EVALUATION OF MATERNAL IMMUNE ACTIVATION WITH POST-WEANING SOCIAL ISOLATION AS A POTENTIAL RAT MODEL FOR SCHIZOPHRENIA

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Abstract

Schizophrenia is a disabling neurodevelopmental disorder whose risk factors are diverse, aetiology is unclear and symptoms remain inadequately treated. The development of animal models with improved face, construct and predictive validity would facilitate our understanding of its neurobiology, and aid development of novel therapeutics. In this thesis, the combination of maternal immune activation (MIA) in pregnant dams and post-weaning social isolation (SI) of resultant rat offspring is evaluated to determine whether this 'dual-hit' neurodevelopmental model produces a more comprehensive array of behavioural and neurochemical changes akin to those observed in schizophrenia than either intervention alone. At gestational day (GD) 15, pregnant dams received a single intraperitoneal (i.p., 10 mg kg^{-1}) or intravenous (i.v., 6 mg kg^{-1}) injection of a viral mimetic, polyinosinic:polycytidylic acid (poly(I:C)), or vehicle saline (1 ml kg^{-1}). Resultant offspring were housed in groups or alone (in isolation) from weaning. In adulthood, male offspring underwent comprehensive behavioural analysis using paradigms relevant to the positive, negative and multiple cognitive domains of schizophrenia and potential mechanisms underlying behavioural abnormalities were studied. Regardless of route of administration, poly(I:C) alone had relatively subtle effects. Interestingly, i.p. poly(I:C) conferred an unexpected resilience towards SI-induced behavioural (locomotion, aggressive behaviour and conditioned fear) and immune abnormalities. Investigations into potential early molecular mechanisms showed a sophisticated interplay between stress experience and gender on long-term behavioural and immune outcomes. When combined with SI, i.v. poly(I:C) resulted in some additive effects on certain behavioural measures related to the positive, negative and cognitive symptoms (locomotion and ultrasonic communication). The same combination, however, promoted resilience against SI-induced immune changes, suggesting an alternative underlying mechanism than with i.p. poly(I:C) administration. Neither poly(I:C) nor SI had any effect on working or spatial memory, or sensorimotor gating. Therefore, despite producing

some behavioural phenotypes with translational relevance to schizophrenia, further improvements and validation of the MIA and SI protocols are required to identify any benefit of or molecular mechanism involved in the ‘dual-hit’ model assessed in this thesis. Finally, the effects of SI on cortical gene expression were examined and interesting changes in genes associated with pathways including the circadian clock, mitogen-activated protein kinase (MAPK) signalling and dopaminergic neurotransmission were observed. These altered genes are discussed in the context of previous SI studies and their potential translational relevance to schizophrenia is considered.

Publications during enrolment

Paper

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Table of Contents

CHAPTER 1 General introduction 1
1.1 Introduction 2
1.1.1 Epidemiology and impact 2
1.1.2 Onset and course 4
1.1.3 Clinical signs and symptoms 5
1.1.4 Aetiology 6
1.1.5 Pathophysiology 12
1.1.6 Current treatment and therapies 20
1.2 Animal models of schizophrenia 24
1.3 Maternal immune activation 27
1.3.1 The placenta: the maternal-foetal interface 27
1.3.2 The prenatal cytokine hypothesis 27
1.3.3 The physiological roles of cytokines 28
1.3.4 Innate and adaptive immunity 28
1.3.5 Indirect infectious mechanisms of MIA 30
1.3.6 Non-infectious mechanisms of MIA 32
1.3.7 Animals models of MIA 34
1.3.8 The poly(I:C) model 38
1.4 Post-weaning social isolation 98
1.5 Aims and objectives of the thesis 104

CHAPTER 2 The combined effects of gestational poly(I:C) exposure via i.p. administration and post-weaning social isolation on behaviour and neurochemistry 107
2.1 Introduction 108
2.2 Aims 116
2.3 Materials and methods 116
2.3.1 Animals 116
2.3.2 Drug preparation 117
2.3.3 Experimental design 117
2.3.4 Sickness behaviour 122
2.3.5 Behavioural apparatus and procedure 123
2.3.6 Tissue collection and handling 134
2.3.7 Neurochemical detection by HPLC-ED 135
2.3.8 Multiplex cytokine analysis 137
2.3.9 mTOR analysis 141
2.3.10 Statistical analysis 141
2.4 Results 144
2.4.1 Experiment 1: The effect of poly(I:C) via i.p. administration on non-pregnant Lister-hooded rats 144
2.4.2 Experiment 2: The combined effects of gestational poly(I:C) via i.p. administration and post-weaning social isolation on behaviour and neurochemistry 147

2.5 Discussion 196
2.5.1 Experiment 1: The effect of poly(I:C) via i.p. administration on non-pregnant Lister-hooded rats 196
2.5.2 Experiment 2: The combined effects of gestational poly(I:C) via i.p. administration and post-weaning social isolation on behaviour and neurochemistry 200

CHAPTER 3 The combined effects of gestational poly(I:C) exposure via i.v. administration and post-weaning social isolation on behaviour and neurochemistry 215
3.1 Introduction 216
3.2 Aims 218
3.3 Materials and methods 218
3.3.1 Animals 218
3.3.2 Experimental design 219
3.3.3 Statistical analysis 222
3.4 Results 224
3.4.1 Experiment 1: The effect of poly(I:C) via i.v. administration on non-pregnant Lister-hooded rats 224
3.4.2 Experiment 2: The combined effects of gestational poly(I:C) via i.v. administration and post-weaning social isolation on behaviour and neurochemistry 227
3.5 Discussion 257
3.5.1 Experiment 1: The effect of poly(I:C) via i.v. administration on non-pregnant Lister-hooded rats 257
3.5.2 Experiment 2: The combined effects of gestational poly(I:C) via i.v. administration and post-weaning social isolation on offspring behaviour and neurochemistry 258

CHAPTER 4 The effect of post-weaning social isolation on cortical gene expression 276
4.1 Introduction 277
4.2 Aims 280
4.3 Materials and methods 281
4.3.1 Animals 281
4.3.2 Experimental design 281
4.3.3 Total RNA extraction and purification 282
4.3.4 cDNA library generation and RNA sequencing 282
4.3.5 Bioinformatics analysis 283
4.3.6 Statistical analysis 286
4.4 Results 290
4.4.1 Body weight analysis: whole cohort and rats selected for RNA-Seq 290
4.4.2 Quality control of RNA-Seq data: mapping 290
4.4.3 Differential gene expression: relationship between samples 292

4.4.4 SI-induced changes in gene expression 292
4.4.5 Pathway analyses 293
4.4.6 Gene ontology enrichment analysis 298
4.5 Discussion 304
4.5.1 Differential gene expression analysis 304
4.5.2 Pathway analyses 307
4.5.2.1 Common DEGs shared by multiple enriched pathways . 308
4.5.2.2 Enriched pathways 312
4.5.2.3 Limitations and future directions 331
4.6 Conclusion 334

CHAPTER 5 General discussion 335
5.1 Animal models for neurodevelopmental disorders 336
5.2 Principal findings 338
5.2.1 The 'dual-hit' model 338
5.2.2 Behaviour and potential early mechanisms in adolescence 338
5.2.3 Cortical gene expression 339
5.3 Impact of findings 339
5.3.1 The poly(I:C) model 339
5.3.2 The SI model 341
5.3.3 The 'dual-hit' model 344
5.3.4 The effect of gender 345
5.4 Future studies 346
5.4.1 The SI model 346
5.4.2 The poly(I:C) model 348
5.5 Conclusion 350

APPENDIX 1 352
Radioligand binding 352
1.1 Introduction 353
1.2 Methods 355
1.2.1 Animals and tissue collection 355
1.2.2 Membrane preparation 355
1.2.3 Saturation binding assay 355
1.2.4 Analysis 356
1.3 Results 357
1.3.1 Optimisation 1 357
1.3.2 Optimisation 2 357
1.4 Discussion 360
APPENDIX 2 361

References 371

Abbreviations

3Rs	Replacement, Reduction and Refinement
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
7-OH-DPAT	7-hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin
AMPH	Amphetamine
AP-1	Activator protein-1
ASST	Attentional set-shifting task
BDNF	Brain-derived neurotrophic factor
BSU	Bio-Support Unit
CD	Compound discrimination
cDNA	Complementary DNA
CFR	Conditioned freezing response
CHD	Chromodomain helicase DNA-binding
CNS	Central nervous system
CNV	Copy number variation
CS	Conditioned stimulus
DAMPs	Damage-associated molecular patterns
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein of 32 kDa
DEGs	Differentially expressed genes
DNA	Deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
DPM	Disintegrations per minute
dsRNA	Double-stranded RNA
ED	Extra-dimensional shift
EGFR	Epidermal growth factor receptor
ERKs	Extracellular signal-regulated kinases
FDR	False discovery rate
GABA	Gamma-aminobutyric acid
GD	Gestational day
GH	Group-housed

GO	Gene Ontology
GOrilla	GO Enrichment Analysis and Visualisation Tool
GTPases	Guanosine triphosphatases
GWAS	Genome-wide association study
HPA	Hypothalamic-pituitary-adrenal
HPLC-ED	High performance liquid chromatography with electrochemical detection
HVA	Homovanillic acid
ID	Intra-dimensional shift
IEGs	Immediate early genes
IFNs	Interferons
IKK	IκB kinase
IL	Interleukin
iPSCs	Induced pluripotent stem cells
IP_3R1	Inositol 1,4,5-trisphosphate receptor type 1
IRF-3/7	Interferon regulatory factor 3/7
ISG15	Interferon-stimulated gene-15
ISH	*In situ* hybridisation
ITI	Inter-trial interval
i.p.	Intraperitoneal (route of administration)
i.v.	Intravenous (route of administration)
JNK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LMA	Locomotor activity
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDA-5	Melanoma differentiation-associated protein 5
mGluR	Metabotropic glutamatergic receptor
MIA	Maternal immune activation
miRISC	MicroRNA-induced silencing complex
miRNAs	MicroRNAs
mRNA	Messenger RNA

mTOR	Mammalian target of rapamycin
MWM	Morris water maze
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NLD	Novel location discrimination
NLRs	Nucleotide oligomerisation domain-like receptors
NMDA	N-methyl-D-aspartate receptor
NOD	Novel object discrimination
NSB	Non-specific binding
PAMPs	Pathogen-associated molecular patterns
PAMs	Positive allosteric modulators
PCP	Phencyclidine
PFC	Prefrontal cortex
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PND	Postnatal day
Poly(I:C)	Polyinosinic:polycytidylic acid
PPI	Pre-pulse inhibition of acoustic startle
PRRs	Pattern recognition receptors
qPCR	Real-time quantitative polymerase chain reaction
Rev1/2/3	Reversal 1/2/3 (first, second or third reversal)
RIG-I	Retinoic acid-inducible gene I
RIPA	Radioimmunoprecipitation assay
RLHs	Retinoic acid-inducible gene I-like helicase
RM	Repeated measures
RNA-Seq	RNA-Sequencing
SD	Simple discrimination
SEM	Standard error of the mean
SI	Social isolation *(note: not to be confused with 'social interaction', which is given in full throughout this thesis)*
SNPs	Single nucleotide polymorphisms
s.c.	Subcutaneous (route of administration)

TBK1	TANK binding kinase 1
TICAM-1	Toll-like receptor adaptor molecule 1
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF3	TNF receptor associated factor 3
US	Unconditioned stimulus
USVs	Ultrasonic vocalisations

CHAPTER 1

General introduction

1.1 Introduction

1.1.1 Epidemiology and impact

Schizophrenia is a chronic, disabling mental disorder which affects more than 23 million people worldwide (World Health Organisation, 2018a). It is associated with increased premature mortality, mostly attributed to natural causes of death such as circulatory and respiratory diseases, and its global burden, measured by incidence, prevalence and years lived with disability, remains consistently high in the last two decades (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018; Lee et al., 2018; Olfson et al., 2015). Impaired cognitive and social functioning in schizophrenia impose tremendous socioeconomic and human burden not only on the afflicted individuals, but also on their families, caregivers and the wider society (Figure 1.1). For instance, in a recent study, the employment rate of working-age individuals with schizophrenia was found to be 10% (Evensen et al., 2016). Individuals with schizophrenia are also more prone to internalised stigmatisation, depression, social anxiety and negative discrimination, which can collectively lead to increased suicide ideation (Farrelly et al., 2015b; Vrbova et al., 2018). Furthermore, poverty, SI, imprisonment and crime victimisation are prevalent in schizophrenia and the heightened risk of aggressive behaviour in patients raises public safety concerns (Albers et al., 2018; Hodgins & Klein, 2017). Therefore, there is a pressing need to improve treatment for this chronic disorder.

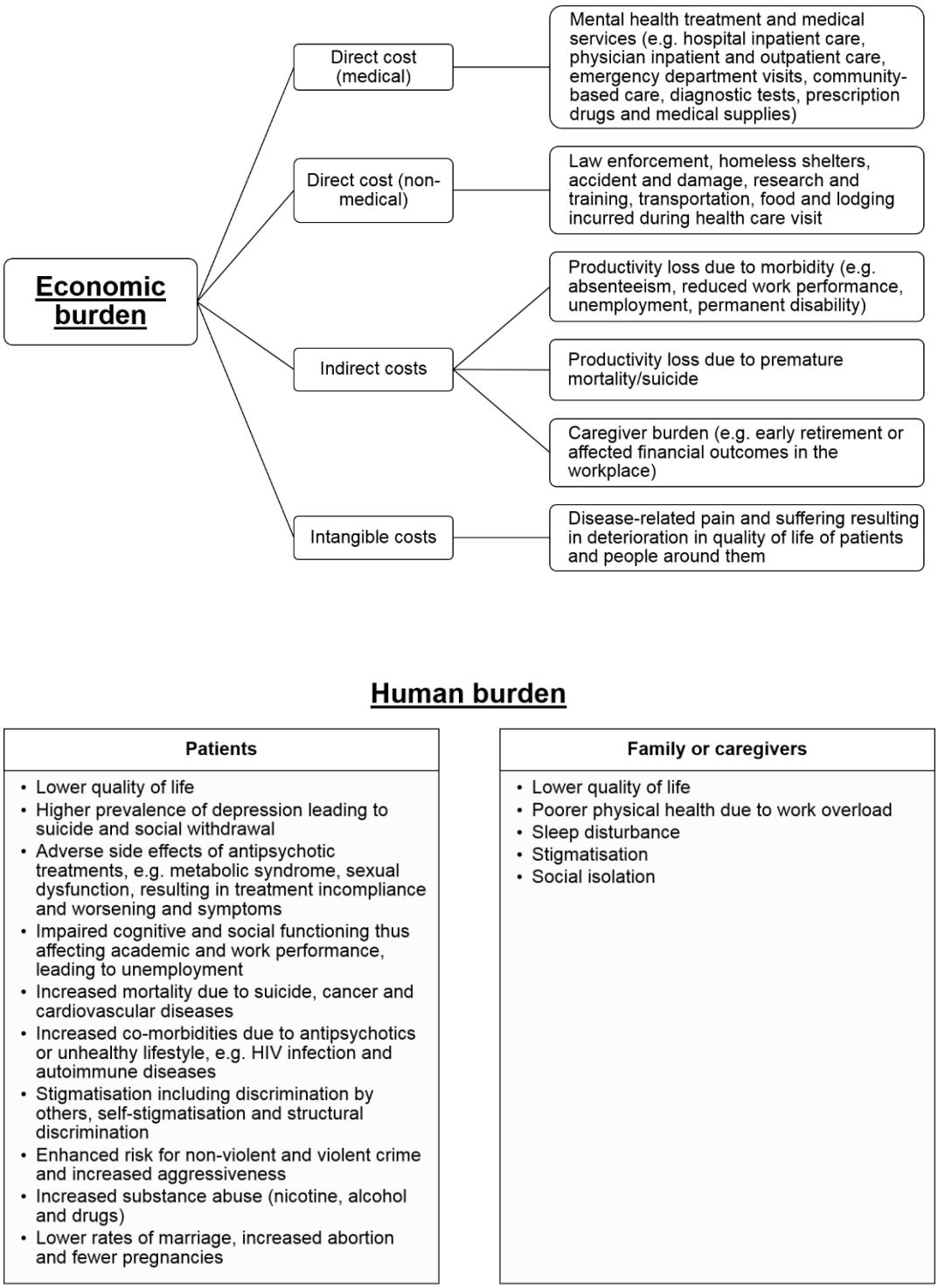


Figure 1.1. Economic and human burden of schizophrenia. The different domains of the economic (direct, indirect and intangible costs; top) and humanistic (bottom) impacts of schizophrenia are presented. Figure was generated based on information from Chong et al. (2016), Cloutier et al. (2016), Csoboth et al. (2015), Desai et al. (2013), Fasseeh et al. (2018), Kovács et al. (2018), Leucht et al. (2007), Millier et al. (2014).

1.1.2 Onset and course

Symptoms of schizophrenia generally appear in late adolescent or early adulthood (16-30 years) and persist throughout life (Mueser & McGurk, 2004). Onset in women seems to be bimodal, with an early phase (25-35 years) slightly later than that in men and a later phase (>40 years) following menopause, probably due to falling oestrogen levels, especially estradiol (Ochoa et al., 2012; Searles et al., 2018). Although rare, there are also cases of childhood-onset (<13 years), early-onset (13-18 years), late-onset (40-60 years) and very-late-onset schizophrenia (>60 years; Howard et al., 2000; Millan et al., 2014).

Symptoms differ extensively among afflicted individuals and in comparison to neurodegenerative diseases like Alzheimer's disease which follow a gradual course, the progression of schizophrenia is variable in that (i) it can manifest in either an abrupt or insidious manner, (ii) the psychosis experienced may be either persisting or episodic with varying remission intervals, and (iii) the clinical condition can either worsen, remain unchanged or fully recover (Austin et al., 2015; Thaker & Carpenter Jr., 2001). Patients typically experience a prodromal state ranging from weeks to months before becoming clinically diagnosed and key features during such a state include social withdrawal, personality alteration and possessing paranoid thoughts (Meyer & MacCabe, 2012). While a majority of these "high-risk" individuals (70%) will not transit into full-blown psychosis and some may progress to related disorders such as depression and anxiety, once established, patients typically undergo repeated cycles of remissions and relapses (Fusar-Poli et al., 2014; Millier et al., 2014). Full remission is rare although studies have reported considerable symptomatic, psychosocial and functional remissions in patients (45, 32 and 53% of total subjects, respectively; Valencia et al., 2015).

1.1.3 Clinical signs and symptoms

Symptoms of schizophrenia fall into three categories: positive, negative and cognitive. Positive or psychotic symptoms are associated with the inability to perceive reality, including hallucinations (can be auditory, gustatory, olfactory, tactile or visual), delusions, bizzare or disorganised thoughts, or behaviour including intentional self-harm and attempted suicide. It has been observed that 97% of individuals with schizophrenia showed anosognosia (lack of insight) and 74% suffer from auditory hallucination, the commonest type of hallucination in schizophrenia (Meyer & MacCabe, 2012; Picchioni & Murray, 2007).

Negative symptoms reflect impairment in general emotional and behavioural states and include blunted affect, impoverished communication, avolition (passivity), apathy, anhedonia, active or passive social withdrawal, psychomotor retardation and impaired mental focus (Möller, 2007; Picchioni & Murray, 2007). They are generally more prevalent and sustained than psychotic symptoms, i.e. present for >6 months, and either manifest with or between psychotic events (Meyer & MacCabe, 2012). These symptoms can be subdivided into primary or secondary, and as the severity of negative symptoms can vary considerably over time, they greatly affect social and functional outcomes in patients (Möller, 2007).

Cognitive deficits are observed in up to 85% of in-patients and are reflected by shortfalls in attention, memory (declaration/explicit, working and episodic), processing speed, problem solving, executive function and social cognition (Meyer & MacCabe, 2012; Millier et al., 2014). Like negative symptoms, they are disabling in that they can intensely disrupt social and occupational roles, leading to stigmatisation of various types (personal, social, familial, occupational and marital), diminished self-esteem and discrimination (Millier et al., 2014; Shrivastava et al., 2011).

In general, earlier symptom onset is associated with more severe course of illness with increased hospitalisations, negative symptoms and relapses, in addition to worsened social or occupational capacity and global outcome (Immonen et al., 2017). Later-onset, in contrast, is correlated to increased positive symptoms, including visual, tactile and olfactory hallucinations, but less formal thought disorder and negative symptoms (Howard et al., 2000). Negative and cognitive symptoms typically emerge ahead of the positive symptoms, and instead of acting as separate entities, the three symptom domains are possibly inter-related, one leading to the manifestation or exacerbation of the other (Figure 1.2; Millan et al., 2014). Whilst positive symptoms are generally responsive to current dopamine D_2 receptor-based antipsychotics, there is an unmet need in developing novel treatments and therapies for combating the treatment-resistant negative and cognitive symptoms (Mueser & McGurk, 2004; Millan et al., 2014; Uno & Coyle, 2019). Current treatments and therapies for schizophrenia are described in section 1.1.6.

1.1.4 Aetiology

Schizophrenia liability is subjected to a complicated interaction between genetic and non-genetic factors during critical windows of neurodevelopment.

1.1.4.1 *Genetic causes*

Schizophrenia has a strong genetic basis in that lifetime risk increases with positive family history, from ~1% in the general population to 3-5% in first or second-degree relatives, 7-12% in siblings and dizygotic twins, and 33-50% in monozygotic twins of afflicted individuals (Hilker et al., 2018; Picchioni & Murray, 2007; Thaker & Carpenter Jr., 2001). Additionally, studies have consistently revealed a heritability estimate of approximately 80% for schizophrenia, i.e. around 80% of schizophrenia risk is attributable to genetic factors (Harrison & Weinberger, 2005; Hilker

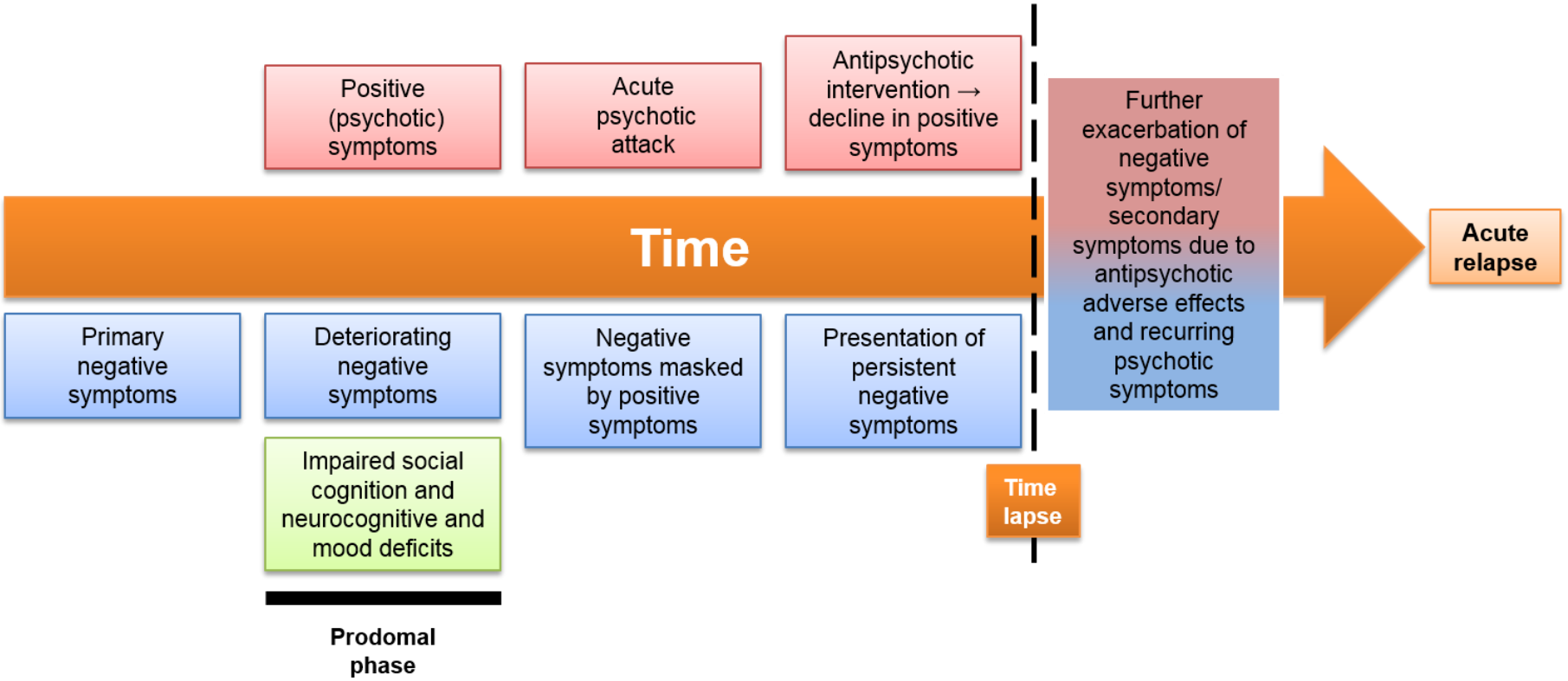


Figure 1.2. Conceptual timeline of symptom presentation in individuals with schizophrenia. The course of schizophrenia typically begins with the manifestation of sub-clinical negative symptoms which deteriorates when positive symptoms appear (prodomal phase). The prodomal phase is also characterised by the presence of cognitive deficits, which persist and greatly impair functional outcomes. Positive symptoms worsen over time until an acute psychotic episode finally occurs and conceals the co-existing negative symptoms. The use of drug intervention to manage positive symptoms could worsen negative symptoms. Repeating psychosis and/or antipsychotic adverse effects aggravate the condition even further. Figure was generated based on information from Millan et al. (2014) and Möller, et al. (2007).

et al., 2018). While conventional linkage analyses have linked several chromosome loci to schizophrenia, e.g. 6p22-24 and 8p21-22, such findings lack reproducibility, potentially due to limited power and factors such as imprecise diagnosis (Harrison & Weinberger, 2005). Association studies, which offer increased spatial resolution and power, have associated multiple candidate genes with schizophrenia, including those related to neurotransmission (e.g. *DRD4* "dopamine receptor D4", *COMT* "catechol-O-methyltransferase", *NRG1* "neuregulin 1" and *RGS4* "regulator of G protein signalling 4"), deoxyribonucleic acid (DNA) methylation (e.g. *MTHFR* "methylenetetrahydrofolate reductase") and neurodevelopment (e.g. *TP53* "tumour protein p53" and *DISC1* "disrupted-in-schizophrenia 1"; Chen et al., 2004; Chowdari et al., 2002; Schumacher et al., 2009; Shi et al., 2008; Stefansson et al., 2002). Genome-wide association studies (GWAS) allow the identification of common single nucleotide polymorphisms (SNPs) whereas copy number variant (CNV) analysis enables detection of rare chromosomal rearrangements (Foley et al., 2017). For instance, an extensive schizophrenia GWAS study in 2014 revealed 108 independent gene loci, of which 83 were novel, including genes involved in dopaminergic and glutamatergic neurotransmission, synaptic plasticity, calcium signalling and immunity (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Meanwhile, CNV analysis has linked various chromosomal duplications and deletions, notably chromosome 22q11.2 deletion, to increased schizophrenia risk (The International Schizophrenia Consortium, 2008). Although rare, CNVs have been found to be highly penetrant, i.e. each variant has a high influence on the risk of schizophrenia (The International Schizophrenia Consortium, 2008). In comparison, the effect sizes of individual SNPs are relatively small (The International Schizophrenia Consortium, 2009). Whole-genome and whole-exome sequencing enable the detection of rare single-nucleotide variants and small insertions or deletions, as well as ultra-rare variants (Foley et al., 2017). It is now appreciated that schizophrenia is a polygenic disorder, of which genetic contribution consists of a mixture of common, rare and de novo mutations, with many risk genes overlapping

considerably with those of bipolar disorder and major depression (Foley et al., 2017; Kavanagh et al., 2015). Moreover, a potential contribution of somatic single-nucleotide variants towards schizophrenia liability has been suggested recently (Fullard et al., 2019).

1.1.4.2 *Environmental factors*

Adverse prenatal exposures can have long-term destructive effects on the foetal brain, as posited by the neurodevelopmental theory of schizophrenia (Weinberger et al., 1987). People born during winter or early spring, when infections like colds and influenza are more prevalent, are more likely to develop schizophrenia such that a 5-8% winter or spring birth excess has been demonstrated (Cheng et al., 2013; Torrey et al., 1997). Maternal stress, diet, metabolic function and gut microbiome are other potential risk factors to the offspring (Delaney & Hornig, 2018; Rivera et al., 2015; Schaefer et al., 2000). Furthermore, excessive exposure to pollutants and tobacco use during pregnancy have been implicated, as have numerous obstetric complications (Mueser & McGurk, 2004; van Os et al., 2010). Intriguingly, advanced paternal but not maternal age increases risk of schizophrenia (Torrey et al., 2009).

Early-life adversities in the developing offspring and postnatal environment are also thought to enhance the development of schizophrenia. Higher-latitude bands are associated with greater schizophrenia prevalence in both genders and urbanicity as a factor has been robustly observed across various ethnic groups (Mueser & McGurk, 2004; Saha et al., 2006). Higher rates of schizophrenia have been consistently demonstrated in minority ethnic populations or migrants and their children, in a manner such that risk corresponds to the degree of minority position (Picchioni & Murray, 2007; van Os et al., 2010). The same is true in subgroups associated with social maladjustment, e.g. single parent family, single person and residential instability, and those in poverty or lower social class (Mueser & McGurk, 2004; van Os et al., 2010). SI in early years and/or adulthood reduces mental ability and

predisposes someone to schizophrenia (Cannon et al., 1997; Selten et al., 2013; van Os et al., 2010). Lastly, childhood developmental trauma and substance abuse are also implicated (Meyer & MacCabe, 2012; Rapoport et al., 2012; van Os et al., 2010). Table 1.1 displays a summary of the developmental and environmental risk factors of schizophrenia.

1.1.4.3 *Epigenetic factors*

Epigenetic mechanisms are fundamental in neurodevelopment where they dictate tissue-specific gene expression patterns (Ovenden et al., 2018). In schizophrenia, various abnormalities in epigenetic mechanisms, including DNA methylation, DNA hydroxymethylation, histone post-translational modifications and nucleosome remodelling are known (Cariage-Martinez & Alelú-Paz, 2017; Ovenden et al., 2018). Moreover, dysregulated expression of microRNAs (miRNAs), which regulate gene expression, have been reported (Shorter & Miller, 2015). As pre- and postnatal environmental alterations can confer long-lasting effects on epigenetic factors and the latter can either act on single genes or gene networks, epigenetic mechanisms provide a link between genetic and environmental factors leading to the development of neurodevelopmental disorders such as schizophrenia (Ovenden et al., 2018; Shorter & Miller, 2015). Given their involvement in the pathology of schizophrenia, miRNAs have become attractive therapeutic targets for intervention (Cao & Zhen, 2018).

Table 1.1. Pre-, peri- and postnatal environmental risk factors of schizophrenia. Exposure to adverse surroundings before, during and after birth is linked to higher risk of developing schizophrenia. Specific examples of such risk factors are presented.

Type of adversity	Examples	References
Prenatal environmental influences	**Maternal infection** • **Viral**, e.g. influenza, rubella, measles, polio, cytomegalovirus, herpes simplex virus, human parvovirus B19, human papillovirus type 16, Epstein-Barr virus, varicella-zoster virus • **Bacterial**, e.g. urinary tract infections (cystitis), upper respiratory tract infections (sinusitis, tonsillitis), pneumonia, pyelonephritis, veneral infection (gonococcal infection, chlamydia) • **Parasitic**, e.g. *Toxoplasma gondii*	Boksa, 2010; Brown & Patterson, 2011; Delaney & Hornig, 2018; Hantsoo et al., 2019; Kneeland & Fatemi, 2013; Meyer & MacCabe, 2012; Meyer, 2014 ; Mueser & McGurk, 2004; Reisinger et al., 2015; Rivera et al., 2015; Schwartz, 2011; Sørensen et al., 2009 ; van Os et al., 2010.
	Maternal stress • **Stressful life events**, e.g. death of partner, divorce, daily hassles • **Chronic stress**, e.g. caregiving, unemployment, extended work stress, racial discrimination, poverty • **Traumatic stress**, e.g. war, natural disasters, nuclear disaster • **Adverse childhood experience**, e.g. physical or emotional abuse, emotional neglect, economic hardship	
	Maternal diet • **Obesity and metabolic dysfunction**, e.g. diabetes, hypertension, unhealthy diet • **Malnutrition**, e.g. folate, iron, vitamin D and vitamin B12 deficiencies	
	Smoking during gestation	
	Exposure to environmental toxins, e.g. lead	
	Maternal gut microbiome	
Obstetric complications	**During pregnancy**, e.g. bleeding (due to placenta abruption, placenta praevia and incompetent cervices), rhesus incompatibility, pre-eclampsia	Cannon et al., 2002; Jones et al., 1998; Meli et al., 2012 ; Picchioni & Murray, 2007.
	During labour, e.g. uterine atony, asphyxia, emergency Caesarean section	
Perinatal complications	Low birth weight (LBW; <2500 g), combined LBW and pre-term birth (<37 weeks), perinatal hypoxia, smaller head circumference, congenital abnormalities	
Postnatal environment influences	Urbanicity	Meyer & McCabe, 2012; Mueser & McGurk, 2004; Picchioni & Murray, 2007; van Os et al., 2010
	Ethnic minority	
	Social maladjustment or isolation	
	Trauma, e.g. sexual or physical abuse, neglect, head injury	
	Substance abuse, e.g. cannabis, amphetamine, lysergic acid diethylamide (LSD)	
Others	Advanced paternal age	Meli et al., 2012; Torrey et al., 2009

1.1.5 Pathophysiology

Structural imaging studies have revealed various brain abnormalities in schizophrenia, including ventricular enlargement, reduced brain volumes and cortical grey matter deficits (Johnstone et al., 1976; Vita et al., 2012; Wright et al., 2000). Functional neuroimaging studies, on the other hand, reported altered neural activity and brain functional connectivity, such as diminished prefrontal activation during episodic encoding and retrieval, abnormal default mode network and reduced connectivity of auditory processing and language networks (Karbasforoushan & Woodward, 2012; Ragland et al., 2009). Given that neurotransmitter systems are found extensively throughout the brain, altered neurotransmission potentially underlies the neurobiology of schizophrenia (Kesby et al., 2018). Central neurotransmitter function can be evaluated by measuring the levels of neurotransmitter precursors, metabolites and enzymes responsible for neurotransmitter biosynthesis and breakdown in the urine, blood or cerebrospinal fluid (Hanin, 1978). Consequently, various *in vivo* and *in vitro* neurotransmitter detection methods have been developed, such as nuclear medicine tomographic neuroimaging (i.e. positron emission tomography and single-photon emission computed tomography), optical sensing techniques (e.g. surface-enhanced Raman spectroscopy, fluorescence and optical fiber biosensing), as well as electrochemistry-based methods (e.g. fast-scan cyclic voltammetry and amperometry). Additional techniques that can be employed to analyse neurotransmitter function are high performance liquid chromatography (HPLC), microdialysis and tandem mass spectrometry (see Niyonambaza et al., 2019 for a concise, up-to-date review of these techniques). Here, the major hypotheses of schizophrenia and alternative mechanisms are reviewed.

1.1.5.1 *The dopamine hypothesis*

The discovery that antipsychotics work through the blockade of postsynaptic dopamine receptors, together with the findings that

dopaminergic stimulants such as amphetamine (AMPH) and methylphenidate are capable of inducing and worsening psychosis in healthy subjects and individuals with schizophrenia, respectively, laid the foundation for the dopamine hypothesis of schizophrenia. Such hypothesis posits that augmented dopamine transmission underlies the symptomatology of schizophrenia (Angrist & van Kammen, 1984; Creese et al., 1976; Lieberman et al., 1987; Seeman et al., 1975). A refined version of the hypothesis, which remains the central dogma until recently (see later), states that mesocortical hypodopaminergia and mesolimbic hyperdopaminergia in schizophrenia accounts for the negative and positive symptoms, respectively (Davis et al., 1991). Imaging and preclinical findings further show that antipsychotics act by blocking ventral striatal D_2 receptors and eventually pinpointing the principal locus of dopamine dysregulation to the presynaptic level (Frankle & Laruelle, 2002; Howes et al., 2007; Meyer-Linderberg, 2002). Consistent with these findings, postmortem and neuroimaging studies revealed elevated densities of dopamine $D_{2/3}$ receptors in brain regions such as the caudate, putamen and striatum in patients with schizophrenia (Howes et al., 2012; Lee & Seeman, 1980; Wong et al., 1986). Moreover, meta-analysis of imaging findings suggested increased presynaptic dopamine synthesis and release in the striatum in patients (Howes et al., 2012). More recently, both the cell bodies and striatal terminals of dopamine neurons are implicated in schizophrenia pathophysiology (Howes et al., 2013). Nevertheless, instead of intrinsic dopaminergic abnormalities, newer theoretical models assert that the dysregulation of striatal dopamine system by afferent structures, such as the hippocampus, contribute to the three symptom domains (Grace, 2016; Kesby et al., 2018; McCutcheon et al., 2019). Furthermore, lately, neuroimaging findings imply a role of the dorsal striatum (nigrostriatal pathway) in schizophrenia, challenging the long-established mesolimbic theory (McCutcheon et al., 2019). Abnormal dopaminergic activity in the caudal striatum has also been associated with disorganised behaviour, motor passivity or thought echo, and auditory hallucinations (Gangarossa et al., 2013; McCutcheon et al., 2018). Finally, in patients, abnormal expression of dopamine D_1, D_4 and

D_5 receptors has been demonstrated (Howes et al., 2015; Meygooni et al., 2019; Seeman et al., 1993).

1.1.5.2 *The glutamatergic hypothesis*

The poor efficacy of D_2 receptor-based antipsychotics on the negative and cognitive symptoms suggests the involvement of signalling pathways other than dopamine in the pathophysiology of schizophrenia (Millan et al., 2014). The glutamatergic system, which is implicated in cognitive domains such as memory, working memory and executive function, constitutes most of the excitatory neurotransmission in the brain and is associated with up to 80% of total brain oxidative energy production (Rothman et al., 2003; Thomas et al., 2017). Early observations that ketamine and phencyclidine (PCP), non-competitive antagonists at the *N*-methyl-D-aspartate (NMDA) receptor subtype of glutamate ionotropic receptors, induced psychosis or thought disorder reminiscence of schizophrenia, as well as negative and cognitive symptoms, prompted the reasoning that schizophrenia was a consequence of NMDA receptor hypofunction (Coyle, 1996; Javitt & Zukin, 1991; Moghaddam & Krystal, 2012; Snyder, 1980). Indeed, hypofunction of NMDA receptors localised on gamma-aminobutyric acid (GABA)ergic interneurons in the prefrontal cortex (PFC) or hippocampus is thought to enhance downstream glutamatergic signalling to the midbrain, promoting mesolimbic hyperdopaminergia and hence psychotic symptoms (Howes et al., 2015). Various glutamatergic abnormalities, also including those of metabotropic glutamatergic receptor (mGluR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and kainate receptor, gathered from genetic, neurophysiological and post-mortem neurochemical studies, support the glutamate hypothesis (Howes et al., 2015; Rubio et al., 2012; Uno & Coyle, 2019). For instance, a recent large-scale GWAS study has highlighted the association of multiple genes involved in glutamatergic transmission and synaptic plasticity with schizophrenia, namely *GRM3* (glutamate metabotropic receptor 3), *GRIN2A* (glutamate ionotropic receptor NMDA type subunit 2A), *SRR*

(serine racemase) and *GRIA1* (glutamate ionotropic receptor AMPA type subunit 1; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

1.1.5.3 *The serotonin hypothesis*

The serotonin (5-hydroxytryptamine; 5-HT) hypothesis was based on the initial findings that the psychomimetic effects of 5-HT-like psychedelic hallucinogens, such as lysergic acid diethylamide, mescaline and psilocybin were due to altered 5-HT function in the central nervous system (CNS; Meltzer et al., 2006; Shaw & Woolley, 1956; Woolley & Shaw, 1954). Furthermore, as binding at 5-HT receptors is required for the therapeutic effects of antipsychotics, the serotonergic system becomes an attractive drug target (Richtand et al., 2007). Although both dopamine and glutamate hypotheses make considerable sense, they do not account for the observed cortical atrophy, peripheral phospholipid abnormalities and therapeutic efficacy of serotonergic blockade by atypical antipsychotics in schizophrenia, albeit dopamine-based antipsychotics do relieve positive symptoms (Eggers, 2013). Moreover, it remains unclear how dysregulation of dopaminergic and glutamatergic systems are associated with stress, a prominent risk factor of schizophrenia (Howes et al., 2017; Popoli et al. 2013). These limitations led to the formation of the current 5-HT model, which proposes that stress-induced hyperactivation of 5-HT_{2A} receptors in the cerebral cortex enhances glutamatergic signalling in the visual cortex and ventral striatum, causing psychosis. Excessive glutamatergic drive could also result in hypometabolism of cortical neurons, contributing to synaptic atrophy and grey matter loss (Eggers, 2013; Stahl, 2018). In schizophrenia, numerous 5-HT transmission abnormalities have been reported (Abi-Dargham et al., 1997). A recent meta-analysis, for example, revealed increased and decreased prefrontal 5-HT_{1A} and 5-HT_{2A} receptors, respectively, with minimal changes in 5-HT transporter availability or other 5-HT receptors (Selvaraj et al., 2014).

1.1.5.4 *The GABA hypothesis*

Converging evidence have suggested a role for GABAergic abnormalities in schizophrenia. For instance, reduced transcription of glutamic acid decarboxylase 67, the GABA synthetic enzyme, has been consistently demonstrated in the dorsolateral PFC (de Jonge et al., 2017). It is now clear that such altered expression occurs exclusively in a subset of parvalbumin-containing GABAergic neurons, the basket neurons, without neuronal cell loss (Hashimoto et al., 2003; Rocco et al., 2016). Dysregulation of such neurons is thought to impair inhibitory gamma frequency oscillation, disrupting synchronisation of excitatory pyramidal neurons required for intact working memory (McNally & McCarley, 2016). Apart from cognitive deficits, altered gamma responses are also associated with positive and negative symptoms. Positive and negative symptoms are linked to increased and reduced gamma activity, respectively (Herrman & Demiralp, 2005). Reduced density of cortical somatostatin messenger RNA (mRNA)-containing GABAergic neurons and somatostatin mRNA expression per neuron, potentially due to disrupted tyrosine kinase B receptor signalling (previously linked to impaired inhibitory prefrontal circuitry), have also been demonstrated (Hashimoto et al., 2005; Morris et al., 2008). Nevertheless, it remains obscure whether dysregulation of GABAergic neurotransmission is due to dampened GABA synthesis or excessive inhibition secondary to reduced GABA reuptake (Lewis et al., 2005). It is also possible that NMDA hypofunction triggers a compensatory reduced inhibitory tone, disinhibiting pyramidal neurons leading to increased gamma oscillations (McNally & McCarley, 2016). Other GABAergic abnormalities include lower cortical GABA levels, decreased GABA transporter-1 in chandelier neurons and upregulated $GABA_A$ α2-receptor subunits at the axon initial segments of pyramidal neurons, which are postsynaptic targets of parvalbumin-containing GABAergic neurons (de Jonge et al., 2017; McNally & McCarley, 2016). Figure 1.3 summarises the mechanisms underlying the major neurotransmitter hypotheses of schizophrenia, as well as the link between the hypotheses.

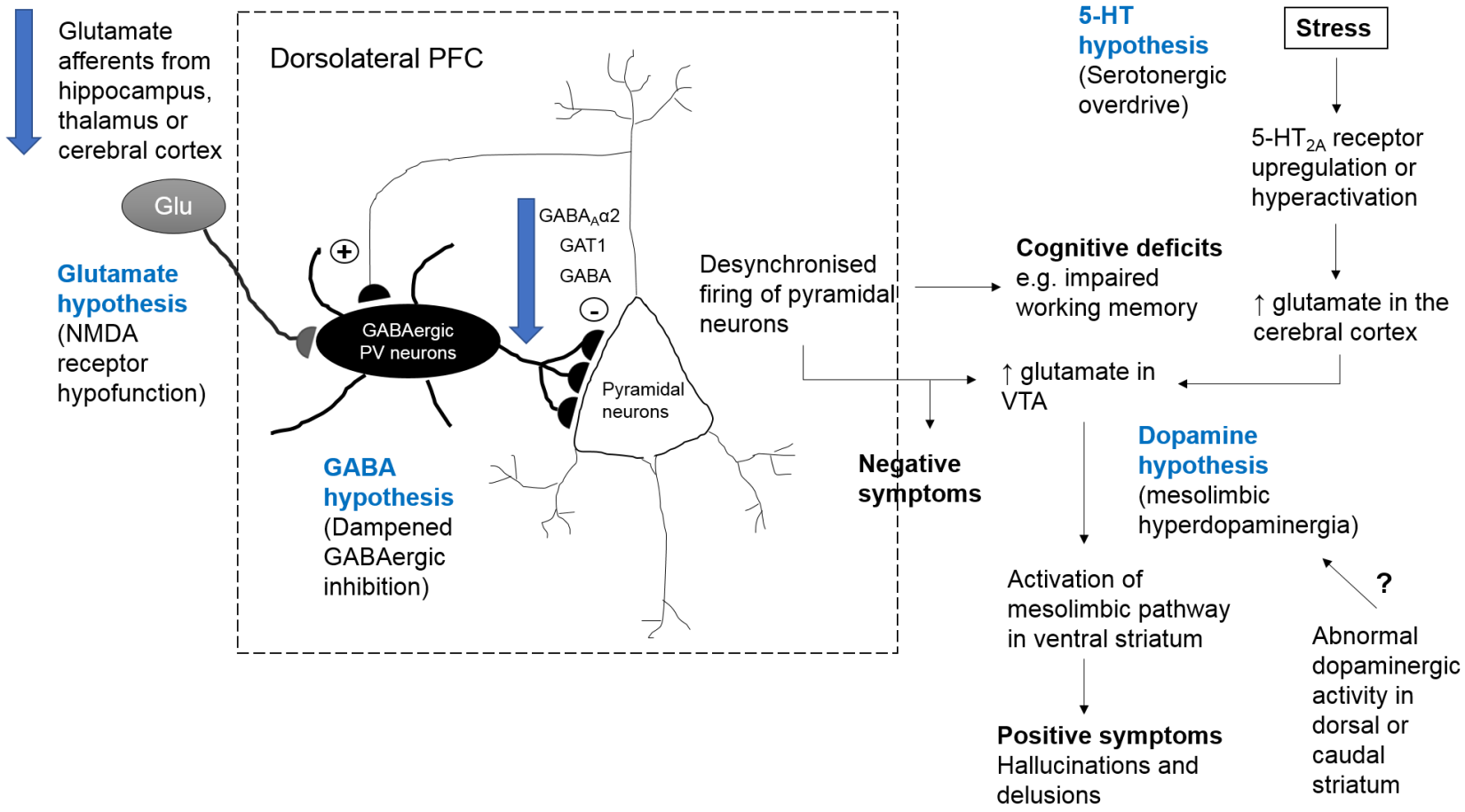


Figure 1.3. Neurotransmitter hypotheses of schizophrenia. This schematic diagram shows the key alterations associated with dopaminergic, glutamatergic, GABAergic and serotonergic systems leading to the positive, negative and cognitive symptoms of schizophrenia. GABA, gamma-aminobutyric acid; GAT, GABA transporter; Glu, glutamate; NMDA, N-methyl-D-aspartate; PFC, prefrontal cortex; PV, parvalbumin; VTA, ventral tegmental area; 5-HT, serotonin.

1.1.5.5 *Cholinergic System*

Cholinergic neurotransmission is closely related to CNS functions such as cognitive processing, memory, motivation, mood and psychosis (Raedler et al., 2007). As cognitive impairment is a consistent domain affected in schizophrenia that is worsened by muscarinic antagonists, it is likely that disruption of this system contributes to schizophrenia (Ellis et al., 2006; Minzenberg et al., 2004). Individuals with schizophrenia were found to have fewer cholinergic interneurons in the ventral striatum, and reduced muscarinic receptors, particularly of M_1 and M_4 subtypes, have been identified in the frontal cortex, anterior cingulate cortex, superior temporal gyrus, hippocampus, caudate and putamen. Furthermore, direct and indirect (via GABAergic system) interactions between the muscarinic and dopaminergic systems in various brain regions have been established (Raedler et al., 2007).

Nicotinic acetylcholine receptors are localised throughout the CNS, peripheral nervous system, immune system and various peripheral tissues, with the α7 subunit-containing receptors being one of the predominantly expressed subtypes in the brain (Dineley et al., 2015). In schizophrenia, hippocampal α7 subunit-containing nicotinic receptor dysfunction has been associated with attenuated sensorimotor gating (Schaaf, 2014).

1.1.5.6 *Other neurotransmitter systems*

The discovery that schizophrenia is linked to reduced frontal cortical histamine H_1 receptors and increased levels of *tele*-methylhistamine, a metabolite of histamine, in the cerebrospinal fluid (indicative of increased central histamine release and metabolism) suggested the involvement of the histaminergic system in the pathology of the disorder (Iwabuchi et al., 2005; Nakai et al., 1991; Prell et al., 1995). Moreover, increased histamine release has been demonstrated in several animal models of

schizophrenia, and histaminergic neurotransmission is functionally linked to both glutamatergic and dopaminergic systems (Faucard et al., 2006; Morisset et al., 2002). Given that histamine H_3 receptors function as auto- and hetero-receptors to regulate CNS synthesis and release of histamine and neurotransmitters such as acetylcholine and dopamine, they are potential drug targets for treatment (Mahmood, 2016). These receptors, which upon stimulation trigger the inhibition of histaminergic neurons via $G\alpha_{i/o}$-dependent signalling, are also thought to modulate cognition and arousal, which are both impaired in schizophrenia (Mahmood, 2016; Nieto-Alamilla, 2016; Passani et al., 2004). The potential of H_3 receptor antagonists or inverse agonists to enhance cognition as well as to rescue disruptions of the circadian rhythm resulted in a renewed interest in histamine H_3 receptors as a therapeutic target for schizophrenia (Alachkar et al., 2017; Mahmood, 2016; Passani et al., 2004). Meanwhile, dysregulated adrenergic system impair cognitive function in schizophrenia. Blockade and activation of α_1-receptor and α_{-2A} receptor signalling, respectively, are possible avenues for cognition enhancement (Arnsten, 2004). Abnormalities in endocannabinoid levels (e.g. increased anandamide levels in the blood and cerebrospinal fluid) and cannabinoid CB_1 receptor signalling in cortical areas associated with cognition and memory plausibly contribute to the aetiology of schizophrenia (Parolaro et al., 2010). Likewise, disrupted oxytocin signalling, which has roles in social or affiliative behaviour, stress-related mechanisms and neuroadaptation, has been implicated (Sarnyai & Kovács, 2014). Finally, purinergic $P2Y_1$ receptors localised on parvalbumin-positive interneurons are thought to mediate hypofunction of NMDA receptors (Krügel, 2016).

1.1.5.7 *Neuroinflammation and oxidative stress*

More recent research has highlighted the possibility that neuroinflammation and oxidative stress are potential mediators of schizophrenia pathogenesis. Consequently, topics such as microglia activation, mitochondrial dysfunction, redox dysregulation, and the roles of heat shock response and toll-like receptors (TLRs) are now the focus

of many studies (Calabrese et al., 2017; Calcia et al., 2016; Koga et al., 2016; Steullet et al., 2016; Venkatasubramanian & Debnath, 2013). Potential mechanisms underlying MIA-induced neuroinflammation in offspring will be discussed later.

1.1.6 Current treatment and therapies

Currently, pharmacological treatment is the mainstay option for managing schizophrenia and preventing relapse (Mueser & McGurk, 2004). Antipsychotics can be broadly segregated into first (typical) and second (atypical) generations based on their clinical outcome and mechanism of actions (Meltzer, 2013). Whilst typical antipsychotics reduce positive symptoms and prevent relapse by predominantly antagonising dopamine D_2 receptors, they have limited effects on the negative and cognitive symptoms, with one-third of patients being resistant to these drugs (Keltner & Johnson, 2002; Millan et al., 2014). Activity of these drugs at D_2 receptors in the nigrostriatal pathway and off-site targets like H_1 and muscarinic receptors leads to adverse effects including extrapyramidal symptoms and weight gain, resulting in reduced compliance and treatment discontinuation (Lieberman et al., 2005; Mailman & Murthy, 2010).

Atypical antipsychotics mainly function as D_2 and 5-HT_{2A} receptor antagonists (Mailman & Murthy, 2010). Their more potent occupancy at 5-HT_{2A} receptors compared to D_2 receptors may be responsible for attenuating extrapyramidal symptoms, thus accounting for some superiority over typical antipsychotics (Carbon et al., 2018). However, accumulating evidence have suggested that with the exception of clozapine, atypical drugs are not more efficacious than their predecessors at treating the symptoms of schizophrenia (Leucht et al. 2013; Lieberman et al., 2005). Aripiprazole, an atypical drug also referred to as a third generation antipsychotic, principally functions as a D_2/D_3 partial agonist and can work under both hypodopaminergia and hyperdopaminergia states. Its partial agonism at 5-HT_{1A} receptor is

associated with improved negative and cognitive symptoms whereas antagonism of 5-HT_{2A} receptors alleviates both psychosis and extrapyramidal symptoms (Keltner & Johnson, 2002). Other third generation antipsychotics are cariprazine and brexpiprazole, of which both also possess partial agonism and/or antagonism activity at dopamine $D_{2/3}$ and 5-HT receptor subtypes (Das et al., 2016; Meltzer, 2013). Akin to aripiprazole, brexpiprazole has low risk of inducing extrapyramidal symptoms (Das et al., 2016). Cariprazine, on the other hand, is known to trigger these symptoms, albeit at a lower cumulative incidence rate compared to patients treated with risperidone (Durgam et al., 2014).

In terms of abnormal glutamatergic activity, metabotropic glutamate receptor agonists and positive allosteric modulators (PAMs; facilitate agonist-mediated metabotropic glutamate receptor activation) have been tested in the hope of advancing to clinical use but without much success (Nicoletti et al., 2015). Kainate, AMPA and NMDA receptors, as well as glycine transporter-1, are potential drug targets (Millan et al., 2014). Given the high localisation of 5-HT receptors in the PFC and hippocampus, which are pivotal for cognitive functions, compounds targeting 5-HT receptors could potentially serve as adjunct therapy for cognition enhancement in schizophrenia (King et al., 2008; Roth et al., 2004). Meanwhile, agents targeting α2 subunit-containing $GABA_A$ receptors, cholinesterase inhibitors and muscarinic receptor agonists have been shown to alleviate symptoms of schizophrenia (Lewis et al., 2005; Raedler et al., 2007). Since nicotine exposure via smoking, nicotine gum, nasal spray or patch seemingly reverses or improves sensory and/or cognitive deficit in schizophrenia, α7 subunit-containing nicotinic receptor agonists and PAMs are currently under clinical investigation as novel therapies (Dineley et al., 2015). With the potential role of neuroinflammation and oxidative stress in schizophrenia, anti-inflammatory agents and antioxidants are intensively researched. There is also ongoing research on hormone-based treatment, e.g. oxytocin (Tomasik et al., 2016; Veerman et al., 2017).

Psychosocial interventions, physical exercise and repetitive transcranial magnetic stimulation have also proven to be useful in preventing relapse and managing symptoms including negative symptoms, leading to improved quality of life (Mueser & McGurk, 2004; Veerman et al., 2017). Finally, with the increase in technology ownership, digital applications to help manage schizophrenia have been developed and trialled (Berry et al., 2019; Thomas et al., 2019). Figure 1.4 illustrates the treatments that are currently available or under development in schizophrenia.

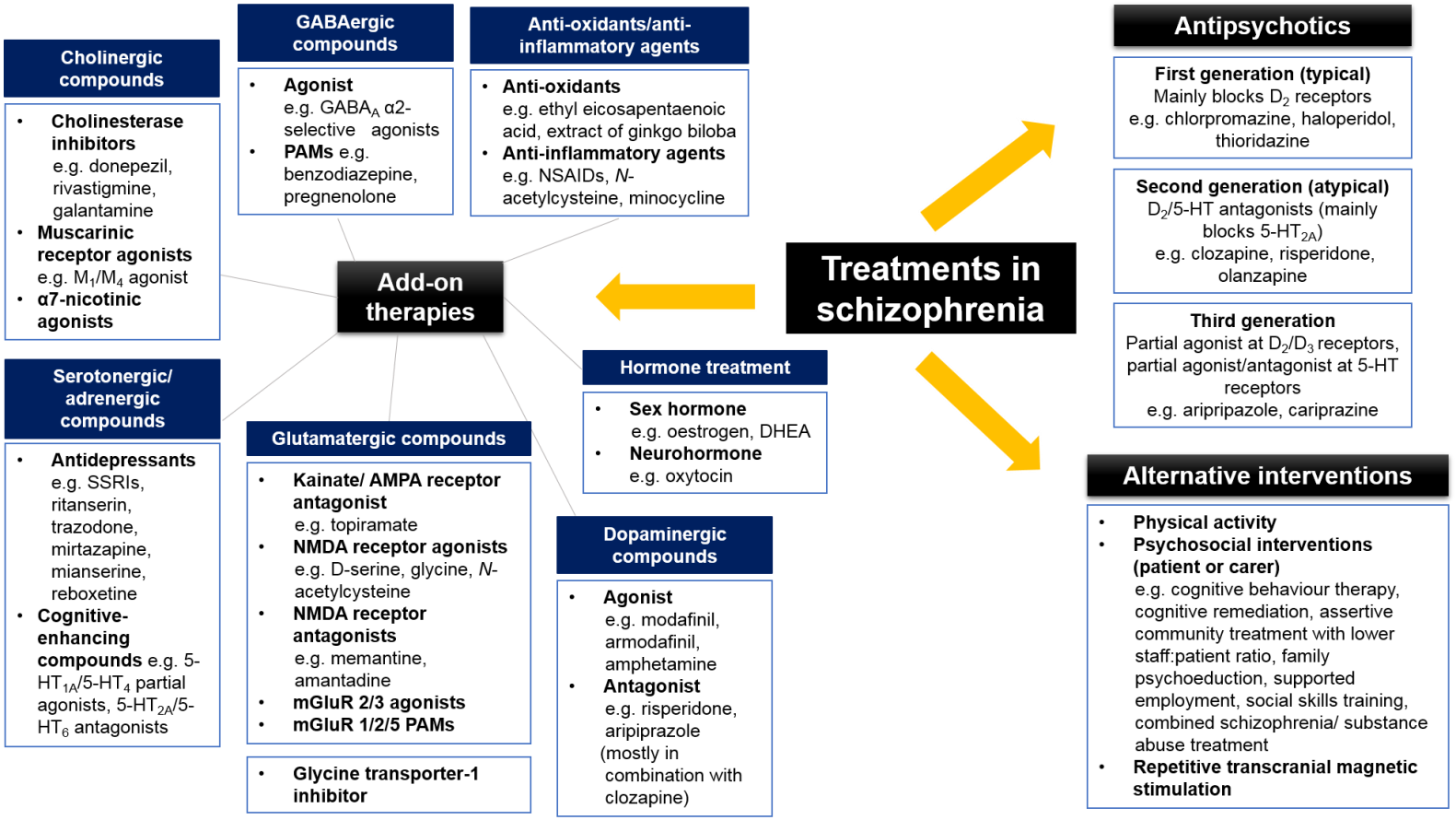


Figure 1.4. Pharmacological and non-pharmacological interventions in schizophrenia. Antipsychotics are primarily used for managing schizophrenia. Various add-on therapies have been investigated for their abilities to further relieve the symptoms of schizophrenia. Non-pharmacological interventions appear to be beneficial in preventing relapses and improving functionality in patients, in addition to providing support for their families or carers. DHEA, dehydroepiandrosterone; GABA, gamma-aminobutyric acid; mGluR, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate; PAMs, positive allosteric modulators; SSRIs, selective serotonin reuptake inhibitors; 5-HT, serotonin.

1.2 Animal models of schizophrenia

Animal models are indispensable preclinical tools that allow rapid monitoring of disease progression compared to in humans, the ability to conduct invasive investigation of disease-associated structural and molecular alterations, and evaluation of novel therapeutics (Jones et al., 2011b). The validity of such models for understanding human psychiatric disorders, however, remains debatable. For example, the lack of biological markers in schizophrenia renders direct assessment of symptoms including hallucinations and thought disorders, which are uniquely human, challenging in animals. Moreover, the translational relevance of biomarker and endophenotype assessments from animal models to schizophrenia might not be clear-cut, given the lack of complete homology between animal and human CNS, as well as insufficient knowledge on the relevance of animal behaviour to human schizophrenia (Canetta & Kellendonk, 2018; Powell & Miyakawa, 2006). Although various genetic and environmental factors (section 1.1.4), as well as theoretical models (section 1.1.5) have been suggested, the precise neurobiology of schizophrenia remains poorly understood, impeding the development of an "ideal" animal model with appropriate face (model exhibits similar symptoms to those observed in schizophrenia), construct (model is created based on the aetiology or theoretical rationale of schizophrenia) and predictive validity (ability of the model to predict the effects of treatments in humans; Jones et al., 2011b; Powell & Miyakawa, 2006). There is no single animal model which is able to reproduce all the behavioural abnormalities in schizophrenia (Gaskin et al., 2014; Howland et al., 2012). Furthermore, there is no available treatment capable of reversing all core symptoms that can serve as a conclusive positive control in animal studies (Jones et al., 2011b).

Albeit the above pitfalls, animal models are not expected to replicate the full symptomatology of disorders, especially in the context of heterogeneous disorders like schizophrenia (Powell & Miyakawa, 2006).

Despite differences in the time scale of brain development, the major events involved as well as gross brain anatomy (e.g. long range projections and neuronal or mechanisms underlying brain functions) can be largely conserved between species (Canetta & Kellendonk, 2018; Semple et al., 2013). Thus, in schizophrenia research, animal models provide useful means to test hypotheses on causative mechanisms underlying particular symptoms or pathophysiological changes, and to determine novel biomarkers that might aid diagnosis and therapeutics development (Canetta & Kellendonk, 2018). Additionally, they enable investigation of the link between potential mechanisms and particular risk factors, or interaction between risk factors, which might not be feasible in human studies (Moran et al., 2016).

Animal models of schizophrenia can be classed as neurodevelopmental, pharmacological or genetic. Neurodevelopmental models, which involve perinatal or early postnatal drug administration or environmental manipulations, are based on the theory that early-life stressors increase the risk of schizophrenia. Besides permitting examination of pathological trajectory from early development to symptom manifestation in adulthood and potentially reproducing developmentally-driven genetic predisposition, these models are superior as experiments can be performed without confounding drug or surgical interventions. Furthermore, they can be used for developing novel multi-target drugs for preventing or managing schizophrenia (Floresco et al., 2005; Jones et al., 2011b; Winship et al., 2019).

Pharmacological models, typically generated via chronic administration of dopamine enhancers (e.g. AMPH) and non-competitive NMDA receptor antagonists (PCP, ketamine, MK-801), in contrast, lack aetiological basis and do not reflect the multifactorial nature of schizophrenia despite being beneficial in improving understanding of the association between specific neurotransmitter system dysfunction and schizophrenia. Moreover, repeated AMPH administration for instance,

does not reliably replicate negative and cognitive symptoms (Floresco et al., 2005; Winship et al., 2019).

While genetic models based on single gene manipulation are useful in enhancing understanding of the genetic mechanisms of schizophrenia, schizophrenia is polygenetic and constitutive knock-out of a particular target gene can trigger neurodevelopmental changes and compensatory mechanisms in the genetic model (Jones et al., 2011b). Additionally, models developed using small-effect allelic variants, e.g. Val/Met SNPs, might not generate schizophrenia-related phenotype and genetic models have limited utility in drug discovery and screening (Jones et la., 2011b; Nestler & Hyman, 2010).

With the increased appreciation that gene x environment interaction is pivotal in schizophrenia development, numerous “multiple-hit” models have been developed, including the risk gene *DISC1* mutation x adolescent cannabis exposure model, to more closely replicate the aetiological basis of schizophrenia (Moran et al., 2016). However, models involving genetic manipulations are typically conducted using mutant mice (Abazyan et al., 2010; O’Leary et al., 2014; Vuillermot et al., 2012). Given the obvious advantages of neurodevelopmental models, the current thesis examines the combination of two neurodevelopmental models, MIA and post-weaning isolation rearing of rats, as an improved model for schizophrenia. Although it can be argued that a “multiple-hit” model established based on both genetic and environmental risk factors would more closely reflect the pathological basis of schizophrenia, schizophrenia is a highly variable disorder and therefore the combination of two environmental risk factors would generate an equally useful model representing a subpopulation of schizophrenia (Powell & Miyakawa, 2006).

1.3 Maternal immune activation

1.3.1 The placenta: the maternal-foetal interface

Bidirectional interactions between the foetus and maternal tissues are crucial for normal foetal growth and development (Reisinger et al., 2015). The highly specialised organ of pregnancy, the placenta, works in conjunction with the foetal membranes and amniotic fluid to fulfil such purposes (Gude et al., 2004). The placenta provides a platform for gaseous exchanges, passage of water as well as the transport and metabolism of nutrients (Gude et al., 2004; Thellin & Heinen, 2003). It also synthesises a range of endocrine, paracrine and/or autocrine factors, including oestrogen, platelet-derived growth factor and cytokines to regulate placental and foetal growth and distant maternal organs (Aagaard-Tillery et al., 2006; Gude et al., 2004; Napso et al., 2018). Moreover, it is instrumental in the acquisition of passive immunity in the foetus by transferring maternal immunoglobulin G through the neonatal Fc receptors expressed on the syncytiotrophoblasts starting from 16 weeks of pregnancy (Saji et al., 1999). Given the vital role of placenta in pregnancy, dysregulation in any aspects in the maternal and foetal milieu, and the bilateral communication between the two would unequivocally have adverse effects on the foetal brain.

1.3.2 The prenatal cytokine hypothesis

Maternal infection of various natures, i.e. viral, bacterial and parasitic, as well as maternal stress, gut microbiome and diet are linked to elevated incidence of schizophrenia, thus suggesting a common pathological mechanism underlying such risk factors (Table 1.1). The prenatal cytokine hypothesis proposes that the inability of the premature foetal immune system to cope with excessive pro-inflammatory cytokine infiltration during MIA, generated through activation of maternal innate and adaptive immunity, causes inflammation in the foetal brain, leading

to abnormal neurodevelopment and eventually schizophrenia (Gilmore & Jarskog, 1997; Kneeland & Fatemi, 2013). The prenatal cytokine hypothesis provides a likely explanation for the link between the above risk factors and schizophrenia, although the exact mechanism underlying such hypothesis remains undetermined. Several mechanisms, which can be classed as indirect infectious or non-infectious, have been proposed to mediate MIA-induced cytokine imbalance in the foetus.

1.3.3 The physiological roles of cytokines

Cytokines are indispensable components of the host defence system that are involved in immunity and inflammation (Turner et al., 2014). Secreted by the immune and non-immune cells in response to environmental stimuli, these biologically active low-molecular weight polypeptides play major roles in immunoregulation (Borish & Steinke, 2003; Meyer et al., 2009a). Cytokines also act as neuromodulators to influence the action, differentiation and survival of neurons whilst their activities are simultaneously regulated by neurotransmitters and neuropeptides released by neurons (Szelényi, 2001). Moreover, constitutive expression of multiple cytokines, including interleukin (IL)-1β and tumour necrosis factor alpha (TNF)-α, and their receptors during foetal brain development in rodents and humans indicates a pivotal role of cytokine signalling in normal brain development (Dame & Juul, 2000; Meyer et al., 2009a; Mousa et al., 1999). These multifunctional intercellular messengers can be defined as pro-inflammatory or anti-inflammatory, or classified based on their target cell and perceived function, or cognate signalling receptors (Meyer et al., 2009a; Turner et al., 2014). Table 1.2 shows the classification of cytokines by their immune responses.

1.3.4 Innate and adaptive immunity

In the innate immunity, the synthesis of cytokines is potently evoked via stimulation of the pattern recognition receptors (PRRs) by pathogen-

Table 1.2. Classification of cytokines by immune response. Cytokines can be classed based on their involvements in adaptive immunity or pro-inflammatory or anti-inflammatory signalling. CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; LIF, leukaemia inhibitory factor; OPN, osteopontin; OSM, oncostatin M; TNF-α, tumour necrosis factor-α; TSLP, thymic stromal lymphopoietin (Adapted from Turner et al., 2014).

Type of immune response	Family	Members
Adaptive immunity	Common Ɣ chain receptor ligands	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21
	Common β chain (CD131) receptor ligands	IL-3, IL-5, GM-CSF
	Shared IL-2β chain (CD122)	IL-2, IL-15
	Shared receptors	IL-13 (IL-13R-IL-14R complex)
		TSLP (TSLPR-IL-7R complex)
Pro-inflammatory signalling	IL-1	IL-1α, IL-1β, IL-1ra, IL-18, IL-33, IL-36α, IL-36β, IL-36Ɣ, IL-36ra, IL-37, IL-1Hy2
	IL-6	IL-6, IL-11, IL-31, CNTF, CT-1, LIF, OPN, OSM
	TNF-α	TNF-α, TNF-β, BAFF, APRIL
	IL-17	IL-17A-F, IL-25 (IL-17E)
	Type I IFN	IFN-α, IFN-β, IFN-ω, IFN-κ, Limitin
	Type II IFN	IFN-Ɣ
	Type III IFN	IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B)
Anti-inflammatory signalling	IL-12	IL-12, IL-23, IL-27, IL-35
	IL-10	IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29

associated molecular patterns (PAMPS) and damage-associated molecular patterns (DAMPS; Borish & Steinke, 2003). The acute innate cytokines, mainly IL-1, TNF-α, IL-6, IL-12, chemokine (C-X-C motif) ligand 8 (previously IL-18), granulocyte-colony stimulating factor and granulocyte macrophage colony stimulating factor, once released, assemble a multitude of immunological factors to drive local inflammation. This can be followed by acute phase systemic reaction, and the initiation of humoral cascades in the case of continued presence of the initial inflammatory stimulus (Holdsworth & Gan, 2015).

In adaptive immunity, antigen recognition and presentation of processed antigen as peptides on the major histocompatibility complex molecules of antigen presenting cells constitute an important step leading to cytokine secretion. The recognition of the antigenic peptides by the T-cell receptors and the association of T-cell receptor CD154 or CD28 with co-

stimulatory proteins on the antigen-presenting cells (e.g. CD40, CD80 and CD86) promote the differentiation of naïve T cells into $CD4^+$ T cells (also T_h cells); Akdis et al., 2016; Holdsworth & Gan, 2015). The innate cytokine in the immediate vicinity dictates the specific differentiation pathway of the activated T cells. Consequently, such T cells can be characterised into distinct phenotypes according to their cytokine secretion profiles, *viz*. T_h1, T_h2, T_h9, T_h17, T_h22, T-follicular helper cells and regulatory T cells. Each subset drives the development of a distinct effector response, which can be cytotoxic, humoral, cell-mediated, allergic or immunosuppressive. Furthermore, T_h17 is associated with autoimmunity whereas T-follicular helper cells with B cell maturation (Akdis et al., 2016; Borish & Steinke, 2003; Holdsworth & Gan, 2015).

1.3.5 Indirect infectious mechanisms of MIA

Three possible indirect infectious mechanisms have been postulated: (i) cross-placenta transfer of maternally-derived cytokines into the foetal compartment, (ii) placental production and secretion of cytokines into the foetal system and (iii) foetal immune system activation.

During an infection, MIA elevates the levels of pro-inflammatory cytokines, disrupting placental barrier integrity enabling passage of maternally-derived cytokines into the foetal bloodstream (Meyer et al., 2009a; Reisinger et al., 2015). However, cross-placenta transfer of cytokines remains obscure and appears to be restricted to specific types of cytokines and is complicated by factors such as gestational stage (Meyer et al., 2009a).

Placental production and secretion of cytokines into the foetal system might explain altered levels of foetal cytokines. PRRs including the TLRs and cytoplasmic-based nucleotide oligomerisation domain-like receptors (NLRs), as well as their cognate co-receptors and accessory proteins are widely expressed in cells of the placenta (Meyer et al., 2009a; Mor et al., 2017). For instance, in the human placenta, the expression of TLRs 1-10

and NLRs, NOD1 and NOD2, has been reported (Costello et al., 2006; Patni et al., 2009). As aforementioned, while placental release of cytokines are determinants of successful pregnancy, stimulation of the placental PRRs during maternal infection could lead to an aberrant perturbation of cytokine homeostasis in the foetal microenvironment. Urakubo et al. (2001) demonstrated increased placental and amniotic fluid cytokines a few hours following administration of the bacterial cell wall component, lipopolysaccharide (LPS), into pregnant rats. Moreover, TNF-α levels were altered in the foetal brain. The placenta has also been unveiled as a major pool of foetal haematopoietic stem cells with equal potential to generate myelo-erythroid and lymphoid cells. Therefore, gestational insults affecting placental integrity might reshape postnatal immunity (Hsiao & Patterson, 2012; Rhodes et al., 2008).

Cytokine imbalance in the foetal system could be a corollary of foetal immune system activation. However, despite its development being initiated early in gestation and is associated with cytokine synthesis, the foetal immune system as a source of cytokine during maternal infection remains debatable as the immune system is relatively immature even at birth (Holt & Jones, 2000). It is also possible that two or more of the above proposed mechanisms work in combination to impact foetal growth. For instance, transplacental crossing of maternal cytokines into the foetal circulation might trigger cytokine production by the foetus (Scola & Duong, 2017).

Despite uncertainties over the mechanisms underlying the prenatal cytokine hypothesis, clinical findings throughout the years have supported cytokine involvement in schizophrenia pathology. For example, in a large birth cohort study, elevated maternal pro-inflammatory IL-8 levels during the second or third trimester of pregnancy were found to induce brain structural alterations relevant to schizophrenia (Ellman et al., 2010). Meanwhile, TNF-α is linked to increased psychosis risk and poorer childhood neurocognitive functioning in offspring (Buka et al., 2001; Ghassabian et al., 2018). Consistent with these, an extensive birth cohort

study conducted in the United States showed that augmented levels of maternal serum levels of anti-inflammatory T_h2 cytokines (IL-4, IL-5 and IL-13) at the time of parturition were linked to lower odds of developing psychosis in later life (Allswede et al., 2016).

1.3.6 Non-infectious mechanisms of MIA

The human gut microbiome, which is dependent upon genetic and environmental factors, harbours a variety of microorganisms including bacteria, archaea, fungi, protozoans, helminth parasites and viruses (Lee & Mazmanian, 2010). Playing an important role in preserving intestinal permeability, the gut flora constitute part of an extended network alongside the enteric nervous system, autonomic nervous system and the neuroimmune and neuroendocrine elements of the CNS, which is collectively termed the 'gut-immune-brain axis' (Rea et al., 2016). This axis is therefore capable of influencing brain and behaviour by converting environmental stimuli into various metabolic, immune and nervous system signals (Delaney & Hornig, 2018). In the context of MIA, it is thought that impaired maternal gut wall during gastrointestinal inflammation, permits the transfer of microbially-derived metabolic substances including LPS into the maternal circulation, thus stimulating PRRs such as TLR4 leading to cytokine production (Delaney & Hornig, 2018). Such maternally-derived cytokines then cross the placental barrier to precipitate enduring neurological outcomes on the foetal brain (Choi et al., 2016; Kim et al., 2017). Moreover, gut microbiota could profoundly influence the balance between pro- and anti-inflammatory responses by affecting regulatory T cell and B cell differentiation, thus reinforcing its potential as a source of immune activation (Delaney & Hornig, 2018; Minakova & Warner, 2018). Alternatively, MIA could prime for altered intestinal integrity and gut microbial composition and metabolome in offspring (Hsiao et al.; 2013). Given that colonisation of the foetal gut by maternal microbiota during birth is necessary for the proper development and functioning of the foetal immune system, modified maternal microbiota by diet and medications such as antibiotics may predispose

the newborn to developing schizophrenia by compromising the various mechanisms of the gut-brain axis (Delaney & Hornig, 2018; Estes & McAllister, 2016; Romano-Keeler & Weitkamp, 2015). In addition, altered gut microbiota in infants born via caesarean section, compared to vaginal delivery, is linked to increased risk of psychosis (O'Neill et al., 2016).

Maternal obesity and metabolic complications including gestational diabetes and maternal hyperinsulinaemia, which are associated with increased exposure of the foetus to nutrients, hormones and inflammatory factors, are linked to heightened risk of schizophrenia and related disorders (Rivera et al., 2015; Schaefer et al., 2000). The chronic low-grade inflammation in maternal obesity, characterised by aberrant cytokine production, modified adipokine profile and stimulation of inflammatory molecules such as the NLR family, also aids the development of maternal insulin resistance and metabolic disorders. Together, these abnormalities promote inflammation in offspring tissue, compromising not just metabolic (e.g. induce hypertrophy of adipocytes and hence facilitating the development of type 2 diabetes), but also cardiovascular and neural functions (Segovia et al., 2014). Growing evidence from animal studies have discerned profound changes in the brain structure and gene expression in the foetuses and offspring of dams fed with high-fat diet, e.g. impaired hippocampal neurogenesis and dysregulated genes in vascular, immune and insulin signalling; the latter is in support of the vascular theory of schizophrenia (Edlow et al., 2016; Hanson & Gottesman, 2005; Tozuka et al., 2009). Various cognitive impairments and behavioural abnormalities of relevance to schizophrenia have also been reported, such as impaired social behaviour and increased anxiety (Buffington et al., 2016; Edlow, 2017; Kang et al., 2014). Offspring of diet-induced obese dams also showed dysbiosis of the gut microbiota, which as aforementioned, can lead to disruption of a myriad of mechanisms of the gut-brain-axis, increasing schizophrenia liability (Buffington et al., 2016; Delaney & Hornig, 2018; Guo et al., 2018). Apart from interfering with 5-HT and dopamine neural circuits, maternal obesity also increases the risk for obstetric complications including gestational

diabetes mellitus, pre-eclampsia and pre-mature birth, which are all risk factors of schizophrenia (Table 1.1).

Stress during pregnancy can trigger immune responses leading to reprogramming of the foetal brain (Hantsoo et al., 2019). As such, proper maintenance of the hypothalamic-pituitary-adrenal (HPA) axis, which governs responses to stress, and its reciprocal relationship with the immune system is critical (Hantsoo et al., 2019; Minakova & Warner, 2018). Stress-induced activation of HPA results in the release of glucocorticoid (corticosterone in rats), a hormone which acts via genomic and non-genomic mechanisms to exert anti-inflammatory effects (Deak et al., 2017; Zen et al., 2011). Increasingly appreciated to also possess pro-inflammatory properties, glucocorticoids modulate both innate and adaptive immunity by coordinating the function of immune cells including dendritic cells, granulocytes, mast cells, monocytes and macrophages (Yeager et al., 2011; Zen et al., 2011). Thus, simultaneous upsurge in cytokine and glucocorticoid levels in the maternal circulation due to stress will undoubtedly upset the intricate glucocorticoid-immune feedback mechanism (Hoffman et al., 2011). Stress is also linked to compromised placental barrier, leading to altered placenta endocrine functions, e.g. reduced third trimester placental vascular endothelial growth factor expression in the labyrinth zone, and downregulation of placental barrier enzymes such as 11β-hydroxysteroid dehydrogenase type 2, which normally limits foetal exposure to excess maternal cortisol. Consequently, increased foetal exposure to cortisol and stress-associated augmentation of maternal catecholamines perturbs foetal growth and HPA axis development, conferring increased risk of schizophrenia (Bronson & Bale, 2016). Figure 1.5 displays the indirect mechanisms of MIA.

1.3.7 Animals models of MIA

Based on epidemiological findings that gestational exposure to infectious pathogens such as influenza, rubella, *toxoplasma gondii* and pneumonia increases the incidence of schizophrenia, a variety of animal models

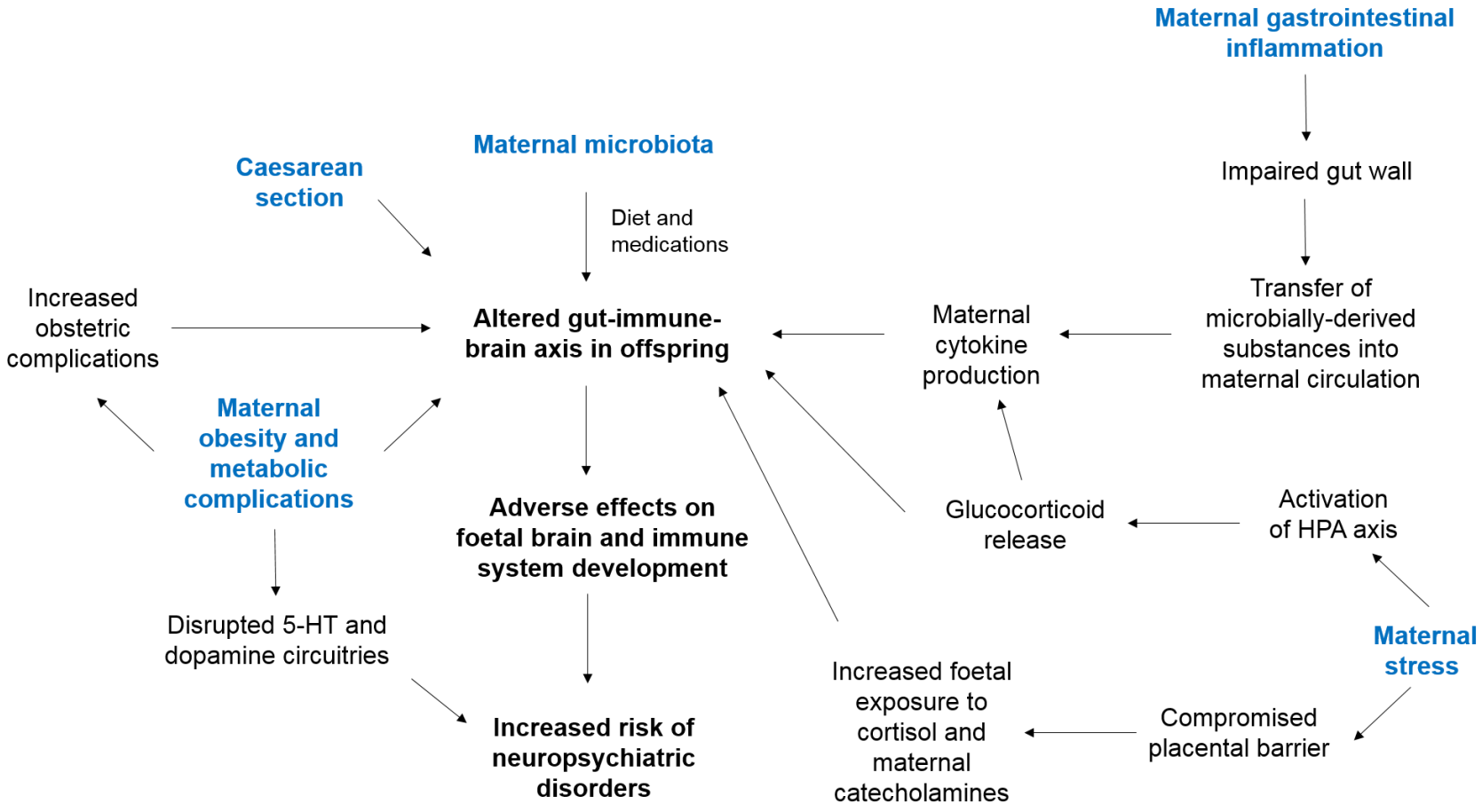


Figure 1.5. Indirect mechanisms of maternal immune activation leading to increased risk of neuropsychiatric disorders. Risk factors such as maternal stress, gastrointestinal inflammation and obesity, altered maternal microbiota and caesarean section are associated with disrupted 'gut-immune-brain' axis in the resultant offspring. This leads to cognitive and behavioural abnormalities, increasing risk of mental disorders including schizophrenia. HPA, hypothalamic-pituitary-adrenal; 5-HT, serotonin.

have been established to substantiate the causality between maternal infection and schizophrenia (Estes & McAliister, 2016; Meyer et al., 2009b; Solek et al., 2018). Gestational exposure to human influenza virus in early or mid-pregnancy has been shown to induce various pathological abnormalities in the developing brains of neonatal mice that is reminiscent of schizophrenia, including increased hippocampal neuronal nitric oxide synthase expression and gliosis, and decreased reelin expression in the cerebral cortex and hippocampus (Fatemi et al., 1998; Fatemi et al., 1999; Fatemi et al., 2002). Whilst live virus-induced MIA would most accurately replicate human viral infection, live viruses are associated with health and safety concerns that restrict their use in laboratory settings (Reisinger et al., 2015). Moreover, a more recent study demonstrated that the presence of viral RNAs was not a requirement to cause schizophrenia-like structural and gene expression abnormalities in the placenta and brain of offspring exposed to prenatal influenza virus (Fatemi et al., 2012). To examine the prenatal cytokine hypothesis, numerous animal models have been established using various pathogen-related immunogens or other immunostimulants (Figure 1.6). Much success have been demonstrated using these models, many reporting altered acute and long-term changes in offspring brain and behaviour that are relevant to schizophrenia (see excellent reviews by Boksa, 2010; Meyer, 2014; Minakova & Warner, 2018; Solek et al., 2018).

LPS and poly(I:C) are the two most widely used agents for stimulating MIA in pregnant rodents (Reisinger et al., 2015). These agents can be easily handled compared to live pathogens and importantly, they induce transfer of maternal plasma or placental cytokines into the foetal unit without crossing the placenta themselves (Ashdown et al., 2006; Reisinger et al., 2015). The former, a gram-negative bacterial cell wall component which activates TLR2 and TLR4, is used for mimicking the innate acute phase response to bacterial infection. Meanwhile, poly(I:C) is used for replicating viral infection (discussed later; Boksa, 2010; Meyer et al., 2009b). Application of LPS or poly(I:C) to animal models does not

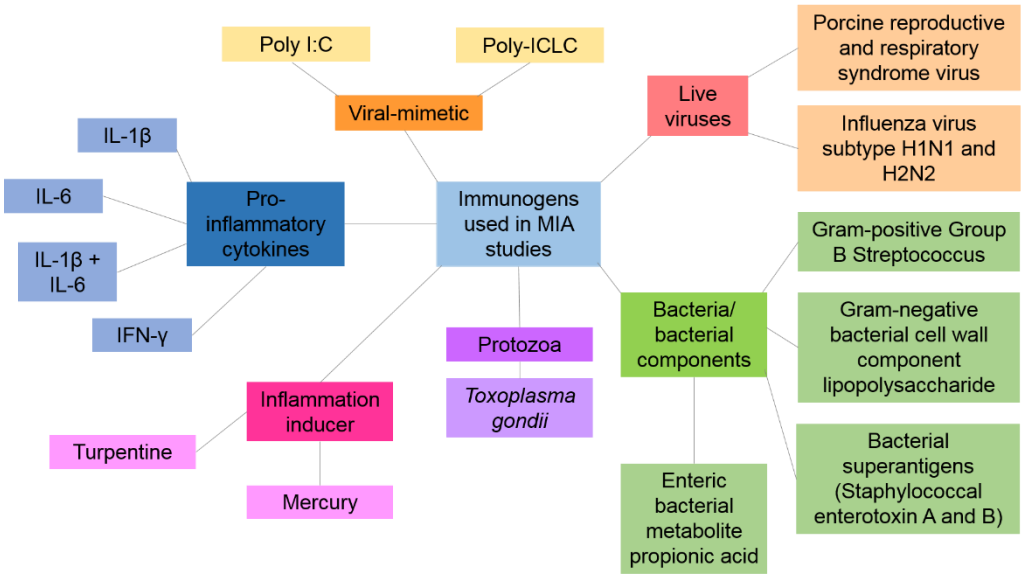


Figure 1.6. Immunogenic substances employed in MIA studies. Several forms of pathogens and pathogen-related immunogens including live viruses, bacteria or bacterial components, protozoans and the viral-mimetic polyinosinic:polycytidylic acid (poly(I:C)) have been used to induce cytokine-induced acute phase reactions in rodents. A modified derivative of poly(I:C), which is generated by complexing poly(I:C) with poly-$_L$-lysine in carboxymethycellulose (poly-ICLC), has lower toxicity and markedly increased resistance to enzymatic degradation than its parent form. It evokes strong interferon production and is used to research the effects of MIA in higher primates such as rhesus monkeys. Inflammatory responses associated with MIA have also been stimulated by directly administrating pro-inflammatory cytokines (IL-1β, IL-6, or a combination of both, and IFN-Ɣ) or inflammatory agents such as turpentine and mercury. Specific examples of each class of immunogen are given. Figure generated based on information from Allard et al. (2017), Bergeron et al. (2013), Caskey et al. (2011), Cotter et al. (1995), Foley et al. (2015), Glass et al. (2019), Kneeland & Fatemi (2013), Levy et al. (1975), Martins et al. (2015), Meyer (2014), Reisinger et al. (2015), Solek et al. (2018).

reproduce the whole time course of bacterial or viral infection, however, it offers the advantage of enabling precise control over the timing and intensity of immunogenic challenge. Factors including route of administration, source, storage and injection conditions, as well as serotype (LPS) or molecular composition (poly(I:C)) can also influence outcome in offspring (Boksa, 2010; Kentner et al., 2019; Mueller et al., 2019). Injections of both immunogens are associated with modified pro- and anti-inflammatory cytokine expression in the placenta, amniotic fluid and the foetal brain, therefore producing similar behavioural and

neurochemical changes relevant to schizophrenia. Nonetheless, appreciable differences have been noted between the immunological profiles of these immunogens (Meyer et al., 2009b; Meyer, 2014; Solek et al., 2018). In a mouse macrophage cell line, poly(I:C) but not LPS potently induces the production of type I interferons (IFNs) whereas LPS causes more robust IL-6 expression compared to poly(I:C) (Kimura et al., 1994). Such differences are thought to explain the differential brain and behavioural effects observed in offspring following prenatal LPS or poly(I:C) exposure (Arsenault et al., 2014; Meyer, 2014). Unlike LPS, poly(I:C) does not induce tolerance with successive daily dosing (Boksa, 2010).

MIA studies have mostly been conducted using rodents (rats and mice). However, more recently, studies have extended into the higher order mammals, such as pigs, rhesus macaque and ferrets (Antonson et al., 2017; Bauman et al., 2014; Li et al., 2018a; Rose et al., 2017). This leap is of significance as the more advanced prenatal corticogenesis in rhesus monkeys compared to rodents might enable better translation of findings to human schizophrenia (Meyer, 2014). The surge in MIA research prompted the inception of the best practice guidelines recommended for this model, to ensure robustness and reproducibility (Kentner et al., 2019).

1.3.8 The poly(I:C) model

Prenatal exposure to different types of maternal infection can have serious consequences on the developing foetal brain, increasing risk of schizophrenia in the unborn child in later life (Table 1.1). A vast amount of literature on infectious diseases in pregnancy has reported adverse pregnancy and perinatal outcomes, including low birth weight, foetal growth restriction and maternal anaemia, which are risk factors of schizophrenia (de Araújo et al., 2018; Rogerson et al., 2018). In the recent “Managing epidemics” publication by the World Health Organisation, eleven out of the fifteen infectious diseases which are deemed potential international threats are caused by viruses, including

Ebola virus disease, Lassa fever, yellow fever, Zika virus infection and seasonal influenza (World Health Organisation, 2018b). Thus, there is a compelling need to understand the association between maternal viral infection and mental disorders such as schizophrenia. As the administration of live viruses into rodents is associated with biosafety concerns (discussed above), the current thesis examines this association using the poly(I:C) MIA model, which mimics viral infection.

1.3.8.1 Poly(I:C), signalling mechanisms and immunogenicity

Double-stranded RNA (dsRNA) is generated during replication of viruses and intracellular bacteria and can also be released by necrotic cells (Chattopadhyay & Sen, 2014). These extracellular or cytoplasmic dsRNAs serve as PAMPS and DAMPS, respectively, which upon recognition by cellular dsRNA-binding proteins such as PRRs, stimulate anti-viral responses in the host by increasing pro-inflammatory cytokines and type I IFNs (IFN α/β) production (Alexopoulou et al., 2001; Chattopadhyay & Sen, 2014; Sprokholt et al., 2017; Tatematsu & Matsumoto, 2014). The activation of the endosomal PRR, TLR3, however, has also been shown to exacerbate infection induced by viruses such as the West Nile virus, thus implying a complex role of PRRs in viral pathogenesis (Wang et al., 2004).

Poly(I:C) is the most potent IFN inducer among synthetic dsRNA analogues, which include the polyanions, polyacrylic acid and polymethacrylic acid (Figure 1.7; De Clercq, 2006). It is capable of activating both innate and adaptive immunity (Huang et al., 2006; Martins et al., 2015; Tatematsu & Matsumoto, 2014), and can trigger the production of type I IFN (particularly IFN-β), cytokines, chemokines and dendritic cell maturation (Matsumoto & Seya, 2008).

Poly(I:C)-mediated antiviral activity has been described both *in vitro* and *in vivo* (Alexopoulou et al., 2001; Field et al., 1967). Akin to virus-derived dsRNA, poly(I:C) mainly acts via TLR3. Poly(I:C) also activates the

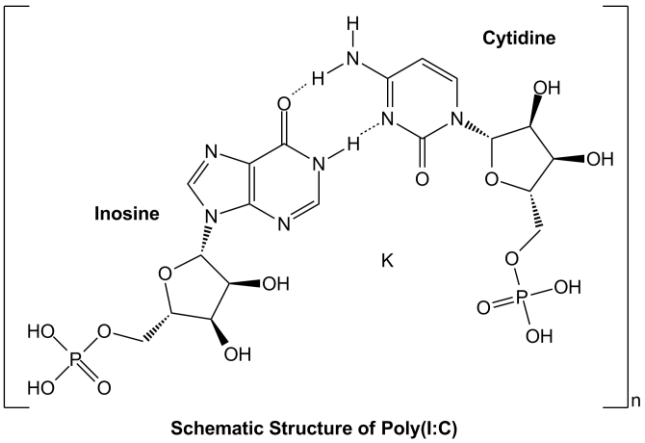


Figure 1.7. Structure of polyinosinic:polycytidylic acid (poly(I:C)). Acquired from AdipoGen Life Sciences website [https://adipogen.com/iax-200-021-polyinosinic-polycytidylic-acid-poly-i-c-endotoxin-free-sterile.html; accessed 1st February 2019].

retinoic acid-inducible gene-I (RIG-I)-like RNA helicases (RLHs), a class of PRRs which sense cytoplasmic dsRNA in myeloid cells, epithelial cells and cells in the CNS (Loo & Gale Jr, 2011). In particular, melanoma-differentiation-associated gene 5 (MDA-5; also HELICARD) and RIG-I (also DDX58) have been implicated whereas the third member of the RLH family, laboratory of genetics and physiology 2, is deemed a dominant-negative regulator of MDA-5 or RIG-1-mediated signalling (Martins et al., 2015; Matsumoto & Seya, 2008; Yoneyama et al, 2005). Although a functionally dominant role of MDA-5 over TLR3 has been postulated for poly(I:C)-triggered type I IFN responses in mice, and that the recognition of poly(I:C) by RIG-I remains debatable, altering the length of poly(I:C) resulted in differential activation of MDA-5 and RIG-I, such that they preferentially recognised the long and short forms of poly(I:C), respectively (Gitlin et al., 2006; Kato et al., 2008; Martins et al., 2015). This knowledge is critical as the inosine poly(I) and cytidine poly(C) strands of poly(I:C) are synthesised enzymatically and therefore each resultant dsRNA can vary substantially in size (Chattopadhyay & Sen, 2014; Kowash et al., 2019). Here, brief summaries of poly(I:C)-induced TLR3 and RIG-I-like signalling are illustrated.

1.3.8.2 Poly(I:C)/TLR3 signalling

In humans, TLR3 is expressed intracellularly in monocyte-derived dendritic cells, $CD11c^+$ blood myeloid dendritic cells, but both extracellularly and intracellularly in fibroblasts and epithelial cells (Matsumoto et al., 2002; Matsumoto et al., 2003).TLR3 has a horseshoe-like ectodomain that is made up of 23 leucine-rich repeats flanked by cysteine-rich N- and C-terminal motifs on each end (Bell et al., 2005; Chattopadhyay & Sen, 2014).

Detection of the 2' hydroxyl groups in the ribose residues in poly(I:C) by TLR3 leads to TLR3 ectodomain homodimerisation, followed by the recruitment of the adaptor protein Toll-IL-1 receptor (TIR) domain-containing adaptor molecule-1 (TICAM-1) to TLR3 cytoplasmic TIR domain (Chattopadhyay & Sen, 2014; Matsumoto & Seya, 2008; Okahira et al., 2005; Tatematsu & Matsumoto, 2014). TICAM-1 binding is dependent on dsRNA-binding induced exposure of the TLR3 cytoplasmic linker region and the subsequent recruitment of the tyrosine kinases epidermal growth factor receptor (EGFR) and Src, which then phosphorylate the tyrosine residues, Tyr^{858} and Tyr^{759}, respectively, in the TLR3 TIR region (Sarkar et al., 2004; Yamashita et al., 2012a). From here, phosphorylated Tyr^{858} leads to TICAM-1 oligomerisation. Such event recruits the serine/ threonine-protein kinase, TANK-binding kinase 1 (TBK1), or IκB kinase-ε (IKKε or IKK-i), which then assembles the transcription factor, interferon regulatory factor 3 (IRF-3), to the N-terminus of TICAM-1. This process is aided by the nucleosome assembly protein, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activating kinase-associated protein 1 (NAP1) and TNF receptor-associated factor 3 (TRAF3; Matsumoto & Seya, 2008). Activation of IRF-3 results in its translocation into the nucleus and subsequently, IFN-β gene transcription (Chattopadhyay & Sen, 2014; Matsumoto & Seya, 2008). Meanwhile, phosphorylated Tyr^{759} activates phosphoinositide 3-kinase (PI3K)/Akt signalling, which overlaps with

TICAM-1 signalling by completing the activation of IRF-3 (Sarkar et al., 2004).

IRF-3 also participates in the complementary NF-κB signalling, which is also heavily reliant upon dual phosphorylation of the same tyrosine residues (Sarkar et al., 2007). Phosphorylated Tyr^{858} is responsible for releasing NF-κB from the repressor I-κB and its translocation to the nucleus, via TICAM-1/TRAF6, TICAM-1/receptor-interacting protein 1 (RIP1) or TICAM-1/transforming growth factor beta-activated kinase 1-mediated activation of IKK complex (Chattopadhyay & Sen, 2014; Matsumoto & Seya, 2008; Sarkar et al., 2007). Phosphorylated Tyr^{759}, on the other hand, is necessary for NF-κB complex p65 phosphorylation, which drives transcription of genes such as A20 or IL-8 (Sarkar et al., 2007).

Finally, TLR3 activity is also coupled with non-genomic cellular effects via an adaptor-independent mechanism preceding TICAM-1 recruitment. Upon binding to the TLR3-EGFR complex, autophosphorylation of Src triggers transient cellular events which are rapidly terminated by sequestration of phospho-Src to lipid rafts. As activating signals for innate immune responses are generally brief, such Src-dependent pathway possibly bridges the gap between innate immunity and cell growth regulation (Yamashita et al., 2012b). A schematic diagram of poly(I:C)/TLR3 signalling is shown in Figure 1.8.

1.3.8.3 Poly(I:C)/RIG-I-like receptor signalling

Poly(I:C) binds to both RIG-I and MDA5, which recognise distinct viral genera (Chattopadhyay & Sen, 2014). Binding of poly(I:C) to the helicase domain of RIG-I exposes the N-terminal caspase activation and recruitment domains for engagement with the adaptor protein mitochondrial antiviral signalling. This is followed by the formation of a signalosome complex comprising TRAF3, TBK1 and IKKε, prompting dimerisation and nuclear entry of the transcription factors IRF-3 and IRF-

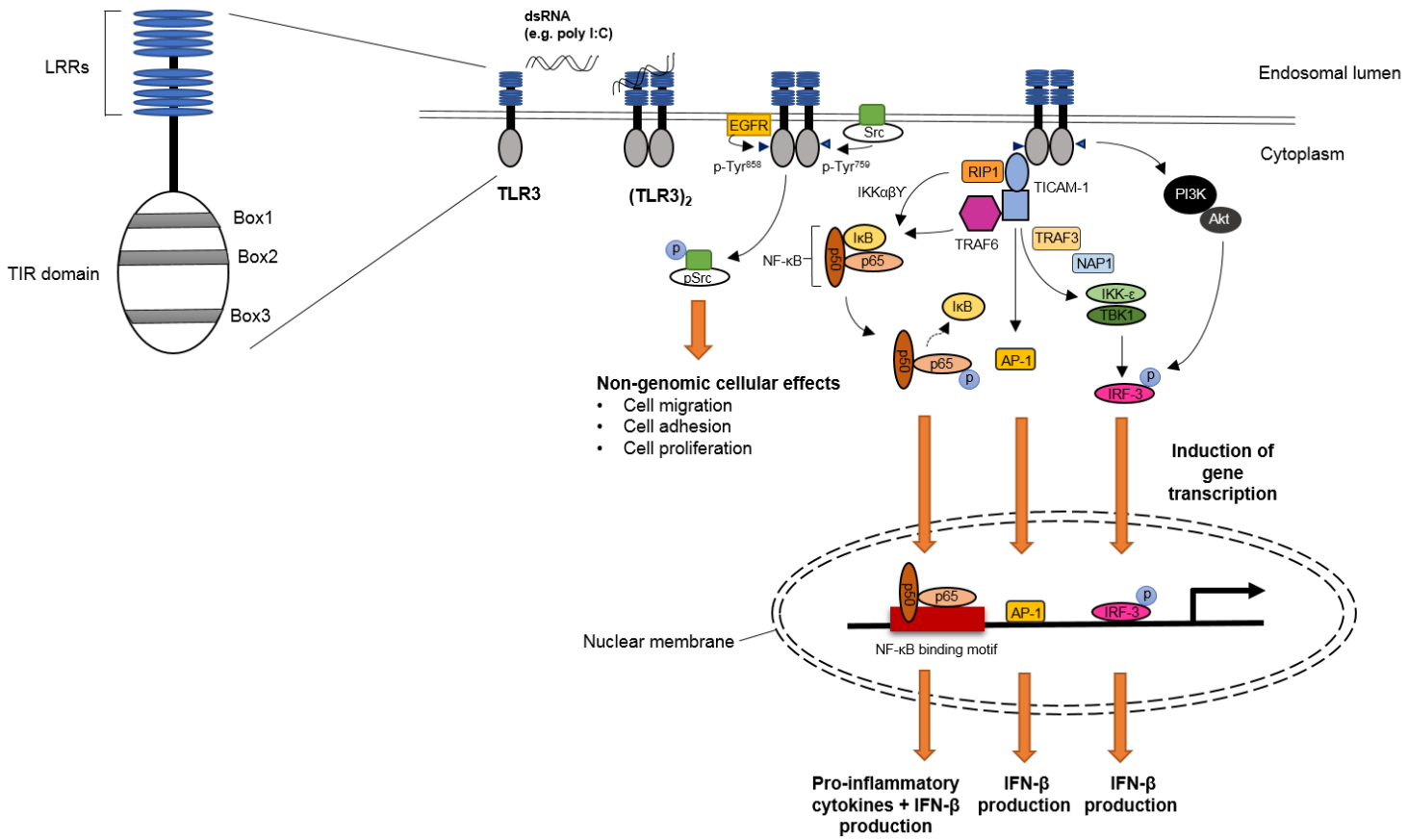


Figure 1.8. Toll-like receptor 3 (TLR3) structure and signalling. A schematic diagram of TLR3 structure and downstream signalling upon recognition of double-stranded RNA (dsRNA) is illustrated. TLR signalling pathways appear to be highly conserved across species (Roach et al., 2005). EGFR, epidermal growth factor receptor; IFN-β, interferon-beta; IKK-ε, IκB kinase-ε; IRF-3, interferon regulatory factor 3; NAP1, NF-κB activating kinase-associated protein 1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; LRRs, leucine-rich repeats; p, phosphorylated; PI3K, phosphoinositide 3-kinase; RIP1, receptor-interacting protein 1; TBK1, TANK-binding kinase 1; TICAM-1, toll-IL-1 receptor domain-containing adaptor molecule-1; TIR, toll-IL-1 receptor; TRAF, TNF receptor-associated factor; Tyr, tyrosine. Figure generated based on information from Akira & Takeda (2004), Chattopadhyay & Sen (2014) and Matsumoto & Seya (2008).

7 and anti-viral gene transcription (Chattopadhyay & Sen, 2014; Loo & Gale Jr, 2011). IRF-3 activation also triggers the pro-apoptotic RIG-I-induced IRF-3-mediated pathway of apoptosis. It seems that viral entry transduces both anti-viral and pro-apoptotic arms of RIG-I-induced IRF-3 signalling. Furthermore, RIG-I activation signals anti-viral and pro-inflammatory defence via IKK/NF-κB and c-Jun N-terminal kinase (JNK)/ activator protein 1 (AP-1) signalling (Chattopadhyay & Sen, 2014; Dey et al., 2011). MDA-5 appears to induce IRF-3-dependent gene transcription similarly to RIG-I (Chattopadhyay & Sen, 2014; Yoneyama et al., 2005). Figure 1.9 shows a simplified diagram of RIG-I downstream signalling following dsRNA detection.

1.3.8.4 Application of poly(I:C) in animal models

Poly(I:C) is widely injected into pregnant rodent females at critical stages of gestation to mimic viral infection and to dissect the effects of such inoculation on development of the resultant offspring. Nevertheless, the physiological and immunological effects of poly(I:C) are species- and strain-specific (Boksa, 2010; Meyer, 2014; Reisinger et al., 2015; Solek et al., 2018). Although the seminal work in this field was performed in mice (Cunningham et al., 2007; Kentner et al., 2019), rats were used in the experiments in this thesis. The use of rats will be justified in Chapter 2. In this section, the application of the poly(I:C) MIA rat model in preclinical schizophrenia research will be described in detail. For an excellent review of the equivalent model in mice, see Meyer & Feldon (2012).

The poly(I:C) MIA model offers several advantages in investigating the neuropathology of schizophrenia. Of paramount importance, the model fulfils at least two of the three criteria for the validity of animal models: the delayed appearance of poly(I:C)-induced behavioural, cognitive and pharmacological abnormalities, which are similar to those seen in human schizophrenia (face validity), corresponds with the post-pubertal clinical onset in humans (construct validity; Meyer, 2014). Whilst the predictive

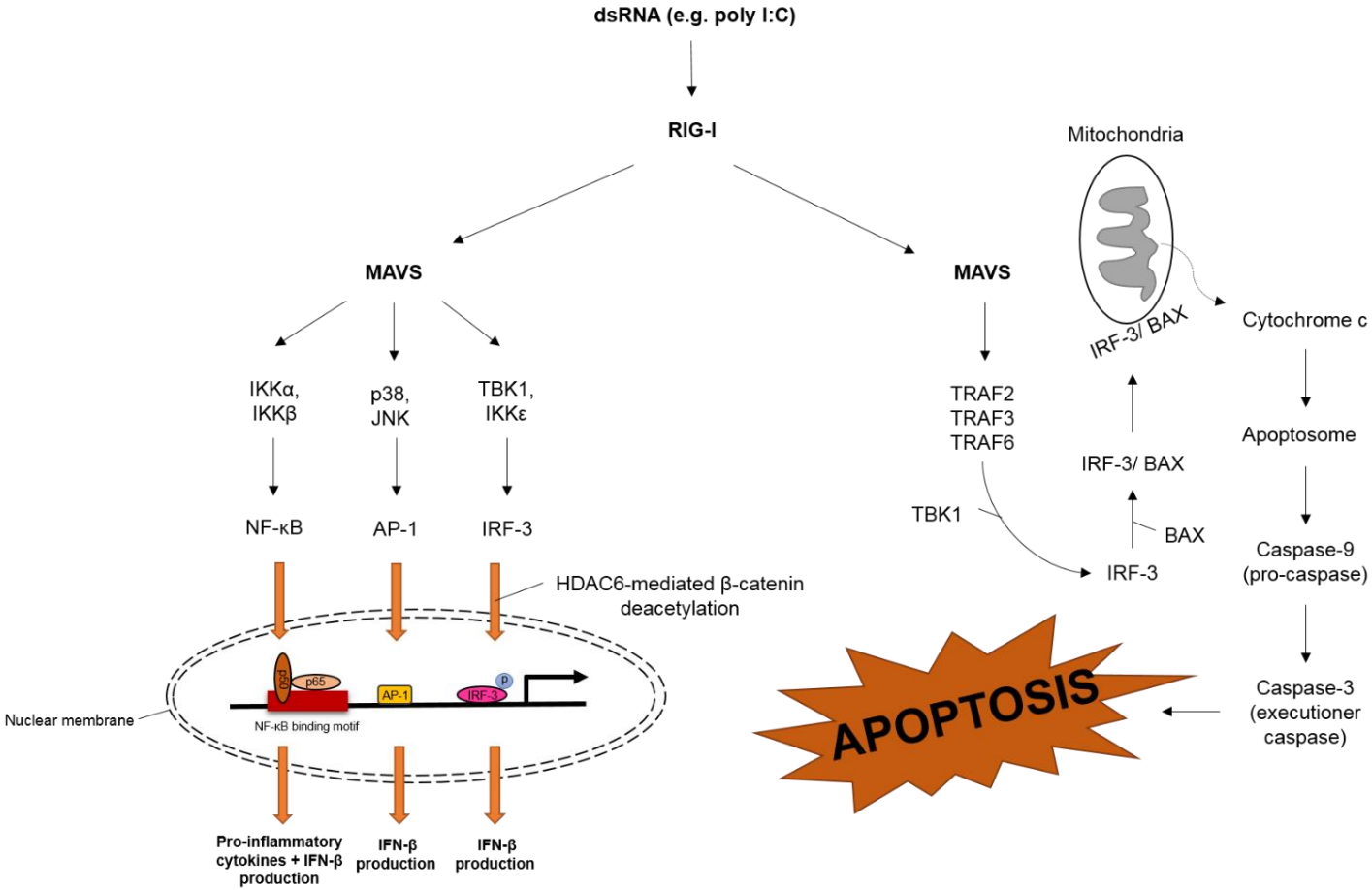


Figure 1.9. dsRNA-activated RIG-I signalling. A schematic diagram of the two branches of MAVS-dependent RIG-I downstream signalling upon recognition of dsRNA is illustrated. HDAC6, histone deacetylase 6; IFN-β, interferon-beta; IKK, IκB kinase; IRF-3, interferon regulatory factor 3; JNK, c-Jun N-terminal kinase; MAVS, mitochondrial antiviral signalling; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RIG-I, retinoic acid-inducible gene-I; RIPA, RIG-I-induced IRF-3-mediated pathway of apoptosis; TBK1, TANK-binding kinase 1; TRAF, TNF receptor-associated factor. Adapted from Chattopadhyay & Sen (2014).

validity of this model cannot be ascertained at the moment owing to the lack of a 'gold-standard' drug capable of reversing all core symptoms of schizophrenia, numerous poly(I:C)-induced behavioural and neurochemical abnormalities appear to be readily normalised by treatment with antipsychotics, including clozapine, risperidone and paliperidone, thus revealing promise for the poly(I:C) model as a preclinical model for schizophrenia (Piontkewitz et al., 2012; Richtand et al., 2012a; Zuckerman & Weiner, 2003). Other advantages and drawbacks of this model, and factors influencing the outcome of this model, have been discussed alongside the LPS model in section 1.3.7.

Effects of poly(I:C) administration on time-mated females

When injected into pregnant females, poly(I:C) induces a range of cytokine-induced sickness behaviour (Dantzer, 2001), which typically resolves within 24-48 h. These include increased production of pro-inflammatory immunological factors, altered body temperature, reduced weight gain and increased plasma corticosterone (Ballendine et al., 2015; Dalton et al., 2012; Gray et al., 2019; Lins et al., 2018). Of note, interferon-stimulated gene-15 (ISG-15) proteins, which have anti-viral activity and induce dendritic impairments, become elevated in the serum (Hu et al., 2019). In terms of overt behaviours, piloerection, hunched posture, lethargy and ptosis (drooping eyelids) have been reported (Clark et al., 2019; Yee et al., 2011). In line with the prenatal cytokine hypothesis, gestational poly(I:C) dosing is linked to increased expression of placental TLR3, pro-inflammatory cytokines and T cell activation marker, as well as reduced placenta weights (McColl & Piquette-Miller, 2019; Murray et al., 2019; Tinsley et al., 2009). Moreover, expression of placental nerve growth factor (NGF), brain-derived neurotropic factor (BDNF) and amino acid transporters was altered (Gilmore et al., 2005; McColl & Piquette-Miller, 2019). There were no discernible effects on maternal licking and nursing behaviour (Zhang et al., 2012; Zhao et al., 2019). Summaries of the acute effects of poly(I:C) on pregnant females are presented in Tables 1.3 (publications available from 2003 to November 2015) and 1.4

(publications available from December 2015 to May 2019; see Chapter 2 for reasons for presenting summaries in two separate tables).

Table 1.3. Acute effects of poly(I:C) administration on pregnant rat dams (2003 – November 2015). As the current thesis defines the day of parturition as gestational day (GD)1, GDs presented in this table were adjusted to match the definition used in this thesis whenever possible. Publications that did not provide a definition for GD are marked with the symbol ^. Abbreviations used are provided at the end of the table.

Strain	Dose, route of administration, injection day	Effect of poly(I:C) on dams (or non-pregnant females if otherwise stated)	Reference
SD	2.5 or 5 mg kg^{-1}, i.p., GD17-18^	24 h post-injection: both doses ↑ plasma IFN-ϒ levels. 5 mg kg^{-1} poly(I:C) also ↑ IL-6 and TNF-α levels. ↓ mRNA expression of placental and hepatic drug transporters, and hepatic transporter protein levels. ↑ total bile acids in plasma.	Petrovic & Piquette-Miller, 2010
SD	8 mg kg^{-1}, i.p., GD15	Weight loss 24 h post-injection.	Bronson et al., 2011
SD	8 mg kg^{-1}, i.p., GD15	Weight loss 24 h post-injection (3 grams or more).	Richtand et al., 2011
SD	8 mg kg^{-1}, i.p., GD15	Weight loss 24 h post-injection.	Richtand et al., 2012a
SD	8 mg kg^{-1}, i.p., GD15	Weight loss 24 h post-injection (3 grams or more).	Richtand et al., 2012b
SD	8 mg kg^{-1}, i.p., GD15	Weight loss 24 h post-injection (average 4 grams).	Roenker et al., 2011
SD	8 mg kg^{-1}, i.p., GD15	Dams divided into two groups based on magnitude of weight change: low-gain and high-gain. Low-gain but not high-gain group gained significantly less weight on GD19 and 21 compared to saline-treated dams.	Vorhees et al., 2012
SD	8 mg kg^{-1}, i.p., GD15	As Vorhees et al. (2012).	Vorhees et al., 2015
WIS	10 mg kg^{-1}, i.p., single or three separate injections (GD14,16,18^)	No observable sickness behaviour (hyperpyrexia, lethargy, hyper-sensitivity and anorexia) following single or repeated administration. 5 h following single poly(I:C) injection on GD18: ↑ plasma monocyte chemoattractant protein-1, unaltered IL-1β or TNF-α levels.	Forrest et al., 2012
WIS	Experiment 1: 10 mg kg^{-1}, i.p., GD14, 16, 18^ Experiment 2: 10 mg kg^{-1}, i.p., GD18 (brain collected from embryo 5 h post-injection)	No observable adverse effects. Trend towards ↓ kynurenine and anthranilic acid levels in plasma and brain.	Khalil et al., 2013

Strain	Dose, route of administration, injection day	Effect of poly(I:C) on dams (or non-pregnant females if otherwise stated)	Reference
WIS, LH, SD	Acute systemetic inflammation (non-pregnant females): 5, 10 or 15 mg kg^{-1}, i.p. Repeated systemic inflammation (non-pregnant females): 2.5, 5 or 10 mg kg^{-1}, i.p., once daily over five consecutive days MIA study: 10 mg kg^{-1}, i.p., GD15	Acute study: At 3 h post-injection, 10 mg kg^{-1} poly(I:C) ↑ body temperature and plasma IL-6 levels in all strains; the latter with lowest spread of data in WIS and fewest non-responders. Thus, WIS was used in further studies. Repeated administration: ↑ body temperature 3 h and 6 h post-injection on all 5 days, and plasma IL-6 levels on first day of dosing only. MIA study: ↓ body weight 6 h and 24 h post-injection. ↑ plasma IL-6 levels 3 h post-injection. No effect on core body temperature 3, 6 or 24 h post-injection and plasma IL-6 levels at PND21.	Murray et al., 2019 (available as abstract at time of literature review)
SD	10 mg kg^{-1}, i.p., GD10, 12, 14, 16, 18^	↑ systolic blood pressure at GD18, urinary protein excretion (indicating endothelial dysfunction), serum IFN-Ɣ and IL-10, and placental TLR3 expression and IFN-Ɣ/IL-10 ratio. ↓ acetylcholine-induced aortic relaxation.	Tinsley et al., 2009
SD	Acute study: 20 mg kg^{-1}, i.p., GD16^ Subacute study: 10 mg kg^{-1}, i.p., GD16^	Acute study: ↑ TNF-α protein levels in plasma and placenta 2 h but not 8 or 24 h post-injection. No effect on TNF-α in amniotic fluid. ↓ nerve growth factor (2 and 24 h) and BDNF (24 h) protein levels in the placenta. Subacute study: Repeated or higher doses resulted in maternal death	Gilmore et al., 2005
WIS	4 mg kg^{-1}, i.v., GD11 or 20	↑ serum IL-6 levels 2 h post-injection.	Duchatel et al., 2016
LE	4 mg kg^{-1}, i.v., GD15^	↓ body weight for 48 h. No effect on body temperature. ↑ serum pro-inflammatory cytokine (IL-1β and TNF) and CXCL1 protein levels 3 h post-injection.	Ballendine et al., 2015
SD	4 mg kg^{-1}, i.v., GD15^	↓ weight gain 24 h post-injection.	Chou et al., 2015b
WIS	4 mg kg^{-1}, i.v., GD15^	No effect on body weight in the four days after injection. ↑ plasma TNF-α, IL-6 and corticosterone protein levels 2 h post-injection, which normalised after 27 days.	Dalton et al., 2012
LE	4 mg kg^{-1}, i.v., GD15^	One dam had bloody discharge from its vagina 24 h post-injection but successfully gave birth to pups. ↓ body weight gain 8, 24 and 48 h post-injection. No effect on body temperature.	Howland et al., 2012
WIS	4 mg kg^{-1}, i.v., GD15	Weight loss for approximately 1 day.	Patrich et al., 2016a
WIS	4 mg kg^{-1}, i.v., GD15	Weight loss for approximately 1 day.	Piontkewitz et al., 2009

Strain	Dose, route of administration, injection day	Effect of poly(I:C) on dams (or non-pregnant females if otherwise stated)	Reference
WIS	4 mg kg^{-1}, i.v., GD15	Weight loss for approximately 1 day.	Piontkewitz et al., 2011
WIS	4 mg kg^{-1}, i.v., GD15	Weight loss for approximately 1 day.	Piontkewitz et al., 2012
LE	4 mg kg^{-1}, i.v., GD15^	↑ weight loss 8 h post-injection, weighed less 24 and 48 h post-treatment. No effect on rectal temperature.	Sangha et al., 2014
WIS	4 mg kg^{-1}, i.v., GD15^	↑ plasma cytokines 2 h post-injection (subset of offspring from dams in study by Dalton et al., 2012).	Verdurand et al., 2014
SD	4 mg kg^{-1}, i.v., GD15^	No effect on mean body weight.	Vernon et al., 2015
SD	4 mg kg^{-1}, i.v., GD15	Trend towards ↓ weight gain.	Wolff & Bilkey, 2010
SD	4 mg kg^{-1}, i.v., GD15	Piloerection and hunched posture were observed but overt behaviour (e.g. coordination, movement) was normal. All dams returned to visibly normal behaviour 4 h post-injection.	Yee et al., 2011
LE	4 mg kg^{-1}, i.v., GD15^	↓ weight gain at 24 and 48 h post-injection. ↑ body temperature at 8 h but not 24 or 48 h. Blanket and passive nursing were infrequently observed on PND2-8. No effect on arch-back nursing/ licking and grooming and off nest time.	Zhang et al., 2012
WIS	4 mg kg^{-1}, i.v., GD15	Weight loss for approximately 1 day.	Zuckerman et al., 2003
WIS	4 mg kg^{-1}, i.v., GD15 or 17, eventually proceeded with GD15 only	Weight loss for approximately 1 day.	Zuckerman & Weiner, 2005
SD	5 mg kg^{-1}, i.v., GD9 (i.p. saline injection 30 min before poly(I:C) injection)	↑ serum IL-10 and TNF-α protein levels 3 h post-injection.	Song et al., 2011
W-H	Dose response study: 2, 4 or 8 mg kg^{-1}, s.c., GD9 or 15^ MIA study: 4 mg kg^{-1}, s.c., GD15^	Dose response study: 4 mg kg^{-1} poly(I:C) induced largest ↑ in blood IL-1β and TNF-α mRNA expression at GD15. No effect on IL-6 and IL-10 at GD9 or 15. MIA study: variable weight responses. Weight loss were accompanied by ↑ serum TNF-α protein levels 6 h post-injection.	Missault et al., 2014

Abbreviations: i.p., intraperitoneal; i.v., intravenous; LE, Long Evans; LH, Lister-hooded; SD, Sprague-Dawley; WIS, Wistar; W-H, Wistar-Hannover; s.c., subcutaneous.

Table 1.4. Acute effects of poly(I:C) administration on pregnant rat dams (December 2015 – May 2019). As the current thesis defines the day of parturition as gestational day (GD)1, GDs presented in this table were adjusted to match the definition used in this thesis whenever possible. Publications that did not provide a definition for GD are marked with the symbol ^. † denotes studies that were reviewed during the literature search in Chapter 3. Abbreviations used are identical to Table 1.3.

Strain	Dose, route of administration, injection day	Effect of poly(I:C) on dams	Reference
WIS	1, 5 or 10 mg kg^{-1}, i.p., GD18^; highest dose used in later experiments.	12 h post-injection: dose-dependent ↑ in serum IL-6 protein levels. ↑ serum TNF-α following 10 mg kg^{-1} poly(I:C). No effect on placental IL-6 and TNF-α levels. 10 mg kg^{-1} poly(I:C): No effect on body weight within the 3 days post-injection and nursing behaviour (nursing, licking and grooming and being out of nest) in the first seven PNDs.	Zhao et al., 2019
SD	10 mg kg^{-1}, i.p., GD14^	↑ serum IL-6 protein concentrations 6 h post-injection, which normalised by 24 h. ↑ placental TNF-α and IL-6 mRNA levels at 6 and 48 h whereas 4F2hc (T-cell activation marker) at 24 h. Altered mRNA and protein expression of various placental amino acid transporters. ↓ branched-chain amino acid concentrations in serum at 48 but not 24 h.	McColl & Piquette-Miller, 2019
WIS	4 mg kg^{-1}, i.v., GD11 or 20	No effect on body weight in the 48 h post-injection. ↑ plasma IL-6 and corticosterone concentrations 2h post-injection.	Meehan et al., 2017 †
WIS	4 mg kg^{-1}, i.v., GD11 or 20	↑ serum IL-6 levels 2 h post-injection.	Duchatel et al., 2018b
SD	4 mg kg^{-1}, i.v., GD15^	This study uses data from Vernon et al. (2015).	Crum et al., 2017
SD	4 mg kg^{-1}, i.v., GD15	Weight loss in the 24 h after injection.	De Felice et al., 2019
WIS	4 mg kg^{-1}, i.v., GD15^	Blood samples were collected at 2 h post-injection for cytokine analysis but data were not shown.	Hollins et al., 2016 †
SD	4 mg kg^{-1}, i.v., GD15	Seven out of forty-three dams were euthanised within 48 h following injection due to hypothermia. Four additional dams experienced body temperature of $<36^0$C but showed alert behaviour. ↓ body weight 8, 24 and 48 h post-injection. No effect on body temperature. ↑ CXCL1 and IL-6 but not CXCL2 or TNF-α protein levels in serum 3 h post-injection.	Lins et al., 2018
SD	4 mg kg^{-1}, i.v., GD15	As Lins et al. (2018).	Lins et al., 2019
SD	4 mg kg^{-1}, i.v., GD15	Weight loss 24 post-injection.	Luchicchi et al., 2016 †
SD	4 mg kg^{-1}, i.v., GD15^	Weight loss in the 24 h after injection.	Millar et al., 2017 †

LE	4 mg kg^{-1}, i.v., GD15^	↑ weight loss 24 h post-injection. No effect on rectal temperature.	Murray et al., 2017
LE	4 mg kg^{-1}, i.v., GD15^	↓ weight gain. No effect on rectal temperature.	Paylor et al., 2016
WIS	5 mg kg^{-1}, i.v., GD15^	↓ temperature and overt symptoms of sickness behaviour that resolved in 24 h. ↑ circulating IL-6 levels 3 h post-injection.	Clark et al., 2019
WIS	5 mg kg^{-1}, i.v., GD15	↑ IL-6 protein levels in plasma collected 2 h post-injection.	Gray et al., 2019
WIS	5 mg kg^{-1}, i.v., GD11 and 20	↑ plasma IL-6 levels 2 h post-injection (GD11 and 20).	Hollins et al., 2018
SD	10 mg kg^{-1}, i.v., GD17	↑ interferon-stimulated gene-15 protein levels in matrix serum 12 h post-injection and on delivery day.	Hu et al., 2019
W-H	4 mg kg^{-1}, s.c., GD15^	Variable weight response. Dams which lost weight were significantly lighter than dams which gained weight.	Missault et al., 2019
WIS	8 mg kg^{-1}, s.c., GD15^	No effect on body weight.	Horska et al., 2017

Effects of gestational poly(I:C) exposure on offspring

Prenatal poly(I:C) exposure is associated with a gamut of behavioural, structural, neurochemical and molecular alterations of translational relevance to schizophrenia later in development. Due to the pace of development in the field, a truly inclusive review is beyond the scope of this thesis. Therefore, only those that are directly pertaining to the experiments presented in Chapters 2 and 3, mainly behavioural effects, will be discussed in detail. Comprehensive summaries of the effects of maternal poly(I:C) injection on resultant litter and offspring can be found in Tables 1.5 & 1.6 (publications available before and after November 2015, respectively).

In rodent preclinical schizophrenia research, stress (i.e. exposure to novel arena) or psychostimulant-induced locomotor hyperactivity is a proxy indicator of enhanced mesolimbic dopamine function that is considered reflective of certain aspects of the positive symptoms, namely psychomotor agitation and grossly disorganised behaviour (Jones et al., 2011b; van den Buuse, 2009). The former can be characterised by examining locomotor responses to novelty whereas the latter by analysing patterns of motor activity (Powell et al., 2009). NMDA receptor

antagonists (MK-801 and PCP) and dopamine receptor agonists (AMPH and apomorphine) are typically used as stimulants in drug-based locomotor activity (LMA) evaluations. Regardless of the context of stimulation, different forms of motor activity are generally taken into account, including horizontal ambulation, rearing and stereotyped behaviours; the last-mentioned typically occur in drug-induced assays at higher doses (Jones et al., 2011b; van den Buuse, 2009). In the poly(I:C) rat model, most studies have found no effect of gestational MIA on offspring spontaneous or novelty-induced LMA (selected papers cited from here onwards; Gray et al., 2019; Richtand et al., 2011; Zuckerman et al., 2003), although a few groups reported increased (Howland et al., 2012; Li et al., 2018b) or blunted (Van den Eynde et al., 2014) activity. The lack of consensus on locomotor responses could be attributed to strain or methodological differences, e.g. maternal poly(I:C) injection at GD15 versus GD17 (Tables 1.5 & 1.6). Early treatment with risperidone, paliperidone, aripiprazole or fluoxetine is capable of rescuing MIA-induced LMA alterations (Richtand et al., 2011; Richtand et al., 2012b).

The novel object discrimination (NOD) task measures visual episodic memory by evaluating the ability of rodents to differentiate between novel and familiar objects (Jones et al., 2011b). A modified version of the task, novel location discrimination (NLD), assesses spatial object memory (Antunes & Biala, 2012). Object recognition is associated with cortex (insular cortex, perirhinal cortex and ventromedial PFC) and albeit controversial, the hippocampus. Location memory, on the other hand, is dependent on dorsal hippocampal CA1 region (Vogel-Ciernia & Wood, 2014). Several studies have found impaired discrimination and reduced total object exploration time in rats prenatally exposed to MIA (Gray et al., 2019; Luchicchi et al., 2016; Osborne et al., 2017; Vernon et al., 2015; Wolff et al., 2011). However, these are contradicted by a study that observed no deficits in NOD performance regardless of sex, but rather male-specific impairment in integration of object and spatial information (Howland et al., 2012).

Social interaction serves as a readout for negative symptoms such as asociality (social withdrawal due to indifference or lack of desire; Neill et al., 2010; Wilson & Koenig, 2014). In rat social interaction, observable behaviour can be defined as social and environmental-directed activities. The former can be further divided into investigative (body and anogenital sniffing) and affiliative behaviours, solicitation and social play, which occur in turn over the course of social interaction. Affiliative behaviour can be active (allogrooming, i.e. cleaning of the partner's fur, or social rest, when being allogroomed by partner) or passive (social inactivity, i.e. rats lying next to each other in close physical contact). Soliciting behaviour, including crawling over or pushing past, is indicative of a 'gaming request' whereas social play, which involves movements such as punching and wrestling, represents the 'game'. Non-social behaviours, e.g. rearing, digging, self-grooming or inactivity (towards conspecific and environment), are also noticeable during normal social interaction. It is important to note that social experience during adolescence, e.g. play behaviour, are crucial for the proper development of social dominance in rats in later life (Chirico et al., 2017). Rats born to poly(I:C)-inoculated dams were found to have reduced sociability or altered social behaviour, such as reduced sniffing and following, compared to vehicle counterparts (Lins et al., 2018; Luchicchi et al. 2016; Osborne et al., 2017). Again, interpretation of findings is complicated by the lack of effect reported by some (Gray et al., 2019).

Measuring social communication in the form of ultrasonic vocalisations (USVs) provides an alternative means to decipher altered social functioning in rodents (Neill et al., 2010). Depending on the surrounding condition and affective state, adolescent and adult rats emit two main types of USVs defined according to frequency and temporal parameters: 22-kilohertz (kHz; in response to aversive stimuli or as alarm calls to warn conspecifics of possible threats) and 50-kHz (non-aversive situations) vocalisations (Portfors, 2007). One study has found poly(I:C)-induced reduction in maternal separation-induced USVs on postnatal day (PND) 11 pups of both sexes (4 mg/kg, i.v., GD15; Chou et al., 2015b). Although

another study observed increased emission of aversive 22-kHz USVs by MIA offspring during fear conditioning (Yee et al., 2012), no studies have examined USVs in a social context.

Intact sensorimotor gating, which occurs in the first few hundred milliseconds before conscious attention, is vital for filtering out redundant or irrelevant incoming sensory input in order to prevent overloading of the higher cortical centres (Cromwell et al., 2008; Jones et al., 2011b). Pre-pulse inhibition of acoustic startle (PPI), a cross-species measure of pre-attentive filtering process, is used to characterise disrupted sensorimotor gating, which is widely impaired in schizophrenia (Cromwell et al., 2008; Jones et al., 2011b; Powell et al., 2009). In this regard, MIA has been consistently shown to impair PPI in resultant rat offspring (Ballendine et al., 2015; Bates et al., 2018; Howland et al., 2012; Wolff & Bilkey, 2010). Furthermore, poly(I:C)-induced PPI suppression has been linked to abnormal neural synchronisation between medial PFC and hippocampus (Dickerson et al., 2010). Prior treatment with minocycline, a tetracycline antibiotic with anti-inflammatory, immunomodulatory and neuroprotective effects (mechanisms include inhibiting key pro-inflammatory enzymes such as inducible nitric oxide synthase and cyclooxygenase-2 and also activation and proliferation of immune cells), was capable of reverting such deficits in adulthood (Garrido-Mesa et al., 2013; Mattei et al., 2014). Multiple studies, however, found no effect of MIA on PPI or startle (Chou et al., 2015b; Gray et al., 2019; Lins et al., 2018). One group reported only dampened basal startle response (Van den Eynde et al., 2014).

Conditioned freezing response (CFR) is a paradigm used for elucidating associative learning, which is impaired in schizophrenia. In this task, a harmless conditioned stimulus (CS) is contingently paired with an aversive unconditioned stimulus (US) that reflexively stimulates unconditioned responses, e.g. fear. Acquisition of such CS-US association evokes CFR in rodents, which could reflect innate fear responses (Kim & Jung, 2006). In rats born to poly(I:C)-treated dams, several groups detected no alterations in fear conditioning to auditory

stimuli, or when a thirst-motivated CFR paradigm was used (Vorhees et al., 2012; Yee et al., 2012; Zuckerman & Weiner, 2005). Notwithstanding, others have reported impaired cued and contextual freezing (Sangha et al., 2014; Vorhees et al., 2015). A study which explicitly assessed the ability of rats to differentiate among cues reflecting fear, reward and safety and hence reflective of behavioural flexibility, revealed impaired freezing response during extinction and extinction recall, thus showing impaired fear regulation (Sangha et al., 2014).

The attentional set-shifting task (ASST), a rodent analogue of the Wincosin card sorting task, is used to assay PFC-mediated attention and cognitive flexibility (Birrell and Brown, 2000; Jones et al., 2011b). Deficits in both set-shifting and reversal learning have been demonstrated in offspring of poly(I:C)-treated dams (Wallace et al., 2014; Zhang et al., 2012). In particular, they showed substantial inability to learn from correct responses during reversal learning (Wallace et al., 2014). When errors are broken down into subtypes, MIA-exposed male offspring are associated with increased perseverative errors during set-shifting and fewer regressive errors during reversal (Zhang et al., 2012). There were no effect of poly(I:C) on perseverative errors throughout reversal (Wallace et al., 2014). Table 1.7 summarises the core behavioural deficits in schizophrenia that are relevant to the work in the current thesis, behavioural tests used for evaluating these deficits in humans and rodent preclinical models, and whether these deficits have been examined in the poly(I:C) model.

Prenatal poly(I:C) insult interferes with offspring early neurodevelopment, as evidenced by perturbed serotonergic neuron development during gestation, as well as altered microglial migration and maturation at PND2 (Ohkawara et al., 2015; Zhang et al., 2019). Changes in brain expression of various proteins, including doublecortin, neuregulin-1, NMDA receptor subunits and BDNF, at particular developmental stages or across development, as well as reduced NGF levels in GD16 foetal liver and spleen, have been characterised (Forrest et al., 2012; Gilmore et al.,

2005; Hemmerle et al., 2015; Khalil et al., 2013). Numerous changes pertaining to neurochemistry and neurotransmitter systems including dopaminergic, serotonergic, glutamatergic and cannabinoid systems, have also been reported (Luchicchi et al., 2016; Ohkawara et al., 2015; Verdurand et al., 2014). In terms of molecular changes, whole-genome miRNA expression analysis revealed altered miRNA expression (including miR-352 and miR-300) in the entorhinal cortex, a key region for cognitive functioning, has been demonstrated (Hollins et al., 2014). Lastly, abnormalities in synaptic transmission, micro-vascularisation, brain arginine and glucose metabolism, kynurenine pathway, perineuronal net, interstitial white matter density, myelination and immune function have been described amongst others (Tables 1.5 & 1.6).

Quantification of blood or brain cytokine levels in offspring could provide insight into MIA-induced neuroinflammatory programming in the offspring. While studies have attempted to characterise gene and protein expression of pro-inflammatory cytokines in MIA offspring during development (foetal liver, spleen, brain), neonatal period (cortex), weaning (plasma), adolescence (plasma, serum, brain) and adulthood (plasma, serum, microglia, brain), equivalent longitudinal studies on cytokine and cytokine receptor levels are lacking (Clark et al., 2019; Duchatel et al., 2018b; Gilmore et al., 2005; Han et al., 2011; Tables 1.5 & 1.6). Only one study has examined the expression of cytokine receptors, TNF-α receptor 1 and 2, in the brain of poly(I:C)-exposed offspring (Mattei et al., 2014).

Table 1.5. Effects of prenatal poly(I:C) exposure on resultant offspring (2003 – November 2015). As the current thesis defines the day of parturition as gestational day (GD)1, GDs presented in this table were adjusted to match the definition used in this thesis whenever possible. Publications that did not provide a definition for GD are marked with the symbol ^. Main abbreviations are provided at the end of the table.

Litter characteristics and offspring postnatal growth			
Strain	Dose, route of administration, injection day	Effect of poly(I:C) (relative to control)	Reference
WIS	4 mg kg^{-1}, i.p., GD15 and GD17 (originally GD15-18)	Three of the four dams could not give birth to living pups when injected daily for 4 consecutive days. When injected every other day (GD15 and 17), all rats gave birth to living pups. Thus, the latter condition was adopted.	Oh-Nishi et al., 2010
SD	8 mg kg^{-1}, i.p., GD15	No malformations or sensorimotor deficits. 6.4% miscarriage rate. **(Adulthood)** ↓ body weight in female offspring of dams with weight loss compared to offspring of dams with weight gain and vehicle-treated dams. No effect on body weight in pubertal and adult male offspring.	Bronson et al., 2011
SD	8 mg kg^{-1}, i.p., GD15	No effect on miscarriage rate, litter size or offspring mortality.	Richtand et al., 2012a
SD	8 mg kg^{-1}, i.p., GD15	No effect on gestation length, litter number, sex ratio within litters, and body weight at PND1, pre-weaning (PND7, 14, 21, 28) and post-weaning (PND35-91).	Vorhees et al., 2012
SD	8 mg kg^{-1}, i.p., GD15-19	No effect on gestation length, litter size, sex ratio within litters, and body weight at PND1, pre-weaning and post-weaning (PND35-91).	Vorhees et al., 2015
SD	10 mg kg^{-1}, i.p., GD10,12,14,16,18^	No effect on litter size. ↑ number of malformed foetuses.	Tinsley et al., 2009
WIS	10 mg kg^{-1}, i.p., GD14,16,18^	No effect on litter size.	Forrest et al., 2012
WIS	10 mg kg^{-1}, i.p., GD14,16,18^	No effect on litter size.	Khalil et al., 2013
WIS	10 mg kg^{-1}, i.p., GD15	No effect on litter size. **GD21:** ↓ placenta weight. No effect on pup body weight or brain weight regardless of gender. **PND21:** ↓ body weight in male offspring but no effect on brain weight; ↑ brain:body weight ratio in male and female offspring. No effect on plasma IL-6 levels.	Murray et al., 2019 (available as abstract at time of literature review)
SD	Subacute study: 10 mg kg^{-1}, i.p., GD16^	Repeated or higher doses resulted in miscarriage (however 20 mg kg^{-1} were used in the acute study).	Gilmore et al., 2005

LE	4 mg kg^{-1}, i.v., GD15^	↓ litter size. No effect on pup weight.	Ballendine et al., 2015
LE	4 mg kg^{-1}, i.v., GD15^	No effect on litter size or pup weight on PND1, 8, 14 or 21 (PND0= day of parturition).	Howland et al., 2012
SD	4 mg kg^{-1}, i.v., GD15^	No effect on body weight (age when weighed not known).	Jing et al., 2013
WIS	4 mg kg^{-1}, i.v., GD15	No effect on miscarriage rate.	Patrich et al., 2016a
WIS	4 mg kg^{-1}, i.v., GD15	No effect on miscarriage rate.	Piontkewitz et al., 2012
LE	4 mg kg^{-1}, i.v., GD15^	No effect on litter size and pup weight from PND1 to PND21 (PND0= day of parturition).	Sangha et al., 2014
WIS	4 mg kg^{-1}, i.v., GD15^	No effect on body weight at adolescence or in adulthood.	Verdurand et al., 2014
SD	4 mg kg^{-1}, i.v., GD15^	No effect on gestation length, litter size and body weight prior to weaning. Slower weight gain post-weaning.	Vernon et al., 2015
LH	4 mg kg^{-1}, i.v., GD15^	Slightly heavier than control animals since birth (not statistically significant).	Wallace et al., 2014
SD	4 mg kg^{-1}, i.v., GD15	No effect on litter size.	Wolff & Bilkey, 2008
SD	4 mg kg^{-1}, i.v., GD15	No effect on litter size.	Wolff & Bilkey, 2010
SD	4 mg kg^{-1}, i.v., GD15	22.7% miscarriage rate. ↓ litter size. ↑ pup weight at PND2 which normalised by PND21. No effect on body weight from PND27 to 68.	Yee et al., 2011
WIS	4 mg kg^{-1}, i.v., GD19^	No effect on gestation length and body weight during PND35-46.	Zavitsanou et al., 2014
LE	4 mg kg^{-1}, i.v., GD15^	No effect on litter size and pup weight on PND1, 8, 14 or 21 (PND0= day of parturition).	Zhang et al., 2012
WIS	4 mg kg^{-1}, i.v., GD15	No effect on miscarriage rate.	Zuckerman et al., 2003
WIS	4 mg kg^{-1}, i.v., GD15	No effect on gestation length or litter size.	Zuckerman & Weiner, 2005
		Behaviour	
Strain/ Sex	Dose, route of administration and injection day	Effect of poly(I:C) on offspring relative to control (age during behavioural testing or at commencement given whenever available)	Reference
SD/M	0.5 mg kg^{-1}, i.p., GD16-19 (daily injections)	**Adolescence (PND35)** No effect on visual ability and spatial learning in MWM; impaired reversal learning.	Han et al., 2011

SD/ M&F	8 mg kg^{-1}, i.p., GD15	**Adulthood (PND56; males only)** No effect on novelty-stimulated LMA. Maternal weight loss: ↓ MK-801-stimulated LMA; ↓ total crossovers for 3 h following MK-801 stimulation. Maternal weight gain: ↑ MK-801-stimulated LMA. **Adulthood (PND90; both genders)** No effect on novelty-stimulated LMA. Maternal weight loss: ↓ AMPH-induced LMA regardless of gender; ↓ total crossovers for 2 h following AMPH stimulation. Maternal weight gain: no effect on AMPH-stimulated LMA.	Bronson et al., 2011
SD/ M&F	8 mg kg^{-1}, i.p., GD15	**Adulthood (3 months old)** No effect on novelty and saline injection-stimulated LMA. **1 mg kg^{-1} AMPH**: ↓ LMA; **5 mg kg^{-1} AMPH**: enhanced transition from initial elevated LMA into stereotypy (i.e. ↓ LMA); more rapid emergence from stereotypy.	Richtand et al., 2011
SD/M	8 mg kg^{-1}, i.p., GD15	**Adulthood (3-8 months old)** No effect on conditioned place preference to 0.5 or 2 mg kg^{-1} AMPH, retention and extinction. ↑ drug-induced reinstatement of preference for drug-paired chamber in rats conditioned to 2 mg kg^{-1} AMPH at 7-8 months old. **~6-7 months old (rats conditioned to 2 mg kg^{-1} AMPH)**: Intact spatial learning and probe trial performance; impaired MWM reversal learning.	Richtand et al., 2012a
SD/ M&F	8 mg kg^{-1}, i.p., GD15	**Adulthood (3 months old)** No effect on novelty or saline injection-stimulated LMA. Attenuated LMA 30-60 min following 1 mg kg^{-1} AMPH injection). Significantly elevated LMA/stereotypy following 5 mg kg^{-1} AMPH injection.	Richtand et al., 2012b
SD/ M&F	8 mg kg^{-1}, i.p., GD15	**Adulthood (PND60-87)** **Maternal high weight gain**: ↓ PPI at 80 dB in females; ↑ errors in Cincinnati water maze; ↓ MK-801-stimualted hyperactivity in the first 10 min. **Maternal low weight gain** ↑ errors in Cincinnati water maze; ↑ AMPH-stimulated hyperactivity 30-50 post-treatment. ↓ MK-801-induced hyperactivity during multiple intervals. No effect on elevated zero maze, open-field LMA, marble burying, straight channel swimming, MWM matching-to-sample, conditioned fear (contextual or cued), latent inhibition, light-dark test, MWM hidden platform acquisition, reversal or shift, or probe trials	Vorhees et al., 2012

SD/ M&F	8 mg kg^{-1}, i.p., GD15-19	**Adulthood (PND65-107)** **<u>Maternal high weight gain</u>**: ↓ startle amplitude in males, ↑ AMPH-stimulated hyperactivity. **<u>Maternal low weight gain</u>**: trend towards↓ startle amplitude and ↑ PPI in females and ↑ PPI in males. Impaired MWM hidden platform acquisition and probe performance. ↓ cued conditioned freezing in males. ↑ AMPH-stimulated hyperactivity (only at the beginning). ↓ MK-801-stimulated hyperactivity. ↓ time spent in dark in the light-dark test regardless of maternal weight response. No effect on elevated zero-maze, open-field LMA, marble burying, straight swimming channel, Cincinnati water maze, MWM matching-to-sample, MWM hidden platform reversal or shift phases or cued testing.	Vorhees et al., 2015
SD/F	4 mg kg^{-1}, i.v., GD13-15^	**Adulthood (>PND94)** ↑ avoidance in the last phase of conditioned avoidance response test (5 mg kg^{-1} clozapine challenge).	Chou et al., 2015a
LE/M	4 mg kg^{-1}, i.v., GD15^	**Puberty and adulthood** ↓ %PPI (PND35-36, PND56-57), impaired associative and crossmodal recognition memory (PND60-80) and altered behavioural flexibility in operant testing (PND80-100).	Ballendine et al., 2015
SD/ M&F or M	4 mg kg^{-1}, i.v., GD15^	**<u>Experiment 1 (males & females)</u>** **Postnatal (PND11)** ↓ maternal separation-induced USVs (significantly ↓ long calls). **Adulthood (PND84)** ↑ AMPH-stimulated LMA. **<u>Experiment 2 (males only)</u>** **Pre-adolescence (PND31-43)** No effect on conditioned avoidance response training, PPI and startle response.	Chou et al., 2015b
SD/M	4 mg kg^{-1}, i.v., GD15^	**Adulthood (~3 months old)** ↓ PPI. No effect on baseline startle reactivity.	Dickerson et al., 2010
WIS/M	4 mg kg^{-1}, i.v., GD15^	**Adolescence and adulthood (PND35, 60, 100)** ↓ %PPI at PND100 but not PND35 and 60.	Hadar et al., 2015

LE/ M&F	4 mg kg^{-1}, i.v., GD15^	**PND0 = day of parturition** **Pre-puberty (PND35 & 36)** M&F: ↓ PPI for trials with 6 and 12 dB pre-pulses and locomotor response to MK-801. Pre-pulse facilitation for 30-ms interval trials (also in controls). No effect on startle amplitudes, PPI for 50-, 80 or 140-ms pre-pulse intervals and novelty-stimulated LMA. **Young adulthood (PND56 & 57)** M&F: ↓ PPI at 3, 6, 12 dB pre-pulse intensities. Pre-pulse facilitation for 30-ms interval trials. ↑ novelty-stimulated LMA. No effect on startle amplitudes, PPI for 50-, 80 or 140-ms pre-pulse intervals and locomotor response to MK-801. **PND60-90** Both genders: ↑ exploration of objects in NOD test phase without effect on memory. No effect on object location recognition. **>PND60** ↑ exploration during sample phase of object-in-place memory task with impaired memory in poly(I:C) males, poly(I:C) females and saline females.	Howland et al., 2012
WIS/ M&F	4 mg kg^{-1}, i.v., GD15	**Adulthood (PND90)** ↓ PPI which was normalised by deep brain stimulation of the mPFC, dorsomedial thalamus and globus pallidus but not the entopenduncularis nucleus or subthalamic nucleus.	Klein et al., 2013
WIS/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (>PND90)** ↓ PPI that was rescued by minocycline treatment.	Mattei et al., 2014
WIS/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (PND90)** Loss of latent inhibition and ↑ AMPH-stimulated LMA. No effect on spontaneous LMA and water maze acquisition.	Piontkewitz et al., 2009
WIS/ M&F	4 mg kg^{-1}, i.v., GD15	**PND70:** Loss of latent inhibition in males. **PND90:** Loss of latent inhibition in both genders. **PND 51, 61, 75, 95:** Spontaneous activity: No effect at PND51, 61 or 75. ↑ in both genders at PND95. AMPH-stimulated activity: no effect at PND51. ↑ and ↓ in males at PND61 and PND75, respectively. ↑ in both genders at PND95.	Piontkewitz et al., 2011

LE/M	4 mg kg^{-1}, i.v., GD15^	**(Age unknown)** Non-discriminative cued paradigm: normal fear responses to cue during conditioning and extinction acquisition. ↑ contextual freezing during extinction recall. Discriminative cued fear, safety and reward conditioning paradigm: Subtly altered fear response during conditioning. ↑ extinction acquisition for both fear and reward cues. Altered response in extinction recall.	Sangha et al., 2014
SD/ M&F	4 mg kg^{-1}, i.v., GD15^	**Adulthood (PND56, 90, 180)** ↓ basal startle response. ↓ spontaneous LMA in both genders. No effect on PPI.	Van den Eynde et al., 2014
SD/M	4 mg kg^{-1}, i.v., GD15^	**Adulthood (PND170)** Impaired discrimination in NOD with ↓ total object exploration time. No effect on novelty-stimulated LMA.	Vernon et al., 2015
LH/M	4 mg kg^{-1}, i.v., GD15^	**Adulthood (3 months old)** Attentional set-shifting task: impaired reversal learning. ↑ correct responses before successfully learning the rule at reversal stages. No effect on simple discrimination, intra-dimensional shift, extra-dimensional shift and number of perseverative errors.	Wallace et al., 2014
SD/M	4 mg kg^{-1}, i.v., GD15	**Adolescence (PND34-35)** No effect on startle reactivity on startle-only or pre-pulse-only trials, habituation of startle response and PPI. **Adulthood (>PND56)** ↓ PPI. No effect of startle reactivity on startle-only or pre-pulse-only trials and habituation of startle response.	Wolff & Bilkey, 2008
SD/M	4 mg kg^{-1}, i.v., GD15	**Adolescence (PND35)** ↓ PPI at 72, 76 and 80 dB. No effect on pre-pulse only and no-stimulus trials. **Adulthood (3 months old)** ↓ PPI at 72, 76, 80 and 84 dB. No effect on pre-pulse only and no-stimulus trials.	Wolff & Bilkey, 2010
SD/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (>3 months old)** NOD task: ↓ total exploration time in test trial. Impaired recognition memory that was not due to impaired object discrimination. Reversal learning: No effect on number of trials to criterion during discrimination training (day 1) and restraining (day 2). Enhanced reversal (day 2). Novel context recognition: ↑ within-trial habituation following contextual manipulation.	Wolff et al., 2011
SD/M	4 mg kg^{-1}, i.v., GD15	**Adulthood** ↓ entries into and time spent in open arms in the elevated plus maze test at PND61. ↓ PPI (76 and 80 dB) at PND68. No effect on startle response.	Yee et al., 2011

SD/M	4 mg kg^{-1}, i.v., GD15	**Early adulthood (PND71 for 3 days)** No effect on overt behaviour (rearing, grooming, defaecation or unconditioned stimulus-induced jumps) during habituation, fear conditioning or fear testing. <u>Changes in affective ultrasonic communication</u>: ↑ 22-kHz USVs after third conditioned stimulus/unconditioned stimulus (CS/US) pairing during fear conditioning. No effect on latency to call, 50-kHz USVs (mainly emitted before first CS/US presentation) and audible calls emitted in response to US application. 22-kHz USVs not observed during habituation or fear testing. ↑ 22-kHz USVs per bout without affecting number of bouts emitted. Most calls were <1 s (*cf.* controls >1 s).	Yee et al., 2012
LE/ M&F	4 mg kg^{-1}, i.v., GD15^	**Adulthood (PND60)** **Operant training**: no effect on days required for fixed-ratio 1 training, retractable lever training, or omitted trials. **Visual-cue discrimination**: no effect on task acquisition. **Strategy set-shift**: ↑ trials to criterion and errors (significantly more perseverative errors) in males. No effect on females. **Reversal learning**: ↓ regressive errors in males. No effect on females.	Zhang et al., 2012
WIS/ M&F	4 mg kg^{-1}, i.v., GD15	**Adolescence (PND35)** No effect on latent inhibition and spontaneous and AMPH-stimulated activity. **Adulthood (3 months old)** Disrupted latent inhibition. ↑ AMPH-stimulated activity. No effect on spontaneous activity.	Zuckerman et al., 2003
WIS/ M&F	4 mg kg^{-1}, i.v., GD15	**Adolescence (PND35)** No effect on latent inhibition in both genders. **Adulthood (3 months old)** Loss of latent inhibition in both genders.	Zuckerman & Weiner, 2003
WIS/M	4 mg kg^{-1}, i.v., GD15 or 17	**Adulthood (3 months old)** Excessive behavioural switching (loss of latent inhibition and rapid reversal learning) and required less time to find repositioned platform in the water maze test. ↑ MK-801-stimulated activity. No effect on fear conditioning, active avoidance, spontaneous activity, water maze acquisition and spatial memory.	Zuckerman & Weiner, 2005

SD/ M&F	5 mg kg^{-1}, i.v., GD9 (i.p. saline injection 30 min before poly(I:C) injection)	**Adulthood (3 months old)** ↓ PPI. Impaired performance in passive avoidance (acquisition and retention) and active avoidance (fewer conditioned responses) tasks.	Song et al., 2011
W-H/M	4 mg kg^{-1}, s.c., GD15^	**Adulthood (PND76-97)** No effect on PPI, basal startle response and spontaneous, AMPH or MK-801-stimulated LMA regardless of maternal body weight change. Maternal weight loss was linked to ↓ sucrose preference in offspring.	Missault et al., 2014
Neurochemical or post-mortem effects			
Strain/ (Sex)	Dose, route of administration and injection day	Effect of poly(I:C)	Reference
SD/M	0.5 mg kg^{-1}, i.p., GD16-19 (daily injections)	**Adolescence (PND35)** ↑ serum TNF-α protein levels 24 h after completion of behavioural tests.	Han et al., 2011
WIS/M	4 mg kg^{-1}, i.p., GD15 and17	**Adolescence (PND28-31)** Dysfunction in basal synaptic transmission and short/long-term synaptic plasticity in hippocampal CA1. ↓ presynaptic proteins (synaptophysin). No increased neuronal loss and apoptosis in the hippocampus.	Oh-Nishi et al., 2010
SD	2.5 or 5 mg kg^{-1}, i.p., GD17-18^	Trend towards increased total bile acids in foetuses harvested 24 h after injection.	Petrovic & Piquette-Miller, 2010
SD/M	8 mg kg^{-1}, i.p., GD15	*In situ* hybridisation **PND14**: ↑ ErbB4 (PFC); ↓ BDNF (ACC); ↑ trkB (PC). **PND30**: ↑ ErbB4 (ACC, frontal and parietal cortices); ↓ ErbB4 (PC). **PND60**: ↓ NRG-1 (PFC); ↑ ErbB4 (ACC); ↑ BDNF (PFC); ↑ trkB (striatum).	Hemmerle et al., 2015
SD/M	8 mg kg^{-1}, i.p., GD15	**Adulthood (PND55-58)** Blunted MK-801-induced increase in extracellular glutamate. ↑ basal extracellular glutamate.	Roenker et al., 2011
WIS/?	10 mg kg^{-1}, i.p., GD9 or 10^	**Adulthood (PND50)** ↓ hippocampal 5-HT levels; no effect on striatal 5-HT, dopamine and their metabolites. **Prenatal (GD15)** ↑ number of serotonergic neurons in the rostral cluster of hindbrains. No effect on the caudal cluster. **Prenatal (GD12 embryos)** No effect on mRNA expression of *Shh*, *Fgf8*, *Gata8*, *Pet-1* in cranial regions.	Ohkawara et al., 2015

WIS/?	10 mg kg^{-1}, i.p., GD14, 16, 18^	**PND21** ↑ VAMP-1 and Sox2, ↓ NMDA receptor GluN1 subunit. No effect on protein expression of synaptophysin, synaptotagmin, small GTPases (RhoA and RhoB), GluN2A, GluN2B, post-synaptic density marker PSD-95, Proliferating Cell Nuclear Antigen, doublecortin and NMDA receptor modulator EphA4.	Forrest et al., 2012
WIS/?	Experiment 1: 10 mg kg^{-1}, i.p., GD14, 16, 18^ Experiment 2: 10 mg kg^{-1}, i.p., GD18 (brain collected from embryo 5 h post-injection)	**PND21 (brain hemispheres)** ↓ sonic hedgehog (SHH) and α-synuclein, ↑ tyrosine hydroxylase (TH). No effect on expression of DISC-1, 5-HT_{2C} receptor, Deleted in Colorectal Cancer (DCC), Uncoordinated-5 protein subtypes Unc5H1 and UncH3, NF-κB, cyclo-oxygenase-2 (COX-2). **GD18 (brains from embryos)** ↑ GluN2B, ↓ doublecortin, ↓ DCC. No effect on expression of NF-κB, COX-2, SHH, PCNA, Unc5H1, UncH3, tyrosine hydroxylase, α-synuclein, DISC-1, EphA4, PSD-95, GluN2A (trend towards decrease). Unaltered levels of tryptophan, kynurenine, kynurenic acid and anthranillic acid.	Khalil et al., 2013
WIS/ M	10 mg kg^{-1}, i.p., GD15	**PND21** ↑ microglia activation in male hippocampi.	Murray et al., 2019 (available as abstract at time of literature review)
SD/?	Acute study: 20 mg kg^{-1}, i.p., GD16^ Subacute study: 10 mg kg^{-1}, i.p., GD16^	Acute study: **Prenatal (GD16)** ↓ TNF-α and NGF protein levels in liver/spleen but not brain. No effect on BDNF and NGF levels in liver/spleen and brain. Subacute study: **1 day after birth** ↓ TNF-α protein levels in neonatal whole brain. No effect on NGF or BDNF. **7 days after birth** No effect on TNF-α, NGF or BDNF protein levels in the neonatal cortex.	Gilmore et al., 2005
WIS/ M&F	4 mg kg^{-1}, i.v., GD11 or 20	**Adulthood (PND84)** White matter of corpus callosum: Trend towards ↑ neuronal nuclear antigen interstitial white matter neuron (IWMN) density when exposed to poly(I:C) at GD20 but not GD11. ↑ somatostatin (SST^+) IWMN density at both GDs with more regionally widespread effects when exposed at GD20.	Duchatel et al., 2016
SD/M	4 mg kg^{-1}, i.v., GD15^	**BrdU injections on PND43 or PND49** No effect on hippocampal cell proliferation or short term cell survival.	Chou et al., 2015b

WIS/M	4 mg kg^{-1}, i.v., GD15^	**Late adolescence/ early adulthood** ↑ plasma TNF-α levels on PND55 (adolescence) but not PND65. No effect on plasma corticosterone and IL-6 levels on PND55 or 65. ↑ in 5-HT_{1A} receptor binding (15-18%) on PND55, 65 and 90.	Dalton et al., 2012
SD/M	4 mg kg^{-1}, i.v., GD15^	**Adulthood (>5 months old)** ↓ mPFC-hippocampus electroencephalogram coherence due to reduced clustering of mPFC firing to hippocampus gamma cycle and altered mPFC phase firing. ↓ firing within a putative population of theta-modulated, gamma-entrained mPFC neurons.	Dickerson et al., 2010
SD/M	4 mg kg^{-1}, i.v., GD15^	**Adulthood (> 5 months old)** Disrupted coherence between mPFC and hippocampus at rest which was rescued by acute clozapine administration.	Dickerson et al., 2012
SD/M	4 mg kg^{-1}, i.v., GD15^	**Adulthood (>3 months old)** ↓ electroencephalogram coherence between mPFC and dorsal hippocampus (not ventral hippocampus) alongside ↓ GAD67 expression within parvalbumin-positive cells without loss of parvalbumin-positive neurons.	Dickerson et al., 2014
WIS/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (PFC collected at PND120)** Dysregulation of core metabolic and myelin-associated proteins, as well as MAPK signalling-related kinases.	Farrelly et al., 2015a
WIS/M	4 mg kg^{-1}, i.v., GD15^	mPFC and hippocampus: abnormal neurochemical and glucose metabolism prior to (i.e. PND35) and with the development of PPI deficits (i.e. PND100). NAcc and globus pallidus: biochemical alterations developed with age.	Hadar et al., 2015
WIS/M	4 mg kg^{-1}, i.v., GD15^	**Late adolescence (brain collected at PND55)** Differential expression of miRNAs predominantly in the entorhinal cortex of the left hemisphere. High proportion of such miRNAs were mapped to chromosome 6q32.	Hollins et al., 2014
SD/M	4 mg kg^{-1}, i.v., GD15^	**Adulthood (3 months old)** ↑ levels of L-arginine (CA1, CA2/3 but not DG, PFC or CE), L-ornithine (CA1, CA2/3, DG, PFC but not CE) and putrescine (DG, PFC but not CA1, CA2/3, CE). ↓ agmatine level (PFC but not CA1, CA2/3, DG or CE). No effect on L-citrulline, glutamate, GABA, spermidine and spermine in all five regions examined.	Jing et al., 2013

WIS/M	4 mg kg^{-1}, i.v., GD15	**Adulthood** ↓ neurogenesis in the DG. ↑ microglia density in NAcc core but no effect in ventral striatum, mPFC, cingulate cortex, DG and CE. ↓ microglia Iba-1 reactivity in the DG and CE. ↑ IL-1β mRNA in hippocampal microglia with no effect on TNF-α mRNA. No effect on cytokine mRNA levels in cerebellar microglia. ↓ TNF-α receptor 2 but not TNF-α receptor 1 expression in hippocampal neuronal progenitor cells.	Mattei et al., 2014
WIS/ M&F	4 mg kg^{-1}, i.v., GD15	Males: ↓ hippocampal excitatory transmission in juvenile period (PND18-20), which persisted in adulthood (PND70 and 90). Females: Abnormalities only emerged in adulthood (PND70). Both genders: ↓ hippocampal size in adulthood. No effect on short-term synaptic plasticity.	Patrich et al., 2016a
WIS/M	4 mg kg^{-1}, i.v., GD15	**Adolescence (PND35)** No effect on lateral ventricle and hippocampal volumes. **Adulthood (PND120)** ↑ lateral ventricle volume, ↓ hippocampal volume.	Piontkewitz et al., 2009
WIS/ M&F	4 mg kg^{-1}, i.v., GD15	**PND35, 46, 56, 70 and 90** ↓ hippocampal volume in both genders at all PNDs except PND35. ↑ lateral ventricle in male offspring from PND56 onwards. No effect on female lateral ventricle at all PNDs. ↓ PFC volume in males and females from PND56 and PND70, respectively: ↓ volume in males at PND70 and 90 whereas females at PND90. ↓ striatal volumes in both genders at all PNDs. No effect on total brain volume in both genders at PND35 and 95.	Piontkewitz et al., 2011

WIS/M	4 mg kg^{-1}, i.v., GD15	**BrdU+ cells generated on PND14-16 or PND34-36 and survived for 21 days** ↓ neurogenesis in DG. **PND37, 57 and 72** ↓ calretinin-positive cells. **PND72 and 100** ↓ parvalbumin-expressing neurons in the entire hippocampus. **PND100** ↓ DG granule cell density in both hemispheres, disturbed micro-vascularisation. No effect on % DG cells double-stained for BrdU and NeuN (assessed PND 72 and 100) and non-neuronal markers GFAP, Galac or OX42 (assessed PND 37, 57, 72 and 100).	Piontkewitz et al., 2012
SD/ M&F	4 mg kg^{-1}, i.v., GD15^	**Adulthood (PND180)** ↑ OX-42 positive intensity of staining and microglial density and ↓ microglial activation in the cortex (motor and somatosensory areas), striatum, corpus collasum, dorsal hippocampus, thalamus and pons. No difference in CD68 receptor (ED-1) staining across the brain regions. Together data suggested non-reactive microglial activation.	Van den Eynde et al., 2014
WIS/M	4 mg kg^{-1}, i.v., GD15^	**Adolescence (PND32-42)** ↓ CB_1R expression in the globus pallidus. **Adulthood (PND75-79)** ↑ CB_1R expression in the sensory cortex and hypothalamus; trend towards ↑ in cingulate cortex.	Verdurand et al., 2014
SD/M	4 mg kg^{-1}, i.v., GD15^	**Adolescence and adulthood (PND50, 100, 180)** ↓ glutathione and taurine at PND180. No effect on N-acetyl-aspartate + N-acetyl-aspartyl-glutamate at any time point.	Vernon et al., 2015
SD/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (PND69)** No effect on adrenal weight and plasma IL-6 and corticosterone concentrations.	Yee et al., 2011
WIS/ M&F	4 mg kg^{-1}, i.v., GD15	**Adolescence (PND42)** No effect on basal and KCl-stimulated [^{3}H]dopamine release in the striatum. **Adulthood** ↑ KCl-induced but not basal [^{3}H]dopamine release in the striatum. Moderate to severe cell loss in hippocampal CA1 and CA3 regions, DG, and entorhinal cortex with neurons exhibiting pyknotic-like profiles.	Zuckerman et al., 2003
WIS/ M&F	4 mg kg^{-1}, i.v., GD15	**Adulthood** Altered hippocampal CA1 and CA3 morphology, with neurons exhibiting a pyknotic-like profile.	Zuckerman & Weiner, 2003

WIS/M	4 mg kg^{-1}, i.v., GD19^	**Pre-adolescence (serum collected at PND31-33)** ↓ kynurenine and kynurenic acid and kynurenine/tryptophan ratio. ↑ quinolinic acid and picolinic acid. No effect on tryptophan and neuroprotective kynurenic acid/kynurenine ratio.	Zavitsanou et al., 2014
W-H/M	4 mg kg^{-1}, s.c., GD15^	**Adulthood (PND90-104)** No effect on Cd11b or Iba-1 microglial activation scores in the frontotemporal cortex, corpus callosum, hippocampus, thalamus, striatum and pons.	Missault et al., 2014

Abbreviations: ACC, anterior cingulate cortex; AMPH, amphetamine; CE, cerebellum; DG, dentate gyrus; F, female; i.p., intraperitoneal; i.v., intravenous; LE, Long Evans; LH, Lister-hooded; LMA, locomotor activity; M, male; mPFC, medial PFC; MWM, Morris water maze; NAcc, nucleus accumbens; NOD, novel object discrimination; PC, piriform cortex; PPI, pre-pulse inhibition of acoustic startle; SD, Sprague-Dawley; USV, ultrasonic vocalisations; WIS, Wistar; W-H, Wistar-Hannover; sc, subcutaneous; ?, gender unspecified.

Table 1.6. Effects of prenatal poly(I:C) exposure on resultant offspring (December 2015 – May 2019). As the current thesis defines the day of parturition as gestational day (GD)1, GDs presented in this table were adjusted to match the definition used in this thesis whenever possible. Publications that did not provide a definition for GD are marked with the symbol ^. **†** denotes studies that were reviewed during the literature search in Chapter 3. Main abbreviations are identical to Table 1.5.

Litter characteristics and offspring postnatal growth or development			
Strain	Dose, route of administration, injection day	Effect of poly(I:C) (relative to control unless otherwise stated)	Reference
WIS	10 mg kg^{-1}, i.p., GD18^	No effect on gestation length, sex ratio, litter size and body weight (PND1 and 21).	Zhao et al., 2019
WIS	4 mg kg^{-1}, i.p., GD15-18 (daily injections)	All dams gave birth to living pups. No effect on litter size.	Oh-Nishi et al., 2016
SD/F	10 mg kg^{-1}, i.p., GD13 or 15	↑ body mass at weaning when exposed to MIA at GD13 compared to GD15 up to 4 weeks post-weaning (~25% difference). Earlier puberty and completion of puberty in rats exposed to MIA at GD13, compared to GD15 and control rats. No effect on length of oestrous cycles.	Cakan et al., 2018
SD	4 mg kg^{-1}, i.v., GD15	No effect on miscarriage rate or litter size. Disrupted pregnancy in 2 out of 15 control dams. Abnormal offspring growth in 2 out of 18 poly(I:C)-treated dams.	De Felice et al., 2019
SD	4 mg kg^{-1}, i.v., GD15	Three dams did not produce viable litters. No effect on pup weight at PND1, 7, 14, 21 (PND0= birth of pups).	Lins et al., 2018
WIS/ M&F	4 mg kg^{-1}, i.v., GD11 or 20	No effect on weight at PND68-72.	Meehan et al., 2017 †

LE	4 mg kg^{-1}, i.v., GD15^	Increased litter size. No effect on pup weight at PND1, 8, 14 and 21 (PND0= day of parturition).	Murray et al., 2017
SD/M	4 mg kg^{-1}, i.v., GD15^	No effect on basic metabolic parameters (body weight, food and water intake (PND56-80; PND0= day of birth).	Osborne et al., 2017
LE	4 mg kg^{-1}, i.v., GD15^	No effect on litter size and pup weight at birth.	Paylor et al., 2016
WIS	5 mg kg^{-1}, i.v., GD15	No effect on litter size, sex ratio within litter and offspring weight (PND21 and 70).	Gray et al., 2019
WIS	8 mg kg^{-1}, s.c., GD15^	No effect on litter size and offspring body weight at PND76 and105.	Horska et al., 2017
		Behaviour	
Strain/ Sex	Dose, route of administration and injection day	Effect of poly(I:C) on offspring relative to control (age during behavioural testing or at commencement given whenever available)	Reference
WIS	10 mg kg^{-1}, i.p., GD18^ (dose selected following dose-response study)	**Pre-puberty (PND27)** No effect on LMA. Anxiety-like behaviour in elevated plus maze test. **Pre-puberty (PND28)** Impaired spatial and reversal learning.	Zhao et al., 2019
WIS/ M&F	4 mg kg^{-1}, i.v., GD11 or 20	**Adulthood (>PND70-84)** ↓ PPI in males regardless of timing of exposure. Impaired working memory in rats exposed to poly(I:C) at GD20 but not GD11. No effect on PPI in females, as well as startle responses and LMA (spontaneous and saline, AMPH- or MK-801-stimulated) in either gender regardless of timing of exposure.	Meehan et al., 2017 †
SD/M	4 mg kg^{-1}, i.v., GD15^	**Adulthood (>3 months old)** ↓ PPI without effect on basal startle response. No effect on learning and performance in the signalled probability sustained attention task, which examined motivation and cognition.	Bates et al., 2018
WIS/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (>PND90)** ↓ PPI and loss of latent inhibition; both were normalised by deep brain stimulation in the mPFC or NAcc.	Bikovsky et al., 2016 †
SD/ M&F	4 mg kg^{-1}, i.v., GD15	**Early adulthood (PND60-70)** ↓ PPI in males but not females. No effect on startle amplitude, latency to peak and startle habituation.	De Felice et al., 2019
SD/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (3 months old)** ↓ overall sensitivity to time in the temporal bisection task (overestimation of time passage). No effect on task acquisition.	Deane et al., 2017
WIS/M	4 mg kg^{-1}, i.v., GD15	To verify the poly(I:C) model, PPI was tested. ↓ PPI (68 and 72 dB pre-pulses).	Hadar et al., 2017
SD/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (between PND56-105)** ↓ sociability. ↑ MK-801-stimulated LMA. Intact tactile object recognition memory but impaired crossmodal memory and oddity discrimination. Facilitated set shifting with ↓ trials to criterion and regressive errors in operant set-shifting. Impaired touchscreen-based reversal learning with ↑ days/trials to criterion, correction trials and errors. No effect on startle and PPI.	Lins et al., 2018

SD/F	4 mg kg^{-1}, i.v., GD15	**Adulthood (between PND56-105)** ↓ sociability. Intact tactile but not visual recognition memory. ↓ oddity preference. No effect on startle, PPI or MK-801-stimulated LMA. Like control offspring, unable to demonstrate crossmodal recognition memory.	Lins et al., 2019
SD/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (PND60-70)** ↓ PPI and social interaction time with conspecific and impaired short-term memory. No effect on startle response, object exploration in NOD familiarisation phase and number of contact with conspecific during social interaction test.	Luchicchi et al., 2016 **†**
SD/M	4 mg kg^{-1}, i.v., GD15^	**Adulthood (>3 months old)** ↑ total response, breakpoint and rewards received during the progressive ratio task (increased motivation to work) due to less sensitivity to the contingency between their behaviour and the resulting outcome. No effect on performance under a random ratio schedule or autoshaping paradigm.	Millar et al., 2017 **†**
LE/M	4 mg kg^{-1}, i.v., GD15^	**Young adulthood (2-3 months old)** No effect on the acquisition of an odour-based nonmatching-to-sample task prior to the odour span task. ↓ span capacity in acquisition and performance in the odour span task thus indicative of impaired working memory.	Murray et al., 2017
SD/M	4 mg kg^{-1}, i.v., GD15^	**PND0= day of birth** **PND72:** impaired recognition memory in the NOD task. **PND78:** ↓ percentage of correct entries in the rewarded T-maze alternation test. No effect on latency to first entry. **PND79:** ↓ social interaction.	Osborne et al., 2017
WIS/ M&F	5 mg kg^{-1}, i.v., GD15	**Adulthood (PND70)** Impaired discrimination in NOD task. ↑ AMPH-stimulated LMA. No effect on elevated plus maze, social interaction (social exploration, grooming and rearing), PPI, acoustic startle, sucrose preference, spontaneous LMA or MK-801-stimulated LMA.	Gray et al., 2019
WIS/ M&F	5 mg kg^{-1}, i.v., GD11 and 20	**Adulthood (PND100)** Anxiety-like behaviour in the elevated plus maze test (↓ time spent in open arms; ↑ time in centre zone and closed arms). No effect on distance travelled.	Hollins et al., 2018
SD/ M&F	10 mg kg^{-1}, i.v., GD9	**Adulthood (PND56)** ↑ LMA. Impaired performance in the passive avoidance and active avoidance tasks. ↓ PPI.	Li et al., 2018b
SD/M	10 mg kg^{-1}, i.v., GD17	**PND30 (PND0= day of birth)** Depression-like behaviour: ↑ immobility time in forced swimming test, ↓ sucrose consumption in sucrose preference test, and ↓ total distance and duration/distance travelled in the centre zone. Unaltered average speed in the open field test.	Hu et al., 2019

W-H	4 mg kg^{-1}, s.c., GD15^	**Adulthood (between PND84-91)** Anxiety-like behaviour: ↓ entry into centre, time spent in centre and % distance travelled in centre in the open field test. No effect on MK-801-stimulated hyperactivity, total distance travelled or velocity in the open field test, startle and % PPI, and sucrose preference.	Missault et al., 2019
Neurochemical or post-mortem effects			
Strain/ (Sex)	Dose, route of administration and injection day	Effect of poly(I:C)	Reference
WIS	1, 5 or 10 mg kg^{-1}, i.p., GD18^; highest dose used in later experiments.	**GD18 (12 h post-injection)** Foetal brain: ↑ IL-6 (10 mg kg^{-1}) and TNF-α (1 and 10 mg kg^{-1}) protein levels. 10 mg kg^{-1} poly(I:C): ↑ IL-6 in hippocampus. ↑ TNF-α in PFC and hippocampus. No effect on cytokine levels in the cerebellum. Thus, this dose was selected. **MIA study: Tissues collected at PND28** Pro-inflammatory activation of M1 microglia potentially promote ↓ hippocampal neurogenesis leading to behavioural deficits (see 'behaviour').	Zhao et al., 2019
WIS/ M	4 mg kg^{-1}, i.p., GD15-18 (daily injections)	↑ serum Ig-κ light chain protein levels at neonatal (PND3) and adulthood (9-16 weeks old). Increased Ig-κ light chain and Ig-λ light chain levels demonstrated in sera of patients with sporadic schizophrenia, thus validating Ig light chain as a potential biomarker for MIA-associated disorders.	Oh-Nishi et al., 2016
SD/M	8 mg kg^{-1}, i.p., GD14^	GAD67 mRNA expression was examined in the PFC, frontal, parietal and piriform cortices, striatum, hippocampus and thalamus. **PND14:** No effect on expression in all regions examined. **PND30:** ↓ expression in hippocampal CA2 region. **PND60:** ↑ expression in DG, CA2 region and thalamic reticular nucleus. ↓ expression in prelimbic cortex.	Cassella et al., 2016
SD/F	10 mg kg^{-1}, i.p., GD13 or 15	**PND21** ↑ number of atretic follicles in rats exposed to MIA at GD13 compared to GD13 controls. No effect on histology of ovary and serum levels of progesterone, follicle stimulating hormone, luteinising hormone, leptin and corticosterone regardless of timing of MIA exposure.	Cakan et al., 2018

SD/ M&F	10 mg kg^{-1}, i.p., GD14^	**Foetal brain collected 6, 24 and 48 h after maternal poly(I:C) injection (GD14-16)** ↑ IL-6 mRNA levels at 6 h whereas 4F2hc (T-cell activation marker) at 48 h. Altered expression of various amino acid transporters (mRNA and protein) and amino acid metabolising enzymes (mRNA), as well as amino acid concentrations.	McColl & Piquette-Miller, 2019
SD/ M&F	4 mg kg^{-1}, i.v., GD15	**Pre-weaning age (PND12-20)** Dopamine neurons in the ventral tegmental area: *Ex vivo* electrophysiology: ↓ firing and paired-pulse facilitation of excitatory and inhibitory synapses in males, hence suggesting ↓ transmitter release. No effect on females. **Adulthood (PND70-90)** *In vivo* electrophysiology: ↓ number of neurons and firing with altered electrophysiological parameters in males. No effect on females.	De Felice et al., 2019
WIS /M&F	4 mg kg^{-1}, i.v., GD11 or 20 (as Duchatel et al., 2016)	**Adulthood (PND72-84)** ↑ *C4* gene expression in the anterior cingulate cortex at GD20 but not GD11; when divided by sex, such ↑ was only observed in males.	Duchatel et al., 2018a
WIS/ M&F	4 mg kg^{-1}, i.v., GD11 or 20	**Adulthood (PND70-84)** No effect on *Iba1*, *Gfap, TNF-α* or *IL-1β* expression in the cingulate cortex regardless of timing of exposure. **Adulthood (PND84)** Subtle alterations in microglia and astrocytes: Exposure at GD20 but not GD11 ↑ microglia density in the white matter of corpus callosum. No effect on microglia density in the cingulate cortex regardless of timing of exposure. Strong trend towards ↑ astrocyte density in the cingulate cortex in GD20 rats. No effect on astrocyte density in the cingulate cortex in GD11 rats, as well as in corpus callosum; the latter regardless of timing of exposure.	Duchatel et al., 2018b
WIS/ M	4 mg kg^{-1}, i.v., GD11 or 20	**Adulthood (tissue collection at PND70-84)** Gene expression of D_1 and D_2 receptors were examined in the NAcc, caudate putamen, substantia nigra and ventral tegmental area. Dopamine transporter and tyrosine hydroxylase expression were examined in the latter two regions: ↑ D_1 receptor mRNA levels in the NAcc when exposed to poly(I:C) at GD11. No other effects were found.	Meehan et al., 2017 †

WIS/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (>PND90)** **Deep brain stimulation (mPFC):** ↑ glucose metabolism in parietal cortex, ventral hippocampus, striatum and NAcc. ↓ metabolism in brainstem and hypothalamus. **Deep brain stimulation (NAcc):** No effect on brain metabolism.	Bikovsky et al., 2016 †
SD/M	4 mg kg^{-1}, i.v., GD15^	**PND50, 100, 180** ↓ volume of several cortical regions, hippocampus, amygdala, striatum, NAcc and lateral ventricles. ↑ volumes of the thalamus, ventral mesencephalon, brain stem and major white matter tracts. Maximal changes between PND50 and 100 with no between-group differences thereafter.	Crum et al., 2017
WIS/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (>PND90)** ↑ microglia density and soma size in hippocampus and NAcc (not in mPFC), as well as MHC II mRNA expression in microglia derived from mPFC (not hippocampus). No effect on cytokine mRNA expression (IL-1β, TNF-α, IL-6) in microglia from hippocampus and mPFC, and Cd11b and Cd54 expression.	Hadar et al., 2017
WIS/M	4 mg kg^{-1}, i.v., GD15^	**Late adolescence (PND55)** No effect on 5-HT_{1A} receptor density and relatively few differentially expressed genes in the left entorhinal cortex.	Hollins et al., 2016 †
WIS/?	4 mg kg^{-1}, i.v., GD15^	**Early adulthood (PND65)** No effect on mRNA and protein levels of epidermal growth factor receptor, ErbB4 and PI3K p110δ in the mPFC, orbital PFC, dorsal striatum and NAcc.	Idrizi et al., 2016 †
SD/M	4 mg kg^{-1}, i.v., GD15	**Adulthood (PND70-90)** ↑ basal extracellular dopamine levels in the NAcc. No effect on NAcc DOPAC levels and mPFC dopamine or DOPAC levels. ↓ number of dopamine neurons in the ventral tegmental area. ↓ discharge activity in putative dopamine neurons (i.e. ↓ percentage of spikes in burst, burst episodes and spikes).	Luchicchi et al., 2016 †
LE/?	4 mg kg^{-1}, i.v., GD15^	**<u>Abnormal perineuronal nets (PNNs):</u>** **Adolescence (PND35)** ↓ perineuronal net (PNN) density in amygdala. **Adulthood (PND90)** ↓ PNN density and % parvalbumin cells with PNN in medial prelimbic cortex. No effect on PNN density, parvalbumin cell density and % parvalbumin cells with PNN in the frontal association cortex and auditory cortex at PND7, 21, 35 and 90. No effect on microglial or astrocytic density in the frontal association cortex, medial prelimbic cortex, amygdala and primary auditory cortex.	Paylor et al., 2016

SD/ M&F	10 mg kg^{-1}, i.v., GD9	**Adulthood (>PND56)** Micro-PET/CT scan and immunohistochemistry: ↑ activated microglia in the PFC and hippocampus.	Li et al., 2018b
SD/ M&F	4 mg kg^{-1}, i.v., GD15	**Altered brain arginine metabolism during neonatal development (PND2)** Forebrain: no effect on L-arginine and downstream metabolites (L-citrulline, L-ornithine, glutamate, glutamine, GABA, glutamate/GABA ratio, glutamine/glutamate ratio, agmatine, putrescine, spermidine and spermine). Frontal cortex: ↓ glutamine/glutamate ratio and ↑ spermidine in females. ↑ spermine in males. No effect on other metabolites. Hippocampus: ↑ spermine in males. No effect on other metabolites. Cerebellum: No changes in metabolite levels.	Zhang et al., 2018a
SD/ M&F	4 mg kg^{-1}, i.v., GD15	**Neonatal (PND2)** ↑ neuronal nitric oxide synthase-positive cells in somatosensory cortex, striatum and hippocampal CA3 and DG, but not FC and hippocampal CA1 region. No effect of sex in any of the regions examined except for striatum, where males had increased staining compared to their treatment-matched females.	Zhang et al., 2018b
SD/ M&F	4 mg kg^{-1}, i.v., GD15	**Neonatal (PND2; same rats used in Zhang et al., 2018b)** Altered microglial migration and maturation: ↓ tangential migration in the corpus callosum and radial migration from corpus callosum into striatum and somatosensory cortex, thus fewer primitive ramified and ramified migrolia in the latter. ↓ tangential migration along hippocampal fissure and velum interpositum and fimbria, and radial migration to hippocampal subregions CA1, CA3 and DG. ↑ amoeboids and/or ↓ ramified microglia in hippocampal fissure, velum interpositum and fimbria.	Zhang et al., 2019
WIS/M	4 mg kg^{-1}, i.v., GD15	**Neonatal (PND0-2)** ↓ intrinsic excitability and spontaneous firing in cultured hippocampal neurons, and ↑ spike frequency adaptation, spontaneous inhibitory post-synaptic currents and amplitude of miniature inhibitory post-synaptic currents. **Adolescence (PND14-16)** ↓ intrinsic excitability in CA1 pyramidal neurons of prepared hippocampal slices.	Patrich et al., 2016b †

WIS/ M	5 mg kg^{-1}, i.v., GD11 and 20	**Autonomic nervous system activation:** Adrenal glands: no effect on tyrosine hydroxylase and pSer40 protein levels on PND7 and PND84. **Gastrointestinal inflammation and integrity disruptions:** **Neonatal (PND7)** Colon: ↑ gene expression of tight junction proteins (Tjp1, Tjp2 and Ocln) and inflammatory marker Il6. No effect on Cldn4, Tnf and Il1b expression. Small intestine: ↑ lymphoid aggregates without architectural damage or inflammation. No effect on protein expression of Tjp2 or the inflammasome markers, NLRP3 and NLRP6. **Young adulthood (PND84)** Colon: ↓ *Tjp2* gene expression. No effect on expression of other gastrointestinal barrier (Tjp1, Ocln and Cldn4) or inflammatory markers (Tnf, Il1b and Il6).	Hollins et al., 2018
WIS/M	5 mg kg^{-1}, i.v., GD15^	**Adolescence (PND35)** **Brain cytokines** Basal mRNA expression: ↑ TNF-α. ↓ IL-4 and IL-10. No effect on IL-1β, IL-6, IFN-Ɣ. Following lipopolysaccharide (LPS) challenge: ↑ IL-1β. ↓ IL-4, IL-6 and IL-10. No effect on TNF-α and IFN-Ɣ. **Brain kynurenine pathway enzymes and metabolites** Basal expression: ↑ indoleamine 2,3-dioxygenase (IDO1) and↓ tryptophan 2,3-dioxygenase (TDO2) mRNA levels. No effect on kynurenine aminotransferase II (KAT II) and kynurenine 3-monooxygenase (KMO) mRNA levels, and kynurenine pathway metabolites. Following LPS challenge: ↓ TDO2 and KATII. No effect on IDO1 and KMO. **Early adulthood (PND60)** **Brain cytokines** No effect on basal mRNA expression. Following LPS challenge: ↓ TNF-α, IL-6, IL-10 and IFN-Ɣ. No effect on IL-1β and IL-4. **Kynurenine pathway enzymes and metabolites** No effect on basal expression. Following LPS challenge: ↓ IDO1, TDO2, tryptophan and kynurenine levels.	Clark et al., 2019

SD/M	10 mg kg^{-1}, i.v., GD17	**1 month old** ↓ total basal dendrite length, basal or apical dendritic crossing and spine density in the PFC neurons. **GD17 (12 h after maternal injection) and PND0 (day of birth)** ↑ serum ISG-15 expression and p-STAT1/STAT1 ratio in the PFC. Activation of STAT1/ISG-15-mediated mechanism likely caused MIA-related depression-like behaviour and dendritic deficits, of which the latter involved suppression of NEDD4 activity.	Hu et al., 2019
W-H	4 mg kg^{-1}, s.c., GD15^	**Adulthood (between PND84-91)** ↑ functional connectivity in the default mode network. ↓ blood-oxygen-level-dependent signal in the striatum and thalamus in response to MK-801 administration. No effect on microstructural alterations, and GluN1 protein expression in the FC, cortex, corpus callosum, striatum, hippocampus and thalamus.	Missault et al., 2019
WIS/M	8 mg kg^{-1}, s.c., GD15^	**Adulthood (serum collected at PND105)** Altered lipid profile: ↑ total cholesterol, low-density lipoprotein and high-density lipoprotein. No effect on triacylglycerides, atherogenic index, atherogenic index of plasma, and levels of adipokines, cytokines and hormones (leptin, ghrelin, glucagon-like peptide 1, glucagon, IL-1, IL-6, TNF-α and fibroblast growth factor 21).	Horska et al., 2017

Table 1.7. Core behavioural deficits in schizophrenia, behavioural tests for assessing such deficits in humans and rodent preclinical models, and whether the rodent tests have been conducted using the poly(I:C) model. For details on all the behavioural tests that were carried out using the poly(I:C) model, see Tables 1.9 and 1.12.

Behavioural deficits in schizophrenia	Relevant behavioural test(s) in humans	Equivalent behavioural test(s) in rodents	Tested in the Poly(I:C) model?	References
Positive symptoms				
Psychomotor agitation	N/A	Locomotor activity in response to novelty or stress	Yes	Jones et al., 2011b; Powell & Miyakawa, 2006
Increased sensitivity to psychostimulants	N/A	Psychostimulant-induced locomotor activity or responsiveness to psychostimulants in other behavioural tasks, e.g. pre-pulse inhibition of acoustic startle	Yes	

Behavioural deficits in schizophrenia	Relevant behavioural test(s) in humans	Equivalent behavioural test(s) in rodents	Tested in the Poly(I:C) model?	References
Negative symptoms				
Social withdrawal and impaired social cognition/ behaviour	N/A	Social interaction, social recognition, social conditioned place preference and social empathy	Yes	Powell & Miyakawa, 2006; Wilson & Koenig, 2014
Deficits in social/ communication skills	Role-play-based social skills assessment, e.g. the Maryland Assessment of Social Competence	Ultrasonic vocalisations	Yes	Dickinson et al., 2007; Wilson & Koenig, 2014
Cognitive symptoms				
Impaired visual learning and working memory	Visual paired comparisons task, n-back task, visual patterns test, Sternberg item recognition task	Novel object discrimination/	Yes	Antunes & Biala, 2012; Song et al., 2013
Impaired spatial memory	Computerised spatial recall task, spatial delayed-response task, spatial working memory task	Novel location discrimination	Yes	Dreher et al., 2001; Park & Holzman 1992
Deficits in sensorimotor gating/pre-attentive processing	Pre-pulse inhibition of acoustic startle, P50 gating	Pre-pulse inhibition of acoustic startle	Yes	Jones et al., 2011b; Powell & Miyakawa, 2006
Impaired associative learning	Fear conditioning	Conditioned freezing response	Yes	Critchley et al., 2002; Jones et al., 2011b
Deficits in executive function (reasoning and problem solving)	Wisconsin Card Sorting Test	Attentional set-shifting	Yes	Jones et al., 2011b

The reproducibility of MIA-associated effects is an important factor to consider when establishing the poly(I:C) model in the laboratory for the first time. Tables 1.8-1.13 present the reproducibility of such effects in resultant offspring.

Table 1.8. Reproducibility of effects of poly(I:C) administration on litter or offspring growth (2003 – November 2015). For each measure, the amount of replication (in percentage) between individual studies and the number of available studies are presented according to the gestational day when dams received poly(I:C) injection.

Gestation, litter and offspring growth				
Measure	**GD**	**Affected? (% of study)**	**Number of study**	**Reference**
Miscarriage rate	GD15	Yes (33%); No (67%)	6	Bronson et al., 2011; Patrich et al., 2016a; Piontkewitz et al., 2012; Richtand et al., 2012a; Yee et al., 2011; Zuckerman et al., 2003
	GD16	No (100%)	1	Gilmore et al., 2005
Gestation length	GD15	No (100%)	3	Vernon et al., 2015; Vorhees et al., 2012; Zuckerman & Weiner, 2005
	GD15-19	No (100%)	1	Vorhees et al., 2015
	GD19	No (100%)	1	Zavitsanou et al., 2014
Pup mortality	GD15-18	Yes (100%)	1	Oh-Nishi et al., 2010
	GD15 and 17	No (100%)	1	Oh-Nishi et al., 2010
	GD15	No (100%)	1	Richtand et al., 2012a
Litter size	GD10, 12, 14, 16, 18	No (100%)	1	Tinsley et al., 2009
	GD14, 16, 18	No (100%)	2	Forrest et al., 2012; Khalil et al., 2013
	GD15	Yes (17%); No (83%)	12	Ballendine et al., 2015; Howland et al., 2012; Murray et al., 2019; Richtand et al., 2012a; Sangha et al., 2014; Vernon et al., 2015; Vorhees et al., 2012; Wolff & Bilkey, 2008; Wolff & Bilkey, 2010; Yee et al., 2011; Zhang et al., 2012; Zuckerman & Weiner, 2005
	GD15-19	No (100%)	1	Vorhees et al., 2015
Sex ratio within litters	GD15	No (100%)	1	Vorhees et al., 2012

	GD15-19	No (100%)	1	Vorhees et al., 2015
Malformations	GD10, 12, 14, 16, 18	Yes (100%)	1	Tinsley et al., 2009
	GD15	No (100%)	1	Bronson et al., 2011
Pup/offspring body weight	GD15	Yes (33%); No (67%)	12	Ballendine et al., 2015; Bronson et al., 2011; Howland et al., 2012; Jing et al., 2013; Murray et al., 2019; Sangha et al., 2014; Verdurand et al., 2014; Vernon et al., 2015; Vorhees et al., 2012; Wallace et al., 2014; Yee et al., 2011; Zhang et al., 2012
	GD15-19	No (100%)	1	Vorhees et al., 2015
	GD19	No (100%)	1	Zavitsanou et al., 2014
Placenta weight (at birth)	GD15	Yes (100%)	1	Murray et al., 2019
Brain weight (at birth and weaning)		No (100%)		
Brain:body weight ratio in offspring (weaning)		Yes (100%)		
Plasma IL-6 levels (weaning)		No (100%)		

Table 1.9. Reproducibility of effects of poly(I:C) administration on offspring behaviour (2003 – November 2015). For each behaviour, the amount of replication (in percentage) between individual studies and the number of available studies are presented according to the gestational day when dams received poly(I:C) injection.

Offspring behaviour				
Behavioural test	**GD**	**Affected? (% of study)**	**Number of study**	**Reference**
Locomotor changes				
Spontaneous LMA	GD15	Yes (33%); No (67%)	6	Missault et al., 2014; Piontkewitz et al., 2009; Piontkewitz et al., 2011; Van den Eynde et al., 2014; Zuckerman et al., 2003; Zuckerman & Weiner, 2005
	GD17	No (100%)	1	Zuckerman & Weiner, 2005

Novelty-stimulated LMA	GD15	Yes (20%); No (80%)	5	Bronson et al., 2011; Howland et al., 2012; Richtand et al., 2011; Richtand et al., 2012b; Vernon et al., 2015
Saline injection-stimulated LMA	GD15	No (100%)	2	Richtand et al., 2011; Richtand et al., 2012b
AMPH-stimulated LMA	GD15	Yes (89%); No (11%)	9	Bronson et al., 2011; Chou et al., 2015b; Missault et al., 2014; Piontkewitz et al., 2009; Piontkewitz et al., 2011; Richtand et al., 2011; Richtand et al., 2012b; Vorhees et al., 2012; Zuckerman et al., 2003
	GD15-19	Yes (100%)	1	Vorhees et al., 2015
MK-801-stimulated LMA	GD15	Yes (80%); No (20%)	2	Bronson et al., 2011; Howland et al., 2012; Missault et al., 2014; Vorhees et al., 2012; Zuckerman & Weiner, 2005
	GD17	Yes (100%)	1	Zuckerman & Weiner, 2005
	GD15-19	Yes (100%)	1	Vorhees et al., 2015
MK-801-stimulated LMA	GD15	Yes (100%)	2	Howland et al., 2012; Zuckerman & Weiner, 2005
	GD17	Yes (100%)	1	Zuckerman & Weiner, 2005
Learning and memory/motivation				
NOD	GD15	Yes (67%); No (33%)	3	Howland et al., 2012; Vernon et al., 2015; Wolff et al., 2011
NLD	GD15	No (100%)	1	Howland et al., 2012
Associative (object-in-place) recognition memory	GD15	Yes (100%)	2	Ballendine et al., 2015; Howland et al., 2012
Crossmodal recognition memory	GD15	Yes (100%)	1	Ballendine et al., 2015
Novel context recognition	GD15	Yes (100%)	1	Wolff et al., 2011
Position discrimination and reversal (T-maze)	GD15	Yes (100%)	2	Wolff et al., 2011; Zuckerman & Weiner, 2005
	GD17	Yes (100%)	1	Zuckerman & Weiner, 2005
Fear response	GD15	Yes (33%); No (67%)	1	Sangha et al., 2014; Vorhees et al., 2012; Zuckerman & Weiner, 2005
	GD15-19	Yes (100%)	1	Vorhees et al., 2015

	GD17	No (100%)	1	Zuckerman & Weiner, 2005
Latent inhibition	GD15	Yes (83%); No (17%)	6	Piontkewitz et al., 2009; Piontkewitz et al., 2011; Vorhees et al., 2012; Zuckerman et al., 2003; Zuckerman & Weiner, 2003; Zuckerman & Weiner, 2005
	GD17	Yes (100%)	1	Zuckerman & Weiner, 2005
Attentional set-shifting: simple discrimination, intra-dimensional shift, extra-dimensional shift and number of perseverative errors	GD15	No (100%)	1	Wallace et al., 2014
Attentional set-shifting: reversal learning		Yes (100%)		
Cincinnati water maze (egocentric learning)	GD15	Yes (100%)	1	Vorhees et al., 2012
	GD15-19	No (100%)	1	Vorhees et al., 2015
Straight channel swimming (control procedure for Cincinnati water maze)	GD15	No (100%)	1	Vorhees et al., 2012
	GD15-19	No (100%)	1	Vorhees et al., 2015
Conditioned avoidance response	GD9	Yes (100%)	1	Song et al., 2011
	GD13-15	Yes (100%)	1	Chou et al., 2015a
	GD15	No (100%)	2	Chou et al., 2015b; Zuckerman & Weiner, 2005
	GD17	No (100%)	1	Zuckerman & Weiner, 2005
Operant-based task	GD15	Yes (100%)	2	Ballendine et al., 2015; Zhang et al., 2012
Conditioned place preference - acquisition, retention and extinction	GD15	No (100%)	1	Richtand et al., 2012a
Conditioned place preference - drug-induced reinstatement		Yes (100%)		
Learning and memory: Morris water maze				

MWM: spatial acquisition	GD15	No (100%)	4	Piontkewitz et al., 2009; Richtand et al., 2012a; Vorhees et al., 2012; Zuckerman & Weiner, 2005
	GD15-19	Yes (100%)	1	Vorhees et al., 2015
	GD16-19	No (100%)	1	Han et al., 2011
	GD17	No (100%)	1	Zuckerman & Weiner, 2005
MWM: reversal learning	GD15	Yes (67%); No (33%)	3	Richtand et al., 2012a; Vorhees et al., 2012; Zuckerman & Weiner, 2005
	GD15-19	No (100 %)	1	Vorhees et al., 2015
	GD16-19	Yes (100%)	1	Han et al., 2011
	GD17	Yes (100%)	1	Zuckerman & Weiner, 2005
MWM procedure: cued testing	GD15-19	No (100 %)	1	Vorhees et al., 2015
MWM: probe trials (reference memory)	GD15	No (100%)	1	Vorhees et al., 2012
	GD15-19	Yes (100%)	1	Vorhees et al., 2015
MWM: visible platform task (visual ability)	GD16-19	No (100%)	1	Han et al., 2011
Matching-to-sample (MWM-MTS; working or trial-dependent learning and memory)	GD15	No (100%)	1	Vorhees et al., 2012
	GD15-19	No (100 %)	1	Vorhees et al., 2015
Ultrasonic vocalisations				
Maternal separation-induced USVs	GD15	Yes (100%)	1	Chou et al., 2015b
Affective ultrasonic communication	GD15	Yes (100%)	1	Yee et al., 2012
Sensorimotor gating				
PPI	GD9	Yes (100%)	1	Song et al., 2011
	GD15	Yes (71%); No (29%)	14	Ballendine et al., 2015; Bronson et al., 2011; Chou et al., 2015b; Dickerson et al., 2010; Hadar et al., 2015; Howland et al., 2012; Klein et al., 2013; Mattei et al., 2014; Missault et al., 2014; Van den Eynde et al., 2014; Vorhees et al., 2012; Wolff & Bilkey, 2008; Wolff & Bilkey, 2010; Yee et al., 2011
	GD15-19	Yes (100%)	1	Vorhees et al., 2015

Startle amplitude	GD15-19	Yes (100%)	1	Vorhees et al., 2015
Startle response	GD15	Yes (14%); No (86%)	7	Chou et al., 2015b; Dickerson et al., 2010; Howland et al., 2012; Missault et al., 2014; Van den Eynde et al., 2014; Wolff & Bilkey, 2008; Yee et al., 2011
Habituation of startle response	GD15	No (100%)	1	Wolff & Bilkey, 2008
Anxiety				
Open-field LMA	GD15	No (100%)	1	Vorhees et al., 2012
	GD15-19	No (100%)	1	Vorhees et al., 2015
Elevated zero maze	GD15	No (100%)	1	Vorhees et al., 2012
	GD15-19	No (100%)	1	Vorhees et al., 2015
Marble burying	GD15	No (100%)	1	Vorhees et al., 2012
	GD15-19	No (100%)	1	Vorhees et al., 2015
Light-dark test	GD15	No (100%)	1	Vorhees et al., 2012
	GD15-19	Yes (100%)	1	Vorhees et al., 2015
Elevated plus maze	GD15	Yes (100%)	1	Yee et al., 2011
Anhedonia				
Sucrose preference test	GD15	Yes (100%)	1	Missault et al., 2014
Others				
Overt behaviour during habituation, fear conditioning or fear testing	GD15	No (100%)	1	Yee et al., 2012

Table 1.10. Reproducibility of effects of poly(I:C) administration on offspring neurochemistry and post-mortem evaluations (2003 – November 2015). For each measure, the amount of replication (in percentage) between individual studies and the number of available studies are presented according to the gestational day when dams received poly(I:C) injection.

Neurochemical or post-mortem changes				
Measure	**GD**	**Affected? (% of study)**	**Number of study**	**Reference**
Blood changes				
Plasma corticosterone concentration	GD15	No (100%)	2	Dalton et la., 2012; Yee et al., 2011
Plasma IL-6 concentration		No (100%)		
Plasma TNF-α levels	GD15	Yes (100%)	1	Dalton et al., 2012

Serum TNF-α protein levels (adolescence)	GD16-19	Yes (100%)	1	Han et al., 2011
Serum levels of kynurenine pathway metabolites	GD19	Yes (100%)	1	Zavitsanou et al., 2014
Neurochemical, metabolic and molecular changes				
5-HT_{1A} receptor binding in hippocampus	GD15	Yes (100%)	1	Dalton et al., 2012
CB_1R expression in the brain	GD15	Yes (100%)	1	Verdurand et al., 2014
Hippocampal 5-HT levels	GD9 or 10	Yes (100%)	1	Ohkawara et al., 2015
Striatal 5-HT, dopamine and their metabolites		No (100%)		
Basal [^{3}H]dopamine release in the striatum	GD15	No (100%)	1	Zuckerman et al., 2003
KCl-stimulated [^{3}H]dopamine release in the striatum		Yes (100%)		
Brain dopamine and serotonin changes	GD15	Yes (100%)	1	Hadar et al., 2015
Brain glucose uptake and metabolism		Yes (100%)		
MK-801-induced increase in extracellular glutamate and basal extracellular glutamate in PFC	GD15	Yes (100%)	1	Roenker et al., 2011
PFC levels of glutathione and taurine	GD15	Yes (100%)	1	Vernon et al., 2015
PFC levels of glutamate or any other metabolite		No (100%)		
Brain levels of L-arginine and its downstream metabolites	GD15	Yes (100%)	1	Jing et al., 2013
Core metabolic and myelin-associated proteins, and MAPK signalling-related kinases	GD15	Yes (100%)	1	Farrelly et al., 2015a
Gene expression of neuregulin-1, BDNF and their receptors, ErbB4 and trkB, respectively (PFC, ACC, PC, striatum)	GD15	Yes (100%)	1	Hemmerle et al., 2015
Levels of TNF-α, BDNF and NGF (foetal liver/spleen and brain)	GD16	Yes (100%)	1	Gilmore et al., 2005

Levels of TNF-α, BDNF and NGF (neonatal whole brain/cortex)		Yes (100%)		
Levels of kynurenine pathway metabolites (embryo brains collected 5 h after MIA)	GD18	No (100%)	1	Khalil et al., 2013
miRNA expression in entorhinal cortex	GD15	Yes (100%)	1	Hollins et al., 2014
Structural and morphological changes				
Total brain volume	GD15	No (100%)	1	Piontkewitz et al., 2011
Volume of PFC		Yes (100%)		
Volume of striatum		Yes (100%)		
Volume of lateral ventricle	GD15	Yes (100%)	2	Piontkewitz et al., 2009; Piontkewitz et al., 2011
Hippocampal size	GD15	Yes (100%)	3	Patrich et al., 2016a; Piontkewitz et al., 2009; Piontkewitz et al., 2011
Morphology in hippocampal CA1 and CA3 regions	GD15	Yes (100%)	2	Zuckerman et al., 2003; Zuckerman & Weiner, 2003
Morphology in the entorhinal cortex	GD15	Yes (100%)	1	Zuckerman et al., 2003
Hippocampal changes				
Hippocampal cell proliferation or short term cell survival	GD15	No (100%)	1	Chou et al., 2015b
Hippocampal excitatory neurotransmission	GD15	Yes (100%)	1	Patrich et al., 2016a
Hippocampal short-term synaptic plasticity		No (100%)	1	
Hippocampal neurogenesis	GD15	Yes (100%)	2	Mattei et al., 2014; Piontkewitz et al., 2012
Number of calretinin-positive DG cells	GD15	Yes (100%)	1	Piontkewitz et al., 2012
Number of parvalbumin-positive neurons in the hippocampus	GD15	Yes (50%); No (50%)	2	Dickerson et al., 2014; Piontkewitz et al., 2012
GAD67 protein expression within parvalbumin-positive cells in the dorsal hippocampus	GD15	Yes (100%)	1	Dickerson et al., 2014
DG granule cell density	GD15	Yes (100%)	1	Piontkewitz et al., 2012

Hippocampal micro-vascularisation		Yes (100%)		
% DG cells co-expressing BrdU and NeuN, GFAP, Galac, or OX42		No (100%)		
TNF-α receptor 1 mRNA expression in hippocampal neuronal progenitor cells	GD15	No (100%)	1	Mattei et al., 2014
TNF-α receptor 2 mRNA expression in hippocampal neuronal progenitor cells		Yes (100%)		
Neural activity and firing				
Medial PFC-hippocampus EEG coherence	GD15	Yes (100%)	3	Dickerson et al., 2010; Dickerson et al., 2012; Dickerson et al., 2014
Firing of theta-modulated, gamma-entrained mPFC neurons	GD15	Yes (100%)	1	Dickerson et al., 2010
Neurodevelopmental and neurotransmission-related changes				
Embryonic development of serotonergic neurons	GD9 or 10	Yes (100%)	1	Ohkawara et al., 2015
Expression of genes involved in the differentiation and maturation of serotonergic neurons (rostral cluster)		No (100%)		
Expression of molecules with critical roles in neuronal development or synaptic transmission	GD14, 16, 18	Yes (100%)	1	Forrest et al., 2012
Expression of neurodevelopmental proteins (cerebral hemispheres at PND21)	GD14, 16, 18	Yes (100%)	1	Khalil et al., 2013
Expression of neurodevelopmental proteins (embryo brains collected 5 h after MIA)	GD18	Yes (100%)		
Basal synaptic transmission, short or long-term synaptic plasticity (hippocampal CA1)	GD15 & 17	Yes (100%)	1	Oh-Nishi et al., 2010
Expression of pre-synaptic protein, synaptophysin (hippocampal CA1)		Yes (100%)		

Expression of post-synaptic proteins, GluR1, GluR2/3 and NR1 (hippocampal CA1)		No (100%)		
Hippocampal CA1/CA3 neuronal loss and apoptosis		No (100%)		
Microglia density, activation and cytokine production				
Microglia density in NAcc	GD15	Yes (100%)	1	Mattei et al., 2014
Microglia density in ventral striatum and CE		No (100%)		
Microglia density in striatum and thalamus	GD15	No (100%)	1	Van den Eynde et al., 2014
Microglia density in pons and corpus callosum		Yes (100%)		
Microglia density in hippocampus/DG	GD15	No (100%)	2	Mattei et al., 2014; Van den Eynde et al., 2014
Microglia density in the cortex/mPFC/cingulate cortex		No (100%)		Mattei et al., 2014; Van den Eynde et al., 2014
Microglia activation in DG and CE	GD15	Yes (100%)	1	Mattei et al., 2014
Microglia activation in hippocampus	GD15	Yes (67%); No (33%)	3	Missault et al., 2014; Murray et al., 2019; Van den Eynde et al., 2014
Microglia activation in pons, cortex, corpus callosum, thalamus and striatum	GD15	No (100%)	2	Missault et al., 2014; Van den Eynde et al., 2014
CD68 receptor (ED-1) staining in the cortex, striatum, corpus collasum, dorsal hippocampus, thalamus and pons	GD15	No (100%)	1	Van den Eynde et al., 2014
Cytokine production by hippocampal microglia	GD15	Yes (100%)	1	Mattei et al., 2014
Cytokine production by cerebellar microglia		No (100%)		
Other changes				
Density of neuronal nuclear antigen ($NeuN^+$) interstitial white matter neuron	GD11	No (100%)	1	Duchatel et al., 2016
	GD20	No (100%)		
Density of somatostatin (SST^+) interstitial white matter neuron	GD11	Yes (100%)		
	GD20	Yes (100%)		

Adrenal weight	GD15	No (100%)	1	Yee et al., 2011
Total bile acids in foetuses (24 h post-injection)	GD17-18	No (100%)	1	Petrovic & Piquette-Miller, 2010

Table 1.11. Reproducibility of effects of poly(I:C) administration on litter or offspring growth (December 2015 – May 2019). For each measure, the amount of replication (in percentage) between individual studies and the number of available studies are presented according to the gestational day when dams received poly(I:C) injection.

Gestation, litter and offspring growth				
Measure	**GD**	**Affected? (% of study)**	**Number of study**	**Reference**
Gestation length	GD18	No (100%)	1	Zhao et al., 2019
Pup mortality	GD15	Yes (100%)	1	Lins et al., 2018
	GD15-18	No (100%)	1	Oh-Nishi et al., 2016
Litter size	GD15	Yes (20%); No (80%)	5	De Felice et al., 2019; Gray et al., 2019; Horska et al., 2017; Murray et al., 2017; Paylor et al., 2016
	GD15-18	No (100%)	1	Oh-Nishi et al., 2016
	GD18	No (100%)	1	Zhao et al., 2019
Sex ratio within litters	GD15	No (100%)	1	Gray et al., 2019
	GD18	No (100%)	1	Zhao et al., 2019
Pup/offspring body weight	GD13	Yes (100%)	1	Cakan et al., 2018
	GD15	No (100%)		
	GD18	No (100%)	1	Zhao et al., 2019
Pup/offspring body weight	GD11	No (100%)	1	Meehan et al., 2017
	GD15	No (100%)	5	Gray et al., 2019; Horska et al., 2017; Lins et al., 2018; Murray et al., 2017; Paylor et al., 2016
	GD20	No (100%)	1	Meehan et al., 2017
Basic metabolic parameters (body weight, food and water intake)	GD15	No (100%)	1	Osborne et al., 2017

Puberty and completion of puberty	GD13	Yes (100%)	1	Cakan et al., 2018
	GD15	No (100%)		
Length of oestrous cycles	GD13	No (100%)		
	GD15	No (100%)		

Table 1.12. Reproducibility of effects of poly(I:C) administration on offspring behaviour (December 2015 – May 2019). For each behaviour, the amount of replication (in percentage) between individual studies and the number of available studies are presented according to the gestational day when dams received poly(I:C) injection.

Offspring behaviour				
Behavioural test	**GD**	**Affected? (% of study)**	**Number of study**	**Reference**
Locomotor changes				
Spontaneous LMA	GD9	Yes (100%)	1	Li et al., 2018b
	GD11	No (100%)	1	Meehan et al., 2017
	GD15	No (100%)	1	Gray et al., 2019
	GD18	No (100%)	1	Zhao et al., 2019
	GD20	No (100%)	1	Meehan et al., 2017
Saline injection-stimulated LMA	GD11	No (100%)	1	Meehan et al., 2017
	GD20	No (100%)		
AMPH-stimulated LMA	GD11	No (100%)	1	Meehan et al., 2017
	GD15	Yes (100%)	1	Gray et al., 2019
	GD20	No (100%)	1	Meehan et al., 2017
MK-801-stimulated LMA	GD11	No (100%)	1	Meehan et al., 2017
	GD15	Yes (25%); No (75%)	4	Gray et al., 2019; Lins et al., 2018; Lins et al., 2019; Missault et al., 2019
	GD20	No (100%)	1	Meehan et al., 2017
Cognition and motivation				
Delayed non-match to position (working memory)	GD11	No (100%)	1	Meehan et al., 2017
	GD20	Yes (100%)		
NOD	GD15	Yes (100%)	3	Gray et al., 2019; Luchicchi et al., 2016; Osborne et al., 2017
Y-maze task (visual recognition memory)	GD15	Yes (100%)	2	Lins et al., 2018; Lins et al., 2019
Y-maze task (tactile object recognition memory)		No (100%)		

Y-maze task (crossmodal recognition memory battery)		Yes (100%; although in Lins et al. (2019) also impaired in control)		
Spontaneous oddity task		Yes (100%)		
Rewarded T-maze alternation test	GD15	Yes (100%)	1	Osborne et al., 2017
Signalled probability sustained attention task (examines motivation and cognition)	GD15	No (100%)	1	Bates et al., 2018
Latent inhibition	GD15	Yes (100%)	1	Bikovsky et al., 2016
Temporal bisection task	GD15	Yes (100%)	1	Deane et al., 2017
Operant set-shifting	GD15	Yes (100%)	1	Lins et al., 2018
Touchscreen pairwise discrimination and reversal learning		Yes (100%)		
Operant-based progressive ratio task	GD15	Yes (100%)	1	Millar et al., 2017
Operant-based random ratio schedule testing		No (100%)		
Operant-based autoshaping paradigm		No (100%)		
Operant-based contigency degradation paradigm		Yes (100%)		
Odour-based nonmatching-to-sample task - acquisition	GD15	No (100%)	1	Murray et al., 2017
Odour span task (working memory)		Yes (100%)		
Passive avoidance task	GD9	Yes (100%)	1	Li et al., 2018b
Active avoidance task		Yes (100%)		
MWM - spatial and reversal learning	GD18	Yes (100%)	1	Zhao et al., 2019
Social behaviour				
Sociability task	GD15	Yes (100%)	2	Lins et al., 2018; Lins et al., 2019
Social interaction	GD15	Yes (67%); No (33%)	3	Gray et al., 2019; Luchicchi et al., 2016; Osborne et al., 2017
Sensorimotor gating				

PPI	GD9	Yes (100%)	1	Li et al., 2018b
	GD11	Yes (100%)	1	Meehan et al., 2017
	GD15	Yes (56%); No (44%)	9	Bates et al., 2018; Bikovsky et al., 2016; De Felice et al., 2019; Gray et al., 2019; Hadar et al., 2017; Lins et al., 2018; Lins et al., 2019; Luchicchi et al., 2016
	GD20	Yes (100%)	1	Meehan et al., 2017
Startle response	GD11	No (100%)	1	Meehan et al., 2017
	GD15	No (100%)	7	Bates et al., 2018; De Felice et al., 2019; Gray et al., 2019; Missault et al., 2019; Lins et al., 2018; Lins et al., 2019; Luchicchi et al., 2016
	GD20	No (100%)	1	Meehan et al., 2017
PPI - latency to peak	GD15	No (100%)	1	De Felice et al., 2019
Startle habituation		No (100%)		
Anxiety				
Open field test - entry into centre, duration or distance travelled in central zone	GD15	Yes (100%)	1	Missault et al., 2019
	GD17	Yes (100%)	1	Hu et al., 2019
Open field test - total distance travelled or velocity	GD15	No (100%)	1	Missault et al., 2019
	GD17	No (100%)	1	Hu et al., 2019
Elevated plus maze - behaviour	GD11 & 20	Yes (100%)	1	Hollins et al., 2018
	GD15	No (100%)	1	Gray et al., 2019
	GD18	Yes (100%)	1	Zhao et al., 2019
Elevated plus maze - distance travelled	GD11 & 20	No (100%)	1	Hollins et al., 2018
Anhedonia				
Sucrose preference test	GD15	No (100%)	2	Gray et al., 2019; Missault et al., 2019
	GD17	Yes (100%)	1	Hu et al., 2019
Depressive-like behaviour				
Forced swimming test	GD17	Yes (100%)	1	Hu et al., 2019

Table 1.13. Reproducibility of effects of poly(I:C) administration on offspring neurochemistry and post-mortem evaluations (December 2015 – May 2019). For each measure, the amount of replication (in percentage) between individual studies and the number of available studies are presented according to the gestational day when dams received poly(I:C) injection.

Neurochemical or post-mortem changes				
Measure	**GD**	**Affected? (% of study)**	**Number of study**	**Reference**
Serum changes				
Serum levels of progesterone, follicle stimulating hormone, luteinising hormone, leptin and corticosterone	GD13 or 15	No (100%)	1	Cakan et al., 2018
Serum lipid profile	GD15	Yes (100%)	1	Horska et al., 2017
Serum levels of adipokines, gastrointestinal hormones and cytokine		No (100%)		
Serum Ig-κ light chain protein levels	GD15-18	Yes (100%)	1	Oh-Nishi et al., 2016
Neurochemical and molecular changes				
mRNA expression of dopamine D_1 receptor (NAcc)	GD11	Yes (100%)	1	Meehan et al., 2017
mRNA expression of dopamine D_1 (caudate putamen and substantia nigra) and D_2 (NAcc, caudate putamen, substantia nigra and VTA) receptors, and dopamine transporter and tyrosine hydroxylase (substantia nigra and VTA)		No (100%)		
mRNA expression of dopamine D_1 and D_2 receptors [NAcc, caudate putamen, substantia nigra (both) and VTA (D_2 only)], and dopamine transporter and tyrosine hydroxylase (substantia nigra and VTA)	GD20	No (100%)		
5-HT_{1A} receptor density in the entorhinal cortex	GD15	No (100%)	1	Hollins et al., 2016

Gene expression in the left entorhinal cortex		Yes (100%)		
GluN1 protein expression in the FC, cortex, corpus callosum, striatum, hippocampus and thalamus	GD15	No (100%)	1	Missault et al., 2019
Baseline extracellular dopamine levels in shell of NAcc	GD15	Yes (100%)	1	Luchicchi et al., 2016
Baseline extracellular dopamine levels in mPFC		No (100%)		
DOPAC levels in the NAcc and mPFC		No (100%)		
GAD67 mRNA expression in hippocampus, thalamus and PFC (prelimbic cortex)	GD14	Yes (100%)	1	Cassella et al., 2016
GAD67 mRNA expression in frontal, parietal and piriform cortices and striatum		No (100%)		
Expression of amino acid transporters (mRNA and protein) and amino acid metabolising enzymes (mRNA), and amino acid concentrations in foetal brain	GD14	Yes (100%)	1	McColl & Piquette-Miller, 2019
mRNA and protein levels of epidermal growth factor receptor, ErbB4 and PI3K p110δ in the mPFC, orbital PFC, dorsal striatum and NAcc	GD15	No (100%)	1	Idrizi et al., 2016
Structural and neurodevelopmental changes				
Whole brain volume	GD15	No (100%)	1	Crum et al., 2017
Volumes of several cortical regions, hippocampus, amygdala, striatum, NAcc, lateral ventricles, thalamus, ventral mesencephalon, brain stem and major white matter tracts		Yes (100%)	1	
Haemodynamic response to MK-801 administration	GD15	Yes (100%)	1	Missault et al., 2019
Brain microstructural changes		No (100%)		

Dendritic development	GD17	Yes (100%)	1	Hu et al., 2019
Hippocampal neurogenesis	GD18	Yes (100%)	1	Zhao et al., 2019
Neural activity and neurophysiological properties				
Functional connectivity in default mode network	GD15	Yes (100%)	1	Missault et al., 2019
Neurocircuitry influenced by deep brain stimulation of mPFC	GD15	Yes (100%)	1	Bikovsky et al., 2016
Neurocircuitry influenced by deep brain stimulation of NAcc		No (100%)		
Number and firing rate of ventral tegmental area dopamine neurons	GD15	Yes (100%)	2	De Felice et al., 2019; Luchicchi et al., 2016
Inhibitory and excitatory synaptic properties of VTA dopamine neurons	GD15	Yes (100%)	1	De Felice et al., 2019
Neurophysiological properties of cultured pyramidal-like hippocampal neurons (neonatal) and CA1 pyramidal neurons of prepared hippocampal slices (adolescence)	GD15	Yes (100%)	1	Patrich et al., 2016b
Density of perineuronal nets in amygdala and medial prelimbic cortex	GD15	Yes (100%)	1	Paylor et al., 2016
% parvalbumin cells with perineuronal nets in medial prelimbic cortex		Yes (100%)		
Perineuronal net density, parvalbumin cell density and % parvalbumin cells with perineuronal net in frontal association cortex and auditory cortex		No (100%)		
Cytokine and immunological factor expression				
C4 gene expression in cingulate cortex	GD11	No (100%)	1	Duchatel et al., 2018a
	GD20	Yes (100%)		
IL1-β and *TNF-α* mRNA levels in cingulate cortex	GD11	No (100%)	1	Duchatel et al., 2018b
	GD20	No (100%)		
Basal mRNA expression of pro-inflammatory cytokines (left or right brain hemispheres)	GD15	Yes (100%)	1	Clark et al., 2019

Lipopolysaccharide-stimulated mRNA expression of brain pro-inflammatory cytokines		Yes (100%)		
IL-6 and TNF-α protein levels in foetal brain (GD18) - PFC and/or hippocampus	GD18	Yes (100%)	1	Zhao et al., 2019
IL-6 and TNF-α protein levels in foetal brain (GD18) - cerebellum		No (100%)		
Cytokine (IL-1β, TNF-α and IL-6) mRNA expression of isolated microglia from hippocampus and mPFC, and Cd11b and Cd54 expression	GD15	No (100%)	1	Hadar et al., 2017
MHCII expression in microglia from mPFC		Yes (100%)		
MHCII expression in microglia from hippocampus		No (100%)		
Interferon-stimulated gene-15 mRNA and protein expression and p-STAT1/STAT1 ratio in PFC	GD17	Yes (100%)	1	Hu et al., 2019
Microglia and astrocytic changes				
Microglia density and soma size in hippocampus and NAcc	GD15	Yes (100%)	1	Hadar et al., 2017
Microglia density and soma size in mPFC		No (100%)		
Microglial density in frontal association cortex, medial prelimbic cortex, amygdala and primary auditory cortex	GD15	No (100%)	1	Paylor et al., 2016
Microglia density in DG, number of microglial primary processes, cell soma size of microglia, and mRNA expression of Cd11b (microglial activation marker)	GD18	Yes (100%)	1	Zhao et al., 2019
mRNA and protein expression of microglial M1 and M2 markers in hippocampus				
Microglial migration and maturation	GD15	Yes (100%)	1	Zhang et al., 2019

Microglia activation in PFC and hippocampus	GD9	Yes (100%)	1	Li et al., 2018b
IBA1+ immunoreactive microglia in cingulate cortex	GD11	No (100%)	1	Duchatel et al., 2018b
	GD20	No (100%)		
IBA1+ immunoreactive microglia in white matter of corpus callosum	GD11	No (100%)		
	GD20	Yes (100%)		
Iba1 and *Gfap*mRNA levels in cingulate cortex	GD11	No (100%)		
	GD20	No (100%)		
GFAP+ immunoreactive astrocytes in cingulate cortex and white matter of corpus callosum	GD11	No (100%)		
	GD20	No (100%)		
Astrocytic density in frontal association cortex, medial prelimbic cortex, amygdala and primary auditory cortex	GD15	No (100%)	1	Paylor et al., 2016
Density of astrocytes in DG	GD18	No (100%)	1	Zhao et al., 2019
Metabolic changes				
Basal expression of brain kynurenine pathway enzymes and metabolites	GD15	Yes (100%)	1	Clark et al., 2019
Lipopolysaccharide-stimulated expression of brain kynurenine pathway enzymes and metabolites		Yes (100%)		
Arginine metabolism in the forebrain	GD15	No (100%)	1	Zhang et al., 2018a
Arginine metabolism in FC		Yes (100%)		
Arginine metabolism in whole hippocampus		Yes (100%)		
Arginine metabolism in cerebellum		No (100%)		
Other changes				
Autonomic nervous system activation	GD11 and 20	No (100%)	1	Hollins et al., 2018
Gastrointestinal function		Yes (100%)		
Expression of intestinal tight junction proteins		Yes (100%)		
Number of atretic follicles	GD13	Yes (100%)	1	Cakan et al., 2018
	GD15	No (100%)		

Neuronal nitric oxide synthase-positive cells in FC and hippocampal CA1 region	GD15	No (100%)	1	Zhang et al., 2018b
Neuronal nitric oxide synthase-positive cells in somatosensory cortex, striatum and hippocampal CA3 and DG		Yes (100%)		

In short, gestational poly(I:C) exposure in rats is capable of reproducing various abnormalities akin to schizophrenia at the cellular, molecular and structural levels, as well as behavioural alterations in paradigms relevant to the positive (e.g. LMA, PPI), negative (e.g. social interaction) and cognitive (e.g. NOD, CFR, ASST) domains of schizophrenia. Several such abnormalities have been successfully attenuated by antipsychotic treatment. This model, however, has its drawbacks. For instance, poly(I:C) administration into dams does not reproduce the whole time course of viral infection, mostly mimicking only the cytokine-associated viral-like acute phase responses (Meyer et al., 2009b). Furthermore, MIA is unlikely a lone risk factor leading to the development of complex neuropsychiatric disorders. Thus, combining it with a further intervention could potentially expand its usefulness. Given the urgent need to clarify the link between maternal viral infection and neuropsychiatric disorders such as schizophrenia, prenatal poly(I:C) was selected as the first 'hit' of the novel 'dual-hit' model for this thesis.

1.4 Post-weaning social isolation

Social isolation, defined as ''the diminution or total lack of social interaction, usually for an extended period of time, has been established as a predisposing risk factor of schizophrenia since the 1930s (Faris, 1934). At the same time, various social deficits have been documented in schizophrenia, including impaired social cognition, skills and motivation (Table 1.14; Fulford et al, 2018; Green et al., 2015). Furthermore, subjects with schizophrenia are more prone to loneliness

and are linked to increased social deprivation, anxiety, anhedonia and disadvantage, resulting in vastly reduced quality of life (Seeman, 2016; Shioda et al., 2016; Stilo et al., 2017). Therefore, there is an unmet need to investigate the mechanisms instrumental to these impairments.

Table 1.14. Domains of social behaviour frequently studied in schizophrenia. The three domains are presented with their definitions and examples.

Domain	Definition	Examples	References
Social skills	Learned, observable behaviours that contribute to social competence	Reciprocity, problem solving, active listening, appropriate eye contact, affective expression	Fulford et al., 2018
Social cognition	Cognitive and emotional facets underlying and supporting social behaviour	Social cue perception (face and voice perception), mentalising, experience sharing (motor resonance and affect sharing), emotion experience and regulation	Green et al., 2015
Social motivation	The drive to form and maintain social bonds	Initiative to approach others, awareness of socially rewarding cues, maintenance of positive affect	Fulford et al., 2018

Laboratory housing can profoundly influence social interaction and structure in rats, which live in large hierarchical groups in nature (Arakawa, 2018; Ellenbroek & Youn, 2016). Chronic social deprivation during critical periods of neurodevelopment, such as post-weaning isolation rearing of rats, typically from weaning (PND21-28) over prolonged periods (weeks), represents a well-established model thought to replicate early life SI, which is a risk factor (Table 1.1), as well as an undesired outcome in schizophrenia (Fone and Porkess, 2008). This model is developed by housing rats in individual cages without environmental enrichment and with minimal handling, while group-housed (GH) control rats (typically littermates) are maintained in small social groups under the same conditions. Such individually reared rats (referred hereafter as ‘isolates’) reliably exhibit the ‘isolation syndrome’, which includes abnormal reactivity (Fone & Porkess, 2008). Whilst readily abrogated by repeated handling and testing, this model produces various persistent brain and behavioural changes which are reminiscent of the core features of schizophrenia (Fone & Porkess, 2008; Lapiz et al., 2003). As with the poly(I:C) model, only alterations that are directly relevant to the work in this thesis will be discussed in depth.

Apart from reduced habituation, isolates consistently exhibited hyperactivity, particularly in horizontal ambulation, compared to GH rats from 2 weeks after isolation commencement when placed in a new environment (Amitai et al., 2014; Simpson et al., 2010; Sun et al., 2017). Such hyperactivity, which may be indicative of enhanced neophobia (increased propensity to escape), elevated state of arousal, limited experience due to prolonged isolation, or abnormal behavioural inhibition, can be reversed by administration of atypical antipsychotic risperidone or quetiapine and the selective mGluR2/3 agonist LY379268, hence confirming the validity of the current model as a tool for schizophrenia research (Jones et al., 2011a; Ko & Liu, 2016; Lapiz et al., 2003; McIntosh et al., 2013). Moreover, it has been correlated to altered expression of immediate early genes (IEGs) and genes that regulate cell differentiation and apoptosis in the medial PFC, and sensitisation of mesolimbic dopamine projections to the nucleus accumbens (Lapiz et al., 2003; Levine et al., 2007). Novelty-induced hyperactivity is typically used as a marker to confirm the development of the 'isolation syndrome' (Fone & Porkess, 2008), although there exist discrepant findings which described reduced locomotion or no change in activity when placed in a new environment (Leng et al., 2004; Okudan & Belviranli, 2017; Weiss et al., 2004). Such differential observations can be conceivably ascribed to methodological differences (e.g. lighting or duration of isolation and evaluation) or strain-specific effects (Fone & Porkess, 2008; Robbins, 2016; Weiss et al., 2004).

Isolates show blunted preference for the novel object during the choice trial when an inter-trial (ITI) delay of 1-2 h is spanned between the familiarisation and choice trial (Bianchi et al., 2006; Gaskin et al., 2014; King et al., 2009; Uys et al., 2016). In a study examining the influence of ITI on rat recognition memory, isolates were found to only be able to differentiate between the familiar and novel objects up to 1h ITI (*cf*. GH animals up to 4 h; McLean et al., 2010). Preferred exploration of novel over the familiar object was restored by acute risperidone (0.2 or 0.5 mg kg^{-1}, i.p., 30 min prior to test), clozapine, LY379268, the α_{2c}-adrenoceptor

antagonist, ORM-10921, or ORM-10921+haloperidol treatment, thus showing the potential of the isolation rearing model to mirror cognitive deficits associated with schizophrenia (Jones et al., 2011a; McIntosh et al., 2013; Uys et al., 2016). However, conflicting results have been reported, for instance, Dunphy-Doherty et al. (2018) found intact episodic memory in isolates during the choice trial following a 2h ITI. No known studies have examined the performance of isolates in the NLD task, however, in other spatial learning tasks, such as Morris water maze (MWM), Olton's spatial memory task, Y-maze task and 17-arm radial maze task, mixed findings on performance have been reported (Einon, 1980; Juraska et al., 1984; Quan et al., 2010; Schrijver et al., 2004; Shao et al., 2015; Wongwitdecha & Marsden, 1996a).

Social deprivation also reduces pro-social behaviour in rats assessed in the social interaction test. For example, isolates spent less time engaging in social interactive behaviours (rearing, anogenital sniffing, approaching and staying with conspecific) and more time in self-grooming; these abnormalities were completely reversed with clozapine treatment (Möller et al., 2011). Whilst reported behavioural alterations are largely consistent across research groups (Kokare et al., 2010; Regenass et al., 2018; Strauss et al., 2014), one study demonstrated SI-induced increase in social exploration (Grotewold et al., 2014). Isolates also showed increased aggression and pinning towards an unfamiliar naïve rat (Grotewold et al., 2014). Furthermore, heightened defensiveness and aggressiveness (increased attacks and biting) in resident-intruder tests, which persist even after resocialisation or fluoxetine treatment, have been described (Biro et al., 2017; Mikics et al., 2018; Tulogdi et al., 2014). Finally, they displayed reduced huddling with cage mates during sleep when being put through resocialisation (Tulogdi et al., 2014).

Prolonged deprivation of post-weaning social play has been shown to impair social communication in rats. For instance, male rats demonstrated deficits in recognising social and reward cues from sexually receptive females by emitting fewer 50-kHz USVs compared to

GH counterparts after chronic single housing (Inagaki et al., 2013). Isolates also emitted more USV distress calls (22-kHz) in response to aggression (von Frijtag et al., 2002). In rats, reception of 50-kHz and 22-kHz USVs induces social approach and freezing behaviour, respectively (Parsana et al., 2012; Wöhr & Schwarting., 2007). When exposed to the playback of the pro-social 50-kHz, but not to 22-kHz or noise, prolonged post-weaning for 28 days resulted in an inhibition of approach behaviour that was attenuated by week-long resocialisation. Such social avoidance was not replicated in rats exposed to post-weaning short-term isolation (24h before testing) or by equivalent short and long-term isolation in post-adolescent rats (Seffer et al., 2015). Moreover, isolation from weaning age resulted in increased pro-social vocalisations during social contact and tickling, thus indicating that such interactions are particularly rewarding to isolates (Burgdorf & Panksepp, 2001; Knutson et al., 1998).

Post-weaning isolation-induced disrupted PPI has been widely reproduced across studies (Jones et al., 2011a; Marsden et al., 2011; Schubert et al., 2009). However, this effect is strain-dependent, with consistent effects most commonly seen in Lister-hooded, Long-Evans and Sprague-Dawley rats (Cilia et al., 2001; Fone & Porkess, 2008). Wistar and Lewis strains are associated with less consistent impairments whereas impairments could be absent when Fischer rats are used (Fone & Porkess, 2008). Caging conditions could also be crucial in the precipitation of such impairment, as isolates kept in grid-floor but not sawdust cages developed PPI deficits (Weiss et al., 1999). Isolation-induced weakened PPI is developmentally specific, i.e. only happens when rats are deprived of social contact from weaning but not in adulthood, and only emerges during or after puberty (Bakshi & Geyer, 1999; Wilkinson et al., 1994). Such deficits remain stable with weekly testing and can be resilient to daily handling, resocialisation and LMA testing (Cilia et al., 2001). Treatment with atypical antipsychotics (clozapine, olanzapine, risperidone, quetiapine), mGluR2/3 agonist LY379268, selective α7-nAChR agonist (*R*)-*N*-(1-Azabicyclo[2.2.2]oct-3-yl)(5-(2-pyridyl)thiophene-2-carboxamide) and α2c-adrenoceptor

antagonist ORM-10921 successfully attenuated isolation-induced PPI alterations (Cilia et al., 2001; Cilia et al., 2005; Jones et al., 2011a; Ko & Liu, 2016; Möller et al., 2011; Uys et al., 2016). The observations that disrupted PPI can be partially normalised by acute raclopride (D_2 receptor antagonist) treatment and 6-hydroxydopamine depletion of nucleus accumbens dopamine imply hyperdopaminergia as a potential cause underlying such impairment (Geyer et al., 1993; Powell et al., 2003).

Chronic isolation of rats during adolescence results in diminished associative learning in the CFR paradigm (Dunphy-Doherty et al., 2018; Gaskin et al., 2014; Jones et al., 2011a). CFR deficits were resistant to treatment with risperidone and the selective mGluR2/3 agonist, LY379268 (Jones et al., 2011a; McIntosh et al., 2013).

Isolates also show cognitive inflexibility, which is mediated by the PFC cortico-striatal pathways, when tested in various rule learning tasks, including bowl-digging ASST (McLean et al., 2010; Powell et al., 2015; Schrijver et al., 2004), the radial arm maze ED/ID paradigm (Schrijver & Würbel, 2001), reversal learning in a MWM paradigm (Quan et al., 2010), rotating T-maze reversal learning task (Li et al., 2007), simultaneous light/dark discrimination and serial reversal learning (Jones et al., 1991) and also the probabilistic reversal learning task (Amitai et al., 2014). Chronic administration of clozapine restored reversal learning in the rotating T maze task (Li et al., 2007). Nonetheless, improved reversal was observed in one MWM paradigm (Wongwitdecha & Marsden, 1996a).

Various neurobiological and neurochemical alterations that are translationally relevant to schizophrenia have been demonstrated in the post-weaning SI model (see review by Fone & Porkess, 2008). Some of the most consistently reported alterations include hyperactive mesolimbic dopaminergic system, hypoactivity of mesocortical dopaminergic system, compromised 5-HT activity in the PFC and hippocampus, and elevated striatal high-affinity D_2 receptors (Fone & Porkess, 2008; King et al., 2009). Selective decrease in PFC volume, decreased dendritic spine

density and parvalbumin- and calbindin-containing interneurons in the PFC and hippocampus, compromised redox regulation and altered gut microbiota have also been demonstrated (Dunphy-Doherty et al., 2018; Jones et al., 2011b; Shao et al., 2015). Moreover, alterations in cortical and hippocampal cytokine levels have been reported (Corsi-Zuelli et al., 2019; Dunphy-Doherty et al., 2018).

Similar to the poly(I:C) model, post-weaning isolation rearing of rats is associated with numerous abnormalities resembling the core features of schizophrenia. Considering that SI does not only act as a predisposing factor of schizophrenia (Table 1.1), but also represents a common, undesired consequence of schizophrenia which can dramatically reduce quality of life in affected individuals (Shioda et al., 2016), it is of utmost importance to study the mechanisms underlying such stress. Much have been learnt from this established model, however, akin to the poly(I:C) model, it is not without limitations. Notably, SI-induced behavioural alterations can be reversed by repeated handling or testing and such changes are not consistently reproduced across cohorts (Jones et al., 2011b). Since the SI model is well characterised in our group, it is selected to be combined with gestational poly(I:C) challenge, in the hope of generating a more robust and useful preclinical model which encompasses the relative strengths of the two models.

1.5 Aims and objectives of the thesis

Given the major burden that schizophrenia imposes on patients and the society as a whole, there is a pressing need to develop new drugs that could alleviate symptoms of this disorder. In this regard, animal models serve as a valuable preclinical tool to examine the pathobiology of the disorder, with the aim of identifying new therapeutic targets and screening novel compounds for therapeutic potential. The combination of two neurodevelopmental interventions, prenatal poly(I:C) exposure and post-weaning SI might potentially produce a wider spectrum of more

severe behavioural and cognitive impairments than either intervention alone. To this end, the work presented in Chapter 2 of this thesis sought to examine if combining these manipulations would produce an improved preclinical model for schizophrenia.

As it was the first time a MIA model was used in our laboratory, a pilot study was conducted to validate the effects of a selected dose of poly(I:C) (i.p.) on non-pregnant females. In the follow-up study, time-mated females were given a single poly(I:C) or vehicle saline injection on GD15 and resultant offspring were subjected to social or individual housing from weaning. Following confirmation of successful MIA and SI and gender selection, a separate cohort of offspring were evaluated in a series of behavioural paradigms relevant to schizophrenia in adulthood. Brain cytokine and mammalian target of rapamycin (mTOR) levels were investigated for potential mechanisms underlying behavioural changes. Finally, potential early mechanisms leading to behavioural alterations in adulthood were studied.

The route of administration can influence the robustness of poly(I:C)-stimulated immune response (Kentner et al., 2019). Therefore, in Chapter 3, the study presented in Chapter 2 was repeated using an alternative dosing method that is commonly used in the field, i.v. administration, without altering other experimental variables. The overall aim was to determine whether a more comprehensive model would be produced by i.v. poly(I:C) administration. A pilot study was conducted in non-pregnant females before the actual study to select an appropriate dose.

In Chapters 2 and 3, SI alone was associated with more robust effects than poly(I:C) alone. As the Nottingham-Monash Joint PhD programme requires the third year of study to be spent at Monash University, Australia, a decision was made to focus on the post-weaning SI model (Chapter 4). RNA-Sequencing (RNA-Seq) and radioligand binding experiments were carried out to examine any alterations in cortical gene

expression and dopamine D_3 receptor expression, respectively, in isolates.

CHAPTER 2

The combined effects of gestational poly(I:C) exposure via i.p. administration and post-weaning social isolation on behaviour and neurochemistry

2.1 Introduction

Maternal viral infection and early-life SI are risk factors of schizophrenia. In rodents, these risk factors have been independently studied and each repeatedly produces a gamut of behavioural and brain abnormalities relevant to schizophrenia (Fone & Porkess, 2008; Solek et al., 2018). Thus, the overall aim of this chapter is to generate an improved animal model for schizophrenia with a more comprehensive profile of behavioural and brain abnormalities, especially those relevant to the treatment-resistant cognitive and negative impairments, by subjecting rats to gestational MIA exposure followed by post-weaning SI. As post-weaning SI is a well-established model in our laboratory, this section will primarily focus on the poly(I:C)-based MIA model, as it was the first time our laboratory has used this approach.

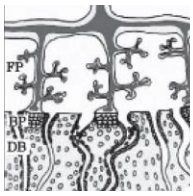
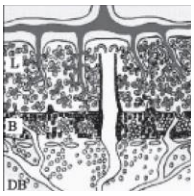
The timing of exposure, mode of delivery, dose and source of poly(I:C) are important considerations that dictate the nature and severity of MIA outcomes (Kentner et al., 2019; Reisinger et al., 2015). Furthermore, such outcomes are species- and strain-dependent (Cunningham et al., 2007; Kentner et al., 2019). Although the huge body of seminal work in poly(I:C)-based MIA research has been carried out in mice, we decided to use rats in our experiments for three primary reasons. Firstly, rats have more anatomically developed brains, display increased intrinsic receptiveness to social interaction and exhibit greater spectrum of social and non-social behaviour than mice (Ellenbroek & Youn, 2016; Kentner et al., 2019; Whishaw et al., 2001). While both species form large hierarchical groups in the wild, in a laboratory setting, rats are less territorial and aggressive than mice, and are more likely to find social behaviour rewarding (Ellenbroek & Youn, 2016; Kondrakiewicz et al., 2019). Rats also show enhanced habituation to standard laboratory environments and are less susceptible to stress following repeated handling in comparison to mice (Ellenbroek & Youn, 2016). These are particularly relevant to the current study as we are interested in

examining any alterations in social behaviour and cognition that are induced by our proposed ‘double-hit’ model, which could be translationally relevant to human schizophrenia, through conducting a series of behavioural tests. Secondly, the rat placenta, like the human placenta, is also of haemochorial type, and is strikingly similar in terms of gross organisation at the placentation site, specifically concerning trophoblast-directed spiral artery remodelling in early placental development (Furukawa et al., 2011; Soares et al., 2012; Table 2.1). Meanwhile, in mice, intrauterine trophoblast invasion is shallow and placentation is superficial (Soares et al., 2012). Thirdly, using rats, our laboratory has repetitively found robust behavioural and neurochemical alterations relevant to schizophrenia following prolonged SI post-weaning (Fone & Porkess, 2008; Lapiz et al., 2003; Marsden et al., 2011).

A thorough literature search was conducted to select the most appropriate experimental design and conditions for stimulating immune activation in pregnant rats. At the time of literature review (October – November 2015), fifty-three poly(I:C)-based MIA studies conducted in rats, not including abstracts, were found (Tables 1.3 & 1.5; studies published after November 2015 are summarised in Tables 1.4 & 1.6). Apart from one study which did not specify the strain, the following strains were used: Sprague-Dawley (26 studies), Wistar (21 studies), Long-Evans (4 studies), Lister-hooded (1 studies) and Wistar-Hannover (1 study). During an infection, the production of peripheral and brain pro-inflammatory cytokines induces “sickness behaviours”, including fever, lethargy and anorexia (Dantzer, 2001). Of the published literature, merely 32 studies reported such physical effects of poly(I:C) on dams, with reduced weight gain (20 studies) and increased cytokine and chemokine levels (11 studies) being the commonest findings. Only five studies examined body temperature of dams post-injection and all but one observed no effect of poly(I:C) on body temperature (Ballendine et al., 2015; Howland et al., 2012; Murray et al., 2019; Sangha et al., 2014; Zhang et al., 2012). Most studies, irrespective of protocol, observed no effect of poly(I:C) on miscarriage rate, gestation length, litter number, sex

ratio within litters or pup weight (Howland et al., 2012; Patrich et al., 2016a; Richtand et al., 2012a; Vorhees et al., 2012).

Table 2.1. Comparison between the human and rat placenta. Table generated based on Furukawa et al. (2011) and Soares et al. (2012).

Parameter	Human	Rat
Uterine and vascular structure	Simplex uterus. Bidirectional blood supply from aortic and internal iliac arteries; internally located arcuate and radial arteries	Duplex uterus. Bidirectional blood supply from aortic and internal iliac arteries; externally located arcuate and radial arteries
Maternal recognition of pregnancy	Placental-derived luteotropin (chorionic gonadotropin)	Mating-stimulated luteotropin secretion (pituitary prolactin)/ placental-derived luteotropin (secondary)
Embryo implantation:	Interstitial; embryonic	Eccentric (secondarily interstitial); abembryonic
i) Uterine modifications	Decidualisation (hormone-dependent)	Decidualisation (hormone-dependent + implantation stimulus)
ii) Immune cell trafficking	Large population of natural killer cells associated with uterine spiral arteries	
Yolk sac placenta	Temporary organ required for organogenesis; degenerates at the end of the 1st trimester	Role throughout gestation
Placental structure	Haemomonochorial (discoid-chorioallantoic)	Haemotrichorial (discoid-chorioallantoic)
i) Chorioallantoic placenta	Villous type: • Foetal placenta (FP) ○ Syncytiotrophoblast • Basal plate (BP) ○ Cytotrophoblastic cell column ○ Interstitial trophoblast ○ Endovascular trophoblast • Decidua basalis (DB) 	Labyrinthe type: • Labyrinth zone (L) ○ Syncytiotrophoblast ○ Cytotrophoblast • Basal zone (B) ○ Spongiotrophoblast ○ Glycogen cell ○ Trophoblastic giant cell • Decidua basalis (DB) 
ii) Placental barrier	Maternal circulation > Syncytiotrophoblast > Basement membrane > Foetal capillary > Foetal circulation	Maternal circulation > Cytotrophoblast > Syncytiotrophoblast I > Syncytiotrophoblast II > Basement membrane > Foetal capillary > Foetal circulation
iii) Trophoblast invasion	Deep intrauterine – endovascular and interstitial	
iv) Spiral artery remodelling	Disappearance of smooth muscle, basement membrane restructuring, trophoblast cell replacement of endothelium and acquisition of a pseudo-endothelial phenotype	
Hormonal maintenance of pregnancy	1st trimester: corpus luteum (source: placenta). 2nd & 3rd trimesters: progesterone and oestrogen	Primary hormone: corpus luteum (source: placenta). Secondary hormones: progesterone and androgens
Gestation length	Long (~ 9 months)	Short (~ 21 days)
Embryo/ Foetal period	1 (Embryo)/ 4 (Foetus)	3 (Embryo)/ 1 (Foetus)
Number of offspring at birth	Typically monotocous/ ditocous	Polytocous

Rats with pigmented eyes such as Lister-hooded have better visual acuity and are adept in visual discrimination tasks (Aggleton et al., 1996; Bussey et al., 2008; Prusky et al., 2002). Using Lister-hooded rats, our laboratory has routinely characterised various behavioural phenotypes akin to schizophrenia, including hyperactivity, impaired NOD and PPI, in addition to abnormal neuronal and endocrine functions, such as altered serotonergic and cholinergic neurotransmission, reduced neurogenesis and modified gut microbiota following post-weaning SI (Dunphy-Doherty et al., 2018; Fone et al., 1996; King et al., 2009; McIntosh et al., 2013; Muchimapura et al., 2003). Consistent with others which have demonstrated persistent PPI deficits in Lister-hooded rats following post-weaning SI, analysis of behavioural profiles of rats across thirteen studies conducted in our laboratory confirmed the high reproducibility of such SI-induced behavioural alterations in the same strain, observing hyperactivity in a novel arena and NOD impairments in 92% and 69% of the studies, respectively (Cilia et al., 2001; Cilia et al., 2005; Fone & Porkess, 2008). Given that SI-induced behavioural alterations can be strain-dependent, such that locomotor hyperactivity for instance is more marked in Lister-hooded rats compared to Sprague-Dawley, we therefore selected Lister-hooded as the most appropriate strain for implementing the 'dual-hit' poly(I:C)-SI model (Hall et al., 1998). The usage of Lister-hooded rats in the implementation of our newly proposed model would also enable phenotypical comparisons with other recent 'dual-hit' models established using the same strain, including the neonatal PCP-SI and MK-801-SI models (Gaskin et al., 2014; Gaskin et al., 2016; Gilabert-Juan et al., 2013). As sexual dimorphism has been observed for both infection and SI-associated outcomes, it is vital to examine the effects of the combined approach in both genders (Estes & McAllister, 2016; Fone & Porkess, 2008).

Epidemiological observations have demonstrated that maternal viral infection during the second trimester in particular, is associated with heightened risk of schizophrenia (*cf.* maternal bacterial infection during first trimester (Brown et al., 2000; Mednick et al., 1988; Sørensen et al.,

2009). This suggests variations in maternal immune response and foetal vulnerability to insults across gestation and highlights the importance of the timing of poly(I:C) challenge (Kentner et al., 2019; Meyer et al., 2009b). In rats, gestation lasts around 21 days, with GD1-10 and GD11-20 corresponding to the first and second trimesters in humans, respectively (Qiang et al., 2002). Research groups have used a variety of timings and frequency of poly(I:C) administration, ranging from repetitive daily to single injections in early (typically GD9-11), mid (GD15) or late gestation (GD19; where GD1 is designated as day after overnight mating; Tables 1.3 & 1.5). As we are interested in the potential mechanisms underlying behavioural alterations induced by a viral-mimetic, GD15, which corresponds to the middle of human second trimester and is employed by most studies (Tables 1.3 & 1.5), would seem the most appropriate timing for immune stimulation. Depending on the objective of the studies, alternate-day (e.g. single injections on GD14, 16 and 18) or chronic dosing regimes have been used (Forrest et al., 2012; Vorhees et al., 2015). As studies have previously shown that a single injection is capable of inducing sickness behaviour in pregnant dams and results in schizophrenia-like behaviour in resultant offspring (Tables 1.3 & 1.5), to minimise stress on dams, we opted for single dosing.

The route of administration can affect the nature and robustness of the elicited immune response (Meyer et al., 2009b). The i.v. and i.p. routes of administration represent the two most common dosing method employed by laboratories conducting MIA research (Tables 1.3 & 1.5). Given that poly(I:C)-induced behavioural or physiological changes have been demonstrated in both dams and their offspring in studies utilising the i.p. route of administration (total 17 studies, doses ranging from 0.5-20 mg kg^{-1}; Tables 1.3 & 1.5), and also in compliance to the 3Rs (Replacement, Reduction, Refinement), we selected this mode of delivery to minimise stress to the pregnant rodents.

As the effects of poly(I:C) could be strain-dependent, we focused on published studies which used Lister-hooded rats and also our desired route of administration. There was only one publication which met this criteria. The study, which eventually proceeded with Wistars conducted a pilot study beforehand to establish a strain and dose of poly(I:C) that triggers a robust systemic inflammatory response in rats (available as abstract at the time of review, now published as Murray et al., 2019). In this study, although 10 mg kg^{-1} poly(I:C) (i.p.) evoked the most robust response, characterised by increased IL-6 and hyperthermia at 3 h post-injection in non-pregnant Wistar females, the equivalent dose elicited comparable responses in Lister-hooded females. In other rat strains, 10 mg kg^{-1} poly(I:C) has also been shown to induce changes in both dams and offspring (5 studies; Tables 1.3 & 1.5). On this basis, we decided that 10 mg kg^{-1} would be a sensible starting point in our studies.

Most studies (40 out of 45 that provided information) sourced poly(I:C) from Sigma, with a minority obtaining it from InvivoGen (4 studies) and Amersham Biosciences (1 study; Table 2.2). Research conducted in the UK has utilised poly(I:C) from Sigma and successfully demonstrated physiological and behavioural abnormalities in dams and/or offspring, justifying this source for the current study. Both poly(I:C) potassium and sodium salts from Sigma have been used, however, despite both are water-soluble, reconstitution of the sodium salts might be challenging, and heating and cooling might be necessary for re-annealing of the double-stranded homopolymer (Sigma-Aldrich, 2012). To avoid such issue, we purchased the potassium salt.

In the first study in this chapter, in order to validate the physiological effects of poly(I:C), non-pregnant Lister-hooded female rats received a single i.p. poly(I:C) (10 mg kg^{-1}) or saline vehicle injection and body temperature and home cage activity were assessed for 4 h, as a similar study which administered 10 mg kg^{-1} (i.p.) in the same strain demonstrated elevated body temperature and plasma IL-6 protein expression at 3 h post-injection (Murray et al., 2019). Brain regions were

Table 2.2. Source and description of poly(I:C) used in poly(I:C) MIA studies. The location where MIA was performed, the vendor of the poly(I:C) and any further details were extracted from 45 publications available at the time of literature review (October to November 2015). Eight other publications (not listed) did not comment on poly(I:C) source. *study available as abstract at time of review; NL, Netherlands; NZ, New Zealand.

Location of MIA	Source	Further description of poly(I:C)	Reference
Australia	Sigma, Australia		Dalton et al., 2012
Australia	Sigma-Aldrich, Australia		Duchatel et al., 2016
Australia	Sigma, Australia		Verdurand et al., 2014
Australia	Sigma, Australia		Zavitsanou et al., 2014
Belgium	Sigma-Aldrich, USA	Sodium salt	Missault et al., 2014
Belgium	Sigma-Aldrich, Switzerland	Potassium salt	Van den Eynde et al., 2014
Canada	InvivoGen, USA	High molecular weight	Ballendine et al., 2015
Canada	InvivoGen, USA	High molecular weight	Howland et al., 2012
Canada	Amersham Biosciences, UK		Petrovic & Piquette-Miller, 2010
Canada	InvivoGen, USA	High molecular weight	Sangha et al., 2014
Canada	InvivoGen, USA	High molecular weight	Zhang et al., 2012
China	Sigma, St. Louis, MO		Han et al., 2011
China	Sigma-Aldrich, Switzerland	Potassium salt	Song et al., 2011
Germany	Sigma, Germany		Klein et al., 2013
Germany	Sigma, Germany		Mattei et al., 2014
Germany	Sigma, Germany		Yee et al., 2012
Germany/ Spain	Sigma, Germany		Hadar et al., 2015
Israel	Sigma, Israel		Patrich et al., 2016a
Israel	Sigma, Israel		Piontkewitz et al., 2009
Israel	Sigma, Israel		Piontkewitz et al., 2011
Israel	Sigma, Israel		Piontkewitz et al., 2012
Israel	Sigma		Zuckerman & Weiner, 2003
Israel	Sigma, Israel		Zuckerman & Weiner, 2005
Israel	Sigma, Israel		Zuckerman et al., 2003
Israel	Sigma, Israel		Farrelly et al., 2015a
Japan	Sigma Aldrich, MO		Ohkawara et al., 2015
Japan	Sigma, St. Louis, MO		Oh-Nishi et al., 2010
NL	Sigma, Germany	P1530 sodium salt	Yee et al., 2011
NZ	Sigma, New Zealand		Dickerson et al., 2014
UK	Sigma, UK	P0913 sodium salt, Y-irradiated	Forrest et al., 2012
UK	Sigma, UK	P0913 sodium salt, Y-irradiated	Khalil et al., 2013
UK	Sigma, UK	P9582 potassium salt	Murray et al., 2019*
UK	Sigma-Aldrich, UK	P9582 potassium salt	Vernon et al., 2015
UK	Sigma, UK	Potassium salt	Wallace et al., 2014
USA	Sigma	P1530 sodium salt	Bronson et al., 2011
USA	Sigma, St. Louis, MO		Chou et al., 2015a
USA	Sigma, St. Louis, MO		Chou et al., 2015b
USA	Sigma, St. Louis, MO		Gilmore et al., 2005
USA	Sigma, St. Louis, MO		Hemmerle et al., 2015
USA	Sigma	P1530 sodium salt	Richtand et al., 2011
USA	Sigma, St. Louis, MO	P1530 sodium salt	Richtand et al., 2012a
USA	Sigma	P1530 sodium salt	Richtand et al., 2012b
USA	Sigma	P1530 sodium salt	Roenker et al., 2011
USA	Sigma, St. Louis, MO	P1530 sodium salt	Vorhees et al., 2012
USA	Sigma, St. Louis, MO	P1530 sodium salt	Vorhees et al., 2015

collected for cytokine analyses to confirm the immunological impact of poly(I:C) in the CNS, and to map the precise area(s) responsible for driving cytokine-induced sickness behaviour (Dantzer et al., 2001).

The second study in this chapter assessed the combined effects of gestational MIA exposure and post-weaning SI on Lister-hooded rats. Time-mated dams received 10 mg kg^{-1} poly(I:C) (i.p.) and sickness behaviour were monitored. Resultant offspring were subjected to social or individual housing from weaning (PND22). Given that SI consistently causes hyperactivity in Lister-hooded rats when placed in a novel arena from two weeks of single housing, LMA was assessed in adolescent male and female rats subjected to single housing for two weeks to confirm such hyperactivity and to determine if there is a gender-specific effect (Fone & Porkess, 2008). Brain tissues were harvested for HPLC with electrochemical detection (HPLC-ED) evaluation of monoamines (dopamine and 5-HT) and their metabolites, as these brain regions and alterations in the said substances are implicated in schizophrenia (Estes & McAllister, 2016; Fone & Porkess et al., 2008; Meyer et al., 2009b). Following a comprehensive test battery relevant to the positive, negative and cognitive symptoms of schizophrenia to identify behavioural impairments induced by MIA and/or SI in a separate group of adult male rats, cytokine and mTOR phenotyping were performed to determine the potential mechanisms underlying behavioural abnormalities. Potential early mechanisms were also probed. We hypothesise that the combined stressors would act synergistically to induce more severe behavioural and brain abnormalities relevant to schizophrenia in Lister-hooded rats than either intervention alone.

2.2 Aims

The aims of the current set of experiments were to:

(1) determine if 10 mg kg^{-1} poly(I:C) (i.p.) induces a short-term sickness response in non-pregnant Lister-hooded female rats without causing detrimental or lethal effects;

(2) examine if combined MIA using poly(I:C) administration to the pregnant rat with subsequent post-weaning isolation rearing produces sex-specific effects on offspring behaviour in adolescence, in order to select the most appropriate gender for behavioural and neurochemical phenotyping in adulthood;

(3) characterise the combined effects of MIA via poly(I:C) administration to the pregnant rat and subsequent post-weaning isolation rearing, on the behaviour and neurochemistry of offspring in adulthood;

(4) assess brain tissues and plasma collected from adolescent offspring exposed to poly(I:C)-induced MIA and post-weaning isolation rearing in order to probe early mechanistic insight preceding behavioural and/or neurochemical alterations in adulthood.

2.3 Materials and methods

2.3.1 Animals

Non-pregnant and time-mated pregnant female Lister-hooded rats were acquired from Charles River UK, Kent. The latter arrived at the Bio-Support Unit (BSU) of the University of Nottingham on GD7-10 (the day when a vaginal plug was found was designated as GD1). Temperature microchips (Bio-Thermo™idENTICHIP®, Animalcare Limited, York, UK) were implanted subcutaneously (s.c.) 24 h before drug treatment in the nape of the neck of the rats to enable temperature measurement. Pups

were born and weaned in the BSU. Rats were kept on a 12:12-h light-dark cycle (lights on 07:00-19:00 h) under constant ambient temperature and humidity (21 ± 2ºC and 55 ± 10%, respectively) with food (2018 Teklad Global 18% Protein Rodent Diet, Envigo (formerly Harlan)) and water available *ad libitum*, unless required for behavioural studies. All experiments conformed to the Animals (Scientific Procedures) Act 1986 and ARRIVE guidelines and were approved by the Animal Welfare and Ethical Review Body of the University of Nottingham.

2.3.2 Drug preparation

All drugs were prepared on the day of injection. Rats received either 10 mg kg^{-1} poly(I:C) acid potassium salt (P9582; Sigma-Aldrich, UK) dissolved in 0.9% saline or 1 ml kg^{-1} saline i.p.. The route of administration and dose were selected from relevant publications as discussed above in the chapter introduction.

2.3.3 Experimental design

2.3.3.1 Experiment 1: The effect of poly(I:C) via i.p. administration on non-pregnant Lister-hooded rats

Injections were carried out between 10:00 and 11:00, in a dimly lighted behavioural suite where eight female rats (140-169 g; housed 4 per group) were habituated for 30 min before injection. Baseline weight, temperature and home cage activity of the rats were recorded prior to injection of poly(I:C) (10 mg kg^{-1}, i.p.) or vehicle control 1 ml kg^{-1} saline. After injection, each rat was returned to its home cage and body temperature monitored every 10 min for 4 h and home cage behaviour scored, as described in section 2.3.4). Rats remained in the same behavioural suite throughout the experiment. Immediately following the last behavioural and body temperature assessment, rats were killed by a schedule 1 procedure (concussion followed by exsanguination) and brain tissues and plasma collected (see section 2.3.6). The experimental protocol is shown in Figure 2.1.

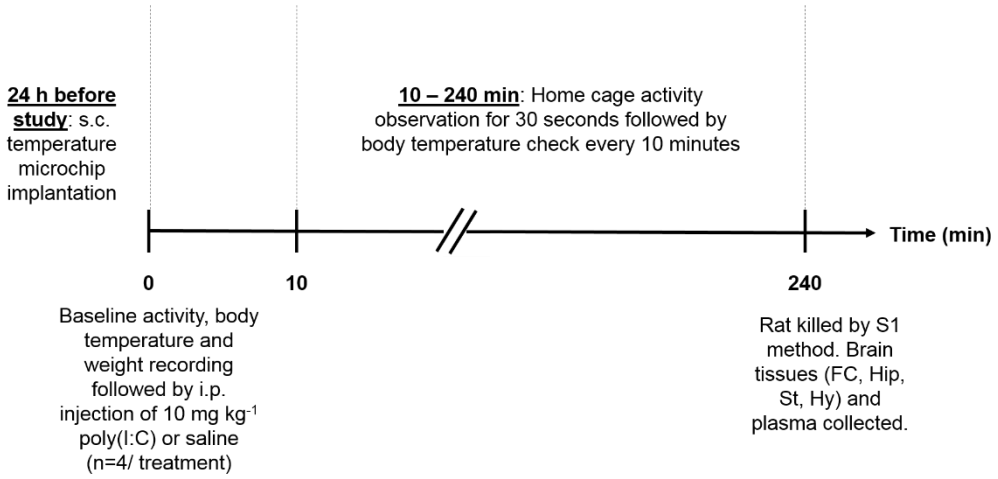


Figure 2.1. Experimental protocol for Experiment 1. Non-pregnant female Lister-hooded rats (n=4 per treatment, housed 4 per cage) were implanted with a subcutaneous (s.c.) temperature microchip 24 h before drug administration. On the experiment day, baseline body temperature was measured prior to intraperitoneal (i.p.) injection of 10 mg kg^{-1} polyinosinic:polycytidylic acid (poly(I:C)) or 1 ml kg^{-1} saline. Rats were returned to their home cages and behavioural and temperature assessments were performed every 10 minutes for 4 hours. Rats were humanely killed by a schedule 1 method (concussion followed by exsanguination) and plasma and brain tissues collected immediately following the final temperature assessment. FC, frontal cortex; Hip, hippocampus; Hy, hypothalamus; St, striatum.

2.3.3.2 Experiment 2: The combined effects of gestational poly(I:C) via i.p. administration and post-weaning social isolation on behaviour and neurochemistry

Seventeen time-mated nulliparous dams were housed individually in double decker individually ventilated cages (GR1800 Double Decker, Techniplast, Italy; floor area: 1862 cm^2). The floor of the cages was lined with sawdust and soft white bedding, and each dam was provided with one wooden block and one cardboard tube. Dams were handled for 5 min per day for three days prior to temperature microchip implantation on GD14. In the morning of GD15 (10 – 11 am), they received 10 mg kg^{-1} poly(I:C) (n=9) or 1 ml kg^{-1} saline (n=8) i.p. and body temperature and home cage or sickness behaviour were assessed hourly for 6 h whereas body weight for three days. In adherence to the 3Rs, blood was not

collected from dams post-injection for cytokine measurements and the use of additional dams for this purpose was not justified.

Nearing the expected delivery dates, dams were checked twice a day for evidence of giving birth. The day on which pups were found was designated PND1. Dams were allowed to nurture their litters without disruption until PND20, when pups were counted and sexed while the dam was temporarily removed from the cage. On PND22, pups (n=128 from 16 litters; n=8 litters per treatment) were weaned and housed four per group with same-sex littermates or alone, giving four treatment x housing groups: vehicle-group (Veh-GH), poly(I:C)-group (poly(I:C)-GH), vehicle-isolation (Veh-SI) and poly(I:C)-isolation (poly(I:C)-SI). Dam composite sickness behavioural scores (see section 2.3.4) were used to assist selection of litters for subsequent behavioural studies and litter from one poly(I:C)-treated dam that did not show any sickness behaviour was excluded. Pups from each litter were assigned to GH and SI wherever possible, however, one Veh litter only contributed GH animals whereas four other Veh litters contributed only SI animals, and 4 poly(I:C) litters gave rise to only SI animals. Rats were housed in polycarbonate cages (GH, 32 x 51 cm; SI, 25 x 42 cm) fitted with metal grid lids and sawdust bedding, and kept in the same holding room to allow visual, auditory and olfactory contact. To establish the effect of gestational poly(I:C) on pregnancy and offspring, the number of pups born to each dam and the weight of pups at weaning were recorded. Handling was restricted to a single weekly cage change and welfare assessment besides behavioural testing.

Behavioural and neurochemical phenotyping in adolescence to assist gender selection for behavioural testing in adulthood

Resultant offspring of both sexes (n=8 per gender x treatment x housing) underwent LMA evaluation at two weeks post-weaning (PND36). Brain regions and plasma from these animals were collected. Neurochemical analyses using HPLC-ED were performed on the brain tissues to provide

additional support for gender selection, i.e. to determine if there are gender-specific differences in changes in monoamine neurotransmitters; sections 2.3.6 & 2.3.7).

Behavioural and neurochemical phenotyping in adulthood

A distinct group of male rats (gender selected based on data from adolescent rats; see results section) underwent comprehensive behavioural testing at weekly intervals commencing around four weeks post-weaning (Figure 2.2): LMA (PND51-57), NOD (PND52-58), NLD (PND59-65), social interaction (PND66-70) and PPI (PND73-78), followed by either associative learning, i.e. CFR (day 1: PND79-83; day 2: PND80-84), or ASST (end dates ~9-15 weeks post-weaning; n=16 per treatment x housing for all tests except for social interaction, where n=8 pairs per treatment x housing, and CFR and ASST, where n=8 per treatment x housing; each test described in detail later). USVs were recorded during the social interaction test to examine any impact on social communication. Tests were conducted in the order of the least to most aversive to minimise unanticipated behavioural alterations due to previous test experience, and also to reduce animal use in compliance with the 3Rs. At roughly eight weeks post-weaning, the ASST subgroup underwent food restriction but all other rats received food *ad libitum*. Tissue collection occurred one week after the final behaviour assessment (PND87-91) for the CFR subgroup (section 2.3.6).

Cytokine and mTOR evaluations to probe early mechanistic insights in adolescence

Brain regions from animals tested for LMA in adolescence were used for cytokine and mTOR analyses (sections 2.3.8.2 and 2.3.9), to probe potential early molecular mechanisms preceding behavioural alterations in adulthood.

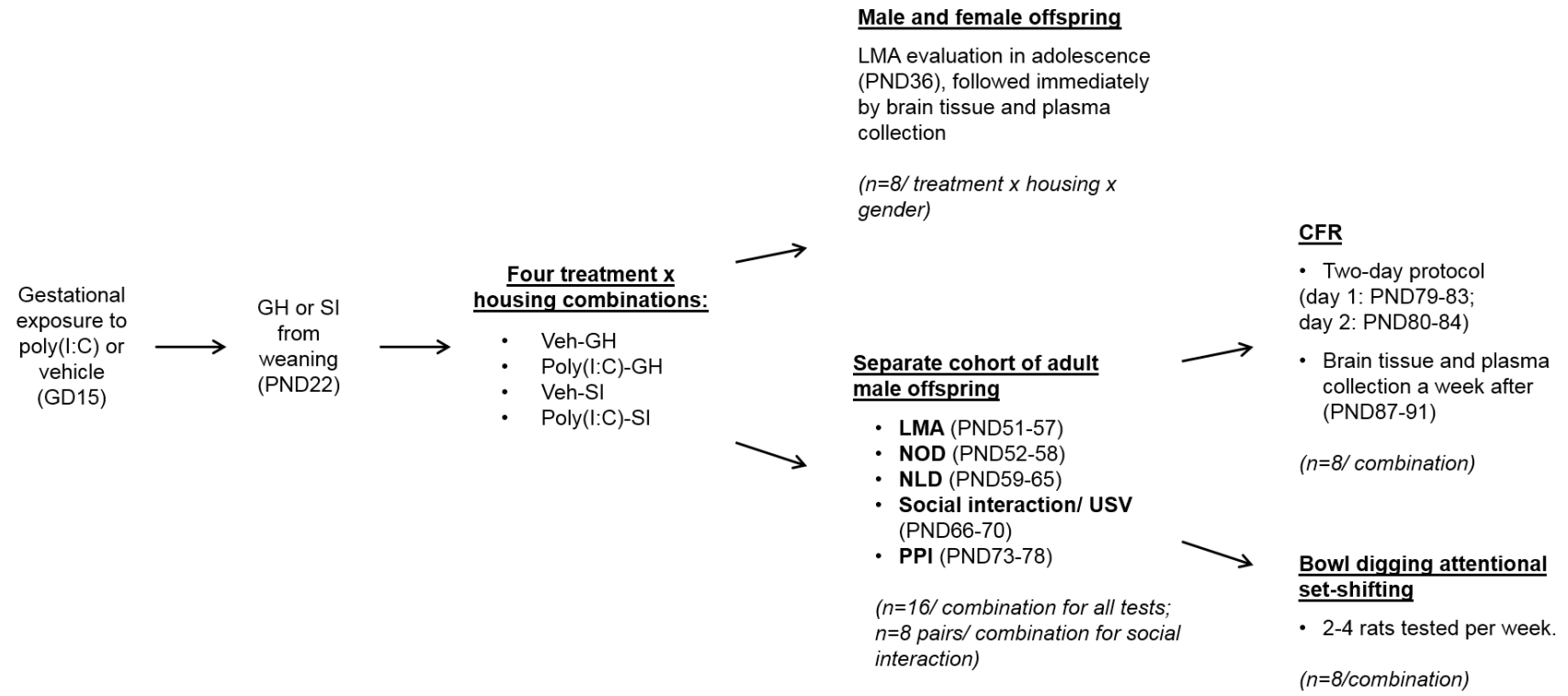


Figure 2.2. Experimental design for Experiment 2. Lister-hooded rats (n=128) were subjected to poly(I:C) or vehicle saline exposure on gestational day (GD) 15, followed by social or individual housing from weaning on postnatal day (PND) 22. At PND36, both genders were assessed for locomotor activity (LMA) and brain tissue and plasma collected immediately after evaluation (n=64). A separate cohort of adult male rats (n=64) underwent a series of behavioural testing, in order of the least to most invasive, at weekly intervals in adulthood: LMA, novel object discrimination (NOD), novel location discrimination (NLD), social interaction and pre-pulse inhibition of acoustic startle (PPI) before being split into two groups to assess either associative learning in a conditioned freezing response (CFR) paradigm or rule learning in bowl digging attentional set-shifting. Tissue collection occurred a week after CFR for the CFR subgroup. The corresponding age of rats when undergoing each task and the *n* numbers are indicated in brackets.

2.3.4 Sickness behaviour

Rat body temperature was taken by positioning the temperature reader (Global Pocket Reader®, Destron Fearing, USA) close to the implanted chip on the outside of the home cage whenever possible, to minimise any acute stress but if required, the cage was gently opened and the temperature reader briefly inserted. Simple home cage behaviours were scored using a sickness behaviour scoring system (Table 2.3) by taking a 30-second activity snapshot prior to temperature measurements to prevent disrupted behaviours caused by the latter. A composite score was calculated from individual sickness behaviours, which included hunched posture, elevated respiration rate, piloerection and shivering. The presence of hunched posture, increased respiration and shivering was each given a score of 1 while piloerection was scored based on phenotypic severity, where mild and marked piloerection were given a score of 1 and 2, respectively. Abdominal pinching was scored based on phenotypic severity as described for piloerection. These scores, however, were not included in the calculation of the composite scores.

Table 2.3. Sickness behaviour scoring system used for monitoring behaviour of non-pregnant and pregnant females following 10 mg kg^{-1} poly(I:C) or vehicle saline i.p. injection. Hunched posture, elevated respiration rate, piloerection and shivering were deemed 'sickness behaviour'. The presence of hunched posture, elevated respiration and shivering was each given a score of 1 whereas 0 indicated absence of these observations. Piloerection was scored based on phenotype severity, where mild presence was denoted by a score of 1 and marked presence by a score of 2.

Home cage behaviour	Score
Locomotor activity	
resting - normal posture	0
resting - hunched posture	1
Respiration rate	
Slow	0
Normal	0
Elevated	1
Piloerection	
None	0
Mild	1
Marked	2
Shivering	
Absence	0
Presence	1

2.3.5 Behavioural apparatus and procedure

All tests were performed during the light phase of the light/dark cycle. All studies were conducted under dim light (80 lux) unless otherwise stated. All equipment were thoroughly wiped with 20% (v/v) ethanol between trials. As drug injections were carried out by an experienced staff from the animal facility, the experimenter was unaware of the treatment received by the non-pregnant (section 2.3.3.1) and pregnant (section 2.3.3.2) females and the neurodevelopment history of the resultant offspring (section 2.3.3.2) until required for data analysis.

2.3.5.1 Locomotor activity

Activity was recorded in a clear Perspex box (39 x 23.5 x 24.5 cm) covered with a wire mesh lid using Photobeam Activity System (San Diego Instruments, CA; Dunphy-Doherty et al., 2018; Gaskin et al., 2014). Two arrays of infra-red beams at 3.2 cm and 7 cm above the floor, respectively, produced counts from beam breaks recorded in 5-min time bins for 1 h for horizontal ambulatory activity (a count required two consecutive lower level beam breaks), fine movement (repeated breaking of one lower level beam break) and rearing (one upper level beam break).

2.3.5.2 Novel object discrimination

NOD was conducted 24 h after LMA in the same box for each rat using a previously published protocol (Bianchi et al., 2006; Gaskin et al., 2014; Watson et al., 2012) with minor modifications, such that an ITI of both 1 and 2 h was used. The 1h ITI was selected based on a previous study demonstrating hippocampal involvement in motor learning in the NOD task using a 1h ITI (Gould et al., 2002). As a 4h ITI was known to disrupt discrimination ability, a shorter ITI of 2 h was included to assess if it would engender more robust difference between GH and SI animals (King et al., 2004). Each rat was acclimatised to the empty box for 3 min, returned to

its home cage for 1 min and re-introduced into the box for two 3-min exploration trials, separated by the 1 or 2 h ITI. Rats were exposed to two identical objects (plastic bottles fully filled with water and covered with white masking tape; height 8 cm x diameter 5 cm; Bibby Sterilin, Staffordshire, UK) affixed with blu-tack to the front left and back right quadrants of the removable box floor during the first (familiarisation) trial. One of the bottles, chosen in a pseudorandom manner (for each group, bottle at the front left quadrant chosen for half of the animals whereas bottle at the back right quadrant chosen for the remaining half), was replaced with a visually novel object (identical bottle with three additional 1.2 cm horizontal black tapes) during the second (choice) trial. Any faeces were removed between trials and familiar object wiped with ethanol. Exploration of each object defined active interest of the object (the nose <1cm from the object with active vibrissae) and sniffing, chewing or licking, was timed using stopwatches during the experiment. Prolonged chewing or climbing, sitting or standing on the object were not deemed active exploration. The discrimination ratio, [novel/(novel + familiar time)], which provides an index of preferential exploration of the novel object in the choice trial, was calculated for further analysis (Gaskin et al., 2014; Jones et al., 2011a). Successful discrimination was defined by a discrimination ratio of >0.5.

2.3.5.3 Novel Location Discrimination

Object location memory was assessed using a variation of the NOD task (Ennaceur et al., 1997; Westbrook et al., 2014). A day prior to NLD testing, each rat was habituated for 1 h in the LMA chamber (38.2 x 22.7 x 24.5 cm) surrounded by different spatial cues on the longer sides. Identical method to NOD was used but with a much shorter 1-min ITI, during which the location of one of the objects, selected in a pseudorandom fashion, was altered such that the new location was at the front right or back left. The 1-min delay was decided on the basis that rats exhibited robust preference for object at the novel location at delays of up to 15 min after

the first trial. Data acquired were analysed using the discrimination ratio, as described for NOD in section 2.3.5.2.

2.3.5.4 Social interaction

The social interaction task was adapted from previous studies (Kohli et al., 2019; Watson et al., 2016). Rats were weighed 24 h before the social interaction test and GH rats were immediately separated for overnight isolation in individual cages (25 x 42 cm). Each rat was paired with a rat from a different litter of similar weight (<30 g difference whenever possible; actual weight difference was between 1 and 33 g, with two poly(I:C)-SI pairs having a difference of >30 g) receiving identical prenatal treatment and housing condition. On the test day, members of each pair were coloured on the back with blue or pink spray (Superdrug Colour Hairspray Blue/Pink, code 221367 and 221362) 30 min before entry into an open-field arena (height 45 cm x diameter 75 cm at 40 lux) positioned below a video recorder. Interaction between rats was video-tracked for 10 min using EthoVision XT 8.5 (Noldus Information Technology, The Netherlands). Each rat was scored for seven distinct behaviours (Table 2.4) during playback of recorded videos. Data analyses were based on mean values obtained for each pair as the behaviour of each rat was dependent on the behaviour of its partner.

Table 2.4. Behaviours scored for the social interaction test. Each rat was manually scored for time spent engaging in the following seven behaviours during playback of social interaction videos. Scoring was done without prior knowledge of the prenatal treatment and housing conditions of rats. For analysis, mean values were calculated for the time spent in each behaviour and total social interaction duration for each rat pair, as rat activity during the social interaction task was not independent of its partner's behaviour (n=8 pairs per treatment x housing).

Social behaviour	Definition
Anogenital sniffing	Sniffing of the anogenital region.
Body sniffing	Nose-to-fur sniffing of body parts except the anogenital region.
Crawling over and under	One rat climbing over the back or under the body of the other rat.
Following	One rat tailing the other rat around the arena.
Lying side by side	Both rats are stationary and lying next to each other for ≥ 2s.
Pinning	One rat has its forepaws placed on another rat that is in submissive position, i.e. rolled on its back.
Boxing and biting	Rats showing pawing or punching movements against another rat and/or biting.

2.3.5.5 Ultrasonic vocalisations

To probe social communication, both 22-kHz and 50-kHz USVs were recorded using an electret microphone (Emkay, Avisoft Bioacoustics, Germany) linked to the computer via the Ultrasound Gate detection unit (customised model 112, Avisoft Bioacoustics, Germany; Kohli et al., 2019; Watson et al., 2016). Audio files were saved as .wav files and analysed using SASLab Pro v 4.38 (Avisoft Bioacoustics, Germany). Files were manually truncated to ensure that only recordings within the 10-min social interaction session remained and batch-processed using the in-built Time Domain Filter function to remove irrelevant sounds. High and low pass filters were set at cut-off frequencies of 22 and 85 kHz, respectively. Spectrograms with a final frequency resolution of 488 Hz and temporal resolution of 0.128 ms were generated with the parameters: 512 FFT length, 75% frame size, flat top window and 93.75% temporal resolution overlap. Spectrograms were subjected to batch identification of calls using the 'Automatic parameter measurements; remove gaps' function using an appropriate threshold of -61dB, 20-ms hold time and a margin of 0.025 s. Manual identification of calls then enabled the number of calls for each treatment x housing combination to be identified based on broad and narrow classifications. Aversive 22-kHz calls were excluded from analyses due to low occurrence (section 2.4.2.3.2). Under the broad classification, 50-kHz calls were divided into three main types – flat (including short calls, upward ramps and downward ramps), step (step up, step down and multi-step calls) and trill (any calls with a trill component; see Figure 2.3 for representative examples). The narrow classification, on the other hand, were mainly based on the classification by Wright et al. (2010), which differentiated 50-kHz calls into 14 subtypes. Unlike the authors who divided trill calls into 'trill', 'flat-trill' and 'trills with jumps' subtypes, the current study has observed very low occurrence of composite calls with a trill component, i.e. flat-trill, step-trill and short-trill, and hence these were grouped together with the standard trill calls as 'trills'.

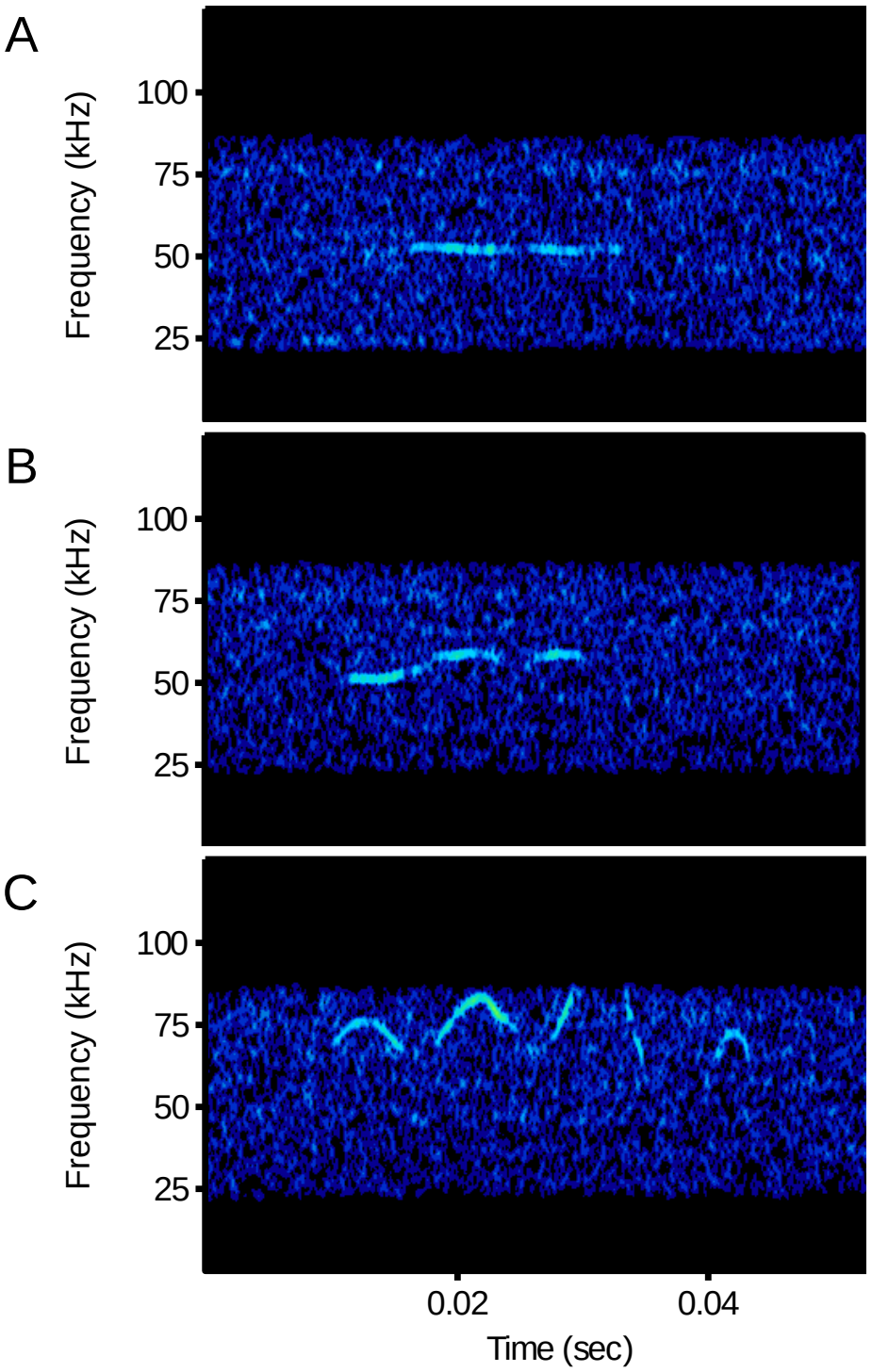


Figure 2.3. Representative examples of the three main types of ultrasonic vocalisation calls from the current experiment. (A) Flat, (B) step and (C) trill calls emitted during social interaction test at six to seven weeks post-weaning. Recordings were acquired using the Avisoft Bioacoustics Ultrasound Gate detection unit customised model 112.

2.3.5.6 Pre-pulse inhibition of acoustic startle

Sensorimotor gating was assessed using SR Lab Startle Response Systems (SD Instrument, San Diego), each comprising of a ventilated, well-lit (15 W) test cabinet (39 x 38 x 58 cm) fitted with a speaker to deliver acoustic stimuli and a transparent cylindrical enclosure (8.8 x 19.5 cm) attached to a solid base and coupled to a piezoelectric accelerometer to detect startle responses. Studies were conducted using a well-established protocol in our laboratory (Gaskin et al., 2014; Jones et al., 2011a; McIntosh et al., 2013). In batches of fours, rats were each assigned a test cabinet in a pseudorandom manner. Following 5-min exposure to background white noise (62 dB), rats received 10 x 120 dB startle tones before 50 trials of either a startle alone or a startle preceded by a pre-pulse (72, 76, 80, 84 dB) presented at unpredictable ITIs (mean 15 s). Each trial ended with 5 x 120 dB trials. Responses were acquired as 100 x 1-ms readings by Startle software (SD Instrument, San Diego) and recorded as area under the curve. % habituation was calculated by comparing the mean responses of the first ten 120 dB trials with that of the final five trials. % PPI was determined for each pre-pulse using the formula [(mean pulse alone-mean pulse)/pulse alone x100]. As the 72 dB pulse acted as a primer to the higher intensities, data from these trials were not included in the analysis. To avoid outlier responses due to rat movement coinciding with tone presentation from skewing the results, individual raw data values of ≥±2 standard deviation from the average startle response of the same trial type were eliminated via conditional formatting in Excel. One Veh-SI rat had outlier responses and therefore n=15 for Veh-SI group. No outlier responses were detected in the other three groups.

2.3.5.7 Conditioned freezing response

A two-compartment shuttle box (51 x 25 x 24 cm internal; PanLab, LE916, Harvard Apparatus Ltd.) comprising one light- and one dark-walled chamber, divided by an automatic door (10 x 10 cm) was used to examine

associative learning. The apparatus was operated using ShutAvoid v.1.8.2. (Harvard Apparatus Ltd.). A protocol that was previously described (Gaskin et al., 2014; Jones et al., 2011a; Watson et al., 2016) was used with slight modifications, where the protocol lasted for two days instead of three in the current study. Rats were placed into the light compartment for 30-s exploration before temporary opening of the door to permit access into the dark compartment whereupon the door was closed. After 30 s in the dark chamber, combined light and tone (5 s, 40 Lux, 89 dB, 3 kHz; CS) was presented, followed by a foot shock (1 s, 0.4 mA; US) in the last second of the CS. The CS-US pairing was administered on two more occasions at 55-s intervals. Latency to cross from the light to dark compartment and the time spent freezing in the 55 s after each CS-US pairing were recorded. Rats were returned to home cage after conditioning. Twenty four hours later, rats were returned to the dark compartment and time spent freezing in the 5 min before (for further analysis, duration spent freezing in the first 55 s and 56-300th seconds were recorded separately) and 5 min after presentation of the CS light and tone was manually timed using a stopwatch. Freezing was defined as state of immobility except for respiration in a hunched posture with inactive vibrissae. CFR data in the current study was collected by Dr Madeleine King (PhD supervisor).

2.3.5.8 Attentional set-shifting task

Behavioural flexibility was evaluated using the ASST adopted from Birrell & Brown (2000). The procedure, which lasted 8 days, consisted of habituation (2x15-min sessions per day for three consecutive days between days 1-6), training on day 7 and complex discrimination testing on day 8. Rats were given 12 g of food on days 1-6 and 10 g after training on day 7 to ensure motivation to dig for food rewards. Each rat was given ten Honey Loops (Honey Cheerios, Nestle, UK) in its home cage on day 1 to allow familiarisation with the new food. The apparatus used was a black, opaque Perspex box (50 x 70 x 50 cm) separated by a removable sliding opaque door into the test (front two-third of the box) and holding

(rear third) areas. The former consisted of two terracotta pots that were sunk into purposely-made holes on the floor, exposing only the rim at the floor level and each separated by a permanent transparent Perspex divider (Figure 2.4). On day 7, rats were trained to make simple discriminations (SD) following scent or digging medium to obtain a food reward. On day 8, rats underwent seven sequential discriminations: SD, compound discrimination (CD), first reversal (Rev1), intra-dimensional shift (ID), second reversal (Rev2), extra-dimensional shift (ED) and the third reversal (Rev3). An example test is shown in Table 2.5. In training and testing, rats were given four discovery trials at the start, i.e. allowed to explore and dig in the correct pot if their initial attempt was incorrect to learn the rule. From the 5th trial onwards, rats were only allowed to dig in one pot and were removed from the test area without receiving a food reward if they dug in the incorrect pot. Six consecutive correct trials had to be achieved before advancing to the next stage. The starting order and subsequent media/odour pairings received were balanced within each treatment-housing group (Table 2.6). The number of trials and errors taken to reach criterion were analysed. ID:ED and CD:Rev 1 ratios were determined based on equations $\frac{\mathrm{ED}}{(\mathrm{ID}+\mathrm{ED})}$ and $\frac{\mathrm{Rev1}}{(\mathrm{CD}+\mathrm{Rev1})}$, respectively. Two rats were unable to reach criterion for testing. Ms Rebecca Hock (PhD student at the University of Nottingham) and Dr Madeleine King collected the majority of ASST data in the current study.

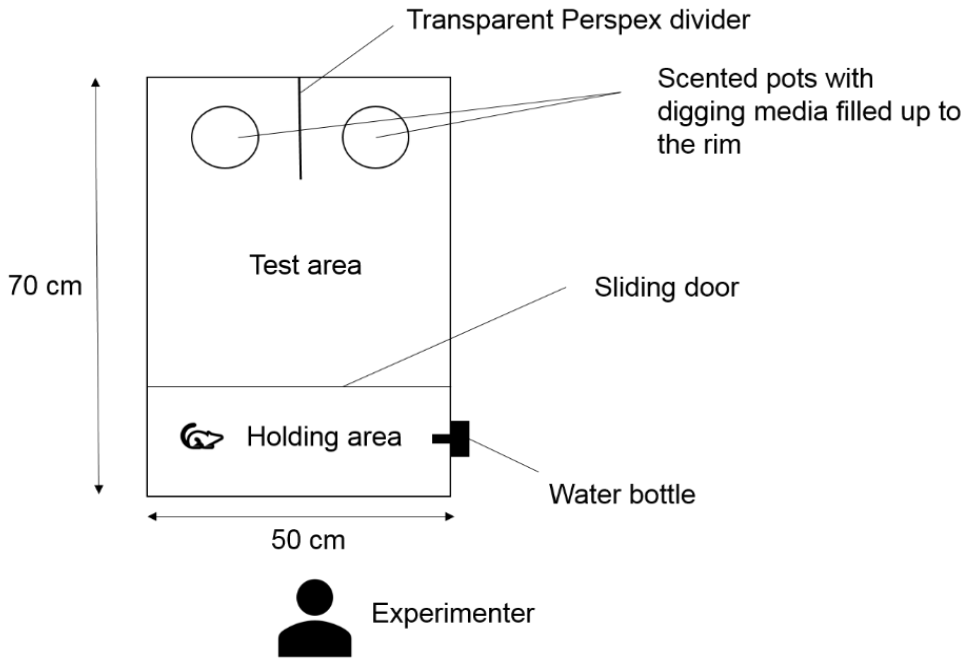


Figure 2.4. Technical set up for bowl digging attentional set shifting. The test apparatus used for attentional set-shifting task was a black opaque Perspex box (50 x 70 x 50 cm), which was divided by a removable sliding door of the same construct material into the holding area and test area. At the start of each trial, the rat was entered into the holding area, which had a fully filled water bottle mounted on the wall, allowing free access to water throughout the task (as testing duration varies depending on the performance of the rats). The sliding door was then lifted and the rat was permitted one-way access into the test area. The test area comprised two terracotta pots sunk into two custom-made holes, exposing only the scented rims. The pots were separated by a transparent Perspex divider, allowing rats to see from one pot to another but not moving swiftly between the two pots. Apart from swapping pots and clearing the test area between trials, the experimenter remained at the position shown in the figure to avoid animals using the experimenter as a visual cue to locate the pot with the rewarded stimuli.

Table 2.5. Media-odour combinations used in attentional set-shifting task. All rats received the same cue combinations during simple discrimination (SD) training but the order of starting cue was balanced in each treatment-housing group. Four media-odour combinations were used in compound discrimination (CD). The rewarded initial cues when media/odour dimension was relevant are shown in bold in the respective columns. Words in brackets represent abbreviations of the cues used.

Pairing	Media	Odour
SD training	**Pink Carefresh recycled paper pulp rodent bedding (Carefresh)**	**Satsuma**
	Medium white paper flake rodent bedding (Paper flake)	Vanilla & Tonka Bean
CD combination 1	**Fish tank gravel (Pebble)**	**Strawberry**
	Chinchilla dusting power (Sand)	Lavender
CD combination 2	**"Catsan" mineral-based cat litter (Mineral)**	**Green tea & Lemon**
	"Bio-catolet" cellulose paper-based cat litter (Cellulose)	Pomegranate & Raspberry
CD combination 3	**"Hama" plastic beads pastel colour mix (Plastic)**	**Exotic**
	White wax candlemaking beads (Wax)	Jasmine & White Frangipani
CD combination 4	**Beech chip reptile substrate (Wood chip)**	**Aloe & soft linen**
	"Tortoise Terrain" reptile substrate (Compost)	Sandalwood & ginger

Table 2.6. Order in which combinations were presented in the attentional set-shifting task. Eight orders of combinations were used for each treatment-housing group. As n=8 per treatment x housing, each order was used once. CD, compound discrimination; ED: extra-dimensional shift; ID: intra-dimensional shift; SD, simple discrimination, Rev1, reversal 1; Rev2, reversal 2; Rev3, reversal 3.

n	SD, CD, Rev1	ID & Rev2	ED & Rev3
1	Combination 1 (odour start)	Combination 2 (odour relevant)	Combination 3 (media relevant)
2	Combination 4 (media start)	Combination 1 (media relevant)	Combination 2 (odour relevant)
3	Combination 3 (odour start)	Combination 4 (odour relevant)	Combination 1 (media relevant)
4	Combination 2 (media start)	Combination 3 (media relevant)	Combination 4 (odour relevant)
5	Combination 1 (media start)	Combination 2 (media relevant)	Combination 3 (odour relevant)
6	Combination 4 (odour start)	Combination 1 (odour relevant)	Combination 2 (media relevant)
7	Combination 3 (media start)	Combination 4 (media relevant)	Combination 1 (odour relevant)
8	Combination 2 (odour start)	Combination 3 (odour relevant)	Combination 4 (media relevant)

2.3.6 Tissue collection and handling

Rats were killed by concussion followed immediately by exsanguination to confirm death. Mixed arteriovenous trunk blood (about 4 ml for each rat) was collected into lithium heparin-lined BD Vacutainer® Plus tubes (BD, New Jersey), immediately centrifuged for 5 min at 4^0C and 1200 G (Eppendorf™ 5702R), and plasma decanted into microcentrifuge tubes and flash-frozen in liquid nitrogen. Brains were dissected from the skull and regions of interest or brain hemispheres were collected on a refrigerated table (BC72: Osborne refrigeration, UK). Samples that were snap-frozen were then stored at -80^0C until further analysis.

2.3.6.1 Experiment 1: The effect of poly(I:C) via i.p. administration on non-pregnant Lister-hooded rats

The frontal cortex, hippocampus, hypothalamus and striatum were dissected, weighed and transferred into Eppendorf microcentrifuge tubes for flash-freezing in liquid nitrogen. Plasma samples and processed brain regions were subsequently used for cytokine analyses (section 2.3.8.1).

2.3.6.2 Experiment 2: The combined effects of gestational poly(I:C) via i.p. administration and post-weaning social isolation on behaviour and neurochemistry

The frontal cortices, hippocampi and striata from both hemispheres were collected from adolescent rats following LMA and flash-frozen in liquid nitrogen. The left brain regions were handled and processed for cytokine and mTOR evaluations, as indicated in sections 2.3.8.2 and 2.3.9, respectively. The right brain regions were used for evaluation of the levels of dopamine and 5-HT and their main metabolites – 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA), respectively, using HPLC-ED; section 2.3.7). In the adult CFR subgroup, the left frontal cortex, hippocampus and striatum were collected and processed for cytokine and mTOR analyses (sections 2.3.8.2 and 2.3.9). Plasma samples from

the adolescent rats and CFR subgroups were also included in the cytokine assays. All samples were analysed within 18 months of collection (Figure 2.5).

2.3.7 Neurochemical detection by HPLC-ED

Thawed samples were sonicated (Soniprep 150; MSE Scientific Instruments) for approximately 20 s in 0.05 M perchloric acid containing 0.4% w/v sodium metabisulfite and 0.2% w/v disodium ethylenediaminetetraacetic acid dehydrate (frontal cortex, 350-1000 µl; hippocampus, 500-550 µl; striatum, 400-1500 µl) then centrifuged at 17,400 x *g* at 4 ºC for 20 min (Centrifuge 5417R, Eppendorf). The resulting supernatant was filtered using a 0.45 µm syringe tip filter (ThermoScientific, India) and 30 µl of each sample was injected onto a Luna® 3 µm column (100 x 2 mm, part number 00D-4251-B0, Phenomenex) by a Perkin Elmer AS200 autosampler. Dopamine, DOPAC, 5-HT, 5-HIAA and HVA were detected using an Antec SenCell comprised of a 2mm glassy carbon working electrode set to +0.75 V, a stainless steel auxiliary electrode for counteracting potential polarisation effects at the electrodes, and three reference electrodes: salt bridge (Ag/AgCl), *in-situ* Ag/AgCl and HyREF™ (Pd/H_2).

Fresh mixed standards solutions were prepared daily from a 1 x 10^{-4} M stock solution and diluted with 0.05 M perchloric acid to concentrations of 1 x 10^{-8}, 4 x 10^{-8} and 1.5 x 10^{-7} M, and for striatal samples only, also 2 x 10^{-7} M. Standards were injected at the start and end of each day, and after every 8 samples. Samples were further diluted and re-evaluated if the concentration of any analyte was beyond the detection peak range. The mobile phase, which consisted of 20 mM potassium dihydrogen orthophosphate anhydrous, 20 mM sodium acetate (anhydrous), 0.1 mM disodium ethylenediaminetetraacetic acid (dihydrate), 0.16 mM 1-octanesulfonic acid and 12% methanol adjusted to pH 3.89 using orthophosphoric acid, was delivered at a flow rate of 0.18 ml min^{-1} via a Dionex P680 pump at 1300 psi. Instrument control, data acquisition,

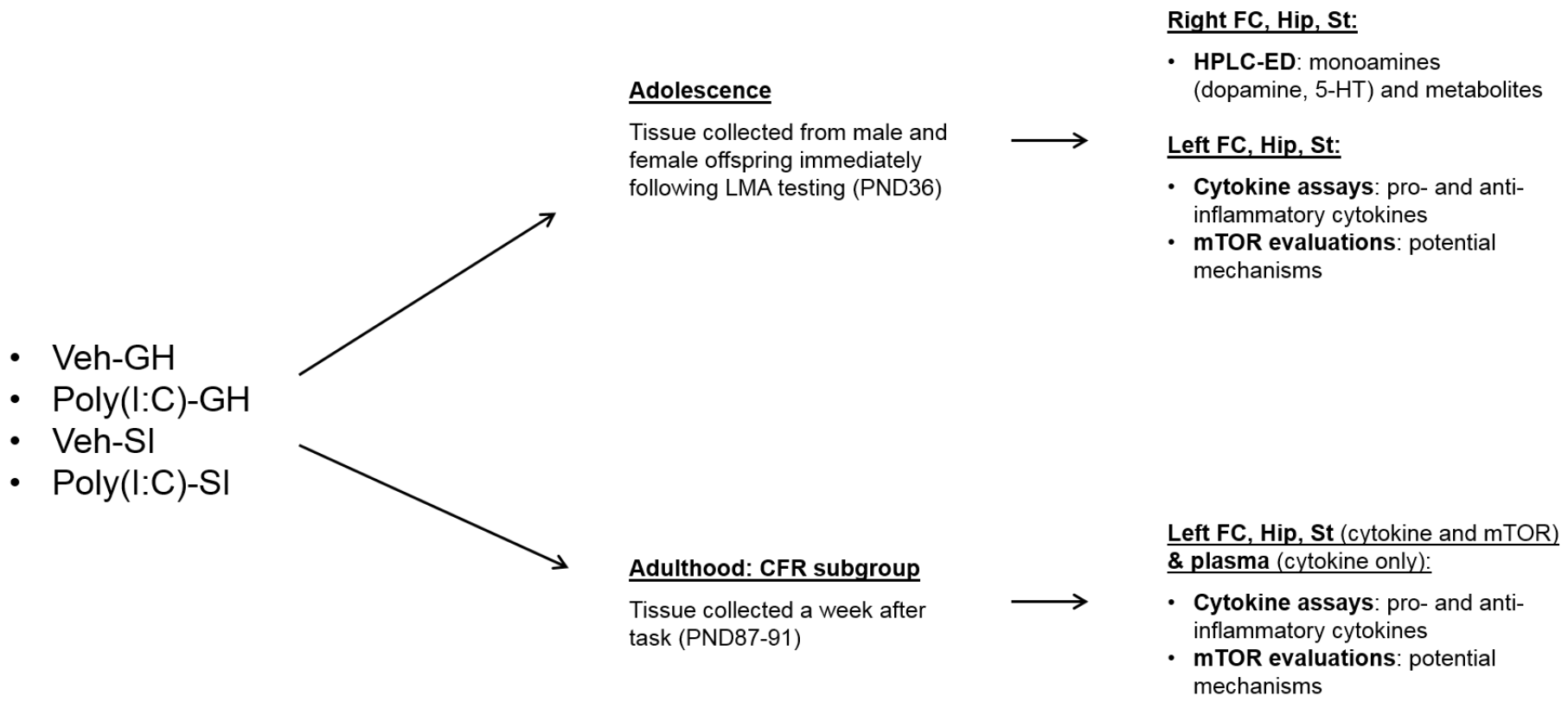


Figure 2.5. *Ex vivo* tissue analyses performed following behavioural testing in Lister-hooded rats exposed to gestational poly(I:C) (10 mg kg^{-1}, i.p.) or vehicle saline on gestational day 15 and/or post-weaning isolation rearing. Brain tissues or plasma were processed for evaluations using techniques such as high performance liquid chromatography with electrochemical detection (HPLC-ED; adolescent offspring) and cytokine and mTOR multiplex assays (adolescent and adult offspring) to probe potential mechanisms underlying behavioural alterations. ASST, attentional set-shifting task; CFR, conditioned freezing response; GH, group-housed; PND, postnatal day; SI, social isolation.

generation of calibration curves and quantification against standards were done using Clarity Chromatography Station (version 5.0.1.461; DataApex, Czech Republic). Representative chromatograms of a set of standards and a striatal sample are shown in Figures 2.6 and 2.7, respectively.

2.3.8 Multiplex cytokine analysis

2.3.8.1 Experiment 1: The effect of poly(I:C) via i.p. administration on non-pregnant Lister-hooded rats

Brain tissues were homogenised in lysis buffer [20 mM Tris, 1 mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 320 mM sucrose, 0.1% Triton X100, 1 mM sodium fluoride, 10 mM beta-glycerophosphate in distilled water adjusted to pH 7.6; 100 mg tissue/1 ml buffer] containing freshly dissolved protease inhibitor cocktail (P8340, Sigma-Aldrich) using a sonic probe for approximately 15 s (Soniprep 150, MSE Scientific Instruments). Tissue homogenates were placed on an orbital shaker at 4^0C for 30 min before centrifugation at 3824 x *g* for 5 min. Total protein concentration in each sample was quantified using Lowry assay (Lowry et al., 1951) and samples were adjusted to final protein concentrations of 5 mg ml^{-1} using appropriate volumes of 5:1 lysis buffer: solubilisation buffer (4% sodium dodecyl sulfate, 5% glycerol, 5% beta-mercaptoethanol, 0.01% bromophenol blue and 0.0625 M Tris hydrochloride). Cytokine analyses (IFN-Ɣ, IL-1β, IL-6, IL-10, TNF-α) were performed using MILLIPLEX$_{MAP}$ Rat Cytokine/Chemokine Magnetic Bead Panel (Cat.#RECYTMAG-65K, Merck Millipore) following instructions in the kit protocol. Neat plasma and also plasma diluted 1:5 in the assay buffer provided by the manufacturer were assayed. Plates were analysed using the MAGPIX® system with xPONENT® 3.1 (Luminex Corporation, Texas) and standard curves for interpolation of cytokine concentrations were fitted using GraphPad Prism 6 (GraphPad Software, Inc., California) with care taken to keep the interpolated values for the two Quality Controls in the ranges provided in the kit catalogue whenever possible (Dunphy-Doherty et al., 2018; Hind et al., 2016). All interpolated

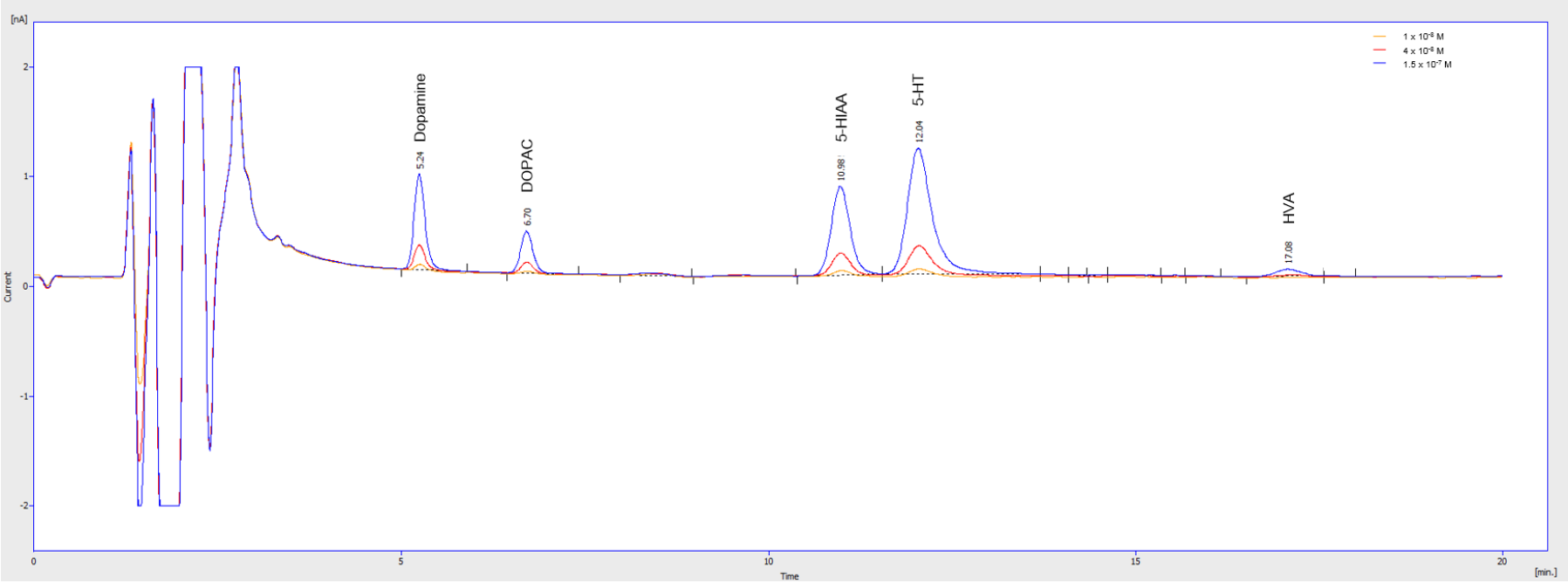


Figure 2.6. Representative chromatogram of standards used for generating calibration curves for quantification of monoamines and their metabolites. Fresh mixed standards solutions were prepared daily from a 1 x 10^{-4} M stock solution and diluted with 0.05 M perchloric acid to concentrations of 1 x 10^{-8} (orange), 4 x 10^{-8} (red) and 1.5 x 10^{-7} M (blue), and for striatal samples only, also 2 x 10^{-7} M (not shown). Standards were injected into a 3 µm column at the start and end of each day, and after every 8 samples. The five labelled peaks correspond to the time window when dopamine, 4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA), 5-hydroxytryptamine (5-HT) and homovanillic acid (HVA), were eluted from the column, respectively.

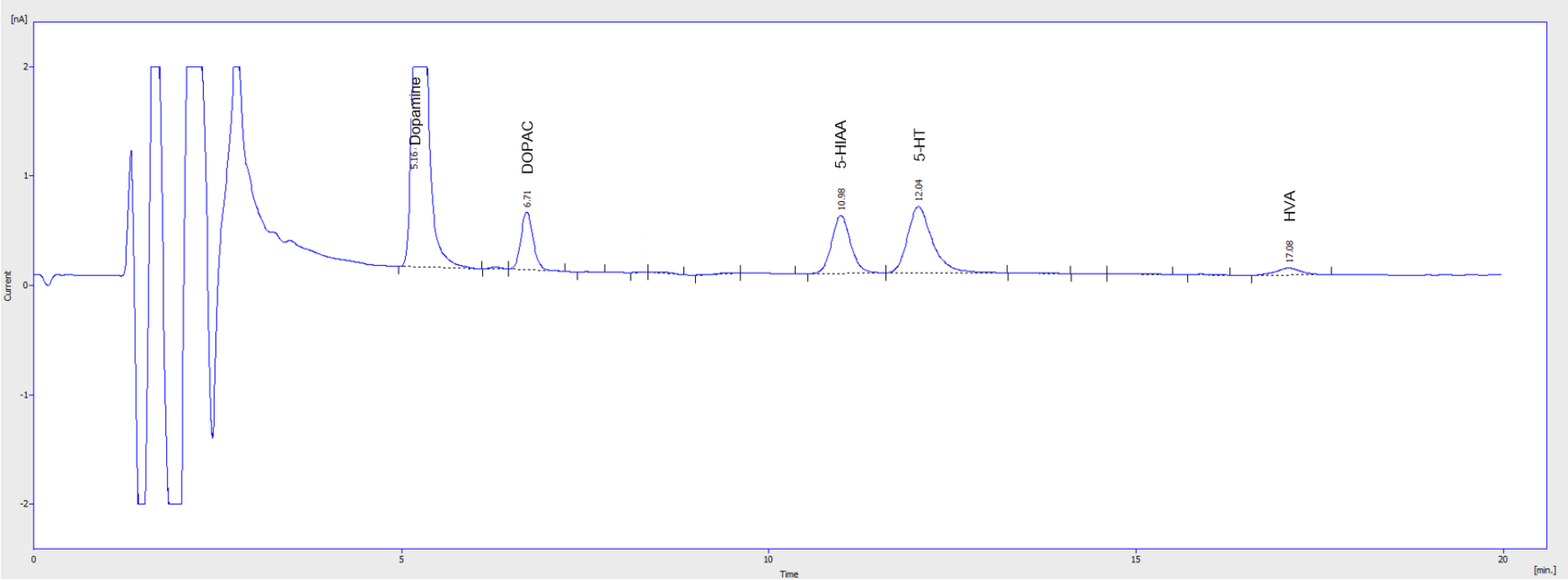


Figure 2.7. Representative chromatogram derived from homogenised striatal tissues from a rat tested for locomotor activity at PND36. *Ex vivo* tissue concentrations of monoamines and their metabolites were evaluated using high performance liquid chromatography with electrochemical detection. Samples were injected into a 3 µm column and the five labelled peaks correspond to the time window when dopamine, 4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA), 5-hydroxytryptamine (5-HT) and homovanillic acid (HVA), were eluted from the column, respectively.

values were corrected to give a total protein concentration of 1 pg mg^{-1} prior to statistical analyses. To avoid outlier readings due to technical errors such as pipetting from skewing the results, for each treatment x housing group, individual raw data values of ≥±2 standard deviation from the average concentration of the same cytokine type were eliminated. A representative standard curve is shown in Figure 2.8.

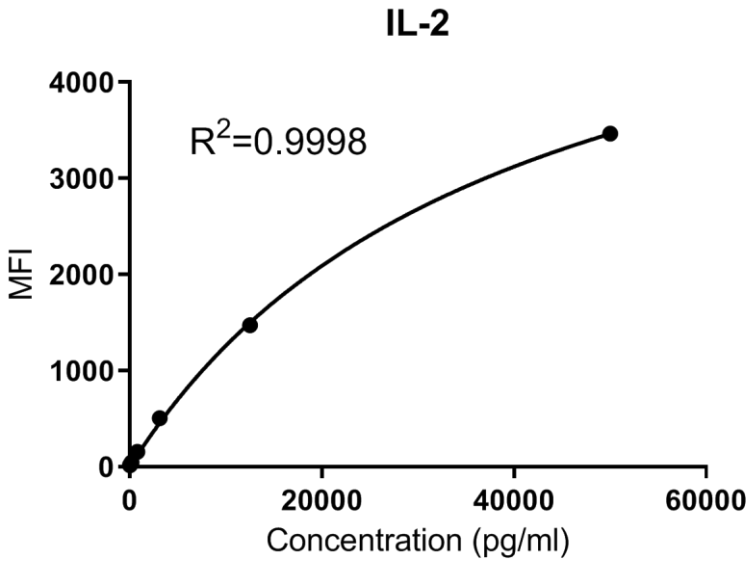


Figure 2.8. Representative cytokine standard curve. Cytokine readings were interpolated from best-fit standard curves generated using GraphPad Prism. In this particular example, the curve was fitted using the hyperbola function. The interpolated values of the two Quality Controls were in the range provided in the kit catalogue. MFI, mean fluorescence intensity.

2.3.8.2 Experiment 2: The combined effects of gestational poly(I:C) via i.p. administration and post-weaning social isolation on behaviour and neurochemistry

Brain tissues were homogenised in radioimmunoprecipitation assay (RIPA) buffer (R0278, Sigma-Aldrich; 120 mg tissue/1 ml buffer) containing freshly dissolved protease inhibitor cocktail (P8340, Sigma-Aldrich) using a sonic probe for approximately 15 s (Soniprep 150, MSE Scientific Instruments). Tissue homogenates were placed on an orbital shaker (4^0C for 1 h) and then centrifuged (3824 x *g* for 5 min). Total

protein concentration in each sample was quantified using Lowry assay (Lowry et al., 1951) and samples were adjusted to final protein concentrations of 8-13 mg ml^{-1} with appropriate volumes of RIPA buffer with protease inhibitor (100 µl protease inhibitor per 10 ml RIPA). Neat plasma from the CFR subgroup was analysed. Cytokine measurements and analyses (IFN-Ɣ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-18, TNF-α) were conducted as described in section 2.3.8.1.

2.3.9 mTOR analysis

Tissue homogenates used in cytokine analyses in Experiment 2 were also analysed for phosphorylated mTOR (Ser2448; phospho mTOR) and total mTOR using the MILLIPLEX® MAP 2-plex Phospho/Total mTOR Magnetic Bead Panel (Cat.#48-625MAG, Merck Millipore). The assays were performed according to the protocol and plates were analysed using the MAGPIX® system with xPONENT® 3.1 software. To avoid outlier readings due to technical errors such as pipetting from skewing the results, for each treatment x housing group, individual raw data values of ≥±2 standard deviation from the average reading of total mTOR or phospho mTOR were eliminated. Data analysis was performed as described previously (Dunphy-Doherty et al., 2018).

2.3.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 and IBM SPSS Statistics 22 (IBM, New York). Terminology of statistical tests used throughout this thesis is consistent with those used in Prism. Temperature (Experiments 1 & 2), sickness behaviour across time (Experiments 1 & 2), abdominal pinching across time (Experiments 1) and body weight (Experiment 2) data were analysed using two-way Repeated Measures (RM) ANOVA (factors treatment and time). Whilst sickness behaviour and abdominal pinching across time should be analysed by non-parametric means given that sickness and abdominal pinching scores are ordinal and therefore rely on the distribution of ranks

rather than assuming a normal or Gaussian distribution, there is no non-parametric equivalent of two-way RM ANOVA. It is arguably possible to perform a Mann-Whitney *U* test, the non-parametric equivalent of an unpaired Student's *t*-test, to compare between sickness or abdominal pinching score of vehicle versus poly(I:C) rats at each time point, however, this would only be sensible if sickness behaviour or abdominal pinching was not assessed in the same animals over time. Moreover, a time x treatment interaction would be unattainable using this method. The total sickness and abdominal pinching scores of vehicle and poly(I:C)-treated females were compared using Mann-Whitney *U*-test (Experiments 1 & 2).

Cytokine data from Experiment 1 were analysed using two-way RM ANOVA (factors treatment and brain region). In Experiment 2, the mean litter sizes of vehicle and poly(I:C)-treated dams were compared using unpaired *t*-test. The same data, and pup weaning weight, were analysed using two-way ANOVA (gender and treatment as between-subject factors). Two weeks post-weaning LMA time courses were analysed using four-way RM ANOVA (with time as the within-subject factor and prenatal treatment, housing and gender as between-subject factors) and cumulative totals for each activity type were analysed using three-way ANOVA (with prenatal treatment, housing and gender as between-subject factors). HPLC data were analysed using two-way ANOVA. For analysis of monoamine:metabolite ratios, Brown-Forsythe and Welch ANOVA tests were performed to ensure homogeneity of variance. Data that did not pass the homogeneity tests were transformed prior to further parametric analyses. Correlation analyses were conducted using Pearson's correlation coefficient.

LMA at around four weeks post-weaning, NOD, NLD, social interaction (pro-social and aggressive behaviours), PPI, CFR and body weight data were analysed by three-way RM ANOVA (with prenatal treatment and housing as between subject factors and time, object, behaviour type, prepulse intensity or shock number/pre- versus post-CS as within subject

factors). Cumulative total LMA, USV, ASST and mTOR data, and certain data from social interaction (total distance travelled, time spent in behaviour and velocity) and CFR (latency to enter dark chamber during acquisition and latency to following cue exposure) were analysed using two-way ANOVA with or without RM. Cytokine data from adolescence and adulthood were analysed by two-way ANOVA (prenatal treatment and housing as between-subject factors). In all analyses, the post-hoc test recommended by Prism was used. All cytokine and mTOR data were checked for normality using the D'Agostino & Pearson normality test recommended by Prism, and the Shapiro-Wilk normality test. All values are mean ± SEM. The significance level employed in all analyses was $p<0.05$.

2.4 Results

2.4.1 Experiment 1: The effect of poly(I:C) via i.p. administration on non-pregnant Lister-hooded rats

In order to establish whether i.p. poly(I:C) induced a short-term sickness response in non-pregnant female rats, body temperature and home cage activity were recorded for 4 h following injection. There was no significant change in body temperature in poly(I:C)-treated rats compared to vehicle controls throughout the monitoring period (Figure 2.9A). Analysis of the time course of sickness behaviour revealed a significant main effect of time ($F_{(24,144)}$= 6.05, $p<0.0001$) and a time x treatment interaction ($F_{(24,144)}$= 2.03, $p<0.01$). Poly(I:C)-treated females had increased sickness behaviour at 3 h post-injection (Figure 2.9B). However, poly(I:C) had no effect on the composite sickness score (Figure 2.9C). Time course of abdominal pinching showed a significant main effect of time ($F_{(24,144)}$= 1.71, $p<0.05$), such that poly(I:C)-treated females demonstrated significant abdominal pinching at 3:30 h post-injection (Figure 2.10A). Comparison of the total abdominal pinching scores between the two treatment groups, however, revealed no significant differences (Figure 2.10B). There was a significant main effect of brain region on IL-1β ($F_{(3,18)}$= 14.50, $p<0.0001$), IL-6 ($F_{(3,18)}$= 7.74, $p<0.01$), IL-10 ($F_{(3,18)}$= 7.09, $p<0.01$) and TNF-α ($F_{(3,18)}$= 3.44, $p<0.05$) levels, but poly(I:C) did not alter cytokine levels in any of the examined brain regions (data not shown). Plasma cytokine levels were below the detection limit. Albeit only four rats were used in this pilot study, significant sickness behaviour and a trend for decreased body temperature were observed in poly(I:C)-treated females. Therefore, further studies were warranted.

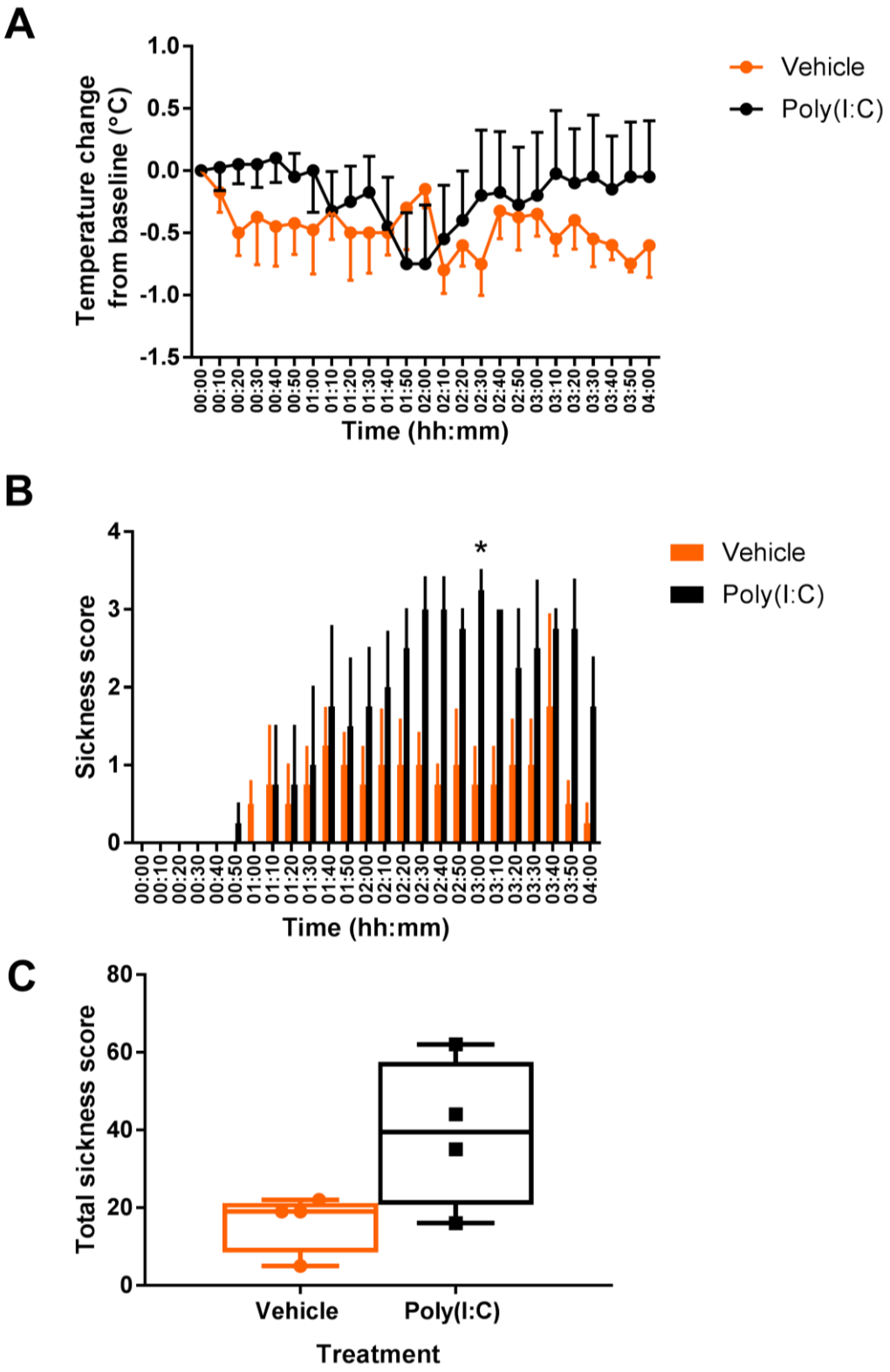


Figure 2.9. Effect of poly(I:C) (10 mg kg^{-1}, i.p.) on body temperature and sickness behaviour in non-pregnant female Lister-hooded rats. (A) Time course of body temperature change relative to baseline, **(B)** sickness score at 10 min intervals and **(C)** box-plot diagram showing total composite sickness score of rats injected with saline (median=19, range=5-22) or poly(I:C) (median=39.5, range=16-62) over the 4 h post-injection (n=4 per treatment). Absolute body temperature readings at baseline for vehicle and poly(I:C)-injected rats were 38.2 ± 0.1°C and 37.8 ± 0.3°C, respectively. Data are mean ± SEM values. *p<0.05 versus vehicle control; two-way RM ANOVA followed by Sidak's post-hoc test (A, B) or Mann-Whitney *U* test (C).

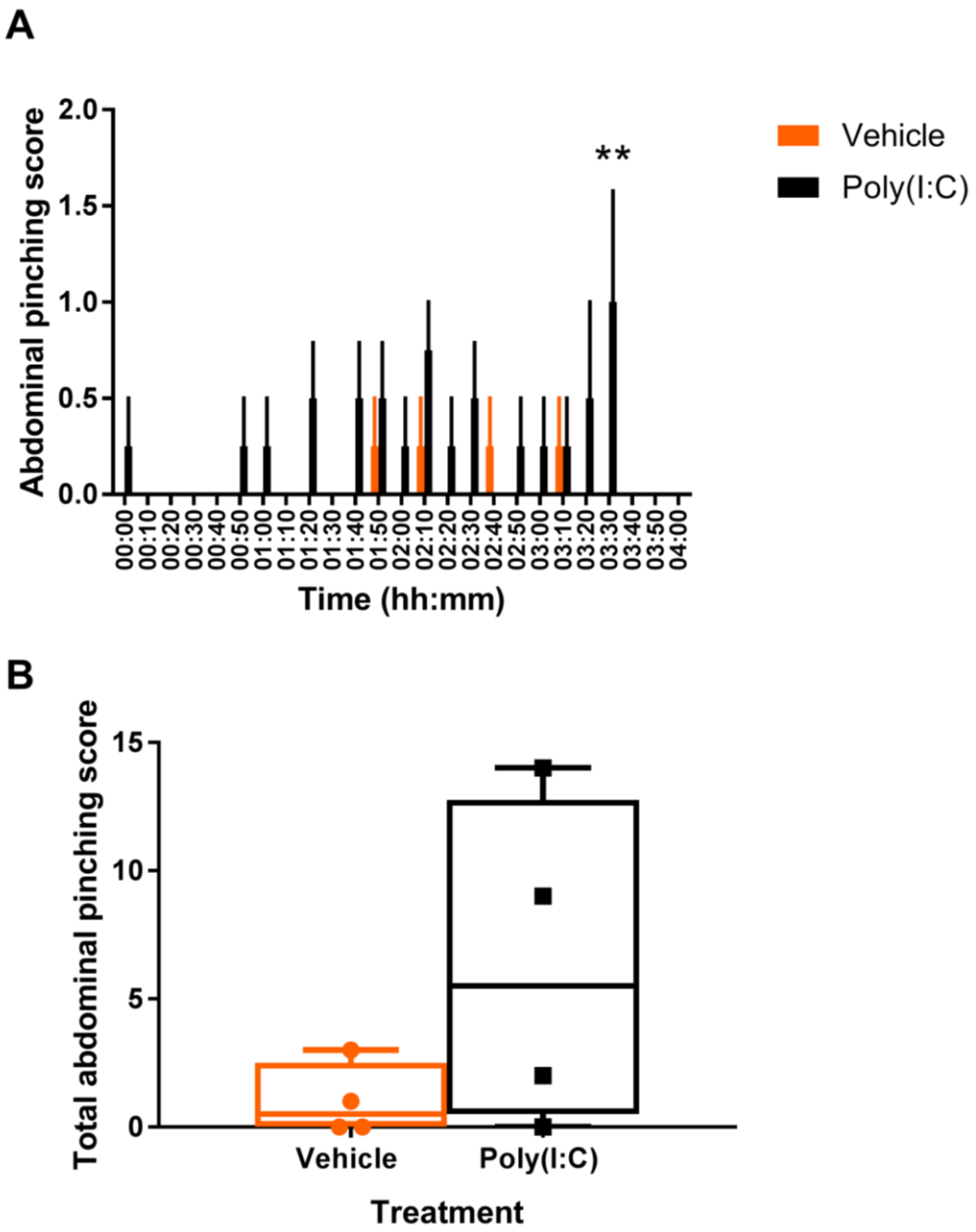


Figure 2.10. Effect of poly(I:C) (10 mg kg^{-1}, i.p.) on abdominal pinching in non-pregnant female Lister-hooded rats. (A) Time course of abdominal pinching and **(B)** total abdominal pinching in rats injected with saline (median=0.5, range=0-3) or poly(I:C) (median=5.5, range=0-14) over the four hours post-injection (n=4 per treatment). Data are mean ± SEM values. **p<0.01 versus Veh control; two-way RM ANOVA followed by Sidak's post-hoc test (A) or Mann-Whitney U test (B).

2.4.2 Experiment 2: The combined effects of gestational poly(I:C) via i.p. administration and post-weaning social isolation on behaviour and neurochemistry

2.4.2.1 Acute behavioural effects of poly(I:C) on dams

To ascertain that poly(I:C) caused acute sickness responses in dams, body temperature and sickness behaviour were monitored for 6 h and body weight for 3 days post-injection. There was a significant main effect of time ($F_{(8,112)}$= 14.06, p<0.0001) and a time x treatment interaction ($F_{(8,112)}$=5.005, p<0.0001) on body temperature, such that poly(I:C) significantly reduced body temperature at 2 h post-injection. Core body temperature returned to baseline level in the two groups by 4 h (Figure 2.11A). Body weight data showed a significant main effect of time ($F_{(2,28)}$= 141.80, p<0.0001) and treatment ($F_{(1,14)}$= 8.49, p=0.01). Poly(I:C)-treated dams showed lower weight gain at 24 h post-injection (Figure 2.11B). Furthermore, poly(I:C)-treated dams exhibited significantly more sickness behaviour, as reflected by an increase in the total composite score (Figure 2.12A). Time course analysis of the same data revealed a significant main effect of time ($F_{(5,70)}$= 5.51, p<0.001), treatment ($F_{(1,14)}$= 14.53, p<0.01) and a time x treatment interaction ($F_{(5,70)}$= 5.09, p<0.001), such that poly(I:C)-treated dams had significantly higher sickness scores at 3 h and 4 h post-injection (Figure 2.12B). Poly(I:C)-treated dams also exhibited increased abdominal pinching (Figure 2.12C).

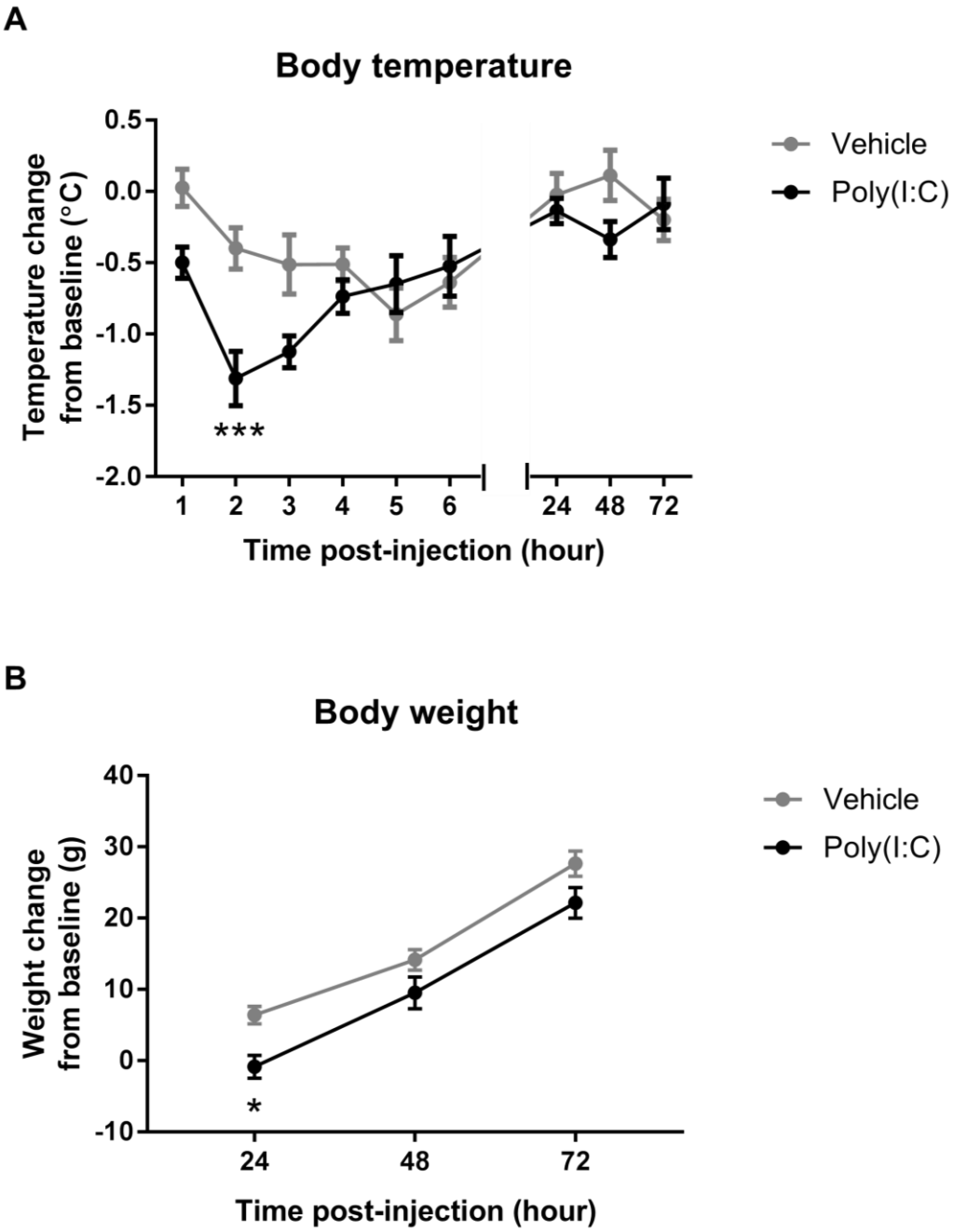


Figure 2.11. Effect of poly(I:C) on body temperature and weight in pregnant Lister-hooded rats. Mean ± SEM **(A)** body temperature and **(B)** weight change from pre-injection baseline following injection of either saline (n=8) or 10 mg kg^{-1} poly(I:C) (n=8) on gestational day 15. Absolute body temperature readings at baseline for vehicle and poly(I:C)-treated dams were 37.2 ± 0.2°C and 37.2 ± 0.1°C, respectively. Absolute baseline body weight for vehicle and poly(I:C)-treated dams were 309.0 ± 7.2 g and 318.0 ± 8.5 g, respectively. *p<0.05 and ***p<0.001 versus vehicle control; two-way RM ANOVA with Sidak's post-hoc tests (A,B).

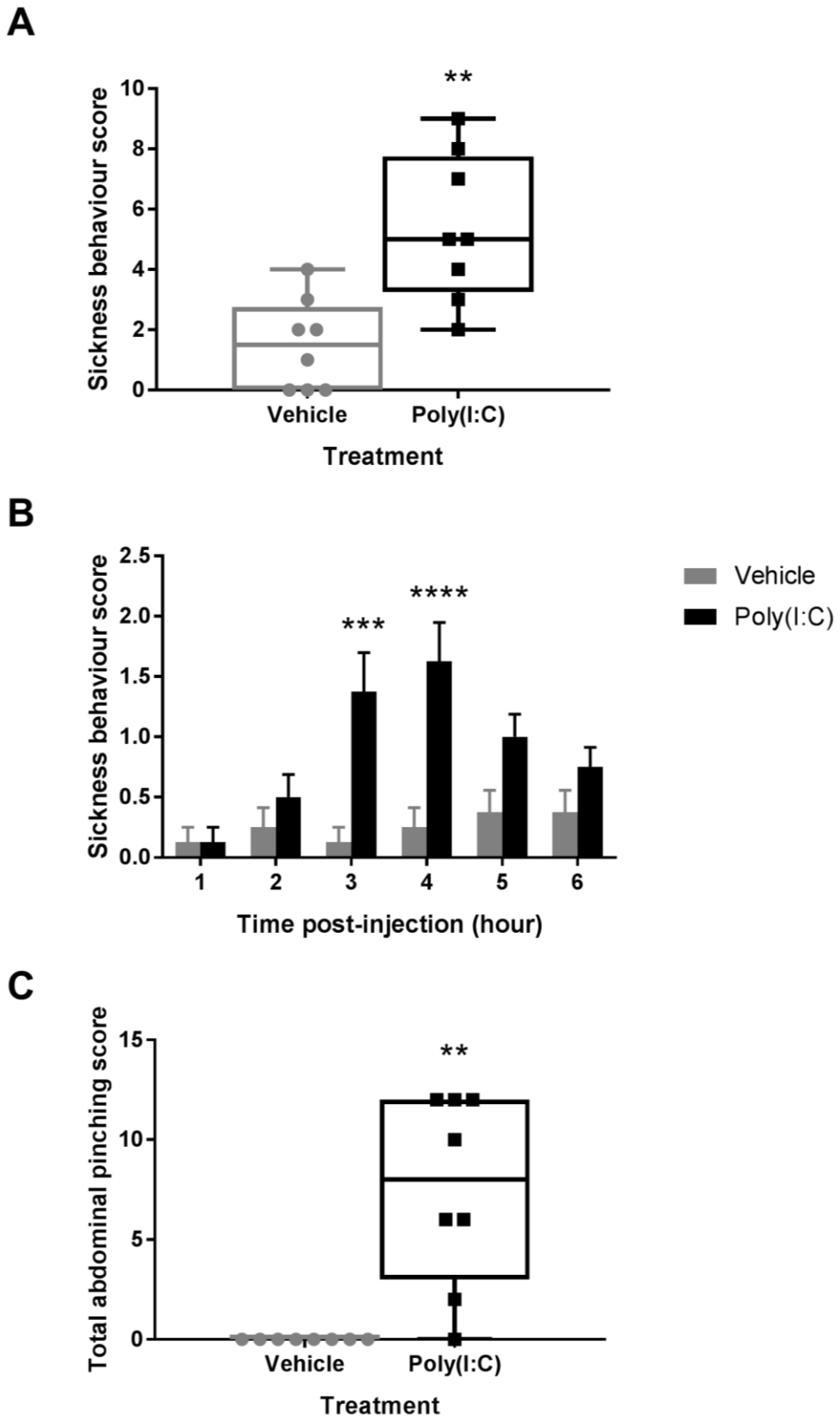


Figure 2.12. Sickness behaviour and abdominal pinching in pregnant Lister-hooded rats following 10 mg kg^{-1} poly(I:C) i.p. injection on gestational day 15. Mean ± SEM **(A)** composite sickness scores of dams injected with vehicle (median=1.5, range=0-4) or poly(I:C) (median=5, range=2-9) across the first 6 h post-injection, **(B)** time course analysis of the same data at hourly intervals and **(C)** total abdominal pinching score of dams treated with vehicle (median=0, range=0-0) or poly(I:C) (median=8, range=0-12; n=8 per treatment). **p<0.01, ***p<0.001 and ****p<0.0001 versus vehicle control; Mann-Whitney *U* test (A,C) or two-way RM ANOVA with Sidak's post-hoc (B).

2.4.2.2 Effect of poly(I:C) on offspring

A total of 215 pups (vehicle, 107; poly(I:C), 108) were born to the sixteen dams. As the dams were to be kept until parturition, it was not possible to examine the number of uterine implantation sites, damaged or dead foetuses, or placental sites without foetuses, to establish the occurrence of any pregnancy loss or foetal malformation. Maternal poly(I:C) injection did not influence mean litter size, male:female ratio or body weight at weaning (Table 2.7).

Table 2.7. Litter characteristics of Lister-hooded dams treated with poly(I:C) (10 mg kg^{-1}, i.p.) or saline vehicle on gestational day 15. Data are mean ± SEM except for total number of pups, number of male and female pups, where absolute numbers are presented (n=8 dams per treatment). Mean litter sizes of vehicle and poly(I:C)-treated dams were compared using unpaired Student's *t*-test. The same data and pup body weight at weaning were analysed by two-way ANOVA followed by Sidak's test, with gender and treatment as between-subject factors.

Prior to selection (n=16 litters):		
	Vehicle	**Poly(I:C)**
Total number of pups	107	108
Number of male pups	51	53
Number of female pups	56	55
Litter size	13.4 ± 0.9	13.5 ± 0.8
Number of male pups per litter	6.4 ± 0.9	6.6 ± 1.0
Number of female pups per litter	7.0 ± 0.8	6.9 ± 0.6
Weight at weaning (male pups)	38.8 ± 0.6	38.4 ± 0.9
Weight at weaning (female pups)	37.0 ± 0.7	38.9 ± 1.0
Following selection (n=16 litters):		
Total number of pups	64	64
Number of male pups	48	48
Number of female pups	16	16
Litter size	8.0 ± 1.8	8.0 ± 1.8
Number of male pups per litter	6.0 ± 1.1	6.0 ± 1.0
Number of female pups per litter	2.0 ± 0.9	2.0 ± 1.1
Weight at weaning (male pups)	38.4 ± 0.6	38.2 ± 0.8
Weight at weaning (female pups)	36.7 ± 0.9	34.1 ± 1.4

2.4.2.3 Effect of poly(I:C) and post-weaning isolation rearing on offspring

2.4.2.3.1 Adolescence (2 weeks post-weaning)

Locomotor activity

To confirm the development of SI behavioural syndrome, hyperactivity in a novel arena was examined together with the influence of gender in adolescent male and female rats. Habituation to the novel arena was reflected by a general decline in ambulation in all groups across time ($F_{(11, 528)}$= 162.88, p=0.001). There was a significant main effect of housing ($F_{(1,48)}$= 20.25, p=0.001) and gender ($F_{(1,48)}$= 15.84, p=0.001) as well as time x housing ($F_{(11,528)}$= 1.81, p=0.05), time x treatment ($F_{(11,528)}$= 1.92, p<0.05), time x gender ($F_{(11,528)}$= 2.23, p<0.05), time x housing x gender ($F_{(11,528)}$= 1.95, p<0.05) and housing x gender ($F_{(1,48)}$= 4.38, p<0.05) interactions on horizontal ambulation. Whilst poly(I:C) alone had no effect, Veh-SI and Poly(I:C)-SI males exhibited enhanced ambulation compared to Veh-GH males (Figure 2.13A). In female rats, no effect of SI or combined poly(I:C)-SI was observed, however, poly(I:C) on its own subtly reduced horizontal ambulation (Figure2.13C). Analysis of cumulative ambulation counts confirmed increased ambulation in Veh-SI males (Figures 2.13B).

Analysis of the time course of fine movement revealed a significant main effect of time ($F_{(11,528)}$= 22.76, p=0.001), housing ($F_{(1,48)}$= 30.71, p=0.001), gender ($F_{(1,48)}$= 21.39, p=0.001), and significant time x gender ($F_{(11,528)}$= 2.72, p<0.01) and housing x treatment x gender ($F_{(1,48)}$= 6.40, p<0.05) interactions. Poly(I:C)-GH, Veh-SI and Poly(I:C)-SI males, as well as Poly(I:C)-SI females exhibited increased fine movement between 40 and 60 min post-injection and in total across the 1 h observation period (Figures 2.14A-D).

There was a significant main effect of time ($F_{(11,528)}$= 36.23, p=0.001), housing ($F_{(1,48)}$= 14.75, p=0.001) and gender ($F_{(1,48)}$= 24.72, p=0.001) and a time x treatment ($F_{(11,528)}$= 1.86, p<0.05) interaction on rearing. SI increased rearing in males regardless of prenatal treatment whereas in females, only Veh-SI rats demonstrated increased rearing (Figures 2.15A & C). Analysis of cumulative rearing counts showed increased rearing in Veh-SI rats of both genders (Figures 2.15B & D).

Neurochemical analysis

To establish if LMA hyperactivity was associated with changes in monoamine neurotransmitters, *ex vivo* levels of dopamine, 5-HT and their metabolites in the frontal cortex, hippocampus and striatum of adolescent male and female rats were measured.

In males, there was a significant main effect of treatment ($F_{(1,28)}$= 12.20, p<0.01) on frontal cortical basal dopamine level. Housing significantly influenced basal HVA level in the hippocampus ($F_{(1,28)}$= 4.23, p<0.05), and dopamine ($F_{(1,28)}$= 5.09, p<0.05) and 5-HIAA ($F_{(1,28)}$= 18.96, p<0.001) levels in the striatum. SI increased striatal 5-HIAA when compared to Veh-GH and Poly(I:C)-GH regardless of prenatal treatment. Combined Poly(I:C)-SI also increased striatal dopamine (Table 2.8). In females, there was a significant treatment x housing interaction ($F_{(1,28)}$= 6.45, p<0.05) on hippocampal 5-HIAA levels but no post-hoc differences were found (data not shown).

Further analyses revealed no effect of treatment or housing on dopamine:DOPAC+HVA and 5HT:5-HIAA ratios in either brain region in males (Table 2.9). While treatment affected hippocampal 5-HT:5-HIAA ratio ($F_{(1,28)}$= 4.94, p<0.05) in females, no between-group differences were found (data not shown).

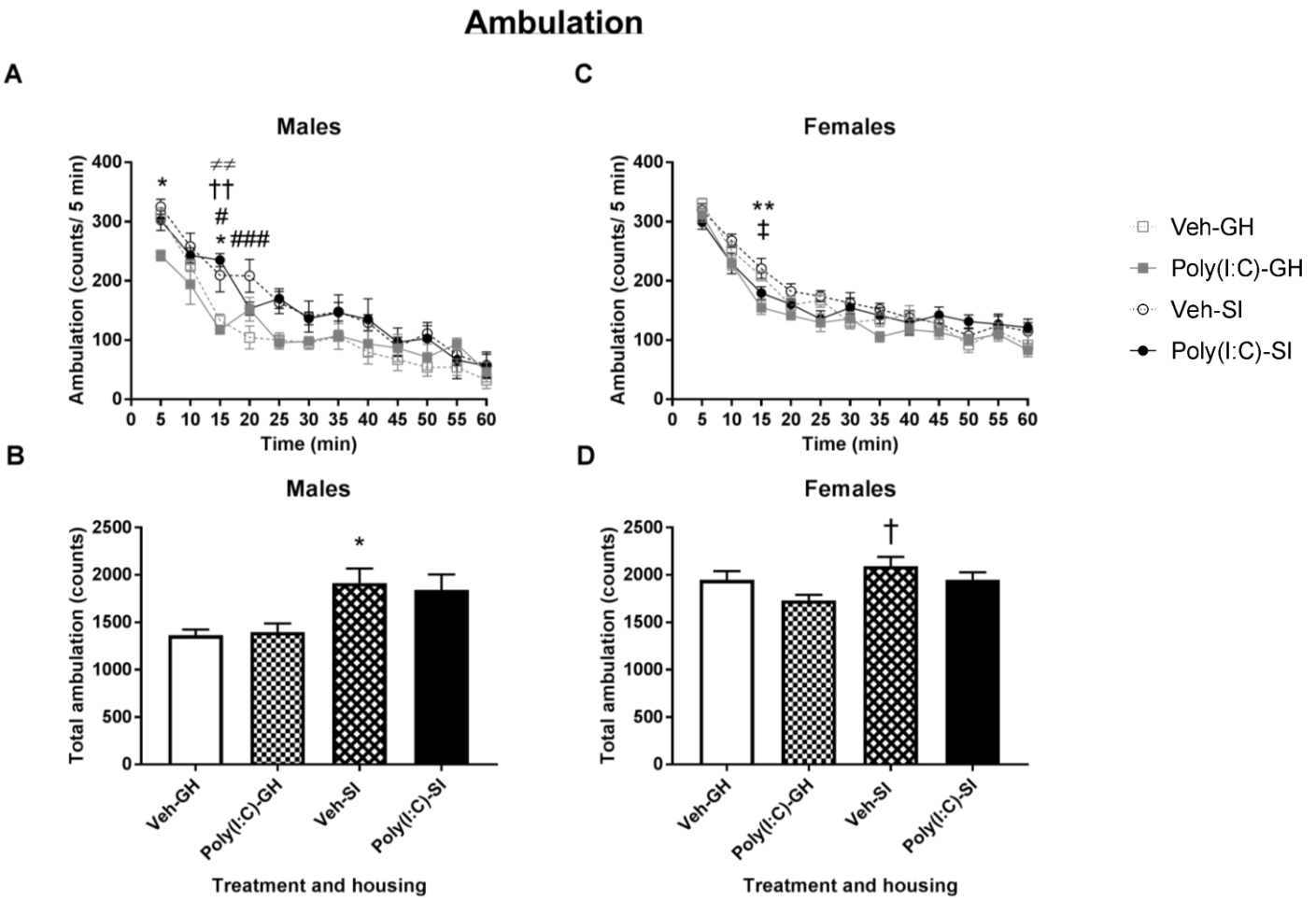


Figure 2.13. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on horizontal ambulation in Lister-hooded rats. Mean ± SEM **(A,C)** horizontal ambulation and **(B,D)** cumulative total ambulation in male (left panel) and female (right panel) adolescent rats (postnatal day 36) in a novel box across one-hour observation period (n= 8 per treatment x housing x gender, except for male Poly(I:C)-GH, n=4; male Poly(I:C)-SI, n=5; and female Poly(I:C)-SI, n=7 due to technical issues). *p<0.05, **p<0.01 Poly(I:C)-GH versus Veh-SI; #p<0.05, ###p<0.001 Veh-GH versus Veh-SI; ††p<0.01 Veh-GH versus Poly(I:C)-SI; ≠≠p<0.01 Poly(I:C)-GH versus Poly(I:C)-SI; ‡p<0.05 Veh-GH versus Poly(I:C)-GH; four-way RM ANOVA with Tukey's post-hoc test (A,C). *p<0.05 versus Veh-GH; †p<0.05 versus Poly(I:C)-GH; three-way ANOVA with Tukey's post-hoc test (B,D).

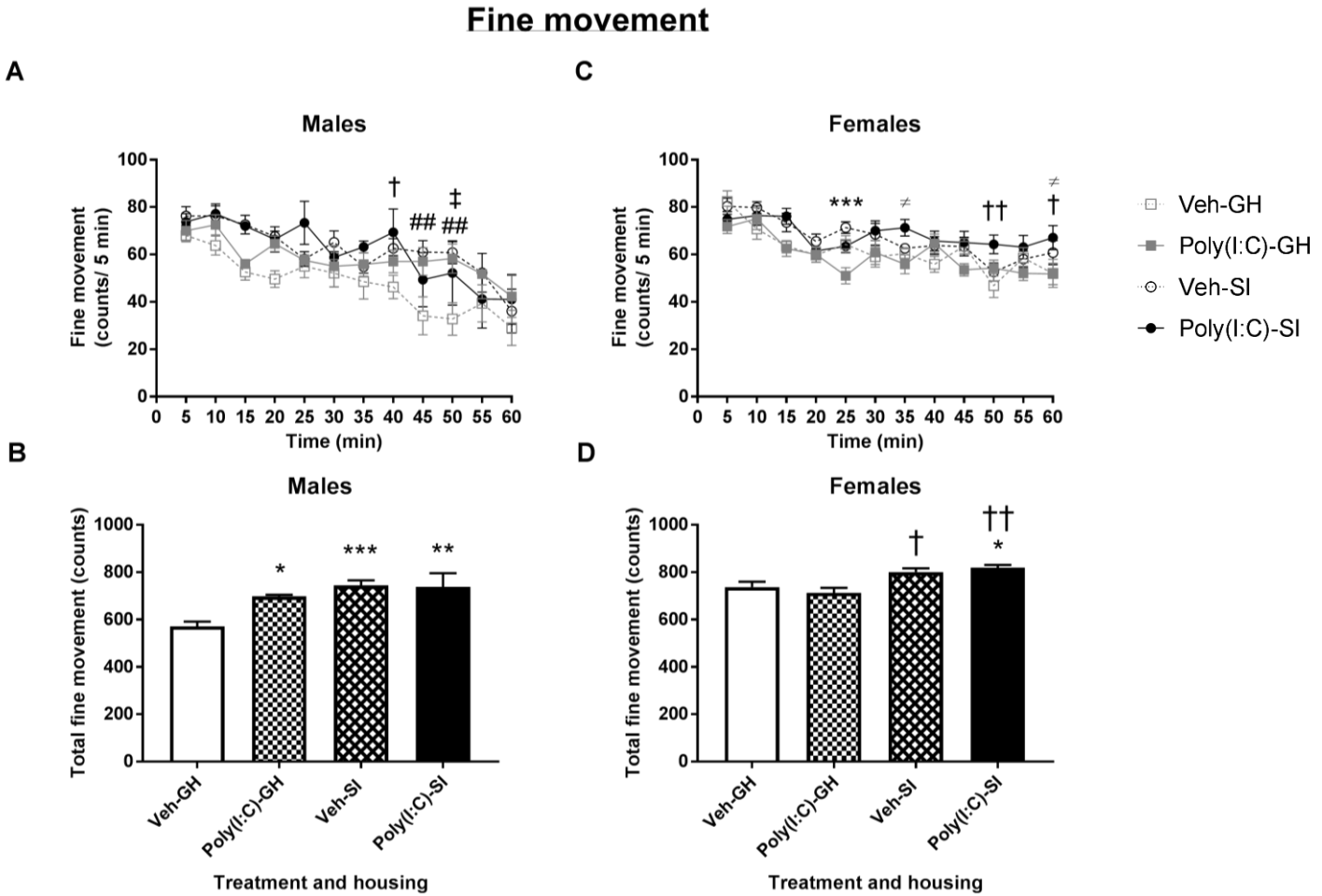


Figure 2.14. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on fine movement in Lister-hooded rats. Mean ± SEM **(A,C)** fine movement and **(B,D)** cumulative total fine movement in male (left panel) and female (right panel) adolescent rats (postnatal day 36) in a novel box across one-hour observation period (n= 8 per treatment x housing x gender, except for male Poly(I:C)-GH, n=4; male Poly(I:C)-SI, n=5; and female Poly(I:C)-SI, n=7 due to technical issues). †p<0.05, ††p<0.01 Veh-GH versus Poly(I:C)-SI; ##p<0.01 Veh-GH versus Veh-SI; ‡p<0.05 Veh-GH versus Poly(I:C)-GH; ***p<0.001 Poly(I:C)-GH versus Veh-SI; ≠p<0.05 Poly(I:C)-GH versus Poly(I:C)-SI; four-way RM ANOVA with Tukey's post-hoc test (A,C). *p<0.05, **p<0.01, ***p<0.001 versus Veh-GH; †p<0.05, ††p<0.01 versus Poly(I:C)-GH; three-way ANOVA with Tukey's post-hoc test (B,D).

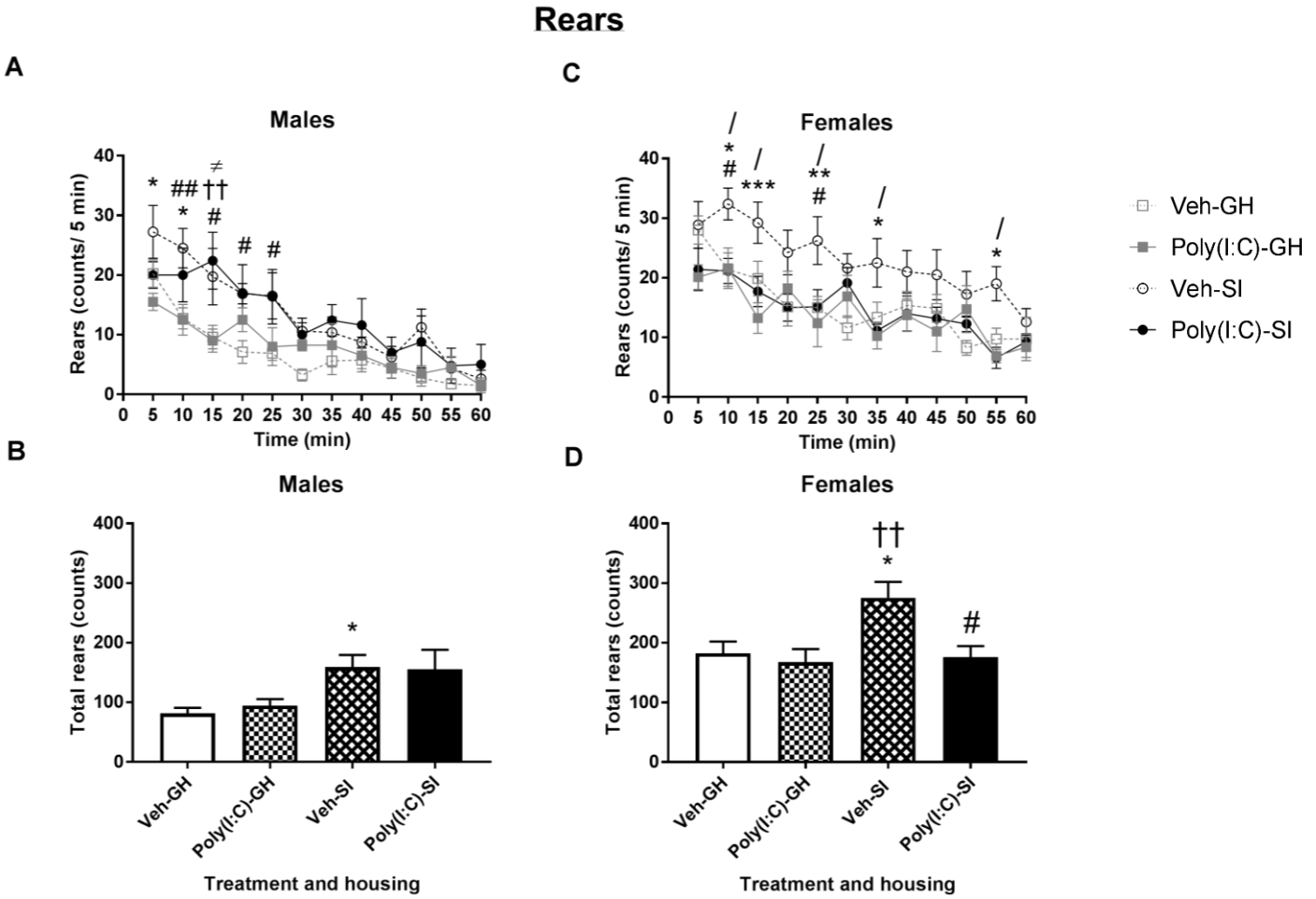


Figure 2.15. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on rears in Lister-hooded rats. Mean ± SEM **(A,C)** rears and **(B,D)** cumulative total rears in male (left panel) and female (right panel) adolescent rats (postnatal day 36) in a novel box across one-hour observation period (n= 8 per treatment x housing x gender, except for male Poly(I:C)-GH, n=4; male Poly(I:C)-SI, n=5; and female Poly(I:C)-SI, n=7 due to technical issues). *p<0.05, **p<0.01, ***p<0.001 Poly(I:C)-GH versus Veh-SI; #p<0.05, ##p<0.01 Veh-GH versus Veh-SI; ††p<0.01 Veh-GH versus Poly(I:C)-SI; ≠p<0.05 Poly(I:C)-GH versus Poly(I:C)-SI; /p<0.05 Veh-SI versus Poly(I:C)-SI; four-way RM ANOVA with Tukey's post-hoc test (A,C). *p<0.05 versus Veh-GH; ††p<0.01 versus Poly(I:C)-GH; #p<0.05 versus Veh-SI; three-way ANOVA with Tukey's post-hoc test (B,D).

Table 2.8. Basal levels of monoamines and their metabolites in brain regions of male Lister-hooded rats subjected to prenatal poly(I:C)-induced MIA (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation for 2 weeks. FC, Hip and St were collected immediately following locomotor activity testing in a novel box at two weeks post-weaning (PND36) and homogenised brain tissues were evaluated for their *ex vivo* levels of monoamines and metabolites using high performance liquid chromatography with electrochemical detection. Data are mean ± SEM (n= 6-8 per treatment x housing). *p<0.05 compared to relevant Veh-GH; #p<0.05 compared to relevant Veh-SI; †p<0.05 compared to relevant Poly(I:C)-GH; two-way ANOVA with Tukey's post-hoc test. DOPAC, 3,4-dihydroxyphenylacetic acid; FC, frontal cortex; Hip, hippocampus; HVA, homovanillic acid; St, striatum; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid.

Ex vivo brain tissue concentrations (pmol mg^{-1} tissue)					
Region	**Monoamine/ metabolite**	**Veh-GH**	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
FC	Dopamine	0.19 ± 0.03	0.25 ± 0.01	0.19 ± 0.02	0.26 ± 0.02#
FC	DOPAC	0.12 ± 0.02	0.13 ± 0.01	0.11 ± 0.02	0.14 ± 0.02
FC	HVA	0.20 ± 0.06	0.12 ± 0.05	0.15 ± 0.06	0.09 ± 0.04
FC	5-HT	3.59 ± 1.31	5.44 ± 1.65	3.91 ± 0.96	7.35 ± 2.22
FC	5-HIAA	1.47 ± 0.09	1.25 ± 0.08	1.33 ± 0.15	1.30 ± 0.08
Hip	Dopamine	0.19 ± 0.07	0.09 ± 0.01	0.31 ± 0.12	0.16 ± 0.05
Hip	DOPAC	0.09 ± 0.03	0.09 ± 0.03	0.12 ± 0.04	0.08 ± 0.02
Hip	HVA	0.02 ± 0.01	0	0.04 ± 0.02	0.03 ± 0.01
Hip	5-HT	5.07 ± 1.43	4.96 ± 1.52	3.94 ± 1.27	7.10 ± 1.58
Hip	5-HIAA	6.16 ± 1.56	5.58 ± 1.50	3.87 ± 1.30	7.10 ± 1.43
St	Dopamine	15.67 ± 6.72	23.81 ± 6.99	26.10 ± 5.45	**39.95 ± 3.88***
St	DOPAC	8.51 ± 1.43	8.57 ± 0.71	7.87 ± 0.95	7.40 ± 0.49
St	HVA	3.85 ± 0.43	3.75 ± 0.25	4.43 ± 0.38	3.92 ± 0.50
St	5-HT	2.43 ± 0.13	2.75 ± 0.15	2.68 ± 0.18	2.95 ± 0.23
St	5-HIAA	1.65 ± 0.59	1.43 ± 0.51	**3.39 ± 0.22*†**	**3.39 ± 0.25*†**

Table 2.9. Monoamine:metabolite ratios in brain regions of male Lister-hooded rats subjected to prenatal poly(I:C)-induced MIA (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation for 2 weeks. FC, Hip and St were collected immediately following locomotor activity testing in a novel box at two weeks post-weaning (PND36) and homogenised brain tissues were evaluated for their *ex vivo* levels of monoamines and metabolites using high performance liquid chromatography with electrochemical detection. Brown-Forsythe and Welch ANOVA tests were run to ensure homogeneity of variance. As striatal ratios did not pass the tests for homogeneity, these data were transformed using the function Y=10^Y in Prism before further parametric analyses. Data are mean ± SEM (n= 6-8 per treatment x housing). **p<0.01 compared to relevant Veh-GH; two-way ANOVA with Tukey's post-hoc test. DOPAC, 3,4-dihydroxyphenylacetic acid; FC, frontal cortex; Hip, hippocampus; HVA, homovanillic acid; St, striatum; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid.

Monoamine : metabolite ratios					
Region	**Monoamine/ metabolite**	**Veh-GH**	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
FC	Dopamine: DOPAC + HVA	0.74 ± 0.14	1.23 ± 0.22	1.16 ± 0.24	1.43 ± 0.26
FC	5-HT:5-HIAA	2.49 ± 0.91	4.21 ± 1.14	3.21 ± 0.84	6.13 ± 2.08
Hip	Dopamine: DOPAC + HVA	2.72 ± 0.95	1.38 ± 0.45	1.80 ± 0.22	1.71 ± 0.63
Hip	5-HT:5-HIAA	1.26 ± 0.50	0.98 ± 0.12	1.07 ± 0.08	1.83 ± 0.75
St	Dopamine: DOPAC + HVA	1039.74 ± 983.38	1532.91 ± 918.71	1462.38 ± 700.29	4935.61 ± 1447.99
St	5-HT:5-HIAA	$1.32 \times 10^{12} \pm 1.32 \times 10^{12}$	$3.04 \times 10^{30} \pm 3.04 \times 10^{30}$	6.29 ± 0.37	7.57 ± 0.54

Correlation between locomotor activity and neurochemistry

Correlation analyses were performed to examine the relationship between total LMA and neurochemical measures in the different treatment x housing groups in adolescence.

In Veh-GH males, hippocampal 5-HT and striatal dopamine levels were positively correlated to total LMA. All striatal neurochemical substrates that were measured in Poly(I:C)-SI males were positively correlated to total LMA (Table 2.10). In females, positive correlations between total LMA and levels of frontal cortical and/or striatal neurochemical substrates were observed across the four groups (Table 2.11).

In males, no significant correlation was found between total LMA and any of the monoamine:metabolite ratios (Table 2.12). In females, total LMA was positively correlated to frontal cortical 5-HT:5-HIAA ratio in both Veh-GH and Veh-SI groups. In Veh-SI females, LMA was also positively correlated with striatal dopamine:DOPAC+HVA ratio (Table 2.13).

Table 2.10. Correlation analysis between total locomotor activity and neurochemical substrates in adolescent males. The relationships between locomotor activity and individual neurochemical compounds (dopamine, 5-HT and their metabolites) in different brain regions were examined. R-squared values are presented. DOPAC, 3,4-dihydroxyphenylacetic acid; FC, frontal cortex; Hip, hippocampus; HVA, homovanillic acid; St, striatum; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; *p<0.05; **p<0.01; Pearson's correlation coefficient.

Adolescent males: total locomotor activity versus neurochemical substrate					
Region	**Monoamine/ metabolite**	**Veh-GH**	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
FC	Dopamine	0.0505	0.00002	0.0014	0.1746
	DOPAC	0.2577	0.1111	0.0007	0.0184
	HVA	0.1153	0.6276	0.0021	0.0360
	5-HT	0.0278	0.0003	0.0147	0.0707
	5-HIAA	0.0033	0.5276	0.4887	0.0091
Hip	Dopamine	0.1532	0.0006	0.0150	0.7195
	DOPAC	0.3998	0.1078	0.0244	0.000001
	HVA	0.0004	-	0.0124	0.0340
	5-HT	**0.6994****	0.0084	0.2837	0.3898
	5-HIAA	0.0183	0.6856	0.3504	0.6184
St	Dopamine	**0.5885***	0.7109	0.0646	**0.8568***
	DOPAC	0.0059	0.2111	0.0335	**0.8382***
	HVA	0.00002	0.6951	0.0085	**0.8513***
	5-HT	0.3254	0.4604	0.0454	**0.8418***
	5-HIAA	0.0773	0.7330	0.0022	**0.8993***

Table 2.11. Correlation analysis between total locomotor activity and neurochemical substrates in adolescent females. The relationships between locomotor activity and individual neurochemical compounds (dopamine, 5-HT and their metabolites) in different brain regions were examined. R-squared values are presented. DOPAC, 3,4-dihydroxyphenylacetic acid; FC, frontal cortex; Hip, hippocampus; HVA, homovanillic acid; St, striatum; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; *p<0.05; **p<0.01; Pearson's correlation coefficient.

Adolescent females: total locomotor activity versus neurochemical substrate					
Region	**Monoamine/ metabolite**	**Veh-GH**	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
FC	Dopamine	0.1163	0.0907	0.4070	0.3534
	DOPAC	0.1413	0.0976	**0.5386***	0.1044
	HVA	0.0058	0.0739	0.0533	**0.5701***
	5-HT	**0.7314****	0.0669	**0.6679***	0.4384
	5-HIAA	0.0004	0.0015	0.0115	0.0313
Hip	Dopamine	0.1126	0.0348	0.0377	0.2843
	DOPAC	0.0473	0.3303	0.0034	0.5125
	HVA	0.1649	0.0873	0.2427	0.1518
	5-HT	0.00004	0.3862	0.0587	0.0091
	5-HIAA	0.3782	0.3130	0.1939	0.0708
St	Dopamine	0.2995	0.3534	**0.5106***	0.0212
	DOPAC	0.1030	0.1081	0.0039	0.1549
	HVA	**0.5346***	0.0592	0.1288	0.0311
	5-HT	0.0691	0.4475	0.0423	0.1657
	5-HIAA	0.1872	**0.5097***	0.1174	0.2214

Table 2.12. Correlation analysis between total locomotor activity and monoamine:metabolite ratios in adolescent males. The relationships between locomotor activity and monoamine:metabolite ratios in different brain regions were examined. R-squared values are presented. DOPAC, 3,4-dihydroxyphenylacetic acid; FC, frontal cortex; Hip, hippocampus; HVA, homovanillic acid; St, striatum; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; *p<0.05; Pearson's correlation coefficient.

Adolescent males: total locomotor activity versus monoamine:metabolite ratio					
Region	**Monoamine/ metabolite**	**Veh-GH**	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
FC	Dopamine:DOPAC + HVA	0.3172	0.6776	0.0028	0.2986
	5-HT:5-HIAA	0.0307	0.2696	0.0003	0.0584
Hip	Dopamine:DOPAC + HVA	0.0012	-	0.0007	0.8681
	5-HT:5-HIAA	0.1472	0.3459	0.2133	0.3112
St	Dopamine:DOPAC + HVA	0.1897	0.7178	0.1096	0.0800
	5-HT:5-HIAA	0.0420	0.0303	0.2049	0.0874

Table 2.13. Correlation analysis between total locomotor activity and monoamine:metabolite ratios in adolescent females. The relationships between locomotor activity and monoamine:metabolite ratios in different brain regions were examined. R-squared values are presented. DOPAC, 3,4-dihydroxyphenylacetic acid; FC, frontal cortex; Hip, hippocampus; HVA, homovanillic acid; St, striatum; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; *p<0.05, **p<0.01; Pearson's correlation coefficient.

Adolescent females: total locomotor activity versus monoamine:metabolite ratio					
Region	**Monoamine/ metabolite**	**Veh-GH**	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
FC	Dopamine:DOPAC + HVA	0.0035	0.0173	0.0661	0.3199
	5-HT:5-HIAA	**0.6223***	0.0800	**0.7063****	0.4019
Hip	Dopamine:DOPAC + HVA	0.3762	0.2232	0.0017	0.0171
	5-HT:5-HIAA	0.0155	0.0379	0.4797	0.1206
St	Dopamine:DOPAC + HVA	0.3541	0.4342	**0.6164***	0.0034
	5-HT:5-HIAA	0.0080	0.0018	0.0001	0.0066

2.4.2.3.2 Adulthood

Locomotor activity

In terms of locomotor activity and neurochemical measures in adolescence, males were more influenced by poly(I:C), SI or combined poly(I:C)-SI compared to females. Therefore, this gender alone was used for more detailed examination of poly(I:C) and/or SI-induced behavioural phenotype in adulthood, using a battery of tests relevant to the positive, negative and cognitive symptom domains of schizophrenia. A cohort of experimentally naïve male rats were used, commencing with LMA evaluations at approximately four weeks post-weaning (PND51-57). Habituation to the novel environment was reflected by a general decline in all activity types in all groups with time (ambulation, $F_{(11,660)}$= 118.50, p=0.001; fine movement, $F_{(11,660)}$= 27.10, p=0.001; rears, $F_{(11,660)}$= 116.25, p=0.001). There were significant treatment x housing interactions for fine movement ($F_{(1,60)}$= 6.32, p<0.05) and rearing ($F_{(1,60)}$= 8.29, p<0.01), together with a time x treatment x housing interaction for rears ($F_{(11,660)}$= 2.03, p<0.05). Post-hoc tests revealed no between-group differences in ambulation and fine movement across time (Figures 2.16A& B) and in total (Figures 2.17A & B) when compared to Veh-GH. Veh-SI reared more than Veh-GH at multiple time points and in total over the whole hour. Poly(I:C) alone produced a subtle increase in rearing, evident at 20 min only. Interestingly, SI-induced increase was absent in the combined Poly(I:C)-SI group (Figures 2.16C & 2.17C).

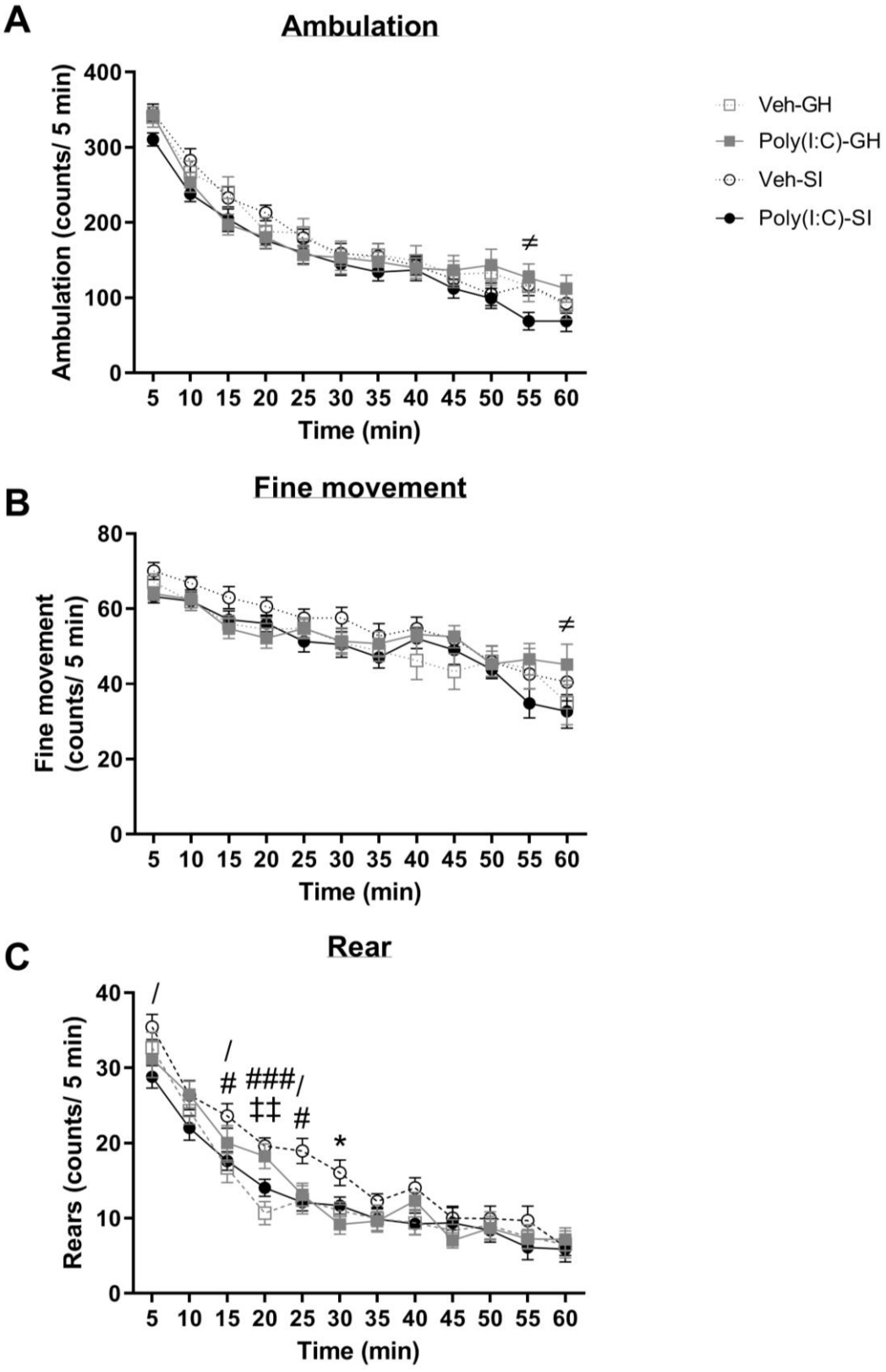


Figure 2.16. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on the different activity types in male Lister-hooded rats at around four weeks post-weaning (postnatal day 51-57). Mean ± SEM **(A)** ambulation, **(B)** fine movement and **(C)** rears in a novel box across an one-hour observation period are presented (n=16 per treatment x housing). ≠p<0.05 Poly(I:C)-GH versus Poly(I:C)-SI; /p<0.05 Veh-SI versus Poly(I:C)-SI; #p<0.05, ###p<0.001 Veh-GH versus Veh-SI; ‡‡p<0.01 Veh-GH versus Poly(I:C)-GH; *p<0.05 Poly(I:C)-GH versus Veh-SI; three-way RM ANOVA with Tukey's post-hoc test (A-C).

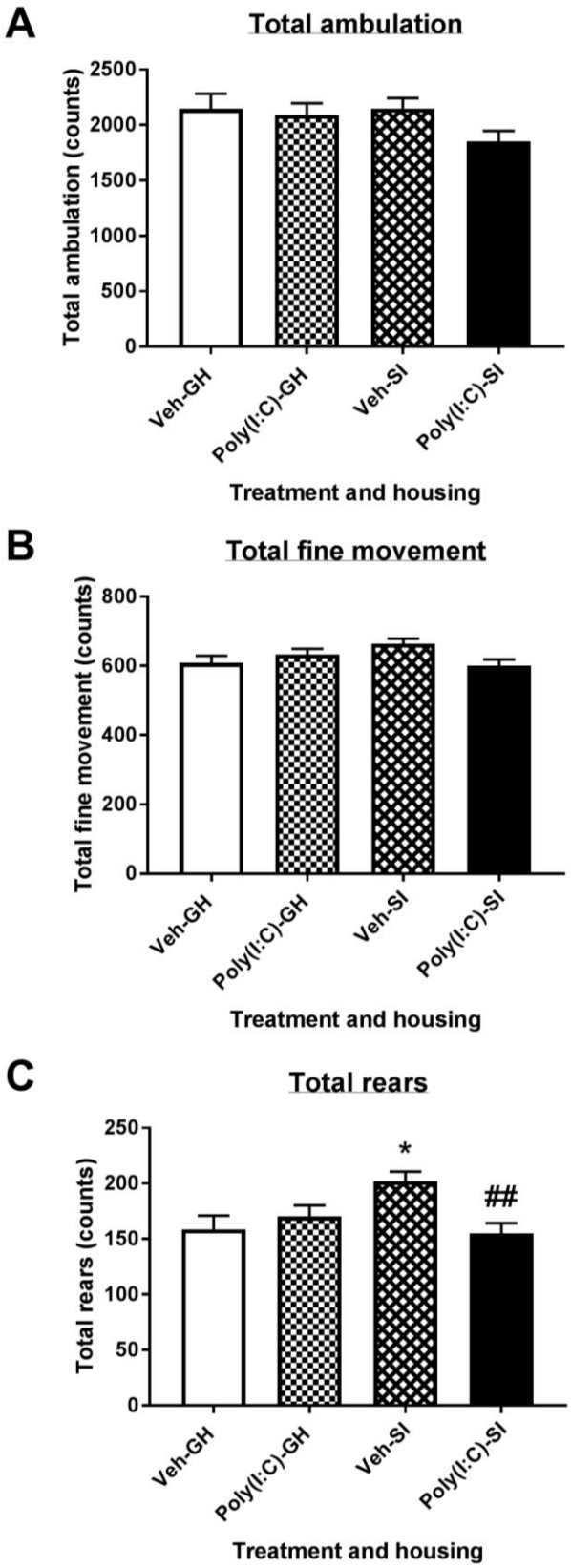


Figure 2.17. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on the cumulative totals of different activity types in male Lister-hooded rats at around four weeks post-weaning (postnatal day 51-57). Mean ± SEM total **(A)** ambulation, **(B)** fine movement and **(C)** rears in a novel box across an one-hour observation period are presented (n=16 per treatment x housing). *p<0.05 compared to Veh-GH; ## p<0.01 compared to Veh-SI; two-way ANOVA with Tukey's post-hoc test (A-C).

Novel object discrimination

To determine if prenatal poly(I:C) exposure and/or SI produced a long-lasting development in visual recognition memory, rats were tested in the NOD task 24 h following LMA. Neither treatment nor housing affected exploration preference for either identical objects in the familiarisation trial (data not shown). When a 1h ITI was used, there was a significant main effect of object ($F_{(1,28)}$= 16.93, p=0.001) and an object x housing interaction ($F_{(1,28)}$= 4.39, p<0.05) on object exploration in the choice trial, such that Veh-SI and Poly(I:C)-SI demonstrated a marked preference for the novel object (Figure 2.18A). When a 2h ITI was used, a significant main effect of object ($F_{(1,28)}$= 14.17, p<0.01) but not treatment or housing was evident, but there were no group differences in the exploration of novel versus familiar objects (Figure 2.18B). Comparison between the discrimination ratios revealed no between-group differences regardless of the ITI used (data not shown). Neither treatment nor housing influenced total time spent in object exploration during the familiarisation or choice trial (data not shown).

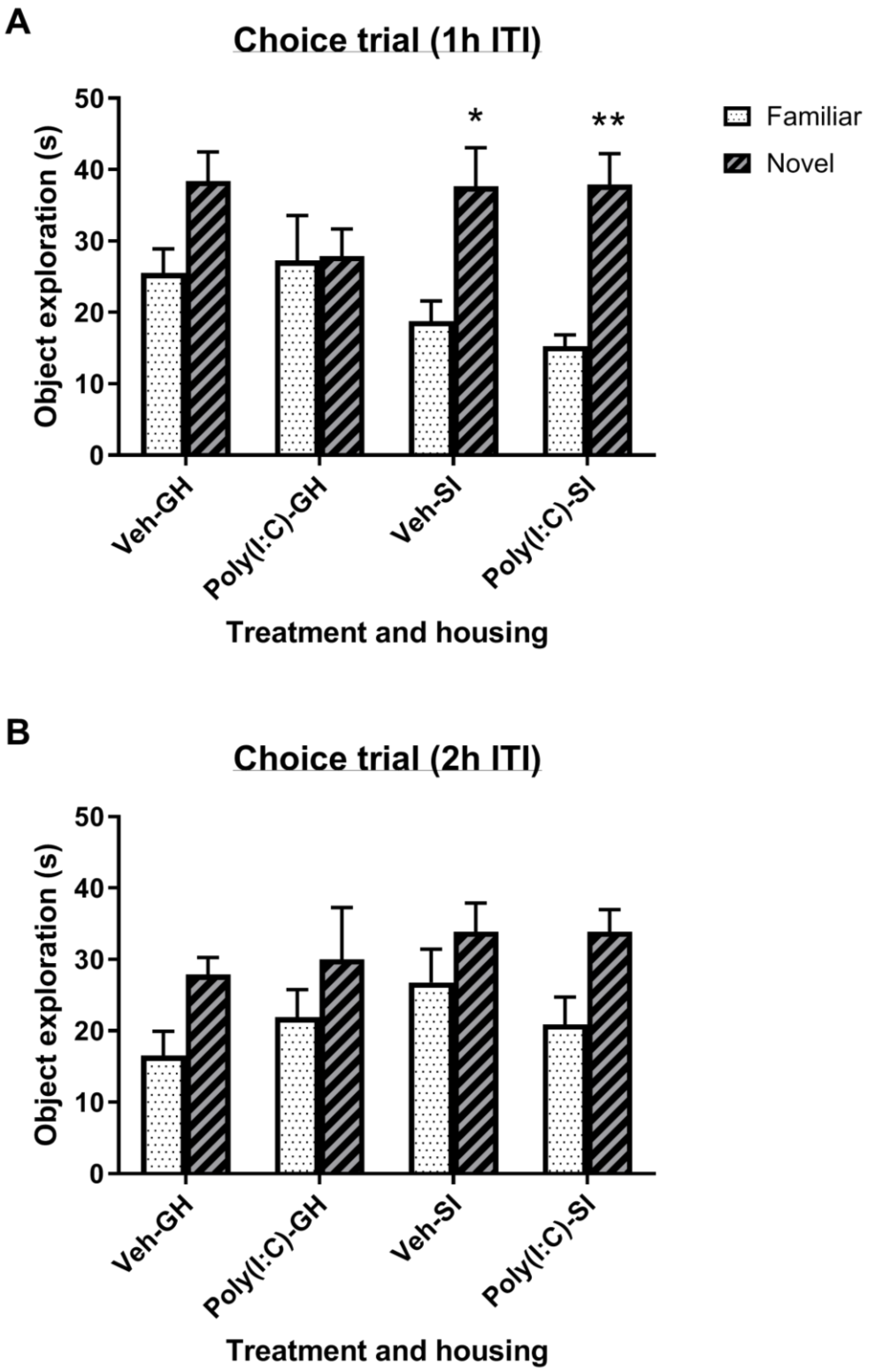


Figure 2.18. Choice trial object exploration in Lister-hooded male rats exposed to prenatal poly(I:C) (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation in the novel object discrimination task at around four weeks post-weaning (postnatal day 52-58). Mean ± SEM time spent exploring the familiar and novel objects during the choice trial **(A)** 1h or **(B)** 2h following the familiarisation trial (n=8 per treatment x housing for each ITI). *p<0.05, **p<0.01 versus familiar object; three-way RM ANOVA with Sidak's post-hoc test (A,B). ITI, inter-trial interval.

Novel location discrimination

In order to assess whether gestational exposure to MIA and/or subsequent SI affected spatial working memory, rats were evaluated in the NLD task. There were no significant main effects of treatment, housing or location on object exploration in the familiarisation trial (data not shown). In the choice trial, there was a significant main effect of location ($F_{(1,60)}$= 38.64, p=0.001) but not treatment or housing on object exploration. Poly(I:C)-GH, Veh-SI and Poly(I:C)-SI demonstrated significant preference for the object at the new location (Figure 2.19A). However, there were no differences in discrimination ratio between the groups (data not shown). Analysis of the total exploration time showed a significant main effect of trial ($F_{(1,60)}$= 66.74, p=0.001). All groups spent significantly less time exploring objects in the choice trial compared to the familiarisation trial (Figure 2.19B).

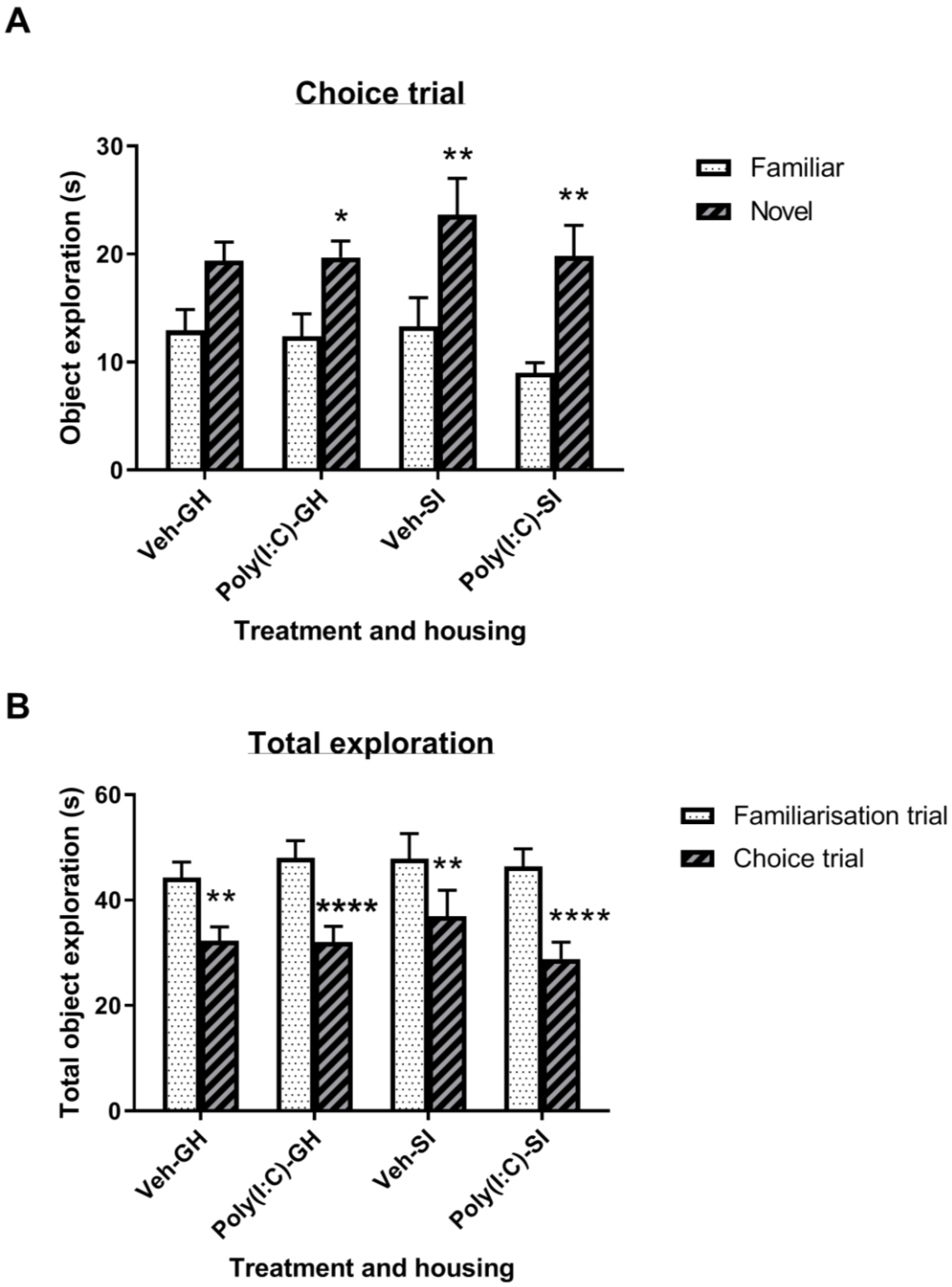


Figure 2.19. Object exploration in Lister-hooded male rats previously exposed to prenatal poly(I:C) (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation in the novel location discrimination task at five to six weeks post-weaning (postnatal day 59-65). Mean ± SEM **(A)** time spent exploring objects at the familiar and novel locations during the choice trial conducted 1 min after the familiarisation trial, and **(B)** total object exploration in the familiarisation and choice trials (n=16 per treatment x housing). *p<0.05, **p<0.01 versus familiar object (A); **p<0.01, ****p<0.0001 versus familiarisation trial (B); three-way RM ANOVA with Sidak's post-hoc test (A,B).

Social interaction

Social interaction test was conducted to study the level and repertoire of social behaviour between rats exposed to distinct neurodevelopmental histories. ANOVA analysis of pro-social behaviour (boxing or biting and pinning excluded) revealed a significant main effect of behaviour subtype ($F_{(4,112)}$= 159.29, p=0.001) and a behaviour subtype x housing ($F_{(4,112)}$= 28.77, p=0.001) interaction. Veh-SI and Poly(I:C)-SI both showed reduced anogenital sniffing, increased body sniffing and increased crawling over and under compared to Veh-GH and/or Poly(I:C)-GH (Figure 2.20A). Aggressive behaviour, which included boxing and biting and pinning, showed a significant main effect of behaviour subtype ($F_{(1,28)}$= 54.46, p=0.001) and behaviour subtype x treatment ($F_{(1,28)}$= 8.09, p<0.01), behaviour subtype x housing ($F_{(1,28)}$= 19.34, p=0.001), behaviour subtype x treatment x housing ($F_{(1,28)}$= 4.90, p<0.05) and treatment x housing ($F_{(1,28)}$= 9.79, p<0.01) interactions. Interestingly, SI-induced increase in boxing and biting was absent in the combined Poly(I:C)-SI group. Meanwhile, gestational poly(I:C) exposure on its own increased pinning compared to Veh-GH and Poly(I:C)-SI (Figure 2.20B). Housing significantly influenced the distance travelled by each pair of rats during the session ($F_{(1,28)}$= 7.94, p<0.01), with post-hoc tests demonstrating increased travel by Veh-SI rat pairs compared to Veh-GH and Poly(I:C)-GH (Figure 2.21). There was a significant housing x treatment interaction on velocity in the open field arena ($F_{(1,28)}$= 10.76, p<0.01). No group was different when compared to Veh-GH, although Poly(I:C)-SI showed reduced velocity relative to Veh-SI (data not shown). No difference in total time spent in behaviour was observed across the four treatment-housing groups (data not shown).

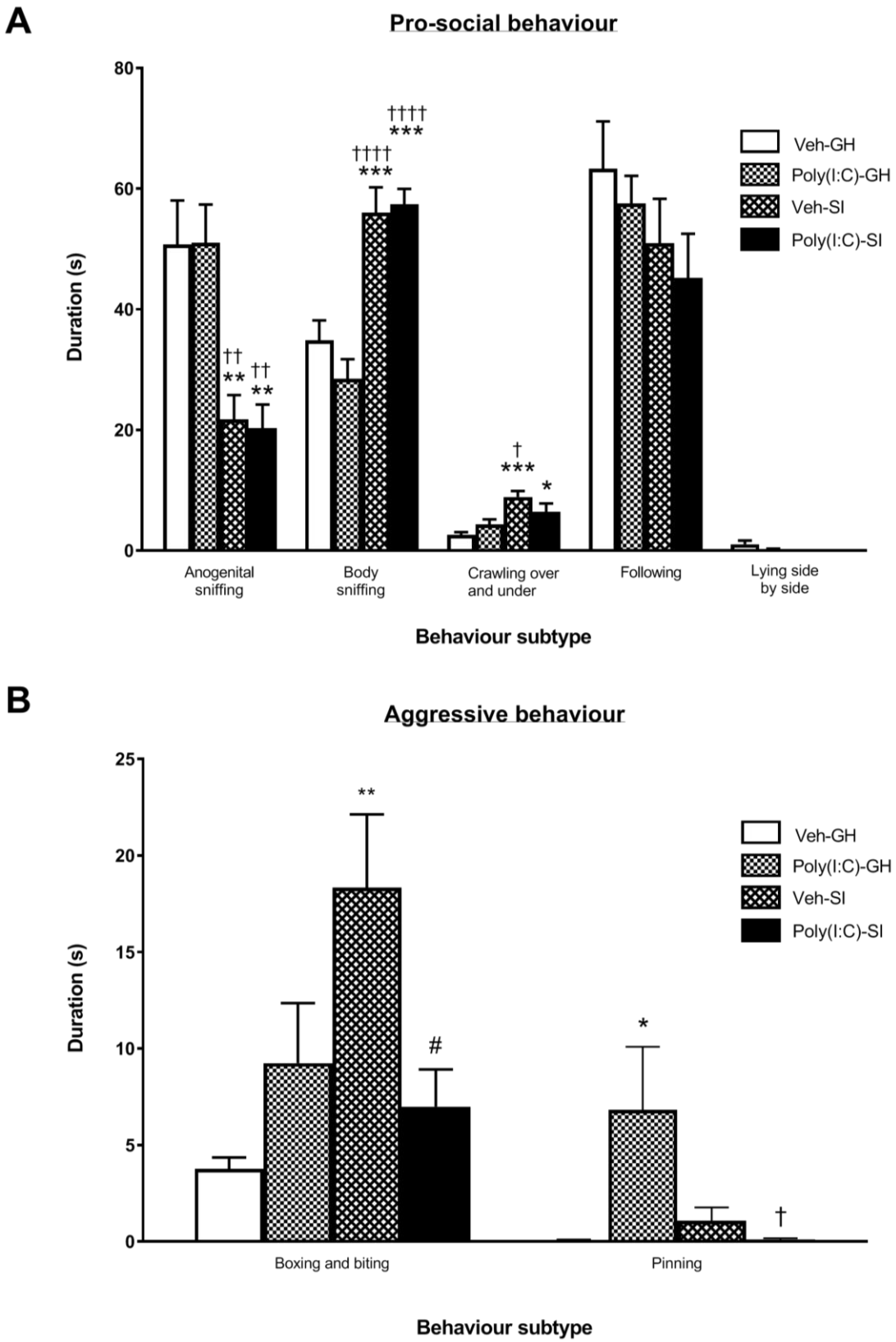


Figure 2.20. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on rat social behaviour at six to seven weeks post-weaning (postnatal day 66-70). Mean ± SEM time spent in **(A)** pro-social and **(B)** aggressive behaviour by pairs of unfamiliar weight- and neurodevelopmental history-matched rats during the 10-min social interaction task (n=8 pairs per treatment x housing). *p<0.05, **p<0.01, ***p<0.001 compared to respective Veh-GH; †p<0.05, ††p<0.01, ††††p<0.0001 compared to respective Poly(I:C)-GH; #p<0.05 versus Veh-SI (A,B); three-way RM ANOVA with Tukey's post-hoc test (A,B).

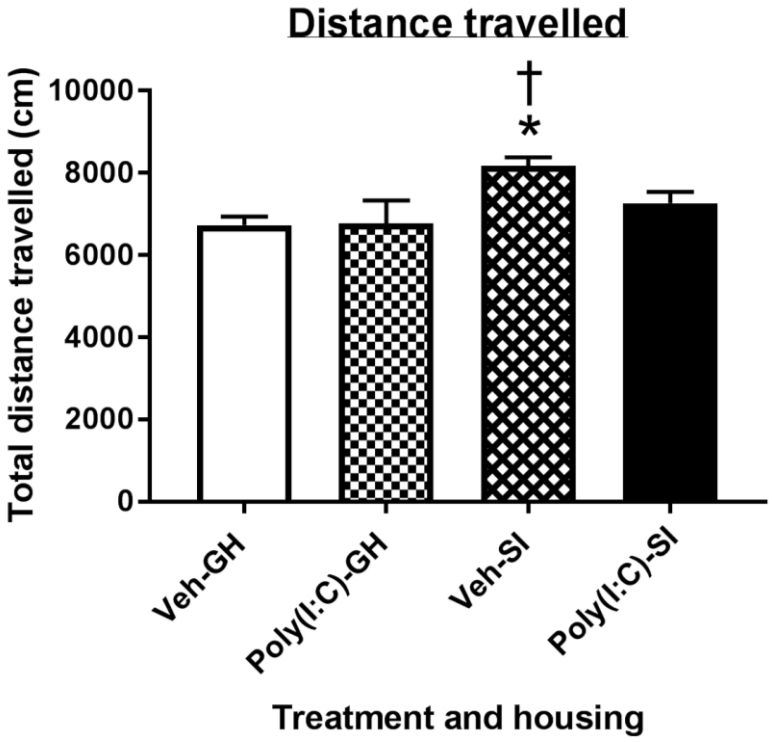


Figure 2.21. Total distance travelled by rats previously exposed to prenatal poly(I:C) (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation during social interaction task at six to seven weeks post-weaning (postnatal day 66-70). Mean ± SEM total distance travelled by pairs of unfamiliar weight- and neurodevelopmental history-matched rats during the 10-min test session (n=8 pairs per treatment x housing). *p<0.05 versus Veh-GH; †p<0.05 versus Poly(I:C)-GH; two-way ANOVA with Tukey's post-hoc test.

Ultrasonic vocalisations

To investigate whether social communication was altered by the two neurodevelopmental manipulations, USVs were recorded during the social interaction test. Aversive 22-kHz calls were exhibited by only six out of the 32 pairs of rats: two poly(I:C)-GH, three Veh-SI and one poly(I:C)-SI. The occurrence of such calls was insufficient to permit statistical analysis. Neither treatment nor housing influenced total 50-kHz USVs but there was a significant treatment x housing interaction ($F_{(1,28)}$= 6.61, $p<0.05$). Veh-SI produced increased total USVs (Figure 2.22). When classified into the three main types of 50-kHz calls (flat, step, trill), ANOVA revealed a significant treatment x housing interaction on total call subtypes (flat, $F_{(1,28)}$= 5.72, $p<0.05$; step, $F_{(1,28)}$= 5.75, $p<0.05$; trill, $F_{(1,28)}$=5.47, $p<0.05$) but no effect of treatment or housing. Veh-SI emitted increased number of flat, step and trill calls (Figure 2.23A-C). Using the narrower classification (section 2.3.5.5), only nine out of the fourteen 50-kHz call subtypes were identified. When the nine subtypes were analysed, ANOVA revealed a significant main effect of housing for downward ramp ($F_{(1,28)}$= 9.91, $p<0.01$) and a significant treatment x housing interaction for flat ($F_{(1,28)}$= 6.29, $p<0.05$), downward ramp ($F_{(1,28)}$= 11.37, $p<0.01$), upward ramp ($F_{(1,28)}$= 5.02, $p<0.05$), trills ($F_{(1,28)}$= 5.39, $p<0.05$), multi-step ($F_{(1,28)}$= 5.08, $p<0.05$) and inverted-U calls ($F_{(1,28)}$=4.94, $p<0.05$). SI alone enhanced trill, multi-step and downward ramp calls whereas poly(I:C) alone only increased upward ramp calls, compared to Veh-GH (Figure 2.24A & B).

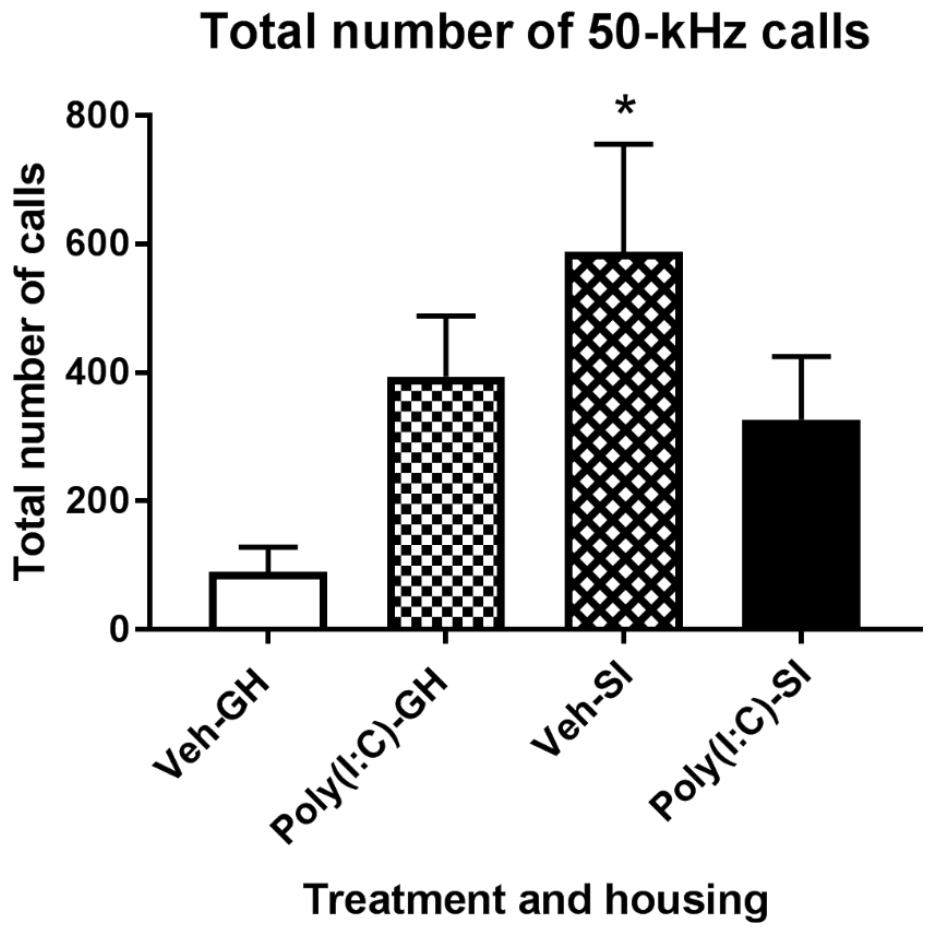


Figure 2.22. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on rat 50-kHz ultrasonic vocalisations at six to seven weeks post-weaning (postnatal day 66-70). Mean ± SEM total number of calls, including unclassifiable calls, recorded in pairs of newly encountered Lister-hooded male rats exposed to the same prenatal treatment and housing conditions during a 10-min social interaction test (n=8 pairs per treatment x housing). *p<0.05 versus Veh-GH; two-way ANOVA with Tukey's post-hoc test.

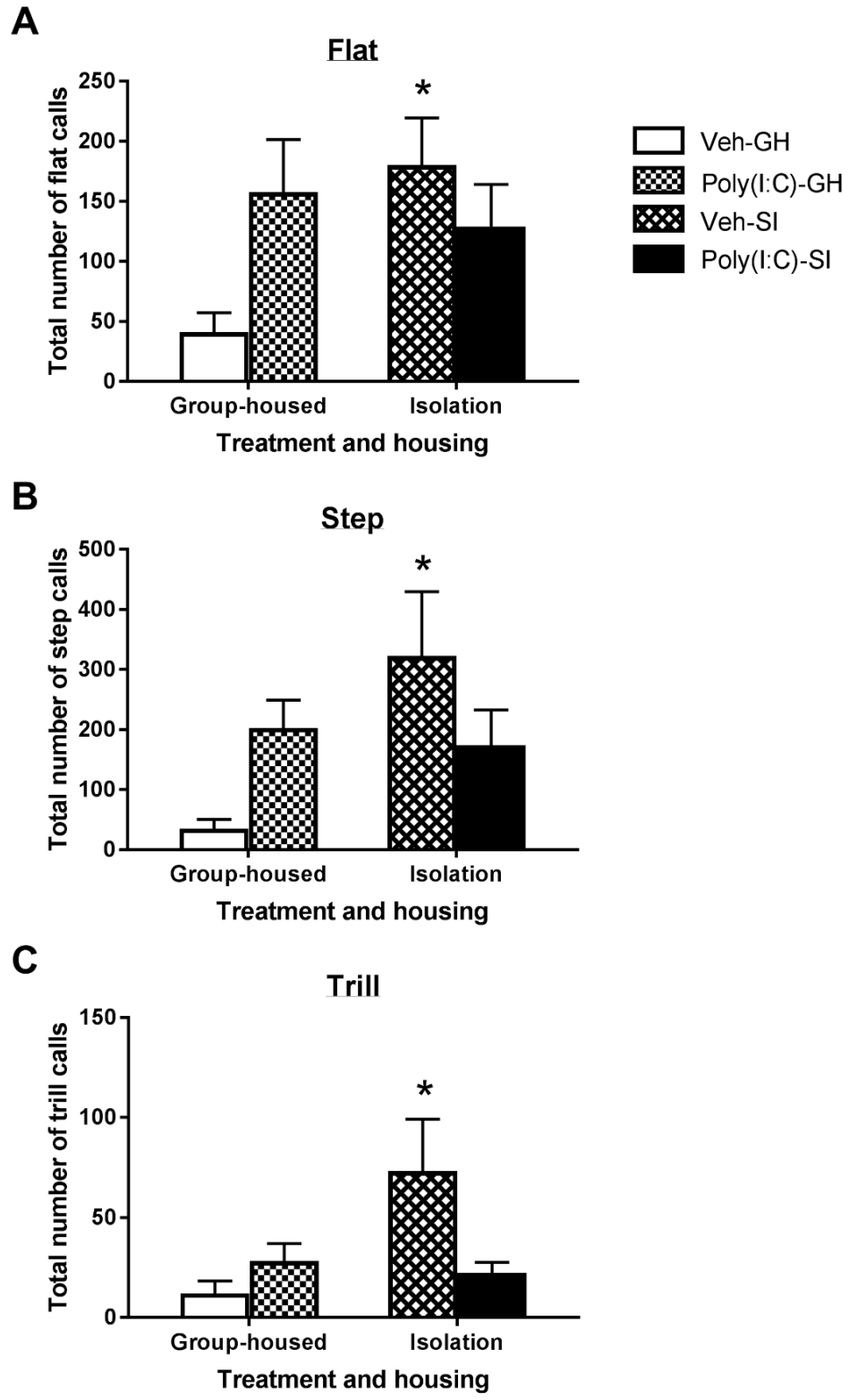


Figure 2.23. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on rat emission of the three main 50-kHz ultrasonic vocalisation subtypes at six to seven weeks post-weaning (postnatal day 66-70). Mean ± SEM total **(A)** flat, **(B)** step and **(C)** trill calls recorded in pairs of newly encountered Lister-hooded male rats exposed to the same prenatal treatment and housing conditions during a 10-min social interaction test (n=8 pairs per treatment x housing). *p<0.05 versus Veh-GH (A-C); two-way ANOVA with Tukey's post-hoc test (A-C).

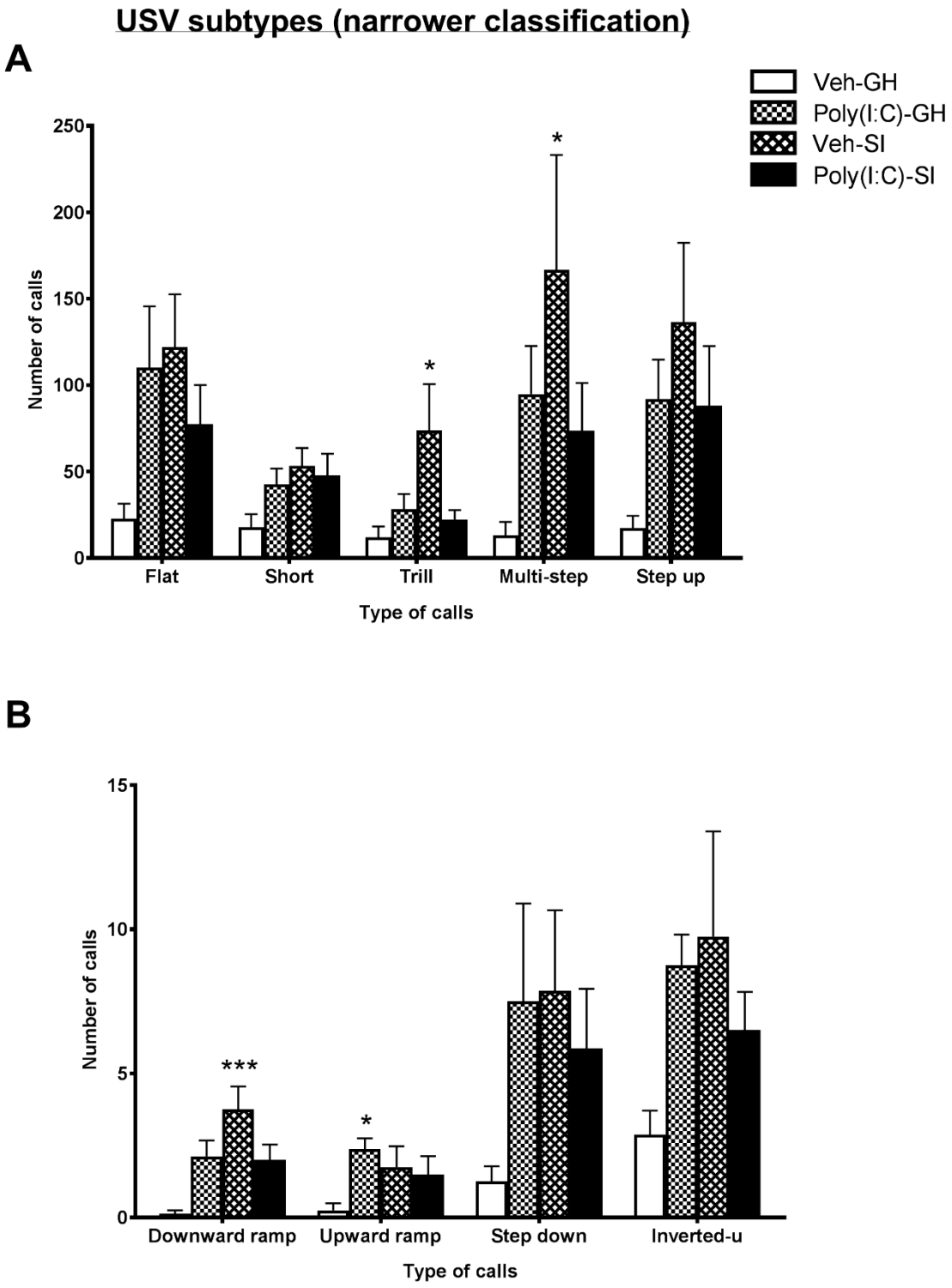


Figure 2.24. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on rat emission of nine 50-kHz ultrasonic vocalisation subtypes at six to seven weeks post-weaning (postnatal day 66-70). Total number of the different call subtypes recorded in pairs of newly encountered Lister-hooded male rats exposed to the same prenatal treatment and housing conditions during a 10-min social interaction test are expressed as mean ± SEM (n=8 pairs per treatment x housing). *p<0.05, ***p<0.001 compared to relevant Veh-GH (A,B); two-way ANOVA with Tukey's post-hoc test (A,B).

Pre-pulse inhibition of acoustic startle

In order to measure any developmental change in sensorimotor gating, rats were analysed in PPI. Expected increase in % PPI with increasing pre-pulse intensity occurred but was not influenced by treatment or housing ($F_{(2,118)}$= 115.79, p=0.001; Figure 2.25). Initial startle showed a significant housing x treatment interaction ($F_{(1,59)}$= 5.52, p<0.05) but no between-group differences were observed (data not shown). Neither treatment nor housing influenced the final startle or habituation to the startle pulse across the test session.

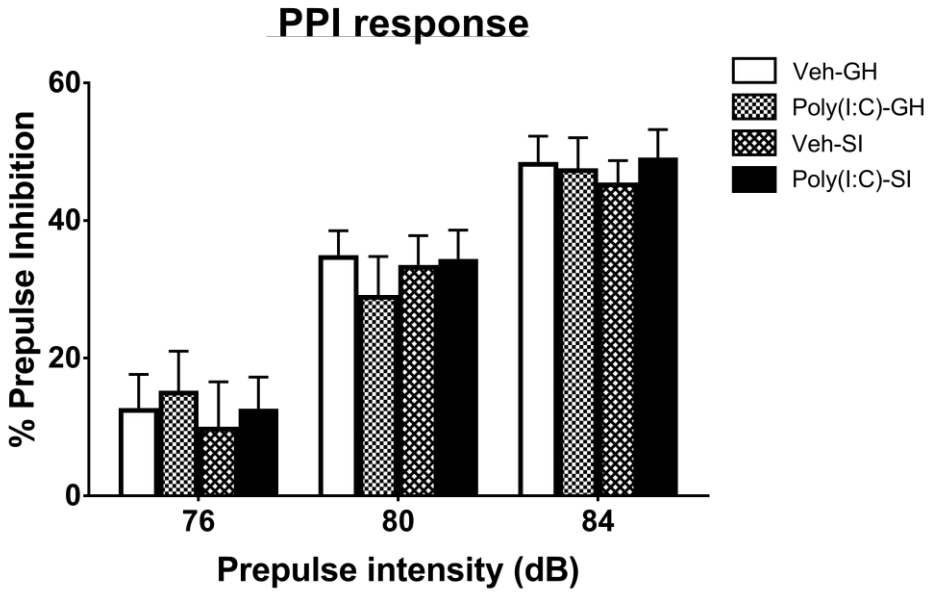


Figure 2.25. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on pre-pulse inhibition at seven to eight weeks post-weaning (postnatal day 73-78). Mean ± SEM % PPI at 76, 80 and 84 dB pre-pulse intensity (n=16 per treatment x housing except for Veh-SI, where n=15); three-way RM ANOVA with Tukey's post-hoc test.

Conditioned freezing response

The impact of prenatal poly(I:C) and/or subsequent SI on associative learning and memory in adult rats was probed using the CFR task. Neither treatment nor housing affected latency to enter the dark compartment. ANOVA revealed a significant main effect of shock number during acquisition on day 1 (0 h; $F_{(2,56)}$= 17.6, p=0.001) but there were no between-group differences in freezing duration following each foot shock. When re-introduced to the context at 24 h in the absence of foot shock, there was a significant main effect of cue ($F_{(1,28)}$ = 14.01, p<0.01) and treatment x housing interaction ($F_{(1,28)}$) = 5.63, p<0.05). All rats froze for a longer duration following cue presentation compared to context exposure alone. Cued freezing decreased in Veh-SI but this deficit was absent in Poly(I:C)-SI (Figure 2.26). Housing influenced the latency to leave on day 2 ($F_{(1,28)}$= 6.70, p<0.05) but there were no post-hoc differences.

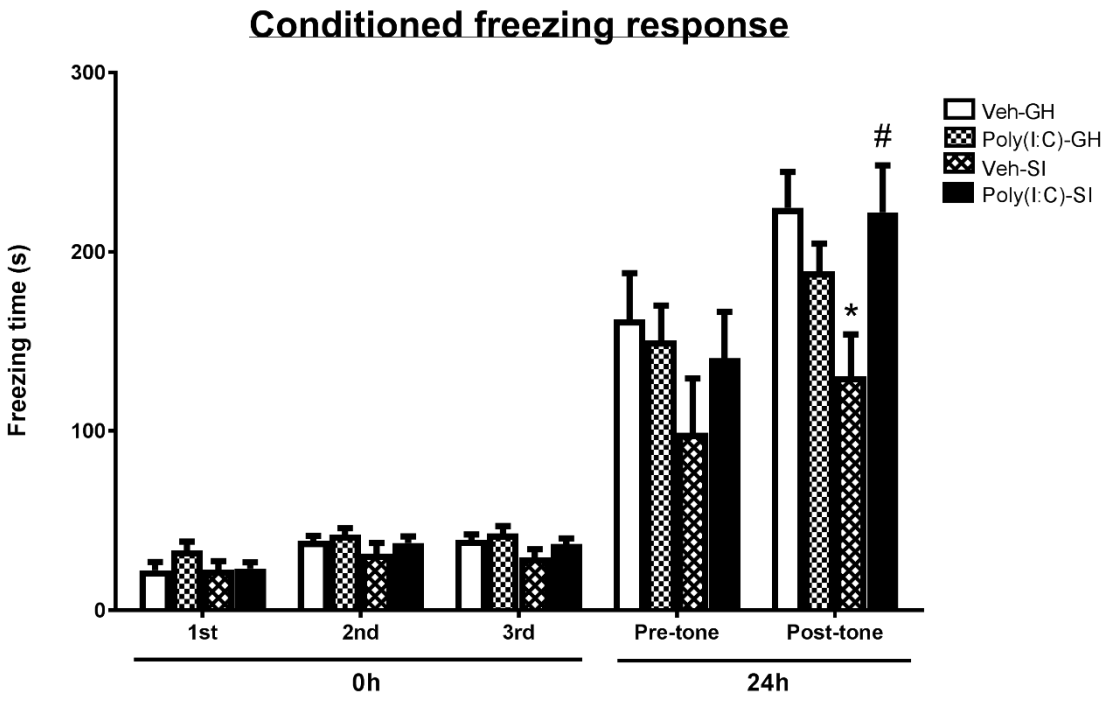


Figure 2.26. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on conditioned freezing response at eight to nine weeks post-weaning (0 h: postnatal day 79-83). Mean ± SEM duration spent freezing (s) following three consecutive presentations of combined light and tone (conditioned stimulus, CS) and foot shock (unconditioned stimulus) at 0 h, and single presentation of CS at 24 h upon re-entry into the dark compartment of the shuttle box, are presented (n=16 per treatment x housing). *p<0.05 compared to relevant Veh-GH; #p<0.05 compared to relevant Veh-SI; three-way RM ANOVA with Tukey's post-hoc test (0 h and 24 h).

Attentional set-shifting task

Rats were assessed in an eight-day long ASST to evaluate their cognitive flexibility. Neither discrimination type (odour or media) nor combined treatment and housing influenced TTC and ETC during day 7 SD training (data not shown). On day 8 complex discrimination, there was a significant effect of discrimination type on TTC (CD versus Rev1, $F_{(1,26)}$= 11.93, $p<0.01$; IDS versus EDS, $F_{(1,26)}$= 4.77, $p<0.05$) and ETC (CD versus Rev1, $F_{(1,26)}$= 13.52, $p<0.01$; IDS versus EDS, $F_{(1,26)}$= 4.85, $p<0.05$), thus confirming that rats generally found Rev1 and EDS harder than CD and IDS, respectively (Figure 2.27A & B). Veh-SI took significantly more trials to reach criteria in Rev1 than CD (TTC, $p<0.01$; ETC, $p<0.05$; Figures 2.27A & B). When all discrimination stages were considered, discrimination type significantly influenced TTC ($F_{(6,156)}$= 2.17, $p<0.05$) and ETC ($F_{(6,156)}$= 2.81, $p<0.05$). Veh-SI required more trials to reach criterion in Rev1 compared to Veh-GH (Figure 2.27A). Cumulative total TTC across all discrimination stages on day 8 showed a significant treatment x housing interaction ($F_{(1,26)}$= 4.48, $p<0.05$) but between-group differences were absent (data not shown).

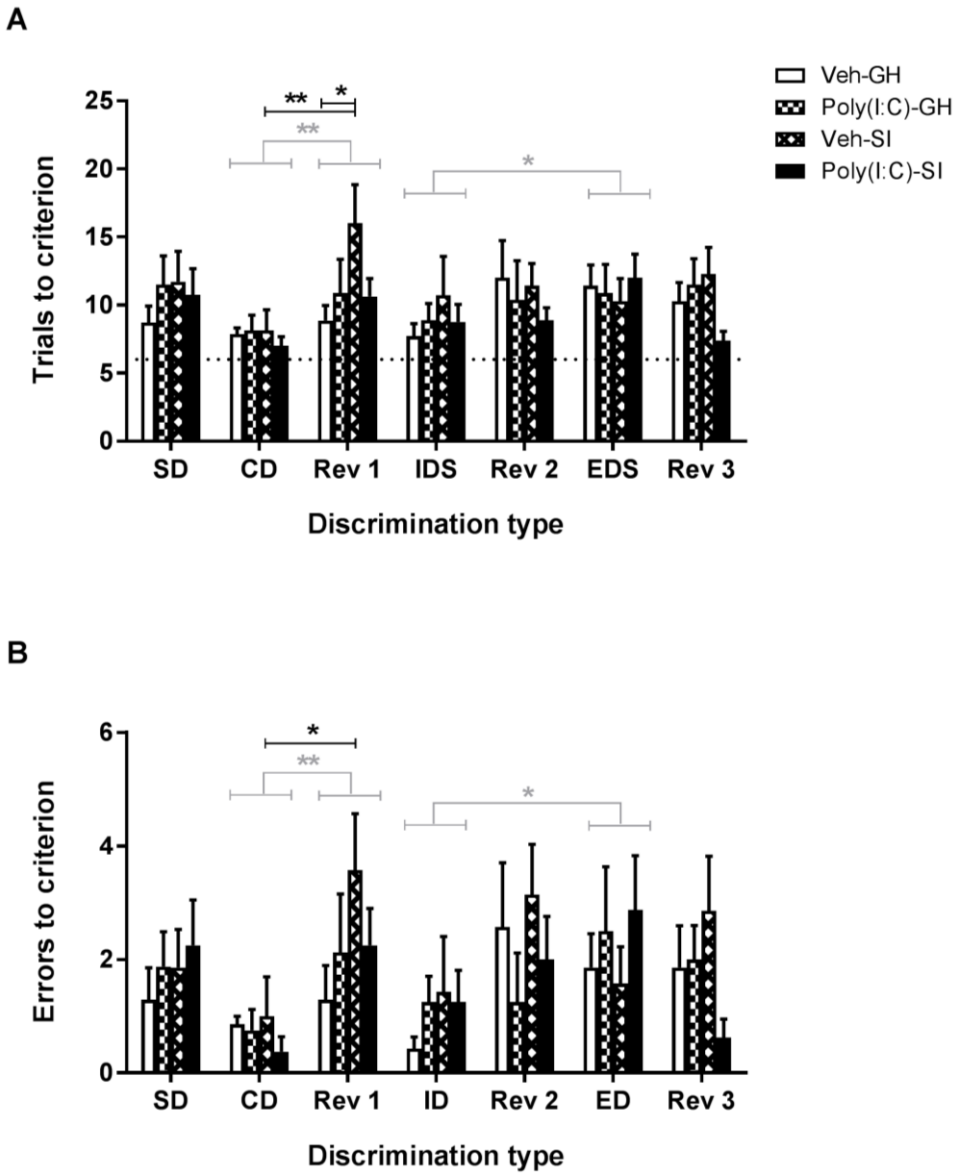


Figure 2.27. Trials to criterion and errors to criterion of male Lister-hooded rats in the attentional set-shifting task. Mean ± SEM **(A)** trials to criterion and **(B)** errors to criterion during SD training on day 7 and the different stages of complex discrimination (CD, Rev1, ID, Rev2, ED, Rev3) on day 8 (n=16 per treatment x housing). *p<0.05, **p<0.01; two-way RM ANOVA with Sidak's post-hoc test (A,B). Black capped lines show comparisons between groups or discrimination types whereas grey capped lines linked with zig-zag lines denote significant main effects of discrimination types. Horizontal dashed line in (A) denotes the criterion to proceed to the next stage, i.e. 6 consecutive correct responses. CD, compound discrimination; ED: extra-dimensional shift; ID: intra-dimensional shift; Rev, reversal; SD, simple discrimination.

Weight analysis: from weaning to young adulthood (7-8 weeks post-weaning)

In order to check if prenatal treatment or housing affected growth, rat body weight was monitored from weaning until the end of the study. As half of the animals began to undergo food restriction for ASST from around eight weeks post-weaning (PND73-78), body weight data are only presented up until this point. There was a main effect of time ($F_{(8,480)}$= 12538.28, p=0.001) and housing ($F_{(1,60)}$= 9.19, p<0.01) as well as time x housing interaction ($F_{(8,480)}$= 4.37, p=0.001). SI rats, regardless of prenatal treatment, were heavier than Veh-GH at several time points across the observation period. Gestational poly(I:C) did not influence rat body weight at any time point (Figure 2.28).

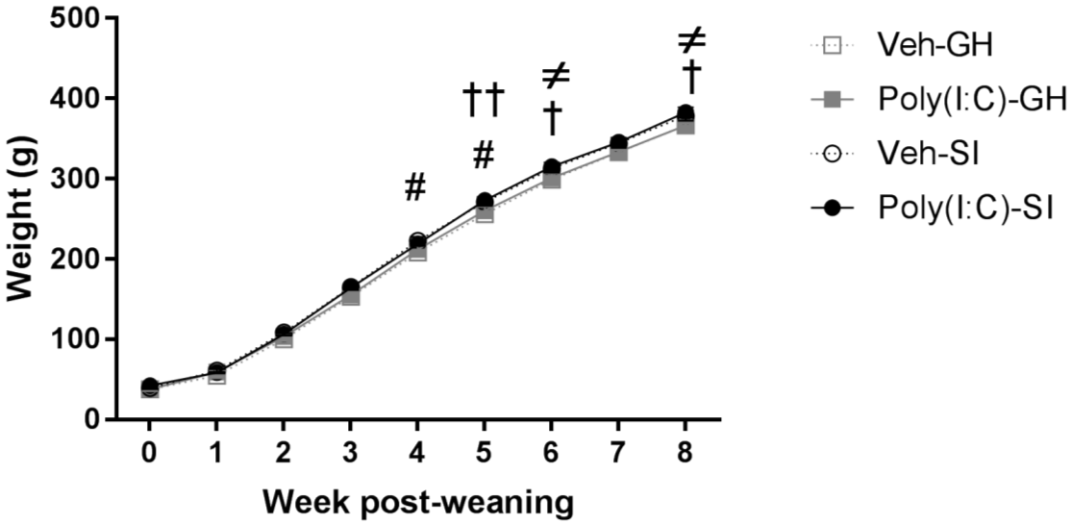


Figure 2.28. Effect of prenatal poly (I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on body growth from weaning till eight weeks post-weaning (postnatal day 73-78). Data are mean ± SEM (n=16 per treatment x housing). #p<0.05 Veh-SI compared to Veh-GH, †p<0.05, ††p<0.01 Poly(I:C)-SI compared to Veh-GH; ≠p<0.05, Poly(I:C)-GH compared to Poly(I:C)-SI; three-way RM ANOVA with Tukey's post-hoc test.

Cytokine analysis

To assess if behavioural alterations in adulthood were accompanied by alterations in cytokine levels, brain regions were collected at PND87-91.

In the frontal cortex, treatment significantly influenced IL-12 levels ($F_{(1,27)}$= 5.59, p<0.05) whereas housing significantly influenced IL-2 ($F_{(1,27)}$= 16.38, p<0.001), IL-6 ($F_{(1,27)}$= 11.06, p<0.01), IL-12 ($F_{(1,27)}$= 10.29, p<0.01) and TNF-α ($F_{(1,27)}$= 9.93, p<0.01) levels. There was a significant treatment x housing interaction on IL-1β ($F_{(1,28)}$= 7.87, p<0.01), IL-2 ($F_{(1,27)}$= 6.83, p<0.05), IL-4 ($F_{(1,28)}$= 5.56, p<0.05), IL-6 ($F_{(1,27)}$= 12.58, p<0.01), IL-10 ($F_{(1,28)}$= 14.43, p<0.001), IL-12 ($F_{(1,27)}$= 7.25, p<0.05) and TNF-α ($F_{(1,27)}$= 5.03, p<0.05) levels. SI alone increased the levels of multiple cytokines, of which some were prevented by gestational poly(I:C) exposure (Figure 2.29).

In the hippocampus, there was a significant main effect of treatment on IL-1β ($F_{(1,27)}$= 6.86, p<0.05), IL-10 ($F_{(1,27)}$= 5.02, p<0.05) and IL-12 ($F_{(1,27)}$= 4.96, p<0.05) levels, and a significant treatment x housing interaction on IL-10 ($F_{(1,27)}$= 6.65, p<0.05) levels. Post-hoc comparisons revealed no differences between any of the cytokines measured compared to Veh-GH (data not shown).

In the striatum, there was a significant main effect of treatment on IL-4 ($F_{(1,27)}$= 7.00, p<0.05), IL-6 ($F_{(1,28)}$= 5.73, p<0.05), IL-12 ($F_{(1,26)}$= 5.26, p<0.05), IFN-Ɣ ($F_{(1,28)}$= 8.84, p<0.01) and TNF-α ($F_{(1,28)}$= 7.29, p<0.05) levels, and a significant main effect of housing on IL-2 ($F_{(1,26)}$= 5.99, p<0.05) and TNF-α ($F_{(1,28)}$= 8.52, p<0.01) levels. There was also a significant treatment x housing interaction on IL-2 ($F_{(1,26)}$= 12.63, p<0.01), IL-4 ($F_{(1,27)}$= 6.97, p<0.05), IL-12 ($F_{(1,26)}$= 21.16, p<0.0001), IFN-Ɣ ($F_{(1,28)}$= 13.27, p<0.01) and TNF-α ($F_{(1,28)}$= 4.50, p<0.05) levels. Poly(I:C) alone increased the levels of several striatal cytokines. These increases were not observed when rats were subjected to SI, alone or combined (Figure 2.30). Plasma cytokines were below the detection limit.

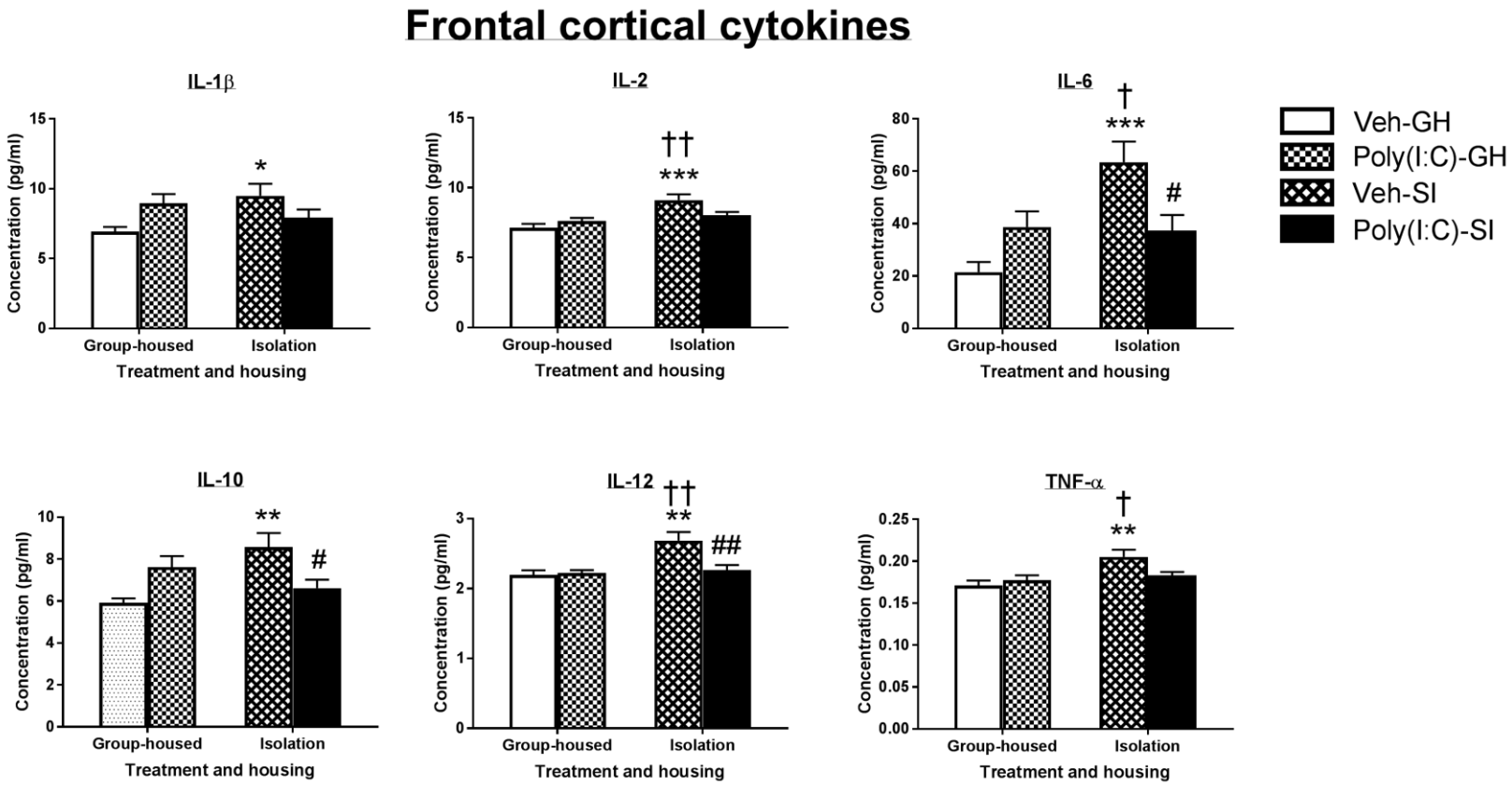


Figure 2.29. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on brain cytokines at nine weeks post-weaning (postnatal day 87-91). Data are mean ± SEM (n=7-8 per treatment x housing). *p<0.05, **p<0.01, ***p<0.001 compared to Veh-GH; †p<0.05, ††p<0.01 compared to Poly(I:C)-GH; #p<0.05, ##p<0.01 compared to Veh-SI; two-way ANOVA with Tukey's post-hoc test.

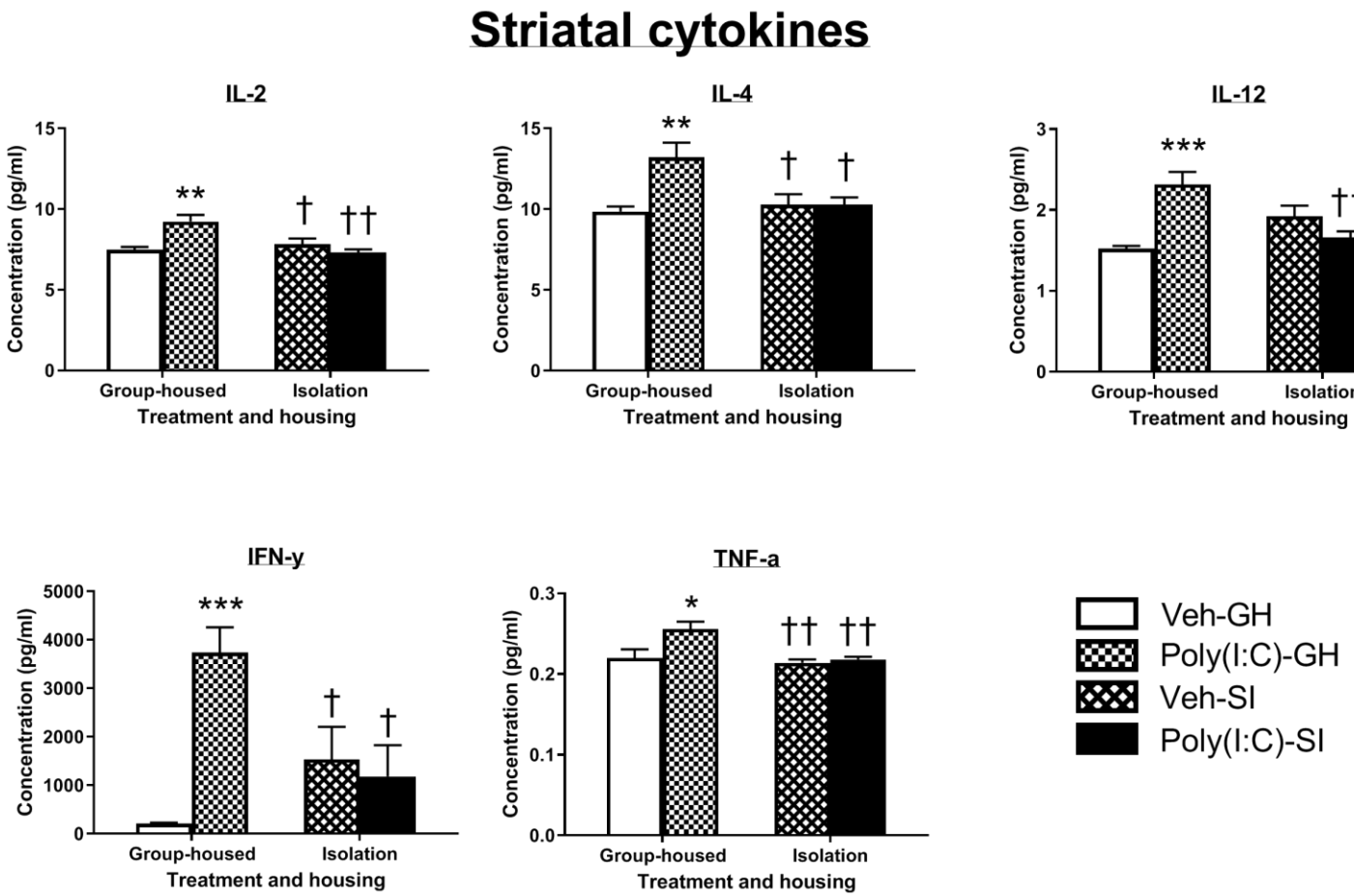


Figure 2.30. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on brain cytokines at nine weeks post-weaning (postnatal day 87-91). Data are mean ± SEM (n=7-8 per treatment x housing). *p<0.05, **p<0.01, ***p<0.001 compared to Veh-GH; †p<0.05, ††p<0.01 compared to Poly(I:C)-GH; two-way ANOVA with Tukey's post-hoc test.

mTOR analysis

To probe region-specific perturbations in mTOR signalling caused by MIA and/or SI, phospho mTOR and total mTOR were analysed in different brain regions implicated in schizophrenia.

In the frontal cortex, analysis of phospho mTOR revealed a significant main effect of housing ($F_{(1,28)}$= 15.16, p<0.001) and treatment ($F_{(1,28)}$= 5.87, p<0.05), as well as a treatment x housing interaction ($F_{(1,28)}$= 40.40, p<0.0001). Poly(I:C) alone and SI alone but not combined poly(I:C)-SI increased phospho mTOR compared to Veh-GH (Figure 2.31A). Housing ($F_{(1,28)}$= 6.65, p<0.05) and treatment x housing ($F_{(1,28)}$= 15.83, p<0.001) significantly influenced total mTOR, such that Poly(I:C)-GH and Veh-SI showed increased total mTOR (Figure 2.31B). For phospho:total mTOR, a significant effect of housing ($F_{(1,28)}$= 13.03, p<0.01), treatment ($F_{(1,28)}$= 6.76, p<0.05) and their interaction ($F_{(1,28)}$= 36.58, p<0.0001) were found. SI alone significantly increased phospho:total mTOR ratio, which was significantly reversed by prior poly(I:C) exposure (Figure 2.31C).

In the hippocampus, there was a significant effect of treatment ($F_{(1,27)}$= 6.11, p<0.05) and treatment x housing interaction ($F_{(1,27)}$= 10.08, p<0.01) on phospho:total mTOR, and a treatment x housing interaction for phospho mTOR ($F_{(1,27)}$= 8.70, p<0.01). Neither group was different to Veh-GH for any measures (data not shown).

In the striatum, there was a significant treatment x housing interaction for phospho mTOR ($F_{(1,27)}$= 7.25, p<0.05) and total mTOR ($F_{(1,26)}$= 4.42, p<0.05) but no between-group differences were found.

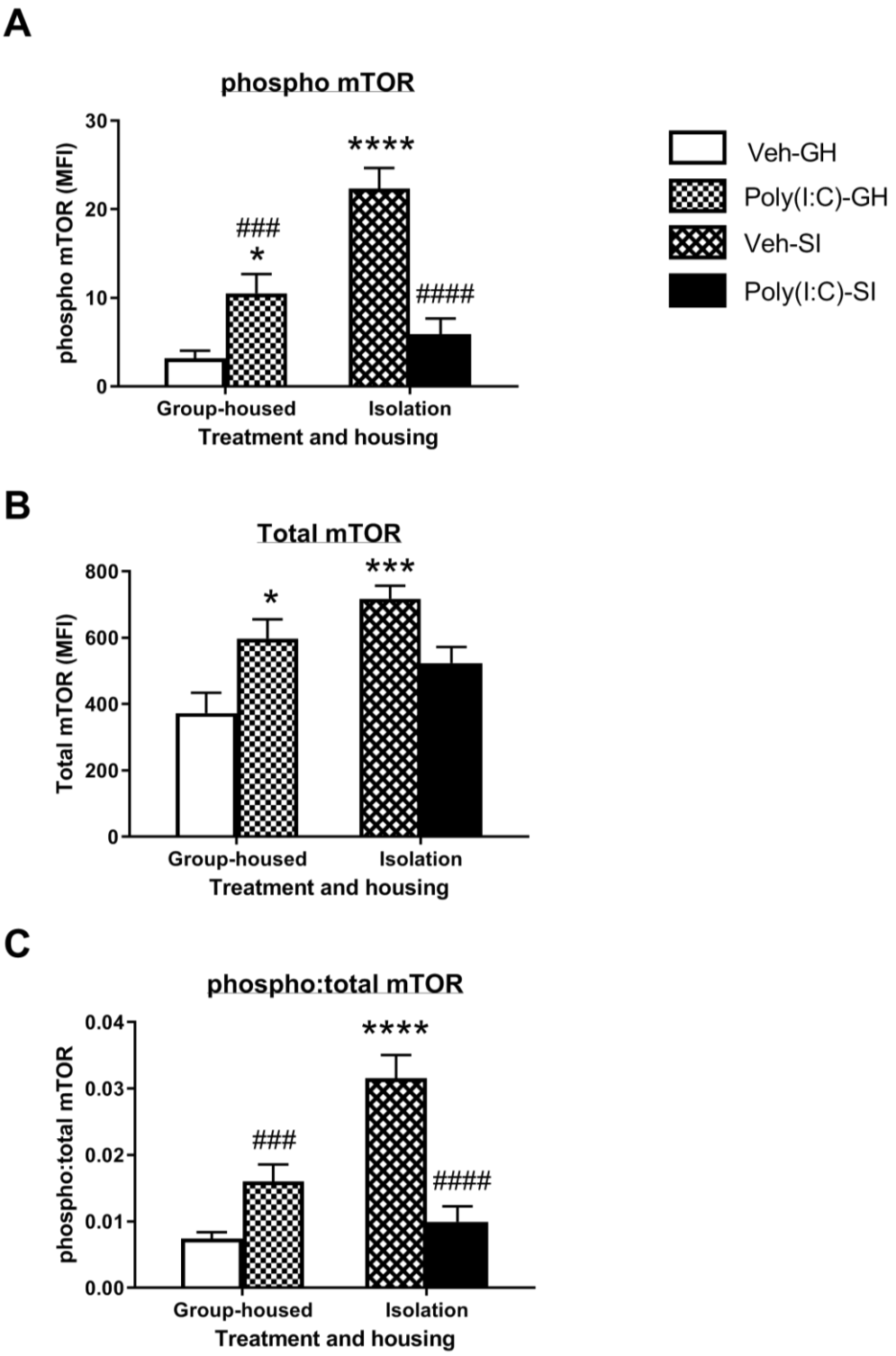


Figure 2.31. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on mTOR levels in the frontal cortex at nine weeks post-weaning (postnatal day 87-91). Mean ± SEM **(A)** phospho mTOR, **(B)** total mTOR and **(C)** phospho:total mTOR ratio (n=8 per treatment x housing). mTOR, mammalian target of rapamycin; phospho, phosphorylated. *p<0.05, ***p<0.001, ****p<0.0001 compared to Veh-GH; ###p<0.001, ####p<0.0001 compared to Veh-SI; two-way ANOVA with Tukey's post-hoc test.

2.4.2.3.3 Potential early mechanisms underlying behavioural alterations in adulthood

Cytokine analysis

In order to track gender-specific cytokine changes in adolescence that might prime behavioural alterations in adulthood, *ex vivo* cytokine analyses were performed on male and female brain regions dissected following LMA at two weeks post-weaning.

In males, there was a significant main effect of housing on IL-1β ($F_{(1,27)}$= 5.15, $p<0.05$) and a treatment x housing interaction on IL-18 ($F_{(1,27)}$= 5.51, $p<0.05$) in the frontal cortex. SI alone caused increased IL-18 compared to Veh-GH (Table 2.14). Treatment significantly influenced hippocampal IL-2 ($F_{(1,28)}$= 9.25, $p<0.01$), IL-4 ($F_{(1,25)}$= 7.65, $p<0.05$), IL-6 ($F_{(1,28)}$= 8.61, $p<0.01$), IL-10 ($F_{(1,27)}$= 6.16, $p<0.05$), IL-12 ($F_{(1,27)}$= 8.24, $p<0.01$) and TNF-α ($F_{(1,27)}$= 7.03, $p<0.05$), such that poly(I:C) alone was associated with reduced hippocampal IL-6 levels (Table 2.14). A significant effect of treatment was evident for striatal IFN-Ɣ ($F_{(1,27)}$= 6.71, $p<0.05$) and TNF-α ($F_{(1,25)}$= 5.43, $p<0.05$). Poly(I:C) alone reduced the levels of these cytokines regardless of housing (Table 2.14).

In females, neither treatment nor housing influenced any of the cytokines measured (Table 2.15).

Table 2.14. *Ex vivo* brain cytokine concentrations of male Lister-hooded rats subjected to gestational poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or 2-week-long post-weaning social isolation. Data are mean ± SEM (n=7-8 per treatment x housing).*p<0.05 compared to respective Veh-GH; two-way RM ANOVA with Tukey's post-hoc test.

Frontal cortical *ex vivo* concentrations (pg mg^{-1} total protein)				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
IL-1β	11.77 ± 0.63	11.17 ± 0.53	13.06 ± 0.25	12.34 ± 0.61
IL-2	8.48 ± 0.21	8.53 ± 0.30	8.84 ± 0.37	8.51 ± 0.23
IL-4	10.59 ± 0.29	10.33 ± 0.57	11.11 ± 0.51	10.41 ± 0.55
IL-6	89.91 ± 2.01	95.67 ± 3.82	96.46 ± 4.02	93.68 ± 3.00
IL-10	10.46 ± 0.65	9.30 ± 0.41	10.54 ± 0.36	10.46 ± 0.51
IL-12	1.98 ± 0.03	1.95 ± 0.03	2.03 ± 0.04	1.96 ± 0.04
IL-18	43.82 ± 2.92	46.66 ± 3.13	**57.12 ± 3.53***	45.50 ± 2.65
IFN-Ɣ	199.10 ± 31.43	176.70 ± 12.86	198.10 ± 11.04	199.80 ± 13.77
TNF-α	0.24 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.24 ± 0.01
Hippocampal *ex vivo* concentrations (pg mg^{-1} total protein)				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
IL-1β	12.56 ± 1.43	10.48 ± 0.86	11.04 ± 1.24	11.44 ± 0.59
IL-2	15.08 ± 2.75	8.45 ± 0.32	13.13 ± 2.02	9.31 ± 0.26
IL-4	24.00 ± 7.22	9.90 ± 0.61	20.18 ± 5.55	9.93 ± 0.21
IL-6	164.70 ± 27.52	**95.79 ± 4.05***	133.80 ± 16.55	107.20 ± 3.57
IL-10	13.62 ± 2.36	9.33 ± 0.65	13.61 ± 2.12	10.10 ± 0.32
IL-12	2.87 ± 0.41	2.05 ± 0.05	2.94 ± 0.39	2.18 ± 0.03
IL-18	46.97 ± 3.20	37.01 ± 3.20	41.13 ± 4.18	44.66 ± 4.06
IFN-Ɣ	209.30 ± 24.99	160.80 ± 38.05	170.50 ± 31.04	217.00 ± 16.60
TNF-α	0.33 ± 0.04	0.25 ± 0.01	0.32 ± 0.03	0.27 ± 0.01
Striatal *ex vivo* concentrations (pg mg^{-1} total protein)				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
IL-1β	36.50 ± 9.96	17.44 ± 0.78	18.64 ± 1.51	17.18 ± 0.93
IL-2	16.18 ± 3.03	10.36 ± 0.39	10.74 ± 0.46	10.16 ± 0.42
IL-4	75.19 ± 32.02	29.33 ± 2.69	32.61 ± 3.33	29.65 ± 2.85
IL-6	246.10 ± 45.97	183.60 ± 9.97	207.80 ± 19.59	176.50 ± 12.32
IL-10	36.19 ± 10.48	15.93 ± 0.85	16.87 ± 1.25	15.48 ± 1.00
IL-12	5.92 ± 2.47	2.81 ± 0.15	2.83 ± 0.14	2.74 ± 0.13
IL-18	40.64 ± 4.12	35.92 ± 4.23	41.42 ± 4.17	39.77 ± 3.70
IFN-Ɣ	452.10 ± 133.90	**137.70 ± 21.51***	222.20 ± 46.88	**149.60 ± 16.16***
TNF-α	0.70 ± 0.13	**0.38 ± 0.01***	0.43 ± 0.03	**0.39 ± 0.01***

Table 2.15. *Ex vivo* brain cytokine concentrations of female Lister-hooded rats subjected to gestational poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or 2-week-long post-weaning social isolation. Data are mean ± SEM (n=7-8 per treatment x housing); two-way RM ANOVA with Tukey's post-hoc test. It was not possible to obtain readings for striatal cytokines.

Frontal cortical *ex vivo* concentrations (pg mg^{-1} total protein)				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
IL-1β	5.25 ± 0.38	6.15 ± 0.39	5.29 ± 0.26	5.02 ± 0.75
IL-2	8.39 ± 0.07	8.45 ± 0.29	8.33 ± 0.10	8.18 ± 0.19
IL-4	7.07 ± 1.19	7.17 ± 0.85	7.29 ± 0.64	5.97 ± 1.23
IL-6	3.25 ± 2.10	14.95 ± 6.09	13.82 ± 6.44	11.64 ± 5.69
IL-10	1.87 ± 0.32	2.26 ± 0.28	2.25 ± 0.40	1.88 ± 0.42
IL-12	3.51 ± 0.15	3.51 ± 0.15	3.60 ± 0.09	3.58 ± 0.10
IL-18	49.98 ± 1.50	57.84 ± 4.57	53.90 ± 3.29	58.83 ± 5.90
IFN-Ɣ	209.40 ± 18.77	238.10 ± 16.90	218.00 ± 14.68	195.90 ± 14.70
TNF-α	0.22 ± 0.00	0.22 ± 0.01	0.22 ± 0.00	0.22 ± 0.00
Hippocampal *ex vivo* concentrations (pg mg^{-1} total protein)				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
IL-1β	13.15 ± 2.61	13.83 ± 2.97	10.64 ± 1.78	9.74 ± 1.90
IL-2	18.98 ± 1.20	17.84 ± 1.06	16.28 ± 0.35	16.79 ± 0.77
IL-6	180.50 ± 12.65	179.50 ± 13.32	163.30 ± 13.48	169.80 ± 13.38
IL-10	-	0.28 ± 0.28	-	-
IL-12	3.92 ± 0.25	3.57 ± 0.08	3.32 ± 0.07	3.55 ± 0.20
IL-18	54.13 ± 3.28	55.60 ± 6.72	55.83 ± 5.68	47.46 ± 3.59
IFN-Ɣ	249.20 ± 34.61	309.90 ± 65.49	229.20 ± 21.40	237.90 ± 22.42
TNF-α	0.42 ± 0.04	0.41 ± 0.02	0.35 ± 0.02	0.37 ± 0.02

Correlation between locomotor activity and cytokines in adolescence

Correlation analyses were performed to examine the relationship between total LMA and brain cytokine levels in the different treatment x housing groups in adolescence.

In males, total LMA in each group was positively correlated to cytokine levels in distinct brain regions (Table 2.16). Meanwhile, in females, only LMA in the Poly(I:C)-GH and Poly(I:C)-SI groups were significantly correlated to cytokine levels, in the frontal cortex and/or hippocampus (Table 2.17).

Table 2.16. Correlation analysis between total locomotor activity and brain cytokine levels in adolescent males. The relationships between locomotor activity and individual cytokines in different brain regions were examined. R-squared values are presented. *p<0.05; Pearson's correlation coefficient.

Adolescent males: total locomotor activity versus brain cytokine level				
Frontal cortex				
Cytokine	**Veh-GH**	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
IL-1β	0.1162	0.7464	0.2830	0.2068
IL-2	0.2855	0.6168	0.1818	0.2431
IL-4	0.0009	0.7128	0.1621	0.0689
IL-6	0.0611	0.5676	0.0492	0.4234
IL-10	0.0787	0.2732	0.1506	0.1795
IL-12	0.0039	0.0477	0.0010	0.0422
IL-18	0.0001	0.0355	**0.5631***	0.2817
IFN-ϒ	0.0497	0.6989	0.2920	0.0068
TNF-α	0.0929	0.5676	0.2250	0.0640
Hippocampus				
Cytokine	**Veh-GH**	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
IL-1β	0.2429	0.1785	0.0259	0.4174
IL-2	0.0647	0.0657	0.0017	0.6646
IL-4	0.5334	0.4240	0.0740	0.0003
IL-6	0.1032	0.1576	0.0003	0.1201
IL-10	0.4950	0.0183	0.0001	0.2487
IL-12	**0.5766***	0.00009	0.0034	0.0139
IL-18	0.0009	0.3067	0.0291	0.3802
IFN-ϒ	0.0764	0.1374	0.0058	0.4432
TNF-α	0.5251	0.0468	0.0465	0.0486
Striatum				
Cytokine	**Veh-GH**	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
IL-1β	0.0033	**0.9309***	0.0505	0.0553
IL-2	0.0134	0.4822	0.0615	0.3114
IL-4	0.1121	0.0318	0.0033	0.2904
IL-6	0.1939	0.1237	0.0400	0.0078
IL-10	0.0081	**0.9399***	0.0329	0.1510
IL-12	0.0856	0.0942	0.0678	0.3511
IL-18	0.0616	0.8439	0.1000	0.3240
IFN-ϒ	0.0196	0.4298	0.0057	**0.9123***
TNF-α	0.0095	0.8850	0.0022	0.3074

Table 2.17. Correlation analysis between total locomotor activity and brain cytokine levels in adolescent females. The relationships between locomotor activity and individual cytokines in different brain regions were examined. R-squared values are presented. *p<0.05; Pearson's correlation coefficient.

Adolescent females: total locomotor activity versus brain cytokine level				
Frontal cortex				
Cytokine	**Veh-GH**	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
IL-1β	0.0008	0.0517	0.000002	0.4942
IL-2	0.0128	0.3087	0.0480	0.1742
IL-4	0.0550	**0.6123***	0.0668	**0.6668***
IL-6	0.2795	0.4693	0.0130	0.2929
IL-10	0.0003	0.0312	0.0344	0.0038
IL-12	0.0334	**0.5787***	0.2485	0.2316
IL-18	0.0137	0.1047	0.0056	0.1262
IFN-Ɣ	0.1206	0.0166	0.0452	0.5392
TNF-α	0.0226	0.2394	0.1337	0.3462
Hippocampus				
Cytokine	**Veh-GH**	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
IL-1β	0.3636	0.2336	0.0415	0.3333
IL-2	0.1662	**0.5398***	0.2717	0.0785
IL-6	0.1648	0.3649	0.0234	0.0939
IL-10	-	0.1739	-	-
IL-12	0.2805	0.0364	0.0956	0.0218
IL-18	0.0511	0.1944	0.0009	0.0544
IFN-Ɣ	0.3553	0.3166	0.0021	0.0073
TNF-α	0.3623	0.4895	0.1327	0.0758

mTOR analysis

To investigate if mTOR signalling was altered, total mTOR, phospho mTOR and the phospho:total mTOR ratio were derived using a 2-plex magnetic bead assay. There was a significant main effect of treatment on the frontal cortical phospho mTOR ($F_{(1,28)}$= 6.23, p<0.05) and phospho:total mTOR ($F_{(1,28)}$= 6.15, p<0.05) in males but there were no differences between groups. Neither treatment nor housing influenced either measure in the hippocampus and striatum (Table 2.18).

In females, there was a significant main effect of housing on frontal cortical phospho mTOR ($F_{(1,28)}$= 5.38, p<0.05), total mTOR ($F_{(1,26)}$= 11.73, p<0.01) and phospho:total mTOR ($F_{(1,27)}$= 6.33, p<0.05), as well as a treatment x housing interaction on total mTOR ($F_{(1,26)}$= 5.21, p<0.05).

Poly(I:C)-SI significantly reduced frontal cortical total mTOR (Table 2.19). In the hippocampus, a significant treatment x housing interaction was revealed for phospho mTOR ($F_{(1,27)}$= 9.86, p<0.01) and phospho:total mTOR ($F_{(1,27)}$= 4.59, p<0.05), such that SI alone increased phospho mTOR (Table 2.19). Neither treatment nor housing influenced mTOR signalling in the striatum of female offspring.

Table 2.18. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on mTOR signalling in the male brain at two weeks post-weaning (postnatal day 36). mTOR readings were corrected to a final protein concentration of 1 pg mg^{-1}. For each group, individual raw data values of ≥±2 standard deviation from the average total mTOR, phospho mTOR or phospho:total mTOR were eliminated. Data are mean ± SEM (n=7-8 per treatment x housing); two-way ANOVA with Tukey's post-hoc test. MFI, mean fluorescence intensity; mTOR, mammalian target of rapamycin; phospho, phosphorylated.

Frontal cortex (MFI)				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
phospho mTOR	2.45 ± 0.43	3.28 ± 0.22	2.72 ± 0.35	3.46 ± 0.19
total mTOR	923.50 ± 66.87	989.70 ± 41.89	972.3 ± 46.50	993.90 ± 21.81
phospho:total mTOR	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Hippocampus (MFI)				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
phospho mTOR	3.29 ± 0.61	3.30 ± 0.69	2.82 ± 0.58	2.81 ± 0.71
total mTOR	986.20 ± 62.15	809.50 ± 88.79	825.70 ± 80.69	689.20 ± 97.49
phospho:total mTOR	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Striatum (MFI)				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
phospho mTOR	0.79 ± 0.23	2.10 ± 0.51	0.92 ± 0.28	0.85 ± 0.24
total mTOR	551.30 ± 121.60	887.70 ± 127.90	595.20 ± 112.50	545.10 ± 108.20
phospho:total mTOR	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 2.19. Effect of prenatal poly(I:C) exposure (10 mg kg^{-1}, i.p., gestational day 15) and/or post-weaning social isolation on mTOR signalling in the female brain at two weeks post-weaning (postnatal day 36). mTOR readings were corrected to a final protein concentration of 1 pg mg^{-1}. For each group, individual raw data values of ≥±2 standard deviation from the average total mTOR, phospho mTOR or phospho:total mTOR were eliminated. Data are mean ± SEM, n=7-8 per treatment x housing. *p<0.05 compared to Veh-GH; ††p<0.01 compared to Poly(I:C)-GH; two-way ANOVA with Tukey's post-hoc test. MFI, mean fluorescence intensity; mTOR, mammalian target of rapamycin; phospho, phosphorylated.

Frontal cortex (MFI)				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
phospho mTOR	3.32 ± 0.41	3.53 ± 0.51	2.70 ± 0.28	2.41 ± 0.24
total mTOR	968.70 ± 27.51	1014.00 ± 38.12	926.60 ± 17.97	**804.00 ± 54.88*††**
phospho:total mTOR	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Hippocampus (MFI)				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
phospho mTOR	3.48 ± 0.49	5.93 ± 0.85	**6.60 ± 0.61***	4.68 ± 0.75
total mTOR	809.70 ± 93.17	991.40 ± 59.27	965.50 ± 56.81	870.10 ± 99.13
phospho:total mTOR	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Striatum (MFI)				
phospho mTOR	1.09 ± 0.38	0.63 ± 0.20	1.09 ± 0.30	1.22 ± 0.43
total mTOR	559.90 ± 122.70	622.40 ± 73.53	620.30 ± 97.52	649.90 ± 109.90
phospho:total mTOR	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Correlation between locomotor activity and mTOR in adolescence

Correlation analyses were performed to examine the relationship between total LMA and brain mTOR levels in the different treatment x housing groups in adolescent rats.

In Poly(I:C)-GH males, total LMA showed a positive correlation to phospho or total mTOR levels in the striatum. No other significant correlations were evident in males (Table 2.20).

In Veh-GH females, there was a significant positive correlation between total LMA and hippocampal phospho or total mTOR levels. In both the

Poly(I:C)-GH and Veh-SI groups, striatal phospho mTOR levels were positively correlated with total LMA. Neither mTOR measures showed correlation with total LMA in Poly(I:C)-SI females (Table 2.20).

Table 2.20. Correlation analysis between total locomotor activity and brain mTOR levels in adolescent males and females. The relationships between locomotor activity and phosphorylated mTOR, total mTOR or phospho:total mTOR ratios in different brain regions were examined. R-squared values are presented. *p<0.05, **p<0.01; Pearson's correlation coefficient.

Total locomotor activity versus mTOR Adolescent males				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
Frontal cortex				
phospho mTOR	0.0227	0.2234	0.3596	0.0108
total mTOR	0.3476	0.3854	0.1334	0.2274
phospho:total mTOR	0.0000	0.1615	0.1086	0.0002
Hippocampus				
phospho mTOR	0.0668	0.0094	0.0049	0.1144
total mTOR	0.0198	0.0459	0.2265	0.0437
phospho:total mTOR	0.4368	0.0158	0.4053	0.0368
Striatum				
phospho mTOR	0.4009	**0.8040****	0.3606	0.5069
total mTOR	0.3550	**0.8313****	0.0091	0.3232
phospho:total mTOR	0.0092	0.0294	0.0307	0.0080
Adolescent females				
	Veh-GH	**Poly(I:C)-GH**	**Veh-SI**	**Poly(I:C)-SI**
Frontal cortex				
phospho mTOR	0.1177	0.3726	0.5077	0.4329
total mTOR	0.0259	0.0585	0.0326	0.1275
phospho:total mTOR	0.0288	0.0001	0.0023	0.0013
Hippocampus				
phospho mTOR	**0.6389***	0.2013	0.0051	0.1101
total mTOR	**0.8663****	0.2662	0.0904	0.0032
phospho:total mTOR	0.1794	0.2061	0.3766	0.0798
Striatum				
phospho mTOR	0.0176	**0.6185***	**0.5707***	0.0324
total mTOR	0.1143	0.5634	0.0049	0.0771
phospho:total mTOR	0.0079	0.0581	0.1677	0.0097

2.5 Discussion

Poly(I:C) administration (10 mg kg^{-1}, i.p.) to non-pregnant female rats significantly increased sickness behaviour but did not affect body temperature and brain cytokine levels. In pregnant dams, the same dose reduced body temperature (peak 2 h post-injection), induced sickness behaviour (peak 3-4 h) and weight loss at 24 h, all of which returned to control values by 48 h. At two weeks post-weaning, male Veh-SI and poly(I:C)-SI were both hyperactive but LMA alterations were less robust in females. In adolescent male poly(I:C)-SI, locomotor hyperactivity was accompanied by increased striatal dopamine and 5-HIAA. Interestingly, prenatal exposure to poly(I:C) prevented or attenuated several SI-induced behavioural alterations in adulthood (LMA, aggressive behaviour and CFR), as well as cytokine and mTOR abnormalities. These findings will now be discussed in the context of previous research on adulthood (Experiment 1) or gestational (Experiment 2) poly(I:C) exposure via i.p. administration, or post-weaning isolation rearing (not adult-only SI), and other 'dual-hit' studies.

2.5.1 Experiment 1: The effect of poly(I:C) via i.p. administration on non-pregnant Lister-hooded rats

In the pilot study (n=4 rats per group), albeit sickness behaviour was transiently increased, there was little evidence of any major change in body temperature or cytokine function in poly(I:C)-treated non-pregnant rats over 4 h post-injection. This is in contrast with the study by Murray et al. (2019), which has observed significantly increased core body temperature and plasma IL-6 levels in adult non-pregnant Lister-hooded females 3 h after injection of 10 mg kg^{-1} poly(I:C). In contrast to the current study, which monitored core body temperature in free-moving rats via s.c. implanted microchips, Murray and colleagues measured core body temperature at baseline, 3 h and 6 h post-injection using a rectal thermistor probe, with a seven-day habituation to the procedure preceding the experiment. Insertion of a thermosensitive device into the

rectum of a rat, which necessitates handling and restraint of animals, is generally considered a stressful procedure (Kort et al., 1998). Both handling and rectal probe insertion, together with other common stressors in an experimental setting including transportation or isolation of animals, have been shown to induce hyperthermia, which are typically long-lasting. Rectal probe insertion for instance has been reported to cause sustained elevation of core body temperature even at 50-70 min after probe removal (Bae et al., 2007; Dallmann et al., 2006; Dilsaver et al., 1992; Eikelboom, 1985; Poole & Stephenson, 1977). Moreover, repeated rectal probe insertions do not appear to diminish such hyperthermic response (Bae et al., 2007; Dangarembizi et al., 2017). Collectively, these factors could possibly explain the differential body temperature outcome in the present study compared to the study conducted by Murray et al. (2019), considering their experimental procedure involved multiple rectal temperature recordings as well as a prior habituation period.

Another possible reason for the disparities in body temperature data could be ascribed to the location of temperature measurement. In the current study, transponders were implanted in the nape of the neck of the rats. Consequently, the resultant subcutaneous temperatures are reflective of peripheral temperatures, i.e. those of the skin and subcutaneous tissues, rather than core body temperature, are highly variable and can be easily influenced by ambient temperature (Meyer et al., 2017). Rectal temperatures, on the other hand, are thought to be more reflective of core body temperature, as when the rectal probe is inserted into an appropriate depth of the rectum, the tip would lie close to the liver, of which temperature is deemed the best approximation to the body core temperature (Lomax, 1966). Following poly(I:C) administration, reduced skin temperature has been shown to occur concomitantly with increased core body temperature in male rats, suggesting activation of heat conservation mechanisms including peripheral constriction (Bastos-Pereira et al., 2015). Given that in the current study a trend towards reduced “subcutaneous temperature” was observed, this could well be

the case. However, it is also likely that the inclination towards hypothermia observed in the current study is indicative of potential development of an overall stress-induced hypothermia on a systemic level, rather than a reduction in temperature that is restricted to the peripheral level. Indeed, close agreement between subcutaneous and rectal temperatures have been previously demonstrated (Cilia et al., 1998; Kort et al., 1998). The manifestation of stress-induced hypothermia is dictated by the ambient temperature, with lower and higher ambient temperatures linked to increased possibilities of developing hypothermia and hyperthermia, respectively (Amar & Sanyal, 1981; Wanner et al., 2013; Yokoi, 1966). Thus, it is possible that the ambient temperature in the experimental room where injections and body temperature monitoring were performed in the current study promoted the development of hypothermia, which confers a protective, energy-saving function under stressful situations (Romanovsky & Székely, 1998). Stress-induced hypothermia has been described by many others (Amar & Sanyal, 1981; Wanner et al., 2013; Yokoi et al., 1966).

It should also be appreciated that stress-induced thermal responses (hyperthermia, hypothermia or no effect) and their magnitude and recovery are dependent on a sophisticated interplay between individual, environmental and social factors, including age, sex, severity and duration of stress, prior stress experiences and stress coping strategies (Oka, 2018). Thus, albeit in opposition to the reference work by Murray et al. (2019) and studies using male rats, which found that lower doses of poly(I:C) (0.03 – 4 mg kg^{-1}) induced fever with a typical onset at 2-2.5 h post-injection, with peaks observed at 2.5-7 h, the lack of significant change in body temperature in the current study does not necessarily suggest a lack of efficacy of poly(I:C) (Bastos-Pereira et al., 2015; Fortier et al., 2014; Hopwood et al., 2009; Katafuchi et al., 2003; Yamato et al., 2014). Assessment of the purity and molecular weight of poly(I:C) could provide valuable information to aid interpretation of data in the current experiment (Kowash et al., 2019; Mueller et al., 2019).

The current study observed a transient increase in sickness behaviour at 3 h following poly(I:C) injection. However, in contrast to studies using male rats which have characterised other physiological and behavioural effects of poly(I:C) including weight loss and decreased food intake, saccharin preference, wheel running activity (indicative of fatigue) and spontaneous LMA, no known studies have examined such effects in non-pregnant female rats (Fortier et al., 2004; Gibney et al., 2013; Hopwood et al., 2009; Katafuchi et al., 2003 ; Yamato et al., 2014). As gender could influence behavioural and physiological responses to a particular stress or treatment, it would be inappropriate to compare the current observation against prior studies conducted using male rats (Archer, 1975; Robison et al., 2017).

Murray et al. (2019) found significantly increased plasma IL-6 levels 3 h post-injection following 10 mg kg^{-1} poly(I:C) administration to non-pregnant Lister-hooded females. They also commented that some of the Lister-hooded females were “non-responders” (the authors defined “non-responders” as rats which had an IL-6 level of 50 pg ml^{-1} or less following poly(I:C) injection). In the present study, the levels of five cytokines (IFN-Ɣ, IL-1β, IL-6, IL-10 and TNF-α) were measured in the frontal cortex, hippocampus, hypothalamus and striatum as well as in the plasma. Although plasma cytokines were below the detection limit thus preventing comparison with the findings by Murray and colleagues, with only n=4 for the poly(I:C) group in the current study, it is not impossible that a substantial proportion of the group were “non-responders”, thus yielding non-significant results for brain cytokine levels. Furthermore, stressors such as handling and rectal temperature measurement have been shown to influence serum cytokine levels (Hale et al., 2003). This could provide an alternative explanation for the contrasting findings between our study and that by Murray et al. (2019), considering the differences in experimental procedure.

Human pregnancy is associated with altered innate and adaptive immune responses at the decidua and in the systematic circulation (Svensson-

Arvelund et al., 2014). Akin to humans, pregnant rats have been consistently found to exhibit increased sensitivity to pro-inflammatory stimuli compared to non-pregnant rats (Cipolla et al., 2011; Faas et al., 2000; Faas et al., 2004). Thus, the use of non-pregnant animals in the present study, due to considerations of 3Rs, reflects a major drawback. Considering the enhanced immune sensitivity in pregnancy, and given that we observed a trend towards hypothermia at around 2 h post-injection together with increased sickness behaviour without obvious adverse effects in the poly(I:C) group, we decided to extend our study into pregnant rats using identical dose and route of administration.

2.5.2 Experiment 2: The combined effects of gestational poly(I:C) via i.p. administration and post-weaning social isolation on behaviour and neurochemistry

2.5.2.1 Physiological and behavioural effects of poly(I:C) on dams

In the current study, poly(I:C) induced hypothermia, sickness behaviour and weight loss which were normalised by 4-48 h compared to saline-treated dams. Contrary to our findings, our reference MIA study found no effect of poly(I:C) (10 mg kg^{-1}) on core body temperature (Murray et al., 2019). As aforementioned, stress-induced thermal responses are reliant upon an array of complicated factors and therefore the dissimilar findings are unsurprising. Moreover, Murray and colleagues used pregnant Wistar rats, as opposed to Lister-hooded rats in our study. Thus, the differences in thermal response to poly(I:C) could simply be attributed to strain differences. Murray et al. (2019) also observed significantly elevated plasma IL-6 levels in poly(I:C)-treated dams 3 h post-injection. However, blood was not collected in the current study and therefore prevented comparison (see later). Several other studies have found a general increase in maternal plasma or placental pro-inflammatory cytokines and chemokines 2-24 h following acute poly(I:C) injection of 2.5-20 mg kg^{-1}, despite some variations between studies (Forrest et al., 2012; Gilmore et al., 2005; Petrovic & Piquette-Miller, 2010). Chronic 10 mg kg^{-1} poly(I:C) dosing on GD10, 12, 14, 16 and 18 increased maternal serum IFN-ϒ and

IL-10 on GD20, in addition to placental TLR3 expression and IFN-Ɣ/IL-10 ratio (Tinsley et al., 2009). Apart from cytokines, the effects of viral-mimetic on the kynurenine pathway and BDNF and NGF expression in maternal placenta and amniotic fluid have also been studied (Gilmore et al., 2005; Khalil et al., 2003). The current observation that poly(I:C) induces weight loss in dams over the 24 h post-injection is consistent with previous findings (Bronson et al., 2011; Murray et al., 2019; Richtand et al., 2011). Maternal weight loss following poly(I:C) administration has been suggested to predict behavioural abnormalities in offspring (Bronson et al., 2011). However, it was later found that offspring of dams with weight gain was also associated with behavioural abnormalities that were relevant to schizophrenia (Vorhees et al., 2012; Vorhees et al., 2015). Therefore, maternal weight response to poly(I:C) challenge might not be a reliable predictor of functional outcome in resultant offspring. Whilst no studies have reported sickness behaviour other than reduced weight gain in dams following poly(I:C), the manifestation of sickness behaviour in the current study is indicative of successful immune activation by poly(I:C) (Dantzer, 2001).

It has been speculated that hypothermia may compromise host defence by influencing cytokine production (Fairchild et al., 2010; Matsui et al., 2006; Russwurm et al., 2002). Hence, the lack of evaluation of blood and brain cytokine levels in poly(I:C)-treated dams, due to constraints of the project licence and attempts to minimise stress in pregnant rodents, is a shortfall in the present study. The inclusion of additional pregnant females as positive controls, of which their blood, placentae and foetuses could be harvested for further analyses, including maternal corticosterone levels, placental integrity marker expression and foetal blood brain-barrier or brain cytokine levels, would provide important insights into the mechanisms of MIA. However, this was impeded by the associated cost and would require justification and amendment of the exisiting project licence.

2.5.2.2 Effect of poly(I:C) on offspring

Poly(I:C) did not influence litter size, gender distribution within litters and pup body weight at weaning. This is in agreement with most previous studies which employed i.p. administration (Bronson et al., 2011; Murray et al., 2019; Vorhees et al., 2015). However, one study has observed poly(I:C)-induced miscarriage (8 mg kg^{-1}, GD15; Bronson et al., 2011) whereas another reported increased malformation in foetuses (10 mg kg^{-1}, GD10, 12, 14, 16, 18; Tinsley et al., 2009). The disparity in pregnancy or postnatal outcomes between studies can be attributed to differences in strain, molecular composition of poly(I:C) and protocol (e.g. dose and timing and frequency of dosing; Kentner et al., 2019). In addition, differences in housing condition and husbandry procedures such as cage cleaning could alter stress levels in dams, thereby influencing pregnancy and/or postnatal outcome (Castelhano-Carlos & Baumans, 2009). Reduced placental but not body or brain weight in both genders at GD21 have also been shown, in conjunction with increased brain to body weight ratio in both genders and increased activated microglia in male hippocampi at PND21 (Murray et al., 2019). In the current study, however, this was not feasible as dams were to be kept until parturition and the use of additional dams for this purpose was prevented by cost.

2.5.2.3 Effect of poly(I:C) and post-weaning isolation rearing on offspring

As isolates consistently demonstrate exaggerated response when placed in a novel environment from 2 weeks after isolation commencement, locomotor hyperactivity is considered a suitable marker for validating the development of ‘isolation syndrome’ before further behavioural or neurochemical phenotyping is performed (section 1.4). In the current study, hyperactivity in a novel arena was observed in adolescent male isolates two weeks post-weaning, regardless of gestational treatment, but this effect was less robust in females. SI-induced hyperactivity, which reflects positive symptoms in schizophrenia, is in agreement with

previous studies (Bakshi & Geyer, 1999; King et al., 2009; Sun et al., 2017). The less robust effects of SI on LMA in females have been previously documented, potentially due to slower development of the 'isolation syndrome' in this gender (Fone & Porkess, 2008). Taken together, the observed hyperactivity confirmed successful implementation of the SI protocol in the current study.

SI-induced augmentation in activity has been linked to enhanced mesolimbic dopaminergic function in the striatum, specifically at the presynaptic level, and is thought to be modulated by the serotonergic system (Jones et al., 1990; Lapiz et al., 2003). Numerous SI-induced dysregulation of dopaminergic and serotonergic functions have been proposed, including excessive firing of dopaminergic neurons, altered striatal pre- and postsynaptic dopamine functions, and decreased basal dopamine and 5-HT turnover in the medial PFC and nucleus accumbens, respectively (Fabricius et al., 2010; Hall et al., 1998; Heidbreder et al., 2000). Whilst findings on basal levels of dopamine, DOPAC, 5-HT and 5-HIAA remain controversial (see review by Fone & Porkess, 2008), the fact that the current study observed altered basal levels of 5-HIAA in the striatum of both adolescent Veh-SI and Poly(I:C)-SI males, with the latter also associated with elevated basal level of striatal dopamine, confirms the robustness of the SI protocol in stimulating neurochemical-related alterations. Of note, increased striatal dopamine activity corresponds to clinical observations in schizophrenia (Abi-Dargham et al., 1998; Howes et al., 2012). The modest influence of poly(I:C) alone is not surprising, as previous findings revealed no effect on spontaneous or novelty-stimulated locomotion (Bronson et al., 2011; Vorhees et al., 2015). The lack of effect of gestational poly(I:C) on neurochemical substrates, however, is in disagreement with prior research. For instance, injection of 10 mg kg^{-1} poly(I:C) into pregnant dams at GD9 has been shown to significantly decrease post-mortem 5-HT levels in the hippocampus of PND50 offspring, as well as to interfere with early development of serotonergic neurons (Ohkawara et al., 2015). In sum, the more robust effect of SI on male locomotion and neurochemistry supported the use of

male rats in subsequent behaviour testing in adulthood. Although the effects of poly(I:C) alone were rather modest, enhanced striatal dopamine activity which was specific to Poly(I:C)-SI males indicated that there was an interaction between the two neurodevelopmental 'hits', showing initial promise of our novel 'dual-hit' model.

In early adulthood, SI alone and poly(I:C) alone caused enhanced rearing but no additional hyperactivity observed when SI was combined with prenatal poly(I:C) treatment, nor there was any effect on horizontal ambulation or fine movements. Elevated rearing is consistent with preceding research (Dalrymple-Alford & Benton, 1981; Fone et al., 1996). Whilst increased horizontal ambulation is typical in rats following SI of at least four weeks (Bakshi & Geyer, 1999; Dalrymple-Alford & Benton, 1981; McIntosh et al, 2013), the occurrence of such enhanced locomotion is not absolute, as a previous behavioural characterisation of thirteen isolation rearing studies in our laboratory has suggested (Fone & Porkess, 2008). The same conclusion was found when newer studies by the laboratory (conducted post-2008; nine in total) were added to the behavioural characterisation (Table 2.21). Several studies have also reported absence of SI-induced increase in horizontal ambulation (Bianchi et al., 2006; Okudan & Belviranli, 2017; Tanaś et al., 2015; Weiss et al., 2004). Additionally, a recent study reported higher ambulation only at the 10 min time point, indicating that ambulatory activity in isolates can be variable (Shortall et al., 2018). No published studies have reported the impact of SI on fine (stereotyped) movements. The fact that hyperactivity was observed, although not across all three activity types, confirmed successful development of the 'isolation syndrome'. The subtle impact of poly(I:C) on its own on rearing is not unexpected, as previous findings revealed no effect of MIA on spontaneous or novelty-induced LMA (Bronson et al., 2011; Richtand et al., 2011; Richtand et al., 2012b). Interestingly, the current finding implies a resilience effect that was conferred by poly(I:C) exposure against SI-induced increase in rearing.

Table 2.21. Behavioural characterisation of 22 distinct isolation rearing studies. An updated version of the characterisation published by Fone & Porkess (2008) is provided. A total of nine studies conducted post-2008 were added to the characterisation. Numbers presented denote the percentage of experiments where a significant change in behaviour was observed in comparison to GH animals.

SI duration (weeks to first behavioural test)	Hyperactivity in a novel arena	NOD impairment	PPI deficit
2 (1 study)	100	100	-
4 (9 studies; NOD examined in 8, PPI examined in 5)	100	88	60
5 (8 studies)*	88	50	88
6 (4 studies; LMA examined in 3; PPI examined in 2)	100	100	50
Overall	95	76	73

Neither poly(I:C) or SI alone nor combined poly(I:C)-SI impaired recognition memory in the NOD task. SI of rats has been repeatedly linked to subsequent impairments in episodic memory, which is implicated in schizophrenia (Bianchi et al., 2006; King et al., 2009; McIntosh et al., 2013; McLean et al., 2010). Nonetheless, the absence of SI-induced memory deficits in our hands is not unforeseen as such impairments, like hyperactivity, is not consistently reproduced across studies (Fone & Porkess, 2008; Table 2.21). Coincidently, a recent study by our laboratory also failed to observe impaired NOD after an interval of two hours (Dunphy-Doherty et al., 2018). The lack of effect of housing on the NOD task has also been reported by another group, but a much longer 24h ITI was employed (Liu et al., 2017). There were no published studies on the effect of poly(I:C) on recognition memory. As neither SI alone or poly(I:C) alone influenced the discrimination index, it is unsurprising that the Poly(I:C)-SI group did not show any NOD deficits.

Rats from all groups demonstrated intact spatial working memory in the NLD task. This contradicts the spatial working memory deficits seen in schizophrenia (Park & Holzman, 1992). No studies have assessed spatial working memory in isolates in a NLD paradigm to our best knowledge. However, mixed findings on performance of isolates have

been demonstrated using other paradigms, such as MWM, modified Olton's spatial memory task and the Y-maze task (see section 1.4). There are also no published studies on the effect of poly(I:C)-induced MIA on offspring performance in the NLD task. In the relevant MWM task, no indication of retarded spatial learning in adolescent offspring of dams treated with 0.5 mg kg^{-1} poly(I:C) from GD16-19 was found (Han et al., 2011). While single injection of 8 mg kg^{-1} poly(I:C) injection to dams at GD15 did not affect offspring spatial learning and memory in two variations of the MWM task (matching-to-sample and fixed platform procedures), repetitive administration at GD15-19 abated acquisition in the fixed platform procedure and also in the probe trial (Vorhees et al., 2012; Vorhees et al., 2015). Another study further backs the absence of effect conferred by single injection of poly(I:C) to dams on spatial acquisition by offspring (Richtand et al., 2012a).

The current experiment saw SI-related alterations in pro-social behaviours regardless of prenatal treatment. Furthermore, SI alone and poly(I:C) alone increased aggression, with the former causing increased boxing and biting which was diminished by gestational poly(I:C) exposure. Poly(I:C) on its own also increased pinning. SI-induced alterations in social interactive behaviour mirror previous studies (Möller et al., 2011; Regenass et al., 2018; Strauss et al., 2014). Increased aggression associated with SI in the form of pinning and aggressive grooming, and increased distance travelled, have been reported by others (Grotewold et al., 2014; Regenass et al., 2018; Strauss et al., 2014; Wongwitdecha & Marsden, 1996b). However, the lack of influence of social deprivation on total interaction observed in the current study was unexpected, as prior studies reported reduced or increased social interaction (Grotewold et al., 2014; Kokare et al., 2010). As these studies have used rats of similar age to our study, likely reasons for the discrepancy in findings include the size and shape of arena (e.g. square arena versus circular in the current study), as well as strain differences (Rex et al., 1996). Moreover, prior experience of rats such as cannulation and vehicle intracerebroventricular injection, and the application of differing

experimental protocol, e.g. measured during conditioning trials in a conditioned place preference paradigm or the usage of a dummy partner, could influence social interaction measures (Grotewold et al., 2014; Kokare et al., 2010). Unlike the SI model, no studies have investigated social interaction with conspecifics in the poly(I:C) model. Behaviour of rats subjected to the combined approach mirrored those of Veh-SI closely but attenuated SI-induced aggression, thus suggesting a protective effect of prior stress experience. Although rodent behaviour cannot be directly mapped onto humans, similarly complex alteration in social functioning in schizophrenia has been reported by many (Fulford et al., 2018; Green et al., 2015).

Worsened social skills have been repeatedly described in individuals with schizophrenia (Fulford et al., 2018). USV profiling provides a way to examine any alterations in social functioning in rodents (Neill et al., 2010). Poly(I:C) alone caused a selective increase in upward ramp calls whereas SI alone significantly increased multiple USV subtypes. The observed effects of SI correspond to previous findings, which associated isolates with enhanced tickle and social contact-induced pro-social vocalisations, implying USVs as a general index of positive social affect in rodents (Burgdorf & Panksepp, 2001; Knutson et al., 1998). Impaired recognition of social and reward cues, as evidenced by decreased emission of 50-kHz affective calls, have also been demonstrated in male rats following post-weaning SI (Inagaki et al., 2013). Moreover, isolates have been associated with increased distress calls which paralleled higher number of injuries and increased agitation compared to social rats in the presence of an aggressor, suggestive of altered responses to an agonistic challenge (von Frijtag et al., 2002). Finally, diminished approach behaviour in response to the playback of pro-social contact calls was evident in rats subjected to chronic SI from weaning, showing altered social communication in isolates (Seffer et al., 2015). No known poly(I:C) studies have investigated USVs in a social interaction context. In the current study, only 6 out of the 32 pairs of rats emitted 22-kHz vocalisations. As 22-kHz vocalisations are typically emitted during

aversive or stressful situations (e.g. exposure to predator and social defeat), such low occurrence, together with the observation that all groups emitted the pro-social 50-kHz vocalisations, suggests that rats did not find the social interaction task aversive or stressful (Portfors, 2007).

In schizophrenia, impaired pre-attentive filtering has been linked to cognitive disorganisation (Geyer et al., 1993). While the lack of effect of SI on offspring PPI was unanticipated as PPI deficits have been widely replicated in the SI model (Jones et al., 2011a; Ko & Liu, 2016; Schubert et al., 2009), there also exist studies using identical protocols which have suggested otherwise (Gaskin et al., 2014; McIntosh et al., 2013). Such variable PPI responses could be attributed to factors such as strain differences (Weiss et al., 2000). For instance, Sprague-Dawley rats were found to exhibit more robust SI-induced PPI deficits than Lister-hooded rats (Weiss et al., 2000). Furthermore, in Lister-hooded rats, the reproduction of SI-induced PPI deficits are less robust compared to locomotor and NOD alterations (Fone & Porkess, 2008; Table 2.21). Poly(I:C)-induced PPI deficits have been widely demonstrated and strain and protocol differences could again explain the differential outcomes between the current study and prior studies (Vorhees et al., 2012; Vorhees et al., 2015). Given that neither poly(I:C) alone nor SI alone influenced PPI, it is not unexpected that poly(I:C)-SI did not alter PPI.

The detrimental effects of post-weaning SI on CFR in the current study are in keeping with previous reports, and are translationally relevant to impaired associative learning seen in patients with schizophrenia (Diwadkar et al., 2008; Gaskin et al., 2014; Jones et al., 2011a). Nonetheless, mixed findings have been previously reported for conditioning in isolates. For instance, impaired acquisition was found in isolates when assessed at PND79-81 (Dunphy-Doherty et al., 2018) whereas two separate studies suggested no housing-induced differences on initial responses (Jones et al., 2011a; Weiss et al., 2004). Dissimilar to others which have found impaired contextual and cued-mediated learning and memory, the current data only revealed alteration in the

latter (Dunphy-Doherty et al., 2018; Gaskin et al., 2014; Jones et al., 2011a; McIntosh et al., 2013). Meanwhile, in male rats that were isolation-reared for 13 weeks from weaning, SI was found to have little effect on freezing to context and during pre-tone and the 8 min of continuous tone presentations (Weiss et al., 2004). Maternal poly(I:C) treatment did not influence conditioned contextual or cued fear in the present experiment. Whilst this agrees with a previous study (Vorhees et al., 2012), a distinct study reported impairments in cued conditioned freezing in male offspring of poly(I:C)-treated dams (Vorhees et al., 2015). It is unexpected that prenatal poly(I:C) exposure was capable of reversing reduced cued freezing in isolates.

In the ASST, the present data implies a selective impairment in reversal learning in isolates, which was not evident when rats were exposed to poly(I:C), alone or in combination. Impaired performance in bowl digging ASST has been previously described (McLean et al., 2010; Powell et al., 2015; Schijver et al., 2004), and is thought to be an index of cognitive flexibility, which is frequently observed in individuals with schizophrenia (Birrell and Brown, 2000). With the poly(I:C) model, one group has observed blunted reversal learning in MIA-exposed offspring that was not attributed to visual impairment (0.5 mg kg^{-1}, GD16-19; Han et al., 2011), which was supported by a further study (8 mg kg^{-1}, GD15; Richtand et al., 2012a). However, in alignment with the current findings, two other studies did not detect altered performance in offspring prenatally exposed to poly(I:C) (8 mg kg^{-1}, GD15 or GD15-19; Vorhees et al., 2012; Vorhees et al., 2015).

In adulthood, there was a pronounced SI-induced elevation in pro- and anti-inflammatory cytokines in the frontal cortex. Some of such increases were not discernible in rats which were also exposed to gestational poly(I:C). The observed elevations in cytokines are in contrary with a recent study which revealed reduced hippocampal IL-10 and frontal cortical IL-6 using identical analysis method. In particular, hippocampal IL-10 was negatively correlated with hyperactivity in a novel arena,

prompting the authors to suggest abnormal behaviour in adulthood as a consequence of reduced anti-inflammatory signalling (Corsi-Zuelli et al., 2019). This is clearly not the case in the current study, which suggested a general activation of the immune system in isolates. Other SI studies have found increased pro-inflammatory cytokine levels in rats isolated for 6 or 9 weeks (Ko & Liu, 2016; Wang et al., 2017a). Meanwhile, a recent study in our laboratory revealed reduced hippocampal IL-6 and IL-10 following 9 weeks of SI (Dunphy-Doherty et al., 2018). Poly(I:C) alone is linked to increases of several striatal cytokines in the current research. While cytokines have been measured in GD16 foetal brain, neonatal brain at PND1 or 7, as well as serum of adolescent rats, no poly(I:C) studies have examined the effects on cytokines in adult rats (Gilmore et al., 2005; Han et al., 2011). Consistent with the pattern seen with several behaviours, MIA prevented some of the SI-induced cytokine increases in the frontal cortex.

Simultaneous activation of mTOR signalling was observed alongside elevated cytokine levels in the frontal cortex of adult isolates, as well as rats exposed to poly(I:C) alone. In isolates, increased 5-HT_6 receptor-mediated mTOR signalling via PI3K/Akt/Pheb in the PFC has been linked to impaired cognition (Meffre et al., 2012). It is therefore likely that the same pathway is responsible for the perturbed cognition in the current study, given that we observed defective reversal learning in isolates in the ASST. Whilst a study has probed the potential involvement of kynurenine pathway in the poly(I:C)-based MIA model, no groups have examined other possible signalling mechanisms in this model (Khalil et al., 2013). Apart from its role in neurodevelopment and synaptic plasticity, mTOR also plays a role in innate immunity regulation, including pro-inflammatory and anti-inflammatory cytokine production. Regulation of cytokine secretion is complex as it depends on the immune cell type, the target tissue and extracellular and intracellular metabolic status, and different mechanisms involving shared and non-shared mediators have been described for dendritic cells, neutrophils, mast cells and natural killer cells (Weichhart et al., 2015). It is possible that altered behavioural

responses in the our SI and poly(I:C) models are due to defective function or regulation of one or more of such mediators, e.g. PI3K, TSC1/TSC2, or downstream translation inhibitors such as eIF4E-binding protein or ribosomal protein S6 kinase. It is also likely that there is instead a deficit in metabolic reprogramming in the immune cells following activation by gestational or chronic SI stress in the current study, resulting in disrupted glycolysis, mitochondrial biogenesis and cholesterol or fatty acid synthesis, processes which are key for normal effector functions such as cytokine synthesis and secretion (Weichhart et al., 2015). The concomitant observations that cytokine production and mTOR signalling are abnormal support the use of our poly(I:C) and SI models to model symptoms of schizophrenia, as these abnormalities have been associated with schizophrenia (Gururajan & van den Buuse, 2014; Rodrigues-Amorim et al., 2018). Consistent with cytokine data, SI-induced increase in mTOR activity was prevented by prior poly(I:C) exposure.

Social deprivation resulted in greater body weight in rats regardless of prenatal treatment at multiple time points. Hyperphagy have been previously demonstrated in isolates, and could be attributed to factors including inactivity due to the absence of conspecifics, or altered food motivation or leptin-hypothalamic axis induced by housing (Hall et al., 1998; Hermes et al., 2011; Mainardi et al., 2010; Morgan, 1973; Shelley, 1965). This is of particular relevance as altered metabolic function has been documented in schizophrenia, alongside cytokine perturbations (Leonard et al., 2012). Weight gain, however, is not consistently observed with SI and may depend on the protocol used (Fone & Porkess, 2008; Smith et al., 1997; Weiss et al., 2004). Maternal poly(I:C) treatment, on the other hand, does not appear to affect body weight of male offspring in puberty or adulthood (Bronson et al., 2011; Vorhees et al., 2012; Vorhees et al., 2015).

Collectively, behavioural (LMA, aggressive behaviour and CFR), cytokine and mTOR data from adulthood suggest that prior stress exposure

confers a beneficial effect against further adversity in later life. This is in support of theoretical theories such as the stress inoculation theory, which posit that stressful experiences promote resilience in the face of subsequent adversity instead of worsening outcomes (Seery, 2011). An example in humans which supports this perspective is the fact that stressful experiences in childhood do not necessarily correlate to manifestation of mental disorders in later life (Kentner et al., 2019). Building upon the multifactorial basis of schizophrenia, various 'dual-hit' models have been generated in the hope of better reproducing symptoms which are translationally relevant to schizophrenia and related disorders. For instance, in our laboratory, the combination of neonatal PCP and post-weaning SI resulted in a wider range of more severe cognitive impairments, with additive effects seen in the PPI and CFR paradigms (Gaskin et al., 2014). The same model induced locomotor hyperactivity and NOD deficits that were attenuated by acute administration of the D_3-preferring D_3/D_2 receptor partial agonist cariprazine, the atypical antipsychotic aripiprazole and the sodium channel blocker lamotrigine (Gaskin et al., 2016; Watson et al., 2016). Similar additive effects have been reported for different combinations of early life stressors, including neonatal LPS treatment/adulthood restraint stress, prenatal poly(I:C) exposure/adulthood MK-801, post-weaning SI/sub-chronic MK-801 treatment and neonatal maternal separation/adolescent corticosterone treatment, although such effects appear to selective and do not affect all behaviours (Lim et al., 2012; Simpson et al., 2010; Walker et al., 2009; Zavitsanou et al., 2014). Moreover, rat genetic models exposed to interventions that mimic human environmental stressors have produced more robust models in aiding the understanding of mechanisms of stress (Gómez-Galán et al., 2016; van der Doelen et al., 2014). Whilst the majority of 'dual-hit' studies back the cumulative stress theory, there are also studies (maternal separation with early adulthood chronic stress or post-weaning SI) which akin to the current study, sustain the stress inoculation theory (Biggio et al., 2014; Zalosnik et al., 2014). Naturally, groups have also observed subtle or no additive effect of combined stressors (e.g. post-weaning SI with modified single prolonged stress,

pubertal chronic methamphetamine exposure or subchronic MK-801 treatment, and maternal separation with post-weaning SI) on behaviour, physiology or neurochemistry (Gilles & Polston, 2017; Hawken et al., 2013; Hofford et al., 2018; Strauss et al., 2014). As is shown in the current study, sex-specific effects have been described for some of the 'dual-hit' models (Hill et al., 2014).

Investigations into the early molecular mechanisms that potentially precede behavioural changes in adulthood revealed a sexually dimorphic effect of poly(I:C) or SI on cytokine and mTOR levels. Cytokine but not mTOR alterations were found in male offspring regardless of neurodevelopment history, which corresponded to altered LMA responses. Taking into account data from adulthood, it seems that in rats subjected to SI alone, aberrant cytokine signalling long preceded behavioural abnormalities in adulthood. This is in support of the 'two-hit' hypothesis of schizophrenia, where a build-up of subsequent stress is required to propel disease progression following the initial insult. As mTOR signalling was not altered in adolescent males, it is likely that there is a dissociation of mechanisms responsible for immune alterations at adolescence and in adulthood. On the other hand, SI alone and combined poly(I:C)-SI modified mTOR signalling but not cytokines in adolescent females. Whilst aberrant mTOR signalling could be responsible for LMA alterations associated with the two models, females exposed to poly(I:C) alone demonstrated altered LMA in the absence of mTOR disruptions. It would be interesting to investigate the longitudinal effects of poly(I:C) and/or SI on females. The brain is subjected to various stressors, which are capable of modifying the brain architecture and hence behaviour, and is actively engaged in facilitating adaptations to maintain survival (McEwen et al., 2015). It is increasingly clear that stress coping strategies are contingent upon complex interaction of factors such as individual attributes (e.g. life time stress history, personal control and optimism), the surrounding environment (e.g. social network and socioeconomic conditions), sex and genomic responses to environmental factors (Dantzer et al., 2018; McEwen et al., 2015). Thus,

it is possible that poly(I:C) alone, SI alone and poly(I:C)-SI represent three distinct “stress histories”, and given the influence of sex, resulting in differential long-term outcomes in the two genders.

2.5.2.4 Conclusion and future direction

In contrast to the hypothesis, instead of exacerbating SI-induced alterations in offspring, findings from the current study suggest a protective effect of gestational poly(I:C) against SI-induced behavioural (LMA, aggressive behaviour and CFR), cytokine and mTOR abnormalities. Our poly(I:C)-SI model supports the stress inoculation theory, which posits that prior stressful experiences promote increased adaptation to subsequent difficulties. Meanwhile, poly(I:C) and SI independently produced behavioural, cytokines and mTOR alterations in adulthood that are relevant to the positive, negative and cognitive symptoms of schizophrenia. Our findings on early molecular mechanisms revealed a complex interaction between neurodevelopment history and gender on the long-term regulation of behaviour and immune function.

Since the route of administration can profoundly influence MIA outcomes, in the next chapter we sought to profile whether administrating poly(I:C) to dams via i.v. administration could produce more comprehensive behavioural abnormalities that are relevant to the three symptom domains of schizophrenia in resultant offspring.

CHAPTER 3

The combined effects of gestational poly(I:C) exposure via i.v. administration and post-weaning social isolation on behaviour and neurochemistry

3.1 Introduction

The previous chapter unexpectedly demonstrated that poly(I:C) exposure, via i.p. administration, attenuated SI-induced behavioural (LMA, aggressive behaviour and CFR), cytokine and mTOR alterations. Although we are keen to adopt i.p. administration as a potential refinement, route of poly(I:C) administration can influence the magnitude of MIA and outcome in offspring (Kentner et al., 2019). Given that the majority of gestational poly(I:C) studies have used i.v. administration and demonstrated various significant responses (see below), this chapter examines whether i.v. administration, combined with post-weaning SI, produces a more comprehensive additive 'dual-hit' model as per our original overall aim.

Similar to Chapter 2, an extensive literature search was conducted before the experiments. At the time of review (September 2016), 42 out of 62 MIA studies conducted in rats which administered poly(I:C) via i.v. administration were found (Tables 1.3-1.6). Four rat strains were used in these studies: Wistar (21 studies), Sprague-Dawley (16 studies), Long Evans (4 studies) and Lister-hooded (1 study). All but one study administered poly(I:C) at a dose of 4 mg kg^{-1} (Tables 1.3-1.6). In terms of injection timings, GD15 was the most common choice (37 studies; one study which piloted GD15 or 17 found no behavioural differences in resultant offspring and proceeded with GD15 in subsequent studies). Other timings that were used are GD9, 11, 13-15, 19 and 20 (Chou et al., 2015a; Duchatel et al., 2016; Meehan et al., 2017; Song et al., 2011; Zavitsanou et al., 2014). Only 22 studies reported the acute effects of poly(I:C), of which thirteen found reduced weight gain 24-48 h post-treatment (Ballendine et al., 2015; Howland et al., 2012; Piontkewitz et al., 2009) and four reported no change (Dalton et al., 2012; Meehan et al., 2017; Vernon et al., 2015; Wolff & Bilkey, 2010). Of the four studies which evaluated body temperature in dams, three revealed no differences (Ballendine et al., 2015; Howland et al., 2012; Sangha et al., 2014) whereas one found an elevation 8 h post-injection (Zhang et al.,

2012). A number of studies also found increased pro-inflammatory cytokines and chemokines, including IL-1β, IL-6, TNF-α and (C-X-C motif) ligand 1, as well as corticosterone in plasma or serum collected 2-3 h after MIA (Ballendine et al., 2015; Dalton et al., 2012; Duchatel et al., 2016; Meehan et al., 2017; Song et al., 2011). One study which monitored changes in fur condition, overt behaviour and posture observed short-term piloerection and hunched posture which resolved within 4 h post-injection (Yee et al., 2011). Moreover, maternal behaviour was not affected (Zhang et al., 2012). Irrespective of protocol, most studies reported no effect of poly(I:C) on miscarriage rate, gestation length, litter size or pup weight (Tables 1.5-1.6).

To allow for comparison with our previous i.p. study (Chapter 2), the current chapter used a consistent experimental design. As there could be a strain-dependent effect of poly(I:C) (section 2.1), any pre-existing literature which administered poly(I:C) into pregnant Lister-hooded rats via i.v. administration was particularly valuable. In the only publication meeting this criterion, male offspring exposed to poly(I:C) at GD15 (4 mg kg^{-1}) were selectively impaired in reversal learning in the ASST when tested at around three months old, thus indicative of cognitive impairments (Wallace et al., 2014). Notwithstanding, variables such as the source of animals, rearing environment, conditions during dosing, poly(I:C) storage conditions, as well as the molecular composition of poly(I:C), could engender disparities in output measures in both the pregnant rodents and their offspring (Kentner et al., 2019; Mueller et al., 2019). Thus, taking these observations into consideration, a pilot study was conducted to select an appropriate dose for the subsequent actual study.

3.2 Aims

The aims of the current set of experiments were to:

(1) determine an appropriate dose of poly(I:C) that can be administered intravenously to non-pregnant female Lister-hooded rats without severe adverse effects;

(2) examine if combined MIA via i.v. poly(I:C) administration to the pregnant rat and subsequent post-weaning isolation rearing produces sex-specific developmental effects on offspring behaviour in adolescence;

(3) characterise the combined effects of MIA via poly(I:C) administration (i.v.) to the pregnant rat and subsequent post-weaning isolation rearing on the behaviour of male offspring in adulthood, and to probe potential mechanisms underlying behavioural alterations.

3.3 Materials and methods

3.3.1 Animals

Non-pregnant and time-mated pregnant female Lister-hooded rats were obtained from Charles River UK, Kent. The latter arrived at the BSU of the University of Nottingham on GD8-10 (the day when a vaginal plug was found was designated as GD1). Microchips (Bio-Thermo™idENTICHIP®, Animalcare Limited, York, UK) were implanted s.c. 24 h before drug treatment in the nape of the neck of the rats to enable temperature measurement. Pups were born and weaned in the BSU. Rats were housed under constant environmental conditions as stated in section 2.3.1. All experiments conformed to the Animals (Scientific Procedures) Act 1986 and ARRIVE guidelines and were approved by the Animal Welfare and Ethical Review Body of the University of Nottingham.

3.3.2 Experimental design

3.3.2.1 Experiment 1: The effect of poly(I:C) via i.v. administration on non-pregnant Lister-hooded rats

The design was similar to the corresponding study in Chapter 2 (Section 2.3.3.1). Briefly, injections were carried out between 10:00 and 11:00 in the rat holding room. Twelve rats (100-124 g; housed four per cage) were placed in a heat box (Thermacage MK3, Datesand Ltd., UK) for approximately 10 min before single i.v. injection of either 4 mg kg^{-1} poly(I:C), 6 mg kg^{-1} poly(I:C) or 1 ml kg^{-1} saline while being restricted in a restraint tube exposing only the tail. The dose of 4 mg kg^{-1} was selected based on previous findings (see above) whereas 6 mg kg^{-1} was included to determine whether a higher dose would evoke a more robust sickness response. All drugs were prepared on the day of injection and injections were carried out by an experienced staff from the BSU. Rats were immediately returned to their home cages following injection. Home cage behaviour were monitored every 10 min for 190 min using the same method described in section 2.3.4. The original plan was to monitor body temperature for 4 h post-injection, but due to human error, data from the pre-injection baseline and first 20 min post-injection were not collected, rendering subsequent temperature data unusable. Rats were killed immediately after the final assessment and brain regions (frontal cortex and hippocampus) and plasma collected for cytokine and mTOR analyses. The experimental protocol is shown in Figure 3.1.

3.3.2.2 Experiment 2: The combined effects of gestational poly(I:C) via i.v. administration and post-weaning social isolation on behaviour and neurochemistry

The experimental design of the current experiment is identical to Experiment 2 of Chapter 2 (section 2.3.3.2), unless stated otherwise herein. Briefly, seventeen time-mated nulliparous dams which had been acclimatised to handling received a temperature microchip implant on GD14. On GD15 (10:00-11:00), baseline home cage activity, body

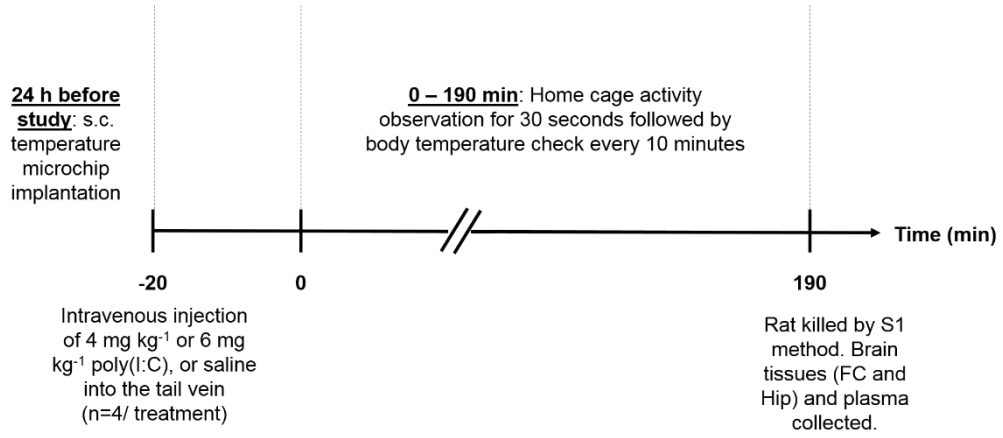


Figure 3.1. Experimental protocol for Experiment 1. Non-pregnant female Lister-hooded rats (n=4 per treatment, housed 4 per cage) were implanted with a subcutaneous (s.c.) temperature microchip 24 h before drug administration. On the experiment day, rats received 4 mg kg^{-1} or 6 mg kg^{-1} polyinosinic:polycytidylic acid (poly(I:C)), or 1 ml kg^{-1} saline (i.v.). Rats were immediately returned to their home cage and behavioural and temperature assessments were performed every 10 min from 20 min post-injection for 190 min. Rats were humanely killed by a schedule 1 method (concussion followed by exsanguination) and plasma and brain tissues collected immediately following the final temperature assessment. FC, frontal cortex; Hip, hippocampus.

temperature and body weight were recorded, then animals were gently warmed in a heat box for 10-15 min to facilitate i.v. administration of 6 mg kg^{-1} poly(I:C) (n=8) or 1 ml kg^{-1} saline (n=9) via the tail vein, during brief confinement in a restraint tube. Body temperature and home cage sickness behaviour were assessed. It would have been desirable to collect blood from the dams 4 h post-injection, however this could not be performed as it was not authorised by our Home Office Project Licence. Since four rats (two vehicle-treated and two poly(I:C)-treated) had been placed into the heat box at 3 h 50 min in preparation for the blood collection, data from these rats were excluded in body temperature and home cage activity analyses to ensure accuracy in data interpretation. Only fifteen dams (Veh, n=8; Poly(I:C), n=7) were maintained until parturition, as one vehicle-treated rat showed no increase in body weight from GD15 (weight measured prior to vehicle administration) whereas one poly(I:C)-treated rat showed little weight gain from GD11. As the rat supplier, Charles River, does not guarantee pregnancy for time-mated rats before 13 days of gestation, it is likely that these two rats were not pregnant on arrival and their data were excluded from all analyses. Dams

were allowed to nurture their litters without disruption until PND13-14, when pups were counted and sexed while the dam was temporarily removed from the cage. On PND22, pups (n=110 from 15 litters) were weaned and assigned to four treatment x housing groups (section 2.3.3.2). Eleven litters contributed both GH and SI animals, one Veh and two poly(I:C) litters contributed only SI animals and one Veh litter gave rise to only GH animals. Resultant offspring were subjected to the exact same housing conditions and handling described in section 2.3.3.2.

Behavioural phenotyping in adolescence to assist gender selection for behavioural studies in adulthood

Due to inadequate number of male pups born to the fifteen remaining dams, only female offspring were tested for LMA at two weeks post-weaning to confirm the SI syndrome (PND36-37; n=8 per treatment x housing). An additional LMA study was conducted using male and female offspring (PND36-37; n=8 per gender x treatment x housing) from a separate batch of eight time-mated females (n=4 per treatment) which underwent identical handling and housing conditions. Counting and sexing of pups in this later study occurred at PND14. Three out of the four Veh litters contributed both GH and SI animals, with the remaining litter contributed only SI animals. All Poly(I:C) litters contributed both GH and SI animals. Data collected from dams and female offspring from both studies were pooled together for analysis.

Behavioural and neurochemical phenotyping in adulthood

A distinct group of male rats to those tested for LMA in adolescence underwent comprehensive behavioural test testing at weekly intervals commencing around five weeks post-weaning (Figure 2.2): LMA (PND57-58), NOD (PND58-59), NLD (PND65-66), social interaction with concomitant monitoring of USVs (PND69-72) and PPI (PND78-82), followed by either associative learning, i.e. CFR (day 1: PND82-85; day 2: PND83-86), or ASST (end dates ~9-15 weeks post-weaning). For each

treatment x housing, n=18 or 20 rats for LMA, NOD, NOL and PPI, n=10 rats for CFR, n=8 or 10 rats for ASST and n=9-10 pairs for social interaction. Table 3.1 provides a summary of the age of rats during each test as well as the sample size. Tests were conducted as described in section 2.3.5. At roughly eight weeks post-weaning, the ASST subgroup underwent food restriction but all other rats received food *ad libitum*. The left frontal cortices, hippocampi and striata, and plasma were collected from the CFR subgroup one week after CFR (PND90-93), for cytokine (brain regions and plasma) or mTOR (brain regions only) analyses (sections 2.3.8.2 and 2.3.9).

3.3.3 Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 and IBM SPSS Statistics 22 (IBM, New York). The composite sickness scores of vehicle and poly(I:C)-treated females were compared using Kruskal-Wallis test (Experiment 1) or Mann-Whitney *U*-test (Experiment 2). Cytokine and mTOR data from Experiment 1 were analysed using one-way ANOVA. All other data were analysed as described in section 2.3.10. All values are mean ± SEM and $p<0.05$ was considered significant.

Table 3.1. Number of animals and age of animals during each behavioural test in Experiment 2 and the additional LMA study. Only female offspring (n=8 per treatment x housing) were included in LMA evaluations at two weeks post-weaning in Experiment 2 due to insufficient male offspring. A separate LMA study was conducted using male and female offspring from eight time-mated females (n=8 per gender x treatment x housing). An additional cohort of 78 male rats underwent consecutive behavioural testing at weekly intervals from five weeks post-weaning, before being split into two groups to be tested in CFR and ASST, respectively. The number of animals used for each test is indicated under the relevant column for each treatment x housing group.

Experiment 2: LMA at 2 weeks post-weaning (PND36-37)	Number of rats			
	Veh-GH	Poly(I:C)-GH	Veh-SI	Poly(I:C)-SI
Female	8	8	8	8
Male	-	-	-	-
Additional study: LMA at 2 weeks post-weaning (PND36-37)	Number of rats			
	Veh-GH	Poly(I:C)-GH	Veh-SI	Poly(I:C)-SI
Female	8	8	8	8
Male	8	8	8	8
Experiment 2: behavioural testing commencing 5 weeks post-weaning (males)	Number of rats			
	Veh-GH	Poly(I:C)-GH	Veh-SI	Poly(I:C)-SI
LMA (PND57-58)	20	20	20	18
NOD (PND58-59)	20	20	20	18
NLD (PND65-66)	20	20	20	18
Social interaction/ USVs recording (PND69-72)	20	20	20	18
PPI (PND78-82)	20	20	20	18
CFR (Day 1: PND82-85; Day 2: PND83-86)	10	10	10	10
Bowl digging ASST (completed 9-15 weeks post-weaning)	10	10	10	8

Abbreviations: ASST, attentional set-shifting task; CFR, conditioned freezing response; GH, group-housed; LMA, locomotor activity; NLD, novel location discrimination; NOD, novel object discrimination; PND, postnatal day; PPI, pre-pulse inhibition of acoustic startle; SI, social isolation; USVs, ultrasonic vocalisations.

3.4 Results

3.4.1 Experiment 1: The effect of poly(I:C) via i.v. administration on non-pregnant Lister-hooded rats

In order to establish whether i.v. poly(I:C) induced a short-term sickness response in non-pregnant female rats, home cage activity were recorded for 190 min following injection. The original plan was to monitor body temperature for 4 h post-injection, but due to human error data from the pre-injection baseline and first 20 min post-injection were not collected, rendering subsequent temperature data unusable.

There was a significant main effect of time ($F_{(19,171)}$= 3.93, p<0.0001) and a time x treatment interaction ($F_{(38,171)}$= 1.84, p<0.01) on sickness behaviour, such that both doses of poly(I:C) significantly increased sickness score at multiple time points (Figure 3.2A). Nevertheless, neither dose altered composite sickness score (Figure 3.2B). There were no between-group differences in abdominal pinching scores across time and total abdominal pinching scores (data not shown).

A significant main effect of treatment was observed for frontal cortical IL-1β ($F_{(2,9)}$= 14.14, p<0.01), IL-10 ($F_{(2,9)}$= 5.93, p<0.05) and IL-18 ($F_{(2,9)}$= 7.07, p<0.05), such that 4 mg kg^{-1} poly(I:C) significantly reduced frontal cortical IL-1β and IL-10 (Figure 3.3A). Hippocampal total mTOR showed a significant main effect of treatment ($F_{(2,9)}$= 5.64, p<0.05), such that 4 mg kg^{-1} and 6 mg kg^{-1} poly(I:C) each caused a significant reduction (Figure 3.3B).

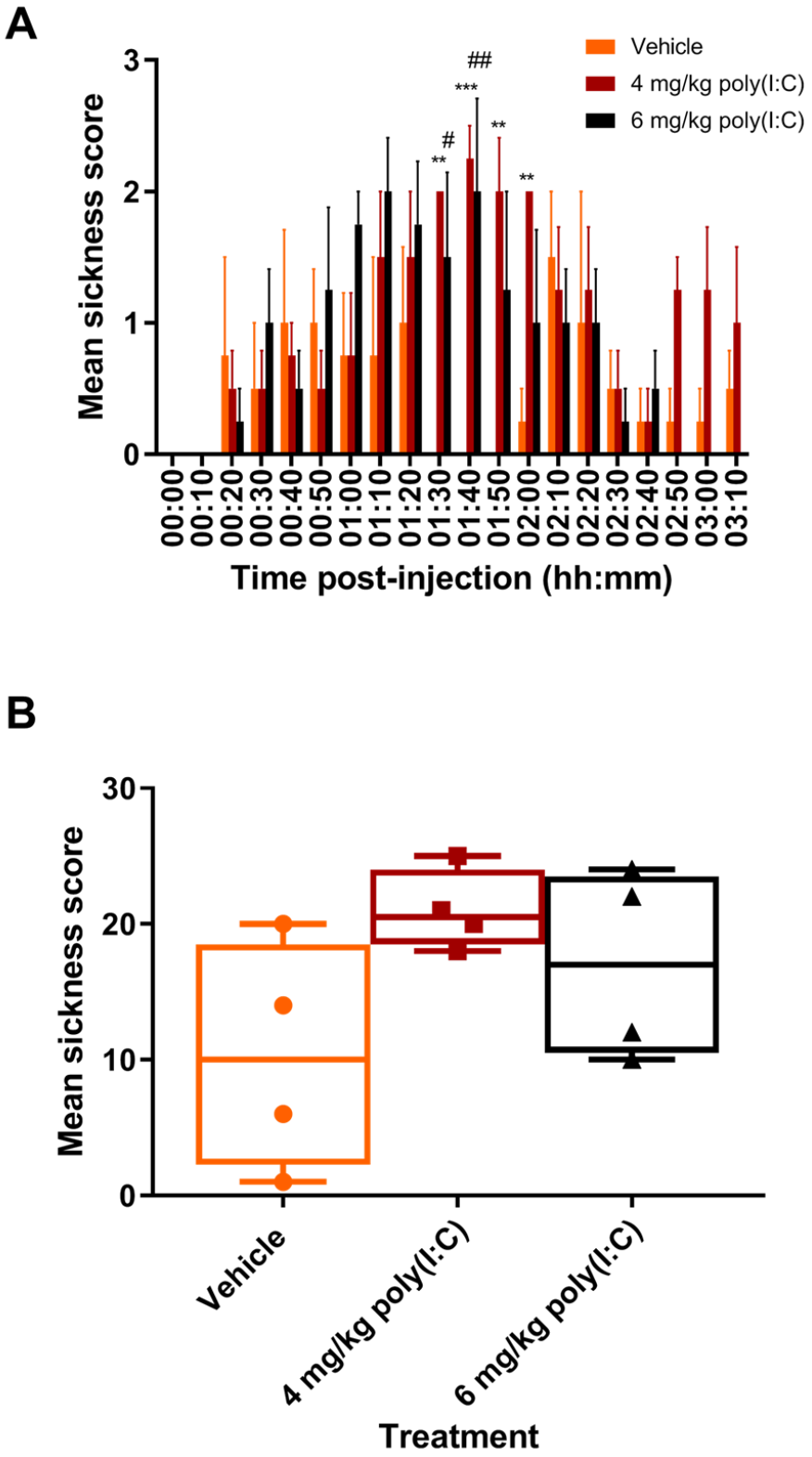


Figure 3.2. Effect of poly(I:C) (4 mg kg^{-1} and 6 mg kg^{-1}, i.v.) on sickness behaviour in non-pregnant female Lister-hooded rats. (A) Time course of sickness score at 10 min intervals and **(B)** box-plot diagram showing total composite sickness score of rats injected with saline (median=10, range=1-20), 4 mg kg^{-1} poly(I:C) (median=20.5, range=18-25) or 6 mg kg^{-1} poly(I:C) (median=17, range=10-24) over the 190 min post-injection (n=4 per treatment). Data are mean ± SEM. **p<0.01, ***p<0.001 4 mg kg^{-1} versus vehicle control; #p<0.05, ##p<0.01 6 mg kg^{-1} versus vehicle control; two-way RM ANOVA with Dunnett's post-hoc test (A) or Kruskal-Wallis test (B).

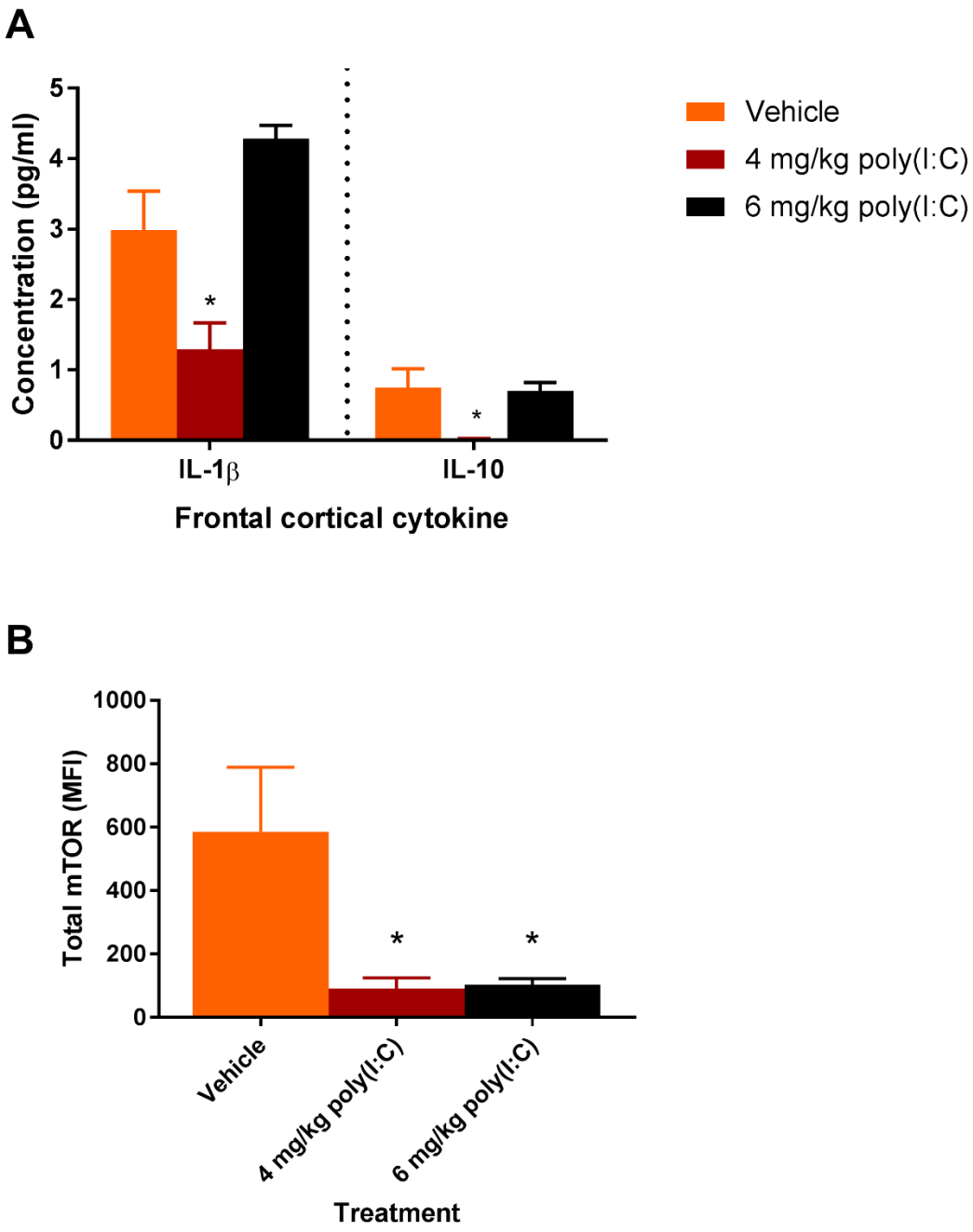


Figure 3.3. Effect of poly(I:C) (4 mg kg^{-1} and 6 mg kg^{-1}, i.v.) on frontal cortical cytokines and hippocampal total mTOR in non-pregnant female Lister-hooded rats. Mean ± SEM **(A)** *ex vivo* concentrations of frontal cortical IL-1β and IL-10 and **(B)** hippocampal total mTOR (n=4 per treatment). *p<0.05 versus vehicle control; one-way ANOVA with Dunnett's post-hoc test (A, B).

3.4.2 Experiment 2: The combined effects of gestational poly(I:C) via i.v. administration and post-weaning social isolation on behaviour and neurochemistry

3.4.2.1 Physiological and behavioural effects of poly(I:C) on dams

To ascertain that 6 mg kg^{-1} poly(I:C) (i.v.) caused acute sickness responses in dams, body temperature and sickness behaviour were monitored for 6 h and body weight for 3 days post-injection. There was a significant main effect of time ($F_{(9,153)}$= 7.56, p<0.0001), treatment ($F_{(1,17)}$= 7.81, p<0.05) and a time x treatment interaction ($F_{(9,153)}$= 2.63, p<0.01) on body temperature, such that poly(I:C)-treated dams had significantly reduced body temperature at 2, 5 and 6 h post-injection (Figure 3.4A). Analysis of body weight data revealed a significant main effect of time ($F_{(2,42)}$= 291.90, p<0.0001) and treatment ($F_{(1,21)}$= 7.93, p<0.05). Poly(I:C)-treated dams gained significantly less weight at 24 h post-injection (Figure 3.4B). Poly(I:C)-treated dams also exhibited significantly more sickness behaviour, as reflected by an increase in total composite score (Figure 3.5A). Time course analysis of the same data revealed a significant main effect of time ($F_{(6,102)}$= 6.17, p<0.0001), treatment ($F_{(1,17)}$= 4.69, p<0.05) and a time x treatment interaction ($F_{(6,102)}$= 2.32, p<0.05), such that poly(I:C) treatment caused increased sickness scores at 3 h post-injection (Figure 3.5B). Regardless of treatment, no abdominal pinching was observed.

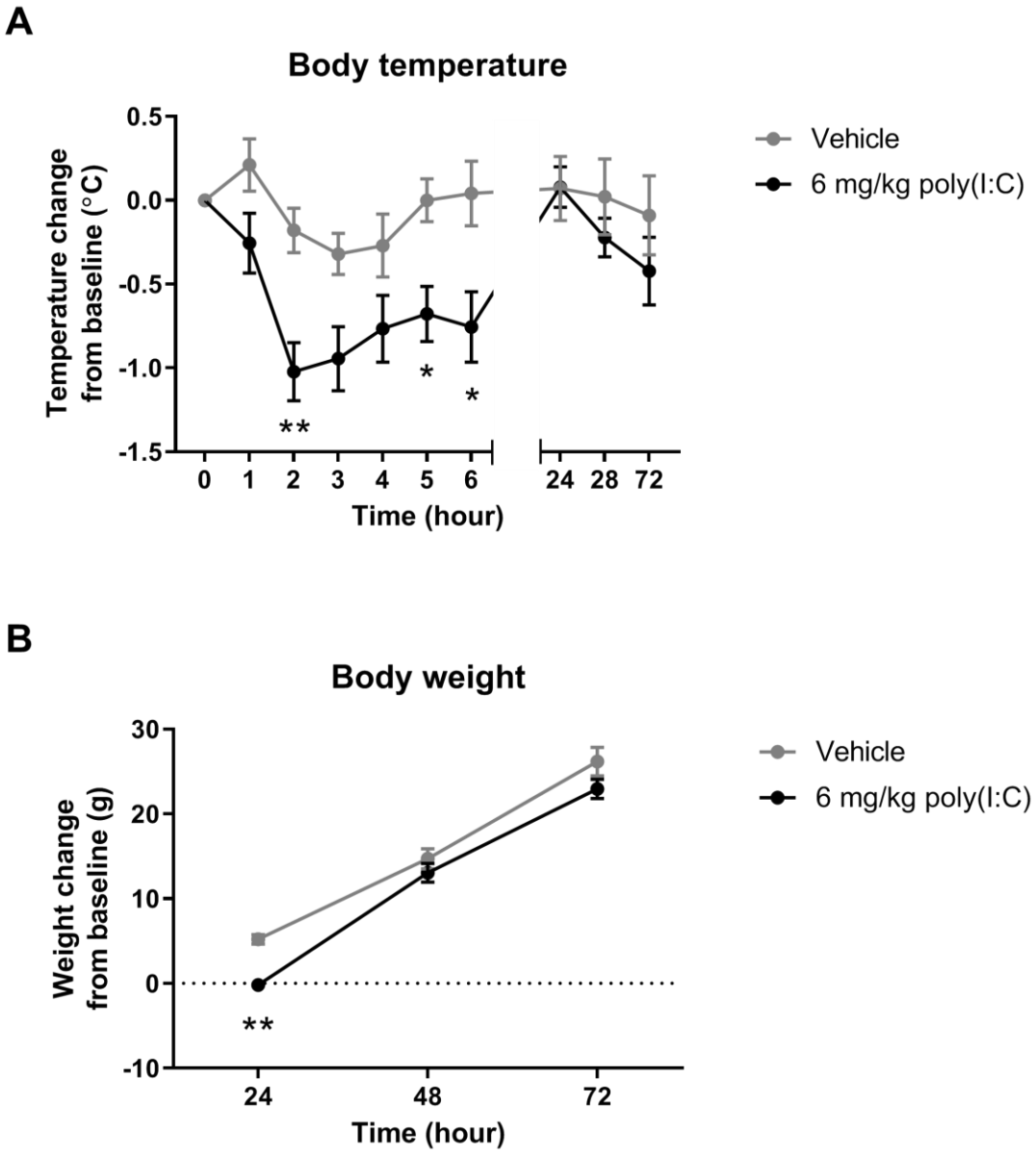


Figure 3.4. Effect of poly(I:C) on body temperature and weight in pregnant Lister-hooded rats. Mean ± SEM **(A)** temperature and **(B)** weight change from pre-injection baseline following intravenous injection of either saline (n=10 for temperature and n=12 for weight) or 6 mg kg^{-1} poly(I:C) (n=9 for temperature and n=11 for weight) on gestational day 15. Absolute body temperature readings at baseline for vehicle and poly(I:C)-treated dams were 36.8 ± 0.1°C and 37.1 ± 0.1°C, respectively. Absolute baseline body weight for vehicle and poly(I:C)-treated dams were 295.4 ± 4.7 g and 279.4 ± 7.0 g, respectively. *p<0.05, **p<0.01 versus vehicle control; two-way RM ANOVA with Sidak's post-hoc test (A, B).

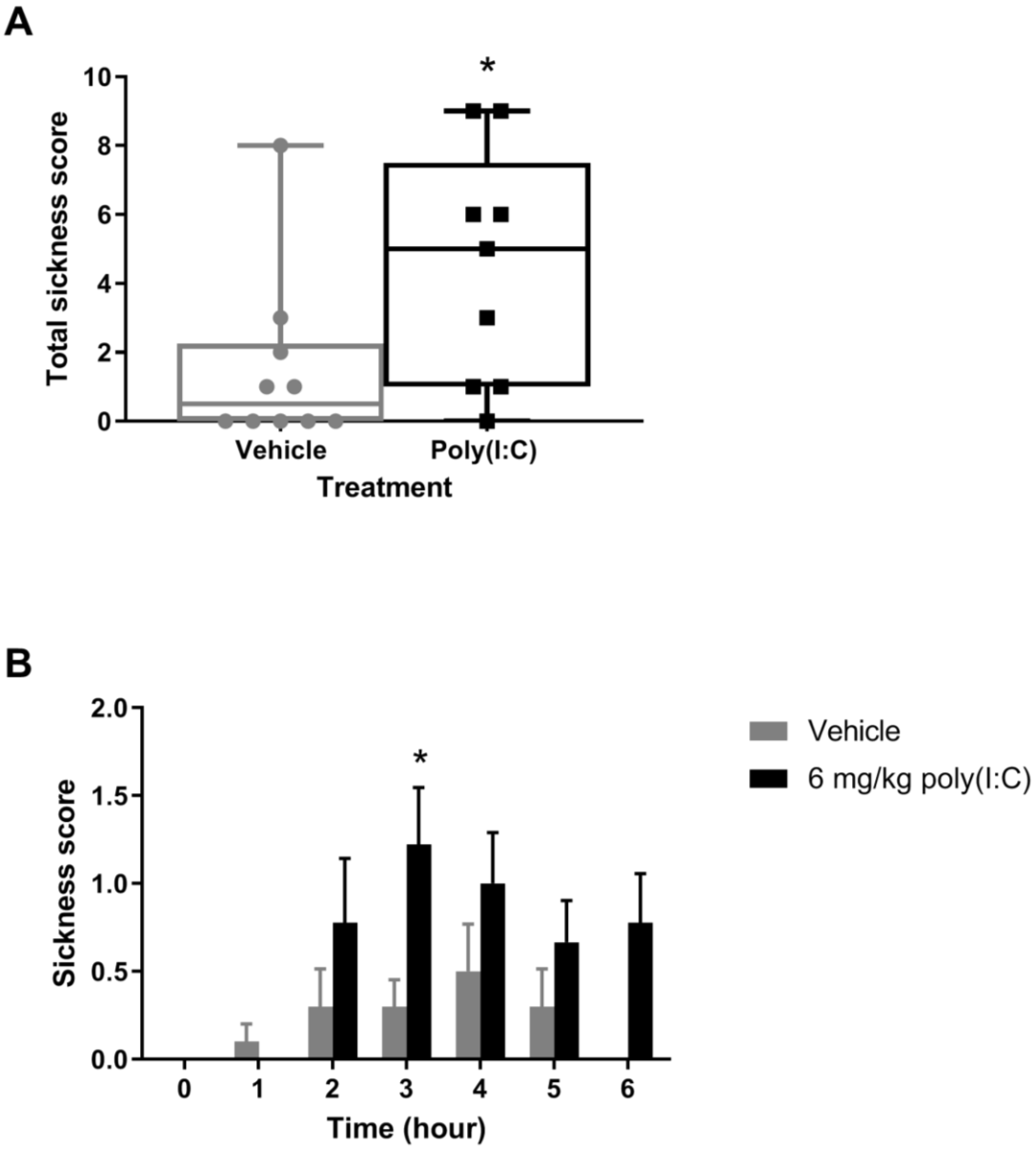


Figure 3.5. Sickness behaviour in pregnant Lister-hooded rats following 6 mg kg^{-1} poly(I:C) i.v. injection on gestational day 15. (A) Box-plot diagram showing total composite sickness score of rats injected with saline (median=0.5, range=0-8) or poly(I:C) (median=5.0, range=0-9) across the first 6 h post-injection and **(B)** time course analysis of the same data at hourly intervals (n=10 for vehicle and n=9 for poly(I:C)). Data are mean ± SEM. *p<0.05 versus vehicle control; Mann-Whiney *U* test (A) or two-way RM ANOVA with Sidak's post-hoc test (B).

3.4.2.2 Effect of poly(I:C) on offspring

A total of 277 pups (vehicle, 147; poly(I:C), 130) were born to the twenty-three dams, including those of the additional study. There was a significant main effect of gender on pup body weight at weaning ($F_{(1,273)}$= 10.89, p<0.01), however, post-hoc analyses revealed no differences between the two genders. Maternal poly(I:C) injection did not influence mean litter size and male:female ratio (Table 3.2). While there was a significant main effect of treatment ($F_{(1,170)}$= 4.71, p<0.05) and gender ($F_{(1,170)}$= 5.50, p<0.05) on the weaning body weight of pups selected for subsequent behavioural testing, no between-group differences were found. As the dams were kept until parturition, it was not possible to examine the number of uterine implantation sites, damaged or dead foetuses, or placental sites without foetuses, to establish the occurrence of any pup loss or foetal malformation.

3.4.2.3 Effect of poly(I:C) and post-weaning isolation rearing on offspring behaviour

3.4.2.3.1 Adolescence (2 weeks post-weaning)

Locomotor activity

To confirm the development of SI-induced behavioural syndrome and to determine if there was an effect of gender, hyperactivity in a novel arena was assessed in adolescent male and female offspring. As expected, habituation was reflected by a general decline in ambulatory activity across time ($F_{(11,968)}$= 195.17, p=0.001). There was a significant main effect of housing ($F_{(1,88)}$= 46.83, p=0.001), as well as significant time x treatment ($F_{(11,968)}$= 2.24, p<0.05) and time x housing x gender ($F_{(11,968)}$= 1.93, p<0.05) interactions on ambulation. While SI alone transiently increased ambulation in females at 15 min post-injection, combined poly(I:C)-SI increased ambulation in females at multiple time points. The effect of poly(I:C)-SI was less robust in males (Figures 3.6A & C).

Table 3.2. Litter characteristics of Lister-hooded dams treated with poly(I:C) (6 mg kg^{-1}, i.v.) or saline vehicle on gestational day 15. Data are mean ± SEM except for total number of pups, number of male and female pups, where absolute numbers are presented (vehicle-treated dams, n=11; poly(I:C)-treated dams, n=12). Mean litter sizes of vehicle and poly(I:C)-treated dams were compared using unpaired Student's *t*-test. The same data and pup body weight at weaning were analysed by two-way ANOVA followed by Sidak's test, with gender and treatment as between-subject factors.

Prior to selection (n=23 litters):		
	Vehicle	**Poly(I:C)**
Total number of pups	147	130
Number of male pups	66	63
Number of female pups	81	67
Litter size	12.3 ± 0.8	11.8 ± 0.8
Number of male pups per litter	5.5 ± 0.4	5.7 ± 0.6
Number of female pups per litter	6.8 ± 0.6	6.1 ± 0.7
Weight at weaning (male pups)	40.1 ± 0.7	41.4 ± 0.7
Weight at weaning (female pups)	38.0 ± 0.6	38.9 ± 0.7
Following selection (n=23 litters):		
Total number of pups	88	86
Number of male pups	56	54
Number of female pups	32	32
Litter size	7.3 ± 0.8	7.8 ± 0.7
Number of male pups per litter	4.7 ± 0.5	4.9 ± 0.6
Number of female pups per litter	4.0 ± 0.8	3.6 ± 0.6
Weight at weaning (male pups)	40.1 ± 0.7	40.9 ± 0.8
Weight at weaning (female pups)	37.0 ± 1.0	39.9 ± 0.9

Analysis of the cumulative total ambulation counts further confirmed a significant main effect of housing ($F_{(1,88)}$= 46.83, p=0.001), such that Veh-SI females, Poly(I:C)-SI males and Poly(I:C)-SI females demonstrated increased total ambulation compared to Veh-GH (Figures 3.6B & D).

Analysis of fine movement revealed a significant main effect of time ($F_{(11,968)}$= 50.00, p=0.001), housing ($F_{(1,88)}$= 51.97, p=0.001) and gender ($F_{(1,88)}$= 13.06, p<0.01), and significant time x housing ($F_{(11,968)}$= 4.93, p=0.001), time x gender ($F_{(11,968)}$= 1.86, p<0.05), time x treatment x gender ($F_{(11,968)}$= 2.59, p<0.01) and time x treatment x housing x gender ($F_{(11,968)}$= 2.05, p<0.05) interactions. SI increased fine movement regardless of prenatal treatment and gender. However, such effect was less robust in males (Figures 3.7A & C). In females, poly(I:C) alone either reduced or increased fine movement (Figure 3.7C). Housing ($F_{(1,88)}$= 51.97, p=0.001) and gender ($F_{(1,88)}$= 13.06, p<0.01) significantly influenced total fine movement, such that Veh-SI females, Poly(I:C)-SI males and Poly(I:C)-SI females had increased total fine movement compared to Veh-GH (Figures 3.7B & D).

For rearing, there was a significant main effect of time ($F_{(11,968)}$= 76.19, p=0.001), housing ($F_{(1,88)}$= 32.56, p=0.001) and gender ($F_{(1,88)}$= 25.49, p=0.001). While poly(I:C)-SI increased rearing in both genders, SI alone only increased rearing in males (Figures 3.8A & C). Analysis of the cumulative total rears showed a similar profile, such that increased total rearing was evident in Veh-SI males, Poly(I:C)-SI males and Poly(I:C)-SI females (Figures 3.8B & D).

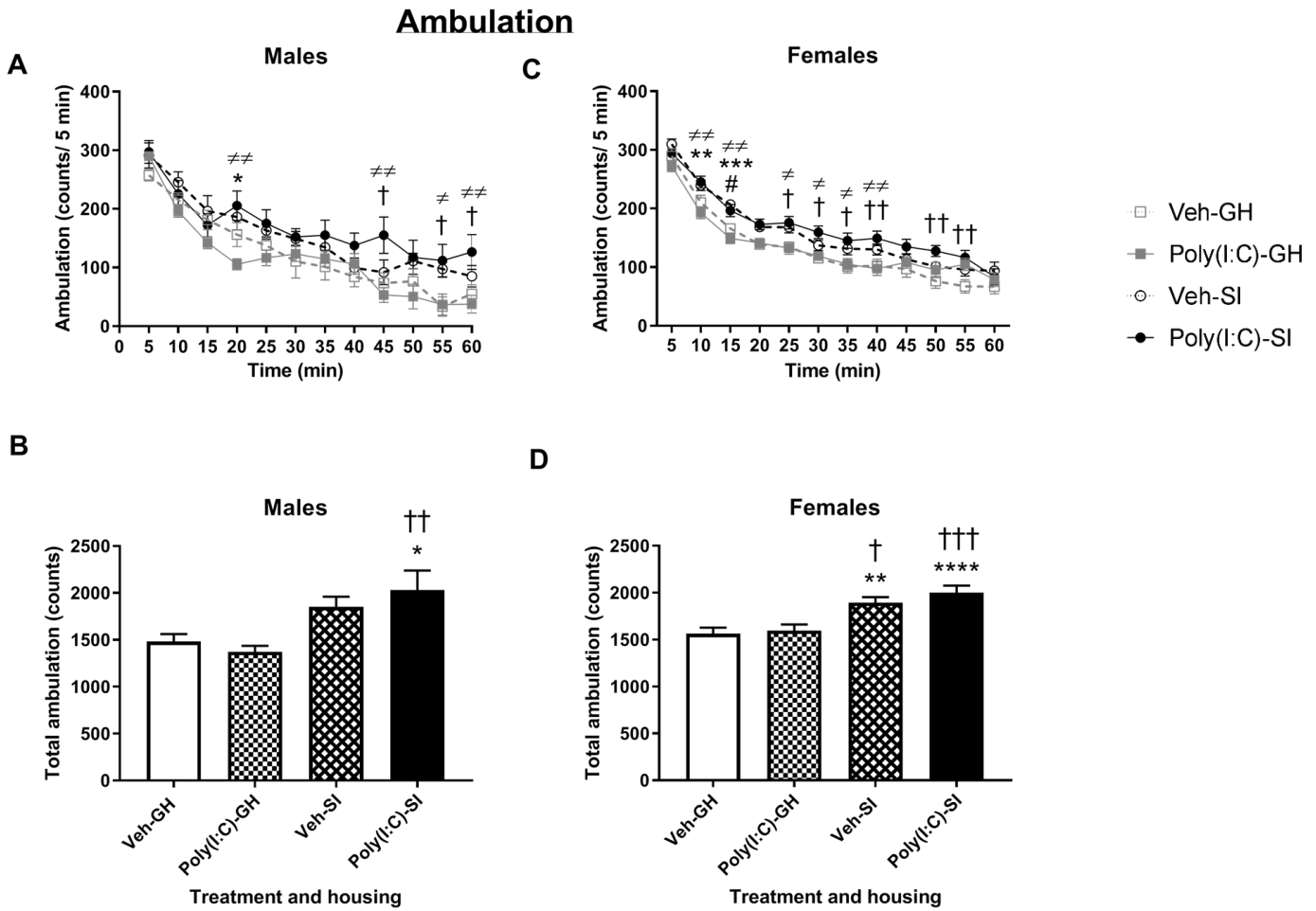


Figure 3.6. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on horizontal ambulation in Lister-hooded rats. Mean ± SEM **(A,C)** horizontal ambulation and **(B,D)** cumulative total ambulation in male (left panel) and female (right panel) adolescent rats (postnatal day 36-37) in a novel box across one-hour observation period (males n=8 per treatment x housing; females, n=16 per treatment x housing). *p<0.05, **p<0.01, ***p<0.001 Poly(I:C)-GH versus Veh-SI; ≠p<0.05, ≠≠p<0.01 Poly(I:C)-GH versus Poly(I:C)-SI; #p<0.05 Veh-GH versus Veh-SI; †p<0.05, ††p<0.01 Veh-GH versus Poly(I:C)-SI; four-way RM ANOVA with Tukey's post-hoc test (A,C). *p<0.05, **p<0.01, ****p<0.0001 versus Veh-GH; †p<0.05, ††p<0.01, †††p<0.001 versus Poly(I:C)-GH; three-way ANOVA with Tukey's post-hoc test (B,D).

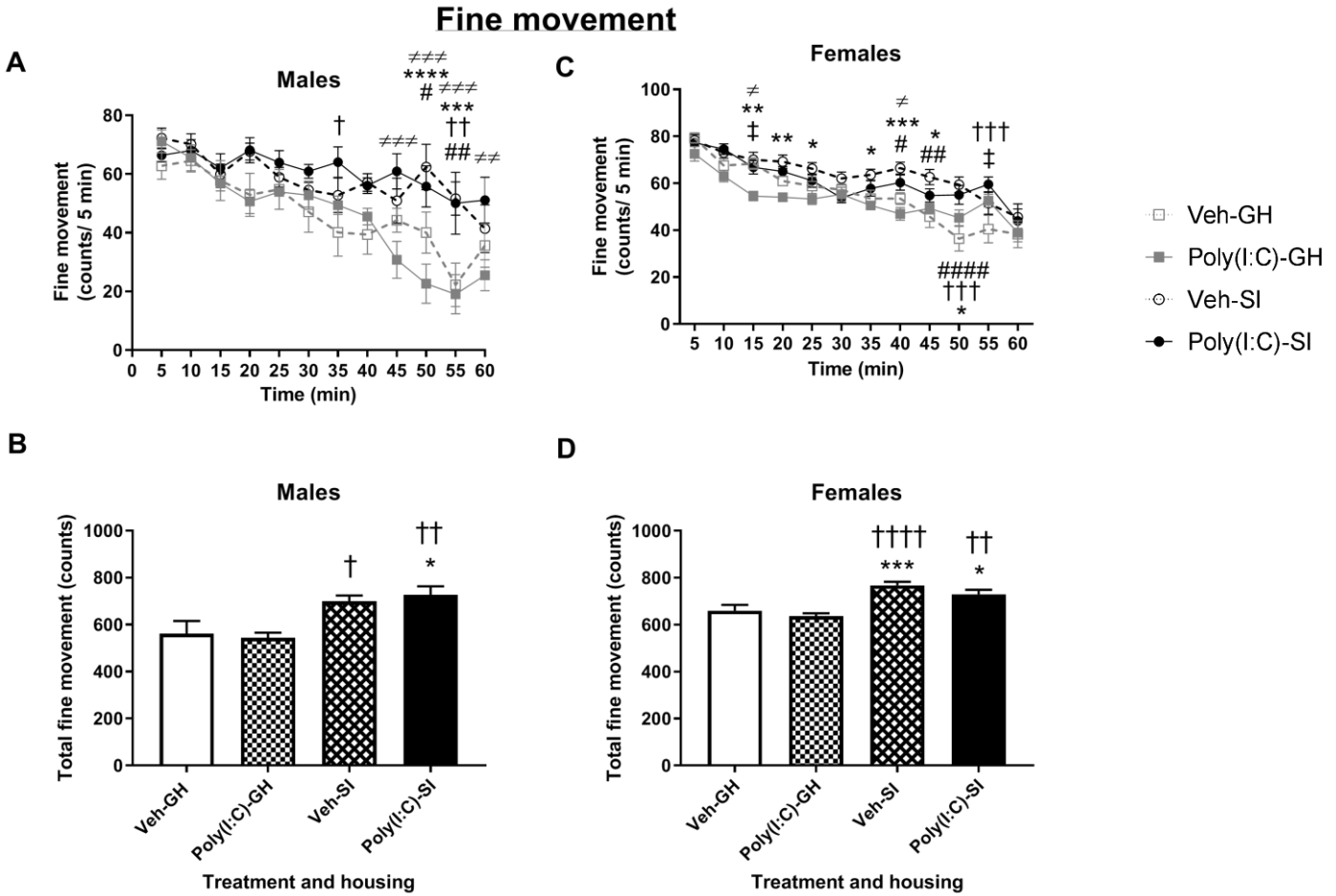


Figure 3.7. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on fine movement in Lister-hooded rats. Mean ± SEM **(A,C)** fine movement and **(B,D)** cumulative total fine movement in male (left panel) and female (right panel) adolescent rats (postnatal day 36-37) in a novel box across one-hour observation period (males n=8 per treatment x housing; females, n=16 per treatment x housing). †p<0.05, ††p<0.01, †††p<0.001 Veh-GH versus Poly(I:C)-SI; ≠p<0.05, ≠≠p<0.01, ≠≠≠p<0.001 Poly(I:C)-GH versus Poly(I:C)-SI; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Poly(I:C)-GH versus Veh-SI; #p<0.05, ##p<0.01, ####p<0.0001 Veh-GH versus Veh-SI; ‡p<0.05 Veh-GH versus Poly(I:C)-GH; four-way RM ANOVA with Tukey's post-hoc test (A,C). *p<0.05, ***p<0.001 versus Veh-GH; †p<0.05, ††p<0.01, ††††p<0.0001 versus Poly(I:C)-GH; three-way ANOVA with Tukey's post-hoc test (B,D).

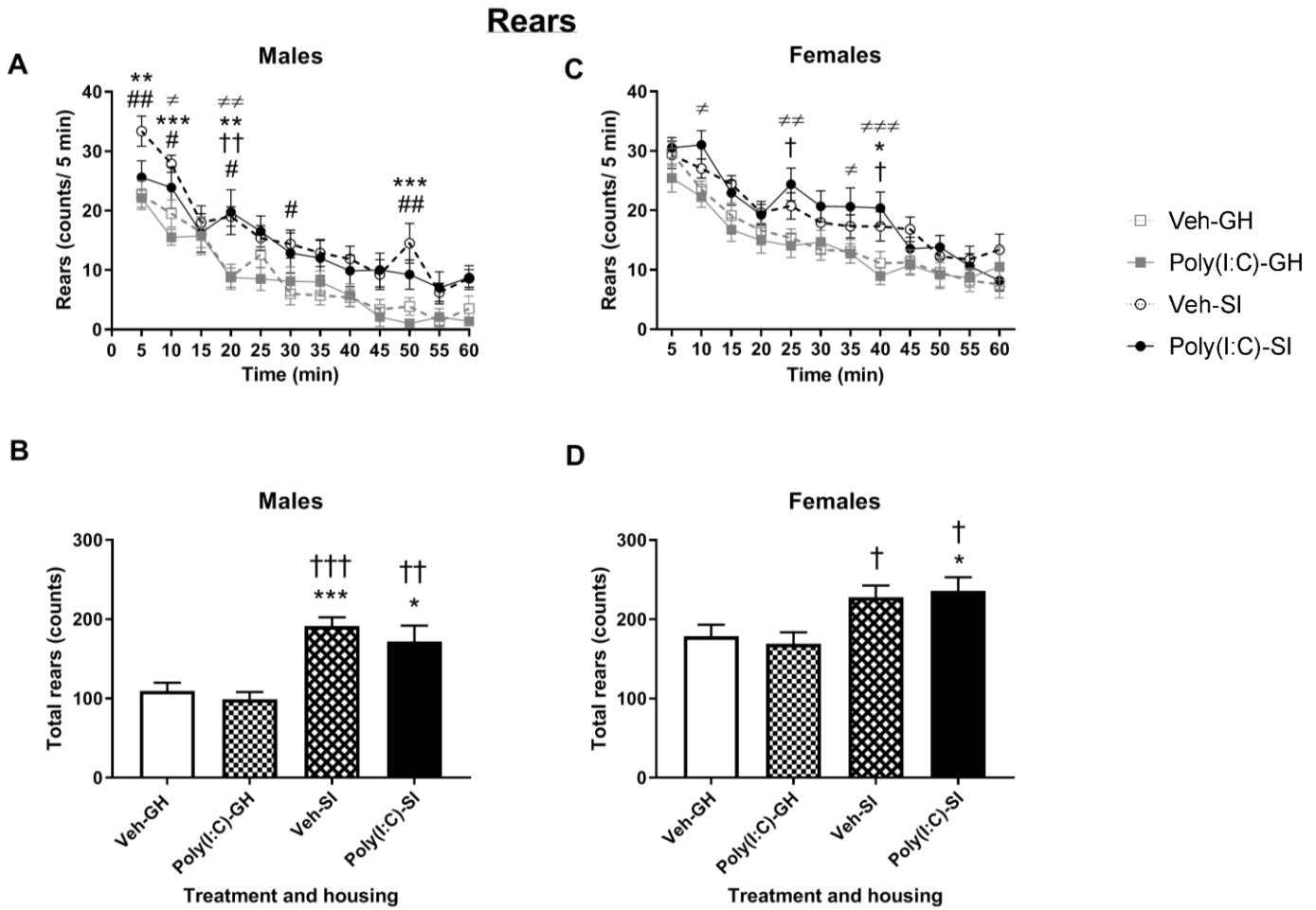


Figure 3.8. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on rears in Lister-hooded rats. Mean ± SEM **(A,C)** rears and **(B,D)** cumulative total rears in male (left panel) and female (right panel) adolescent rats (postnatal day 36-37) in a novel box across one-hour observation period (males n=8 per treatment x housing; females, n=16 per treatment x housing). #p<0.05, ##p<0.01 Veh-GH versus Veh-SI; *p<0.05, **p<0.01, ***p<0.001 Poly(I:C)-GH versus Veh-SI; ≠p<0.05, ≠≠p<0.01, ≠≠≠p<0.001 Poly(I:C)-GH versus Poly(I:C)-SI; †p<0.05, ††p<0.01 Veh-GH versus Poly(I:C)-SI; four-way RM ANOVA with Tukey's post-hoc test (A,C). *p<0.05, ***p<0.001 versus Veh-GH; †p<0.05, ††p<0.01, †††p<0.001 versus Poly(I:C)-GH; three-way ANOVA with Tukey's post-hoc test (B,D).

3.4.2.3.2 Adulthood

Locomotor activity

Despite both genders showed significantly enhanced activity during the LMA task, only male offspring were used for more detailed examination of poly(I:C) and/or SI-induced behavioural phenotype in adulthood using a battery of tests relevant to the positive, negative and cognitive symptom domains of schizophrenia.

Habituation to the novel arena was reflected by a decline in horizontal ambulation ($F_{(11,814)}$= 121.16, p=0.001), fine movement ($F_{(11,814)}$= 38.98, p=0.001) and rears ($F_{(11,814)}$= 140.58, p=0.001) with time. There were significant main effects of housing on all activity types (ambulation, $F_{(1,74)}$= 8.61, p<0.01; fine movement, $F_{(1,74)}$= 7.98, p<0.01; rears, $F_{(1,74)}$= 9.55, p<0.01), as well as time x housing interactions for ambulation ($F_{(11,814)}$= 1.80, p=0.05) and fine movement ($F_{(11,814)}$= 1.86, p<0.05), and a main effect of treatment on ambulation ($F_{(1,74)}$= 10.17, p<0.01). SI alone subtly increased rears and fine movement while poly(I:C)-SI increased all activity types at various time points across the observation period (Figure 3.9A-C). Such findings were confirmed by analyses of the cumulative total counts, which revealed increased total ambulation, fine movement and rears in Poly(I:C)-SI rats compared to Veh-GH (Figure 3.10A-C).

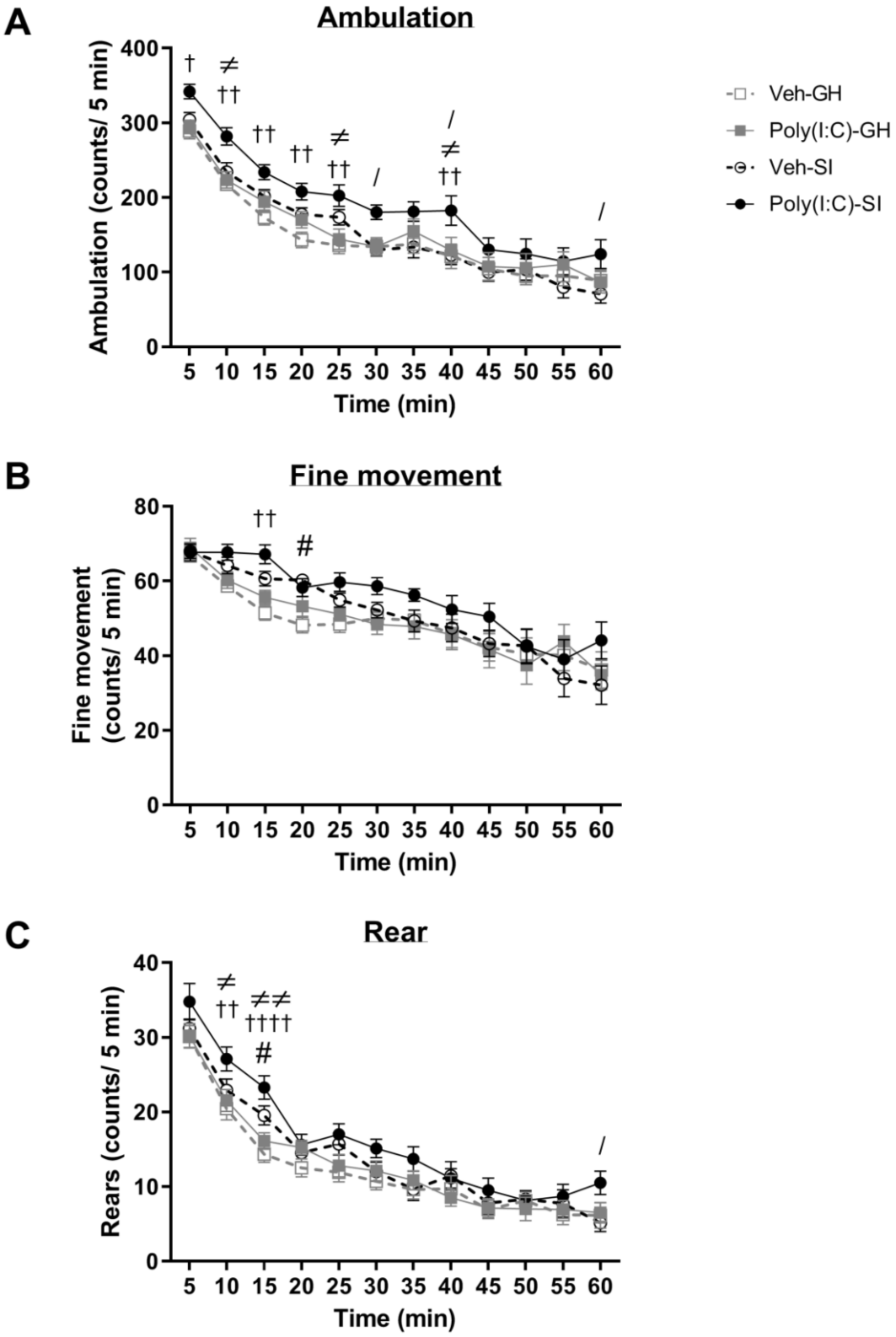


Figure 3.9. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on the different activity types in male Lister-hooded rats at five weeks post-weaning (postnatal day 57-58). Mean ± SEM **(A)** ambulation, **(B)** fine movement and **(C)** rears in a novel box across an one-hour observation period are presented (n=18-20 per treatment x housing). †p<0.05, ††p<0.01, ††††p<0.0001 Veh-GH versus Poly(I:C)-SI; #p<0.05 Veh-GH versus Veh-SI; ≠p<0.05, ≠≠p<0.01 Poly(I:C)-GH versus Poly(I:C)-SI; /p<0.05 Veh-SI versus Poly(I:C)-SI; three-way RM ANOVA with Tukey's post-hoc test (A-C).

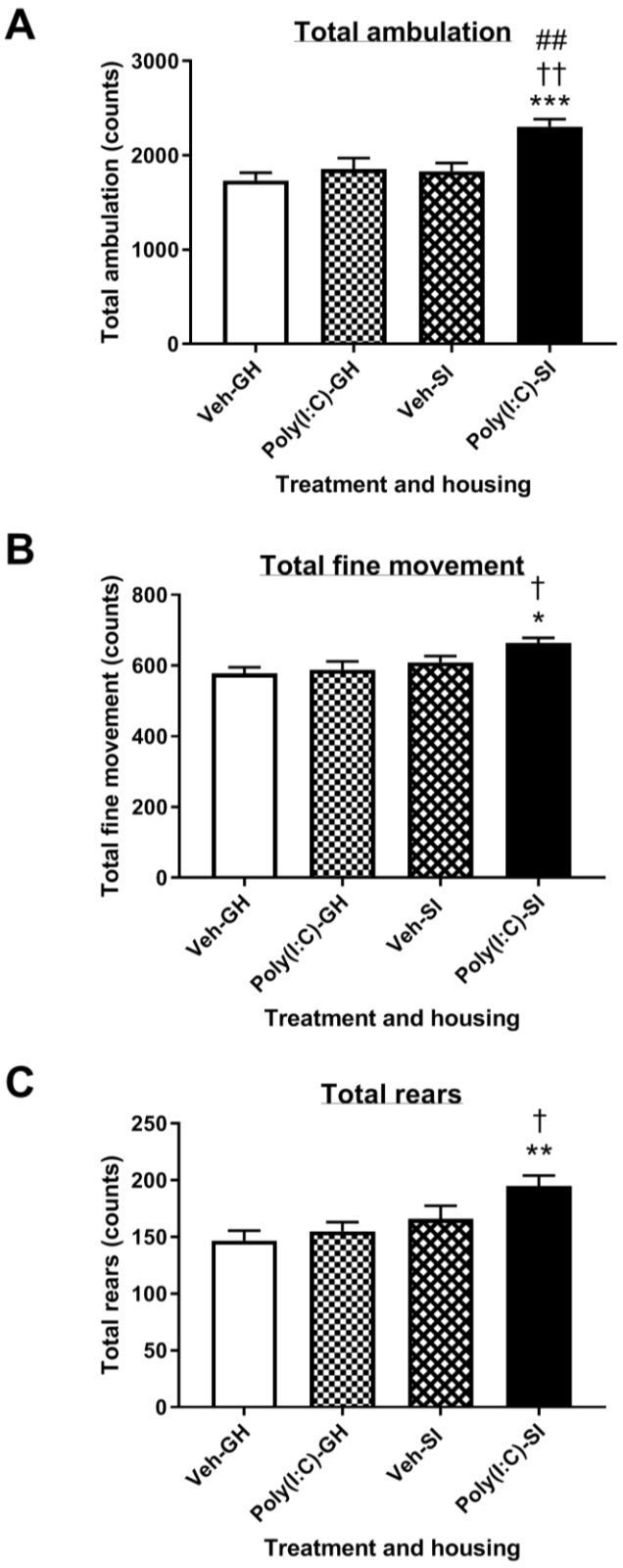


Figure 3.10. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on the cumulative totals of the different activity types in male Lister-hooded rats at five weeks post-weaning (postnatal day 57-58). Mean ± SEM total **(A)** ambulation, **(B)** fine movement and **(C)** rears in a novel box across an one-hour observation period are presented (n=18-20 per treatment x housing). *p<0.05, **p<0.01, ***p<0.001 compared to Veh-GH; †p<0.05, ††p<0.01 compared to Poly(I:C)-GH; ## p<0.01 compared to Veh-SI; two-way ANOVA with Tukey's post-hoc test (A-C).

Novel object discrimination

To determine if prenatal poly(I:C) exposure and/or SI produced long-lasting deficits in visual recognition memory, rats were tested in the NOD task 24 h following LMA. There was no significant main effect of object, treatment or housing on the familiarisation trial (data not shown). In contrast, there was a significant main effect of object but not treatment or housing on choice trial object exploration following either a 1h ($F_{(1,36)}$= 8.83, p<0.01) or 2h ($F_{(1,34)}$= 22.7, p=0.001) ITI. Poly(I:C)-SI and Veh-GH rats showed significant preferential exploration of the novel object during the choice trial after an ITI of 1 h and 2 h, respectively (Figure 3.11). Trial number significantly affected total object exploration (1 h ITI, $F_{(1,36)}$= 25.52, p=0.001; 2 h ITI, $F_{(1,34)}$= 40.79, p=0.001). In the task with a 1h ITI, Veh-GH, Poly(I:C)-GH and Veh-SI rats spent significantly less time exploring during the choice trial. In the task with a 2h ITI, reduction in choice trial exploration was evident in Veh-GH, Poly(I:C)-GH and Poly(I:C)-SI rats (data not shown).

Novel location discrimination

To assess whether spatial working memory was affected, rats were evaluated in the NLD task. Location, treatment or housing did not affect object exploration during familiarisation trial but there was a significant location x treatment x housing interaction ($F_{(1,74)}$= 4.13, p<0.05; data not shown). Rats explored objects at both locations equally during the familiarisation trial. Although location influenced object exploration in the choice trial ($F_{(1,74)}$= 10.11, p<0.01), rats demonstrated similar preference for object exploration at the novel and familiar locations (data not shown). Trial number significantly affected the total object exploration time ($F_{(1,74)}$= 50.70, p=0.001), such that all groups spent a shorter duration exploring objects in the choice trial compared to the familiarisation trial (data not shown).

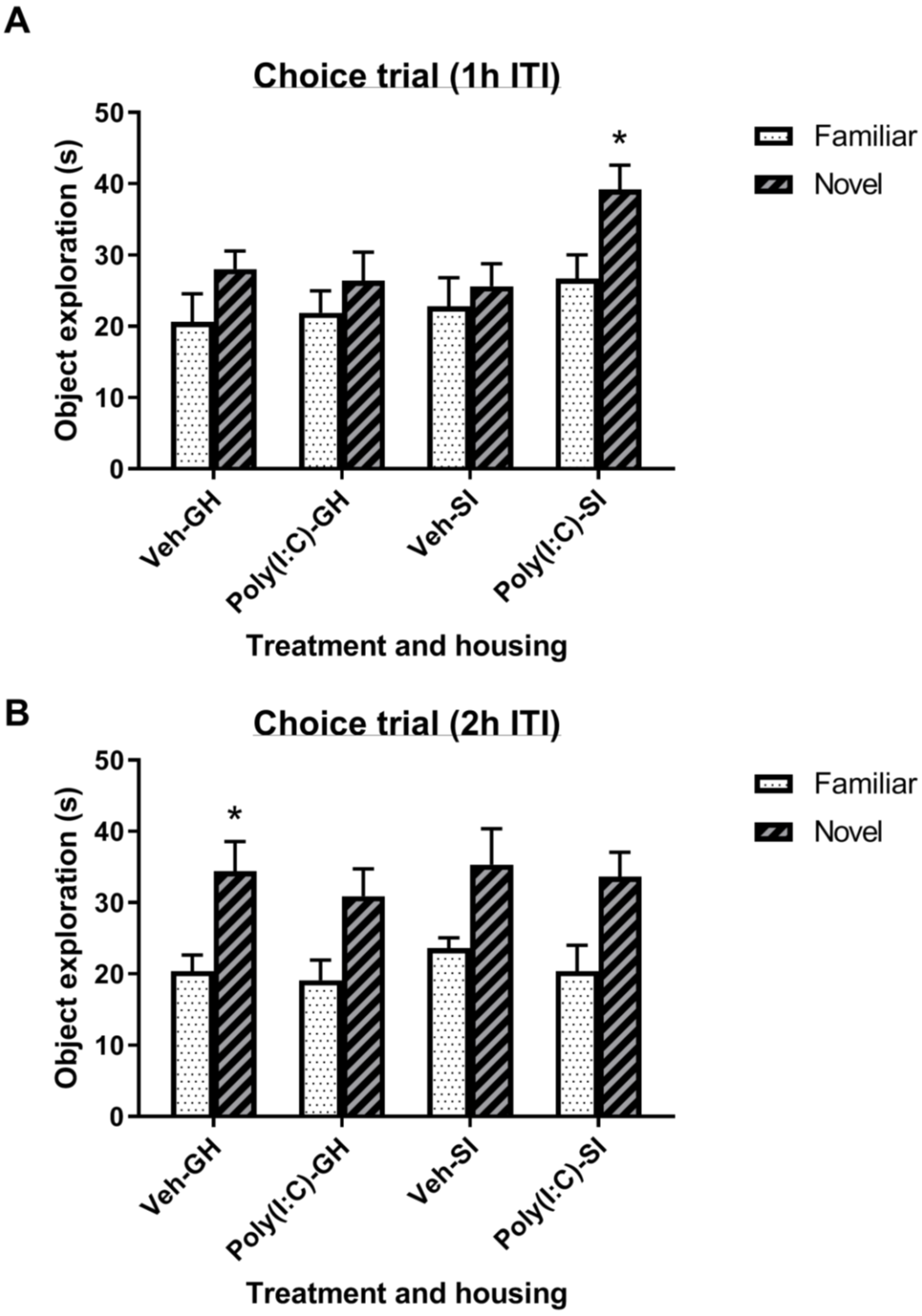


Figure 3.11. Choice trial object exploration of Lister-hooded male rats exposed to prenatal poly(I:C) (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation in the novel object discrimination task at five weeks post-weaning (postnatal day 58-59). Mean ± SEM time (s) spent exploring the familiar and novel objects during the choice trial **(A)** 1h or **(B)** 2h following the familiarisation trial (n=8-10 per treatment x housing for each ITI). *p<0.05 versus familiar object; three-way RM ANOVA with Sidak's post-hoc test (A,B). ITI, inter-trial interval.

Social interaction

Social interaction test was conducted to examine any impact of MIA and/or SI on social functioning in rats. Of particular note, each rat pair consisted of weight-matched non-littermates that were unfamiliar to each other. There was a significant main effect of behaviour subtype ($F_{(4,140)}$= 249.46, p=0.001) and a behaviour subtype x housing interaction ($F_{(4,140)}$= 74.38, p=0.001) on pro-social behavioural components. Veh-SI and Poly(I:C)-SI demonstrated significantly reduced anogenital sniffing but a corresponding increase in body sniffing compared to Veh-GH and Poly(I:C)-GH. Poly(I:C)-SI also showed increased crawling over and under (Figure 3.12A). Aggressive behaviour showed a significant main effect of behaviour subtype ($F_{(1,35)}$= 19.03, p=0.001) and housing ($F_{(1,35)}$= 8.56, p<0.01), alongside a behaviour subtype x housing interaction ($F_{(1,35)}$= 7.89, p<0.01). There were however no between-group differences in either boxing and biting or pinning (Figure 3.12B). Housing significantly influenced total distance travelled ($F_{(1,35)}$= 11.82, p<0.01) and velocity in the open field arena ($F_{(1,35)}$= 11.34, p<0.01), but there were no differences compared to Veh-GH (data not shown). Neither treatment nor housing affected the total time spent in all interactive behaviours (data not shown).

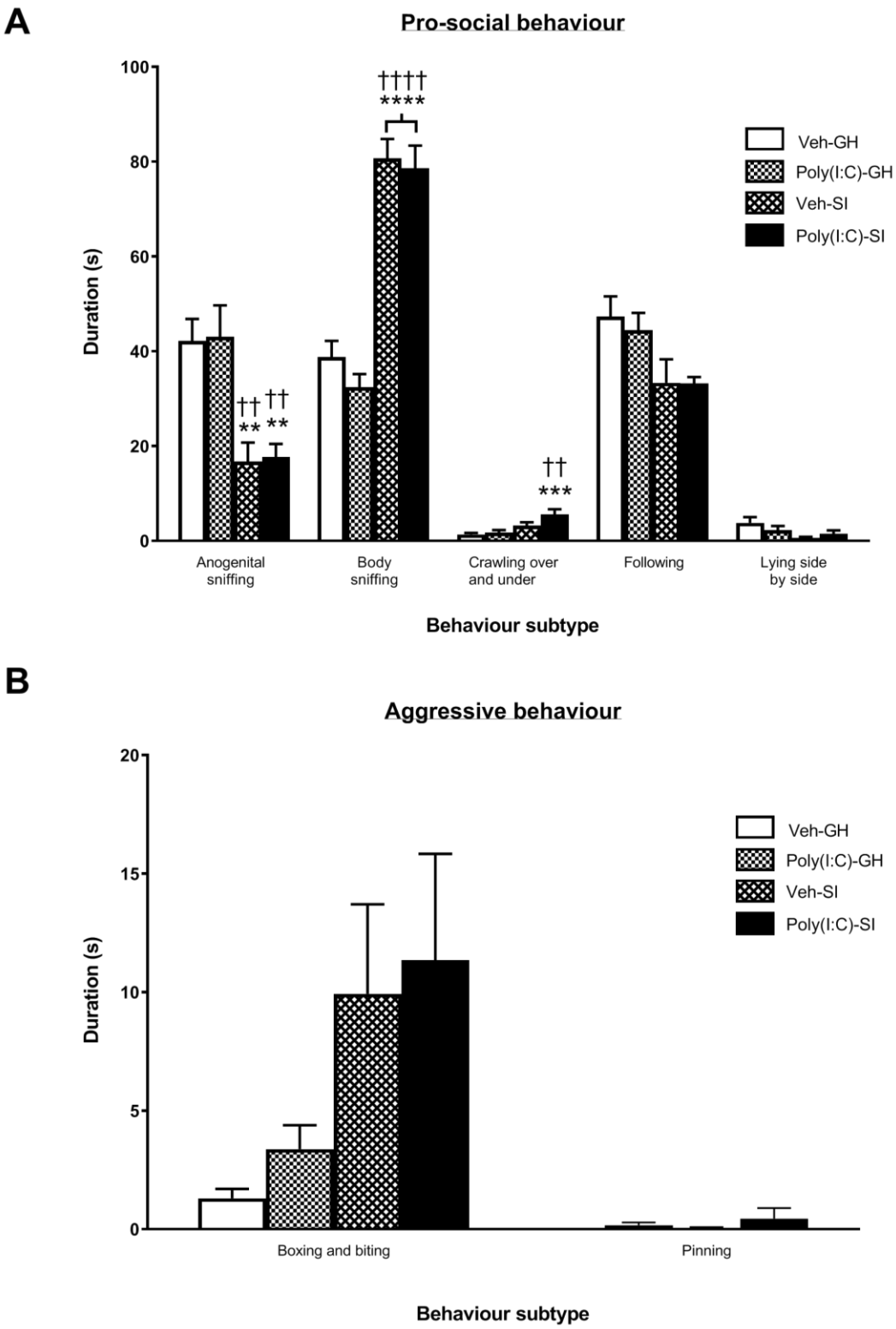


Figure 3.12. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on rat social behaviour at around seven weeks post-weaning (postnatal day 69-72). Mean ± SEM time spent in **(A)** pro-social and **(B)** aggressive behaviour by pairs of unfamiliar weight- and neurodevelopmental history-matched rats during the 10-min social interaction task (n=9-10 pairs per treatment x housing). **p<0.01, ***p<0.001, ****p<0.0001 compared to respective Veh-GH (A); ††p<0.01, ††††p<0.0001 compared to respective Poly(I:C)-GH; three-way RM ANOVA with Tukey's post-hoc test (A,B).

Ultrasonic vocalisations

Concomitant recording of USVs was carried out during the social interaction test to investigate any alterations in social communication. Aversive 22-kHz calls were exhibited by only five of the 39 pairs of rats: one Veh-GH, one Poly(I:C)-GH, one Veh-SI and two Poly(I:C)-SI. The occurrence of such calls was insufficient to warrant statistical analysis. In contrast, all rat pairs exhibited a variety of complex 50-kHz calls that were analysed in detail. Housing significantly affected the total number of 50-kHz calls ($F_{(1,35)}$= 43.80, p<0.0001), such that Veh-SI and Poly(I:C)-SI produced significantly more 50-kHz calls (Figure 3.13). When categorised into the three main types of 50-kHz calls, significant main effects of housing were revealed for flat ($F_{(1,35)}$= 48.29, p<0.0001), step ($F_{(1,35)}$= 35.91, p<0.0001) and trill ($F_{(1,35)}$= 12.01, p<0.01), in addition to a significant main effect of treatment ($F_{(1,35)}$= 7.35, p<0.05) and a housing x treatment ($F_{(1,35)}$= 6.05, p<0.05) interaction on flat calls. Veh-SI produced significantly more flat, step and trill calls whereas Poly(I:C)-SI only showed enhanced flat and step (Figure 3.14A-C). Using the narrower classification (section 2.3.5.5), nine out of the fourteen 50-kHz call subtypes were identified. Housing significantly influenced all identified subtypes: flat ($F_{(1,35)}$= 41.58, p<0.0001), downward ramp ($F_{(1,35)}$= 11.22, p<0.01), upward ramp ($F_{(1,35)}$= 14.32, p<0.001), short ($F_{(1,35)}$= 43.34, p<0.0001), trills ($F_{(1,35)}$= 12.01, p<0.01), multi-step ($F_{(1,35)}$= 24.40, p<0.0001), step up ($F_{(1,35)}$= 35.20, p<0.0001), step down ($F_{(1,35)}$= 21.80, p<0.0001) and inverted-U ($F_{(1,35)}$= 16.58, p<0.001). There was also a significant main effect of treatment on flat ($F_{(1,35)}$= 6.91, p<0.05), upward ramp ($F_{(1,35)}$= 4.77, p<0.05) and short ($F_{(1,35)}$= 5.18, p<0.05) calls, alongside a housing x treatment interaction on flat ($F_{(1,35)}$= 7.04, p<0.05) and step up ($F_{(1,35)}$= 5.15, p<0.05). SI caused a marked increase in flat, short, multi-step and step down calls regardless of prenatal treatment. SI alone significantly increased trill calls whereas Poly(I:C)-SI enhanced step up, upward ramp and inverted-U calls (Figure 3.15A & B).

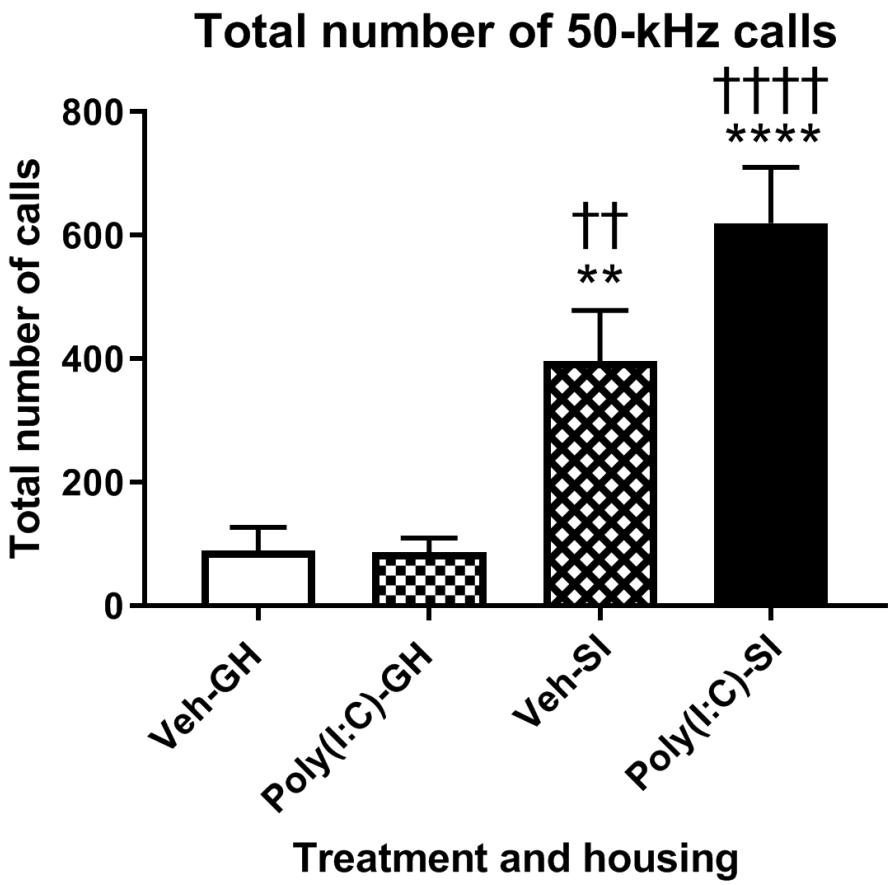


Figure 3.13. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on rat 50-kHz ultrasonic vocalisations at around seven weeks post-weaning (postnatal day 69-72). Mean ± SEM total number of calls, including unclassifiable calls, recorded in pairs of weight-matched, newly encountered Lister-hooded male rats (from different litters) exposed to the same prenatal treatment and housing conditions during a 10-min social interaction test (n=9-10 pairs per treatment x housing). **p<0.01, ****p<0.0001 compared to Veh-GH; ††p<0.01, ††††p<0.0001 compared to Poly(I:C)-GH; two-way ANOVA with Tukey's post-hoc test.

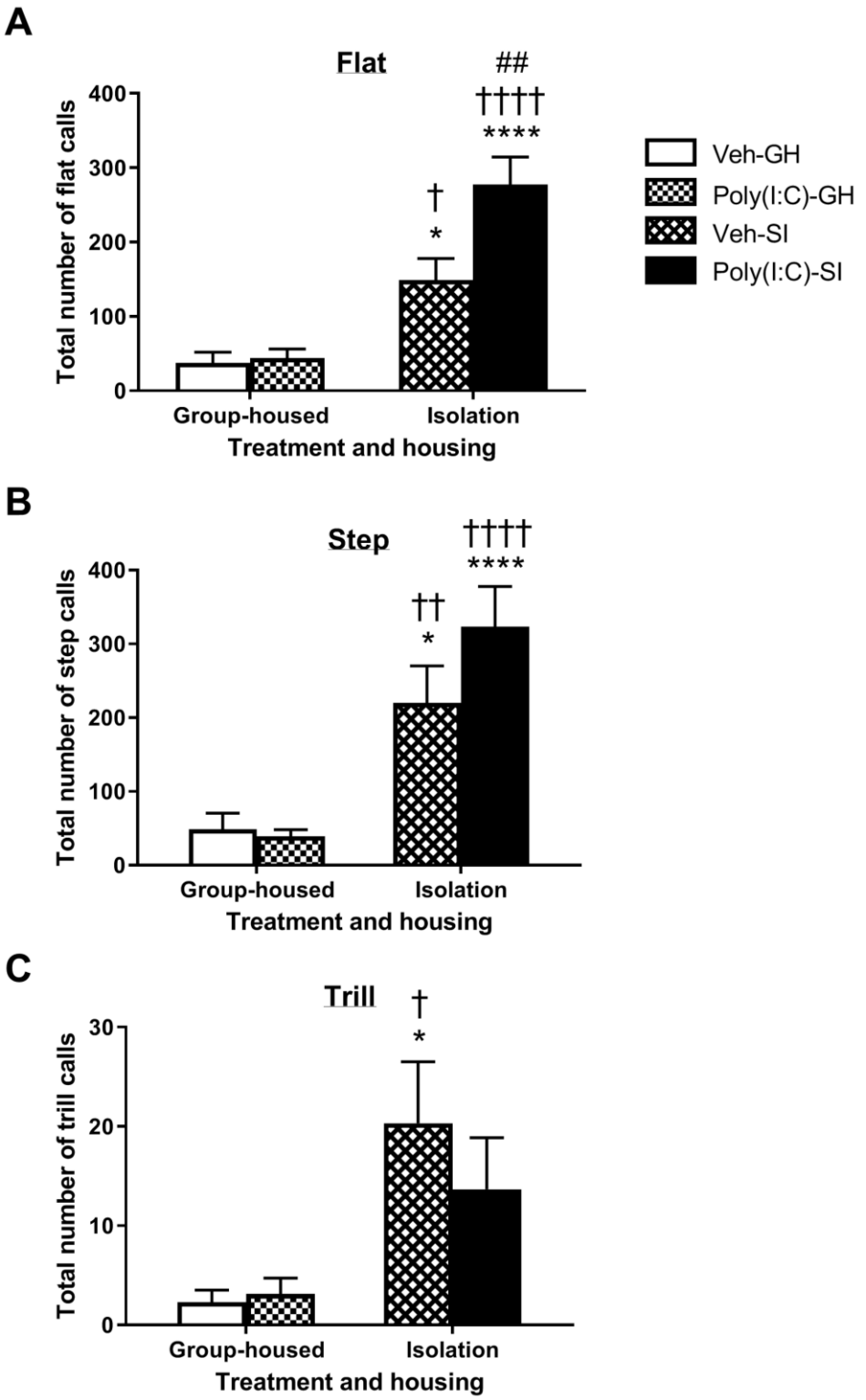


Figure 3.14. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on rat emission of the three main 50-kHz ultrasonic vocalisation subtypes at around seven weeks post-weaning (postnatal day 69-72). Mean ± SEM total **(A)** flat, **(B)** step and **(C)** trill calls recorded in pairs of newly encountered Lister-hooded male rats exposed to the same prenatal treatment and housing conditions during a 10-min social interaction test (n=9-10 pairs per treatment x housing). *p<0.05, ****p<0.0001 versus Veh-GH; †p<0.05, ††p<0.01, ††††p<0.0001 compared to Poly(I:C)-GH; ##p<0.01 versus Veh-SI; two-way ANOVA with Tukey's post-hoc test (A-C).

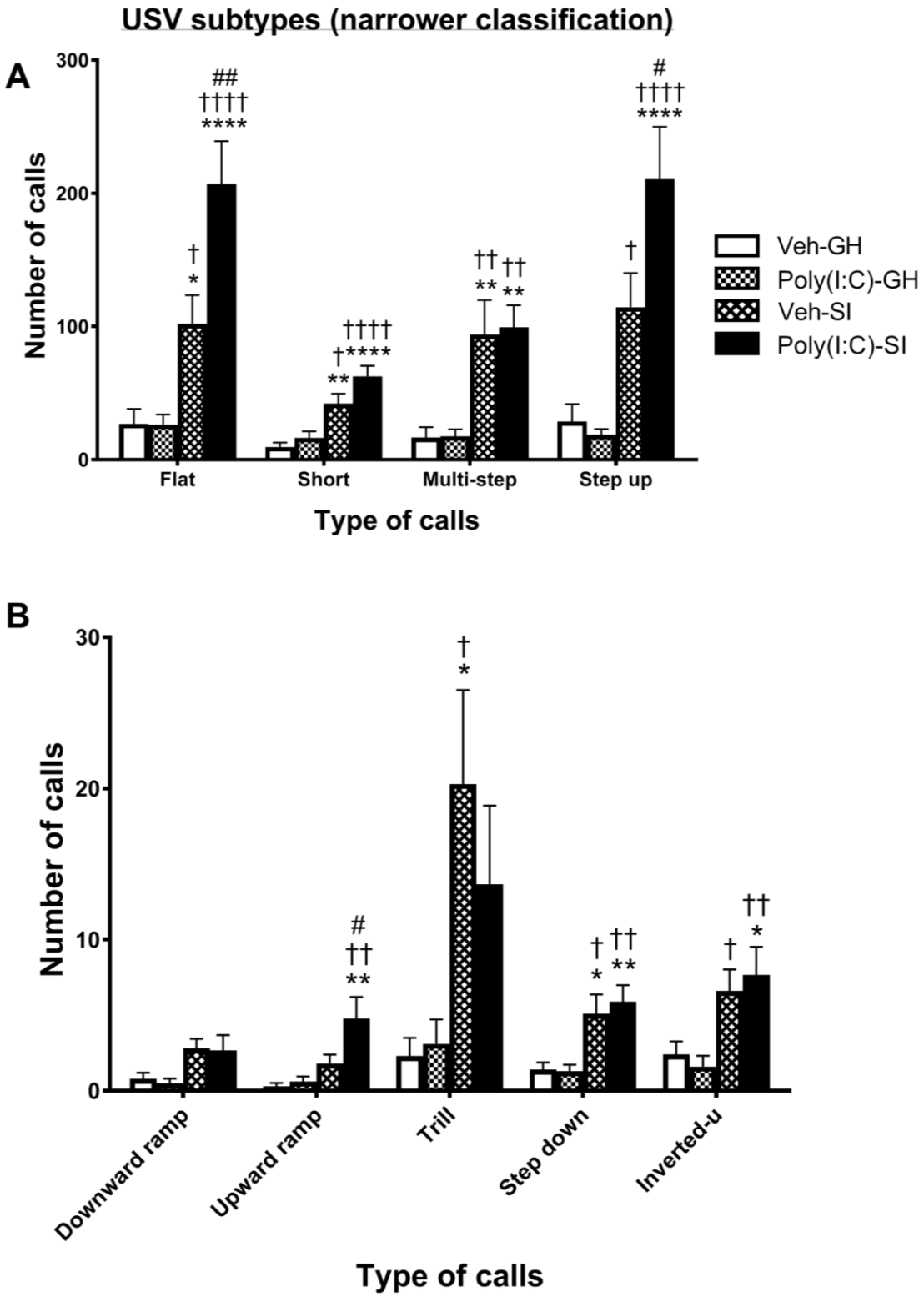


Figure 3.15. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on rat emission of nine 50-kHz ultrasonic vocalisation subtypes at around seven weeks post-weaning (postnatal day 69-72). Total number of the different call subtypes recorded in pairs of newly encountered Lister-hooded male rats exposed to the same prenatal treatment and housing conditions during a 10-min social interaction test are expressed as mean ± SEM (n=9-10 pairs per treatment x housing). *p<0.05, **p<0.01, ****p<0.0001 compared to relevant Veh-GH, †p<0.05, ††p<0.01, ††††p<0.0001 compared to relevant Poly(I:C)-GH; #p<0.05, ##p<0.01 compared to relevant Veh-SI; two-way ANOVA with Tukey's post-hoc test (A,B).

Pre-pulse inhibition of acoustic startle

There was an expected increase in % PPI with increasing pre-pulse intensity that was not influenced by treatment or housing ($F_{(2,148)}$= 152.06, p=0.001). Neither treatment nor housing influenced initial startle, final startle and habituation to the startle pulse across the test session (Figure 3.16).

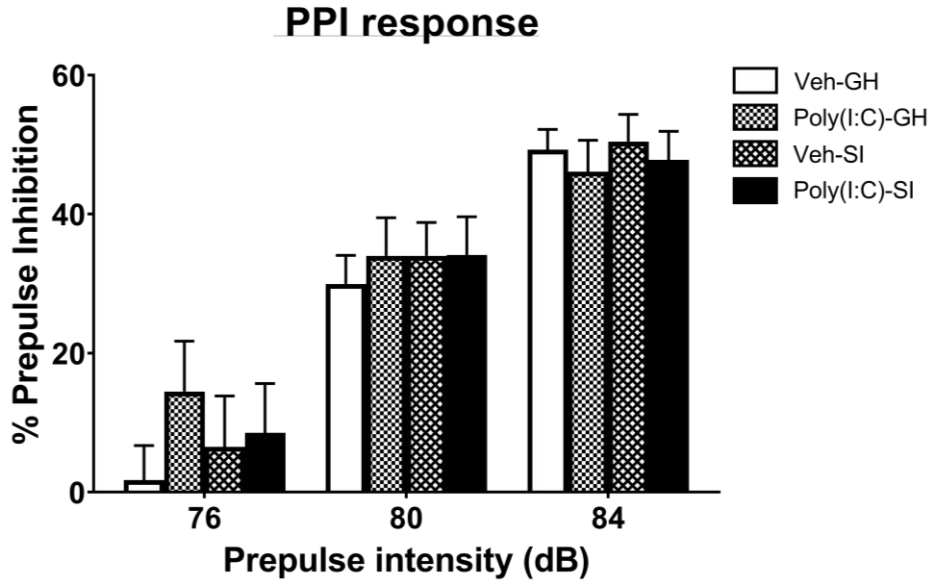


Figure 3.16. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on pre-pulse inhibition at eight weeks post-weaning (postnatal day 78-82). Mean ± SEM % PPI at 76, 80 and 84 dB pre-pulse intensity (n=18-20 per treatment x housing); three-way RM ANOVA with Tukey's post-hoc test.

Conditioned freezing response

The impact of prenatal poly(I:C) and/or subsequent SI on associative learning and memory was probed using a previously validated CFR task. Data from one Poly(I:C)-GH rat was excluded due to the inter-chamber wall falling twice during the acquisition phase of the task, resulting in n=9 for Poly(I:C)-GH and n=10 for the other groups. Neither treatment nor housing influenced latency to enter the dark chamber (data not shown). There was a significant main effect of shock number ($F_{(2,70)}$= 7.46, p<0.01) and housing ($F_{(1,35)}$= 17.54, p=0.001) on freezing duration in the acquisition phase on day one conditioning (0 h). Veh-SI showed reduced freezing following the second and third foot shocks whereas Poly(I:C)-SI froze less after the first shock only. When re-introduced to the context at 24 h in the absence of foot shock (US), there was a significant main effect of CS cue ($F_{(1,35)}$= 43.34, p=0.001) and housing ($F_{(1,35)}$= 6.68, p<0.05) but there were no between-group differences (Figure 3.17). Housing and treatment did not affect latency to leave the dark compartment 5 min post-cue presentation (data not shown).

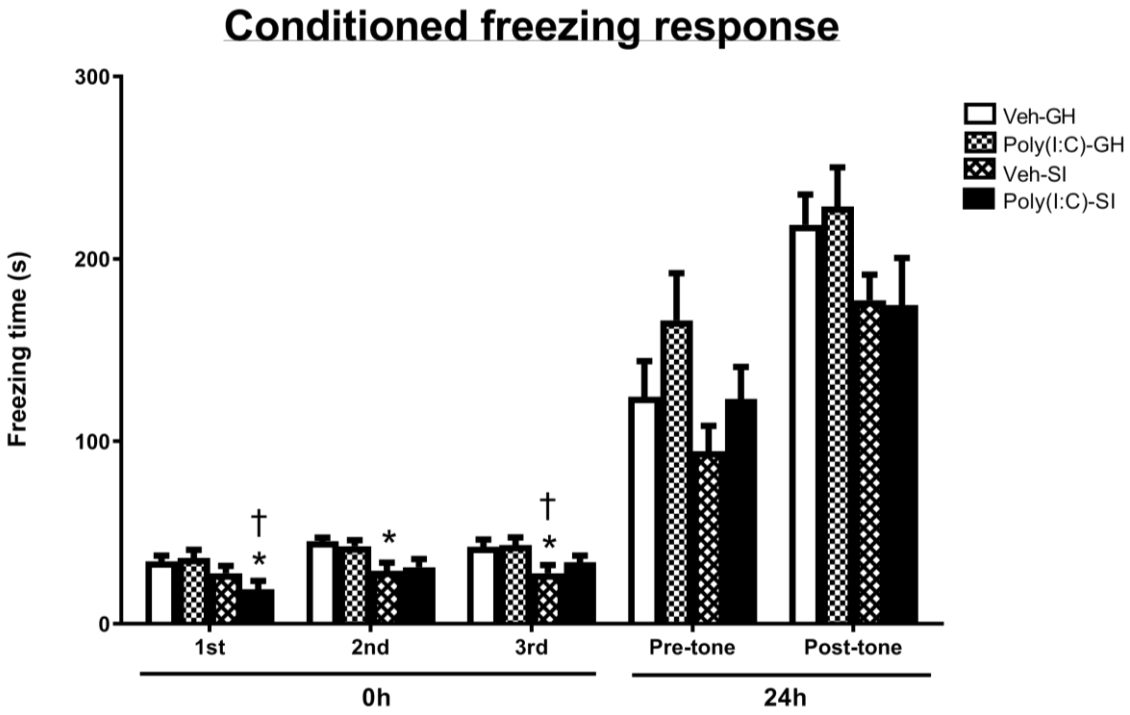


Figure 3.17. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on conditioned freezing response at around nine weeks post-weaning (0 h: postnatal day 82-85). Mean ± SEM duration spent freezing (s) following three consecutive presentations of combined light and tone (conditioned stimulus, CS) and foot shock (unconditioned stimulus) at 0 h, and before and after single presentation of CS at 24 h upon re-entry into the dark compartment of the shuttle box, are presented (n=9-10 per treatment x housing). *p<0.05 compared to relevant Veh-GH; †p<0.05 compared to relevant Poly(I:C)-GH; three-way RM ANOVA with Tukey's post-hoc test (0 h and 24 h).

Attentional set-shifting task

Rats were assessed in an eight-day long ASST to evaluate their cognitive flexibility. Two Veh-GH littermates could not perform the task. To avoid confounding factors such as heritability from skewing the results, data from their littermates were also excluded from the analysis. Data from four additional rats were excluded from analysis due to accidental over-training of one Poly(I:C)-GH rat and inability to complete reversal on first attempt by two Veh-SI and one Poly(I:C)-SI rats, resulting in n=7-9 per treatment x housing.

Neither discrimination type (odour or media) nor combined treatment and housing influenced TTC and ETC during day 7 SD training (data not shown). On day 8 CD, there was a significant effect of discrimination type on TTC (CD versus Rev1, $F_{(1,27)}= 4.24$, $p<0.05$; IDS versus EDS, $F_{(1,27)}= 5.64$, $p<0.05$) and ETC (CD versus Rev1, $F_{(1,27)}= 6.36$, $p<0.05$; IDS versus EDS, $F_{(1,27)}= 8.40$, $p<0.01$), thus showing that rats generally found Rev1 and EDS harder than CD and IDS, respectively. Poly(I:C)-GH rats made significantly more errors in EDS compared to IDS (Figure 3.18). When all discrimination stages were considered, there was a significant main effect of discrimination type on ETC ($F_{(6,162)}= 3.03$, $p<0.01$) but all groups performed similarly. Neither treatment nor housing influenced cumulative total TTC and ETC across all discrimination stages on day 8 (data not shown).

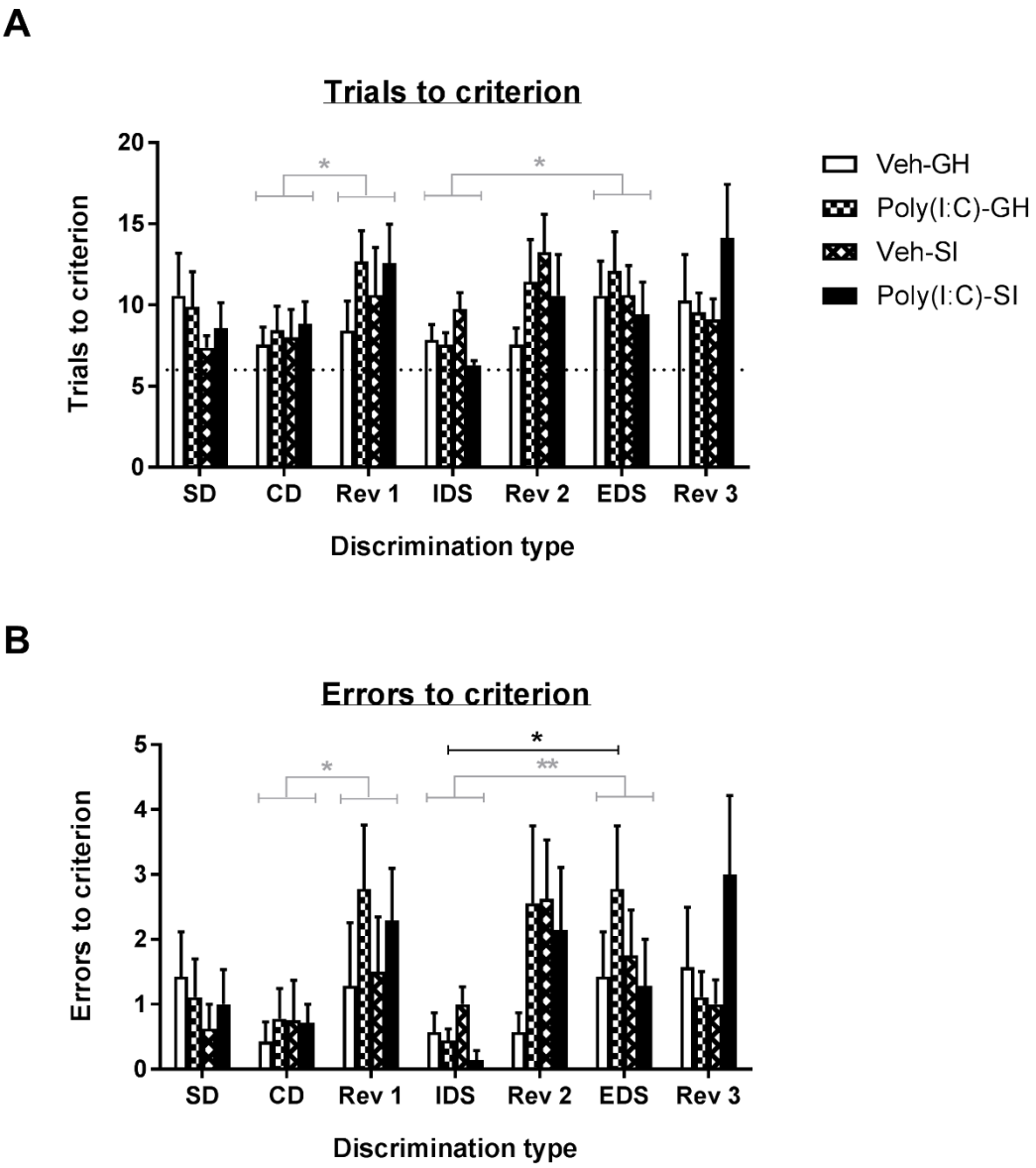


Figure 3.18. Trials to criterion and errors to criterion of male Lister-hooded rats in the attentional set-shifting task. Mean ± SEM **(A)** trials to criterion and **(B)** errors to criterion during SD training on day 7 and the different stages of complex discrimination (CD, Rev1, ID, Rev2, ED, Rev3) on day 8 (n=7-9 per treatment x housing). *p<0.05, **p<0.01; two-way ANOVA with Sidak's post-hoc test (A,B). Black capped line shows comparison between discrimination types whereas grey capped lines linked with zig-zag lines denote significant main effects of the discrimination types. Horizontal dashed line in (A) denotes the criterion to proceed to the next stage, i.e. 6 consecutive correct responses. CD, compound discrimination; ED: extra-dimensional shift; ID: intra-dimensional shift; Rev, reversal; SD, simple discrimination.

Weight analysis: from weaning to young adulthood (8 weeks post-weaning)

To determine whether prenatal treatment or housing affected growth, body weight was monitored from weaning until the end of the study. As half of the animals began to undergo food restriction for ASST from approximately eight weeks post-weaning, body weight data are only presented up until this point. There was a significant main effect of time ($F_{(8,592)}$= 15018.78, p=0.001) and time x treatment interaction ($F_{(8,592)}$= 7.10, p=0.001) but no between-group differences were evident until eight weeks post-weaning, when Poly(I:C)-GH gained significantly less weight than Veh-GH (Figure 3.19).

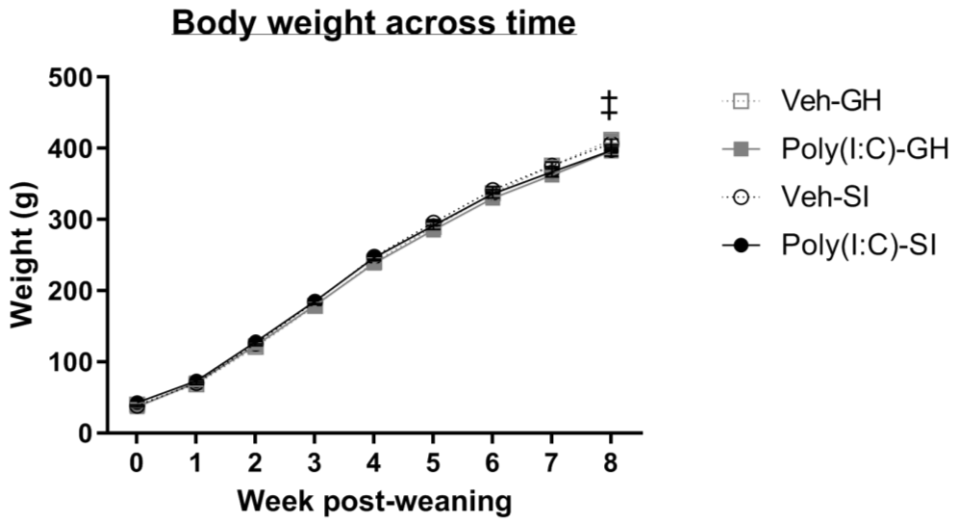


Figure 3.19. Effect of prenatal poly (I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on body growth from weaning till approximately eight weeks post-weaning. Data are mean ± SEM (n=18-20 per treatment x housing). ‡p<0.05 Poly(I:C)-GH compared to Veh-GH; three-way RM ANOVA with Tukey's post-hoc test.

Cytokine analysis

To investigate if cytokine changes accompanied behavioural changes, brain tissues were collected and analysed.

In the frontal cortex, there was a significant main effect of housing on IL-2 levels ($F_{(1,35)}$= 4.57; p<0.05), and a significant treatment x housing interaction on IL-1β ($F_{(1,35)}$= 11.78; p<0.01), IL-2 ($F_{(1,35)}$= 10.83; p<0.01), IL-6 ($F_{(1,35)}$= 19.04; p<0.001), IL-10 ($F_{(1,35)}$= 17.18; p<0.001), IL-12 ($F_{(1,33)}$= 13.44; p<0.001) and TNF-α ($F_{(1,36)}$= 11.90; p<0.01) levels. Gestational poly(I:C) exposure alone increased IL-1β and IL-10 levels whereas SI alone elevated IL-2, IL-10, IL-12 and TNF-α levels, compared to controls. SI-induced increases were prevented by prior poly(I:C) exposure (Figure 3.20B-E).

In the hippocampus, treatment and housing significantly influenced IL-1β ($F_{(1,36)}$= 5.78; p<0.05) and IL-2 levels ($F_{(1,34)}$= 4.58; p<0.05), respectively, but no between-group differences were found (data not shown).

In the striatum, there was a significant main effect of treatment on IL-4 levels ($F_{(1,34)}$= 4.65; p<0.05) and housing on IL-2 levels ($F_{(1,34)}$= 4.60; p<0.05), as well as a significant treatment x housing interaction on IL-1β ($F_{(1,35)}$= 4.60; p<0.05), IL-4 ($F_{(1,34)}$= 10.43; p<0.01) and IL-6 ($F_{(1,35)}$= 4.49; p<0.05) levels. Gestational poly(I:C) exposure increased IL-4 levels compared to Veh-GH (Figure 3.20F).

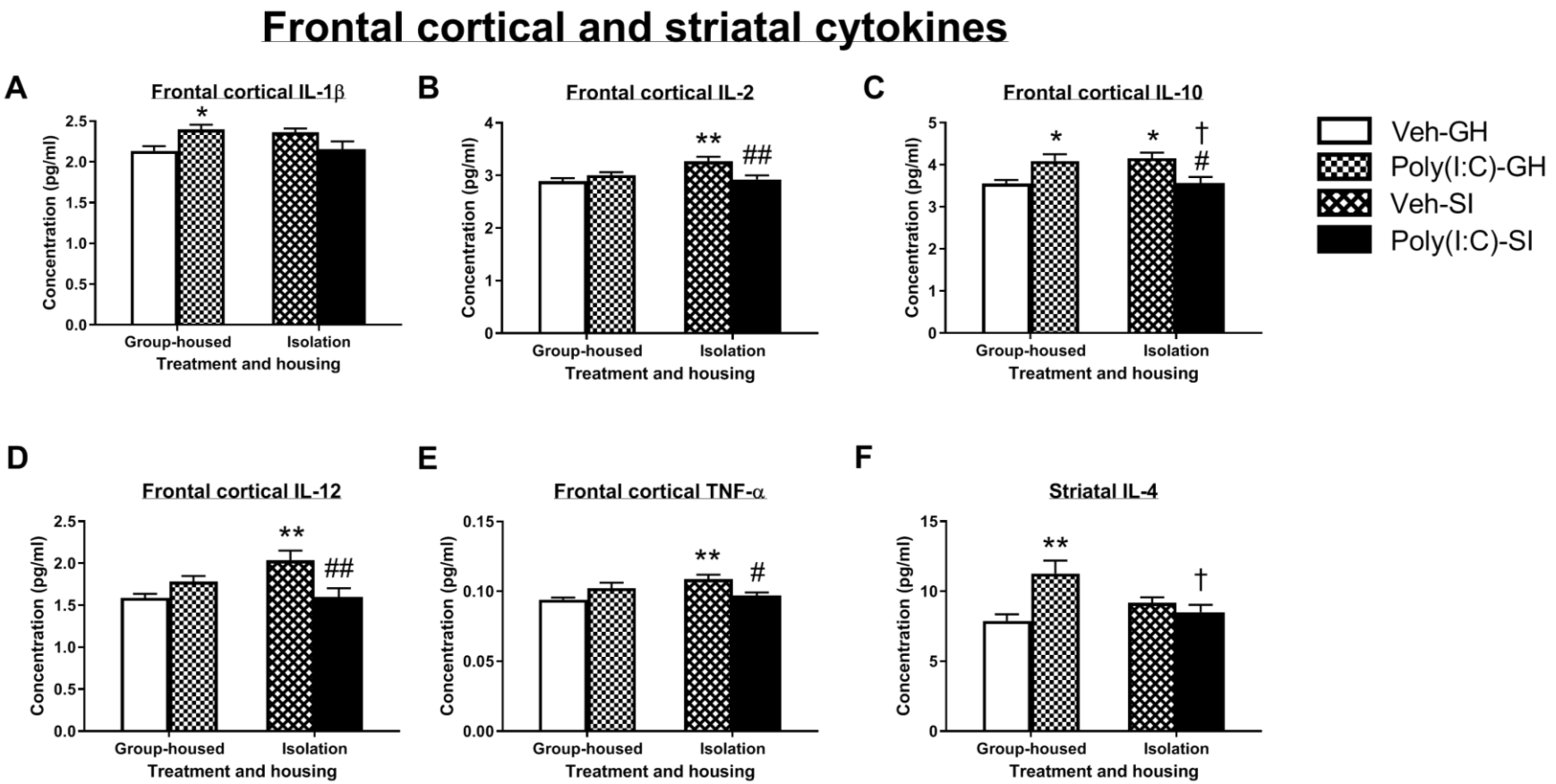


Figure 3.20. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on brain cytokines at around ten weeks post-weaning (postnatal day 90-93). Gestational poly(I:C) exposure alone and/or isolation alone caused elevation in several frontal cortical and striatal cytokines in male Lister-hooded rats. Data are mean ± SEM (n=9-10 per treatment x housing). *p<0.05, **p<0.01 compared to Veh-GH; #p<0.05, ##p<0.01 compared to Veh-SI; †p<0.05 compared to Poly(I:C)-GH; two way ANOVA with Tukey's post-hoc test.

mTOR analysis

To investigate if mTOR signalling was altered by developmental alterations, brain tissues were analysed using the 2-plex magnetic bead assay. In the striatum, housing significantly influenced phospho mTOR levels ($F_{(1,34)}$= 11.09; $p<0.01$). There was also a significant main effect of treatment (phospho, $F_{(1,34)}$= 6.17, $p<0.05$; total, $F_{(1,36)}$= 4.44, $p<0.05$), as well as a significant treatment x housing interaction (phospho, $F_{(1,34)}$= 8.63, $p<0.01$; total, $F_{(1,36)}$= 4.96, $p<0.05$) on mTOR levels. Poly(I:C) alone elevated both phospho and total mTOR levels in the striatum (Figure 3.21A & B). Neither treatment nor housing influenced phospho mTOR, total mTOR or the phospho:total mTOR ratio in the frontal cortex and hippocampus (data not shown).

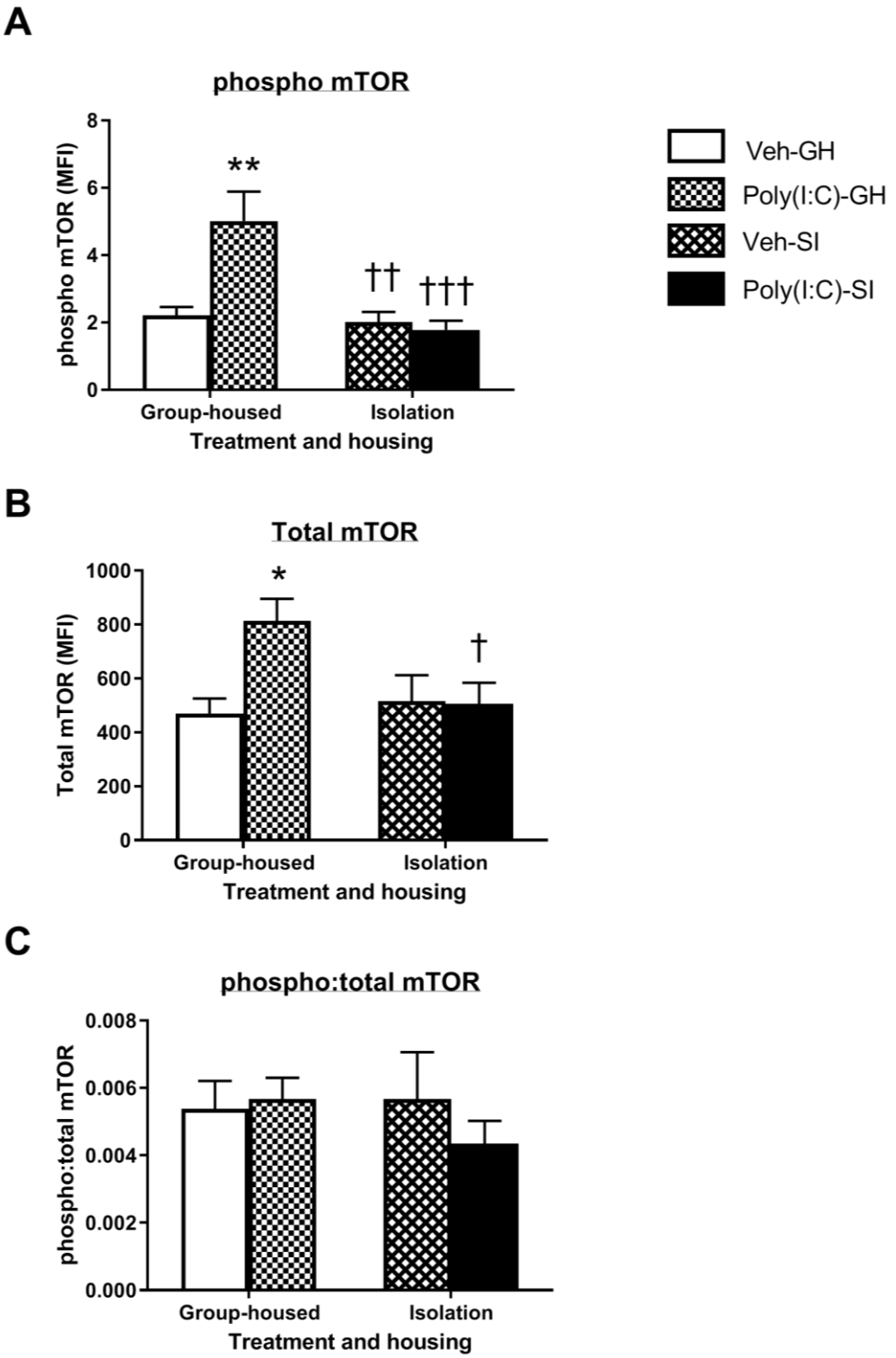


Figure 3.21. Effect of prenatal poly(I:C) exposure (6 mg kg^{-1}, i.v., gestational day 15) and/or post-weaning social isolation on mTOR levels in the striatum at ten weeks post-weaning (postnatal day 90-93). Mean ± SEM **(A)** phospho mTOR, **(B)** total mTOR and **(C)** phospho:total mTOR ratio (n=9-10 per treatment x housing). mTOR, mammalian target of rapamycin; phospho, phosphorylated. *p<0.05, **p<0.01 compared to Veh-GH; †p<0.05, ††p<0.01, †††p<0.001 compared to Poly(I:C)-GH; two-way ANOVA with Tukey's post-hoc test.

3.5 Discussion

In the current chapter, the aims were to identify an appropriate dose of poly(I:C) for i.v. administration to pregnant rodents, and to determine whether the selected dose would produce more robust effects on resultant offspring in combination with post-weaning SI. Both 4 mg kg^{-1} and 6 mg kg^{-1} poly(I:C) induced sickness behaviour in non-pregnant female rats. Whilst both doses caused a significant reduction in hippocampal total mTOR, only 4 mg kg^{-1} poly(I:C) altered frontal cortical cytokine levels. When administered to dams at GD15, 6 mg kg^{-1} poly(I:C) induced hypothermia, sickness behaviour (peak 3 h post-injection) and weight loss at 24 h, which returned to baseline levels by 48 h post-injection. At two weeks post-weaning, male and female Veh-SI and Poly(I:C)-SI were both hyperactive in a novel arena but this effect was more robust in females. In adulthood, in contrast to the protective effect conferred by i.p. poly(I:C) against several SI-induced behavioural alterations (Chapter 2), in the current chapter, i.v. poly(I:C) resulted in additive effects on novelty-induced hyperactivity and USVs when combined with SI but attenuated SI-induced cytokine elevations in the frontal cortex. These findings will now be discussed in the context of previous research on gestational poly(I:C) exposure via i.v. route of administration or post-weaning isolation rearing (not adult-only SI), and compared against the 'Poly(I:C)-SI' model generated in Chapter 2. The translational relevance of the two developmental interventions have been discussed in Chapter 2 and will not be addressed again.

3.5.1 Experiment 1: The effect of poly(I:C) via i.v. administration on non-pregnant Lister-hooded rats

No published studies have investigated the acute effects of i.v. poly(I:C) on non-pregnant female rats, preventing comparison with the current data. This study implies that 4 mg kg^{-1} might be the dose of choice as it induced more robust effects than 6 mg kg^{-1}, including significantly increased sickness score at more time points across the monitoring

period and significantly reduced frontal cortical IL-1β and IL-10. However, at the time when a dose had to be decided, a technical fault prevented the planned cytokine and mTOR analyses and therefore results did not become available until Experiment 2 had commenced. Although prior MIA rat studies served as a useful starting point, pregnancy in rats, akin to human, is associated with various adaptations leading to altered immune sensitivity (Cipolla et al., 2011; Faas et al., 2000; Svensson-Arvelund et al., 2014). Additionally, previous experiments have demonstrated distinct behavioural and pharmacological effects of the same drug in different rat strains (Cadoni et al., 2013; López-Rubalcava & Lucki, 2000; Porsolt et al., 1978). Therefore, predicting the effects of 4 mg kg^{-1} poly(I:C) on non-pregnant or even pregnant Lister-hooded females based on studies in pregnant rats of other strains is challenging, especially when experimental variables such as the source, housing environment and poly(I:C) batch variation also have a substantial influence (Kentner et al., 2019; Mueller et al., 2019). Taking these factors into consideration, and the previous finding that non-pregnant Lister-hooded rats were associated with less robust effects of poly(I:C) compared to Wistar rats (Murray et al., 2019), the higher dose of 6 mg kg^{-1} was selected for administration into the MIA model. Importantly, in the current study, no undesired effects were observed in rats injected with this dose. Moreover, no studies have explored the effects of 6 mg kg^{-1} poly(I:C) in the MIA rat model. Hence, it would be valuable to determine if this dose would result in more robust schizophrenia-related behavioural phenotypes in the resultant offspring.

3.5.2 Experiment 2: The combined effects of gestational poly(I:C) via i.v. administration and post-weaning social isolation on offspring behaviour and neurochemistry

3.5.2.1 Physiological or behavioural effects of poly(I:C) on dams

In the current study, 6 mg kg^{-1} poly(I:C) induced hypothermia, weight loss and sickness behaviour compared to saline-treated dams. All poly(I:C)-associated effects were normalised by 48 h post-injection. The

hypothermia observed in the current study agrees with a previous study which administered 5 mg kg^{-1} poly(I:C) to pregnant Wistar rats (Clark et al., 2019). Meanwhile, all but one study which administered 4 mg kg^{-1} poly(I:C) to time-mated females showed no effect on body temperature (Ballendine et al., 2015; Howland et al., 2012; Lins et al., 2018; Murray et al., 2017; Paylor et al., 2016; Sangha et al., 2014; Zhang et al., 2012). Clark et al. (2019) did not report the method of temperature monitoring used whereas all studies which administered 4 mg kg^{-1} poly(I:C) were conducted by the same research group and have assessed rectal temperature using the Homeothermic Blanket System from Harvard Instruments, USA. Apart from Lins et al. (2018) who used Sprague-Dawley rats, all other studies used Long-Evans rats and one study using Long-Evans rats found significantly increased rectal temperature in poly(I:C)-treated dams 8 h post-injection (Zhang et al., 2012). This confirms the current view that a wide spectrum of variables, including those related to experimental procedures, strain, housing and husbandry, can profoundly affect the impact of poly(I:C) immunogenicity in dams (Castelhano-Carlos & Baumans, 2009; Kentner et al., 2019; Mueller et al., 2019). Furthermore, varying behavioural responses have been recorded in different batches of rats acquired from the same vendor, as well as within a single batch (Fitzpatrick et al., 2013; Näslund et al., 2015). All of the mentioned poly(I:C) studies also anaesthetised dams with isoflurane prior to drug injection. As exposure to general anaesthesia has been linked to both hypothermia and hyperthermia, this may also influence the thermogenic impact of poly(I:C), regardless of the dose that was administered (Bindu et al., 2017; Joseph et al., 1982). The direction of temperature change in pregnant rats in the current experiment is in contrast with poly(I:C)-induced hyperthermia in non-pregnant females in the pilot study. This could be attributed to modified immune responses in pregnancy (discussed above). Reduced body temperature following poly(I:C) challenge in the current study matches the observation in Chapter 2.

Poly(I:C)-induced weight loss is consistent with several previous observations (De Felice et al., 2019; Howland et al., 2012; Zuckerman et al., 2003), although others found no effect with the same poly(I:C) dose (Meehan et al., 2017; Vernon et al., 2015; Wolff & Bilkey, 2010). These differences again could be due to variation in experimental procedures or the surrounding environment, or strain-or batch-specific response to poly(I:C) stimulation. In some studies, prenatal poly(I:C) treatment was performed by the vendor and the resultant offspring were only sent to research laboratories following weaning (Vernon et al., 2015; Yee et al., 2011; Yee et al., 2012). Transportation is associated with stress, causing alterations in not only behaviour (e.g. water intake, LMA, social interaction) but also physiological parameters such as body weight, heart rate and blood corticosterone levels, which could persist up till 16 days post-transportation (Arts et al., 2012; Capdevila et al., 2007; van Ruiven et al., 1998). Therefore, the lack of transportation stress during gestation could explain the absence of weight loss in the study by Vernon et al. (2015). Moreover, another study which performed in-house mating found no poly(I:C)-associated weight change in dams (Meehan et al., 2017). While maternal weight loss following i.p. or s.c. poly(I:C) challenge has been proposed as an indicator of successful MIA, such functional link has not been assessed in studies using i.v. poly(I:C) administration (Bronson et al., 2011; Missault et al., 2014). Nevertheless, if route of administration is not important in this regard, given the similar findings in studies using i.p. or s.c. administration, it is likely that maternal weight response does not accurately predict behavioural outcome of poly(I:C) in offspring, as maternal weight gain has been shown to induce a different set of behavioural abnormalities that are equally relevant to schizophrenia in offspring, when compared to maternal weight loss (Vorhees et al., 2012; Vorhees et al., 2015).

Only two studies have reported poly(I:C)-associated sickness behaviour. Following poly(I:C) injection (4 mg kg^{-1}, GD15), one study noticed piloerection and hunched posture in dams which normalised by 4 h post-injection, being in keeping with the current data. The same study also

revealed no effect of poly(I:C) on overt behaviour such as excessive grooming and restlessness (Yee et al., 2011). On the other hand, a separate study (5 mg kg^{-1}, GD15) found increased sickness behaviour (piloerection, lethargy and ptosis) in poly(I:C)-injected dams up until 24 h post-injection (Clark et al., 2019). Although maternal weight loss might not be an absolute predictor of successful MIA, the occurrence of cytokine-induced sickness behaviour (Dantzer, 2001) provided confidence that the MIA protocol had worked in the current study.

In contrast to Chapter 2, poly(I:C)-treated dams in the current study did not exhibit increased abdominal pinching compared to saline-treated dams. This implies that rather than an acute effect of poly(I:C), abdominal pinching in Chapter 2 may result from peritoneal irritation associated with i.p. injection.

As discussed in Chapter 2, project licence restrictions prevented cytokine data measurement from poly(I:C)-treated dams. Previous studies which administered 4-5 mg kg^{-1} poly(I:C) have observed significantly increased levels of pro-inflammatory cytokines and/or chemokines in the maternal blood at 2-3 h following poly(I:C) injection, regardless of the injection timing (GD9, 11, 15 or 20; Ballendine et al., 2015; Meehan et al., 2017; Song et al., 2011; Verdurand et al., 2014), and elevated corticosterone (Dalton et al., 2012; Hollins et al., 2016; Meehan et al., 2017). A recent study assessing the role of ISG15 (critical for inflammation and neural development) in MIA observed a significant increase in the maternal serum 12 h after 10 mg kg^{-1} poly(I:C) administration at GD17, which persisted until delivery day (Hu et al., 2019).

3.5.2.2 Effect of poly(I:C) on offspring

Akin to Chapter 2, poly(I:C) did not influence litter size, gender distribution within litters and pup body weight at weaning, consistent with most studies which administered poly(I:C) via i.v. administration (Gray et al., 2019; Vernon et al., 2015; Wolff & Bilkey, 2010; Zuckerman & Weiner,

2005). Conversely, other studies found undesired effects of poly(I:C) injection, including miscarriage, bloody discharge from vagina and abnormal offspring growth (De Felice et al., 2019; Howland et al., 2012; Lins et al., 2018; Yee et al., 2011). Both increased and decreased litter sizes have been reported, in addition to reduced and increased body weight of pups exposed to prenatal poly(I:C), at PND1 and PND2, respectively (Ballendine et al., 2015; Lins et al., 2018; Murray et al., 2017; Yee et al., 2011). Most studies have administered a single injection of 4 mg kg^{-1} poly(I:C) on GD15, however, factors such as the genetic background of animals, poly(I:C) administration conditions and methodological differences, e.g. blood collection following injection, could influence MIA intensity and hence pregnancy outcome (Kentner et al., 2019). Furthermore, environmental factors including light, sound, cage size and routine procedures such as cage cleaning and in-house transport could affect stress levels in rats, altering pregnancy and postnatal outcomes (Castelhano-Carlos & Baumans, 2009). The enhanced appreciation that numerous variables could impact MIA or pregnancy outcome led to the establishment of the MIA model reporting guidelines checklist, to ensure rigour and reproducibility of the model (Kentner et al., 2019). Lastly, exposure to 10 mg kg^{-1} poly(I:C) at GD17 resulted in increased ISG15 mRNA and protein in the PFC of GD17 embryos at 12 h post-exposure, and at birth (Hu et al., 2019).

3.5.2.3 Effect of poly(I:C) and post-weaning isolation rearing on offspring behaviour

The ’isolation syndrome’ is typically confirmed by assessing activity in a novel environment, as isolates consistently demonstrate exaggerated response under such circumstances from two weeks post-isolation commencement (section 1.4). In the current study, hyperactivity in a novel arena was observed in male and female isolates at two weeks post-weaning. SI increased different activity types in the two genders: fine movement and rearing in males, and ambulation and fine movement in females. While enhanced horizontal ambulation and rearing have been

demonstrated in isolates by previous studies (Bakshi & Geyer, 1999; Dalrymple-Alford & Benton, 1981; King et al., 2009; Fone et al., 1996) and effects of SI on fine movement are yet to be characterised, novelty-stimulated hyperactivity is not 100% reproduced across studies (Fone & Porkess; Okudan & Belviranli, 2017; Weiss et al., 2004; Table 2.21). Moreover, methodological differences including lighting and duration of isolation and evaluation, or strain-specific effects could influence locomotor response (Fone & Porkess, 2008; Robbins, 2016; Weiss et al., 2004). Taking these into consideration, the fact that both male and female isolates demonstrated hyperactivity, although not across all activity types, confirmed successful implementation of the SI protocol.

While Veh-SI and Poly(I:C)-SI rats of both genders demonstrated hyperactivity in adolescence, in contrast to Chapter 2, females were more robustly affected than males (Table 3.3). The finding that SI caused more robust effects on female rats is unexpected, as it is generally acknowledged that male rats are more prone to SI-induced alterations than female rats (Fabricius et al., 2010; Fone & Porkess, 2008; Weiss et al., 2001; Weiss et al., 2004). However, female rats have been associated with more robust SI-induced alterations in PPI, adrenal gland weight and social behaviour and oxytocinergic activity (Hatch et al., 1963; Powell et al., 2002; Tanaka et al., 2019). As the strain, animal source, housing and testing conditions, as well as experimenter, were identical to the i.p. study, differential locomotor outcomes in the two genders are potentially due to inherent variability between batches. Batch-to-batch variability within rats from the same source, as well as within-batch effects have been previously characterised (Näslund et al., 2015; O'Bryant et al., 2011). Moreover, despite efforts to standardise testing protocols and environment across three laboratories, laboratory-specific effects were found on behaviour in several tasks, including cocaine-stimulated activity and elevated plus maze (Crabbe et al., 1999). Although in our case both i.p. and i.v. studies were performed at the same site, the fact that there was roughly a year's gap between these two studies, it is possible that subtle differences in the testing environment,

Table 3.3. Summary of findings from resultant offspring in Chapters 2 and 3. Effects of poly(I:C) and post-weaning social isolation (SI), alone or in combination, on behaviour, neurochemistry, cytokines and mammalian target of rapamycin (mTOR) in adolescence. All findings are relative to Veh-GH.

	Locomotor activity (Adolescence)	Neurochemistry	Cytokines (Adolescence)	mTOR (Adolescence)
Chapter 2 (i.p.)				
Poly(I:C)-GH	**Males**: ↑ fine movement **Females**: ↓ ambulation	No effect on either gender.	**Males**: ↓ hippocampal IL-6 and striatal IFN-γ and TNF-α	No effect
Veh-SI	**Males**: ↑ ambulation, fine movement and rears **Females**: ↑ rears	**Males**: ↑ striatal 5-HIAA	**Males**: ↑ frontal cortical IL-18	**Female**: ↑ hippocampal phospho mTOR
Poly(I:C)-SI	**Males**: ↑ ambulation, fine movement and rears **Females**: ↑ fine movement	**Males**: ↑ striatal 5-HIAA and dopamine	**Males**: ↓ striatal IFN-γ and TNF-α	**Female**: ↓ frontal cortical total mTOR
Chapter 3 (i.v.)				
Poly(I:C)-GH	**Females**: ↑ or ↓ fine movement	-	-	-
Veh-SI	**Males**: ↑ fine movement and rears **Females**: ↑ ambulation and fine movement	-	-	-
Poly(I:C)-SI	**Males**: ↑ ambulation, fine movement and rears **Females**: ↑ ambulation, fine movement and rears	-	-	-

e.g. replacement of new light bulb in testing room, resulted in behavioural differences. Seasonality, which can affect shipping conditions and laboratory context, has also been reported to influence training between batches of rats in a reaching task (O'Bryant et al., 2011). Both the current study and i.p. studies were carried out around winter and thus seasonal differences were unlikely to have significantly contributed to the disparity in findings. Poly(I:C) alone had minimal effect on females and did not

influence novelty-induced locomotor response in males. Previous findings on the impact of gestational poly(I:C) exposure on spontaneous or novelty-induced LMA have been mixed, with hypoactivity, hyperactivity and no change all being described (Gray et al., 2019; Li et al., 2018; Van den Eynde et al., 2014; Zavitsanou et al., 2014; Zuckerman et al., 2003). In particular, increased total LMA was observed in MIA offspring in young adulthood (PND57-58) but not pre-puberty (PND36-37; Howland et al., 2012). This could well reflect the modest or lack of poly(I:C) influence on LMA in adolescent females and males, respectively, in the current study. As discussed in Chapter 2, SI-induced hyperactivity is associated with aberrant dopaminergic and serotonergic activity particularly in mesolimbic neurons. The lack of data on brain monoamine levels in the current study, therefore limits comprehensive characterisation of the neurochemical changes in rats exposed to either developmental intervention alone or combined. As the purpose of this study was to determine if poly(I:C) (i.v.) generated a more robust model compared to poly(I:C) (i.p.), when combined with SI, only male rats were used in subsequent studies.

In adulthood, SI produced very subtle changes in fine movement and rearing in a novel arena whereas combined poly(I:C)-SI consistently increased all three activity types. As stated above, the reproduction of hyperactivity in a novel arena is not absolute. Therefore, despite only modest increases in fine movement and rearing were observed, such observation confirmed successful development of the ‘isolation syndrome’. Poly(I:C) did not exert any noticeable effects on its own. Strain, methodological and environmental differences (see above) possibly contributed to such lack of effect, as previous studies reported elevated or reduced spontaneous or novelty-stimulated LMA (Gray et al., 2019; Li et al., 2018; Van den Eynde et al., 2014; Zavitsanou et al., 2014; Zuckerman et al., 2003). Unexpectedly, poly(I:C) interacted with SI to cause robust increases in all activity types, thus showing potential as a novel ‘dual-hit’ model. This interactive effect is in contrary with the resilience effect seen with the i.p. poly(I:C)-SI model (Table 3.4), where

Table 3.4. Summary of findings from resultant offspring from Chapters 2 and 3. Effects of poly(I:C) and post-weaning social isolation (SI), alone or in combination, on behaviour, cytokines, mammalian target of rapamycin (mTOR) and body weight in adulthood. All findings are relative to Veh-GH. In Chapter 2, poly(I:C)-SI promoted resilience against SI-induced changes (blue: significant difference compared to Veh-SI, bold(black): significant changes in the equivalent measure in Veh-SI). In Chapter 3, poly(I:C)-SI resulted in additive effects on several behavioural components (red: significant increase compared to Veh-SI) and promoted resilience against SI-induced cytokine alterations. LMA, locomotor activity; NLD, novel location discrimination; NOD, novel object discrimination; PPI, pre-pulse inhibition of acoustic startle; USV, ultrasonic vocalisation.

	LMA	NOD	NLD	Social interaction	USVs	PPI
Chapter 2 (i.p.)						
Poly(I:C)-GH	↑ rears	No impairments	No impairments	↑ pinning	Narrower classification: ↑ upward ramp	No effect
Veh-SI	↑ rears	No impairments	No impairments	↓ anogenital sniffing, ↑ body sniffing, crawling over and under, boxing and biting, and total distance travelled	↑ 50-kHz calls Three main call types: ↑ flat, step and trill Narrower classification: ↑ trill, multi-step, downward ramp	No effect
Poly(I:C)-SI	**No effect**	No impairments	No impairments	↓ anogenital sniffing, ↑ body sniffing and crawling over and under, **no effect on total distance travelled (↓ boxing and biting)**	**No effect**	No effect
Chapter 3 (i.v.)						
Poly(I:C)-GH	No effect	No impairments	No impairments	No effect	No effect	No effect
Veh-SI	↑ fine movement and rears	No impairments	No impairments	↓ anogenital sniffing, ↑ body sniffing	↑ 50-kHz calls Three main call types: ↑ flat, step and trill Narrower classification: ↑ flat, short, multi-step, trill and step down	No effect
Poly(I:C)-SI	↑ **ambulation**, fine movement and **rears**	No impairments	No impairments	↓ anogenital sniffing, ↑ body sniffing, ↑ crawling over and under	↑ 50-kHz calls Three main call types: ↑ **flat** and step Narrower classification: ↑ **flat**, short, multi-step, **step up**, **upward ramp**, step down and inverted-u	No effect

Table 3.4. Summary of findings from resultant offspring from Chapters 2 and 3 (continued).

	Conditioned freezing response	Attentional set-shifting	Cytokines	mTOR	Body weight
Chapter 2					
Poly(I:C)-GH	No effect	No effect	↑ striatal IL-2, IL-4, IL-12, IFN-ϒ and TNF-α	↑ frontal cortical phospho and total mTOR	No effect
Veh-SI	↓ cued freezing 24 h after acquisition	Found Reversal 1 significantly harder than compound discrimination; required more trials to reach criterion in Reversal 1	↑ frontal cortical IL-1β, IL-2, IL-6, IL-10, IL-12 and TNF-α	↑ frontal cortical phospho and total mTOR, and phospho:total mTOR ratio	↑ body weight at several time points across development
Poly(I:C)-SI	**No effect on cued freezing (↑ cued freezing)**	**No effect**	**↓ frontal cortical IL-6, IL-10 and IL-12, no effect on other frontal cortical cytokines**	**No effect on mTOR levels (↓ frontal cortical phospho mTOR and phospho:total mTOR)**	↑ body weight at several time points across development
Chapter 3					
Poly(I:C)-GH	No effect	↑ errors in extra-dimensional shift compared to intra-dimensional shift	↑ frontal cortical IL-1β and IL-10 and striatal IL-4	↑ striatal phospho and total mTOR	↓ body weight at around eight weeks post-weaning
Veh-SI	Impaired acquisition	No effect	↑ frontal cortical IL-2, IL-10, IL-12 and TNF-α	No effect	No effect
Poly(I:C)-SI	Slower acquisition	No effect	**No effect on cytokines (↓ frontal cortical IL-2, IL-10, IL-12 and TNF-α)**	No effect	No effect

i.p. poly(I:C) prevented SI-induced increase in rearing. The discrepant findings can be attributed to procedural differences associated with i.v. versus i.p. administration of poly(I:C). For example, the necessity to restrain dams in a device prior to i.v. administration could produce more severe gestational stress, altering interaction between poly(I:C) and SI and hence outcome in offspring.

Akin to the i.p. study, rats from all groups demonstrated intact object recognition memory in the NOD task. While single housing of rats from weaning has been shown to induce working memory deficits, such deficits are not always observed in every cohort (Bianchi et al., 2006; Dunphy-Doherty et al., 2018; Fone & Porkess, 2008; McLean et al., 2010; Table 2.21). Contrary to the current finding, gestational poly(I:C) exposure has been linked to reduced novel object preference in rats indicative of impaired recognition memory (Gray et al., 2019; Luchicchi et al., 2016). Another study further revealed that lowered novelty preference was due to defective memory (Wolff et al., 2011). In contrast, Howland et al. (2012) failed to observe poly(I:C)-associated memory deficits. Given that neither SI alone nor poly(I:C) alone altered the discrimination index, it is not surprising that the combination did not induce NOD deficits.

Spatial memory in the NLD task was not influenced by poly(I:C) alone, SI alone or combined poly(I:C)-SI, as observed in the i.p. study. No known studies have examined the impact of SI on spatial working memory in a NLD paradigm, but mixed findings have been published in regard to the performance of isolates in other spatial memory tasks, including the MWM and Y-maze task (Shao et al., 2015; Wongwitdecha & Marsden, 1996a). Rats exposed to gestational poly(I:C) (4 mg kg^{-1}, GD15), on the other hand, had intact object location memory when assessed in a similar NLD task to the current study with a 24 h ITI (Howland et al., 2012).

In this study, SI induced alterations in pro-social behaviour regardless of prenatal treatment. Poly(I:C)-SI rats showed increased crawling over and

under which was not evident in Veh-SI rats. SI-induced alteration in anogenital sniffing is consistent with previous studies (Möller et al., 2011; Regenass et al., 2018; Strauss et al., 2014). The same studies also reported significantly reduced staying together in SI rats, which contradicts the current findings on lying side by side. Possible reasons for the differences in behaviours observed have been discussed in Chapter 2. Consistent with the i.p. study, SI alone had no effect on total interaction in the current study, but rather produced subtle changes in component behaviours, showing importance of thorough analysis of ethological behaviour. Other studies have reported reduced or increased total social interaction in isolates (Grotewold et al., 2014; Kokare et al., 2010). The current study also observed no effect of MIA on total time spent in social interaction. Whilst this is backed by a previous finding (Gray et al., 2019), in several other studies, prenatal poly(I:C) exposure was associated with reduced social interaction in pairs of unfamiliar rats (Lucchichi et al., 2016; Osborne et al., 2017). Moreover, in a sociability task where rats had a choice to explore between a cage with a stranger rat or an empty cage, offspring of poly(I:C)-treated dams spent less time exploring the former, suggestive of sociability deficit (Lins et al., 2018). Although poly(I:C) and SI did interact during social interaction to significantly increase crawling over and under, unlike the corresponding i.p. study, the current study did not observe SI-induced increased aggression in the form of boxing and biting, or poly(I:C)-induced increase in pinning. Abnormal aggression has been repeatedly observed in isolates and therefore the lack of replication of such behavioural alteration in the current study again shows that behavioural abnormalities are not always reproducible across experiments (Oliveira et al., 2019; Tulogdi et al., 2014; Wongwitdecha & Marsden, 1996b). Moreover, the current data suggests that i.v. administration of poly(I:C) may result in less robust social behavioural alterations compared to i.p. administration.

In terms of USVs, prenatal poly(I:C) on its own had no effect whereas SI increased USVs regardless of prenatal treatment. Consistent with the current results, increased tickle and social contact-induced pro-social

vocalisations occur in rats housed singly since weaning (Burgdorf & Panksepp, 2001; Knutson et al., 1998). Alterations in social communication in isolates have also been demonstrated, including impaired social and reward cue recognition (Inagaki et al., 2013; Seffer et al., 2015; von Frijtag et al., 2002). In the poly(I:C) model, impaired social communication was reported in PND11 pups, as they produced significantly less USVs (particularly long calls) when separated from their respective dams (Chou et al., 2015b). When assessed during a fear conditioning session in adulthood, rats exposed to prenatal poly(I:C) exhibited altered affective communication such that there was increased emission of aversive 22-kHz USVs that were shorter in duration and occurred more per bout (Yee et al., 2012). While SI-induced increase in USVs complements findings of Chapter 2, unlike Chapter 2, the present experiment observed no effects of poly(I:C) on USVs. Combined poly(I:C)-SI caused more robust increase in USVs compared to SI alone, complementing the additive effects observed in LMA in adulthood.

Similar to Chapter 2, neither gestational treatment nor housing influenced PPI in the current experiment. Given that PPI deficits have been widely described in the SI model, the lack of such phenomenon in the current study is unexpected (Jones et al., 2011a; Ko & Liu, 2016; Marsden et al., 2011; Möller et al., 2011; Schubert et al., 2009; Uys et al., 2016). Nevertheless, in Lister-hooded rats in particular, SI-induced PPI deficits appear to be less robust and reproducible compared to locomotor and NOD alterations (Fone & Porkess, 2008; Table 2.21). The absence of MIA influence on PPI was unpredicted as most MIA studies reported disrupted PPI in rats born to poly(I:C)-treated dams (Howland et al., 2012; Luchicchi et al., 2016; Wolff & Bilkey, 2010; Yee et al., 2011). Given that there are also studies which failed to observe altered PPI, it is possible that PPI deficits represent a phenotype that is not consistently reproduced in the poly(I:C) model (Gray et al., 2019; Lins et al., 2018; Van den Eynde et al., 2014). As neither intervention conferred any effects independently, it is reasonable that the combined approach did not cause any sensorimotor gating alterations.

SI impaired acquisition regardless of prenatal treatment but did not affect contextual and cued-mediated learning and memory. Albeit impaired acquisition is consistent with a recent SI study, mixed findings have been published for the responses of isolates in both the acquisition and retention phases of the CFR task (Dunphy-Doherty et al., 2018; Gaskin et al., 2014; Jones et al., 2011a; McIntosh et al., 2013; Weiss et al., 2004). The lack of robust replication of CFR deficits could therefore explain the discrepant CFR findings in isolates in the current and i.p. studies. In a classical non-discriminative auditory cue paradigm, prenatal poly(I:C) exposure was associated with normal freezing responses during acquisition and extinction phases but abnormal contextual freezing during extinction recall (Sangha et al., 2014). Using a more advanced paradigm that required behavioural flexibility, the same group showed impaired fear behaviour regulation in offspring of poly(I:C)-injected dams. Nonetheless, a further study which utilised a fear conditioning protocol consisting of habituation, fear conditioning and fear testing on days 1, 2 and 3 respectively, failed to notice behavioural alterations at any stage in rats prenatally exposed to poly(I:C) (Yee et al., 2012). Inconsistent results on the impact of maternal poly(I:C) challenge on similarly fear-motivated tasks to assess latent inhibition in resultant offspring also exist (Song et al., 2011; Zuckerman & Weiner, 2005). With such varying effects of prenatal poly(I:C) exposure, the lack of poly(I:C) effect on freezing behaviour in the current paradigm is unsurprising.

Poly(I:C) alone but not SI or combined poly(I:C)-SI impaired learning in the ASST. As opposed to the current findings, impaired set-shifting has been described in socially-deprived rats (McLean et al., 2010; Powell et al., 2015; Schijver et al., 2004). In the poly(I:C) model, one study reported a selective deficit in reversal learning (Wallace et al., 2014). A second study which administered the same poly(I:C) dose to dams found that resultant male offspring made fewer regressive errors during reversal learning, and demonstrated blunted set-shifting due to increased perseverative errors, when compared to saline-exposed offspring (Zhang et al., 2012). In an operant set-shifting task, prior poly(I:C) exposure has

been linked to either increased or reduced regressive errors during set-shifting, and unaltered reversal learning (Ballendine et al., 2015; Lins et al., 2018). MIA-associated improvement in reversal learning is interestingly supported by two other studies employing a position discrimination and reversal task (Wolff et al., 2011; Zuckerman & Weiner, 2005). The current ASST findings are inconsistent with Chapter 2, which revealed selective impairment in reversal learning in isolates.

Maternal poly(I:C) treatment significantly reduced body weight gain in resultant offspring at approximately eight weeks post-weaning. By comparison, SI had no effect on body weight gain, although previous studies have suggested SI-induced excessive eating, potentially as a consequence of inactivity due to altered food motivation or leptin-hypothalamic axis activity, or lack of conspecifics (Hall et al., 1998; Hermes et al., 2011; Mainardi et al., 2010; Morgan, 1973; Shelley, 1965). However, weight changes are generally modest with SI (Fone & Porkess, 2008; Smith et al., 1997; Weiss et al., 2004). Although poly(I:C)-based MIA has been shown to either decrease or increase post-weaning weight gain in male offspring, such changes did not reach post-hoc significance (Vernon et al., 2015; Wallace et al., 2014). Other studies found no effect of poly(I:C) on offspring growth in puberty or adulthood (Meehan et al., 2017; Verdurand et al., 2014; Yee et al., 2011). Poly(I:C) exposure does not appear to alter total food or water intake in late adolescence or adulthood (Osborne et al., 2017).

Poly(I:C) alone and SI alone increased frontal cortical pro- and anti-inflammatory cytokine levels. Poly(I:C) alone also increased striatal IL-4 levels. The pattern of cytokine changes in the current study is distinct to that observed in Chapter 2, where SI and poly(I:C) exclusively elevated cytokine levels in the FC and striatum, respectively. Akin to the i.p. study, the current data supports a general activation of the immune system associated with SI. Although a previous study has suggested abnormal behaviour in adulthood as an outcome of reduced anti-inflammatory signalling (Corsi-Zuelli et al., 2019), other studies have found increased

pro-inflammatory cytokine levels in the plasma and hippocampus of adult isolates (Ko & Liu, 2016; Wang et al., 2017a). A further study reported reduced hippocampal IL-6 and IL-10 in adult isolates (Dunphy-Doherty et al., 2018). In the poly(I:C) model, elevated *TNF-α* mRNA levels, as well as reduced anti-inflammatory *IL-4* and *IL-10* mRNA levels have been observed in the brain of adolescent but not adult MIA offspring (Clark et al., 2019). Meanwhile, assessment of *TNF-α* and *IL-1β* gene expression in the cingulate cortex of adult offspring revealed no poly(I:C)-related altered expression regardless of poly(I:C) exposure at GD10 or GD19 (Duchatel et al., 2018b). It is interesting that poly(I:C)-SI did not alter cytokine levels in the brain regions investigated, given that this combination interacted to alter LMA, social interaction, USVs and acquisition of CFR. As poly(I:C)-SI conferred an additive effect on adulthood LMA and USVs compared to SI alone while promoted resilience towards SI-induced elevation in cytokines, it is likely that in this model, immune changes in adulthood is unrelated to the observed behavioural changes.

Gestational poly(I:C) alone but not SI altered mTOR levels in the current study. Intact mTOR signalling in isolates is unexpected, as mTOR is implicated in the synthesis of pro-inflammatory and anti-inflammatory cytokines (Weichhart et al., 2015), which were altered in the current study. Nevertheless, cytokine expression is also governed by transduction mechanisms involving mediators such as NF-κB, the MAPKs JNK and p38, as well as janus kinases and signal transducers and activators of the transcription family of proteins (Turner et al., 2014). In isolates, altered expression of several components of the JNK and p38 MAPK signalling pathways has been characterised (Shortall et al., 2018). Hence, it is possible that in the current study, aberrant behaviour and cytokine regulation occurs through a non-mTOR-dependent mechanism. The present study also suggests mTOR-mediated behavioural and cytokine alterations in rats exposed to MIA. No prior studies have probed the impact of gestational poly(I:C) challenge on mTOR signalling. However, one study showed that NF-κB inhibition could suppress poly(I:C)-induced

MIA and partially rescue MIA-associated behavioural alterations in offspring (Song et al., 2011). Another study demonstrated upregulation of ISG15 which paralleled suppressed NEDD4/Rap2A signalling in offspring exposed to gestational MIA (Hu et al., 2019). ISG15, an ubiquitin-like protein that binds LFA-1 integrin receptor, promotes IFN-Ɣ and IL-10 secretion through activation of Src family kinases (Swaim et al., 2017).

As indicated above, poly(I:C)-SI did not alter cytokine or mTOR signalling and therefore behavioural alterations are most likely independent of immune regulation. A potential alternative mechanism underlying behavioural changes in this model is the dysregulation of HPA axis, as apart from stress response, the HPA axis is also involved in cognition and social functioning (Fairchild et al., 2018; Wingenfeld & Wolf, 2010). Apart from LMA, alterations associated with poly(I:C)-SI, i.e. social interaction, USVs and acquisition during CFR, are related to either cognition or social functioning. The HPA axis is also known to interact with mesolimbic dopaminergic system (Wand et al., 2007) and thus might account for the enhanced LMA observed in Poly(I:C)-SI rats. Cytokine and/or behavioural alterations in Veh-SI rats can also be ascribed to disruption in the HPA axis. In particular, altered HPA axis stress response have been demonstrated in isolates (Serra et al., 2005; Weiss et al., 2004). Moreover, the HPA axis has immunomodulatory effects (section 1.3.6).

3.5.2.4 Conclusion and future direction

Poly(I:C) administration (6 mg kg^{-1}, i.v., GD15) induced hypothermia, weight loss and sickness behaviour in pregnant Lister-hooded dams compared to saline-treated controls. Contrary to the protective effect conferred by i.p. gestational poly(I:C) exposure against several SI-induced adulthood behavioural alterations (Chapter 2), i.v. poly(I:C) in combination with SI resulted in additive effects on behaviour relevant to the positive (LMA) and negative or cognitive (USVs) domains of schizophrenia. Combined i.v. poly(I:C) and SI was also associated with

abnormal learning in the CFR paradigm. Behavioural abnormalities in the poly(I:C)-SI model most likely occured via a non-cytokine and non-mTOR-dependent mechanism. A non-mTOR-dependent pathway was likely responsible for the behavioural and/or cytokine changes seen in rats exposed to SI alone. In rats exposed to poly(I:C) alone, such alterations were likely mediated by mTOR-dependent signalling. One alternative mechanism which could underlie the observed abnormalities in the poly(I:C)-SI and Veh-SI models is HPA axis dysregulation.

The promising potential of the i.v. poly(I:C)-SI model warrants further development and validation. However, due to the requirement of the Nottingham-Monash Joint PhD programme, the third year of study was spent at Monash University. Given that SI produced more robust changes compared to poly(I:C), in the next chapter, we decided to focus on the effects of post-weaning SI of rats on cortical gene expression.

CHAPTER 4

The effect of post-weaning social isolation on cortical gene expression

4.1 Introduction

Both the preceding experimental chapters reveal a more substantial impact of post-weaning SI than gestational poly(I:C) alone or in combination, on behaviour, neurochemistry or cytokine levels in Lister-hooded rats. Therefore, in this chapter, we decided to focus on the post-weaning SI model.

Since its implementation in the 1970s, various aspects of the SI rat model have been robustly investigated (Nishikawa et al., 1976). These range from neuroanatomy and major neurotransmitter systems (e.g. neurotransmitter levels and receptor density) to metabolic changes and behaviour, and encompass themes such as oxidative stress, endocrinology and circadian behaviour (Fone & Porkess, 2008; Lapiz et al., 2003; Shao et al., 2015; Teo et al., 2017; Weiss et al., 2004). Multiple studies have reported the impact of SI on gene expression of functional proteins including receptors and signalling mediators, as well as expression of IEGs and epigenetic regulation (Iwata & Yamamuro, 2016; Ko & Liu, 2016; Levine et al., 2007; Li et al., 2016; Wang et al., 2017a). Nevertheless, only a handful of genes, typically those directly relevant to the specific research interests, are investigated at a time. No group has yet probed the effect of isolation since weaning on the whole transcriptome. Given that SI of rats results in various behavioural and neurochemical abnormalities which are relevant to schizophrenia (section 1.4), altered gene expression associated with this model would provide valuable insight into the biological basis of neuropsychiatric disorders such as schizophrenia, which could hopefully drive the development of novel therapies through a targeted pharmacogenomics approach (Mirnics et al., 2001). The current study, therefore, aims to capture the genetic effects of post-weaning isolation rearing in rats by performing whole-transcriptome analysis with total RNA-Seq. The initial aim was to also examine the effects of SI on D_3 receptor density by radioligand binding, given the predominant localisation of such receptors in the limbic striatum and their potential involvement in cognition

(Nakajima et al., 2013). However, binding work was not pursued due to high non-specific binding (NSB) with the radiolabel [^{3}H]-7-hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin ([^{3}H]7-OH-DPAT; Appendix 1).

RNA-Seq, which uses the start-of-the-art next generation sequencing technologies, has been utilised to infer gene expression changes in disorders such as schizophrenia, both in post-mortem tissue and pluripotent stem cells used for modelling the disorder (Li & Teng, 2015; Wu et al., 2017). This high-throughput sequence-based approach confers many advantages compared to the conventional hybridisation-based DNA microarrays and tag-based sequencing approaches. These include a relatively quick turnaround time, low RNA requirement and background noise, high reproducibility, unbiased transcriptome analysis with single-base resolution and a large dynamic detection range (Li & Teng, 2015; Wang et al., 2009). Besides global expression changes, RNA-Seq, unlike microarrays, permits the detection of splicing events, disease-related SNPs and novel or rare transcripts, and could inform knowledge on transcriptional start sites and regulatory elements (Han et al., 2015; Wang et al., 2009). Whilst the cost of RNA-Seq is relatively low in comparison to its predecessors, it is still more costly than many other molecular techniques, costing up to a few thousand dollars (Li & Teng, 2015). Thus, for cost purposes and time involved in data analysis, it is vital to select an appropriate brain region which could most potentially reflect schizophrenia-related changes for such transcriptome analysis.

In the previous two chapters, SI induced locomotor hyperactivity in response to novelty, altered social behaviour and impaired learning, all of which are supported by previous studies in rats (Bakshi & Geyer, 1999; Dunphy-Doherty et al., 2018; Inagaki et al., 2013; McLean et al., 2010; Möller et al., 2011). In rats, SI-induced locomotor hyperactivity and social impairments are accompanied by abnormalities in the PFC, such as decreased presynaptic serotonergic function, and altered expression of synaptic-related proteins (postsynaptic density protein 95 and synapsin I) and glutamate receptor subunits (GluR1 and NR1), respectively

(Bickerdike et al., 1993; Hermes et al., 2011). Also considering the fact that in Chapters 2 and 3, isolates showed deficits in both CFR and ASST, behavioural paradigms involving PFC-mediated associative learning and cognitive flexibility, respectively, it is clear that PFC dysfunction is a key region in schizophrenia pathogenesis (Birrell & Brown, 2000; Rozeske et al., 2015). In patients with schizophrenia, deficits in various PFC-mediated functions have been characterised, including problem solving, attention, motivation and social cognition, together with structural and cellular changes such as thinning of the cortex and reduced oligodendroglial density (Jones et al., 2011b; Knöchel et al., 2016; Szczepanski & Knight, 2015; Uranova et al., 2004). Moreover, perturbations in frontal cortical cytokine levels and/or mTOR signalling observed in the previous chapters have been previously demonstrated in the SI model, and mirror clinical findings (Ko & Liu, 2016; Meffre et al., 2012; Paterson et al., 2006; Ryskalin et al., 2018). Thus, collectively, these prompted our selection of the PFC as an appropriate brain region for RNA-Seq analysis in the current study.

Whilst experiments in Chapters 2 and 3 were conducted using Lister-hooded rats, the current study utilises Sprague Dawley rats. This was due to the unavailability of the Lister-hooded strain in Australia, where the current study was conducted. Similar to Lister-hooded, Sprague Dawley is an outbred rat and has been widely used in the characterisation of the SI model (Hofford et al., 2018; Weiss et al., 2004). However, strain differences have been reported for certain SI-induced behavioural abnormalities. For example, Sprague-Dawley rats showed more persistent PPI deficits compared to Lister-hooded rats whereas Lister-hooded rats exhibited more pronounced hyperactivity in a novel environment compared to Sprague-Dawley rats (Weiss et al., 2000).

In the SI model, rats subjected to weeks of single housing are typically examined for LMA changes to confirm the isolation syndrome before behavioural evaluations or tissue collection for further analysis (Fone & Porkess, 2008; Gaskin et al., 2016). In the current study, however,

behavioural characterisation was not performed due to unavailability of facilities for rat behavioural testing at Monash Institute of Pharmaceutical Sciences. Although this reflects a potential caveat, significantly greater weight gain (see later) and increased defensiveness and reactivity to handling were observed in isolates, thus providing support that the SI protocol has worked (Fone & Porkess, 2008; Tulogdi et al., 2014).

A typical RNA-Seq workflow comprises RNA sample preparation, complementary DNA (cDNA) library construction, sequencing and bioinformatics analysis (Han et al., 2015; Wu et al., 2017). Following sequencing, a quality check is typically performed to ensure low-quality sequences, adaptor contaminations and overrepresented sequences are eliminated, prior to mapping of sequences to a reference genome and downstream differential gene expression and functional pathway analyses (Han et al., 2015). Various methods or tools have been developed for each of these steps and selecting the appropriate method or tool to employ depends on the sample quality, sequencing system, RNA subtype of interest and experiment objective (Han et al., 2015). The selection of the method or tool(s) used for each process in the current experiment is justified in the methods section.

We hypothesised that post-weaning SI of rats would result in altered gene expression in the PFC compared to GH rats.

4.2 Aims

The current experiments were designed to:

(1) profile cortical genetic alterations associated with post-weaning isolation rearing in Sprague Dawley rats;

(2) identify the molecular functions and pathways of differentially expressed genes (DEGs) in the SI model relative to GH controls and determine their translational relevance to schizophrenia.

4.3 Materials and methods

4.3.1 Animals

Sixteen male Sprague Dawley rats (from four different dams) were acquired from Monash Animal Research Platform and were 22-24 days old on arrival at the Monash Institute of Pharmaceutical Sciences Animal Facility, Australia. Rats were either housed individually or in a group of four in open-top cages (floor area 1500 cm^2) with bedding (Breeders Choice cat litter, FibreCycle). GH rats were from two different dams and were housed with a mix of littermates and non-littermates. SI rats originated from the two other dams. GH rats were provided with an empty tissue box and shredded paper in the home cage whereas SI rats did not receive any form of environmental enrichment. Food (Barastoc WEHI Mouse Breeder Cubes, Ridley AgriProducts) and drinking water were available *ad libitum*. All rats were kept in the same holding room on a 12:12-h light-dark cycle (lights on 07:00-19:00 h), at room temperature of 21 ± 3ºC and relative humidity 40-70%. All rats received minimal handling, during weekly cage cleaning (twice a week for GH animals whereas once per week for SI) and weight measurements. The study conformed to the Australian code for the care and use of animals for scientific purposes 8th edition (2013) and the Prevention of Cruelty to Animals Act (1986) and Regulations (2008) and was approved by the local animal ethics committee (approval number MIPS.2017.48) under the licence number SPPL20181.

4.3.2 Experimental design

Following six weeks of housing (PND63-66), rats were subjected to terminal anaesthesia with 5% isoflurane. The head was removed using a guillotine and the PFC rapidly collected, snap frozen on dry ice and stored at -80^0C.

4.3.3 Total RNA extraction and purification

Total RNA extraction and purification was carried out using Qiagen RNeasy® Mini Kit (Qiagen, Cat.#74104) according to the protocol provided (Part 1 and Part 2; version January 2011). Total RNA was extracted from the PFC of nine rats (n=4 per housing; one used for preliminary RNA quality assessment). Of these rats, three of the four GH rats were littermates whereas SI rats comprised two pairs of littermates. For preliminary RNA quality assessment, a purified RNA sample was selected at random from the available samples. RNA quality was determined using two fluorescence-based methods, capillary gel electrophoresis (AATI Fragment Analyzer) and Invitrogen Qubit assay, of which outputs have been shown to correlate with each other (Advanced Analytical, 2018). Following confirmation of suitable RNA quality, identical evaluation was carried out using the remaining eight samples. The total RNA was at least 200 ng and with a RNA integrity number of above 8, indicative of suitable quality. RNA purification for preliminary quality assessment was performed by Dr Patricia Rueda. Quality and quantity assessment of all RNA samples were performed by staff at Dr Nicholas Huntington's laboratory at the Walter and Eliza Hall Institute, Australia. PFC samples from the remaining cohort were not evaluated due to time and financial constraints.

4.3.4 cDNA library generation and RNA sequencing

Purified RNA samples were sent to Micromon Genomics, Monash University for RNA sequencing. For whole transcriptome analysis in eukaryotic cells, poly-A selection of protein-coding RNA using poly-T oligo beads is generally preferred over poly-T oligo priming (typically reserved for low-input samples) and ribosomal RNA depletion (required if RNA is partly degraded or interested in non-coding RNAs; Hrdlickova et al., 2017). Confirmation of suitable RNA quality (section 4.3.3) enabled commencement of poly-T bead-based purification and library generation based on the Illumina TruSeq Stranded mRNA kit protocol. Briefly,

protein-coding RNAs (i.e. mRNAs) were isolated using poly-T oligo beads and subjected to fragmentation using divalent cations under increased temperature. The RNA fragments were primed for first strand cDNA synthesis via reverse transcription using random hexamer primers in the presence of Actinomycin D, resulting in a blunted end cDNA. Addition of a single 'A' nucleotide to the 3' ends of the blunt fragments prevented ligation of the first and second strands. Illumina adapters were attached to the 5' and 3' ends (P5 and P7, respectively) of the cDNA to capture directionality of the RNA in the resulting cDNA libraries, and to allow library fragments to bind to the flow cells of the sequencer. Fragments with adapters on both ends were enriched using polymerase chain reaction. Only the first strand cDNA was amplified as the second strand comprised dUTP in place of dTTP and therefore cannot be read. Consequently, a new complementary strand of the amplified first strand, with dTTP incorporated, was synthesised. The newly formed double-stranded cDNA ('library') was purified, checked and quantified, before being multiplexed and sequenced using Illumina NextSeq 500.

4.3.5 Bioinformatics analysis

Data pre-processing, mapping and quality control

Single-end 75 base pair sequence reads, available as FastQ files, were checked for quality using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) before being converted to BAM files of unaligned reads. The reads were mapped to the Ensembl rat genome assembly (Rnor_6.0, INSDC Assembly, July 2014) using STAR aligner (https://github.com/alexdobin/STAR), which demonstrated improved mapping accuracy and speed compared to other aligners (Dobin et al., 2013). Samtools (http://www.htslib.org/) and Picard (http://broadinstitute.github.io/picard/) were used for manipulating alignments (e.g. sorting, merging and indexing and duplicate removal) whereas QualiMap (http://qualimap.bioinfo.cipf.es/) and FeatureCounts (http://bioinf.wehi.edu.au/featureCounts/) were used to assess the quality

of mapped sequences and summarise reads mapped to genomic features, respectively (García-Alcalde et al., 2012; Li et al., 2009; Liao et al., 2014). Multiple tools were used as they offer distinct features and analyses, with QualiMap for example reveals coverage profile along genes but not FeatureCounts. Furthermore, this approach allows comparison to confirm validity of quality assignment. Output from the different tools were entered into MultiQC (https://multiqc.info/) to generate a single report summarising statistical output across all the tools, for each individual sample, allowing identification of outlier samples (Ewels et al., 2016).

Differential gene expression analysis

To estimate differential expression, a matrix of gene count was produced before the sequenced libraries were demultiplexed, filtered and normalised for quality using the trimmed mean of M values method to prevent bias due to discrepancies in library size (Robinson & Oshlack, 2010). The output gene count matrix was then fed into Degust version 3.1.0 (http://degust.erc.monash.edu/) and analysed using the voom/limma function, where a linear model was fitted to the genes, transforming counts into normally distributed data via variance shrinkage. This method is deemed to perform as well as or better than common alternatives including edgeR and DESeq2, which use a log-linear negative-binomial-based model to shrink dispersion (Law et al., 2014). A moderated *t*-test was then performed for each gene to compare expression between the two housing conditions. The $\log_2$ fold-change of genes in isolates was expressed relative to those of GH animals.

Functional analysis

To characterise the molecular functions and pathways in which the DEGs are involved, a list of DEGs was generated using a false discovery rate (FDR) cut-off value of 0.05. The list was used for Reactome (https://reactome.org/) and Kyoto Encyclopedia of Genes and Genomes

(KEGG) (https://www.genome.jp/kegg/pathway.html) pathway analyses, besides Gene Ontology (GO) enrichment analysis using the GO Enrichment Analysis and Visualisation Tool (GOrilla). Reactome and KEGG PATHWAY are manually-curated peer-reviewed databases of known biological pathways and processes. Whilst Reactome offers interactive, scalable pathway visualisations, arranged in a hierarchical manner, it lacks drug- or target-based, stress-activated or lipid-mediated signaling pathways, which is available in KEGG (Chowdhury & Sarkar, 2015; Sidiropoulos et al., 2017). Thus, the two tools complement each other and were both used in the current study. However, unlike Reactome pathways which is human-based, KEGG pathways lack organism specificity (Fabregat et al., 2018; Kanehisa et al., 2019). In GO analysis, a dynamic but structured vocabulary is used to define the function of gene products in respect to three biological domains: molecular function, cellular component and biological processes (Ashburner et al., 2000). Apart from the standard approach of using a hypergeometric model where ranking is ignored, GOrilla permits the detection of enriched GO terms in ranked lists using mHG statistics (http://cbl-gorilla.cs.technion.ac.il/#ref). Moreover, apart from being highly interactive and requires little running time, GOrilla outperforms similar web-based applications including Fatiscan and GoMiner by providing a threshold-free approach, resulting in an exact mHG p-value and using a colour-coded directed acyclic graph to represent significantly enriched terms (Eden et a., 2009). Therefore, GOrilla is used in the current study. All functional analyses were conducted by Dr Patricia Rueda. A summary of the work flow is shown in Figure 4.1. Figure 4.2 shows an overview of the pathways available for *Rattus norvegicus* in Reactome whereas Figure 4.3 represents an output from GOrilla in the form of a colour-coded directed acyclic graph from the current study.

4.3.6 Statistical analysis

Body weight data were analysed by two-way RM ANOVA (housing and time as factors) followed by Sidak's post-hoc test. All values are mean ± SEM and $p<0.05$ was taken as significant.

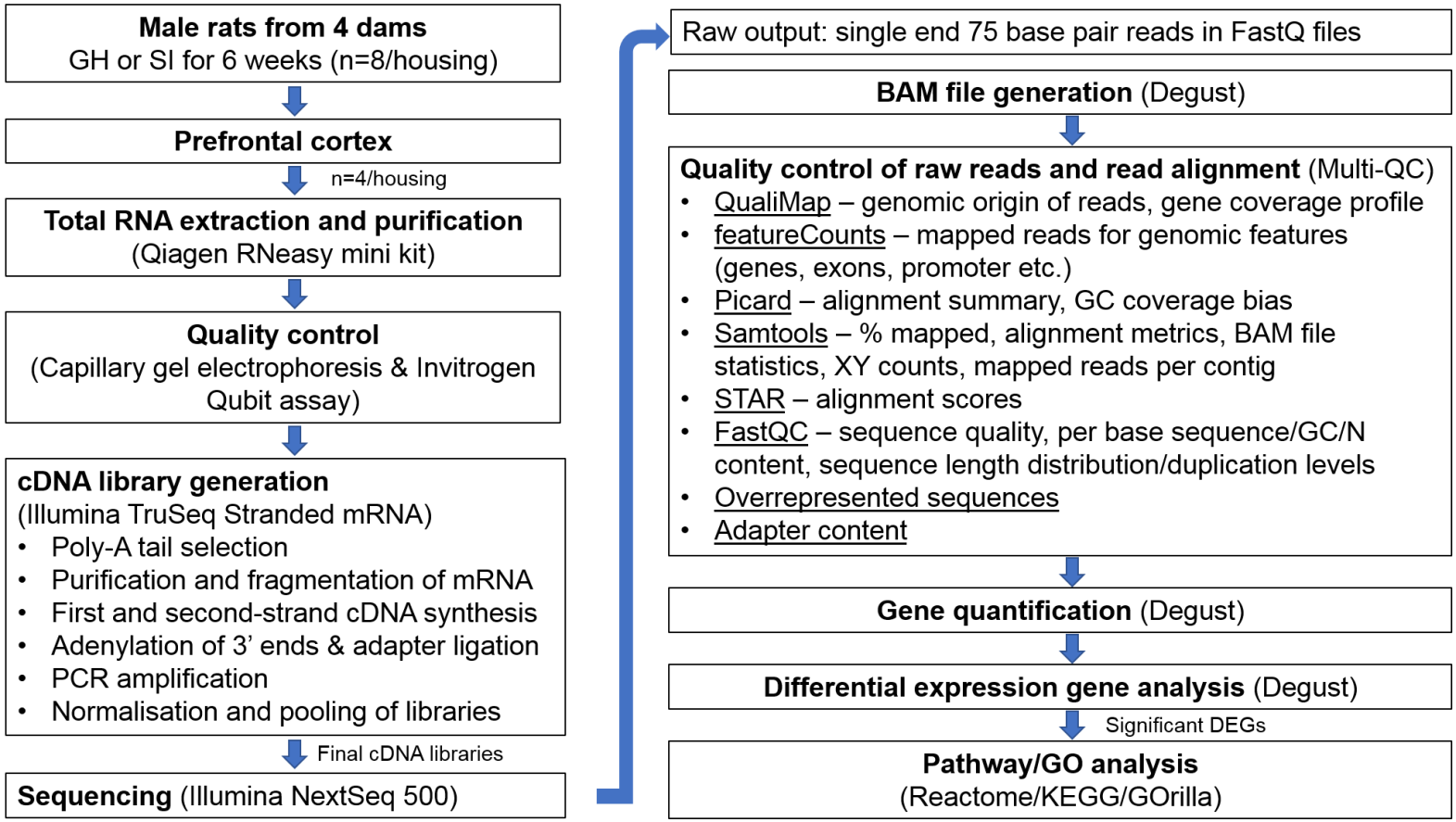


Figure 4.1. Overview of RNA-sequencing analysis pipeline. The main steps for the RNA-sequencing study in post-mortem frontal cortices of Sprague-Dawley male rats (four GH and four SI) are illustrated. The kit, system or software used in each step is given in brackets. BAM, Binary Alignment Map; cDNA, complementary deoxyribonucleic acid; DEGs, differentially expressed genes; GH, group-housed; GOrilla, Gene Ontology enrichment analysis and visualization tool; KEGG, Kyoto Encyclopedia of Genes and Genomes; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; SI, post-weaning social isolation

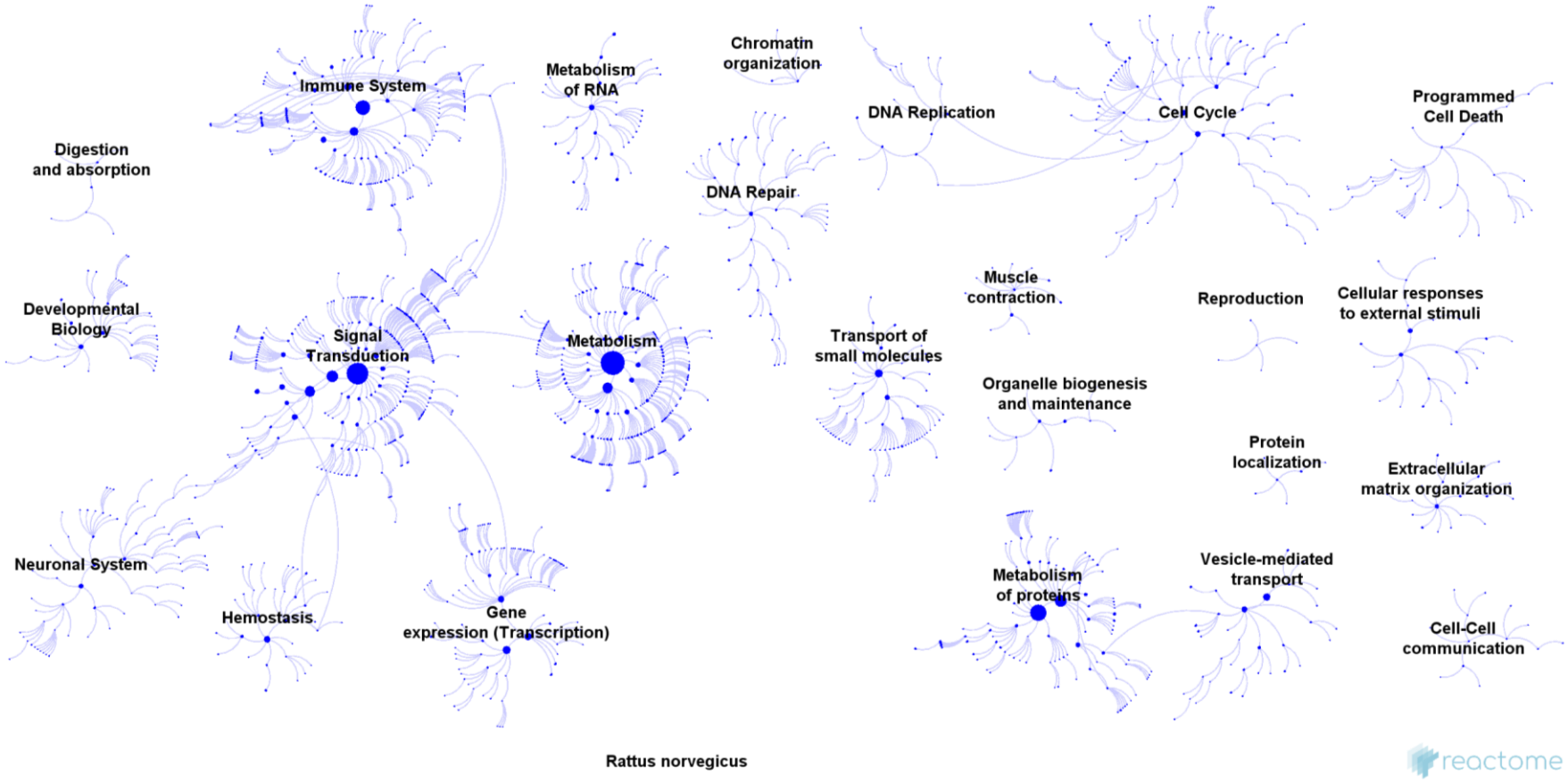


Figure 4.2. Overview of pathways available for *Rattus norvegicus* in the Reactome software. Twenty-four biological pathways are available at their highest hierarchical level, which can be interrogated by interactive illustrations (intermediate levels) progressively leading to the lower levels represented by detailed pathway diagrams. Diagram and information were acquired from Reactome website [https://reactome.org/PathwayBrowser/#/R-RNO-71064; accessed 5th June 2019].

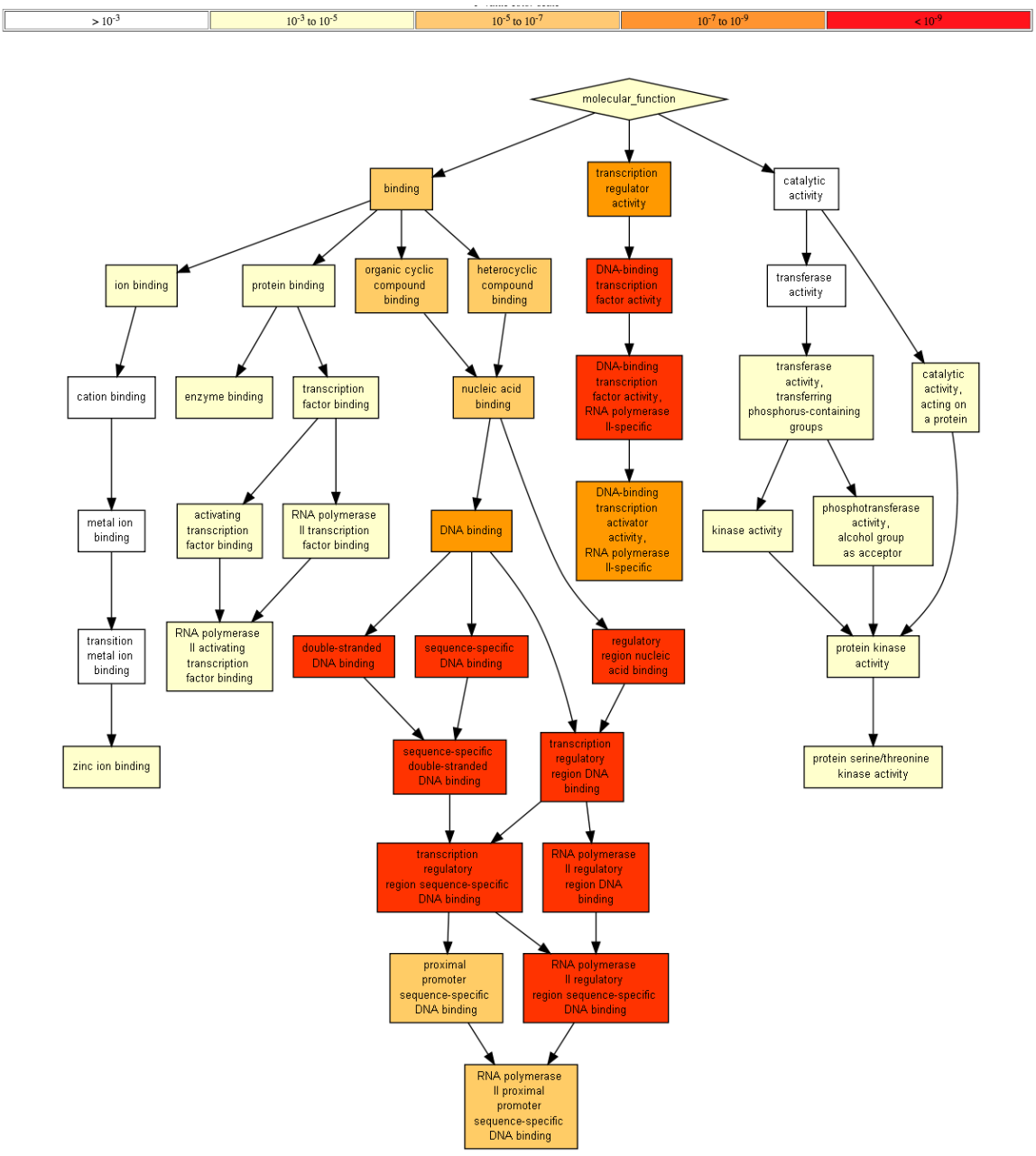


Figure 4.3. An example of GOrilla output from the current study. Significantly enriched gene ontology terms under the 'molecular function' arm are colour-coded according to their enrichment p-values.

4.4 Results

4.4.1 Body weight analysis: whole cohort and rats selected for RNA-Seq

Analysis of body weight of the whole rat cohort from weaning (PND21) to young adulthood (PND63-66) revealed a significant main effect of time ($F_{(5,70)}$= 1642, p<0.0001) and a time x housing interaction ($F_{(5,70)}$= 18.75, p<0.0001). SI rats were significantly heavier than GH rats at six weeks post-weaning, thus confirming development of the isolation syndrome (Figure 4.4A).

As advised by bioinformaticians at Micromon Genomics, four rats were randomly selected from each housing condition for RNA-Seq. There was a significant main effect of time ($F_{(5,30)}$= 234.9, p<0.0001) and a time x housing interaction ($F_{(5,30)}$= 7.98, p<0.0001) on the body weight of selected rats. SI rats were significantly heavier than GH rats at six weeks post-weaning (Figure 4.4B).

4.4.2 Quality control of RNA-Seq data: mapping

For each sample, approximately 22.5 million reads were generated (range: 20.8 to 26.2 million reads). On average 22.2 million reads per sample were mapped to the reference genome (range 20.6 to 25.8 million reads, approximately 99% of the generated reads were mapped). The average uniquely mapped reads per sample was 19.6 million, corresponding to about 87% of the total sequence. All samples had less than 1% of reads made up of overrepresented sequences and no samples were associated with adapter contamination of greater than 0.1%.

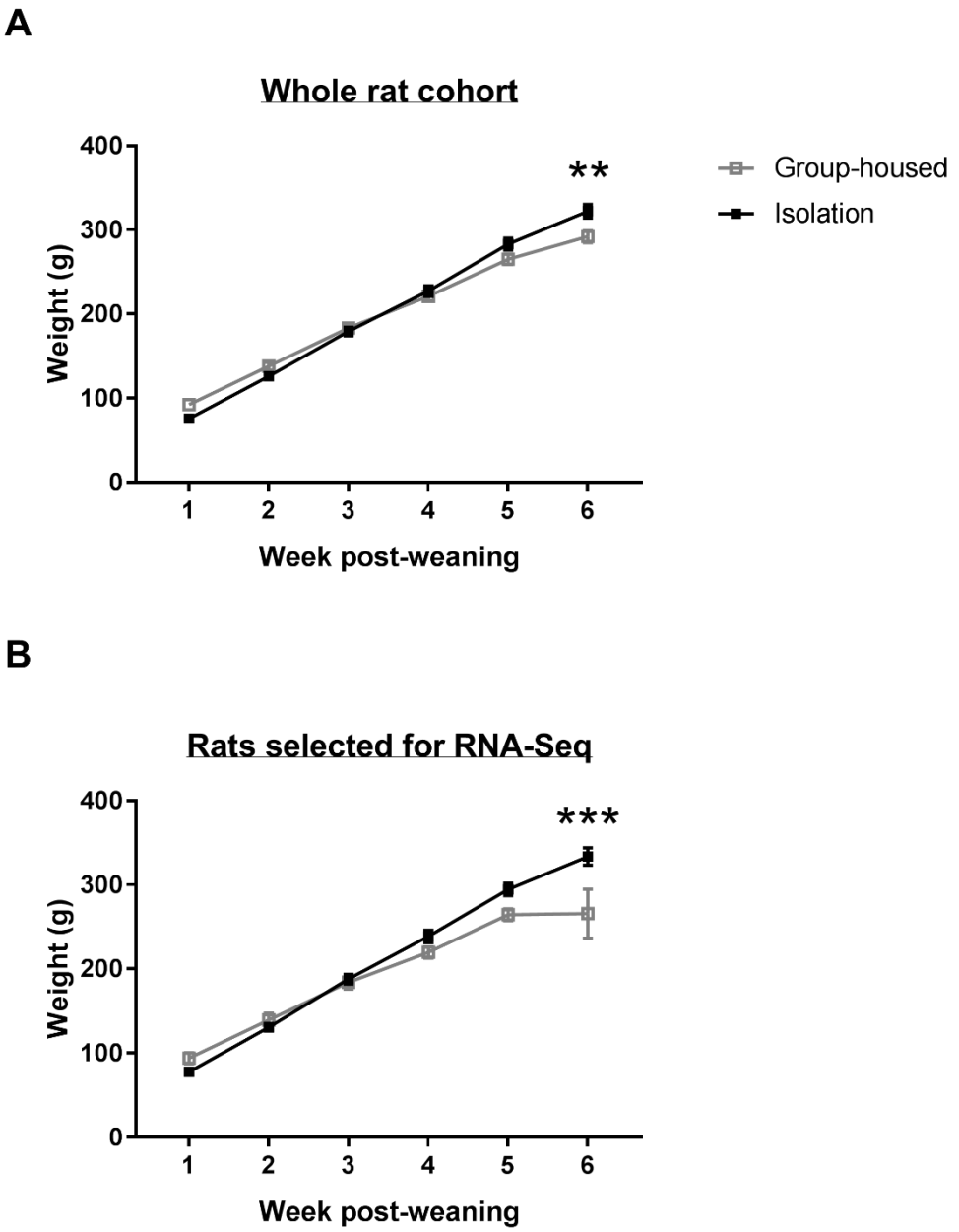


Figure 4.4. Effect of post-weaning social isolation on rat body growth from weaning (PND21) to 6 weeks post-weaning (PND63-66). Mean ± SEM body weight of the **(A)** whole rat cohort (n=8 per housing) and **(B)** rats selected for RNA-Seq (n=4 per housing). **p<0.01, ***p<0.001 compared to group-housed; two-way RM ANOVA with Sidak's post-hoc test (A,B).

4.4.3 Differential gene expression: relationship between samples

A visual representation of the variation between samples or RNA-seq libraries when the FDR cut-off was set at 0.05 is illustrated in Figure 4.5. From this it was clear that the transcriptome of GH and isolated rats formed distinct clusters, warranting further analysis.

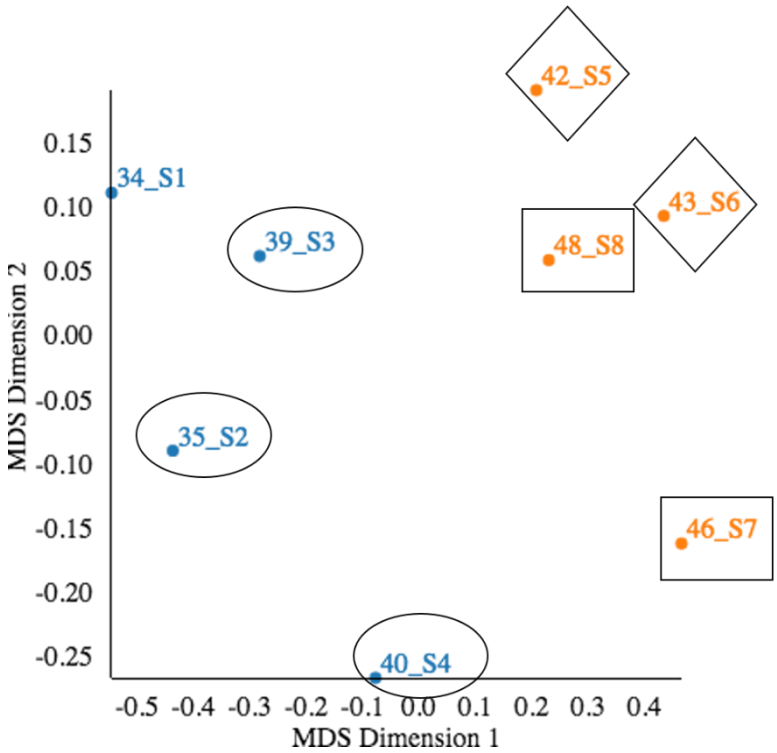


Figure 4.5. Multidimensional plot. Relationship between individual samples from group-housed (blue) and isolated rats (orange; n=4 per housing condition). The x-axis and y-axis denote the first and second dimensions of the Euclidean distances between the samples, respectively. Individual labels are in the format of 'rat identification number_sample number'. Same shape denotes same maternal-paternal source.

4.4.4 SI-induced changes in gene expression

Profiling in the PFC identified a total of 183 genes that were differentially expressed in isolates by the criteria defined in the methods. Of these genes, only twelve were up-regulated (log_2 fold change: 0.27 to 5.65) whereas 171 were down-regulated (log_2 fold change: -3.43 to -0.26; Appendix 2, Table 2.1).

4.4.5 Pathway analyses

To identify the pathways altered by long-term isolation rearing in rats, functional analyses were carried out using Reactome or KEGG.

Pathway analysis using Reactome identified 34 canonical pathways in which DEGs were enriched (Table 4.1). Classified at the highest hierarchical levels, these pathways belong under gene expression (transcription), cellular response to external stimuli, developmental biology, circadian clock, neuronal system, immune system and signal transduction. Fifty-five downregulated genes were implicated. Further interrogation of the DEGs revealed extensive links between most significant pathways (Figure 4.6). For example, *Tnrc6c* and *Tnrc6b* (gene names given in full in Appendix 2, Table 2.1) were associated with fourteen of the pathways in which transcriptome changes were identified. These two genes were the only ones altered in two pathways ('Post-transcriptional silencing by small RNAs' and 'Competing endogenous RNAs regulate PTEN translation') and apart from *Kmt2a* and *Nr4a3* in a third pathway ('Runx1 regulates genes involved in megakaryocyte differentiation and platelet function'). The remaining eleven pathways formed complicated network with each other, and also pathways without *Tnrc6c* and *Tnrc6b* involvement, through common downregulated signalling substrates. Examples of such substrates are the immediately early genes *Fos* and *Jun*, shared between five to six *Tnrc6c*/*Tnrc6b*-associated pathways (including 'MAPK family signalling cascades') and two non-*Tnrc6c*/*Tnrc6b*-associated pathways. Among these pathways, some genes were further shared, e.g. *Tnik* and *Kdm6b* between 'oxidative stress induced senescence' and 'cellular senescence'. 'Cellular senescence' was also linked to 'oncogene-induced senescence' via *Erf*. Meanwhile, 'oestrogen-dependent gene expression' and 'oestrogen receptor-mediated signalling' shared the DEGs *Jun* and *Ncoa3*, which also participated in MAPK family signalling cascades. Whilst certain DEGs were exclusive to MAPK signalling, others (*Grin2b*, *Dusp6* and *Sptb*) also engaged in seven other pathways under developmental

biology (‘L1CAM interactions’ and ‘interaction between L1 and ankyrins’), innate immune system (‘MAPK targets/nuclear events mediated by MAP kinases’), gene transcription (‘transcriptional regulation by MeCP2’) and neuronal system (‘neurexins and neuroligins’, ‘protein-protein interactions at synapse’, and ‘assembly and cell surface presentation of NMDA receptors’). A similar example is *Prkca*, which was involved in five pathways involved in signal transduction, cell cycle and developmental biology. One of the pathways, ‘signalling by Rho GTPases’ was linked to three further pathways: ‘Rho GTPases activate CIT’ (via *Cit*), ‘Rho GTPase cycle’ (via *Arhgef12*, *Arhgap5*, *Trio*, *Srgap3*, *Arap2* and *Myo9a*) and ‘Sema4D induced cell migration and growth-cone collapse’ (via *Arhgef12* and *Rock2*).

Three out of the 34 total significant pathways were relatively independent (two circadian-related pathways grouped together). DEGs in ‘RNA polymerase III transcription termination’ (*Nfix*, *Nfib*) and ‘Runx2 regulates genes involved in cell migration’ (*Itgbl1*) were not associated with other pathways. Albeit the two circadian clock pathways shared common DEGs (*Bhlhe40* and *Chd9*), they were not linked to other pathways via other DEGs.

Table 4.1. Significant Reactome pathways sorted by p-value. 183 differentially expressed genes found in the prefrontal cortex of isolation-reared rats compared to group-housed controls following 6 weeks of differential housing were entered into Reactome to identify isolation-induced pathway perturbations. The p-value reflects the likelihood that the associated pathway is enriched by random chance.

Reactome pathway	**p-value**	**Genes**
TP53 regulates transcription of additional cell cycle genes whose exact role in the p53 pathway remain uncertain	0.0001	*Btg2*, *Plk3*, *Cnot6*
Oxidative Stress Induced Senescence	0.0003	*Jun*, *Tnrc6c*, *Fos*, *Tnik*, *Tnrc6b*, *Kdm6b*
Circadian Clock	0.0010	*Per1*, *Bhlhe40*, *Chd9*, *Sik1*
Interaction between L1 and Ankyrins	0.0016	*Kcnq2*, *Ank3*, *Ank2*, *Sptb*
Neurexins and neuroligins	0.0021	*Cask*, *Apba1*, *Syt7*, *Dlgap2*, *Grin2b*
Cellular Senescence	0.0030	*Jun*, *Tnrc6c*, *Erf*, *Fos*, *Tnik*, *Tnrc6b*, *Kdm6b*
TP53 Regulates Transcription of Cell Cycle Genes	0.0030	*Btg2*, *Plk3*, *Cnot6*
Post-transcriptional silencing by small RNAs	0.0050	*Tnrc6c*, *Tnrc6b*
RUNX1 regulates genes involved in megakaryocyte differentiation and platelet function	0.0064	*Tnrc6c*, *Kmt2a*, *Nr4a3*, *Tnrc6b*
MAPK family signalling cascades	0.0110	*Jun*, *Tnrc6c*, *Ncoa3*, *Dusp1*, *Irs2*, *Ksr2*, *Sptb*, *Tnrc6b*, *Dusp6*, *Grin2b*
Protein-protein interactions at synapses	0.0129	*Cask*, *Apba1*, *Syt7*, *Dlgap2*, *Grin2b*
Transcriptional Regulation by TP53	0.0139	*Btg2*, *Plk3*, *Jun*, *Dyrk2*, *Tnrc6c*, *Cnot6*, *Sesn3*, *Tsc1*, *Fos*, *Tnrc6b*, *Hipk2*
Activation of the AP-1 family of transcription factors	0.0139	*Jun*, *Fos*
MAPK targets/ Nuclear events mediated by MAP kinases	0.0155	*Jun*, *Fos*, *Dusp6*
Transcriptional Regulation by MECP2	0.0171	*Tnrc6c*, *Tnrc6b*, *Hipk2*, *Grin2b*
RUNX2 regulates genes involved in cell migration	0.0186	*Itgbl1*
Regulation of MECP2 expression and activity	0.0206	*Tnrc6c*, *Tnrc6b*, *Hipk2*
Rho GTPase cycle	0.0209	*Arhgap5*, *Trio*, *Srgap3*, *Arhgef12*, *Arap2*, *Myo9a*
Oncogene Induced Senescence	0.0249	*Tnrc6c*, *Erf*, *Tnrc6b*
BMAL1:CLOCK,NPAS2 activates circadian gene expression	0.0249	*Bhlhe40*, *Chd9*
Oestrogen-dependent gene expression	0.0278	*Jun*, *Tnrc6c*, *Ncoa3*, *Fosb*, *Fos*, *Tnrc6b*
Regulation of KIT signalling	0.0295	*Prkca*, *Cbl*

Reactome pathway	**p-value**	**Genes**
Ca2+ pathway	0.0317	*Tnrc6c*, *Prkca*, *Tnrc6b*, *Itpr1*
Competing endogenous RNAs (ceRNAs) regulate PTEN translation	0.0326	*Tnrc6c*, *Tnrc6b*
Assembly and cell surface presentation of NMDA receptors	0.0349	*Cask*, *Apba1*, *Grin2b*
ESR-mediated signalling	0.0369	*Jun*, *Tnrc6c*, *Ncoa3*, *Fosb*, *Strn*, *Fos*, *Tnrc6b*
TP53 Regulates Metabolic Genes	0.0391	*Tnrc6c*, *Sesn3*, *Tsc1*, *Tnrc6b*
Signalling by Rho GTPases	0.0401	*Fmnl2*, *Arhgap5*, *Trio*, *Srgap3*, *Taok1*, *Arhgef12*, *Rock2*, *Prkca*, *Arap2*, *Myo9a*, *Cit*
DAG and IP3 signalling	0.0426	*Adcy1*, *Prkca*, *Itpr1*
L1CAM interactions	0.0449	*Dcx*, *Kcnq2*, *Ank3*, *Ank2*, *Sptb*
RHO GTPases activate CIT	0.0460	*Cit*
Depolymerisation of the Nuclear Lamina	0.0460	*Prkca*, *Lpin1*
RNA Polymerase III Transcription Termination	0.0460	*Nfix*, *Nfib*
Sema4D induced cell migration and growth-cone collapse	0.0496	*Arhgef12*, *Rock2*

Abbreviations: AP-1, activator protein 1; BMAL1:CLOCK, brain and muscle Arnt-like protein-1:clock circadian regulator; CIT, Citron Rho-interacting kinase; DAG, diacylglycerol; ESR, oestrogen receptor; IP3, inositol triphosphate; KIT, KIT proto-oncogene receptor tyrosine kinase; L1 (also L1CAM), L1 cell adhesion molecule; MAPK, mitogen-activated protein kinase; MECP2, methyl-CpG binding protein 2; NMDA, N-methyl-D-aspartate; NPAS2, neuronal PAS domain protein 2; PTEN, phosphatase and tensin homolog; RUNX1/2, runt related transcription factor 1/2; Sema4D, semaphorin 4D; TP53, tumour protein P53.

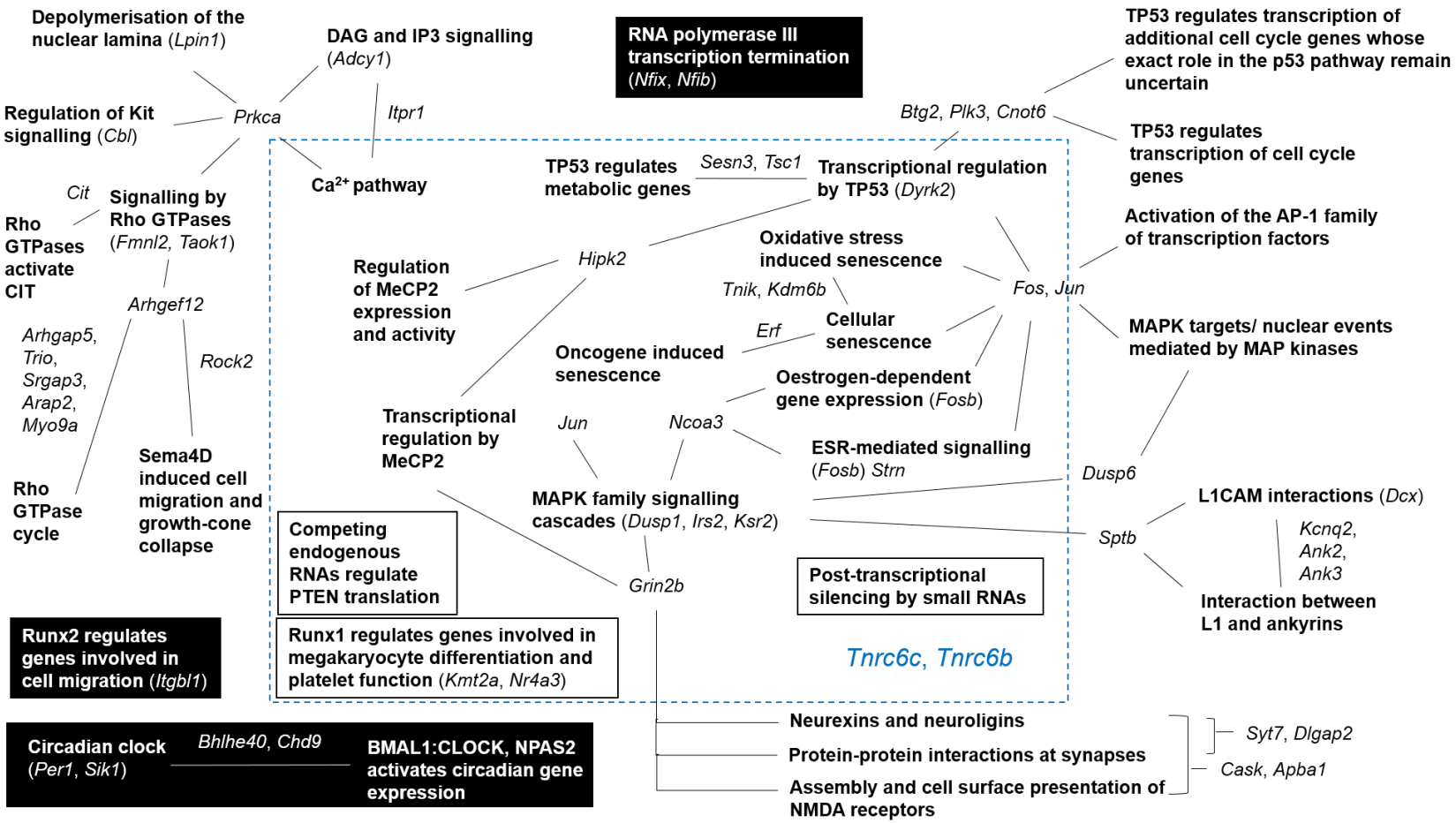


Figure 4.6. Summary diagram depicting the relationship between Reactome significant pathways. Fifty-five downregulated genes were implicated in 34 significant pathways, of which 14 share the common downregulated genes *Tnrc6c* and *Tnrc6b* (blue dotted line box). These two genes were the only ones altered in two pathways and apart from *Kmt2a* and *Nr4a3* in a third pathway (black-bordered boxes). Altered genes shared between pathways are connected by a line whereas genes in brackets are exclusive to their associated pathways. For example, *Dusp1*, *Irs2* and *Ksr2* were only altered in 'MAPK family signalling cascades' while *Dusp6* was altered in both 'MAPK family signalling cascades' and 'MAPK targets/nuclear events mediated by MAP kinases'. Three of the pathways are relatively independent (filled black boxes, two pathways related to circadian clock grouped as one). Gene descriptions are given in Table 2.1 of Appendix 2.

KEGG enrichment analysis revealed fourteen significant pathways in which 30 downregulated genes were enriched (Table 4.2). Some of the enriched DEGs were heavily shared among the different enriched pathways, including general signalling components and transcription factors such as protein kinase C alpha (thirteen pathways), inositol 1,4,5-triphosphate receptor type 1 (nine pathways), Fos (nine pathways), Jun (seven pathways) and adenylate cyclase 1 (seven pathways). Very few genes were specific to their associated enriched pathways, with examples being *Per1*, whose gene product is essential in circadian entrainment and *Chrm4*, which encodes for muscarinic acetylcholine receptor 4 (M_4; Figures 4.7 & 4.8). Nineteen of the 30 enriched genes were also identified by Reactome, including *Adcy1*, *Dusp1*, *Fos*, *Grin2b* and *Jun*. Those not identified by Reactome are *Arc*, *Cacna1e*, *Chrm4*, *Dgkh*, *Egr2*, *Egr3*, *Gadd45b*, *Itgb8*, *Kcnj6*, *Nr4a1* and *Xiap*.

4.4.6 Gene ontology enrichment analysis

To decipher the attributes of the products of DEGs, GO analysis was performed. Enrichment of terms were summarised in the three main categories as described in section 4.3.5.

For biological processes, the DEGs were significantly enriched for terms under the parent term ‘biological regulation’, including terms related to regulation of molecular function (e.g. regulation of tau-kinase and MAPK activity) and regulation of biological process. For the latter, enriched terms can be subcategorised into terms relevant to the regulation of cellular or developmental processes (e.g. cell death, proliferation, communication, differentiation and migration), multicellular organismal process, and metabolic process. Significant enrichment is evident in the regulation of multiple metabolic processes, such as protein phosphorylation and protein modification, as well as regulation of biosynthetic process. In particular, enriched terms were found for regulation of gene expression, and macromolecule biosynthetic and metabolic processes, particularly those of RNA. Furthermore, SI is linked

to GO terms belonging to behaviour and rhythmic or developmental processes, including locomotion, cognition, learning, long-term memory and anatomical structural development. Numerous terms associated with cellular response to stimuli including endogenous hormones, organic and inorganic substances, drugs, oxidative stress and abiotic factors such as radiation have also been significantly enriched.

Enriched GO terms for molecular function are related to serine/threonine kinase activity or transcription factor binding and transcription regulation. In terms of cellular components, DEGs appear to be localised in the nucleus, axon part, neuronal cell body and cytoplasm, as well as forming part of a transcription factor complex, specifically the AP-1 complex (Figure 4.9).

Table 4.2. Significant KEGG pathways sorted by the p-value. A total of 183 differentially expressed genes found in the frontal cortices of rats that were isolation-reared for 6 weeks since weaning, relative to group-housed controls, were used in KEGG pathway analysis.

KEGG term	Genes	p-value
rno04713:Circadian entrainment	*Prkca*, *Fos*, *Adcy1*, *Kcnj6*, *Grin2b*, *Per1*, *Itpr1*	0.0003
rno05031:Amphetamine addiction	*Prkca*, *Fos*, *Arc*, *Grin2b*, *Jun*, *Fosb*	0.0003
rno04725:Cholinergic synapse	*Prkca*, *Fos*, *Adcy1*, *Kcnj6*, *Chrm4*, *Kcnq2*, *Itpr1*	0.0007
rno04010:MAPK signaling pathway	*Prkca*, *Fos*, *Dusp1*, *Taok1*, *Jun*, *Nr4a1*, *Cacna1e*, *Gadd45b*, *Dusp6*	0.0030
rno04921:Oxytocin signaling pathway	*Prkca*, *Fos*, *Adcy1*, *Kcnj6*, *Rock2*, *Jun*, *Itpr1*	0.0032
rno05205:Proteoglycans in cancer	*Prkca*, *Ank2*, *Rock2*, *Ank3*, *Cbl*, *Arhgef12*, *Itpr1*	0.0121
rno04915:Estrogen signaling pathway	*Fos*, *Adcy1*, *Kcnj6*, *Jun*, *Itpr1*	0.0131
rno05231:Choline metabolism in cancer	*Prkca*, *Fos*, *Tsc1*, *Jun*, *Dgkh*	0.0156
rno04720:Long-term potentiation	*Prkca*, *Adcy1*, *Grin2b*, *Itpr1*	0.0239
rno04270:Vascular smooth muscle contraction	*Prkca*, *Adcy1*, *Rock2*, *Arhgef12*, *Itpr1*	0.0289
rno04728:Dopaminergic synapse	*Prkca*, *Fos*, *Kcnj6*, *Grin2b*, *Itpr1*	0.0345
rno05161:Hepatitis B	*Prkca*, *Fos*, *Egr3*, *Egr2*, *Jun*	0.0435
rno04925:Aldosterone synthesis and secretion	*Prkca*, *Adcy1*, *Nr4a1*, *Itpr1*	0.0460
rno04510:Focal adhesion	*Prkca*, *Arhgap5*, *Xiap*, *Rock2*, *Itgb8*, *Jun*	0.0498

Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase.

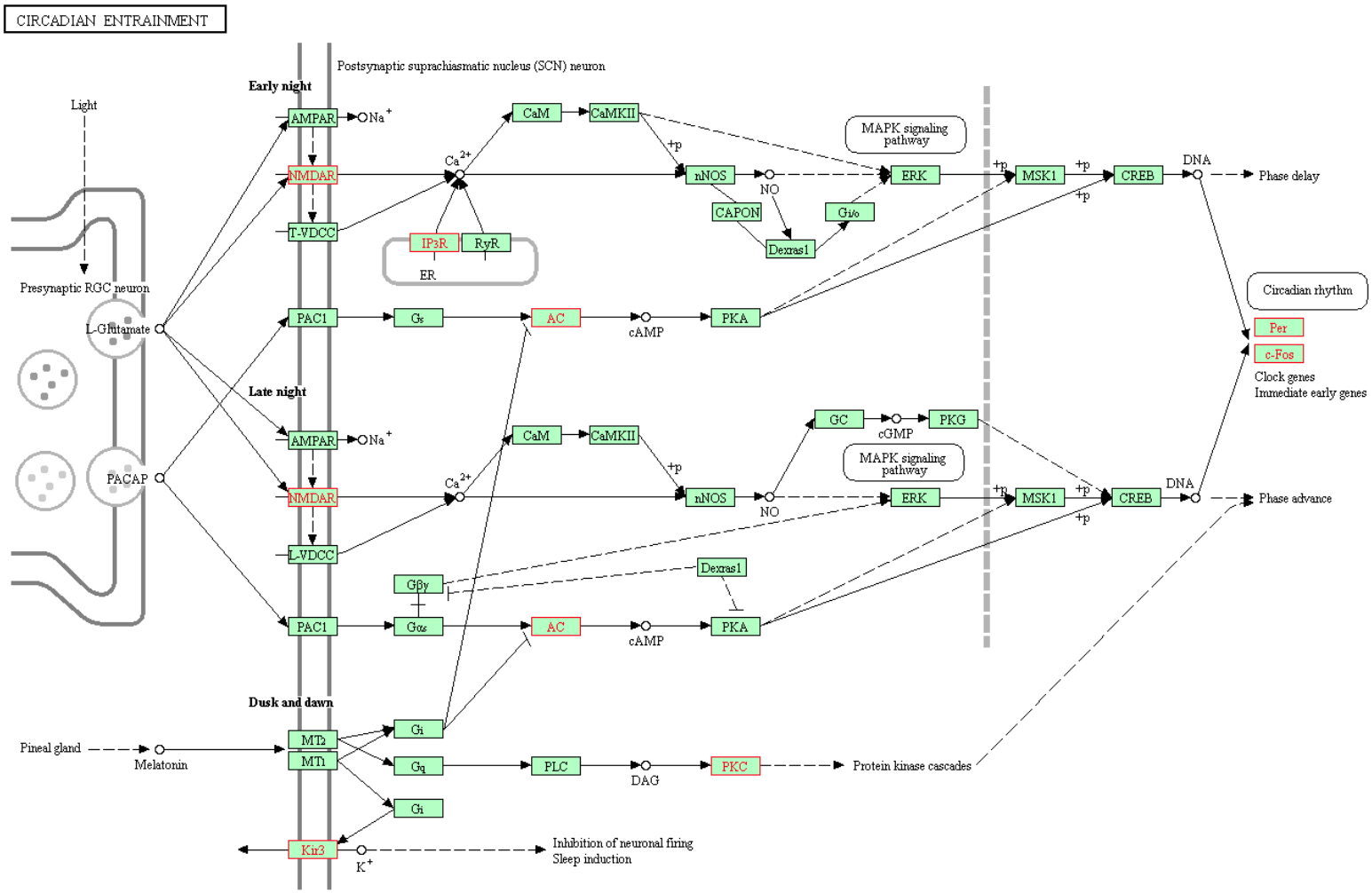


Figure 4.7. Enriched KEGG pathway 'Circadian entrainment'. Most differentially expressed genes (marked in red) involved in this pathway encode signalling substrates common to multiple pathways, e.g. adenylyl cyclase (AC) and the transcription factor c-fos, rather than components exclusive to this pathway. In this example, the only altered gene that is specific to circadian entrainment is *Per1*.

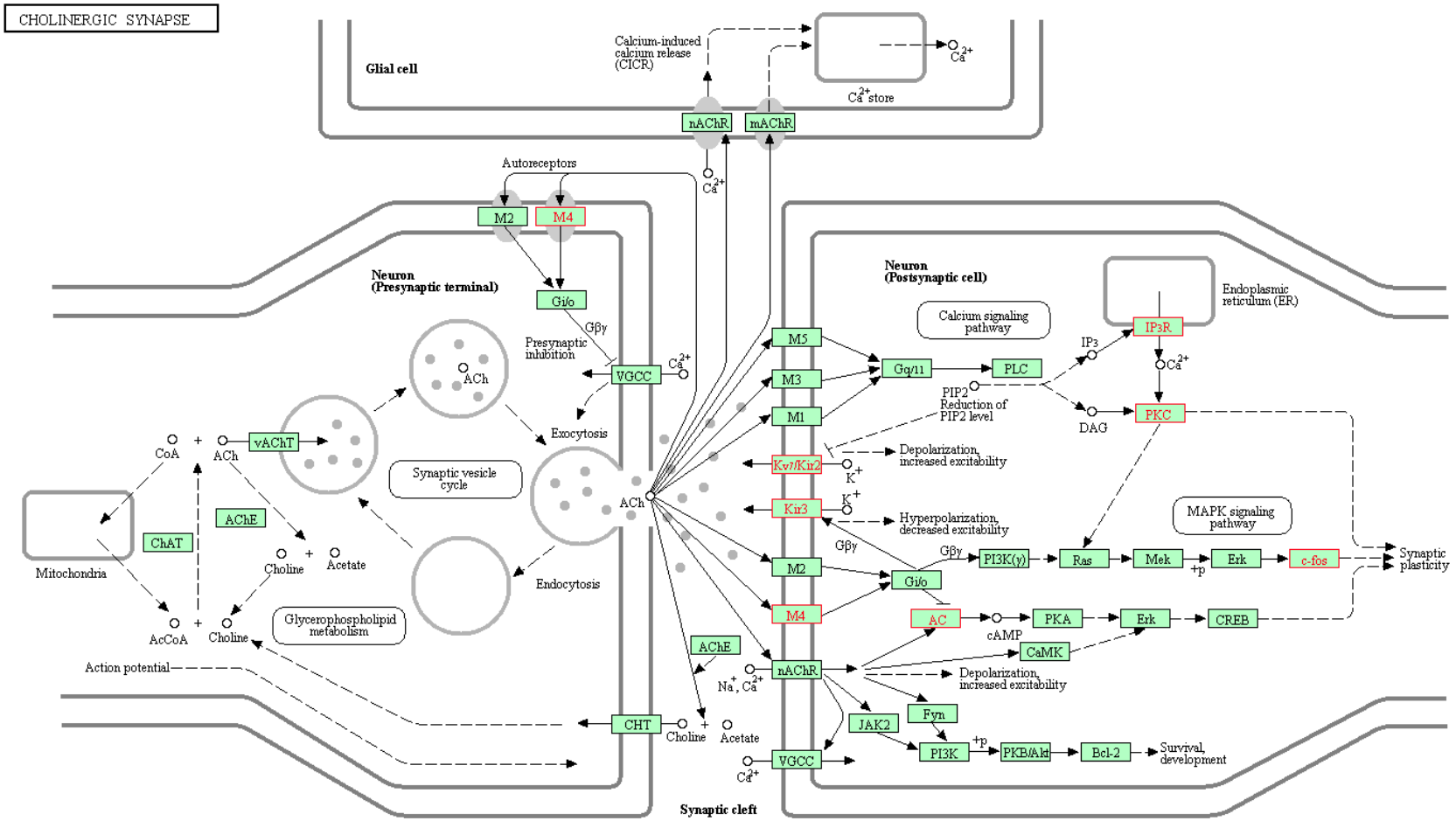


Figure 4.8. Enriched KEGG pathway 'Cholinergic synapse'. This example further confirms that most differentially expressed genes (marked in red) enriched in this pathway encode proteins also involved in other enriched pathways, e.g. adenylyl cyclase (AC) and the transcription factor c-fos, rather than components specific to this pathway, e.g. muscarinic acetylcholine receptors M_1-M_3 and M_5.

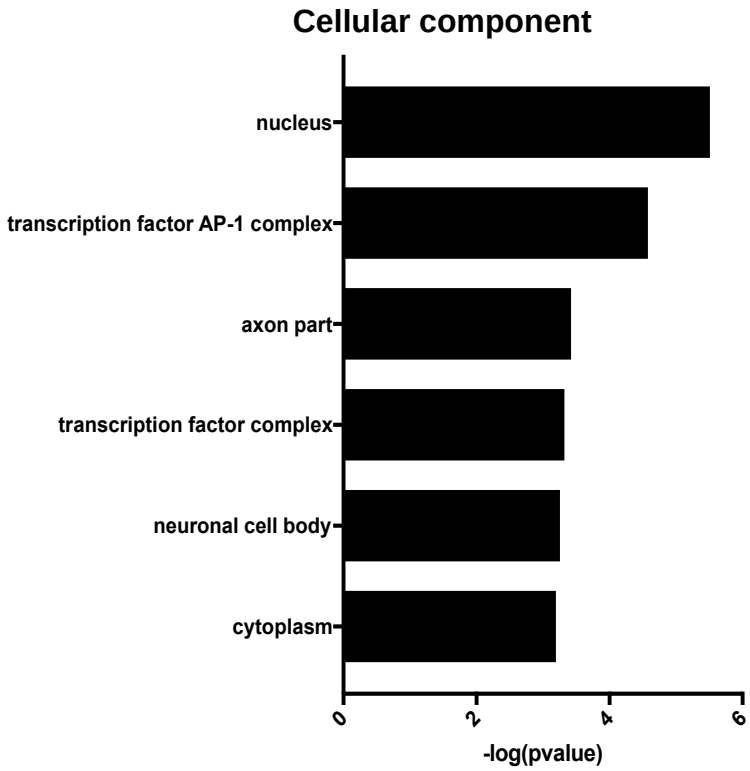


Figure 4.9. Gene Ontology enrichments in terms of cellular component. 183 differentially expressed genes in rat prefrontal cortex compared to group-housed controls following 6 weeks of differential housing, were analysed for gene ontology enrichment using GOrilla. Significant enrichment is denoted by $p<0.05$.

4.5 Discussion

The current study shows that early-life stress in the form of post-weaning SI alters gene expression in the PFC of Sprague-Dawley rats. Many of the altered genes are common to multiple signalling pathways, and have been implicated in schizophrenia. Furthermore, functional pathway analyses revealed several enriched pathways that are relevant to the putative aetiological mechanisms of schizophrenia, including pathways related to cholinergic and dopaminergic transmission, and oxytocin signalling. To our best knowledge, this is the first whole transcriptome study conducted in rats that have been isolation reared since weaning.

4.5.1 Differential gene expression analysis

In the current study, decreased expression of multiple IEGs including *Arc*, *Junb*, *Nr4a1* (also *NGFI-B*), *Klf2*, *Fos* (*c-Fos*), *Egr2* and *Egr4*, as well as apoptosis-related genes including *Mkp1* (*Dusp1*) and *Btg2*, were observed in isolated rats. This finding corresponds to a previous study utilising Affymetrix GeneChip microarray to analyse the expression of IEGs and genes that modulate cell differentiation and apoptosis in the medial PFC of SI rats (Levine et al., 2007). Whilst the current data suggest downregulation of further IEGs, e.g. *Klf10*, *Fosb*, *Egr1*, *Egr3 and Ier5*, as well as *Mkp3* (*Dusp6*), we failed to replicate SI-induced altered expression of the IEG *Homer1*, genes related to apoptosis (*Gadd45g*, *Id1*, *Tmpo*, *Nfkb1*, *Mad2l1* and *Sertad1*) or endocytosis (*Ap1g1* and *Itsn*), the oncogene *Mas1* and other genes (including *Asb8* and *Rars*), which have prospective roles in protein modification (ubiquitination) and myelination (Andresen et al., 2014; Levine et al., 2007; Wolf et al., 2014). The current study also failed to detect altered expression of *Grik2* and *Gria3*, which encode glutamate receptor kainate type subunit 2 and AMPA type subunit 3, respectively (Levine et al., 2007). Perturbed expression of *Syt7*, related to synapse integrity, is consistent with a prior microarray analysis, which also revealed transcriptional dysregulation of transcripts related to GABA transmission and dopamine signalling in the medial PFC (Murphy

et al., 2010). Using real-time quantitative polymerase chain reaction (qPCR), a previous study has demonstrated that 8-week SI reduced PFC expression of *Grin2a* (encodes NMDA receptor subunit NR2A; Zhao et al., 2009). In the current experiment, while SI marginally downregulated *Grin2b* (encodes NR2B subunit; fold change= -0.56; FDR=0.049), *Grin2*a expression was not significantly affected, albeit the direction of change corresponds to that of the previous study (fold change= -0.73; FDR=0.06). Nevertheless, the method used to assess the changes seems to be critical, as both microarray analysis and *in situ* hybridisation (ISH) revealed increased, rather than decreased, NR2A transcript in the PFC of SI rats subjected to similar length of SI before behavioural testing and RNA extraction (Turnock-Jones et al., 2009; discussed later). Using various SI protocols, qPCR studies have also found increased frontal cortical mRNA expression of genes related to blood-brain barrier formation and maintenance of its integrity (including *ORLs*, *Vof21* and *Leng8*) and oxidative stress (e.g. *Nox2* and genes encoding its subunits), and genes encoding serine proteases (e.g. *Prep* which encodes prolyl endopeptidase), which was not reflected in the current study (Schiavone et al., 2009; Schiavone et al., 2017b; Zubkov et al., 2019). Similarly, the present study did not observe decreased frontal cortical IL-6, as well as altered *Bdnf* and *TrkB* transcripts, as have previously shown (Corsi-Zuelli et al., 2019; Li et al., 2016; Mikics et al., 2018). The lack of effect of SI on several genes implicated in schizophrenia, such as *Erbb4*, *Nrg1* and *Nr1a* in the current study, however, matches prior findings in the PFC (Hall et al., 2002; Swerdlow et al., 2013).

Whilst all the aforementioned studies share the same aim of measuring SI-associated differential gene expression, the studies have either used qPCR, DNA microarrays or ISH. Technology differences could account for the discrepancies in findings between the present experiment and prior studies. For instance, a recent study has demonstrated poor correlation between RNA-Seq read counts and microarray intensity, as well as fold change values from the two technologies (Trost et al., 2015). Although deriving a geometric mean from multiple microarrays appears

to improve fold change correlation between the two platforms, SI experiments typically only hybridise sample from each rat to one microarray (Trost et al., 2015; Turnock-Jones et al., 2009). Trost et al. (2015) also proposed better concordance, albeit not statistically significant, between RNA-Seq and qPCR fold change values, compared to those of microarrays. While we are unable to comment specifically on the agreement between the current data with prior studies on a statistical basis, the dissimilarities between the current data, acquired through RNA-Seq, and those generated by qPCR or DNA microarray, as outlined above, could be plausibly explained by low data concordance. Possible reasons include the limitations associated with microarrays, e.g. inability to discern splice variants and low abundance transcripts, non-specific hybridisation and cross-hybridisation, and qPCR-related challenges including inconsistent results due to inadequately standardised protocols (Costa et al., 2013; Trost et al., 2015; Zhao et al., 2014). Nevertheless, there exist studies which support consistent performance between RNA-Seq and either microarrays or qPCR, as well as those which found RNA-Seq superior than microarrays (Everaert et al., 2017; Fu et al., 2009; Marioni et al., 2008; Zhao et al., 2014). No known studies have shown that qPCR outperforms RNA-Seq or vice-versa. Despite offering greater sensitivity than non-radioactive ISH and allowing for examination of gene spatial expression and differential gene expression by semi-quantitative mRNA measurements, the inability to detect lowly expressed genes is a drawback of radioactive ISH (Carter et al., 2010; Jensen, 2014). Thus, this could explain any variability between the current results and those of ISH studies. It is vital to note that RNA-Seq is not without potential flaws, e.g. uneven coverage of target transcriptome attributed to positional bias introduced by RNA fragmentation and random hexamer priming, and short sequence reads leading to mapping uncertainty (Hansen et al., 2010; Zhao et al., 2014). Furthermore, RNA-Seq output is hugely dependent on the genome annotation database used and also method or packages used for reads counting, normalisation, differential gene expression analysis, pathway analysis and so forth (Evans et al., 2018; Han et al., 2015; Law et al., 2014).

Rat strain could also contribute to the discrepancy in findings. For instance, in one study comparing the effects of SI on *Nr1a* mRNA expression in Fawn-hooded and Wistar rats, apart from strain-specific decreased expression in all four hippocampal subregions examined (CA1, CA2, CA3 and dentate gyrus), a significant housing x strain interaction was revealed for most hippocampal subregions in the former, with SI seemingly normalised such reduced expressions (Hall et al., 2002). Thus, it would prove challenging comparing the current findings to prior studies using different rat strains. While the duration of SI could potentially yield differential outcomes between studies, a recent review found no association between SI duration and BDNF expression (Murínová et al., 2017). Additionally, in some studies, SI is followed by a resocialisation period to enable continued neurodevelopment after the critical isolation period, which may influence gene expression (Li et al., 2016). Hence, caution must be exercised when comparing results.

4.5.2 Pathway analyses

Various significant Reactome and KEGG pathways that we identified are implicated in schizophrenia, hence showing promise for SI as a model for schizophrenia. Enriched GO terms further confirmed the roles of significantly altered genes. Several genes, such *Tnrc6b*, *Tnrc6c*, *Fos*, *Jun*, *Prkca*, *Grin2b*, *Adcy1*, *Kcnj6* and *Itpr1* stood out as they connected multiple Reactome and KEGG pathways. These genes will be discussed in the context of prior gene expression findings in the SI rat model and their translational relevance to schizophrenia will be considered. Individual enriched pathways, grouped together based on function whenever possible, will then be explored.

4.5.2.1 Common DEGs shared by multiple enriched pathways

Tnrc6b and Tnrc6c

These two DEGs were implicated in 14 of the 34 significant Reactome pathways and were the sole genes altered in the pathways 'post-transcriptional silencing by small RNAs' and 'competing endogeneous RNAs regulate PTEN translation'. MiRNAs are a conserved class of endogenous small RNAs of 21-25 nucleotides that regulate a multitude of cellular processes through post-transcriptional repression of partially or fully complementary mRNAs (Chen et al., 2009; Pillai, 2005). To do so, the formation of a miRNA-induced silencing complex (miRISC), comprising mature miRNA and Argonaute proteins, and other proteins such as the miRNA-processing enzyme Dicer, is pivotal (Chen et al., 2009; Ding & Han, 2007). *Tnrc6b* and *Tnrc6c* encode for two of the three paralogues of *Drosophila melanogaster* GW182, trinucleotide repeat containing adaptor 6B and 6C, respectively. These proteins are proposed to form part of the miRISC by interacting with Argonaute proteins before association with the 3' untranslated region of target mRNA, thereby facilitating ribosome release and translocation of the miRISC-mRNA complex to the cytoplasmic granules P bodies or messenger ribonucleoproteins. Tnrc6b/Tnrc6c-mediated recruitment of deadenylation and decapping complexes then takes place to induce mRNA cleavage (Chen et al., 2009). Although no links have been made between *TNRC6B*/*TNRC6C* with schizophrenia, these proteins have been linked to altered cell adhesion, proliferation and migration, which are noted in schizophrenia (Hou et al., 2018; Murakami et al., 2013). Moreover, Tnrc6b is linked to disrupted speech, which could be relevant to schizophrenia, and is a potential competing endogenous RNA of the tumour suppressor gene phosphatase and tensin homologue deleted on chromosome 10 (PTEN; Eising et al., 2019; Zarringhalam et al., 2017). Thus, the fact that *Tnrc6b* and *Tnrc6c* are implicated in fourteen significant Reactome pathways suggests modified post-transcriptional regulation in the SI model. Post-transcriptional regulation has not been

probed in the SI model, however, this corresponds to clinical and post-mortem findings in schizophrenia, where dysregulated miRNA expression is observed both centrally and peripherally (Beveridge & Cairns, 2012).

Fos and Jun

Both *Fos* (*c-Fos*, *FosB*, *Fra1* and *Fra2*) and *Jun* (*JunB* and *JunD*) are IEGs of which rapid activation is stimulated following exposure to cellular stimuli such as growth factors, hormones and cytokines (Bejjani et al., 2019). Through common leucine-zipper domain interactions, proteins of *Fos* and *Jun*, alongside those of the activating transcription factor family and musculoaponeurotic fibrosarcoma family, form various combinations of heterodimers and homodimers, collectively termed the AP-1 transcription factor, to regulate diverse cellular processes such as cell proliferation, differentiation and apoptosis (Eferl & Wagner, 2003; Karin et al., 1997). Depending on dimer composition, AP-1 complex preferentially binds to distinct DNA response elements, e.g. 3'-5'-cyclic adenosine monophosphate-response element, to regulate activity of nearby promoters (Eferl & Wagner, 2003). Increasing AP-1 protein levels serve as a negative feedback mechanism to control AP-1 activity at the gene level, as well as regulation by transcriptional co-activators (Karin et al., 1997). At the protein level, AP-1 activity is modulated by phosphorylation (e.g. by MAPKs – extracellular signal-regulated kinases [ERKs], JNKs, p38 and Fos-regulating kinases), ubiquitylation and SUMOylation (Bejjani et al., 2019; Deng & Karin, 1994). In the SI model, while no groups have measured expression of AP-1, altered gene and protein expressions of *Fos* and *Jun*, have been shown (discussed above; Biro et al., 2017; Schiavone et al., 2009; Soga et al., 2015). Clinically, several SNPs in *FOS* and *JUN* have been associated with schizophrenia (Boyajyan et al., 2015).

Prkca

Ubiquitously expressed in all tissues, protein kinase C alpha is a lipid-sensitive serine/threonine protein kinase that is involved in numerous cellular responses such as cell division, adhesion, motility and apoptosis (Singh et al., 2017). In alignment with this perception, *Prkca* connects five distinct significant Reactome pathways and thirteen KEGG pathways involved in multiple cellular functions (Figure 4.6). Protein kinase C is speculated to play a role in prefrontal structural plasticity and cognitive function, as well as episodic memory, of which any dysregulation of these processes is highly relevant to psychiatric disorders like schizophrenia (Hains et al., 2009; MacLeod & Donaldson, 2014). The impact of SI on this specific protein kinase has not been assessed. Nonetheless, both rare and common genetic variants of *PRKCA* have been linked to schizophrenia, with dampened protein kinase C-mediated signalling also described (Carroll et al., 2010; Wang et al., 1999).

Grin2b

Owing to the glutamate dysfunction hypothesis of schizophrenia (section 1.1.5.2), ionotropic glutamate receptors including kainate, AMPA and NMDA receptors, as well as glycine transporter-1, have been extensively studied as potential drug targets (section 1.1.6). So far studies are largely in support of the potential involvement of *Grin2b* (encodes NMDA receptor subunit NR2B), which is crucial for neural development and learning and memory, in schizophrenia pathophysiology (Ohtsuki et al., 2001; Yang et al., 2015). In patients, altered methylation of *GRIN2B* promoter is linked to cognitive deficits whereas simultaneous alterations of polymorphic loci in both *GRIN2B* and *DRD2* (encodes dopamine D_2 receptor) impaired social adaptation (Alfimova et al., 2019; Fachim et al., 2019). The effect of SI on *Grin2b* expression has been discussed above under ‘differential gene expression analysis’.

Adcy1

Adcy1 encodes adenylate cyclase 1, a transmembrane enzyme that catalyses the conversion of adenosine triphosphate to the second messenger cyclic adenosine monophosphate, which in turn stimulates effector molecules such as protein kinase A (PKA) to elicit various physiological processes via different signalling mechanisms (Sassone-Corsi, 2012). Highly expressed in neurons, adenylate cyclase 1 is positively modulated by $G\alpha_s$, forskolin, calmodulin and protein kinase C whereas negatively modulated by $G\alpha_i$, Gβϒ and calmodulin kinase IV. It is associated with functions including circadian rhythm, synaptic plasticity, learning and memory and long-term potentiation (Hanoune & Defer, 2001). In schizophrenia, while genetic evidence is lacking, altered cAMP production due to a defect in the adenylate cyclase-G protein complex has been speculated (Kaiya, 1992). Moreover, reduced density of adenylate cyclase in post-mortem dentate gyrus and expression of miRNA-320a, which regulates *ADCY1*, have been reported (Dean et al., 1997; Vachev et al., 2016). It is unclear how SI influence adenylate cyclase activity, however, increased NMDA synaptic plasticity in the ventral tegmental area, likely mediated by PKA-dependent mGluR/IP_3 calcium signalling, has been previously demonstrated and could reflect aberrant adenylate cyclase activity (Whitaker et al., 2013).

Itpr1

Itpr1 encodes for inositol 1,4,5-triphosphate receptor type 1 (IP_3R1), a calcium channel expressed in virtually all cells that modulates intracellular calcium dynamics. Upon activation by IP_3 (formed following G-protein-coupled receptor-mediated phospholipase C stimulation) and calcium, IP_3Rs trigger calcium release from intracellular stores eliciting numerous cellular responses (Egorova & Bezprozvanny, 2018). Predominantly expressed in the brain, IP_3R1 is involved in synaptic plasticity. DISC1, mutation of which predisposes the afflicted individual to schizophrenia, is known to regulate both *Itpr1* mRNA expression and

IP_3R-mediated calcium release (Hisatsune & Mikoshiba, 2017). There is no direct evidence of aberrant IP_3R1-mediated calcium signalling in schizophrenia, however, altered phospholipase C expression has been demonstrated (Kim et al., 2015). In the SI model, increased IP_3 sensitivity, potentially due to altered PKA-dependent phosphorylation of IP_3R have been reported (Whitaker et al., 2013). Given that IP_3R1 is modulated by PKA, it is likely that this protein is altered by social deprivation, as the current data suggests (Taylor, 2017).

Kcnj6

This gene encodes a G protein-coupled inwardly rectifying potassium channel subunit known as KCNJ6, Kir3.2 or GIRK2, which is essential in neuronal excitability through maintaining the resting membrane potential and moderating action potential duration (Hibino et al., 2010). KCNJ6 is localised in the brain and either form heterotetramers with KCNJ3 (Kir3.1) or KCNJ9 (Kir3.3), or homotetramers. It plays a role in GABA transmission and tonic adrenergic activity and hypermethylation upstream of its gene was detected in schizophrenia subjects (Mill et al., 2008; Torrecilla et al., 2013). SNPs in the promoter region of *KCNJ6* are linked to alcohol and nicotine dependence (Clarke et al., 2011; Nishizawa et al., 2014). Whilst voltage-dependent potassium channels and small-conductance calcium-activated potassium channels have been researched in the SI model, no groups have probed SI-associated effects on G protein-coupled inwardly rectifying potassium channels (Martin et al., 2010; Quan et al., 2010).

4.5.2.2 Enriched pathways

P53 tumour suppressor-related pathways (Reactome)

The genes *Btg2*, *Plk3* and *Cnot6*, which encode the BTG anti-proliferation factor 2, polo like kinase 3 and CCR4-NOT transcription complex subunit 6, respectively, are shared by four tumour suppressor p53-associated

pathways. Other DEGs implicated in p53-associated pathways are *Fos*, *Jun*, *Dyrk2* (encodes dual specificity tyrosine phosphorylation regulated kinase 2), *Sesn3* (sestrin3), *Tsc1* (TSC complex subunit 1) and *Hipk2* (homeodomain interacting protein kinase 2); the latter links the p53-related pathways with pathways in which the methyl-CpG binding protein 2 (MeCP2) is involved (see later). The influence of SI on the genes mentioned is unknown. Haplotype analysis revealed positive association of the IEG *BTG2* with schizophrenia whereas qPCR following RNA-Seq validated reduced *BTG2* in the post-mortem superior temporal gyrus of schizophrenia subjects compared to controls (Deng et al., 2011; Wu et al., 2012). Gene clustering identified the potential involvement of Plk3, a serine/threonine kinase involved in cell cycle arrest in response to genotoxic (DNA damage) and non-genotoxic (mitotic spindle disruption) stress, in schizophrenia pathophysiology (Bahassi et al., 2002; Gilman et al., 2012). Whilst the contributions of *CNOT6* (regulates RNA lifecycle from synthesis to decay) and *HIPK2* (serine/threonine kinase that regulates transcription by integrating various stress signals) towards schizophrenia are yet to be defined, downregulation of miRNA-183-5p, which regulates these genes, has been reported in the peripheral blood of patients (Collart, 2016; Schmitz et al., 2014; Vachev et al., 2016). Dyrk2, which akin to Hipk2 belongs to the CMGC group of protein kinases and is involved in neuronal development and function, has been found located near a differentially methylated position in patients when compared to controls (Montano et al., 2016; Schmitz et al., 2014). In the context of schizophrenia, the contribution of Sestrin-3, a known regulator of pro-inflammatory genes in macrophages, microglia and neurons, is unknown (Johnson et al., 2015). However, significant downregulation in its expression has been observed following knockdown of ZNF804A, a risk gene of schizophrenia (Chapman, 2013). The tumour suppressor Tsc1 is a component of the tuberous sclerosis complex which inhibits mTOR signalling. Disrupted mTOR transduction leading to compromised neural development and function has been implicated in schizophrenia and other neurodevelopmental or psychiatric disorders, as well as in the SI model (Gururajan & van den Buuse, 2014; Meffre et al., 2012).

The tumour suppressor p53, a transcription factor, is thought to be involved in cellular events fundamental to tumour suppression, including induction of transient cell cycle arrest, cellular senescence, apoptosis and DNA repair, following activation by stressors such as DNA damage, oxidative stress, hypoxia and nerve growth factor withdrawal (Tedeschi & Di Giovanni, 2009). It also participates in non-apoptotic processes including aging, nervous system development, maintenance of mature neurons and post-injury neuron regeneration, and is linked to numerous developmental syndromes (Bowen & Attardi, 2019; Jacobs et al., 2006; Pentimalli, 2018; Tedeschi & Di Giovanni, 2009). Given its role in neurodevelopment, the TP53 gene has been put forward as a candidate gene for schizophrenia. No known studies have explicitly examined the impact of p53 expression in SI rats, but besides dysregulated stress responses, SI of female Sprague-Dawley rats has been linked to increased mammary tumour burden and tumour malignancy (Hermes et al., 2009). The facts that p53 activation is triggered by chronic inflammation and SI is associated with chronic inflammation provide confidence for the current findings on TP53 (Möller et al., 2013; Staib et al., 2005). Nonetheless, human genetic studies have yielded inconclusive findings. For instance, a case-control study in the Chinese population has revealed positive association of rs2078486, a SNP of the *TP53* gene, with schizophrenia (Yang et al., 2004). While two case-control and family-based studies conducted in Caucasian subjects backed such positive association (Ni et al., 2005), other studies were in opposition (Chiu et al., 2001; Lung et al., 2009).

Cellular senescence (Reactome)

Reactome analysis revealed several significant pathways related to cellular senescence, including those induced by oxidative stress or oncogene. Apart from *Fos* and *Jun*, DEGs that were implicated are *Tnik*, *Kdm6b* and *Erf*. *Tnik* encodes for the TRAF2 and NCK interacting kinase (MAP4K), a highly expressed DISC1-interacting serine/threonine kinase in the brain that is enriched in postsynaptic densities and synaptosomes.

It is implicated in stress response, postsynaptic glutamatergic signalling, dendritic spine formation and cognitive function (Baltussen et al., 2018; Coba et al., 2012). A GWAS study has identified a SNP in *TNIK* as one of the top twelve hits in African-American subjects with schizophrenia (Shi et al., 2009). Such linkage between *TNIK* and schizophrenia is supported by convergent functional genomics analyses (Ayalew et al., 2012). In addition, SNPs in *TNIK* has been linked to prefrontal perturbations (Potkin et al., 2009). The product of *Kdm6b* is lysine demethylase 6B, a histone demethylase for H3K27. Upon activation by inflammatory substrates and cellular or environmental stressors, it engages in the regulation of cellular senescence and immune responses via demethylase-dependent and independent mechanisms (Salminen et al., 2014). Albeit *KDM6B* has been proposed to contribute to neuron survival and is associated with autism spectrum disorders, no links have been established between *KDM6B* and schizophrenia (Sanders et al., 2015; Wijayatunge et al., 2014). ETS2 repressor factor, a ubiquitously expressed ETS-domain transcriptional repressor encoded by *Erf*, is a direct effector of the ERK MAPKs. It is implicated in processes including cell cycle regulation, apoptosis, haematopoiesis, macrophage differentiation, embryonic development and osteogenesis (Oikawa & Yamada, 2003; Papadaki et al., 2007; Peraki et al., 2017; Sharrocks, 2001). Although *ERF* is neither a candidate gene nor known to be differentially expressed in schizophrenia, it is a putative susceptibility gene for domesticated traits in schizophrenia (e.g. facial asymmetry and aggressive behaviour), possibly due to hypofunction of the neuronal crest (Benítez-Burraco & Lattanzi, 2017).

No studies have examined cellular senescence in SI rats. Multiple studies, however, have focused on the effect of SI on oxidative stress, which is known to contribute to cellular senescence or ageing (Möller et al., 2011; Möller et al., 2013; Schiavone et al., 2009; Shao et al., 2015). Multiple lines of evidence support cellular senescence and accelerated ageing in schizophrenia. For instance, schizophrenia is linked to reduced life expectancy, accelerated age-related decline in white matter integrity,

increased prevalence of metabolic abnormalities associated with ageing and shortened telomere length (Okusaga, 2014; Yu et al., 2008). Nonetheless, decreased life expectancy is also related to factors such as adverse effects of antipsychotic treatment, unhealthy lifestyle, somatic comorbidities, suboptimal treatment of somatic disorders and genetic factors, apart from unnatural causes of death including suicide and accidental deaths (Laursen, 2019). Researchers have also argued for schizophrenia as a whole body disorder termed segmental progeria, given the resemblance of its clinical, biochemical and molecular features to premature ageing diseases (Papanastasiou et al., 2011). More recently, schizophrenia is considered to potentially reflect a state of both accelerated and cell senescence (Fernandez-Egea & Kirkpatrick, 2017).

Circadian clock (Reactome and KEGG)

Prolonged SI appears to perturb the circadian clock, with several genes specific to circadian entrainment being significantly downregulated. One such example is the circadian pacemaker *Per1*, which is overrepresented by both Reactome and KEGG pathways. *Per1* is vital for proper circadian entrainment and rhythmicity of other clock genes (Numano et al., 2006). Consistent with the current data, altered *PER1* gene expression has been demonstrated in post-mortem temporal cortices of patients with schizophrenia (Aston et al., 2004). Reactome analysis also revealed three further significant genes in the circadian pathway. BHLHE40, a known circadian modulator, is thought to modulate hippocampal neuronal excitability and synaptic plasticity, both of which abnormalities are implicated in schizophrenia (Hamilton et al., 2018; Sanderson et al., 2012). Moreover, it interacts with the zinc finger domain containing 804A, where a significant association between a SNP in this gene with schizophrenia is found (O'Donovan et al., 2008). Chromodomain helicase DNA-binding 9 (CHD9) is an ATP-dependent chromatin remodeller associated with transcriptional regulation, cellular proliferation and DNA repair (Mills, 2017). While its role in schizophrenia and circadian regulation remains obscure, CHD7, of the same family subgroup, is a

putative risk gene linked to cognitive deficits in schizophrenia (Whitton et al., 2016). Mutations of CHD8, another subgroup member, are implicated in both schizophrenia and autism (McCarthy et al., 2014). SIK1, a serine/threonine protein kinase implicated in cell proliferation and apoptosis, suppresses the shifting effects of light on the suprachiasmatic nuclei through attenuation of CREB-mediated *Per1* and *Per2* transcription, thus preventing rapid entrainment to abnormal light exposure (Jagannath et al., 2017). While it is currently linked to rare CNVs, further study of its role in schizophrenia has been suggested (Lang et al., 2014). Together, these support potential roles of these DEGs in schizophrenia pathology. Of note, numerous light-induced genes in the circadian clock, e.g. *Fos*, *Nr4a1*, *Egr1*, *Egr3* and *Dusp1* are also overrepresented by Reactome or KEGG analysis, thereby implying further complex interactions that are not revealed by currently available pathway analysis tools.

One study has examined the impact of SI on the circadian clock, although it was on the potential involvement of CLOCK in SI-induced alterations in gonadotropin-inhibitory hormone neurons in the dorsomedial hypothalamus, concerning sexual dysfunction (Teo et al., 2017). Meanwhile, disrupted sleep inhibited startle habituation in SI rats in a PPI paradigm, thus indicating impaired response to novel stimulus, linking SI-induced circadian abnormalities to schizophrenia-related phenotype (Chang et al., 2014). In schizophrenia, disrupted circadian rhythm (including irregular sleep-wake patterns, delayed or advanced sleep phase and free-running sleep-wake cycles), altered sleep architecture (e.g. reduced rapid eye movement latency) and abnormal melatonin rhythms are well described, with up to 80% of patients having issues with sleep onset and maintenance, thus potentially perturbed circadian pathway is of translational relevance (Cohrs et al., 2008; Monti et al., 2013). Furthermore, various neurotransmitters and neuropeptides (e.g. GABA, dopamine, glutamate, serotonin and pituitary adenylate cyclase-activating polypeptide) are involved in the regulation of the mammalian circadian clock whereas neurotransmitter receptors, including

dopaminergic and muscarinic cholinergic receptors which pathways are overrepresented by the current KEGG analysis, are influenced by circadian rhythm (Hegazi et al., 2019; Wirz-Justice, 1987). Considering also that SI is potentially a cause of sleep and circadian rhythm disruption in schizophrenia, which can profoundly influence emotion, cognition and somatic responses, the above evidence collectively support the SI model as a model for schizophrenia (Pritchett et al., 2012).

Cell/focal adhesion, synaptic function and plasticity (Reactome/KEGG)

Reactome analysis revealed 'Interactions between L1 and Ankyrin' as one of the top significant pathways, together with the genes *Kcnq2*, *Ank3*, *Ank2* and *Sptb*. No SI studies have investigated the impact of SI on these genes. *Kcnq2* encodes for a potassium voltage-gated channel localised in neuronal cells, which exerts inhibitory modulation over neuronal excitability via the generation of slow activating and deactivating M-current (Selyanko et al., 1999). Importantly, potassium M-currents appear to be modulated by the muscarinic receptors M_1 and M_3, which are both implicated in schizophrenia (Fukuda et al., 1988). In a genetic model for schizophrenia (22q11.2 deletion), compromised KCNQ2 function in parvalbumin-containing interneurons led to dampened D_2 receptor-mediated inhibition of pyramidal neurons (Choi et al., 2018). *KCNQ2* variants have also been implicated in schizophrenia and related disorders such as bipolar disorder (Lee et al., 2013). Apart from participating in membrane biogenesis and subcellular protein migration, ankyrins organise and maintain specialised membrane domains by connecting membrane proteins such as ion channels and transporters (e.g. sodium/calcium exchanger, IP_3R and voltage-gated potassium channel subunits KCNQ2/3), cell adhesion molecule (L1 family of CAMs) and signalling components with actin or β-spectrin cytoskeletons. *Ank2* and *Ank3* represent splice isoforms of the ankyrin gene, which undergo further alternative splicing encoding various Ankyrin-B and Ankyrin-G isoforms, respectively (Cunha & Mohler, 2009). Despite recent emerging evidence supporting potential *ANK2* association with schizophrenia,

information pertaining *ANK2* contribution towards schizophrenia is relatively lacking thus hindering conclusive interpretation (Cai et al., 2018; Yazdani et al., 2018). Nevertheless, Ankyrin-B is known to be repressed by miRNA-101, which expression is altered in schizophrenia, to restrict excessive synapse formation (Lippi et al., 2016). Although *ANK3*, whose protein is essential for the assembly of axon initial segment and nodes of Ranvier, normal forebrain GABAergic synaptic connectivity and hippocampal-prefrontal synchrony, has been strongly associated with schizophrenia, such findings are not consistently replicated (Gella et al., 2011; Jenkins et al., 2015; Nelson et al., 2018; Yuan et al., 2012). The spectrin skeleton, composed of α and β-spectrin subunits, interacts with proteins such as L1 and ankyrins to perform cellular functions including cell morphology regulation, plasma membrane organisation and synapse stabilisation as described above (Machnicka et al., 2012). In schizophrenia, a variant of the nonerythroid alpha-spectrin gene has been researched but polymorphisms of *SPTB*, which encodes β-spectrin, remain unexplored (Murakami et al., 1999).

The significant Reactome pathways ‘neurexins and neuroligins’, ‘protein-protein interaction at synapses’ and ‘assembly and cell surface presentation of NMDA receptors’ further imply altered synaptic cell adhesion molecule properties and therefore synaptic dysfunction in the SI model. Several downregulated genes are common to these pathways, including *Grin2b*, *Cask* and *Apba1*. *Syt7* and *Dlgap2* are shared between the first two pathways. To ensure proper glutamatergic transmission, newly assembled NR2B-containing NMDA receptors in the soma have to be transported to the dendrites. This is achieved by direct interaction between KIF17, a microtubule-dependent molecular motor, and APBA1, a neuron-specific scaffolding protein which complexes with further scaffolding proteins CASK and LIN-7 (Setou et al., 2000). Altered *CASK* and *APBA1* expressions have been previously demonstrated in schizophrenia, suggesting abnormal NR2B trafficking as a potential mechanism of NMDA receptor hypofunction (Kristiansen et al., 2010). Transcriptional dysregulation of neurotransmission-related genes *SYT7*

and *DLGAP2*, encoding synaptotagmin 7 and Discs large-associated protein 2, respectively, has also been observed (Bowden et al., 2008; Soler et al., 2018). The former, a pre-synaptic calcium sensor, is implicated in synaptic facilitation related to short-term plasticity whereas the latter is indispensable for glutamatergic postsynaptic density organisation (Jackman et al., 2016; Soler et al., 2018).

KEGG analysis provided further validation of modified long-term potentiation, with *Grin2b* similarly implicated, together with signalling-associated genes *Prkca*, *Adcy1* and *Itpr1*. *Prkca*, *Arhgap5*, *Xiap*, *Rock2*, *Jun* and *Itgb8* are associated with focal adhesion pathway. Rho GTPase activating protein 5 (p190B; encoded by *Arhgap5*) represents a principal negative regulator of the Rho family of small guanosine triphosphatases (GTPases), e.g. RhoA, which play crucial roles in actin-based processes including cell division, gene transcription regulation and membrane trafficking. Located at the nexus of multiple signalling pathways, it is implicated in neuronal and mammary development, and cancer progression (Héraud et al., 2019). Whilst another isoform encoded by the gene *ARHGAP35* has been identified as part of the mechanism underlying suicide in schizophrenia, the link between ARHGAP5 and schizophrenia remains uncertain (Li et al., 2017). Notwithstanding, a refined CNV analysis associated *ARHGAP5* loss-of-function with developmental delay, which is highly relevant to schizophrenia (Coe et al., 2014; Niemi et al., 2003). *Xiap* encodes X-linked inhibitor of apoptosis, which blocks apoptosis by directly suppressing the proteolytic activity of activated effector caspases and participates in multiple transduction pathways, e.g. MAPK pathway, as an E3 ligase (Latour & Aguilar, 2015). In female but not male patients with schizophrenia, hypermethylation in the promoter region of *XIAP* was found, thus implying increased apoptosis (Rukova et al., 2014). *Rock2*, on the other hand, encodes Rho associated coiled-coil containing protein kinase 2, a ubiquitous serine/threonine kinase shown to control dendritic spine morphology, of which abnormalities are widely demonstrated in schizophrenia (Swanger et al., 2015). Finally, *ITGB8* (encodes integrin subunit beta 8, which

constitutes heterodimeric transmembrane receptors enabling cell-extracellular matrix linkages) is one of the top 50 DEGs identified in patients with 7-18 years of diagnosed schizophrenia (Huttenlocher & Horwitz, 2011; Narayan et al., 2008).

MAPK signalling (Reactome/KEGG)

The two MAPK-related Reactome pathways share common DEGs between themselves and with multiple other pathways including oestrogen and synaptic function-associated pathways. Implicated DEGs are *Dusp1*, *Dusp6*, *Irs2*, *Ksr2*, *Ncoa3*, *Jun*, *Fos*, *Sptb*, *Grin2b*, *Tnrc6b* and *Tnrc6c*. *Dusp1* and *Dusp6* encode for dual specificity phosphatases 1 and 6, respectively. The resultant protein tyrosine phosphatases dephosphorylates MAPKs, which are involved in immune and inflammatory responses and regulation of processes in the brain (Pérez-Sen et al., 2019; Seternes et al., 2019). Consistent with the current data, *DUSP1* is downregulated in the superior temporal gyrus of patients with schizophrenia (Wu et al., 2012). DUSP6, in contrast, is associated with bipolar disorder but not schizophrenia (Lee et al., 2006). Insulin receptor substrate 2 (encoded by *Irs2*) is a cytoplasmic adaptor protein that transduces extracellular stimuli into the intracellular environment upon activation by upstream tyrosine kinase, cytokine or hormone receptors. This stimulates downstream signalling such as the PI3K/Akt/mTOR and MAPK signalling pathways, promoting cell proliferation and transcription activation and other biological processes (Machado-Neto et al., 2018). Insulin dysregulation is a potential cause of schizophrenia (Ghasemi et al., 2013). Although not associated with schizophrenia, one SNP in the *IRS2* gene appears to influence the manifestation or lack of auditory hallucinations in subjects with schizophrenia (Kim et al., 2013). *Ksr2* encodes kinase suppressor of Ras 2, a scaffolding protein highly expressed in the brain which contributes towards 5’ AMP-activated protein kinase and Raf/MEK/ERK-dependent modulation of energy balance and glucose homeostasis (Guo et al, 2017). While linkage analysis has linked the locus where *KSR2* is located to schizophrenia,

association analysis did not reveal significant association between a *KSR2* variant and schizophrenia (Sprooten et al., 2015). *Ncoa3* encodes nuclear receptor coactivator 3, which has roles in somatic growth, female reproductive function, mammary gland development and tumorigenesis (Xu et al., 2000). It is unclear whether *Ncoa3* is associated with schizophrenia.

Apart from *Fos*, *Jun*, *Dusp1* and *Dusp6*, KEGG analysis implicated the involvement of different DEGs: *Prkca*, *Nr4a1*, *Taok1*, *Cacna1e* and *Gadd45b*. *Taok1* encodes TAO kinase 1, a member of the sterile 20-like family capable of stimulating JNK and p38 MAPK pathways and is involved in developmental and regenerative tissue growth, apoptosis, microtubule dynamics, neurodevelopment and IL-17-mediated inflammatory signalling (Poon et al., 2011; Zhang et al., 2018c). In cortical excitatory neurons derived from induced pluripotent stem cells (iPSCs) from a schizophrenia subject presenting a rare *DISC* mutation, altered TAOK1 expression and activity were observed (Bentea et al., 2019). Nevertheless, various drawbacks are associated with the use of iPSCs in disease modelling, including high rate of apoptosis and insertional mutagenesis (Singh et al., 2015). Albeit *TAOK1* was found to be enriched for *de novo* mutations in individuals with autism spectrum disorder or intellectual disability and/or developmental delay, no direct association between *TAOK1* and schizophrenia has been made (Coe et al., 2019; Dulovic-Mahlow et al., 2019). *Cacna1e* encodes calcium voltage-gated channel subunit alpha1 E (also $Ca_v2.3$), the principal pore-forming components of the high voltage-activated R-type calcium channels which are implicated in various forms of epilepsies (Helbig et al., 2018). With both pre- and postsynaptic functions such as neurotransmitter release and long-term potentiation, $Ca_v2.3$ has also been linked to the regulation of cardiac and neuronal oscillations, as well as insulin (Holmkvist et al., 2007; Schneider et al., 2015). Whilst genes encoding voltage-gated calcium channel α-subunits such as *CACNA1C* (encodes L-type $Ca_v1.2$) and *CACNA1I* (T-type $Ca_v3.3$) are acknowledged risk genes of schizophrenia, it was not until recently that a molecule-based genetic

association study revealed the potential involvement of *CACNA1E* alongside *CACNA1G* (T-type $Ca_v3.1$; Li et al., 2018c; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Lastly, increased expression and reduced *BDNF* promoter binding of growth arrest and DNA-damage-inducible beta, which is encoded by *Gadd45b* and coordinates epigenetic regulation through DNA demethylation, has been linked to psychosis (Gavin et al., 2012). Given that BDNF influences neuronal function and synaptic plasticity and altered methylation at *BDNF* promoters has been demonstrated in schizophrenia, it is plausible that modified GADD45B function underlies the pathology of schizophrenia (Ikegame et al., 2013). No known studies have investigated these DEGs in the SI model, however, altered BDNF expression and components of MAPK signalling, which bears translational relevance to schizophrenia, have been demonstrated (Funk et al., 2012; Murínová et al.,2017; Nieto et al., 2013; Shortall et al., 2018).

Oestrogen-dependent gene expression/oestrogen signalling (Reactome/KEGG)

The DEGs *Jun*, *Tnrc6b*, *Tnrc6c*, *Ncoa3*, *Fosb*, *Fos* and *Strn* are implicated in the two significant oestrogen-related pathways revealed by Reactome. *Strn* encodes striatin, a subunit of the serine/threonine phosphatase, PP2A, which is mainly concentrated in the dendritic spines of striatal medium spiny neurons. PP2A in turn constitutes part of the striatin-interacting phosphatase and kinase complex responsible for processes including the regulation of motor function and dendritic development (Bartoli et al., 1999; Li et al., 2018c). Of note, PP2A is capable of regulating dopaminergic and glutamatergic signalling by dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32; Ahn et al., 2007). The connection between striatin-1 and schizophrenia is unknown, but pathogens responsible for infections including rubella virus and *Toxoplasma gondii* are demonstrated to express proteins with homology to striatin, a known autoantigen in schizophrenia. Therefore, it is likely that homologous proteins from the pathogens trigger host

antibody-mediated knockdown of striatin, contributing to the pathophysiology of schizophrenia (Carter, 2011). Since abnormal DARPP-32 phosphorylation or expression and dopaminergic signalling in the medium spiny neurons are described in schizophrenia, altered striatin-1 expression could underlie such abnormalities (McCutcheon et al., 2019; Wang et al., 2017b). No studies have investigated the effects of SI on striatins or oestrogen signalling, however, oestrogen dysfunction is widely implicated in schizophrenia (Gogos et al., 2015).

DEGs implicated in the significant KEGG 'oestrogen signalling pathway' (*Fos*, *Adcy1*, *Kcnj6*, *Itpr1* and *Jun*) are components of general signalling or cell excitability and have been covered above.

Pathways involving Prkca and related pathways (Reactome)

The gene *Prkca* was implicated in five pathways. DEGs participating in those pathways that have not been discussed previously include *Fmnl2*, *Trio*, *Srgap3*, *Arhgef12*, *Arap2*, *Myo9a*, *Cit*, *Cbl* and *Lpin1*. Related significant pathways ('Rho GTPases activate CIT',' Rho GTPase cycle' and 'Sema4D induced cell migration and growth-cone collapse'), revealed repeated participation of several DEGs, such as *Cit* and *Srgap3*. Fmnl2 encodes formin like 2, an effector of actin and microtubule regulation widely expressed in the neurons and glia, which is modulated by the Rho family GTPase Cdc42. It is involved in processes such as cell migration and invasion and dendritic spine formation (Galbraith & Kengaku, 2019). FMNL2 is deemed to be clinically relevant to schizophrenia, with one GWAS study in a Japanese population revealing a SNP in the *FMNL2* as one of the top 20 SNPs (Sundararajan et al., 2018; Yamada et al., 2011). Furthermore, *FMNL2* mRNA is regulated by miRNA-137, a replicated genetic risk factor of schizophrenia (Liang et al., 2013; The Schizophrenia Psychiatric GWAS Consortium, 2011). *Trio* encodes for Rho guanine nucleotide exchange factor, a large multi-domain protein with three enzymatic domains which facilitates GTP substitution of GDP on Rho GTPases. Trio has been ascribed to play a

part in nervous system development, particularly regarding axon outgrowth and guidance, and is implicated in cell adhesion and mitosis (Schmidt & Debant, 2014). *TRIO* variants are associated with neurodevelopmental disorders such as schizophrenia and intellectual disability (Katrancha et al., 2017). *Srgap3* encodes SLIT-ROBO Rho GTPase activating protein 3, which mediates actin-based cytoskeletal remodelling, leading to neurodevelopmental events including neuronal differentiation and dendritic spine growth (Dai et al., 2014). A duplication in the *SRGAP3* gene has been linked to childhood onset schizophrenia (Wilson et al., 2011). ARHGEF12, like TRIO, activates Rho GTPases by promoting the exchange of GDP for GTP whereas *Arap2* encodes an Arf6-specific GTPase-activating protein involved in RhoA-mediated focal adhesion (Schmidt & Debant, 2014; Yoon et al., 2006). The involvement of *ARHGEF12* and *ARAP2* in schizophrenia is unknown. *Myo9a*, on the other hand, encodes myosin IXA, an actin-based motor protein implicated in synaptic transmission, long-term potentiation and cognition (Folci et al., 2016). A previous RNA-Seq study revealed differentially expressed *Myo9a* in the post-mortem cerebellar cortex of patients with schizophrenia (Mudge et al., 2008). Citron Rho-interacting serine/threonine kinase, encoded by *Cit*, is long known to take part in cytokinesis, the last stage of cell division. However, recently, novel functions such as spindle orientation during metaphase, DNA damage control, Golgi complex organisation and epigenetic gene silencing have been assigned to this kinase (D'Avino, 2017). *CIT* gene is associated with schizophrenia (Johnstone et al., 2015; Xu et al., 2008). Additionally, CIT interacts with DISC1, which expression is disrupted in schizophrenia (Nicodemus et al., 2010). *Cbl* gene encodes for Cbl proto-oncogene, a RING domain-containing E3 ubiquitin-protein ligase and adaptor protein involved in a wide range of biological functions, e.g. neurite growth, innate and adaptive immunity , regulation of actin cytoskeleton and bone resorption (Swaminathan & Tsygankov, 2006). No literature has reported the link between *CBL* and schizophrenia. Nonetheless, mutations in the genes of various E3 ligases including those consisting a RING catalytic domain are known to contribute to schizophrenia (George et al., 2018).

Although decreased expression of Sprouty2, a prominent binding partner of the Cbl proto-ontogene has been observed in schizophrenia alongside BDNF changes, such changes are thought to reflect effects of antipsychotics rather than being intrinsic to the pathology (Fong et al., 2003; Pillai, 2008). Lastly, *Lpin1* encodes lipin 1, a dual-function phosphatidate phosphatase enzyme essential for normal lipid homeostasis (Chen et al., 2015). There is no evidence that *Lpin1* is clinically important in schizophrenia, however, disrupted lipid metabolism is well established in schizophrenia (Solberg et al., 2016; Yang et al., 2017). In the SI model, adipose tissue dysfunction has been identified by a previous research (Schiavone et al., 2017a). Consistent to the current data, alterations in signalling mechanisms involving Rho GTPases, IP_3/calcium and semaphorin, as well as nuclear lamins (important for neuronal migration) are implicated in schizophrenia (Berridge, 2014; Coffinier et al., 2011; Gilabert-Juan et al., 2015; Ide & Lewis, 2010).

Molecular functions (Reactome)

In the pathway 'RNA polymerase III transcription termination', the genes *Nfix* and *Nfib*, encoding nuclear factor I X and nuclear factor I B, respectively, are implicated. The resultant proteins, both belonging to the nuclear factor I family of transcription factors, are vital for normal development in terms of cellular proliferation, differentiation and migration as well as lineage progression. In particular, both proteins are involved in astroglial differentiation and astroglia dysfunction potentially underlie schizophrenia (Chen et al., 2017; Trépanier et al., 2016). No known studies have inquired into the impact of SI on these transcription factors and *NFIX* has not been investigated in the context of schizophrenia. SNPs in *NFIB*, however, is connected to abnormal corpus callosum morphology in schizophrenia (Gupta et al., 2015). Although not exclusive to schizophrenia, both NFIX and NFIB have been proposed as strong candidate genes of intellectual disability and/or brain abnormalities (Schanze et al., 2018).

DEGs implicated in the two MeCP2-related pathways (*Trnc6b*, *Tnrc6c*, *Hipk2* and *Grin2b*) have been discussed and therefore will not be explored further. MeCP2 protein, a member of the methylated DNA-binding protein family, is highly expressed in the brain and operates as both an activator and repressor of gene transcription. In addition, it also regulates RNA splicing (Chin & Goh, 2019). There is no available data on SI-associated effects on MeCP2 expression and function. However, *MECP2* is implicated in schizophrenia and alterations in its expression is found to influence aggression (Cohen et al., 2002; McCarthy et al., 2014; Tantra et al., 2014).

Two other pathways related to molecular functions are 'Runx1 regulates genes involved in megakaryocyte differentiation and platelet function' and 'Runx2 regulates genes involved in cell migration'. The DEGs *Tnrc6c*, *Tnrc6b*, *Kmt2a* and *Nr4a3* were implicated in the former whereas *Itgbl1* were associated with the latter. *Kmt2a* encodes lysine methyltransferase 2A, which methylates histone H3 on lysine 4 (H3K4) to drive gene transcription. In schizophrenia, *KMT2A* deficiency has been shown to affect cognition and emotion including working memory and anxiety, besides reducing glutamate decarboxylase 1, a GABA-synthetic enzyme (Bharadwaj et al., 2014; Black & Whetstine, 2013). Therefore, altered *KMT2A* expression could play an important role in schizophrenia pathogenesis. In support of this, patients with schizophrenia were reported to have elevated blood *KMT2A* mRNA levels (Huang et al., 2016). Furthermore, miRNA-320a which regulates the transcription of *KMT2A* is downregulated in schizophrenia (Vachev et al., 2016). Encoded by the *Nr4a3* gene, nuclear receptor subfamily 4 group A member 3 is an IEG that has functions in inner ear and hippocampal development, immune homeostasis and T-cell development, as well as neutrophil survival and dendritic cell migration amongst others (Park et al., 2016; Prince et al., 2017; Safe et al., 2016). SNPs within NR4A3 is linked to heavy smoking in schizophrenia while reduced NR4A3 mRNA levels have been detected in patients showing elevated inflammatory makers in the dorsolateral PFC (Novak et al., 2010; Tsai et al., 2017). A

very rare CNV overlapping a miRNA resulting in loss of function of *ITGBL1*, which encodes the integrin subunit beta like 1, has been detected in subjects with schizophrenia (Warnica et al., 2015). No known SI studies have examined the DEGs associated with these pathways.

Other significant KEGG pathways

Several KEGG pathways, such as 'proteoglycans in cancer', 'choline metabolism in cancer', 'vascular smooth muscle contraction', 'hepatitis B' and 'aldosterone synthesis and secretion' are irrelevant to schizophrenia and therefore will not be reviewed.

Similar to those that overlap with Reactome findings and have been discussed above (circadian entrainment, MAPK signalling pathway, oestrogen signalling pathway, long-term potentiation and focal adhesion), in the remaining KEGG pathways (amphetamine addiction, cholinergic synapse, oxytocin signalling pathway and dopaminergic synapse), several DEGs were implicated repeatedly, namely *Prkca*, *Fos*, *Itpr1*, *Adcy1*, *Kcnj6*, *Grin2b* and *Jun*. These DEGs, together with *FosB* (implicated in amphetamine addiction), *Rock2* (oxytocin signalling), *Nr4a1* (aldosterone synthesis and secretion) as well as *Kcnq2* (cholinergic synapse) have been described above. Thus, only two DEGs associated with amphetamine addiction (*Arc*) and cholinergic synapse (*Chrm4*) will be explored here.

Amphetamine addiction

Arc encodes an immediately early protein, activity-regulated cytoskeleton-associated protein. The resultant protein is only expressed in calcium/calmodulin-dependent protein kinase II-positive principal neurons and have roles in long-term potentiation and memory consolidation, long-term depression and homeostatic synaptic scaling (Managò & Papaleo, 2017). Consistent with the current study, downregulation of Arc has been reported by prior SI studies (Levine et

al., 2007; Pisu et al., 2011; Wall et al., 2012). Mutations, hypermethylation and potentially reduced expression of *ARC*, as well as SNP of the gene, are associated with schizophrenia (Chuang et al., 2016; Fromer et al., 2014; Guillozet-Bongaarts et al., 2014; Huentelman et al., 2015).

Substance abuse including AMPH abuse is considered to be at least 10 times more prevalent in individuals with early psychosis or schizophrenia in comparison to the general population (Fowler et al., 1998; Sara et al., 2014). In concordance with this notion, AMPH use is associated with increased risk for readmission in schizophrenia patients, as well as elevated rates of non-mental health admission, self-harm, homelessness and infectious diseases (Sara et al., 2014; Thomsen et al., 2018). Similar to schizophrenia, the SI rat model is associated with increased AMPH self-administration (Bardo et al., 2001). Isolates also have altered profile of AMPH-induced neurotransmitter release and consistently exhibit AMPH-induced locomotor hyperactivity compared to social controls (Fone & Porkess, 2008).

Cholinergic synapse

Chrm4 encodes for muscarinic acetylcholine receptor M_4, a pre-/ postsynaptic autoreceptor which decreased expression is consistently observed in the post-mortem brains of schizophrenia patients (Carruthers et al., 2015). M_4 receptors are highly expressed in brain areas rich in dopamine receptors, especially the striatum, with numerous evidence converging to support its role in modulating abnormal dopamine neurotransmission in schizophrenia (Jeon et al., 2010; Tzavara et al., 2004). As such, M_4 receptor PAMs have been developed and show promise as novel antipsychotics and cognitive enhancers (Carruthers et al., 2015). Besides M_4 receptors, M_1 receptors have been linked to schizophrenia whereas altered transcripts of M_5 and α_7-nicotinic receptors confer susceptibility jointly (Scarr, 2012). No groups have evaluated M_4 receptor expression in SI rats, despite hippocampal

dysfunction attributed to modified muscarinic activity has been described (Muchimapura et al., 2002). Hippocampal M_1 receptor mRNA expression was found to be unaltered (Del-Bel et al., 2002).

Oxytocin signalling

Oxytocin represents a nonapeptide neurohormone synthesised in the hypothalamus which is known to play a part in social or affiliative behaviour, stress-related mechanisms and neuroadaptation, including drug addiction, learning and memory (Sarnyai & Kovács, 2014). It also interacts with other neurotransmitter systems, such as the dopaminergic, glutamatergic and serotonergic system, which are dysregulated in schizophrenia (Feifel et al., 2016; Sarnyai & Kovács, 2014). In patients with schizophrenia, changes in plasma oxytocin and oxytocin receptor levels have been reported, thus indicating potentially disrupted oxytocin signalling (Aydin et al., 2018; Uhrig et al., 2016). Considering the plethora of studies supporting a beneficial effect of oxytocin on social behaviour and cognition, there is a surge in clinical studies assessing oxytocin administration, primarily through the intranasal route, as a potential treatment for schizophrenia, on its own or as an adjunct therapy (Bradley & Woolley, 2017). Oxytocin-related alleviations of the three symptom domains of schizophrenia have been rather encouraging, however, clinical findings are hugely variable and is challenged by issues including uncertainty regarding intranasal oxytocin penetration of the brain (as oxytocin is unable to traverse the blood brain barrier) and publication bias (Feifel et al., 2016; Lee et al., 2019a; Leng & Ludwig, 2016). Regardless of sex, SI rats are associated with reduced plasma oxytocin and striatal oxytocin receptor binding and enhanced *Oxt* mRNA expression in the paraventricular nucleus (Harvey et al., 2019; Oliveira et al., 2019). Socially-deprived female rats also demonstrated reduced number and activation of oxytocinergic neurons (Tanaka et al., 2019). One study, however, failed to observe effects of oxytocin on SI-induced social deficits (Lavoie et al., 2018).

Dopaminergic synapse

Aberrant dopaminergic signalling in schizophrenia has been intensively studied in schizophrenia (Howes et al., 2015; Kesby et al., 2018). Patients are known to have exaggerated dopamine synthesis and release as well as abnormal expression of all five subtypes of dopamine receptors, which all are implicated in social and cognitive functions (Howes et al., 2015; Meygooni et al., 2019; Seeman et al., 1993). Despite evidence backing unaltered densities of dopamine transporter and presynaptic dopaminergic neurons, altered function of DARPP-32, a key integrator of dopaminergic and glutamatergic inputs that is vital for synaptic plasticity, has been demonstrated (Howes et al., 2015; Wang et al., 2017b). In contrast to mesolimbic hyperdopaminergia, the longstanding central dogma of the dopamine hypothesis, recent evidence are in support of dopaminergic dysfunction in the nigrostriatal pathway (McCutcheon et al., 2019). Isolation rearing of rats causes various changes in the dopaminergic system, including alterations in basal and drug-induced dopamine release, D_1/D_2 receptor expression and levels of the presynaptic protein CDCrel-1, which modulates dopaminergic signalling (Fone & Porkess, 2008).

4.5.2.3 Limitations and future directions

There are several limitations in this study: (1) although the current study has observed increased defensiveness and sensitivity to handling in isolates, which are typical of rats reared in prolonged isolation following weaning (section 1.4), further behavioural, biochemical or molecular characterisation would provide additional validation for successful implementation of the SI protocol. This can be achieved through for example, subjecting rats to LMA or PPI testing prior to tissue collection, or by performing immunohistochemical analysis of parvalbumin and calbindin-containing GABAergic interneurons in the post-mortem hippocampus (Fone & Porkess, 2008); (2) for each housing, despite samples were randomly selected from a total of eight available choices,

rats were derived from only two distinct maternal-paternal sources. Thus, three out of the four GH rats which contributed samples were littermates whereas SI rats consisted of two pairs of littermates. There are reasonable variations in the transcriptome of rats within the same group (Figure 4.5), however, there is still a chance that litter effects affect the current findings to some extent. Using rats with more heterogeneous genetic backgrounds could overcome this issue, potentially increasing robustness of data; (3) the brain is composed of various cell types including neuronal, glial and vascular cells (Darmanis et al., 2015). Furthermore, differential gene expression has been demonstrated in different cell types, and even within a single cell type (Kamme et al., 2003; Lein et al., 2007; Zawar et al., 1999). Therefore, altered gene expression in a particular cell type in the PFC could have been masked by other cell types. For instance, in schizophrenia, reduced expression of the gene encoding glutamic acid decarboxylase 67, has been found to occur exclusively in a subset of parvalbumin-containing GABAergic neurons, the basket cells (Hashimoto et al., 2003; Rocco et al., 2016). While GABAergic neurons constitute 15% of the total neuron population in the frontal cortex and neocortical areas (occipital and parietal lobes), parvalbumin-containing neurons only make up 5.6% of the total neuron population in the medial PFC (Beaulieu, 1993; Gabbott et al., 1998). Thus, the low relative abundance of parvalbumin-containing basket cells in the PFC could explain the lack of alteration in *GAD67* expression in the current study; (4) at steady state, mRNA transcript levels typically only predict approximately 40% of protein abundance, with post-transcriptional and translational regulation and protein degradation accounting for the remaining 60% (Vogel & Marcotte, 2012). Moreover, gene expression can be influenced by variations in epigenetic mechanisms including DNA methylation (Ovenden et al., 2018). Therefore, parallel assessments of protein expression and/or epigenetic mechanisms would enhance the understanding of schizophrenia in a wider, functional context. Examples of proteomics technologies that can be used are reverse-phase protein microarray and Western blotting (Shortall et al., 2018). Epigenetic changes can be probed using

techniques such as chromatin immunoprecipitation (histone modification), CpG island microarray (DNA methylation) or quantitative real-time polymerase chain reaction (small noncoding RNAs such as miRNAs; Stephens et al., 2013); (5) albeit the current results have revealed various DEGs that are clinically relevant to schizophrenia, multiple gene loci of low penetrance most likely contribute to the expression of such complex disorder (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). As schizophrenia is a heterogeneous disorder, the current findings might reflect genetic changes in only a subset of patient population.

Whilst the current data show great promise for the SI model, as mentioned above, the availability of numerous methods or tools developed for each step of the RNA-Seq workflow could influence RNA-Seq outcome. For instance, in a study comparing between tools utilising different statistical methods to identify DEGs across treatments or conditions, the tested tools exhibit variations in the power to detect true differential expression (Law et al., 2014). With advances in RNA-Seq technology, increasingly more new tools and methods for data analysis have become available (Gao et al., 2018; Yang et al., 2016). Using such new approaches to re-analyse RNA-Seq output from the current experiment could therefore shed light on the robustness of the current interpretation, thus improving understanding of the SI rat model. Moreover, enhanced knowledge of the genetics of the SI model can be achieved through the use of improved sequencing technologies, such as nanopore direct RNA-Seq, a real-time sequencing approach that generates long, strand-specific RNA sequences. Besides bypassing the need for reverse transcription or amplification during library preparation and hence potential PCR or reverse transcription biases, this method is also capable of detecting splice variants and modified bases, which might be hindered by previous RNA-Seq methods (Garalde et al., 2018).

4.6 Conclusion

In summary, post-weaning SI of Sprague-Dawley rats caused differential expression of genes implicated in a wide spectrum of biological functions compared to GH controls. Such altered expression has been mostly demonstrated by prior studies in the SI rat model and many are relevant to the clinical phenotype observed in human patients. Functional analyses revealed various pathways with significant DEG involvements. Common DEGs shared by multiple pathways (e.g. *Prkca* and *Fos*), rather than DEGs encoding proteins exclusive in certain pathways (e.g. dopamine receptors in 'dopaminergic synapse', Runx2 in 'Runx2 regulates genes involved in cell migration), appear to predominantly influence pathway analysis outcome. That being said, few genes are exclusive to their respective pathways, for example *Per1* in 'circadian clock' and *Chrm4* in 'cholinergic synapse'. Regardless, many of the implicated pathways are known to be dysregulated in schizophrenia. This further confirms high translational relevance of the SI rat model to human schizophrenia, justifying its use in further enhancing pre-existing knowledge on the neurobiology of schizophrenia and as a tool for evaluating novel or existing compounds and/or treatments for schizophrenia.

CHAPTER 5

General discussion

5.1 Animal models for neurodevelopmental disorders

Post-mortem tissues from patients who have died from neurodevelopmental disorders or patient biopsies are associated with several obvious limitations. For instance, post-mortem studies typically only capture the cellular or molecular alterations at disease endpoint and are lacking in the ability to uncover dynamic cellular changes across disease progression (Lee et al., 2019b; Wang et al., 2019). Moreover, chronic drug treatment such as with antipsychotic treatment is accompanied by metabolic changes and neuroadaptation, which could influence brain structure and function independent of disease state (Amato et al., 2017). A more contemporary modelling approach is enabled by advances in the iPSC technology, which offers the advantage of capturing genetic composition of individual patients (Lee et al., 2019b; Wang et al., 2019). By introducing defined transcription factors (Oct3/4, Sox2, c-Myc and Klf4), patient-derived somatic cells can be reprogrammed to generate iPSCs (Yamanaka, 2007). Such iPSCs can then undergo directed differentiation into various regional and functional-specific neuronal subtypes (e.g. cortical excitatory neurons and hippocampal dentate gyrus neurons) and glia, via exposure to different combinations of small molecules, growth factors and morphogens (Wang et al., 2019). While attempts have been made to increase the complexity of iPSC-based methods, as evidenced by the shift from two-dimensional culture systems to microfluidic-device based reconstruction of functional circuitry, co-culturing of iPSC-derived neurons with glia and three-dimensional brain organoid models, these *in vitro* systems still do not adequately replicate the complex *in vivo* environment (Lee et al., 2019b; Wang et al., 2019). For example, the lack of microenvironment, neuronal circuits, vascular circulation and immune system could restrict the development and long-term survival of brain organoids (Mansour et al., 2018). These cellular models are also associated with issues including scalability and reproducibility, due to technical variability and pre-existing genetic and epigenetic variations within single parent cells (Lee et al., 2019b; Wang et al., 2019). Thus, animal models of CNS disorders, which

attempt to resemble humans in terms of genetics, anatomy and physiology, serve as invaluable platforms for enhancing our understanding of the pathogenesis of schizophrenia (Simmons, 2008). They are also useful in identifying novel therapies, especially those capable of alleviating the currently treatment-resistant negative and cognitive symptoms (Jones et al., 2011b).

While numerous neurodevelopmental, pharmacological and genetic models have been developed, each model typically produces only a few changes that mirror the core behavioural and neurochemical alterations in schizophrenia. Given the diverse nature of the disorder, animal models based on a single intervention to mimic an identified risk factor often fail to replicate the full symptomatology and are associated with shortcomings related to validity (section 1.2). Therefore, the main aim of the thesis was to generate and validate a novel 'dual-hit' model for schizophrenia by combining two pre-existing neurodevelopmental models for the disorder. SI of rat pups is a well-established protocol that has been evaluated in our laboratory over the last 30 years. However, when used alone, SI-induced behavioural alterations can be prevented by repeated handling or partially reversed by testing, and such changes are not consistently reproduced across cohorts. Meanwhile, the high prevalence of exposure to viral infection during pregnancy prompted the selection of prenatal poly(I:C) administration to induce MIA as a complementary 'hit', to investigate the contribution of prenatal cytokine changes to the development of schizophrenia. Our hypothesis was that the combination of MIA and SI would result in a rodent model with a wider gamut of more severe abnormalities that may have translational relevance to the changes observed in schizophrenia than either 'hit' alone.

5.2 Principal findings

5.2.1 The ‘dual-hit’ model

As reported in Chapters 2 and 3 and discussed in Chapter 3 (Tables 3.3 & 3.4), poly(I:C) alone produced relatively subtle changes compared to SI alone or in combination with SI. However, poly(I:C) (10 mg kg^{-1}, i.p., GD15) conferred an unexpected resilience, rather than additive effects, against several SI-induced alterations in behaviour (LMA, aggressive behaviour and CFR) and cytokine and mTOR levels. Consistent with our original hypothesis, poly(I:C) (6 mg kg^{-1}, i.v., GD15) demonstrated an additive effect on SI-induced behavioural changes (LMA and USVs). Conversely, rather than causing more robust changes, i.v. poly(I:C) attenuated SI-induced cortical cytokine changes.

5.2.2 Behaviour and potential early mechanisms in adolescence

In adolescence, poly(I:C) (i.p. and i.v.) and SI, independently or combined, induced hyperactivity in both genders in a novel environment (Table 3.3). Overall, in Chapter 2 (i.p. poly(I:C)), LMA was more robustly modified in males than females. Hyperactivity in Veh-SI and Poly(I:C)-SI males was accompanied by elevated striatal 5-HIAA, with the latter also associated with an increase in basal level of striatal dopamine. Concurrent examination of potential early mechanisms preceding behavioural changes in adulthood revealed a sexually dimorphic effect of i.p. poly(I:C) and/or SI on cytokine and mTOR levels. In particular, behavioural abnormalities in males subjected to i.p. poly(I:C) and/or SI appeared to be associated with aberrant cytokine signalling via a non-mTOR-dependent mechanism. In contrast, in females, exposure to i.p. poly(I:C) and SI, alone or in combination, differentially altered behaviour, potentially via distinct non-cytokine-dependent signalling mechanisms. In Chapter 3 (i.v. poly(I:C)), LMA was more robustly modified in females than males.

5.2.3 Cortical gene expression

Given the more robust effects associated with SI than poly(I:C) (Tables 3.3 & 3.4), in Chapter 4, we reported the effects of SI alone on cortical gene expression to identify potential pathways or molecular processes underlying the behavioural abnormalities produced. As expected, we found altered expression of numerous genes, many of which have been individually associated with schizophrenia. We also observed that a number of the DEGs are shared between functional pathways relevant to the putative aetiological mechanisms of schizophrenia (section 1.1.5), including pathways involved in dopaminergic, muscarinic and oxytocinergic signalling.

5.3 Impact of findings

The main question arising from findings of the current thesis is how they can be used to improve our knowledge of the neurobiology of schizophrenia, to benefit drug discovery and development. We believe that the current thesis has improved knowledge in the following ways:

5.3.1 The poly(I:C) model

Through Chapters 2 and 3, we demonstrated that route of administration of poly(I:C) to pregnant rats resulted in varying outcomes in resultant offspring (Tables 3.3 and 3.4). For instance, when behaviour was assessed in adulthood, rats exposed to gestational poly(I:C) (i.p.) alone exhibited increased rears and pinning and emitted more upward ramp calls whereas rats exposed to poly(I:C) (i.v.) alone merely showed impaired cognitive flexibility. Route of administration is a well acknowledged factor which could influence MIA outcome (Meyer et al., 2009b; Voss et al., 2006). However, factors including source and storage conditions of poly(I:C) and the rearing environment have also been implicated (Kentner et al., 2019), rendering comparison of MIA outcomes from different laboratories utilising distinct routes of administration

challenging. In guinea pigs, the injection method modulated the strength of poly(I:C)-induced fever (Voss et al., 2006). Meanwhile, there were no differences in PPI, latent inhibition or open field behaviour in offspring of pregnant mice injected via the i.p. or i.v. route (Ito et al., 2010). These findings imply that the effect of route of administration could be species-dependent. No equivalent study has been conducted in rats. Thus, our work suggests that future studies should consider the method of poly(I:C) delivery at the planning stage, possibly by conducting a pilot study to compare between two or multiple intended routes, to select the most appropriate option.

The i.p. poly(I:C) model. Whilst previous studies have examined the impact of gestational poly(I:C) exposure on LMA, NOD, NLD, PPI, CFR and ASST (Tables 1.5 and 1.6), no one has assessed USV emissions in a social context. The current thesis is also the first to profile the longitudinal effects of MIA on cytokine and mTOR levels. We demonstrated that administration of 10 mg kg^{-1} poly(I:C) at GD15 to pregnant Lister-hooded rats produced behavioural abnormalities in resultant offspring in adulthood, which were relevant to the positive (LMA) and negative or cognitive (social interaction/USVs) symptom domains of schizophrenia. Of particular note, poly(I:C) increased aggression during social interaction. This is especially relevant to human schizophrenia, as heightened aggressiveness and violent perpetration have been observed in patients (Figure 1.1). Although modest, we observed an increase in upward ramp calls, indicative of altered social communication (Neill et al., 2010). Therefore, this model shows the potential to replicate a phenotype akin to the negative or cognitive symptoms of schizophrenia, which are currently resistant to treatment, in addition to positive symptoms. We also demonstrated that abnormal behaviour in adolescent male rats were potentially mediated by cytokines through a non-mTOR-dependent mechanism. In contrast, mTOR-dependent cytokine signalling was responsible for aberrant behaviour in adulthood. Altered immune function is increasingly thought to contribute to the aetiology of schizophrenia, as patients have been shown to have abnormal levels of cytokines, including

IL-1β, IL-6, IL-10, IL-23, IFN-γ and TGF-β (Borovcanin et al., 2013; Kim et al., 2004; Miller et al., 2011; Xiu et al., 2014). However, cytokine production can be modulated by several signalling pathways, including mTOR, NF-κB and JNK pathways (Turner et al., 2014). While prior studies have focused on the effects of i.p. poly(I:C) on cytokine changes during gestation (GD16), neonatal period (PND1 or 7) and adolescence (Gilmore et al., 2005; Han et al., 2011), no one has evaluated the mechanisms underlying such alterations. Thus, apart from suggesting a potential mechanism for the long-term effects of MIA on immune function in resultant offspring, the current thesis also revealed that behaviour at different developmental stages could be mediated by distinct signalling mechanisms. This could have important implications for the development of preventative treatments, as early mechanisms could be different to those causing behavioural alterations in later life. This model could also be particularly useful for investigating the prospects of immunomodulatory agents in managing or preventing schizophrenia. It is worth mentioning that enhanced mTOR activity is linked to perturbed cognition, thus further supporting the potential of i.p. poly(I:C) to model cognitive symptoms of schizophrenia (Dunphy-Doherty et al., 2018; Meffre et al., 2012).

The i.v. poly(I:C) model. Intravenous injection of poly(I:C) to dams resulted in relatively modest effects on resultant offspring compared to i.p. injection (Table 3.4). Nonetheless, this protocol shows the potential for modelling the cognitive domain of schizophrenia, as evidenced by impaired performance in the ASST. This protocol was also associated with elevated frontal cortical as well as striatal cytokines and mTOR and therefore can be further developed to assess the therapeutic potential of immunomodulatory drugs and mTOR inhibitors.

5.3.2 The SI model

In both Chapters 2 and 3, SI consistently induced abnormalities in LMA, social interaction, USVs, CFR and cytokines. Apart from showing the

robustness of our SI model, this also suggests that the model could be useful for screening therapeutics to combat the positive (LMA), negative (social interaction/USVs) and cognitive (CFR) domains of schizophrenia, as well as for evaluating the prospects of immunomodulatory drugs. The impact of SI on NOD, NLD and PPI, however, was minimal. While alterations in LMA, social interaction and CFR have been previously characterised by many groups (Jones et al., 2011b), no groups have investigated the impact of SI alone on USVs in adult rats, although our group has previously shown a change in USVs with the combined PCP-SI model, consistent with the current findings (Watson et al., 2016). Knutson et al. (1998) reported that pairs of adolescent isolates emitted more pro-social 50-kHz USVs compared to GH during social play. Here, we provided an extension to such findings, showing increased 50-kHz calls in adult isolates, as well as a comprehensive classification of the 50-kHz call subtypes. To the best of our knowledge, no research groups have evaluated spatial memory in isolates using the NLD paradigm. In a previous study, where rats reared in social groups were handled once a week and given six 5 min habituation sessions to explore the NLD arena before the experiment, rats were only able to discriminate between the novel and familiar locations in the first minute of the 3-min test session (Dix & Aggleton, 1999). This prompted the authors to suggest that extending the test phase beyond 1 min might reduce task sensitivity. While we could dissociate discrimination before and after the first minute of the test phase, the protocol used by the authors is not suitable for our studies, as GH animals had to be exposed to the same handling conditions as isolates. Using an alternative paradigm, e.g. MWM and Y-maze, might be able to recapitulate spatial memory impairments seen in schizophrenia (Quan et al., 2010; Shao et al., 2015). The limited ability in modelling NOD and PPI deficits are not surprising, given that these alterations are less robustly reproduced across SI cohorts (Fone & Porkess, 2008; Table 2.21). Additionally, a single intervention is not expected to induce behavioural abnormalities akin to the full syndrome of human schizophrenia.

We also provided insights into the mechanisms underlying cytokine alterations in isolates, despite some inconsistencies between Chapters 2 and 3. In Chapter 2, we demonstrated that mTOR signalling was responsible for cytokine changes in the FC of adult isolates, thus confirming potential to model cognitive deficits. While this agrees with a previous study by our laboratory, rats used in that study underwent testing in a different set of behavioural paradigms, including elevated plus maze and restraint stress (Dunphy-Doherty et al., 2018). The inconsistency between Chapters 2 and 3 can be explained by factors such as inherent differences between batches (Näslund et al., 2015). Distinct prior stress experience (i.p. vs i.v injection) might have also shaped immune function in isolates differently. Another novel finding of this thesis is that in isolates, cytokine function at distinct developmental stage is mediated by different mechanisms. As described for the i.p. poly(I:C) model, this could have important implications for the development of preventative treatments.

Body weight was measured in isolates and compared to GH controls in Chapters 2-4. In Chapter 2, while rats subjected to SI alone were significantly heavier than GH controls four to five weeks post-weaning, in Chapter 3, no weight differences were found in rats subjected to the same housing up to eight weeks post-weaning. In Chapter 4, isolates had increased body weight six weeks post-weaning (experiment terminated at this point). Hence, similar to individual behavioural components of LMA and social interaction in Chapters 2 and 3 (Table 3.4), SI-induced changes in body weight were not consistently replicated across studies and tended to be rather modest when observed, although findings from Chapter 4 somewhat correspond to those of Chapter 2. Existing literature has suggested that weight gain may be dependent on the SI protocol used (Fone & Porkess, 2008; Weiss et al., 2004).

Lastly, our whole-transcriptome analysis of the PFC in SI rats provided a starting point for understanding changes at the gene level in isolates. More comprehensive gene expression profiling at the single-cell level, for

example, could potentially shed light on the contribution of specific cell types to behavioural abnormalities relevant to schizophrenia (Kamme et al., 2003). This could also enable the identification of novel putative biomarkers or drug targets for schizophrenia (Hwang et al., 2018).

5.3.3 The ‘dual-hit’ model.

In Chapter 2, an unexpected resilience effect of poly(I:C) was observed against SI-induced alterations in behaviour (LMA, aggressive behaviour and CFR) and cytokine and mTOR levels. In Chapter 3, poly(I:C) conferred a mixture of additive (behaviours including LMA and USVs) and protective (cytokines) effects on SI-induced alterations. Numerous ‘dual-hit’ models have been developed using SI of rats or gestational poly(I:C) administration as one of the ‘hits’. For instance, neonatal PCP challenge, single or chronic MK-801 treatment in adolescence, neonatal ventral hippocampal lesion, neonatal domoic acid (AMPA/kainite agonist) administration and peripubertal poly(I:C) challenge have each been combined with post-weaning isolation rearing (Alquicer et al., 2008; Ashby et al., 2010; Gaskin et al., 2014; Gilabert-Juan et al., 2013; Hickey et al., 2012; Lukasz et al., 2013; Marriott et al., 2016). SI of rats have also been applied in conjunction with environmental stressors such as single prolonged stress (e.g. 2 h restraint followed by cold swim stress), chronic social defeat stress and maternal separation (Gilles & Polston, 2017; Hofford et al., 2018; Riga et al., 2017). Prenatal poly(I:C) exposure, on the other hand, has been combined with juvenile stress, adolescent cannabinoid exposure and adolescent or adulthood LPS challenge (Clark et al., 2019; Hollins et al., 2016; Idrizi et al., 2016; Richtand et al., 2012a; Yee et al., 2011). As discussed in depth in section 2.5.2.3, while most ‘dual-hit’ studies back the cumulative stress theory, there also exist studies which support the stress inoculation theory, as well as those which found no effect following combination of ‘hits’. Our findings in Chapter 2 are undoubtedly consistent with the stress inoculation theory. However, in Chapter 3, we demonstrated that combination of two stressors does not necessarily produce either an additive effect or a

resilience effect on a strict basis. Given the dissociation between cytokine and behavioural alterations, this supports the need for polypharmacy in schizophrenia management, e.g. prescribing dopamine-based antipsychotics with immunotherapy as adjunct treatment, or the development of novel multi-purpose drugs, such as dopamine D_2-based antipsychotics which can also modulate inflammatory response.

5.3.4 The effect of gender

The current thesis also revealed an influence of gender on behaviour and biochemical alterations in adolescence. Overall, in Chapter 2, we observed more robustly modified LMA response, neurochemistry and cytokine levels in male compared to female rats. These findings are particularly relevant to schizophrenia, as onset in women is typically later than in men, possibly due to the protective effects of oestrogen (section 1.1.2). Meanwhile, more robust LMA changes were evident in females in Chapter 3. Sexually dimorphic effects are consistent with prior studies using the SI model, with males reported to be more affected in behaviours including PPI and latent inhibition, as well as neuroendocrine state such as basal levels of adrenocorticotropic hormone and stress-induced increase in plasma corticosterone (Chmelova et al., 2019; Pisu et al., 2016; Weiss et al., 2001; Weiss et al., 2004). However, other studies reported a limited effect of gender on social behaviour, forced swim test and sucrose preference test (Gilles & Polston, 2017). In the poly(I:C) model, the effects of gender have also been extensively studied (Tables 1.5 & 1.6). Studies have mainly focused on behaviour (Gray et al., 2019; Howland et al., 2012; Vorhees et al., 2015), neurophysiology (De Felice et al., 2019; Patrich et al., 2016a) and neurochemical, structural or histological changes (McColl & Piquette-Miller, 2019; Piontkewitz et al., 2011; Zuckerman & Weiner, 2003). Microglia density, maturation and activation (Duchatel et al., 2018b; Li et al., 2018b; Zhang et al., 2019) and complement component 4 gene expression (Duchatel et al., 2018a) have been studied, but no one has yet dissected the effects of gender on mTOR signalling and its potential link to cytokines. It is vital to note that

gender typically only affects a particular subset of behaviour or functional readouts. For instance, in a recent publication which reported the long-term consequences of SI on behaviour (PPI, open-field test and elevated plus maze), VGF/BDNF/tyrosine receptor kinase B pathway and gene expression of two isoforms of nitric oxide synthase in the hippocampus, gender only influenced mRNA levels of VGF and neuronal nitric oxide synthase (Chmelova et al., 2019). Our findings in Chapter 2 support these results, as behaviour, neurochemistry and cytokine levels were mainly modified in males and mTOR levels were selectively modified in Veh-SI and Poly(I:C)-SI females (Table 3.3). Additionally, different combinations of prenatal treatment and housing conditions produced slightly distinct behavioural and neurochemical outcomes in both genders. Therefore, our findings stress the importance of conducting simultaneous behavioural and/or neurochemical phenotyping in both genders, and ideally multiple endpoint measurements should be included to prevent misleading interpretation of results. Further studies should also specify the gender used in their experiments, as have suggested by the recent reporting guidelines for the MIA model (Kentner et al., 2019). Good examples which incorporated this practice are the work by Zuckerman & Weiner (2003) and Oliveira et al. (2019).

5.4 Future studies

As discussed above, both poly(I:C) and SI model show great promise for modelling neuropsychiatric disorders such as schizophrenia. Further modification of the independent protocols could produce more robust alterations associated with each paradigm and help understand the developmental consequences of these independent factors.

5.4.1 The SI model

Isolation rearing of rats from weaning induces a wide range of behavioural abnormalities relevant to schizophrenia (see section 1.4). However, such deficits are of rather fragile nature, as they are not

consistently seen across studies (e.g. differential effects on ASST and mTOR in Chapters 2 and 3; Table 3.4). Furthermore, behavioural characterisation of twenty-two separate SI studies revealed an overall 95%, 76% and 73% replication of SI-induced hyperactivity in a novel environment, NOD and PPI deficits, respectively (Table 2.21). It has been previously shown that plasma corticosterone levels are only elevated when isolates are reared in more austere conditions, e.g. in grid-floor versus sawdust cages, or in hanging metal cages versus larger plastic cages (Heidbreder et al., 2000; Holson et al., 1991). Augmented circulating corticosterone has also been demonstrated in rats housed individually in soundproof chambers, in addition to disrupted hypothalamic 5-HT system (Greco et al., 1989). Therefore, subjecting isolates to more austere housing conditions might serve as a potential refinement leading to more robust changes in relevant stress-related behaviour and gene expression than those observed in this thesis. This is relevant to schizophrenia as reduced glucocorticoid receptor expression, potentially due to hypercortisolemia, has been reported in afflicted individuals (Sinclair et al., 2011; Webster et al., 2002). Furthermore, the development of specific behavioural deficits can be sensitive to prior test experience. For instance, exposure to prior LMA testing has been found to abolish SI-induced PPI deficits in subsequent testing (Domeney & Feldon, 1998). Such findings could explain the lack of SI-induced PPI impairments in Chapters 2 and 3, as rats underwent LMA, NOD, NLD and social interaction testing prior to PPI evaluation. However, each paradigm was conducted at one-week interval, in increasing order of aversiveness, to minimise impact on subsequent paradigms. Thus, allocating rats to distinct subgroups, each assigned for evaluation of a single behaviour, might be useful in producing robust impairments specific to a particular symptom domain of schizophrenia. However, this would not conform to the 3Rs unless it is absolutely justified and makes correlation of neurochemical measures with multiple behavioural endpoint changes more restrictive.

5.4.2 The poly(I:C) model

Factors pertaining to the protocol (e.g. exposure timing, route of administration, dose, storage and administration conditions), animal (e.g. strain and genetic background) and environment (e.g. caging system) can influence the robustness of poly(I:C) paradigm (Estes & McAllister, 2016; Kentner et al., 2019; Mueller et al., 2018). Research groups have utilised poly(I:C) from different vendors (Table 2.2), as well as varying dosing protocols and strains (Tables 1.3-1.6), rendering comparison of results between groups challenging. Recently, poly(I:C) products from different suppliers as well as different batches originating from the same supplier have been shown to vary in the molecular weight of dsRNA fragments and hence immunogenicity, differentially altering maternal physiology (e.g. thermoregulation and immune response) and pregnancy outcomes (e.g. rate of spontaneous abortion and litter size; Kowash et al., 2019; Mueller et al., 2019). Moreover, contamination of poly(I:C) by the endotoxin LPS, which could occur during manufacturing or reconstitution in the laboratory, has been suggested to interact with poly(I:C) molecular weight to modulate the resultant maternal cytokine response and therefore influence developmental outcomes (Kowash et al., 2019). As such, assessment of the purity and molecular composition of poly(I:C) through spectrophotometry (to measure UV light absorbance ratio at 260 nm relative to 280 nm) and electrophoresis or multi-angle light scattering (for molecular weight quantification), and any contamination with LPS via enzyme-linked immunosorbent assay and/or limulus amebocyte lysate assay would assist interpretation of the poly(I:C) outcomes in the current thesis (Kowash et al., 2019; Mueller et al., 2019). This work is currently being undertaken.

In this thesis, a single injection of poly(I:C) was administered regardless of route of administration. Given the subtle effects of poly(I:C), and especially because additive effects of poly(I:C) and SI were evident in resultant offspring in adulthood in Chapter 3 (i.v. poly(I:C)), using a repeated dosing protocol could potentially elicit stronger effects on the

pregnant dams and resultant offspring. However, sub-chronic or chronic dosing is associated with increased stress (Meyer et al., 2009b). In prior i.p. studies, inconsistent maternal or gestational outcomes following repeated dosing have been reported (Forrest et al., 2012; Gilmore et al., 2005; Oh-Nishi et al., 2010; Tinsley et al., 2009). As for i.v. administration, repeated dosing is rare, only one study has administered poly(I:C) (5 mg kg^{-1}) on two occasions, at GD11 and 20 (Hollins et al., 2018). As we are particularly interested in dissecting the relationship between maternal viral infection and offspring behaviour, and increased risk of schizophrenia is linked to maternal viral infection particularly during the second trimester, injecting a second dose of poly(I:C) within GD11-20 would be an interesting protocol (Qiang et al., 2002). Hollins et al. (2018) observed 'anxiety-like' behaviour in the elevated plus maze as well as gastrointestinal inflammation and altered expression of colonic tight junction proteins in resultant offspring. The findings that up to 65% of patients with schizophrenia experience anxiety symptoms (Temmingh & Stein, 2015) and gastrointestinal inflammation is a relevant pathology in schizophrenia (Severance et al., 2012) support the idea of further evaluating this dosing protocol.

In mice, the type of caging system has been shown to affect maternal immune responses to poly(I:C) and offspring phenotypes (Mueller et al., 2018). In particular, early gestational poly(I:C) administration (GD9) to dams housed in open cages were linked to robust MIA, as evidenced by increased maternal cytokine and chemokine levels, without adverse pregnancy outcomes (spontaneous abortion, litter size and sex ratio) whereas resultant offspring, also housed in the same caging system, exhibited deficits in PPI and social interaction at adulthood. When housed in individually ventilated cages, while MIA was successfully confirmed, a dose-dependent increase in spontaneous abortion was observed in dams whereas no poly(I:C)-induced behavioural abnormalities were seen in offspring. Interestingly, caging type did not appear to affect pregnancy outcomes when MIA was stimulated at a later gestational age (GD12), although deficits in working memory, social interaction and PPI were

seen in offspring housed in individually ventilated cages. No equivalent studies have been conducted in rats, however, caging system as an environmental factor could be equally relevant. In the current thesis, dams were housed in individually ventilated cages upon arrival until pups were weaned. Pups were then transferred to open-top cages. It would be interesting to examine whether housing dams in open-top cages would result in more robust phenotypes in offspring, especially when combined with subsequent SI of offspring. However, this would require justification and alteration of the existing project licence.

A further measure which can be taken to improve validity of results is to employ cross-fostering, which involves exchanging newly born pups or whole litters between lactating dams, hence separating environmental influences from genetic effects (McCarty, 2017; Winney et al., 2015). Although such manipulation provides the advantage of partially controlling for potential confounds including litter size, sex ratio, lactation and maternal behaviour, it may introduce changes to the social and physical early environment by altering within-litter competitive hierarchy, transferring pathogens and parasites between litters and influencing dam and offspring behaviour, depending on the method used (Kentner et al., 2019; Winney et al., 2015). However, switching litters between dams of identical treatment groups could control for the effects of handling as well as maternal-pup disruption (Kentner et al., 2019).

5.5 Conclusion

The current thesis aimed to develop and validate a novel ‘dual-hit’ model for schizophrenia to improve understanding of the aetiological basis of neurodevelopmental disorders such as schizophrenia. This was achieved by combining two widely used neurodevelopmental interventions, MIA and post-weaning SI in rats, which are thought to replicate known risk factors for human schizophrenia.

Gestational poly(I:C) exposure alone produced relatively subtle effects on behaviour in adolescent and adult rats regardless of route of administration. When combined with SI, prior poly(I:C) exposure (10 mg kg^{-1}, i.p., GD15) conferred an unexpected resilience against SI-induced behavioural (LMA, aggressive behaviour and CFR), cytokine and mTOR alterations in adulthood. This could indicate a role of aberrant mTOR signalling in mediating abnormal behaviour and immune function seen with SI alone. Investigations into potential early molecular mechanisms preceding behavioural changes produced by poly(I:C) and/or SI revealed a complex interaction between distinct "stress histories" (i.e. differential effects of poly(I:C) alone, SI alone or poly(I:C)-SI) and gender on the modulation of long-term immune and behavioural outcomes. Poly(I:C) exposure (6 mg kg^{-1}, i.v., GD15) in combination with SI resulted in some additive effects on paradigms relevant to the positive (LMA) and negative or cognitive (USVs) symptom domains, warranting further development to increase validity of this 'dual-hit' model. Interestingly, the same combination attenuated SI-induced increases in frontal cortical cytokines while having no effect on mTOR signalling, suggesting a dissociation between behavioural changes in adulthood and altered immune function. Furthermore, SI-induced cytokine changes most likely occurred via a non-mTOR-dependent mechanism.

Lastly, post-weaning SI of rats altered expression of numerous genes in the PFC. Many of the DEGs have been implicated in schizophrenia and are common to functional pathways relevant to the putative aetiological mechanisms of schizophrenia, including dopaminergic, muscarinic and oxytocinergic neurotransmission. These findings support the usefulness of post-weaning isolation rearing of rats in modelling neuropsychiatric disorders such as schizophrenia.

APPENDIX 1

Radioligand binding

1.1 Introduction

Dopamine receptors exist as five distinct subtypes, $D_{1\text{-}5}$. They are further categorised based on their downstream coupling to either $G\alpha_{s,olf}$ (D_1-class receptors; D_1 and D_5) or $G\alpha_{i/o}$ (D_2-class receptors; D_2, D_3 and D_4) proteins, which leads to activation and inhibition of the production of the second messenger cyclic adenosine monophosphate, respectively (Beaulieu et al., 2015). Functioning as both autoreceptors on dopaminergic neurons and postsynaptic receptors, D_3 receptors display a much higher binding affinity for dopamine compared to other dopamine receptor subtypes, e.g. approximately 70-fold greater affinity compared to D_1 or D_2 receptors (Sokoloff et al., 1992). They are densely localised in the ventral striatum (island of Calleja, olfactory tubercles and nucleus accumbens) and cerebellum, and are also found in alternative limbic areas including the hippocampus, septum or mammillary nuclei in the hypothalamus, indicative of a significant role in dopamine-mediated regulation of cognitive, emotional, reward and endocrine functions (Nakajima et al., 2013; Sokoloff et al., 1992). As blockade of D_3 receptors is associated with improved cognition, numerous D_3 receptor-selective partial agonists or antagonists, e.g. aripiprazole, blonanserin and cariprazine have been evaluated for potential as cognitive enhancers in neuropsychiatric disorders such as schizophrenia (Leggio et al., 2016; Nakajima et al., 2013). Furthermore, D_3 receptors are expressed in very low levels in the caudate, putamen and pituitary gland, areas linked to the undesired extrapyramidal and neuroendocrine effects of most D_2-based typical antipsychotics, making them attractive targets for the development of novel antipsychotics (Levant, 1997). Overall, distribution of D_3 receptor mRNA and putative sites in the human brain is similar to the rat brain, albeit in humans distribution of putative D_3 sites appears to be more widespread, e.g. moderately expressed throughout the cerebellar cortex and in the dentate nucleus, which is not observed in rats (Herroelen et al., 1994; Levant, 1997).

In the SI rat model, selective blockade of D_3 receptors is capable of reversing cognitive impairments as well as locomotor hyperactivity (proxy marker for positive symptoms), backing the involvement of D_3 receptors in schizophrenia pathophysiology (Watson et al., 2012). Whilst findings on the level and activity of striatal D_2 receptors remain controversial (Fone & Porkess, 2008; Han et al., 2012; Lukkes et al., 2009; Yorgason et al., 2013), no studies have investigated the effects of SI on striatal D_3 receptors. As such, one of the initial aims of Chapter 4 was to examine D_3 receptor protein expression by saturation binding.

Radioligand binding experiments involve incubating samples containing the receptor of interest with a suitable radioligand for an appropriate length of time, followed by measurements of radioactivity bound to the receptor. Such experiments can be divided into saturation, inhibition and kinetic experiments. Of relevance to the current study, a saturation assay allows for the determination of receptor affinity for the radioligand (K_d or dissociation constant), as well as total receptor density (B_{max}; Bylund & Toews, 1993).

Since its identification as a selective probe at dopamine D_3 receptors, [^{3}H]7-OH-DPAT has been widely used in receptor binding studies to characterise the pharmacology of putative D_3 sites in membrane preparations from the rat brain (Hillefors & von Euler, 2001; Levant, 1995; Lévesque et al., 1992; Liu et al., 1994; Wallace & Booze, 1995; Wallace & Booze, 1996). A further radioligand, [^{3}H]PD 128907 has also been used in such studies (Bancroft et al., 1998; Hillefors et al., 1999). In particular, putative D_3 sites appear to have similar affinity for 7-OH-DPAT and PD 128907, and sites labelled by [^{3}H]7-OH-DPAT and [^{3}H]PD 128907 are characteristic of D_3 receptors, in terms of pharmacological profile, insensitivity to guanyl nucleotide regulation and regional distribution (Bancroft et al., 1998). As more information on [^{3}H]7-OH-DPAT binding were available at the time of setting up binding assay conditions, [^{3}H]7-OH-DPAT was chosen as the radioligand in the current study.

1.2 Methods

1.2.1 Animals and tissue collection

Tissues used for optimisation of the saturation binding assay were from rats kindly donated by Dr Ling Yeong Chia or Dr Patricia Rueda, or from a different batch of Sprague-Dawley rats to those used for RNA-Seq in Chapter 4. For tissue collection, rats were terminally anaesthetised with 5% isoflurane and decapitated. The striatum was promptly dissected from each brain hemisphere, snap frozen on dry ice and stored at -80^{0}C.

1.2.2 Membrane preparation

Striatal tissues were pooled and homogenised in ice-cold membrane preparation buffer (50 mM Tris HCl, 1 mM EDTA and 5 mM $MgCl_2$, pH 7.4) using Bio-Gen Series PRO200 and then centrifuged for 1 h at 40,000 x g at 4^{0}C (Sorvall Evolution RC, ThermoScientific; rotor: SA-600). The resulting pellet was resuspended in membrane preparation buffer. To remove endogenous biogenic amines, membranes were incubated for 30 min at 37^{0}C, centrifuged for 1 h at 40,000 x g and 4^{0}C. The resulting pellet was resuspended in buffer. The final solution was stored at -80^{0}C in aliquots of 10 µl for protein concentration determination using Thermo Scientific Pierce BCA Protein Assay Kit (Cat # 23225) and EnVision multimode plate reader (PerkinElmer), or 50 µl for radioligand binding assays.

1.2.3 Saturation binding assay

As it was the first time D_3 receptor binding was carried out in the lab using rat brain tissues, assay conditions had to be set up from scratch. To begin with, appropriate concentrations of [^{3}H]7-OH-DPAT (Optimisation 1) and membrane preparation (receptor) concentration (Optimisation 2) were determined. However, due to time constraints (two months left prior to returning to the UK), no further optimisations were carried out.

1.2.3.1 Optimisation 1

Striatal membrane preparations (final concentration 30 µg/500 µl) were incubated in assay buffer (50 mM Tris-HCl, 1 mM EDTA, 2 mM $MgCl_2$, 50 mM NaCl, 0.01% ascorbic acid, 0.5 mM carbetapentane, 1 mM GTPγS, pH 7.4) containing 0-300 nM [^{3}H]7-OH-DPAT for 2 h on a shaker at room temperature (in duplicates, total two experiments). As it has been previously shown that [^{3}H]7-OH-DPAT might also bind to σ receptors, carbetapentane, an opioid antitussive drug which binds σ receptors, was included in the assay buffer to increase specific binding of [^{3}H]7-OH-DPAT to D_3 receptors (Wallace & Booze, 1995). NSB was determined in the presence of 10 µM (+)-butaclamol hydrochloride (Sigma D033-5MG, Lot # 063M4608V), as was previously shown (Kreiss et al., 1995; Mulder et al., 1987). Radioligand binding was terminated by the addition of ice-cold 0.9% NaCl and rapid filtration through Whatman Grade GF/B Glass microfiber filters (GE Healthcare Life Sciences; Cat # 1821-915, Lot # 9837289). Radioligand bound was quantified using scintillation spectrometry.

1.2.3.2 Optimisation 2

Binding reactions were initiated by the addition of eight different concentrations of striatal membrane preparations (30-100 µg/500 µl protein) to 10 nM [^{3}H]7-OH-DPAT and either 10 µM assay buffer (total binding) or 10 µM (+)-butaclamol (NSB; duplicates, total two experiments). 10 nM [^{3}H]7-OH-DPAT was selected based on findings from Optimisation 1 (section 1.3.1). Incubation conditions and harvesting of membranes with bound radioligand were identical to Optimisation 1.

1.2.4 Analysis

For each concentration of [^{3}H]7-OH-DPAT that was tested in Optimisation 1, total radioactivity in disintegrations per minute (DPM) was acquired in the absence of membrane preparations and assay buffer or

(+)-butaclamol. DPM values were converted to molar concentrations using the equation: actual concentration of radioligand= DPM/(127.4 Ci/mmol x 2.22 DPM/Ci x 50 µl x 10), where 127.44 Ci/mmol represents the specific activity of [^{3}H]7-OH-DPAT, 50 µl is the volume of radioligand aliquot and 10 is the dilution factor used in the binding assay. Total and NSB were plotted against increasing concentrations of [^{3}H]7-OH-DPAT. Specific binding was calculated as the difference between total and NSB.

1.3 Results

1.3.1 Optimisation 1

To determine a [^{3}H]7-OH-DPAT concentration which would give the "biggest window between total binding and NSB", 0.1-30 nM (final) [^{3}H]7-OH-DPAT were incubated with a constant membrane concentration and NSB was defined in the presence of 10 µM (+)-butaclamol hydrochloride. There was negligible separation between total binding and NSB, with a possible window at 10 nM [^{3}H]7-OH-DPAT (Figure 1.1). Therefore, this radioligand concentration was selected for further study.

1.3.2 Optimisation 2

Using 10 nM [^{3}H]7-OH-DPAT, further experiments were performed using increasing membrane concentrations to determine the lowest concentration of membrane preparation that could result in a window between NSB and total binding; data from each experiment are presented separately (Figure 1.2). Whilst low, there was some evidence of a window of around 1000 DPM at lower membrane protein concentrations, i.e. 30-50 µg (n=2 per membrane concentration; Figure 1.2B).

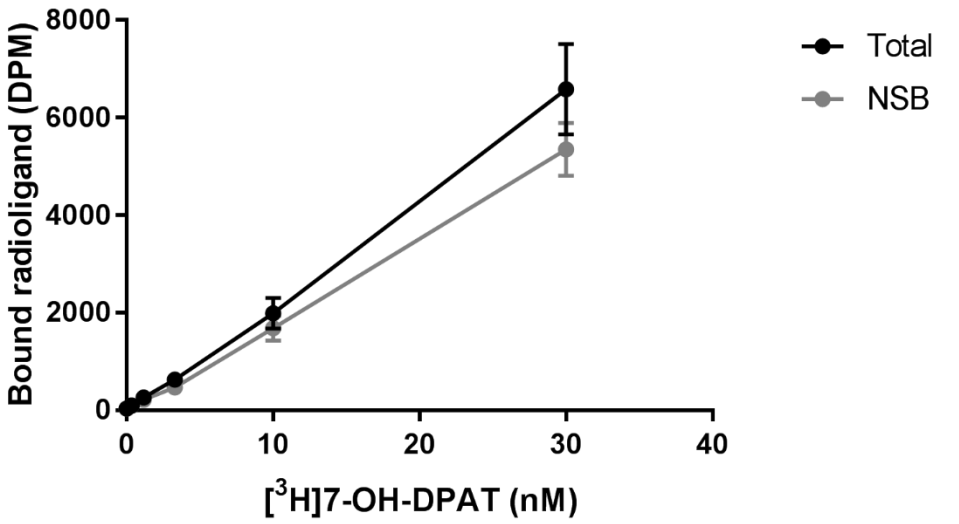


Figure 1.1. Saturation binding of ^{3}[H]7-OH-DPAT at D_3 receptors on membrane preparation from rat striatum. Increasing [^{3}H]7-OH-DPAT concentrations (0-300 nM) were incubated with 30 µg membrane preparation in 500 µl total assay volume. NSB was defined in the presence of 10 µM (+)-butaclamol hydrochloride. Results are from two experiments, where n=2 for each [^{3}H]7-OH-DPAT concentration.

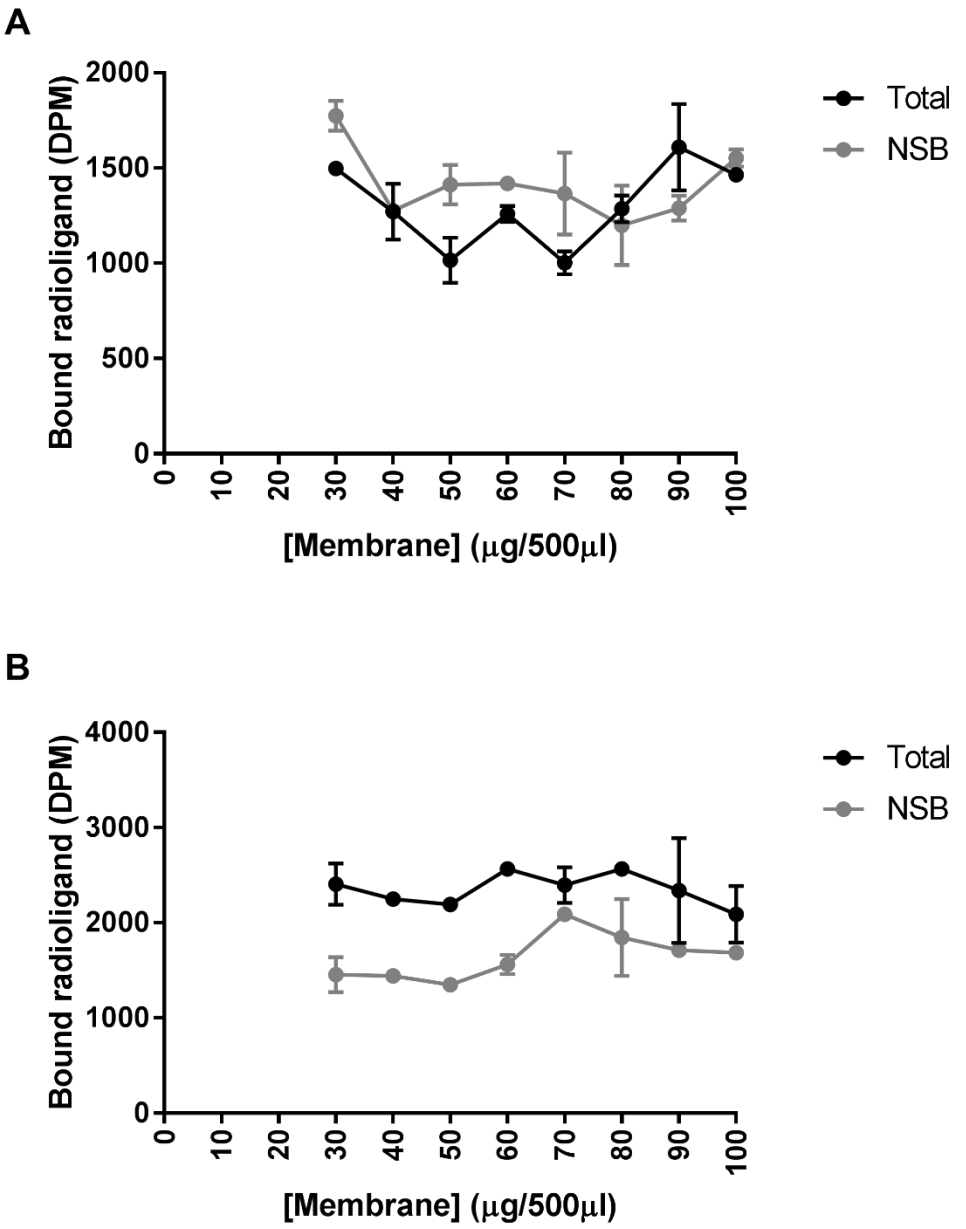


Figure 1.2. Total binding and non-specific binding (NSB) of [^{3}H]7-OH-DPAT at rat striatal D_3 receptors versus membrane protein concentration. In Optimisation 2 (total two experiments), 10 nM [^{3}H]7-OH-DPAT was incubated with different concentrations of membrane preparation (30-100 µg) in 500 µl total assay volume. NSB was determined in the presence of 10 µM (+)-butaclamol hydrochloride. As there was a slight miscalculation during membrane preparation in the second experiment leading to slightly increased final membrane concentrations, data from the two experiments were presented separated in **(A)** and **(B)**, respectively (n=1-2 per membrane concentration).

1.4 Discussion

In a saturation binding experiment, NSB should ideally constitute only 10-20% of the total binding to ensure reliable results (Motulsky & Neubig, 2010). In Optimisation 1, although increased binding (total and non-specific) due to increases in [^{3}H]7-OH-DPAT concentration was as expected (Motulsky & Neubig, 2010), NSB was close to total binding, hence indicative of inhibition of specific binding. To confirm NSB, an experiment can be conducted in the absence of membranes (Auld et al., 2012). High NSB compared to total binding could be due to too little signal or too much noise within the assay. Whilst the former can be attributed to too few receptors, potentially due to very low expression levels in tissue of interest, the latter can arise due to greater intrinsic NSB of radioligands, to certain surfaces or filters (Hein et al., 2005). In the rat striatum, specific binding of [^{3}H]7-OH-DPAT to putative D_3 sites has been previously shown to only account for 10-20% of total binding (Lévesque et al., 1992). Therefore, increasing the membrane preparation might overcome high NSB (Hein et al., 2005). Alternatively, using a different type of filter (e.g. GF/C), pre-treating filters with solutions (e.g. Tween 80 or polyethylenimine), washing filters with more buffer, changing the temperature of buffer, or switching to a different radioligand might address such issue (McBride et al., 1997; Motulsky & Neubig, 2010; Wallace & Booze. 1996).

In Optimisation 2, increasing the concentration of membrane preparation should result in a linear increase in [^{3}H]7-OH-DPAT binding until a plateau is reached (Ahmadi et al., 2014; Claro, 2006). This was clearly not the case, and NSB was greater than total binding at several membrane protein concentrations in one of the replicates (Figure 1.2A). NSB can be minimised as discussed above, but further optimisations were not carried out due to time constraints.

APPENDIX 2

Supplementary Data for Chapter 4

Table 2.1. List of differentially expressed genes (DEGs) sorted by false discovery rate (FDR). Raw sequencing data were entered into Degust for differential gene expression analysis. A total of 183 DEGs were identified, of which 12 were up-regulated whereas 171 were down-regulated. The gene identity, symbol, name or description, expression in isolates (expressed as log_2 fold change relative to group-housed controls), FDR and p-values associated with each DEG are given. An FDR adjusted p-value (q-value) cut-off of 0.05 was used.

Gene ID	Gene symbol	Gene name or description	Log_2 fold change	FDR	p-value
Up-regulated genes					
ENSRNOG00000010079	*Car3*	carbonic anhydrase 3	5.65	0.0149	0.0000
ENSRNOG00000014365	*Gne*	glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase	0.47	0.0188	0.0000
ENSRNOG00000008656	*Snca*	synuclein alpha	0.29	0.0221	0.0001
ENSRNOG00000012684	*Bloc1s2*	biogenesis of lysosomal organelles complex 1 subunit 2	0.29	0.0303	0.0002
ENSRNOG00000021067	*Fxyd7*	FXYD domain containing ion transport regulator 7	0.37	0.0358	0.0003
ENSRNOG00000017829	*Ssu72*	SSU72 homolog, RNA polymerase II CTD phosphatase	0.26	0.0378	0.0003
ENSRNOG00000028884	*Atp5l*	ATP synthase membrane subunit g (also *Atp5mg*)	0.30	0.0378	0.0003
ENSRNOG00000037720	*Rita1*	RBPJ interacting and tubulin associated 1	0.30	0.0412	0.0004
ENSRNOG00000026211	*Mri1*	methylthioribose-1-phosphate isomerase 1	0.34	0.0423	0.0004
ENSRNOG00000019221	*Gstm4*	glutathione S-transferase mu 4	0.47	0.0457	0.0005
ENSRNOG00000002115	*Sod1*	superoxide dismutase 1	0.27	0.0486	0.0005
ENSRNOG00000061153	*Hrh3*	histamine receptor H3	0.35	0.0486	0.0005
Down-regulated genes					
ENSRNOG00000003977	*Dusp1*	dual specificity phosphatase 1	-1.63	0.0001	0.0000
ENSRNOG00000042838	*Junb*	junB proto-oncogene, AP-1 transcription factor subunit	-1.20	0.0006	0.0000
ENSRNOG00000043465	*Arc*	activity regulated cytoskeleton associated protein	-2.83	0.0006	0.0000

Gene ID	Gene symbol	Gene name or description	Log_2 fold change	FDR	p-value
ENSRNOG00000007607	*Nr4a1*	nuclear receptor subfamily 4 group A member 1	-1.90	0.0021	0.0000
ENSRNOG00000033433	*Csrnp1*	cysteine and serine rich nuclear protein 1	-1.11	0.0021	0.0000
ENSRNOG00000007830	*Apold1*	apolipoprotein L domain containing 1	-0.93	0.0033	0.0000
ENSRNOG00000001189	*Sik1*	serine/threonine-protein kinase SIK1	-1.87	0.0036	0.0000
ENSRNOG00000022395	*Tnrc6c*	trinucleotide repeat-containing gene 6C protein	-0.55	0.0036	0.0000
ENSRNOG00000006118	*Klf10*	Kruppel like factor 10	-1.28	0.0108	0.0000
ENSRNOG00000046547	*Rbm24*	RNA binding motif protein 24	-0.72	0.0125	0.0000
ENSRNOG00000018484	*Plk3*	serine/threonine-protein kinase PLK3	-1.26	0.0132	0.0000
ENSRNOG00000056984	*Baz2b*	bromodomain adjacent to zinc finger domain 2B	-0.48	0.0132	0.0000
ENSRNOG00000008015	*Fos*	fos proto-oncogene, AP-1 transcription factor subunit	-2.09	0.0149	0.0000
ENSRNOG00000011238	*Tiparp*	TCDD inducible poly(ADP-ribose) polymerase	-1.25	0.0149	0.0000
ENSRNOG00000019422	*Egr1*	early growth response 1	-1.19	0.0149	0.0000
ENSRNOG00000018714	*Arl5b*	ADP ribosylation factor like GTPase 5B	-0.80	0.0149	0.0000
ENSRNOG00000015692	*Taok1*	thousand and one amino acid protein kinase 1	-0.68	0.0149	0.0000
ENSRNOG00000009795	*Nfib*	nuclear factor I B	-0.65	0.0149	0.0000
ENSRNOG00000010680	*Med12l*	mediator complex subunit 12 like	-0.61	0.0149	0.0000
ENSRNOG00000007152	*Bhlhe40*	basic helix-loop-helix family member E40	-0.60	0.0149	0.0000
ENSRNOG00000042519	*Peak1*	pseudopodium enriched atypical kinase 1	-0.59	0.0149	0.0000
ENSRNOG00000018841	*Sox8*	SRY-box 8	-0.56	0.0149	0.0000
ENSRNOG00000008425	*Nav1*	neuron navigator 1	-0.51	0.0149	0.0000
ENSRNOG00000024101	*Phkb*	phosphorylase kinase regulatory subunit beta	-0.48	0.0149	0.0000
ENSRNOG00000011460	*Arfgef3*	ARFGEF family member 3	-0.82	0.0152	0.0000

Gene ID	Gene symbol	Gene name or description	Log_2 fold change	FDR	p-value
ENSRNOG00000053240	*Soga1*	suppressor of glucose, autophagy associated 1	-0.66	0.0172	0.0000
ENSRNOG00000004100	*Trib1*	tribbles pseudokinase 1 (G-protein-coupled receptor-induced gene 2 protein)	-1.73	0.0181	0.0000
ENSRNOG00000054391	*Snurf*	SNRPN upstream reading frame	-1.62	0.0181	0.0000
ENSRNOG00000000640	*Egr2*	early growth response 2	-3.43	0.0188	0.0001
ENSRNOG00000023509	*Irs2*	insulin receptor substrate 2	-0.71	0.0188	0.0000
ENSRNOG00000008415	*Nab2*	NGFI-A binding protein 2	-0.56	0.0188	0.0000
ENSRNOG00000008173	*Sesn3*	sestrin 3	-0.52	0.0188	0.0000
ENSRNOG00000012422	*Tnik*	TRAF2 and NCK interacting kinase	-0.49	0.0188	0.0000
ENSRNOG00000004057	*Ccdc88a*	coiled-coil domain containing 88A	-0.47	0.0188	0.0000
ENSRNOG00000010217	*Prrc2b*	proline rich coiled-coil 2B	-0.43	0.0188	0.0000
ENSRNOG00000001143	*Cit*	citron Rho-interacting serine/threonine kinase	-0.37	0.0188	0.0000
ENSRNOG00000059479	*Adcy1*	adenylate cyclase 1	-0.55	0.0198	0.0001
ENSRNOG00000047386	*Smg1*	SMG1 nonsense mediated mRNA decay associated PI3K related kinase	-0.55	0.0204	0.0001
ENSRNOG00000007126	*Gpr19*	G protein-coupled receptor 19	-0.53	0.0204	0.0001
ENSRNOG00000020770	*Arl4d*	ADP ribosylation factor like GTPase 4D	-0.62	0.0207	0.0001
ENSRNOG00000016950	*Otud1*	OTU deubiquitinase 1	-0.59	0.0207	0.0001
ENSRNOG00000052894	*Epg5*	ectopic P-granules autophagy protein 5 homolog	-0.45	0.0207	0.0001
ENSRNOG00000006515	*Klhl15*	kelch like family member 15	-1.61	0.0221	0.0001
ENSRNOG00000059406	*Ier5*	immediate early response 5	-0.67	0.0221	0.0001
ENSRNOG00000015133	*Kmt2a*	lysine methyltransferase 2A	-0.67	0.0221	0.0001

Gene ID	Gene symbol	Gene name or description	Log_2 fold change	FDR	p-value
ENSRNOG00000004823	*Dock4*	dedicator of cytokinesis 4	-0.45	0.0221	0.0001
ENSRNOG00000049302	*Chd9*	chromodomain helicase DNA binding protein 9	-0.48	0.0224	0.0001
ENSRNOG00000023969	*Herc6*	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6	-0.57	0.0231	0.0001
ENSRNOG00000017556	*Chrm4*	cholinergic receptor muscarinic 4	-0.37	0.0233	0.0001
ENSRNOG00000019677	*Arid3b*	AT-rich interaction domain 3B	-0.96	0.0237	0.0001
ENSRNOG00000061121	*Wdfy3*	WD repeat and FYVE domain containing 3	-0.52	0.0237	0.0001
ENSRNOG00000018012	*Tulp4*	tubby like protein 4	-0.43	0.0237	0.0001
ENSRNOG00000031760	*Fmn1*	formin 1	-0.96	0.0240	0.0001
ENSRNOG00000028493	*Ppp1r15b*	protein phosphatase 1 regulatory subunit 15B	-0.34	0.0240	0.0001
ENSRNOG00000023896	*Dusp6*	dual specificity phosphatase 6	-1.15	0.0244	0.0001
ENSRNOG00000028630	*Ksr2*	kinase suppressor of ras 2	-0.74	0.0244	0.0001
ENSRNOG00000000635	*Arid5b*	AT-Rich interaction domain 5b	-0.54	0.0244	0.0001
ENSRNOG00000010065	*Dgkh*	diacylglycerol kinase eta	-0.94	0.0247	0.0001
ENSRNOG00000011470	*Tsc1*	TSC1 complex subunit 1	-0.38	0.0248	0.0001
ENSRNOG00000056150	*Purb*	purine rich element binding protein B	-0.50	0.0248	0.0001
ENSRNOG00000014205	*Klf2*	Kruppel Like Factor 2	-0.97	0.0256	0.0001
ENSRNOG00000054891	*Cnot6*	CCR4-NOT transcription complex subunit 6	-0.35	0.0257	0.0001
ENSRNOG00000003028	*Dnah17*	dynein axonemal heavy chain 17	-2.42	0.0288	0.0001
ENSRNOG00000013281	*Mib1*	mindbomb E3 ubiquitin protein ligase 1	-0.40	0.0288	0.0001
ENSRNOG00000013623	*Amer2*	APC membrane recruitment protein 2	-0.74	0.0289	0.0001
ENSRNOG00000002053	*Fras1*	Fraser extracellular matrix complex subunit 1	-0.61	0.0289	0.0001

Gene ID	Gene symbol	Gene name or description	Log_2 fold change	FDR	p-value
ENSRNOG00000047712	*Dcx*	doublecortin	-0.47	0.0289	0.0001
ENSRNOG00000008924	*Arhgef12*	Rho guanine nucleotide exchange factor 12	-0.36	0.0289	0.0001
ENSRNOG00000007472	*Setd5*	SET domain containing 5	-0.29	0.0289	0.0001
ENSRNOG00000020344	*Sntb2*	syntrophin beta 2	-1.35	0.0300	0.0002
ENSRNOG00000006509	*Srgap3*	SLIT-ROBO Rho GTPase activating protein 3	-0.43	0.0300	0.0002
ENSRNOG00000007821	*Dyrk2*	dual specificity tyrosine phosphorylation regulated kinase 2	-0.41	0.0300	0.0002
ENSRNOG00000000956	*Rasl11a*	RAS like family 11 member A	-0.83	0.0302	0.0002
ENSRNOG00000007387	*Per1*	period circadian regulator 1	-0.86	0.0303	0.0002
ENSRNOG00000024846	*Ier5l*	immediate early response 5 like	-0.71	0.0303	0.0002
ENSRNOG00000001255	*Mlxip*	MLX interacting protein	-0.48	0.0304	0.0002
ENSRNOG00000061764	N/A	AABR07021812.1 (clone-based (Ensembl) gene)	-0.42	0.0304	0.0002
ENSRNOG00000056106	*Dip2b*	disco interacting protein 2 homolog B	-0.39	0.0304	0.0002
ENSRNOG00000053288	*Ank3*	ankyrin 3	-0.33	0.0308	0.0002
ENSRNOG00000001730	*Acap2*	ArfGAP with coiled-coil, ankyrin repeat and PH domains 2	-0.49	0.0310	0.0002
ENSRNOG00000001844	*Klhl42*	Kelch like family member 42	-0.47	0.0310	0.0002
ENSRNOG00000004377	*Lpin1*	lipin1	-0.44	0.0310	0.0002
ENSRNOG00000006911	*Sptb*	spectrin beta, erythrocytic	-0.33	0.0310	0.0002
ENSRNOG00000005802	*Usp24*	ubiquitin specific peptidase 24	-0.27	0.0310	0.0002
ENSRNOG00000024907	*Tnrc6b*	trinucleotide repeat-containing gene 6B protein	-0.37	0.0322	0.0002
ENSRNOG00000025160	*Tmem56*	transmembrane protein 56	-0.54	0.0322	0.0002
ENSRNOG00000011268	*Chd5*	chromodomain helicase DNA binding protein 5	-0.30	0.0340	0.0002
ENSRNOG00000006893	*Ppm1k*	protein phosphatase, Mg2+/Mn2+ dependent 1K	-0.42	0.0342	0.0002

Gene ID	Gene symbol	Gene name or description	Log_2 fold change	FDR	p-value
ENSRNOG00000055567	*Fmnl2*	formin like 2	-0.34	0.0342	0.0002
ENSRNOG00000020372	*Hdac4*	histone deacetylase 4	-0.43	0.0352	0.0002
ENSRNOG00000014928	*Apba1*	amyloid beta precursor protein binding family A member 1	-0.32	0.0354	0.0002
ENSRNOG00000046667	*Fosb*	fosB proto-oncogene, AP-1 transcription factor subunit	-1.17	0.0355	0.0002
ENSRNOG00000040287	*Cyp1b1*	cytochrome P450 family 1 subfamily B member 1	-0.82	0.0355	0.0003
ENSRNOG00000002750	*Rc3h1*	ring finger and CCCH-type domains 1	-0.60	0.0355	0.0002
ENSRNOG00000003491	*Prkca*	protein kinase C alpha	-0.55	0.0355	0.0003
ENSRNOG00000013257	*Hecw2*	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2	-0.52	0.0355	0.0003
ENSRNOG00000004696	*Arhgap5*	Rho GTPase activating protein 5	-0.46	0.0355	0.0002
ENSRNOG00000018477	*Otud4*	OTU deubiquitinase 4	-0.45	0.0355	0.0003
ENSRNOG00000017828	*Egr3*	early growth response 3	-0.45	0.0355	0.0002
ENSRNOG00000006338	*Lrp6*	LDL receptor related protein 6	-0.39	0.0355	0.0003
ENSRNOG00000024535	*Zzef1*	zinc finger ZZ-type and EF-hand domain containing 1	-0.38	0.0355	0.0003
ENSRNOG00000012573	*Dlgap2*	DLG associated protein 2	-0.36	0.0355	0.0002
ENSRNOG00000014548	*Nedd9*	neural precursor cell expressed, developmentally down-regulated 9	-0.50	0.0357	0.0003
ENSRNOG00000009253	*Igsf9b*	immunoglobulin superfamily member 9B	-0.90	0.0363	0.0003
ENSRNOG00000052583	*Zfp106*	zinc finger protein 106	-0.34	0.0363	0.0003
ENSRNOG00000020009	*Npas4*	neuronal PAS domain protein 4	-1.18	0.0372	0.0003
ENSRNOG00000002863	*Cacna1e*	calcium voltage-gated channel subunit alpha1E	-0.84	0.0372	0.0003
ENSRNOG00000013851	*Spry4*	sprouty RTK signaling antagonist 4	-1.02	0.0377	0.0003

Gene ID	Gene symbol	Gene name or description	Log_2 fold change	FDR	p-value
ENSRNOG00000008471	*Kif21b*	kinesin family member 21 B	-0.35	0.0377	0.0003
ENSRNOG00000005964	*Nr4a3*	nuclear receptor subfamily 4 group A member 3	-1.39	0.0378	0.0003
ENSRNOG00000052840	*Tanc2*	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2	-0.55	0.0378	0.0003
ENSRNOG00000004806	*Strn*	striatin	-0.44	0.0378	0.0003
ENSRNOG00000052157	*Nav3*	neuron navigator 3	-0.38	0.0378	0.0003
ENSRNOG00000000275	*Fam13c*	family with sequence similarity 13 member C	-0.31	0.0378	0.0003
ENSRNOG00000023453	*Lrba*	LPS responsive beige-like anchor protein	-0.28	0.0378	0.0003
ENSRNOG00000026432	*Syt7*	synaptotagmin 7	-0.26	0.0378	0.0003
ENSRNOG00000002894	*Mnt*	MAX network transcriptional repressor	-0.52	0.0391	0.0003
ENSRNOG00000004828	*Acvr1c*	activin A receptor type 1C	-0.85	0.0393	0.0004
ENSRNOG00000037613	*Kdm6b*	lysine demethylase 6B	-0.66	0.0393	0.0004
ENSRNOG00000009590	*Stox2*	storkhead box 2	-0.49	0.0393	0.0004
ENSRNOG00000018226	*Zcchc14*	zinc finger CCHC-type containing 14	-0.39	0.0393	0.0003
ENSRNOG00000011076	*Ank2*	ankyrin 2	-0.37	0.0393	0.0004
ENSRNOG00000004048	*Lrrk2*	leucine rich repeat kinase 2	-0.35	0.0393	0.0003
ENSRNOG00000007104	*Itpr1*	inositol 1,4,5-triphosphate receptor type 1	-0.26	0.0393	0.0003
ENSRNOG00000022305	*Mrp1*	multidrug resistance-associated protein 1 (also ATP binding cassette subfamily C member 1; *Abcc1*)	-0.39	0.0393	0.0004
ENSRNOG00000003300	*Btg2*	BTG anti-proliferation factor 2	-1.17	0.0402	0.0004
ENSRNOG00000006378	*Mga*	MGA, MAX dimerization protein	-0.61	0.0402	0.0004
ENSRNOG00000011358	*Hipk3*	homeodomain interacting protein kinase 3	-0.44	0.0402	0.0004

Gene ID	Gene symbol	Gene name or description	Log_2 fold change	FDR	p-value
ENSRNOG00000015121	*N4bp1*	NEDD4 binding protein 1	-0.32	0.0402	0.0004
ENSRNOG00000008444	*Cbl*	cbl proto-oncogene	-0.66	0.0405	0.0004
ENSRNOG00000024998	*Trps1*	transcriptional repressor GATA binding 1	-0.54	0.0408	0.0004
ENSRNOG00000011619	*Myo9a*	myosin IXa	-0.53	0.0432	0.0004
ENSRNOG00000053635	*Zfp192*	zinc finger with KRAB and SCAN domains 8 (also *Zkscan8*)	-0.44	0.0432	0.0004
ENSRNOG00000014285	*Ssh2*	slingshot protein phosphatase 2	-0.40	0.0432	0.0004
ENSRNOG00000005616	*Ncoa3*	nuclear receptor coactivator 3	-0.39	0.0432	0.0004
ENSRNOG00000056826	*Arap2*	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	-0.31	0.0448	0.0005
ENSRNOG00000061262	*Huwe1*	HECT, UBA and WWE domain containing 1, E3 ubiquitin protein ligase	-0.34	0.0454	0.0005
ENSRNOG00000057806	*Trpm7*	transient receptor potential cation channel subfamily M member 7	-0.37	0.0454	0.0005
ENSRNOG00000010379	*Celf1*	CUGBP Elav-like family member 1	-0.42	0.0456	0.0005
ENSRNOG00000002983	*Nfix*	nuclear factor I X	-0.40	0.0457	0.0005
ENSRNOG00000060946	*Cask*	calcium/calmodulin dependent serine protein kinase	-0.39	0.0459	0.0005
ENSRNOG00000027151	*Lrrc58*	leucine rich repeat containing 58	-0.42	0.0462	0.0005
ENSRNOG00000001324	*Asmt*	acetylserotonin O-methyltransferase	-1.70	0.0470	0.0005
ENSRNOG00000000942	*Pan3*	poly(A) specific ribonuclease subunit PAN3	-0.39	0.0482	0.0005
ENSRNOG00000004516	*Itgbl1*	integrin subunit beta like 1	-1.18	0.0486	0.0005
ENSRNOG00000006569	*Itgb8*	integrin subunit beta 8	-0.69	0.0486	0.0005
ENSRNOG00000014486	*Rfx3*	regulatory factor X3	-0.66	0.0486	0.0005
ENSRNOG00000019822	*Gadd45b*	growth arrest and DNA damage inducible beta	-0.65	0.0486	0.0006
ENSRNOG00000003882	*Cep350*	centrosomal protein 350	-0.49	0.0486	0.0006

Gene ID	Gene symbol	Gene name or description	Log_2 fold change	FDR	p-value
ENSRNOG00000016680	*Nsd1*	nuclear receptor binding SET domain protein 1	-0.47	0.0486	0.0006
ENSRNOG00000014142	*Ogfrl1*	opioid growth factor receptor like 1	-0.40	0.0486	0.0006
ENSRNOG00000038365	*Plekha2*	pleckstrin homology domain containing A2	-0.33	0.0486	0.0005
ENSRNOG00000009137	*Ubxn2b*	UBX domain protein 2b	-0.33	0.0486	0.0006
ENSRNOG00000001658	*Kcnj6*	potassium voltage-gated channel subfamily J member 6	-0.44	0.0487	0.0006
ENSRNOG00000026293	*Jun*	jun proto-oncogene, AP-1 transcription factor subunit	-0.35	0.0487	0.0006
ENSRNOG00000015719	*Egr4*	early growth response 4	-0.50	0.0489	0.0006
ENSRNOG00000047873	*Sec22c*	SEC22 homolog C, vesicle trafficking protein	-0.49	0.0489	0.0006
ENSRNOG00000007992	*Srsf10*	serine and arginine rich splicing factor 10	-0.36	0.0489	0.0006
ENSRNOG00000011624	*Kcnq2*	potassium voltage-gated channel subfamily Q member 2	-0.35	0.0489	0.0006
ENSRNOG00000003434	*Trove2*	TROVE domain family member 2	-0.61	0.0491	0.0006
ENSRNOG00000012148	*Trio*	trio Rho guanine nucleotide exchange factor	-0.26	0.0491	0.0006
ENSRNOG00000012386	*Zbtb38*	zinc finger and BTB domain containing 38	-0.47	0.0491	0.0006
ENSRNOG00000017092	*Zfp169*	zinc finger protein 169	-0.69	0.0492	0.0006
ENSRNOG00000032656	*Ptprt*	protein tyrosine phosphatase, receptor type T	-0.59	0.0492	0.0006
ENSRNOG00000008766	*Grin2b*	glutamate ionotropic receptor NMDA type subunit 2B	-0.56	0.0492	0.0006
ENSRNOG00000006967	*Xiap*	x-linked inhibitor of apoptosis	-0.42	0.0492	0.0006
ENSRNOG00000025634	*Zfhx2*	zinc finger homeobox 2	-0.32	0.0492	0.0006
ENSRNOG00000020426	*Erf*	ETS2 repressor factor	-0.32	0.0492	0.0006
ENSRNOG00000007034	*Hipk2*	homeodomain interacting protein kinase 2	-0.42	0.0492	0.0006
ENSRNOG00000004496	*Rock2*	Rho associated coiled-coil containing protein kinase 2	-0.30	0.0495	0.0006
ENSRNOG00000030247	*Ankhd1*	ankyrin repeat and KH domain containing 1	-0.29	0.0497	0.0006

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