

**Effects of VEGF-A₁₆₅b and SRPK1
inhibition on pain behaviour,
cyclooxygenase expression and glial
activation in the CNS in a model of
osteoarthritis**

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AUTHOR'S DECLARATION

I declare that the work submitted in this thesis was conducted according to University's Regulations and Code of Practice, and it is the result of original research conducted by me, except where indicated by specific reference in the text. This thesis has not been submitted for any other academic award.

ABSTRACT

Background: Osteoarthritis (OA) is the most common musculoskeletal disease worldwide and a major cause of chronic pain. Treatment of OA pain is still suboptimal due to limited efficacy and considerable side effects of available analgesics. Pain in OA has a significant central component. Cyclooxygenases (COXs) and glial cells in the spinal cord and the periaqueductal gray (PAG) play a significant role in central sensitisation and pain modulation.

Vascular endothelial growth factor-A (VEGF-A) is a key molecule in normal and pathological angiogenesis. Serine arginine protein kinase 1 (SRPK1), which phosphorylates serine arginine splice factor 1 (SRSP1), controls VEGF gene alternative splicing. This results in two splice variants; VEGF-A_{165a}, which is pro-angiogenic and pro-nociceptive, and anti-angiogenic VEGF-A_{165b}, which showed anti-nociceptive effects in models of neuropathic and inflammatory pain.

Objective: This thesis investigated changes in COX expression and glial activation in the spinal cord and the PAG in the monosodium iodoacetate (MIA) model of OA. It also addressed the effects of VEGF-A_{165b} and SRPK1 inhibitor SPHINX-31 on pain behaviour and joint pathology, as well as COX expression and glial activation in the spinal cord and the PAG in the same model.

Hypothesis: VEGF-A_{165b} and SPHINX-31 can prevent and/or reverse enhanced pain behaviour in the MIA model of OA, through involvement of spinal glial cells and COXs in the PAG and spinal cord. Vascular-astrocyte association in the PAG is enhanced in the MIA model of OA, and this effect is reversed by administration of SPHINX-31.

Methods: Rats received an intra-articular injection of MIA (1 mg) in the knee. In one study, animals were treated with VEGF-A_{165b} (i.p. 20 ng/g body weight twice weekly) on days 0-13 (VEGF(d0-13) group) or days 14-28 (VEGF(d14-28) group) after MIA injection; in another study, rats received SPHINX-31 (i.p. 0.8 µg/g body weight twice weekly; (MIA/SPHINX group)) for 19 days after induction of the model. Pain behaviour was monitored throughout the studies, at the end of which (day 28) tissues were collected for the assessment of joint histopathology and the evaluation of spinal COX-2 mRNA expression by PCR.

In addition, immunofluorescence (IF) was used to assess COX-2 expression and glial activation in the spinal cord, as well as astrocyte activation and vascular-astrocyte association in the PAG.

Results: VEGF-A₁₆₅b significantly attenuated weight bearing asymmetry (%) in MIA rats on day 28 (29.58 ± 1.803 in MIA/VEGF(d0-13) group vs. 22.95 ± 2.088 in MIA/PBS group, $p < 0.01$; 29.23 ± 1.49 in VEGF(d14-28) vs. 22.95 ± 2.088 in MIA/PBS, $p < 0.05$). VEGF-A₁₆₅b reversed mechanical withdrawal thresholds to the naïve level, but without reaching statistical significance. No significant changes in knee joint pathology were observed in VEGF-A₁₆₅b treated MIA rats compared to the MIA/PBS counterparts. In the MIA/VEGF(d0-13) group, contralateral deep laminae of the dorsal horn had a higher percentage (%) of non-neuronal cells expressing COX-2 than the corresponding superficial laminae (3.26 ± 1.16 vs 1.12 ± 0.43 , $p < 0.05$), while no difference was observed in the MIA/PBS group. Administration of VEGF-A₁₆₅b did not significantly affect spinal microglia and astrocyte activation, nor COX-2 expression in the PAG.

SPHINX-31 had no significant effects on pain behaviour, joint pathology or spinal COX-2 expression in the MIA model of OA. On the other hand, MIA/SPHINX group exhibited a higher activation of spinal microglia than MIA controls (% of CD11b +ve cells in MIA/SPHINX-31 vs MIA/vehicle groups: 6.36 ± 0.89 vs 1.72 ± 0.38 , $p < 0.01$). In addition, SPHINX-31 significantly increased astrocyte activation in the ipsilateral dorsolateral (DL) PAG relative to corresponding ventrolateral (VL) PAG (GFAP IF intensity: 12.89 ± 1.52 vs 8.46 ± 0.84 , $p < 0.05$), and it increased vascular-astrocyte association (%) in the contralateral DL PAG relative to corresponding VL PAG (70.35 ± 7.68 vs 38.92 ± 8.19 , $p < 0.05$). Interestingly, naïve rats had a significantly higher astrocyte activation and vascular-astrocyte association in the VL PAG than in DL PAG.

Conclusions: VEGF-A₁₆₅b exerted a significant antinociceptive effect in the MIA model of OA without affecting joint pathology, spinal glial cell activation or COX-2 expression in the PAG. SPHINX-31 did not reverse pain behaviour, but showed a potential effect on astrocyte activation and vascular-astrocyte association in the DL PAG relative VL PAG in the MIA model of OA.

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

- Drake RAR, Leith JL, Almahasneh F, Martindale J, Wilson AW, Lumb B, Donaldson LF. Periaqueductal grey EP3 receptors facilitate spinal nociception in arthritic secondary hypersensitivity. *The Journal of Neuroscience*. 2016; 36(35):9026-40.
- Beazley-Long N, Moss CE, Ashby WR, Bestall SM, Almahasneh F, Durrant AM, Benest AV, Blackley Z, Ballmer-Hofer K, Hirashima M, Hulse RP, Bates DO, Donaldson LF. VEGFR2 promotes central endothelial activation and the spread of pain in inflammatory arthritis. *Brain, Behavior, and Immunity*. 2018. [Epub ahead of print].

PRESENTATIONS

POSTER

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- Almahasneh F, Beazley-Long N, Blackley Z, Bates DO, Donaldson LF. Effects of vascular endothelial growth factor-A_{165b} on pain behaviour in a model of osteoarthritis in rat. Pharmacology, London, UK, December 2016.
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ABBREVIATIONS

20-HETE	20-Hydroxyeicosatetraenoic acid
5-HT	5-hydroxytryptamine (serotonin)
AC3	Activated caspase 3
ACLT	Anterior cruciate ligament transection
ACR	American College of Rheumatology
ADAMTS	A Disintegration and Metalloproteinase with Thrombospondin-like Motifs
AMPA	α -amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid
ANOVA	Analysis of variance
AQ	(Cerebral) aqueduct
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
CB2	Cannabinoid receptor 2
CBF	Cerebral blood flow
CCI	Chronic constriction injury
CD11b	Cluster of differentiation 11b
CD31	Cluster of differentiation 31
CeA	Central nucleus of the amygdala
CFA	Complete Freund Adjuvant
CGRP	Calcitonin gene related peptide
CI	Confidence interval

CNS	Central nervous system
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
CR3	Type 3 complement receptor
CT	Computed tomography
CXCL1	Chemokine (C-X-C motif) ligand 1
DAPI	4',6-diamidino-2-phenylindole
DAMPs	Damage-associated molecular pattern proteins
DEG/ENaC	Degenerin/Epithelial amiloride-sensitive Na ⁺ channel
DLF	Dorsolateral funiculus
DL PAG	Dorsolateral PAG
DMM	Destabilization of the medial meniscus
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribose nucleoside triphosphate
DP	PGD ₂ receptor
dPCR	Digital PCR
DR	Dorsal nucleus raphe
DRG	Dorsal root ganglion
DRt	Dorsal reticular nucleus
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EET	Epoxyeicosatrienoic acid
EMG	Electromyography
EP	PGE ₂ receptor

EPSC	Excitatory post-synaptic current
ERK	Extracellular signal–regulated kinase
ESE	Exonic sequence enhancer
EULAR	European League against Rheumatism
FBS	Fetal bovine serum
fMRI	Functional MRI
FP	PGF _{2α} receptor
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
GLY _B /NMDA	Glycine site on the NMDA receptor
H&E	Haematoxylin and Eosin
HFS	High-frequency stimulation
HIF-1-α	Hypoxia inducible factor-1-α
HUVEC	Human umbilical vein endothelial cell
i.a.	Intra-articular
ICC	Intraclass correlation
IF	Immunofluorescence
IFN	Interferon
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
IP	PGI ₂ receptor

LC	Locus coeruleus
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MC4R	Melanocortin 4 receptor
MCP-1	Monocyte chemoattractant protein-1
MEK	Mitogen-activated protein kinase kinase
MIA	Monosodium iodoacetate
MMP	Matrix metalloproteinase
MMT	Medial meniscal tear
MNX	Meniscal transection
MRI	Magnetic resonance imaging
NaV	Voltage-operated sodium channel
NFκB	Nuclear factor kappa B
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NGF	Nerve growth factor
NMDA	N-Methyl-D-aspartate
NO	Nitric oxide
NRP	Neuropilin
NS	Normal saline
NSAIDs	Non-steroidal anti-inflammatory drugs
NWR	Nociceptive withdrawal reflexes
O ₂	Oxygen
OA	Osteoarthritis

OARSI	Osteoarthritis Research Society International
OCT	Optimal cutting temperature
PAG	Periaqueductal gray
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PAG	Periaqueductal gray
PCR	Polymerase chain reaction
PGD ₂	Prostaglandin D ₂
PGE ₂	Prostaglandin E ₂
PGF _{2α}	Prostaglandin F _{2α}
PGI ₂	Prostaglandin I ₂
PKA	Protein kinase A
PKC γ	Protein kinase C γ
PLA ₂	Phospholipase A ₂
PlGF	Placenta growth factor
PRRs	Pattern recognition receptors
PVP	Polyvinylpyrrolidone
qPCR	Real time PCR
RT	Reverse transcription / reverse transcriptase
RTK	Receptor tyrosine kinase
RVM	Rostral ventromedial medulla
SEM	Standard error of mean
SFA	Société Française D'Arthroscopie (French society of arthroscopy)
Src	Proto-oncogene tyrosine kinase

SRPK1	Serine arginine-rich protein kinase 1
SRSF1	Serine arginine rich splicing factor 1
SRT	Spinoreticular tract
STT	Spinothalamic tract
STZ	Superficial tangential zone
STZ	Streptozotocin
TAE	Tris-acetate-EDTA buffer
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TP	Thromboxane receptor
TrkA	Tropomyosin receptor kinase A
TRP	Transient receptor potential
TRPA1	Transient receptor potential cation channel subfamily A member 1
TRPM8	Transient receptor potential cation channel subfamily M member
TRPV1	Transient receptor potential vanilloid 1
TTX	Tetrodotoxin
TxA ₂	Thromboxane A ₂
VEGF	Vascular endothelial growth factor
VL PAG	Ventrolateral PAG
VSMCs	Vascular smooth muscle cells

Chapter 1. Introduction

1.1. Osteoarthritis

Osteoarthritis (OA) is the most common arthritic disease and it is considered a major cause of pain, disability, decreased quality of life and economic burden. (Laupattarakasem et al., 2008, Perrot, 2015, Chen et al., 2012).

OA Research Society International (OARSI) defines OA as “a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodelling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness” (Kraus et al., 2015). Clinically, the condition is characterized by joint pain, tenderness, limitation of movement, crepitus, occasional effusion, and variable degrees of local inflammation (Symmons et al., 2003). OA can be described based on the radiographic and histological changes of the joint as well as the signs and symptoms; however, these rarely correlate exactly, which makes it challenging to find a general agreed on definition of OA.

OA is sometimes considered a collection of disorders with different underlying pathophysiological mechanisms that have a similar final common pathway (Aigner and McKenna, 2002) or a "process" rather than a disease, because it is observed in all the conditions displaying an imbalance between mechanical stresses applied to the articular cartilage and its ability to withstand that stress (Slemenda et al., 1998).

1.1.1. Epidemiology

OA is the most common joint disorder in the world (Vos et al., 2012). Prevalence of OA varies greatly due to differences in diagnostic criteria, populations and joints involved (Palazzo et al., 2016). Literature reported OA prevalence ranges from 12.3% in France (Palazzo et al., 2014) to 21.6% in the United States (Helmick et al., 2008). In the UK, an estimated 8.75 million people aged 45 years and over (33%) have sought treatment for osteoarthritis, sixty percent of whom were female and 40% were male (Arthritis Research, 2013). In England, 18.2% of adults (4.11 million people) aged 45 and above have OA of the knee, 6.1% of which are affected by the severe form of the condition. Within the next 20 years, one quarter of the UK population will be over the age of 65, 50% will be clinically obese and the number of patients with OA is predicted to double (Briggs et al., 2012).

OA is associated with significant disability, pain and economic burden. Studies addressing quality of life found the impact of OA to be comparable to that of cardiac, neurological and pulmonary diseases (Briggs et al., 2012). Hip and knee OA was ranked as the 11th highest contributor to global disability (Cross et al., 2014).

OA is also one of the leading causes of chronic pain in the world. In Europe, 20% of chronic pain is related to OA (O'Brien and Breivik, 2012). In the UK, nearly three quarters of people with osteoarthritis report constant pain, with 1 in 8 describing their pain as often unbearable (Conaghan et al., 2015).

The economic cost of OA is huge. In 2010, the average direct care cost per person with OA in UK was £783 per year or some £5.2 billion per annum in total (Oxford Economics, 2010). OA and other musculoskeletal diseases account for almost one tenth of the total annual NHS budget (£10 billion annually) and 12 % of primary care consultations. OA is also the major cause of absence from work, costing the UK economy up to £18 billion annually (Briggs et al., 2012, Arthritis Research, 2009) and it leads in many cases to permanent retirement, with an estimated cost - along with rheumatoid arthritis - of £10 billion (Oxford Economics, 2010).

1.1.1.1. Risk factors

The exact underlying cause of OA is not known. However, several risk factors have been associated with the disease, including:

- 1- Genetic predisposition. Epidemiological and twin studies have established that OA is to a large extent genetically determined, but the underlying genetic variants remain mostly unknown (Loughlin, 2005, van Meurs, 2017).
- 2- Constitutional factors, such as:
 - a. Age. Age is the strongest predictor of osteoarthritis development (Du et al., 2005). Incidence of osteoarthritis increases almost exponentially with age (Petersson, 1996). Knee osteophytes were found to increase by 20% per 5-year age increase (Hart et al., 1999) and age > 50 was found as a risk factor for knee, hip and hand osteoarthritis (Andrianakos et al., 2003).
 - b. Female sex. Females present higher risk of developing the disease than men, and this is probably related to oestrogen hormones (Abramson and Attur, 2009). There is a significant increase in the prevalence of OA after the age of 50 years, particularly in the knee. Articular chondrocytes possess functional oestrogen receptors, and oestrogen can upregulate proteoglycan synthesis. Human and animal studies indicate that oestrogen replacement therapy reduces the incidence of OA, although the evidence for a relation between oestrogen deficiency and OA is inconsistent (Abramson and Attur, 2009)
 - c. Obesity. Overweight and obesity are recognised risk factors for the development and progression of osteoarthritis (Pereira et al., 2015), and this is probably associated with increased load and stress in the joints and/or systemic changes (Cooper et al., 2000, Felson and Zhang, 1998). The effect of body mass index (BMI) on osteoarthritis could also be explained by the pro-inflammatory action of fat (van der Kraan, 2012, Madry et al., 2012). In mice, a high-fat diet induced OA and increased inflammatory mediators proportional to body fat, and short-term wheel

running protected against obesity-induced OA by modulating systemic inflammation (van der Kraan, 2012).

- d. High bone density. High bone mineral density is thought to be related with osteoarthritis (Goerres et al., 2005, Hochberg et al., 2004). However, this association is not always definite, as it can change in different populations and joint sites, and can be mediated by other factors such as vitamin D status (Zhang and Jordan, 2010).

3- Biomechanical risk factors:

- a. Occupational/recreational usage. Excessive joint activity or stress is linked to the aetiology of OA (Rossignol et al., 2003). Manual and physical work, as well as occupations that require repetitive, load-bearing activities (Felson and Nevitt, 2004), were found to be risk factors for knee (Yoshimura et al., 2004), hip (Juhakoski et al., 2009) and hand osteoarthritis (Kalichman and Hernandez-Molina, 2010). Sports that involve rapid acceleration with instant deceleration, or continuous training with a high impact on joints, or prolonged competition at elite level increase the likelihood of developing early OA (Vannini et al., 2016).
- b. Joint injury and reduced muscle strength. A history of joint injury (Lau et al., 2000) and muscle weakness (Goldring and Goldring, 2007) are associated with an increased risk for the development of OA.
- c. Joint instability / malalignment. This factor is most evident in the knee and ankle joints. A shift from normal joint alignment can change load distribution across the knee, so malalignment may contribute to abnormal mechanical forces (Johnson and Hunter, 2014). Knee malalignment is one of the strongest predictors of knee OA progression (Felson et al., 2003), but its effect on the incidence of OA is less clear (Hunter et al., 2007).

The relative importance of these risk factors vary largely between the different sites of OA in the body (Pereira et al., 2015).

1.1.2. Pathophysiology of OA

OA had historically been seen as a degenerative disorder exclusively characterised by wear and tear of the joint (Shapiro et al., 2014). However, recent understanding of OA pathophysiology has revealed the role of remodelling of joint tissues driven by metabolic and inflammatory mediators within the affected joint (Mobasher and Batt, 2016).

The main characteristics of OA are loss of articular cartilage and subchondral bone sclerosis (van der Kraan, 2012); figure 1.1. Cartilage pathology, known as chondropathy, is manifested as early fragmentation, chondrocyte cluster formation, vertical clefts, crystal deposition, remodelling, and eventual invasion of the tidemark by blood vessels (Madry et al., 2012); refer to figure 1.2. Bone remodelling and attrition occur early in the development of the disease caused by fibrocartilage degeneration, chondro-osteophytes, protrusions, subsynovial inflammatory cells and synovium cell hyperplasia (Hernandez-Molina et al., 2008). Activated synovium secretes excess synovial fluid, producing capsular swelling (Neogi et al., 2009).

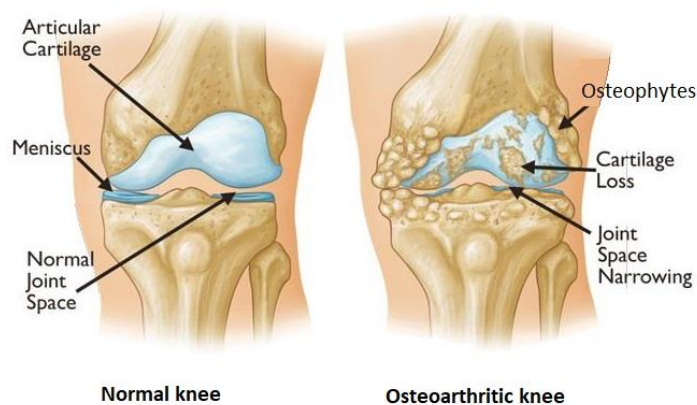


Figure 1.1. Normal and osteoarthritic knees. OA knee shows cartilage loss, joint space narrowing and osteophytes. *Picture reproduced from (OrthoInfo, 2018).*

1.1.2.1. Articular cartilage

The articular cartilage is a protective connective tissue covering the ends of long bones, which acts as a shock absorber and reduces friction during movement (Nwosu, 2015). Cartilage consists of 1) chondrocytes and 2) the extracellular matrix (ECM), which contains water, collagen fibres (mainly type II collagen), hyaluronic acid, and hydrophilic sulfated proteoglycans (Table 1.1). The network of type II collagen fibres maintains the volume and shape of the tissue, whereas proteoglycans provide elasticity (Sulzbacher, 2013).

Table 1.1. Cartilage components

Fluid extracellular matrix	Water
Solid extracellular matrix	Collagen fibres Type II collagen Small amounts of type V, VI, IX, XI collagen Hyaluronic acid Proteoglycans Chondroitin 4-6 and keratin sulfate Decorin, fibromodulin, biglycan
Cellular matrix	Chondrocytes

Cartilage consists of four layers that differ in chondrocyte shape, proteoglycan content and arrangement of collagen fibres. The first three layers form the non-calcified cartilage and are separated from the fourth calcified cartilage layer by a tidemark (figure 1.2).

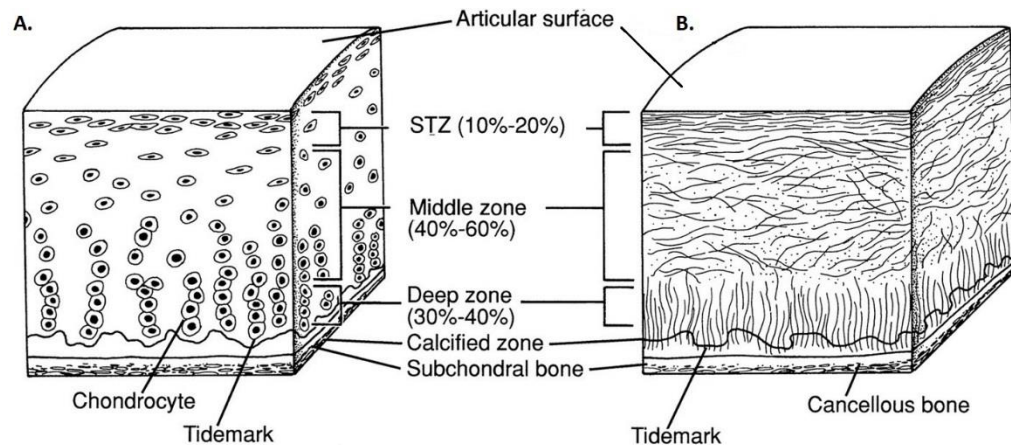


Figure 1.2. The articular cartilage. (A) Chondrocyte and (b) collagen fibre organisation in the articular cartilage. (STZ = Superficial tangential zone; figure taken from (Steward et al., 2011)).

In normal cartilage, chondrocytes are relatively inactive, and matrix undergoes little turnover. In OA, these cells become activated and exhibit proliferation, cloning and increased production of matrix proteins and degradation enzymes. These changes, which may be considered a response to injury, eventually lead to matrix remodelling, abnormal hypertrophy of chondrocytes and calcification of the cartilage (Goldring and Marcu, 2009). Degradation of cartilage matrix is a complex process that involves several interacting genetic, environmental, mechanical and biochemical factors. These include the degrading enzymes matrix metalloproteinases (MMPs), which break down collagen and proteoglycans. Of this family, the aggrecanase (A Disintegration and Metalloproteinase with Thrombospondin-like Motifs) ADAMTS5 and the stromelysin MMP3, which degrade aggrecan, and the collagenase MMP13, whose target is type II collagen, are responsible for matrix breakdown in early OA (Loeser et al., 2012). ADAMTS4 and -10, MMP1 and -8, and the gelatinase MMP2 have also been reported to be elevated in OA (Hassanali and Oyoo, 2011, Loeser et al., 2012, Murphy and Nagase, 2008).

Chondrocytes express receptors for the components of extracellular matrix. Mechanical stimulation of these receptors contributes to the synthesis and release of matrix-degrading proteinases, as well as inflammatory cytokines and chemokines, which may be produced by the synovium. Interleukins (IL) -1, 17, 18 and tumour necrosis factor (TNF)- α stimulate MMPs and decrease the activity of their inhibitors during cartilage degradation (Martel-Pelletier, 2004). The activity of MMPs is controlled by tissue inhibitors of metalloproteinases (TIMPS). TIMP1 inhibits MMP1 in normal cartilage, while TIMP3 inhibits ADAMTS4 and -5 (Davidson et al., 2006, Kashiwagi et al., 2001). An imbalance in the activity of MMPs and TIMPS may lead to increased chondropathy (Fransès et al., 2010).

Angiogenesis plays a key role in OA cartilage pathology (Ashraf et al., 2011a). In normal conditions, adult cartilage is an avascular tissue (Ashraf et al., 2011a), but in OA cartilage neovascularization occurs, probably as a result of inflammation (Suri et al., 2007). Angiogenesis can also be observed during endochondral bone development. This involves the expression of angiogenic factors, such as vascular endothelial growth factor (VEGF), which control not only angiogenesis but also chondrocyte metabolism (Murata et al., 2008).

1.1.2.2. Subchondral bone

Bone contributes significantly to the development of chondropathy. Articular cartilage and subchondral bone form a functional unit that maintain normal joint function. Disrupted balance in the mechanical or biological state of the subchondral bone may cause changes in the articular cartilage and vice versa (Goldring and Goldring, 2010b). During the course of OA, remodelling of the cortical and trabecular bone is observed. This process is initiated by osteoclast-mediated bone resorption, followed by bone formation by osteoblasts. In normal bone physiology, the processes of bone resorption and formation are balanced (Goldring and Goldring, 2010a), while OA is characterized by an increase in anabolic activity in the subchondral bone (Sharma et al., 2013).

Subchondral bone can undergo several changes in OA, including thickening of the bone plate, trabecular bone remodelling, osteophytosis, subchondral bone sclerosis or cyst formation (Goldring and Goldring, 2010a) and tidemark duplication, which results from the vascular invasion of the calcified cartilage from the subchondral bone (Walsh et al., 2007). These changes may indirectly result in joint transformations that further contribute to an adverse biomechanical environment (Burr, 2004, Bullough, 2004). The subchondral bone may be a source of pain in OA as it is richly innervated by sensory nerves (Hunter et al., 2008).

1.1.2.3. Synovium

The synovium is a specialised connective tissue lining the spaces of diarthrodial joints, tendon sheaths and bursae, where it has an immune function and plays a role in phagocytosis, lubrication and cartilage nutrition (Smith, 2011). The synovium is an important component of the knee and its inflammation contributes to cartilage degeneration (Scanzello and Goldring, 2012). Synovitis occurs early in OA, when it is characterised by macrophage infiltration (Benito et al., 2005), but the prevalence of synovitis increases with advancing OA (Krasnokutsky et al., 2011, Scanzello et al., 2011). Synovial inflammation also includes lymphocyte infiltration (Pearle et al., 2007), fibrosis, lining and villous hyperplasia, cartilage and bone detritus (Scanzello and Goldring, 2012) and increased vascularity (Ashraf et al., 2011b). Chemokines and cytokines, such as IL-1, IL-6 and TNF- α promote synovitis and are upregulated from the early stages of OA (Benito et al., 2005) and after joint injury (Furman et al., 2014). Released cytokines can inhibit matrix synthesis and promote cartilage break down (Goldring and Marcu, 2009). Phagocytosis of shed cartilage debris by synovial cells also contributes to inflammation (Nwosu, 2015) .

Synovial angiogenesis is also a feature of OA, and this process is thought to contribute to chronic synovitis (Wenham and Conaghan, 2010). One of the proposed mechanisms of synovial angiogenesis and inflammation is the production of VEGF by synovial macrophages (Haywood et al., 2003); indeed,

macrophages and mast cells, which abound in chronically inflamed synovium, have been shown to produce VEGF (Bonnet and Walsh, 2005). However, the exact regulation of angiogenesis in OA is not yet completely understood (Ashraf and Walsh, 2008).

Figure 1.3 summarises the signalling pathways involved in OA pathology and the structural changes of osteoarthritic joint.

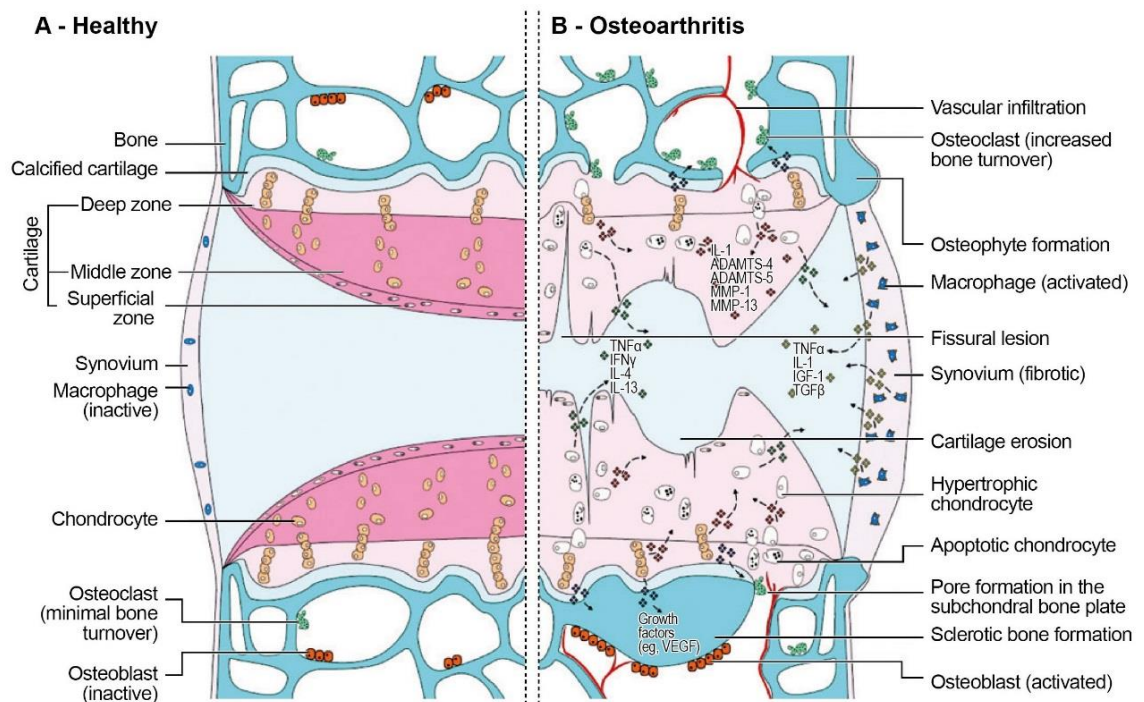


Figure 1.3. (A) Healthy joint and (B) osteoarthritic joint showing the signalling pathways involved in OA pathology and structural changes in osteoarthritic joint. (ADAMTS = a disintegrin and metalloproteinase with a thrombospondin-like motifs; IL = interleukin; MMP = matrix metalloproteinase; TNF = tumour necrosis factor; IFN = interferon; IGF = insulin-like growth factor; TGF = transforming growth factor; VEGF = vascular endothelial growth factor). *Picture reproduced from (Glyn-Jones et al., 2015).*

1.1.3. Signs and symptoms

OA occurs mainly in the large weight bearing joints, such as knees, hip and lower spine, as well as in hands and feet (Ashford and Williard, 2014). OA can be classified as 1) primary (idiopathic), which affects previously healthy individuals over the age of 40 years, or 2) secondary, which is a result of joint injury.

The main manifestation of OA is joint pain, which is initially related to activity, and becomes constant later in the disease. The condition is also characterized by tenderness, crepitus, stiffness and limitation of movement, with occasional effusion and variable degrees of local inflammation (Pereira et al., 2015). Later in the disease, joint deformities, altered gait and muscle weakness or atrophy may occur. OA patients experience functional disability, which is mainly caused by pain and associated with articular limitation, stiffness and crepitus (Arden and Nevitt, 2006). Radiographic findings in OA include osteophyte formation, joint space narrowing, subchondral sclerosis and cysts (Goldring and Goldring, 2007). The presence of an osteophyte is the most specific radiographic marker for OA, although it indicates a relatively advanced disease.

1.1.4. Diagnosis

The diagnosis of OA is based on the observation of signs and symptoms through clinical examination and, sometimes, joint changes by imaging. Different recommendations have been proposed for the diagnosis of OA. The European League Against Rheumatism (EULAR) stated that detection of the following three symptoms and three signs is sufficient to diagnose 99% of knee OA (Altman, 2010). The symptoms are persistent knee pain, limited morning stiffness and reduced function, and the 3 signs are crepitus, restricted movement and bony enlargement (Zhang et al., 2010a). The American College of Rheumatology (ACR) describes OA based on history and physical examination of knee pain in addition to 3 of the following 6 criteria: > 50 years of age, < 30 minutes of morning stiffness, crepitus on active motion, tenderness of the bony

margins of the joint, bony enlargement, or absence of palpable warmth of synovium (Altman et al., 1986).

X-ray radiography is the simplest, least expensive and most widely used imaging technique for the diagnosis of OA (Gignac et al., 2006), although it does not detect the earliest pathological features of the disease and findings do not always complement the clinical presentation of pain (McAlindon et al., 1992).

Magnetic resonance imaging (MRI) is not routinely used to diagnose OA, but has recently gained importance in the study of OA due to its ability to image the entire knee as a whole organ (cartilage, meniscus, subarticular bone marrow, and synovium) and to provide a direct three-dimension assessment of cartilage morphology and composition (Guermazi et al., 2013). MRI can detect abnormalities to the cartilage, menisci, bone, synovium and ligaments in OA patients with no radiographic changes (Conaghan et al., 2006).

Ultrasound, computed tomography (CT) and arthroscopy can also be useful in evaluating early OA, but are rarely used for diagnosis.

1.1.5. Treatment

A definitive cure for OA has not been found yet, and the development of disease-modifying therapies for OA is considered an important unmet clinical need. Therefore, treatment of OA is currently focused on symptomatic relief of pain and inflammation, improvement of quality of life, joint function and mobility and delay of disease progression (Altman, 2010). Recommended therapy includes both non-pharmacological and pharmacological treatments (table 1.2).

Non-pharmacological treatments include physical exercise, weight loss, injury prevention, orthotic devices and patient education. These approaches, in combination with pharmacological treatments, help in managing OA (Roubille et al., 2013). Transcutaneous electrical stimulation (Zhang et al., 2008) and acupuncture (Zhang et al., 2010b) have shown efficacy in the relief of short term

pain, but the NICE guidelines recommend against the use of the latter (National Clinical Guideline, 2014).

Pharmacological treatments include:

- 1- Analgesics
 - a. Paracetamol
 - b. Opioids
 - c. Duloxetine
 - d. Capsaicin
- 2- Anti-inflammatory agents with analgesic properties
 - a. Non-selective non-steroidal anti-inflammatory drugs (NSAIDs)
 - b. COX-2 selective inhibitors
 - c. Topical NSAIDs
 - d. Intra-articular corticosteroids
- 3- Slow acting drugs for OA
 - a. Hyaluronic acid
 - b. D-glucosamine sulphate
 - c. Chondroitin sulphate
 - d. Diacerein

Paracetamol and/or topical NSAIDs are considered the first-line of treatment for mild to moderate OA pain (Zhang et al., 2010b) ahead of oral NSAIDs, COX-2 inhibitors or opioids (National Clinical Guideline, 2014). NSAIDs are one of the most used classes of drugs and are currently indicated in case of poor response to paracetamol (Zhang et al., 2008). A recent review, however, identified reduced effectiveness of paracetamol in the management of osteoarthritis compared with what was previously thought (da Costa et al., 2017), which may lead in future to an advancement of NSAIDs in the lines of OA treatment. COX-2 inhibitors are used alternatively to non-selective NSAIDs. Compared to the latter, they exhibit lower GI toxicity (Patrignani and Patrono, 2015), but have retained nephrotoxicity and have been associated with increased cardiovascular risk (McKenna et al., 2001). Tramadol and stronger

opioids are indicated for moderate to severe OA pain in patients intolerant or unresponsive to NSAIDs and paracetamol (Zhang et al., 2008). Intra-articular injection of corticosteroids is effective for the symptomatic treatment of OA patients with synovitis (Pasquali Ronchetti et al., 2001) and it is particularly recommended in patients with moderate to severe pain not responding to oral analgesic/anti-inflammatory agents and in patients with symptomatic knee OA with effusions or other physical signs of local inflammation (Zhang et al., 2010b).

Glucosamine and hyaluronic acid had showed variable degrees of efficacy in the control of pain and function improvement in knee OA (Zhang et al., 2008), and they are not included in the latest NICE guidelines for the treatment of OA (National Clinical Guideline, 2014).

Joint surgery should be considered for patients who experience joint symptoms (pain, stiffness and reduced function) that have a significant impact on their quality of life and are refractory to non-surgical treatment (National Clinical Guideline, 2014). Surgical treatment of OA may be conservative, with the damaged cartilage left in place, and radical, where the cartilage is replaced by an artificial prosthesis. The latter procedure is called joint arthroplasty. Arthrodesis is another surgical intervention in cases of OA. This procedure involves the artificial induction of joint ossification between two bones by surgery. It sacrifices the articular function of the joint and is performed on small joints, such as wrists and ankles (de l'Escalopier et al., 2016, DeHeer et al., 2012).

1.2. Pain

Pain is defined as “an unpleasant sensory or emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. *Nociception* is a physiological term and it indicates the neural processes of encoding and processing noxious stimuli (Loeser and Treede, 2008).

1.2.1. Peripheral mechanisms of nociception

Nociception originates in the periphery through the activation by noxious stimuli of specialised sensory neurons called nociceptors (Kandel et al., 1991). Nociceptors are the free endings of primary afferent A ($A\beta$ and $A\delta$) and C fibres and are distributed throughout the body (skin, viscera, muscles, joints). Nociceptors transduce mechanical, thermal and chemical stimuli into depolarizing sensory potentials and transmit the resulting nerve signals to the dorsal horn of the spinal cord, where they synapse with second-order neuronal cells. These neurons then project to centres in the brain stem or have other functions (Schaible, 2007).

A and C fibres differ in many aspects; $A\beta$ fibres are large myelinated fibres with conduction velocity of up to 75 m/s. $A\delta$ fibres are lightly myelinated, have conduction rates of 5-30 m/s, and are associated with well-localised sharp, pricking pain. C fibres are smaller in diameter, unmyelinated, conduct at rates of 0.5-2 m/s and evoke poorly-localised slow, burning pain (Kandel et al., 1991, Djouhri and Lawson, 2004).

Nociceptor transduction mechanisms depend on 1) non-selective cation channels of the transient receptor potential channel superfamily (TRP), namely TRPV1 (vanilloid or capsaicin sensitive) receptor channel for noxious heat transduction, TRPM8 (methanol-sensitive) receptor for noxious cold transduction and TRPA1 receptor for chemical nociception; 2) MeT receptors for mechanical nociception, including mechanically activated Na^+ -channels of the DEG/ENaC class [such as acid sensing (ASIC) receptor], TRPV2 and TRPA1 receptors and mechanically sensitive 'two-pore' potassium channels (K_{2P} channels); 3) K_{2P} potassium channel ionophores for thermal nociception and ASIC channels in acidic tissue conditions (Hudspith, 2016).

Transmission of action potentials in primary afferent neurons is dependent on voltage-operated sodium channel (NaV) opening. Multiple NaV isoforms exist: NaV 1.1 -1.9, of which the tetrodotoxin (TTX)-sensitive NaV 1.1, 1.6, 1.7 and the TTX-resistant NaV 1.8 and 1.9 are expressed in adult mammalian sensory neurons (Hudspith, 2016). After the generation of action potentials, resting

membrane potential and repolarization is determined by a complex array of voltage-operated potassium channels, including Kv (delayed rectifier channels) and K_{2p} channels (Hudspith, 2016).

In the dorsal horn of the spinal cord, primary afferent nociceptors synapse with second-order neurons, by means of Ca⁺² dependent release of chemical neurotransmitters, such as glutamate, substance P and calcitonin gene-related peptide (CGRP) (Ashmawi and Freire, 2016). Neuronal voltage operated Ca²⁺-channels Ca_v2.1 (P- and Q-channels) and Ca_v2.2 (N-channels) mediate the synaptic vesicle release associated with fast synaptic transmission in the dorsal horn. Ca_v3 (T-channels) modulate dorsal horn neuronal threshold excitability and contribute to low threshold exocytosis and ‘volume transmission’ of neurotransmitters and neuromodulators (Simms and Zamponi, 2014). Spinal neurons can respond exclusively to low-threshold stimuli, can be nociceptive-specific (e.g. limited to noxious stimuli from A δ or C fibers) or wide dynamic range (e.g. respond to A β , A δ and C fiber-mediated innocuous and noxious stimuli) (Almeida et al., 2004).

Projections of some spinal neurons convey nociceptive information to the brain through ascending pathways, including the spinothalamic (STT), spinoreticular spinoreticular (SRT), spinomesencephalic (SMT) and spinoparabrachial tracts (Almeida et al., 2004). Neurons in the STT end in the thalamus, where sensory perception is encoded, or ramify into brain stem. SRT neurons project into the lateral and dorsal reticular nucleus, the nucleus reticularis gigantocellularis and others, while SMT neurons end in the periaqueductal gray and other midbrain structures (Dostrovsky and Craig, 2003).

Tissue injury causes cellular disruption, local sympathetic efferent discharge and activation of macrophages, lymphocytes and mast cells, which results in the release of intracellular contents such as protein kinases, arachidonic acid and histamine (Dinakar and Stillman, 2016). This initiates a cascade of events that lead to a peripheral inflammatory response and generate a mixture of inflammatory sensitizing molecules (bradykinin, H⁺, ATP, purines, prostaglandin E₂, leukotrienes, cytokines and nerve growth factor (NGF)). The resulting effect of some, or all of these molecules on neurons is referred to as

peripheral sensitisation. NGF produced by activated macrophages binds to tropomyosin-related kinase (TrkA) receptors on peptidergic C fibers activating them. This interaction results in TRPV1 phosphorylation and development of thermal hyperalgesia. Macrophages also release interleukin-6 (IL-6), IL-1 β and tumour necrosis factor α (TNF α), which increase the local production of proalgesic agents, such as bradykinin, prostaglandins and further release of NGF by inflammatory cells. Peripheral sensitisation also induces hyperexcitability of nociceptive neurons in the central nervous system (CNS), which results in allodynia (pain in response to non-nociceptive stimulus) and hyperalgesia (increased pain sensitivity) (Hudspeth, 2016).

1.2.2. Central mechanisms of nociception

Central sensitisation refers to “increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input, including increased responsiveness due to dysfunction of endogenous pain control systems” (Loeser and Treede, 2008). This process involves both spinal and supra-spinal mechanisms.

1.2.2.1. Spinal sensitisation

Excitatory post-synaptic currents (EPSCs) in second order neurons result from Ca²⁺-mediated release of glutamate from synaptic vesicles in the dorsal horn and subsequent Na⁺ entry following activation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors. High-intensity and/or sustained C and A δ fibre activation associated with injury and inflammation depolarizes N-methyl-D-aspartate (NMDA) receptors, stimulating the release of glutamate and neurokinins, such as substance P (Murphy et al., 2012). This results in increased intraneural calcium levels due to Ca²⁺ entry through NMDA receptors and release from intraneural stores. Elevated Ca²⁺ levels activate a number of intracellular and cytosolic kinases, such as protein kinase A (PKA), protein kinase C γ (PKC γ), mitogen-activated protein kinase (MAPK) and proto-

oncogene tyrosine kinase (Src). These signalling pathways mediate changes in gene expression and modulate descending and facilitating pathways of the CNS, leading to lower excitation thresholds of spinal cord neurons, expanded receptor fields and increased excitability (Lee et al., 2011b, Kidd, 2012).

In spinal sensitisation, disinhibition of most superficial spinal dorsal horn laminae is observed, with decreased GABAergic and glycinergic transmission, in part due to apoptosis (Moore et al., 2002). In addition, increased expression of NMDA receptors and their activation lead to cytotoxicity, which results in neuronal death (Scholz et al., 2005). Wind-up, activation of collateral synapses and sprouting of A β -fibres in lamina II have been suggested as functional changes in spinal sensitisation, but these are debated (Woolf, 2011).

Among the molecules involved in spinal sensitisation, prostaglandins and cyclooxygenases are of paramount importance. These molecules are synthesized from arachidonic acid, a 20-carbon unsaturated fatty acid, through the action of prostaglandin G/H synthase, also known as cyclooxygenase (COX), and respective synthases (Ricciotti and FitzGerald, 2011) (figure 1.4). Two isoforms of COX have been identified: COX-1, which is constitutively expressed in most body tissues and controls physiological processes requiring immediate and continuous regulation; and COX-2, which is usually induced by inflammation or other stimuli, but was also found to be constitutively expressed in CNS and kidney (Narumiya, 2007, Samad et al., 2002). Different prostaglandin synthases give rise to four types of molecules: PGI₂, PGD₂, PGE₂ and PGF_{2 α} . Each type is expressed in certain tissues and exerts different functions (figure 1.4). Prostaglandins have specific receptors, which were classified in 4 types: DP, IP, FP and EP receptors, specific for PGD₂, PGI₂, PGF_{2 α} and PGE₂, respectively. EP receptors have 4 subtypes (EP1, EP2, EP3, and EP4), with various splice variants of EP3 occurring. All these receptors are G protein-coupled, rhodopsin-type receptors with seven transmembrane domains and each is encoded by a specific gene (Ricciotti and FitzGerald, 2011).

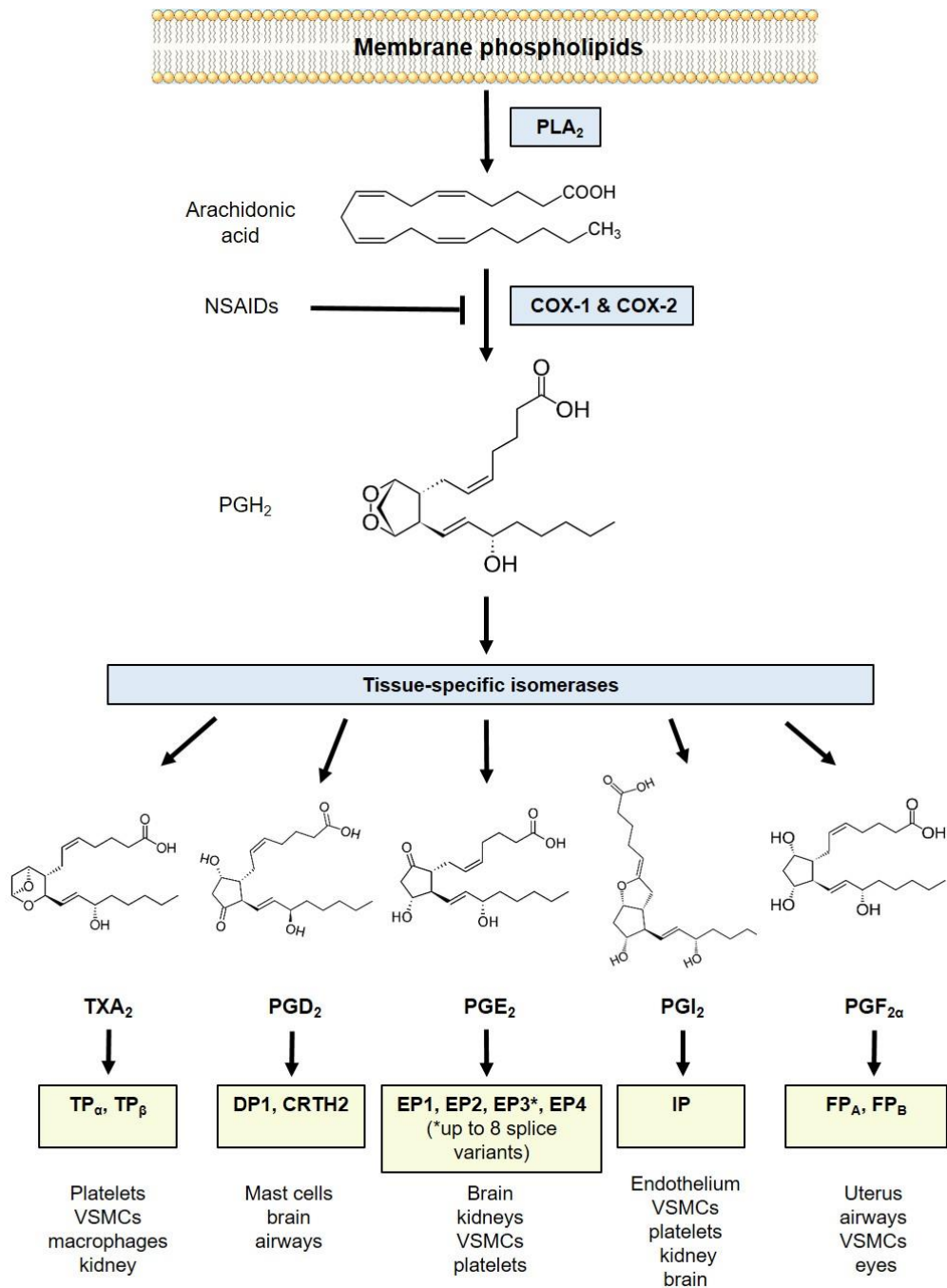


Figure 1.4. Synthetic pathway of prostanoids. (PLA₂: phospholipase A₂, COX: cyclooxygenase, PG: prostaglandin, TxA₂: thromboxane A₂, TP: thromboxane receptor, DP: PGD₂ receptor, EP: PGE₂ receptor, IP: PGI₂ receptor, FP: PGF_{2α} receptor, VSMCs: vascular smooth muscle cells). *Adapted from (Ricciotti and FitzGerald, 2011).*

COX-1 and COX-2 are constitutively expressed in the spinal cord (Willingale et al., 1997, Beiche et al., 1996), where high densities of PG receptors are found

(Matsumura et al., 1992). Peripheral inflammation, spinal injury and persistent activation of C fibres increase expression of COX-2 in the spinal cord (Samad et al., 2001, Adachi et al., 2005, Hay and de Belleruche, 1997). This enhances production of PGE₂ and PGI₂, which by binding to their receptors on primary afferents and intrinsic spinal neurons, activate cAMP and pkA pathway. As a result, TTX-R Na⁺ current and voltage-dependent Ca²⁺ inflow are enhanced and voltage dependent K⁺ current is decreased in nociceptive afferents. This leads to 1) lower firing thresholds and higher firing rate; 2) release of glutamate, substance P, CGRP (which in turn stimulate the release of PGs) and NO; 3) facilitated membrane currents and 4) enhanced release of substance P and CRGP induced by low pH, bradykinin and capsaicin (Vanegas and Schaible, 2001). In this way, spinal prostaglandins play a major role in central sensitisation and the pathogenesis of chronic pain. In addition, it was found that intrathecal administration of PGs causes thermal, mechanical and chemical hyperalgesia, while intrathecal COX inhibitors attenuated behavioural responses and spinal neuron excitability associated with peripheral inflammation (Svensson and Yaksh, 2002, Seybold et al., 2003).

It is increasingly clear now that mechanisms of central sensitisation are not limited to neuronal-neuronal interactions, but they also involve glial cells (Bradesi, 2010, Ji et al., 2013). Glial cells refer to distinct non-neuronal cell types including oligodendrocytes, microglia, astrocytes, ependymal cells, Schwann cells and satellite cells. Oligodendrocytes and Schwann cells play an important role in axon myelination in the central and peripheral nervous system, respectively (Baumann and Pham-Dinh, 2001, Jessen and Mirsky, 2005). Ependymal cells are involved in the production, secretion and circulation of cerebrospinal fluid (CSF) and formation of the blood-CSF barrier (Johansson et al., 1999). Satellite glial cells are small cells surrounding neurons in sensory, sympathetic, and parasympathetic ganglia that help regulate the external chemical environment (Ohara et al., 2008). Microglia comprehend heterogeneous cell types capable of phagocytosis that share common lineage with macrophages and represent 5-12% of all cells in the CNS. These cells exhibit mobile protrusions and several surface receptors, through which they surveil, interpret and promptly respond to changes in the surrounding

environment (Brodal, 2016). Microglia become activated in response to stimuli that endanger CNS integrity (trauma, ischemia, stress or pathogens). Astrocytes constitute 40-50% of all glial cells and they have numerous projections that link to neurons and blood vessels. They have an active role in synaptic transmission, control the external chemical environment of neurons and regulate blood flow to the brain (see section 1.6). Astrocytes are activated by the same stimuli as microglia, and by by-products released from activated microglia themselves (Bradesi, 2010). Microglia and astrocytes were historically thought to play a passive supportive role in the CNS, but were later observed to express a variety of receptors including P2X3 ATP, ionotropic and metabotropic glutamate receptors, neurokinin receptors, CB2 cannabinoid and opioid receptors (Bradesi, 2010). Evidence indicates that pathological activation of microglia and astrocytes in the spinal cord and DRGs contributes to the induction and maintenance of neuropathic and inflammatory pain states (Gosselin et al., 2010). Glial activation can present as: 1) glial reaction (upregulation of glial markers, such as IBA1 and glial fibrillary acidic protein (GFAP) and/or morphological changes); 2) phosphorylation of mitogen-activated protein kinase (MAPK) signalling pathways; 3) upregulation of ATP and chemokine receptors and downregulation of glutamate transporters; and 4) synthesis and release of glial mediators (e.g., cytokines, chemokines, growth factors, and proteases). Peripheral injuries evoke rapid reaction of satellite glial cells, followed by microglial and astrocytic reaction in the CNS. Glial cells are activated by neuronal signals involving neurotransmitters (substance P or glutamate), purinergic agents, chemokines (monocyte chemoattractant protein-1 (MCP-1) and CXCL1 (for microglia only)), and opioids (Bradesi, 2010). Activation of hemichannels (Cx43 and PNX1) and P2X7 leads to release of ATP and glutamate from astrocytes; in microglia, P2X7 and P2X4 receptors are thought to be involved in pain facilitation. Glial cells also express toll-like receptors (TLRs), which are up-regulated in neuropathic pain and, when activated, lead to the release of proinflammatory cytokines such as IL-1 β , IL-6, TNF α . As a result, glia synthesise and release proinflammatory cytokines (TNF- α , IL-1 β and IL-6), chemokines, growth factors and neuromodulators (ATP and NO), which activate key signalling pathways, such as MAPK, p38, ERK1/2, JNK or nuclear factor

kappa B (NFκB) pathways. This can modulate excitatory and inhibitory synaptic transmission and activate other glial cells (Ji et al., 2013).

The role of spinal glial cells is especially evident in neuropathic pain resulting from nerve injury. This condition is associated with a significant activation of astrocytes and microglia in the spinal cord and the release of proinflammatory mediators (Bradesi, 2010). Glial activation inhibitors, such as minocycline and fluorocitrate inhibit glial activation, prevent and/or reverse increased pain behaviour and reduce the release of proinflammatory mediators (Raghavendra et al., 2003). Similar results were observed in models of peripheral inflammation, such as subcutaneous CFA injection, or intra-articular CFA injection in a model of arthritis (Cao and Zhang, 2008), as well as OA (see section 1.3.2.2).

1.2.2.2. Supraspinal mechanisms of central sensitisation

Besides those involving spinal cord, other mechanisms at the supraspinal level have been involved in central sensitisation. These include: 1) reduced function of descending inhibitory pathways (Meeus et al., 2008) or increased function of descending facilitatory pathways (Meeus and Nijs, 2007, Staud et al., 2007), 2) increased levels of excitatory neurotransmitters (e.g. glutamate, substance P, NGF) or decreased levels of inhibitory neurotransmitters (norepinephrine and/or serotonin, GABA, cannabinoids) and 3) changes in the size or connectivity of brain structures (Seifert and Maihofner, 2009, Zhuo, 2007).

Pharmacological or anatomical disruption of descending facilitation pathways attenuates behavioural hypersensitivity associated with inflammatory, nerve injury and visceral pain, and reduces the excitability of wide dynamic range dorsal horn neurones (Rahman et al., 2009). These pathways arise mainly from the rostral ventromedial medulla (RVM), the midbrain periaqueductal gray (PAG) and the dorsal reticular nucleus (Figure 1.5).

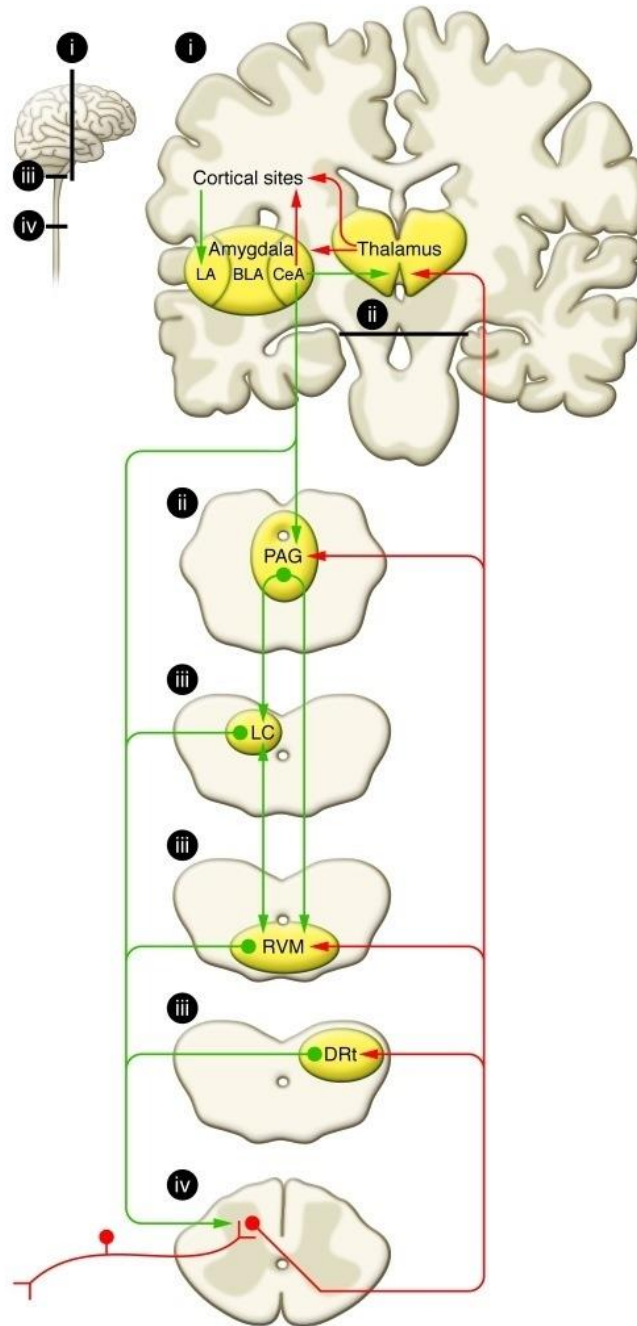


Figure 1.5. Schematic representation of the pain modulatory system. Red and green lines represent ascending and descending pathways, respectively. (DRt: dorsal reticular nucleus, RVM: rostral ventromedial medulla, LC: locus coeruleus, PAG: periaqueductal gray, LA: lateral, BLA: basolateral, CeA: central nucleus of the amygdala). *Image from (Ossipov et al, 2010).*

The RVM receives inputs from the cortex, hypothalamus, the limbic system and the PAG (Ossipov et al., 2010). Neurons in the RVM then project along the dorsolateral funiculus (DLF) to the dorsal horn of spinal cord (Ossipov et al., 2010). The involvement of RVM (and PAG) in stimulation-produced analgesia, and the recognition of these two sites in opioid-induced analgesia (Tsou and Jang, 1964) highlight their antinociceptive actions. However, the RVM has also been implicated in hyperalgesia and allodynia in inflammation and neuropathy (Porreca et al., 2002). Facilitative output from the RVM as a part of positive feedback loop has been observed in rodents in several conditions, including headache, visceral pain and post-surgical pain (Heinricher and Fields, 2003). The RVM is also involved in top-down facilitation, as seen in case of opioid withdrawal, mild or chronic stress, and sickness response to immune system activation (Heinricher and Fields, 2003). The RVM contains 3 different types of neurons: 1) ON-cells, which discharge just before withdrawal from noxious heat and facilitate nociception mechanisms at the spinal dorsal horn (Gebhart, 2004, Ossipov et al., 2010); 2) OFF-cells, which stop firing just before a withdrawal effect and contribute in descending inhibition of nociception and 3) NEUTRAL-cells, which show no difference in activity before withdrawal reflex (Fields and Heinricher, 1985). Some NEUTRAL cells contain serotonin and could therefore have pain modulatory effects (Heinricher and Fields, 2003), and they may also play a role by adopting ON-like or OFF-like functionality (Khasabov et al., 2015). RVM function depends on phasic response of cells to noxious stimuli (Mason, 2012). ON- and OFF-cells are significantly involved in the action of opioids in the PAG-RVM system (Heinricher et al., 1994). It is still not very clear what determines the inhibitory or facilitatory effect to dominate in the RVM; one hypothesis was that acute pain is associated with central inhibition, while chronic pain, such as that caused by sustained inflammation, activates brain facilitatory mechanisms (Vanegas and Schaible, 2004). Other researchers suggested that spinal cord could have different neuronal pools for different sources of input in the body (Vanegas, 2004). In models of inflammation, descending inhibition outweighs facilitation in pain circuits with input from the inflamed tissue, thus attenuating primary hyperalgesia, while facilitation predominates over inhibition in circuits originating from neighbour tissues, and

thus facilitates secondary hyperalgesia (Vanegas and Schaible, 2004).

Descending modulation also involves other sites in the brain, such as the amygdala, and different neurotransmitters, such as serotonin and noradrenaline (Ossipov et al., 2010).

1.2.2.3. The periaqueductal gray (PAG)

The PAG is the interface between the forebrain and the lower brainstem and it participates in integrated autonomic and somatic responses to stress, pain modulation, and other adaptive functions (Cersosimo and Benarroch, 2013). The PAG has roles in thermoregulation (de Menezes et al., 2009), cardiovascular responses associated with pain modulation (Heinricher et al., 2009), micturition reflex (Holstege, 2005), respiratory rhythm and airway resistance (Haxhiu et al., 2002, Zhang et al., 2007, Subramanian et al., 2008), arousal (Lu et al., 2006), and regulation of REM sleep (Fuller et al., 2007).

The PAG receives direct nociceptive inputs from collateral ascending projection fibers, as well as projections from the central nucleus of the amygdala (CeA), medial prefrontal cortex, hypothalamus and RVM (Ossipov et al., 2010, Heinricher and Fields, 2003). A major output from PAG is to the RVM. Indeed, the PAG is a vital part of the system that modulates pain transmission at the spinal cord level through a relay in the RVM (Fields and Heinricher, 1985). This connection is considered critical, since PAG output to the spinal cord is minimal. The PAG is functionally divided into distinct longitudinal columns (dorsolateral, lateral, dorsomedial, ventrolateral and ventromedial; figure 1.6) that have different cortical and brainstem connections, coordinate different patterns of behaviours (Bandler et al., 2000) and exhibit different sensitivity to the action of analgesics (Bandler and Keay, 1996, Li and Mitchell, 2003). The ventrolateral (VL) PAG is sensitive to opioids and is considered a site for the antinociceptive effects of NSAIDs (Leith et al., 2007). PGE₂ receptor 1 (EP1) in the VL PAG facilitates thermal nociception in normal and neuropathic pain (Palazzo et al., 2011).

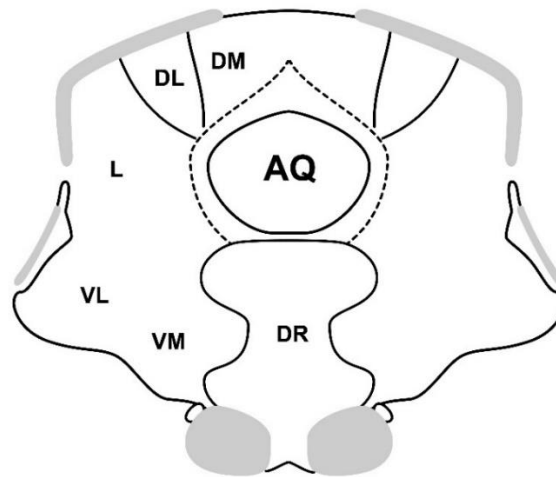


Figure 1.6. Representative image of a coronal section of the PAG showing the functional columns. (DL, dorsolateral; L, lateral; DM, dorsomedial; VL, ventrolateral; VM, ventromedial; AQ, cerebral aqueduct; and DR, dorsal nucleus raphe). *Reproduced from (Li and Mitchell, 2003).*

The discovery of a “pain-modulating system” firstly occurred with the observation of stimulation-produced analgesia upon electrical stimulation of rat PAG (Reynolds, 1969). This stimulation inhibits simple noxious stimulus-evoked reflexes organized mainly at the spinal level and is selective to nociceptive neurons in the dorsal horn of spinal cord (Waters and Lumb, 1997). This effect was reversed by naloxone, indicating the involvement of an endogenous opioidergic inhibitory system (Hosobuchi et al., 1977). Microinjection of morphine into rat PAG also produced an antinociceptive effect (Tsou and Jang, 1964). It was also found that the administration of an opioid systemically or locally into PAG activates OFF-cells in RVM, producing analgesia (Jensen and Yaksh, 1989, Cheng et al., 1986). Other studies showed inhibitory projections in the spinal cord originating from or travelling through PAG (Lewis and Gebhart, 1977a, Lewis and Gebhart, 1977b, Yaksh and Rudy, 1978); other neuronal outputs from PAG directly or indirectly reach the RVM. Inactivation of RVM by injection of local anaesthetics blocked stimulation-induced inhibition from the PAG (Gebhart, 2004). Interestingly, the PAG-RVM system exerts its inhibitory effect on nociceptive inputs mediated by C fibres,

while it retains sensory information transmitted by A fibers (Heinricher et al., 2009).

Facilitatory functions of the PAG have been demonstrated in several studies and they were in most cases directly related to RVM activity. Microinjection of PGE₂ into the PAG activated RVM ON-cells, suppressed OFF-cells and produced significant hyperalgesia (Heinricher et al., 2004). COX-1 in the PAG is known to be involved in pain descending facilitation and COX-1 inhibition disrupted the neuronal encoding of C-nociceptor information without affecting the protective A-nociceptor information (Leith et al., 2014). Activation of the dorsal PAG through the glycine site at the NMDA type (GLY_B/NMDA) receptor can produce nociception facilitation after injection of formalin in the hindpaw, while hyponociception occurred if formalin was injected in the knee. This difference could depend on the degree of stress/aversion caused by the nociceptive model (Martins et al., 2010). Melanocortin 4 receptor (MC4R) in the PAG was found to contribute to pain facilitation after peripheral nerve injury by regulating glial activation and inflammatory cytokine secretion (Chu et al., 2012).

Prostaglandins exert relevant effects in the PAG, where expression of prostanoid receptors (Ek et al., 2000, Matsumura et al., 1992), as well as COX-1 and COX-2 (Breder et al., 1995). Injection of COX inhibitors in PAG and other brain sites produced anti-nociceptive effects reversible by naloxone (Björkman et al., 1992), while intraventricular administration of PGE₂ produced hyperalgesia (Oka et al., 1994). Electrophysiological experiments demonstrated that injection of PGE₂ into PAG caused hyperalgesia and strengthened the modulatory circuit in RVM (Heinricher et al., 2004). It has been demonstrated that COX-1 isoform is responsible for acute antinociceptive effects of NSAIDs in PAG and that this effect is evoked exclusively from the opioid-sensitive ventrolateral (VL) PAG (Leith et al., 2007). In addition, EP1 receptors within the VL PAG have a facilitatory effect on thermonociception in normal and neuropathic pain (Palazzo et al., 2011). The mechanisms by which PGs produce hyperalgesia in the PAG are still unclear, but one study showing formalin-induced hyperalgesia suggests increased glutamate release and biphasic changes in the release of GABA.

Involvement of TTX-R Na⁺ channels, substance P and bradykinin has also been proposed. (Oliva et al., 2006).

PAG is also significantly involved in other modalities of pain modulation, including serotonergic and noradrenergic pathways (Ossipov et al., 2010).

1.3. Pain in OA

Pain is the major manifestation of OA and it significantly impacts the function and quality of life of affected patients. The safe and effective treatment of OA pain is still suboptimal (Adatia et al., 2012).

Both peripheral and central mechanisms contribute to OA pain. Events in peripheral tissues generate nociceptive signals, which are further modulated through the activation of central pain-processing pathways (Sofat et al., 2011). Nociceptive input in OA may arise from the synovium, bone marrow, soft tissues and even cartilage in the advanced stages of the disease (Walsh et al., 2010). Pain severity in OA correlates poorly with the extent of cartilage damage, and it persists in some patients after joint replacement (Kidd, 2012), suggesting a significant role for pain modulation at the central level. The mechanisms of this modulation include central sensitization at the spinal level, activity of discrete areas of the brain, and cortical sensitization (Kidd, 2012).

1.3.1. Peripheral mechanisms of pain in OA

A large body of evidence on peripheral sensitisation in OA was obtained from experimental models, using pain behavioural tests (Syx et al., 2018), in vivo electrophysiology studies (Schuelert et al., 2010) and calcium imaging techniques (Kim et al., 2016).

Sensitisation of afferent neurons in OA is mediated by a number of molecules, such as NGF, cytokines, chemokines and neuropeptides, and it involves low-grade inflammation and innate immunity, immune cell infiltration and activation and damage-associated molecular patterns.

NGF plays a significant role in nociceptor sensitization after tissue injury, mainly through the TrkA receptor. In rat models of OA, administration of NGF into the knee caused prolonged weight-bearing asymmetry compared to NGF injection in non-OA knees (Ashraf et al., 2013), and pain behaviours coincided with NGF upregulation (Driscoll et al., 2016, McNamee et al., 2010, Orita et al., 2011). Subcutaneous or intramuscular injection of NGF induces local hyperalgesia in humans (Petty et al., 1994, Deising et al., 2012). Clinically, NGF levels are increased in synovial fluid of OA patients (Aloe et al., 1992) and synovial NGF expression is associated with pain (Stoppiello et al., 2014).

Low-grade inflammation contributes to OA pathogenesis and pain generation (Syx et al., 2018). Nociceptors express receptors for a number of inflammatory mediators that excite and sensitise them (Ji et al., 2014). Joint trauma, such as in OA (Miller et al., 2015, Agalave and Svensson, 2015), results in the release of damage-associated molecular pattern proteins (DAMPs), such as fibronectin, alarmins and α 2-microglobulin, which interact with pattern recognition receptors (PRRs) and activate the innate immune system. This results in low-grade inflammation through the release of a large number of pro-inflammatory cytokines and chemokines, including IL-1 β , IL-6 and TNF- α (Goldring and Otero, 2011). Several studies have demonstrated the contribution of these molecules to pain generation and nociceptor sensitisation in OA (Miller et al., 2012, Scanzello et al., 2011, Nair et al., 2015, Tran et al., 2017). Recently, infiltration of macrophages, mast cells and T lymphocytes into the synovium and infrapatellar fat pad has been reported (Syx et al., 2018). These inflammatory cells are involved in synovitis and promotion of joint damage, but little is known about their role in OA pain (Scanzello and Goldring, 2012).

The neuropeptide CGRP is implicated in the peripheral mechanisms of OA pain. It is released from the peripheral terminals of nociceptors and it acts on vascular cells, among others, to produce vasodilation and neurogenic inflammation, and on peripheral terminals of sensory neurons, causing sensitisation of joint afferents (Walsh et al., 2015). Synovial expression of CGRP was associated with pain in knee OA patients (Takano et al., 2017).

Changes in the innervation of OA joint are also thought to contribute to OA pain. Both a decrease in synovial sensory innervation (Murakami et al., 2015) and ectopic sprouting of sensory and sympathetic nerves in arthritic joint (Jimenez-Andrade and Mantyh, 2012) were reported. In addition, both human and experimental OA joints exhibited vascular channels breaching the osteochondral junction (Suri et al., 2007, Mapp et al., 2013). These channels may contain sensory neurons and may therefore constitute a potential source of peripheral sensitization and pain (Suri et al., 2007). Vascular penetration and nerve growth have been observed in the human meniscus (Ashraf et al., 2011b), osteophytes, and subchondral bone (Mapp and Walsh, 2012).

In addition to nociceptive pain and peripheral sensitization, more evidence has now been gained about the neuropathic component of OA pain (Dimitroulas et al., 2014). Neuropathic pain is defined as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory nervous system” (Loeser and Treede, 2008). Numerous studies have detected neuropathic components in OA pain (Chappell et al., 2011, Duarte et al., 2014, Harvey and Dickenson, 2009, Hawker et al., 2008, Hochman et al., 2010, Im et al., 2010, Ivanavicius et al., 2007). Development of neuropathic pain in OA involves the generation of ectopic impulses due to increased expression of voltage-gated sodium channels (Koltzenburg and Scadding, 2001) and cross excitation (Bridges et al., 2001). Nerve injury results in the release of abundant immune and inflammatory mediators, which cause nociceptor sensitisation and diminished number and function of GABA receptors, reducing dorsal horn inhibition (Moore et al., 2002). A number of nociceptive TRP channels, including TRPV1 and TRPA1, have been implicated in the development of neuropathic pain (Cortright et al., 2007). Ectopic impulses can also be generated from affected neurons in the central nervous system, which then develops central sensitisation as explained above (section 1.2.2). Non-neuronal cells, like glia and immune cells, have a significant role in neuropathic pain development. In peripheral neuropathies, for example, signals released by damaged axons and Schwann cells cause neutrophils, lymphocytes and macrophages to move to the site of injury and to the distal end of the affected nerve. Immune cells also infiltrate the DRG and spinal glial cells proliferate and develop morphological changes. This

microgliosis may also extend to higher centres like the brainstem and the thalamus (Calvo and Bennett, 2012).

1.3.2. Central mechanisms of pain in OA

A number of clinical presentations in OA patients indicate the presence of a central modulation of pain (Lluch et al., 2014). For example, the severity of pain is poorly correlated with the degree of joint damage or inflammation, and pain does not always resolve after total joint replacement. In addition, patients develop increased sensitivity to noxious and non-noxious stimuli in tissues away from the affected joints (Lluch et al., 2014) and exhibit lower pressure pain thresholds in the leg and higher pain summation scores upon repetitive stimulation of the OA knee (Schaible, 2012). OA patients often complain of fatigue, sleep disturbance and cognitive difficulties (Clauw and Hassett, 2017).

As explained in section 1.2.2, central sensitisation results from sustained peripheral nociceptive input, which increases neuronal sensitivity at the spinal or supra-spinal levels in a host of complex mechanisms (Murphy et al., 2012). Studies on experimental models of OA revealed similar changes, including enhanced spinal responses to paw mechanical stimulation and increased levels of substance P and CGRP in the spinal cord (Zhang et al., 2013). Intra-articular MIA (monosodium iodoacetate) injection resulted in increased production of spinal pro-inflammatory cytokines and chemokines, such as IL-1 α/β , IL-13, IL-17, TNF- α , L-selectin, TIMP-1 and VEGF (Zhang et al., 2013). In the destabilization of the medial meniscus (DMM) model of OA, it was demonstrated that DRG MCP-1 and its receptor, CCR2, participate in the development and maintenance of mechanical allodynia (Zhang et al., 2013). In the MIA model of OA, spinal TRPV1 activity is enhanced and TRPV1 antagonists decrease glutamate and CGRP release in the spinal cord, alleviate pain behaviour and block thermal hypersensitivity (Zhang et al., 2013). In addition, increased expression of activating transcription factor-3 (ATF3) in DRG neurons (Orita et al., 2011) and activation of spinal glial cells (Sagar et al., 2011), typical of neuropathic pain, were observed. Spinal sensitization is often

counteracted by local and descending inhibitory mechanisms, including the endocannabinoids, which provide tonic spinal inhibition in OA (Sagar et al., 2010).

At the supraspinal level, serotonergic facilitation is one of the mechanisms that contribute to OA pain. Spinal serotonin (5-HT) is supraspinally produced (largely in the RVM) and in MIA rats mechanical and thermal stimulation-evoked responses are facilitated by spinal pronociceptive 5-HT₃ receptors (Rahman et al., 2009). In the same model, alterations in the noradrenergic descending inhibitory pathway were observed (Burnham and Dickenson, 2013). RVM lidocaine blocks MIA-induced ongoing pain (Havelin et al., 2016), again implying a facilitatory action from RVM.

Imaging studies in humans have recognised a number of areas in the brain involved in the generation of pain. This “pain matrix” was found to be activated during arthritis and includes the thalamus, the putamen, the PAG, amygdala and secondary somatosensory, insular and cingulate cortices (Sofat et al., 2011). An increase in supraspinal functional connectivity between nucleus accumbens and ventral posterior lateral thalamus, both involved in pain processing, was detected in the medial meniscal tear (MMT) model of OA (Upadhyay et al., 2013). Interestingly, a decrease in brain gray matter, which is reversed after arthroplasty, was observed in OA patients (Perrot, 2015).

1.3.2.1. The periaqueductal gray

Studies utilizing functional magnetic resonance imaging (fMRI) showed enhanced activity of the PAG and changes in PAG connectivity with brain cortex and hippocampus, which are associated with chronic pain in OA patients (Gwilym et al., 2009, Egorova et al., 2015, Howard et al., 2012).

MIA rats that received intra-articular capsaicin injection exhibited significant activation of the periaqueductal gray (Abaei et al., 2016), and periaqueductal gray metabolism was significantly increased in OA cats compared with non-OA cats (Guillot et al., 2015).

Several experimental studies addressed the role of PAG in inflammatory arthritis (see section 1.2.2.3), but very few have been performed on animal models of OA (Yang et al., 2005).

1.3.2.2. Glial cells

Spinal glial cells are involved in the establishment of chronic pain in neuropathic and inflammatory conditions (Gosselin et al., 2010). The same effects were observed in models of OA pain. A microglial response was detected 7 days after injection of MIA in mouse (Ogbonna et al., 2013) and after 8 weeks in mouse DMM model (Tran et al., 2017). In rats, spinal microglia showed significant hyperactivity between days 7-28 post-MIA injection (Orita et al., 2011, Sagar et al., 2011), while astrogliosis was observed at day 28. Glial activation inhibitors minocycline and nimesulide attenuated pain behaviour and glial activation in the ipsilateral spinal cord (Sagar et al., 2011). In addition, MIA rats showed a rapid activation of p38 MAPK, which was also expressed by microglia, bilaterally in the dorsal horn (Lee et al., 2011a). In the collagenase model of OA, rats developed movement-evoked nociception and activation of glial cells in both the DRG and the spinal cord six weeks after model induction; inhibition of glial cell activation by fluorocitrate reversed these OA-associated pain behaviours (Adaes et al., 2017). Li et al found that miR-146a, a microRNA that modulates Toll-like receptor (TLR) and NF κ B-dependent inflammatory responses, regulates expression of pain-related molecules (COX-2, TRPV1, TNF- α , IL-6, IL-8) in microglia and astrocytes in the MIA model of OA (Li et al., 2011).

1.4. Animal models of osteoarthritis

Preclinical animal models are essential tools for the study of OA onset and progression, and for the evaluation of novel therapeutic interventions. Animal models are induced by different stimuli and therefore exhibit several differences in the pathology, progression and presentation of the disease (McCoy, 2015). OA is a heterogeneous disease in humans, which no single animal model can

completely reproduce; however, each model can be useful to comprehend particular pathways (van den Berg, 2009).

When choosing an animal model of OA, the following criteria should be taken into account: (a) efficiency for therapeutic prediction in humans, (b) similarity to human disease in all tissues of the joint, (c) an inexpensive, easy to use and house mammalian species, (d) reproducible disease that develops in a suitable time frame to allow for productive studies and for reproducibility of data and, (e) reasonable test duration period, to allow for the study of early, mid and late pathophysiology and treatment effects (Bendele, 2001, Little and Smith, 2008).

Generally, the models used to study the pathophysiology of OA and mechanisms of joint damage are distinct from those dedicated to the study of OA pain (Little and Zaki, 2012). The latter include: 1) spontaneous (age- or obesity- associated), 2) surgically induced instability (such as anterior cruciate ligament transection [ACLT], medial meniscal tear (MNX) and medial meniscal tear [MMT] in rats, among others), 3) intra-articular injection of carrageenan, complete Freund's adjuvant, urate crystals or the most commonly used MIA, 4) enzymatic instability by collagenase, and 5) genetic modifications (IL1B over expression and Col9a1^{-/-}) (Bendele, 2001, Mogil, 2009, Little and Zaki, 2012).

Spontaneous models of OA are better than surgical or chemical models in reproducing the causes, histopathological features, and slow progression of human disease (Poole et al., 2010). However, they also show significant variability in the onset and the rate and extent of structural progression between animals. This leads to heterogeneous populations, the need for larger experimental group sizes, and difficult recruitment of appropriate age-matched, non-arthritic controls. Therefore, induced models of OA pain which share pathological features with human OA can be used instead (Sagar et al., 2013).

1.4.1. The MIA model of OA

MIA is an inhibitor of the enzyme glyceraldehyde-3-phosphate dehydrogenase. It inhibits glycolysis in chondrocytes, leading to their death (Little and Smith,

2008). Intra-articular injection of MIA results in the destruction of cartilage, formation of chondrophytes and osteophytes, progressive proteoglycan loss and atrophy of collagenous matrix. At later stages of the disease, cartilage fibrillation and resorption and subchondral bone degradation occur.

Different degrees of chondrocyte killing and thus cartilage destruction are obtained depending on the concentration of MIA used. Two intra-articular injections of 0.3 mg MIA at 24 hour intervals caused the death of all chondrocytes in the tibial plateaux and femoral condyles (Bendele, 2001)

Although the MIA model is widely used, its validity as a clinical model for OA has been questioned (Poole et al., 2010, Teeple et al., 2013). The histopathology of the degenerating joint is similar to that in the human disease (Combe et al., 2004), but MIA causes widespread cell death and rapid joint destruction, which are not typically observed in spontaneous or post-traumatic OA (Little and Hunter, 2013). On the other hand, this model can be easily and inexpensively induced in small animals and it produces pain behaviour in a rapid and reproducible way, which makes it useful for the study of OA related pain and analgesic screening (Fernihough et al., 2004, Combe et al., 2004, Gregory et al., 2012).

1.4.2. The collagenase model of OA

Intra-articular injection of collagenase in mice has been used since 1990 as a model of OA structural change (van der Kraan et al., 1990). Collagenase digests collagen type I in the extracellular matrix of cartilage and in tendons, ligaments and menisci. This produces osteoarthritic lesions within a few weeks (severe cartilage lesions, patellar dislocation, sclerosis of subchondral bone and osteophyte formation) and consequent deformity of the knee joints (van der Kraan et al., 1990). A correlation between the degree of instability of the joint and the amount of cartilage damage was demonstrated (Smith et al., 2007).

This model is widely used to study OA pathophysiology, since it produces histopathological changes similar to those found in human, and because it

mimics the slow and continuous progress of the disease in human (Adães et al., 2014). On the other hand, it was also found to induce a significant acute inflammation at the site of injection and to produce a greater level of inflammation than the one observed in human OA (Smith et al., 2007). The collagenase model has recently been evaluated as a model of pain in OA (Adães et al., 2014). Animals injected with collagenase showed increased expression of TRPV1 in DRG and increased nociceptive behaviour, which was reversed by subcutaneous morphine and intra-articular lidocaine. These results support the potential for collagenase injection as a model for OA pain. Indeed, the collagenase model had been utilized for the evaluation of OA pain in the past (Anderson et al., 2010), but the pain mechanisms involved in this model were not fully elucidated, especially at the central level.

1.5. Measurements of pain behaviour

Pain is the key symptom of OA; therefore, assessment and quantification of pain in animal models is essential to investigate OA mechanisms and evaluate the effects of potential analgesics. A number of behavioural tests have been developed for the assessment of pain in rodent models of osteoarthritis. These include; weight bearing by the affected foot, paw withdrawal threshold to punctuate stimulation (indicating distal mechanical allodynia), paw withdrawal latency to heat stimuli (secondary thermal hyperalgesia), foot position and gait analysis, paw elevation time, and locomotor activity scoring either subjectively or by computer (Yu et al., 2002, Min et al., 2001, Tonussi and Ferreira, 1992, Malfait et al., 2013).

Weight bearing asymmetry in rodents is considered a correlate of standing pain in patients and it is caused by the tendency to move away from or put less weight on the affected joint (Christiansen and Stevens-Lapsley, 2010), while mechanical withdrawal thresholds represent diffuse or referred pain (secondary hypersensitivity) (Arendt-Nielsen et al., 2010). Hindpaw thermal latency to either radiant or conductive thermal stimuli describes secondary thermal hyperalgesia (Banik and Kabadi, 2013). Weight bearing alteration is both

peripherally and centrally driven, while hindpaw withdrawal to mechanical and thermal stimuli is provoked by a peripheral stimulus but has a predominant central component (Graven-Nielsen and Arendt-Nielsen, 2002, Arendt-Nielsen et al., 2010). Both parameters can be easily measured in small animals to provide reliable indications of joint discomfort (Bove et al., 2003) and secondary allodynia (Sagar et al., 2010), respectively.

1.6. Astrocyte-vascular interactions

1.6.1. Astrocytes and brain vascular system

There is a close connection between astrocytes and blood vessels in the brain. Astrocytes are a major cell type in blood-brain barrier (BBB); in their mature form, they modulate and maintain BBB once it is formed (Daneman and Prat, 2015). Astrocyte end feet, which consist of perivascular processes, completely enclose cerebral blood vessels, producing multiple bidirectional interactions. These processes express several receptors and channels (e.g., potassium channels, aquaporin 4, and glucose transporters) (Tanigami et al., 2012). Astrocytes provide a cellular link between the neuronal circuitry and blood vessels, which regulates blood flow in response to neuronal activity through contraction and dilation of arteriole smooth muscles (Gordon et al., 2007). This neurovascular coupling is thought to be mediated by a number of mechanisms involving glial cells. Increased intracellular Ca^{2+} in astrocytes induces the production of arachidonic acid, which can be then converted to 20-hydroxyeicosatetraenoic acid (20-HETE), causing vasoconstriction, or to PGE_2 or epoxyeicosatrienoic acid (EET), leading to dilation. The level of NO determines which of these patterns is recruited (Zonta et al., 2003). Activation of K^+ channels in astrocyte end feet and K^+ release into blood vessels modify vascular tone by hyperpolarization and relaxation of smooth muscle cell (Iadecola and Nedergaard, 2007). Nitric oxide (NO) is also released by astrocytes to control vessel constriction and blood flow (Iadecola, 1993). In pathological conditions, astrocyte dysfunction can compromise the regulation of blood flow (MacVicar and Newman, 2015).

Aberrant astrocyte function in certain pathologies reduces their ability to maintain the homeostasis and integrity of neurovascular units, impairing critical neuronal functions. These conditions include hepatic neuropathy, which is considered a primary astrocytopathy, and where excessive ammonia can pass the BBB and accumulate in astrocytes (Tanigami et al., 2012). Disruption of the neurovascular unit has also been observed in stroke, brain trauma (Lok et al., 2015), hypertension, Alzheimer's disease (Lecrux and Hamel, 2011), infection and inflammation (Radu et al., 2013). The coupling between the capillary cell membrane and the surrounding glial end feet is reduced in pathological conditions, such as major depressive disorder, where a significant reduction in the coverage of gray matter vessels by astrocyte processes was also observed (Rajkowska and Stockmeier, 2013). Altered neuron-glia-vascular communication is involved in cognitive decline in Alzheimer's disease (Rossi, 2015).

1.6.2. Glial-vascular interactions and pain

Glial-vascular interactions also play a key role in pain. Stimulation of astrocytes cocultured with brain endothelial cells (ECs) with 5-hydroxytryptamine (5-HT) induces intracellular Ca^{2+} release in the presence of inflammatory or pain-mediating activators, such as substance P, CGRP, lipopolysaccharide (LPS) and leptin; mu-opioid agonists inhibit augmented intracellular Ca^{2+} responses in astrocytes activated by inflammation and cocultured with brain ECs (Hansson et al., 2008). Overexpression of the vasoconstricting peptide endothelin-1 in astrocytes improves inflammatory pain response after hindpaw formalin injection (Hung et al., 2012). Recent findings indicate that microglial $\text{TNF-}\alpha$ stimulates glio-vascular activation in the spinal cord, which results in the expression of endothelial PGI_2 synthase and COX-2, contributing to the generation of neuropathic pain (Kanda et al., 2017). In the CFA model of peripheral inflammation, a significant increase in the number of astrocyte end feet wrapping blood vessels was observed in the spinal cord. This suggests an astrocyte-mediated mechanism for microvessel activation (Beazley-Long et al., 2018).

Models of pain have shown BBB disruption or change in permeability in the spinal cord (neuropathic pain) and brain (inflammatory pain), which were, in some cases, associated with glial activation (Radu et al., 2013). In a carrageenan model of inflammatory pain, concomitant BBB permeability and glial activation was observed in the frontal and parietal cortices and in thalamus (Huber et al., 2006). Glial cells, especially astrocytes, are essential for maintaining the BBB; therefore, glial-endothelial interactions are hypothesised to play a relevant role in the development of chronic neuropathic and inflammatory pain (Willis et al., 2008).

1.7. VEGF

1.7.1. Overview

Vascular endothelial growth factor (VEGF) is a key signal protein involved in vasculogenesis (the *de novo* formation of the embryonic vascular system) and in both physiological and pathological angiogenesis (the growth of blood vessels from pre-existing vasculature). VEGF, a 45 kD heparin-binding homodimeric glycoprotein, was identified while investigating potential factors affecting tumour growth (Ferrara and Henzel, 1989, Senger et al., 1983, Keck et al., 1989). VEGF in humans includes five families of proteins: 1) VEGF-A, usually referred to as VEGF 2) placenta growth factor (PlGF), which has a role in vasculogenesis, 3) VEGF-B, involved in embryonic angiogenesis, 4) VEGF-C and VEGF-D, which regulate lymphatic angiogenesis (Ferrara et al., 2003).

VEGF exerts several functions, mainly on vascular endothelial cells (EC), although mitogenic effects of VEGF have been observed on non-ECs, such as retinal pigment epithelial cells, pancreatic duct cells, alveolar type II cells, neurons (Oosthuyse et al., 2001), astroglia and Schwann cells (Krum et al., 2002, Silverman et al., 1999, Mani et al., 2005). VEGF induces a potent angiogenic response in several *in vivo* models and it is considered a survival factor for ECs, mainly by prevention of apoptosis. VEGF is also known as vascular permeability factor; it can enhance vascular permeability and induce vascular

leakage, a process involved in inflammation and other pathological circumstances. VEGF also affects bone marrow-derived cells (monocytes, macrophages, dendritic cells) and haematopoiesis (Ferrara et al., 2003).

VEGF plays a central role in several pathologies. In many human tumours, VEGF mRNA and protein is upregulated (Dvorak et al., 1995). Antibodies to VEGF and other anti-VEGF molecules exert a potent inhibitory effect on the growth of several tumour cell lines in vitro and in vivo (Ferrara et al., 2003). Combination of anti-VEGF treatments with chemotherapy (Klement et al., 2000) or radiation therapy (Lee et al., 2000) produces greater antitumor effect than either treatment alone.

VEGF was also found to be involved in the pathophysiology of retinal diseases, such as exudative age-associated macular degeneration (ARMD) and diabetic macular oedema, in which angiogenesis and inflammation act interdependently with the critical role of VEGF (Ajlan et al., 2016). In animal models of diabetic nephropathy, VEGF is upregulated in the kidney, it can reproduce some aspects of diabetic glomerulopathy, and VEGF antagonists attenuate diabetic albuminuria (Chen and Ziyadeh, 2008). Advanced heart failure exhibits dysfunctional blood vessel formation, in which VEGF is the cornerstone cytokine (Taimeh et al., 2013). Diabetic neuropathy and OA also involve VEGF in their pathological processes (see below).

VEGF induces cellular responses by binding to two related receptor tyrosine kinases (RTKs), known as VEGFR1 and VEGFR2. These receptors have an extracellular domain consisting of seven immunoglobulin-like domains, a single transmembrane spanning region, and an intracellular portion containing a split tyrosine-kinase domain. VEGFR2 mediates most of known cellular responses of VEGF, including mitogenic, angiogenic and permeability-enhancing effects. (Ferrara et al., 2003). In endothelial cells, VEGFR2 undergoes dimerization and causes ligand-dependent tyrosine phosphorylation of several proteins, including phospholipase C- γ , PI-3 kinase, Ras GTPase-activating protein and the Src family (Ferrara, 2004). The function of VEGFR1 is still under debate, but it is thought to modulate VEGFR2 signalling or to act as a “decoy” receptor, which prevents VEGF binding to VEGFR2. Under certain circumstances, it may also

generate mitogenic signals (Maru et al., 1998). VEGF also interacts with a family of co-receptors, the neuropilins, NRP1 and NRP2. NRP1 is thought to enhance the effectiveness of VEGFR2-mediated signal transduction of VEGF₁₆₅ (Soker et al., 1998).

VEGF and its receptors exert major functions in nociception. VEGF and VEGFR2 are involved in the pathogenesis of neuropathic pain, primarily potentiating pain responses mediated by P2X_{2/3} receptor on DRG neurons. VEGF antagonism in models of neuropathic pain may reduce chronic pain by decreasing the expressions of VEGFR2 and P2X_{2/3} receptors on DRG neurons, which leads to inhibition of neuropathic pain signalling (Lin et al., 2010b). In a model of inflammatory arthritis, a new VEGFR2-mediated spinal cord gliovascular mechanism of chronic pain was identified (Beazley-Long et al., 2018). Briefly, gliovascular activation in the spinal cord enhances transendothelial migration of pro-nociceptive immune cells into neural parenchyma and contributes to central sensitization and the spread of inflammatory pain. This mechanism was proved to be mediated by VEGF-A through VEGFR2 (Beazley-Long et al., 2018). In human cancer and mouse models, tumour derived VEGF-A augmented pain sensitivity through selective activation of VEGFR1 expressed in sensory neurons; in addition, blockade of VEGFR1 prevented tumour-induced nerve remodelling and attenuated cancer pain (Selvaraj et al., 2015).

Of particular importance is the link between VEGF and COX-2. Treatment of human umbilical vein endothelial cells (HUVEC) with VEGF increased COX-2 protein and activity (Akarasereenont et al., 2002). COX-2 expression is upregulated in several types of malignancy, inducing angiogenesis and other processes involved in tumorigenesis (Toomey et al., 2009). A correlation was found between COX-2 gene expression and angiogenesis in premalignant tissues and cancer. PGE₂ stimulates angiogenesis via the transcription factor hypoxia inducible factor-1-alpha (HIF-1 alpha), leading to the induction of VEGF. At the same time, VEGF can stimulate the expression of COX-2, showing a mutual influence of COX-2 and VEGF on each other; this indicates a positive feedback amplification mechanism (Zarghi and Arfaei, 2011). Both COX-2 and VEGF are known to be upregulated in injured spinal cord. In addition, injection of PGF₂

into injured spinal cord induced the expression of VEGF and its receptors (Skold et al., 2000).

1.7.2. VEGF isoforms

The human VEGF-A gene is organized in eight exons, separated by seven introns and it is localized in chromosome 6p21.3. Alternative exon splicing generates different isoforms, VEGF_{xxx}a (or simply VEGF_{xxx}) and VEGF_{xxx}b, where xxx is the number of amino acid residues (Harper and Bates, 2008). VEGF₁₆₅, the predominant isoform, lacks the residues encoded by exon 6 (figure 1.7).

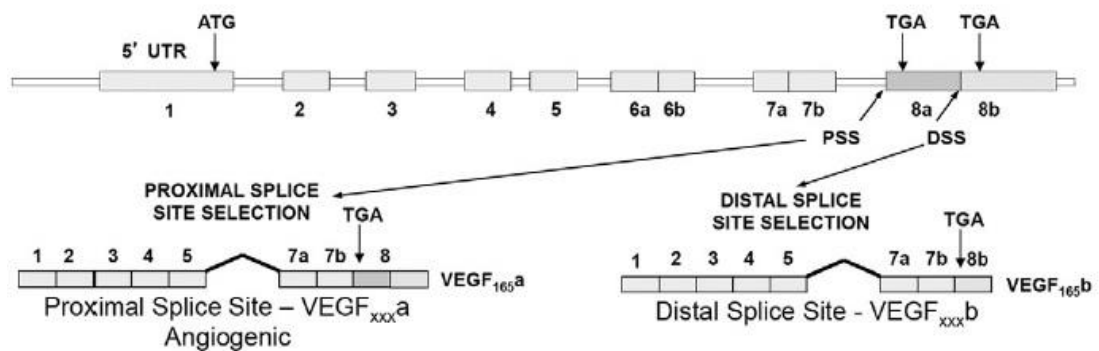


Figure 1.7. VEGF-A gene splice variant isoforms. Alternative splicing of VEGF-A pre-mRNA results in two families of mRNAs: VEGF-A_{xxx}a and VEGF-A_{xxx}b (represented in this figure by VEGF-A₁₆₅a and VEGF-A₁₆₅b). Translation of mRNAs that use proximal splice site (PSS) and include the entire exon 8 produces VEGF-A_{xxx}a proteins, while VEGF-A_{xxx}b results from mRNAs that use the distal splice site (DSS) and contain only the b part of exon 8. *Reproduced from (Hulse et al., 2014).*

The resulting splice variants exhibit different structure, receptor binding properties and physiological functions. VEGF-A₁₆₅a, for example, is known to be pro-angiogenic (Nowak et al., 2010) and pro-nociceptive (Hulse et al., 2016, Nesic et al., 2010), while VEGF-A₁₆₅b is anti-angiogenic (Varey et al., 2008)

and anti-nociceptive in a number of pain states (Hulse et al., 2014, Hulse et al., 2015, Beazley-Long et al., 2018).

1.7.3. VEGF in OA

VEGF is an important mediator of bone development and bone repair. In OA, increased expression of VEGF was detected in the articular cartilage, synovium, synovial fluid, subchondral bone and serum during later stages of disease. Increased synovial fluid VEGF is correlated with both OA severity and the degree of OA pain. A meta-analysis of nine genome-wide association studies found that the VEGF gene was found to be significantly correlated with OA (Hamilton et al., 2016).

Angiogenesis contributes to the progression of OA. Cartilage, usually an avascular and aneural tissue, undergoes vascular invasion with increasing OA severity (Walsh et al., 2010, Fransès et al., 2010). Indices of angiogenesis (vascular density and endothelial cell (EC) proliferation) have been found to be increased in OA synovium (Haywood et al., 2003, Walsh et al., 2007, Im et al., 2010), and enhanced vascular invasion has been observed in the meniscus (Ashraf et al., 2011b). Administration of VEGF-A into the knee and the temporomandibular joints independently caused characteristic features of OA. In the first case, bone sclerosis and synovial hyperplasia resolved upon discontinuation of VEGF injections (Ludin et al., 2013, Shen et al., 2015). A number of studies demonstrated that VEGF signalling at chondrocytes influences pro-catabolic mediators; VEGF acts directly through VEGFRs expressed by OA chondrocytes to mediate a catabolic response (MMP expression, NO production and release of IL-1 β , IL-6, and TNF- α) (Pufe et al., 2004, Blanco et al., 1995). Aberrant VEGF signalling in the OA joint may contribute to both abnormal endochondral ossification at the articular surface and subchondral bone remodelling, leading to cartilage degeneration, osteophyte formation and subchondral bone cysts and sclerosis (Hamilton et al., 2016). Osteophyte formation is well known to be stimulated by VEGF, which is

produced by hypertrophic chondrocytes and ECs, leading to vascular invasion into the cartilage matrix, and bone formation (Hashimoto et al., 2002).

VEGF also contributes to the development of inflammation in the joint, which is featured as synovitis and synovial hyperplasia. VEGF expression is increased in synovial macrophages and fibroblast-like synovial cells, which in turn release inflammatory mediators (such as IL-6, TNF- α and IL-8) when stimulated by VEGF. Inflammation also results in the sensitisation of fine unmyelinated sensory nerves, which mediate OA pain (Bonnet and Walsh, 2005). In addition, pathologic angiogenesis contributes to pain in OA promoting the growth of sensory nerves along the newly formed blood vessels in the osteochondral junction, osteophytes, synovium, and meniscus, facilitating OA pain (Walsh et al., 2007, Im et al., 2010, Ashraf et al., 2011b). This process involves increased expression of NGF (Walsh et al., 2010, McKelvey et al., 2013) and direct effects of VEGF and VEGFRs on hyperexcitability of sensory neurons (Malykhina et al., 2012, Lin et al., 2010b, Selvaraj et al., 2015). VEGF acts upon the sensory neuron by inducing sensory neuron axonal outgrowth and survival (Beazley-Long et al., 2013, Hulse et al., 2015) and the VEGF-A_{165a} isoform sensitizes them through actions on VEGFR2 and a TRPV1-dependent mechanisms (Hulse et al., 2014). VEGFR2 is expressed on myelinated A fibre and unmyelinated C fibre sensory neurons and is activated in rodent models of neuropathic pain (Hulse et al., 2016). Several lines of evidence demonstrated a significant role in pain also for VEGFR1 (Selvaraj et al., 2015). Targeting NGF and VEGF in clinical studies and OA models, respectively, led to a significant reduction in OA pain (Lane et al., 2010, Nagai et al., 2014); however, the effects of anti-VEGF antibody directed at VEGF-influenced nociceptive neuron signalling in OA pain are still unclear (Hamilton et al., 2016). Figure 1.8 summarises the pathways of VEGF-mediated pathology and pain in OA.

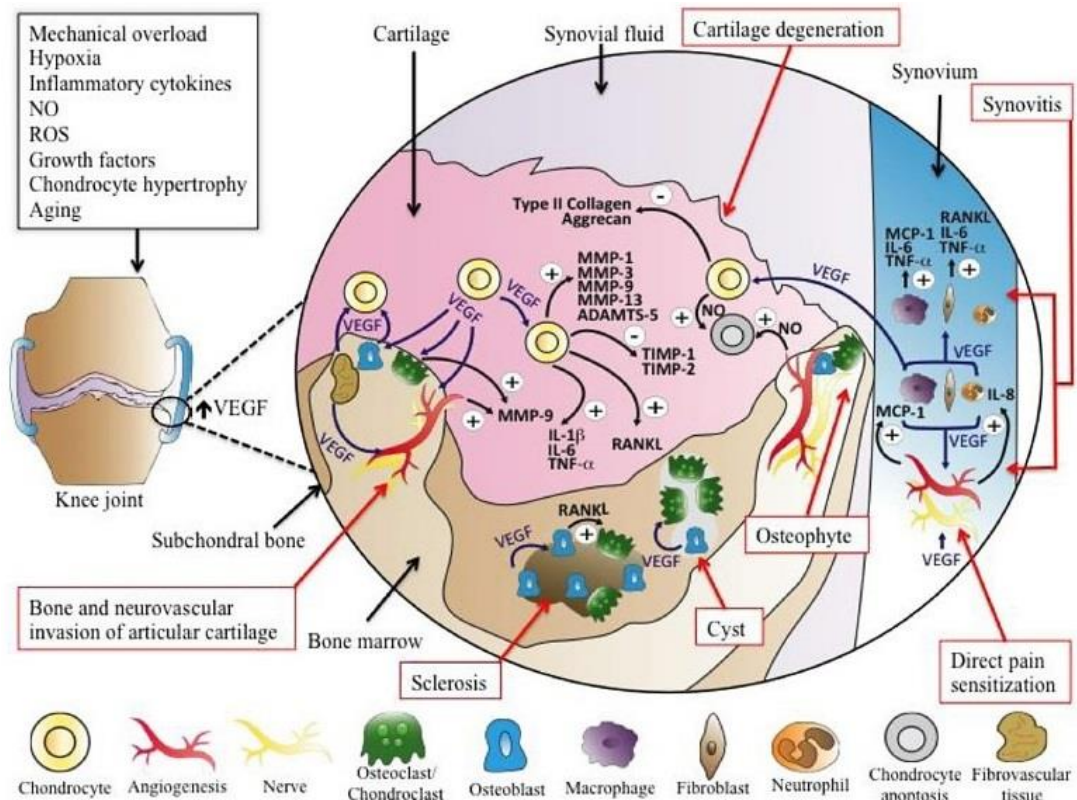


Figure 1.8. Potential pathways of VEGF-mediated OA progression and pain. (ADAMTS = A Disintegration and Metalloproteinase with Thrombospondin-like Motifs, IL = interleukin, MCP-1 = Monocyte chemoattractant protein-1, MMP = Matrix metalloproteinase, RANKL = Receptor activator of nuclear factor kappa-B ligand, TIMP = Tissue inhibitor of metalloproteinase, TNF- α = Tumour necrosis factor α , VEGF = vascular endothelial growth factor). *Reproduced from (Hamilton et al., 2016).*

1.7.4. VEGF-A_{165b}

Splicing of the VEGFA gene at the distal splice site of exon 8b results in the production of VEGF-A_{165b}. This splice variant exhibits anti-angiogenic (Bates et al., 2002, Woolard et al., 2004), neuroprotective and anti-nociceptive properties.

VEGF-A_{165b} was first recognised in renal cell carcinoma. Using reverse-transcription PCR primers that would differentiate between VEGF splice variants, a new VEGF isoform was detected whose transcription would result in a 165-amino acid peptide with an alternative terminal 6 amino acids, followed

by a stop codon. This isoform, named VEGF_{165b}, was found to inhibit VEGF₁₆₅-mediated proliferation, migration of ECs and vasodilation of mesenteric arteries (Bates et al., 2002). Woolard et al. observed that VEGF_{165b} binds to VEGFR2 with the same affinity as VEGF₁₆₅ (Woolard et al., 2004) and it activates both the receptor and different signalling pathways (Cebe Suarez et al., 2006), such as mitogen-activated protein kinase kinase MEK 1/2 (Beazley-Long et al., 2013); in addition, it prevents VEGF₁₆₅-mediated VEGFR2 phosphorylation and signalling in cultured cells. VEGF-A_{165b} was found to inhibit in vivo experimental and physiological angiogenesis and tumour growth (Woolard et al., 2004, Qiu et al., 2009, Cebe Suarez et al., 2006).

VEGF-A_{165a} isoform is neuroprotective for retinal (Brar et al., 2010), cortical (Rosenstein et al., 2003), hippocampal (Jin et al., 2000a, Jin et al., 2000b, Nicoletti et al., 2008) and DRG (Lin et al., 2010a) neurons. VEGF-A_{165b} was also found to exert cytoprotective effects on a variety of cells, including endothelial and epithelial cells of the retina (Magnussen et al., 2008) and kidney (Oltean et al., 2015) and neuronal cells. Recombinant human VEGF-A_{165b} effectively protected peripheral and central neurons in vivo and in vitro through VEGFR2, (MEK)1/2, and inhibition of caspase-3 induction (Beazley-Long et al., 2013). In diabetic neuropathic pain, VEGF-A_{165b} reduced hyperglycaemia-induced activated caspase 3 (AC3) levels in sensory neurons, epidermal sensory nerve fibre loss and aberrant sciatic nerve morphology, inducing neuroprotection (Hulse et al., 2015, Beazley-Long et al., 2013).

Of paramount importance is the role of VEGF-A_{165b} in nociception. VEGF is known to enhance nociception, but this is believed to be achieved mainly by VEGF-A_{xxx}a. Mediated by TRPV1 and TRPA1 channel activity, VEGF_{xxx}a sensitises mechanically sensitive C fibres, leading to increased transmission of pain signals and mechanical allodynia (Hulse et al., 2014). VEGF-A_{165a} was found to potentiate capsaicin-induced DRG sensory neuronal responses and TRPV1 phosphorylation; in addition, ablation of TRPV1 signalling by TRPV1 knockout or pharmacological inhibition completely inhibited VEGF-A_{165a} mediated mechanical hyperalgesia. On the other hand, VEGF-A_{165b} suppresses TRPV1 and TRPA1 mediated activation of DRG sensory neurons, C fibre

sensitisation and neuropathic pain (Hulse et al., 2014). Systemic administration of VEGF-A_{165b} reversed diabetes-induced changes in mechanical allodynia and heat hyperalgesia and prevented the onset of cold allodynia in the streptozotocin (STZ) model of diabetic neuropathy (Hulse et al., 2015). In the CFA model of inflammatory arthritis, two systemic doses of VEGF-A_{165b} had a significant inhibitory effect on weight bearing asymmetry and inhibited the development of secondary mechanical allodynia in the ipsilateral hind paw (Beazley-Long et al., 2015).

1.7.5. SRPK1 inhibition

VEGF-A alternative splicing is controlled by the enzyme serine arginine rich protein kinase 1 (SRPK1), which phosphorylates serine arginine rich splice factor 1 (SRSF1) (Amin et al., 2011, Nowak et al., 2010, Nowak et al., 2008). Inactive SRSF1 is located in the cytoplasm, and after phosphorylation it translocates to the nucleus, where it interacts with an exonic sequence enhancer (ESE) upstream of the VEGF exon 8a proximal splice site, which results in VEGF_{xxx} splicing (Gammons et al., 2013b, Amin et al., 2011). Inhibition of SRPK1 was found to prevent SRSF1 phosphorylation and VEGF-A_{165a} upregulation in vivo (Nowak et al., 2008). A number of SRPK1 inhibitors have been developed and tested as potential therapies in angiogenic-related diseases. Although older classes of SRPK1 inhibitors are neither specific nor potent (Batson et al., 2017), a few agents were effective as anti-angiogenic in neoplasms (Mavrou and Oltean, 2016, Mavrou et al., 2015) and ophthalmologic disorders (Gammons et al., 2013a, Gammons et al., 2013b). One of these molecules, SRPIN340, was found to produce hypoalgesia in naïve mice when injected in the hindpaw plantar surface and reduced the amount of VEGF-A_{165a} mRNA as a proportion of the total VEGF-A mRNA in plantar skin (Hulse et al., 2014). In addition, spinal SRPIN340 attenuated SRSF1 activity, reversed pain behaviour and suppressed central sensitization in a model of neuropathic pain (Hulse et al., 2016). This indicates a role of alternative pre-mRNA splicing in nociceptive processing (Hulse et al., 2014).

1.7.5.1. SPHINX-31

SPHINX-31 is a highly specific and potent inhibitor of SRPK1 (Batson et al., 2017). This molecule was developed based on the scaffold structure of SPHINX, a molecule with moderate potency but high selectivity for SRPK1. The methyl group on the furan ring of the SPHINX structure was replaced with a 4-pyridine group, and the substituent on piperazine ring was extended by replacement of oxygen by (NCH₂-2-pyridine). The resulting compound occupies a binding pocket created by the unique helical insert of SRPK1 and triggers a backbone flip in the hinge region, which improves potency ($IC_{50} = 5.9$ nM), maintaining high SRPK1 selectivity and affinity ($K_d = 72 \pm 5$ nM; figure 1.9) (Batson et al., 2017).

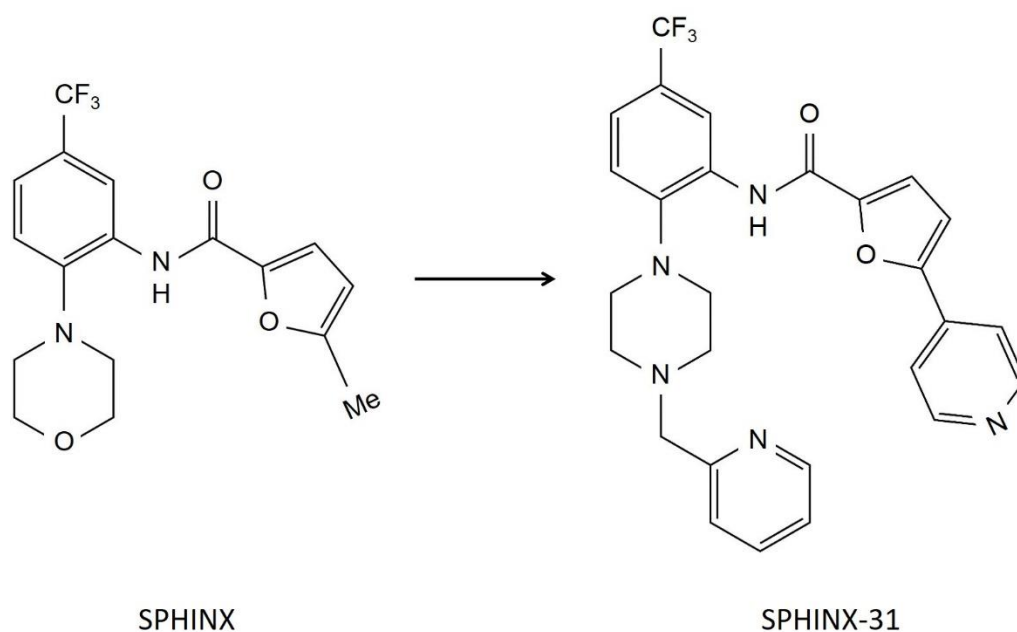


Figure 1.9. Chemical structure of SPHINX-31. The methyl substituent on the furan ring of the SPHINX structure was replaced with a 4-pyridine group, and the oxygen of the piperazine ring was substituted by (N-CH₂-2-pyridine), improving potency and maintaining SRPK1 affinity and selectivity.

SPHINX-31 caused inhibition of SRSF1 phosphorylation in PC3 prostate cells and switched splicing from VEGF-A_{165a} to VEGF-A_{165b} in retinal pigmented epithelials (RPE), a major source of VEGF in the retina (Batson et al., 2017).

Topical application of SPHINX-31 to the eye resulted in a potent inhibition of blood vessel growth and macrophage infiltration in models of choroidal angiogenesis in vivo, and it caused a switch in splicing toward anti-angiogenic VEGF (Batson et al., 2017). Recently, systemic administration of SPHINX-31 in mice was also found to inhibit SRSF1 phosphorylation in kidney and favour VEGF-A splicing towards VEGF-A_{165b} (Babaedi-Jadidi, Bates et al, unpublished data).

1.8. Hypotheses and objectives of the thesis

It was hypothesised that:

- 1- VEGF-A_{165b} can prevent and / or reverse enhanced pain behaviour in the MIA model of osteoarthritis, through involvement of spinal glial cells and COXs in the PAG and spinal cord.
- 2- SPHINX-31 can inhibit the development of augmented pain behaviour in the MIA model of OA, by affecting the expression of spinal COX and activation of glial cells in the PAG and the spinal cord.
- 3- Vascular-astrocyte association in the PAG is enhanced in the MIA model of OA, and this effect is reversed by administration of SPHINX-31.

The main objectives of this thesis were therefore:

- 1- To investigate changes in COX expression and glial activation in the spinal cord and the PAG in the MIA model of OA.
- 2- To study the effects of VEGF-A_{165b} and SRPK1 inhibitor SPHINX-31 on pain behaviour and joint pathology, as well as COX expression and glial activation in the spinal cord and the PAG in the MIA model of OA.

Chapter 2. Methods

2.1. Animals

All animal procedures in this project, including housing, handling and testing, were conducted according to the United Kingdom Animals (Scientific Procedures) Act 1986 / Amendment Regulations 2012 and with the approval of the University of Nottingham Animal Welfare and Ethical Review Group. Male Wistar rats provided by Charles River UK were used. The rats, weighing ~ 250 g at the beginning of each experiment, were kept in groups of four per cage and left to habituate for at least five days before handling. They were housed at a temperature of 20-24°C and 45 to 65% humidity, with a 12-hour light/dark cycle, standard food and water ad libitum. Body weights were checked at the beginning of each study, before pain behaviour test and after invasive procedures.

All tests were conducted in a randomised manner and with the experimenter blind to the rat arthritis and treatment condition.

2.2. Models of osteoarthritis

Two models of knee osteoarthritis were induced in this study; the MIA model of osteoarthritic pain and the collagenase model of joint destabilisation. Anaesthesia was induced with 5% inhaled isoflurane in O₂, delivered to the rat in an anaesthesia box, and maintained through a nasal cone delivering 2% isoflurane. Anaesthesia was confirmed by the absence of response to plantar pinching. The right hindpaw was then shaved up to the groin using a hair trimmer (Wella, UK) and the knee swabbed with chlorhexidine gluconate (4% w/v, Ecolab Ltd, Leeds, UK). With the paw bent, an injection was given into the tibiofemoral joint through the suprapatellar ligament using an insulin syringe with an attached 29 G needle (Myjector[®], Terumo Medical Corporation, Elkton, USA); figure 2.1. Injection contained 500 U active collagenase type II from

Clostridium histolyticum (Sigma-Aldrich, St. Louis, MO, USA) in 250 µl normal saline (Adães et al., 2014) or MIA (Sigma-Aldrich, UK) 1 mg in 50 µl sterile normal saline (Guzman et al., 2003). The rat was then transferred to a recovery box until fully awake. Animals in the collagenase study received intra-articular injections of the denatured enzyme as control. Denatured collagenase was obtained by heating the enzyme at 90°C for 1 hour. The MIA control animals underwent the same procedure without receiving the injection and will be referred to as “naïve” throughout this thesis. Sterile material was used throughout the procedure, which was carried in a clean environment.

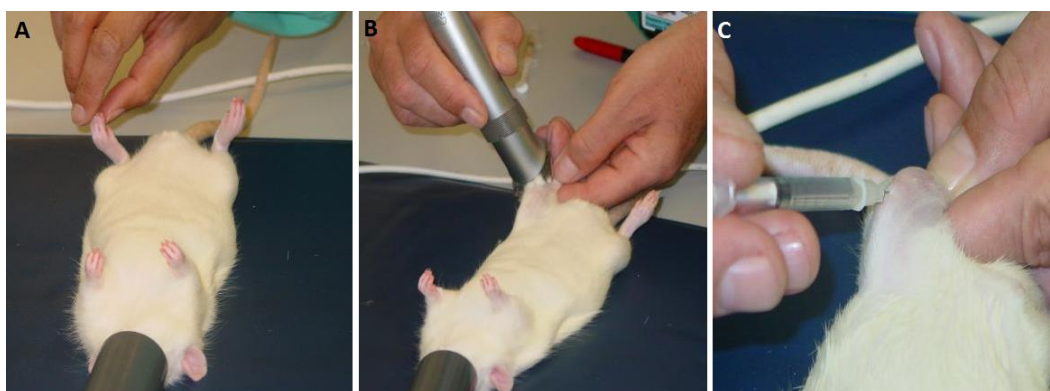


Figure 2.1. Intra-articular injection into the knee joint. A) Depth of anaesthesia was assessed by the absence of response to plantar pinching. (B) The skin over the joint was shaved and swabbed with chlorhexidine gluconate (4% w/v). (C) Injection was given using a 29-gauge needle inserted through the suprapatellar ligament whilst the joint was held in 90° flexion. *Pictures taken from (Ashraf, 2011).*

2.3. Intra-peritoneal injection

Intra-peritoneal injection was administered while another person held the rat with both hands. A 25 G, 0.5 inch needle was used to administer a maximum volume of 10 ml/kg body weight (specific volumes are detailed in individual experiment). It was inserted at 45° angle in the lower abdominal quadrant of the rat between the midline and leg (figure 2.2). Gentle aspiration was performed

prior to injection to check that the needle was not inserted into a blood vessel, intestine or urinary bladder.



Figure 2.2. Intraperitoneal injection in the rat. While one person is holding the rat in the correct position, another one gives the injection in the lower abdominal quadrant with needle placed at a 45° angle. *Picture reproduced from (AWHLA, 2018).*

2.4. Pain behaviour tests

Pain was indirectly tested through the measurement of weight bearing asymmetry and withdrawal thresholds to mechanical and, in one occasion, thermal stimuli. On the days preceding the study, rats were handled by the researcher for 10-15 minutes, and then kept in their cages in the test room for 30 minutes. Subsequently, they were habituated to the test equipment for 15 minutes.

2.4.1. Distal thermal allodynia

Thermal hypersensitivity was measured using the plantar test (Hargreaves method, Linton Instruments, Palgrave, UK) (Hargreaves et al., 1988). The

system consists of a movable source of infrared light connected to a controller and a transparent Perspex floor (figure 2.3). Rats were placed into the Perspex testing arena and allowed to settle for 10 minutes. The infrared heat source was positioned under the plantar surface of the hindpaw and the heat source activated. The latency to respond was automatically recorded by the device on removal of the hindpaw/beam break. An upper time limit of 20 seconds was set, after which the heat source had to be cut in order to prevent tissue damage. The process was repeated three times per hindpaw, with a minimum of 5 minutes between each testing to avoid local sensitization and possible tissue damage.



Figure 2.3. Plantar test (Hargreaves method). Rats are placed in Perspex boxes and the source of infrared heat is positioned under the plantar surface of the hindpaw. The controller automatically records hindpaw withdrawal latency. *Picture from (BIHEC, 2005-2018).*

2.4.2. Distal mechanical allodynia

Distal mechanical allodynia was tested measuring hindpaw withdrawal thresholds by von Frey filaments (Linton Instrumentation, Norfolk, UK) (Kim

and Chung, 1992). Each of these nylon hairs delivers a specific and fixed amount of force, according to its length and diameter. A typical aesthesiometer set contains 20 filaments, with forces ranging from 0.008 g to 300 g (Bradman et al., 2015). Each rat was placed in one of the mesh bottom Perspex test cages (figure 2.4) and left 15 minutes to habituate. The test was conducted using the full battery paradigm (Kim and Chung, 1992). Each filament was applied for three seconds to the hindpaw mid-plantar region, avoiding the raised tori, five times for each force value. Presence or absence of response, defined as the abrupt withdrawal of the hindpaw, was recorded for each application and the response percentage was calculated. The following filament order was used: 4g, 15 g, 8 g and 26 g. Values down to 1 g and up to 65 g were occasionally used.

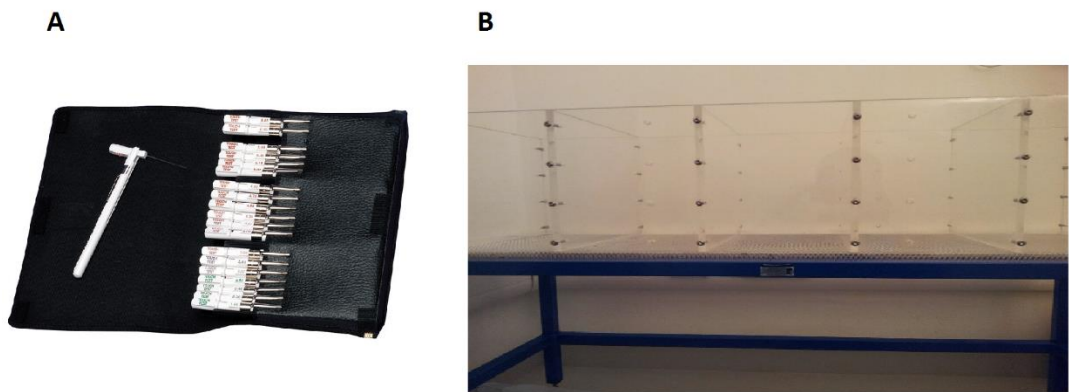


Figure 2.4. von Frey test for mechanical withdrawal thresholds. A) von Frey hairs. **B)** Wire bottom test cages for measuring mechanical withdrawal thresholds. *Pictures taken from (Stoelting, 2018, Ashraf, 2011).*

Using Prism V.7 (GraphPad, San Diego, California, USA), response percentages were plotted against the logarithm (base 10) of applied forces and the value at which each animal responded to the stimulus 50% of the time (EC50) was calculated using a non-linear regression test (variable slope, bottom constrain = 0, top constraint = 100; figure 2.5).

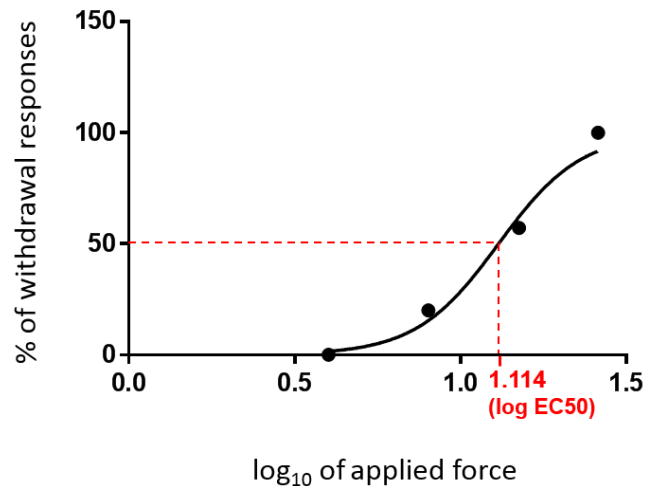


Figure 2.5. Example graph showing data from a rat von Frey test and determination of mechanical withdrawal threshold. Response percentages to each applied force were plotted against the logarithm (base 10) of forces. Using a non-linear regression test, the value at which the animal responded to the stimulus 50% of the time (EC50) was calculated (red dotted line).

2.4.3. Weight bearing

This test was used to measure the percentage of weight borne on the hindpaw ipsilateral to the MIA injection, which is considered an indirect measurement of knee pain (Neugebauer et al., 2007). Rats were positioned in a Perspex box placed on an Incapacitance Meter (Linton Instruments Cambridge UK), with each paw lying on a separate transducer pad. Animals were kept in the box for 10 minutes to habituate and reduce stress levels, and the weight borne on each paw for a period of three seconds was recorded (figure 2.6). An average of three readings for each animal was calculated. Results were then showed as 1) percentage of the weight borne on the ipsilateral hindpaw over the sum of the ipsi- and contralateral paws and 2) the percentage of the weight borne on the ipsilateral hindpaw over the rat total body weight.



Figure 2.6. Incapacitance meter for the measurement of weight bearing asymmetry. Rats are positioned in a Perspex box placed on the Incapacitance Meter, with each paw lying on a separate transducer pad. The weight borne on each paw for a period of three seconds is recorded. *Picture from (Gregersen et al., 2013).*

2.5. Acute inflammation (swelling)

Joint swelling was measured as a follow-up of the injection procedure, and as an indicator of inflammation. While holding the rat with one hand, a carbon fibre digital electronic calliper (Control Company, Friendswood, USA) was placed around the injected knee to measure its diameter in millimetres.

2.6. Rearings

Rearings are a measurement of exploration, locomotion, arousal and non-specific excitability (Lever et al., 2006). A decreased number of rearings may indicate sedation (Sousa et al., 2017), or movement-provoked pain in models of osteoarthritis (Miller et al., 2012, Inglis et al., 2008, More et al., 2013, Nagase et al., 2012). Each rat was positioned in an open Perspex wire bottom box and left to habituate for 5 minutes. After that, the number of rearings over a period

of two minutes was counted. Rearings included standing of the rat on his hindpaws, jumping on the box wall, and walking on the box edge for 5 seconds.

2.7. Recording of EMG activity

Evoked thresholds for electromyographic (EMG) activity were recorded in the hind leg bicep femoris and used to calculate noxious thermal withdrawal thresholds. This included preferential activation of A- and C-heat nociceptors. Rats underwent surgical preparation under isoflurane anaesthesia (2–3% in O₂). This included the insertion of an external jugular vein cannula for anaesthetic administration, external carotid artery cannulation for blood pressure measurement and tracheal cannulation to maintain open airways. Once the intravenous cannula had been inserted, anaesthesia was switched to a continuous infusion of alphaxalone (Alfaxan[®], Jurox) 5 mg/ml physiological saline at a rate of 40 mg/kg/h for the length of experiment. Body temperature was maintained within 37–38°C by a feedback-controlled heating mat and rectal probe. To record EMG activity, a custom-made bipolar electrode was prepared from a Teflon-coated steel wire (0.075 mm diameter; Advent Research Materials) and inserted into the bicep femoris of the left hind leg. The signal detected by the electrodes was amplified (x1K, A-B configuration; Neurolog NL104 amplifier, Digitimer), and filtered (50 Hz–5 kHz, NL125, Neurolog). Raw data were digitized via a CED 1401 and stored for off-line analysis using Spike2 (CED). A- and C-heat nociceptors were preferentially activated using a custom-made heat lamp, which consisted of a stainless-steel casing containing a sputter-coated 12 V bulb (halogen lamp, 15 v, 150 w; Phillips) positioned over a copper disk (45 mm² contact area) (McMullan et al., 2004, Leith et al., 2007, Leith et al., 2014). The heat lamp was placed on the surface of the paw and a constant voltage applied to provide fast or slow rates of heating. Fast rates of heating (7.5°C ± 1°C/s) preferentially activated myelinated A-fibres, while slow rates (2.5°C ± 1°C/s) activated unmyelinated C-fibres (McMullan et al., 2004, Leith et al., 2007). A T-type thermocouple attached to the copper disk monitored temperature at the heat lamp-skin border. The heat ramp was applied on the paw

for at least 60 seconds before the start of heating to allow for adaptation of low-threshold mechanoreceptors. An interval of eight minutes was left between stimuli to prevent tissue damage and sensitization (McMullan et al., 2004, McMullan and Lumb, 2006, Leith et al., 2007, Hughes et al., 2013, Leith et al., 2014). A feedback-controlled cut-off was set at 58°C for fast thermal ramps and 55°C for slow thermal ramps, and thresholds were recorded as cut-off + 2°C if no EMG activity was recorded at cut-off temperature (Leith et al., 2007). The use of heat ramp is a non-invasive method that reliably reproduces the previously described subcutaneous heating rates that preferentially activate A-versus C-nociceptors at the single-fibre level. This method removes the potential confound of different stimulus modalities (thermal/mechanical) from the interpretation of the findings and it activates populations of neurons rather than individual fibres, which is a more physiological stimulus than, for example, electrical stimulation.

2.8. Tissue collection and processing

At the end of each in vivo study, rats were sacrificed in accordance to the Schedule 1 methods of humane killing, and according to the expected use of tissues.

2.8.1. Systemic transcardiac perfusion fixation

To induce anaesthesia, each rat received an intra-peritoneal (i.p.) injection of 0.5 ml pentobarbital (Euthatal[®], Merial, UK; 200 mg/ml). The animal was left until the absence of corneal and hind paw withdrawal reflexes, but before the cessation of heart beat, which was essential for the perfusion process. The rat was then restrained with needles on a dissection tray. The skin was cut at the level of the sternum and the underlying muscles dissected. The sternum cartilage was raised by forceps and the ribs gently cut to expose the heart without damaging the lungs. Holding the heart with forceps, a needle connected to the perfusion equipment was inserted in the left ventricle up to the ascending aorta, and the right atrium was cut to allow the release of circulating blood and

perfusion buffer. Around 300 ml of phosphate buffer in normal saline (PBS) containing 10 U/ml heparin was perfused until the blood cleared, followed by the same amount of 4% paraformaldehyde (PFA) in PBS as fixative. Tissues were then harvested as explained below (section 2.8.3).

2.8.2. Anaesthetic overdose

When fresh tissues were needed, rats were killed by an overdose of pentobarbital (Euthatal[®], 200 mg/ml, 1 ml). When hindpaw and corneal reflexes became absent, and circulation and breathing ceased, death was confirmed by cervical dislocation. Required tissues were dissected and processed as explained in section 2.8.3.

2.8.3. Tissue collection and processing

2.8.3.1. Brain and periaqueductal gray (PAG)

To dissect the brains, skulls were open on the sagittal suture and cranial bones removed. Olfactory nerves were cut, and the spinal cord severed just below the brain stem to release each brain. For fresh tissues, brains were transferred immediately to dry ice for snap freezing. In case of perfused fixed animals, the brains were first post-fixed in 4% PFA for 24 hours at 4°C, then cryoprotected in 30% sucrose in PBS for other 24 hours, and then embedded and frozen in Optimum Cutting Temperature (OCT[®]) mounting medium (VWR Chemicals, Leuven, Belgium). All brains were stored at -80°C until required, and later used to obtain PAG tissue for RNA extraction (see section 2.11) or PAG sections for immunofluorescence (as in section 2.9.2). Samples of the ventrolateral and dorsolateral PAG columns were obtained from the brains using a custom-built tissue puncher (Drake et al., 2016). Using a Dremel (Dremel 300, UK) and a cutting disk, the sharp tip of a 21 G needle (internal diameter 0.5 mm; BD Microlance[™], Drogheda, Ireland) was cut and the edge was aligned and sharpened, making a tissue micropuncher of 0.5 mm internal diameter.

Throughout the study, precautions were taken to prevent contamination. Equipment and working area were cleaned with 70% alcohol before and after work, and new disposables, sterile whenever possible, were used for each sample, including blades, slides, syringes and gloves. To obtain the PAG, a cut was made just behind the superior colliculus using a single edge razor blade to remove the cerebellum. Other layers were removed as needed to reach approximately 0.5 mm from the PAG. Brain was then placed on a freezing microtome (Leica SM 2010 R, Leica Microsystems, UK) and when sufficiently frozen, 50 µm sections were cut from the caudal extremity of the PAG to the end of the ventrolateral columns, as shown in the images from the atlas of rat brain (Paxinos and Watson, 2009). At -8.28 mm bregma, a 1-mm-thick section containing (VL) PAG columns was carefully cut using the microtome, placed onto a glass slide, and flash frozen on dry ice. This step was immediately repeated at 7.20 mm caudal to bregma to obtain a (DL) PAG containing section of the same thickness. VL and DL PAG samples from both ipsilateral and contralateral parts were punched out from the respective sections using the tissue puncher and with the help of a dissecting microscope. The coordinates of micropunching sites were determined with reference to the rat brain atlas (Paxinos and Watson, 2009) to ensure that each section contained the relevant PAG columns throughout its thickness. Extracted tissues were finally stored at -80°C until further processing.

To obtain PAG sections, brains were equilibrated at -20°C overnight, and then transferred to a cryostat (Leica CM1900, Leica Biosystems, Nussloch, Germany). Moving in a caudal-rostral direction, 20 µm coronal sections were cut starting at around -7.92 mm bregma. Sections were placed on SuperFrost Plus® slides (Thermo Scientific, Waltham, USA) (three sections/slide), allowed to dry for five minutes at room temperature and then stored in air-tight slide boxes at -80°C.

2.8.3.2. Spinal cord

After perfusion (when necessary), vertebral columns were dissected with the surrounding tissues. Spinal cord was then exposed by opening the vertebral column and excess tissues trimmed. Samples were post-fixed in 4% PFA in PBS for 24 hours, and subsequently kept for 24 hours in 30% sucrose in PBS for cryoprotection. Spinal cords were then dissected to obtain the L3 – L5 segments, embedded in OCT and stored at -80°C. To obtain sections for immunofluorescence (see section 2.9.2), samples were transferred to the -20°C freezer overnight, and sectioned the next day using a cryostat (Leica CM1900). The L3-L5 portion of spinal cord was excised and fixed by OCT on the cryostat metal chock. Ten micrometre thick sagittal sections were cut in a caudal to rostral direction and placed on SuperFrost Plus® slides (5-6 sections/slide). Sections were left to dry at room temperature for 10 minutes, and then stored in air-tight slide boxes at -80°C.

2.8.3.3. Tibiofemoral joint

Tibiofemoral joints were harvested by cutting mid-femur and tibia with a bone cutter and trimming excess skeletal muscle. The joints were then stored in 4% PFA until needed. Knee joints were first disarticulated for macroscopic assessment of chondropathy (see section 2.10.1.1). Using small scissors, the patellar tendon was cut at the level of the tibiofemoral space and then cut again above the patella to excise it. Subsequently, anterior cruciate ligament and lateral and medial collateral ligaments were cut using Vannas scissors. Posterior cruciate ligament was severed by a scalpel, and with a gentle rotating movement, femur and tibia were set apart. Using tweezers, meniscus was removed, paying attention not to damage the cartilage. The separate bones and patella were stored in PBS at 4°C until scoring was performed.

The obtained tibiae were then processed for the microscopic assessment of chondropathy (as explained in section 2.10.1.2.). First, bones underwent decalcification, a process by which heavily mineralised tissues are softened to

enable sectioning at the appropriate thickness and quality (Bancroft and Gamble, 2008). This is achieved using solutions that react with calcium, including weak acids, such as formic acid, and chelating agents, such as ethylenediaminetetraacetic acid [EDTA] (Schmitz et al., 2010). EDTA is considered slower and less harsh than acids for decalcification and it has less effects on immunohistochemical stains (Bancroft and Gamble, 2008), so it was used in this study. Bones were immersed in a decalcification solution consisting of 10% w/v EDTA (Sigma Aldrich, Poole, UK) and 7.5% w/v polyvinylpyrrolidone (PVP; Sigma Aldrich, Poole, UK) in 10 mM Tris buffer (pH 6.95-7.0). The solution was changed twice weekly for 5 weeks, at the end of which the ammonium oxalate test was performed to confirm that the process was complete (Clayden, 1952). A 5-ml sample of used decalcifying fluid was first neutralized by ammonium hydroxide, and 5 ml ammonium oxalate (5 ml) was then added; absence of any precipitation in 30 minutes indicated that the last change of decalcifying fluid was free of calcium ions, and that full decalcification was achieved. At this point, the tibiae were split frontally, processed by standard histological techniques, and subsequently mounted in paraffin wax blocks for sectioning. Briefly, the samples were placed flat side down in labelled plastic cassettes and transferred to the Shandon Pathcentre enclosed tissue processor (Shandon Thermo Scientific, Leicestershire, UK). There, bones underwent a series of dehydration and clearing steps in alcohol and xylene, respectively. Subsequently, molten wax was dispensed from a Tissue Tek[®] embedding centre (model TEC5 EME2; Sakura Finetek Europe, The Netherlands) into metal moulds placed on a hot-plate. Tibia tissues were positioned flat side down into the centre of the mould and moved briefly to a cold-plate to allow the wax to solidify slightly at the bottom of the mould. At the same time, the section was pressed to obtain an even flat tissue surface with the wax. Labelled plastic cassettes were then placed on top of the moulds and pressed down. More wax was poured on top of the cassettes to evenly fill them. Moulds were then transferred to the cold-plate for quick cooling of the wax. The wax blocks were removed from the metal moulds and the excess wax removed. The blocks were stored at room temperature until required for sectioning. This tissue processing was performed at King's Mill Hospital by an experienced laboratory technician, Roger Hill.

2.9. Histochemistry

Histochemistry is the study of the identification of chemical components of cells and tissues using histological techniques (Bancroft and Gamble, 2008). Histochemistry is useful in the evaluation of diseases that involve the bone, like osteoarthritis.

2.9.1. Histological stains

Tibiae obtained from the in vivo studies and processed as in section 2.8.3.3 were stained with haematoxylin and eosin (H&E) to analyse joint pathology. H&E is often used to analyse tissue morphology. The basic haematoxylin dye has a high affinity for acidic structures, such as cell nuclei, which are stained blue/black. The eosin dye is acidic and stains basic structures, including the cell cytoplasm and connective tissue fibres in different shades of pink, red or orange (Bancroft and Gamble, 2008).

Paraffin wax embedded tibiae were sectioned using a sliding microtome. Sections were cut at three 200 µm intervals, so representative samples from all areas of the bone could be evaluated. Eight 7 µm sections were obtained at each level. Sections were mounted on polylysine slides (Fisher Scientific Ltd. Loughborough, UK) and dried at 50°C for two hours. For the staining process, one section per level was used. Sections were first dewaxed in xylene and then rehydrated in graded ethanol (100% and 75%). After washing in tap water for 5 minutes, slides were immersed in Mayer's haematoxylin for 10 minutes, rinsed in running tap water, dipped in 1% acid alcohol solution for 10 seconds and washed again in tap water for 3 minutes. Slides were then immersed in 1% eosin for 1 minute and placed in running tap water to differentiate staining. Subsequently, sections were dehydrated with graded ethanol (75% and 100%), followed by xylene. Finally, sections were mounted in DPX (Fisher Scientific UK Limited, Loughborough, UK), covered with cover-slips and allowed to air-dry (figure 2.7).

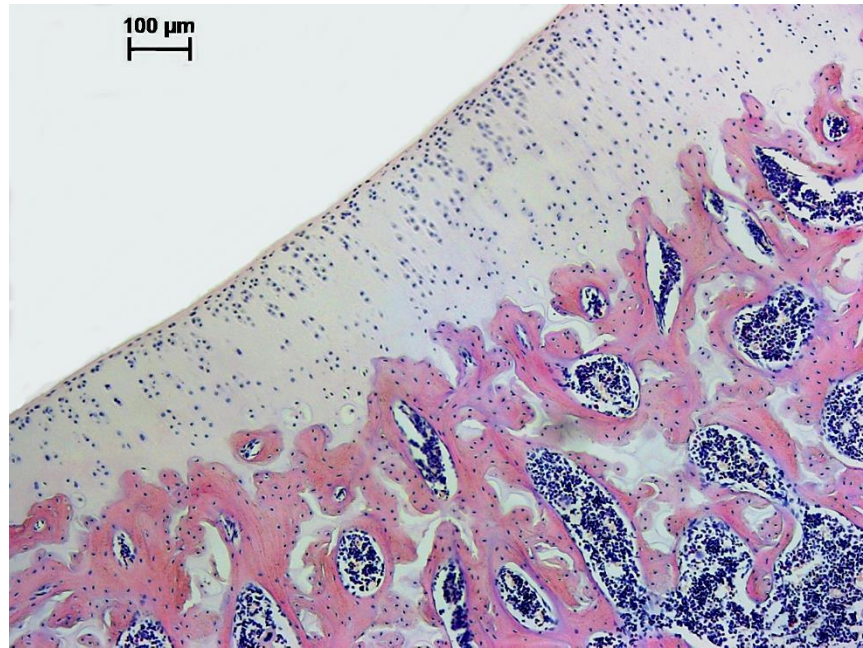


Figure 2.7. Haematoxylin and eosin stained section of a tibia.

2.9.2. Immunofluorescence

2.9.2.1. Introduction

Immunohistochemistry (IHC) is a “technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions, the site of antibody binding being identified by direct labelling of the antibody, or by use of a secondary labelling method” (Bancroft and Gamble, 2008). Immunofluorescence (IF) is an IHC technique in which a specific target antigen is labelled by a fluorescent dye (called fluorophore or fluorochrome), chemically conjugated to antibodies. The fluorophore allows visualization of the target distribution in the sample under a fluorescent microscope, such as epifluorescence and confocal microscopes. IF can use a single primary antibody directed against the target of interest; in this case, the antibody is directly conjugated to a fluorophore, and the technique is called direct IF. In indirect IF, the fluorophore is conjugated to the secondary antibody directed against the unconjugated primary antibody (figure 2.8). Direct IF is faster and simpler, but it is more expensive and produces a weaker signal than indirect IF (Donaldson, 2015).

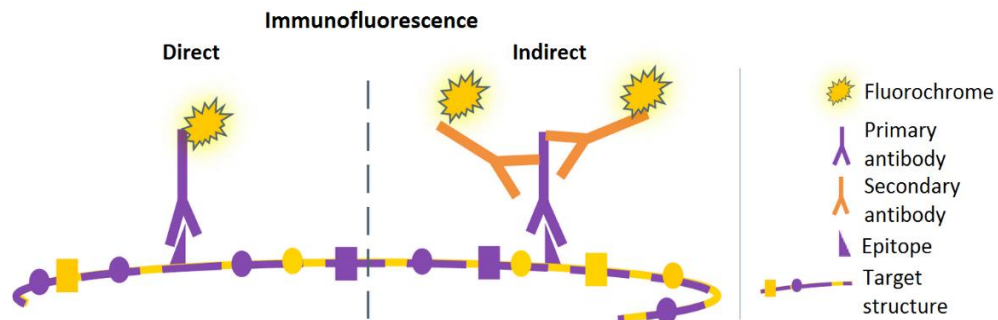


Figure 2.8. Direct and indirect immunofluorescence. Schematic representation of the direct and indirect IF methods (reproduced from (Hoff, 2015)).

To amplify IF signal, streptavidin-biotin complex can be used. Streptavidin is a ~56 kDa homotetramer protein obtained from the bacterium *Streptomyces avidinii*. It binds with very high affinity to four molecules of biotin, a water-soluble B vitamin that functions as a coenzyme for carboxylase enzymes (Dundas et al., 2013). In this case, the secondary antibodies used are conjugated with biotin, and a third step in the IF protocol includes incubation with streptavidin-fluorophore conjugates (figure 2.9).

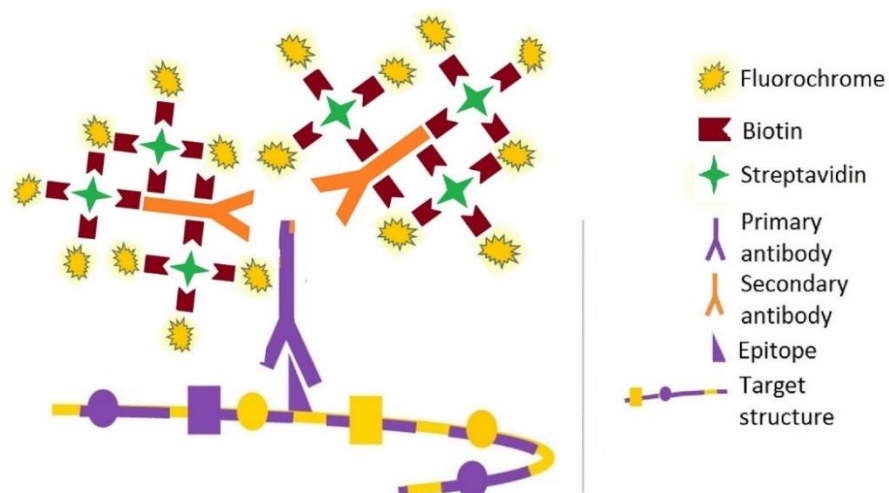


Figure 2.9. Schematic representation of the indirect method of IF involving streptavidin-biotin complex amplification. Biotinylated secondary antibody binds the unconjugated primary antibody; streptavidin fluorophore conjugate is added to bind to

secondary antibodies. Four biotin molecules bind with high affinity to one streptavidin molecules, intensifying the antigen-antibody signal.

2.9.2.2. Protocols

After tissues were collected and processed (section 2.8), thin sections were cut and stained with labelled antibodies. Tissue processing is needed to preserve tissue architecture, to prevent deterioration and loss of target antigens and to prepare tissues for sectioning. Sections were cut from either perfused fixed tissue samples or unfixed frozen samples, which were followed in one occasion by a fixation step using PFA.

In the first protocol, immunofluorescence was used to detect the expression of COX-2 and its co-localisation with neuronal cells. Sections were treated using rabbit anti-COX-2 antibodies (Cayman Chemical, Ann Arbor, USA) and mouse anti-NeuN antibodies (Millipore, Temecula, USA) as primary antibodies, biotinylated anti-rabbit IgG (R&D Systems, Abingdon, UK) and anti-mouse IgG Alexa Fluor® 555 (Cell Signaling Technology, Leiden, The Netherlands) as secondary antibodies, and streptavidin Alexa Fluor® 488 conjugate (Invitrogen, Eugene, USA) for signal detection of COX-2.

After hydration with PBS (5 minutes, three times), sections were permeabilised with 0.2% Triton-X 100® (Sigma-Aldrich, UK) in PBS (PBS-X 0.2%) for 10 minutes three times, and then blocked with a solution of 5% bovine serum albumin (BSA) and 10% fetal bovine serum (FBS) in PBS-X 0.2% for two hours at room temperature. Sections were then incubated with rabbit anti-COX-2 (1:200 dilution, 1 µg/ml) and anti-NeuN (1:100 dilution, 10 µg/ml) antibodies at 4°C overnight. The following day, sections were washed with PBS-X 0.2% 10 minutes for 5 times and blocked first with the 5% BSA and 10% FBS solution for 30 minutes, then with streptavidin and biotin solutions (Vector Laboratories, Burlingame, USA) for 15 minutes each. Biotinylated goat anti-rabbit IgG (1:100, 2 µg/ml) were added and left overnight at 4°C. After washing with PBS-X 0.2% (10 minutes, 5 times), filtered streptavidin Alexa Fluor® 488 conjugate (1:500) and anti-mouse IgG Alexa Fluor® 555 (1:1000) were applied for two

hours and then washed with PBS (10 minute, 3 times). Sections were finally mounted with Fluoroshield[®] containing DAPI (Sigma, USA) and stored away from light in air-tight containers at -80°C.

In another protocol, antibodies against CD11b (clone OX-42, mouse, BioRad, UK), GFAP (rabbit GFAP anti-serum; Abcam, Cambridge, UK) and CD31 (goat CD31 antibody; R&D Systems, Minneapolis, USA) were used to mark microglia, astrocytes and blood vessels, respectively, in both spinal cord and PAG. PAG sections were fixed on the slides by addition of 4% PFA on the section for 15 minutes. Spinal cord and fixed PAG sections were hydrated for 10 minutes in PBS, and then permeabilised with 0.2% Triton-X[®] in PBS for 3 times 15 minutes each. Blocking was performed with a solution of 5% BSA and 10% FBS in 0.2% Triton-X[®] in PBS (PBS-X 0.2%) for two hours at room temperature. Primary antibodies were diluted in the blocking solution at the following concentrations (mouse anti-CD11b 1:500, 2 µg/ml; rabbit anti-GFAP 1:1000; goat anti-CD31, 1:100, 2 µg/ml) and added to the sections, which were incubated at 4°C overnight. After washing three times with PBS-X 0.2% for 15 minutes each, sections were incubated with secondary antibodies [chicken anti-mouse Alexa Fluor[®] 488 (Invitrogen), donkey anti-rabbit CF 633 (Sigma Aldrich), and donkey anti-goat Alexa Fluor[®] 555 (Life Technologies)] 1:1000 dilution for 4 hours. Subsequently, sections were washed with PBS five times for 5 minutes each, mounted with Fluoroshield[®] with DAPI, covered with coverslips and stored in air-tight containers at -80°C and away from light until imaging was performed.

Each of the conducted immunofluorescence tests included at least a negative control, which was obtained by not adding primary antibodies or using species and concentration matched IgG antibodies. IgG control of anti-GFAP antibody was whole rabbit serum, since this antibody comes as whole anti-serum.

NeuN, also known as Fox-3, is a 46 kDa protein specifically expressed in the nucleus of the vast majority of neurons. Antibodies against this antigen were first produced in 1992 (Mullen et al., 1992), although the protein structure was only identified in 2009. NeuN plays a role in neurospecific alternative splicing and contributes to the regulation of neuron differentiation (Gusel'nikova and

Korzhevskiy, 2015). It is now extensively used to immunohistochemically mark neuronal nuclei and differentiate them from other CNS cell types. CD11b (cluster of differentiation 11b) protein is a subunit of the integrin type 3 complement receptor (CR3). It is located on the plasma membranes of neutrophils, granulocytes and most mononuclear phagocytes and natural killer cells. CD11b is not a highly specific microglial marker, but IHC reactions for this protein are often used as a method for detecting activated microglia. In rats, CD11b is most commonly detected in microglia using monoclonal antibody OX-42 (Korzhevskii and Kirik, 2016). CD31 (cluster of differentiation 31), also known as platelet endothelial cell adhesion molecule (PECAM-1), is a protein expressed on endothelial cells, platelets, macrophages, granulocytes, and lymphocytes, among others, and it plays a role in angiogenesis and leukocyte transmigration. CD31 has been used in IHC to mark endothelial cells (Miettinen, 2014). GFAP (glial fibrillary acid protein) is one of a family of intermediate filament proteins involved in the structure and function of cell's cytoskeleton. It is expressed on several types of CNS cells, including astrocytes and ependymal cells. Use of GFAP in the IHC identification of astrocytes has some limitations; it is not an absolute marker of all non-reactive astrocytes and it is often not immunohistochemically detectable in healthy CNS tissue. However, it is extensively used as a marker of reactive astrocytes (Sofroniew and Vinters, 2010). DAPI (4',6-diamidino-2-phenylindole) is a dye that penetrates through intact cell membrane and binds firmly to A-T rich regions in the DNA. When bound to double-stranded DNA and excited with ultra-violet light (wavelength of 358 nm), DAPI emits a blue light (wavelength of 461 nm), and is therefore used to mark and count cellular nuclei in fluorescence microscopy and flow cytometry (Tarnowski et al., 1991).

2.10. Image analysis and quantification

2.10.1. Cartilage and subchondral bone

Histological scoring is of paramount importance to investigate pathological changes in osteoarthritis. Many methods for the measurement of structural

changes were proposed, mainly based on histology detection (Janusz et al., 2002, Ostergaard et al., 1999, Pritzker et al., 2006). In this study, both macroscopic and microscopic histologic scoring of chondropathy were measured.

2.10.1.1. Macroscopic scoring

Macroscopic scoring of chondropathy provides a gross evaluation of the appearance of the joint and it was mainly developed from arthroscopic studies in man (Oakley and Lassere, 2003). As most assessment methods, it is based on the Collin's classification (Collins, 1950), which mainly considered cartilage destruction, marginal proliferation and changes in bony contour. This classification was also the base for the Société Française D'Arthroscopie (SFA) system (Dougados et al., 1994) and its revised version (Ayrat et al., 1996). The SFA system involved reporting the observed severity score on an articular diagram dependent on localization, depth and size. Another macroscopic scoring system involved the use of Indian ink, as described by Meachim (Meachim, 1972), which makes areas of mild damage more apparent and helps to distinguish between real and artefactual irregularities of the joint surface. In this study, the severity of cartilage damage was first assessed using the macroscopic method described by Guingamp (Guingamp et al., 1997). The tibiofemoral joints were disarticulated as explained in section 2.8.3.3. For each joint, patella, femoral condyle (medial and lateral), tibial plateau (medial and lateral) and femoral groove were inspected by naked eye and assessed for the degree of chondropathy according to the grading system in table 2.1. An additional score of 1 was added in case of osteophyte presence, making the maximum possible total score 25. Macroscopic scoring was performed by a single observer blinded to study groups. To validate the method, assessment was performed twice in separate occasions and the two sets of scores were compared using Spearman's correlation test in Prism V.7 (GraphPad, San Diego, California, USA).

Table 2.1. Chondropathy macroscopic scoring system.

Grade	Finding
Grade 0	Normal appearance
Grade 1	Slight yellowish discolouration of the chondral surface
Grade 2	Small cartilage erosions in load bearing areas
Grade 3	Large cartilage erosions extending down to subchondral bone
Grade 4	Large cartilage erosions with large areas of subchondral bone exposure

2.10.1.2. Microscopic scoring

Microscopic evaluation of lesions of OA in rats is usually performed following the modified Mankin score system (Gerwin et al., 2010). The Mankin score was originally developed from a grading system using femoral heads removed at arthroplastic surgery in humans (Mankin et al., 1971). The modified version was revised for other species and include specific adaptations for rats. Most of these schemes limit evaluation of OA to changes in cartilage, while a few including OA-related changes in bone and synovium.

In this study, the method described by Janusz (Janusz et al., 2002) was used. H&E stained tibia sections were visualised through a 10x objective lens of a light microscope (Zeiss Axioscop-50; Carl Zeiss Ltd, Welwyn Garden City, UK), and images taken using a camera (AxioCam MRc Zeiss, Carl Zeiss Ltd, Gottingen, Germany).

The degree of cartilage damage in lateral and medial tibial plateau was scored according to the scale in table 2.2. The score was then multiplied by the portion

of plateau involved by damage (1, 2 or 3 out of 3) to obtain the overall chondropathy score. The method was validated before scoring the experimental samples.

Table 2.2. Chondropathy microscopic scoring system

Grade	Description
Grade 0	Cartilage of normal appearance
Grade 1	Minimal fibrillation in superficial zone only
Grade 2	Mild, extends into the upper middle zone
Grade 3	Moderate, well into the middle zone
Grade 4	Marked, into the deep zone but not to the tidemark
Grade 5	Severe, full thickness degeneration to the tidemark

Osteophyte and synovitis were also assessed. Osteophytes were given scores of 1, 2 or 3 for mild (<40 µm), moderate (40–160 µm) or severe (>160 µm), respectively. Although not available in most samples as a result of disarticulation, when present synovia were assessed for lining thickness and cellularity (Mapp et al., 2008) as summarised in table 2.3. Synovitis scoring was performed using a 20× objective lens of a Zeiss Axioscop-50 microscope (Carl Zeiss Ltd, Welwyn Garden City, UK).

Table 2.3. Synovitis microscopic scoring system

Score	Description
0	Lining cell layer 1-2 cells thick
1	Lining cell layer 3-5 cells thick
2	Lining cell layer 6-8 cells thick and/or mild increase in cellularity
3	Lining cell layer >9 cells thick and/or severe increase in cellularity

2.10.2. Spinal cord and PAG

Images of stained spinal cord sections were obtained using a X20 objective lens on Leica TCS SP5 X confocal microscope (Leica Microsystems). One image was taken of the ipsilateral and contralateral dorsal horn in three sections per animal, except in the MIA characterisation study (chapter 3), where one section per animal was analysed. For cell count and fluorescence measurements, section images were analysed using ImageJ (NIH open software). Areas of interest were determined that included laminae I and II, laminae III-V, and in case of the MIA characterisation study, in the ventral horn, too. COX-2 expression was quantified by manually counting the cells, indicated by DAPI staining, that showed COX-2 immunoreactivity in both ipsilateral and contralateral quadrants. Cells that were both COX-2 and NeuN immunoreactive were also counted. DAPI positive cells were automatically counted by ImageJ, and the numbers were used to calculate the percentage of COX-2 positive cells. Microglia in spinal cord were identified by a morphologically matching positive CD11b staining. Number of microglia was manually counted and expressed as percentage of total cells. In addition, CD11b average intensity in the area of interest was measured. GFAP expression, as indicator of astrocytes, was also measured as average intensity. In both cases, background fluorescence intensity

was measured in control sections with no primary antibodies and it was then subtracted from the intensities measured in stained sections to obtain the final values for analysis.

For the analysis of PAG immunofluorescence, three sections per animal were used. One image of dorsolateral and ventrolateral PAG on both ipsilateral and contralateral sides was obtained, using a 10x objective lens on Leica TCS SP5 X confocal microscope (Leica Microsystems; figure 2.10). GFAP fluorescence was assessed measuring the average intensity of GFAP by ImageJ. Blood vessels were identified with positive CD31 staining in a vessel-like shape structure (a chain of two or more elongated nuclei for longitudinal vessels and two or more curved nuclei forming a crescent or complete ring for cross-sectional vessel sections), and were counted manually. The number of vessels associated with astrocytes was obtained by the manual counting of the blood vessels combined with GFAP immunoreactive astrocytes. The percentage of the latter was then calculated out of the total number of blood vessels. All image analysis was performed off-line on captured images of the stained sections by a blinded examiner.

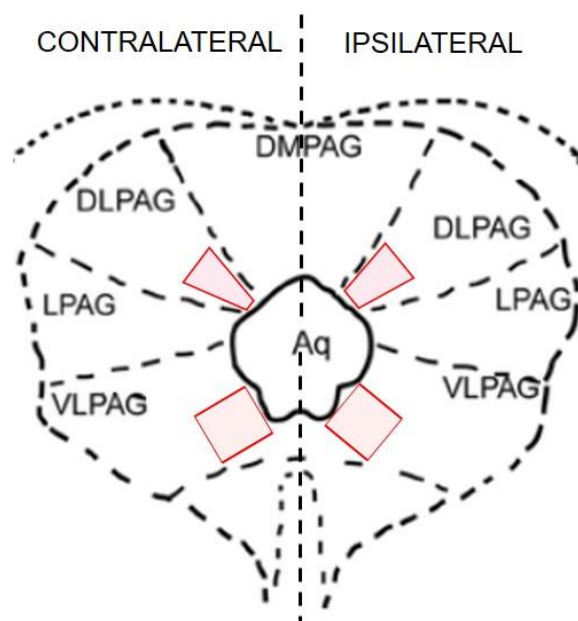


Figure 2.10. Graphic representation of a coronal section of the PAG showing the regions considered for astrocyte activation analysis. (Aq = aqueduct, DL =

dorsolateral, DM = dorsomedial, L = lateral, VL = ventrolateral). *Reproduced with modifications from (Messanvi et al., 2013).*

2.11. RNA extraction

RNA was extracted from PAG samples using Trizol (Fisher Scientific Ltd., Loughborough, UK) and following manufacturer instructions. First, 500 µl of Trizol reagent was added to each sample for 10 minutes, with frequent vortexing, to lyse the tissues. Chloroform (100 µl) was then added, followed by vigorous shaking of the samples for 15 seconds and incubation at room temperature for 15 minutes. Samples were then centrifuged for 15 minutes at 12,000 rpm and 4°C. The upper aqueous layer containing RNA was carefully separated and mixed with 250 µl isopropanol. After a 10-minute incubation at room temperature, tubes were centrifuged again at 12,000 rpm and 4°C for 10 minutes. The supernatant was discarded, and the remaining RNA pellets were washed with 500 µl 75% ethanol. Samples were centrifuged for other 5 minutes at 10,000 rpm and 4°C and ethanol discarded. Samples were allowed to air dry for 10 minutes, and the pellets re-suspended in 20 µl RNase-free water (Promega, Southampton, UK). For further purification, 2 µl of 3 M sodium acetate, 1 µl of glycogen RNA grade (Thermo Scientific, Carlsbad, USA; 1 µg/µl) and 60 µl of 100% ethanol were added. Samples were kept at -80°C overnight. The next day, samples were centrifuged for 20 minutes at 12,000 rpm and 4°C and the supernatant removed. The pellets were then rinsed with 75% ethanol and left to air dry for 10 minutes. Pellets were finally reconstituted in 9 µl RNase-free water, and stored at -80°C. RNA concentration was measured using a Nanodrop® 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA).

2.12. cDNA synthesis

In order to produce cDNA to be used in PCR reactions, reverse transcription (RT) was performed. Due to the low yield of RNA extracted from the PAG

samples, all the amount of RNA from each sample was used in the RT process. To eliminate any genomic DNA contamination, DNase and its respective 10X buffer (1 µl each; Promega, Madison, USA) were added to the RNA and incubated at 37°C for 30 minutes. The reaction was halted adding 2 µl DNase stopping solution (Promega, USA) followed by 10-minute incubation at 65°C. Subsequently, 500 ng of both oligo dT (Thermo Fisher Scientific, USA) and random primers (Promega, USA) were added to the solution, which was heated to 95°C for 10 minutes. Immediately after that, samples were immersed in an ice bath for 5 minutes. Finally, dNTP (Promega, USA; at a final concentration of 0.5 mM), 200 U of MMLV reverse transcriptase and 4 µl of 5X buffer (Promega, USA) were added and the solution incubated at 37°C for 60 minutes and 42°C for 30 minutes, followed by 10 minutes at 70°C to inactivate reverse transcriptase. For every RT experiment, a “no RT” control was prepared, running the above-mentioned protocol without the addition of the reverse transcriptase in the last step. This would exclude the presence of any DNA not produced by the RT process.

2.13. PCR

2.13.1. Conventional PCR

2.13.1.1. Protocol

Conventional PCR was performed as a semi-quantitative method of analysis of COX-1 and COX-2 gene expression in the PAG. Several runs were tried to determine the optimal primer design and PCR conditions. The primer sequences and expected product size are summarised in Table 2.4. This table also contains information about GAPDH, a housekeeping gene that was used as a reference gene throughout the study. GAPDH is routinely used to normalise for differences in the cDNA content of samples, because it is mostly expressed in a stable and non-regulated constant level with little variability between tissues and physiological states of the organism (Kozera and Rapacz, 2013). PCR solution was prepared adding 10 µl of MasterMix (Promega, USA), 1 µl of each primer

(0.5 μ M) and 1 μ l of cDNA. The following protocols was run for COX-2: denaturation 94°C 30 seconds, annealing 45°C 30 seconds and extension 72°C 1 minute for 35 cycles. COX-1 was amplified with 45 cycles of 94°C 30 seconds, 50°C 30 seconds and 72°C 1 minute. PCR reactions were run using a PTC-200 Peltier Thermal Cycler (MJ Research), and products run in a gel of 2% (w/v) agarose in 1X TAE buffer containing ethidium bromide. Images of the gel were obtained using Bio-Rad Gel Doc® Ez Imager.

Table 2.4. Primer sequences and expected amplicon size of GAPDH and COX isoforms.

Target	Primer	Sequence (5' $\rightarrow$ 3')	Amplicon size
GAPDH	Upstream	CAACTACATGGTTTACATGTTC	181 bp
	Downstream	GCCAGTGGACTCCACGAC	
COX-1	Upstream	GGCGTTGCTCATCCATCTACTC	116 bp
	Downstream	AGCATCTGTGAGCAGTACCGG	
COX-2	Upstream	GATGACGAGCGACTGTTCCA	98 bp
	Downstream	TGGTAACCGCTCAGGTGTTG	

2.13.1.2. Analysis

Results from conventional PCR were reported as samples positive or negative for the expression of COX-1 and COX-2. The number of samples in each group and in each area of the PAG were tested for contingency using Fisher's exact test or Chi-Square test, as indicated.

2.13.2. Digital PCR

Digital PCR (dPCR) is a molecular biology technique that combines the classical PCR reaction kinetics and the fluorescence-based detection methods used in real-time PCR (qPCR). It is particularly useful for the accurate detection and analysis of low abundance nucleic acids. The term "digital PCR" was first used

in 1999 (Vogelstein and Kinzler, 1999), but similar techniques had been described since 1988 (Saiki et al., 1988), being referred to as “single molecule PCR” or “limiting dilution PCR” (Jeffreys et al., 1990, Simmonds et al., 1990). Initially, the use of this innovative technique was hindered by cost and lack of easily available equipment. Recently, these limitations have been overcome and dPCR is now largely used in nucleic acid quantification (Geuensleben et al., 2013), viral and pathogen detection and quantification (Strain et al., 2013, Kelley et al., 2013), rare mutation abundance detection (Hindson et al., 2011), single cell analysis and many other biological applications (Tewhey et al., 2009). In dPCR, a reaction solution is prepared using the usual components (primers, dNTPs, polymerase, buffers, etc.) and a reporter molecule (dye or probe). The mixture is then physically partitioned at the level of single molecules (figure 2.11) and standard PCR cycling conditions applied to amplify the samples. An all-or-none signal is recorded, and the number of molecules is calculated using a Poisson distribution (Morley, 2014, Bizouarn, 2014).

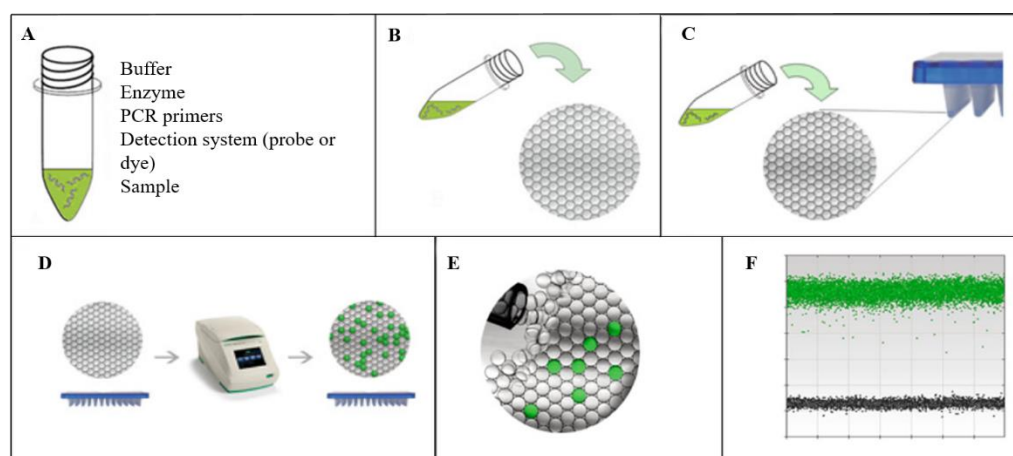


Figure 2.11. Digital PCR. (A) Components of digital PCR solution. (B) Partitioning of the sample. (C) Partitioned sample is transferred to a standard PCR plate. (D) Samples undergo amplification in a thermocycler. (E) Subreactions containing DNA will host DNA amplification (in green). (F) A graph produced by the dPCR software showing the negative (black) and positive (green) subreactions. *Adapted from (Bizouarn, 2014).*

Standard reference curves are not needed in dPCR. This is because quantitation depends on the attributes of each reaction on a positive versus negative basis of the subreactions, making it an absolute result. For the same reason, results are

not affected by assay efficiency or variability in mixture preparation, minor reaction inhibition, or user inconsistencies. This is an extremely useful advantage for samples that are analysed at different time points, or have low levels of target molecules, and for samples for which it is difficult or impractical to generate an accurate absolute standard curve.

The total target molecule count is calculated from the positive and negative subreaction counts using the following formula:

$$\lambda = -\ln(1-p)$$

where (λ) is the average number of targets per subreaction and (p) the ratio of positives to total. The result is then multiplied by the number of subreactions per μl . These calculations are usually performed by the analysis software packages provided with dPCR equipment.

In my study, use of digital PCR was attempted for the detection and quantification of COX-1 and COX-2 mRNA in PAG. QX100 Droplet Digital PCR (ddPCR™, Bio-Rad) equipment and validated TaqMan® primer/probe assays from Applied Biosystems™ were used. These assays consisted of a pair of unlabeled PCR primers and a TaqMan® probe with a FAM™ or VIC™ dye label on the 5' end and minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end.

Reactions solutions were prepared by adding 5 μl cDNA, 12.5 μl X2 MasterMix®, 2.5 μl of primers/probes and water to add to 25 μl for each sample. Samples were then transferred to a droplet generation cartridge, and droplet generation oil was loaded into the corresponding channels. The cartridge was then placed into the QX100 droplet generator, where each sample was transformed into an emulsion and partitioned into 20,000 one nanolitre droplets. At the end of the process, the emulsified samples were transferred to a standard 96-well PCR plate and loaded into a thermocycler to undergo amplification with the following protocol: 95°C 10 minutes, then 40 cycles of 94°C 30 seconds and 59°C 1 minute, then 98°C 10 minutes. The plate was then placed in the droplet reader, which detected the fluorescence emitted by each droplet. As fluorescence was detected, results were recorded as positive and negative by the software and

presented on the screen as graphs. Absolute concentration was calculated by the accompanying software.

2.13.3. Real-time PCR

Real-time PCR (qPCR) was used to determine the relative mRNA levels of COX-1 and COX-2 genes in PAG. For each sample, a reaction solution was prepared by adding 10 µl PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Austin, USA) and one microlitre of each primer (final concentration of 0.5 µM) to 100-250 ng of cDNA, made up to a final volume of 20 µl. A LightCycler® 480 System (Roche Diagnostics Ltd, UK) was used with the following conditions: 95°C for 5 minutes, then 45 cycles of 95°C 10 seconds, 60°C 10 seconds, 72°C 10 seconds, then 72°C 10 min. GAPDH was used as a housekeeping/control gene, and the reactions were performed in duplicates. The same primers used in conventional PCR, and shown in Table 1, were used here. To confirm that the reactions were producing the expected targets, in the first trials of qPCR, the amplified products were run in a 2% agarose gel in 1X TAE buffer to check the band size and the DNA was then extracted from the gel to be sent for DNA sequencing. DNA extraction from agarose gel was performed using QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer protocol. The DNA fragment was excised from the gel with a clean scalpel and three volumes Buffer QG was added to one volume gel. Tubes were incubated at 50°C for 10 minutes to completely dissolve the gel, and 1 volume of isopropanol was added with mixing. The sample was then applied to the QIAquick® column and centrifuged for one minute. Flow-through was discarded and the column placed back into the same tube. Buffer QG (500 µl) was added and the tube centrifuged again for 1 minute. After discarding the flow-through, 750 µl of Buffer PE was added to wash the column. The buffer was left to stand for two minutes, followed by one-minute centrifugation twice. Finally, 50 µl water was added to the centre of the column and let to stand for one minute, and the sample was centrifuged to elute DNA. DNA was then sent to the DNA sequencing facility in the School of Life Sciences. The produced sequences were aligned with RNA sequences referenced in BLAST (Basic Local Alignment

search tool) using the online DNA alignment tool ALIGN. In addition, the melting temperatures (T_m) of qPCR products were obtained at the end of the reaction. These were compared with the expected melting temperatures calculated using uMeltSM software v.2.0.2 (Dwight et al., 2011). Corresponding temperatures were used as a reference to determine the target qPCR products and exclude excess co-products from analysis. Changes in gene expressions were calculated using the $\Delta\Delta C_t$ comparative quantification method, which is expressed in the following equations:

$$\text{Fold difference} = 2^{-\Delta\Delta C_t}$$

$$\Delta\Delta C_t = \Delta C_{t \text{ sample}} - \Delta C_{t \text{ calibrator}}$$

$$\Delta C_{t \text{ sample}} = C_{t \text{ GOI}}^s - C_{t \text{ norm}}^s$$

$$\Delta C_{t \text{ calibrator}} = C_{t \text{ GOI}}^c - C_{t \text{ norm}}^c$$

where (C_t) is the threshold cycle, (GOI) the gene of interest and (norm) the normaliser gene (GAPDH, in this case); calibrator refers to the control group of the study.

2.14. Statistical analysis

Data for each experiment were analysed and presented graphically using Prism V.7 (GraphPad Software, San Diego, California, USA). Parametric data were analysed using unpaired t-test, when comparing two groups, or analysis of variance (ANOVA) followed by Bonferroni post hoc test for multiple comparisons. Non-parametric data were analysed using Mann-Whitney U test (for two groups) or Kruskal-Wallis test with post hoc Dunn's test (for more than two groups). Associations between variables were expressed as Spearman's correlation coefficients. Contingency tables were analysed using Fisher's exact test, when possible, or Chi-Square test. Numerical data were reported as mean $\pm$ standard error of the mean (SEM) in the text and graphically, unless otherwise stated. A two-tailed P value of less than 0.05 was considered significant in all cases.

Chapter 3. MIA model characterisation

3.1. Introduction

The MIA model is one of the most commonly used models of osteoarthritic pain (Kuyinu et al., 2016) due to its ability to produce sustained pain behaviour (Combe et al., 2004) and pathologic changes similar to those seen in humans (Guzman et al., 2003). It is particularly useful in the study of OA drug efficacy (Fernihough et al., 2004). This chapter contains the experiments performed to reproduce the MIA model of osteoarthritis in its pain behaviour and joint pathology. It also includes the assessment of reflex sensitisation, activation of spinal glial cells and expression of COX isoforms in the spinal cord and PAG in this model of OA.

3.2. Methods

3.2.1. Induction of the MIA model of OA and assessment of pain behaviour

Thirty male Wistar rats were used in the MIA characterisation study. Animals were handled and habituated to the test equipment, and baseline readings of each pain test was obtained. Under isoflurane anaesthesia (2% in O₂), twenty rats were then injected with MIA (1 mg / 50 µl sterile NS) in the right tibiofemoral joint. The other animals were anaesthetised but received no injection (naïve control). Rats were then tested twice weekly for thermal latency, mechanical withdrawal threshold and weight bearing (see section 2.4 for more details on how these tests are performed). Figure 3.1 shows the time course of the in vivo study.

Time course of the experiment

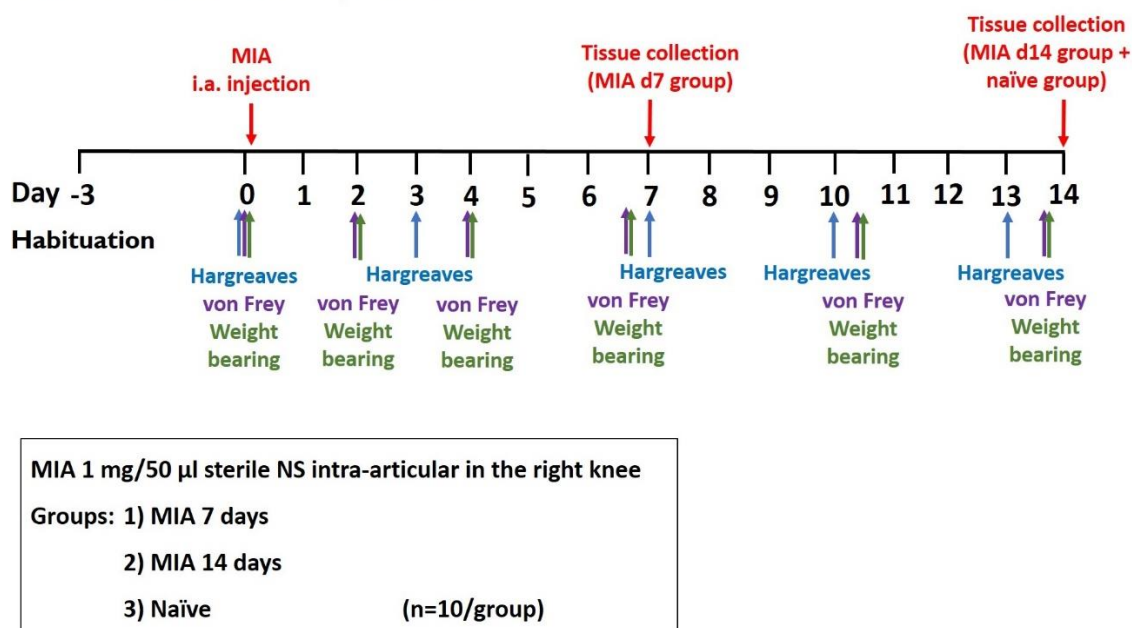


Figure 3.1. Time course of the MIA model characterisation study and pain behaviour.

3.2.2. Recording of thermal withdrawal thresholds

An EMG study was designed to test the hypothesis that intra-articular injection of MIA in the knee produces secondary hyperalgesia through spinal sensitisation, expressed as a decrease in the thermal withdrawal thresholds of the bicep femoris upon stimulation of A- and C- nociceptors under anaesthesia. Using the method explained in section 2.7, a separate set of rats, divided into naïve and MIA day 7 groups (n=9/group) were anaesthetised and cannulated. An electrode connected to an amplifier for data processing and recording was inserted into the bicep femoris of the left hind leg. The heat lamp was then applied to the dorsal surface of the hindpaw at different heating rates to preferentially activate A- or C-nociceptors, and the thermal hind leg withdrawal thresholds were recorded.

3.2.3. Tissue collection

On day 7, four MIA rats were perfused fixed (section 2.8.1) and six were killed with an overdose of pentobarbital (section 2.8.2). The remaining animals were sacrificed on day 14 by either method (eight and twelve rats, respectively). Brains, spinal cords and knees were dissected from all animals and processed as explained in section 2.8.3. Brains were used to micropunch PAG (section 2.8.3.1), from which RNA was extracted using the Trizol[®] method (section 2.11). Reverse transcription was performed (as in section 2.12), and the resulting cDNA amplified with conventional PCR to detect changes in COX-1 and COX-2 expression (section 2.13.1). Spinal cords were cut into 10 µm sections using a cryostat (section 2.8.3.2) and stained for COX-2 / NeuN and GFAP and CD11b immunofluorescence (section 2.9.2). Rat knee joints were disarticulated for the macroscopic assessment of chondropathy and then decalcified using a solution of 10% w/v EDTA and 7.5% w/v polyvinylpyrrolidone (PVP) in 10 mM Tris buffer. Tibiae were then embedded in paraffin wax and sectioned to be treated with H&E stain for microscopic evaluation (sections 2.8.3.3, 2.9.1 and 2.10.1).

3.3. Results

3.3.1. Pain behaviour

3.3.1.1. Paw thermal latency (Hargreaves)

The MIA group showed a significant decrease in the ipsilateral paw thermal latency (s) compared to the control group on days 3 and 13 post-MIA injection (Figure 3.2.A; 7.53 ± 0.59 vs 11.44 ± 1.27 , $p < 0.001$ on day 3; 5.70 ± 0.47 vs 8.66 ± 1.07 , $p < 0.05$ on day 13). No significant difference in contralateral thermal latency between MIA injected rats and the control group was detected at any time point (figure 3.2.B).

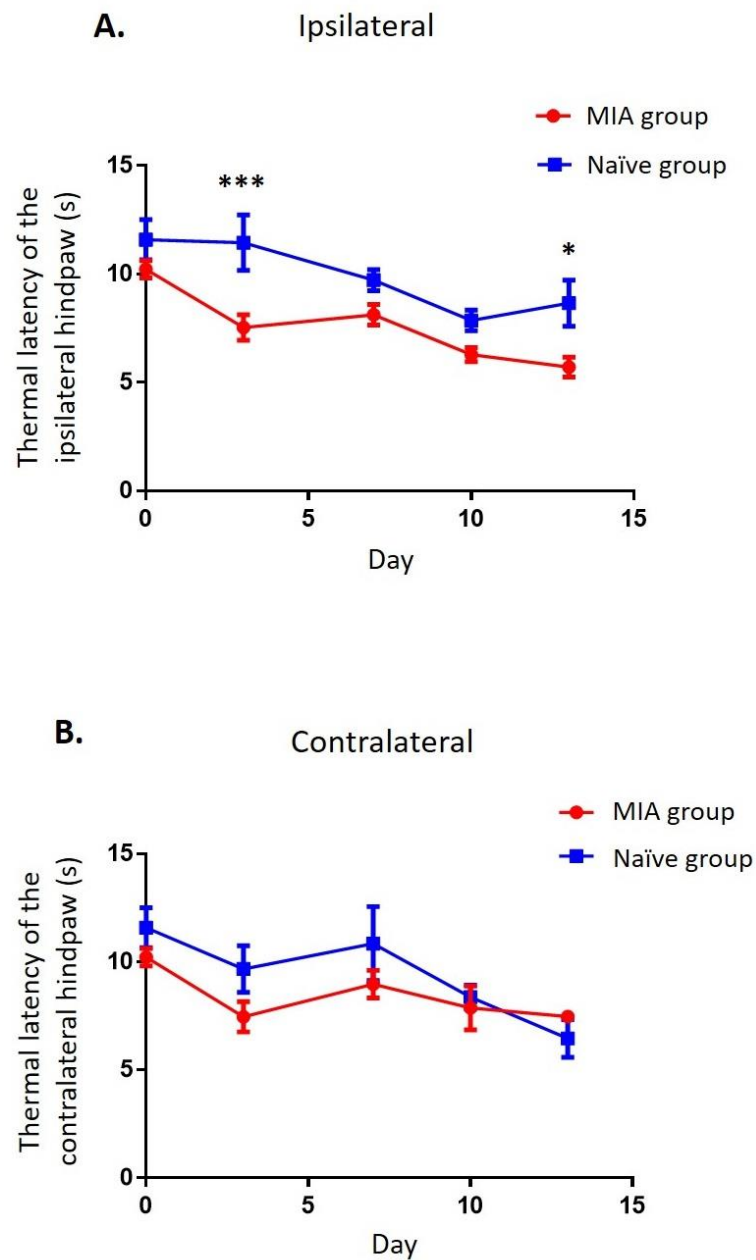


Figure 3.2. Thermal latency of the ipsilateral paw significantly decreased in the MIA group compared to the naïve control group. **A)** On days 3 and 13 post MIA injection the MIA rats showed a significant decrease in the ipsilateral thermal latency compared to the rats that were not injected. **B)** No statistical difference was found between the thermal latency of contralateral paw between the MIA injected group and the control group. (MIA group $n=8$ on days 0, 3 and 7 and $n=4$ on days 10 and 13; control group $n=4$. Statistical analysis: two-way ANOVA with post-hoc Bonferroni; $*p<0.05$, $***p<0.001$).

3.3.1.2. Mechanical withdrawal thresholds

MIA injected animals showed a decrease in mechanical withdrawal threshold of the ipsilateral hindpaw compared to the naïve group all over the duration of the study; however, this decrease was not statistically significant (figure 3.3).

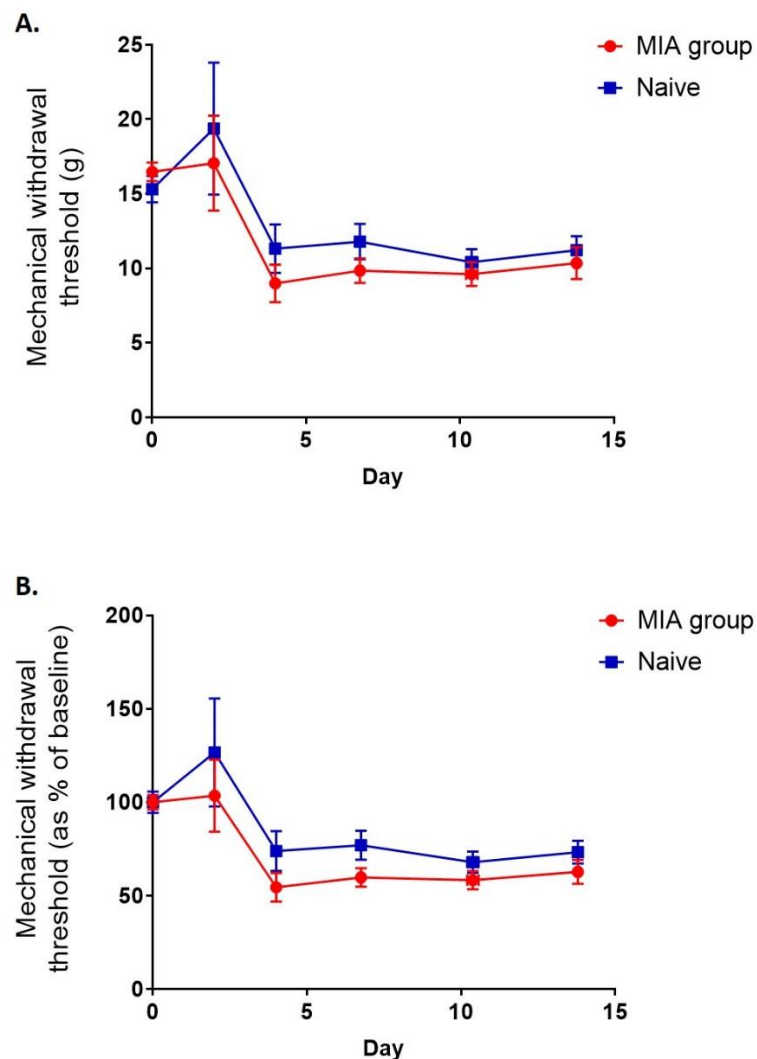


Figure 3.3. Mechanical withdrawal thresholds of the ipsilateral hindpaws in MIA injected rats. **(A)** No significant decrease in ipsilateral mechanical withdrawal thresholds was found at any time point between the MIA rats and the naïve control group. **(B)** Mechanical withdrawal thresholds of the ipsilateral hindpaw showed as percentage of baseline. (MIA group n= 4-28; two-way ANOVA).

Note: to analyse this parameter and the weight bearing asymmetry, the samples from the naïve and MIA groups of the MIA/VEGF-A_{165b} study were pooled with the samples of the current study. The pain behaviour was recorded on different days in the two studies, so weighted arithmetic means of pairs of days were plotted to get the following time points: 6.75, 10.38 and 13.79, in addition to fixed days 0, 2 and 4. This also explains the difference in the sample size (n) of each group.

3.3.1.3. Weight bearing

Two days after MIA injection, the percentage of weight borne on the ipsilateral hindpaw over the weight of both hindpaws was significantly lower in the MIA group than in the control group (38.24 ± 2.39 vs 47.76 ± 3.49 ; $p < 0.01$). Significant weight bearing asymmetry was detected over the study duration on days 4, 6.75 and 13.79 (MIA vs naïve 38.24 ± 2.39 vs 47.76 ± 3.49 , $p < 0.01$ on day 4; 34.31 ± 1.61 vs 47.74 ± 0.97 , $p < 0.0001$ on day 4; 42.38 ± 0.96 vs 47.46 ± 0.97 , $p < 0.001$ on day 6.75; 43.07 ± 1.27 vs 48.37 ± 0.75 , $p < 0.01$ on day 13.79; figure 3.4).

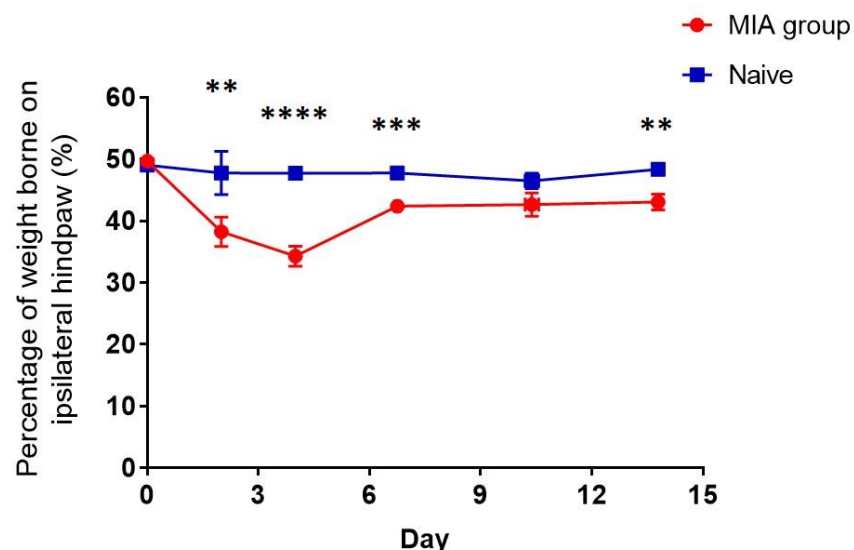


Figure 3.4. MIA injected rats developed weight bearing asymmetry. The percentage of weight borne on the ipsilateral hindpaw over the sum of weights borne on both paws significantly decreased in the MIA group compared to the control group starting on day

2 after MIA injection (n=4-28/group; two-way ANOVA with Bonferroni's post hoc test; **p<0.01, ***p<0.001, ****p<0.0001).

3.3.2. Chondropathy, synovial inflammation and osteophytosis

To assess the extent of osteoarthritic pathologic changes in the MIA injected rats, knee joints were assessed macroscopically using the Guingamp score system (Guingamp et al., 1997) and then microscopically as described by Janusz (Janusz et al., 2002).

3.3.2.1. Macroscopic chondropathy score

Tibiofemoral joints were disarticulated as explained in section 2.8.3.3 to expose tibial and femoral cartilage. Patella, femoral condyle (medial and lateral), tibial plateau (medial and lateral) and femoral groove were observed by naked eye and assigned a score according to the Guingamp score system (table 2.1; figure 3.6.A). The scores were finally summed, including an additional score of one in case of osteophyte presence (refer to section 2.10.1.1 for more details).

To validate the efficacy and consistency of the scoring process, the samples were scored twice, on two different occasions by the same blinded researcher, and the two sets of scores were analysed for correlation, using Spearman's correlation coefficient ($r = 0.7311$; 95% CI 0.4951 to 0.8666; p value < 0.0001; figure 3.5).

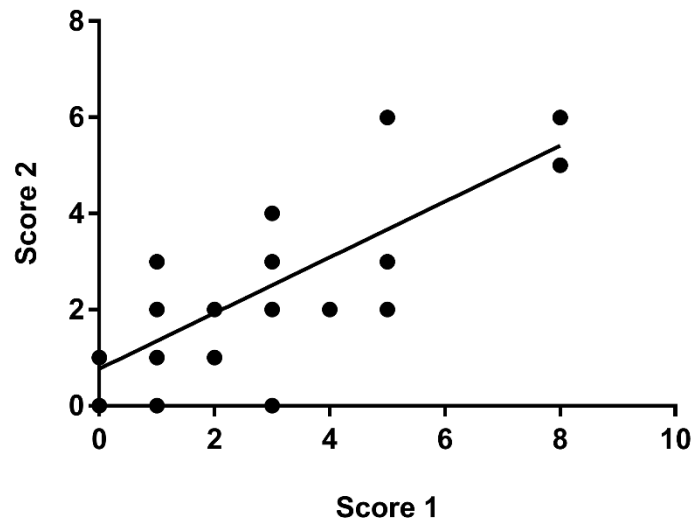
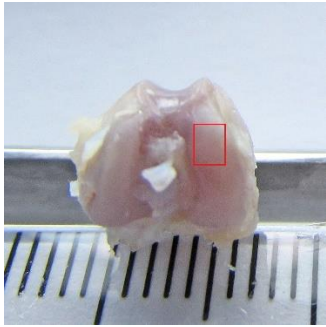
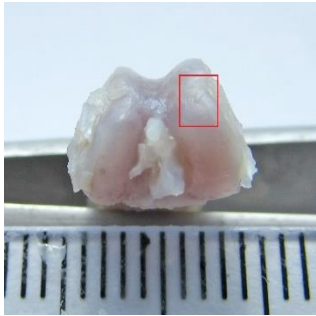
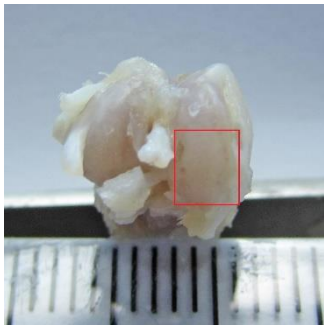
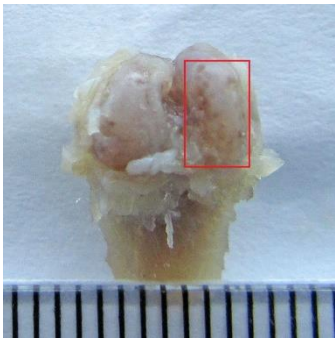
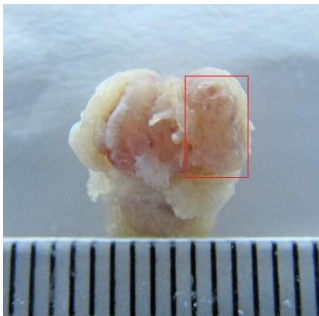


Figure 3.5. Correlation of two sets of macroscopic chondropathy scores in naïve and MIA rats obtained by the same blinded researcher at two different times. The two sets of scores obtained on two separate occasions significantly correlated (Spearman's correlation coefficient $r = 0.7311$; 95% CI 0.4951 to 0.8666; $P < 0.0001$).

Intra-articular injection of MIA did not cause a significant increase in knee macroscopic chondropathy score at day 7 or day 14 compared to the control group (figure 3.6.B), but all MIA injected rats showed some evidence of joint damage.

A.

Grade 0	
Grade 1	
Grade 2	
Grade 3	
Grade 4	

B.

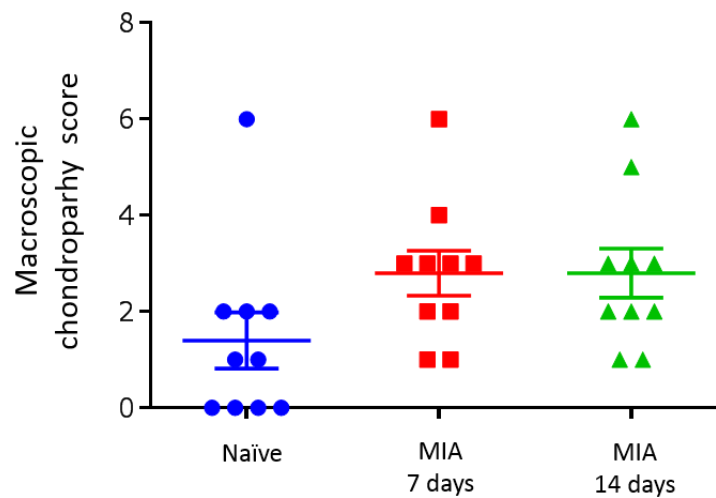
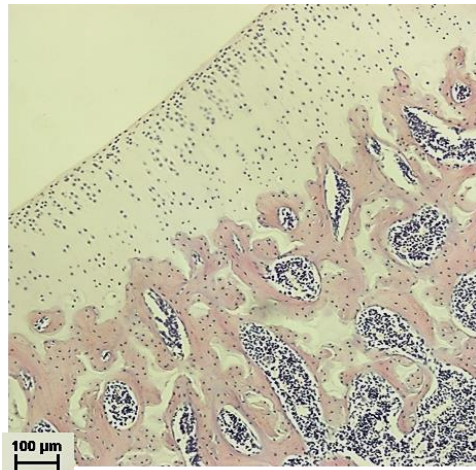


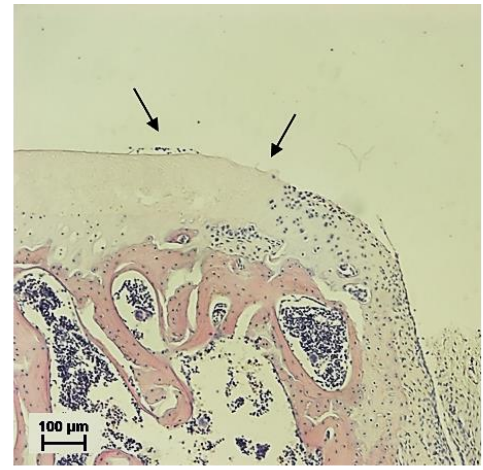
Figure 3.6. A) Example pictures of macroscopic cartilage damage in the femoral condyle in naïve and MIA rats and corresponding Guingamp scores. 1: Slight yellowish discolouration of the chondral surface. 2: Little cartilage erosions in load bearing areas. 3: Large erosions extending down to subchondral bone. 4: Large erosions with large areas of subchondral bone exposure [scale bar 1 mm]. **B) Macroscopic chondropathy score in naïve and MIA rats at day 7 and 14.** No significant difference in knee macroscopic chondropathy score was detected between any of study groups (n=10/group; Kruskal-Wallis test).

3.3.2.2. Microscopic chondropathy, synovitis and osteophyte scores

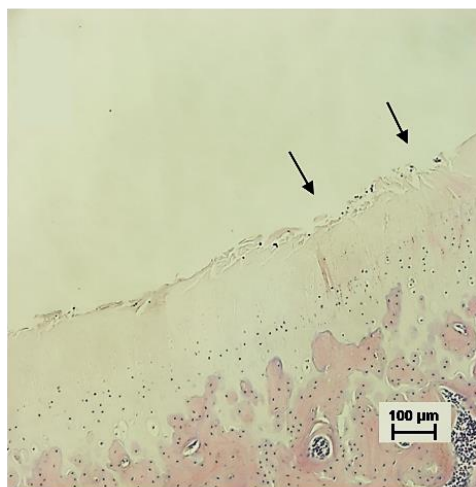
Microscopic evaluation of chondropathy, synovial inflammation and osteophyte formation was conducted as explained in section 2.10.1.2 Briefly, paraffin embedded sections of decalcified tibiae were treated for H&E staining and visualised using a X10 objective lens of a light microscope. Chondropathy, synovitis and osteophytosis were then scored according to the method described by Janusz and Mapp (Janusz et al., 2002, Mapp et al., 2008). Chondropathy in both lateral and medial tibial plateaux was scored according to the scale in table 2.2. The score was then multiplied by the portion of plateau involved by damage (1, 2 or 3 out of 3 zones of the cartilage) to obtain the overall chondropathy score. Figure 3.7 shows microscopic images of tibial cartilage.



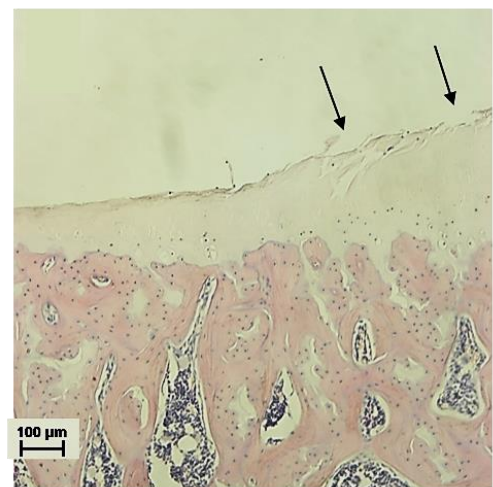
No cartilage degeneration



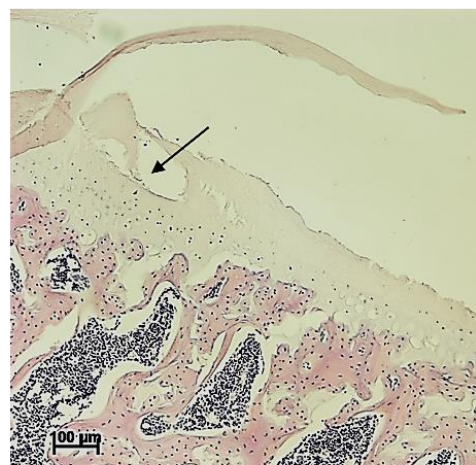
Minimal / Surface only



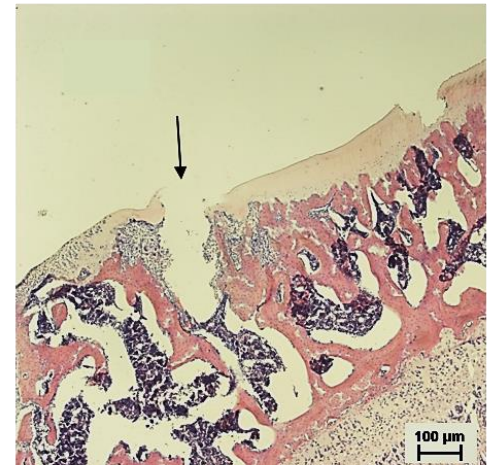
Mild / upper middle zone



Moderate / into midzone



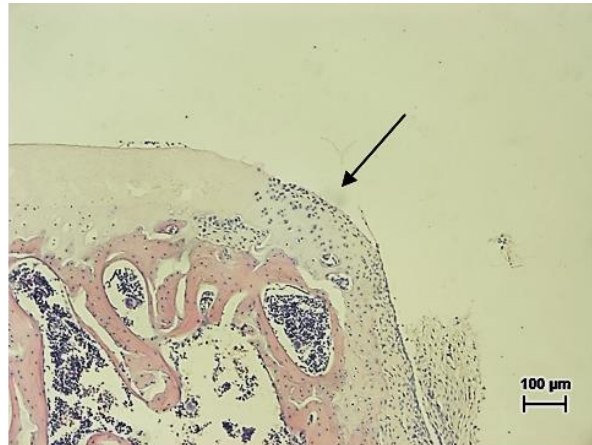
Marked / into deep zone



full thickness degeneration

Figure 3.7. Microscopic photographs of H&E stained rat tibial sections. Microscopic images of H&E stained rat tibial sections showing different degrees of chondropathy (scale bar = 100 μm).

Osteophytes were given scores of 1, 2 or 3 for mild ($<40\text{ }\mu\text{m}$), moderate ($40\text{--}160\text{ }\mu\text{m}$) or severe ($>160\text{ }\mu\text{m}$), respectively (figure 3.8).



No osteophyte



Osteophyte $< 40\text{ }\mu\text{m}$



Osteophyte $> 160\text{ }\mu\text{m}$

Figure 3.8. Microscopic photographs of H&E stained rat tibial sections. Microscopic images of H&E stained rat tibial sections showing different sizes of osteophyte in the tibia (scale bar = $100\text{ }\mu\text{m}$).

When present, synovia were assessed for lining thickness and cellularity using a X20 objective lens of a Zeiss Axioscop-50 microscope, as in table 2.3 (Mapp et al., 2008).

To evaluate the ability of the researcher to perform the microscopic scoring of chondropathy, synovitis and osteophyte in a correct and consistent way, a set of knee sections from a previous study were scored and the intraclass correlation coefficient (ICC) was calculated using the results previously obtained from an experienced technician. The two sets of scores positively correlated [ICC (95% CI) for average score for each animal = 0.87 (0.69 – 0.94), $p < 0.001$].

Microscopic chondropathy score of the MIA rats was significantly higher than the naïve animals on day 14 only (4.175 ± 0.586 MIA d14 vs 1.658 ± 0.434 naïve, $p < 0.05$; figure 3.9.A). Both MIA groups exhibited a higher osteophyte score than the naïve control group (0.43 ± 0.16 vs 0 ± 0 MIA d7 vs naïve groups, $p < 0.05$; 0.46 ± 0.16 vs 0 ± 0 , $p < 0.05$; figure 3.9.B). No significant changes in synovial inflammation score were detected in any of the MIA groups compared to the control group (figures 3.9.C).

It should be noticed that the naïve group in the microscopic scoring tests consists of the pooled readings of naïve animals from the current study, the MIA/VEGF- A_{165b} study (chapter 4) and the MIA/SPHINX-31 study (chapter 5), because some samples were too damaged to be assessed, and some were lost during sectioning or due to broken slides. This group is therefore the same in all the three in vivo studies. In addition, the group size of synovitis microscopic scores is smaller than the initial numbers because synovium was removed from most of the samples during disarticulation. This also applies to the studies in the following chapters.

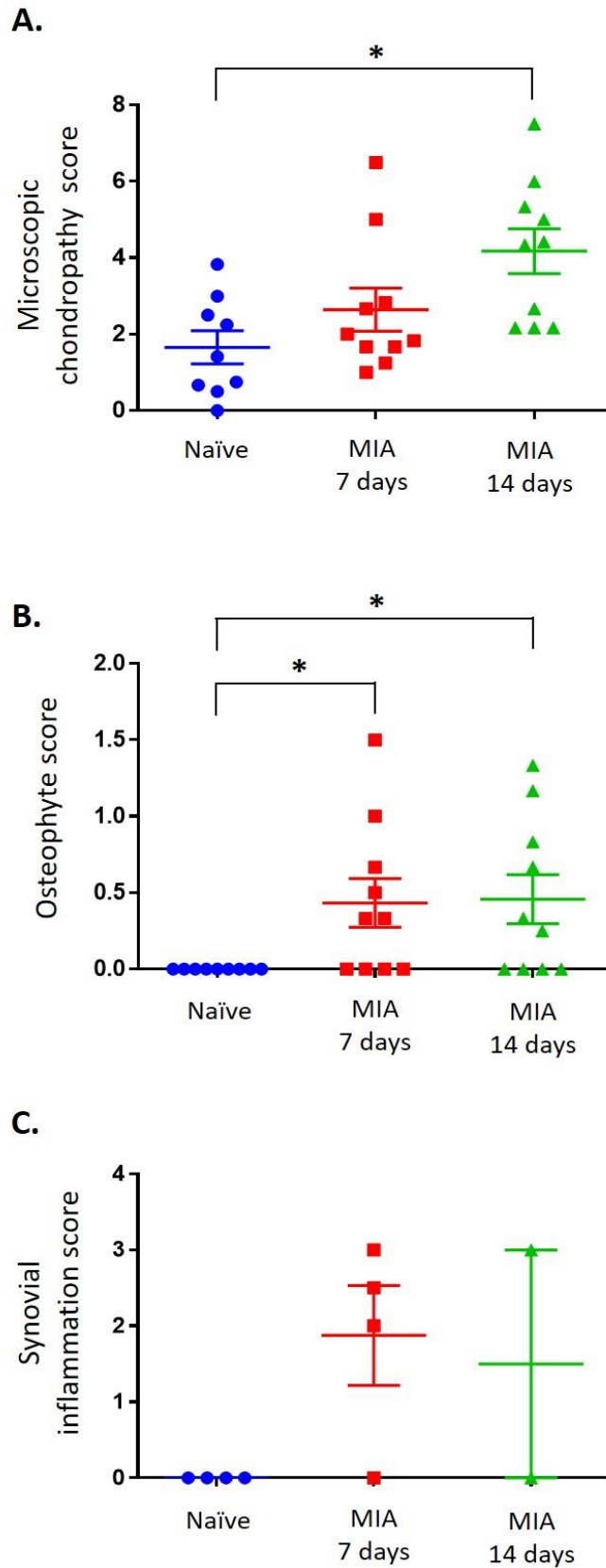


Figure 3.9. Microscopic scores of chondropathy, osteophyte and synovial inflammation in naïve and MIA animals at days 7 and 14. A) A significant increase

in microscopic chondropathy index was detected 14 days after MIA injection relative to naïve control. **B)** Both MIA groups showed a significantly higher osteophyte score compared to the naïve group. **C)** No significant differences in synovial inflammation was detected between any of the study groups. [n=9-10/group (except for synovial inflammation score, where n= 2-4); Kruskal-Wallis test with Dunn's post-hoc, *p<0.05].

3.3.3. Thermal withdrawal thresholds

Thermal withdrawal thresholds (°C) of the bicep femoris were measured in naïve and MIA rats (at day 7 post-injection) after the preferential activation of the hindpaw A- and C-nociceptors. No significant differences in the evoked thermal withdrawal thresholds were detected between the MIA and naïve groups upon activation of A- or C-fibres (figure 3.10).

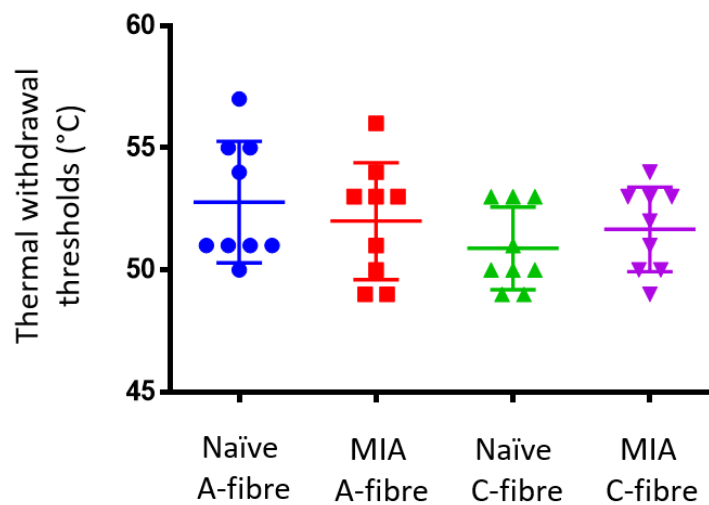


Figure 3.10. Evoked thermal withdrawal thresholds in naïve and MIA injected rats at day 7. No significant difference in thermal withdrawal thresholds was detected between MIA (day 7) group and control group for each of the A-fibre and C-fibre evoked stimuli. (n=9/group, one-way ANOVA).

3.3.4. Glial cell activation in the spinal cord

Fixed perfused sections from the spinal cords of rats with MIA arthritis were stained for activated microglia and astrocytes using anti-CD11b and anti-GFAP antibody, respectively (see section 2.9.2.2). CD31 was also used as a marker of vascular endothelial cells; however, its signal was weak and inconsistent, so no quantification of this protein expression was subsequently performed. DAPI was used to identify cell nuclei. Figure 3.11 contains representative images of CD11b, GFAP, CD31 and DAPI immunofluorescence in spinal cord.

Figure 3.11

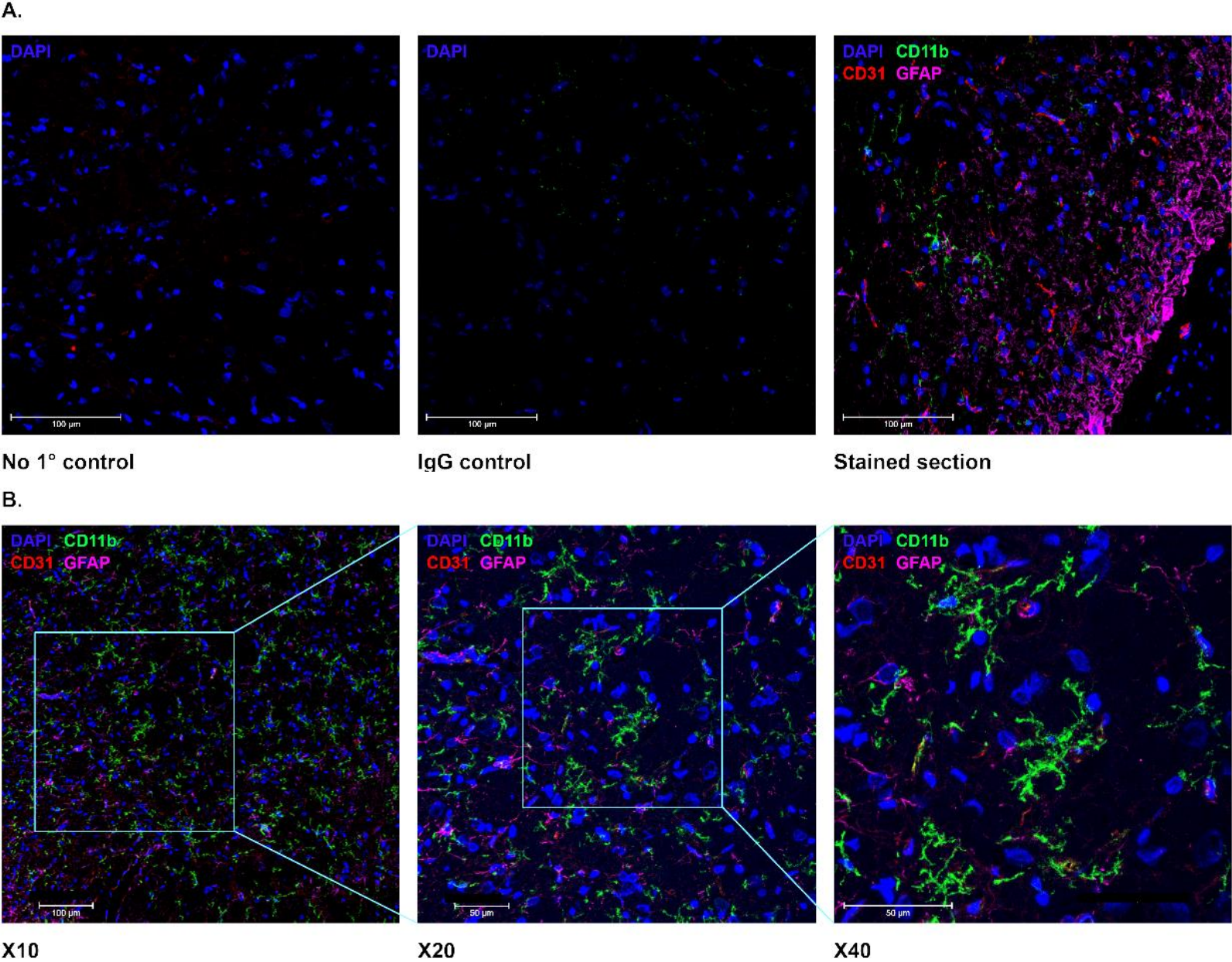


Figure 3.11

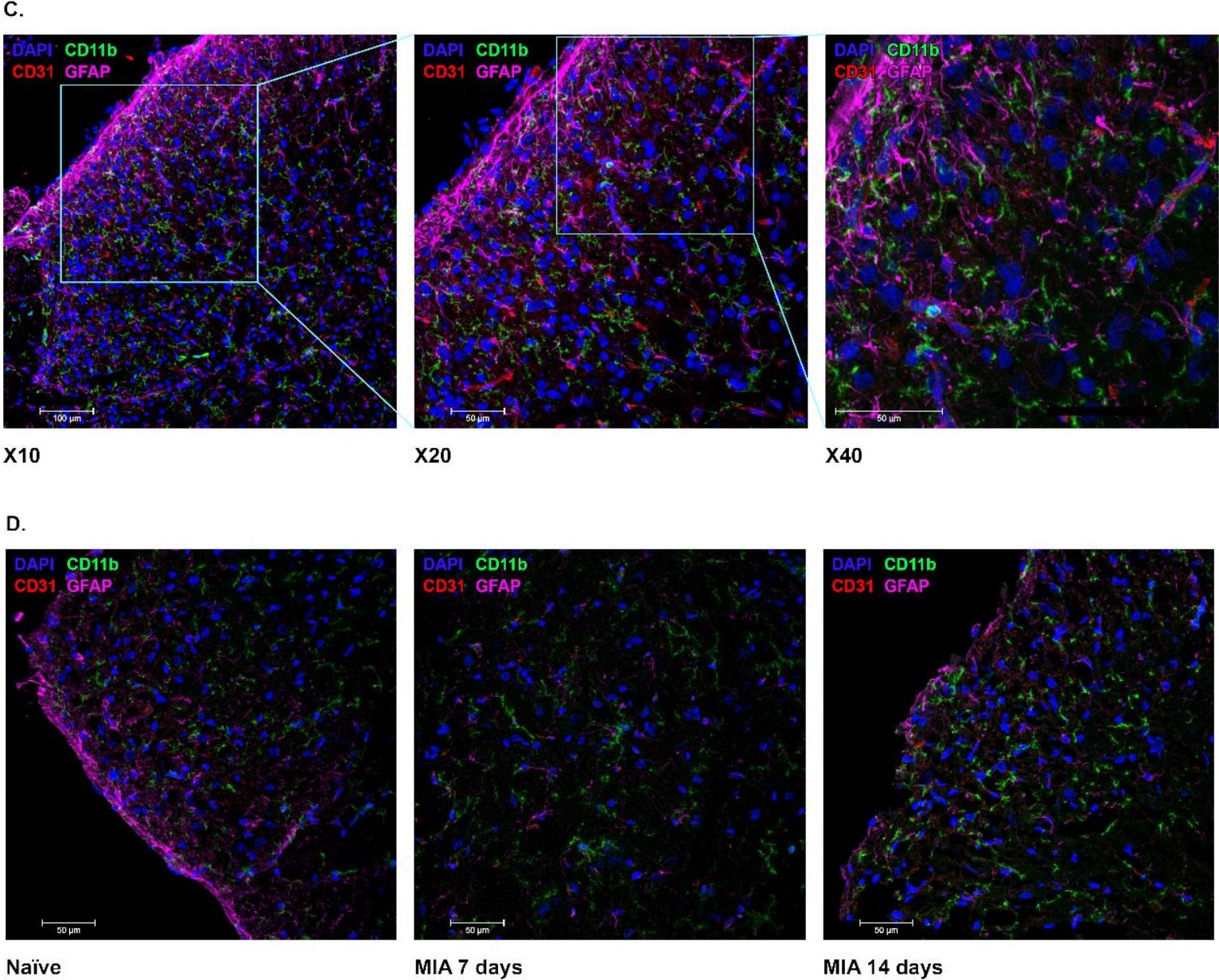


Figure 3.11

E.

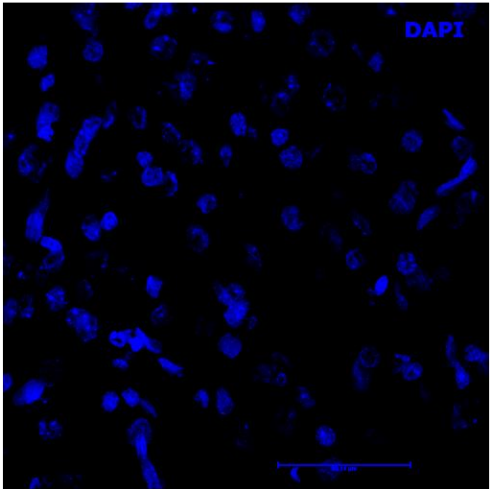
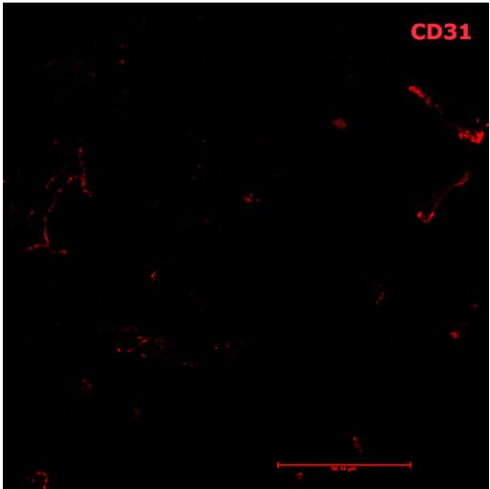
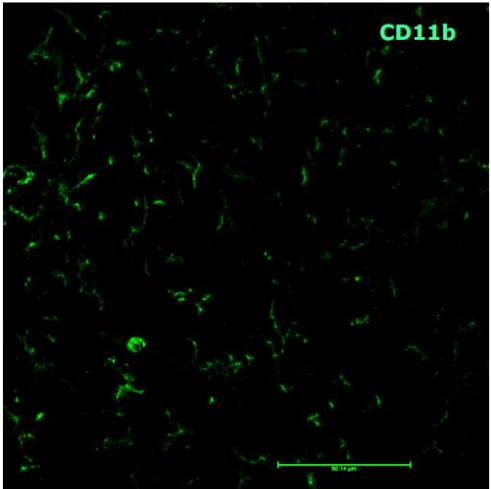
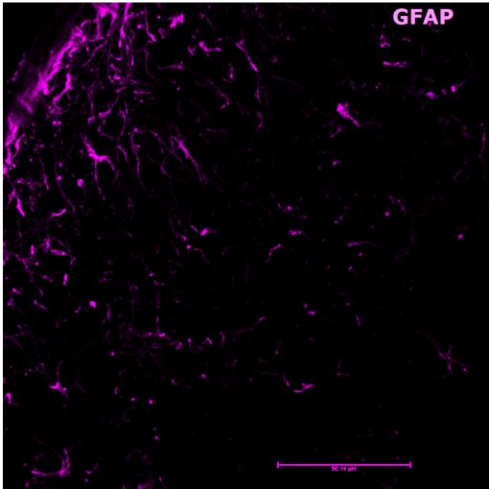
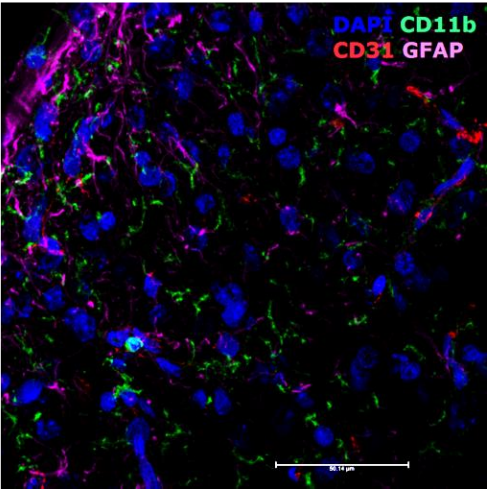


Figure 3.11. Immunofluorescence of CD11b, GFAP, CD31 and DAPI in laminae I, II of the spinal cords from rats of the MIA characterisation study. **A)** Confocal images of two controls sections (no primary antibodies and type and concentration matching IgG) and a section treated with all primary antibodies (CD11b = green, GFAP = magenta, CD31 = red and DAPI = blue). **B)** Confocal images of a stained spinal cord section at increasing magnifications (10x, 20x, 40x) showing evident CD11b immunofluorescence. **C)** Confocal images of a stained spinal cord section at increasing magnifications (10x, 20x, 40x) showing GFAP immunofluorescence. **D)** Representative sections from the naïve, MIA day 7 and MIA day 14 groups showing CD11b, GFAP, CD31 and DAPI immunofluorescence. **E)** Immunofluorescence of DAPI, CD31, CD11b and GFAP showed as separate channels and merged image (scale bar in **(E)** = 50 μ m).

3.3.4.1. Microglia

Activated microglia were detected in laminae I, II of the spinal cord using CD11b as a marker, as explained in section 2.9.2.2. Activation was quantified by both counting of cell number and measurement of average fluorescence intensity (see section 2.10.2). No significant difference in the percentage of CD11b +ve cells was found between any groups in both the ipsilateral and contralateral dorsal horn (figure 3.12.A). On the other hand, MIA rats at 14 days showed an increase in the CD11b immunofluorescence intensity compared to the MIA rats at day 7 in both ipsilateral and contralateral dorsal horn (ipsilateral MIA d14 1.95 ± 0.40 vs MIA d7 0.15 ± 0.24 , $p < 0.05$; contralateral MIA d14 1.98 ± 0.36 vs MIA d7 0.32 ± 0.31 ; $p < 0.05$; figure 3.12.B).

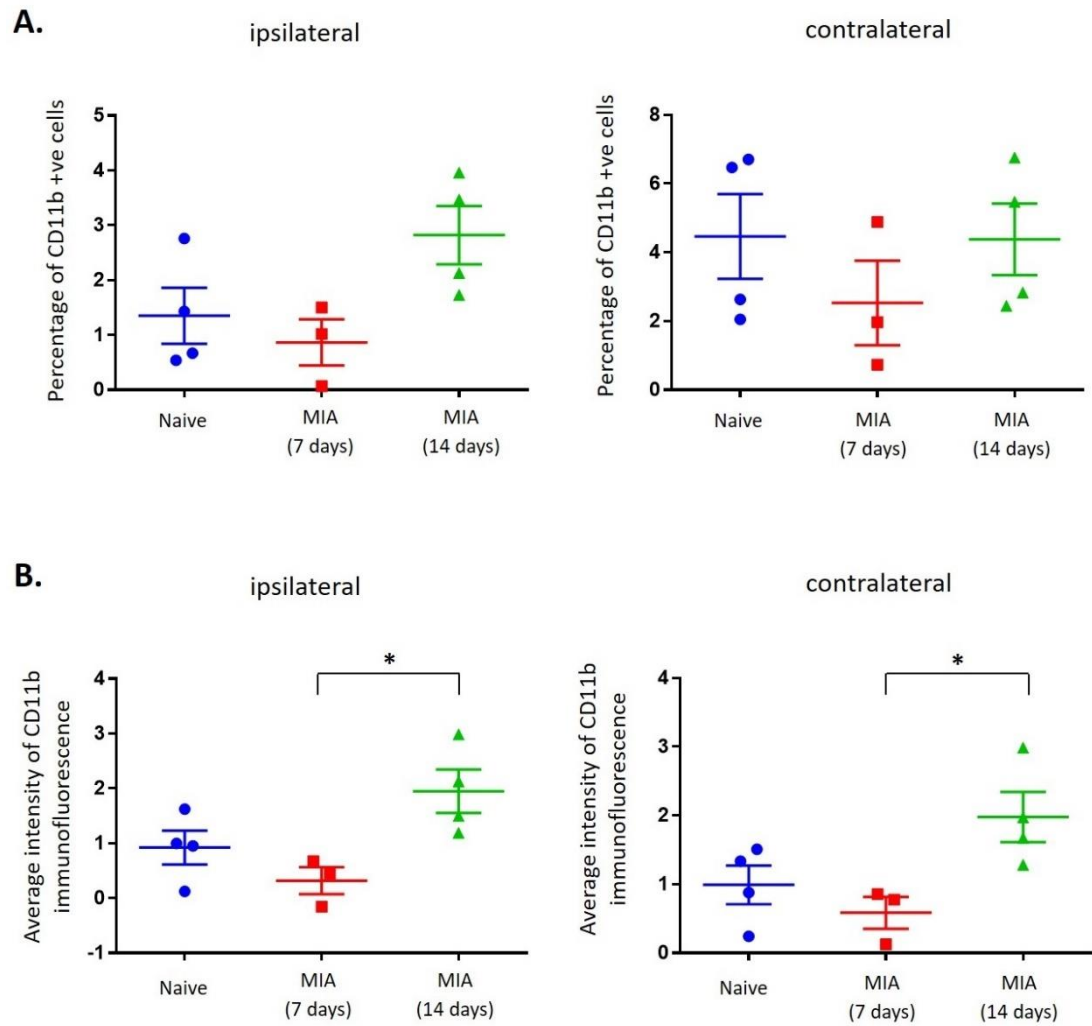


Figure 3.12. Microglia activation in the ipsilateral and contralateral spinal cord of naïve and MIA rats at days 7 and 14 post-injection. A) No significant difference in the percentage of CD11b +ve cells was found between any groups in the ipsilateral and contralateral dorsal horn. **B)** MIA rats at 14 days showed a significantly increased CD11b fluorescence intensity in the ipsilateral and contralateral dorsal horns compared to the MIA rats at day 7. (n= 4/group [except for MIA day 7 group, where n=3 due to failed staining]; Kruskal-Wallis analysis with Dunn's multiple comparisons test, *p<0.05).

3.3.4.2. Astrocytes

Changes in the average intensity of glial fibrillary acidic protein (GFAP) immunofluorescence were used to detect changes in the activation of astrocytes in the spinal cord of naïve rats and MIA injected counterparts at days 7 and 14

post model induction. Intra-articular injection of MIA caused no significant changes in the fluorescence intensity of GFAP in both the ipsilateral and contralateral portions of spinal cord at either day 7 or 14 (figure 3.13). A post-test for linear trend was performed to detect whether the fluorescence intensity of GFAP increased systematically with time, but it resulted not significant in both ipsi- and contralateral spinal cords.

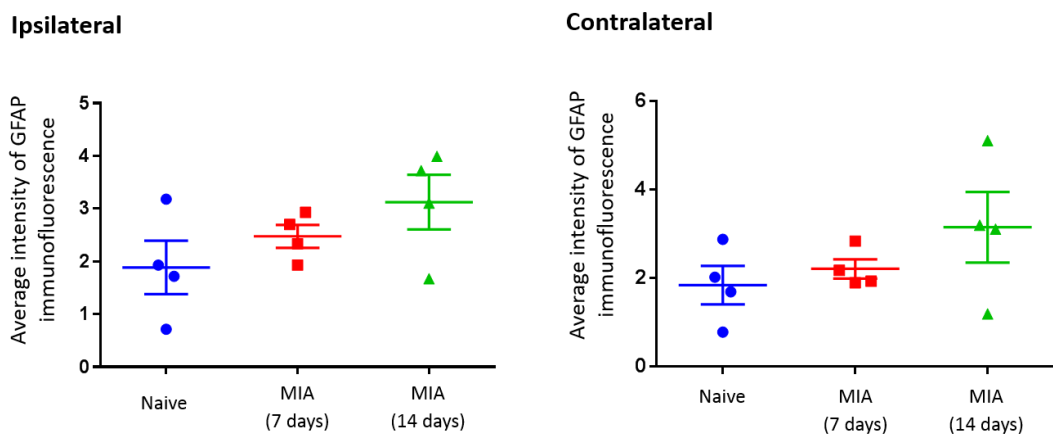


Figure 3.13. Astrocyte activation in ipsilateral and contralateral spinal cord of naïve and MIA rats at days 7 and 14 post-MIA injection. MIA rats at 7 and 14 days showed no significant changes in GFAP immunofluorescence in either ipsilateral or contralateral dorsal horn compared to the naïve group. (n= 4/group, Kruskal-Wallis analysis).

3.3.5. COX-2 expression in spinal cord

COX-2 in spinal cord was detected using immunofluorescence (figures 3.14, 3.15 and 3.16). Anti-NeuN antibodies were used to mark neuronal nuclei and therefore detect co-localisation of COX-2 with neuronal cells. For this study, one image was taken for each area of the ipsilateral and contralateral spinal cord (laminae I-II, laminae III-V and ventral horn). The total number of cells was estimated by the number of DAPI +ve cells, which were automatically counted using ImageJ. Cells expressing COX-2 (with or without NeuN) were counted manually.

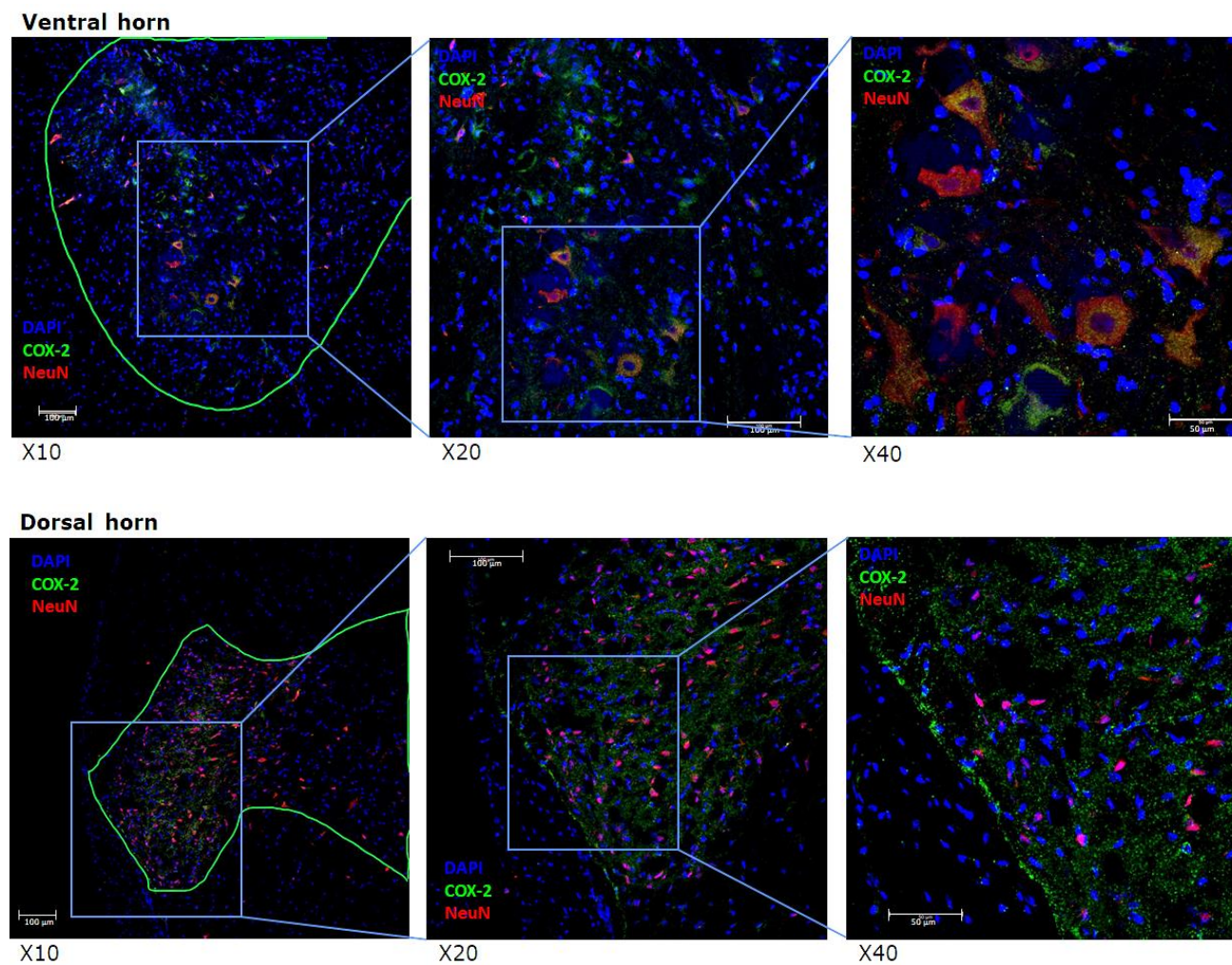


Figure 3.14. COX-2 and NeuN immunofluorescence in the spinal cord of naïve and MIA rats. Representative confocal images of COX-2 (green), NeuN (red) and DAPI (blue) immunofluorescence in the dorsal horn and ventral horn of the spinal cord in MIA and naïve rats, respectively, at increasing magnifications (10X, 20X, 40X).

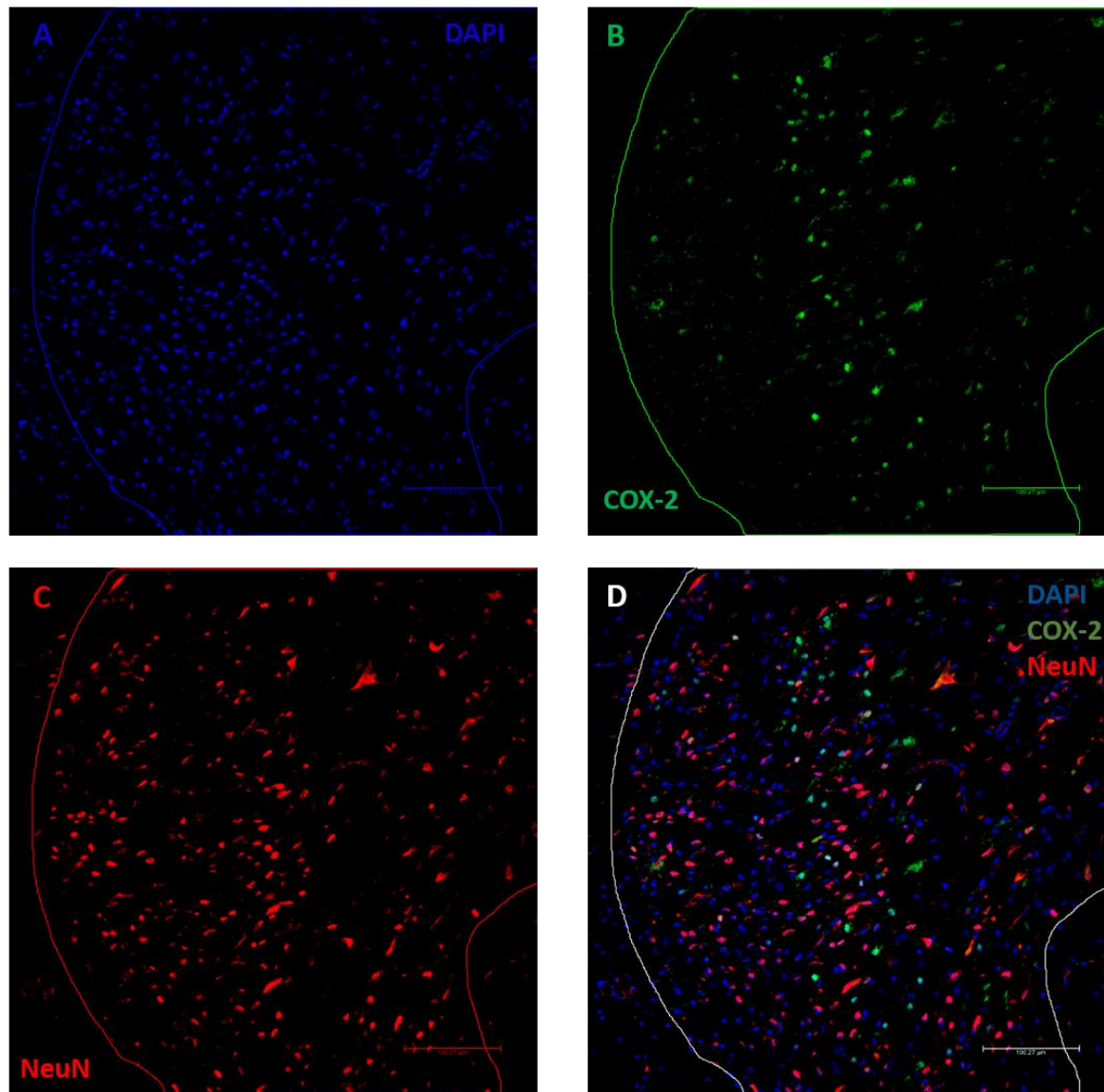
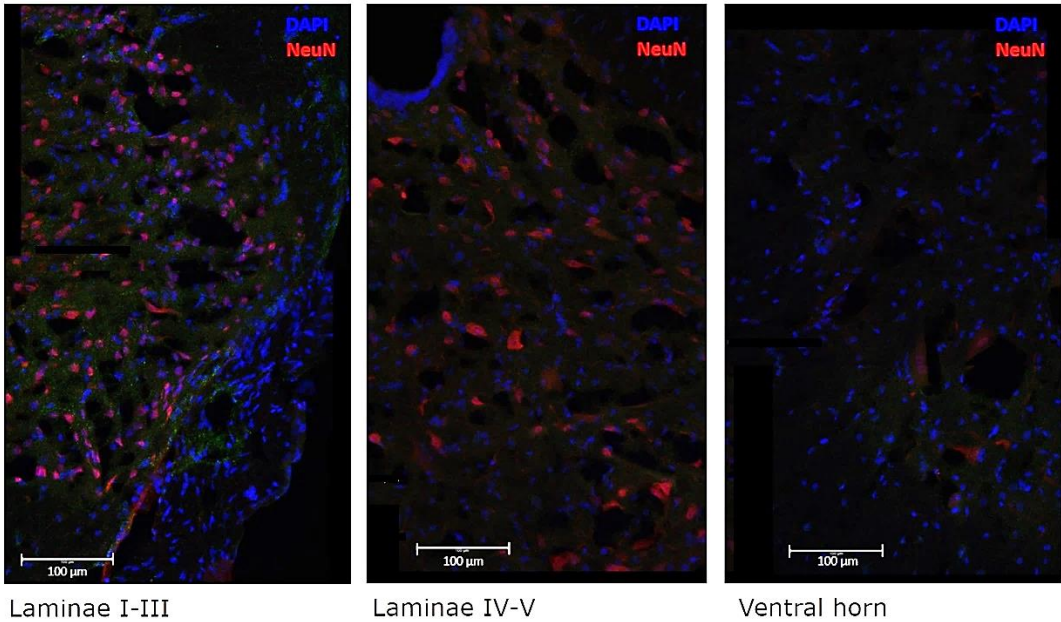


Figure 3.15. Spinal cord COX-2 and NeuN immunofluorescence. Confocal images of a spinal cord section showing immunofluorescence of (A) DAPI (blue), (B) COX-2 (green), (C) NeuN (red) and (D) a merged image of the three channels.

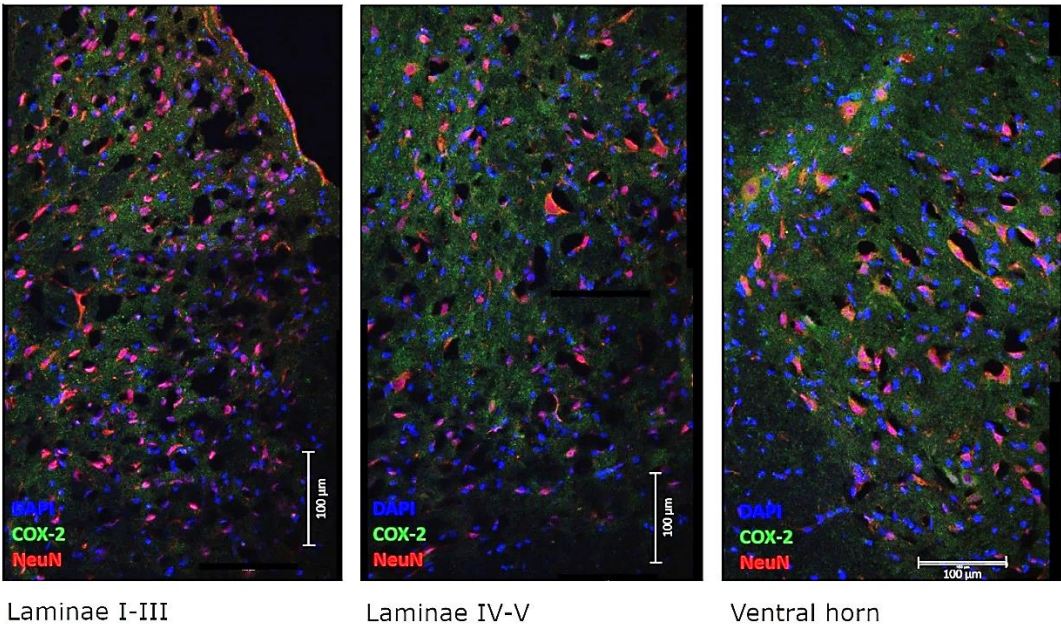
(Scale bar = 100 μm)

Figure 3.16

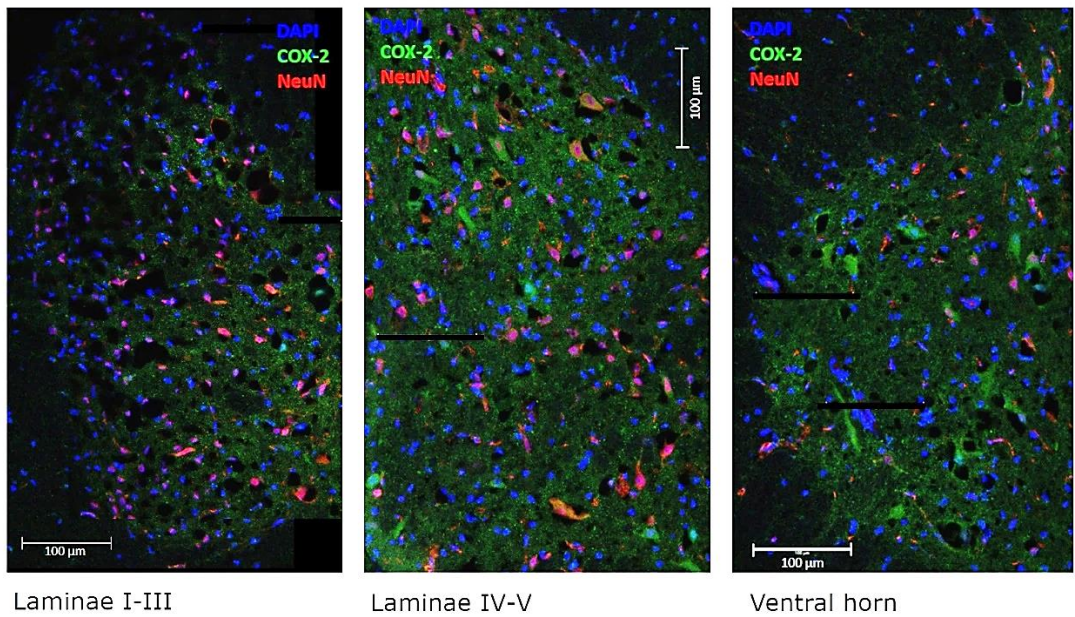
No 1° control



Naïve



MIA day 7



MIA day 14

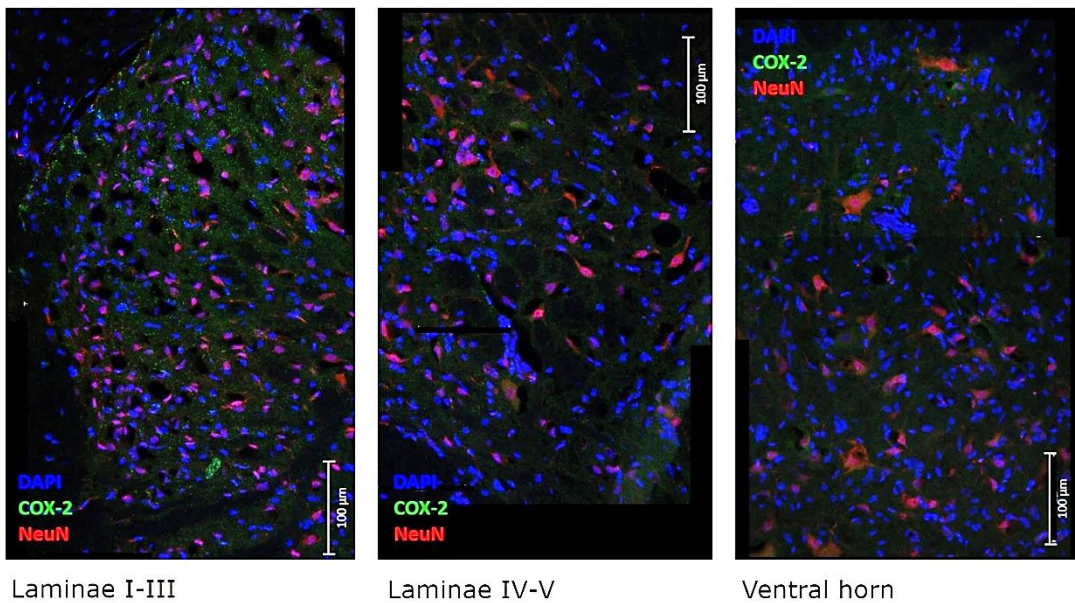


Figure 3.16. COX-2 and NeuN immunofluorescence in the spinal cord of naïve and MIA rats. Representative images of COX-2 (green), NeuN (red) and DAPI (blue) immunofluorescence in three areas of the spinal cord (laminae I – III, laminae IV,V and ventral horn) in naïve, MIA day 7 and MIA day 14 groups. The first row shows a negative control of the IF process, consisting of sections not treated with anti-COX-2 primary antibodies.

No significant differences in the percentage of COX-2 +ve / NeuN +ve or COX-2 +ve / NeuN -ve cells was found between the naïve and MIA groups in any of the ipsilateral (figure 3.17) or contralateral laminae (figure 3.18).

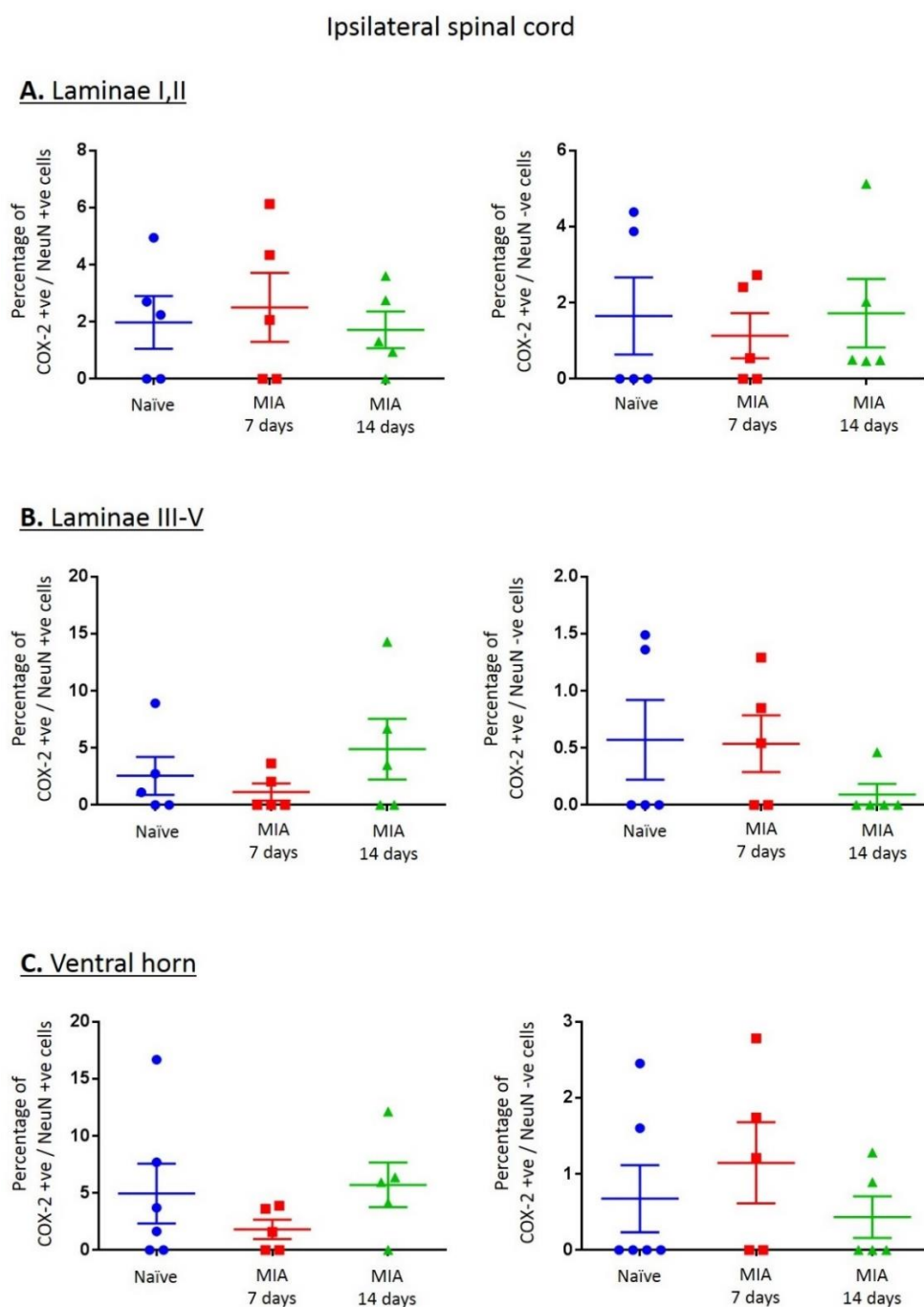


Figure 3.17. Expression of COX-2 in neuronal (NeuN +ve) and non-neuronal (NeuN -ve) cells in laminae I-II, laminae III-V and ventral horn of the ipsilateral spinal cords. No significant differences in the percentage of COX-2 +ve / NeuN +ve

cells or COX-2 +ve / NeuN –ve cells were found between the naïve and MIA groups in (A) laminae I-III, (B) laminae IV, V or (C) ventral horn. (n= 5-6/group; Kruskal-Wallis test).

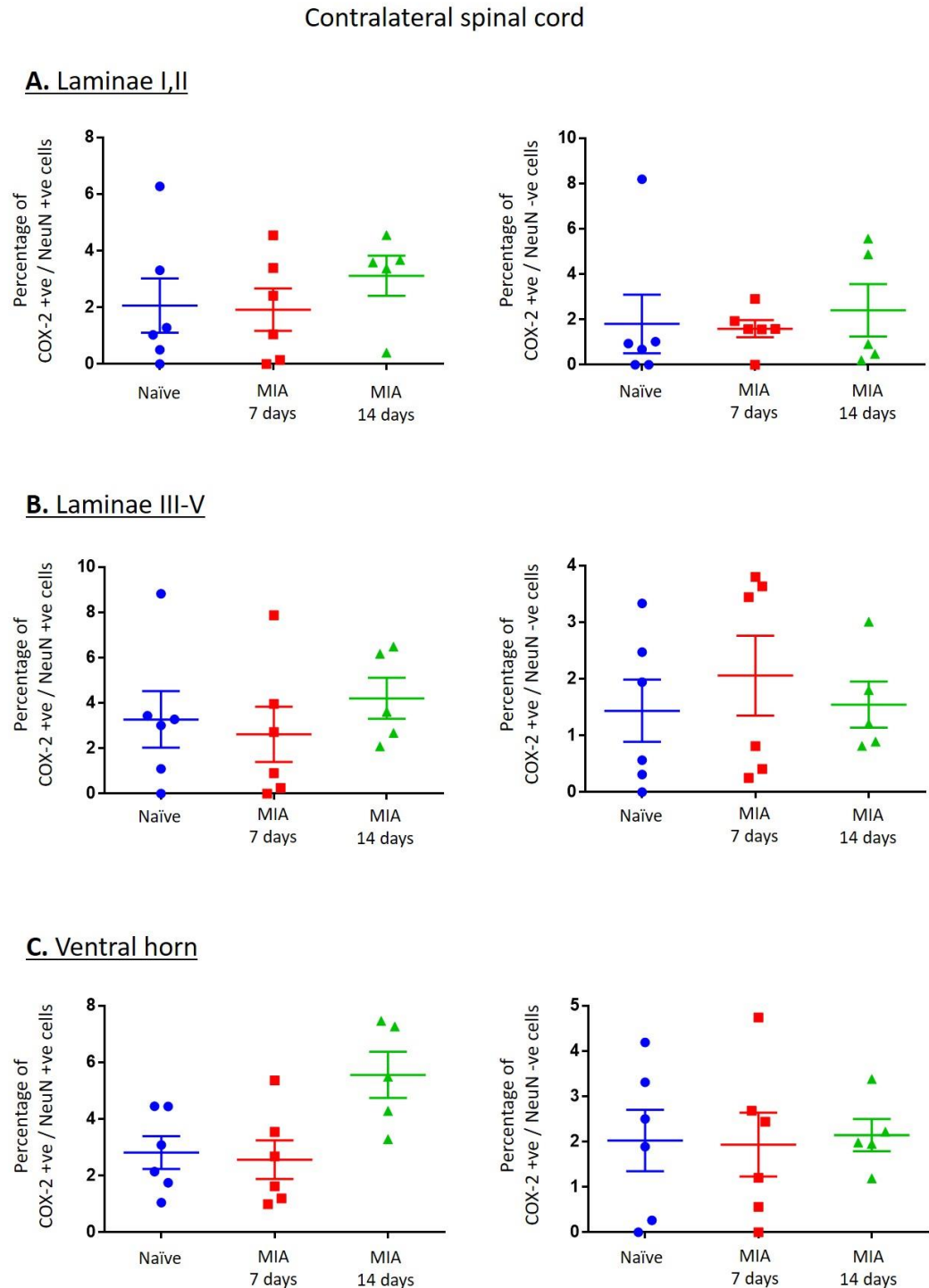


Figure 3.18. Expression of COX-2 in neuronal (NeuN +ve) and non-neuronal (NeuN -ve) cells in laminae I-II, laminae III-V and ventral horn of the contralateral

spinal cords of MIA injected rats on days 7 and 14 after injection. No significant differences in the percentage of COX-2 +ve / NeuN +ve cells or COX-2 +ve / NeuN –ve cells were found between the naïve and MIA groups in **(A)** laminae I-II, **(B)** laminae III-V and **(C)** ventral horn. (n= 5-6/group; Kruskal-Wallis test).

3.3.6. COX-2 expression in PAG

A trial of digital PCR (dPCR) was attempted to quantify changes in the expression of COX-2 mRNA in the PAG. However, the results were not conclusive. Therefore, conventional PCR was performed to detect the expression of COX-2 in the obtained samples. Sixty-five PAG samples from 18 animals were available for analysis, including samples from the dorsolateral (DL) and ventrolateral (VL) PAG from both ipsilateral and contralateral sides. PCR for GAPDH was run and 52 samples amplified it, indicating the presence of cDNA (figure 3.19).

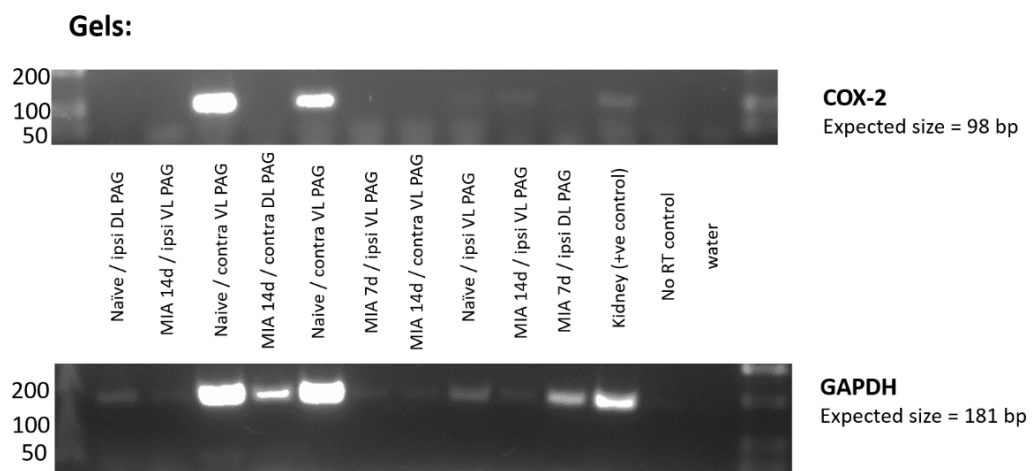


Figure 3.19. Agarose gel electrophoresis of PCR assay for COX-2 (and GAPDH) of a representative set of samples from the MIA characterisation study.

Distribution of samples between the groups and the number of samples expressing COX-2 are summarised in figure 3.20.A. A significantly higher percentage of samples expressing COX-2 was detected in the control group

compared to the MIA groups in the contralateral VL PAG (100.0% naïve vs 33.33% MIA; difference between proportions 0.6667, CI 95% 0.1315 to 0.8794; Fisher's exact test, $p < 0.05$). No significant changes in the percentage of samples expressing COX-2 were found between the MIA groups and the naïve control group in the other regions of the PAG (figure 3.20.B).

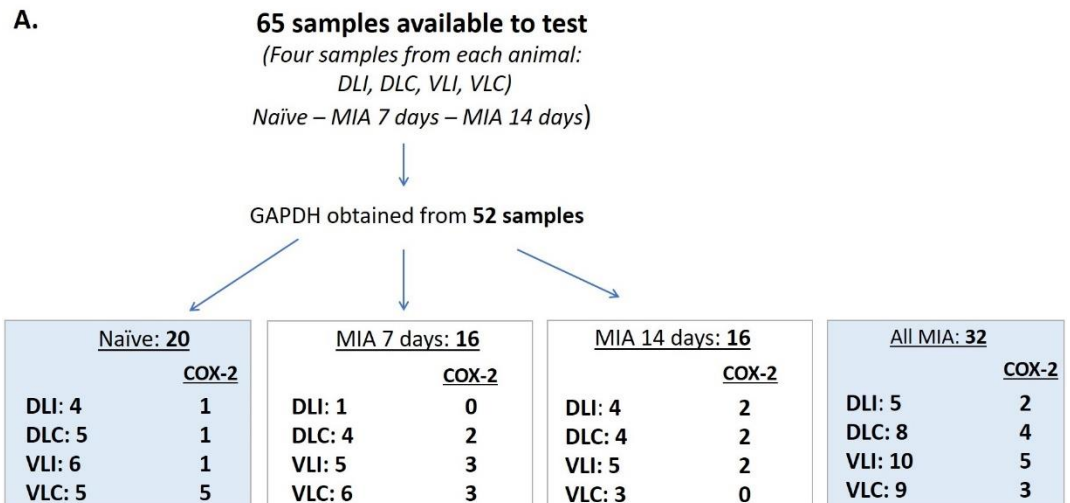
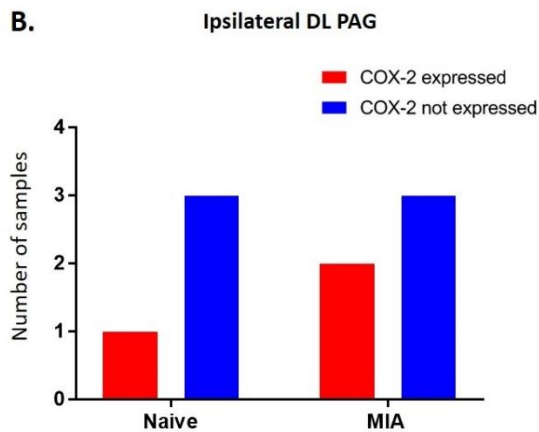
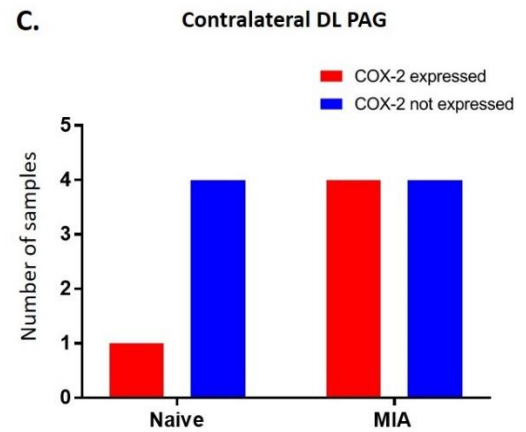
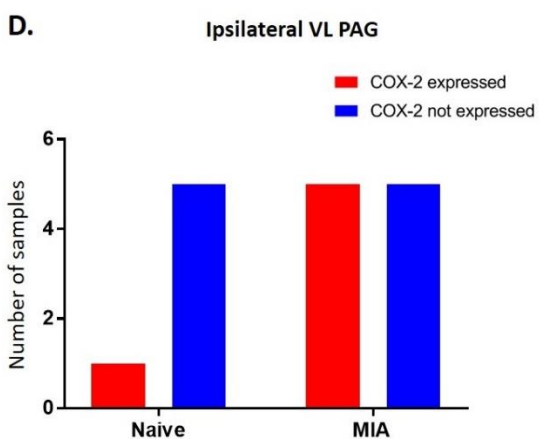
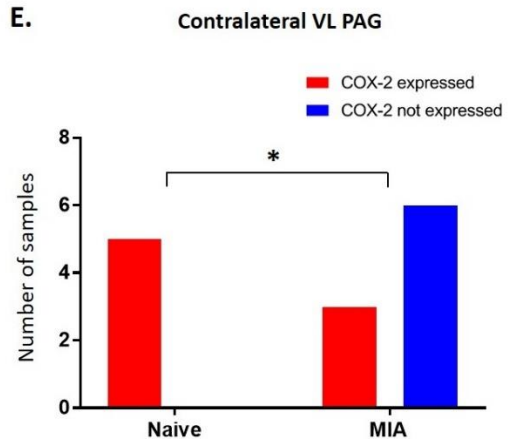


Figure 3.20. COX-2 expression in the PAG in the MIA characterisation study. A)
 Diagram showing the numbers and distribution of PAG samples within the groups

representing four PAG sites. No significant difference in the percentage of samples expressing COX-2 was found between naïve and MIA groups in the ipsilateral **(B)** and contralateral DL PAG **(C)** or the ipsilateral VL PAG **(D)**. In the contralateral VL PAG **(E)** the percentage of samples expressing COX-2 was significantly lower in the MIA group than the naïve control group. (n=4-10/group; Fisher's exact test, *p<0.05).

3.4. Discussion

3.4.1. Pain behaviour in the MIA model of OA

A number of behaviour tests are used for the assessment of pain in models of arthritis, including 1) the incapacitance meter test for weight bearing asymmetry, 2) plantar hindpaw stimulation by von Frey hairs, which measures mechanical withdrawal thresholds and 3) the plantar test (Hargreaves' method) for the measurement of thermal hyperalgesia. Weight bearing asymmetry in rats and mice is considered a correlate of standing pain in patients and it is caused by the tendency to move away from or put less weight on the affected joint (Christiansen and Stevens-Lapsley, 2010). On the other hand, mechanical withdrawal thresholds represent diffuse or referred pain (secondary hypersensitivity) (Arendt-Nielsen et al., 2010). Hindpaw thermal latency describes secondary hyperalgesia to thermal stimuli. Both weight bearing and withdrawal thresholds can be measured in small animals to provide easy and reliable indications of joint discomfort (Bove et al., 2003) and secondary allodynia (Sagar et al., 2010), respectively. Weight bearing is both peripherally and centrally driven while paw withdrawal threshold is mainly centrally driven (Graven-Nielsen and Arendt-Nielsen, 2002, Arendt-Nielsen et al., 2010).

The main objective of this part of the study was to ensure that the MIA model of OA could be effectively reproduced by the researcher. This was assessed by tests of pain behaviour and evaluation of joint pathology. The patterns of pain behaviour reported in the MIA model of OA varied considerably among studies, and this may be attributed to different doses of MIA, rat strains and assessment techniques (Mapp et al., 2013). In general, intra-articular injection of 1 mg of

MIA in the rat knee was found to induce 1) significant weight bearing asymmetry starting as early as 3 days after injection and lasting up to 35 days, 2) a significant decrease in ipsilateral mechanical withdrawal thresholds over the same time frame, but with more variability in onset and significance (Nwosu et al., 2016b, Nwosu et al., 2016a, Burston et al., 2016, Sagar et al., 2014, Kelly et al., 2013, Ashraf et al., 2013) and 3) significant reduction in ipsilateral hindpaw withdrawal latency to noxious thermal stimuli from day 3 to day 35 post-MIA injection (Im et al., 2010). MIA injected rats in the current study showed a significant decrease in weight bearing asymmetry starting from day 2 and persisting throughout the study (14 days), and this was comparable with the amount and timing of weight bearing imbalance reported in previous literature (see above). On the other hand, the extent of mechanical withdrawal sensitisation in the current study was not as pronounced as previously reported; MIA rats showed lower mechanical withdrawal thresholds since day 2 and for the duration of the study, but this decrease did not reach statistical significance at any time point. This was probably due to poor technique and inexperience of the researcher. MIA injection also caused a significant decrease in thermal withdrawal latency on days 3 and 13 of the current study. This reproduces the results found in literature (Im et al., 2010), although in other studies thermal hypersensitisation was not observed (Vermeirsch et al., 2007), or only developed with a high dose (4.8 mg) of MIA (Okun et al., 2012, Liu et al., 2011, Kim and Kim, 2013).

3.4.2. Chondropathy, synovial inflammation and osteophytosis

Extent of cartilage damage in the MIA injected rats was assessed using both macroscopic and microscopic scoring systems. Some researchers (Vonsy et al., 2009, Rahman and Dickenson, 2015, Burnham and Dickenson, 2013) do not check the development of chondropathy, assuming that this model will cause joint damage, and therefore findings related to OA are discussed in the absence of evidence of OA-like damage. Although assessment of chondropathy is

important to ascertain the actual development of OA pathology, it is known that pain in OA is poorly correlated to the degree of joint damage (Kidd, 2012, Sofat et al., 2011, Hart and Spector, 1995, Conaghan and Felson, 2004). In addition, pain in this model of OA can develop before significant cartilage damage can be detected (Mapp et al., 2013, Ivanavicius et al., 2007), so the need to verify joint damage is not essential when focusing on pain studies. In this project, it was chosen to assess the extent of cartilage pathology both macroscopically and microscopically to obtain a wider understanding of the MIA model of OA and to investigate the potential effects of VEGF-A₁₆₅b (chapter 4) and SPHINX-31 (chapter 5) on OA pathology.

In the current study, macroscopic scores of the MIA animals were not significantly higher than those in the naïve group at either time points (day 7 and 14), but evidence of mild joint damage was noted in the MIA injected joints. On the other hand, the microscopic chondropathy score was significantly higher in the MIA rats 14 days post-injection, indicating a significant worsening of cartilage pathology compared to the naïve control. Although not significantly different from the saline control, Mapp et al (Mapp et al., 2013) found a similar microscopic score (~6) in MIA rats on day 14. Bove et al (Bove et al., 2003) also found moderate collapse of cartilage matrix with loss of cellular detail in the knees of rats injected with 1 mg MIA 7 days before. At 14 days post-injection, joints showed extensive areas of partial to full thickness cartilage damage with complete loss of cellular detail and occasional osteophyte growth. However, histopathological changes were not quantified in that study. To the best of our knowledge, Guingamp method was not used to evaluate macroscopic chondropathy in the first 14 days after OA induction. However, Udo et al proposed an alternative macroscopic score system based on the evaluation of punctuate depression and erosion of cartilage (Udo et al., 2016). It was found that two weeks after MIA injection, cartilage erosion occupied most of the tibial and femoral articular surfaces, and they had a macroscopic cartilage score of 3.8 (out of a maximum of 5). This is higher than the results of the current study. The fact that the increase was significant in microscopic, but not macroscopic scores could be explained by the early progression of the disease at these time points,

in which pathologic changes are too subtle to be observed by the naked eye, but could be detected with histological staining and microscopy.

Osteophytes are “fibrocartilage-capped bony outgrowths” (van der Kraan and van den Berg, 2007) that are commonly seen in human osteoarthritis and related animal models. They most often emerge at the margins of the joint, originally as outgrowths of cartilage and subsequently undergo endochondral ossification (Felson et al., 2005), through chondrogenesis of cells and replacement of mature chondrocytes by osteoblasts (Kaneko et al., 2013). Osteophyte development is triggered by mechanical factors, growth factors, such as transforming growth factor beta (TGF β) (Scharstuhl et al., 2002), and cytokines that trigger the proliferation of mesenchymal stem cells in the periosteum (Kaneko et al., 2013). Osteophyte formation was evaluated in this study histologically in H&E stained sections of the tibiae using the scoring system suggested by Janusz (Janusz et al., 2002). MIA injected rats had significantly higher osteophyte scores at both time points compared to the naïve group. This finding agrees with the study by Mapp et al (Mapp et al., 2013), where osteophyte score was significantly higher than the saline control on day 14 post- MIA injection, although higher in value than the current study result (1.2 vs 0.46). Guingamp et al also observed large osteophytes at the margins of femoropatellar joint 15 days after MIA intra-articular injection (Guingamp et al., 1997). Other studies, however, found different time patterns in the development of osteophytes. Yamairi et al, for example, identified no signs of osteophytes 7 days after MIA injection in the rat knee (Yamairi et al., 2011), while they were reported on day 28 by Ferland et al (Ferland et al., 2011). Takahashi et al detected osteophyte formation only 6–8 weeks after MIA administration (Takahashi et al., 2017). These discrepancies in osteophyte development may be explained by the complexity of regulatory factors involved in this process [cells, growth factors, cytokines, bone morphogenetic protein secreted by chondrocytes and synovial cells (van der Kraan and van den Berg, 2007)], which can be affected differently by MIA injection (Takahashi et al., 2017). In addition, different scoring systems and MIA doses were adopted in different studies.

Most tibia samples did not contain synovium, since it was removed during the process of disarticulation. The samples that contained enough synovial tissue to be assessed were scored (n=4 for the naïve and MIA d7 groups, n=2 for the MIA d14 group). No significant difference in synovitis score was found between the MIA groups and the naïve control. This may be due to the small sample size or an actual resolution of synovial inflammation, as previously found by Bove et al, who observed the highest inflammation score one day after MIA injection, followed by a rapid decrease on day 3 and a complete resolution of inflammation on day 7 to day 14 (Bove et al., 2003). A similar trend of synovial inflammation, but with a peak on day 8, was observed in the study by (Liu et al., 2011). Other studies, however, showed contrasting results. Mapp et al, for example, found a significantly higher synovitis score in rats injected with 1 mg MIA compared to saline controls on day 14 post-injection (Mapp et al., 2013). Similarly, Udo et al detected synovial inflammation since day 3 post-MIA injection, increasing over time to reach its peak value on day 14 (Udo et al., 2016). The variable results reported in previous studies may be explained by different doses of MIA injected and/or various scoring systems used to assess synovitis.

3.4.3. Thermal withdrawal thresholds

Previous electrophysiology studies demonstrated that spinal nociceptive reflex pathways are sensitized in the MIA model of OA, suggesting the presence of central sensitization. MIA injected rats showed an overall enhanced neuronal response to mechanical stimulation, greater mechanical evoked neuronal after-discharge and enhanced thermal evoked neuronal responses compared to naïve controls (Rahman et al., 2009). Baseline levels of spontaneous firing for wide dynamic range (WDR) neurons were significantly higher in OA rats compared to sham animals in the study by (Chu et al., 2011).

A limited number of studies included EMG experiments in the MIA model of OA. Kelly et al assessed the effects of knee injection of MIA on the excitability of spinal nociceptive withdrawal reflexes (NWRs) and found that MIA injected rats had reduced mechanical thresholds in the biceps femoris at day 28, which

were maintained at the spinal level, while reflexes were largely unaltered 14 days post-MIA (Kelly et al., 2013). Similar findings were observed in case of inflammatory arthritis, but at earlier time points (Alvarez et al., 2014, Drake et al., 2016, Nieto et al., 2016). In the current study, evoked thresholds for EMG activity recorded in the hindleg bicep femoris were assessed and used to determine noxious thermal withdrawal thresholds for A and C nociceptors. No significant difference in thermal withdrawal thresholds of either A- or C- fibres in the ipsilateral hindpaw was detected between the anaesthetised MIA injected rats at day 7 and naïve controls. This indicates a lack of spinal sensitisation to thermal stimuli at this time point, which is early in the development of the disease. This result agrees with the findings by (Kelly et al., 2013), although thermal thresholds were addressed here compared to mechanical ones in the former. On the other hand, in the CFA model of inflammatory arthritis, arthritic animals had significantly lower withdrawal thresholds to thermal A-nociceptor stimulation on day 7 than naïve counterparts (Drake et al., 2016).

3.4.4. Glial cell activation in the spinal cord

Central sensitisation plays a critical role in the processing of pain in osteoarthritis, as demonstrated by the presence of distal mechanical allodynia following joint pathology (Fernihough et al., 2004, Hendiani et al., 2003) and enhanced spinal neuronal activity (Sagar et al., 2010). Once considered a purely neuronal event, central sensitisation is now known to involve glial cells, particularly in neuropathic pain (Zhuang et al., 2005). Spinal microglia activation has been observed early in the MIA model of OA pain (Orita et al., 2011) and has been associated with pain behaviour at later time points (Lee et al., 2011a), when astrocyte activation also occurred (Sagar et al., 2011).

In the current study, microglia activation in the spinal cord was assessed counting the number of CD11b reactive cells and measuring the average intensity of CD11b immunofluorescence. In both ipsilateral and contralateral spinal cords, MIA rats showed a significant increase in CD11b immunofluorescence intensity at 14 days compared to the MIA rats at day 7;

however, no significant increase was found in the MIA group at day 7 compared to the naïve controls. These findings are therefore partially contrasting to what reported by Sagar et al, where levels of activated microglia in ipsilateral spinal cord were significantly elevated as early as day 7 post-MIA injection and until day 28 (Sagar et al., 2011). In the study by Orita et al, significant microglia activation was observed on days 14, 21 and 28 (Orita et al., 2011). Comparable results were found in other pain models known to involve spinal sensitisation, including neuropathic (Echeverry et al., 2017 , Zhuo et al., 2011) and inflammatory pain (Fu et al., 2000, Aumeerally et al., 2004). Glial cell activation in the contralateral dorsal horn has been observed in some chronic pain states, such neuropathic pain (Cheng et al., 2014, Obata et al., 2010) and peripheral inflammation (Schreiber et al., 2008, Gao et al., 2010). These changes involved astrocytes and/or microglia and were involved in the development of contralateral allodynia or mirror-image pain. To the best of our knowledge, these effects were not investigated in the MIA model of osteoarthritis, so the activation of microglia detected on day 14 of the current study provides an interesting novel finding.

Spinal astrocyte activation was investigated in this study measuring GFAP immunofluorescence. No significant increase in GFAP fluorescence was detected in the MIA injected groups compared to the naïve control. This finding is consistent with results of previous studies, which found astrocytes to be not activated after MIA injection (Ogbonna et al., 2013, Lee et al., 2011a) or activated late in the course of the disease (Sagar et al., 2011, Yu et al., 2013), although early activation was demonstrated in neuropathic, inflammatory and cancer pain (Gao and Ji, 2010). The slower onset of spinal glial activation in the MIA model may reflect the development of structural changes within the joint (Sagar et al., 2011).

Microglia signalling to astrocytes and their subsequent activation is well established (Hartung et al., 1989, Milligan and Watkins, 2009) and this is a probable explanation of the distinct time courses of different glial cell activation.

3.4.5. COX-2 expression in the spinal cord

Spinal prostaglandins and their synthetic enzymes cyclooxygenases play a critical role in central sensitisation and the pathogenesis of chronic pain (Hoffmann, 2000, Svensson and Yaksh, 2002, Seybold et al., 2003), and this is particularly true in models of inflammatory arthritis. In a kaolin/carrageenan model of arthritis, for example, upregulation of spinal COX-2 was observed 3 hours after induction of joint inflammation (with a peak at 12 hours), resulting in a tonic release of PGE₂ within the dorsal and ventral horn (Ebersberger et al., 1999). Application of PGE₂ (Vasquez et al., 2001) and EP receptor agonists (Bar et al., 2004) to the spinal cord surface promoted responses of spinal cord neurons to mechanical stimulation of knee joint inflamed by kaolin. In addition, spinal administration of a COX inhibitor, indomethacin, reduced the development of hyperexcitability, but it did not affect the enhanced responses of spinal cord neurons after the establishment of inflammation and spinal hyperexcitability (Vasquez et al., 2001). This effect is thought to be mediated by the transcription factor nuclear factor- κ B (NF- κ B), because the activation of COX-2 depends on the activation of this molecule (Barnes and Karin, 1997, Lee et al., 2004). The status of the spinal cord may determine which EP receptor subtype is likely to influence the neuronal response to mechanical stimulation of the inflamed knee. After the establishment of central sensitisation and inflammation, for example, only EP1 receptor agonists further increased such response, while an EP3 α receptor agonist reduced responses to mechanical stimulation of the knee when it was spinally applied. Similarly, spinal application of PGE₂ facilitated NMDA or AMPA-mediated neuronal firing during induction and not maintenance of inflammation (Bar et al., 2004). Other studies, however, showed that endogenous cyclooxygenase products in the spinal cord contributed to the maintenance of increased spinal neuronal excitability during peripheral inflammation (Seybold et al., 2003, Samad et al., 2001). Another major PG in the central nervous system, PGD₂, has been involved in central sensitisation by exerting an opposite effect to PGE₂ (Telleria-Diaz et al., 2008).

COX-2 is constitutively expressed in the spinal cord (Samad et al., 2001), mainly by neurons and radial astrocytes (Ghilardi et al., 2004). Many different types of

spinal neurons express COX-2, such as the majority of lamina I NK-1-expressing neurons (Todd et al., 2002), which are involved in the ascending conduction of pain. On the other hand, only <1% of the GFAP +ve astrocytes in the gray matter co-express COX-2 and most glial cells such as oligodendrocytes and microglia do not express detectable baseline levels of COX-2 in the spinal cord (Ghilardi et al., 2004). However, after significant tissue injury, COX-2 upregulation is detected in a number of cell types, including neurons (Cao and Zhang, 2008), endothelial cells (Kanda et al., 2017, Hoffmann, 2000), macrophages (Durrenberger et al., 2006) and glial cells (Ikeda-Matsuo et al., 2005, Vardeh et al., 2009, Goppelt-Strube and Beiche, 1997). In the current study, COX-2 was co-localised in the spinal cord with both neurons (marked by the neuronal nuclear marker NeuN) and non-neuronal cells (marked by the absence of NeuN co-staining).

The MIA model of OA produces a relatively low level of inflammation, mainly in the synovium, which peaks on the first three to eight days after MIA injection (Bove et al., 2003, Liu et al., 2011). This process is followed by thinning of articular cartilage and subsequent lesion of subchondral bone, making it crucial for the development of the disease (Ivanavicius et al., 2007). A similar cascade of events is noticed in human OA (Golding and Goldring, 2010a), where inflammation is known to contribute significantly to pain (Bonnet and Walsh, 2005). A number of studies investigated the expression of COX isoform and their products in the spinal cord of MIA animals, and their effects on hyperalgesia. In the work by Prochazkova et al, spinal COX-2 mRNA reached maximum expression on day 5 post-MIA injection and remained increased until day 31. Expression of spinal COX-1 mRNA increased gradually reaching maximum on the day 31, when the relative expression of both genes was almost equal. The highest production of COX-2 protein was observed on day 5 after the induction of osteoarthritis, while the levels of COX-1 protein increased gradually with maximum on day 31. This also indicated a role of spinal COX-1 in osteoarthritis pain, in contrast to inflammatory pain (Prochazkova et al., 2009). Both systemic and spinal administration of the COX-2 inhibitor nimesulide attenuated spinal neuronal responses to both noxious and innocuous stimuli in the MIA model of OA, indicating the role of peripheral and spinal

COX-2 products in neuronal responses in this model (Staniaszek et al., 2010). Interestingly, nimesulide attenuated pain behaviour (assessed with weight bearing and mechanical hindpaw allodynia) and microglia and astrocyte activation in the ipsilateral spinal cord (Sagar et al., 2011).

In the current study, no significant changes in the expression of COX-2 were detected in neuronal or non-neuronal cells in any of the addressed areas of the spinal cord. This implies that MIA injection did not cause an upregulation of COX-2 expression as hypothesised, which could indicate a more limited role of COX-2 in the establishment of hyperalgesia in the MIA model of osteoarthritis. In addition, previous studies investigated the overall expression of COXs mRNA and protein (by Western blot) in the spinal cord (Prochazkova et al., 2009) or the effect on hyperalgesia as proved by efficacy of COX-inhibitors (Sagar et al., 2011, Staniaszek et al., 2010), while in the current study expression of COX-2 protein was evaluated by immunofluorescence, in very specific areas of the dorsal horn and in association with neuronal or non-neuronal cells. COX-2 mRNA was found to be up-regulated bilaterally at the spinal cord level during peripheral inflammation (Beiche et al., 1996, Ichitani et al., 1997); however, such changes were not detected in the current study involving the MIA model of OA, probably due to different inflammatory status of the two conditions.

3.4.6. COX-2 expression in the PAG

The midbrain PAG plays a crucial role in the descending control of nociception. PAG receives ascending nociceptive stimuli that have been processed in the spinal cord, as well as descending inputs from the amygdala. It then sends ascending stimuli to locus coeruleus (LC) and rostral ventromedial medulla (RVM), which then transmit ascending inputs into the spinal cord, modulating pain processing in the spinal cord (Ossipov et al., 2010). COX enzymes and their main products, prostaglandins, are involved in this function. Prostanoid receptors (Ek et al., 2000, Matsumura et al., 1992) and both COX isoforms (Breder et al., 1992) are expressed in the PAG. Injection of PGE₂ into the PAG caused hyperalgesia (Oka et al., 1994) and strengthened the modulatory circuit

in the RVM (Heinricher et al., 2004). Prostaglandins or synthetic analogues inhibit glutamatergic (Lu et al., 2007) and potentiate GABAergic and glycinergic transmission in the PAG (Vaughan, 1998, Shin et al., 2003). EP1 receptors within the VL PAG have a facilitatory effect on thermonociception in normal conditions and in neuropathic pain (Palazzo et al., 2011). It has been demonstrated that COX-1 isoform is responsible for acute antinociceptive effects of NSAIDs in the PAG and that this effect is evoked exclusively from the opioid-sensitive VL PAG (Leith et al., 2007); in addition, inhibition of COX in the PAG promotes an opioid-mediated descending pain inhibition (Björkman et al., 1992). Based on the observations of regulation of COX expression in the PAG in inflammatory arthritis (Drake et al., 2016), this study investigated a potential increase in COX-2 expression in the PAG of MIA rats. No significant changes in the percentage of samples expressing COX-2 were found in osteoarthritic rats compared to the naïve group in any of the PAG areas except in the contralateral VL PAG, where the percentage of COX-2 expressing samples was significantly lower in the MIA injected animals than in naïve controls. These results conflict with those observed in inflammatory arthritis, where expression of COX-2 mRNA was significantly lower in arthritic rats compared to naïve counterparts in the dorsolateral PAG only (Drake et al., 2016). However, these findings are insufficient to draw a conclusion regarding the role of PAG COX-2 in central sensitisation in the MIA model of OA.

Chapter 4. Effects of VEGF-A_{165b} in the MIA model of osteoarthritis

4.1. Introduction

Vascular endothelial growth factor A (VEGF-A, also known as VEGF) is a key molecule in angiogenesis (Ferrara and Henzel, 1989, Senger et al., 1983, Keck et al., 1989) and it has been associated with a number of pathologies, including neoplasms (Hicklin and Ellis, 2005, Ferrara, 2004, Amini et al., 2012), eye (Ajlan et al., 2016, Penn et al., 2008), heart (Taimeh et al., 2013, Chen and Liu, 2014) and kidney (Chen and Ziyadeh, 2008, Schrijvers et al., 2004) diseases, diabetic neuropathy (Leininger et al., 2004, Wirostko et al., 2008) and osteoarthritis (Mapp and Walsh, 2012), among others.

VEGF-A can be found in two families of splice variants, VEGF-A_{xxx}a and VEGF-A_{xxx}b (where xxx is the length of amino acid chain) as a result of alternative pre-mRNA splicing of the *vegfa* gene (Harper and Bates, 2008). VEGF-A_{165a} is known to be pro-angiogenic (Nowak et al., 2010) and pro-nociceptive (Hulse et al., 2016, Nesic et al., 2010), while VEGF-A_{165b} was found to be anti-angiogenic (Varey et al., 2008) and showed anti-nociceptive effects in neuropathic pain (Hulse et al., 2014), diabetic neuropathy (Hulse et al., 2015) and inflammatory arthritis (Beazley-Long et al., 2018). A hypothesis was therefore proposed that VEGF-A_{165b} can exert an analgesic effect in the MIA model of osteoarthritis, involving spinal glial cells and cyclooxygenases in the PAG and spinal cord. The work performed to test this hypothesis is presented in this chapter.

4.2. Methods

A total of 32 male Wistar rats weighing 250-300 g were used in this 28-day cross-over study. Refer to chapter 2 for detailed methodology.

4.2.1. Induction of the MIA model of osteoarthritis

The MIA model of OA was induced in 23 rats by an intra-articular injection of MIA in the right knee (see section 2.2). The remaining animals were anaesthetised but not injected to be used as naïve controls (naïve group). Of the MIA animals, one group (n=9) received systemic VEGF-A₁₆₅b (i.p. 20 ng/g rat body weight; injected volume = 1 µl/g body weight) twice weekly for the first 14 days of the study and PBS (i.p. 1 µl/g body weight) twice weekly on days 14-28 [VEGF(d0-13) group; figure 4.1]. A second group (n=5) received PBS on days 0-13 and VEGF-A₁₆₅b in the last two weeks of the study [VEGF(d14-28) group]. Another nine MIA injected rats received PBS injections at the same times, for the study duration (MIA/PBS group). It should be noticed that, since animals from the VEGF(d14-28) group received PBS before day 14, they were considered part of the MIA/PBS group on days 0-13 for pain behaviour analysis. Figure 4.1 shows the time course of the study.

Time course of the experiment

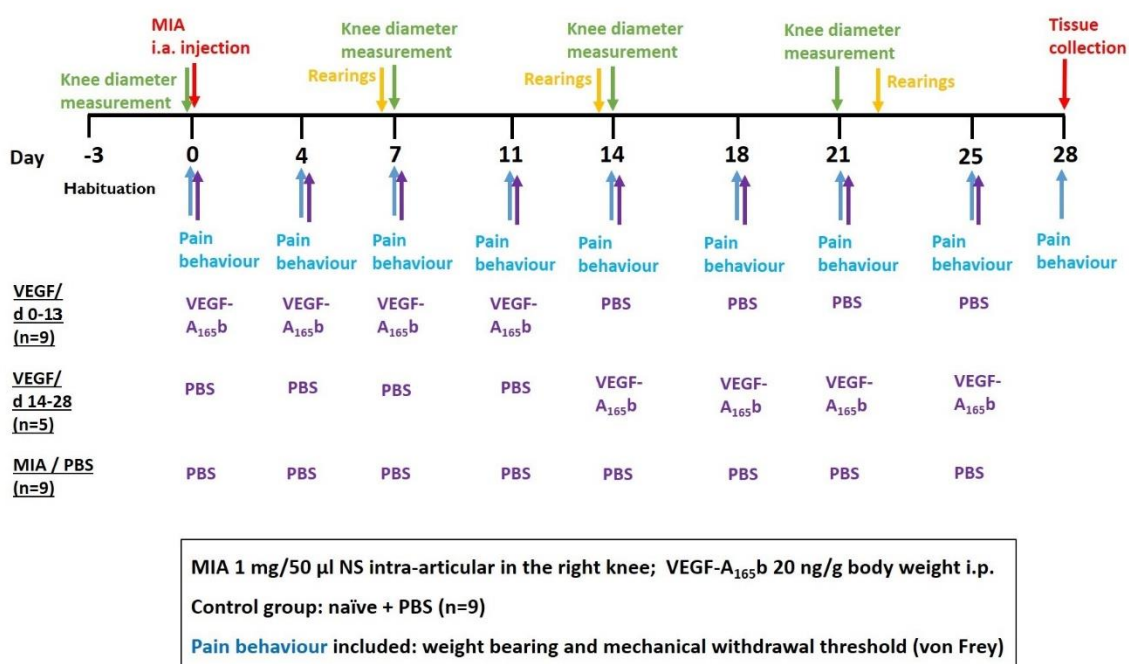


Figure 4.1. Time course of the MIA/VEGF-A₁₆₅b in vivo study and pain behaviour.

4.2.2. Pain behaviour, rearings and knee swelling

Pain behaviour in rats was assessed measuring 1) weight bearing imbalance using an incapacitance test meter and 2) mechanical withdrawal thresholds by von Frey hairs (for more details refer to section 2.4). Knee diameters were measured using electronic callipers to assess evident joint inflammation. The number of rearings that rats performed during a two-minute session were counted (see section 2.6). Rats were first handled and habituated to the test equipment, and baseline readings were obtained (referred to as day 0). Pain behaviour tests were performed twice weekly on days 4, 7, 11, 14, 18, 21, 25, and 28. Ipsilateral knee diameter was measured before the intra-articular injection of MIA (day 0) and weekly after that (days 7, 14, 21). The numbers of rearings were measured on different days in the two parts of the study, so weighted arithmetic means of pairs of days were plotted (6.67, 13.67, 22.31).

4.2.3. Tissue collection and processing

On day 28, all rats were killed by an overdose of pentobarbital (Euthatal[®], 200 mg/ml, 1 ml). Death was confirmed by cervical dislocation. Brains were dissected and immediately snap frozen in dry ice. Knee joints were harvested and kept in 4% PFA until needed. Spinal cords were also dissected, then post-fixed in 4% PFA for 24 hours, cryoprotected in 30% sucrose in PBS solution for 24 hours and finally embedded in OTC and kept at -80°C until sectioned.

Knees were later washed in PBS and disarticulated for the macroscopic assessment of chondropathy. The tibiae were then processed for sectioning, H&E staining and microscopic evaluation of joint damage (see section 2.8.3, 2.9.1 and 2.10.1). Spinal cords were sectioned using a cryostat (Leica 1900), stained for immunofluorescence (COX-2/NeuN and GFAP/CD11b; section 2.9.2) and analysed as in section 2.10.2. PAG tissue was micropunched as explained in section 2.8.3.1, and RNA extracted for reverse transcription (section 2.11). Real-time PCR was used to determine changes in COX-1 and COX-2 expression in the PAG (section 2.13.3).

4.3. Results

4.3.1. Acute inflammation (swelling)

No significant difference in the diameter of the ipsilateral knee joint was found between the study groups at any time point (figure 4.2).

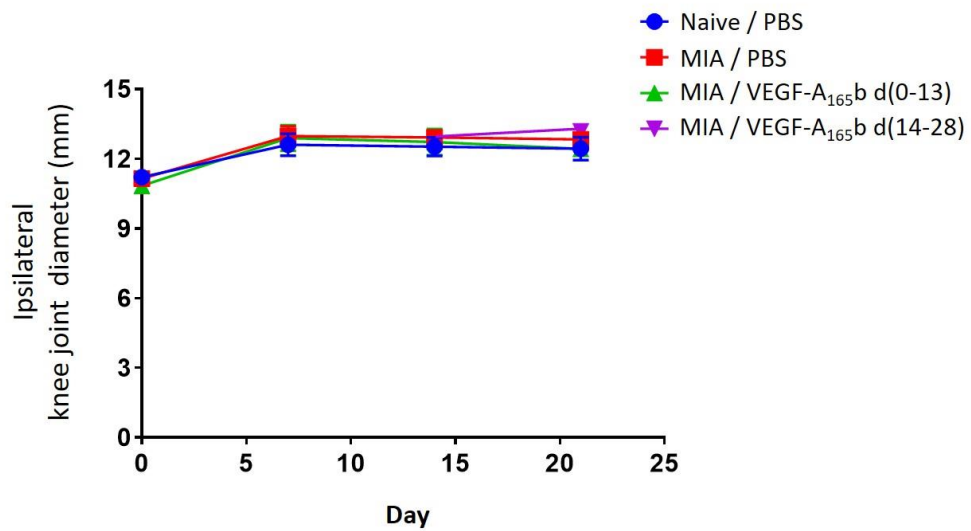


Figure 4.2. Rat ipsilateral knee diameter in the VEGF-A₁₆₅b treated MIA animals and control groups. No significant differences in knee diameter were found between the study groups at any time point. (n=5 for VEGF(d14-28) group, n=14 for MIA/PBS group until day 14, n=9 for other groups; one-way ANOVA).

4.3.2. Rearings

No significant difference was found in the number of rearings performed by the rats between any of the study groups at any time point (figure 4.3).

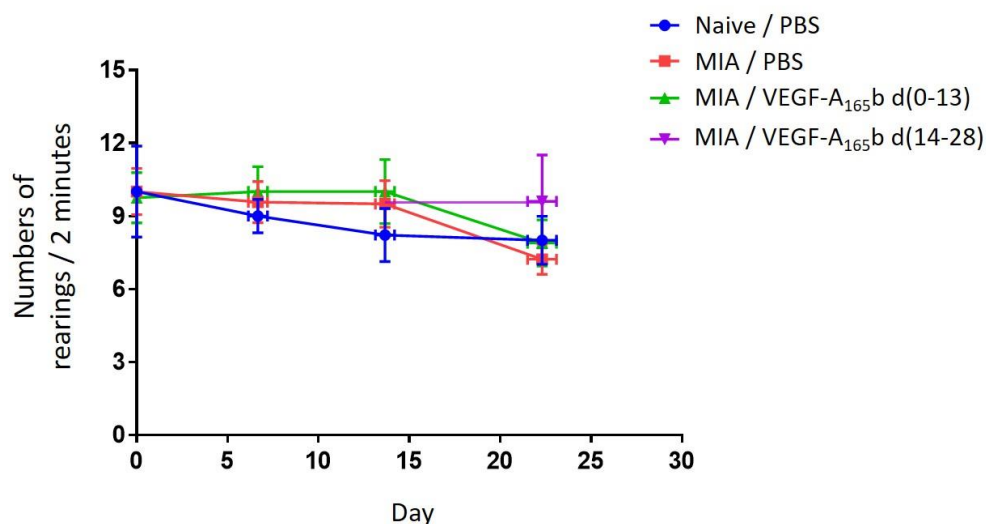


Figure 4.3. Number of rearings performed by rats in the MIA / VEGF-A₁₆₅b study.

No significant changes in the number of rearings were detected at any time in the VEGF-A₁₆₅b treated animals compared to the controls groups. (n=5 for VEGF(d14-28) group, n=14 for MIA/PBS group until day 14, n=9 for the other groups, one-way ANOVA).

4.3.3. Pain behaviour

4.3.3.1. Mechanical withdrawal threshold

Mechanical withdrawal thresholds of MIA injected rats showed a reversal to the levels of the naïve group with administration of VEGF-A₁₆₅b, but these changes were not statistically significant (figure 4.4).

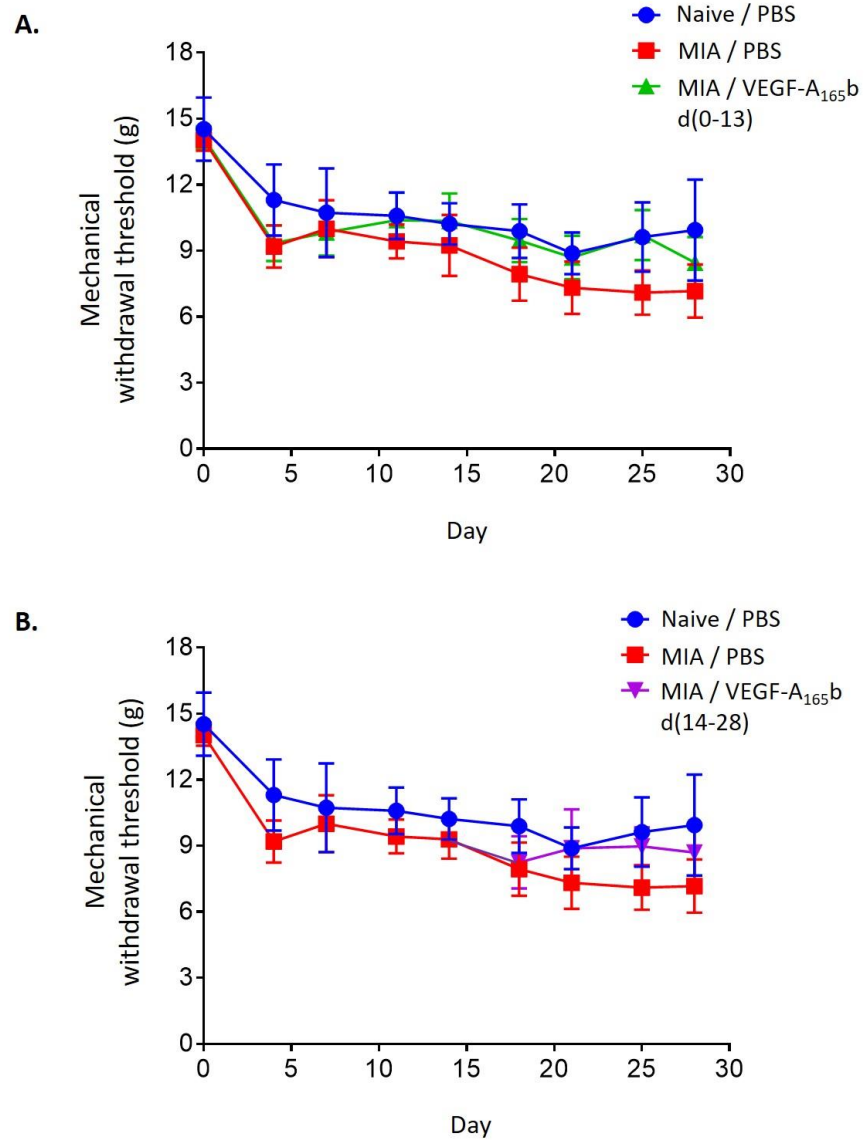


Figure 4.4. Mechanical withdrawal thresholds of the ipsilateral hindpaw of the VEGF-A₁₆₅b treated MIA rats and control groups. No significant changes in the mechanical withdrawal thresholds of ipsilateral hindpaw were detected between control groups and groups treated with **(A)** early or **(B)** late VEGF-A₁₆₅b, although a reversal of withdrawal thresholds to naïve group values can be noticed starting on day 11 in **(A)** and day 21 in **(B)**. (n=9, except for MIA/VEGF-A₁₆₅b d(14-28), where n=5 and MIA/PBS, where n=14 until day 14; two-way ANOVA).

4.3.3.2. Weight bearing

The weight borne by the ipsilateral hindpaw is expressed here as 1) percent of weight borne by both hindpaws and 2) percent of rat body weight. The first value is calculated according to the following formula:

$$\frac{\text{weight borne on the ipsilateral hindpaw}}{\text{weight borne on the (ipsilateral hindpaw + contralateral hindpaw)}} \times 100 \%$$

and it indicates a lateral shift of weight bearing away from the affected paw towards the unaffected one (Pomonis et al., 2005). However, animals can also relieve some of the weight load on the arthritic limb by shifting towards the tail or forelimbs (Fernihough et al., 2004). Hence, the use of a second measurement of weight bearing, calculated as follows:

$$\frac{\text{weight borne on the ipsilateral hindpaw}}{\text{rat body weight}} \times 100 \%$$

All rats injected with MIA developed a significant weight bearing asymmetry compared to the naïve control 4 days after injection (MIA/PBS vs naïve/PBS 37.29 ± 1.67 vs 47.74 ± 0.97 , $p < 0.0001$; VEGF(d0-13) vs naïve/PBS 38.75 ± 1.28 vs 47.74 ± 0.97 , $p < 0.001$; figure 4.5). Systemic administration of VEGF-A_{165b} on days 0-13 caused a significant reduction in weight bearing asymmetry (as a percentage of weight on hindpaws) of MIA rats compared to the vehicle injected counterparts starting on day 18 and till the end of the study (VEGF(d0-13) vs MIA/PBS $46.48\% \pm 1.68$ vs. $41.16\% \pm 2.15$, $p < 0.05$ on day 18; VEGF(d0-13) vs MIA/PBS $45.46\% \pm 1.86$ vs. $36.59\% \pm 2.165$, $p < 0.001$ on day 28; figure 4.5.A). No significant effect on weight bearing asymmetry was found in MIA rats when injected with VEGF-A_{165b} on days 14-28 of the study (figure 4.5.B).

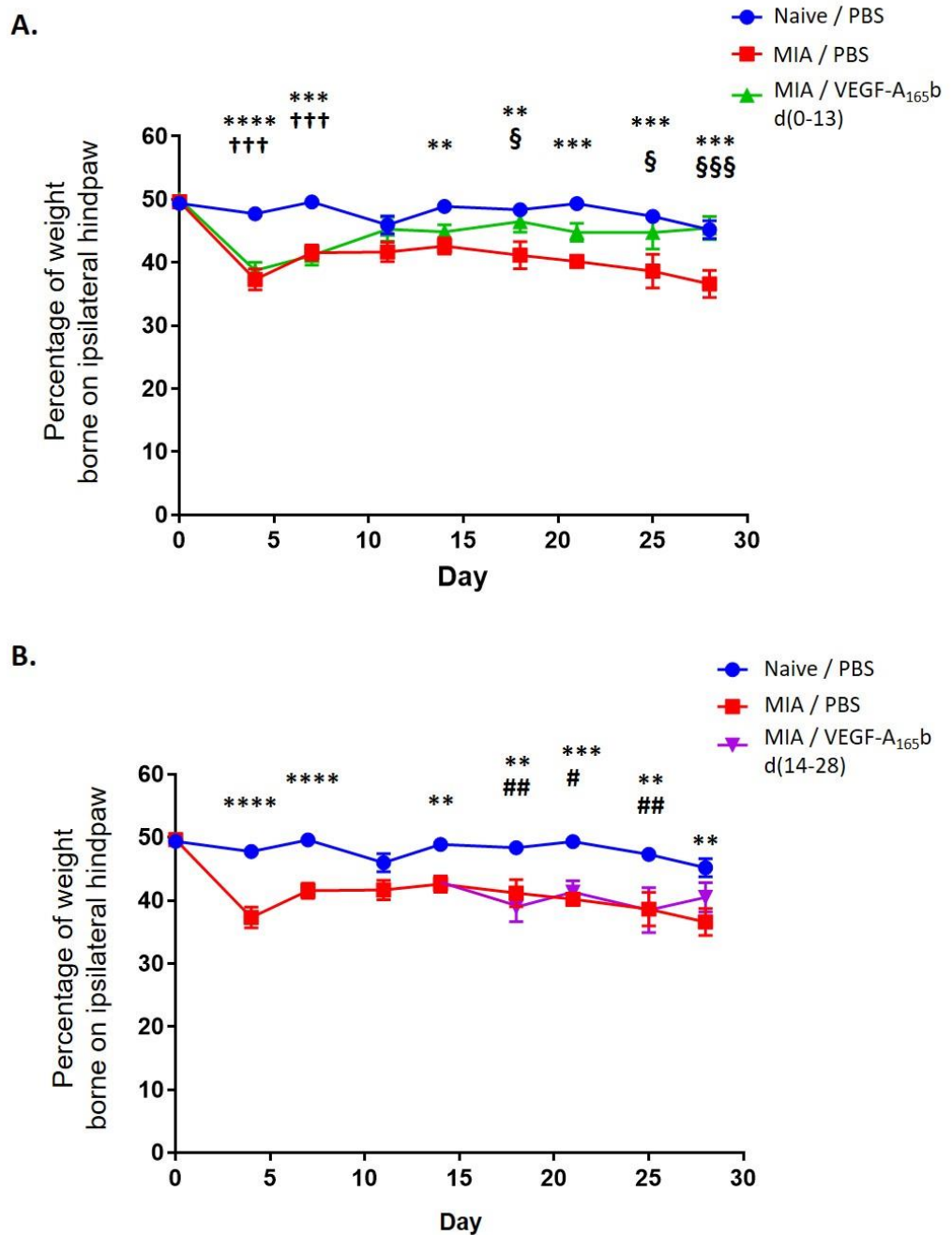


Figure 4.5. Early administration of systemic VEGF-A₁₆₅b reduces weight bearing asymmetry in the MIA model of OA in rats. MIA injected rats developed a significant weight bearing imbalance compared to the naïve control group since day 4. **A)** MIA injected rats treated with VEGF-A₁₆₅b on days 0-13 showed a significant reduction in weight bearing asymmetry compared to vehicle injected MIA rats on days 18, 25 and 28. **B)** No significant difference in weight bearing imbalance was found between MIA rats injected with VEGF-A₁₆₅b on days 14-28 and MIA/vehicle controls. (n=5 for VEGF(d14-28) group, n=14 for MIA/PBS group until day 14, n=9 for other

groups; two-way ANOVA with Bonferroni multiple comparisons test; ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ for MIA/PBS vs naïve groups; ††† $p<0.001$ for VEGF(d0-13) vs naïve groups; § $p<0.05$, §§§ $p<0.01$ for VEGF(d0-13) vs MIA/PBS groups; # $p<0.05$, ## $p<0.01$ for VEGF(d14-28) vs naïve groups).

Weight bearing asymmetry, when calculated as percentage of weight borne on the ipsilateral hindpaw over rat body weight, was significantly reversed with both early and late administration of VEGF-A_{165b}. MIA injection caused a significant weight bearing asymmetry compared to the naïve/PBS group starting on day 4 (MIA/PBS vs naïve/PBS 21.83 ± 1.33 vs 30.03 ± 0.94 , $p<0.0001$; VEGF(d0-13) vs naïve/PBS 23.64 ± 1.01 vs 30.03 ± 0.94 , $p<0.01$; figure 4.6). Arthritic rats treated with VEGF-A_{165b} on the first 14 days had a significant increase in the percentage (%) of weight borne on the ipsilateral hindpaw over body weight on days 25 and 28 of the study compared to the MIA/PBS group (29.58 ± 1.803 vs. 22.95 ± 2.088 , $p<0.01$ on day 28; figure 4.6.A). When VEGF-A_{165b} was administered on days 14-28, a significant increase in the same parameter was found on day 28 (VEGF(d14-28) vs MIA/PBS 29.23 ± 1.49 vs. 22.95 ± 2.088 , $p<0.05$; figure 4.6.B).

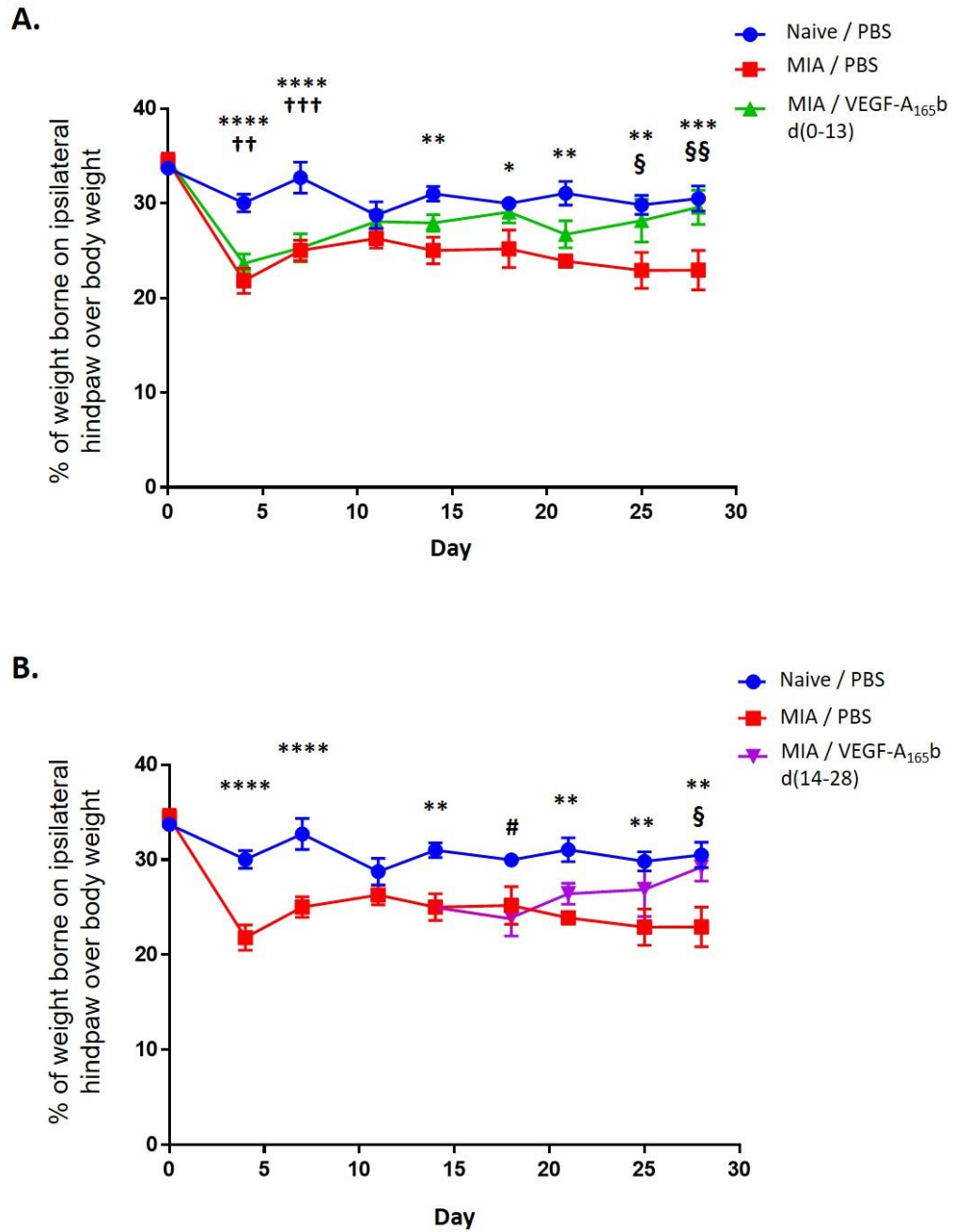


Figure 4.6. Both early and late administration of VEGF-A₁₆₅b significantly reversed the decreased percentage of weight borne on the ipsilateral hindpaw over total body weight. MIA injected rats developed a significant weight bearing asymmetry compared to the naïve control group since day 4. **A)** MIA rats treated with VEGF-A₁₆₅b on the first 14 days had a significant increase in the percentage (%) of weight borne on the ipsilateral hindpaw over total body weight on days 25 and 28 compared to the MIA/PBS group. **B)** Administration of VEGF-A₁₆₅b on days 14-28 significantly reversed weight bearing asymmetry on day 28. (n=5 for VEGF(d14-28) group, n=14 for MIA/PBS until day 14, n=9 for other groups; two-way ANOVA with

Bonferroni for multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ for MIA/PBS vs naïve/PBS groups; †† $p < 0.01$, ††† $p < 0.001$ for VEGF(d0-13) vs naïve groups; § $p < 0.05$, §§ $p < 0.01$ for VEGF group vs MIA/PBS group).

4.3.4. Joint pathology and histology

4.3.4.1. Macroscopic chondropathy score

After disarticulation, knee joints were assessed for chondropathy as explained in section 2.10.1. To validate the efficacy of scoring, the samples were scored twice in two different occasions by the researcher, and the two sets of scores were then analysed for correlation using Spearman's correlation coefficient (figure 4.7). The two sets of scores correlated significantly (Spearman's correlation coefficient $r = 0.8751$; 95% CI 0.7529 to 0.939; P value < 0.0001).

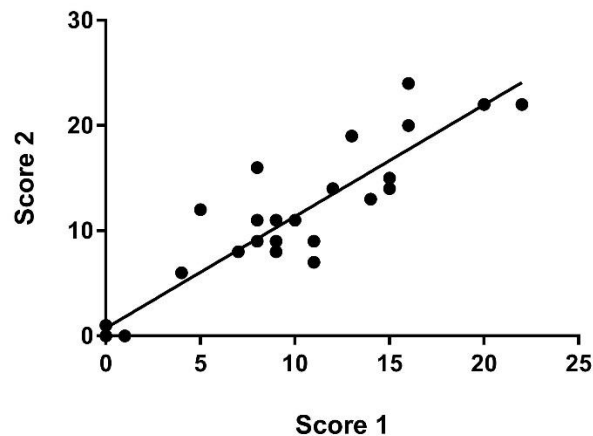


Figure 4.7. Correlation of two sets of macroscopic chondropathy scores in MIA / VEGF-A₁₆₅b rats obtained by the researcher at two different times. The two sets of scores obtained in two separate occasions significantly correlated (Spearman's correlation test; $p < 0.0001$).

Intra-articular injection of MIA produced significant chondropathy in the knee joint, which was reflected in a significantly higher macroscopic score in the MIA groups compared to the naïve control group (MIA/PBS vs naïve 15.22 ± 2.00 vs 0.22 ± 0.15 , $p < 0.001$; MIA/VEGF(d0-13) vs naïve 11.22 ± 1.34 vs 0.22 ± 0.15 , $p < 0.05$; MIA/VEGF(d14-28) vs naïve group 12.4 ± 2.32 vs 0.22 ± 0.15 , $p < 0.05$). VEGF-A_{165b} administration to MIA rats did not significantly affect the macroscopic chondropathy score of rats compared to the MIA/PBS group, whether it was given in the first or second two weeks of the study (figure 4.8).

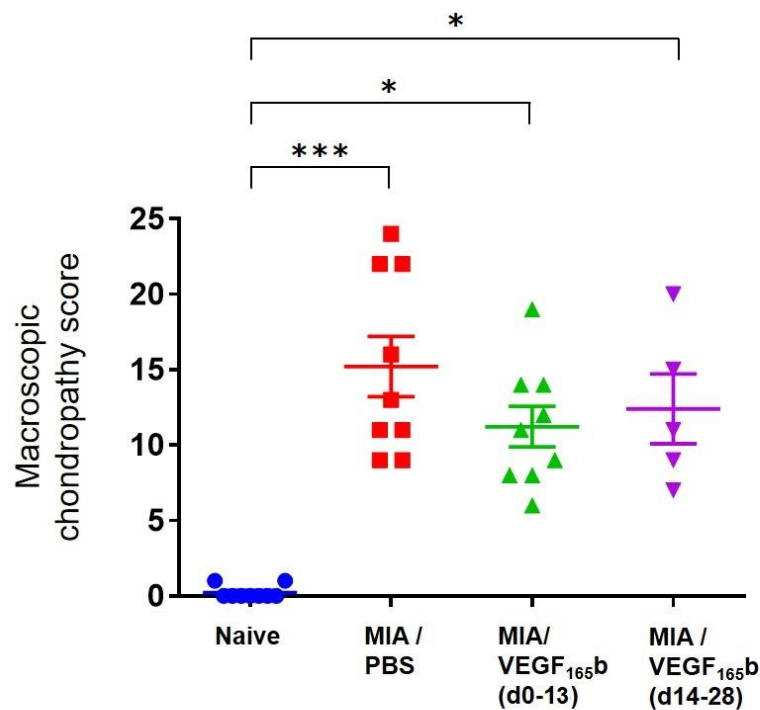


Figure 4.8. Knee macroscopic chondropathy score of rats from the MIA / VEGF-A_{165b} study. All MIA groups exhibited a significantly higher macroscopic chondropathy score compared with the naïve control group. Administration of VEGF-A_{165b} did not cause significant changes in the macroscopic score of MIA rats compared to the MIA/PBS control. (Kruskal-Wallis with post-hoc Dunn's tests; * $p < 0.05$, *** $p < 0.001$; $n = 9$, except for MIA/VEGF(d14-28) group, where $n = 5$).

4.3.4.2. Microscopic chondropathy, synovitis and osteophyte scores

Microscopic evaluation of chondropathy, synovial inflammation and osteophyte formation was performed as explained in section 2.10.1. The ability of the researcher to perform the microscopic scoring in a correct and consistent way had already been assessed using the intraclass correlation coefficient (ICC; refer to section 3.3.2.2). MIA injected rats showed a significantly higher microscopic chondropathy score (MIA/PBS vs naïve/PBS groups 12.17 ± 0.92 vs 1.66 ± 0.43 , $p < 0.001$; MIA/VEGF(d0-13) vs naïve/PBS 10.68 ± 1.01 vs 1.66 ± 0.43 , $p < 0.01$), which was not reversed by the administration of VEGF-A₁₆₅b (figure 4.9.A). The synovial inflammation score of the MIA/PBS group was significantly higher than the naïve control group (MIA/PBS vs naïve/PBS 3 ± 0 vs 0 ± 0 , $p < 0.05$), while no significant effect of VEGF-A₁₆₅b on synovitis score was detected (figures 4.9.B). No significant differences in osteophyte score were found between the study groups (figure 4.9.C); however, 55.56% of the MIA/PBS samples developed osteophytes, while none were observed in the naïve group (Chi-square test; $p < 0.01$; figure 4.9.D).

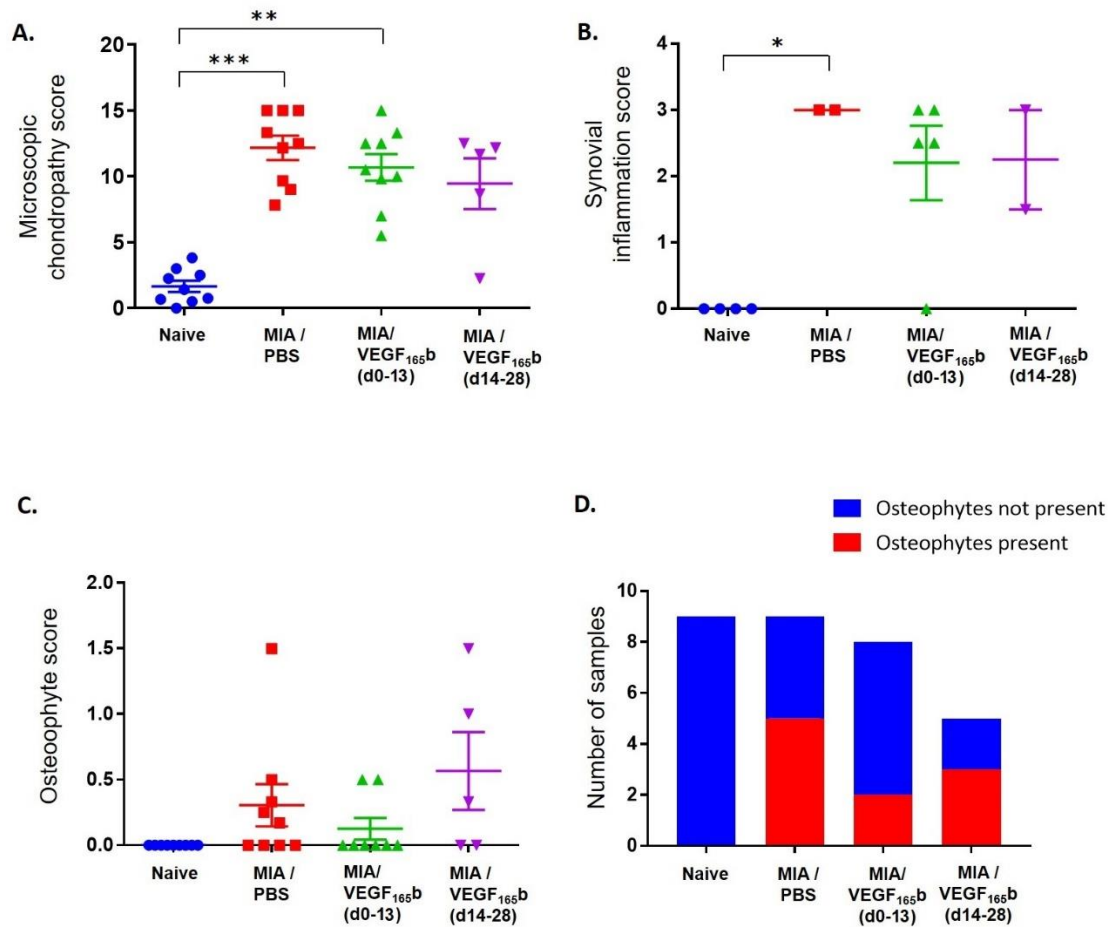


Figure 4.9. Microscopic scores of chondropathy, synovial inflammation and osteophyte in the MIA / VEGF-A_{165b} study. (A) MIA/PBS and VEGF(d0-13) groups had significantly higher microscopic chondropathy scores than the naïve control. No significant changes were detected between the MIA rats injected with early or late VEGF-A_{165b} and the MIA/vehicle control. (B) MIA/PBS rats showed significant synovial inflammation compared to the naïve group. No significant differences were found between the VEGF-A_{165b} treated groups and the MIA/PBS groups. (C) No significant differences in the osteophyte score were found between any of the study groups; however, (D) contingency analysis showed a significant change in the percentage of samples presenting osteophytes in the study. [n=5 or 9 (except for synovial inflammation score, where n= 2-4 and VEGF(d14-28) group in graphs C and D, where n=8); Kruskal-Wallis test with Dunn's post-hoc for A, B and C, *p<0.05, **p<0.01, ***p<0.001; Chi-square test for the contingency table in D, *p<0.05]. Note: lower n numbers are due to missing samples or samples not intact enough to be scored.

4.3.5. Glial cell activation in the spinal cord

4.3.5.1. Microglia

Spinal microglia activation was assessed by measuring the percentage of CD11b reactive cells and the average intensity of CD11b immunofluorescence (refer to section 2.9.2). No significant difference in the percentage of CD11b reactive cells (figure 4.10.A and 4.10.B) or the average intensity of CD11b immunofluorescence (figure 4.10.C and 4.10.D) in ipsilateral or contralateral dorsal horn was found between any of the study groups.

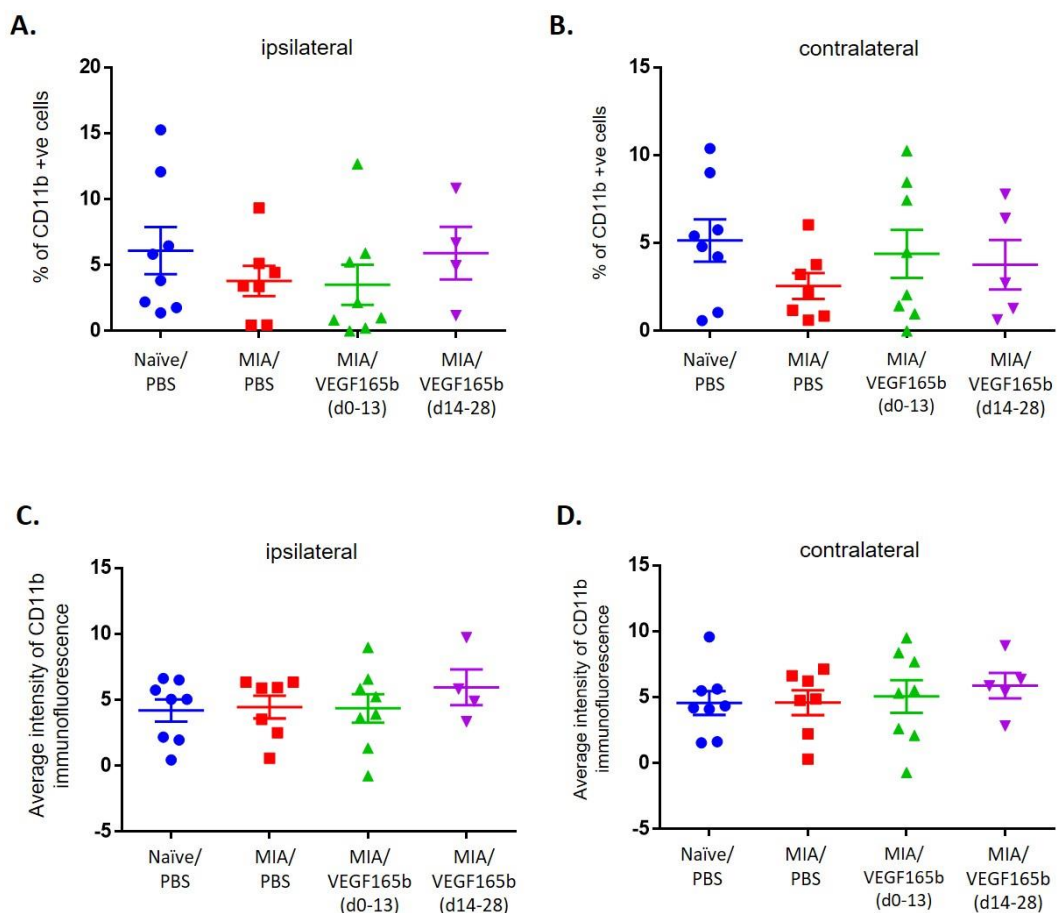


Figure 4.10. Microglia activation in the ipsilateral and contralateral laminae I and II of VEGF-A₁₆₅b treated MIA rats measured as percentage of CD11b immunoreactive cells and average intensity of CD11b fluorescence. No significant difference was found between the study groups in the percentage of CD11b positive cells in both **(A)** ipsilateral and **(B)** contralateral spinal cord. CD11b average intensity was not significantly different between any of the study groups in **(C)** ipsilateral and **(D)** contralateral spinal cord. (n= 4-8 / group; Kruskal-Wallis analysis).

4.3.5.2. Astrocytes

Activation of astrocytes measured by the average fluorescence intensity of GFAP was not significantly different between any of the study groups (figure 4.11).

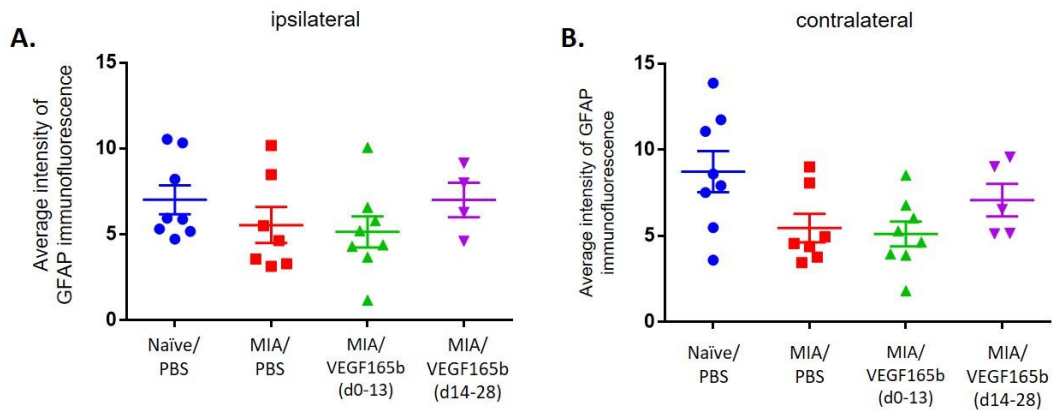


Figure 4.11. Astrocyte activation in the ipsilateral and contralateral laminae I and II of MIA rats treated with VEGF-A₁₆₅b measured as average intensity of GFAP fluorescence. No significant changes were detected in GFAP average intensity between the study groups in (A) ipsilateral or (B) contralateral spinal cord. (n= 4-8 / group; Kruskal-Wallis test).

4.3.6. COX-2 expression in the spinal cord

Spinal cord sections were stained using anti-COX-2 and anti-NeuN antibodies, as explained in section 2.9.2.2. NeuN is a marker for neuronal nuclei (Gusel'nikova and Korzhevskiy, 2015), and it was used to identify COX-2 expression in neuronal cells (NeuN +ve) or non-neuronal cells (NeuN -ve; figure 4.12). The number of cells expressing COX-2 (with and without NeuN co-localisation) were counted and expressed as percentage of cells out of the total number of DAPI positive cells. Counting was performed in two regions of the spinal cord (laminae I, II and laminae III-V) in both the ipsi- and contralateral sides.

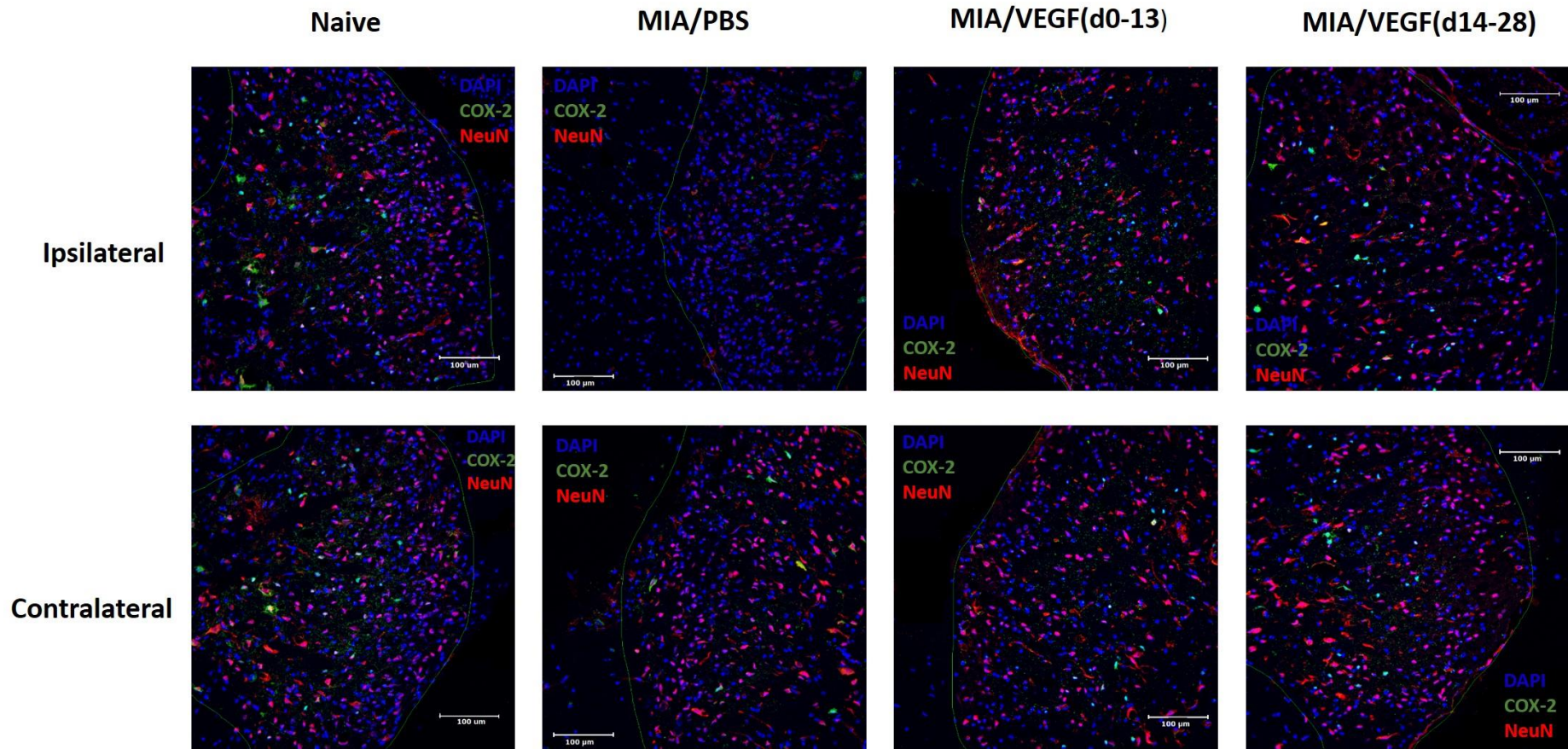


Figure 4.12. COX-2 and NeuN immunofluorescence in the spinal cord of rats from the MIA/VEGF-A_{165b} study. Representative images of COX-2 (green), NeuN (red) and DAPI (blue) immunofluorescence in the ipsilateral and contralateral dorsal horn of the spinal cord in the study groups. (Scale bar = 100 μm).

No significant difference in the percentage of NeuN +ve/COX-2 +ve cells or NeuN -ve/COX-2 +ve cells was found between the study groups in any of the studied regions of the spinal cord (ipsilateral and contralateral laminae I,II and III-V; figure 4.13 and 4.14).

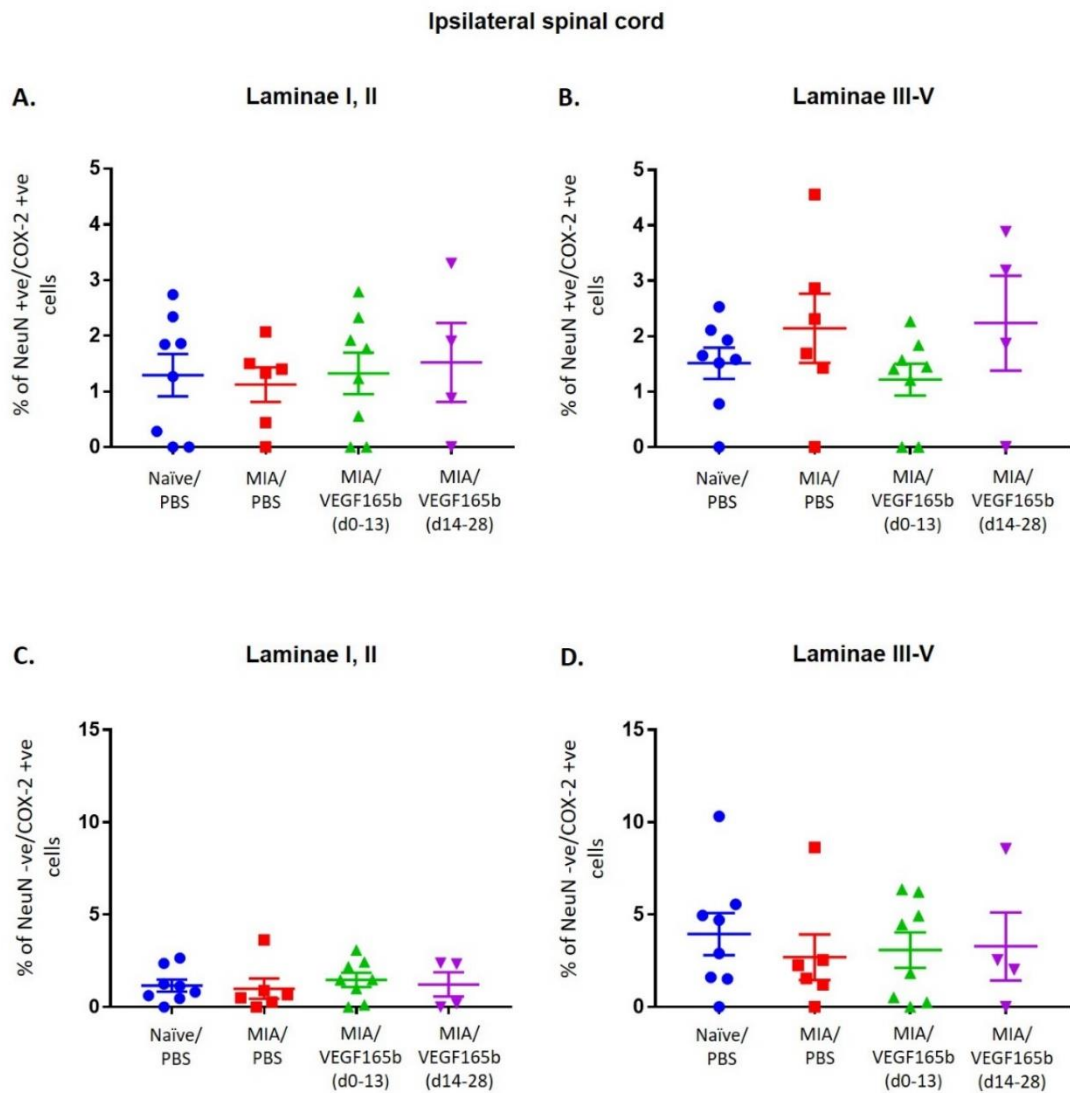


Figure 4.13. Percentage of neuronal and non-neuronal cells expressing COX-2 in the ipsilateral spinal cord of rats in the MIA/VEGF-A_{165b} study. No significant differences were detected between any of the study groups in the percentage of NeuN +ve/COX-2 +ve cells or NeuN -ve/COX-2 +ve cells in any of the studied laminae (I,II and III-V). (n= 4-8/group; Kruskal-Wallis test).

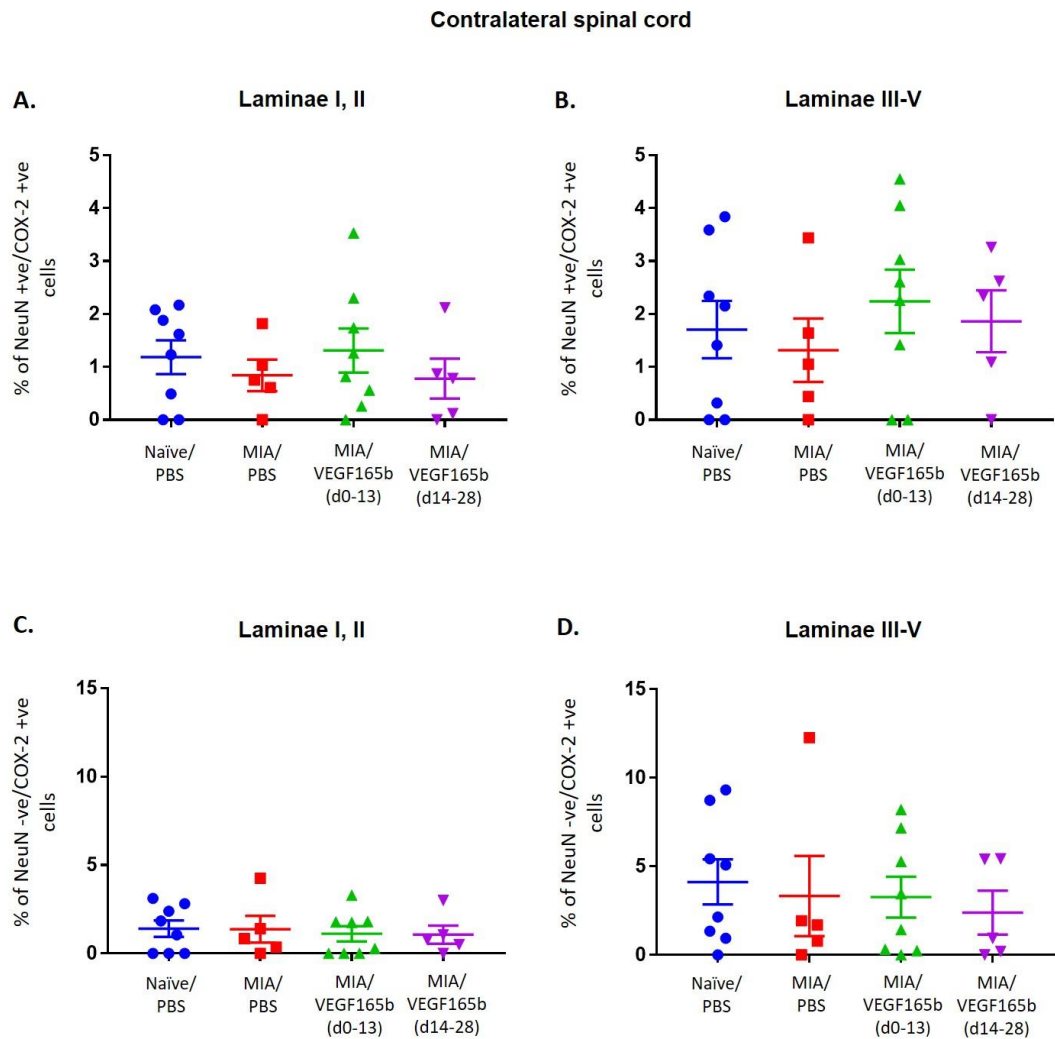


Figure 4.14. Percentage of neuronal and non-neuronal cells expressing COX-2 in the contralateral spinal cord of MIA rats treated with VEGF-A₁₆₅b and naïve and vehicle controls. No significant difference was detected between any of the study groups in the percentage of NeuN +ve/COX-2 +ve cells or NeuN -ve/COX-2 +ve cells in any of the studied laminae (I,II and III-V). (n= 5-8/group; Kruskal-Wallis analysis).

Differences in COX-2 expression between the superficial and deep laminae of the spinal dorsal horn were also addressed. No significant differences were detected in the expression of neuronal COX-2 between the superficial and the deep laminae in any study group (figure 4.15). On the other hand, the percentage of non-neuronal cells expressing COX-2 was significantly higher in the deep laminae than the superficial laminae in both the ipsilateral and contralateral dorsal horn of naïve animals (ipsilateral 3.94 ± 1.14 vs 1.16 ± 0.33 , $p < 0.05$; contralateral 4.12 ± 1.27 vs 1.41 ± 0.47 , $p < 0.05$; figures 4.16.A and 4.16B) and in the contralateral dorsal horn of the MIA/VEGF(d0-13) animals (3.26 ± 1.16 vs 1.12 ± 0.43 , $p < 0.05$; figure 4.16B).

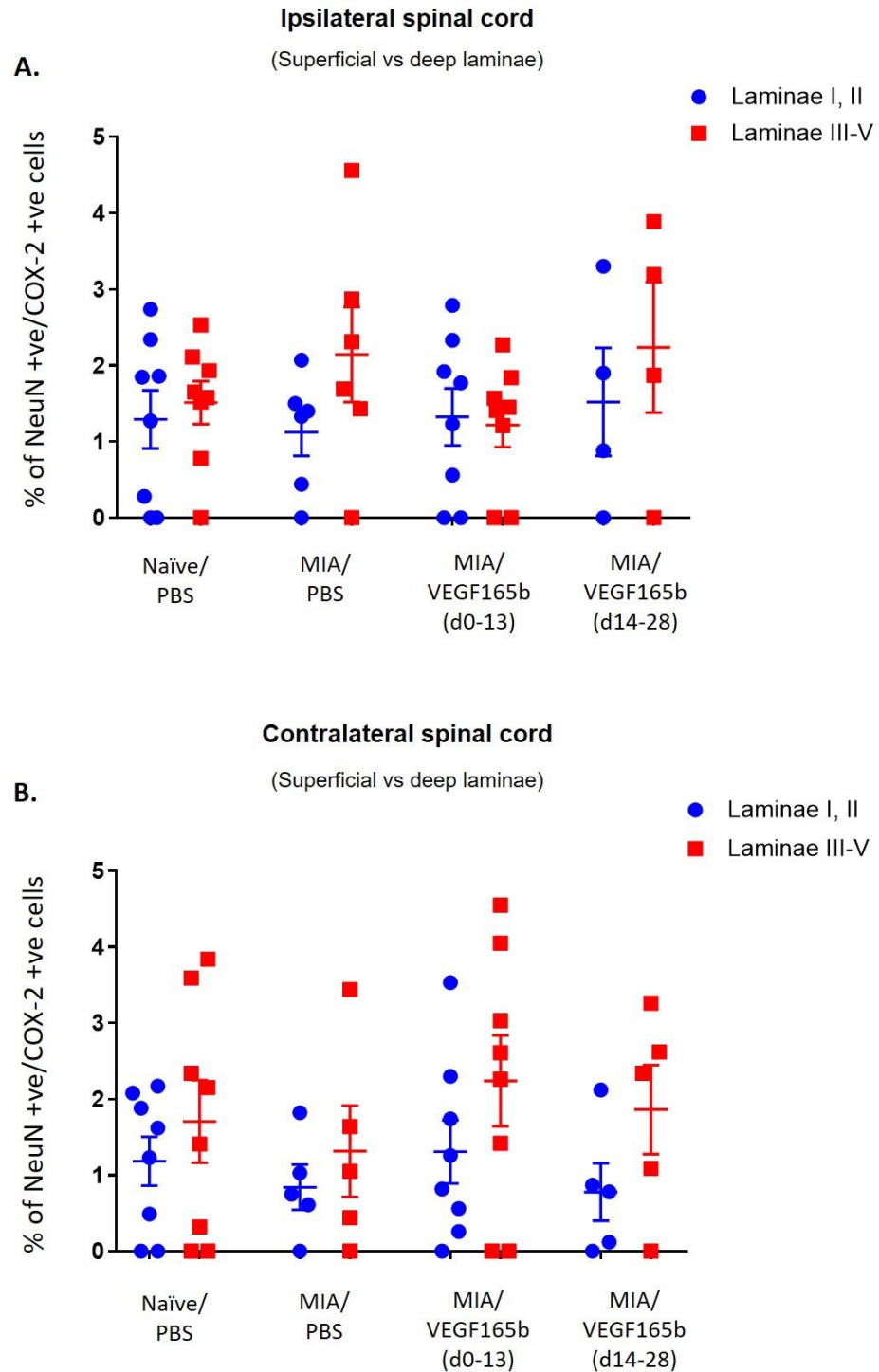


Figure 4.15. Expression of COX-2 in neuronal cells in the superficial and deep laminae of the ipsilateral and dorsolateral spinal cord in MIA/VEGF- A_{165b} rats and controls. No significant differences in the expression of neuronal COX-2 were observed between the superficial and deep laminae for each study group in both **(A)** ipsilateral and **(B)** contralateral spinal cord. (n=4-8; Wilcoxon test).

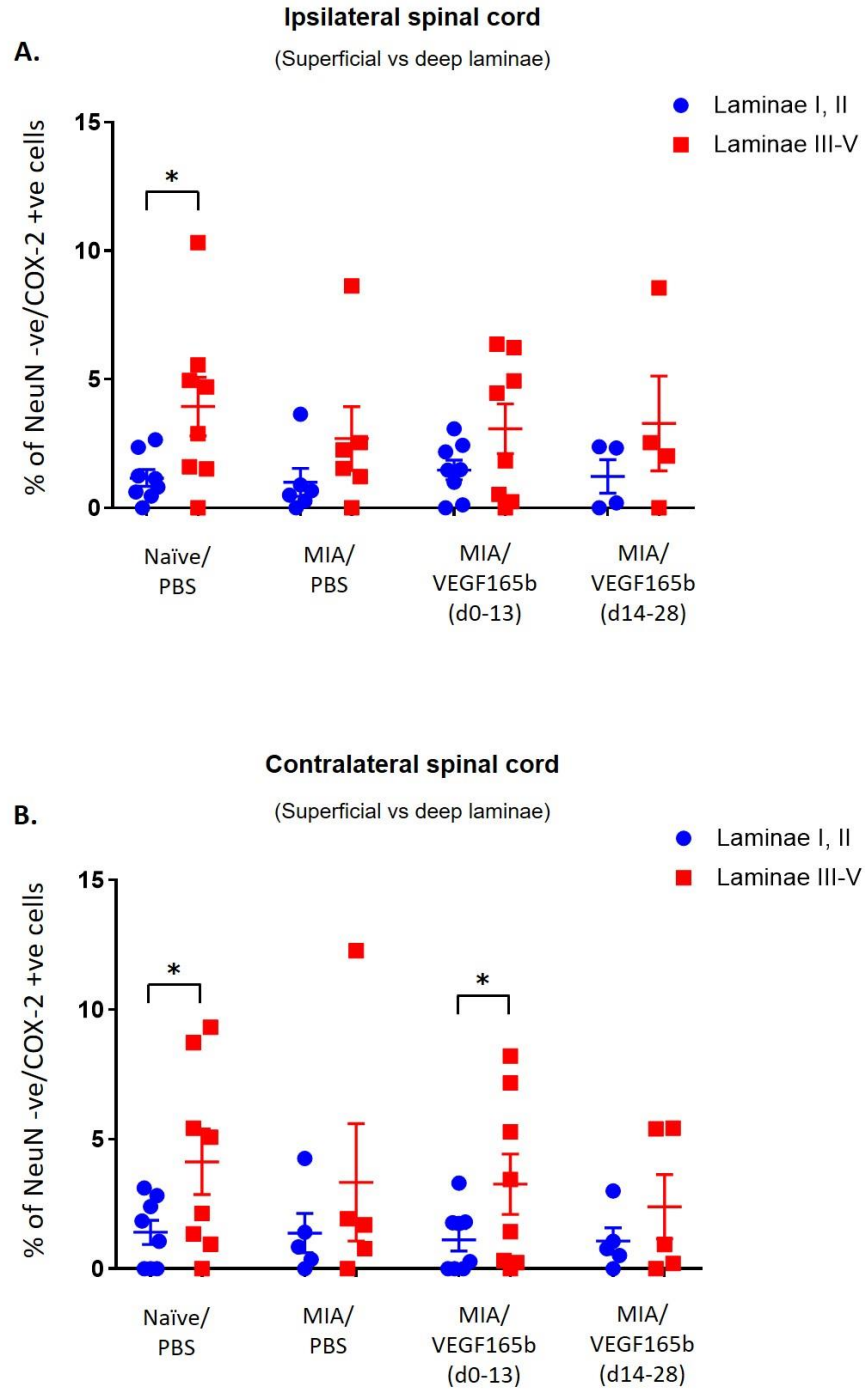


Figure 4.16. Expression of COX-2 in non-neuronal cells in the superficial and deep laminae of the ipsilateral and dorsolateral spinal cord in MIA/VEGF-A₁₆₅b rats and control. (A) In the naïve group, ipsilateral laminae III-V showed a significantly higher expression of non-neuronal COX-2 compared to ipsilateral laminae I,II. **(B)** In the naïve and MIA/VEGF(d0-13) groups, contralateral deep laminae had a higher percentage of non-neuronal cells expressing COX-2 than the corresponding superficial laminae. (n=4-8; Wilcoxon test, *p<0.05).

4.3.7. COX isoform expression in the PAG

To assess changes in the expression of COX isoforms in the PAG of MIA rats treated with VEGF-A₁₆₅b, four regions of the PAG (DL and VL PAG; ipsilateral and contralateral) were micropunched as described in section 2.8.3.1. After RNA extraction and cDNA synthesis (sections 2.11, 2.12), qPCR was run (section 2.13.3). Due to the small quantity of reliable samples obtained, results were recorded as number of samples in each group - regardless of the location in the PAG - that expressed the COX isoform or did not express it (figure 4.17).

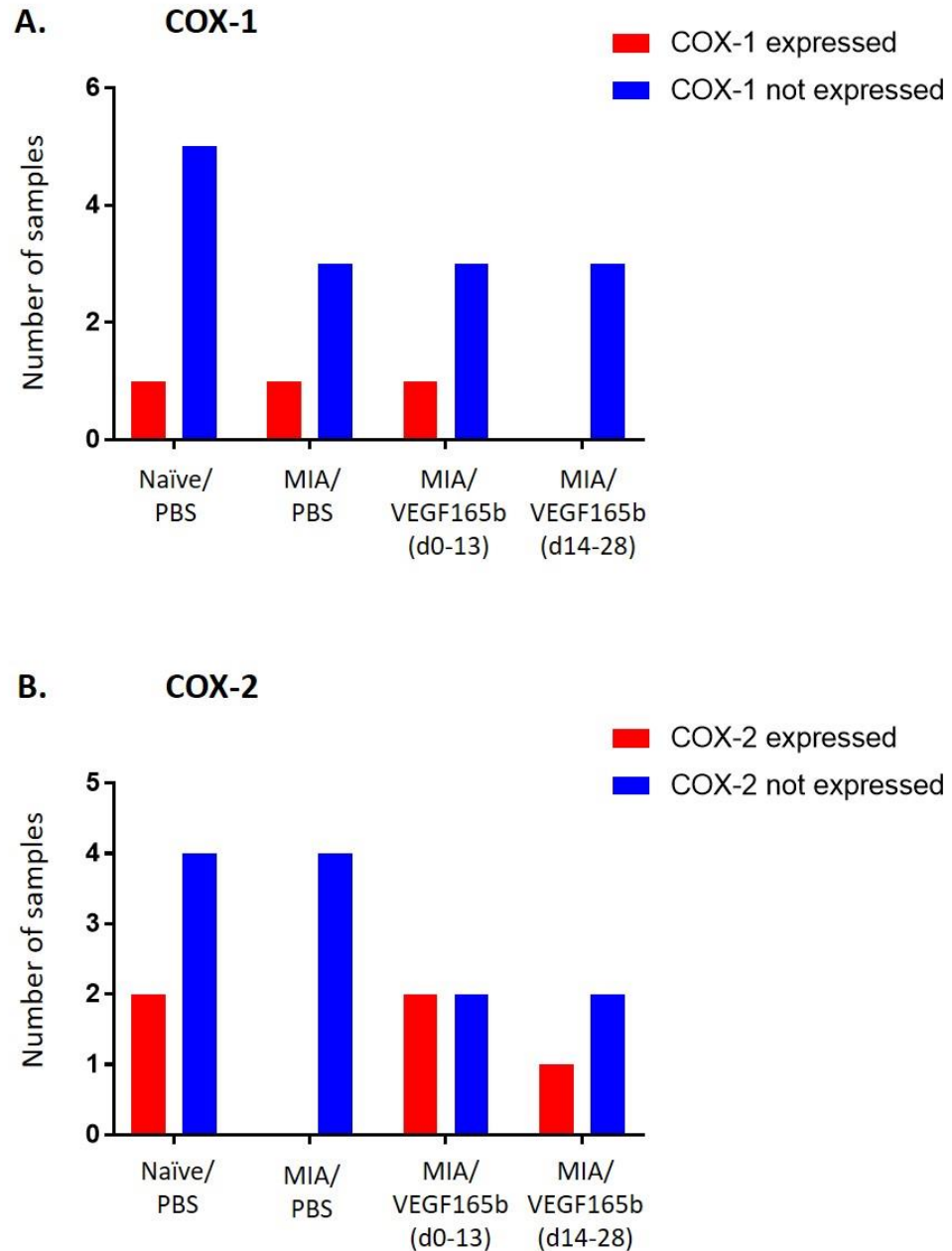


Figure 4.17. Number of samples expressing and not expressing COX-1 and COX-2 mRNA in the PAG of MIA rats treated with VEGF-A₁₆₅b. No significant difference was found in the percentage of samples expressing (A) COX-1 or (B) COX-2 mRNA between any of the study groups (n= 3-6/group; Chi-square test for trend).

4.4. Discussion

4.4.1. Acute inflammation (swelling)

Synovial inflammation is a feature of knee OA in humans (Wenham and Conaghan, 2010, Scanzello and Goldring, 2012). In the MIA model of OA, it has been observed as mild joint swelling in the early phases of the disease, as well as synovial thickening and infiltration by immune cells (Bove et al., 2003, Clements et al., 2009). In this study, no significant difference in knee diameter was found between the study groups at any time point. This is probably because the transient swelling observed after MIA injection had already resolved by day 7, when the first measurement after baseline was taken. These findings are also consistent with other several studies, where joint swelling was observed on the first 1-3 days post MIA injection, and completely resolved by day 7-10 (Nwosu et al., 2016b, Mapp et al., 2013, Al-Saffar et al., 2009). The increase in knee diameter over time is due to the increase in rat weight and size.

4.4.2. Rearings

Rearings are vertical spontaneous movements (Nagase et al., 2012) used as measurement of exploration, locomotion, arousal and non-specific excitability in rodents (Lever et al., 2006). The number of rearings was positively correlated to ambulation in different strains of mice (van Abeelen, 1977). A decreased rearing frequency may indicate sedation (Sousa et al., 2017) or stress (Masood et al., 2003) and it has been considered a movement-provoked pain behaviour in models of osteoarthritis (Miller et al., 2012, Inglis et al., 2008, More et al., 2013, Nagase et al., 2012).

In this study, no significant differences in the number of rearings performed in a two-minute time frame were recorded between any of the groups. This finding rules out any potential effect of sedation, stress or changes in excitability or ambulation on changes in evoked pain behaviour. At the same time, it indicates the absence of this spontaneous pain behaviour in the MIA injected rats. This

finding contrasts with previous literature, where MIA injected rats showed reduced number of rearings. However, those studies utilised an automated technique to count vertical movements overnight (Quinn et al., 2003), involved bilateral MIA model (Nagase et al., 2012, Bryden et al., 2015) or used a higher MIA dose [3 mg; (More et al., 2013)]. These factors may explain the discrepancies of results with the current study.

Administration of VEGF-A_{165b} had no significant effect on the frequency of rearings. This may indicate an actual lack of action of VEGF-A_{165b} on vertical spontaneous behaviour or is probably explained by the lack of such behaviour in the MIA rats in the first place.

4.4.3. Pain behaviour

The main objective of this part of the study was to assess the potential analgesic effects of VEGF-A_{165b} in OA. For this purpose, hindpaws mechanical withdrawal thresholds and weight bearing asymmetry were measured. Weight bearing is both peripherally and centrally driven, while paw withdrawal threshold is mainly centrally driven (Graven-Nielsen and Arendt-Nielsen, 2002, Arendt-Nielsen et al., 2010). MIA rats that received VEGF-A_{165b} did not show a significant reversal of decreased mechanical withdrawal thresholds. This is probably because the decrease in withdrawal thresholds of the MIA/PBS animals compared to the naïve control group was not significant in the first place. The MIA model of OA is well-known to feature secondary sensitisation, expressed as a significant decrease in hindpaw withdrawal threshold (Combe et al., 2004, Liu et al., 2011). In the current study, such finding was not significant, although OA pathology did develop, as showed by increases in macroscopic and microscopic scores comparable to those in literature (Mapp et al., 2013, Nwosu et al., 2016b, Sagar et al., 2014). Therefore, the absence of significant withdrawal sensitisation is probably due to a failure to detect pain behaviour using von Frey hairs and this may be due to inexperience, poor technique or different method of calculation of withdrawal thresholds. In this study, the test was conducted using the full battery paradigm (Kim and Chung, 1992) (see

section 2.4.2 for details), while other OA studies used different methods, such as 1) application of hairs in ascending order until the first withdrawal is observed, or 2) the staircase (“up and down”) assay (Bradman et al., 2015).

In the current study, rats developed significant weight bearing asymmetry 4 days after the intra-articular injection of MIA and for the whole duration of the study. This pattern of pain behaviour is extensively found in literature, with weight bearing imbalance observed as soon as 3 days post-MIA injection and lasting up to 10 weeks (Combe et al., 2004, Fernihough et al., 2004, Liu et al., 2011, Nwosu et al., 2016a).

Administration of VEGF-A_{165b} significantly reversed weight bearing asymmetry. When given twice weekly for the first two weeks of the study, VEGF-A_{165b} caused a significant increase in the percentage of weight borne on the ipsilateral hindpaw by day 28. When weight bearing imbalance was calculated as percentage of weight borne on the ipsilateral hindpaw over total body weight to account for any transfer of weight from hindpaws to forepaws, both early and late administration of VEGF-A_{165b} reversed weight bearing asymmetry. When comparing the effects on pain behaviour in diabetic neuropathy, inflammatory arthritis and the current MIA model of OA, it was found that biweekly systemic VEGF-A_{165b} treatment significantly inhibits pain behaviour in all models. VEGF-A_{165b} treatment reversed diabetes-induced changes in mechanical allodynia and heat hyperalgesia starting from weeks 2 and 4, respectively and prevented the onset of cold allodynia in the streptozotocin (STZ) model of diabetic neuropathy (Hulse et al., 2015). In the CFA model of inflammatory arthritis, two systemic doses (on days 0 and 3 post-CFA injection) of VEGF-A_{165b} had a significant inhibitory effect on weight bearing asymmetry and inhibited the development of secondary mechanical allodynia in the ipsilateral hind paw (Beazley-Long et al., 2015). Therefore, the current study replicated the effect exerted by VEGF-A_{165b} on the weight bearing imbalance in different models of pain but failed to show a similar effect on mechanical withdrawal thresholds (see above).

The evident effect of VEGF-A_{165b} on weight bearing asymmetry, especially with administration in the early course of the disease, suggests an effect on

peripheral sensitisation, along with prevention of central sensitisation. This would go with a proposed mechanism for VEGF-A_{165b} anti-nociception involving TRP channels, mainly TRPV1 and TRPA1 (Hulse et al., 2015, Hulse et al., 2014).

TRPV1 (transient receptor potential vanilloid) channel is a calcium-permeable channel expressed primarily in small-diameter neurons of the sensory ganglia (Caterina et al., 1997) and in some cortical areas and epithelia (Toth et al., 2005, Fernandes et al., 2012). TRPV1 can be activated by noxious heat (greater than ~42 °C) (Sun and Zakharian, 2015), some endogenous fatty acid metabolites (Cao et al., 2013), acidic solutions (Tominaga et al., 1998, Julius and Basbaum, 2001) and some pungent chemicals and food ingredients such as capsaicin (Clapham, 2003, Zheng, 2013), as well as by toxins, such as resiniferatoxin (Raisinghani et al., 2005) and vanillotoxins (Siemens et al., 2006). TRPV1 is known to be involved in peripheral sensitisation of neurons, leading to chronic pain states (Levine and Alessandri-Haber, 2007).

VEGF-A is thought to modulate TRPV1 sensitivity through PLC/PKC signalling pathway (Hulse et al., 2014), or neuropeptides involved in nociception, such as somatostatin, angiotensin and kappa opioids (Pan et al., 2008). VEGF-A_{165a} variant exerts direct effects on agonist-induced TRPV1 channel opening, TRPV1-evoked calcium signalling and TRPV1 phosphorylation in isolated DRG neurons, resulting in peripheral mechanical sensitisation (Hulse et al., 2014). VEGF-A_{165a} could also exert central sensitising effects, as suggested by central TRPV1 receptor responses in both TRPV1 knockout and antagonist interventions (Fernandes et al., 2012). VEGF-A_{165b} blocks the effect of VEGF-A_{165a} (Hulse et al., 2014) and it is thus believed to have actions on nociception that involves TRPV1.

TRPA1 is a non-specific cation channel involved in the sensitisation of peripheral nociceptors to mechanical (Dunham et al., 2008), cold (Barriere et al., 2012) and chemical (Koivisto et al., 2012) pain. In vitro studies showed that VEGF-A_{165b} induced blockade of TRPA1-mediated calcium entry and/or handling in hyperglycaemic conditions, thus reducing mechanical and thermal pain in diabetic neuropathy (Hulse et al., 2015). In addition, VEGF-A_{165b} also

affects nociception through TRPV1, as mentioned above. These findings are of particular interest, given the role of TRP channels in neuropathic pain (Honore et al., 2005, Puttfarcken et al., 2010, McGaraughty et al., 2010) and the recently highlighted neuropathic aspects of pain in OA (Konttinen et al., 2012, Ivanavicius et al., 2007, Im et al., 2010, Tsuda, 2016). Indeed, both TRPV1 and TRPA1 have been shown to play a key role in models of osteoarthritis (Barton et al., 2006, Kelly et al., 2015, Fernandes et al., 2011).

Of paramount importance is the role of VEGF in the pathophysiology of osteoarthritis. In the inflamed synovium, activated immune and endothelial cells release angiogenic growth factors, including VEGF, which contribute to the neovascularisation of articular cartilage. The formation and the innervation of these vessels are important in the stimulation of primary sensory neurons and the development of deep pain described by some OA patients (Bonnet and Walsh, 2005). Angiogenesis also maintains chondrocyte hypertrophy, endochondral ossification and osteophyte formation in the osteochondral junction, contributing to the exaggeration of pain (Mease et al., 2011, Dimitroulas et al., 2014). These observations indicate that VEGF indirectly contributes to OA pain through its angiogenic effects. Therefore, it cannot be excluded that the administration of the anti-angiogenic variant VEGF-A_{165b} impaired nociception by antagonising the pro-angiogenic effects of endogenous VEGF.

4.4.4. Joint pathology and histology

Both macroscopic and microscopic scores obtained in this study indicated that intra-articular injection of 1 mg MIA produced significant chondropathy 28 days after injection. This confirms the findings of several previous studies (Nwosu et al., 2016b, Sagar et al., 2014, Ashraf et al., 2013), although the degree of chondropathy was more accentuated in others (Guingamp et al., 1997, Guzman et al., 2003, Udo et al., 2016). These discrepancies may be explained by the use of different rat strain, age or weight, doses of MIA or multiple methods of chondropathy assessment. Interestingly, chondropathy extent in the MIA

characterisation study (chapter 3) was not significant in the MIA rats compared to the naïve control, and this difference is due to the different time point at which cartilage damage was assessed (days 7 and 14 in the MIA characterisation study vs day 28 in the MIA/VEGF-A_{165b} study). Neither early nor late administration of systemic VEGF-A_{165b} affected chondropathy of MIA injected rats. This indicates that VEGF-A_{165b} did not interfere with the pathology of OA, which is known in the MIA model to be a chemically- initiated death of chondrocytes and subsequent cartilage destruction (Pitcher et al., 2016), and that the analgesic effect of VEGF-A_{165b} was not associated with improvement of chondropathy.

Osteophytes are a feature of OA in humans (van der Kraan and van den Berg, 2007) and they were found to develop significantly in rat models of OA as early as two weeks after induction of OA (Janusz et al., 2002, Mapp et al., 2013). In the current study, no significant difference in osteophyte score was detected between MIA groups and the naïve control group; however, the percentage of samples presenting osteophytes was significantly higher in the MIA/PBS group than that in the naïve control group. VEGF-A_{165b} administration to MIA rats in the early or late phase of the study had no significant effect on osteophyte score.

Synovial inflammation is a key feature of human OA and it is clearly reproduced in the animal models of OA, including the MIA model. Although most synovial tissues were missing in the current study because they were lost during knee disarticulation, synovial inflammation score of the available samples was significantly higher in the MIA/PBS group than the naïve control on day 28. This is compatible with the findings of previous studies (Mapp et al., 2013, Guingamp et al., 1997, Sagar et al., 2014), in which MIA animals showed significant synovial inflammation at the same time point. Administration of systemic VEGF-A_{165b}, on the other hand, did not significantly affect synovitis score of the MIA injected rats.

Both osteophyte formation and synovial inflammation in OA involve angiogenesis (Ashraf et al., 2011a), in which VEGF is an essential molecule (Murata et al., 2008). Increased angiogenesis in the synovium is associated with chronic synovitis in OA (Haywood et al., 2003). Normal adult human articular cartilage is avascular and aneural, but in OA neo-vascularisation and generation

of sympathetic and sensory nerves are observed in the cartilage, subchondral bone and newly formed osteophytes (Walsh et al., 2007, Suri et al., 2007). Local intra-articular injection of VEGF in the knee (Ludin et al., 2013) and temporomandibular joint (Shen et al., 2015) directly produced OA characteristic features. Inhibition of VEGF by different mechanisms in models of OA or related pathologies (rheumatoid arthritis, osteochondral defects) reduced angiogenesis, improved cartilage repair and reduced osteophyte formation and synovitis (Hamilton et al., 2016). It should be noticed here that VEGF previously studied in the context of OA and just mentioned above refers to the whole VEGF-A protein (pan-VEGF) and does not differentiate between the splice variants. As mentioned before, VEGF protein is found in two isoform families, pro-angiogenic VEGF-A_{165a} and anti-angiogenic VEGF-A_{165b} (Harper and Bates, 2008). VEGF-A_{165a} isoform, which is known to be upregulated in pathological conditions, is therefore responsible for the angiogenic activity of VEGF and subsequent effects in OA. These previous findings could suggest a potential effect of the anti-angiogenic VEGF-A_{165b} in the prevention of disease progression and development of synovitis and osteophyte, but that was not the case in the current study. Possible explanations of these findings include 1) an actual lack of effect due to systemic rather than topical administration or insufficient dosing, and 2) inadequate statistical power due to the small group size (n=2-4 for synovitis scoring).

4.4.5. Spinal glial cell activation

Spinal glial cells play a relevant role in central sensitisation and development of allodynia and secondary hyperalgesia in various models of chronic pain, including neuropathic and inflammatory pain (Bradesi, 2010). Neuropathic pain has been extensively studied in animal models, such as nerve transection or resection, ligation of the sciatic nerve, spinal nerves or lumbar spinal cord roots, and spinal cord injury. Neuropathic pain is known to involve primary and secondary hyperalgesia associated with a robust activation of spinal microglia and astrocytes and the release of pro-inflammatory mediators (Zhuang et al., 2005, Ren and Dubner, 2008). Inhibitors of glial cells, such as minocycline and

fluorocitrate, reverse increased pain behaviour while reducing release of inflammatory mediators in models of neuropathic pain (Raghavendra et al., 2003, McMahon et al., 2005). Models of inflammatory pain, such as CFA induced arthritis, also show activation of spinal microglia and astrocytes along with hyperalgesia, which is inhibited by glial blockers (Cao and Zhang, 2008). However, conflicting results on the timing of glial cell activation in inflammatory pain were reported (Bradesi, 2010). In the study by Cao and Zhang, for example, a robust microglial activation was observed at days 1, 3, and 10 post-CFA injection, with the maximum on day 3, and significant astrocytic activation occurred on days 3 and 10. Other groups demonstrated that hindpaw injection of 2.5-5% formalin produces mild microglial activation after an interval of 3–7 days (Aumeerally et al., 2004, Fu et al., 2000, Sweitzer et al., 1999). One study found an increase in OX-42 immunostaining with a concomitant increase in mRNA expression for other microglia markers (Mac-1, TLR4 and CD14) in rat spinal cord starting four hours after injection of CFA (Raghavendra et al., 2004). However, other studies failed to demonstrate increased OX-42 immunoreactivity over a similar time course of peripheral inflammation (Honore et al., 2000, Zhang et al., 2003).

Sagar et al investigated the contribution of spinal glial cells to chronic pain behaviour in the MIA model of OA (Sagar et al., 2011). Microglia in the ipsilateral spinal cord were activated from day 7 post MIA injection and up to day 28, when the extent of microglia activation was also significantly correlated with distal allodynia. Astrocyte activation was only observed on day 28. Anti-inflammatory nimesulide and glial activation blocker minocycline attenuated distal allodynia and reversed activation of microglia and astrocytes.

In the current study, analysis of CD11b and GFAP immunofluorescence in spinal cord revealed no significant activation of microglia or astrocytes 28 days after injection of MIA. These results are not consistent with the previous findings mentioned above and may be due to differences in species or models of OA used, or by differences in methods of analysis (detection of cell activation and analysis of data). However, a decline in microglia activation by day 28 post MIA injection was also suggested (Sagar et al., 2011), which may explain the

lack of significant activation of spinal microglia at this time point in the current study.

In addition to its well-known angiogenic role, VEGF is also known for its important effects in the nervous system, where it affects astrocytes, Schwann cells and motor neurons (Rosenstein et al., 2010). VEGF is known to have trophic effects on neurons, which may be mediated indirectly by glial cells. For example, VEGF enhances survival of primary motor neurons on glial feeder layers in oxidative stress, an effect that is inhibited by VEGF receptor blocker antibodies (Oosthuyse et al., 2001). In addition, VEGF is mitogenic for astroglia and Schwann cells (Krum et al., 2002, Silverman et al., 1999, Mani et al., 2005). Both types of cells produce many growth factors that support neuronal growth, including VEGF itself. Glial expression of VEGF occurs when glial end feet begin to enclose blood vessels and VEGF signals are then used to maintain vasculature (Lambrechts and Carmeliet, 2006). VEGF is also expressed in neurons and glial cells of the spinal cord, and it increases proliferation and migration of astrocytes (Silverman et al., 1999, Krum et al., 2002) and microglia (Forstreuter et al., 2002). Figure 4.18 summarises the interactions between VEGF and some types of cells, including glial cells.

Previous literature investigated the neuroprotective effects of VEGF-A_{165b} splice variant, but the role of glial cells in this process is not known (Beazley-Long et al., 2013). VEGF-A_{165a} isoform is known to be neuroprotective in retinal (Brar et al., 2010), cortical (Rosenstein et al., 2003), hippocampal (Jin et al., 2000a, Jin et al., 2000b, Nicoletti et al., 2008) and dorsal root ganglia (Lin et al., 2010a) neurons, where endogenous expression of VEGF-A_{165b} was also detected. Recombinant human VEGF-A_{165b} effectively protected both peripheral and central neurons in vivo and in vitro through VEGFR2, mitogen-MEK1/2, and inhibition of caspase-3 induction (Beazley-Long et al., 2013).

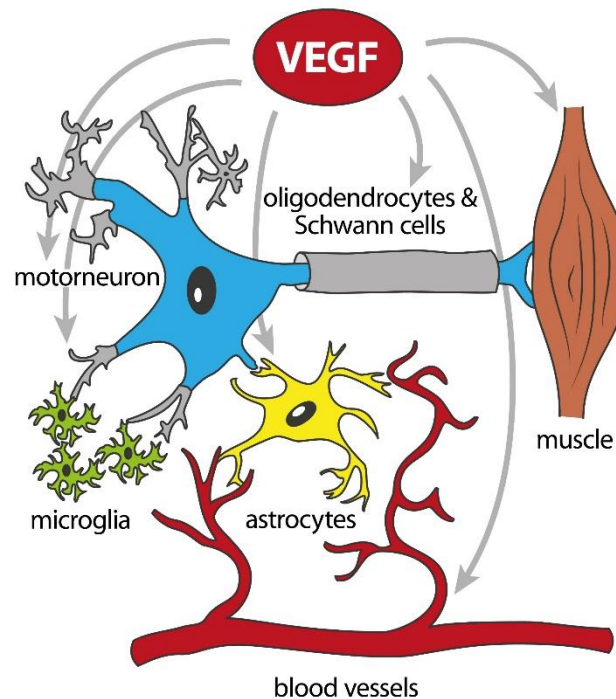


Figure 4.18. Image representing the cells affected by VEGF in the nervous system and their interactions. (Reproduced with modifications from Lambrechts and Carmeliet, 2006).

VEGF-A_{165b} had neuroprotective effects in diabetic neuropathic pain through reduction of hyperglycaemia-induced activated caspase 3 (AC3) levels in sensory neurons, epidermal sensory nerve fibre loss and aberrant sciatic nerve morphology (Hulse et al., 2015, Beazley-Long et al., 2013).

To the best of our knowledge, effects of VEGF-A_{165b} variant on spinal glial cell activation in models of pain were not previously investigated, so this would be the first study to address the issue. It was found that administration of VEGF-A_{165b} did not cause any change in the activation of spinal glial cells in the MIA model of osteoarthritis.

4.4.6. COX-2 expression in the spinal cord

Cyclooxygenases and their main reaction products, prostaglandins, are of paramount importance in the development of central sensitisation in the spinal cord (Hoffmann, 2000, Svensson and Yaksh, 2002, Seybold et al., 2003), as well as synaptic plasticity (Yang and Chen, 2008). Numerous studies in models of inflammatory arthritis demonstrated upregulation of COX-2 and release of PGE₂ in the spinal cord (Ebersberger et al., 1999, Goppelt-Strube and Beiche, 1997), increased spinal neuronal responses upon application of PGE₂ (Vasquez et al., 2001) and EP receptor agonists (Bar et al., 2004) and reduced spinal hyperexcitability after the administration of a COX inhibitor (Vasquez et al., 2001). COX-2 is also involved in synaptic plasticity, as demonstrated by the upregulation of COX-2 expression by high-frequency stimulation (HFS) associated with the induction of long-term potentiation (LTP), a form of synaptic plasticity and cellular model of learning and memory (Bliss and Collingridge, 1993). In addition, HFS-induced LTP and long-term depression was reduced by selective COX-2 inhibitors at hippocampal synapses (Chen et al., 2002, Murray and O'Connor, 2003, Slanina et al., 2005). It was also observed that administration of COX-2 inhibitors impairs passive avoidance task (Holscher, 1995), memory acquisition, memory retention (Rall et al., 2003, Teather et al., 2002), and spatial memory consolidation (Sharifzadeh et al., 2006, Shaw et al., 2003), which supports the involvement of COX-2 in long-term synaptic plasticity and cognition.

Although the level of inflammation in the MIA model of OA is relatively low (Bove et al., 2003, Liu et al., 2011), inflammation is known to contribute significantly to pain (Bonnet and Walsh, 2005). A few studies investigated the expression of COX isoforms in the spinal cord of MIA animals, and their effects on hyperalgesia. Prochazkova et al found a maximum increase in the expression of spinal COX-2 mRNA and COX-2 protein on day 5 post-MIA injection, while expression of spinal COX-1 mRNA and protein increased gradually reaching maximum on the day 31. This indicated a role of spinal COX-1 in osteoarthritis pain, in contrast to inflammatory pain (Prochazkova et al., 2009). In addition, systemic and spinal administration of the COX-2 inhibitor nimesulide attenuated

spinal neuronal responses to both noxious and innocuous stimuli in the MIA model of OA, indicating a role for peripheral and spinal COX-2 products in neuronal responses in this model (Staniaszek et al., 2010). Nimesulide also attenuated pain behaviour and microglia and astrocyte activation in the ipsilateral spinal cord of MIA injected rats (Sagar et al., 2011). In the current study, no significant changes in the spinal expression of COX-2 were observed in the MIA injected rats on day 28 post-MIA injection compared to the naive group in any of the studied areas of the spinal cord (superficial or deep laminae of the ipsilateral or contralateral dorsal horn). These findings could indicate a more limited role of COX-2 in the establishment of hyperalgesia in the MIA model of osteoarthritis. In addition, previous studies investigated the overall expression of COXs mRNA and protein (by Western blot) in the spinal cord (Prochazkova et al., 2009) or the effect on hyperalgesia as proved by efficacy of COX-inhibitors (Sagar et al., 2011, Staniaszek et al., 2010), while in the current study expression of COX-2 protein was evaluated by immunofluorescence, in very specific areas of the dorsal horn and in association with neuronal or non-neuronal cells. COX-2 mRNA (Beiche et al., 1996, Ichitani et al., 1997, Goppelt-Struebe and Beiche, 1997) and protein (Ebersberger et al., 1999) were found to be up-regulated bilaterally at the spinal cord level during peripheral inflammation; however, such changes were not detected in the current study involving the MIA model of OA, probably due to the different inflammatory status of the two conditions. Interestingly, in naïve rats, the expression of COX-2 in non-neuronal cells in both the ipsilateral and contralateral spinal cord was significantly higher in the deep laminae (III-V) than the superficial ones (laminae I-II), which suggests a greater involvement of COX-2 in non-neuronal (probably glial or endothelial) cells in the transmission of noxious information to supra-spinal pain centres.

VEGF has been shown to play important roles in inflammation and interact with several inflammatory mediators. The link between COX-2 and VEGF has been widely investigated in the inflammatory context of cancer pathophysiology. Clinical evidence suggest that COX-2 expression is upregulated in several types of malignancy, inducing angiogenesis and other processes involved in tumorigenesis (Toomey et al., 2009). In human umbilical vein endothelial cells

(HUVEC) treated with VEGF, COX-2 protein and activity increased in a dose-dependent, protein tyrosine kinase mediated manner (Akarasereenont et al., 2002). A correlation was found between COX-2 gene expression and angiogenesis in premalignant tissues and cancer. PGE₂ stimulates angiogenesis via the transcription factor hypoxia inducible factor-1 (HIF-1 α), leading to the induction of VEGF. Interestingly, VEGF also stimulates the expression of COX-2. The mutual influence of COX-2 and VEGF on each other indicates a positive feedback amplification mechanism (Zarghi and Arfaei, 2011).

Both COX-2 and VEGF are known to be upregulated in injured spinal cord. It has been shown that astrocytes (Krum and Rosenstein, 1998, Papavassiliou et al., 1997) and inflammatory cells (Bartholdi et al., 1997) express VEGF following CNS trauma. Injection of PGF₂ into injured spinal cord induced the expression of VEGF and its receptors; the same effect was observed *in vitro* using cell cultures of meningeal fibroblast and astrocyte origin, even in the absence of inflammatory cells (Skold et al., 2000).

As mentioned above, COX-2 plays a role in synaptic plasticity, a phenomenon in which VEGF has also been implicated (Licht et al., 2011, Zhang et al., 2015, Vivar et al., 2013). However, no direct relation between these molecules was observed in this context.

VEGF-A_{165b} expression and use as a potential therapeutic agent have been investigated in a number of cancer types (Tayama et al., 2011, Bates et al., 2002, Rennel et al., 2008a, Rennel et al., 2008b, Mavrou et al., 2015), but very little is known about its interactions with COX-2. In the current study, VEGF-A_{165b} did not affect the expression of COX-2 in the spinal cords of MIA animals. This finding is particularly interesting when compared to the mutual upregulating effects of endogenous VEGF and COX-2. These actions, however, are exerted by the pan-VEGF protein, including both splice variants, while only VEGF-A_{165b} was used in this study. This may explain the lack of effect on the expression of spinal COX-2. To differentiate between the effects of the two variants on COX-2 expression in the spinal cord, it would be useful to administer VEGF-A_{165a} and pan-VEGF in separate studies. Systemic VEGF-A_{165b} crosses BBB and penetrates the spinal cord, as showed in the study by Beazley Long et

al, where systemic, but *not* intra-articular VEGF-A_{165b} significantly reduced CFA-induced ipsilateral mechanical allodynia, indicating a central rather than local anti-nociceptive effect of VEGF-A_{165b} (Beazley-Long et al., 2018). Therefore, the lack of VEGF-A_{165b} effect on spinal COX-2 expression may be due to impaired penetration to spinal cord. In this case, the sites of effect would be the vasculature itself or, less likely, locally in the joint.

4.4.7. COX isoform expression in the PAG

The PAG is a key component of the pain control system in the CNS. One descending facilitatory system from the PAG depends on endogenous prostaglandins (Leith et al., 2007). COX enzymes and PGE₂ receptors (known as EP receptors) are reportedly expressed in the PAG (Breder et al., 1992, Ek et al., 2000, Matsumura et al., 1992). The COX-1-PGE₂ system within the ventrolateral PAG (VL PAG) facilitates spinal processing of C-nociceptor inputs in naive rats, as indicated by the development of hyperalgesia upon PGE₂ injection in the VL PAG (Heinricher et al., 2004, Leith et al., 2007) and analgesia due to COX-1 inhibition in the PAG (Tortorici and Vanegas, 1995, Leith et al., 2007). EP receptor signalling in the PAG is involved in primary inflammatory hyperalgesia and neuropathic pain in the rat (Oliva et al., 2006, Palazzo et al., 2008). PGE₂ in the VL PAG modulates ON cell activity within the rostral ventral medulla (RVM), which is critical in the development of secondary hypersensitivity (Urban et al., 1999, Vanegas, 2004, Ambriz-Tututi et al., 2011).

Based on the observations of regulation of COX expression in the PAG in inflammatory arthritis (Drake et al., 2016), this study investigated a potential increase in COX enzyme expression in the PAG of MIA rats, as well as an inhibitory effect of VEGF-A_{165b} on PAG COX expression. However, no significant changes in the expression of COX-1 or COX-2 were found in osteoarthritic rats or in the animals treated with VEGF-A_{165b}. These findings are inadequate to clearly indicate the role of COX enzymes in the PAG in the MIA model of OA and any potential related effect of VEGF-A_{165b}.

Chapter 5. Effects of SPHINX-31 in the MIA model of osteoarthritis

5.1. Introduction

Alternative splicing of VEGF-A into its variants VEGF-A_{165a} (also denoted VEGF-A₁₆₅) and VEGF-A_{165b} is regulated by serine arginine rich protein kinase 1 (SRPK1), which phosphorylates serine rich splice factor 1 (SRSF1) in the cytoplasm. After SRSF1 nuclear translocation, this auxiliary splicing factor interacts with an exonic sequence enhancer (ESE) upstream of the VEGF exon 8a proximal splice site, enhancing VEGF_{xxx} splicing (Gammons et al., 2013b, Amin et al., 2011). Generally, basal expression of VEGF is dominated by the VEGF-A_{xxx}b variant (Bevan et al., 2007), but this was found to be down-regulated in most investigated cancers (Bates et al., 2002, Woolard et al., 2004, Varey et al., 2008, Pritchard-Jones et al., 2007) and other pathologies associated with abnormal angiogenesis, such as proliferative diabetic neuropathy (Perrin et al., 2005). On the other hand, upregulation of VEGF-A_{165b} was detected in diabetic patients with preserved kidney function (Oltean et al., 2015), in obese patients (Ngo et al., 2014) and post-ST-elevation myocardial infarction (Hueso et al., 2017). Upregulation of VEGF-A_{165a} in the absence of change in VEGF-A_{165b} was also observed in prostate cancer (Mavrou et al., 2015). Inhibition of SRPK1 prevented SRSF1 phosphorylation and VEGF-A_{165a} upregulation in vivo (Nowak et al., 2008, Nowak et al., 2010). A number of SRPK1 inhibitors were developed and successfully tested as anti-angiogenic agents in neoplasms (Mavrou and Oltean, 2016, Mavrou et al., 2015) and ophthalmologic disorders (Gammons et al., 2013a, Gammons et al., 2013b). Intraplantar injection of one of these agents, SRPIN340, raised mechanical withdrawal thresholds (i.e. resulted in hypoalgesia) in naïve mice and reduced the amount of VEGF-A_{165a} mRNA as a proportion of the total VEGF-A mRNA in plantar skin (Hulse et al., 2014). In addition, spinal SRPIN340 attenuated SRSF1 activity, reversed pain behaviour and suppressed central sensitization in a model of neuropathic pain (Hulse et al., 2016), indicating a role of alternative pre-mRNA splicing in nociceptive processing (Hulse et al., 2014). SPHINX-31 is a highly specific and

potent inhibitor of SRPK1 that was recently developed (Batson et al., 2017). Topical application of SPHINX-31 to the eye resulted in potent inhibition of blood vessel growth in models of choroidal angiogenesis in vivo (Batson et al., 2017). Recently, systemic administration of SPHINX-31 was found to favour VEGF-A splicing towards VEGF-A_{165b} through inhibition of SRSF1 in mouse kidney (Babaedi-Jadidi et al, unpublished).

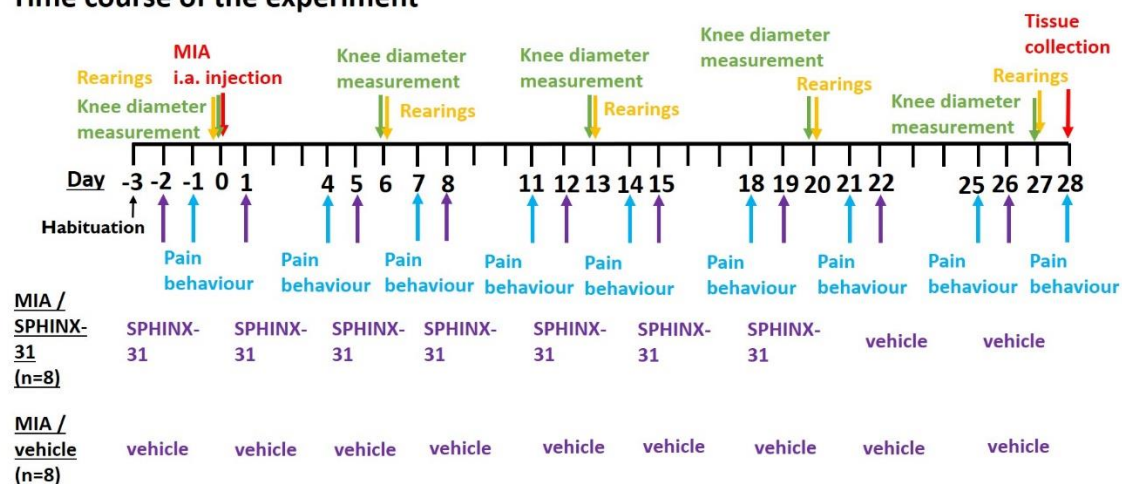
This is the first study to evaluate SRPK1 inhibitor SPHINX-31 as a potential analgesic agent. It tested the hypothesis that SPHINX-31 would prevent and/or reverse pain behaviour in the MIA model of OA, involving spinal glial cells and cyclooxygenases in the PAG and the spinal cord. In addition, activation of astrocytes in the PAG and their interaction with blood vessels were investigated. These two elements were addressed based on the growing body of evidence that glial/endothelial interactions play a relevant role in the development of chronic pain after peripheral inflammation (Willis et al., 2008, Huber et al., 2006), and that this effect may involve VEGF (Beazley-Long et al., 2018). The work performed to test these hypotheses is presented in this chapter.

5.2. Methods

5.2.1. Induction of the MIA model of OA

Knee osteoarthritis was induced in sixteen male Wistar rats with intra-articular injection of MIA, as explained in section 2.2. Half of them received SPHINX-31 (i.p. 0.8 µg/g body weight in 1% DMSO 8 µl/g body weight, twice weekly until day 19; “MIA/SPHINX-31”), while the others received vehicle only (i.p. 1% DMSO 1 µl/g body weight, twice weekly; “MIA/vehicle”). Eight rats were anaesthetised without receiving MIA injection and were treated with vehicle as above (“Naïve/vehicle”). Figure 5.1 shows the time course of the study.

Time course of the experiment



MIA 1 mg/50 μ l NS intra-articular in the right knee; SPHINX-31 0.8 μ g/g body weight i.p.
Control group: naïve + vehicle (n=9)
Pain behaviour included: weight bearing and mechanical withdrawal threshold (von Frey)

Figure 5.1. Time course of the MIA/SPHINX-31 in vivo study.

The dose of SPHINX-31 is the one used in the study by (Babaedi-Jadidi et al, unpublished), which effectively changed VEGF-A splicing towards VEGF-A_{165b} by inhibiting SRSF1 phosphorylation in mouse kidney (figure 5.2).

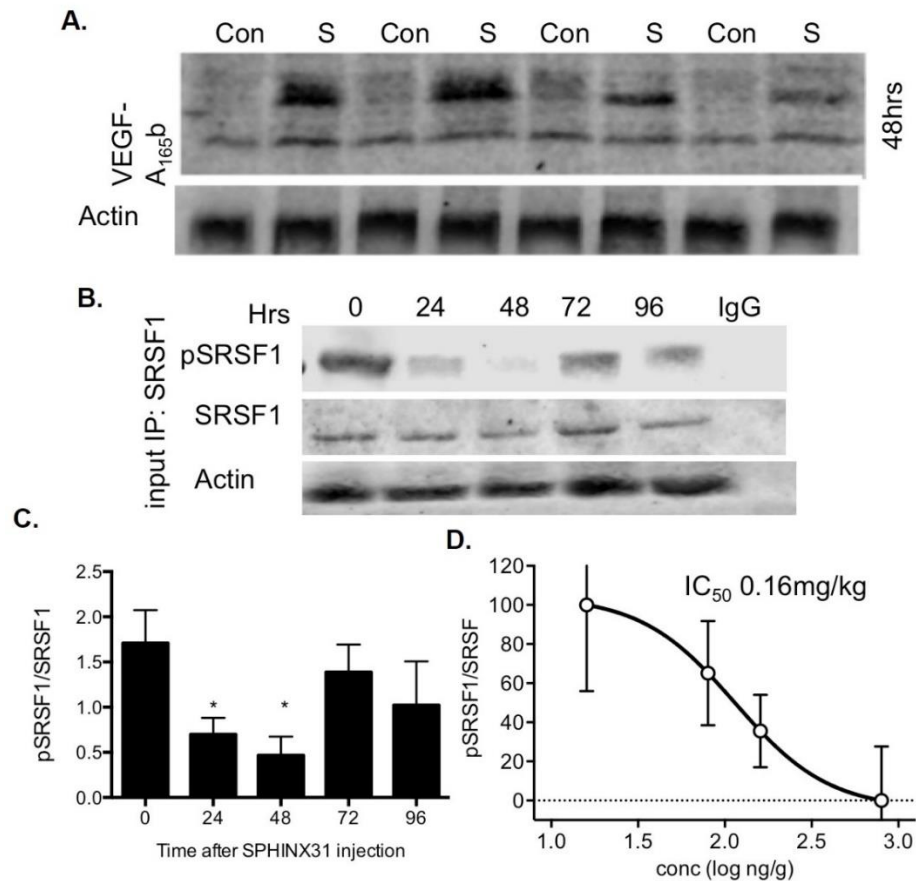


Figure 5.2. Systemic SPHINX-31 prevents kidney SRSF1 phosphorylation and alters VEGF-A splicing. (A) Single intraperitoneal injection of SPHINX-31 (0.8 µg/g) alters VEGF-A splicing in favour of VEGF-A_{165b}. **(B)** SRSF1 phosphorylation is reduced in kidney at 24 hours by SPHINX-31 and **(C)** maintained up to 48 hours, with an IC₅₀ of 0.16 mg/kg **(D)**. Animals experienced no obvious adverse effects. *(Experiments done by Dr Babaedi-Jadidi).*

5.2.2. Pain behaviour, rearings and knee swelling

Pain behaviour was determined by measurement of 1) weight bearing asymmetry using an incapacitance test meter and 2) mechanical withdrawal thresholds by von Frey hairs (for more details refer to section 2.4). Knee joint swelling and number of rearings were recorded as explained in sections 2.5 and 2.6. After habituation, baseline pain behaviour was assessed (day 0). Tests were then performed twice weekly on days 4, 7, 11, 14, 18, 21, 25, and 28. Ipsilateral

knee diameter and rearing frequency were measured before the intra-articular injection of MIA (day 0) and then on days 6, 13, 20 and 27. One rat in the MIA/vehicle group was culled on day 18 for developing a severe reaction (as defined in the project protocol), so n=7 for the MIA/vehicle group from day 18 on.

5.2.3. Tissue collection and processing

On day 28 post-MIA injection, rats were killed and tissues collected as explained in details in section 2.8. Knees were disarticulated for the macroscopic assessment of chondropathy (section 2.10.1.1), and the tibiae were then processed for sectioning, H&E staining and microscopic evaluation of joint damage (see sections 2.8.3.3, 2.9.1 and 2.10.1.2). Using a cryostat (Leica CM-1900), midbrain PAGs and spinal cords at the L3-L5 level were sectioned to obtain 20 µm and 10 µm sections, respectively (see section 2.8.3). The sections were then stained for immunofluorescence (COX-2/NeuN and GFAP/CD11b/CD31; section 2.9.2) and analysed as explained in section 2.10.

5.3. Results

5.3.1. Acute inflammation (knee joint swelling)

No significant differences in ipsilateral knee diameter were found between the study groups at any time point (figure 5.3).

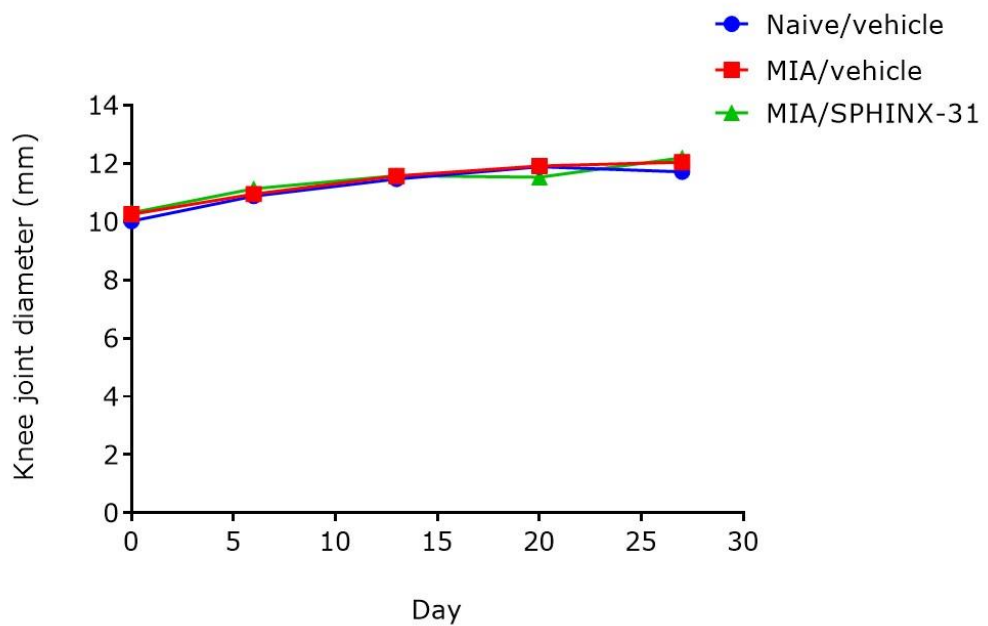


Figure 5.3. Ipsilateral knee joint diameter in the MIA/SPHINX-31 study. No significant changes were found in the diameter of ipsilateral knee between the study groups at any time point (n=8, except for MIA/vehicle group where n=7 from day 18; two-way ANOVA).

5.3.2. Rearings

The numbers of rearings that rats performed in a two-minute session did not differ between the study groups at any time point (figure 5.4)

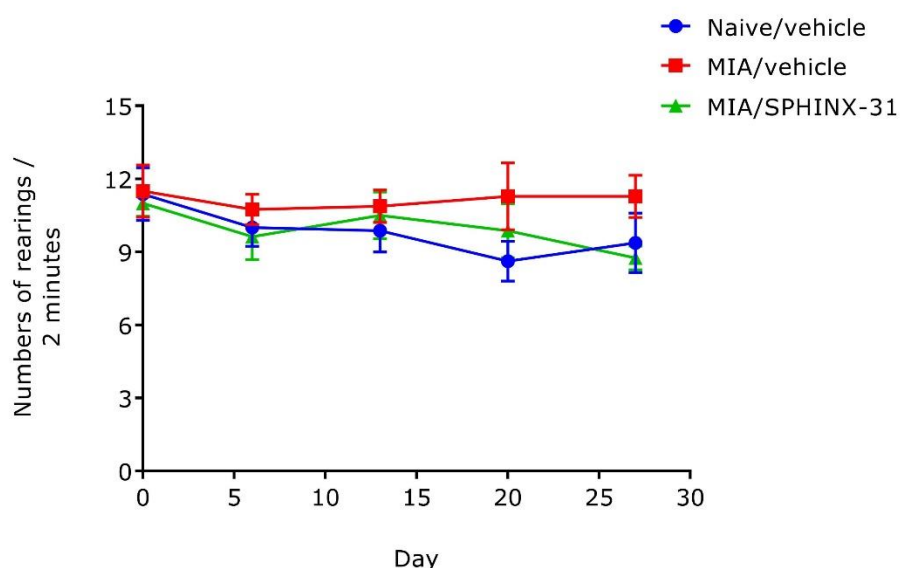


Figure 5.4. Number of rearings performed by rats in two minutes in the MIA/SPHINX-31 study. No differences between the study groups were found in the number of rearings performed over two minutes at any time point (n=8, except for MIA/vehicle group, where n=7 from day 18; two-way ANOVA).

5.3.3. Pain behaviour

5.3.3.1. Mechanical withdrawal thresholds (von Frey)

Mechanical withdrawal thresholds (g) of ipsilateral hindpaw were significantly lower in the MIA/vehicle group and in the MIA/SPHINX-31 group compared to the naïve controls starting on day 4 and 7, respectively (MIA/vehicle vs naïve/vehicle 4.15 ± 0.60 vs 7.11 ± 0.41 , $p < 0.05$; MIA/SPHINX-31 vs naïve/vehicle 4.66 ± 0.54 vs 9.75 ± 0.86 , $p < 0.0001$). This effect lasted until day 28 in the MIA/vehicle group and day 18 in the MIA/SPHINX-31 group

(MIA/vehicle vs naïve/vehicle 5.40 ± 0.78 vs 8.47 ± 0.52 , $p < 0.05$; MIA/SPHINX-31 vs naïve/vehicle 4.09 ± 0.76 vs 7.62 ± 0.36 , $p < 0.01$). No significant differences in the ipsilateral mechanical withdrawal thresholds were found between the MIA group treated with SPHINX-31 and the MIA/vehicle group at any time point (figure 5.5).

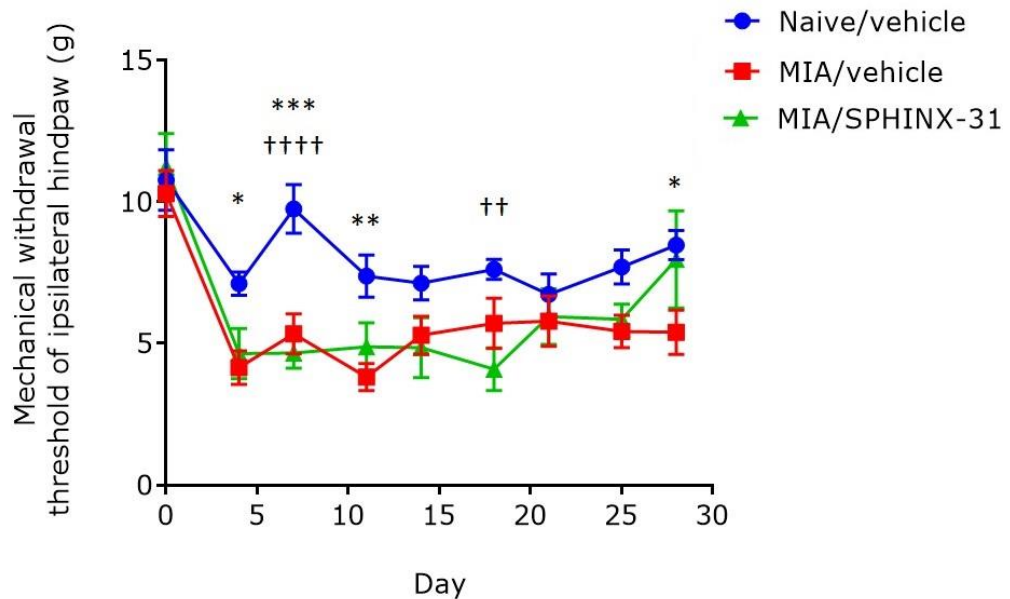


Figure 5.5. Ipsilateral mechanical withdrawal thresholds in the MIA/SPHINX-31 study. MIA/vehicle group showed significantly lower withdrawal thresholds of the ipsilateral hindpaw compared to the naïve control group on days 4, 7, 11 and 28. Ipsilateral withdrawal thresholds of the MIA/SPHINX-31 group also decreased significantly compared to the naïve/vehicle group on days 7 and 18. No significant difference in withdrawal thresholds was found between the MIA/SPHINX-31 and MIA/vehicle groups at any time point. (n=8/ group, except for MIA/vehicle group, where n=7 from day 18; two-way ANOVA with post-hoc Bonferroni test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for MIA/vehicle vs naïve group; †† $p < 0.01$, †††† $p < 0.0001$ for MIA/SPHINX-31 vs naïve group).

5.3.3.2. Weight bearing asymmetry

MIA rats injected with vehicle showed a significant weight bearing asymmetry

(%) all over the study duration compared to the naïve control group (MIA/vehicle vs naïve/vehicle 35.98 ± 1.62 vs 48.78 ± 0.65 , $p < 0.0001$ on day 4 and 42.71 ± 1.23 vs 49.17 ± 0.24 , $p < 0.01$ on day 28). MIA/SPHINX-31 group also exhibited weight bearing imbalance (%) compared to the naïve animals on days 4, 7, 14, 25 and 28 (MIA/SPHINX-31 vs naïve/vehicle 37.89 ± 2.13 vs 48.78 ± 0.65 , $p < 0.0001$ on day 4 and 44.41 ± 1.33 vs 49.17 ± 0.24 , $p < 0.05$ on day 28). No significant difference in weight bearing asymmetry between MIA/vehicle and MIA/SPHINX-31 groups was detected at any time point (figure 5.6).

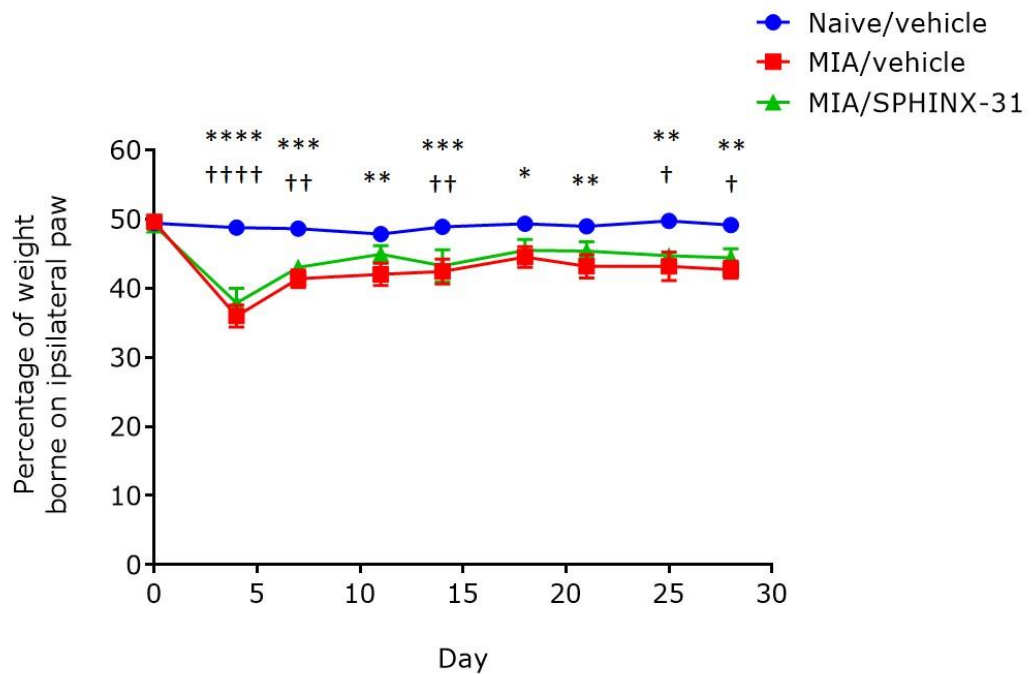


Figure 5.6. Weight bearing asymmetry calculated as percentage of weight borne on the ipsilateral hindpaw over the sum of weights borne on both hindpaws in the MIA/SPHINX-31 study. MIA/vehicle group showed significant weight bearing asymmetry compared to the naïve control group throughout the study duration. MIA/SPHINX-31 group also had a significant weight bearing imbalance compared to the naïve/vehicle group on days 4, 7, 14, 25 and 28. No significant difference in weight bearing asymmetry was found between the MIA/SPHINX-31 and MIA/vehicle groups at any time point. (n=8/group, except for MIA/vehicle group, where n=7 from day 18; two-way ANOVA with post-hoc Bonferroni test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ for MIA/vehicle vs naïve group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.0001$ for MIA/SPHINX-31 vs naïve group).

5.3.4. Joint pathology and histology

5.3.4.1. Macroscopic chondropathy score

Intra-articular injection of MIA in the knee produced significant chondropathy 28 days after injection, which was not reversed by the administration of SPHINX-31. This was indicated by the significant increase in macroscopic score in both MIA/vehicle and MIA/SPHINX-31 groups compared to the naïve control group (MIA/vehicle vs naïve/vehicle 11.71 ± 1.29 vs 1.00 ± 0.57 , $p < 0.05$; MIA/SPHINX-31 vs naïve/vehicle 14.25 ± 0.92 vs 1.00 ± 0.57 , $p < 0.001$; figure 5.7).

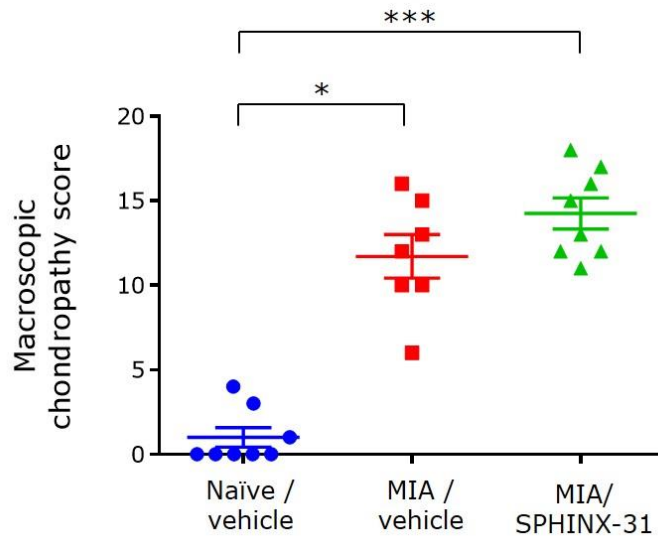


Figure 5.7. Macroscopic chondropathy score in the MIA/SPHINX-31 study. Both MIA groups had significantly higher macroscopic scores compared to the naïve/vehicle group. No significant difference in macroscopic chondropathy score was found between the MIA/vehicle and MIA/SPHINX-31 groups. (n=8/group, except for MIA/vehicle group, where n=7; Kruskal-Wallis with post-hoc Dunn's test; * $p < 0.05$, *** $p < 0.001$).

5.3.4.2. Microscopic chondropathy, osteophyte and synovial inflammation scores

Both MIA groups were associated with a significant increase in microscopic chondropathy score compared to the naïve group (MIA/vehicle vs naïve/vehicle 6.79 ± 0.90 vs 1.66 ± 0.43 , $p < 0.01$; MIA/SPHINX-31 vs naïve/vehicle 8.24 ± 1.35 vs 1.66 ± 0.43 , $p < 0.01$). No significant change was detected in the MIA/SPHINX-31 group compared to the MIA/vehicle group, indicating that SPHINX-31 treatment had no effect on MIA-induced chondropathy (figure 5.8).

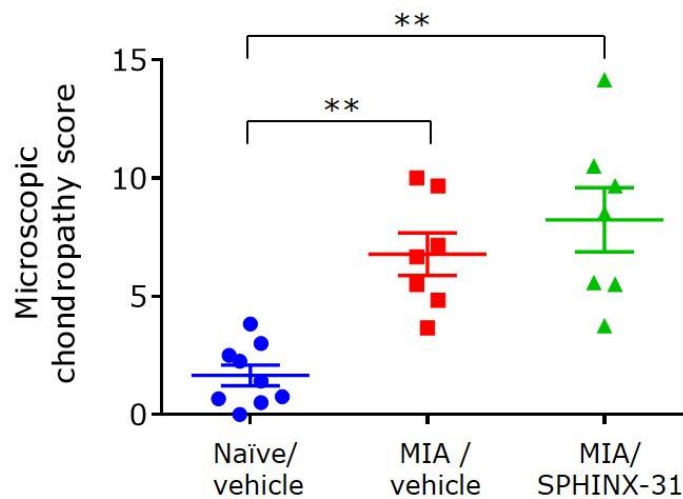


Figure 5.8. Microscopic chondropathy score in the MIA/SPHINX-31 study. Both MIA/vehicle and MIA/SPHINX-31 groups had significantly higher chondropathy scores compared to the naïve/vehicle group (n=7/group, except for naïve group, where n=9; Kruskal-Wallis analysis followed by Dunn's post-hoc; ** $p < 0.01$).

Scores of osteophyte and synovial inflammation did not significantly differ between the study groups (figure 5.9). It should be noticed that group size for the latter is variable due to the loss of synovial tissue from some samples during joint disarticulation.

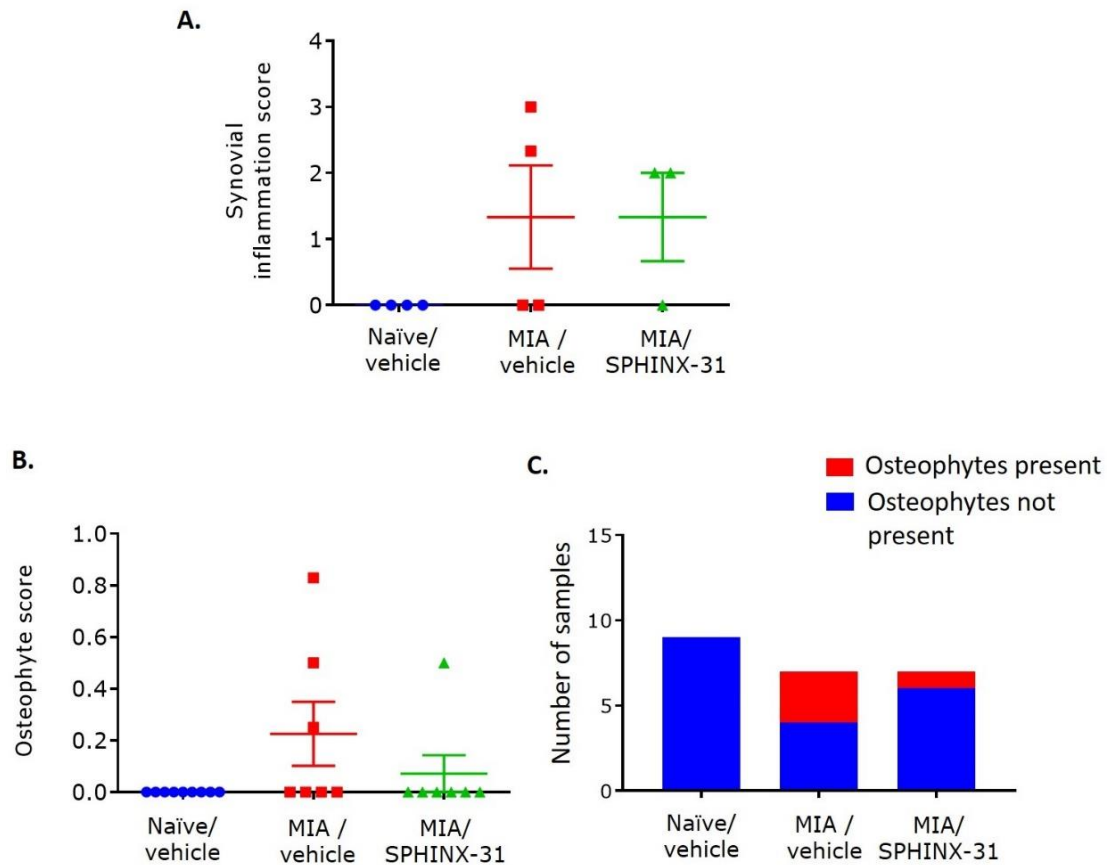


Figure 5.9. Synovial inflammation and osteophyte scores in the MIA/SPHINX-31 study. No significant difference in **(A)** synovitis and **(B)** osteophyte scores were found between study groups. **(C)** Contingency analysis of osteophytes did not show significant changes in the percentage of samples exhibiting osteophytes (n=3-9; Kruskal-Wallis test for **A** and **B**; Chi-square analysis for **C**).

5.3.5. Spinal COX-2 expression

Spinal cord sections were treated with anti-COX-2 and anti-NeuN (marker of neuronal nuclei) antibodies for the detection of COX-2 and its co-localisation with neuronal cells. Two areas (laminae I-II and laminae III-V) of both ipsilateral and contralateral spinal cords were analysed. No significant changes in the expression of COX-2 in neuronal or non-neuronal cells were detected in any of the spinal cord areas (figures 5.10 and 5.11).

Ipsilateral spinal cord

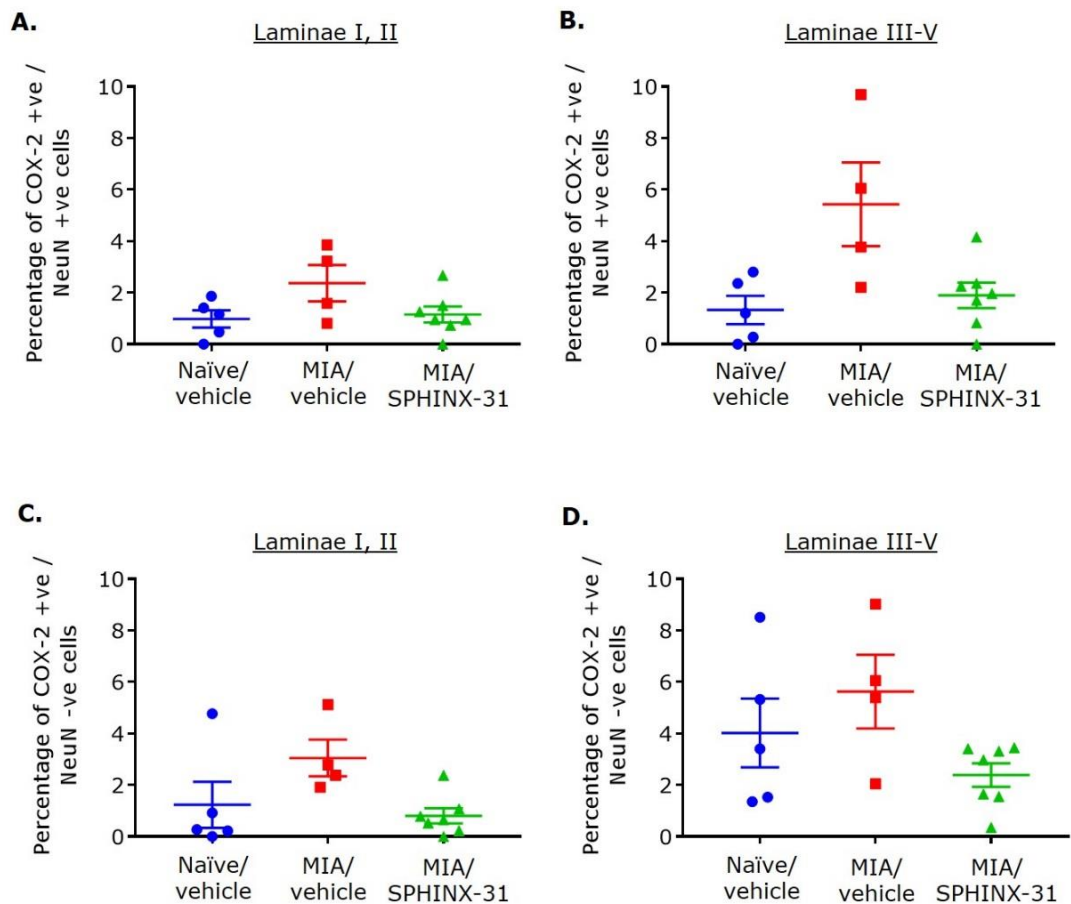


Figure 5.10. Percentage of neuronal and non-neuronal cells expressing COX-2 in laminae I,II and laminae III-V of the ipsilateral spinal cords in the MIA/SPHINX-31 study. In the ipsilateral spinal cord, no significant difference was found in the percentage of COX-2 +ve / NeuN +ve cells in **(A)** laminae I,II and **(B)** laminae III-V, or in the percentage of COX-2 +ve / NeuN -ve cells in **(C)** laminae I,II and **(D)** laminae III-V. (n=4-7/group; Kruskal-Wallis test).

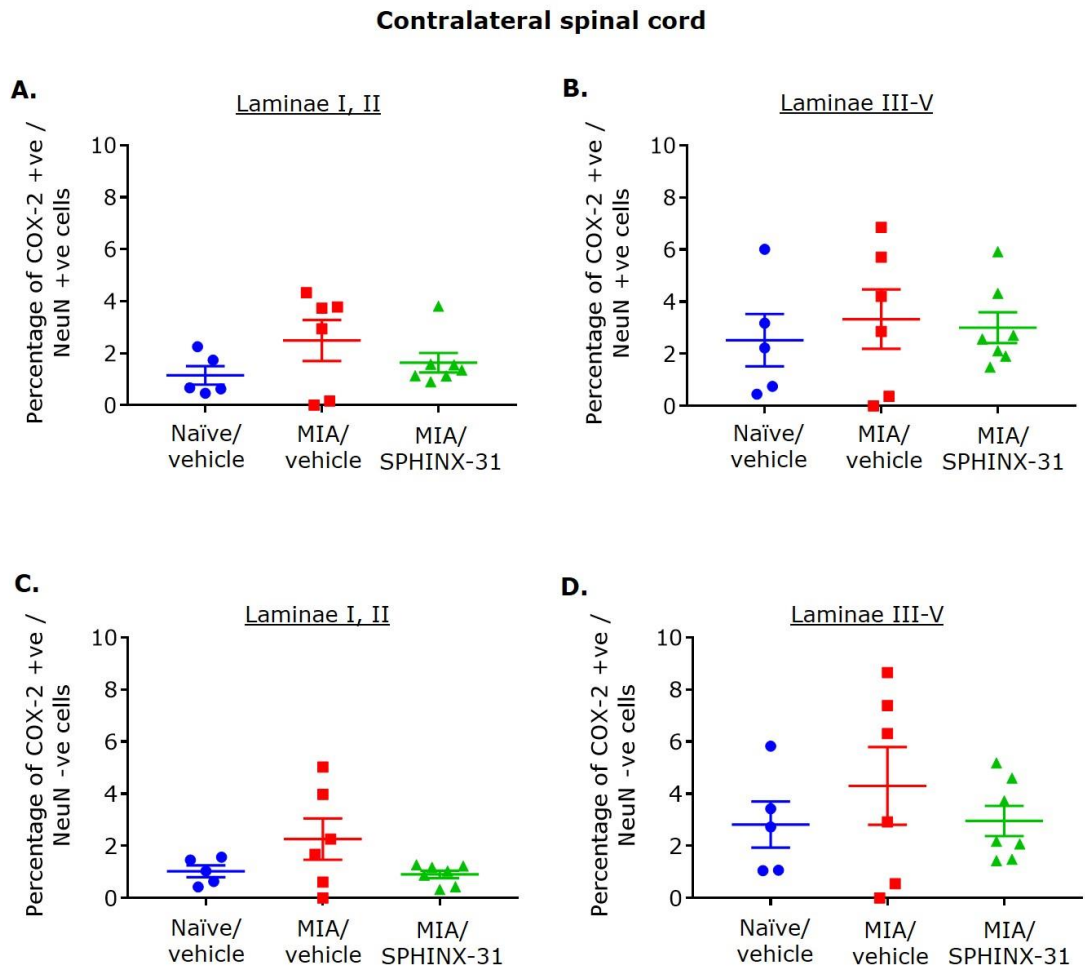


Figure 5.11. Percentage of neuronal and non-neuronal cells expressing COX-2 in laminae I,II and laminae III-V of the contralateral spinal cords in the MIA/SPHINX-31 study. In the contralateral spinal cord, no significant differences were found in the percentage of COX-2 +ve / NeuN +ve cells in **(A)** laminae I,II and **(B)** laminae III-V, or in the percentage of COX-2 +ve / NeuN -ve cells in **(C)** laminae I,II and **(D)** laminae III-V. (n=5-7/group, Kruskal-Wallis analysis).

5.3.6. Spinal glial cell activation

Activation of microglia was assessed by counting the number of CD11b +ve cells and measuring the average intensity of CD11b fluorescence in laminae I,II of ipsilateral and contralateral spinal cord. No significant difference in the activation of microglia was detected in the ipsilateral spinal cord. However, in the contralateral dorsal horn, the percentage of CD11b +ve cells was

significantly higher in the MIA/SPHINX-31 group compared with MIA/vehicle group (MIA/SPHINX-31 vs MIA/vehicle 6.36 ± 0.89 vs 1.72 ± 0.38 , $p < 0.01$), but was not different from the control group (figure 5.12).

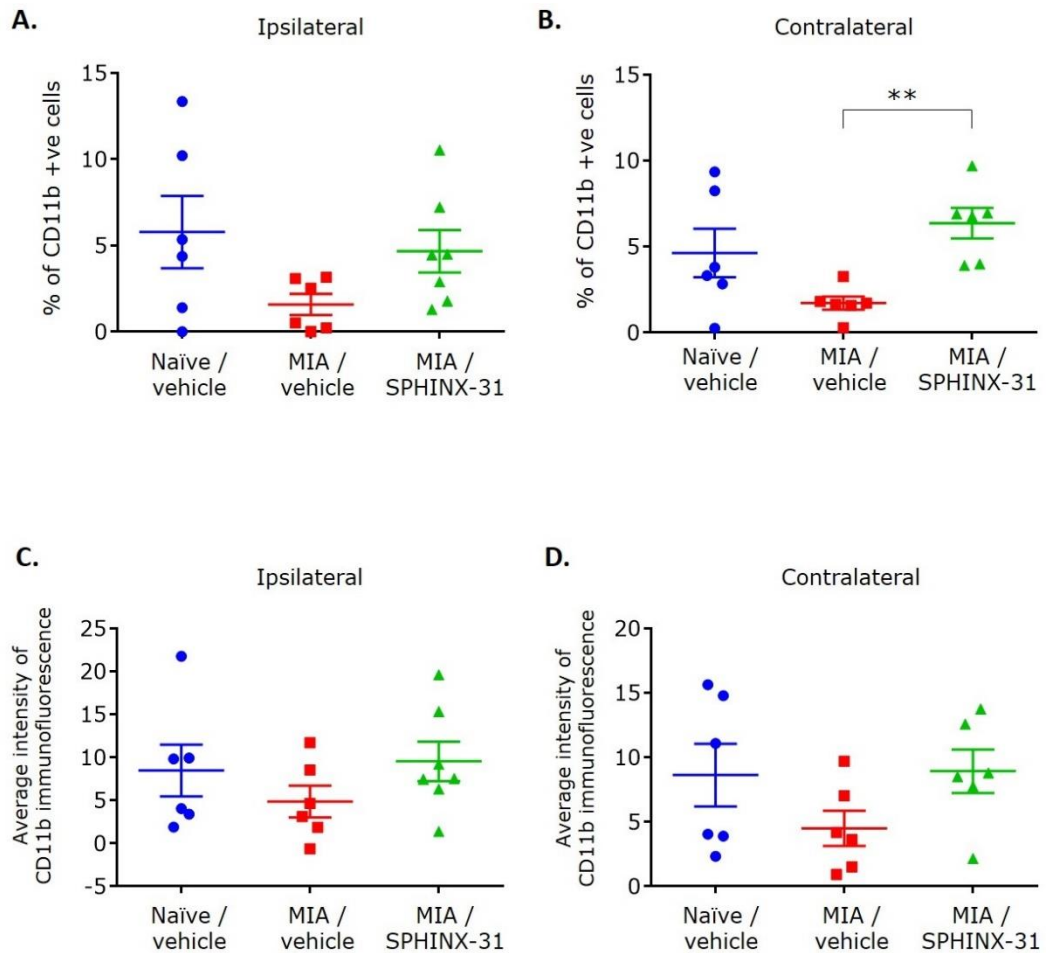


Figure 5.12. Spinal microglia activation in the MIA/SPHINX-31 study. (A) No significant changes in the percentage of CD11b +ve cells in the ipsilateral dorsal horn were detected between the study groups. (B) The percentage of CD11b +ve cells in the contralateral spinal cord is significantly higher in the MIA/SPHINX-31 groups compared to the MIA/vehicle group. No significant differences in the average intensity of CD11b immunofluorescence was found between study groups in both (C) ipsilateral and (D) contralateral dorsal horn of the spinal cord. (n=6-7/group; Kruskal-Wallis with post-hoc Dunn's analysis; ** $p < 0.01$).

Spinal astrocyte activation, measured as average intensity of GFAP fluorescence, was found not significantly different between the study groups (figure 5.13).

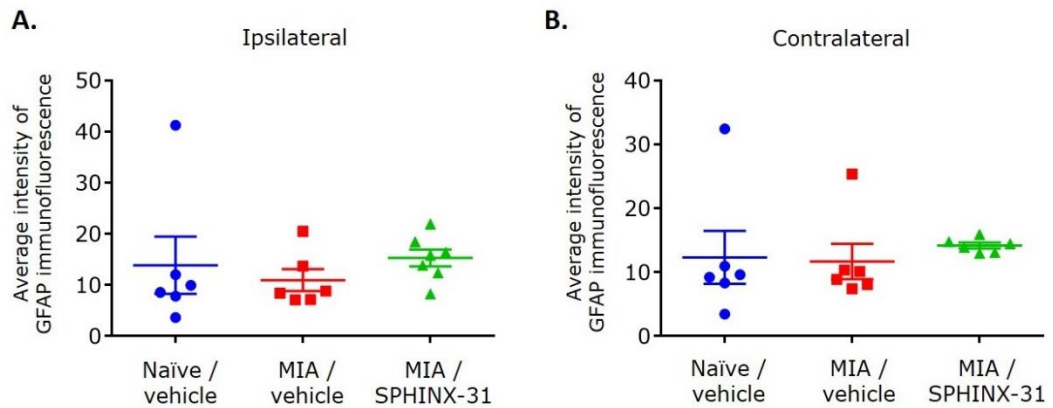


Figure 5.13. Spinal astrocyte activation in the MIA/SPHINX-31 study. No significant differences in average intensity of GFAP immunofluorescence was found between study groups in both **(A)** ipsilateral and **(B)** contralateral dorsal horn of the spinal cord. (n=6-7/group; Kruskal-Wallis test).

5.3.7. Glial cell activation in the PAG

In this part of the study, PAG sections were obtained and treated with antibodies against CD11b, GFAP and CD31 as markers of microglia, astrocytes and blood vessels, respectively. CD11b immunofluorescence was poor and the signal in treated sections did not differ from the negative control (figure 5.14); therefore, CD11b fluorescence was not analysed, and only astrocyte activation was addressed (figure 5.15).

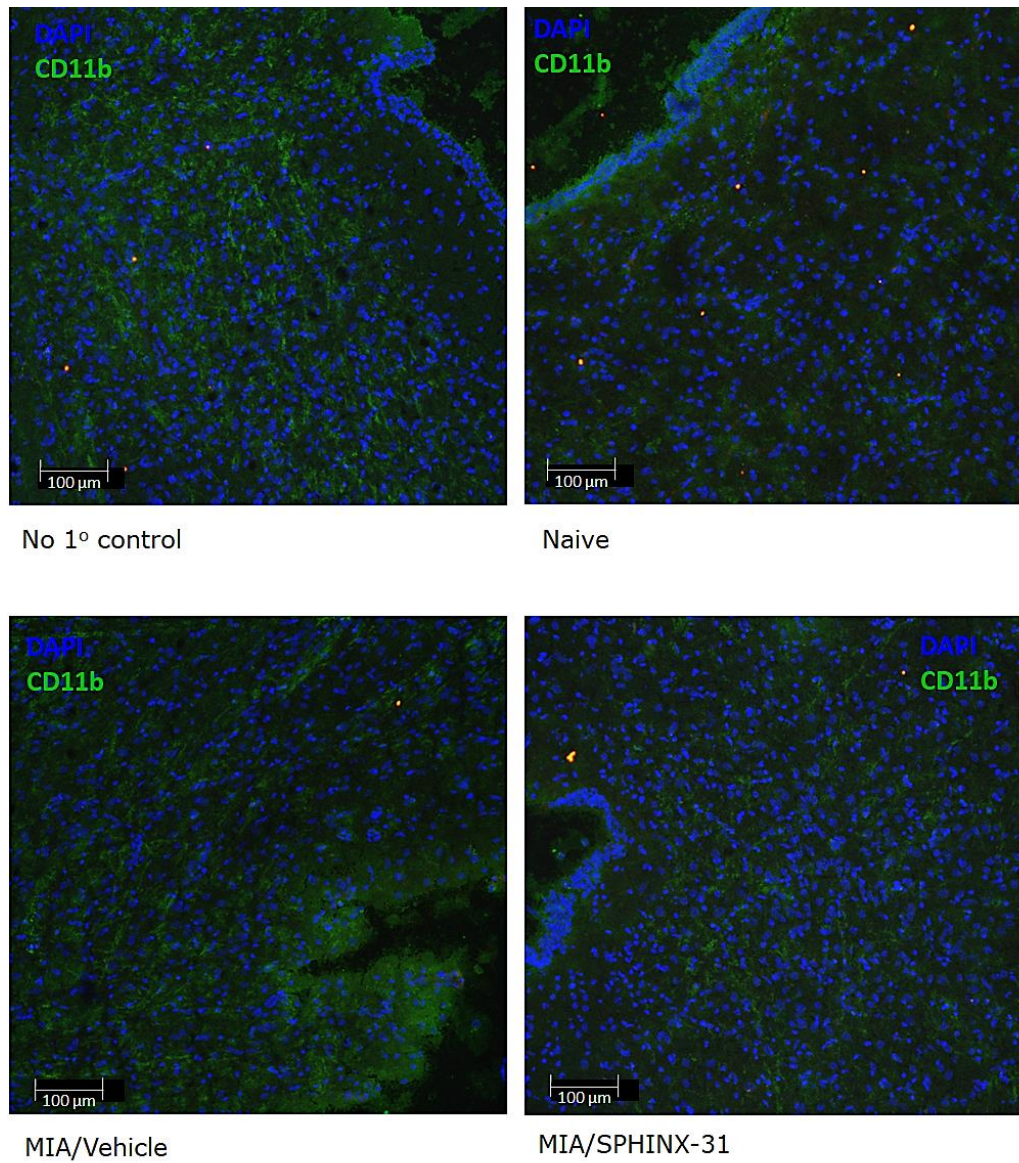


Figure 5.14. Confocal images of CD11b immunofluorescence and negative control in the PAG of rats from the MIA/SPHINX-31 study. Representative confocal images of a negative control (no primary antibodies) and CD11b immunofluorescence in the rat PAG from naïve, MIA/vehicle and MIA/SPHINX-31 groups. Immunofluorescence was poor and the signal in the negative control was the same as in the treated sections, so microglia activation was not addressed in this study. (10x magnification; scale bar = 100 μm).

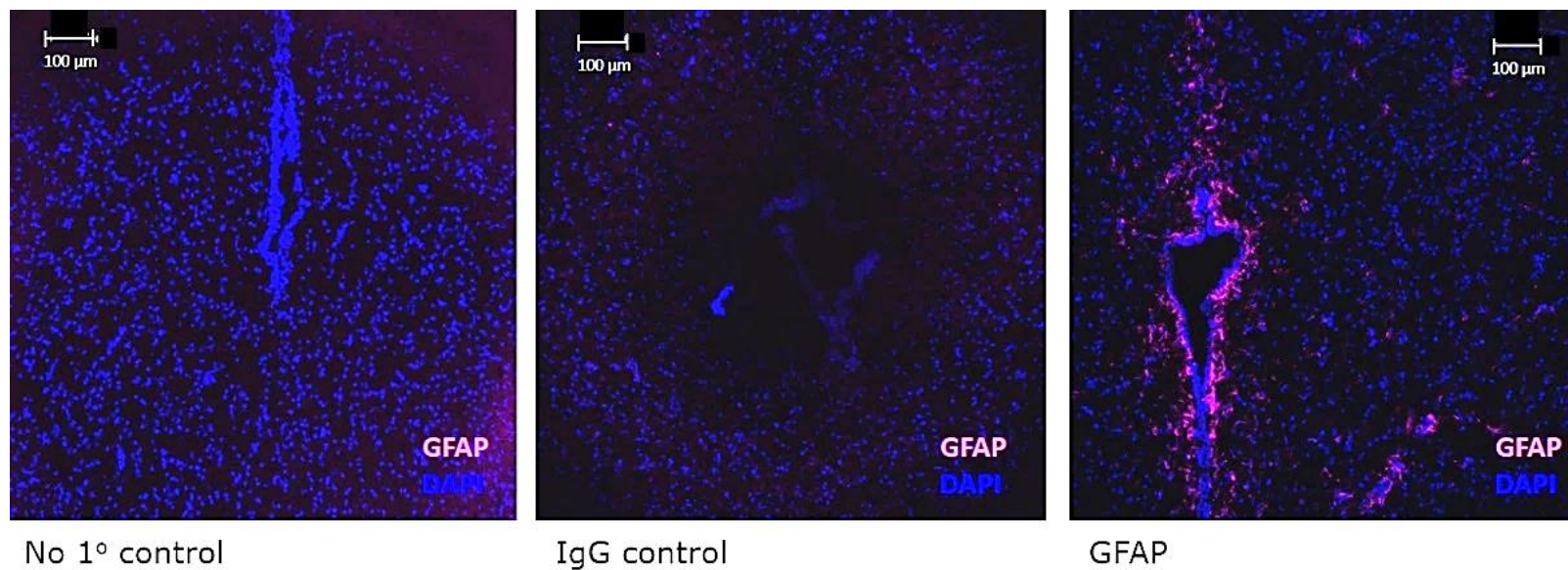
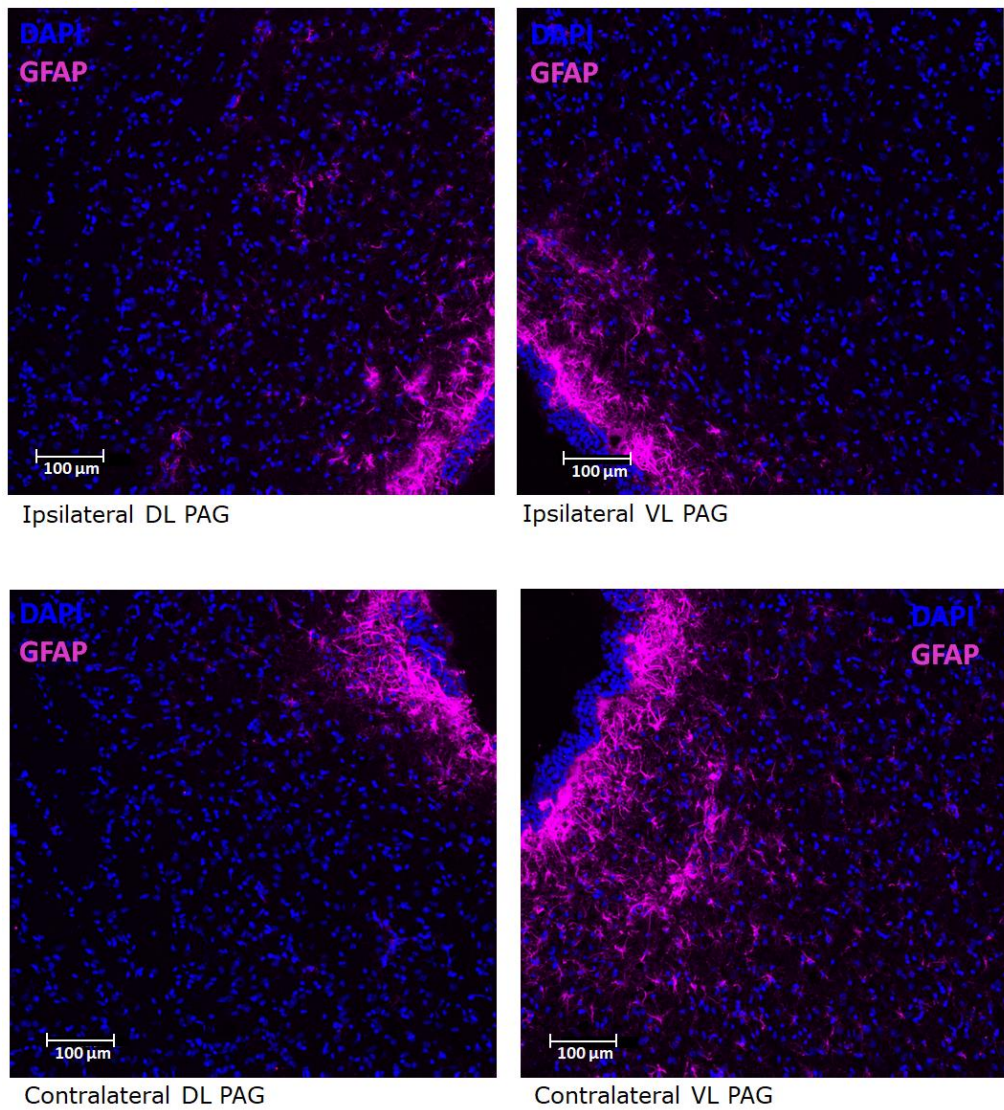


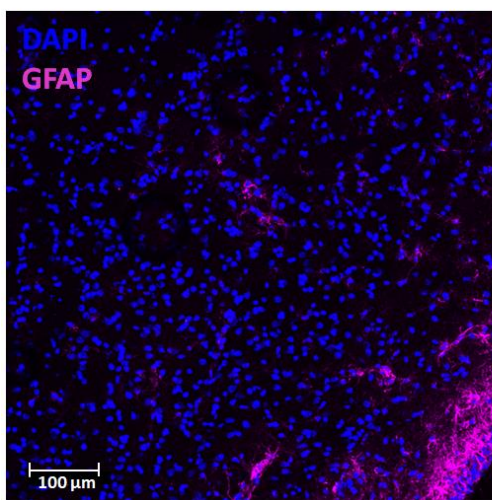
Figure 5.15. Images of GFAP immunofluorescence and two negative controls in PAG sections. Confocal microscopy images of PAG sections treated with no primary antibodies, species and concentration matching IgG (IgG control) and anti-GFAP antibodies. (X10 magnification, scale bar 100 µm).

No significant difference in the activation of astrocytes, expressed as average intensity of GFAP immunofluorescence, was found between the study groups in any given PAG region (figures 5.16 and 5.17).

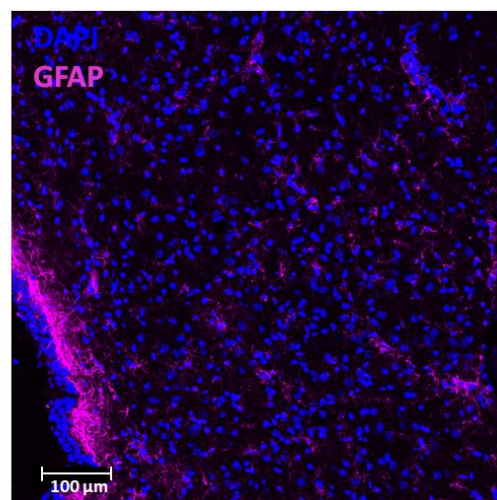
A. Naïve



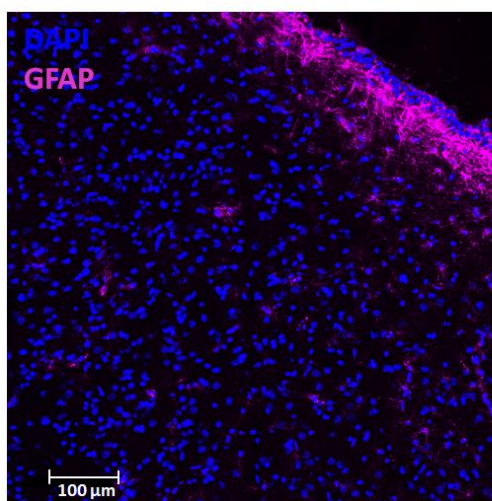
B. MIA/vehicle



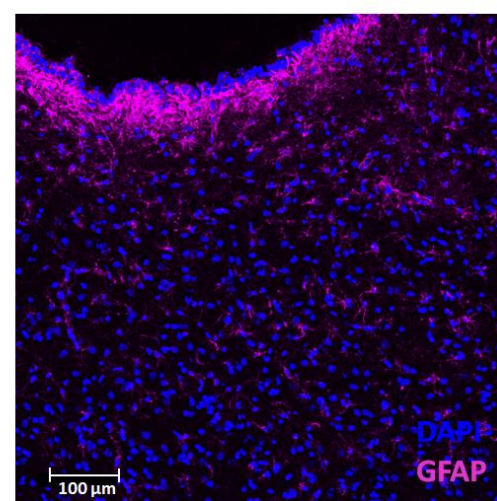
Ipsilateral DL PAG



Ipsilateral VL PAG



Contralateral DL PAG



Contralateral VL PAG

C. MIA/SPHINX-31

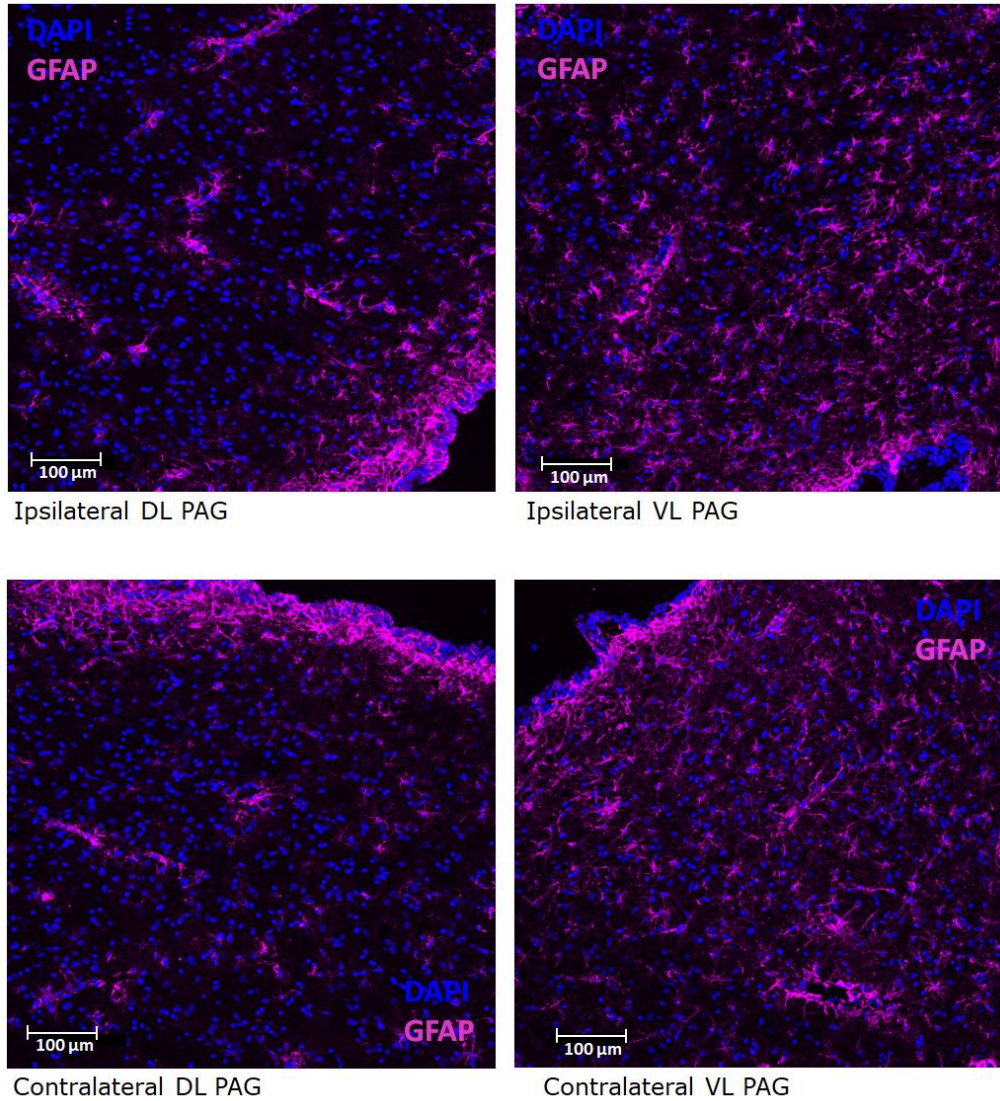


Figure 5.16. Confocal images of GFAP immunofluorescence in the rat PAG from the MIA/SPHINX-31 study. Representative confocal images of GFAP immunofluorescence (magenta) in four different areas of the PAG (ipsilateral and contralateral DL and VL) of rats from the **(A)** naïve, **(B)** MIA/vehicle and **(C)** MIA/SPHINX-31 groups (X20 magnification, scale bar 100 µm).

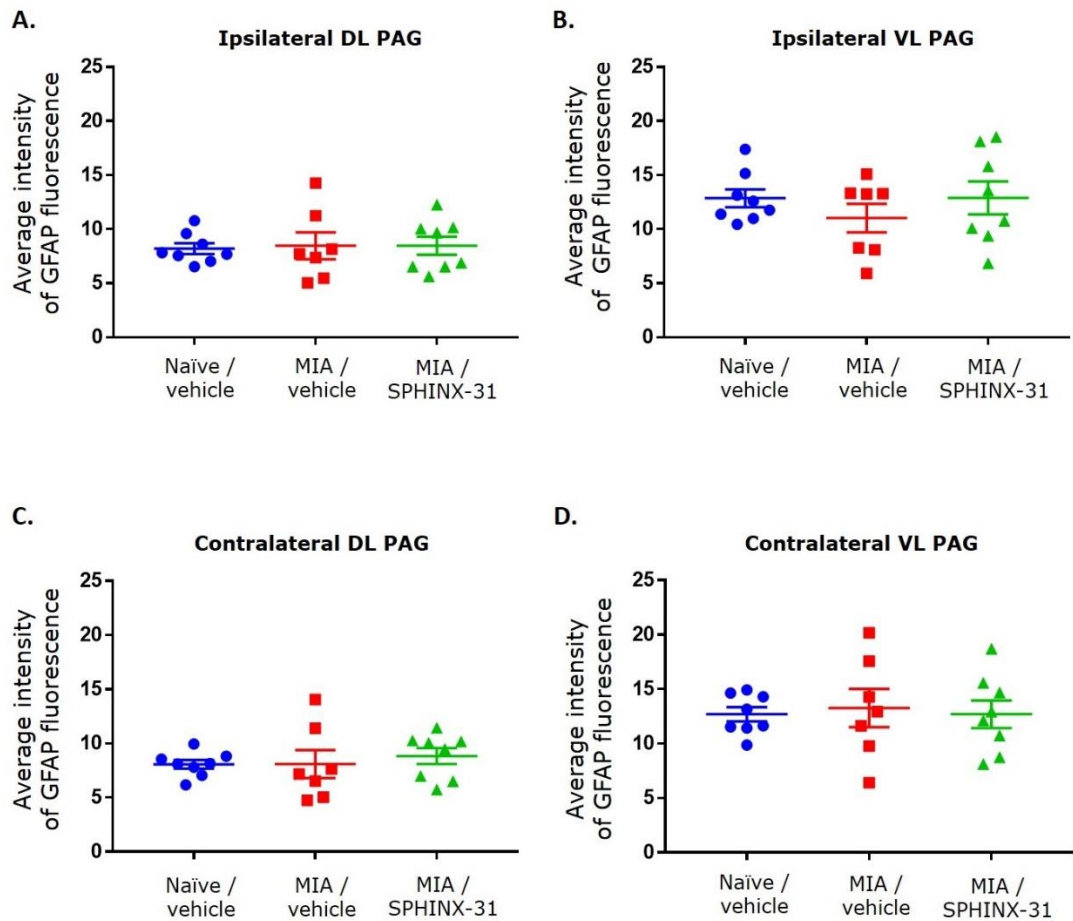


Figure 5.17. Astrocyte activation, expressed as average intensity of GFAP fluorescence, in the PAG of rats from the MIA/SPHINX-31 study. No significant differences were found in GFAP fluorescence intensity between the study groups in any of the PAG areas. (n= 8 / group, except for MIA/vehicle group, where n=7; Kruskal-Wallis analysis).

On the other hand, the average intensity of GFAP fluorescence was significantly higher in the VL region compared to the corresponding DL region, except in the ipsilateral PAG of the MIA/vehicle animals (Naïve/vehicle group: ipsilateral VL vs DL PAG 12.86 ± 0.83 vs 8.2 ± 0.50 , $p < 0.001$; contralateral VL vs DL PAG 12.69 ± 0.65 vs 8.08 ± 0.40 , $p < 0.0001$. MIA/vehicle group: contralateral VL vs DL PAG 13.25 ± 1.75 vs 8.09 ± 1.29 , $p < 0.05$. MIA/SPHINX-31 group: ipsilateral VL vs DL 12.89 ± 1.52 vs 8.46 ± 0.84 , $p < 0.05$; contralateral VL vs DL 12.7 ± 1.26 vs 8.83 ± 0.74 , $p < 0.05$. Figure 5.18).

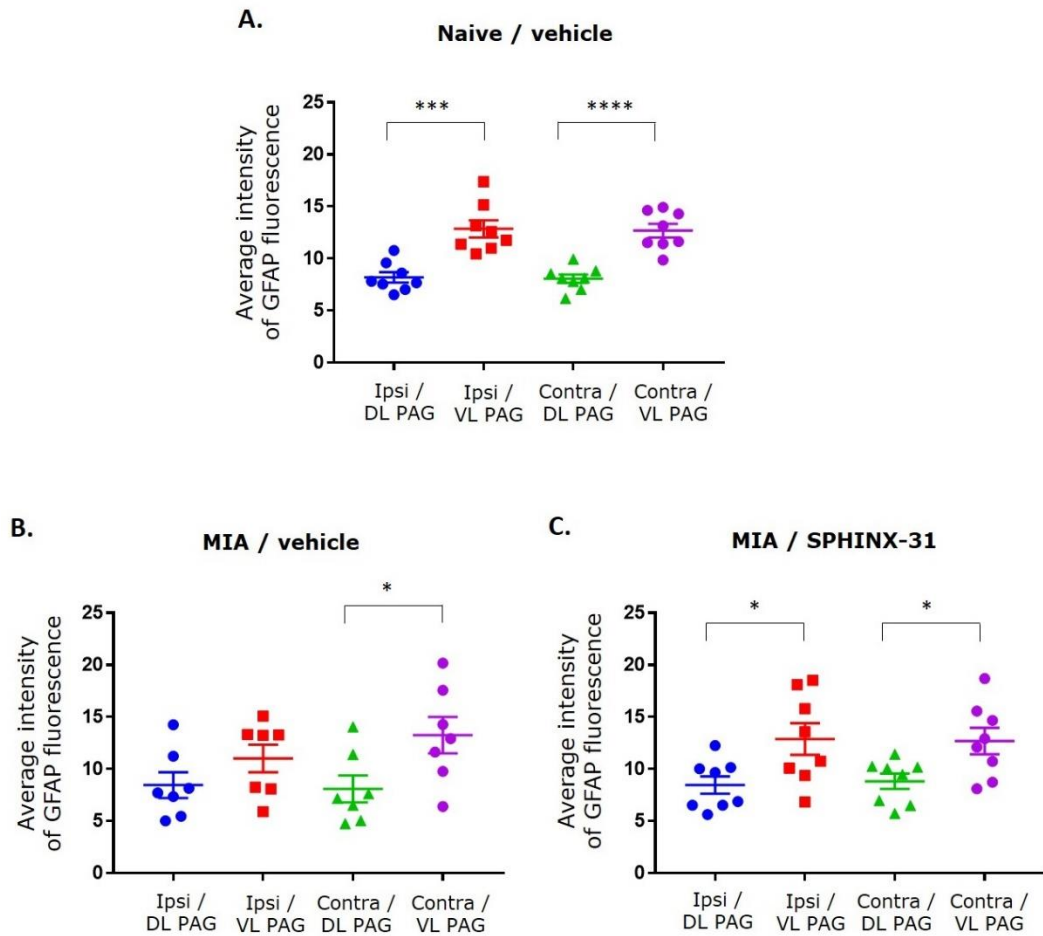


Figure 5.18. Astrocyte activation in the PAG in the MIA/SPHINX-31 study. In both ipsilateral and contralateral sides of PAG, the VL region showed significantly higher intensity of GFAP fluorescence compared to the DL counterpart in **(A)** naïve rats and **(C)** MIA/SPHINX-31 group. **(B)** In the MIA/vehicle group, VL PAG in contralateral side only showed a significant increase in GFAP fluorescence intensity compared to the DL counterpart. (n= 8 / group, except for MIA/vehicle group, where n=7, unpaired t-test; *p<0.05, ***p<0.001, ****p<0.0001).

To quantify the difference in astrocyte activation between the VL and DL portions of the PAG, the ratio of GFAP fluorescence intensity in VL PAG to DL PAG was calculated. However, no significant difference in this parameter was found between the study groups (figure 5.19).

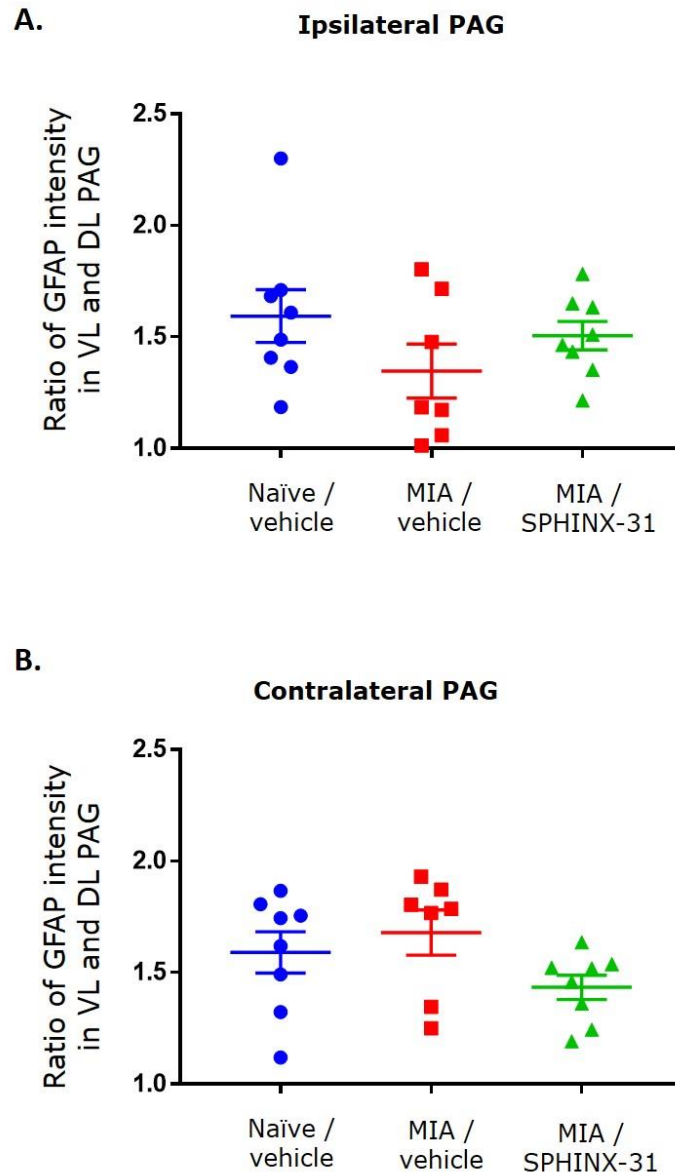


Figure 5.19. Ratio of astrocyte activation in VL PAG to DL PAG in the MIA/SPHINX-31 study. No significant differences were found between the study groups in the ratios of GFAP fluorescence intensity in VL to DL PAG in both **(A)** ipsilateral and **(B)** contralateral portions of the PAG (n= 8 / group, except for MIA/vehicle group, where n=7; Kruskal-Wallis analysis).

5.3.8. Astrocyte – blood vessels association in the PAG

In the current study, the association of blood vessels with astrocytes in the PAG was investigated. CD31 immunoreactivity was used to identify and count blood vessels. The number of blood vessels associated with astrocytes (indicated by GFAP positive morphologically matching cells) was then counted, and the results expressed as percentage of vessels associated with astrocytes over the total number of blood vessels. The previously mentioned PAG regions were analysed (figure 5.20).

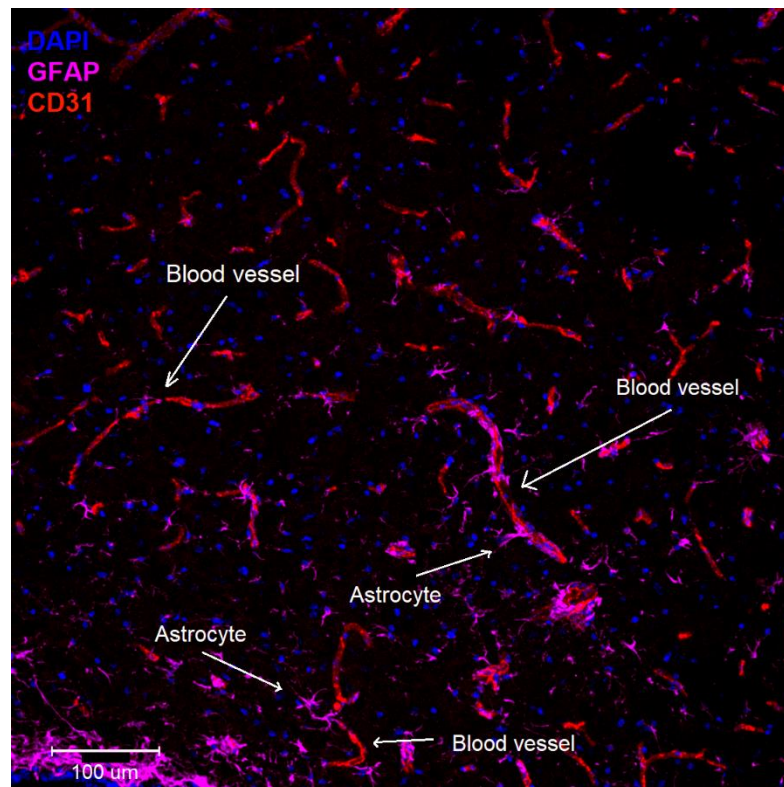


Figure 5.20. Representative image of CD31 and GFAP immunofluorescence in a PAG section. Confocal microscopy image of a PAG section treated with anti-CD31 and anti-GFAP antibodies. Blood vessel-like structures (in red) and astrocytes (in purple) can be recognised. (X40 magnification, scale bar 100 μm).

MIA/SPHINX-31 group exhibited a significantly higher percentage of vessels associated with astrocytes than the naïve/vehicle group in the ipsilateral DL PAG

(MIA/SPHINX-31 vs naïve/vehicle 49.17 ± 3.38 vs 26.39 ± 3.49 , $p < 0.05$). No significant differences were found between the study groups in the other PAG regions (figure 5.21).

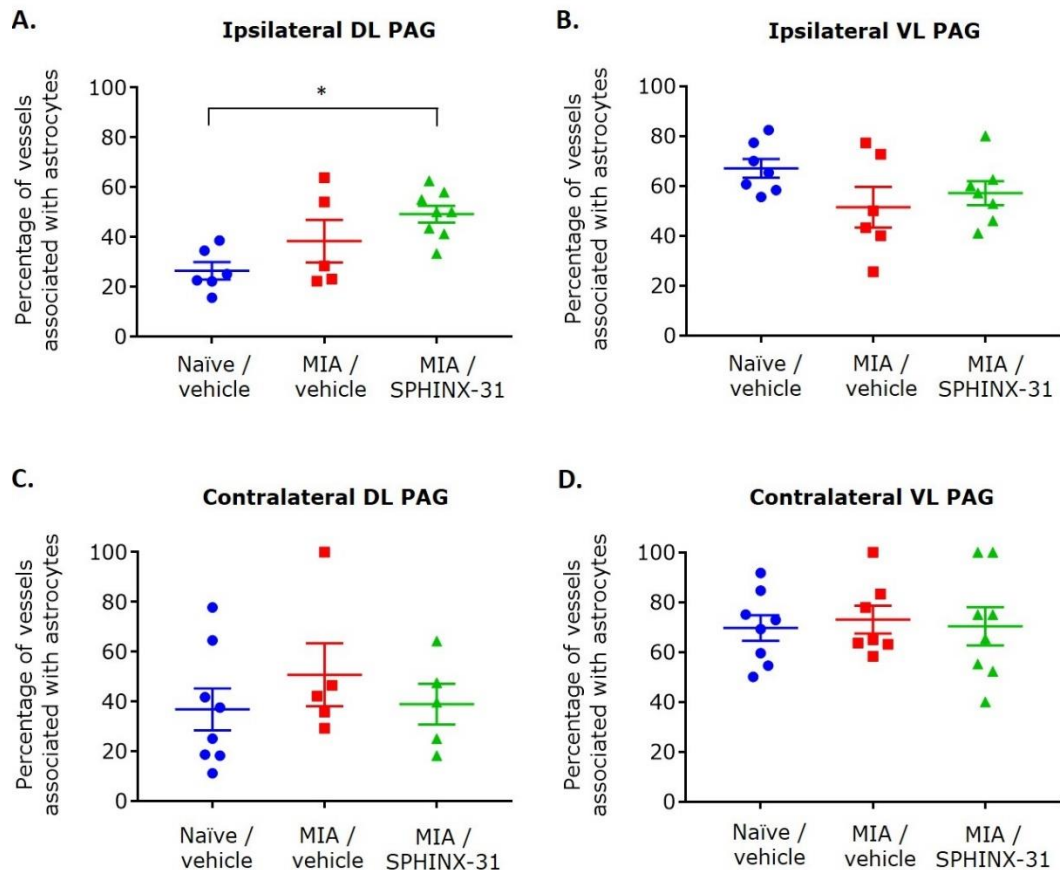


Figure 5.21. Percentage of blood vessels associated with astrocytes in the PAG in the MIA/SPHINX-31 study. (A) In ipsilateral DL PAG, the MIA/SPHINX-31 showed a significantly higher percentage of blood vessels associated with astrocytes compared to the naïve/vehicle group. No significant differences were observed between the study groups in (B) ipsilateral VL, (C) contralateral DL and (D) contralateral VL PAG (n=5-8/group, Kruskal-Wallis with post-hoc Dunn's tests; * $p < 0.05$).

Interestingly, blood vessel-astrocyte association in the naïve/vehicle group was significantly higher in the VL region of the PAG compared to the corresponding DL part in both the ipsilateral and contralateral sides of PAG (ipsilateral VL vs DL PAG 67.05 ± 3.78 vs 26.39 ± 3.49 , $p < 0.01$; contralateral VL vs DL PAG 69.68

± 5.11 vs 36.80 ± 8.40 , $p < 0.05$). This difference was only seen in the contralateral, but not ipsilateral PAG of MIA/SPHINX-31 animals (contralateral VL vs DL PAG 70.35 ± 7.68 vs 38.92 ± 8.19 , $p < 0.05$), and no significant differences were found between the corresponding DL and VL regions of both ipsilateral and contralateral PAG in the MIA/vehicle group (figure 5.22).

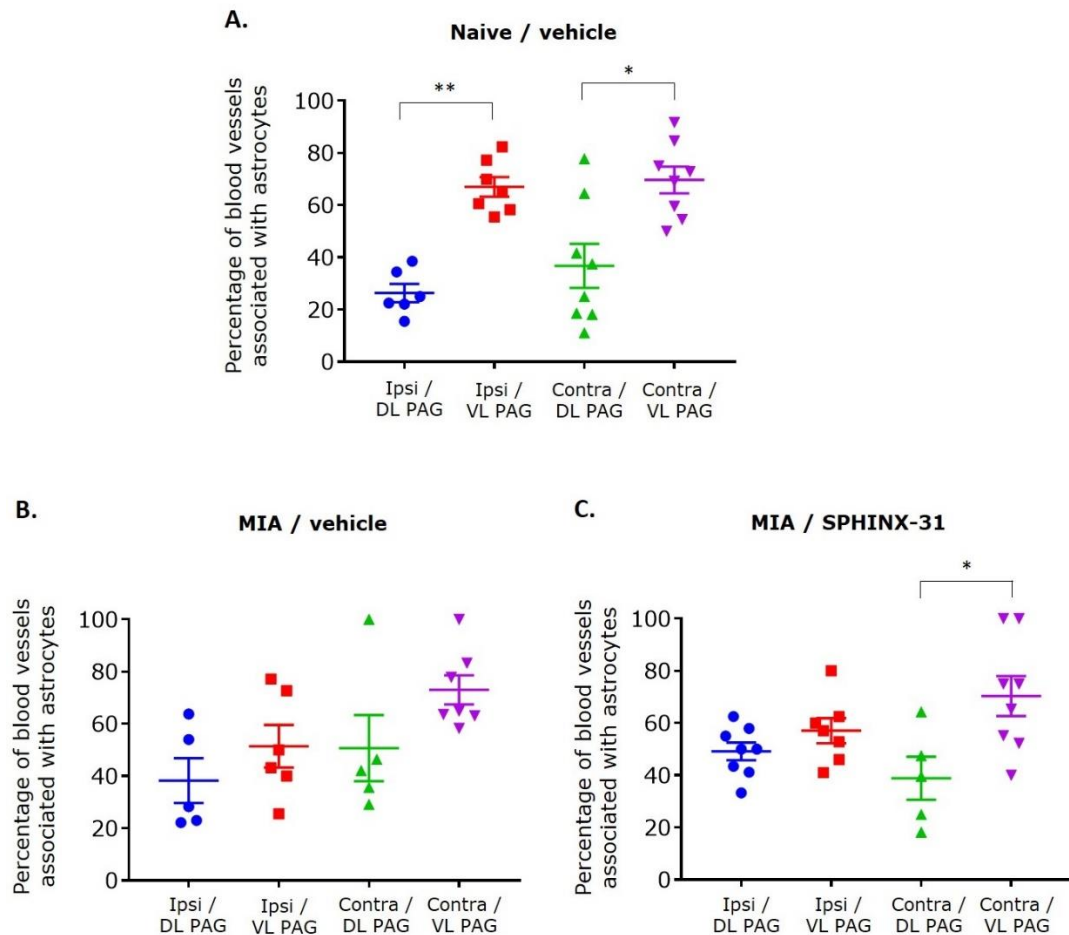


Figure 5.22. Astrocyte association with blood vessels in the PAG in the MIA/SPHINX-31 study. (A) In both ipsilateral and contralateral sides of the PAG in the naïve/vehicle group, the VL region showed a significantly higher percentage of blood vessels associated with astrocytes compared to the DL counterpart. (B) In the MIA/PBS group, no significant changes were found between the DL and VL PAG in ipsilateral or contralateral sides. (C) In the MIA/SPHINX-31 group, only contralateral VL PAG had a significant higher percentage of vessels associated with astrocyte compared with the DL counterpart. (n= 8 / group, except for MIA/vehicle group, where n=7, Mann-Whitney U test; * $p < 0.05$, ** $p < 0.01$).

The ratio of the percentage of blood vessels associated with astrocytes in VL PAG to DL PAG was calculated in order to quantify the difference in astrocyte-vessel association between the VL and DL portions of the PAG. No significant difference was found between the naïve and MIA/vehicle groups, or the MIA/vehicle and MIA/SPHINX-31 groups (figure 5.23).

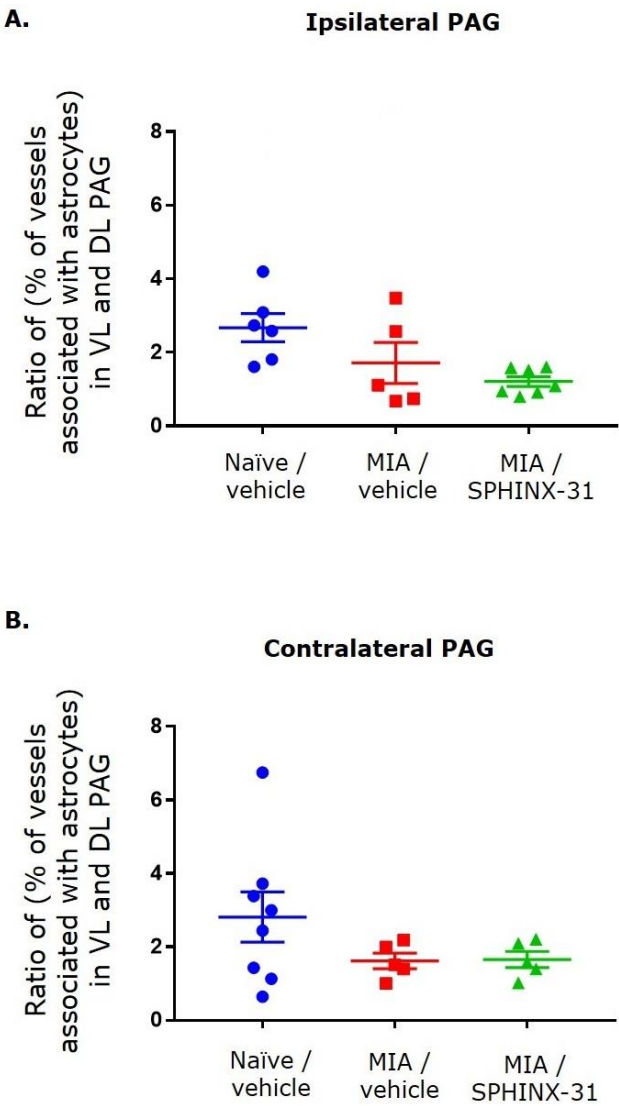


Figure 5.23. Ratio of percentage of blood vessels associated with astrocytes in VL PAG to DL PAG in the MIA/SPHINX-31 study. No significant differences were found between study groups in the ratio of blood vessel-astrocyte association in VL to

DL PAG in both **(A)** ipsilateral and **(B)** contralateral portions of the PAG (n= 5-8 / group; Kruskal-Wallis analysis).

5.4. Discussion

5.4.1. Acute inflammation (knee joint swelling)

In previous studies of MIA model of OA, mild joint swelling has been observed on the first 1-3 days post MIA injection, and it completely resolved by day 7-10 (Nwosu et al., 2016b, Mapp et al., 2013, Al-Saffar et al., 2009). Early knee swelling was accompanied by synovial thickening and immune cell infiltration (Bove et al., 2003, Clements et al., 2009). In the current study, no significant swelling of the ipsilateral knee compared to the naïve group was detected, since the first measurement of knee diameter was performed on day 7 after injection, when the obvious inflammation would have cleared.

The group treated with SPHINX-31 showed no significant differences in ipsilateral knee diameter compared to the naïve and vehicle treated animals, which is explained by a lack of significant swelling in the MIA rats in the first instance.

5.4.2. Rearings

Changes in rearing frequency are considered movement-provoked pain behaviour in models of osteoarthritis (Miller et al., 2012, Inglis et al., 2008, More et al., 2013, Nagase et al., 2012). They were also associated with sedation (Sousa et al., 2017), stress (Masood et al., 2003) and changes in ambulation (van Abeelen, 1977) and excitability (Lever et al., 2006).

In the MIA/SPHINX-31 study, no significant changes in the frequency of rearings were observed between any of the groups. This finding rules out differences in sedation, stress or ambulation, and it indicates the absence of this spontaneous pain behaviour in the MIA injected rats. This result differs from reports in

previous literature, where MIA injected rats showed reduced number of rearings, and it may be explained by different techniques of rearing counting (Quinn et al., 2003), use of bilateral vs unilateral MIA model (Nagase et al., 2012, Bryden et al., 2015) or the use of a higher MIA dose (More et al., 2013). The administration of SPHINX-31 had no effect on this parameter either. This may indicate an actual lack of action of SPHINX-31 on vertical spontaneous behaviour, or it is explained by the lack of such behaviour in the MIA rats in the first place.

5.4.3. Pain behaviour

Injection of MIA in the rat knee joint produced significant decrease in mechanical withdrawal thresholds and weight bearing asymmetry starting on day 4 post-injection and persisting for the whole duration of the study (28 days). These findings reflect the pain behaviour patterns known to develop in the MIA model of OA (Combe et al., 2004, Fernihough et al., 2004, Liu et al., 2011, Nwosu et al., 2016a). Systemic administration of SPHINX-31 did not reverse these altered patterns of pain behaviour. SPHINX-31 was hypothesised to exert anti-nociceptive effects in the MIA model of OA by inhibiting the action of SPRK1, thus increasing the production of endogenous VEGF-A_{165b} and its ratio to total VEGF-A. These effects were previously observed upon intrathecal administration of the SRPK1 inhibitor SRPIN340 in a murine model of neuropathic pain (Hulse et al., 2016). The lack of anti-antinociceptive action of systemic SPHINX-31 is difficult to explain, since systemic use of SPHINX-31 has been limited, especially in the setting of pain studies, and its exact mechanism of action as analgesic is still unknown. However, some probable explanations include:

1) Systemic administration; as in previous studies of neuropathic pain SRPIN340 was used intrathecally (Hulse et al., 2016) or topically to the site of nerve injury (Hulse et al., 2014). However, in the study performed by Dr Babaedi-Jadidi, a single intra-peritoneal injection of 0.8 mg/kg SPHINX-31 prevented SRSF1 phosphorylation and altered VEGF-A splicing in favour of VEGF-A_{165b} in mouse kidney, which demonstrates the expected effect of SPHINX-31 in vivo.

- 2) Insufficient dose; the dose of SPHINX-31 in this study (refer to section 5.2.1) was previously used for mice in studies of cancer xenograft and kidney disease (Batson, oral communication, Babaei-Jadidi et al, unpublished), so its appropriateness to produce antinociceptive effect in rats cannot be confirmed.
- 3) A relatively short $T_{1/2}$ (95.79 min), which would not provide adequate plasma concentrations, seen the limit of intra-peritoneal injections to twice/week.
- 4) Differences in nociceptive mechanisms between neuropathic pain and OA pain.

5.4.4. Joint pathology and histology

Rats injected with intra-articular MIA developed significant cartilage deterioration, as demonstrated by a significant increase in both macroscopic and microscopic chondropathy scores compared to the naïve group. This is consistent with the findings of the MIA/VEGF-A_{165b} study (section 4.3.1) and the course of joint pathology in the MIA model reported in the literature, which is considered to be already established by day 20 after MIA injection (Sagar et al., 2010, Bove et al., 2003). No significant differences in the macroscopic and microscopic scores were observed between the MIA/SPHINX-31 group and the MIA/vehicle group. This indicates that systemic SPHINX-31 had no effect on the extent of chondropathy in MIA rats.

Previous literature reported significant osteophytosis two weeks (Janusz et al., 2002, Mapp et al., 2013, Guingamp et al., 1997) or four weeks (Ferland et al., 2011) after the induction of MIA model. This was not found in the current study, where osteophyte scores showed no significant differences between the MIA and naïve groups. However, no osteophytes were observed in the naïve animals whatsoever, while they developed in a number of MIA injected animals. SPHINX-31 administration to MIA rats had no significant effect on osteophyte score. These results partly replicate what found in the MIA/VEGF-A_{165b} study (section 4.3.1.2).

The extent of synovial inflammation did not differ significantly between any of the study groups. This is in contrast with the findings of previous studies (Mapp et al., 2013, Guingamp et al., 1997, Sagar et al., 2014), in which MIA animals showed significant knee synovial inflammation at the same time point (day 28). As mentioned in previous chapters (sections 3.4.2 and 4.4.4), the group sizes for this parameter were small (n= 3-4 for the MIA/SPHINX-31 study) due to loss of synovial tissue during knee disarticulation, and that could have prevented the detection of a statistically significant change in synovitis score. Again, SPHINX-31 had no significant effect on the degree of synovial inflammation when administered to MIA rats. The lack of effect of SPHINX-31 on osteophyte and synovitis scores may be due to an absence of significant difference between the MIA and naïve groups in the first place, or an actual lack of action of this agent on the development of osteophyte and inflammation in this model of OA.

5.4.5. Spinal COX-2 expression

Unlike most peripheral tissues, COX-2 is constitutively expressed in neurons and radial astrocytes of the spinal cord (Samad et al., 2001, Ghilardi et al., 2004), while most astrocytes in the gray matter, oligodendrocytes and microglia do not express detectable baseline levels of COX-2 in the spinal cord (Ghilardi et al., 2004). After significant tissue injury, however, COX-2 expression is upregulated in a number of cell types, including neurons (Cao and Zhang, 2008), glial cells (Ikeda-Matsuo et al., 2005, Vardeh et al., 2009, Goppelt-Struebe and Beiche, 1997), endothelial cells (Kanda et al., 2017, Hoffmann, 2000) and macrophages (Durrenberger et al., 2006). In the current study, COX-2 was co-localised in the spinal cord with both neurons (marked by the neuronal nuclear marker NeuN) and non-neuronal cells (marked by the absence of NeuN co-staining).

Spinal expression of COX-2 is known to be upregulated in models of peripheral inflammation (Beiche et al., 1996, Ichitani et al., 1997, Hay and de Belleruche, 1997, Seybold et al., 2003). A number of studies addressed the role of spinal COX enzymes and PGs in the development of central sensitisation in the MIA model of OA (Sagar et al., 2011, Staniaszek et al., 2010). An increase in COX-2 mRNA

expression and protein production in the spinal cord was detected from day 5 to day 31 post-MIA injection (Prochazkova et al., 2009). In the current study, no significant increase in the expression of COX-2 was detected in the MIA injected rats compared to the naïve controls in any of the selected areas of the spinal cord (superficial and deep laminae of the ipsilateral and contralateral dorsal horn). The discrepancy of these findings with those found in literature are probably explained by the different techniques used to detect COX-2 expression; in the study by (Prochazkova et al., 2009), mRNA and protein expression of COX-2 was quantified, using qPCR and Western blot, respectively, in the whole lumbar section of the spinal cord. In the current study, however, immunofluorescence was used to detect COX-2 expression and co-localisation with neuronal and non-neuronal cells in very specific areas of the spinal cord.

5.4.6. Spinal glial cell activation

In previous studies investigating spinal glial cell activation in the MIA model of OA, significant activation of microglia in the spinal cord was observed on day 14 after model induction (Orita et al., 2011) and was directly correlated with pain behaviour on day 28 (Lee et al., 2011a), when astrocyte activation also occurred (Sagar et al., 2011). In the current study, no significant differences in the activation of microglia in ipsilateral dorsal horn were detected between the study groups, while the MIA/SPHINX-31 group had a significantly higher percentage of CD11b +ve cells compared to the MIA/vehicle group in the contralateral dorsal horn. These results contrast with those reported in literature, where levels of activated microglia in ipsilateral spinal cord were significantly elevated as early as 2 weeks after MIA injection and up to 4 weeks (Sagar et al., 2011, Orita et al., 2011). However, a decline in microglia activation by day 28 post MIA injection was also suggested (Sagar et al., 2011), which may account for the lack of microglia activation in the current study.

Chronic pain conditions, such as neuropathic and inflammatory pain, are known to produce glial cell activation in the contralateral spinal cord (Obata et al., 2010, Schreiber et al., 2008), contributing to the development of contralateral allodynia

or mirror-image pain. A similar effect in the MIA model of OA was not reported in literature; however, in the MIA model characterisation study (section 3.3.4), microglia were significantly activated on day 14 compared to day 7 post-MIA injection in both ipsilateral and contralateral spinal cord. In this part of the study, the MIA rats treated with SPHINX-31 showed a significant activation of microglia compared with the MIA rats that received vehicle. This finding indicates that SPHINX-31 can activate microglia in MIA rats, but it is challenging to propose a mechanism to explain this effect. Future works could investigate potential trophic and protective effects of SPHINX-31 or VEGF-A_{165b} on glial cells, based on the known neurotrophic actions of VEGF-A_{165b}.

No significant difference in the activation of spinal astrocytes was detected between any of the study groups. This result replicates what found in the MIA/VEGF-A_{165b} study at the same time point (see section 4.3.2.2), but differs from previous reports, which found spinal astrocytes to be activated 28 days after MIA injection (Sagar et al., 2011, Yu et al., 2013). These discrepancies between current results and previous literature regarding astrocyte and ipsilateral microglia activation may be explained by the differences in species, models of OA or methods of analysis (detection of cell activation and analysis of data) among the studies.

5.4.7. Astrocyte activation in the PAG

As previously explained, microglia and astrocytes in the CNS are largely involved in the development of central sensitisation and chronic pain (Gosselin et al., 2010). Both cell types are known to react to various insults (trauma, inflammation or neurodegeneration) by acquiring a state of activation including morphological changes and/or the up-regulation of various markers (Suter et al., 2007, Beggs and Salter, 2010). Glial cells are activated by neurotransmitters, purines, substance P, cytokines (chemokines) and excitatory amino acids released from primary afferent terminals. Glia also influence the activity of the nociceptive pathway through the release of neuronally active cytokines and other inflammatory

mediators and the synaptic reuptake of released neurotransmitters by astrocytes (Panigada and Gosselin, 2011).

Marked changes in glial cells also occur in various regions of the brain, including the thalamus after spinal cord injury (Hains and Waxman, 2006, Zhao et al., 2007), the cingulate cortex, following sciatic nerve ligation (Kuzumaki et al., 2007) and in the nucleus of the tractus solitarius after colonic inflammation (Sun et al., 2005). Interestingly, these areas are not directly involved in nociception, but rather in limbic circuitry, suggesting a role of brain glial cells in the regulation of the affective component of pain (Gosselin et al., 2010). This finding is also supported by evidence that brain astrocytes and microglia react in the upper brain structures that mediate mood and cognitive dysfunctions in chronic pain (Panigada and Gosselin, 2011). Clinically, thalamic glial cells were activated in patients with low back pain (Loggia et al., 2015).

Regarding the PAG, it was found that neuropathic pain induced by chronic constriction injury (CCI) is associated with glial activation in the VL PAG, which presumably participates in descending pain facilitation through the p38 MAPK signalling pathway (Ni et al., 2016). Following CCI, intra-VL-PAG injection of microglial or astrocytic inhibitors or mitogen-activated protein kinase (p-p38 MAPK) inhibitor abolished microglial activation and produced a significant time-dependent attenuation of mechanical and thermal hyperalgesia. Melanocortin 4 receptor (MC4R) activation has also been involved in glial modulation in the PAG (Chu et al., 2012). In another study, astrocyte activation was observed in the VL PAG of rats exhibiting persistent disability with neuropathic pain after CCI (Mor et al., 2010). Glial-neuron interactions have been involved in opioid withdrawal syndrome (Ouyang et al., 2012, Hao et al., 2011).

Very little is known regarding changes in brain glial cells, as opposed to spinal cord glia, and their potential effects on pain development in arthritis. Investigating the effect of persistent pain on morphine tolerance and the activation of VL PAG glial cells, Eidson and Murphy found that peripheral inflammation induced by intraplantar CFA did not illicit significant increases in VL PAG glial cell activity (Eidson and Murphy, 2013). In the current study, activation of astrocytes in different areas of the PAG was investigated in the MIA model of OA. No

significant changes in astrocyte activation were detected between the study groups in any given area of the PAG. However, it was found that the activation of astrocytes in the VL region was significantly higher compared to the correspondent DL region in both sides of the PAG in all study groups, except for the ipsilateral PAG of the MIA/vehicle group. These findings indicate that the basal activity of astrocytes is higher in the VL PAG than in the DL PAG. This difference was no longer observed in the ipsilateral PAG of rats 28 days after the injection of MIA, but it was reversed in the SPHINX-31 treated animals. This would suggest that intra-articular injection of MIA increases astrocyte activation in the DL PAG, where the nociceptive input is received, while SPHINX-31 reverses this activation. However, when the difference in astrocyte activation between the VL and DL PAG was quantified as the ratio of GFAP intensity in VL PAG to DL PAG, no significant difference in this parameter was detected between the study groups.

5.4.8. Blood vessel and astrocyte association in the PAG

There is a close relationship between astrocytes and blood vessels in the brain, as their end feet completely enclose cerebral blood vessels and they exhibit multiple bidirectional interactions. Astrocytes are a major cell type in BBB (Daneman and Prat, 2015) and are involved in the regulation of cerebral blood flow (CBF) (Gordon et al., 2007) through Ca^{2+} -dependent mechanisms involving arachidonic acid derivatives (Zonta et al., 2003), K^+ release onto blood vessels (Iadecola and Nedergaard, 2007) and NO production (Iadecola, 1993). Astrocytes contribute to the generation of vascular tone, and their dysfunction in pathological conditions can compromise the regulation of blood flow (MacVicar and Newman, 2015).

A significant reduction in the coverage of gray matter vessels by astrocyte processes was observed in major depressive disorder (Rajkowska and Stockmeier, 2013) and altered neuron-glia-vascular communication is also involved in cognitive decline in Alzheimer's disease (Rossi, 2015).

Glial-vascular interactions have also been studied in the field of pain. Recent research indicates that glio-vascular activation stimulated by microglial TNF- α promotes endothelial PGI₂ synthase and COX-2 expression, contributing to the generation of neuropathic pain (Kanda et al., 2017). A significant increase in the number of GFAP positive astrocytic end-feet wrapping blood vessels in the spinal cord was observed in the CFA model of peripheral inflammation, suggesting an astrocyte-mediated mechanism for microvessel activation (Beazley-Long et al., 2018). Interestingly, this mechanism involved endothelial VEGFR2. Chronic pain resulting from peripheral inflammation has been associated with concomitant disruption of the blood brain barrier (BBB) and glial activation in specific regions of the brain (frontal and parietal cortices and thalamic region) (Huber et al., 2006); seen the role of glia in maintaining the BBB (Prat et al., 2001), glial/endothelial interactions are hypothesised to play a relevant role during inflammatory pain (Willis et al., 2008).

To the best of our knowledge, cerebral glio-vascular interaction has not been investigated in models of pain, nor in arthritic disorders. In the current study, the percentage of blood vessels associated with astrocytes was calculated and it was found that this percentage is significantly increased in the MIA/SPHINX-31 group compared to the naïve control group in the ipsilateral DL PAG, while no significant differences were observed in the other PAG areas. The effect of SPHINX-31 in blood vessel activation in MIA rats is limited to the ipsilateral DL PAG, and this suggests a link to the nociceptive inputs to the PAG. In addition, the percentage of blood vessels associated with astrocytes was significantly higher in the VL region of the PAG compared to the correspondent DL area in both the ipsilateral and contralateral sides of the PAG in naïve/vehicle group, while this difference was no longer apparent in the MIA injected animals. This could indicate that after MIA injection, vessel-astrocyte association increases in the DL PAG, which constitutes the input area of the PAG. In the contralateral PAG of the MIA/SPHINX-31 animals, VL PAG showed a higher percentage of astrocyte-associated vessels than that in the DL PAG, suggesting that SPHINX-31 decreased vessel-astrocyte association in the contralateral DL PAG, probably through vascular activation. However, the ratio of percentage of blood vessels associated with astrocytes in the VL PAG to DL PAG was calculated, and no

significant difference in vessel-astrocyte association between the VL and DL PAG was detected between the study groups.

SPHINX-31 is known to inhibit SRPK1, which in turn suppresses phosphorylation of SRSF1. This promotes alternative splicing of VEGF-A from the pro-angiogenic isoform to the anti-angiogenic isoform, producing a potent inhibition of blood vessel growth (Batson et al., 2017). SRPK1 also controls the mRNA alternative splicing of other molecules, including myc, RON, TEAD1 and TAU (see figure 5.24, which summarises the known targets of SRPK1 alternative pre-mRNA splicing). Although none of these were directly implicated in nociception, and SRPK1 inhibitors tested to date only affected VEGF-A, it would be interesting in the future to address changes in the PAG expression of these molecules' alternative splice variants in the MIA model of osteoarthritis and after administration of SPHINX-31. More information could also be gained from the investigation of astrocyte activation and astrocyte-vessel association in VEGF-A_{165b} treated MIA rats. These points could hint at some interesting future work to clarify the role of SRPK1 inhibition in the PAG.

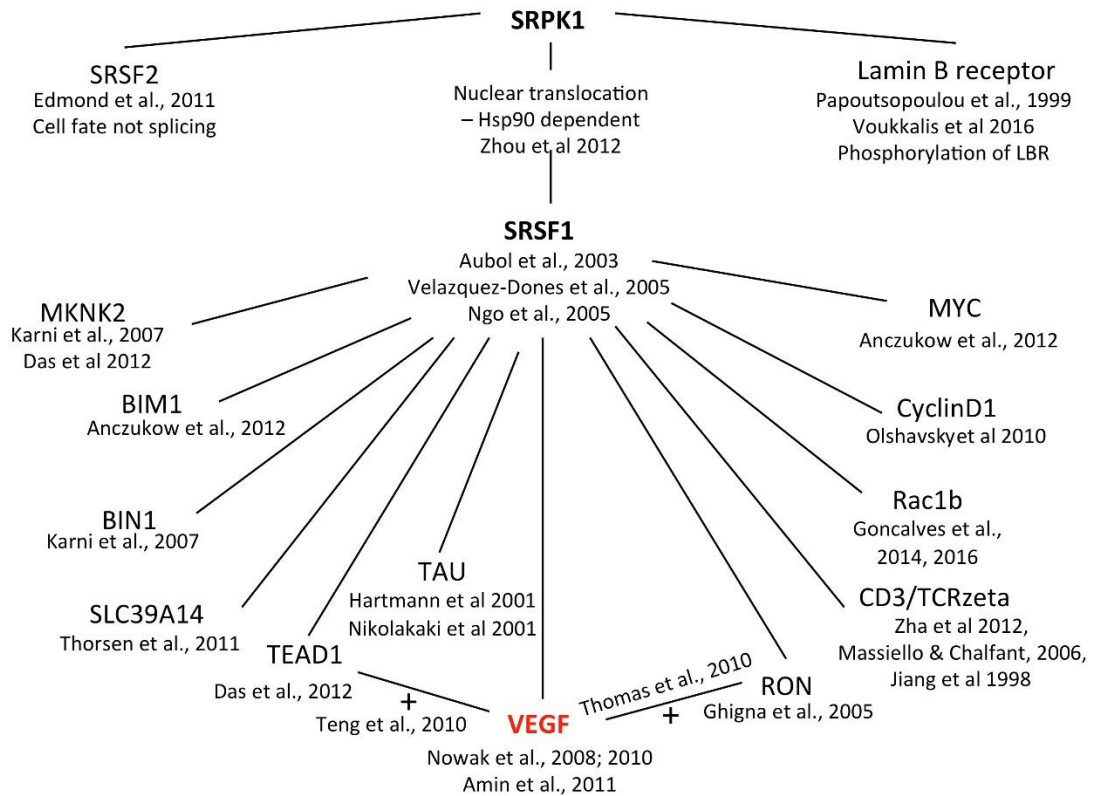


Figure 5.24. Known targets of SRPK1 alternative pre-mRNA splicing. The serine-arginine protein kinase SRPK1 has three major downstream targets, the RNA splicing factor SRSF1 (Edmond et al., 2011), SRSF2 (Aubol and Adams, 2011, Ngo et al., 2005, Velazquez-Dones et al., 2005) and the laminin B receptor (Papoutsopoulou et al., 1999). SRSF1 controls alternative RNA splicing of:

- 1) the **proto-oncogenes** myc (Anczukow et al., 2012), cyclinD1 (Olshavsky et al., 2010), Rac1b (Goncalves et al., 2014, Goncalves et al., 2016), CD3/TCRzeta (Zha et al., 2012, Massiello and Chalfant, 2006, Jiang et al., 1998), caspases 2 and 9, and SLC39A14 (Thorsen et al., 2011);
- 2) the **tumour suppressors**: MKNK2 (Karni et al., 2007, Das et al., 2012), BIM (Anczukow et al., 2012) and BIN (Karni et al., 2007);
- 3) the **Alzheimer's** associated protein Tau (Hartmann et al., 2001);
- 4) the **pro-angiogenesis** related genes RON (Ghigna et al., 2005, Thomas et al., 2010) and TEAD1 (Das et al., 2012, Teng et al., 2010).

(Summary prepared by Dr Lucy Donaldson).

Chapter 6. Discussion

6.1. Main study findings

Using the MIA model of OA, the data collected in this thesis show that VEGF-A_{165b} can constitute a potential analgesic agent for OA pain. In addition, they indicate a role for PAG astrocytes in OA, which involves, at least in part, blood vessels and is affected by SPHINX-31.

Administration of systemic VEGF-A_{165b} (i.p. 20 ng/g rat body weight) twice weekly in either early (days 0-13) or late (days 14-28) MIA model of OA significantly reversed weight bearing asymmetry, and it did not affect the extent of knee chondropathy, osteophytosis or synovitis. This indicates an anti-nociceptive effect of VEGF-A_{165b} that is not related to OA structural pathology and probably depends on inhibition of peripheral sensitisation. No significant changes in spinal microglial activation or spinal COX expression could be observed in this study after treatment with VEGF-A_{165b}. However, it was found that in naïve rats, non-neuronal COX-2 expression in laminae III-V of the ipsilateral and contralateral dorsal horn is significantly higher than that in laminae I & II. The same finding was observed in the contralateral spinal cord of MIA rats that received VEGF-A_{165b} in the first two weeks of the study. This may suggest a greater involvement of non-neuronal, probably glial, COX-2 in the transmission of noxious information to supra-spinal pain centres and in the descending modulation of pain.

Administration of SPHINX-31 (i.p. 0.8 µg/g body weight in 1% DMSO 8 µl/g body weight) twice weekly for 19 days did not prevent enhanced pain behaviour in the MIA model of OA, nor did it reverse the disease pathology observed in the tibia. However, administration of SPHINX-31 to MIA rats caused a significant activation of microglia in the contralateral dorsal horn compared to MIA rats that received vehicle. This finding indicates that SPHINX-31 can activate microglia in MIA rats and it suggests a potential effect of microglia activation on the descending facilitation of pain.

This study investigated for the first time the activation of PAG astrocytes and their association with blood vessels in a model of OA. It was found that in naïve rats, astrocyte activation in the VL PAG was significantly higher compared to the correspondent DL region in both ipsilateral and contralateral portions of the PAG. Similarly, the percentage of blood vessels associated with astrocytes was significantly higher in the VL PAG compared to the correspondent DL area in both the ipsilateral and contralateral sides of the PAG in the naïve animals.

6.1.1. The MIA model of osteoarthritis

To date, treatment of OA is still suboptimal. No disease modifying agents are available, so therapy aims at symptom relief, especially pain. However, achievement of successful treatment is impeded by inadequate efficacy of used agents and their adverse effects. As a result, there is a need for new effective analgesics to be used in OA, and animal models represent an essential tool for the development and testing of these agents. In this study, MIA (1 mg / 50 ml sterile NS) was injected under isoflurane anaesthesia in the tibiofemoral joint of Wistar male rats to induce the MIA model of OA. This model was chosen because it produces pain behaviour in a rapid and reproducible way (Fernihough et al., 2004, Combe et al., 2004, Gregory et al., 2012) and histopathologic changes similar to those seen in the human disease (Combe et al., 2004).

6.1.1.1. Knee joint inflammation and rearing frequency

Knee joint diameter was assessed as a measurement of local joint inflammation. MIA rats had the same knee diameter as the naïve control on day 7 post-injection and for the whole duration of the study. This can be explained by the resolution of joint swelling by day 3, as reported in literature (Nwosu et al., 2016b).

Rearings are vertical spontaneous movements related to exploration, locomotion and arousal (Lever et al., 2006). Changes in the frequency of rearings may indicate sedation (Sousa et al., 2017) or movement-provoked pain behaviour in models of

osteoarthritis (Miller et al., 2012, Inglis et al., 2008). No significant difference in the frequency of rearings were observed between the naïve rats and the MIA injected counterparts. This excludes differences in sedation, stress, ambulation or excitability that could have affected evoked pain behaviour, but also contradicts previous studies, which reported reduced number of rearings in MIA rats (More et al., 2013, Nagase et al., 2012). These discrepancies may be explained by differences in the MIA dose and counting technique used.

6.1.1.2. Pain behaviour

Considering all the three *in vivo* studies performed, it was found that these animals developed a significant decrease in thermal latency of the ipsilateral paw on days 3 and 13 after injection and a significant weight bearing asymmetry starting two days after induction of the model until day 28. MIA injected rats also showed a decrease in ipsilateral mechanical withdrawal thresholds compared to the naïve counterparts on days 4-11 and day 28; this decrease, however, did not reach statistical significance in the first two studies, probably due to inexperience or operator technique. The pain behaviour exhibited after MIA injection indicates the development of peripheral sensitisation (weight bearing imbalance) and central hyperalgesia (weight bearing imbalance, decreased thermal latency and mechanical withdrawal thresholds). These results reproduced the pain behaviour patterns of the MIA model of OA reported in literature, which include 1) significant reduction in ipsilateral hindpaw withdrawal latency to noxious thermal stimuli from day 3 to day 35 post-MIA injection (Im et al., 2010), 2) significant weight bearing asymmetry starting as early as 3 days after injection and lasting up to 35 days, and 3) significant decrease in ipsilateral mechanical withdrawal thresholds over the same time frame, but with more variable onset and significance (Nwosu et al., 2016b, Nwosu et al., 2016a, Burston et al., 2016, Sagar et al., 2014, Kelly et al., 2013, Ashraf et al., 2013).

6.1.1.3. Joint pathology

To assess the extent of joint pathology, chondropathy was first assessed macroscopically based on the Guingamp method (Guingamp et al., 1997). After obtaining H&E stained tibial sections, chondropathy and osteophytosis were scored microscopically as described by (Janusz et al., 2002). Finally, synovial inflammation scores were assigned as reported by (Mapp et al., 2008). MIA injected rats developed significant macroscopic chondropathy by day 28 post-injection, while significant microscopic cartilage pathology was already observed on day 14; this can be explained by the enhanced ability to detect smaller cartilage changes with H&E staining and microscopy. Significant synovitis in MIA injected rats was observed on day 28 in one of the *in vivo* studies, and this is compatible with previous literature reports (Mapp et al., 2013, Guingamp et al., 1997, Sagar et al., 2014), in which MIA animals showed significant synovial inflammation at the same time point. Other studies, however, showed that at this point synovitis was not present or reduced (Guzman et al., 2003). Earlier synovitis (days 3 to 14 post injection) was reported in some studies (Udo et al., 2016, Mapp et al., 2013), and it was not observed in the current study. This may be due to small group sizes, which resulted from the loss of synovium during joint disarticulation, or may replicate other previous results that did not observe synovitis on days 7 or 14 (Guzman et al., 2003, Bove et al., 2003, Liu et al., 2011). Osteophyte formation has been observed in the literature in very diverse time patterns, which could start on day 14 (Mapp et al., 2013, Guingamp et al., 1997), day 28 (Ferland et al., 2011) or no earlier than 6 weeks (Takahashi et al., 2017). In this study, a significantly higher synovitis score was found in the MIA animals compared to the naïve at days 7 and 14. On day 28 a significant difference in the proportion of samples that exhibited osteophytes between the study groups was detected.

6.1.1.4. Thermal withdrawal thresholds

In a pilot study in a separate small group of rats, thermal withdrawal thresholds were assessed for naïve rats and rats injected with intra-articular 1 mg MIA seven

days before. The A- and C- nociceptors in the rat hindpaw were preferentially stimulated by heat and the withdrawal temperatures of the bicep femoris muscles recorded. No significant differences in the evoked thermal withdrawal thresholds were detected between the MIA and naïve groups upon activation of A- or C- fibres. Differences between A and C nociceptor contributions to the MIA model have previously been reported (Kelly et al 2012). In that study, A-mechanonociceptors showed reduced activation thresholds and increased mechanically-evoked responses; in contrast C-mechanonociceptors exhibited spontaneous activity, but no threshold or evoked changes. In contrast, lower withdrawal thresholds to thermal A-nociceptor stimulation were recorded on day 7 in the CFA model of inflammatory arthritis (Drake et al., 2016). Reduced mechanical thresholds in the biceps femoris were observed at day 28, but not day 14, post-MIA injection (Kelly et al., 2013). Together these results support the conclusion that central sensitisation to either A- or C-nociceptive input is not evident in the MIA model of OA at day 7 post MIA injection, unlike in a model where profound inflammation is present at the same time point (Drake et al 2016).

6.1.1.5. Spinal glial activation

Microglia and astrocytes in the spinal cord are involved in central sensitisation and chronic pain in neuropathic and inflammatory pain (Zhuang et al., 2005, Gosselin et al., 2010). This involvement was also demonstrated in the MIA model of OA, where spinal microglia showed significant hyperactivity between days 7-28 (Orita et al., 2011, Sagar et al., 2011), and astrocytes at day 28 post-injection. At this time point (day 28), microgliosis has been associated with pain behaviour (Lee et al., 2011a, Sagar et al., 2011). Glial activation inhibitors minocycline and nimesulide attenuated pain behaviour and glial activation in the ipsilateral spinal cord (Sagar et al., 2011). The current study only partly reproduces the findings reported in literature; significant bilateral microglia activation was only detected in the MIA rats at day 14 compared to MIA counterparts at day 7, and no astrocyte activation was detected at any time point. These findings can be explained by the different methods of detection and data analysis, and, regarding microglia, the resolution of activation at day 28 (Sagar et al., 2011).

6.1.1.6. Spinal COX-2 expression

No significant differences between naïve and MIA injected animals were found in the percentage of neuronal and non-neuronal cells expressing COX-2 in any of the tested areas at any time point. These results go against findings in previous literature, which indicated enhanced expression of spinal COX-2 mRNA and protein after intra-articular injection of MIA (Prochazkova et al., 2009), and attenuated spinal neuronal responses in the MIA model of OA upon systemic and spinal administration of the COX-2 inhibitor nimesulide (Staniaszek et al., 2010).

Interestingly, this study showed that the non-neuronal expression of COX-2 in both the ipsilateral and contralateral dorsal horn of naïve animals is significantly higher in the deep laminae than the superficial laminae, while this difference was not detected in the MIA group. This indicates an increase in the expression of non-neuronal COX-2 in the superficial laminae relative to deep laminae as a result to MIA injection and suggests a bilateral involvement of non-neuronal COX-2 in spinal sensitisation.

6.1.1.7. COX expression in the PAG

COX enzymes and PGs in the PAG play a role in the supraspinal modulation of pain in inflammatory conditions, but their effects have not been investigated in models of OA. In the current study, expression of COX-2 in different columns of the PAG was assessed using PCR techniques. Data showed that induction of the MIA model of OA significantly reduced the percentage of samples in which COX-2 mRNA could be detected in the contralateral VL PAG on days 7 and 14 compared to the naïve animals. Involvement of the contralateral PAG may be due to activation of descending inputs into the PAG more on the side of input (i.e. contralaterally to the side of damage). On the other hand, no significant effect was observed for MIA injection at day 28 on the proportion of samples expressing COX-1 or COX-2 in any of the examined columns of the PAG. This may indicate a minor role for PAG COX enzymes at late time points of the MIA model of OA.

6.1.1.8. Vascular-astrocyte association in the PAG

Recent studies have demonstrated the role of glio-vascular activation in the generation of neuropathic (Kanda et al., 2017) and inflammatory pain (Beazley-Long et al., 2018). Data from the current study showed that in naïve animals the association of astrocytes with blood vessels is higher in the VL PAG than the DL PAG, while no difference is detected 28 days after the induction of MIA model. This suggests that intra-articular MIA injection can induce the association of astrocytes with blood vessels in the DL PAG, where nociceptive inputs are received.

6.1.2. Effects of VEGF-A_{165b}

6.1.2.1. Local inflammation and rearings

Knee diameter was not significantly different between MIA rats treated with VEGF-A_{165b} and those that received vehicle. VEGF-A_{165b} had no significant effect on rearing frequency either. The lack of effect of VEGF-A_{165b} on these two parameters can be explained by the absence of a significant difference between the MIA and naïve rats in the first place.

6.1.2.2. Pain behaviour

Both early (days 0-13) and late (days 14-28) administration of VEGF-A_{165b} significantly attenuated the weight bearing asymmetry of MIA rats. Injection of MIA rats with VEGF-A_{165b} also reversed the decreased mechanical withdrawal thresholds to the naïve levels; this effect, however, was not significant. This is probably because the decrease in mechanical withdrawal thresholds in MIA rats compared to the naïve controls was not significant in the first place, presumably due to experimenter inexperience. Previously, it was found that VEGF-A_{165b} significantly inhibits mechanical allodynia in diabetic neuropathy (Hulse et al., 2015) and reverses weight bearing asymmetry and mechanical allodynia in the

CFA model of inflammatory arthritis (Beazley-Long et al., 2015). Therefore, the current study replicated the effect exerted by VEGF-A_{165b} on the weight bearing imbalance in different models of pain, but failed to show a similar effect on mechanical withdrawal thresholds. This pattern of pain behaviour suggests that the analgesic effect of VEGF-A_{165b} may involve more peripheral than central mechanisms of pain (Graven-Nielsen and Arendt-Nielsen, 2002, Arendt-Nielsen et al., 2010).

6.1.2.3. Joint pathology

VEGF-A is known to contribute to the pathology of osteoarthritis, mainly due to its roles in angiogenesis and inflammation (Ashraf et al., 2011a). VEGF-A_{165b} isoform is anti-angiogenic, so an inhibitory effect on joint pathology was hypothesised. However, the administration of VEGF-A_{165b} to MIA animals had no significant effect on chondropathy, osteophytosis or synovial inflammation of the involved tibia. This is probably explained by an actual lack of effect possibly due to systemic rather than topical administration, or insufficient dosing, or inadequate statistical power due to the relatively small group size. However, this finding is relevant, as it indicates that the anti-nociceptive effect of VEGF-A_{165b} was not related to an inhibitory effect of structural joint pathology.

6.1.2.4. Spinal glial activation

Previous literature has reported trophic effects of pan-VEGF on neurons and glial cells and neuroprotective properties of VEGF-A_{165b}, while little is known about the effects of VEGF-A_{165b} on glial cells. In the current study, VEGF-A_{165b} had no significant effect on the activation of microglia or astrocytes in the dorsal horn of MIA rats, which was not significantly different from the naïve counterparts at the same time point (day 28).

6.1.2.5. Spinal COX expression

The MIA rats that received VEGF-A_{165b} on days 0-13 had a higher percentage of non-neuronal cells expressing COX-2 in the contralateral deep laminae than the corresponding superficial laminae, while the expression was not significantly different between superficial and deep laminae in the MIA rats that received vehicle. Since naïve rats also exhibited higher expression of non-neuronal COX-2 in the contralateral deep laminae than the superficial ones, this finding can indicate an increase in COX-2 expression in the superficial laminae relative to the deep laminae after MIA injection, which is inhibited by early administration of VEGF-A_{165b}. These changes in COX-2 expression occurred in non-neuronal cells of the contralateral spinal cord, which suggests a potential involvement of non-neuronal COX-2 in the transmission of noxious information to supra-spinal pain centres and in the descending modulation of pain.

6.1.3. Effects of SPHINX-31

6.1.3.1. Local inflammation and rearings

Administration of SPHINX-31 to MIA rats did not significantly affect knee diameter or rearing frequency, which may be explained by the absence of a significant difference in these parameters between the MIA and naïve rats in the first place.

6.1.3.2. Pain behaviour

Systemic administration of SPHINX-31 did not significantly reverse the enhanced pain behaviour showed by MIA rats (mechanical allodynia and weight bearing asymmetry). This is the first study to investigate the antinociceptive effects of SPHINX-31. Previously, intraplantar injection of the SRPK1 inhibitor SRPIN340 produced hypoalgesia in naïve mice (Hulse et al., 2014), and spinal SRPIN340 reversed pain behaviour and suppressed central sensitization in a model of neuropathic pain (Hulse et al., 2016). The lack of antinociceptive effect of SPHINX-31 in the MIA model of OA may be due to several factors, including 1)

systemic, rather than local, administration; 2) chemistry and dosing frequency, as this molecule was not designed to be systemically available, and does not have a prolonged half-life, which might result in insufficient plasma concentrations for analgesia; 3) insufficient dose, since the dose chosen for this study was previously used in studies of cancer xenograft and kidney disease in mice, and was based on the maximum solubility of the compound (Batson, oral communication, Babaei-Jadidi et al, unpublished); and 4) differences between the mechanisms of neuropathic pain and OA pain.

6.1.3.3. Joint pathology

SPHINX-31 is an inhibitor of SRPK1 that at the administered dose is known to inhibit phosphorylation of SRSF1 and shift VEGF-A splicing towards anti-angiogenic VEGF-A_{165b} (Hulse et al., 2014). Considering the role of VEGF in the development of osteophytes, synovitis and cartilage pathology, it was hypothesised that OA pathology could be inhibited by treatment of MIA rats with SPHINX-31. However, the current study did not detect any significant change in tibial chondropathy, synovitis and osteophytosis in the MIA rats after treatment with SPHINX-31. This finding may be explained by an actual lack of action of SPHINX-31 in these processes or small group size, especially for synovitis.

6.1.3.4. Spinal glial cell activation

On day 28 post-MIA injection, it was found that MIA animals treated with SPHINX-31 showed increased microglial activation in the contralateral dorsal horn compared to the vehicle injected counterparts. This indicates that SPHINX-31 can enhance the activation of contralateral spinal microglia in the MIA model of OA. However, since SPHINX-31 did not affect pain behaviour, it is unlikely that this activation had any significant effect on nociception.

6.1.3.5. Astrocyte activation in the PAG

MIA rats that received SPHINX-31 had a higher astrocyte activation in the VL PAG than in the corresponding DL PAG in both ipsilateral and contralateral portions. Interestingly, astrocyte activation showed the same pattern in naïve rats, while astrocyte activation in MIA injected rats was higher in the VL PAG than DL PAG in the contralateral side only. This suggests that intra-articular injection of MIA increases astrocyte activation in the ipsilateral DL PAG, where nociceptive inputs are received, compared to VL PAG. This effect is reversed by SPHINX-31, and is probably related to the production of VEGF-A_{165b} through inhibition of SRSF1. However, the ratios of astrocyte activation in VL PAG to that in DL PAG were not significantly different after MIA injection or SPHINX-31 administration.

6.1.3.6. Vascular-astrocyte association in the PAG

The results of this study showed that SPHINX-31 treated MIA rats had a higher vascular-astrocyte association in the contralateral VL PAG than the corresponding DL PAG. Naïve rats showed the same pattern of astrocyte activation, but in both ipsilateral and contralateral portions of the PAG, while no differences were detected in the vehicle injected rats. Although the ratios of vascular-astrocyte association did not differ significantly between MIA rats and naïve or SPHINX-31 injected rats, this finding may imply that MIA can induce the association of astrocytes with blood vessels in the DL PAG, while SPHINX-31 inhibits this effect. Inhibition of vascular activation may be involved in the action of SPHINX-31 on vascular-astrocyte association.

6.2. Limitations of current research

The conclusions presented in this thesis are limited by a number of factors:

1- The use of animal models. The MIA model of OA that was used in this study display histological changes and patterns of pain similar to those observed in humans (Combe et al., 2004, Sagar et al., 2013). However, this is only one model of OA, which cannot reproduce every aspect of the disease, so the results cannot be generalised, especially to human OA. Rat models develop rapidly and pain mechanisms may differ in early and long established OA, so the efficacy of drugs may not directly reflect that observed in humans. In addition, many animal models of OA are produced mostly by mechanical factors (although not the MIA model), therefore some factors that contribute to human OA may not be detected. Treatment may be effective in animal models because they are initiated at to the time of injury or close to it, in contrast to human clinical trials, where treatment usually begins after disease is well established (Martel-Pelletier and Pelletier, 2013). However, preclinical research is essential in understanding the pathological mechanisms of OA and the identification of potential treatment targets.

2- The extent of joint pathology in this study was assessed by scoring of chondropathy, osteophytosis and synovial inflammation. Although these parameters provide an adequate representation of joint pathology, the study did not address angiogenesis in knee joint, which is known to be involved in the development of OA and could have been affected by the administration of VEGF-A_{165b} and SPHINX-31. In addition, most samples did not contain synovium, which was removed during disarticulation, so it was difficult to draw conclusions on synovitis due to tissue loss.

3- The evaluation of local knee inflammation, measured as knee diameter, was performed once weekly only, and this probably caused the transient inflammation observed in the first three days of model induction to go overlooked.

4- The immunofluorescence study performed to detect COX-2 expression in the spinal cord differentiated between the enzyme expressed by neurons and non-neuronal cells, using anti-NeuN antibodies as markers of neuronal nuclei. However, no staining was performed to identify the type(s) of non-neuronal cells

involved in the expression of spinal COX-2. These cells may include endothelial or glial cells.

5- The results regarding COX isoform expression in the PAG should be interpreted with caution due to some limitations in analysis. First, results were showed as percentage of samples expressing the enzymes and calculated using association (contingency) statistical tests, rather than changes in expression. This was due to complications faced during qPCR and digital PCR, which resulted from small tissue volumes in punches used to limit analysis to specific PAG regions. As a result, RNA was insufficient for analysis in multiple samples and this led to a low number of valid samples, decreasing the statistical power of the study. For the same reason, the samples obtained from MIA rats on both day 7 and 14 were analysed as a single group.

Despite these limitations, the work contained in this thesis supports an anti-nociceptive effect of VEGF-A_{165b} in the MIA model of OA that is separate from structural pathology. It also demonstrates a role for PAG astrocytes in OA, which involves, at least in part, blood vessels and is affected by the SRPK1 inhibitor SPHINX-31.

6.3. Directions for future research

1- Spinal COX-2 is known to be expressed in different types of cells, including neurons (Cao and Zhang, 2008), glial cells (Ikeda-Matsuo et al., 2005, Vardeh et al., 2009, Goppelt-Struebe and Beiche, 1997), endothelial cells (Kanda et al., 2017, Hoffmann, 2000) and macrophages (Durrenberger et al., 2006). In the current study, anti-NeuN antibody was utilised to stain neuronal nuclei and therefore differentiate neuronal and non-neuronal COX-2 expression. Future studies should determine the type of non-neuronal (endothelial or glial) cells that express COX-2 in the spinal cord by co-staining with specific markers.

2- Although endogenous VEGF and COX-2 have mutual upregulating effects, VEGF-A_{165b} splice variant did not affect the expression of COX-2 in the

spinal cord of MIA animals. In the future, VEGF-A_{165a} and pan-VEGF should be administered in separate studies to differentiate between the effects of the two variants on COX-2 expression in the spinal cord.

3- Further studies should be performed to assess changes in the expression of COX-2 in the DL and VL PAG using qPCR or dPCR.

4- In the current study, VEGF-A_{165b} attenuated enhanced pain behaviour in the MIA model of OA. According to previous literature, these effects could be mediated by TRPV1 and TRPA1 receptors (Hulse et al., 2015, Hulse et al., 2014). Further studies should be planned to clarify the mechanisms through which antinociceptive effects are exerted.

5- Future work involving VEGF-A_{165b} in the MIA model of OA should investigate synovial and osteochondral angiogenesis (endothelial cell proliferation index, vascular density and channels positive for vessels) and inflammation (by measuring macrophage fractional area and thickness of the synovial lining) in the knee to determine a potential effect of this splice variant on these two components of OA pathophysiology.

6- Administration of SPHINX-31 did not show anti-nociceptive effects in the MIA model of OA, and this may be due to inadequate dosing. Future work should determine the concentration of SPHINX-31 and phosphorylated SRSF1 in different tissues (blood, DRG, spinal cord and brain) to confirm the presence and efficacy of SPHINX-31.

7- SPHINX-31 showed significant effects on the activation of PAG astrocytes. SPHINX-31 may exert its effect directly on cells, through inhibition of angiogenesis or by regulation of mRNA splicing of different genes. Further studies should be planned to determine the exact mechanisms of these interactions. SRPK1 is known to control the mRNA alternative splicing of other molecules, so new studies should explore changes in the PAG expression of these molecules' alternative splice variants in the MIA model of osteoarthritis and after administration of SPHINX-31.

8- The current study addressed astrocyte-vascular association in the PAG in a model of OA, which had also been investigated in the spinal cord of the CFA model of inflammation. Future work should study gliovascular interactions in the spinal cord of the MIA model of OA, and after administration of SPHINX-31, investigate the potential mechanisms involved and assess potential correlations with nociception. Further studies should also address astrocyte-vascular association in the spinal cord and PAG after treatment with VEGF-A_{165b}.

6.4. Concluding remarks

OA is the most common musculoskeletal disease and a major cause of chronic pain worldwide. Treatment of OA related pain is still suboptimal, due to limited efficacy of available analgesics and their unfavourable side effect profiles. Therefore, there is still a need to clarify mechanisms of pain in OA and develop new effective analgesics.

VEGF-A_{165b} is an antiangiogenic splice variant of VEGF-A, which results from the alternative splicing of VEGF-A gene at distal splice site. This is controlled by the action of SRPK1, which phosphorylates the splicing factor SRSF1. VEGF-A_{165b} has showed antinociceptive effects in models of neuropathic and inflammatory pain, which involved TRPV1 and TRPA1 receptors and vascular activation, respectively. Similarly, pharmacological inhibition of SRPK1 was found to reverse pain behaviour in neuropathic pain.

The findings outlined in this thesis have provided evidence that VEGF-A_{165b} is anti-nociceptive in the MIA model of OA and that astrocytes in the PAG play a role in OA, which involves blood vessels and is affected by SRPK1 inhibitor SPHINX-31.

Future work will investigate the mechanisms of VEGF-A_{165b} antinociceptive effects, as well as the mechanisms of SPHINX-31 actions on PAG astrocytes and vascular-astrocyte association.

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APPENDICES

Materials and Reagents

Accu-Edge® disposable microtome blades	Sakura, Japan
Agarose	Bioline, London, UK
Albumin, Bovine	Sigma Aldrich, Poole, UK
Ammonium hydroxide	VWR Ltd, UK
Ammonium oxalate	VWR Ltd, UK
Axio Vision Imaging system	Carl Zeiss Ltd. Welwyn Garden City, UK
Axiocam MRc Zeiss	Carl Zeiss Ltd. Welwyn Garden City, UK
Chlorhexidine gluconate	Ecolab Ltd, Leeds, UK
Chloroform	VWR Ltd, UK
Coverslips 22mm x 64mm	VWR Ltd, Lutterworth, UK
DPX™	VWR Ltd, Lutterworth, UK
EDTA disodium salt dihydrate	Sigma Aldrich, Poole, UK
Electronic Calliper	Control Company, Friendswood, USA
Eosin (1%)	Thermo Fisher, UK
Ethanol, absolute	Sigma Aldrich, Poole, UK
Ethidium bromide	Sigma Aldrich, Poole, UK
Fetal bovine serum	Sigma Aldrich, Poole, UK

Leica SM 2010 R sliding microtome	Leica Microsystems, UK
Incapacitance Meter	Linton Instruments, Norfolk, UK
Isopropanol	Sigma Aldrich, Poole, UK
Leica TCS SP5 X confocal microscope	Leica Microsystems, UK
Mayer's haematoxylin	Thermo Fisher, UK
OCT [®] mounting medium	VWR Chemicals, Leuven, Belgium
Paraformaldehyde (P6148)	Sigma Aldrich, Poole, UK
Phosphate Buffered Saline, tablets	Oxoid Ltd, Basingstoke, UK
Polylysine [™] Microscope adhesion slides	Fisher Scientific Ltd. Loughborough, UK
Polyvinylpyrrolidone (PVP40T)	Sigma Aldrich, Poole, UK
Shandon Pathcentre Tissue Processor	Shandon Thermo Scientific, Leicester, UK
Sodium acetate	Sigma Aldrich, Poole, UK
Sterile scalpel blades	Swann-Morton Sheffield, UK
Syringe insulin (1 ml, 29G)	Myjector [®] , Terumo Medical Corporation, Elkton, USA
Tissue Tek [®] embedding centre	Sakura Finetek Europe, The Netherlands
Tris base	Sigma Aldrich, Poole, UK
Triton-X 100	Sigma-Aldrich, UK
von Frey filaments	Linton Instrumentation, Norfolk, UK
Wistar rats	Charles River, Kent, UK

Xylene

Sigma Aldrich, Poole, UK

Zeiss Axioscop-50 microscope

Carl Zeiss Ltd. Welwyn Garden City,
UK