Understanding the role of hepatic Neuronal PAS Domain Protein 2 (NPAS2) in circadian rhythms and cognition

Ruby Chrisp, MBiol.

Submitted to the University of Nottingham for the degree of Doctor of Philosophy

September 2022

Abstract

Circadian rhythms are highly conserved, endogenous biological oscillations with a periodicity of approximately 24 hours. These cycles have significant impacts on the sleep wake cycle, metabolism, mood, and cognition. A defining characteristic of these rhythms is their ability to be entrained by external stimuli, such as light and temperature, known as zeitgebers. Such rhythms are generated by a hierarchy of biological clocks which are centrally controlled by the suprachiasmatic nucleus (SCN) within the anterior hypothalamus. The SCN orchestrates phase synchronisation of clocks in peripheral tissues to allow for rhythmic regulation of endogenous processes throughout the day. Further understanding of circadian rhythms is important, since disruption of the clock and modern day non-circadian light schedules have been associated with cancers, metabolic diseases, and ageing.

One gene central to the rhythmic control of physiological processes is neuronal PAS domain protein 2 (NPAS2). NPAS2 is a bHLH-PAS domain containing protein which is the lesser-known paralog of CLOCK. Both CLOCK and NPAS2 are transcription factors that can form heterodimeric complexes with BMAL1 in the positive limb of the core circadian transcription-translation feedback loop to initiate the transcription of clock-controlled genes. The presence of one of the two, NPAS2 or CLOCK, is vital to maintain rhythmicity, but in the absence of one, the other will compensate in circadian functionality. In addition to circadian roles, NPAS2 has been implicated in cognition and emotional long-term memory. Knockdown of NPAS2 has also been seen to cause anxiolytic effects in behavioural paradigms, and striatal NPAS2 levels increase following acute and chronic stress, showing an additional role for the circadian gene in stress responses. Here, we utilised conditional peripheral knockouts of NPAS2 in mouse models to elucidate the role of hepatic NPAS2 in global circadian

rhythmicity, locomotor activity, recognition memory and anxiety. These experiments were originally performed as a peripheral control for brain specific knockout mice.

To investigate peripheral NPAS2 roles in circadian period, phase and locomotor activity, pre- and post-natal hepatic NPAS2 knockout mice were employed in cycling and constant lighting conditions. These mouse models displayed no effect of loss of liver NPAS2 on entrainment or adaptation to constant lighting conditions, however, significant female-specific weight loss following social isolation was observed that was absent in C57BL/6J wild type controls. Most notably, significant sexual dimorphism in circadian parameters following extended time in constant light were revealed in control mice, indicating female superiority in light condition adaptation. This effect was lost in hepatic NPAS2 deleted models. Furthermore, novel object recognition tests uncovered significant differences in cognitive performance in constant light, in favour of post-natal hepatic NPAS2 knockout mice. Recognition tests also showed male superiority in the cognitive performance of control mice that was abolished in liver deleted NPAS2 mice. These results suggest a sex-specific mechanism by which hepatic NPAS2 may influence recognition memory.

A proposed method by which cognition is altered following hepatic NPAS2 loss is through aberrant circadian gene expression within the brain. Downstream investigations into circadian gene expression in the frontal lobe revealed altered expression and phase of core clock genes *Bmal1*, *Clock* and *Rev-erbß* in animals with hepatic NPAS2 loss. Additionally, loss of sexually dimorphic central circadian gene expression was also noted in post-natal NPAS2 liver knockouts. Taken together, these results suggest, first, that hepatic loss of NPAS2 may influence centrally mediated behaviours such as cognition, and second, suggest this may represent a sex-specific mechanism. To complement the findings of this work, brain specific NPAS2 knockout mice could be employed to investigate the impact of direct NPAS2 loss in the brain on cognition and circadian gene expression.

Acknowledgements

I owe many thanks to my supervisors Dr Paula Moran and Prof David Heery. I am very grateful for their consistent support, guidance and advice throughout the last four years of my PhD. I would also like to thank my additional supervisors, Dr Hilary Collins and Prof Claire Gibson. I feel very fortunate to have had such a versatile and engaged supervisory team who helped motivate and support me so significantly.

A huge thank you is also due to Dr Mitchell Masterson. During the first two years of my PhD his continued advice and training was greatly appreciated. He set the foundations for this project, and I am very lucky to have been welcomed into this line of work by him. I must also acknowledge Rebecca Pope for her role in this work. She has had a significant impact both on my life and my PhD, one that I will never forget and will always be grateful for.

I would also like to thank the members of DMH who have helped me so much over the past four years, Ryan Morris, Dr Chris Roberts and Luc Costello-Heaven. Not only have they been helpful for troubleshooting, advice, and general lab assistance, but they have also been great friends. Additionally, a huge thank you goes to the wider GRRB lab groups. As lab members came and went, the GRRB community always made for a wonderful place to work. I would also like to give a huge thank you to my favourite women in science who have helped me so significantly along the way. Eilidh Mackenzie, Claire Pearce, Megan Wilde and Temiloluwa Daike – doing this PhD with and alongside them has been so special, I am so proud of them all.

Finally, I would like to thank my wonderful family. Though they won't understand much of this thesis, I could not have written it without them.

List of Abbreviations

NPAS2 – Neuronal PAS domain protein 2

CLOCK – Circadian locomotor output cycles kaput

BMAL1 – Brain muscle Arnt-like 1

PER – Period

CRY – Cryptochrome

SCN – Suprachiasmatic nucleus

RHT – Retino-hypothalamic tract

FEO – Food entrainable oscillator

TTFL – transcription-translation feedback loop

PAS – Per-Arnt-Sim

RORα – Retinoic acid-related orphan receptor alpha

RORE – Retinoic acid-related orphan receptor response element

LD – light dark

DD – double darkness, constant dark

LL – constant light

bHLH – Basic helix loop helix

HAT – histone acetyltransferase

NAD+ – nicotinamide adenine dinucleotide

HDAC – histone deacetylase

CREB – cAMP response element-binding protein

CBP – CREB binding protein

CT – Circadian time

ZT – Zeitgeber time

IR – Infrared

EEG – Electroencephalography

NOR – Novel object recognition

NOL – Novel object location

ROS – Reactive oxygen species

LTP – Long-term potentiation

LTM – Long-term memory

CCF – Cued and contextual fear

NREM – Non-rapid eye movement

SNP – Single nucleotide polymorphism

DRD1 – Dopamine D1 receptor

NAc – Nucleus accumbens

SAD – Seasonal affective disorder

CFS – Chronic fatigue syndrome

PBMC – Peripheral blood mononuclear cells

MDD – Major depressive disorder

VIP – Vasoactive intestinal polypeptide

AR – Androgen receptors

ER – Oestrogen receptors

HPG – Hypothalamic pituitary gonadal

HPA – Hypothalamic pituitary adrenal

LH – Luteinizing hormone

AVPV – Antero-ventral periventricular nucleus

FSH – Follicle stimulating hormone

RT-qPCR – Reverse transcription quantitative polymerase chain reaction

RNA – Ribonucleic acid

mRNA – Messenger RNA

DNA – Deoxyribonucleic acid

cDNA – Complementary DNA

NAFLD – Non-alcoholic fatty liver disease

cKO – Conditional knockout

Afp – α-fetoprotein

Alb – Albumin

FFT-NLLS – Fast-Fourier transform non-linear least squares

Bp – Base pairs

RAE – Relative amplitude error

GCL – Granule cell layer

DI – Discrimination index

OF – Open field

LD box – Light-dark box

SSI – Semi-social isolation

ASPA – Animals (Scientific Procedures) Act

PBS – Phosphate buffered saline

SDS-PAGE – Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis

EDTA – Ethylenediaminetetraacetic acid

TEMED – N,N,N,N-tetramethylethylenediamine

PFC – Pre-frontal cortex

LIV – Liver

ACC – Anterior cingulate cortex

ANOVA – Analysis of variance

NTC – No template control

DMH – Dorsal medial hypothalamus

BSA – Bovine serum albumin

Table of Contents

Abstract 2
Acknowledgements 4
List of Abbreviations 5
Table of Figures 13
Table of Tables 17
Chapter 1: Introduction 18
1.1 The circadian clock 19
1.2 Molecular transcription-translation feedback loops 21
1.3 CLOCK/NPAS2 compensation and circadian paralogs 23
1.4 Post-translational modification: regulation of the circadian clock 25
1.5 The clinical relevance of circadian studies 28
1.6 *In vivo* circadian methodology 32
1.7 Circadian clock mutations and cognition 35
1.8 NPAS2 in cognition, mood and anxiety 38
1.9 Peripheral clocks 43
1.10 Sexual dimorphism of the SCN in humans and rodent models 46
1.11 Sex differences in circadian behaviour 50
1.12 Aims and objectives 53
Chapter 2: The effects of pre- and post-natal hepatic NPAS2 loss, and sex, on global circadian rhythmicity and locomotor activity 55
2.1 Introduction 56
2.1.1 Global impacts of hepatic circadian clocks 56
2.1.2 Circadian rhythmicity in *Npas2* knockout models 58
2.1.3 Circadian sex differences of C57BL/6J wild type mice 60
2.1.4 An introduction to Alb and Afp conditional knockout mice 61
2.1.5 Aims and Objectives 64
2.2 Materials and Methodology 65
2.2.1 Animals 65
2.2.2 *In vivo* husbandry and general care 66
2.2.3 Experimental set up and circadian recording 66
2.2.4 Afp cKO and Afp fl/fl experiments 68
2.2.5 cKO and fl/fl experiments 68
2.2.6 Circadian data analysis 69
2.2.7 Statistical analysis 69
2.2.8 Molecular and biochemical reagents 70
2.2.9 Genotyping 70

2.2.10 Purification of genomic DNA from tissues 71
2.2.11 Polymerase chain reaction (PCR) 71
2.2.12 Agarose gel electrophoresis 71
2.3 Results 72
2.3.1 Generation and genotyping of conditional knockout models 72
2.3.2 Confirmation of liver specific NPAS2 knockout 73
2.3.3 Locomotor activity of Afp fl/fl and Afp cKO mice 74
2.3.4 Locomotor activity of fl/fl and cKO mice 77
2.3.5 Locomotor activity of C57BL/6J wild type mice 80
2.3.6 Circadian rhythmicity and sexual dimorphism in Afp fl/fl and Afp cKO mice 83
2.3.7 Circadian rhythmicity and sexual dimorphism in fl/fl and cKO mice 86
2.3.8 Circadian rhythmicity and sexual dimorphism in C57BL/6J mice 88
2.4 Discussion 93
2.4.1 Pre-natal hepatic NPAS2 deletion influences locomotor activity but not circadian rhythmicity 93
2.4.2 Post-natal hepatic NPAS2 deletion does not influence locomotor activity or circadian rhythmicity 96
2.4.3 Sex differences in NPAS2 transgenic lines 98
2.4.4 Sex differences in C57BL/6J wild type mice 99
Chapter 3: The effects of post-natal hepatic NPAS2 loss, and sex, on recognition memory 101
3.1 Introduction 102
3.1.1 Clock mutations and cognition 102
3.1.2 *Npas2* and cognition 103
3.1.3 Testing object recognition memory 104
3.1.4 Sexual dimorphism in spatial and recognition memory 106
3.1.5 Memory under conditions of constant dark and constant light 109
3.1.6 Aims and Objectives 111
3.2 Materials and Methodology 113
3.2.1 Animals 113
3.2.2 *In vivo* husbandry and general care 113
3.2.3 Circadian box experimental set-up 113
3.2.4 NOR test protocol 114
3.2.5 NOR data analysis 115
3.2.6 Statistical analysis 116
3.3 Results 117
3.3.1 Assessing object and spatial biases in NOR sample trials 117
3.3.2 The effects of NPAS2 cKO, sex, and lighting condition on NOR test interaction 119
3.3.3 The effects of NPAS2 cKO and lighting condition on recognition memory 120
3.3.4 The effects of sex and NPAS2 cKO on recognition memory 122

3.4 Discussion 124
3.4.1 Sex and genotype differences in total test interaction in cKO and fl/fl mice 125
3.4.2 Post-natal hepatic NPAS2 deletion improves recognition memory in constant light 127
3.4.3 cKO memory superiority in constant light is likely a result of fl/fl sex differences 129
Chapter 4: The effects of pre- and post-natal hepatic NPAS2 loss, and sex, on anxiety 132
4.1 Introduction 133
4.1.1 *Npas2* and anxiety 133
4.1.2 Testing anxiety: light/dark box and open field arena 134
4.1.3 Anxiety under constant lighting conditions 137
4.1.4 Social isolation and anxiety 140
4.1.5 Aims and Objectives 142
4.2 Materials and Methodology 144
4.2.1 Animals for LD box tests 144
4.2.2 Animals for OF arena tests 145
4.2.3 *In vivo* husbandry and general care 146
4.2.4 Experimental set up and lighting conditions for LD box tests 146
4.2.5 Experimental set up and lighting conditions for OF 147
4.2.6 LD box test protocol 147
4.2.7 LD box analysis 147
4.2.8 OF arena trial protocol 148
4.2.9 OF arena analysis 148
4.2.10 Statistical analysis 148
4.3 Results 149
4.3.1 Significant sex-linked weight loss in Afp fl/fl and Afp cKO mice following SSI 149
4.3.2 Significant sex-linked weight loss in fl/fl and cKO mice following SSI 151
4.3.3 Latency to enter the dark and time spent in the light chamber showed effects of sex and genotype in Afp transgenic lines 153
4.3.4 LD box chamber transitions and aborted entries were increased in Afp fl/fl and Afp cKO following SSI 156
4.3.5 OF show increased anxiety in both Afp transgenic lines following SSI 162
4.3.6 Sexual dimorphism of latency to enter the dark chamber seen in fl/fl and cKO transgenic lines 166
4.3.7 Significant sex and isolation condition effects on number of aborted LD box entries in cKO and fl/fl mice 171
4.3.8 OF showed increased anxiety in cKO and fl/fl lines following SSI 176
4.3.9 Sex and genotype differences in OF performed following SSI in LL conditions 183
4.3.10 Genotype differences in OF performed following extended SSI 186
4.4 Discussion 189
4.4.1 Significant sex-linked weight loss observed in all transgenic lines was not present in C57BL/6J mice 190

4.4.2 SSI reliably reduced time spent in the central zone of an OF arena in all genotypes 192
4.4.3 LD box tests revealed variable results of SSI, genotype, and sex on anxiety in Afp transgenic lines 194
4.4.4 LD box tests revealed variable results of SSI, genotype and sex on anxiety in fl/fl and cKO mice 197
4.4.5 Extended SSI and LL SSI do not appear to be anxiogenic in comparison to no SSI and SSI conditions 200
Chapter 5: Post-mortem confirmation of hepatic *Npas2* loss and variable circadian gene expression within the pre-frontal cortex 203
5.1 Introduction 204
5.1.1 The effects of *Npas2* knockout on circadian gene expression 204
5.1.2 Circadian gene expression aberrancies as a mechanism for cognitive change 205
5.1.3 Aims and Objectives 206
5.2 Materials and Methodology 207
5.2.1 Animals and experimental conditions 207
5.2.2 *In vivo* husbandry 209
5.2.3 Schedule 1 and tissue collection 209
5.2.4 General laboratory chemicals 209
5.2.5 Molecular reagents and antibodies 209
5.2.6 Genomic extraction 211
5.2.7 PCR 211
5.2.8 Agarose gel electrophoresis and DNA band extraction 211
5.2.9 Sequencing DNA 211
5.2.10 RNA extraction 211
5.2.11 cDNA synthesis 212
5.2.12 Quantitative reverse-transcription PCR (RT-qPCR) primer validation 213
5.2.13 RT-qPCR protocol 213
5.2.14 RT-qPCR analysis and statistical analysis 215
5.2.15 Protein extraction and concentration determination 215
5.2.16 Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) 216
5.2.17 Western blot 216
5.3 Results 217
5.3.1 Validation of *Npas2* exon 3 loss in the liver and maintained *Npas2* exon 3 in the frontal lobe of cKO and Afp cKO mice 217
5.3.2 Investigating *Npas2* expression levels in cKO and fl/fl tissues using RT-qPCR 219
5.3.3 Confirmation of reduced NPAS2 protein in cKO livers of both sexes 223
5.3.4 Altered *Clock* expression in the liver and frontal lobe of hepatic *Npas2* cKO male and female mice 225
5.3.5 Variable *Bmal1* expression in the frontal lobe of hepatic *Npas2* cKO male and female mice 230
5.3.6 Sexual dimorphism of *Rev-erbß* expression in hepatic *Npas2* cKO and fl/fl control mice 235

5.4 Discussion 241
5.4.1. *Npas2* exon 3 loss and protein reduction confirmed in male and female cKO mice 241
5.4.2 Differential circadian expression in the frontal lobe as a possible mechanism for circadian behavioural and cognitive changes 243
5.4.3. Sexual dimorphism in circadian gene expression in C67BL/6J mice 244
Concluding remarks 246
Appendix 250
A.1 Additional investigations into sex-linked weight loss in SSI: maternal genotype influence and food consumption 251
A.1.1 Maternal genotype did not influence weight loss in SSI conditions 251
A.1.2 Adequate consumption of dry diet in addition to intervention diet 252
A.2 No innate preferences were seen between objects within NOR sets 253
A.2.1 Materials and Methodology 253
A.2.2 Results 253
A.3. Averaged actograms for fl/fl and cKO mice 254
A.3.1 Methods 254
A.3.2 Results 254
A.4 Oligonucleotides 255
A.5 RT-qPCR primer validation 255
A.6 Normalisation of RT-qPCR groups 258
A.7 Additional cDNA confirmation gel for validation on full length Npas2 in fl/fl frontal lobe 258
A.8 Statistical analysis of C57BL/6J period and phase in comparison to transgenic lines 259
A.8.1 C57BL/6J, Afp fl/fl and Afp cKO 259
A.8.2 C57BL/6J, fl/fl and cKO 259
A.9 Professional Internship Placement (PIP) Reflective Statement 260
Bibliography 262

Table of Figures

Figure 1.1: A schematic detailing the core TTFL along with the modulatory secondary loop. 23
Figure 1.2: Double-plotted actograms depicting the compensation of CLOCK and NPAS2, adapted from DeBruyne et al., 2007. 24
Figure 1.3: Survival of *Clock*$^{-/-}$ and *Bmal1*$^{-/-}$ mice, adapted from Kondratov et al., 2006b. 29
Figure 1.4: Reduced anxiety in *Npas2* mutant mice, adapted from Ozburn et al., 2017. 42
Figure 2.1: Double plotted actograms and average wheel revolutions depicting differential activity of Npas2$^{-/-}$ mice. 59
Figure 2.2: Mechanism of Cre recombinase removing the transgene. 62
Figure 2.3: Schematic detailing the timeline of NPAS2 knockout in both Afp cKO and cKO. 63
Figure 2.4: Circadian chambers for recording behaviour. 67
Figure 2.5: Genotyping cKO and fl/fl mice. 73
Figure 2.6: Confirmation of liver specific *Npas2* exon 3 loss in cKO and Afp cKO mice. 74
Figure 2.7: Average hourly activity patterns did not differ between Afp cKO and Afp fl/fl in LD, DD or LL. 75
Figure 2.8: Afp cKO subjects displayed increased distance travelled in DD compared to Afp fl/fl, and a significant reduction in locomotor activity in LL. 76
Figure 2.9: Average hourly activity patterns of cKO mice did not significantly differ from fl/fl controls in LD, DD or LL. 78
Figure 2.10: cKO and fl/fl subjects displayed a significant reduction in locomotor activity in LL. 79
Figure 2.11: Average hourly activity patterns of C57BL/6J mice appear similar to Afp fl/fl, fl/fl, Afp cKO and cKO transgenic lines. 81
Figure 2.12: C57BL/6J mice displayed a significant reduction in locomotor activity in LL. 82
Figure 2.13: No significant differences in circadian parameters between Afp cKO and Afp fl/fl controls in any lighting condition. 83
Figure 2.14: Significant sex differences in period and phase between male and female Afp cKO subjects. 85
Figure 2.15: No significant differences in circadian parameters seen between cKO and fl/fl controls in any lighting condition. 86
Figure 2.16: Significant effects of sex on period in cKO, and fl/fl mice in LL. 88
Figure 2.17: Experimental conditions replicated a 24-hour entrained period in C57BL/6J mice. 89
Figure 2.18: Significant differences in period and phase of Afp transgenic lines compared to C57BL/6J mice. 90
Figure 2.19: Significant differences in period and phase of cKO and fl/fl, compared to C57BL/6J mice. 91
Figure 2.20: Significant sex differences in C57BL/6J period and phase in constant light conditions. 92
Figure 3.1: Sexual dimorphism in C57BL/6J recognition memory varies in the literature. 108

Figure 3.2: A schematic depicting the NOR procedure employed in this study. Created with Biorender.com. 114
Figure 3.3: No location preference shown in either genotype during sample trial. 117
Figure 3.4: No significant differences in sample trial DI between sexes or genotypes. 118
Figure 3.5: NOR test interaction does not differ between genotypes but differs between cKO and fl/fl males. 119
Figure 3.6: NOR test interaction differs between fl/fl sexes in LD and DD. 120
Figure 3.7: Both genotypes show a preference for novelty in all lighting conditions. 121
Figure 3.8: NPAS2 cKO mice show a significantly improved memory performance in constant light. . 122
Figure 3.9: Sexual dimorphism in recognition memory is lost in cKO mice. 123
Figure 3.10: Sexual dimorphism in recognition memory is lost in cKO mice. 124
Figure 4.1: SSI induced weight loss in Afp fl/fl and Afp cKO was more severe in female mice. 150
Figure 4.2: SSI induced weight loss was only present in fl/fl and cKO female mice. 152
Figure 4.3: No significant differences in latency to enter dark were seen between genotypes, or across isolation conditions in Afp lines. 153
Figure 4.4: Significant effect of genotype on male latency to enter dark in Afp lines. 154
Figure 4.5: A significant preference for the light chamber was seen in both Afp fl/fl and Afp cKO irrespective of housing condition. 155
Figure 4.6: Significant sex effect on time spent in the light chamber for Afp fl/fl mice. 156
Figure 4.7: Both LD box chamber transitions and aborted entries are increased following SSI in Afp transgenic lines. 158
Figure 4.8: Significant effects of SSI on number of LD box transitions in Afp fl/fl mice and female Afp cKO mice. 159
Figure 4.9: Significant interaction of SSI and genotype on the number of LD box aborted entries in Afp fl/fl and Afp cKO mice. 161
Figure 4.10: Afp fl/fl and Afp cKO mice both preferred the peripheral area of an OF arena. 162
Figure 4.11: Time spent in the central zone of an OF arena was reduced following SSI in Afp fl/fl and Afp cKO mice. 163
Figure 4.12: Time spent in the central zone of an OF arena was reduced following SSI in Afp fl/fl females and Afp cKO male and female mice. 165
Figure 4.13: No significant differences in latency to enter dark were seen between genotypes, or across isolation conditions in cKO or fl/fl lines. 166
Figure 4.14: Significant sexual dimorphism in latency to enter dark in cKO and fl/fl mice. 168
Figure 4.15: A significant preference for the light chamber was seen in both fl/fl and cKO mice in no SSI and SSI. 169
Figure 4.16: Significant sex effect on time spent in the light chamber by cKO mice. 170
Figure 4.17: Significant effect of SSI on aborted entries but not LD box transitions in fl/fl and cKO mice. 171

Figure 4.18: Significant interaction of isolation condition and sex on fl/fl LD box chamber transitions that was not seen in cKO mice. 173
Figure 4.19: Number of aborted entries showed significant sex effects in fl/fl mice and significant isolation effects in female mice. 175
Figure 4.20: Fl/fl and cKO mice both preferred the peripheral area of an OF arena. 176
Figure 4.21: Time spent in the central zone of an OF arena was reduced following SSI in fl/fl and cKO mice. 177
Figure 4.22: Time spent in the central zone of an OF arena was reduced following SSI in fl/fl mice and cKO females. 179
Figure 4.23: Distance travelled in OF trials showed significant differences between fl/fl and cKO males. 180
Figure 4.24: Distance travelled in OF trials showed significant differences between fl/fl and cKO males. 182
Figure 4.25: Fl/fl and cKO mice both preferred the peripheral area of an OF arena in LL SSI conditions. 183
Figure 4.26: LL SSI had a sex-specific effect on time spent in the central zone. 184
Figure 4.27: Genotype and isolation condition effects seen on distance travelled in LL SSI. 186
Figure 4.28: Fl/fl and cKO mice both preferred the peripheral area of an OF arena in extended SSI conditions. 187
Figure 4.29: Time spent in the central zone of an OF arena was not significantly reduced in extended SSI compared to no SSI. 188
Figure 4.30: Distance travelled in the OF arena was not significantly different in extended SSI compared to other conditions. 189
Figure 5.1: *Npas2* exon 3 is only absent in the liver of cKO and Afp cKO mice. 219
Figure 5.2: *Npas2* expression levels in cKO and fl/fl tissues do not significantly differ between genotypes. 220
Figure 5.3: cKO females showed significantly reduced hepatic *Npas2* expression compared to fl/fl females. 222
Figure 5.4: Significantly reduced NPAS2 levels in male and female cKO mice compared to fl/fl controls. 224
Figure 5.5: Loss of sexual dimorphism in Clock expression within cKO mice. 227
Figure 5.6: Hepatic *Npas2* loss appears to abolish cyclical Clock expression in the frontal lobe. 228
Figure 5.7: Similar patterns of Clock expression in the frontal lobe and liver of C57BL/6J, compared to fl/fl mice. 229
Figure 5.8: All sex and genotype groups demonstrate cyclical *Bmal1* expression in the liver. 231
Figure 5.9: Loss of significant cyclical expression of *Bmal1* in the frontal lobe of Npas2 cKO female mice. 233
Figure 5.10: Significant effects of sex on frontal lobe *Bmal1* expression in C57BL/6J mice. 234
Figure 5.11: Significant sex differences in hepatic *Rev-erbß* expression in cKO mice. 236

Figure 5.12: Significant sex differences in frontal lobe *Rev-erbß* expression in cKO and fl/fl mice. 238
Figure 5.13: Sex differences in liver and frontal lobe *Rev-erbß* expression in C57BL/6J mice. 240
Figure A.1: Maternal genotype does not have a significant influence on weight loss. 251
Figure A.2: Average daily dry food consumption of fl/fl and cKO mice over five weeks. 252
Figure A.3: No innate object preferences in Set 1 or Set 2. 253
Figure A.4: Average distance travelled actograms for fl/fl and cKO mice do not reveal significant differences. 254
Figure A.5: RT-qPCR primer validations. 256
Figure A.6: Product sizes from RT-qPCR correspond to predicted sizes. 257
Figure A.7: Product size for RT-qPCR using *Npas2* primers. 257
Figure A.8: Repeat gel image to confirm absence of 91 bp band in fl/fl frontal lobe. 259

Table of Tables

Table 2.1: PCR reaction recipe. 71
Table 5.2: n numbers of fl/fl, cKO and C57BL/6J mice that were used in gene expression analysis 209
Table 5:3: Reverse transcription for cDNA synthesis step one. 212
Table 5.4: Reverse transcription for cDNA synthesis step two...... 212
Table 5.5: RT-qPCR reaction mix volumes. 214
Table 5.6: RT-qPCR protocol...... 214
Table 5.7: SDS-PAGE gel recipe. 216

Chapter 1: Introduction

1.1 The circadian clock

Circadian rhythms are highly conserved, endogenous biological oscillations with a periodicity of approximately 24 hours. They are generated by a hierarchy of biological clocks which are centrally controlled by the suprachiasmatic nucleus (SCN) within the anterior hypothalamus. The SCN obtains ambient light cues from the retina via the retino-hypothalamic tract (RHT) and projects to the dorsal medial hypothalamus (DMH), which subsequently projects to various other hypothalamic regions (Gerstner and Yin, 2010, Bertolucci et al., 2008). This results in the SCN orchestrating phase synchronisation of clocks in peripheral tissues, allowing for rhythmic regulation of endogenous processes (Glaser and Stanewsky, 2005, Landgraf et al., 2016). These processes include: the sleep/wake cycle, body temperature, metabolism and hormone secretion. Via this mechanism, organisms benefit from increased fitness due to their ability to predict fluctuations in the environment and adapt to novelty (Bertolucci et al., 2008, Takahashi et al., 2008). Understanding circadian rhythms is clinically relevant since disruption of the clock has been associated with cancers, metabolic diseases, and ageing (Dupuis et al., 2010, Fu and Lee, 2003, Libert et al., 2012).

Core parameters of circadian output are period, phase, and amplitude. Period refers to the interval of time between two successive points of an oscillation, with an endogenous circadian period being 24 hours. Phase is defined as the point of peak activity within that oscillation, meaning whereabouts within a period a subject is most active, whilst amplitude dictates the maximum extent of the oscillation, indicative of the strength of the rhythm (Zielinski et al., 2014). A defining characteristic of these rhythms is their ability to be entrained by external stimuli, such as light and temperature, known as zeitgebers (DeBruyne et al., 2007). Entrainment is characterised by the alignment of an internal rhythm (often referred to as *tau* (τ)) to an external environmental cue, such that the period of this internal rhythm synchronises to the cue given. The most critical zeitgeber for SCN entrainment is the light/dark cycling of

night and day. The SCN presents as two functionally disparate nuclei; the ventrolateral SCN (the core) and the dorsomedial SCN (the shell). The ventrolateral SCN is the component that receives light input from the external environment via the RHT, whilst the dorsomedial SCN is independent of light entrainment. Thus, the dorsomedial SCN requires light entrained signals from the ventrolateral SCN to maintain rhythmicity (Gonzalez, 2008). Adaptation to the daily light schedules of the environment confers an evolutionary advantage for many organisms, for example, plant species can orchestrate transcriptional activation of photosynthetic machinery to mirror that of day-light hours, and some rodents present nocturnality as a method of avoiding diurnal predators in a bid to improve survival (Panda et al., 2002, Takahashi et al., 2008).

Circadian clocks can also be entrained by feeding cues via a food entrainable oscillator (FEO) which is independent of, and at times out of synchrony with, the central SCN oscillator. In most instances where light schedules dictate feeding times, this assists the synchronisation by the SCN of peripheral tissues involved in feeding and metabolism to light cycles (Damiola et al., 2000, Hirota et al., 2002). Whilst the tissue origin of the FEO is largely unknown, restricted feeding paradigms in rodents depict peripheral clocks in the liver, kidney, heart and pancreas entraining to feeding cues without SCN synchrony. Additionally, metabolic state, cellular redox state and glucose levels are a key influence of cellular circadian rhythmicity that also link to food intake (Damiola et al., 2000, Hirota et al., 2002, Lamia et al., 2009, Rutter et al., 2001). In nature, this translates to preservation of energy in some mammals by being most active during times of high food availability (Panda et al., 2002, Takahashi et al., 2008). Though it is important to emphasise that any cyclical rhythm that does not persist in the absence of entrainment, either by feeding or light schedules, is not a true circadian rhythm; this would be merely a response to an environmental cue. Circadian periods of behaviour and physiology are maintained under conditions of constant lighting or variable feeding schedules (Panda et al., 2002)

1.2 Molecular transcription-translation feedback loops

The molecular basis of periodic rhythmicity is well understood; circadian rhythms are produced by interlocking, negative transcription-translation feedback loops (TTFL) which involve post-translational modifications of transcriptional repressors throughout the day (DeBruyne et al., 2007). This was elucidated in *Drosophila* by Nobel Prize winning researchers Hall, Rosbash and Young (Edery et al., 1994, Hall, 1994, Myers et al., 1996). The positive limb of the mammalian circadian clock consists of three basic helix-loop-helix (bHLH) transcription factors; circadian locomotor output cycle kaput (CLOCK), neuronal PAS domain protein 2 (NPAS2) and brain and muscle ARNT-Like 1 (BMAL1). These three proteins contain Per-Arnt-Sim (PAS) domains that allow for their heterodimerisation in the forms of CLOCK:BMAL1 and NPAS2:BMAL1 (hereafter referred to as the CLOCK/NPAS2:BMAL1 heterodimer) (Bertolucci et al., 2008, DeBruyne et al., 2006). Throughout the day, these heterodimers act as transcriptional activators by binding conserved E-box enhancer elements (CACGTG) in regulatory regions of core circadian genes *Period* (*Per1, Per2,* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*), as well as other clock-controlled genes. PER and CRY then comprise the negative limb of the TTFL; during the dark phase, they heterodimerise and translocate back into the nucleus to repress their own transcription via inhibition of CLOCK/NPAS2:BMAL1 heterodimer activity. CRY is seen as the principal repressor, it employs a mode of blocking repression by binding the CLOCK/NPAS2:BMAL1 heterodimer at the promoter of a circadian target gene and inhibits the transcription without dissociating the complex. Alternatively, PER engages in displacement repression; this entails the removal of CLOCK/NPAS2:BMAL1 from the promoter of target genes (Ye et al., 2014, Bertolucci et al., 2008). Degradation of the PER:CRY complex then relieves this inhibition, which allows the

CLOCK/NPAS2:BMAL1 heterodimer to resume transcriptional activity as another 24-hour cycle begins (Figure 1.1) (Landgraf et al., 2016).

Aside from the core TTFL involving the CLOCK/NPAS2:BMAL1 heterodimer, another interconnecting modulatory TTFL exists involving *Rev-erbα*, *Rev-erbß* and retinoic acid-related orphan receptor alpha (*Rorα*). The oscillatory transcription of *Rev-erb* genes is initiated by the CLOCK/NPAS2:BMAL1 heterodimer and is repressed by the PER:CRY heterodimer. REV-ERBs act as inhibitors of *Bmal1* transcription by binding the retinoic acid-related orphan receptor response element (RORE) within the *Bmal1* promoter (DeBruyne et al., 2007, Guillaumond et al., 2005). Conversely, RORα is an oscillating activator of *Bmal1* transcription that is periodically expressed within the SCN. RORα competes with REV-ERB proteins for the RORE within the *Bmal1* promoter which results in the circadian oscillation of *Bmal1* expression. This is a vital contribution to the circadian network, since REV-ERBα deficient mice show abnormal circadian rhythms that have amplified BMAL1 and CLOCK levels, as well as a reduction in period duration. Similar uncharacteristic rhythms are noted in *Rorα* mutant mice (Guillaumond et al., 2005, Preitner et al., 2002). In addition, NPAS2 contains two ROREs and is also a target of REV-ERB proteins and RORα, which demonstrates the extent of integration between this modulatory secondary loop and the core TTFL (Figure 1.1) (Crumbley et al., 2010, Guillaumond et al., 2005, Solt et al., 2011).

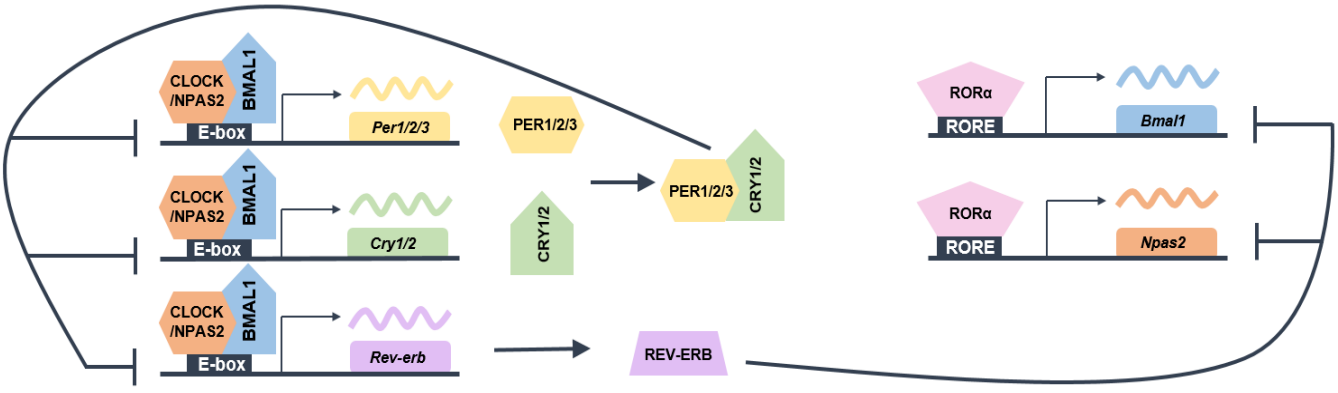


Figure 1.1: A schematic detailing the core TTFL along with the modulatory secondary loop.

CLOCK/NPAS2:BMAL1 heterodimers initiate the transcription of core circadian genes by binding E-boxes within regulatory regions. PER1/2/3:CRY1/2 form a heterodimer that translocates to the nucleus and prevents CLOCK/NPAS2:BMAL1 mediated transcription (Landgraf et al., 2016). *Rev-erb* transcription is also induced via the CLOCK/NPAS2:BMAL1 heterodimer, REV-ERB then attenuates the transcription of *Bmal1* and *Npas2*. *Bmal1* and *Npas2* transcription resumes when RORα competes with REV-ERBα for the RORE within the *Bmal1* and *Npas2* promoters (Solt et al., 2011).

1.3 CLOCK/NPAS2 compensation and circadian paralogs

Homozygous mutations in CLOCK (the first identified mammalian clock gene) cause behavioural arrhythmia and depleted molecular rhythm amplitudes in the SCN (Landgraf et al., 2016, Vitaterna et al., 1994). These discoveries led to the initial assumption that CLOCK is an indispensable element within the TTFL, and that its absence results in an inoperative circadian clock. However, DeBruyne et al. disproved this concept some 10 years later; they showed *Clock* knockout mice have strong, robust locomotor activity rhythms even after 4 weeks in complete darkness (DD) and display only slight alterations in molecular cycling. This confirmed that CLOCK was not a prerequisite for circadian oscillations, and that there is likely another BMAL1 binding partner that allows for rhythmic regulation of gene expression (DeBruyne et al., 2006). NPAS2 has considerable sequence homology to CLOCK, particularly in both the bHLH and PAS domains. It was later revealed that NPAS2 can form transcriptionally active complexes with BMAL1, much like CLOCK (Landgraf et al., 2016). It was believed that NPAS2 had a solely SCN-specific role and lacked the ability to substitute for CLOCK in peripheral tissues (Landgraf et al., 2016). Nevertheless, recent research shows NPAS2 upholds its ability to compensate for CLOCK in peripheral oscillators such as the liver, and in cells extracted from the peripheral tissues of *Clock*$^{-/-}$ mice which continue to display cell-autonomous circadian rhythms independent of the SCN (Bertolucci et al., 2008, Landgraf et al., 2016). Furthermore, *Clock*$^{-/-}$ mice show upregulated NPAS2 in both peripheral and

central tissues. Similar to *Clock*$^{-/-}$ mice, *Npas2*$^{-/-}$ mice have slightly shorter circadian periods, yet molecular rhythms persist with only subtle variations. Contrastingly, CLOCK-deficient mice with non-functional NPAS2 (*Clock*$^{-/-}$;*Npas2*$^{-/-}$) were completely arrhythmic when placed in a free-running environment, meaning that at least one of the BMAL1 dimerisation partners is required to maintain circadian rhythmicity (Figure 1.2) (DeBruyne et al., 2006, DeBruyne et al., 2007). NPAS2 and CLOCK knockout mice have also demonstrated that the alternate BMAL1 dimerisation partners also amount to different gene expression profiles and thus must have disparate affinities for certain circadian promoters. These findings suggest that NPAS2 is a compensatory BMAL1 dimerisation partner in the absence of CLOCK, but that they have diverse roles, and CLOCK has a more central function within the SCN (DeBruyne et al., 2007).

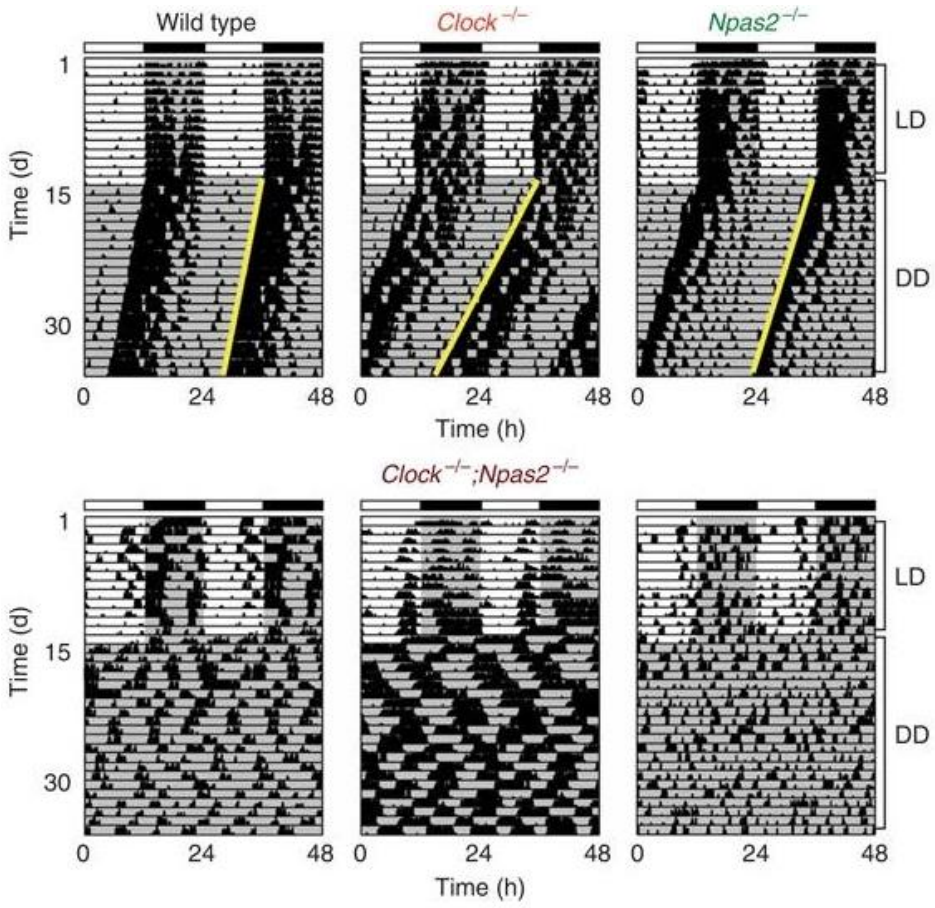


Figure 1.2: Double-plotted actograms depicting the compensation of CLOCK and NPAS2.

Actograms depict wheel-running activity; dense black areas indicate the most activity whilst blank white areas represent inactivity. The top panel portrays *Clock*$^{-/-}$ and *Npas2*$^{-/-}$ mutants showing marginally altered circadian periods in free-running, DD (complete darkness) conditions, but masking during light/dark cycling (LD). The bottom panel shows three *Clock*$^{-/-}$*;Npas2*$^{-/-}$ double mutant mice displaying complete arrhythmicity in DD, and considerably perturbed rhythms during LD (adapted from DeBruyne et al., 2007).

Almost all core circadian proteins have a compensatory paralog pair, including *Per1/2/3* and *Cry1/2*, therefore both genes must be mutated to observe a total abolition of rhythmicity (Bunger et al., 2000). Since BMAL1 is capable of causing arrhythmicity with a single mutation, it was assumed that BMAL1 lacks a similar compensatory partner. This is supported by the fact that *Bmal1*$^{-/-}$ mice exhibit more severe characteristics than other circadian mutants; they show an immediate and complete loss of circadian rhythms, as well as non-circadian phenotypes such as reduced activity and diminished longevity (Bunger et al., 2000, Kondratov et al., 2006b, Shi et al., 2010). It is known that BMAL1 has a homologous partner recognised as BMAL2 that is capable of forming transcriptionally active complexes with CLOCK. Since *Bmal1*$^{-/-}$ mice exhibit a total loss of rhythmicity, BMAL1 and BMAL2 clearly lack functional redundancy (Bunger et al., 2000, Shi et al., 2010). Whilst ectopic expression of *Bmal2* permits the rescue of both metabolic and behavioural phenotypes within *Bmal1* knockout mice, wild type endogenous *Bmal2* levels are insufficient. It has subsequently been concluded that *Bmal1* employs a regulatory role in *Bmal2* expression, and that a knockout of *Bmal1* is a functional double knockout of both paralogs. Therefore, the fact that a single gene mutation results in such extensive phenotypic consequences infers BMAL1 is uniquely vital in the maintenance of circadian rhythms (Bunger et al., 2000, Shi et al., 2010).

1.4 Post-translational modification: regulation of the circadian clock

The circadian protein CLOCK is reported to have intrinsic histone acetyltransferase (HAT) activity and appears to influence chromatin structure by acetylation of H3K9 and H3K14 (Doi

et al., 2006, Nakahata et al., 2008, Nakahata et al., 2009). This impacts chromatin-remodelling events and thus further influences gene expression. CLOCK also possesses the ability to acetylate BMAL1 at Lys537 – a highly conserved residue present in all vertebrates. Notably, this acetylation is also rhythmic and coincides with the downregulation of clock-controlled gene transcription (Hirayama et al., 2007). This BMAL1 acetylation is reported to be necessary for the dimerisation of CLOCK and BMAL1, and also for the recruitment of CRY1 to the CLOCK:BMAL1 heterodimer to facilitate transcriptional repression. Since Lys537 mutants are unable to cause cyclic gene expression, it suggests that BMAL1 acetylation is essential for rhythmic transcription of circadian genes (Doi et al., 2006, Hirayama et al., 2007). Thus, CLOCKs role as a HAT opens chromatin and causes transcriptional activation, therefore encouraging the positive limb of the TTFL. Whilst BMAL1 acetylation allows for CRY1 recruitment and subsequent transcriptional repression, which therefore mediates the negative TTFL limb (Hirayama et al., 2007). As of yet, no evidence suggests NPAS2 possesses this HAT ability.

Another example of the impacts of post-translational modifications on the circadian clock is elucidated in the role of Sirtuin (SIRT1). SIRT1 is a histone deacetylase (HDAC) that can remove acetyl residues from histone tails in order to silence gene expression and condense chromatin. SIRT proteins are a unique class of HDACs that require the co-enzyme nicotinamide adenine dinucleotide (NAD+), these enzymes therefore act as functional links between gene expression and metabolic activity (Nakahata et al., 2008). SIRT1 is thought to be the HDAC that deacetylates BMAL1 following its acetylation by CLOCK, which amounts to oscillatory levels of BMAL1 activation. It has also been demonstrated that SIRT1 is able to physically associate with CLOCK and subsequently assist the deacetylation of circadian targets. SIRT1 colocalizes with the CLOCK/NPAS2:BMAL1 heterodimer to form a chromatin regulatory complex at the promoters of circadian target genes (Etchegaray et al., 2003,

Nakahata et al., 2008). The necessity of this regulation is demonstrated using the pharmacological loss of SIRT1 activity, which leads to broader phases and higher oscillatory amplitudes, signifying loss of strict circadian control. This not only demonstrates a model of post-translational, time-dependent circadian regulation, but also depicts how metabolic influences can impact the clock (Nakahata et al., 2008).

It is known that transcriptional coactivators and HATs, such as p300/CREB binding protein (CBP), can interact with CLOCK and NPAS2 to facilitate circadian gene expression. There is significant evidence that chromatin remodelling complexes form an association with endogenous clock proteins, and therefore have a role in temporal regulation (Curtis et al., 2004, Kwapis et al., 2018, Nakahata et al., 2008). Studies have shown rhythmic acetylation of histone H3 and histone H4 at the E-box of *Per1*. Interestingly, periodic acetylation of lysine 9 within histone H3 at *Per1*/*2* promoters appears to coincide with the transcriptional activation of both genes (Asher et al., 2008, Curtis et al., 2004). This time-of-day specific acetylation is performed by p300, which is a HAT known to associate with CLOCK. This proposes a model whereby the CLOCK/NPAS2:BMAL1 complex employs HAT coactivators, as well as HDACs, to influence transcriptional activation of circadian targets (Nakahata et al., 2008, Curtis et al., 2004). This was proven using overexpression of CBP, which leads to amplified activation of CLOCK/NPAS2:BMAL1 target genes. Since transcriptional activators CLOCK and BMAL1, and repressors PER1 and PER2, only transiently bind their applicable target gene, there must be an alternative process that sustains the repressed state after said transcription factors have left the site (Ripperger and Merrow, 2011). Since clock genes remain repressed even after the degradation of CRY/PER, evidence suggests temporary epigenetic silencing via histone modifications continue the repression until CLOCK/NPAS2:BMAL1 reemployment (Duong and Weitz, 2014).

1.5 The clinical relevance of circadian studies

The importance of furthering knowledge of circadian rhythms is clinically relevant in many scientific fields including, ageing, cancer biology, and studies of sleep and shift work. During ageing, physiological processes display a steady deterioration in efficiency. Examples of such processes include; metabolic function, sleep/wake cycles, immune responses and cognitive function (Banks et al., 2016). Importantly, as one ages, sleeping patterns become fragmented; older people tend to display increased awakening during the night, and sleep more frequently during the day. They also show a reduction in actual sleep time and sleep efficiency (Huang et al., 2002). Additionally, perturbations of sleep and circadian rhythms are one of the earliest symptoms displayed in Alzheimer's and Parkinson's patients (Pace-Schott and Spencer, 2011, Wulff et al., 2010). Within the SCN, age-related losses in rhythmic amplitude, neuronal connectivity and electrical characteristics are seen. There are also perturbations in GABAergic signalling, which is implicated in neuronal synchrony and thus has severe downstream repercussions on cell communication (Banks et al., 2016). Significantly, there is also an age-related failing of multiple forms of memory, such as spatial and declarative memory (Kondratova and Kondratov, 2012).

In mouse models, a clear reduction in circadian rhythmicity and amplitude is seen in aged mice, as well as a decreased expression of core clock proteins (Banks et al., 2016, Musiek et al., 2013). In an aged subject, the clock has a reduced sensitivity to light and thus impaired entrainment via light cues. Additionally, mice deficient in CLOCK show a 15% decrease in average lifespan, and a 20% decrease in maximum lifespan in comparison to wild type mice (Figure 1.3). CLOCK deficiency also amounts to an increased incidence of cataracts and dermatitis, which are both characteristic age-specific pathologies (Dubrovsky et al., 2010). Interestingly, this phenotype is markedly less severe than the progeria-like phenotype described in *Bmal1*$^{-/-}$ mice. *Bmal1*$^{-/-}$ mice have significantly shortened lifespans and exhibit acute

symptoms of premature ageing, including sarcopenia, organ shrinkage and cataracts. The average lifespan of C57BL/6J mice is considered to be around 2 years, whilst the average lifespan of the *Bmal1*$^{-/-}$ mutant mice was seen to be half this (Figure 1.3) (Kondratov et al., 2006b). At approximately 16-weeks-old, *Bmal1*$^{-/-}$ mice begin to exhibit growth retardation and reduced body weight, by 52 weeks, wild type mice are double the size of *Bmal1*$^{-/-}$ mice.

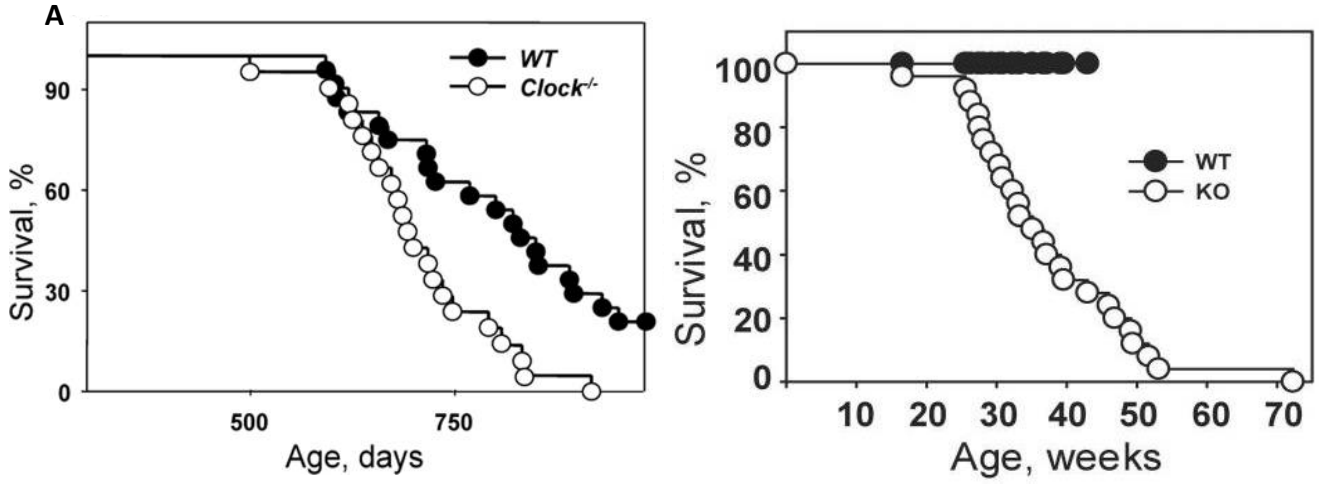


Figure 1.3: Survival of *Clock*$^{-/-}$ and *Bmal1*$^{-/-}$ mice.

A) A Kaplan-Meyer survival curve detailing the lifespan of wild type and *Clock*$^{-/-}$ mice of both sexes that died of natural causes. Adapted from Dubrovsky et al., 2010. B) A Kaplan-Meyer survival curve detailing the lifespan of wild type and BMAL1 knockout mice. Wild type mice were culled at 1 year of age (adapted from Kondratov et al., 2006b).

It has been hypothesized that *Bmal1*$^{-/-}$ mice display a more extreme ageing phenotype than CLOCK deficient mice since BMAL1 lacks a binding paralog that functionally compensates. Since CLOCK and NPAS2 are dispensable, a more acute ageing phenotype is displayed when mice are deficient for both proteins (Musiek et al., 2013). Double deletion of *Clock* and *Npas2* induced age-dependent astrogliosis – a rise in astrocyte number due to neuronal damage – that was equivalent to the astrogliosis seen in *Bmal1* deletion. *Clock* and *Npas2* double deletion phenocopies BMAL1 deficiency within the cortex, with both mutations displaying alike neuropathology (Musiek et al., 2013). Interestingly, age-related increases in astrogliosis and neuropathology were not observed in mice deficient in *Per1* or *Per2*. Since

PER1/2 are negative-limb TTFL proteins, this could suggest it is the positive limb that has a role in age-related neuropathology prevention (Musiek et al., 2013).

Additionally, circadian rhythms have been implicated in the tumorigenesis of breast cancer, colorectal cancer, liver cancer, lung cancer, prostate cancer and many more. There are multiple mechanisms by which the circadian clock can influence the incidence of cancer. Primarily, clock components both directly and indirectly regulate transcription of genes employed in DNA damage repair, cell secretion, autophagy and protein folding (Sulli et al., 2020, Zhou et al., 2022). Homeostasis of these processes is maintained by rhythmic gene expression, thus disruption of the clock and these pathways can result in redox imbalance, metabolic variations, cell cycle breaches and inflammation, all of which may amount to favourable tumorigenic environments. For example, mouse knockouts of *Per2* show increased incidence of cancer, due to a lack of cell cycle regulation (Lee et al., 2010, Sulli et al., 2020, Zhou et al., 2022). Similarly, mice lacking just one copy of the *Cry1/2* or *Per1/2* homologs depict increased tumour development both in spontaneous and radiation-induced conditions. This leads to the conclusion that tumour suppression is a clock-regulated process, and that repeated and sustained circadian dysfunction gives a tumour promoting effect (Lee et al., 2010).

Atypical circadian cycles have even been recorded as a carcinogen by the World Health Organisation. Circadian aberrancies are also linked to poorer prognosis for survival and early mortality within cancer patients. Numerous studies have detailed the role of circadian dysfunction in shift workers with the increased risk of breast and prostate cancer, depicting a robust correlation between >20 years as a shift worker and cancer prevalence (Sulli et al., 2020, Zhou et al., 2022). Specifically, cancer risk was seen to increase in direct relation to the number of years spent as a shift-worker, the frequency in which a worker rotated from day to night shift working, and the number of nightshift hours a week worked. Though, it is worth noting that

shift work often coincides with additional intersecting factors including smoking, alcohol consumption, non-stereotypical food choices, altered activity levels and exposure to other carcinogens (Fu and Kettner, 2013). Additionally, breast and prostate cancer risk was also seen to increase in those who consumed food across a full 24-hour day or excessively ate around midnight. This potentially implicates another zeitgeber, namely feeding rhythms, in cancer incidence. Feeding/fasting, sleep/wake and activity/rest cycles have all been implicated in cancer occurrence, with diet being a crucial determinant of cancer risk, and physical activity being a known protective factor in reduction of cancer occurrence (Zhou et al., 2022).

Similarly, the converse is true. Cancers are seen to reciprocally disrupt cellular circadian clocks using mechanisms such as promoter methylation to induce epigenetic silencing, dysregulation of transcription, and circadian gene polymorphisms leading to aberrant clock proteins (Fu and Kettner, 2013). Breast cancer biopsies display abnormal expression of core clock genes *Bmal1, Clock, Cry1/2, Per1/2/3* and *Npas2*. Additional cancers showing variations in circadian gene expression include colorectal cancer, acute myeloid leukaemia, non-Hodgkin's lymphoma, ovarian, lung and prostate cancer, amongst others. Often, aberrant expression of specific circadian genes, such as *Bmal1* and *Cry1*, is considered a poor prognostic marker in cancer patients (Fu and Kettner, 2013, Taniguchi et al., 2009).

Studying circadian biology in non-disease models is also of worth to general society, as modern-day life has introduced shift-work, intensive care hospital units and long-distance travel – all of which expose humans to prolonged, non-circadian periods of light, or light at night. Shift work is often accompanied by heightened blood pressure, cholesterol and body mass index, sleep disturbances, obesity and increased risk of cardiovascular disease (Ohta et al., 2005, Fonken et al., 2010). In addition, >99% of Americans and Europeans are subjected to light at night from electronic devices, streetlamps, and light overspill from surrounding buildings. Exposure to light at night is correlated with metabolic decline, perturbed sleep/wake

cycles and may even impact cognition (Fonken et al., 2010, Fonken and Nelson, 2014). One of the most frequent experiences of transient circadian desynchrony in modern life is jetlag, which occurs during long-distance travel in which a person crosses several time zones rapidly. This can result in irritability, fatigue, misaligned sleep/wake cycles with daylight hours, gastrointestinal issues and disorientation (Manfredini et al., 1998). Mimicking jetlag in *Drosophila* has also been shown to alter the expression of core circadian genes, and increases degradation of the clock gene *Timeless*. Thus, people who commonly experience jetlag, such as air hosts and hostesses, are considered amongst shift workers as most at risk for suffering circadian desynchrony (Koh et al., 2009).

1.6 *In vivo* circadian methodology

Three distinct kinds of photoreceptors are present within the mammalian system; rods, cones, and the more recently uncovered intrinsically photosensitive retinal ganglion cells (ipRGCs). The traditional photoreceptors, rods and cones, are accountable for producing image-forming vision, which allows us to create an image of the surrounding environment. Conversely, ipRGCs assess environmental irradiance and are vital for non-image producing vision (Alves-Simoes et al., 2016, Foster et al., 1991, Freedman et al., 1999). Mice lacking rods and cones respond to light with normal circadian parameters, which is indicative of a role for ipRGCs in photoentrainment within the mammalian retina. ipRGCs project both directly onto the SCN, and also to numerous brain regions required in governing functions such as sleep. It is thought that ipRGCs have a crucial role in circadian regulation and entrainment through their detection of the principal zeitgeber, light (Alves-Simoes et al., 2016, Paul et al., 2009). Foetal mammals cannot receive light cues from the external environment, therefore, their rhythmicity is primarily influenced by maternal metabolic and hormonal signals. The foetal circadian system then finishes developing in post-natal life. Following birth, ipRGCs establish far earlier than rods and cones as external light/dark zeitgebers override maternal cues. Clock gene

rhythmicity is then seen to be influenced by zeitgeber time at approximately post-natal day 10 (Alves-Simoes et al., 2016, O'Neil et al., 2017).

Typically, laboratory conditions that mimic the external environment consist of 12h:12h light dark (LD) cycling to entrain mice. Often when employing artificial conditions of LD cycling, researchers will align the light phase with natural daytime, but there are also instances where reverse light is employed. Reverse light still maintains animals on 12h:12h schedules, but the dark phase is instead aligned to natural daytime. Arguably, this allows for more accurate behavioural and cognitive testing, as the nocturnal rodents are in their active phase during experimental hours (Beeler et al., 2006, Munn et al., 2011). Though, this does confer disadvantages to the researchers themselves who may struggle to complete their husbandry checks and experimental tasks under such conditions. Studies directly comparing the use of reverse light and normal LD cycling have revealed no significant differences in several behavioural parameters, including activity and anxiety, but differences in ease of handling were noted (Beeler et al., 2006, Munn et al., 2011).

Following this period of LD entrainment, if external zeitgebers are eliminated, a subject will begin to diverge from a maintained 24-hour circadian period to exhibit their endogenous free-running rhythm that could continue indefinitely. This free running period length is variable between species but often subtly deviates from the 24-hour circadian period displayed during entrainment. This internal period length (τ) is referred to in circadian time (CT) rather than zeitgeber time (ZT) due to the lack of external influence. To induce these intrinsic free-running rhythms, chronobiologists often employ constant lighting conditions to eliminate entrainment cues and unmask endogenous period lengths (Jud et al., 2005, Zheng et al., 2001). The most popular way to expose free-running rhythms within rodents is constant darkness (DD). In DD, wild type, nocturnal mice typically display a free-running period of 23.7-hours ± 0.1 hour. Additionally, nocturnal rodent activity peaks shortly after the onset of darkness in an entrained

LD environment, whilst mice exposed to DD display a phase advance, where their peak of activity moves to earlier in the subjective day (Dudley et al., 2003, Jud et al., 2005, Zheng et al., 2001). DD is generally achieved using one of two lighting methods: red light, or infrared (IR) light. Mice are significantly less sensitive to red light than white light, and at low intensities it can mimic DD as most rodent vision is lost. Experimenters often prefer this method due to the ease of handling and set up, since a human's vision is not considerably impaired by red light (Peirson et al., 2018, Winters and Reid, 2010). However, it has been shown that mice are indeed receptive to red light, and whilst their vision may be compromised, it is not entirely absent. Thus, IR lighting is a more robust mimic of constant darkness that allows for visual recording of rodent mice (Albasser et al., 2010, Peirson et al., 2018, Ruby et al., 2013).

Another non-invasive experimental manipulation of the environment is constant light. Constant light is generally less widely employed as a method of free-running in comparison to DD, this is because the condition is least favourable for nocturnal rodents, who are active and feed throughout the dark phase. As a result, utilising LL in research conditions is more highly regulated than DD methods of free-running. Nevertheless, studying the consequences of LL environments has significant clinical relevance. Modern-day life has brought many scenarios in which exposure to non-circadian periods of light or light at night have dramatically increased (Ohta et al., 2005, Fonken et al., 2010). Induction of LL experimentally produces a significantly longer circadian period, a phase delay – where peak activity moves to later in the elongated period –, and reduced locomotor activity levels in nocturnal mice. LL can even provoke behavioural and physiological arrhythmicity by desynchronising individual clock neurons within the SCN (Alves-Simoes et al., 2016, Chen et al., 2008).

To measure rhythmic activity in these environments, researchers often employ behavioural methods such as locomotor activity, or wheel-running parameters. Wheel-running relies on the

assumption that any time an animal is not actively spinning the wheel, it is asleep or inactive (Fisher et al., 2012, Pendergast et al., 2014). Whilst this method is frequently used to determine sleep/wake cycles and circadian rhythmicity, it does not accurately represent an animal's total wake period. Locomotor activity recorded using cameras mounted above cages can give a more detailed depiction of sleep/wake cycles, as well as offering insight into distance travelled and location preference. Video tracking software can be employed to store such data continuously (Fisher et al., 2012, Pendergast et al., 2014). More invasive, surgical methods can be used to assess sleep/wake cycles via electrical activity. Electroencephalography (EEG) is the recording of brain activity commonly used in human studies. EEGs have been adapted to be light-weight and mobile, so that they can be utilised in freely moving mice (Ly et al., 2015, Rozov et al., 2016).

1.7 Circadian clock mutations and cognition

Mutations in core clock genes not only cause perturbed rhythms, they also have severe impacts on memory and other cognitive functions. Memory acquisition, consolidation and retrieval demonstrate temporal regulation instilled by the endogenous circadian clock. The genes implicated in the core TTFL are ubiquitously expressed in brain regions commonly associated with memory and cognition, such as the amygdala and the hippocampus (Hampp et al., 2008, Jilg et al., 2010, Lamont et al., 2005). Furthermore, disruption of the SCN via surgical ablation has significant consequences on both memory acquisition and maintenance of recognition memory (Ruby et al., 2008). This modulation of learning and memory could result from numerous avenues of regulation, including: neurotransmission, kinase signalling cascades, hormone secretion and epigenetic histone modification (Krishnan and Lyons, 2015). Mice with mutations in critical components of the circadian clock thus display impairments in various types of learning and memory paradigms. This cognitive impact of

circadian dysfunction has also been noted in humans, where substantial cognitive difficulties have been observed in long-term shift workers (Kondratova et al., 2010, Krishnan and Lyons, 2015). In humans, cognitive performance peaks during midday, where sleep inertia is relieved, and alertness steadily increases. This is also seen in nocturnal rodents, where their cognitive capabilities in the Morris water maze, contextual fear conditioning and the radial arm maze peak during their active phase – the night (Valentinuzzi et al., 2001, Valentinuzzi et al., 2004). It has therefore been suggested that there is a phylogenetically conserved mechanism by which organisms demonstrate oscillating capacities for learning and memory based upon a temporal framework (Gerstner et al., 2009).

The novel object location (NOL) is a hippocampal-dependent recall test that reveals temporal variations in memory. Mice deficient for forebrain *Bmal1* (fKO) appear to lose the time-of-day deviations in memory performance during this test (Snider et al., 2016). In fact, the results of *Bmal1* fKO models mirror those of novel object recognition (NOR) tests from completely arrhythmic mice. These impairments imply that additional clocks within the forebrain may have an indispensable role in learning and memory (Snider et al., 2016). In addition, mice with BMAL1 deficiency exhibit hyperactivity in novel environments and diminished habituation, which can be symptomatic of impeded long- and short-term memory capacities. Habituation occurs when a subject becomes familiarised with neutral stimuli and is capable of detecting novelty in that environment (Kondratova et al., 2010). This impaired habituation is thought to be a result of aberrant reactive oxygen species (ROS) homeostasis, since BMAL1 deficiency causes an overabundance of ROS. It is thought that the surplus of ROS causes persistent oxidative stress within the brain and may affect functions such as memory formation (Kondratova et al., 2010). In contrast, mice deficient in CRY1 and CRY2 have been shown to exhibit reduced activity and more rapid habituation to the environment (Kondratova et al., 2010). However, *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice lack the ability to learn time-place

associations, which is vital for the fitness and reproductive success of an organism. During this assessment, the mice had to remember that, depending on the time of day, different arms of the maze were either negatively or positively reinforced (Van der Zee et al., 2008). *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, which are arrhythmic in a free running environment, but masked in LD, failed to learn which arms would be negatively/positively enforced at which times of day. This proposes that *Cry* genes, or a functioning circadian clock system, are necessary for time-place learning and memory. Nonetheless, global *Cry* mutation cannot control for the influence of *Cry* in peripheral tissues, therefore brain specific knockouts would be ideal for conclusive results on memory aberrancies (Van der Zee et al., 2008).

Long-term potentiation (LTP) in the hippocampus is a type of synaptic plasticity that varies dependent on time-of-day and is believed to have a critical role in learning and memory (Gerstner and Yin, 2010). Mice with mutations in the circadian gene *Per2* demonstrate altered LTP with reduced magnitude; these mice have shortfalls in memory recall of trace fear conditioning, hippocampal LTP and display an gradual loss of circadian rhythmicity (Wang et al., 2009). Interestingly, *Per2* mutant mice display aberrant levels of phosphorylated cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein expression. Phosphorylation of CREB is vital in signalling cascades that result in strong LTP, as well as particular types of long-term memory (LTM). This suggests a role for *Per2* in LTP regulation, and recall memory (Wang et al., 2009). The hypothesis that these cognitive deficits are a result of shorter endogenous period lengths is also a possibility. Since humans living with shortened period lengths have compromised learning and memory, it is plausible that the shorter rhythm that results from *Per2* mutation is capable of causing the memory deficits (Wang et al., 2009, Wright et al., 2006).

In general, whether these circadian mutations cause impacts on learning and memory via sleep-dependent vs sleep-independent methods is debatable. Sleep disruption is a potential

mechanism by which circadian mutations may influence learning and memory, since sleep promotes memory consolidation. For validity, future studies should aim to include similar data on sleep records to see if circadian mutations may have sleep-dependent effects (Ruby et al., 2008, Van der Zee et al., 2008).

1.8 NPAS2 in cognition, mood and anxiety

Mice globally deficient in NPAS2 show deficits in LTM during cued and contextual fear (CCF) tasks. Garcia et al. exposed male NPAS2 knockout mice to auditory cues combined with foot shocks to associate the two. Both long and short interval times were used to assess the recall of this association, where an auditory cue was repeated, known as cued fear conditioning, or the subject was placed back in the original conditioning chamber, known as contextual fear conditioning (Garcia et al., 2000). No differences attributed to phenotype were noted following a short retention time, indicative of no short-term memory failings in NPAS2 knockout mice. Though, 24 hours after the initial association, NPAS2 knockout mice displayed reduced freezing responses to both tone and conditioning context in comparison to control mice. This suggests that NPAS2 is a requirement for conditioned fear responses after a 24-hour retention interval, and that NPAS2 loss impairs long-term memory in emotional tasks (Garcia et al., 2000).

Additionally, mice fed a western diet show significant hippocampal-dependent memory impairments and altered circadian gene expression in the hippocampus, namely, changes in *Npas2*, *Bmal1* and *Per2* levels. The western diet consisted of 40% fat chow and sugar water *ad libitum* for 16-weeks. Western diet fed mice gained significantly more weight that controls and showed a poorer memory performance in both Y maze and NOR cognitive tasks, thought to be a result of altered circadian gene expression in the hippocampus (Woodie et al., 2020). This links recognition and spatial memory impairments with induced changes in core circadian gene expression within the hippocampus.

Furthermore, global *Npas2*$^{-/-}$ mice display a reduction in the power of sleep spindles, a neural oscillatory activity generated by thalamic nuclei during non-rapid eye-movement (NREM) sleep (Franken et al., 2009). These sleep spindles have an essential role in LTM consolidation and sensory processing, thus, a reduction in these oscillations gives impairments in cortical plasticity and significant memory processes. This is a potential mechanism by which *Npas2* could influence long-term memory in rodents (Franken et al., 2009, Gais et al., 2002, Timofeev et al., 2002). Additionally, 19 *Npas2* single nucleotide polymorphisms (SNPs) have been identified to correlate with the risk of cognitive ageing, along with one *Bmal1* SNP and three *Clock* SNPs. *Npas2* SNPs were also seen to have an interaction with *Ror-α* and *Ror-β* SNPs to increase the risk of cognitive ageing. Cognitive ageing refers to the age-related decline in memory, recognition, reasoning and processing speed that occurs in late adulthood (Lin et al., 2017). This further implicates *Npas2* and its interactions with cognitive capabilities, and adds to evidence of a role for circadian proteins in ageing and cognition.

Npas2 is also highly enriched in dopamine D1 receptor (DRD1) containing neurons (Ozburn et al., 2015). These neurons are of high density in the striatum, the nucleus accumbens (NAc) and the substantia nigra, as well as being widely expressed in many other brain regions such as the hippocampus. DRD1 containing neurons have been implicated in the modulation of learning and memory (Fazio et al., 2016, Puighermanal et al., 2016, Wass et al., 2013). DRD1 sensitivity within the pre-frontal cortex (PFC) dictates wide-ranging cognitive capabilities as well as modulating working memory. Working memory training within CD-1 mice was reported to increase DRD1-mediated neuronal activation. Also, dopamine D1 signalling within the PFC can influence attentional abilities, which is thought to coincide with selective attention, a component of working memory (Wass et al., 2013). Additionally, within the dorsal hippocampus, D1 receptors have roles in synaptic plasticity and memory processes. Consolidation of long-term memory relies on dopamine D1 receptors within the paravalbumin

cells in the CA1 subfield of the dorsal hippocampus (Kern et al., 2015, Puighermanal et al., 2017). Dopamine and DRD1s are also seen to have significant roles in the persistence of memory within the hippocampus; where hippocampal-mediated, long-term memory requires activation of D1 and D5 receptors at the time of encoding (Bethus et al., 2010). Therefore, increased expression of *Npas2* in these DRD1 containing neurons correlates with an important role for *Npas2* in cognition and memory. Interestingly, cognitive deficits in *Per2* KO mice in Barnes maze and Y-maze tasks suggest impairments in spatial memory (Kim et al., 2022, Wang et al., 2009). DRD1 levels were higher in *Per2* KO mice than in wild type, and were inversely proportional to PER2 levels. Following administration of DRD1 antagonist SCH-23390, *Per2* KO mice showed rescued spatial memory, which implicates DRD1 in circadian regulation of spatial working memory (Kim et al., 2022). This presents a mechanism by which circadian proteins might alter cognitive processes in a DRD1-dependent manner, which could include roles for *Npas2*.

In addition to memory, NPAS2 appears to have a role in anxiety and mood. Whilst Garcia et al. demonstrated no anxiety phenotypes in global NPAS2 KO mice in open field (OF) or light-dark (LD) box tests, the circadian gene is present in the forebrain cortices that assimilate sensory information, as well as areas of the limbic system and basal ganglia which are associated with the regulation of anxiety and emotion. NPAS2 is also abundant in brain regions implicated in behavioural responses to stressful stimuli, such as the striatum and the forebrain (Dudley et al., 2003, Garcia et al., 2000, Ozburn et al., 2017). Contrary to Garcia et al., Ozburn et al., discovered significantly reduced anxiety phenotypes in *Npas2* mutant mice (Figure 1.4). Loss of global *Npas2* resulted in increased time spent in the open arms in elevated plus maze tests, as well as increased entries to the open arms, though this was not statistically significant (Ozburn et al., 2017). In LD box trials, Ozburn et al. found that global *Npas2* mutants had a significantly reduced latency to enter the light side of the box. This is considered the anxiogenic

side, as the bright light is unfavourable for nocturnal animals, thus speedy entry to this chamber is indicative of reduced anxiety. Additionally, OFs revealed an increased distance travelled in the central area and increased time spent in the central area of global *Npas2* mutant mice. The centre of an OF arena is also considered the most anxiogenic, as this is where the animal is most vulnerable, so increased time and distance travelled in this area is also indicative of reduced anxiety (Figure 1.4) (Ozburn et al., 2017). This presents loss of *Npas2* as having an anxiolytic effect.

Both acute and chronic stress increases the levels of striatal *Npas2* expression, showing the circadian gene is intrinsically linked to stress responses in the striatum (Ozburn et al., 2017). Additionally, mice deficient in the *Npas2* gene also exhibit a behavioural lack of sensitivity to diazepam treatment. The suggested mechanism by which this occurs, is the implication of *Npas2* in GABAergic neurotransmission within the ventral striatum (Ozburn et al., 2017, Partonen et al., 2007, Soria et al., 2010). NAc specific siRNA knockdown of *Npas2* also amounts to reductions in anxiety (Ozburn et al., 2017, Spencer et al., 2013). Specifically, the NAc within the striatum contains DRD1 containing neurons which receive input from midbrain dopaminergic regions. *Npas2* is highly enriched in DRD1 containing neurons, and in the NAc. As well as an anxiolytic effect, *Npas2* knockdown in the NAc abolishes diurnal mRNA expression of dopamine receptors *Drd1*, *Drd2* and *Drd3*. This suggests *Npas2* related memory and anxiety phenotypes may be dependent on dopamine signalling pathways within these brain regions (Ozburn et al., 2017).

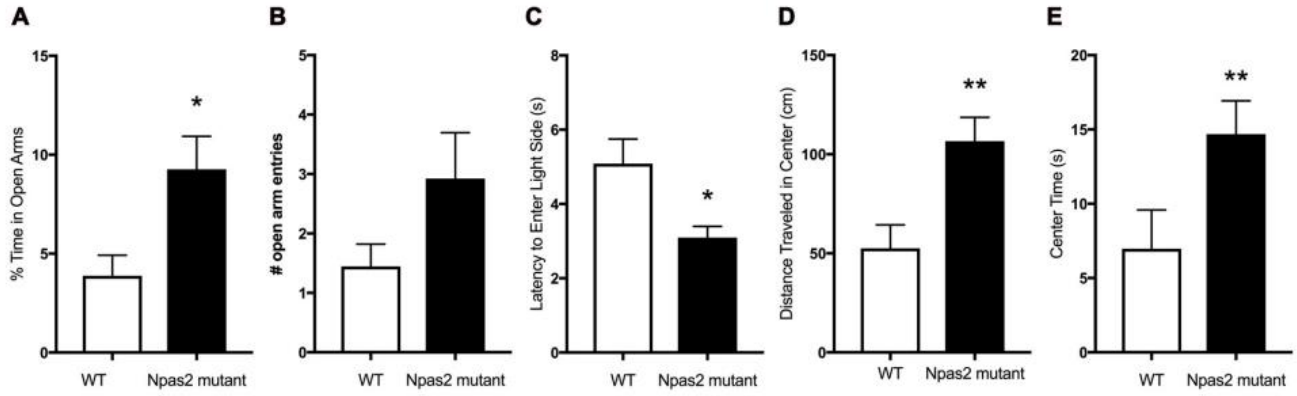


Figure 1.4: Reduced anxiety in *Npas2* mutant mice.

A) Significantly increased percentage time spent in the open arms of the elevated plus maze for *Npas2* mutants compared to WT. B) Slight, though not significant, increase in number of open arm entries in elevated plus maze for *Npas2* mutants compared to WT. C) Significantly lower latency to enter the light side in LD box trials for *Npas2* mutants compared to WT. D) Significantly higher distance travelled in the centre of an OF arena for *Npas2* mutants compared to WT. E) Significantly higher centre time in the OF arena for *Npas2* mutants compared to WT (adapted from Ozburn et al., 2017).

Furthermore, SNPs in *Npas2* are frequently linked to seasonal affective disorder (SAD). *Per2*, *Bmal1* and *Npas2* SNPs were seen to have an additive effect on SAD prevalence and were identified as a genetic risk factor for the condition (Partonen et al., 2007). *Npas2* has also been implicated in other conditions, such as bipolar disorder and chronic fatigue syndrome (CFS). CFS is notoriously hard to diagnose, with no known biomarkers or diagnostic tests. Patients with CFS present immune dysregulation, as well as sleep and cognitive impairments. Smith et al. noted people with particular *Npas2* SNPs were more likely to develop CFS, and CFS mice also displayed a 10-fold increase in *Npas2* expression in peripheral blood mononuclear cells (PBMCs) (Smith et al., 2011). In addition to CFS, SNPs of *Npas2* or increased expression of *Npas2* have been seen in bipolar disorder and unipolar major depressive disorder (MDD). Bipolar disorder is characterised as episodes of major depression and episodes of mania, whilst MDD is defined as clinical depression (Geoffroy et al., 2015, Ojeda and Forero, 2014, Soria et al., 2010). 14 SNPs in five circadian genes, including *Npas2*, are implicated in seasonal bipolar disorder, with a notable additive effect of *Cry1* and *Npas2*

SNPs. Geoffroy et al. determined that alterations in the *Npas2* gene may be a genetic biomarker for seasonal bipolar disorder. Similarly, associations in *Cry1* and *Npas2* SNPs are noted in MDD (Geoffroy et al., 2015, Ojeda and Forero, 2014, Soria et al., 2010). There is also evidence of *Npas2* SNPs giving increased incidence of schizophrenia and schizoaffective disorder, as well as elevated *Npas2* expression seen in patients suffering from schizophrenia (Hader et al., 2009, Ojeda and Forero, 2014). Together, this data proposes a role for *Npas2* in the regulation of cognition, anxiety and mood.

1.9 Peripheral clocks

Initially, it was theorised that the SCN was the only circadian pacemaker within the mammalian body, and that peripheral tissue timing was determined by this central pacemaker. The term 'peripheral clocks' denotes any oscillators that are not the SCN, and thus can refer to additional clock tissue within the brain. Peripheral clocks were first discovered in 1958 when Bunning demonstrated cultured mammalian intestinal tissue expressing 24-hour rhythms of muscle contraction (Bunning, 1958). Later, several studies also revealed rhythmic metabolism, hormonal secretion or enzyme activity in additional cultured mammalian tissues including the heart, liver and adrenal glands (Andrews and Edgar, 1964, Brown et al., 2019, Langner and Rensing, 1972). Balsalobre et al. then became the first to reveal cyclical gene expression of core clock genes *Per1* and *Per2* in rat fibroblasts and hepatocellular carcinoma cells. Such cell lines showed maintained rhythmicity for up to three consecutive days following serum shock, and showed a periodicity of 22.5 hours independent of the synchronising effect of the SCN (Balsalobre et al., 1998). Additionally, molecular biology advances allowed Plautz et al. to utilise luciferase reporters in the promoter regions of *Drosophila Per* genes. Using this method, transgenic *Drosophila* demonstrated self-sufficient, entrained, circadian rhythms in almost all cultured tissues, including antenna, legs and wings (Plautz et al., 1997b). Together, these

findings presented a new hypothesis, whereby peripheral, self-governing clocks may be present in all tissues, which have individual roles and cycle independently of the SCN.

The concept of the SCN as a synchroniser of peripheral tissues then brought into question the theory of circadian misalignment. Circadian misalignment describes either internal or external desynchrony – where internal represents descynchrony either between central and peripheral oscillators or amongst peripheral oscillators, and external depicts desynchrony between oscillators and the external environment or zeitgebers (Brown et al., 2019, Scheer et al., 2009). Stokkan et al. demonstrated circadian misalignment using a restricted feeding paradigm in rats. Nocturnal rats were provided food for only four hours a day during the light phase, when they would naturally eat during the dark phase; this meant they received contradictory environmental cues. As a result, despite the SCN maintaining rhythmicity coupled to light/dark cycles, the liver independently entrained to the restricted feeding programme, thus shifting 10 hours in only two days (Stokkan et al., 2001). Circadian misalignment was also shown to increase insulin, glucose and arterial pressure, and decrease leptin and sleep efficiency in human models replicating jet lag and shift work, as well as significantly altering the rhythmic levels of cortisol. All of these phenotypes of circadian misalignment can be precursors to disease states such as insulin resistance, hyperglycaemia, obesity and hypertension (Scheer et al., 2009). Together, this could suggest coupling of central and peripheral oscillators may actually be due to aligned environmental cues rather than the strength of SCN synchrony (Brown et al., 2019, Stokkan et al., 2001).

The biological necessity of peripheral clocks is due to their highly specialised, individual roles. To investigate such roles, *Bmal1* – the only circadian TTFL member without a functional, compensatory paralog – is often knocked out within specific tissues. Loss of *Bmal1* is seen to have tissue-specific circadian effects in the retina, liver, pancreas, skeletal muscle, smooth muscle, cardiomyocytes, adipocytes, adipose tissue and ovaries (Brown et al., 2019, Ehlen et

al.,. 2017, Hodge et al., 2015, Lamia et al., 2009, Marcheva et al., 2010, Young et al., 2014). In pancreatic islet cells, phase-shifts of glucose metabolism and insulin signalling are seen following *Clock* or *Bmal1* deletions. Defects also include, age-related decline in pancreatic islet size and proliferation, reduced insulin secretion and diminished glucose tolerance. Most remarkably, abolition of the pancreatic circadian clock amounts to the onset of diabetes mellitus (Brown et al., 2019, Marcheva et al., 2010). Skeletal muscle specific loss of *Bmal1* also gives disrupted glucose metabolism, hyperglycaemia and glucose intolerance. Such mice show no obvious differences in circadian behaviour, but demonstrate significantly dampened expression of circadian genes utilized in glucose metabolism. Ehlen et al. (2017) also found that skeletal muscle clocks also have unique roles in sleep loss recovery and sleep amount regulation (Ehlen et al., 2017, Hodge et al., 2015). Another notable peripheral clock role is that found by Cre-lox mediated deletion of *Bmal1* in cardiomyocytes. Loss of *Bmal1* in these contractile heart cells results in reduced glucose usage, altered responses to metabolic stresses such as fasting, accelerated age-dependent cardiomyopathy and significantly reduced lifespan (Brown et al., 2019, Young et al., 2014). Together, these studies demonstrate specialist, local roles of peripheral clocks that, when ablated, cause significant phenotypes despite intact SCN clock function. As well as reinforcing links between circadian cycles and ageing, even at peripheral levels, studies of tissue-specific *Bmal1* losses also demonstrate robust links between circadian clocks and metabolism.

The hepatic circadian clock is pivotal for carbohydrate, lipid and amino acid metabolic processes, oxidative pathways and detoxification (Damiola et al., 2000, Koronowski et al., 2019, Stokkan et al., 2001). The liver regulates blood glucose levels based upon a diurnal feeding/fasting schedule of dietary glucose intake. Hepatic glucose is produced during feeding and degraded throughout fasting to preserve blood glucose within a fine margin, this gives increased concentrations of circulating carbohydrates during the active phase (Koronowski et

al., 2019). Loss of *Bmal1* in the liver, and the subsequent impacts on local circadian rhythmicity results in hypoglycaemia during the fasting phase, and loss of rhythmic glucose regulatory gene expression (Lamia et al., 2009). However, global *Bmal1* knockout with exclusive, liver specific *Bmal1* restoration (known as Liver-RE) shows partial rescue of cyclical hepatic glucose levels. This suggests the hepatic clock is the autonomous driver of glucose concentration, independent of the SCN. Since recovery of the liver clock does not fully restore glucose homeostasis, external inputs from other clocks must also be partly necessary. Global *Bmal1* knockout with intact liver *Bmal1* gives insight into how the hepatic clock acts autonomously. Within Liver-RE mice, molecular oscillatory components cycle similarly to wild type except for marginally reduced amplitude, as well as, phase advancements in clock gene expression, peak *Bmal1* phosphorylation and *Bmal1* recruitment to promoter regions. This demonstrates an autonomous hepatic clock that can maintain rhythmicity in the absence of any other peripheral clocks, or input from the SCN (Koronowski et al., 2019). However, Liver-RE mice were only able to sustain the rhythmic expression of 10% of usually rhythmic transcripts, and only 20% of metabolic oscillatory transcripts. Interestingly, all of these cyclic oscillations were abolished in DD, suggesting that despite some autonomous capabilities of the hepatic clock, the role is significantly dampened when all other clocks are lost, and is completely eliminated in the absence of light as an external cue. This could show that in the absence of additional peripheral clocks, and the SCN, daily light/dark cycles are utilised for liver entrainment (Koronowski et al., 2019, Miyake and Doi, 2019).

1.10 Sexual dimorphism of the SCN in humans and rodent models

Within scientific research, males are frequently exclusively used to maintain increased group sizes, decrease cost, and prevent the influence of sex (Andreano and Cahill, 2009, Beery and Zucker, 2011, Sechzer et al., 1994). There are obvious anatomical, hormonal, and

metabolic differences between species of opposite sexes, but there are also less recognized variations in sleep and circadian rhythms. The SCN itself has significant morphological and cellular sexual dimorphism. Electron microscopy has revealed that male rats have higher total SCN volume, more axo-spinal synapses, increased postsynaptic density material, additional asymmetrical synapses, and neurons with greater numbers of nucleoli than female rats. Opposing sexual dimorphism of the SCN is seen in humans, whereby females have a greater total SCN volume and increased length of rostrocaudal axis (Güldner, 1982, Güldner, 1983, Güldner, 1984, Hofman et al., 1988, Nicolaides and Chrousos, 2020). Additionally, vasoactive intestinal polypeptide (VIP) is manufactured by cells situated in the SCN core. This polypeptide is central to coupling SCN neurons and coordinating rhythmicity. Notably, there are increased numbers of VIP expressing neurons within the human male SCN than compared to female, and there is also sexual dimorphism seen in rhythmic *Vip* mRNA within the SCN (Bailey and Silver, 2014, Zhou et al., 1995). The SCN is also seen to be sexually dimorphic in electrical activity across variable lighting conditions; during the light phase, or 'daytime', male SCN shell neurons are seen to have higher rates of spontaneous activity compared to females. As well as this, during the dark phase, female SCN *shell* neurons have higher thresholds for evoking action potentials, whilst male SCN *core* cells have higher action potential thresholds, faster rates of rising and greater amplitude (Bailey and Silver, 2014, Kuljis et al., 2013, Maywood et al., 2011).

Along with electrophysiological and anatomical differences in male and female SCNs, there are also variations in steroid receptor localisation and abundance. Ovarian steroids, and less-so testicular steroids, modulate a multitude of circadian parameters including: the sleep-wake cycle, amplitude of rhythms, free-running period, melatonin rhythms and daily rhythmic phase (Cain et al., 2013, Mong et al., 2011). Androgen receptors (ARs), which bind hormones such as testosterone, are more abundant in the core region of the mouse SCN, whilst oestrogen

receptors (ERs) are predominantly localised in the shell region. In humans, there is increased ERα expression in female SCN neurons, and males have a 2-fold greater SCN expression level of AR (Fernández-Gausti et al., 2000, Iwahana et al., 2008, Kruijver and Swaab, 2002). Gonadectomy – removal of either the testes or ovaries – abolishes said sex differences in steroid receptor expression, which can be restored using androgen treatment, thus creating male-like patterns of AR expression in female mice (Iwahana et al., 2008). Female mice also show a five-fold increase of ERß expression within SCN neurons compared to males, whilst sexual dimorphism in ERß levels is not apparent in human equivalents. This exemplifies how variable gonadal hormone expression can influence the core circadian oscillator to potentially give sexual dimorphism in many clock controlled parameters. It also interestingly demonstrates significant variability between human and rodent SCNs (Bailey and Silver, 2014, Vida et al., 2008).

There are additional significant sex differences in the hypothalamic pituitary gonadal (HPG) and hypothalamic pituitary adrenal (HPA) axes (Walton et al., 2022). The HPG axis is a reference to the hypothalamus, pituitary gland and the gonadal glands; it plays a crucial role in the regulation of reproduction, immunity, development and ageing via hormonal signalling. The SCN controls the rhythmic release of luteinizing hormone (LH) from the pituitary gland. As well as menstrual cycle surges of LH which cause ovulation, daily cycles of LH in females coincide with the onset of activity. This daily LH surge is absent in males, possibly due to a reduced number of kisspeptin neurons within the antero-ventral periventricular nucleus (AVPV). Lower AVPV kisspeptin cell volume is likely engineered by steroid hormones, since testosterone treatment during development in female mice results in loss of sex disparities in AVPV kisspeptin cell volume (Clarkson and Herbison, 2006, Walton et al., 2022). Conversely, the HPA axis consists of the hypothalamus, pituitary gland and the adrenal glands. This axis has significant roles in mood, stress and immunity. Sexual dimorphism within the receptiveness

of the HPA axis to stress in rodent models present females as more responsive to adverse stimuli (Handa et al., 1994, Walton et al., 2022). Additionally, SCN-HPA axis connections modulate rhythmic glucocorticoid levels. Glucocorticoids are steroid hormones synthesized in the adrenal glands that display circadian expression which peaks at the time of activity onset. This peak of serum glucocorticoids is higher in females than males, as is the amplitude of rhythmic glucocorticoid expression (Balsalobre et al., 2000, Nicolaides and Chrousos, 2020). Glucocorticoid receptors bind glucocorticoid response elements (GREs) which can subsequently regulate the circadian expression of *Per* genes. The glucocorticoid receptor can also modulate additional circadian targets such as *Rev-erbα* via communication with the CLOCK:BMAL1 heterodimer, as well as glucocorticoid receptors themselves being regulated by CRY1/2 (Lamia et al., 2011, Yamamoto et al., 2005, Murayama et al., 2019). Glucocorticoids are also a method of peripheral clock synchrony, demonstrated by the glucocorticoid dexamethasone's synchronising capabilities in *in vitro* cultures (Balsalobre et al., 2000, Walton et al., 2022). Together, this presents a potential mechanism for sex differences in stress responses, as well as a sexually dimorphic mode in which peripheral clocks may be synchronised.

Sexually dimorphic reproductive organs also show circadian clock differences at a peripheral level. The ovaries demonstrate autonomous circadian oscillations that are modulated by gonadotrophin hormones released from the pituitary gland. LH and follicle-stimulating hormone (FSH) have been shown to act as endocrine signals to influence cultured ovarian cells resulting in significant phase shifts. FSH and LH also induce the rhythmic expression of *Per2* within ovarian granulosa cells (He et al., 2007, Yoshikawa et al., 2009). Additionally, surgical removal of the pituitary gland, known as a hypophysectomy, results in reduced oscillatory expression of the core circadian genes *Per2* and *Bmal1* in female rats. This can be rescued using an analog of LH which redeems the rhythmic expression, suggesting gonadotrophin

hormones can regulate autonomous, peripheral circadian clocks (He et al., 2007, Karman and Tischkau, 2006, Yoshikawa et al., 2009). In contrast, the testes are not thought to be fundamentally rhythmic since they do not show oscillatory expression of core circadian genes. However, accompanying male reproductive tissues such as the epididymis, prostate, seminal vesicles and the vas deferens, do show cyclical gene expression (Bebas et al., 2009).

1.11 Sex differences in circadian behaviour

Behavioural experiments employing rodent models have long been biased in favour of male mice. This is often justified using the assumption that variation in behavioural parameters throughout the oestrous cycle would be too great unless experimenters control for cycle phase. Meta-analysis by Prendergast et al. (2014) used 293 literature articles to discount this assumption on the basis that it has no scientific grounding. Throughout the data analysed, comparing female mice at various stages in the oestrous cycle did not show significantly increased deviations in behavioural, morphological or physiological parameters in comparison to male counterparts. This argues for the inclusion of female mice in future experiments to explore the prevalence of sex differences throughout all stages of development (Krizo and Mintz, 2015, Prendergast et al., 2014).

Sex differences in circadian period are normally minor and greatly species-specific. Male rats and hamsters show longer free-running periods than female equivalents, whilst females of the *Octodon degus* (the common degu) species have significantly elongated free-running period compared to males. C57BL/6J mice and mutants of a C57BL/6J background have so far shown no sexual dimorphism in free-running period in the literature (Davis et al., 1983, Kuljis et al., 2013, Labyak and Lee, 1995, Lee and Labyak, 1997, Schull et al., 1989). Though, many species show significant impacts of gonadal hormones on circadian period. Gonadectomy of male mice gives a lengthened circadian period, as does ovariectomy in female rats and hamsters, but not

female mice. Both of these period elongations are reversible by testosterone or estradiol (an oestrogen steroid hormone) treatment, in male and female rodents, respectively (Butler et al, 2012, Krizo and Mintz, 2015, Morin et al., 1977). Though this elongated period effect is not seen in ovariectomized female mice, they do show a shortened period length when treated with either estradiol, ERα agonist or ERß agonist (Royston et al., 2014). Similarly, female mice display increased phase shifts to light pulses than male counterparts, and females with depleted ERα also demonstrate a more significantly increased phase response to light. Additionally, gonadectomised males also show larger phase shifts in comparison to males with intact testes, exemplifying the role of gonadal hormones in phase resetting and the effects of light on behaviour (Blattner and Mahoney, 2013, Karatsoreos et al., 2011, Kuljis et al., 2013).

Generally, female mice are shown to have higher levels of activity than males, though this effect diminishes with ages (Bartling et al., 2017, Konhilas et al., 2015). Onset of activity is earlier in female rodents and is also seen to significantly vary across the oestrous cycle in many rodent species, which amounts to additional sexual dimorphisms in behavioural patterns. The beginning of activity is earliest during ovulation, which parallels to heightened estradiol levels, though the mechanism by which this occurs is not fully understood (Krizo and Mintz, 2015, Kuljis et al., 2013, Morin et al., 1977). Males also show significant variation of activity onset when tested over consecutive days, however it is not known why this behaviour is variable in the male sex as well. Androgen treatment in mice following gonadectomy shows a positive correlation between testosterone concentration and activity onset, and negative correlation between testosterone concentration and the length of activity duration, specifically under free-running DD conditions (Bailey and Silver, 2014, Butler et al., 2012). Similarly, gonadectomy alone diminishes the accuracy of activity onset and reduces activity in the early evening. Since testosterone is readily converted into estradiol in the brain, it is thought that estrogenic effects in males may modulate sex differences in circadian activity (Mong et al., 2011).

Sexual dimorphism within circadian parameters in rodents is paralleled in humans. The most significant sex difference noted is chronotype. Chronotype refers to the natural, preferential time frame of sleep and activity, with people either identifying colloquially as a 'night owl' that goes to sleep later, or an 'early bird' who wakes earlier and rests earlier. Chronotype is variable over developmental stages and also between sexes. Women usually have earlier sleep phases than men of the same age, meaning they have an earlier chronotype. This is the case up until the age of menopause/andropause, where the sex difference is abolished. This finding is in line with women demonstrating a shorter circadian period than men (Cain et al., 2010, Duffy et al., 2010, Nicolaides and Chrousos, 2020). Chronotype also alters through life stage, with a significant shift occurring during adolescence towards an 'evening' chronotype. The adolescent alteration of chronotype generally occurs one year earlier in females, which parallels their earlier puberty, as well as their peak delay in sleep onset, which is generally one year sooner than male counterparts. Additionally, in adulthood, women go to bed earlier, spend more overall time in bed, and get increased total sleep than males (Bailey and Silver, 2014, Manber et al., 2006). Whilst these sexual dimorphisms may have societal and environmental influences, their strength during adolescence and loss following menopause/andropause implicates additional hormonal factors. Circadian sexual dimorphisms are clinically relevant since conditions such as insomnia and SAD demonstrate circadian links as well as disproportionate sex ratios in humans. Both conditions are more prevalent in females, and show greater sex disparities during the reproductive years than following menopause (Kasper et al., 1989, Rosenthal et al., 1984, Tonetti et al., 2007, Zhang and Wing, 2006). This coincides with the point of most significant sex differences in chronotype and sleep phase, furthering the argument that reproductive hormones are implicated in male and female circadian divergence.

1.12 Aims and objectives

The overarching aim of this project was to compare NPAS2 loss in central and peripheral mouse knockout models to investigate its role in global circadian rhythmicity, activity, and recognition memory. Due to the COVID-19 pandemic, having established a peripheral NPAS2 knockout mouse colony it was not viable within this work's timeframe to continue to create the central brain knockout model. Instead, the following body of work therefore focused on the peripheral model to continue the original aims. This was to investigate any influence of hepatic NPAS2 on global circadian rhythmicity, activity and cognition, with a view to continuation of the original aims in future work.

The first goal was to establish both conditional knockout mouse colonies and confirm the model using genotyping. This involved crossing homozygous floxed *Npas2* mice with liver specific Albumin (Alb) Cre or α-fetoprotein promoter-driven (Afp) Cre, whereby Afp is expressed from the foetal stage and Alb is expressed at post-natal day two. These models were then used to investigate the first aim of this work: to elucidate the role of hepatic NPAS2 on global circadian rhythmicity. Since peripheral clocks are vital for metabolism, glucose homeostasis, alternative entrainment methods and are implicated in sleep efficiency, this work aimed to investigate the role of hepatic clocks in global circadian parameters. To do this, light-tight circadian boxes were employed under three lighting conditions: 12h:12h light dark cycling or entrainment conditions, free running constant darkness and free running constant light. This allowed measurements of locomotor activity, sleep/wake cycles, circadian period, phase of activity and amplitude of circadian rhythms.

The second aim of this work was to explore the role of hepatic NPAS2 in cognition and memory. *Npas2* has been implicated in LTM memory paradigms, and this work aimed to compare peripheral NPAS2 and central NPAS2 loss in NOR tasks under cycling light conditions, as well as constant light and constant dark.

Prompted by findings in object recognition memory, which detailed poorer recognition memory in controls in conditions of constant light, the project next focused on investigating the levels of basal and induced anxiety following hepatic NPAS2 loss under differing lighting conditions. This was achieved using open-field arena and light-dark box tests, as well as phenotypic parameters such as weight, hair loss and faecal frequency. A final aim was to use post-mortem time point frontal lobe and liver tissue to identify potential molecular mechanisms for cognitive and anxiety changes in hepatic NPAS2 knockout mice. This was in tandem with the investigation of circadian gene expression in central and peripheral tissues following hepatic NPAS2 loss.

Chapter 2: The effects of pre- and post-natal hepatic NPAS2 loss, and sex, on global circadian rhythmicity and locomotor activity

2.1 Introduction

2.1.1 Global impacts of hepatic circadian clocks

As noted in section 1.9, the circadian clock within the liver has been shown to be involved in carbohydrate, lipid and amino acid metabolism, in addition to oxidative roles (Damiola et al., 2000, Koronowski et al., 2019, Stokkan et al., 2001). Clock functions within the liver also include rhythmic production of cholesterol and bile acids (Nohara et al., 2019). Disruption of the hepatic clock by means of *Bmal1* deletion has been demonstrated to result in hypoglycaemia during the fasting phase and loss of rhythmic expression of genes involved in glucose homeostasis (Lamia et al., 2009). The theory of circadian misalignment also demonstrates that hepatic clocks are capable of causing desynchrony from the SCN over conflicting environmental cues such as feeding schedules. Restricted feeding paradigms can preferentially entrain the liver to feeding/fasting timing over the SCN light/dark entrained rhythms. This exemplifies the significance of the hepatic clock in global rhythmic output, and also demonstrates its autonomy (Damiola et al., 2000, Stokkan et al., 2001).

Circadian clocks in the liver are capable of maintaining autonomous rhythms in the absence of any other peripheral or central clocks. Liver-RE mice with intact hepatic clocks but *Bmal1* loss in all other tissues show maintenance of 10% of rhythmic liver transcripts and 20% of rhythmic metabolite oscillations without any entrainment or influence of the SCN. Only minor changes are seen in rhythmic amplitude along with slight phase advances in clock gene expression (Koronowski et al., 2019). However, time point RT-qPCR and RNA sequencing revealed in the absence of other clocks, the liver can only maintain autonomous rhythms of a small percentage of transcripts, and even this rhythmicity is lost in free-running conditions. This suggests the liver still has a reliance on distal clocks to uphold the majority of rhythmic transcripts during LD cycling, and to preserve circadian rhythmicity in free running conditions. These findings also insinuate that in the absence of additional clocks, the liver clock is

synchronised using LD cycling and removal of this cue causes arrhythmicity at a molecular level (Koronowski et al., 2019). In mice with SCN-specific *Bmal1* loss, liver clocks maintain circadian rhythmicity in LD conditions, displaying their autonomy from intact SCN synchrony. Though, these oscillations significantly dampen in DD, substantiating the role of the core oscillator in maintaining hepatic rhythmicity in free running conditions (Husse et al., 2014). To bypass the SCN in order to entrain the liver of animals with central clock deletions, light must activate hepatic cycling in an alternate manner. Proposed mechanisms include light activating the adrenal clock which results in corticosterone and *Per1* expression in the liver, though this does also rely on a functional adrenal clock which is absent in the case of Liver-RE mice (Koronowski et al., 2019). An additional hypothesis involves a light-transducing axis from the brain-liver facilitated by sympathetic innervation of the paraventricular nucleus of the hypothalamus (PVN), which supports *Per1, Per2* and *Bmal1* rhythmic expression in the liver (Oster et al., 2006, Terazono et al., 2003).

Desynchrony or disruption of hepatic clocks can also exacerbate and influence the morbidity of: non-alcoholic fatty liver disease (NAFLD), liver cancer, cirrhosis and hepatitis (Shi et al., 2019, Tahara and Shibata, 2016). Conversely, these diseases also bring about clock disarray, exemplifying the bidirectional role of the hepatic clock in maintaining liver health. *Per1* and *Per2* double knockout mice have raised hepatic bile acid and cholestatic livers, meaning the flow of bile acid from the liver is impaired. Additionally, bile-duct-ligated rats kept in constant darkness showed reduced symptoms of cirrhosis, thought to result from increased melatonin synthesis. In hepatitis, CRY2 and PER1 proteins are reduced in infected cell lines, and PER2 overexpression reduces hepatitis viral replication (Benegiamo et al., 2013, Ma et al., 2009, Meng et al., 2014). Furthermore, NAFLD patients show SNPs in peroxisome proliferator activated receptor gamma (*PPARG*), a metabolic circadian target, and mice with mutations in core clock genes demonstrate more severe hepatic steatosis (an accumulation of

fat in liver cells) (Mazzoccoli et al., 2014, Tahara and Shibata, 2016). Together, this data exhibits a role for hepatic rhythmicity in health and disease.

2.1.2 Circadian rhythmicity in *Npas2* knockout models

Loss of NPAS2 does not significantly alter global circadian behavioural parameters, since the dominant BMAL1 binding partner, CLOCK, is still present and able to maintain rhythmicity (Landgraf et al., 2016). *Npas2*$^{-/-}$ mice remain rhythmic in entrainment and retain this rhythmicity adequately in free running conditions, mirroring that of *Clock*$^{-/-}$ mice (DeBruyne et al., 2007). Though *Clock*$^{-/-}$ mice show a reduced circadian period in comparison to wild type, the reduction in circadian period of *Npas2*$^{-/-}$ mice in LD conditions is less noteworthy. Wild type mice tend to free run at a period of 23.7 h ± 0.1 hour, whilst *Npas2*$^{-/-}$ mice demonstrate a free-running period of 23.5 h (Dudley et al., 2003). In addition to this, *Npas2*$^{-/-}$ mice do not display the middle of the night cease of wheel-running activity which is prominent in wild type mice. *Npas2*$^{-/-}$ mice remain robustly active past 7.8 h into DD, where wild type mice demonstrate significantly reduced activity to an average of 10-wheel revolutions compared to 60 at peak activity. *Npas2*$^{-/-}$ mice maintain activity for a further two hours after wild type mice reduce their activity (Dudley et al., 2003) (Figure 2.1). This could demonstrate differences in activity profiles amounting from *Npas2* loss. Wild type mice then recover this activity in a small secondary peak during the final hour of DD, whilst *Npas2*$^{-/-}$ mice steadily decrease activity at this point (Dudley et al., 2003). As well as this, *Npas2*$^{-/-}$ mice display an improved response to lighting changes. Wild type mice exposed to a four-hour phase advance took multiple days to synchronise their activity to the new LD timing, yet *Npas2*$^{-/-}$

mice shifted their activity patterns within the first day. This could suggest that *Npas2* loss improves the efficiency of cycle synchrony to light schedules (Dudley et al., 2003).

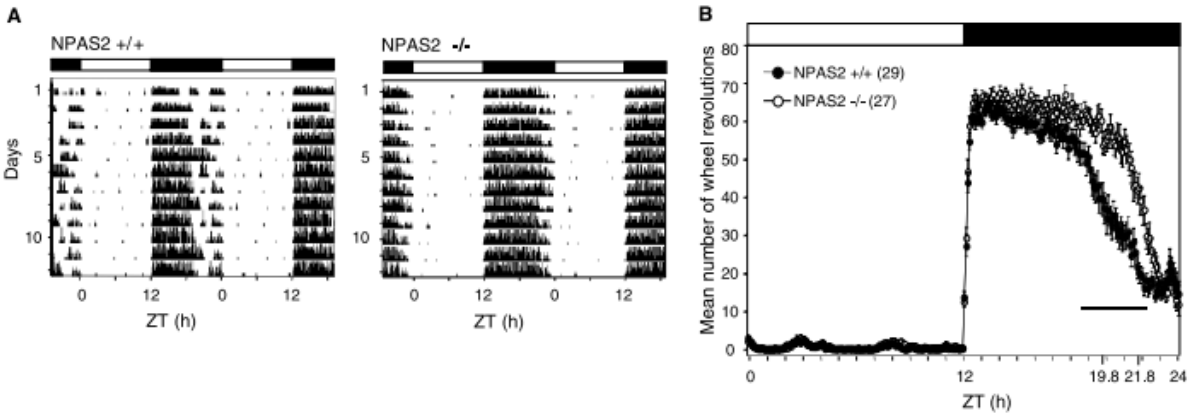


Figure 2.1: Double plotted actograms and average wheel revolutions depicting differential activity of *Npas2*$^{-/-}$ mice.

A) Representative double plotted actograms of wild type and *Npas2*$^{-/-}$ mice showing a late-night cease of wheel-running activity in wild type mice which is absent in *Npas2*$^{-/-}$ mice. B) Mean number of wheel-running revolutions in six-minute bins over a 24-hour period. Wild type mice significantly reduced wheel revolutions at approximately 19.8 hours, whilst *Npas2*$^{-/-}$ mice remained active until later, where their activity fell at 21.8 hours. Taken from Dudley et al., 2003.

Within the SCN, *Npas2*$^{-/-}$ mice showed reductions in *Per2* rhythmic expression, and increased *Bmal1* expression throughout the day in comparison to wild type mice. Interestingly, the molecular aberrancies in *Npas2*$^{-/-}$ mice are less severe than *Clock*$^{-/-}$ mice, which assists the theory that *Clock* has a more dominant role in the TTFL and that *Npas2* has compensatory capabilities in its absence. These differences in gene expression suggest that the CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers have varying affinities to clock-controlled gene promoters (DeBruyne et al., 2007). Additionally, liver specific KOs of *Npas2* demonstrate altered circadian gene expression over their lifespan. At post-natal day two, conditional *Npas2* knockouts (cKOs) show reduced *Bmal1*, *Clock*, *Rorα*, and *Rorγ*, as well as increased expression of *Per1*, *Reverbβ* and *Cry2* in the liver, compared to control mice (O'Neil et al., 2013). In adult livers, these cKOs show decreased expression of *Per1*, and increased

expression of *Bmal1* and *Rorα*, in comparison to control mice. Though, it is worth noting that this study did not use time point tissue to give an accurate reflection of cycling gene expression, and also used only three mice of undisclosed sex to determine these outcomes (O'Neil et al., 2013). As of yet, there is no information on sexual dimorphism in circadian parameters following *Npas2* loss. Additionally, how peripheral *Npas2* loss can alter global circadian behavioural parameters, such as locomotor activity, period and phase, has also not been thoroughly assessed.

2.1.3 Circadian sex differences of C57BL/6J wild type mice

The circadian rhythmicity of C57BL/6J wild type mice is of particular significance to our studies as they are the background strain in which all our transgenic *Npas2* mice were generated (O'Neil et al., 2017). C57BL/6J mice have been shown to have a shortened free-running period of 23.7-hours ± 0.1 hour in DD conditions, as well as significant phase advances (Dudley et al., 2003). Similarly, they also demonstrate elongated periods, reduced locomotor activity and delayed phase in LL conditions (Alves-Simoes et al., 2016, Chen et al., 2008). However, to date, limited research has been performed on the sex differences between circadian parameters in C57BL/6J mice. Furthermore, sexual dimorphism in free-running period is seen to be very species specific, and whilst there are opposing significant differences in rats, hamsters and degus, C57BL/6J mice have not been seen to demonstrate sex differences in free-running period (Davis et al., 1983, Kuljis et al., 2013, Labyak and Lee, 1995, Lee and Labyak, 1997, Schull et al., 1989). However, female mice do show significantly higher levels of general activity and display earlier onset of activity than male counterparts. As a result of this varying activity onset, circadian phase may exhibit sexual dimorphism also, though this has yet to be explored (Bartling et al., 2017, Konhilas et al., 2015) (see section 1.11 for additional details).

Since this work is largely limited, it warrants further investigation into sex effects on behaviour, locomotor activity, and circadian period and phase in C57BL/6J mice.

2.1.4 An introduction to Alb and Afp conditional knockout mice

Cre-lox technology was devised in the late 1980s and has since been further developed to enable vast possibilities in yeast, plants and rodents. The strategy involves directing a site-specific Cre recombinase enzyme, from P1 bacteriophage, to precise target sequences of DNA to allow for the splicing and removal of said sequence (Sauer, 1987, Sauer and Henderson, 1988). Sites used for this specific targeting are known as *loxP* sites – a 34-base sequence comprising an 8-base core sandwiched between two asymmetric 13-base repeats. Typically, two *loxP* sites will flank a DNA region for deletion, consequently known as the floxed locus. Cre enzymes dimerise and bind to both 13-base repeats on one *loxP* site and form a tetramer with a dimer on the alternate *loxP* site, the DNA is then cut at both sites and re-joined by DNA ligase, excluding the excised region (Figure 2.2) (Sauer, 1987, Sauer and Henderson, 1988). To produce a mouse with an excised gene of interest, Cre-expressing mice must be crossed with mice carrying a floxed locus. These Cre-expressing mice could have the enzyme commanded by a tissue-specific promoter or could have inducible Cre that is susceptible to dietary intake of doxycycline, for example (Nagy, 2000, Utomo et al., 1999).

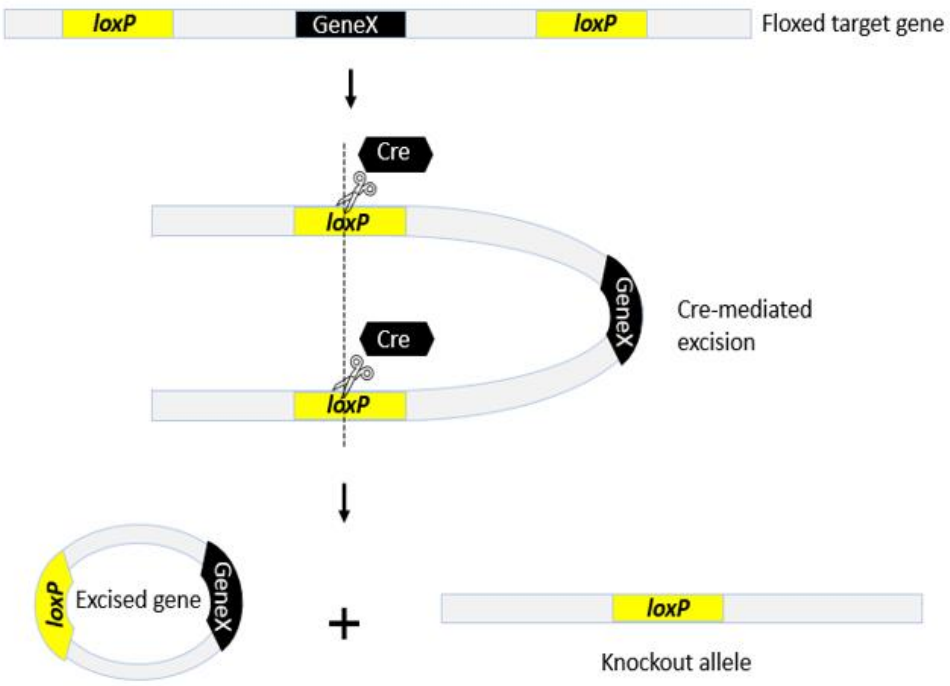


Figure 2.2: Mechanism of Cre recombinase removing the transgene.

Cre enzymes bind a floxed target gene at the *loxP* sites. Dimers form at each *loxP* site and cause the excision of the looped gene of interest. A lone *loxP* site remains as a knockout allele.

Generally, several phenotypes displayed in constitutive knockouts are absent in inducible knockouts. This suggests that some characteristics result from specific effects of protein loss during development, as opposed to the actual impact of circadian disturbance in adulthood (Banks et al., 2016). For future work, inducible knockouts should be favoured over those with deficits during developmental stages. α-fetoprotein (Afp) and albumin (Alb) gene promoters can drive liver specific Cre-recombinases to give differential gene expression; Afp is expressed from the foetal stage, whilst Alb is not expressed until post-natal day two (Figure 2.3). The NPAS2^{TM2AAGAA} mouse model contains floxed *Npas2* exon 3 and was generated by the Aagaard group to investigate the action of NPAS2 within the microbiome (O'Neil et al., 2017). Research by O'Neil et al. (2017) crossed mice expressing Afp- or Alb-driven Cre recombinases with mice containing a floxed *Npas2*. This produces pre- and post-natal NPAS2 deletions to study the necessity of liver NPAS2 in foetal development and any post-birth

compensatory effects (Jin et al., 2009, O'Neil et al., 2017, Postic and Magnuson, 2000). Use of the canonical ATG start site following loss of *Npas2* exon 3 would cause a frameshift mutation and generate a non-functional protein (Garcia et al., 2000).

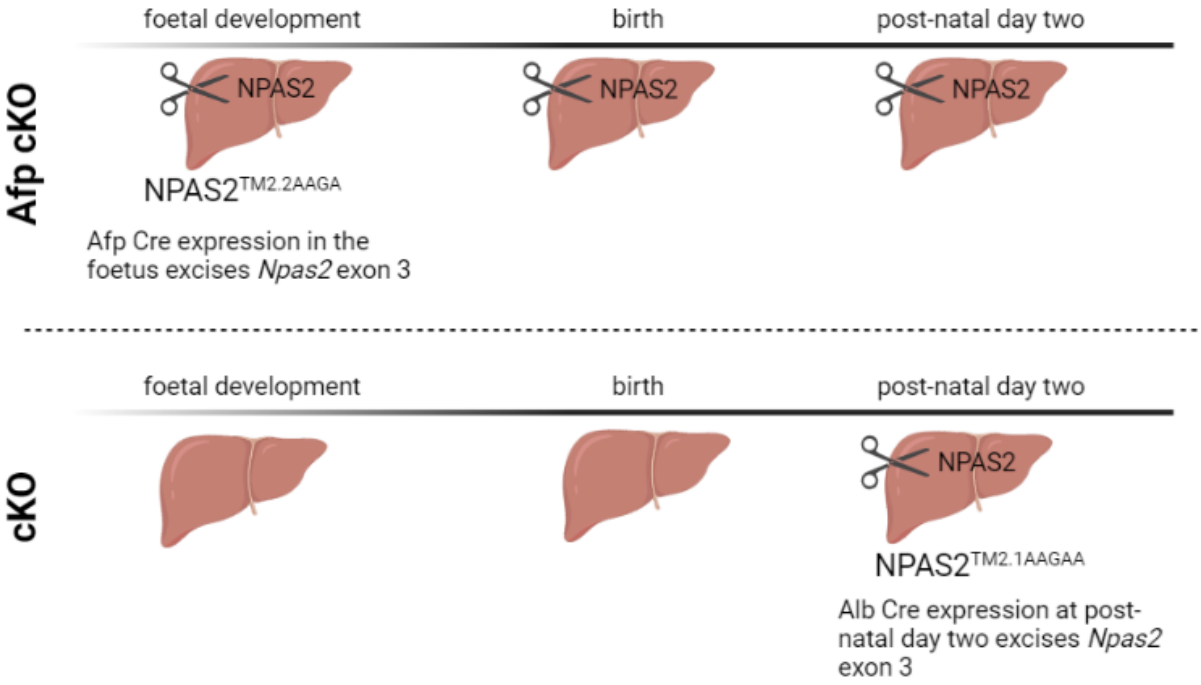


Figure 2.3: Schematic detailing the timeline of NPAS2 knockout in both Afp cKO and cKO.

Afp Cre is expressed during foetal development, resulting in loss of *Npas2* exon 3. NPAS2 is maintained in cKO – which is under the control of Alb Cre – until post-natal day two, where Alb Cre is expressed and *Npas2* exon 3 is lost. Created with Biorender.com.

Using the Alb Cre-expressing mice, O'Neil et al. (2017), investigated the role of NPAS2 in the gut microbiome following a period of restricted feeding. The circadian clock is thought to be intrinsically linked to the gut microbiome, with a bidirectional modulation between the two. Components of the gut microbiome demonstrate circadian rhythmicity that is absent in mice lacking core clock genes. Similarly, mice that lack gut microbes display altered peripheral circadian gene expression in the liver (O'Neil et al., 2017). This study concluded that NPAS2 has an epigenomic role in metabolic homeostasis governed by the maternal diet, and that this role manifests itself primarily during restricted feeding. In addition, the result was

seen to be sexually dimorphic, since NPAS2 expression within the liver is not essential for adaptations to dietary stress in females (O'Neil et al., 2017). Research employing this peripheral *Npas2* knockout has predominantly centred on clock links to the microbiome and gut health. Previous groups have not investigated global circadian rhythmicity or circadian behavioural parameters, which could be potentially insightful to peripheral *Npas2* roles in global clocks.

2.1.5 Aims and Objectives

This study aimed to characterise the effects of pre- (hereafter referred to as Afp cKO) and post-natal (hereafter referred to as cKO) hepatic NPAS2 deletion on circadian parameters, to specifically look at the role of NPAS2 in liver clocks and how this globally impacts circadian behavioural outputs. Additionally, this would make clear the effects of NPAS2 loss in foetal development and whether this has implications on global circadian rhythmicity. Additional lines of fl/fl control mice were bred alongside Afp cKO and cKO mice, hereafter referred to as Afp fl/fl and fl/fl respectively. Though genetically these fl/fl lines are identical, they were bred separately alongside cKO counterparts for the duration of this work. These experiments aimed to use locomotor activity tracking to investigate circadian period, phase and amplitude, as well as hourly distance travelled and inactivity data throughout conditions of cycling light, DD and LL. Cohorts of large enough size were investigated so that they could be sub-divided into sex groups to unveil any sexual dimorphism in the above parameters, since no sexually dimorphic studies on hepatic NPAS2 knockouts have been performed thus far. Previously published studies of circadian rhythmicity show varied n numbers, though most mouse cohorts sit within a range of eight to 16 per genotype, some show group sizes as low as five (Fujioka et al., 2010, Alves-Simoes et al., 2015, Zhou et al., 2018, Fonken et al., 2010, Zhang et al., 2017, Hasan et al., 2018). In order to allow for valuable and reliable statistical analysis, a minimum of 16 mice

per genotype were tested. Originally, these experiments were designed to be compared to brain specific NPAS2 knockouts in order to assess differences in central and peripheral knockouts.

This study also employed an additional control of C57BL/6J wild type mice. The aim of this additional control was to assess any background strain divergence and/or phenotypes of floxing in the fl/fl control strains. Additionally, these experiments aimed to investigate sexual dimorphism in circadian parameters in C57BL/6J wild type mice in addition to the conditional knockouts and their relevant control lines.

The hypothesis of these experiments was that both peripheral NPAS2 knockout mouse genotypes would maintain rhythmicity in LL and DD free running conditions, based on evidence showing global NPAS2 knockout mice are capable of maintaining rhythmicity due to *Clock* gene expression (DeBruyne et al., 2007, Dudley et al., 2003). It was expected that neither conditional knockout line would deviate in circadian period or phase from the fl/fl control or wild type mice. In reference to sexual dimorphism, it was hypothesized that there would be no sex differences in circadian free running or entrainment period, as C57BL/6J and mutants of that lineage have previously shown no sex differences under these conditions (Kuljis et al., 2013). However, sex differences in circadian parameters of C57BL/6J and mutants of C57BL/6J background have not yet been investigated under conditions of constant light.

2.2 Materials and Methodology

2.2.1 Animals

Floxed control ($NPAS2^{TM2AAGAA}$), Afp cKO ($NPAS2^{TM2.2AAGA}$) and cKO ($NPAS2^{TM2.1AAGAA}$) transgenic lines were imported from the Baylor Institute, Texas, USA and bred to generate in house colonies. Afp fl/fl and fl/fl control mice are genetically identical and are homozygous floxed at exon 3 of *Npas2*. Cre expression is generated under the Afp or Alb

promoters exclusively in the liver, resulting in a loss of functional NPAS2 in both cases either from foetal development or post-natal day two, respectively (O'Neil et al., 2017). All animals used in the following experiments were between three and six months of age, and individual experiments had age matched cohorts. Age matched C57BL/6J wild type mice were purchased from Charles River Labs UK and were acclimatized to the animal unit for at least seven days before being used in experiments.

2.2.2 *In vivo* husbandry and general care

Experimental and holding rooms were kept at 22^{o}C ± 2^{o}C with a humidity of 50-55%. All animals had *ad libitum* access to food (2018 Rodent diet, Envigo) and water. In holding rooms, lights cycled with a 12h:12h schedule ON at 7:00am (ZT0), and OFF at 7:00pm (ZT12). Whilst in holding rooms, mice were only disturbed for cage cleaning, food and water replacement, and weight tracking. All experiments were carried out in compliance with the Home Office regulations on animal experimentation, with appropriate personal and project license authority under the Animals (Scientific Procedures) Act (ASPA), UK 1986. UK Home Office Project License No: P16F842EF. UK Home Office Personal License No: I9355DEF9

2.2.3 Experimental set up and circadian recording

Locomotor activity recording was measured inside four light-tight ventilated chambers (80cm x 61cm x 71cm), which house four identical cages (16 cm x 19 cm x 26 cm) suitable for a total of 16 mice. Each individual chamber is equipped with a fluorescent white bulb (150 lux), an IR light and roof-mounted recording camera (ENV-018, MED associates inc, St Albans, VT, USA) (Figure 2.4). The camera acts in conjunction with the Video Tracking Interface Software (Version 1, MED associates inc, VT, USA) thus recording each individual mouse from the four boxes within a chamber. The footage is then converted into distance

travelled (cm) by the Activity Monitor Software (Version 5, MED associates inc, VT, USA). Data is stored in 10-minute bins and analysed thereafter.

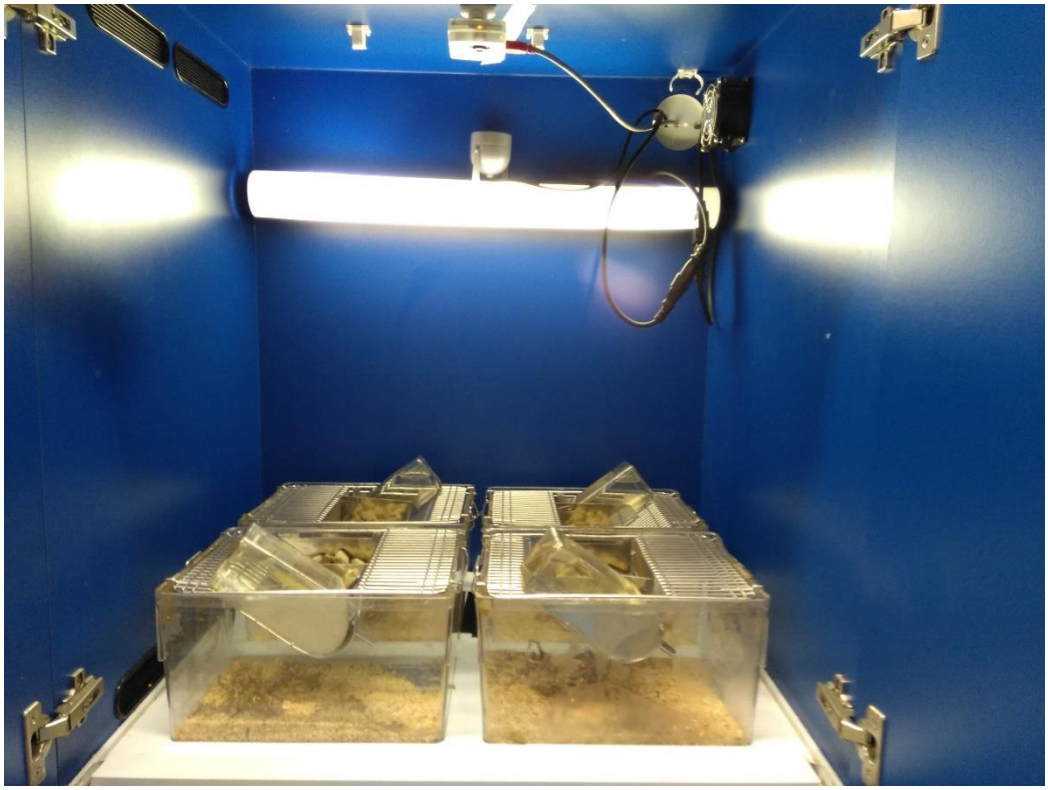


Figure 2.4: Circadian chambers for recording behaviour.

Four individual cages within a circadian chamber. Chambers contain a white light, an IR light, a mounted camera for recording, and a fan.

Two red lamps are situated within the experimental room to allow for daily welfare checks, and a light-proof curtain is placed around the door frame to eliminate exposure to external photic cues. During the experiment, mice were only disturbed for water and food replacement, cage cleaning and weight tracking, unless otherwise stated. Individual cages contained bedding, sizzle nesting, food pellets and a water bottle. Throughout experiments, a radio played constantly at 79 dB to remove any external time cues from building noise and surrounding experimental rooms. All experiments began with seven days LD cycling

entrainment, followed by 10 days of IR light, replicating DD, and then 10 days of LL using constant white light (150 lux), unless stated otherwise.

2.2.4 Afp cKO and Afp fl/fl experiments

Afp cKO and Afp fl/fl experiments used n = 39 mice (Afp cKO n = 19, seven males and 12 females, Afp fl/fl n = 20, 13 males and seven females). Each experimental cohort is limited to n = 16 due to the sizes of the circadian chambers, so in order to generate this n number, three cohorts containing both genotypes were used. To justify combining multiple cohorts, experimental conditions were kept identical and all experimental results were analysed for normality, homogeneity of variances and outliers. The first cohort consisted of a seven-day LD entrainment period (12h light: 12h dark) followed by 10 days of DD, and the latter two cohorts included an additional 10 days of LL after DD. An identical experiment was also performed using a cohort of n = 16 C57BL/6J mice (eight male, eight female) to act as a secondary control. This experiment was performed under identical conditions as the latter two cohorts of Afp cKO and Afp fl/fl experiments.

2.2.5 cKO and fl/fl experiments

Experiments using the post-natal NPAS2 knockout used n =32 mice (cKO n = 16, eight males and eight females, fl/fl n = 16, eight males and eight females). To justify combining multiple cohorts, experimental conditions were kept identical and all experimental results were analysed for normality, homogeneity of variances and outliers. Both cohorts consisted of a seven-day LD entrainment period (12h light: 12h dark) followed by 10 days of DD, followed by 10 days of LL. As mentioned in 2.2.4, an additional n = 16 C57BL/6J mice (eigh male, eight female) underwent an experiment in identical conditions to act as a secondary control measure.

2.2.6 Circadian data analysis

Distance travelled data was used to generate double plotted actograms using ActogramJ, for visual representation of locomotor activity (Schmid et al., 2011). Distance travelled data was analysed using BioDare2, found at biodare2.ed.ac.uk (Zielinski et al., 2014). BioDare2 employs Fast Fourier transform-nonlinear least squares (FFT-NLLS) analysis whereby a cosine curve is fit to the raw data to produce period, phase and amplitude estimates. Relative amplitude errors (RAEs) are generated for each period estimate, these values are derived from dividing the 95% confidence interval by the amplitude estimate. This gives a value between 0-1; 0 indicates an infinitely precise rhythm with no error, whilst 1 indicates a non-statistically significant rhythm (error is probable) (Plautz et al., 1997a).

2.2.7 Statistical analysis

Power calculations were performed in Gpower and were calculated for repeated measures ANOVAs across lighting conditions (Faul et al., 2007). Using a Cohen's f of 0.8 based on a large effect size, for an ANOVA alpha value of 0.05 and power of 0.9 with two groups, a total of 14 animals equal to seven per genotype would be needed. To include sex as a variable, using a Cohen's f of 0.8 based on a large effect size, for an ANOVA alpha value of 0.05 and a power of 0.9 with four groups, a total of 20 animals equal to five per sex would be needed.

All statistics were performed in Prism (GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). All data was graphed in Prism. All data was tested for normality and homogeneity of variance on SPSS (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp), and all data is shown as mean ± SEM, unless otherwise stated. Repeated measures two-way ANOVA was performed to analyse; phase/period across all lighting conditions in genotype and

sex subgroups. Mixed-effects analysis was used in replacement of repeated measures two-way ANOVAs when not all mice completed every repeated measure, e.g. due to FFT-NLLS failing to identify a reliable circadian period. Independent T-tests were performed to assess periods/phases of different genotypes within one lighting condition. A probability value of $p<0.05$ was used as the criteria to determine statistical significance with $p<0.05$ as '*', $p<0.01$ as '**', $p<0.001$ as '***', and $p<0.0001$ as '****'.

2.2.8 Molecular and biochemical reagents

DNA polymerase enzymes with appropriate buffers and dNTPs were purchased from New England Biolabs (NEB). DNA ladders were purchased from Invitrogen. Oligonucleotides were purchased from Sigma-Aldrich as pellets which were resuspended in sterile diH_2O to a concentration of 100 µM and stored at -20^{o}C. Agarose gel loading dye (10x) was made as follows: 3.9 ml glycerol, 500 µl of 10% (w/v) SDS, 200 µl 0.5 M EDTA, 0.025 g bromophenol blue, made to 10 ml with sterile diH_2O and used at 1x. Tris-borate-EDTA (TBE) is made as follows: 40 mM Tris-base, 40 mM boric acid, 0.5 mM EDTA (at pH 8.0), made to 1 L with sterile diH_2O.

2.2.9 Genotyping

Genotyping was initially performed in house using ear notches collected from weaned mice. Genomic extraction and polymerase chain reaction (PCR) were performed (as detailed in 2.2.9 and 2.2.10) to identify the presence of Cre expression and heterozygous or homozygous floxed *Npas2* exon 3. Once the colony was established, genotyping was subsequently outsourced to Transnetyx, Inc., Cordova, TN, USA whom use automated genotyping services using our recognised primer sequences.

2.2.10 Purification of genomic DNA from tissues

Tissues were extracted, immediately frozen in dry ice and stored at -80°C. To obtain genomic DNA, ~75 mg of tissue was homogenised and DNA was extracted as per manufacturer's instructions in the GenElute™ Mammalian Genomic DNA Miniprep Kit. Concentration was measured using a NanoDrop 1000 Spectrophotometer, and DNA was then stored at -20°C.

2.2.11 Polymerase chain reaction (PCR)

PCR was used to analyse genomic DNA from mammalian tissues. PCR was also used for genotyping transgenic mice and for post-mortem confirmation of gene expression. A typical procedure is shown below, performed in an Applied Biosystems 2720 thermal cycler. Primers used for PCRs can be found in the appendix (A.4)

DNA template	50 ng	95°C – 30 seconds	
10x Taq reaction buffer	5 µl (1x)	95°C – 10 seconds 55°C – 30 seconds 68°C – 30 seconds/kb	35 cycles
10 µM forward primer	1 µl (0.2 µM)		
10 µM reverse primer	1 µl (0.2 µM)		
10 mM dNTP mix	1 µl (0.2 mM)	68°C – 10 minutes	
Taq DNA polymerase	0.25 µl		
Nuclease-free sterile diH_2O	to a total of 50 µl		

Table 2.1: PCR reaction recipe.

2.2.12 Agarose gel electrophoresis

For visualisation of PCR products. Agarose of a relevant mass (w/v) for the required percentage gel was mixed with 1x TBE buffer and heated until clear. 1 µl of 10 mg/ml ethidium bromide solution was added per 50 ml of agarose. The liquid was then poured and left to solidify. 10 µl of the DNA ladder was loaded alongside samples mixed with the necessary volume of 10x DNA loading buffer at a final concentration of 1x. Gels were than ran at 90 V in 1x TBE buffer for an appropriate length of time, before being visualized and imaged using a BIO-RAD Universal Hood II UV transilluminator.

2.3 Results

2.3.1 Generation and genotyping of conditional knockout models

To confirm the genotypes of offspring, DNA was extracted from ear notches and used in PCR amplification experiments. The first round of breeding involved using homozygous floxed *Npas2* mice, which contained two copies of the *loxP* flanked *Npas2* gene, with mice containing Alb or Afp driven Cre expression. This offspring would be heterozygous floxed, meaning they carried one copy of the *loxP* flanked *Npas2*, and liver specific Cre expressing. Inter-breeding of this offspring in a second round of breeding generates the desired offspring genotypes; homozygous floxed controls (fl/fl and Afp fl/fl) and homozygous floxed, heterozygous liver specific Cre expressing mice (cKO and Afp cKO).

The first PCR performed identified whether mice contained homozygous or heterozygous floxed *Npas2* using primers either side of the 5' *loxP* site of *Npas2* exon 3. Amplifying this region gives different product sizes for floxed and non-floxed *Npas2*; mice containing wild type exon 3 only will show a smaller band and mice containing floxed *Npas2* only will show a larger band, indicative of the *loxP* insertion. Heterozygous mice containing a copy of both will display bands of DNA at both sizes. A second PCR confirmed the presence or absence of Cre expression by amplifying a region within the Cre recombinase sequence. The presence of Cre gives a positive PCR, showing a DNA band, and the absence gives no band at all. Examples of both PCRs are depicted below (Figure 2.5).

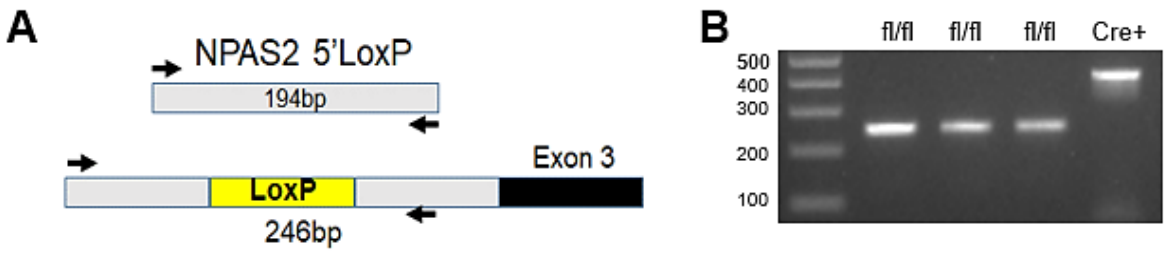


Figure 2.5: Genotyping cKO and fl/fl mice.

A) A schematic detailing the change in size between homozygous floxed *Npas2* and wild type *Npas2*. B) Agarose gel of PCR genotyping showing three homozygous floxed samples with strong bands at 246 bp, and an example of a Cre^{+} band at 408 bp. Primers used were NPAS2 loxP 5' F and R and Cre primer F and R.

2.3.2 Confirmation of liver specific NPAS2 knockout

Next, PCR was employed to validate that there has been specific loss of *Npas2* exon 3 solely in the liver tissue of cKO and Afp cKO mice. Genomic DNA was extracted from both liver and brain tissue of fl/fl, cKO, Afp cKO and C57BL/6J mice. This PCR was performed using a forward primer upstream of the 5' *loxP* site and a reverse primer downstream of the 3' *loxP* site. Both tissues in C57BL/6J mice displayed a 650 bp band, indicative of wild type *Npas2*. Both fl/fl tissues displayed a band at 825 bp, which details wild type *Npas2* with the additional *loxP* sequences. Both cKO and Afp cKO displayed the 825 bp band in brain tissue, but both displayed a significantly reduced band of 220 bp in the liver, revealing Cre recombination of the *loxP* sites and subsequent *Npas2* exon 3 loss only in the liver (Figure 2.6).

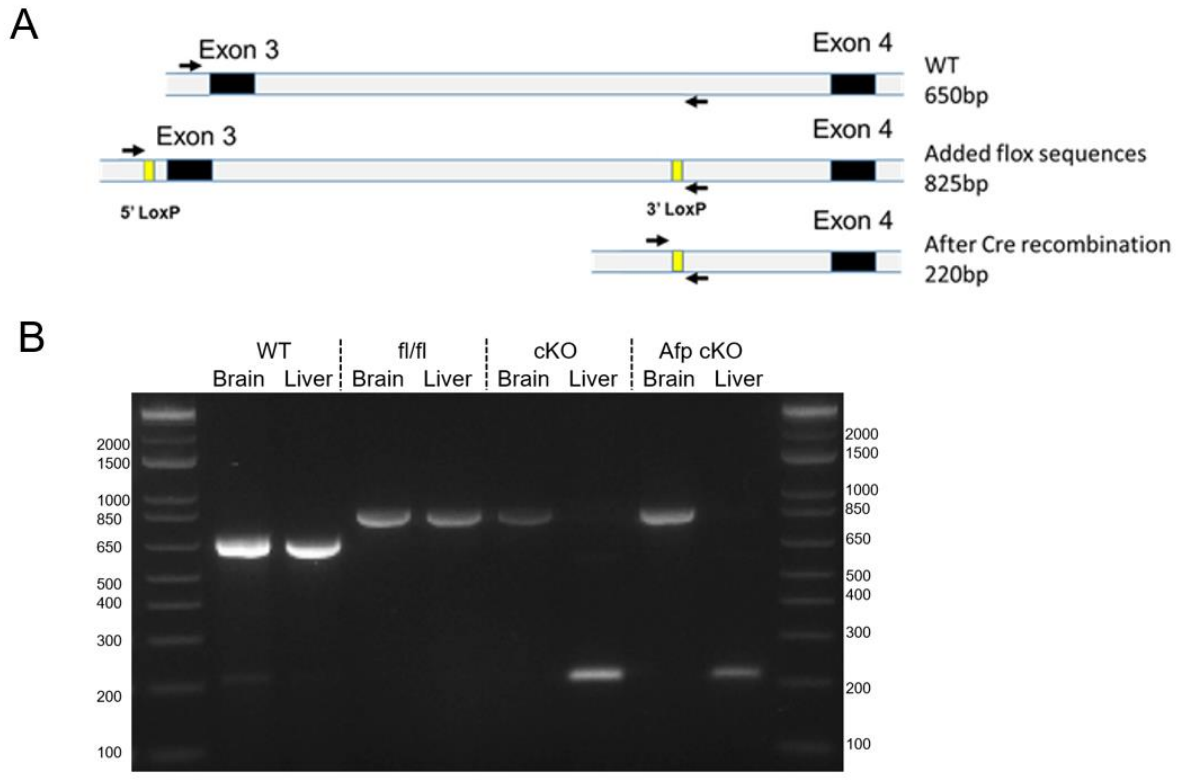


Figure 2.6: Confirmation of liver specific *Npas2* exon 3 loss in cKO and Afp cKO mice.

A) A schematic detailing the change in DNA band size between wild type *Npas2*, floxed *Npas2* and Cre excision of *Npas2* exon 3. B) Agarose gel of PCR products amplified from the DNA of liver and brain tissue taken from a representative male C57BL/6J mouse, a representative male fl/fl mouse, a representative male cKO mouse and a representative female Afp cKO mouse. No loss of *Npas2* exon 3 was visible in any brain tissue extracted DNA, but cKO and Afp cKO representative mice demonstrated loss of exon 3 *Npas2* in the liver.

2.3.3 Locomotor activity of Afp fl/fl and Afp cKO mice

No significant differences were noted in the average hourly activity patterns between genotypes in LD, DD or LL conditions. Since LD induces a constant output, all days in LD are averaged to generate locomotor activity data. In contrast, since every additional day in DD and LL gives further sliding from 24-hour periods, only one day at the end of the condition is utilised in the presented data to exemplify the altered timing of activity. In LD, for Afp fl/fl controls and Afp cKO, onset of activity begun almost immediately after the lights were turned off at ZT12 and ended promptly after the lights were turned back on at ZT0. The distance travelled in the light phase was comparable between genotypes, yet the dark phase showed an increased distance travelled in Afp cKO mice (Figure 2.7A). Following the onset of DD, both genotypes became generally more active across a 24-hour period, with activity dispersing into the day and peaking significantly earlier than in LD entrainment, indicative of a phase advance. Again, the Afp cKO mice showed increased distance travelled compared to Afp fl/fl (Figure 2.7B). After the induction of LL, both genotypes displayed dispersed patterns of activity; both showed activity peaking significantly later than in LD entrainment – known as a phase delay. Additionally, Afp cKO showed similar distance travelled to Afp fl/fl mice during the LL condition (Figure 2.7C).

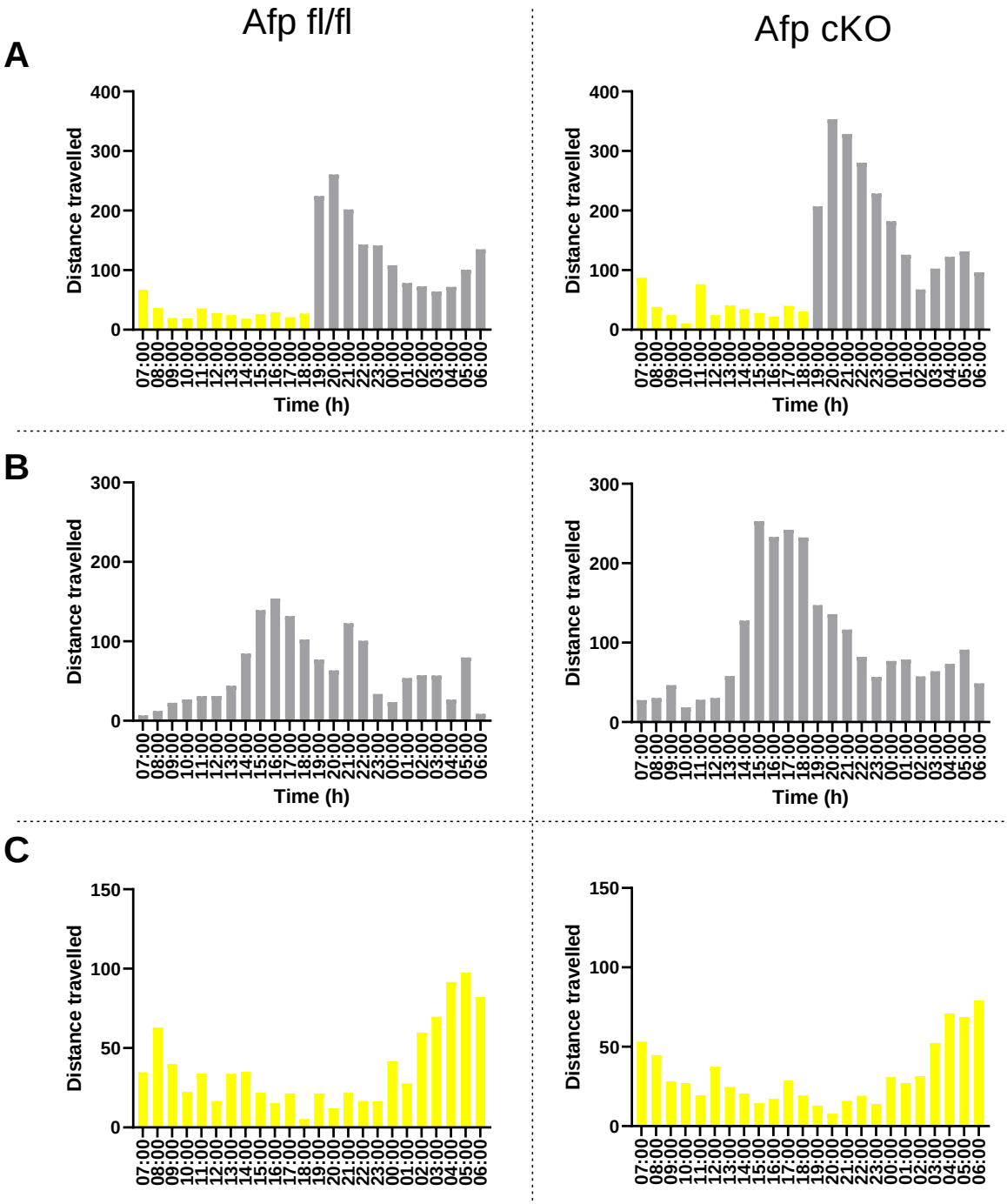


Figure 2.7: Average hourly activity patterns did not differ between Afp cKO and Afp fl/fl in LD, DD or LL.

A) Peaks of average hourly activity of the whole Afp fl/fl (n = 20) and Alb cKO (n = 19) experimental cohort averaged over five days in LD cycling. Genotypes showed comparable patterns of activity, but increased distance travelled was seen in Afp cKO in the dark phase. B) Peaks of average hourly activity of the whole experimental cohort taken from one day at the end of DD conditions. Comparable patterns of activity were seen, but Afp cKO showed increased distance travelled between 15:00-19:00. C) Peaks of average hourly activity of the final two cohorts (Afp fl/fl n = 16, Afp cKO n = 16) taken from one day at the end of LL conditions. Comparable activity patterns and distance travelled were seen.

Since Figure 2.7 displayed disparities in distance travelled between Afp cKO mice compared to Afp fl/fl controls in DD, distance travelled from LD entrainment, the final 24 hours of DD and the first and final 24 hours of LL was compared between genotypes. Two-way ANOVA analysis revealed that distance travelled was significantly altered depending on lighting condition ($F(3,184)=14.76$, $p<0.0001$). Independent t-tests revealed that in DD, Afp cKO mice showed significantly increased distance travelled compared to Afp fl/fl controls within the sampled 24 hours (independent t-test, $t(46)=-2.065$, $p=0.0446$). However, within the first 24 hours of LL conditions, Afp cKO locomotor activity dropped significantly (independent t-test, $t(46)=4.358$, $p<0.0001$). In comparison, fl/fl controls also slightly reduced locomotor activity in constant light, though this was not significant and was seen more as a gradual reduction in activity (Figure 2.8). This could potentially indicate an anxiety phenotype in Afp cKO under LL conditions that is absent in Afp fl/fl.

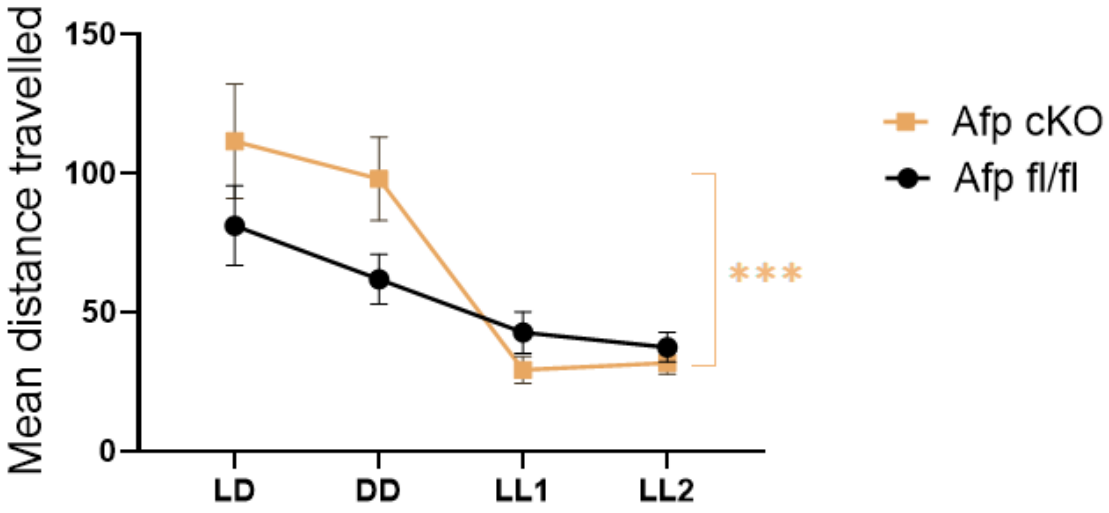


Figure 2.8: Afp cKO mice displayed increased distance travelled in DD compared to Afp fl/fl, and a significant reduction in locomotor activity in LL.

Mean hourly distance travelled from all mice within the latter two cohorts (Afp fl/fl n = 16, Afp cKO n = 16). LD represents hourly locomotor activity across five days. DD represents the final 24 hours of DD, LL1 represents the first 24 hours of LL, and LL2 represents the final 24 hours of LL. Afp cKO locomotor activity in DD was significantly higher than Afp fl/fl controls (*, $p=0.0446$). Afp cKO locomotor activity in LL1 and LL2 was significantly reduced from DD (***, $p=0.0002$, $p=0.0004$ respectively), whereas Afp fl/fl locomotor activity was not significantly reduced.

2.3.4 Locomotor activity of fl/fl and cKO mice

No significant differences were noted in the average hourly activity patterns of each genotype in LD, DD or LL conditions. In LD, for both fl/fl controls and cKO, onset of activity begun almost as soon as the lights were turned off at ZT12 and activity ended immediately after the lights were turned back on at ZT0. The distance travelled in both the light phase and the dark phase was comparable between genotypes (Figure 2.9A). Following the onset of DD, both genotypes became generally more active across a 24-hour period, with activity dispersing into the day (Figure 2.9B). After the induction of LL, both genotypes displayed a phase delay, where their activity peaked much later in the 24-hour period, with both genotypes most active at 03:00am. In both genotypes, distance travelled was reduced in LL in comparison to other lighting conditions (Figure 2.9C). Averaged actograms generated using this distance travelled data can be found in A.3.2.

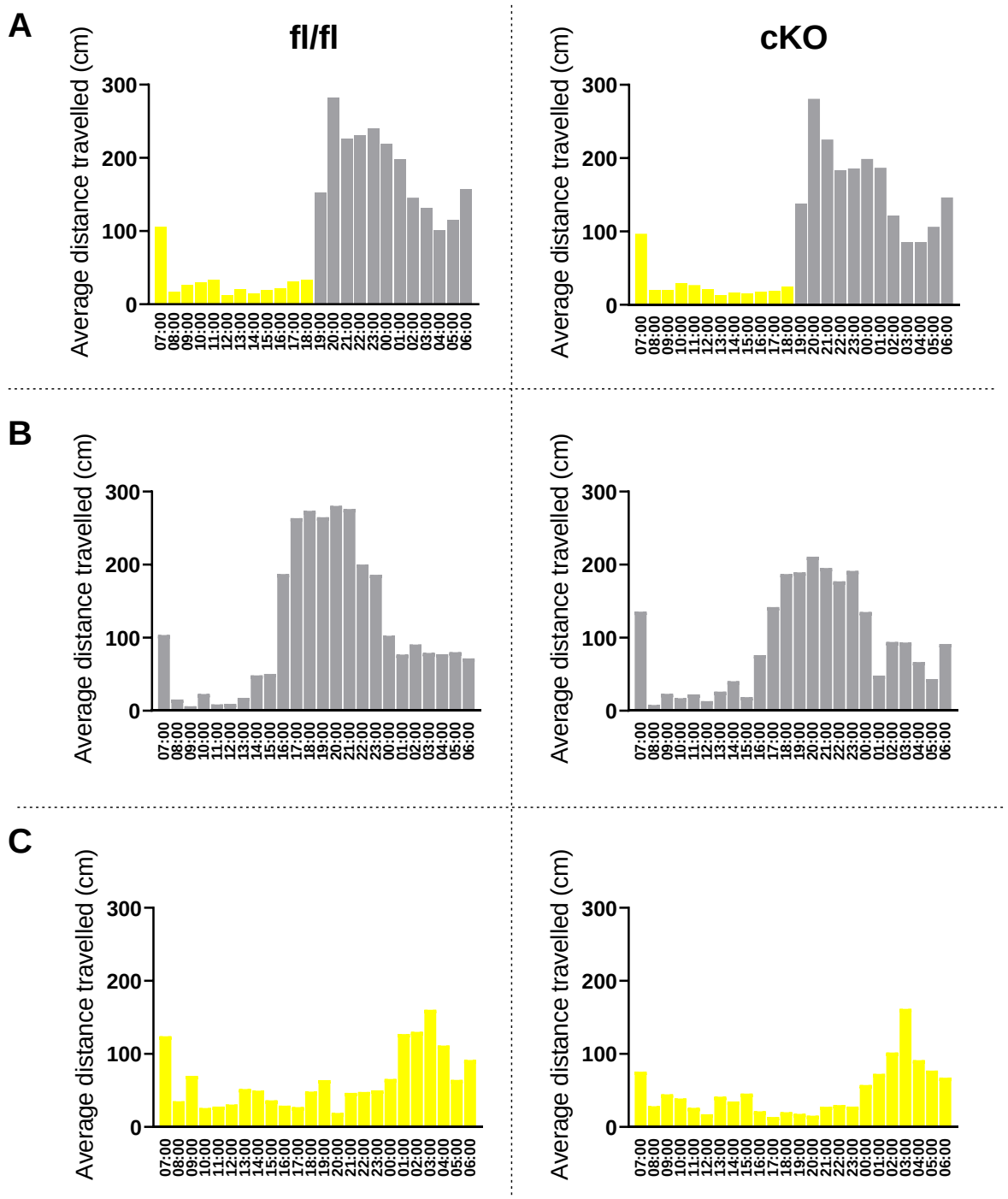


Figure 2.9: Average hourly activity patterns of cKO mice did not significantly differ from fl/fl controls in LD, DD or LL.

A) Peaks of average hourly activity of the whole fl/fl (n = 16) and cKO (n = 16) experimental cohort averaged over five days in LD cycling. Genotypes showed comparable patterns of activity and distance travelled. B) Peaks of average hourly activity of the whole experimental cohort taken from one day at the end of DD conditions. Comparable patterns of activity were seen, though distance travelled was marginally reduced in cKO mice. C) Peaks of average hourly activity of the whole experimental cohort taken from one day at the end of LL conditions. Comparable patterns of activity and distance travelled were seen.

As Figure 2.9 showed a decrease in distance travelled in LL for both genotypes, distance travelled in LD entrainment and the final 24 hours of DD was compared to the first and final 24 hours of LL. Two-way ANOVA analysis revealed lighting condition significantly affected locomotor activity ($F(3,138)=9.953$, $p<0.0001$). Though, there were no significant differences in either genotype between hourly distance travelled in LD or DD. However, both genotypes showed a reduction of activity in LL compared to DD. Fl/fl mice significantly reduced activity in LL within the first 24 hours (Tukey's post-hoc, LL1 $p=0.0384$) and this was maintained in the final 24 hours of LL (Tukey's post-hoc, LL2 $p=0.0107$). cKO mice showed reduced distance travelled in the first 24 hours of LL, though this was not statistically significant. This reduction in activity was maintained until the final 24 hours of LL, where activity was significantly reduced from DD distance travelled (Tukey's post-hoc, LL2 $p=0.0334$) (Figure 2.10).

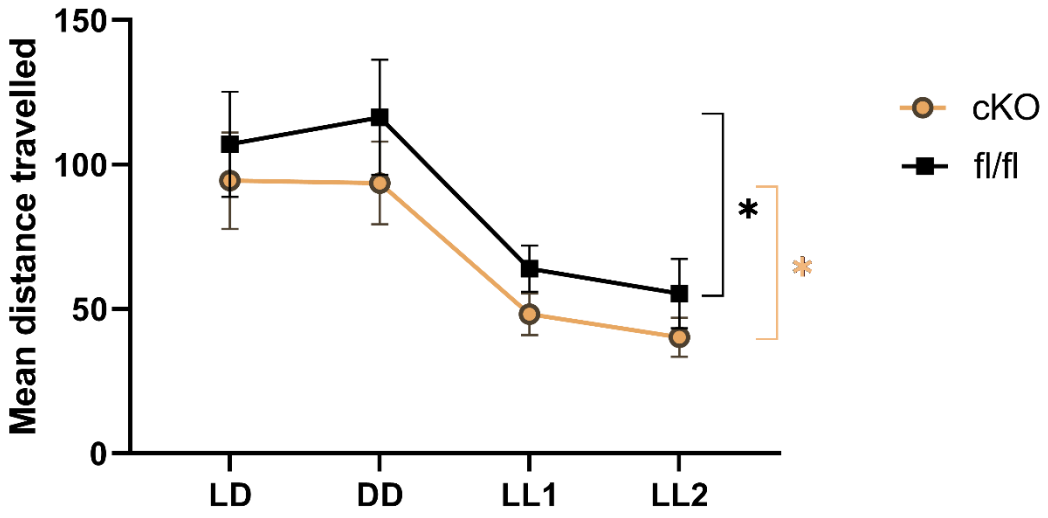


Figure 2.10: cKO and fl/fl mice displayed a significant reduction in locomotor activity in LL.

Mean hourly distance travelled from all mice within the experimental cohort (fl/fl n = 16, cKO n = 16). LD represents average hourly activity over a five day period. DD represents the final 24 hours of DD, LL1 represents the first 24 hours of LL, and LL2 represents the final 24 hours of LL. Both genotypes

showed a reduction in activity in LL compared to DD. Fl/fl mice reduced activity significantly between DD and LL1 (*, p=0.0384) and between DD and LL2 (*, p=0.0107). cKO mice significantly reduced activity between DD and LL2 (*, p=0.0334).

2.3.5 Locomotor activity of C57BL/6J wild type mice

In a separate experiment, C57BL/6J animals underwent identical conditions to give locomotor activity output in LD, DD and LL conditions. Patterns of activity from this experiment can be visually equated to those in the transgenic lines, though because C57BL/6J were not integrated into those exact experiments, they are not directly comparable in terms of distance travelled calibration. C57BL/6J mice became active at ZT12, coordinating with the time of lights off, and reduced this activity at ZT0 when the lights come back on (Figure 2.11A). Activity then moved to earlier in the day in DD, indicating a phase advance of locomotor activity (Figure 2.11B). In LL, activity was visually reduced in comparison to DD, and was so significantly phase delayed that it peaked in what would be the middle of the light phase of cycling light, at 11:00AM (Figure 2.11C). All patterns of activity are comparable to those seen in transgenic lines (Figure 2.7, Figure 2.9).

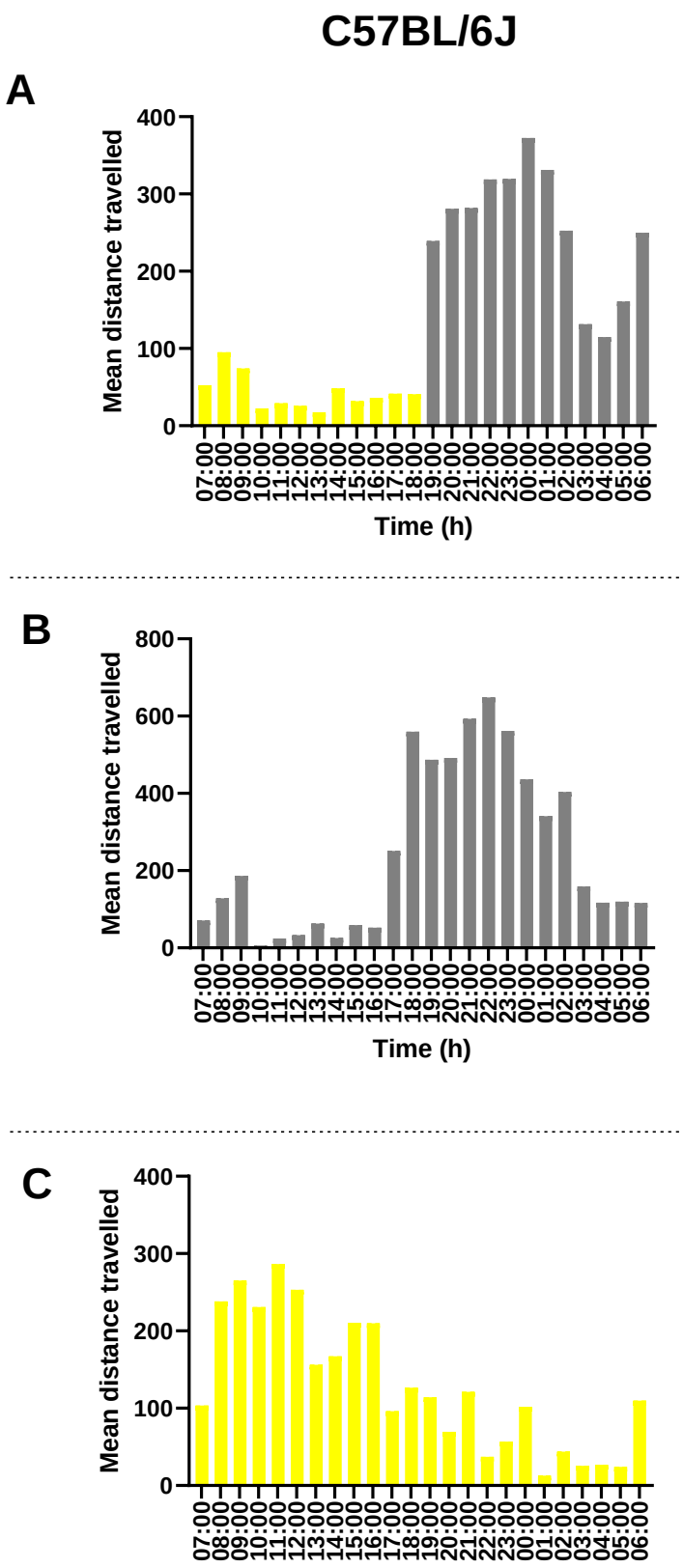


Figure 2.11: Average hourly activity patterns of C57BL/6J mice appear similar to Afp fl/fl, fl/fl, Afp cKO and cKO transgenic lines.

A) Peaks of average hourly activity of the whole C57BL/6J experimental cohort (n = 16) taken averaged over five days in LD cycling. B) Peaks of average hourly activity of the whole experimental cohort taken from one day at the end of DD conditions. C) Peaks of average hourly activity of the whole experimental cohort taken from one day at the end of LL conditions.

Since Figure 2.11 showed a decrease in distance travelled in LL for C57BL/6J mice, distance travelled in LD entrainment and the final 24 hours of DD was compared to the first and final 24 hours of LL. One-way ANOVA analysis revealed lighting condition significantly affected locomotor activity ($F(3,92)=5.371$), $p=0.0019$). C57BL/6J mice displayed a slight increase in activity in DD, though this was not statistically significant. Wild type mice also showed a significant reduction in activity in LL1 compared to DD (Tukey's post-hoc, LL1 $p=0.0055$) and this was maintained in the final 24 hours of LL (Tukey's post-hoc, LL2 $p=0.0034$) (Figure 2.12). Meaning all genotypes reduced activity significantly in LL, with the exception of Afp fl/fl which showed marginal activity reductions that were not statistically significant (Figure 2.8, Figure 2.10, Figure 2.12).

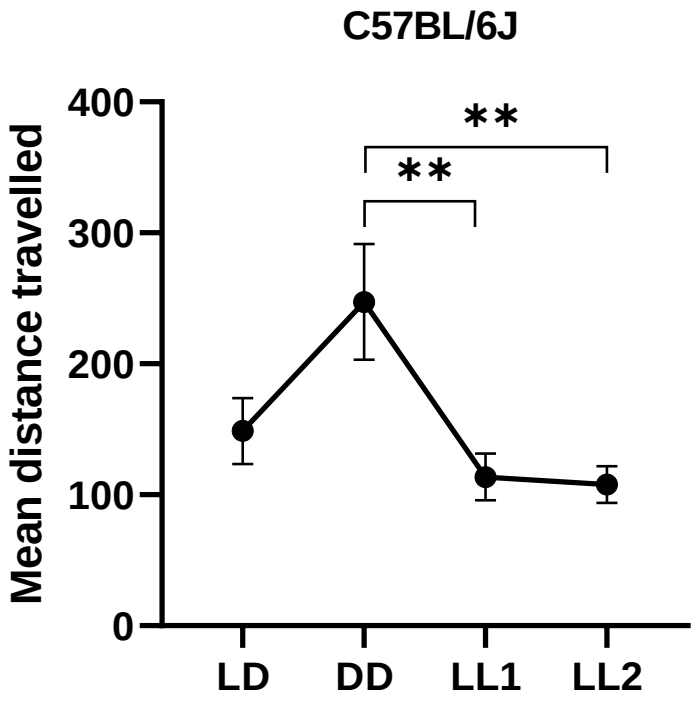


Figure 2.12: C57BL/6J mice displayed a significant reduction in locomotor activity in LL.

Mean hourly distance travelled from all mice within the experimental cohort (n = 16). LD represents average hourly activity over a five-day period. DD represents the final 24 hours of DD, LL1 represents the first 24 hours of LL, and LL2 represents the final 24 hours of LL. C57BL/6J mice showed a significant reduction in activity between DD and LL1 (**, $p=0.0050$) and between DD and LL2 (**, $p=0.0034$).

2.3.6 Circadian rhythmicity and sexual dimorphism in Afp fl/fl and Afp cKO mice

Locomotor activity recordings of distance travelled were taken in 10-minute bins during all three lighting treatments. These were then examined using FFT-NLLS cosine analysis to give period, phase and amplitude estimates.

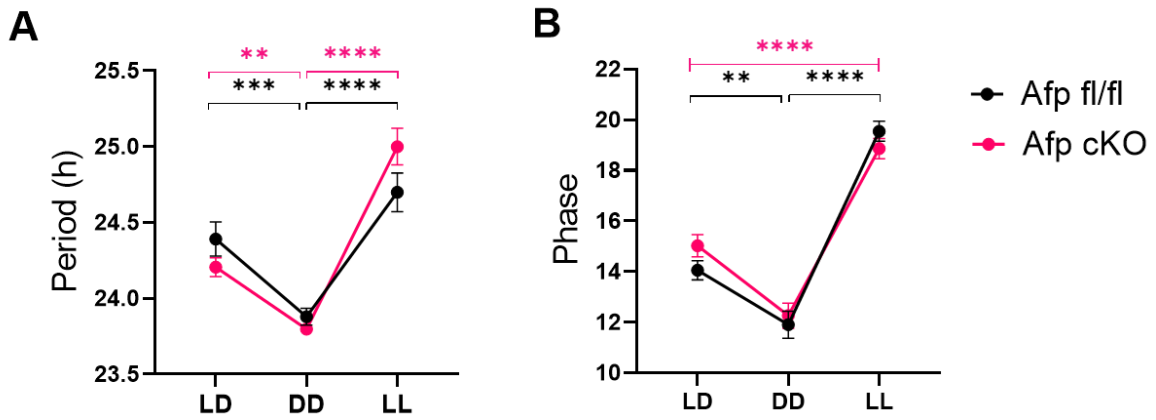


Figure 2.13: No significant differences in circadian parameters between Afp cKO and Afp fl/fl controls in any lighting condition.

A) No significant difference was seen in period length between Afp cKO (n = 19 in LD, n = 18 in DD and n = 17 in LL) and Afp fl/fl controls (n = 20 in LD, n = 17 in DD, and n = 15 in LL) in any lighting conditions. Significant decreases in period length were seen comparably in both genotypes in DD (Afp fl/fl: ***, p=0.0003, Afp cKO: **, p=0.0047), and significant increases in period length were seen in LL from DD (****, both p<0.0001). B) No significant differences in phase were seen between genotypes in any condition. Afp cKO phase was significantly altered across all conditions (all p<0.0001). Afp fl/fl phase was significantly altered throughout all conditions (LD-DD; **, p=0.0024, DD-LL and LD-LL; ****, p<0.0001).

Mixed-effects analysis revealed that lighting condition was a significant influence on circadian period (F(2,100)=57.59, p<0.0001), but genotype was not (F(1,100)=0.02274, p=0.8804). Additionally, two-way ANOVA analysis showed lighting condition was also a significant influence on circadian phase (F(2,99)=121.5, p<0.0001), but genotype was not (F(1,99)=0.3376, p=0.5625). LD period length and phase were not significantly different between genotypes, as expected due to the external cue entrainment. There was also no significant difference in DD or LL period length between Afp cKO and Afp fl/fl controls. In

both genotypes, DD induced a significant period reduction (Tukey's post-hoc; Afp fl/fl: p=0.0003, Afp cKO: p=0.0047) and LL induced a significant period length extension (Tukey's post-hoc; Afp fl/fl: p<0.0001, Afp cKO: p<0.0001) (Figure 2.13A). Furthermore, no significant divergences in phase were noted between genotypes in any condition. Both genotypes successfully demonstrated a significant phase advance as a consequence of DD (Tukey's post-hoc; Afp fl/fl: p=0.0024, Afp cKO: p<0.0001), and a significant phase delay as a result of constant light (Tukey's post-hoc; Afp fl/fl: p<0.0001, Afp cKO: p<0.0001) (Figure 2.13B). This demonstrates that NPAS2 within the liver, both in adulthood and throughout development, is not necessary for maintained global circadian rhythmicity.

Each genotype was then split by sex and comparisons of circadian period and phase were made. No significant differences were noted between sexes in the period and phase of Afp fl/fl controls in any lighting condition (Figure 2.14A, 2.14B). However, the interaction of sex and lighting condition had a significant impact on Afp cKO period (F(2,47)=8.176, p=0.0009) and phase (F(2,48)=3.908, p=0.0268). Afp cKO females showed significantly elongated period in LL and significantly increased phase in LD, in comparison to Afp cKO males (Šídák's post-hoc, p=0.0057, p=0.0193 respectively) (Figure 2.14C, 2.14D). This is atypical, as LD entrainment involves a light cue to which locomotor activity synchronises. It also suggests that LL conditions can induce sexually dimorphic circadian responses, a phenotype that warrants further investigation.

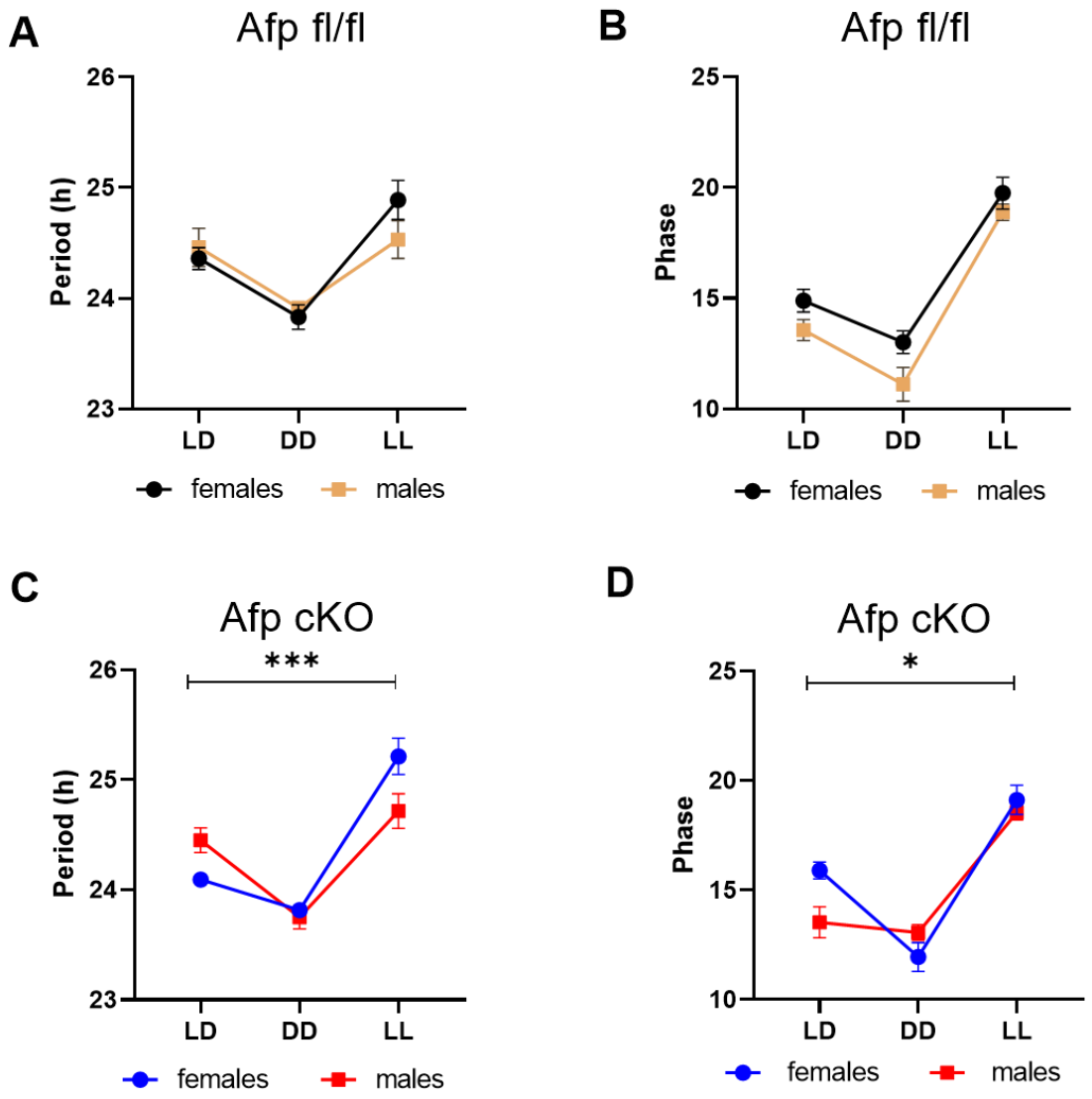


Figure 2.14: Significant sex differences in period and phase between male and female Afp cKO mice.

A) No significant difference was seen in period length between Afp fl/fl males (LD n = 12, DD n = 10, LL n = 9) and females (all n = 7) in any condition. B) No significant differences in phase were seen between Afp fl/fl males and females in any condition. C) There was a significant interaction between sex and lighting condition on period in Afp cKO mice. Afp cKO females (LD, DD n = 13, LL n = 9) had a significant longer period in LL than Afp cKO males (LD, DD n = 6, LL n = 7) (**, p=0.0057). D) There was a significant interaction between sex and light condition on phase in Afp cKO mice. Afp cKO females had a significantly higher phase in LD than Afp cKO males (*, p=0.0193).

2.3.7 Circadian rhythmicity and sexual dimorphism in fl/fl and cKO mice

Locomotor activity recordings of distance travelled were taken in 10-minute bins during all three lighting treatments. These were then examined using FFT-NLLS cosine analysis to give period, phase and amplitude estimates.

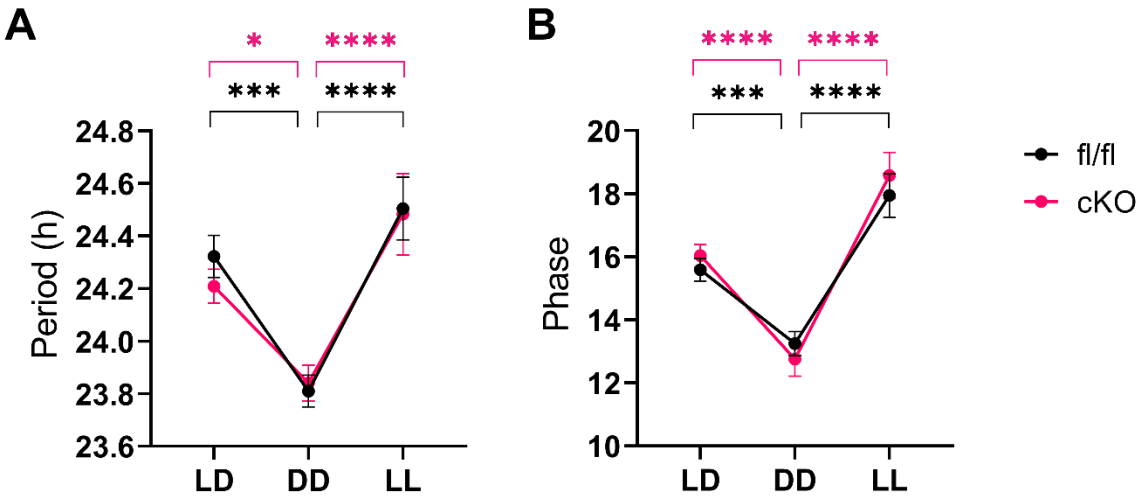


Figure 2.15: No significant differences in circadian parameters seen between cKO and fl/fl controls in any lighting condition.

A) No significant difference was seen in period length between cKO (LD n = 16, DD and LL n = 15) and fl/fl controls (n = 16 in all) in any lighting conditions. Period significantly decreased in DD in both genotypes (fl/fl: ***, p=0.0008, cKO: *, p=0.0298), and significantly increased in LL in both genotypes (both: ****, p<0.0001). B) No significant differences in phase were seen between genotypes in any lighting condition. Phase was significantly lower in DD in both genotypes (fl/fl: ***, p=0.0003, cKO: ****, p<0.0001), and was significantly higher in LL in both genotypes (both: ****, p<0.0001).

Mixed-effects analysis revealed lighting condition significantly impacts circadian period ($F(2,87)=24.02$, $p<0.0001$), but genotype does not ($F(1,87)=0.1936$, $p=0.6610$). Analysis also revealed that circadian phase is significantly influenced by lighting condition ($F(1.575,44.89)=61.82$, $p<0.0001$), but not by genotype ($F(1,30)=0.1896$, $p=0.6664$). DD shortened circadian period in both genotypes (Šídák's post-hoc, fl/fl: $p=0.0008$, cKO: $p=0.0298$) and LL elongated circadian period in both genotypes (Šídák's post-hoc, both: $p<0.0001$) (Figure 2.15A). Both genotypes demonstrated a significant phase advance in DD

(Šídák's post-hoc, fl/fl: p=0.0003, cKO: p<0.0001), and a significant phase delay in LL (Šídák's post-hoc, fl/fl: p<0.0001, cKO: p<0.0001) (Figure 2.15B). However, neither parameter demonstrated variation between genotypes. This is in agreement with Afp cKO results which suggest that loss of hepatic NPAS2 does not alter global rhythmicity, as hypothesized.

Each genotype was then split by sex and comparisons of circadian period and phase were made. Two-way ANOVA analysis revealed that sex had no significant effect on circadian period in fl/fl mice (F(1,14)=3.150, p=0.0977), however, there was a significant difference between fl/fl male and female periods in LL (Šídák's post-hoc, p=0.0149) (Figure 2.16A). This is of particular interest as it represents potentially the most anxiogenic condition, suggesting possible sexual dimorphism in anxiety. Despite this, there were no significant sex differences between fl/fl phases (Figure 2.16B). In cKO mice, the interaction between sex and lighting condition was shown to significantly effect period (F(2,26)=4.699, p=0.0181). However, post-hoc tests revealed no significant sex differences in periods or phases of any lighting conditions (Figure 2.16C, Figure 2.16D).

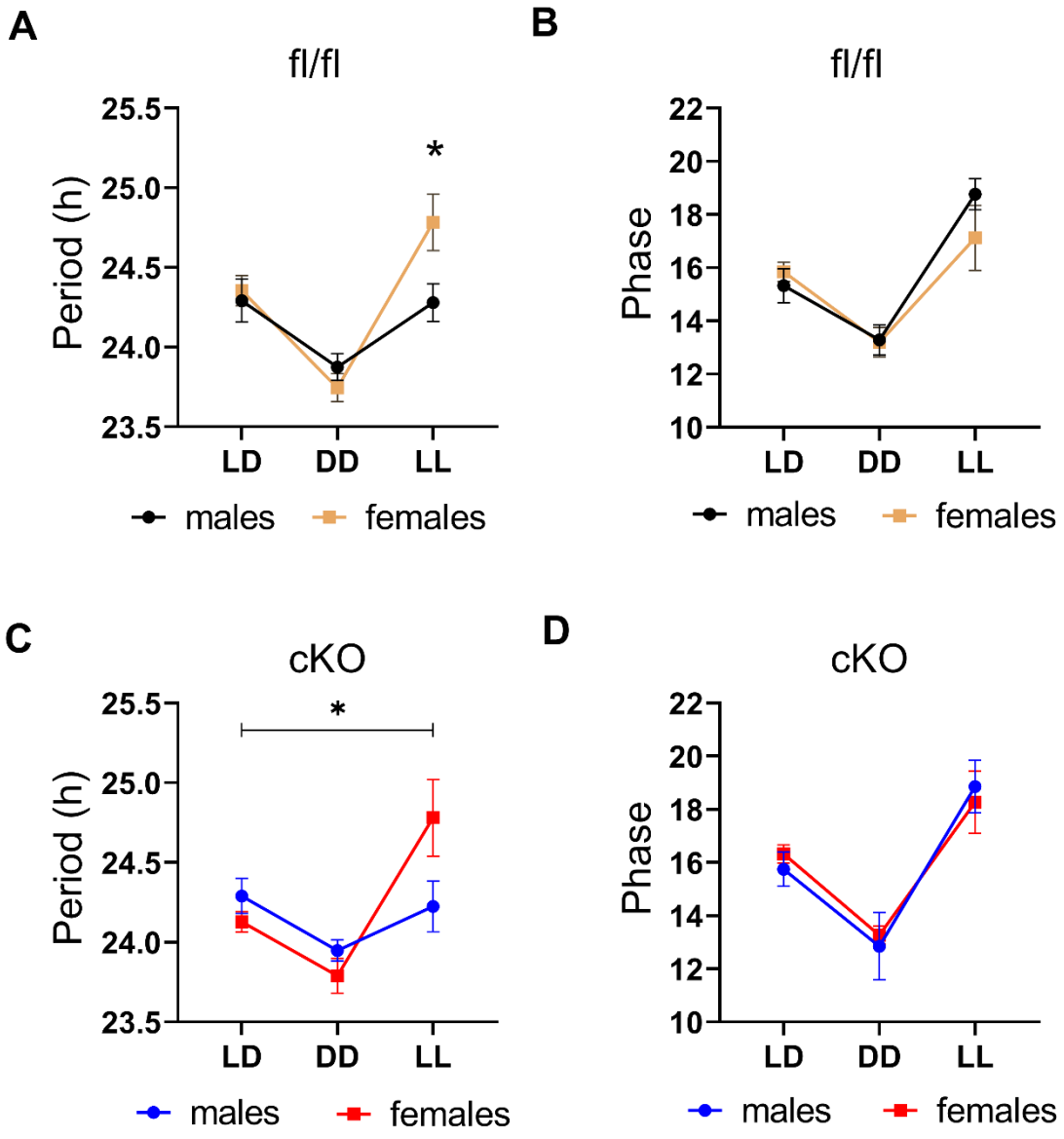


Figure 2.16: Significant effects of sex on period in cKO, and fl/fl mice in LL.

A) No significant difference was seen in period length between fl/fl males (n = 8) and females (n = 8) in LD or DD, but females showed a significantly longer period in LL (*, p=0.0149). B) No significant differences in phase were seen between fl/fl males and females in any lighting condition. C) No significant differences were seen in period length between cKO males (n = 8 in LD and LL, n = 7 in DD) and females (n = 8 in LD and DD, n = 7 in LL) in any lighting condition, though the interaction of lighting condition and sex on period length was significant (*, p=0.0181). D) No significant differences were seen in phase between cKO males and females in any lighting condition.

2.3.8 Circadian rhythmicity and sexual dimorphism in C57BL/6J mice

Since all transgenic lines showed a period significantly longer than 24 hours in entrained LD conditions, C57BL/6J mice were utilised as a control, since they have a known

24-hour period in cycling conditions. The aim of this secondary control experiment was to confirm that the experimental set up was not artificially elongating LD circadian period, and thus to determine if this is a phenotype of the transgenic lines.

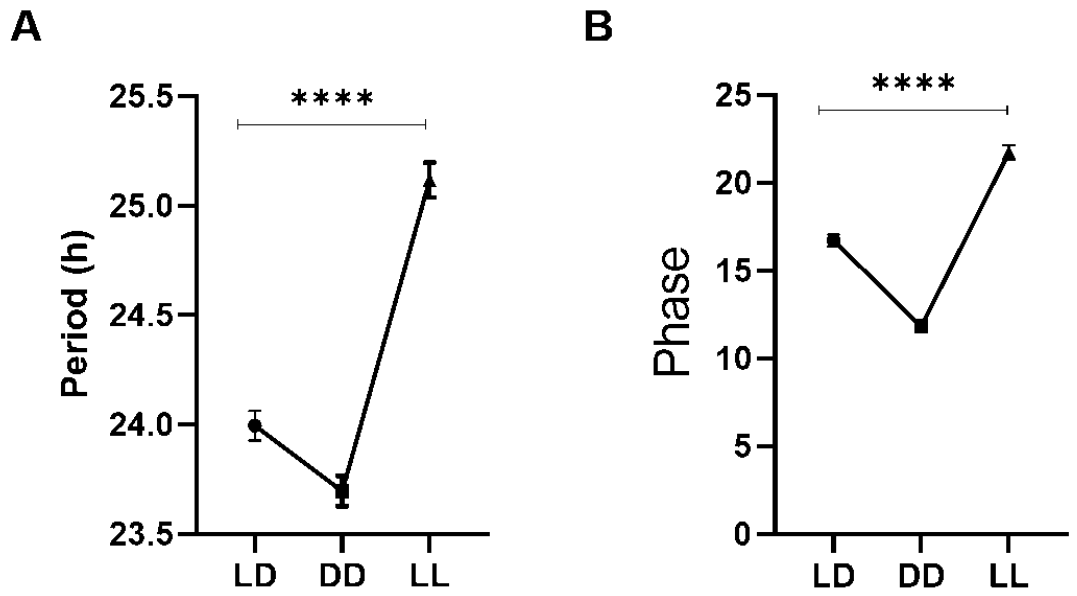


Figure 2.17: Experimental conditions replicated a 24-hour entrained period in C57BL/6J mice.

A) LD period in C57BL/6J mice is 23.995 hours. Period significantly decreased in DD (p=0.0164), and significantly increased in LL (****, p<0.0001) (n = 16 in LD and LL, n = 15 in DD). B) Phase was significantly lower in DD (****, p<0.0001) and was significantly higher in LL (****, p<0.0001).

The C57BL/6J control experiment revealed C57BL/6J mice exhibited a LD period average of 23.995 hours, which supports literature detailing their 24-hour period (Figure 2.17A) (DeBruyne et al., 2007, Hofstetter et al., 2007). One-way ANOVA analysis showed lighting condition had a significant effect on C57BL/6J period length (F(2,44)=105.9, p<0.0001) and C57BL/6J phase (F(2,44)=218.4, p<0.0001). C57BL/6J period significantly shortened in DD (Tukey's post-hoc, p=0.0164) and significantly elongated in LL (Tukey's post-hoc, p<0.0001) (Figure 2.17A). C57BL/6J phase shifted to significantly earlier in DD (Tukey's post-hoc, p<0.0001) and later in the subjective day in LL (Tukey's post-hoc, p<0.0001) (Figure 2.17B).

This confirmed that the experimental parameters adequately created an entrained period of ~24 hours in C57BL/6J wild type mice. C57BL/6J mice were then directly compared to previous experiments utilising the transgenic lines. Firstly, Afp fl/fl and Afp cKO were compared to C57BL/6J circadian parameters (Figure 2.18).

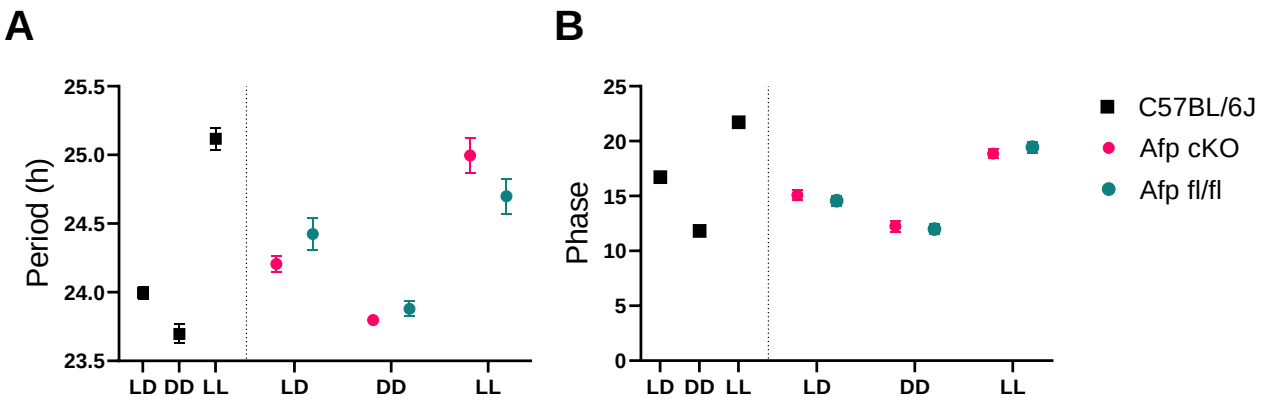


Figure 2.18: Differences in period and phase of Afp transgenic lines compared to C57BL/6J mice.

A) Differences between the mean periods of C57BL/6J (n = 16 in LD and LL, n = 15 in DD), and Afp cKO (n = 19 in LD, n = 18 in DD and n = 17 in LL) and between C57BL/6J and Afp fl/fl controls (n = 20 in LD, n = 17 in DD, and n = 15 in LL) were observed in LD. Differences between the mean periods of C57BL/6J and Afp fl/fl mice were observed in LL. B) Differences between the mean phases of C57BL/6J and both transgenic lines were observed in LD and in LL.

Whilst C57BL/6J mice underwent identical experimental conditions as Afp fl/fl and Afp cKO mice, they were not integrated into the Afp cohorts. Inspection of the means suggested differences between C57BL/6J and both Afp transgenic lines. For indicative purposes, the effect of genotype on period and phase was analysed and significant differences were seen in LD period and phase, as well as LL period and phase between transgenic groups and C57LB/6J (see A.8.1 for further details). These differences were suggestive of significant divergence of the transgenic lines from their background strains, or an effect of floxing. However, C57BL/6J mice would need to be integrated into experimental cohorts before conclusions could be drawn.

Next, C57BL/6J circadian parameters were compared to those of cKO and fl/fl mice, since both cKO and fl/fl mice also presented an entrained period significantly greater than 24 hours in LD.

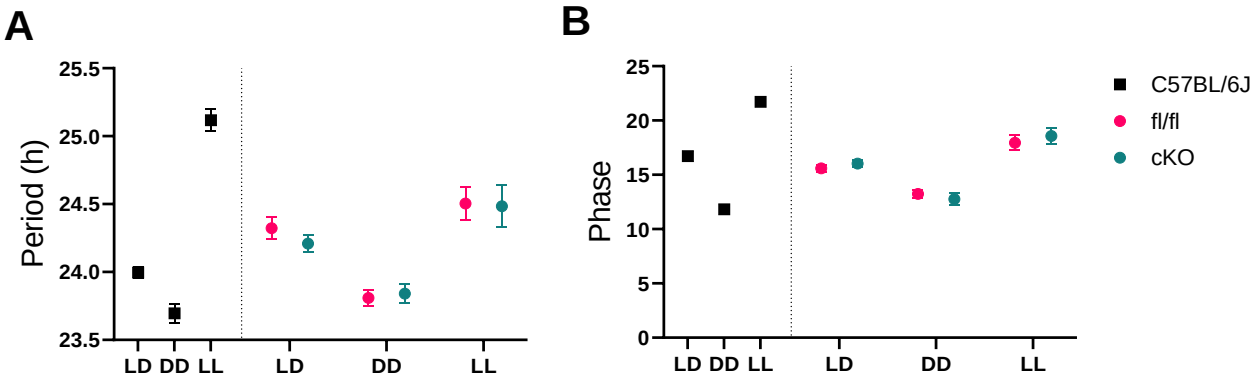


Figure 2.19: Differences in period and phase of cKO and fl/fl, compared to C57BL/6J mice.

A) Differences between the mean periods of C57BL/6J (n = 16 in LD and LL, n = 15 in DD) and cKO (n = 16 in LD, n = 15 in DD and LL) and between C57BL/6J and fl/fl controls (n = 16 in LD, DD, and LL) were observed in LD and in LL. B) Differences between the mean phases of C57BL/6J and fl/fl controls were observed in DD. Differences between the mean phases of C57BL/6J and both transgenic lines were observed in LL.

Similarly, despite C57BL/6J mice undergoing identical experiments as fl/fl and cKO mice, they were not integrated into the same cohorts. Inspection of the means suggested differences between C57BL/6J and both cKO and fl/fl lines. For indicative purposes, the effect of genotype on period and phase was analysed and significant differences were seen in LD period and DD phase, as well as LL period and phase between transgenic groups and C57LB/6J (see A.8.2 for further details). This again demonstrates significant deviations of transgenic lines from their background C57BL/6J strain. However, as is the case with Afp transgenic lines, C57BL/6J mice would need to be integrated into cKO/fl/fl experimental cohorts before conclusions could be drawn.

Since sexual dimorphism was noted in the transgenic lines, sex differences were also evaluated in the C57BL/6J wild type control experiment (Figure 2.20). Mixed-effects analysis

revealed lighting conditions had a significant impact on period (F(1.533,20.70)=190.5, p<0.0001), as did sex (F(1,14)=5.952, p=0.0286), and that there was a significant interaction between sex and lighting condition (F(2,27)=4.847, p=0.0159). Females had a significantly longer period in LL (Šídák's post-hoc, p=0.0027) (Figure 2.20A). Additionally, a significant interaction between lighting condition and sex was seen on phase (F(2,27)=15.69, p<0.0001), specifically identifying an elevated phase in females in LL (Šídák's post-hoc, p=0.0001) (Figure 2.20B). Sex differences in C57BL/6J mice were only seen in constant light, in agreement with the transgenic line findings.

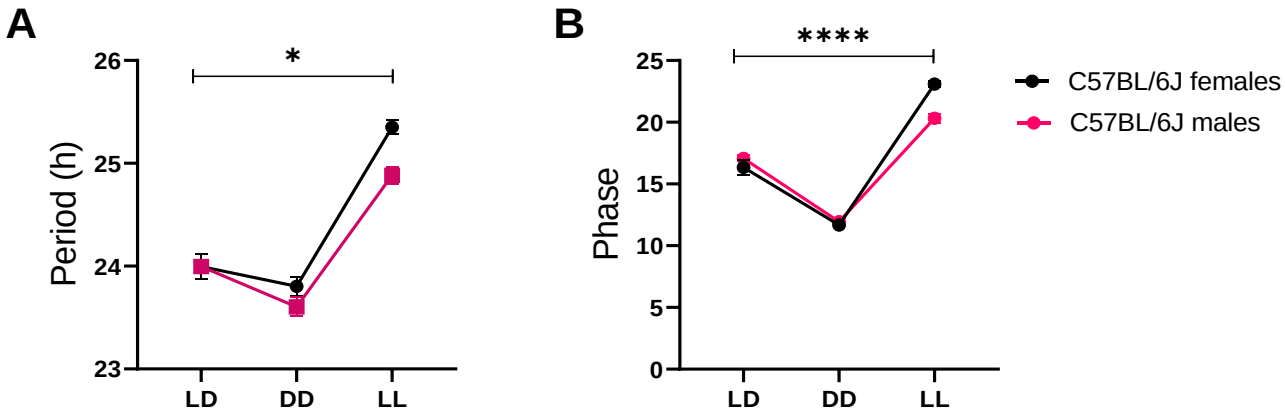


Figure 2.20: Significant sex differences in C57BL/6J period and phase in constant light conditions.

A) There was a significant interaction between sex and lighting condition on C57BL/6J period (*, p=0.0159). No significant difference was seen in period length between C57BL/6J males (n = 8) and females (n = 8 in LD and LL, n = 7 in DD) in LD or DD, but females displayed a significantly longer period in LL (**, p=0.0027). B) There was a significant interaction between sex and lighting condition on C57BL/6J phase (****, p<0.0001). No significant difference was seen in phase between C57BL/6J males (n = 8) and females (n = 8 in LD and LL, n = 7 in DD) in LD or DD, but females displayed a significantly higher phase in LL (***, p=0.0001).

Addendum:

This analysis could have been improved by performing three-way ANOVAs which included sex, genotype and lighting condition as variables, in replacement of two way ANOVAs individually assessing the effect of genotype and then sex. Without demonstration

of an interaction between sex X genotype, any claim that the two genotypes show variable sex differences has limited statistical support.

2.4 Discussion

In summary, these experiments revealed all transgenic lines displayed reductions in locomotor activity following the onset of LL conditions. Secondly, no effect of hepatic NPAS2 loss was seen on circadian period or circadian phase in either Afp cKO and Afp fl/fl mice, or cKO and fl/fl mice, in any lighting condition. However, abnormal entrainment periods and adaptation to altered lighting conditions were noted in the transgenic lines when compared to a separate cohort of C57BL/6J wild type mice. Finally, significant sex differences were noted specifically under conditions of constant light, in fl/fl, Afp cKO and C57BL/6J mice, where female mice had elongated periods.

2.4.1 Pre-natal hepatic NPAS2 deletion influences locomotor activity but not circadian rhythmicity

The hourly activity patterns of both Afp cKO and Afp fl/fl mice were similar in all lighting conditions; however, disparities were seen in mean distance travelled. In DD, Afp cKO showed increased distance travelled in comparison to Afp fl/fl controls, but under constant light Afp cKO mice substantially reduced their locomotor activity. In comparison, the reduction in activity of Afp fl/fl mice in LL was not statistically significant. Reduced activity is the typical response to constant light as nocturnal rodents usually rest during the light phase of a LD cycle. Additionally, these locomotor activity reductions following the onset of constant light are frequently observed (Chen et al., 2008, Dudley et al., 2003, Ma et al., 2007, Marin et al., 2015). Nevertheless, this does not clarify why locomotor activity reductions were significantly more exaggerated in Afp cKO mice. One hypothesis is a disproportionate response to stress between the two genotypes. LL exposure is known to induce stress hormones, such as corticosterone, and evoke stress responses within animal models. This stress induction can even induce

hippocampal changes at a neuronal level and inhibit cell proliferation (Fujioka et al., 2010, Jameie et al., 2016, Ma et al., 2007, Ruby et al., 2013). However, mice exposed to constant light have also been shown to have reduced anxiety parameters within OF tests, and thus the effects of LL on stress induction are conflicting (Castro et al., 2005, Fonken et al., 2009). To investigate further, anxiety tests should be performed to assess basal and LL-induced anxiety levels in hepatic NPAS2 deleted mice compared to controls.

No significant differences in period or phase were seen between Afp cKO and Afp fl/fl controls in any lighting condition. LD entrainment presented no disparities in circadian parameters between the two genotypes, as predicted since this is a peripheral knockout model. It was hypothesized that the hepatic knockout of NPAS2 would not be capable of inducing central circadian changes distally, especially considering CLOCK is the dominant BMAL1 binding partner in circadian roles and is able to compensate for NPAS2 loss (Dudley et al., 2003, Landgraf et al., 2016). Interestingly, both Afp cKO and fl/fl mice displayed a LD circadian period above 24 hours, which is unexpected due to the entrainment conditions of 12h:12h LD that should enforce a 24-hour period (Jud et al., 2005, Zheng et al., 2001). Due to these disparities, C57BL/6J wild type mice were utilised to confirm there were no failings in the experimental set-up inducing an accurate entrainment period. When means were compared between transgenic lines and C57BL/6J mice, both Afp cKO and Afp fl/fl genotypes appeared to diverge from wild type in LD period length. C57BL/6J mice displayed an entrained period of 23.995 h, whilst Afp cKO mice averaged at 24.424 h and Afp fl/fl controls averaged at 24.206 h, both significantly variable from 24 hours. This could suggest that both transgenic lines have an inability to adequately entrain to a 12h:12h LD cycle. This may be a product of floxing *Npas2* since both Afp cKO and Afp fl/fl lines display the same irregularity. On the other hand, this could simply detail the disparities between lines bred in different facilities

diverging from their background strain, since the transgenic lines were reared over numerous generations in-house, and the C57BL/6J were purchased externally.

Each subsequent lighting condition was successful in inducing comparable phase and period changes within both genotypes. Constant darkness caused a substantial reduction in Afp cKO mice' period to 23.79 h and a similarly shorter period of 23.87 h was seen in Afp fl/fl controls. This is aligned with literature which notes that DD ordinarily shortens period length to approximately 23.7 ± 0.1 h (Dudley et al., 2003). In this study by Dudley et al. it is seen that constitutive NPAS2 knockouts display a shorter free-running period than C57BL/6J, at approximately 23.5 ± 0.1 h, which is a phenotype not present in the peripheral knockout mice. This could suggest that brain and/or global *Npas2* loss is required in order to generate a shortened DD period phenotype, and peripheral *Npas2* loss alone mirrors wild type in this parameter (Dudley et al., 2003). Additionally, mice exposed to DD typically display a phase advance, where their peak of activity moves to earlier in the subjective day (Dudley et al., 2003, Jud et al., 2005, Zheng et al., 2001). Both genotypes presented a significant phase advance, and no statistical differences were noted between DD induced phase. Furthermore, LL conditions successfully increased circadian period in both genotypes equally, and also provoked a significant phase delay that was comparable between genotypes – both of which are characteristic of LL treatment (Alves-Simoes et al., 2016, Chen et al., 2008). This suggests that pre-natal hepatic NPAS2 deletion has no significant effect on circadian period or phase, either in entrainment or free-running conditions.

LL again showed disparities in both transgenic lines in comparison to C57BL/6J wild type mice. C57BL/6J mice were far more significantly affected by conditions of constant light and displayed a more substantial phase delay and period elongation. However, it is important to note that C57BL/6J mice were not integrated into the Afp cohorts and therefore further experiments are needed to confirm differences between genotypes. Despite this, both

transgenic lines still generated significant circadian responses to LL, meaning they are still receptive to the change in conditions, though this appeared to be dampened compared to their background strain. This further highlights a potential circadian phenotype as a result of floxing *Npas2*, or a weakened response to constant light due to divergence from the C57BL/6J background strain. Disparities between the transgenic lines and C57BL/6J mice in circadian parameters emphasises the need for further confirmation of *Npas2* expression levels, stability of *Npas2* exon 3, and NPAS2 protein presence in Afp fl/fl and fl/fl controls.

2.4.2 Post-natal hepatic NPAS2 deletion does not influence locomotor activity or circadian rhythmicity

As seen in Afp cKO and Afp fl/fl mice, cKO and fl/fl hourly activity patterns depicted similar behaviour within all lighting conditions. Additionally, there was no significant differences in circadian period between either genotype in any lighting condition. Both genotypes showed shortened periods in constant darkness, and elongated periods in constant light, as predicted by previous results in Afp cKO and Afp fl/fl lines and demonstrated previously in literature (Alves-Simoes et al., 2016, Chen et al., 2008, Dudley et al., 2003). Additionally, mice exposed to DD typically display a phase advance, where their peak of activity moves to earlier in the subjective day (Dudley et al., 2003, Jud et al., 2005, Zheng et al., 2001). Both cKO and fl/fl mice displayed a similar phase advance in DD. As well as this, LL conditions successfully increased circadian period in both genotypes comparably, and also provoked a significant phase delay that was equivalent between genotypes – both of which are characteristic of LL treatment (Alves-Simoes et al., 2016, Chen et al., 2008). This suggests that neither pre-, nor post-natal hepatic NPAS2 deletion, have a significant effect on circadian parameters, either in entrainment or free-running conditions. This is in line with the experimental hypothesis which thought peripheral rhythm perturbation would not significantly

alter global rhythmicity, and even local rhythmic changes in the liver would be compensated for by the presence of the *Clock* gene (Debruyne et al., 2007).

As seen in Afp cKO and Afp fl/fl mice, cKO and fl/fl mice also displayed significant differences in circadian period and phase in entrainment compared to C57BL/6J. Both Alb transgenic lines behaved very similarly to Afp transgenic lines under 12h:12h LD conditions, with a cKO LD period of 24.209 h and fl/fl LD period of 24.323 h, both of which are considerably different from C57BL/6J LD period of 23.995 h. This phenotype is discussed further in 2.4.1. Additionally, Alb transgenic lines also replicated the reduced impact of LL on circadian parameters that are seen in Afp lines, in comparison to C57BL/6J. As well as this, fl/fl controls displayed a significantly reduced phase advance in DD in comparison to C57BL/6J mice. This is especially interesting given both fl/fl lines are supposedly identical, and this effect was not seen in Afp fl/fl equivalents. Again, it is important to observe that C57BL/6J mice were not integrated into cKO and fl/fl experiments, and therefore cohorts of mixed genotypes would be required to confirm these phenotypes.

Given differences in fl/fl lines were also noted in circadian parameters, it supports the theory that separating strains over multiple cohorts of breeding can amount to divergence of phenotypes that are not a product of genotype. However, it could also demonstrate that the deviation from the background strain is potentially reducing the impact of variable lighting conditions on circadian parameters, which would be noteworthy (Bryant et al., 2013, Sultana et al., 2019). Ultimately, since all genotypes still significantly adapted to variations in lighting conditions, the experiment remains adequate in providing the expected outcome. However, this does not negate the necessity to further prove floxing exon 3 does not impact *Npas2* expression or NPAS2 protein levels.

Finally, since this study demonstrates that hepatic NPAS2 is not necessary for maintenance of rhythmicity in LD, DD or LL, and previous work has shown that global NPAS2 is not needed for circadian rhythmicity in free-running DD conditions (Debruyne et al., 2007), future work in this area could explore central NPAS2 loss under conditions of constant light.

2.4.3 Sex differences in NPAS2 transgenic lines

Sex differences in circadian literature are very variable, which is likely due to insufficient research into sexual dimorphisms in rhythmicity. There are obvious anatomical, hormonal, and metabolic differences between sexes in different species, but there are also less recognized variations in sleep and circadian rhythms (Cain et al., 2013, Mong et al., 2011). In our experiments, fl/fl females showed an increase in period compared to males in conditions of constant light. Afp cKO female mice also showed a significantly increased period in LL compared to males, as do Afp fl/fl female controls, though not significantly. This therefore presents a trend of elongated period length in female mice following conditions of constant light. Since this effect was significant in Afp cKO but not cKO, and fl/fl but not Afp fl/fl, it may be the case that higher sex group sizes are required to unmask the significance of this effect in all transgenic lines. Fl/fl control lines are genetically identical and it is assumed that they do not significantly vary between groups. Additionally, thus far no significant circadian differences have been noted between Afp cKO and Alb cKO groups, therefore it is possible that with increased group sizes this sexually dimorphic effect will be consistent across all groups. Despite this, this work proposes the novel finding that female mice are more susceptible to circadian changes induced by constant light conditions.

Additionally, both Afp cKO and cKO mice showed significant overall interactions between lighting condition and sex on period (Afp cKO, cKO) and phase (Afp cKO). This interaction was not significant in either fl/fl line, which could suggest a role for hepatic *Npas2* in the sexual dimorphism of circadian parameters irrespective of the developmental knockout

stage. This is also interesting since the results in the transgenic lines are contradictory. Elongated period and phase delays are characteristic of constant light and are induced together; if one sex were to be more affected by LL conditions than the other, it is expected that both parameters would be exaggerated in that sex (Alves-Simoes et al., 2016, Chen et al., 2008). Additionally, literature notes that females are more susceptible to light-induced phase shifts, which furthers the expectation that we would see an equivalently significant phase delay in females following LL exposure (Karatsoreos et al., 2011, Krizo et al., 2015, Kuljis et al., 2013, Schull et al., 1989). Whilst these deviations are largely unexplained, future studies into the sex differences of stress and anxiety induction resultant of lighting condition changes could help explain these disparities. Additionally, to ensure more accurate unveiling of sexual dimorphisms in circadian rhythmicity, influences on parameters such as oestrous cycle, sleep, and corticosterone levels should be controlled for.

2.4.4 Sex differences in C57BL/6J wild type mice

To date, limited research has been performed on the sex differences between circadian parameters in C57BL/6J mice. Sexual dimorphism in free-running period is seen to be very species specific, with C57BL/6J mice showing no sex differences in free-running period in the literature (Davis et al., 1983, Kuljis et al., 2013, Labyak and Lee, 1995, Lee and Labyak, 1997, Schull et al., 1989). This experiment supports the literature findings of a lack of sexual dimorphism under LD conditions, as no differences in period or phase were seen between C57BL/6J males and females in entrainment. Moreover, literature states that female mice show significantly higher levels of general activity and display earlier onset of activity than male counterparts. As a result of varying activity onset, it was thought that circadian phase may exhibit sexual dimorphism also (Bartling et al., 2017, Konhilas et al., 2015). Our study found significant interactions between sex and lighting condition on circadian phase and circadian period, with a significant difference between males and females in LL conditions. This work

therefore presents the novel finding of significant circadian sexual dimorphism within C57BL/6J mice, specifically under conditions of constant light. This suggests that sexual dimorphism in circadian parameters is irrespective to NPAS2 loss in the liver and is instead a naturally occurring phenomenon. Most notably, wild type C57BL/6J mice displayed a sexually dimorphic adaptation to constant light, and effect that was replicated in transgenic NPAS2 lines.

This therefore presents a trend of elongated period length in female mice following conditions of constant light. Interestingly, C57BL/6J mice also displayed significantly higher phase in females in LL, which was not noted in Afp or Alb lines. As described in 2.4.3, it is predicted that one significantly altered parameter of circadian rhythmicity would coincide with the other, however, this was only seen in C57BL/6J mice. The C57BL/6J genotype generally depicted a higher circadian phase in LL than the transgenic genotypes, which could suggest a longer period in LL would be needed to potentially unmask this effect in the transgenic lines. It could also further imply that the transgenic lines are less effected by the conditions of LL because of background strain divergence, which could result in reduced sexual dimorphism in LL phase within these genotypes.

Chapter 3: The effects of post-natal hepatic NPAS2 loss, and sex, on recognition memory

3.1 Introduction

3.1.1 Clock mutations and cognition

As discussed at length in 1.7, mutations in many clock genes can result in severe impacts on memory and cognition. The circadian clock is thought to be involved in memory acquisition, consolidation and retrieval, and genes implicated in the core TTFL are ubiquitously expressed in brain regions commonly associated with memory and cognition, such as the amygdala and the hippocampus. (Hampp et al., 2008, Jilg et al., 2010, Lamont et al., 2005). As well as this, disruption to the SCN impairs memory acquisition and maintenance of learning and memory (Ruby et al., 2008). Mice with mutations in critical components of the circadian clock thus display impairments in various types of learning and memory paradigms. Mice deficient of forebrain *Bmal1* lose time-of-day deviations in NOL memory performance, mirroring that of arrhythmic mice (Snider et al., 2016). BMAL1 knockouts also exhibit hyperactivity and inhibited habituation, which could be an outcome of impeded memory capabilities (Kondratova et al., 2010, Snider et al., 2016). However, CRY1 and CRY2 double knockouts display reduced activity and increased environmental habituation, though they lose the ability to memorise time-place associations during maze tasks (Van der Zee et al., 2008). Similarly, mice with mutations in *Per2* demonstrate atypical LTP in the hippocampus, causing impaired memory recall of fear conditioning (Wang et al., 2009). Additionally, cognition is regulated in a circadian manner as demonstrated in time-of-day correlated memory performance. In humans, cognitive functioning peaks during midday, where sleep inertia is relieved, and alertness steadily increases. Cognitive impact of lifestyle on circadian dysfunction has also been noted in humans, where substantial cognitive difficulties have been observed in long-term shift workers (Kondratova et al., 2010, Krishnan and Lyons, 2015). Time-of-day dependent learning is also seen in nocturnal rodents, where their cognitive capabilities in the Morris water maze, contextual fear conditioning and the radial arm maze peak during their

active phase – the night (Valentinuzzi et al., 2001, Valentinuzzi et al., 2004). This demonstrates a significant influence of circadian rhythmicity and time of day on learning and memory in both mouse and human models.

3.1.2 *Npas2* and cognition

As discussed in more depth in 1.8, mice deficient in NPAS2 show deficits in LTM during cued and contextual fear (CCF) tasks. In contrast, Garcia et al. demonstrated that such mice showed intact short-term memory, and no abnormalities in non-emotional behavioural assays. This suggests that NPAS2 is a requirement for the processing of complicated sensory stimuli (Garcia et al., 2000). However, the investigation of sex in the context of NPAS2 loss on memory was not explored, therefore it is unknown if there are sex dimorphisms within NPAS2 LTM impairments (Garcia et al., 2000). Furthermore, *Npas2*$^{-/-}$ mice display a reduction in the power of sleep spindles, which have an essential role in long-term memory consolidation and sensory processing. Thus, a reduction in these oscillations gives impairments in cortical plasticity and significant memory processes. This is a potential mechanism by which *Npas2* could influence long-term memory in rodents (Franken et al., 2009, Gais et al., 2002, Timofeev et al., 2002). Additionally, 19 *Npas2* SNPs have been identified which correlate with the risk of cognitive ageing, specifically found within the Taiwanese population. *Npas2* SNPs were also seen to have an interaction with *Ror-α* and *Ror-β* SNPs to increase the risk of cognitive ageing (Lin et al., 2017). This further implicates *Npas2* and its interactions with cognitive capabilities and a potential role for circadian proteins in ageing and cognition.

Npas2 is also highly enriched in DRD1 containing neurons. These neurons are principally located in the striatal regions and the olfactory bulb, as well as the VTA within the midbrain, the substantia nigra and the hypothalamus. DRD1 neurons have significant roles in the modulation of learning and memory (Fazio et al., 2016, Fremeau et al., 1991, Olguín et al.,

2016, Puighermanal et al., 2016, Wass et al., 2013). DRD1 receptors are implicated in general cognitive processes, working memory modulation, PFC attentional and working memory roles, synaptic plasticity, and consolidation and persistence of LTM (Bethus et al., 2010, Puighermanal et al., 2017, Wass et al., 2013). Increased expression of *Npas2* in these DRD1 containing neurons may suggest an important role for *Npas2* in cognition and memory. Interestingly, DRD1 levels were higher in *Per2* KO mice than in wild type and were inversely proportional to PER2 levels. Following administration of DRD1 antagonist SCH-23390, *Per2* KO mice showed rescued spatial memory, which implicates DRD1 in circadian regulation of spatial working memory (Kim et al., 2022). This presents a mechanism by which circadian proteins might alter cognitive processes in a DRD1-dependent manner, which could include roles for *Npas2*.

3.1.3 Testing object recognition memory

Broadly, LTM can be divided into two subtypes: declarative and non-declarative. Declarative, or explicit, memory refers to the conscious recall of personal history, facts, and events. Whereas non-declarative, or implicit, memory encompasses skills and habits, and does not utilise conscious thought (Birzniece et al., 2005, Cohen and Stackman, 2015). Recognition memory is a form of declarative memory that has been demonstrated in many species including mice. The NOR task is a well-substantiated behavioural paradigm which employs an animal's innate preference to explore a novel stimulus in order to evoke conclusions on cognition and memory. The premise of the test involves presenting an animal with two identical objects in a sample trial, in which interaction should be comparable, followed by a protocol-dependent retention time, and a test trial where a previous object is substituted for a novel item (Albasser et al., 2010, Ruby et al., 2013). The foundation of the test is a spontaneous predilection for the novel object, which requires no prior training and no motivational reward or punishment. The NOR task is also advantageous from a welfare perspective simply because it is non-aversive;

with satisfactory habituation to an open arena the test itself should not evoke any stressful responses within the subject. However, the NOR test is prone to considerable variability most likely due to disparities in protocols, which can also lead to conflicting results (Sutcliffe et al., 2007).

The fundamental neuronal circuitry that the NOR task relies upon is frequently debated. Some literature demonstrates support for hippocampal involvement in object recognition. For example, hippocampal lesions in rats have diminished the retrieval of object recognition memory that was encoded prior to the lesion being imposed, and pharmacological temporary inactivation of the hippocampus hinders NOR results (Cohen and Stackman, 2015, Cohen et al., 2013, Gaskin, Tremblay and Mumby, 2003). However, other work suggests the perirhinal cortex is central to the NOR task, and the hippocampus is only essential in a particular variant of the task where an object used in the sample trial moves locations for the test trial (Brown and Aggleton, 2001, Cohen and Stackman, 2015). Remembering the locations of objects, rather than the novelty of the object, employs spatial memory as well as recognition memory (Labban et al., 2021, Roustazade et al., 2021). Whilst there is support for the theory that the hippocampus contributes to encoding and consolidation of object recognition memory, perirhinal lesions in rats and monkeys lead to far more profound impairments in recognition memory than equivalent lesions in the hippocampus. The principal view is therefore that the perirhinal cortex is mostly accountable for rodent recognition memory (Cohen and Stackman, 2015, Cinalli et al., 2020, Cohen et al., 2013). Subsequently, a theory has evolved by which the two brain regions act cooperatively. Hippocampal inactivation studies have shown significant deficiencies in the NOR task if the retention time exceeds 10 minutes. Similar studies have likewise demonstrated hippocampal redundancy if the retention time is less than 10 minutes, leading to the hypothesis that engagement of the hippocampus is in fact dependent on the interlude between sample and test trials (Cohen and Stackman, 2015, Cohen et al., 2013, Cinalli

et al., 2020). Thus, the theory suggests extra hippocampal structures, such as the perirhinal cortex, provisionally retain the object memory and that stronger, more deeply encoded recollection is maintained by the hippocampus. Although, this concept of an integrated recognition memory network is similarly disputed (Cohen and Stackman, 2015, Cohen et al., 2013, Cinalli et al., 2020).

3.1.4 Sexual dimorphism in spatial and recognition memory

In addition to sex differences noted in circadian rhythmicity and behaviour, explored in 1.10 and 1.11, there are also significant sexual dimorphisms in cognition within laboratory rodents and humans. In healthy human participants, females have been shown to demonstrate superior episodic memory compared to males, though this declines in age more rapidly than male episodic memory (Loprinzi and Frith, 2018). Female humans tend to dominate males in autobiographical, semantic, auditory, object location and recognition memory, as well as having faster memory retrieval and increased hippocampal volume (Dixon et al., 2004, Herlitz and Yonker, 2002, Maitland et al., 2014, Pauls et al., 2013, Voyer et al., 2007). It has also been demonstrated in numerous studies that males outperform females in spatial-based memory paradigms in human models (Fernandez-Baizan et al., 2019, Jones and Healy, 2006, Piber et al., 2018). Additionally, two-thirds of dementia diagnoses are given to women, and dementia generates greater memory impairments in females than males. Furthermore, females show accelerated rates of whole brain and temporal lobe atrophy (Beam et al., 2018).

Despite this, in animal models there are discrepancies. Sex differences in cognition have been found in abundance in laboratory rats, yet this is often absent or contradictory to results found in laboratory mice (Bettis and Jacobs, 2012, Bettis and Jacobs, 2013). Female rats show superior discrimination of the novel object in recognition memory trials, yet male rats reliably outperform female rats in radial maze working memory trials, radial maze

reference memory trials and water maze protocols. Thus, the most robust sex differences in laboratory rat cognition is found in spatial memory. It is thought that these sex differences in maze trials result from organisational effects of steroid hormones (Endo et al., 1994, Frick and Gresack, 2003, Berger-Sweeney et al., 1995, Jonasson, 2005, Seymoure et al., 1996, Voikar et al., 2001).

Equivalent, robust sexual dimorphisms have not been replicated in mouse models. In a meta-analysis of sex differences in learning and memory, four mouse water maze experiments revealed male superiority in one study, female superiority in another, and no recorded sex differences in the remaining two studies (Berger-Sweeney et al., 1995, Frick et al., 2000, Jonasson, 2005). Two studies exploring radial maze trials that employed working memory found no sex differences in one study, whilst the other demonstrated that males learned the maze task significantly faster, and with fewer errors, than female mice (Jonasson, 2005, LaBuda et al., 2002, Mishima et al., 1986). Additionally, no sex differences were found in radial maze trials employing reference memory in mice (Jonasson, 2005, LaBuda et al., 2002). These studies taken together show a contradiction between the robust recognition and spatial memory findings observed in laboratory rats, and a lack of consistent sexual dimorphism seen in mice. There are a multitude of factors which can prevent similarities across models and laboratory experiments, namely, significant protocol variation between research groups. For example, in NOR trials alone, variance can be introduced by duration of habituation, length of sample trial object exposure, length of retention time, the shape/size of the arena and features of the objects (Sutcliffe et al., 2007, Bettis and Jacobs, 2013).

More recent studies of NOR paradigms in laboratory mice have generated similarly mixed results. Some reports suggest no sex differences are present, while others either state male or female superiority (Benice et al., 2006, Bettis and Jacobs, 2012, Bettis and Jacobs, 2013, Frick and Gresack, 2003). Both sexes of CD-1 mice demonstrate robust preference for

novel objects in object recognition paradigms, and displaced objects in object location trials, depicting no sexual dimorphism of recognition or spatial memory. Though, a lack of sex differences in this strain may be due to the fact their gonads are 16 times less responsive to oestrogen, and they are an outbred strain. This means their oestrogen insensitivity may mask hormonal-induced sex differences present in inbred strains (Ricceri et al., 2000). In contrast, Bettis and Jacobs, 2013, show that female C57BL/6J mice preferentially explored the novel object while male mice did not. However, this study also noted an increased exploration of both objects and thus an increased test interaction in female mice which may impact cognitive output (Bettis and Jacobs, 2013). Nevertheless, as depicted in Figure 3.1A, differences between sexes were negligible, the effect is seen simply because females show a significant contrast between novel and familiar object exploration and males do not. Frick and Gresack demonstrate opposing results. This study showed that male C57BL/6J mice had a significant preference for the novel object, and female mice did not (Figure 3.1B). However, this study continued until animals met an interaction time threshold, meaning total test interaction time was not controlled for and could not be compared. As a result, the influence of test exploration on recognition memory cannot be ruled out (Frick and Gresack, 2003).

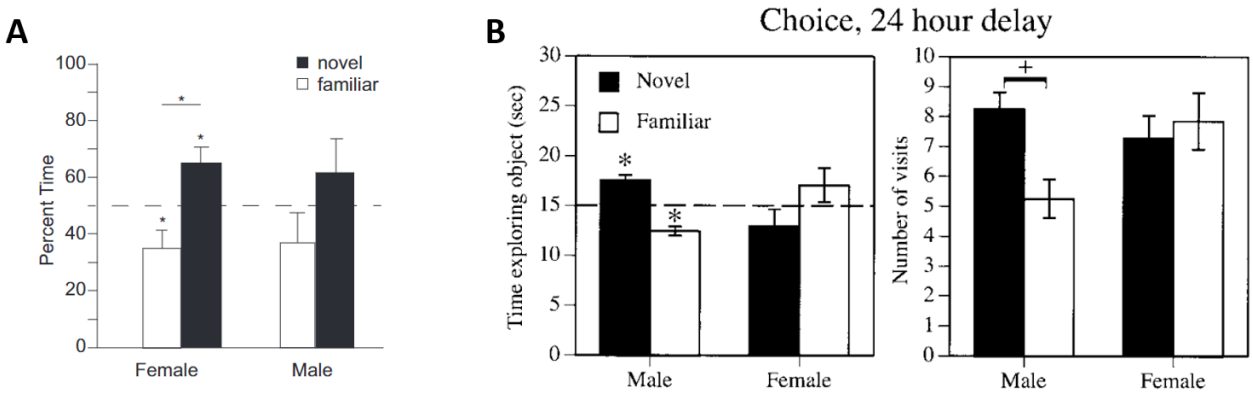


Figure 3.1: Sexual dimorphism in C57BL/6J recognition memory varies in the literature.

A) Percentage time spent exploring novel and familiar objects by C57BL/6J male and female mice, depicting a significant preference for the novel object in female but not male mice. Adapted from Bettis

and Jacobs, 2012. B) Time spent exploring novel and familiar objects and total number of object visits in C57BL/6J male and female mice, depicting a significant preference for the novel object in both parameters in male mice but not female mice. Adapted from Frick and Gresack, 2013. (Bettis and Jacobs, 2012, Frick and Gresack, 2003).

Research by Bettis and Jacobs, 2013, suggested disparities between results could be attributed to social housing, and that more accurate depictions of male mouse cognition are unveiled when they are singly housed, as this replicates their solitary preference in the wild (Bettis and Jacobs, 2013). Additionally, Bettis and Jacobs, 2012, suggested object similarity is a factor for correct novel object discrimination in male mice; insinuating females have superior recognition memory and can discriminate similar objects to the familiar object, but male mice require a significant visual difference between novel and familiar objects (Bettis and Jacobs, 2012, Simeonovska-Nikolova, 2016).

3.1.5 Memory under conditions of constant dark and constant light

In addition to phenotypes mentioned in 1.6, different lighting conditions can also influence memory and cognition. Constant darkness has been found to impair spatial memory in C57BL/6J mice and Wistar rats (Jameie et al., 2015, Zhou et al., 2018). Following eight weeks of total light deprivation, a significant decrease in neurogenesis was seen in both male and female rats, with increased severity in males. Additionally, escape latency time from a Morris water maze was increased in mice held under DD conditions, indicative of impaired spatial memory (Jameie et al., 2015). However, this experiment employed eight weeks of total darkness, which began one week pre-natally, meaning that mice employed had never been exposed to light. This may have had significant implications on the development of the visual and circadian systems that are more deleterious than general constant darkness (Jameie et al., 2015). In support of this, recent findings by Asadian et al. (2022) show that three weeks of constant darkness impairs spatial memory in male Wistar rats during Morris maze water tests. This study also revealed significant differences in fear conditioning following DD, whereby rats in constant darkness froze for a shorter amount of time. This could indicate a memory

failing in which the DD mice display enhanced extinction of auditory fear memories (Asadian et al., 2022). Zhou et al. demonstrate similar findings in C57BL/6J mice following four weeks of DD. Mice in constant darkness spent less time in the target quadrant and had longer latencies to find the platform of a Morris water maze, both of which indicate impairments in spatial memory (Zhou et al., 2018). However, many of these studies were performed in light conditions *after* periods of light deprivation, rather than performed *under* the conditions of light deprivation. This allows the influence of light on the testing day and prevents accurate understanding of light deprivation on memory. Similarly, data on recognition memory trial performance in DD conditions is sparse; this is in part due to the common misconception that the NOR task is visuospatial and therefore cannot be conducted in the dark (Tam et al., 2017). Multiple studies have since been performed that dismiss this idea. Under IR or red light, rats have been shown to use both tactile and tactile-to-visual cross modal object recognition to give significant results in DD NOR tasks. This multimodal novelty recognition is facilitated by both the perirhinal cortex and the posterior parietal cortex (Albasser et al., 2010, Winters and Reid, 2010).

Likewise, LL has been shown to negatively influence learning and memory in nocturnal rodents (Fujioka et al., 2011, Jameie et al., 2016, Ma et al., 2007, Zhou et al., 2018). In male mice, exposure to LL for three weeks significantly altered hippocampal morphology by reducing the proliferation of hippocampal cells in the granule cell layer (GCL) of the dentate gyrus. As well as this, an increased latency to escape during water maze trials indicated impaired spatial memory following conditions of LL (Fujioka et al., 2011). This is supported by Zhou et al. who also show impairments in spatial memory in mice following exposure to LL for four weeks (Zhou et al., 2018). This effect is similarly noted in rats by Ma et al., utilising three weeks of LL exposure in Wistar rats, who subsequently spent significantly less time in the target quadrant of the Morris water maze task (Ma et al., 2007). LL can also alter time-of-

day peaks of olfactory memory performance, inverting the optimal memory performance from ZT6 in LD to ZT18 in mice that underwent repeated exposure to constant light, as well as desynchronising recognition memory (Tam et al., 2017). Impaired memory in Barnes maze tasks were also noted in mice under conditions of sustained dim light at night, replicating modern day living conditions as opposed to continuous, harsh, bright light. This lighting schedule was not enough to modify circadian rhythmicity or locomotor activity patterns, therefore giving insight into how prolonged, non-circadian periods of light which do not alter other circadian parameters, could affect memory in human scenarios (Fonken et al., 2012). In contrast, daily exposure to significant bright light is seen to increase spatial memory in mice. Two hours of bright light, daily, for three weeks resulted in improved NOL performance and enhanced Morris water maze tests. This could demonstrate that prolonged LL is detrimental to memory, but consistent bright light pulses during the subjective day may alter alertness and have positive outcomes on memory paradigms (Huang et al., 2021, Milosavljevic et al., 2021).

Memory in both DD and LL has not yet been assessed in the context of NPAS2 loss, either centrally or peripherally. As NPAS2 is a circadian protein that has been shown to demonstrate LTM impairments, the consequences of its loss on memory under variable lighting conditions would be insightful (Garcia et al., 2000).

3.1.6 Aims and Objectives

The following experiments aimed to assess cognition, specifically recognition memory, in mice deficient of hepatic NPAS2. This work was performed since impairments in LTM are seen following global NPAS2 loss in male mice, and therefore gaining insights into peripheral versus central NPAS2 knockouts on recognition memory would be valuable (Garcia et al., 2000). Preliminary experiments using just hepatic NPAS2 knockouts revealed interesting findings and justified repeated cohorts. The hypothesis of this experiment was that no

significant differences in recognition memory would be noted between post-natal hepatic NPAS2 knockout mice, and controls. This is because the NPAS2 knockout is peripheral, and general brain function was predicted to be unaltered, unlike in Garcia et al.'s global knockout (Garcia et al., 2000).

Another aim of this study was to assess recognition memory changes through variable lighting conditions, and any alterations due to post-natal NPAS2 knockout in the liver. Conditions of DD and LL have been shown to have detrimental effects on memory in rodents (Fujioka et al., 2011, Jameie et al., 2015, Ma et al., 2007, Zhou et al., 2018). As NPAS2 is a circadian protein, it was of interest to assess recognition memory under different lighting conditions. The hypothesis of this experiment was that no significant differences would be noted between genotypes, however, it was hypothesized that memory would be impaired in both DD and LL conditions in comparison to cycling light conditions, within both genotypes.

In addition, we planned to perform analysis of sex differences, since our cohorts were of mixed sex. This is especially interesting since sex differences in C57BL/6J recognition memory are highly contradictory and regularly disputed (Bettis and Jacobs, 2012, Bettis and Jacobs, 2013, Frick and Gresack, 2003, Jonasson, 2005). This work aimed to clarify natural sexual dimorphism in fl/fl mice of C57BL/6J background and to investigate if hepatic NPAS2 loss influenced sex differences in memory. Since both fl/fl and hepatic NPAS2 deficient mice demonstrated global circadian rhythmicity effects in a sex-dependent manner, it was hypothesized that these changes in rhythmicity, paired with variable lighting conditions during NOR testing, may amount to sex differences in cognition. Previous work by Bettis and Jacobs, 2012, suggested solitary housed males may depict a more male-typical recognition memory performance and that this would be desirable testing conditions. Due to our circadian box experimental set up, this study aimed to test the recognition memory of singly housed mice under multiple lighting conditions. (Bettis and Jacobs, 2012).

3.2 Materials and Methodology

3.2.1 Animals

A total of 17 fl/fl mice and 15 cKO, made up of two cohorts, were used in NOR experiments (see Figure 2.3 for cKO explanation). Each cohort performed NOR trials under three different lighting conditions. Both cohorts contained eight cKO mice (four male, four female) and eight fl/fl mice (four male, four female) initially. Post-mortem genomic analysis revealed an incorrectly genotyped female within the second cohort, meaning a total of nine fl/fl females and seven cKO females were used in these experiments. The n number chosen for this study was based on previously published literature and limitations of experimental chambers. Previous NOR tests of this nature utilised subject group sizes between n = five and n = 20, with the most common n number per group being n = 10 (Arqu*é* et al., 2008, Botton et al., 2010, Carlini et al., 2008, Denninger et al., 2019, Hansen et al., 2010, Leger et al., 2013, Tam et al., 2010). Since this NOR paradigm can often result in test failures through lack of interaction with the objects, this experiment utilised increased group size from average publication n numbers to n = 16, which became n = 15-17. To justify combining multiple cohorts, experimental conditions were kept identical, and all experimental results were analysed for normality, homogeneity of variances and outliers. Any discrepancies in n numbers across conditions was a result of failed NOR tests by experimenter, such as providing an incorrect object set or recording errors. No mice failed to interact with the objects during NOR trials.

3.2.2 *In vivo* husbandry and general care

As described in 2.2.2.

3.2.3 Circadian box experimental set-up

Locomotor activity recording was measured as described in 2.2.3. Circadian recording was used to confirm that the mice were adapting to each lighting condition/cycling light during NOR trials. Circadian data analysis was performed using the methods described in 2.2.6.

3.2.4 NOR test protocol

The NOR protocol was based on previous literature (Gresack et al., 2007, Zhao et al., 2012). Tests were performed over three consecutive days, with 24 hours in between each session. Day 1 habituation: mice were removed from their home cages and placed inside clear, acrylic open field arenas (16 cm x 19 cm x 26 cm) within the ventilated chamber for five minutes to habituate to the new cage. Day 2 sample trial: mice were rehabituated to the arena for one minute, then two identical objects were placed in the top left and bottom right corners of the arena, the mice' interaction was recorded for five minutes. Day 3 test trial: mice were rehabituated to the arena for one minute, then one familiar object from day 2 was placed in the bottom right corner, and one novel object was placed in the top left corner. The mice' interaction was recorded for 10 minutes.

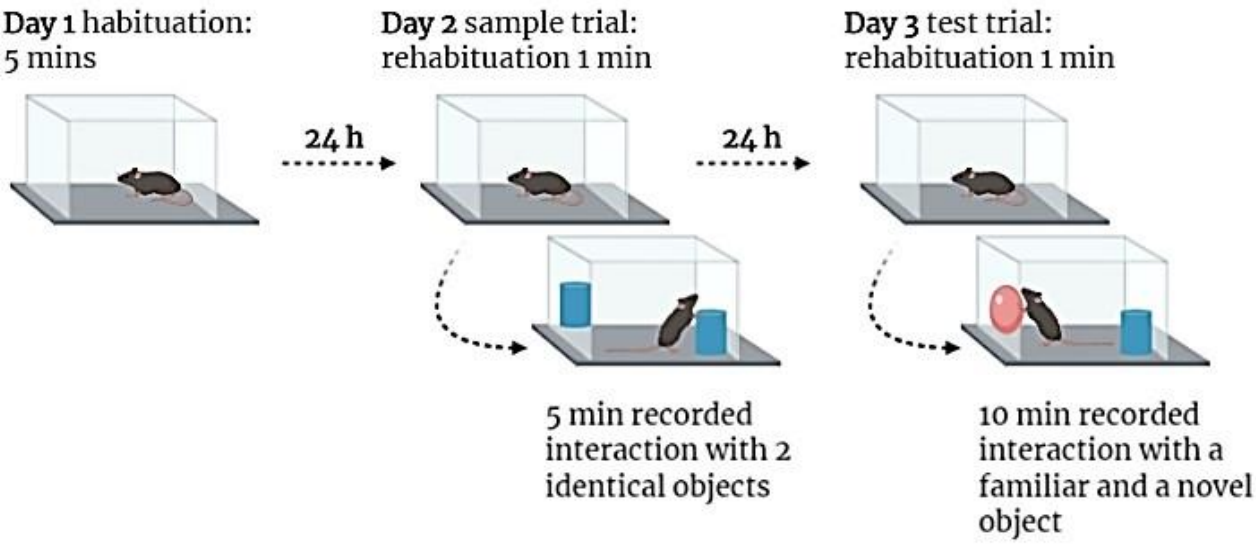


Figure 3.2: A schematic depicting the NOR procedure employed in this study.

Created with Biorender.com.

A threshold of >15 second interaction in all sample trials was set in order to discount any mice that did not interact with the objects adequately. Objects had a maximum height of 12 cm and had a base area no larger than 6 cm x 6 cm. Examples include salt/pepper mills, filled falcon tubes and miniature bottles. Pairs of objects were comparable in height and width but varied in colour and shape for novelty discrimination. Objects had identical replicas to be used in subsequent test trials and were washed in apple cider vinegar to remove any odour cues, and 70% ethanol for sterilisation, as were the test arenas.

Each cohort was subjected to a seven-day LD entrainment period, followed by 14-days of DD, and 14 days of LL. After five days in LD entrainment, a NOR task was performed on three consecutive days using the protocol described in Figure 3.2, after 10 days in DD and LL, a second and third NOR task were performed as described in Figure 3.2. Thus, the order of NOR tests for the first cohort went: LD, DD, LL. Although testing order of lighting conditions was not completely counterbalanced, testing order was at least varied and the order was counterbalanced between LL and LD conditions. The second cohort of mice underwent the same experiment but with the lighting conditions reversed, in order to avoid an influence of repeated testing on cognitive output. Thus, the order of NOR tests for the second cohort went: LL, DD, LD. The same three separate object sets were used in both cohorts, so no mice encountered the same set twice, and sets were randomised between mice and over each lighting condition so that no set was used more in one condition than another.

3.2.5 NOR data analysis

NOR results were analysed by manually timing interaction with both objects. This was performed by two independent, blinded assessors. Inter-rater reliability was on average >90% (Pearson's correlation coefficient) and a very high level of consistency was repeatedly observed using Cronbach's alpha (>0.94). Using these scores, a discrimination index (DI) was

calculated utilising Equation 3.1:

$$DI = \frac{(\text{time spent with novel object}) - (\text{time with spent familiar object})}{(\text{time spent with novel object}) + (\text{time spent with familiar object})}$$

Equation 3.1: Discrimination index calculation based on time spent with each object.

DIs give a value between -1 and +1. 0 indicates no statistically significant preference. -1 shows a perfect preference for the familiar object, and +1 shows a perfect preference for the novel object. To verify that mice were performing above chance, DIs were compared to zero using a one-sample T test using SPSS.

3.2.6 Statistical analysis

Power calculations were performed in Gpower and were calculated for repeated measures ANOVAs across lighting conditions (Faul et al., 2007). Using a Cohen's f of 0.8 based on a large effect size, for an ANOVA alpha value of 0.05 and power of 0.9 with two groups, a total of 14 animals equal to seven per genotype would be needed. To include sex as a variable, using a Cohen's f of 0.8 based on a large effect size, for an ANOVA alpha value of 0.05 and a power of 0.9 with four groups, a total of 20 animals equal to five per sex would be needed.

All statistics were performed in Prism (GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). All data was graphed in Prism. All data was tested for normality and homogeneity of variance on SPSS (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp), and all data is shown as mean ± SEM, unless otherwise stated. Repeated measures two-way ANOVA was performed to analyse interaction time with objects between treatments and discrimination indices of multiple NORs. Mixed-effects analysis was used in replacement of repeated measures two-way and three-way ANOVAs when not all mice completed every

repeated measure, e.g. mice that were removed from one condition due to experimenter protocol error. Independent T-tests were performed to assess total interaction time in one lighting condition, and discrimination indexes in one lighting condition. One-sample T-tests were used to compare discrimination indices to 0, to identify if the result was 'above chance'. Paired T-tests analysed sample bias, and object preference. Non-parametric equivalents of one-way ANOVAs (Friedman test), independent T-tests (Mann-Whitney test) and paired T-tests (Wilcoxon test) were performed on non-normal data, where stated. A probability value of $p<0.05$ was used as the criteria to determine statistical significance with $p<0.05$ as '*', $p<0.01$ as '**', $p<0.001$ as '***', and $p<0.0001$ as '****'.

3.3 Results

3.3.1 Assessing object and spatial biases in NOR sample trials

Genotype was confirmed by PCR analysis, as described in 2.2.9 and 2.3.1. Mice first underwent a sample trial with two identical objects to confirm there is no spatial bias in the open arena.

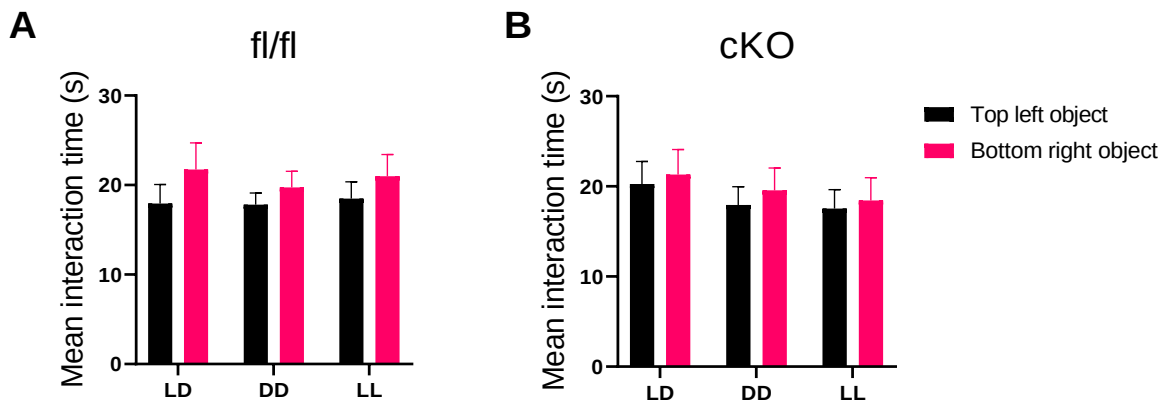


Figure 3.3: No location preference shown in either genotype during sample trial.

A) Fl/fl controls (LD n = 15, DD n = 17, LL n = 17) showed no significant preference in object location in sample trials under any lighting condition. B) cKO mice (n = 14 in LD, n = 15 in DD and LL) showed no significant preference in object location in sample trials under any lighting condition.

No significant location preferences were noted in either genotype (fl/fl: $F(1, 92)=2.571$, $p=0.1160$, cKO: $F(1,82)=0.3691$, $p=0.5491$), in all conditions. Though, there was a very slight increase in bottom right object exploration in both genotypes within all conditions (Figure 3.3). Due to this, novel objects within the test trial were placed in the top left corner to ensure any discrimination indices in favour of the novel object were not biased by modest positioning preferences.

Sample trial results were then split by sex to ensure there was no spatial bias in any sex groups. A discrimination index was generated for the sample trials using Equation 3.1.

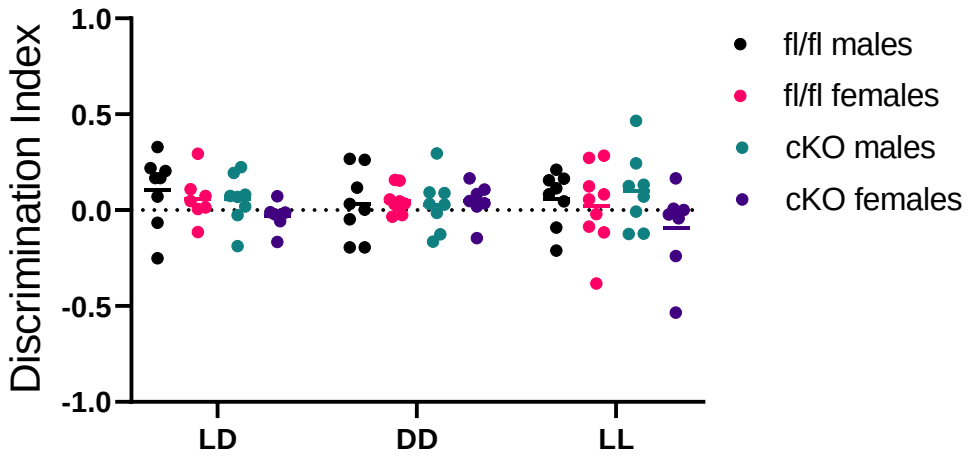


Figure 3.4: No significant differences in sample trial DI between sexes or genotypes.

DIs of all sex/genotype groups (fl/fl males: n = 8 in all conditions, fl/fl females n = 7 in LD, n = 9 in DD and LL, cKO males n = 8 in all conditions, cKO females n = 6 in LD, n = 7 in DD and LL) revealed no significant object or spatial preferences in LD, DD or LL.

There was no significant effect of sex/genotype group on spatial preference in the sample trials ($F(3,81)=1.577$, $p=0.2013$). There was also no significant effect of lighting condition on DI within the sample trials ($F(2,81)=0.2159$, $p=0.8063$), nor was there an interaction between lighting condition and sex/genotype group on sample trial DIs

($F(6,81)=0.7205$, $p=0.6342$) (Figure 3.4). In addition to sample trials, innate object preference was assessed to ensure no biases within object sets were seen (see A.2).

3.3.2 The effects of NPAS2 cKO, sex, and lighting condition on NOR test interaction

24 hours later, the 10-minute test trial was performed. Mean total interaction time, where interaction with both the novel and the familiar object were combined, revealed no significant differences in total test interactivity between genotypes or between lighting conditions (Figure 3.5A). However, a sex X genotype effect was seen by mixed-effects analysis ($F(1,75)=4.786$, $p=0.0318$). This also revealed fl/fl males showed generally increased total test object exploration, with a specific significant increase in exploration in LD (Šídák's post-hoc, $p=0.0304$) (Figure 3.5B). No significant influence of genotype or significant total interaction time differences were seen in females (Figure 3.5C).

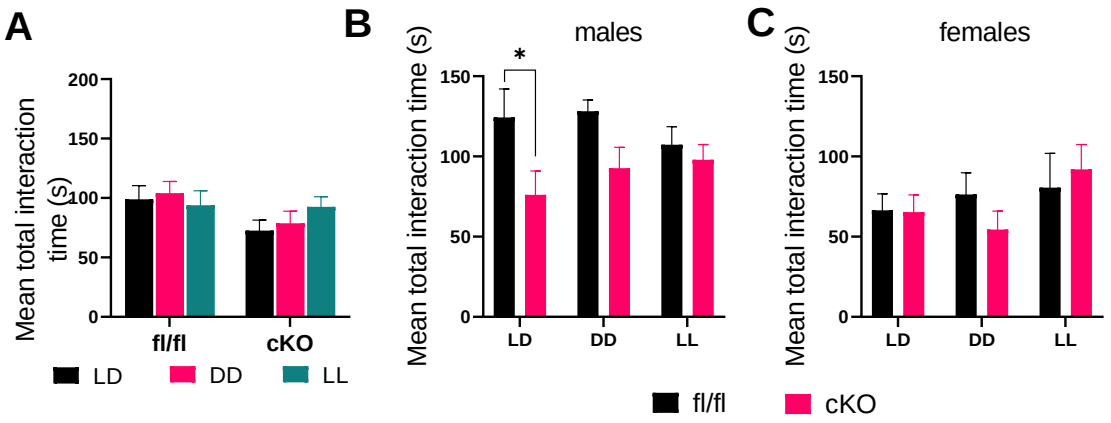


Figure 3.5: NOR test interaction does not differ between genotypes but differs between cKO and fl/fl males.

A) Mean total interaction time of cKO (n = 14 all conditions) and fl/fl controls (LD n = 16, DD n = 15, LL n = 14) in all lighting conditions. No significant differences in total interaction were seen between genotypes or lighting conditions. B) Mean total interaction time of fl/fl males (LD and DD n = 8, LL n = 7) and cKO males (LD, DD n = 8, LL n = 7) in all lighting conditions. There was a significant effect of genotype on male total interaction time (**, $p=0.0055$). Fl/fl males showed significantly more exploration in LD than cKO males (*, $p=0.0304$). C) Mean total interaction time of fl/fl females (LD n = 8, DD and LL n = 7) and cKO females (LD, DD n = 6, LL n = 7) in all lighting conditions. No genotype differences in female test interactivity were seen.

Since an interaction of sex X genotype was seen on total test exploration, sexual dimorphism within genotypes was assessed. In both LD (Šídák's post-hoc, p=0.0485) and DD (Šídák's post-hoc, p=0.0227), fl/fl males had higher total test interaction than fl/fl females, but not in LL. However, sex was seen to have no influence on test interactivity in the cKO strain (Figure 3.6A, 3.6B).

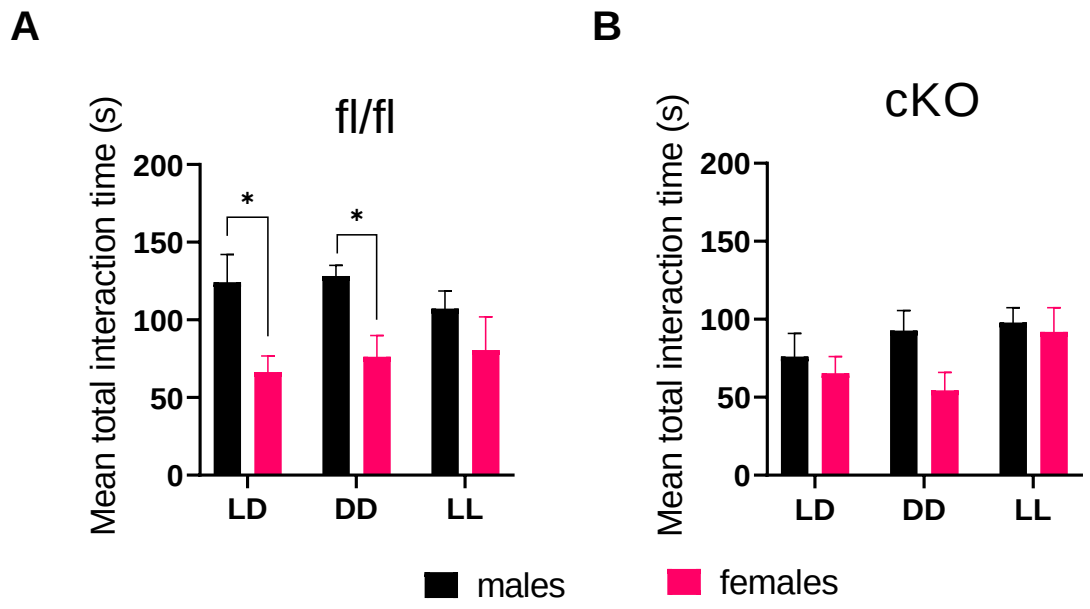


Figure 3.6: NOR test interaction differs between fl/fl sexes in LD and DD.

A) Mean total interaction time of fl/fl males (LD and DD n = 8, LL n = 7) and fl/fl females (LD n = 8, DD and LL n = 7) in all lighting conditions. Fl/fl males were significantly more interactive with the NOR test than fl/fl females in LD (*, p=0.0485) and DD (*, p=0.0227). There was a significant effect of sex on total interaction time in fl/fl mice (***, p=0.0003). B) Mean total interaction time of cKO males (LD, DD n = 8, LL n = 7) and cKO females (LD, DD n = 6, LL n = 7) in all lighting conditions. No sex differences in test interactivity were seen.

3.3.3 The effects of NPAS2 cKO and lighting condition on recognition memory

Both genotypes displayed significantly more interaction with the novel object than the familiar object in LD (Šídák's post-hoc, fl/fl: p<0.0001, cKO: p<0.0001), DD (Šídák's post-

hoc, fl/fl: p<0.0001, cKO: p=0.0003), and LL (Šídák's post-hoc, fl/fl: p=0.0028, cKO: p<0.0001). This increased interaction with the novel object was significantly above chance in both genotypes, under all lighting conditions (LD: fl/fl t(15)=5.893, p<0.0001, cKO t(13)=6.103, p<0.0001), (DD: fl/fl t(14)=7.554, p<0.0001, cKO t(12)=7.342, p<0.0001), (LL: fl/fl t(13)=2.837, p=0.0140, cKO t(13)=10.50, p<0.0001) (Figure 3.7A, 3.7B, 3.7C). This demonstrates that both genotypes remembered the familiar object in an above chance manner in LD, DD and LL. However, there were no significant differences between genotypes on time spent with either object across lighting conditions.

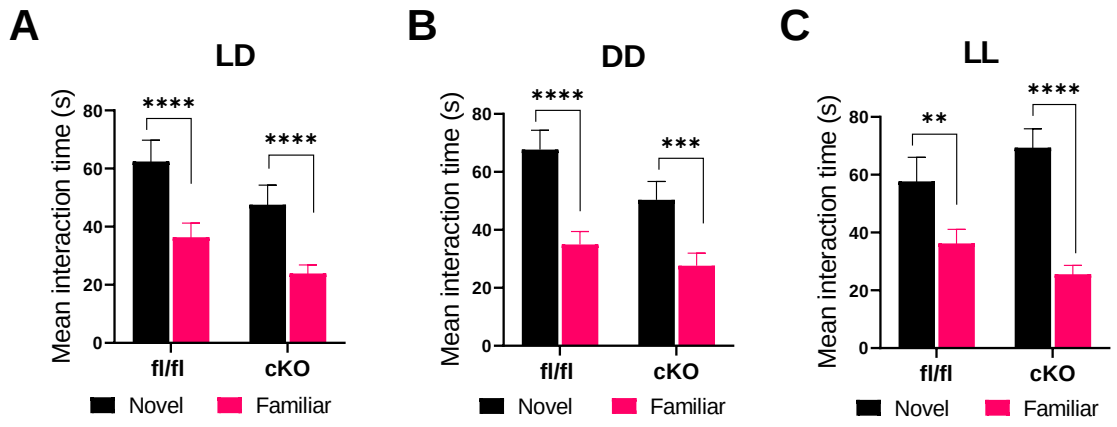


Figure 3.7: Both genotypes show a preference for novelty in all lighting conditions.

A) Mean interaction time of cKO (n = 14) and fl/fl controls (n = 16) with novel and familiar objects in LD conditions of both cohorts combined. Both genotypes performed better than chance (both: ****, p<0.0001). B) Mean interaction time of both genotypes (cKO: n = 14, fl/fl: n = 15) with novel and familiar objects in DD conditions of both cohorts combined. Both genotypes performed better than chance (both: ****, p<0.0001). C) Mean interaction time of both genotypes (cKO: n = 14, fl/fl: n = 14) with novel and familiar objects in LL conditions of both cohorts combined. Both genotypes performed better than chance (fl/fl: *, p=0.0140, cKO: ****, p<0.0001).

Memory performance was measured by DI, which either showed a positive preference for novelty, or a negative preference for familiarity. Mixed-effects analysis revealed that DI is influenced by the interaction of genotype and lighting condition (F(2,80)=6.548, p=0.0023), and also by genotype itself (F(1,80=6.141, p=0.0153). DIs revealed almost identical preferences for novelty between cKO and fl/fl controls in LD and DD, which were also

comparable across both lighting conditions, but showed a significant disparity in recognition memory in LL (Šídák's post-hoc, p=0.0001). cKO showed a significantly higher mean DI than fl/fl controls in LL, which is indicative of superior recognition memory (Figure 3.8). Intra-genotype differences revealed no significant difference in fl/fl memory performance across lighting conditions but did show that cKO had a significantly higher DI in LL than in DD (Tukey's post-hoc, p=0.0444) and LD (Tukey's post-hoc, p=0.0199) (Figure 3.8). This displays a light condition specific improved memory performance in NPAS2 cKO mice.

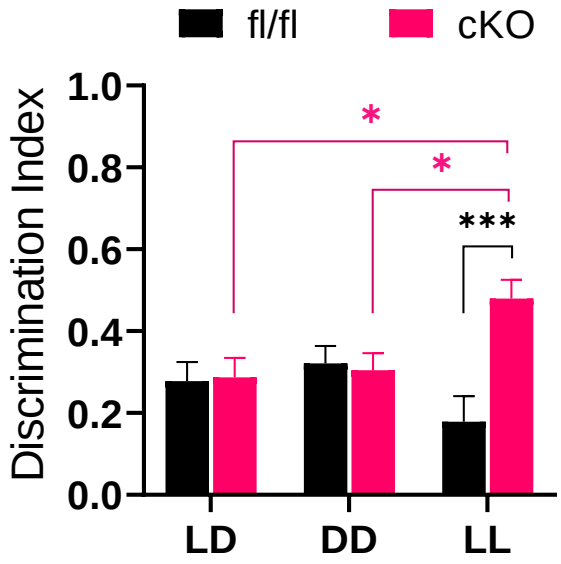


Figure 3.8: NPAS2 cKO mice showed a significantly improved memory performance in constant light.

DI of cKO (n = 14 all conditions) and fl/fl controls (LD n = 16, DD n = 15, LL n = 14) in all lighting conditions. No significant differences in DIs were seen between fl/fl and cKO mice in LD or DD. cKO mice showed a significantly higher DI in LL compared to fl/fl (***, p=0.0001). No significant differences in DIs across lighting treatment were seen in fl/fl controls. CKO mice showed a significantly higher DI in LL than DD (*, p=0.0444), and LD (*, p=0.0199).

3.3.4 The effects of sex and NPAS2 cKO on recognition memory

Previous literature has also shown object memory can vary by sex (Benice et al., 2006, Bettis and Jacobs, 2012, Bettis and Jacobs, 2013, Frick et al., 1999, Frick and Gresack, 2003). Mixed-effects analysis showed a significant sex X genotype X lighting condition interaction was seen (F(1,41)=7.366, p=0.0097), specifically showing a significantly lower DI of fl/fl females in LL compared to fl/fl males (Tukey's post-hoc, p=0.0021). Female fl/fl mice were also seen to perform significantly worse in LL compared to DD (Tukey's post-hoc, p=0.0002)

and compared to LD (Tukey's post-hoc, p=0.0430) (Figure 3.9A, 3.9B). This demonstrates significant sexual dimorphism in the recognition memory of fl/fl mice that was absent in cKO mice.

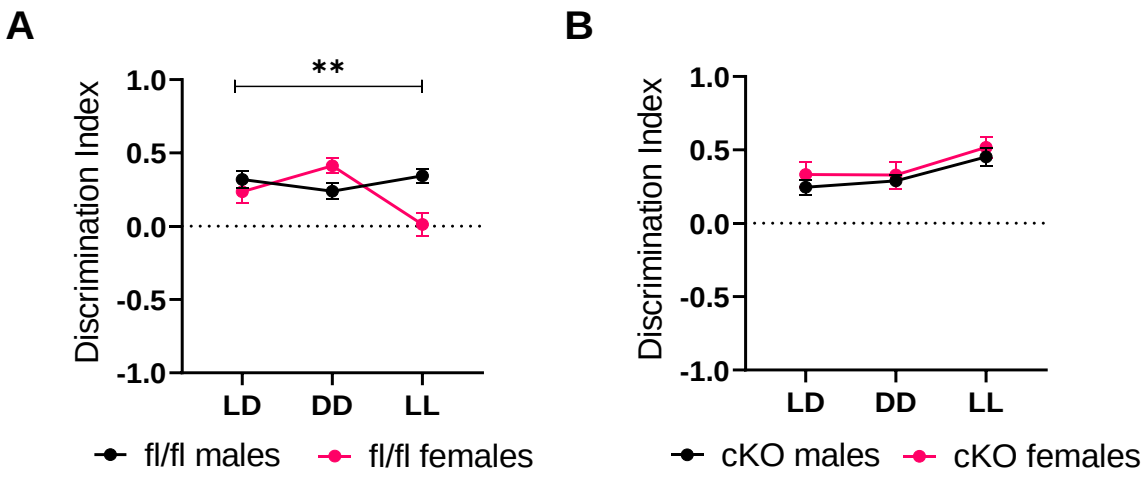


Figure 3.9: Sexual dimorphism in recognition memory was lost in cKO mice.

A) DIs of fl/fl females (LD n = 8, DD, LL n = 7) and fl/fl males (LD, DD n = 8, LL n = 7) revealed no disparities in recognition memory in LD or DD, but a significantly worse DI of fl/fl females in LL (*, p=0.0260). An interaction between lighting condition and sex on fl/fl DI was seen (**, p=0.0012). Fl/fl females also performed significantly worse in LL than in DD (***, p=0.0002) and LD (*, p=0.0430). B) DI of cKO females (LD, DD n = 6, LL n = 7) and cKO males (LD, DD n = 8, LL n = 7) showed no significant differences in any lighting condition.

The DIs of females between each genotype were then compared; no significant differences were seen in LD or DD, but fl/fl females performed significantly worse in LL compared to cKO females (Šídák's post-hoc, p=0.0001). Additionally, there was a significant interaction of lighting condition and genotype on female DI (F(2,33)=7.513, p=0.0020) (Figure 3.10A). No significant differences were seen in any condition when cKO and fl/fl male DIs were compared (Figure 3.10B).

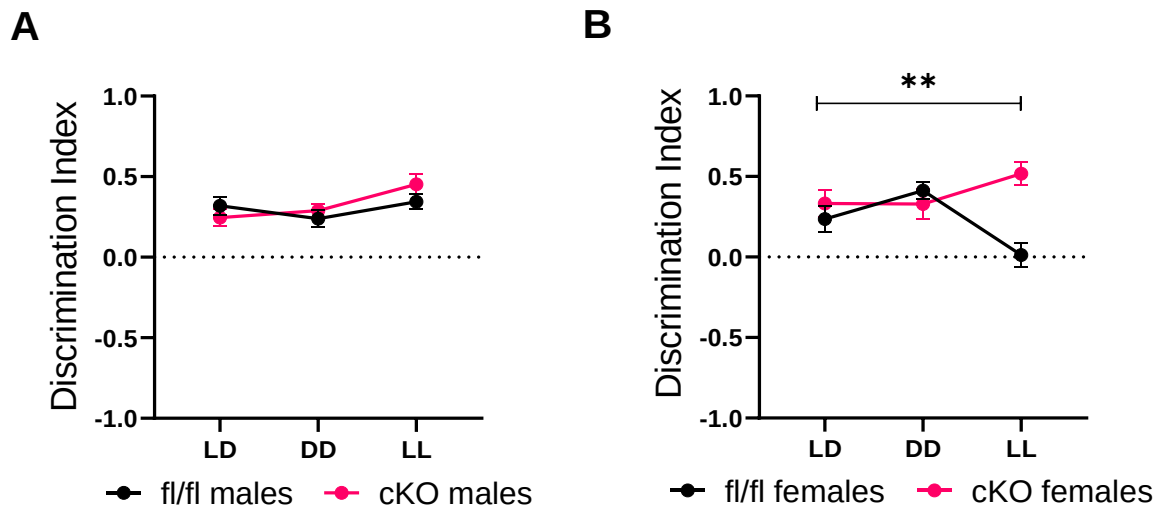


Figure 3.10: cKO females displayed superior recognition memory in LL compared to fl/fl females.

A) Fl/fl males (LD, DD n = 8, LL n = 7) and cKO males (LD, DD n = 8, LL n = 7) showed no significant differences in DI in any lighting condition. B) A significant interaction between genotype and lighting condition was seen on female DI (**, p=0.0020). Fl/fl females (LD n = 8, DD, LL n = 7) had a significantly lower DI than cKO females (LD, DD n = 6, LL n = 7) in LL (**, p=0.0050) but showed no significant differences in LD or DD.

Addendum:

This analysis could have been improved by performing three-way ANOVAs which included sex, genotype and lighting condition as variables, in replacement of two way ANOVAs individually assessing the effect of genotype and then sex. Without demonstration of an interaction between sex X genotype, any claim that the two genotypes show variable sex differences has limited statistical support.

3.4 Discussion

In summary, this work found significant sex differences in NOR test interaction between fl/fl males and fl/fl females, where males were more interactive in LD and DD, which were absent in cKO mice. Most notably, hepatic NPAS2 loss improved recognition memory performance under conditions of constant light. This effect is likely attributed to sexual

dimorphism in fl/fl recognition memory, where fl/fl females performed significantly worse than fl/fl males in LL conditions; these sex differences were similarly abolished in hepatic NPAS2 mutants.

3.4.1 Sex and genotype differences in total test interaction in cKO and fl/fl mice

The NOR task repeatedly showed an above chance performance in all genotypes, across all conditions. This confirms that the LTM NOR protocol is successful in maintaining mouse memory over three successive days and gives significant preference for novelty irrespective of external lighting conditions. Whilst NOR protocols across laboratories show considerable variability, our data supports that this protocol is reproducible and reliably evokes adequate interaction with the test and significant memory responses (Sutcliffe et al., 2007, Tam et al., 2017). Sample test analysis revealed no significant preferences for either the top left or bottom right object in either genotype, or sex groups. This showed that identical objects evoke almost identical exploration in the mice regardless of their placement, ruling out object or spatial preferences.

No significant differences were seen between genotypes in exploration time across lighting conditions. This is in contrast to previous studies which show significant differences in interaction that correlate with cognitive output and therefore suggest confounding effects of test exploration (Bettis and Jacobs, 2012, Bettis and Jacobs, 2013, Frick and Gresack, 2003). However, when the genotype groups were divided in sex sub-groups, significant differences between fl/fl and cKO males were unmasked. There is a significant influence of genotype on total test exploration in males, which showed fl/fl males to be more exploratory in the NOR paradigm. This could be indicative of increased anxiety in cKO males leading to reduced exploration of the test objects compared to fl/fl male counterparts. NPAS2 is highly expressed in areas linked to stress responses, such as the striatum, and brain regions involved in anxiety

and emotion like the basal ganglia and limbic system (Dudley et al., 2003, Garcia et al., 2000, Ozburn et al., 2017). Constitutive NPAS2 null mice demonstrate reduced anxiety in open field tests and elevated plus maze trials. Specific NPAS2 knockdown in the ventral striatum using viral vector approaches also produces anxiolytic effects (Dudley et al., 2003, Garcia et al., 2000, Ozburn et al., 2017). This could propose that loss of NPAS2 solely in the liver can have implications for global anxiety responses, though anxiety phenotypes in hepatic cKO mice must be explored further to validate this hypothesis. As well as this, it is additionally important to note that equivalent effects were not seen in females.

Furthermore, when interaction was explored between sexes, a significant effect of sex on total test exploration was seen in fl/fl controls, with specific variation in cycling light conditions and constant darkness. Fl/fl males explored the test objects significantly more so than fl/fl females in LD and DD. This is unusual since locomotor activity and actogram data robustly showed females are more active than males in general activity (data not shown), and literature also supports females being the most active of the two sexes over an average 24-hour period (Konhilas et al., 2014, Oydanich et al., 2019). This suggests the male fl/fl mice increase their activity in comparison to females within the test paradigm, irrespective of generalised locomotor activity. Additionally, previous literature has reported increased female activity and exploration in NOR tests in comparison to males, meaning the results of this study oppose those previously noted (Bettis and Jacobs, 2013, Frick and Gresack, 2003). Some discrepancies may be due to the lighting conditions employed but contrasting sex differences are also noted in normal cycling conditions. This furthers the hypothesis of increased anxiety or stress responses within female mice which could amount to a reduction in total exploration of the test objects. Female mice are generally the more anxious of the two sexes and thus females could be responding to the stress of the testing conditions, or semi-social isolation (SSI), more significantly than males (Dugandzic et al., 2019, Li et al., 2016, Marchette et al., 2018, Mulvey

et al., 2018). Still, these significant differences in test exploration are lost in constant light, which is considered the more anxiogenic of the three conditions used. This is due to both a slight reduction in male test object exploration and a slight increase in female test object exploration, making the sex differences between the two negligible. Additionally, cKO mice do not display this sexual dimorphism in total test interaction, and instead show equal levels of exploration between sexes throughout all lighting conditions. Thus, loss of superior male exploration in the cKO group generates genotype differences and abolishes sexual dimorphism in this parameter.

3.4.2 Post-natal hepatic NPAS2 deletion improves recognition memory in constant light

NOR task results revealed similar object recognition memory in both genotypes in LD and DD, with no significant differences between genotype or lighting condition. Interestingly, there were no improvements or deteriorations of recognition memory under DD conditions compared to LD, which furthers the argument that NOR paradigms are not visuospatial, since it can successfully be performed in constant darkness using non-visual methods of novelty discrimination (Tam et al., 2017). Constant darkness is seen to impair spatial memory in both Wistar rats and C57BL/6J mice undergoing various memory paradigms, though the DD period they were exposed to was in excess of four weeks and therefore memory impairments may require longer DD exposure (Jameie et al., 2015, Zhou et al., 2018). It is also important to reiterate that these paradigms explored spatial memory, as opposed to recognition memory, and discrepancies in outcome may be a result of this. Additionally, Jameie et al. employed DD conditions pre-natally until testing, which would likely have had more severe impacts than inducing DD after a period of LD cycling post-birth (Jameie et al., 2015). Also, perhaps for purposes of ease, these experiments were often performed in light conditions following DD treatment, rather than maintaining the DD conditions for the test. This could potentially induce

anxiety and/or memory failings as a result of bright light induction following long stretches of constant darkness. It is therefore not necessarily applicable to assume DD would generate NOR memory impairments based on these experiments, since they are significantly variable from the test conditions employed here. As discussed in 3.1.5, NOR tests are rarely performed in DD conditions due to the assumption they are visuospatial, and therefore there is limited data to compare these results to. Ruby et al. previously showed that NOR scores are higher during the dark phase – which correlates to an animal's 'awake' period – demonstrating robust NOR performances in darkness (Ruby et al., 2008). Nevertheless, there is no further evidence of NOR performance following elongated periods of DD.

The LL NOR task revealed a significant difference in recognition memory between cKO and fl/fl controls. cKO mice showed significantly improved DIs compared to their performance in LD and DD, whilst fl/fl showed a significantly worse performance in LL compared to cKOs. This is particularly surprising given the cKO line are a hepatic only NPAS2 knockout, inferring loss of NPAS2 in the liver can effect recognition memory performance. Fl/fl controls showed almost identical interaction with the test to cKO mice in LL, meaning their poorer memory performance was not a result of lack of engagement in the trial. It is commonly observed that LL negatively influences learning and memory in nocturnal rodents, with most noted deficits seen in spatial memory (Fujioka et al., 2011, Jameie et al., 2016, Ma et al., 2007, Zhou et al., 2018). Therefore, the reduced ability to discriminate novelty in fl/fl controls is consistent with literature, and since LL significantly affected fl/fl period and phase, it is reasonable to assume the lighting condition was successful in evoking memory impairments. This suggests that the cKO mice were less affected by the LL-induced memory deficits, and even demonstrated a significant improvement in recognition memory from prior conditions. Global NPAS2 deficient mice show deficits in long term memory, meaning results in the hepatic NPAS2 cKO model are contradictory to the global knockout (Garcia et al., 2000).

However, the paradigms used to determine cognitive outcomes between Garcia et al. and the tests performed in this study are not directly comparable (Garcia et al., 2000).

Since a NOR task does not reward the correct result nor punish incorrect responses, the cKO mice could not have been trained into discriminating the novel object better. Additionally, once results from the first cohort displayed significant differences in memory performance under LL conditions, the NOR test order was reversed in a secondary cohort to confirm this was not artefact of test order. The second cohort also displayed significant differences in recognition memory in constant light and upheld the improved performance in cKO mice. An interesting addition to this study would be a cognitive comparison to central NPAS2 loss. Future work could include examining recognition memory under LD, DD and LL conditions to investigate if NPAS2 loss in the brain replicates the improved memory performance seen in LL conditions in hepatic NPAS2 knockouts. This would determine if the effect is in fact liver specific.

3.4.3 cKO memory superiority in constant light is likely a result of fl/fl sex differences

Previous literature was inconclusive on whether NOR tests show male, or female, or no superiority in mice (Bettis and Jacobs, 2012, Bettis and Jacobs, 2013, Frick and Gresack, 2003, Jonasson, 2005). Within our study, fl/fl and cKO mice demonstrate no sex differences in normal cycling conditions. Similarly, in conditions of constant dark, no sex differences are noted in either strain. Fl/fl controls are of a C57BL/6J background, and they should demonstrate no significant phenotype of floxing, therefore this suggests no sex differences in recognition memory are present in normal cycling light conditions. This test employed familiar and novel objects that were very visually different, both in colour, texture and shape, which could explain why no sexual dimorphism was seen. Bettis and Jacobs, 2012 hypothesize that both male and female mice can perform accurate novel object discrimination if the novel object

is very dissimilar to the familiar, but that only female mice can correctly identify a 'similar' familiar object (Bettis and Jacobs, 2012). Additionally, our results were noted in singly housed mice, which is inconsistent with the hypothesis that male superior recognition memory is unveiled when males are housed singly to better reflect their natural, solitary living conditions (Bettis and Jacobs, 2013).

Despite this, conditions of constant light induced sex differences in recognition memory in fl/fl mice, where male superiority was seen in NOR tests under LL conditions. This sex effect was not seen in cKO mice. This furthers the hypothesis of increased anxiety in fl/fl females, explored in 2.4.3, which could influence both test exploration and memory performance. Though it is important to note, reduced fl/fl female test exploration was seen in LD and DD in comparison to males, and poorer memory performance in fl/fl females was seen in LL, meaning it is likely the two are not concomitant. This suggests cognitive performance in anxiogenic conditions is sex-dependent, and that hepatic NPAS2 deletion prevents this reduction in recognition memory perhaps through methods of reduced anxiety. This body of work repeatedly argues the case for use of both sexes within cognitive and circadian experiments, since significant differences are frequently found between sexes and unmasked in genotype groups when they are divided into sex sub-groups.

Fl/fl males did not show a significantly worse memory performance in LL in comparison to cKO males, meaning the reduction in fl/fl genotype DI was due to fl/fl female performance, which was not above chance levels. Since this cognitive effect was seen under conditions of constant light, it suggests a circadian response might be the cause of sexual dimorphism in recognition memory. In section 2.3.7, significant circadian sex differences in fl/fl mice were seen under constant light conditions. As discussed in 2.4.3, female fl/fl mice show a significantly longer period in LL than males, suggesting they are more significantly affected by the condition. This presents the hypothesis that *circadian* sex differences seen in

LL conditions in fl/fl mice may amount to *cognitive* sex differences within the same condition. If fl/fl females are more significantly affected by the LL condition, this may result in diminished recognition memory, since effective LL treatment has previously been noted to impair memory (Fujioka et al., 2011, Jameie et al., 2016, Ma et al., 2007, Zhou et al., 2018). This also presents a mechanism where loss of circadian sexual dimorphism in hepatic NPAS2 knockout mice leads to loss of cognitive sexual dimorphism, thus implicating liver NPAS2 in sex-dependent cognitive changes. Further assessment of central circadian changes such as brain circadian gene expression is needed to assess alterations in hepatic NPAS2 knockout male and females in comparison to fl/fl controls. Additionally, anxiety paradigms using both transgenic lines could assist understanding of how genotypes and sexes may differ in anxiety phenotype.

Chapter 4: The effects of pre- and post-natal hepatic NPAS2 loss, and sex, on anxiety

4.1 Introduction

4.1.1 *Npas2* and anxiety

Next, it was important to investigate whether improved memory in hepatic NPAS2 cKO mice in LL conditions might be explained by reduced anxiety, since LL conditions may be considered to be an anxiogenic environment. In addition to memory phenotypes which are explored in 3.1.2, NPAS2 has been shown to have a role in anxiety and mood. *Npas2* is found ubiquitously in areas that assimilate sensory information, as well as areas of the limbic system and basal ganglia, which are associated with the regulation of anxiety and emotion. NPAS2 is also abundant in brain regions implicated in behavioural responses to stressful stimuli, such as the striatum in the forebrain (Dudley et al., 2003, Garcia et al., 2000, Ozburn et al., 2017). In LD box, elevated plus maze tasks and OF, Garcia et al. demonstrated no significant anxiety phenotypes in global NPAS2 KO mice. Contrary to this, Ozburn et al., discovered significantly reduced anxiety phenotypes in *Npas2* mutant mice in all three of the above listed anxiety trials. Loss of global *Npas2* resulted in increased time spent in the open arms in elevated plus maze tests and reduced latency to enter the anxiogenic light side of the LD box (Ozburn et al., 2017). Additionally, OFs using NPAS2 knockout mice revealed an increased distance travelled in the central area and increased time spent in the central area (Ozburn et al., 2017). This suggests loss of *Npas2* has an anxiolytic effect, but since both studies are contradictory, it is unclear whether these results are reproducible. Both studies used the same three anxiety trials for their conclusions, but Garcia et al. gave little detail on methodology so direct comparison of protocols is limited (Garcia et al., 2000, Ozburn et al., 2017).

Ozburn et al. also noted that specific knockdown of *Npas2* in the ventral striatum leads to a mirrored performance of anxiety reduction as that seen in *Npas2* null mice. Additionally, both acute and chronic stress increases the levels of striatal *Npas2* expression, suggesting the circadian gene is intrinsically linked to stress responses in the striatum (Ozburn et al., 2017).

Mice deficient in the *Npas2* gene have also been shown to exhibit a behavioural lack of sensitivity to diazepam treatment when tested for motor consequences of anxiolytics. The suggested mechanism by which this occurs is via an interaction between *Npas2* and GABAergic neurotransmission within the ventral striatum (Ozburn et al., 2017, Partonen et al., 2007, Soria et al., 2010). Moreover, NAc specific siRNA knockdown of *Npas2* also amounts to reductions in anxiety (Ozburn et al., 2017, Spencer et al., 2013). As well as an anxiolytic effect, *Npas2* knockdown in the NAc abolishes diurnal mRNA expression of dopamine receptors *Drd1*, *Drd2* and *Drd3*. Since NPAS2 is upregulated in DRD1 containing neurons, which are heavily involved in learning and memory, and abolishing *Npas2* expression results in loss of rhythmic *Drd1* expression, it suggests a regulatory role for *Npas2* in cognition. (Ozburn et al., 2017). Though, despite a multitude of evidence for *Npas2* functions in anxiety, as evidenced above, the literature on global and tissue specific *Npas2* loss in anxiety trials such as LD box tests and OF trails is contradictory and limited. Given *Npas2* expression and *Npas2* SNPs show links with SAD, bipolar disorder, and clinical depression prevalence, investigating *Npas2* loss in the context of anxiety could be of interest, clinically (Geoffroy et al., 2015, Ojeda and Forero, 2014, Partonen et al., 2007, Soria et al., 2010).

4.1.2 Testing anxiety: light/dark box and open field arena

The LD box test is amongst the most popular innate anxiety tests, meaning no prior training is required to facilitate the test. It is based on approach-avoidance behaviour and measures the subject's innate aversion to bright light and unfamiliar areas – known as neophobia – and general preference for dark, sheltered areas (Bourin and Hascoët, 2003, Kulesskaya and Voikar, 2014). The experimental set-up consists of a large arena containing two chambers, one brightly illuminated, and one <10 lux and covered; these chambers are often identical in size, but occasionally protocols employ a larger dark chamber. This is based on the hypothesis that a large open field is aversive due to the sizable space, so by reducing the light

chamber size to a third of the total arena, you may prevent the induction of anxiety that is not caused by the actual behavioural paradigm itself (Bourin and Hascoët, 2003, Heredia et al., 2014, Miller et al., 2011). The subject is placed in either chamber, most commonly the light chamber, and latency to enter the opposing chamber is timed, as well as time spent in each chamber and other behavioural parameters including rearing, exploration, and defecation. Tests are often recorded using a camera mounted above the arena and can either be manually scored or analysed using a programme (Costall et al., 1989, Crawley et al., 1997, Heredia et al., 2014, Kulesskaya and Voikar, 2014).

The LD box test relies on a natural conflict where the subject is implored to spontaneously explore a new environment, but also feels exposed and has the innate urge to retreat from the anxiogenic chamber into the preferential dark chamber. The paradigm has been employed to assess the effects of anxiolytic and anxiogenic drugs. C57BL/6J mice show higher levels of exploratory behaviour and significantly more transitions between chambers when treated with diazepam (Crawley and Davis, 1982). Additional factors indicative of reduced anxiety include slower latency to enter the dark chamber and less time spent in the dark chamber (Borsini et al., 2002, Bourin and Hascoët, 2003, Kulesskaya and Voikar, 2014). The advantages of using this test is that it was specifically designed for use in mice and therefore presents no adaptation problems unlike some paradigms designed for rats. The test is also unconditioned and quick to perform, with very little experimental set-up required. However, LD box tests are extremely variable when performed across multiple different laboratories, experimental set ups and arena types. Also, when using disparate analysis techniques, an abundance of inconsistencies between data can be seen (Borsini et al., 2002, Bourin and Hascoët, 2003, Ennaceur, 2014, Heredia et al., 2014). LD box protocol and analysis discrepancies, as well as advances in understanding, may have been factors in observed

differences between anxiety phenotypes noted in NPAS2 knockout mice (Garcia et al., 2000, Ozburn et al., 2017).

Due to repeated inconsistencies in anxiety experiment results, and significant varibility in protocols between research groups, it is considered best to explore anxiety using more than one paradigm. OF are another standard test in behavioural neuroscience commonly used to phenotype anxiety levels in mice. They are performed in either a large rectangular, square, or circular arena that is previously unknown to the subject, meaning no habituation has occurred. Rodents have a robust aversion to large, brightly lit, unknown, open spaces, and therefore would consider the OF arena a dangerous environment (Choleris et al., 2001, Siebenhener and Wooten, 2015). The arena is mapped into two areas; a central area within the middle of the arena where an animal is considered more exposed, and a peripheral area closest to the walls of the arena, where the subject is more sheltered. This mapping is done either using video imaging or stencils on the arena surface, there are no physical barriers between areas. Increased time spent in the central zone of the OF arena is considered indicative of reduced anxiety, since the central zone is more exposed, and a rodent would feel more vulnerable here. Additionally, OF allow for analysis of locomotor activity and exploration (Choleris et al., 2001, Siebenhener and Wooten, 2015). The subject is placed into either the centre or the peripheral area, depending on experimental design, and then parameters such as time spent in each area and distance travelled are measured over a set period of time (often five to 10 minutes); these parameters can be measured either using automated software or by manual scoring (Sullens et al., 2021, Walsh and Cummings, 1976, Yesudhas et al., 2021). The OF arena trial is preferable for experimenters as it does not require any prior training, is quick to perform and easy to set up experimentally; it is therefore one of the most commonly used anxiety diagnostic tests performed in laboratory rodents.

Often, discrepancies in OF arena results occur between research laboratories. For example, of 52 studies assessing diazepam use in OFs, 55.8% of the literature demonstrates an anxiolytic effect of diazepam, whilst others suggest opposing results (Pádua-Reis et al., 2021). Such inconsistencies are likely to be due to protocol deviations between laboratories, which may also partially explain opposing OF results in Garcia et al. and Ozburn et al. involving NPAS2 knockout mice (Garcia et al., 2000, Ozburn et al., 2017). For more accurate comparisons between research groups, full transparency of protocol should be given, and consistent procedures should be employed.

It should also be noted that these methods of assessing anxiety in rodents may not directly translate to human behaviours. There tend to be few characteristics within mice and rats that are medically relevant to human populations, and many rodent behaviours need to be anthropomorphised in order to be applicable in human contexts. A caveat of this is that rodent data is therefore often overinterpreted. Whilst spending longer time in the centre of an OF test following diazepam treatment may indicate reduced anxiety-like behaviour in mice, it is not clear how these measures of innate anxiety in rodent models relate to clinical anxiety disorders in people (Pádua-Reis et al., 2021).

4.1.3 Anxiety under constant lighting conditions

When performing *in vivo* studies, increased anxiety induced by handling or environmental conditions can adversely affect the results of non-anxiety related trials. When previously exploring the memory of hepatic NPAS2 knockout mice in Chapter 3, anxiety was hypothesized as a factor which could alter cognition under specific lighting conditions. Hepatic NPAS2 knockouts showed enhanced recognition memory in LL, however, the specificity of the effects in constant light may suggest anxiety is the mediating factor (Coleman and Canal, 2017, Coleman et al., 2016, Lu et al., 2017, Zhang et al., 2016a, Zhang et al., 2016b). In

addition to phenotypes mentioned in 3.1.5, light deprivation conditions can evoke detrimental anxiety and depression phenotypes in many strains of rats and mice, often with more notable symptoms in females than males. In male Sprague-Dawley rats, DD leads to delayed sleep phases, reduced amplitudes of rhythmic sleep-wake cycles, depressive-behavioural phenotypes, hyperactivity in novel environments, increased anxiety, and heightened sensitivity to acute stress. Similarly, in male Wistar rats, DD induces an anxious and depressive behavioural phenotype and reduces body weight gain (González and Aston-Jones, 2006, González and Aston-Jones, 2008, Jameie et al., 2016, Marin et al., 2015, Tapia-Osorio et al., 2013). In C57BL/6J mice, DD can lead to reduced body weight, depressive phenotypes, and increased plasma corticosterone – which is often seen to be elevated in response to stress. Interestingly, this weight loss phenotype that often coincides with depressive characteristics is not seen in all studies, Namgyal et al. noted no significant weight disparities between control and DD exposed mice following three weeks of constant dark (Namgyal et al., 2021). Furthermore, other strains of mice mirror this depressive phenotype and also display reduced locomotor activity (Coleman and Canal, 2017, Coleman et al., 2016, Lu et al., 2017, Zhang et al., 2016a, Zhang et al., 2016b). This depression-like phenotype is demonstrated by increased immobility in forced swim tests, reduced wakefulness and arousal, heightened responses to stressors, modified expression of inflammatory cytokines (as seen in depression-replicating models), and a diminished sucrose preference (González, 2018, Lu et al., 2017, Zhang et al., 2016a, Zhou et al., 2018). Furthermore, DD-induced deleterious effects occur rapidly after the induction of constant darkness and many of these hallmark depressive physiological and behavioural consequences remain long-term (González, 2018, Lu et al., 2017, Zhang et al., 2016a, Zhang et al., 2016b). Anxiety effects in rats were also noted in OFs, where reduced locomotor activity and quadrant entry was seen, though other studies saw no significant differences in DD vs LD OF outcomes (Marin et al., 2015, Tapia-Osorio et al., 2013). In mice,

OFs have shown increased freezing time and reduced grooming in DD exposed mice within some studies, indicative of increased anxiety. Though, other studies noted no significant differences in anxiety parameters in elevated plus maze trials (Namgyal et al., 2021, Zhou et al., 2018).

Similarly to DD, LL has been shown to induce depression-like characteristics. Mice under LL for three or four-weeks showed reduced glucose intake and increased immobility in force swim tests and tail suspension tests, which is indicative of behavioural despair (Fonken et al., 2009, Zhou et al., 2018). However, these two studies demonstrate conflicting results on anxiety. Fonken et al. (2009) showed that, following three weeks of constant light, mice have decreased anxiety in elevated-plus maze tests. Said mice show no significant differences in LL compared to LD in OF arena parameters but have significantly more open arm entries and a significantly reduced latency to enter the open arms of an elevated plus maze (Fonken et al., 2009). Whilst Zhou et al. suggest LL causes significant increases in anxiety-like behaviours within the same tests. These mice demonstrated significantly reduced time spent in the open arms and less open arm entries in the elevated plus maze trial following four weeks of LL conditions (Castro et al., 2005, Fonken et al., 2009, Zhou et al., 2018). Similar inconsistencies are seen in studies of LL and the induction of stress; some literature suggests LL elevates stress hormones such as corticosterone, others show no significant changes, whilst some suggest LL produces reductions in stress responses and corticosterone levels. Variation in these studies may amount from differences in LL treatment duration, with shorter lengths of LL perhaps causing less severe or even downregulated stress responses (Alves-Simoes et al., 2016, Fujioka et al., 2011, Jameie et al., 2016, Ma et al., 2007, Ruby et al., 2013). Interestingly, mice housed in LL conditions have also been shown to have significantly increased body mass. This body mass increase in comparison to LD mice was evident after only seven days in constant light conditions and was maintained throughout the eight-week study (Fonken et al., 2010). To

summarise, anxiety induction under conditions of LL is variable between mice and rats, is often contradictory, and is shown to be dependent on LL treatment length.

4.1.4 Social isolation and anxiety

Throughout the course of our study, mice have been held in a form of social isolation within circadian chambers (see Figure 2.4). It is generally well-accepted that in addition to variable lighting conditions, social isolation can also induce anxiety. Mice in social isolation are normally kept in singly housed cages with no ability to interact with other mice. It has been widely reported that elongated periods of social isolation or socially isolated rearing from birth can lead to increased aggression, anxiety, depression-like characteristics, and hyperactivity (Berry et al., 2011, Ieraci et al., 2016, Koike et al., 2011, Ma et al., 2011). Whilst these findings are well-documented, the neuronal changes that underpin isolation induced phenotypes are not well understood. It is hypothesized that developmental social deprivation impairs the development of the PFC. Social isolation through adolescence can worsen plasticity in the PFC and correlates with immature dendritic spine morphology. Though, it is not known how non-developmental social isolation in adulthood also amounts to similar behavioural characteristics (Medendorp et al., 2018).

Elevated plus maze studies demonstrate significantly reduced time spent in the open arms following social isolation in mice, consistent with increased anxiety (Koike et al., 2009). Additionally, rats have demonstrated fear-like behaviour following 20-days of social isolation, or 60-days of social isolation from birth, when tested in elevated plus maze trials. They show increased time spent in enclosed arms and reduced open arm entries, both indicative of increased anxiety (Molina-Hernandez et al., 2001, de Silva et al., 1996). Additionally, anxiety phenotypes are noted in OFs following prolonged social isolation. Ieraci et al. utilised eight-week-old C57BL/6J male mice whom, following one week of normal social housing, were

either socially isolated or group housed for 28 days (Ieraci et al., 2016). Isolated mice displayed a reduced time spent in the centre area of an OF compared to group housed mice, which indicates an increase in anxiety (Ieraci et al., 2016). Similarly, significantly reduced time in the central area of an OF arena was seen in male NMRI mice following social isolation. These mice also demonstrated increased immobility in forced swim tests and increased distance travelled in the OF arena (Haj-Mirzaian et al., 2019). Hyperactivity and increased distance travelled in OFs has been noted in multiple studies using rodents isolated for five days or longer (Bakshi and Geyer, 1999, Haj-Mirzaian et al., 2019, Heidbreder et al., 2000, Hilakivi et al., 1989, Ieraci et al., 2016, Molina-Hernandez et al., 2001).

Similar results of increased anxiety were noted in LD box tests in socially isolated adult mice. Interestingly, Rivera-Irizarry et al. demonstrate that both male and female adult mice with six weeks of social isolation spend less time in the light chamber of a LD box. This is contrary to adolescent mice which demonstrate no significant differences in anxiety parameters between group and single housing (Rivera-Irizarry et al., 2020). Though, LD box tests following social isolation tend to depict an anxiolytic effect of social isolation, as demonstrated by reduced time spent in the dark chamber for both male and female isolated Swiss-Kunming mice. This anxiolytic effect was maintained from week one to week four of single housing (Guo et al., 2004). Similarly, Lopez and Laber employed male and female C57BL/6J mice socially isolated from weaning to demonstrate a reduced time spent in the dark side of a LD box, indicative of a reduction in anxiety (Lopez and Laber, 2015). This furthers the imperative to employ more than one measure to diagnose robust anxiety phenotypes.

As well as anxiety phenotypes, socially isolated mice increased behaviours of aggression such as attacking, biting, wrestling, tail rattling and aggressive grooming when met with an ‘intruder’ mouse (Koike et al., 2009). This may be due to natural male hierarchies which are formed amongst male cage-mates; removal of these hierarchies by social isolation

could present the ‘intruder’ as a threat to dominance in the single-housed environment, and therefore instigate aggressive behaviours. This hyper-aggression phenotype is robustly demonstrated in mice following social isolation, for even as little as seven days (Lagerspetz and Lagerspetz, 1971, Locci et al., 2017, Rodgers and Cole, 1973, Tan et al., 2019). Some of these studies also note reduced immobility in tail suspension tests, indicative of behavioural despair and depression-like characteristics following social isolation (Ieraci et al., 2016). Furthermore, often significant weight loss or weight gain can be seen following conditions of prolonged stress. Social isolation can lead to less body weight gain compared to group housed individuals over the same time period, and even significant weight loss in aged female and male mice (Abramov et al., 2004, Guo et al., 2004, Yamada et al., 2015).

Significantly, housing conditions are seen to have effects on novel object recognition memory. Voikar et al., demonstrated seven weeks of isolation in C57BL/6J males gives impaired novel object recognition (Voikar et al., 2015). Similarly, other studies suggest long-term spatial, social and recognition memory cannot be adequately formed, or is significantly impaired, following even a few days in socially deprived conditions (Almeida-Santos et al., 2019, Huang et al., 2011, Leser and Wagner, 2015). Overall, despite the abundance of literature on social isolation effects, there is a lot of variability of developmental stage, age of mice, sex, length of isolation and strains of rodent. As a result, experimental outcomes vary significantly between research groups.

4.1.5 Aims and Objectives

Following the discovery of significant NOR differences in controls compared to hepatic NPAS2 knockouts, it was hypothesized that there may be significant differences in anxiety between fl/fl and cKO lines, and sexes. To investigate if there were any significant differences in baseline anxiety levels between genotypes and sexes, OF and LD box tests were employed.

This aimed to investigate whether females demonstrated increased anxiety in comparison to males, as has been demonstrated in other strains, and also to explore whether hepatic NPAS2 loss can alter basal levels of anxiety (Dugandzic et al., 2019, Li et al., 2016, Marchette et al., 2018, Mulvey et al., 2018). Previously, global *Npas2* loss has been shown to reduced anxiety. Since our previous experiments have demonstrated hepatic NPAS2 loss is capable of causing recognition memory change, it was also of interest to assess if hepatic NPAS2 loss could influence anxiety, perhaps as a means by which cognitive change occurs (Ozburn et al., 2017).

Secondly, this work investigated if the significant sex-linked weight loss, which was noted in both circadian and cognitive experiments, could be a product of the experimental set up in which semi-social isolation (SSI) is employed. SSI consists of individually housed mice within a chamber containing four cages. This means each mouse can see, hear, and smell three other mice but cannot physically interact with them. To investigate if SSI induced anxiety during previous studies, OF and LD box texts were employed to evaluate differences in induced anxiety levels between cKO and fl/fl, and Afp cKO and Afp fl/fl mice. Genotype groups were also evaluated at the level of sex to assess if weight loss could be induced in a sex-linked manner due to disproportionate effects of anxiety. The hypothesis was that changes in living conditions and subsequent isolation was inducing a sex-specific increase anxiety in all genotypes of female mice, which was significant enough to result in weight loss. This is based on the robust finding that isolation and social deprivation in nocturnal rodents can amount to substantial anxiety phenotypes, which can in turn amount to changes in weight (Abramov et al., 2004, Guo et al., 2004, Yamada et al., 2015).

Finally, following significant changes in NOR output specifically under conditions of SSI and LL, this work aimed to assess if LL or prolonged SSI induces anxiety responses between genotypes and/or sexes. This was measured using open field arena and light/dark box trials following LL SSI, and extended SSI to match the duration of isolation experienced by

mice within the cognitive trials. This was based on the hypothesis that LL negatively influences cognition in fl/fl mice through means of increased anxiety, as previously demonstrated in other genotypes (Fujioka et al., 2011, Jameie et al., 2016, Ma et al., 2007, Zhou et al., 2018).

4.2 Materials and Methodology

4.2.1 Animals for LD box tests

A total of 24 cKO mice and 24 fl/fl mice, and a total of 18 Afp cKO and 18 Afp fl/fl mice were used for LD box experiments (see Figure 2.3 for genotype explanation). These groups were divided into two sub-groups; one that would be tested in group housing (hereafter referred to as no SSI), and one that would be tested under SSI conditions. The n number chosen for this study was based on previously published literature. Additionally, these were the final *in vivo* experiments performed with these colonies and therefore n numbers were also restrained by the last litter sizes. Previous LD box tests of this nature utilised subject group sizes between n = 5 and n = 18, with the most common n number per group being n = 8 (Bouayed et al., 2007, De Moraes Pultrini et al., 2006, Heredia et al., 2014, Kulesskaya and Voikar, 2014, Malmberg-Aiello et al., 2002, Park et al., 2021, Walia et al., 2019). All animals used in the following experiments were between three and six months of age, and individual experiments had age matched cohorts. Age matched C57BL/6J wild type mice were purchased from Charles River Labs UK and were acclimatized to the animal unit for at least seven days before being used in experiments.

	cKO	fl/fl
SSI	n = 13 (5 female, 8 male)	n = 12 (6 female, 6 male)
No SSI	n = 11 (5 female, 6 male)	n = 12 (5 female, 7 male)

Table 4.1: n numbers of cKO and fl/fl mice used in LD box tests.

	Afp cKO	Afp fl/fl
SSI	n = 8 (4 female, 4 male)	n = 8 (4 female, 4 male)
No SSI	n = 10 (4 female, 6 male)	n = 10 (5 female, 5 male)

Table 4.2: n numbers of Afp cKO and Afp fl/fl mice used in LD box tests.

4.2.2 Animals for OF arena tests

A total of 19 Afp cKO and 17 Afp fl/fl mice were used in OF (see Figure 2.3 for genotype explanation). In Afp mice, these groups were divided into two sub-groups; one that would be tested in no SSI, and one that would be tested following seven days of SSI. The n number chosen for this study was based on previously published literature. Additionally, these were the final *in vivo* experiments performed with these colonies and therefore n numbers were also restrained by the last litter sizes. Previous OF of this nature utilised subject group sizes between n = 6 and n = 20, with most common group sizes between n = 7 and n = 9 (Kalueff et al., 2006, Pairojana et al., 2022, Strutz et al., 2018, Sullens et al., 2021, Takahashi et al., 2020, Yesudhas et al., 2021).

	Afp cKO	Afp fl/fl
SSI	n = 8 (4 female, 4 male)	n = 8 (4 female, 4 male)
No SSI	n = 11 (5 female, 6 male)	n = 9 (4 female, 5 male)

Table 4.3: n numbers of Afp cKO and Afp fl/fl mice that underwent OF.

A total of 46 cKO mice and 46 fl/fl mice were used in OF. In Alb mice, these groups were divided into four sub-groups; one that would be tested in no SSI conditions, one that would be tested following seven days of SSI in normal cycling light conditions, one that would be tested following seven days of SSI in constant light conditions (hereafter referred to as LL SSI), and one that would be tested following four weeks of SSI in variable lighting conditions

(LD for five days, DD for 10 days, LL for 10 days, and then seven days of LD before testing) (hereafter referred to as extended SSI). This was to replicate the elongated isolation and variable lighting conditions mice underwent in NOR experiments in 3.2.4. LL SSI was employed to replicate the most anxiogenic lighting condition faced during the NOR experiments in 3.2.4, were variable cognition between genotypes was seen. All animals used in the following experiments were between three and six months of age, and individual experiments had age matched cohorts. Age matched C57BL/6J wild type mice were purchased from Charles River Labs UK and were acclimatized to the animal unit for at least seven days before being used in experiments.

	cKO	fl/fl
SSI	n = 14 (6 female, 8 male)	n = 12 (6 female, 6 male)
No SSI	n = 16 (5 female, 11 male)	n = 18 (5 female, 13 male)
LL SSI	n = 8 (4 female, 4 male)	n = 8 (4 female, 4 male)
Extended SSI	n = 8 (4 female, 4 male)	n = 8 (4 female, 4 male)

Table 4.4: n numbers of cKO and fl/fl mice that underwent OF.

4.2.3 *In vivo* husbandry and general care

As described in 2.2.2. In addition to *ad libitum* dry food, diet was supplemented with ~3.5 g 2019s Teklad ‘mash’ (Envigo) daily to prevent further weight loss. The ‘mash’ is similar in nutritional makeup to the main diet but is soaked in water for ease of consumption. ‘Mash’ was provided to all animals, three times a week, for the duration of this experiment.

4.2.4 Experimental set up and lighting conditions for LD box tests

For LD box tests utilising SSI, mice were individually housed in circadian chambers. Chambers, lighting and recording equipment are as described in 2.2.3.

4.2.5 Experimental set up and lighting conditions for OF

For OF following any form of SSI under normal or constant lighting conditions, mice were individually housed in circadian chambers. Chambers, lighting and recording equipment are as described in 2.2.3.

4.2.6 LD box test protocol

The LD box protocol was based on similar tests performed in the literature (Costall et al., 1989, Heredia et al., 2014, Kulesskaya and Voikar, 2014, Miller et al., 2011). A clear, acrylic arena (16 cm x 19 cm x 26 cm) was split into two, equal chambers. One chamber was brightly lit (150 lux) and the other chamber was dark (<10 lux) and covered. There was a small opening between the two chambers for the mouse to transition. Mice were placed in the light chamber of the LD box, and activity was recorded for five minutes. Parameters recorded include: time spent in light chamber, time spent in dark chamber, number or transitions, latency to dark chamber, false entries (stretches from one chamber into the other, where not all four feet enter) and defaecation (Crawley et al., 1997, Heredia et al., 2014, Kulesskaya and Voikar, 2014). All testing occurred between 10:00 am and 12:00 pm to reduce any impact of time of day. The test arena was washed in apple cider vinegar to remove any odour cues, and 70% ethanol was used for sterilisation.

4.2.7 LD box analysis

These parameters were analysed by manual timing/counting based on recordings of the LD box trials. Time spent in each chamber was timed, and number of entries into the opposing chamber and false entries (two or more feet entering the opposing chamber) were tallied. Recordings were analysed by two blinded assessments. Inter-rater reliability measured by Pearson's correlation coefficient maintained >99% average, additionally, a very high level of consistency was observed using Cronbach's alpha (>0.99).

4.2.8 OF arena trial protocol

The OF arena trial protocol was based on similar tests performed in the literature (Sullens et al., 2021, Yesudhas et al., 2021). A clear, acrylic arena (16 cm x 19 cm x 26 cm) was divided into two sections, a central area and a peripheral area. The central area was a third of the total arena size, directly in the centre of the arena. Mice were released into the central area and were recorded navigating the arena for five minutes. Time spent in each area was documented. Distance travelled was also tracked using EthoVision (Noldus EthoVision, Wageningen, Netherlands, versions 1.96 through XT11.5). All testing occurred between 10:00 am and 12:00 pm, to reduce any impact of time of day. The test arena was washed in apple cider vinegar to remove any odour cues, and 70% ethanol was used for sterilisation.

4.2.9 OF arena analysis

These parameters were measured by manual timing/counting based on recordings of the OF. Time spent in each area was manually timed and total distance travelled within the five-minute test was analysed by EthoVision (Noldus EthoVision, Wageningen, Netherlands, versions 1.96 through XT11.5). Manual timing of time spent in each area was timed by two blinded assessments. Inter-rater reliability measured by Pearson's correlation coefficient maintained >80% average, additionally, a very high level of consistency was observed using Cronbach's alpha (>0.93).

4.2.10 Statistical analysis

Power calculations were performed in Gpower and were calculated for two-way ANOVAs with genotype and sex as factors (Faul et al., 2007). Power calculations were performed in Gpower and were calculated for two-way ANOVAs with genotype and sex as factors (Faul et al., 2007). Using a Cohen's f of 0.8 based on a large effect size, for an ANOVA

alpha value of 0.05, power of 0.85 with two groups, and a numerator df of one, a total of 17 animals equal to eight to nine per genotype would be needed. To include sex as a variable, using a Cohen's f of 0.8 based on a large effect size, for an ANOVA alpha value of 0.05, a power of 0.9 with four groups, and a numerator df of three, a total of 24 animals equal to six per sex would be needed.

All statistics were performed in Prism (GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). All data was graphed in Prism. All data was tested for normality and homogeneity of variance on SPSS (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp), and all data is shown as mean ± SEM, unless otherwise stated. Three-way ANOVA was performed to analyse distance travelled between isolation conditions and sex sub-groups within genotype groups. Two-way ANOVA was performed to analyse; latency to enter dark and time spent in the dark chamber in multiple conditions. Mixed-effects analysis was used to compare weight of all genotypes. Tukey's and Sidak's post-hoc tests were used for individual multiple comparisons following ANOVA analysis. A probability value of $p<0.05$ was used as the criteria to determine statistical significance with $p<0.05$ as '*', $p<0.01$ as '**', $p<0.001$ as '***', and $p<0.0001$ as '****'.

4.3 Results

4.3.1 Significant sex-linked weight loss in Afp fl/fl and Afp cKO mice following SSI

As discussed in 2.2.2, during circadian and cognitive experiments, animals were weighed weekly in line with Home Office ASPA standards. Within the experimental circadian chambers, the mice were under SSI – meaning they were able to see, hear and smell three other mice, but they could not interact with them. Following the separation from group housing into SSI, a severe weight loss was noticed in both Afp genotypes within the first seven days. This

was most prominent in the females of both Afp transgenic lines but was not seen in replicated experimental conditions with C57BL/6J wild type mice. Thus, part of the rationale for investigating anxiety in these transgenic lines came from the discovery of this weight loss phenotype.

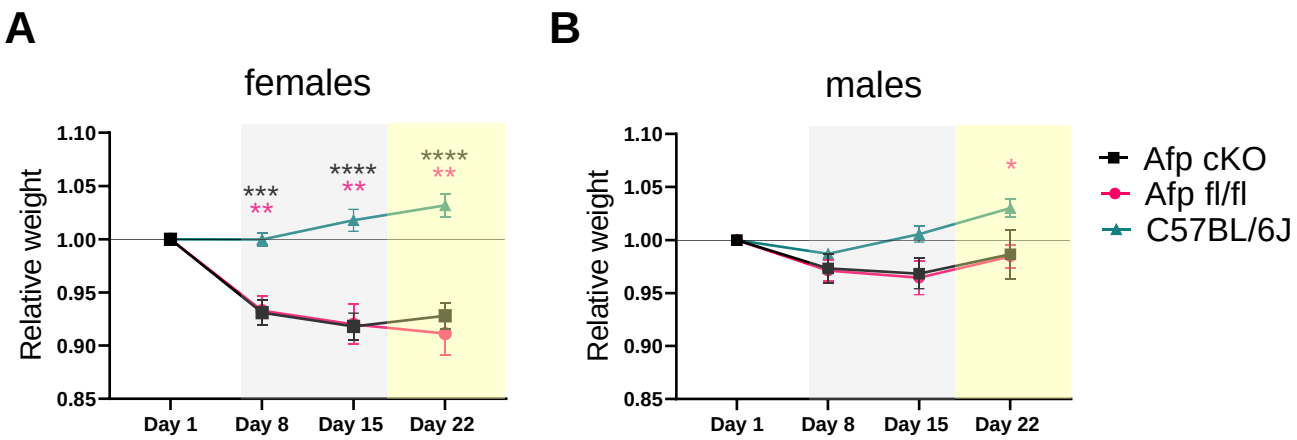


Figure 4.1: SSI induced weight loss in Afp fl/fl and Afp cKO was more severe in female mice.

A) Relative weight loss of all Afp cKO (n = 10) and Afp fl/fl females (n = 7) compared to a separate cohort of C57BL/6J females (n = 8). Afp cKO females showed significantly lower weight than C57BL/6J females on Day 8, Day 15 and Day 22 (***, p=0.0004, ****, p<0.0001, ****, p<0.0001, respectively) and Afp fl/fl females showed significantly lower weight than C57BL/6J females on Day 8, Day 15 and Day 22 (**, p=0.0047, **, p=0.0031, **, p=0.0014, respectively). Genotype had a significant influence on weight (****, p<0.0001). B) Relative weight loss of Afp cKO (n = 6) and Afp fl/fl (n = 9) males compared to a separate cohort of C57BL/6J males (n = 8). C57BL/6J males only differed from Afp fl/fl males on Day 22 (*, p=0.0142).. Genotype had a significant influence on weight (*, p=0.0306). Lighting condition changes are indicated using colour blocks: white refers to LD cycling, grey refers to DD and yellow refers to LL

In the first seven days, Afp fl/fl females lost an average of 6.62% of their body weight and Afp cKO females lost an average of 6.91%. Afp fl/fl males lost an average of 2.86% and Afp cKO males lost an average of 2.77% of their body weight. This initial weight loss was during LD cycling, before experimental lighting conditions begin on Day 7. Males of both transgenic genotypes had almost identical weight loss, as did females of both transgenic genotypes, though females showed far more severe weight loss than males. Two-way ANOVA analysis of just the transgenic lines revealed sex significantly affected weight loss

($F(3,120)=4.971$, $p=0.0028$), but there was no implication of genotype. Mixed-effects analysis between all genotypes of females, including C57BL/6J females, revealed genotype significantly affected weight loss ($F(2,29=23.61)$, $p<0.0001$). C57BL/6J females had a significantly higher weight than Afp cKO females on Day 8, Day 15, and Day 22 (Tukey's post-hoc, $p=0.0004$, $p<0.0001$, $p<0.0001$, respectively) and Afp fl/fl females on Day 8, Day 15 and Day 22 ($p=0.0047$, $p=0.0031$ and $p=0.0014$, respectively). Genotype also had a significant effect on weight in males when all genotypes, including C57BL/6J, were compared ($F(2,29)=3.943$, $p=0.0306$), with a significant difference between C57BL/6J and Afp fl/fl males on Day 22 (Tukey's post-hoc, $p=0.0142$). Weight marginally recovered in Afp cKO males and females, and Afp fl/fl males at Day 22 after LL conditions began, though it continued to drop in Afp fl/fl females (Figure 4.1).

4.3.2 Significant sex-linked weight loss in fl/fl and cKO mice following SSI

After clear sex-linked weight loss was noticed in Afp cKO and Afp fl/fl mice under SSI, weight was closely monitored in cKO and fl/fl mice. The same pattern of weight loss was observed in these experiments, with more severe weight loss in female mice of both transgenic lines. The weight loss in cKO and fl/fl experiments was also compared to C57BL/6J controls under identical conditions (Figure 4.2).

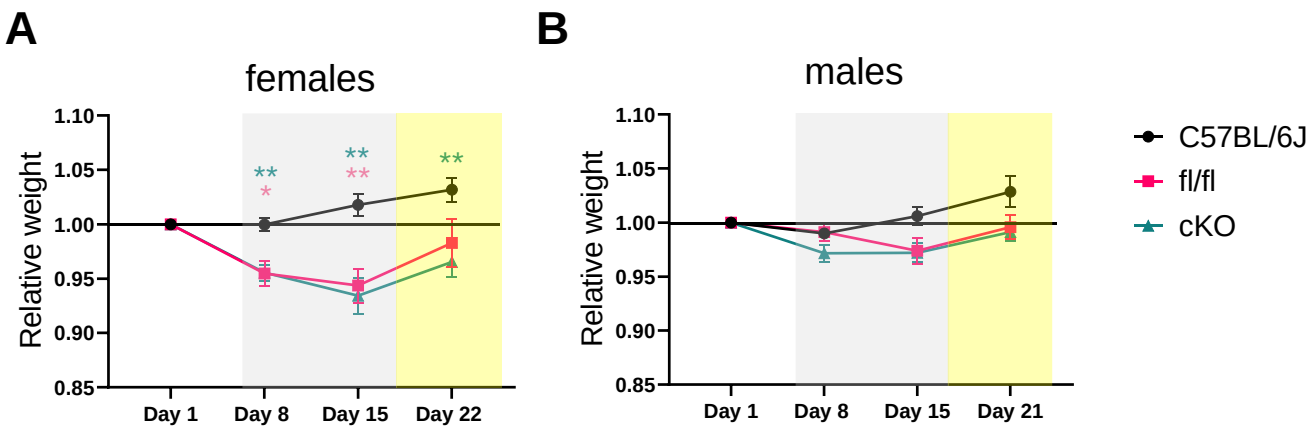


Figure 4.2: SSI induced weight loss was only present in fl/fl and cKO female mice.

A) Relative weight loss of all cKO (n = 8) and fl/fl females (n = 8) compared to a separate cohort of C57BL/6J females (n = 8). cKO females showed significantly lower weight than C57BL/6J females on Day 8, Day 15 and Day 22 (**, p=0.0012, **, p=0.0031, **, p=0.0062, respectively) and fl/fl females showed significantly lower weight than C57BL/6J females on Day 8 and Day 15 (*, p=0.0128, **, p=0.0048, respectively). B) Relative weight loss of cKO (n = 8) and fl/fl (n = 8) males compared to a separate cohort of C57BL/6J males (n = 8). No significant differences were seen between any genotypes. Lighting condition changes are indicated using colour blocks: white refers to LD cycling, grey refers to DD and yellow refers to LL.

In the first seven days, fl/fl females lost an average of 4.52% of their body weight and cKO females lost an average of 4.45%. Fl/fl males lost an average of 0.90% and cKO males lost an average of 2.84% of their body weight. This weight loss was during LD cycling, before experimental lighting conditions begin on Day 7. Two-way ANOVA analysis comparing all genotypes of males, including C57BL/6J males, showed no significant differences between any genotypes, over any days measured. Comparing all genotypes of females, including C57BL/6J females, revealed genotype had a significant influence on weight loss (F(2,21)=9.006, p=0.0015), specifically detailing differences between C57BL/6J female controls with cKO females on Day 8, Day 15 and Day 22 (Tukey's post-hoc, p=0.0012, p=0.0031, p=0.0062, respectively), and fl/fl females on Day 8 and Day 15 (Tukey's post-hoc, p=0.0128, p=0.0048, respectively). There were no significant differences between cKO males and fl/fl males, nor between cKO females and fl/fl females. Additionally, there were no significant sex differences in weight loss between fl/fl male and females or cKO male and females. Weight in all four transgenic groups improved on Day 22, after LL conditions began (Figure 4.2). These results, taken with those in 4.3.1, demonstrate sex-linked weight loss irrespective of hepatic NPAS2 loss, that are not seen in the background strain C57BL/6J and that occur before experimental lighting conditions begin. The likely trigger of this weight loss is therefore SSI conditions, which may induce anxiety sufficiently enough to alter weight.

4.3.3 Latency to enter the dark and time spent in the light chamber showed effects of sex and genotype in Afp transgenic lines

To investigate phenotypes of anxiety that may have implications on weight maintenance, two cohorts of Afp cKO and Afp fl/fl mice underwent LD box trials following either no SSI, or seven days of SSI. Firstly, latency to enter the dark chamber was measured. Latency to enter dark refers to the amount of time it takes the subject to enter the dark chamber after being placed in the light chamber (Crawley et al., 1997, Heredia et al., 2014, Kulesskaya and Voikar, 2014).

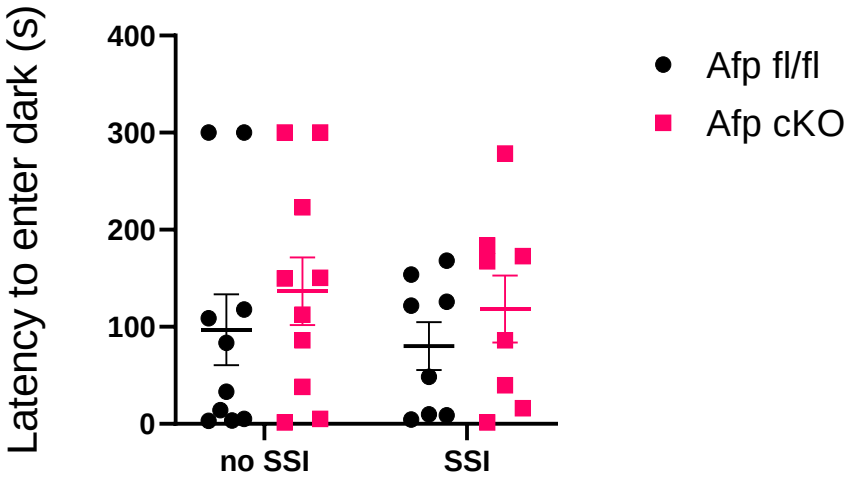


Figure 4.3: No significant differences in latency to enter dark were seen between genotypes, or across isolation conditions in Afp lines.

Latency to enter the dark chamber for Afp cKO mice (no SSI n = 10, SSI n = 8) and Afp fl/fl mice (no SSI n = 10, SSI n = 8). No differences between genotypes or isolation condition were found.

Two-way ANOVA analysis revealed no significant effects of genotype or SSI, nor a genotype X isolation condition effect on latency to enter dark (interaction ($F(1,32)=0.0005582$, $p=0.9814$) (Figure 4.3). To look at this parameter further, genotype groups were divided in sex sub-groups. There was also no significant effects of sex, isolation, or an interaction between the two on Afp fl/fl or Afp cKO latency to enter dark (interaction ($F(1,14)=0.3395$, $p=0.5694$), ($F(1,14)=2.521$, $p=0.1347$), respectively) (Figure 4.4A, 4.4B). Nor was there any significant

interactions between genotype and isolation on male or female latency to enter dark ((*F*(1,15)=0.3537, *p*=0.5609), (*F*(1,13)=0.2608, *p*=0.6181), respectively). However, there was a significant effect of genotype on male latency to enter dark (*F*(1,15)=6.189, *p*=0.0251). Afp cKO males had a longer latency to enter the dark chamber in both no SSI and SSI conditions, indicative of reduced anxiety following hepatic NPAS2 loss in males (Figure 4.4.C, 4.4D).

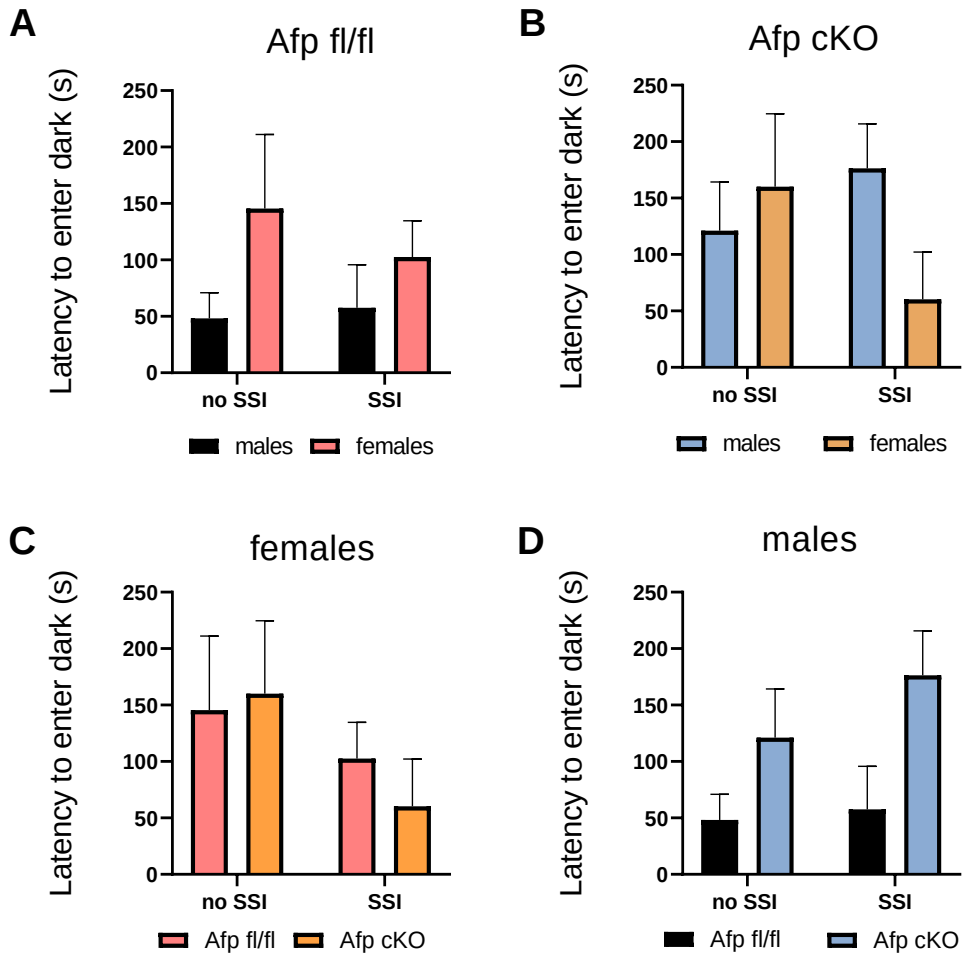


Figure 4.4: Significant effect of genotype on male latency to enter dark in Afp lines.

A) Latency to enter the dark chamber for Afp fl/fl mice in no SSI (females n = 5, males n = 5) and in SSI (females n = 4, males n = 4). B) Latency to enter the dark chamber for Afp cKO mice in no SSI (females n = 4, males n = 6) and in SSI (females n = 4 and males n = 4). C) Latency to enter the dark chamber for females in no SSI (Afp fl/fl n = 5, Afp cKO n = 4) and in SSI (Afp fl/fl n = 4, Afp cKO n = 4). D) Latency to enter the dark chamber for males in no SSI (Afp fl/fl n = 5, Afp cKO n = 6) and in SSI (Afp fl/fl n = 4, Afp cKO n = 4). Genotype had a significant effect on latency to enter the dark in males across conditions (*, *p*=0.0251).

The second parameter analysed in the LD box test was the time spent in the light chamber versus time spent in the dark chamber. Two-way ANOVA revealed there was a significant effect of chamber on exploration time in both no SSI and SSI conditions (($F(1,36)=45.48$, $p<0.0001$), ($F(1,28)=33.64$, $p<0.0001$), respectively). Both genotypes showed a robust preference for the light chamber in SSI (Šídák's post-hoc, both $p<0.0001$) and no SSI (Šídák's post-hoc, Afp fl/fl $p=0.0005$, Afp cKO $p=0.0047$) (Figure 4.5A, 4.5B). Time spent in each chamber did not show a genotype effect in either condition.

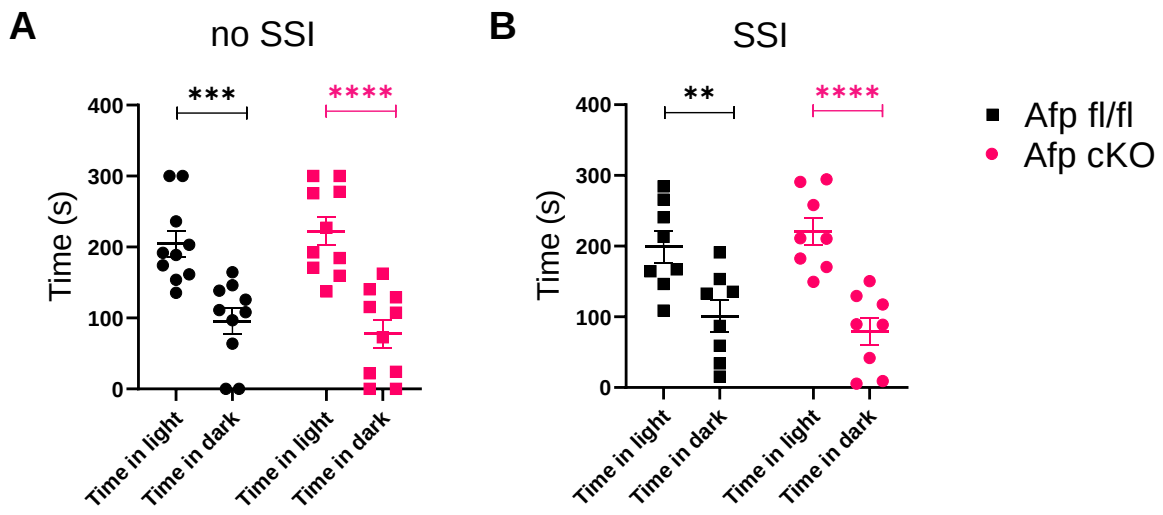


Figure 4.5: A significant preference for the light chamber was seen in both Afp fl/fl and Afp cKO irrespective of housing condition.

A) Time spent in both the light and dark chamber of Afp fl/fl (n = 10) and Afp cKO (n = 10) in no SSI. Afp fl/fl and Afp cKO spent significantly more time in the light chamber than the dark chamber in no SSI (***, $p=0.0005$, ****, $p<0.0001$, respectively). B) Time spent in both the light and dark chamber of Afp fl/fl (n = 8) and Afp cKO (n = 8) in SSI. Afp fl/fl and Afp cKO spent significantly more time in the light chamber than the dark chamber in SSI (**, $p=0.0047$ and ****, $p<0.0001$, respectively).

To look at this parameter further, genotype groups were divided in sex sub-groups as a supplementary analysis. Time spent in the light chamber did not show a significant interaction of sex and isolation for either Afp fl/fl or Afp cKO (($F(1,14)=0.04702$, $p=0.8315$), ($F(1,14)=2.431$, $p=0.1413$), respectively). However, there was a significant effect of sex on

time spent in the light chamber in Afp fl/fl mice across conditions ($F(1,14)=0.04918$, $p=0.0316$). Afp fl/fl males consistently showed a lower time spent in the light chamber compared to Afp fl/fl females (Figure 4.6A, 4.6B).

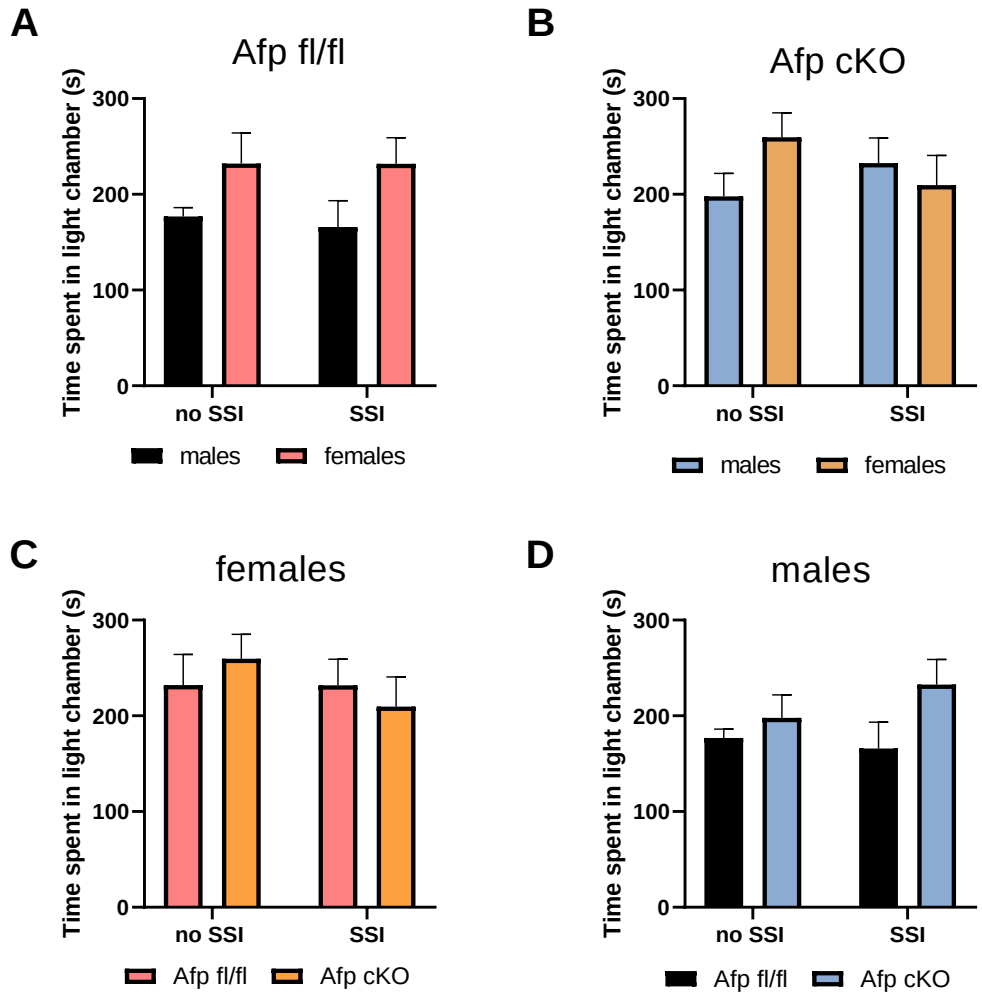


Figure 4.6: Significant sex effect on time spent in the light chamber for Afp fl/fl mice.

A) Time spent in the light chamber for Afp fl/fl mice in no SSI (females n = 5, males n = 5) and under SSI conditions (females n = 4, males n = 4). A significant influence of sex was seen on time spent in the light chamber across conditions (*, $p=0.0316$). B) Time spent in the light chamber for Afp cKO mice in no SSI (females n = 4, males n = 6) and under SSI conditions (females n = 4 and males n = 4). C) Time spent in the light chamber for females in no SSI (Afp fl/fl n = 5, Afp cKO n = 4) and under SSI conditions (Afp fl/fl n = 4, Afp cKO n = 4). D) Time spent in the light chamber for males in no SSI (Afp fl/fl n = 5, Afp cKO n = 6) and under SSI conditions (Afp fl/fl n = 4, Afp cKO n = 4).

4.3.4 LD box chamber transitions and aborted entries were increased in Afp fl/fl and Afp cKO following SSI

An additional parameter measured in LD box trials is the numbers of transitions between chambers and the number of false entries – where a mouse fails to fully enter the opposing chamber and retreats (Crawley et al., 1997, Heredia et al., 2014, Kulesskaya and Voikar, 2014). Transitions are considered an index of activity and exploration. Increased transitions is indicative of heightened exploratory behaviour. Frequent alternation between chambers suggests the subject is inquisitive of both and is not hiding in the dark chamber for prolonged periods of time. Reduced transitions may therefore a key indicator of anxiety within this test (Bourin and Hascoët, 2003, Kulesskaya and Voikar, 2014). Aborted entries are also of interest, these are defined as less than all four paws entering a specific chamber, followed by a retreat or avoidance responses such as stretching. Increased aborted attempts to aversive areas demonstrates heightened anxiety, and reduced aborted entry attempts represents the converse (Griebel et al., 1996, Bourin and Hascoët, 2003).

The number of transitions demonstrated a significant effect of isolation condition ($F(1,32)=7.339$, $p=0.0107$). This effect showed a general increase in number of chamber transitions following SSI treatment (Figure 4.7A). Additionally, there was a significant effect of isolation condition on the number of aborted entries ($F(1,32)=9.874$, $p=0.0036$). This demonstrated a general increase in number of aborted entries following SSI treatment, and a specific significant increase in aborted entries in Afp fl/fl mice between no SSI and SSI (Šídák's post-hoc, $p=0.0319$) (Figure 4.7B). This is contradictory as one characteristic implies reduced anxiety whilst the other infers increased anxiety.

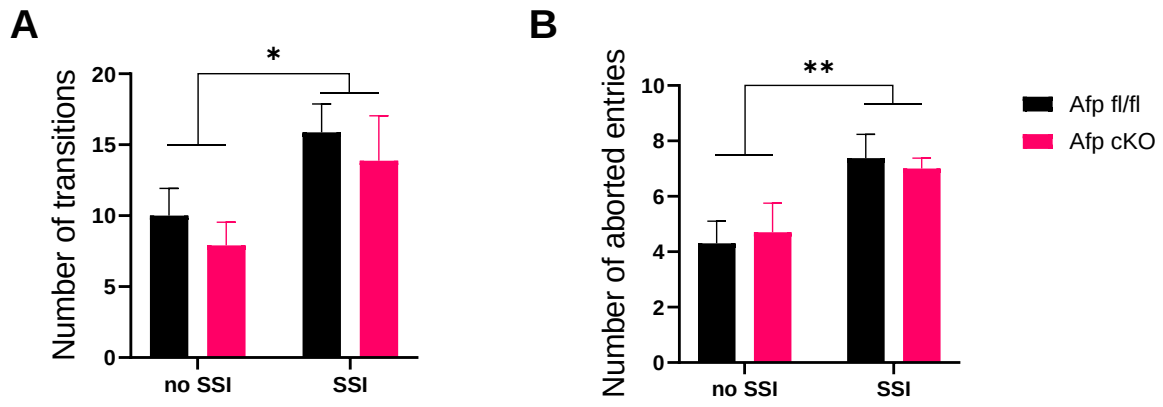


Figure 4.7: Both LD box chamber transitions and aborted entries are increased following SSI in Afp transgenic lines.

A) Number of transitions between chambers in Afp fl/fl (no SSI n = 10, SSI n = 8) and Afp cKO mice (no SSI n = 10, SSI n = 8). There was a significant effect of isolation condition on the number of transitions across genotypes (*, p=0.0107). B) Number of aborted entries between chambers in Afp fl/fl (no SSI n = 10, SSI n = 8) and Afp cKO mice (no SSI n = 10, SSI n = 8). There was a significant effect of isolation condition on the number of aborted entries (**, p=0.0036). Afp fl/fl mice also showed a significant increase in aborted entries in SSI (*, p=0.0319).

To look at the effects of LD box transitions further, genotype groups were divided in sex sub-groups as part of a supplementary analysis. Supplementary two-way ANOVA analysis revealed no significant interaction of sex and isolation condition on the number of transitions between chambers in Afp fl/fl or Afp cKO mice ((F(1,14)=0.02570, p=0.8749), (F(1,14)=3.878, p=0.0690), respectively). Though, Afp fl/fl mice did show a significant effect of isolation condition on number of chamber transitions, where there are increased transitions following SSI (F(1,14)=4.911, p=0.0438) (Figure 4.8A). Similarly, Afp cKO females demonstrated a significant increase in number of transitions following SSI treatment (Šídák's post-hoc, p=0.0341) (Figure 4.8B). Increased transitions following SSI suggests a reduction in anxiety.

In addition, females demonstrated a significant effect of isolation condition on number of chamber transitions, where there are increased transitions following SSI (F(1,13)=6.911, p=0.0208) (Figure 4.8C). Males demonstrated a significant effect of genotype on number of chamber transitions, where Afp cKO males consistently showed less transitions between

chambers across conditions, indicative of heightened anxiety in Afp cKO males ($F(1,15)=6.360$, $p=0.0235$) (Figure 4.8D).

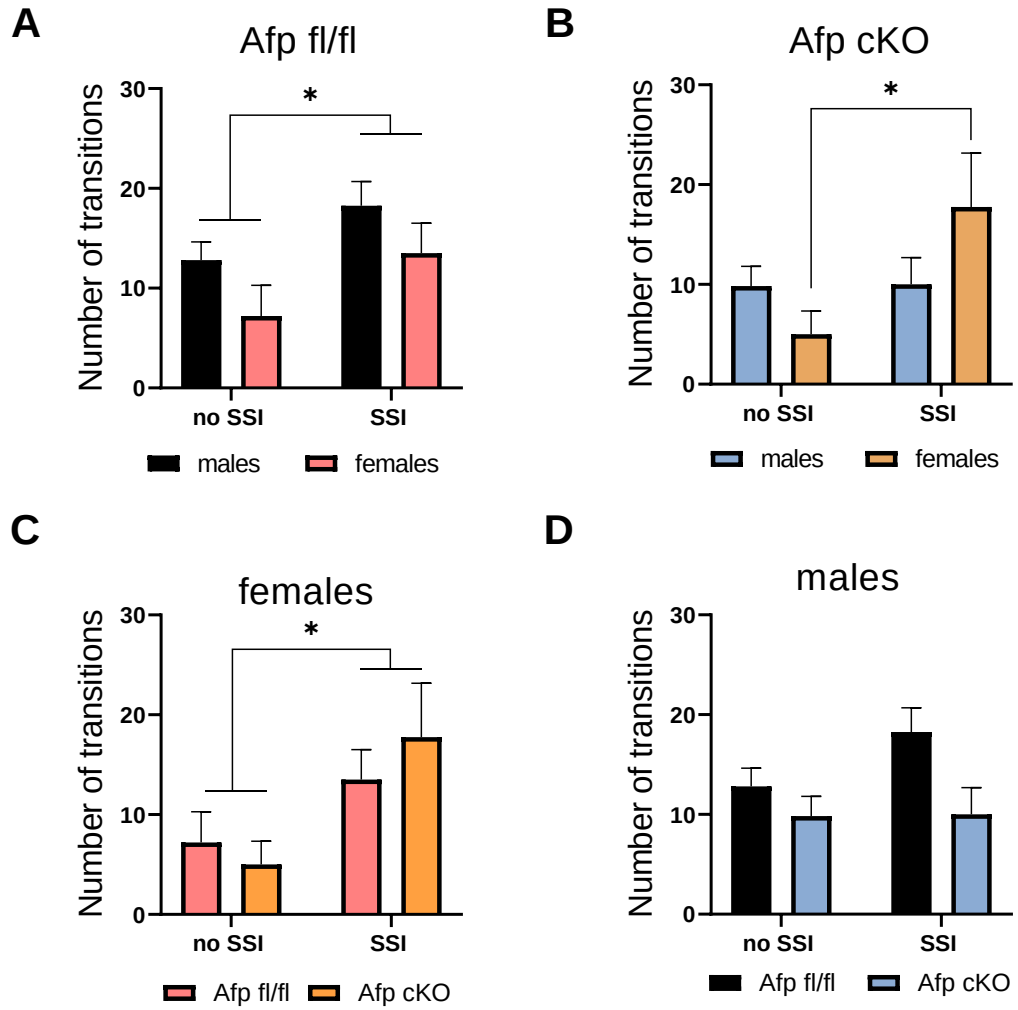


Figure 4.8: Significant effects of SSI on number of LD box transitions in Afp fl/fl mice and female Afp cKO mice.

A) Number of transitions between chambers in Afp fl/fl mice in no SSI (females n = 5, males n = 5) and under SSI conditions (females n = 4, males n = 4). A significant influence of isolation condition was seen on number of transitions across sexes (*, $p=0.0438$). B) Number of transitions between chambers in Afp cKO mice in no SSI (females n = 4, males n = 6) and under SSI conditions (females n = 4 and males n = 4). Females showed a significantly higher number of transitions in SSI than no SSI (*, $p=0.0341$). C) Number of transitions between chambers in females in no SSI (Afp fl/fl n = 5, Afp cKO n = 4) and under SSI conditions (Afp fl/fl n = 4, Afp cKO n = 4). A significant influence of isolation condition was seen on number of transitions across sexes (*, $p=0.0208$). D) Number of transitions between chambers in males in no SSI (Afp fl/fl n = 5, Afp cKO n = 6) and under SSI conditions (Afp fl/fl n = 4, Afp cKO n = 4). A significant influence of genotype was seen on number of transitions (*, $p=0.0235$).

Next, aborted entries in Afp fl/fl and Afp cKO mice were explored. Supplementary two-way ANOVA analysis demonstrated a significant interaction of sex and isolation condition on the number of aborted entries in Afp fl/fl mice ($F(1,14)=18.44$, $p=0.0007$), that was not seen in Afp cKO mice ($F(1,14)=3.081$, $p=0.1010$). Afp fl/fl mice also showed a significant effect of isolation condition on number of aborted entries ($F(1,14)=14.44$, $p=0.0020$), with significantly higher aborted entries in Afp fl/fl females in SSI compared to no SSI (Šídák's post-hoc, $p=0.0001$). This is indicative of increased anxiety in Afp fl/fl females following SSI. Additionally, significantly lower aborted entries were seen in Afp fl/fl females compared to Afp fl/fl males in no SSI (Šídák's post-hoc, $p=0.0032$), suggesting females were less anxious than males in normal housing conditions (Figure 4.9A).

Afp cKO males demonstrated a significant increase in number of aborted entries following SSI treatment, which proposes that SSI increased their anxiety (Šídák's post-hoc, $p=0.0412$). Afp cKO males also showed significantly lower number of aborted entries in no SSI compared to Afp cKO females, the converse of Afp fl/fl mice (Šídák's post-hoc $p=0.0412$), thus presenting Afp cKO males as less anxious than Afp cKO females in normal group housing conditions (no SSI) (Figure 4.9B).

When exploring genotype differences at a sex level, a significant interaction of genotype and isolation condition was seen on the number of aborted entries in both males and females (($F(1,15)=8.608$, $p=0.0103$), ($F(1,13)=7.570$, $p=0.0165$, respectively). Females also showed a significant effect of isolation condition on number of aborted entries ($F(1,13)=7.570$, $p=0.0165$). Afp cKO females displayed significantly increased aborted entries compared to Afp fl/fl females in no SSI, suggesting Afp cKO females were more anxious than controls in no SSI conditions (Šídák's post-hoc, $p=0.0233$) (Figure 4.9C).

Males also demonstrated a significant effect of isolation condition on number of aborted

entries ($F(1,15)=5.662$, $p=0.0310$), as well as a significantly higher number of aborted entries in Afp fl/fl males in no SSI compared to Afp cKO males, indicative of higher anxiety in Afp fl/fl males (Šídák's post-hoc, $p=0.0072$). Additionally, significantly higher aborted entries were seen in Afp cKO males in SSI compared to no SSI, suggesting anxiety was induced in SSI conditions (Šídák's post-hoc, $p=0.0033$) (Figure 4.9D). Though sex groups were underpowered, these results suggest they merit further investigation.

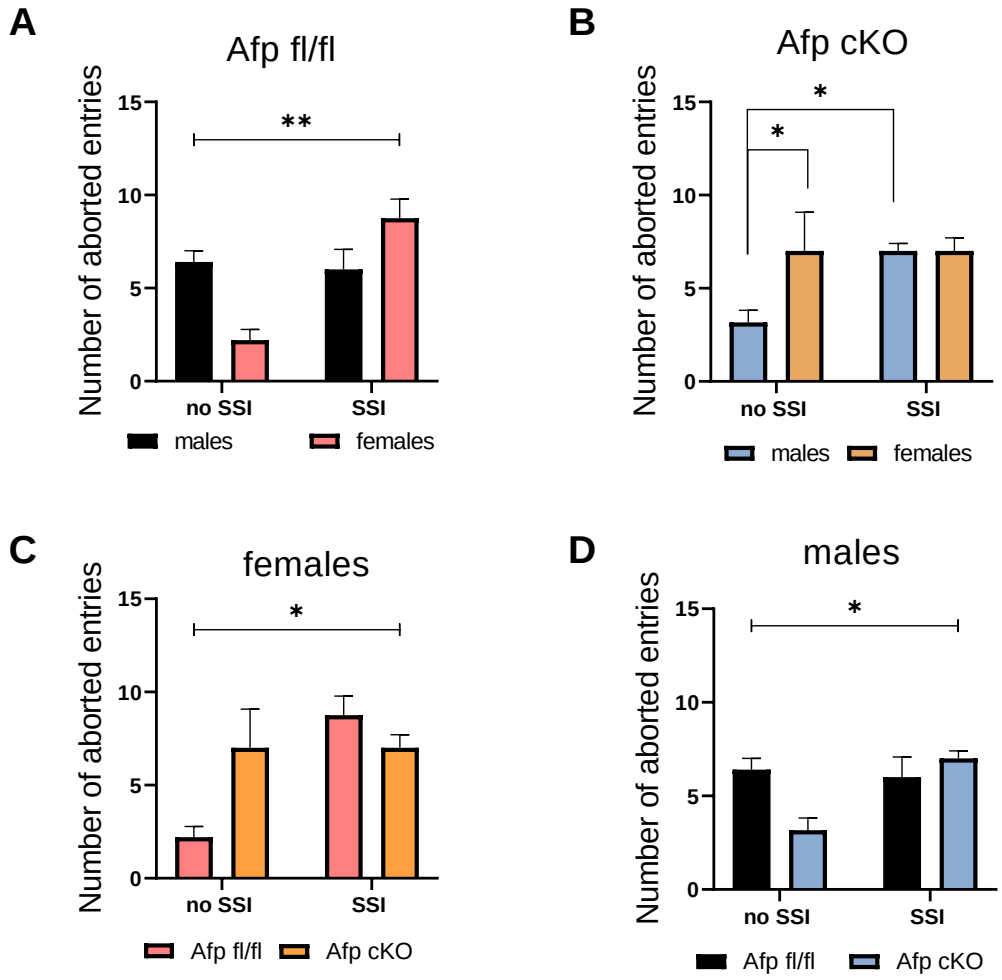


Figure 4.9: Significant interaction of SSI and genotype on the number of LD box aborted entries in Afp fl/fl and Afp cKO mice.

A) Number of aborted entries in Afp fl/fl mice in no SSI (females n = 5, males n = 5) and in SSI (females n = 4, males n = 4). A significant interaction of sex and isolation condition was seen on number of aborted entries in Afp fl/fl mice (**, $p=0.0020$). B) Number of aborted entries in Afp cKO mice in no SSI (females n = 4, males n = 6) and in SSI (females n = 4 and males n = 4). Females showed a significantly higher number of aborted entries in no SSI compared to Afp cKO males (*, $p=0.0412$). Afp cKO males also showed an increase in number of aborted entries in SSI compared to no SSI (*,

p=0.0412). C) Number of aborted entries in females in no SSI (Afp fl/fl n = 5, Afp cKO n = 4) and in SSI (Afp fl/fl n = 4, Afp cKO n = 4). A significant interaction of genotype and isolation condition was seen on number of aborted entries (*, *p*=0.0165). D) Number of aborted entries in males in no SSI (Afp fl/fl n = 5, Afp cKO n = 6) and in SSI (Afp fl/fl n = 4, Afp cKO n = 4) . A significant interaction of genotype and isolation condition was seen on number of aborted entries (*, *p*=0.0103).

4.3.5 OF shows increased anxiety in both Afp transgenic lines following SSI

Another common assessment of anxiety is the OF arena trial (Bourin and Hascoët, 2003, Kulesskaya and Voikar, 2014). This was performed in addition to the LD box trials explored in 4.3.3 and 4.3.4. Two cohorts of Afp cKO and Afp fl/fl mice underwent OFs following either no SSI, or seven days of SSI. Firstly, time spent in the peripheral and central zones was compared between genotypes. Two-way ANOVA revealed a significant interaction between genotype and zone type on time spent in each zone in SSI conditions ($F(1,28)=4.235$, $p=0.0490$). There was also a significant effect of zone type on exploration in no SSI ($F(1,36)=2915$, $p<0.0001$) and in SSI ($F(1,28)=5067$, $p<0.0001$). Both Afp fl/fl and Afp cKO mice spent more time in the peripheral zone in no SSI and in SSI (Šídák's post-hoc, all $p<0.0001$) (Figure 4.10A, 4.10B).

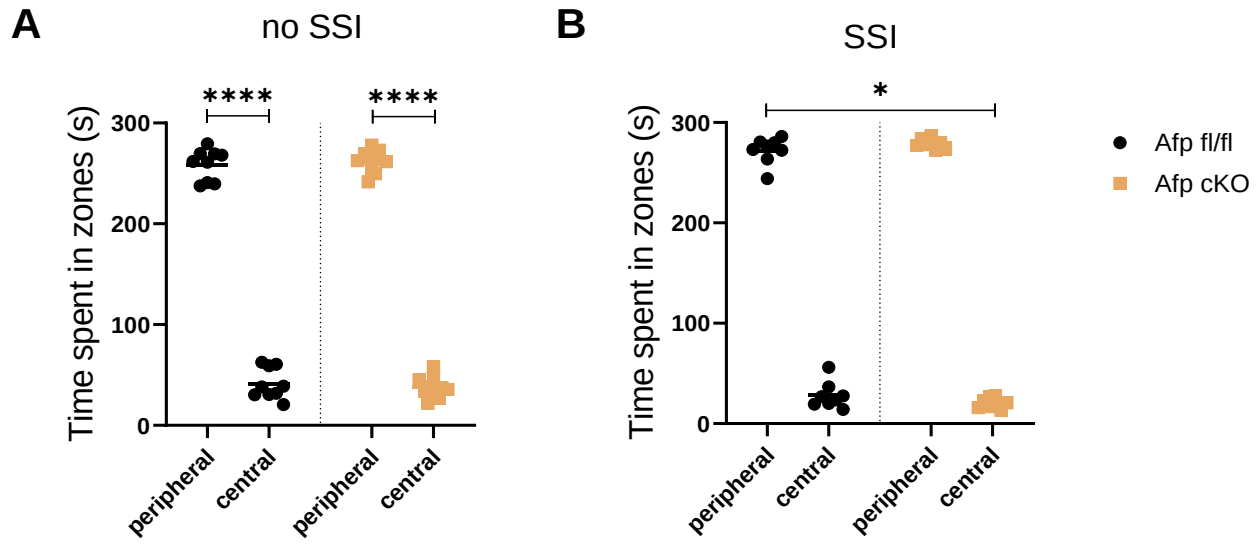


Figure 4.10: Afp fl/fl and Afp cKO mice both preferred the peripheral area of an OF arena.

A) Time spent in the central versus peripheral zones of the OF arena by Afp fl/fl (n = 9) and Afp cKO (n = 11) mice in no SSI conditions. Both Afp fl/fl and Afp cKO mice spent significantly more time in the peripheral zone compared to the central zone (both: ****, $p<0.0001$). B) Time spent in the central versus peripheral zones of the OF arena by Afp fl/fl (n = 8) and Afp cKO (n = 8) mice in SSI conditions. A significant effect of the interaction between genotype and zone type was seen on zone exploration across genotypes (*, $p=0.0490$).

There was no significant interaction between genotype and isolation condition on time spent in the central zone ($F(1,32)=0.2012$, $p=0.6568$). However, there was a significant effect of isolation condition ($F(1,32)=14.80$, $p<0.0001$). Both genotypes significantly reduced time spent in the central zone of the OF arena when housed in SSI conditions, indicative of increased anxiety (Afp fl/fl: Šídák's post-hoc, $p=0.0495$, Afp cKO: Šídák's post-hoc, $p=0.0079$) (Figure 4.11).

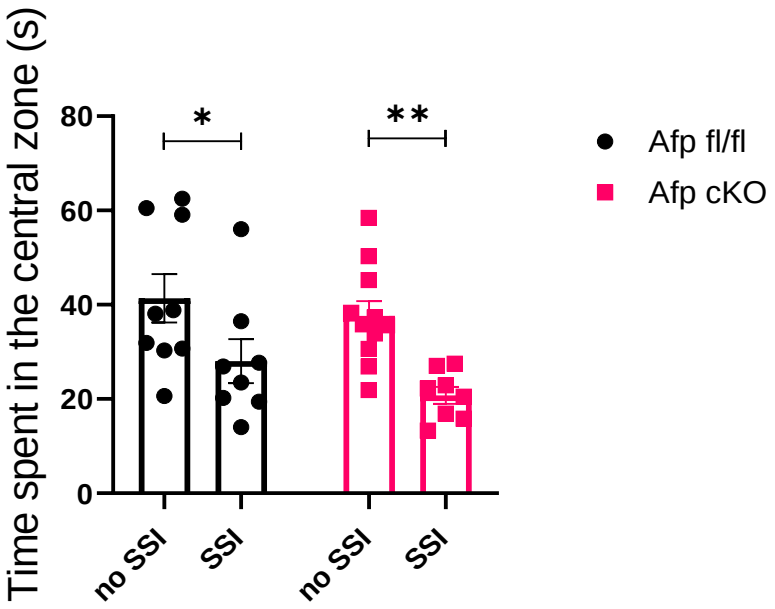


Figure 4.11: Time spent in the central zone of an OF arena was reduced following SSI in Afp fl/fl and Afp cKO mice.

Both Afp fl/fl (no SSI n = 9, SSI n = 8) and Afp cKO (no SSI n = 11, SSI n = 8) showed a significant reduction in time spent in the central zone in SSI compared to no SSI conditions (*, $p=0.0495$, **, $p=0.0079$, respectively). There was a significant effect of isolation condition on time spent in the central zone (***, $p=0.0005$).

To look at the effects of time spent in the central zone further, genotype groups were

divided in sex sub-groups. Supplementary two-way ANOVA analysis revealed a significant reduction in time spent in the central zone of the OF arena in Afp fl/fl females in SSI compared to no SSI (Šídák's post-hoc, p=0.0031). Interestingly, Afp cKO mice showed a significant influence of isolation treatment on time spent in the central zone (F(1,15)=20.33, p=0.0004). Both Afp cKO males and females significantly reduced their time spent in the central zone of the OF arena in SSI compared to no SSI (Šídák's post-hoc, p=0.0032, p=0.0353, respectively) (Figure 4.12A, 4.12B). Furthermore, female mice showed a significant influence of isolation treatment on time spent in the central zone (F(1,13)=22.59, p=0.0004). Both Afp fl/fl females and Afp cKO females significantly reduced their time spent in the central zone of the OF arena in SSI (Šídák's post-hoc, p=0.0031, p=0.0353, respectively) (Figure 4.12C, 4.12D). This demonstrates that only Afp fl/fl males maintained similar time spent in the central zone of the OF arena following SSI treatment, and that all other groups reduced their exploration of the central zone in SSI, indicating heightened anxiety.

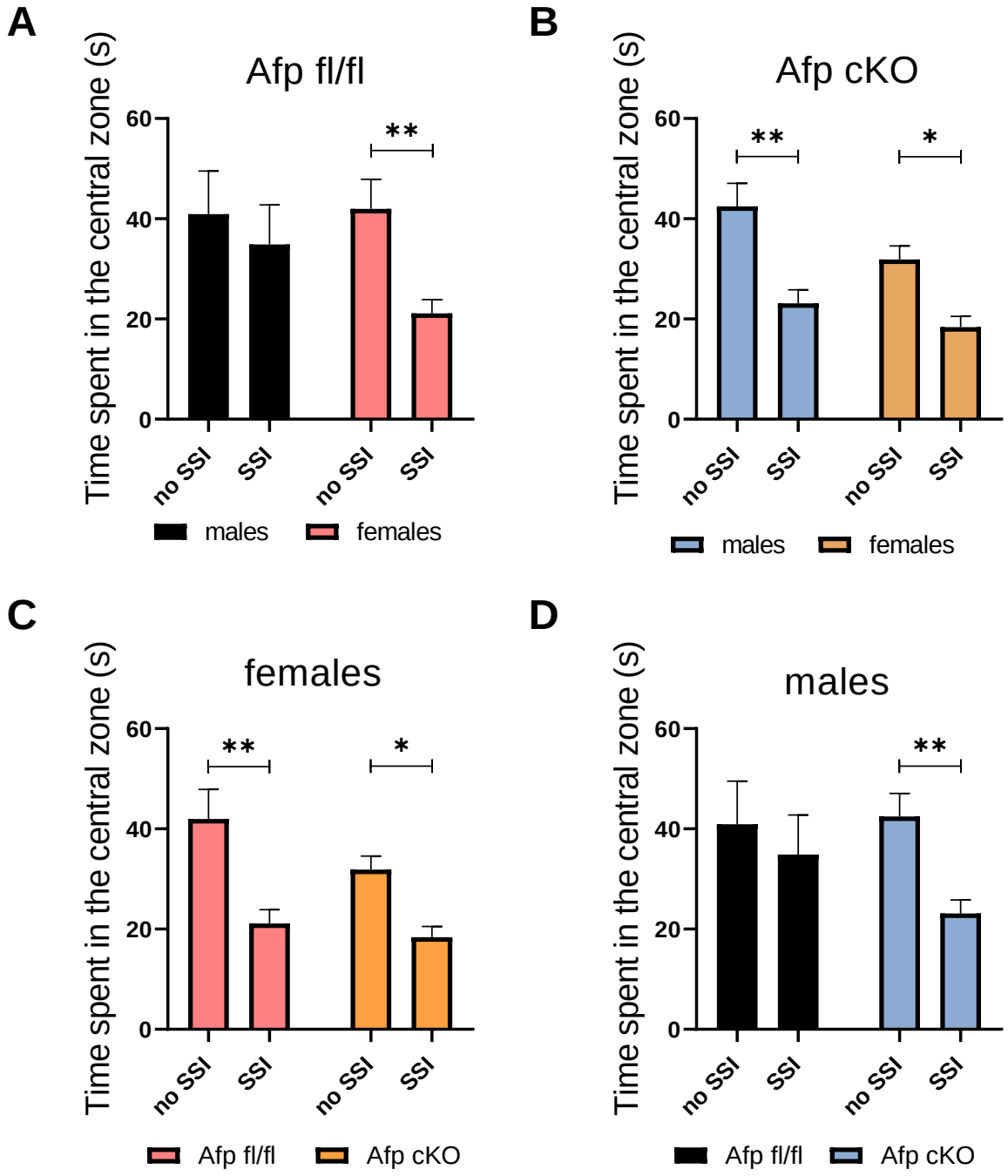


Figure 4.12: Time spent in the central zone of an OF arena was reduced following SSI in Afp fl/fl females and Afp cKO male and female mice.

A) Time spent in the central zone of the OF arena in Afp fl/fl mice in no SSI (females n = 4, males n = 5) and under SSI conditions (females n = 4, males n = 4). Afp fl/fl females significantly reduced time spent in the central zone in SSI compared to no SSI (**, p=0.0031). B) Time spent in the central zone of the OF arena in Afp cKO mice in no SSI (females n = 4, males n = 6) and under SSI conditions (females n = 4 and males n = 5). Both males and females showed a significant reduction in time spent in the central zone in SSI compared to no SSI (**, p=0.0032, *, p=0.0353, respectively). C) Time spent in the central zone of the OF arena in females in no SSI (Afp fl/fl n = 4, Afp cKO n = 4) and under SSI conditions (Afp fl/fl n = 4, Afp cKO n = 4). D) Time spent in the central zone of the OF arena in males

in no SSI (Afp fl/fl n = 5, Afp cKO n = 6) and under SSI conditions (Afp fl/fl n = 4, Afp cKO n = 4).

4.3.6 Sexual dimorphism of latency to enter the dark chamber seen in fl/fl and cKO transgenic lines

To investigate phenotypes of anxiety that may have implications on weight maintenance and potentially even recognition memory, two cohorts of cKO and fl/fl mice underwent LD box trials following either no SSI, or seven days of SSI. Firstly, the latency to enter the dark chamber was measured.

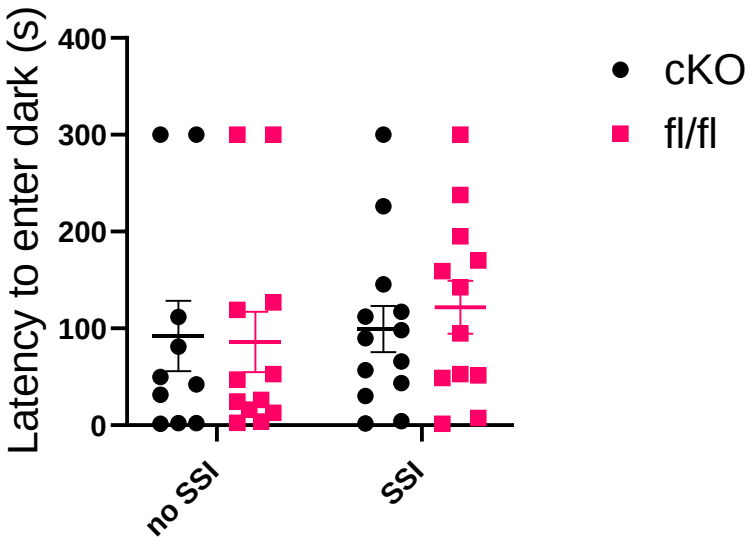


Figure 4.13: No significant differences in latency to enter dark were seen between genotypes, or across isolation conditions in cKO or fl/fl lines.

Latency to enter the dark chamber for cKO mice (no SSI n = 11, SSI n = 13) and Afp fl/fl mice (no SSI n = 12, SSI n = 12). No differences between genotypes or isolation condition were found.

Two-way ANOVA analysis revealed no significant effects of genotype, SSI or interaction between the two on latency to enter dark (interaction ($F(1,43)=0.2375$, $p=0.6285$) (Figure 4.13). To look at this parameter further, genotype groups were divided in sex sub-groups. There was no significant interaction of sex and isolation on fl/fl or cKO latency to enter dark (supplementary two-way ANOVA ($F(1,20)=2.322$, $p=0.1432$), ($F(1,19)=3.133$,

p=0.0927), respectively). However, fl/fl females did show significantly higher latency to enter dark in no SSI than fl/fl males, suggesting they were less anxious than male counterparts (Šídák's post-hoc, *p*=0.0410). In addition, cKO mice showed an effect of sex on latency to enter dark ($F(1,19)=5.239$, $p=0.0337$), and demonstrated that cKO females to have a significantly higher latency to enter dark in no SSI than cKO males (Šídák's post-hoc, *p*=0.0263) (Figure 4.14A, 4.14B). This showed that in no SSI conditions, males of both genotypes entered the dark chamber significantly faster than female counterparts, indicating heightened anxiety in males.

There was no significant interaction of genotype and isolation on male or female latency to enter dark (($F(1,22)=0.0.01299$, $p=0.9103$), ($F(1,17)=0.06018$, $p=0.8091$), respectively). However, there was a significant effect of isolation condition on male latency to enter dark ($F(1,22)=7.807$, $p=0.0106$). Both males increased their latency to enter dark in SSI conditions compared to no SSI (Figure 4.14.C, 4.14D). Interestingly, this infers reduced anxiety in males following SSI conditions.

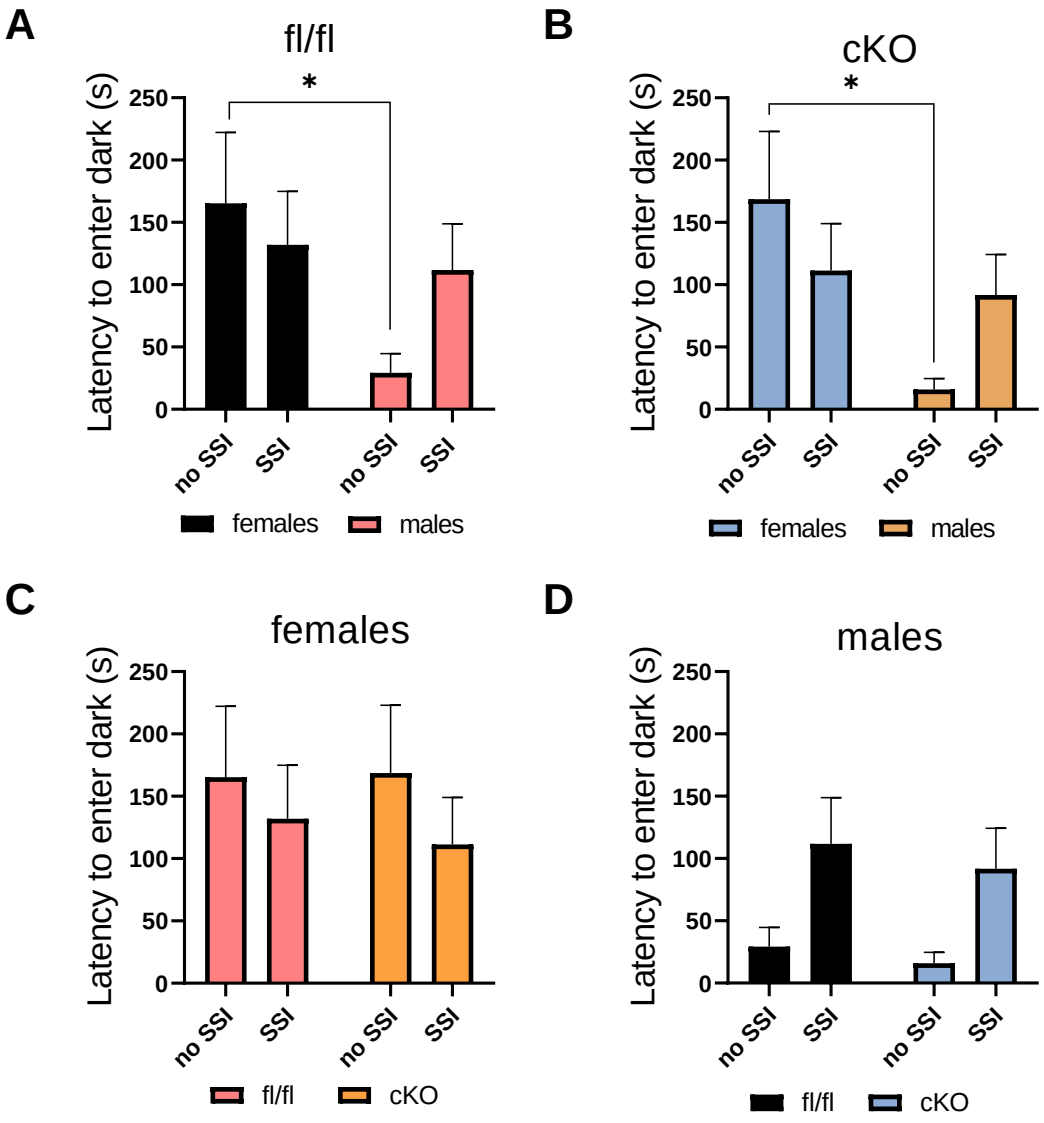


Figure 4.14: Significant sexual dimorphism in latency to enter dark in cKO and fl/fl mice.

A) Latency to enter the dark chamber for fl/fl mice in no SSI (females n = 5, males n = 7) and under SSI conditions (females n = 6, males n = 6). Females showed a significantly longer latency to enter dark in no SSI compared to males (*, p=0.0410). B) Latency to enter the dark chamber for cKO mice in no SSI (females n = 5, males n = 6) and under SSI conditions (females n = 5 and males n = 8). A significant effect of sex on latency was seen (*, p=0.0337). C) Latency to enter the dark chamber for females in no SSI (fl/fl n = 5, cKO n = 5) and under SSI conditions (fl/fl n = 6, cKO n = 5). D) Latency to enter the dark chamber for males in no SSI (fl/fl n = 7, cKO n = 6) and under SSI conditions (fl/fl n = 6, cKO n = 8). Isolation condition had a significant effect on latency to enter the dark in males across conditions (*, p=0.0106).

The second parameter analysed in the LD box test was the time spent in the light versus the dark chamber. There was a significant effect of chamber on exploration time in both no SSI

and SSI conditions ((*F*(1,42)=37.68, *p*<0.0001), (*F*(1,46)=78.70, *p*<0.0001), respectively). Both genotypes showed a robust preference for the light chamber in both SSI and no SSI conditions (Šídák's post-hoc, all *p*<0.0001 except cKO in no SSI: *p*=0.0026) (Figure 4.15A, 4.15B).

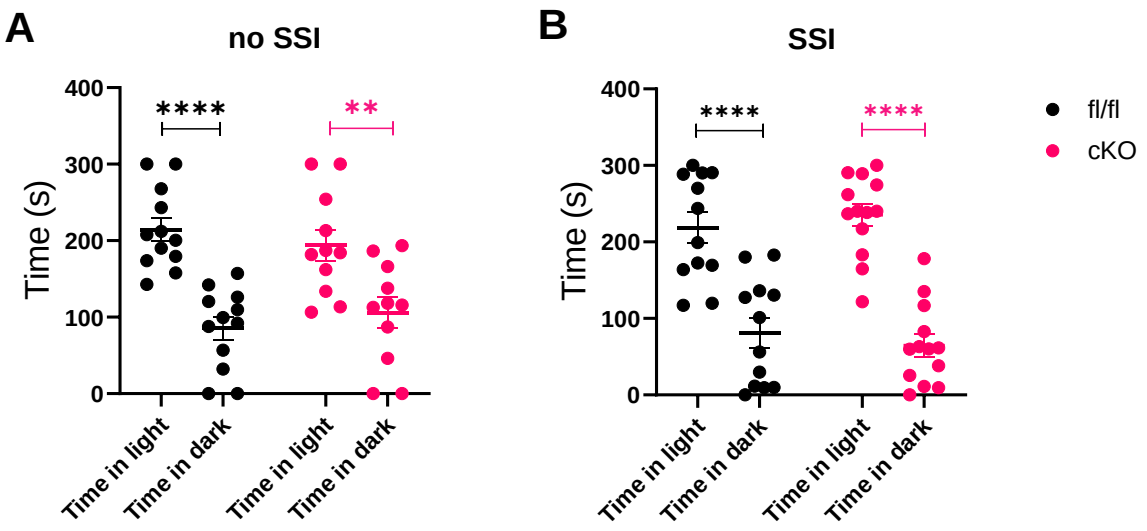


Figure 4.15: A significant preference for the light chamber was seen in both fl/fl and cKO mice in no SSI and SSI.

A) Time spent in both the light and dark chamber of fl/fl (no SSI n = 12) and cKO (no SSI n = 11) in no SSI. Fl/fl and cKO spent significantly more time in the light chamber than the dark chamber in no SSI (****, *p*<0.0001, **, *p*=0.0026 respectively). B) Time spent in both the light and dark chamber of fl/fl (SSI n = 12) and cKO (SSI n = 13) in SSI. Fl/fl and cKO spent significantly more time in the light chamber than the dark chamber in SSI (both: ****, *p*<0.0001).

To look at this parameter further, genotype groups were divided into sex sub-groups. Time spent in the light chamber did not show a significant interaction of sex and isolation in either fl/fl or cKO (supplementary two-way ANOVA (*F*(1,20)=1.798, *p*=0.8315), (*F*(1,20)=1.357, *p*=0.2578), respectively). However, there was a significant effect of sex on time spent in the light chamber in cKO mice (*F*(1,20)=5.129, *p*=0.0348). cKO males spent less time in the light chamber compared to cKO females in no SSI conditions, suggesting males had higher anxiety levels compared to females in group-housed conditions (Šídák's post-hoc,

p=0.0482) (Figure 4.16A, 4.16B). Additionally, males demonstrated a significant influence of isolation condition on time spent in the light chamber, with increased time in the light following SSI ($F(1,23)=5.312$, $p=0.0305$) (Figure 4.16.C, 4.16D). This suggests both male groups had reduced anxiety in SSI conditions. Thus, though sex groups were underpowered, these results suggest they merit further investigation.

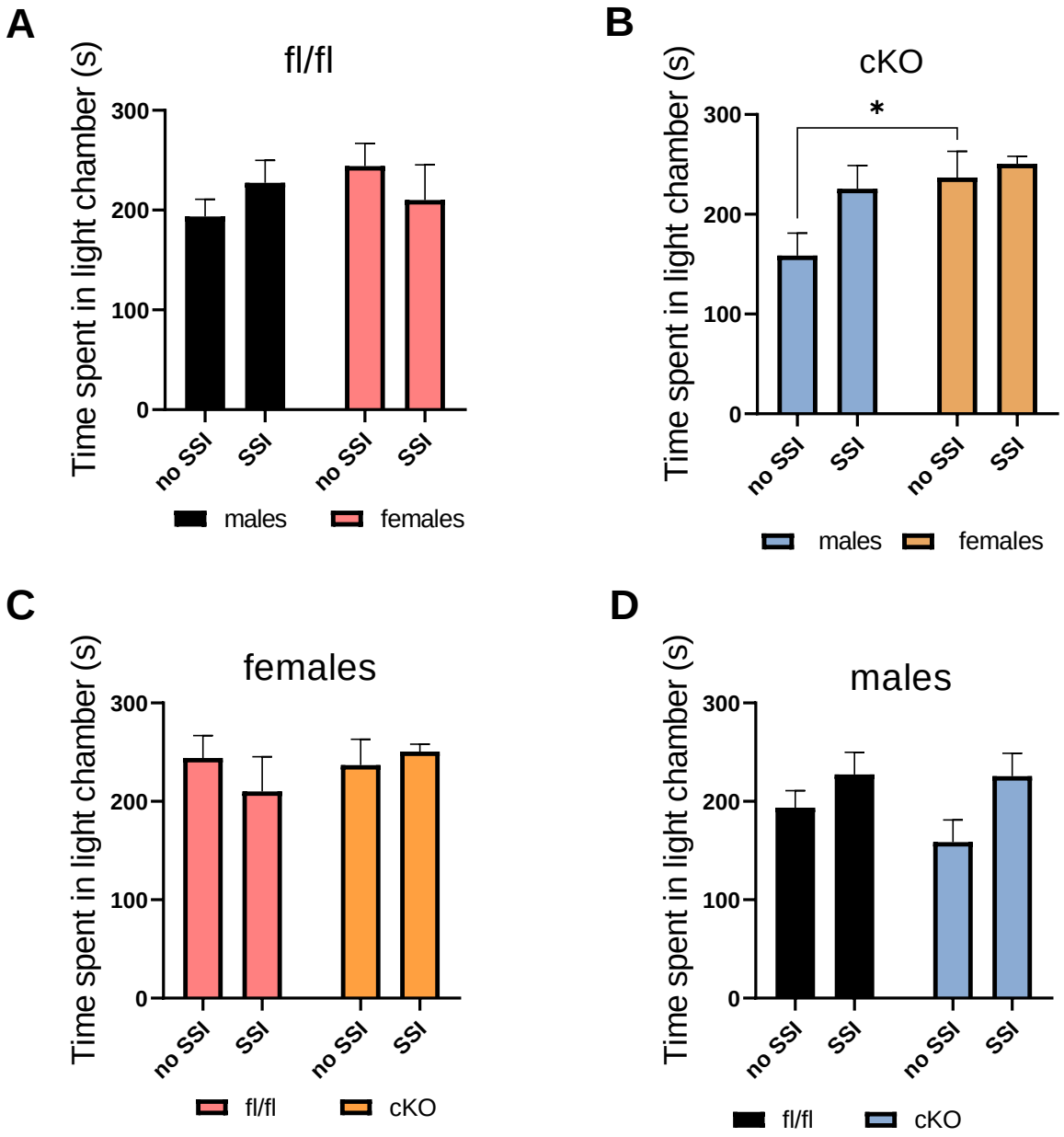


Figure 4.16: Significant sex effect on time spent in the light chamber by cKO mice.

A) Time spent in the light chamber for fl/fl mice in no SSI (females n = 5, males n = 7) and under SSI conditions (females n = 6, males n = 6). B) Time spent in the light chamber for cKO mice in no SSI (females n = 5, males n = 6) and under SSI conditions (females n = 5 and males n = 8). A significant influence of sex was seen on time spent in the light chamber (*, p=0.0348). C) Time spent in the light chamber for females in no SSI (fl/fl n = 5, cKO n = 5) and under SSI conditions (fl/fl n = 6, cKO n = 5). D) Time spent in the light chamber for males in no SSI (fl/fl n = 7, cKO n = 6) and under SSI conditions (fl/fl n = 6, cKO n = 8). Males showed a significant influence of isolation condition on time spent in the light chamber (*, p=0.0305).

4.3.7 Significant sex and isolation condition effects on number of aborted LD box entries in cKO and fl/fl mice

As discussed in 4.3.4, other noteworthy parameters of LD box trials are the numbers of chamber transitions and the number of false entries (Crawley et al., 1997, Heredia et al., 2014, Kulesskaya and Voikar, 2014).

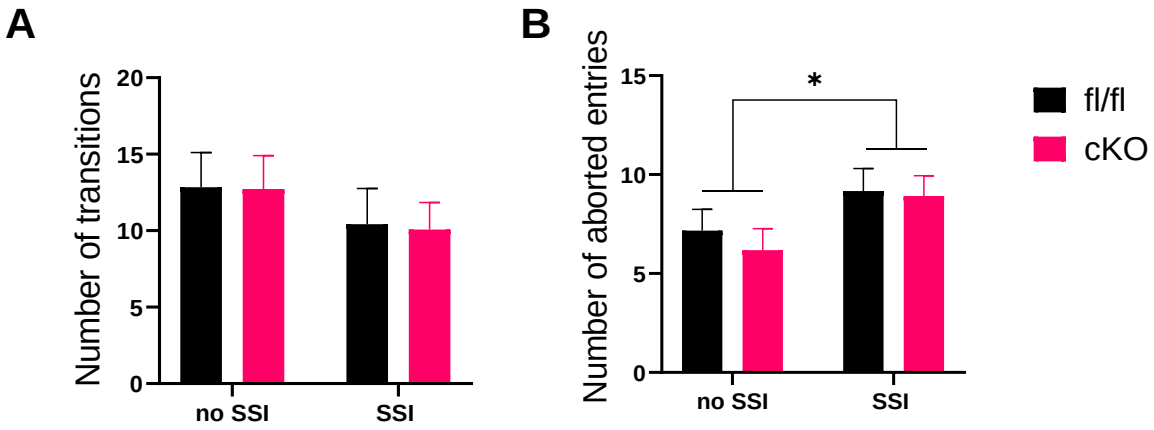


Figure 4.17: Significant effect of SSI on aborted entries but not LD box transitions in fl/fl and cKO mice.

A) Number of transitions between chambers in fl/fl (no SSI n = 12, SSI n = 12) and cKO mice (no SSI n = 11, SSI n = 13). B) Number of aborted entries between chambers in fl/fl (no SSI n = 12, SSI n = 12) and cKO mice (no SSI n = 11, SSI n = 13). Number of aborted entries showed an influence of isolation condition across genotypes (*, p=0.0338).

Two-way ANOVA analysis revealed the number of transitions did not demonstrate an interaction between genotype and isolation condition (F(1,44)=0.0002981, p=0.9567) (Figure

4.17A). Additionally, the number of aborted entries into the opposing chamber did not show an interaction between genotype and isolation condition ($F(1,44)=0.1174$, $p=0.7336$). Nevertheless, there was a significant effect of isolation condition on the number of aborted entries ($F(1,44)=4.801$, $p=0.0338$). This demonstrated a general increase in the number of aborted entries between chambers following SSI treatment, indicative of increased anxiety (Figure 4.17B).

To look at the effects of LD box transitions further, genotype groups were divided in sex sub-groups. No significant interaction of sex and isolation condition was seen on the number of transitions between chambers in cKO mice (supplementary two-way ANOVA ($F(1,20)=0.3839$, $p=0.5396$). Despite this, fl/fl mice did display a significant interaction between sex and isolation condition on the number of chamber transitions ($F(1,20)=0.4.384$, $p=0.0492$). Fl/fl females increased the number of transitions in SSI conditions, whilst fl/fl males reduced the number of transitions in SSI (Šídák's post-hoc, $p=0.0475$). This suggests fl/fl females were less anxious, and fl/fl males were more anxious following SSI (Figure 4.18A, 4.18B).

No significant interaction of genotype and isolation condition was seen on the number of transitions between chambers in males or females (($F(1,23)=0.5627$, $p=0.4608$), ($F(1,17)=0.6326$, $p=0.4374$), respectively). However, males demonstrated a significant effect of isolation condition on number of chamber transitions, with reduced transitions in SSI compared to no SSI ($F(1,13)=7.381$, $p=0.0123$) (Figure 4.18C, 4.18D)

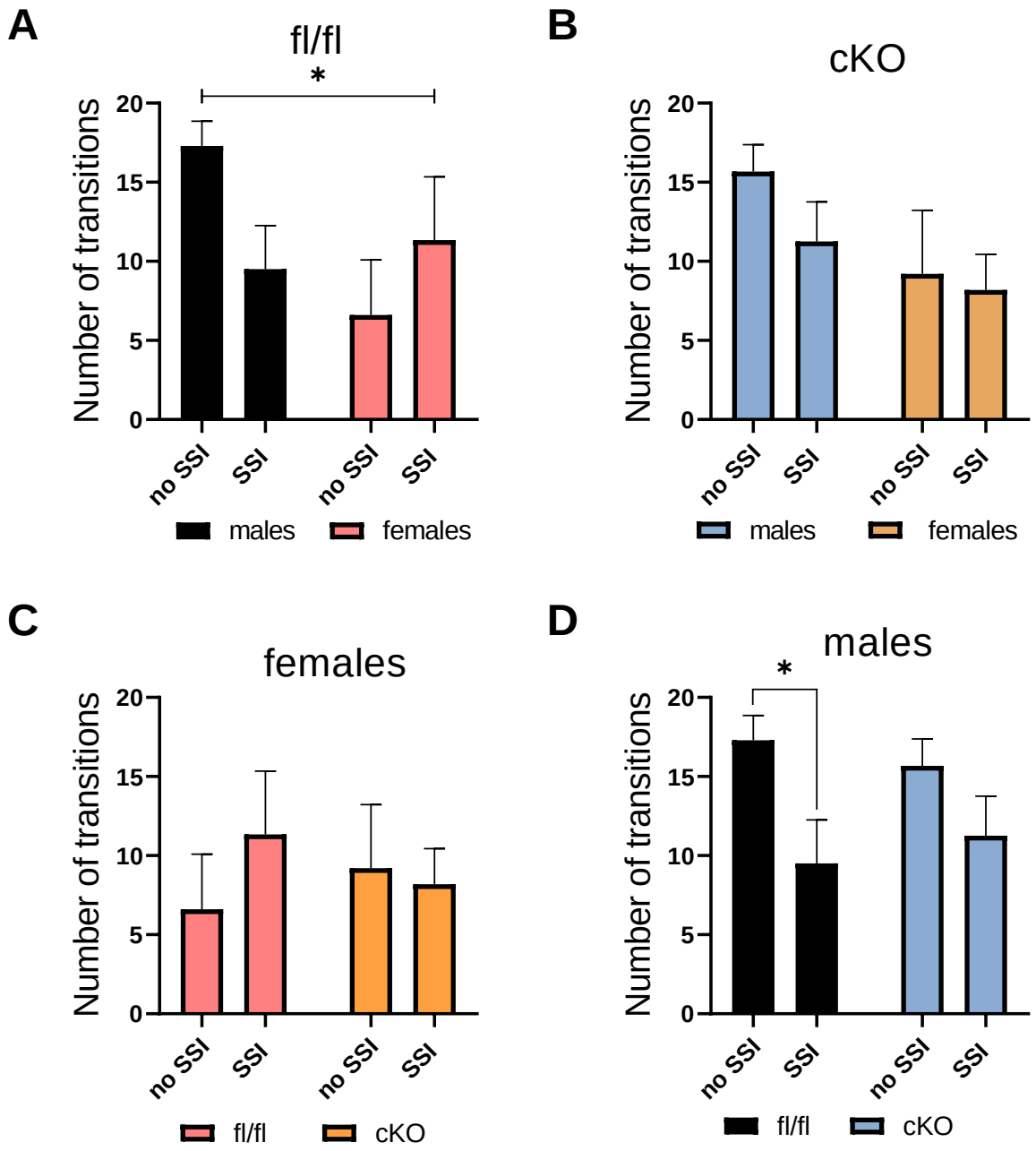


Figure 4.18: The significant interaction betwen isolation condition and sex on fl/fl LD box chamber transitions that was not seen in cKO mice.

A) Number of transitions between chambers in fl/fl mice in no SSI (females n = 5, males n = 7) and under SSI conditions (females n = 6, males n = 6). A significant interaction between sex and isolation condition was seen on number of transitions in fl/fl mice (*, p=0.0492). B) Number of transitions between chambers in cKO mice in no SSI (females n = 5, males n = 6) and under SSI conditions (females n = 5 and males n = 8). C) Number of transitions between chambers in females in no SSI (fl/fl n = 5, cKO n = 5) and under SSI conditions (fl/fl n = 6, cKO n = 5). D) Number of transitions between chambers in males in no SSI (fl/fl n = 7, cKO n = 6) and under SSI conditions (fl/fl n = 6, Afp cKO n = 8) . Males showed an influence of isolation condition on number of transitions (*, p=0.0123).

Furthermore, no significant interaction of sex and isolation condition was seen on the number of aborted entries in fl/fl or cKO mice (supplementary two-way ANOVA analysis ($F(1,20)=0.5825$, $p=0.4542$), ($F(1,20)=3.404$, $p=0.2746$), respectively). However, fl/fl mice did show a significant effect of sex on number of aborted entries ($F(1,20)=8.754$, $p=0.0078$), with higher number of aborted entries in males compared to fl/fl females in no SSI (Šídák's post-hoc, $p=0.0330$) (Figure 4.19A).

There was also no significant interaction of genotype and isolation condition on the number of aborted entries in either male or female mice (($F(1,23)=0.008466$, $p=0.9275$), ($F(1,17)=0.02860$, $p=0.8677$, respectively). Despite this, females showed a significant effect of isolation condition on number of aborted entries ($F(1,17)=5.606$, $p=0.0300$) (Figure 4.19C). Thus, despite sex groups being underpowered, these results infer they warrant additional investigation with larger cohort sizes.

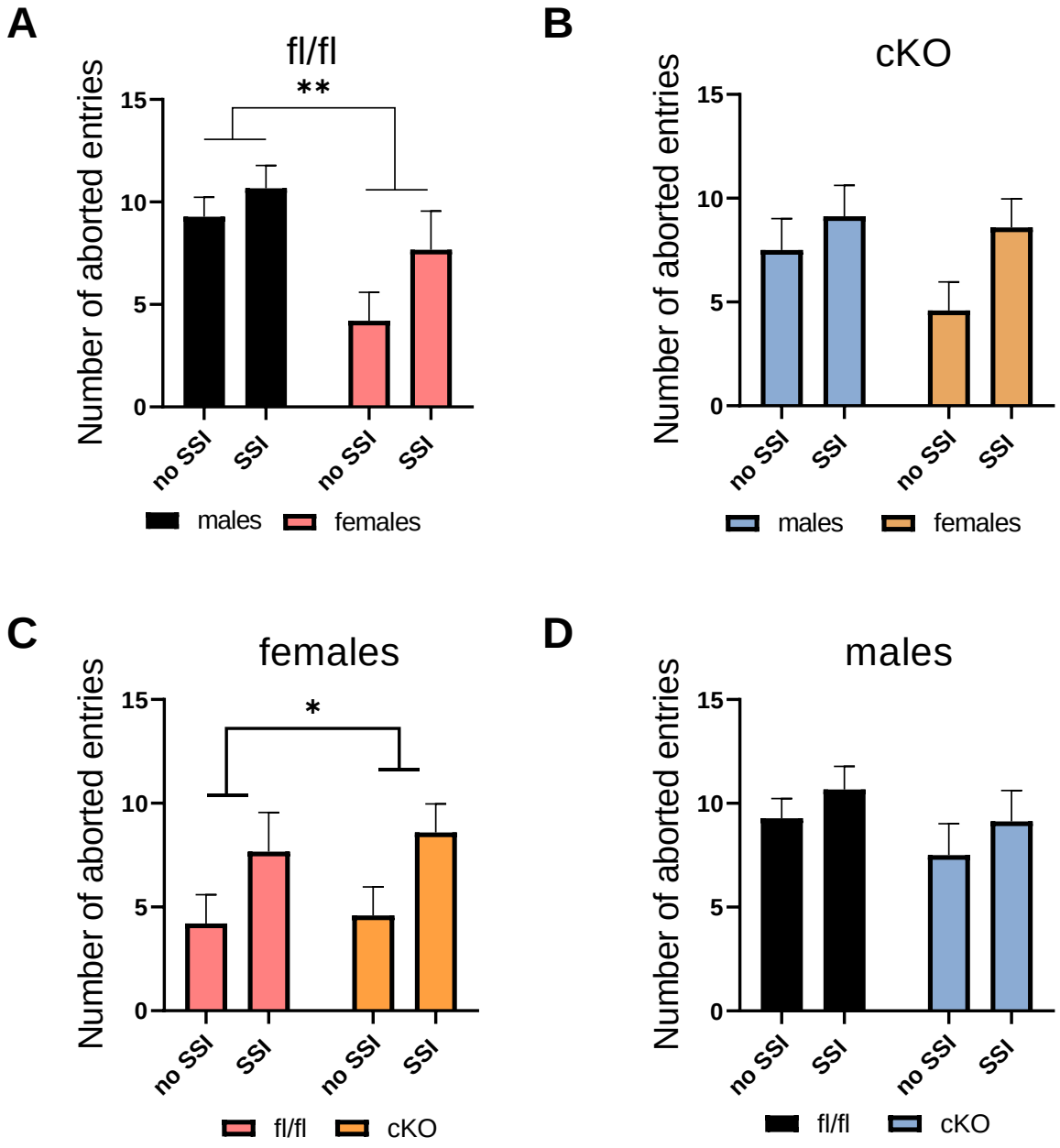


Figure 4.19: Number of aborted entries showed significant sex effects in fl/fl mice and significant isolation effects in female mice.

A) Number of aborted entries in fl/fl mice in no SSI (females n = 5, males n = 7) and under SSI conditions (females n = 6, males n = 6). A significant effect of sex was seen on number of aborted entries in fl/fl mice (**, p=0.0078). B) Number of aborted entries in cKO mice in no SSI (females n = 5, males n = 6) and under SSI conditions (females n = 5 and males n = 8). C) Number of aborted entries in females in no SSI (fl/fl n = 5, cKO n = 5) and under SSI conditions (fl/fl n = 6, cKO n = 5). Females showed a significant effect of isolation condition on number of aborted entries (*, p=0.0300). D) Number of aborted entries in males in no SSI (fl/fl n = 7, cKO n = 6) and under SSI conditions (fl/fl n = 6, Afp cKO n = 8).

4.3.8 OF showed increased anxiety in cKO and fl/fl lines following SSI

An OF arena trial was performed alongside the LD box test; two cohorts of cKO and fl/fl mice underwent OF following either no isolation, or seven days of SSI. Firstly, time spent in the peripheral and central zones was compared between genotypes. Two-way ANOVA analysis revealed both no SSI and SSI conditions showed an effect of zone type on time spent in each zone (($F(1,64)=8492$, $p<0.0001$), ($F(1,48)=0.12036$, $p<0.0001$), respectively). This demonstrated that in both conditions, fl/fl and cKO spent more time in the peripheral zone (Šídák's post-hoc, all $p<0.0001$) (Figure 4.20A, 4.20B).

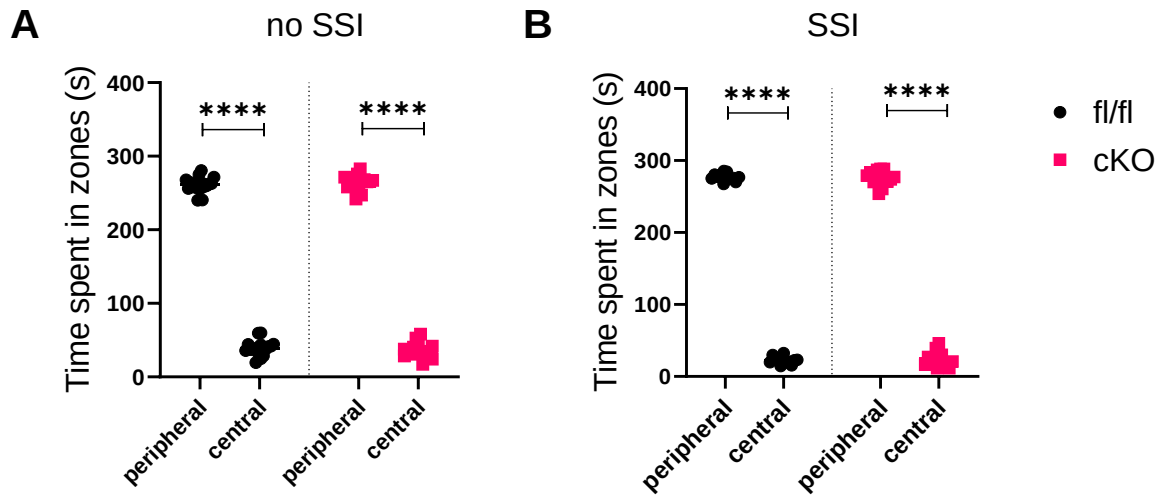


Figure 4.20: Fl/fl and cKO mice both preferred the peripheral area of an OF arena.

A) Time spent in the central versus peripheral zones of the OF arena by fl/fl (n = 18) and cKO (n = 16) mice in no SSI conditions. A significant effect of zone type on zone exploration was seen (****, $p<0.0001$). B) Time spent in the central versus peripheral zones of the OF arena by fl/fl (n = 12) and cKO (n = 14) mice in SSI conditions. A significant effect of zone type was seen on zone exploration across genotypes (****, $p<0.0001$).

No significant interaction between genotype and isolation condition on time spent in the central zone was seen ($F(1,56)=0.8919$, $p=0.3490$). However, there was a significant effect of isolation condition on time spent in the central zone ($F(1,56)=33.69$, $p<0.0001$). Both genotypes significantly reduced the time spent in the central zone of the OF arena when housed in SSI conditions (Šídák's post-hoc, fl/fl: $p<0.0001$, cKO: $p=0.0020$) (Figure 4.21). This is

indicative of increased anxiety following SSI irrespective of hepatic NPAS2 loss.

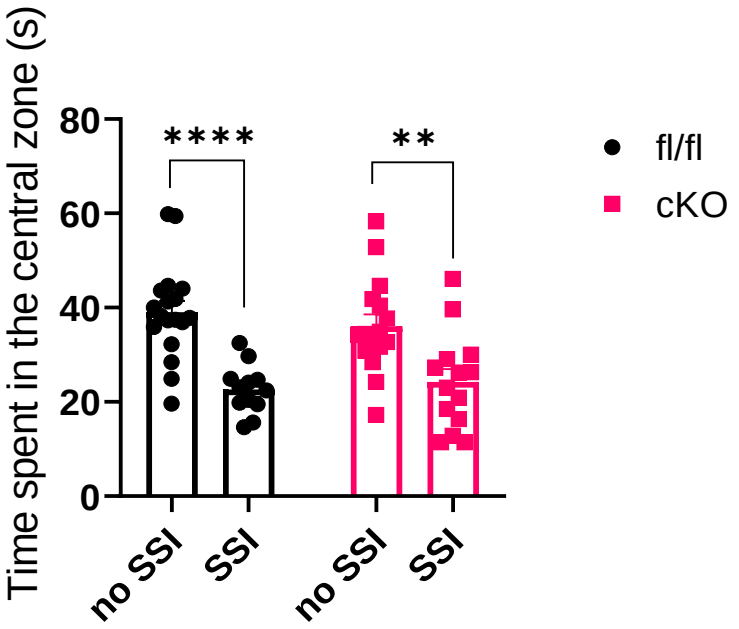


Figure 4.21: Time spent in the central zone of an OF arena was reduced following SSI in fl/fl and cKO mice.

Both fl/fl (no SSI n = 18, SSI n = 12) and cKO (no SSI n = 16, SSI n =14) showed a significant reduction in time spent in the central zone in SSI compared to no SSI conditions (****, $p<0.0001$, **, $p=0.0020$, respectively). There was a significant effect of isolation condition on time spent in the central zone (****, $p<0.0001$).

To look at the effects of time spent in the central zone further, genotype groups were divided into sex sub-groups. Supplementary two-way ANOVA analysis revealed no significant interaction between sex and isolation condition on time spent in the central zone in either fl/fl or cKO mice (($F(1,26)=0.5822$, $p=0.4523$), ($F(1,26)=0.05117$, $p=0.8228$), respectively). However, there was a significant influence of isolation treatment on time spent in the central zone by both fl/fl and cKO mice (($F(1,26)=21.01$, $p=0.0001$), ($F(1,26)=8.743$, $p=0.0065$), respectively). Fl/fl males and fl/fl females both spent significantly less time in the central zone following SSI (Šídák's post-hoc, $p=0.0005$, $p=0.0408$, respectively). In addition, cKO females spent significantly less time in the central zone following SSI conditions (Šídák's post-hoc,

p=0.0355) (Figure 4.22A, 4.22B). Furthermore, there was a significant influence of isolation treatment on time spent in the central zone by male and by female mice ((*F*(1,34)=16.56, *p*=0.0003), (*F*(1,18)=15.05, *p*=0.0011), respectively) (Figure 4.22C, 4.22D). This demonstrates that only cKO males maintained similar time spent in the central zone of the OF following SSI treatment, and that all other genotype/sex sub-groups reduced their exploration of the central zone in isolated conditions, suggestive of heightened anxiety. Though sex groups were underpowered, these results merit additional investigation.

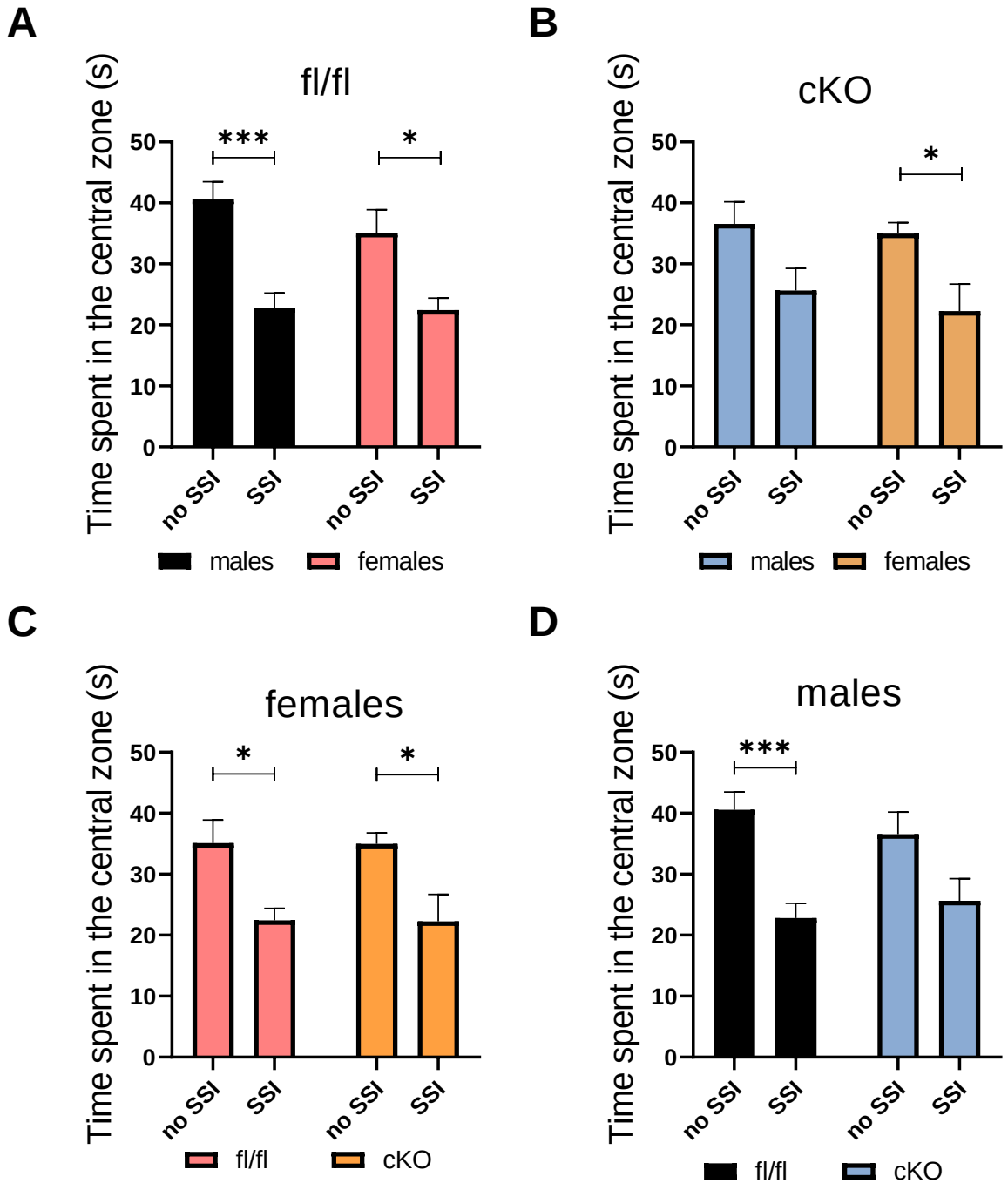


Figure 4.22: Time spent in the central zone of an OF arena was reduced following SSI in fl/fl mice and cKO females.

A) Time spent in the central zone of the OF arena in fl/fl mice in no SSI (females n = 5, males n = 13) and under SSI conditions (females n = 6, males n = 6). There was a signficant effect of isolation condition on time spent in the central zone (***, *p*=0.0001). B) Time spent in the central zone of the OF arena in cKO mice in no SSI (females n = 5, males n = 11) and under SSI conditions (females n = 6 and males n = 8). There was a signficant effect of isolation condition on time spent in the central zone (**, *p*=0.0065). C) Time spent in the central zone of the OF arena in females in no SSI (fl/fl n = 5, cKO n = 5) and under SSI conditions (fl/fl n = 6, cKO n = 6). There was a signficant effect of isolation condition on time spent in the central zone (**, *p*=0.0011). D) Time spent in the central zone of the OF

arena in males in no SSI (fl/fl n = 13, cKO n = 11) and under SSI conditions (fl/fl n = 6, cKO n = 8). There was a significant effect of isolation condition on time spent in the central zone (***, p=0.0003).

Another parameter of OF that is commonly analysed is the total distance travelled throughout the five minute test (Sullens et al., 2021, Yesudhas et al., 2021). A lower distance travelled is considered indicative of increased anxiety, whilst a higher distance travelled can suggest hyperactivity (Ieraci et al., 2016, Molina-Hernandez et al., 2001, Seibenhener and Wooten, 2015). There was no significant interaction between genotype and isolation condition on distance travelled in the OF arena (F(1,57)=0.7646, p=0.3856). However, there was a significant effect of genotype on distance travelled (F(1,57)=5.777, p=0.0195). cKO showed a higher distance travelled compared to fl/fl mice, irrespective of isolation condition (Šídák's post-hoc, p=0.0293) (Figure 4.23).

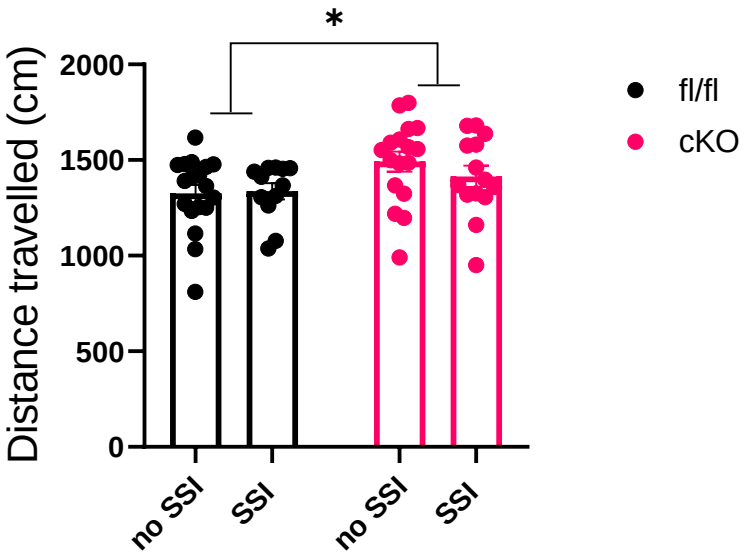


Figure 4.23: Distance travelled in OFs showed significant differences between fl/fl and cKO males.

Distance travelled in the OF arena in fl/fl and cKO mice in no SSI (fl/fl n = 18, cKO n = 16) and in fl/fl and cKO mice under SSI (fl/fl n = 12, cKO n = 14). There was a significant effect of genotype on distance travelled across conditions (*, p=0.0195).

To investigate if this genotype difference varies by sex, genotypes were split into sex sub-groups. Supplementary two-way ANOVA analysis showed no significant effects of sex, isolation condition or the interaction between the two on distance travelled in either fl/fl or

cKO mice (interaction ($F(1,26)=0.006828$, $p=0.9348$), ($F(1,26)=3.273$, $p=0.0816$), respectively). Additionally, no significant effect of genotype, isolation or the interaction between the two was seen on distance travelled in either male or female mice (interaction ($F(1,35)=0.08013$, $p=0.7788$), ($F(1,18)=2.227$, $p=0.1530$, respectively). However, male mice did show a significant effect of genotype on distance travelled within the OF arena, where cKO males had heightened activity under both conditions ($F(1,35)=7.120$, $p=0.0115$) (Figure 4.24C, 4.24D).

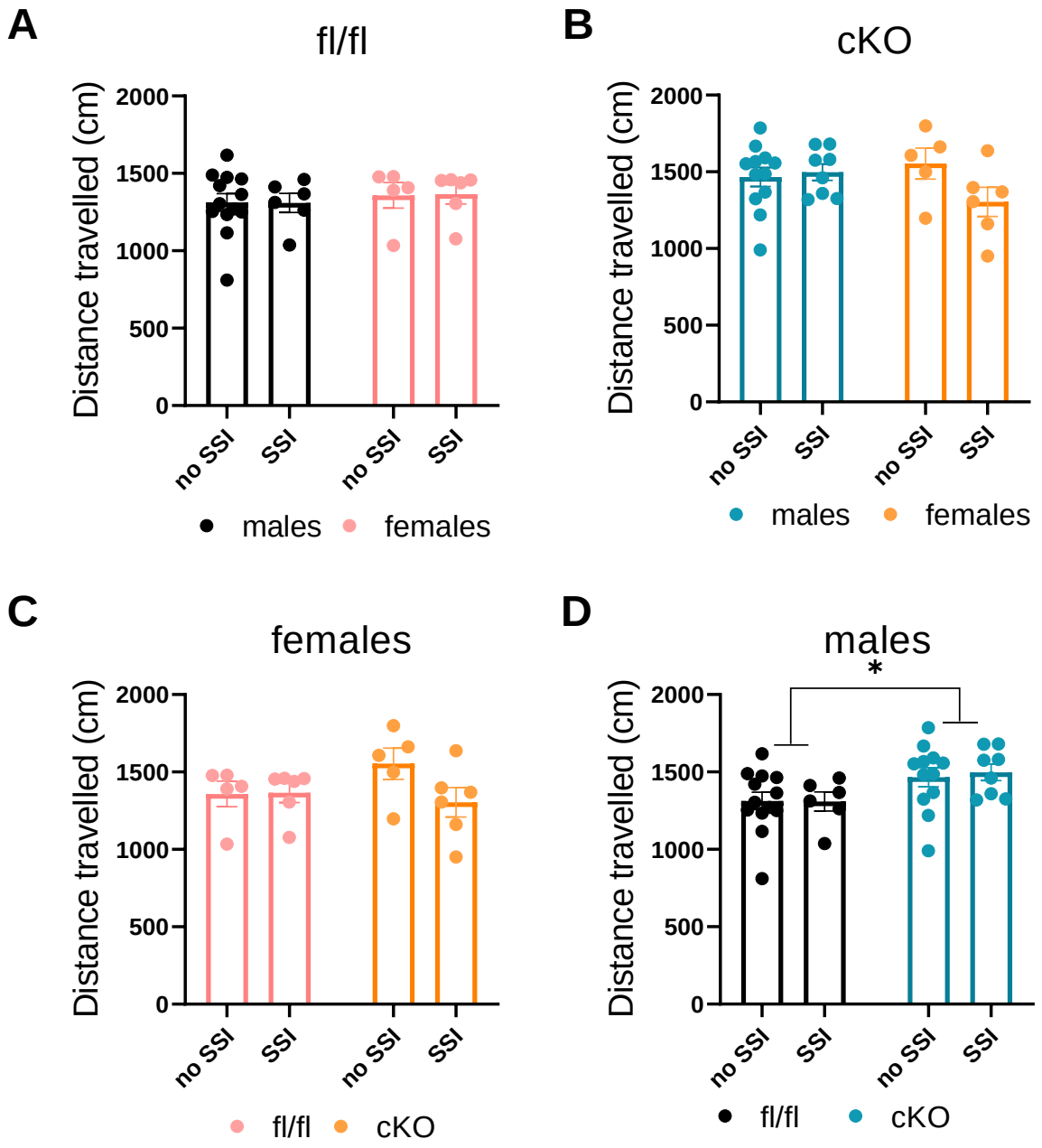


Figure 4.24: Distance travelled in OFs showed significant differences between fl/fl and cKO males.

A) Distance travelled in the OF arena in fl/fl mice in no SSI (females n = 5, males n = 13) and under SSI conditions (females n = 6, males n = 6). B) Distance travelled in the OF arena in cKO mice in no SSI (females n = 5, males n = 11) and under SSI conditions (females n = 6 and males n = 8). C) Distance travelled in the OF arena in females in no SSI (fl/fl n = 5, cKO n = 5) and under SSI conditions (fl/fl n = 6, cKO n = 6). D) Distance travelled in the OF arena in males in no SSI (fl/fl n = 13, cKO n = 11) and under SSI conditions (fl/fl n = 6, cKO n = 8). There was a significant effect of genotype on distance travelled across conditions (*, p=0.0115).

4.3.9 Sex and genotype differences in OF performed following SSI in LL conditions

A cohort of cKO and fl/fl mice also performed OF following seven days of LL conditions. LL SSI was employed to replicate the most anxiogenic lighting condition utilised during the NOR experiments in 3.2.4. Firstly, two-way ANOVA analysis revealed no significant interaction between genotype and zone type on time spent in each zone in LL SSI ($F(1,28)=0.4417$, $p=0.5117$). However, there was a significant effect of zone type ($F(1,28)=5145$, $p<0.0001$). Both fl/fl and cKO mice spent more time in the peripheral zone in LL SSI (Šídák's post-hoc, both $p<0.0001$) (Figure 4.25).

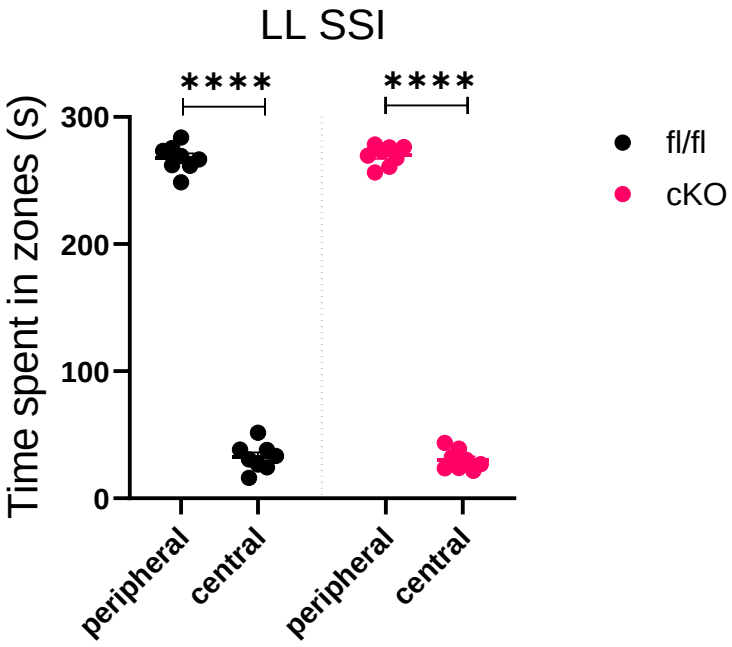


Figure 4.25: Fl/fl and cKO mice both preferred the peripheral area of an OF arena in LL SSI conditions.

Time spent in the central versus peripheral zones of the OF arena by fl/fl (n = 8) and cKO (n = 8) mice in LL SSI conditions. There was a significant effect of zone type on time spent in zones (****, $p<0.0001$).

There was also no significant interaction between genotype and isolation condition on time spent in the central zone ($F(2,70)=0.4712$, $p=0.6262$). However, a significant effect of isolation condition on time spent in the central zone was seen ($F(2,70)=16.83$, $p<0.0001$). The

significant reduction of time spent in the central zone noted following SSI in 4.3.6 was not seen following LL SSI conditions in either genotype. In fact, genotypes increased their time spent in the central zone following LL SSI compared to SSI in normal LD cycling conditions, though only fl/fl mice did this significantly (Šídák's post-hoc, p=0.0131). This suggests LL SSI was not as anxiogenic as SSI in LD conditions. (Figure 4.26A).

When assessed in sex sub-groups using supplementary two-way ANOVA analysis, LL SSI showed a significant effect of sex on time spent in the central zone (F(1,12)=7.296, p=0.0193). Females of both genotypes show reduced central zone exploration compared to males, which indicates that females may be more anxious that males irrespective of genotype. Following seven days of LL SSI, fl/fl males spent significantly longer in the OF central zone than fl/fl females (Šídák's post-hoc, p=0.0236) (Figure 4.26B).

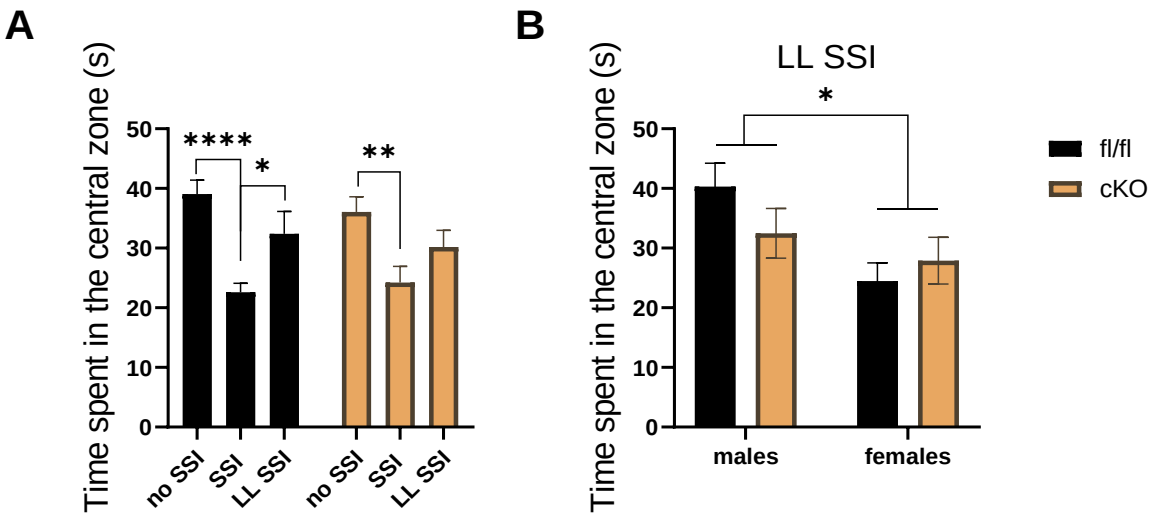


Figure 4.26: LL SSI had a sex-specific effect on time spent in the central zone.

A) Time spent in the central zone of the OF arena of fl/fl (no SSI n = 18, SSI n = 12, LL SSI n = 8) and cKO (no SSI n = 16, SSI n = 14, LL SSI n = 8). There was a significant effect of isolation condition on time spent in the central zone (****, p<0.0001). Fl/fl mice significantly increased their time spent in the central zone in LL SSI compared to SSI in LD cycling conditions (*, p=0.0131). B) Time spent in the central zone of the OF arena in LL SSI conditions of fl/fl mice (males n = 4, females n = 4) and cKO mice (males n = 4, females n = 4). A significant effect of sex on time spent in the central zone was seen across genotypes (*, p=0.0193).

Distance travelled was then compared from no SSI, SSI and LL SSI conditions. A significant interaction between genotype and isolation condition was seen on distance travelled in the OF arena ($F(2,70)=4.035$, $p=0.0219$). There was also a significant effect of isolation condition on OF distance travelled ($F(2,70)=8.493$, $p=0.0005$). cKO distance travelled remained relatively similar in all three isolation conditions, whilst fl/fl distance travelled significantly increased in LL SSI compared to no SSI, and SSI conditions (Šídák's post-hoc, $p<0.0001$, $p=0.0003$, respectively) (Figure 4.27A).

In supplementary three-way ANOVA analysis of sex sub-groups, there remained an interaction between isolation condition and genotype (fl/fl males and fl/fl females versus cKO males and cKO females) ($F(2,70)=4.115$, $p=0.0208$). There was also a significant effect of isolation condition on OF distance travelled within sex groups ($F(2,70)=8.762$, $p=0.0004$). Fl/fl males significantly increased their distance travelled in LL SSI compared to no SSI and SSI OFs (Šídák's post-hoc, $p=0.0089$, $p=0.0212$, respectively), as did fl/fl females (Šídák's post-hoc, $p=0.0069$, $p=0.0057$, respectively). This suggests an anxiolytic effect of LL SSI in fl/fl mice. cKO males demonstrated a reduced distance travelled in LL SSI open field trials compared to fl/fl males (Šídák's post-hoc, $p=0.0363$). Whilst cKO females only increased their distance travelled in LL SSI compared to SSI conditions (Šídák's post-hoc, $p=0.0125$). (Figure 4.27B). Though sex groups were underpowered, these results suggest future investigation is waranted.

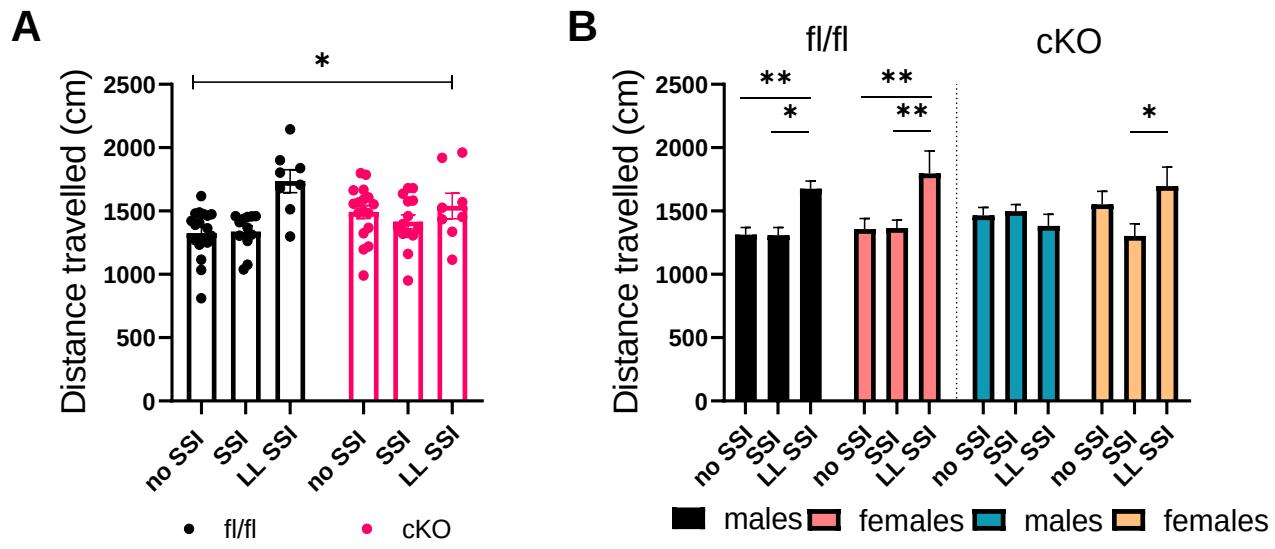


Figure 4.27: Genotype and isolation condition effects were seen on distance travelled in LL SSI.

A) Distance travelled in the OF arena in no SSI (fl/fl n = 18, cKO n = 16), SSI (fl/fl n = 12, cKO n = 14) and LL SSI (fl/fl n = 8, cKO n = 8) conditions. There was a significant interaction between isolation condition and genotype on distance travelled (*, p=0.0219). Fl/fl mice increased their distance travelled in LL SSI compared to no SSI and SSI OFs (****, p<0.0001, ***, p=0.0003, respectively). B) Distance travelled in the OF arena in fl/fl males (no SSI n = 13, SSI n = 6, LL SSI n = 4), fl/fl females (no SSI n = 5, SSI n = 6, LL SSI n = 4), cKO males (no SSI n = 11, SSI n = 8, LL SSI n = 4) and cKO females (no SSI n = 5, SSI n = 6, LL SSI n = 4). A significant effect of sex X genotype X isolation condition was seen (*, p=0.0208).

4.3.10 Genotype differences in OF performed following extended SSI

A cohort of cKO and fl/fl mice also performed OF following extended SSI of variable lighting conditions (LD for five days, DD for 10 days, LL for 10 days, and then seven days of LD before testing). This was to replicate the elongated isolation and variable lighting conditions mice underwent in NOR experiments in 3.2.4. Firstly, two-way ANOVA analysis revealed no significant interaction between genotype and zone type on time spent in each zone in extended SSI conditions (F(1,28)=0.3718, p=0.5117). However, there was a significant effect of zone type (F(1,28)=2567, p<0.0001). Both fl/fl and cKO mice spent more time in the peripheral zone in extended SSI (Šídák's post-hoc, both p<0.0001) (Figure 4.28).

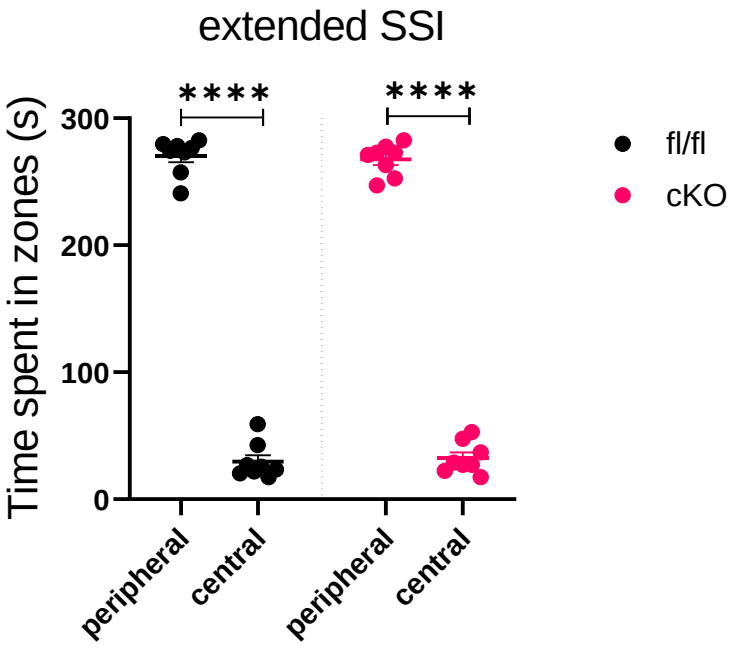


Figure 4.28: Fl/fl and cKO mice both preferred the peripheral area of an OF arena in extended SSI conditions.

Time spent in the central versus peripheral zones of the OF arena by fl/fl (n = 8) and cKO (n = 8) mice in extended SSI conditions. There was a significant effect of zone type on time spent in zones (****, $p<0.0001$).

Furthermore, there was no significant interaction between genotype and isolation condition on time spent in the central zone ($F(2,70)=0.5951$, $p=0.5543$). However, there was a significant effect of isolation condition ($F(2,70)=13.99$, $p<0.0001$). No significant reduction in time spent in the central zone was seen following extended SSI conditions in comparison to no SSI in either genotype, suggesting extended SSI was not as anxiogenic as seven days of LD SSI (Figure 4.29A). When assessed in sex sub-groups, extended SSI showed no significant effects of genotype, sex, or interaction between sex and genotype (supplementary two-way ANOVA, interaction ($F(1,12)=0.4638$, $p=0.5088$) (Figure 4.29B).

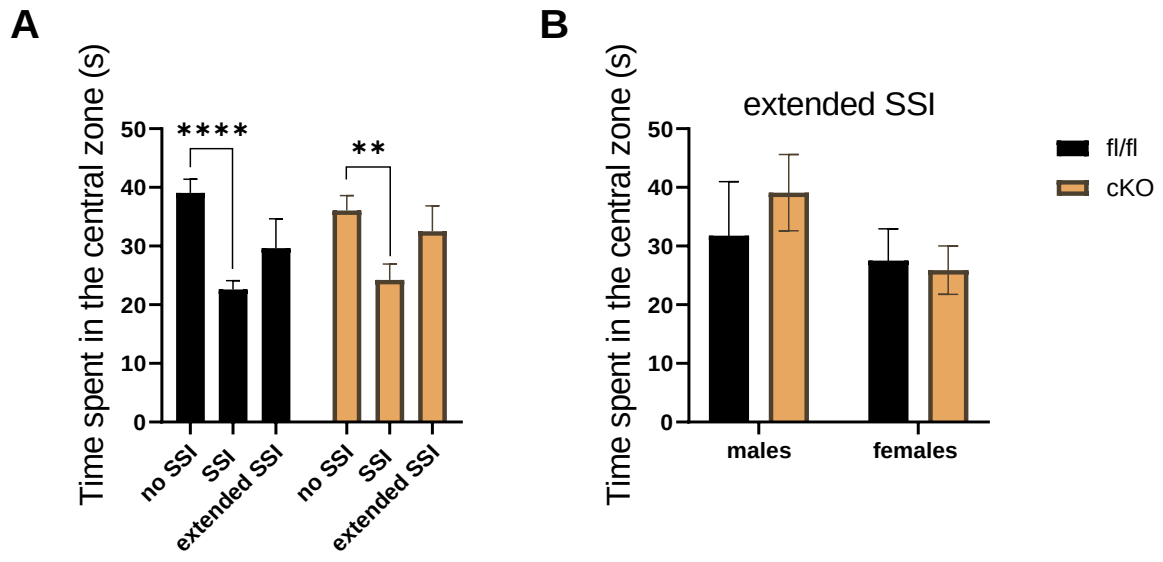


Figure 4.29: Time spent in the central zone of an OF arena was not significantly reduced in extended SSI compared to no SSI.

A) Time spent in the central zone of the OF arena of fl/fl (no SSI n = 18, SSI n = 12, extended SSI n = 8) and cKO (no SSI n = 16, SSI n = 14, extended SSI n = 8) mice. There was a significant effect of isolation condition on time spent in the central zone (****, p<0.0001). B) Time spent in the central zone of the OF arena in extended SSI conditions of fl/fl mice (males n = 4, females n = 4) and cKO mice (males n = 4, females n = 4).

Distance travelled was then compared from no SSI, SSI and extended SSI conditions. A significant effect of genotype on OF distance travelled (F(2,70)=6.057, p=0.0163) (Figure 4.30A). When compared in sex sub-groups, there was also a significant effect of genotype (fl/fl males and females versus cKO males and females) on distance travelled (supplementary three-way ANOVA, F(1,70)=5.481, p=0.0223) (Figure 4.30B). These results suggest underpowered sex groups warant additional investigation.

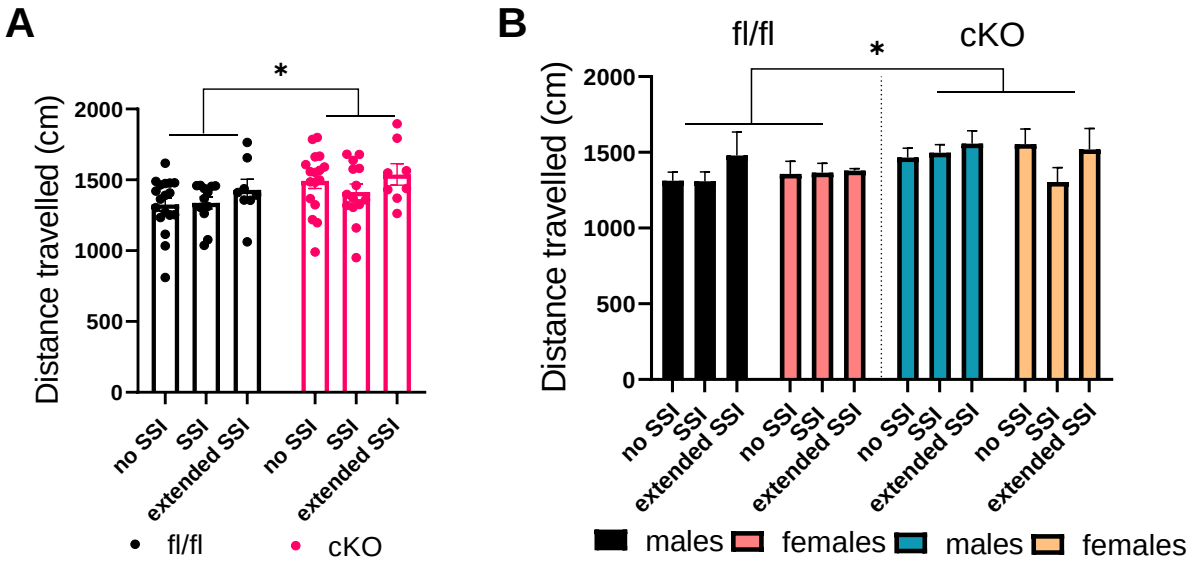


Figure 4.30: Distance travelled in the OF arena was not significantly different in extended SSI compared to other conditions.

A) Distance travelled in the OF arena in no SSI (fl/fl n = 18, cKO n = 16), SSI (fl/fl n = 12, cKO n = 14) and extended SSI (fl/fl n = 8, cKO n = 8) conditions. There was a significant effect of genotype on time spent in the OF arena across conditions (*, p=0.0163). B) Distance travelled in the OF arena in fl/fl males (no SSI n = 13, SSI n = 6, LL SSI n = 4), fl/fl females (no SSI n = 5, SSI n = 6, LL SSI n = 4), cKO males (no SSI n = 11, SSI n = 8, LL SSI n = 4) and cKO females (no SSI n = 5, SSI n = 6, LL SSI n = 4). A significant effect of genotype (fl/fl male and female versus cKO male and female) was seen (*, p=0.0223).

Addendum:

This analysis could have been improved by performing three-way ANOVAs which included sex, genotype and lighting condition as variables, in replacement of two way ANOVAs individually assessing the effect of genotype and then sex. Without demonstration of an interaction between sex X genotype, any claim that the two genotypes show variable sex differences has limited statistical support.

4.4 Discussion

To summarise, significant female-specific weight loss was noted in all four transgenic lines, yet not within C57BL/6J mice. Secondly, OF results represented a clear induction of anxiety within all transgenic lines following SSI conditions whilst LD box tests showed more varied

outcomes. Neither LL SSI, nor extended SSI, were seen to exacerbate SSI phenotypes and anxiety characteristics were seen to be reduced in comparison to seven days of SSI conditions. Key results are summarised in Table 4.4.

LD box	• Afp cKO males' latency to enter the dark chamber was longer in both no SSI and SSI compared to fl/fl males • Afp cKO and Afp fl/fl showed increased transitions and aborted entries in SSI • cKO and fl/fl (Alb) showed increased aborted entries after SSI exposure • fl/fl (Alb) females show reduced aborted entries in both no SSI and SSI
OF arena	• Afp cKO and Afp fl/fl showed reduced time in the central zone following SSI • cKO and fl/fl mice (Alb) reduced time spent in the central zone following SSI • cKO (Alb) demonstrated increased distance travelled in both conditions compared to fl/fl controls

Table 4.4: Summary table of key results from Chapter 4.

4.4.1 Significant sex-linked weight loss observed in all transgenic lines was not present in C57BL/6J mice

Significant weight loss was noticed in females of all four transgenic lines following the commencement of experimental conditions prior to lighting condition alterations. Minor weight loss was also seen in the males of all four transgenic lines, though C57BL/6J mice displayed consistent weight maintenance and/or weight gain throughout identical conditions. The significant weight loss noticed in both Afp cKO and Afp fl/fl mice was far more severe in females than males. Identical effects were noted in cKO and fl/fl mice, who also displayed significant weight loss within seven days of being placed in SSI. Previous work with these mutant mice has revealed that hepatic NPAS2 is vital in determining prolonged metabolic homeostasis, and that altering hepatic NPAS2 expression amounts to gut microbiome dysbiosis. The circadian clock is thought to be intrinsically linked to the gut microbiome, with a bidirectional modulation between the two. Components of the gut microbiome demonstrate circadian rhythmicity that is absent in mice lacking core clock genes; similarly, mice that lack

gut microbes display altered peripheral circadian gene expression in the liver (O'Neil et al., 2017). O'Neil et al. (2017) concluded that NPAS2 has an epigenomic role in metabolic homeostasis governed by the maternal diet, and that this role manifests itself primarily during restricted feeding. In addition, the result was sexually dimorphic, since hepatic NPAS2 expression was not seen to be essential for adaptations to dietary stress in females (O'Neil et al. 2013, O'Neil et al., 2017).

In contrast, whilst no dietary stress was induced in our experiments, the results show females are more severely affected rather than protected from the weight loss. Additionally, mice were separated into groups of those with an Afp cKO/cKO mother, and those with a Afp fl/fl or fl/fl mother to assess if maternal genotype influenced weight loss, but no significant differences were seen (see A.1.1). Since NPAS2 in the liver is linked to both metabolism and the gut microbiome, it is possible that the weight loss is a consequence of hepatic NPAS2 deletion, however, no significant difference was noted between fl/fl controls and Afp cKO/cKO mice. All four transgenic lines demonstrate similar weight loss. Additionally, to rule out the influence of reduced consumption, food was weighed daily to confirm that all animals in SSI conditions were consuming enough. Food intake was maintained at >3 g a day during isolation (see A.1.2), meaning adequate consumption was displayed despite the weight loss. Additionally, Fonken et al. demonstrated significant weight gain in mice held in LL conditions. This effect was replicated in the above experiments when variable light conditions entered constant light. This is likely a product of the significantly reduced locomotor activity seen in LL conditions in 2.3.3 and 2.3.4, and maintained food intake resulting in weight stabilisation or weight gain in the transgenic lines (Fonken et al., 2010). The significant weight loss is also not seen in experiments using C57BL/6J mice, thus bringing into question the effects of floxing NPAS2. Whilst a genomic post-mortem confirmation concluded that fl/fl controls do not show the reduced band size of *Npas2* displayed within deleted tissues, this does not rule out potential

splicing problems. Thus, a confirmation of protein expression, and an mRNA confirmation are needed to ensure maintained NPAS2 expression in fl/fl tissues and to rule out splicing events in fl/fl mice.

Since the weight loss seen in the transgenic lines is triggered upon SSI, another potential explanation is the induction of anxiety that is disproportionately acting on the females of both genotypes. A generally well-accepted product of social isolation is anxiety, which could result in a reduction in weight (González and Aston-Jones, 2008, Ieraci et al., 2016, Jameie et al., 2016, Kwak et al., 2009, Marin et al., 2015, Tapia-Osorio et al., 2013). To test this hypothesis, anxiety trials were performed in both group housing and SSI conditions.

4.4.2 SSI reliably reduced time spent in the central zone of an OF arena in all genotypes

Anxiety trials were performed to explore a possible explanation for significant sex-linked weight loss in SSI, and also poorer cognitive performances of fl/fl females in anxiogenic lighting conditions. In OF, a robust preference for the peripheral zone was demonstrated by all sexes and genotypes in both no SSI and SSI conditions. This is in line with literature which suggests mice feel less exposed and vulnerable remaining close to the periphery of the arena, and often predominantly explore the area closest to the arena walls (Choleris et al., 2001, Siebenhener and Wooten, 2015). Additionally, all four transgenic lines demonstrated a significant reduction in time spent in the central zone of the OF arena following one week of SSI conditions. The central zone is considered the most anxiogenic area for the mice, as this is where they are most exposed and vulnerable. To significantly reduce the time spent in the central zone following a period of SSI is indicative of increased anxiety under those conditions. This mirrors previous studies which have demonstrated significant increases in anxiety phenotypes following social deprivation and isolation in OFs (Berry et al., 2011, Haj-Mirzaian et al., 2019, Ieraci et al., 2016, Koike et al., 2011, Ma et al., 2011). However, SSI, rather than

complete social isolation was employed in our experiments which is where animals can see, hear, and smell three other mice but cannot directly interact with them. As a result, this is considered less severe to complete social isolation, especially given social odour cues coming from the mice and their urine are still present (Berry et al., 2012). This suggests that even lack of physical social interaction is sufficient to increase anxiety parameters in OF within these lines.

When this was explored in the context of sex, Afp fl/fl females, Afp cKO males and Afp cKO females demonstrated significant reductions in time spent in the central zone of an OF arena following SSI, whilst Afp fl/fl males reduced their time non-significantly. This could demonstrate that Afp fl/fl males were less effected by the SSI conditions, though no significant Afp fl/fl sex differences were noted. Additionally, fl/fl males, fl/fl females, and cKO females within the Alb lines demonstrated significantly reduced time spent in the central zone of the OF arena following SSI, whilst cKO males reduced their time non-significantly. Again, no sex differences were noted between cKO mice, so this is unlikely to be a sex effect in anxiety induction following SSI. The same pattern of central zone time reduction following SSI was seen in both groups that failed to reach statistical significance (cKO males and Afp fl/fl males) and therefore it may be that reduced n numbers within these experiments restricted the significance of this effect in those sex sub-groups.

Furthermore, OF performed in cKO and fl/fl mice were also analysed for total distance travelled within the test. Many studies employing either rats or mice have shown increased distance travelled in the OF arena trial as a result of social deprivation, which can indicate hyperactivity (Bakshi and Geyer, 1999, Haj-Mirzaian et al., 2019, Heidbreder et al., 2000, Hilakivi et al., 1989, Ieraci et al., 2016, Molina-Hernandez et al., 2001). Interestingly, distance travelled in the OF arena demonstrated a genotype effect, where distance travelled across isolation conditions was higher in cKO mice than fl/fl mice. When this was assessed at sex

sub-group level, this effect of genotype on distance travelled was seen to be significant between fl/fl males and cKO males as well. General locomotor activity does not significantly differ between cKO and fl/fl mice (see 2.3.4), therefore this genotype effect in distance travelled in the OF arena could indicate that cKO mice are more hyperactive under such test conditions. This is in line with Ozburn et al. who discovered significantly reduced anxiety phenotypes in global *Npas2* mutant mice in LD box tests, OF and elevated plus maze tests. Loss of global *Npas2* in OFs revealed an increased distance travelled in the central area and increased time spent in the central area. Though, Garcia et al. contradictorily concluded that there was no anxiety phenotype in global NPAS2 knockout mice under the same tests (Garcia et al., 2000, Ozburn et al., 2017). Thus, more anxiety parameters would need to be assessed to conclude this phenotype. Additionally, as this is a hepatic NPAS2 knockout demonstrating modest anxiolytic characteristics, it furthers the need for more post-mortem confirmation of intact *Npas2* expression within the brain.

In summary, all genotypes tested by OF in this study robustly demonstrated increased characteristics of anxiety following SSI, in line with literature detailing social deprivation amounts to heightened anxiety (Bakshi and Geyer, 1999, Haj-Mirzaian et al., 2019, Heidbreder et al., 2000, Hilakivi et al., 1989, Ieraci et al., 2016, Molina-Hernandez et al., 2001). This suggests SSI could be capable of inducing significant weight loss prior to variable lighting conditions; though there were no sex differences displayed in OF arena parameters, meaning the increased severity of weight loss in female mice is yet to be explained.

4.4.3 LD box tests revealed variable results of SSI, genotype, and sex on anxiety in Afp transgenic lines

Alongside OFs, Afp fl/fl and Afp cKO mice underwent LD box tests in normal group housing and following seven days of SSI conditions. One of the key parameters assessed in LD

box tests is latency to enter dark. The faster a subject enters the dark chamber, the more anxious they are considered to be (Borsini et al., 2002, Costall et al., 1989, Heredia et al., 2014). LD box tests performed on Afp fl/fl and Afp cKO mice revealed no significant genotype or isolation condition differences in latency to enter dark. This suggests that SSI was not able to induce anxiety within this LD test parameter. It also displays no evidence of hepatic NPAS2 loss amounting to reduced anxiety, either basally in normal group housing conditions, or in SSI. Ozburn et al. (2017) noted a reduced latency to enter the dark chamber in global *Npas2* knockouts compared to wild type mice. This suggests a hepatic knockout is not capable of reproducing such a result of reduced anxiety in this parameter. However, Garcia et al. noted no significant differences in global *Npas2* knockout mice compared to wild type within this test, so it is disputed than even global *Npas2* loss can lead to increased anxiety (Garcia et al., 2000, Ozburn et al., 2017). Despite the lack of an overall genotype effect, there was a significant effect of genotype on male latency to enter dark, with cKO mice displaying increased latency indicative of heightened anxiety.

Another key parameter of the LD box test is time spent in each chamber. Most literature suggests a nocturnal rodent will favour the dark chamber, as this has preferential conditions when anxious (Borsini et al., 2002, Heredia et al., 2014, Kulesskaya and Voikar, 2014, Miller et al., 2011). However, this study found mice spent significantly more time in the light chamber compared to the dark chamber, regardless of genotype, sex, or isolation condition. This is not an abnormal finding for protocols which use the light chamber as the starting chamber. Kulesskaya and Voikar (2014) noted that initial placement of mice in either chamber influences chamber preference, when a LD box test uses the light chamber as the starting point, the subject tends to spend longer in the light chamber (Kulesskaya and Voikar, 2014).

Furthermore, there were no genotype or isolation effects on time spent in the light chamber, which furthers the argument that loss of hepatic *Npas2* does not alter anxiety

characteristics in LD box tests. However, it also demonstrates that SSI has not altered anxiety characteristics in this trial. Findings on anxiety in LD box trials are very variable, often due to large disparities in protocol. Previous studies have demonstrated reduced time spent in the light chamber following social isolation in adult male and female mice. Since Rivera-Irizarry et al. employed six weeks of social isolation, it suggests that either SSI is not as unfavourable as complete social isolation, or that seven days is inadequate at inducing anxiety (Rivera-Irizarry et al., 2020). However, other studies present an anxiolytic effect of social deprivation on LD box trials, with an increased time spent in the light chamber and less time spent in the less exposed dark chamber (Guo et al., 2004, Lopez and Laber, 2015). Interestingly, time spent in the light chamber did demonstrate significant sex differences in Afp fl/fl mice across isolation conditions. Afp fl/fl females spent significantly longer in the light chamber than Afp fl/fl males, indicative of reduced anxiety in Afp fl/fl females in comparison to male counterparts. This is surprising, since female mice are typically considered to be more anxious than male mice, and Afp fl/fl females demonstrated more severe weight loss (Dugandzic et al., 2019, Li et al., 2016, Marchette et al., 2018, Mulvey et al., 2018).

Furthermore, there was a significant effect of isolation condition on both LD box transitions and aborted entries. Both genotypes displayed increased aborted entries and increased transitions between chambers following SSI conditions, which is somewhat contradictory. Increased aborted entries is indicative of heightened anxiety, where the animal is retreating from aversive areas. On the other hand, increased transitions suggest anxiolytic effects of SSI, and elevated exploration (Crawley and Davis, 1982). Interestingly, males demonstrate a genotype effect on transitions, with Afp fl/fl males showing higher transitions compared to Afp cKO males across isolation conditions. This suggests increased anxiety in Afp cKO males, which is not seen in Afp cKO females compared to Afp fl/fl females. Since there was also a male genotype effect on latency to enter the dark, where Afp cKO males

demonstrated an increased latency and thus heightened anxiety, this combination makes a case for increased anxiety in Afp cKO males in comparison to Afp fl/fl males; an effect that is absent in females. This increased anxiety contradicts the findings of reduced anxiety in global *Npas2* knockouts noted by Ozburn et al., and no anxiety phenotype noted by Garcia et al. (Garcia et al., 2000, Ozburn et al., 2017).

Additionally, there are significant sex and isolation differences in aborted entries throughout all Afp genotype/sex sub-groups. Afp fl/fl females increase their number of aborted entries following SSI, indicating elevated anxiety, whilst Afp fl/fl maintain similar numbers of aborted entries between conditions. Afp cKO males show increased aborted entries in SSI, whilst Afp cKO females do not. This follows the phenotype of heightened anxiety in Afp cKO males within the LD box test. As well as this, this parameter demonstrates heightened anxiety in Afp fl/fl females compared to males, as commonly seen in the literature, though this is the only parameter that demonstrates sexual dimorphism (Dugandzic et al., 2019, Li et al., 2016, Marchette et al., 2018, Mulvey et al., 2018). Unlike the OF arena trial, the LD box test does not robustly demonstrate induced anxiety following SSI. This suggests SSI may not be the sole factor at play in significant weight loss prior to variable lighting conditions. Especially given neither anxiety test demonstrated sex differences, meaning the increased severity of weight loss in female mice is still unexplained.

4.4.4 LD box tests revealed variable results of SSI, genotype and sex on anxiety in fl/fl and cKO mice

LD box tests performed on fl/fl and cKO mice revealed no significant genotype or isolation condition differences in latency to enter dark. This suggests that SSI was not able to induce anxiety within this LD test parameter. It also displays no evidence of hepatic NPAS2 loss amounting to reduced anxiety, either in normal group housing conditions, or in SSI. This suggests a hepatic knockout is not capable of reproducing the result of reduced anxiety

demonstrated in global *Npas2* knockouts in Ozburn et al., though this is disputed (Garcia et al., 2000, Ozburn et al., 2017). However, comparing genotypes overall was masking significant sexual dimorphism in LD box anxiety parameters that persisted in hepatic NPAS2 absence. Under no SSI conditions males of both genotypes had a significantly higher latency to enter the dark chamber than females, but following seven days of SSI, sexes of both genotypes had similar latency to enter the dark chamber. This suggests a significantly increased anxiety response in both genotypes of males when group housed that is abolished under conditions of isolation. Since it is generally accepted that social isolation can increase anxiety parameters in many behavioural paradigms such as plus maze trials, and forced swim tests, this is an unexpected finding (Ieraci et al., 2016, Kwak et al., 2009, McCool and Chappell, 2009). A potential explanation for this result is the excessive aggression in male group housing. In males, a strict dominance hierarchy is put in place in which subordinate males tend to have reduced weight and bald patches in their fur as a result of the aggressive grooming behaviours. In this condition, a subordinate male cannot escape the hierarchical structure, whilst SSI allows freedom from said aggressor and could potentially reduce anxiety despite the isolation (Lidster et al., 2019). Previous studies involving isolation have displayed similar results to these findings. Lopez and Labour (2014) found males spent significantly more time in the dark chamber of the LD box test when group housed, and that anxiety parameters were notably reduced when males were socially isolated. However, other studies have indicated a reduction in time spent in the dark chamber following isolation to be in both sexes, and thus contradicting this male group housed theory (Lopez and Labour, 2015, Rivera-Irizarry et al., 2020). What is also of interest is that Afp transgenic lines did not demonstrate this effect.

Additionally, there was also a significant effect of genotype on male latency to enter dark, with cKO mice displaying increased latency, indicative of heightened anxiety. This was also seen in Afp transgenic lines (see 4.4.3). Together, these findings make a case for increased

anxiety in Afp cKO/cKO males in comparison to Afp fl/fl and fl/fl males; a male-specific effect of hepatic NPAS2 loss on anxiety that is absent in females. This increased anxiety contradicts the findings of reduced anxiety noted by Ozburn et al., which depicted a slower latency to enter the dark chamber in global *Npas2* knockouts, and the phenotype of no anxiety changes noted by Garcia et al. (Garcia et al., 2000, Ozburn et al., 2017). However, as mentioned previously, since this is a hepatic NPAS2 knockout demonstrating modest anxiolytic characteristics, it furthers the need for post-mortem confirmation of intact *Npas2* expression within the brain. These findings of male disparities in anxiety following hepatic NPAS2 loss also do not help to explain fl/fl female NOR failings and sexual dimorphism in cognition.

No noteworthy differences in time spent in the light chamber were seen in fl/fl and cKO mice, other than a moderate sex difference in cKO no SSI, where female cKO mice spent longer in the light chamber than males. However, as seen in Afp fl/fl and Afp cKO mice, number of aborted entries in the LD box test was increased following SSI in cKO and fl/fl mice. This indicates that SSI was capable of inducing moderate anxiety phenotypes in all transgenic lines based on this parameter (Griebel et al., 1996, Bourin and Hascoët, 2003). Interestingly, there was also a significant interaction between sex and isolation condition on fl/fl transitions between chambers. Fl/fl males had significantly higher transitions in no SSI conditions compared to fl/fl females, though under SSI conditions, females increased transitions and males reduced transitions. In addition, fl/fl females displayed significantly reduced aborted entries in comparison to fl/fl males regardless of isolation condition, adding to the argument that they were in fact less anxious than male counterparts (Griebel et al., 1996, Bourin and Hascoët, 2003). This suggests SSI is anxiolytic for fl/fl females and amounts to increased exploration, which contradicts the hypothesis that fl/fl females may be more susceptible to isolation stress and subsequently perform poorly in NOR trials (Bourin and Hascoët, 2003, Kulesskaya and Voikar, 2014).

In summary, all four transgenic lines demonstrated increased anxiety following SSI in OF. However, LD box trials gave varied and inconclusive results, with some parameters displaying increased anxiety, and others reduced anxiety. Similarly, sex and genotype differences were modest and fluctuated across conditions. It is therefore unlikely that significant sex-linked weight loss is entirely dependent on anxiety induction from SSI conditions. Likewise, lack of signficiant or consistent anxiety sex differences in LD box and OF results suggest sex-specific anxiety is unlikely to be the explanation behind significant cognitive disparities in fl/fl mice. Since LD box results did not robustly replicate increased anxiety seen in OFs following SSI, future work should aim to add additional anxiety tests, such as elevated plus maze and social interaction trials.

4.4.5 Extended SSI and LL SSI do not appear to be anxiogenic in comparison to no SSI and SSI conditions

Since significant differences in cognitive output were noted between genotypes and sexes during conditions of LL (see 3.3.3), it was hypothesized that LL may disproportionately effect fl/fl females, giving elevated anxiety and amounting to poorer cognitive performance. This was based on prior findings which suggest female mice are generally more anxious than males, a sexual dimorphism that was hypothesized to be lost in cKO mice, and also that LL is capable of inducing anxiety and depression-like phenotypes (Dugandzic et al., 2019, Fonken et al., 2009, Li et al., 2016, Marchette et al., 2018, Mulvey et al., 2018, Zhou et al., 2018). OF were performed following seven days of SSI in LL conditions, which mimicked the conditions of social deprivation seen in LL NOR experiments. These trials showed no significant reduction in time spent in the central zone following LL SSI, in fact, fl/fl mice demonstrated an increased in time spent in the central zone during LL SSI compared to no SSI and SSI conditions. This suggests that LL SSI did not induce anxiety within either genotype, and may have even been less anxiogenic than normal LD cycling SSI conditions for fl/fl mice. This is in line with Fonken et al. who displayed no

increased anxiety-like characteristics in OFs following three weeks of constant light, though this did not employ social isolation in combination with LL conditions (Fonken et al., 2009). However, it contradicts other studies which demonstrate significant anxiety induction in LL conditions within OFs and elevated-plus maze tests (Castro et al., 2005, Zhou et al., 2018).

Another noteworthy finding was that LL SSI had a significant sex effect of time spent in the central zone irrespective of genotype. Both fl/fl and cKO females spent significantly less time in the central zone under this condition than male counterparts, indicating elevated anxiety in females. This supports the hypothesis that female mice are more anxious (Dugandzic et al., 2019, Li et al., 2016, Marchette et al., 2018, Mulvey et al., 2018). It also significantly contributes to the hypothesis that fl/fl female failures in recognition memory under conditions of constant light may be a result of sexual dimorphism in anxiety. This is contrasted by distance travelled findings, which display both fl/fl males and fl/fl females as significantly increasing their distance travelled in LL SSI OF in comparison to both no SSI and SSI OF. This increase in distance travelled proposes a reduction in anxiety and elevated exploration (Ieraci et al., 2016, Molina-Hernandez et al., 2001, Seibenhener and Wooten, 2015).

To summarise, LL SSI did not robustly induce anxiety in OF in the same way that SSI in cycling light conditions did. This suggests LL is perhaps not as anxiogenic as first anticipated. However, it is worth noting than females spent significantly less time in the central zone of the OF arena in this condition in comparison to males, as this may somewhat explain NOR failings in fl/fl females under LL. Despite this, no genotype differences were found, which suggests elevated female anxiety would proportionately effect cKO mice as well, though this was not evident in LL NOR trials.

To further assess if the conditions in which NOR trials were performed could have impacted cognitive output, a cohort of cKO and fl/fl mice also performed OF following extended SSI of variable lighting conditions (LD for five days, DD for 10 days, LL for 10 days, and then seven days of LD before testing), to replicate conditions mice underwent in

NOR experiments in 3.2.4. No significant effects of extended SSI were noted on anxiety, unlike in normal LD cycling SSI conditions. Neither genotype, nor sex sub-groups, increased their time spent in the central zone following extended SSI compared to either no SSI or SSI conditions. Similarly, distance travelled was not significantly altered in extended SSI in any genotype or sex sub-group compared to no SSI or SSI conditions. This suggests that extended SSI conditions do not induce anxiety in any groups. However, a noteworthy outcome when comparing no SSI, SSI and extended SSI distance travelled was that cKO consistently showed increased distance travelled irrespective of isolation condition. This genotype effect was maintained when groups were divided by sex, demonstrating higher distance travelled in cKO males and cKO females in comparison to fl/fl males and fl/fl females. This could indicate general hyperactivity and reduced anxiety in cKO mice (Haj-Mirzaian et al., 2019, Heidbreder et al., 2000, Ieraci et al., 2016, Molina-Hernandez et al., 2001). Though this contradicts results in SSI suggesting cKO males are more anxious than fl/fl males, meaning LD box results are inconsistent on genotype anxiety effects. However, it is unlikely that an anxiety effect in this condition could generate or contribute to sex-specific cognitive failings, since there are no significant anxiety phenotypes in fl/fl females. Despite being underpowered, results found in LL SSI and extended SSI sex groups warrant further investigation. Future work would aim to increase sex group sizes to properly investigate sex differences in anxiety induced throughout various lighting conditions and isolation lengths.

Chapter 5: Post-mortem confirmation of hepatic *Npas2* loss and variable circadian gene expression within the pre-frontal cortex

5.1 Introduction

5.1.1 The effects of *Npas2* knockout on circadian gene expression

Loss of global *Npas2* amounts to modest variations in core clock genes within the SCN. *Per2* rhythms are seen to dampen over a 24-hour period, and expression of *Bmal1* is shown to increase throughout the day compared to wild type. However, other genes assessed including *Per1* and *Rev-erbs* showed no significant differences from wild type expression (Debruyne et al., 2007). Additionally, the core circadian gene *Clock*, of which *Npas2* is a paralog of, showed no significant alterations in expression within the forebrain following global *Npas2* loss (Dudley et al., 2003). Similar studies utilising *Npas2* deficient mice have also demonstrated loss of cyclical *Per2* mRNA within the frontal cortex (Reick et al., 2001). Together, these findings suggest global *Npas2* loss has subtle impacts on central circadian gene expression.

As explored in 2.1.2, knockout of *Npas2* has been shown to have age-dependent effects on hepatic, circadian gene expression. Two-day-old *Npas2* knockout mice displayed reduced *Clock* and *Ror*γ expression, as well as increased *Cry2* and *Rev-erbß* expression compared to wild type, though none of these effects are maintained in adult, 25-day old *Npas2* knockout mice. Additionally, contrasting effects are seen in *Bmal1*, *Per1* and *Rora* expression between two and 25-day old mice (O'Neil et al., 2013). *Npas2* knockout in two-day old mice gives reduced *Bmal1* and *Rora* expression, however, both of these genes are then increased in adult *Npas2* knockout mice compared to wild type. Similarly, two-day old *Npas2* knockout mice show increased *Per1* compared to wild type, an effect which is reversed in 25-day old mice. However, it is important to reiterate that this study did not employ time points to analyse circadian genes, nor did it disclose the sex of the animals used, and n numbers were limited (O'Neil et al., 2013). This work does, however, demonstrates significant circadian gene expression changes in the liver governed by global *Npas2* loss, which brings into question how local *Npas2* knockout could influence hepatic, circadian gene expression.

5.1.2 Circadian gene expression aberrancies as a mechanism for cognitive change

Snider et al. employed mice deficient for forebrain *Bmal1* (*Bmal1* fKO) in memory trials to further understand implications of the forebrain clock on memory. *Bmal1* fKO mice had normal *Bmal1* expression in the SCN, and thus are considered a peripheral *Bmal1* knockout. This model demonstrated significant deficits in acquisition and recall when undergoing the Barnes maze trial, and also appeared to lose the time-of-day deviations in NOL memory performance (Snider et al., 2016). These impairments imply that additional clocks within the forebrain may have an indispensable role in learning and memory. Peak performance in rodent memory is commonly observed during the subjective night, as they are nocturnal, but this increase in DI during the circadian night is lost in *Bmal1* fKO mice (Snider et al., 2016, Takahashi et al., 2013). Since time-of-day NOL memory was compromised, *Bmal1* fKO mice show that peripheral knockout models (with intact *Bmal1* expression within the SCN master clock) can have significant circadian impacts (Snider et al., 2016). This generates the hypothesis that expression of *Bmal1* in the forebrain may be a mechanism by which recognition memory changes are induced.

In contrast to this, Wang et al. (2021) demonstrate that microglia-specific reduction of *Bmal1* (microglia$^{Bmal1\text{-}KD}$) can improve memory. NOR trials employing male mice showed improved long-term recognition memory following reduction of *Bmal1* in microglia. Improvements in LTM were also seen in microglia$^{Bmal1\text{-}KD}$ mice in Morris water maze trials, and in memory persistence tests, where microglia$^{Bmal1\text{-}KD}$ mice spent longer in the target quadrants (Wang et al., 2021). As a result of *Bmal1* knockdown, microglia$^{Bmal1\text{-}KD}$ mice displayed increased *Cry1* and *Cry2* expression in microglia. This is of particular interest, since *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice are seen to lack the ability to learn time-place associations; *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice failed to learn which arms would be negatively/positively enforced at which times of day

in a novel reward and punishment paradigm (Van der Zee et al., 2008). This suggests reduction in the expression of core circadian genes within specific areas of the brain can have downstream effects on additional TTFL genes, leading to wider implications in memory outputs. Taken together, this implicates *Bmal1* in recognition memory – whether that be improved memory due to microglia-specific knockdown, or worsened memory following forebrain deletion (Van de Zee et al., 2008, Wang et al., 2021).

5.1.3 Aims and Objectives

Following the revelation that both fl/fl control and knockout lines lose significant amounts of weight in SSI, and that both present a >24-hour circadian period in entrainment, it was important to confirm the knockout of exon 3 of *Npas2* is only present in cKO and Afp cKO livers. The aim of this group of experiments was to confirm that only liver tissue in knockout lines demonstrated a lack of NPAS2, and that fl/fl controls presented intact *Npas2* in both frontal lobe and liver tissue. This evidence was largely absent in previously published research using these transgenic mouse models (O'Neil et al., 2017), so it was imperative to confirm that *Npas2* expression was maintained in the brain of cKO lines and in all tissues in fl/fl control mice. It was important to also demonstrate that fl/fl phenotypes observed in these studies which do not correlate to C57BL/6J results, were not a consequence of floxing *Npas2* exon 3 (O'Neill et al., 2017). The hypothesis being that there may be some impact of floxing on either *Npas2* exon 3 presence, *Npas2* transcript levels, or NPAS2 protein levels, in order for deviations in weight and circadian period and phase from C57BL/6J to materialise. Additionally, since sex seems to influence many parameters seen throughout this study, both sexes were used for confirmations of knockout and intact *Npas2*.

To do this, this work aimed to use cDNA to confirm *Npas2* exon 3 presence or absence in all strains' frontal lobe and liver tissues at an mRNA level. In doing this, any implication of floxing *Npas2* on downstream splicing events would be evident within the

cDNA transcript. Following this, expression levels of *Npas2* were to be checked using RT-qPCR. This study aimed to use mice of each sex and genotype to assess *Npas2* expression levels in both the frontal lobe and liver, using primers designed across the exon 5 – exon 6 boundary, downstream of exon 3 loss. The final aim of confirmation studies included assessment of NPAS2 protein levels by western blot, to confirm reduced protein in NPAS2 cKO mice.

A secondary aim of molecular work utilising cKO and fl/fl mice was to assess expression of core circadian genes in both the frontal lobe and liver. Previous studies have described significant downstream gene expression changes following central, global, and hepatic *Npas2* knockouts (Debruyne et al., 2007, Dudley et al., 2003, O'Neil et al., 2013, Reick et al., 2001). Additionally, some studies propose loss of indispensable circadian components, such as *Bmal1*, can amount to significant cognitive changes (Snider et al., 2017, Wang et al., 2021). As a result, this work aimed to assess the expression of core circadian genes, *Clock*, *Bmal1* and *Rev-erbß*, in the frontal lobe as a possible mechanism by which hepatic NPAS2 loss can amount to changes in circadian behaviour and cognition. Liver gene expression was also assessed to investigate the impacts of local NPAS2 loss on peripheral circadian rhythmicity.

Additionally, given that fl/fl differ from C57BL/6J wild type mice in circadian period, circadian phase and SSI induced weight loss, this work aimed to compare both fl/fl and cKO circadian gene expression to C57BL/6J mice as a secondary line of control.

5.2 Materials and Methodology

5.2.1 Animals and experimental conditions

All cKO and fl/fl animals used in the following experiments were between three and six months of age, and individual experiments had age matched cohorts. Age matched C56BL/6J wild type mice were purchased from Charles River Labs UK and were acclimatized

to the animal unit for at least seven days before being used in experiments. Animals were either held in circadian boxes employing LD cycling: 8am lights ON/8pm lights OFF, or reverse LD cycling employing 8am lights OFF/8pm lights ON. Mice in LD cycling conditions were culled at zeitgeber time 0 (ZT0) and tissue was collected. Mice in reversed LD cycling conditions were culled at zeitgeber time 12 (ZT12) and tissue was collected. ZT refers to the influence of the zeitgeber, which in this case is light. ZT0 coordinates with lights coming on in the morning, and ZT12 is equivalent to lights going off in the evening. Animals held in LD cycling conditions were kept in circadian chambers for five days, since they did not need to adapt to the lighting schedule. Animals held in reverse LD cycling conditions were kept in the circadian chambers for ten days to adapt to reverse lighting. Locomotor activity recording, as described in 2.2.3, was employed to ensure mice in reverse LD cycling were fully adjusted to the conditions before culling.

This experiment aimed to employ n = 14-16 mice per genotype, per time point for RT-qPCR gene expression analysis. Previous studies using the same transgenic mouse lines utilised only three mice per group (O'Neil et al., 2013). Some studies investigating gene expression changes in mouse tissues employed slightly higher n numbers between five and seven, though frequently just three or four mice are used for molecular confirmations (DeBruyne et al., 2007, Mongrain et al., 2011, Ozburn et al., 2017, Shi et al., 2011, Woodie et al., 2020). Due to variability in literature findings and between mice within the same cohort, significantly higher n numbers were chosen for molecular gene expression analysis in this study. This also allowed for adequate sex sub-group numbers to determine any sexual dimorphism in expression.

	fl/fl	cKO	C57BL/6J
ZT0 liver tissue	n = 15 (8 male, 7 female)	n = 15 (8 male, 7 female)	n = 16 (8 of each sex)
ZT0 brain tissue	n = 10 (6 male, 4 female)	n = 10 (6 male, 4 female)	n = 16 (8 of each sex)
ZT12 liver tissue	n = 16 (8 male, 8 female)	n = 16 (8 male, 8 female)	n = 16 (8 of each sex)
ZT12 brain tissue	n = 14 (8 male, 6 female)	n = 14 (8 male, 6 female)	n = 16 (8 of each sex)

Table 5.2: n numbers of fl/fl, cKO and C57BL/6J mice that were used in gene expression analysis

5.2.2 *In vivo* husbandry

As described in 2.2.2

5.2.3 Schedule 1 and tissue collection

All mice were culled using CO_2 and were confirmed using cervical dislocation, as per Home Office guidelines. The liver and brain were extracted, immediately frozen on dry ice and stored at -80^{o}C.

5.2.4 General laboratory chemicals

General laboratory chemicals of analytical grade were purchased from either Fisher Scientific or Sigma-Aldrich, unless otherwise indicated. Phosphate buffered saline (1x PBS) was made using one PBS tablet purchased from OXOID Ltd per 100 ml water. Double deionised water purified by passing through an Elixir essential water purification system was used to prepare all solutions. The pH of solutions was measured using a Jenway 3510 pH meter, calibrated using standards of known pH.

5.2.5 Molecular reagents and antibodies

DNA polymerase enzymes with appropriate buffers and dNTPs were purchased from New England Biolabs (NEB), as were Monarch® gel extraction kits. DNA ladders were

purchased from Invitrogen. GenElute™ mammalian genomic DNA miniprep kits were purchased from Sigma-Aldrich. RNeasy® Plus Mini kits and QuantiTect Reverse Transcription kits were purhcased from Qiagen Ltd. Oligonucleotides were purchased from Sigma-Aldrich as pellets which were resuspended in sterile diH_2O to a concentration of 100 μM and stored at -20ºC. Agarose gel loading dye (10x) was made as follows: 3.9 ml glycerol, 500 μl of 10% (w/v) Sodium-dodecyl-sulphate (SDS), 200 μl 0.5 M EDTA, 0.025 g bromophenol blue, made to 10 ml with sterile diH_2O and used at 1x. Tris-borate-ethylenediaminetetraacetic acid (TBE) was made as follows: 40 mM Tris-base, 40 mM boric acid, 0.5 mM ethylenediaminetetraacetic acid (EDTA) (at pH 8.0), made to 1 L with sterile diH_2O. Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight protein ladder was purchased from NEB. Nitrocellulose membrane for western blotting was purchased from Bio-Rad. N,N,N,N-tetramethylethylenediamine (TEMED) was purchased from Sigma-Aldrich. 30% (w/v) ProtoGel acrylamide was purchased from National Diagnostics.

Primary antibody rabbit anti-NPAS2 (HPA019674) was purchased from Sigma-Aldrich and mouse anti-β actin (66009-1-IG) was sampled from Protein Tech. Horseradish peroxidise (HRP) conjugated secondary antibody chicken anti-mouse (sc-2954) and secondary antibody goat anti-rabbit (sc-2004) were purchased from Santa Cruz Biotechnology. Pierce™ ECL Western Blotting Substrate was purchased from ThermoFisher Scientific. 4x SDS-PAGE loading buffer was made as follows: 2 ml 1 M Tris-HCl pH 6.8, 0.8 g SDS, 4 ml 100% glycerol, 0.4 ml 14.7 M β-mercaptoethanol, 1.0 ml 0.5 M EDTA and 8 mg bromophenol blue, made to 10 ml with sterile diH_2O. Ponceau solution was made as follows: 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid made to 1 L with sterile water. 5% milk solution for blocking was made using 5% (w/v) Marvel 0% fat milk powder in 1x PBS. Buffers were made as follows: Tris-glycine-SDS (TGS) buffer 10x: 250 mM Tris base, 2 M glycine, 35 mM SDS, pH 8.3. Transfer buffer

(high glycine buffer): 48 mM Tris base, 39 mM glycine, 0.037% (w/v) SDS, 20% (v/v) methanol. RIPA buffer: 150 mM NaCl, 50 mM Tris HCl (pH 7.3), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, to 100 ml with diH_2O. Ammonium persulfate (APS) solution was made as follows: 10% (w/v) APS in sterile water, stored at -20^oC until use. SDS solution was made using 10% (w/v) SDS in sterile water.

5.2.6 Genomic extraction

As described in 2.2.10.

5.2.7 PCR

As described in 2.2.11.

5.2.8 Agarose gel electrophoresis and DNA band extraction

Agarose gel electrophoresis was performed as described in 2.2.12. DNA to be extracted was separated on an agarose gel alongside empty wells, to prevent contamination. These bands were then excised using a clean scalpel and RAD Universal Hood II UV transilluminator for visualisation. DNA was purified using Monarch® DNA Gel Extraction Kit, as per manufacturer's instructions. The concentration of DNA was then determined by a NanoDrop 1000 Spectrophotometer.

5.2.9 Sequencing DNA

DNA was purified from gels and sequenced by Source Bioscience Ltd using an Applied Biosystems 3730 DNA Analyzer and PeakTrace™ software. 5 µl of PCR product was sent for sequencing at a concentration of 10 ng/µl. 5 µl of appropriate primer was sent at 3.2 pmol/µl. Primers can be found in the appendix (A.4).

5.2.10 RNA extraction

~100 mg of liver or frontal lobe was minced on ice and immediately placed in 1 ml of cold Trizol reagent (Sigma). It was then sonicated in a Diagenode Bioruptor® in cold water

and ice for two rounds of 15 seconds for tissue disruption. RNA isolation was then performed according to the manufacturer's protocol (Sigma). RNA was separated using chloroform, and the RNA phase was precipitated using isopropanol, RNA was then washed using 70 % ethanol and resuspended in 30 µl of RNase free water for frontal lobe RNA, and 150 µl of RNase free water for more concentrated liver RNA.

5.2.11 cDNA synthesis

cDNA synthesis was performed using the QuantiTect Reverse Transcription kit (QIAGEN Ltd). The first stage involved removal of genomic DNA, to prevent contamination:

7x gDNA Wipeout buffer	1 µl (1x)
Template RNA	500 ng
Sterile RNase-free water	To a total volume of 7 µl

Table 5:3: Reverse transcription for cDNA synthesis step one.

For the second step, to synthesise cDNA, reverse transcription reactions were formulated as shown below:

Quantiscript Reverse Transcriptase	0.5 µl
5x Quantiscript RT buffer	2 µl (1x)
RT Primer mix	0.5 µl
Genomic DNA wipeout reaction	7 µl
Total volume	10 µl

Table 5.4: Reverse transcription for cDNA synthesis step two.

These preparations were incubated for 15 minutes at 42 °C, then for three mins at 95 °C to inactivate the QuantiScript Reverse Transcriptase enzyme. Samples were then stored at -20 °C until required.

5.2.12 Quantitative reverse-transcription PCR (RT-qPCR) primer validation

All RT-qPCR primers were designed using NCBI primer BLAST software, or were taken from published literature. All were purchased from Sigma-Aldrich as lyophilised and desalted pellets which were then resuspended in sterile water to 100 µM and stored at -20 °C. Primers were intentionally designed across exon-exon boundaries to prevent amplification of residual genomic DNA. They are all 18-23 bases long and use melting temperatures between 58-60 °C.

All RT-qPCR primers were validated by examining the reaction efficiency and specificity of the products. A dilution series was performed to confirm the reaction gives a linear association with input cDNA concentrations. For this dilution series, either cDNA extracted from NIH3T3 cells or C57BL/6J liver was employed at concentrations of 1x, 0.5x, 0.1x, 0.05x and 0.025x. The association between critical threshold (Ct) value and relative quantity of cDNA was investigated using semi-log least squares fit on Prism (GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). The output of this interpolation analysis was an equation of a linear line, and an R squared value. R squared values > 0.95 display high correlation between input cDNA and the Ct value given, meaning the reaction efficiency is irrespective of cDNA concentration (Bustin and Huggett, 2017). Thus, primers were only employed in this study if the gave an R squared value of > 0.95 (see A.5). Products were also separated on an agarose gel to confirm band size matches the predicted size, and that there were no contaminants or non-specific products.

5.2.13 RT-qPCR protocol

RT-RT-qPCR was performed using Brilliant II SYBR® Green RT-QPCR Master Mix from Agilent Technologies. RT-qPCRs were performed using a Stratagene Mx3005P RT-qPCR machine, with data collected through the MXPRO software. Three replicates of a no

template control (NTC) (reaction mix lacking cDNA) were used per master mix to confirm the absence of contaminants that would affect Ct value. Dissociation curves and amplification plots of each experiment were observed in the MXPRO software, to confirm singular products and no contaminants. The software calculated a Ct value for each run and quantified the cycle number when the Ct was met for each sample. RT-qPCR analysis was performed in technical triplicates using a number of biological repeats (specific experimental n numbers are in figure legends). *18S* rRNA (*Rn18s*) expression was used as a normalisation gene. This is because common housekeeping genes such as GAPDH have been shown to demonstrate temporal variation (Matsumura et al., 2014).

Each RT-qPCR reaction was prepared as shown below:

Sterile dH_2O	2 µl
10 µM Forward primer	0.5 µl
10 µM Reverse primer	0.5 µl
Brilliant II SYBR® Green RT-QPCR Master Mix	5 µl
cDNA	2 µl
Total volume	10 µl

Table 5.5: RT-qPCR reaction mix volumes.

The experimental set up, as performed on the Stratagene Mx3005P RT-qPCR machine, was as presented below:

No. Cycles	**Time**	**Temperature**
x 1	10 mins (initial denaturation)	95 °C
x 40	30 secs (denaturation)	95 °C
	1 min (primer annealing and extension)	60 °C
x 1	melt curve analysis	60-95 °C

Table 5.6: RT-qPCR protocol.

5.2.14 RT-qPCR analysis and statistical analysis

Each technical triplicate value was compared to ensure no variation above 1.5 Ct values was detected. Data was then normalised to the results of a parallel 18S rRNA internal control using the $2^{-\Delta Ct}$ method. Average 18S rRNA Ct values were subtracted from the experimental transcript values and converted to copy number. Copy numbers were then normalised to the average copy number of control samples of that specific experiment, to give a relative change.

A power calculation using a Cohen's f of 0.908, based on a pilot study of gene expression in the liver of control and cKO mice by Mitchell Masterson was performed in G power for an ANOVA with an alpha value of 0.05, power of 0.9 with four groups, and a numerator df of 1 (Mitchell Masterson, PhD thesis 2020). As a result, 16 mice are required for molecular analysis equal to four per genotype X sex group. All RT-qPCR statistics were performed in Prism (GraphPad Prism version 8 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Two-way ANOVAs were used to investigate the effect of time and genotype, time and sex, or sex and genotype on gene expression. Unpaired t-tests were used to compare genotypes at one time point. A probability value of $p<0.05$ was used as the criteria to determine statistical significance with $p<0.05$ as '*', $p<0.01$ as '**', $p<0.001$ as '***', and $p<0.0001$ as '****'.

5.2.15 Protein extraction and concentration determination

Protein was extracted from tissues stored at -80 °C. ~100 mg of tissue was taken with a scalpel and placed in 500 µl of RIPA buffer containing 1x protease inhibitors added on the day. Samples were then placed in a cell disruptor (Disruptor Genie, Scientific Industries) for two hours at 4°C before being sonicated for five minutes (30s ON, 30s OFF) in a Diagenode

Bioruptor® with cold water and ice. Cell debris was then pelleted by centrifugation at 20,000 G for 10 minutes at 4°C and the supernatant contained extracted protein.

Protein concentration was determined using the Bradford assay method. Reactions were made up as follows: 2 µl of protein lysate, 800 µl diH_2O & 200 µl of Sigma-Aldrich Bradford dye in cuvettes. Samples were mixed using gentle vortexing and left for 15 minutes before the absorbance was read at 595 nm. Concentration was calculated based on values of known-concentration bovine serum albumin (BSA) standards.

5.2.16 Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were prepared as shown below. Different percentages of resolving gel were used depending on protein size, but a 5% stacking gel was consistently used. Generally, 50 µg of protein in 7 µl of 4x SDS-PAGE loading buffer made to a total volume of 28 µl with sterile diH_2O was loaded in to a 10% or 12% SDS-PAGE gel and ran at 160 V with 1x TGS buffer.

	5% stacking gel	10% resolving gel	12% resolving gel
diH_2O	3.40	1.9	1.6
30% (w/v) Acrylamide	0.83	1.7	2.0
1.5 M Tris pH 8.8	-	1.3	1.3
1.0 M Tris pH 6.8	0.63	-	-
10% SDS solution	0.05	0.05	0.05
10% APS solution	0.05	0.05	0.05
TEMED	0.005	0.002	0.002

Table 5.7: SDS-PAGE gel recipe.

5.2.17 Western blot

Protein was separated on an SDS-PAGE gel at 160 V for two hours. Samples were then transferred from SDS-PAGE gels onto nitrocellulose membranes using transfer buffer, either at 30 V overnight at 4°C, or at room-temperature for two hours at 60 V. 1% Ponceau S solution

was used to stain the membrane to confirm protein transfer. The membrane was blocked for one hour with 5% milk (Marvel), at room temperature with agitation, before being incubated overnight at 4^{o}C in 5 ml of 5% milk containing primary antibody – typically at a concentration of 1:500. The membrane was then washed three times at room temperature for five minutes, with agitation, using PBS containing 1% Tween ®80 (PBST). It was then incubated at room temperature with secondary antibody – typically at a concentration of 1:5000). Three PBST washes were then repeated. Pierce™ ECL developing solution (Thermo Scientific) was then added to the nitrocellulose membrane, after one minute the membrane was imaged using a Fujifilm LAS-4000 system to detect chemiluminescent signal. The protein ladder was also imaged for reference of molecular weight.

Western blots were quantified using densitometry measurements taken from ImageJ (Schneider et al., 2012). Five repeated measurements of the density of each band, including loading controls, were taken and subtracted from the background density. The protein bands of interest were then normalised to averaged densities of the loading control band of their genotype (Butler et al., 2019, Gassmann et al., 2009).

5.3 Results

5.3.1 Validation of *Npas2* exon 3 loss in the liver and maintained *Npas2* exon 3 in the frontal lobe of cKO and Afp cKO mice

Following the observation that both control and both knockout lines of Afp and Alb strains lose significant amounts of weight in SSI, and that all transgenic lines present a >24-hour circadian period in entrainment, it was important to confirm the deletion of *Npas2* exon 3 only occurs in cKO and Afp cKO livers. Additionally, since sex seems to influence many parameters seen in this study, both sexes were used for confirmations of knockout and intact *Npas2*. Total cDNA from three male mice (one fl/fl, one cKO, and one C57BL/6J) was used in endpoint PCR utilising a forward primer in *Npas2* exon 2 and a reverse primer in *Npas2* exon

4 to confirm the presence or absence of exon 3 in the liver and frontal lobe. Fl/fl, Afp fl/fl and C57BL/6J mice produced a single 240 bp product consistent with wild type *Npas2* transcripts containing exon 3, in both the liver and frontal lobe. The 240 bp band was also maintained in the frontal lobe of both cKO and Afp cKO, as expected, whereas a single band below 100 bp was observed in cKO and Afp cKO liver cDNA. This is consistent with the expected size (91 bp) of *Npas2* transcripts lacking exon 3, indicating successful liver specific loss of this sequence (Figure 5.1A).

To confirm that this lower band size is due to *Npas2* exon 3 loss, the bands of cKO and Afp cKO frontal lobe and livers were excised and DNA was extracted and sequenced. Sequences from amplicons of all transgenic frontal lobe's were identical to the sequence taken from C57BL/6J wild type frontal lobe, confirming the inclusion of exon 3 (Figure 5.1B). The sequence of livers from both cKO and Afp cKO were then compared to *Npas2* C57BL/6J liver sequence, which confirmed the reduction in band size shown in Figure 5.1A corresponds to a loss of *Npas2* exon 3 (Figure 5.1C).



Figure 5.1: *Npas2* exon 3 was only absent in the liver of cKO and Afp cKO mice.

A) An agarose gel showing both tissues of fl/fl, Afp fl/fl and C57BL/6J mice presented a ~240 bp band of *Npas2*. This ~240 bp band was also present in the frontal lobe (labelled PFC) of cKO and Afp cKO mice, but a much smaller band of ~91 bp was shown in the liver of cKO and Afp cKO mice. Primers used: *Npas2* ex2 F and *Npas2* ex4 R. Each band indicates one male representative mouse. B) Sequencing results detailing intact *Npas2* exon 3 in cKO frontal lobe (labelled PFC), compared to C57BL/6J brain. C) Sequencing results detailing loss of *Npas2* exon 3 in cKO liver, compared to intact *Npas2* exon 3 in C57BL/6J liver.

5.3.2 Investigating *Npas2* expression levels in cKO and fl/fl tissues using RT-qPCR

The next line of enquiry was to assess if floxing impacts *Npas2* gene expression levels in the cKO frontal lobe and in fl/fl control tissues. This was in order to explore whether *LoxP* insertions flanking exon 3 might inadvertently impact on *Npas2* expression levels in fl/fl mice, which may generate phenotypes dissimilar to C57BL/6J mice. We therefore designed RT-

qPCR primers targeting the *Npas2* exon 5 – exon 6 boundary, downstream of excised exon, were employed. Although deletion of exon 3 would remove the AUG start codon and thus be detrimental for expression of full-length NPAS2 protein, it is unclear whether transcription of the *Npas2* gene would be affected. Indeed, as exon 3 is distant from the transcriptional start site, it may not impact on the overall levels of the *Npas2* transcripts.

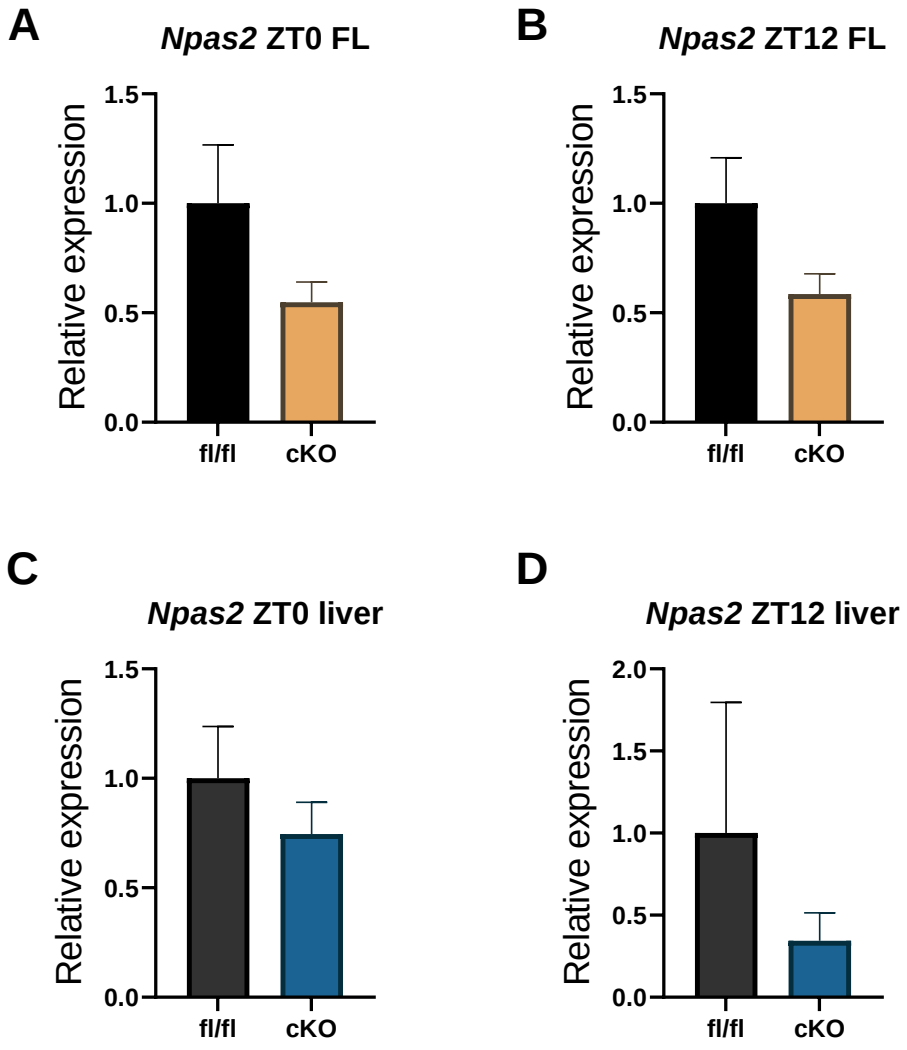


Figure 5.2: *Npas2* expression levels in cKO and fl/fl tissues do not significantly differ between genotypes.

A) *Npas2* expression levels in fl/fl (n = 14) and cKO (n = 14) mice in the frontal lobe (FL) at ZT0. B) *Npas2* expression levels in fl/fl (n = 14) and cKO (n = 14) mice in the frontal lobe (FL) at ZT12. C) *Npas2* expression levels in fl/fl (n = 15) and cKO (n = 15) mice in the liver at ZT0. D) *Npas2* expression levels in fl/fl (n = 16) and cKO (n = 16) mice in the liver at ZT12. No significant genotype differences are seen throughout any time points or tissues. Fl/fl were normalised to one, and cKO were normalised

to fl/fl (see A.6 for details). Relative expression values were based on RT-qPCR reads after $2^{-\Delta Ct}$ transformation based on 18S results.

Analysis of this data revealed no significant differences between cKO and fl/fl *Npas2* expression levels in the frontal lobe at either ZT0 or ZT12 (Figure 5.2A, 5.2B). Additionally, no significant differences between cKO and fl/fl *Npas2* expression levels were noted in the liver at either ZT0 or ZT12 (Figure 5.2C, 5.2D).

To investigate if combining sexes masked a sex-effect on *Npas2* expression levels in either tissue, RT-qPCR was also assessed in sex sub-groups. This was because phenotypes observed in this model, such as weight loss, circadian period and phase, and recognition memory have demonstrated sex-specific genotype effects.

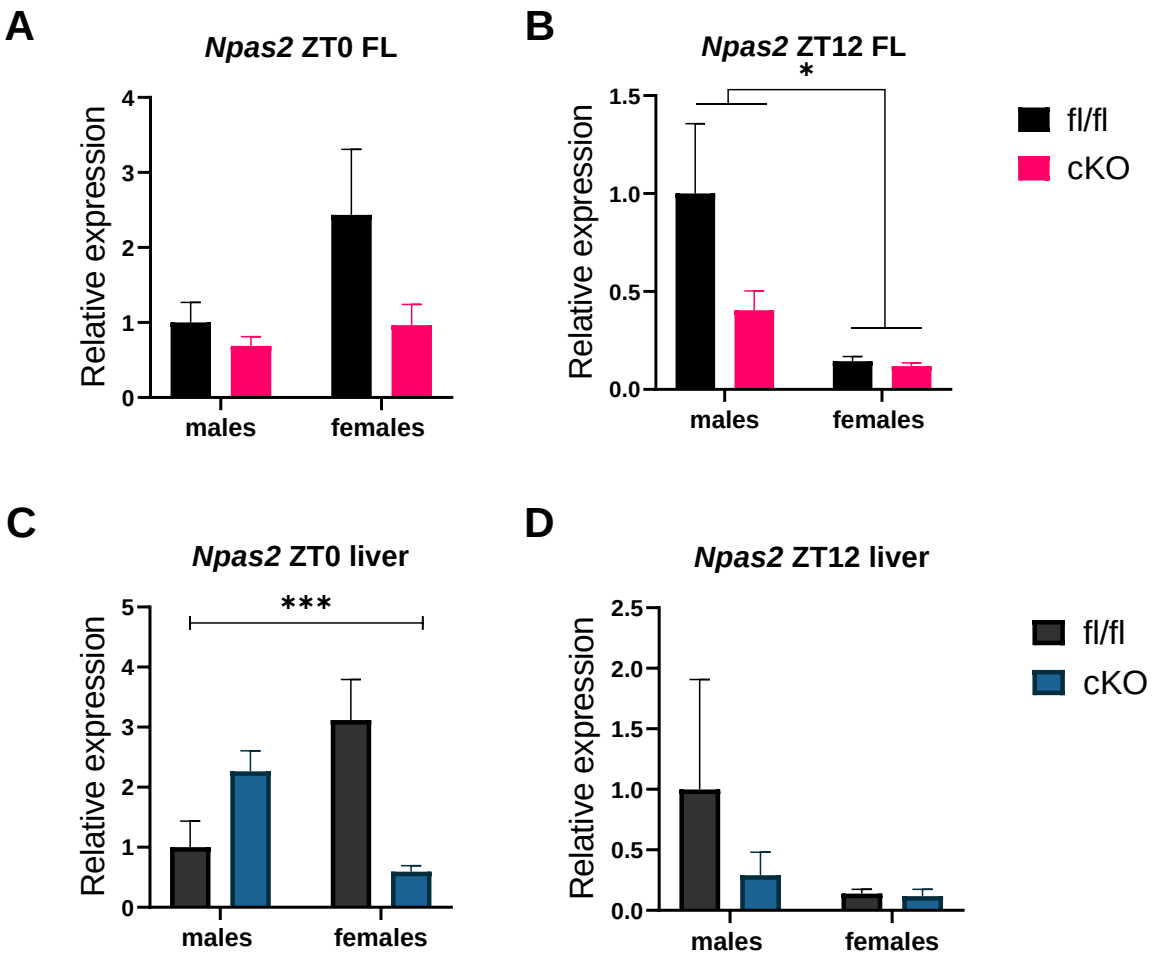


Figure 5.3: cKO females showed significantly reduced hepatic *Npas2* expression compared to fl/fl females.

A) RT-qPCR of *Npas2* expression levels in fl/fl (males n = 6, females n = 4) and cKO (males n = 6, females n = 4) mice in the frontal lobe (FL) at ZT0. There was a significant effect of genotype on *Npas2* expression (*, p=0.0475). B) RT-qPCR of *Npas2* expression levels in fl/fl (males n = 8, females n = 6) and cKO (males n = 8, females n = 6) mice in the frontal lobe (FL) at ZT12. There was a significant effect of sex on *Npas2* expression (*, p=0.0146). Fl/fl males had significantly higher frontal lobe *Npas2* expression at ZT12 than fl/fl females (*, p=0.0201). C) RT-qPCR of *Npas2* expression levels in fl/fl (males n = 8, females n = 7) and cKO (males n = 8, females n = 7) mice in the liver at ZT0. There was a significant interaction between sex and genotype on *Npas2* expression (***, p=0.0002). Fl/fl females had significantly higher hepatic *Npas2* expression compared to cKO females at ZT0 (**, p=0.0010). Fl/fl males had lower hepatic *Npas2* expression compared to fl/fl females (**, p=0.0039) and cKO males had higher hepatic *Npas2* expression compared to cKO females (*, p=0.0232). D) RT-qPCR of *Npas2* expression levels in fl/fl (males n = 8, females n = 8) and cKO (males n = 8, females n = 8) mice in the liver at ZT12. Relative expression values were based on RT-qPCR reads after $2^{-\Delta Ct}$ transformation based on 18S results. See A.6 for details.

No significant interaction between sex and genotype on frontal lobe *Npas2* expression at ZT0 was seen (F(1,24)=1.837, p=0.1879) or ZT12 (F(1,24)=1.737, p=0.2000), though a significant effect of genotype was seen at ZT0 (F(1,24)=4.362, p=0.0475). Despite this, Šídák's multiple comparisons test found no significant genotype differences. Additionally, a significant effect of sex was seen on frontal lobe *Npas2* expression at ZT12, with females showing reduced expression (F(1,24)=6.927, p=0.0146). Fl/fl females showed significantly reduced frontal lobe *Npas2* expression at ZT12 compared to fl/fl males (Šídák's post-hoc, p=0.0201). The results appear to depict cyclical *Npas2* levels in female frontal lobes, and relatively static *Npas2* levels in male frontal lobes between ZT0 and ZT12 time points (Figure 5.3A, 5.3B).

Two-way ANOVA analysis revealed a significant interaction between sex and genotype on hepatic *Npas2* expression at ZT0 (F(1,26)=18.94, p=0.0002). Fl/fl females had higher hepatic *Npas2* than fl/fl males (Šídák's post-hoc, p=0.0309), whilst cKO females had lower hepatic *Npas2* than cKO males, suggesting opposing sexual dimorphism of *Npas2* expression (Šídák's post-hoc, p=0.0232). Most significantly, cKO females showed significantly lower hepatic *Npas2* expression compared to fl/fl females (Šídák's post-hoc, p=0.0010) (Figure 5.3C, 5.3D).

To summarise, no significant differences were seen between cKO and fl/fl *Npas2* expression at either ZT0 or ZT12 within the frontal lobe. There was, however, a significant effect of sex on frontal lobe *Npas2* expression at ZT12, where both groups of females displayed reduced expression. Most notably, cKO females displayed significantly reduced *Npas2* expression in the liver at ZT0 compared to fl/fl females. However, this effect was not seen in the males.

5.3.3 Confirmation of reduced NPAS2 protein in cKO livers of both sexes

Next, levels of NPAS2 protein were measured between genotypes, and across sexes. Following the confirmation that *Npas2* exon 3 was deleted in the livers of Afp cKO and cKO mice, it was important to confirm this resulted in reduction or loss of NPAS2 protein.

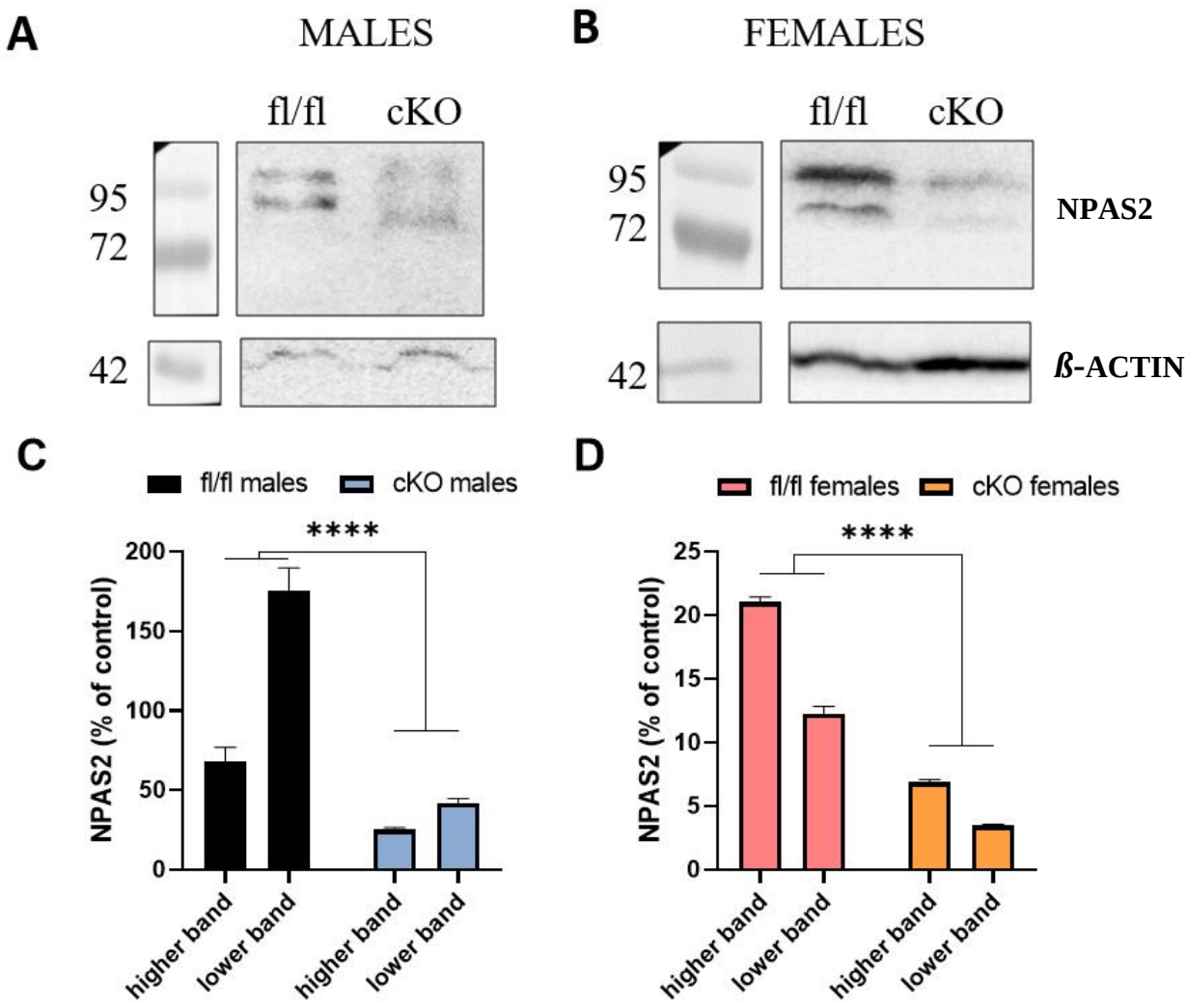


Figure 5.4: Significantly reduced NPAS2 levels in male and female cKO mice compared to fl/fl controls.

A) Western blots detecting NPAS2 in extracts from male and female mouse liver revealed a reduction in protein level in the cKO liver compared to the fl/fl liver. Expected NPAS2 band size is 92 kDa. A slight reduction in band size of the lower band was also seen in cKO male liver. B) Female cKO and fl/fl livers blotted for NPAS2 revealed a reduction in protein level in the cKO liver compared to the fl/fl liver. Each sample was one representative liver. C) Five densitometry repeated measurements showed significantly higher NPAS2 levels in fl/fl males compared to cKO males (****, $p<0.0001$). D) Five densitometry repeated measurements showed significantly higher NPAS2 levels in fl/fl females compared to cKO females (****, $p<0.0001$).

A double band was detected when blotting for the NPAS2 protein in all samples. Both male and female cKO mice displayed a reduction in NPAS2 protein in the liver, compared to fl/fl controls (Figure 5.4A, 5.4B). This was quantified using densitometry which revealed a

significant effect of genotype on NPAS2 protein level in both male and female mice (two-way ANOVA, (F(1,16)=106.5, p<0.0001), (F(1,16)=1027, p<0.0001), respectively). This demonstrated that cKO males and cKO females have significantly reduced NPAS2 protein levels in the liver compared to fl/fl controls, confirming their status as hepatic NPAS2 knockout mice. Given that the gene is still present, we can suggest that a non-functional *Npas2* transcript is produced within these mice.

5.3.4 Altered *Clock* expression in the liver and frontal lobe of hepatic *Npas2* cKO male and female mice

Next, transcript levels of core circadian genes in both the frontal lobe and liver were analysed between genotypes and sex groups using RT-qPCR. This was measured at ZT0 and ZT12, to additionally assess if cyclical gene expression was maintained in hepatic *Npas2* cKO mice. This is because previous findings in global *Npas2* KO mice have noted dampened oscillatory gene expression in some circadian targets (Debruyne et al., 2007, Reick et al., 2001).

Firstly, *Clock*, the paralog of *Npas2*, was assessed. Two-way ANOVA revealed a significant effect of genotype on male hepatic *Clock* expression (F(1,28)=17.07, p=0.0003). cKO males showed higher *Clock* expression at both ZT0 and ZT12, compared to fl/fl males (ZT0 Šídák's post-hoc, p=0.0134, ZT12 Šídák's post-hoc, p=0.0138). This could represent compensatory *Clock* expression in the absence of *Npas2* in the liver (Figure 5.5A). Females showed a significant interaction between genotype and time point on hepatic *Clock* expression (Two-way ANOVA, F(1,26)=6.869, p=0.0145)). Fl/fl females showed cycling hepatic *Clock* expression between ZT0 and ZT12 (Šídák's post-hoc, p=0.0014), whilst cKO females did not (Figure 5.5B).

Furthermore, significant sexual dimorphism in fl/fl hepatic *Clock* gene expression was noted. A significant interaction of sex and time point on fl/fl hepatic *Clock* expression was seen (F(1,27)=9.421, p=0.0048), as well as individual effects of time point (F(1,27)=13.99,

p=0.0009) and sex (*F*(1,27)=4.316 *p*=0.0474). Fl/fl females showed significantly higher expression of *Clock* in the liver at ZT0 compared to fl/fl males (Šídák's post-hoc, *p*=0.0027). Fl/fl males do not appear to demonstrate cyclical hepatic *Clock* expression (Figure 5.5C). This sexual dimorphism was absent in cKO mice, who displayed no significant differences between sex or time point, indicating abolished sex differences and lack of cyclical expression of hepatic *Clock* (Figure 5.5D).

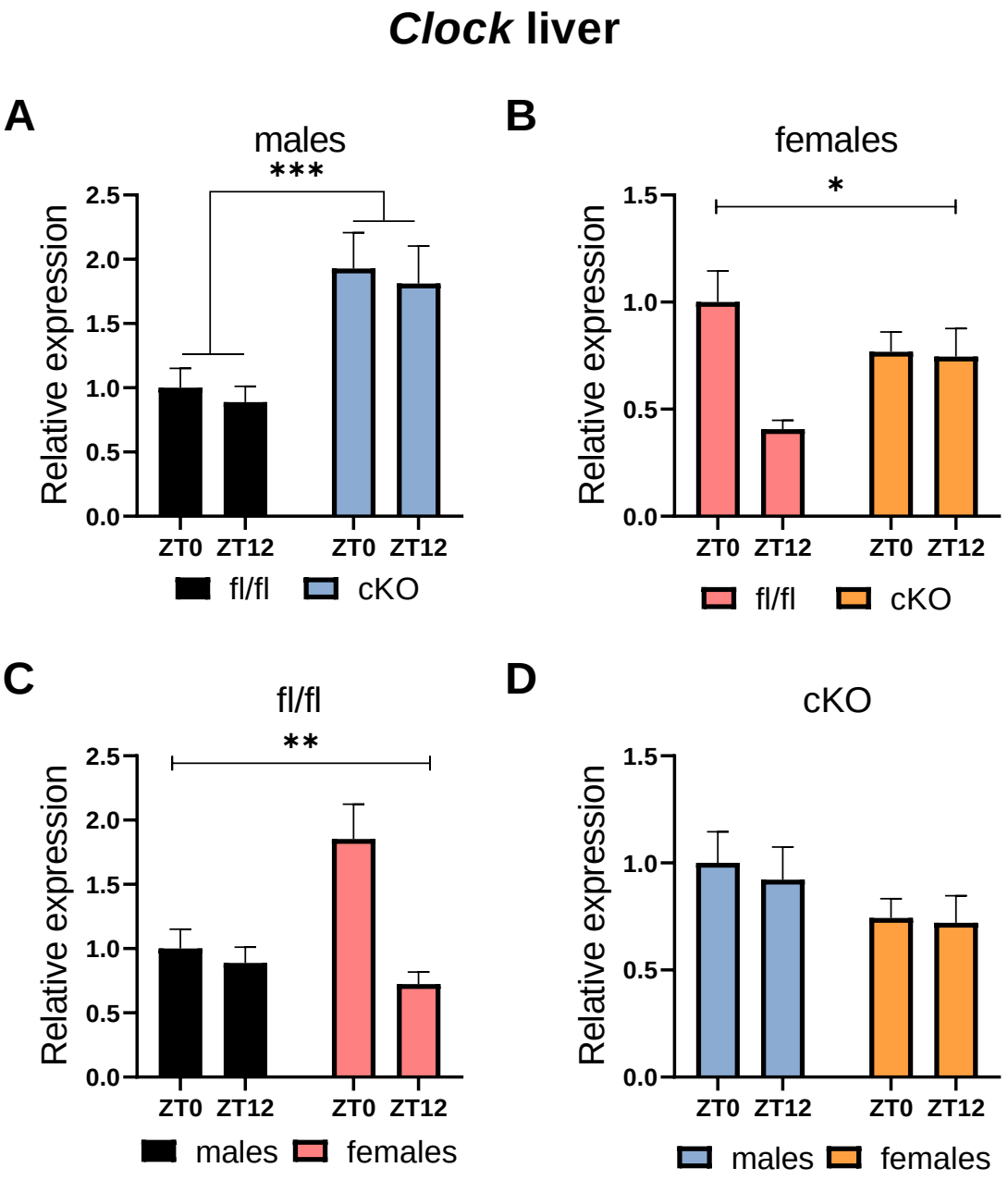


Figure 5.5: Loss of sexual dimorphism in *Clock* expression within cKO mice.

A) RT-qPCR of *Clock* expression levels in fl/fl males and cKO males (ZT0 both n = 8, ZT12 both n = 8) in the liver. There was a significant effect of genotype on *Clock* expression (***, p=0.0003). B) RT-qPCR of *Clock* expression levels in fl/fl females and cKO females (ZT0 both n = 7, ZT12 both n = 8) in the liver. There was a significant interaction of genotype and time point on *Clock* expression (*, p=0.0145). Fl/fl females had significantly higher hepatic *Clock* expression at ZT0 than ZT12 (**, p=0.0014). C) RT-qPCR of *Clock* expression levels in fl/fl male and female mice (males n = 8 in both, females ZT0 n = 7, ZT12 n = 8) in the liver. There was a significant interaction between sex and time point (**, p=0.0048). Fl/fl females had significantly higher *Clock* expression at ZT0 compared to fl/fl males (**, p=0.0027). D) RT-qPCR of *Clock* expression levels in cKO mice (males: n = 8 in both, females: ZT0 n = 7, ZT12 n = 8) in the liver. Relative expression values were based on RT-qPCR reads after $2^{-\Delta Ct}$ transformation based on 18S results. See A.6 for details.

Considerable genotype and sex differences were revealed in *Clock* gene expression within the frontal lobe. There was a significant interaction between genotype and time point on male *Clock* gene expression in the frontal lobe (F(1,24)=4.384, p=0.0470). Fl/fl males showed *Clock* expression to peak in the frontal lobe at ZT12 (Šídák's post-hoc, p=0.0290), whilst cKO males show no significant cycling (Figure 5.6A). Females displayed a significant effect of time point on frontal lobe *Clock* gene expression (two-way ANOVA, (F(1,16)=8.328, p=0.0108)), with higher expression in ZT0 for fl/fl females (Šídák's post-hoc, p=0.0057). Fl/fl females also demonstrate significantly higher *Clock* expression at ZT0 compared to cKO females (Šídák's post-hoc, p=0.0406) (Figure 5.6B).

When investigating sex differences, a significant interaction between fl/fl sex and time point was seen on frontal lobe *Clock* expression (F(1,20)=6.633, p=0.0181), with females showing significantly higher frontal lobe *Clock* expression at ZT0 compared to fl/fl males (Šídák's post-hoc, p=0.0235) (Figure 5.6C). Similarly, cKO mice showed a significant effect on sex on frontal lobe Clock gene expression (F(1,20)=18.63, p=0.0003), with cKO males showing consistently higher expression across time points (ZT0 Šídák's post-hoc, p=0.0194, ZT12 Šídák's post-hoc, p=0.0070) (Figure 5.6D). This demonstrates that fl/fl male and fl/fl female frontal lobe *Clock* expression peaks antiphase to one another, and that neither cKO males nor cKO females demonstrate significant cycling expression between time points.

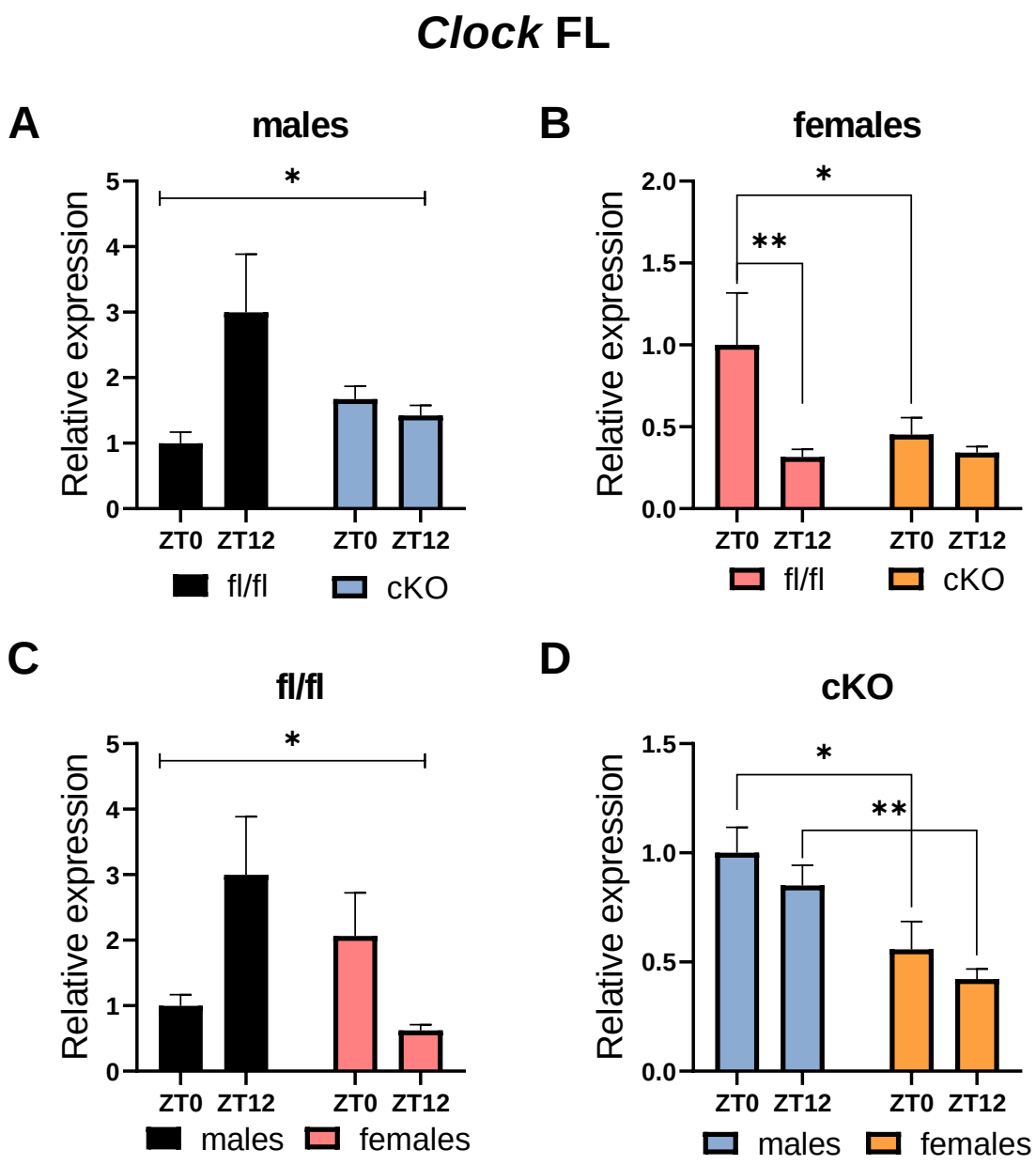


Figure 5.6: Hepatic *Npas2* loss appears to abolish cyclical *Clock* expression in the frontal lobe.

A) RT-qPCR of *Clock* expression levels in fl/fl males and cKO males (ZT0 both n = 6, ZT12 both n = 8) in the frontal lobe. There was a significant interaction between genotype and time point (*, *p*=0.0470). B) RT-qPCR of *Clock* expression levels in fl/fl females and cKO females (ZT0 both n = 4, ZT12 both n = 6) in the frontal lobe. There was a significant effect of time point on *Clock* expression (*, *p*=0.0108). Fl/fl females had significantly higher frontal lobe *Clock* expression at ZT0 than cKO females (*, *p*=0.0406), and fl/fl females at ZT12 (**, *p*=0.0057). C) RT-qPCR of *Clock* expression levels in fl/fl male and female mice (ZT0 males n = 6, females n = 4. ZT12 males n = 8, females n = 6) in the frontal lobe. There was a significant interaction between sex and time point (*, *p*=0.0181). Fl/fl males had significantly higher *Clock* expression at ZT12 compared to fl/fl females (*, *p*=0.0235). D) RT-qPCR of *Clock* expression levels in cKO mice (ZT0 males n = 6, females n = 4. ZT12 males n = 8, females n = 6) in the frontal lobe. A significant effect of sex was seen on *Clock* gene expression (***, p=0.0003). cKO males had higher expression at ZT0 and ZT12 than cKO females (*, p=0.0194, **,

p=0.0070, respectively). Relative expression values were based on RT-qPCR reads after $2^{-\Delta Ct}$ transformation based on 18S results. See A.6 for details.

To assess if the expression patterns in fl/fl mice equate to those of C57BL/6J mice, *Clock* gene expression was investigated in the frontal lobe and liver of wild type mice as a secondary control.

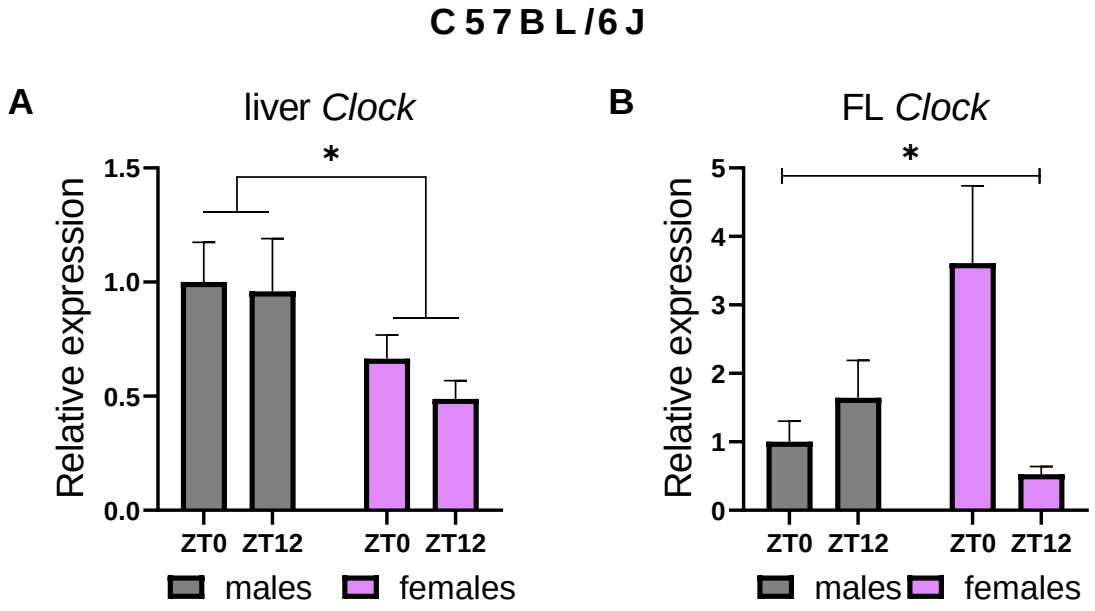


Figure 5.7: Similar patterns of *Clock* expression in the frontal lobe and liver of C57BL/6J, compared to fl/fl mice.

A) RT-qPCR of *Clock* expression in C57BL/6J mice (males n = 8, females n = 8) in the liver at ZT0 and ZT12. A significant effect of sex was seen (*, p=0.0168). B) RT-qPCR of *Clock* expression in C57BL/6J mice (males: ZT0 n = 6, ZT12 n = 8, females n = 8) in the frontal lobe (FL). A significant interaction between timepoint and sex was seen (*, p=0.0120). At ZT0 females have higher frontal lobe *Clock* expression than males (*, p=0.0316) and compared to ZT12 females (**, p=0.0058). Relative expression values were based on RT-qPCR reads after $2^{-\Delta Ct}$ transformation based on 18S results. See A.6 for details.

As seen in fl/fl male mice, cyclical expression of hepatic *Clock* was not seen in C57BL/6J mice. However, an effect of sex was seen on *Clock* expression in the liver, where C57BL/6J females had lower expression compared to males (F(1,28)=6.470, p=0.0168). No significant interaction was seen between sex and time point in C57BL/6J hepatic *Clock*

expression (F(1,28)=0.1818, p=0.6730) (Figure 5.7A). However, significant interaction between sex and time point was noted on frontal lobe *Clock* gene expression (F(1,26)=7.295, p=0.0120). This was identical to the effect noted in fl/fl frontal lobe *Clock* gene expression, whereby C57BL/6J females have higher expression of *Clock* in the frontal lobe at ZT0 (Šídák's post-hoc, p=0.0316), and antiphase cycling was seen between sexes (Figure 5.7B).

In summary, this depicts both significant sex and genotype differences between fl/fl and cKO mice in hepatic and central *Clock* gene expression. This therefore infers that the loss of liver *Npas2* is capable of causing altered *Clock* expression in the brain. In addition, sexual dimorphism in fl/fl mice were similar to those seen in C57BL/6J, further validating fl/fl mice as an appropriate control for *Npas2* cKO. However, some C57BL/6J results were variable from fl/fl mice, such as less cyclical frontal lobe *Clock* expression in C57BL/6J females compared to fl/fl females; this may be due to background strain divergence by the *Npas2* transgenic lines.

5.3.5 Variable *Bmal1* expression in the frontal lobe of hepatic *Npas2* cKO male and female mice

Another core circadian gene is *Bmal1*, this forms the binding partner of CLOCK or NPAS2 in order to initiate transcription of clock-controlled genes (Bertolucci et al., 2008, DeBruyne et al., 2006). Firstly, no significant interactions between genotype and time point were seen on hepatic *Bmal1* expression in either male or female mice ((F(1,27)=0.0579, p=0.8117), (F(1,26)=1.816, p=0.1895), respectively). However, males demonstrated a significant effect of time point on hepatic *Bmal1* expression, (F(1,27)=11.95, p=0.0018), as do females (F(1,27)=52.32, p<0.0001). cKO and fl/fl males showed significantly higher *Bmal1* expression in the liver at ZT0 (Šídák's post-hoc, p=0.0258, p=0.0183, respectively). Similarly, fl/fl females and cKO females both show heightened *Bmal1* expression in the liver at ZT0 (Šídák's post-hoc, p<0.0001, p=0.0006, respectively). No genotype differences were noted in either males or females (Figure 5.8A, 5.8B). No significant interactions between sex and time

point on hepatic *Bmal1* expression were seen in either fl/fl or cKO mice ((*F*(1,26)=0.2230, *p*=0.6407), (*F*(1,27)=0.07230, *p*=0.7901), respectively) (Figure 5.8C, 5.8D).

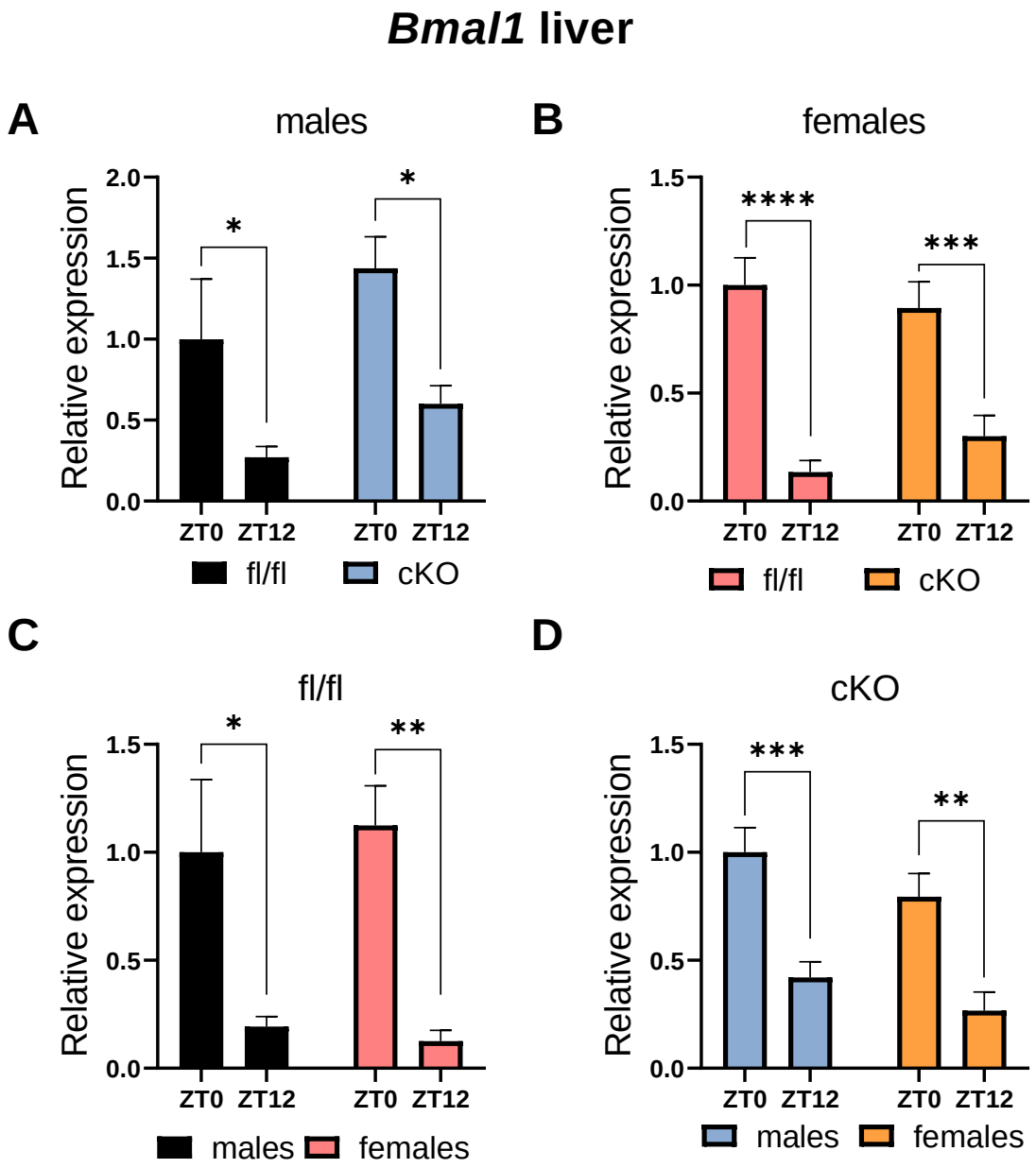


Figure 5.8: All sex and genotype groups demonstrate cyclical *Bmal1* expression in the liver.

A) RT-qPCR of *Bmal1* expression levels in fl/fl males and cKO males (ZT0 fl/fl n = 7, cKO n = 8, ZT12 both n = 8) in the liver. There was a significant effect of time point (**, *p*=0.0018). B) RT-qPCR of *Bmal1* expression levels in fl/fl females and cKO females (ZT0 both n = 7, ZT12 both n = 8) in the liver. There was a significant effect of time point (****, *p*<0.0001).C) RT-qPCR of *Bmal1* expression

levels in fl/fl male and female mice (ZT0 both n = 7, ZT12 both n = 8) in the liver. There was a significant effect of time point (***, p=0.0001). D) RT-qPCR of *Bmal1* expression levels in cKO mice (males: n = 8 in both, females: ZT0 n = 7, ZT12 n = 8) in the liver. There was a significant effect of time point (****, p<0.0001). Relative expression values were based on RT-qPCR reads after $2^{-\Delta Ct}$ transformation based on 18S results. See A.6 for details.

Next, *Bmal1* expression was explored in the frontal lobe of *Npas2* cKO and fl/fl mice. Firstly, males demonstrated a significant influence of time point on *Bmal1* expression in the frontal lobe (F(1,24)=8.097, p=0.0089). Both fl/fl males and cKO males demonstrated a significant difference between ZT0 and ZT12 (fl/fl males: Šídák's post-hoc, p=0.0234, cKO males: unpaired t-test, t(12)=2.676, p=0.0202) (Figure 5.9A). Two-way ANOVA revealed females demonstrated both a significant interaction between genotype and time point (F(1,16)=7.539, p=0.0144), and a significant effect of genotype (F(1,16)=6.783, p=0.0192), on frontal lobe *Bmal1* expression. Fl/fl females had higher *Bmal1* expression in the frontal lobe at ZT0 than cKO females (Šídák's post-hoc, p=0.0065), and compared to fl/fl females at ZT12 (Šídák's post-hoc, p=0.0087). This demonstrated that fl/fl females showed cyclical *Bmal1* expression in the frontal lobe, but that cKO females did not (Figure 5.9B).

No significant interaction of sex and time point was seen on fl/fl frontal lobe *Bmal1* expression levels (F(1,20)=3.713, p=0.0683). Interestingly, frontal lobe *Bmal1* expression in fl/fl females was seen to be antiphase to males and significantly lower at ZT12 (Šídák's post-hoc, p=0.0303) (Figure 5.9C). In addition, a significant interaction of sex and time point was seen on cKO frontal lobe *Bmal1* expression levels (F(1,20)=4.414, p=0.0485), as well as individual effects of sex (F(1,20)=6.324, p=0.0206), and time point (F(1,20)=5.368, p=0.0312). cKO males displayed significantly higher frontal lobe *Bmal1* expression at ZT12 compared to cKO females (Šídák's post-hoc, p=0.0036). This demonstrates that cKO females, but not males, displayed abolished cyclical *Bmal1* expression in the frontal lobe between ZT0 and ZT12 (Figure 5.9D).

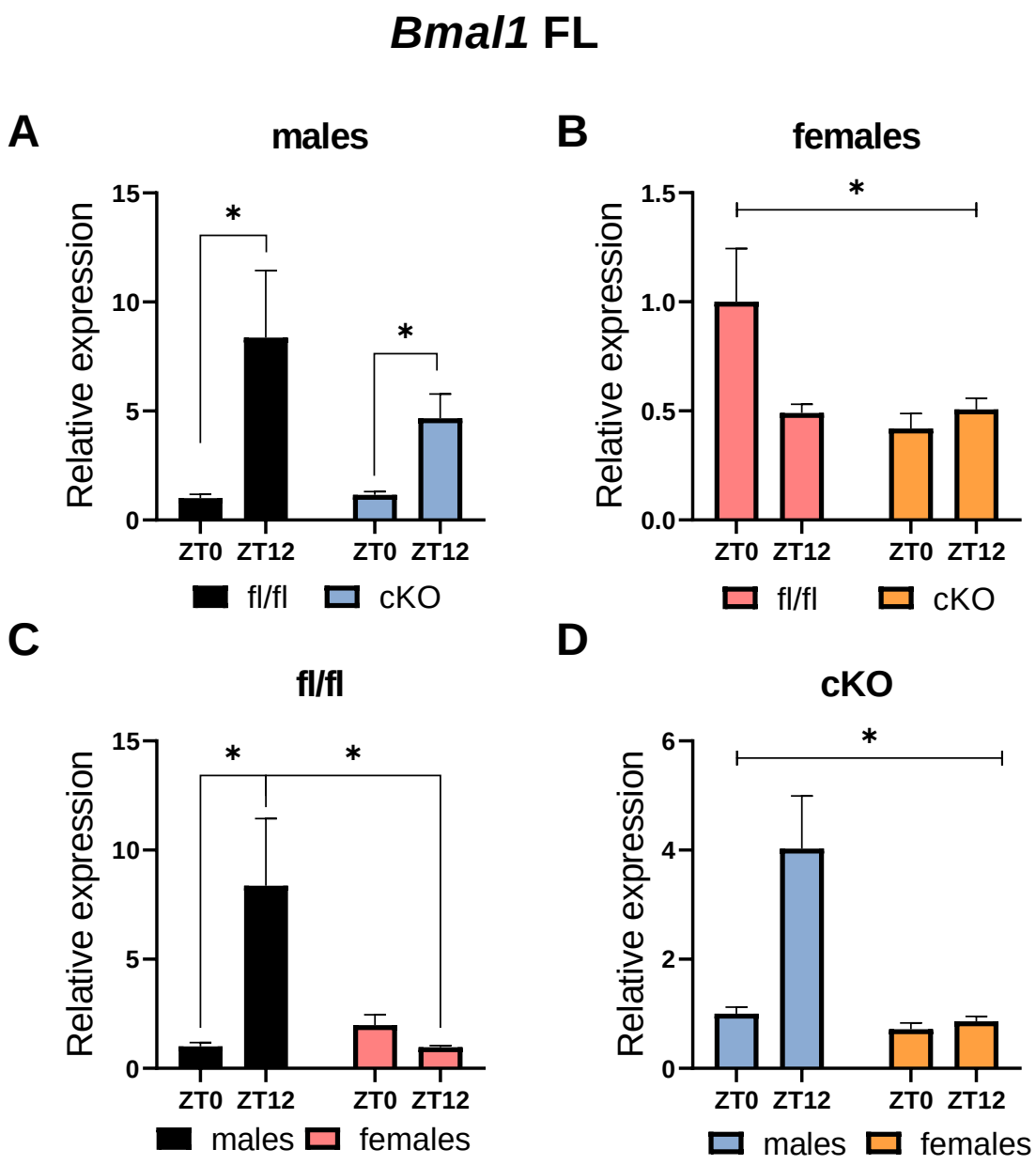


Figure 5.9: Loss of significant cyclical expression of *Bmal1* in the frontal lobe of *Npas2* cKO female mice.

A) RT-qPCR of *Bmal1* expression levels in fl/fl males and cKO males (all n = 8) in the frontal lobe (FL). There was a significant effect of time point on *Bmal1* expression (**, p=0.0089). Both fl/fl males and cKO males showed peak expression at ZT12 (*, p=0.0234, *, p=0.0202, respectively). B) RT-qPCR of *Bmal1* expression levels in fl/fl females and cKO females (ZT0 both n = 7, ZT12 both n = 8) in the frontal lobe. There was a significant interaction of genotype and time point on *Bmal1* expression (*, p=0.0144). Fl/fl females had significantly higher *Bmal1* expression at ZT0 than cKO females (**, p=0.0065) and compared to fl/fl females at ZT12 (**, p=0.0087). C) RT-qPCR of *Bmal1* expression levels in fl/fl male and female mice (males n = 8 in both, females ZT0 n = 7, ZT12 n = 8) in the frontal lobe. Fl/fl males had significantly higher *Bmal1* expression at ZT12 compared to fl/fl females (*, p=0.0303) and compared to fl/fl males at ZT0 (*, p=0.0310). D) RT-qPCR of *Bmal1* expression levels in cKO mice (males: n = 8 in both, females: ZT0 n = 7, ZT12 n = 8) in the frontal lobe. A significant

interaction between sex and time point was seen (*, p=0.0485). cKO males show peak expression at ZT12 (**, p=0.0051). Relative expression values were based on RT-qPCR reads after $2^{-\Delta Ct}$ transformation based on 18S results. See A.6 for details.

Bmal1 expression was also measured between C57BL/6J sexes under identical conditions. No significant interaction of sex and time point was seen on hepatic *Bmal1* expression (F(1,28)=0.825, p=0.3714). However, there was a significant effect of time point on hepatic *Bmal1* expression (F(1,28)=58.93, p<0.0001). Both males and females showed higher expression of *Bmal1* in the liver at ZT0 (Šídák's post-hoc, both p<0.0001), an effect which mirrors expression patterns noted in fl/fl mice (Figure 5.10A). Furthermore, an interaction between sex and time point was seen on frontal lobe *Bmal1* expression (F(1,25)=4.399, p=0.0462). A significant effect of time point was also noted (F(1,25)=8.982, p=0.0061). Females showed stronger cyclical expression, peaking at ZT12 (Šídák's post-hoc, p=0.0033) (Figure 5.10B).

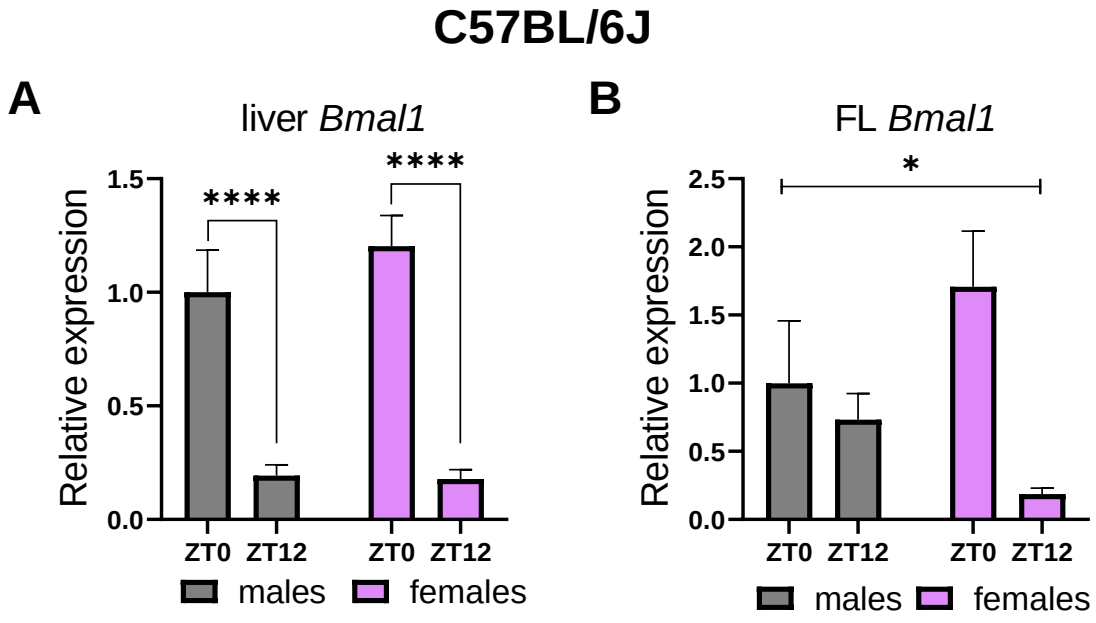


Figure 5.10: Significant effects of sex on frontal lobe *Bmal1* expression in C57BL/6J mice.

A) RT-qPCR of *Bmal1* expression in C57BL/6J mice (males n = 8, females n = 8, in both) in the liver at ZT0 and ZT12. A significant effect of time point was seen (****, p<0.0001). Both males and females showed elevated *Bmal1* expression in the liver at ZT0 (both: ****, p<0.0001). B) RT-qPCR of *Bmal1*

expression in C57BL/6J mice (males: ZT0 n = 7, ZT12 n = 6, females n = 8 in both) in the frontal lobe (FL). A significant interaction between timepoint and sex was seen (*, p=0.0462). At ZT0 females had higher frontal lobe *Bmal1* expression than ZT12 females (**, p=0.0033). Relative expression values were based on RT-qPCR reads after $2^{-\Delta Ct}$ transformation based on 18S results. See A.6 for details.

In summary, this analysis demonstrated significant sex and genotype effects on frontal lobe *Bmal1* expression as a result of hepatic *Npas2* loss. Interestingly, equivalent sex and genotype effects are not seen in hepatic *Bmal1* expression. Comparable expression patterns to fl/fl mice were seen in C57BL/6J *Bmal1* in the liver, though C57BL/6J males appear not to show cycling *Bmal1* expression in the frontal lobe, unlike fl/fl males.

5.3.6 Sexual dimorphism of *Rev-erbß* expression in hepatic *Npas2* cKO and fl/fl control mice

The *Rev-erb* genes also have significant roles in the negative limb of the circadian TTFL. REV-ERBs act as inhibitors of *Bmal1* transcription by binding the RORE within the *Bmal1* promoter. Conversely, RORα competes with REV-ERB proteins for the RORE within the *Bmal1* promoter which results in the circadian oscillation of BMAL1 expression (DeBruyne et al., 2007, Guillaumond et al., 2005, Preitner et al., 2002). Cyclical expression of *Rev-erbß* was therefore assessed in the frontal lobe and liver of *Npas2* cKO and fl/fl mice.

Two-way ANOVA revealed no significant interactions between genotype and time point on hepatic *Rev-erbß* expression in either males or females ((F(1,26)=1.097, p=0.3046), (F(1,26)=2.757, p=0.1088), respectively) (Figure 5.11A). Fl/fl females showed higher hepatic *Rev-erbß* expression at ZT12 than ZT0 (unpaired t-test, t(13)=3.206, p=0.0069), whilst cKO females show no temporal changes (Figure 5.11B). No significant interaction between sex and time point was seen on hepatic *Rev-erbß* expression in either fl/fl or cKO mice ((F(1,25)=0.2988, p=0.5895), (F(1,27)=3.226, p=0.0837), respectively). However, fl/fl mice demonstrated a significant effect of time point (F(1,25)=9.676, p=0.0046), where expression is higher at ZT12 (Figure 5.11C). Additionally, cKO mice displayed a significant effect of sex

on hepatic *Rev-erbß* expression ($F(1,27)=9.245$, $p=0.0052$), where cKO females showed lower *Rev-erbß* expression at ZT12 (Šídák's post-hoc, $p=0.0034$) (Figure 5.11D). It appears as though *Npas2* cKO females have lost cyclical expression of hepatic *Rev-erbß*, thus generating sex differences compared to cKO males.

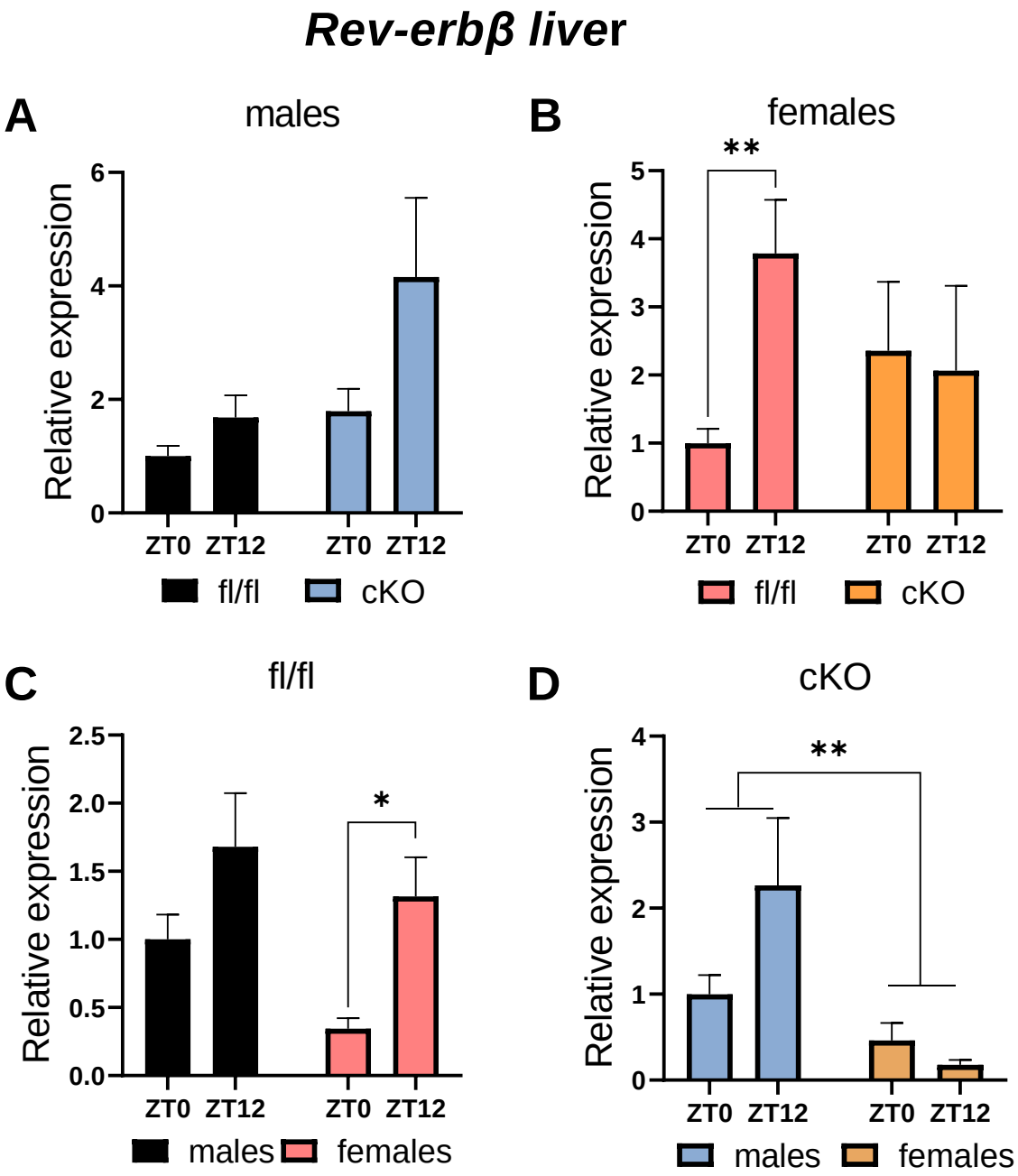


Figure 5.11: Significant sex differences in hepatic *Rev-erbß* expression in cKO mice.

A) RT-qPCR of *Rev-erbß* expression levels in fl/fl males and cKO males (fl/fl both n = 7, cKO both n = 8) in the liver at ZT0 and ZT12. B) RT-qPCR of *Rev-erbß* expression levels in fl/fl females and cKO females (ZT0 both n = 7, ZT12 both n = 8) in the liver. Fl/fl female *Rev-erbß* expression was higher at ZT12 than ZT0 (**, p=0.0069). C) RT-qPCR of *Rev-erbß* expression levels in fl/fl male and female mice (ZT0 both n = 7, ZT12 females n = 8, males n = 7) in the liver. There was a significant effect of time point (**, p=0.0046). Fl/fl female *Rev-erbß* expression was higher at ZT12 (**, p=0.0069). D) RT-qPCR of *Rev-erbß* expression levels in cKO mice (males: n = 8 in both, females: ZT0 n = 7, ZT12 n = 8) in the liver. There was a significant effect of sex on hepatic *Rev-erbß* expression (**, p=0.0052). Relative expression values were based on RT-qPCR reads after $2^{-\Delta Ct}$ transformation based on 18S results. See A.6 for details.

Next, *Rev-erbß* expression was explored in the frontal lobe of *Npas2* cKO and fl/fl mice. No significant interaction between genotype and time point was noted in frontal lobe *Rev-erbß* expression in either males of females ((F(1,24)=2.421, p=0.1328), (F(1,20)=0.7893, p=0.3849), respectively). However, both males and females demonstrated a significant effect of time point on *Rev-erbß* expression ((F(1,24)=8.306, p=0.0082), (F(1,20)=12.49, p=0.0021), respectively). Both fl/fl males and cKO males showed heightened frontal lobe *Rev-erbß* expression at ZT12 (unpaired t-tests, (t(12)=2.250, p=0.0440), (t(12)=4.028, p=0.0017), respectively), indicative of cyclical expression (Figure 5.12A). cKO females also demonstrated a heightened frontal lobe *Rev-erbß* expression at ZT12, in line with males (t(10)=5.529, p=0.0003). Fl/fl females showed a similar pattern of elevated frontal lobe *Rev-erbß* expression at ZT12, but this was not statistically significant (Figure 5.12B).

Furthermore, two-way ANOVA revealed no significant interaction between sex and time point on frontal lobe *Rev-erbß* expression in fl/fl mice (F(1,22)=4.003, p=0.0579), however, cKO mice did demonstrate a significant interaction between sex and time point (F(1,22)=9.390, p=0.0057). Both fl/fl and cKO revealed a significant effect of time point on frontal lobe *Rev-erbß* expression ((F(1,22)=4.660, p=0.0420), (F(1,22)=18.89, p=0.0003), respectively), and cKO mice also displayed a significant effect of sex (F(1,22)=15.43, p=0.0007). Both fl/fl males and cKO males had higher frontal lobe *Rev-erbß* expression at ZT12 than female counterparts (Šídák's post-hoc, p=0.0153, p<0.0001, respectively) (Figure

5.12C, 5.12D). This demonstrates higher amplitude of frontal lobe *Rev-erbß* oscillations in males compared to females, and no significant genotype differences.

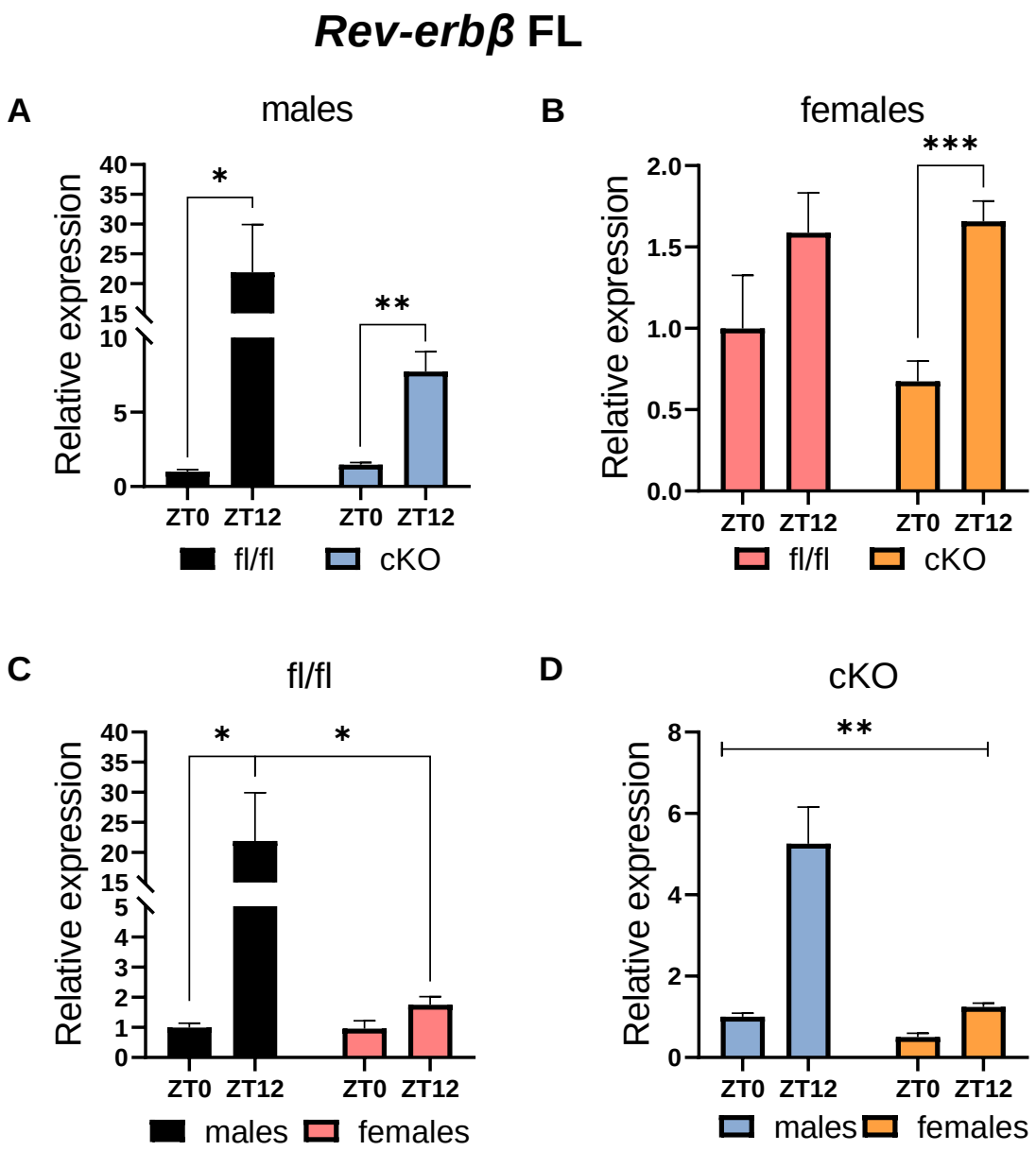


Figure 5.12: Significant sex differences in frontal lobe *Rev-erbß* expression in cKO and fl/fl mice.

A) RT-qPCR of *Rev-erbß* expression levels in fl/fl males and cKO males (ZT0 both n = 6, ZT12 both n = 8) in the frontal lobe (FL). There was a significant effect of time point on *Rev-erbß* expression (**, p=0.0082). Both fl/fl males and cKO males showed peak expression at ZT12 (*, p=0.0440, **, p=0.0017, respectively). B) RT-qPCR of *Rev-erbß* expression levels in fl/fl females and cKO females (all groups n = 6) in the frontal lobe. There was a significant effect of time point (**, p=0.0021). cKO females showed peak *Rev-erbß* expression at ZT12 (***, p=0.0003). C) RT-qPCR of *Rev-erbß* expression levels in fl/fl male and female mice (males: n = 6 in ZT, n = 8 in ZT12, females n = 6 in

both) in the frontal lobe. Fl/fl males had significantly higher *Rev-erbß* expression at ZT12 compared to fl/fl females (*, p=0.0153) and compared to fl/fl males at ZT0 (*, p=0.0440). D) RT-qPCR of *Rev-erbß* expression levels in cKO mice (males: n = 6 in ZT0, n = 8 in ZT12, females: n = 6 in both) in the frontal lobe. A significant interaction between sex and time point was seen (**, p=0.0057). cKO males showed higher expression at ZT12 compared to cKO females (p<0.0001) and compared to cKO males at ZT0 (p=0.0017). Relative expression values were based on RT-qPCR reads after $2^{-\Delta Ct}$ transformation based on 18S results. See A.6 for details.

Rev-erbß expression was also measured between C57BL/6J sexes under identical conditions. No significant interaction between sex and time point was seen in hepatic *Rev-erbß* expression (F(1,27)=3.598, p=0.0686). However, there was a significant effect of time point (F(1,27)=12.32, p=0.0016). Both males and females show elevated hepatic *Rev-erbß* expression at ZT12 (unpaired t-test, t(14)=2.838, p=0.0132, t(13)=4.875, p=0.0003, respectively) (Figure 5.13A). Additionally, analysis revealed a significant interaction between sex and time point in frontal lobe *Rev-erbß* expression (F(1,27)=7.640, p=0.0102). C57BL/6J males and females presented antiphase frontal lobe *Rev-erbß* expression across the two time points, an effect which was not seen in fl/fl mice. Additionally, fl/fl and cKO mice presented a significant difference in ZT12 expression of frontal lobe *Rev-erbß* between male and female mice; this significant sexual dimorphism was also noted in C57BL/6J (Šídák's post-hoc, p=0.0076) (Figure 5.13B).

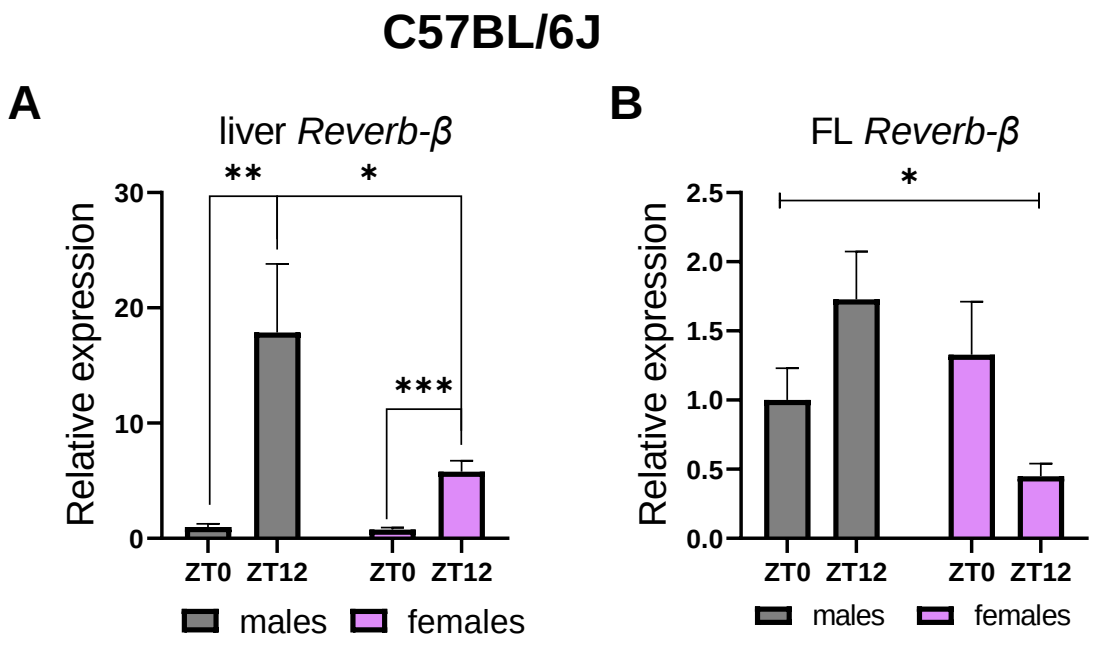


Figure 5.13: Sex differences in liver and frontal lobe *Rev-erbß* expression in C57BL/6J mice.

A) RT-qPCR of *Rev-erbß* expression in C57BL/6J mice (males n = 8, females: ZT0 n = 7, ZT12 n = 8) in the liver at ZT0 and ZT12. A significant effect of time point was seen (**, p=0.0016). Both males and females show elevated *Rev-erbß* expression in the liver at ZT0 (*, p=0.0132, ***, p=0.0003, respectively). Males had significantly higher expression at ZT12 than females (*, p=0.0191). B) RT-qPCR of *Rev-erbß* expression in C57BL/6J mice (males: ZT0 n = 7, ZT12 n = 8, females n = 8) in the frontal lobe. A significant interaction between timepoint and sex was seen (*, p=0.0102). At ZT12 males have higher frontal lobe *Rev-erbß* expression than ZT12 females (**, p=0.0076). Relative expression values were based on RT-qPCR reads after $2^{-\Delta Ct}$ transformation based on 18S results. See A.6 for details.

In summary, significant sex differences were seen in both hepatic and frontal lobe expression levels of *Rev-erbß*. However, neither tissue showed an effect of *Npas2* cKO on *Rev-erbß* expression. In addition, sexual dimorphism, and *Rev-erbß* gene expression patterns in fl/fl mice are not too dissimilar to C57BL/6J, suggesting that fl/fl mice are likely an appropriate equivalent to wild type for molecular control purposes. C57BL/6J female mice also show increased *Rev-erbß* expression at ZT12, like fl/fl mice, though so do C57BL/6J males.

Addendum:

This analysis could have been improved by performing three-way ANOVAs which included sex, genotype and lighting condition as variables, in replacement of two way

ANOVAs individually assessing the effect of genotype and then sex. Without demonstration of an interaction between sex X genotype, any claim that the two genotypes show variable sex differences has limited statistical support.

5.4 Discussion

To summarise, this work confirmed the liver specific reduction in NPAS2 protein levels in both male and female hepatic knockouts. However, only female cKO mice displayed a reduction in *Npas2* transcripts within the liver. Analysis of circadian gene expression revealed cyclical *Clock* expression in the frontal lobe was lost within hepatic NPAS2 knockout males and females. Furthermore, expression of *Bmal1* and *Clock* appears to be antiphase in male and female fl/fl control mice; this sexual dimorphism was abolished in NPAS2 liver knockouts. Finally, it was noted that hepatic NPAS2 loss in females impaired cyclical *Bmal1* expression in the frontal lobe.

5.4.1. *Npas2* exon 3 loss and protein reduction confirmed in male and female cKO mice

To date, little confirmation of this model other than genotyping had been performed. It was important to assess *Npas2* expression and protein levels within these mice; this was in order to rule out floxing implications on fl/fl mouse phenotypes, and *Npas2* loss in the brain on recognition memory differences. cDNA sequencing of male and female cKO mice confirmed exon 3 of *Npas2* was absent in the liver, further validating the knockout. It also confirmed exon 3 was still present in cKO frontal lobe and in fl/fl liver and frontal lobe. Similarly, significant reductions in NPAS2 protein levels were observed in cKO mice by western blot, additionally proving the model. Together, this suggests floxing has not impacted NPAS2, inferring that phenotypes seen in circadian period, phase, and weight loss in fl/fl mice are likely a product of background strain divergence.

Interestingly, only cKO females showed a significant reduction in hepatic *Npas2* expression by RT-qPCR. Males did not display a genotype difference in hepatic *Npas2* expression. Since the primer pair used for this analysis was downstream of exon 3, it suggests a transcript is still made specifically in males, despite the deleted exon 3. Garcia et al. removed the bHLH domain of *Npas2* that is upstream of exon 3 and added in a LacZ reporter in this region (Garcia et al., 2000). They found that an alternate ATG in exon 2 could act as an in-frame start codon, causing an *Npas2* transcript to be made and transcribed without exon 3. However, they found that without exon 3 and the bHLH domain, this transcript formed a truncated protein which was functionally null. Cells transfected with this mutant *Npas2* could not heterodimerise and cause transcriptional activation (Garcia et al., 2000). This suggests that generation of a transcript that does not contain exon 3 does not compromise our cKO mice; thus, confirmation of NPAS2 protein reduction and sequencing depicting exon 3 loss are sufficient to confirm the model. However, this is still an interesting finding, as it would propose a sex-linked effect of *Npas2* transcript production (Garcia et al., 2000). Notably, our western blot analysis appears to depict a reduced lower band size in cKO male mice. This could potentially depict the truncated transcript displayed in Garcia et al., 2000. To investigate this finding further, the marginally lower sized band seen in our cKO male western blot could be extracted from the nitrocellulose membrane and analysed by mass spectrometry to determine exact molecular weight. Garcia et al. demonstrate this truncated protein to have a lower molecular weight on western blots than intact *Npas2*, therefore extraction could conclude a sizing difference between male and female cKO mice, thus suggesting a sex-specific use of the alternate ATG start codon (Garcia et al., 2000).

5.4.2 Differential circadian expression in the frontal lobe as a possible mechanism for circadian behavioural and cognitive changes

Molecular expression analysis revealed significant effects of sex and/or hepatic *Npas2* loss on *Clock*, *Bmal1* and *Rev-erbß* expression in the liver and the frontal lobe. Hepatic *Clock* gene expression was shown to be higher in cKO males compared to fl/fl males. Since *Npas2* is a paralog of *Clock*, an upregulation of *Clock* in the liver following *Npas2* loss could be a mechanism of functional compensation, perhaps in a sex-specific manner. The converse has been shown in *Clock*$^{-/-}$ mice, where *Npas2* was seen to be significantly upregulated in the liver (Debruyne et al., 2006, Landgraf et al., 2016). However, *Clock* showed no significant alterations in expression within the forebrain following global *Npas2* loss (Dudley et al., 2003). Since upregulation of the opposing paralog was noted in hepatic tissue, but not within the brain following global KO, it may be that this compensatory effect is unique to the liver.

Similar findings were shown in *Bmal1* expression in the frontal lobe, though predominantly in females. cKO females showed no significant cycling of *Bmal1* at ZT0 and ZT12 time points, unlike both males and fl/fl females. Previously, loss of *Bmal1* in the forebrain has been shown to result in NOL recognition memory deficits. Contrastingly, *Bmal1* knockdown in microglia amounted to significant improvements in long-term recognition memory through the NOR paradigm (Snider et al., 2016, Wang et al., 2021). This presents a role for *Bmal1* expression in recognition memory. Since cKO females demonstrate loss of cyclical *Bmal1* expression between ZT0 and ZT12, and significantly improved memory performance from fl/fl females in LL, there may be a mechanism by which aberrant *Bmal1* expression improves cognitive output. Antiphase patterns in fl/fl mice were also noted in frontal lobe *Clock* expression, where opposing cycling meant fl/fl females had peak expression at ZT0, whilst fl/fl males had peak expression at ZT12, and neither cKO sex demonstrated cyclical expression. This reiterates that a peripheral knockout is capable of causing central

circadian gene expression to vary, which is a significant and novel finding. Additionally, this presents fl/fl females as having irregular frontal lobe circadian gene expression compared to all other groups tested, which may be an explanation for their behavioural circadian sex differences from fl/fl males, and their cognitive deficits. Loss of this anti-phase male versus female *Clock* and *Bmal1* expression may be why hepatic *Npas2* knockout mice fail to present sex differences in circadian period and phase, and cognition, under conditions of constant light.

Another noteworthy finding was significantly higher frontal lobe *Rev-erbß* expression in males compared to females, irrespective of genotype. This is especially interesting since enhanced *Rev-erbß* expression in Alzheimer's disease mouse models has been shown to improve cognitive deficits in NOR trials (Roby et al., 2019). A proposed mechanism for this cognitive improvement is the reduction in amyloid-ß plaques within the brains of Alzheimer's model mice, meaning this is not necessarily applicable to young mice with healthy brains. Still, other markers such as improved synaptic health and strengthened axoskeletal structures were noted as a result of enhanced *Rev-erbß* expression, meaning heightened *Rev-erbß* may have more widespread benefits within the brain (Roby et al., 2019). This could potentially be a mechanism that brings about cognitive sex differences in fl/fl mice, though the same reduction in expression was noted in cKO females and there was no cognitive dimorphism between cKO sexes.

Whilst these gene expression findings are both significant and interesting, they are limited to only exploring the positive limb of the TTFL and one gene from the additional loop. Future studies should investigate negative limb genes such as *Per1/Per2/Per3* and *Cry1/Cry2/Cry3*, as well as a wider range of additional loop genes, for example *Rev-erba* and *Rora*.

5.4.3. Sexual dimorphism in circadian gene expression in C67BL/6J mice

This study presents novel findings of sex differences in central and peripheral circadian gene expression within C57BL/6J mice. Molecular analysis revealed C57BL/6J sexual dimorphism in *Clock* and *Bmal1* expression in the frontal lobe, and *Rev-erbß* expression in both the liver and the frontal lobe. Within the frontal lobe, *Rev-erbß* and *Clock* expression were antiphase between males and females, and *Bmal1* showed a lack of rhythmicity in male mice between ZT0 and ZT12 time points. Sex differences in molecular rhythms in C57BL/6J mice have not been thoroughly explored, however, disparities in male and female circadian gene expression have been noted in humans. Three core clock genes, *Per2*, *Per3* and *Bmal1*, have been shown to have misaligned phases between aged men and women, with female gene expression peaking between four to six hours earlier in the frontal lobe than males (Lim et al., 2014). Interestingly, fl/fl, cKO and C57BL/6J mice in our study displayed significant influences of sex on *Bmal1* expression at ZT0 and ZT12 in the frontal lobe, perhaps as a result of asynchronous phase between sexes. Additionally, studies have shown that men and women show varied rhythmic transcriptomes in the frontal lobe and anterior cingulate cortex (ACC) (Logan et al., 2022). Females expressed almost four times as many cyclical transcripts within the ACC compared to males, whilst males had nearly double the volume of rhythmic transcripts in the frontal lobe. Interestingly, in males, ACC and frontal lobe expression was in phase, yet in females the regions presented significant phase asynchrony (Logan et al., 2022). In order to explore this in more depth, additional time points such as ZT6 and ZT18 should be utilised to assess phase shifts in peak expression. Additionally, wider screening of the C57BL/6J rhythmic transcriptome between males and females is necessary, perhaps employing additional brain regions and peripheral tissues with strong rhythmicity. Nonetheless, this proposes that C57BL/6J exhibit sexual dimorphism in molecular rhythms within the frontal lobe, a finding that has not yet been noted.

Concluding remarks

This project aimed to elucidate the roles of the circadian transcription factor NPAS2 in global circadian rhythmicity and cognition through a conditional knockout approach in mice. NPAS2 has been shown to have roles in the maintenance of circadian cycles within the body through its ability to heterodimerise with BMAL1 and cause transcriptional activation of clock-controlled genes. This role is predominantly utilized in the absence of CLOCK, for which NPAS2 is a paralog of and can functionally compensate for (DeBruyne et al., 2007). Despite this, peripheral roles of NPAS2 on global rhythmicity have not been well characterised.

This work aimed to assess the influence of hepatic NPAS2 globally, utilising locomotor behaviour measurements of knockout mouse models. Additionally, research into NPAS2 in cognition is limited, and specifically fails to explore NPAS2 and memory in the context of altered zeitgeber conditions. This work therefore also aimed to assess the role of NPAS2 in recognition memory through cycling and constant conditions. Initial revelations in hepatic NPAS2 knockouts, which were originally planned as control experiments for central NPAS2 loss, created the basis for the continuation of this project.

To investigate the impact of peripheral NPAS2 on global circadian rhythmicity and locomotor activity, pre- and post-natal hepatic NPAS2 knockout lines were established from $NPAS2^{TM2AAGAA}$ and $NPAS2^{TM2.1AAGAA}$ mice generated at the Baylor Institute (O'Neil et al., 2017). Our circadian analysis of locomotor behaviour under cycling and constant conditions found no significant differences in circadian period or circadian phase between either pre- or post-natal knockout and fl/fl control counterparts. This suggests that hepatic NPAS2 is not critical in maintaining global circadian rhythmicity in cycling conditions, nor is it necessary for adaptation to constant lighting conditions. This supports hypotheses which theorized that maintained global expression of *Clock* and intact NPAS2 within the SCN would be sufficient

to sustain behavioural rhythmicity (Debruyne et al., 2007). Additionally, even global loss of NPAS2 has not been shown to significantly alter circadian parameters (Landgraf et al., 2016). Furthermore, we found that in conditions of constant light, both C57BL/6J and fl/fl control mice displayed significant sexual dimorphism in period length, with females showing more elongated periods. This effect was absent in hepatic NPAS2 knockout mice, where no significant difference in constant light period was noted. This suggests the novel hypothesis that NPAS2 may be a factor underlying sex differences in circadian changes in constant light conditions. Since this work was completed, it has been found that NPAS2 is critical for sex differences in opioid tolerance, withdrawal, and analgesia (Puig et al., 2022). Our data suggests that this role may extend to circadian changes under abnormal lighting conditions. Circadian analysis of period and phase also revealed significant variability of transgenic lines compared to C57BL/6J wild type mice, likely as a result of background strain divergence over multiple generations of breeding.

Loss of NPAS2 has previously been shown to induce deficits in emotional long-term memory in cued and contextual fear paradigms, as well as significantly reduced anxiety in open field and elevated plus maze trials (Garcia et al., 2000, Ozburn et al., 2017). Research into NPAS2 in cognition is limited, and specifically fails to explore NPAS2 and memory in the context of altered zeitgeber conditions. This work therefore also aimed to assess the role of hepatic NPAS2 in recognition memory through cycling and constant light conditions. Most notably, our *in vivo* cognitive experiments found significantly improved recognition memory following post-natal hepatic NPAS2 loss, specifically under conditions of constant light. Molecular analysis confirmed intact *Npas2* within the brains of hepatic NPAS2 knockout mice, meaning this effect was due to liver specific NPAS2 loss. Closer examination of sexual dimorphism revealed that fl/fl control females demonstrated inferior recognition memory during constant light conditions in comparison to both control males and NPAS2 deleted

females. Thus, loss of NPAS2 within the liver abolishes sex differences in recognition memory specifically under conditions of constant light. We subsequently hypothesized that, as effects were confined to constant light conditions, differences in anxiety may be responsible. In particular, the potential confounding effects of experimental housing conditions in inducing anxiety, since the evaluation of circadian parameters under different lighting necessitated 'semi-socially isolated' housing conditions. We simultaneously considered the hypothesis that variations in circadian parameters at a sex level could impact cognition.

Furthermore, mice in semi-social isolation demonstrated significant sex-linked weight loss in all transgenic lines. Taken together with significant cognitive deficits in control mice within conditions of constant light, we hypothesized that anxiety phenotypes may be present in the transgenic models, either as a result of hepatic NPAS2 loss or a product of experimental conditions. In open field trials, anxiety was significantly increased in all groups following semi-social isolation. However, it is worth noting that this effect was not seen as robustly in light-dark box tests. This anxiogenic effect of isolation was not exacerbated by elongated semi-social isolation, nor semi-social isolation under constant light conditions. This suggests constant light is not more anxiogenic that other conditions within this mouse model, and also that a habituation effect may occur over time to offset the anxiety induced by isolation. Since no effect of hepatic NPAS2 loss was noted on anxiety parameters, it was concluded that anxiety did not have a role in significant recognition memory impairments noted in fl/fl control mice. Additionally, this proposes that hepatic NPAS2 loss does not generate an anxiety phenotype, and that NPAS2 knockouts which demonstrate alleviated anxiety are likely due to reduced NPAS2 within the brain (Ozburn et al., 2017).

Next, given the effect of NPAS2 cKO on novel object recognition memory, expression of core circadian genes within the pre-frontal cortex and liver of hepatic NPAS2 mice and controls were analysed. This was to determine if a peripheral knockout could induce central

circadian gene expression impacts. Firstly, hepatic *Clock* levels were found to be significantly higher in males with hepatic NPAS2 loss in comparison to control males, perhaps due to functional compensation. Secondly, it was noted that hepatic NPAS2 loss in females ablated cyclical *Bmal1* expression in the frontal lobe. Finally, expression of *Bmal1* and *Clock* appears to be antiphase in male and female fl/fl control mice; this sexual dimorphism was absent in the central circadian gene expression of NPAS2 liver knockouts. Thus, a proposed mechanism by which sexual dimorphism in recognition memory may be abolished in cKO mice in constant light conditions is through changes in central circadian gene expression, potentially in combination with loss of sexual dimorphism in period length and adaptation to constant light conditions.

Taken together, this work presents multiple novel conclusions. Primarily, peripheral NPAS2 loss is capable of having significant influences on recognition memory, specifically under conditions of constant light. Additionally, loss of NPAS2 in the liver has central effects on circadian gene expression within the frontal lobe. These findings likely centre around naturally occurring sexual dimorphism in circadian gene expression, period length and recognition memory which are diminished following NPAS2 loss in the liver. Finally, this work also presents novel findings in C57BL/6J mice, namely, significant sexual dimorphism in circadian behavioural parameters and circadian gene expression, whereby males and females present antiphase cycling of circadian genes within the brain.

Addendum:

The statistical analysis throughout this work could have been improved by performing three-way ANOVAs which included sex, genotype and lighting condition as variables. Without demonstration of an interaction between sex X genotype, any claim that the two genotypes show variable sex differences has limited statistical support.

Appendix

A.1 Additional investigations into sex-linked weight loss in SSI: maternal genotype influence and food consumption

A.1.1 Maternal genotype did not influence weight loss in SSI conditions

Findings in O'Neil et al. which utilised *Npas2* cKO and fl/fl mice demonstrated significant influence of maternal genotype on gut microbiome (O'Neil et al., 2013). O'Neil et al. concluded that NPAS2 has an epigenomic role in metabolic homeostasis governed by the maternal diet, and that this role manifests itself primarily during restricted feeding. In addition, the result was sexually dimorphic, since hepatic NPAS2 expression was not seen to be essential for adaptations to dietary stress in females (O'Neil et al. 2013, O'Neil et al., 2017). To investigate if their findings had merit within our study, fl/fl and cKO mice were pooled and weight loss was compared between groups with a hepatic *Npas2* knockout mother, and those with a fl/fl control mother. The genotype of the breeding mother was shown to have no significant effect on weight of pooled genotypes over a three-week period ($F(1,42)=0.5144$, $p=0.4772$) (Figure A.1). This is supporting evidence that maternal genotype was not a factor in significant sex-linked weight loss, and that this was likely a result of the experimental conditions (SSI) or a novel phenotype due to background strain divergence from C57BL/6J mice.

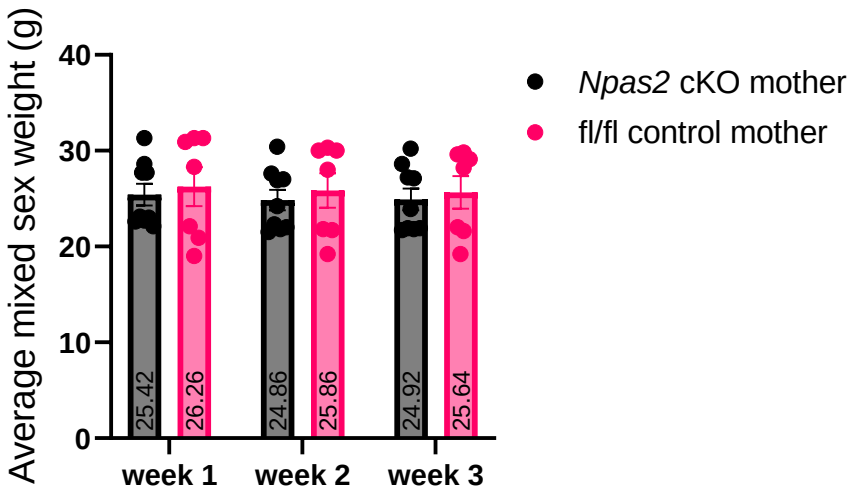


Figure A.1: Maternal genotype did not have a significant influence on weight loss.

Weights of pooled male and female cKO and fl/fl mice over a three-week period, with either an *Npas2* cKO mother (n = 9) or a fl/fl mother (n = 7). No significant influence of maternal genotype was seen.

A.1.2 Adequate consumption of dry diet in addition to intervention diet

Food consumption was measured by weighing out dry diet over multiple days. Many sources, including the biomedical support unit (BSU) that housed our animals, suggested a C57BL/6J adult mouse requires ~3 g of food daily (Bachmanov et al., 2002, BSU pers. comms, Species Scientific Information: Mouse).

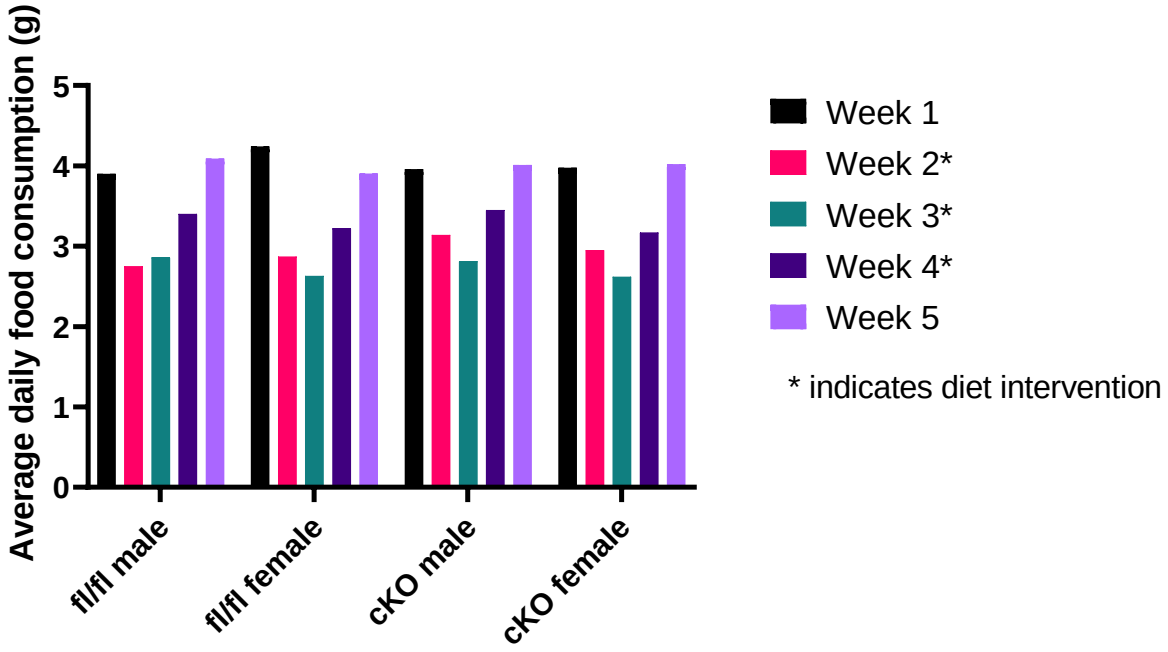


Figure A.2: Average daily dry food consumption of fl/fl and cKO mice over five weeks.

Averaged across n = 5 for all groups. Week one shows higher than 3 g food consumption for all groups despite significant sex-linked weight loss. Adequate food consumption is maintained throughout the five-week experimental period.

When combining the dietary intervention ‘mash’ and daily dry food intake, all groups consumed far more than an average of 3 g daily (Figure A.2). This is additional evidence that significant sex-linked weight loss was likely a result of the experimental conditions (SSI) or a novel phenotype due to background strain divergence from C57BL/6J mice.

A.2 No innate preferences were seen between objects within NOR sets

A.2.1 Materials and Methodology

Innate preferences were tested using C57BL/6J females (n = 8). A standard NOR test trial was performed, as discussed in 3.2.4, but without the sample trial 24 hours beforehand. Animals were given both unique objects of the set, one in the top left corner, one in the bottom right, and were allowed to explore both for ten minutes. Time spent with each object was then manually timed.

A.2.2 Results

Neither Set 1 nor Set 2 revealed a significant, innate preference for either object (paired t-test, $t(7)=0.6585$, $p=0.5313$, $t(7)=0.6972$, $p=0.5082$, respectively). This shows that results in the NOR were not skewed by natural object favourites (Figure A.3). Set 3 was not tested but was balanced across conditions, as well as between sexes and genotypes. All animals underwent a NOR test with all object sets.

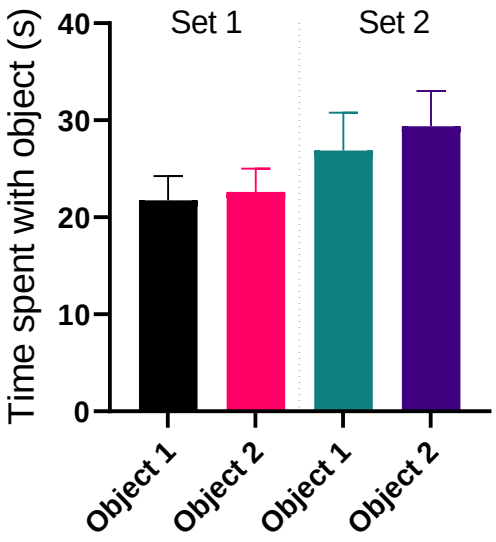


Figure A.3: No innate object preferences in Set 1 or Set 2.

Object preferences examined in C57BL/6J females (n = 8). No significant preference between objects in either Set 1 or Set 2 were seen.

A.3. Averaged actograms for fl/fl and cKO mice

A.3.1 Methods

Locomotor activity is recorded as described in 2.2.3. Using this distance travelled data, double-plotted actograms can be made using ActogramJ, a plugin for ImageJ, for visual representation of locomotor activity (Schmid et al., 2011, Schneider et al., 2012). Actograms demonstrated below are averaged across an entire cohort.

A.3.2 Results

Actograms detailed no visual differences in activity patterns in averaged fl/fl versus averaged cKO mice (Figure A.4).

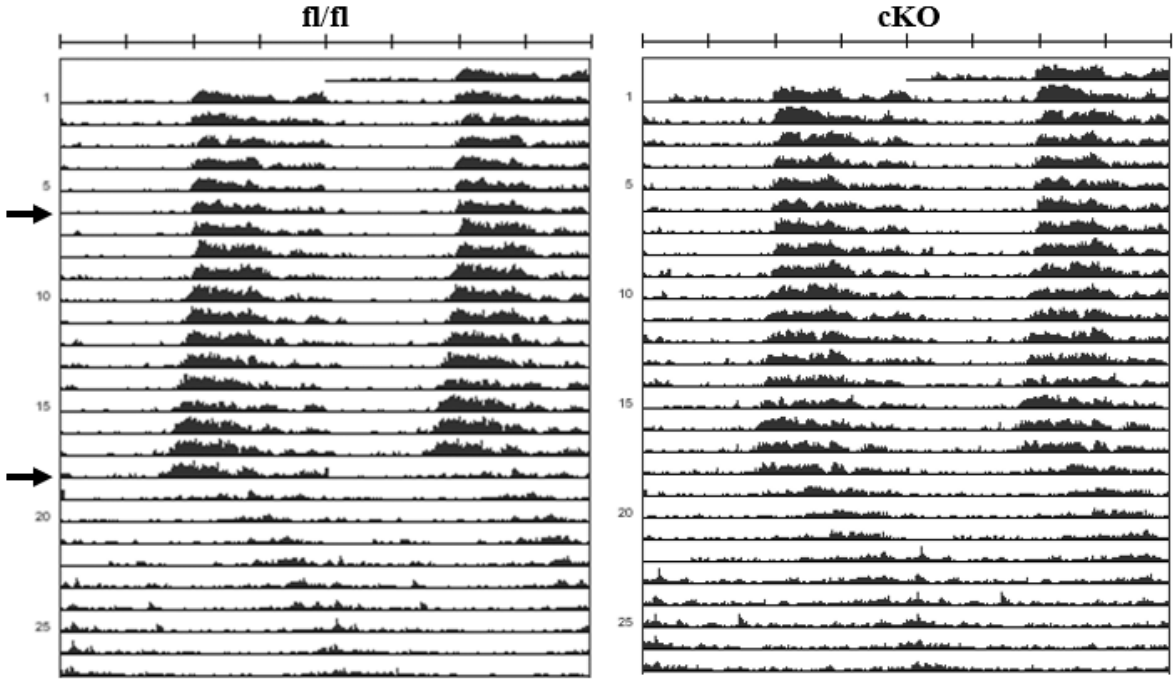


Figure A.4: Average distance travelled actograms for fl/fl and cKO mice did not reveal significant differences.

Average locomotor activity actograms for fl/fl mice (n = 8), and cKO mice (n = 8). Similar patterns of distance travelled were seen. The first five days were in LD conditions. The first arrow at six days indicates the beginning of DD conditions, and the second arrow at 17 days indicates LL conditions.

A.4 Oligonucleotides

Name	Specifics	Sequence
Cre F	Primer F (genotyping)	GCATTACCGGTCGATGCAACGAGTGATGAG
Cre R	Primer R (genotyping)	GAGTGAACGAACCTGGTCGAAATCAGTGCG
Npas2 loxP 5’	5’ F primer (genotyping)	TGGCCAAAGTCTAGCAAAA
Npas2 loxP 5’	5’ R primer (genotyping)	GGAACACACAAGGCACAGGTTA
Npas2 loxP 3’	3’ R primer (genotyping)	GGATGAGTTCCAGGGGATTATGA
Npas2 exon2 F	Ex2 F primer (confirmation)	GGACGAAGATGAGAAGGATAGAG
Npas2 exon4 R	Ex4 R primer (confirmation)	CTGAGGAATGATGGCTTCCAGT
Clock F	*Clock* primer F (RT-qPCR)	CGGCGAGAACTTGGCATT
Clock R	*Clock* primer R (RT-qPCR)	AGGAGTTGGGCTGTGATCA
Bmal1 F	*Bmal1* primer F (RT-qPCR)	TGACCCTCATGGAAGGTTAGAA
Bmal1 R	*Bmal1* primer R (RT-qPCR)	GGACATTGCATTGCATGTTGG
Rev-erbß F	*Rev-erbß* primer F (RT-qPCR)	GGAGTTCATGCTTGTGAAGGCTGT
Rev-erbß R	*Rev-erbß* primer R (RT-qPCR)	CAGACACTTCTTAAAGCGGCACTG
Npas2 F	*Npas2* primer F (RT-qPCR)	ATCGTCGTGACAACAGACGG
Npas2 R	*Npas2* primer R (RT-qPCR)	CTTGGACATTTACCGGCGGA
18S rRNA F	*18S rRNA* primer F (RT-qPCR)	GTAACCCGTTGAACCCCATT
18S rRNA R	*18S rRNA* primer R (RT-qPCR)	CAAGCTTATGACCCGCACTT

Table A.1: RT-qPCR and PCR primers.

A.5 RT-qPCR primer validation

The RT-qPCR primers used in Chapter 5 were validated by investigation of the reaction efficiency to ensure proportional, linear relationships between Ct values and cDNA input concentration was noted. Serial dilutions and primer validations for *18s rRNA*, *Clock*, *Bmal1* and *Rev-erbß* were performed by Mitchell Masterson, using NIH3T3 cell cDNA. *Npas2* RT-

qPCR primers were validated as part of this study, using C57BL/6J liver cDNA. The association between Ct value and relative quantity of cDNA was investigated using semi-log least squares fit on Prism. The output of this interpolation analysis was an equation of a linear line, and an R squared value. R squared values > 0.95 display high correlation between input cDNA and the Ct value given, meaning the reaction efficiency is irrespective of cDNA concentration (Bustin and Huggett, 2017). Thus, primers were only employed in this study if the gave an R squared value of > 0.95 (Figure A.5).

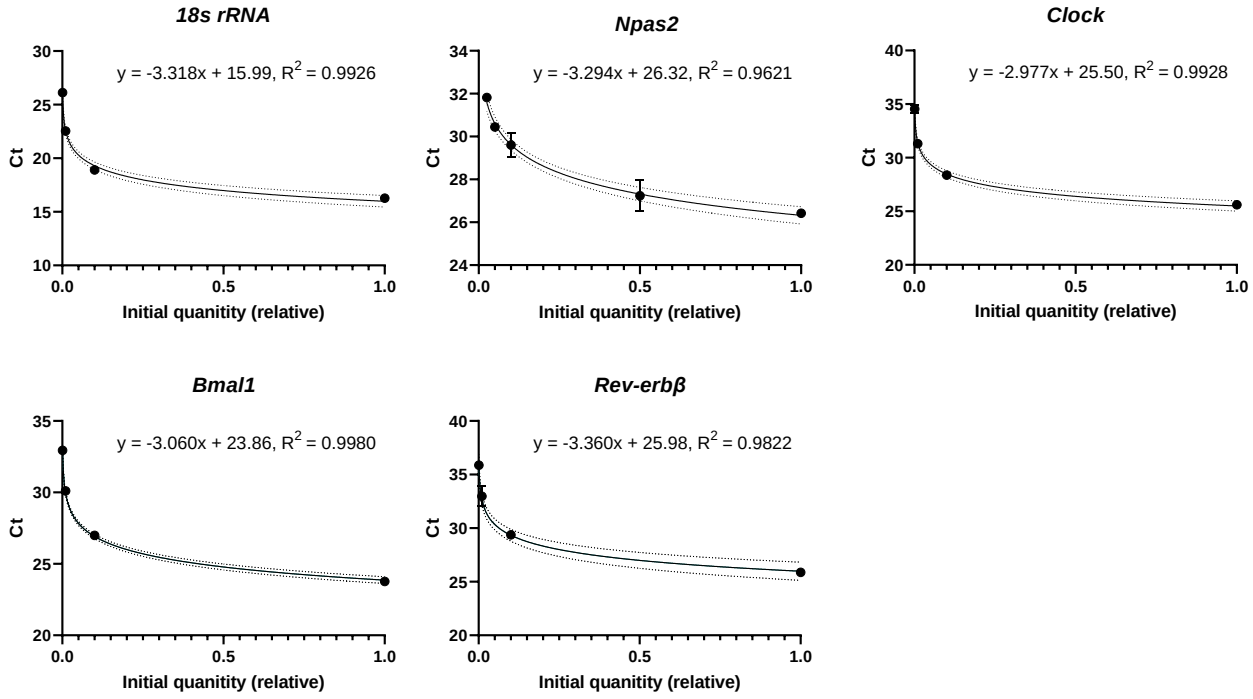


Figure A.5: RT-qPCR primer validations.

Serial dilutions and primer validations for *18s rRNA*, *Clock*, *Bmal1* and *Rev-erbß* were performed by Mitchell Masterson, using NIH3T3 cell cDNA (Mitchell Masterson, PhD thesis, 2020). *Npas2* RT-qPCR primers were validated as part of this study, using C57BL/6J liver cDNA. All primer sets used had and R squared value >0.95.

To validate primer specificity, RT-qPCR primer products were ran on an agarose gel, as described in 2.2.11. For all primer sets the expected product sizes were observed (Figure A.6).

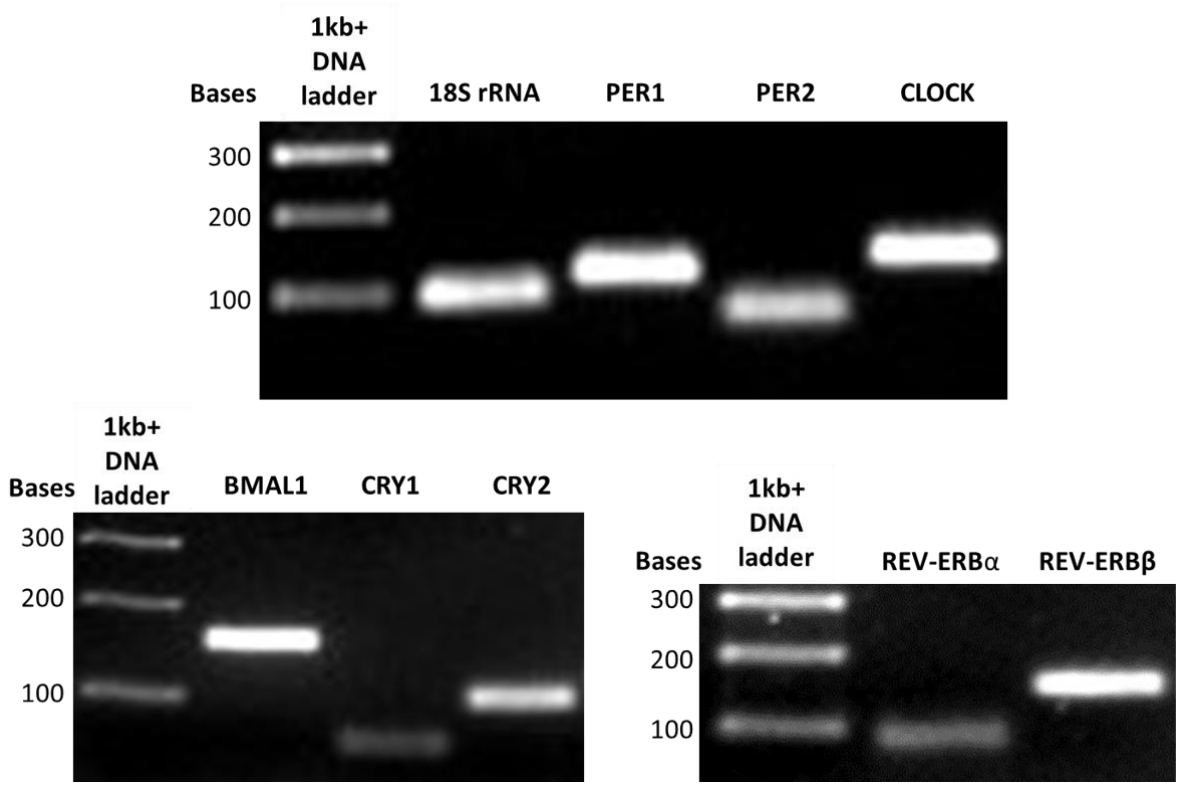


Figure A.6: Product sizes from RT-qPCR correspond to predicted sizes.

Experiment performed by Mitchell Masterson, figure taken from Mitchell Masterson, PhD thesis, 2020. Only *18s rRNA*, *Clock*, *Bmal1* and *Rev-erbß* primer sets were used in this study. Predicted sizes were: *18s rRNA* 93 bp, *Clock* 135 bp, *Bmal1* 154 bp and *Rev-erbß* 150 bp.

Additionally, this was the case for the *Npas2* primer set which also showed the predicted band size when ran on a gel (Figure A.7).

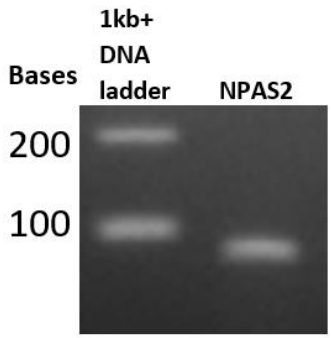


Figure A.7: Product size for RT-qPCR using *Npas2* primers.

Product size showed below 100 bp, consistent with a 77 bp product for our *Npas2* primer sets.

A.6 Normalisation of RT-qPCR groups

When comparing fl/fl males and females, all samples are normalised to fl/fl males thus making them equal to one. When comparing fl/fl males and cKO males, all samples are also normalised to fl/fl males thus making them equal to one. When comparing fl/fl females and cKO females, all samples are normalised to fl/fl females thus making them equal to one. When comparing cKO males and cKO females, all samples are normalised to cKO males thus making them equal to one. In cases where all sex and genotype groups are compared, all samples are normalised to fl/fl males. C57BL/6J RT-qPCRs comparing male and female expression were always normalised to C57BL/6J males, making them equal to one.

A.7 Additional cDNA confirmation gel for validation on full length Npas2 in fl/fl frontal lobe

The gel image represented in Figure 5.1 (section 5.3.1) displays the *Npas2* transcript for all genotypes and tissues but showed some contamination of fl/fl frontal lobe. Fl/fl frontal lobe displays the expected band of 240 bp, indicative of full length, intact *Npas2* transcript, but also a faint present of the 91 bp band which likely represents exon 3 deleted *Npas2*. Using gel electrophoresis and PCR methods detailed in 2.2.10 and 2.2.11, this gel was repeated using only cKO and fl/fl samples to confirm this 91 bp band was not present in fl/fl frontal lobe (Figure A.8).

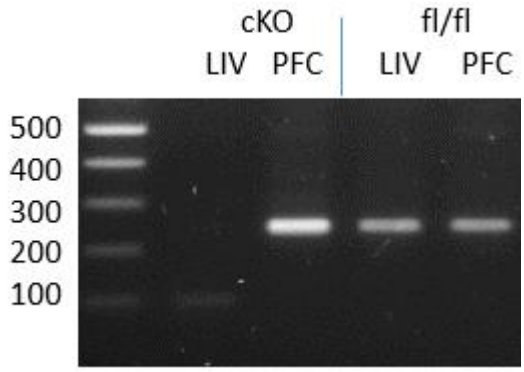


Figure A.8: Repeat gel image to confirm absence of 91 bp band in fl/fl frontal lobe.

Fl/fl frontal lobe demonstrated a single band when amplified with exon 2 – exon 4 *Npas2* primers. This band represents intact exon 3.

This figure depicts only a single 240 bp band for fl/fl frontal lobe, confirming no loss of exon 3 within the *Npas2* transcript. Due to time constraints, the entire gel represented in Figure 5.1 could not be repeated, but this study concludes fl/fl frontal lobe has only full length *Npas2* transcripts.

A.8 Statistical analysis of C57BL/6J period and phase in comparison to transgenic lines

A.8.1 C57BL/6J, Afp fl/fl and Afp cKO

Supplementary mixed-effects analysis of Afp fl/fl, Afp cKO and C57BL/6J genotypes revealed a significant interaction between genotype and lighting condition on circadian period ($F(4,142)=6.382$, $p<0.0001$), which specifically showed C57BL/6J period to be lower than Afp cKO and Afp fl/fl in LD (Tukey's post-hoc, $p=0.0220$ and $p=0.0048$, respectively) and higher than Afp fl/fl in LL (Tukey's post-hoc, $p=0.0274$) (Figure 2.18A). Mixed-effects analysis also showed genotype to have a significant effect on phase ($F(2,52)=11.92$, $p<0.0001$), specifically C57BL/6J having a higher phase in LD than Afp cKO and Afp fl/fl (Tukey's post-hoc, $p=0.0131$, $p<0.0001$, respectively) and a higher phase in LL than Afp cKO and Afp fl/fl (Tukey's post-hoc, $p<0.0001$ and $p=0.0008$, respectively) (Figure 2.18B).

A.8.2 C57BL/6J, fl/fl and cKO

Supplementary mixed-effects analysis of fl/fl, cKO and C57BL/6J genotypes revealed a significant interaction between genotype and lighting condition ($F(4, 86)=11.23$, $p<0.0001$), which specifically showed both transgenic lines had a higher circadian period than C57BL/6J mice in LD (Tukey's post-hoc, cKO: $p=0.0472$ and fl/fl: $p=0.0016$) and a significantly lower period in LL compared to C57BL/6J mice (Tukey's post-hoc, cKO: $p=0.0096$, fl/fl: $p=0.0009$) (Figure 2.19A). Mixed-effects analysis also showed genotype to have a significant effect on phase ($F(2,45)=3.879$, $p=0.0279$), specifically C57BL/6J had a lower phase in DD than fl/fl (Tukey's post-hoc, $p=0.0085$) and a higher phase in LL than cKO and fl/fl (Tukey's post-hoc, cKO: $p=0.0035$ and fl/fl: $p=0.0002$) (Figure 2.19B).

A.9 Professional Internship Placement (PIP) Reflective Statement

The Professional Internship Placement is a compulsory assignment which must be undertaken by Doctoral Training Programme (DTP) students. This reflective statement is designed to capture the skills development during the student's placement and the impact on their career plans it has had:

During the COVID-19 Pandemic, the lab I worked in was closed for six months. Following that closure, it reopened at half capacity meaning all students were allowed in the lab for one to two days a week. Despite this significant loss of experimental time, I was determined to do an in-person, immersive PIP that could assist my future career decisions and grant me additional molecular biology skills. In order to ensure I could do an in-person PIP, I refrained from interrupting my studies during the second and third year, which is where a PIP is normally recommended, due to nation-wide lockdowns. Instead, I undertook my placement nine months before the end of my PhD. From January to March of this year (2022), I worked at QIAGEN in their Molecular Assay Development team for Clinical Diagnostics in

Manchester. My role involved trialling a diagnostic kit which screened for HPV strains within cervical cancer tissue. This involved RT-qPCRs, RNA extractions and an immense amount of EU and FDA regulated paperwork. My work helped advance the trial stage of this kit and also developed more troubleshooting methods as a result of Guard-band studies – which assessed the conformity and robustness of the diagnostic kit.

This work was a welcome break from intense *in vivo* experiments throughout my PhD and helped me come back to my studies with a clear mind, ready to begin writing. However, it was not quite the opportunity I had hoped for. I felt my PIP was somewhat repetitive, since I performed the same procedures, namely RT-qPCR, two or three times a day for almost two months. From a scientific skills perspective, I did not gain the experience of using new equipment and methodologies that I was hoping for. However, the working environment was very insightful. Having been a Master's student and then a PhD student of small labs, I am used to being largely independent and self-sufficient. My time at QIAGEN aided my communication skills, as well as helping me learn to work cooperatively in a team and rely on others. It also allowed me to see the downstream effects of research processes, where a significant laboratory finding can result in a diagnostic method going to market. This gave me a wider picture of the necessity of scientific research and the benefits to the general public which are somewhat less visible during academic research.

Bibliography

Abramov, U., Raud, S., Kõks, S., Innos, J., Kurrikoff, K., Matsui, T., & Vasar, E. (2004). Targeted mutation of CCK2 receptor gene antagonises behavioural changes induced by social isolation in female, but not in male mice. *Behavioural Brain Research*, *155*(1), 1–11.

Albasser, M. M., Chapman, R. J., Amin, E., Iordanova, M. D., Vann, S. D., & Aggleton, J. P. (2010). New behavioral protocols to extend our knowledge of rodent object recognition memory. *Learning and Memory*, 17(8), 407–419.

Almeida-Santos, A. F., Carvalho, V. R., Jaimes, L. F., de Castro, C. M., Pinto, H. P., Oliveira, T. P. D., Vieira, L. B., Moraes, M. F. D., & Pereira, G. S. (2019). Social isolation impairs the persistence of social recognition memory by disturbing the glutamatergic tonus and the olfactory bulb-dorsal hippocampus coupling. *Scientific Reports 2019 9:1*, *9*(1), 1–13.

Alves-Simoes, M., Coleman, G., & Canal, M. M. (2016). Effects of type of light on mouse circadian behaviour and stress levels. *Laboratory Animals*, 50(1), 21–29.

Amiri, S., Haj-Mirzaian, A., Rahimi-balaei, M., Razmi, A., Kordjazy, N., Shirzadian, A., Ejtemaei Mehr, S., Sianati, H., & Dehpour, A. R. (2015). Co-occurrence of anxiety and depressive-like behaviors following adolescent social isolation in male mice; possible role of nitrergic system. Ph*ysiology & Behavior*, 145, 38–44.

Andreano, J. M., & Cahill, L. (2009). Sex influences on the neurobiology of learning and memory. *Learning and Memory*, 16(4), 248–266.

Andrews, R. V., & Edgar Folk, G. (1964). Circadian Metabolic Patterns in Cultured Hamster Adrenal Glands. *Comparative Biochemistry and Physiology*, *11*(4), 393–409.

Antunes, M., & Biala, G. (2012). The novel object recognition memory: Neurobiology, test procedure, and its modifications. *In Cognitive Processing* (Vol. 13, Issue 2, pp. 93–110). Springer.

Arqué, G., Fotaki, V., Fernández, D., de Lagrán, M. M., Arbonés, M. L., & Dierssen, M. (2008). Impaired spatial learning strategies and novel object recognition in mice haploinsufficient for the dual specificity tyrosine-regulated kinase-1A (Dyrk1A). *PLoS ONE*, 3(7), e2575.

Asadian, N., Parsaie, H., Vafaei, A. A., Dadkhah, M., Omoumi, S., & Sedaghat, K. (2022). Chronic light deprivation induces different effects on spatial and fear memory and hippocampal BDNF/TRKB expression during light and dark phases of rat diurnal rhythm. *Behavioural Brain Research*, *418*.

Asher, G., Gatfield, D., Stratmann, M., Reinke, H., Dibner, C., Kreppel, F., Mostoslavsky, R., Alt, F. W., and Schibler, U. (2008). "SIRT1 Regulates Circadian Clock Gene Expression through PER2 Deacetylation." *Cell* 134 (2): 317–28.

Aten, S., Hansen, K. F., Snider, K., Wheaton, K., Kalidindi, A., Garcia, A., Alzate-Correa, D., Hoyt, K. R., and Obrietan, K. (2018). "miR-132 Couples the Circadian Clock to Daily Rhythms of Neuronal Plasticity and Cognition." *Learning & Memory* 25 (5): 214–29.

Bachmanov, A. A., Reed, D. R., Beauchamp, G. K., & Tordoff, M. G. (2002). Food Intake, Water Intake, and Drinking Spout Side Preference of 28 Mouse Strains. *In Behavior Genetics* (Vol. 32, Issue 6).

Bailey, M., & Silver, R. (2014). Sex differences in circadian timing systems: implications for disease. *Frontiers in Neuroendocrinology*, *35*(1), 111–139.

Balsalobre, A., Brown, S. A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H. M., Schütz, G., Schibler, U. (2000). Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science (New York, N.Y.)*, *289*(5488), 2344–2347.

Balsalobre, A., Damiola, F., & Schibler, U. (1998). A Serum Shock Induces Circadian Gene Expression in Mammalian Tissue Culture Cells. *Cell*, *93*(6), 929–937.

Banks, G., Nolan, P. M., & Peirson, S. N. (2016). "Reciprocal interactions between circadian clocks and ageing." *Mammalian Genome.* 27: 332–340

Bartling, B., Al-Robaiy, S., Lehnich, H., Binder, L., Hiebl, B., & Simm, A. (2017). Sex-related differences in the wheel-running activity of mice decline with increasing age. *Experimental Gerontology*, *87*(Pt B), 139–147.

Beam, C. R., Kaneshiro, C., Jang, J. Y., Reynolds, C. A., Pedersen, N. L., & Gatz, M. (2018). Differences Between Women and Men in Incidence Rates of Dementia and Alzheimer's Disease. *Journal of Alzheimer's Disease : JAD*, *64*(4), 1077.

Bebas, P., Goodall, C. P., Majewska, M., Neumann, A., Giebultowicz, J. M., & Chappell, P. E. (2009). Circadian clock and output genes are rhythmically expressed in extratesticular ducts and accessory organs of mice. *The FASEB Journal*, *23*(2), 523–533.

Becker-Krail, D. D., Parekh, P. K., Ketchesin, K. D., Yamaguchi, S., Yoshino, J., Hildebrand, M. A., Dunham, B., Ganapathiraju, M. K., Logan, R. W., Colleen McClung, C. A. (2022). Circadian transcription factor NPAS2 and the NAD +-dependent deacetylase SIRT1 interact in the mouse nucleus accumbens and regulate reward. *European Journal of Neuroscience.* ;55(3):675-693.

Beeler, J. A., Prendergast, B., & Zhuang, X. (2006). Low amplitude entrainment of mice and the impact of circadian phase on behavior tests. *Physiology and Behavior*, *87*(5), 870–880.

Beery, A. K., & Zucker, I. (2011). Sex bias in neuroscience and biomedical research. In *Neuroscience and Biobehavioral Reviews* (Vol. 35, Issue 3, pp. 565–572). NIH Public Access.

Bélanger, V., Picard, N., and Cermakian, N. (2006). "The Circadian Regulation of Presenilin-2 Gene Expression." *Chronobiology International* 23 (4): 747–66.

Benegiamo, G., Mazzoccoli, G., Cappello, F., Rappa, F., Scibetta, N., Oben, J., Greco, A., Williams, R., Andriulli, A., Vinciguerra, M., Pazienza, V. (2013). Mutual Antagonism between

Circadian Protein Period 2 and Hepatitis C Virus Replication in Hepatocytes. *PLOS ONE*, *8*(4), e60527.

Benice, T. S., Rizk, A., Kohama, S., Pfankuch, T., & Raber, J. (2006). Sex-differences in age-related cognitive decline in C57BL/6J mice associated with increased brain microtubule-associated protein 2 and synaptophysin immunoreactivity. *Neuroscience*, 137(2), 413–423.

Berger-Sweeney, J., Arnold, A., Gabeau, D., & Mills, J. (1995). Sex Differences in Learning and Memory in Mice: Effects of Sequence of Testing and Cholinergic Blockade. *Behavioral Neuroscience*, *109*(5), 859–873.

Berry, A., Bellisario, V., Capoccia, S., Tirassa, P., Calza, A., Alleva, E., & Cirulli, F. (2012). Social deprivation stress is a triggering factor for the emergence of anxiety- and depression-like behaviours and leads to reduced brain BDNF levels in C57BL/6J mice. *Psychoneuroendocrinology*, *37*(6), 762–772.

Bertolucci, C., Cavallari, N., Colognesi, I., Aguzzi, J., Chen, Z., Caruso, P., Foá, A., Tosini, G., Bernardi, F., and Pinotti, M. (2008). "Evidence for an Overlapping Role of CLOCK and NPAS2 Transcription Factors in Liver Circadian Oscillators." *Molecular and Cellular Biology* 28 (9): 3070–75.

Bethus, I., Tse, D., & Morris, R. G. M. (2010). Dopamine and Memory: Modulation of the Persistence of Memory for Novel Hippocampal NMDA Receptor-Dependent Paired Associates. *Behavioral/Systems/Cognitive* 30(5):1610 –1618

Bettis, T. J., & Jacobs, L. F. (2012). Sex differences in object recognition are modulated by object similarity. *Behavioural Brain Research*, *233*(2), 288–292.

Bettis, T. J., & Jacobs, L. F. (2013). Sex differences in memory for landmark arrays in C57BL/J6 mice. *Animal Cognition*, *16*(6), 873–882.

Bilkei-Gorzó, A., Gyertyán, I., & Lévay, G. (1998). mCPP-induced anxiety in the light-dark box in rats - A new method for screening anxiolytic activity. *Psychopharmacology*, 136(3), 291–298.

Blattner, M. S., & Mahoney, M. M. (2013). Photic phase-response curve in 2 strains of mice with impaired responsiveness to estrogens. *Journal of Biological Rhythms*, *28*(4), 291–300.

Borsini, F., Podhorna, J., & Marazziti, D. (2002). Do animal models of anxiety predict anxiolytic-like effects of antidepressants? *In Psychopharmacology* (Vol. 163, Issue 2, pp. 121–141). Springer.

Botton, P. H., Costa, M. S., Ardais, A. P., Mioranzza, S., Souza, D. O., da Rocha, J. B. T., & Porciúncula, L. O. (2010). Caffeine prevents disruption of memory consolidation in the inhibitory avoidance and novel object recognition tasks by scopolamine in adult mice. *Behavioural Brain Research*, 214(2), 254–259.

Bouayed, J., Rammal, H., Younos, C., & Soulimani, R. (2007). Positive correlation between peripheral blood granulocyte oxidative status and level of anxiety in mice. *European Journal of Pharmacology*, *564*(1–3), 146–149.

Bourin, M., & Hascoët, M. (2003). The mouse light/dark box test. *In European Journal of Pharmacology* (Vol. 463, Issues 1–3, pp. 55–65). Elsevier.

Breen, D. P., Vuono, R., Nawarathna, U., Fisher, K., Shneerson, J. M., Reddy, A. B., and Barker, R. A. 2014. "Sleep and Circadian Rhythm Regulation in Early Parkinson Disease." *JAMA Neurology* 71(5):589-595.

Brown, A. J., Pendergast, J. S., & Yamazaki, S. (2019). Peripheral Circadian Oscillators. In *Yale Journal of Biology and Medicine* (Vol. 92), 327-335.

Bryant, C. D., Zhang, N. N., Sokoloff, G., Fanselow, M. S., Ennes, H. S., Palmer, A. A., & McRoberts, J. A. (2008). Behavioral differences among C57BL/6 substrains: Implications for transgenic and knockout studies. *Journal of Neurogenetics*, 22(4), 315–331.

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., Simon, M. C., Takahashi, J. S., and Bradfield, C. A. (2000). "Mop3 Is an Essential Component of the Master Circadian Pacemaker in Mammals." *Cell* 103 (7): 1009–17.

Bunning, E. (1958) Das weiterlaufen der "physiologischen uhr" in sugerdarm ohne zentrale steuerung. "Das weiterlaufen der "physiologischen Uhr" im Saugerdarm ohne zentrale Steuerung," can be translated into "The persistence of "physiological clock" in intestine without central control". *Naturwissenschaften* 45:68.

Bustin, S., & Huggett, J. (2017). qPCR primer design revisited. *In Biomolecular Detection and Quantification* (Vol. 14, pp. 19–28). Elsevier GmbH.

Butler, M. P., Karatsoreos, I. N., Sauter, J. Le, & Silver, R. (2012). Dose-Dependent Effects of Androgens on the Circadian Timing System and Its Response to Light. *Endocrinology*, *153*(5), 2344.

Butler, T. A. J., Paul, J. W., Chan, E. C., Smith, R., & Tolosa, J. M. (2019). Misleading Westerns: Common Quantification Mistakes in Western Blot Densitometry and Proposed Corrective Measures. *BioMed Research International*, *2019*.

Cain, S. W., Dennison, C. F., Zeitzer, J. M., Guzik, A. M., Khalsa, S. B. S., Santhi, N., Schoen, M. W., Czeisler, C. A., & Duffy, J. F. (2010). Sex differences in phase angle of entrainment and melatonin amplitude in humans. *Journal of Biological Rhythms*, *25*(4), 288–296.

Carlini, V. P., Martini, A. C., Schiöth, H. B., Ruiz, R. D., Fiol de Cuneo, M., & de Barioglio, S. R. (2008). Decreased memory for novel object recognition in chronically food-restricted mice is reversed by acute ghrelin administration. *Neuroscience*, 153(4), 929–934.

Castro, J. P. M. V., Frussa-Filho, R., Fukushiro, D. F., Chinen, C. C., Abílio, V. C., & Silva, R. H. (2005). Effects of long-term continuous exposure to light on memory and anxiety in mice. *Physiology and Behavior*, 86(1–2), 218–223.

Chang, H. C., & Guarente, L. (2013). "SIRT1 mediates central circadian control in the SCN by a mechanism that decays with ageing." Cell, 153(7), 1448–1460.

Chen, R., Seo, D. O., Bell, E., Von Gall, C., & Lee, D. C. (2008). Strong resetting of the mammalian clock by constant light followed by constant darkness. *Journal of Neuroscience,* 28(46), 11839–11847.

Choleris, E., Thomas, A. W., Kavaliers, M., & Prato, F. S. (2001). A detailed ethological analysis of the mouse open field test: Effects of diazepam, chlordiazepoxide and an extremely low frequency pulsed magnetic field. *Neuroscience and Biobehavioral Reviews, 25*(3), 235–260.

Cinalli, D. A., Cohen, S. J., Guthrie, K., & Stackman, R. W. (2020). Object Recognition Memory: Distinct Yet Complementary Roles of the Mouse CA1 and Perirhinal Cortex. *Frontiers in Molecular Neuroscience*, *13*, 192.

Clarkson, J., & Herbison, A. E. (2006). Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone (GnRH) neurons. *Endocrinology*, *147*(12), 5817.

Coleman, G., & Canal, M. M. (2017). Postnatal light effects on pup stress axis development are independent of maternal behavior. *Frontiers in Neuroscience*, *11*(FEB), 46.

Coleman, G., Gigg, J., & Canal, M. M. (2016). Postnatal light alters hypothalamic-pituitary-adrenal axis function and induces a depressive-like phenotype in adult mice. *European Journal of Neuroscience*, *44*(10), 2807–2817.

Costall, B., Jones, B. J., Kelly, M. E., Naylor, R. J., & Tomkins, D. M. (1989). Exploration of mice in a black and white test box: Validation as a model of anxiety. *Pharmacology, Biochemistry and Behavior,* 32(3), 777–785.

Crawley, J. N. (1981). Neuropharmacologic specificity of a simple animal model for the behavioral actions of benzodiazepines. Pharmacology, Biochemistry and Behavior, 15(5), 695–699.

Crawley, J. N., & Davis, L. G. (1982). Baseline exploratory activity predicts anxiolytic responsiveness to diazepam in five mouse strains. Brain Research Bulletin, 8(6), 609–612.

Crumbley, C., Wang, Y., Kojetin, D. J., and Burris, T. P. (2010). "Characterization of the Core Mammalian Clock Component, NPAS2, as a REV-ERBalpha/RORalpha Target Gene." *The Journal of Biological Chemistry* 285 (46): 35386–92.

Curtis, A. M., Seo, S., Westgate, E. J., Rudic, R. D., Smyth, E. M., Chakravarti, D., FitzGerald, G. A., and McNamara, P. (2004). "Histone Acetyltransferase-Dependent Chromatin Remodeling and the Vascular Clock." *The Journal of Biological Chemistry* 279 (8): 7091–97.

Da Silva, N. L., Ferreira, V. M. M., De Padua Carobrez, A., & Morato, G. S. (1996). Individual Housing From Rearing Modifies the Performance of Young Rats on the Elevated Plus-Maze Apparatus. *Physiology & Behavior*, *60*(6), 1391–1396.

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., & Schibler, U. (1996). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes & Development* 14:2950–296.

Davis, F. C., Darrow, J. M., & Menaker, M. (1983). Sex differences in the circadian control of hamster wheel-running activity. *The American Journal of Physiology*, *244*(1).

De Moraes Pultrini, A., Almeida Galindo, L., & Costa, M. (2006). Effects of the essential oil from Citrus aurantium L. in experimental anxiety models in mice. *Life Sciences*, *78*(15), 1720–1725.

DeBruyne, J. P., Noton, E., Lambert, C. M., Maywood, E. S., Weaver, D. R., and Reppert, S. M. (2006). "A Clock Shock: Mouse CLOCK Is Not Required for Circadian Oscillator Function." *Neuron* 50 (3): 465–77.

DeBruyne, J. P., Weaver, D. R., and Reppert, S. M. (2007). "CLOCK and NPAS2 Have Overlapping Roles in the Suprachiasmatic Circadian Clock." *Nature Neuroscience* 10 (5): 543–45.

Denninger, J. K., Smith, B. M., & Kirby, E. D. (2018). Novel object recognition and object location behavioral testing in mice on a budget. *Journal of Visualized Experiments*, 2018(141).

Dixon, R. A., Wahlin, Å., Maitland, S. B., Hultsch, D. F., Hertzog, C., & Bäckman, L. (2004). Episodic memory change in late adulthood: Generalizability across samples and performance indices. *Memory & Cognition 2004 32:5*, *32*(5), 768–778.

Doi, M., Hirayama, J., and Sassone-Corsi, P. (2006). "Circadian Regulator CLOCK Is a Histone Acetyltransferase." *Cell* 125 (3): 497–508.

Dubrovsky, Y. V., Samsa, W. E., and Kondratov, R. V. (2010). "Deficiency of Circadian Protein CLOCK Reduces Lifespan and Increases Age-Related Cataract Development in Mice." *Ageing* 2 (12): 936–44.

Dudley, C. A., Erbel-Sieler, C., Estill, S. J., Reick, M., Franken, P., Pitts, S., and McKnight, S. L. (2003). "Altered Patterns of Sleep and Behavioral Adaptability in NPAS2-Deficient Mice." *Science* 301 (5631): 379–83.

Duffy, J. F., Cain, S. W., Chang, A. M., Phillips, A. J. K., Muñch, M. Y., Gronfier, C., Wyatt, J. K., Dijk, D., Wright, K. P., Czeisler, C. A. (2011). Sex difference in the near-24-hour intrinsic period of the human circadian timing system. *Proceedings of the National Academy of Sciences of the United States of America*, *108 Suppl 3*(Suppl 3), 15602–15608.

Dugandzic, A., Ratko, M., & Habek, N. (2020). Anxiety-like behavior in female mice changes by feeding, possible effect of guanylate cyclase C. *European Journal of Neuroscience*, 52(1), 2781–2790.

Duong, H. A., and Weitz, C. J. (2014). "Temporal Orchestration of Repressive Chromatin Modifiers by Circadian Clock Period Complexes." *Nature Structural & Molecular Biology* 21 (2): 126–32.

Dupuis, J., Langenberg, C., Prokopenko, I., Saxena, R., Soranzo, N., Jackson, A. U., Wheeler, E., et al. (2010). "New Genetic Loci Implicated in Fasting Glucose Homeostasis and Their Impact on Type 2 Diabetes Risk." *Nature Genetics* 42 (2): 105–16.

Eckel-Mahan, K. L. (2012). “Circadian Oscillations within the Hippocampus Support Memory Formation and Persistence”. *Frontiers in Molecular Neuroscience* 5: 46.

Eckel-Mahan, K. L., Phan, T., Han, S., Wang, H., Chan, G. C. K., Scheiner, Z. S., and Storm, D. R. (2008). “Circadian Oscillation of Hippocampal MAPK Activity and cAmp: Implications for Memory Persistence.” *Nature Neuroscience* 11 (9): 1074–82.

Edery, I., Rutila, J. E., & Rosbash, M. (1994). “Phase shifting of the circadian clock by induction of the Drosophila period protein.” *Science*, 263(5144), 237–240.

Ehlen, J. C., Brager, A. J., Baggs, J., Pinckney, L., Gray, C. L., DeBruyne, J. P., Esser, K. A., Takahashi., J. S., Paul, K. N. (2017). Bmal1 function in skeletal muscle regulates sleep. *ELife*, *6*.

Endo, Y., Mizuno, T., Fujita, K., Funabashi, T., & Kimura, F. (1994). Soft-diet feeding during development enhances later learning abilities in female rats. *Physiology & Behavior*, *56*(4), 629–633.

Ennaceur, A. (2014). Tests of unconditioned anxiety - Pitfalls and disappointments. *In Physiology and Behavior* (Vol. 135, pp. 55–71). Elsevier Inc.

Etchegaray, J., Lee, C., Wade, P. A., and Reppert, S. M. (2003). “Rhythmic Histone Acetylation Underlies Transcription in the Mammalian Circadian Clock.” *Nature* 421 (6919): 177–82.

Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods, 39,* 175-191.

Fazio, L., Pergola, G., Papalino, M., Carlo, P. Di, Monda, A., Gelao, B., Amoroso, N., Tangaro, S., Rampino, A., Popolizio, T, Bertolino, A., Blasi, G. (2018). Transcriptomic context of DRD1 is associated with prefrontal activity and behavior during working memory. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(21), 5582–5587.

Feillet, C., Guérin, S., Lonchampt, M., Dacquet, C., Gustafsson, J. Å., Delaunay, F., & Teboul, M. (2016). Sexual dimorphism in circadian physiology is altered in LXRα deficient mice. *PLoS ONE*, 11(3).

Fernandez-Baizan, C., Arias, J. L., & Mendez, M. (2019). Spatial memory in young adults: Gender differences in egocentric and allocentric performance. *Behavioural Brain Research*, *359*, 694–700.

Fernández-Gausti, A., Kruijver, F. P., Fodor, M., & Swaab, D. F. (2000). Sex Differences in the Distribution of Androgen Receptors in the Human Hypothalamus. *J. Comp. Neurol*, *425*, 422–435.

Fisher, S. P., Godinho, S. I. H., Pothecary, C. A., Hankins, M. W., Foster, R. G., & Peirson, S. N. (2012). Rapid Assessment of Sleep-Wake Behavior in Mice. *Journal of Biological Rhythms*, *27*(1), 48–58.

Fonken, L. K., & Nelson, R. J. (2014). The effects of light at night on circadian clocks and metabolism. *Endocrine Reviews*, 35(4), 648–670.

Fonken, L. K., Finy, M. S., Walton, J. C., Weil, Z. M., Workman, J. L., Ross, J., & Nelson, R. J. (2009). Influence of light at night on murine anxiety- and depressive-like responses. *Behavioural Brain Research*, 205(2), 349–354.

Fonken, L. K., Workman, J. L., Walton, J. C., Weil, Z. M., Morris, J. S., Haim, A., & Nelson, R. J. (2010). Light at night increases body mass by shifting the time of food intake. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(43), 18664–18669.

Foster, R. G., Provencio, I., Hudson, D., Fiske, S., De Grip, W., & Menaker, M. (1991). Circadian photoreception in the retinally degenerate mouse (rd/rd). *Journal of Comparative Physiology A*, 169(1), 39–50.

Franken, P., Dudley, C. A., Estill, S. J., Barakat, M., Thomason, R., O'Hara, B. F., & McKnight, S. L. (2006). NPAS2 as a transcriptional regulator of non-rapid eye movement sleep: Genotype and sex interactions. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(18), 7118–7123.

Freedman, M. S., Lucas, R. J., Soni, B., Von Schantz, M., Muñoz, M., David-Gray, Z., & Foster, R. (1999). Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. *Science*, 284(5413), 502–504.

Fremeau, R. T., Duncanm, G. E., Fornarettol, M., Dearryii, A., Gingrichii, J. A. Y. A., Breeset, G. R., & Caronii, M. G. (1991). Localization of D1 dopamine receptor mrRNA in brain supports a role in cognitive, affective, and neuroendocrine aspects of dopaminergic neurotransmission. 88(May), 3772–3776.

Frick, K. M., & Gresack, J. E. (2003). Sex Differences in the Behavioral Response to Spatial and Object Novelty in Adult C57BL/6 Mice. *Behavioral Neuroscience*, 117(6), 1283–1291.

Frick, K. M., Burlingame, L. A., Arters, J. A., & Berger-Sweeney, J. (2000). Reference memory, anxiety and estrous cyclicity in C57BL/6NIA mice are affected by age and sex. *Neuroscience*, 95(1), 293–307.

Fu, L., & Kettner, N. M. (2013). *The Circadian Clock in Cancer Development and Therapy*. *Prog Mol Biol Transl Sci*. 2013 ; 119: 221–282.

Fu, L., and Lee, C. C. (2003). "The Circadian Clock: Pacemaker and Tumour Suppressor." *Nature Reviews. Cancer* 3 (5): 350–61.

Fu, S., Zhu, M., Wang, C., & Le, Y. Z. (2014). Efficient induction of productive Cre-mediated recombination in retinal pigment epithelium. *Molecular Vision*, 20, 480–487.

Fujioka, A., Fujioka, T., Tsuruta, R., Izumi, T., Kasaoka, S., & Maekawa, T. (2011). Effects of a constant light environment on hippocampal neurogenesis and memory in mice. *Neuroscience Letters*, 488(1), 41–44.

Gais, S., Mö, M., Helms, K., & Born, J. (2002). *Learning-Dependent Increases in Sleep Spindle Density*. *Journal of Neuroscience*, 22 (15) 6830-6834.

Gao, J., Wang, W., Mao, Y., Gräff, J., Guan, J., Pan, L., Mak, G., Kim, D., Su, S. C., and Tsai, L. (2010). "A Novel Pathway Regulates Memory and Plasticity via SIRT1 and miR-134." *Nature* 466 (7310): 1105–9.

Garcia, J. A., Zhang, D., Estill, S. J., Michnoff, C., Rutter, J., Reick, M., Scott, K., Diaz-Arrastia, R., McKnight, S. L. (2000). "Impaired cued and contextual memory in NPAS2-deficient mice." *Science*, *288*(5474), 2226–2230.

Gassmann, M., Grenacher, B., Rohde, B., & Vogel, J. (2009). Quantifying Western blots: Pitfalls of densitometry. *Electrophoresis*, *30*(11), 1845–1855.

Geoffroy, P. A., Lajnef, M., Bellivier, F., Jamain, S., Gard, S., Kahn, J.-P., Henry, C., Leboyer, M., Etain, B., Pepsy, D. (2015). Genetic association study of circadian genes with seasonal pattern in bipolar disorders OPEN. *Nature Publishing Group*. 5:10232.

Gerstner, J. R., and Yin, J. C. P. (2010). "Circadian Rhythms and Memory Formation." Nature Reviews. *Neuroscience* 11 (8): 577–88.

Gerstner, J. R., Lyons, L. C., Wright, K. P. Jr, Loh, D. H., Rawashdeh, O., Eckel-Mahan, K. L., and Roman, G. W. (2009). "Cycling Behavior and Memory Formation." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 29 (41): 12824–30.

Glaser, F. T., and Stanewsky, R. (2005). "Temperature Synchronization of the Drosophila Circadian Clock." *Current Biology:* CB 15 (15): 1352–63.

González, M. M. C. (2018). Dim light at night and constant darkness: Two frequently used lighting conditions that jeopardize the health and well-being of laboratory rodents. *Frontiers in Neurology*, 9(AUG), 1–17.

González, M. M. C., & Aston-Jones, G. (2006). Circadian Regulation of Arousal: Role of the Noradrenergic Locus Coeruleus System and Light Exposure. *Sleep*, *29*(10), 1327–1336.

González, M. M. C., & Aston-Jones, G. (2008). Light deprivation damages monoamine neurons and produces a depressive behavioral phenotype in rats. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(12), 4898–4903.

Gresack, J. E., Kerr, K. M., & Frick, K. M. (2007). Life-long environmental enrichment differentially affects the mnemonic response to estrogen in young, middle-aged, and aged female mice. *Neurobiology of Learning and Memory*, 88(4), 393–408.

Griebel, G., Blanchard, D. C., Rettori, M. C., Guardiola-Lemaître, B., & Blanchard, R. J. (1996). Preclinical profile of the mixed 5-HT1A/5-HT2A receptor antagonist S 21357. *Pharmacology Biochemistry and Behavior*, *54*(2), 509–516.

Gritton, H. J., Stasiak, A. M., Sarter, M., & Lee, T. M. (2013). Cognitive Performance as a Zeitgeber: Cognitive Oscillators and Cholinergic Modulation of the SCN Entrain Circadian Rhythms. *PLoS ONE*, 8(2).

Guillaumond, F., Dardente, H., Giguère, V., and Cermakian, N. (2005). “Differential Control of Bmal1 Circadian Transcription by REV-ERB and ROR Nuclear Receptors.” *Journal of Biological Rhythms* 20 (5): 391–403.

Güldner, F. H. (1982). Sexual dimorphisms of axo-spine synapses and postsynaptic density material in the suprachiasmatic nucleus of the rat. *Neuroscience Letters*, *28*(2), 145–150.

Güldner, F. H. (1983). Numbers of neurons and astroglial cells in the suprachiasmatic nucleus of male and female rats. *Experimental Brain Research*, *50*(2–3), 373–376.

Güldner, F. H. (1984). Suprachiasmatic nucleus: numbers of synaptic appositions and various types of synapses. A morphometric study on male and female rats. *Cell and Tissue Research*, *235*(2), 449–452.

Guo, M., Wu, C. F., Liu, W., Yang, J. Y., & Chen, D. (2004). Sex difference in psychological behavior changes induced by long-term social isolation in mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *28*(1), 115–121.

Gurkar, A. U., & Niedernhofer, L. J. (2015). “Comparison of mice with accelerated ageing caused by distinct mechanisms.” *Experimental Gerontology*, 68, 43–50.

Haj-Mirzaian, A., Nikbakhsh, R., Ramezanzadeh, K., Rezaee, M., Amini-Khoei, H., Haj-Mirzaian, A., Ghesmati, M., Afshari, K., Haddadi, N. S., & Dehpour, A. R. (2019). Involvement of opioid system in behavioral despair induced by social isolation stress in mice. *Biomedicine & Pharmacotherapy*, 109, 938–944.

Hall, J. C. (1994). “The mating of a fly.” *Science*, 264(5166), 1702–1714.

Hampp, G., Ripperger, J. A., Houben, T., Schmutz, I., Blex, C., Perreau-Lenz, S., Brunk, I., Spanagel, R., Ahnert-Hilger, G., Meijer, J. H., Albrecht, U. (2008). “Regulation of Monoamine Oxidase A by Circadian-Clock Components Implies Clock Influence on Mood.” *Current Biology: CB* 18 (9): 678–83.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. *Journal of Molecular Biology*, *166*(4), 557–580.

Hansen, K. F., Sakamoto, K., Aten, S., and Snider, K. H. (2016). “Targeted Deletion of miR-132/-212 Impairs Memory and Alters the Hippocampal Transcriptome.” *Learning & Memory*. 23 (2): 61–71.

Handa, R. J., Burgess, L. H., Kerr, J. E., & O’keefe, J. A. (1994). Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. *Hormones and Behavior*, *28*(4), 464–476.

Hansen, K. F., Sakamoto, K., Wayman, G. A., Impey, S., & Obrietan, K. (2010). Transgenic miR132 alters neuronal spine density and impairs novel object recognition memory. *PLoS ONE*, 5(11), 15497.

Hases, L., Archer, A., Indukuri, R., Birgersson, M., Savva, C., Korach-André, M., & Williams, C. (2020). High-fat diet and estrogen impacts the colon and its transcriptome in a sex-dependent manner. *Scientific Reports*, *10*(1).

He, P. J., Hirata, M., Yamauchi, N., Hashimoto, S., & Hattori, M. A. (2007). Gonadotropic regulation of circadian clockwork in rat granulosa cells. *Molecular and Cellular Biochemistry*, *302*(1–2), 111–118.

Heffner, C. S., Herbert Pratt, C., Babiuk, R. P., Sharma, Y., Rockwood, S. F., Donahue, L. R., Eppig, J. T., & Murray, S. A. (2012). Supporting conditional mouse mutagenesis with a comprehensive cre characterization resource. *Nature Communications*, 3(1), 1–9.

Heredia, L., Torrente, M., Colomina, M. T., & Domingo, J. L. (2014). Assessing anxiety in C57BL/6J mice: A pharmacological characterization of the open-field and light/dark tests. *Journal of Pharmacological and Toxicological Methods*, 69(2), 108–114.

Herlitz, A., & Yonker, J. E. (2002). Sex differences in episodic memory: the influence of intelligence. *Journal of Clinical and Experimental Neuropsychology*, *24*(1), 107–114.

Heyward, F. D., Walton, R. G., Carle, M. S., Coleman, M. A., Garvey, W. T., & Sweatt, J. D. (2012). Adult mice maintained on a high-fat diet exhibit object location memory deficits and reduced hippocampal SIRT1 gene expression. *Neurobiology of Learning and Memory*, 98(1), 25–32.

Hirayama, J., Sahar, S., Grimaldi, B., Tamaru, T., Takamatsu, K., Nakahata, Y., and Sassone-Corsi, P. (2007). "CLOCK-Mediated Acetylation of BMAL1 Controls Circadian Function." *Nature* 450 (7172): 1086–90.

Hirota, T., Okano, T., Kokame, K., Shirotani-Ikejima, H., Miyata, T., & Fukada, Y. (2002). Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured rat-1 fibroblasts. *Journal of Biological Chemistry*, *277*(46), 44244–44251.

Hodge, B. A., Wen, Y., Riley, L. A., Zhang, X., England, J. H., Harfmann, B. D., Schroder, E. A., Esser, K. A. (2015). The endogenous molecular clock orchestrates the temporal separation of substrate metabolism in skeletal muscle. *Skeletal Muscle 2015 5:1*, *5*(1), 1–16.

Hofman, M. A., Fliers, E., Goudsmit, E., & Swaab, D. F. (1988). Morphometric analysis of the suprachiasmatic and paraventricular nuclei in the human brain: sex differences and age-dependent changes. *Journal of Anatomy*, *160*, 127.

Hofstetter, J. R., Svihla-Jones, D. A., & Mayeda, A. R. (2007). A QTL on mouse chromosome 12 for the genetic variance in free-running circadian period between inbred strains of mice. *Journal of Circadian Rhythms*, 5.

Huang, H. J., Liang, K. C., Ke, H. C., Chang, Y. Y., & Hsieh-Li, H. M. (2011). Long-term social isolation exacerbates the impairment of spatial working memory in APP/PS1 transgenic mice. *Brain Research*, *1371*, 150–160.

Huang, X., Huang, P., Huang, L., Hu, Z., Liu, X., Shen, J., Xi, Y., Yang, Y., Fu, Y., Tao, Q., Lin, S., Xu, A., Xu, F., Xue, T., So, K. F., Li, H., & Ren, C. (2021). A Visual Circuit Related to the Nucleus Reuniens for the Spatial-Memory-Promoting Effects of Light Treatment. *Neuron*, *109*(2), 347-362.e7.

Huang, Y.-L., Liu, R.-Y., Wang, Q.-S., Van Someren, E. J. W., Xu, H., & Zhou, J.-N. (2002). "Age-associated difference in circadian sleep-wake and rest-activity rhythms." *Physiology & Behavior*, *76*(4–5), 597–603.

Husse, J., Leliavski, A., Tsang, A. H., Oster, H., & Eichele, G. (2014). The light-dark cycle controls peripheral rhythmicity in mice with a genetically ablated suprachiasmatic nucleus clock. *The FASEB Journal*, *28*(11), 4950–4960.

Ieraci, A., Mallei, A., & Popoli, M. (2016). Social Isolation Stress Induces Anxious-Depressive-Like Behavior and Alterations of Neuroplasticity-Related Genes in Adult Male Mice. *Neural Plasticity,* 2016.

Iwahana, E., Karatsoreos, I., Shibata, S., & Silver, R. (2008). Gonadectomy reveals sex differences in circadian rhythms and suprachiasmatic nucleus androgen receptors in mice. *Hormones and Behavior*, *53*(3), 422–430.

Jameie, S. B., Mousavi, M., Farhadi, M., Mehraein, F., Ababzadeh, S., Soleimani, M., Kerdari, M., & Jalilpouraghdam, M. (2015). Effects of Total Light Deprivation on Hippocampal Neurogenesis and Memory of Adult Rats: A Sexual Dimorphic Study. *Thrita,* 5(3).

Jilg, A., Lesny, S., Peruzki, N., Schwegler, H., Selbach, O., Dehghani, F., and Stehle, J. H. (2010). "Temporal Dynamics of Mouse Hippocampal Clock Gene Expression Support Memory Processing." *Hippocampus* 20 (3): 377–88.

Jin, L., Long, L., Green, M. A., & Spear, B. T. (2009). The alpha-fetoprotein enhancer region activates the albumin and alpha-fetoprotein promoters during liver development. *Developmental Biology*, 336(2), 294–300.

Jonasson, Z. (2005). Meta-analysis of sex differences in rodent models of learning and memory: a review of behavioral and biological data. *Neuroscience and Biobehavioral Reviews*, *28*(8), 811–825.

Jones, C. M., & Healy, S. D. (2006). Differences in cue use and spatial memory in men and women. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1598), 2241.

Joseph, R., Orlov, Y. L., Huss, M., Sun, W., Li Kong, S., Ukil, L., Pan, Y. F., Li, G., Lim, M., Thomsen, J. S., Ruan, Y., Clarke, N. D., Prabhakar, S., Cheung, E., & Liu, E. T. (2010). Integrative model of genomic factors for determining binding site selection by estrogen receptor-α. *Molecular Systems Biology*, *6*(1), 456.

Jud, C., Schmutz, I., Hampp, G., Oster, H., & Albrecht, U. (2005). A guideline for analyzing circadian wheel-running behavior in rodents under different lighting conditions. *Biological Procedures Online*, 7(1), 101–116.

Kalueff, A. V., Keisala, T., Minasyan, A., Kuuslahti, M., & Tuohimaa, P. (2006). Temporal stability of novelty exploration in mice exposed to different open field tests. *Behavioural Processes*, *72*(1), 104–112.

Karatsoreos, I. N., Butler, M. P., Lesauter, J., & Silver, R. (2011). Androgens Modulate Structure and Function of the Suprachiasmatic Nucleus Brain Clock. *Endocrinology*, *152*(5).

Karman, B. N., & Tischkau, S. A. (2006). Circadian clock gene expression in the ovary: Effects of luteinizing hormone. *Biology of Reproduction*, *75*(4), 624–632.

Kasper, S., Wehr, T. A., Bartko, J. J., Gaist, P. A., & Rosenthal, N. E. (1989). Epidemiological findings of seasonal changes in mood and behavior. A telephone survey of Montgomery County, Maryland. *Archives of General Psychiatry*, *46*(9), 823–833.

Kern, A., Mavrikaki, M., Ullrich, C., Albarran-Zeckler, R., Brantley, A. F., & Smith, R. G. (2015). Hippocampal Dopamine/DRD1 Signaling Dependent on the Ghrelin Receptor. *Cell*, *163*(5), 1176–1190.

Kim, M., Custodio, R. J., Lee, H. J., Sayson, L. V., Ortiz, D. M., Kim, B. N., Kim, H. J., Cheong, J. H. (2022). Per2 Expression Regulates the Spatial Working Memory of Mice through DRD1-PKA-CREB Signaling. *Molecular Neurobiology*, *59*(7), 4292–4303.

Kitagawa, K. (2007). "CREB and cAMP Response Element-Mediated Gene Expression in the Ischemic Brain: CREB-Mediated Gene Expression in Cerebral Ischemia." *The FEBS Journal* 274 (13): 3210–17.

Koh, K., Zheng, X., & Sehgal, A. (2006). JETLAG Resets the Drosophila Circadian Clock by Promoting Light-Induced Degradation of TIMELESS. *Science (New York, N.Y.)*, *312*(5781), 1809.

Koike, H., Ibi, D., Mizoguchi, H., Nagai, T., Nitta, A., Takuma, K., Nabeshima, T., Yoneda, Y., & Yamada, K. (2009). Behavioral abnormality and pharmacologic response in social isolation-reared mice. *Behavioural Brain Research*, *202*(1), 114–121.

Kondratov, R. V., Kondratova, A. A., Gorbacheva, V. Y., Vykhovanets, O. V., and Antoch, M. P. (2006B). "Early ageing and Age-Related Pathologies in Mice Deficient in BMAL1, the Core Component of the Circadian Clock." *Genes & Development* 20 (14): 1868–73.

Kondratov, R. V., Kondratova, A. A., Lee, C., Gorbacheva, V. Y., Chernov, M. K., and Antoch, M. P. (2006A). "Posttranslational Regulation of Circadian Transcriptional Clock (NPAS2)/BMAL1 Complex by Cryptochromes." *Cell Cycle* 5 (8): 890–95.

Kondratov, R. V., Vykhovanets, O., Kondratova, A. A., & Antoch, M. P. (2009). "Antioxidant N-acetyl-L-cysteine ameliorates symptoms of premature ageing associated with the deficiency of the circadian protein BMAL1." *Ageing,* 1(12), 979–987.

Kondratova, A. A., & Kondratov, R. V. (2012). "The circadian clock and pathology of the ageing brain. Nature Reviews." *Neuroscience,* 13(5), 325–335.

Kondratova, A. A., Dubrovsky, Y. V., Antoch, M. P., and Kondratov, R. V. (2010). "Circadian Clock Proteins Control Adaptation to Novel Environment and Memory Formation." *Ageing* 2 (5): 285–97.

Konhilas, J. P., Chen, H., Luczak, E., McKee, L. A., Regan, J., Watson, P. A., Stauffer, B. L., Khalpey, Z. I., McKinsey, T. A., Horn, T., LaFleur, B., & Leinwand, L. A. (2015). Diet and sex modify exercise and cardiac adaptation in the mouse. *American Journal of Physiology - Heart and Circulatory Physiology*, 308(1), H135–H145.

Koronowski, K. B., Kinouchi, K., Welz, P. S., Smith, J. G., Zinna, V. M., Shi, J., Samad, M., Chen, S., Magnan, C. N., Kinchen, J. M., Li, W., Baldi, P., Benitah, S. A., Sassone-Corsi, P. (2019). Defining the Independence of the Liver Circadian Clock. *Cell*, *177*(6), 1448-1462.e14.

Korzus, E., Rosenfeld, M. G., & Mayford, M. (2004). CBP histone acetyltransferase activity is a critical component of memory consolidation. *Neuron*, 42(6), 961–972.

Krishnan, H. C., and Lyons, L. C. (2015). "Synchrony and Desynchrony in Circadian Clocks: Impacts on Learning and Memory." *Learning & Memory* 22 (9): 426–37.

Krizo, J. A., Mintz, E. M., Vaudry, H., & Antle, M. (2015). Sex differences in behavioral circadian rhythms in laboratory rodents. *Frontiers in Endocrinology*, *5*(DEC).

Kruijver, F. P. M., & Swaab, D. F. (2002). Sex hormone receptors are present in the human suprachiasmatic nucleus. *Neuroendocrinology*, *75*(5), 296–305.

Kulesskaya, N., & Voikar, V. (2014). Assessment of mouse anxiety-like behavior in the light-dark box and open-field arena: Role of equipment and procedure. *Physiology and Behavior*, 133, 30–38.

Kuljis, D. A., Loh, D. H., Truong, D., Vosko, A. M., Ong, M. L., McClusky, R., Arnold, A. P., Colwell, C. S. (2013). Gonadal- and Sex-Chromosome-Dependent Sex Differences in the Circadian System. *Endocrinology*, *154*(4), 1501–1512.

Kwak, C., Lee, S. H., & Kaang, B. K. (2009). Social isolation selectively increases anxiety in mice without affecting depression-like behavior. *Korean Journal of Physiology and Pharmacology*, 13(5), 357–360.

Kwapis, J. L., Alaghband, Y., Kramár, E. A., López, A. J., Vogel Ciernia, A., White, A. O., Shu, G., Rhee, D., Michael, C. M., Montellier, E., Liu, Y., Magnan, C. N., Sassone-Corsi, P., Baldi, P., Matheos, D. P., Wood, M. A. (2018). "Epigenetic regulation of the circadian gene Per1 contributes to age-related changes in hippocampal memory." *Nature Communications* 9(1):3323.

Labban, S., Alshehri, F. S., Kurdi, M., Alatawi, Y., & Alghamdi, B. S. (2021). Melatonin Improves Short-Term Spatial Memory in a Mouse Model of Alzheimer's Disease. *Degenerative Neurological and Neuromuscular Disease*, *11*, 15.

LaBuda, C. J., Mellgren, R. L., & Hale, R. L. (2002). Sex differences in the acquisition of a radial maze task in the CD-1 mouse. *Physiology and Behavior*, *76*(2), 213–217.

Labyak, S. E., & Lee, T. M. (1995). Estrus- and steroid-induced changes in circadian rhythms in a diurnal rodent, Octodon degus. *Physiology & Behavior*, *58*(3), 573–585.

Lagerspetz, K. M. J., & Lagerspetz, K. Y. H. (1971). Changes in the aggressiveness of mice resulting from selective breeding, learning and social isolation. *Scandinavian Journal of Psychology*, *12*(1), 241–248.

Lamia, K. A., Papp, S. J., Yu, R. T., Barish, G. D., Uhlenhaut, N. H., Jonker, J. W., Downes, M., Evans, R. M. (2011). Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature*, *480*(7378), 552–556.

Lamia, K. A., Sachdeva, U. M., Di Tacchio, L., Williams, E. C., Alvarez, J. G., Egan, D. F., Vasquez, D. S., Juguilon, H., Panda, S., Shaw, R. J., Thompson, C. B., & Evans, R. M. (2009). AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. *Science*, *326*(5951), 437–440.

Lamont, E. W., Robinson, B., Stewart, J., and Amir, S. (2005). "The Central and Basolateral Nuclei of the Amygdala Exhibit Opposite Diurnal Rhythms of Expression of the Clock Protein Period2." *Proceedings of the National Academy of Sciences of the United States of America* 102 (11): 4180–84.

Lander, S. S., Linder-Shacham, D., & Gaisler-Salomon, I. (2017). Differential effects of social isolation in adolescent and adult mice on behavior and cortical gene expression. *Behavioural Brain Research*, 316, 245–254.

Landgraf, D., Wang, L. L., Diemer, T., and Welsh, D. K. (2016). "NPAS2 Compensates for Loss of CLOCK in Peripheral Circadian Oscillators." *PLoS Genetics* 12 (2): e1005882.

Langner, R., & Rensing, L. (1972). Circadian Rhythm of Oxygen Consumption in Rat Liver Suspension Culture: Changes of Pattern. *Zeitschrift Fur Naturforschung - Section B Journal of Chemical Sciences*, *27*(9), 1117–1118.

Laws, Maxwell T., Robin E. Bonomi, Swatabdi Kamal, David J. Gelovani, Jeremy Llaniguez, Shreya Potukutchi, Xin Lu, Thomas Mangner, and Juri G. Gelovani. 2019. "Molecular Imaging HDACs Class IIa Expression-Activity and Pharmacologic Inhibition in Intracerebral Glioma Models in Rats Using PET/CT/(MRI) with [18F]TFAHA." *Scientific Reports* 9 (1): 3595.

Lee, S., Donehower, L. A., Herron, A. J., Moore, D. D., & Fu, L. (2010). Disrupting Circadian Homeostasis of Sympathetic Signaling Promotes Tumor Development in Mice. *PLoS ONE* 5(6): e10995

Lee, T. M., & Labyak, S. E. (1997). Free-running rhythms and light- and dark-pulse phase response curves for diurnal Octodon degus (Rodentia). *The American Journal of Physiology*, *273*(1 Pt 2).

Leger, M., Quiedeville, A., Bouet, V., Haelewyn, B., Boulouard, M., Schumann-Bard, P., & Freret, T. (2013). Object recognition test in mice. *Nature Protocols*, 8(12), 2531–2537.

Leser, N., & Wagner, S. (2015). The effects of acute social isolation on long-term social recognition memory. *Neurobiology of Learning and Memory*, *124*, 97–103.

Li, K., Nakajima, M., Ibañez-Tallon, I., & Heintz, N. (2016). A Cortical Circuit for Sexually Dimorphic Oxytocin-Dependent Anxiety Behaviors. *Cell*, 167(1), 60-72.e11.

Libert, S., Bonkowski, M. S., Pointer, K., Pletcher, S. D., and Guarente, L. (2012). "Deviation of Innate Circadian Period from 24 H Reduces Longevity in Mice." *Ageing Cell* 11 (5): 794–800.

Lidster, K., Owen, K., Browne, W. J., & Prescott, M. J. (2019). Cage aggression in group-housed laboratory male mice: an international data crowdsourcing project. *Scientific Reports*, 9(1), 1–12.

Lim, A. S. P., Myers, A. J., Yu, L., Buchman, A. S., Duffy, J. F., De Jager, P. L., & Bennett, D. A. (2013). Sex Difference in Daily Rhythms of Clock Gene Expression in the Aged Human Cerebral Cortex. *Journal of Biological Rhythms*, *28*(2), 117.

Lin, E., Kuo, P.-H., Liu, Y.-L., Yang, A. C., Kao, C.-F., & Tsai, S.-J. (2017). Effects of circadian clock genes and environmental factors on cognitive ageing in old adults in a Taiwanese population. *Oncotarget* 11;8(15):24088-24098.

Locci, A., Geoffroy, P., Miesch, M., Mensah-Nyagan, A. G., & Pinna, G. (2017). Social isolation in early versus late adolescent mice is associated with persistent behavioral deficits that can be improved by neurosteroid-based treatment. *Frontiers in Cellular Neuroscience*, *11*, 208.

Logan, R. W., Xue, X., Ketchesin, K. D., Hoffman, G., Roussos, P., Tseng, G., McClung, C. A., & Seney, M. L. (2022). Sex Differences in Molecular Rhythms in the Human Cortex. *Biological Psychiatry*, 91(1), 152–162.

Lopez, M. F., & Laber, K. (2015). Impact of social isolation and enriched environment during adolescence on voluntary ethanol intake and anxiety in C57BL/6J mice. *Physiology and Behavior*, 148, 151–156.

Loprinzi, P. D., & Frith, E. (2018). The role of sex in memory function: Considerations and recommendations in the context of exercise. *Journal of Clinical Medicine*, *7*(6).

Lu, C., Li, Q. Q., Li, Y. Y., Lin, H. Z., Qu, J., Wang, Y., & Zhang, Y. F. (2017). Light deprivation produces distinct morphological orchestrations on RGCs and cortical cells in a depressive-like YFP-H mouse model. *Neuroscience Letters*, *659*, 60–68.

Ly, J. Q. M., Gaggioni, G., Chellappa, S. L., Papachilleos, S., Brzozowski, A., Borsu, C., Rosanova, M., Sarasso, S., Middleton, B., Luxen, A., Archer, S. N., Phillips, C., Dijk, D., Maquet, P., Massimini, M., Vandewalle, G. (2016). *ARTICLE Circadian regulation of human cortical excitability*.

Ma, K., Xiao, R., Tseng, H. T., Shan, L., Fu, L., & Moore, D. D. (2009). Circadian Dysregulation Disrupts Bile Acid Homeostasis. *PLOS ONE*, *4*(8), e6843.

Ma, W. P., Cao, J., Tian, M., Cui, M. H., Han, H. L., Yang, Y. X., & Xu, L. (2007). Exposure to chronic constant light impairs spatial memory and influences long-term depression in rats. *Neuroscience Research,* 59(2), 224–230.

Maitland, S. B., Herlitz, A., Nyberg, L., Bäckman, L., & Nilsson, L. G. (2004). Selective sex differences in declarative memory. *Memory & Cognition 2004 32:7*, *32*(7), 1160–1169.

Malmberg-Aiello, P., Ipponi, A., Bartolini, A., & Schunack, W. (2002). Mouse light/dark box test reveals anxiogenic-like effects by activation of histamine H1 receptors. *Pharmacology Biochemistry and Behavior*, 71(1–2), 313–318.

Manber, R., Baker, F. C., Gress, J. L. (2006) Sex differences in sleep and sleep disorders: a focus on women's sleep. *Int J Sleep Disorders.* 1:7–15.

Manfredini, R., Manfredini, F., Fersini, C., Conconi, F., Manfredini, R., Fersini, C., & Di, S. (1998). Reviews Circadian rhythms, athletic performance, and jet lag. *Br J Sports Med*, *32*, 101–106.

Mansour, H. A., Talkowski, M. E., Wood, J., Chowdari, K. V., McClain, L., Prasad, K., Montrose, D., Fagiolini, A., Friedman, E. S., Allen, M. H., Bowden, C. L., Calabrese, J., El-Mallakh, R. S., Escamilla, M., Faraone, S. V., Fossey, M. D., Gyulai, L., Loftis, J. M., Hauser, P., Ketter, T. A., Marangell, L. B., Miklowitz, D. J., Nierenberg, A. A., Patel, J., Sachs, G. S., Sklar, P., Smoller, J. W., Laird, N., Keshavan, M., Thase, M. E., Axelson, D., Birmaher, B., Lewis, D., Monk, T., Frank, E., Kupfer, D. J., Devlin, B., Nimgaonkar, V. L. (2009). Association study of 21 circadian genes with bipolar I disorder, schizoaffective disorder, and schizophrenia. *Bipolar Disorders*, *11*(7), 701.

Marchette, R. C. N., Bicca, M. A., Santos, E. C. da S., & de Lima, T. C. M. (2018). Distinctive stress sensitivity and anxiety-like behavior in female mice: Strain differences matter. *Neurobiology of Stress*, 9, 55–63.

Marcheva, B., Ramsey, K. M., Buhr, E. D., Kobayashi, Y., Su, H., Ko, C. H., Ivanova, G., Omura, C., Mo S., Vitaterna, M. H, Lopez, J. P., Philipson, L. H., Bradfield, C. A., Crosby, S. D., JeBailey, L., Wang, X., Takahashi, J. S., Bass, J. (2010). Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature* (Vol. 466), 627-631.

Marin, M. T., Araújo, T. T., Rezende, M. M. F., & Ribeiro-Barbosa, É. R. (2015). Behavioral alterations induced by absence of circadian light rhythm: Effects of constant light or constant dark on depression-like behaviors and locomotor activity in rats. *Bioscience Journal*, *31*(6), 1837–1843.

Matsumura, R., Okamoto, A., Node, K. & Akashi, M. 2014. Compensation for intracellular environment in expression levels of mammalian circadian clock genes. *Scientific reports,* 4**,** 4032-4032.

Maywood, E. S., Chesham, J. E., O'Brien, J. A., & Hastings, M. H. (2011). A diversity of paracrine signals sustains molecular circadian cycling in suprachiasmatic nucleus circuits. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(34), 14306–14311.

Mazzoccoli, G., Vinciguerra, M., Oben, J., Tarquini, R., & De Cosmo, S. (2014). Non-alcoholic fatty liver disease: the role of nuclear receptors and circadian rhythmicity. *Liver International : Official Journal of the International Association for the Study of the Liver*, *34*(8), 1133–1152.

McCool, B. A., & Chappell, A. M. (2009). Early social isolation in male long-evans rats alters both appetitive and consummatory behaviors expressed during operant ethanol self-administration. Alcoholism: *Clinical and Experimental Research,* 33(2), 273–282.

Meng, F., Onori, P., Han, Y., DeMorrow, S., Venter, J., Francis, H., Franchitto, A., Ray, D., Kennedy, L., Greene, J., Renzi, A., Mancinelli, R., Gaudio, E., Glaser, S., Alpini, G. (2014). Prolonged exposure of cholestatic rats to complete dark inhibits biliary hyperplasia and liver fibrosis. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *307*(9), G894.

Michán, S., Li, Y., Chou, M. M. H., Parrella, E., Ge, H., Long, J. M., Allard, J. S., Lewis, K., Miller, M., Xu, W., Mervis, R. F., Chen, J., Guerin, K. I., Smith, L. E. H., McBurney, M. W., Sinclair, D. A., Baudry, M., De Cabo, R., & Longo, V. D. (2010). SIRT1 is essential for normal cognitive function and synaptic plasticity. *Journal of Neuroscience,* 30(29), 9695–9707.

Miller, S. M., Piasecki, C. C., & Lonstein, J. S. (2011). Use of the light-dark box to compare the anxiety-related behavior of virgin and postpartum female rats. *Pharmacology Biochemistry and Behavior*, 100(1), 130–137.

Milosavljevic, N., Brown, T. M., & Lucas, R. J. (2021). A Bright Idea for Improving Spatial Memory. *Neuron*, *109*(2), 197–199.

Mishima, N., Higashitani, F., Teraoka, K., & Yoshioka, R. (1986). Sex differences in appetitive learning of mice. *Physiology & Behavior*, *37*(2), 263–268.

Miyake, T., & Doi, M. (2019). Reconstitution of Organismal Liver Clock Function Requires Light. *Trends in Endocrinology & Metabolism*, *30*(9), 569–571.

Molina-Hernández, M., Tellez-Alcantara, P., & Perez-Garcia, J. (2001). Isolation rearing induced fear-like behavior without affecting learning abilities of Wistar rats. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *25*(5), 1111–1123.

Mong, J. A., Baker, F. C., Mahoney, M. M., Paul, K. N., Schwartz, M. D., Semba, K., & Silver, R. (2011). Mini-Symposium Sleep, Rhythms, and the Endocrine Brain: Influence of Sex and Gonadal Hormones.

Mongrain, V., La Spada, F., Curie, T., & Franken, P. (2011). Sleep Loss Reduces the DNA-Binding of BMAL1, CLOCK, and NPAS2 to Specific Clock Genes in the Mouse Cerebral Cortex. *PLOS ONE*, *6*(10), e26622.

Morin, L. P., Fitzgerald, K. M., & Zucker, I. (1977). Estradiol shortens the period of hamster circadian rhythms. *Science (New York, N.Y.)*, *196*(4287), 305–307.

Mulvey, B., Bhatti, D. L., Gyawali, S., Lake, A. M., Kriaucionis, S., Ford, C. P., Bruchas, M. R., Heintz, N., & Dougherty, J. D. (2018). Molecular and Functional Sex Differences of Noradrenergic Neurons in the Mouse Locus Coeruleus. *Cell Reports*, 23(8), 2225–2235.

Munn, E., Bunning, M., Prada, S., Bohlen, M., Crabbe, J. C., & Wahlsten, D. (2011). Reversed light – dark cycle and cage enrichment effects on ethanol-induced deficits in motor coordination assessed in inbred mouse strains with a compact battery of refined tests. *Behavioural Brain Research*, *224*(2), 259–271.

Murayama, Y., Yahagi, N., Takeuchi, Y., Aita, Y., Mehrazad Saber, Z., Wada, N., Li, E. N., Piao X., Sawada, Y., Shikama, A., Masuda, Y., Nishi-Tatsumi, M., Kubota, M., Izumida, Y., Miyamoto, T., Sekiya, M., Matsuzaka, T., Nakagawa, Y., Sugano, Y., Iwasaki, H., Kobayashi, K., Yatoh, S., Suzuki, H., Yagyu, H., Kawakami, Y., Shimano, H. (2019). Glucocorticoid receptor suppresses gene expression of Rev-erbα (Nr1d1) through interaction with the CLOCK complex. *FEBS Letters*, *593*(4), 423–432.

Musiek, E. S., Lim, M. M., Yang, G., Bauer, A. Q., Qi, L., Lee, Y., Roh, J. H., Ortiz-Gonzalez, X., Dearborn, J. T., Culver, J. P., Herzog, E. D., Hogenesch, J. B., Wozniak, D. F., Dikranian, K., Giasson, B. I., Weaver, D. R., Holtzman, D. M., FitzGerald, G. A. (2013). "Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration." *Journal of Clinical Investigation.* 123(12): 5389–5400.

Myers, M. P., Wager-Smith, K., Rothenfluh-Hilfiker, A., & Young, M. W. (1996). "Light-induced degradation of TIMELESS and entrainment of the drosophila circadian clock." *Science*, *271*(5256), 1736–1740.

Nagy, A. (2000). Cre recombinase: The universal reagent for genome tailoring. In *Genesis* (Vol. 26, Issue 2, pp. 99–109).

Nakahata, Y., Kaluzova, M., Grimaldi, B., Sahar, S., Hirayama, J., Chen, D., Guarente, L. P., and Sassone-Corsi, P. (2008). "The NAD+-Dependent Deacetylase SIRT1 Modulates CLOCK-Mediated Chromatin Remodeling and Circadian Control." *Cell* 134 (2): 329–40.

Namgyal, D., Chandan, K., Ali, S., Ahmad, A., Hashim, M. J., & Sarwat, M. (2021). Aberrant Lighting Causes Anxiety-like Behavior in Mice but Curcumin Ameliorates the Symptoms. *Animals : An Open Access Journal from MDPI*, *11*(9), 2590.

Nicolaides, N. C., & Chrousos, G. P. (2020). Sex differences in circadian endocrine rhythms: Clinical implications. *European Journal of Neuroscience*, *52*(1), 2575–2585.

Nohara, K., Nemkov, T., D'Alessandro, A., Yoo, S. H., & Chen, Z. (2019). Coordinate Regulation of Cholesterol and Bile Acid Metabolism by the Clock Modifier Nobiletin in Metabolically Challenged Old Mice. *International Journal of Molecular Sciences*, *20*(17).

Juárez Olguín, H., Calderón Guzmán, D., Hernández García, E., & Barragán Mejía, G. (2016). The role of dopamine and its dysfunction as a consequence of oxidative stress. *Oxidative Medicine and Cellular Longevity*, *2016*. https://doi.org/10.1155/2016/9730467

O'Neil, D. S., Stewart, C. J., Chu, D. M., Goodspeed, D. M., Gonzalez-Rodriguez, P. J., Shope, C. D., & Aagaard, K. M. (2017). Conditional postnatal deletion of the neonatal murine hepatic circadian gene, Npas2, alters the gut microbiome following restricted feeding. *American Journal of Obstetrics and Gynecology*, 217(2), 218.e1-218.e15.

O'Neil, D., Mendez-Figueroa, H., Mistretta, T. A., Su, C., Lane, R. H., & Aagaard, K. M. (2013). Dysregulation of Npas2 leads to altered metabolic pathways in a murine knockout model. *Molecular Genetics and Metabolism*, 110(3), 378–387.

Ohta, H., Yamazaki, S., & McMahon, D. G. (2005). Constant light desynchronizes mammalian clock neurons. *Nature Neuroscience*, 8(3), 267–269.

Ojeda, D. A., & Forero, D. A. (2014). Study of five novel non-synonymous polymorphisms in human brain-expressed genes in a Colombian sample. *Annals of Neurosciences, 21*.

Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., Hoffmann, M. W., Eichele, G. (2006). The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metabolism*, *4*(2), 163–173.

Oydanich, M., Babici, D., Zhang, J., Rynecki, N., Vatner, D. E., & Vatner, S. F. (2019). Mechanisms of sex differences in exercise capacity. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 316(6), R832–R838.

Ozburn, A. R., Falcon, E., Twaddle, A., Nugent, A. L., Gillman, A. G., Spencer, S. M., Arey, R. N., Mukherjee, S., Lyons-Weiler, J., Self, D. W., McClung, C. A. (2015). Direct regulation of diurnal Drd3 expression and cocaine reward by NPAS2. *Biological Psychiatry*, 77(5), 425–433.

Ozburn, A. R., Kern, J., Parekh, P. K., Logan, R. W., Liu, Z., Falcon, E., Becker-Krail, D., Purohit, K., Edgar, N. M., Huang, Y., McClung, C. A. (2017). "NPAS2 regulation of anxiety-like behavior and GABAA receptors." *Frontiers in Molecular Neuroscience*, 10.

Pace-Schott, E. F., & Spencer, R. M. C. (2011). "Age-related changes in the cognitive function of sleep." *Progress in Brain Research* (Vol. 191, pp. 75–89).

Pádua-Reis, M., Nôga, D. A., Tort, A. B. L., & Blunder, M. (2021). Diazepam causes sedative rather than anxiolytic effects in C57BL/6J mice. *Scientific Reports.* 11:1, 11, 1, 4.

Pairojana, T., Phasuk, S., Suresh, P., & Liu, I. Y. (2022). Behavioral and Synaptic Phenotypes of Female Prdx6 −/− Mice. *Antioxidants* (Basel). 2022 Jun; 11(6): 1201.

Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., Schultz, P. G., Kay, S. A., Takahashi, J. S., & Hogenesch, J. B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell*, *109*(3), 307–320.

Park, K., Park, S., Nagappan, A., Ray, N., Kim, J., Yoon, S., Moon, Y., & Galvez, J. (2021). Probiotic Escherichia coli Ameliorates Antibiotic-Associated Anxiety Responses in Mice. *Nutrients.* 2021 Mar; 13(3): 811.

Partonen, T., Treutlein, J., Alpman, A., Frank, J., Johansson, C., Depner, M., Aron, L., Rietschel, M., Wellek, S., Soronen, P., Paunio, T., Koch, A., Chen, P., Lathfrop, M., Adolfsson, R., Persson, M. L., Kasper, S., Schalling, M., Peltonen, L., Schumann, G. (2007). "Three Circadian Clock Genes Per2, Arntl, and Npas2 Contribute to Winter Depression." *Annals of Medicine* 39 (3): 229–38.

Paul, K. N., Saafir, T. B., & Tosini, G. (2009). The role of retinal photoreceptors in the regulation of circadian rhythms. *In Reviews in Endocrine and Metabolic Disorders* (Vol. 10, Issue 4, pp. 271–278).

Pauls, F., Petermann, F., & Lepach, A. C. (2013). Gender differences in episodic memory and visual working memory including the effects of age. *Memory (Hove, England), 21*(7), 857–874.

Peirson, S. N., Brown, L. A., Pothecary, C. A., Benson, L. A., & Fisk, A. S. (2018). Light and the laboratory mouse. *Journal of Neuroscience Methods,* 300, 26–36.

Pendergast, J. S., Branecky, K. L., Huang, R., Niswender, K. D., & Yamazaki, S. (2014). Wheel-running activity modulates circadian organization and the daily rhythm of eating behavior. *Frontiers in Psychology*, *5*(MAR), 177.

Piber, D., Nowacki, J., Mueller, S. C., Wingenfeld, K., & Otte, C. (2018). Sex effects on spatial learning but not on spatial memory retrieval in healthy young adults. *Behavioural Brain Research*, *336*, 44–50.

Plautz, J. D., Kaneko, M., Hall, J. C., & Kay, S. A. (1997a). Independent photoreceptive circadian clocks throughout Drosophila. *Science (New York, N.Y.), 278*(5343), 1632–1635.

Plautz, J. D., Straume, M., Stanewsky, R., Jamison, C. F., Brandes, C., Dowse, H. B., Hall, J. C., and Kay, S. A. (1997b). "Quantitative Analysis of Drosophila Period Gene Transcription in Living Animals." *Journal of Biological Rhythms* 12 (3): 204–17.

Postic, C., & Magnuson, M. A. (2000). DNA excision in liver by an albumin-Cre transgene occurs progressively with age. *Genesis,* 26(2), 149–150.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002). "The Orphan Nuclear Receptor REV-ERBα Controls Circadian Transcription within the Positive Limb of the Mammalian Circadian Oscillator." *Cell* 110 (2): 251–60.

Prendergast, B. J., Onishi, K. G., & Zucker, I. (2014). Female mice liberated for inclusion in neuroscience and biomedical research. *Neuroscience and Biobehavioral Reviews*, *40*, 1–5.

Puighermanal, E., Cutando, L., Boubaker-Vitre, J., Honoré, E., Longueville, S., Hervé, D., & Valjent, E. (2017). Anatomical and molecular characterization of dopamine D1 receptor-expressing neurons of the mouse CA1 dorsal hippocampus. *Brain Structure and Function*, *222*(4), 1897–1911.

Puig, S., Shelton, M. A., Barko, K., Seney, M. L., & Logan, R. W. (2022). Sex-specific role of the circadian transcription factor NPAS2 in opioid tolerance, withdrawal and analgesia. *Genes, Brain, and Behavior*, *21*(7).

Ramos, A., Pereira, E., Martins, G. C., Wehrmeister, T. D., & Izídio, G. S. (2008). Integrating the open field, elevated plus maze and light/dark box to assess different types of emotional behaviors in one single trial. *Behavioural Brain Research,* 193(2), 277–288.

Rawashdeh, O., Jilg, A., Jedlicka, P., Slawska, J., Thomas, L., Saade, A., Schwarzacher, S. W., Stehle, J. H. (2014). "PERIOD1 coordinates hippocampal rhythms and memory processing with daytime." *Hippocampus, 24*(6), 712–723.

Reick, M., Garcia, J. A., Dudley, C. & McKnight, S. L. 2001. NPAS2: An Analog of Clock Operative in the Mammalian Forebrain. *Science,* 293**,** 506-509.

Ricceri, L., Colozza, C., & Calamandrei, G. (2000). Ontogeny of spatial discrimination in mice: A longitudinal analysis in the modified open-field with objects. *Developmental Psychobiology*, 37, 109–118.

Ripperger, J. A., and Merrow, M. (2011). "Perfect Timing: Epigenetic Regulation of the Circadian Clock." *FEBS Letters* 585 (10): 1406–11.

Rivera-Irizarry, J. K., Skelly, M. J., & Pleil, K. E. (2020). Social Isolation Stress in Adolescence, but not Adulthood, Produces Hypersocial Behavior in Adult Male and Female C57BL/6J Mice. *Frontiers in Behavioral Neuroscience*, 14, 129.

Roby, D. A., Ruiz, F., Kermath, B. A., Voorhees, J. R., Niehoff, M., Zhang, J., Morley, J. E., Musiek, E. S., Farr, S. A., Burris, T. P. (2019). Pharmacological activation of the nuclear receptor REV-ERB reverses cognitive deficits and reduces amyloid-β burden in a mouse model of Alzheimer's disease. *PLoS ONE*, *14*(4).

Rodgers, R. J., & Cole, J. C. (1993). Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice. *Physiology & Behavior*, *54*(4), 729–736.

Rosenthal, N. E., Sack, D. A., Gillin, J. C., Lewy, A. J., Goodwin, F. K., Davenport, Y., Mueller, P. S., Newsome, D. A., Wehr, T. A. (1984). Seasonal affective disorder. A description of the syndrome and preliminary findings with light therapy. *Archives of General Psychiatry*, *41*(1), 72–80.

Roustazade, R., Radahmadi, M., & Yazdani, Y. (2021). Therapeutic effects of saffron extract on different memory types, anxiety, and hippocampal BDNF and TNF-α gene expressions in sub-chronically stressed rats. *An international Journal on Nutrition, Diet and Nervous System. 25*(1), 192–206.

Royston, S. E., Yasui, N., Kondilis, A. G., Lord, S. V., Katzenellenbogen, J. A., & Mahoney, M. M. (2014). ESR1 and ESR2 Differentially Regulate Daily and Circadian Activity Rhythms in Female Mice. *Endocrinology*, *155*(7), 2613.

Rozov, S. V, Zant, J. C., Gurevicius, K., Porkka-Heiskanen, T., Panula, P. (2016). Altered Electroencephalographic Activity Associated with Changes in the Sleep-Wakefulness Cycle of

C57BL/6J Mice in Response to a Photoperiod Shortening. *Frontiers in Behavioral Neuroscience | Www.Frontiersin.Org*, *1*, 168.

Ruby, N. F., Fernandez, F., Garrett, A., Klima, J., Zhang, P., Sapolsky, R., & Heller, H. C. (2013). Spatial Memory and Long-Term Object Recognition Are Impaired by Circadian Arrhythmia and Restored by the GABAAAntagonist Pentylenetetrazole. *PLoS ONE*, 8(8), e72433.

Ruby, N. F., Hwang, C. E., Wessells, C., Fernandez, F., Zhang, P., Sapolsky, R., & Heller, H. C. (2008). "Hippocampal-dependent learning requires a functional circadian system." *Proceedings of the National Academy of Sciences of the United States of America*, 105(40), 15593–15598.

Rutter, J., Reick, M., Wu, L. C. & Mcknight, S. L. 2001. Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science,* 293**,** 510-4.

Sack, R. L., Auckley, D., Auger, R. R., Carskadon, M. A., Wright, K. P., Vitiello, M. V., and Zhdanova, I. V. (2007). "Circadian Rhythm Sleep Disorders: Part II, Advanced Sleep Phase Disorder, Delayed Sleep Phase Disorder, Free-Running Disorder, and Irregular Sleep-Wake Rhythm." *Sleep* 30(11):1484-501.

Sauer, B., & Henderson, N. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1 (genome rearrangement/herpesviruses/Cre-lox/metalothionein). In *Proc. Nail. Acad. Sci.* USA (Vol. 85).

Sauer, B., (1987). Functional Expression of the cre-lox Site-Specific Recombination System in the Yeast Saccharomyces cerevisiae. In *Molecular and Cellular Biology* (Vol. 7, Issue 6).

Scheer, F. A. J. L., Hilton, M. F., Mantzoros, C. S., & Shea, S. A. (2009). Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(11), 4453–4458.

Schmid, B., Helfrich-Förster, C. & Yoshii, T. 2011. A New ImageJ Plug-in "ActogramJ" for Chronobiological Analyses. *Journal of Biological Rhythms*, 26**,** 464-467.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675.

Schull, J., Walker, J., Fitzgerald, K., Hiilivirta, L., Ruckdeschel, J., Schumacher, D., Stranger, D., McEachron, D. L. (1989). Effects of sex, thyro-parathyroidectomy, and light regime on levels and circadian rhythms of wheel-running in rats. *Physiology & Behavior*, *46*(3), 341–346.

Sechzer, J. A., Rabinowitz, V. C., Denmark, F. L., McGinn, M. F., Weeks, B. M., & Wilkens, C. L. (1994). Sex and Gender Bias in Animal Research and in Clinical Studies of Cancer, Cardiovascular Disease, and Depression. *Annals of the New York Academy of Sciences*, 736(1), 21–48.

Seibenhener, M. L., & Wooten, M. C. (2015). Use of the Open Field Maze to Measure Locomotor and Anxiety-like Behavior in Mice. *Journal of Visualized Experiments : JoVE*, *96*, 52434.

Seymoure, P., Dou, H., & Juraska, J. M. (2013). Sex differences in radial maze performance: Influence of rearing environment and room cues. *Psychobiology 1996 24:1*, *24*(1), 33–37.

Shi, D., Chen, J., Wang, J., Yao, J., Huang, Y., Zhang, G., & Bao, Z. (2019). Circadian clock genes in the metabolism of non-alcoholic fatty liver disease. *Frontiers in Physiology*, *10*(MAY), 423.

Shi, S., Hida, A., McGuinness, O. P., Wasserman, D. H., Yamazaki, S., and Johnson, C. H. (2010). "Circadian Clock Gene Bmal1 Is Not Essential; Functional Replacement with Its Paralog, Bmal2." *Current Biology* 20 (4):316-21.

Smith, A. K., Fang, H., Whistler, T., Unger, E. R., & Rajeevan, M. S. (2011). Genomic Studies Identify Association of GRIK2 and NPAS2 with Chronic Fatigue Syndrome. *Neuropsychobiology*, *64*, 183–194.

Snider, K. H., Dziema, H., Aten, S., Loeser, J., Norona, F. E., Hoyt, K., and Obrietan, K. (2016). "Modulation of Learning and Memory by the Targeted Deletion of the Circadian Clock Gene Bmal1 in Forebrain Circuits." *Behavioural Brain Research* 308 (July): 222–35.

Solt, L. A., Kojetin, D. J., and Burris, T. P. (2011). "The REV-ERBs and RORs: Molecular Links between Circadian Rhythms and Lipid Homeostasis." *Future Medicinal Chemistry* 3 (5): 623–38.

Soria, V., Martínez-Amoró, rika, rgia Escaramís, G., Valero, J., Pé rez-Egea, R., García, C., Gutierrez-Zotes, A., Puigdemont, D., Bayes, M., Crespol, J. M., Martorell, L., Vilella, E., Labad, A., Vallejol, J., Perez, V., Menchon, J. M., Estivill, X., Gratacos, M., Urretavizcaya, M. (2010). Differential Association of Circadian Genes with Mood Disorders: CRY1 and NPAS2 are Associated with Unipolar Major Depression and CLOCK and VIP with Bipolar Disorder. *Neuropsychopharmacology*. 35, 1279-1289.

Soria, V., Martínez-Amorós, E., Escaramís, G., Valero, J., Pérez-Egea, R., García, C., Gutiérrez-Zotes, A., Puigdemont, D., Bayés, M., Crespo, J. M., Vilella, E., Labad, A., Vallejo, J., Pérez V,. Menchón, J. M., Estivill, X., Gratacòs, M., Urretavizcaya, M. (2010). "Differential Association of Circadian Genes with Mood Disorders: CRY1 and NPAS2 Are Associated with Unipolar Major Depression and CLOCK and VIP with Bipolar Disorder." Neuropsychopharmacology: *Official Publication of the American College of Neuropsychopharmacology* 35 (6): 1279–89.

Species Specific Information: Mouse. (n.d.). Retrieved August 16, 2022, from https://web.jhu.edu/animalcare/procedures/mouse.html

Spencer, S., Falcon, E., Kumar, J., Krishnan, V., Mukherjee, S., Birnbaum, D. G., and McClung, C. A. (2013). "Circadian Genes Period 1 and Period 2 in the Nucleus Accumbens Regulate Anxiety-Related Behavior." *The European Journal of Neuroscience* 37 (2): 242–50.

Stokkan, K. A., Yamazaki, S., Tei, H., Sakaki, Y., & Menaker, M. (2001). Entrainment of the circadian clock in the liver by feeding. *Science (New York, N.Y.)*, *291*(5503), 490–493.

Struntz, K. H., & Siegel, J. A. (2018). Effects of methamphetamine exposure on anxiety-like behavior in the open field test, corticosterone, and hippocampal tyrosine hydroxylase in adolescent and adult mice. *Behavioural Brain Research*, *348*, 211–218.

Sullens, D. G., Gilley, K., Jensen, K., Vichaya, E., Dolan, S. L., & Sekeres, M. J. (2021). Social isolation induces hyperactivity and exploration in aged female mice. *PLOS ONE*, *16*(2), e0245355.

Sulli, G., Lam, M. T. Y., & Panda, S. (2019). Interplay between Circadian Clock and Cancer: New Frontiers for Cancer Treatment. *Trends in Cancer*, *5*(8), 475–494.

Sultana, R., Ogundele, O. M., & Lee, C. C. (2019). Contrasting characteristic behaviours among common laboratory mouse strains. *Royal Society Open Science*, 6(6).

Sutcliffe, J. S., Marshall, K. M., & Neill, J. C. (2007). Influence of gender on working and spatial memory in the novel object recognition task in the rat. *Behavioural Brain Research*, 177(1), 117–125.

Tahara, Y., & Shibata, S. (2016). Circadian rhythms of liver physiology and disease: experimental and clinical evidence. *Nature Reviews Gastroenterology & Hepatology 2016 13:4*, *13*(4), 217–226.

Takahashi, J. S., Hong, H., Ko, C. H., and McDearmon, E. L. (2008). "The Genetics of Mammalian Circadian Order and Disorder: Implications for Physiology and Disease." *Nature Reviews. Genetics* 9 (10): 764–75.

Takahashi, M., Sugiyama, A., Wei, R., Kobayashi, S., Fukuda, K., Nishino, H., Takahashi, R., Tsutsumi, K., Kita, I., Ando, K., Manabe, T., Kamiguchi, H., Tomomura, M., & Hisanaga, S. ichi. (2020). Hyperactive and impulsive behaviors of LMTK1 knockout mice. *Scientific Reports 2020 10:1*, *10*(1), 1–13.

Takahashi, Y., Sawa, K., & Okada, T. (2013). The diurnal variation of performance of the novel location recognition task in male rats. *Behavioural Brain Research*, *256*, 488–493.

Tam, S. K. E., Hasan, S., Choi, H. M. C., Brown, L. A., Jagannath, A., Hughes, S., Hankins, M. W., Foster, R. G., Vyazovskiy, V. V., Bannerman, D. M., & Peirson, S. N. (2017). Constant light desynchronizes olfactory versus object and visuospatial recognition memory performance. *Journal of Neuroscience*, 37(13), 3555–3567.

Tan, O., Musullulu, H., Raymond, J. S., Wilson, B., Langguth, M., & Bowen, M. T. (2019). Oxytocin and vasopressin inhibit hyper-aggressive behaviour in socially isolated mice. *Neuropharmacology*, *156*, 107573.

Taniguchi, H., Fernández, A. F., Setién, F., Ropero, S., Ballestar, E., Villanueva, A., Yamamoto, H., Imai, K., Shinomura, Y., & Esteller, M. (2009). Epigenetic inactivation of the circadian clock gene BMAL1 in hematologic malignancies. *Cancer Research*, *69*(21), 8447–8454.

Tapia-Osorio, A., Salgado-Delgado, R., Angeles-Castellanos, M., & Escobar, C. (2013). Disruption of circadian rhythms due to chronic constant light leads to depressive and anxiety-like behaviors in the rat. *Behavioural Brain Research*, *252*, 1–9.

Terazono, H., Mutoh, T., Yamaguchi, S., Kobayashi, M., Akiyama, M., Udo, R., Shigehiro, O, Okamura, H., Shibata, S. (2003). Adrenergic regulation of clock gene expression in mouse liver. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(11), 6795–6800.

Timofeev, I., Grenier, F., Bazhenov, M., Houweling, A. R., Sejnowski, T. J., & Steriade, M. (2002). Short- and medium-term plasticity associated with augmenting responses in cortical slabs and spindles in intact cortex of cats in vivo. *The Journal of Physiology*, *542*(2), 583–598.

Tonetti, L., Barbato, G., Fabbri, M., Adan, A., & Natale, V. (2007). Mood seasonality: a cross-sectional study of mice aged between 10 and 25 years. *Journal of Affective Disorders*, *97*(1–3), 155–160.

Turlo, K. A., Gallaher, S. D., Vora, R., Laski, F. A., & Iruela-Arispe, M. L. (2010). When Cre-mediated recombination in mice does not result in protein loss. *Genetics*, 186(3), 959–967.

Utomo, A. R. H., Nikitin, A. Y., & Lee, W. H. (1999). Temporal, spatial of and cell type-specified control of cre-mediated DNA recombination in transgenic mice. *Nature Biotechnology*, *17*(11), 1091–1096.

Valentinuzzi, V. S., Kolker, D. E., Vitaterna, M. H., Ferrari, E. A. M., Takahashi, J. S., & Turek, F. W. (2001). Effect of circadian phase on context and cued fear conditioning in C57BL/6J mice. *Animal Learning and Behavior*, 29(2), 133–142.

Valentinuzzi, V. S., Menna-Barreto, L., and Xavier, G. F. (2004). "Effect of Circadian Phase on Performance of Rats in the Morris Water Maze Task." *Journal of Biological Rhythms* 19 (4): 312–24.

Van der Zee, E. A., Havekes, R., Barf, R. P., Hut, R. A., Nijholt, I. M., Jacobs, E. H., and Gerkema, M. P. (2008). "Circadian Time-Place Learning in Mice Depends on Cry Genes." *Current Biology: CB* 18 (11): 844–48.

Vanhooren, V., & Libert, C. (2013). "The mouse as a model organism in ageing research: usefulness, pitfalls and possibilities". *Ageing Research Reviews*, 12(1), 8–21.

Vecsey, C. G., Hawk, J.D., Lattal, K. M., Stein, J. M., Fabian, S. A., Attner, M. A., Cabrera, S. M., McDonough, C. B., Brindle, P. K., Abel., T., Wood, M. A. (2007). "Histone Deacetylase Inhibitors Enhance Memory and Synaptic Plasticity via CREB: CBP-Dependent Transcriptional Activation". *Journal of Neuroscience* 27(23):6128-40.

Vida, B., Hrabovszky, E., Kalamatianos, T., Coen, C. W., Liposits, Z., & Kalló, I. (2008). Oestrogen receptor alpha and beta immunoreactive cells in the suprachiasmatic nucleus of mice: distribution, sex differences and regulation by gonadal hormones. *Journal of Neuroendocrinology*, *20*(11), 1270–1277.

Vitaterna, M. H., King, D. P., Chang, A. M., Kornhauser, J. M., Lowrey, P. L., McDonald, J. D., Dove, W. F., Pinto, L. H., Turek, F. W., and Takahashi, J. S. (1994). "Mutagenesis and Mapping of a Mouse Gene, Clock, Essential for Circadian Behavior." *Science* 264 (5159): 719–25.

Võikar, V., Kõks, S., Vasar, E., & Rauvala, H. (2001). Strain and gender differences in the behavior of mouse lines commonly used in transgenic studies. *Physiology & Behavior*, *72*(1–2), 271–281.

Võikar, V., Polus, A., Vasar, E., & Rauvala, H. (2005). Long-term individual housing in C57BL/6J and DBA/2 mice: assessment of behavioral consequences. *Genes, Brain and Behavior*, *4*(4), 240–252.

Voyer, D., Postma, A., Brake, B., & Imperato-McGinley, J. (2007). Gender differences in object location memory: a meta-analysis. *Psychonomic Bulletin & Review*, *14*(1), 23–38.

Wahid, F., Shehzad, A., Khan, T., and Kim, Y. Y. (2010). "MicroRNAs: Synthesis, Mechanism, Function, and Recent Clinical Trials." *Biochimica et Biophysica Acta* 1803 (11): 1231–43.

Walia, V., Garg, C., & Garg, M. (2019). NO-sGC-cGMP signaling influence the anxiolytic like effect of lithium in mice in light and dark box and elevated plus maze. *Brain Research*, *1704*, 114–126.

Walsh, R.N., Cummins, R, A. (1976). The open field test: a critical review. *Psychol. Bull.* 1976;83:482–504.

Walton, J. C., Bumgarner, J. R., & Nelson, R. J. (2022). Sex Differences in Circadian Rhythms. *Cold Spring Harbor Perspectives in Biology*, a039107.

Wang, L. M., Dragich, J. M., Kudo, T., Odom, I. H., Welsh, D. K., O'Dell, T. J., and Colwell, C. S. (2009). "Expression of the Circadian Clock Gene Period2 in the Hippocampus: Possible Implications for Synaptic Plasticity and Learned Behaviour." *ASN Neuro* 1 (3).

Wang, X. L., Kooijman, S., Gao, Y., Tzeplaeff, L., Cosquer, B., Milanova, I., Wolff, S. E. C., Korpel, N., Champy, M. F., Petit-Demoulière, B., Goncalves Da Cruz, I., Sorg-Guss, T., Rensen, P. C. N., Cassel, J. C., Kalsbeek, A., Boutillier, A. L., & Yi, C. X. (2021). Microglia-specific knock-down of Bmal1 improves memory and protects mice from high fat diet-induced obesity. *Molecular Psychiatry*, *26*(11), 6336–6349.

Wass, C., Pizzo, A., Sauce, B., Kawasumi, Y., Sturzoiu, T., Ree, F., Otto, T., Matzel, L. D. (2013). Dopamine D1 sensitivity in the prefrontal cortex predicts general cognitive abilities and is modulated by working memory training. *Learning & Memory*, *20*(11), 617–627.

Winters, B. D., & Reid, J. M. (2010). A distributed cortical representation underlies crossmodal object recognition in rats. *Journal of Neuroscience*, 30(18), 6253–6261.

Woodie, L. N., Johnson, R. M., Ahmed, B., Fowler, S., Haynes, W., Carmona, B., Reed, M., Suppiramaniam, V., Greene, M. W. (2020). Western diet-induced obesity disrupts the diurnal

rhythmicity of hippocampal core clock gene expression in a mouse model. *Brain, Behavior, and Immunity*, *88*, 815–825.

Wright, K. P., Jr, Hull, J. T., Hughes, R. J., Ronda, J. M., and Czeisler, C. A. (2006). "Sleep and Wakefulness out of Phase with Internal Biological Time Impairs Learning in Humans." *Journal of Cognitive Neuroscience* 18 (4): 508–21.

Wulff, K., Gatti, S., Wettstein, J. G., & Foster, R. G. (2010). "Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease." *Nature Reviews Neuroscience,* Vol. 11, pp. 589–599.

Xie, X., Yang, H., An, J. J., Houtz, J., Tan, J. W., Xu, H., Liao, G. Y., Xu, Z. X., & Xu, B. (2019). Activation of Anxiogenic Circuits Instigates Resistance to Diet-Induced Obesity via Increased Energy Expenditure. *Cell Metabolism*, 29(4), 917-931.e4.

Xu, Liu, Shuang Xu, Lifang Lin, Xi Gu, Congcong Fu, Yingying Fang, Xiaowen Li, and Xuemin Wang. 2018. "High-Fat Diet Mediates Anxiolytic-like Behaviors in a Time-Dependent Manner Through the Regulation of SIRT1 in the Brain." *Neuroscience* 372 (February): 237–45.

Yamada, C., Saegusa, Y., Nahata, M., Sadakane, C., Hattori, T., & Takeda, H. (2015). Influence of ageing and Gender Differences on Feeding Behavior and Ghrelin-Related Factors during Social Isolation in Mice. *PLOS ONE*, *10*(10), e0140094.

Yamamoto, T., Nakahata, Y., Tanaka, M., Yoshida, M., Soma, H., Shinohara, K., Yasuda, A., Mamine, T., Takumi, T. (2005). Acute physical stress elevates mouse period1 mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. *The Journal of Biological Chemistry*, *280*(51), 42036–42043.

Yang, Wei, Xiaomin Kang, Jiali Liu, Huixia Li, Zhengmin Ma, Xinxin Jin, Zhuang Qian, et al. 2016. "Clock Gene Bmal1 Modulates Human Cartilage Gene Expression by Crosstalk With Sirt1." *Endocrinology* 157 (8): 3096–3107.

Yates, G., Panksepp, J., Ikemoto, S., Nelson, E., & Conner, R. (1991). Social isolation effects on the "behavioral despair" forced swimming test: Effect of age and duration of testing. *Physiology & Behavior*, *49*(2), 347–353.

Ye, R., Selby, C. P., Chiou, Y., Ozkan-Dagliyan, I., Gaddameedhi, S., and Sancar, A. (2014). "Dual Modes of CLOCK:BMAL1 Inhibition Mediated by Cryptochrome and Period Proteins in the Mammalian Circadian Clock." *Genes & Development* 28 (18): 1989–98.

Yesudhas, A., Radhakrishnan, R. K., Sukesh, A., Ravichandran, S., Manickam, N., & Kandasamy, M. (2021). BOTOX® counteracts the innate anxiety-related behaviours in correlation with increased activities of key antioxidant enzymes in the hippocampus of ageing experimental mice. *Biochemical and Biophysical Research Communications*, *569*, 54–60.

Yoshikawa, T., Sellix, M., Pezuk, P., & Menaker, M. (2009). Timing of the Ovarian Circadian Clock Is Regulated by Gonadotropins. *Endocrinology*, *150*(9), 4338.

Yoshitane, H., & Fukada, Y. (2009). CIPC-dependent phosphorylation of CLOCK and NPAS2 in circadian clockwork. *Sleep and Biological Rhythms*, 7(4), 226–234.

Zhang, B., & Wing, Y. K. (2006). Sex differences in insomnia: a meta-analysis. *Sleep*, *29*(1), 85–93.

Zhang, W., and Liu, H. T. (2002). "MAPK Signal Pathways in the Regulation of Cell Proliferation in Mammalian Cells." *Cell Research* 12 (1): 9–18.

Zhang, Y. F., Li, Q. Q., Qu, J., Sun, C. M., & Wang, Y. (2017). Alterations of motor cortical microcircuit in a depressive-like mouse model produced by light deprivation. *Neuroscience*, *341*, 79–94.

Zhang, Y., Zhao, Y., Pan, F., & Zhang, P. (2017). EGb761 attenuates depressive-like behaviours induced by long-term light deprivation in C57BL/6J mice through inhibition of NF-κB-IL-6 signalling pathway. *Central European Journal of Immunology*, *41*(4), 350–357.

Zhao, Z., Fan, L., Fortress, A. M., Boulware, M. I., & Frick, K. M. (2012). Hippocampal histone acetylation regulates object recognition and the estradiol-induced enhancement of object recognition. *Journal of Neuroscience*, 32(7), 2344–2351.

Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Sun., Z. S., Eichlele, G., Bradley, A., & Lee, C. C. (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell*, 105(5), 683–694.

Zhou, J. N., Hofman, M. A., & Swaab, D. F. (1995). VIP neurons in the human SCN in relation to sex, age, and Alzheimer's disease. *Neurobiology of ageing*, *16*(4), 571–576.

Zhou, L., Zhang, Z., Nice, E., Huang, C., Zhang, W., & Tang, Y. (2022). Circadian rhythms and cancers: the intrinsic links and therapeutic potentials. In *Journal of Hematology and Oncology* (Vol. 15, Issue 1). BioMed Central.

Zhou, Y., Zhang, H. K., Liu, F., Lei, G., Liu, P., Jiao, T., & Dang, Y. H. (2018). Altered light conditions contribute to abnormalities in emotion and cognition through HINT1 dysfunction in C57BL/6 Mice. *Frontiers in Behavioral Neuroscience,* 12.

Zielinski, T., Moore, A. M., Troup, E., Halliday, K. J., & Millar, A. J. (2014). Strengths and Limitations of Period Estimation Methods for Circadian Data. *PLoS ONE*, 9(5