# THE INFLUENCE OF ANAESTHESIA ON DIFFUSE NOXIOUS INHIBITORY CONTROLS IN THE RAT

**KATE L WHITE FRCVS**

**Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy**

**November 2020**

## ABSTRACT

In healthy animals and humans, a test stimulus will attenuate to a noxious conditioning stimulus applied elsewhere on the body; a phenomenon termed diffuse noxious inhibitory controls (DNIC). This endogenous pain inhibitory system can be used to study impairments in descending pain modulation, which may underlie some chronic pain conditions. The descending modulation and spinal nociceptive processing can be studied in anaesthetised rodents by examining nociceptive withdrawal reflexes (NWR) which are nocifensive, polysynaptic, multisegmental spinal reflexes designed to withdraw a limb from a damaging insult. These reflexes can be studied by measuring the electromyographic activity in limb muscles. Refining the anaesthetic for studying the NWR paradigm was the first aim of the study. The ideal anaesthetic for nociceptive studies will provide immobility, unconsciousness and be devoid of cumulative and antinociceptive effects. The pharmacokinetics and pharmacodynamics (PK/PD) of the anaesthetic agent alfaxalone were determined in male and female Sprague Dawley rats. These data informed subsequent DNIC studies. Nitrous oxide is also used commonly as a component of anaesthesia in animals and humans and has been used in laboratory rodent experiments for decades. In view of this, parsing out the modulating effects of $N_2O$ on an NWR model in naïve animals was undertaken to determine whether preconditioning with $N_2O$ could influence descending controls and DNIC. Experiments were performed in naïve Sprague Dawley rats with an intact neuraxis and rats decerebrated at the pre-collicular level to evaluate the suprapsinal contribution. Lewis rats resistant to $N_2O$ antinociceptive effects were also studied. $N_2O$ preconditioning had a significant effect on the level of DNIC which differed

depending on the reflex studied. Lewis rats were resistant to $N_2O$'s effects on DNIC. The final series of experiments evaluated preconditioning with $N_2O$ on DNIC in an induced osteoarthritis model demonstrating differences in the magnitude of DNIC between the controls and diseased animals albeit reflex specific differences. In conclusion based on these results it would be advisable to omit $N_2O$ from anaesthesia protocols interrogating the descending control of reflexes in rats. It is evident that $N_2O$ is able to modulate descending controls and further work is required to investigate the translational possibilities of this intervention.

## DEDICATION

*I dedicate this PhD to my parents for their unswerving support and encouragement throughout my life*

## ACKNOWLEDGEMENTS

I would like to thank the School of Veterinary Medicine and Science and the School of Biosciences at the University of Nottingham for giving me the opportunity to undertake a PhD alongside my full-time job. In particular I'd like to thank Professor Gary England and Professor David Gardner at SVMS for their encouragement and grounded advice, and Dr Stuart Paine for his patience with my learning of pharmacokinetics.

I cannot thank my supervisor Dr John Harris enough for taking me on, and teaching me so much neuroscience, and putting up with me! Thank you also to Dr Carl Stevenson for encouragement and sound advice, all the staff in BSU for help and care of the animals, and Victoria Simmonds for her patient teaching and friendship in the electrophysiology laboratory.

My family have also been a constant source of encouragement and tolerated my early morning working. I will forever associate this PhD with the shipping forecast.

*Viking, North Utsire South Utsire, Forties, Cromarty, Forth, Tyne, Dogger, Fisher, German Bight, Humber, Thames, Dover, Wight, Portland, Plymouth, Biscay, Trafalgar, Fitzroy, Sole, Lundy, Fastnet, Irish Sea, Shannon, Rockall, Malin, Hebrides, Bailey, Fairisle, Faroes, SE Iceland*

# TABLE OF CONTENTS

ABSTRACT ..... ii

DEDICATION ..... iv

ACKNOWLEDGEMENTS ..... v

TABLE OF CONTENTS ..... vi

ABBREVIATIONS AND ACRONYMS ..... xi

LIST OF TABLES ..... xv

LIST OF FIGURES ..... xvi

1. LITERATURE REVIEW ..... 1

1.1. INTRODUCTION ..... 1

1.2. RECEIPT, ENCODING AND TRANSMISSION OF NOCICEPTIVE INFORMATION 2

1.2.1. Biology of sensory transduction ..... 2
1.2.2. Sensitisation ..... 6
1.2.3. Initiation of a generator potential ..... 8
1.2.4. Transmission of the action potential ..... 9
1.2.5. Anatomy of the dorsal horn ..... 10
1.2.6. Anatomy of the ascending pathways ..... 15
1.2.7. Anatomy of the descending pathways ..... 16
1.2.8. The pharmacology of the descending controls ..... 19
1.2.8.1. Noradrenaline ..... 20
1.2.8.2. Serotonin ..... 22
1.2.8.3. Dopamine ..... 23
1.2.8.4. Opioids ..... 24
1.2.8.5. Endocannabinoids ..... 26
1.2.8.6. GABA and glycine ..... 27
1.2.9. Tonic control ..... 28
1.2.10. The spino-bulbo-spinal loop ..... 29
1.2.11. Conditioned pain modulation (CPM) and Diffuse Noxious Inhibitory Controls (DNIC) ..... 29

1.3. THE MECHANISMS OF GENERAL ANAESTHESIA ..... 33

1.4. ANAESTHESIA TECHNIQUES IN LABORATORY ANIMALS ..... 41

1.4.1. Inhalation anaesthetics ..... 41
1.4.2. Nitrous oxide ($N_2O$) ..... 43
1.4.3. Injectable anaesthetics ..... 46
1.4.4. Methods avoiding the use of anaesthesia ..... 48

1.5. STUDYING PAIN ELECTROPHYSIOLOGICALLY ..... 50

1.5.1. Electroencephalography ..... 50
1.5.2. Nociceptive withdrawal reflexes (NWRs) ..... 50
1.5.2.1. Studying nociceptive withdrawal reflexes in rodent models: reflex arrangement ..... 52

1.6. DELIVERING CLINICAL IMPACT ..... 53

1.6.1. Osteoarthritis ..... 53
1.6.1.1. Preclinical models of osteoarthritis ..... 55
1.7. THE MODEL ..... 58
1.7.1. The Subject ..... 60
1.7.2. The assay ..... 61
1.7.3. The measure ..... 61
1.8. SUMMARY ..... 63
2. MATERIALS AND METHODS ..... 65
2.1. ETHICAL APPROVAL ..... 65
2.2. ANIMALS ..... 65
2.3. ANAESTHESIA ..... 66
2.3.1. Isoflurane ..... 66
2.3.2. Alfaxalone infusion ..... 66
2.3.3. Monitoring anaesthesia ..... 67
2.3.4. Haematology, biochemistry and blood gases ..... 67
2.4. SURGERY ..... 68
2.5. STABILISATION ..... 69
2.6. ELECTROPHYSIOLOGY ..... 70
2.6.1. Earthing electrode ..... 70
2.6.2. Hind limb stimulation and recording ..... 70
2.6.3. Conditioning stimulus ..... 73
2.7. EUTHANASIA ..... 73
2.8. STATISTICAL ANALYSIS ..... 73
2.8.1. Group sizes ..... 74
2.8.2. Cardiopulmonary data, haematology, biochemistry and blood gases ..... 74
2.8.3. Reflex data ..... 75
2.8.3.1 Electrical thresholds required to elicit reflexes ..... 75
2.8.3.2. Raw control responses between sexes and other groups ..... 75
2.8.3.3. The effect of capsaicin on the reflex ..... 75
2.8.4. Behavioural data (Chapter 5) ..... 76
3. THE EFFECT OF SEX ON ALFAXALONE ANAESTHESIA AND DNIC ..... 77
3.1. SEX ..... 77
3.2. ALFAXALONE ..... 77
3.2.1. History of the use of alfaxalone ..... 77
3.2.2. The use of alfaxalone in biomedical research ..... 78
3.2.3. The pharmacokinetics of alfaxalone ..... 78
3.2.4. Aims of study ..... 79
3.3. MATERIALS AND METHODS ..... 80
3.3.1. Animals ..... 80
3.3.2. Group sizes for pharmacokinetic/pharmacodynamic (PK/PD) studies ..... 80
3.3.3. General anaesthesia and surgery ..... 81

3.3.4. Alfaxalone infusion (both sexes) ... 83
3.3.5. Alfaxalone infusion (females only) ... 83
3.3.6. Blood sampling protocol for PK study ... 83
3.3.7. Sample analyses ... 84
3.3.8. PK analysis ... 85
3.3.9. Electrophysiological recordings ... 86
3.3.10 Vaginal smears ... 86
3.3.11 Statistical analysis ... 87

3.4. RESULTS ... 88

3.4.1. Anaesthesia (both sexes study) ... 88
3.4.2. Anaesthesia (female only study) ... 88
3.4.3. PK of alfaxalone in both sexes ... 88
3.4.4. PD of alfaxalone in both sexes ... 92
3.4.5. Vaginal smears ... 100
3.4.6. Demographics and stimulus intensities to elicit reflexes ... 100
3.4.7. Raw control responses ... 101
3.4.8. The effect of capsaicin on reflex responses ... 103

3.5. DISCUSSION ... 107

3.5.1. PD and sex differences ... 107
3.5.2. PD and plane of anaesthesia ... 109
3.5.3. Electrophysiology ... 111

3.6. CONCLUSION ... 117

4. THE EFFECTS OF $N_2O$ ON DNIC ... 118

4.1. DESCENDING CONTROLS ... 118

4.1.1. Counter irritation paradigm for studying descending controls ... 118
4.1.2. Studying DNIC under the influence of $N_2O$ ... 118
4.1.3. Rat strain variability in response to $N_2O$ ... 120
4.1.4. Aims of study ... 120

4.2. MATERIALS AND METHODS ... 122

4.2.1. Animals ... 122
4.2.2. Anaesthesia for animals with intact neuraxis ... 122
4.2.3. Anaesthesia for decerebration ... 124
4.2.4. Mechanical decerebration ... 126
4.2.5. Hind limb stimulation and recording of NWR ... 127
4.2.6. DNIC conditioning stimulus ... 127
4.2.7. Statistical analysis ... 127

4.3. RESULTS ... 128

4.3.1. Experimental groups ... 128
4.3.2. Haematology, blood biochemistry and blood gases ... 129
4.3.3. Demographics of groups ... 132
4.3.4. Electrical thresholds required to elicit reflexes ... 134
4.3.5. Stimulation strengths for eliciting NWRs ... 136
4.3.6. Effect of $N_2O$ on DNIC of reflexes in naïve Sprague Dawley rats ... 138
4.3.7. Effect of $N_2O$ on DNIC of reflexes in decerebrated Sprague Dawley rats ... 140
4.3.8. Effect of $N_2O$ on DNIC of reflexes in Lewis rats ... 143

4.4. DISCUSSION ... 145

4.4.1. Global impact of $N_2O$ on the model ... 145
4.4.2. Differential effects of $N_2O$ preconditioning on NWR ... 146

4.4.3. Anaesthesia stability ..... 147
4.4.4. Strain susceptibility and tolerance to $N_2O$ ..... 148
4.4.5. Decerebration versus intact neuraxis ..... 149

4.5. IMPLICATIONS OF THE STUDY ..... 150

4.6. CONCLUSIONS ..... 152

5. $N_2O$ MODULATES DNIC IN A MODEL OF OSTEOARTHRITIS ..... 153

5.1 THE PATHOPHYSIOLOGY OF OSTEOARTHRITIS ..... 153

5.1.1. The role of disordered central pain modulation ..... 154
5.1.2. DNIC in chronic pain ..... 154
5.1.3. Studying DNIC and osteoarthritis preclinically ..... 156
5.1.4. Aims of the study ..... 156

5.2. MATERIALS AND METHODS ..... 157

5.2.1. Animals ..... 157
5.2.2. Induction of osteoarthritis ..... 159
5.2.3. Examination and gait analysis ..... 159
5.2.4. Habituation ..... 160
5.2.5. Weight bearing paradigm ..... 160
5.2.6. Mechanical threshold paradigm ..... 160
5.2.7. Anaesthesia and reflex recordings ..... 161
5.2.8. Gross anatomical pathology of the knee joints ..... 163
5.2.9. Statistical analysis ..... 163

5.3. RESULTS ..... 164

5.3.1. General health of the animals ..... 164
5.3.2. MIA-evoked pain behaviours ..... 165
5.3.2.1. Weight bearing ..... 165
5.3.2.2. Tactile mechanical allodynia ..... 166
5.3.3. Anaesthesia for electrophysiology ..... 167
5.3.3.1. Cardiopulmonary parameters ..... 167
5.3.3.2. Blood gases and biochemistry ..... 169
5.3.4. Hind limb stimulation and recording of NWRs ..... 171
5.3.5. The effect of capsaicin on reflex responses ..... 173
5.3.5.1. Animals with induced osteoarthritis (MIA) ..... 173
5.3.5.2. Saline injected (control) animals ..... 178
5.3.6. Pathology ..... 181

5.4 DISCUSSION ..... 183

5.4.1. Induction of hyperexcitability ..... 183
5.4.2. Stimulation thresholds ..... 183
5.4.3. Inhibition and sensitisation of reflexes ..... 184
5.4.4. The influence of $N_2O$ ..... 186
5.4.5. DNIC differences between MIA and saline controls, and the influence of $N_2O$ ..... 187
5.4.6. Gait control ..... 190
5.4.7. The MIA model ..... 191
5.4.8. DNIC dysfunction ..... 192

5.5 CONCLUSION ..... 193

6. GENERAL DISCUSSION ..... 195

6.1. DNIC SUMMARY ..... 195

6.2. THE IDEAL MODEL TO STUDY NOCICEPTION ..... 201

6.3. FUTURE RESEARCH ..... 202

REFERENCES ..... 205

APPENDICES ..... 272

Appendix 1. Haematology and biochemistry for male and female rats (8–12) weeks ..... 272
Appendix 2. Abstracts presented at international conferences ..... 276
Appendix 3. List of published peer reviewed papers ..... 278

## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| 5-HT | 5-hydroxytryptamine |
| ACL | anterior cruciate ligament |
| ABP | arterial blood pressure |
| ACC | anterior cingulate cortex |
| ALFX | alfaxalone |
| AMPA | $\alpha$-amino-3-hydroxy-5methyl-4-isoxazoleproprionic |
| ANOVA | analysis of variance |
| AP | action potential |
| ASIC | acid sensing ion channel |
| ASPA | Animal Scientific Procedures Act |
| ATP | adenosine triphosphate |
| AUC | area under the curve |
| BF | *biceps femoris* |
| CGRP | calcitonin gene related peptide |
| CRI | constant rate infusion |
| CRF | corticotrophin releasing factor |
| CL | clearance |
| Cmax | maximum plasma concentration |
| CNS | central nervous system |
| $CO_2$ | carbon dioxide |
| CPM | conditioned pain modulation |
| CRI | constant rate infusion |
| DAP | diastolic arterial pressure |
| DC | decerebrated |
| DEG/ENaC | degenerin/epithelial sodium channel |
| DNIC | diffuse noxious inhibitory controls |
| DRG | dorsal root ganglion |
| ECG | electrocardiogram |
| EEG | electroencephalogram |
| EMG | electromyography |
| $F_{50}$ | EEG median frequency |
| fMRI | functional magnetic resonance imaging |

| | |
|---|---|
| GABA | γ-aminobutyric acid |
| GM | monosialotetrahexosylganglioside1 (GM1) lipids (sometimes called lipid rafts or lipid domains) |
| GP | generator potential |
| GPCR | G protein coupled receptor |
| HR | heart rate |
| IA | intraarticular |
| IM | intramuscular |
| IP | intra peritoneal |
| IPPV | intermittent positive pressure ventilation |
| IQR | inter quartile ranges |
| ISO | isoflurane |
| IV | intravenous |
| K2P | 2 pore potassium channel |
| LC | locus coeruleus |
| LC-MS/MS | liquid chromatography-mass spectrometry |
| LEW | Lewis rats |
| LTP | long term potentiation |
| MAC | minimal alveolar concentration |
| MAP | mean arterial pressure |
| MG | *medial gastrocnemius* |
| MIA | monosodium iodoacetate |
| MRT | mean residence time |
| NK-1 | neurokinin-1 |
| NMDA | N-methyl-D-aspartate |
| $N_2O$ | nitrous oxide |
| NOP | opioid receptor-like 1 receptor |
| NRM | nucleus raphe magnus |
| NS | nociceptive specific |
| NSAID | non-steroidal anti-inflammatory drug |
| NWR | nociceptive withdrawal reflex |
| OA | osteoarthritis |
| OD | outside diameter |
| ORL1 | opioid receptor like 1 receptor |

| | |
|---|---|
| $P_aCO_2$ | partial pressure of carbon dioxide in arterial blood |
| $P_aO_2$ | partial pressure of oxygen in arterial blood |
| PA | parabrachial area |
| PC | personal computer |
| PAG | periaqueductal grey |
| PAR | protease activated receptor |
| PB | parabrachial |
| PD | pharmacodynamic |
| PLD2 | phospholipase D2 |
| PK/PD | pharmacokinetic/pharmacodynamic |
| PNS | peripheral nervous system |
| PPA | phosphatidic acid |
| PUFA | polyunsaturated fatty acid |
| QC | quality control |
| RVM | rostral ventral medulla |
| S1 | Schedule 1 procedure (appropriate method of humane killing) |
| SAP | systolic arterial pressure |
| SD | Sprague Dawley |
| SEM | standard error of the mean |
| SRD | subnucleus reticularis dorsalis |
| $t_{1/2}$ | terminal half-life |
| TA | *tibialis anterior* |
| TASK | potassium family subfamily K member 3 |
| TRAAK | TWIK subfamily K member 4 |
| TRP | transient receptor potential |
| TRPA | transient receptor potential subfamily ankyrin |
| TRPC | transient receptor potential subfamily canonical |
| TRPM | transient receptor potential subfamily melastatin |
| TRPV | transient receptor potential subfamily vanilloid |
| TREK | TWIK subfamily K |
| TWIK | tandem of P domains in a weak inward rectifying channel |
| UVB | ultra violet B |
| Vdss | volume of distribution at steady state |
| VGSC | voltage gated sodium channels |

| | |
|---|---|
| VLM | ventrolateral medulla |
| WDR | wide dynamic range |

# LIST OF TABLES

Table 1.1 A selection of mammalian mechanosensory and thermosensory transduction channels associated with the skin ..... 4
Table 1.2 Effects of anaesthetics on ion channels or receptor targets. ..... 35
Table 1.3 A summary of the different osteoarthritis models used in rats ..... 57
Table 1.4 Construct of the rodent experimental model..... 59
Table 3.1 Pharmacokinetic parameters after alfaxalone infusion ..... 90
Table 3.2 Cardiopulmonary parameters prior to and during alfaxalone infusion..... 93
Table 3.3 Blood gas variables and clinical biochemistry parameters..... 98
Table 3.4 Demographics, electrical thresholds and stimulation strengths ..... 101
Table 4.1 Numbers of animals in each group of the experiment..... 128
Table 4.2 Blood gas variables and clinical biochemistry parameters..... 130
Table 4.3 Raw control responses of reflexes from heel and toes ..... 133
Table 5.2 Measured blood gas variables and clinical biochemistry parameters..... 170
Table 5.3 Thresholds and stimulation strengths..... 172
Table 5.4 Raw control responses of reflexes elicited from the heel and toes..... 173

# LIST OF FIGURES

Figure 1.1 Schematic diagram of ascending and descending pain pathways. ..... 13
Figure 1.2 Activation of descending diffuse noxious inhibitory controls (DNIC) results in pain modulation..... 32
Figure 1.3 Periodic table of molecular elements in human and veterinary anaesthesia. ..... 37
Figure 1.4 Schematic depicting palmitate-mediated localisation. This is a central component of TREK-1 anaesthesia and mechano-sensitisation. ..... 40
Figure 2.1 Raw trace of reflex evoked in *medial gastrocnemius* by electrical stimulation of the heel..... 71
Figure 3.1 Timeline for PK and DNIC studies in male and female rats. ..... 82
Figure 3.2 Plasma concentration-time profiles of male and female rats ..... 89
Figure 3.3 Plasma concentration-time profiles for all rats during alfaxalone infusion..... 91
Figure 3.4 Mean arterial blood pressure of all rats during the experiment. ..... 96
Figure 3.5 Raw control responses for heel and toes' reflexes prior to injection of capsaicin in naïve male and female Sprague Dawley rats. ..... 102
Figure 3.6 Effect of capsaicin on heel- and toe-evoked reflexes in male and female Sprague Dawley rats ..... 106
Figure 4.1 Timeline for the 2 groups of male Sprague Dawley rats and 2 groups of Lewis rats with intact neuraxes undergoing DNIC studies. ..... 123
Figure 4.2 Timeline for the male Sprague Dawley rats undergoing pre-collicular decerebration prior to DNIC studies. ..... 125
Figure 4.3 Electrical thresholds ..... 135
Figure 4.4 Stimulation strength ..... 137
Figure 4.5 Effect of capsaicin on reflexes elicited from heel and toes in male Sprague Dawley rats with intact neuraxes..... 139
Figure 4.7 Effect of capsaicin injection on reflexes elicited from heel and toes in male Lewis rats. ..... 144
Figure 5.1 Timeline for the Sprague Dawley rats undergoing induction of osteoarthritis with MIA or saline (controls) ..... 158
Figure 5.2 Timeline for the 2 groups of male Sprague Dawley rats (MIA and saline (control)) undergoing terminal anaesthesia for DNIC studies. ..... 162
Figure 5.3 Bodyweight change for all animals over the course of the experiment ..... 164

Figure 5.4 Behavioural profile of joint hypersensitivity indicated by differences in weight bearing in pelvic limbs from 0–28 days. ..... 165
Figure 5.5 The effect of osteoarthritis model induction on tactile allodynia. ..... 166
Figure 5.6 Systolic, diastolic and mean blood pressure values during alfaxalone anaesthesia (with or without $N_2O$ preconditioning) and prior to capsaicin injection ..... 168
Figure 5.7 Effect of capsaicin injection on reflexes elicited from heel and toes in the induced (MIA) model and saline controls. ..... 177
Figure 5.8 Negative area under the curve (AUC) for reflexes elicited from heel and toes for MIA and saline controls. ..... 180
Figure 5.9 Gross joint pathology. ..... 182

# 1. LITERATURE REVIEW

## 1.1. INTRODUCTION

For over two centuries general anaesthetics have been used to render humans and animals unconscious for surgical procedures. The transmission of signals conveying the pain from the site of injury is conveyed and processed via the same sites affected by the anaesthesia drugs. Drugs capable of inducing anaesthesia display a varied array of receptor targets also involved in many homeostatic mechanisms. So, although the use of anaesthesia in animal pain studies obtunds the influence of other variables such as fear, expectation and attention, its presence adds a layer of complexity as the influence of anaesthesia can impact the nociceptive pathway at many levels.

The purpose of this literature review is twofold: firstly, to provide a concise account of the neuronal pathways involved in the receipt and transmission of nociceptive information from the periphery to the central nervous system (CNS), with an emphasis on the control exerted by the descending pathways; and secondly to summarise the mechanisms, use and effect of anaesthesia on the models utilised to study nociception. The first chapter of the thesis concludes with a justification for studying aspects of nociception in an anaesthetised rodent model, the detail of which is expanded on in Chapter 2.

## 1.2. RECEIPT, ENCODING AND TRANSMISSION OF NOCICEPTIVE INFORMATION

### 1.2.1. Biology of sensory transduction

The majority of pain experiences stem from activity in nociceptive primary afferent (sensory) neurones caused by mechanical, thermal or/and chemical stimuli. The membrane of the peripheral terminal is populated with transduction proteins capable of responding to the stimuli. The definition of transduction is the conversion of the noxious stimulus into an electrical signal (Cesare and Mcnaughton 1996; Reichling and Levine 1997; Thut et al. 2003; Gold and Gebhart 2010). The transducers or ionotropic receptors can crudely be described by the type and origin of stimulus (mechanical, thermal, chemical), their tissue type association and whether they are extrinsic or intrinsic to the primary afferent. However, thereafter the classification breaks down because these transducers lack a single criterion to reliably identify them (Jordt et al. 2003; Lumpkin and Caterina 2007). Additionally, as a population they share anatomical, biochemical, physical and functional heterogeneity which may have hindered efficacy of therapy and contributed to a failure of translation of preclinical data to clinical use.

So, for example a selection of the mechanosensory and thermosensory transduction channels in mammalian skin is outlined in Table 1.1. The activators and modality of the channels is characterized in the table but *in vivo* the activator may also initiate other types of cells to release chemicals and the functional implications of these concurrent events are still to be properly understood. This area

of research is rapidly expanding, and the polymodality of receptors makes classification challenging. Chemical or chemo transducers are well characterised, numerous and can respond to exogenous and endogenous compounds. The direct form of chemotransducer is an ion channel that has a binding site for a ligand; also known as ionotropic receptors. An indirect form known as a metabotropic receptor also exists; which is slower, with the chemotransduction driven by an intracellular signalling cascade (Goudet et al. 2009) (see below).

Table 1.1 A selection of mammalian mechanosensory and thermosensory transduction channels associated with the skin

| Identity | Family | Modality | Activators | Temperature range | Location | References |
|---|---|---|---|---|---|---|
| TRPA1 | TRPA | Thermal, Mechanical | Isothiocyanates, $Ca^{2+}$, icilin | <18°C | C fibres | Bandell et al., 2004; Karashima et al., 2007 |
| TRPC1 | TRPC | Mechanical | Receptor/store operated | N/A | C & Aδ fibres, keratinocytes | Garrison et al., 2012; Maroto et al., 2005 |
| TRPC5 | TRPC | Thermal | Thioredoxin | 25–37°C | C & Aδ fibres | Zimmermann et al., 2011 |
| TRPM3 | TRPM | Thermal | Pregnenolone sulphate | >30°C | C & Aδ fibres | Paricio-Montesinos et al., 2020 |
| TRPM8 | TRPM | Thermal | Menthol, icilin | < 28°C | C fibres | Acosta et al., 2014; Bautista et al., 2007; McKemy et al., 2002; Tajino et al., 2011 |
| TRPV1 | TRPV | Thermal, Osmotic | Capsaicin, $H^+$ Endocannabinoids, diphenyl compounds | >42°C | C & Aδ fibres, keratinocytes | Caterina and Julius, 2001; Szallasi et al., 2006; Woodbury et al., 2004 |
| TRPV2 | TRPV | Thermal, Osmotic, Mechanical | 2-aminoethoxydiphenyl borate | >52°C | Aδ & Aβ fibres, immune cells | Caterina and Julius, 2001; Woodbury et al., 2004 |
| TRPV3 | TRPV | Thermal | Camphor, carvacrol, diphenyl compounds | >34–39°C | C fibres Keratinocytes | Lee and Caterina, 2005; Moqrich et al., 2005; Peier et al., 2002 |
| TRPV4 | TRPV | Thermal, Osmotic, Mechanical (in injury) | PUFAs, 4αPDD, epoxyeicosatrienoic acids | >27–34°C | Keratinocytes, Merkel cells, Aδ and C fibres | Güler et al., 2002; Liedtke, 2005 |
| ASIC1 | DEG/ ENaC | Mechanical (touch) | $H^+$ | N/A | Aδ, Aβ & C fibres | Calavia et al., 2010; Page et al., 2004 |
| ASIC2 | DEG/ ENaC | Mechanical (touch) | $H^+$ | N/A | Aδ & Aβ fibres | Bianchi and Driscoll, 2002; Drew et al., 2004 |
| ASIC3 | DEG/ ENaC | Mechanical (touch) | $H^+$ | N/A | Aδ & Aβ fibres | Mogil et al., 2005 |
| TREK-1 | 2P $K^+$ channel | Thermal Mechanical | PUFAs, $H^+$ | Noxious cold and noxious heat | Aδ & C fibres, Aβ ? | Franks and Honoré, 2004; Honoré, 2007; Maingret, 2000 |
| TREK-2 | K2P channel | Thermal Mechanical | PUFAs, $H^+$ | 40–46°C 20–25°C | C fibres Dorsal root ganglia | Pereira et al., 2014 Acosta et al., 2014 |
| TRAAK | K2P channel | Thermal Mechanical | PUFAs, $H^+$ | Noxious cold and noxious heat | C fibres Dorsal root ganglia | Noël et al., 2009; Schneider et al., 2014 |
| $Na_v$ 1.8 | VGNC | Thermal | No subfamily activator | Noxious cold | C fibres Dorsal root ganglia | Akopian et al., 1999; Luiz et al., 2019; Wood et al., 2004 |
| Piezo 1 | Piezo | Mechanical | Pyrazine? | N/A | Dorsal root ganglia, (less than Piezo 2) Merkel cells | Coste et al., 2010; Parpaite and Coste, 2017; Volkers et al., 2014 |
| Piezo 2 | Piezo | Mechanical | Pyrazine? | N/A | Dorsal root ganglia Aδ fibres | Bron et al., 2014; Lee et al., 2017 |

TRP: transient receptor potential, TRPA: transient receptor potential subfamily ankyrin, TRPC: transient receptor potential subfamily canonical, TRPM: transient receptor potential subfamily melastatin, TRPV: TRPA: transient receptor potential subfamily vanilloid, DEG/ENaC: degenerin/epithelial sodium channel, ASIC: acid sensing ion channel, VGNC: voltage gated sodium channel, PUFA: polyunsaturated fatty acid, 4αPDD: 4α–phorbol 12, 13-didecanoate, K2P channel: 2 pore potassium channel, TREK: TWIK (TWIK: tandem of P domains in a weak inward rectifying channel) subfamily K, TRAAK: TWIK subfamily K member 4, $H^+$: protons.
Modified after (Lumpkin and Caterina 2007).

The cloning of the transient receptor potential cation vanilloid subfamily member 1 (TRPV1) was pivotal not only in furthering the understanding of chemotransduction in nociceptive afferents, but also in thermotransduction too (Messeguer et al. 2005; Holzer and Izzo 2014). Other mammalian transient receptor potential (TRP) family receptors, TRPA (TRP subfamily ankyrin) and TRPM (TRP subfamily melastatin), share a thermotransduction role with a number of the 2 pore potassium channels (K2P), (Noël et al. 2009; Nikolaev et al. 2019), as well as the $Na_v1.8$ channel (Zimmermann et al. 2007).

Despite the overwhelming evidence that mechanosensation is the most common component of somatosensation, it is the least well understood. The transducers known to play a role in mammalian mechanotransduction include the Piezo channels, TRPA1 and TRPV4, transient $Ca^{2+}$ channels and at least three K2P channels (Maingret et al. 1999; Liedtke 2005; Amato et al. 2012; Gottlieb and Sachs 2012; François et al. 2015; Woo et al. 2015). There will be overlap of the contribution of the mechanosensitive properties with the chemosensitive properties as chemicals are released from cells in response to mechanical stimuli. This overlap and lack of transducer specificity makes interpretation from intact preparations difficult.

### 1.2.2. Sensitisation

The definition of sensitisation is a decreased threshold for response, and/or can also be characterised by an increased response to a suprathreshold stimulus. Additionally, an increase in spontaneous activity can occur in the nociceptors, and an increase in receptor field size in the dorsal horn can be detected (Cook et al.

1987; Woolf and King 1990). Peripheral sensitisation is defined as an increased responsiveness in the nociceptive neurones in the periphery, whereas central sensitisation is increased responsiveness of nociceptive neurones in the CNS to the normal or to subthreshold afferent input. It is within these spinal neurones that 'windup' (the progressive repeated stimulation of C fibres) occurs (Staud et al. 2003; Cuellar et al. 2005). All primary afferents release glutamate, and the three inotropic receptors for glutamate (N-methyl-D-aspartate (NMDA), $\alpha$-amino-3-hydroxy-5methyl-4-isoxazoleproprionic (AMPA) and kainate) are present in the dorsal horn. Windup can be considered a product of the temporal summation of NMDA and neurokinin-1 (NK-1) receptor-mediated cumulative depolarisations caused by primary afferent release of glutamate and the excitatory neuropeptide substance P respectively (Davies and Lodge 1987; Dickenson and Sullivan 1987a; Herrero et al. 2000). Temporal summation describes an increased perception of pain in response to repetitive painful stimuli.

Sensitisation is a neurophysiological construct that can only definitively be determined if both the neural input and output are known. Clinically, the term sensitisation is inferred in cases of reported allodynia (pain due to a stimulus that would not normally provoke pain) and hyperalgesia (increased pain from a stimulus that normally provokes pain). Both these terms do not infer a mechanism *per se* (IASP Task Force on Taxonomy 1994; Treede et al. 2015).

In inflammatory states, a large number of endogenous factors released in the vicinity of the free endings of the nociceptors can cause sensitisation (Herrero et al. 2000). The nociceptors express characteristic patterns of the different inflammatory

mediator receptors; G protein coupled receptors (GPCRs), ligand gated ion channels, tyrosine kinase or cytokine receptors in response to the inflammation. These indirect forms of chemotransducers known as metabotropic receptors operate more slowly (compared to the inotropic receptors), with the chemotransduction driven by an intracellular signalling cascade (Goudet et al. 2009). The commonest example of this metabotropic receptor is the GPCR which includes the μ, κ, and δ opioid receptors, which can be targeted for pain relief (Malfait and Miller 2016). There is plentiful evidence of chemicals being released from thermally and mechanically stimulated tissue too, making this an overlapping and complicated area of study that is being fervently researched. There is an ever increasing list of metabotropic receptors in the sensory neurones. These include receptors for cytokines, chemokines, and neurotrophins, albeit the number normally active is small, including those for bradykinin ($B_1$ and $B_2$) (Maurer et al. 2011), histamine ($H_1$) (Mobarakeh et al. 2000), ATP (Bodin and Burnstock 2001; Burnstock 2013), endothelin 1 (Gokin et al. 2001), proteases (PAR–2) (Rattenholl and Steinhoff 2003) and prostacyclin (IP) (Smith et al. 1998). This area of study is further complicated by the fact that a neurone can express ionotropic and metabotropic receptors for the same ligand (Nandigama et al. 2010).

### 1.2.3. Initiation of a generator potential

The resting potential of the nociceptive afferent is -40 mV, decreasing to between -50 and -75 mV at the cell body. The action potential threshold range is between -35mV and -55mV (Baccaglini and Hogan 1983). For the sensory information to be propagated to the CNS an action potential (AP) must be generated by membrane

depolarisation caused by the transduction process. This event is termed a generator potential (GP) and can be initiated in three main different ways. Firstly, via the opening of an ion channel which pushes the membrane potential above threshold and causes an AP that can be propagated towards the CNS. One such example of this is the TRPV1 channel which can be activated by both thermal and chemical stimuli (Caterina et al. 1997; Lumpkin and Caterina 2007). Secondly, the GP can be indirectly elicited by the closing of $K^+$ channels (potassium family subfamily K member 2 (TREK-1), potassium family subfamily K member 3 (TASK-1) and others) normally responsible for hyperpolarising currents. TREK-1 channels can be activated by mechanical stimulation, hydrogen ions and warm temperature demonstrating their polymodal behaviour (Honoré 2007). Thirdly, also indirectly, via activation of low threshold voltage gated ion channels that are in close association with ion channels capable of driving membrane depolarization (Liu et al. 2010).

Transduction at the nociceptor terminal results in a generator potential that travels passively to the spike initiation zone. At this point an action potential can be generated that is transmitted via nociceptive afferents.

### 1.2.4. Transmission of the action potential

Propagation of the action potential along the axon of the nociceptor is caused by the initial depolarisation that spreads in one direction as adjacent voltage gated sodium channels (VGSC) open. Primary sensory neurones exhibit a rich heterogenous morphology (Koerber et al. 1988) and the most common way to classify these neurones is based upon their speed of conduction. The Aα/β group

are myelinated axons with the fastest speed of conduction (33–75 m/s). The Aδ group are thinner myelinated fibres with an intermediate speed (5–30 m/s), and the C fibres are the smallest unmyelinated fibres with the slowest conducting speed (0.5–2 m/s). Most of the neurones conducting within the Aα/β group do not encode noxious stimuli but respond to innocuous mechanical stimuli and are classified as low threshold mechanoreceptors. The majority of the Aδ and C fibres do encode noxious stimuli and are classified as nociceptors.

### 1.2.5. Anatomy of the dorsal horn

The dorsal horn is divided into parallel laminae based on the packing density of the neurones (Figure 1.1)(Rexed 1952). The termination of the primary afferents in the dorsal horn laminae is determined by fibre diameter and receptive field modality (Wall 1967; Light and Perl 1979). Smaller diameter nociceptive afferents synapse with nociceptive specific (NS) cells in laminae I–II of the superficial dorsal horn, with a small number terminating deeper in the spinal cord. Swett and Woolf demonstrated an orderly somatotopic arrangement of the small afferent (Aδ and C) fibers from the hindlimb of the rat terminating in laminae I and II with arborisations to the deeper layers (Swett and Woolf 1985). Lamina I (marginal zone) neurones contribute to the spinothalamic and spinoreticular tracts both responsible for processing pain and temperature information (Craig and Kniffki 1985; Ikeda et al. 2003). Lamina II (substantia gelatinosa) is the major site for nociceptive inputs (C and Aδ fibres) arriving at the spinal cord (Swett and Woolf 1985) and in the main consists of densely packed unmyelinated interneurones (Woodbury et al. 2001). Lamina II has been shown to have explicit organization of modules of neurones that

differentially modify and transmit the afferent input from the Aδ and C fibres (Lu and Perl 2005). Lamina II central neurones, with C fibre input, were shown to excite lamina II vertical neurones with Aδ input. Lamina II outer vertical neurones with Aδ input excited lamina I neurones (Lu and Perl 2003). The function of this arrangement is postulated to be an ability of the afferent input to be amplified or modulated. The C fibres can be divided into two groups, based on phenotype and sensitivity to neurotrophins, although functionally the differentiation is less clear. One group is sensitive to nerve growth factor (NGF) and usually contain substance P, calcitonin gene related peptide (CGRP) and galanin (peptidergic fibres); the second group is distinguished by binding sites for lectin isolectin B4 and usually possess the purinergic receptors $P2X_3$ and $P2Y_1$ (non-peptigergic fibres) (Zhang et al. 1993, 1995; Molliver et al. 1995). The central projections of these two populations of C fibres differ in their arborisation, the peptidergic C fibres branching in lamina I and the outer portion of lamina II with a small number extending to deeper terminals. The non-peptidergic fibres project to the central portion of lamina II (Saeed and Ribeiro-da-Silva 2012). The non-peptidergic C fibres are chiefly associated with the superficial layers of the skin (Taylor et al. 2009), whereas peptidergic fibres tend to innervate other tissues in addition to the deeper layers of the skin (Perry and Lawson 1998).

Laminae III-VI are known as the deep dorsal horn, and receive less nociceptive input. Transmission of innocuous stimuli is predominantly through large diameter, myelinated Aβ fibres which terminate in laminae III–VI, hence within these laminae are proprioceptive neurones responding exclusively to touch (Light and Perl 1979). In addition to the NS and the proprioceptive neurones, a third class of spinal neurone

is described, known as wide dynamic range (WDR). The WDR neurones can receive noxious or non-noxious input from Aδ, Aβ or C fibres and respond in a graded manner (i.e. frequency of action potentials) from low through to high threshold noxious input (Figure 1.1). The WDR neurones (also known as lamina V type, trigger type, Class 2, multireceptive or convergent neurones) are found in superficial layers but more so in lamina V and include interneurones involved in the polysynaptic reflexes and projection neurones (Le Bars and Cadden 2008). For Aδ fibres terminating in lamina V, the interneurones they synapse with decussate and ascend in the spinothalamic tract, and those terminating more superficially will synapse with interneurones forming the spinoparabrachial tract (Figure 1.1). The ventral and intermediate laminae are less important in terms of inputs from primary afferents. Laminae VII and X receive a proportion of nociceptive input that is mainly visceral in origin (Sugiura et al. 1989; Olivar et al. 2000). Muscle afferents feed into laminae V, VI and VII.

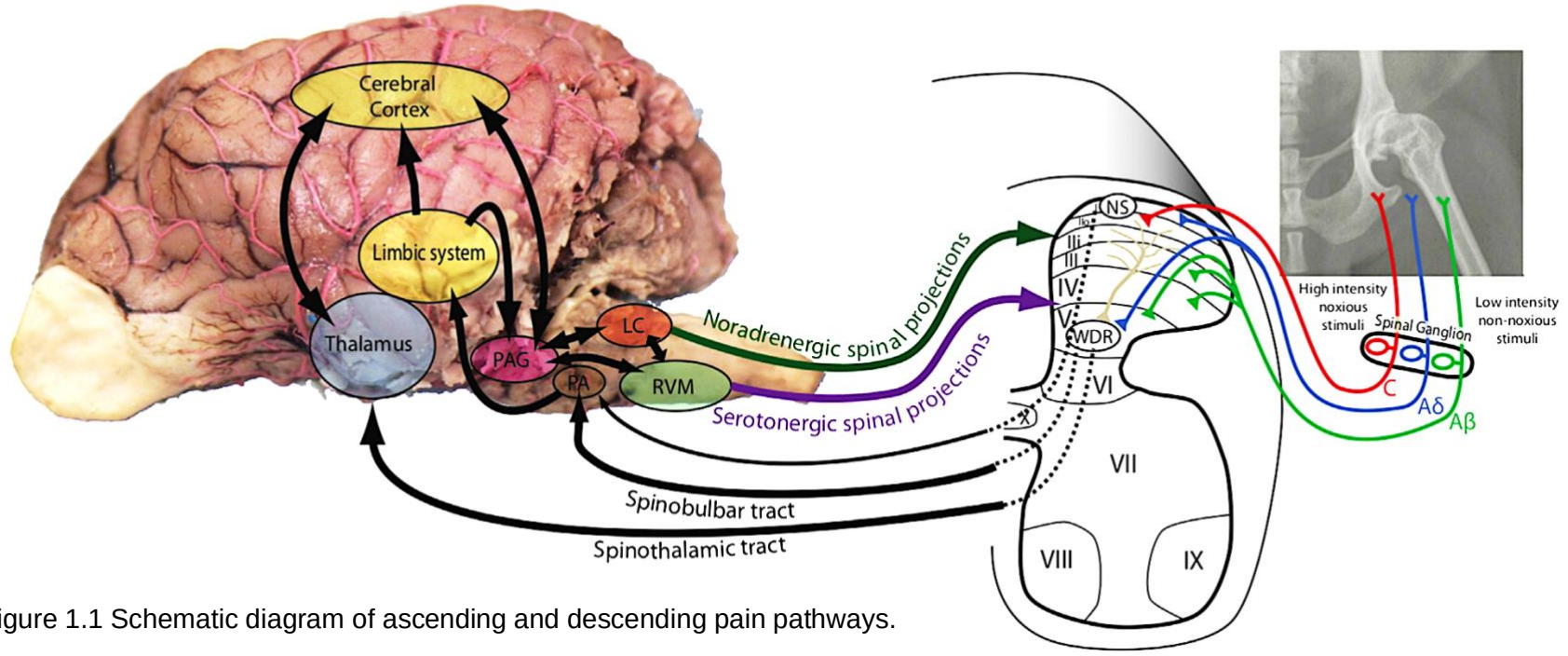



Figure 1.1 Schematic diagram of ascending and descending pain pathways.

Primary afferent fibres (Aβ, Aδ, and C fibres) transmit impulses from the periphery to the dorsal horn of the spinal cord. Secondary nociceptive specific (NS) cells are predominantly located in the superficial dorsal horn (laminae I–II) projecting to the parabrachial area (PA), with most wide dynamic range (WDR) neurones located in the deeper dorsal horn (e.g. lamina V). Lamina V neurones project to the thalamus (via the spinothalamic tract) where there are subsequent neuronal projections to various cortical regions which form part of a 'pain matrix' (primary and secondary somatosensory, insular, anterior cingulate, and prefrontal cortices).

PAG: periaqueductal grey, DRG: dorsal root ganglion, RVM: rostroventromedial medulla, LC: locus coeruleus, LS: limbic system, NS: nociceptive specific cells, PA: parabrachial area, WDR: wide dynamic range neurones.

The primary afferents form excitatory synapses with either WDR projection cells (convey information to various parts of the brain) or interneurones (responsible for local circuitry within the spinal cord, an example is represented in Figure 1.1. connecting the WDR neurones of lamina V with the superficial dorsal horn). All primary afferents use glutamate as their primary excitatory transmitter although arrangements do differ. Non-peptidergic C fibres and Aδ hair-follicle afferents can form the central axons of a synaptic glomerulus. A synaptic glomerulus is a complex structure consisting of a central axonal bouton (of primary afferent origin) that is in synaptic contact with dendrites and peripheral axons. The nonpeptidergic C fibres and Aδ hair-follicle can be arranged such that they are presynaptic to several dendrites, or postsynaptic at axo-axonic synapses (Ribeiro-Da-Silva and Coimbra 1982). The central terminals of Aβ afferents and Aδ nociceptors have a simpler synaptic arrangement. In contrast, the peptidergic primary afferents have relatively few axoaxonic synapses (Ribeiro-da-silva et al. 1989).

Interneurones can be classified as either excitatory (predominantly glutamatergic) or inhibitory (mainly γ-aminobutyric acid (GABA) or glycinergic). The morphology of the interneurones has been most closely studied in lamina II, with a classification scheme that includes islet, central, radial and vertical interneurones (Grudt and Perl 2002; Lu and Perl 2005; Maxwell et al. 2007; Yasaka et al. 2010). The identity and morphology of the glutamatergic axons was greatly facilitated by the discovery of vesicular glutamate transporters (Naito and Ueda 1983).

An alternative approach to classification is to use immunocytochemistry and stratify the interneurones based on neuropeptide and protein localisation (Todd and Spike

1993; Todd 2010). A large number of neuropeptides have been identified, and consequently several neuropeptide receptors expressed by the interneurones including NK1 and NK3 tachykinin receptors, μ opioid receptor 1 (MOR-1), somatostatin receptor $sst_{2A}$, and the NPY receptor Y1 are being studied. It is not possible to use the neuropeptides to definitively define populations of interneurones, although some markers show a restricted distribution. The dorsal horn also receives input from axons descending from various parts of the brain (Figure 1.1). The $GABA_A$ and glycine receptors are distributed throughout the spinal cord and most likely expressed on all dorsal horn neurones. Many studies do not distinguish between the interneurones and the projection neurones when describing receptors for glutamate, GABA and glycine. GABA is present in ~25%, 30% and 40% of neurones in laminae I, II and III, respectively (Polgár et al. 2003). Spinal cord wiring is complex and little understood, the main spinal signaling pathways in the rodent are comprehensively reviewed elsewhere (Chédotal 2019).

### 1.2.6. Anatomy of the ascending pathways

It is within the spinal cord that substantial transformation and modulation of the nociceptive signal can occur before it ascends to higher centres (Kayalioglu 2009), due to the discrete populations of intrinsic interneurones that can alter responses of NS and WDR neurones, with astrocytes and microglia also modulating, particularly in disease states (Hains and Waxman, 2006; Scholz and Woolf, 2007; Ji et al., 2013). Superficially in lamina I of the dorsal horn there is a large population of projection neurones which preferentially express the neurokinin (NK1) receptor for substance P (Doyle and Hunt 1999; Todd 2002), thereby are nociceptive neurones.

These neurones project to the periaqueductal grey (PAG), thalamus and particularly the parabrachial area (PA). These cells also project to the rostral ventral medualla (RVM) and can influence descending controls (Suzuki et al. 2002; Gauriau and Bernard 2010).

The ascending tracts are usually defined according to where they terminate in the brain (Dostrovsky and Craig 2013). Briefly, the spinothalamic tract (terminating in the thalamus) integrates the thalamic traffic (and other signals) and is responsible for the discriminative/localisation component of pain via projections to the sensorimotor cortex, insular cortex and the anterior cingulate originating mainly from laminae I, IV, V and X (Dum et al. 2009; Sengul and Watson 2014). The other major ascending partner tract is the spinobulbar tract (terminating in both the hindbrain and midbrain regions associated with pain processing) and this conveys the affective/intensity component, and projects to the amygdala and hypothalamus via the parabrachial nucleus (Craig 2003). As mentioned above, this spinobulbar pathway can influence and recruit descending pathways via the PAG, pontine locus coeruleus (LC) and RVM, thereby dictating the output passing through the spinal cord (Benarroch 2008; Waters and Lumb 2008) (Figure 1.1).

### 1.2.7. Anatomy of the descending pathways

The PAG is one of the most important structures associated with the descending pain control system (Behbehani 1995; Keay and Bandler 2015) and assimilates information from the somatosensory and cingulate cortices, the thalamus, amygdala and hypothalamus as well as directly receiving nociceptive input from the ascending

pathways. However, although there is evidence for the PAG having direct projections to the spinal cord (Mantyh and Peschanski 1982), spinal analgesia following its stimulation is considered to be due to its projections to the nucleus raphe magnus (NRM) and neighbouring structures of the RVM (Vanegas and Schaible, 2004; Heinricher et al., 2009). In general, the descending pathways ascendancy can be considered to originate at the periaqueductal grey-rostral ventromedial medulla (PAG-RVM) and the ventrolateral medulla (VLM) (Basbaum and Fields 1979). Within the spinal cord the descending inhibitory influences are arranged in the dorsolateral funiculi with the facilitatory influences tending to be centred in the ventral/ventrolateral cord (Zhuo & Gebhart 1997; Zhuo & Gebhart 1990). The PAG-RVM exerts a degree of selective inhibition of C fibre mediated nociceptive impulses, but preserves A fibre messages coding sensory and discriminatory information (Lu et al., 2004; McMullan and Lumb, 2006a; Heinricher et al., 2009) and the RVM can be considered the final relay point through which facilitation or inhibition of the nociceptive message passes (Villanueva and Le Bars, 1995; Calejesan et al., 2000).

The degree of inhibition or facilitation of the pain signals in some part is controlled in the RVM by at least two different types of neurones known as ON cells and OFF cells and these cells are inextricably connected to the higher brain centres involved in a large number of emotions, psychological states, stresses, and pathologies. Briefly, the ON, OFF and neutral cells are characterized by their response to nociceptive input (Fields et al. 1983). The ON cells show a burst of activity prior to nociceptive input and are considered a component of excitatory drive. The OFF cells show a short lived decrease in firing rate before nociceptive input and are

considered inhibitory. Neutral cells do not respond to nociceptive input. The role of these cells may be more complicated than previously thought (Cleary et al. 2008; Lau and Vaughan 2014; Salas et al. 2016). Despite the overwhelming evidence for the major role of the RVM as a relay station, it is without doubt that the descending pathways also require a forebrain loop (Millan 2002). There is also evidence for anterior cingulate cortex (ACC) projections regulating spinal neurones (Gu et al. 2015; Kang et al. 2015) and being able to selectively modulate the pain experience. The ACC is involved in the processing and modulation of pain affect, and offers a further target for manipulating the pain signature. Understanding the role the ACC plays requires more sophisticated paradigms rather than relying primarily on the reflexive behaviour from an aversive stimulus. The role of the ACC and pain processing is comprehensively described in a recent review article (Fuchs et al. 2014).

The dorsolateral and lateral sector of the midbrain PAG has differential downstream effects on the nociceptive reflexes evoked by activity in both the unmyelinated (C fibre) and the myelinated (A fibre) nociceptors (McMullan and Lumb 2006a,b). This differential control of nociceptive reflexes is also seen following RVM stimulation (Lu et al. 2004) demonstrating that these descending endogenous systems have A and C fibre specificity offering further complexity but nonetheless attractive targets for analgesia interventions (Waters and Lumb 2008). In the mature adult rat, descending inhibition is targeted to spinal neurones with a strong afferent C fibre input. However, in the first few weeks postnatally the system is controlled differently, with greater descending facilitation particularly targeted to the A fibre input (Koch and Fitzgerald 2014). The evolutionary reason proposed for this A fibre input is to

provide the dorsal horn with low level, non-noxious, tactile input thereby promoting a development of the animal's sensory networks. The switch from facilitation to inhibition as the animal matures is primarily dependent on endogenous opioid levels in the RVM (Hathway et al. 2009) with GABA and endocannabinoid levels also playing a role (Hathway et al. 2012; Li et al. 2015). It is possible that immature nervous systems could be at risk of excessive sensory overload and peripheral injury in the first few weeks of life where facilitation is favoured (Schwaller et al. 2016).

In summary, this balance of inhibition and facilitation of descending pathways is a dynamic product of the afferent evoked activity, the age of the animal, but also the excitability of the dorsal horn cell too (Koch and Fitzgerald 2014). Notwithstanding the anatomical arrangement, the descending pathways appear to lack specificity, influencing all portions of the spinal cord and it is this widespread and intrinsic influence that is responsible for the ability to facilitate or inhibit transmission. Indeed, this widespread arrangement also results in not only pain perception being affected but other senses too, potentially explaining why some chronic pain states have clinical signs distinct from pain alone but can manifest with debilitating effects on sleep and other emotions (Tracey 2010; Zhuo 2016). Moreover, the perception of pain is sensitive to many mental processes therefore not exclusively driven by or maintained by the noxious input (Wiech et al. 2008); heightened anxiety and fear for example can then often exacerbate the suffering of pain (Wiech and Tracey 2009).

### 1.2.8. The pharmacology of the descending controls

The major descending pathways use the monoamines noradrenaline and 5-hydroxytryptamine (5-HT; serotonin) as transmitters (Millan 2002; Bannister and Dickenson 2016a). These bidirectional monoaminergic systems exert a complex modulating role over the outputs of the dorsal horn neurones and the current understanding is that descending control is overwhelmingly determined by noradrenaline and serotonin activity. The inhibition or facilitation have been shown to be mediated by distinctly different receptors (Zhuo & Gebhart 1990; Zhuo & Gebhart 1990; Zhuo & Gebhart 1991), and the balance of power between the systems is determined by the type of noxious input and the response to it. The vast multiplicity of the transmitter pharmacology involved in the descending controls is comprehensively covered elsewhere (Millan 2002).

*1.2.8.1. Noradrenaline*

Noradrenaline was discovered in the brain in the 1950s. It is the major neurotransmitter released by sympathetic postganglionic nerve fibres and is involved in autonomic regulation of numerous organs. The noradrenergic cell groups were mapped in 1964, and are prefixed by the letter A (for aminergic) (Dahlstroem and Fuxe 1964). Areas A1 to A7 inclusive contain noradrenaline; areas A8 to A14 contain dopamine. Group A1 is located in the caudal ventrolateral part of the medulla and is responsible for aspects of body fluid metabolism (Antunes-Rodrigues et al. 2004), group A2 plays a role in stress and food intake and is located in the solitary nucleus (Rinaman 2011). Groups A5 (close to the superior olivary complex) and A7 (in the pontine reticular formation) project to the spinal cord (Felten and Sladek 1983).

Noradrenaline plays a major role in modulating nociception (Pertovaara 2006, 2013; Gyires et al. 2009); the majority of descending projections originating from the pontine nucleus LC are noradrenergic (Areas A6 and adjoining A4), and microstimulation of this nucleus was shown to produce antinociceptive effects via spinal $\alpha$2 adrenoceptors (Jones and Gebhart 1986). The LC is small in absolute terms but projects to all parts of the brain and spinal cord (Sara and Bouret 2012). In healthy subjects the noradrenergic system serves to regulate pain thresholds, with its role becoming more prominent in cases of injury or inflammation (Pertovaara 2013). Noradrenaline released from the LC is also involved in the regulation of vigilance, attention, and cognitive functions but it is still not possible to surmise on the net effect of the noradrenergic system on supraspinal structures; evidence exists for both anti-nociceptive and pro-nociceptive actions (Llorca-Torralba et al. 2016). However, in the main, the central noradrenergic system inhibits pain, and drugs acting on the $\alpha$2 adrenoceptor, alone (or as an adjuvant) have proved effective to varying degrees as analgesics. In humans topically applied $\alpha$2 agonists may have a role in treating neuropathies (Wrzosek et al. 2015) and intra-articular administration has reduced postoperative knee pain (Al-Metwalli et al. 2008). Patients with intractable cancer pain have benefitted from intrathecal administration of $\alpha$2 agonists (Eisenach et al. 1995). The $\alpha$2 agonists are widely utilised in wild, domestic and laboratory animals for sedation, analgesia and muscle relaxation. Continuous rate infusions of the $\alpha$2 agonists dexmedetomidine and medetomidine can also be useful as a component of multimodal analgesic regimens during anaesthesia in horses, dogs and cats (Ansah et al., 2000; Murrell and Hellebrekers, 2005; Ringer et al., 2007; Valtolina et al., 2009; Kalchofner et al., 2009). In general,

spinally administered $\alpha$2 adrenoceptor agonists have an enhanced antinociceptive potency in animal models with a persistent injury (Yaksh et al. 1995; Mansikka et al. 1996; Xu et al. 1999), and what is becoming clear is that the intensity, duration and type of noxious injury will ultimately determine the noradrenergic response (Poree et al., 1998; Kingery et al., 2000; Malmberg et al., 2001; Lähdesmäki et al., 2003; Mansikka et al., 2004). Likewise, abnormal noradrenergic activity (for example a reduction in the inhibitory influences) can contribute to the hypersensitivity seen in models of both acute inflammation and nerve injury (Green et al. 1998; Xu et al. 1999; Rahman et al. 2008; De Felice et al. 2011) hence the interest in the noradrenergic system as a target for analgesics. This central role is further evidenced by studies that have demonstrated restoration of diminished noradrenergic control after use of a selective noradrenaline reuptake inhibitor following the development of a neuropathic pain phenotype (Hughes et al. 2015). In these experiments, chronic intrathecal reboxetine alleviated the evoked hypersensitivity produced by tibial nerve transection.

*1.2.8.2. Serotonin*

Serotonin has a diverse and widespread distribution throughout the body and even though most serotonin is located outside of the CNS, it is commonly considered one of the most important neurotransmitters. Studies have shown that approximately 20% of the neurones in the RVM are serotonergic, and there was early evidence for serotonin involvement in descending modulation (Le Bars 1988). These descending pain modulatory pathways arising from the RVM exert a bidirectional influence upon nociception through activation of different serotonergic receptors in the spinal cord

(Dogrul et al. 2009). Both acute and chronic noxious stimuli can activate these RVM 5-HT neurones and increase the expression of 5-HT receptors in the spinal cord (Zhang et al. 2000; Cai et al. 2014). The 5-HT neurones can influence nociceptive processing directly but also indirectly by influencing other non-serotonergic neurones involved in the descending pathways. The collaterals of the 5-HT neurones have been shown to regulate the bidirectional control from the aforementioned ON and OFF cells in the RVM (Braz and Basbaum 2008). Unlike the adrenergic system which is largely considered a pain inhibitory system the serotonergic system is more complex with pain inhibition or facilitation attributable to different subtypes of 5-HT receptors (Suzuki et al. 2004; Dogrul et al. 2009; Viguier et al. 2013). Spinal 5-HT2 and 5-HT3 receptors are considered facilitatory and 5-HT1A , 5-HT2A and 5-HT7 receptors classified as inhibitory. On balance, it would seem the serotonergic facilitation assumes more influence than the inhibition. It is also now known that tonic activation of 5-HT (and non 5-HT) mediated brainstem facilitatory influences is one of a multitude of contributors to the development and maintenance of central sensitisation in sustained pain states (Urban and Gebhart 1999; Porreca et al. 2002).

*1.2.8.3. Dopamine*

The monoamine dopamine also plays a role in nociception and pain (Bannister and Dickenson 2016a) with mesolimbic, mesocortical and nigrostriatal dopaminergic pathways identified as capable of inhibiting nociception, primarily its affective component (Magnusson and Fisher 2000; Gao et al. 2001) more by influences in the brain rather than direct spinal projections *per se*. The dopamine system is

embedded in the pain matrix circuitry (Wood 2008) but also features importantly in the movement system (Cenci 2007) and in areas of the brain associated with reward (Baik 2013), learning and cognition (Robbins and Arnsten 2009; Werlen and Jones 2015). Studies have shown that dopamine therapies may offer promise as analgesics (Evans et al. 2008; Park et al. 2016) and can play a role in descending modulation of sensory processing too (Garcá-Ramrez et al. 2014) but efforts to develop dopamine based therapies are complicated by its intrinsic role in locomotion, learning and reward.

*1.2.8.4. Opioids*

The descending modulatory pathways can also be considered components of an opioid sensitive matrix. Many of the drugs interacting with components of the descending pathways influence or mimic the production of endogenously produced opioids, and there is evidence of a bulbospinal opioidergic pathway (Gjerstad et al. 2000). Numerous animals studies have shown that the PAG is one of the major sources of opioid mediated inhibition of the ascending nociceptive impulses (Waters and Lumb 1997). The PAG receives cortical inputs mediating a 'top down' endogenous pain inhibition system, and these projections from the PAG extend to the RVM and noradrenergic pontine nuclei which in turn modulate nociceptive input at the spinal cord through the aforementioned release of noradrenaline and serotonin. It is likely that the communication from the PAG to these noradrenergic and serotoninergic fibres can be both direct but also invoked through intermediate relays (Odeh and Antal 2001). Different regions of the PAG have different functions; with respect to the descending control of pain, the dorsal-dorsolateral portions of

the PAG play a role in stress-induced analgesia, which is independent of opioids but depends on endocannabinoids (see below); the lateral-ventrolateral portions of the PAG are involved in opioid analgesia and analgesia induced by non-steroidal anti-inflammatory drugs (NSAIDs) (Vanegas et al. 2010).

One example of this 'top down' modulating pathway is the placebo effect – an analgesic construct that can be elicited in certain people that has its mechanisms firmly rooted, but likely not limited to activation of the μ opioid receptor and changes in blood flow to these areas; particularly the rostral anterior and pregenual cingulate cortices, the dorsolateral prefrontal cortex and anterior insular cortex (Zubieta et al. 2005; Petrovic et al. 2010). In the case of placebo it is now apparent that other non-opioidergic mechanisms are also likely recruited since expectation, reward, learning and memory play a part in this response, and the once simplistic view that the μ opioid receptor was solely responsible has been superseded by a complex web of interwoven processes (Eippert et al. 2009). The recent finding that opioidergic descending pathways are also players in potential interventions such as distraction and hypnosis, offers potential for developing further analgesic interventions. Functional magnetic resonance imaging (fMRI) studies have shown (particularly in placebo) that the descending pathways are the conduits through which the cognitive influences affect a pain experience. Nocebo studies (defined as studies where the negative expectations of a treatment have a more negative effect than it otherwise would have) also have their mechanisms rooted the descending opioidergic controls. This experience is also subsequently modulated by the individual's control and on-going response to the pain experience exerted by pre-frontal and limbic brain regions (Wiech et al. 2008; Krummenacher et al. 2010). The exact mechanisms

involved in the descending controls are not yet fully understood. There is a consensus on their importance, and research into their endogenous modulating attributes is a worthwhile ambition that may result in useful patient interventions.

*1.2.8.5. Endocannabinoids*

There is also widespread evidence of endocannabinoid involvement in areas involved in the processing of nociceptive inputs (Jenkins et al. 2004). The expression of the endocannabinoid receptors, presence of ligands and metabolites is dynamic and dependent on the type of pain being expressed. The endocannabinoid receptors offer potentially attractive targets for analgesic opportunities (Jhaveri et al. 2007; Sagar et al. 2009) but are also involved in motor function, cognition, and many signalling pathways, making isolation of the analgesic properties of molecules without psychoactive side effects a challenging conundrum. It has been shown that analgesia produced by NSAIDs in the descending pain control system also requires an activation of the cannabinoid 1 receptor (Vanegas et al. 2010). Furthermore, numerous experiments suggest that opioids, NSAIDs and the cannabinoids in the PAG and RVM interact to potentially decrease GABA-ergic inhibition and thus enhance the descending flow of impulses that inhibit pain as a mechanism of analgesia (Tham et al., 2005; Guindon et al., 2006; Guindon and Hohmann, 2009). There is also evidence that the endocannabinoid system can express adaptive changes in the face of persistent pain states such as osteoarthritis (Sagar et al. 2010) highlighting one of the many potential systems to target in the face of spinal hyperexcitability. However, to date the translation of promising results with the analgesia afforded by cannabinoid drugs in the laboratory, to humans, is

seemingly hampered by the likelihood that the drugs so far trialled modulate the affective component but not the sensory component of the pain (Lötsch et al. 2018).

*1.2.8.6. GABA and glycine*

Electrophysiological studies have also highlighted a major role for GABA and glycine as inhibitory transmitters through their ability to influence descending activity via their presence within interneurones (McLaughlin et al. 1975; Harvey et al. 2004; Harvey and Rigo 2010). Projections from the PAG also synapse with GABAergic spinopetal neurones (Vanegas and Schaible 2004). These GABAergic or glycinergic projections can inhibit noxious inputs into the dorsal horn of the spinal cord, and recent elegant tracer studies have identified that the neurones can express either or both neurotransmitters (Hossaini et al. 2012). Their role in modulating the noxious input is without doubt, but parsing out the exact role of each system and how they interact remains unfinished. Studies have also demonstrated that the ON, OFF and neutral cells have varying expression of GABA and glycine, but globally the cell populations and expression is determined by the context of the pain experience; for example in peripheral inflammation the gene expression and the phenotype of the ON and OFF cells was altered (Miki et al. 2002). This study demonstrated during continuous neuronal recordings (3–6.5 h), a phenotypic switch of RVM neurones during the development of inflammation. This was characterised by an increase in the percentage of ON and OFF cells and a reduction in the percentage of neutral cells, and this phenotypic change was mediated via NMDA receptor activation in response to the inflammation (Miki et al. 2002).

### 1.2.9. Tonic control

In the absence of nociceptive input spinal nociceptive neurones are under both tonic and stimulus evoked (phasic) descending controls (Sandkuhler et al. 1987; Gilbert and Franklin 2001). Some of the evidence for the tonic control comes from studies investigating the development of long term potentiation (LTP) as a model for central sensitisation. In anaesthetised rodents LTP can be induced by stimulating the spinal dorsal horn neurones with high intensity electrical pulses and the effect is manifest as an increased postsynaptic response to a single stimulus applied to the afferent once a minute. The stimulus used is not representative of a natural noxious stimulus, and indeed it is very challenging to elicit LTP in natural circumstances. However, in animals that have undergone rostral spinalisation, LTP could be induced with natural noxious stimuli such as pinch or intense heat. In these cases this is allied to primary hyperalgesia and illustrates that spinalisation has removed the tonic descending inhibitory control (Sandkühler and Liu 1998). The structures responsible for tonic descending inhibition can be probed by destroying the descending serotonergic neurones of the NRM and comparing the paw inflammation and withdrawal responses in lesioned rats to the normal rats; hyperalgesia was more marked in the rats lesioned 4-14 days earlier (Ren and Dubner 1996). Lesions in the LC also result in an increased hyperalgesia caused by carrageenan compared to non-lesioned rats confirming again the loss of tonic descending control (Tsuruoka et al. 2004). Characterisation of the receptors involved in the tonic control mechanisms has also been shown through many elegant experiments using antagonists (Soja and Sinclair 1983; Rivot et al. 1987; Clarke and Harris 2004). One important caveat to these experiments involving discrete lesions and anaesthesia is

they may inadvertently overlook the contribution of the plasticity of the spinal cord in response to inflammation or indeed the differences in the free ranging conscious animal compared to anaesthetised animal, and these should be considered when drawing conclusions.

### 1.2.10. The spino-bulbo-spinal loop

A pain experience consists of both a somatosensory component and a psychological, affective component. The term nociception refers to the neural activity in the PNS and CNS caused by a painful stimulus, and the term pain itself is used to describe both this and the emotional and autonomic responses to the insult. These different components of pain are processed in separate, discrete areas of the brain. In most cases the nociceptive insult is the cause of pain, but this insult may be absent and its magnitude is not linearly related to the pain that is reported or behaviours that are displayed (Loeser and Treede 2008). This is, in part, a consequence of a feedback loop between the brain and spinal cord. This spino-bulbo-spinal loop can alter the extent to which pain signals are amplified or inhibited within the spinal cord.

### 1.2.11. Conditioned pain modulation (CPM) and Diffuse Noxious Inhibitory Controls (DNIC)

Normally in healthy naïve animals and humans, a conditioning noxious stimulus will attenuate the response to a test stimulus applied to a remote (extra segmental) body area, a phenomenon termed diffuse noxious inhibitory controls (DNIC) (Le Bars et

al. 1979b) or ‘pain inhibiting pain’. DNIC are powerful, long-lasting controls which inhibit spinal as well as trigeminal nociceptive neurones (Dickenson et al. 1980). DNIC operate via a spinobulbospinal loop with the afferent and efferent pathways coursing through the ventrolateral quadrant and the dorsolateral funiculus of the spinal cord (Figure 1.2) (Villanueva and Le Bars 1995). The neural pathways responsible for DNIC appear to be partially distinct from the aforementioned descending pathways; the spinoparabrachial and the spinoreticular pathways activate DNIC (Villanueva et al. 1986; Lapirot et al. 2009) whereas the NK1 neurones projecting to the parabrachial area (responsible for recruiting both pontospinal, and bulbospinal pathways) are key for the descending controls (Suzuki et al. 2002). Early experiments have demonstrated that sectioning the spinal cord abolishes DNIC, with supraspinal areas such as the caudal medulla and the subnucleus reticularis dorsalis (SRD) involved (Bouhassira et al. 1992) and with the spinoparabrachial and the hypothalamic dopaminergic descending pathways facilitating the ascending and descending components of the loop (Figure 1.2) (Lapirot et al. 2009, 2011). The monoamines play pivotal roles in DNIC and offer potentially attractive targets for interventions (Le Bars et al. 1979b; Bannister et al. 2015; Harris 2016).

What has become apparent across many different persistent painful conditions (migraine, irritable bowel disease, and idiopathic pain states) is that some humans have been shown to have impaired DNIC (Yarnitsky 2010) thereby suggesting that a DNIC paradigm could be used in prediction of chronic pain susceptibility (van Wijk and Veldhuijzen 2010). As previously mentioned the descending pathways consist of inhibitory and facilitatory controls; experimentally these opposing pathways can

be studied, but in a patient, currently it is only possible to infer the aggregate of the descending control, and so the DNIC terminology has been superseded by Conditioned Pain Modulation (CPM) for humans. CPM describes the phenomenon by which a conditioning stimulus affects the test stimulus, and can be further subdivided into non painful, inhibitory and facilitatory CPM (Yarnitsky et al. 2010). Employing CPM tools in human pain research is still in its infancy, but reliability and consensus on testing is available (Nir and Yarnitsky 2015; Yarnitsky et al. 2015). For both client owned animals and laboratory animals, there are only a very small number of studies published evaluating DNIC, as the methodology is defined and refined in view of the fact that sedation or anaesthesia must be used because of the necessity to apply a potentially aversive stimulus (Hunt et al. 2016; White et al. 2017).

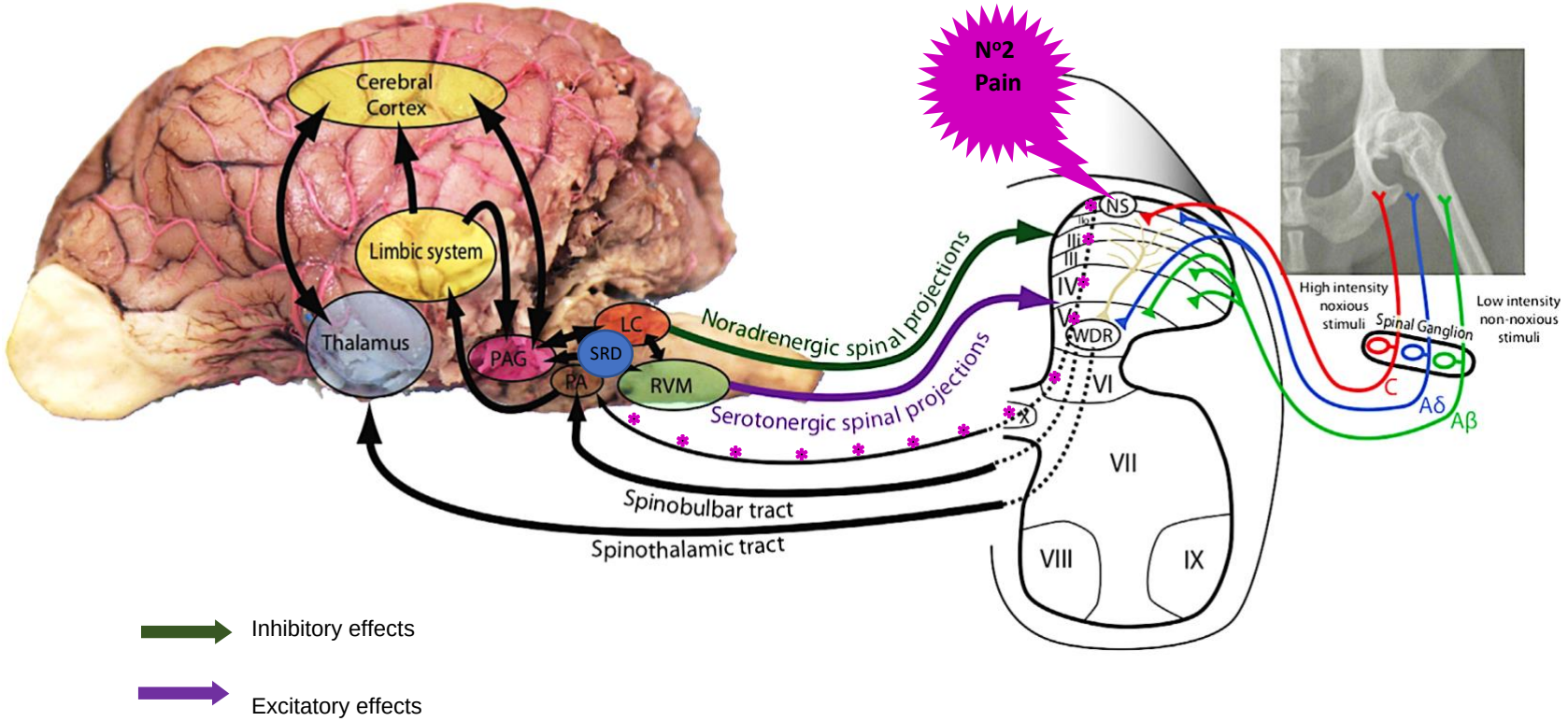



Figure 1.2 Activation of descending diffuse noxious inhibitory controls (DNIC) results in pain modulation.

The drive originates in the parabrachial area (PA) from the superficial dorsal horn (NK-1 expressing neurones; pink asterisks) labelled NS (nociceptive specific) activated by Aδ and C fibres. DNIC are mediated by ascending pathways in the ventrolateral quadrant of the spinal cord (spinoreticular tracts). DNIC involve brain structures confined but not limited to the caudal most part of the medulla, in particular the subnucleus reticularis dorsalis (SRD). The descending pathways (noradrenergic and serotoninergic) course in the dorsolateral funiculi. DNIC activation results in wide dynamic range neurone hyperpolarisation. PAG: periaqueductal grey, DRG: dorsal root ganglion, RVM: rostroventromedial medulla, LC: locus coeruleus, LS: limbic system, NS: nociceptive specific cells, PA: parabrachial area, WDR: wide dynamic range neurones.

## 1.3. THE MECHANISMS OF GENERAL ANAESTHESIA

General anaesthesia is usually produced by gaseous or injectable regimens or a combination of both; and can rely on a single drug to produce loss of consciousness, reflex suppression, muscle relaxation, amnesia or multiple agents, each contributing to anaesthesia. Analgesia is often supplied in addition to the anaesthetic agents, since most volatile and many injectable induction agents lack antinociceptive properties.

General anaesthesia alters the neural responses in many areas of the CNS and in the PNS especially with respect to reflex responses. To date the search for a unitary or simple mechanism for general anaesthesia has failed (Kopp Lugli et al. 2009). At the beginning of the 19$^{th}$ century, Overton and Meyer identified a strong correlation between the potency of an anaesthetic and its solubility in olive oil. The correlation suggested a common unitary mechanism and it was proposed that the lipid solubility held the key to the functional mechanism of anaesthetics. However subsequent experiments highlighted that the explanation was not so simple, as compounds capable of inducing anaesthesia were discovered that failed to follow the correlation line (Fang et al. 1996; Koblin et al. 2006), and size and stereoselectivity of molecules challenged the theory (Lysko et al. 1994; Dickinson et al. 2000; Raines and Miller 2006).

A change of emphasis then ensued with proteins (ion channels) becoming a more plausible target (Franks and Lieb 1994). However, with the human genome able to code for between 20,000 and 21,000 proteins (Pennisi 2007) a reductionist

approach was necessary to target likely protein candidates. The criteria used were as follows: (1) anaesthetics must produce a reversible effect at a functional site with clinically relevant concentrations; (2) a functional site must be situated at a plausible anatomical location to mediate the specific behavioural effects of an anaesthetic; (3) stereoselectivity of anaesthetic effects *in vivo* should duplicate the stereoselective effects observed *in vitro*; (4) a functional site should be insensitive to the effects of non-immobilisers (Hemmings et al. 2005). The main contenders for mediating the anaesthetic action are $GABA_A$ receptors, glycine receptors, nicotinic cholinergic receptors, glutamate receptors, voltage-gated potassium channels, ATP-sensitive potassium channels, background potassium channels, adenosine receptors and 5-HT receptors (Sonner et al. 2003). Clues to the potential sites of anaesthetic action can be gleaned from those drugs that produce anaesthesia by acting primarily on a single signaling system for example, propofol, alfaxalone, and etomidate which enhance the $GABA_A$ receptor (Table 1.2) (Krasowski et al. 2001). Implicating proteins as a site for anaesthetic action is based upon the hypothesis they contain a specific binding site within their folded structure that can mediate a functional interaction with the anaesthetic. However, the variety of chemical structures able to induce immobility, amnesia and loss of consciousness challenges such functional specificity. It is possible that the anaesthetic drugs wield their effects by controlling gaps or pockets within the proteins (Eckenhoff 2001). These proteins retain lipophilic domains, so the relevance of the Meyer Overton hypothesis persists (Franks and Lieb 1994).

Table 1.2 Effects of anaesthetics on ion channels or receptor targets.

| **Drug** | **$GABA_A$** | **$K_{2P}$ channel** | **Glycine** | **NMDA** |
|---|---|---|---|---|
| Barbiturates | + | 0 | + | - |
| Propofol | + | 0 | + | - |
| Alfaxalone | + | 0 | 0 | 0 |
| Benzodiazepines | + | 0 | - | 0 |
| Etomidate | + | 0 | + | 0 |
| Ketamine | 0 | 0 | 0 | - |
| Sevoflurane, ether, isoflurane, desflurane, halothane | + | + | + | - |
| Xenon | 0 | + | + | - |
| Nitrous oxide | 0 | + | + | - |

- inhibitory effect; + potentiating effect; 0 no effect. $GABA_A$: γ-aminobutyric acid A receptor, $K_{2P}$ channel: two-pore potassium channel, NMDA: N-methyl-D-aspartate.

The lack of hypotheses for the mechanisms of anaesthesia is not for want of trying but a symptom of the complexity of the problem. Indeed, the definition of general anaesthesia itself is still debated because of the unknown mechanisms. The target sites range from the membrane proteins in the lipid bilayer to neurones and dendrites, glial cells, myocytes, immune and endocrine cells. There is no homogeneity or common substructure that yet explains anaesthetic action. The periodic table of anaesthetics with the addition of the drugs used in veterinary and laboratory anaesthesia demonstrates this construct (Figure 1.3) (Urban 2002).

| ELG | ILG | | | | | | | | | | | INR | JUN | VLG | MIS | ENDO | Inert |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $5HT_3$ | | | | | | | | | | | | | | | | | He |
| ATP | Rya | | | | | | | | | | | ATP-i | CON | Ca | MEC | NO | Ne |
| AMPA | Insp3 | | | | | | **IUPHAR receptors** | | | | | G/Ach | | Cl | MIT | CO | Ar |
| NMDA | cAMP | mACh | Ade | Adr | Ang | Bra | Can | Che | Cho | Cor | Dop | $K_{ir}$ | | $K_a$ | NUC | CO2 | Kr |
| nACh | cGMP | End | EAA | HIS | 5HT | Mlc | Mlt | Neu | Nuc | Opi | Pros | IfhQ | | $K_v$ | OSM | End | Xe |
| $GABA_A$ | CFTR | | Prot | Som | VIP | Oxy | | | | | | | | Na | SYN | Enk | Rn |
| Gly | KCa | | | | | | | | | | Bil | | | | | | |
| | | | | | | | | | | | | | | | | | |
| | **GAS** | $N_2O$ | Eth | Chl | Hal | Enf | Iso | Des | Sev | Cyc | Div | mFl | Flx | Etc | tcE | Alc | |
| | **IV** | Thi | Amo | MHx | Pro | Eto | Ket | Mid | Flu | Dro | Mor | Fen | Rem | Coc | Lid | Bup | |
| | | Xyl | Det | Dexmed | Rom | Alfax | Ure | Pen | | | | | | | | | |

Figure 1.3 Periodic table of molecular elements in human and veterinary anaesthesia. This table contains the gaseous agents and a broad selection of intravenous anaesthetics, endogenous compounds (ENDO), noble gases (Inert), receptors according to the International Union of Pharmacology (IUPHAR) and ion channels as defined by the Ion Channel Network, including extracellular ligand-gated (ELG), intracellular ligand-gated (ILG), inward rectifying potassium (INR), junctional (JUN), voltage-gated (VLG), and miscellaneous (MIS) ion channels.

GAS: (from left to right) nitrous oxide, diethyl ether, chloroform, halothane, enflurane, isoflurane, desflurane, sevoflurane, cyclopropane, divinyl ether, methoxyflurane, fluroxene, ethyl-chloride, trichloroethylene, alcohol.

I.V.: (from left to right) thiopental, amobarbital, methohexital, propofol, etomidate, ketamine, midazolam, flunitrazepam, droperidol, morphine, fentanyl, remifentanil, cocaine, lidocaine, bupivacaine, xylazine, detomidine, dexmedetomidine, romifidine, alfaxalone, urethane, pentobarbitone

ENDO: (Top to bottom) nitric oxide, carbon monoxide, carbon dioxide, endorphin, enkephalin.

Ion channels (from the top of each column):

ELG: 5-$HT_3$, ATP-gated (P2X), AMPA, and kainate, NMDA glutamate receptor, nicotinic ACh receptor, $GABA_A$ receptor, glycine receptor.

ILG: ryanodine, InsP3-sensitive $Ca^{2+}$-release receptor, cAMP-activated cation channel, cGMP-activated cation channel, CFTR channel, $Ca^{2+}$-activated $K^+$ channel.

INR: ATP-inhibited $K^+$ channel, G/ACh muscarinic-activated $K^+$ channel, $K_{ir}$ inwardly rectifying $K^+$ channel, Ifhq native hyperpolarization- activated cation channel.

JUN: connexins. VLG: $Ca^{2+}$ channel, $Cl^-$ channel, $K_e$ ($K_{eag}$, $K_{elk}$, $K_{erg}$) ether a-go-go $K^+$ channel, $K_v$ delayed rectifier $K^+$ channel, $Na^+$ channel.

MIS: mechanosensitive channel; mitochondrial membrane channel, nuclear membrane channel; aquaporins; synaptophysin channel.

IUPHAR receptors: (left to right, first row) muscarinic ACh receptor, adenosine receptor, adrenoceptors, angiotensin receptor, bradykinin receptor, cannabionoid receptor, chemokine receptor, cholecystokinin receptor, corticotropin-releasing factor receptor, dopamine receptor; (left to right, second row) endothelin receptor, excitatory amino acid receptor, histamine receptor, serotonin receptor, melanocortin receptor, melatonin receptor, neuropeptide Y receptor, nucleotide receptor (P2X receptor, P2Y receptor), opioid receptor, prostanoid receptor; (left to right, third row) protease-activated receptor, somatostatin, vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide receptor, vasopressin and oxytocin receptor. Figure adapted from Urban 2002.

Plentiful evidence exists for anaesthetic action on microcircuits within the brain and spinal cord, and recent advances in functional imaging modalities and electrophysiology prove that the anaesthetics can affect almost all areas of the CNS, but also the PNS and immune and endocrine systems (Traynor and Hall 1981; Taylor 1989, 1998; Mcbride 1996). The focus has tended towards seeking out and evaluating putative targets of action of the molecules and drawing conclusions from structure-activity associations within the homologous groups.

An alternative is to prioritise studying the molecular characteristics that define their potency (Sewell and Halsey 1997; Good and Richards 1998). Computer modelling can correlate activity with molecular bulk and its electrostatic potential. Interestingly in one such study characterising 11 diverse hypnotic substances and comparing potency, a single activity model was derived for chiral and non-chiral intravenous (IV) agents. The model derived was more accurate when the intravenous agent alfaxalone was omitted as an outlier (Sewell and Sear 2002), although potentially this may have been a result of the underestimation of plasma concentrations which were unavailable at the time for alfaxalone alone in any mammalian species. These studies support the construct of a common molecular basis for the mechanism of general anaesthesia but not a common site of action. Indeed*, in vitro* studies indicate that halothane's and isoflurane's mechanisms of immobilisation are distinctly different with isoflurane's site of action within the spinal cord distinctly more ventral when compared to halothane (Jinks et al. 2003b). Additionally, $GABA_A$, neuronal nicotinic acetylcholine and NMDA receptors are all influenced by the diverse array of IV anaesthetic agents but the involvement of these ligand-gated ion channels in induction and maintenance of general anaesthesia *in vivo* is less clear.

One recent study hypothesised anaesthetics could activate TREK-1 channels (TREK-1 channels are TWIK-related $K^+$ channels; TWIK stands for tandem of P domains in a weak inward rectifying channel, these are 2 pore potassium channels, a large family of resting or background $K^+$ channels) indirectly by disruption of adjacent lipid rafts (Pavel 2020). This mechanism, distinct from the usual receptor-ligand interaction, could establish a definitive membrane-mediated mechanism for inhaled anaesthesia (Petersen et al. 2020). The elegant experiments showed that anesthetics disrupt lipid rafts and activate TREK-1 through a two-step phospholipase D2 (PLD2) enzyme dependent mechanism (Pavel 2020) rather than the previously reported direct activation of TREK-1 (Bertaccini et al. 2014). Direct binding may be the mechanism for other channels, but with the discovery of PLD2, and the process of palmitoylation (which is required to localize it to the lipid rafts, and is widespread), this indirect process may explain many other mechanisms (Figure 1.4) (Huang and El-Husseini 2005; Shipston 2011; Weinrich and Worcester 2018).

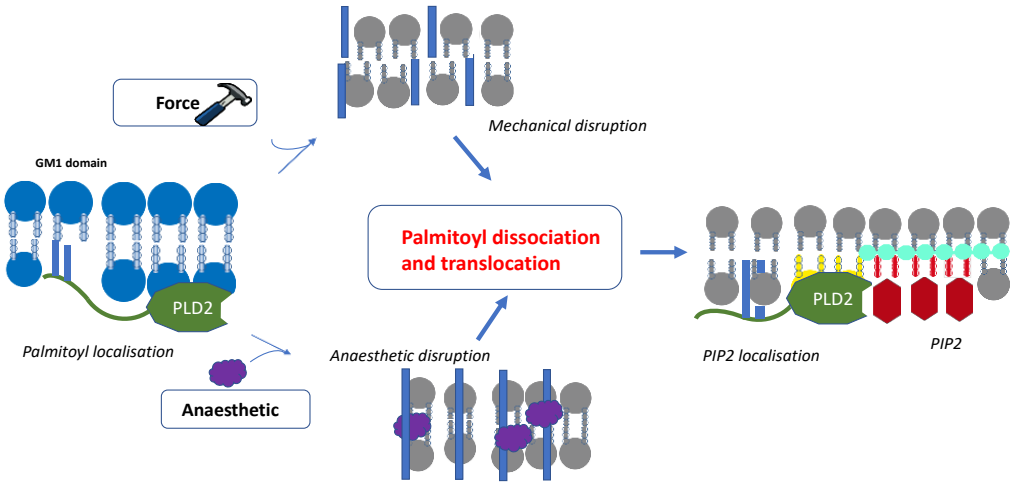



Figure 1.4 Schematic depicting palmitate-mediated localisation. This is a central component of TREK-1 anaesthesia and mechano-sensitisation. The phospholipase D2 is shown with two palmitoylation sites (PLD2, green) and it localises the enzyme to GM1 lipids (blue spheres). Mechanical force (depicted by a hammer picture) disrupts lipid domains resulting in smaller dispersed domains in the membrane (indicated by disordered royal blue rectangles). Anaesthetics (depicted by purple cloud shapes) disrupt lipid domains causing them increase in size but remaining ordered (indicated by intact royal blue rectangles). Both types of raft disruption cause PLD2 translocation and PLD2 binding to PIP2 lipids (red hexagons), substrate (phosphatidylcholine, grey spheres) presentation, and phosphatidic acid (PPA, yellow spheres) production. TREK-1 (not shown) is activated by PPA. The cyan spheres indicate clustered lipids on the inner leaflet (PIP2 domain).
GM: monosialotetrahexosylganglioside1 (GM1) lipids (sometimes called lipid rafts or lipid domains).
Modified from (Petersen et al. 2020).

## 1.4. ANAESTHESIA TECHNIQUES IN LABORATORY ANIMALS

### 1.4.1. Inhalation anaesthetics

Induction and maintenance of rodent anaesthesia can be performed using the inhaled volatile anaesthetic agents: halothane, isoflurane, sevoflurane or desflurane. These agents differ in potency, cardiovascular effects, endocrine and immune responses, and neuroprotection and neurotoxicity. The volatile agents are typically administered in oxygen or an oxygen/nitrous oxide (typically 1:2 ratio) or oxygen/medical air mixture. Although the selection of the agent is typically based on the laboratory or vaporisers available, the agents do differ in speed of induction, recovery and the change in depth or plane of anaesthesia [based on potency, blood-gas partition coefficients (the solubility of inhaled agents in blood at equilibrium) and the concentration delivered]. The potency of the agents is compared using the minimal alveolar concentration (MAC) (Quasha et al. 1980; Sonner et al. 2003). MAC is defined as the concentration at which 50% of subjects respond to a supramaximal noxious stimulus with a purposeful movement response (Urban and Bleckwenn 2002). The limitation of MAC is that it fails to reflect the hypnotic properties and is more appropriate to describe the anaesthetic effects on the spinal cord rather than the effects in the brain (Antognini and Schwartz 1993). The CNS effects of the volatile agents do differ, and there is a consensus that halothane offers advantages over the other agents for neurophysiological studies (Murrell et al. 2008). The minimal anaesthesia model uses the electroencephalogram (EEG) response to noxious stimulation is one tool to evaluate analgesia in veterinary research and halothane is the volatile of choice. Halothane is usually used as it does

not possess antinociceptive effects, plus additional analgesics are avoided. This model can also be used to compare efficacy of different analgesic regimens (Murrell et al. 2003, 2005, 2008; Haga and Ranheim 2005; Johnson et al. 2005).

Halothane and isoflurane also differ in their effects on windup within the spinal cord. Both suppress the C fibre latency, however, in contrast, absolute windup was accentuated by isoflurane but not by halothane (Cuellar et al. 2005). Therefore, if immobility is used as a surrogate marker for general suppression of neuronal excitability it does not predict the degree of windup. It is precisely these types of differences that must be understood particularly in animal models studying pain pathways during anaesthesia.

There are fewer studies investigating the effect of general anaesthesia on peripheral nociceptors compared with central effects. However recent studies have shown that many of the volatile anaesthetics can activate peripheral nociceptors. Isoflurane has been shown to activate and sensitise the TRPA1 receptor (Matta et al. 2008). In this murine model neurogenic inflammation was greater in those mice anaesthetised with isoflurane compared to sevoflurane (Matta et al. 2008) proving that the volatile agents can continue to sensitise these receptors during maintenance of anaesthesia which may exacerbate inflammation caused by the surgical insult. Halothane, isoflurane and enflurane activate C fibre nociceptors *in vitro* at 1 MAC, whereas Aδ fibres were depressed at comparable concentrations (MacIver and Tanelian 1990). Elucidation of these mechanisms responsible for the pain and inflammation further highlights the impact the anaesthesia model could have on the experimental results;

and poses the question of whether clinically in humans and animals the potential deleterious effects of the anaesthetic regimen need closer scrutiny.

Currently the availability of halothane is scarce resulting in isoflurane and sevoflurane use being commonplace The volatile agents typically ensure rapid induction of anaesthesia and recovery from anaesthesia, and the plane of anaesthesia or 'depth' is easily controlled (Grosenbaugh and Muir 1998; Flecknell 2015). These agents lack obvious antinociceptive effects, and additional analgesia should be provided (Coulter et al. 2011; Flecknell 2018).

### 1.4.2. Nitrous oxide ($N_2O$)

The gas nitrous oxide ($N_2O$) has analgesic, anaesthetic, anxiolytic and psychoactive actions. The analgesia provided by $N_2O$ is similar to plasma concentrations of 2 ng $ml^{-1}$ of remifentanil (a short acting $\mu$ opioid agonist) when administered at 66–70% of the fresh gas flow (Lee et al. 2005). The inclusion of $N_2O$ in anaesthesia protocols has been common for many years based on the theory that the analgesic effect is additive and it provides a useful supplement to the volatile agents (Saidman and Eger 1964; Quasha et al. 1980; Eger 2001). Nitrous oxide is considered a volatile sparing drug working synergistically with the volatile agents that potentiate $GABA_A$ and inhibit TREK-1 channels (Duke et al. 2006; Hendrickx et al. 2008; Voulgaris et al. 2013). However this additive effect is not proved definitively and there exists evidence of both non-linearity of additive interaction: that is the predicted reduction in MAC is less than expected (Cole et al. 1989, 1990; Katoh et al. 1997; Russell and

Graybeal 1998; Vahle-Hinz et al. 2007) and definitive infra additivity or antagonism of the volatiles with $N_2O$ (Goto et al. 1996; Katoh et al. 1997; Eger et al. 2008).

Nitrous oxide is also an NMDA receptor antagonist (Jevtovic-Todorovic et al. 1998; Georgiev et al. 2008) and this mechanism of action at NMDA receptors may support its use in persistent pain (Bessière et al. 2010). Other ion channels which may be targets include the T-type calcium channels specifically the $Ca_V3.2$ isoform (Todorovic et al. 2001). This is abundantly expressed in the peripheral sensory neurones and substantia gelatinosa in the spinal cord (Talley et al. 1999) and further implicated in knock out and knock-down studies (Bourinet et al. 2005; Choi et al. 2007; Na et al. 2008).

Nitrous oxide activates noradrenergic neurones, causing noradrenaline release in the brain and the spinal cord (Zhang et al. 1999; Sawamura et al. 2000). This is supported by the findings that both antagonism of adrenergic receptors or diminishment of spinal cord noradrenaline reduces the antinociceptive effect of $N_2O$ (Guo et al. 1996; Zhang et al. 1999). Furthermore the $N_2O$ antinociception is also inhibited by sectioning of brainstem noradrenergic neurones (Sawamura et al. 2000). It has been shown that stimulation of the descending inhibitory pathways by $N_2O$ is facilitated by opioid peptide release in the PAG (Zuniga et al. 1987; Fang et al. 1997; Fujinaga et al. 2000). The administration of the opioid antagonist naloxone or ablation of the PAG reverses these actions (Berkowitz et al. 1977a; Quock et al. 1990). The opioid peptides modulate these descending inhibitory pathways by absolving them of their tonic control by the GABA-ergic pathway overseen by area A7 of the pons, enhancing antinociception via $\alpha$2-adrenergic receptors and spinal

inhibitory GABA interneurones (Guo et al. 1999; Sawamura et al. 2000; Orii et al. 2002). Furthermore, one study has shown that $N_2O$ can also cause the release of corticotrophin releasing factor (CRF) in the LC thereby offering a further mechanism for stimulating the noradrenergic neurones (Sawamura et al. 2003). Additionally nitric oxide has a role mediating $N_2O$'s antinociceptive effects in the midbrain (McDonald et al. 1994; Ishikawa and Quock 2003; Emmanouil et al. 2008; Chung et al. 2016).

The endogenous agonist of the opioid receptor-like (ORL) 1 receptor (NOP) is nociceptin. Nociceptin and NOP are extensively expressed in the CNS, and nociceptin has pain modulating actions (New and Wong 2002). There are conflicting reports of the effects of nociceptin on the MAC of the volatile agents (Dahan et al. 2001; Himukashi et al. 2005), but nociceptin has been shown to be involved in altering the antinociceptive effects of $N_2O$ (Himukashi et al. 2006; Koyama and Fukuda 2009).

The duration of action of $N_2O$ reported is variable depending on the species, paradigm and endpoints used (Zuniga et al. 1986; Hashimoto et al. 2001; Ducassé et al. 2013). As an inhaled analgesic for labour pain, or dental surgery, its peak time to onset and duration of action are usually quoted as between one and two minutes. Tolerance to the effects of the gas have been documented in different rodent strains (Berkowitz et al. 1979; Fender et al. 2000) although the magnitude of this can be reduced by inhibiting enkephalinases thereby maintaining higher levels of endogenous peptides (Rupreht et al. 1984).

### 1.4.3. Injectable anaesthetics

Induction and maintenance of anaesthesia is possible using one or more of the hypnotic agents. Injections can be administered via the intravenous, intramuscular, subcutaneous or intraperitoneal route. Most injectable combinations for rats are based on a combination of the dissociative drugs (see below) or opiates with $\alpha 2$ agonists and sedatives (Flecknell and Mitchell 1984; Nevalainen et al. 1989; Hu et al. 1992; Brammer et al. 1993; Whelan and Flecknell 1994; Antunes et al. 2003; Flecknell 2015; Jiang et al. 2015; Arenillas and Gomez de Segura 2018).

Propofol (2,6-disopropylphenol) is a short acting sedative-hypnotic that is not suitable for intramuscular or subcutaneous administration (McKune et al. 2008) but can be administered intravenously or intraperitoneally (Ihmsen et al. 2002; Alves et al. 2010; Jiang et al. 2015; Moheban et al. 2016). Propofol is associated with cardiopulmonary depression and vasodilation during its infusion (Park et al. 1995; Sinha et al. 2015) and is less commonly used than ketamine based regimens or the volatiles. Propofol is shown to activate the TRPA1 receptors; TRPA1-null mice demonstrate a lack of behaviours such as nose wiping associated with application of propofol to the nasal epithelium (Matta et al. 2008); nocifensive behaviours are protective in origin, and wiping the propofol applied to the nose would be a normal behaviour. The activation of the TRPA1 and TRPV1 receptors is believed to be the mechanism responsible for the pain on intravenous injection with propofol (Fischer et al. 2010). The activation of the TRPA1 receptor by the general anaesthetics, in combination with the neurogenic inflammation caused by surgery through release

of mediators such as bradykinin, is postulated to be additive and potentially a disadvantage of these drugs but the clinical significance of this is unknown.

Alfaxalone (or alphaxalone) is a neuroactive steroid, its use in laboratory rodents is uncommon. Alfaxalone has been used in a small number of nociceptive withdrawal reflex (NWR) and cardiovascular studies (Kelly et al. 2013; Weerasinghe et al. 2014; Holmes et al. 2018) because of the cardiopulmonary and reflex stability. In some neuroendocrine studies alfaxalone may offer advantages for preserving forebrain activity (Sarkar et al. 1976; Sherwood et al. 1980) and protect CNS development and myelination (Yawno et al. 2014). Alfaxalone's effects on the peripheral nociceptors are as yet unknown.

Ketamine is a commonly used dissociative anaesthetic, that is used in combination with other drugs because of poor muscle relaxation associated with its administration alone. The term dissociative anaesthetic describes a functional and electrophysiological dissociation between the thalamo-neocortical and limbic systems (Pai and Heining 2007). Ketamine combinations can cause poor quality recoveries but this depends on the length of the procedure, total ketamine administered and additional drugs used (Hacker et al. 2005). Ketamine offers varying degrees of antinociception in rats (Nadeson et al. 2002) but its interaction at the NMDA receptor may make it unsuitable for neurophysiological or pain studies. Combinations using the $\alpha$2 agonist medetomidine and ketamine or $\mu$ agonist fentanyl and medetomidine offer rapid recoveries through the use of an $\alpha$2 antagonist such as atipamezole (Hu et al. 1992). Neuroleptanalgesic combinations of fast-acting opiates and $\alpha$2 agonists usually require high doses of both compounds

to achieve surgical anaesthesia, potentially resulting in profound cardiopulmonary depression (Hu et al. 1992), and are more commonly used prior to maintenance of anaesthesia with a volatile agent, or for relatively short procedures.

The use of older anaesthetic regimens (chloral hydrate, α-chloralose pentobarbitone, urethane) cannot be recommended as the drugs can cause unnecessary pain or distress (muscle necrosis or peritonitis). These regimens also have a narrow safety margin (Richardson and Flecknell 2005; Flecknell et al. 2015). Mechanoreceptor afferent responses to pentobarbital and isoflurane were shown to be indistinguishable in rats (Cheng et al. 2013). However the safety profile of isoflurane would favour its use over pentobarbital in animals (Sahaghian et al. 2009). The choice of anaesthetic protocol is based upon a wide range of factors and should consider practicalities, animal welfare issues and potential interactions with the specific research being undertaken.

### 1.4.4. Methods avoiding the use of anaesthesia

In view of the effects anaesthesia *per se* has on the animal, researchers have sought ways to record from single neurones or reflexes without the background 'contamination' or 'confusion' of the anaesthesia. Brain slice preparations have been used to facilitate the study of neuronal integration and synapse biology (Dingledine et al. 1980; Korn and Dingledine 1986). These studies offer control of the extracellular milieu and the ability to apply different drugs, record from and visualise cells without anaesthesia. A further refinement to study larger areas of tissue

involved preparation of perfused *in vitro* models (Bourque and Renaud 1983; Randle et al. 1986; Mühlethaler et al. 1990).

To this end researchers have then utilised perfused *in situ* whole animal models; allowing the study of nociceptive and autonomic processes whilst being able to control the external environment still without the need for anaesthesia (Pickering and Paton 2006). These models are particularly useful, for example for integrative physiological studies such as bladder voiding studies and cardiac control studies (Nalivaiko et al. 2010; Sadananda et al. 2011). The disadvantages of the *in situ* rat model is the limitation to the use of juvenile animals (to ensure adequate perfusion) and such models are not ideal for studies involving acute immune reactions or models of acute and chronic or neuropathic pain. An alternative to the juvenile *in situ* model is to use an *in vivo* decerebrated adult model. The decerebrate preparation was introduced over a century ago (Sherrington 1897) and is still commonly used in cardiovascular and neurological research. This preparation can be used for spinal cord studies, and hindbrain and cerebellum research. A detailed methodology of the approach has been published and reports success rates greater than 95% in the hands of experienced operators (Dobson and Harris 2012) although some workers have reported higher rates of haemorrhage and complications (Fouad and Bennett 1998). Other drawbacks of the decerebrate preparation are the removal of the projections descending from the forebrain or the facility to examine structures rostral to the midbrain. These challenges can also be overcome by implanting electrode arrays chronically under anaesthesia to record from the animal while it is awake. The experiments use head restraints in the conscious animal and have been used in a variety of species (Muniak et al. 2012; Sandiego et al. 2013).

## 1.5. STUDYING PAIN ELECTROPHYSIOLOGICALLY

### 1.5.1. Electroencephalography

The EEG provides a unique insight into how the nociceptive pathways contribute to pain perception. This technique utilises electrodes placed at various locations on the head producing the summated electrical activity of populations of neurones and glial cells (Murrell et al., 2005; 2008). Electroencephalography has numerous research and clinical applications (Teplan 2002). The technique can be used in humans and animals to measure the plane of anaesthesia, and antinociceptive effect of different drugs during anaesthesia (Rampil and Matteo, 1987; Johnson and Taylor, 1998). For example, in ponies undergoing halothane anaesthesia for surgical castration, lidocaine obtunded the EEG median frequency ($F_{50}$) change, which is the frequency below which 50% of the power of the EEG is located. This use of intraoperative EEG monitoring and examination of $F_{50}$ during application of a noxious surgical stimulus, can be used as a tool to investigate the antinociceptive action of different agents (Murrell et al., 2003; 2005).

Direct recording of the activity of individual neurones during application of a noxious or non-noxious stimulus can provide information about the intensity, quality, duration and velocity of the stimulus and may involve recording from peripheral afferent sensory fibres or from WDR neurones (Dickenson and Le Bars, 1983; Bee and Dickenson, 2007; Kelly et al., 2012).

### 1.5.2. Nociceptive withdrawal reflexes (NWRs)

Recording of the NWR is also an established neurophysiological technique which consists of applying a noxious stimulus, for example to the limb of an animal, and then undertaking measurement of electromyographic activity in the muscles contributing to limb withdrawal (Levinsson et al., 1999; Clarke et al., 1992; Clarke and Harris, 2004). The withdrawal response, far from being a simplistic monolithic reflex, is a modular combination of reflexes to individual muscles arranged in a matrix best placed to withdraw or remove the limb from the inciting injury (Harris and Clarke, 2003; 2007). Nociceptive withdrawal reflexes can be used as a direct measure of spinal cord hyperexcitability and thus a biomarker of central sensitisation (Harris and Clarke 2007). Central sensitisation's hallmark of an increase in excitability of neurones in the central nociceptive pathways to normal or subthreshold input (Loeser and Treede 2008), is manifest as altered pain sensitivity (Woolf 2011). In models of persistent pain, central sensitisation causes a decrease in the threshold required to elicit a response and is also characterised by enhanced temporal summation of NWRs; both measures are used to evaluate changes in spinal cord nociceptive processing (Kelly et al. 2013).

The anaesthetic regimen used can have profound influences on the results of the NWR. A number of studies have demonstrated techniques using the neurosteroid alfaxalone to be particularly useful for studying NWR during anaesthesia (Kelly et al. 2013; Hsieh et al. 2015; Hunt et al. 2016) in view of the fact that traditional gaseous anaesthesia (e.g. isoflurane) can increase NWR thresholds and reduce DNIC (Petersen-Felix et al. 1996; Jinks et al. 2003a,b; Spadavecchia et al. 2010). These techniques have been used in anaesthetised rodents but NWR thresholds and temporal summation have also been measured in awake and anaesthetised

dogs and horses, using electrical stimuli to evaluate central sensitisation, study the pharmacology of descending controls and probe the antinociceptive effects of analgesic drugs (Peterbauer et al. 2008; Bergadano et al. 2009; Levionnois et al. 2010; Hunt et al. 2016).

*1.5.2.1. Studying nociceptive withdrawal reflexes in rodent models: reflex arrangement*

Applying a noxious insult to the toes or heel of the hindlimb of a rat evokes a short latency polysynaptic reflex. This results in the limb withdrawing from the noxious stimulus. The limb withdrawal consists of multiple, synchronised reflex contractions and relaxations in each individual muscles of the limb. Stimulation of the heel evokes responses in the ankle extensor muscle *medial gastrocnemius* (MG) to lift the heel, transferring the weight bearing to the toes. In contrast toes stimulation evokes responses in the ankle flexor muscle *tibialis anterior* (TA) which raises the toes shifting weight bearing to the heel. Furthermore, the knee flexor and hip extensor muscle *biceps femoris* (BF) exhibits a dual role, and is both activated by heel and toes stimulation. The function of BF is to lift the foot completely. These reflexes can be considered protective in origin to move the limb away from potential injury. As defined earlier these are termed nocifensive reflex responses (Gebhart 2013). The cutaneous excitatory receptive field for TA and MG corresponds to the areas of the foot that the activated reflex is withdrawing from the ground/noxious stimulus (Schouenborg et al. 1994). The NWR to individual muscles was found to be evoked by distinct and separate reflex pathways discovered by probing and mapping receptive fields, time courses and thresholds (Schouenborg and Kalliomäki 1990;

Schouenborg et al. 1992). Furthermore, the withdrawal reflexes to different hindlimb muscles are differentially controlled by descending pathways; it is a complex, and dynamic influence (Sandrini et al. 2005).

## 1.6. DELIVERING CLINICAL IMPACT

The role played by the descending pathways and matrix of transmitters in different pain states has yet to be fully elucidated but consensus is that progress in understanding these areas will profit pain management. The mechanism of one pain inhibiting another pain (DNIC/CPM) has been shown to be a valuable tool, that can probe the integrity of the descending systems and translate into clinical interventions.

### 1.6.1. Osteoarthritis

Osteoarthritis is a chronic debilitating disease in humans and animals, the treatment of the disease remains an unmet need. Osteoarthritis is characterised by progressive erosion of cartilage, fibrillation, chondrocyte proliferation and osteophyte formation as osteoblasts proliferate along the joint margin in response to inflammation and joint instability. The disease consists of cartilage, synovial and bone pathologies. Initially there is hydration of the extracellular matrix. The synthesis of proteoglycans is then upregulated (Mankin et al. 1981) with stimulation of normally quiescent chrondrocytes (Brocklehurst et al. 1984). The synovium is also involved (Wenham and Conaghan 2010); synovial cells proliferate, this is manifest as hyperplasia of the synovial cells with infiltration of macrophages and

fibroblasts and the development of fibrosis. With time sclerosis of subchondral bone occurs too (Mansell et al. 2007).

The articular damage results in pain and loss of function and the pain is further aggravated by movement and the patient's quality of life is impaired. Pain is undoubtedly the major reason that people seek medical and/or surgical intervention. The medical treatment usually involves NSAIDs, intra articular hyaluronic acid viscosupplemention (Abate et al. 2010) or corticosteroids (McCabe et al. 2016), but treatment failure is common (Smith et al. 2016). Hence joint replacements are ever increasing, but are an economic burden (Ruiz et al. 2013; Losina et al. 2015) and not without problems such as prosthesis failure.

The treatment failure is likely a result of the pain being driven not only by peripheral sensitising inputs but also by a central component. This central sensitisation is believed to be responsible for altering the balance in the serotonergic and noradrenergic pathways originating in the PAG-RVM and increasing the barrage of nociceptive impulses reaching the brain resulting in increased pain. Pre-clinical osteoarthritis studies have demonstrated an increased serotonergic facilitation in the descending pathways resulting in increased spinal cord hyperexcitability (Rahman et al. 2009) but also some of the aforementioned transmitters such as the endocannabinoids are also altered (Sagar et al. 2010). In the vast majority of painful disease states, damage to tissue and/or nerves in the periphery is the inciting cause, leading to enhanced transmitter release in the spinal cord and central sensitisation (Woolf 2011; Hunt et al. 2018). The central sensitisation is then maintained by continuing input from the periphery with modulation from inhibitory and facilitatory

descending control from the midbrain and brainstem (Harris and Clarke 2003). In many cases it is difficult to parse out the degree of central or peripheral input so to effectively treat the disease, both the peripheral input and the central modulation will need to be addressed to effectively improve the patient's quality of life.

#### *1.6.1.1. Preclinical models of osteoarthritis*

No single animal model is able to mirror all the variants of human osteoarthritis. As for human osteoarthritis there are different subset models in animals; primary idiopathic models of naturally occurring osteoarthritis related to ageing and secondary induced disease models. In view of the fact that no model completely reproduces the signs and symptoms of osteoarthritis in humans, the model selection should be based upon the most appropriate to answer the question being posed rather than familiarity and precedence. The models have a wide range of severity and progression of the pathogenic changes. Naturally occurring models such as the Dunkin-Hartley guinea pig (Jimenez et al. 1997; McDougall et al. 2009) and C57B16 mice models (Chillingworth and Donaldson 2003) develop osteoarthritis over the animals' lifetime whereas the surgical injuries resulting in severe structural changes occur more rapidly and can be more appropriate for studying cartilage degradation (Zhang et al. 2013). The inflammatory models are also acute in onset and more rapidly progressing and whilst they may be of use in drug development, they also may not be appropriate for studying less potent drugs that may be suitable for the slowly developing condition in humans (Schuelert and McDougall 2009; Ferland et al. 2011; Orita et al. 2011).

There are a range of rat models available and this species is particularly suitable for undertaking osteoarthritis research incorporating electrophysiology components to the study (Stanfa and Dickenson 2004). The advantages of using an osteoarthritis model in rats is that rat cartilage is thicker than mouse cartilage, so it is easier to create partial and full-thickness cartilage defects (Bendele 2002; Gregory et al. 2012) and rats rarely experience post-operative infections (Bendele 2002). The management and maintenance costs of rats is low and it is easier to undertake surgery in rats compared to mice (Little and Smith 2008; Gregory et al. 2012). Furthermore the full rat genome is available for study (Little and Smith 2008). The rat medial meniscus tear/transection model, cruciate ligament transection and iodoacetate models are very useful to study pain (Table 1.3) (Little and Smith 2008; Gregory et al. 2012). Additionally rat models are useful in toxicology testing and studying cartilage restoration techniques (Bendele 2002; Teeple et al. 2013). In all these models the influence of the anaaesthesia must also be considered (Vahle-Hinz and Detsch 2002; Hirsch et al. 2014).

Table 1.3 A summary of the different osteoarthritis models used in rats

| Induced or naturally occurring | Description | Comments | References |
|---|---|---|---|
| **Naturally occurring disease** | Aged OA | Rat generally devoid of naturally occurring OA<br>Variation in results between strains, disease severity age; older rats exhibit more severe OA | Gerwin et al., 2010; McCoy, 2015 |
| **Surgically induced model** | Anterior cruciate ligament transection | OA that progresses very fast<br>Severe lesions<br>Reproduces traumatic OA | Appleton et al., 2007; Hayami et al., 2006; Lampropoulou-Adamidou et al., 2014; Pickarski et al., 2011 |
| | Medial meniscectomy or partial medial meniscectomy | Severity of onset of disease usually higher than in humans after same injury | Lampropoulou-Adamidou et al., 2014; Pickarski et al., 2011; Teeple et al., 2013 |
| | Articular groove model | OA development depends on cartilage lesion produced | Ahern et al., 2009; Anraku et al., 2009 |
| | Medial meniscal transection | Mimics nociceptive and neuropathic OA | Bendele, 2002; Bove et al., 2006; Lampropoulou-Adamidou et al., 2014 |
| | Immobilization | Less commonly used model, induces atrophic changes in articular cartilage mimics OA pathology | Little and Smith, 2008; Troyer, 1982 |
| | Anterior cruciate ligament (ACL) injury | ACL damage caused by tibial compression | Maerz et al., 2015 |
| | Ovariectomy | Mature ovariectomised rats - accurate animal model of human postmenopausal OA | Høegh-Andersen et al., 2004 |
| | Monosodium-iodoacetate (MIA) injection | Well established and resembles<br>the histological and pain-related behaviour of human degenerative OA | Bove et al., 2003; Fernihough et al., 2004; Marker and Pomonis, 2012; Pomonis et al., 2005 |
| | Collagenase | An alternative model to MIA for studying nociception associated with OA | Blom et al., 2007; van Osch et al., 1994 |
| | Papain | Papain studies enable different stages of OA to be studied depending on papain dose administered<br>Less commonly used than other models | Murat et al., 2007; Pomonis et al., 2005 |
| | Immunotoxin | Loss of joint afferents precedes OA and predisposes the animal to OA | Salo et al., 2002; Teeple et al., 2013 |

OA: osteoarthritis.

## 1.7. THE MODEL

To date in this review the focus has been on the model, and the definition of this term warrants further clarification. The model in fact refers to the subject, the assay and the measure (Figure 1.4) (Mogil 2009). All of these variables can influence the model.

Table 1.4 Construct of the rodent experimental model

| **Subject** | **Assay** | **Measure** |
|---|---|---|
| Species<br>Rat, mouse, guinea pig | Aetiology<br>Nociceptive (thermal, chemical, mechanical, electrical)<br>Inflammatory<br>Disease state development<br>Neuropathic (surgical or chemical) | Reflex<br>Heat or cold or mechanical stimulus inducing the reflex response |
| Strain/mutant<br>Different strains have different pain phenotypes<br>Transgenic mice | Body part being tested<br>Nervous system<br>Cutaneous<br>Visceral<br>Muscular<br>Orofacial | Spontaneous<br>Directed behavious such as biting, licking, flinching, guarding, shaking, excessive grooming, writhing<br>Altered gait |
| Sex<br>Male only studies still most common | Time frame of testing<br>During and after induction of the model, intervals and duration of testing | Associative Learning<br>Learned aversion<br>Place preference<br>Reinforcement conflict |
| Age<br>Juvenile versus adult or aged | | Complex behaviours<br>Anxiety-like, depression-like, disability, sleep deprivation, sociability |
| Husbandry<br>Cage stocking density, diet, and enrichment are influencing factors | | |
| Method of testing<br>Operator influence (sex) and handling,<br>Conspecifics presence during testing | | |

Adapted from (Mogil 2009)

### 1.7.1. The Subject

For basic pain experiments using rats the Sprague Dawley (SD) strain remains the mainstay. The reluctance to select a more heterogenous population is based on: the previous experiments all using SD precluding comparison, cost, familiarity and perceived variability by using other strains. Yet, in humans, the final target, chronic pain manifests itself most commonly in the older female (Berkley 1997; Gagliese and Melzack 1997; Greenspan et al. 2007), and this 'gender gap' with distinct racial and ethnic differences (Edwards et al. 2001; Campbell and Edwards 2012) is likely to have contributed to the poor translation (see below). Many laboratories have switched to using transgenic mice in pain experiments and this has resulted in a huge number of pain phenotypes from the null mutation of target genes (LaCroix-Fralish et al. 2007), but again these expensive, genetically altered animals do not often offer normal pain sensitivity alongside the protein of study (Lariviere et al. 2001) often because of the altered pain phenotype of the background strain. This trend may reverse with the use of small interfering RNA technology (Hasuwa et al. 2002; Mogil 2009).

Sex differences in pain have been known for some time (Unruh 1996; Berkley 1997; Unruh et al. 1999) but most basic science pain studies still use young adult, male rats, implicitly assuming that the biology of pain is conserved across the sexes or the restriction of variability is a primary aim (Zucker and Beery 2010; Mccarthy et al. 2012). This incorrect assumption may be one of the reasons the success of translating the basic science data into efficacious analgesics has been slower than anticipated (Mogil 2009; Berge 2011). Omitting studying the effect of drugs in

females can result in costly, epic and dangerous failures in drug translation (Galer et al. 2005; Mak et al. 2014; Criado-Perez 2019), hence the need for more heterogenous animal populations (Greenspan et al. 2007) and optimised study design (McGrath et al. 2010; Kilkenny et al. 2014). The same can be said of age; although costly to use older animals, the study of pain phenotype in older animals and multiple strains, would likely translate better and serve the target population which is often aged and diverse (Gagliese and Melzack 1997; LaCroix-Fralish and Mogil 2009; Da Silva et al. 2020). Other husbandry components of the model such as social groups, sole housing, diet and cage environment (presence/absence of enrichment) can have effects on the result and must be considered, and should be reported (Kilkenny et al. 2014).

### 1.7.2. The assay

The variety of assays can be divided into inflammatory, neuropathic, nociceptive or disease states, for example cancer, and will target different parts of the body. The time points for measurements will have a profound effect on the results and must be reported.

### 1.7.3. The measure

The measure(s) of the experiment can be divided into reflex, associative learning and spontaneous. Reflex includes the potentially aversive/noxious stimulus (heat/cold/mechanical) from which the animal exhibits nocifensive behaviours. The spontaneous behaviours include directed responses, for example, biting the

affected limb, writhing, licking etc, or in some cases shedding of the limb (autotomy). The changes may be subtler as indicated by altered gait and a change in weight bearing. Operant measures, include place preference and escape measures. In addition, as for humans, the animals can be influenced by sleep, anxiety-like and depression-like behaviours. The dominant measure in pain studies is tending towards these awake behaviours. A further refinement although complex is to evaluate 'quality of life' like behaviours. Advances have been made to assess these behaviours through more global assessment of the individual animals. In the home cage it is possible using individual telemetry to assess rats' behaviours and movements. Furthermore, software is able to collate time spent grooming, moving bedding, interaction with cage mates, playing, fighting and incidence of spontaneous behaviours. These technologies facilitate a more holistic approach to assessing the animal's affective state rather than focusing on individual operant dependent static measures of behaviour.

In view of the ever expanding number of pain conditions it is unlikely that a single model would demonstrate adequate predictive, face and construct validity for chronic pain (Blackburn-Munro 2004). The poor record of translation from laboratory to clinical intervention for pain, has raised the possibility of the current measures being inadequate; ongoing forward and back translation is necessary to develop more comprehensive paradigms for developing interventions for pain.

## 1.8. SUMMARY

Much of what we know about pain to date has been discovered utilising various pharmacological, and neurophysiological techniques in animal models and these discoveries have more recently been confirmed with genomics and advanced imaging (Rainville 2002). Neurophysiological modalities, have been used to study both afferent ascending and descending pain pathways and cortical representation of pain (Murrell and Johnson 2006). Whilst some may argue that it is preferable to use behavioural outcome measures in conscious animals in order to better capture behavioural/learned and homeostatic mechanisms in response to a noxious insult, anaesthetised animal models can reduce the subjectivity and bias associated for example with quantifying a withdrawal response. Nonetheless, the emphasis in both anaesthetized and conscious patients, particularly in the study of chronic pain, should be that an appropriate parameter is measured (Mogil and Crager 2004), but we are still some way off having a validated set of tools for testing all components of the pain experience.

Studying reflexes can serve as a surrogate for studying a population of neurones. In view of the fact that the reflex pathways are entirely within the spinal cord they offer a convenient, organised target for manipulations of spinal cord activity. These studies, in turn, lead to a clearer understanding of how spinally mediated events are restrained. From an ethical standpoint, numbers, and reproducibility the laboratory rat offers the most appropriate model for studying these reflexes. The very nature of the stimulus (noxious) means that ethically anaesthesia is required to prevent awareness, nociception and potentially pain perception.

The descending control of the spinal nociception has evolved from a simplistic construct of the supraspinal and segmental influences having a direct effect on the sensory processes and being able to influence the acute or chronic pain experience. We now consider a more complex Bayesian matrix where the nociceptive input will be concurrently constrained by behavioural, learned, predicted, homeostatic mechanisms together with the peripheral incoming signals. These, in turn, must be interpreted against the backdrop of variables associated with the model such as anaesthesia, sex and strain.

The present studies have therefore investigated:

i) the suitability of alfaxalone anaesthesia for studying NWR and DNIC in male and female rats

ii) the influence of preconditioning with $N_2O$ on DNIC in Sprague Dawley and Lewis rats with intact neuraxes and in decerebrated rats

iii) the influence of preconditioning with $N_2O$ on DNIC in rats with induced osteoarthritis (MIA model)

# 2. MATERIALS AND METHODS

## 2.1. ETHICAL APPROVAL

Studies were performed in accordance with Project Licence (PPL) 30/3156 issued under the Animal (Scientific) Procedures Act 2013 (EU Directive 2010/63/EU) and local ethics committee approval.

## 2.2. ANIMALS

All rats underwent 10 days acclimatisation to the animal unit after arrival from the breeding establishment. The strains of rats used included Sprague Dawley, and Lewis rats depending on the study design. Both sexes were used wherever possible. Animals were housed in single sex groups of 4 in double layer, ventilated cages (Sealsafe Double Decker, Tecniplast, UK) given access to food (Teklad 2018, Harlan) and tap water *ad libitum* and maintained on a 12-hour light/dark cycle (19-23°C) with one hour of dusk and 55 ± 10% humidity. All cages had play tubes, bedding material and chew blocks for enrichment.

The experiments were carried out on two surgically distinct preparations; intact neuraxis, or decerebrated at the mid collicular level. The anaesthesia protocol differed depending on the particular study (see below). All experiments started between 0900 and 10 00h.

## 2.3. ANAESTHESIA

### 2.3.1. Isoflurane

Anaesthesia was induced using 3% isoflurane (Isoflo, Abbott, Maidenhead, UK) in $O_2$ with or without $N_2O$ (depending on study design, see sections 4.2.3 and 5.2.7). Once the rat had lost its righting reflex it was transferred to a homeothermic heating blanket (Harvard Apparatus Ltd., Edenbridge, UK) coupled to a rectal probe for maintenance of body temperature (37.5 ± 0.5°C). Anaesthesia was maintained using 2–2.25% (vaporiser setting) isoflurane in $O_2/N_2O$ or $O_2$ (depending on study design; see below) delivered initially via a nosecone.

### 2.3.2. Alfaxalone infusion

Following instrumentation (see section 2.3.3 below), anaesthesia was continued with either intravenous alfaxalone (Alfaxan, Jurox, Malvern, UK) (section 3.2) with a loading dose of 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by a constant rate infusion (CRI) (0.75 mg $kg^{-1}$ $min^{-1}$) for the remainder of the electrophysiological experiment using a calibrated syringe driver (SP100iz, WPI, Hitchin, UK), or using isoflurane.

One set of experiments (section 3.3.5.) using female Sprague Dawley animals used a reduced rate of infusion with a loading dose of 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes

followed by a constant rate infusion of 0.56 mg $kg^{-1}$ $min^{-1}$ for 60 min then a further reduction to 0.42 mg $kg^{-1}$ $min^{-1}$ for the remainder of the experiment.

### 2.3.3. Monitoring anaesthesia

Respiratory rate and effort were assessed by observing chest excursion and measuring end tidal carbon dioxide and end tidal isoflurane (Capnotrue MG, Masimo, Basingstoke, UK). In animals exhibiting respiratory depression as judged by a low respiratory rate and rising end tidal carbon dioxide values, intermittent positive pressure ventilation was initiated (SAV04, Vetronic, Abbotskerswell, UK) to maintain end tidal carbon dioxide between 35 and 45 mmHg. Arterial blood pressure was monitored by an arterial pressure transducer (SensoNor 840; SensoNor, Horten, Norway) and recorded using a PC running Spike2 software (version 2.08) via a CED micro1401 interface (CED Ltd, Cambridge, UK). Heart rate was recorded via two 25g needles inserted subcutaneously on the lateral sides of the thoracic wall. The ECG signal was amplified and used to trigger an instant rate meter (Neurolog NL253, Digitimer, Welwyn Garden City, UK) and again recorded using Spike2 software via a CED micro1401 interface. The hypnotic characteristics of the anaesthetic were evaluated by monitoring paw withdrawal reflex in response to pinch, corneal reflex in response to light brushing, spontaneous blinking, gross purposeful movement and cardiopulmonary parameters.

### 2.3.4. Haematology, biochemistry and blood gases

For blood gases, haematology and biochemistry, 0.2 ml carotid blood was withdrawn anaerobically and analysed with a hand held blood gas analyser (EPOC, Woodley, Bolton, UK). Analyses were carried out at intervals during the experiment (see Figures 3.1, 4.1, 4.2 and 5.2). The EPOC cartridges measured blood pH, partial pressure of oxygen, ($P_aO_2$), partial pressure of carbon dioxide ($P_aCO_2$), sodium, potassium, chloride, glucose, lactate, haematocrit, creatinine, and calculated bicarbonate, total $CO_2$, anion gap, base excess, and haemoglobin. Results were stored on the handheld machine and blue toothed to a laptop and stored as an Excel file for further off-line analysis.

## 2.4. SURGERY

The animal was placed in dorsal recumbency and a rectangular area from the manubrium sternum to the angle of the jaw clipped and surgically prepared (Virusolve, Farla, London, UK). A sterile surgical fenestrated drape was placed over the surgical site and lidocaine 0.5% (Lignol, Dechra, Shrewsbury, UK, 2% diluted with sterile saline to 0.5%) 4 mg $kg^{-1}$ was infiltrated subcutaneously prior to skin incision. A surgical plane of anaesthesia was confirmed prior to starting surgery. A 4 cm midline incision was made over the trachea. The subcutaneous tissue was undermined and the neck muscles exposed. Further lidocaine (2 mg $kg^{-1}$ ) was infiltrated into the muscle before transection of the sternohyoid muscle. Mosquito forceps were applied to the edges of the transected muscle to facilitate exposure of the trachea. Fascia overlying the trachea was removed. Two silk stay sutures were placed encircling the trachea, and mid-way between the sutures an incision between the tracheal rings was made and a polyethylene 2.42 mm outside diameter (OD)

tubing (Fisher Scientific, Loughborough, UK) inserted 2 cm and secured in place. The nose cone was removed and anaesthesia was then maintained via the tracheal cannula connected to a paediatric T piece with Jackson-Rees modification breathing system and pressure cycled ventilator attached (SAV04, Vetronic, Abbotskerswell, UK). The left carotid artery was then exposed and surgically cannulated using heparinised (10 IU $ml^{-1}$) mm OD polyethylene tubing (Fisher Scientific, Loughborough, UK) in order to monitor arterial blood pressure and for blood gases or pharmacokinetic sampling. Care was taken to avoid damage to the vagus encircling the carotid artery. The left jugular vein was also surgically cannulated using heparinised 0.63 mm OD polyethylene tubing (Fisher Scientific, Loughborough, UK) for administration of alfaxalone. Both cannulae were secured in the vessels using 3-0 (2 M) silk. Two small portions of hemostatic sponge were placed either side of the vessel incisions. Topical anaesthetic (EMLA 5% Astra Zeneca, Cambridge, UK) was applied to the mucosa, and several sutures placed to appose the edges of the surgical wound to reduce heat and moisture loss.

## 2.5. STABILISATION

Animals were allowed to stabilise for 60 minutes after commencing the alfaxalone infusion, prior to electrophysiological recordings being carried out. For those animals undergoing decerebration in Chapter 4 (section 4.2.4.), all surgery was carried out under isoflurane anaesthesia. Anaesthesia was then maintained with alfaxalone infusion as detailed above or isoflurane for the remainder of the experiment.

## 2.6. ELECTROPHYSIOLOGY

### 2.6.1. Earthing electrode

Hair was removed from the midthoracic area in the midline and the skin surgically prepared. Lidocaine (4 mg $kg^{-1}$) was infiltrated subcutaneously and a silver-silver chloride earthing electrode was placed via a 3 cm incision overlying the epaxial (dorsal) muscles in the region of thoracic vertebra T8-T11, and embedded within the muscle tissue. The incision was closed with two simple interrupted sutures.

### 2.6.2. Hind limb stimulation and recording

The left hind limb was clipped and cleaned with Virusolve (Farla, London, UK) soaked cotton wool. Paired varnish insulated copper wire electrodes were inserted percutaneously into the 3 hind limb muscles, knee flexor *biceps femoris* (BF), ankle flexor *tibialis anterior* (TA) and ankle extensor *medial gastrocnemius* (MG) using a 23G needle as an introducer. Electrical stimulation of the plantar aspect of the heel (MG), and the two most lateral toes (BF, TA) was elicited via two 27G stainless steel needle electrodes, the position of which was based on previous cutaneous mapping studies (Weng and Schouenborg 1996) using ISO-flex stimulators (A.M.P.I, KF Technology Srl, Italy) using 8 stimuli of 2 ms duration at 1 Hz. Stimulation strengths were 1–5 times the threshold, with a maximum of 10 mA possible although the actual criterion used was that the stimulus evoked a response of sufficient amplitude to allow an unequivocal decrease to be observed following capsaicin conditioning (see section 2.6.3). Short latency (0–25 ms) compound EMGs were amplified (x 5000),

filtered (10 Hz – 5 kHz) and fed to a CED 1401 connected to a PC running Signal software (Version 2.08, CED Ltd, Cambridge, UK) where the rectified responses to each block of 8 stimuli were averaged and integrated. Responses at 0 mA were also collected prior to each evoked response, and subtracted offline.

An example of a raw data trace (generated every two minutes) is shown below (Figure 2.1). The plot represents an average of eight sweeps. Cursor position was kept constant throughout the 64 minutes after injection of capsaicin. Area under the curve (AUC) was calculated after cursors were positioned and baseline reflex activity subtracted offline.

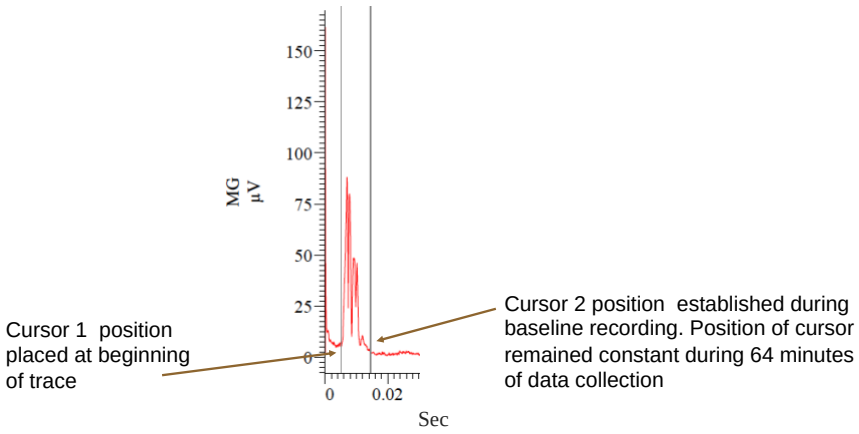



Figure 2.1 Raw trace of reflex evoked in *medial gastrocnemius* by electrical stimulation of the heel.

Recording electrodes were repositioned within the muscles if the baseline was not flat. Prior to the experiment starting it was also important to remove all sources of 50Hz interference, and ensure that background muscle activity was minimal. If spontaneous activity was evident (reflexes being generated without stimulation), a

further period of waiting ensued, typically 15–20 minutes. In cases of intractable 50 Hz interference with no obvious cause, a mini Faraday shield was constructed around the animal using aluminum foil and interlocking metal rectangles, while still allowing access to the rat. Following baseline reflex recordings for at least 30 minutes (and establishment of 3 sequential measurements of each reflex within 10% of each other), an acute noxious conditioning stimulus (capsaicin; see below) was administered. Reflexes were then collected and measured every 2 min for a minimum of 63 min (i.e. a total of 32 stimulations, 16 heel and 16 toes).

### 2.6.3. Conditioning stimulus

The stock solution was made by dissolving 25 mg capsaicin powder (Tocris, Abingdon, UK) in 250 μL ethanol (Sigma-Aldrich, Gillingham, UK) and 250 μL Tween-80 (Sigma-Aldrich Gillingham, UK). For each experiment 30 μL stock capsaicin was then added to 270 μL Ringers' solution (Vetivex 9, Dechra, Shrewsbury, UK) to yield a final concentration of 5 mg $ml^{-1}$. This volume was sufficient for each experiment and surplus was discarded at the end of the experiment. The capsaicin (100 μl) was injected via a 25G needle into the muscle mass of *extensor carpi radialis* and *pronator teres* of the right (contralateral) forelimb.

## 2.7. EUTHANASIA

At the end of the electrophysiology experiments animals were euthanised by intravenous injection of pentobarbitone 150 mg $kg^{-1}$ (Pentobarbital, Ayrton Saunders Ltd, Runcorn, UK). Cessation of cardiac output was confirmed by two methods (cessation of arterial blood pressure trace, lack of heart beat) followed by cervical dislocation (as required by UK Home Office regulations).

## 2.8. STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism software (version 8.4.3, GraphPad Software Inc., USA). The level of significance was set as $P < 0.05$.

### 2.8.1. Group sizes

Group sizes in these studies were based on effect size data from previous published electrophysiological/pharmacological experiments conducted in the supervisor's laboratory (Kelly et al. 2013). Calculations indicate a group size of n = 8 assuming 80% power (i.e. 1 – β error probability of 0.8) and a significance of $P < 0.05$ (i.e. α error probability of 0.05) of detecting a difference for the AUC for toes-BF reflex with or without the treatment. Group sizes of 12 were determined with a greater power (control: $\mu_1 = 8.6$; $\sigma_1 = 3.3$; drug: $\mu_2 = 5.1$; $\sigma_2 = 2.4$). Variance in the effects size can give groups of 8–12 animals.

### 2.8.2. Cardiopulmonary data, haematology, biochemistry and blood gases

Normality assumptions were tested with Kolmogorov-Smirnov or Shapiro-Wilk tests. Differences in heart rate, mean/systolic/diastolic arterial blood pressure, blood gas variables, lactate, glucose and electrolytes between sexes in the first study were compared using an unpaired *t*-test for data that was normally distributed and data shown as mean and standard error. Nonparametric data were compared with Mann-Whitney test and shown as median and range.

For a more detailed analysis of changes over time, these variables were also analysed by two-way repeated-measures ANOVA (one factor repetition) for the time points between baseline anaesthesia (isoflurane) and 120 minutes after alfaxalone infusion with Sidak's correction for multiple comparisons.

The pharmacokinetic parameters in Chapter 3 (section 3.3.8.) for log transformed parameter data for both sexes were compared using an unpaired, two tailed Student's *t*-test. With the introduction of a third group (female animals receiving a reduced constant rate infusion CRI), plasma concentrations were tested with a two-way ANOVA with Tukey's multiple comparison test.

### 2.8.3. Reflex data

#### *2.8.3.1 Electrical thresholds required to elicit reflexes*

Electrical thresholds to elicit reflexes were compared between groups using Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test for *post hoc* contrasts.

#### *2.8.3.2. Raw control responses between sexes and other groups*

Raw control responses prior to capsaicin injection were compared between sexes using a Mann-Whitney test. With the introduction of a third group in Chapter 3, of female animals (section 3.3.5.) receiving a reduced CRI, and multiple groups in Chapter 4 (4.2.1.) and Chapter 5 (5.2.1.), thresholds and raw control reflex values were compared using the Kruskal-Wallis test.

#### *2.8.3.3. The effect of capsaicin on the reflex*

The effects of capsaicin were assessed by converting each reflex response to a percentage of the mean of the three control values prior to capsaicin administration, then expressing pooled data as medians with inter-quartile ranges (IQR). Reflex data collected in the 64 minutes after capsaicin injection were compared to baseline using Friedmann's ANOVA followed by Dunn's multiple comparison test. Comparison between groups at set time points was analysed using a Mann-Whitney test or Kruskal-Wallis test. In Chapter 4 the cited percentage (%) inhibition values are at 17 and 19 min post-capsaicin for heel-MG and toes-BF/TA, respectively.

In addition, negative AUC from baseline to 64 minutes was analysed between groups for each reflex using a Mann-Whitney test in the first study, and using a Kruskal-Wallis with the introduction of a third group.

### 2.8.4. Behavioural data (Chapter 5)

Behavioural data (weight bearing and mechano-allodynia) were compared between ipsilateral and contralateral limbs and control and saline and MIA-injected groups at the same time points using a mixed model ANOVA followed by Sidak's multiple comparison test for post hoc contrasts.

# 3. THE EFFECT OF SEX ON ALFAXALONE ANAESTHESIA AND DNIC

## 3.1. SEX

The majority of animals used in basic pain research are young healthy male laboratory rodents. The inclusion of female rodents is to be encouraged despite the additional perceived complexities that the variability of the oestrous cycle and sexual dimorphism poses (Mogil and Chanda 2005); well-designed studies can include both sexes without needless increase in animal numbers (Clayton and Collins 2014).

## 3.2. ALFAXALONE

### 3.2.1. History of the use of alfaxalone

Alfaxalone (or alphaxalone) is a neuroactive steroid that modulates neurotransmission through interaction with a steroid recognition site on the $GABA_A$ receptor complex causing a positive allosteric modulation of this ligand gated chloride channel resulting in inhibition of neuronal excitability (Harrison and Simmonds 1984; Turner et al. 1989). Such agents therefore have roles in anaesthesia, epilepsy, anxiety, insomnia, migraine and drug dependence (Rupprecht and Holsboer 1999). Alfaxalone had been used as an anaesthetic induction agent in humans and veterinary species for almost half a century but anaphylactoid reactions attributed to the polyethoxylated castor oil (Cremophor EL)

vehicle (Tammisto et al. 1973) made its use redundant. Subsequent formulations of alfaxalone incorporating a cyclodextrin have hitherto been devoid of the previous side effects and Alfaxan (alfaxalone dissolved in 2-hydroxypropyl-*β*-cyclodextrin) is now registered for induction and maintenance of anaesthesia in dogs, cats and rabbits (Navarrete-Calvo et al. 2014) and has been used in horses (Goodwin et al. 2011), sheep (Andaluz et al. 2012; del Mar Granados et al. 2012) and other more exotic species (Bouts and Karunaratna 2011; McMillan and Leece 2011; Bauquier et al. 2013; Knotek et al. 2013; Villaverde-Morcillo et al. 2014).

### 3.2.2. The use of alfaxalone in biomedical research

The use of alfaxalone for research and clinical veterinary medicine is gaining popularity as it may offer some selective advantages over other anaesthetic combinations in terms of safety, reflex suppression, cardiopulmonary depression, interaction with receptors involved in pain pathways/modulation and pain on injection (Child et al. 1972; Michou et al. 2012; Santos González et al. 2013). Alfaxalone has been suggested as a suitable anaesthetic for neuroendocrine studies in view of its sparing effects on the forebrain (Sarkar et al. 1976; Sherwood et al. 1980). Preliminary trials of alfaxalone in humans have been undertaken. The phase 1 ‘first in human’ trial used young male adults and showed promising results when compared to propofol (Monagle et al. 2015).

### 3.2.3. The pharmacokinetics of alfaxalone

The pharmacokinetics of a single IV dose have been defined in dogs (Ferré et al. 2006), thoroughbred horses (Wakuno et al. 2019), cats (Whittem et al. 2008; Muir et al. 2009), rabbits (Marín et al. 2020), female rats (Lau et al. 2013) and male rats after a 5 minute infusion (Visser et al. 2002).

### 3.2.4. Aims of study

The novelty and primary aim of this study was therefore to characterise the pharmacokinetics, pharmacodynamics and hypnotic characteristics of a constant rate infusion of alfaxalone in male versus female rats in order to then study potential differences in pain mechanisms against the backdrop of anaesthesia and sex.

## 3.3. MATERIALS AND METHODS

The study was performed in two blocks; the first consisted of pharmacokinetic/pharmacodynamic (PK/PD) and electrophysiology experiments in both the sexes. This was followed several months later by a second block of PK/PD and electrophysiology experiments in female rats with a reduced alfaxalone CRI in order to test DNIC at similar plasma concentrations to male rats.

### 3.3.1. Animals

Twenty-five (9–12-week-old) Sprague Dawley rats, comprising 14 males (399 ± 14 g) and 11 females (283 ± 6 g) were used in the first electrophysiology study, of these, six males (397 ± 14 g) and six females (286 ± 6 g), were also used in an initial (PK/PD) study. A second series of experiments used a further eight female rats (307 ± 4 g) for a PK/PD and electrophysiological study. Animals were housed in groups of 4 in double layer ventilated cages as detailed in Chapter 2 (section 2.2.).

### 3.3.2. Group sizes for pharmacokinetic/pharmacodynamic (PK/PD) studies

Group sizes of six animals were deemed appropriate for evaluating a drug exhibiting within subject variability of less than 30% coefficient of variation of pharmacokinetic measures (Rowland and Tozer 2011) and recent studies comparing cardiovascular effects of anaesthetic drugs have successfully used five rats per group (Bencze et al. 2013). These numbers informed the sample size for the PK arm of the study,

however larger groups were used, influenced by the sample size calculation (section 2.8.1.) for detecting differences between the sexes for the DNIC assessment.

### 3.3.3. General anaesthesia and surgery

Anaesthesia and surgical techniques are described in detail in Chapter 2 (section 2.3. and 2.4. respectively). Cardiopulmonary data were collected continuously and analysed at the pharmacokinetic time points (section 3.3.6). A timeline for the experiment is shown in Figure 3.1

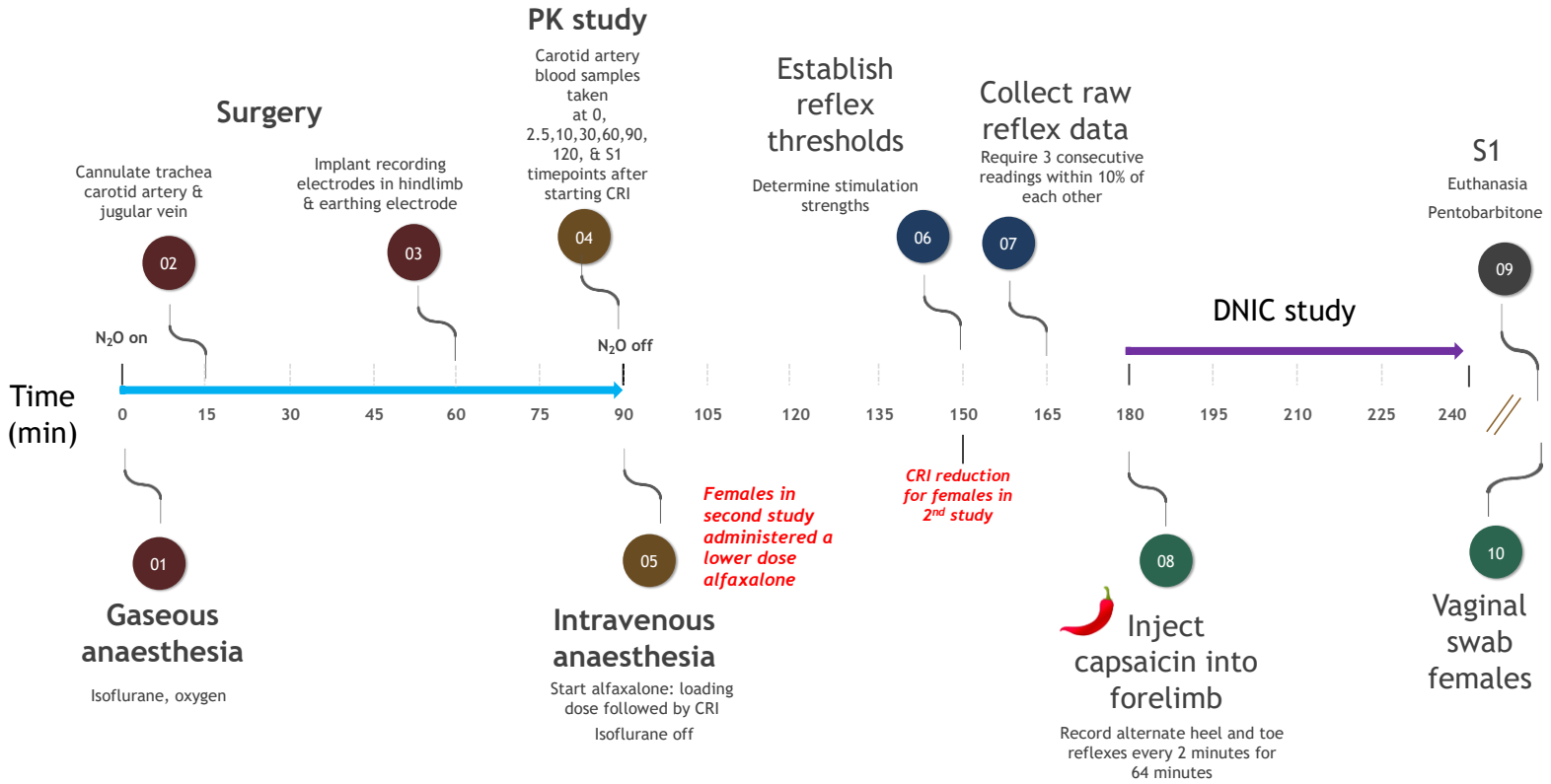



Figure 3.1 Timeline for PK and DNIC studies in male and female rats. In the first experiment rats were administered the higher alfaxalone dose: 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by a constant rate infusion of 0.75 mg $kg^{-1}$ $min^{-1}$. In the second set of experiments female rats were administered 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by 0.56 g $kg^{-1}$ $min^{-1}$ for 60 minutes followed by 0.42 mg $kg^{-1}$ $min^{-1}$ for the remainder of the experiment. CRI: constant rate infusion, DNIC: diffuse noxious inhibitory controls, $N_2O$: nitrous oxide, PK: pharmacokinetic, S1 = Schedule 1 (euthanasia).

### 3.3.4. Alfaxalone infusion (both sexes)

Following completion of surgery, an infusion of alfaxalone via the jugular cannula was started (time 0) at a loading dose of 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by a constant rate infusion (0.75 mg $kg^{-1}$ $min^{-1}$) for the remainder of the electrophysiological experiment. Isoflurane was stopped 2.5 minutes after starting the alfaxalone infusion, $N_2O$ was stopped after a total of 90 minutes.

### 3.3.5. Alfaxalone infusion (females only)

Based on the findings of the first experiment (both sexes), a second experiment was performed several months later using a female population of Sprague Dawley rats that received a reduced infusion of alfaxalone; 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by a constant rate infusion 0.56 mg $kg^{-1}$ $min^{-1}$ for 60 min followed by 0.42 mg $kg^{-1}$ $min^{-1}$ for the remainder of the experiment.

### 3.3.6. Blood sampling protocol for PK study

Arterial blood was withdrawn from the carotid cannula into lithium heparin tubes and placed on ice. Blood samples (200 µl) were collected (prior to alfaxalone) time 0, end of loading dose (2.5 min) and at 10, 30, 60, 90, 120 minutes after commencing the infusion. A final sample at the end of the subsequent electrophysiology experiment was also taken prior to termination (S1). Arterial blood gases, biochemistry and haematology parameters (pH, $P_aCO_2$, $P_aO_2$, bicarbonate, sodium, potassium, chloride, calcium, glucose, lactate and creatinine concentrations) were

also measured (EPOC, Woodley Instrumentation, Bolton, Lancashire, UK). All rats received an equal volume of balanced electrolyte solution after sampling (Vetivex 11 (Hartmann's), Dechra, Shrewsbury, UK). Samples were centrifuged (4000*g* for 10 minutes) within 30 minutes of collection. Plasma was harvested and stored at -20°C until determination of plasma alfaxalone concentration (Xenogesis, Biocity, Nottingham, UK).

### 3.3.7. Sample analyses

Plasma samples were analysed for alfaxalone using a liquid chromatography-mass spectrometry (LC-MS/MS) method (Xenogesis, Biocity, Nottingham, UK). Methanolic standard curve and quality control (QC) spiking solutions were produced for alfaxalone from separate accurate weighings of solid compound. Standards and QCs were prepared by spiking 10 µl spike solution into a solution of 20 µl plasma + 30 µl water + 40 µl methanol + 150 µl methanol containing 1000 nM tolbutamide as internal standard. Plasma standard curves were prepared from 100 – 5000 ng $ml^{-1}$ and QCs were prepared for 250 and 2500 ng $ml^{-1}$. Blank male or female plasma was used for the standards and QC solutions (Charles River, Margate, UK). The plasma samples were prepared by adding 30 µl water + 50 µl methanol + 150 µl methanol containing 1000 nM tolbutamide as internal standard to 20 µl plasma. Samples, standards and QCs were then mixed and stored in a freezer at -20°C for a minimum of 120 minutes prior to centrifugation at 4000*g* for 20 minutes. The samples were extracted and analysed using a Micromass Quattro Premier mass spectrometer incorporating an Acquity autosampler (Waters, Elstree, UK). An ACE Excel 2 C18-AR 50 x 2.1mm column was used with the following LC conditions:

solvent A = water + 0.1% formic acid, solvent B = methanol + 0.1% formic acid, flow rate = 0.8 ml $min^{1}$, column temperature = 60°C. LC gradient went from 95 % solvent A:5 % solvent B to 5 % solvent A:95 % solvent B over a 1.5 minute interval. The MS/MS method used electrospray positive mode with a 333.16 >107.01 transition for the detection of alfaxalone. The lower limit of quantification (LLOQ) was 100 ng $ml^{-1}$. The coefficients of variation of concentrations over the range studied from the LLOQ to the upper limit of quantification (ULOQ) (10000 ng $ml^{-1}$) varied between 0.2 and 16%. All samples were run in triplicate. Two separate LC-MS/MS runs were performed for the male and female samples, respectively. Samples were analysed within 28 days of collection based on data from analytical validation study file supporting stability of alfaxalone in rat plasma at -20°C for 30 days (Jurox, 2010).

### 3.3.8. PK analysis

Pharmacokinetic analysis was carried out using Phoenix WinNonlin 6.3 (Pharsight, Sunnyvale, CA, USA). The pharmacokinetic parameters (clearance, volume of distribution and half-life) for each individual rat were estimated according to best fit from an IV infusion one compartmental pharmacokinetic model, based on previous published data showing a single exponential decay, with appropriate weighting for best fit. The appropriate weighting for best fit in this study was based on examination of the residuals showing random scatter around predicted values using 1/^ weighting by 1/reciprocal of the predicated value (Rowland and Tozer 2011).

The pharmacokinetic parameters for log transformed parameter data for both sexes were compared using an unpaired, two tailed Student's *t*-test or a two-way ANOVA with Tukey's multiple comparison test (section 2.8.).

### 3.3.9. Electrophysiological recordings

Electrophysiological recordings commenced at least 90 minutes after the alfaxalone infusion started and EMG responses were recorded in MG, BF and TA as described in Chapter 2 (section 2.6.2.). Subsequently an acute noxious conditioning stimulus comprising 100 µl capsaicin (preparation as described in section 2.6.3.) was injected via a 25G needle into the muscle mass of *extensor carpi radialis* and *pronator teres* of the right (contralateral) forelimb. Reflexes were then collected and measured every 2 min for a minimum of 63 min (i.e. a total of 32 stimulations, 16 heel and 16 toes). Experiments were terminated by an overdose of pentobarbitone, followed by cervical dislocation.

### 3.3.10 Vaginal smears

All female rats in the PK arm of the initial study underwent vaginal swabbing immediately after euthanasia to characterise vaginal smear cell types. Slides were examined under x40 and then x100 magnifications (BH2 microscope, Olympus, Southend-on-Sea, UK) after staining with modified Giemsa (Diff Quik, Vet Direct, Newcastle upon Tyne, UK) and cell types and numbers were recorded (Cora et al. 2015). The proeostrus phase was defined by a majority of round, nucleated cells, and an absence of leucocytes. The oestrus phase was signified by a majority of

cornified cells and an absence of leucocytes and the metoestrus phase was signified by the presence of round nucleated cells, cornified cells, and leucocytes. The dioestrus phase was defined by a presence of all three cell types but a reduced number of cells compared to the other phases.

### 3.3.11 Statistical analysis

Statistical analysis was performed as detailed in Chapter 2 (section 2.8.).

## 3.4. RESULTS

### 3.4.1. Anaesthesia (both sexes study)

All animals underwent a total of 95 ± 2 minutes of isoflurane in $O_2$ and $N_2O$ anaesthesia followed by 230 ± 10 minutes of alfaxalone anaesthesia. The plane of anaesthesia was continually evaluated by serial cardiopulmonary measurements, blood gas analysis and reflex responses. Subjective evaluation of the hypnotic effect of alfaxalone in all rats in the first study was excellent. No rat demonstrated gross purposeful movement or required a change in infusion rate to improve the plane of anaesthesia.

### 3.4.2. Anaesthesia (female only study)

Of the eight female rats included in the second study, seven successfully completed electrophysiological measurements using the reduced alfaxalone infusion rates. For one animal the plane of anaesthesia was insufficient (as evidenced by twitching, piloerection, increased heart rate and respiratory rate and withdrawal of the hindlimb in response to electrode placement) to allow collection of baseline data or injection of capsaicin into the forelimb.

### 3.4.3. PK of alfaxalone in both sexes

The shape of the concentration-time curve following a loading dose and then constant rate infusion was typical of those observed for anaesthetic induction drugs

exhibiting an initial steep phase after the loading dose followed by a gradual increase until steady state was achieved (Figure 3.2) (Hill 2004).

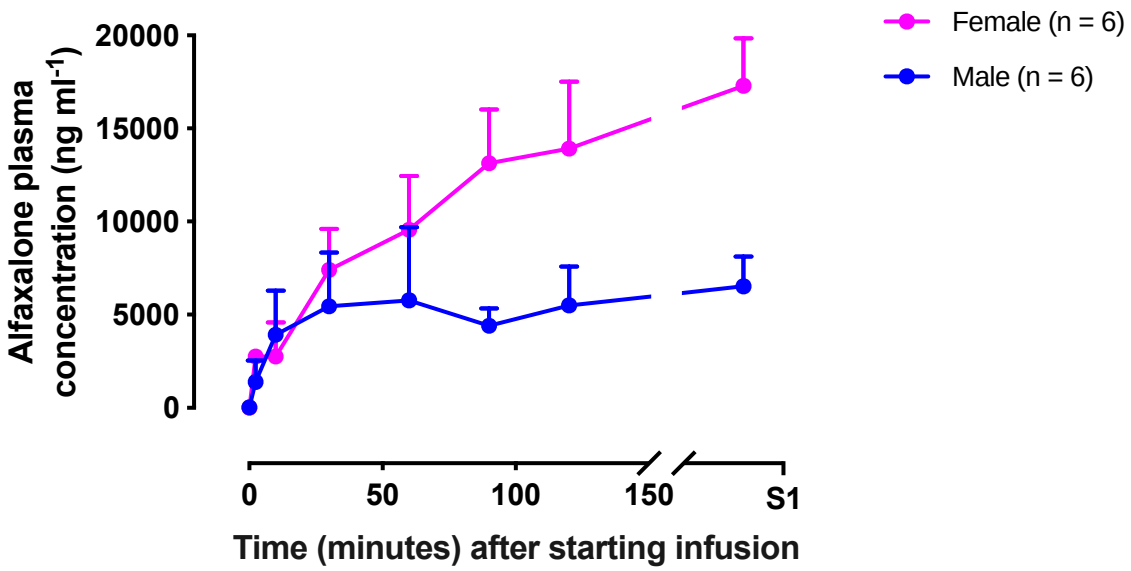



Figure 3.2 Plasma concentration-time profiles of male and female rats during alfaxalone infusion. A loading dose of 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by a constant rate infusion of 0.75 mg $kg^{-1}$ $min^{-1}$ was administered to male and female rats. S1 = euthanasia (230 minutes ± 10). Data are mean ± SEM.

Pharmacokinetic parameters calculated by an IV infusion, one compartmental model, are shown in Table 3.1. Logarithmic transformed data for clearance (CL) and half-life ($t_{1/2}$) was significantly increased for male rats compared to female rats (P = 0.0008, P = 0.03 respectively, Student's *t*-test). Furthermore, because of the relationship between CL, mean residence time (MRT) and volume of distribution at steady state (Vdss) [Vdss = CL x MRT], MRT was also significantly greater in the female rats compared to the male rats (P = 0.03, Student's *t*-test). There was no significant difference between the sexes for volume of distribution and maximum plasma concentration.

Table 3.1 Pharmacokinetic parameters after alfaxalone infusion

| **Female animals** | **CL (ml $kg^{-1}$ $min^{-1}$)** | **t ½ (min)** | **Vdss (L $Kg^{-1}$)** | **MRT (min)** | **Cmax (mg $L^{-1}$)** |
|---|---|---|---|---|---|
| 1 | 66.3 | 33.8 | 3.24 | 48.8 | 1.3 |
| 2 | 40.4 | 29.8 | 1.74 | 43.0 | 2.3 |
| 3 | 46.9 | 76.7 | 5.19 | 110.7 | 0.8 |
| 4 | 58.2 | 30.9 | 2.60 | 44.6 | 1.6 |
| 5 | 31.9 | 69.2 | 3.19 | 99.9 | 1.3 |
| 6 | 42.3 | 34.1 | 2.08 | 49.2 | 2.0 |
| Mean (± SEM) | 47.7 (5.13) | 45.8 (8.68) | 3.00 (0.5) | 66.0 (12.5) | 1.5 (0.2) |
| **Male animals** | **CL (ml $kg^{-1}$ $min^{-1}$)** | **t ½ (min)** | **Vdss (L $Kg^{-1}$)** | **MRT (min)** | **Cmax (mg $L^{-1}$)** |
| 1 | 65.8 | 52.1 | 4.94 | 75.1 | 0.8 |
| 2 | 79.9 | 13.2 | 1.53 | 19.1 | 2.6 |
| 3 | 78.5 | 17.3 | 1.95 | 24.9 | 2.0 |
| 4 | 117.9 | 10.3 | 1.75 | 14.8 | 2.2 |
| 5 | 106.2 | 18.6 | 2.85 | 26.8 | 1.4 |
| 6 | 101.2 | 26.5 | 3.87 | 38.3 | 1.0 |
| Mean (± SEM) | 91.6 (8.11)*** | 23.0 (6.24)* | 2.82 (0.55) | 33.2 (9.0)* | 1.7 (0.29) |

All rats were administered 1.67 mg $kg^{-1}$ $min^{-1}$ alfaxalone for 2.5 minutes followed by a 0.75 mg $kg^{-1}$ $min^{-1}$ constant rate infusion of alfaxalone for 120 minutes. CL = clearance, $t_{1/2}$= terminal half-life, Vdss = volume of distribution at 120 minutes, MRT = mean residence time, Cmax = maximum plasma concentration. Asterisks denote significant difference between sexes * $P < 0.05$, *** $P < 0.001$ (Student's *t* test).

The pharmacokinetic parameters from the first study were used to model an appropriate reduction in infusion rate for female rats to produce plasma concentrations comparable with the males. The plasma concentration time profiles from the second study (reduced infusion rate) are shown in Figure 3.3. The plasma concentrations from the first (both sexes) experiments are shown for comparison.

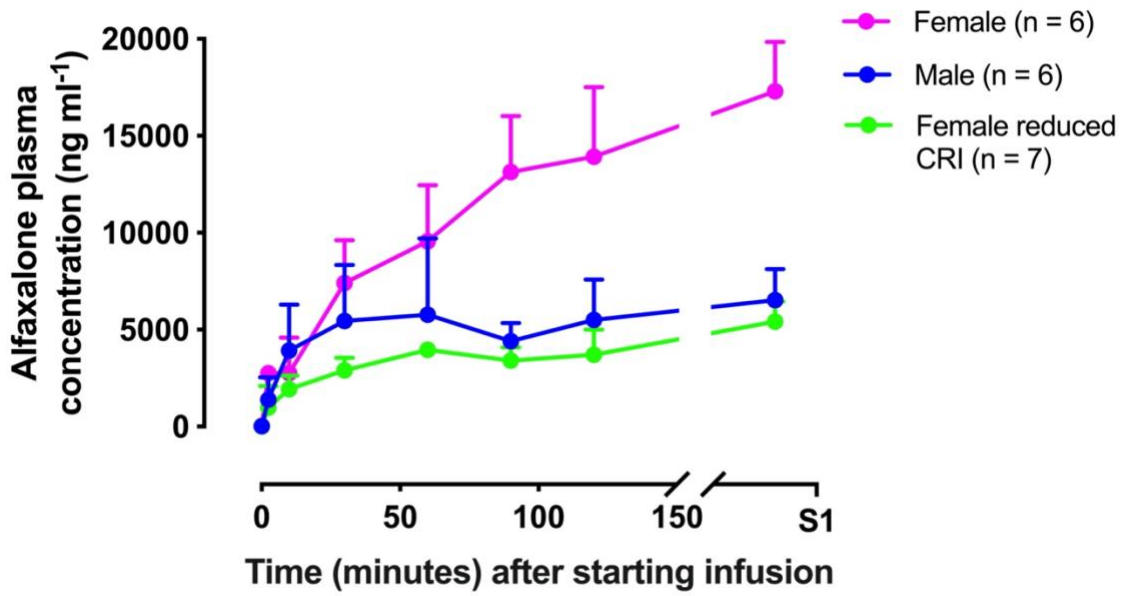



Figure 3.3 Plasma concentration-time profiles for all rats during alfaxalone infusion The green line represents the female rats that received a reduced infusion of alfaxalone: loading dose of 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by 0.56 mg $kg^{-1}$ $min^{-1}$ for 60 minutes followed by 0.42 mg $kg^{-1}$ $min^{-1}$ for the remainder of the experiment. The male and female rats (blue and pink lines) administered the higher alfaxalone dose (1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by an infusion of 0.75 mg $kg^{-1}$ $min^{-1}$) are included for comparison. S1 = euthanasia (230 minutes ± 10). Data are mean ± SEM.

### 3.4.4. PD of alfaxalone in both sexes

Table 3.2 includes cardiopulmonary data from both experiments. After a 2.5-minute loading dose all rats showed an initial short-lived decrease in heart rate, arterial blood pressure and respiratory rate as a result of concomitant administration of inhalant and alfaxalone. Thereafter heart rate remained stable during alfaxalone anaesthesia and there was no significant difference between the sexes at any time points up to 120 minutes ($P > 0.05$, Two-way ANOVA). Heart rate, blood pressure (mean, systolic, diastolic) and respiratory rate were not significantly different between male and female rats during isoflurane (baseline) anaesthesia ($P > 0.05$, ANOVA). In 4/12 animals (2 males and 2 females) cardiopulmonary depression, indicated by a decrease in blood pressure or respiratory rate, necessitated discontinuation of isoflurane and $N_2O$ before the 2.5 minute time point. Within 60 seconds of discontinuation of isoflurane and $N_2O$, heart rate and blood pressure began to rise in all animals. Subsequently systolic, mean and diastolic arterial pressures all increased under alfaxalone anaesthesia, reached a peak (between 60 and 90 minutes) and thereafter showed a trend of decreasing with time. Within the next 5 minutes following discontinuation of isoflurane and $N_2O$, all male rats demonstrated a significant increase in blood pressure from baseline under isoflurane and $N_2O$ anaesthesia (Figure 3.4).

Table 3.2 Cardiopulmonary parameters prior to and during alfaxalone infusion

| Variable | Sex | Minutes relative to alfaxalone infusion start time | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | -5 | 2.5 | 10 | 30 | 60 | 90 | 120 | S1 |
| MAP (mmHg) | Male | 79 ± 3 | 71 ± 3 | 110 ± 13* | 127± 7**** | 138 ± 5**** | 135 ± 7**** | 132 ± 4**** | 112 ± 8** |
| | Female | 89 ± 5 | 90 ± 8 | 110 ± 7 | 114 ± 7 | 121 ± 6** | 113 ± 9 | 114 ± 9 | 105 ± 5 |
| | Female R | 103 ± 10 | 109 ± 10 | 96 ± 20 | 85 ± 17 | 138 ± 9 | 130 ± 5 | 95 ±11 | 109 ± 7 |
| SAP (mmHg) | Male | 98 ± 3 | 93 ± 4 | 132 ± 13** | 153 ± 9**** | 167 ± 7**** | 167 ± 7**** | 158 ± 5**** | 137 ± 3** |
| | Female | 108 ± 5 | 111 ± 7 | 127 ± 6 | 133 ± 6 | 144 ± 6** | 138 ± 10* | 138 ± 10* | 133 ± 4 |
| | Female R | 106 ± 11 | 99 ± 9 | 145 ± 6 | 137 ± 8 | 149 ± 6 | 148 ± 9 | 160 ± 11 | 155 ± 5 |
| DAP (mmHg) | Male | 69 ± 3 | 60 ± 4 | 98 ± 13* | 114 ± 6*** | 124 ± 4**** | 119 ± 8**** | 120 ± 3**** | 100 ± 10* |
| | Female | 79 ± 6 | 79 ± 9 | 101 ± 8 | 104 ± 8 | 109 ± 6* | 100 ± 9 | 101 ± 8 | 91 ± 5 |
| | Female R | 74 ± 9 | 62 ± 7 | 105 ± 5 | 99 ± 6 | 113 ± 7 | 124 ± 9 | 121 ± 10 | 119 ± 4 |
| HR (beats $min^{-1}$) | Male | 440 ± 25 | 442 ± 28 | 440 ± 15 | 455 ± 23 | 447 ± 28 | 438 ± 34 | 423 ± 23 | 455 ± 23 |
| | Female | 440 ± 31 | 440 ± 31 | 436 ± 35 | 455 ± 23 | 445 ± 22 | 415 ± 12 | 422 ± 16 | 418 ± 18 |
| | Female R | 445 ± 16 | 450 ± 14 | 432 ± 12 | 430 ± 17 | 436 ± 23 | 420 ± 18 | 400 ± 20 | 410 ± 23 |
| RR (breaths $min^{-1}$) | Male | 58 ± 12 | 55 ± 10 | 56 ± 10 | 68 ± 10 | 57 ± 14 | 65 ± 7 | 71 ± 9 | 69 ± 15 |
| | Female | 56 ± 10 | 54 ± 7 | 56 ± 6 | 55 ± 5 | 63 ± 5 | 68 ± 4 | 69 ± 4 | 69 ± 8 |
| | Female R | 52 ± 8 | 50 ± 12 | 56 ± 8 | 52 ± 12 | 58 ± 13 | 60 ± 10 | 54 ± 12 | 58 ± 12 |

Mean (MAP), systolic (SAP) and diastolic (DAP) arterial blood pressures (mmHg), heart rate (HR) (beats $min^{-1}$) and respiratory rate (RR) (breaths $min^{-1}$) during isoflurane anaesthesia (-5), then at time points after cessation of isoflurane and during administration of alfaxalone. Both studies are included, the first with the higher infusion of alfaxalone 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by constant rate infusion at 0.75 mg $kg^{-1}$ $min^{-1}$ in males and females, and the second experiment in females with a lower infusion rate (Female R) 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by 0.56 mg $kg^{-1}$ $min^{-1}$ for 60 minutes followed by 0.42 mg $kg^{-1}$ $min^{-1}$ for the remainder of the experiment. All values are means ± SEM. S1 = euthanasia. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ or **** $P < 0.0001$ denote significant difference from baseline (5 min prior to starting alfaxalone infusion) (Two way ANOVA).

Overall mean arterial blood pressure was significantly higher in male than female rats ($P = 0.026$, Two-way ANOVA), however the interaction with time ($P < 0.0001$) differed between the sexes and makes the interpretation of these data difficult (Figure 3.4). Significant differences were analysed *post hoc* using Sidak's multiple comparison test. Mean arterial blood pressure was significantly increased compared to baseline in male rats at 30, 60, 90 and 120 minutes from starting the alfaxalone ($P < 0.0005$). Mean arterial blood pressure was only significantly greater than baseline (during isoflurane anaesthesia) in the female rats at 60 minutes. Systolic and diastolic blood pressures showed similar trends with time and between the sexes. Mean arterial blood pressure was not significantly different to the previous group of male and female rats that received alfaxalone at the higher infusion rate at any time point ($P > 0.05$, Two-way ANOVA) (Figure 3.4). Blood pressure rose steadily during the first hour of alfaxalone anaesthesia and was maintained well above values recorded during isoflurane anaesthesia. There was a marked individual variation but no difference between the groups. Most animals showed an acute short-lived blood pressure rise immediately after injection of capsaicin into the contralateral forelimb. The median duration of the rise was 12 minutes (range 4–16 min), and the median increase was 46 mmHg (range 30–62 mmHg) for systolic and 30 mmHg (range 8–44 mmHg) for diastolic pressure. Occasionally a second rise in blood pressure was seen between 20 and 30 minutes after the first rise of a smaller magnitude and shorter lived than the first. Some animals exhibited a biphasic rise then fall to values below the baseline values measured before capsaicin injection.

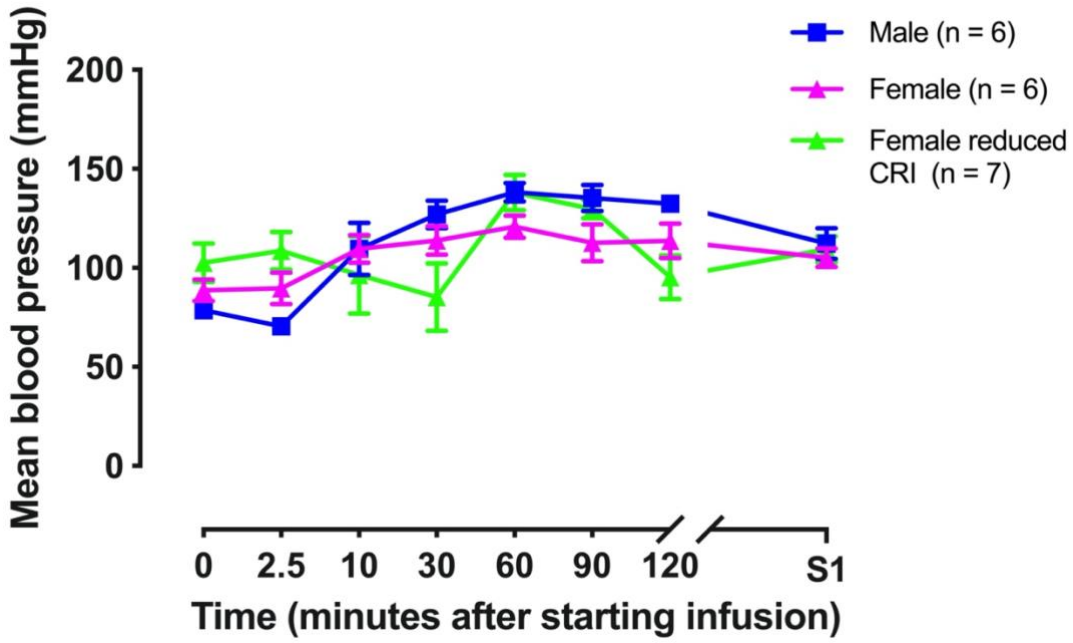



Figure 3.4 Mean arterial blood pressure of all rats during the experiment. Female rats received a reduced alfaxalone CRI: loading dose of 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by 0.56 mg $kg^{-1}$ $min^{-1}$ for 60 minutes followed by 0.42 mg $kg^{-1}$ $min^{-1}$ for the remainder of the experiment. The male and female rats were administered the higher alfaxalone dose: 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by a constant rate infusion of 0.75 mg $kg^{-1}$ $min^{-1}$. S1 = euthanasia (230 min ± 10). Data are mean ± SEM.

Four male rats and four female rats required transient intermittent positive pressure ventilation (IPPV) for varying amounts of time during anaesthesia in response to a decreasing respiratory rate and in order to maintain end tidal $CO_2$ within normal range (40–45 mmHg).

Blood gas parameters and biochemistry values from the first experiment are presented in Table 3.3. There were no significant differences between sexes for these parameters except for pH, which was significantly lower in female rats in conjunction with higher partial pressures of carbon dioxide ($P$ = 0.0027, Student's $t$ test). Partial pressures of oxygen ($P_aO_2$ mmHg) tended to be different between baseline and subsequent time points, trending towards higher values under total

intravenous anaesthesia using alfaxalone compared to inhalational anaesthesia. Glucose concentrations tended to decrease with time as would be expected with glucose consumption. Blood gas samples at the same timepoints were not available for the second experiment.

Table 3.3 Blood gas variables and clinical biochemistry parameters

| Variable | Sex | Minutes relative to the alfaxalone infusion start time | | | | |
|---|---|---|---|---|---|---|
| | | -5 | 30 | 60 | 90 | 120 |
| pH* | Male | 7.36 ± 0.04 | 7.42 ± 0.05 | 7.44 ± 0.03 | 7.41 ± 0.03 | 7.40 ± 0.02 |
| | Female | 7.36 ± 0.06 | 7.36 ± 0.06 | 7.35 ± 0.04 | 7.32 ± 0.04 | 7.37 ± 0.03 |
| $P_aCO_2$ (mmHg) | Male | 36 ± 2 | 35 ± 5 | 39 ± 2 | 40 ± 2 | 30 ± 2 |
| | Female | 40 ± 7 | 44 ± 9 | 44 ± 7 | 43 ± 5 | 37± 3 |
| $P_aO_2$ (mmHg) | Male | 140 ± 5 | 218 ± 8 | 249 ± 5 | 258 ± 7 | 276 ± 7 |
| | Female | 190 ± 13 | 318 ± 53 | 265 ± 22 | 263 ± 15 | 219 ± 29 |
| Bicarbonate (mmol $L^{-1}$) | Male | 23 ± 2 | 25 ± 0.8 | 23 ± 1.2 | 23 ± 1.2 | 21 ± 1.2 |
| | Female | 24 ± 1.6 | 23 ± 1.6 | 22 ± 1.2 | 21 ± 1.2 | 21 ± 4 |
| Sodium (mmol $L^{-1}$) | Male | 142 ± 0.1 | 143 ± 0.1 | 142 ± 1.6 | 143 ± 0.1 | 145 ± 1 |
| | Female | 144 ± 0.4 | 143 ± 1.2 | 147 ± 0.8 | 148 ± 2 | 150 ± 2 |
| Potassium (mmol $L^{-1}$) | Male | 4.6 ± 0.04 | 4.8 ± 0.08 | 4.8 ± 0.45 | 4.7 ± 0.37 | 4.5 ± 0.9 |
| | Female | 5.2 ± 0.37 | 4.6 ± 0.5 | 4.8 ± 0.37 | 4.7 ± 0.45 | 5 ± 0.65 |
| Ionized Calcium (mmol $L^{-1}$) | Male | 1.50 ± 0.04 | 1.50 ± 0.07 | 1.47 ± 0.01 | 1.37 ± 0.14 | 1.34 ± 0.2 |
| | Female | 1.46 ± 0.1 | 1.50 ± 0.05 | 1.51 ± 0.07 | 1.43 ± 0.1 | 1.37 ± 0.14 |
| Chloride (mmol $L^{-1}$) | Male | 110 ± 2 | 116 ± 2 | 116 ± 2 | 114 ± 3 | 117 ± 2 |
| | Female | 114 ± 3 | 110 ± 3 | 116 ± 2 | 117 ± 3 | 114 ± 2 |
| Glucose (mmol $L^{-1}$) | Male | 12.4 ± 1.2 | 7.1 ± 1.5 | 7.02 ± 1.3 | 5.8 ± 0.08 | 5.8 ± 1.1 |
| | Female | 13 ± 1 | 7.8 ± 0.98 | 6.3 ± 1.3 | 5.4 ± 0.4 | 5.9 ± 1.6 |
| Lactate (mmol $L^{-1}$) | Male | 1.1 ± 0.29 | 1.0 ± 0.01 | 1.0 ± 0.04 | 1.1 ± 0.08 | 0.6 ± 0.04 |
| | Female | 1.0 ± 0.04 | 1.1 ± 0.16 | 1.0 ± 0.12 | 1.1 ± 0.2 | 0.6 ± 0.04 |
| Creatinine (µmol $L^{-1}$) | Male | 20 ± 2.9 | 35 ± 6.5 | 28 ± 5.3 | 37 ± 5.3 | 33 ± 8.2 |
| | Female | 27 ± 5 | 33 ± 5.7 | 29 ± 5.7 | 37 ± 7.8 | 34 ± 5.7 |

All rats (6 female, 6 male) were administered alfaxalone 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by 0.75 mg $kg^{-1}$ $min^{-1}$ constant rate infusion of alfaxalone for 230 ± 10 minutes. Data are mean ± SEM. Derived variables reported by the EPOC analyzer (actual bicarbonate, total $CO_2$, base excess of extra cellular fluid, base excess of blood, oxygen saturation, anion gap, anion gap potassium, haemoglobin) were calculated but are not included in the table. The baseline (-5 minute) samples were all measured under isoflurane (2% vaporizer setting) in $N_2O$ and $O_2$ (2:1). $P_aCO_2$: partial pressure of carbon dioxide, $P_aO_2$: partial pressure of oxygen. * $P < 0.05$ indicates a significant difference between sexes (Student's *t* test).

### 3.4.5. Vaginal smears

All slides were evaluated from the six females included in the first PK study to give an impression of the smear, rather than exact cell counts. Three rats were characterized as in dioestrus, one in proestrus, one in oestrus, and one in metoestrus.

### 3.4.6. Demographics and stimulus intensities to elicit reflexes

Male rats (399 ± 14g) were significantly heavier than the female rats (283 ± 6g), (304 ± 4g) in both studies ($P < 0.0001$, one-way ANOVA). No significant differences were apparent between groups with respect to electrical thresholds at the heel and toes or stimulation strength (Table 3.4). The maximum stimulus strength (10 mA) was not required for any female undergoing a reduced infusion whereas this was required for eliciting a heel-MG reflex in one male and one female from groups receiving the higher infusion rate.

Table 3.4 Demographics, electrical thresholds and stimulation strengths

| | Male | Female | Female (reduced CRI) | *P* value |
|---|---|---|---|---|
| Number of animals | 14 | 12 | 7 | - |
| Weight (g) | 399 ± 14 | 283 ± 6 | 304 ± 4 | 0.0001 |
| MG electrical threshold, heel (mA) | 2.2 (1.1–3.9) | 2.8 (1.2–3.8) | 1.2 (1.0–1.5) | 0.097 |
| Heel stimulation strength (mA) | 5.0 (3.9–6.5) | 6.0 (3.2–7.9) | 5.0 (5.0–5.0) | 0.95 |
| BF/TA electrical threshold, toes (mA) | 1.0 (0.8–2.0) | 1.5 (1.0–2.3) | 1.0 (0.7–1.4) | 0.32 |
| Toes stimulation strength (mA) | 3.5 (2.5–4.5) | 4.3 (2.8–5.6) | 6.0 (5.0–7.0) | 0.053 |

Demographics (weight, electrical thresholds, electrical stimulation strengths) for male and female Sprague Dawley rats with equivalent alfaxalone infusion rates and females with a reduced alfaxalone infusion. CRI: constant rate infusion, MG: *medial gastrocnemius*, BF: *biceps femoris*, TA: *tibialis anterior*. Data are mean ± SEM or median (IQR). Significance tested using ANOVA or Kruskal-Wallis test followed by Dunn's multiple comparison test.

### 3.4.7. Raw control responses

Median raw control responses for heel-MG for the female animals receiving the reduced infusion were 389 μV.ms (IQR 279–698 μV.ms) and were significantly greater ($P$ = 0.0021, Kruskal Wallis) than those raw control responses in the male 140 μV.ms (IQR 78–233 μV.ms) or the female animals 160 μV.ms (180–197 μV.ms) receiving the higher infusion. There were no significant differences between groups for median control responses for toes-BF or toes-TA (Figure 3.5).

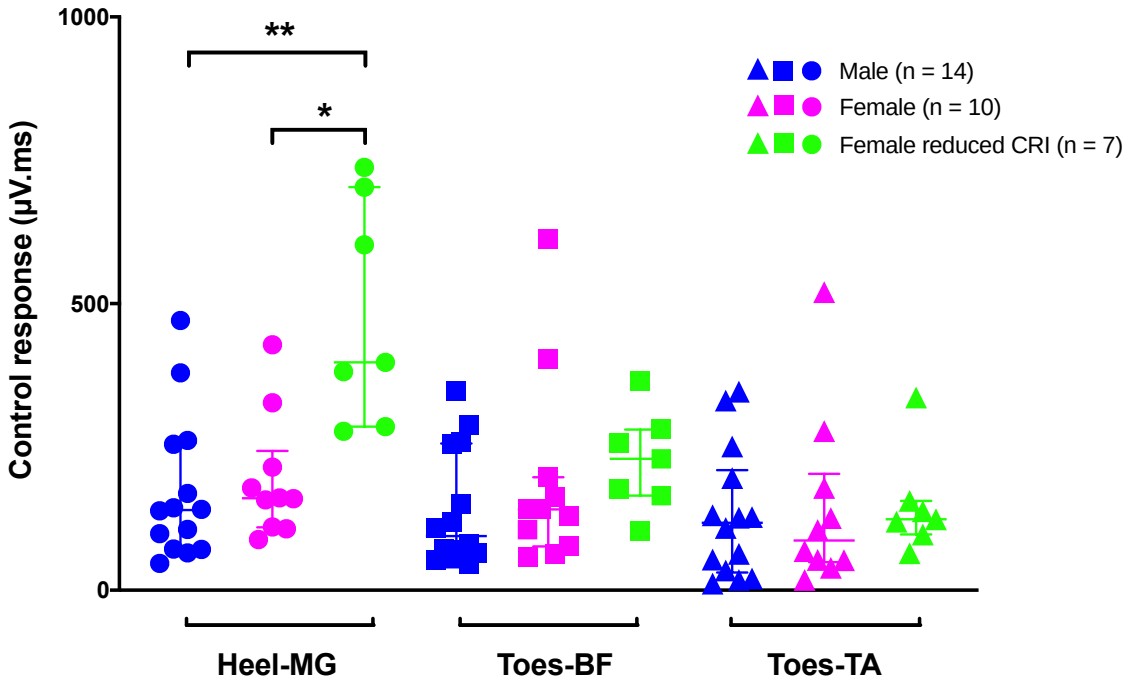



Figure 3.5 Raw control responses for heel and toes' reflexes prior to injection of capsaicin in naïve male and female Sprague Dawley rats. One male and one female group (designated by blue and pink symbols respectively) received an alfaxalone loading dose of 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by 0.75 mg $kg^{-1}$ $min^{-1}$ maintenance rate. A second female group (green symbols) received a loading dose of 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by 0.56 mg $kg^{-1}$ $min^{-1}$ maintenance rate for 60 minutes followed by 0.42 mg $kg^{-1}$ $min^{-1}$ for the remainder of the experiment. Heel-MG: heel-*medial gastrocnemius*, Toes-BF: toes-*biceps femoris*, Toes-TA: toes-*tibialis anterior*. Data are median and IQR.

The raw control responses for the heel-MG reflex during the reduced infusion rate was significantly greater (Kruskal-Wallis test, followed by Dunn's multiple comparison test) when compared to the higher male and female infusion rates. $P = 0.0015$ and $P = 0.033$ respectively.

### 3.4.8. The effect of capsaicin on reflex responses

No significant inhibition by the conditioning injection of capsaicin was seen for heel-MG in both male and female rats ($P > 0.05$, Friedman's ANOVA) (Figure 3.6A). In contrast to males and females receiving the higher dose of alfaxalone where no heel-MG inhibition was observed, capsaicin induced a significant inhibition ($P = 0.0003$, Friedman's ANOVA) of heel-MG reflexes to a median of 71% (IQR 69–77%) compared to baseline at 19 minutes in the female rats undergoing the reduced CRI (Figure 3.6A). The median recovery time was 61 minutes (IQR 38–61 min).

For male rats, toes-BF reflexes were significantly inhibited ($P < 0.0001$, Friedman's ANOVA) to a median of 55% (IQR 33–69%) of pre-capsaicin controls at 19 min post-injection whereas significant inhibition ($P = 0.013$ Friedman's ANOVA) of this reflex in females was less profound (median inhibition of 74% (IQR 71–114%) at 19 min) (Figure 3.6B). Furthermore, a comparison of the negative area under the curve (AUC) (Mann-Whitney test) indicated a significant difference between male and female animals receiving the same infusion rate of alfaxalone for toes-BF reflexes ($P = 0.024$) (Figure 3.6E). For the females receiving the reduced CRI, significant inhibition ($P = 0.028$ Friedman's ANOVA) was apparent for toes-BF with a median inhibition of 72% (IQR 51–79%). (Figure 3.6B). A comparison of the negative AUC indicated a significant difference (Mann-Whitney test) between females receiving the same rate as the males and the females receiving the reduced CRI for toes-BF ($P = 0.033$). Duration of inhibition of the reflex was not significantly different between the sexes.

Significant capsaicin-induced inhibition of toes-TA reflexes in males ($P < 0.0001$, Friedman's ANOVA) and females ($P < 0.02$, Friedman's ANOVA) was evident to a median of 57% (IQR 42–67%) and 65% (IQR 24–84%) respectively at 19 min post-injection (Figure 3.6C). Duration of inhibition of the reflex was not significantly different between sexes. For the females receiving the reduced infusion toes-TA was significantly inhibited ($P = 0.0099$, Friedman's ANOVA) to a median of 74% (IQR 64-85%) of pre-capsaicin controls at 19 minutes. A comparison of the negative AUCs (Kruskal-Wallis test) indicated no significant differences between male and female animals and female animals receiving a reduced dose for heel-MG and toes-TA.

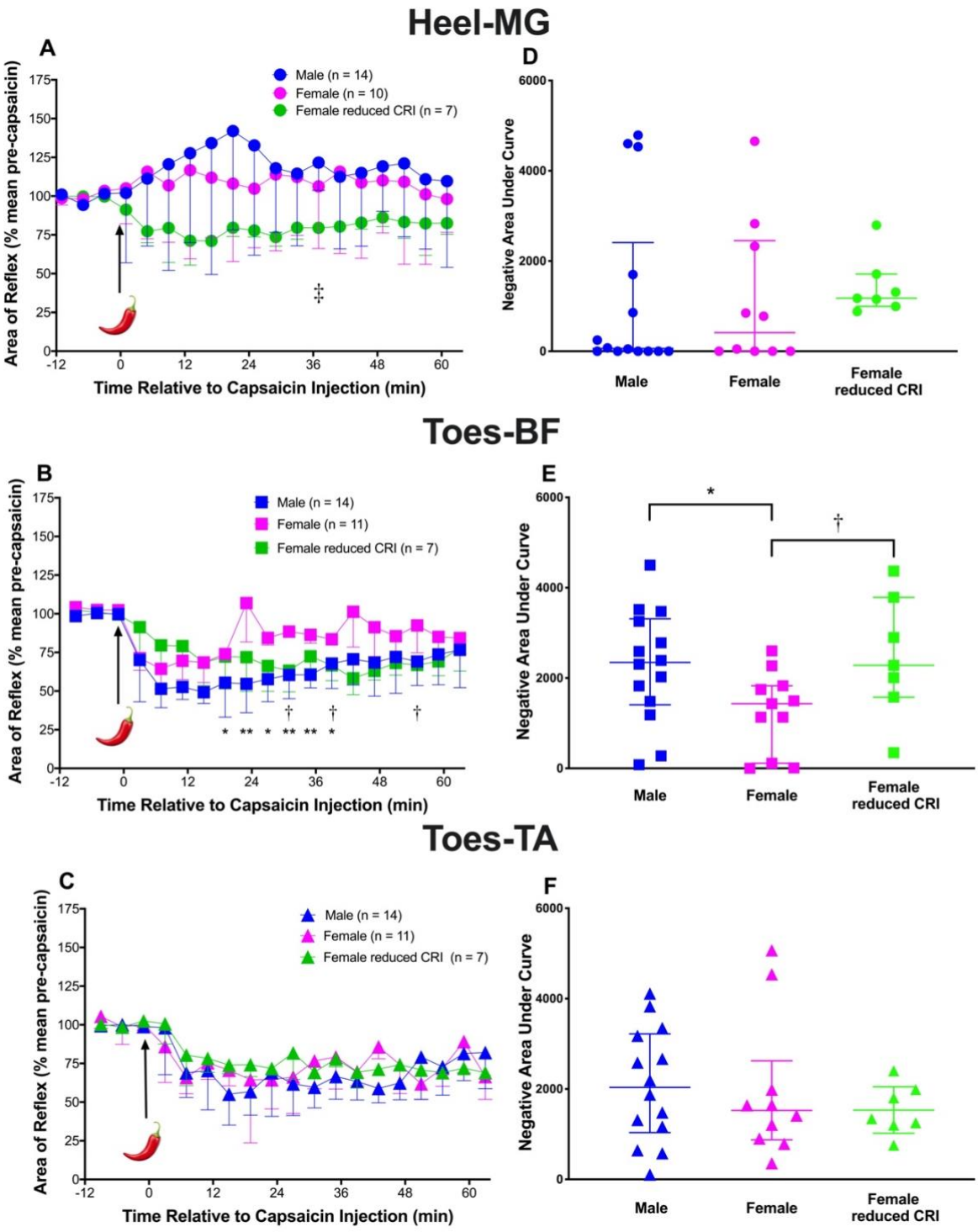

Heel-MG
A
Male (n = 14)
Female (n = 10)
Female reduced CRI (n = 7)
Area of Reflex (% mean pre-capsaicin)
Time Relative to Capsaicin Injection (min)
D
Negative Area Under Curve
Male
Female
Female reduced CRI
Toes-BF
B
Male (n = 14)
Female (n = 11)
Female reduced CRI (n = 7)
Area of Reflex (% mean pre-capsaicin)
Time Relative to Capsaicin Injection (min)
E
Negative Area Under Curve
Male
Female
Female reduced CRI
Toes-TA
C
Male (n = 14)
Female (n = 11)
Female reduced CRI (n = 7)
Area of Reflex (% mean pre-capsaicin)
Time Relative to Capsaicin Injection (min)
F
Negative Area Under Curve
Male
Female
Female reduced CRI

Figure 3.6 Effect of capsaicin on heel- and toe-evoked reflexes in male and female Sprague Dawley rats. Capsaicin was injected (arrow) into the contralateral forelimb and effects measured on heel-*medial gastrocnemius* (heel-MG) (A), toes-*biceps femoris* (toes-BF) (B) and toes-*tibialis anterior* (toes-TA) (C) reflexes in the male and female groups. The first male and female groups received a loading dose of 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by 0.75 mg $kg^{-1}$ $min^{-1}$ maintenance rate, and a second female group received a loading dose of 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by 0.56 mg $kg^{-1}$ $min^{-1}$ maintenance rate for 60 minutes followed by 0.42 mg $kg^{-1}$ $min^{-1}$ for the remainder of the experiment. Negative area under the curve (AUC) values following capsaicin injection into the contralateral forelimb on heel-MG (D), toes-BF (E) and toes-TA reflexes (F) for all three reflexes in male and female animals and female animals receiving the reduced infusion of alfaxalone after injection of capsaicin into the contralateral forelimb.

* $P < 0.05$, ** $P < 0.01$, indicates a significant difference between males and females (same infusion) at the designated time point (Mann-Whitney test). † $P < 0.05$ indicates a significant difference between females receiving different infusions at the designated time point (Mann-Whitney test). ‡ $P < 0.05$ designates differences between males and females receiving the reduced CRI (Mann-Whitney test).

The negative AUC for toes-BF was significantly greater (Mann-Whitney test; $P = 0.02$) in male rats compared to female rats receiving the same rate of infusion of alfaxalone. The negative AUC for toes-BF was also significantly greater (Mann-Whitney test; $P = 0.03$) for female rats receiving the reduced dose of alfaxalone when compared to the females receiving the standard male dose. * $P < 0.05$, † $P < 0.05$.

## 3.5. DISCUSSION

The major finding from this study is that consideration must be given to the dose of anaesthetic delivered to male and female subjects. Without this interrogation of the experimental model there remains the danger that studies will be carried out under what is assumed to be identical ‘planes’ or depths of anaesthesia, when in reality one sex may be more or less profoundly anaesthetized such that, for example, neurophysiological, hormonal or neuroendocrine responses will be affected.

### 3.5.1. PD and sex differences

Differences in efficacy of the older alfaxalone steroid (Alphathesin/Althesin) in male and female rats have been demonstrated and it was concluded that the influence of sex hormones was responsible for this discrepancy (Fink et al. 1982). Sex differences have also been found with alfaxalone administered intraperitoneally too (Arenillas and Gomez de Segura 2018). However one recent study disputed the sex differences and postulated that observed differences are more likely a result of the different formulations of alfaxalone and assay methodologies than differences between sexes (Lau et al., 2013). Sex based differences in drug metabolism are the primary cause of sex-dependent pharmacokinetics and reflect differences in the expression of hepatic enzymes active in the metabolism of many extrinsic and intrinsic chemicals, including cytochrome P450 (Waxman and Holloway 2009). Rodent studies have identified more than a 1000 genes whose expression is dependent on sex and these genes modulate liver metabolic function and create sexual dimorphism in liver function (Tanaka 1999). Differences in bioavailability, distribution, metabolism, and/or excretion in different sexes are multifactorial and

complicated (Soldin and Mattison 2009). Drug distribution can also be sex linked, influenced by factors such as body fat, plasma volume and differential perfusion of organs. However, in this study no significant difference was recorded between male and female Vdss suggesting this was not an issue. In general, however, sex differences in metabolism are thought to be the primary determinant of variation in pharmacokinetics and this is most likely the reason for the differences seen in this study (White et al. 2017). This contrasts to previous published studies, which have dismissed a sex difference (Ferré et al. 2006; Berry 2015).

Notwithstanding these views it has also been shown that formulation is hugely influential; the toxicity of alfaxalone in Wistar rats was less in those animals receiving alfaxalone dissolved in 7-sulfobutyl-ether-β-cyclodextrin compared to alfaxalone in Cremophor EL (Goodchild et al. 2015). The current study directly comparing male and female Sprague Dawley rats receiving a 2-hydroxypropyl-β-cyclodextrin alfaxalone formulation seemingly favours a true sex difference due to pharmacokinetics, pharmacodynamics or both as the explanation. Sex based studies using rats anaesthetised with pentobarbitone have also demonstrated differences (Zambricki and D'Alecy 2004). Sex differences have been detected in studies comparing intraperitoneal (IP) and IV routes in rats (Estes et al. 1990) postulating that the lack of obvious sex differences with single IV dosing may be a result of the short duration of effect. Other species for example, cats, display similar pharmacokinetic characteristics (Whittem et al. 2008) to this rat study. The characteristic rapid hepatic metabolic clearance of alfaxalone by the liver has been identified *in-vivo* and *in-vitro* in rats (Sear and McGivan 1980, 1981).

### 3.5.2. PD and plane of anaesthesia

The quality of anaesthesia during IV infusion of the neuroactive steroid alfaxalone was subjectively judged as excellent in all rats. Previous studies (Brammer et al. 1993) albeit using alphaxalone and alphadolone reported apnoea and providing a suitable surgical plane of anaesthesia as problematic especially at 180 minutes and beyond. In this current study, alfaxalone provided very good conditions for electrophysiological recordings, and survival beyond 180 minutes. A small number of other studies have used alfaxalone alone for assessing mechanisms underlying chronic pain control to good effect. Weersinghe et al. used 0.38–0.6 mg $kg^{-1}$ $min^{-1}$ alfaxalone IV in male rats after halothane anaesthesia for a UVB and heat rekindling model (Weerasinghe et al. 2014) and Kelly et al. used 0.68 mg $kg^{-1}$ $min^{-1}$ IV after isoflurane and $N_2O$ (Kelly et al. 2013) for EMG recording in male rats. One paper also reports the use of 0.42 mg $kg^{-1}$ $min^{-1}$ IV alfaxalone after halothane in male animals for EMG recordings (Hsieh et al. 2015). None of these papers however reported heart rates, blood pressures or respiratory rates, making evaluation of the plane of anaesthesia difficult.

In the current study there was no significant difference in heart rates between groups; blood pressure measurements were different in the face of different plasma concentrations, but differences were absent when plasma concentrations were reduced for the female rats. It should also be noted that the presence of isoflurane and $N_2O$ at the outset of the loading dose is likely to have affected cardiopulmonary parameters in the very early stages of the infusion and conclusions drawn about differences at these time points are likely to be tenuous, but could be compared with

those identical experiments where $N_2O$ is omitted (Chapter 4) in order to parse out the contributing effects of the $N_2O$ and isoflurane. In the current experiment, both sex groups underwent identical protocols so this influence would have been similar for both groups. The use of a volatile agent such as isoflurane with minimal metabolism and rapid elimination ensured that the period of time from ceasing administration was as short as possible; maximum possible duration of initial concurrent administration was 2.5 minutes.

The consistency of the haematology, biochemistry and blood gas values in all rats demonstrates the stability of the alfaxalone protocol for electrophysiology experiments. Blood gas values are infrequently reported for rodent anaesthetics, in part due to the technical nature of artery cannulation, and previously the volume of blood required (2 ml) made repeated sampling impossible due to a deleterious depletion of the blood volume of the animal (Nigam 2016). Newer point of care analysers are able to process much smaller volumes and can utilize capillary blood as well as arterial/venous samples (Cao et al. 2017). Total blood volume removed was well below the limit (Wolfensohn and Lloyd 2013) of 10% blood volume and the removal of blood in conjunction with a replenishment of balanced electrolyte solution clearly had no impact on the animals. All biochemistry and haematology values except chloride were similar to those provided by the supplier of the Sprague Dawley rats in age-matched conscious subjects. These findings demonstrate the healthy status of the animals during the 230 ± 10 minutes of anaesthesia. Invariably these samples were analysed with different machines, but even with slight discrepancies usually seen between laboratories, plasma chloride values in the study were different to those in conscious aged-matched Sprague Dawley rats (Appendix 1). A

moderate corrected hyperchloraemia was present when measured sodium values were also taken into consideration. The formula [corrected chloride = measured chloride x (normal sodium/measured sodium)] was used. The most likely cause was the use of heparinised saline during cannula placement and through flushing of the carotid cannula with heparinised saline to maintain patency for sampling and blood pressure measurement. No concurrent acidosis was observed, and sodium values were closely aligned between the supplied conscious values and those measured (Appendix 1). The side effects of normal saline administration have been discussed (Handy and Soni 2008) and the use of non-physiological saline and balanced electrolyte solutions is to be recommended. The impact of hyperchloraemia is unknown in this current study. For humans there is an increasing awareness that hyperchloraemia and hyperchloraemic acidosis can result in clinical consequences (Handy and Soni 2008).

### 3.5.3. Electrophysiology

Electrophysiological studies of DNIC of NWR found differences between the sexes. It is likely these differences were in the main a result of the PK differences of alfaxalone between the sexes; with terminal half-life and mean residence time significantly shorter in the males and a clearance of the alfaxalone approaching a rate twice as rapid in the males compared to the females. Blood pressure values were lower for female rats during identical alfaxalone infusion rates compared to males, as were heart rates (albeit not significantly) and these are also useful as markers of nociception (Haga et al. 2001; Rigaud et al. 2011). The conclusion that the female rats were more profoundly anaesthetised and therefore the reflexes more

impaired is a logical conclusion. The blood pH was significantly less in females as a result of the increased $P_aCO_2$ values, a likely consequence of the deeper plane of anaesthesia reducing the respiratory rate.

The NWRs in females were inhibited less profoundly and for a shorter duration compared to the males. These findings highlight the risk that using one sex may exacerbate the failure of Phase 1 trials or result in misleading conclusions. In countless studies the influence of the anaesthetic is ignored, or so poorly reported that repeating the experiment would be impossible. Although rats are commonly used in laboratory studies, it is typically males that are used to reduce the perceived experimental variability (Zucker and Beery 2010). The confines of this methodology is that translational basic science is potentially compromised by the use of one sex (Clayton and Collins 2014). This perceived variability associated with females because of the oetrous cycle is often used as a reason for excluding females from studies. A meta-analysis of 293 studies in both sexes in which murine behavioural, morphological, physiological, and molecular traits were closely evaluated showed variability was not significantly greater in females for any endpoint, but several traits contributed to substantially greater variability in the males in this analysis, including the influence of group housing (Prendergast et al. 2014).

Commendably, there is a sea change happening and a conscious effort to include more female subjects in studies. The International Association for the Study of Pain consensus report (Sex, Gender and Pain special interest group) suggested that pain research be conducted in both sexes and if this was restricted by practical considerations then the work should be tested in female animals only (Greenspan

et al. 2007). There are reports of sex affecting the response to acute nociceptive test procedures in the conscious animal; female rodents are often more sensitive to the nociceptive-producing effects of various types of stimuli including both thermal (hot-plate, warm water and radiant heat models) and mechanical pressure stimuli after acute presentation (Kest et al. 1999; Barrett et al. 2002; Terner et al. 2003; Cook 2004). Sex linked differences have been detected in WDR neurones in response to subcutaneous formalin, an effect that has been shown to be stimulus dependent and modulated by supraspinal centres (You et al. 2006). Furthermore, a difference in the frequency of the conditioning stimulus required for suppression, was also found between the sexes (You et al. 2006). Similarly individual variations have been shown between men and women's responses of pain and sensory thresholds to high frequency transcutaneous electrical nerve stimulation (Lund et al. 2005), and sex differences in the temporal characteristic of DNIC in human volunteers have been demonstrated too (Ge et al. 2004).

Historically the modulatory effects of the oestrogens have received most attention (Amandusson and Blomqvist 2013; Traub and Ji 2013; Rosen et al. 2017). However, there are also studies that demonstrate less sensitivity in females, thereby highlighting results are paradigm dependent and can be influenced by timing, dosing of analgesics and strain of animal as well as sex (Bartok and Craft 1997; Cook et al. 2000), and indeed some studies report no difference. Far fewer studies have reported on the involvement of testosterone in pain and analgesia (Claiborne et al. 2006); although it has been known for some time that castration augments the symptoms of adjuvant arthritis in rats (Harbuz et al. 1995) and testosterone administration to females can slow down the progression of some autoimmune

diseases (Liva and Voskuhl 2001; Gold and Voskuhl 2009). By virtue of the distribution of androgen receptors in brain regions associated with pain modulation (Murphy et al. 1999; Loyd and Murphy 2008) and a variety of testosterone mediated mechanisms proposed (Sorge et al. 2011; Niu et al. 2012; Torres-Chávez et al. 2012), it is likely testosterone plays more of role and may offer more promise than has been realised to date.

In view of the DNIC differences observed between the sexes in the first part of the study the next logical step was to perform a study with female rats receiving an appropriately modelled reduction in alfaxalone infusion to aim for similar plasma concentrations between the sexes, and then repeat the DNIC experiment. In this second part of the study, the reduced alfaxalone infusions used in the females resulted in similar plasma concentrations to the male animals and unmasked larger heel-MG raw reflexes and a more profound and long lasting DNIC in heel-MG and toes-BF in these females. The reduced plasma concentrations resulted in less change in DNIC in toes-TA compared to heel-MG and toes-BF. This true difference in DNIC observed between the sexes in the second experiment supports the hypothesis of true sexual dimorphism in pain processing but highlights how the anaesthesia *per se* can profoundly influence results.

Much of the work to date on sex differences has focused on the CNS, particularly opioid receptors and opioid analgesics (Craft 2003; Bodnar and Kest 2010) and more recently microglia sexual dimorphism has been shown (Doyle et al. 2017). Differences in the peripheral site processing of the nociceptive insult have been implicated too; sex hormones are able to influence effects of nerve growth factor

and other enzymes for example, protein kinase C family in the processing of inflammatory pain (Dina et al. 2001). Increasingly, the findings are suggesting that sex differences in nociception are not entirely responsible and more likely are common sets of signalling pathways differentially modulated by the hormonal environment (Hucho et al. 2006; Rosen et al. 2017). Unfortunately, the persistent under-representation of females in pain studies (basic science and clinical) may have resulted in differences being overlooked for a long time. For example during a human volunteer study men but not women showed a pain-induced increase in PAG functional connectivity with the amygdala caudate and putamen, and as a result of this finding it became apparent that female animals had been ignored when the connections between the amygdala and PAG were investigated (Linnman et al. 2012). The anatomical and physiological characterisation of the descending periaqueductal gray-rostral ventral medulla region (PAG-RVM) as described in Chapter 1 (section 1.2.7.) are now well understood across the species (Behbehani and Fields 1979; Beitz 1982) but in fact in the past these studies were naively perfomred in males only, under the misguided supposition that the neural architecture would be conserved between sexes. The sexual dimorphism of the PAG-RVM is now known to differ during persistent inflammatory states (Loyd and Murphy 2006; Loyd et al. 2007) and it is possible that differences in these descending pathways and connectivity influenced the results in the current study. One group reported increased connectivity from the PAG to the pre limbic cortex in a functional magnetic imaging (fMRi) DNIC study in males compared to females and castrated male rats and suggested this was responsible for the more efficient DNIC in males (Da Silva et al. 2018); an observation seen in men and women too (Labus et al. 2008; Wang et al. 2014). Although in the animal studies (Da Silva et al. 2018),

the backdrop of isoflurane anaesthesia and the imaging timepoint (only up to 15 minutes) and conclusions drawn about the response to the capsaicin injection will have influenced the results and the overall impact. A parallel in this study is the preconditioning with isoflurane and $N_2O$.

## 3.6. CONCLUSION

In summary, there were sex-linked pharmacokinetic and pharmacodynamic differences with alfaxalone in cyclodextrin; terminal half-life, clearance and mean residence time were significantly different between male and female rats. Sex differences in DNIC were observed in animals with similar alfaxalone plasma concentrations confirming sexual dimorphism of DNIC, with a more efficient DNIC seen in male animals, although this was dependent on the reflex being studied. The results in this study emphasize the importance of including both sexes when studying basic mechanisms of pain processing. In the research community there remains a collective responsibility for a thoroughness of reporting anaesthesia conditions in order that the anaesthesia is not 'the elephant in the room'.[a]

[a] ***"Elephant in the room"*** *is an English metaphorical idiom for an obvious truth that is either being ignored or going unaddressed. The idiomatic expression also applies to an obvious problem or risk no one wants to discuss.*

# 4. THE EFFECTS OF $N_2O$ ON DNIC

## 4.1. DESCENDING CONTROLS

Before transmission to higher centres, nociceptive information goes through extensive modulation in the spinal cord via a network of descending pathways extending from the midbrain, pons and medulla which can inhibit as well as facilitate nociceptive signalling (Millan 2002). Specifically the midbrain periaqueductal gray (PAG) inputs onto major noradrenergic and serotoninergic bulbospinal pathways from the pontine LC and the RVM respectively (Figure 1.1) (Millan 2002; Bannister and Dickenson 2016a), which together exert a complex modulation of dorsal horn neurones via specific receptor subtypes (Zhuo and Gebhart 1991; Millan 2002).

### 4.1.1. Counter irritation paradigm for studying descending controls

These pathways can also be recruited by counter-irritation paradigms such that a nociceptive stimulus applied to a heterotopic site on the body (the 'conditioning' stimulus) can inhibit another source of pain (the 'test' stimulus) a distance away, termed DNIC (Le Bars et al. 1979b; Villanueva et al. 1984) or CPM in humans (Yarnitsky 2010).

### 4.1.2. Studying DNIC under the influence of $N_2O$

Historically, many of the seminal basic science experiments that have informed mechanisms of DNIC have been performed under the influence of $N_2O$ (Carstens

et al. 1980; Villanueva et al. 1984; Dickenson and Le Bars 1987; Dickenson and Sullivan 1987b; Sandkuhler et al. 1987; Bing et al. 1990; Harris and Clarke 2003). However, oftentimes studies are devoid of detail concerning use of this gas and no indication of duration of administration is included. Furthermore its potential effects on experimental outcomes are not considered (Chung et al. 1979; Gebhart et al. 1983; Schouenborg and Dickenson 1985; Zhuo and Gebhart 1997; Zhou et al. 1998). Nitrous oxide boasts numerous pharmacological properties, namely as an analgesic, anaesthetic, anxiolytic and psychoactive substance, and the mechanisms of action underlying these are discrete and varied (Novelli et al. 1983; Fujinaga and Maze 2002; Bessière et al. 2007; Koyanagi et al. 2008; Sanders et al. 2008; Jinks et al. 2009; Imberger et al. 2014; Savage and Ma 2014). Nitrous oxide is able to influence the descending pathways via the noradrenergic system (Komatsu et al. 1981; Zhang et al. 1999) and through release of opioid peptides in the PAG (Zuniga et al. 1987; Fang et al. 1997). Its actions can be antagonised by ablation of the PAG or by systemic administration of opioid or $\alpha$2 receptor antagonists (Berkowitz et al. 1977a; Quock et al. 1990). Interestingly, intrathecally administered opioid receptor antagonists are devoid of effects in contrast to the intracerebroventricular route, and the reverse is true for $\alpha$2 receptor antagonists (Guo et al. 1996). The apparent lack of effect of the intrathecally administered opioid receptor antagonists can be attributed to the powerful descending inhibitory pathways masking the effect of the antagonist. This is demonstrated by the large increase in reflexes in spinalised rabbits given naloxone, supporting the construct of strong opioidergic inhibition by local spinal opioidergic interneurones. In experiments with combined opioid/$\alpha$2 receptor antagonists in intact rabbits this strong inhibitory effect of descending noradrenergic pathways and local opioidergic

pathways was revealed (Clarke et al. 1988). Antinociception produced in rats by administration of morphine supraspinally is blocked by intrathecal injection of $\alpha$2 receptor antagonists (Wigdor and Wilcox 1987; Suh et al. 1989), and yet intensified by the intrathecal administration of reuptake inhibitors blocking the reuptake of noradrenaline (Larsen and Arnt 1984). These experiments support the concept that noradrenergic mechanisms mediate the analgesia action of exogenous and endogenous opioids. Guo et al. (1996) established that the analgesic action of $N_2O$ in rats was mediated through supraspinal, but not spinal opiate receptors and spinal but not supraspinal $\alpha$2 receptors

### 4.1.3. Rat strain variability in response to $N_2O$

Different rat strains have been shown to differ in their antinociceptive effects of $N_2O$ and tolerance to $N_2O$ (Berkowitz et al. 1977b, 1979; Shingu et al. 1985). In view of these conflicting results the strain selection for pain studies is important (Quock et al. 1993, 1996). Some groups advise to select an in-bred strain to minimise the pain phenotype (Fender et al. 2000). The in-bred Lewis strain has been shown to have no antinociceptive effect to $N_2O$. The mechanism behind this lack of antinociception may be attributable to lower levels of opioids in the PAG of the Lewis rat, resulting in less activation of the descending adrenergic system (Beitner-Johnson et al. 1991; Guitart et al. 1993; Fender et al. 2000).

### 4.1.4. Aims of study

Given the potential for $N_2O$ to affect the balance of descending inhibition in studies of pain, the aims of this study were twofold; to evaluate the influence of $N_2O$ preconditioning on DNIC reflexes following a heterotopic noxious conditioning stimulus in Sprague Dawley and Lewis rats; also, to probe the influence of the $N_2O$ preconditioning in the decerebrate model.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Animals

Experiments were performed using male SD and Lewis rats (Charles River Laboratories, Margate, UK). Animals were housed in groups of 4 as detailed in Chapter 2 (section 2.2.). The rats were randomly selected and allocated to treatment groups after ten days acclimatisation to the animal unit. Data are reported from 47 rats with intact neuraxes and 28 decerebrated animals (Table 4.1).

### 4.2.2. Anaesthesia for animals with intact neuraxis

The timeline for the experiment is outlined in Figure 4.1. Anaesthesia was induced using 3% isoflurane (Isoflo, Abbott, Maidenhead, UK) in either $O_2$ or an $O_2/N_2O$ mixture (1:2) as described in chapter 2 (section 2.3.). Anaesthesia was maintained with a CRI of alfaxalone following instrumentation. A carotid blood sample for blood gases, haematology and biochemistry was collected 60 minutes after starting the alfaxalone infusion (see section 2.3.4).

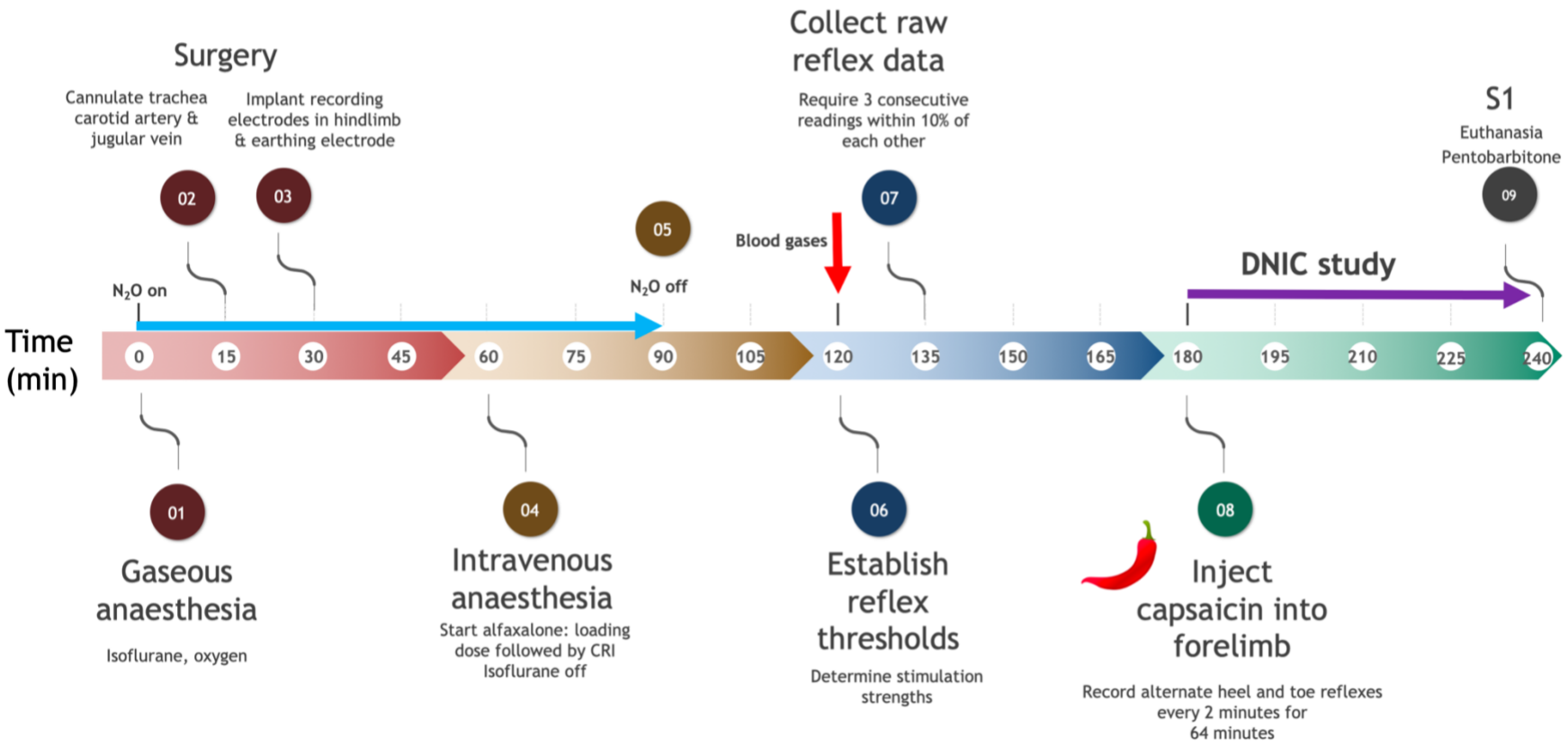



Figure 4.1 Timeline for the 2 groups of male Sprague Dawley rats and 2 groups of Lewis rats with intact neuraxes undergoing DNIC studies. One group of SD and one group of Lewis rats received $N_2O$ preconditioning. All animals were administered alfaxalone dose: 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by a constant rate infusion of 0.75 mg $kg^{-1}$ $min^{-1}$ at time point 5. The DNIC study started at time point 8. CRI: constant rate infusion, DNIC: diffuse noxious inhibitory controls, $N_2O$: nitrous oxide, SD: Sprague Dawley, S1 = euthanasia.

### 4.2.3. Anaesthesia for decerebration

The study timeline for the decerebration experiments is shown in Figure 4.2. Anaesthesia was induced using 3% isoflurane (Isoflo, Abbott, Maidenhead, UK) in either $O_2$ or an $O_2/N_2O$ mixture (1:2) as described in chapter 2 (section 2.3.). Isoflurane anaesthesia was continued for the duration of the decerebration. Following decerebration, the isoflurane was reduced, and the rat immobilised using either a sub MAC concentration of isoflurane or an alfaxalone CRI depending on the study design throughout electrophysiological recording. All animals received $O_2$. Animals were allowed to stabilise for 60 minutes prior to electrophysiological recordings. A carotid blood sample for blood gases, haematology and biochemistry was collected 60 minutes after starting the alfaxalone infusion (see section 2.3.4)

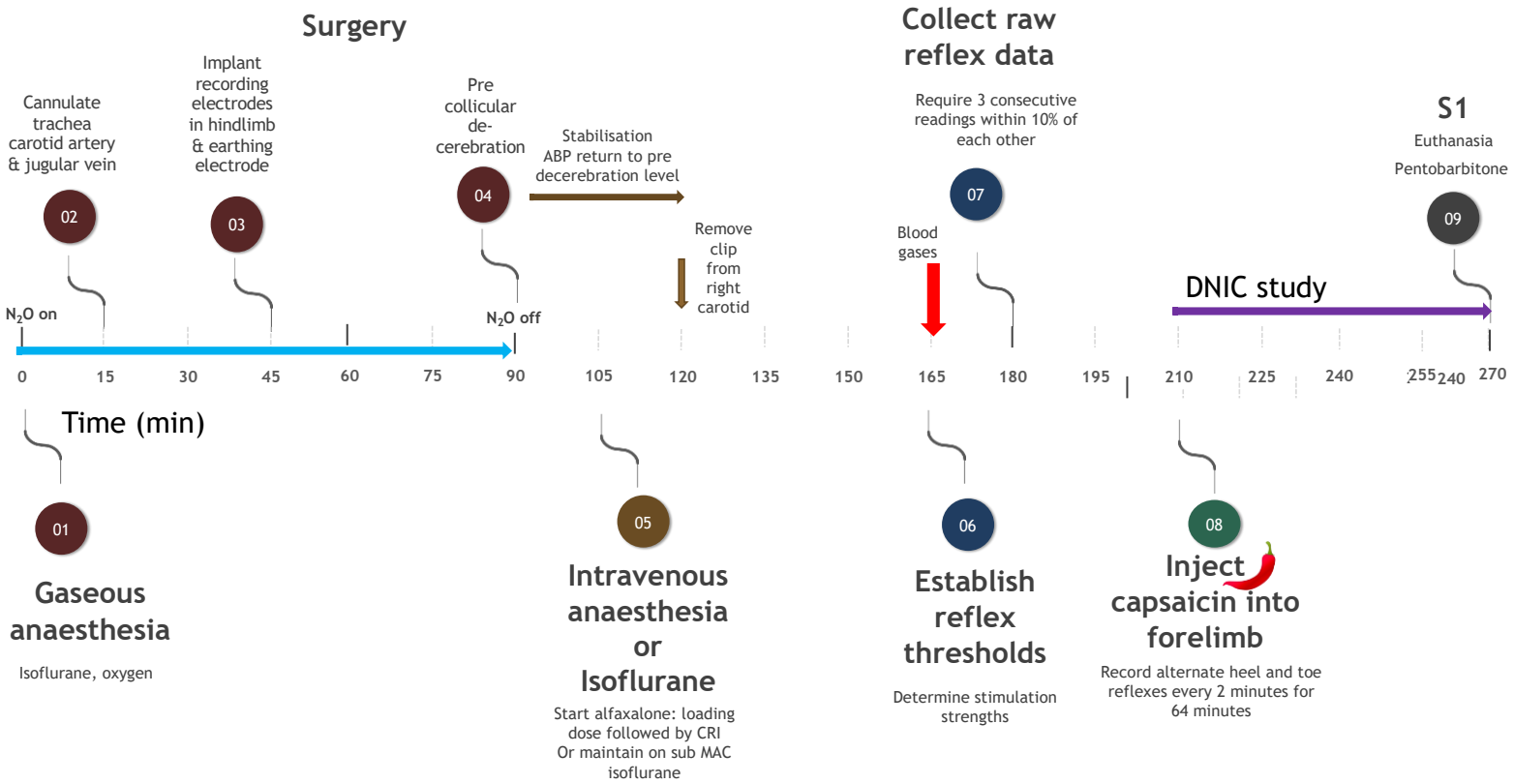



Figure 4.2 Timeline for the male Sprague Dawley rats undergoing pre-collicular decerebration prior to DNIC studies. Two groups were administered alfaxalone after decerebration (dose: 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by a constant rate infusion of 0.75 mg $kg^{-1}$ $min^{-1}$) one of which received 60 minutes of preconditioning with $N_2O$. Two groups received isoflurane for the duration of the experiment, one of which received preconditioning with $N_2O$. The DNIC study started at time point 8. ABP: arterial blood pressure, CRI: constant rate infusion, DNIC: diffuse noxious inhibitory controls, MAC: minimal alveolar concentration, $N_2O$: nitrous oxide, SD: Sprague Dawley, S1 = euthanasia.

### 4.2.4. Mechanical decerebration

For animals undergoing decerebration a temporary occluding clip was also placed on the right carotid artery prior to the decerebration to reduce haemorrhage during the procedure, and removed approximately 30 minutes after decerebration was completed. For the decerebration procedure (Dobson and Harris 2012), the animal was placed in sternal recumbency with the head supported. The hair over the cranium was clipped and the skin surgically prepared. A longitudinal incision was made midline from midway along the snout to the cervical area. The pericranium was scraped and the sagittal, coronal and transverse sutures of the cranium exposed. On either side of the sagittal suture, at a midway point between the coronal and transverse sutures, two small holes were drilled through the cranium. The holes were carefully enlarged using ronguers either side but leaving a bridge of bone midline approximately 0.5cm wide. Two silk sutures were then placed at the rostral and caudal end of this bridge of parietal bone and were passed under the bone avoiding the sinus. These sutures prevented hemorrhage from the sinus when the bridge of parietal bone was removed piecemeal. Once the bone had been removed the dura was carefully retracted from the surface of the cerebrum. The cerebral hemispheres were then carefully removed with a suction machine fitted with a microtip, followed by a rapid coronal sectioning, leaving the colliculi intact. Inevitable haemorrhage from the cavernous sinus and the basilar artery was reduced by a combination of suction and then placing multiple suitable sized pieces of haemostatic gelatin sponge (Spongostan, Johnson & Johnson, Belgium) soaked in tissue glue (Vetbond, 3M, Bracknell, UK) against the cranial vault, until the cranial vault was packed with sponge to promote haemostasis. During coronal section,

arterial blood pressure decreased, and then rose in the ensuing 30–60 minutes. Once the blood pressure had regained the pre-decerebration or cortical aspiration values the clip placed on the right carotid artery was removed to maintain circulation to the right carotid sinus and preserve baroreceptor reflexes. The animal was then secured to the table using magnetic bases and plaster of Paris supports to reduce the effects of the motor movements that are commonly observed in decerebrate rats.

### 4.2.5. Hind limb stimulation and recording of NWR

The earthing electrode and the recording electrodes were inserted as described in Chapter 2 (section 2.6.2.).

### 4.2.6. DNIC conditioning stimulus

A fresh stock of capsaicin solution was prepared prior to each experiment group (section 2.6.3.). The acute noxious conditioning stimulus comprising 100 μL (0.5 mg) capsaicin was injected via a 25G needle into the muscle mass of *extensor carpi radialis* and *pronator teres* of the right (contralateral) forelimb. Reflexes were then collected and measured for a further 64 minutes.

At the end of experiments animals were euthanised by intravenous injection of 150 mg $kg^{-1}$ pentobarbitone followed by cervical dislocation.

### 4.2.7. Statistical analysis

Statistical analysis was performed as outlined in Chapter 2 (section 2.8.).

## 4.3. RESULTS

### 4.3.1. Experimental groups

The experimental groups are detailed in Table 4.1.

Table 4.1 Numbers of animals in each group of the experiment

| Group | Sprague Dawley (naïve) | | Lewis (naïve) | | Sprague Dawley (decerebrated) | | | |
|---|---|---|---|---|---|---|---|---|
| Number of animals entering study | 14 | 10 | 15 | 8 | 9 | 7 | 9 | 10 |
| Nitrous oxide preconditioning | Yes | No | Yes | No | Yes | No | Yes | No |
| Alfaxalone CRI | Yes | Yes | Yes | Yes | Yes | Yes | No | No |
| Number of animals generating MG reflexes of sufficient size | 14 | 10 | 11 | 8 | 7 | 6 | 6 | 2 |
| Number of animals generating BF reflexes of sufficient size | 14 | 10 | 12 | 8 | 7 | 6 | 6 | 7 |
| Number of animals generating TA reflexes of sufficient size | 14 | 10 | 9 | 7 | 6 | 5 | 6 | 7 |
| Number of animals not completing study | 0 | 0 | 0 | 0 | 2[#] | 1[#] | 2[#] | 3[#] |

Group Allocation and outcomes. Nitrous oxide preconditioning is designated by columns shaded in grey. MG, *medial gastrocnemius*; BF, *biceps femoris*; TA, *tibialis anterior*; CRI, constant rate infusion.

# Experiment terminated due to sustained hypotension following decerebration

### 4.3.2. Haematology, blood biochemistry and blood gases

Blood gases, haematology and biochemistry results for all animals are presented in Table 4.2. A significant difference was apparent for plasma partial pressure of oxygen ($P_aO_2$ mmHg) across groups ($P$ = 0.0007, one way-ANOVA) such that the $P_aO_2$ was significantly greater in decerebrated Sprague Dawley rats not exposed to $N_2O$ when compared to preconditioned Lewis rats ($P$ = 0.01, Sidak's multiple comparison test), Lewis rats not preconditioned with $N_2O$ ($P$ = 0.002, Sidak's multiple comparison test) and Sprague Dawley rats with intact neuraxes preconditioned with $N_2O$ ($P$ = 0.0019, Sidak's multiple comparison test). All values were within expected ranges. Haematocrit values tended to be lower in those rats that underwent decerebration.

Table 4.2 Blood gas variables and clinical biochemistry parameters

| Group | Sprague Dawley (naïve) | | Lewis (naïve) | | Sprague Dawley (decerebrated) | | | |
|---|---|---|---|---|---|---|---|---|
| Number of animals | 14 | 10 | 15 | 8 | 7 | 6 | 7 | 7 |
| $N_2O$ duration (min) | 95 ± 2 | None given | 87 ± 4 | None given | 90 ± 3 | None given | 90 ± 4 | None given |
| Alfaxalone CRI | Yes | Yes | Yes | Yes | Yes | Yes | No | No |
| pH | 7.44 ± 0.08 | 7.35 ± 0.05 | 7.34 ± 0.06 | 7.38 ± 0.07 | 7.35 ± 0.04 | 7.34 ± 0.02 | 7.33 ± 0.02 | 7.38 ± 0.07 |
| $P_aCO_2$ (mm Hg) | 44 ± 16 | 53 ± 12 | 51 ± 9 | 51 ± 13 | 45 ± 5 | 51 ± 4 | 46± 4 | 37 ± 5 |
| $P_aO_2$ (mm Hg) *** | 350 ± 71 | 518 ± 109 | 376 ± 98 | 428 ± 103 | 512 ± 35 | 586 ± 17 [a b c] | 468 ± 37 | 449 ± 50 |
| $HCO_3$ (mmol $L^{-1}$) | 23 ± 3 | 28 ± 5 | 26 ± 5 | 29 ± 3 | 24 ± 1.8 | 26 ± 1.7 | 25 ± 2.3 | 23 ± 1.3 |
| Base Excess (mmol $L^{-1}$) | 1.3 ± 5 | 2.9 ± 5 | 0.2 ± 5 | 4.1 ± 2.8 | -1.7 ± 1.8 | 0.5 ± 1.6 | -1.1 ± 2.5 | -2.0 ± 2 |
| Sodium (mmol $L^{-1}$) | 142 ± 4 | 142 ± 3 | 142 ± 3 | 145 ± 3 | 140 ± 1.2 | 142 ± 2 | 142 ± 1 | 144 ± 0.3 |
| Potassium (mmol $L^{-1}$) | 4.8 ± 1.1 | 4.2 ± 2 | 3.8 ± 0.7 | 4 ± 0.6 | 5.2 ± 0.3 | 5.3 ± 0.3 | 5.2 ±0.2 | 4.9 ± 0.6 |
| Ionized Calcium (mmol $L^{-1}$) | 1.47 ± 0.24 | 1.26 ± 0.12 | 1.2 ± 0.12 | 1.28 ± 0.08 | 1.26 ± 0.05 | 1.32 ± 0.03 | 1.3 ± 0.03 | 1.3 ± 0.04 |
| Chloride (mmol $L^{-1}$) | 116 ± 6 | 108 ± 7 | 110 ± 5 | 107 ± 3 | 109 ± 2 | 107 ± 0.8 | 108 ± 1.9 | 109 ± 2.1 |
| Anion Gap (mmol $L^{-1}$) | 8 ± 6 | 11 ± 3 | 10 ± 3 | 13 ± 3 | 12.2 ± 1.1 | 13.3 ± 1.8 | 13.3 ± 1.2 | 15 ± 2.1 |
| Haematocrit (%) | 34 ± 8 | 34 ± 8 | 30 ± 7 | 35 ± 5 | 30 ± 2 | 34 ± 1.1 | 28 ± 1.8 | 26 ± 3.5 |
| Haemoglobin (g $dl^{-1}$) | 11.0 ± 3.2 | 11.0 ± 3 | 10.0 ± 2.5 | 11.8 ± 1.8 | 8.7 ± 1.3 | 11.4 ± 0.3 | 10 ± 0.7 | 9.0 ± 1.2 |
| Glucose (mmol $L^{-1}$) | 6.3 ± 3.2 | 6 ± 1.4 | 6 ± 1.8 | 6.2 ± 1.2 | 7.3 ± 1.2 | 6.3 ± 0.6 | 8.8 ± 1.9 | 7.1 ± 0.4 |
| Lactate (mmol $L^{-1}$) | 1.0 ± 0.3 | 0.7 ± 0.4 | 0.68 ± 0.29 | 0.6 ± 0.2 | 1.3 ± 0.3 | 2.7 ± 1.1 | 2.2. ± 0.7 | 2.4 ± 1.4 |
| Creatinine (mmol $L^{-1}$) | 29 ± 14 | 50 ± 28 | 25 ± 17 | 13 ± 13 | 61 ± 7 | 77 ± 14 | 73 ± 8 | 63 ± 25 |

Blood gas and biochemistry values prior to establishing reflex thresholds. Groups received alfaxalone infusion (1.67 mg $kg^{-1}$ $min^{-1}$ alfaxalone for 2.5 minutes followed by 0.75 mg $kg^{-1}$ $min^{-1}$) or isoflurane 2% immediately prior to baseline reflex data collection. CRI: constant rate infusion, $P_aCO_2$: partial arterial carbon dioxide pressure, $P_aO_2$: partial arterial oxygen pressure, $HCO_3$: bicarbonate. Data are mean ± SEM. *** $P < 0.001$ (one way ANOVA, followed by Sidak's multiple comparisons test for post hoc contrasts).

[a] designates $P_aO_2$ significantly greater in decerebrated rats receiving the alfaxalone CRI and no exposure to $N_2O$ when compared to Lewis rats preconditioned with $N_2O$ ($P = 0.01$).

[b] designates $P_aO_2$ significantly greater in decerebrated rats receiving the alfaxalone CRI and no exposure to $N_2O$ when compared to Lewis rats not exposed to $N_2O$ ($P < 0.0002$).

[c] designates $P_aO_2$ significantly greater in decerebrated rats receiving the alfaxalone CRI and no exposure to $N_2O$ when compared to Sprague Dawley rats with intact neuraxes exposed to $N_2O$ ($P < 0.0019$).

### 4.3.3. Demographics of groups

The demographics of the eight groups are presented in Table 4.3. Anaesthesia was standardised with similar duration of exposure to $N_2O$ for preconditioned groups. Comparison of body weights indicated a significant difference between groups ($P$ = 0.0001, one way ANOVA) such that Lewis rats receiving $N_2O$ were significantly smaller than those not exposed to $N_2O$ ($P$ = 0.0071, Sidak's multiple comparison test) but CRIs of alfaxalone administered for the duration of the electrophysiological recordings were standardised and based on body weight. The two decerebrate groups that did not receive alfaxalone, but received isoflurane (with or without $N_2O$ preconditioning) for the whole experiment, had comparable end-tidal isoflurane %. There were no significant differences ($P$ > 0.05, Kruskal-Wallis one-way ANOVAs) between groups with respect to the size of raw control baseline reflexes prior to administration of capsaicin.

Table 4.3 Raw control responses of reflexes from heel and toes

| Group | Sprague Dawley (naïve) | | Lewis (naïve) | | Sprague Dawley (decerebrated) | | | |
|---|---|---|---|---|---|---|---|---|
| Number of animals | 14 | 10 | 15 | 8 | 7 | 6 | 7 | 7 |
| $N_2O$ (min) | 95 ± 2 | None given | 87 ± 4 | None given | 90 ± 3 | None given | 90 ± 4 | None given |
| Alfaxalone CRI | Yes | Yes | Yes | Yes | Yes | Yes | No | No |
| Weight (g) | 399 ± 14 | 372 ± 10 | 337 ± 7 | 389 ± 5 | 356 ± 9 | 379 ± 25 | 372 ± 13 | 322 ± 9 |
| Raw control response heel-MG (μV.ms) | 140 (78–233) | 195 (122–575) | 261 (202–354) (n=11) | 300 (173–415) | 216 (180–332) | 216 (132–406) | 127 (113–163) (n=6) | 437 (337–538) (n=2) |
| Raw control response toes-BF (μV.ms) | 95 (66–229) | 103 (61–144) | 87 (72–133) (n=12) | 145 (106–168) | 96 (93–252) | 137 (84–185) | 53 (47–88) (n=6) | 81 (54–112) |
| Raw control response toes-TA (μV.ms) | 118 (39–180) | 97 (45–203) | 84 (65–99) (n=9) | 250 (94–307) (n=7) | 59 (42–72) (n=6) | 71 (63–115) (n=5) | 86 (73–98) (n=6) | 92 (74–112) |

Demographics for the eight treatment groups used in the present study. Grey shading denotes preconditioning with $N_2O$. Occasionally reflexes were too small to reliably measure resulting in a lower n number as indicated. CRI: constant rate infusion, MG: *medial gastrocnemius*, BF: *biceps femoris*, TA: *tibialis anterior*. Data are mean ± SEM or median (IQR).

### 4.3.4. Electrical thresholds required to elicit reflexes

The electrical thresholds required to elicit reflexes were significantly different between the four groups preconditioned with $N_2O$ for both heel ($P$ = 0.0024, Kruskal Wallis ANOVA) and toes ($P$ = 0.0001, Kruskal Wallis ANOVA) but not for those animals not exposed to $N_2O$ (Figure 4.3). The current required to elicit heel withdrawal reflexes was significantly greater in decerebrated Sprague Dawley animals receiving isoflurane ($P$ = 0.034, Kruskal Wallis ANOVA followed by Dunn's multiple comparison test) compared to the same strain with intact neuraxes. For the reflexes elicited from the toes, the preconditioned Sprague Dawley animals with intact neuraxes required the smallest current when compared to all other groups ($P$ = 0.001, Kruskal Wallis ANOVA followed by Dunn's multiple comparison test).

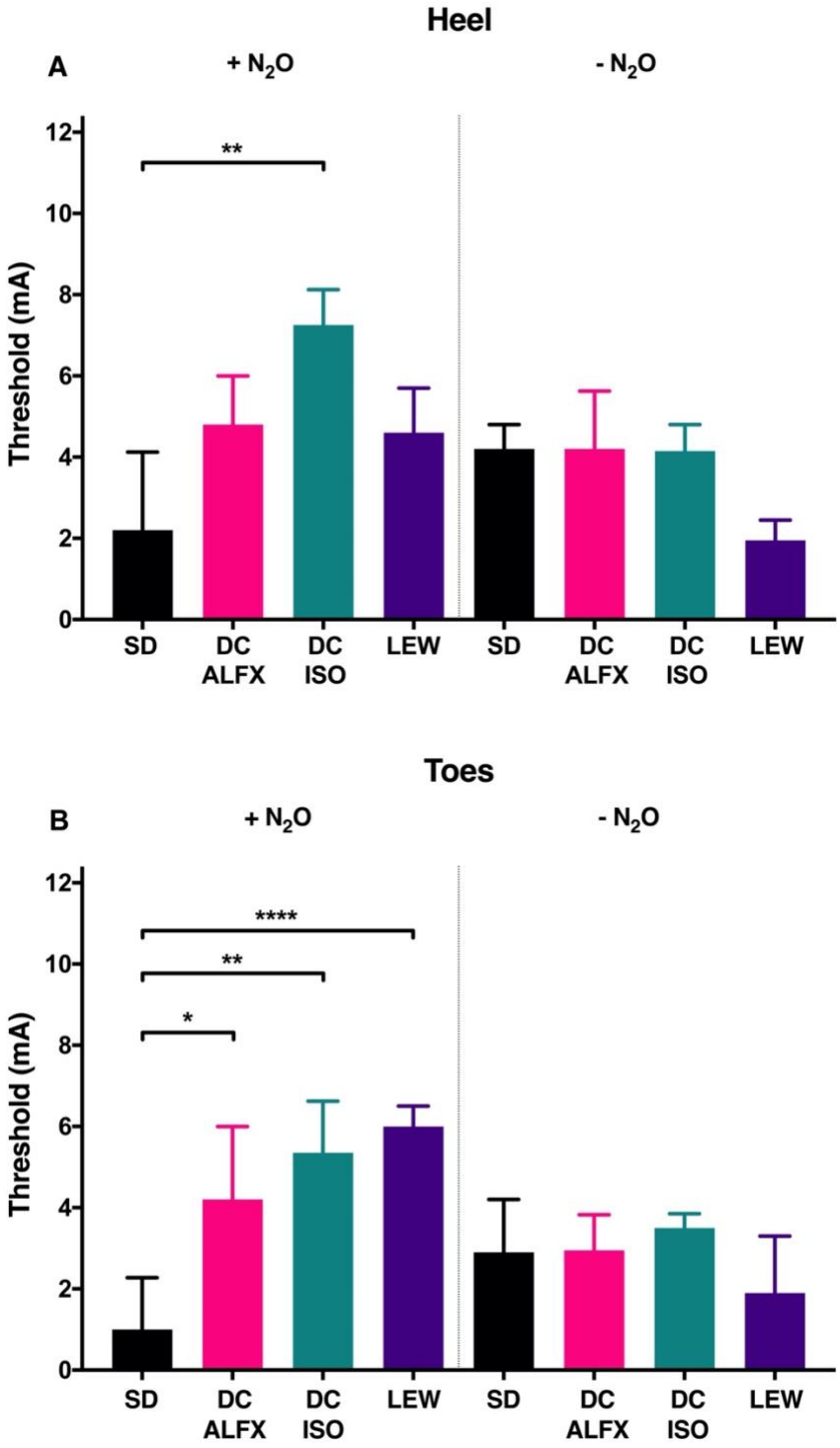



Figure 4.3 Electrical thresholds required to elicit nociceptive withdrawal reflexes from the heel (A) and toes (B) with (+ $N_2O$) or without $N_2O$ (- $N_2O$) preconditioning.

SD, Sprague Dawley; DC, decerebrated; ALFX, alfaxalone; ISO, isoflurane; LEW, Lewis. Data are expressed as median (IQR). *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. (Kruskal-Wallis one way ANOVA followed by Dunn's multiple comparison test for post hoc contrasts).

### 4.3.5. Stimulation strengths for eliciting NWRs

The stimulation strengths used in the preparations were determined by the operator in order to produce a reflex of sufficient magnitude that could unequivocally be inhibited or enhanced in response to the capsaicin injection. The stimulation strength for eliciting the heel-MG reflex of $N_2O$ preconditioned rats was significantly different between groups ($P$ = 0.012, Kruskal Wallis ANOVA) with decerebrate preparations receiving isoflurane receiving significantly larger stimuli than the intact Sprague Dawley animals preconditioned with $N_2O$ ($P$ = 0.0074, Dunn's multiple comparison test). Similarly, for the toes reflexes', there was a significant difference between stimulation strengths used for the groups preconditioned with $N_2O$ ($P$ = 0.0074, Kruskal Wallis ANOVA) such that a significantly larger current was used for the decerebrate preparations receiving isoflurane ($P$ = 0.0011) and the Lewis rats ($P$ = 0.016) when compared to the intact Sprague Dawley rats (Dunn's multiple comparison test) (Figure 4.4).

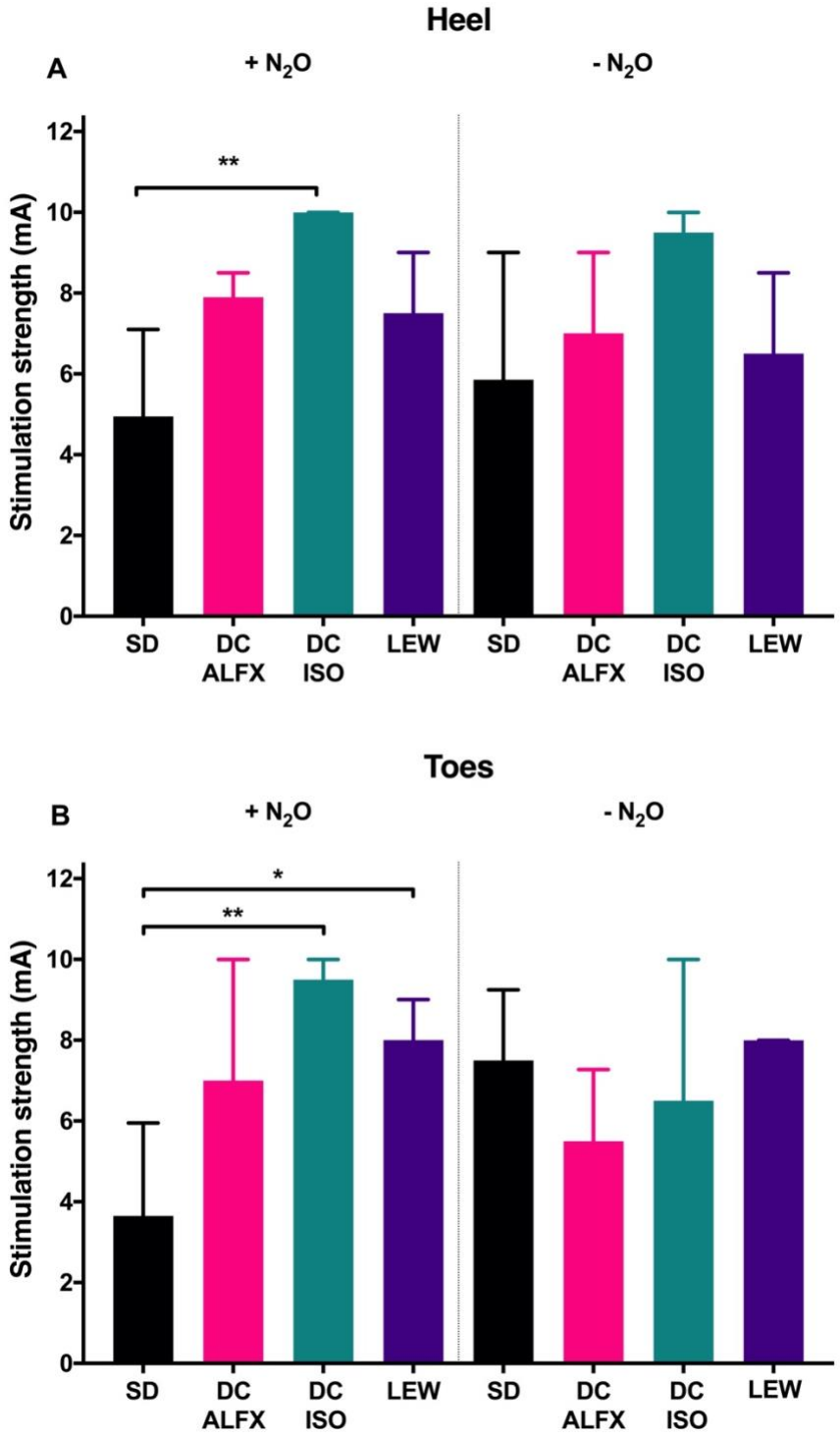



Figure 4.4 Stimulation strength applied to the heel (A) and toes (B) in the four groups with (+ $N_2O$) or without $N_2O$ (- $N_2O$) preconditioning.

Stimulation strengths used were 1–5 times the threshold (Figure 4.3). SD, Sprague Dawley; DC, decerebrated; ALFX, alfaxalone; ISO, isoflurane; LEW, Lewis. Data are expressed as median (IQR). * $P < 0.05$, ** $P < 0.01$, (Kruskal-Wallis one way ANOVA followed by Dunn's multiple comparison test for post hoc contrasts).

### 4.3.6. Effect of $N_2O$ on DNIC of reflexes in naïve Sprague Dawley rats

In the $N_2O$ preconditioned group, the capsaicin stimulus overall tended to increase responses in heel–MG to a median of 134% (IQR 49–232%) however this was not significant (*P* = 0.33, Friedman's ANOVA, n = 14) (Figure 4.5). On the other hand, capsaicin administration significantly decreased toes–BF and toes–TA reflexes (both *P* < 0.0001, Friedman's ANOVA, n = 14) to a median of 55% (IQR 33–70%) and 57% (42–67%) of pre-capsaicin levels and inhibition was maintained for at least 63 min (IQR 63–63 min) and at least 63 min (IQR 15–63 min), respectively. In contrast, in animals not exposed to $N_2O$, only heel-MG responses significantly decreased following capsaicin injection (*P* < 0.0001, Friedman's ANOVA, n = 10) to 66% (IQR 46–72%) of pre-capsaicin controls for a duration of 53 min (IQR 45–61 min). These differences were further supported by comparison of negative area under the curve measurements which showed toes-BF (P = 0.0045, Mann-Whitney test) and toes-TA (*P* = 0.0358, Mann-Whitney test) responses were significantly more inhibited when $N_2O$ had been present (Figure 4.5).

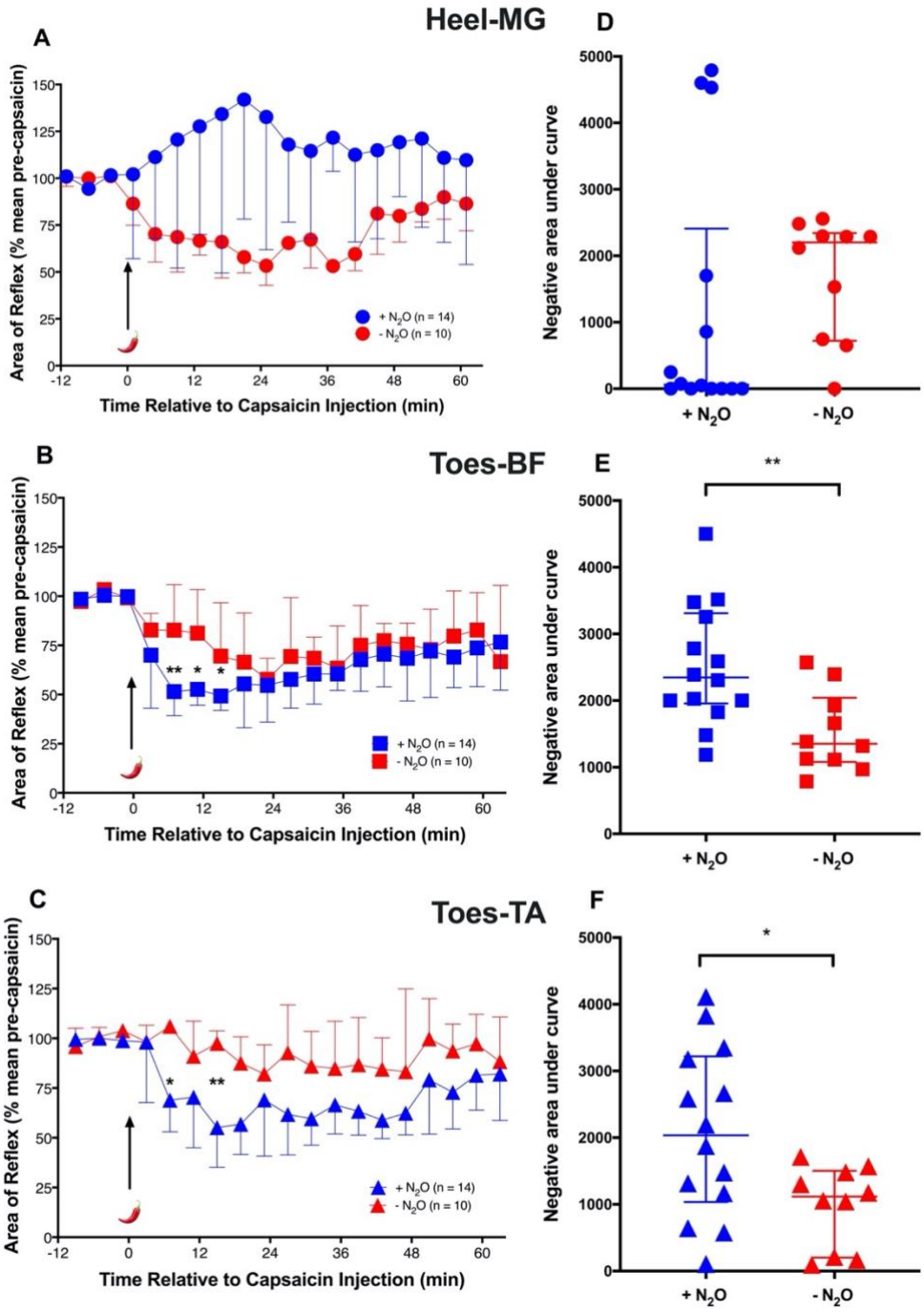



Figure 4.5 Effect of capsaicin on reflexes elicited from heel and toes in male Sprague Dawley rats with intact neuraxes. Capsaicin was injected into the contralateral forelimb (arrow) and effects measured on (A) heel-*medial gastrocnemius* (heel-MG), (B) toes-*biceps femoris* (toes-BF) and (C) toes-*tibialis anterior* (toes-TA) reflexes. Responses were measured under alfaxalone anaesthesia (1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes then 0.75 mg $kg^{-1}$ $min^{-1}$) following surgery using isoflurane 2% (vaporiser setting) in the absence or presence of $N_2O$ preconditioning. (D-F) Negative AUC values following capsaicin injection on heel-MG (D), toes-BF (E) and toes-TA reflexes (F). * $P < 0.05$ and ** $P < 0.01$ (Mann-Whitney test).

### 4.3.7. Effect of $N_2O$ on DNIC of reflexes in decerebrated Sprague Dawley rats

In decerebrated animals receiving alfaxalone no significant inhibition of any of the three reflexes ($P > 0.05$, Friedman's ANOVAs) was evident following the acute capsaicin conditioning stimulus after preconditioning with $N_2O$ during surgery (Figure 4.6). In the group that did not receive $N_2O$, there was only significant inhibition of the toes-BF reflex ($P = 0.0009$, Friedman's ANOVA) to a median inhibition of 66% (IQR 62–77%) with inhibition lasting 63 min (IQR 45–63 min). Area under the curve comparisons did not detect any significant differences ($P < 0.05$, Mann-Whitney test) between post-capsaicin responses with and without $N_2O$ preconditioning for heel-MG. Significant differences were evident for toes-BF reflexes during alfaxalone infusion with and without $N_2O$ preconditioning ($P = 0.0017$).

For decerebrated animals that were maintained on isoflurane anaesthesia throughout surgery and electrophysiological recording (no alfaxalone CRI), there was significant inhibition of the heel-MG reflex in the $N_2O$ preconditioned group to a median of 60% (IQR 32–77%), with inhibition lasting for a median duration of 63 min (IQR 31–63 min). However, it was apparent that heel-MG reflexes were difficult to elicit (up to maximum 10 mA stimulation strength) in the non-$N_2O$ preconditioned group making comparisons difficult in view of the low number of responses obtained (n = 2). In contrast, flexor responses could be reliably recorded with or without prior exposure to $N_2O$ and significant inhibition of toes-BF and toes-TA reflexes following capsaicin was seen under both conditions ($P = 0.0001$ for both BF and TA with $N_2O$ preconditioning; $P = 0.0001$ (BF) and $P = 0.0019$ (TA) without $N_2O$ preconditioning,

Friedman's ANOVAs). Following $N_2O$ preconditioning, toes-BF and toes-TA were reduced to a median of 34% (IQR 26–38%) and to 43% (IQR 27–74%) of controls with inhibition lasting for a median duration of at least 63 min (IQR 63–63 min) and 41 min (IQR 25–54 min) respectively. For animals not exposed to $N_2O$, corresponding inhibitions were to 19% (IQR 15–33%) and 39% (IQR 15–51%) of controls for toes-BF and toes-TA respectively, with a duration of inhibition of at least 63 min (IQR 63–63) for both reflexes (Figure 4.6). Comparison of negative AUC values found no significant difference ($P > 0.05$, Mann-Whitney test) in capsaicin-induced inhibition with and without $N_2O$ preconditioning for either flexor response in decerebrated animals undergoing isoflurane anaesthesia. However, differences in inhibition were evident between $N_2O$ preconditioned animals receiving alfaxalone or isoflurane ($P = 0.026$) for toes-BF (Figure 4.6).

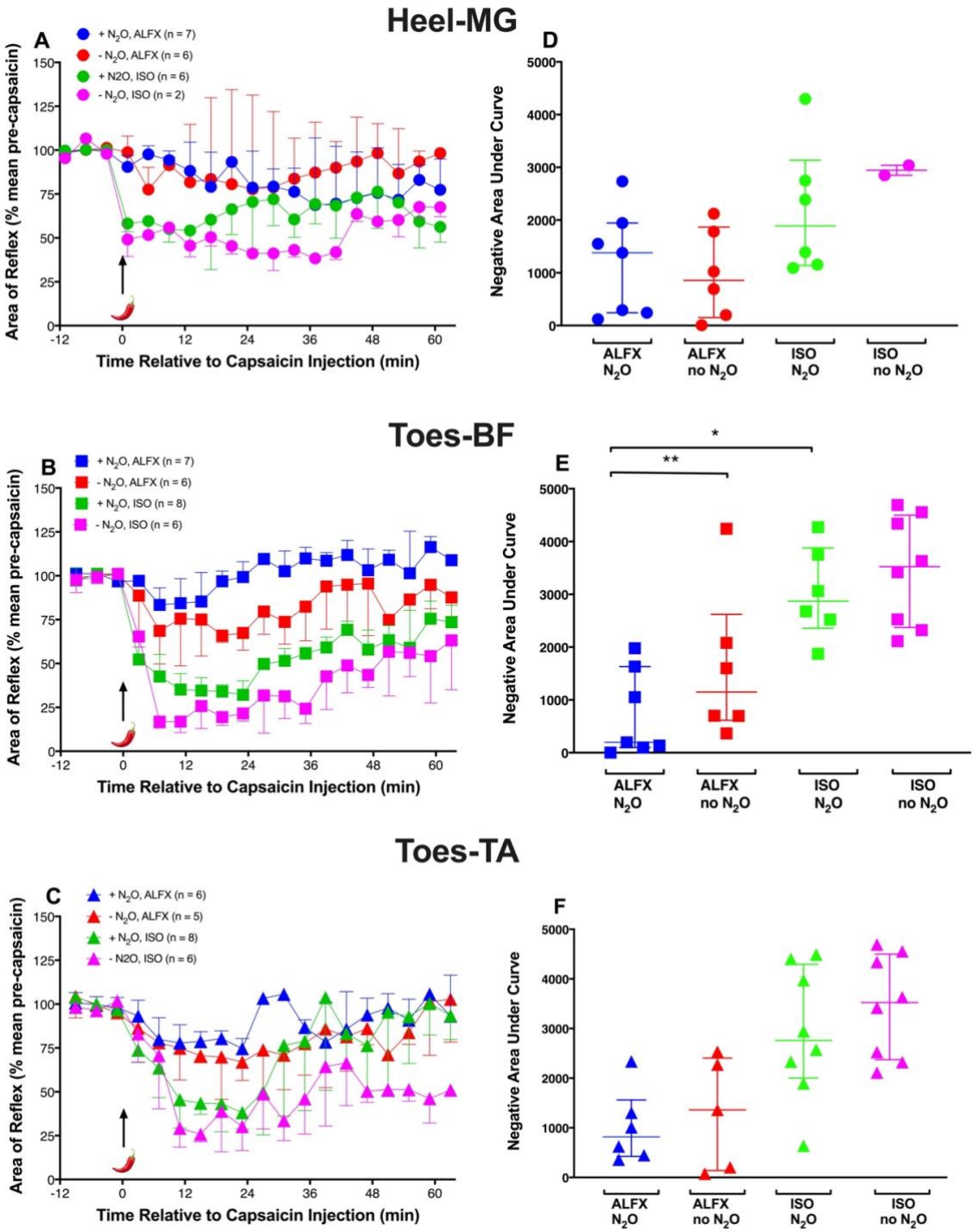



Figure 4.6 Effect of capsaicin injection on reflexes elicited from heel and toes in decerebrated male Sprague Dawley rats. The effects of the capsaicin injection (arrow) into the contralateral forelimb on (A) heel-*medial gastrocnemius* (heel-MG), (B) toes-*biceps femoris* (toes-BF) and (C) toes-*tibialis anterior* (toes-TA) reflexes were measured with or without $N_2O$ preconditioning in decerebrated animals followed by administration of 1.67 mg $kg^{-1}$ $min^{-1}$ alfaxalone for 2.5 minutes then 0.75 mg $kg^{-1}$ $min^{-1}$ alfaxalone or isoflurane 2%. (D-F) Negative AUC values for heel-MG (D), toes-BF (E) and toes-TA reflexes (F). ALFX alfaxalone; ISO, isoflurane. *$P$ < 0.05 and ** $P$ < 0.01 (Kruskal-Wallis test).

### 4.3.8. Effect of $N_2O$ on DNIC of reflexes in Lewis rats

For the $N_2O$ resistant Lewis rats, following preconditioning with $N_2O$, capsaicin injection significantly inhibited heel-MG ($P$ = 0.0017, Friedman's ANOVA) and toes-BF reflexes ($P$ < 0.0001, Friedman's ANOVA) compared to pre-capsaicin controls to a median of 75% (IQR 71–93%) for a median duration of 61 min (IQR 7–61 min) and 49% (IQR 23–81%) for 63 min (IQR 63–63 min), respectively. In contrast toes-TA responses were not significantly inhibited ($P$ = 0.2314, Friedman's ANOVA) (Figure 4.7). In the Lewis group not exposed to $N_2O$, capsaicin-induced significant decreases in the heel-MG ($P$ = 0.0186; Friedman's ANOVA) and toes-BF reflexes ($P$ < 0.0001; Friedman's ANOVA) to 78% (IQR 76–85%) and 48% (IQR 31–56%) of controls with inhibition lasting for 61 min (IQR 10–61 min) and 63 min (IQR 61–63 min) respectively. Again, there was no significant inhibition of toes-TA responses. Comparison of negative AUC values found no significant differences ($P$ > 0.05, Mann-Whitney test) with or without preconditioning for any of the three reflexes using Lewis rats (Figure 4.7).

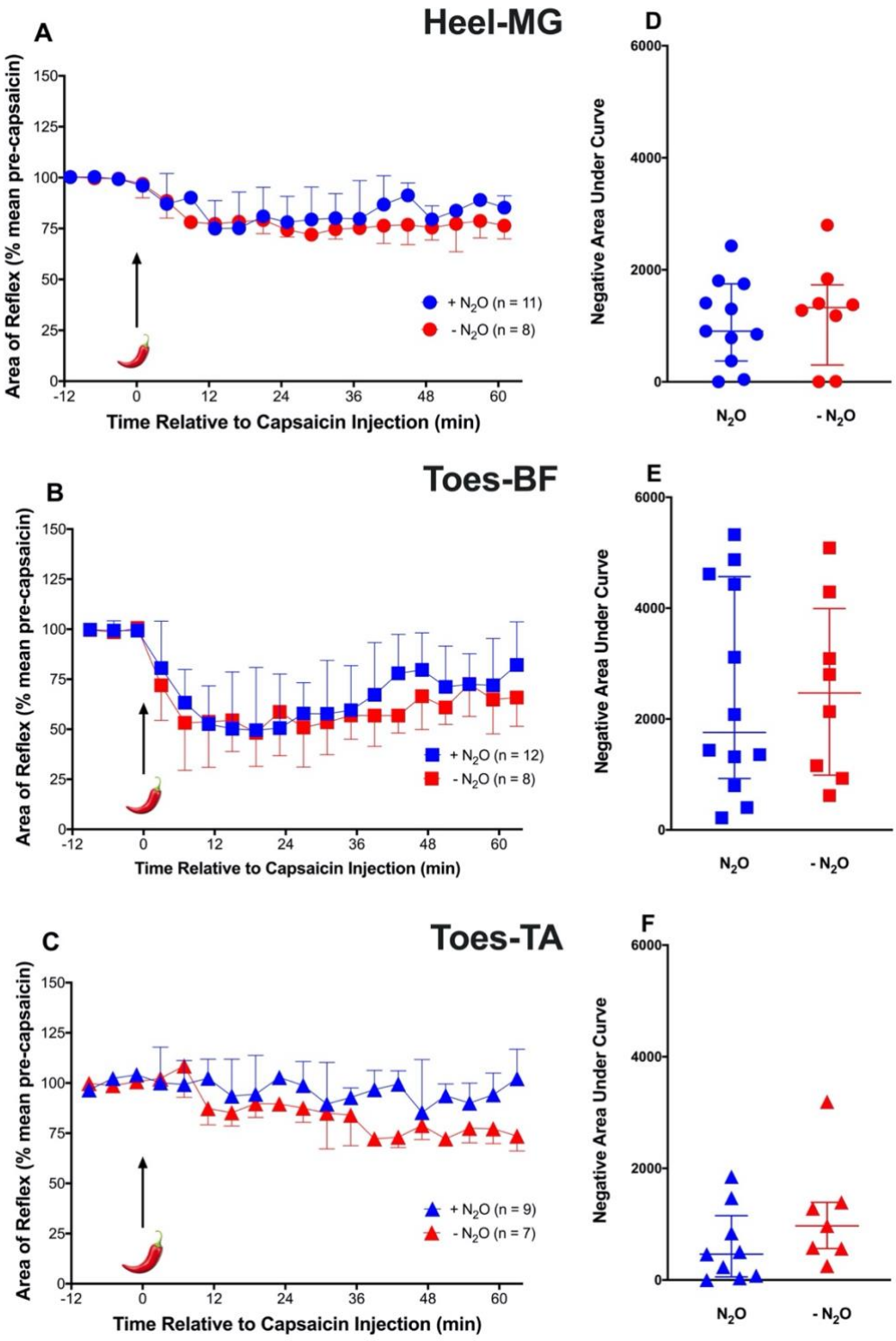



Figure 4.7 Effect of capsaicin injection on reflexes elicited from heel and toes in male Lewis rats. The effects of the capsaicin injection (arrow) into the contralateral forelimb on (A) heel–*medial gastrocnemius* (heel-MG), (B) toes–*biceps femoris* (toes-BF) and (C) and toes–*tibialis anterior* (toes-TA) reflexes were measured in Lewis animals with or without $N_2O$ preconditioning followed by administration of 1.67 mg $kg^{-1}$ $min^{-1}$ alfaxalone for 2.5 minutes then 0.75 mg $kg^{-1}$ $min^{-1}$ alfaxalone. (D-F) Negative AUC following capsaicin injection to the contralateral forelimb on heel–MG (D) toes–BF (E) and toes–TA (F).

## 4.4. DISCUSSION

This study has demonstrated that in the anaesthetised rat, sustained inhibition of hindlimb withdrawal reflexes in response to a remote injection of capsaicin (DNIC) can be modulated by preconditioning with $N_2O$.

### 4.4.1. Global impact of $N_2O$ on the model

Anaesthesia protocols incorporating $N_2O$ can have a significant effect on electrophysiological findings. Preconditioning with $N_2O$ for 90 minutes resulted in differential effects on reflex thresholds and modulation of DNIC that extended to beyond the period of delivery and persisted for up to at least 2.5 hours following cessation of administration. Studies evaluating duration of action or effect vary considerably depending on study design. For example early research in rats found the β-endorphin release stimulated by 60–80% inhaled $N_2O$ diminished to pre-exposure levels in 15–30 minutes (Zuniga et al. 1986). In contrast, the expression of c-Fos positive cells (as markers of neuronal activity in the spinal cord) peaked between 90 and 120 minutes, after 90 minutes of exposure (to 75% $N_2O$). In humans it is perceived to offer almost immediate analgesia, and has been widely used in dental practice, ambulances, trauma cases, during labour and as an adjunct to the volatile anaesthetics (Buhre et al. 2019). In view of the fact that countless preclinical studies have been performed during or following anesthetic regimes including $N_2O$, and a lasting effect on the measured outcomes is possible (Le Bars et al. 2001; Hirsch et al. 2014), interrogation of the naïve model is imperative to understand the impact this gas can have. Sex differences were not apparent when studying $N_2O$

neurotoxicity (Jevtovic-Todorovic et al. 2001), hence the use of males in this study with multiple groups; however this does not preclude sex differences being present in the study of modulating descending controls.

### 4.4.2. Differential effects of $N_2O$ preconditioning on NWR

Each individual muscle NWR is evoked by a separate reflex pathway (Schouenborg and Kalliomäki 1990; Kalliomäki et al. 1992; Schouenborg 2002; Harris and Clarke 2003), a concept discovered through iterative experiments, starting with the idea of a monolithic 'flexion reflex' through Sherrington's pioneering work (Sherrington 1910). This theory persisted with flexors being considered a single entity as 'flexor reflex afferents' (Eccles and Lunberg 1959), but with time this developed into a modular theory (Schouenborg et al. 1992, 1994) as it became apparent the simplistic organization was much more complicated (Hagbarth 1952; Megirian 1962; Wilson 1963; Hielm-Björkman et al. 2007). This modular theory supports differences in receptive fields, thresholds and time course of withdrawal reflexes measured from individual flexors and extensors, albeit some reflexes elicited from the toes don't quite fit into the framework (Schomburg 1997; Schomburg et al. 2001). Nitrous oxide preconditioning enhanced inhibition of toes-TA and toes-BF flexor reflexes and appeared to facilitate the heel-MG extensor reflex in intact Sprague Dawley rats. This reflex differentiation is not unforeseen since hind limb reflex pathways can be differentially sensitised and altered by descending pathways and can exhibit inequitable sensitivity to analgesic drugs (Kalliomäki et al. 1992; Harris and Clarke 2003, 2007; Clarke and Harris 2004; Jenkins et al. 2004).

### 4.4.3. Anaesthesia stability

The use of isoflurane was inferior to the alfaxalone protocol. During isoflurane anaesthesia of decerebrate animals, baseline reflexes (particularly for heel-MG) were difficult to elicit even with maximal stimulation (10 mA). Although these data are not reported here, comparable studies during isoflurane anaesthesia in Sprague Dawley rats with an intact neuraxis, affirmed it was not possible to elicit NWR and demonstrate DNIC, even at a light plane of surgical anaesthesia. This finding is supported by studies showing DNIC is depressed by isoflurane anaesthesia (Jinks et al. 2003a). Furthermore, other workers have reported that NWR thresholds are significantly increased by isoflurane anaesthesia (Petersen-Felix et al. 1996; Spadavecchia et al. 2010) which is also associated with depression of heat evoked withdrawal responses in rats (Jinks et al. 2003b), and explained by a preferential depression of ventral horn motoneurones contributing to immobility at the expense of a greater depression of nociceptive responses (Kim et al. 2007). Furthermore arterial blood pressure and partial pressure of oxygen are lower during isoflurane anaesthesia compared to alfaxalone supporting the superiority of an alfaxalone CRI for electrophysiology experiments (White et al. 2017). Alfaxalone's pharmacokinetic profile supports its use as a constant rate infusion (White et al. 2017) and the drug has been used successfully for electrophysiology experiments (Kelly et al. 2013; Hsieh et al. 2015; Hunt et al. 2016).

### 4.4.4. Strain susceptibility and tolerance to $N_2O$

Studies have shown $N_2O$ to exhibit strong acute antinociceptive effects in Sprague Dawley rats and resistance in Lewis rats, with tolerance evident between 60–90 minutes of administration and a paucity of tolerance in the Lewis' (Fender et al. 2000). The administration of $N_2O$ can result in a degree of tolerance, the magnitude of which is strain dependent and related to endogenous opioid peptide levels (Berkowitz et al. 1979; Fender et al. 2000). This is further supported by studies that demonstrate maintaining high levels of endogenous opioid peptides by inhibiting enkephalinase reduces tolerance (Rupreht et al. 1984). In the current study the duration of $N_2O$ preconditioning remained constant as tolerance was not an outcome measure being studied, but the rodent strain susceptibility to $N_2O$ was confirmed in comparing the DNIC paradigm exposed to oxygen or a $N_2O/O_2$ mixture. The current study showed no difference in DNIC intensity or duration in Lewis animals, for all three reflexes, with or without $N_2O$ preconditioning. This suggests that in strains where no $N_2O$ resistance is apparent, DNIC is modulated by preconditioning with $N_2O$. The findings in this current study are supported by other studies evidencing Lewis rats having lower basal opioid levels in the PAG, and failing to increase opioid levels in response to the administration of morphine when compared to the in-bred Fischer rat (Nylander et al. 1995); and also in the lack of antinociceptive effect of $N_2O$ in the tail flick test (Fender et al. 2000). Collectively these findings support the prominent view that $N_2O$'s antinociceptive effect are in the main mediated through activation of opiate receptors in the PAG (Fang et al. 1997).

### 4.4.5. Decerebration versus intact neuraxis

Nitrous oxide can demonstrate widespread effects across the neuraxis, directly and indirectly. In the midbrain nitric oxide contributes to $N_2O$'s antinociceptive effect (McDonald et al. 1994; Ishikawa and Quock 2003; Emmanouil et al. 2008; Chung et al. 2016), and $N_2O$ is also known to stimulate the release of CRF in the LC leading to a stimulation of its noradrenergic neurones (Sawamura et al. 2003). Nitrous oxide causes release of opioid peptides in the brain stem (Orii et al. 2002, 2003; Ohashi et al. 2003) which modulate descending noradrenergic inhibitory pathways by absolving them of their tonic control by the GABA-ergic interneurones (i.e. disinhibition). The knock–on effect is these descending pathways can then enhance antinociception at the spinal level via $\alpha$2-adrenergic receptors and inhibitory GABA interneurones (Guo et al. 1999; Orii et al. 2002) with the $\alpha 2_B$-receptor subtype being the most likely candidate for mediating the effect (Sawamura et al. 2000). Currently it remains unclear the role each of these mechanisms contribute to the global antinociception and activity timeframe.

The inclusion of a decerebrate preparation enabled investigation of the forebrain influence on DNIC and NWR. The anaesthesia (necessary to prevent involuntary movement) in the decerebrates had profound effects on the results. Capsaicin injections resulted in profound NWR inhibition in decerebrated animals receiving isoflurane, but not alfaxalone, re-affirming the distinctly different molecular targets for anesthetics (Rudolph and Antkowiak 2004) and the necessity to continually refine the model as we strive to answer the question of where and how anesthetics

cause anaesthesia (Vahle-Hinz and Detsch 2002). As mentioned above, unpublished data (White & Harris) in intact animals receiving isoflurane found the current paradigm unable to reliably elicit NWRs and study DNIC without subjecting animals to potentially aversive stimuli, confirming alfaxalone but not isoflurane facilitates electrophysiological recordings of NWR in intact rats. Interestingly, under isoflurane only in the decerebrate model, obtaining measurable responses in heel-MG proved more difficult compared to evoking reflexes from the toes, possibly a result of a greater reduction in descending inhibitory control of flexors when compared to extensor reflexes, a similar situation observed in rabbits (Harris and Clarke 2003).

## 4.5. IMPLICATIONS OF THE STUDY

The findings from this current study have real implications in three areas: basic pre-clinical experiments incorporating $N_2O$ in the protocol; clinically where the anaesthesia protocol (presence or absence of $N_2O$) could potentially influence descending modulatory pathways and subsequent post-operative pain; and in clinical antinociceptive studies evaluating CPM efficiency. The present studies demonstrated preconditioning with $N_2O$ (albeit through co-administration with isoflurane) can modulate DNIC in naïve animals and antagonised the inherent neuronal plasticity induced by an acute nociceptive input.

Previous preclinical DNIC studies have used $N_2O$ as a component of the anaesthesia, and in view of what researchers now know about the action of $N_2O$, the conclusions drawn require revisiting. Notwithstanding $N_2O$'s effects, studies

have demonstrated that manipulation of endogenous descending controls can enhance DNIC and offer varying degrees of pain relief (Niesters et al. 2014; Bannister et al. 2015; Martini et al. 2015). In view of the fact that it is now believed that those patients suffering from chronic pain may have impaired DNIC, it is interesting to hypothesise whether inhaled $N_2O$ may also be able to enhance DNIC. Future studies investigating this are warranted. It is known that hyperalgesia is common in the postoperative period as a result of surgical nociception (and opioid use too) and is debilitating and unpleasant, negatively impacting on the intended surgery and potentially developing into chronic pain; but what is clear is that in this preclinical model the anaesthesia regimen *per se* was able to modulate descending controls. What mechanisms are responsible is still to be properly elucidated in view of $N_2O$ having multiple target sites. It is tempting to favour a matrix of effects including but not limited to antagonism at NMDA receptors (Jevtovic-Todorovic et al. 1998) and opioid peptide release in the brain stem activating descending noradrenergic inhibitory neurones (Orii et al. 2002; Ohashi et al. 2003; Georgiev et al. 2008). The mechanism of action at NMDA receptors supports its use in persistent pain (Bessière et al. 2010) and a limited number of studies have shown that subgroups of the population may experience less post-operative pain after the use of $N_2O$ during anaesthesia (Leslie et al. 2011; Chan et al. 2016) although the question of neurotoxicity still remains (Savage and Ma 2014; Buhre et al. 2019). Interestingly this gas may also offer promise in treatment of cases of resistant depression (Nagele et al. 2015); a comorbidity frequently occurring in chronic pain patients.

## 4.6. CONCLUSIONS

Nitrous oxide influences DNIC in Sprague Dawley rats (differentially on the three reflexes) but in Lewis ($N_2O$ resistant) rats the effect on DNIC is less marked. Inhibition and facilitation were not evident in decerebrate animals undergoing alfaxalone immobilisation (compared to isoflurane) which suggests the anaesthetic's effects *per se* on forebrain is important. The duration of influence of $N_2O$ is surprising, likely manifesting as a persistent effect on descending controls.

# 5. $N_2O$ MODULATES DNIC IN A MODEL OF OSTEOARTHRITIS

## 5.1 THE PATHOPHYSIOLOGY OF OSTEOARTHRITIS

Osteoarthritis is no longer considered a single disease but a pathological state, a final common pathway of different aetiologies including genetic or acquired cartilage abnormalities, biomechanical stresses, obesity and joint instability. It can affect any articular joint in humans, but most commonly it is the knee, hip, spine and hands that predominate and the aetiological factors can be joint specific too (Glyn-Jones et al. 2015). Newer therapies are being developed but translation to clinical trials is slow, and the latter also require improved design in order to reduce placebo effects, allowing definition of the most appropriate outcome measures and stratification of patients (Conaghan 2013; Zhang et al. 2015; Watt and Gulati 2017). Notwithstanding the peripheral pathologies, modulation in the CNS is essential to the regulation of pain perception. In view of the peripheral and central mechanisms there is a need to employ a mechanism-based therapy in order to tailor the treatment to the condition. The pain reported by patients fails to correlate well with joint pathology and is likely a product of many other inputs into the CNS either facilitating or inhibiting the pain (Bannister et al. 2017). There is an urgent need to better understand the aetiopathogenesis of this osteoarthritic condition preclinically in order to design interventions that could be translated for human utilisation.

### 5.1.1. The role of disordered central pain modulation

Animal studies have shown that some painful conditions may have their origins in disordered central pain modulation (Boyer et al. 2014; Itomi et al. 2016) but also altered descending controls can be responsible for the conservation of persistent inflammatory and neuropathic pain (Nir et al. 2009; Yarnitsky 2010; Bannister and Dickenson 2016b). In many chronic pain states, including osteoarthritis, there is compelling evidence that sustained debilitating effects of pain are fueled by inappropriate activity in descending pathways. Pain intensity is often mismatched by the degree of pathology. Pre-clinical studies have displayed that the balance of power between inhibition and facilitation is overwhelmingly in favor of inhibition in the healthy animal but in chronic, persistent (including neuropathic pain), there is proof that this balance is altered and may in some part contribute to the ongoing pain signature (Porreca et al. 2002; Suzuki et al. 2004; White et al. 2018). The true complexity of descending controls remains unknown, and a multitude of additional neurotransmitters have been implicated (van Wijk and Veldhuijzen 2010; Bannister and Dickenson 2016a) with the actions of inhibitory descending pathways mimicked by commonly used analgesic interventions such as opioids and $\alpha$2 agonists in acute and chronic pain (White et al. 2018).

### 5.1.2. DNIC in chronic pain

As stated in Chapter 1 (section 1.2.11.), in many patients with different persistent painful conditions, for example, migraine, irritable bowel disease, idiopathic pain

states and osteoarthritis, an impaired DNIC, or the human equivalent CPM has been identified (Yarnitsky 2010). The DNIC paradigm can be used in prediction of chronic pain susceptibility (van Wijk and Veldhuijzen 2010). For a clinician, the ability to reliably identify patients with impaired DNIC could help devise a more efficacious and personalised treatment plan, targeting the impaired descending pathways (Arendt-Nielsen and Yarnitsky 2009). One study showed that in patients suffering from diabetic neuropathy, the baseline DNIC correlated with efficacy of the selective serotonin-norepinephrine reuptake inhibitor (SSNRI) duloxetine (Yarnitsky et al. 2012). Testing DNIC/CPM and identifying impairments preoperatively enabled clinicians to predict the likelihood of developing chronic post-operative pain in people undergoing knee replacement (Petersen et al. 2016) and in those having a procedure via thoracotomy (Yarnitsky et al. 2008). Altered CPM is also a characteristic in patients with migraine, chronic tension type headaches, medication overuse headache and headache following traumatic brain injury too (Pielsticker et al. 2005; Perrotta et al. 2010, 2013; Defrin 2014). Moreover, in one study of cluster type headache, DNIC inhibition was absent during the active phase of the disease and evident in the remission phase confirming DNIC dysfunction (Perrotta et al. 2013). Earlier studies in human patients with osteoarthritis undergoing joint salvage showed an absence of pressure pain modulation (induced by heterotropic noxious conditioning stimulation) before surgery suggesting a dysfunction in systems subserving DNIC (Kosek and Ordeberg 2000). Following surgery when the patients were pain free, normal pressure pain modulation returned indicating that the dysfunction of DNIC had been maintained by chronic nociceptive pain. Age and sex matched pain free controls did demonstrate normal pressure pain modulation and DNIC like mechanisms.

### 5.1.3. Studying DNIC and osteoarthritis preclinically

In order to study osteoarthritis and DNIC preclinically, and develop effective therapies, a reliable inducible model that mimics the human disease is required as described in Chapter 1 (section 1.6.1.1). The chondrocyte metabolism inhibitor monosodium iodoacetate (MIA) disrupts glycolysis, causing cell death and resultant changes in the knee joint closely resembling human osteoarthritis (Kalbhen 1987; Williams and Thonar 1989; Guingamp et al. 1997). The mechanical allodynia that results, mimics pain from the non-injured sites reported in humans with osteoarthritis; furthermore, this paradigm results in activated spinal microglia and an upregulated response of spinal neurones. These characteristics represent indicators of central sensitisation providing a unique model for study of central pain mechanisms during osteoarthritis (Sagar et al. 2010, 2011). The MIA model is widely studied and accepted as a model with outcome measures attributable to pain associated with osteoarthritis, namely thermal and mechanical analgesia, mechanical sensitivity and changes in the gait, hyperalgesia and allodynia and hind limb grip force test (Ferreira-Gomes et al. 2008; Liu et al. 2011; Tremoleda et al. 2011; Little and Zaki 2012).

### 5.1.4. Aims of the study

Given the results from Chapter 4 and an obvious effect of $N_2O$ on naïve Sprague Dawley rats undergoing DNIC studies, the aim in this set of experiments was to evaluate DNIC in an induced model of osteoarthritis and to investigate the effect 60 minutes of preconditioning with $N_2O$ could have on the paradigm.

## 5.2. MATERIALS AND METHODS

### 5.2.1. Animals

Forty-eight (75–125g) 4–5 week-old male Sprague Dawley rats were used in this experiment. Animals were housed in single sex groups of 4 as detailed in Chapter 2 (section 2.2.). Animals were acclimatised in the animal unit for ten days prior to entering the study. In each cage two animals were randomly assigned to receive MIA, and two saline (control). Animals were clearly identified by indelible marker pen lines applied to the tail base. The timeline for the experiments is outlined in Figure 5.1.

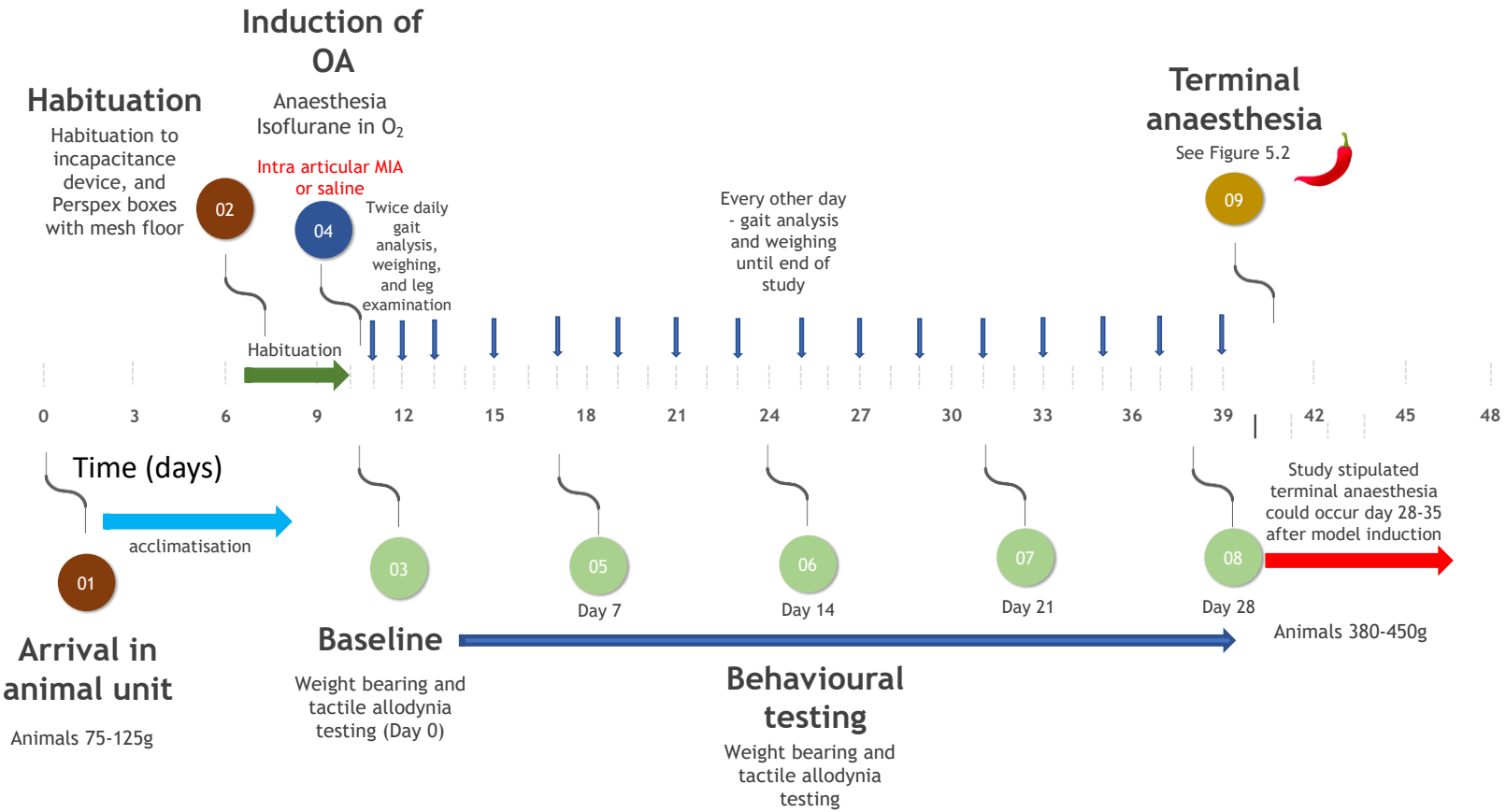



Figure 5.1 Timeline for the Sprague Dawley rats undergoing induction of osteoarthritis with MIA or saline (controls) from arrival at the animal unit to terminal anaesthesia. MIA: monosodium-iodoacetate, OA: osteoarthritis

### 5.2.2. Induction of osteoarthritis

Anaesthesia was induced with 3% isoflurane in $O_2$ and reduced to 2% for maintenance of anaesthesia. The rat was positioned in dorsal recumbency and the left knee was shaved and prepared in an aseptic manner. The animal's leg was flexed 90° at the knee joint and the patellar ligament was gently palpated below the patella. A 29G needle was inserted perpendicular to the ligament in the midline and placement in the articular space confirmed by a loss of resistance. In cases where the joint space was not located the needle was withdrawn and re-positioned. Once placement was confirmed, 1 mg monosodium-iodoacetate (MIA) solution dissolved in 50 µl sterile saline or 50 µl sterile saline alone was injected. After the intra articular (IA) injection was completed the isoflurane was discontinued and the animal recovered in a heated cage. No analgesics or anti-inflammatory drugs were administered.

### 5.2.3. Examination and gait analysis

Twice daily for the first three days following intra articular injections animals' hindlegs were carefully examined for pain, swelling or signs of infection. Animals underwent gait analysis on a flat surface and were observed moving in their double layer cages. The movement/ambulation of the rat on the flat surface as they explored and moved unimpeded was assigned a score: 0 = no limp, normal gait, 1 = slight limp, 2 = marked limp with a decreased use of the ipsilateral limb and 3 = complete avoidance of use of ipsilateral limb. Thereafter gait analyses, weight

gain/loss and overall health were evaluated on alternate days for 28–35 days. All observations and handling were performed by the same individual.

### 5.2.4. Habituation

All testing was carried out in a separate room to the home cages. Animals were habituated to the weight bearing device and the Perspex boxes with mesh floors prior to being used in behavioural testing to minimise stress induced by exposure to a novel, confined environment.

### 5.2.5. Weight bearing paradigm

An incapacitance tester (Linton instrumentation, Norfolk, UK) was used to evaluate weight bearing on the hind limbs prior to joint injection and then at 7, 14, 21 and 28 days after IA injection as described elsewhere (Jhaveri et al. 2007; Sagar et al. 2010). Animals were place in an angled Perspex box and allowed to acclimatise for 5 minutes. The hind limbs were positioned squarely on the right and left force plates with the animal in a normal relaxed position. The weight distribution to each hind limb was measured over a three second period. The difference in weight distribution between the hind limbs was determined.

### 5.2.6. Mechanical threshold paradigm

Tactile mechanical allodynia was assessed using von Frey monofilaments (Stoelting, IL, USA). Rats were removed from their home cages and placed in a

Plexiglas cage with a wire mesh bottom and allowed to acclimatise for 10–15 min (judged by exploratory and grooming behaviours reduced to a level compatible with behavioural testing). The von Frey monofilaments were applied to the mid-plantar left hind paw first, using a series that ranged in stiffness from 0.4 to 28.9 g (0.4, 0.6, 1, 2, 4, 6, 8, 15, 28.9 g). Filaments were applied once for 3 sec with 1 minute of rest between filament application. Rats were tested using the up-down method (Chaplan et al. 1994). Briefly, animals were tested beginning with the 2 g monofilament. If the rat did not emit a positive response (foot lifting/licking), the next highest filament in the sequence was applied until the rat showed a positive response. If the rat did respond to the 2 g monofilament, the next lowest filament was used until the rat stopped emitting a positive response. The test was continued for 5 further stimuli after the first change in response occurred. Tactile allodynia was assessed prior to induction of the osteoarthritis model, and then at 7, 14, 21 and 28 days after IA injection.

### 5.2.7. Anaesthesia and reflex recordings

Between day 28 and 35 after joint injection, all rats underwent terminal anaesthesia for electrophysiology experiments and DNIC evaluation. The anaesthesia and surgical techniques are described in detail in Chapter 2 (sections 2.3., 2.4. and 2.6.2.). The timeline for this part of the study is outlined in Figure 5.2. Twenty four animals (12 control (saline) and 12 induced model (MIA)) received 60 minutes $N_2O$ preconditioning prior to electrophysiology recordings and 24 animals were anaesthetised without $N_2O$ preconditioning (12 control (saline) and 12 induced model (MIA)).

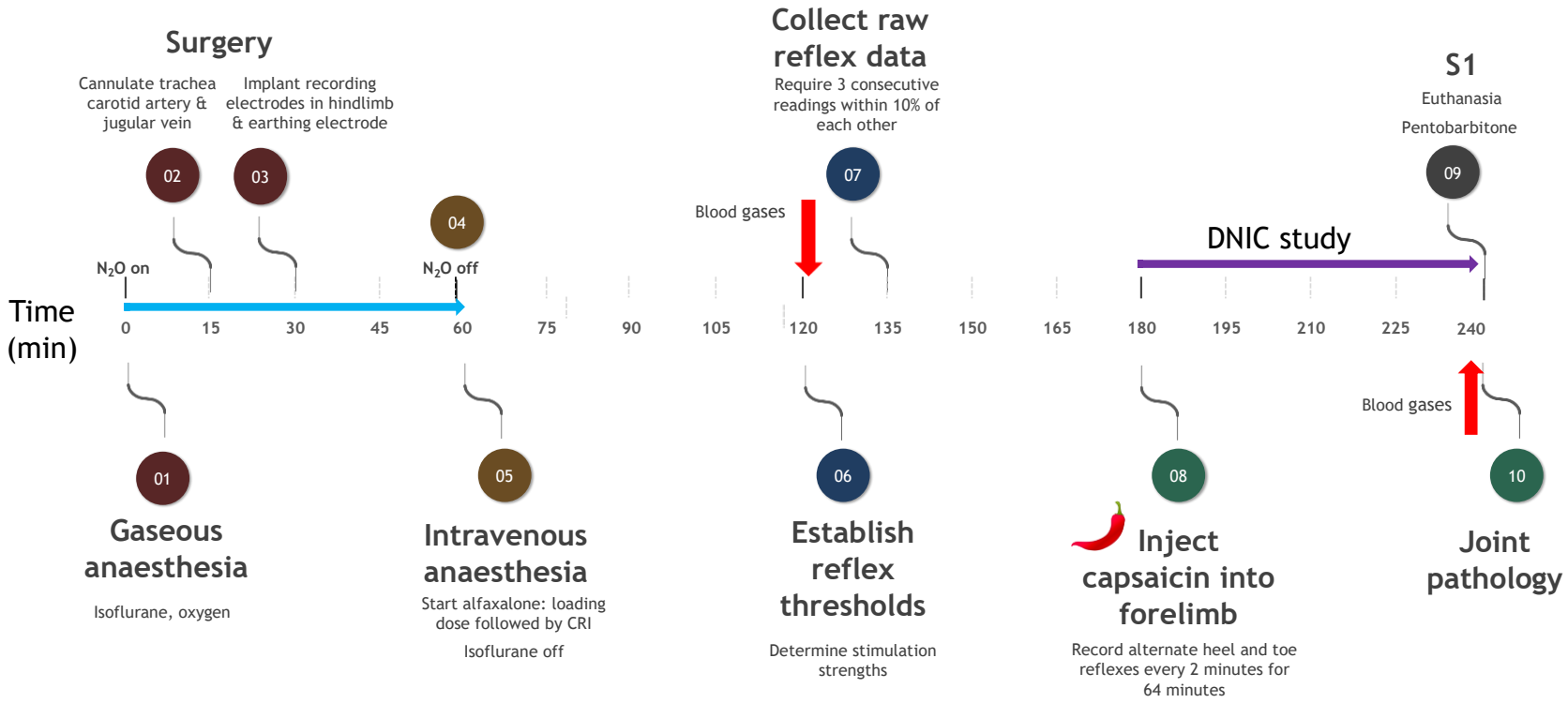



Figure 5.2 Timeline for the 2 groups of male Sprague Dawley rats (MIA and saline (control)) undergoing terminal anaesthesia for DNIC studies. Half of the MIA injected animals and half of the saline injected animals received $N_2O$ preconditioning. All animals were administered alfaxalone dose: 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by a constant rate infusion of 0.75 mg $kg^{-1}$ $min^{-1}$ at time point 5. The DNIC study started at time point 8. CRI: constant rate infusion, DNIC: diffuse noxious inhibitory controls, $N_2O$: nitrous oxide, SD: Sprague Dawley, S1 = euthanasia.

### 5.2.8. Gross anatomical pathology of the knee joints

At the end of the electrophysiology experiment the animal was euthanised with pentobarbitone and underwent cervical dislocation. The left and right knee joints were examined and a gross anatomical assessment was made of the articular surfaces to confirm induction of osteoarthritis. The criteria used for grading the pathology is detailed in Table 5.1.

Table 5.1 Descriptors used to grade the degree of joint chrondropathy

| Grade | Surface Appearance |
|---|---|
| 0 | Normal<br>Smooth surface, homogenous white to off-white colour |
| 1 | Swelling and softening, a little brown homogeneous colouration |
| 2 | Superficial fibrillation, lightly broken surface, white to off-white/light brown in colour |
| 3 | Deep fibrillation – coarsely broken cartilage surface, dark brown, grey or red colour |
| 4 | Subchondral bone exposed – stippled white and dark brown/red in colour |

Descriptors used to grossly grade the degree of chrondropathy macroscopically in the left knee joint (loss of surface grading scale as component of modified Collins scoring scheme, the Outerbridge scale and the SFA (Société Française d'Arthroscopie) scale (Collins 1949; Outerbridge 1961; Dougados et al. 1994)

### 5.2.9. Statistical analysis

Behavioural data (weight bearing and mechano-allodynia) were compared between saline and MIA-injected groups at the same time points using a two way ANOVA. The analyses of the reflex data are described in detail in Chapter 2 (section 2.8.)

## 5.3. RESULTS

### 5.3.1. General health of the animals

No differences in body weight were discernible between MIA-injected and the matched saline control animals over the four-week period (Figure 5.3). Within the first 72 hours the MIA injected animals exhibited knee swelling and occasional mild lameness on a flat surface (1/3 gait analysis score). The 4-point grading system for evaluation is used by our group, and approved by the local Named Veterinary Surgeon, and used elsewhere (Rahman et al. 2009). After 72 hours, until terminal anaesthesia at 28–35 days, the general health of the animals was good. There were no signs of spontaneous nociceptive behaviour, distress or alterted or impaired locomation. All 48 animals completed the study.

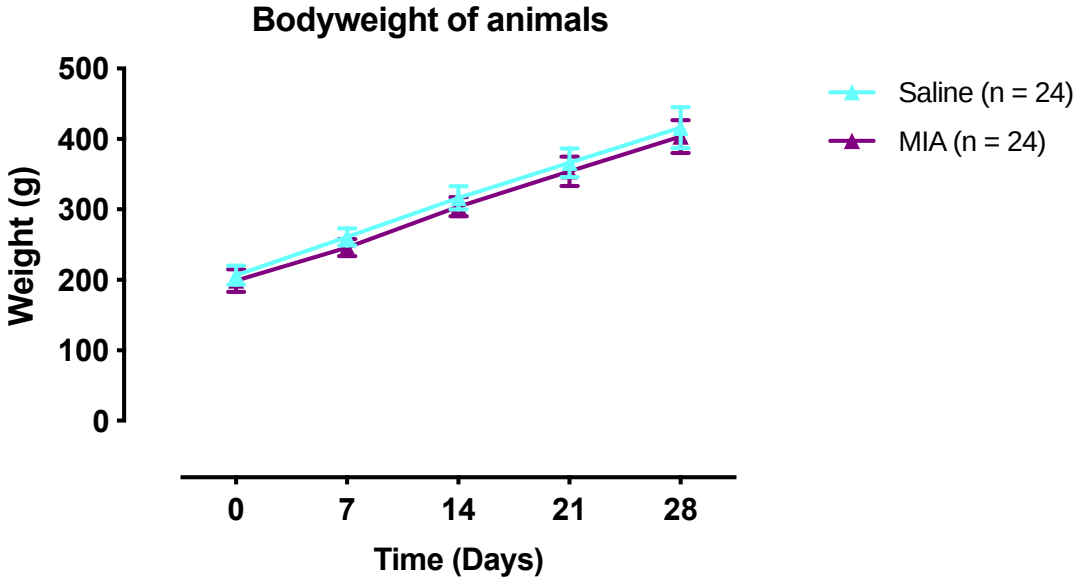



Figure 5.3 Bodyweight change for all animals over the course of the experiment. Intra-articular injections (MIA 1mg in 50 ul, or 50 ul sterile saline) were performed on day 0 under isoflurane (2%) anaesthesia.

### 5.3.2. MIA-evoked pain behaviours

#### *5.3.2.1. Weight bearing*

MIA injection into the left knee significantly decreased ($P < 0.0001$, mixed model ANOVA) ipsilateral hind limb weight bearing compared with saline at 7, 14, 21 and 28 days ($P < 0.0001$, Sidak's multiple comparison test for post hoc contrasts at all time points) confirming development of osteoarthritis induced pain behaviour in this model (Figure 5.4). At all time points, the weight borne by contralateral paws of both saline-injected controls and MIA-treated animals were not statistically different ($P > 0.05$ mixed model ANOVA) from each other.

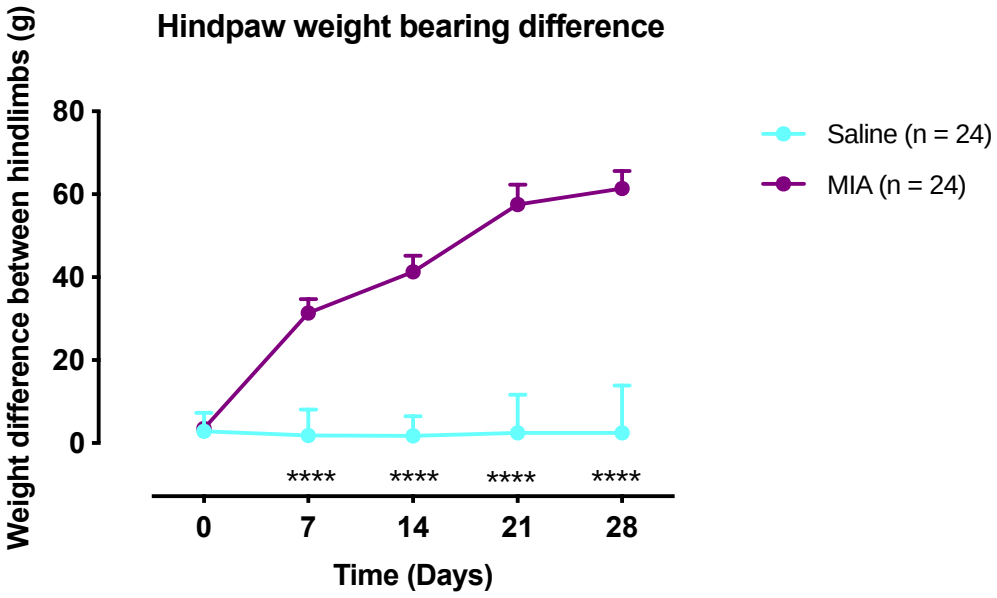



Figure 5.4 Behavioural profile of joint hypersensitivity indicated by differences in weight bearing in pelvic limbs from 0–28 days. A significant reduction in weight bearing of the ipsilateral hind limb was observed in MIA-treated rats (n = 24) compared to the ipsilateral saline-treated limb (control) rats (n = 24). **** $P < 0.0001$ (Mixed model ANOVA followed by Sidak's

multiple comparison test for post hoc contrasts). Each point represents mean ± SEM (24 animals per group, per timepoint).

*5.3.2.2. Tactile mechanical allodynia*

A threshold of 6 g has been suggested (Fernihough et al. 2004), below which a response signifies pronounced allodynia. Figure 5.5 demonstrates a significant reduction ($P < 0.0001$, mixed model ANOVA) in mechanical threshold in the left hind paw all animals following intra-articular MIA from day 7 onwards ($P < 0.0001$, Sidak's multiple comparison test for post hoc contrasts) at 7, 14, 21 and 28 days compared to the saline injected groups. This result confirms development of tactile mechanical allodynia in the injected limb and confirmation of behavioural hypersensitivity.

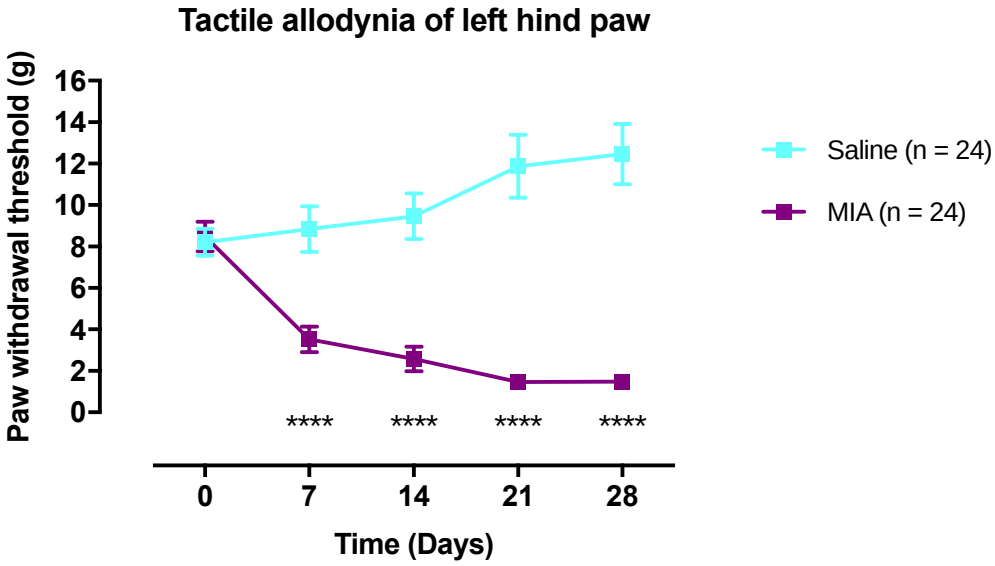



Figure 5.5 The effect of osteoarthritis model induction on tactile allodynia.
Animals injected with intraarticular MIA (n = 24) compared to saline-treated control rats (n = 24). **** $P < 0.0001$ (Mixed model ANOVA followed by Sidak's multiple comparison test for post hoc contrasts). Each point represents mean ± SEM (24 animals per group, per timepoint.

### 5.3.3. Anaesthesia for electrophysiology

#### *5.3.3.1. Cardiopulmonary parameters*

Blood pressure rose steadily during the first hour of alfaxalone anaesthesia from a nadir of 113/68 mmHg (mean 83 mmHg) during inhalational anaesthesia to a baseline of 188/122 mmHg (mean 144 mmHg). Blood pressure was maintained well above values recorded during isoflurane anaesthesia after capsaicin injection (Figure 5.6), and thereafter until S1 as for Chapter 3 and 4. There was a marked individual variation but no difference between the four groups ($P$ = 0.73, mixed model ANOVA). Most animals showed an acute short-lived blood pressure rise immediately after injection of capsaicin into the contralateral forelimb. The median duration of the rise was 8 minutes (IQR 4–10 min), and the median increase was 40 mmHg (IQR 30–60 mmHg) for systolic and 22 mmHg (IQR 8–34 mmHg) for diastolic pressure. Occasionally a second rise in blood pressure was seen between 20 and 45 minutes after the first rise of a smaller magnitude and shorter lived than the first. Some animals exhibited a biphasic rise then fall to values below the baseline values measured before capsaicin injection.

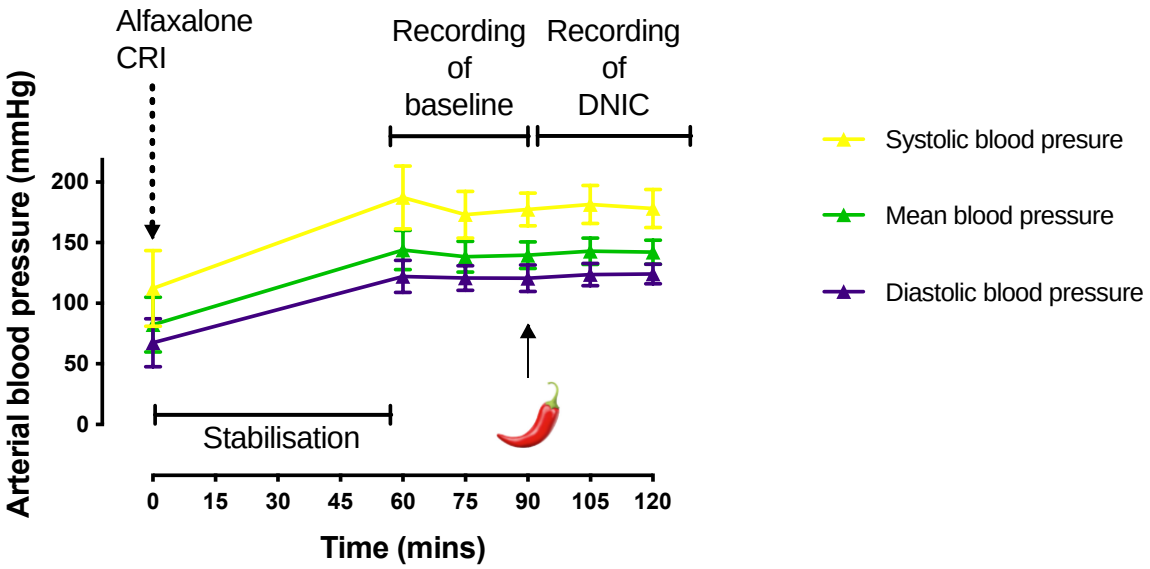



Figure 5.6 Systolic, diastolic and mean blood pressure values during alfaxalone anaesthesia (with or without $N_2O$ preconditioning) and prior to capsaicin injection (chilli pepper symbol) into the contralateral forelimb and thereafter for 30 minutes. The dotted arrow designates the start of the alfaxalone infusion (loading dose of 1.67 mg $kg^{-1}$ $min^{-1}$ for 2.5 minutes followed by a constant rate infusion of 0.75 mg $kg^{-1}$ $min^{-1}$). Isoflurane (and $N_2O$) were switched off 2.5 minutes after the infusion of alfaxalone started. The solid arrow designates the time of injection of capsaicin into the contralateral forelimb. No significant differences (P = 0.73, mixed model ANOVA) were detected between the saline and MIA groups, or between the $N_2O$ preconditioned group or those animals not exposed to $N_2O$, therefore the blood pressure measurements have been pooled from all animals included in the electrophysiology study (n = 46). Two animals did not complete the study. Values are (mean ± SEM).

*5.3.3.2. Blood gases and biochemistry*

In this set of experiments carotid arterial blood samples (0.2 ml) were taken 60 and 180 minutes after the start of alfaxalone CRI for routine monitoring of arterial blood gases, haematology and biochemistry. No significant differences (two-way ANOVA) were observed between the 4 groups at 60 or 180 minutes, and the data has therefore been pooled. A significant increase in lactate was observed in all animals between 60 and 180 minutes ($P < 0.0001$, Mann-Whitney test) resulting in a mild-moderate hyperlactaemia (typically defined as 2–4 mmol $L^{-1}$) likely caused by the catecholamines associated with the noxious injection of capsaicin (Table 5.2). In animals with $P_aCO_2$ > 55 mmHg following the 180-minute sample, intermittent positive pressure ventilation (IPPV) was initiated. Between 3 and 5 animals per group required IPPV. In all of the ventilated animals, injection of capsaicin resulted in spontaneous ventilation resuming and the ventilator was switched off.

Table 5.2 Measured blood gas variables and clinical biochemistry parameters

| Variable | Minutes after the alfaxalone infusion start time | |
|---|---|---|
| | 60 | 180 |
| pH | 7.33 ± 0.01 | 7.34 ± 0.02 |
| $P_aCO_2$ (mm Hg) | 55 ± 2 | 63 ± 4 |
| $P_aO_2$ (mm Hg) | 504 ± 21 | 477 ± 64 |
| $HCO_3$ (mmol $L^{-1}$) | 29 ± 1 | 34 ± 1 |
| Sodium (mmol $L^{-1}$) | 142 ± 1 | 140 ± 1 |
| Potassium (mmol $L^{-1}$) | 4.3 ± 0.3 | 4.7 ± 0.1 |
| Ionized Calcium (mmol $L^{-1}$) | 1.3 ± 0.02 | 1.3 ± 0.01 |
| Chloride (mmol $L^{-1}$) | 107 ± 1 | 101 ± 0.5 |
| Glucose (mmol $L^{-1}$) | 9.2 ± 0.7 | 9.3 ± 3.2 |
| Lactate (mmol $L^{-1}$) | 0.7 ± 0.1 | 3.4 ± 0.8 **** |
| Creatinine (µmol $L^{-1}$) | 44 ± 6 | 43 ± 9 |

Arterial blood was sampled at 60 minutes and 180 minutes after administration of 1.67 mg $kg^{-1}$ $min^{-1}$ alfaxalone for 2.5 minutes followed by 0.75 mg $kg^{-1}$ $min^{-1}$ alfaxalone for all animals included in the electrophysiology study (n = 46). Data were pooled from all animals as there were no significant differences between groups for any variable. Data are mean ± SEM. Derived variables reported by the EPOC analyser (actual bicarbonate, total $CO_2$, base excess of extra cellular fluid, base excess of blood, oxygen saturation, anion gap, anion gap potassium, haemoglobin) were calculated but are not included in the table. **** $P < 0.0001$ (Mann-Whitney test).

### 5.3.4. Hind limb stimulation and recording of NWRs

Reflexes were difficult to elicit and measure in one animal in both saline groups; the reasons for this were unclear, and therefore only 11 animals were included in each of these groups.

There were significant differences in electrical thresholds for eliciting heel-MG reflexes between groups ($P$ = 0.004, Kruskal-Wallis test; Table 5.3), specifically between the two saline groups ($P$ = 0.0482, Dunn's multiple comparisons test), however subsequent stimulation strengths applied to the heel did not differ between groups ($P$ = 0.2163, Kruskal-Wallis test). Similarly, electrical thresholds at the toes for evoking flexor responses differed between groups ($P$ = 0.0004, Kruskal-Wallis test), specifically between the saline animals exposed or not to $N_2O$ ($P$ = 0.0011, Dunn's multiple comparisons test), and MIA animals exposed to $N_2O$ and saline animals without exposure to $N_2O$ ($P$ = 0.0042, Dunn's multiple comparisons test); but stimulation strengths applied to the toes were similar between groups ($P$ = 0.1198, Kruskal-Wallis test).

Table 5.3 Thresholds and stimulation strengths

| | MIA | | Control (saline) | | *P* value |
|---|---|---|---|---|---|
| $N_2O$ duration (min) | Yes (60) | None | Yes (60) | None | - |
| Number of animals | 12 | 12 | 11 | 11 | - |
| Weight (day 28–35) (g) | 412 ± 7 | 379 ± 7 | 421 ± 5 | 372 ± 11 | < 0.0001 |
| MG electrical threshold, heel (mA) | 1.2 (0.7–1.8)[b] | 1.6 (1.4–2.1) | 1.2 (1.1–1.6)[a] | 2.0 (1.7–3.1) | 0.004 |
| Heel stimulation strength (mA) | 6.0 (5.0–6.3) | 6.0 (5.0–7.0) | 6.0 (5.0–6.0) | 7.0 (6.0–7.0) | 0.22 |
| BF/TA electrical threshold, toes (mA) | 1.2 (1.0–1.2)[b] | 1.9 (1.3–3.0) | 1.0 (1.0–1.4)[a] | 3.6 (2.1–4.4) | 0.0004 |
| Toes stimulation strength (mA) | 6.6 (6.0–7.0) | 7.0 (6.0–9.0) | 6.0 (5.5–6.5) | 7.0 (6.0–8.5) | 0.12 |

Demographics (weight, electrical thresholds, and electrical stimuli) for all rats. Data are mean ± SEM or median (IQR). Grey shading denotes $N_2O$ preconditioning. MG: *medial gastrocnemius*, BF: *biceps femoris*, TA: *tibialis anterior*.

[a] designates significant difference between saline controls with and without $N_2O$ preconditioning.

(Kruskal-Wallis one way ANOVA followed by Dunn's multiple comparison test for post hoc contrasts).

For heel-MG and toes-BF reflexes there were no significant differences ($P$ = 0.47 and $P$ = 0.95 respectively, Kruskal-Wallis test) between groups regarding the size of raw control responses prior to the injection of capsaicin (Table 5.4). Raw control reflexes for toes-TA however did differ between groups ($P$ = 0.02, Kruskal-Wallis test) with responses in saline preconditioned group being larger ($P$ = 0.045, Dunn's multiple comparison test) than the saline group not exposed to $N_2O$ (Table 5.4).

Table 5.4 Raw control responses of reflexes elicited from the heel and toes.

| Group | MIA | | Control (saline) | |
|---|---|---|---|---|
| $N_2O$ duration (min) | Yes (60) | None | Yes (60) | None |
| Raw control response heel-MG (μV.ms) | 397 (203–502) | 291 (192–602) | 399 (274–522) | 241 (175–368) |
| Raw control response toes-BF (μV.ms) | 172 (125–256) | 176 (99–275) | 189 (129–437) | 215 (111–247 |
| Raw control response toes-TA (μV.ms) * | 164 (88–271) | 86 (70–167) | 189 (128–282) [a] | 80 (70–163) |

Raw control responses for reflexes in all four groups prior to capsaicin injection. Grey shading denotes $N_2O$ preconditioning. Data are median (IQR). * $P < 0.05$ (Kruskal-Wallis one way ANOVA followed by Dunn's multiple comparison test for post hoc contrasts). MG: *medial gastrocnemius*, BF: *biceps femoris*, TA: *tibialis anterior*.

[a] designates significant between saline controls with and without $N_2O$ preconditioning.

### 5.3.5. The effect of capsaicin on reflex responses

#### *5.3.5.1. Animals with induced osteoarthritis (MIA)*

No overall significant inhibition by the conditioning injection of capsaicin was seen for heel-MG in MIA animals without $N_2O$ preconditioning ($P > 0.05$, Friedman's ANOVA) and in fact in 4/12 animals' reflexes increased in size for the duration of the 64 minutes. In contrast, a period of 60 minutes of preconditioning with $N_2O$ did result in significant capsaicin-induced inhibition ($P < 0.0001$, Friedman's ANOVA) of this response despite the fact that 3/12 responses were facilitated in this group as well. At 17 minutes post-capsaicin, heel-MG responses were inhibited to a median of 81% (IQR 72–95%) of raw controls with preconditioning, and to 87% (IQR 63–

102%) of raw control values for animals not preconditioned to $N_2O$ (Figure 5.7A). There were no significant differences (Kruskal-Wallis) in negative AUC between $N_2O$ or no $N_2O$ groups for heel-MG reflexes ($P$ = 0.0768; Figure 5.8). No significant differences in duration of DNIC were evident either between the groups (Dunn's multiple comparison test).

For the toes-BF reflex, all MIA animals irrespective of preconditioning exhibited significant inhibition from baseline following capsaicin injection ($P$ < 0.0001, Friedman's ANOVA; Figure 5.7B). At 19 minutes post-injection toes-BF was inhibited to a median of 43% (IQR 30–59%) of controls with $N_2O$ preconditioning and 48% (IQR 23–57%) without $N_2O$, with respective median recovery times of 63 min (IQR 63–33min) and 63 min (IQR 58–63min). Although recovery times were not significantly different between groups, there did appear to be some level of recovery in the non-preconditioned group. There were no significant differences (Kruskal-Wallis) in negative AUC between $N_2O$ or no $N_2O$ groups for toes-BF reflexes ($P$ = 0.8687; Figure 5.8). No significant differences in duration of DNIC were evident either between the groups for toes-BF reflexes (Dunn's multiple comparison test).

There was significant inhibition compared to pre-injection controls of reflexes by capsaicin for toes-TA responses in MIA injected animals that had received $N_2O$ preconditioning ($P$ = 0.0029, Friedman's ANOVA); MIA injected animals not exposed to $N_2O$ also exhibited significant inhibition ($P$ = 0.0005, Friedman's ANOVA) (Figure 5.7C). At 19 minutes after capsaicin injection the median inhibition of toes-TA in $N_2O$ preconditioned animals was to 58% (IQR 48–72%), and in those rats not exposed to $N_2O$ 60%, of controls (IQR 48–72%). There were no significant

differences (Kruskal-Wallis) in negative AUC between $N_2O$ or no $N_2O$ groups for toes-TA reflexes (Figure 5.8). No significant differences in duration of DNIC were evident either between the groups for toes-TA reflexes (Dunn's multiple comparison test).

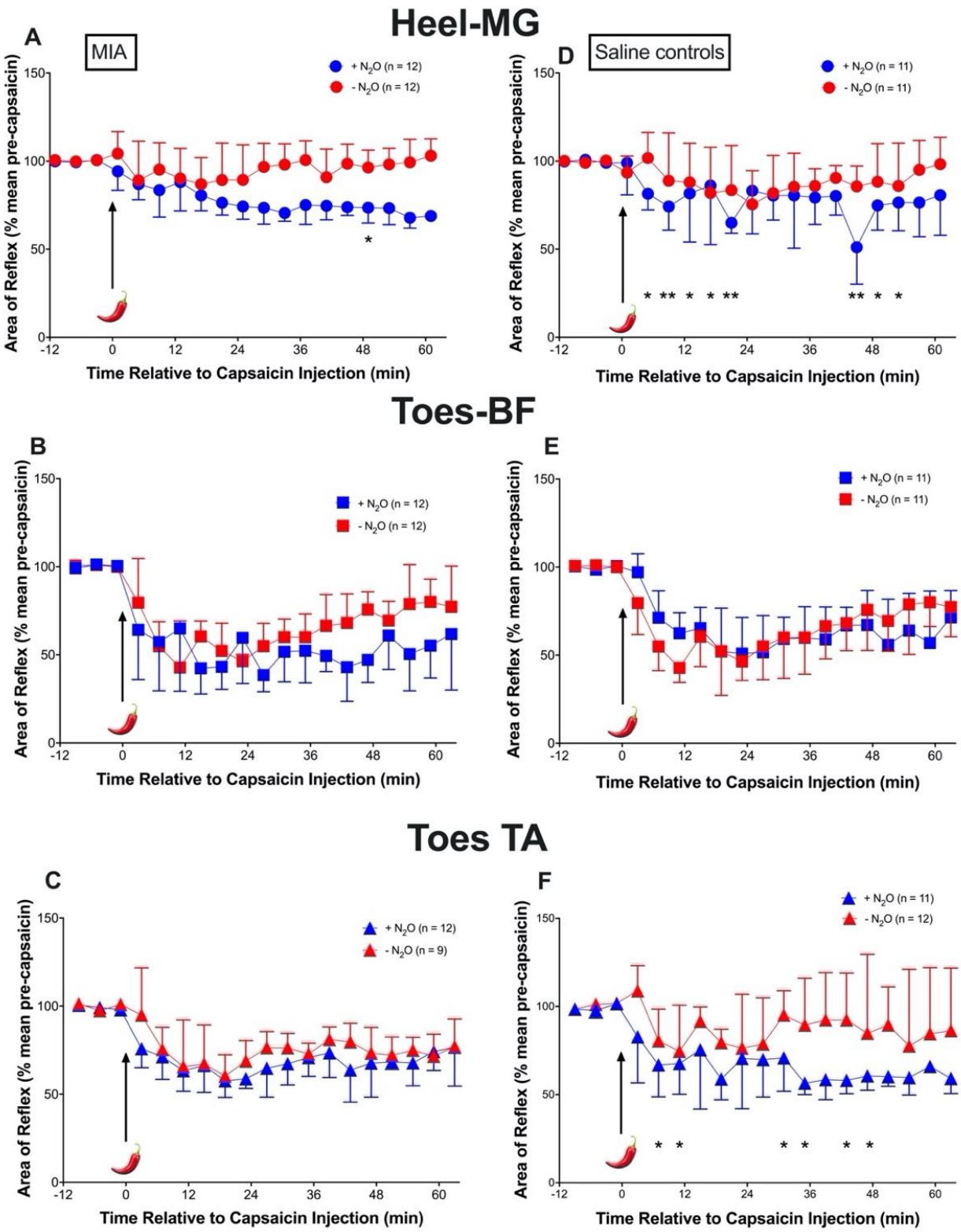

Heel-MG
A
MIA
+ N2O (n = 12)
- N2O (n = 12)
Area of Reflex (% mean pre-capsaicin)
Time Relative to Capsaicin Injection (min)
D
Saline controls
+ N2O (n = 11)
- N2O (n = 11)
Toes-BF
B
+ N2O (n = 12)
- N2O (n = 12)
E
+ N2O (n = 11)
- N2O (n = 11)
Toes TA
C
+ N2O (n = 12)
- N2O (n = 9)
F
+ N2O (n = 11)
- N2O (n = 12)

Figure 5.7 Effect of capsaicin injection on reflexes elicited from heel and toes in the induced (MIA) model and saline controls.

Effects of capsaicin (arrow) to the contralateral forelimb on (A) heel–medial gastrocnemius (MG), (B) toes–biceps femoris (BF) and (C) toes–tibialis anterior (TA) reflexes. Responses on the left-hand side (A-C) were in the MIA model with and without 60 minutes of N2O. Those on the right-hand side (D-F) were obtained in saline-injected control animals with and without 60 minutes of $N_2O$. A significant difference in the effect of capsaicin was found between ± $N_2O$ groups at equivalent time points after capsaicin injection (Mann-Whitney test), * $P < 0.05$, ** $P < 0.01$.

*5.3.5.2. Saline injected (control) animals*

For the saline-injected group, significant inhibition by the conditioning injection of capsaicin was seen for heel-MG responses in animals with $N_2O$ preconditioning ($P$ > 0.0001, Friedman's ANOVA) but was absent ($P$ = 0.1300, Friedman's ANOVA; Figure 5.7D) in those animals anaesthetised with isoflurane in 100% oxygen. At 17 minutes post-capsaicin, median inhibition of heel-MG was 86% (IQR 53–97%) of controls with preconditioning and 82% (IQR 77–124%) without. There were no significant differences (Kruskal-Wallis) in negative AUC between $N_2O$ or no $N_2O$ groups for heel-MG reflexes ($P$ = 0.247; Dunn's multiple comparison test; Figure 5.8). Sixty minutes of preconditioning with $N_2O$ induced significant differences (Mann-Whitney) in the extent of inhibition at the same time points in the saline controls for heel-MG (Figure 5.7D). No significant differences in duration of DNIC were evident between the groups for the heel-MG reflex.

For toes-BF reflexes, all animals irrespective of preconditioning exhibited significant inhibition from baseline following capsaicin injection ($P$ < 0.0001, Friedman's ANOVA; Figure 5.7E). At 19 minutes post-capsaicin, toes-BF was inhibited to a median of 52% (IQR 41–74%) of controls with $N_2O$ preconditioning and 60% (IQR 41–67%) without $N_2O$ and median recovery times were 63 min (IQR 63–63min) and 63 min (IQR 51–63min) respectively. There was no significant difference (Kruskal-Wallis) in negative AUC between $N_2O$ or no $N_2O$ groups for toes-BF reflexes ($P$ > 0.999); Dunn's multiple comparison test; Figure 5.8). No significant difference in duration of DNIC was evident either between the groups for the toes-BF reflex.

For toes-TA reflexes, there was no significant inhibition by the capsaicin injection following $N_2O$ preconditioning ($P$ = 0.1252, Friedman's ANOVA), whereas the group not exposed to $N_2O$ exhibited significant inhibition ($P$ = 0.0026, Friedman's ANOVA; Figure 5.7F). At 19 minutes after capsaicin injection the median inhibition of toes-TA in $N_2O$ preconditioned animals was 59% (IQR 47–8%) of controls and 79% (IQR 48–87%) in those rats not exposed to $N_2O$. There was no significant difference (Kruskal-Wallis) in negative AUC between $N_2O$ or no $N_2O$ groups for toes-TA reflexes ($P$ > 0.1746); Dunn's multiple comparison test; Figure 5.8). No significant differences in duration of DNIC were evident either between the groups for toes-TA reflexes (Dunn's multiple comparison test).

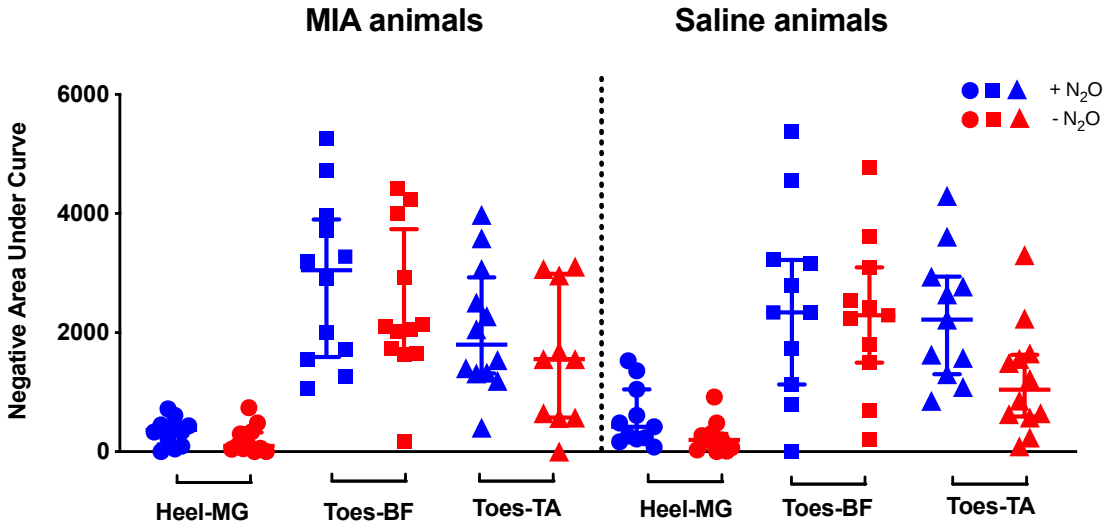



Figure 5.8 Negative area under the curve (AUC) for reflexes elicited from heel and toes for MIA and saline controls.

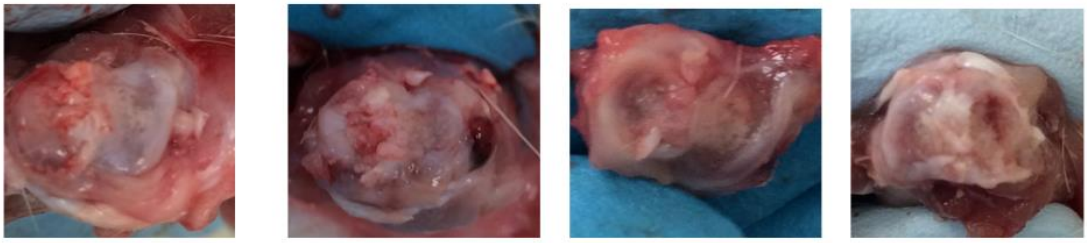
All three reflexes are depicted: heel–*medial gastrocnemius* (MG), toes–*biceps femoris* (BF) and toes–*tibialis anterior* (TA) after injection of capsaicin into the contralateral forelimb in the MIA injected animals and the saline (control) animals with $N_2O$ (blue symbols) or without $N_2O$ (red symbols) preconditioning. Data are median (IQR). There were no significant differences in negative AUC in either the MIA or saline groups ($P$ = 0.077, Kruskal-Wallis one way ANOVA).

For all animals recording of NWR ceased at 64 minutes, and a median recovery time of either 61 minutes (for heel-MG) or 63 minutes (for toes-TA, toes-BF) was recorded if the reflex had not recovered to the control value prior to capsaicin injection. It is therefore likely that the inhibition or attenuation (or in some cases with MG, facilitation) could persist beyond 64 minutes.

### 5.3.6. Pathology

Examination of gross joint pathology was undertaken after euthanasia of the animals to confirm induction of osteoarthritis. All knee joints injected with MIA showed obvious gross changes including extensive loss of cartilage, bony erosion and were classified as 3 or 4 (Figure 5.9A; see Table 5.1). All knee joints injected with saline were unremarkable showing a smooth, off white shiny surface (grade 0) (Figure 5.9B).

A



B

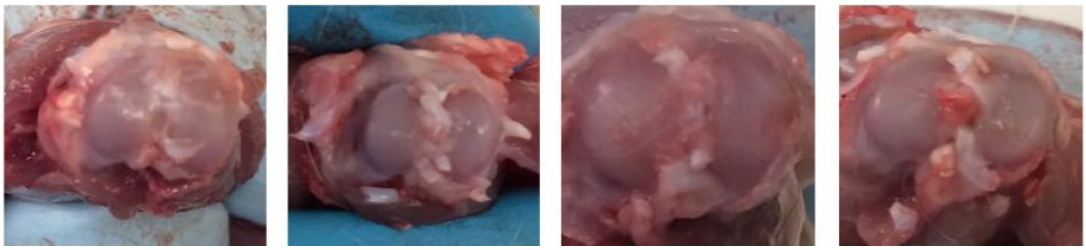


Figure 5.9 Gross joint pathology. A selection of the photographs of the articular surface of the left tibia. The top panel (A) represents joints injected with monosodium-iodoacetate (MIA). The lower panel (B) represents joints injected with saline (controls).

## 5.4 DISCUSSION

### 5.4.1. Induction of hyperexcitability

Intra articular injection into the knee joint with MIA induced hyperexcitability and altered descending control of the nociceptive matrix. Gross pathology confirmed induction of osteoarthritis. The hyperexcitability is inferred by the induced tactile allodynia that was recorded from day seven. This behavioural hypersensitivity is characteristic of secondary hyperalgesia, a feature frequently reported by patients suffering from osteoarthritis (Bajaj et al. 2001; Vonsy et al. 2009).

### 5.4.2. Stimulation thresholds

In these experiments with intact neuraxes, in contrast to other studies (Harris and Clarke 2003), the threshold stimuli required to evoke reflexes in MG from the heel were the same as in flexor muscles from the toes, in both the MIA and saline controls; differential threshold stimuli could indicate differential descending control from higher centres but this was not evident in this study. It is possible that the species difference (rat versus rabbit) and the distinctly different locomotor patterns may be responsible for these contrasts.

It was anticipated that due to central sensitisation, thresholds to elicit a NWR would be less in the MIA animals compared to the controls (Rhudy and France 2007; Courtney et al. 2009; Terry et al. 2011; Kelly et al. 2013) but this was not significant in the populations that received $N_2O$ preconditioning. The decrease in threshold was evident in MIA animals in both reflexes elicited from stimulating the heel and toes

(albeit not reaching significance) when $N_2O$ was omitted. Interestingly, in one recent canine osteoarthritis study, thresholds in dogs with joint disease were increased compared to controls (Hunt et al. 2018) potentially a result incurred by the contribution afforded by the Aβ fibre hypoaesthesia induced by central sensitisation. One defining feature in subsets of human patients with neuropathic pain is a coexistence of sensory gain and sensory loss (Baron et al. 2010; Hochman et al. 2013) which could explain these observations.

### 5.4.3. Inhibition and sensitisation of reflexes

For this experiment in the rat; functionally the reflex can be considered to comprise of the flexors (toes-BF, and toes-TA) and the extensors (heel-MG) with BF having both knee flexor and hip extensor actions. The descending modulation of the flexors and extensors is via the reticulospinal tract and can be modulated as mentioned by a number of factors such as stimulus, pathophysiology, and balance of supraspinal and spinal control. For example, in this experiment heel–MG was both inhibited and sensitised in response to capsaicin, but changes that were manifest were to a far lesser extent when compared to the toes-BF reflex. Similar findings with cannabinoids resulting in differential effects on heel-MG and toes-TA have been seen in other studies (Jenkins et al. 2004), but again the results may have been affected by the use of $N_2O$ (see below for discussion).

In the main, for heel-MG, inhibition of the reflex dominated rather than sensitisation, and this is evident in other preparations too (Harris and Clarke 2003), but the magnitude of changes was limited with heel-MG even with such a potent noxious

stimulus, possibly as a result of the changes being primarily central in origin. This is also suggested in experiments using a less noxious stimulus (mustard oil) in rabbits (Harris and Clarke 2003) where expansion of the sensitisation fields was not evident, suggesting sites able to sensitise heel-MG reflex is rather a product of the spinal cord interneurones rather than descending systems *per se*. In decerebrated animals the tonic inhibition directed at heel-MG compared to those in flexors (toes-TA, toes-BF) is less marked, with flexors eliciting larger amplitude reflexes and more susceptible to inhibition (Harris and Clarke 2003). There is altered DNIC for the toes-TA reflex when comparing the MIA injected animals with the saline controls (in the absence of $N_2O$); the magnitude of the inhibition is less profound and of a shorter duration in the saline animals compared to the MIA animals, the latter assumed to have significant central sensitisation. Kelly et al. (2013) studied spinal nociceptive reflexes in a MIA rat model and found no effect on NWR for toes-TA in the MIA animals, but revealed hyperexcitability on spinalisation. The conclusion drawn was that the descending inhibition had normalised the TA NWR by day 28. The altered DNIC for MIA toes-TA supports these findings. Furthermore, both these studies demonstrate the importance of studying multiple reflexes to understand spinal nociceptive processing. Dysfunctional DNIC has been shown to be maintained in humans by a chronic nociceptive input, and resolved following surgery (Kosek and Ordeberg 2000).

The mechanisms of development of central sensitisation are not well understood. What is known is that excitability of joint derived C and Aδ nociceptors is enhanced by MIA (Kelly et al. 2012) and this enhanced excitability is a key feature of central sensitisation in knee osteoarthritis (Schaible et al. 2011; Schaible 2012). The NWR

findings from the current study are in agreement with human knee osteoarthritis studies where altered lower limb reflexes can be elicited in the disease (Courtney et al. 2009; Suokas et al. 2012).

It has also been shown that spinal neurones can receive convergent input from both the knee joint and hind paw (Schaible and Grubb 1993) and this can manifest as enhanced synaptic efficacy. Stimulation of the low threshold mechanoreceptors of the hind paw (a distal non injured site) in conjunction with the sensitised C and Aδ nociceptors from the joint results in enhanced reflex activity.

Many studies have highlighted the contribution of descending facilitation to central sensitisation in chronic pain as outlined in Chapter 1 (sections 1.2.8.2 and 1.2.11). Pharmacological disruption of the descending facilitatory pathways can attenuate the behavioural hypersensitivity and modulate the activity of WDR neurones (Calejesan et al. 1998; Urban and Gebhart 1999; Burgess et al. 2002; Suzuki et al. 2002; Bee and Dickenson 2008; Rahman et al. 2009).

### 5.4.4. The influence of $N_2O$

Animals preconditioned with $N_2O$ exhibited lower thresholds for both heel-MG and toes-BF/TA in the saline control animals compared to those not exposed to $N_2O$. In Chapter 4 (section 4.3.3.) this was also evident in the naïve male Sprague Dawley rats; a significantly lower threshold was evident for the toes of those animals preconditioned with $N_2O$ (90 minutes) compared to those animals with no exposure to the gas. These findings of reduced thresholds after $N_2O$ exposure is unexpected

and fails to support the claim that the gas provides analgesia or contributes to immobility. Furthermore any effects the gas causes has historically been assumed to occur only during the administration of the gas (Eger et al. 1990), again this is inconsistent with the findings in this experiment.

### 5.4.5. DNIC differences between MIA and saline controls, and the influence of $N_2O$

Differences in the magnitude of DNIC were evident between the induced model and the saline controls but the differences were reflex specific and also influenced by the inclusion of 60 minutes of $N_2O$ pre-conditioning. The preconditioning with $N_2O$ facilitated DNIC in the heel-MG of control and MIA animals but in the absence of $N_2O$, DNIC was absent in heel-MG. In the light of these findings repeating some of the seminal studies (Kalliomäki et al. 1992) without $N_2O$ would be of interest.

Descending noradrenergic inhibitory neurones are fundamental elements of the endogenous pain matrix, and at rest are tonically inhibited by GABA-ergic neurones (Basbaum 1984). It has been proposed that these GABA-ergic interneurones are inhibited by $N_2O$, which activates, or leads to disinhibition of the descending noradrenergic inhibitory neurones (Orii et al. 2003), facilitating in this case DNIC. Activation by $N_2O$ of the descending noradrenergic inhibitory neurones leads to the release of noradrenaline in the dorsal horn. Consequently nociceptive processing is modulated by at least two neuronal pathways (Zhang et al. 1999; Hashimoto et al. 2001; Orii et al. 2003) potentially explaining the different observations. In Schouenborg's modular theory of withdrawal it was proposed that each module

(within a withdrawal reflex) could potentially be differentially affected by exogenous or endogenous substances (Schouenborg et al. 1994). This could incur an added degree of complexity in experiments where for example, nociceptive responsiveness was being evaluated as the individual reflexes could respond in different ways depending on the intensity and proximity of the noxious insult (Kalliomäki et al. 1992; Clarke and Harris 2004) but actually also in response to test drugs or anaesthesia (Jenkins et al. 2004; White et al. 2017). In this experiment the influence of $N_2O$ had differential effects on DNIC of the heel-MG, toes-BF, and toes-TA, and is worthy of further discussion; interestingly those original experiments undertaken by Kalliomaki et al. (1992) and Jenkins et al. (2004) also used $N_2O$ but failed to consider that this gas could be responsible for any differential effects seen in the reflexes. Of the aforementioned studies interrogating facilitation of descending controls $N_2O$ was not used by one group, (Burgess et al. 2002), the effects of $N_2O$ were not discussed or implicated in three sets of experiments (Suzuki et al. 2002; Bee and Dickenson 2008; Rahman et al. 2009). For two studies it was impossible to decide if $N_2O$ had been incorporated since the carrier gas or gases were not stated (Urban et al. 1999; Calejesan et al. 2000).

The spinal cord interneurones may also be a target for the $N_2O$ preconditioning, unmasking and facilitating more inhibition from the descending pathways and more effective DNIC seen in both reflexes elicited from stimulating the toes in Chapter 4 and in this study in heel-MG and toes-TA reflexes. Studies have demonstrated that $N_2O$ exposure increases the expression of GABA-ergic neurones in the spinal cord and is both duration and dose dependent (Hashimoto et al. 2001). Exposure of rats to $N_2O$ induced c-Fos expression in the spinal cord, and it was blocked by prazosin

(α1 adrenergic receptor antagonist) and naloxone (μ opioid antagonist). Additionally c-Fos expression was found to be co-localised with α1 adrenoceptor immunoreactivity in laminae III–IV of the dorsal horn (Orii et al. 2002). This supports the hypothesis that one method of antinociception is produced by $N_2O$ activating GABA-ergic interneurones through α1 adrenoceptors. However, the heel-MG results from the control animals in this study (section 5.3.6.2.) contrast to those in the identical DNIC paradigm tested in Chapter 4 (section 4.3.4.) for heel-MG with naïve Sprague Dawley rats. The most likely reason for the differences observed between the two studies is the duration of exposure to $N_2O$. The longer 90 minute exposure (as opposed to 60 minutes in this experiment) to $N_2O$ may, for the heel-MG have caused a degree of tolerance resulting in sensitisation rather than inhibition of the NWR. The proposed mechanism underlying tolerance is believed to be a result of depletion of enkephalins in the PAG (Berkowitz et al. 1979; Fender et al. 2000); supported too by the finding that enkephalinase inhibitors prevent this development of tolerance to the analgesic effects of $N_2O$ (Rupreht et al. 1984). Following 60–90 minutes of $N_2O$ exposure, activation of the noradrenergic descending inhibitory pathways, leading to modulation of nociceptive processing via α2 adrenoceptors in the dorsal horn of the spinal cord may start to wane as opioids are depleted (Fender et al. 2000). This is further supported by the finding that neonatal rats (up to 3 weeks old) have descending noradrenergic neurones that are not yet anatomically or functionally developed, and thereby lack an antinociceptive effect to $N_2O$ in the tail flick test (Fujinaga et al. 2000). In the past it was assumed that all reflexes were under similar controlling influences and would be equally sensitive to agonists/antagonists, but what has become apparent is the reflex modules differ in their response, depending on paradigm, descending controls,

reflex in question, status of neuraxis (intact versus spinalised versus decerebrated) and strength and location of noxious stimulus, as well as the administration of the agonists, antagonists and sex differences (Chapter 3).

### 5.4.6. Gait control

Moreover, a further consideration in these DNIC studies is the natural gait of the animal being studied as differences in locomotion will necessitate different patterns of limb flexion and extension. Understanding how the flexors and extensors work in the species being studied during locomotion and at rest is necessary since this will invariably influence the tonic control and behaviour of the reflexes (Cazalets et al. 1990) contexualising their control by supraspinal structures (Viala 2006). Studies have shown that the gait phenotype can be altered by modifying the inhibitory and excitatory balance across the midline in the spinal cord; the normal alternating gait of mice can be changed to the hopping gait typical of rabbits by changing the balance in the central pattern generators of the spinal cord (Restrepo et al. 2011). Furthermore it has been shown that the sensory input will ultimately influence the spatiotemporal motor reflex output which means the very nature of the stimulus could alter the reflex generated (Zhuo and Gebhart 1997; Kauppila et al. 1998; Hayes et al. 2009). Additionally, it is not possible to extrapolate across species when considering these arrangements; one comparative study between decerebrate and decerebrate spinalised rabbits found that the spinally intact animal still displayed the characteristic hopping-type motion whereas spinally-transected rabbits resembled the rat and showed the alternating pelvic limb actions (Viala, 2006). Experiments

such as these suggest diversity in the supraspinal control of reflexes may be responsible at least for the observed differences in the rat and the rabbit.

Based on the results in this study it is not currently possible to generalise about the findings, since the paradigm will be influenced by species, type and intensity of the stimulus, sensory processing, degree of pre-existing hyperalgesia, and balance of inhibition/facilitation to each reflex. However, this complexity should not be a deterrent for understanding the role descending controls have in modulating pain perception, or the search for interventions that could modulate the pain signature.

### 5.4.7. The MIA model

The rat monosodium-iodoacetate (MIA) model itself (and also the rat meniscal tear model) are considered quite aggressive models of osteoarthritis (Bove et al. 2009). Intra articular MIA causes chrondrocyte death by inhibiting glyceraldehyde-3-phosphate dehydrogenase. The MIA model has been widely characterised and used as a model for human osteoarthritis. Many studies have shown that in the early stages of development of the model NSAIDs are efficacious, confirming the role played by prostaglandins in the acute stages of the disease development (Bove et al. 2003; Pomonis et al. 2005). However the effectiveness of the NSAIDs begins to wane with time (Ivanavicius et al. 2007) necessitating the acute administration of opiates, gabapentin and tricyclic antidepressant drugs in order to provide on-going relief. The ineffectiveness of the NSAIDs as the disease progresses suggests that the inflammatory component is less dominant and it is likely the peripheral drive develops a neuropathic component and in view of the diversity and multitude of

structures implicated in osteoarthritis this is a logical conclusion (Ivanavicius et al. 2007). This theory is further supported by studies that have identified TRPV1 and CGRP expression in the nerves that originate in the knee joint with induction of activating transcription factor expression, a potential marker of neuronal damage in the L5 dorsal root ganglion (DRG) (Fernihough et al. 2005; Ivanavicius et al. 2007).

The efficacy of gabapentin and amitriptyline in reducing pain associated behaviours confirms a peripheral and central effect; both are effective in alleviating behaviours associated with neuropathic pain (Mao and Chen 2000; Bomholt et al. 2005). The role the neuropathic pain and central sensitisation play in osteoarthritis is not well defined but nonetheless acknowledged as typically occurring in the later stages (Bajaj et al. 2001; Dimitroulas et al. 2015; French et al. 2017). In models such as MIA, the mechanical allodynia that manifests itself late in the disease can be reversed with drugs designed to treat neuropathic pain, reaffirming support for the MIA model for analgesic intervention testing but also emphasizing the central mechanisms at work in the disease. (Combe et al. 2004; Fernihough et al. 2004; Kalff et al. 2010; Lee et al. 2011). Impaired endogenous pain mechanisms have been implicated in driving the pain associated behaviours in some populations of humans in chronic pain states such as osteoarthritis (Kosek and Ordeberg 2000).

### 5.4.8. DNIC dysfunction

Impaired DNIC in this study was not obvious in the heel-MG and toes-BF reflexes, but was evident some of the toes-TA reflexes of the MIA groups. (Borsook et al. 2018; Hunt et al. 2018). It is possible that the noxious stimulus strength may have

been inappropriate for reliably inducing an inhibitory/facilitatory response in all reflexes. DNIC dysfunction was evident in toes-TA when MIA and saline groups were compared in the absence of $N_2O$. Since DNIC, or conditioned pain modulation (CPM) is influenced by cognitive processes in man (Nir et al. 2009) this DNIC paradigm may have been heavily influenced by the anaesthetised state. Despite best efforts to utilise the most appropriate anaesthesia (Kelly et al. 2013; Hunt et al. 2016) and tightly control this variable between individuals, the DNIC dysfunction was less than expected.

This MIA model provides a paradigm for studying the DNIC phenomenon in a chronic disease model, and the MIA model is widely accepted by researchers for mimicking the development of human osteoarthritis. One major drawback of the MIA model is that killing the chondrocytes effectively eliminates one of the major contributors to osteoarthritis pathophysiology. In view of this, in conjunction with possible MIA induced sensory nerve damage it is possible the model is too severe for accurate study of subtleties associated with DNIC.

## 5.5 CONCLUSION

In summary this study is the first to describe the comparative effects of preconditioning with $N_2O$ on DNIC in an induced osteoarthritis model demonstrating long lasting effects well beyond the period of administration. Differences in the magnitude of DNIC were evident between the saline and induced model but the differences were reflex specific and also influenced by the inclusion of 60 minutes of $N_2O$ pre-conditioning. Based on these results it would be advisable to omit $N_2O$

from anaesthesia protocols interrogating the descending control of reflexes. It is evident that $N_2O$ is able to modulate descending controls and further work is required to investigate the translational possibilities of this intervention.

# 6. GENERAL DISCUSSION

## 6.1. DNIC SUMMARY

The excitability of spinal nociceptive pathways is dynamically governed by a number of supraspinal sites, namely the PAG-RVM, subnucleus reticularis dorsalis and ventrolateral medulla (Heinricher et al. 2010). These neurones form part of the descending controls, and have large inhibitory or excitatory receptive fields in the dorsal horn of the spinal cord, where they form projections with WDR neurones (Le Bars 2002). Activation of these systems following application of a noxious stimulus, in this case capsaicin applied to the contralateral forelimb, selectively inhibits the WDR neurones. This mechanism, termed diffuse noxious inhibitory controls, was first reported over 40 years ago (Le Bars et al. 1979b,a; Dickenson et al. 1980). The DNIC have a profound effect on the excitatory nociceptive pathways (Schouenborg and Dickenson 1985; Kalliomäki et al. 1992; Schouenborg 1997) and the fact they are implicated in contributing to aspects of chronic pain necessitates their study. DNIC affect the NWR in a specialised manner, confirming the complexity of the system, and highlighting that separate nociceptive pathways exist to the different muscles of the hindlimb (Schouenborg and Kalliomäki 1990; Kalliomäki et al. 1992; Schouenborg et al. 1992, 1995). This is clearly shown in the studies in this thesis, as the response of heel-MG, toes-BF and toes-TA exhibited distinct and differing patterns of mainly inhibition, in response to a heterotopic conditioning stimulus.

The choice of the potent drug capsaicin injected intramuscularly as the noxious stimulus was an iterative choice building on previous studies in our group (Harris

and Clarke 2003, 2007; Dobson 2013; Kelly et al. 2013). Capsaicin, the pungent ingredient of chilli or capsicum peppers is a selective activator of the thermo and mechano-sensitive TRPV1 cation channel (Caterina et al. 1997; Szallasi et al. 1999). Eliciting a reliable, long lasting and reproducible DNIC was the aim with the reported model. An intra muscular injection of capsaicin into a heterotropic site away from the recording of the NWR was chosen, albeit potentially at the expense of repeated injections. Our laboratory has refined the rodent NWR experimental paradigm (Kelly et al. 2013) from extensive work in rabbits and rats, mapping organization of the withdrawal fields (Clarke et al. 1989; Harris and Clarke 2003, 2007; Clarke and Harris 2004). The duration of inhibition recorded in these experiments in Chapters 3, 4 and 5 was consistent and predictable, and aligns with some other studies particularly using an injectable chemical conditioning stimulus such as capsaicin to elicit DNIC (Itomi et al. 2016). In many of the experiments reported in this thesis, DNIC were still evident at least 64 minutes after injection of the capsaicin, albeit fading. Studies using heat, $CO_2$ laser, or mechanical stimuli such as pinch or tourniquet report varying durations of DNIC lasting from only the duration of application of the stimulus to up to 60 minutes and in some cases unrelated to the intensity of the stimulus (Kakigi 1994; Graven-Nielsen et al. 1998; Svensson et al. 1999; Fujii et al. 2006). However, it is impossible to extrapolate across paradigms, especially with human DNIC studies due the additional psychophysical variable, so currently the duration of DNIC generally, remains undefined.

As for many laboratories the habit for rodent anaesthesia has been to rely on the combination of a volatile agent such as halothane or more latterly isoflurane in

combination with $N_2O$ in many cases. The tendency has been to ignore the influence of the anaesthesia protocol on the research in question, despite what we now know about the volatile agents, $N_2O$ and the injectable anaesthetic drugs. It was imperative to select a regimen that could offer unconsciousness, immobility, and a lack of toxicity or cumulating effect in view of the duration of the experiments (4-8 hours), but also importantly lacking in antinociceptive effects. Alfaxalone administration to rats is not new, but is not common, and prior to these studies the kinetics were unknown in male Sprague Dawley rats, thereby necessitating extrapolation from other species, and a degree of trial and error in its use. The use of alfaxalone IP is unreliable for providing a surgical plane of anaesthesia, with 3/10 rats in one study failing to become anaesthetised, and bolus dosing rats IV was complicated by apnoea, potentially unacceptable in the absence of being able to secure an airway (Lau et al. 2013). These challenges were surmountable by inducing anaesthesia with a volatile agent and infiltrating lidocaine subcutaneously to undertake the surgery. Once the trachea, carotid artery and jugular vein were cannulated, the alfaxalone infusion was started and isoflurane terminated in the majority of studies (the exceptions were the two groups of decerebrated rats that received isoflurane for the duration of the study).

Prior to starting the study, there was a paucity of data pertaining to sex differences with alfaxalone. One older study had identified a sex difference with the alphaxalone/alphadolone mixture in cremophor EL (Saffan) (Fink et al. 1982). Following our publication of the PK/PD differences in male and female rats (White et al. 2017) one other research group published findings on sex difference studies using alfaxalone in rats (Arenillas and Gomez de Segura 2018). The rate of infusion

of alfaxalone for the PK study and subsequent DNIC studies was chosen after evaluating planes of anaesthesia, blood pressure and necessity for IPPV in other related studies in the laboratory. Whilst it may have been preferable to have had a larger number of blood samples for the PK study, the limiting factor was the total amount of blood removed from the rat over the course of the experiment; therefore, the experiment was modelled to ensure as many samples as possible were taken during the predicted steep upstroke of plasma concentrations seen with an IV bolus followed by an infusion. The sex differences found in the PK study (White et al. 2017) informed a subsequent experiment in Chapter 3 to evaluate DNIC in females with similar plasma concentrations to the males by infusing alfaxalone at a reduced rate. This experiment identified a difference in the magnitude of DNIC between the sexes, only confirmed by performing the PK study and repeating the electrophysiology experiments in a second group of female animals. This difference in DNIC observed between the sexes in the second experiment supports the hypothesis of true sexual dimorphism in pain processing but raises the question of whether the plasma concentration is a suitable surrogate marker to compare 'depths' of anaesthesia under which to compare the DNIC paradigm. Had the plasma concentrations and PK data been ignored, the conclusion with respect to DNIC between the sexes, would have been different.

Further studies incorporating electroencephalograms and fMRI may go some way to answer this question, as would inclusion of gonadectomised male and female rats to evaluate the effect of sex hormones on DNIC (Da Silva et al. 2018). Furthermore, the results may have bearing on the surgical neutering of companion animals, which

is commonplace in Western society but the influence this process may have on endogenous pain control is unknown.

However, all of these studies necessitate the use of anaesthesia and this introduces a confounding factor. Reducing the exposure to the volatile agents and maintaining as short a period of anaesthesia possible is commendable in the preparation of the model, but the effects remain to varying degrees. Anaesthesia is not innocuous, and its detrimental effects can persist for years (Evered et al. 2018), or in some cases can be utilised; for example a proof of concept trial found inhaling 50% $N_2O$ for an hour could have a long term modulation on refractory depression (Nagele et al. 2015). The investigations in Chapters 4 and 5 detailing the effects of $N_2O$ preconditioning on DNIC were necessary in the reductionist approach taken to understanding the DNIC pathway in the naïve and induced model of disease. The irrefutable evidence that the presence of $N_2O$ has modulating effects has implications for both past results and future planning of experiments. We know that the long-lasting inhibition of the spinal reflexes following the application of a noxious insult such as capsaicin is mediated through activation of the supraspinal noradrenergic and serotonergic pathways. The evidence that $N_2O$ is able to modulate the descending pathways too through its actions complicates conclusions drawn if the gas was used in the anaesthetic protocol. The exact modulation and magnitude requires further study. It is likely to be a matrix of effects, both central and peripheral. Although digressing slightly, it is worth mentioning that although the centrally mediated endogenous analgesia afforded by DNIC is often the focus of studies, DNIC analgesia is also mediated through peripheral mechanisms too such as opioids and somatostatin (Pintér et al. 2006; Stein et al. 2009).

Returning to the studies in this thesis, further scrutiny of the experiments also begs the question of the role of the isoflurane preconditioning. One argument could support the premise that a short period of controlled exposure to a minimally metabolised volatile agent such as isoflurane followed by a period of at least 2 hours washout before data collection goes a long way to address this concern. In these experiments this approach was taken, with close attention paid to standardising end tidal isoflurane percentages, and end tidal $CO_2$ values, instigating IPPV where necessary to maintain the values within normal ranges prior to the alfaxalone anaesthesia. However, the caveat remains, that isoflurane may have modulated the effect in all the studies. The serial blood gas measurements and haematology and biochemistry results further ensured standardisation and monitoring to a rigorous level uncommon in rodent anaesthesia experiments, furthermore these data are compelling evidence of the stability and safety afforded by the alfaxlone CRI. Indeed, alfaxalone offered superior anaesthesia when compared to isoflurane for studying NWR and DNIC in intact neuraxes and in the decerebrate model (Chapter 4).

The investigation of DNIC in an induced model of osteoarthritis in Chapter 5 paves the way for future studies. A subsection of the animals with the induced disease will potentially have impaired DNIC either as a result of the osteoarthritis, or an underlying predisposition. Identifying this subset was not a primary aim, and beyond the scope of this study.

## 6.2. THE IDEAL MODEL TO STUDY NOCICEPTION

The characteristics of an ideal model evaluating nociception in animals have been comprehensively reviewed and evaluated (Le Bars et al. 2001; Berge 2011, 2014; McKune et al. 2015). The focus in most cases is a conscious animal model, but many considerations are equally applicable to the anaesthetised paradigm.

Briefly, the stimulus applied must possess input specificity that is, must be nociceptive. This is not always obvious, and the presence of a flexion reflex does not confirm it is a nociceptive flexion reflex. In this series of experiments the specificity of the noxious conditioning stimulus (capsaicin) is well recognised as noxious (Bevan and Szolcsányi 1990; Lynn et al. 1992) and the nociceptive input from the electrical stimuli causing the NWR is a validated method of studying the spinal nociceptive processing (Harris and Clarke 2003, 2007) and therefore this also by inference confirms the output specificity because of the anaesthesia and immobility.

The sensitivity of the experiments was demonstrated by being able to quantify the magnitude of the response (inhibition/sensitisation), nadir of response and the reliable duration of effect of at least 64 minutes. Furthermore, the sensitivity was demonstrated as illustrated by the manipulation of DNIC by $N_2O$, alfaxalone and isoflurane. The validity of the model was confirmed by the long term stability (up to 8 hours), and minimal change in physiological parameters over the time course of the experiment and the measurement of NWR in response to a heterotopic stimulus, that is DNIC. The reproducibility was demonstrated over the time course of the

experiments only within our laboratory, and replicated in other laboratories albeit in other species (dog and cat) (Hunt et al. 2018). These proven characteristics verify the NWR exemplar.

### 6.3. FUTURE RESEARCH

The NWR paradigm described here presents an excellent whole animal model for evaluating the spinal nociceptive processing, descending controls and DNIC. The skills involved in undertaking electrophysiology recordings *in vivo* is an important training tool for researchers in neuroscience and pain research and should be continued to be taught. The anaesthetic refinements that were achieved throughout the studies are critical to the baseline recordings and interpretation of results. Future studies should omit the confounding factor of $N_2O$ as a routine component of the anaesthetic, but instead regard it a possible intervention and pursue understanding whether the gas may have a role in modulating impaired DNIC in chronic pain scenarios. There are a multitude of follow on studies possible to gain further insight into the mechanism of DNIC in both the naïve and induced disease model following on from this series of experiments. For example, in the first instance the role of alfaxalone peripherally requires further scrutiny in view of its infusion throughout the DNIC study. It is unknown whether alfaxalone and the cyclodextrin it is formulated in have effects on the peripheral nociceptors, particularly the TRP receptors being recruited by the capsaicin in this model. Propofol, etomidate and the volatile anaesthetics activate peripheral noicieptors in particular TRPA1 (Campbell et al. 1984; MacIver and Tanelian 1990; Matta et al. 2008), but it is currently not known what effect alfaxalone has on the range of peripheral nociceptors, transduction or

modulatory effects at a spinal cord level. With the recent finding of the ability of anaesthetics to disrupt the lipid rafts in much the same way as mechanical force does to cause a PLD2 translocation and activation of TREK-1, the molecular actions of drugs such as alfaxalone on TREK channels is also unknown, since to date there has been acceptance that its actions are confined to activating the $GABA_A$ receptor to cause anaesthesia and no definitive antinociceptive effects. The central effects of alfaxalone with respect to the pain pathways is an area where there is again a paucity of data and research.

The stability of the model lends itself to pharmacological experiments such as intrathecal administration of agonists and antagonists targeting but not limited to the noradrenergic and serotonergic receptors involved in the tonic control and the descending inhibition and sensitization. The decerebration paradigm enables removal of forebrain structures involved in the endogenous pain pathways eliminating their influence in controlled studies. Additionally the effects of the anaesthesia on forebrain structures can be interrogated too using this same model.

The reflex data in this thesis were focused on the A fibre activity, after electrical stimulation and this would benefit from investigating concurrent C fibre activity too. Furthermore, it would be interesting to understand if subsequent injections of capsaicin induce a subsequent sensitisation of reflexes.

It is likely that chronic pain research involving animal models will evolve further to include but not be limited to tests of CPM, central sensitisation (measured with quantitative sensory testing), peripheral sensitisation (measured with tools

evaluating allodynia and hyperalgesia) and more comprehensive ethograms. Furthermore, tests that can evaluate spontaneous pain behaviours are desirable, in addition to being able to assess the complex relationship between chronic pain and quality of life through aspects of sleep, mood, cognitive ability, appetite and the negative affect. These tools can be back and forward translated and used in tandem with the electrophysiology experiments such as NWR, EEG and advanced imaging and genomics. Despite all of these challenges in the non-verbal species, there is more need than ever to correlate the altered pain phenotypes that occur in pain states in laboratory and domestic animals used in research with human clinical behaviours to ensure therapy is targeted and efficacious and pain interventions can be better developed, and finally, to forever question and scrutinise the model being used.

# REFERENCES

Abate, M., D. Pulcini, A. Di Iorio and C. Schiavone. 2010. Viscosupplementation with intra-articular hyaluronic acid for treatment of osteoarthritis in the elderly. Curr. Pharm. Des. 16: 631–40.

Acosta, C., L. Djouhri, R. Watkins, C. Berry, K. Bromage and S. N. Lawson. 2014. TREK2 expressed selectively in IB4-binding C-fiber nociceptors hyperpolarizes their membrane potentials and limits spontaneous pain. J. Neurosci. 34: 1494–1509.

Ahern, B. J., J. Parvizi, R. Boston and T. P. Schaer. 2009. Preclinical animal models in single site cartilage defect testing: a systematic review. Osteoarthr. Cartil. 17: 705–713.

Akopian, A. N., V. Souslova, S. England, K. Okuse, N. Ogata, J. Ure, A. Smith, B. J. Kerr, S. B. McMahon, S. Boyce, R. Hill, L. C. Stanfa, A. H. Dickenson and J. N. Wood. 1999. The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. Nat Neurosci 2: 541–548.

Al-Metwalli, R. R., H. A. Mowafi, S. A. Ismail, A. K. Siddiqui, A. M. Al-Ghamdi, M. A. Shafi and A. R. El-Saleh. 2008. Effect of intra-articular dexmedetomidine on postoperative analgesia after arthroscopic knee surgery. Br. J. Anaesth. 101: 395–399.

Alves, H. N. C., A. L. M. Da Silva, I. A. S. Olsson, J. M. G. Orden and L. M. Antunes. 2010. Anesthesia with intraperitoneal propofol, medetomidine, and fentanyl in rats. J. Am. Assoc. Lab. Anim. Sci. 49: 454–459.

Amandusson, Å. and A. Blomqvist. 2013. Estrogenic influences in pain processing. Front. Neuroendocrinol. 34: 329–349.

Amato, V., E. Viña, M. G. Calavia, M. C. Guerrera, R. Laurà, M. Navarro, F. de Carlos, J. Cobo, A. Germanà and J. A. Vega. 2012. TRPV4 in the sensory organs of adult zebrafish. Microsc. Res. Tech. 75: 89–96.

Andaluz, A., N. Felez-Ocaña, L. Santos, L. Fresno and F. García. 2012. The effects on

cardio-respiratory and acid-base variables of the anaesthetic alfaxalone in a 2-hydroxypropyl-β-cyclodextrin (HPCD) formulation in sheep. Vet. J. 191: 389–92.

Anraku, Y., H. Mizuta, A. Sei, S. Kudo, E. Nakamura, K. Senba and Y. Hiraki. 2009. Analyses of early events during chondrogenic repair in rat full-thickness articular cartilage defects. J. Bone Miner. Metab. 27: 272–286.

Ansah, O. B., M. Raekallio and O. Vainio. 2000. Correlation between serum concentrations following continuous intravenous infusion of dexmedetomidine or medetomidine in cats and their sedative and analgesic effects. J. Vet. Pharmacol. Ther. 23: 1–8.

Antognini, J. F. and K. Schwartz. 1993. Exaggerated anesthetic requirements in the preferentially anesthetized brain. Anesthesiology 79: 1244–1249.

Antunes-Rodrigues, J., M. De Castro, L. L. K. Elias, M. M. Valença and S. M. McCann. 2004. Neuroendocrine Control of Body Fluid Metabolism. Physiol. Rev. 84: 169–208.

Antunes, L. M., H. D. R. Golledge, J. V Roughan and P. A. Flecknell. 2003. Comparison of electroencephalogram activity and auditory evoked responses during isoflurane and halothane anaesthesia in the rat. Vet. Anaesth. Analg. 30: 15–23.

Appleton, C. T., D. D. McErlain, J. L. Henry, D. W. Holdsworth and F. Beier. 2007. Molecular and histological analysis of a new rat model of experimental knee osteoarthritis. Ann N Y Acad Sci 1117: 165–174.

Arendt-Nielsen, L. and D. Yarnitsky. 2009. Experimental and clinical applications of quantitative sensory testing applied to skin, muscles and viscera. J Pain 10: 556–572.

Arenillas, M. and I. A. Gomez de Segura. 2018. Anaesthetic effects of alfaxalone administered intraperitoneally alone or combined with dexmedetomidine and fentanyl in the rat. Lab. Anim. 52: 588–598.

Baccaglini, P. I. and P. G. Hogan. 1983. Some rat sensory neurons in culture express characteristics of differentiated pain sensory cells. Proc. Natl. Acad. Sci. U. S. A. 80: 594–598.

Baik, J.-H. 2013. Dopamine signaling in reward-related behaviors. Front. Neural Circuits 7: 1–16.

Bajaj, P., T. Graven-Nielsen and L. Arendt-Nielsen. 2001. Osteoarthritis and its association with muscle hyperalgesia: an experimental controlled study. Pain 93: 107–114.

Bandell, M., G. M. Story, S. W. Hwang, V. Viswanath, S. R. Eid, M. J. Petrus, T. J. Earley and A. Patapoutian. 2004. Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron 41: 849–857.

Bannister, K. and A. H. Dickenson. 2016a. What do monoamines do in pain modulation? Curr. Opin. Support. Palliat. Care 10: 143–148.

Bannister, K. and A. H. Dickenson. 2016b. What the brain tells the spinal cord. Pain 157: 2148–2151.

Bannister, K., M. Kucharczyk and A. H. Dickenson. 2017. Hopes for the Future of Pain Control. Pain Ther. 6: 117–128.

Bannister, K., R. Patel, L. Goncalves, L. Townson and A. H. Dickenson. 2015. Diffuse noxious inhibitory controls and nerve injury: restoring an imbalance between descending monoamine inhibitions and facilitations. Pain 156: 1803–1811.

Baron, R., A. Binder and G. Wasner. 2010. Neuropathic pain: Diagnosis, pathophysiological mechanisms, and treatment. Lancet Neurol. 9: 807–819.

Barrett, A. C., E. S. Smith and M. J. Picker. 2002. Sex-related differences in mechanical nociception and antinociception produced by μ- and κ-opioid receptor agonists in rats. Eur. J. Pharmacol. 452: 163–173.

Bartok, R. E. and R. M. Craft. 1997. Sex differences in opioid antinociception. J. Pharmacol. Exp. Ther. 282: 769–78.

Basbaum, A. 1984. Endogenous Pain Control Systems: Brainstem Spinal Pathways and Endorphin Circuitry. Annu. Rev. Neurosci. 7: 309–338.

Basbaum, A. I. and H. L. Fields. 1979. The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: further studies on the

anatomy of pain modulation. J. Comp. Neurol. 187: 513–531.

Bauquier, S. H., J. Greenwood and T. Whittem. 2013. Evaluation of the sedative and anaesthetic effects of five different concentrations of alfaxalone in goldfish, Carassius auratus. Aquaculture 396–399: 119–123.

Bautista, D. M., J. Siemens, J. M. Glazer, P. R. Tsuruda, A. I. Basbaum, C. L. Stucky, S. E. Jordt and D. Julius. 2007. The menthol receptor TRPM8 is the principal detector of environmental cold. Nature 448: 204–209.

Bee, L. A. and A. H. Dickenson. 2008. Descending facilitation from the brainstem determines behavioural and neuronal hypersensitivity following nerve injury and efficacy of pregabalin. Pain 140: 209–223.

Bee, L. A. and A. H. Dickenson. 2007. Rostral ventromedial medulla control of spinal sensory processing in normal and pathophysiological states. Neuroscience 147: 786–793.

Behbehani, M. M. 1995. Functional characteristics of the midbrain periaqueductal gray. Prog. Neurobiol. 46: 575–605.

Behbehani, M. M. and H. L. Fields. 1979. Evidence that an excitatory connection between the periaqueductal gray and nucleus raphe magnus mediates stimulation produced analgesia. Brain Res. 170: 85–93.

Beitner-Johnson, D., X. Guitart and E. J. Nestler. 1991. Dopaminergic brain reward regions of Lewis and Fischer rats display different levels of tyrosine hydroxylase and other morphine- and cocaine-regulated phosphoproteins. Brain Res. 561: 147–150.

Beitz, A. J. 1982. The organization of afferent projections to the midbrain peiaquaductal gray of the rat. Neurosci Res 7: 133–159.

Benarroch, E. E. 2008. Descending monoaminergic pain modulation: Bidirectional control and clinical relevance. Neurology 71: 217–221.

Bencze, M., M. Behuliak and J. Zicha. 2013. The impact of four different classes of anesthetics on the mechanisms of blood pressure regulation in normotensive and spontaneously hypertensive rats. Physiol. Res. 62: 471–8.

Bendele, A. M. 2002. Animal models of osteoarthritis in an era of molecular biology. J Musculoskelet Neuronal Interact 2: 501–503.

Bergadano, A., O. K. Andersen, L. Arendt-Nielsen and C. Spadavecchia. 2009. Modulation of nociceptive withdrawal reflexes evoked by single and repeated nociceptive stimuli in conscious dogs by low-dose acepromazine. Vet Anaesth Analg 36: 261–272.

Berge, O. G. 2014. Behavioral pharmacology of pain. Curr. Top. Behav. Neurosci. 20: 33–56.

Berge, O. G. 2011. Predictive validity of behavioural animal models for chronic pain. Br. J. Pharmacol. 164: 1195–1206.

Berkley, K. J. 1997. Sex differences in pain. Behav. Brain Sci. 20: 371–380.

Berkowitz, B. A., A. D. Finck and S. H. Ngai. 1977a. Nitrous oxide analgesia: resemblance to opiate action. Science 203: 967–8.

Berkowitz, B. A., A. D. Finck and S. H. Ngai. 1977b. Nitrous oxide analgesia: reversal by naloxone and development of tolerance. J Pharmacol Exp Ther 203: 539–547.

Berkowitz, B., A. Finck, M. Hynes and S. Ngai. 1979. Tolerance to Nitrous Oxide Analgesia in Rats and Mice. Anesthesiology 51: 30-9–312.

Berry, S. 2015. Injectable Anesthetics. P. in Veterinary Anesthesia and Analgesia (K. Grimm, L. Lamont, W. Tranquilli, S. Greene, and S. Robertson, eds). Wiley Blackwell, Ames, Iowa, 277-296.

Bertaccini, E. J., R. Dickinson, J. R. Trudell and N. P. Franks. 2014. Molecular modeling of a tandem two pore domain potassium channel reveals a putative binding site for general anesthetics. ACS Chem. Neurosci. 5: 1246–1252.

Bessière, B., E. Laboureyras, J. Chateauraynaud, J. P. Laulin and G. Simonnet. 2010. A Single Nitrous Oxide (N2O) Exposure Leads to Persistent Alleviation of Neuropathic Pain in Rats. J. Pain 11: 13–23.

Bessière, B., P. Richebé, E. Laboureyras, J. P. Laulin, A. Contarino and G. Simonnet. 2007. Nitrous oxide (N2O) prevents latent pain sensitization and long-term anxiety-

like behavior in pain and opioid-experienced rats. Neuropharmacology 53: 733–740.

Bevan, S. and J. Szolcsányi. 1990. Sensory neuron-specific actions of capsaicin: mechanisms and applications. Trends Pharmacol. Sci. 11: 331–333.

Bianchi, L. and M. Driscoll. 2002. Protons at the gate: DEG/ENaC ion channels help us feel and remember. Neuron 34: 337–340.

Bing, Z., L. Villanueva and D. Le Bars. 1990. Acupuncture and diffuse noxious inhibitory controls: Naloxone-reversible depression of activities of trigeminal convergent neurons. Neuroscience 37: 809–818.

Blackburn-Munro, G. 2004. Pain-like behaviours in animals - How human are they? Trends Pharmacol. Sci. 25: 299–305.

Blom, A. B., P. L. Van Lent, S. Libregts, A. E. Holthuysen, P. M. Van Der Kraan, N. Van Rooijen and W. B. Van Den Berg. 2007. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: Involvement of matrix metalloproteinase 3. Arthritis Rheum. 57: 147–157.

Bodin, P. and G. Burnstock. 2001. Purinergic signalling: ATP release. Neurochem. Res. 26: 959–969.

Bodnar, R. J. and B. Kest. 2010. Sex differences in opioid analgesia, hyperalgesia, tolerance and withdrawal: Central mechanisms of action and roles of gonadal hormones. Horm. Behav. 58: 72–81.

Bomholt, S., J. Mikkelsen and G. Blackburn-. 2005. Antinociceptive effects of the antidepressants amitriptyline, duloxetine, mirtazapine and citalopram in animal models of acute, persistent and neuropathic pain. Neuropharmacology 48: 252–263.

Borsook, D., A. M. Youssef, L. Simons, I. Elman and C. Eccleston. 2018. When pain gets stuck: the evolution of pain chronification and treatment resistance. Pain 159: 2421–2436.

Bouhassira, D., L. Villanueva, Z. Bing and D. le Bars. 1992. Involvement of the subnucleus reticularis dorsalis in diffuse noxious inhibitory controls in the rat. Brain Res. 595: 353–357.

Bourinet, E., A. Alloui, A. Monteil, C. Barrère, B. Couette, O. Poirot, A. Pages, J. McRory, T. P. Snutch, A. Eschalier and J. Nargeot. 2005. Silencing of the Cav3.2 T-type calcium channel gene in sensory neurons demonstrates its major role in nociception. EMBO J. 24: 315–324.

Bourque, C. W. and L. P. Renaud. 1983. In vitro neurophysiology of identified rat hypothalamic 'neuroendocrine' neurons. Neuroendocrinology 36: 161–164.

Bouts, T. and D. Karunaratna. 2011. Evaluation of medetomidine-alfaxalone and medetomidine-ketamine in semi-free ranging Bennett's wallabies (Macropus rufogriseus). J. Zoo Wildl. Med. 42: 617–622.

Bove, S. E., S. L. Calcaterra, R. M. Brooker, C. M. Huber, R. E. Guzman, P. L. Juneau, D. J. Schrier and K. S. Kilgore. 2003. Weight bearing as a measure of disease progression and efficacy of anti-inflammatory compounds in a model of monosodium iodoacetate-induced osteoarthritis. Osteoarthr. Cartil. 11: 821–830.

Bove, S. E., S. J. L. L. Flatters, J. J. Inglis and P. W. Mantyh. 2009. New advances in musculoskeletal pain. Brain Res Rev 60: 187–201.

Bove, S. E., K. D. Laemont, R. M. Brooker, M. N. Osborn, B. M. Sanchez, R. E. Guzman, K. E. Hook, P. L. Juneau, J. R. Connor and K. S. Kilgore. 2006. Surgically induced osteoarthritis in the rat results in the development of both osteoarthritis-like joint pain and secondary hyperalgesia. Osteoarthr. Cartil. 14: 1041–1048.

Boyer, N., R. Dallel, A. Artola and L. Monconduit. 2014. General trigeminospinal central sensitization and impaired descending pain inhibitory controls contribute to migraine progression. Pain 155: 1196–1205.

Brammer, A., C. D. West and S. L. Allen. 1993. A comparison of propofol with other injectable anaesthetics in a rat model for measuring cardiovascular parameters. Lab. Anim. 27: 250–257.

Braz, J. M. and A. I. Basbaum. 2008. Genetically expressed transneuronal tracer reveals direct and indirect serotonergic descending control circuits. J. Comp. Neurol. 507: 1990–2003.

Brocklehurst, R., M. T. Bayliss, A. Maroudas, H. L. Coysh, M. A. Freeman, P. A. Revell and S. Y. Ali. 1984. The composition of normal and osteoarthritic articular cartilage from human knee joints. With special reference to unicompartmental replacement and osteotomy of the knee. J. Bone Jt. Surg. 66: 95–106.

Bron, R., R. J. Wood, J. A. Brock and J. J. Ivanusic. 2014. Piezo2 expression in corneal afferent neurons. J. Comp. Neurol. 522: 2967–2979.

Buhre, W., N. Disma, J. Hendrickx, S. DeHert, M. W. Hollmann, R. Huhn, J. Jakobsson, P. Nagele, P. Peyton and L. Vutskits. 2019. European Society of Anaesthesiology Task Force on Nitrous Oxide: a narrative review of its role in clinical practice. Br. J. Anaesth. 122: 587–604.

Burgess, S. E., L. R. Gardell, M. H. Ossipov, T. P. Malan, T. W. Vanderah, J. Lai and F. Porreca. 2002. Time-dependent descending facilitation from the rostral ventromedial medulla maintains, but does not initiate, neuropathic pain. J. Neurosci. 22: 5129–36.

Burnstock, G. 2013. Purinergic mechanisms and pain - An update. Eur. J. Pharmacol. 716: 24–40.

Cai, Y. Q., W. Wang, Y. Y. Hou and Z. Z. Pan. 2014. Optogenetic activation of brainstem serotonergic neurons induces persistent pain sensitization. Mol Pain 10: 70.

Calavia, M. G., J. A. Montaño, O. García-Suárez, J. Feito, M. A. Guervós, A. Germanà, M. Del Valle, P. Pérez-Piñera, J. Cobo and J. A. Vega. 2010. Differential localization of acid-sensing ion channels 1 and 2 in human cutaneus pacinian corpuscles. Cell. Mol. Neurobiol. 30: 841–848.

Calejesan, A. A., M. H. C. Ch'ang and M. Zhuo. 1998. Spinal serotonergic receptors mediate facilitation of a nociceptive reflex by subcutaneous formalin injection into the hindpaw in rats. Brain Res., doi: 10.1016/S0006-8993(98)00394-1.

Calejesan, A. A., S. J. Kim and M. Zhuo. 2000. Descending facilitatory modulation of a behavioral nociceptive response by stimulation in the adult rat anterior cingulate cortex. Eur. J. Pain 4: 83–96.

Campbell, C. M. and R. R. Edwards. 2012. Ethnic differences in pain and pain

management. Pain Manag. 2: 219–230.

Campbell, J. N., S. N. Raja and R. A. Meyer. 1984. Halothane sensitizes cutaneous nociceptors in monkeys. J. Neurophysiol. 52: 762–770.

Cao, J., R. Edwards, J. Chairez and S. Devaraj. 2017. Validation of capillary blood analysis and capillary testing mode on the epoc Point of Care system. Pract. Lab. Med. 9: 24–27.

Carstens, E., D. Klumpp and M. Zimmermann. 1980. Differential inhibitory effects of medial and lateral midbrain stimulation on spinal neuronal discharges to noxious skin heating in the cat. J Neurophysiol 43: 432–442.

Caterina, M. J. and D. Julius. 2001. The Vanilloid Receptor: A Molecular Gateway to the Pain Pathway. Annu. Rev. Neurosci. 24: 487–517.

Caterina, M. J., M. A. Schumacher, M. Tominaga, T. A. Rosen, J. D. Levine and D. Julius. 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389: 816–824.

Cazalets, J. R., P. Grillner, I. Menard, J. Cremieux and F. Clarac. 1990. Two types of motor rhythm induced by NMDA and amines in an in vitro spinal cord preparation of neonatal rat. Neurosci. Lett. 111: 116–121.

Cenci, M. A. 2007. Dopamine dysregulation of movement control in l-DOPA-induced dyskinesia. Trends Neurosci. 30: 236–243.

Cesare, P. and P. Mcnaughton. 1996. A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. Proc. Natl. Acad. Sci. U. S. A. 93: 15435–15439.

Chan, M. T. V., P. J. Peyton, P. S. Myles, K. Leslie, N. Buckley, J. Kasza, M. J. Paech, W. S. Beattie, D. I. Sessler, A. Forbes, S. Wallace, Y. Chen, Y. Tian and W. K. K. Wu. 2016. Chronic postsurgical pain in the Evaluation of Nitrous Oxide in the Gas Mixture for Anaesthesia (ENIGMA)-II trial. Br. J. Anaesth. 117: 801–811.

Chaplan, S. R., F. W. Bach, J. W. Pogrel, J. M. Chung and T. L. Yaksh. 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53: 55–63.

Chédotal, A. 2019. Roles of axon guidance molecules in neuronal wiring in the developing spinal cord. Nat. Rev. Neurosci. 20: 380–396. Springer US.

Cheng, J.-W., A. I. Weber and S. J. Bensmaia. 2013. Comparing the effects of isoflurane and pentobarbital on the responses of cutaneous mechanoreceptive afferents. BMC Anesthesiol. 13: 10.

Child, K. J., B. Davis, M. G. Dodds and D. J. Twissell. 1972. Anaesthetic, cardiovascular and respiratory effects of a new steroidal agent CT 1341: a comparison with other intravenous anaesthetic drugs in the unrestrained cat. Br. J. Pharmacol. 46: 189–200.

Chillingworth, N. L. and L. F. Donaldson. 2003. Characterisation of a Freund's complete adjuvant-induced model of chronic arthritis in mice. J. Neurosci. Methods 128: 45–52.

Choi, S., H. S. Na, J. Kim, J. Lee, S. Lee, D. Kim, J. Park, C. C. Chen, K. P. Campbell and H. S. Shin. 2007. Attenuated pain responses in mice lacking CaV3.2 T-type channels. Genes, Brain Behav. 6: 425–431.

Chung, E., Y. Ohgami and R. M. Quock. 2016. Increasing the availability of L-arginine and nitric oxide increases sensitivity of nitrous oxide (N2O)-insensitive inbred mice to N2O-induced antinociception. Brain Res. Bull. 125: 218–221.

Chung, J. M., D. R. Kenshalo, K. D. Gerhart and W. D. Willis. 1979. Excitation of primate spinothalamic neurons by cutaneous C-fiber volleys. J. Neurophysiol. 42: 1354–1369.

Claiborne, J., S. Nag and S. S. Mokha. 2006. Activation of Opioid Receptor Like-1 Receptor in the Spinal Cord Produces Sex-Specific Antinociception in the Rat: Estrogen Attenuates Antinociception in the Female, whereas Testosterone Is Required for the Expression of Antinociception in the Male. J. Neurosci. 26: 13048–13053.

Clarke, R. W., T. W. Ford and J. S. Taylor. 1988. Adrenergic and opioidergic modulation of a spinal reflex in the decerebrated rabbit. J. Physiol. 404: 407–417.

Clarke, R. W., T. W. Ford and J. S. Taylor. 1989. Reflex actions of selective stimulation of sural nerve c fibres in the rabbit. Q. J. Exp. Physiol. 74: 681–690.

Clarke, R. W. and J. Harris. 2004. The organization of motor responses to noxious stimuli. Brain Res Rev 46: 163–172.

Clarke, R. W., J. Harris, T. W. Ford and J. S. Taylor. 1992. Prolonged potentiation of transmission through a withdrawal reflex pathway after noxious stimulation of the heel in the rabbit. Pain 49: 65–70.

Clayton, J. A. and F. S. Collins. 2014. Policy: NIH to balance sex in cell and animal studies. Nature 509: 282–3.

Cleary, D. R., M. J. Neubert and M. M. Heinricher. 2008. Are opioid-sensitive neurons in the rostral ventromedial medulla inhibitory interneurons? Neuroscience 151: 564–571.

Cole, D. J., M. W. Kalichman and H. M. Shapiro. 1989. The Nonlinear Contribution of Nitrous Oxide at Sub-MAC Concentrations to Enflurane MAC in Rats. Anesth. Analg. 68: 556–562.

Cole, D. J., M. W. Kalichman, H. M. Shapiro and J. C. Drummond. 1990. The nonlinear potency of sub-MAC concentrations of nitrous oxide in decreasing the anesthetic requirement of enflurane, halothane, and isoflurane in rats. Anesthesiology 73: 93–9.

Collins, D. 1949. The pathology of articular and spinal diseases. Eward Arnold and Co, London, UK.

Combe, R., S. Bramwell and M. J. Field. 2004. The monosodium iodoacetate model of osteoarthritis: a model of chronic nociceptive pain in rats? Neurosci Lett 370: 236–240.

Conaghan, P. G. 2013. Parallel evolution of OA phenotypes and therapies. Nat. Rev. Rheumatol. 9: 68–70.

Cook, A. J., C. J. Woolf, P. D. Wall and S. B. Mcmahon. 1987. Dynamic receptive field plasticity in rat spinal cord dorsal horn following C-primary afferent input. Nature 325: 151–153.

Cook, C. D. 2004. Nociceptive Sensitivity and Opioid Antinociception and Antihyperalgesia in Freund's Adjuvant-Induced Arthritic Male and Female Rats. J. Pharmacol. Exp. Ther. 313: 449–459.

Cook, C. D., A. C. Barrett, E. L. Roach, J. R. Bowman and M. J. Picker. 2000. Sex-related differences in the antinociceptive effects of opioids: Importance of rat genotype, nociceptive stimulus intensity, and efficacy at the mu opioid receptor. Psychopharmacology (Berl). 150: 430–442.

Cora, M. C., L. Kooistra and G. Travlos. 2015. Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. Toxicol. Pathol. 43: 776–793.

Coste, B., J. Mathur, M. Schmidt, T. J. Earley, S. Ranade, M. J. Petrus, A. E. Dubin and A. Patapoutian. 2010. Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. Science 330: 55–60.

Coulter, C. A., P. A. Flecknell, M. C. Leach and C. A. Richardson. 2011. Reported analgesic administration to rabbits undergoing experimental surgical procedures. BMC Vet. Res. 7: 2–6.

Courtney, C. A., M. D. Lewek, P. O. Witte, S. J. Chmell and T. G. Hornby. 2009. Heightened flexor withdrawal responses in subjects with knee osteoarthritis. J Pain 10: 1242–1249.

Craft, R. M. 2003. Sex differences in opioid analgesia: 'from mouse to man'. Clin J Pain 19: 175–86.

Craig, A. D. (Bud). 2003. Pain Mechanisms: Labeled Lines Versus Convergence in Central Processing. Annu. Rev. Neurosci. 26: 1–30.

Craig, A. D. and K. D. Kniffki. 1985. Spinothalamic lumbosacral lamina I cells responsive to skin and muscle stimulation in the cat. J. Physiol. 365: 197–221.

Criado-Perez, C. 2019. The drugs don't work. Pp. 195–216 in Invisible Women: Data Bias in a World Designed for Men. Chatto & Windus, London, UK.

Cuellar, J. M., R. C. Dutton, J. F. Antognini and E. Carstens. 2005. Differential effects of

halothane and isoflurane on lumbar dorsal horn neuronal windup and excitability. Br. J. Anaesth. 94: 617–625.

Da Silva, J. T., C. Tricou, Y. Zhang, D. A. Seminowicz and J. Y. Ro. 2020. Brain networks and endogenous pain inhibition are modulated by age and sex in healthy rats. Pain 161: 1371–1380.

Da Silva, J. T., Y. Zhang, J. Asgar, J. Y. Ro and D. A. Seminowicz. 2018. Diffuse noxious inhibitory controls and brain networks are modulated in a testosterone-dependent manner in Sprague Dawley rats. Behav. Brain Res. 349: 91–97.

Dahan, A., E. Sarton, L. Teppema, C. Olievier, D. Nieuwenhuijs, H. W. D. Matthes and B. L. Kieffer. 2001. Anesthetic potency and influence of morphine and sevoflurane on respiration in μ-opioid receptor knockout mice. Anesthesiology 94: 824–832.

Dahlstroem, A. and K. Fuxe. 1964. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. Acta Physiol. Scand. Suppl. 232:1–55. England.

Davies, S. N. and D. Lodge. 1987. Evidence for involvement of N-methylaspartate receptors in ‘wind-up’ of class 2 neurones in the dorsal horn of the rat. Brain Res. 424: 402–406.

De Felice, M., R. Sanoja, R. Wang, L. Vera-Portocarrero, J. Oyarzo, T. King, M. H. Ossipov, T. W. Vanderah, J. Lai, G. O. Dussor, H. L. Fields, T. J. Price and F. Porreca. 2011. Engagement of descending inhibition from the rostral ventromedial medulla protects against chronic neuropathic pain. Pain 152: 2701–2709.

Defrin, R. 2014. Chronic post-traumatic headache: clinical findings and possible mechanisms. J. Man. Manip. Ther. 22: 36–44.

del Mar Granados, M., J. M. Dominguez, A. Fernandez-Sarmiento, F. J. Funes, J. Morgaz, R. Navarrete, J. Ma Carrillo, M. Rubio, P. Munoz-Rascon, I. A. Gomez de Segura and R. J. Gomez-Villamandos. 2012. Anaesthetic and cardiorespiratory effects of a constant-rate infusion of alfaxalone in desflurane-anaesthetised sheep. Vet Rec 171: 125–125.

Dickenson, A. H. and D. Le Bars. 1983. Diffuse noxious inhibitory controls (DNIC) involve trigeminothalamic and spinothalamic neurones in the rat. Exp. Brain Res. 49: 174–180.

Dickenson, A. H. and D. Le Bars. 1987. Supraspinal morphine and descending inhibitions acting on the dorsal horn of the rat. J. Physiol. 384: 81–107.

Dickenson, A. H., D. Le Bars and J. M. Besson. 1980. Diffuse noxious inhibitory controls (DNIC). Effects on trigeminal nucleus caudalis neurones in the rat. Brain Res. 200: 293–305.

Dickenson, A. H. and A. F. Sullivan. 1987a. Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following c fibre stimulation. Neuropharmacology 26: 1235–1238.

Dickenson, A. H. and A. F. Sullivan. 1987b. Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurones. Neurosci. Lett. 83: 207–211.

Dimitroulas, T., R. V. Duarte, A. Behura, G. D. Kitas and J. H. Raphael. 2015. Neuropathic pain in osteoarthritis: A review of pathophysiological mechanisms and implications for treatment. Semin. Arthritis Rheum. 44: 145–154.

Dina, O. A., K. O. Aley, W. Isenberg, R. O. Messing and J. D. Levine. 2001. Sex hormones regulate the contribution of PKCepsilon and PKA signalling in inflammatory pain in the rat. Eur. J. Neurosci. 13: 2227–2233.

Dingledine, R., J. Dodd and J. S. Kelly. 1980. The in vitro brain slice as a useful neurophysiological preparation for intracellular recording. J. Neurosci. Methods 2: 323–362.

Dobson, K. L. 2013. Descending control in sensitisation of reflexes in the rat. University of Nottingham PhD Thesis.

Dobson, K. L. and J. Harris. 2012. A detailed surgical method for mechanical decerebration of the rat. Exp Physiol 97: 693–698.

Dogrul, A., M. H. Ossipov and F. Porreca. 2009. Differential mediation of descending pain

facilitation and inhibition by spinal 5HT-3 and 5HT-7 receptors. Brain Res. 1280: 52–59.

Dostrovsky, J. and A. Craig. 2013. Ascending Projection Systems. Pp. 182–197 in Textbook of Pain (S. McMahon, M. Koltzenburg, I. Tracey, and D. Turk, eds). Elsevier Saunders, Philadelphia, PA.

Dougados, M., X. Ayral, V. Listrat, A. Gueguen, J. Bahuaud, P. Beaufils, J. A. Beguin, J. P. Bonvarlet, T. Boyer, H. Coudane, C. Delaunay, H. Dorfmann, J. P. Dubos, A. Frank, J. F. Kempf, B. Locker, J. L. Prudhon and J. Thiery. 1994. The SFA system for assessing articular cartilage lesions at arthroscopy of the knee. Arthroscopy 10: 69–77.

Doyle, C. A. and S. P. Hunt. 1999. Substance P receptor (neurokinin-1)-expressing neurons in lamina I of the spinal cord encode for the intensity of noxious stimulation: A c-Fos study in rat. Neuroscience 89: 17–28.

Doyle, H. H., L. N. Eidson, D. M. Sinkiewicz and A. Z. Murphy. 2017. Sex Differences in Microglia Activity within the Periaqueductal Gray of the Rat: A Potential Mechanism Driving the Dimorphic Effects of Morphine. J. Neurosci. 37: 3202–3214.

Drew, L. J., D. K. Rohrer, M. P. Price, K. E. Blaver, D. A. Cockayne, P. Cesare and J. N. Wood. 2004. Acid-sensing ion channels ASIC2 and ASIC3 do not contribute to mechanically activated currents in mammalian sensory neurones. J. Physiol. 556: 691–710.

Ducassé, J. L., G. Siksik, M. Durand-Béchu, S. Couarraze, B. Vallé, N. Lecoules, P. Marco, T. Lacombe and V. Bounes. 2013. Nitrous oxide for early analgesia in the emergency setting: A randomized, double-blind multicenter prehospital trial. Acad. Emerg. Med. 20: 178–184.

Duke, T., N. A. Caulkett and J. M. Tataryn. 2006. The effect of nitrous oxide on halothane, isoflurane and sevoflurane requirements in ventilated dogs undergoing ovariohysterectomy. Vet. Anaesth. Analg. 33: 343–350.

Dum, R. P., D. J. Levinthal and P. L. Strick. 2009. The Spinothalamic System Targets

Motor and Sensory Areas in the Cerebral Cortex of Monkeys. J. Neurosci. 29: 14223–14235.

Eccles, R. M. and A. Lunberg. 1959. Synaptic actions in motoneurones by afferents in which may evoke the flexion reflex. Arch. Ital. Biol. 97: 199–221.

Edwards, R. R., D. M. Doleys, R. B. Fillingim and D. Lowery. 2001. Ethnic differences in pain tolerance: Clinical implications in a chronic pain population. Psychosom. Med. 63: 316–323.

Eger, E. 2001. Age, Minimum Alveolar Anesthetic Concentration, and Minimum Alveolar Anesthetic Concentration-Awake. Anesth Analg 93: 947–53.

Eger, E. I., G. H. Lampe, L. Z. Wauk, P. Whitendale, M. K. Cahalan and J. H. Donegan. 1990. Clinical pharmacology of nitrous oxide: an argument for its continued use. Anesth. Analg. 71: 575–585.

Eger, E. I., M. Tang, M. Liao, M. J. Laster, K. Solt, P. Flood, A. Jenkins, D. Raines, J. F. Hendrickx, S. L. Shafer, T. Yasumasa and J. M. Sonner. 2008. Inhaled anesthetics do not combine to produce synergistic effects regarding minimum alveolar anesthetic concentration in rats. Anesth. Analg. 107: 479–485.

Eippert, F., U. Bingel, E. D. Schoell, J. Yacubian, R. Klinger, J. Lorenz and C. Büchel. 2009. Activation of the Opioidergic Descending Pain Control System Underlies Placebo Analgesia. Neuron 63: 533–543.

Eisenach, J. C., S. DuPen, M. Dubois, R. Miguel and D. Allin. 1995. Epidural clonidine analgesia for intractable cancer pain. Pain 61: 391–399.

Emmanouil, D. E., A. S. Dickens, R. W. Heckert, Y. Ohgami, E. Chung, S. Han and R. M. Quock. 2008. Nitrous oxide-antinociception is mediated by opioid receptors and nitric oxide in the periaqueductal gray region of the midbrain. Eur. Neuropsychopharmacol. 18: 194–199.

Estes, K. S., M. E. Brewster, A. I. Webb and N. Bodor. 1990. A non- surfactant formulation for alfaxalone based on an amorphous cyclodextrin: Activity studies in rats and dogs. Int. J. Pharm. 65: 101–107.

Evans, M. S., C. Lysakowski and M. R. Tramèr. 2008. Nefopam for the prevention of postoperative pain: Quantitative systematic review. Br. J. Anaesth. 101: 610–617.

Evered, L., B. Silbert, D. S. Knopman, D. A. Scott, S. T. DeKosky, L. S. Rasmussen, E. S. Oh, G. Crosby, M. Berger, R. G. Eckenhoff, L. Evered, R. G. Eckenhoff, D. Ames, A. Bekker, M. Berger, D. Blacker, J. Browndyke, G. Crosby, S. G. Deiner, D. van Dijk, S. T. DeKosky, M. F. Eckenhoff, L. Eriksson, D. Galasko, K. Hogan, S. Inouye, D. S. Knopman, C. Lyketsos, E. Marcantonio, P. Maruff, M. Maze, E. S. Oh, B. A. Orser, T. Ottens, C. Price, L. S. Rasmussen, P. Sachdev, K. Schenning, D. A. Scott, F. E. Seiber, B. Silbert, J. Silverstein, J. Steinmetz, N. Terrando, P. Trzapacz, R. Whittington and Z. Xie. 2018. Recommendations for the nomenclature of cognitive change associated with anaesthesia and surgery—2018. Br. J. Anaesth. 121: 1005–1012.

Fang, F., T. Z. Guo, M. F. Davies and M. Maze. 1997. Opiate receptors in the periaqueductal gray mediate analgesic effect of nitrous oxide in rats. Eur. J. Pharmacol. 336: 137–141.

Felten, D. L. and J. R. Sladek. 1983. Monoamine distribution in primate brain V. Monoaminergic nuclei: Anatomy, pathways and local organization. Brain Res. Bull. 10: 171–284.

Fender, C., M. Fujinaga and M. Maze. 2000. Strain differences in the antinociceptive effect of nitrous oxide on the tail flick test in rats. Anesth. Analg. 90: 195–199.

Ferland, C. E., S. Laverty, F. Beaudry and P. Vachon. 2011. Gait analysis and pain response of two rodent models of osteoarthritis. Pharmacol Biochem Behav 97: 603–610.

Fernihough, J., C. Gentry, S. Bevan and J. Winter. 2005. Regulation of calcitonin gene-related peptide and TRPV1 in a rat model of osteoarthritis. Neurosci Lett 388: 75–80.

Fernihough, J., C. Gentry, M. Malcangio, A. Fox, J. Rediske, T. Pellas, B. Kidd, S. Bevan and J. Winter. 2004. Pain related behaviour in two models of osteoarthritis in the rat knee. Pain 112: 83–93.

Ferré, P. J., K. Pasloske, T. Whittem, M. G. Ranasinghe, Q. Li and H. P. Lefebvre. 2006. Plasma pharmacokinetics of alfaxalone in dogs after an intravenous bolus of Alfaxan-CD RTU. Vet. Anaesth. Analg. 33: 229–36.

Ferreira-Gomes, J., S. Adães and J. M. Castro-Lopes. 2008. Assessment of Movement-Evoked Pain in Osteoarthritis by the Knee-Bend and CatWalk Tests: A Clinically Relevant Study. J. Pain 9: 945–954.

Fields, H. L., H. Vanegas, I. D. Hentall and G. Zorman. 1983. Evidence that disinhibition of brain stem neurones contributes to morphine analgesia. Nature 306: 684–686.

Fink, G., D. Sarkar, R. Dow, H. Dick, N. Borthwick, S. Malnick and M. Twine. 1982. Sex difference in response to alphaxalone anaesthesia may be oestrogen dependent. Nature 298: 270–272.

Fischer, M. J. M., A. Leffler, F. Niedermirtl, K. Kistner, M. Eberhardt, P. W. Reeh and C. Nau. 2010. The general anesthetic propofol excites nociceptors by activating TRPV1 and TRPA1 rather than GABAA receptors. J. Biol. Chem. 285: 34781–34792.

Flecknell, P. 2018. Rodent analgesia: Assessment and therapeutics. Vet. J. 232: 70–77.

Flecknell, P. A. 2015. Laboratory animal anaesthesia. 4th ed. Academic Press Elsevier, New York, NY:

Flecknell, P. A. and M. Mitchell. 1984. Midazolam and fentanyl-fluanisone: assessment of anaesthetic effects in laboratory rodents and rabbits. Lab. Anim. 18: 143–146.

Flecknell, P., J. L. S. Lofgren, M. C. Dyson, R. R. Marini, M. Michael Swindle and R. P. Wilson. 2015. Chapter 24 - Preanesthesia, Anesthesia, Analgesia, and Euthanasia. Pp. 1135–1200 in Laboratory Animal Medicine: Third Edition (J. Fox, G. Otto, M. Whary, L. Anderson, and K. Pritchett-Corning, eds). Academic Press, New York, NY:

Fouad, K. and D. J. Bennett. 1998. Decerebration by global ischemic stroke in rats. J. Neurosci. Methods 84: 131–137.

François, A., N. Schüetter, S. Laffray, J. Sanguesa, A. Pizzoccaro, S. Dubel, A. Mantilleri, J. Nargeot, J. Noël, J. N. Wood, A. Moqrich, O. Pongs and E. Bourinet. 2015. The Low-Threshold Calcium Channel Cav3.2 Determines Low-Threshold

Mechanoreceptor Function. Cell Rep. 10: 370–382.

Franks, N. P. and E. Honoré. 2004. The TREK K2P channels and their role in general anaesthesia and neuroprotection. Trends Pharmacol. Sci. 25: 601–8.

French, H. P., K. M. Smart and F. Doyle. 2017. Prevalence of neuropathic pain in knee or hip osteoarthritis: A systematic review and meta-analysis. Semin. Arthritis Rheum. 47: 1–8.

Fuchs, P. N., Y. B. Peng, J. A. Boyette-Davis and M. L. Uhelski. 2014. The anterior cingulate cortex and pain processing. Front. Integr. Neurosci. 8: 1–10.

Fujii, K., K. Motohashi and M. Umino. 2006. Heterotopic ischemic pain attenuates somatosensory evoked potentials induced by electrical tooth stimulation: Diffuse noxious inhibitory controls in the trigeminal nerve territory. Eur. J. Pain 10: 495–504.

Fujinaga, M., R. Doone, M. F. Davies and M. Maze. 2000. Nitrous oxide lacks the antinociceptive effect on the tail flick test in newborn rats. Anesth. Analg. 91: 6–10.

Fujinaga, M. and M. Maze. 2002. Neurobiology of nitrous oxide-induced antinociceptive effects. Mol. Neurobiol. 25: 167–189.

Gagliese, L. and R. Melzack. 1997. Chronic pain in elderly people. Pain 70: 3–14.

Galer, B. S., D. Lee, T. Ma, B. Nagle and T. G. Schlagheck. 2005. MorphiDex® (morphine sulfate/dextromethorphan hydrobromide combination) in the treatment of chronic pain: Three multicenter, randomized, double-blind, controlled clinical trials fail to demonstrate enhanced opioid analgesia or reduction in tolerance. Pain 115: 284–295.

Gao, X., Y. Q. Zhang and G. cheng Wu. 2001. Effects of dopaminergic agents on carrageenan hyperalgesia after intrathecal administration to rats. Eur. J. Pharmacol. 418: 73–77.

Garcá-Ramrez, D. L., J. R. Calvo, S. Hochman and J. N. Quevedo. 2014. Serotonin, dopamine and noradrenaline adjust actions of myelinated afferents via modulation of presynaptic inhibition in the mouse spinal cord. PLoS One 9(2): e89999.

Garrison, S. R., A. Dietrich and C. L. Stucky. 2012. TRPC1 contributes to light-touch

sensation and mechanical responses in low-threshold cutaneous sensory neurons. J. Neurophysiol. 107: 913–922.

Gauriau, C. and J. F. Bernard. 2010. Physiological Society Symposium Nociceptors as Homeostatic Afferents : Central Processing Pain pathways and parabrachial circuits in the rat. Exp. Physiol. 87: 251–258.

Ge, H.-Y., P. Madeleine and L. Arendt-Nielsen. 2004. Sex differences in temporal characteristics of descending inhibitory control: An evaluation using repeated bilateral experimental induction of muscle pain. Pain 110: 72–78.

Gebhart, G. F. 2013. Nocifensive Behavior. Pp. 2274–2275 in Encyclopedia of Pain (G. F. Gebhart and R. F. Schmidt, eds). Springer Berlin Heidelberg.

Gebhart, G. F., J. Sandkühler, J. G. Thalhammer and M. Zimmermann. 1983. Inhibition of spinal nociceptive information by stimulation in midbrain of the cat is blocked by lidocaine microinjected in nucleus raphe magnus and medullary reticular formation. J. Neurophysiol. 50: 1446–59.

Georgiev, S. K., T. Kohno, M. Ikoma, T. Yamakura and H. Baba. 2008. Nitrous oxide inhibits glutamatergic transmission in spinal dorsal horn neurons. Pain 134: 24–31.

Gerwin, N., A. M. Bendele, S. Glasson and C. S. Carlson. 2010. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the rat. Osteoarthr. Cartil. 18: S24–S34.

Gilbert, A. K. and K. B. J. Franklin. 2001. GABAergic modulation of descending inhibitory systems from the rostral ventromedial medulla (RVM). Dose-response analysis of nociception and neurological deficits. Pain 90: 25–36.

Gjerstad, J., A. Tjølsen, F. Svendsen and K. Hole. 2000. Inhibition of spinal nociceptive responses after intramuscular injection of capsaicin involves activation of noradrenergic and opioid systems. Brain Res. 859: 132–136.

Glyn-Jones, S., A. J. R. Palmer, R. Agricola, A. J. Price, T. L. Vincent, H. Weinans and A. J. Carr. 2015. Osteoarthritis. Lancet 386: 376–387.

Gokin, A. P., M. U. Fareed, H. L. Pan, G. Hans, G. R. Strichartz and G. Davar. 2001.

Local injection of endothelin-1 produces pain-like behavior and excitation of nociceptors in rats. J. Neurosci. 21: 5358–5366.

Gold, M. S. and G. F. Gebhart. 2010. Nociceptor sensitization in pain pathogenesis. Nat Med 16: 1248–1257.

Gold, S. M. and R. R. Voskuhl. 2009. Estrogen and testosterone therapies in multiple sclerosis. Prog. Brain Res. 175: 239–251.

Goodchild, C. S., J. M. Serrao, A. Kolosov and B. J. Boyd. 2015. Alphaxalone Reformulated: A Water-Soluble Intravenous Anesthetic Preparation in Sulfobutyl-Ether-β-Cyclodextrin. Anesth. Analg. 120: 1025–1031.

Goodwin, W. A., H. L. Keates, K. Pasloske, M. Pearson, B. Sauer and M. G. Ranasinghe. 2011. The pharmacokinetics and pharmacodynamics of the injectable anaesthetic alfaxalone in the horse. Vet. Anaesth. Analg. 38: 431–8.

Goto, T., J. J. Marota and G. Crosby. 1996. Volatile anaesthetics antagonize nitrous oxide and morphine-induced analgesia in the rat. Br. J. Anaesth. 76: 702–6.

Gottlieb, P. A. and F. Sachs. 2012. Piezo1: Properties of a cation selective mechanical channel. Channels 6: 214–219.

Goudet, C., V. Magnaghi, M. Landry, F. Nagy, R. W. Gereau IV and J. P. Pin. 2009. Metabotropic receptors for glutamate and GABA in pain. Brain Res. Rev. 60: 43–56.

Graven-Nielsen, T., V. Babenko, P. Svensson and L. Arendt-Nielsen. 1998. Experimentally induced muscle pain induces hypoalgesia in heterotopic deep tissues, but not in homotopic deep tissues. Brain Res. 787: 203–210.

Green, G. M., L. Lyons and A. H. Dickenson. 1998. Alpha-2-Adrenoceptor antagonists enhance responses of dorsal horn neurones to formalin induced inflammation. Eur. J. Pharmacol. 347: 201–204.

Greenspan, J. D., R. M. Craft, L. LeResche, L. Arendt-Nielsen, K. J. Berkley, R. B. Fillingim, M. S. Gold, A. Holdcroft, S. Lautenbacher, E. A. Mayer, J. S. Mogil, A. Z. Murphy and R. J. Traub. 2007. Studying sex and gender differences in pain and analgesia: A consensus report. Pain 132: S26–S45.

Gregory, M. H., N. Capito, K. Kuroki, A. M. Stoker, J. L. Cook and S. L. Sherman. 2012. A Review of Translational Animal Models for Knee Osteoarthritis. Arthritis 2012: 1–14.

Grosenbaugh, D. A. and W. Muir. 1998. Cardiorespiratory effects of sevoflurane, isoflurane, and halothane anesthesia in horses. Am. J. Vet. Res. 59: 101–106.

Grudt, T. J. and E. R. Perl. 2002. Correlations between neuronal morphology and electro-physiological features in the rodent superficial dorsal horn. J. Physiol. 540: 189–207.

Gu, L., M. L. Uhelski, S. Anand, M. Romero-Ortega, Y. T. Kim, P. N. Fuchs and S. K. Mohanty. 2015. Pain inhibition by optogenetic activation of specific anterior cingulate cortical neurons. PLoS One 10(2): e0117746.

Guindon, J. and A. G. Hohmann. 2009. The endocannabinoid system and pain. CNS Neurol Disord Drug Targets 8: 403–421.

Guindon, J., J. LoVerme, A. De Lean, D. Piomelli and P. Beaulieu. 2006. Synergistic antinociceptive effects of anandamide, an endocannabinoid, and nonsteroidal anti-inflammatory drugs in peripheral tissue: a role for endogenous fatty-acid ethanolamides? Eur J Pharmacol 550: 68–77.

Guingamp, C., P. Gegout-Pottie, L. Philippe, B. Terlain, P. Netter and P. Gillet. 1997. Mono-iodoacetate-induced experimental osteoarthritis: a dose-response study of loss of mobility, morphology, and biochemistry. Arthritis Rheum 40: 1670–1679.

Guitart, X., J. H. Kogan, M. Berhow, R. Z. Terwilliger, G. K. Aghajanian and E. J. Nestler. 1993. Lewis and Fischer rat strains display differences in biochemical, electrophysiological and behavioral parameters: studies in the nucleus accumbens and locus coeruleus of drug naive and morphine-treated animals. Brain Res. 611: 7–17.

Güler, A. D., H. Lee, T. Iida, I. Shimizu, M. Tominaga and M. Caterina. 2002. Heat-evoked activation of the ion channel, TRPV4. J. Neurosci. 22: 6408–6414.

Guo, T. Z., M. F. Davies, W. S. Kingery, A. J. Patterson, L. E. Limbird and M. Maze. 1999. Nitrous oxide produces antinociceptive response via alpha2B and/or alpha2C adrenoceptor subtypes in mice. Anesthesiology 90: 470–476.

Guo, T. Z., L. R. Poree, W. Golden, J. Stein, M. Fujinaga and M. Maze. 1996. Antinociceptive response to nitrous oxide is mediated by supraspinal opiate and spinal alpha 2 adrenergic receptors in the rat. Anesthesiology 85: 846–852.

Gyires, K., Z. S. Zádori, T. Török and P. Mátyus. 2009. Alpha 2-Adrenoceptor subtypes-mediated physiological, pharmacological actions. Neurochem. Int. 55: 447–453.

Hacker, S. O., C. E. White and I. H. Black. 2005. A comparison of target-controlled infusion versus volatile inhalant anesthesia for heart rate, respiratory rate, and recovery time in a rat model. Contemp Top Lab Anim Sci 44: 7–12.

Haga, H. A. and B. Ranheim. 2005. Castration of piglets: The analgesic effects of intratesticular and intrafunicular lidocaine injection. Vet. Anaesth. Analg. 32: 1–9.

Haga, H. A., A. Tevik and H. Moerch. 2001. Electroencephalographic and cardiovascular indicators of nociception during isoflurane anaesthesia in pigs. Vet. Anaesth. Analg. 28: 126–131.

Hagbarth, K. E. 1952. Excitatory and inhibitory skin areas for flexor and extensor motoneurons. Acta Physiol. Scand. Suppl. 26: 1–58.

Hains, B. C. and S. G. Waxman. 2006. Activated Microglia Contribute to the Maintenance of Chronic Pain after Spinal Cord Injury. J. Neurosci. 26: 4308–4317.

Handy, J. M. and N. Soni. 2008. Physiological effects of hyperchloraemia and acidosis. Br. J. Anaesth. 101: 141–150.

Harbuz, M. S., Z. Perveen-gill, S. L. Lightman and D. S. Jessop. 1995. A protective role for testosterone in adjuvant-induced arthritis. Rheumatology 34: 1117–1122.

Harris, J. 2016. Involvement of spinal alpha2 -adrenoceptors in prolonged modulation of hind limb withdrawal reflexes following acute noxious stimulation in the anaesthetized rabbit. Eur. J. Neurosci. 43: 834–845.

Harris, J. and R. W. Clarke. 2003. Organisation of sensitisation of hind limb withdrawal reflexes from acute noxious stimuli in the rabbit. J Physiol 546: 251–265.

Harris, J. and R. W. Clarke. 2007. Site-specific, inflammation-induced adaptations in withdrawal reflex pathways in the anesthetized rabbit. Brain Res 1131: 106–111.

Harrison, N. and M. Simmonds. 1984. Modulation of the GABA receptor complex by a steroid anesthetic. Brain Res. 323: 287–292.

Harvey, R., U. Depner, H. Wässle, S. Ahmadi, C. Heindl, H. Reinold, T. Smart, K. Harvey, B. Schütz, O. Abo-Salem, A. Zimmer, P. Poisbeau, H. Welzl, D. Wolfer, H. Betz, H. Zeilhofer and U. Müller. 2004. GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. Science 304: 884–887.

Harvey, R. J. and J.-M. Rigo. 2010. Glycinergic transmission: physiological, developmental and pathological implications. Front. Mol. Neurosci. 3: 1–2.

Hashimoto, T., M. Maze, Y. Ohashi and M. Fujinaga. 2001. Nitrous oxide activates GABAergic neurons in the spinal cord in Fischer rats. Anesthesiology 95: 463–469.

Hasuwa, H., K. Kaseda, T. Einarsdottir and M. Okabe. 2002. Small interfering RNA and gene silencing in transgenic mice and rats. FEBS Lett. 532: 227–230.

Hathway, G. J., S. Koch, L. Low and M. Fitzgerald. 2009. The changing balance of brainstem-spinal cord modulation of pain processing over the first weeks of rat postnatal life. J Physiol 587: 2927–2935.

Hathway, G. J., D. Vega-Avelaira and M. Fitzgerald. 2012. A critical period in the supraspinal control of pain: Opioid-dependent changes in brainstem rostroventral medulla function in preadolescence. Pain 153: 775–783.

Hayami, T., M. Pickarski, Y. Zhuo, G. A. Wesolowski, G. A. Rodan and T. Duong le. 2006. Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. Bone 38: 234–243.

Hayes, H. B., Y.-H. Chang and S. Hochman. 2009. An In Vitro Spinal Cord–Hindlimb Preparation for Studying Behaviorally Relevant Rat Locomotor Function. J. Neurophysiol. 101: 1114–1122.

Heinricher, M. ., I. Tavares, J. L. Leith and B. M. Lumb. 2010. Descending control of nociception. Brain 60: 214–225.

Heinricher, M. M., I. Tavares, J. L. Leith and B. M. Lumb. 2009. Descending control of

nociception: Specificity, recruitment and plasticity. Brain Res. Rev. 60: 214–225.

Hendrickx, J. F. A., E. I. Eger, J. M. Sonner and S. L. Shafer. 2008. Is synergy the rule? a review of anesthetic interactions producing hypnosis and immobility. Anesth. Analg. 107: 494–506.

Herrero, J. F., J. M. A. Laird and J. A. Lopez-Garcia. 2000. Wind-up of spinal cord neurones and pain sensation: Much ado about something? Prog. Neurobiol. 61: 169–203.

Hielm-Björkman, A., V. Reunanen, P. Meri and R. M. Tulamo. 2007. Panax Ginseng in combination with brewers' yeast (Gerivet®) as a stimulant for geriatric dogs: A controlled-randomized blinded study. J. Vet. Pharmacol. Ther. 30: 295–304.

Hill, S. A. 2004. Pharmacokinetics of drug infusions. Contin. Educ. Anaesthesia, Crit. Care Pain 4: 76–80.

Himukashi, S., Y. Miyazaki, H. Takeshima, S. Koyanagi, K. Mukaida, T. Shichino, H. Uga and K. Fukuda. 2005. Nociceptin system does not affect MAC of volatile anaesthetics. Acta Anaesthesiol. Scand. 49: 771–773.

Himukashi, S., H. Takeshima, S. Koyanagi, T. Shichino and K. Fukuda. 2006. The involvement of the nociceptin receptor in the antinociceptive action of nitrous oxide. Anesth. Analg. 103: 738–741.

Hirsch, S., A. Dickenson and L. Corradini. 2014. Anesthesia influences neuronal activity and drug effectiveness in neuropathic rats. Pain 155: 2583–90.

Hochman, J. R., A. M. Davis, J. Elkayam, L. Gagliese and G. A. Hawker. 2013. Neuropathic pain symptoms on the modified painDETECT correlate with signs of central sensitization in knee osteoarthritis. Osteoarthr. Cartil. 21: 1236–1242.

Høegh-Andersen, P., L. B. Tankó, T. L. Andersen, C. V Lundberg, J. a Mo, A.-M. Heegaard, J.-M. Delaissé and S. Christgau. 2004. Ovariectomized rats as a model of postmenopausal osteoarthritis: validation and application. Arthritis Res Ther 6(2): R169–R180.

Holmes, A. P., C. J. Ray, S. A. Pearson, A. M. Coney and P. Kumar. 2018. Ecto-5′-

nucleotidase (CD73) regulates peripheral chemoreceptor activity and cardiorespiratory responses to hypoxia. J. Physiol. 596: 3137–3148.

Holzer, P. and A. A. Izzo. 2014. The pharmacology of TRP channels. Br. J. Pharmacol. 171: 269–2473.

Honoré, E. 2007. The neuronal background K2P channels: focus on TREK1. Nat. Rev. Neurosci. 8: 251–261.

Hossaini, M., J. A. C. Goos, S. K. Kohli and J. C. Holstege. 2012. Distribution of glycine/GABA neurons in the ventromedial medulla with descending spinal projections and evidence for an ascending glycine/GABA projection. PLoS One 7(4): e35293.

Hsieh, M. T., L. F. Donaldson and B. M. Lumb. 2015. Differential contributions of A- and C-nociceptors to primary and secondary inflammatory hypersensitivity in the rat. Pain 156: 1074–1083.

Hu, C., P. A. Flecknell and J. H. Liles. 1992. Fentanyl and medetomidine anaesthesia in the rat and its reversal using atipamazole and either nalbuphine or butorphanol. Lab. Anim. 26: 15–22.

Huang, K. and A. El-Husseini. 2005. Modulation of neuronal protein trafficking and function by palmitoylation. Curr. Opin. Neurobiol. 15: 527–535.

Hucho, T. B., O. A. Dina, J. Kuhn and J. D. Levine. 2006. Estrogen controls PKCε-dependent mechanical hyperalgesia through direct action on nociceptive neurons. Eur. J. Neurosci. 24: 527–534.

Hughes, S., L. Hickey, L. F. Donaldson, B. M. Lumb and A. E. Pickering. 2015. Intrathecal reboxetine suppresses evoked and ongoing neuropathic pain behaviours by restoring spinal noradrenergic inhibitory tone. Pain 156: 328–334.

Hunt, J., J. Murrell, D. Knazovicky, J. Harris, S. Kelly, T. G. Knowles and B. D. X. Lascelles. 2016. Alfaxalone anaesthesia facilitates electrophysiological recordings of nociceptive withdrawal reflexes in dogs (Canis familiaris). PLoS One 11(7): 0158990.

Hunt, J. R., M. Goff, H. Jenkins, J. Harris, T. G. Knowles, B. D. X. Lascelles, M. Enomoto,

M. Mendl, H. R. Whay and J. C. Murrell. 2018. Electrophysiological characterisation of central sensitisation in canine spontaneous osteoarthritis. Pain 159: 2318–2330.

IASP Task Force on Taxonomy. 1994. Part III: Pain Terms, A Current List with Definitions and Notes on Usage. P. in Classification of Chronic Pain.

Ihmsen, H., A. Tzabazis, M. Schywalsky and H. Schwilden. 2002. Propofol in rats: testing for nonlinear pharmacokinetics and modelling acute tolerance to EEG effects. Eur. J. Anaesthesiol. 19: 177–188.

Ikeda, H., B. Heinke, R. Ruscheweyh and J. Sandkühler. 2003. Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. Science (80-. )., doi: 10.1126/science.1080659.

Imberger, G., A. Orr, K. Thorlund, J. Wetterslev, P. Myles, A. M. Miller and J. G. Hardman. 2014. Does anaesthesia with nitrous oxide affect mortality or cardiovascular morbidity? A systematic review with meta-analysis and trial sequential analysis. Br. J. Anaesth. 112: 410–426.

Ishikawa, M. and R. M. Quock. 2003. Role of nitric-oxide synthase isoforms in nitrous oxide antinociception in mice. J. Pharmacol. Exp. Ther. 306: 484–489.

Itomi, Y., Y. Tsukimi and T. Kawamura. 2016. Impaired diffuse noxious inhibitory controls in specific alternation of rhythm in temperature-stressed rats. Eur. J. Pharmacol. 784: 61–68. Elsevier.

Ivanavicius, S. P., A. D. Ball, C. G. Heapy, F. R. Westwood, F. Murray and S. J. Read. 2007. Structural pathology in a rodent model of osteoarthritis is associated with neuropathic pain: Increased expression of ATF-3 and pharmacological characterisation. Pain 128: 272–282.

Jenkins, S., M. Worthington, J. Harris and R. W. Clarke. 2004. Differential modulation of withdrawal reflexes by a cannabinoid in the rabbit. Brain Res 1012: 146–153.

Jevtovic-Todorovic, V., S. M. Todorovic, S. Mennerick, S. Powell, K. Dikranian, N. Benshoff, C. F. Zorumski, J. W. Olney, V. Jevtović-Todorović, S. M. Todorović, S. Mennerick, S. Powell, K. Dikranian, N. Benshoff, C. F. Zorumski and J. W. Olney.

1998. Nitrous oxide (laughing gas) is an NMDA antagonist, neuroprotectant and neurotoxin. Nat Med 4: 460–463.

Jevtovic-Todorovic, V., D. F. Wozniak, N. D. Benshoff and J. W. Olney. 2001. A comparative evaluation of the neurotoxic properties of ketamine and nitrous oxide. Brain Res. 895: 264–267.

Jhaveri, M. D., D. R. Sagar, S. J. R. Elmes, D. A. Kendall and V. Chapman. 2007. Cannabinoid CB2 receptor-mediated anti-nociception in models of acute and chronic pain. Mol. Neurobiol. 36: 26–35.

Ji, R.-R., T. Berta and M. Nedergaard. 2013. Glia and pain: Is chronic pain a gliopathy? Pain 154: S10–S28.

Jiang, Y. H., X. Q. Ni, W. W. Xiong, H. Wang, Y. Tan, Z. H. Huang and X. Y. Yao. 2015. Different effects of etomidate and propofol on memory in immature rats. Int. J. Neurosci. 125: 66–69.

Jimenez, P. A., S. S. Glasson, O. V Trubetskoy and H. B. Haimes. 1997. Spontaneous osteoarthritis in Dunkin Hartley guinea pigs: histologic, radiologic, and biochemical changes. Lab Anim Sci 47: 598–601.

Jinks, S., J. F. Antognini and E. Carstens. 2003a. Isoflurane depresses Diffuse Noxious Inhibitory Controls in rats between 0.8 and 1.2 minimum alveolar anesthetic concentration. Anesth. Analg. 97: 111–116.

Jinks, S. L., E. Carstens and J. F. Antognini. 2009. Nitrous oxide-induced analgesia does not influence nitrous oxide’s immobilizing requirements. Anesth. Analg. 109: 1111–1116.

Jinks, S., J. T. Martin, E. Carstens, S.-W. Jung and J. F. Antognini. 2003b. Peri-MAC depression of a nociceptive withdrawal reflex is accompanied by reduced dorsal horn activity with halothane but not isoflurane. Anesthesiology 98: 1128–1138.

Johnson, C. B. and P. M. Taylor. 1998. Comparison of the effects of halothane, isoflurane and methoxyflurane on the electroencephalogram of the horse. Br. J. Anaesth. 81: 748–753.

Johnson, C. B., P. R. Wilson, M. R. Woodbury and N. A. Caulkett. 2005. Comparison of analgesic techniques for antler removal in halothane-anaesthetized red deer (Cervus elaphus): Electroencephalographic responses. Vet. Anaesth. Analg. 32: 61–71.

Jones, S. L. and G. F. Gebhart. 1986. Characterization of coeruleospinal inhibition of the nociceptive tail-flick reflex in the rat: Mediation by spinal alpha 2-adrenoceptors. Brain Res. 364: 315–330.

Jordt, S. E., D. D. McKemy and D. Julius. 2003. Lessons from peppers and peppermint: The molecular logic of thermosensation. Curr. Opin. Neurobiol. 13: 487–492.

Kakigi, R. 1994. Diffuse noxious inhibitory control. Reappraisal by pain-related somatosensory evoked potentials following CO2 laser stimulation. J. Neurol. Sci. 125: 198–205.

Kalbhen, D. A. 1987. Chemical model of osteoarthritis - A pharmacological evaluation. J. Rheumatol. 14 Spec No: 130–131.

Kalchofner, K. S., S. Picek, S. K. Ringer, M. Jackson, M. Hässig and R. Bettschart-Wolfensberger. 2009. A study of cardiovascular function under controlled and spontaneous ventilation in isoflurane-medetomidine anaesthetized horses. Vet. Anaesth. Analg. 36: 426–435.

Kalff, K. M., M. El Mouedden, J. van Egmond, J. Veening, L. Joosten, G. J. Scheffer, T. Meert and K. Vissers. 2010. Pre-treatment with capsaicin in a rat osteoarthritis model reduces the symptoms of pain and bone damage induced by monosodium iodoacetate. Eur J Pharmacol 641: 108–113.

Kalliomäki, J., J. Schouenborg and A. H. Dickenson. 1992. Differential effects of a distant noxious stimulus on hindlimb nociceptive withdrawal reflexes in the rat. Eur J Neurosci 4: 648–652.

Kang, S. J., C. Kwak, J. Lee, S.-E. Sim, J. Shim, T. Choi, G. L. Collingridge, M. Zhuo and B.-K. Kaang. 2015. Bidirectional modulation of hyperalgesia via the specific control of excitatory and inhibitory neuronal activity in the ACC. Mol. Brain 8: 81.

Karashima, Y., N. Damann, J. Prenen, K. Talavera, A. Segal, T. Voets and B. Nilius.

2007. Bimodal action of menthol on the transient receptor potential channel TRPA1. J. Neurosci. 27: 9874–9884.

Katoh, T., K. Ikeda and H. Bito. 1997. Does nitrous oxide antagonize sevoflurane-induced hypnosis? Br. J. Anaesth. 79: 465–468.

Kauppila, T., V. K. Kontinen and A. Pertovaara. 1998. Influence of spinalization on spinal withdrawal reflex responses varies depending on the submodality of the test stimulus and the experimental pathophysiological condition in the rat. Brain Res. 797: 234–242.

Kayalioglu, G. 2009. Projections from the Spinal Cord to the Brain. Pp. 148–167 in The spinal cord : a Christopher and Dana Reeve Foundation text and atlas (C. Watson, G. Paxinos, and Kayalioglu. G, eds). Elsevier/Academic Press, New York, NY:

Keay, K. A. and R. Bandler. 2015. Periaqueductal Gray. Pp. 207–221 in The Rat Nervous System (G. Paxinos, ed). Elsevier, New York, NY:

Kelly, S., K. L. Dobson and J. Harris. 2013. Spinal nociceptive reflexes are sensitized in the monosodium iodoacetate model of osteoarthritis pain in the rat. Osteoarthr. Cartil. 21: 1327–1335.

Kelly, S., J. P. Dunham, F. Murray, S. Read, L. F. Donaldson and S. N. Lawson. 2012. Spontaneous firing in C-fibers and increased mechanical sensitivity in A-fibers of knee joint-associated mechanoreceptive primary afferent neurones during MIA-induced osteoarthritis in the rat. Osteoarthr. Cartil. 20: 305–313.

Kest, B., S. G. Wilson and J. S. Mogil. 1999. Sex differences in supraspinal morphine analgesia are dependent on genotype. J. Pharmacol. Exp. Ther. 289: 1370–5.

Kilkenny, C., W. Browne, I. Cuthill, M. Emerson and D. Altman. 2014. Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. Animals 4: 35–44.

Kim, J., A. Yao, R. Atherley, E. Carstens, S. L. Jinks and J. F. Antognini. 2007. Neurons in the ventral spinal cord are more depressed by isoflurane, halothane, and propofol than are neurons in the dorsal spinal cord. Anesth. Analg. 105: 1020–1026.

Kingery, W. S., T. Z. Guo, M. F. Davies, L. Limbird and M. Maze. 2000. The α(2A) adrenoceptor and the sympathetic postganglionic neuron contribute to the development of neuropathic heat hyperalgesia in mice. Pain 85: 345–358.

Knotek, Z., A. Hrdá, Z. Knotková, Š. Trnková and V. Babák. 2013. Alfaxalone anaesthesia in the green iguana (Iguana iguana). Acta Vet. Brno 82: 109–114.

Koch, S. C. and M. Fitzgerald. 2014. The selectivity of rostroventral medulla descending control of spinal sensory inputs shifts postnatally from A fibre to C fibre evoked activity. J. Physiol. 592: 1535–1544.

Koerber, H. R., R. E. Druzinsky and L. M. Mendell. 1988. Properties of somata of spinal dorsal root ganglion cells differ according to peripheral receptor innervated. J. Neurophysiol. 60: 1584–1596.

Komatsu, T., K. Shingu, N. Tomemori, N. Urabe and K. Mori. 1981. Nitrous oxide activates the supraspinal pain inhibition system. Acta Anaesthesiol. Scand. 25: 519–522.

Korn, S. J. and R. Dingledine. 1986. Inhibition of GABA uptake in the rat hippocampal slice. Brain Res. 368: 247–255.

Kosek, E. and G. Ordeberg. 2000. Lack of pressure pain modulation by heterotopic noxious conditioning stimulation in patients with painful osteoarthritis before, but not following, surgical pain relief. Pain 88: 69–78.

Koyama, T. and K. Fukuda. 2009. Nociceptin receptor antagonist JTC-801 inhibits nitrous oxide-induced analgesia in mice. J. Anesth. 23: 301–303.

Koyanagi, S., S. Himukashi, K. Mukaida, T. Shichino and K. Fukuda. 2008. Dopamine D2-Like receptor in the nucleus accumbens is involved in the antinociceptive effect of nitrous oxide. Anesth. Analg. 106: 1904–1909.

Krummenacher, P., V. Candia, G. Folkers, M. Schedlowski and G. Schönbächler. 2010. Prefrontal cortex modulates placebo analgesia. Pain 148: 368–374.

Labus, J. S., B. N. Naliboff, J. Fallon, S. M. Berman, B. Suyenobu, J. A. Bueller, M. Mandelkern and E. A. Mayer. 2008. Sex differences in brain activity during aversive

visceral stimulation and its expectation in patients with chronic abdominal pain: A network analysis. Neuroimage 41: 1032–1043.

LaCroix-Fralish, M. L., J. B. Ledoux and J. S. Mogil. 2007. The Pain Genes Database: An interactive web browser of pain-related transgenic knockout studies. Pain 131: 3.e1-3.e4.

LaCroix-Fralish, M. L. and J. S. Mogil. 2009. Progress in Genetic Studies of Pain and Analgesia. Annu. Rev. Pharmacol. Toxicol. 49: 97–121.

Lähdesmäki, J., M. Scheinin, A. Pertovaara and H. Mansikka. 2003. The α2A-adrenoceptor subtype is not involved in inflammatory hyperalgesia or morphine-induced antinociception. Eur. J. Pharmacol. 468: 183–189.

Lampropoulou-Adamidou, K., P. Lelovas, E. V. Karadimas, C. Liakou, I. K. Triantafillopoulos, I. Dontas and N. A. Papaioannou. 2014. Useful animal models for the research of osteoarthritis. Eur. J. Orthop. Surg. Traumatol. 24: 263–271.

Lapirot, O., R. Chebbi, L. L. Monconduit, A. Artola, R. Dallel and P. Luccarini. 2009. NK1 receptor-expressing spinoparabrachial neurons trigger diffuse noxious inhibitory controls through lateral parabrachial activation in the male rat. Pain 142: 245–254.

Lapirot, O., C. Melin, A. Modolo, C. Nicolas, Y. Messaoudi, L. Monconduit, A. Artola, P. Luccarini and R. Dallel. 2011. Tonic and phasic descending dopaminergic controls of nociceptive transmission in the medullary dorsal horn. Pain 152: 1821–1831.

Lariviere, W. R., E. J. Chesler and J. S. Mogil. 2001. Transgenic studies of pain and analgesia: Mutation or background genotype? J. Pharmacol. Exp. Ther. 297: 467–473.

Larsen, J. -J and J. Arnt. 1984. Spinal 5-HT or NA Uptake Inhibition Potentiates Supraspinal Morphine Antinociception in Rats. Acta Pharmacol. Toxicol. (Copenh)., doi: 10.1111/j.1600-0773.1984.tb01897.x.

Lau, B. K. and C. W. Vaughan. 2014. Descending modulation of pain: The GABA disinhibition hypothesis of analgesia. Curr. Opin. Neurobiol. 29: 159–164.

Lau, C., M. G. Ranasinghe, I. Shiels, H. Keates, K. Pasloske and M. . Bellingham. 2013.

Plasma pharmacokinetics of alfaxalone after a single intraperitoneal or intravenous injection of Alfaxan in rats. J Vet Pharmacol Ther 36: 516–520.

Le Bars, D. 1988. Serotonin and Pain. Pp. 171–229 in Neuronal Serotonin (N. N. Osborne and M. Hamon, eds). Wiley, New York.

Le Bars, D. 2002. The whole body receptive field of dorsal horn multireceptive neurones. Brain Res. Rev. 40: 29–44.

Le Bars, D. and S. W. Cadden. 2008. What is a Wide-Dynamic-Range Cell? Senses A Compr. Ref. 5: 331–338.

Le Bars, D., A. H. Dickenson and J.-M. Besson. 1979a. Diffuse noxious inhibitory controls (DNIC). II. Lack of effect on non-convergent neurones, supraspinal involvement and theoretical implications. Pain 6: 305–327.

Le Bars, D., A. H. Dickenson and J.-M. M. Besson. 1979b. Diffuse noxious inhibitory controls (DNIC) .1. Effects on dorsal horn convergent neurons in the rat. Pain 6: 283–304.

Le Bars, D., M. Gozariu and S. W. Cadden. 2001. Animal models of nociception. Pharmacol. Rev. 53: 597–652.

Lee, H. and M. J. Caterina. 2005. TRPV channels as thermosensory receptors in epithelial cells. Pflugers Arch. Eur. J. Physiol. 151: 160–167.

Lee, L. H. Y., M. G. Irwin and S. K. Lui. 2005. Intraoperative remifentanil infusion does not increase postoperative opioid consumption compared with 70% nitrous oxide. Anesthesiology 102: 398–402.

Lee, W., F. Guilak and W. Liedtke. 2017. Role of Piezo Channels in Joint Health and Injury. Pp. 263–273 in Piezo Channels (P. Gottlieb, ed). Academic Press, London, UK.

Lee, Y., M. Pai, J. D. Brederson, D. Wilcox, G. Hsieh, M. F. Jarvis and R. S. Bitner. 2011. Monosodium iodoacetate-induced joint pain is associated with increased phosphorylation of mitogen activated protein kinases in the rat spinal cord. Mol. Pain 7 39: 2–10.

Leslie, K., P. S. Myles, M. T. V. Chan, A. Forbes, M. J. Paech, P. Peyton, B. S. Silbert and E. Williamson. 2011. Nitrous oxide and long-term morbidity and mortality in the ENIGMA trial. Anesth. Analg. 112: 387–393.

Levinsson, A., M. Garwicz and J. Schouenborg. 1999. Sensorimotor transformation in cat nociceptive withdrawal reflex system. Eur. J. Neurosci. 11: 4327–4332.

Levionnois, O. L., M. Menge, W. Thormann, M. Mevissen and C. Spadavecchia. 2010. Effect of ketamine on the limb withdrawal reflex evoked by transcutaneous electrical stimulation in ponies anaesthetised with isoflurane. Vet. J. 186: 304–311.

Li, M. H., K. L. Suchland and S. L. Ingram. 2015. GABAergic transmission and enhanced modulation by opioids and endocannabinoids in adult rat rostral ventromedial medulla. J. Physiol. 593: 217–230.

Liedtke, W. 2005. TRPV4 as osmosensor: A transgenic approach. Pflugers Arch. Eur. J. Physiol. 451: 176–180.

Light, A. R. and E. R. Perl. 1979. Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. J. Comp. Neurol. 186: 133–150.

Linnman, C., J. C. Beucke, K. B. Jensen, R. L. Gollub and J. Kong. 2012. Sex similarities and differences in pain-related periaqueductal gray connectivity. Pain 153: 444–454.

Little, C. B. and M. M. Smith. 2008. Animal Models of Osteoarthritis. Curr. Rheumatol. Rev. 4: 1–8.

Little, C. B. and S. Zaki. 2012. What constitutes an animal model of osteoarthritis the need for consensus? Osteoarthr. Cartil. 20: 261–267.

Liu, B., J. E. Linley, X. Du, X. Zhang, L. Ooi, H. Zhang, N. Gamper and 1Department. 2010. The acute nociceptive signals induced by bradykinin in rat sensory neurons are mediated by inhibition of M-type K+ channels and activation of Ca2+-activated Cl– channels. J. Clin. Invest. 120: 1240–1252.

Liu, P., A. Okun, J. Ren, R. C. Guo, M. H. Ossipov, J. Xie, T. King and F. Porreca. 2011. Ongoing pain in the MIA model of osteoarthritis. Neurosci Lett 493: 72–75.

Liva, S. M. and R. R. Voskuhl. 2001. Testosterone Acts Directly on CD4+ T Lymphocytes to Increase IL-10 Production. J. Immunol. 167: 2060–2067.

Llorca-Torralba, M., G. Borges, F. Neto, J. A. Mico and E. Berrocoso. 2016. Noradrenergic Locus Coeruleus pathways in pain modulation. Neuroscience 338: 93–113.

Loeser, J. D. and R. D. Treede. 2008. The Kyoto protocol of IASP Basic Pain Terminology. Pain 137: 473–477.

Losina, E., A. D. Paltiel, A. M. Weinstein, E. Yelin, D. J. Hunter, S. P. Chen, K. Klara, L. G. Suter, D. H. Solomon, S. A. Burbine, R. P. Walensky and J. N. Katz. 2015. Lifetime medical costs of knee osteoarthritis management in the United States: Impact of extending indications for total knee arthroplasty. Arthritis Care Res. 67: 203–215.

Lötsch, J., I. Weyer-Menkhoff and I. Tegeder. 2018. Current evidence of cannabinoid-based analgesia obtained in preclinical and human experimental settings. Eur. J. Pain 22: 471–484.

Loyd, D. R., M. M. Morgan and A. Z. Murphy. 2007. Morphine preferentially activates the periaqueductal gray-rostral ventromedial medullary pathway in the male rat: A potential mechanism for sex differences in antinociception. Neuroscience 147: 456–468.

Loyd, D. R. and A. Z. Murphy. 2008. Androgen and estrogen (α) receptor localization on periaqueductal gray neurons projecting to the rostral ventromedial medulla in the male and female rat. J. Chem. Neuroanat. 36: 216–226.

Loyd, D. R. and A. Z. Murphy. 2006. Sex differences in the anatomical and functional organization of the periaqueductal gray-rostral ventromedial medullary pathway in the rat: A potential circuit mediating the sexually dimorphic actions of morphine. J. Comp. Neurol. 496: 723–738.

Lu, Y. and E. R. Perl. 2003. A specific inhibitory pathway between substantia gelatinosa neurons receiving direct C-fiber input. J. Neurosci. 23: 8752–8758.

Lu, Y. and E. R. Perl. 2005. Modular organization of excitatory circuits between neurons

of the spinal superficial dorsal horn (laminae I and II). J. Neurosci. 25: 3900–3907.

Lu, Y., S. M. Sweitzer, C. E. Laurito and D. C. Yeomans. 2004. Differential opioid inhibition of C- and A delta- fiber mediated thermonociception after stimulation of the nucleus raphe magnus. Anesth. Analg. 98: 414–419.

Luiz, A. P., D. I. MacDonald, S. Santana-Varela, Q. Millet, S. Sikandar, J. N. Wood and E. C. Emery. 2019. Cold sensing by Na V 1.8-positive and Na V 1.8-negative sensory neurons. Proc. Natl. Acad. Sci. U. S. A. 116: 3811–3816.

Lumpkin, E. A. and M. J. Caterina. 2007. Mechanisms of sensory transduction in the skin. Nature 445: 858–865.

Lund, I., T. Lundeberg, J. Kowalski and E. Svensson. 2005. Gender differences in electrical pain threshold responses to transcutaneous electrical nerve stimulation (TENS). Neurosci. Lett. 375: 75–80.

Lynn, B., W. Ye and B. Cotsell. 1992. The actions of capsaicin applied topically to the skin of the rat on C-fibre afferents, antidromic vasodilatation and substance P levels. Br. J. Pharmacol. 107: 400–406.

MacIver, M. B. and D. L. Tanelian. 1990. Volatile anesthetics excite mammalian nociceptor afferents recorded in vitro. Anesthesiology 72: 1022–1030.

Maerz, T., M. D. Kurdziel, A. A. Davidson, K. C. Baker, K. Anderson and H. W. T. Matthew. 2015. Biomechanical Characterization of a Model of Noninvasive, Traumatic Anterior Cruciate Ligament Injury in the Rat. Ann. Biomed. Eng. 43: 2467–2476.

Magnusson, J. E. and K. Fisher. 2000. The involvement of dopamine in nociception: The role of D1 and D2 receptors in the dorsolateral striatum. Brain Res. 855: 260–266.

Maingret, F. 2000. TREK-1 is a heat-activated background K+ channel. EMBO J. 19: 2483–2491.

Maingret, F., M. Fosset, F. Lesage, M. Lazdunski and E. Honoré. 1999. TRAAK is a mammalian neuronal mechano-gated K+ channel. J. Biol. Chem. 274: 1381–1387.

Mak, I. W. Y., N. Evaniew and M. Ghert. 2014. Lost in translation: Animal models and

clinical trials in cancer treatment. Am. J. Transl. Res. 6: 114–118.

Malfait, A. M. and R. J. Miller. 2016. Emerging Targets for the Management of Osteoarthritis Pain. Curr. Osteoporos. Rep. 14: 260–268.

Malmberg, A. B., L. R. Hedley, J. R. Jasper, J. C. Hunter and A. I. Basbaum. 2001. Contribution of α 2 receptor subtypes to nerve injury-induced pain and its regulation by dexmedetomidine. Br. J. Pharmacol. 132: 1827–1836.

Mankin, H. J., M. E. Johnson and L. Lippiello. 1981. Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. III. Distribution and metabolism of amino sugar-containing macromolecules. J. Bone Jt. Surg. 63: 131–139.

Mansell, J. P., C. Collins and A. J. Bailey. 2007. Bone, not cartilage, should be the major focus in osteoarthritis. Nat. Clin. Pract. Rheumatol. 3: 306–307.

Mansikka, H., J. J. Idänpään-Heikkilä and A. Pertovaara. 1996. Different roles of α2-adrenoceptors of the medulla versus the spinal cord in modulation of mustard oil-induced central hyperalgesia in rats. Eur. J. Pharmacol. 297: 19–26.

Mansikka, H., J. Lähdesmäki, M. Scheinin and A. Pertovaara. 2004. Alpha(2A) adrenoceptors contribute to feedback inhibition of capsaicin-induced hyperalgesia. Anesthesiology 101: 185–190.

Mantyh, P. W. and M. Peschanski. 1982. Spinal projections from the periaqueductal grey and dorsal raphe in the rat, cat and monkey. Neuroscience 7: 2769–2776.

Mao, J. and L. L. Chen. 2000. Gabapentin in pain management. Anesth. Analg. 91: 680–687.

Marín, P., E. Belda, F. G. Laredo, C. A. Torres, V. Hernandis and E. Escudero. 2020. Pharmacokinetics and sedative effects of alfaxalone with or without dexmedetomidine in rabbits. Res. Vet. Sci. 129: 6–12.

Marker, C. L. and J. D. Pomonis. 2012. The monosodium iodoacetate model of osteoarthritis pain in the rat. Methods Mol. Biol. 851: 239–248.

Maroto, R., A. Raso, T. G. Wood, A. Kurosky, B. Martinac and O. P. Hamill. 2005. TRPC1

forms the stretch-activated cation channel in vertebrate cells. Nat. Cell Biol. 7: 179–185.

Martini, C., M. Van Velzen, A. Drewes, L. Aarts, A. Dahan and M. Niesters. 2015. A randomized controlled trial on the effect of tapentadol and morphine on conditioned pain modulation in healthy volunteers. PLoS One 10: e0128997.

Matta, J. a, P. M. Cornett, R. L. Miyares, K. Abe, N. Sahibzada and G. P. Ahern. 2008. General anesthetics activate a nociceptive ion channel to enhance pain and inflammation. Proc. Natl. Acad. Sci. U. S. A. 105: 8784–8789.

Maurer, M., M. Bader, M. Bas, F. Bossi, M. Cicardi, M. Cugno, P. Howarth, A. Kaplan, G. Kojda, F. Leeb-Lundberg, J. Lötvall and M. Magerl. 2011. New topics in bradykinin research.

Maxwell, D. J., M. D. Belle, O. Cheunsuang, A. Stewart and R. Morris. 2007. Morphology of inhibitory and excitatory interneurons in superficial laminae of the rat dorsal horn. J. Physiol. 584: 521–533.

Mcbride, W. T. 1996. Immunomodulation: An important concept in modern anaesthesia. Anaesthesia 51: 465–473.

McCabe, P. S., N. Maricar, M. J. Parkes, D. T. Felson and T. W. O'Neill. 2016. The efficacy of intra-articular steroids in hip osteoarthritis: a systematic review. Osteoarthr. Cartil. 24: 1509–1517.

Mccarthy, M. M., A. P. Arnold, G. F. Ball, J. D. Blaustein, J. Geert and G. J. De Vries. 2012. Sex Differences in the Brain : The Not So Inconvenient Truth. J Neurophysiol 32: 2241–2247.

McCoy, A. M. 2015. Animal Models of Osteoarthritis: Comparisons and Key Considerations. Vet. Pathol. 52: 803–818.

McDonald, C. E., M. J. Gagnon, E. A. Ellenberger, B. L. Hodges, J. K. Ream, S. A. Tousman and R. M. Quock. 1994. Inhibitors of nitric oxide synthesis antagonize nitrous oxide antinociception in mice and rats. J Pharmacol Exp Ther 269: 601–608.

McDougall, J. J., B. Andruski, N. Schuelert, B. Hallgrimsson and J. R. Matyas. 2009.

Unravelling the relationship between age, nociception and joint destruction in naturally occurring osteoarthritis of Dunkin Hartley guinea pigs. Pain 141: 222–232.

McGrath, J. C., G. B. Drummond, E. M. McLachlan, C. Kilkenny and C. L. Wainwright. 2010. Editorial: Guidelines for reporting experiments involving animals: The ARRIVE guidelines. Br. J. Pharmacol. 160: 1573–1576.

McKemy, D. D., W. M. Neuhausser and D. Julius. 2002. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature 416: 52–58.

McKune, C. M., R. J. Brosnan, M. J. Dark and G. J. Haldorson. 2008. Safety and efficacy of intramuscular propofol administration in rats. Vet. Anaesth. Analg. 35: 495–500.

McKune, C. M., J. Murrell, A. Nolan, K. White and B. Wright. 2015. Nociception and Pain. Pp. 584–623 in Lumb & Jones' Veterinary Anaesthesia & Analgesia: (K. Grimm, L. Lamont, W. Tranquilli, S. Greene, and S. Robertson, eds). Wiley Blackwell, Ames, Iowa.

McLaughlin, B. J., R. Barber, K. Saito, E. Roberts and J. Y. Wu. 1975. Immunocytochemical localization of glutamate decarboxylase in rat spinal cord. J. Comp. Neurol. 164: 305–321.

McMillan, M. W. and E. A. Leece. 2011. Immersion and branchial/transcutaneous irrigation anaesthesia with alfaxalone in a Mexican axolotl. Vet. Anaesth. Analg. 38: 619–23.

McMullan, S. and B. M. Lumb. 2006a. Midbrain control of spinal nociception discriminates between responses evoked by myelinated and unmyelinated heat nociceptors in the rat. Pain 124: 59–68.

McMullan, S. and B. M. Lumb. 2006b. Spinal dorsal horn neuronal responses to myelinated versus unmyelinated heat nociceptors and their modulation by activation of the periaqueductal grey in the rat. J. Physiol. 576: 547–56.

Megirian, D. 1962. Bilateral facilitatory and inhibitory skin areas of spinal motoneurones of cat. J. Neurophysiol. 25: 127–37.

Messeguer, A., R. Planells-Cases and A. Ferrer-Montiel. 2005. Physiology and

Pharmacology of the Vanilloid Receptor. Curr. Neuropharmacol. 4: 1–15.

Michou, J. N., E. A. Leece and J. C. Brearley. 2012. Comparison of pain on injection during induction of anaesthesia with alfaxalone and two formulations of propofol in dogs. Vet. Anaesth. Analg. 39: 275–281.

Miki, K., Q. Q. Zhou, W. Guo, Y. Guan, R. Terayama, R. Dubner and K. Ren. 2002. Changes in gene expression and neuronal phenotype in brain stem pain modulatory circuitry after inflammation. J Neurophysiol 87: 750–760.

Millan, M. J. 2002. Descending control of pain. Prog. Neurobiol. 66: 355–474.

Mobarakeh, J. I., S. Sakurada, S. Katsuyama, M. Kutsuwa, A. Kuramasu, Z. Y. Lin, T. T. Watanabe, Y. Hashimoto, T. T. Watanabe and K. Yanai. 2000. Role of histamine H1 receptor in pain perception: A study of the receptor gene knockout mice. Eur. J. Pharmacol. 391: 81–89.

Mogil, J. S. 2009. Animal models of pain: progress and challenges. Nat Rev Neurosci 10: 283–294.

Mogil, J. S., N. M. Breese, M. F. Witty, J. Ritchie, M. L. Rainville, A. Ase, N. Abbadi, C. L. Stucky and P. Séguéla. 2005. Transgenic expression of a dominant-negative ASIC3 subunit leads to increased sensitivity to mechanical and inflammatory stimuli. J. Neurosci. 25: 9893–9901.

Mogil, J. S. and M. L. Chanda. 2005. The case for the inclusion of female subjects in basic science studies of pain. Pain 117: 1–5.

Mogil, J. S. and S. E. Crager. 2004. What should we be measuring in behavioral studies of chronic pain in animals? Pain 112: 12–15.

Moheban, A. A., H. H. Chang and L. A. Havton. 2016. The Suitability of Propofol Compared with Urethane for Anesthesia during Urodynamic Studies in Rats. J. Am. Assoc. Lab. Anim. Sci. 55: 89–94.

Molliver, D. C., M. J. Radeke, S. C. Feinstein and W. D. Snider. 1995. Presence or absence of TrKA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. J. Comp. Neurol. 361: 404–

416.

Monagle, J., L. Siu, J. Worrell, C. Goodchild and J. Serrao. 2015. A Phase 1c Trial Comparing the Efficacy and Safety of a New Aqueous Formulation of Alphaxalone with Propofol. Anesth Analg 121: 914–924.

Moqrich, A., S. W. Hwang, T. J. Earley, M. J. Petrus, A. N. Murray, K. S. R. Spencer, M. Andahazy, G. M. Story and A. Patapoutian. 2005. Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. Science 307: 1468–1472.

Mühlethaler, M., P. Cand, R. Henauer and M. Serafin. 1990. Design of a microprocessor-controlled thermoregulated chamber for perfusing in vitro mammalian brain preparations. J. Neurosci. Methods 31: 215–223.

Muir, W., P. Lerche, A. Wiese, L. Nelson, K. Pasloske and T. Whittem. 2009. The cardiorespiratory and anesthetic effects of clinical and supraclinical doses of alfaxalone in cats. Vet. Anaesth. Analg. 36: 42–54.

Muniak, M. A., Z. M. Mayko, D. K. Ryugo and C. V. Portfors. 2012. Preparation of an awake mouse for recording neural responses and injecting tracers. J. Vis. Exp. 64: 1–7.

Murat, N., B. Karadam, S. Ozkal, V. Karatosun and S. Gidener. 2007. Quantification of papain-induced rat osteoarthritis in relation to time with the Mankin score. Acta Orthop. Traumatol. Turc. 41: 233–237.

Murphy, A. Z., M. A. Shupnik and G. E. Hoffman. 1999. Androgen and estrogen (α) receptor distribution in the periaqueductal gray of the male rat. Horm. Behav. 36: 98–108.

Murrell, J. C. and L. J. Hellebrekers. 2005. Medetomidine and dexmedetomidine: a review of cardiovascular effects and antinociceptive properties in the dog. Vet. Anaesth. Analg. 32: 117–127.

Murrell, J. C. and C. B. Johnson. 2006. Neurophysiological techniques to assess pain in animals. J. Vet. Pharmacol. Ther. 29: 325–335.

Murrell, J. C., C. B. Johnson, K. L. White, P. M. Taylor, Z. L. Haberham and A. E.

Waterman-Pearson. 2003. Changes in the EEG during castration in horses and ponies anaesthetized with halothane. Vet. Anaesth. Analg. 30: 138–146.

Murrell, J. C., D. Waters and C. B. Johnson. 2008. Comparative effects of halothane, isoflurane, sevoflurane and desflurane on the electroencephalogram of the rat. Lab. Anim. 42: 161–170.

Murrell, J., K. White, C. Johnson, P. Taylor, T. Doherty and A. Waterman-Pearson. 2005. Investigation of the EEG effects of intravenous lidocaine during halothane anaesthesia in ponies. Vet. Anaesth. Analg. 32: 212–221.

Na, H. S., S. Choi, J. Kim, J. Park and H.-S. Shin. 2008. Attenuated Neuropathic Pain in CaV3.1 Null Mice. Mol Cells 25: 242–246.

Nadeson, R., A. Tucker, E. Bajunaki and C. S. Goodchild. 2002. Potentiation by ketamine of fentanyl antinociception. I. An experimental study in rats showing that ketamine administered by non-spinal routes targets spinal cord antinociceptive systems. Br. J. Anaesth. 88: 685–691.

Nagele, P., A. Duma, M. Kopec, M. A. Gebara, A. Parsoei, M. Walker, A. Janski, V. N. Panagopoulos, P. Cristancho, J. P. Miller, C. F. Zorumski and C. R. Conway. 2015. Nitrous oxide for treatment-resistant major depression: A proof-of-concept trial. Biol. Psychiatry 78: 10–18.

Naito, S. and T. Ueda. 1983. Adenosine triphosphate-dependent uptake of glutamate into protein I-associated synaptic vesicles. J. Biol. Chem. 258: 696–699.

Nalivaiko, E., V. R. Antunes and J. F. R. Paton. 2010. Control of cardiac contractility in the rat working heart-brainstem preparation. Exp. Physiol. 95: 107–119.

Nandigama, R., M. Bonitz, T. Papadakis, U. Schwantes, T. Bschleipfer and W. Kummer. 2010. Muscarinic acetylcholine receptor subtypes expressed by mouse bladder afferent neurons. Neuroscience 168: 842–850.

Navarrete-Calvo, R., R. J. Gómez-Villamandos, J. Morgaz, J. Manuel Domínguez, A. Fernández-Sarmiento, P. Muñoz-Rascón, I. López Villalba and M. Del Mar Granados. 2014. Cardiorespiratory, anaesthetic and recovery effects of morphine

combined with medetomidine and alfaxalone in rabbits. Vet. Rec. 174: 95.

Nevalainen, T., L. Pyhala, H. M. Voipio and R. Virtanen. 1989. Evaluation of anaesthetic potency of medetomidine-ketamine combination in rats, guinea-pigs and rabbits. Acta Vet. Scand. Suppl. 85: 139–143.

New, D. C. and Y. H. Wong. 2002. The $ORL_1$ Receptor: Molecular Pharmacology and Signalling Mechanisms. Neurosignals 11: 197–212.

Niesters, M., P. L. Proto, L. Aarts, E. Y. Sarton, A. M. Drewes and A. Dahan. 2014. Tapentadol potentiates descending pain inhibition in chronic pain patients with diabetic polyneuropathy. Br. J. Anaesth. 113: 148–156.

Nigam, P. K. 2016. Correct Blood Sampling for Blood Gas Analysis. J. Clin. diagnostic Res. 10: BL01–BL02.

Nikolaev, Y. A., C. D. Cox, P. Ridone, P. R. Rohde, J. F. Cordero-Morales, V. Vásquez, D. R. Laver and B. Martinac. 2019. Mammalian TRP ion channels are insensitive to membrane stretch. J. Cell Sci. 132.

Nir, R. R., M. Granot, E. Sprecher and D. Yarnitsky. 2009. Defining Diffuse Noxious Inhibitory Controls (DNIC) as a trait. Eur. J. Pain 13: S53–S54.

Nir, R. R. and D. Yarnitsky. 2015. Conditioned pain modulation. Curr. Opin. Support. Palliat. Care 9: 131–137.

Niu, K. Y., Y. Zhang and J. Y. Ro. 2012. Effects of gonadal hormones on the peripheral cannabinoid receptor 1 (CB1R) system under a myositis condition in rats. Pain 153: 2283–2291.

Noël, J., K. Zimmermann, J. Busserolles, E. Deval, A. Alloui, S. Diochot, N. Guy, M. Borsotto, P. Reeh, A. Eschalier and M. Lazdunski. 2009. The mechano-activated K± channels TRAAK and TREK-1 control both warm and cold perception. EMBO J. 28: 1308–1318.

Novelli, G. P., V. A. Peduto, E. Bertol, F. Mari and E. Pieraccioli. 1983. Analgesic interaction between nitrous oxide and delta-9-tetrahydrocannabinol in the rat. Br. J. Anaesth. 55: 997–1000.

Nylander, I., M. Vlaskovska and L. Terenius. 1995. Brain dynorphin and enkephalin systems in Fischer and Lewis rats: effects of morphine tolerance and withdrawal. Brain Res. 683: 25–35.

Odeh, F. and M. Antal. 2001. The projections of the midbrain periaqueductal grey to the pons and medulla oblongata in rats. Eur. J. Neurosci. 14: 1275–1286.

Ohashi, Y., T. Guo, R. Orii, M. Maze and M. Fujinaga. 2003. Brain stem opioidergic and GABAergic neurons mediate the antinociceptive effect of nitrous oxide in fischer rats. Anesthesiology 99: 947–954.

Olivar, T., F. Cervero and J. M. A. Laird. 2000. Responses of rat spinal neurones to natural and electrical stimulation of colonic afferents: Effect of inflammation. Brain Res. 866: 168–177.

Orii, R., Y. Ohashi, T. Guo, L. E. Nelson, T. Hashimoto, M. Maze and M. Fujinaga. 2002. Evidence for the involvement of spinal cord alpha1 adrenoceptors in nitrous oxide-induced antinociceptive effects in Fischer rats. Anesthesiology 97: 1458–1465.

Orii, R., Y. Ohashi, S. Halder, M. Giombini, M. Maze and M. Fujinaga. 2003. GABAergic interneurons at supraspinal and spinal levels differentially modulate the antinociceptive effect of nitrous oxide in Fischer rats. Anesthesiology 98: 1223–1230.

Orita, S., T. Ishikawa, M. Miyagi, N. Ochiai, G. Inoue, Y. Eguchi, H. Kamoda, G. Arai, T. Toyone, Y. Aoki, T. Kubo, K. Takahashi and S. Ohtori. 2011. Pain-related sensory innervation in monoiodoacetate-induced osteoarthritis in rat knees that gradually develops neuronal injury in addition to inflammatory pain. BMC Musculoskelet Disord 12: 134.

Outerbridge, R. E. 1961. The etiology of chrondromalacia patellae. J. Bone Jt. Surg. 43-B: 752–757.

Page, A. J., S. M. Brierley, C. M. Martin, C. Martinez-Salgado, J. A. Wemmie, T. J. Brennan, E. Symonds, T. Omari, G. R. Lewin, M. J. Welsh and L. A. Blackshaw. 2004. The ion channel ASIC1 contributes to visceral but not cutaneous mechanoreceptor function. Gastroenterology 127: 1739–1747.

Pai, A. and M. Heining. 2007. Ketamine. Contin. Educ. Anaesthesia, Crit. Care Pain 7: 59–63.

Paricio-Montesinos, R., F. Schwaller, A. Udhayachandran, F. Rau, J. Walcher, R. Evangelista, J. Vriens, T. Voets, J. F. A. Poulet and G. R. Lewin. 2020. The Sensory Coding of Warm Perception. Neuron 106: 830–841.

Park, H. J., J. U. Park, W. Yoo and Y. E. Moon. 2016. Analgesic effects of nefopam in patients undergoing bimaxillary osteotomy: A double-blind, randomized, placebo-controlled study. J. Cranio-Maxillofacial Surg. 44: 210–214.

Park, K. W., H. B. Dai, E. Lowenstein and F. W. Selke. 1995. Profol-Associated Dilation of Rat Distal Coronary Arteries Is Mediated by Multiple Substances, Including Endothelium Derived Nitric Oxide. Anesth Analg 81: 1191–1196.

Parpaite, T. and B. Coste. 2017. Piezo channels. Curr. Biol. 27: R250–R252. Elsevier.

Pavel. 2020. Studies on the mechanism of general anesthesia. PNAS 117: 13757–13766.

Peier, A. M., A. Moqrich, A. C. Hergarden, A. J. Reeve, D. A. Andersson, G. M. Story, T. J. Earley, I. Dragoni, P. McIntyre, S. Bevan and A. Patapoutian. 2002. A TRP channel that senses cold stimuli and menthol. Cell 108: 705–715.

Pereira, V., J. Busserolles, M. Christin, M. Devilliers, L. Poupon, W. Legha, A. Alloui, Y. Aissouni, E. Bourinet, F. Lesage, A. Eschalier, M. Lazdunski and J. Noël. 2014. Role of the TREK2 potassium channel in cold and warm thermosensation and in pain perception. Pain 155: 2534–2544.

Perrotta, A., M. Serrao, A. Ambrosini, M. Bolla, G. Coppola, G. Sandrini and F. Pierelli. 2013. Facilitated temporal processing of pain and defective supraspinal control of pain in cluster headache. Pain 154: 1325–1332.

Perrotta, A., M. Serrao, G. Sandrini, R. Burstein, G. Sances, P. Rossi, M. Bartolo, F. Pierelli and G. Nappi. 2010. Sensitisation of spinal cord pain processing in medication overuse headache involves supraspinal pain control. Cephalalgia 30: 272–284.

Perry, M. J. and S. N. Lawson. 1998. Differences in expression of oligosaccharides

neuropeptides, carbonic anhydrase and neurofilament in rat primary afferent neurons retrogradely labelled via skin, muscle or visceral nerves. Neuroscience 85: 293–310.

Pertovaara, A. 2006. Noradrenergic pain modulation. Prog. Neurobiol. 80: 53–83.

Pertovaara, A. 2013. The noradrenergic pain regulation system: A potential target for pain therapy. Eur. J. Pharmacol. 716: 2–7.

Peterbauer, C., P. M. Larenza, M. Knobloch, R. Theurillat, W. Thormann, M. Mevissen and C. Spadavecchia. 2008. Effects of a low dose infusion of racemic and S-ketamine on the nociceptive withdrawal reflex in standing ponies. Vet. Anaesth. Analg. 35: 414–423.

Petersen-Felix, S., L. Arendt-Nielsen, P. Bak, D. Roth, M. Fischer, P. Bjerring and A. M. Zbinden. 1996. Analgesic effect in humans of subanaesthetic isoflurane concentrations evaluated by evoked potentials. Br. J. Anaesth. 76: 38–42.

Petersen, E. N., M. A. Pavel, H. Wang and S. B. Hansen. 2020. Disruption of palmitate-mediated localization; a shared pathway of force and anesthetic activation of TREK-1 channels. Biochim. Biophys. Acta - Biomembr. 1862: 183091.

Petersen, K. K., T. Graven-Nielsen, O. Simonsen, M. B. Laursen and L. Arendt-Nielsen. 2016. Preoperative pain mechanisms assessed by cuff algometry are associated with chronic postoperative pain relief after total knee replacement. Pain 157: 1400–1406.

Petrovic, P., E. Kalso, K. M. Petersson, J. Andersson, P. Fransson and M. Ingvar. 2010. A prefrontal non-opioid mechanism in placebo analgesia. Pain 150: 59–65.

Pickarski, M., T. Hayami, Y. Zhuo and L. T. Duong. 2011. Molecular changes in articular cartilage and subchondral bone in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. BMC Musculoskelet. Disord. 12: 197.

Pickering, A. E. and J. F. R. Paton. 2006. A decerebrate, artificially-perfused in situ preparation of rat: Utility for the study of autonomic and nociceptive processing. J. Neurosci. Methods 155: 260–271.

Pielsticker, A., G. Haag, M. Zaudig and S. Lautenbacher. 2005. Impairment of pain

inhibition in chronic tension-type headache. Pain 118: 215–223.

Pintér, E., Z. Helyes and J. Szolcsányi. 2006. Inhibitory effect of somatostatin on inflammation and nociception. Pharmacol. Ther. 112: 440–456.

Polgár, E., D. I. Hughes, J. S. Riddell, D. J. Maxwell, Z. Puskár and A. J. Todd. 2003. Selective loss of spinal GABAergic or glycinergic neurons is not necessary for development of thermal hyperalgesia in the chronic constriction injury model of neuropathic pain. Pain 104: 229–239.

Pomonis, J. D., J. M. Boulet, S. L. Gottshall, S. Phillips, R. Sellers, T. Bunton and K. Walker. 2005. Development and pharmacological characterization of a rat model of osteoarthritis pain. Pain 114: 339–346.

Poree, L. R., T. Z. Guo, W. S. Kingery and M. Maze. 1998. The analgesic potency of dexmedetomidine is enhanced after nerve injury: A possible role for peripheral alpha-2-adrenoceptors. Anesth. Analg. 87: 941–948.

Porreca, F., M. H. Ossipov and G. F. Gebhart. 2002. Chronic pain and medullary descending facilitation. Trends Neurosci. 25: 319–325.

Prendergast, B. J., K. G. Onishi and I. Zucker. 2014. Female mice liberated for inclusion in neuroscience and biomedical research. Neurosci. Biobehav. Rev. 40: 1–5.

Quasha, A., E. Eger and J. Tinker. 1980. Determinations and Applications of MAC. Anesthesiology 53: 315–334.

Quock, R. M., J. A. Best, D. C. Chen, L. K. Vaughn, P. S. Portoghese and A. E. Takemori. 1990. Mediation of nitrous oxide analgesia in mice by spinal and supraspinal -opioid receptors. Eur. J. Pharmacol. 175: 97–100.

Quock, R. M., J. L. Mueller and L. K. Vaughn. 1993. Strain-dependent differences in responsiveness of mice to nitrous oxide (N2O) antinociception. Brain Res. 614: 52–56.

Quock, R. M., J. L. Mueller, L. K. Vaughn and J. K. Belknap. 1996. Nitrous oxide antinociception in BXD recombinant inbred mouse strains and identification of quantitative trait loci. Brain Res. 725: 23–29.

Rahman, W., C. S. Bauer, K. Bannister, J. L. Vonsy, A. C. Dolphin and A. H. Dickenson. 2009. Descending serotonergic facilitation and the antinociceptive effects of pregabalin in a rat model of osteoarthritic pain. Mol Pain 5: 45.

Rahman, W., R. D'Mello and A. H. Dickenson. 2008. Peripheral nerve injury-induced changes in spinal alpha(2)-adrenoceptor-mediated modulation of mechanically evoked dorsal horn neuronal responses. J Pain 9: 350–359.

Rainville, P. 2002. Brain mechanisms of pain affect and pain modulation. Curr. Opin. Neurobiol. 12: 195–204.

Rampil, I. J. and R. S. Matteo. 1987. Changes in EEG spectral edge frequency correlate with the hemodynamic response to laryngoscopy and intubation. Anesthesiology 67: 139–142.

Randle, J. C., C. W. Bourque and L. P. Renaud. 1986. Alpha 1-adrenergic receptor activation depolarizes rat supraoptic neurosecretory neurons in vitro. Am. J. Physiol. Integr. Comp. Physiol. 251: R569–R574.

Rattenholl, A. and M. Steinhoff. 2003. Role of proteinase-activated receptors in cutaneous biology and disease. Drug Dev. Res. 59: 408–416.

Reichling, D. B. and J. D. Levine. 1997. Heat transduction in rat sensory neurons by calcium-dependent activation of a cation channel. Proc. Natl. Acad. Sci. U. S. A. 94: 7006–7011.

Ren, K. and R. Dubner. 1996. Enhanced descending modulation of nociception in rats with persistent hindpaw inflammation. J. Neurophysiol. 76: 3025–3037.

Restrepo, C. E., G. Margaryan, L. Borgius, L. Lundfald, D. Sargsyan and O. Kiehn. 2011. Change in the balance of excitatory and inhibitory midline fiber crossing as an explanation for the hopping phenotype in EphA4 knockout mice. Eur. J. Neurosci. 34: 1102–1112.

Rexed, B. 1952. The cytoarchitectonic organization of the spinal cord of the cat. J. Comp. Neurol. 415–495.

Rhudy, J. L. and C. R. France. 2007. Defining the nociceptive flexion reflex (NFR)

threshold in human participants: A comparison of different scoring criteria. Pain 128: 244–253.

Ribeiro-Da-Silva, A. and A. Coimbra. 1982. Two types of synaptic glomeruli and their distribution in laminae I–III of the rat spinal cord. J. Comp. Neurol. 209: 176–186.

Ribeiro-da-silva, A., P. Tagari and A. C. Cuello. 1989. Morphological characterization of substance P-like immunoreactive glomeruli in the superficial dorsal horn of the rat spinal cord and trigeminal subnucleus caudalis: A quantitative study. J. Comp. Neurol. 281: 497–515.

Richardson, C. A. and P. A. Flecknell. 2005. Anaesthesia and Post-operative Analgesia Following Experimental Surgery in Laboratory Rodents : Are We Making Progress ? Altern. to Lab. Anim. 33: 119–127.

Rigaud, M., G. Gemes, S. E. Abram, C. Dean, F. A. Hopp, C. L. Stucky, D. Eastwood, S. Tarima, J. Seagard and Q. H. Hogan. 2011. Pain tests provoke modality-specific cardiovascular responses in awake, unrestrained rats. Pain 152: 274–284.

Rinaman, L. 2011. Hindbrain noradrenergic A2 neurons: Diverse roles in autonomic, endocrine, cognitive, and behavioral functions. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 300: R222–R235.

Ringer, S. K., K. Kalchofner, J. Boller, A. Fürst and R. Bettschart-Wolfensberger. 2007. A clinical comparison of two anaesthetic protocols using lidocaine or medetomidine in horses. Vet. Anaesth. Analg. 34: 257–268.

Rivot, J. P., B. Calvino and J. M. Besson. 1987. Is there a serotonergic tonic descending inhibition on the responses of dorsal horn convergent neurons to C-fibre inputs? Brain Res. 403: 142–146.

Robbins, T. W. and A. F. T. Arnsten. 2009. The neuropsychopharmacology of fronto-executive function: monoaminergic modulation. Annu. Rev. Neurosci. 32: 267–287.

Rosen, S., B. Ham and J. S. Mogil. 2017. Sex differences in neuroimmunity and pain. J. Neurosci. Res. 95: 500–508.

Rowland, M. and T. N. Tozer. 2011. Clinical pharmacokinetics and pharmacodynamics :

concepts and applications. Wolters Kluwer Health/Lippincott William & Wilkins. pp 333-355.

Rudolph, U. and B. Antkowiak. 2004. Molecular and neuronal substrates for general anaesthetics. Nat. Rev. Neurosci. 5: 709–720.

Ruiz, D., L. Koenig, T. M. Dall, P. Gallo, A. Narzikul, J. Parvizi and J. Tongue. 2013. The direct and indirect costs to society of treatment for end-stage knee osteoarthritis. J. Bone Jt. Surg. 95: 1473–1480.

Rupprecht, R. and F. Holsboer. 1999. Neuroactive steroids: mechanisms of action and neuropsychopharmacological perspectives. Trends Neurosci. 22: 410–6.

Rupreht, J., O. E. Ukponmwan, B. Dworacek, P. V. Admiraal and M. R. Dzoljic. 1984. Enkephalinase inhibition prevented tolerance to nitrous oxide analgesia in rats. Acta Anaesthesiol. Scand. 28: 617–620.

Russell, G. B. and J. M. Graybeal. 1998. Nonlinear additivity of nitrous oxide and isoflurane potencies in rats. Can. J. Anaesth. 45: 466–470.

Sadananda, P., M. J. Drake, J. F. R. Paton and A. E. Pickering. 2011. An exploration of the control of micturition using a novel in situ arterially perfused rat preparation. Front. Neurosci. 5: 1–14.

Saeed, A. W. and A. Ribeiro-da-Silva. 2012. Non-peptidergic primary afferents are presynaptic to neurokinin-1 receptor immunoreactive lamina I projection neurons in rat spinal cord. Mol. Pain 8: 1–10.

Sagar, D. R., J. J. Burston, G. J. Hathway, S. G. Woodhams, R. G. Pearson, A. J. Bennett, D. A. Kendall, B. E. Scammell and V. Chapman. 2011. The contribution of spinal glial cells to chronic pain behaviour in the monosodium iodoacetate model of osteoarthritic pain. Mol Pain 7: 88.

Sagar, D. R., M. Jhaveri and V. Chapman. 2009. Targeting the Cannabinoid system to produce Analgesia. Curr. Top. Behav. Neurosci. 1: 275–287.

Sagar, D. R., L. E. Staniaszek, B. N. Okine, S. Woodhams, L. M. Norris, R. G. Pearson, M. J. Garle, S. P. H. Alexander, A. J. Bennett, D. A. Barrett, D. A. Kendall, B. E.

Scammell and V. Chapman. 2010. Tonic modulation of spinal hyperexcitability by the endocannabinoid receptor system in a rat model of osteoarthritis pain. Arthritis Rheum. 62: 3666–3676.

Sahaghian, R., N. G. Faith and C. Czuprynski. 2009. Comparison of systemic Listeria monocytogenes infection in esophageally inoculated mice anesthetized with isoflurane or pentobarbital. Lab Anim. (NY). 38: 126–130.

Saidman, L. J. and E. I. Eger. 1964. Effect of nitrous oxide and of narcotic premedication on the alveolar concetration of halothane required for anesthesia. Anesthesiology 25: 302–3026.

Salas, R., K. Ramirez, H. Vanegas and E. Vazquez. 2016. Activity correlations between on-like and off-like cells of the rostral ventromedial medulla and simultaneously recorded wide-dynamic-range neurons of the spinal dorsal horn in rats. Brain Res. 1652: 103–110.

Salo, P. T., T. Hogervorst, R. A. Seerattan, D. Rucker and R. C. Bray. 2002. Selective joint denervation promotes knee osteoarthritis in the aging rat. J. Orthop. Res. 20: 1256–1264.

Sanders, R. D., J. Weimann and M. Maze. 2008. Biologic effects of nitrous oxide. Anesthesiology 109: 707–722.

Sandiego, C. M., X. Jin, T. Mulnix, K. Fowles, D. Labaree, J. Ropchan, Y. Huang, K. Cosgrove, S. A. Castner, G. V Williams, L. Wells, E. A. Rabiner and R. E. Carson. 2013. Awake nonhuman primate brain PET imaging with minimal head restraint: evaluation of GABAA-benzodiazepine binding with 11C-flumazenil in awake and anesthetized animals. J Nucl Med 54: 1962–1968.

Sandkuhler, J., Q. G. Fu and M. Zimmermann. 1987. Spinal pathways mediating tonic or stimulation-produced descending inhibition from the periaqueductal gray or nucleus raphe magnus are separate in the cat. J Neurophysiol 58: 327–341.

Sandkühler, J. and X. Liu. 1998. Induction of long-term potentiation at spinal synapses by noxious stimulation or nerve injury. Eur. J. Neurosci. 10: 2476–2480.

Sandrini, G., M. Serrao, P. Rossi, A. Romaniello, G. Cruccu and J. C. Willer. 2005. The lower limb flexion reflex in humans. Prog. Neurobiol. 77: 353–395.

Santos González, M., B. T. Bertrán de Lis and F. J. Tendillo Cortijo. 2013. Effects of intramuscular alfaxalone alone or in combination with diazepam in swine. Vet. Anaesth. Analg. 40: 399–402.

Sara, S. J. and S. Bouret. 2012. Orienting and Reorienting: The Locus Coeruleus Mediates Cognition through Arousal. Neuron 76: 130–141. Elsevier Inc.

Sarkar, D. K., S. A. Chiappa, G. Fink and N. M. Sherwood. 1976. Gonadotropin-releasing hormone surge in pro-oestrous rats. Nature 264: 469–463.

Savage, S. and D. Ma. 2014. The neurotoxicity of nitrous oxide: the facts and 'putative' mechanisms. Brain Sci. 4: 73–90.

Sawamura, S., W. S. Kingery, M. F. Davies, G. S. Agashe, J. D. Clark, B. K. Kobilka, T. Hashimoto and M. Maze. 2000. Antinociceptive action of nitrous oxide is mediated by stimulation of noradrenergic neurons in the brainstem and activation of alpha2B adrenoceptors. J. Neurosci. 20: 9242–9251.

Sawamura, S., M. Obara, K. Takeda, M. Maze and K. Hanaoka. 2003. Corticotropin-releasing factor mediates the antinociceptive action of nitrous oxide in rats. Anesthesiology 99: 708–715.

Schaible, H. G. 2012. Mechanisms of chronic pain in osteoarthritis. Curr. Rheumatol. Rep. 14: 549–556.

Schaible, H. G., A. Ebersberger and G. Natura. 2011. Update on peripheral mechanisms of pain: Beyond prostaglandins and cytokines. Arthritis Res. Ther. 13: 1–8.

Schaible, H. G. and B. D. Grubb. 1993. Afferent and spinal mechanisms of joint pain. Pain 55: 5–54.

Schneider, E. R., E. O. Anderson, E. O. Gracheva and S. N. Bagriantsev. 2014. Temperature Sensitivity of Two-Pore (K2P) Potassium Channels. Curr. Top. Membr. 74: 113–115.

Scholz, J. and C. J. Woolf. 2007. The neuropathic pain triad: neurons, immune cells and

glia. Nat. Neurosci. 10: 1361–1368.

Schomburg, E. D. 1997. Restrictions on the interpretation of spinal reflex modulation in pain and analgesia research. Pain Forum 6: 101–109.

Schomburg, E. D., H. Steffens and N. Wada. 2001. Parallel nociceptive reflex pathways with negative and positive feedback functions to foot extensors in the cat. J. Physiol. 536.2: 605–613.

Schouenborg, J. 2002. Modular organisation and spinal somatosensory imprinting. Brain Res. Rev. 40: 80–91.

Schouenborg, J. 1997. Nociceptive withdrawal reflexes as a model for spinal nociceptive transmission. Pain Forum 6: 116–118.

Schouenborg, J. and A. Dickenson. 1985. The effects of a distant noxious stimulation on A and C fibre-evoked flexion reflexes and neuronal activity in the dorsal horn of the rat. Brain Res. 328: 23–32.

Schouenborg, J., H. Holmberg and H. R. Weng. 1992. Functional organization of the nociceptive withdrawal reflexes. II. Changes of excitability and receptive fields after spinalization in the rat. Exp Brain Res 90: 469–478.

Schouenborg, J. and J. Kalliomäki. 1990. Functional organization of the nociceptive withdrawal reflexes. I. Activation of hindlimb muscles in the rat. Exp Brain Res 83: 67–78.

Schouenborg, J., H.-R. R. Weng and H. Holmberg. 1994. Modular organization of spinal nociceptive reflexes: A new hypothesis. Physiology 9: 261–265.

Schouenborg, J., H. R. Weng, J. Kalliomäki and H. Holmberg. 1995. A survey of spinal dorsal horn neurones encoding the spatial organization of withdrawal reflexes in the rat. Exp. Brain Res. 106: 19–27.

Schuelert, N. and J. J. McDougall. 2009. Grading of monosodium iodoacetate-induced osteoarthritis reveals a concentration-dependent sensitization of nociceptors in the knee joint of the rat. Neurosci Lett 465: 184–188.

Schwaller, F., C. Kwok and M. Fitzgerald. 2016. Postnatal maturation of the spinal-bulbo-

spinal loop: Brainstem control of spinal nociception is independent of sensory input in neonatal rats. Pain 157: 677–686.

Sear, J. W. and J. D. McGivan. 1981. Metabolism of Alphaxalone in the Rat: Evidence for the Limitation of the Anaesthetic Effect By the Rate of Degradation Through the Hepatic Mixed Function Oxygenase System. Br. J. Anaesth. 53: 417–424.

Sear, J. W. and J. D. McGivan. 1980. The metabolism of alphaxalone by isolated rat hepatocytes. Biochem Pharmacol 29: 248.

Sengul, G. and C. Watson. 2014. Ascending and Descending Pathways in the Spinal Cord. Pp. 115–130 in The Rat Nervous System: Fourth Edition (G. Paxinos, ed). Academic Press, New York, NY:

Sherrington, C. S. 1897. Decerebrate rigidity, and reflex coordination of movements. J Physiol 22: 319–332.

Sherrington, C. S. 1910. Flexion-reflex of the limb, crossed extension-reflex, and reflex stepping and standing. J Physiol 40: 28–121.

Sherwood, N. M., S. A. Chiappa, D. K. Sarkar and G. Fink. 1980. Gonadotropin-Releasing Hormone (GnRH) in Pituitary Stalk Blood from Proestrous Rats: Effects of Anesthetics and Relationship Between Stored and Released GnRH and Luteinizing Hormone. Endocrinology 107: 1410–1417.

Shingu, K., M. Osawa, K. Fukuda and K. Mori. 1985. Acute tolerance to the analgesic action of nitrous oxide does not develop in rats. Anesthesiology 62: 502–504.

Shipston, M. J. 2011. Ion channel regulation by protein palmitoylation. J. Biol. Chem. 286: 8709–8716.

Sinha, S., P. Sinharoy, I. N. Bratz and D. S. Damron. 2015. Propofol causes vasodilation in vivo via TRPA1 ion channels: Role of nitric oxide and BKCa channels. PLoS One 10: e0122189.

Smith, J. A. M., S. M. Amagasu, R. M. Eglen, J. C. Hunter and K. R. Bley. 1998. Characterization of prostanoid receptor-evoked responses in rat sensory neurones. Br. J. Pharmacol. 124: 513–523.

Smith, S. R., B. R. Deshpande, J. E. Collins, J. N. Katz and E. Losina. 2016. Comparative pain reduction of oral non-steroidal anti-inflammatory drugs and opioids for knee osteoarthritis: Systematic analytic review. Osteoarthr. Cartil. 24: 962–972.

Soja, P. J. and J. G. Sinclair. 1983. Evidence that noradrenaline reduces tonic descending inhibition of cat spinal cord nociceptor-driven neurones. Pain 15: 71–81.

Soldin, O. P. and D. R. Mattison. 2009. Sex Differences in Pharmacokinetics and Pharmacodynamics. Clin. Pharmacokinet. 48: 143–157.

Sonner, J. M., J. F. Antognini, R. C. Dutton, P. Flood, A. T. Gray, R. A. Harris, G. E. Homanics, J. Kendig, B. Orser, D. E. Raines, I. J. Rampil, J. Trudell, B. Vissel and E. I. Eger. 2003. Inhaled anesthetics and immobility: mechanisms, mysteries, and minimum alveolar anesthetic concentration. Anesth. Analg. 97: 718–740.

Sorge, R. E., M. L. LaCroix-Fralish, A. H. Tuttle, S. G. Sotocinal, J.-S. Austin, J. Ritchie, M. L. Chanda, A. C. Graham, L. Topham, S. Beggs, M. W. Salter and J. S. Mogil. 2011. Spinal Cord Toll-Like Receptor 4 Mediates Inflammatory and Neuropathic Hypersensitivity in Male But Not Female Mice. J. Neurosci. 31: 15450–15454.

Spadavecchia, C., O. Levionnois, P. Kronen and O. K. Andersen. 2010. The effects of isoflurane minimum alveolar concentration on withdrawal reflex activity evoked by repeated transcutaneous electrical stimulation in ponies. Vet J 183: 337–344.

Stanfa, L. C. and A. H. Dickenson. 2004. In vivo electrophysiology of dorsal-horn neurons. Methods Mol. Med. 99: 139–53.

Staud, R., R. C. Cannon, A. P. Mauderli, M. E. Robinson, D. D. Price and C. J. Vierck. 2003. Temporal summation of pain from mechanical stimulation of muscle tissue in normal controls and subjects with fibromyalgia syndrome. Pain 102: 87–95.

Stein, C., J. D. Clark, U. Oh, M. R. Vasko, G. L. Wilcox, A. C. Overland, T. W. Vanderah and R. H. Spencer. 2009. Peripheral mechanisms of pain and analgesia. Brain Res. Rev. 60: 90–113.

Sugiura, Y., N. Terui and Y. Hosoya. 1989. Difference in distribution of central terminals between visceral and somatic unmyelinated (C) primary afferent fibers. J.

Neurophysiol. 62: 834–840.

Suh, H. H., J. M. Fujimoto and L. L. F. Tseng. 1989. Differential mechanisms mediating β-endorphin- and morphine-induced analgesia in mice. Eur. J. Pharmacol. 168: 61–70.

Suokas, A. K., D. A. Walsh, D. F. McWilliams, L. Condon, B. Moreton, V. Wylde, L. Arendt-Nielsen and W. Zhang. 2012. Quantitative sensory testing in painful osteoarthritis: a systematic review and meta-analysis. Osteoarthr. Cartil. 20: 1075–1085.

Suzuki, R., S. Morcuende, M. Webber, S. P. Hunt and A. H. Dickenson. 2002. Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. Nat. Neurosci. 5: 1319–1326.

Suzuki, R., W. Rahman, S. P. Hunt and A. H. Dickenson. 2004. Descending facilitatory control of mechanically evoked responses is enhanced in deep dorsal horn neurones following peripheral nerve injury. Brain Res. 1019: 68–76.

Svensson, P., C. H. Hashikawa and K. L. Casey. 1999. Site- and modality-specific modulation of experimental muscle pain in humans. Brain Res. 851: 32–38.

Swett, J. E. and C. J. Woolf. 1985. The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. J. Comp. Neurol. 231: 66–77.

Szallasi, A., P. M. Blumberg, L. L. Annicelli, J. E. Krause and D. N. Cortright. 1999. The cloned rat vanilloid receptor VR1 mediates both R-type binding and C-type calcium response in dorsal root ganglion neurons. Mol. Pharmacol. 56: 581–587.

Szallasi, A., F. Cruz and P. Geppetti. 2006. TRPV1: a therapeutic target for novel analgesic drugs? Trends Mol. Med. 12: 545–554.

Tajino, K., H. Hosokawa, S. Maegawa, K. Matsumura, A. Dhaka and S. Kobayashi. 2011. Cooling-sensitive TRPM8 is thermostat of skin temperature against cooling. PLoS One 6: e17504 1-6.

Talley, E. M., L. L. Cribbs, J. H. Lee, A. Daud, E. Perez-Reyes and D. A. Bayliss. 1999. Differential distribution of three members of a gene family encoding low voltage-

activated (T-type) calcium channels. J. Neurosci. 19: 1895–1911.

Tammisto, T., S. Takki, I. Tigerstedt and A. Kauste. 1973. A comparison of althesin and thiopentone in induction of anaesthesia. Br. J. Anaesth. 45: 100–107.

Tanaka, E. 1999. Gender-related differences in pharmacokinetics and their clinical significance. J. Clin. Pharm. Ther. 24: 339–46.

Taylor, A. M. W., J. C. Peleshok and A. Ribeiro-Da-Silva. 2009. Distribution of P2X3-immunoreactive fibers in hairy and glabrous skin of the rat. J. Comp. Neurol. 514: 555–566.

Taylor, P. M. 1998. Effects of surgery on endocrine and metabolic responses to anaesthesia in horses and ponies. Res. Vet. Sci. 64: 133–140.

Taylor, P. M. 1989. Equine stress responses to anaesthesia. Br. J. Anaesth. 63: 702–709.

Teeple, E., G. D. Jay, K. A. Elsaid and B. C. Fleming. 2013. Animal Models of Osteoarthritis: Challenges of Model Selection and Analysis. AAPS J. 15: 438–446.

Teplan, M. 2002. Fundamentals of EEG measurement. Meas. Sci. Rev. 2: 1–11.

Terner, J. M., L. M. Lomas, E. S. Smith, A. C. Barrett and M. J. Picker. 2003. Pharmacogenetic analysis of sex differences in opioid antinociception in rats. Pain 106: 381–91.

Terry, E. L., C. R. France, E. J. Bartley, J. L. Delventura, K. L. Kerr, A. L. Vincent and J. L. Rhudy. 2011. Standardizing procedures to study sensitization of human spinal nociceptive processes: comparing parameters for temporal summation of the nociceptive flexion reflex (TS-NFR). Int J Psychophysiol 81: 263–274.

Tham, S. M., J. A. Angus, E. M. Tudor and C. E. Wright. 2005. Synergistic and additive interactions of the cannabinoid agonist CP55,940 with μ opioid receptor and α2-adrenoceptor agonists in acute pain models in mice. Br. J. Pharmacol. 144: 875–884.

Thut, P. D., D. Wrigley and M. S. Gold. 2003. Cold transduction in rat trigeminal ganglia neurons in vitro. Neuroscience 119: 1071–1083.

Todd, A. J. 2002. Anatomy of primary afferents and projection neurones in the rat spinal dorsal horn with particular emphasis on substance P and the neurokinin 1 receptor.

Exp. Physiol. 87: 245–249.

Todd, A. J. 2010. Neuronal circuitry for pain processing in the dorsal horn. Nat. Rev. Neurosci. 11: 823–836.

Todd, A. J. and R. C. Spike. 1993. The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. Prog. Neurobiol. 41: 609–645.

Todorovic, S. M., V. Jevtovic-Todorovic, S. Mennerick, E. Perez-Reyes, C. F. Zorumski, P. Orestes, D. Bojadzic, J. H. Lee, E. Leach, R. Salajegheh, M. R. DiGruccio, M. T. Nelson, S. M. Todorovic, V. Jevtovic-Todorovic, S. Mennerick, E. Perez-Reyes and C. F. Zorumski. 2001. Cav3.2 Channel is a molecular substrate for inhibition of T-type calcium currents in rat sensory neurons by nitrous oxide. Mol. Pharmacol. 60: 603–610.

Torres-Chávez, K. E., J. M. Sanfins, J. T. Clemente-Napimoga, A. Pelegrini-Da-Silva, C. A. Parada, L. Fischer and C. H. Tambeli. 2012. Effect of gonadal steroid hormones on formalin-induced temporomandibular joint inflammation. Eur. J. Pain 16: 204–216.

Tracey, I. 2010. Getting the pain you expect: mechanisms of placebo, nocebo and reappraisal effects in humans. Nat. Med. 16: 1277–83.

Traub, R. J. and Y. Ji. 2013. Sex differences and hormonal modulation of deep tissue pain. Front. Neuroendocrinol. 34: 350–366.

Traynor, C. and G. M. Hall. 1981. Endocrine and metabolic changes during surgery: Anaesthetic implications. Br. J. Anaesth. 53: 153–160.

Treede, R. D., W. Rief, A. Barke, Q. Aziz, M. I. Bennett, R. Benoliel, M. Cohen, S. Evers, N. B. Finnerup, M. B. First, M. A. Giamberardino, S. Kaasa, E. Kosek, P. Lavand'homme, M. Nicholas, S. Perrot, J. Scholz, S. Schug, B. H. Smith, P. Svensson, J. W. S. Vlaeyen and S. J. Wang. 2015. A classification of chronic pain for ICD-11. Pain 6: 1003–1007.

Tremoleda, J. L., M. Khalil, L. L. Gompels, M. Wylezinska-Arridge, T. Vincent and W. Gsell. 2011. Imaging technologies for preclinical models of bone and joint disorders.

EJNMMI Res. 1: 1–14.

Troyer, H. 1982. Experimental models of osteoarthritis: A review. Semin. Arthritis Rheum. 11: 362–374.

Tsuruoka, M., M. Maeda and T. Inoue. 2004. Persistent hindpaw inflammation produces coeruleospinal antinociception in the non-inflamed forepaw of rats. Neurosci. Lett. 367: 66–70.

Turner, D. M., R. W. Ransom, J. S. Yang and R. W. Olsen. 1989. Steroid anesthetics and naturally occurring analogs modulate the gamma-aminobutyric acid receptor complex at a site distinct from barbiturates. J. Pharmacol. Exp. Ther. 248: 960–966.

Unruh, A. M. 1996. Gender variations in clinical pain experience. Pain 65: 123–167.

Unruh, A. M., J. Ritchie and H. Merskey. 1999. Does gender affect appraisal of pain and pain coping strategies? Clin. J. Pain 15: 31–40.

Urban, B. W. and M. Bleckwenn. 2002. Concepts and correlations relevant to general anaesthesia. Br. J. Anaesth. 89: 3–16.

Urban, M. O. and G. F. Gebhart. 1999. Supraspinal contributions to hyperalgesia. Proc. Natl. Acad. Sci. 96: 7687–7692.

Urban, M. O., P. K. Zahn and G. F. Gebhart. 1999. Descending facilitatory influences from the rostral medial medulla mediate secondary, but not primary hyperalgesia in the rat. Neuroscience 90: 349–352.

Vahle-Hinz, C. and O. Detsch. 2002. What can in vivo electrophysiology in animal models tell us about mechanisms of anaesthesia? Br. J. Anaesth. 89: 123–142.

Vahle-Hinz, C., O. Detsch, C. Hackner and E. Kochs. 2007. Corresponding minimum alveolar concentrations of isoflurane and isoflurane/nitrous oxide have divergent effects on thalamic nociceptive signalling. Br. J. Anaesth. 98: 228–235.

Valtolina, C., J. H. Robben, J. Uilenreef, J. C. Murrell, J. Aspegrén, B. C. McKusick and L. J. Hellebrekers. 2009. Clinical evaluation of the efficacy and safety of a constant rate infusion of dexmedetomidine for postoperative pain management in dogs. Vet. Anaesth. Analg. 36: 369–383.

van Osch, G. J., P. M. van der Kraan and W. B. van den Berg. 1994. Site specific inhibition of cartilage proteoglycan synthesis in the murine knee joint. Differences between 3 metabolic stimuli. J Rheumatol 21: 1107–1112.

van Wijk, G. and D. S. Veldhuijzen. 2010. Perspective on Diffuse Noxious Inhibitory Controls as a Model of Endogenous Pain Modulation in Clinical Pain Syndromes. J. Pain 11: 408–419.

Vanegas, H. and H. G. Schaible. 2004. Descending control of persistent pain: Inhibitory or facilitatory? Brain Res. Rev. 46: 295–309.

Vanegas, H., E. Vazquez and V. Tortorici. 2010. NSAIDS, opioids, cannabinoids and the control of pain by the central nervous system. Pharmaceuticals 3: 1335–1347.

Viala, D. 2006. Evolution and behavioral adaptation of locomotor pattern generators in vertebrates. Comptes Rendus - Palevol 5: 667–674.

Viguier, F., B. Michot, M. Hamon and S. Bourgoin. 2013. Multiple roles of serotonin in pain control mechanisms--implications of 5-$HT_7$ and other 5-HT receptor types. Eur. J. Pharmacol. 716: 8–16.

Villanueva, L., S. W. Cadden and D. Le Bars. 1984. Evidence that diffuse noxious inhibitory controls (DNIC) are mediated by a final post-synaptic inhibitory mechanism. Brain Res. 298: 67–74.

Villanueva, L. and D. Le Bars. 1995. The activation of bulbo-spinal controls by peripheral nociceptive inputs: Diffuse noxious inhibitory controls. Biol. Res. 28: 113–125.

Villanueva, L., M. Peschanski, B. Calvino and D. Le Bars. 1986. Ascending pathways in the spinal cord involved in triggering of diffuse noxious inhibitory controls in the rat. J. Neurophysiol. 55: 34–55.

Villaverde-Morcillo, S., J. Benito, R. García-Sánchez, O. Martín-Jurado and I. a Gómez de Segura. 2014. Comparison of isoflurane and alfaxalone (Alfaxan) for the induction of anesthesia in flamingos (Phoenicopterus roseus) undergoing orthopedic surgery. J. Zoo Wildl. Med. 45: 361–6.

Visser, S. A. G., C. J. G. M. Smulders, B. P. R. Reijers, P. H. Van der Graaf, L. A. Peletier

and M. Danhof. 2002. Mechanism-based pharmacokinetic-pharmacodynamic modeling of concentration-dependent hysteresis and biphasic electroencephalogram effects of alphaxalone in rats. J. Pharmacol. Exp. Ther. 302: 1158–1167.

Volkers, L., Y. Mechioukhi and B. Coste. 2014. Piezo channels: from structure to function. Pflugers Arch. Eur. J. Physiol. 467: 95–99.

Vonsy, J. L., J. Ghandehari and A. H. Dickenson. 2009. Differential analgesic effects of morphine and gabapentin on behavioural measures of pain and disability in a model of osteoarthritis pain in rats. Eur J Pain 13: 786–793.

Voulgaris, D. A., C. M. Egger, M. R. Seddighi, B. W. Rohrbach, L. C. Love and T. J. Doherty. 2013. The effect of nitrous oxide on the minimum alveolar concentration (MAC) and MAC derivatives of isoflurane in dogs. Can. J. Vet. Res. 77: 131–5. Canadian Veterinary Medical Association.

Wakuno, A., M. Aoki, A. Kushiro, N. Mae, T. Maeda, Y. Yamazaki, Y. Minamijima, S. I. Nagata and M. Ohta. 2019. Clinical pharmacokinetics and pharmacodynamics of intravenous alfaxalone in young Thoroughbred horses premedicated with medetomidine and midazolam. Vet. Rec. 184: 411.

Wall, P. D. 1967. The laminar organization of dorsal horn and effects of descending impulses. J Physiol 188: 403–423.

Wang, G., N. Erpelding and K. D. Davis. 2014. Sex differences in connectivity of the subgenual anterior cingulate cortex. Pain 155: 755–763.

Waters, A. J. and B. M. Lumb. 2008. Descending control of spinal nociception from the periaqueductal grey distinguishes between neurons with and without C-fibre inputs. Pain 134: 32–40.

Waters, A. J. and B. M. Lumb. 1997. Inhibitory effects evoked from both the lateral and ventrolateral periaqueductal grey are selective for the nociceptive responses of rat dorsal horn neurones. Brain Res 752: 239–249.

Watt, F. E. and M. Gulati. 2017. New Drug Treatments for Osteoarthritis: What Is on the Horizon? Eur. Med. J. 2: 50–58.

Waxman, D. J. and M. G. Holloway. 2009. Sex Differences in the Expression of Hepatic Drug Metabolizing Enzymes. Mol. Pharmacol. 76: 215–228.

Weerasinghe, N. S., B. M. Lumb, R. Apps, S. Koutsikou and J. C. Murrell. 2014. Objective validation of central sensitization in the rat UVB and heat rekindling model. Eur. J. Pain 18: 1–8.

Weinrich, M. and D. L. Worcester. 2018. The actions of volatile anesthetics: a new perspective. Acta Crystallogr. Sect. D Struct. Biol. 74: 1169–1177. International Union of Crystallography.

Weng, H. R. and J. Schouenborg. 1996. Cutaneous inhibitory receptive fields of withdrawal reflexes in the decerebrate spinal rat. J. Physiol. 493: 253–265.

Wenham, C. Y. J. and P. G. Conaghan. 2010. The role of synovitis in osteoarthritis. Ther. Adv. Musculoskelet. Dis. 2: 349–359.

Werlen, E. and M. W. Jones. 2015. Modulating the map: dopaminergic tuning of hippocampal spatial coding and interactions. Prog. Brain Res. 219: 187–216.

Whelan, G. and P. A. Flecknell. 1994. The use of etorphine/methotrimeprazine and midazolam as an anaesthetic technique in laboratory rats and mice. Lab. Anim. 28: 70–77.

White, K. L., S. Paine and J. Harris. 2017. A clinical evaluation of the pharmacokinetics and pharmacodynamics of intravenous alfaxalone in cyclodextrin in male and female rats following a loading dose and constant rate infusion. Vet. Anaesth. Analg. 44: 865–875.

White, K., M. Targett and J. Harris. 2018. Gainfully employing descending controls in acute and chronic pain management. Vet. J. 237: 16–25.

Whittem, T., K. S. Pasloske, M. C. Heit and M. G. Ranasinghe. 2008. The pharmacokinetics and pharmacodynamics of alfaxalone in cats after single and multiple intravenous administration of Alfaxan at clinical and supraclinical doses. J. Vet. Pharmacol. Ther. 31: 571–579.

Wiech, K., M. Ploner and I. Tracey. 2008. Neurocognitive aspects of pain perception.

Trends Cogn. Sci. 12: 306–313.

Wiech, K. and I. Tracey. 2009. The influence of negative emotions on pain: Behavioral effects and neural mechanisms. Neuroimage 47: 987–994.

Wigdor, S. and G. L. Wilcox. 1987. Central and systemic morphine-induced antinociception in mice: Contribution of descending serotonergic and noradrenergic pathways. J. Pharmacol. Exp. Ther. 242: 90–95.

Williams, J. A. and E. J. M. A. Thonar. 1989. Early osteophyte formation after chemically induced articular cartilage injury. Am. J. Sports Med. 17: 7–15.

Wilson, V. J. 1963. Ipsilateral excitation of extensor motoneurones. Nature 198: 290–291.

Wolfensohn, S. and M. Lloyd. 2013. Handbook of Laboratory Animal Management and Welfare. 4th Ed. Wiley Blackwell, Oxford, UK.

Woo, S.-H., V. Lukacs, J. C. De Nooij, D. Zaytseva, C. R. Criddle, A. Francisco, T. M. Jessell, K. A. Wilkinson, A. Patapoutian and N. N. Author. 2015. Piezo2 is the principal mechanotransduction channel for proprioception. Nat Neurosci 18: 1756–1762.

Wood, J. N., J. P. Boorman, K. Okuse and M. D. Baker. 2004. Voltage-gated sodium channels and pain pathways. J. Neurobiol. 61: 55–71.

Wood, P. B. 2008. Role of central dopamine in pain and analgesia. Expert Rev. Neurother. 8: 781–797.

Woodbury, C. J., M. Zwick, S. Wang, J. J. Lawson, M. J. Caterina, M. Koltzenburg, K. M. Albers, H. R. Koerber and B. M. Davis. 2004. Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. J. Neurosci. 24: 6410–6415.

Woodbury, J. C., A. M. Ritter and H. R. Koerber. 2001. Central anatomy of individual rapidly adapting low-threshold mechanoreceptors innervating the ‘Hairy’ skin of newborn mice: Early maturation of hair follicle afferents. J. Comp. Neurol. 436: 304–323.

Woolf, C. J. 2011. Central sensitization : Implications for the diagnosis and treatment of pain. Pain 152: S2–S15.

Woolf, C. J. and A. E. King. 1990. Dynamic alterations in the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat spinal cord. J. Neurosci. 10: 2717–2726.

Wrzosek, A., J. Woron, J. Dobrogowski, J. Jakowicka-Wordliczek and J. Wordliczek. 2015. Topical clonidine for neuropathic pain. Cochrane Database Syst. Rev. CD010967.

Xu, M., V. K. Kontinen and E. Kalso. 1999. Endogenous noradrenergic tone controls symptoms of allodynia in the spinal nerve ligation model of neuropathic pain. Eur. J. Pharmacol. 366: 41–45.

Yaksh, T. L., J. W. Pogrel, Y. W. Lee and S. R. Chaplan. 1995. Reversal of nerve ligation-induced allodynia by spinal alpha-2 adrenoceptor agonists. J. Pharmacol. Exp. Ther. 272: 207–214.

Yarnitsky, D. 2010. Conditioned pain modulation (the diffuse noxious inhibitory control-like effect): its relevance for acute and chronic pain states. Curr. Opin. Anaesthesiol. 23: 611–615.

Yarnitsky, D., L. Arendt-Nielsen, D. Bouhassira, R. R. Edwards, R. B. Fillingim, M. Granot, P. Hansson, S. Lautenbacher, S. Marchand and O. Wilder-Smith. 2010. Recommendations on terminology and practice of psychophysical DNIC testing. Eur. J. Pain 14: 339.

Yarnitsky, D., D. Bouhassira, A. M. Drewes, R. B. Fillingim, M. Granot, P. Hansson, R. Landau, S. Marchand, D. Matre, K. B. Nilsen, A. Stubhaug, R. D. Treede and O. H. G. Wilder-Smith. 2015. Recommendations on practice of conditioned pain modulation (CPM) testing. Eur. J. Pain 19: 805–806.

Yarnitsky, D., Y. Crispel, E. Eisenberg, Y. Granovsky, A. Ben-Nun, E. Sprecher, L. A. Best and M. Granot. 2008. Prediction of chronic post-operative pain: Pre-operative DNIC testing identifies patients at risk. Pain 138: 22–28.

Yarnitsky, D., M. Granot, H. Nahman-Averbuch, M. Khamaisi and Y. Granovsky. 2012. Conditioned pain modulation predicts duloxetine efficacy in painful diabetic

neuropathy. Pain 153: 1193–1198.

Yasaka, T., S. Y. X. Tiong, D. I. Hughes, J. S. Riddell and A. J. Todd. 2010. Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. Pain 151: 475–488.

Yawno, T., M. Mortale, A. E. Sutherland, G. Jenkin, E. M. Wallace, D. W. Walker and S. L. Miller. 2014. The effects of betamethasone on allopregnanolone concentrations and brain development in preterm fetal sheep. Neuropharmacology 85: 342–8.

You, H.-J., D.-Y. Cao, B. Yuan and L. Arendt-Nielsen. 2006. Sex differences in the responses of spinal wide-dynamic range neurons to subcutaneous formalin and in the effects of different frequencies of conditioning electrical stimulation. Neuroscience 138: 1299–1307.

Zambricki, E. a. and L. G. D'Alecy. 2004. Rat Sex Differences in Anesthesia. Comp. Med. 54: 49–53.

Zhang, C., M. F. Davies, T. Z. Guo and M. Maze. 1999. The analgesic action of nitrous oxide is dependent on the release of norepinephrine in the dorsal horn of the spinal cord. Anesthesiology 91: 1401–1407.

Zhang, R. X., K. Ren and R. Dubner. 2013. Osteoarthritis pain mechanisms: Basic studies in animal models. Osteoarthr. Cartil. 21: 1308–1315.

Zhang, W., K. Zou and M. Doherty. 2015. Placebos for knee osteoarthritis: Reaffirmation of 'Needle is better than pill'. Ann. Intern. Med. 163: 392–393.

Zhang, X., R. -R Ji, S. Nilsson, M. War, R. Ubink, G. Ju, Z. Wiesenfeld-Hallin and T. Hökfelt. 1995. Neuropeptide Y and Galanin Binding Sites in Rat and Monkev Lumbar Dorsal Root Ganalia and Spinal Cord and Effect of Peripheral Axotomy. Eur. J. Neurosci. 7: 367–380.

Zhang, X., G. Ju, R. Elde and T. Hökfelt. 1993. Effect of peripheral nerve cut on neuropeptides in dorsal root ganglia and the spinal cord of monkey with special reference to galanin. J. Neurocytol. 22: 342–381.

Zhang, Y. Q., X. Gao, Y. L. Huang and G. C. Wu. 2000. Expression of 5-HT1A receptor mRNA in rat dorsal raphe nucleus and ventrolateral periaqueductal gray neurons after peripheral inflammation. Neuroreport 11: 3361–5.

Zhou, H. H., T. T. Jin, B. Qin and H. Turndorf. 1998. Suppression of spinal cord motoneuron excitability correlates with surgical immobility during isoflurane anesthesia. Anesthesiology 88: 955–961.

Zhuo, M. 2016. Neural Mechanisms Underlying Anxiety-Chronic Pain Interactions. Trends Neurosci. 39: 136–145.

Zhuo, M. and G. Gebhart. 1990a. Characterization of descending inhibition and facilitation from the nuclei reticularis-gigantocellularis and gigantocellularis pars alpha in the Rat. Pain 42: 337–350.

Zhuo, M. and G. E. Gebhart. 1990b. Spinal cholinergic and monoaminergic receptors mediate descending inhibition from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. Brain Res 535: 67–78.

Zhuo, M. and G. F. Gebhart. 1997. Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat. J. Neurophysiol. 78: 746–758.

Zhuo, M. and G. F. Gebhart. 1991. Spinal serotonin receptors mediate descending facilitation of a nociceptive reflex from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. Brain Res 550: 35–48.

Zimmermann, K., A. Leffler, A. Babes, C. M. Cendan, R. W. Carr, J. I. Kobayashi, C. Nau, J. N. Wood and P. W. Reeh. 2007. Sensory neuron sodium channel Nav1.8 is essential for pain at low temperatures. Nature 447: 855–858.

Zimmermann, K., J. K. Lennerz, A. Hein, A. S. Link, J. Stefan Kaczmarek, M. Delling, S. Uysal, J. D. Pfeifer, A. Riccio and D. E. Clapham. 2011. Transient receptor potential cation channel, subfamily C, member 5 (TRPC5) is a cold-transducer in the peripheral nervous system. Proc. Natl. Acad. Sci. U. S. A. 108: 18114–18119.

Zubieta, J.-K., J. A. Bueller, L. R. Jackson, D. J. Scott, Y. Xu, R. A. Koeppe, T. E. Nichols and C. S. Stohler. 2005. Placebo Effects Mediated by Endogenous Opioid Activity on

μ-Opioid Receptors. J. Neurosci. 25: 7754–7762.

Zucker, I. and A. K. Beery. 2010. Males still dominate animal studies. Nature 465: 690.

Zuniga, J., S. Joseph and K. Knigge. 1987. Nitrous oxide analgesia: partial antagonism by naloxone and total reversal after periaqueductal gray lesions in the rat. Eur. J. Pharmacol. 142: 51–60.

Zuniga, J. R., K. K. Knigge and S. A. Joseph. 1986. Central beta-endorphin release and recovery after exposure to nitrous oxide in rats. J. Oral Maxillofac. Surg. 44: 719–723.

# APPENDICES

## Appendix 1. Haematology and biochemistry for male and female rats (8–12) weeks

| Test (Unit) | N | Mean | Range |
|---|---|---|---|
| Erythrocytes, $10^6/\mu l$ | 549 | 7.96 | 7.77 - 8.19 |
| Hematocrit, % | 549 | 43.93 | 41.20 - 47.30 |
| Hemoglobin, g/dl | 549 | 15.07 | 14.40 - 16.00 |
| MCHC, % | 544 | 34.38 | 32.70 - 35.70 |
| MCH, pg | 544 | 18.96 | 18.30 - 20.00 |
| MCV, fl | 544 | 55.17 | 53.00 - 59.50 |
| Leukocytes, $10^3/\mu l$ | 549 | 11.57 | 10.09 - 14.01 |
| Neutrophils, % | 529 | 9.89 | 8.20 - 14.50 |
| Neutrophils, $10^3/\mu l$ | 529 | 1.18 | 0.91 - 1.70 |
| Lymphocytes, % | 529 | 84.39 | 80.20 - 86.00 |
| Lymphocytes, $10^3/\mu l$ | 529 | 9.73 | 8.50 - 12.10 |
| Monocytes, % | 529 | 2.58 | 2.10 - 3.40 |
| Monocytes, $10^3/\mu l$ | 529 | 0.28 | 0.10 - 0.46 |
| Eosinophils, % | 529 | 1.20 | 1.00 - 1.50 |
| Eosinophils, $10^3/\mu l$ | 529 | 0.13 | 0.10 - 0.16 |
| Basophils, % | 529 | 0.48 | 0.30 - 1.00 |
| Basophils, $10^3/\mu l$ | 529 | 0.05 | 0.00 - 0.10 |
| Large Unstained Cells, % | 529 | 1.68 | 1.10 - 2.80 |
| Large Unstained Cells, $10^3/\mu l$ | 215 | 0.18 | 0.14 - 0.27 |
| Platelets, $10^3/\mu l$ | 260 | 792.00 | 379 - 967 |

Summary of haematology, historical control data from male Sprague Dawley rats, 8-12 weeks of age (Charles River)

| Test (Unit) | N | Mean | Range |
|---|---|---|---|
| Total Protein, g/dl | 723 | 6.16 | 5.90 - 6.60 |
| A/G Ratio | 693 | 1.42 | 1.10 - 2.70 |
| Albumin, g/dl | 724 | 3.59 | 3.30 - 4.60 |
| Alkaline Phosphatase, IU/l | 719 | 160.00 | 136.00 - 188.00 |
| Total Bilirubin, mg/dl | 723 | 0.55 | 0.10 - 1.00 |
| Urea Nitrogen, mg/dl | 724 | 14.30 | 13.00 - 16.00 |
| Creatinine, mg/dl | 724 | 0.54 | 0.50 - 0.60 |
| Glucose, mg/dl | 724 | 146.30 | 112.00 - 176.00 |
| Alanine Aminotransferase, IU/l | 725 | 34.40 | 28.00 - 40.00 |
| Aspartate Aminotransferase, IU/l | 725 | 100.60 | 87.00 - 114.00 |
| Creatine Kinase, IU/l | 724 | 362.00 | 344.00 - 380.00 |
| Lactate Dehydrogenase, IU/l | 724 | 389.00 | 360.00 - 418.00 |
| Gamma glutamyl Transpeptidase, IU/l | 579 | 0.50 | 0.00 - 1.00 |
| Calcium, mg/dl | 718 | 10.04 | 9.40 - 11.00 |
| Chloride, meq/l | 720 | 104.00 | 102.00 - 105.00 |
| Phosphorus, mg/dl | 672 | 8.11 | 7.30 - 10.00 |
| Potassium, meq/l | 725 | 5.15 | 4.70 - 6.20 |
| Sodium, meq/l | 725 | 144.00 | 141.00 - 150.00 |
| Total Cholesterol, mg/dl | 709 | 61.70 | 54.00 - 74.00 |
| Triglycerides, mg/dl | 653 | 73.00 | 61.00 - 99.00 |

Summary of serum biochemistry, historical control data from male Sprague Dawley rats, 8-12 weeks of age (Charles River)

| Test (Unit) | N | Mean | Range |
|---|---|---|---|
| Erythrocytes, $10^6/\mu l$ | 554 | 7.53 | 7.21 - 7.92 |
| Hematocrit, % | 554 | 38.67 | 9.60 - 46.00 |
| Hemoglobin, g/dl | 554 | 14.53 | 13.70 - 15.70 |
| MCHC, % | 551 | 34.90 | 32.80 - 36.20 |
| MCH, pg | 551 | 19.30 | 18.60 - 20.00 |
| MCV, fl | 551 | 55.35 | 53.60 - 58.10 |
| Leukocytes, $10^3/\mu l$ | 554 | 9.17 | 7.44 - 10.70 |
| Neutrophils, % | 554 | 8.93 | 6.80 - 13.00 |
| Neutrophils, $10^3/\mu l$ | 554 | 0.85 | 0.54 - 1.40 |
| Lymphocytes, % | 554 | 85.94 | 82.30 - 88.40 |
| Lymphocytes, $10^3/\mu l$ | 554 | 7.89 | 6.42 - 9.47 |
| Monocytes, % | 554 | 2.29 | 1.90 - 3.10 |
| Monocytes, $10^3/\mu l$ | 554 | 0.19 | 0.10 - 0.24 |
| Eosinophils, % | 554 | 1.18 | 1.00 - 1.50 |
| Eosinophils, $10^3/\mu l$ | 554 | 0.10 | 0.09 - 0.12 |
| Basophils, % | 554 | 0.35 | 0.30 - 0.40 |
| Basophils, $10^3/\mu l$ | 554 | 0.03 | 0.00 - 0.05 |
| Large Unstained Cells, % | 232 | 1.34 | 1.00 - 1.70 |
| Large Unstained Cells, $10^3/\mu l$ | 257 | 0.13 | 0.11 - 0.17 |
| Platelets, $10^3/\mu l$ | 554 | 875.90 | 797 - 1112 |

Summary of haematology, historical control data from female Sprague Dawley rats, 8-12 weeks of age (Charles River)

| Test (Unit) | N | Mean | Range |
|---|---|---|---|
| Total Protein, g/dl | 724 | 6.36 | 6.10 - 7.00 |
| A/G Ratio | 724 | 1.61 | 1.30 - 2.90 |
| Albumin, g/dl | 724 | 3.88 | 3.50 - 5.10 |
| Alkaline Phosphatase, IU/l | 695 | 113.30 | 90.00 - 147.00 |
| Total Bilirubin, mg/dl | 724 | 0.60 | 0.20 - 1.00 |
| Urea Nitrogen, mg/dl | 724 | 13.40 | 11.00 - 16.00 |
| Creatinine, mg/dl | 724 | 0.59 | 0.50 - 0.60 |
| Glucose, mg/dl | 724 | 160.30 | 113.00 - 185.00 |
| Alanine Aminotransferase, IU/l | 724 | 29.00 | 25.00 - 36.00 |
| Aspartate Aminotransferase, IU/l | 724 | 96.40 | 85.00 - 123.00 |
| Creatine Kinase, IU/l | 364 | 443.00 | 420.00 - 466.00 |
| Lactate Dehydrogenase, IU/l | 354 | 411.50 | 401.00 - 422.00 |
| Gamma glutamyl Transpeptidase, IU/l | 579 | 0.20 | 0.00 - 0.40 |
| Calcium, mg/dl | 724 | 10.08 | 9.50 - 11.00 |
| Chloride, meq/l | 723 | 105.40 | 103.00 - 107.00 |
| Phosphorus, mg/dl | 674 | 6.98 | 6.20 - 9.10 |
| Potassium, meq/l | 723 | 4.78 | 4.20 - 6.10 |
| Sodium, meq/l | 723 | 143.00 | 141.00 - 149.00 |
| Total Cholesterol, mg/dl | 708 | 75.20 | 67.00 - 87.00 |
| Triglycerides, mg/dl | 642 | 59.60 | 42.00 - 74.00 |

Summary of serum biochemistry, historical control data from female Sprague Dawley rats, 8-12 weeks of age (Charles River)

## Appendix 2. Abstracts presented at international conferences

17th IASP World congress on Pain (12th-16th September 2018, Boston Massachusetts, USA)

**Effect of nitrous oxide preconditioning on diffuse noxious inhibitory controls of hindlimb nociceptive withdrawal reflexes in control, osteoarthritic and Lewis rats**

White, K.L., Simmonds, V. and Harris, J.

17th IASP World congress on Pain (12th-16th September 2018, Boston Massachusetts, USA)

**Differential effects of alfaxalone and isoflurane on diffuse noxious inhibitory controls of hind limb nociceptive withdrawal reflexes in the rat**

White, K.L. and Harris, J.

Association of Veterinary Anaesthetists Spring Conference (10-13th March 2018, Grenada, West Indies)

**Differential effects of alfaxalone and isoflurane on diffuse noxious inhibitory controls of hind limb nociceptive withdrawal reflexes in the precollicular decerebrated rat**

White, K.L. and Harris, J.

Association of Veterinary Anaesthetists Spring Conference (10-13th March 2018, Grenada, West Indies)

**Mechanical nociceptive threshold (MNT) testing in rats: effects of probe tip configuration and cage floor characteristics for electronic von Frey (EvF) compared to traditional filaments (Fil)**

White, K.L., Taylor P.M. and Harris, J.

16th IASP World congress on Pain (26th- 30th September 2016, Yokohama, Japan)

**Differential effect of nitrous oxide preconditioning on diffuse noxious inhibitory controls of hindlimb nociceptive withdrawal reflexes in the rat**

White, K.L. and Harris, J.

**Appendix 3. List of published peer reviewed papers**

White, K. L., S. Paine and J. Harris. 2017. A clinical evaluation of the pharmacokinetics and pharmacodynamics of intravenous alfaxalone in cyclodextrin in male and female rats following a loading dose and constant rate infusion. Vet. Anaesth. Analg. 44: 865–875.

White, K., M. Targett and J. Harris. 2018. Gainfully employing descending controls in acute and chronic pain management. Vet. J. 237: 16–25.