Understanding the role of Neuregulin 1-erbB signalling in microglia in the context of neuropathic pain.

Sanam Sikander

Green Templeton College

University of Oxford

Supervisor: Professor Dr. David Bennett

Thesis submitted for the degree of Doctor of Philosophy

Trinity Term 2015

Abstract

Understanding the role of Neuregulin1-erbB signalling in microglia in the context of neuropathic pain.

Sanam Sikander, Green Templeton College, University of Oxford

Submitted for the degree of Doctor of Philosophy, Trinity Term 2015

Neuregulin 1 (NRG1) is a growth factor required for peripheral nerve development and functional recovery following nerve injury. However, its importance in regulating neuropathic pain via microglial signaling remains unclear. Previous pharmacological studies suggest NRG1 regulates microglial proliferation, mechanical allodynia and cold hypersensitivity through binding to extracellular tyrosine kinase receptors (e.g. erbB2, erbB3 and erbB4) found on microglia.

The aim of this thesis was to further dissect the role of NRG1 in regulating pain behaviour during neuropathic pain by using transgenic systems that conditionally ablate NRG1 expression in adulthood or erbB receptor expression specifically within microglia. In our hands it was determined that the CX3CR1 Cre is more efficient than the Cd11b Cre system in effectively targeting tissue specific gene ablation.

Using animal models of nerve injury, gene expression analysis showed that NRG1 and erbB gene expression levels are dysregulated in the peripheral nervous system and the spinal cord, in neuropathic pain models. A novel cold pain behaviour assay was optimised to measure cold pain behaviour. With conditional NRG1 ablation, and the use of the spared nerve injury model, it was determined that NRG1 regulates cold hypersensitivity in the delayed stages of nerve injury but does not regulate mechanical hypersensitivity or attenuate microglial proliferation. Similarly, conditional ablation of erbB3 and erbB4 receptors in microglia suggests that the NRG1-erbB signaling pathway does not regulate mechanical hypersensitivity or microglial proliferation. However, NRG1-erbB signaling does regulate cold hypersensitivity in the delayed stages of nerve injury through microglia. The work presented in this thesis has further refined the role of the NRG1-erbB signaling pathway in the context of peripheral nerve injury neuropathic pain.

Declaration

The work presented in this thesis was conducted by the author (Sanam Sikander) unless stated otherwise. The research was carried out during 2011-2014 at the WOLFSON Centre for Age related diseases, Guys Campus, Kings College London, London and the Nuffield Department of Clinical Neuroscience, John Radcliffe Hospital, University of Oxford, Oxford. This work was funded by the Wellcome Trust. No part of this thesis has been submitted for any other degree at this or any other university or institute of learning.

Acknowledgements

I would like to acknowledge my colleagues, friends and family who have supported me over the past four years in my PhD studies and have made it such a memorable and life changing experience.

First and foremost, I would like to thank my supervisor Dr. David Bennett. You are a true inspiration and have made a long-lasting impression on me. You have taught me many skills both inside and outside of the laboratory. You have taught me to remain optimistic, passionate and opportunistic at the adversities faced over the years. Thank you for your infallible support, guidance, enthusiasm and patience with me. Thank you for taking me under your wing and making the last few years a pleasant experience. In particular, thank you for your thought provoking conversations and guidance on the preparation of this thesis.

Thank you Dr. Florence (Rose) Fricker, Dr. Margarita Calvo, and Dr. Ning Zhu. Margarita, you are a great inspiration and infected me with your enthusiasm for this research project. Rose, you helped the transition from Kings College London to Oxford a lot easier. Thank you for being my informal mentor and taking the time to teach me new skills and for all the exciting conversations about the Great British Bake Off and Masterchef. Ning, where would I be without you? The research group could simply not function without you. Thank you for support, both in the office and the laboratory, thank you for being a great friend. In particular, thank you to Rose, Margarita and Ning for teaching me how to conduct surgery, rodent behaviour, histology and generating transgenic colonies (Chapter 4, 5 and 6).

Dr. John Dawes and Dr. Alex Clark – it has been an absolute pleasure meeting you and working with you over the past few years. I enjoyed every day that I spent learning from you. John, thank you for teaching me quantitative PCR and for your support though the behavioural and histological studies (Chapter 2 and 3). Working with you has always been an entertaining experience. Alex, thank you for your support and advice on the *in vitro* cultures and solving my nonsensical calculations (Chapter 5 and 6). You both taught me to be confident and to never give up. Thank

you John and Alex for your everlasting patience, your infectious enthusiasm and for including me in all the sport related activities.

Dr. Jorge Galino and Dr. Ilaria Cervillini – how I will miss our many “lost in translation” conversations. I thoroughly enjoyed deciphering your Spanglish and Italian-English conversations. Each and every day I enjoyed learning from you both. Thank you Jorge and Ilaria for your educational discussions and advice on experimental design. In particular, thank you for your support in the generation of transgenic mice, histology and western blotting experiments (Chapter 6). Your companionship will be deeply missed.

A special thanks to Steven West, whom I met before starting my PhD studies. In particular, thank you for teaching me histology and for your assistance in confocal microscopy. I really valued your insightful conversations and suggestions in improving my experimental designs. Thank you for your time and patience in helping me whilst you also attempting to complete your PhD studies.

The camaraderie within the Bennett Lab will be sorely missed. Thank you to Dr. Andreas Themistocleous. Meeting you has taught me so much more about life beyond academic research. I thoroughly enjoyed your cat videos, our yoga sessions and weekend brunch(s) around Oxford. Thank you to the present lab members Annina, Juan, Greg, Simon, and Lucy for making it a great environment to work in. Many thanks go to the students, Thomas, Liam and Dominique for their assistance. Thank you Dominique for your assistance in Chapter 6 behavioural studies.

My close friends outside of the lab have also been a great support throughout my PhD. They have listened to all high’s and low’s and always managed to cheer me up. Thank you Meghan, Nick, Ify, Jess, and Zee. You were sorely missed when I moved to Oxford. Thank you for being my good friends, for your patience and understanding when I wasn't able to visit often. Thank you for all the memorable adventures that we had.

My parents cannot go without mention. Ammi and Abu, I am ever so grateful for your faith in me. Thank you for educating me, instilling such drive and determination to

work hard and succeed. My brother Iqbal and sisters Saba, Hira and Bhabi – thank you for my ‘planning my wedding’. I simply could not have fulfilled both my academic and personal ambitions without you. You were my greatest allies but also my toughest critics. Sorry I couldn't’ visit more often. Zara, you are the greatest new addition and motivation to complete my studies so that I can finally come to meet you.

Finally but most importantly, I wish to thank my Husband Suleman (and his family). Suleman, I cannot begin to express my utmost gratitude for you. You have moved around the country with me, sacrificed our holidays, and gone hungry many a ‘nights because I was in the library. Thank you for motivating me to make that ‘one last push’ and complete my studies. You have been my greatest companion and showered me with your with kindness, tough love and friendship. Alhamdulillah, I am ever so grateful to each and every one of you.

This thesis is dedicated to my late Nanni Ammi, Kaneez Akhter (1944 – 2011), Nanna Abu, Raja Zafar Ali Khan (1934 – 2015), Daddy Ammi and Dadda Abu.

TABLE OF CONTENTS

1 Introduction 25

1.1 Neuropathic pain 25

1.1.1 Prevalence of neuropathic pain 25

1.2 Measuring pain related behaviour in animals 27

1.3 Models of neuropathic pain in animals 31

1.3.1 Peripheral nerve injury models 31

1.4 Mechanisms of Neuropathic pain 32

1.4.1 Peripheral sensitisation 32

1.4.2 Peripheral nerve and DRG ectopic changes after injury 33

1.4.3 Spinal cord: Central sensitisation and disinhibition 34

1.4.4 Descending modulation by the brainstem 37

1.4.5 Immune cell contribution to neuropathic pain 37

1.5 Neuro-immune interactions in the CNS 40

1.5.1 Astrocytes 40

1.5.2 Oligodendrocytes 41

1.5.3 Microglia 42

1.5.4 Are microglia always bad? 60

1.6 Neuregulins 62

1.6.1 Isoforms 62

1.6.2 Proteolytic cleavage 66

1.6.3 NRG1 Signaling through extracellular tyrosine kinase (erbB) receptors 66

1.6.4 Paracrine and juxtacrine Signaling 66

1.6.5 ‘Forward Signaling’ 67

1.6.6 ‘Backward Signaling’ 68

1.6.7 Neuregulin-1 function in the Peripheral Nervous System 68

1.7 NRG1/erbB signaling in the CNS 70

1.7.1 NRG1/erbB signaling in microglia 70

1.7.2 Limitations to using pharmacological approaches to understand NRG1- erbB signalling 72

1.7.3 Conditional transgenic mice 75

1.7.4 Inducible Transgenic Cre systems 76

1.8 Thesis Aims 78

2 Investigating changes in Neuregulin-1 and extracellular tyrosine kinase (erbB) receptor gene expression after peripheral nerve injury in mice. 79

2.1 Introduction 79
2.2 Methods 80
2.2.1 Animals 80
2.2.2 Experimental animals 80
2.2.3 Surgery 81
2.2.4 Animal behavioural experiments 81
2.2.5 Tissue extraction 82
2.2.6 Quantitative PCR 83
2.2.7 Statistical Analysis 85
2.3 Results 86
2.3.1 Mice develop significant mechanical hypersensitivity after nerve injury 86
2.3.2 Mice develop significant thermal hypersensitivity after nerve injury 86
2.3.3 Up-regulation in mRNA expression of injury markers is detected after injury 88
2.3.4 NRG1 mRNA expression is dysregulated after peripheral nerve injury 93
2.3.5 NRG2 and NRG3 mRNA expression levels remain unchanged in the spinal cord and DRG after nerve injury 96
2.3.6 ErbB mRNA gene expression levels are dysregulated after nerve injury 99
2.4 Discussion 103
2.4.1 Altered expression of NRG1 isoforms and their receptors in the injured nerve 103
2.4.2 Altered gene expression of NRG1 isoforms and their receptors in the injured DRG 106
2.4.3 Altered expression of NRG1 isoforms and their receptors in the injured spinal cord 108
2.4.4 Technical Limitations 112
2.5 Considerations 113
3 Using the Place Preference Assay to measure thermal and cold hypersensitivity in rodents 114
3.1 Introduction 114
3.2 Methods 116
3.2.1 Animals 116
3.2.2 Experimental Animals 116
3.2.3 Surgery 117
3.2.4 Mexilitine treatment to SCN9A$^{F1449V/+}$mice 117
3.2.5 Behavioural Assays 117
3.2.6 Cold Place Preference 117

3.2.7 Statistical analysis 119
3.3 Results 120
3.3.1 Animals spend approximately 30% of their time at 16°C versus room temperature (22°C) 120
3.3.2 The 16°C vs. 22°C paradigm demonstrates that animals develop cold hypersensitivity after peripheral nerve injury 122
3.3.3 SCN9A$^{F1449V-/+}$ animals exhibit heat hypersensitivity to 37°C 125
3.3.4 Mexilitine is capable of attenuating thermal temperature preference differences measured between the SCN9A$^{F1449V+/+}$ WT and SCN9A$^{F1449V-/+}$ (Het) cohorts 129
3.4 Discussion 132
3.4.1 Limitations with current assays used to measure cold hypersensitivity 133
3.5 Conclusion 138

4 NRG1 ablation attenuates cold hypersensitivity after nerve injury but does not attenuate mechanical hypersensitivity 139

4.1 Introduction 139
4.2 Methods 141
4.2.1 Animals 141
4.2.2 Tamoxifen administration 142
4.2.3 Surgery 143
4.2.4 Immunohistochemistry 143
4.2.5 Antibodies 143
4.2.6 Image analysis 144
4.2.7 Animal Behavioural Tests 144
4.2.8 Quantitative PCR 146
4.2.9 Statistical Analysis 147
4.3 **Results** 148
4.3.1 NRG1 mRNA levels are significantly down regulated using the Tamoxifen inducible CAG-CreER^{TM} system 148
4.3.2 Mechanical hypersensitivity is not attenuated with conditional NRG1$^{-/-}$ knockdown 150
4.3.3 Thermal hypersensitivity post nerve injury is not altered in NRG1 $^{-/-}$ animals 152
4.3.4 NRG1$^{-/-}$ animals develop delayed recovery of motor coordination (proprioception) after peripheral nerve injury 154
4.3.5 The Cold Plate assay suggests that cold hypersensitivity is reduced in Global NRG1$^{-/-}$ animals after peripheral nerve injury 157

4.3.6 The Cold Place Preference assay also demonstrates that cold hypersensitivity is attenuated in NRG1$^{-/-}$ animals after peripheral nerve injury 159
4.3.7 Microgliosis is not down regulated after injury with the conditional ablation of NRG1$^{-/-}$ 162
4.3.8 Astrocyte proliferation is not attenuated after nerve injury with global NRG1 knockdown 164
4.4 **DISCUSSION** 166
4.4.1 NRG1 ablation impairs motor function in the naïve state which is accentuated following nerve injury. 166
4.4.2 Studying the role of NRG1 in the development of pain related hypersensitivity following peripheral nerve injury in the mouse 168
4.4.3 Other erbB ligands may be capable of activating intracellular signaling in microglia to induce microglial mediated mechanical hypersensitivity 171
4.4.4 NRG1 release after nerve injury could regulate cold allodynia 174
4.4.5 Does NRG1 play a role in astrocytes? 175
4.5 Considerations 176
5 Targeting the selective ablation of erB3 and erbB4 in the myeloid lineage in order to investigate the role of NRG-1-erbB signaling after peripheral nerve injury 177
5.1 Introduction 177
5.2 Methods 179
5.2.1 Animals 179
5.2.2 Tamoxifen dosing 182
5.2.3 Surgery 183
5.2.4 Tissue extraction 183
5.2.5 Polymerase chain reaction: Genotyping 185
5.2.6 Histology 187
5.2.7 Image analysis 188
5.2.8 Animal Behavioural Tests 188
5.2.9 Statistical Analysis 190
5.3 Results 191
5.3.1 ErbB3 and erbB4 mRNA levels are not down-regulated in the Cre (+) group 191
5.3.2 ErbB4 protein is not suppressed in the conditional knockout group. 194
5.3.3 The R26$^{tdTomato\ (+)}$ strain is a highly efficient reporter strain 195
5.3.4 Cd11b Cre is inefficient in effectively targeting microglial cells for Cre-mediated recombination 197

5.3.5 Mechanical and thermal reflex withdrawal thresholds do not differ between the Cre (-) and Cre (+) group 200
5.3.6 Cold pain (8ºC) thresholds do not differ between the Cre (-) and Cre (+) group. 203
5.3.7 Motor function is normal between the Cre (-) and Cre (+) groups 205
5.3.8 There is no change in microglial number or morphology between the Cre (-) and Cre (+) genotype 206
5.4 Discussion 208
5.4.1 The Cd11b Cre strain is an inefficient tool that fails to effectively ablate the erbB3 and erbB4 gene in monocytes. 208
5.4.2 Cd11b Cre shows poor Cre activity within microglia in our hands and exhibits low levels of gene recombination 210
5.4.3 The CX3CR1-Cre strain can be used as a microglial specific Cre to target erbB3 and erbB4 on microglia. 212
5.5 Considerations 212

6 Using CX3CR1 CreERTM to selectively ablate erbB3 and erbB4 expression in microglia to understand NRG1-erbB signaling in neuropathic pain 213

6.1 Introduction 213
6.2 Methods 215
6.2.1 Animals 215
6.2.2 *In vivo* Tamoxifen treatment 217
6.2.3 Surgery 217
6.2.4 Tissue extraction 217
6.2.5 Quantitative PCR 218
6.2.6 Immunohistochemistry 218
6.2.7 Antibodies 219
6.2.8 Image analysis 219
6.2.9 Animal Behavioural Tests 220
6.2.10 Statistical Analysis 221
6.3 Results 222
6.3.1 YFP expression is clearly identified in CX3CR1 CreER™ (+): erbB3$^{fl/fl}$B4$^{fl/fl}$ microglia 222
6.3.2 The CX3CR1CreER™ strain can be used to target gene ablation in microglia 225
6.3.3 ErbB3 and erbB4 mRNA gene expression levels are significantly reduced using the Tamoxifen inducible CX3CR1 CreER™ system. 230

6.3.4 ErbB2 mRNA levels are significantly upregulated when erbB3 and erbB4 receptors are down-regulating using Tamoxifen. 232
6.3.5 Mechanical hypersensitivity is not attenuated with erbB3 and erbB4 receptor knock-down 234
6.3.6 Thermal hypersensitivity is not attenuated in animals that lack erbB3 and erbB4 receptors on microglia 236
6.3.7 Proprioception is not affected by erbB3 and erbB4 receptor knockdown in microglia 238
6.3.8 Cold hypersensitivity is regulated by NRG1-erbB signaling in microglia 240
6.3.9 Microglial numbers are not attenuated after SNI with erbB3 and erbB4 gene ablation 243
6.3.10 Astrocyte numbers are not attenuated after SNI, with erbB3 an erbB4 ablation in microglia. 245
6.4 Discussion 247
6.4.1 CX3CR1 CreER™ is highly specific to microglia and can be used to distinguish the differences between macrophages and microglia in neuropathic pain 247
6.4.2 It is unclear how cold hypersensitivity is regulated by NRG1-erbB signaling in microglia given that microglial morphology and proliferation rate are unaffected 248
6.4.3 Technical limitations 251
6.5 Considerations 252
7 General Discussion 254
7.1 Summary of findings 254
7.2 NRG1- erbB signaling in microglia modulates cold but not mechanical hypersensitivity following nerve injury 255
7.3 Future Work 257
7.4 The complexities of using transgenic systems to understand signaling mechanisms 261
7.5 Are microglia involved in human chronic pain? 262
7.6 Can microglial neurotoxic functions be modulated without disturbing neuroprotective functions? 265
7.7 Conclusion 266

LIST OF TABLES

Table 1.1: Example of pharmacological interventions to treat neuropathic pain.

Table 1.2 Assessment of evoked pain related behaviour in animals.

Table 1.3 Examples of common peripheral nerve injury models in animals.

Table 1.4: Examples of microglial receptors and their function in the context of neuropathic pain.

Table 1.5: Examples of molecules that regulate microglial function.

Table 1.6: Examples of receptors and molecules capable of inducing microglial induced phagocytosis.

Table 1.7 The role played by soluble/transmembrane NRG1 isoforms in the PNS during development and after nerve injury determined using transgenic and conditional knockout mice.

Table 5.1 Sequence of primers used in genotyping PCR.

Table 5.2 Sequence of primers used for qPCR.

LIST OF FIGURES

Figure 1-1 The functional diversity of the microglial response.

Figure 1-2: The six types of Neuregulin 1 (NRG1) isoforms are classified according to their distinct amino-terminal sequences.

Figure 1-3: The role played by soluble/transmembrane NRG1 isoforms in the PNS during development and after nerve injury determined using transgenic and conditional knockout mice.

Figure 1-4: Examples of the erbB family of receptors and their cognate ligands.

Figure 2-1: Mechanical hypersensitivity develops in mice after spared nerve injury.

Figure 2-2: Thermal hypersensitivity develops in mice after spared nerve injury.

Figure 2-3: ATF3 is significantly upregulated in the spinal cord and DRG after injury.

Figure 2-4: PR2X4 mRNA levels fluctuate in the spinal cord after nerve injury.

Figure 2-5: Aif-1 mRNA expression is significantly upregulated in the DRG and sciatic nerve after injury.

Figure 2-6: Pan-NRG1 is significantly upregulated in the spinal cord after injury

Figure 2-7: NRG1 is significantly upregulated in the DRG after injury.

Figure 2-8: NRG1 Type I is significantly upregulated in the sciatic nerve after injury.

Figure 2-9: NRG-2 and NRG-3 mRNA levels remain unchanged in the spinal cord.

Figure 2-10: NRG2 and NRG3 mRNA levels remain unchanged in the DRG after nerve injury.

Figure 2-11: NRG-2 mRNA levels are significantly down-regulated in the sciatic nerve after injury.

Figure 2-12: erb2 mRNA levels are significantly down-regulated in the spinal cord after injury.

Figure2-13: erbB2 mRNA levels are significantly down-regulated in the DRG after injury.

Figure 2-14: erbB2, 3 and 4 are significantly down-regulated in the sciatic nerve immediately after injury.

Figure 3-1: Experimental apparatus for the thermal place preference assay.

Figure 3-2: The cold place preference temperature range.

Figure 3-3: Animals develop significant cold hypersensitivity after SNI.

Figure 3-4: The number of crossings in the cold place preference assay remain unchanged after SNI.

Figure 3-5: The $SCN9A^{F1449V-/+}$ (hets) develop thermal hypersensitivity to 37°C and thus spend a higher proportion of their time on the 32°C environment.

Figure 3-6: $SCN9A^{F1449V-/+}$ (hets) prefer 22°C instead of 37°C.

Figure 3-7: Mexilitine reverses thermal hypersensitivity seen in $SCN9A^{F1449V-/+}$ heterozygotes when given the choice to habituate either 22ºC or 37ºC.

Figure 4-1: NRG1 mRNA levels are significant down regulated in the PNS and CNS after Tamoxifen administration.

Figure 4-2: Mechanical hypersensitivity is not attenuated in Global NRG1-/- mice

Figure 4-3: Thermal hypersensitivity is not attenuated with global ablation of NRG1

Figure 4-4: Global NRG1-/- animals develop impaired motor coordination with delayed motor recovery after peripheral nerve injury

Figure 4-5: Global NRG1 animals display significantly fewer cold pain like behaviours in the delayed stages of peripheral nerve injury

Figure 4-6: Global ablation of NRG1 leads to delayed attenuation of cold hypersensitivity

Figure 4-7: Microglial numbers are not attenuated after nerve injury with NRG1 ablation

Figure 4-8: GFAP expression does not differ after SNI in the absence of NRG1.

Figure 5-1: Targeting scheme of the erbB3 and erbB4 locus.

Figure 5-2: mRNA expression does not significantly vary between Cre (-) and Cre (+) group

Figure 5-3: erbB4 protein levels are not significantly down-regulated using Cd11b Cre mediated excision

Figure 5-4: The R26tdTomato strain is a highly efficient reporter strain to that can be used to measure Cre activity. PLP-Cre (+): R26TFP(+) were used as a positive control

Figure 5-5: The Cd11b Cre (+) strain does not express tdTomato in the PNS or CNS tissue after SNI

Figure 5-6: Mechanical sensitivity thresholds are indifferent between Cre (-) and Cre (+) group

Figure 5-7: Thermal hypersensitivity thresholds do not differ between the Cre (-) and Cre (+) group

Figure 5-8: Heat and cold pain threshold levels are similar between the control and experimental group

Figure 5-9 Motor function is also unaffected between the Cre (-) and Cre (+) group.

Figure 5-10: Microglial morphology and numbers do not differ between Cre (-) and Cre (+) genotypes

Figure 6-1: YFP expression is detected in microglia upon the addition of CX3CR1 Cre system

Figure 6-2: CX3CR1CreERTM is a robust Cre transgenic system that can be used to target gene modification in microglia

Figure 6-3: tdTomato+ expression is not detected in CX3CR1 Cre ERTM (+) monocytes in the PNS after SNI

Figure 6-4: There is a significant reduction in erbB3 and erbB4 mRNA expression using CX3CR1 CreERTM Tamoxifen inducible system

Figure 6-5: erbB3 and erbb4 mRNA levels are significantly reduced when compared to IBA1 relative mRNA expression

Figure 6-6: erbB2 mRNA levels are significantly upregulated after Cre mediated excision of erbB3 and erbB4 gene expression

Figure 6-7: erbB3 and B4 receptor knock down does not attenuate mechanical hypersensitivity

Figure 6-8: erbB3 and erbB receptor knockdown in microglia does not attenuate thermal hypersensitivity after peripheral nerve injury

Figure 6-9: cKO animals (Cre (+) Tx.) exhibit normal proprioceptive repair after peripheral nerve injury

Figure 6-10: Cold allodynia is attenuated in cKO animals at 22 and 29 days after injury.

Figure 6-11: Microglial numbers are not attenuated after injury with erbB3 and erbB4 receptor blockade

Figure 6-12: GFAP expression does not differ after SNI in the absence of erbB3 and erbB4 in microglia.

ABBREVIATIONS

Akt	Protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC	Antigen-presenting cell
ARIA	Acetylcholine receptor-induced activity
ATF3	Activation transcription factor 3
ATP	Adenosine triphosphate
BACE	β-site of amyloid precursor protein cleaving enzyme
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BL	Baseline
BTC	Betacellulin
C5	Complement component-5
C5aR	Complement component-5 receptor
CAG	Glutamine
CCI	Chronic constriction injury
CCL2	Chemokine ligand 2
CCL21	Chemokine ligand 21
CCR2	Chemokine receptor type 2
Cd11b	Integrin alpha M
CD4	T-cell surface glycoprotein
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene regulated protein
CHO	Chinese hamster ovary cell
CIBP	Cancer induced bone pain

cKO	Conditional knockout
CNS	Central nervous system
CO_2	Carbon dioxide
CRD	Cysteine rich domain
CSF	Cerebrospinal fluid
Ct	Cycle threshold
CX3CL1	Fraktalkine
CX3CR1	CX3C chemokine receptor 1
Cy-3	Anti-rabbit Cyanine-3
DAPI	4', 6-diamidino-2-phenylindole
ddC	2′,3′-dideoxycytidine
DH	Dorsal horn
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EPG	Epiregulin
ER	Human oestrogen receptors
erbB	Extracellular tyrosine kinase protein
ERK	Extracellular tyrosine kinase
ERK1/2	Extracellular signal-regulated kinase 1/2
ESC	Embryonic stem cells
eYFP	Enhanced yellow fluorescent protein

FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FKN	Fraktalkine
GABA	Gamma Aminobutyric acid
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GGF	Glial growth factor
GPCR	G-protein coupled receptors
HBSS	Hank's Balanced Salt Solution
HET	Heterozygotes
HIV	Human immunodeficiency virus
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRG	Heregulin
HSPG	Heparin-sulphate proteoglycans
IB4	Isolectin B4
IBA1	Ionised calcium-binding adapter molecule 1
ICD	Intracellular domain
IEM	Inherited erythromelalgia
IFN- γ	Interferon γ
IHH	Immunohistochemistry
IL-1β	Interleukin 1β
IL-4	Interleukin 4
JNK	c-Jun N-terminal kinase

KCC2	Potassium-chloride transporter member 5
L3	Lumbar segment 3
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase
Mex	Mexilitine
MHC II	Major histocompatibility complex class II
MMPS	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NaV	Sodium ion channel
NGF	Nerve growth factor
NK-1	Neurokinin 1
NMDA	N-Methyl-D-aspartic acid
NO	nitric oxide
NRG	Neuregulin
NS	Nociceptive specific
OCT	Optimal Cutting Temperature compound
OEG	Olfactory ensheathing glia
OHT	4-Hydroxytamoxifen
OX42	Complement receptor C3bi
P2X	Purinoeceptor
P2X3	Purinergic receptor
P2X4R	Purinergic receptor 4
P2YR	Metabotropic P2Y receptors
P38 MAPK	p38 mitogen-activated protein kinases

PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PNI	Peripheral nerve injury
PNS	Peripheral nervous system
PSL	Partial sciatic nerve ligation
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT	Room temperature
RTX	Resinferatoxin
RVM	Rostral ventromedial medulla
SC	Spinal cord
SCDH	Spinal cord dorsal horn
SCI	Spinal cord injury
SCN9A	Sodium channel, voltage gated, type IX alpha subunit
SCs	Schwann cells
siRNA	Silence ribonucleic acid
SNI	Spared nerve injury
SNL	Spared nerve ligation
SOD1	Superoxide dismutase
STZ	Streptozotocin
TACE or ADAM17	Tumour factor-α converting enzyme

Th1	T helper cells
TLR	Toll-like receptors
TMD	Transmembrane domain
TNFα	Tumour necrosis factor α
TRAAK	Potassium channel subfamily K member 4
TREK	Potassium channel subfamily K member
TREM-2	Triggering Receptor Expressed On Myeloid Cells 2
TrkA	Tyrosine kinase A
TrkB	Tyrosine receptor kinase B
TRP	Transient receptor potential
TRPA1	Transient receptor potential cation channel, subfamily A
TRPM	Transient receptor potential ion channels
TRPV1	Transient receptor potential channel 1
Tx	Tamoxifen
VEGFr	Vascular endothelial growth factor receptor
VFL	Ventral funiculus lesion
VGLUT3	Vesicular glutamate transporter 3
Vh	Vehicle
VMSC	Vascular smooth muscle cells
WDR	Wide dynamic range
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
WT	Wild type
YFP	Yellow fluorescent protein

GENE AND PROTEINS

Gene Name	Protein name
ADAM17	Disintegrin and metalloproteinase domain-containing protein 17
AIF-1	Allograft inflammatory factor 1 (AIF-1) also known as ionized calcium-binding adapter molecule 1 (IBA1)
ATF3	Cyclic AMP-dependent transcription factor ATF-3
Bace1	Beta-secretase 1
CALCA	Calcitonin gene-related peptide 1
CX3CL1	Fractalkine
CX3CR1	CX3C chemokine receptor 1
EGFR	Epidermal Growth Factor Receptor
erbB2	Receptor tyrosine-protein kinase erbB-2
erbB3	Erb-B2 Receptor Tyrosine Kinase 3
erbB4	Erb-B2 Receptor Tyrosine Kinase 4
GABA	Gamma-aminobutyric acid receptor-associated protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	GFP-like fluorescent chromoprotein FP538
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase
IFNG	Interferon gamma
IL1B	Interleukin-1 beta
IL4R	Interleukin-4 receptor
ITGAM	Integrin alpha-M (Cd11b)
KCC2	K+/Cl-Cotransporter
KCNK4	Potassium channel subfamily K member 4
MAPK1	Mitogen-activated protein kinase 1 (ERK2)
MAPK1	Mitogen-activated protein kinase 1
MAPK3	Mitogen-activated protein kinase 3 (ERK1)
nk-1	NK-1 receptor
NRG1	Pro-neuregulin-1, membrane-bound isoform
NRG2	Pro-neuregulin-2, membrane-bound isoform
NRG3	Pro-neuregulin-3, membrane-bound isoform
P2RX4b	purinergic receptor P2X, ligand-gated ion channel, 4 (P2RX4) P2X purinoceptor
P2ry4	P2Y purinoceptor 4
P2X3	P2X purinoceptor 3
PRB2 or IB-4	Basic proline-rich peptide Isolectin Beta-4
SCN9A	Sodium channel protein type 9 subunit alpha
SLC17A8	Vesicular glutamate transporter 3
TNF	Tumor necrosis factor
TREM2	Triggering receptor expressed on myeloid cells 2
TRPM1	Transient receptor potential cation channel subfamily M member 1
Trpv1	Transient receptor potential cation channel subfamily V member 1
YFP	Yellow fluorescent protein

1 Introduction

1.1 Neuropathic pain

Neuropathic pain is a type of pain triggered by lesion or *disease* of somatosensory nervous system that leads to neuropathic pain, which are capable of permanently altering structure and function to induce spontaneous noxious pain.

Following injury to the nervous system a series of deleterious effects occur within the peripheral (PNS) and central nervous system (CNS) to cause long lasting and chronic neuropathic pain.

Patients suffering from neuropathic pain present common positive (hyperalgesia and allodynia) symptoms that develop as a consequence of decreased threshold levels and negative symptoms that develop after nerve damage due to a loss of function accompanied by on-going spontaneous pain (anaesthesia dolorosa).

The aetiology of neuropathic pain is very diverse and often multifactorial. It can be produced by different lesions or diseases either in the PNS or CNS and often appears in context to other comorbidities (e.g. distal symmetrical polyneuropathy in patients suffering from HIV infection (Verma et al. 2005); inflammatory and neuropathic pain in cancer induced bone pain (CIBP) (Kane et al. 2015).

1.1.1 Prevalence of neuropathic pain

Information on the prevalence and characteristics of those suffering with neuropathic pain is either limited or conflicting. Most of the information is derived from studies that focus on measuring neuropathic pain prevalence within specialised cohorts of people such as those suffering from back pain (Gálvez et al. 2005), diabetic induced

neuropathies (Argoff et al. 2006), multiple sclerosis or nerve entrapment syndrome (Gálvez et al. 2005).

Questionnaire studies suggest the prevalence of neuropathic pain to be approximately 6-8% in the population (Bouhassira et al. 2008). Of those that seek pain relief - only 40-60% of patients receive partial relief (Dworkin et al. 2010). Table 1 summarises some of the existing drug classes used to treat neuropathic pain.

The development of therapeutic options available to neuropathic pain patients is complicated by the diverse and unpredictable aetiology of neuropathic pain, the possibility of genetic components that might predispose an individual to neuropathic pain (Nissenbaum et al. 2010; Bali et al. 2014) and the limitations in translating animal model data into a clinical setting (Ossipov & Porreca 2005).

Table 1-1: Example of pharmacological interventions to treat neuropathic pain

Pharmacological treatment option	Example of drugs	Function
Tricyclic antidepressants	Amitriptyline and serotonin re-uptake inhibitors	• Block serotonin and noradrenaline re-uptake into the CNS increase the activity of the descending inhibitory pain pathways.
Anticonvulsants	Carbamazepine and sodium valproate	• Block voltage gated sodium channel.
	Lamotrigine	
	Gabapentin	• Acts on the α2δ subunit of voltage dependent calcium channels in the dorsal horn of the spinal cord.
Strong opioids	Morphine and fentanyl effective in single dose trials	• Inhibit neurotransmitter release from the primary afferent terminals in the spinal cord and activate descending inhibitory controls in the midbrain.
	Oxycodone, levorphanol, transdermal fentanyl and transdermal buphrorphine	
Cannabinoids	THC and Cannabinol	• Target the CB1 receptors found in the PNS and CNS and CB2 receptors found in the PNS only.

1.2 Measuring pain related behaviour in animals

The absence of verbal communication in animals (unlike in humans) makes evaluation of pain very difficult to quantify. Because pain cannot be directly measured in animals, it is important to use quantifiable, sensitive and specific measures of nociceptive behaviour.

Pain-related behaviour, can be tested in several different ways. The reaction to high intensity stimuli can be investigated using mechanical, thermal, or chemical stimuli applied to skin, deep tissue, or viscera in animal studies. With animals, a reaction to stimuli does not necessarily suggest the sensation of pain in animals but it measures instead the enhanced nociception in response to the stimulus applied (Sandkühler 2009).

Animals that are capable of displaying certain types of behaviours, have been shown to be linked to enhanced nociception. Evoked nociception through painful stimuli is seen through paw or tail withdrawal from the stimulus source, vocalisation, reduced locomotion and agitation during sensory stimulation and motor reflexes elicited by both noxious and innocuous stimuli (Sandkühler 2009).

Signs of spontaneous pain can be detected through behaviours such as audible and ultrasonic vocalisation, conditional place avoidance, analgesic self-administration, excessive grooming of the face, body and hind limbs, and self-mutilation of a limb with autotomy of an affected limb also being considered as sign of spontaneous pain after nerve injury (Mogil & Crager 2004).

In order to quantitatively measure modality specific nociceptive behaviours in both acute and chronic pain states, many behavioural assays have been designed and used. The most well-known types of behavioural assays assessing acute evoked pain are

presented in Table 1-2. Whilst Table 1-2 is not exhaustive, it highlights the key behavioural assays used to measure nociception in animal models.

Beyond these traditional methods of measuring nociception (Von Frey Test (Chaplan et al. 1994), Hargreaves Test (Hargreaves et al. 1988), Cold Plate test (Jasmin et al. 1998), electrical stimulation of the tail or hind paw (Jourdan et al. 1995), more sophisticated behavioural assays, such as the conditioned place preference assay (King et al. 2009), have been produced that are capable of providing further quantitative information on mechanical, thermal, electrical and chemical pain perception within animals.

Table 1-2 assessment of evoked pain related behaviour in animals

Modality	Common Test Name	Method of Testing	Area of testing	Measured Parameter
Mechanical	Von Frey (Chaplan et al. 1994)	Application of calibrate static filaments on skin	Face and Hindpaw	50% withdrawal threshold force (static mechanical allodynia)
	Randal Sellitto	Application of linearly increasing mechanical force in noxious range on skin	Hindpaw	Paw withdrawal threshold force eliciting from noxious stimulus (mechanical hyperalgesia)
	Unnamed	Innocuous brushing, stroking of skin	Hindpaw	Time latency to withdraw paw or to display nociceptive behaviour (dynamic mechanical allodynia)
	Unnamed	Noxious mechanical stimulation to viscera	Visceral organs such as colon and bladder	Threshold/number/strength of muscle contractions/autonomic responses (hyperalgesia)
Heat	Tail Flick	Immersion of tail into hot water or the application of radiant heat on tail	Tail	Time latency to withdraw tail or to display nociceptive behaviour (heat hyperalgesia)
	Hargreaves Plantar Assay (Hargreaves et al. 1988)	Application of radiant heat on skin	Hindpaw	Time latency to withdraw paw or to display nociceptive behaviour (heat hyperalgesia)
	Hot Plate	Animal placed in enclosed chamber onto a heated metal plate	Hindpaw and forepaws	Time latency to display nociceptive behaviour (heat hyperalgesia)
	Thermal Place Preference	Animal placed onto a heated metal with the choice to move between two temperature plates	Hindpaw and forepaws	The % of time spent between the two temperature settings

Cold	Acetone Test	Acetone applied to skin	Hindpaw	Duration/intensity of noxious behaviour (cold hyperalgesia)
	Cold Plate Test (Jasmin et al. 1998)	Animal placed onto cold plate	Hindpaw	Time latency to withdraw paw or to display nociceptive behaviour (cold hyperalgesia)
	Cold Water Test	Animal placed in cold water	Hindpaw	Time latency to elicit nociceptive or escape behaviours, duration and intensity of nociceptive behaviours (cold hyperalgesia)
	Cold Place Preference	Animal placed onto a cold metal with the choice to move between two temperature plates	Hindpaw and forepaws	The % of time spent between the two temperature settings (cold hypersensitivity)
Electrical	Unnamed (Le Bars et al. 2001)	Electrical current application	Tail, paw, viscera, dental pulp	Withdrawal thresholds/vocalisation/ Escape latency (allodynia) (Jourdan et al. 1995)
Chemical	ATP	Intraplantar injection into hindpaw	Hindpaw	Latency/frequency/duration/intensity of noxious behaviour
	Acid			
	Capsaicin			
	Formalin			

1.3 Models of neuropathic pain in animals

There is a need for validated and easily reproducible animal models of neuropathic pain, in combination with nociception assays, to broaden our understanding of neuropathic pain. Different animal models have been established to replicate the diverse aetiology and manifestations seen in neuropathic pain patients. These animal models can be broadly classified as either peripheral nerve injury induced, central pain induced, drug induced, or disease induced neuropathy models which equates to over 40 different models of neuropathic pain (Jaggi et al. 2011).

Whilst the central pain models, drug induced and disease induced models are all as equally useful and informative as the peripheral nerve injury induced models, the discussion of such models are beyond the scope of this thesis and are described elsewhere (Jaggi et al. 2011).

1.3.1 Peripheral nerve injury models

The peripheral nerve injury model is described in **Error! Reference source not ound.**. In this thesis I have used the spared nerve injury model (SNI). Using the SNI model, mechanical and thermal hyperalgesia can be detected as early as 4 days after injury with persistent effects ranging from several weeks to 6 months after injury (Decosterd & Woolf 2000; Bourquin et al. 2006). Cold allodynia is also shown to develop and is maintained until at least 7 weeks after injury (Decosterd & Woolf 2000). The SNI model presents unique quantitative analysis capabilities compared with the CCI, PSL and SNL models because it allows the assessment of the difference in mechanical and thermal sensitivities of non-injured skin regions bordering the denervated areas. With these capabilities, SNI studies have highlighted the important contribution of non-injured neurons to neuropathic pain, including non injured

neurons, abnormal expression of sensory voltage-gated sodium channels and augmentation of the transient receptor potential channel 1 (TRPV1) (Jaggi et al. 2011). The surgical procedure used to conduct SNI is relatively simple with little variation in the degree of damage to produce robust changes in mechanical, thermal and cold sensitivities that closely mimic many clinical features of neuropathic pain (Shields et al. 2003).

1.4 Mechanisms of Neuropathic pain

The mechanisms of neuropathic are very diverse that are manifested by certain autonomic, psychological and behavioural reactions. The various mechanisms of neuropathic pain are summarised in the following way:

1. Peripheral sensitisation
2. Peripheral nerve and DRG ectopic changes after injury
3. Central sensitisation and dis-inhibition in the spinal cord
4. Immune cell contribution to neuropathic pain

1.4.1 Peripheral sensitisation

1.4.1.1 Nociceptors

Nociceptors are the specialised peripheral sensory neurons responsible for detecting potentially harmful stimuli at the skin. They can be classified by an array of anatomical, physiological, biochemical and functional criteria that distinguishes each subtype. Nociceptor endings in tissue are un-encapsulated and commonly associated with either unmyelinated (C) or thinly myelinated (Aδ) axons with small diameter somata in the DRG (and other sensory) ganglia. Subpopulations of nociceptors can be subdivided into peptidergic and non-peptidergic fibres. Peptidergic fibres represent a

subpopulation of C fibres that express either substance P and/or calcitonin gene regulated protein (CGRP) or are capable of expressing nerve growth factor (NGF) receptor tyrosine kinase A (TrkA) (Snider & McMahon 1998). By contrast, non-peptidergic populations of fibres can be classified according to the level of Ret expression (a glial cell derived neurotrophic factor), isolectin B4 (IB4) binding via membrane glycoproteins and purinergic receptor P2X3 expression (Snider & McMahon 1998).

Peripheral sensitisation is a reduction in threshold and an increase in action potential frequency or responsiveness of the peripheral ends of nociceptors to contribute to the pain hypersensitivity found at the site of tissue damage and inflammation (Chen et al. 1999; Guenther et al. 1999; Hucho & Levine 2007; Pethö et al. 2001; Thacker et al. 2007; Basbaum et al. 2009).

1.4.2 Peripheral nerve and DRG ectopic changes after injury

When axons lose connection with target tissue after nerve injury, the retrograde transport of vital growth factors to the cell body is disrupted. This causes a change in the neurochemical and electrical properties of sensory neurons to generate ectopic action potentials and cause spontaneous pain. After peripheral nerve damage, ectopic or spontaneous activity is generated at the neuroma (Wall & Gutnick 1974), the cell body of injured DRG neurons (Shinder et al. 1999; Chung et al. 1996; Zhang et al. 2004) and in neighbouring intact afferents.

Ectopic impulse generation is largely caused by; the activity of voltage-gated sodium channels expressed on axons and DRG's (Dib-Hajj et al. 2007; Dib-Hajj et al. 2009; He et al. 2010); dysregulation of potassium channels (Tsantoulas et al. 2012; D. S. Kim et al. 2002; Chien et al. 2007); nerve inflammation and demyelination of injured

nerves (Kajander & Bennett 1992); and a reduction in threshold of transient receptor potential (TRP) heat sensitive channels to generate spontaneous activation at normal (body) temperatures (reviewed in (Waxman et al. 1999; Waxman & Zamponi 2014)). Phenotypic switches coupled with the expression level changes in ion channels and genes in the DRG, are capable of increasing the capacity of nociceptors to produce central changes in the CNS known as central sensitisation.

1.4.3 Spinal cord: Central sensitisation and disinhibition

1.4.3.1 Dorsal horn anatomy and normal function

The dorsal horn is the major site of termination for primary afferent axons where primary afferent terminals are distributed in an orderly fashion based on fibre size and sensory modality. Lamina I of the dorsal horn corresponds to the marginal layer and lamina II corresponds to the substantia gelantinosa, while the remainder of the dorsal horn consists of 4 further laminae identified as III, IV, V and VI (Rexed 1952). The spinal cord contains various neuronal cell types that are capable of receiving nociceptive input and propagating this information further along the somatosensory relay system. Nociceptive specific cells concentrated in lamina 1 of the dorsal horn receive input from Aδ and C fibres to generate action potentials after application of a noxious stimulus at the periphery. Wide dynamic range neurons (WDR) receive input from all three types of primary afferent fibres and are capable of responding to the full range of stimulation. WDR's generate action potentials in a graded fashion depending on stimulus intensity and also exhibit an unusual characteristic where repetitive stimulation of WDR neurons causes an increase in the evoked response and post discharge of WDR's with each stimulus (wind-up) (Dickenson & Sullivan 1987). Within the dorsal horn, there are also excitatory, glutaminergic, and inhibitory,

GABAergic interneurons within the spinal cord that are capable of increasing or decreasing nociceptive specific (NS) and WDR cell response to subsequently influence the overall output of the dorsal horn (D'Mello & Dickenson 2008).

1.4.3.2 Central sensitisation

Central sensitisation is described as major shift from high-threshold nociception to low threshold pain hypersensitivity via an enhancement of neuronal and circuit functions within CNS nociceptive pathways caused by increased membrane excitability, increased synaptic efficacy and reduced inhibition in CNS systems (Latremoliere & Woolf 2009; Woolf 2011).

Fast excitatory synaptic transmission in pain pathways is mediated by glutamate acting on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainite ligand-gated ion channels. Activation of N-Methyl-D-aspartic acid (NMDA) ion channels requires membrane depolarisation in order to remove its magnesium blockade. Unlike low-threshold primary sensory neurons that use glutamate as their only neurotransmitter, nociceptors may use glutamate, neuropeptides (e.g. substance P) (Afrah et al. 2002) and calcitonin gene-regulated peptide (CGRP). Substance P is co-released with glutamate by unmyelinated peptidergic nociceptors and is involved in the generation of central sensitisation through binding to neurokinin 1 (NK-1) g-protein coupled receptors (GPCR's) expressed on spinothalamic, spinoparabrachial and spino-periaqueductal neurons (Gauriau & Bernard 2002), this causes long lasting membrane depolarization, and temporal summation of C fibre evoked synaptic potentials (Dougherty & Willis 1991; Xu et al. 1992; Juránek & Lembeck 1996). Glutamate also utilises synaptic modulators such as ATP and BDNF where ATP binds to P2X receptors and BDNF binds to TrkB receptor to enhance NMDA receptor C-fibre-evoked responses (Kerr et al. 1999) and causes activation of extracellular

tyrosine kinase (ERK) and Protein kinase C (PKC) signaling pathway in spinothalamic tract neurons (Kawasaki et al. 2004; Pezet et al. 2002; Slack et al. 2005; Slack et al. 2004). These fast and slow excitatory post synaptic potentials all contribute to the temporal summation and cumulative depolarisation of neurons to boost NMDA receptor recruitment (Woolf & Thompson 1991; Woolf & Mannion 1999) and produce a net effect of increased excitability of the dorsal horn neurons. With these central plastic changes, low- threshold sensory fibres are capable of activating neurons in the spinal cord which normally only respond to high threshold noxious stimuli.

1.4.3.3 Disinhibition

The sensory input into the spinal cord is also under regulation of inhibitory circuitry maintained by sensory afferents, spinal interneurons and descending inhibition. This regulatory circuitry is disrupted after peripheral nerve injury to cause misinterpretation of peripheral inputs and thus develop chronic pain. The main inhibitory neurotransmitters in central nervous system are γ-amino butyric acid (GABA) and glycine. GABAergic tone plays a critical role in sensory transmission and integration. Intense noxious stimuli is capable of inducing a functional loss of GABAergic inhibition via the reduction of GABA and GABA- synthesizing enzyme glutamic acid decarboxylase (GAD) release from presynaptic terminals or the trans-synaptic reduction of the potassium-chloride exporter KCC2 (disruption of anion homeostasis, see section on immune cells) to reduce nociceptive threshold after injury. These findings coupled with electrophysiological recordings show overall reduced spinal inhibitory tone in the injured animals (Castro-Lopes et al. 1993; Eaton et al. 1998; Gwak et al. 2006; Ibuki et al. 1997; Moore et al. 2002; Anseloni & Gold 2008; Coull et al. 2003; Funk et al. 2008; Knabl et al. 2008; Staud et al. 2007). This reduced

inhibitory tone in the dorsal horn, when combined with the excitatory glutamate effects, contributes to overall central sensitisation after peripheral nerve damage.

1.4.4 Descending modulation by the brainstem

The facilitatory input from brainstem structures is believed to be critical for the maintenance of neuropathic pain. Spinal transection, hemi section or selective lesions of the ipsi- or contra-lateral dorsal columns has been shown to attenuate the development of nerve injury induced pain (Bian et al. 1998; Sung et al. 1998; Sun et al. 2001) and the lesion of the major pathways connecting the rostral ventromedial medulla (RVM) to spinal cord, dorsolateral funiculus, also abolished development of neuropathic pain (Burgess et al. 2002). Electrophysiological experiments demonstrated that specific population of RVM cells expressing μ-opioid receptor are directly related to the descending facilitatory input (Heinricher & Neubert 2004) where the selective ablation of these cells prevents the development of neuropathic pain (Burgess et al. 2003; Ossipov & Porreca 2005).

1.4.5 Immune cell contribution to neuropathic pain

It is well recognised that neurons are not the only players responsible for driving the onset and maintenance of neuropathic pain after injury. Recent research implicates immune cell activity, not only in inflamed tissues, but also in damaged peripheral nerves and in the central nervous system. It has been determined that peripheral nerve injury provokes immune cell reactions at the periphery (macrophages and Schwann cells), at the DRG (macrophages, lymphocytes and satellite cells) and the CNS (astrocytes and microglia) (Scholz & Woolf 2007a).

1.4.5.1 Immune cell activation in the periphery

Under normal physiological conditions, macrophages are responsible for interstitial homeostasis by removing cellular debris. In primed immune states, resident and blood-recruited macrophages phagocytose foreign material, microbes, other leukocytes and injured tissue i.e., during Wallerian degeneration (Brück 1997). Resident endoneurial macrophages play an early and active role in the immediate cellular events after nerve lesions and predominate at the initial inflammatory reaction after nerve damage (Mueller et al. 2001). Resident macrophages account for up to 9% of the entire endoneurial cell population where their location makes them a prime candidate for early responses to disease or damage. Following nerve damage, resident macrophages respond rapidly without the need for prior activation of precursor cells and are joined by circulating macrophages, a process that can occur for 2‑3 days after damage. Recruited macrophages quickly outnumber the resident cells and this process is vital for nerve regeneration (Griffin et al. 1993; Taskinen & Röyttä 1997). Endogenous (i.e., pro-inflammatory factors released by necrotic cells) and exogenous signals (foreign agents) can activate macrophages, followed by their migration to the site of injury to release pro-inflammatory mediators (cytokines TNF α and IL-1 β, NGF, nitric oxide and prostanoids). Experimental data gives evidence for the crucial contribution of macrophages to the modulation of pain processing. The overt pain behaviour induced by intraperitoneal injections of acetic acid, lipopolysaccharide (LPS) or zymosan in rodents is exacerbated with increasing macrophage concentrations, partly due to the release of TNFα and IL-1β (Ribeiro et al. 2000; Thomazzi et al. 1997). With anti-inflammatory cytokines or a vasodilator drug (pentoxifyline), this inflammatory pain can be significantly reduced with a decrease in production of cytokines from the resident macrophages (Vale et al. 2003). A

reduction in neuropathic pain behaviours correlates with an attenuation of macrophage recruitment into the damaged nerve (Xu et al. 2007; Myers et al. 1996; Ransohoff 1997; Sommer & Schäfers 1998) and mice with delayed Wallerian degeneration show markedly reduced thermal hyperalgesia compared to normal mice with a chronic constriction injury of the sciatic nerve, which is temporally related to the delayed recruitment of macrophages to the injured nerve (Sommer & Schäfers 1998).

Following nerve injury immune cells such as neutrophils, macrophages and T lymphocytes are recruited to the DRG. Immune cells, satellite cells and DRG neurons, up-regulate the expression of pro-inflammatory cytokines and its receptors to increase DRG neuronal excitability (Calvo et al. 2012). Inflammatory mediators increase DRG neuronal excitability by interacting with endogenous sensory transduction mechanisms. Previous studies show that chemokine ligand 2 (CCL2) is expressed on DRG neurons and is capable of binding chemokine receptor type 2 (CCR2) on immune cells which release factors resulting in the sensitisation of TRPV1 and transient receptor potential cation channel, subfamily A (TRPA1) receptors to subsequently increase neuronal excitability (Jung et al. 2008; Jung et al. 2009; White et al. 2005). Additionally, some cytokines such as TNFα and IL-1β can also enhance the excitability of sensory neurons through similar mechanisms (Nicol et al. 1997; Obreja et al. 2002). Sensory neurons can also express toll-like receptors (TLR's), which when activated can have a direct sensitising effect (Diogenes et al. 2011) and are able to induce the secretion of pro-inflammatory mediators from these cells (Qi et al. 2011).

1.5 Neuro-immune interactions in the CNS

Neuroinflammation has a pathogenic role in many neurological conditions including stroke, infections, neurodegenerative disorders (Glass et al. 2010) and more recently we have realised that there is a marked glial response to peripheral nerve injury. These effects are mediated by microglia, astrocytes or oligodendrocytes (glial cells in the CNS) through bi-directional neural-glial interactions to respond to CNS insults.

1.5.1 Astrocytes

Interactions between astrocytes and neurons contribute to the mechanisms of chronic pain (Ji et al. 2006; Ren 2010). Previous research indicates that Glial Fibrillary Acidic Protein (GFAP) upregulation correlates well with nerve injury induced neuropathic pain, where the magnitude of the increase in GFAP is also said to be correlated to the degree of hyperalgesia (Garrison et al. 1991; Colburn et al. 1997; Colburn et al. 1999). GFAP acts as a marker of morphological changes within astrocytes and is not in itself causative for pain pathogenesis given that inhibition of GFAP does not suppress chronic pain (Zhuang et al. 2006a). Persistent GFAP upregulation is reported in different animal models such as bone cancer (Honore et al. 2000; Mantyh et al. 2002), spinal nerve ligation (Tanga et al. 2006; Zhuang et al. 2006a), partial sciatic nerve ligation (Zhuang et al. 2006b), spinal cord injury (Nesic et al. 2005) and adjuvant-induced inflammation (Raghavendra et al. 2004).

Astrocyte activation is accompanied by the initiation of cellular signal transduction pathways (p38-MAPK, ERK, c-Jun). Phosphorylated ERK (pERK), is induced in spinal astrocytes at approximately 3 weeks after SNL and spinal inhibition of ERK where intrathecal mitogen-activated protein kinase (MEK) inhibitor reverses mechanical allodynia (Zhuang et al. 2005), to support a role of astrocytic ERK in the

late phase maintenance of neuropathic pain (Ji et al. 2006). C-Jun N-terminal kinase (JNK) activation also occurs in spinal astrocytes 3 weeks after partial sciatic nerve injury where phosphorylated JNK co-localises with the astroglial marker GFAP (Zhuang et al. 2006a; Zhuang et al. 2005; Ji et al. 2006). Such activation of signaling pathways in astrocytes leads to transcriptional gene regulation and release of a variety of known inflammatory chemical mediators (e.g. IL-1β (Zhang et al. 2005; Guo et al. 2007; Wei et al. 2008; Wasserman et al. 2007; Ji et al. 2006)).

Astrocyte activation also leads to down-regulation of glutamate transporter activity to directly affect synaptic transmission and changes in gap junction proteins (e.g. Connexin-43 (Nagy et al. 2004; Rohlmann et al. 1993; Lee et al. 2005) and gap-junction decoupler carbenxolone (Spataro et al. 2004)). Finally, astrocyte activation leads to increased blood brain barrier (BBB) permeability to contribute nerve and glial cell activation and pain hypersensitivity (Gordh et al. 2006; Ji et al. 2006). These coordinated changes in astrocyte functions are all capable of modulating neuronal activity and facilitating overall pain transmission within the CNS.

1.5.2 Oligodendrocytes

It is not fully clear whether oligodendrocyte spinal cord function or pathology contributes to the onset or maintenance of neuropathic pain. With oligodendrocyte ablation, phenotypic changes in the brain and spinal cord include a rapid loss of labelled oligodendrocytes somata, microglial reaction, astrogliosis, lack of adaptive immunity (absence of invading T cells), progressive and late demyelination and neurological symptoms (ataxia, tremor, ultimately death) (Gritsch et al. 2014). By achieving oligodendrocyte tissue specific knockdown, this study provides evidence for significant reduction in mechanical and cold pain thresholds and loss of

myelination that occurs at later time points from the onset of nociceptive behaviours (Gritsch et al. 2014).

1.5.3 Microglia

1.5.3.1 Microglia in the healthy and injured CNS

In the late nineteenth century, Frank Nissl was the first to describe microglia as rod cells ("Stabchenzellen") that possess reactive glial elements including migration, phagocytosis and proliferation. Santiago Ramon y Cajal renamed the "rod cells" as the "third element of the nervous system" to differentiate them from neurons and neuroglia whilst del Rio-Hortega, in 1919, made a further distinction between various cells within the "third element" based on morphological and functional differences and introduced the term "microglial cell". These were a non-neuronal, non-astrocytic third element distinct from neuro-ectodermal oligodendroglia or oligodendrocytes (reviewed by (Rezaie & Male 2002; Ginhoux et al. 2013)). Despite Rio-Hortega's seminal work, his theories were largely overlooked until a pivotal genetic study revealed that mice lacking PU.1 factor (expressed exclusively in cells of hematopoietic lineage), were also devoid of microglia (McKercher et al. 1996; Beers et al. 2006) and thus microglia are derived from the myeloid lineage.

Whilst microglial origin has only been recently clarified, microglial form and function are better established. Microglia possess intricate and complex morphologies that can cycle reversibly from simple unramified "activated" amorphous blobs to "surveillance state" relatively small soma and long, tortuous primary processes that can be characterised as "spider-like", "jointed" or "thorny" with secondary or tertiary branches endowed with wispy ends or yet further branches (reviewed by (Karperien et al. 2013)). In the normal mature CNS, microglia adopt various morphologies to

actively engage in their physiological roles- they act as vigilant sentinels constantly surveying their environment and ensuring proper functioning of neurons, providing neurotrophic support (Karperien et al. 2013), acting on regulating neurotransmitters (Garcia-Segura et al. 1996), mediating pain (Watkins et al. 2001; Inoue 2006), responses to psychological stress (Hinwood et al. 2013), protecting neurons from damage (Vinet et al. 2012) and responding to changes in the microenvironment (Eder et al. 1998; Lyons & Kettenmann 1998; Polito et al. 2011; Tremblay et al. 2011; Won et al. 2012). In the healthy state, local cell division and antigen presentation at a low level rate maintains the number of resident microglia in the CNS. When CNS homeostasis is disrupted, microglia rapidly adopt a "reactive" morphology and change their gene expression profile to provide both positive (neuroprotective) and negative (neurotoxic) roles seen in CNS injury/diseases including spinal cord injury (Loane & Byrnes 2010), stroke/ischemia (Weinstein et al. 2010), infection (Yadav & Collman 2009), multiple sclerosis (Jack et al. 2005) and neurodegenerative diseases (Streit et al. 2005).

Microglial activation has now been shown to contribute to maintain neuropathic pain via CNS hyperexcitability following peripheral nerve injury (Tsuda et al. 2005; Tsuda et al. 2013).

1.5.3.2 Microglial response to nerve injury

With peripheral nerve injury, microglial cells respond through a series of morphological and physiological events including the adoption of a reactive amoeboid morphology, the proliferation of microglial cells, the migration of such cells to the site of injury in the dorsal horn, phagocytosis of cellular debris, antigen presentation and adoption of an inflammatory response role with the release of well identified inflammatory chemokines and cytokines into the CNS (reviewed in

(Hanisch & Kettenmann 2007; Calvo & Bennett 2012). This series of events is described using an umbrella term identified as "microgliosis". Reactive microgliosis is best defined as microglial response that occurs after the release of neuronal damage signals, to perpetuate further activation and neurotoxicity to generate a self-propelling and progressive cycle between microglial activation and neuronal damage (Lull & Block 2010; Hanisch & Kettenmann 2007).

Microgliosis occurs after nerve damage and there is increasing evidence suggesting that the microglial response to injury directly contributes to neuropathic pain (Scholz & Woolf 2007b; Costigan, Scholz, et al. 2009; Thacker et al. 2007). This was first clearly demonstrated in 2003 by Tsuda et al. where it was shown that intrathecal injection of microglia stimulated by ATP could cause mechanical hypersensitivity and that blockade of the P2X4 receptor expressed in microglia could prevent or reverse mechanical hypersensitivity following nerve injury in the rodent. Intracthecal injections of ATP can elicit pain related responses in naive rats through microglial activation

A number of animal models of chronic pain have been used to further our understanding of the microglial contribution to neuropathic pain. Using a modified version of the SNL model of peripheral nerve injury, it has been demonstrated that microglia accumulate at the ipsilateral side L5 segment of the spinal cord site of injury (dorsal horn) and around motor neurons (ventral horn) (Tsuda et al. 2003; Calvo et al. 2010). Microgliosis is also observed in animal models of CCI (Colburn et al. 1997), SNI (Beggs & Salter 2007) and PNI (Coyle 1998).

The streptozotocin (STZ)-induced models of diabetes (Daulhac et al. 2006; Daulhac et al. 2011; Talbot et al. 2010a; Tsuda et al. 2008) has identified microgliosis as a contributor to neuropathic pain. Wodarszki et al. utilise STZ-diabetic rats that are

known to exhibit significant mechanical hypersensitivity to administer Gabapentin and provide evidence for the attenuation of mechanical allodynia through the reduction in microglial numbers after treatment (Wodarski et al. 2009).

HIV-induced painful neuropathy models (Milligan et al. 2001; Herzberg & Sagen 2001; Wallace et al. 2007) identify microglial activity as a crucial CNS inflammatory mediator. Milligan et al. give evidence for the a HIV-1 envelope glycoprotein gp120 inducing significant thermal hyperalgesia, mechanical allodynia and activating microglial and astrocytic induced proinflammatory cytokine release (Milligan et al. 2001).

Sweitzer at al. used low doses of a cancer chemotherapeutic agent - vincristine to demonstrate vincristine induced mechanical allodynia and mild bilateral microglial activation (Sweitzer et al. 2006) whilst Peters et al. use intravenous infusions of clinically relevant doses of paclitaxel to demonstrate induced mechanical allodynia, cold hyperalgesia coupled with microglial activation in the spinal cord (Peters et al. 2007). These studies highlight the presence of microglial cells and their likely contribution to the onset and maintenance of neuropathic pain caused by PNS/CNS disease or damage.

1.5.3.3 Signals that lead to a microglial response

In the healthy CNS system, microglia are distributed evenly within the spinal cord to fulfil their surveillance roles, but this changes following nerve injury. Damaged sensory neurons enter the dorsal horn of the spinal cord and alter their functional state to release signals in order to initiate spinal microgliosis. Beggs and Salter (2007) used stereological techniques to show microglia accumulation at the central territory of peripherally axotomised afferents with some extensions into the uninjured terminal afferent areas (Beggs & Salter 2007).

After nerve injury, the peripheral input from both large myelinated fibre and C fibre nociceptors has been shown to be involved in the development of microgliosis (Suter et al. 2009). Suter et al. used Bupivacaine to prevent impulse conduction in all sensory and motor axons. This showed significant inhibition of p38 activation and microglial proliferation. Resinferatoxin (RTX) has used to selectively silence C-fibres which generate effective nociceptive block but did not inhibit p38 activation or microglial proliferation in the spinal cord and concluded that spinal microglial activation after nerve damage requires peripheral spontaneous activity from large myelinated (Aβ) fibres (Suter et al. 2009).

Hathway et al. showed that low frequency C-fibre stimulation, in the absence of nerve damage is capable of generating a microglial response coupled with behavioural hyperalgesia and minocycline administration is capable of blocking C- fibre evoked behavioural sensitisation and microglial accumulation (Hathway et al. 2009).

A number of mediators that are known to modulate microglial phenotype are released from damaged sensory neurons. Such mediators that have the capacity to stimulate microglia include ATP, misfolded or aggregated proteins, nuclear factors, ROS, complement components and nitric oxide (NO) (reviewed by (Calvo & Bennett 2012)). I will discuss the various aspects of the microglial response.

1.5.3.4 (a) Microglial pro-inflammatory response

Currently, microglial functions are classed into three distinct function groups. The first is the classical activation state of microglia that is responsible for the pro-inflammatory response. The second is alternatively activated microglia, which are associated with the production of anti-inflammatory cytokines in the resolution phase of the inflammatory response and the third activation state of microglia which overlaps with and complements the alternative activation phase and is called acquired

deactivation to cause immunosuppression and anti-inflammatory and develop functional repair phenotypes (Gordon 2003; Gordon & Taylor 2005). Upon signal release from damaged neurons, microglia switch into a pro-inflammatory phenotype to secrete cytokines, nitric oxide and BDNF, which are all capable of enhancing dorsal horn neuron excitability (Coull et al. 2005a; Kawasaki et al. 2008).

Microglia express receptors that respond to injury signals from endogenous TLRs ligands, complement components, purines and chemokines (Milligan and Watkins, 2009; Scholz and Woolf, 2007). These different chemical mediators are all capable of activating intracellular pathways within microglia including P38 MAPK and ERK1/2 (reviewed in (Jha et al. 2015)).

The mitogen-activated protein kinases (MAPKs) constitute a family of signaling molecules that play a pivotal role in mediating cellular responses to a variety of signals. The phosphorylated p38 MAPK and ERK1/2 kinases are both induced in spinal cord microglia after peripheral nerve injury and are responsible for microglial pro-inflammatory responses. P38 MAPK is described as a stress-induced kinase where phosphorylation of p38 MAPK has been implicated in exaggerated various pain states (Ji et al. 2002; S.-Y. Kim et al. 2002; Jin et al. 2003; Schäfers et al. 2003; Tsuda et al. 2004; Svensson et al. 2005). P38 MAPK can be phosphorylated via a number of cytokine receptors (TNFa receptor, CX3CL1, CCR2) and purinoceptors (P2X4) found on the microglial surface (Ji & Suter 2007; Beggs et al. 2012) to produce a number of inflammatory mediators from microglia including IL-1B, IL-6, TNFa, PGE2, NO and BDNF which are also produced in the spinal cord after injury (Tsuda et al. 2005; Coull et al. 2005a; Watkins et al. 2001; DeLeo & Yezierski 2001; Marchand et al. 2005; Milligan et al. 2003; Sweitzer et al. 2001; Winkelstein et al.

2001). The direct correlation between microglial activation, P38 MAPK phosphorylation and neuropathic pain development has been made using CCI (Chiang et al. 2013; S.-Y. Kim et al. 2002), SNI (Tsuda et al. 2004) and SNL (Jin et al. 2003; Terayama et al. 2008) models of nerve injury.

ERK1/2 are capable of transducing extracellular stimuli into intracellular post transcriptional and translational responses to regulate cell proliferation, differentiation and growth (Schaeffer & Weber 1999). ERK activation has been demonstrated within neurons, microglia and astrocytes after nerve injury to contribute to mechanical hypersensitivities after injury (Zhuang et al. 2005). Within microglia, ERK1/2 has been thought to be involved in microglial proliferation and the release of a number of pro-inflammatory mediators such as TNFa and NO (reviewed by (Calvo & Bennett 2012)).

The different chemical mediators responsible for initiating a microglial pro-inflammatory response after CNS homeostasis disturbance are described in the next section.

I. Toll-like receptors

Toll-like receptors (TLRs) are a group of pattern recognition receptors (PRRs) that recognise conserved motifs called pathogen-associated molecular patterns (PAMPs), which are expressed, on broad classes of microbes (Akira et al. 2006; Kaisho & Akira 2004). A total of 13 TLRs have been identified where each confers recognition of conserved motifs from large subclasses of exogenous bacterial (lipopeptides, lipopolysaccharides (LPS) from Gram-negative bacteria, viruses, yeast, and fungi ligands (Akira et al. 2006; Kaisho & Akira 2004; Carpenter & O'Neill 2007) and endogenous ligands released from cells undergoing non-programmed necrotic death.

Microglia have been shown to express the TLRs 1 to 9 where the TLRs 2, 3 and 4 have been implicated in turning microglia into a pro-inflammatory phenotype after peripheral nerve injury. Genetic ablation studies using mice lacking TLR2, 3 or 4 have shown decreased expression of spinal microglial markers and pro-inflammatory cytokines after nerve injury (Kim et al. 2007; Obata et al. 2008; Tanga et al. 2005) (See Table 1-3).

II. Interferon γ

Interferon γ (IFN- γ) is a type II IFN inflammatory cytokine implicated in early immunological responses to viral and tumour insults, as well as slow developing adaptive responses to infections (Mamane et al. 1999). IFN- γ production in the spinal cord has been identified in T-cells (Schoenborn & Wilson 2007; Schroder et al. 2004). *In vitro* application of IFN- γ is capable of inducing a strong microglial response. In naive conditions, spinal microglia express the IFN- γ receptor cytokine which, when stimulated, activates microglia and generates long-term pain hypersensitivity evoked by non-noxious stimuli (Badie et al. 2000) (see Table 1-3).

III. Complement components

The complement system is formed of a number of soluble and membrane-associated proteins that interact to opsonise microorganisms and to induce an inflammatory response that contributes to the resolution of the infectious process (Walport 2001). Complement activation produces the C5 cleavage fragment C5a, a potent inflammatory mediator, which recruits and activates immune cells. Following nerve damage microglia express the complement component-5 (C5) and its receptor C5aR. The complement component functions described in Table 1-3 indicates that

complement activation converging on C5a and C5aR in microglia contributes to nerve injury-induced cold allodynia (Griffin et al. 2007a; Levin et al. 2008).

IV. Purinergic receptors

Extracellular nucleotides are known to play pivotal roles in regulating glial and neuronal function in the nervous system through P2 purinoceceptors. P2 purinoceceptors are divided into the ionotropic P2X receptors (P2XRs) and metabotropic P2Y receptors (P2YRs) (Tsuda et al. 2010). P2X receptors are family of cation permeable ligand gated ion channels with a role in ATP binding (Khakh et al. 2001; North 2002; Vial et al. 2004). A number of P2XR and P2YR subtypes have been shown to be expressed on microglia to control several properties of microglia, including the motility of their fine processes, the release of cytokines, migration and phagocytosis (Ferrari 1997; Koizumi et al. 2007). Following peripheral nerve injury, dorsal horn microglia are upregulated and display an increased expression of various purinergic receptors.

The first evidence to demonstrate a causal role of microglia was the finding that nerve-injury induced pain hypersensitivity is suppressed with the use of instraspinal P2X4R inhibitors (Tsuda et al. 2003). Conversely, Data showing that spinal administration of P2X4R-stimulated microglia caused normal rats to develop allodynia indicated that P2X4R activation in microglia is not only necessary but is also sufficient to cause tactile allodynia (Tsuda et al. 2003; Tsuda et al. 2005). Further studies demonstrated the necessity of P2X4Rs through pharmacological blockade or conditional ablation of P2X4Rs to cause a marked reduction in neuropathic pain (Tsuda et al. 2003; Ulmann et al. 2008; M. Tsuda et al. 2009). *In vitro* studies show an application of TLR proteins such as nucleotide-binding oligomerisation domain 2

and extracellular matrix (ECM) proteins fibronectin are capable of increasing P2X4R expression in culture (Guo et al. 2006; Toulme et al. 2010; Nasu-Tada et al. 2006; Tsuda et al. 2010). Intrathecal administration of fibronectin leads to PI3K-Akt and MEK-ERK activation, which are known to play distinct roles in P2X4R expression up-regulation in microglia and generate neuropathic pain *in vivo* (Makoto Tsuda et al. 2009). Biber et al. indicate that injured DRG neurons directly contribute to P2X4R expression in microglia through the CCL21 chemokine, which is rapidly expressed in injured DRGs and transported to their central terminals in the dorsal horn (Biber et al. 2011). The activation of P2X4R microglia has been linked to cause activation of the P38 signalling cascade to produce BDNF (Beggs & Salter 2010). This neurotrophic factor acts on dorsal horn neurons to induce neuronal hyperexcitability via the BDNF receptor TrkB (Coull et al. 2005b; Beggs & Salter 2010). Other examples of purinergic receptors and their role in microglial induced neuropathic pain are described in Table 1-3.

V. Fractalkine

Fractalkine is the sole member of the CX3C class of chemokine (Asensio & Campbell 1999) and is capable of acting as either a soluble or membrane-bound mediator or signals through the G protein-coupled chemokine receptor CX3CR1, expressed on monocytes, natural killer cells, T cell and smooth muscle cells. In contrast to many other chemokines, FKN only binds one receptor (CX3CR1) and its receptor only binds FKN (Jung et al. 2000; Hughes et al. 2002). In the nervous system, CX3CR1 is expressed in microglia, by satellite cells in the DRG and human embryonic neurons (Verge et al. 2004). In the spinal cord, FKN is expressed by neurons (LINDIA et al. 2005; Clark et al. 2009) whilst its receptor CX3CR1 is expressed by spinal microglia

(Verge et al. 2004; LINDIA et al. 2005). In neuropathic pain conditions Catepsin S is released from microglia to liberate FKN from dorsal horn sensory neurons to generate a positive feedback loop where FKN binds onto CX3CR1 receptors on microglia and contributes to the overall nociceptive behaviour (Wodarski et al. 2009). Following nerve injury, whilst FKN immunoreactivity remains unaltered in the dorsal horn, CX3CR1 is extensively up-regulated (Verge et al. 2004; LINDIA et al. 2005). The role of FKN in neuropathic pain is described in Table 1-3.

VI. Metalloproteinases

The matrix metallopoteinase's (MMPS) belong to a large family of endopeptidases that require a zinc binding domain to fulfill their enzyme activity. MMPS have been implicated in the pathogenesis of neuro-inflammatory diseases through the cleavage of extracellular matrix proteins, cytokines and chemokines (Rosenberg 2002; Parks et al. 2004). Although there are over 20 MMPs, the gelatinases MMP2 and MMP9 are better understood. MMP2 and MMP9 are found in the ECM with important roles in neuro-inflammation and involvement in CNS diseases including Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis, brain and spinal cord trauma, epilepsy and stroke (Ji et al. 2009). Some examples of metalloproteinase's and their role in microglia are described in Table 1-3.

Table 1-3: Examples of microglial receptors and their function in the context of neuropathic pain.

Chemical mediator	Examples	Function
Toll-like receptors	TLRs 1 to 9	• Switch microglia into a pro-inflammatory phenotype after peripheral nerve injury. • Pro-inflammatory cytokine release.
	TLR2	• Mediate nerve injury induced NAPDH oxidase 2 (Nox2) expression in spinal cord microglia, microglia activation and pain hypersensitivity (Lim et al. 2013).
	TLR3	• Spinal microglial p38 MAPK activation after SNL(Mei et al. 2011).
	TLR4	• CCI-induced microglial activation and pain hypersensitivity (Hutchinson et al. 2008).
Interferon γ		• Activate macrophage phagocytosis and microglia • Generate long-term pain hypersensitivity evoked by non-noxious stimuli - tactile allodynia (M. Tsuda et al. 2009).
Complement components	C5a and its receptor CD88	• Production of inflammatory cytokines, reactive oxygen species, and bioactive amines, among other inflammatory mediators (Woodruff et al. 2010).
	Complement component-5 (C5) and its receptor C5aR	• Blockade of the formation of C3a and C5a or blockade of C5aR attenuates mechanical or cold allodynia in neuropathic rats (Twining et al. 2005; Griffin et al. 2007b). • Intrathecal administration of a peptide that mimics C5a induces cold allodynia in naïve animals (Griffin et al. 2007b). • Depletion of spinal C3 activates C5 and alleviates established allodynia in neuropathic pain models (Levin et al. 2008).
Purinergic receptors	P2X4R	• P2X4R activation in microglia is not only necessary but is also sufficient to cause tactile allodynia (Tsuda et al. 2003; Tsuda et al. 2005). • Activation results in BDNF release by microglia.
	P2XR7	• Regulate mechanical and thermal hypersensitivity after partial sciatic nerve ligation (Chessell et al. 2005). • Induce IL-1B release in LPS-primed microglia *in vitro* and ATP stimulated microglia in vivo to contribute to neuropathic pain (Clark, Staniland, et al. 2010). • High concentrations of extracellular ATP promotes the release of active Cathepsin S from microglia, via P2X7 receptor activation, to contribute to neuropathic pain (Clark, Wodarski, et al. 2010).

	P2Y12R	• Induce tactile allodynia after nerve injury (Tozaki-Saitoh et al. 2008; Kobayashi et al. 2008).
CX3C class of chemokine	Fractalkine	• Intrathecal administration of the chemokine domain of FKN leads to significant mechanical (Milligan et al. 2004; Milligan et al. 2005; Clark et al. 2007) and thermal hypersensitivity (Milligan et al. 2004; Milligan et al. 2005). • IL-1B, IL-6 and nitric oxide release (Milligan et al. 2005).
Metallopoteinase's	MMP 2	• MMP-2 is transiently increased and correlates with the delayed response in DRG satellite cells and spinal astrocytes (also through IL-1β cleavage) which is consistent with the late phase of neuropathic pain (Kawasaki et al. 2008; Ji et al. 2009).
	MMP 9	• Early phase of pain hypersensitivity and induce IL-1β release and microglial activation after SNL (Kawasaki et al. 2008).
	MMP 24	• Regulate mechanical allodynia after nerve injury (Komori et al. 2004).

(b) Microglial proliferation

Glial activation is common in the CNS in response to nerve injury. Following peripheral nerve injury, resident microglia have been shown to undergo extensive proliferation. Recent evidence suggests that microglial numbers increase following peripheral nerve injury to proliferation of resident microglia rather than recruitment of microglia from the blood stream (Ajami et al. 2007; Mildner et al. 2007). Echeverry et al. describe the temporal and spatial pattern of glial cell proliferation in the spinal cord after CCI. A proliferative 'burst' of glial cells occurs in the ipsilateral side of the dorsal and ventral horn following nerve injury. The majority of these dividing cells are identified as microglia which continue to proliferative for up to two weeks with a close correlation to the development of neuropathic pain (Echeverry et al. 2008). Elsewhere, it has been shown that dorsal horn laminae I to III manifest a higher density of microglia after peroneal nerve ligation (F. Zhang et al. 2008).

(c) Microglial migration to the site of injury

Microglial migration can also cause microglial number to accumulate at the site of injury. Microglial migration is triggered by a pathologic insult when ramified microglia undergo activation to transform into the amoeboid form and migrate to the site of injury. There are many candidate molecules that can serve as pathological signals and initiate microglial migration and act as chemoattractant molecules. These molecules include purines, chemokines, bradykinin, and complement components (see Table 1-4).

Table 1-4: Examples of molecules that regulate microglial function

Molecule	Example	Function
Purines	ATP	• Stimulating microglia to release IL-1β (Ferrari 1996; 1997), plasminogen (Inoue 1998), and TNF-α (hide et al. 2000). • Regulating cell motility in microglia *in vitro* (Honda et al. 2001). • P2Y12 receptors mediated chemoattraction (Haynes et al. 2006). • P2Y12 receptor regulates nerve injury-induced mechanical allodynia (Tozaki-Saitoh et al. 2008). • ATP binding with P2X4 to induce microglial chemotaxis (Ohsawa et al. 2007). • ATP binding to P2X4 results in BDNF release.
Chemokines	MCP1/ CCL2	• Up-regulated after peripheral nerve injury in dorsal horn astrocytes (Suter et al. 2009) and DRG neurons and transported to the central terminals of primary afferents in the dorsal horn (Abbadie et al. 2003; Tanaka et al. 2004; White et al. 2005; Zhang & De Koninck 2006). • Regulates tactile allodynia after nerve injury (Abbadie et al. 2003; Tanaka et al. 2004; Thacker et al. 2009).
	Fractalkine	• Causes intracellular calcium release and induces actin rearrangement and morphology change in microglia (proinflammatory) (Maciejewski-Lenoir et al. 1999). • FKN generates directed microglial chemotaxis *in vitro* (Maciejewski-Lenoir et al. 1999; Imai et al. 1997).
Endogenous peptide	Bradykinin B1	• Regulate microglial motility (Ifuku et al. 2007). • Regulate mechanical and thermal hypersensitivity (Ferreira et al. 2005). • Regulate tactile and cold allodynia in diabetic rats (Talbot et al. 2010b).
Complement components	C5a	• C5a intrathecal application produces a dose-dependent slow-onset of cold pain in naive animals (Griffin et al. 2007b) • Participate in inflammatory response (Yao et al. 1990). • Induce immediate intense ruffling of cell membranes with lamellipodia extensions developing within seconds of cell membrane ruffling (Nolte et al. 1996)

(d) Increased phagocytosis

Phagocytosis is normally exerted by phagocytic cells, such as microglia and macrophages. When microglia detect exposed ‘eat me’ signals, they rapidly recognise and engulf neurons or part of neurons. Eat-me signals can be recognised by opsonins, which are soluble, extracellular proteins that promote cell engulfment by binding to eat-me signals on the target cells as well as the phagocytic receptors on the phagocyte (Brown & Neher 2012). In general, Microglial phagocytosis involves two distinctive types of receptors, one with a high affinity to bind to foreign microbial pathogens, such as TLRs, and another recognising apoptotic cellular substances, such as triggering receptors expressed on myeloid cells 2 (TREM-2). Through a sequence of signaling events, morphogenesis, cell movement and phagocytosis are driven by dynamic remodeling of the actin cytoskeleton (see Table 1-5).

Table 1-5: Examples of receptors and molecules capable of inducing microglial induced phagocytosis

Microglial receptors/molecules	Function
$P2Y_{12/13}$	• Membrane ruffling and chemotaxis in microglia (Honda et al. 2001; Nasu-Tada et al. 2005).
P2Y6	• UDP stimulation causes microglial morphology change (filopodia-like protrusions and phagosome-like vacuoles) (Koizumi et al. 2007).
Complement receptor 3 (Mac-1)	• Removal of neuronal debris in the developing brain and synapse elimination in the degenerating CNS (Stevens et al. 2007).
Phosphatidylserine and milk fat globule EGF factor 8 (MFG-E8)	• Exposure of phosphatidylserine onto the cell surface by damaged neurons, acts as a crucial eat-me signal to induce microglial phagocytosis (Neher et al. 2011; Neniskyte & Brown 2013; Neniskyte et al. 2011; Elward & Gasque 2003; Zhu et al. 2012). • MFG-E8 is bound to exposed phosphatidylserine and activates phagocytosis via VNR is a receptor expressed on microglia (Neher et al. 2011; Neniskyte & Brown 2013). • Fraktalkine release by damaged neurons has been linked to increasing the release of the bridging protein MFG-E8 to further perpetuate microglial phagocytosis (Cardona et al. 2006; Noda et al. 2011; Brown & Neher 2014).
TLR agonists: • LPS, • Lipoteichoic acid • Amyloid-β (Aβ))	• Induces slow, progressive loss of neurons by phagocytosis in neuron–microglia co-cultures.

(e) Antigen presentation and recruitment of T-cells

It has been shown that nerve injury causes significant albumin immunoreactivity in the lumbar spinal cord (Gordh et al. 2006) caused by an increase in the blood-spinal cord barrier permeability, which is controlled by both neuronal and immune mechanisms. With nerve injury, it is also known that there is the recruitment of CNS antigen presenting cells and the expression of major histocompatibility complex class II (MHC II) molecules. MHC II molecules are a set of polymorphic genes responsible for presenting antigenic peptides to T lymphocytes, which are found only on antigen

presenting cells (APCs). It has been shown that microglia express MHC II following peripheral nerve injury due to contribute to injury-induced allodynia. Using MHC II deficient mice, the SNT model of nerve injury was used to demonstrate that in the absence of MHC II, mice display attenuated allodynia (Sweitzer & DeLeo 2002). Microglia are also capable of communicating with $CD4^+$ lymphocytes via a CD40 - CD154 interaction. After L5 spinal nerve ligation there is a significant increase of $CD40^+$ microglia in the ipsilateral side of the lumbar spinal cord. CD40 knock-out mice exhibited significantly less mechanical hypersensitivity (Cao et al. 2009).

The antigen presenting feature of microglia seems to have a functional relevance in neuropathic pain as T cells have been shown to infiltrate the spinal cord after nerve injury (Sweitzer & DeLeo 2002) and T cell infiltration has been linked with injury-induced pain behaviours. Functional T-cell deficient mice have been shown to display attenuated mechanical hypersensitivity after axotomy, which can be reversed by transferring exogenous CD4+ lymphocytes (Cao & DeLeo 2008; Costigan, Moss, et al. 2009). T helper 1 (Th1) lymphocytes (pro-inflammatory) secrete interferon γ (IFN γ). Mice that lack the IFN γ receptor showed significant attenuation of nerve injury-evoked microgliosis and allodynia (M. Tsuda et al. 2009). Glatiramer acetate is an immunomodulatory drug that acts partially through shifting the population of T cells from pro-inflammatory Th1 cells into regulatory Th2 cells. Administration of this drug following nerve injury found that changing the balance between Th1 and Th2 cells resulted in a reduction in microgliosis and allodynia (Leger et al. 2011). Taken together, these studies suggest that presentation of antigens by microglia and the consequent T-cell recruitment contributes to the development and maintenance of axotomy-induced allodynia. However, the source of microglial antigen presentation by microglia following nerve injury through MHC class II expression remains to be

addressed. It is hypothesised that it may be derived from the injury site due to prolonged inflammation, or conversely, may be of central origin (Grace et al. 2011). The range of microglial functions involved in neuropathic pain is represented in **Error! Reference source not found.**.

1.5.4 Are microglia always bad?

'Glial activation' per se is not always bad - microglia are neurotoxic but they can also be neuroprotective (Nguyen et al. 2002; Glezer et al. 2007). There is evidence supporting the role of resident microglia and those derived from the bone marrow in the stimulation of myelin repair (Glezer et al. 2006), removal of toxic proteins from the CNS through phagocytosis and the prevention of neurodegeneration in chronic brain diseases (reviewed by (Glezer et al. 2007; Polazzi & Monti 2010)). Schwartz et al. have proposed that a well-controlled glial response induced by signals from injured neurons, T cells and other microglia determines whether glia are neuroprotective or excitotoxic (Schwartz et al. 2006). Depending on the context in which they are stimulated, distinct patterns of microglial activation occur (e.g. LPS induces pro-inflammatory cytokine release, which kills infected cells as well as non-infected bystander cells whilst glia activated by growth factors or anti-inflammatory cytokines, such as IL-4, promote neuronal survival (Butovsky et al. 2001)). In the context of neuropathic pain the aim would be to preserve functions that may help repair such as phagocytosis of redundant synapses and prevent those aspects which may lead to excessive neuropathic pain such as BDNF release.

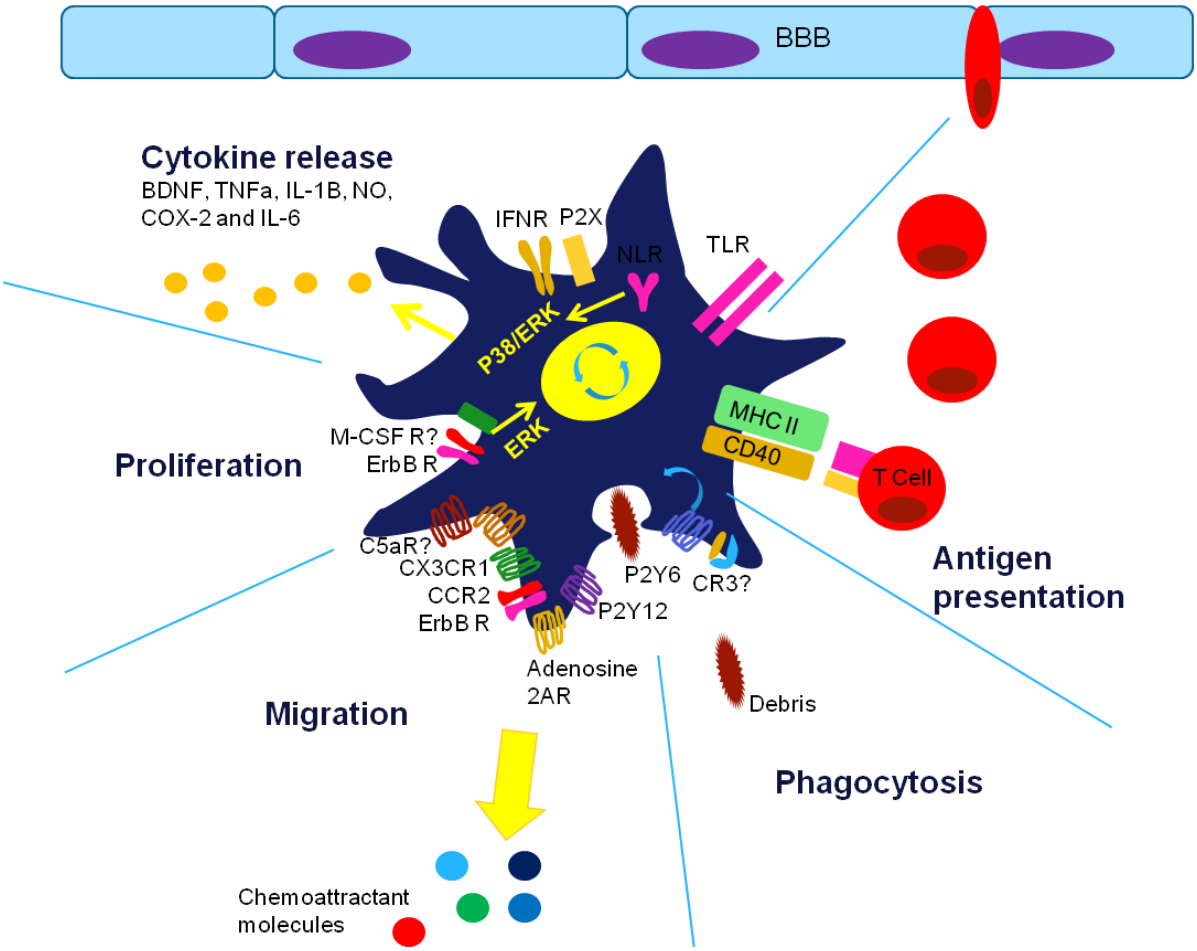


Figure 1.1 The functional diversity of the microglial response. This figure adapted from (Calvo & Bennett 2012) summarises the key capabilities of microgliosis after peripheral nerve injury. Following peripheral nerve damage, signals (e.g. NRG1) are released from damaged primary afferents to can act on microglia through its various ligand binding receptors (e.g. erbB receptors). Upon ligand binding, intracellular signalling cascades are activated in microglia to allow them to migrate to the site of injury where they proliferate in number. Here, they make a significant contribution to the release of pro-inflammatory cytokines, which leads to CNS inflammation. Microglia are also capable of phagocytosis and antigen presentation to encourage T cell infiltration from the damaged Blood brain barrier (BBB).

1.6 Neuregulins

Given our laboratory's research interests in understanding the role of Neuregulin in the PNS and CNS after nerve injury (Fricker et al. 2013; Fricker et al. 2009; Cheret et al. 2013; Calvo et al. 2010; Calvo et al. 2011), our research group previously used pharmacological approaches to understanding Neuregulin 1 (NRG1) signaling in the context of neuropathic pain. The experiments in this thesis now attempt to utilise a transgenic approach and selectively target NRG1 and its receptors to understand their role in microglia after nerve injury.

1.6.1 Isoforms

Neuregulins were first identified in 1992-1993 by four research groups. Two of the research groups described a growth factor (heregulin (HRG) and neu-differentiation factor (Neu)) capable of activating the proto-oncogene erb2 (Holmes et al. 1992; Peles et al. 1992); the third described a factor capable of stimulating the proliferation of Schwann cells (glial growth factor (GGF)) (Goodearl et al. 1993) and the fourth described a factor capable of stimulating acetylcholine activity receptors at neuromuscular synapses (acetylcholine receptor-induced activity (ARIA)) (Peles et al. 1992). Marchionni et al. were the first group to recognise that all of these factors were products of the same gene and proposed a new gene name called Neuregulin (NRG) (Marchionni et al. 1993a).

Since the first identification of the NRG gene, three other genes encoding related proteins were discovered and described as NRG1- 4. All 4 NRG genes encode epidermal growth factor (EGF) like domains and are capable of mediating their effects through erbB tyrosine kinase receptors (Marchionni et al. 1993b; Carraway et

al. 1997; Zhang et al. 1997; Harari et al. 1999a). Whilst the NRG1 gene is extensively studied, much less is understood about the role of NRG's 2, 3 and 4.

The human NRG1 gene is extensive, spanning approximately 1.4 megabases (Falls 2003). This gene, located on the short arm of the human chromosome 8 (Orr-Urtreger et al. 1993), is subject to a series of alternative splicing reactions and differential transcription start sites that have led to over 31 isoforms to be identified. (Mei & Xiong 2008). All isoforms contain either an α or β EGF-like domain, named for its structural homology to epidermal growth factor EGF, which is necessary and essential for biological activity (Lemke 1996; Meyer & Birchmeier 1995). The three structural characteristics that are capable of differentiating isoforms with respect to their *in vivo* functions and cell biological properties are the type of EGF-like domain (α or β), the N-terminal sequence, and whether the isoform is initially synthesised as a transmembrane or non-membrane protein. Given these differences, NRG1 isoforms can be categorised into six types identified as Type I-VI. Functionally, the most widely studied isoform sub-types include Types I-III whilst less is understood about IV-VI types (Mei & Xiong 2008).

Together, Type I and II isoforms are sometimes referred to as "Ig-NRG's because they contain a single transmembrane domain and immunoglobulin-like (Ig-like) domain in their N-terminus, and type II isoforms also have a kringle-like domain in their N-terminus. The function of the Ig-like domains is thought to lie in their high affinity binding to heparan-sulphate proteoglycans (HSPGs) present on the cell surface and in the extracellular matrix. By interacting with HSPGs, Ig- NRGs are thought to be concentrated in the vicinity of erbB receptors and therefore enhance signalling (Loeb & Fischbach 1995; Li & Loeb 2001). Type III isoforms contain two

transmembrane domains and a cysteine rich domain (CRD) in the N- terminus and are thus described as CRD-NRGs (see **Figure 1-1**).

Most NRG1 isoforms are synthesised in the pro-NRG1 form where the pro-NRG1 exists as a membrane anchored precursor with the EGF domain positioned outside of the cell. Pro-NRG1's undergo proteolytic cleavage at the juxtamembrane region that lies on the carboxy-terminal side of the EGF domain. With the induction of proteolytic cleavage, a diffusible mature form of NRG1 is produced. The generally held model is that type III isoforms remain tethered to the membrane because the second transmembrane domain in the N- terminus remains anchored to the protein after cleavage (Wang et al. 2001). There is recent evidence however that these type III NRG1 isoforms can undergo a second proteolytic cleavage event mediated by tumour factor-α converting enzyme (ADAM17) resulting in the release of the EGF domain (Fleck et al. 2013). NRG1 type II-β3 does not have a transmembrane domain and consequently does not have a 'pro' stage, being directly secreted from the cell (Falls 2003).

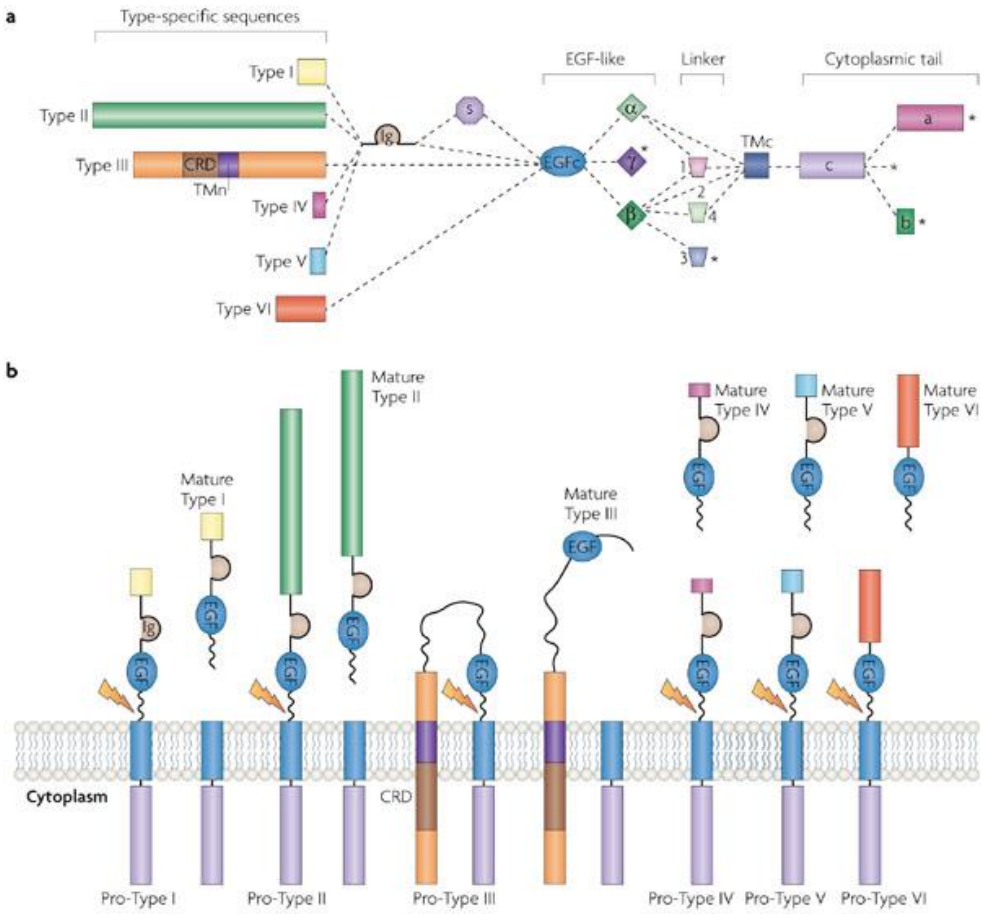


Figure 1-1: The six types of Neuregulin 1 (NRG1) isoforms are classified according to their distinct amino-terminal sequences. (a) Depicts the similarities and differences in the sequences of each of the 6 NRG1 isoforms. (b) Most NRG1 isoforms are synthesised as transmembrane precursor polypeptides (pro-NRG1s) with the EGF domain located in the extracellular region, but in Type III NRG1 both the N- and the C-terminal regions are located inside the cell. Cleavage (indicated by the lightning arrow) generates mature NRG1s that are soluble, except in the case of Type III NRG1, which is thought to function in a manner that requires cell contact. The processing of Type IV, Type V and Type VI pro-NRG1s is less well characterised but is thought to resemble that of Type I and Type II. (Modified from (Mei & Xiong 2008).

1.6.2 Proteolytic cleavage

Proteolytic cleavage of NRGs to produce the mature form has been shown to be catalysed by three type I transmembrane proteases called tumour factor-α converting enzyme (TACE or ADAM17) (Loeb et al. 1998; Montero et al. 2007), β-site of amyloid precursor protein cleaving enzyme (BACE) (Hu et al. 2006; Willem et al. 2006) and meltrin beta (ADAM19) (Yokozeki et al. 2007). Narilysin is a metalloendopeptidase and linker protein that binds ADAM17 to induce NRG1 cleavage and enhances metalloproteinase cleavage of BACE to also cleave NRG1 (Nishi et al. 2006; Hiraoka et al. 2007; Herrlich et al. 2008).

1.6.3 NRG1 Signaling through extracellular tyrosine kinase (erbB) receptors

Initial crystallographic studies have shown that there are four members of the erbB protein family that are capable of forming homodimers, heterodimers or oligomers (Burgess et al. 2003) upon activation by a subset of known growth factor ligands (including NRG1). The EGF like domain of all NRG1 isoforms can only bind the erbB3 and 4 receptors. The erbB2 receptor lacks a ligand binding domain but is a crucial co-receptor responsible for mediating signal transduction (Carraway & Cantley 1994). A strict hierarchy of receptor dimerisation exists where erbB2 is the preferred partner for heterodimerisation (Graus-Porta et al. 1997) and therefore heterodimerises preferentially with erbB3 to transduce its signal downstream (Graus-Porta et al. 1997) (see **Figure 1-**).

1.6.4 Paracrine and juxtacrine Signaling

NRG1 is capable of signalling in a paracrine fashion where proteolytic cleavage of NRG1 type I and II from the membrane releases mature NRG1 as a diffusible protein

signal that activates erbB receptors on nearby cells (Falls 2003). The most well described example of NRG1 mediated paracrine signalling is seen in cardiac development. Normal cardiac development requires NRG1 signalling between the NRG1 expressing endocardium and the developing myocardium, which expresses erbB receptors. The endocardium and myocardium are not physically touching and thus require communication through diffusible factor (i.e. NRG1) (Falls, 2003). Previous studies have shown that NRG1-erbB signalling is indispensable in cardiac development (Gassmann et al. 1995; Lee et al. 1995; Meyer & Birchmeier 1995; Lemmens et al. 2007).

NRG1 type III is not secreted following cleavage; its second transmembrane domain tethers it to the membrane where it remains anchored (Wang et al. 2001). NRG1 type III can only activate erbB receptors on cells it is directly in contact with, and therefore NRG1 type III signals in a juxtacrine fashion. Juxtacrine NRG1 signalling has been shown to be crucial in the peripheral nervous system as axo-glial signalling is contact dependent and this is essential for normal peripheral nervous system development (Falls 2003).

1.6.5 'Forward Signaling'

NRG-1 erbB signalling can involve either forward or backward (non-canonical) signalling routes. Forward signaling by NRG's, involves NRG binding to the extracellular domain of the erbB3 and erbB4 receptors to induce erbB receptor dimerisation. This dimerisation leads to the activation of the erbB kinase domain to induce auto- and trans-phosphorylation of the intracellular domains. This process has been shown to require erbB endocytosis (Gu et al. 2005; Liu et al. 2007; Yang et al.

2005). Forward canonical signaling activates Raf-MEK-ERK, P13K-Akt-S6K, c-Abl, JNK, CDK5, Fyn, and Pyk2 pathways (reviewed by (Mei & Xiong 2008)). In non-canonical forward signaling, the juxtamembrane-a (JMa) isoform of ErbB4 is initially cleaved by TACE to release a soluble extracellular peptide that contains the NRG1 binding site (ecto-erbB4). The remaining membrane-anchored fragment which exists in the transmembrane form is further cleaved by presenilin-dependent γ-secretase to release the erbB4 intracellular domain (Lee et al. 2002; Ni et al. 2001). This domain is then translocated to the nucleus to regulate further transcription (Sardi et al. 2006).

1.6.6 'Backward Signaling'

Backward signaling describes a component of the bidirectional signaling capabilities. NRG1 type III is known to signal 'forwardly' through juxtacrine binding of erbB receptors on nearby cells. Additionally, Bao et al. 2003 provide *in vitro* evidence for non-canonical signaling, where erbB receptors that are either membrane bound or soluble erbB ectodomains bind to induce γ-secretase dependent cleavage of NRG1 type III. This releases an intracellular domain (ICD) of ~50kDa which can translocate to the nucleus and regulate transcription associated with cell survival (Bao et al. 2003) (reviewed by (Mei & Xiong 2008)).

1.6.7 Neuregulin-1 function in the Peripheral Nervous System

In the PNS, the entire program of developmental Schwann cell differentiation and myelination is controlled by NRG1 type III expressed by axons (Garratt et al. 2000; Jessen & Mirsky 2005). In adulthood NRG1 is not required for myelin maintenance but contributes to the response to nerve injury where NRG1 has the capabilities of interacting not only between injured axons and Schwann cells but also between axons

and macrophages and microglia.

Transgenic and conditional ablation studies have demonstrated the roles played by soluble and transmembrane NRG1 isoforms in the PNS during development and after nerve injury. These roles are summarised in **Table 1.** (modified from (Gambarotta et al. 2014)).

NRG1		Developmental myelination	Peripheral nerve regeneration
Soluble	Knockout	+	—
	Overexpression	+	++
Transmembrane	Knockout	—	—
	Overexpression	++	++

Table 1.3 The role played by soluble/transmembrane NRG1 isoforms in the PNS during development and after nerve injury determined using transgenic and conditional knockout mice. NRG1 transgenic models have been generated to understand its role in myelination and regernation (+ symbol equates to 'does not affect' whilst – symbol equates 'impairs', ++ sybmol equates to 'improvement' (modified from (Gambarotta et al. 2013)).

1.7 NRG1/erbB signaling in the CNS

The roles of NRG1- erbB signaling in the CNS are extensively reviewed by (Mei & Xiong 2008) but to summarise, the other roles of NRG1- erbB signaling in the CNS include dendritic spine maturation (Barros et al. 2009), neuronal migration during early brain development, axon guidance and synapse formation. NRG1 has been shown to be released from neurons to promote the formation and maintenance of radial glial cells, which are necessary for the radial migration of neurons from ventricular zones to the pial surface (Rio et al. 1997; Anton et al. 1997). *In vitro* data suggests that the inhibition of erbB signaling in glial cells is sufficient to block neuronal migration along radial glial fibres (Rio et al. 1997). NRG1 has been shown to regulate tangential migration of GABAergic interneurons in the cortical region as well as regulating the thalamocortical axons through the diencephalon by corridor cells expressing NRG1 (López-Bendito et al. 2006). NRG1 has been shown to regulate synapse formation between excitatory synapse formed between glutamatergic terminals and spines (reviewed by (Mei & Xiong 2008)). NRG1 is not essential for oligodendrocyte myelination but may modulate this process (Makinodan et al. 2012).

1.7.1 NRG1/erbB signaling in microglia

Whilst the role of NRG1 in the PNS after peripheral nerve injury are well characterised, the role of NRG1 in the CNS after peripheral nerve injury have been recently investigated. *In vitro* data shows cultured neurons capable of releasing soluble NRG1 in an activity dependant manner (Ozaki et al. 2004) and peripheral nerve injury has been shown to cause the production and/or release of NRG1 into the CNS indicating this could be a source of axoglial signalling. Calvo et al., investigated the role of NRG1 erbB signalling pathway after peripheral nerve injury demonstrating

that NRG1 stimulates microglial survival, proliferation and chemotaxis and IL-1β release *in vitro* (Calvo et al. 2010). After spinal nerve ligation, a marked increase in NRG1 was observed in the dorsal horn with evidence of erbB2 activation within microglia. Using an erbB2 pharmacological inhibitor PD 168393, they demonstrate that blocking the erb2 receptor after SNL prevents proliferation of microglial cells and blocks p-38 activation within microglia (Calvo et al. 2010). Sequestering the endogenous release of NRG1 after SNL also prevents microglial proliferation or P38 activation. Intrathecal administration of NRG1β generated mechanical and cold pain related hypersensitivity in a dose dependant manner and conversely injury related hypersensitivity could be reversed by blockade of NRG1-erbB signalling. Calvo et al. further investigated the intracellular signalling pathways downstream of the initial NRG1- erbB binding on microglia. Their findings show that intrathecal administration of NRG1 induced the phosphorylation of the extracellular signal-regulated kinase (ERK1/2) signalling pathways as well as the protein kinase B (Akt) signalling pathways but did not activate the P38 mitogen-activated protein kinase (P38 MAPK) pathway (Calvo et al. 2011). With the use of specific kinase inhibitors they show that the mitogenic effects of NRG1 on microglia was attributed to the MEK/ERK 1/2 signalling, whilst the chemotactic effects of NRG1 are attributed to PI3K/Akt signalling with microglial survival being dependant on both pathways. Furthermore, a MEK inhibitor U0126 was capable of partially attenuating mechanical hypersensitivity and cold hypersensitivity (Calvo et al. 2011).

Another study has also implicated NRG1- erbB signalling in microglia to contribute to the development of neuropathic pain. Xiang et al. used the paw incision model to demonstrate that injury induced microglial activation and mechanical allodynia

associated with significantly increased NRG1 type I and II mRNA expression levels in the DRG and in the spinal cord at the protein level. Using erbB pharmacological inhibitors and siRNA directed against NRG1 type I and II, the authors demonstrated suppression of incision induced microglial activation and mechanical allodynia (Xiang et al. 2014).

1.7.2 Limitations to using pharmacological approaches to understand NRG1-erbB signalling

With the use of pharmacological inhibitors, certain limitations arise. With the use of small molecule or a mutation targeting the same protein or pathway radically different phenotypes are likely to be produced. More specifically, phenotypic differences have been shown to exist between kinase inhibitors and their relevant knockout models as highlighted by a review on the use of Genetics or pharmacology to probe protein functions in cells (Knight & Shokat 2007). Initially kinases were considered as poor drug targets when studying the effects of pharmacological inhibitors due to the large number of protein kinase enzymes all sharing a common cofactor and similar three-dimensional structure of the catalytic site and thus confounding attempts at targeting selective sites. Whilst some of these issues have been overcome with the development of small molecule inhibitors, developing mono-specific or very highly specific kinase inhibitors for therapeutic use, clinical experience or for understanding kinase biology is still a considerable challenge (Cohen 2002). Additionally, with the use of erbB2 inhibitor PD 168393, it is possible that the erbB2 inhibitor is also targeting the EGF-erbB1 signalling pathway as well as the NRG-1 erbB signalling pathway, and therefore blocking normal cellular responses such as proliferation and cell survival activated by EGF binding.

The limitations arising from the use of pharmacological inhibitors can be overcome with the use of genetic animal models. Previous studies have investigated the effects of global mutation of the 3 erbB receptors and shown that erbB2 and erbB3 are embryonically lethal owing to a lack of ventricular myocyte differentiation along with cranial ganglia defects (Erickson et al. 1997), whilst global erbB4 mutation also leads to lack of cardiac development and a lack of axon guidance in the CNS (Gassmann et al. 1995; Birchmeier 2009). Owing to cardiac complications, via global erbB mutations, the use of conditional mutations/selective ablation of the erbB receptor is likely to prove more useful in understanding the role of NRG1- erbB signalling in microglia after peripheral nerve injury. The erbB2 receptor is essential for NRG-1 erbB signalling pathway but targeted ablation of erbB2 is also likely to interrupt the EGF-erbB1 signalling pathway. Upon NRG1 ligand binding, erbB3 and erbB4 heterodimerise with erbB2. By selectively targeting these two receptors, it would allow us to investigate the NRG1-erbB signalling pathway in microglia without interrupting the EGF-erbB signalling pathway. In order to selectively target erbB3 and erbB4 in the myeloid lineage (i.e. macrophages and microglia) the Cre-Lox-P system was used. A transgenic approach is taken using a Cd11b Cre (where the Cre is expressed under the monocytic Cd11b promotor) and CX3CR1 Cre to selectively target and ablate erbB3 and erbB4 in the myeloid lineage. This selective ablation will allow us to better understand the role of NRG-1-erbB signalling in microglia after peripheral nerve injury.

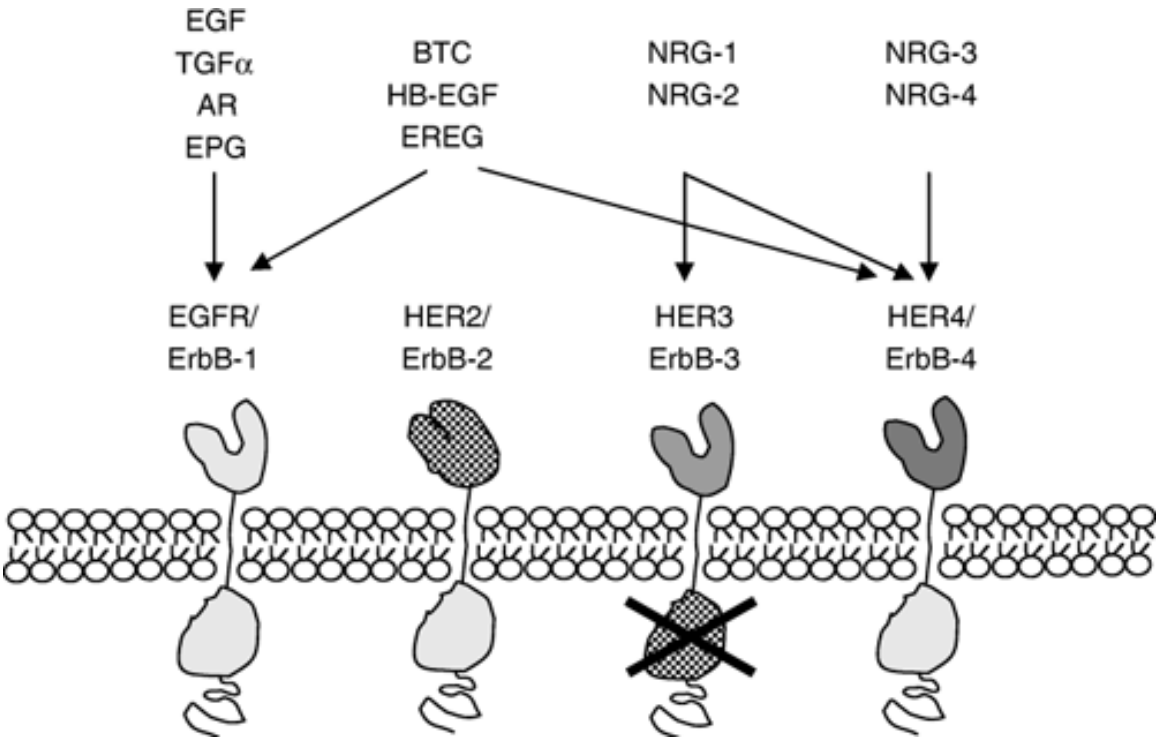


Figure 1-4: Examples of the erbB family of receptors and their cognate ligands. The Neuregulins (NRGs), which can exist in different alternatively spliced isoforms, bind ErbB3 and ErbB4. This pattern of ligand/receptor interactions is complicated by the fact that upon ligand binding, ErbB receptors can homo- or heterodimerise and cross-phosphorylate each other. In these interactions the ligandless ErbB2 and the kinase-defective ErbB3 receptors also participate where the most potent heterodimer in terms of triggering proliferating and survival signals is the ErbB2/ErbB3 heterodimer (modified from Berasain et al. 2007).

1.7.3 Conditional transgenic mice

Cellular diversity within a developing organism is achieved through the spatial and temporal regulation of gene expression, which in turn is, determined by internal genetic programs and inductive signals. As a consequence, genes have multiple functions within spatial and temporal dimensions that make it difficult to dissect (Belteki et al. 2005).

By utilising two members of the integrase family of site specific recombinases, P1 bacteriophage Cre and *Saccharomyces cerevisae* FLP, scientists are now capable of achieving a level of sophistication that allows any spatial and temporal modification of the mouse genome that varies from genetic activation, inactivation, conditional knock-in, and conditional knockout, of genes of interest (Nagy 2000).

Cre and FLP are both capable of regulating tissue specific, site specific and heritable chromosomal DNA recombination events (Orban et al. 1992) but for the purpose of this thesis, only the role of Cre recombinase is discussed.

Lox P sites consist of a 34 base pair (bp) sequence composed of two 13 bp palindromic sequences which are separated by an 8bp spacer region. By flanking a segment of DNA on either side with a Lox-P sequence, Cre recombinase can recognise and bind to these sites to catalyse the reciprocal DNA recombination of the segment between the two sites. By expressing Cre recombinase under the control of tissue specific promoters/enhancers, targeted DNA is only recombined in the desired tissue. This is termed a conditional knockout, allowing spatial control of genetic modifications.

The spatial control of genetic modifications can be activated during embryogenesis to produce a progeny in which the conditional allele is inactivated only in those tissues

or cells that express Cre. An example of such a system used in this thesis is the Cd11b Cre recombinase. Cd11b encodes the α subunit of the Mac-1 leukocyte integrin heterodimer, a major adhesion molecule and a commonly used haematopoietic surface marker (Ferron & Vacher 2005) and induces DNA recombination in monocyte and macrophage cells during development (Ferron & Vacher 2005).

1.7.4 Inducible Transgenic Cre systems

There are certain limitations in inducing the knockdown of gene(s) of interest during development, owing to the complications of such genes being expressed in multiple tissues, with multiple crucial functions, during development (e.g. NRG1). NRG1 has been shown to: regulate the development of crucial functions during development; be indispensible for cardiac development through directed cell formation, growth and survival; be implicated in neural development through axon guidance, myelination and ensheathment, and synapse formation. Generating a global knockout of NRG1 has lead to embryonic lethality owing to cardiac functions (Falls 2003). In order to understand the function of such important genes, the temporal control and ablation of genes can be conducted using drug inducible Cre recombinases system.

1.7.4.1 Drug inducible Cre system

Inducible transgenesis through the administration of Tamoxifen *in vivo* and *in vitro* provides specific temporal and spatial control of gene function. Ligand dependant chimeras such as CreER recombinases have been developed to add inducubility into traditional Cre lox P systems. Here, the Cre recombinase is fused to the mutated hormone binding domains of the human oestrogen receptor (ER). In mice, the mutant ER receptor cannot bind to natural ligands at physiological concentrations. The CreER fusion protein is restricted to the cytoplasm which can only be released and

can gain access into the nucleus with exposure to the synthetic oestrogen receptor ligand- Tamoxifen or 4-OHT (Feil et al. 1996; Feil et al. 2009). Using the drug inducible Cre recombinase system, unwanted Cre activity and associated side effects can be limited, and thus confer a significant advantage over the traditional transient Cre expression during development system (Loonstra et al. 2001).

Another Tamoxifen inducible Cre system used in this thesis is the CX3CR1^{ERTM} (Goldmann et al. 2013) to exclusively target gene ablation in microglia. CX3CR1 is a Fraktalkine G-protein coupled receptor that is exclusively expressed on microglia. A targeting vector is used to replace exon 2 of the chemokine receptor Fraktalkine (CX3CR1) gene with a Cre^{ERTM} coding sequence. Similarly, like the CAG Cre^{ERTM} mechanisms, CX3CR1 Cre^{ERTM} is located in the cytoplasm and unable to transport to the nucleus without Tamoxifen administration. The CX3CR1 is being increasingly used as a promoter for the exclusive visualisation and genetic manipulation of microglia specifically to understand the microglial contribution to various CNS systems (Wolf et al. 2013; Goldmann et al. 2013). Microglia are a self-renewing population (Ajami et al. 2007) with a low turnover rate (Lawson et al. 1992) whilst inflammatory macrophages exhibit a rapid turnover rate (van Furth & Cohn 1968) and are replenished through a bone marrow precursor population that is CX3CR1^{-} (Fogg et al. 2006). Upon Cre-mediated recombination, microglia undergo recombination that persists but peripheral CX3CR1^{+} populations initially undergo recombination and are then quickly replaced by non-recombined infiltrating cells from CX3CR1^{-} progenitors in the absence of sustained Cre activity.

1.8 Thesis Aims

The aim of this thesis is to investigate the role of NRG1-erbB signalling on microglial cells in the development of neuropathic pain following peripheral nerve injury. Using various pharmacological approaches, our research groups have previously studied the functional role of NRG1 and shown that NRG1-erbB signalling in microglia contributes to the development of microgliosis and for regulating mechanical pain related hypersensitivity and cold allodynia after nerve injury (Calvo et al. 2010; Calvo et al. 2011). The objective of this thesis is to now utilise a transgenic approach with new microglial specific transgenic Cre lines to confirm and delve further into the complex behavioural outcomes of NRG1-erbB signalling in microglia in the context of neuropathic pain.

Chapter 2 investigates NRG1 (and its subtypes) expression in the PNS and CNS of mice after nerve injury *in vivo*. Chapter 3 highlights the limitations of the current behavioural tools used to measure cold allodynia and describes a novel behavioural assay to measure cold hypersensitivity/allodynia in mice. Chapter 4 uses the Cre-Lox-P system to generate conditional ablation of NRG1 and then investigates the role of microglia in neuropathic pain in the absence of NRG1. Chapter 5 utilises the Cd11b Cre system to generate tissue specific knockdown of erbB receptors in microglia to measure the behavioural outcomes after peripheral nerve injury. Finally Chapter 6, examines the role of NRG-erbB signalling by conditionally ablating erbB3 and 4 receptors on microglia using CX3CR1 Cre^{ER}.

2 Investigating changes in Neuregulin-1 and extracellular tyrosine kinase (erbB) receptor gene expression after peripheral nerve injury in mice.

2.1 Introduction

Nerve injury results in marked changes in gene expression at multiple levels of the somatosensory system including; the glial and immune cells of the damaged nerve; the cell bodies of sensory and motor neurons; and the dorsal horn of the spinal cord (within neurons and glia). Such changes in gene expression are relevant to nerve repair/regeneration as well as the evolution of neuropathic pain.

Neuregulin's are a family of epidermal growth like factors where NRG's 1-4 are capable of binding and phosphorylating extracellular receptor tyrosine kinases (erbB receptors). Such receptors are found on almost all cell types to mediate downstream intracellular signalling (Kanzaki et al. 2012; Harari et al. 1999b; Carraway & Cantley 1994; Marchionni et al. 1993b) (Please refer to chapter 1 for detailed discussion of NRG1 and its receptors).

Previously, most of the emphasis in studying NRG-erbB signalling has been placed on understanding axoglial interactions within a damaged nerve. Limited information is available on the neuregulin or erbB receptor gene expression changes that occur in a model of neuropathic pain. Thus, in this chapter, I utilise a model of nerve injury in mice to investigate alterations in the expression of mRNA encoding isoforms of NRG 1-4 as well as neuregulin receptors (erbB 2-4) in PNS and CNS targets including the dorsal horn of the spinal cord.

2.2 Methods

2.2.1 Animals

All experimental work on rodents was carried out accordingly to UK Home Office legislation (Scientific Procedures Act 1986). Adult mice (8 weeks or older) were used for tissue extraction and behaviour.

2.2.2 Experimental animals

A cohort 40 wild type mice of C57BL6 origin (acquired from Harlan (UK)) were recruited and split equally into experimental groups (n= 10 sham; n=10 SNI day 3, n=10 SNI day 7, n= 10 SNI day 21). Where possible, equal genders were included into each cohort to minimise bias. Having analysed previous literature, most models used n=8-10 per group to conduct pain behaviour experiments in rodents (experimenters observation). A more practical step in the research protocol involves determining the optimal sample size for a study to ensure adequate power to detect statistical significance but also to minimise the unnecessary waste of animals and resources. To determine the sample size, a power calculation can be used. The power calculation was conducted using an online tool (http://www.biomath.info/power/ttest.htm). Given that there is likely to be control and "treated" group(s), the null hypothesis was that there is no difference between the means of the two groups. The alternative hypothesis was that the means differ in a two sided manner. The group 1 mean was specified to be of 1.0; Group 2 mean was specified to be 1.4; with a standard deviation of 0.2 a power of 0.8 and a significance level of 0.05. Using the aforementioned values, the power calculation reports the minimum sample size as n=6 where if complete data is collected from n=6, we are capable of detecting statistical significance between groups should it occur.

2.2.3 Surgery

The spared nerve injury (SNI) model was used to measure behavioural outcomes and mRNA expression changes before and after injury. Mice were anaesthetised with isoflurane; the left sciatic nerve trunk was exposed; and the three branches of the sciatic nerve were identified. Lidocaine was applied directly onto the nerve for 30 sec. Connective tissue was cleared around the common peroneal and tibial branches of the sciatic nerve. Using non-dissolvable sutures, a ligation was made around the common peroneal and tibial branches individually before a 2mm length of each branch was cut distal to the ligature (to prevent re-innervation). The wound was closed with 5.0 sutures or staples and postoperative analgesia (Marcain and Rimadyl) was given immediately and 24 hours after surgery. With sham surgery, the left sciatic nerve was exposed and then the area was closed using 5.0 sutures or staples. Similarly, post-operative analgesia was given to sham mice.

2.2.4 Animal behavioural experiments

All behavioural tests were carried out whilst the observer was blind to the experimental group of the animal. Animals were tested in a temperature stable room during the light period of their daily day/night cycle, at the same time every day. Where possible, equal numbers of animals and gender were recruited into each cohort.

2.2.4.1 Mechanical withdrawal threshold testing using Von Frey filaments

Unrestrained animals were placed and acclimatised in acrylic cubicles (5 x 5 x 10cm) atop a perforated polymer resin platform with approximately 5 x 5mm square holes. The intervening metal grid is approximately 1mm wide, which is comfortable enough for the animal and allows easy view of the target area of the paw (37450-277, Ugo Basile, Italy). Animals were acclimatised for 60 minutes before testing. Calibrated Von Frey hairs were applied to the plantar surface of the hind paw until the

monofilament fibre was bent. Mice exhibit a paw withdrawal threshold when the paw is touched unexpectedly (Chaplan et al. 1994). The 50% withdrawal threshold was calculated using the up-down method (Dixon 1980). Results were expressed as the mean 50% withdrawal threshold for each genotypic group. The Von Frey test was conducted prior to injury (three baseline measurements) and after sham surgery or spared nerve injury at day 7 and day 21.

2.2.4.2 Thermal nociception threshold testing using Hargreaves' test

Thermal nociception or heat pain thresholds can be determined using the Hargreaves test (Hargreaves et al. 1988). Unrestrained animals were placed into acrylic cubicles (5 x 5 x 14cm) atop a uniform glass plate and allowed to acclimatise for 60 mins before testing. The Hargreaves apparatus (37370, Ugo Basile, Italy) was set to 23 which is equivalent to a radiometer reading of 103±10mW/cm^2. The infrared light source was placed under the plantar surface of the hind paw and the latency to paw withdrawal was measured in seconds. A total of three responses were recorded for each hind paw with an interval of at least 2 mins between each test. To avoid tissue injury, the maximum stimulus latency was set to 30.0 secs. Results were expressed as the mean latency to withdraw for each group. The Hargreaves test was conducted prior to injury (three baseline measurements) and after sham surgery or spared nerve injury at day 7 and day 21.

2.2.5 Tissue extraction

Animals were sacrificed using CO_2 and fresh tissue was dissected. The lumbar L3-L5 spinal cord was extracted and split into ipsilateral (injured) and contralateral (uninjured) blocks of tissue but the spinal cord was not split into dorsal and ventral blocks of tissue. Ipsilateral and contralateral DRG's L3-L4 were extracted and the proximal segments of the sciatic nerve from both sides were also extracted (the distal

nerve was very poorly disintegrated so the immediate proximal site to ligation was extracted for analysis). Fresh Tissue was processed for investigating mRNA expression changes after injury. Tissue was extracted on days 3, 7 and 21 after injury whilst the sham tissue was extracted on day 7.

2.2.6 Quantitative PCR

RNA extracted from the relevant tissues was then used to synthesise into first strand cDNA using the Roche Reverse Transcriptase protocol and kit (Roche Cat no: 03 531 295 001). To summarise, For each 20µl reaction, 5µg of RNA template/samples were added to PCR grade water to make to 11.5µl and mixed with 2µl of random primers/hexamers before being incubated at 65ºC for 10 min before being placed on ice. To this mixture, 4µl of Transcriptor RT reaction buffer, 2µl of dNTPs and 0.5µl of transcriptor reverse transcriptase (all from Roche) were added. The samples were then vortexed and spun briefly before being incubated at 25ºC for 10 min, followed by 55ºC for 30 min and then the enzyme was inactivated by heating to 85ºC for 5 min before being placed on ice. Complementary DNA (cDNA) was then ready to use for qPCR experiments.

The Roche qPCR protocol (LightCycler® 480 SYBR Green I Master Cat no: 04 887 352 001) was used to investigate mRNA expression changes of erbB3 and erbB4 in the transgenic line generated. To briefly summarise, cDNA from appropriate samples was diluted to 2.5ng/µl of which 4µl was added to 10µl SYBR Green I master mix, followed by 2µl of primer pairs and 4µl of PCR grade water. The samples were then loaded onto a MWP plate and into The Roche Life cycler 480 where it was ready to run.

The qPCR cycling protocol was run as follows: Pre-incubation at 95ºC for 5 min, followed by 45 cycles of the Amplification step including 95ºC for 10 sec, 60ºC for

20 sec and 72ºC for 10 sec. Following this, the melting curve step included 95ºC for 5 sec and then 65ºC for 1 min and a final cooling phase of 40ºC for 30 sec. The primer pair sequences used are listed in Table 2.1. Primers for NRG's were selected by using Ensembl (http://www.ensembl.org/index.html) to search for gene sequences and designed using NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) where the sequences were also tested to ensure annealing specificity.

The internal housekeeping genes selected were GAPDH and HPRT to compensate for reverse transcription and amplification efficiency variation. Such housekeeping genes were selected because they are some of the most commonly ideal internal control genes uniformly expressed during all environmental or experimental conditions (Bär et al. 2009). For analysis, mRNA expression levels of genes of interest were normalised to housekeeping genes and measured as fold changes from baseline values. The comparative cycle threshold (Ct) method was used to compare Ct values of the genes of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The Ct values of both the calibrator and the samples of interest are normalised to an appropriate endogenous housekeeping gene. The comparative Ct method is also known as the 2– Δ Δ Ct method, where:

$$\Delta \Delta Ct = \Delta Ct \text{ (sample)} - \Delta Ct \text{ (reference)}$$

Here, Δ CT (sample) is the Ct value for any sample normalised to the endogenous housekeeping gene and Δ Ct (reference) is the Ct value for the calibrator also normalised to the endogenous housekeeping gene.

Control reactions contained water instead of cDNA and therefore produced no amplification whilst melting curve analysis confirmed specificity of all products.

2.2.7 Statistical Analysis

The variable within the behavioural cohort was whether the animals are grouped into the sham or spared nerve injury groups. The statistical test used for behavioural outcomes was one way ANOVA on ranks, post-hoc Dunn's Method. The statistical test used for mRNA expression data was repeated measures one way ANOVA with the post-hoc Holm-Sidalk test. Results are reported as mean values ± SEM.

Table 2.1 primer sequences used in rt-qPCR experiments

Gene name	Forward sequence	Reverse sequence
Pan NRG1	CGTAATGGCCAGCTTCTACA	CCATGTTGTTTCGTTCTGACC
NRG1 Type I	GGAAGGGCAAGAAGAAGGACC	GTTTCACACCGAAGCACGAG
NRG1 Type II	CTGACAACTGGACCCTACTC	TTTCACAGCCCACACCTG
NRG1 Type III	CCAGACACCCACACAGAAGATG	TCCACAAATACCCACTTTAGGC
NRG2	CGACCCTAACGGCAAAAACATC	GTCTGGCTCTTCATCTTCTTCAGC
NRG3	TGCTTTGCTCAACAGGAAGGAG	AGGGTGGCAGACTATTTGGTTTC
erbB2	ATGAAGCGTACGTCATGGCT	CAGGTAGCTCATCCCCTTGG
erbB3	TTGCCTACAGGAACGCTTACCCG	ACCCCCCAAAACCGCAGAATC
erbB4	GGACCCACAGAAAATCACTGCCAG	TCCATAGCACCTGCCATCACATTG
IBA1	CTTGAAGCGAATGCTGGAGAA	GGAGCCACTGGACACCTCTCT
P2X4	TGGCAGCGACAAGGCGAC	GCCTGAGATGTGTACAATGAACC
ATF3	GTGCCTGCAGAAAGAGTCAGA	GAGGTTCCTCTCGTCTTCCG
GAPDH	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG
HPRT	GTCCTGTGGCCATCTGCCTAG	TGGGGACGCAGCAACTGACA

2.3 Results

2.3.1 Mice develop significant mechanical hypersensitivity after nerve injury

Using the Von Frey test, it was evident that mice developed marked mechanical hypersensitivity after spared nerve injury as shown in Figure 2-. The 50% withdrawal threshold at baselines was reported as 0.824 ± 0.047g for the sham group and 0.930 ± 0.061g for the group that was due to undergo nerve injury. There was no difference between the two groups at baseline. After injury, the sham groups retained normal 50% withdrawal threshold values at 0.782 ± 0.073g whilst the SNI group developed significant mechanical hypersensitivity with a marked reduction in 50% withdrawal threshold values maintained through the observation period (0.031 ± 0.007g at day 7 and 0.031 ± 0.008g at day 21 after injury) (n= 8-10 per group, One way ANOVA on ranks, post-hoc Dunn's Method *p<0.05 SNI vs. BL. Sham BL vs. Sham D7 NS).

2.3.2 Mice develop significant thermal hypersensitivity after nerve injury

Using the Hargreaves test, it was also evident that mice developed significant thermal hypersensitivity after spared nerve injury as shown in Figure 2-. The withdrawal latency measured at baseline was reported to be 7.450 ± 0.492s for the sham group and 8.644 ± 0.812s for the group that was due to undergo nerve injury. There was no difference between the two groups at baseline. After injury, the sham groups retained normal latency to withdrawal values at 7.925 ± 0.690s whilst the SNI group developed significant thermal hypersensitivity which was demonstrated through the shorter latency to withdrawal values (4.010 ± 0.380s at day 7 and 4.321 ± 0.633s at day 21 after injury) (n= 8-10 per group, One way ANOVA on ranks, post-hoc Dunn's Method *p<0.05 SNI vs. BL. Sham BL vs. Sham D7 NS).

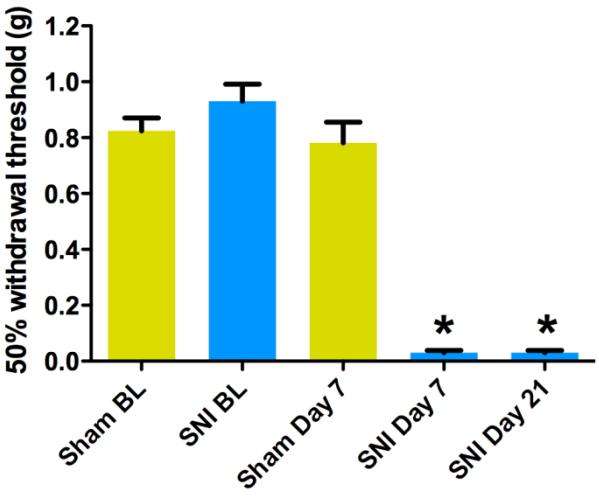


Figure 2-1: Mechanical hypersensitivity develops in mice after spared nerve injury. Mice develop a marked decrease in 50% withdrawal threshold to mechanical stimuli at day 7 and day 21 after injury. Sham animals do not develop mechanical hypersensitivity but maintain normal 50% withdrawal threshold levels. (n= 8-10 per group, One way ANOVA on ranks, post-hoc Dunn's Method *p<0.05 SNI vs. BL. Sham BL vs. Sham D7 NS).

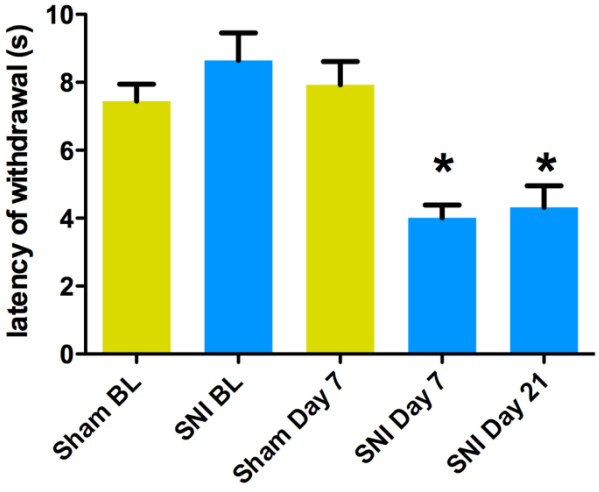


Figure 2-2: Thermal hypersensitivity develops in mice after spared nerve injury. Mice that underwent SNI show a significant reduction in their latency to withdraw to thermal stimuli at day 7 and 21 after injury. Sham animals maintain normal baseline values at day 7 after sham surgery (n= 8-10 per group, One way ANOVA on ranks, post-hoc Dunn's Method *p<0.05 SNI vs. BL. Sham BL vs. Sham D7 NS).

2.3.3 Up-regulation in mRNA expression of injury markers is detected after injury

2.3.3.1 ATF3 mRNA expression is significantly upregulated after nerve injury.

ATF3 is a neuronal damage marker and was implemented as positive control to test for neuronal damage after spared nerve injury (Chen et al. 1996; Drysdale et al. 1996; Yin et al. 1997). Messenger RNA expression of ATF3 was significantly upregulated at days 3, 7 and 21 after injury in the spinal cord and DRG compared to sham as show in Figure 2-.

In the spinal cord, at day 3, there was a significant 10.982 ± 3.295 fold up-regulation compared to sham whilst at day 7 there was also a significant 9.311 ± 1.706 fold up-regulation compared to sham. By day 21, the ATF3 mRNA expression was still elevated compared to sham at 4.099 ± 0.442 fold change. Therefore ATF3 expression changes showed robust neuronal damage in the spinal cord using the spared nerve injury model (n=5 per group, *$p<0.05$, **$p<0.005$ one way ANOVA). The increased expression in ATF3 represents the increased expression from damaged afferents in the dorsal horn and increased expression motor neurons in the ventral horn.

In the DRG, there was also robust neuronal damage as identified by ATF3 mRNA expression changes. At day 3, there was a significant 6.762 ± 31.329 fold up-regulation compared to sham whilst at day 7 there was also a significant 4.891 ± 1.159 fold up-regulation compared to sham. By day 21, the ATF3 mRNA expression was still elevated compared to sham at 3.309 ± 0.654 fold change (n=5 per group, *p<0.05, **p<0.005 one way ANOVA). Using ATF3 as a neuronal injury marker, we can determine that the spared nerve injury was robust in generating neuronal damage that lasts throughout the study. This neuronal damage was likely to correlate with the behavioural pain related sensitivity seen after injury.

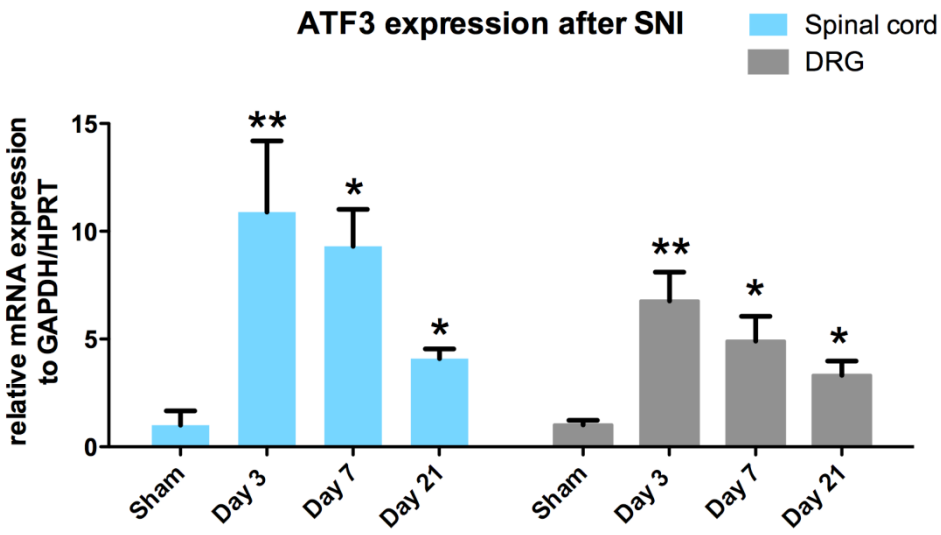


Figure 2-3: ATF3 is significantly upregulated in the spinal cord and DRG after injury. ATF3 levels are significantly elevated at days 3, 7 and 21 in the spinal cord after injury. Similarly, ATF3 mRNA levels are significantly upregulated at days 3, 7 and 21 in the DRG after injury. (n= 5 per group, * p<0.05 repeated measures one way ANOVA post-hoc holm-Sidalk test).

2.3.3.2 PR2X4 mRNA expression was dys-regulated after injury

It has been identified that on-going activation of PR2X4 receptors expressed by microglia is required to maintain never injury induced pain hypersensitivity (Tsuda et al. 2013). Given my interest in microglial function following nerve injury, I therefore investigated expression of this gene.

Figure 2- shows that PR2X4 relative mRNA expression was significantly down regulated at day 3 after injury by a significant 0.673 ± 0.238 fold decrease compared to sham (1.000 ± 0.116). By day 7 however, PR2X4 mRNA expression returned to normal at 1.127 ± 0.264 fold change vs. sham. At day 21, the PR2X4 mRNA expression was measured to be 0.736 ± 0.729 fold change vs sham (n=5 per group, *p<0.05, one way ANOVA).

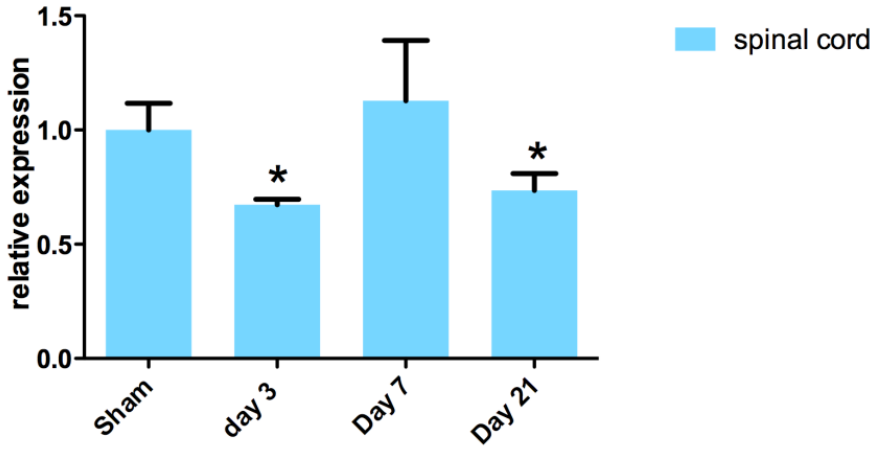


Figure 2-4: PR2X4 mRNA levels fluctuate in the spinal cord after nerve injury. At day 3 and day 21 after injury, P2X4 mRNA levels are significantly reduced when compared to sham in the spinal cord (n= 5 per group, * p<0.05 repeated measures one way ANOVA post-hoc holm-Sidalk test).

2.3.3.3 IBA1 mRNA expression is significantly up-regulated in the DRG and proximal nerve after injury

IBA1 mRNA expression changes were used as a marker for microglial or macrophage expression changes after injury. IBA1 mRNA changes were detected in spinal cord, DRG and sciatic nerve as shown in Figure 2-.

Surprisingly, there was no significant change in IBA1 relative mRNA expression in the spinal cord at day 3, and day 7 after injury vs sham. However, at day 21, there was significant down-regulation of IBA1 in the spinal cord by a 0.412 ± 0.036 fold decrease compared to sham (n=5 per group, *p<0.05, one way ANOVA).

In the DRG however, a significant up-regulation in IBA1 mRNA expression was detected at 3 days post-injury (2.943 ± 0.498) and day 7 (2.604 ± 0.351 fold change) vs. sham (1.000 ± 0.137). IBA1 mRNA expression levels returned to normal levels at day 21 after injury (n=5 per group, *p<0.05, one way ANOVA).

In the proximal segment of the sciatic nerve a significant up-regulation in IBA1 mRNA expression was detected at day 3 (4.972 ± 0.871 fold change) after injury vs. sham (1.000 ± 0.420). IBA1 mRNA expression levels returned to normal levels at day 7 (2.765 ± 0.347 fold change) and day 21 (1.087 ± 0.355 fold change) after injury (n=5 per group, *p<0.05, one way ANOVA).

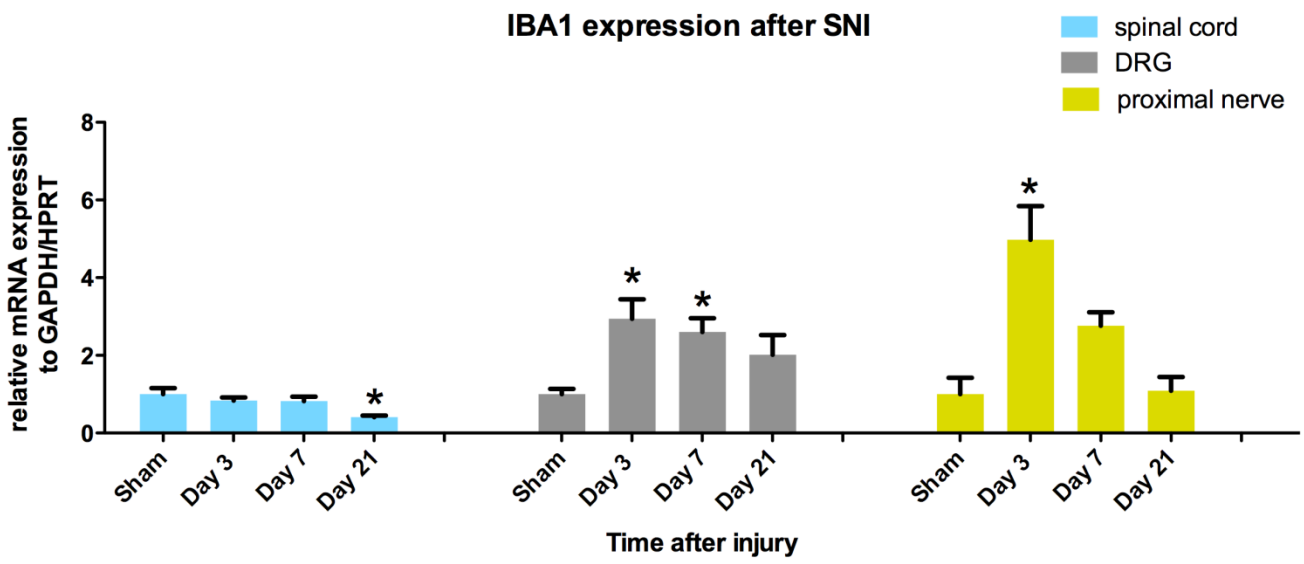


Figure 2-5: Aif-1 mRNA expression is significantly upregulated in the DRG and sciatic nerve after injury. *Aif-1* mRNA levels remain predominantly unchanged in the spinal cord but down-regulate at day 21 after injury. In the DRG, *Aif-1* levels are significantly upregulated until day 7 after injury and in the sciatic nerve, *Aif-1* mRNA levels are upregulated at day 3. These levels return to normal in the sciatic nerve by day 7 after injury (n= 5 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test).

2.3.4 NRG1 mRNA expression is dysregulated after peripheral nerve injury

2.3.4.1 Pan-NRG1 mRNA expression is significantly up-regulated in the spinal cord

NRG1 subtype mRNA expression changes after peripheral nerve injury (PNI) in the spinal cord, DRG and sciatic nerve were investigated. After PNI, NRG1 subtype I, II and III mRNA expression remained unchanged after injury, when compared to sham (**Figure 2-**). However, total NRG1 (Pan NRG1) was significantly upregulated at day 3 (1.483 ± 0.151 fold change) and day 7 (1.557 ± 0.129 fold change) after injury when compared to sham (1.000 ± 0.155). Pan NRG1 mRNA expression returned to normal levels at day 21 (n=5 per group, *p<0.05, one way ANOVA, post-hoc Holm Sidalk test).

2.3.4.2 NRG1 type I is significantly upregulated in the DRG after nerve injury

Within the L3-L4 DRG's, a small but significant up-regulation of NRG1 Type I was detected at day 3 (1.479 ± 0.098 fold change) after injury when compared to sham 1.000 ± 0.135) (**Figure 2-**). At days 7 and 21 there was also an increase in NRG1 type 1 mRNA but this did not reach statistical significance. NRG1 type II and Type III mRNA relative expression did not significantly change after injury and thus total NRG1 levels remained unchanged. (n=5 per group, *p<0.05, one way ANOVA, post-hoc Holm Sidalk test).

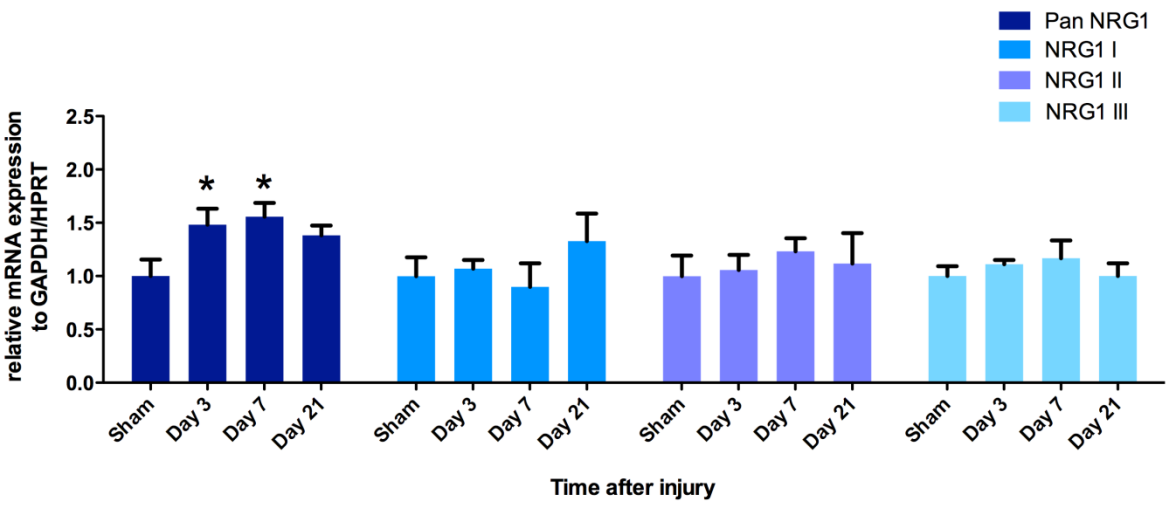


Figure 2-6: Pan-NRG1 is significantly upregulated in the spinal cord after injury. With injury, there is no evident NRG1 subtype that is dysregulated after injury. However, Pan NRG1 levels are significantly upregulated at day 3 and day 7 after injury when compared to sham (n= 5 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test).

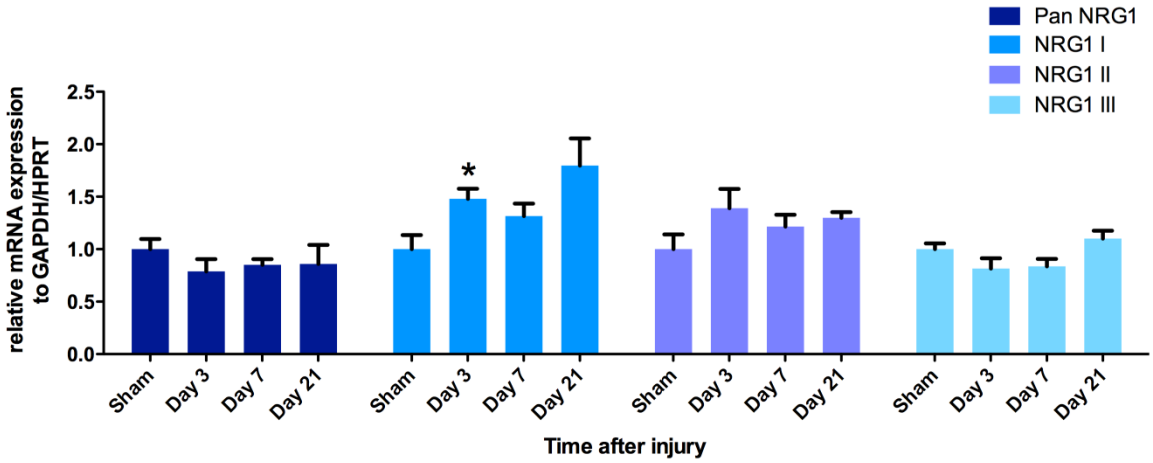


Figure 2-7: NRG1 is significantly upregulated in the DRG after injury. Within the DRG, NRG1 Type I is identified as significantly upregulated at only day 3 after injury when compared to sham. Other subtypes and thus total NRG1 levels remain unchanged after injury (n= 5 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test).

2.3.4.3 NRG1 type I is also significantly upregulated in the sciatic nerve after nerve injury

The proximal segment of the sciatic nerve was also analysed to measure NRG1 subtype mRNA levels (Figure 2-). Within the sciatic nerve, NRG1 type I was significantly upregulated at day 3 (9.423 000 ± 0.1.257 fold change) and day 7 (8.185 ± 2.436 fold change) after injury when compared to sham (1.000 ± 0.291). By day 21, the mRNA levels for NRG1 type I return to normal n=5 per group, *$p<0.05$, one way ANOVA, post-hoc Holm Sidalk test). The source of NRG1 is likely to be Schwann cells that function to repair myelination after nerve damage (Carroll et al. 1997).

2.3.4.4 NRG1 type III is significantly down- regulated in the sciatic nerve after injury

Interestingly, NRG1 type III mRNA levels remained unchanged at day 3 (0.786 ± 0.076 fold change) and day 7 (0.847 ± 0.243 fold change) after injury, but were significantly down regulated at day 21 (0.367 ± 0.102) after injury, when compared to sham (1.000 ± 0.073; Figure 2-) (n=5 per group, *$p<0.05$, one way ANOVA, post-hoc Holm Sidalk test) (Figure 2-). NRG1 type II mRNA levels were very low (with a very high Ct ratio) and could not be consistently detected in either sham or injured groups. NRG-2 levels could not be consistently detected and are not reported here.

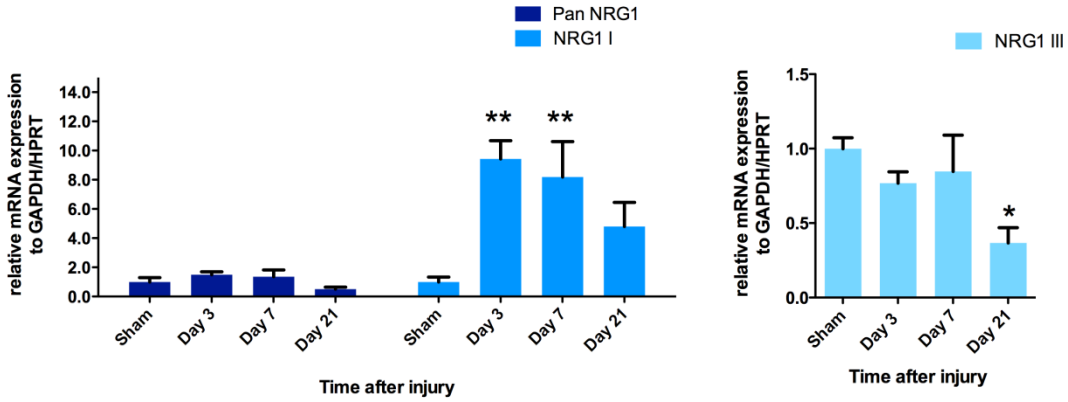


Figure 2-8: NRG1 Type I is significantly upregulated in the sciatic nerve after injury. NRG1-type I is identified as significantly upregulated at days 3 and 7 after injury before returning to normal levels at day 21. NRG1 Type III is significantly down regulated at only day 21 after injury when compared to sham (n= 5 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test).

2.3.5 NRG2 and NRG3 mRNA expression levels remain unchanged in the spinal cord and DRG after nerve injury

Whilst NRG1 is the most studied NRG in association with pain studies, I was interested in determining whether other NRG's may also be regulated after injury in the PNS and CNS. NRG-2 and NRG-3 were found to remain unchanged in the spinal cord after injury when compared to sham (**Figure 2-**). As shown in **Figure 2-**, NRG2 and NRG3 also remained unchanged in the DRG after nerve injury when compared to sham (n= 8-10 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test).

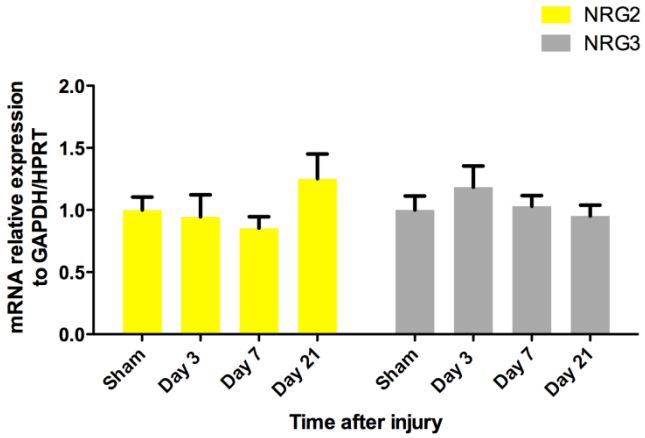


Figure 2-9: NRG-2 and NRG-3 mRNA levels remain unchanged in the spinal cord. NRG-2 and NRG-3 mRNA levels fluctuate after injury but do not significantly differ from sham groups. (n= 5 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test).

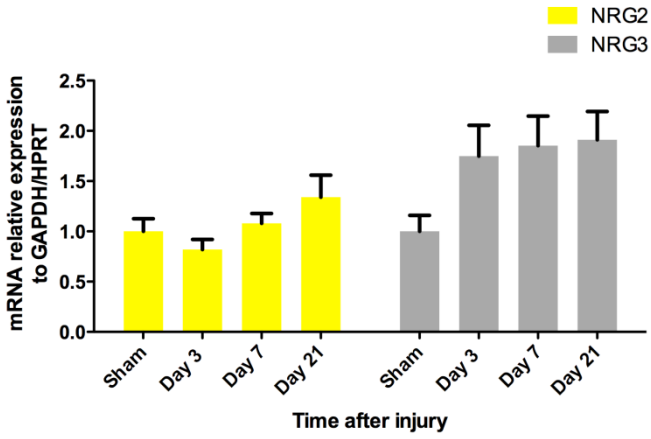


Figure 2-10: NRG2 and NRG3 mRNA levels remain unchanged in the DRG after nerve injury. NRG-2 and NRG-3 mRNA levels fluctuate after injury but do not significantly differ from sham groups. (n= 5 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test).

2.3.5.1 NRG2 is significantly down regulated at day 3 after injury in the sciatic nerve

As shown in Figure 2-, NRG2 levels could be detected and were significantly reduced after nerve injury at day 3 (0.097 ± 0.018 fold change) when compared to sham (1.000 ± 0.636). By day 7 however, NRG2 levels returned to near baseline levels (day 7 - 0.265 ± 0.088 fold change) and thus remained unchanged at day 21 (0.307 ± 0.636) (n=5 per group, *p<0.05, one way ANOVA, post-hoc Holm Sidalk test). The sciatic nerve was also tested for NRG2 and NRG3. NRG3 mRNA levels were out with the range of detection by the qPCR apparatus and experimental setup. NRG3 levels could not be consistently detected and are not reported here.

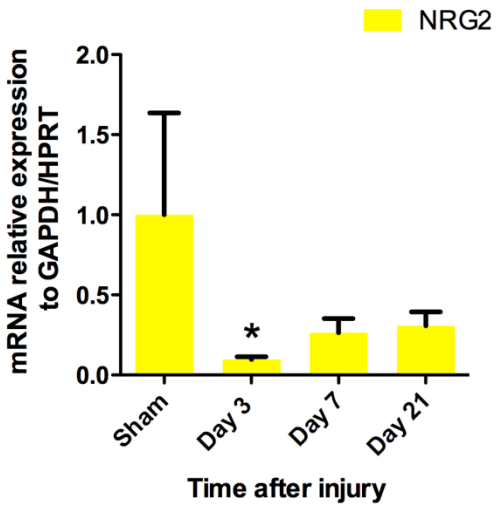


Figure 2-11: NRG-2 mRNA levels are significantly down-regulated in the sciatic nerve after injury. NRG-2 mRNA levels are significantly reduced at day 3 after injury when compared to sham. NRG-2 levels then return to normal by days 7 and 21 (n= 5 per group, * p<0.05 repeated measures one way ANOVA post-hoc holm-Sidalk test).

2.3.6 ErbB mRNA gene expression levels are dysregulated after nerve injury

Microglia are the predominant immune cells expressing erbBRs in the spinal cord (Pearson & Carroll, 2004) whilst primary afferent terminals also express erbB receptors (Mizobuchi et al. 2013). It is worth mentioning that using qPCR as a method to analyse erbB levels in spinal cord tissue, we cannot differentiate between immune cells and primary afferent terminals.

2.3.6.1 ErbB2 is significantly down-regulated in the spinal cord after nerve injury

Very little is known about the level of expression of erbB2, 3 and 4 in the CNS after PNI. Microglia are the predominant immune cells expressing these receptors in the spinal cord. With injury, erbB3 and erbB4 mRNA expression levels remained unchanged relative to sham after injury as shown in Figure 2-. However, erbB2 mRNA levels showed an initial significant reduction in mRNA expression at day 3 (0.643 ± 0.037 fold change) vs. sham (1.000 ± 0.140). This erbB3 mRNA down-regulation returned to normal levels by day 7 and 21 (n= 8-10 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test). As described earlier in section 2.3.6 we cannot differentiate between immune cells and primary afferent terminals using qPCR. Therefore the lack of detection of an increase in microglial markers is difficult to interpret.

2.3.6.2 ErbB2 is significantly down-regulated in the DRG after nerve injury

Similarly, erbB3 and erbB4 levels remained unchanged after injury in the DRG. As shown in Figure 2-, erbB2 mRNA expression levels, were significantly down regulated at day 3 (0.546 ± 0.087 fold change) and day 7 (0.629 ± 0.076 fold change) after injury when compared to sham (1.000 ± 0.127). The mRNA levels of erbB2

returned to normal by day 21 (n= 8-10 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test). The initial downregulation of erbB2 in the DRG after nerve injury is likely to be due to neuronal damage and a loss of Schwann cells which also express erbB2.

2.3.6.3 erbB2, 3 and 4 mRNA levels are significantly down-regulated in the sciatic nerve after nerve injury

At the sciatic nerve, a significant reduction in erbB2 mRNA levels was seen at day 3 (0.249 ± 0.055 fold change) compared to sham (1.000 ± 0.189). The mRNA levels of erbB2 quickly returned to normal levels by day 21 and remained unchanged at day 21. It is interesting to note that erbB3 mRNA levels were also significantly down-regulated at day 3 (0.147 ± 0.034 fold change) after injury compared to sham (1.000 ± 0.232). However, these mRNA levels returned to normal by day 7 and 21 after injury (see **Figure 2-**).

Similarly, erbB4 mRNA levels were also significantly down regulated after injury at day 3 (0.143 ± 0.023 fold change) but not day 7 and 21 after injury when compared to sham (1.000 ± 0.202) (n= 8-10 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test).

Previous studies show that in Schwann cells the expression of Ig-containing isoforms of NRG1 and erbB2 and erbB3 receptors in the distal nerve significantly increases after SNT. This downregulation is first detected 3 days post injury and is sustained for at least 30 days (Carroll et al. 1997; Cohen et al. 1992; Kwon et al. 1997). Guertin and coworkers examined the levels of erbB2 activation immediately following sciatic nerve transection and found that there is also an acute and transient increase in the levels of phosphorylated erbB2 and erbB3 in the first hour following injury, which

decreases again by 3 h postinjury (Guertin et al. 2005). In chronically denervated sciatic nerves, expression levels of erbB receptors are much decreased (Li et al. 1997).

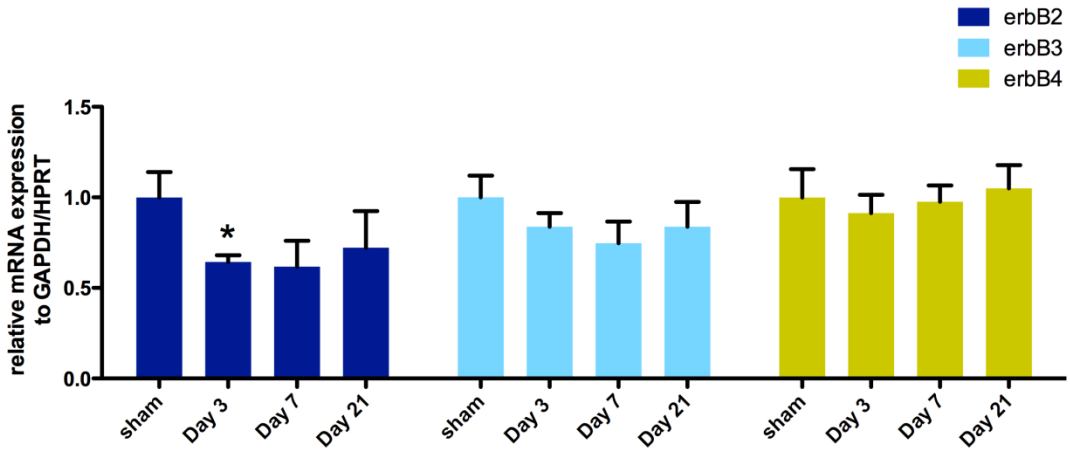


Figure 2-12: erb2 mRNA levels are significantly down-regulated in the spinal cord after injury. mRNA expression levels of erbB2 are significantly decreased at only day 3 after injury and return to levels comparative to sham by day 7. erbB3 and erbB4 levels remain unchanged after injury in the spinal cord (n= 5 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test)

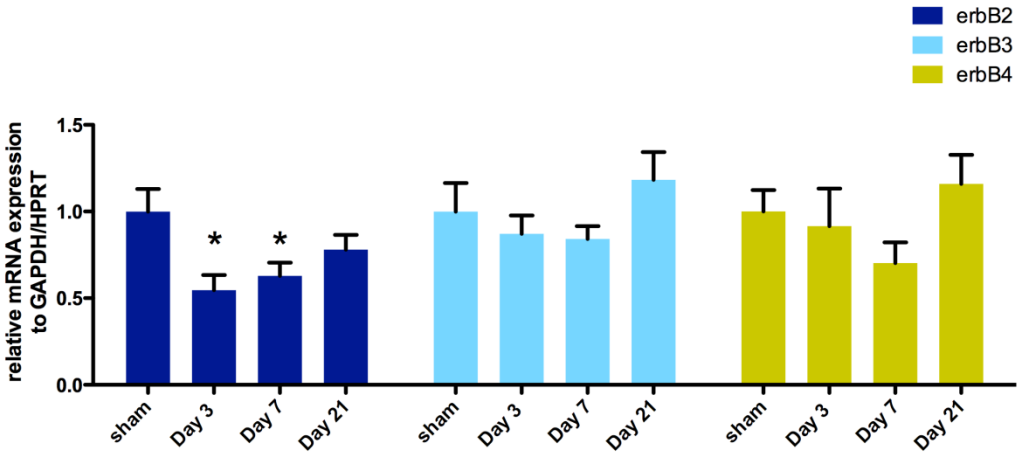


Figure 2-13: erbB2 mRNA levels are significantly down-regulated in the DRG after injury. erbB2 mRNA levels of expression are significantly depleted at day 3 and 7 after injury but return to normal levels by day 21. erbB3 and erbB4 mRNA levels of expression remain unchanged after injury (n= 5 per group, * $p<0.05$ repeated measures one way ANOVA post-hoc holm-Sidalk test).

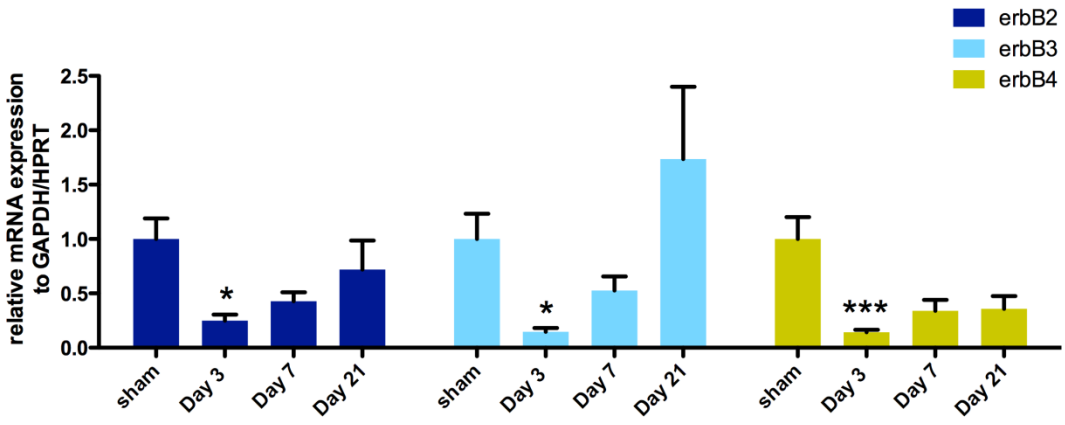


Figure 2-14: erbB2, 3 and 4 are significantly down-regulated in the sciatic nerve immediately after injury. erbB2, 3 and 4 mRNA expression levels seem to only be significantly down regulated at day 3 after injury before returning to normal levels by day 7 after injury. (n= 5 per group, * $p<0.05$, ***$p<0.001$, repeated measures one way ANOVA post-hoc holm-Sidalk test).

2.4 Discussion

The role of NRG1 in nerve injury is likely to be very complex. It is plausible that NRG1 may present some adaptive functions such as mediating nerve repair but it may also present some unwanted effects such as contributing to neuropathic pain. A number of other authors have investigated the expression of components of the NRG signalling pathway in different models of nerve injury. I will draw comparison with these studies to my findings using the SNI model in the different tissues examined. In this chapter, I assessed NRG1 isoform and receptor expression following nerve injury including analysis of both peripheral nervous tissue and the dorsal horn to which injured sensory neurons project. There was significant dysregulation of neuregulin and ErbB expression.

2.4.1 Altered expression of NRG1 isoforms and their receptors in the injured nerve

Within the PNS, an initial mRNA expression increase was detected with IBA1. This is likely to correlate with the immediate major infiltration of macrophages into the site of damage after injury. Macrophages infiltrated the nerve and DRG after injury and levels remained elevated until day 7 before returning to normal at day 21. This timeline correlates with previous studies where the infiltration of IBA1 macrophages into the DRG was prominent at day 3, peaks at day 7 before returning to normal levels by day 14 post-injury (Kim et al. 2011).

Within the sciatic nerve, a significant up-regulation in NRG1 type I was detected at days 3 and 7 and a significant reduction in NRG1 type III was detected at day 21 (when compared to controls). It is known that in Schwann Cells (SCs) the expression of NRG1 isoforms and the erbB2 and 3 receptors in the distal nerve are significantly

increased following SNT until 30 days post-injury (Carroll et al. 1997; Cohen et al. 1992). It is known that during peripheral nerve development, neuronal NRG1 type III is the predominant isoform, capable of regulating the entire lineage of SCs and the amount of NRG1 type III is correlated to the myelin sheath thickness (Taveggia et al. 2010). It has also been reported that at day 3 after nerve injury, SCs undergo injury induced proliferation (Kwon et al. 1997). Here, the NRG1 Type III levels initially decrease and then increase as axons begin to re-innervate their targets (Carroll et al. 1997; Bermingham-McDonogh et al. 1997). Elsewhere, Kerber *et al* use the facial nerve transection model to illustrate a decrease in NRG1 type III in axotomised motor neurons until 14 days post-injury (Kerber et al. 2003). Stasstart *et al* suggest that NRG1 type I is highest at early time points (between days 1 and 4) after injury when axons (and thus neuronal NRG1 type III) are lost by Wallerian degeneration (Stassart et al. 2013).

With these findings, it is likely that immediately after injury, NRG-I type I is overexpressed due to denervated SCs and whilst our study reports a reduction in NRG-I type III only at day 21, it correlates with the denervation pattern of SCs. It is possible that with SNI, the Schwann cell denervation is delayed and NRG1 type III levels only become significantly dysregulated at day 21. It is likely that NRG1 type III is the key instructive signal for sciatic nerve myelination and a reduction initiates SC mediated re-myelination and repair. It is vital to note that the area of the sciatic nerve analysed in this chapter was extracted proximal to the site of injury whilst previous studies investigated the distal segment of the injured nerve. This is likely to explain the differences seen here in comparison to previous studies.

In the injured nerve environment, there are two main sources of erbB gene expression- SCs and macrophages. Given that macrophages infiltrate the site of injury

and the proliferation of SCs after injury, it was hypothesised that there may be a significant upregulation in erbB receptor expression after nerve injury. However, data presented in this chapter suggests that SNI induces a significant reduction in erbB2, 3 and 4 gene expression in the sciatic nerve at 3 days post-injury.

Previous studies suggest that in SCs, the expression of Ig-containing isoforms of NRG1, erbB2 and erbB3 receptors in the distal sciatic nerve significantly increases following nerve transection. This is first detected 3 days post-injury and is persistent for at least 30 days (Carroll et al. 1997; Cohen et al. 1992; Kwon et al. 1997). Kwon *et al* report that erbB2 phosphorylation occurs within SCs and coincides both spatially and temporally (3 days post-injury) with SC mitotic activity (Kwon et al. 1997). Guertin *et al* performed SNT to demonstrate an acute and transient increase in erbB2 and erbB3 phosphorylation within 1 hour post-injury, followed by a decrease at 3 hours post-injury. Elsewhere, Li *et al* used in situ hybridisation, and qualitative immunohistochemistry to compare the expression of c-erbB2 and c-erbB4 receptors in SCs denervated for up to 6 months *in vivo*. They report that in chronically denervated sciatic nerves, expression levels of erbB receptors are significantly reduced (Li et al. 1997). Jonsson *et al* show that erbB3 and erbB4 gene expression is unchanged at 1 month after SNT but modestly reduced at 3 and 6 months after injury whilst erbB2 gene expression levels remain unchanged (Jonsson et al. 2013).

These dissimilar findings raise the possibility of differences in the type of neural damage arising from transection and ligation. Persistent neural damage caused by ligation may lead to continuous increases in erbB2, 3 and 4 expression in a delayed manner. However, nerve transection may only cause an initial decrease in erbB receptor expression before neural damage repair is initiated by SC's. It is also

possible that nerve injury gives rise to initial loss of SCs at the site of damage which is demonstrated through an initial reduction in erbB receptor expression in the early stages after transection. It is also worth noting that previous studies demonstrate there is no detectable erbB1 or erbB4 expression in normal or mutant sciatic nerves, regardless of lesions (Cohen et al. 1992; Levi et al. 1995; Grinspan et al. 1996). An alternative source of erbB4 in the damaged sciatic nerve is likely to be macrophages (Mencel et al. 2013). It is possible that initial injury causes a reduction in resident macrophages (and thus reduction in erbB4 levels) in the early stages of injury before infiltrating macrophages present themselves at the site of injury. These dissimilar findings are also likely to be caused by the issue that in our study, the proximal segment of the injured sciatic nerve was analysed whilst previous studies analysed the distal segment.

2.4.2 Altered gene expression of NRG1 isoforms and their receptors in the injured DRG

The activating transcription factor 3 (ATF3) is often described as an adaptive response gene whose activity is usually modulated by stressful cellular activity. ATF3 is the standard gene marker used for neurons axotomised by PNI (Chen et al. 1996). In this study, the upregulation in ATF3 expression was measured to determine neuronal damage and as an indirect measure of the pre-requisite series of cellular changes that is likely to lead to neuropathic pain. As expected, ATF3 expression was significantly upregulated after SNI at days 3, 7 and 21 (see Figure 2-).

It has been previously shown that DRG neurons express several structurally distinct NRG1 isoforms including NRG1 type I and type III, with Type I being expressed by sensory neurons (Zhang et al. 2006). A recent study reports a decrease in total NRG1 level expression in L5 DRG at day 14 (Wang et al. 2014), which is in accordance with

our data where little difference is seen in total NRG1 levels at days 3, 7 and 21 compared to baseline after injury.

Kanzaki *et al* attempted to characterise NRG1 subtype expression changes using SNL as a model of neuropathic pain in rats. At day 14 after injury, they reported a significant increase in NRG1 Type I and II mRNA levels when compared to contralateral L5 DRG's. This study also suggests that the NRG1 Type III levels are significantly decreased after injury when compared to the contralateral side and thus the total NRG1 mRNA levels remain unchanged (Kanzaki et al. 2012). Although the total NRG1 mRNA levels are not significantly altered, I show a similar significant increase in NRG1 Type I at day 3 after injury; a trend for an increase in Type II; and a trend for a decrease in Type III mRNA levels. These increased soluble NRG1 isoforms in the DRG are likely to be responsible for the total up-regulation of NRG1 seen in the dorsal horn and perhaps the enhancement of neuropathic pain.

Data presented in this chapter suggests that erbB3 and erbB4 are not dysregulated after injury but erbB2 is significantly down-regulated in the inured DRG at days 3 and 7 after SNI. Similar findings are presented in an another study. One study also uses a sciatic nerve axotomy model to measure erbB receptor expression changes in the DRG after injury. After sciatic transection, erbb2 and erbB3 signals are slightly reduced at day 1 after nerve axotomy and more modestly reduced at various post-axotomy intervals for at least 30 days. In contrast to erbB2 and erbB3 expression profile, they show a significant up regulation in erbB4 levels at days 3 and 10 after injury (Pearson & Carroll 2004). This study suggested that the pattern of erbB expression in lumbar DRG sensory and spinal cord motor neurons was variable and complex, with differential expression in each neuronal population and even in distinct intracellular compartments in these cells. Evidence for the expression of all three erbB

receptors in small, medium, and large DRG neurons (Pearson & Carroll 2004) suggests that the function of at least some nociceptive, proprioceptive, and mechanoceptive neurons could be modified by NRG 1.

2.4.3 Altered expression of NRG1 isoforms and their receptors in the injured spinal cord

After SNI, an increase in *Aif-1* protein expression (visualised using immunohistochemistry or western blotting) is normally correlated with the process of microgliosis. However, data in this chapter does not show a significant upregulation on *Aif-1* mRNA expression after nerve injury. As the purinergic receptor P2X, ligand-gated ion, 4 (PR2X4) receptors are located on microglia it was also hypothesised that PR2X4 mRNA expression would also significantly increase in the injured dorsal horn after nerve injury. However, there was a dysregulation of P2XR4 mRNA expression after SNI. These findings do not necessarily suggest an absence of microgliosis in our experiment but it is likely that certain limitations arise when comparing mRNA expression versus protein expression (this is discussed in Section 2.4.4). P2X4 expression is not limited to microglia in the spinal cord. In the absence of change in IBA1 mRNA expression, p2X4 results are un-interpretable. Given that microglia are known to proliferate significantly after peripheral nerve injury. A significant up-regulation in IBA1 and thus P2X4 was expected. Not having seen IBA1 up-regulation, it is possible that p2X4 expression increase is independent of microglial function. Alternatively, it is also possible that the p2X4 expression levels currently measured are underestimated and if IBA1 expression changes were detected, P2X4 levels would be significantly higher than reported here.

There are very few studies that report on the level of NRG1 expression changes in the spinal cord after PNI. In this study, I report a significant up-regulation of Pan NRG1

in the spinal cord at days 3 and day 7 post-injury. A recent study by Xiang *et al* used an incision induced mechanical model to induce neuropathic pain in mice and measured NRG1 Type I and II changes after injury in the L5 spinal cord. After paw incision, they report a 1.76 fold increase in NRG1 type I and II protein levels via western blotting (Xiang et al. 2014). It is interesting to note that in C57BL6 mice, the L4 contribution (55%) to sciatic nerve is greater than L3 (25%) whilst the L5 spinal nerve only gives approximately 15% contribution to the sciatic nerve (Rigaud et al. 2008). Elsewhere, sciatic nerve terminal distribution in mouse is reported. Leadermann et al. (2014) reposterd that using ATF 3 as a marker of spared nerve injury, AT3 upregulation confirmed that most injured fibers find their cell bodies in the L3 and L4 DRG after SNI in C57BL/6 J mice; this contrasts with their L4 and L5 DRG localisation in rats (Laedermann et al. 2014). Whilst Xiang *et al* report an increase in NRG1 increase in the L5 spinal cord, it is likely that there is a more significant increase in the L4 segment of the spinal cord than detected by Xiang *et al*. Elsewhere, Gauthier *et al* used the aneurysm compression clip spinal cord injury (SCI) model in rats to evaluate the gene expression profiles of NRG1 and its receptors in the ventral aspect of lumbar spinal cord at acute (days 1 and 3), subacute (1 and 2 weeks), and chronic (6 weeks) time points. From their findings, they indicate that NRG1 is down-regulated using a SCI injury. They report an 80%, 87% and 90% reduction in NRG1 mRNA levels at day 1, 3 and 6 weeks respectively, when compared to uninjured levels (Gauthier et al. 2013). Similarly, an another study reports a decrease in NRG1 mRNA during the first 5 days in rat motor-neurons after a ventral funiculus lesion (VFL) in the lumbar spinal cord and then a slight upregulation at 3 weeks post-lesion (Lindholm et al. 2002). Whilst these studies contradict our findings it is likely that the differences are due the type of injury model adopted and

the cell types investigated. Using double immuno-labelling Gauthier *et al* show NRG1 protein to be expressed in primarily motor neurons and axons but not $Cd11b^+$ or $GFAP^+$ microglia. It is well reported that SCI results in the degeneration of oligodendrocytes that leads to CNS demyelination and axonal dysfunction (Crowe et al. 1997; Beattie et al. 2002; Karimi-Abdolrezaee et al. 2006). After injury Gauthier *et al* report that NRG1 expression remains confined to the motor neurons and oligodendrocytes and it is therefore likely that the down-regulation in NRG1 reported in that study is due to loss of oligodendrocytes.

The Pan-NRG1 mRNA level increase seen after injury in our study is likely to be attributed to increased NRG1 expression and release from damaged axons and motor neurons after injury. Immune cells within the spinal cord are unlikely to produce NRG1. Instead, NRG1 is synthesised at the DRG and is released into the spinal cord to bind onto and activate microglia and astrocytes.

Data presented in this chapter is unable to directly attribute a specific NRG1 subtype responsible for the total NRG1 gene expression level increase after injury. However, it is possible that whilst the classic NRG1 subtypes do not show a significant change, the new NRG1 subtypes could be significantly upregulated after injury and thus responsible for the total NRG1 increase reported in this study.

Tan *et al* suggest that NRG1 Type IV is associated with schizophrenia and show mRNA and protein to be expressed in the human foetal and adult brain but not the human heart, skeletal muscle lymphocytes and monocytes (Tan et al. 2007). They do not investigate NRG1 Type IV expression in the spinal cord. Parodi *et al* recently reviewed NRG1 isoform tissue and cellular expression and suggest that, to date, there are no studies that show expression of the NRG1 subtypes V and VI (Parodi & Kuhn

2014). Thus, it is unknown whether the new NRG's do play a role in the spinal cord or bear any relevance to nerve injury.

NRG1 directly binds to erbB3 and erbB4 and thus at least one of these receptors must be expressed by a cell to respond to NRG1. Using *in-situ* hybridisation, Pearson and Carroll give evidence for erbB2, 3 and 4 being differentially expressed in the lumbar DRG and spinal cord (Pearson & Carroll 2004). This study reports that erbB2, 3 and 4 present a distinct distribution pattern both in the white matter and grey matter. Within the grey matter, erbB2 like proteins are detected as dense punctate immunoreactivity, whilst erbB3 is localised to intense labelling of neuronal and glial cell bodies that are GFAP negative (and thus most likely to be microglia). The erbB4 antibody stains some of the small neurons and produces intense punctate labelling of the neuropil. Using a sciatic nerve axotomy model, this study concludes that there are no differences in the expression levels of erbB2, 3 and 4 in the non-injured vs. injured tissue. Similarly, I report that there are no major changes in erbB mRNA expression levels in the dorsal horn except the initial reduction in erbB2 at day 3 after injury. This small change combined with the diffuse erbB expression in the spinal cord makes it challenging to identify a specific site or cell type responsible for the lowered erbB2 levels. It is speculated that with microglial proliferation after injury, erbB expression mRNA levels may increase. However it is unlikely that mRNA levels of erbB genes are directly correlated to numbers of receptors or numbers of microglial cells. To date there are no studies that report on erbB expression changes within the CNS in control or lesioned animals. In this study, there was no significant increase in microglial *Aif-1* gene expression at injury but instead a reduction reported at day 21 post injury. Given that a significant upregulation in *Aif-1* after nerve injury could not be identified, it is possible that due to both the lumbar dorsal and ventral areas being

analysed, the initial upregulation effect is diluted out as microglia are normally concentrated in the dorsal horn and site of injury. However, at day 21, when *Aif-1* levels do begin to self-regulate, the decrease is more apparent and thus detected in our study.

2.4.4 Technical Limitations

In this chapter, it was reported that *Aif-1* and PR2X4 mRNA expression increase was not detected after nerve injury. It is suggested that there are discrepancies when comparing mRNA expression and protein expression. To illustrate this point, Griffin *et al* analysed 245 genes in the yeast *Saccharomyces cerevisiae in vitro* to discover that there is a significant number of genes that show large discrepancies between abundance ratios when measuring the levels of mRNA and protein expression (Griffin 2002). It is thought that relative quantification of protein expression is more accurate than measuring mRNA expression ratios because problems such as cross-hybridisation and sequence-specific effects can confound hybridisation-based mRNA measurements but do not limit the accuracy of protein measurements (Griffin 2002). Alternatively, it is also possible that analysing whole tissue raises difficulty in investigating cells that are contributing to expression changes. It is likely that an altered cellular composition of a tissue after nerve injury may impact the expression profile of genes. Consideration was made to conduct these experiments at the protein level but it was determined that many erbB receptor and neuregulin antibodies show poor efficacy in mouse tissue and thus would not prove effective in immunolabelling or protein-analysis experiments. It is also worth noting that fluctuating' levels of *p2rx4* are difficult to interpret because *p2rx4* expression is restricted to microglia. It would have been more appropriate to use a microglial-specific marker as a control, such as *itgam or emr-1*.

When analysing sciatic nerve tissue, previous studies normally analysed the distal segment of the site of injury. However, the distal segment of the sciatic nerve in our experiments was severely deteriorated which resulted in the extraction of the proximal segment instead for tissue analysis. It is therefore likely that any discrepancies seen in the sciatic nerve findings in comparison to previous studies is owing to the difference in the tissue site analysed.

2.5 Considerations

In summary, it is evident that the NRG1-erbB signaling pathway is dysregulated after PNI. As yet, there are no studies that present information on NRG1 subtype expression or erbB receptor gene expression changes after nerve injury in the CNS. In the CNS, the specific NRG1 subtype responsible for binding onto erbB receptors and mediating microglial activity could not be clearly identified but it is likely to be NRG1 type I. As this isoform can be released and signals in a paracrine function, increased expression of this isoform within damaged sensory neurons could lead to increased release into the dorsal horn of the spinal cord. This correlates with increased NRG1 protein levels within cerebrospinal fluid (CSF). The NRG1 type I isoform also shows markedly increased expression within injured nerve where it could signal to glia and infiltrating macrophages. It was hypothesised that there is significant increase in erbB expression after nerve injury owing to microglia proliferation and accumulation at site of injury. Results in this study could not confirm this. Gene expression levels of erbB3 and 4 remain unchanged after nerve injury whilst erbB2 is significantly reduced. The following data chapters describe genetic ablation studies used to better understand the role of NRG1 on microglia in the context of neuropathic pain.

3 Using the Place Preference Assay to measure thermal and cold hypersensitivity in rodents

3.1 Introduction

To measure mechanical, chemical or thermal modalities in rodents, the Von-Frey test (Chaplan et al. 1994), the Randal Selitto Test (RANDALL & SELITTO 1957), the hot plate and the Hargreaves test (Hargreaves et al. 1988) are the standardised behavioural assays used to deepen our understanding of the molecular and cellular mechanisms underlying evoked pain both acutely and following injury.

Measuring cold allodynia bears significant clinical relevance given that cold hypersensitivity is an important symptom in neuropathic pain in humans (Bouhassira et al. 2004; Bouhassira et al. 2008; Attal et al. 2010) both in relation to traumatic nerve injury and after certain chemotherapeutic agents such as Oxaliplatin (Descoeur et al. 2011). Compared to the response to noxious, mechanical or heat stimuli, measuring cold hyperalgesia or hypersensitivity is proving to be technically challenging. In rodents, different methods for testing cold hypersensitivity have been described: paw or tail withdrawal after immersion in cold water (Kayser & Christensen 2000; Kayser et al. 1995), water-alcohol bath (Abbadie et al. 1994), ethyl chloride spray (Hao et al. 1996), direct application of acetone (Carlton et al. 1994; Choi et al. 1994; El-Khoury et al. 2002), contact with a Peltier thermode (Simone & Kajander 1996) or a cold plate (Bennett & Xie 1988; Jasmin et al. 1998). Cold hypersensitivity behavioural data from such assays is often variable, non-reproducible and can create conflict in experimental findings.

Recently, new behavioural models have been devised to explore the molecular mechanisms of cold sensation to complement current cold behavioural assays.

Similar to the thermal place preference assays (Balayssac et al. 2014; Minett et al. 2014), I have devised a unique cold place preference assay protocol in mice to measure changes in cold behaviour following nerve injury. In order to test the hypothesis that NRG-1 may be important in regulating cold behaviour through microglial function as suggested by Calvo et al., in this chapter I utilise an animal model of nerve injury and describe a behavioural assay used to effectively measure cold hypersensitivity in mice.

In this study, I also investigated hypersensitivity to warm stimuli in an animal model of erythromelalgia (Dib-Hajj et al., 2005). This is a primary neuropathic pain condition in humans in which mutations in the ion channel NaV1.7 leads to persistent pain that is exacerbated by warmth. An animal model of this condition was generated by the EUROPAIN IMI consortium and used to study such warm hypersensitivity in rodents.

3.2 Methods

3.2.1 Animals

All experimental work on rodents was carried out accordingly to UK Home office legislation (Scientific Procedures Act 1986). Adult mice (8 weeks or older) were used for tissue extraction and behaviour. Animals were housed in SPF cages with unlimited access to food and water. They were housed in a 12 hour light dark cycle. Observers who performed behavioural experiments and analysis were blind to the experimental group of the animals.

3.2.2 Experimental Animals

Control wild type animals (n=10) of C57BL6 origin were recruited from existing cohorts of experimental animals and used in the cold place preference assay.
I sought to include a cohort of transgenic strain that is likely to display a thermal hypersensitivity phenotype as a positive control. In humans, gain of function mutations in the sodium channel subunit Nav1.7 can lead to a range of pain disorders including inherited erythromelalgia (IEM), paroxysmal extreme pain disorder and small fibre neuropathy. IEM is an autosomal dominant disorder characterised by erythema of the hands and feet, as well as intense burning pain, which can be triggered by warm temperatures. A targeting vector carrying the SCN9A F1449V mutation (Dib-Hajj et al., 2005) was constructed and electroporated into the mouse C57BL6 embryonic stem cells (ESC) as part of the EUROPAIN consortium. Successfully targeted ESC clones were identified and employed to create the mouse model in which the wild type mouse Scn9a gene is replaced by the mutated form. Wild type adult C57BL6 mice ($SCN9A^{+/+}$**;** n=10), and heterozygous mice ($SCN9A^{F1449V/+}$**;** n=10) were recruited into the study. Heterozygous strains were

recruited because IEM is an autosomal dominant disorder where a heterozygous gain-of-function missense mutation in Nav1.7 is sufficient to induce a pain phenotype in IEM patients (Dibb-Hajj et al 2005). It was also observed that generating homozygous SCN9A$^{F1449V/+}$ caused embryonic lethality (unpublished observation). Where possible, equal numbers and sex were assigned into each experimental group. The power calculation conducted is described in Chapter 2, section 2.2.2.

3.2.3 Surgery

The spared nerve injury (SNI) was used as a model of peripheral nerve injury to measure behavioural outcomes after injury. Surgery was performed in the same manner as described in Chapter 2 section 2.2.3.

3.2.4 Mexilitine treatment to SCN9A$^{F1449V/+}$mice

Surgery was not performed on the SCN9A$^{+/+}$ (WT) or SCN9A$^{F1449V/+}$ (het) cohorts but instead, they were treated with vehicle (0.9% saline) or 10mg/kg Mexilitine (dissolved in 0.9% saline) and their behavioural responses were measured at 1 hour post-treatment and compared to pre-treatment. The experimenter was blind to each treatment group when administering and recording behavioural output.

3.2.5 Behavioural Assays

All behavioural experiments were carried out between 10.00 and 16.00, unless otherwise stated. Each animal was recorded using a video camera, from which data was later analysed.

3.2.6 Cold Place Preference

The behavioural apparatus was set up as follows. Two 10mm thick aluminium plates (25 – 37 x 47(h) cm 35100, Ugo Basile, Italy) were used to form two chambers connected using a non-conducting corridor platform. As shown in Figure 3 1, the

additional auxiliary plate is controlled by the main unit, which can be used to define the temperatures on both plates. The auxiliary hot plate is labelled as the temperature reference plate and is continuously set to 22°C whilst the other plate is set at fixed temperatures ranging from temperatures as low as 2°C up to 22°C. The temperature range used in this study varied from 6°C to 22°C when measuring cold hypersensitivity after injury. Each animal is placed onto the fixed temperature plate and the animal is permitted to explore both plates for 10 minutes. Animals were tested on fixed temperature settings that were set in a randomised order to reduce learning or habituation effects. Thermal place preference was calculated by measuring the ratio of time spent on the fixed temperature plate versus the reference room temperature plate. Following experimental set-up and refinement, the optimal 'cold' temperature was identified as 16°C whilst the temperature reference plate was maintained at 22°C. Animals were tested using this experimental set-up at days 3, 7, 14 and 21 post-injury.

3.2.6.1 Thermal Place Preference

In order to test the full functional capabilities of the aforementioned protocol, I used the $SCN9A^{F1449V+/+}$ (WT) and $SCN9A^{F1449V/+}$ (Het) cohorts to measure sensitivity to thermal stimuli. The same behavioural apparatus is used as in section 3.2.6 but the temperature ranged between 22°C and 37°C. Here, the reference plate was set to 37°C and the fixed temperature plate was set to either 22°C or 32°C. Animals were placed onto the reference plate and permitted to explore both chambers for 60 minutes. Thermal place preference was measured in the same manner described in section 3.2.6. Once baseline values were determined, animals were allowed to rest for a week before being treated with either Vehicle or Mexilitine and tested 1 hour after treatment for 60 minutes.

3.2.7 Statistical analysis

The spared nerve injury study behavioural results were analysed and the repeated measures two-way ANOVA statistical test was performed with the post-hoc Bonferronni test. Similarly, in the SCN9A$^{F1449V+/+}$ (WT) and SCN9A$^{F1449V/+}$ (Het) experiments, a repeated measures two-way ANOVA statistical test was performed with the post-hoc Holm-Sidalk test.

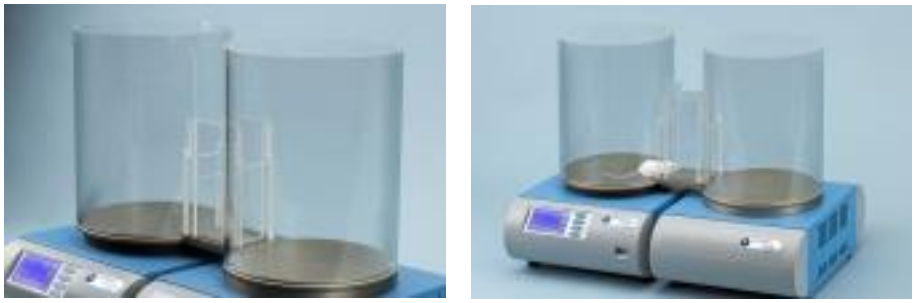


Figure 3-1 Experimental apparatus for the thermal place preference assay. The plate on the left side of the connecting corridor is identified as the fixed temperature plate, whilst the plate on the right end of the corridor is identified as the reference plate. The central connecting corridor does not conduct heat but remains at room temperature. The equipment was purchased from Ugo Basile, Italy (Images used for illustration purposes)

3.3 Results

3.3.1 Animals spend approximately 30% of their time at 16°C versus room temperature (22°C)

Using the place preference experimental set up, I investigated the optimal temperatures at which mice were likely to exhibit a distinct but measurable avoidance to either a cold or "cooler" environment. As Figure 3-2 shows, wild type animals only spent 7.51± 2.82% of their total time (10mins) on the 6°C plate when given the choice between 6°C and the reference room temperature plate (RT - 22°C). This latency was very small and would not prove useful in nerve injury studies. Other temperature ranges were also tested. At 12°C, animals spent 20.33 ± 2.60% of their total time on the 12°C plate in comparison to the reference RT plate. A temperature range was tested until it was determined that animals spent approximately 31.45 ± 4.15% of their total time on the 16°C versus the reference RT plate. From 18°C to 20°C, a similar percentage of time was spent on the fixed plate (33.34 ± 6.02% of total time spent on 18ºC and 34.02 ± 6.83% on 20 ºC) and did not significantly vary ($p>0.05$; repeated measures one way ANOVA). No preference was made when animals were given a choice between 22°C vs. RT (which was also 22°C). They spent 47.31 ± 10.09% of their total time on the 22°C vs. RT. Therefore, 16°C was the lowest temperature that could be sensibly used in order to measure normal animal exploratory behaviour over 10 minutes. It was determined that testing animals under 16°C vs. 22°C conditions is the most optimal assay in order to reliably determine cold pain behaviour changes after nerve injury. This experimental set up provides a reasonable latency to measure the effects of nerve injury on cold related hypersensitivity.

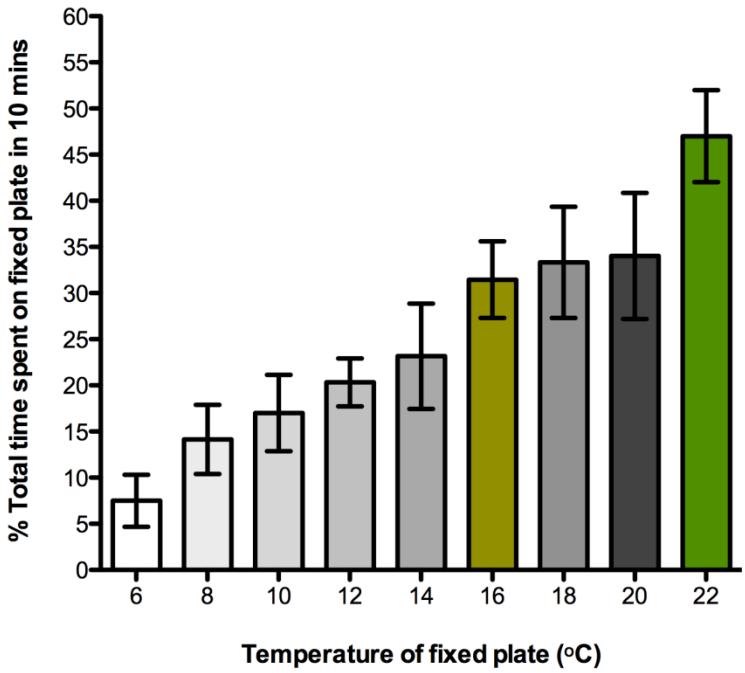


Figure 3-2 The cold place preference temperature range. Data shows the percentage of total time spent on a fixed temperature plate versus the reference plate in 10 mins. Using 16°C (vs. the reference plate temperature of 22°C), animals spend approximately 35% of their time on the 'cold' plate. There is no preference when both plates are set to 22°C as shown in the final column. This temperature range testing was conducted in a randomised order to prevent sensitisation to the experimental set up (n=10 per group; $p>0.05$; repeated measures on way ANOVA).

3.3.2 The 16°C vs. 22°C paradigm demonstrates that animals develop cold hypersensitivity after peripheral nerve injury

Having determined the optimal set up to measure cold related hypersensitivity, I used a model of peripheral nerve injury to measure cold pain related behaviour changes after injury. As shown in Figure 3-3, at baseline, animals spent approximately 35% of their time in the 16°C environment and approximately 33% of their time prior to injury (vs. the reference RT plate). After SNI, animals developed significant cold hypersensitivity to 16°C as early as day 3 after injury. Animals spent significantly less time on the 16°C at day 3 post injury (8.0 ± 2.93%) vs. the reference RT plate. Animals maintained this cold related hypersensitivity until day 21 where they spent only 10.0 ± 3.10 % of their time on the 16°C (vs. the reference RT plate). It is worth noting that animals use the corridor to transverse to the other opposite chamber and did not stop or remain within the corridor. Given that the connecting corridor remained at room temperature (22°C), any time spent there was included in the reference RT plate measurement.

To investigate whether the animal's exploratory behaviour was affected as a result of nerve injury, the total number of crossings between the 16°C and 22°C plate were measured through the course of the study. As shown in Figure 3-4, animals maintained similar number of crossings from one temperature environment to another throughout the study. At Baseline, animals made 37.93 ± 4.34 crossings to the 16°C environment 10 minutes. After injury, the numbers of crossings remained unchanged at day 3, 7, 14 or 21. At day 21, animals made an average of 31.24 ± 5.12 crossings into the 16°C environment. There was no significant difference in the number of crossings throughout the course of the study ($p>0.05$; repeated measures two way ANOVA Bonferroni post-hoc test). It was noted that with extreme temperatures such

as 6°C and 8°C the number of crossings were noticeably reduced (unreported). Animals tended to avoid the cold plate temperatures by displaying certain "avoidance" behaviours after initial exposure of the cold environment. Animals would either hesitate or place their whiskers or paws into the cold chamber and then abruptly retreat to the opposite chamber and refuse to enter the cold chamber again (experimenter's observation).

With these findings I conclude that the cold place preference model (16°C vs. 22°C) is effective in reliably measuring in cold related hypersensitivity after injury that remains stable over a 3-week period following injury.

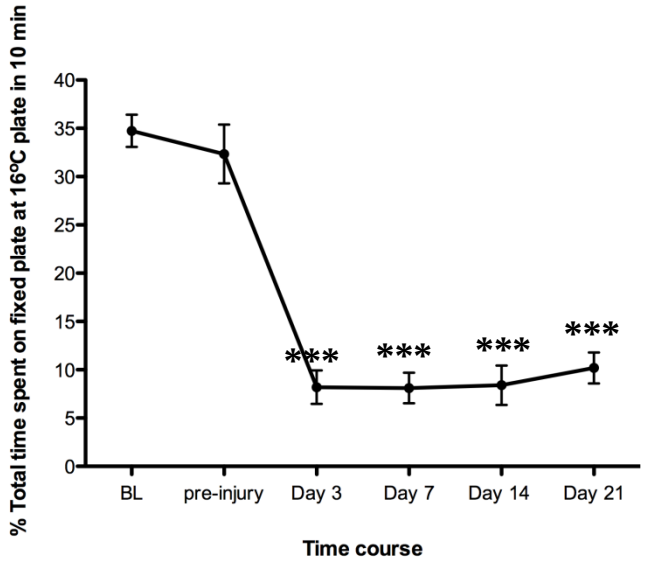


Figure 3-3 animals develop significant cold hypersensitivity after SNI. Using the 16°C vs. 22°C set up, animals spend approximately 35% of their time on the 16°C but after injury spend only approximately 5-10% of their time on the 16°C plate. Animals continue to develop cold related hypersensitivity up until and including day 21. (n=10, ***p<0.001, compared to baseline (BL) repeated measured two way ANOVA, Bonferronni post hoc test)

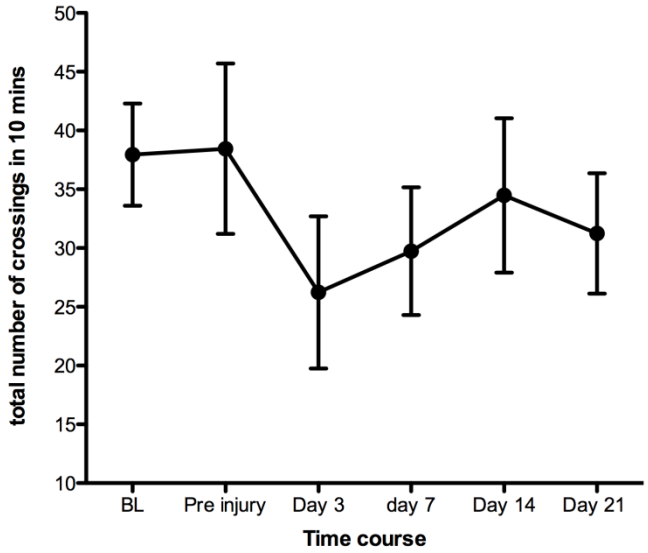


Figure 3-4 the number of crossings in the cold place preference assay remain unchanged after SNI. Animals underwent SNI and their exploratory behaviour was measured through the number of crossings in the cold place preference assay. There is no significant difference in the number of crossings across the study. (n=10, $p>0.05$ compared to baseline (BL), repeated measured two way ANOVA, Bonferronni post hoc test).

3.3.3 SCN9A$^{F1449V-/+}$ animals exhibit heat hypersensitivity to 37°C

As a correlate to measuring changes in cold hypersensitivity I also assessed if thermal place preference could be used in an outcome measure in a transgenic mouse model of a human mutation associated with thermal hypersensitivity: The SCN9A$^{F1449V-/+}$ (Het) transgenic strain has been designed to model human erythromelalgia in animals, where patients suffer from extreme burning pain at their distal extremities, triggered by heat, pressure, mild activity or stress.

Using this transgenic strain, it was hypothesised that the SCN9A$^{F1449V-/+}$ (Hets) would develop sensitivity to thermal stimuli and thus prefer an environment that is cooler than the higher temperature. Animals were tested for 60 minutes and placed in a condition environment of 32°C vs. 37°C and 22°C vs. 37°C.

As shown in Figure 3-5a, wild-type (WT) animals spent on average 44.66 ± 6.23% of their time on the reference plate at 37°C, whilst the heterozygotes (Het) spent a significantly lower ratio (23.27 ± 4.29%) of time on the reference plate. This suggested that heterozygotes have developed thermal sensitivity to 37°C and thus preferred to spend more of their time on the "cooler" temperature at 32°C. In order to understand exploratory behaviour, the percentage of time spent on each plate in 10-minute intervals was measured as shown in Figure 3-5b. Within the first 10 minutes, there was no significant difference between the WT and Het cohorts and their preference of temperatures. Both groups spent similar amounts of time on the 37°C plate (40.09 ±11.96% and 27.81 ± 12.86% respectively). Similarly, no difference was detected between 10-20 minute intervals between groups. However, between 20-30 minutes the Het cohort spent less % of time on the reference 37°C environment (31.83 ±15.81%) in comparison to WT (46.23 ± 10.11%) and continued to do so throughout the course of the study. Between 50-60 minutes, on average the Het cohort

only spent 10.97 ± 3.16% of its time on the reference 37°C environment whilst the WT cohort spent up to 54.18 ± 9.63% of its time there. This shift seen in the Het cohort would suggest that this strain developed sensitivity to warmer temperatures and thus preferred to stay in a "cooler" environment over time (n=10 per group, $p<0.001$ repeated measures two way ANOVA, post hoc Holm Sidalk test).

Following this, a more extreme temperature range was used to test each cohort (22°C vs. 37°C) and similar results were reported. Figure 3-6(a) shows that WT's occupied significantly more of their total time on a 37°C environment instead of 22°C in comparison to Hets (57.47 ± 2.05% and 32.85 ± 2.34% respectively). The percentage of time spent on each plate in 10-minute slots was also measured. Similarly, within the first 10 minutes the WT's and HET's both spent on average 48.91 ± 11.02% and 58.73 ± 2.51% of their time on the 37°C environment. In the 10-20 minute interval the WT strain continued to spend more of its time on the 37°C environment (48.91 ± 6.31%), whilst the Het group spent a significantly lower proportion of its time on the 37°C environment (25.61 ± 10.93%). This trend continued through the remainder of their study. Within the last 10 minutes of the study, heterozygotes only spent on average 30.36 ± 6.23% of their time on the 37°C environment whilst the wild types spent significantly more on average of 67.43 ± 5.12% (n=10 per group, $p<0.001$ repeated measures two way ANOVA, post hoc Holm Sidalk test).

With these findings, it can be concluded that when given an opportunity to choose between two temperatures, the $SCN9A^{F1449V-/+}$ (Hets) prefer to spend a higher proportion of their time in the lower or "cooler" environment. Using the thermal place preference assay, I demonstrate some of the behavioural phenotypes that are normally displayed by patients suffering from erythromelalgia.

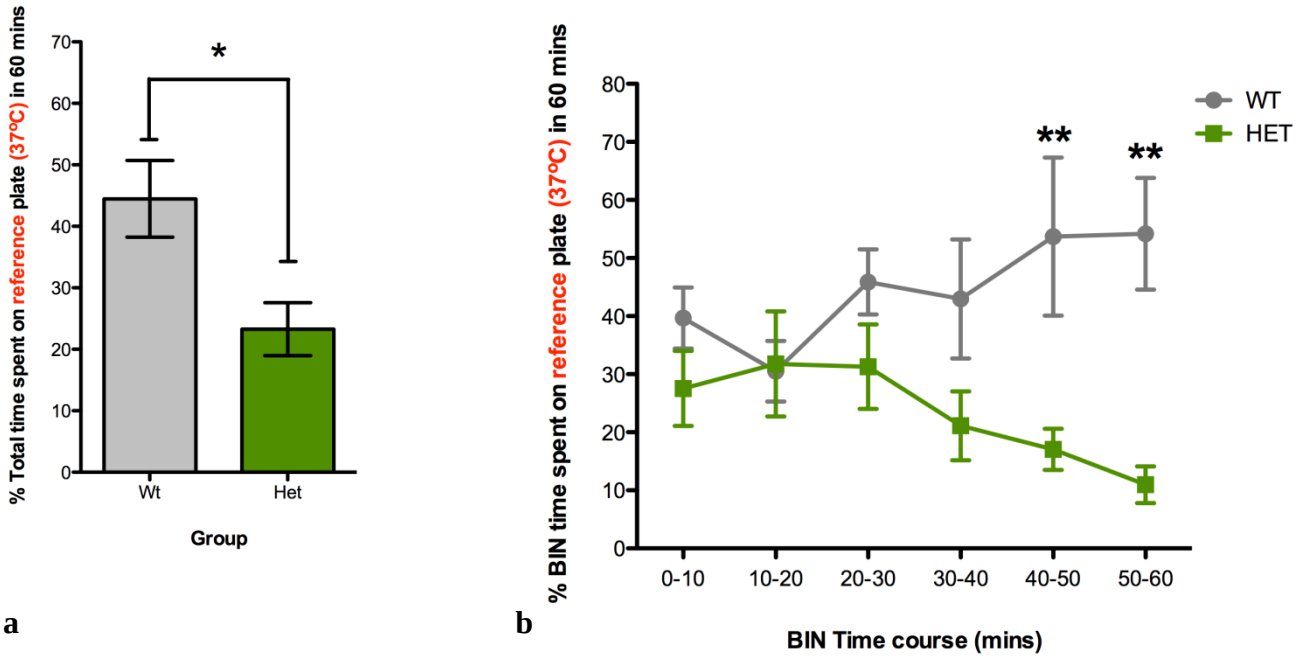


Figure 3-5 the SCN9A$^{F1449V-/+}$ (hets) develop thermal hypersensitivity to 37°C and thus spend a higher proportion of their time on the 32°C environment. (a) The Het cohort spends significantly less time on the 37°C plate when compared to wild-types in 60 mins. (b) Show the interval times in 10 minute slots. In the first 30 mins, the ratio of time spent between the two chambers remains equal. In the last two interval times, the heterozygotes spend significantly less time on the 37°C environment and thus indicate sensitivity to 37°C environment. The Het cohort shows a preference for the 32°C environment. It is worth noting that the animals were introduced into this behavioural apparatus for the first time and therefore spent the first 30 mins exploring both chambers. In this set up, there is no influence of learned behaviour. n=10 per group, **p<0.01, repeated measured two way ANOVA, Holm Sidalk post hoc test).

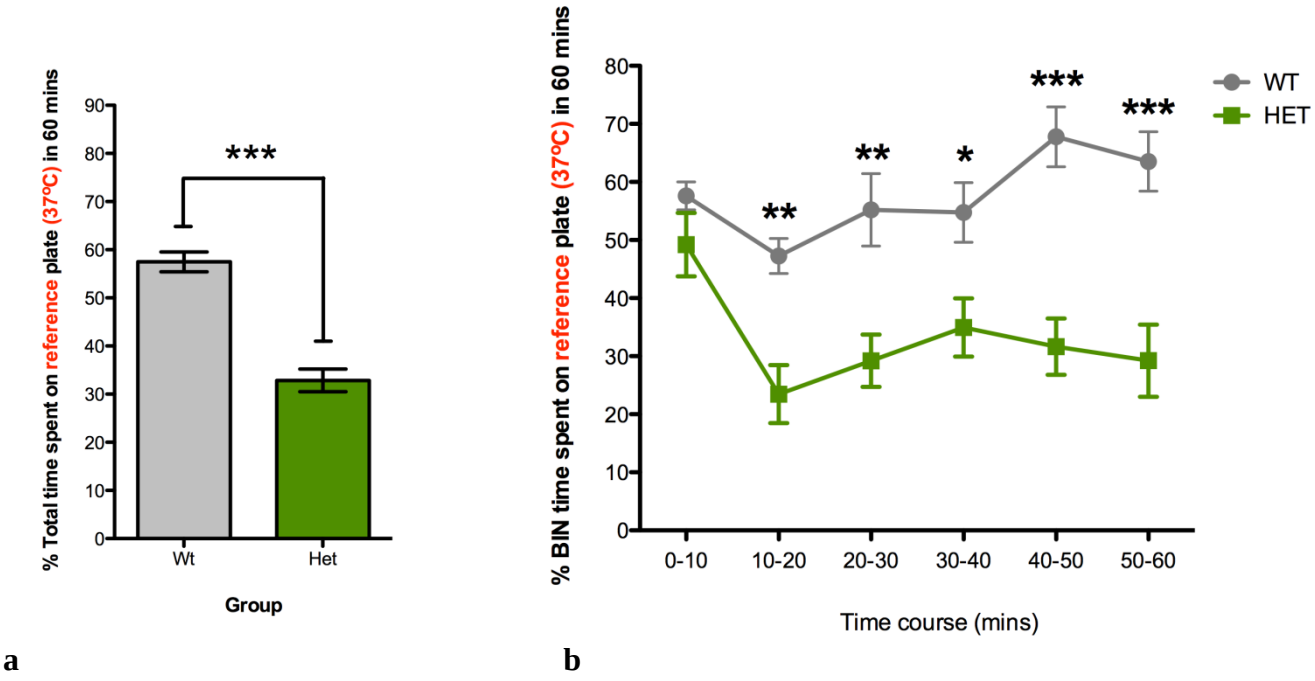


Figure 3-6 SCN9A$^{F1449V-/+}$ (hets) prefer 22°C instead of 37°C. (a) Wild types spend a significantly higher proportion of their total time on the reference (37°C) plate in comparison to the Het cohort. (b) Whilst WT's spend approximately 60-70% of their time on the 37°C environment, the SCN9A Hets prefer to spend approximately 50% of their time within the first 10 minutes but then only spend approximately 20-30% of their time on 37°C through the remainder of the study. The SCN9A Het cohort developed a preference to the "cooler" environment when permitted to choose between two temperatures. (n=10 per group,*p<0.05, **p<0.01, ***p<0.001 repeated measured two way ANOVA, Holm Sidalk post hoc test).

3.3.4 Mexilitine is capable of attenuating thermal temperature preference differences measured between the SCN9A$^{F1449V+/+}$ WT and SCN9A$^{F1449V-/+}$ (Het) cohorts

In order to further test and standardise our experimental set up, I used the positive control study and determine whether the effects of thermal preference seen in the wild types could be recapitulated in the SCN9A$^{F1449V-/+}$ heterozygotes using a pharmacological approach. From Figure 3-5 and Figure 3-6, it was evident that the Het cohort preferred a cooler environment (i.e. either 22°C or 32°C) in comparison to the 37°C environment. By administering either vehicle (Vh) or a non-specific voltage gated sodium channel blocker- Mexilitine (Mex), the % occupancy at 37°C vs. the 22°C temperature of the WT and Het cohort was measured after treatment. Interestingly, with wild types, whether they received Vh or Mex, the % occupancy on the 37°C remained unchanged. On average wild types spent 73.59 ± 2.65% of their total time (60 mins) on the 37°C plate when treated with vehicle and on average 77.97 ± 3.53% of their time on the 37°C when treated with Mexilitine. The Vh treated Het cohort spent significantly less time on the 37°C at only 48.51 ± 3.07% of the total time in comparison to the Vh treated WT cohort ($p<0.05$). With Mex. treatment however, the Het cohort spent on average 92.65 ± 1.45% of their time on the 37°C instead of the 22°C environment (Figure 3-7a). This is significantly greater than the Het group that is treated with vehicle ($P<0.001$).

The % occupancy in 10-minute slots was also calculated (Figure 3-7b and c). It was found that the Het Vh cohort spent on average 24.67 ± 11.62% of their time on the 37°C at 0-10mins; 37.93 ± 13.63% at 10-20mins, and 46.02 ± 14.36% at 20-30 min interval. The Het Mex cohort was indistinguishable from the WT Vh or WT Mex treated groups. The SCN9A$^{F1449V-/+}$ Het cohort treated Mex cohort spent on average

62.42 ± 17.61% of their time in the 0-10 min interval; 70.93 ± 19.68% in the 10-20 min interval and 78.35 ± 17.33% in the 20-30 min interval (Figure 3-7c) in the 37°C environment.

It can therefore be concluded that with Mexilitine treatment, the SCN9A$^{F1449V-/+}$ (Hets) cohort lose the preference to spend most of their time on the lower (22°C) temperature but instead, now show a preference for the warmer 37°C environment. After Mexilitine treatment SCN9A$^{F1449V-/+}$ (Hets) are almost indistinguishable from the WT controls in their perception of thermal stimuli.

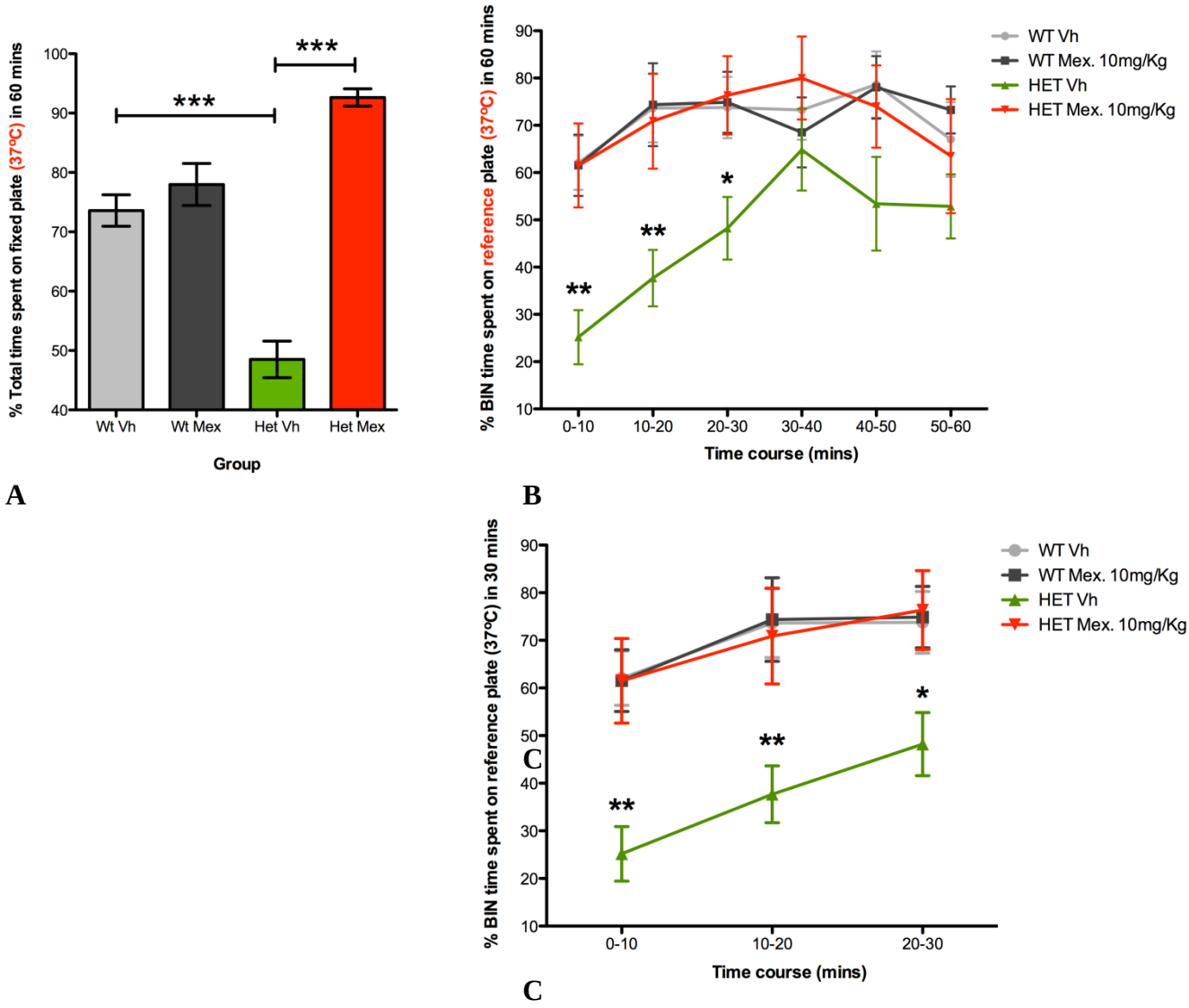


Figure 3-7 Mexilitine reverses thermal hypersensitivity seen in SCN9A$^{F1449V-/+}$ heterozygotes when given the choice to habituate either 22°C or 37°C. (a) Wild type animals spend similar % occupancy at 37°C whether they have been administered with vehicle or Mexilitine. The Het Vh. group spends approximately 45% of its time on 37°C. When administered with Mexilitine, the Het Mex. group spends a significantly higher % of time on 37°C when compared to the Het Vh. group. (b) Shows the overall interval times in the 60 minutes of testing. (c) Shows the first half (0-30 mins) of the study after treatment. Whilst the Het Vh. group spends significantly less time on the 37°C from 0-10, 10-20 and 20-30 minutes, the Het Mex. group is indistinguishable from the WT Vh. or WT Mex. group. With Mexilitine treatment, thermal hypersensitivity is attenuated in the SCN9A Hets. It is worth noting that these animals were previously introduced into this behavioural apparatus a week prior to testing. Therefore it is likely that there is some contribution of learned behaviour and this explains the differences seen within the first 10 minutes. (n=10 per group,*p<0.05, **p<0.01, repeated measured two way ANOVA, Bonferronni post hoc test).

3.4 Discussion

Thermosensation is a vital sensory function that is controlled by a variety of transducer molecules including those from the Transient Receptor Potential (TRP) ion channel family. Both heat and cold evoke thermosensation, which, when evoked with sufficient intensity, can elicit feelings of pain. Temperature detection and internal temperature regulation is a critical function in the maintenance of thermal homeostasis. Importantly and relevant to my thesis, such thermal sensitivity can be sensitised in the context of persistent pain states.

In relation to the detection of cooling and cold pain by sensory neurons, a number of TRP channels have been implicated. The TRPM8 (CMR1) a ligand gated, nonselective cation channel, is activated by both cold and chemical stimuli (e.g. menthol) *in vitro* with some mice studies suggesting TRPM8 to play an important role in certain types of cold induced pain (Chung & Caterina 2007). The noxious cold-activated TRP ankyrin 1 (TRPA1) is activated by pungent compounds such as cinnamaldehyde, mustard oil and garlic oil (Bandell et al. 2004; Jordt et al. 2004; Bautista et al. 2005). Cold perception is often altered in patients suffering from multiple sclerosis, chemotherapy induced neuropathy or stroke. Such phenomena include perception of pain in response to cooling which would normally be non-noxious (cold allodynia) and the perception of heat in response to cooling (paradoxical heat sensation). Such alterations can significantly impair quality of life. Treatment options for these patients have been limited by the progress in dissecting the mechanisms of cold pain sensitisation. The mechanisms of cold pain sensitisation in turn have been slowed by the limitations in the behavioural assays used to study animals for cold perception.

In this chapter I present evidence that the 16°C vs. RT (22°C) cold place preference assay is a useful tool to measure cold related pain behaviour phenotypes at baseline and after peripheral nerve damage. Using an animal model of human IEM, I also demonstrate that this experimental set-up can be used to test not only cold hypersensitivity but also heat hypersensitivity. By administering Mexilitine to the $SCN9A^{F1449V-/+}$ (Hets) I show that the effects of the mutation on the animal's thermal hypersensitivity thresholds can be reversed.

3.4.1 Limitations with current assays used to measure cold hypersensitivity

Previously, it has proved challenging to conduct a behavioural assay on mice that effectively measures cold pain behaviour. Whilst the traditional Acetone test is effective in measuring cold hypersensitivity in rats, it has proved to be very challenging in mice with little success or a failure to replicate results (Brenner et al. 2012). The "Icilin induced jumping" assay requires an animal to be dosed with icilin (a synthetic agonist of the TRPM8 ion channel) and placed into bell jars and monitored for spontaneous jumping behaviour over a set time after injection (Colburn et al. 2007). It is suggested that this assay may be more useful and easier to replicate. However it is difficult to replicate in mice that have undergone surgery, where animals are sometimes limited in their full motor function and repetitive testing can cause pro-longed stress which is known to activate analgesic mechanisms (Pinto-Ribeiro et al. 2004).

Another commonly used test is the tail flick assay. Here the latency of an animal to withdraw its tail from cold water (at set temperatures) is measured. Whilst the animal's behavioural response is distinctly clear, the assay is limited by the method, with which it is conducted. The animal is restrained where this is again likely to cause stress and perhaps cause stress induced-analgesia and most models of nerve injury are

applied to the hind paw instead of the tail. The well-known cold plate assay measures the behavioural responses of mice placed on a pre-cooled plate. Here, many metrics (number of jumps, the latency to withdraw and the number of paw lifts) can be used to measure the effects of the cold temperature but no general consensus exists on the meaning of each parameter.

Another technique recently described is the "cold plantar assay" where animals are placed on a glass plate and dry ice is packed into a pellet and applied to the underside of glass surface under the hind paw. With this, the evaporation of the dry ice cools the glass at a set rate and applies a cold stimulus to the hind paw where the withdrawal latency is measured (Brenner et al. 2012). Using this technique Brenner et al. show that mice respond to consistent changes in temperature in the cold plantar assay. Brenner et al., also employ a model of peripheral nerve injury (SNL) and use the cold plantar assay to measure withdrawal latencies. Following injury, SNL mice present a significant reduction in the withdrawal latency when compared to baseline values or sham (Brenner et al. 2012).

Whilst researching these different cold pain behaviour assays, I used some of the assays described above to determine cold pain thresholds on specific cohorts. The acetone test proved difficult to repeat reproducibly. The volume of acetone exerted on the hind paw; the location of the application site; the rate of evaporation and the mechanical disturbance were difficult to control. The method of measurement and analysis also varies greatly between research groups. Some research groups grade the responses within set time frames after acetone application (Makoto Tsuda et al. 2009). Tsuda et al. describe their grading response as 0, no response; 1, quick withdrawal flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking (more than 2 times) of the paw; 3, repeated flicking of the paw with licking directed to the plantar

surface of the hind paw. Whilst this is extremely informative, it is difficult to measure these events in mice. The mechanical disturbance, coupled with the smell and the rate of evaporation causes the animal to display signs of either distress or in some cases naive mice display no response (Brenner et al. 2012) which makes it difficult to accurately measure cold anaesthesia.

Using the traditional Cold plate assay also proved problematic. In baseline phenotyping studies, the cold plate assay can only be useful if animals are tested once or twice. For example when phenotyping a transgenic strain in chapter 4, we set the temperature to 8°C and measured the first latency of withdrawal (bite, lick, or jump) to the cold stimuli. This is relatively simple to conduct and the animals displayed clear cold like pain behaviour. However, re-testing these animals at baseline or using this test in nerve injury studies presents the problem of a learning effect. Animals quickly learn or adapt to this behaviour assay and often respond before the test has begun or simply continue to jump and avoid the cold temperature. The latency to withdraw is significantly reduced which can result in misinterpretation of results after injury as part of this may be a learned response to the apparatus rather than the cold stimulus per se. More importantly it is difficult to reach a general consensus of which type or response is a true reflection of the animal displaying cold pain.

The cold plantar assay was also tested but due to technical limitations, it could not be fully tested. Animals that underwent SNI were placed onto a glass plate enclosed in a Perspex box. Once habituated, dry ice was packed into a pellet in a syringe with the needle removed and bevelled to produce a flat surface. The hind paw was located and the dry ice pellet was compressed and pressure was applied directly under the hind paw through the glass plate. The pilot data produced varied and non-reproducible results owing to certain technical limitations. The thickness of the glass plate is

important in regulating the latency of withdrawal; the injured hind paw does not lie flat but is instead curled making it difficult to measure a true response; it is difficult to control the rate at which the plate cools and whether the stimuli reaches the hind paw surface at a constant rate.

As will be described in chapter 4 the cold plate assay was adopted and a protocol was devised where each animal was placed on an 8°C cold plate (Ugo Basile) and the total number of pain like behaviours (bite, paw flick, jumps) were counted in a testing period of 6 minutes. This assay highlighted a significant difference in the number of responses between the transgenic line and the controls but could not effectively measure cold pain hypersensitivity. Instead, there was a significant learning effect over time, where the total number of responses continued to decline over time after surgery. It was difficult to dissociate this progressive declining response to changes in cold perception post injury. With this in mind, I developed a cold place preference behaviour assay described in this chapter and customised it to use in nerve injury studies. Similar behavioural assays have recently been published and suggested that a 2 or 4 temperature choice test reflects the animal's temperature preference over a long period of time instead of the spinal reflexes measured in the more traditional cold pain behaviour assays.

Noel et al. were the first to adopt this strategy and show that the mechanogated high temperature sensitive potassium channels TREK-1 and TRAAK are important regulators of nociceptor activation by cold, particularly in the population that is not activated by menthol (Noël et al. 2009). More recently, Minett et al., employ a similar thermal place preference approach by testing animals for 2 mins with a choice of two temperatures to demonstrate that Nav1.8 positive neurons are required for the detection of extreme cold (0°C) but not cooling stimuli (14-16°C) (Minett et al. 2014).

Balayassac et al. Studied the thermal place preference model further and suggest that the thermal place preference test in rats is most reproducible when it is performed in the morning and in the dark for 3 mins with the reference plate set to 25°C (Balayssac et al. 2014).

Whilst the above-mentioned studies conducted a cold behavioural assay in a similar manner to the protocol described here, there is a lack of studies that present data collected from the cold/thermal place preference assay after peripheral nerve injury. With a paradigm of 16°C vs. RT (22°C) used in this chapter, I effectively demonstrate that wild type animals develop cold hypersensitivity after nerve injury which was reproducible and stable. Following nerve injury animals showed greater avoidance of the 16°C chamber consistent with cold allodynia. The effect was mediated by the length of time that the mice spent in the 16°C chamber rather than the number of crossings between chambers (i.e. there did not appear to be a change in exploratory behaviour. I saw no evidence of declining response with repeated testing as has been seen in some other measures of cold sensitivity. In future studies it would be interesting to undertake a pharmacological characterisation in order to determine whether this assay is sensitive to analgesic agents.

It is worth mentioning, that the optimal control for ensuring that the cold hypersensitivity is due to nerve injury and not due to the effects of surgery would have been to include a sham surgery group in which the sciatic nerve trifurcation was exposed but not lesioned. Unfortunately, due to animal availability, I was not able to include a sham group in the study.

The experimental set up was also manipulated to test whether the effects of thermal preference seen in the wild types could be recapitulated into the $SCN9A^{F1449V-/+}$

heterozygotes. This transgenic strain is said to model human erythromelalgia, where in humans, it affects the skin of the feet or hands, or both, and causes visible redness, intense burning pain and heat sensitisation. Precipitating factors include warm temperatures and mild exercise. Cooling the hot body parts relieves the pain, as does elevating the affected areas. A single substitution of phenylalanine by valine (F1449V) at codon 1449 in the sodium channel Nav1.7 causes a single amino acid to be substituted and alters the biophysical properties of Nav1.7 and reduces the threshold for action potential firing and bursting of DRG neurons (Dib-Hajj et al. 2007; Dib-Hajj et al. 2005). These mice become more sensitive to heat stimuli. This was tested using 22°C vs. 37°C set up. Results in this chapter show that the $SCN9A^{F1449V-/+}$ heterozygotes over time develop thermal hypersensitivity to the 37°C environment. They avoid the 37°C environment and prefer to spend more time on the cooler perhaps, pain alleviating 22°C environment. By administering a non-specific voltage gated sodium channel blocker, this effect can be reversed and the mice resemble behaviour that is similar to the wild type group.

3.5 Conclusion

In conclusion, it can be determined that this behaviour paradigm works well in both the cold and heat spectrum. The optimal testing conditions to reliably measure cold allodynia is 16°C vs. 22°C. The optimal testing conditions to reliably measure thermal preference is 22°C vs. 37°C. Using the abovementioned paradigm, it allows for reproducible and repeated measurement of animal nociceptive behaviour throughout the course of nerve injury studies, with little effects of learned behaviour.

In subsequent chapters I use the paradigm of cold place preference as a behavioural paradigm to measure cold related behaviour changes after nerve injury in the context of genetic manipulation.

4 NRG1 ablation attenuates cold hypersensitivity after nerve injury but does not attenuate mechanical hypersensitivity

4.1 Introduction

The role of Neuregulin 1 (NRG1) in the peripheral nervous system (PNS) has been well studied. NRG1 is an important signalling factor involved in Schwann cell development and PNS myelination during development. In adulthood, It has been identified that NRG1 is upregulated in the PNS after nerve injury to promote axonal growth, remyelination and target re-innervation (reviewed by (Fricker & Bennett 2011)).

Whilst NRG1 plays a crucial role in the PNS, the role of NRG1 in the CNS in the context of neuropathic pain remains to be fully elucidated. NRG1 has been shown to be produced in the DRG (Loeb et al. 1999; Falls 2003; Velanac et al. 2012; Stassart et al. 2013) where it is likely that it is released into the CNS after nerve damage. Upon NRG1 release, in the dorsal horn of the spinal cord, NRG1 is able to mediate its effects by binding onto erbB receptors on microglia. In Chapter 2, I present evidence that NRG1 and its erbB receptors are dysregulated after nerve injury whilst previous studies suggest that NRG1 released after nerve injury activates microglial erbB receptors in microglia following which the MEK/ERK1/2 pathway is activated leading to microglial proliferation and contributing to the development of neuropathic pain (Calvo et al. 2011). It has been further demonstrated that using pharmacological inhibition of the erbB2 receptor or sequestration of endogenous NRG1, microglial

proliferation is reduced with attenuated mechanical and cold hypersensitivity (Calvo et al. 2010).

I postulate that conditional ablation of NRG1 in adulthood will attenuate microglial proliferation, mechanical and cold hypersensitivity following nerve injury.
In order to investigate this, I use the Cre-loxP system to conditionally ablate NRG1 and investigate its effects on pain related behaviour changes after peripheral nerve injury.

4.2 Methods

4.2.1 Animals

All experimental work on rodents was carried out accordingly to UK Home office legislation (Scientific Procedures Act 1986). Adult mice (8 weeks or older) were used for cell culture, tissue extraction, behaviour, immunohistochemistry and confocal microscopy unless stated otherwise.

Generating conditional NRG1$^{-/-}$ ablation

NRG1$^{fl/fl}$ mice were crossed with CAG-CreER^{TM} mice (JAX(r) mice 004682) to produce CAG-CreER^{TM+}; NRG1$^{fl/fl}$ or CAG-CreER^{TM-}; NRG1$^{fl/fl}$ strains. Both colonies were on a C57BL/6 background (Hayashi & McMahon 2002) . The generation and genotyping of mutant mice with floxed alleles of NRG1 (NRG1$^{fl/fl}$) mice has previously been described (Yang et al. 2001; Brinkmann et al. 2008; Young et al. 2008)

In CAG-CreER^{TM} mice, a tamoxifen inducible form of Cre recombinase is expressed ubiquitously driven by a chimeric promoter constructed from a cytomegalovirus intermediate-early enhancer and a chicken β actin promoter/enhancer (Hayashi and McMahon, 2002). The CreER^{TM} expression and induction has been previously validated (Fricker et al. 2013) using a reporter strain showing Cre activity in DRG and spinal cord upon Cre induction via tamoxifen. The NRG1$^{fl/fl}$ mice are null for α-isoforms of NRG1 in the absence of Cre recombination as they carry a premature stop codon in exon 7, which encodes the α-EGF domain. The lox-P sites flank exons 7–9, and exon 8 encodes the β-EGF domain. Cre recombination therefore results in

ablation of all remaining β isoforms. Following tamoxifen treatment, there is effective reduction on active NRG1 at both the mRNA and protein level (Fricker et al. 2013).

4.2.2 Tamoxifen administration

10 week old CreER^{TM}; NRG1$^{fl/fl}$ mice were dosed by oral gavage with 0.25 mg/kg of body weight with tamoxifen (Sigma T5648) in corn oil, or with corn oil alone (vehicle) for five consecutive days. The CreER^{TM+}; NRG1$^{fl/fl}$, when treated with tamoxifen, were renamed as CreER^{TM+}; NRG1$^{-/-}$ representing conditional NRG1 ablation. After dosing, an interval of 4 weeks was left before surgery to enable appropriate protein knockdown.

In each experiment CreER^{TM+}; NRG1$^{-/-}$ tamoxifen treated animals (later identified as Cre (+) Tx) were compared with CreER^{TM+}; NRG1$^{fl/fl}$ vehicle treated animals (later identified as Vh control). As a further control, comparison was also made with CreER^{TM-}; NRG1 $^{fl/fl}$ tamoxifen treated controls (later identified as Cre (-) Tx). The rationale used for the tamoxifen dosing regimen was that previous studies in our lab and elsewhere have used this regimen to effectively shown knockdown of NRG1 (Fricker et al. 2013). Whilst the Fricker et al. 2013 shows over 90% reduction in NRG1 levels in the spinal cord, our studies shows only 60% reduction. It is possible that following initial ablation, residual NRG1 resides and begins to accumulate over time.

For labeling purposes animals are described as Vh. Control; Cre (-) TX; and Cre (+) Tx throughout this chapter. Wherever possible, we included equal numbers of animals

of each gender in each experimental group. The power calculation conducted is described in Chapter 2, section 2.2.2.

4.2.3 Surgery

The spared nerve injury (SNI) was used as a model of peripheral injury in mice in order to measure behavioural outcomes after injury. The surgical procedures are described in Chapter 2 section 2.2.3.

4.2.4 Immunohistochemistry

Animals were perfused with 0.9% saline and fixed using paraformaldehyde (4% in 0.1M phosphate buffer). Spinal cord, the DRG and sciatic nerve tissue from the ipsilateral (injured) and contralateral (uninjured) sides were dissected from animals. Spinal cord was fixed in 4% PFA for 24 hours before being dehydrated in 30% sucrose for 48 hours at 4°C. DRG and sciatic nerve were fixed for 30 mins – 1 hour before being dehydrated in 30% sucrose for 48 hours at 4°C. Tissue was embedded in OCT compound on dry ice and stored at -80°C. Spinal cord was cut at 30µm, DRG at 10 µm and sciatic nerve at 10 µm and mounted onto SuperFrost Ultra Plus® slides.

4.2.5 Antibodies

Spinal cord, DRG and sciatic nerve tissue sections were washed with PBS and blocked using normal donkey serum (Chemicon S30 1:10, 30 min). Sections were stained with either rabbit ionised calcium binding adaptor molecule 1 (IBA1 1:1000 Wako, Japan), anti-chicken GFP (to enhance YFP signal 1:100 Santa Cruz) and left overnight before being washed 3 times with PBS and then incubated with either anti-rabbit Cyanine-3 (Cy-3 1;500 Life Technologies) or anti rabbit Alexa 488 at room temperature for 3 hours. All reagents were diluted in PBS containing 0.2% triton X-

100 and 0.1% sodium azide. Slides were washed with PBS and cover slipped in Vectashield® mounting medium with DAPI (Vector Labs).

4.2.6 Image analysis

Immunofluorescence was visualized under a Zeiss Imager.Z1 microscope or a confocal Zeiss LSM 700 laser scanning microscope. Photographs were taken using the Axio Cam and AxioVision LE Rel. 4.2 or the LSM Image Browser software for image analysis.

Aif-1 quantification in the spinal cord was performed by taking photomicrographs of at least 4 sections from adjacent regions of the lumbar L3- L4 region per animal and 5 animals per genotype. Each section (Spinal cord, DRG and sciatic nerve) was imaged using x20 objective using a z-stack throughout the section. Three areas within the dorsal horn were selected by drawing a box and staining was analysed within these selected parameters. The same process was carried out at the ventral horn on the ipsilateral and contralateral sides. Within the defined parameters, the percentage of co-localisation between $IBA1^+$ cells $DAPI^+$ nuclear staining were counted and quantified. Within the DRG, 4 separate areas of the DRG were analysed whilst 4 distinct parameters were measured in the sciatic nerve. Analysis was performed in a similar manner to spinal cord section analysis. The experimenter was blind to the genotype during analysis.

4.2.7 Animal Behavioural Tests

All behavioural tests were carried whilst the observer was blind to the genotype of the animal. Animals were tested in a temperature stable room during the light period of their daily day/night cycle, at the same time every day. Where possible, equal numbers of animals of each gender were selected per group.

4.2.7.1 Mechanical withdrawal threshold testing using Von Frey filaments

Mechanical withdrawal thresholds were measured in the same manner as described in Chapter 2 section 2.2.4.1. Animals were tested at Baseline, prior to injury and days 3, 7, 14, 21 and 28 post injury.

4.2.7.2 Thermal nociception threshold testing using Hargreaves' test

Thermal sensitivity threshold levels were measured in the same manner as described in Chapter 2 section 2.2.4.2. Animals were tested at Baseline, prior to injury and days 3, 7, 14, 21 and 28 post injury.

4.2.7.3 Cold Plate Assay

Cold induced allodynia was measured using the Cold Plate assay. Animals were placed on a 10mm thick aluminium plate (35100, Ugo Basile, Italy) surrounded by 47cm tall plastic enclosure with the temperature plate set to 8°C. Each animal was tested for a total of 6 minutes where the number of pain like behaviours (biting, flicking, licking the hind paw or jumping) was measured. Each animal was only tested once at baseline and then at specific time points after injury.

4.2.7.4 Cold Place Preference

The behavioural apparatus was set up as follows. Two 10mm thick aluminium plates (25 – 37 x 47(h) cm 351001, Ugo Basile, Italy) are used to form two chambers connected using a non-conducting corridor platform. The reference plate is set to room temperature of 22°C whilst the fixed temperature plate is set to 16°C. Each animal is placed onto the fixed temperature plate and the animal is permitted to explore the two plates over a period of 10 minutes. At the end of the study, the ratio of time spent on the fixed temperature plate versus the reference room temperature

plate was calculated. Animals were also tested in a 22°C vs. 22°C condition at baseline to investigate any learning or habituation effects.

4.2.7.5 Assessing proprioception using the Beam Test.

The Balance Beam test can be used to measure proprioception type motor coordination (Goldstein & Davis 1990). This type of behavioural testing was conducted under subdued lighting. A wooden beam of 2.5cm diameter with a length of 122cm was elevated approximately 45cm above the floor of the plastic supports. A cardboard box measuring 25 x 25 x 15cm with a 10cm opening was placed at one end of the beam and was identified as the goal target. A light source (lamp with 75W bulb) was located at the start end of the beam. The light source was identified as the avoidance stimuli whilst the dark box was identified as the "safe" environment. For each trial, an animal was placed approximately 5cm from the light source and the animal was encouraged to transverse across the beam into the dark cardboard box environment. Training consisted of 3 trials on the beam separated by 5 minutes before baseline measurements were measured. Pre-training was only conducted before baseline testing. Each animal's performance was analysed by video recording and measuring the number of correct steps (hind paw placed with grip on beam) versus the number of incorrect steps (hops, skips, falls) which was then represented as a percentage. Animals were tested at 3 days prior to Tamoxifen dosing (BL), 3 days prior to injury (pre-injury) and then at days 4, 8, 15, and 22 post-injury.

4.2.8 Quantitative PCR

The process of RNA extraction from relevant tissues, the conversion of RNA into cDNA and the process of running qPCR experiments were followed in the same manner as described in Chapter 2, Section 2.2.6. The internal housekeeping genes

selected were GAPDH and HPRT to compensate for reverse transcription and amplification efficiency variation. Control reactions contained water instead of cDNA and therefore produced no amplification whilst melting curve analysis confirmed specificity of all products. Primers used to detect NRG1 are described in Chapter 2, section 2.2.6.

4.2.9 Statistical Analysis

As the genotypes of each animal are the only variable, similarities or differences between Vh control, Cre (-) Tx, and Cre (+) Tx were determined using repeated measures two-way ANOVA with post Hoc Bonferronni test for behavioural studies whilst the Students t-test was used for immunohistochemistry and qPCR studies. Results are reported as mean values ± SEM.

4.3 Results

4.3.1 NRG1 mRNA levels are significantly down regulated using the Tamoxifen inducible CAG-CreER^{TM} system

To determine Global NRG1 knockdown, tissue was extracted from spinal cord, lumbar DRG and sciatic nerve. The level of NRG1 mRNA in either the Cre (-) Tx or Cre (+) Tx groups was measured. Due to limitations in the number of animals available, the Vh control could not be included in this experiment. NRG1 mRNA levels were quantified and expressed as mRNA expression relative to housekeeping genes as shown in Figure 4-1.

With Tamoxifen administration, NRG1 mRNA levels were significantly down regulated in the spinal cord, DRG and sciatic nerve of Cre (+) Tx versus Cre (-) Tx. There was approximately 60% reduction in NRG1 mRNA levels in the spinal cord; approximately 70% reduction in the DRG and 80% reduction in the sciatic nerve.

Using mRNA from CreER^{TM}; NRG1$^{fl/fl}$ and CreER^{TM+}; NRG1$^{-/-}$ (conditional NRG1 KO) strains, it can determined that the CAG- CreER^{TM} Cre lox-P system is capable of significantly down regulating NRG1 mRNA levels. Therefore the effects of NRG1 knockdown on pain related behaviour after peripheral nerve injury can be studied. Given the time and resource constraints in this study, further attempts should have been made to improve the degree of knockdown of NRG1. However, it is plausible to state that 60% reduction in NRG1 levels is sufficient for its biological activity.

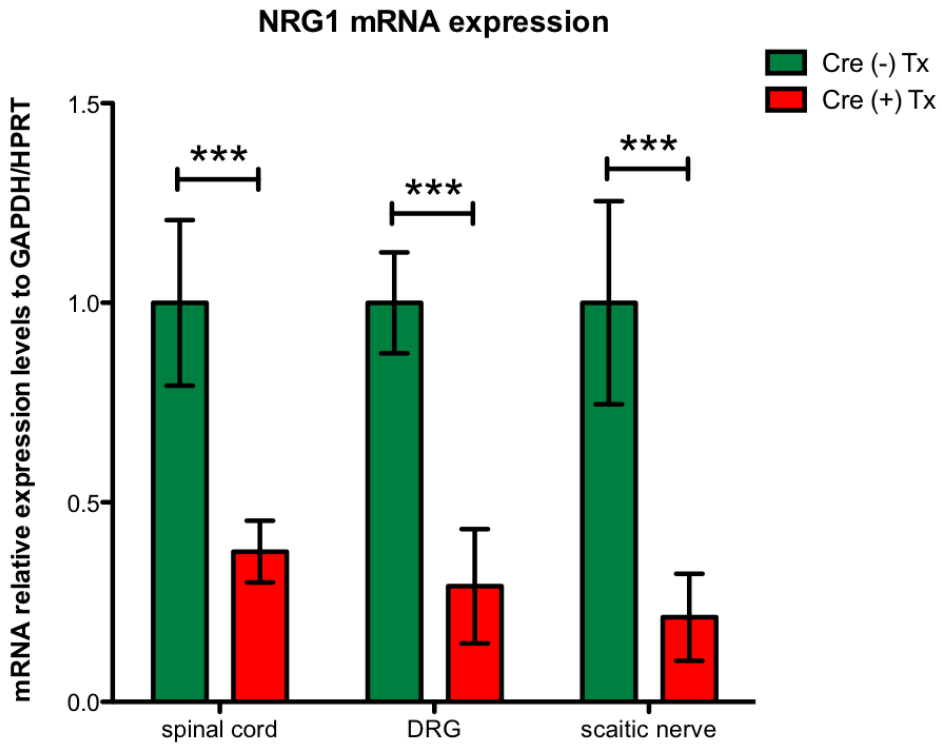


Figure 4-1 NRG1 mRNA levels are significant down regulated in the PNS and CNS after Tamoxifen administration. Animals were treated with Tamoxifen and mRNA was extracted 4 weeks later from lumbar spinal cord, DRG and sciatic nerve. In the presence of Cre recombinase, NRG1 mRNA levels were significantly down regulated in the spinal cord, DRG and sciatic nerve in the $NRG1^{-/-}$ Cre (+) Tx group vs. control (Cre (-) Tx) ($p<0.001$; n=6 per group; Students t-test).

4.3.2 Mechanical hypersensitivity is not attenuated with conditional NRG1$^{-/-}$ knockdown

Animals underwent spared nerve injury and were tested for mechanical hypersensitivity using the Von Frey Test. Figure 4-2 shows the 50% withdrawal threshold to mechanical stimuli at baseline and days 3, 7, 14 and 21 after spared nerve injury. At baseline, animals from all three groups exhibited normal 50% withdrawal thresholds (Vh. Control 0.945 ± 0.135g; Cre (-) Tx. 0.909 ± 0.062g; Cre (+) Tx. 1.042 ± 0.080g). After tamoxifen dosing (pre-injury) animals from all groups maintained normal mechanical sensitivity thresholds. However, 3 days after injury, all three groups developed significant mechanical hypersensitivity ($p<0.0001$) but no significant difference existed between all groups (Vh. Control 0.019 ± 0.006g; Cre (-) Tx. 0.108 ± 0.041g; Cre (+) Tx. 0.023 ± 0 0.005g). Mechanical hypersensitivity was maintained through the course of the study up until day 21 with no significant difference detected between the control(s) and NRG1$^{-/-}$ mice ($p = 0.768$). Whilst the ipsilateral (injured) paw developed extreme mechanical hypersensitivity after spared nerve injury, the contralateral (uninjured) paw maintained normal 50% withdrawal threshold levels (Figure 4-2b). Given that Sorge et al. suggest that microglial induced neuropathic pain was gender specific, each cohort was split by gender and further analysed. It was determined that there is no significant difference in the mechanical hypersensitivity thresholds between genders within each experimental group (Sorge et al. 2015) (please refer to appendix Table 1 and 2).

With these findings, it can be determined that NRG1 release after peripheral nerve injury is not important in regulating mechanical hypersensitivity.

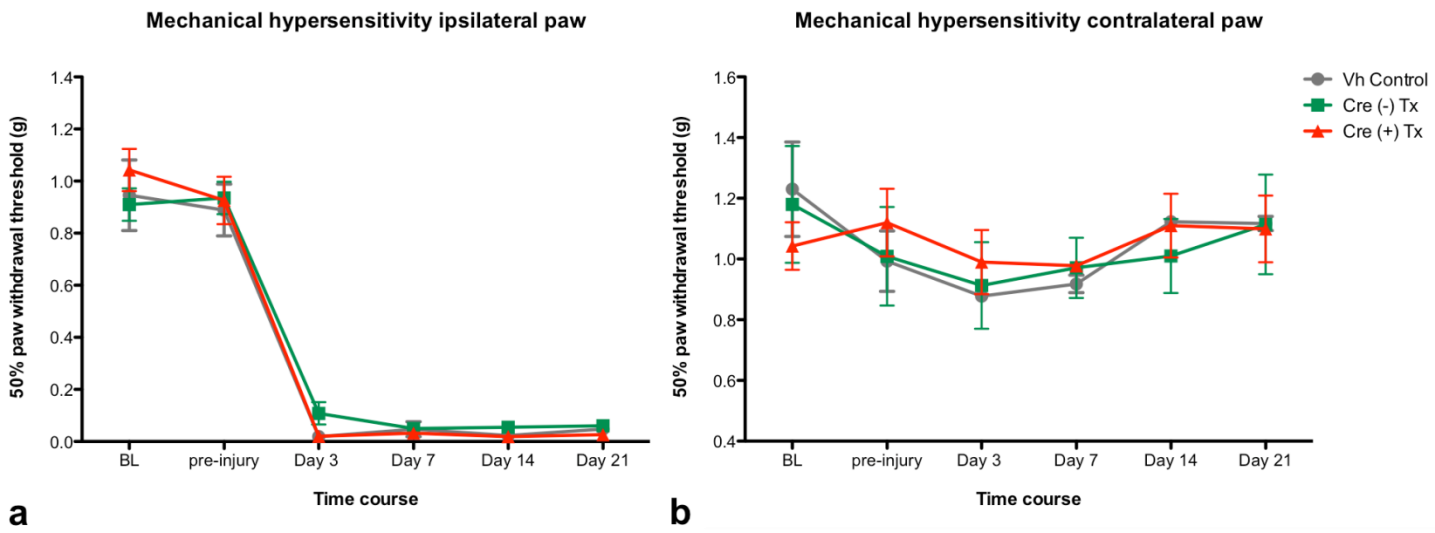


Figure 4-2 mechanical hypersensitivity is not attenuated in Global NRG1-/- mice (a) Shows the 50% withdrawal threshold in the ipsilateral hind (injured) paw before and after nerve injury. Whilst animals from both groups develop significant mechanical hypersensitivity in the ipsilateral paw ($p<0.0001$), there is no significant difference in their 50% withdrawal threshold between the two genotypes ($p=0.768$). (b) Shows the 50% withdrawal threshold in the contralateral (uninjured) hind paw. There is no mechanical hypersensitivity in the contralateral paw through the course of the study ($p=0.897$) (Vh. Control $CreER^{TM+}$; $NRG1^{fl/fl}$ oil treated; Cre (-) Tx. = $CreER^{TM-}$; $NRG1^{fl/fl}$ Tx. treated; Cre (+) Tx = $CreER^{TM+}$; $NRG1^{-/-}$ Tx treated) n= 9-28 per group; repeated measures two way ANOVA; post-hoc Bonferronni test)

4.3.3 Thermal hypersensitivity post nerve injury is not altered in NRG1 $^{-/-}$ animals

The Hargreaves test was used to measure thermal hypersensitivity levels after peripheral nerve injury in the appropriate control(s) and NRG1$^{-/-}$ group. At baseline, all three groups: Vh control, Cre (-) Tx and Cre (+) Tx maintain normal thermal sensitivity thresholds in the ipsilateral paw as demonstrated in Figure 4-3.

The average latency to withdraw from thermal stimuli was measured as 11.316 ± 1.412s in the Vh. control group; 11.131 ± 0.718s in the Cre (-) Tx. group; and 12.042 ± 0.812s in the Cre (+) Tx. group. At 3 days after injury, both control groups and the Cre (+) Tx. group developed significant thermal hypersensitivity ($p<0.001$) but there was no significant difference between the Vh control, Cre (-) Tx and Cre (+) Tx groups. There was also no significant difference between the control groups and global NRG1$^{-/-}$ group at days 3, 7, 14, and 21 after injury. At day 21 the Vh. control group exhibited a withdrawal latency of 5.117 ± 0.563 whilst the Cre (-) Tx. group exhibited 6.118 ± 0.512s and the Cre (+) Tx. group exhibited 5.963 ± 1.414s. Whilst the ipsilateral (injured) paw developed extreme thermal hypersensitivity after spared nerve injury, the contralateral (uninjured) paw maintained normal thermal latency threshold levels (Figure 4-3b). Each cohort was split by gender and further analysed. It was determined that there is no significant difference in the thermal hypersensitivity thresholds between genders within each experimental group (please refer to appendix Table 3 and 4).

NRG1 ablation did not therefore attenuate thermal hypersensitivity. Microglial activation via NRG1 is unlikely to regulate thermal hypersensitivity after peripheral nerve injury.

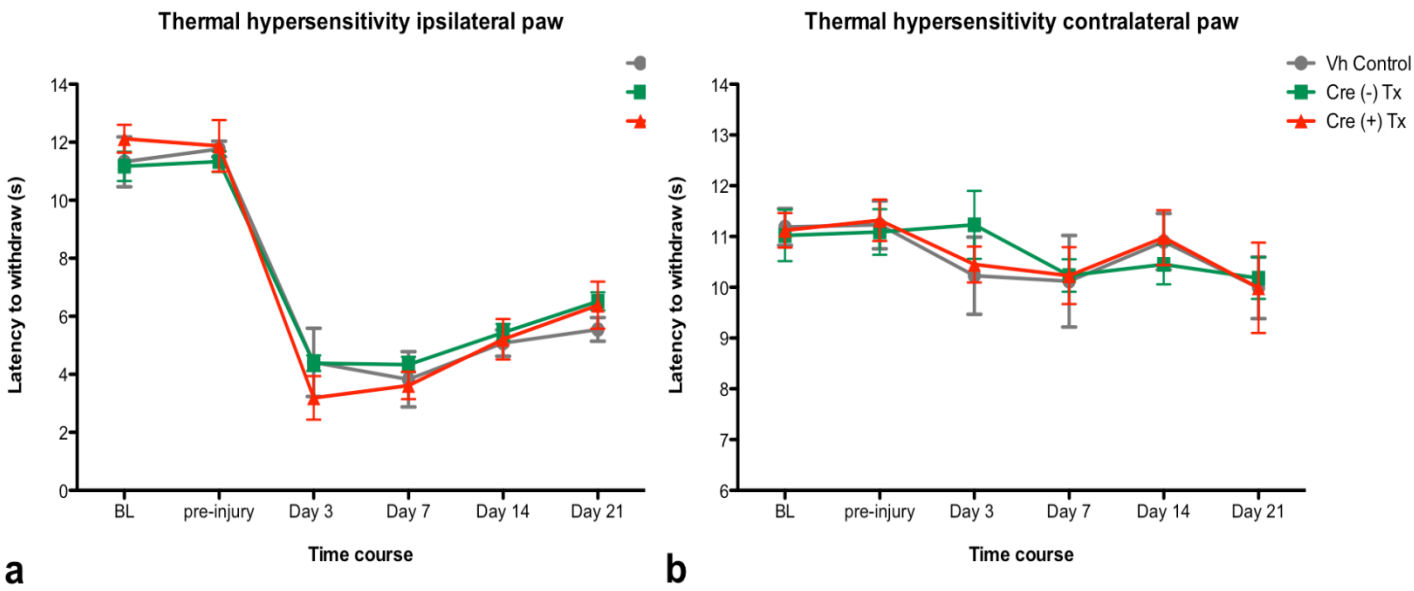


Figure 4-3 thermal hypersensitivity is not attenuated with global ablation of NRG1

(a) The latency to withdraw from a thermal stimuli is measured in the ipsilateral (injured) paw of all three groups. At baseline, all groups maintain normal withdrawal latencies. After injury however, all three groups develop significant thermal hypersensitivity ($p<0.0001$) but all to the same degree. There is no difference in the latency to withdraw from a thermal stimulus between the control(s) and NRG1-/- group at days 3, 7, 14, and 21 after injury ($p= 0.731$). (b) Shows the latency to withdraw from thermal stimuli in the contralateral (uninjured) paw. The contralateral paw does not develop thermal hypersensitivity through the course of the study but maintain normal threshold levels. (Vh. Control CreERTM+; NRG1fl/fl oil treated; Cre (-) Tx. = CreERTM-; NRG1fl/fl Tx. treated; Cre (+) Tx = CreERTM+; NRG1-/- Tx treated. n= 9-28 per group; repeated measures two way ANOVA; post-hoc Bonferronni test)

4.3.4 NRG1$^{-/-}$ animals develop delayed recovery of motor coordination (proprioception) after peripheral nerve injury

It has been proposed that that NRG1 isoforms regulate the differentiation of postsynaptic skeletal muscle fibres at the neuromuscular junction to induce muscle spindle formation (Buonanno & Fischbach 2001; Hippenmeyer et al. 2002). It was hypothesised that NRG1 knockdown leads to loss of muscle spindle formation, which in turn impairs motor coordination. The Beam test was used to measure proprioception after NRG1 knockdown coupled with peripheral nerve injury.

In Figure 4-4, the percentage of correct steps transversed across a beam was quantified. At baseline, the Vh control made on average 93.189 ± 1.716% of correct steps whilst the Cre (-) Tx group spent 93.052 ± 1.698% and the Cre (+) Tx group spent 90.764% of correct steps. After treatment, at pre-injury the control groups exhibited approximately 96% and 92% accuracy whilst the Cre (+) Tx groups exhibited significantly lower accuracy with the number of correct steps (73.231 ± 1.997% $p = 0.045$). After injury all three groups developed significantly impaired motor function but there was no significant difference between all three groups at day 4 and day 8 after injury. By day 15 however, the Cre (+) Tx group exhibited significantly delayed motor function recovery, which was maintained at day 22 in comparison to controls. At day 22 the Vh. control group made on average 97.782 ± 2.362% of correct steps whilst the Cre (-) Tx group made 88.395 ± 2.746% of correct steps. By day 22, the control groups regained normal proprioception and motor function and returned to baseline readings. The Cre (+) Tx. group however made significantly lower % of correct steps at 65.346 ± 7.231% ($p = 0.045$). It seems that after nerve injury, the Cre (+) Tx group that motor impairment does not significantly differ from the Vh Control or Cre (-) Tx group, but is significantly impaired at the

delayed stages of nerve injury (days 15 and 22). Whilst this degree of impairment in Cre (+) Tx reaches the level seen after Tx dosing (pre-injury), it is evident that an ablation of NRG1-/- is vital in controlling motor function. In the absence of NRG1-/-, motor function is impaired and this is independent of nerve injury.

It can therefore be determined that NRG1 is crucial in the PNS and regulating motor function. In the absence of NRG1, motor coordination is significantly impaired in the absence of injury and results in delayed recovery after peripheral nerve injury.

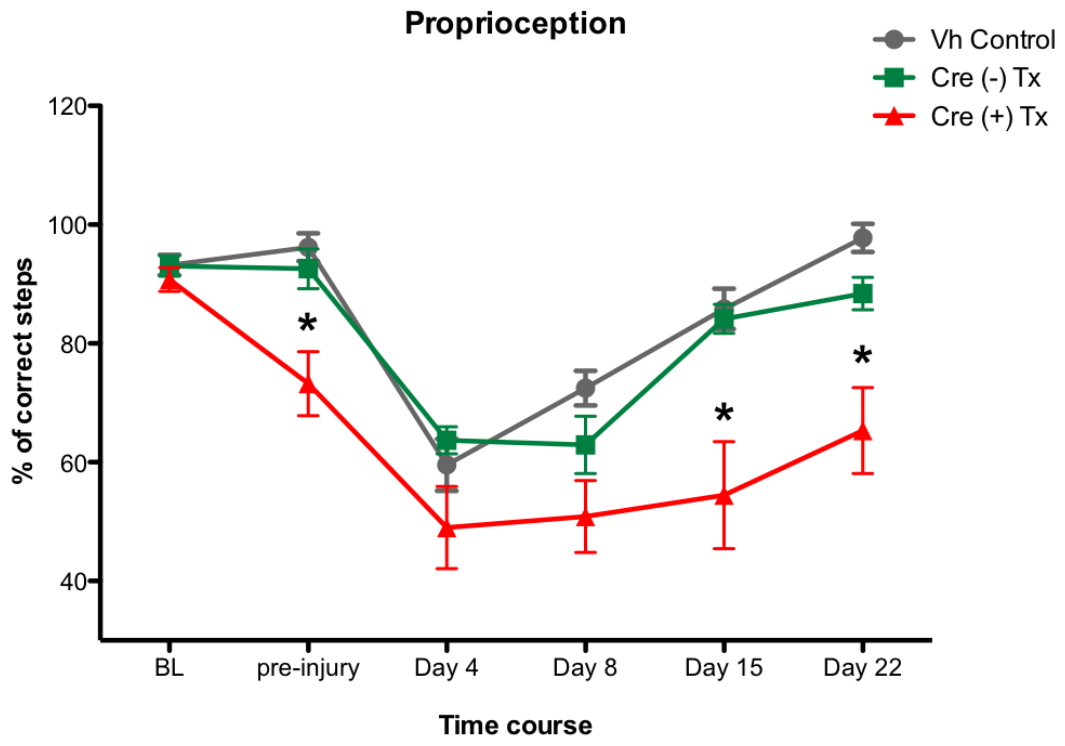


Figure 4-4 Global NRG1-/- animals develop impaired motor coordination with delayed motor recovery after peripheral nerve injury. At baseline all 3 groups exhibit near 100% correct steps when transversing a beam. After Tx. dosing, the Cre (+) group develops a significant reduction in the % of correct steps in comparison to control groups (p=0.042). After injury, all three groups develop marked motor impairment at day 3 onwards. Whilst the control groups return to baseline levels by day 22, the Cre (+) Tx group develops presents significantly lowered % of correct responses, and thus delayed recovery at days 15 and 22 in comparison to controls. (p= 0.045) (Vh. Control CreER^{TM+}; NRG1$^{fl/fl}$ oil treated; Cre (-) Tx. = CreER^{TM-}; NRG1$^{fl/fl}$ Tx. treated; Cre (+) Tx = CreER^{TM+}; NRG1$^{-/-}$ Tx treated. n= 9 per group; repeated measures two way ANOVA; post-hoc Bonferronni test).

4.3.5 The Cold Plate assay suggests that cold hypersensitivity is reduced in Global NRG1$^{-/-}$ animals after peripheral nerve injury

The pharmacological blockade of erbB2 receptors and endogenous NRG1 release has been suggested to regulate cold related hypersensitivity. The Cold Plate test was used to measure cold related pain behaviour after peripheral nerve injury (Figure 4-5). At baseline, control groups presented on average 53.714 ± 2.652 (Vh. Control) and 52.421 ± 4.279 (Cre (-) Tx) pain like behaviours to cold stimuli whilst the Cre (+) Tx group presented on average 49.222 ± 6.436 pain like behaviours. There was no significant difference between the groups at baseline or after Tamoxifen treatment (pre-injury). However, after injury all three groups presented lowered number of pain behaviours and continue to present fewer pain-like behaviours until day 21. At day 21, the Cre (+) Tx. group exhibited significantly fewer number of pain like behaviours from control groups (Figure 4-5). Each cohort was split by gender and further analysed. It was determined that there is no significant difference in the cold hypersensitivity thresholds between genders within each experimental group (please refer to appendix Table 5).

It can be speculated that with NRG1 ablation, cold allodynia is attenuated in the delayed stages of nerve injury. It is worth noting that over time each group presents fewer, pain like responses in comparison to the previous time point. This was most likely due to habituation. Over time, the prior experiences have led the animals to display fewer, pain like behaviours.

Thus, I used an alternative behavioural assay to determine whether NRG1 regulates cold hypersensitivity after nerve injury.

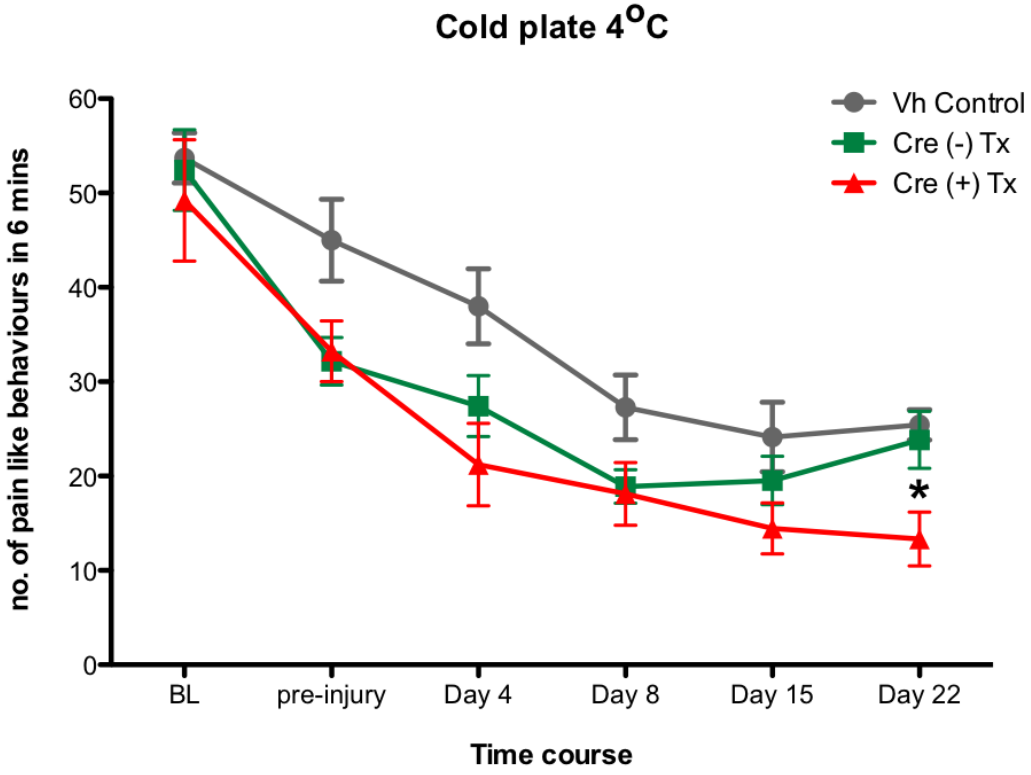


Figure 4-5 Global NRG1 animals display significantly fewer cold pain like behaviours in the delayed stages of peripheral nerve injury. Animals were placed in a 4ºC chamber and the total number of pain like behaviours was quantified. At baseline all three groups exhibited on average approximately 50-55 pain like behaviours to cold stimuli. After injury, all groups exhibited consistently lower no. of pain like behaviours. This was due to a learning effect. However at day 22, the Cre (+) Tx group exhibited significantly lower number of pain like behaviours (13.333 ± 0.285) to cold stimuli vs. the control groups (25.428 ± 1.616 Vh. control; 23.842 ± 3.012 Cre (-) Tx). With NRG1 ablation it seems that animals displayed delayed attenuation of cold allodynia (Vh. Control $CreER^{TM+}$; $NRG1^{fl/fl}$ oil treated; Cre (-) Tx. = $CreER^{TM-}$; $NRG1^{fl/fl}$ Tx. treated; Cre (+) Tx = $CreER^{TM+}$; $NRG1^{-/-}$ Tx treated. p=0.031; n= 9-18 per group; repeated measures two way ANOVA; post-hoc Bonferronni test)

4.3.6 The Cold Place Preference assay also demonstrates that cold hypersensitivity is attenuated in NRG1$^{-/-}$ animals after peripheral nerve injury

Given that the previous behavioural assay caused the animals to display learned responses over time, I used the Cold Place Preference assay described in depth in Chapter 3. Due to limitations in the number of animals available, the Vh. control group could not be included into this experiment.

At baseline the Cre (-) Tx group and the Cre (+) Tx group exhibited reduced % occupancy in the 16°C chamber vs. 22°C chamber which did not differ between the groups (28.738 ±1.216% Cre (-) Tx; 28.330 ± 1.201 Cre (+) Tx). After Tamoxifen treatment, the control group and the Cre (+) Tx group both exhibited unchanged % occupancy in the 16°C in 10 mins (Figure 4-6a). At 4 days after injury, both groups developed significant hypersensitivity to cold stimuli (17.109 2.879% Cre (-) Tx; 19.679 ± 2.678% Cre (+) Tx occupancy of the 16°C chamber). Whilst both groups developed significant hypersensitivity to the cold 16°C environment, there was no significant difference in the % occupancy in the 16°C between the control and global NRG1$^{-/-}$ group at days 4, 8, and 15 after nerve injury.

At days 22 and 29 however the Cre (+) Tx group displayed significantly higher % occupation in the 16°C environment vs. the Cre (-) Tx group. At day 29, the Cre (-) Tx. group only spends approximately 7.418 ± 1.130% of 10 mins in the 16°C whilst the Cre (+) Tx. group spends significantly more time 16.217 ± 1.989% of 10 mins ($p= 0.005$).

To determine whether this behavioural assay led to learning (and therefore avoidance of the 16°C environment) the number of crossings between the 16°C and 22°C environments were quantified. As shown in Figure 4-6b, animals from both groups

maintained stable no. of crossings throughout the course of the study. Therefore it is unlikely that the reduction in % occupancy of 16°C seen after injury is due to learning effects but rather due to the development of cold hypersensitivity. Each cohort was split by gender and further analysed. It was determined that there is no significant difference in the cold hypersensitivity thresholds between genders within each experimental group (please refer to appendix Table 6 and 7).

Given the temporal profile of the delayed attenuation of cold allodynia it is likely that cells other than microglia (e.g. astrocytes) could play a role in the maintenance of cold allodynia. The temporal profile of the delayed attenuation mimics that of the role of astrocytes in neuropathic pain. Alternatively is it also plausible, since NRG1-/- ablation was only 60% efficient, there may some residual effects of basal NRG1 in the initial stages after nerve injury whereby the affect of cold hypersensitivity attenuation is diminished. However, as chapter 6 demonstrates, similar effects in delayed cold attenuation are seen independent of NRG1 ablation. It therefore remains unclear to as why there is a delay in the onset of cold allodynia. As a result, it is also possible that NRG1 function may not be responsible for microglial responses to nerve injury or the pain that ensues. With these findings, it can therefore be determined that in the absence of NRG1 there is attenuation of cold hypersensitivity at later stages of after peripheral nerve injury.

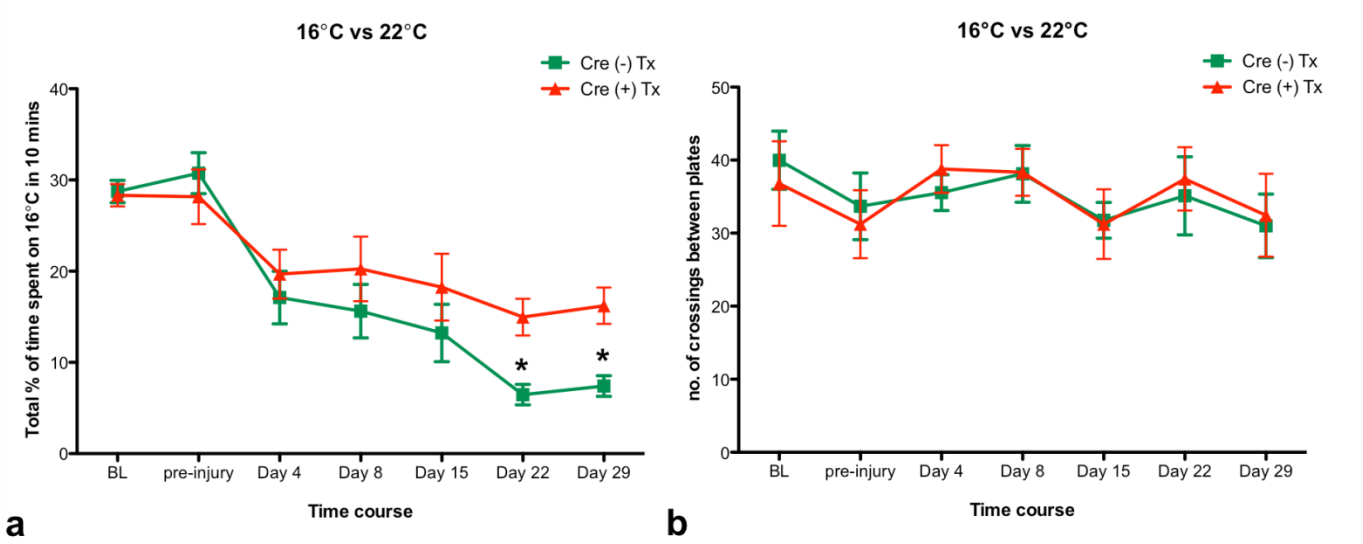


Figure 4-6 global ablation of NRG1 leads to delayed attenuation of cold hypersensitivity (a) Shows the total % occupation at 16°C vs. 22°C in 10 mins. At baseline and after Tamoxifen treatment, the control group (Cre (-) Tx.) and NRG1 knockout group (Cre (+) Tx) exhibit normal % occupancy at 16°C. After injury, both groups develop marked sensitivity to cold stimuli and spend significantly less time on the 16°C environment at days 4, 8, and 15. At day 22 and 29 however, the conditional NRG1 KO group displays a significantly higher % occupancy at 16°C vs. the control group (p= 0.005). With conditional NRG1 ablation, cold allodynia is attenuated in the delayed stages of nerve injury. (b) shows the no. of crossings between the 16°C and 22°C environment with no change in their exploratory behaviour through the course of the study (p=0.871). (Cre (-) Tx. = $CreER^{TM-}$; $NRG1^{fl/fl}$ Tx. Treated and Cre (+) Tx = $CreER^{TM+}$; $NRG1^{-/-}$ Tx treated. n=9 per group; repeated measures two way ANOVA, post-hoc Bonferronni test)

4.3.7 Microgliosis is not down regulated after injury with the conditional ablation of NRG1$^{-/-}$

It was hypothesised that with ablation of NRG1, there may be attenuation or down regulation in microglial proliferation caused by the lack of NRG1-erbB receptor binding because in other model systems - NRG1 can promote proliferation of these cells.

In order to investigate microglial proliferation, spinal cord tissue was extracted and immunostained to quantify the number of IBA1^{+} microglia between the control(s) and conditional NRG1$^{-/-}$ group.

Figure 4-7a and b show that there was no significant change in microglial numbers in all areas of the spinal cord between the Vh control and Cre (-) Tx groups and the Cre (+) Tx (conditional NRG1$^{-/-}$) group after injury.

Whilst there is no difference between groups, there is significantly higher proportion of IBA1^{+} labelled microglial cells in the ipsilateral (injured) side versus the contralateral (uninjured) side. This is attributed to microglial proliferation on the side of injury. Figure 4-7b shows the number of branches from each IBA1^{+} cells analysed. There was no significant difference in the number of microglial branches between controls and the global NRG1$^{-/-}$ group. There was also no significant difference between the ipsilateral (injured) and contralateral (uninjured) sides. The microglial response to nerve injury at a morphological level is therefore not altered following conditional NRG1 ablation.

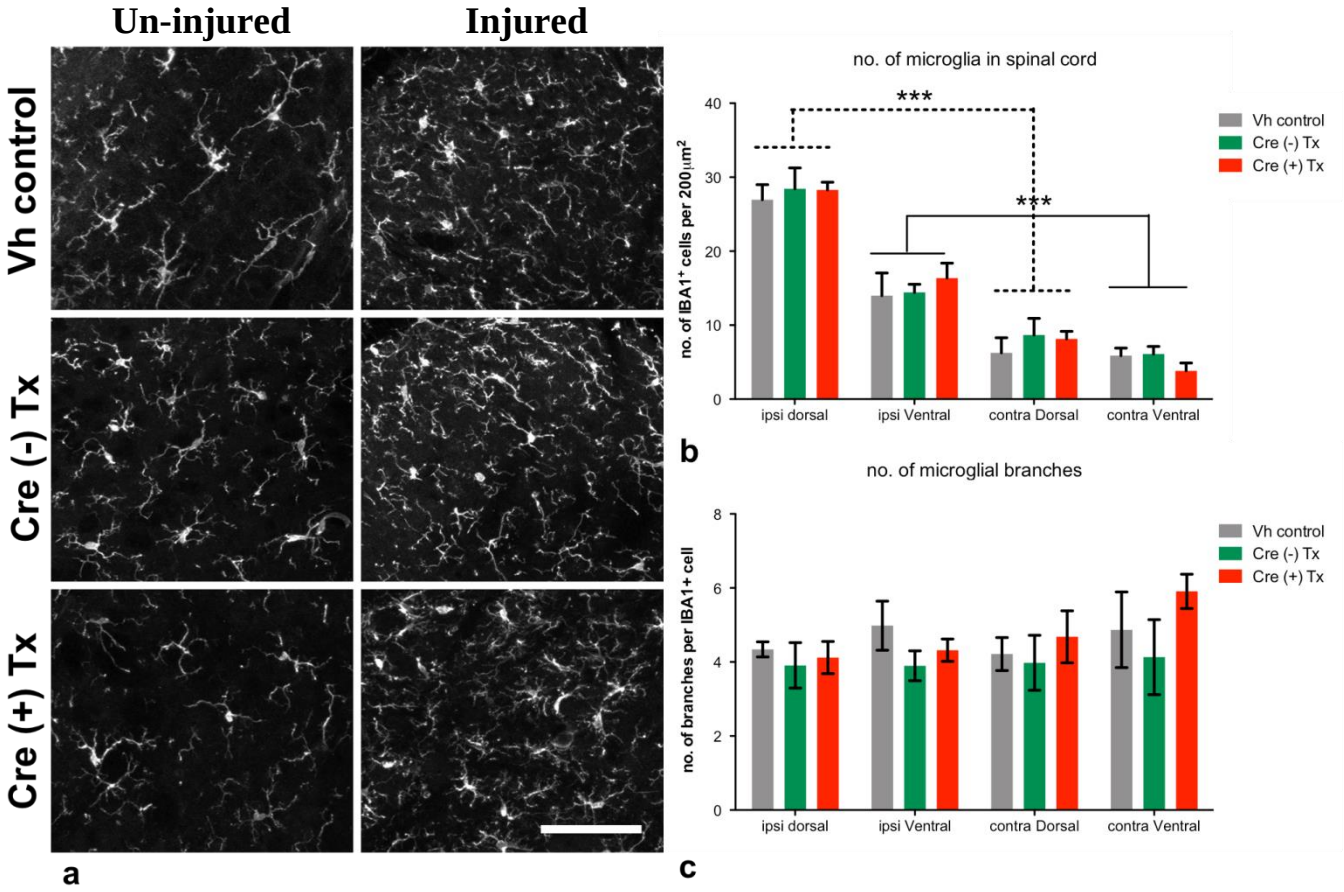


Figure 4-7 microglial numbers are not attenuated after nerve injury with NRG1 ablation (a) representative images of IBA^{+} cells in the dorsal horn of each group. (b) The number of IBA1^{+} microglia was quantified in the lumbar spinal cord. IBA1 quantification was conducted in the dorsal horn ipsilateral and contralateral and the ventral horn ipsilateral and contralateral sides. There is no change in microglial numbers between the control groups and the global NRG1$^{-/-}$ group. Whilst there are significantly more microglia in the ipsilateral side vs. the contralateral side ($p<0.0001$), there is no difference in number between groups ($p=0.408$). (c) Shows that the number of branches does change between groups either and there is no significant difference in the number of branches between the ipsilateral and contralateral sides (ipsi. Dorsal= injured DH; ipsi. Ventral = injured VH; contra. Dorsal = uninjured DH; contra. Ventral = uninjured VH) (Vh. Control CreER^{TM+}; NRG1$^{fl/fl}$ oil treated; Cre (-) Tx. = CreER^{TM-}; NRG1$^{fl/fl}$ Tx. treated; Cre (+) Tx = CreER^{TM+}; NRG1$^{-/-}$ Tx treated. $p=0.661$; n = 6 per groups; repeated measures two way ANOVA; post-hoc Bonferronni test).

4.3.8 Astrocyte proliferation is not attenuated after nerve injury with global NRG1 knockdown

Astrocytes are also known to express erbB receptors (Francis et al. 1999; Galvez-Contreras et al. 2013; Sharif et al. 2009) and therefore warrant investigation into their effects in the CNS after peripheral nerve injury. Whilst I did not detect an attenuation of microglial number through proliferation with nerve injury in the absence of NRG1, I investigated astrocyte proliferation immunofluorescence activity after SNI in the control(s) and Global $NRG1^{-/-}$ strain. As Figure 4.8 demonstrates, there was no significant difference in $GFAP^{+}$ IR between the Cre (-) Tx and Cre (+) Tx groups in the injured dorsal horn. On average the Cre (-) Tx group exhibited 4.501± 0.255 $GFAP^{+}$ IR whilst the Cre (+) Tx group exhibited 4.221 ± 0.201 $GFAP^{+}$ IR. Whilst there were significantly more $GFAP^{+}$ IR in the injured side versus the uninjured side in the dorsal horn ($p<0.0001$), there was no significant difference in the $GFAP^{+}$ IR between genotypes in any area of the spinal cord.

It can therefore be speculated that with NRG1 ablation, does not alter the astrocytic response to nerve injury.

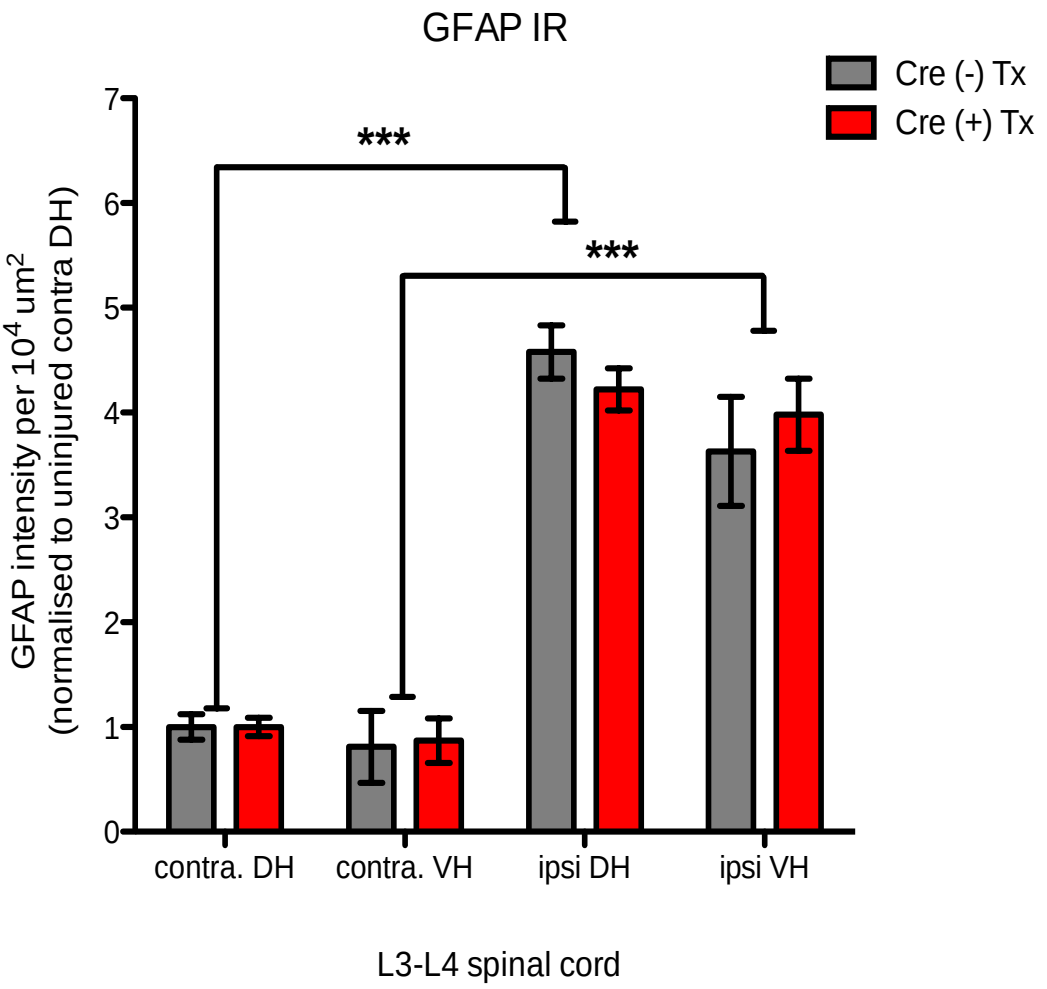


Figure 4-8 GFAP expression does not differ after SNI in the absence of NRG1. GFAP expression does not differ after SNI in the absence of NRG1. The level of GFAP immunofluorescence intensity was measured in the lumbar area of the spinal cord between the Cre (-) Tx and Cre (+) Tx group at day 29 post SNI. The uninjured contralateral DH was used as control and all results were referenced to this point. Within the injured ipsilateral dorsal horn, there is no significant difference in GFAP IR between Cre (-) Tx and Cre (+) Tx group (4.500 ± 0.251 vs. 4.221 ± 0.201 respectively). Whilst there is significantly higher GFAP IR in the ipsilateral DH vs. the contralateral DH in both groups ($P<0.0001$), There is no significant difference in the level of GFAP IR increase between the two groups ($p=0.81$; n= 5 per group; repeated measures two way ANOVA; post hoc-Bonferonni test).

4.4 DISCUSSION

Signals that activate microglial cells to initiate downstream signalling and cause the release of CNS inflammatory mediators are key targets in attenuating the microglial contribution to neuropathic pain. NRG1 is one key target (Calvo et al. 2010).
In chapter 2 evidence shows that NRG1 subtypes and erbB receptors are significantly dysregulated after peripheral nerve injury. In order to directly study the role of NRG1 following nerve injury I conditionally ablated NRG1 in adulthood using a global Cre lox P system. The most significant findings were that NRG1 was required for proprioceptive function both before and after injury and contributed to the development of cold hypersensitivity at later phases following nerve injury. Using this system in contrast to expectation in animals lacking NRG1 I did not observe a reduction in mechanical pain hypersensitivity or microgliosis following nerve injury.

4.4.1 NRG1 ablation impairs motor function in the naïve state which is accentuated following nerve injury.

It was hypothesized that with nerve injury and the absence of NRG1, motor co-ordination may be impaired through the loss of muscle spindle fibers. In this chapter, I ablate the expression of all functional NRG1 isoforms to demonstrate that proprioception is impaired after NRG1 knockdown (Figure 4-4).
Muscle spindles are sensory organs distributed throughout the muscles of vertebrates. They are responsible for detecting muscle stretch, to allow the perception of body position (proprioception) which is important for coordinated movement (Maier 1997). The formation, maturation and maintenance of muscle spindles depend on Bace1 substrates. During development, Bace1 expression has been shown to coincide with NRG1 Type III expression in the DRG and motor neurons of the spinal cord (Willem

et al. 2006). Here, Bace1 is responsible for the cleavage of NRG1 in cells required for full activity of the molecule (Cheret et al. 2013). Mutant mice lacking Bace1 display severe hypomyelination of peripheral nerves similar to that seen in mice lacking NRG1 Type III and recent evidence suggests that BACE-1 is required for cleavage and activation of Ig domain containing NRG1 isoforms (types 1 and 2) required for muscle spindle development and maintenance (Taveggia et al. 2005) and Cheret et al. showed that Bace1 controls the maintenance of muscle spindles during adulthood, as well as their formation during development and is required to maintain motor coordination. Bace 1 mutant mice displayed impaired grip ability and aberrant gait demonstrated through a lack of forelimb and hind limb coordination (Cheret et al. 2013).

A graded reduction in Ig Nrg1 signal strength *in vivo* results in increasingly severe deficits in formation and maturation of muscle spindles. When Bace1 substrate, NRG1, is ablated throughout the PNS and CNS, it leads to severe reduction in spindle fiber numbers (84% reduction), with significantly smaller spindle diameter and fewer intrafusal fibers in the remaining spindles (but sensory innervation is preserved) (Cheret et al. 2013). This results in a more pronounced deficit in motor-coordination. The conditional $NRG1^{-/-}$ mice displayed profoundly impaired grip and hung 3–4 times less long in the inverted grid test than control littermates. Motor coordination was strongly impaired, as assessed using a beam-walking test (Cheret et al. 2013).

In our study, similar results are reported. In the absence of NRG1 prior to injury, animals display significantly impaired proprioception, (likely to be mediated through the loss of muscle spindles as previously demonstrated), in comparison to controls. With injury, this effect is further enhanced. Whilst control group(s) and the $NRG1^{-/-}$

group develop impaired proprioception, the NRG1$^{-/-}$ group presents delayed recovery and significantly impaired motor coordination in the late stages of injury.

4.4.2 Studying the role of NRG1 in the development of pain related hypersensitivity following peripheral nerve injury in the mouse

A number of previous studies have assessed the potential role of NRG1 in the development of persistent pain using a number of different models.

Calvo et al. 2011 demonstrated that intrathecal treatment with NRG1 in naïve animals induces phosphorylation of this pathway within spinal microglia. This was associated with microglial proliferation and development of cold and mechanical pain-related hypersensitivity (Calvo et al. 2011). Additionally, Lacroix-Fralish et al. demonstrated that intrathecal administration of recombinant NRG1-β1 protein significantly decreased mechanical withdrawal threshold in male rats (LaCroix-Fralish et al. 2006).

Wang et al. report that NRG-1 inhibits mechanical hypersensitivity in the SNI model, while it aggravates formalin-induced mechanical pain where the knockdown of erbB receptors relieves the aggravation of NRG1 on formalin induced mechanical hypersensitivity. Dai et al. performed gene knockdown experiments to show that ErbB4 was involved in the inhibition of SNI induced neuropathic, while ErbB2 was involved in the aggravation of formalin-induced pain in response to NRG-1 (Wang et al. 2014).

Ma et al. generated transgenic rats with a small *Sleeping Beauty* transposon mutation in the NRG1 transgene to show a decrease in mechanical threshold on the whisker pads between 2 - 5 weeks after CCI injury (of the trigeminal infrorbital nerve) when compared to control (Ma et al. 2012). Using the paw incision induced pain model,

Xiang et al. 2014 also demonstrated the role of NRG1 on microglia where NRG1 Type I and II mRNA are significantly up regulated in the L5 DRG coupled with microglial proliferation and mechanical allodynia. The pharmacological blockade of erbB2 leads to down-regulation of NRG1 type I and II in the DRG and suppressed incision induced microglial proliferation and mechanical allodynia (Xiang et al. 2014). Kanzaki et al. show that in the SNL model of nerve injury in the rat, the expression of NRG1 type I and type II are significantly increased in the ipsilateral L5 DRG compared to the contralateral side and follow a temporal profile similar to that with the development of neuropathic pain (Kanzaki et al. 2012).

In this chapter, it was determined that with conditional NRG1 ablation in adult mice, mechanical hypersensitivity was not attenuated whilst cold hypersensitivity was reduced in the delayed stages after injury.

There are a number of reasons to as why these findings are not consistent with previous findings. It is often difficult to interpret findings in rat versus mouse. It is possible that NRG1 may play distinct roles in rat and mouse where NRG1 plays a role in modulating mechanical hypersensitivity and cold related allodynia in rat but does not regulate mechanical hypersensitivity in the mouse. Instead, NRG1 may only regulate cold related allodynia in the mouse. To support this argument, it is known that the sciatic nerve contribution from the DRG is identified at different levels in the rat and mouse. Rigaud et al. suggest that strain dependent variability in segmental contributions to the sciatic nerve may account in part for genetic differences in pain behaviour after nerve injury. Rigaud et al. demonstrate that mouse L3 and L4 neuronal segments are anatomically and functionally homologous with rat L4 and L5 segments (Rigaud et al. 2008).

Gender differences may also account for the variability seen in this study in comparison to previous findings. Sorge et al. induced mechanical allodynia in mice of both sexes using SNI followed by intrathecal injections of glial inhibitors at 7 days post injury. Results showed that three different glial inhibitors all produced robust, dose-dependent reversal of allodynia in male mice whilst no reversal of allodynia was observed in female mice at any dose. Similar results were observed for persistent inflammatory pain. This sex difference appeared to be dependent on testosterone whilst reactive microgliosis after SNI was broadly similar between sexes (Sorge et al. 2015). It was hypothesised that the microglial effects of NRG1 regulation in mice within our study may be mitigated by the fact that both sexes were included in the analysis. However, when each group was subdivided into each sex, in the absence of NRG1, there was still no difference detected in either mechanical or thermal hypersensitivity (as shown in the appendix Tables 1-4) after SNI. Given that each group was further subdivided according to gender, it is possible that the small *n* number does not portray the full extent of the role of NRG1 in neuropathic pain after nerve injury in mice.

Differences seen in this study in comparison to previous findings could also be attributed to the various models of nerve injury employed to investigate NRG1 function. From the studies described above, it can be determined that the SNI, SNT and CCI model were used to investigate NRG1 function after nerve injury. It is also important to note that whilst a significant reduction in NRG1 mRNA is reported, it does not equate to 100% gene ablation. It is possible that without 100% down regulation of the gene of interest, it is difficult to detect the role of NRG1 in neuropathic pain.

Finally it is well known that previous pharmacological experiments have lacked specificity and can impact other ligands where it is then difficult to directly attribute a specific function to a specific ligand.

4.4.3 Other erbB ligands may be capable of activating intracellular signaling in microglia to induce microglial mediated mechanical hypersensitivity

Whilst NRG1 has been globally ablated, other ligands are potentially released into the CNS and are capable of binding onto erbB receptors on microglia to activate the ERK1/2 signalling pathway and induce microglial proliferation.

Microglia express all erbB receptors 2, 3 and 4, where erbB lacks a ligand binding domain; erbB3 is capable of binding NRG1, 2 and 6; whilst erbB4 is capable of binding NRG's 1, 2, 3, 4, 5 as well as HB-EGF, BTC and EPG (Iwakura & Nawa 2013).

If NRG1 is not regulating microglial induced mechanical hypersensitivity and microgliosis in the global $NRG1^{-/-}$ strain, then it is likely that one of these other ligands are responsible for activating the MEK/ERK1/2 pathway. Traditionally NRG1 is the most well studied and implicated in neuropathic pain but NRG2 and NRG3 mRNA levels after peripheral nerve injury were also examined in chapter 2. With SNI, I presented evidence that NRG2 and NRG3 mRNA levels, whilst they exist in the spinal cord, are not up-regulated in the spinal cord after peripheral nerve injury (Chapter 2 Figure 2.6). Very little is known about NRG2 but it differs from NRG1. NRG-2 preferentially promotes the heterodimerisation of erbB3 with the EGF receptor (Carraway et al. 1997) whilst NRG1 heterodimerises erbB3 or erbB4 with erbB2. The EGF receptor and signalling pathway is not normally implicated in neuropathic pain and it is unlikely that NRG2 plays a role in activating microglial

proliferation or neuropathic pain after nerve injury. To date, there is no direct evidence to suggest that NRG2 is upregulated after nerve injury.

NRG3 is almost exclusively expressed in the developing and adult CNS and has been implicated in neural development and plasticity with implications in neurodegenerative diseases (Kao et al. 2010). To date, NRG3 has not been implicated in neuropathic pain and according to mRNA expression data presented in Chapter 2; NRG3 is unlikely to be upregulated after nerve injury or induce microglial proliferation. Given that NRG1 is ablated and NRG2 and 3 are not specifically upregulated after peripheral nerve injury, it is possible that these NRG's are perhaps capable of concentrating together to cumulatively regulate mechanical hypersensitivity and microglial proliferation. A similar effect has been reported by De Mello et al. where they investigated whether NRG1β, NRG-2α, and NRG-3 elicit a mitogenic effect on olfactory ensheathing glia (OEG) *in vitro*. When cultured OEGs were treated with NRG1β, NRG-2α, or NRG-3 in the absence of mitogenic agents, NRG1β caused a significant increase in OEG cell proliferation at high concentrations; NRG-2α caused a significant increase in OEG cell proliferation at low concentrations; whilst no effect was observed with NRG-3. The most significant increase in OEG cell proliferation was detected when NRG1β and NRG-2α were added together (De Mello et al. 2007). These findings could explain why microglial proliferation is still detected in the absence of NRG1. It is possible that after nerve injury, minor increases in NRG-2α along with dysregulation of other NRG's could cause overall glial cell proliferation.

Betacellulin (BTC) is a member of the epidermal growth factor (EGF) family of peptide ligands with a unique capability of activating all possible erbB heterodimeric erbB receptors in addition to erbB1/erbB4 homodimers. Saito et al. give evidence that

BTC and EGF are capable of activating ERK2 phosphorylation with distinguishable patterns in CHO cells that do not express appreciable amounts of erBB2 or erbB3. They also demonstrate that both BTC and EGF stimulation of ERK phosphorylation can be completely blocked by administering a specific MEK inhibitor (Saito et al. 2004).

Elsewhere, BTC has been shown to stimulation proliferation and migration of vascular smooth muscle cells (VMSC) via BTC mediated ERK1/2, AKT and p38 MAPK phosphorylation. BTC- induced migration of VMSC is dependent on ERK and AKT with partial p38 MAPK phosphorylation but BTC-induced proliferation requires ERK and partially AKT but not p38 MAPK phosphorylation (Mifune et al. 2004).

If BTC is capable of activating the MEK/ERK signaling pathway through binding on erbB4 receptors and induces proliferation of cells, then it is possible that BTC is compensating for the role of NRG1 in regulating mechanical hypersensitivity and microglial proliferation through MEK/ERK1 activation pathways. Whilst there is no direct evidence to suggest that BTC is expressed or released after nerve injury in the spinal cord- it is a possible candidate that can act as a substitute for NRG1.

One member of the EGF family, epiregulin (EPG), can also activate ERK/MAPK and protein kinase B (Akt) signalling pathways in biological processes (Lindvall et al. 2003). More specifically EPG transcripts have been previously detected in macrophages from mononuclear leucocytes where incubation of peripheral blood cells with silica suspension used to deplete macrophages also decreases the expression of EPG in mononuclear leucocytes. Epiregulin transcript is also deleted in mouse peritoneal macrophages (Toyoda et al. 1997). Given that macrophages and microglia

are of similar lineages it is possible that EPG may also be present in the spinal cord after injury and could regulate microglial function through the EGF receptor.

What is certain from our findings is that a ligand other than NRG1 is capable of inducing microglial activation of the ERK signaling pathway to induce proliferation and mechanical allodynia.

Further work should be conducted using the erbb2 blocking compound PD168393 experiments in the rat model. These should be repeated in the naive mouse strain to validate the erbb2 blocking compound PD168393 results (Calvo et al. 2010; Calvo et al. 2011). Furthermore, the erbb2 blocking compound PD168393 could also be administered to this NRG1$^{-/-}$ to better represent biologically active levels and ensure near 100% ablation of the NRG1- erbB signaling pathway.

4.4.4 NRG1 release after nerve injury could regulate cold allodynia

Whilst results in this chapter demonstrate that blockade of NRG1 release after nerve injury is capable of regulating cold allodynia it is also possible that further modulation of cold allodynia is regulated by distinct molecular signaling changes in microglia. Griffin et al. attempted to identify common molecular mechanism of neuropathic pain by performing a large-scale gene expression profile analysis on the SNI, CCI and SNL models of peripheral nerve injury. Result showed that induction and action of the complement cascade in microglia contributes to neuropathic pain. In particular, the C5a peptide produced a dose-dependent increase in cold pain sensitivity whilst the administration of a C5aR antagonist significantly reduced cold allodynia but did not attenuate mechanical allodynia. This suggests that the C5a cascade is necessary at least for the cold pain sensitivity feature of neuropathic pain (Griffin et al. 2007b).

4.4.5 Does NRG1 play a role in astrocytes?

Whilst microglia are identified as the "first line of defense" and the CNS macrophages, astrocytes have been shown to be responsible for the maintenance of neuropathic pain. Astrocytes are capable of regulating spinal processing of nociceptive signaling induced by; peripheral inflammation, nerve injury, surgery, cancer, chemotherapy and spinal cord injury (as reviewed in Svensson & Brodin 2010). In models of neuropathic pain, an increase in the number of microglia is observed within days of injury but astrocyte activation becomes visible when microglial activation begins to decrease whilst pain hypersensitivity persists throughout. GFAP is recognized as a useful readout of astrocytic activation where GFAP regulation correlates with nerve injury induced neuropathic pain (Eliasson et al. 1999; Ma & Quirion 2002). It has also been demonstrated that GFAP immunoreactivity is correlated to JNK and ERK phosphorylation levels at 3 weeks PSNL (Ma & Quirion 2002).

It is also plausible that NRG1 might play a role in regulating cold allodynia, not through microglial functions but instead through astrocytic functions. Given the temporal profile of the delayed attenuation of cold allodynia it is likely that cells other than microglia (e.g. astrocytes) could play a role in the maintenance of cold allodynia. In this chapter I report no significant difference in astrocyte $GFAP^{+}$ cells between the control(s) and global $NRG1^{-/-}$ strain but do predict that further analysis needs to be conducted into understanding the role of NRG1 in astrocytes after peripheral nerve injury.

4.5 Considerations

In order to understand the locus of action of NRG1 in contributing to cold hypersensitivity I used a transgenic system to selective ablate erbB receptors in microglia.

5 Targeting the selective ablation of erB3 and erbB4 in the myeloid lineage in order to investigate the role of NRG-1-erbB signaling after peripheral nerve injury

5.1 Introduction

In Chapter 4, I investigated the role of NRG1 in the context of neuropathic pain. Using a transgenic mouse in which a tamoxifen inducible Cre recombinase is expressed ubiquitously in all tissues (Hayashi and McMahon, 2002); tamoxifen was administered during adulthood to ablate NRG1 in all cells. With NRG1 ablation mechanical hypersensitivity is not attenuated but cold hypersensitivity is attenuated. It was also found that microglial proliferation following nerve injury is not attenuated in the absence of NRG1.

What remains to be understood is whether NRG1 mediated cold hypersensitivity is truly regulated through microglia. In order to investigate this, the erbB receptors found on microglia can be selectively targeted. To selectively target erbB receptors on microglia it is possible to use an alternative transgenic mouse in which a Cre recombinase is expressed selectively in tissues of monocytic lineage.

Previous studies have investigated the effects of global mutation of the 3 erbB receptors and shown that erbB2 and erbB3 are embryonically lethal owing to a lack of ventricular myocyte differentiation along with cranial ganglia defects (Erickson et al. 1997). Global deletion of erbB4 alone also leads lack of cardiac development and a lack of axon guidance in the CNS (Gassmann et al. 1995; Birchmeier 2009). Owing to these developmental effects in cardiac and neural tissue, selective ablation of the erbB receptors is likely to prove more useful in understanding the role of NRG-1 erbB signalling in microglia after peripheral nerve injury.

Upon NRG1 ligand binding erbB3 and erbB4 heterodimerise with erbB2, this lacks a ligand binding domain). By selectively targeting the erB3 and erbB4 receptors, I can investigate the NRG1-erbB signalling pathway in microglia without interrupting the EGF-erbB signalling pathway (which is dependent on binding to the erbB1 receptor). It is hypothesised that with selective ablation of erbB3 and erbB4 receptors on myeloid cells, NRG-1 is released after injury but is unable to bind and activate microglia. It is hypothesised that certain components of microgliosis may be attenuated and cold hypersensitivity may be attenuated after peripheral nerve injury.

5.2 Methods

5.2.1 Animals

All experimental work on rodents was carried out accordingly to UK Home office legislation (Scientific Procedures Act 1986). Adult mice (8 weeks or older) were used for cell culture, tissue extraction, behaviour, immunohistochemistry and confocal microscopy unless stated otherwise. Where possible, equal numbers of animals and both male and female mice were recruited into each experimental group, to minimise bias. The power calculation conducted is described in Chapter 2 section 2.2.2.

5.2.1.1 Generating Cd11b Cre (+): erbB3$^{fl/fl}$ B4$^{fl/fl}$ mice

The initial generation of mice with alleles homozygous floxed for erbB3 and erbB4 has been previously described (Riethmacher et al. 1997; Gassmann et al. 1995). The erbB3$^{fl/fl}$ strain has been recently validated (Sheean et al. 2014) and lox-P sites are placed spanning exons 10-16. Lox-P sites were inserted around exon 2 of the erbB4 gene to create erbB4$^{fl/fl}$ mice. (This information was kindly provided to us by Carmen Birchmeier research group (Gassmann et al. 1995); see **Figure 5-1**). Boille et al., 2005 generated mice that selectively express Cre under the Cd11b promoter (Boillée et al. 2006; Hickstein et al. 1992), where the expression of Cd11b is limited to cells of myeloid origin (Hickstein et al. 1992). This allows selective targeting of monocytic lineage cells such as microglia and macrophages. Both colonies were bred onto a C57BL6 background by repeated backcrossing over 10 generations to allow these strains to be considered fully congenic.

ErbB3 $^{fl/fl}$ erbB4$^{fl/fl}$ mice were bred with Cd11b Cre (+): erbB3$^{fl/fl}$ B4$^{fl/fl}$ mice were generated whilst Cd11b Cre (-): erbB3$^{fl/fl}$erbB4$^{fl/fl}$ mice were used as littermate controls. For labelling purposes Cd11b Cre until Cd11b Cre (+): erbB3$^{fl/fl}$ B4$^{fl/fl}$ mice

are identified as “Cre (+)” whilst Cd11b-Cre (-): erbB3$^{fl/fl}$erbB4$^{fl/fl}$ mice are identified as “Cre (-)” through the remainder of this chapter.

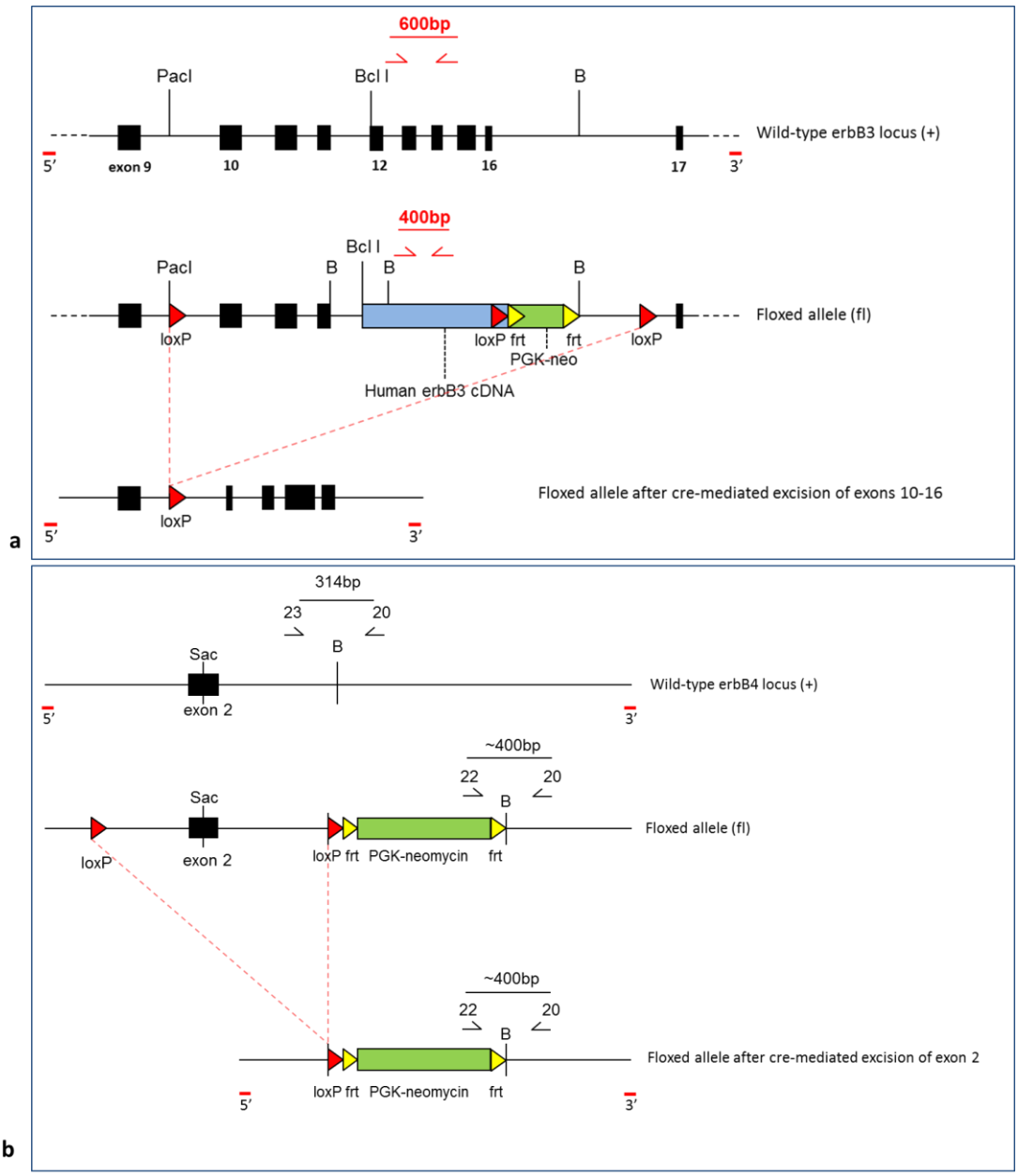


Figure 5-1 Targeting scheme of the erbB3 and erbB4 locus. (a) shows the erbB3 locus and the insertion site of the LoxP sites. Location and length of primers used to genotype is also identified. The human erbB3 cDNA was inserted into exon 12 followed by a LoxP-flanked neomycin resistance cassette; the human ERBB3 cDNA and the neomycin resistance cassette also replaced part of exon 12 and exons 13-16. The 3' homology arm contained exon 17 that encodes the transmembrane domain (TMD) (b) shows the erbB4 locus and the insertion site of the LoxP sites. Location and length of primer sequence used to genotype is also identified. The neomycin resistance cassette was introduced downstream of exon 2 and did not feature a part of the Cre-mediated excision.

5.2.1.2 Generating Cd11b Cre (+):R26^{YFP} mice

The reporter R26^{YFP} strain expresses YFP ubiquitously from the ROSA 26 locus promoter, after a transcriptional termination sequence, flanked by Lox-P sites, has been removed by Cre recombinase activity. To determine the efficiency and specificity of the Cd11b Cre strain, it was bred with R26^{YFP} mice (gifted from Dr. Jefferson's Research Laboratory at Kings College London) to generate offspring of either Cd11b Cre (+):R26$^{YFP\ (+)}$ or Cd11b Cre (-):R26$^{YFP\ (+)}$. Another Cre system CAG-CreER^{TM} was used a positive control to test the functionality of the R26^{YFP} reporter mouse. Using CAG-CreER^{TM} and R26^{YFP}, the CAG-CreER^{TM} (+): R26$^{YFP\ (+)}$ and CAG-CreER^{TM} (-): R26$^{YFP\ (+)}$ strains were selected.

5.2.1.3 Generating Cd11b Cre (+): R26^{tdtomato} mice

The reporter R26^{tdtomato} strain expresses a red fluorescent protein variant under the ROSA 26 locus after the floxed STOP codon has been removed when introducing a Cre strain. To determine Cre activity, the R26^{tdtomato} strain was bred with the Cd11b Cre (+) strain to generate offspring of various genotypes. Cd11b Cre (+): R26$^{tdtomato\ (+)}$ or Cre (-): R26$^{tdtomato\ (+)}$ mice were selected for the study. Another Cre system PLP-CreER^{TM} was used a positive control to test the functionality of the R26 ^{tdtomato} reporter mouse. Cre recombinase is placed under the mouse proteolipid protein (myelin) 1(PLP) promoter to drive transgene expression in myelinating cells (e.g. oligodendrocytes). Using PLP-CreER^{TM} and R26 ^{tdtomato}, the PLP-CreER^{TM} (+): R26 $^{tdtomato\ (+)}$ and PLP-CreER^{TM} (-): R26 $^{tdtomato\ (+)}$ strains were selected.

5.2.2 Tamoxifen dosing

10 week old CAG-CreER^{TM} (+): R26$^{YFP\ (+)}$ or Cre negative R26$^{YFP\ (+)}$ mice were dosed by oral gavage with 0.25 mg/g of body weight with tamoxifen (Sigma T5648) in corn oil for five consecutive days. Upon tamoxifen administration, in the presence of the

Cre enzyme, Cre recombination results in the stop codon being removed from the $R26^{YFP}$ and auto-immunofluorescence can be visualised in promoter specific tissues. Animals were left for 4 weeks post dosing before utilising in further experiments. The PLP-CreER^{TM} (+): R26$^{tdtomato\ (+)}$ and PLP-CreER^{TM} (-): R26$^{tdtomato\ (+)}$ groups were treated in the same manner.

5.2.3 Surgery

The Cd11b Cre (+): R26^{tdtomato} strain underwent surgery to investigate whether injury drivers further Cre activity and expression after nerve injury. The spared nerve injury (SNI) was used as a model of neuropathic pain in mice to measure behavioural outcomes after injury. Surgical procedures were followed as described in Chapter 2 Section 2.2.3

5.2.4 Tissue extraction

5.2.4.1 Primary microglia cell culture

Mixed glial cultures from the cortex of P3-P7 mice of Cre (-) and Cre (+) group were isolated, according to a modified version of the method of Giulian and Baker (Giulian and Baker, 1986). Following mechanical and chemical dissociation, cells were seeded in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% foetal bovine serum (FBS) and 1% penicillin-streptomycin and cultured at 37°C in humidified 5% CO_2/95% air. All reagents used were purchased from Invitrogen, UK. Medium was replaced every 3 days and confluency was typically achieved after 7 days *in vitro*. Confluent mixed glial cultures were shaken for 30 min on a shaker, and the floating cells were pelleted and plated on uncoated T25 flasks. The following day medium was changed to discard all non-adherent cells. This method typically resulted in 96-99% purity, as assessed by immunostaining with Iba1 (to label microglia) and

DAPI (to label all nuclei present). Cells were then incubated overnight in standard medium to allow them to settle down and to firmly attach to the flask surface. Once confluent, microglia were ready to be used in further experiments.

5.2.4.2 Preparation of Bio-Gel 2%

The peritoneal cavity of rodents proves to be a convenient site for macrophage extraction (Edwards et al. 2006). Cell suspensions from un-elicited murine peritoneal cavity is approximately 50%. Bio-Gel is a sterile irritant that can be used to enhance the yield of peritoneal macrophages from ~50% (un-elicited) up to ~75% (elicited). Bio-gel was prepared according to previous publications (X. Zhang et al. 2008). 2g of Bio-Gel P-100 gel (BioRad, cat. no. 150-4174) was washed three times with 30ml of Dulbecco's phosphate-buffered saline (without calcium and magnesium). Bio-Gel was then pelleted by centrifugation for 5 min at 400*g*, and then resuspended in 100ml Dulbecco's phosphate-buffered saline (without calcium and magnesium) to yield 2% Bio-Gel. This solution was autoclaved for 20 min before use.

5.2.4.3 Peritoneal macrophage cell culture

Adult mice from the Cre (-) and Cre (+) group were injected with Bio-Gel beads and left for 4 days before being sacrificed with increasing CO_2 concentrations. Following CO_2 administration, the mice were immediately injected intraperitoneally with 40mL of sterile Hank's Balanced Salt Solution (HBSS). The elicited peritoneal exudates cells were harvested and re-suspended in DMEM 1% penicillin-streptomycin supplemented or not with 10% FBS, as required for the different experimental conditions. Cells were plated at a density of 3 x 105 cells per ml and cultured at 37°C in humidified 5% CO_2/95% air.

5.2.5 Polymerase chain reaction: Genotyping

Genomic DNA was extracted from macrophages and microglial cells and analysed using PCR. To confirm the presence of lox P sites, primers for erbB3 (shown in Table 5-1) were used, the mixture was initially heated at 94ºC for 2 min and then 34 cycles were set to heat at 94ºC for 1 min followed by 55ºC for 2 min and then 72ºC for 1 min. The final step held the samples at 72ºC for 5 min with a final hold at 4ºC.

The lox P sites were also confirmed on the erbB4 gene using primers shown in Table 5-1. The PCR reaction mixture for the erbB4 gene was initially heated at 94ºC for 2 min and then 34 cycles were set to heat at 94ºC for 1 min followed by 58ºC for 2 min and then 72ºC for 2 min. The final step held the samples at 72ºC for 10 min with a final hold at 4ºC. The recombination PCR reaction mixture for erbB4 gene was initially heated at 94ºC for 2 min and then 34 cycles were set to heat at 94ºC for 1 min followed by 55ºC for 2 min and then 72ºC for 1 min. The final step held the samples at 72ºC for 5 min with a final hold at 4ºC.

Primer name	Sequence (5' – 3')	location	Product size
erbB3 WT/floxed allele	TCT CCT TGT TGA TCA TGA AGA ACT TG	Within exon 12	600bp WT 400bp and 600bp floxed 600bp mutant
	TTG CAG TGA GTC ACA CAG ACA CCT	Within exon 13	
erbB4 WT allele	CAA ATG CTC CTG TTC GTG G	Before exon 1 (BamHI site)	314bp WT 400bp floxed allele 200bp mutant allele
	TTT TGC CAA GTT ATT CCA TCA GAA GC	After exon 1 (in neo cassette)	
erbB4 mutant allele	CAA ATG CTC CTG TTC GTG G	Before exon 1 (BamHI site)	
	TAT TGT GTT CAT TCA TTG CAA CCC AG	After exon 1 (in neo cassette)	
CD11b	CAG GTA TGC GAA AAC GCC	Bacteriophage P1 cre recombinase	445 bp
	TGG GCC AAC CCA AGA AAC AAG	CD11b promoter	

Table 5-1 sequence of primers used in genotyping PCR

5.2.5.1 Quantitative PCR

Once cultured microglia and/or macrophages were confluent, RNA was extracted in the same manner as described in Chapter 2 section 2.2.6.

To extract RNA from spinal cord and brain, animals were sacrificed by intraperitoneal injections of 200mg/kg of pentobarbital before being perfused with 0.9% saline injected through the left atrium and into the blood circulation to flush out blood. Lumbar region of the spinal cord and the brain were dissected and immediately frozen using liquid nitrogen. Tissues were then homogenised and RNA was extracted.

Primer name	direction	Sequence (5' – 3')
erbB3	FORWARD	TCA CTG GCC TCA ATG GTG AC
	REVERSE	AGC CTG TAA TCT CCC GGA CT
erbB4	FORWARD	GGG ATC TGA GAC TTG CCA AA
	REVERSE	CCC ATG ACT ACC TCG CAG TT
GAPDH	FORWARD	TGT GTC CGT CGT GGA TCT GA
	REVERSE	TTG CTG TTG AAG TCG CAG GAG
HPRT	FORWARD	GTC CTG TGG CCA TCT GCC TAG
	REVERSE	GCC AGT CGG CAT CGT TTA TG

Table 5-2 Sequence of primers used for qPCR

5.2.6 Histology

5.2.6.1 Tissue extraction

Animals were deeply anaesthetised with pentobaritol and transcardially perfused with 15 ml saline followed by 20 ml of 4% paraformaldehyde in 0.1M phosphate buffer. All tissues that were dissected were post fixed in 4% paraformaldehyde in 0.1M phosphate buffer. Spinal cord was post fixed for 24 hours before being stored in 30% sucrose for 2-3 days and then mounted in OCT embedding compound on dry ice and stored at -80°C. Transverse sections of spinal cord were cut at 30µm thickness using a cryostat (Leica) onto SuperFrost Ultra Plus® slides.

Macrophage and microglial cells, once confluent onto coverslips, were washed with sterile PBS and fixed for 30 minutes with 4% paraformaldehyde in 0.1M phosphate buffer before 100% methanol was applied to cells for 5 minutes. Cells were washed with PBS and coverslips were ready to be used for immunohistochemistry.

5.2.6.2 Immunohistochemistry

Microglial cultures, macrophage cultures and spinal cord sections were washed with PBS and blocked using normal donkey serum (Chemicon S30 1:10, 30 min). Sections were stained with either rabbit ionised calcium binding adaptor molecule 1 (IBA1 1:1000 Wako, Japan), rabbit erbB3 (1; 100 Santa Cruz), rabbit erbB4, or anti-chicken GFP (1:100 Santa Cruz) and left overnight before being washed 3 times with PBS and then incubated with either anti-rabbit Cyanine-3 (Cy-3 1; 500 Life Technologies) or anti-chicken Alexa 488 at room temperature for 3 hours. All reagents were diluted in PBS containing 0.2% triton X-100 and 0.1% sodium azide. Slides were washed with PBS and coverslipped in Vectashield® mounting medium with DAPI (Vector Labs).

5.2.7 Image analysis

Immunofluorescence was visualized under a Zeiss Imager.Z1 microscope or a confocal Zeiss LSM 700 laser scanning microscope. Photographs were taken using the Axio Cam and AxioVision LE Rel. 4.2 or the LSM Image Browser software for image analysis.

IBA1 quantification in the spinal cord was conducted in the same manner as described in chapter 4 section 4.2.6

5.2.8 Animal Behavioural Tests

All behavioural tests were carried whilst the observer was blind to the genotype of the animal. Animals were tested in a temperature stable room during the light period of their daily day/night cycle, at the same time every day. Wherever possible, equal numbers of animals of each gender were selected per group.

5.2.8.1 Mechanical withdrawal threshold testing using Von Frey filaments

Mechanical withdrawal thresholds were measured in the same manner as described in Chapter 2 section 2.2.4.1. Animals were tested at Baseline, prior to injury and days 3, 7, 14, 21 and 28 post injury.

5.2.8.2 Thermal nociception threshold testing using Hargreaves' test:

Thermal sensitivity threshold levels were measured in the same manner as described in Chapter 2 section 2.2.4.2. Animals were tested at Baseline, prior to injury and days 3, 7, 14, 21 and 28 post injury.

5.2.8.3 Thermal nociception testing using the Hot Plate

The Hot Plate test is a common method to measure thermal nociception at a set temperature which has been well described (WOOLFE & MACDONALD 1944; EDDY & LEIMBACH 1953). The common method was modified so that a loose

scruff was used to restrain animals and each paw was placed on the 10mm thick aluminium plate (35100, Ugo Basile, Italy). The plate was set to 53°C and the latency to withdrawal, as demonstrated by the reflex paw flicking was measured. Three baselines were recorded for each animal over a number of days. Results were expressed as the mean latency to withdrawal for each genotypic group.

5.2.8.4 Cold allodynia testing using the Cold Plate

The Cold Plate test can be carried out using the same apparatus as for the Hot plate. The temperature was set to 10°C and unrestrained animals were placed in the Plexiglas cylindrical enclosure atop the aluminium metal plate and the latency to withdrawal was measured. Three baselines were recorded for each animal over a number of days. Results were expressed as the mean latency to withdrawal for each genotypic group.

5.2.8.5 Motor function testing using the Rotorod

Motor function and co-ordination can be tested using the accelerating Rotorod method which has been previously described (JONES & ROBERTS 1968). The rotating rod is a metal bar diameter covered by rubber tubing to give a total diameter of 1½ inches and further divided into five 3 inch compartments by means of spacing discs (47600, Ugo Basile, Italy). Each animal is placed onto the accelerating rotating rod (05 cm/sec increase every 5 sec) and the latency to fall off the rod is measured. This latency to fall is scored automatically with infrared sensors. No pre-training was required for this test and 3 baselines for each animal were taken over a number of days. Results were expressed as the mean latency to fall for each genotypic group.

5.2.8.6 Mechano-sensation testing using the Randal-Selitto Test

The Randal-Selitto test can be used to measure threshold limits to mechanical pressure (RANDALL & SELITTO 1957). Before testing, each animal was trained by handling and placing the animal onto a soft tissue before being carefully immobilised using the same hand as in the test conditions. The other hand was used to hold the tested paw. During testing, the hind paw of each animal was placed under the tip of the device where an increasing mechanical force was applied to the dorsal surface of the paw until a paw withdrawal response was seen. The maximum weight applied to the hind paw was limited to 150g to prevent tissue damage. Both hind paws were tested and three baselines were recorded for each animal over a number of days. Results were expressed as the mean weight tolerated by each genotypic group.

5.2.9 Statistical Analysis

As the genotypes of each animal are the only variable, similarities or differences between Cd11b Cre (+) and erbB3$^{fl/fl}$erbB4$^{fl/fl}$ (control) strains were determined using the Students t-test for validation experiments and phenotyping. Results are reported as mean values ± SEM. Where appropriate, the repeated measures two way ANOVA is used with the post hoc Bonferronni test.

5.3 Results

5.3.1 ErbB3 and erbB4 mRNA levels are not down-regulated in the Cre (+) group

The level of erbB3 and erbB4 mRNA gene expression was also investigated in macrophage and microglial cultures. Given that the forward exons are placed between exon 9-10 and the reverse exons are placed between exons 15-16 for erbB3 and around exon 2 for erbB4 (see **Figure 5-1**), it was hypothesised that with Cre induction, a sequence of mRNA will be excised to render the erbB3 and erbB4 genes non-functional.

Results show that there is no significant reduction in mRNA expression of erbB3 or the erbB4 gene in microglia, macrophages, brain or spinal cord in the presence of Cd11b Cre (**Figure 5-2**a-h). Messenger RNA expression is measured relative to the expression of housekeeping genes – HPRT and GAPDH. The Cre (-) group was normalised to the arbitrary numerical value of 1 and the relative expression of erbB3 and erbB4 of the Cre (+) group was compared. Relative erbB3 mRNA expression between Cre (-) and Cre (+) derived microglia (1.000 ± 0.191 vs. 0.773 ± 0.264), macrophages (1.000 ± 0.348 vs. 0.687 ± 0.151), spinal cord (1.000 ± 0.105 vs. 0.691 ± 0.156) and brain (1.000 ± 0.290 vs. 1.188 ± 0.281) remained unchanged. There was no significant difference between the two groups in any of the tissue analysed (Students t test, $p>0.05$ n=10 animals per group). Relative erbB4 mRNA expression in microglia (1.000 ± 0.215 vs. 1.207 ± 0.293), macrophages (1.000 ± 0.335 vs. 0.767 ± 0.114), spinal cord (1.000 ± 0.201 vs. 0.771 ± 0.261) and brain (1.000 ± 0.280 vs. 1.154 ± 0.202) also remained unchanged and there was no significant difference between the Cre (-) and Cre (+) group (Students t test, $p>0.05$ n=10 animals per group). It is therefore conclusive that the Cd11b Cre transgenic strain is inefficient at

significantly down-regulating mRNA expression of both erbB3 and erbB4 in macrophages and microglia.

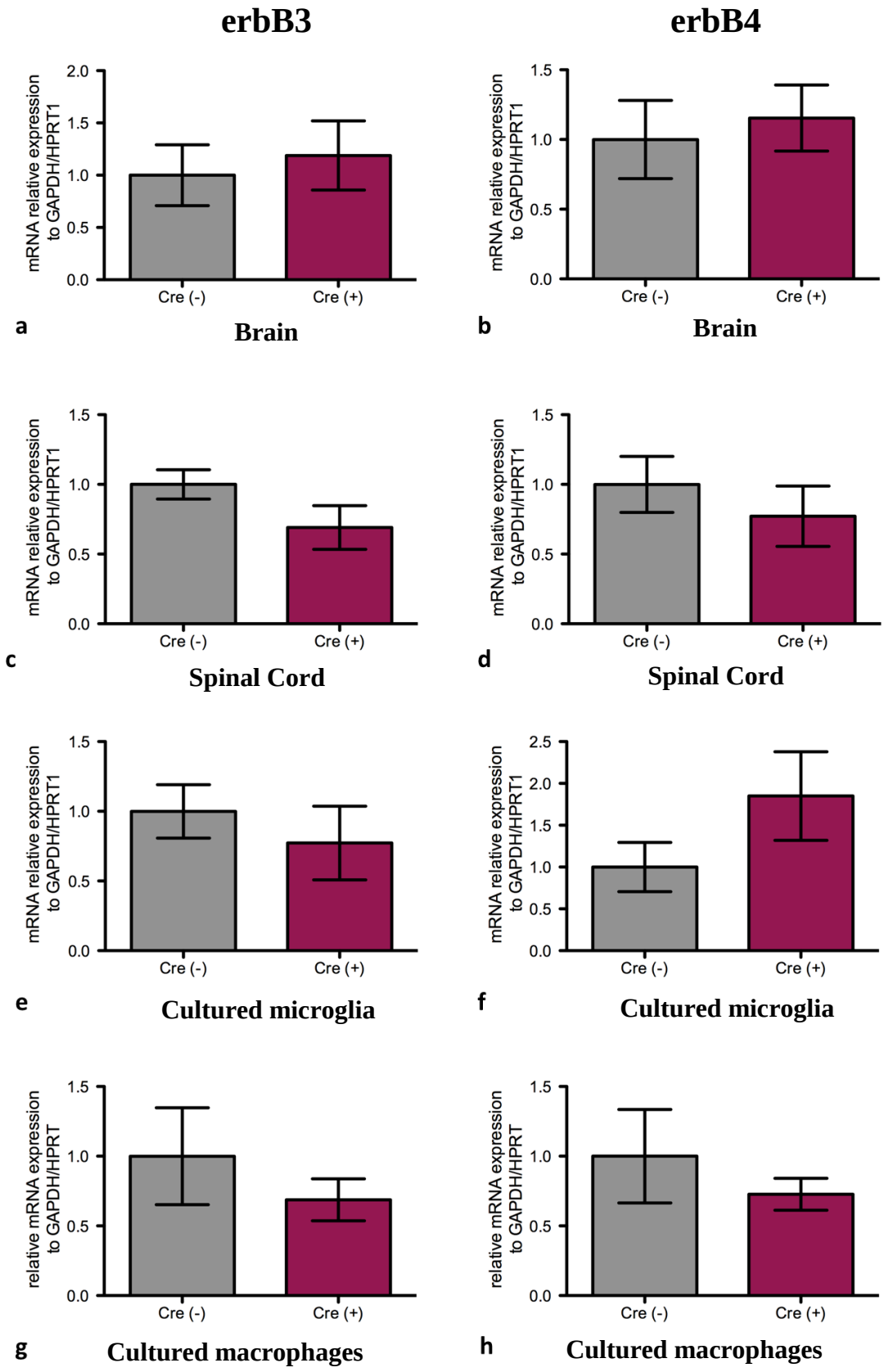


Figure 5-2 mRNA expression does not significantly vary between Cre (-) and Cre (+) group. (a) shows the relative expression of erbB3 mRNA levels to GAPDH/HPRT1 in the brain. There is no difference between the Cre (-) and Cre (+) group (b) shows the relative expression of erbB4 mRNA levels in the brain and similarly, there is no significant difference between the Cre (-) and Cre (+) group. (c) and (d) show the relative mRNA levels of erbB3 and erbB4 in the spinal cord whilst (e) and (f) show the relative mRNA levels of erBB3 and erbB4 in microglial cells and (g) and (h) show the relative mRNA levels of erbB3 and erbB4 in macrophages. Overall there is no significantly distinct difference in the erbB3 or erbB4 levels between the Cre (-) and Cre (+) in the brain, spinal cord, microglia and macrophages. (n=10 per group for brain and spinal cord; n=5 animals per group for microglial and macrophage cultures; $p>0.05$ for all groups; Students t test).

5.3.2 ErbB4 protein is not suppressed in the conditional knockout group.

Microglial cells were isolated from mice P3-7 brains from either Cre (-) or Cre (+) group to measure erbB3 and erbB4 protein levels. All antibodies tested for erbB3 protein expression were poor and inconsistent on western blot analysis (Santa Cruz, Abcam, Millipore and Cell signalling). Whilst erbB3 and erbB4 antibodies worked well in rat tissue, these antibodies failed to specifically label mouse tissue. An erbB4 antibody was produced in the Buonanno Lab and kindly gifted to our research group for use (Buonanno, 2013). Using this antibody, erbB4 protein was detected in both the Cre (-) and Cre (+) group but no significant difference in protein expression could be seen (**Figure 5-3**). Using the loading control protein levels (GAPDH), the target protein levels were normalised using a Bio-rad western blot analysis program. There was no significant difference in the levels of erbB4 protein between Cre (-) and Cre (+) groups (1.000 ± 0.140 vs. 1.069 ± 0.0.049)**.** With these results, it is likely that the Cd11b Cre transgenic strain is inefficient in significantly reducing erbB4 protein levels in the presence of Cre (2 animals pooled per genotype, n=3 per group, Student t-test).

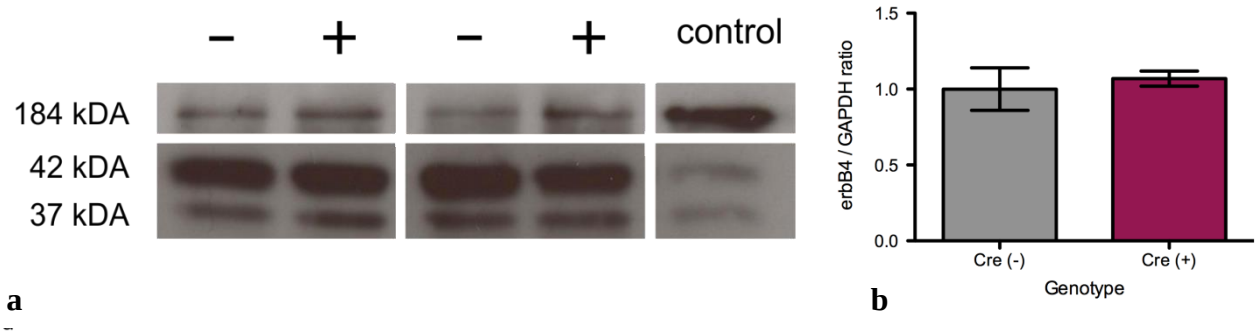


Figure 5-3 erbB4 protein levels are not significantly down-regulated using Cd11b Cre mediated excision. (a) Representative image of an erbB4 western blot. Beta-actin protein is detected at 37kDA, whilst GAPDH protein is detected at 42kDA whilst erbB4 protein is detected at 184kDA in both the Cre (-) and Cre (+) groups. The control sample used was a wild type sciatic nerve. (b) Quantification of erbB4 protein levels in Cre (-) and Cre (+) groups relative to GAPDH protein levels.

5.3.3 The R26$^{tdTomato\ (+)}$ strain is a highly efficient reporter strain

A previously validated Cre line identified as PLP Cre (Leone et al. 2003) was used to measure the level of Cre activity by crossing with R26^{tdTomato} reporter. As shown in **Figure 5-4**, in the PLP Cre (-): R26$^{tdTomato\ (+)}$ strain when Tx. is administered, there is a lack of tdTomato^{+} labelling in the DRG (**Figure 5-4**c and d) and in S100 labelled cells.

When Tamoxifen was administered to the PLP Cre (+): R26$^{tdTomato\ (+)}$ strain, there were high levels of co-localisation of S100^{+} and tdTomato^{+} cells in the DRG (**Figure 5-4** a-b), sciatic nerve (**Figure 5-4** e-f) and the spinal cord (**Figure 5-4**g). With this study, it was determined that the R26$^{tdTomato\ (+)}$ reporter strain is highly efficient in measuring Cre mediated activity. This reporter stain was then used to investigate the functional activity and specificity of the Cd11b Cre line.

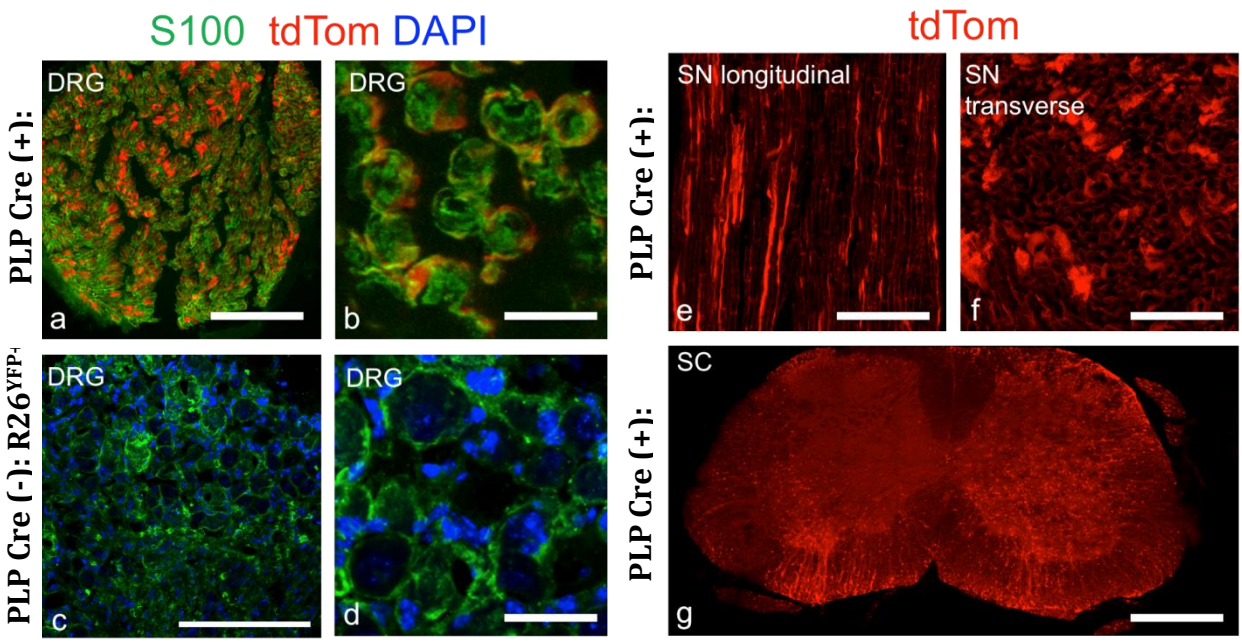


Figure 5-4 the R26tdTomato strain is a highly efficient reporter strain to that can be used to measure Cre activity. PLP-Cre (+): R26TFP(+) were used as a positive control. (a) and (b) illustrate S100 labelled cells that are identified in green.These are Schwann cells which are known to express PLP. Upon Tamoxifen administration to PLP-Cre (+): R26TFP(+), the S100+ cells co-localise with the tdTomato+ marker, whilst in the absence of Cre but with tamoxifen administration, there is a lack of tdTomato+ expression as shown in (c) and (d). (e) shows a representative image of the longitudinal section of the sciatic nerve with tdTomato+ labelling seen in PLP-Cre (+): R26TFP (+) Tx. Administered group. (e) A representative transverse section of sciatic nerve and (f) lumbar spinal cord is also labelled with tdTomato+ in the PLP-Cre (+): R26TFP (+) Tx as PLP is expressed within oligodendrocytes. (Figure a and c scale = 150μm; Figure b and d scale 50 μm; Figure e scale =20 μm; Figure f scale = 100 μm; figure g scale = 10 nm).

5.3.4 Cd11b Cre is inefficient in effectively targeting microglial cells for Cre-mediated recombination.

The Cd11b Cre (+) strain was bred with the $R26^{tdTomato+}$ strain to test for the functionality of Cd11b Cre. Results showed that there was 0% tdTomato expression in the lumbar spinal cord, L3, L4 and L5 DRG's and the sciatic nerve of Cd11b Cre (+) animals. Therefore quantification or experiment analysis could not be conducted. It was hypothesised that peripheral nerve injury might drive Cre expression and induce $R26^{tdTomato+}$ in Cd11b Cre (+) cells (i.e. $IBA1^{+}$ monocytes). SNI was performed and the level of $R26^{tdTomato+}$ in $IBA1^{+}$ cells was investigated.

As **Figure 5-5**a-c shows, after SNI the Cd11b Cre (+): $R26^{tdtomato+}$ strain failed to exhibit any $R26^{tdtomato+}$ expression in $IBA1^{+}$ cells in the spinal cord, DRG or injured sciatic nerve. There was only once instance where one Cre (+) section of sciatic nerve displayed an $IBA1^{+}$: $R26^{tdtomato+}$ cell (**Figure 5-5**d). This was not seen in other tissue or in any other Cre (+) animals.

It was interesting to note that, in rare instances where $R26^{tdtomato+}$ staining was detected, it did not co-localise with IBA1. Upon closer inspection, it is hypothesised that these rare instances of $R26^{tdtomato+}$ expression is likely to have labelled blood vessels because activated granulocytes or neutrophils can also be specifically identified by the expression of the surface antigen Cd11b (Davis et al. 2000; Neirinckx et al. 2014). An example is shown in **Figure 5-5**e where, in the sciatic nerve, $R26^{tdtomato+}$ labelling does not correspond to $IBA1^{+}$ cell but instead resembles the morphology of a blood vessel.

It was ultimately concluded that the Cd11b Cre (+) strain cannot be used to investigate the role of NRG-1-erbB signalling in macrophages and microglia after peripheral nerve injury given the low level of Cre activity in these cell types. I report

behavioural and anatomical phenotyping of the Cre (-) and Cre (+) groups which I had commenced before completing the validation of the Cd11b Cre line. These results need to be interpreted in light of the fact that this Cre is only expressed in a very small minority of microglia and macrophages.

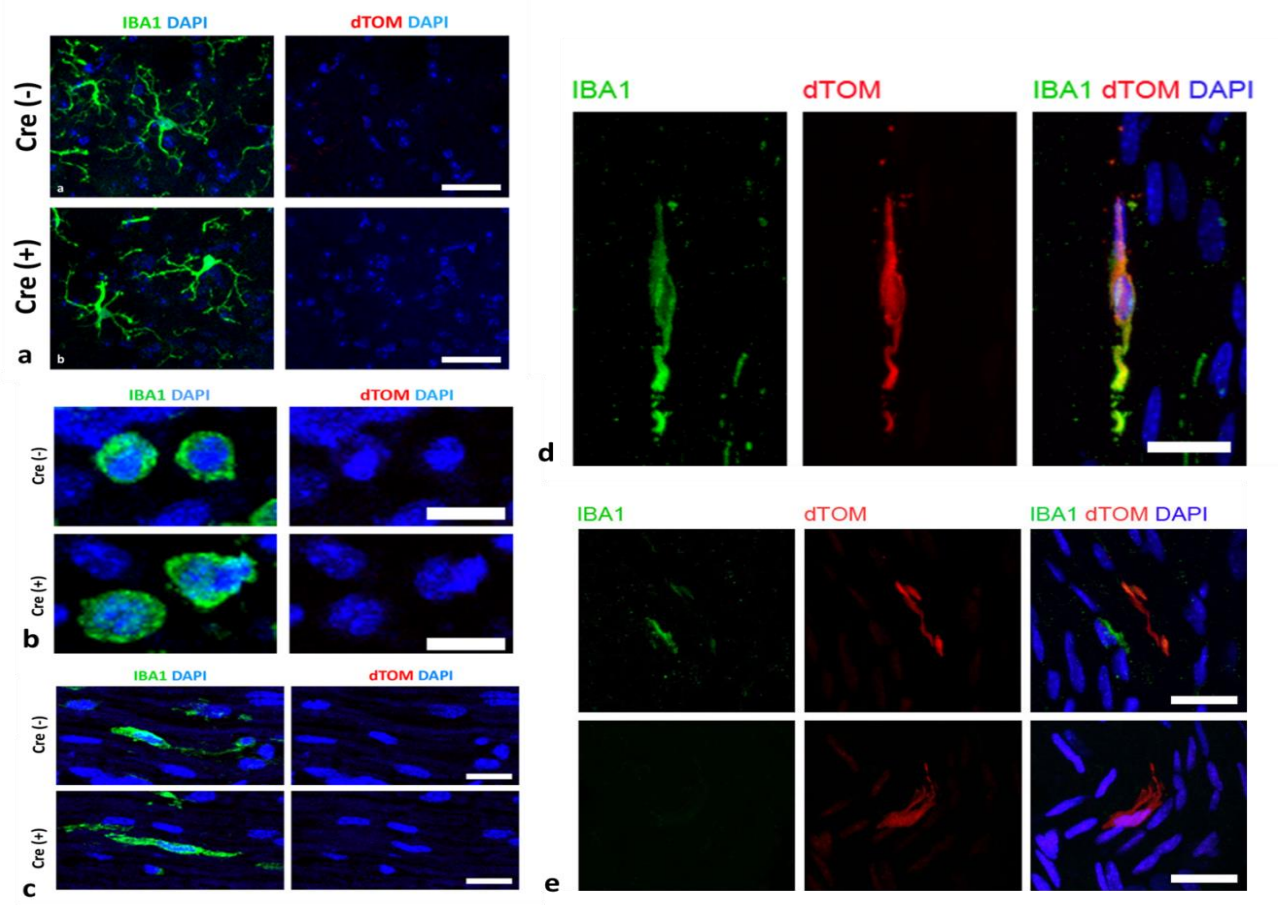


Figure 5-5 The Cd11b Cre (+) strain does not express tdTomato in the PNS or CNS tissue after SNI. (a) Show IBA1^{+} positive microglia in the spinal cord. As expected there are no IBA1^{+} cells that express tdTomato^{+} in the control Cd11b Cre (-): R26$^{tdTomato+}$ group. In the Cre (+) group however, there is also a complete lack of tdTomato^{+} expression in IBA1^{+} cells. Similarly, in the Cre (+) group, there is no tdTomato^{+} expression in IBA1^{+} cells in the (b) DRG or (c) sciatic nerve. (d) In one instance, there was an IBA1^{+} cell in the sciatic nerve that also expressed tdTomato in the Cre (+) group. This was not seen elsewhere. (e) Shows representative images in the Cre (+) group where tdTomato^{+} expression is seen but it does not label IBA1^{+} cells. It is hypothesised that this tdTomato^{+} expression is linked to the surface antigens presented in blood vessel epithelium. Given the lack of tdTomato expression, no quantification or statistical analysis could be conducted. (Scale bars a= 200µm; b = 10 µm; c and d = 25 µm; e = 15 µm)

5.3.5 Mechanical and thermal reflex withdrawal thresholds do not differ between the Cre (-) and Cre (+) group

Mechanical sensitivity threshold values were obtained using the Up-Down Von Frey method. There was no significant difference between the Cre (+) and Cre (-) groups. (**Figure 5-6**a).

The Randal-Selitto test was used to measure reflex withdrawal to mechanical pressure applied to the hind paw of mice recruited in this study. Paw pressure tolerance does not differ between genotypes (**Figure 5-6**b).

Thermal sensitivity threshold values were obtained using the Hargreaves method which demonstrated that there was no significant difference between the two groups (**Figure 5-7**a). The Hot Plate test was set to 53ºC and the latency to withdraw from the stimulus was measured. This did not differ between groups (**Figure 5-7**b).

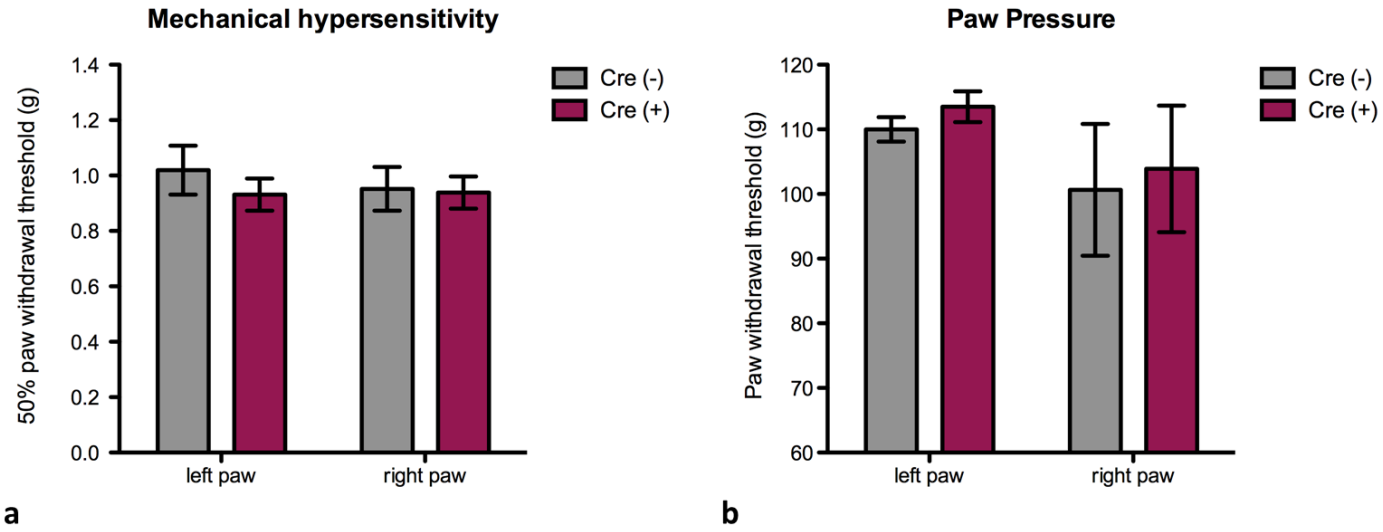


Figure 5-6 mechanical sensitivity thresholds are indifferent between Cre (-) and Cre (+) group. (a) Shows the 50% withdrawal threshold between Cre (-) and Cre (+) group for both the left and right paw. With the left paw, the Cre (-) group exhibited 50% withdrawal threshold to 1.020 ±0.089g whilst the Cre (+) group exhibited 0.931 ± 0.058g. There is no significant difference between the two groups. Similarly, with the right paw there is no significant difference between Cre (-) and Cre (+) groups in regards to the 50% withdrawal threshold levels exhibited (0.952 ± 0.079g vs. 0.938 ± 0.058g respectively). (b) Shows the paw pressure threshold between the Cre (-) and Cre (+) group, measured using the Randal-Selitto test. There is no difference in the left paw withdrawal threshold between the Cre (-) and Cre (+) group (110.0 ± 1.892g vs 113.500 ± 2.376g). The same observation is made for the right paw between the Cre (-) and Cre (+) group (100.650 ± 10.189g vs 103.894 ± 9.781g respectively). Thus both groups exhibit normal mechanical sensitivity thresholds (n=10-11 animals per group; $p>0.05$; Students t-test).

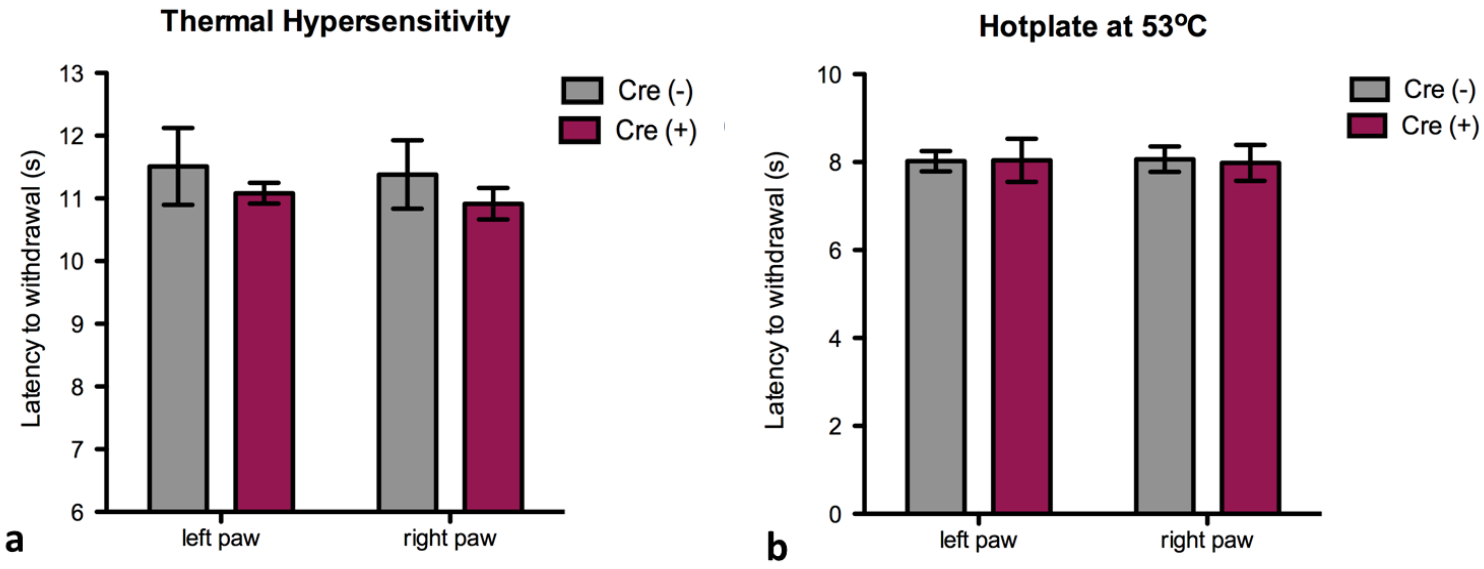


Figure 5-7 Thermal hypersensitivity thresholds do not differ between the Cre (-) and Cre (+) group. (a) shows the latency to withdraw from thermal stimuli of the left and right paw between the Cre (-) and Cre (+) group. In the Cre (-) group, on average the left paw displayed a withdrawal latency of 11.511 ±0.614s whilst the Cre (+) group exhibited 11.083 ± 0.166s. There was no significant differences existed between the two groups. Similarly, with the right paw there was no significant difference between Cre (-) and Cre (+) group (10.381 ± 1.237s vs 10.915 ± 0.250s respectively. n= 10-11; $p>0.05$ students t-test) (b) shows the latency to withdraw from a radiant heat source set to 53ºC. With the left paw, the average latency to withdraw a paw was similar between the Cre (-) and Cre (+) group (8.022 ± 0.230s vs. 8.039 ± 0.490s respectively). There was also no difference between the two groups in the right paw withdrawal to 53ºC temperature (8.067 ± 0.289s vs. 7.984 ± 0.408s respectively; n= 10-11; $p>0.05$ students t-test). Thus thermal sensitivity threshold levels remain normal between the two groups.

5.3.6 Cold pain (8ºC) thresholds do not differ between the Cre (-) and Cre (+) group.

To measure reflex withdrawal to cold, animals were stabilised using the scruff and the right or left paw was placed on the 8ºC plate before the latency to paw withdrawal was measured. This did not significantly differ between groups.

On average, the left paw withdrawal latencies between the Cre (-) and Cre (+) group were indifferent (10.405 ± 0.718s vs. 9.719 ± 0.303s respectively). Similar thresholds of the right paw between the Cre (-) and Cre (+) group were also measured and were measured to be indifferent between groups (**Figure 5-8**) (11.018 ± 0.846s vs. 10.161 ± 0.454s respectively. n=10-11 per group; $p>0.05$; Students t test). Thus, the two genotypic groups exhibit normal sensitivity levels to cold stimuli.

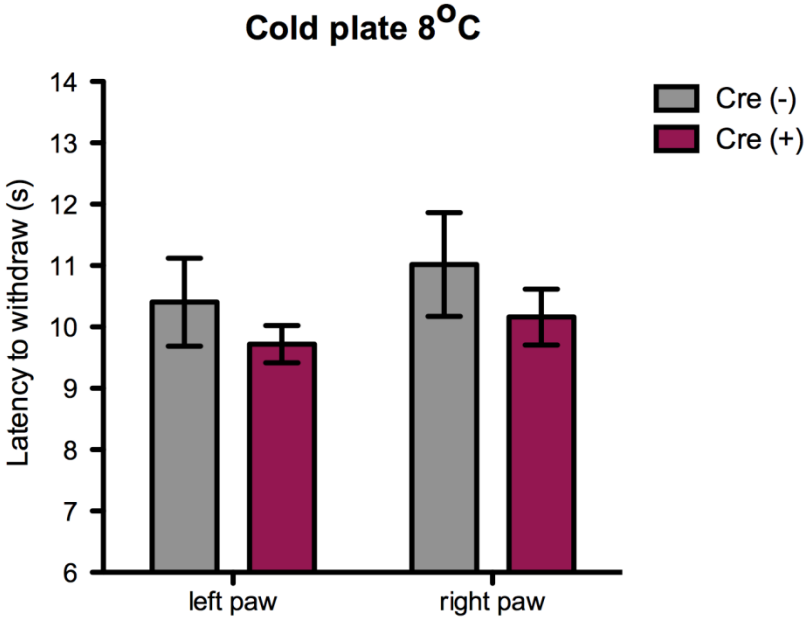


Figure 5-8 Heat and cold pain threshold levels are similar between the control and experimental group. (a) Shows the latency to withdraw to a temperature of 53ºC between the Cre (-) and Cre (+) group for the left and right paw. There is no significant difference in the latency to withdraw, from a thermal stimulus, between the left and right paw within genotypes and between genotypes. (b) Shows the latency to withdraw from a cold stimuli (8ºC) between the Cre (-) and Cre (+) group. There is no significant difference in the latency to withdraw to a cold stimulus between the left and right paw within a genotype and between genotypes. Thus the Cre (-) and Cre (+) groups exhibit normal heat and cold pain thresholds (n=10-11 animals per group; $p>0.05$; Students t-test).

5.3.7 Motor function is normal between the Cre (-) and Cre (+) groups.

The Rotor-rod test was used to measure motor function between the Cre (-) and Cre (+) group. Each animal was tested 5 times on an accelerating rotor-rod. On average the Cre (-) group remained 73.984 ± 2.186s on the rotor-rod before slipping whilst the Cre (+) group remained on average of 73.99 ± 1.794s (n= 10-11, p >0.05 Students t-test). These findings suggest that there is no difference in motor function between groups (**Figure 5-9**).

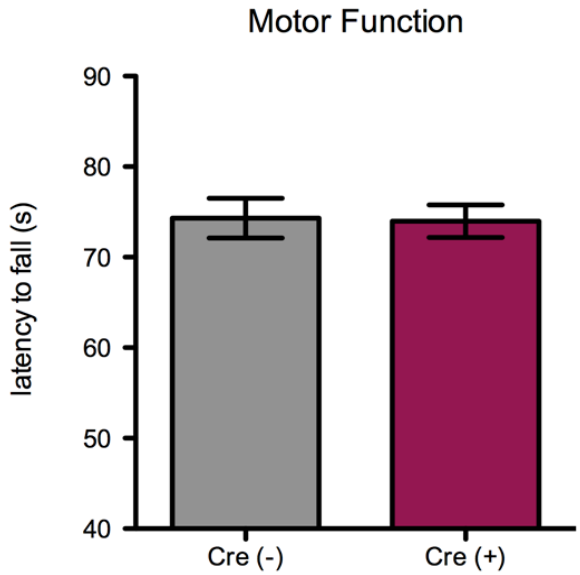


Figure 5-9 Motor function is also unaffected between the Cre (-) and Cre (+) group. This figure shows the motor function between Cre (-) and Cre (+) group using the rotor-rod. The latency to fall from the rotor-rod is measured. There is no significant difference in the latency to fall between the Cre (-) and Cre (+) group. (n=10-11 animals per group; p>0.05; Students t-test).

5.3.8 There is no change in microglial number or morphology between the Cre (-) and Cre (+) genotype

Whilst there was no detectable behavioural phenotype between the two genotypic groups, I investigated whether microglial numbers differ in the Cre (-) and Cre (+) group and whether the microglial morphology itself differs. Spinal cord tissue was extracted from both genotypes and immuno-stained with IBA1. Upon analysis, it was determined that there is no significant difference between the number of microglia seen in the Cre (-) and Cre (+) group in the naïve state (**Figure 5-10**e). Similarly, after peripheral nerve injury (SNI), microgliosis develops between both groups and there is no significant difference in the microglia numbers or morphology between the two groups after injury **Figure 5-10**e - f).

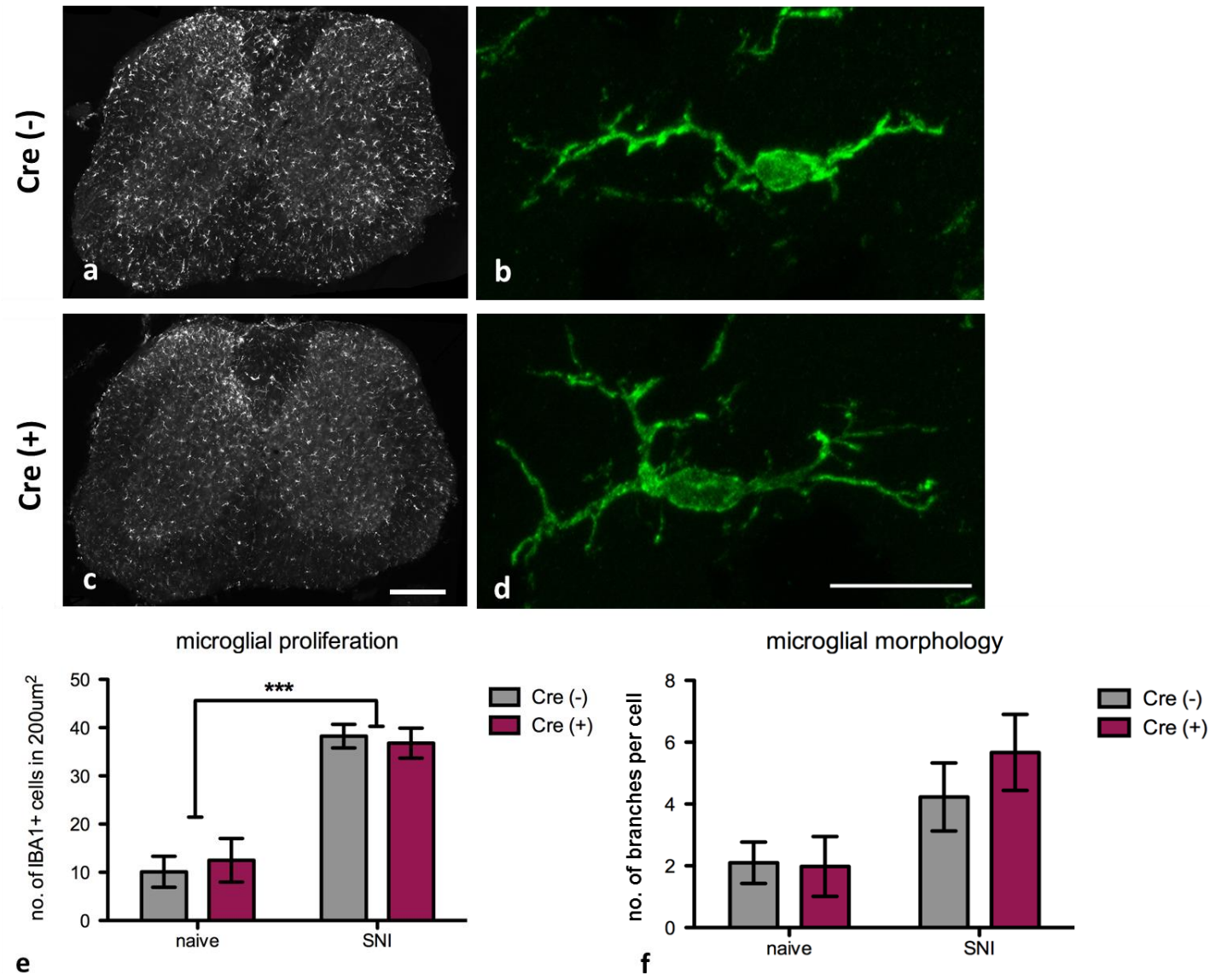


Figure 5-10 microglial morphology and numbers do not differ between Cre (-) and Cre (+) genotypes. (a) shows microglial proliferation after SNI in the Cre (-) group (b) shows an individual microglial profile in the Cre (-) group in the injured state (c) shows that there is a similar level of microglial proliferation in the Cre (+) group where they also have the same morphological profile (d). (e) Quantification of the number of microglial cells shows significant upregulation in no. of microglia after SNI but there is no significant difference between genotypes at the naïve or SNI stage. (n=5 per group, $p<0.001$ repeated measures two way ANOVA, post hoc Bonferronni test).

5.4 Discussion

5.4.1 The Cd11b Cre strain is an inefficient tool that fails to effectively ablate the erbB3 and erbB4 gene in monocytes.

It has been shown that NRG-1 is released after nerve injury into the CNS in the rat and is capable of binding onto erbB receptors on microglia. It has also been suggested that such NRG-1 release may contribute to microgliosis, mechanical and cold relative behavioural responses after nerve injury (Calvo et al. 2010; Calvo et al. 2011). In order to investigate this, I aimed to utilise a myeloid lineage specific Cre to selectively ablate erbB3 and erbB4 gene expression in macrophages and microglia. The principle finding of this chapter is that Cd11b Cre which was reported to have low efficacy in our hands shows very little Cre activity in microglia or macrophages. This Cre line is therefore inadequate to test our original hypothesis that erbB receptor expression contributes to microgliosis and pain following nerve injury. Previous literature suggested Cd11b Cre to be a prime candidate for targeting microglial specific gene modification.

To date, since its first description, the Cd11b Cre strain has been reported and used in 11 research publications. The Cd11b Cre line drives its Cre expression in the monocytic lineage under the Cd11b (monocytic) promoter. Cd11b encodes the α subunit of the Mac-1 leukocyte integrin heterodimer that is used as a surface marker. Cd11b is upregulated during myeloid differentiation and produces the highest levels in mature monocytes, macrophages and neutrophils (Beller et al. 1982; Rosmarin et al. 1989) which makes the Cd11b Cre a suitable transgenic strain to target macrophages and microglia in our study.

For generation of the CD11b Cre mice, a 1.7 kb DNA fragment of the human Cd11b promoter was directly cloned by PCR from genomic DNA derived from the human

leukemic myelomonocytic cell line. A sense-strand Kpn I-Xho I fragment was cloned into the respective restriction sites of pBluescript (Stratagene) already containing a 1.1 kb Cre recombinase gene with a nuclear localization site. Finally a polyadenylation signal from the human growth hormone gene was added (Boillée et al. 2006). Boillée et al. generated this transgenic Cd11b Cre line to selectively target and ablate the superoxide dismutase (SOD1) gene in myeloid cells to determine that SOD1 expression within motor neurons in the early phase of amytrophic lateral sclerosis (ALS).

This study reports that there is approximately 25% Cre dependant decrease in the SOD1 transgene levels in microglia and show B-Gal activity is localised to Cd11b Cre positive cells in the spinal cord (Boillée et al. 2006). Elsewhere Ferrini et al. (2012) use the Cd11b Cre line to selectively ablate BDNF gene expression in microglia to demonstrate that in the absence of BDNF, mice do not develop hyperalgesia to morphine (Ferrini et al. 2013). The author's state: "Microglia, but not neurons or astrocytes, expressed β-gal in Rosa26; Cd11b Cre mice. In addition, Cre expression was detected only in cells that expressed the microglial markers Iba-1 and Cd11b (unpublished observation)". BDNF knockdown however was not directly measured.

Woodling et al. also use Cd11b Cre to selectively ablate the Prostaglandin EP4 receptor on microglia to determine that in the absence of EP4, the toxic microglial inflammatory responses are suppressed in Alzheimer's disease and other neurodegenerative diseases (Woodling et al. 2014). This study also fails to provide evidence for effective knockdown of the gene of interest using the Cd11b Cre system. Whilst the Boillée et al. study reports a 25% reduction in gene expression in microglia

with the use of Cd11b Cre, it remains unclear to as how efficient this transgenic strain was in targeting ablating genes of interest in the remaining published studies. Given the lack of specificity of other transgenic Cre lines such as the Lysosome-M Cre, the Cd11b Cre strain proved to be the most promising at the time and was thus adopted into our study. Whilst extreme caution was taken when backcrossing the Cd11b Cre strain, errors in genotyping may have occurred. The mouse strains most often used in the generation of knockout mice are 129 and B6. It is worth highlighting substantial genetic variability among substrains has been linked to several instances of genetic contamination. In spite of extensive backcrossing, knockout/congenics do not have a 100% B6 background. These strains still harbour discrete regions of residual 129-derived material that may act in isolation or produce epistatic interactions with the ablated gene of interest (reviewed by Eisener-Dorman et al. 2009). It was therefore, crucial that the level of Cre mediated recombination was determined and the selection ablation of erbB3 and erbB4 in monocytes was confirmed before conducting nerve injury studies.

5.4.2 Cd11b Cre shows poor Cre activity within microglia in our hands and exhibits low levels of gene recombination

We attempted to determine the specificity and activity of the CD11b Cre in a number of different ways. Using the $R26^{tdtomato}$ reporter strain, it was determined that there is less than 1% Cd11b Cre mediated activity within IBA1 immunoreactive microglia within the spinal cord. Similarly there was a very low level of Cre activity in macrophages. We showed that the $R26^{tdtomato}$ is an effective reporter strain when we used a tamoxifen inducible Cre as a positive control (and also see the following chapter when this reporter strain is also used.

Using the Cd11b-Cre I sought to selectively target and ablate the erbB3 an erbB4 receptors on macrophages and microglia. We found that was a lack of efficient knockdown in erbb3 and erbB4 levels at the mRNA and protein. These results are therefore at variance to previous published work. We obtained this line from the European Mutant Mouse Archive after they were denoted by the original investigator. It is possible that there could have been some form of mutation within the colony that impacted on Cre activity, these are transgenic animals using the human promoter rather than knocking in Cre to the genomic locus and this may have led to some form of genomic instability. As described below another group has also recently confirmed poor efficiency of this Cre line.

Goldmann et al. compared the LysM Cre, Cd11b Cre and a new microglial specific Cre identified as CX3CR1 Cre to conditionally ablate TGF-β-activated kinase 1 (TAK1) expression in microglia to understand its role in autoimmune inflammation. Within this study, various monocyte specific Cre lines were crossed with the $R26^{YFP}$ reporter strain to examine microglial specific TAK1 gene activation. Goldmann et al. (2013) report that by using the LysM-Cre there is an average recombination rate of IBA1 positive spinal cord microglia of 45.5 ± 2.5% YFP^+ cells (FACS analysis) and 45.6 ±5.3% as determined through histology. The Cd11bCre YFP^+ strain however exhibits a low recombination efficient in microglia with less than 7% of microglia are $YFP^+CD45^{lo}CD11b^+$ (determined by FACS) with an even lower level of expression in brain regions (4.4± 0.5%), and the spinal cord (5.2 ± 4.2%) determined through histology. The CX3CR1 Cre strain however exhibits a very high level of YFP expression (>93%) in microglia in the cortex, hippocampus and spinal cord (Goldmann et al. 2013). It can therefore be concluded that the Cd11b Cre strain does

not effectively ablate genes of interest and it is therefore unsurprising, that in our study the erbB3 and erbB4 genes could not be effectively ablated in microglia and or macrophages. It is also unsurprising, that there are no differences in behavioural or anatomical phenotypes in the presence of Cd11b Cre.

5.4.3 The CX3CR1-Cre strain can be used as a microglial specific Cre to target erbB3 and erbB4 on microglia.

The new microglial specific CX3CR1-Cre has quickly become a popular choice as a tool to effectively target genes of interest on microglia. Parkhurst et al. (2014) recently demonstrated its highly specific and efficient efficacy in targeting the ablation of BDNF in microglia using a CX3CR1 Cre line that is inducible by tamoxifen administration. Using an $R26^{dsred}$ reporter strain bred with the CX3CR1 Cre^{ER} strain, they report that in the absence of tamoxifen, few ($0.3 \pm 0.01\%$) $DsRed^{+}$ microglia are found in the brain of CX3CR1-Cre^{+} $DsRed^{+}$ mice. In contrast, five days after tamoxifen treatment, the majority ($93.9 \pm 0.5\%$) of CX3CR1 Cre^{+} $DsRed^{+}$ mice co-express eYFP and DsRed, thus demonstrating that Cre-mediated recombination in microglia is highly efficient using the CX3CR1 Cre strain (Parkhurst et al. 2013).

5.5 Considerations

In order to investigate the role of NRG-1 and its erbB signalling on microglia, in the next chapter, CX3CR1 Cre line is recruited to selectively target and ablate the erbb3 and 4 genes in microglia. My findings in this chapter emphasise the importance even when Cre lines have been previously reported to check Cre activity in the cell and model system of interest.

6 Using CX3CR1 CreERTM to selectively ablate erbB3 and erbB4 expression in microglia to understand NRG1-erbB signaling in neuropathic pain

6.1 Introduction

In Chapter 5, The Cre-Lox-P system was used to further investigate the role of NRG-1 on microglia by targeting the selective ablation of its receptors (erbB3 and erbB4). The myeloid specific Cre line used was Cd11b Cre. This Cd11b Cre transgenic system proved to be highly inefficient in selectively targeting and ablating erbB3 and erbB4 in monocytes.

In this study, an alternative myeloid specific Cre-Transgenic system is used to investigate NRG1-erbB signalling in microglia. More specifically, it is the first transgenic system that exclusively targets microglial cells in an inducible fashion. Parkhurst et al. (2014) generated a CX3CR1 CreER Tamoxifen inducible transgenic line specific to microglia under the CX3CR1 promoter (Parkhurst et al. 2013). CX3CR1 or Fraktalkine is a $G\alpha_i$ -coupled seven transmembrane receptor synthesised as a membrane anchored protein to promote integrin-independent adhesion. Whilst the CX3CR1 gene is widely expressed throughout the organism, it is highly expressed in specific cell types where CX3CR1 expression in the brain and spinal cord is restricted to microglial cells (Kim et al. 2011).

Utilising this novel CX3CR1 CreER^{TM} system, the aim of this study is to target and selectively ablate erbB3 and erbB4 expression in microglia and investigate the role of NRG-1 release on microglia after injury.

In Chapter 4, the CAG-CreER^{TM} system is used to conditionally ablate NRG1 gene expression. In the absence of NRG1 evidence was given for the attenuation of cold

hypersensitivity after injury. Mechanical hypersensitivity was not attenuated with the absence of NRG1.

With these findings, it is hypothesised that upon erbB3 and erbB4 receptor and gene ablation, NRG-1 is released into the dorsal horn after peripheral nerve injury but is unable to bind and activate microglial cells. By blocking microglial function, it is hypothesised that microglial related cold allodynia is attenuated in mice after nerve injury.

Using the CX3CR1 CreER^{TM} transgenic system, I present evidence for the possible role of NRG1 and its implication in microglial induced cold hypersensitivity.

6.2 Methods

6.2.1 Animals

All experimental work on rodents was carried out accordingly to UK Home office legislation (Scientific Procedures Act 1986). Adult mice (8 weeks or older) were used for cell culture, tissue extraction, behaviour, immunohistochemistry and confocal microscopy unless stated otherwise. The power calculation conducted is described in Chapter 2 section 2.2.2.

6.2.1.1 Generating conditional CX3CR1 CreER^{TM} (+): erbB3$^{-/-}$B4$^{-/-}$ ablation

The initial generation of mice with alleles that are homozygous floxed for erbB3 and erbB4 has been described in Chapter 5. The CX3CR1 CreER^{TM} strain was purchased from Jackson laboratories on a C57BL6 background. The generation and validation of the CX3CR1 CreER^{TM} strain has been previously described (Goldmann et al. 2013; Wolf et al. 2013). To summarise, A targeting vector was designed to replace exon 2 of the chemokine (C-X3-C) receptor 1 (*Cx3cr1*) gene with a CreER^{TM} (Cre recombinase fused to an estrogen receptor ligand binding domain) coding sequence, followed by an internal ribosome entry site (IRES) and an enhanced yellow fluorescent protein (EYFP). CX3CR1 CreER mice express a Cre-ER fusion protein and an enhanced yellow fluorescent protein (EYFP) from endogenous *Cx3cr1* promoter/enhancer elements. EYFP immunofluorescence is observed in *Cx3cr1*-expressing monocytes, dendritic cells, and brain microglia, mimicking endogenous gene expression patterns. The erbB3$^{fl/fl}$B4$^{fl/fl}$ strain was cross-bred with the CX3CRCreER^{TM} strain to generate either CX3CR1 CreER^{TM} (-): erbB3$^{fl/fl}$B4$^{fl/fl}$ or CX3CR1 CreER^{TM} (+): erbB3$^{fl/fl}$B4$^{fl/fl}$ mice. Both groups were administered with Tamoxifen. For labelling purposes the CX3CR1 CreER^{TM} (-): erbB3$^{fl/fl}$B4$^{fl/fl}$ group is

identified as Cre (-) Tx. When Tamoxifen is administered to the CX3CR1 CreER^{TM} (+): erbB3$^{fl/fl}$B4$^{fl/fl}$, the erbB3 and erbB4 gene are selectively ablated and this group is identified as CX3CR1 CreER^{TM} (+): erbB3$^{-/-}$B4$^{-/-}$. For labelling purposes this group is identified as Cre (+) Tx. through the remainder of the chapter. Where possible, equal numbers of animals were recruited into each group and both genders were used to minimise bias.

6.2.1.2 Generating CX3CR1 CreER^{TM} (+): R26$^{tdTomato\ (+)}$ mice

To determine CX3CR1 CreER^{TM} activity, a reporter strain was used to visualize activity. To summarize, the Rosa-CAG-LSL-tdTomato-WPRE targeting vector was designed with (from 5' to 3') a CMV-IE enhancer/chicken beta-actin/rabbit beta-globin hybrid promoter (CAG), an *FRT* site, a *loxP*-flanked STOP cassette (with stop codons in all 3 reading frames and a triple polyA signal), tdTomato sequence (a non-oligomerising DsRed fluorescent protein variant with a 12 residue linker fusing two copies of the protein (tandem dimer)), a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE; to enhance the mRNA transcript stability), a polyA signal, and an *attB*/*attP*-flanked PGK-*FRT*-Neo-polyA cassette. This entire construct was inserted between exons 1 and 2 of the *Gt (ROSA) 26Sor* locus via electroporation of (129S6/SvEvTac x C57BL/6) F1-derived G4 embryonic stem (ES) cells. Correctly targeted ES cells (clone Ai9) were selected and chimeric males were bred to C57BL/6J females. The resulting Ai9 mice were then backcrossed to C57BL/6J background (Goldmann et al. 2013). To evaluate the specificity and efficiency of CX3CR1CreER^{TM}, it was crossed with the R26$^{tdTomato\ (+)}$ reporter to generate either CX3CR1 CreER^{TM} (-): R26$^{tdTomato\ (+)}$ or CX3CR1 CreER^{TM} (+): R26$^{tdTomato\ (+)}$ strains. Both groups were administered with Tamoxifen. It was

hypothesised that in the presence of Cre mediated recombination, any CX3CR1 positive cells will emit tdTomato immunofluorescence. Where possible, equal numbers of animals were recruited into each group and both genders were used to minimise bias.

6.2.2 *In vivo* Tamoxifen treatment

10 week old mice of all strains mentioned above were dosed by oral gavage with 0.25 mg/kg of body weight with Tamoxifen (Sigma T5648) in corn oil for five consecutive days. After dosing, animals were left for 4 weeks before using in further experiments.

6.2.3 Surgery

The spared nerve injury (SNI) was used as a model of peripheral injury on mice in order to measure behavioural outcomes after injury. Surgical and post-operative procedures have been previously described in chapter 2 Section 2.2.3. The same protocol was followed to conduct SNI on cohorts described in this chapter.

6.2.4 Tissue extraction

Mixed glial cultures from the CX3CR1 CreER^{TM} (+): erbB3$^{fl/fl}$B4$^{fl/fl}$ group were isolated from the cortex of P3- P7 mice. Microglial cells were harvested from the mixed glial culture as previously described in chapter 5 section 5.2.4.1.

6.2.4.1 *In vitro* Tamoxifen treatment

Mixed glial culture from CX3CR1 CreER^{TM} (+): erbB3$^{fl/fl}$B4$^{fl/fl}$ or CX3CR1 CreER^{TM} (-): erbB3$^{fl/fl}$B4$^{fl/fl}$ group were both treated with 2µg of 4-OHT every 48 hours for a maximum of 10 days before microglial cells were harvested. Upon harvesting, microglial cells were plated and administered with 2µg of 4-OHT for a further 48 hours before being used for experiments.

6.2.5 Quantitative PCR

Cultured microglia were used to measure erbB3 and erbB4 mRNA levels following Tamoxifen treatment and Cre mediated excision. RNA extraction from microglial cells is previously described in Chapter 5. RNA extracted from microglial cells was used to synthesise into first strand cDNA using the methods previously described in Chapter 2. cDNA was then used to test for mRNA levels of expression of both erbB3 and erbB4 levels relative to either *Aif-1* levels or housekeeping gene expression. The primer sequences used as previously described in chapter 5 Section 5.2.5.2. For analysis, mRNA expression levels of erbB3 and erbB4 were normalised and measured as fold change of the conditional cKO group (Cre (+) Tx.) versus the control group (Cre (-) Tx.).

6.2.6 Immunohistochemistry

Animals were sacrificed in the same manner as described in Chapter 3, Section 3.2.4. Spinal cord, DRG and sciatic nerve tissue from the ipsilateral (injured) and contralateral (uninjured) sides were dissected from animals in each experiment. Spinal cord was fixed in 4% PFA for 24 hours before being dehydrated in 30% sucrose for 48 hours at 4°C. DRG and sciatic nerve were fixed for 30 mins – 1 hour before being dehydrated in 30% sucrose for 48 hours at 4°C. Tissue was embedded in OCT compound on dry ice and stored at -80°C. Spinal cord was cut at 30µm, DRG at 10 µm and sciatic nerve at 10 µm and mounted onto SuperFrost Ultra Plus® slides.

6.2.7 Antibodies

Spinal cord, DRG and sciatic nerve profiles were visualised for YFP, IBA1, GFAP or tdTomato labelling. Sections were washed with PBS and blocked using normal donkey serum (Chemicon S30 1:10, 30 min). Sections were stained with either rabbit ionised calcium binding adaptor molecule 1 (IBA1 1:1000 Wako, Japan), anti-chicken GFP (to enhance YFP signal 1:100 Santa Cruz) and left overnight before being washed 3 times with PBS and then incubated with either anti-rabbit Cyanine-3 (Cy-3 1;500 Life Technologies) or anti-chicken Alexa 488 at room temperature for 3 hours. No antibody was required to visualise tdTomato -A *lox-P*-flanked STOP cassette prevents transcription of the downstream red fluorescent protein variant (tdTomato). When bred to mice that express Cre recombinase, the resulting offspring will have the STOP cassette deleted in the *cre*-expressing tissue(s), resulting in expression of tdTomato. Because this CAG promoter driven reporter construct was targeted for insertion into the *Gt (ROSA) 26Sor* locus, tdTomato expression is determined by which tissue(s) express Cre recombinase. All reagents were diluted in PBS containing 0.2% triton X-100 and 0.1% sodium azide. Slides were washed with PBS and coverslipped in Vectashield® mounting medium with DAPI (Vector Labs).

6.2.8 Image analysis

Immunofluorescence was visualized under a Zeiss Imager.Z1 microscope or a confocal Zeiss LSM 700 laser scanning microscope. Photographs were taken using the Axio Cam and AxioVision LE Rel. 4.2 or the LSM Image Browser software for image analysis.

IBA1 quantification and analysis in the spinal cord, DRG and sciatic nerve was conducted in the same manner as described in Chapter 4 Section 3.2.6. GFAP

immunoreactivity was performed by determining immunofluorescence intensity within 10^4 μm2 boxes using AxioVision LE 4.2 software (Carl Zeiss Imaging Solutions, Rugby, UK) (Clark et al. 2010). The experimenter was blind to the genotype during analysis to minimise bias.

6.2.9 Animal Behavioural Tests

All behavioural tests were carried out whilst the observer was blind to the genotype of each animal tested. Animals were tested in a temperature stable room during the light period of their daily day/night cycle, at the same time every day.

6.2.9.1 Mechanical withdrawal threshold testing using Von Frey filaments

Mechanical withdrawal threshold testing was conducted in the same manner as described in Chapter 2 Section 2.2.4.1. Animals were tested at Baseline, prior to injury and days 3, 7, 14, 21 and 28 post injury

6.2.9.2 Thermal nociception threshold testing using Hargreaves' test

Thermal nociception was carried out in the same manner as described in Chapter 2 Section 2.2.4.2. Animals were tested at Baseline, prior to injury and days 3, 7, 14, 21 and 28 post injury.

6.2.9.3 Cold Place Preference

The Cold Place preference assay was conducted in the same manner as described in Chapter 3 Section 3.2.5.1. The reference plate was set to room temperature of 22°C whilst the fixed temperature plate is set to 16°C. Analysis was perfomed in the same manner.

6.2.10 Statistical Analysis

As the genotypes of each animal are the only variable, similarities or differences between erbB3B4$^{-/-}$CX3CR1^{CreERTM} and erbB3B4$^{fl/fl}$CX3CR1^{CreERTM} littermate controls were determined using repeated measures two way ANOVA with post Hoc Bonferroni statistical test for Behavioural studies and immunohistochemistry experiments. The Students t-test was used for and IHH and qPCR studies where appropriate. Results are reported as mean values ± SEM.

6.3 Results

6.3.1 YFP expression is clearly identified in CX3CR1 CreER^{TM} (+): erbB3$^{fl/fl}$B4$^{fl/fl}$ microglia

The CX3CR1 CreER^{TM} system recruited is said to express a Cre-ER fusion protein and an enhanced yellow fluorescent protein (EYFP) from endogenous *Cx3cr1* promoter/enhancer elements. Jackson Laboratories report that eYFP immunofluorescence is observed in *Cx3cr1*-expressing monocytes, dendritic cells, and brain microglia, mimicking endogenous gene expression patterns.

Upon crossing the erbB3$^{fl/fl}$B4$^{fl/fl}$ strain with the CX3R1CreER^{TM} strain, endogenous eYFP immunofluorescence was investigated in IBA1^{+} cells to determine the capabilities of the Cre-ER eYFP labelling.

As Tamoxifen is not required to induce eYFP expression, spinal cord tissue from the CX3CR1 CreERTM (-): erbB3$^{fl/fl}$B4$^{fl/fl}$ and CX3CR1 CreER^{TM} (+): erbB3$^{fl/fl}$B4$^{fl/fl}$ group was analysed. Upon investigation, endogenous YFP expression could not be clearly identified. Thus a GFP antibody was used to enhance the signal. **Figure 6-1**a-f give examples of IBA1 labelled microglia that did not express YFP (identified using GFP antibody) in the absence of CX3CR1 CreER^{TM}. **Figure 6-1**g-l give examples of IBA1 labelled microglial cells that expressed YFP with the addition of the CX3CR1CreER^{TM}. The number of IBA1^{+} cells between the two groups was quantified in spinal cord (SC), DRG and sciatic nerve (SN) tissue. The number of IBA1^{+} cells did not differ in the SC, DRG or SN depending on the presence or absence of Cre (**Figure 6-1**m). **Figure 6-1**n shows the quantification of the total percentage of IBA1^{+}: YFP^{+} positive in the SC. On average 66.816 ± 5.086% of IBA1^{+} cells express YFP in the presence of CX3CR1 Cre (+) whilst there is 0.00% YFP expression seen in the CX3CR1 Cre (-) strain. The level of YFP expression in DRG

and sciatic nerve was also quantified between genotypes and found to be less than 5% $IBA1^{+}$: YFP co-expression in the CX3CR1 Cre (+) strain and 0% in the CX3CR1 Cre (-) group (see **Figure 6-1**o). This is perhaps owing to a poor level of endogenous YFP expression. Whilst using a GFP labelling antibody makes it markedly easier to identify CX3CR1 Cre (+) microglia in the CNS tissue, less is understood about the level of expression in the sciatic nerve and DRG and thus requires further validation. In summary, the addition of CX3CR1CreER^{TM} to the erbB3$^{fl/fl}$B4$^{fl/fl}$ strain and CreER fusion allowed for endogenous YFP expression to be detected (using an antibody) in microglia.

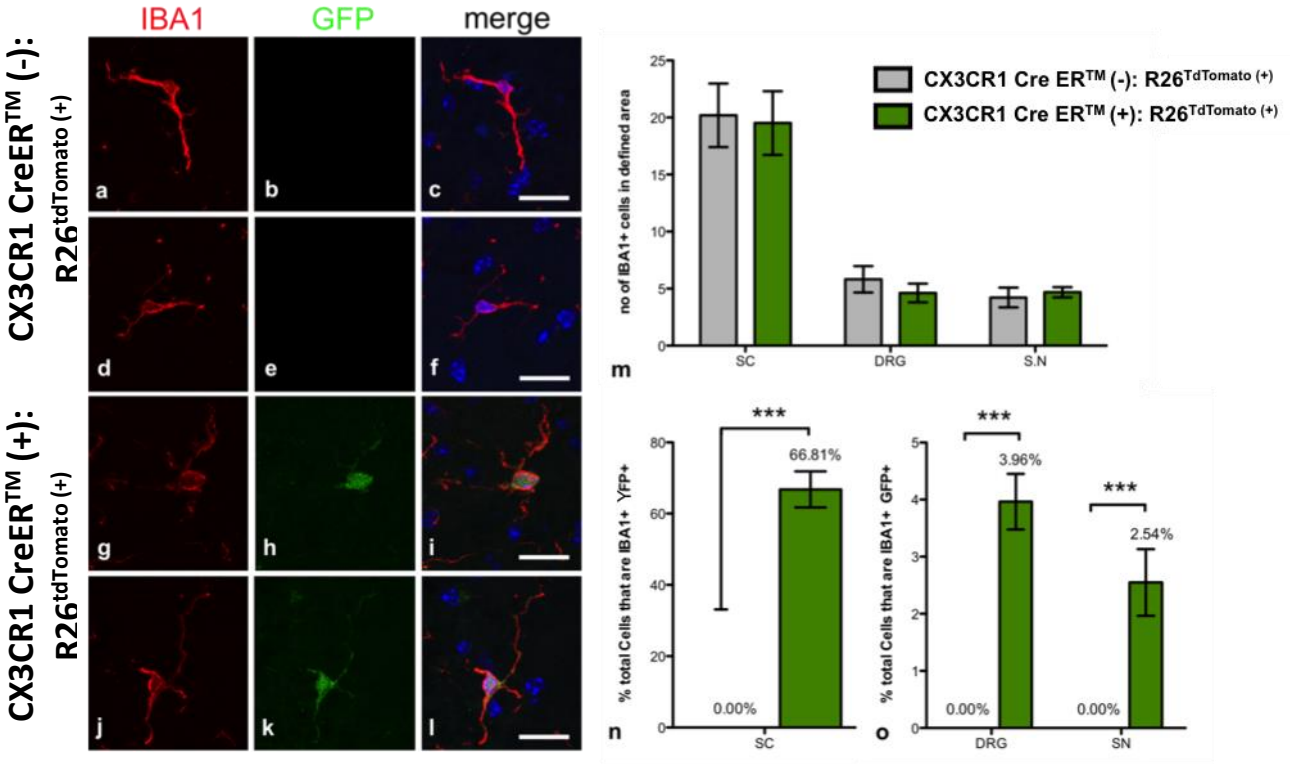


Figure 6-1 YFP expression is detected in microglia upon the addition of CX3CR1 Cre system. (a-f) give examples of an IBA1+ labelled cell in the spinal cord dorsal horn (SCDH) in the absence of CX3CR1 Cre. (b and d) show that IBA1+ cells do not express eYFP in the CX3CR1 CreERTM (-): erbB3fl/flB4fl/fl group (c and f). (g-h) give examples of an IBA1+ labelled cell in the SCDH in the presence of CX3CR1 Cre. (h and k) show YFP expression in the SCDH which is co-expressed with IBA1+ cells in the CX3CR1 CreERTM (+): erbB3fl/flB4fl/fl group (i and l). (m) Quantification of the number of IB1+ cells between genotypes in the SC, DRG and SN tissue. As expected in the naive state, there are few IBA1+ macrophages in the PNS (area size analysed SC=200um, DRG = 100um, SN = 500um; n= 5 per group; $p>0.05$ Students t-test). (n) Quantifies the level of IBA1+/GFP expression in the SC. In the presence of CX3CR1 Cre animals expresses significantly higher levels of IBA1+:eYFP co=expression (n= 5 per group; *$p<0.05$ **$p<0.001$ ***$p<0.0001$; Students t-test). (o) DRG and sciatic nerve tissue was also quantified. (n= 5 per group; *$p<0.05$ **$p<0.001$ ***$p<0.0001$; Students t-test).

6.3.2 The CX3CR1CreER^{TM} strain can be used to target gene ablation in microglia

The endogenous YFP expression seen in the CX3CR1 CreER^{TM} strain could not be confidently verified in PNS tissue and the true level of functionality of the CreER strain could not be clearly identified. Therefore the CX3CR1 CreER^{TM} was bred with the R26$^{tdTomato\ (+)}$ reporter and the level of tdTomato expression in IBA1^{+} cells was quantified through immunohistochemical analysis. According to the eYFP expression findings, it was predicted that at least 66% of IBA1^{+} cells express tdTomato $^{(+)}$ upon Tamoxifen induced Cre mediated recombination.

The CX3CR1 CreER^{TM} (+): R26$^{tdTomato\ (+)}$ and CX3CR1 CreER^{TM} (-): R26$^{tdTomato\ (+)}$ groups were treated with Tamoxifen and a model of nerve injury was performed 4 weeks later. Animal tissue was analysed at 7 days post injury.

As shown in **Figure 6-2** a-c, in the CX3CR1 CreER^{TM} (-): R26$^{tdTomato\ (+)}$ group, when Tamoxifen was administered there was no tdTomato expression detected in IBA1^{+} cells in the SCDH (**Figure 6-2** d-f). However, when Tamoxifen was administered to CX3CR1 CreER^{TM} (+): R26$^{tdTomato\ (+)}$ animals, a high level of cells labelled with both IBA1 and tdTomato were identified in the SCDH. This level of IBA1^{+}: tdTomato^{+} expression was further quantified.

The total number of IBA1 cells did not differ between genotypes in any of the areas of the spinal cord. In the ipsilateral DH, there were on average 33.972 ± 3.562 IBA1^{+} cells in the CX3CR1 CreER^{TM} (-): R26$^{tdTomato\ (+)}$ group whilst there are 35.660 ± 4.283 IBA1^{+} positive cells in the CX3CR1 CreER^{TM} (+): R26$^{tdTomato\ (+)}$ group (per 200um^{2} area). There was also no difference in the number of IBA1 cells in the ventral horn between the two groups (27.718 ± 2.271 vs. 31.282 ± 3.357 respectively).

The percentage of $IBA1^+$: $tdTomato^+$ cells were quantified (**Figure 6-2**h). There was a significantly higher proportion of $IBA1^+$: $tdTomato^+$ cells in the CX3CR1 $CreER^{TM}$ (+): $R26^{tdTomato\ (+)}$ group versus CX3CR1 $CreER^{TM}$ (-): $R26^{tdTomato\ (+)}$ group strain across all 4 areas of the lumbar spinal cord. In the CX3CR1 $CreER^{TM}$ (+): $R26^{tdTomato\ (+)}$ group, there are on average, 87.222 ± 4.288% of $IBA1^+$: $tdTomato^+$ cells in the ipsilateral horn (per 200um $area^2$) of the Cre (+) strain whilst there was 90.812 ± 2.912% $IBA1^+$: $tdTomato^+$ cells in the ipsilateral ventral horn. As expected, there was 0.000% of $IBA1^+$: $tdTomato^+$ cells in the CX3CR1 $CreER^{TM}$ (-): $R26^{tdTomato\ (+)}$ group.

With these findings, it can be determined that the CX3CR1 $CreER^{TM}$ strain is approximately 87% efficient in selectively mediated gene recombination in microglia using the Cre-loxP system. It can therefore be resolved that the $CX3CR1^{CreERTM}$ strain can be used to selectively target and ablate erbB3 and erbB4 gene expression in microglia in order to investigate NRG1-erbB signalling in microglia.

One unique feature of the CX3CR1 $CreER^{TM}$ is that it can be used to distinguish between microglia and macrophages. Microglia and other CX3CR1+ cells have substantially different rates of turnover and are derived from different precursor populations to other CX3CR1+ cells. Microglia are a self-renewing population (Ajami et al. 2007) with a low turnover rate (Lawson et al. 1992) whilst monocytes and inflammatory macrophages exhibit a rapid turnover rate (van Furth & Cohn 1968). After SNI, macrophages quickly infiltrate the site of injury and are replenished through a bone marrow precursor population that is $CX3CR1^-$ in nature. Given this feature it was speculated that after following tamoxifen treatment and in the presence of Cre recombinase, resident macrophages would express tdTomato whilst

replenished macrophages would not. In the context of SNI, it was predicted that IBA1^{+} cells in the PNS would not express tdTomato. Tissue was extracted from both groups at 7 days post SNI which equates to 37 days post tamoxifen treatment. The level of tdTomato expression in IBA1^{+} cells in the PNS was investigated (Figure 6.3). As expected, the CX3CR1 CreER^{TM} (-) strain do not express tdTomato+ in any IBA1^{+} expressing cells in the DRG or sciatic nerve (**Figure 6-3**a-c and g-i) following tamoxifen treatment and SNI. The level of tdTomato expression in the CX3CR1 CreER^{TM} (+) in the injured DRG and sciatic nerve was also measured. There was no tdTomato expression detected in any of the IBA1^{+} cells in the injured DRG's or injured sciatic nerve (quantification could not be carried out as the uninjured contralateral paws also showed absolutely no tdTomato expression in the DRG or sciatic nerve).

With these findings, it can be determined that the CX3CR1 CreER^{TM} strain is highly specific to targeting gene ablation in microglia and can be used to differentiate between macrophages.

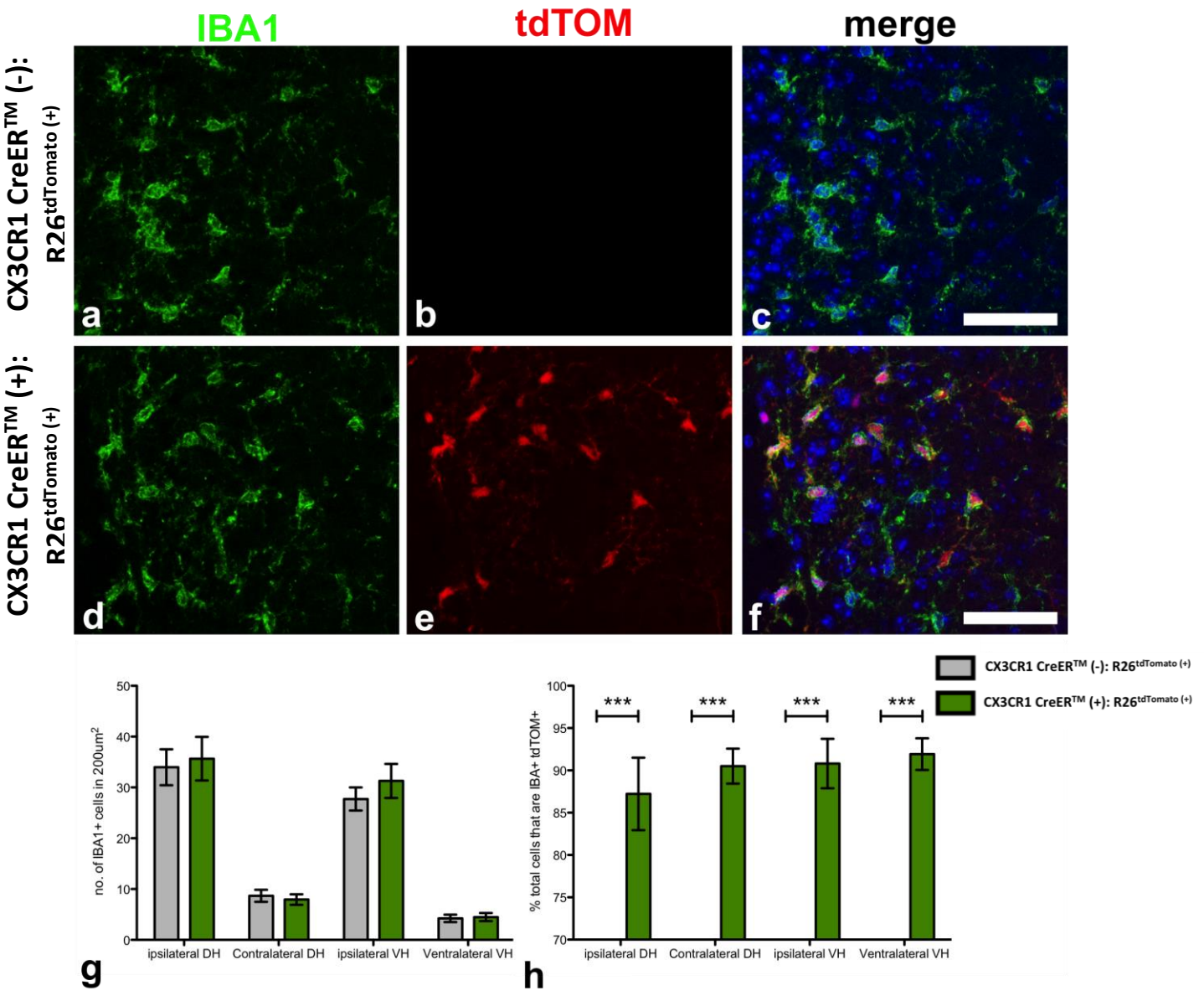


Figure 6-2 CX3CR1CreERTM is a robust Cre transgenic system that can be used to target gene modification in microglia. (a) Shows IBA1 labelled microglia in the SCDH of the CX3CR1 CreERTM (-): R26tdTomato (+) group after nerve injury. (b) As expected, with Tx. Treatment there is a lack of tdTomato expression in IBA1 expressing cells (c). (d) Shows IBA1 labelled microglia in the SCDH of the CX3CR1 CreERTM (+): R26tdTomato (+) group after nerve injury. (e) With Tx treatment, there is significant number of IBA1+: tdTomato+ cells (f). (g) The number of IBA1+ cells is quantified in the ipsilateral (injured) and contralateral (uninjured) sides of the SC. (h) Shows that there are a significantly higher proportion of IBA1+: tdTomato+ cells in the CX3CR1 CreERTM (+): R26tdTomato (+) group in all areas of the SC vs. the CX3CR1 CreERTM (-): R26tdTomato (+) group (n=5 per group, *p<0.01 **p<0.001 ***P<0.0001 Students t-test).

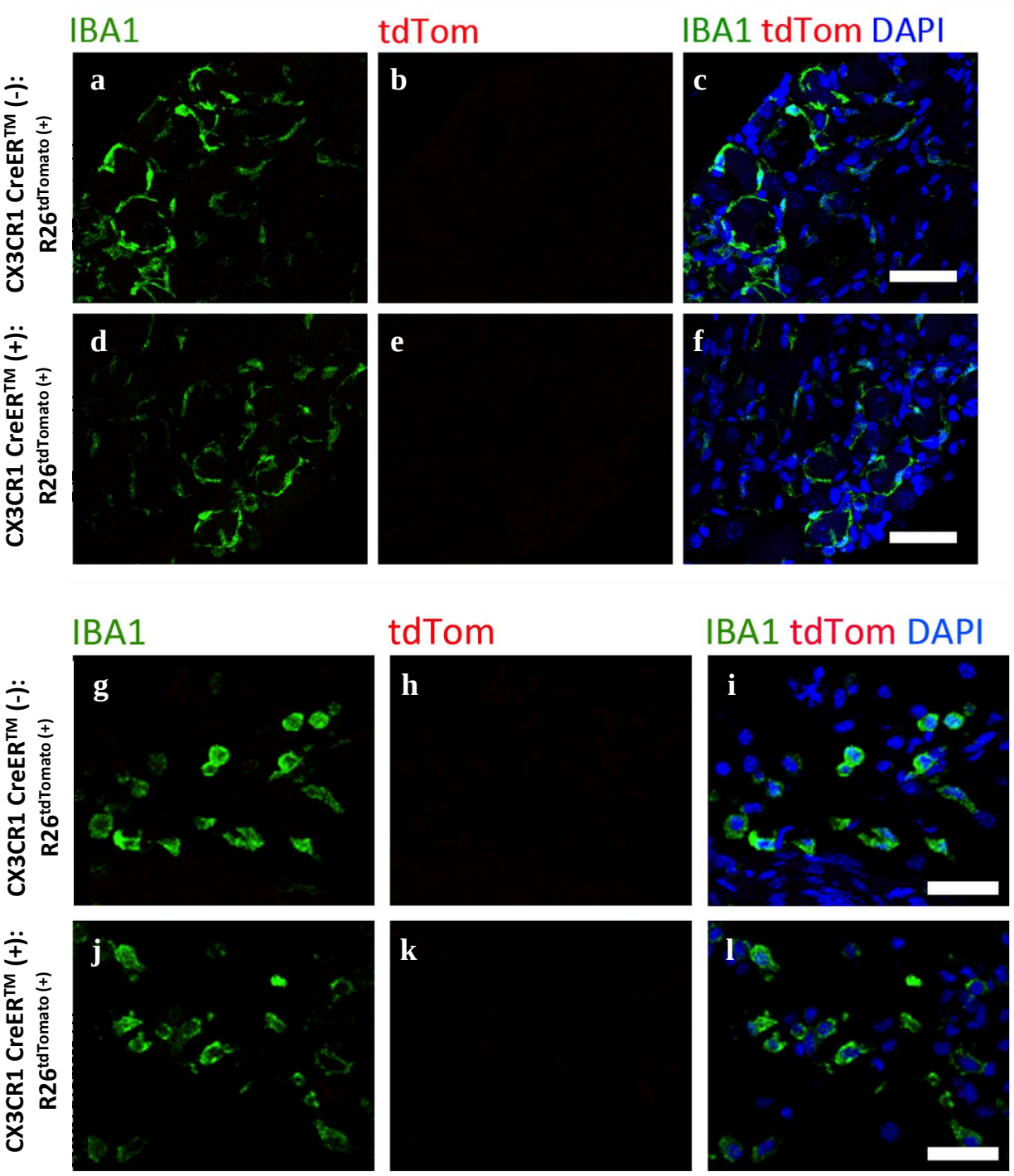


Figure 6-3 tdTomato+ expression is not detected in CX3CR1 Cre ERTM (+) monocytes in the PNS after SNI. (a-c) Shows IBA1+ macrophages in the lumbar injured DRG at 7 days after SNI (37 days) after Tamoxifen treatment. In the absence of Cre, there is no tdTomato expression. (d-f) in the presence of Cre, and Tamoxifen induction, there is also no tdTomato+ expression in any IBA1+ infiltrating macrophages. (g-i) Shows IBA1+ macrophages in the injured sciatic at 7 days after SNI (37 days) after Tamoxifen treatment. In the absence of Cre, there is no tdTomato expression. (j-l) in the presence of Cre, and Tamoxifen induction, there is also no tdTomato+ expression in any IBA1+ infiltrating macrophages in the sciatic nerve. No quantification could be conducted as neither the injured or uninjured PNS tissue exhibited any tdTomato expression (scale bar 50µm).

6.3.3 ErbB3 and erbB4 mRNA gene expression levels are significantly reduced using the Tamoxifen inducible CX3CR1 CreER^{TM} system.

The following experiments now discuss the CX3CR1 CreER^{TM} (-): erbB3$^{fl/fl}$B4$^{fl/fl}$ and the CX3CR1 CreER^{TM} (+): erbB3$^{-/-}$B4$^{-/-}$ groups that were treated with Tamoxifen. As mentioned earlier these groups are respectively labelled as Cre (-) Tx and Cre (+) Tx for simplicity. Microglial cultures used from both groups and Tamoxifen was administered in vitro before mRNA was extracted for gene expression analysis. When Tamoxifen was administered to Cre (+) TX microglial cultures, there was a near 90% reduction in erbB3 levels and erbB4 approximately 90% reduction in erbb4 mRNA levels relative to GAPDH/HPRT housekeeping genes (**Figure 6-4**). mRNA expression of erbB3 and erbB4 was also measured relative to *Aif-1*. As **Figure 6-5** shows, with Tx administration to the Cre (+) Tx. group, there was approximately 75% reduction in erbB3 and 80% reduction in erbB4 levels (relative to *Aif-1*).

It can therefore be determined, that using the CX3CR1CreER^{TM}, gene expression of erbB3 and erbB4 can be selectively ablated. This is turn leads to efficient knock down of erbB3 and 4 receptors in microglia and the role NRG1 after peripheral nerve injury can be investigated.

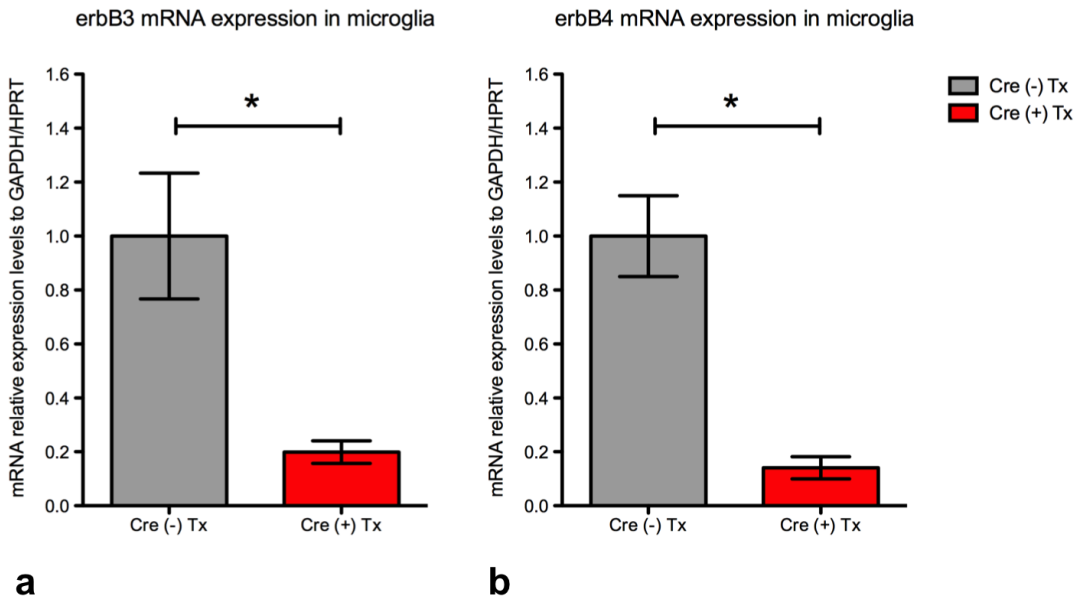


Figure 6-4 there is a significant reduction in erbB3 and erbB4 mRNA expression using CX3CR1 CreERTM Tamoxifen inducible system. (a) The level of erbB3 mRNA expression is compared relative to housekeeping genes GAPDH and HPRT levels. There is a significant reduction in the erbB3 mRNA in the Cre (+) Tx group vs. the Cre (-) Tx group (0.199 ± 0.042 vs. 1.000 ± 0.233 respectively) (b) erbB4 mRNA levels are also significantly reduced in the Cre (+) group when compared to the Cre (-) group (0.140 ± 0.041 vs. 1.000 ± 0.150 respectively) (n= 3 per group p= 0.041 and p = 0.021 respectively, Students t-test).

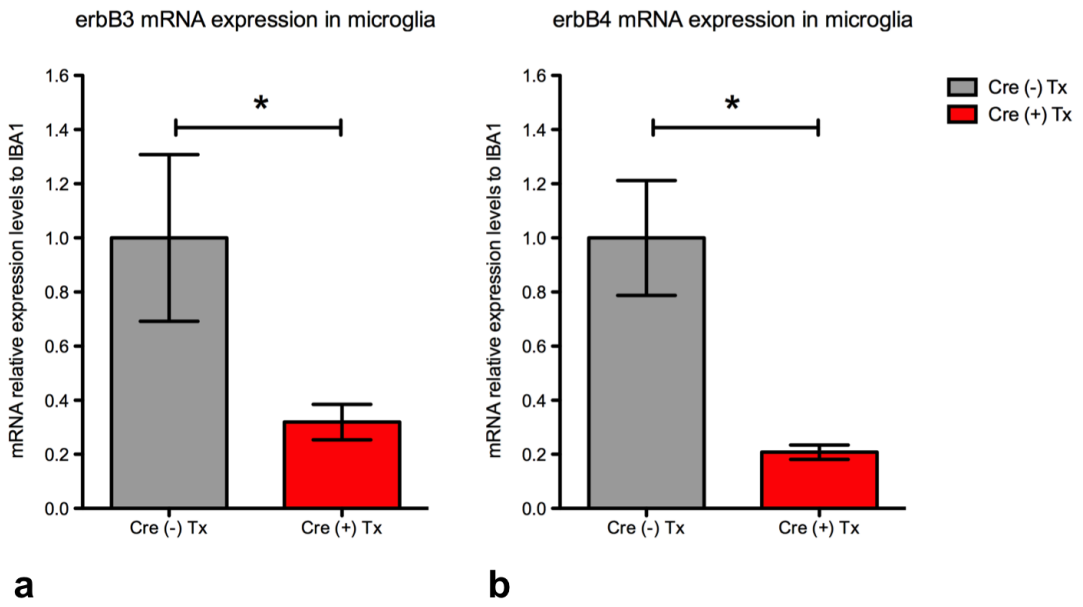


Figure 6-5 erbB3 and erbb4 mRNA levels are significantly reduced when compared to IBA1 relative mRNA expression. (a) erbB3 and (b) erbB4 mRNA levels were also quantified relative to IBA1 expression instead of HK genes, and there is still a significant reduction in erbB3 and erbB4 mRNA levels in Cre (+) Tx group versus the control Cre (-) Tx group (n= 5 per group; p = 0.048 p = 0.017 ; Students t-test).

6.3.4 ErbB2 mRNA levels are significantly upregulated when erbB3 and erbB4 receptors are down-regulating using Tamoxifen.

ErbB2 receptors are also found on microglia but lack a ligand binding domain. ErbB2 receptors heterodimerise with either erbB3 or erbB4 to mediate intracellular downstream signalling in microglia. When erbB3 and erbB4 mRNA levels are significantly down-regulated with Tamoxifen administration, we sought to quantify the level of erbB2 mRNA expression. With Tamoxifen administration, there is approximately 1.5 x fold increase in erbB2 mRNA levels relative to HK genes and approximately 3 x fold increase relative to *Aif-1* gene expression (**Figure 6-6**). It seems with Tamoxifen administration, there is a decrease in erbB3 and erbB4 mRNA levels but a significant increase in erbB2 mRNA levels.

Using the endogenous YFP expression, reporter tdTOM strain and qPCR analysis of erbb3 and erbB4 mRNA levels, we have been able to clearly identify a significant level of Cre-ER activity with a significant reduction in erbB3 and erbB4 mRNA levels. With this validation, we can now induce nerve injury in the control erbB3B4$^{fl/fl}$ Cre (-) and erbB3B4$^{-/-}$ Cre (+) group.

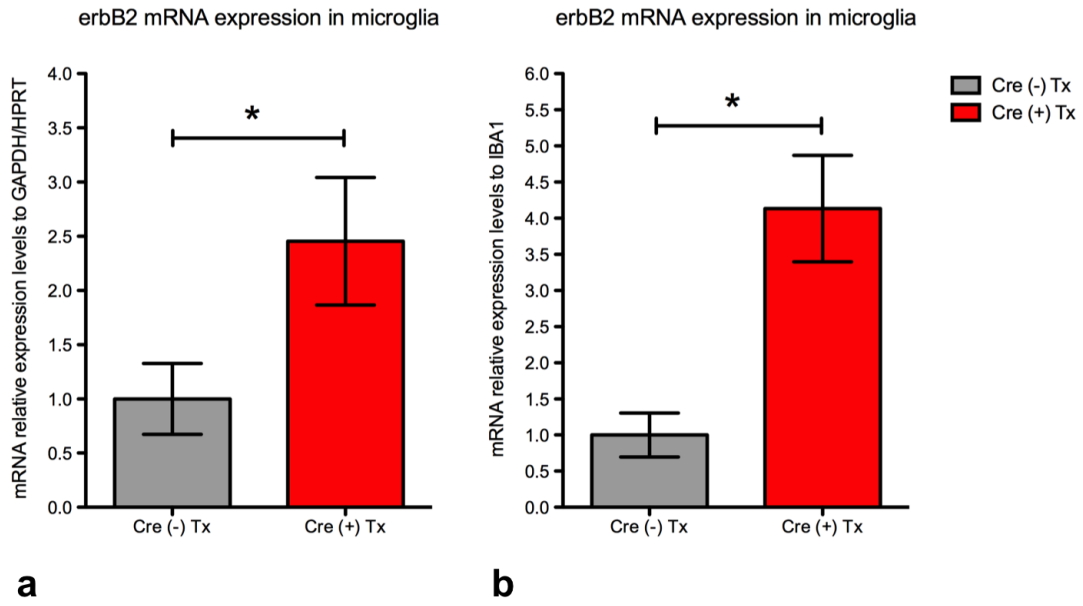


Figure 6-6 erbB2 mRNA levels are significantly upregulated after Cre mediated excision of erbB3 and erbB4 gene expression. (a) erbB2 mRNA levels relative to HK genes are significantly upregulated in the Cre (+) Tx condition versus Cre (-) Tx condition (2.455 ± 0.588 vs. 1.000 ± 0.328 respectively, p= 0.048) (b) erbB2 mRNA levels relative to IBA1 levels are also significantly upregulated in the Cre (+) Tx condition versus Cre (-) Tx condition (4.131± 0.737 vs. 1.000 ± 0.326 respectively ; p = 0.017) (n= 3 animals per group $p<0.05$; Students t-test).

6.3.5 Mechanical hypersensitivity is not attenuated with erbB3 and erbB4 receptor knock-down

Animals were administered with Tamoxifen and then underwent spared nerve injury before behavioural testing was conducted at set time points to measure behavioural changes after injury in the Cre (-) Tx and Cre (+) Tx group (**Figure 6-**).

At baseline both groups exhibited normal 50% withdrawal thresholds which were measured at 1.145 ± 0.128g in the Cre (-) Tx group and 1.248 ± 0.134g in the Cre (+) Tx group. At day 3 after injury, both groups developed significant mechanical hypersensitivity with 50% withdrawal thresholds at 0.071 ± 0.289g in the Cre (-) Tx group and 0.062 ± 0.008g in the Cre (+) Tx group. There was no difference in the level of hypersensitivity between the two groups at days 3, 7, 14, 21 or 28 after injury (**Figure 6-**). At day 28, animals continued to exhibit significantly lowered 50% withdrawal thresholds in both groups (0.073 ± 0.340g in the Cre (-) Tx group and 0.081 ± 0.045g in the Cre (+) Tx group). Given that Sorge et al. suggest that microglial induced neuropathic pain is gender specific, each cohort was split by gender and further analysed. It was determined that there is no significant difference in the mechanical hypersensitivity thresholds between genders within each experimental group (Sorge et al. 2015) (please refer to appendix Table's 16 and 17).

It can be concluded that with selective ablation of erbB3 and erbB4 receptors on microglia mechanical hypersensitivity is not attenuated after nerve injury. The NRG1-erbB signalling pathway is unlikely to regulate mechanical allodynia after nerve injury.

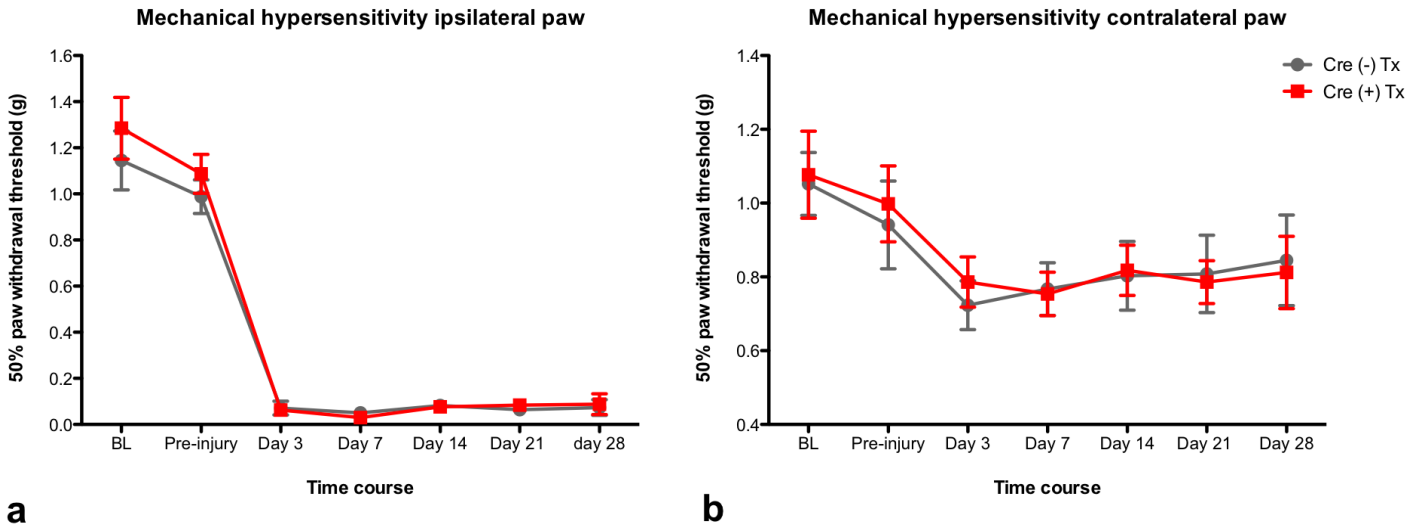


Figure 6-7 erbB3 and B4 receptor knock down does not attenuate mechanical hypersensitivity. (a) shows the 50% withdrawal thresholds to a mechanical stimuli in the ipsilateral paw. Before injury, animals exhibit normal baseline 50% withdrawal thresholds. After injury there is robust mechanical hypersensitivity that develops between both Cre (-) Tx and Cre (+) Tx group. There is no distinct difference between the two groups through the whole time course after injury. (b) shows the contralateral paw 50% withdrawal threshold there is a slight decrease after injury in the 50% withdrawal threshold in bother groups but overall there is no significant change in withdrawal threshold after injury in the contralateral paw (n=8-11 per group; p= 0.308; p = 0.776; repeated measures two way ANOVA Bonferroni post hoc test).

6.3.6 Thermal hypersensitivity is not attenuated in animals that lack erbB3 and erbB4 receptors on microglia

The Hargreaves test was used to test thermal hypersensitivity in Cre (-) Tx and Cre (+) Tx groups (**Figure 6-**). At baseline both groups exhibited normal latency to withdraw from a radiant heat source. The latency to withdraw was measured at 12.730 ± 0.316s in the Cre (-) Tx. group and 12.150 ± 0.567s in the Cre (+) Tx group. At day 3 after injury, both groups developed significant thermal hypersensitivity with lowered latencies to withdraw at 4.391 ± 0.982s in the Cre (-) Tx. group and 4.312 ± 1.193s in the Cre (+) Tx group. There was no difference in the level of hypersensitivity exhibited between the two groups at days 3, 7, 14, 21 or 28 after injury (**Figure 6-**). At day 28, animals continued to exhibit significantly lowered thermal withdrawal latencies of 6.345 ± 0.841s in the Cre (-) Tx. group and 6.566 ± 1.324s in the Cre (+) Tx. group. Each cohort was split by gender and further analysed. It was determined that there is no significant difference in the thermal hypersensitivity thresholds between genders within each experimental group (please refer to appendix Table's 18 and 19).

It can therefore be concluded that with selective ablation of erbB3 and erbB4 receptors on microglia thermal hypersensitivity is not attenuated after nerve injury. Thermal hypersensitivity in animals is likely to develop independently of NRG1-erbB signalling in microglia.

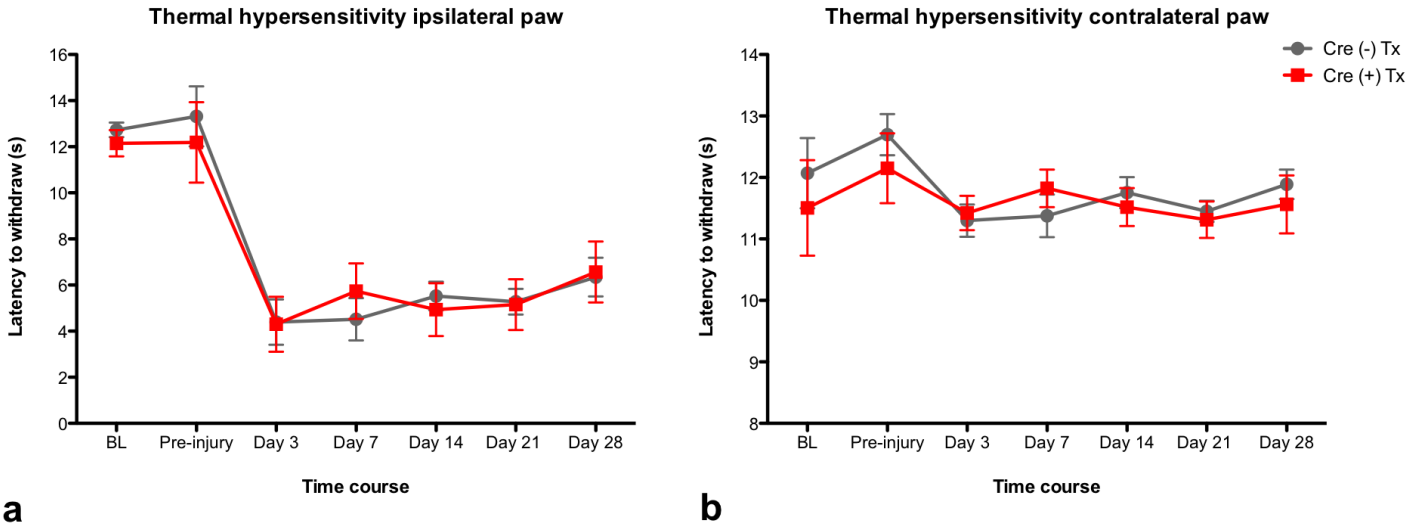


Figure 6-8 erbB3 and erbB receptor knockdown in microglia does not attenuate thermal hypersensitivity after peripheral nerve injury. (a) shows the latency to withdraw from a radiant heat source applied to the ipsilateral paw in the control Cre (-) Tx group and conditional ablation Cre (+) Tx. group. There is no difference in the latency to withdraw at baseline in Cre (-) Tx and Cre (+) Tx group. After injury, both groups develop significant thermal hypersensitivity and withdraw their paws in reduced time. However there is no difference in the latency to withdraw between the two groups at days 3,7,14,21 and 28 after injury. (b) Shows the latency to withdraw from a radiant heat source applied to the right paw. In the contralateral, no thermal hypersensitivity develops (n=8-11 per group; p= 0.797; p = 0.400; repeated measures two way ANOVA Bonferroni post hoc test).

6.3.7 Proprioception is not affected by erbB3 and erbB4 receptor knockdown in microglia

Proprioception was measured in the control Cre (-) Tx and cKO Cre (+) Tx groups. As **Figure 6-** shows, animals were tested for proprioceptive function using the Beam test. Animals were allowed to cross a beam of set diameter and the number of correct hind paw steps vs. the number of incorrect steps (slips and hops) were quantified as percentage values and reported in **Figure 6-**. At baseline, the Cre (-) Tx. and Cre (+) Tx. group both exhibited normal proprioceptive function with almost 100% correct steps. With Tamoxifen induced gene ablation in microglia, the Cre (-) Tx. group exhibited 94.560 ± 2.234% correct steps whilst the Cre (+) Tx. group exhibited 87.125 ± 3.539% correct steps when transversing the beam. After SNI, both groups developed significant motor function impairment ($P<0.0001$) but there was no significant difference in the level of impairment between the two groups ($p= 0.500$). Both groups developed a normal degree of proprioceptive impairment at days 4, 8, 15, 22 and 29 after peripheral nerve injury. At day 29, animals from both groups exhibited some proprioceptive repair where Cre (-) Tx group made on average 68.456 ± 4.328% correct steps whilst Cre (+) Tx group made on average 79.098 ± 6.212% correct steps (n=8-11 per group; $p=0.500$; repeated measures two way ANOVA, post-hoc Bonferroni test).

It can therefore be concluded that motor co-ordination is impaired after peripheral nerve injury independently of NRG1-erbB activity in microglia. As previously described in Chapter 4, NRG1 is important in the PNS for muscle spindle formation/maintenance and whilst NRG1 release after nerve injury remains intact, motor function recovery recurs at a normal rate.

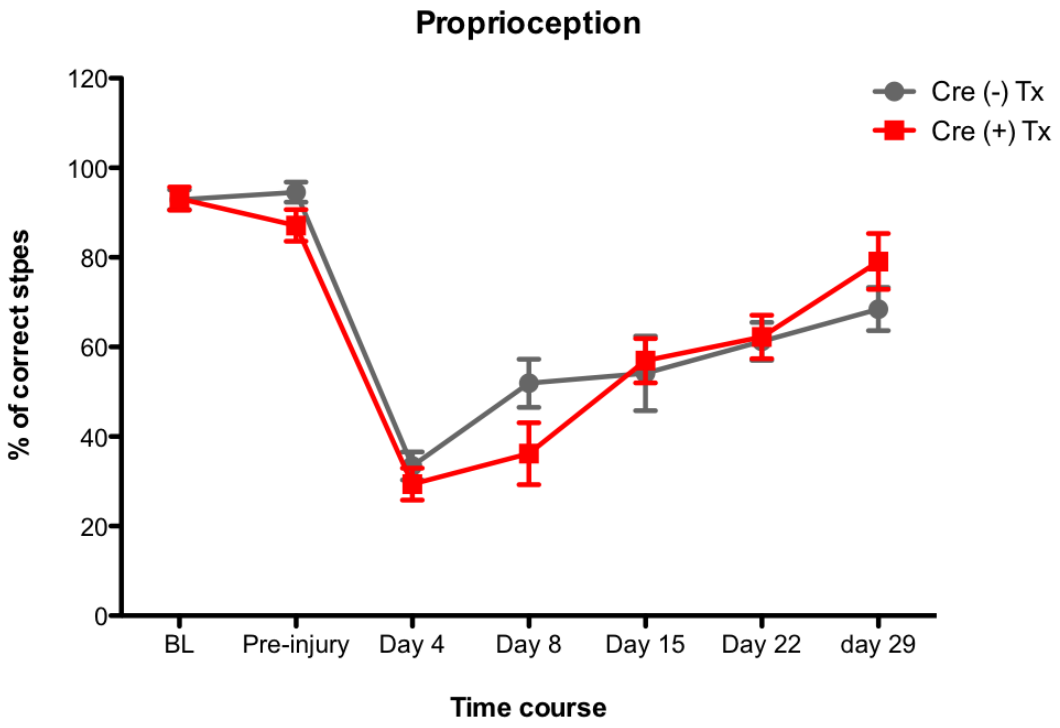


Figure 6-9cKO animals (Cre (+) Tx.) exhibit normal proprioceptive repair after peripheral nerve injury. Cre (-) Tx and Cre (+) Tx animals underwent spared nerve injury and the level of proprioceptive repair was measured using the Beam Test. The % of correct steps vs. falls or slips (incorrect steps) across a beam was measured between both groups. At Baseline, both groups exhibit near 100% correct steps and thus highly co-ordinated motor function. At 4 days after injury, however, Cre (-) Tx animals exhibit a significant reduction in % of correct steps. Whilst there is a significant reduction from baseline ($p<0.0001$), there is no significant difference between genotypes at days, 4, 8, 15, 22 and day 28. (n=8-11 per group; $p=0.500$; repeated measures two way ANOVA, post-hoc Bonferrroni test).

6.3.8 Cold hypersensitivity is regulated by NRG1-erbB signaling in microglia

With NRG1 ablation it was found that whilst mechanical hypersensitivity is not attenuated, cold hypersensitivity is attenuated in the delayed stages after peripheral nerve injury. In order to investigate this further, erbB3 and erbB4 gene were selectively ablated in microglia using the CX3CR1 CreER^{TM} system and the cold place preference assay was used to measure cold pain behaviour changes after injury. The percentage of time spent in the 16°C environment versus the reference room temperature plate (22°C) is presented in **Figure 6**a.

At baseline, the Cre (-) Tx. and Cre (+) Tx. group spent approximately 23-27% of their total 10 mins on the 16°C environment. At 4 days after injury, both groups developed marked hypersensitivity to cold stimuli and spent 14.052 ± 2.167% and 16.951 ± 2.164% of their time on the 16°C environment. There was no significant difference between the Cre (-) Tx and Cre (+) Tx groups at days 4, 8 and 15 after injury. At day 22 however, the Cre (+) Tx. groups parcentage of occupation at 16°C was significantly higher than the control Cre (-) Tx group (19.428 ± 1.408 vs.12.713 ±1.682% respectively). At day 29 the Cre (+) Tx group continued to spend significantly more % of their time in the 16°C vs. the Cre (-) Tx group (22.234 ± 1.107 vs. 13.451 ± 1.129 respectively). The cKO (Cre (+) Tx) group almost returned to baseline levels whilst control group continued to exhibit marked cold allodynia/hypersensitivity to 16°C. The number of crossings between 16% and 22% were also measured to determine whether animals had learned to avoid the cold stimuli, but there was no change in the number of crossings through the course of the study (**Figure 6**b). Animals from either group continued to explore both chambers throughout the course of the study without any prejudice due to previous experiences in the same set up (Figure 6b). Given that Sorge et al. suggest that microglial induced

neuropathic pain is gender specific, each cohort was split by gender and further analysed. It was determined that there is no significant difference in the cold hypersensitivity thresholds between genders within each experimental group (Sorge et al. 2015) (please refer to appendix Table 19).

With these findings, it can be determined that erbB3 and erbB4 receptor knockdown in microglia is likely to regulate NRG1 signalling which in turn regulates microglial induced cold hypersensitivity in mice after nerve injury.

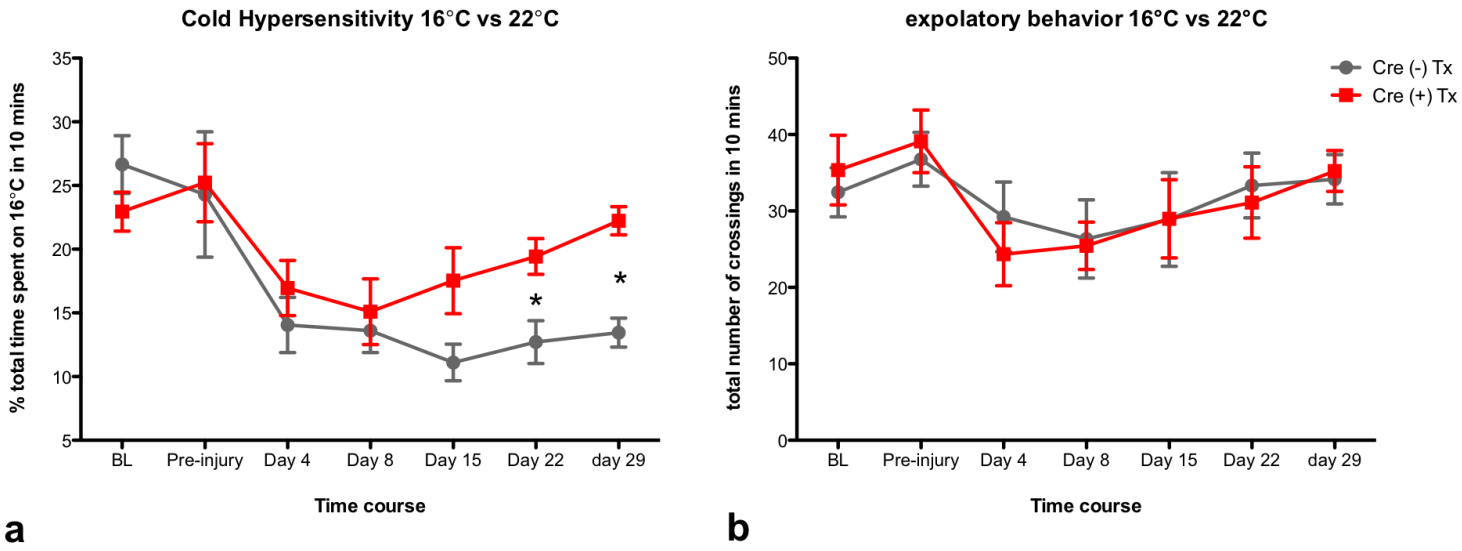


Figure 6-10 cold allodynia is attenuated in cKO animals at 22 and 29 days after injury. (a) Shows the total % of time spent on 16°C vs. 22°C in 10 minutes. At baseline, both the Cre (-) Tx and Cre (+) group spend approximately 23-27% of their time on the cold 16°C environment. At 4 days after injury, both groups develop marked hypersensitivity to the cold stimuli and spend approximately 14 % and 16% of their time on the 16°C environment. There is no significant difference between the Cre (-) Tx Tx and Cre (+) Tx groups at days 4, 8 and 15 after injury. At day 22 however, the Cre (+) Tx groups % occupation at 16°C is significantly higher than the control Cre (-) tx group (approximately 19% vs.12% respectively). At day 29 the Cre (+) Tx group continues to spend significantly more % of its total time in the 16°C vs the Cre (-) Tx group (approx. 22% vs. 13% respectively). (n=8-11 animals per group; p=0.017; repeated measures two way ANOVA, post-hoc Bonferroni test). (b) the number of crossings between the 16°C and 22°C were also measured and do not change through the course of the study. It can therefore be determined there is unlikely to be any learning effects or avoidance behaviour (n=8-11 animals per group; p=0.571; repeated measures two way ANOVA, post-hoc Bonferroni test).

6.3.9 Microglial numbers are not attenuated after SNI with erbB3 and erbB4 gene ablation

After nerve injury, it is well known that proliferation of microglia occurs. This is attributed to NRG1 binding onto erbB receptors in microglia to initiate ERK phosphorylation. Here, the number of microglia after were quantified in the control Cre (-) Tx group and the cKO Cre (+) Tx group.

After injury, there was no significant difference in the number of microglia in the injured (ipsilateral) dorsal horn between the control Cre (-) Tx and cKO Cre (+) Tx strain (**Figure 6-6**). There was on average 29.876 ± 3.121 $IBA1^+$ cells in the DH of the Cre (-) Tx group, whilst there was on average 31.112 ± 2.001 $IBA1^+$ cells in the Cre (+) Tx group. Similarly, in the injured side of the ventral horn, there was also no difference in microglial numbers between the two groups (12.209 ± 1.034 Cre (-) Tx vs. 14.651 ± 1.001 Cre (+) Tx). Whilst there were significantly more $IBA1^+$ microglia in the injured DH vs. the uninjured DH ($P<0.0001$), there was no significant difference in numbers between genotypes in any of the areas analysed (**Figure 6-6**b) (n= 5 per group; p=0.651; student t-test).

After injury, microglial morphology is known to switch from a ramified (with many branches) morphology to an amoeboid shape (with few branches). Results in **Figure 6-6**c show that there was no significant difference in the number of branches between the Cre (-) Tx and Cre (+) Tx groups in the ipsilateral (injured) DH (3.423 ± 0.921 vs. 4.109 ± 0.671 respectively) or the contralateral (uninjured) DH (4.222 ± 0.910 vs. 4.309 ± 1.231 respectively). Interestingly, it seems that although microglial numbers increase on the injured side, most microglial cells in the spinal cord, independent of injury, display ramified morphology instead of the "activated" amoeboid morphology. This is reflected in the number of branches, where all areas of the spinal cord display

similar number of microglial branches (p= 0.823; n= 5 per group; repeated measures two way ANOVA; post-hoc Bonferroni test).

This observation does not seem to be as a result of erbB receptor ablation, as the same effect is seen in the control (Cre (-) Tx) group. It seems microglial cells maintain their "resting" ramified morphology instead of the activated "amoeboid" morphology after SNI.

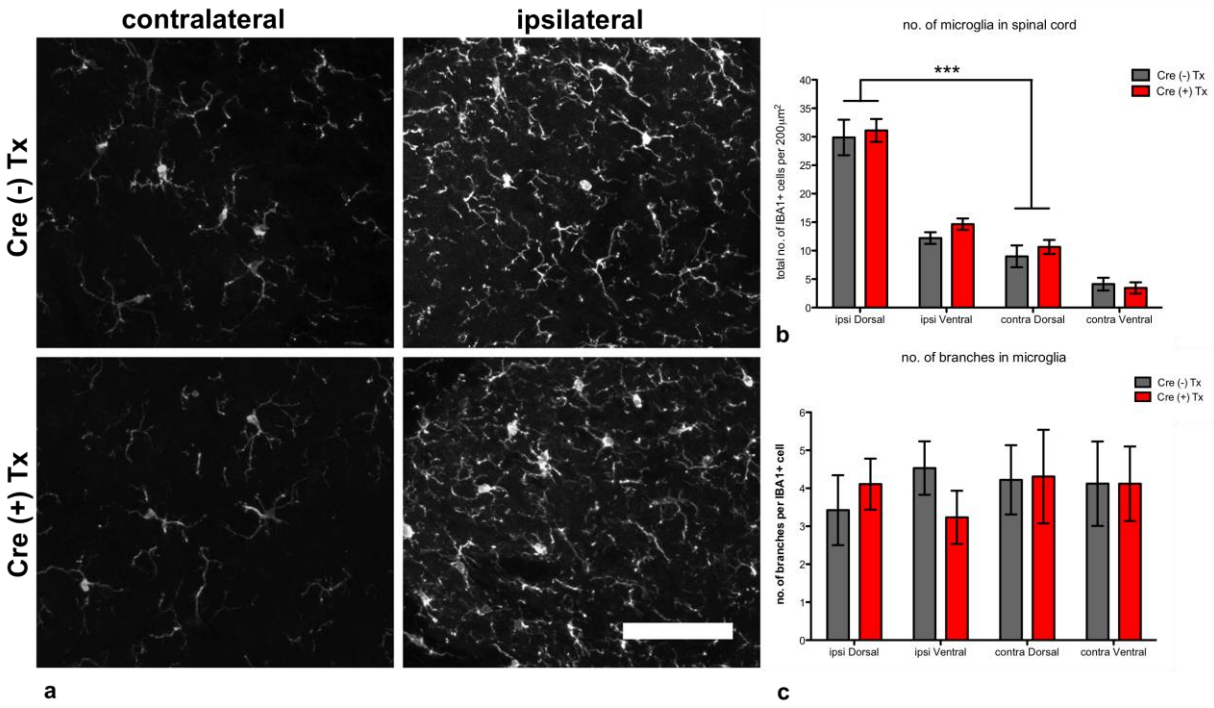


Figure 6-6 microglial numbers are not attenuated after injury with erbB3 and erbB4 receptor blockade (a) representative images of IBA+ labelled microglia in the dorsal horn of Cre (-) Tx control group and the cKO group Cre (+) Tx. There are significantly more IBA1+ labelled microglia in the injured (ipsilateral) side vs. the uninjured (contralateral) side but unexpectedly, the majority of these cells maintain a ramified morphology. (b) The number of IBA1+ cells were quantified in a defined area. Whilst there are significantly more IBA1+ labelled microglia in the ipsilateral dorsal horn vs. the contralateral horn ($p<0.0001$) there is no significant difference between the control (Cre (-) Tx) and cKO (Cre (+) Tx) microglial numbers in any area of the spinal cord ($p=0.651$). (c) Shows the quantification of the total number of branches per microglial cell. There is no significant difference in the number of branches between the control and cKO group in any of the lumbar areas of the spinal cord analysed ($p=0.823$; n= 5 per group; repeated measures two way ANOVA; post-hoc Bonferonni test).

6.3.10 Astrocyte numbers are not attenuated after SNI, with erbB3 an erbB4 ablation in microglia.

Given the delayed onset of cold allodynia attenuation with erbB3 and erbB4 ablation in microglia, astrocytes were labelled using GFAP antibody and the level of immunofluorescence after injury (30 days) was measured in the lumbar spinal cord between the Cre (-) Tx and the Cre (+) Tx groups.

The contralateral (uninjured) DH is used as control because no naive animals were included in this study. All immunofluorescence is normalised to contralateral DH. As Figure 6- demonstrates, there was a significantly higher level of GFAP IR in the ipsilateral side in the injured DH vs. the uninjured (contralateral) DH ($p<0.0001$) in both the Cre (-) Tx group and the Cre (+) Tx group. However, no increase in the intensity of GFAP immunofluorescence was observed in the injured (ipsilateral) dorsal horn of the spinal cord between the Cre (-) Tx or Cre (+) Tx group or the ipsilateral ventral horn. There is no significant difference between genotypes in all areas of the spinal cord analysed ($p>0.05$; $n= 5$ per group; repeated measures two way ANOVA). The intensity of GFAP staining did not reveal any changes between genotypes after nerve injury. Therefore it can be concluded that erbB3 and erbB4 receptor ablation in microglia does not affect astrocyte expression after nerve injury.

areas of the spinal cord analysed (p>0.05; n= 5 per group; repeated measures two way ANOVA). The intensity of GFAP staining did not reveal any changes between genotypes after nerve injury. Therefore it can be concluded that erbB3 and erbB4 receptor ablation in microglia does not affect astrocyte expression after nerve injury

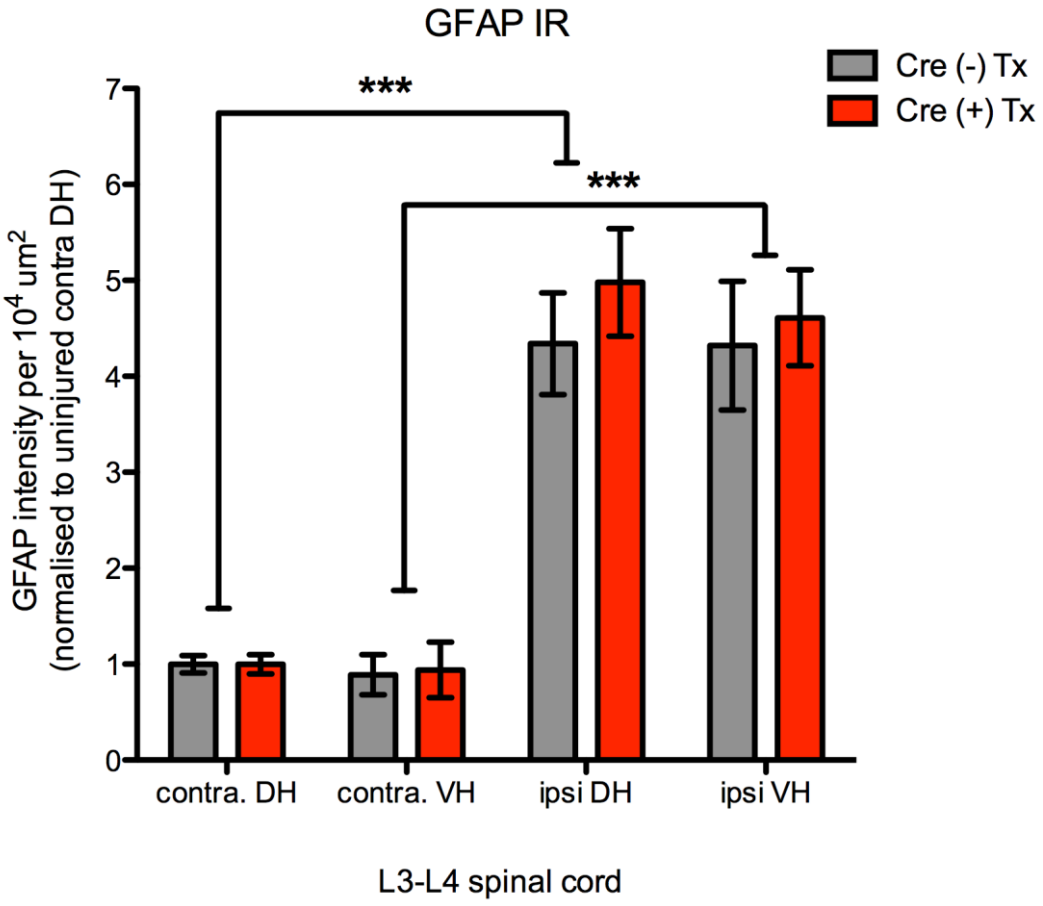


Figure 6-12 GFAP expression does not differ after SNI in the absence of erbB3 and erbB4 in microglia. The level of GFAP immunofluorescence intensity was measured in the lumbar area of the spinal cord between the Cre (-) Tx and Cre (+) Tx group at day 30 post SNI. The uninjured contralateral DH was used as control and all results were referenced to this point. Within the injured ipsilateral dorsal horn, there is no significant difference in GFAP IR between Cre (-) Tx and Cre (+) Tx group (4.25 ± 0.67 vs. 4.86 ± 0.78 respectively). Whilst there is significantly higher GFAP IR in the ipsilateral DH vs. the contralateral DH in both groups (P<0.0001), There is no significant difference in the level of GFAP IR increase between the two groups (p=0.78; n= 5 per group; repeated measures two way ANOVA; post hoc-Bonferonni test).

6.4 Discussion

6.4.1 CX3CR1 CreER^{TM} is highly specific to microglia and can be used to distinguish the differences between macrophages and microglia in neuropathic pain

The lack of tools available to specifically manipulate microglial gene expression at defined time points in a living animal had previously made it difficult to describe the precise functions of microglia in the context of neuropathic pain. Most recently, an exciting new transgenic tool (CX3CR1 CreER^{TM}) has been generated that allows the specific and inducible manipulation of microglial numbers and functions within the CNS (Parkhurst et al. 2013). In order to restrict Cre-mediated recombination exclusively to microglia, advantage was taken of the fact that microglia and other CX3CR1^{+} cells have substantially different rates of turnover and are derived from different precursor populations to other CX3CR1^{+} cells. Microglia are a self-renewing population (Ajami et al. 2007) with a low turnover rate (Lawson et al. 1992). Monocytes and inflammatory macrophages however exhibit a rapid turnover rate (van Furth & Cohn 1968) and are replenished through a bone marrow precursor population that is CX3CR1^{-} (Fogg et al. 2006). Therefore, upon exposure to tamoxifen, microglia undergo recombination that persists once tamoxifen has dissipated but peripheral CX3CR1^{+} populations initially undergo recombination and are then quickly replaced by non-recombined infiltrating cells from CX3CR1^{-} progenitors in the absence of additional tamoxifen.

By taking advantage of CX3CR1 CreER^{TM} mice Figure 6-1, Figure 6-2, and Figure 6-3 show that when crossed with the R26$^{tdTomato\ (+)}$ reporter strain, tdTomato^{+} expression is only detected in microglia in the presence of CX3CR1 Cre (+) and

induction using tamoxifen. The lack of tdTomato^{+} expression in peripheral tissue (sciatic nerve and DRG) from CX3CR1 Cre (+) tamoxifen induced animals confirms the specificity of the CX3CR1 CreER^{TM} strains. It verifies that 37 days after tamoxifen treatment and 7 days after nerve injury non-recombined infiltrating cells are derived from CX3CR1^{-} progenitors and therefore do not express tdTomato^{+} in the absence of additional tamoxifen. The same transgenic Cre system was used by Parkhurst et al. they also results demonstrate that CX3CR1 CreER^{TM} (+) mice, when used 30 days after tamoxifen administration, permit Cre-dependent manipulation of gene expression almost exclusively in microglia in the brain and not in the spleen or blood (Parkhurst et al. 2013).

6.4.2 It is unclear how cold hypersensitivity is regulated by NRG1-erbB signaling in microglia given that microglial morphology and proliferation rate are unaffected

Modulating microglial function after peripheral nerve injury offers a real novel therapeutic opportunity. By reducing the pro-inflammatory functions of microglia whilst retaining its protective mechanisms, it may prove useful in selectively attenuating microglial induced neuropathic pain. Nociceptive inputs from injured or neighbouring uninjured sensory neurons projecting to the dorsal horn are known to be crucial in generating spinal microgliosis. *In vivo* studies show that following spinal nerve ligation (SNL) the soluble form of NRG1 is released from primary afferents in the dorsal horn and activates the erbB2 receptors in microglia. Blockade of erbB2 or sequestration of endogenous NRG1 following SNL attenuates microglial proliferation in the dorsal horn with attenuation in mechanical pain-related hypersensitivity and cold allodynia (Calvo et al. 2010). Inhibition of downstream signalling pathways that

are dependent on NRG1- erbB binding on microglia also results in decreased spinal microgliosis and attenuated mechanical and cold hypersensitivity after peripheral nerve damage (Calvo et al. 2011).

To investigate the precise role of microglia in the context of NRG1-erbB signalling during neuropathic pain, erbB receptors 3 and 4 were conditionally ablated using CX3CR1 CreER^{TM}. The overall findings show that in the absence of erbB receptors 3 and 4 on microglia, NRG1-erbB signalling on microglia does not attenuate mechanical hypersensitivity. Similarly, in chapter 4 it was reported that the conditional ablation of NRG1 does not attenuate mechanical allodynia after nerve injury.

However with erbB gene ablation on microglia, it is evident that cold hypersensitivity is attenuated at the delayed stages of nerve injury. Similarly, it was reported in Chapter 4 that conditional NRG1 ablation also attenuates cold hypersensitivity at the delayed stages of injury. It can now be concluded that NRG1-erbB signalling on microglia is likely to contribute to regulating cold hypersensitivity in the context of neuropathic pain.

Whilst there is some certainty that microglia are responsible for regulating cold through NRG1-erbB binding. It is unclear how they do so. Intracellular signalling pathways such as the ERK and P13/Akt signalling pathway have all been previously described to modulate distinct aspects of the cellular response. ERK signalling is said to promote cell proliferation and PI3K/Akt is said to regulate cellular motility (Eckert et al., 2009; Maurel and Salzer, 2000; Sei et al., 2007) in response to NRG1. It is likely MEK/ERK1/2 is truly dependant on NRG1-erbB signalling, and leads to attenuation of cold hypersensitivity but whether MEK/ERK1/2 is solely responsible for microglial proliferation is unclear in this study.

It is possible that NRG1-erbB signalling does not strictly correlate to microglial proliferation. It is plausible that in the absence of erbB3 and 4 microglial receptors, downstream signalling pathways responsible for microglial proliferation (e.g. MEK/ERK1/2) are activated through alternative upstream signalling pathways, independent of NRG1-erbB signalling. For example, there is evidence stimulation of the Cannabinoid 2 receptor is capable of activating microglial cells by rapid ERK-phosphorylation but prevents over-activation in the presence of a second stimulus (Merighi et al. 2012). Elsewhere it has been shown that the production or reactive oxygen species (ROS) after spinal cord injury is capable of inducing p38MAPK and ERK activation in microglia, and mediating mechanical allodynia and thermal hyperalgesia (Choi et al. 2012).

If MEK/ERK1/2 phosphorylation is still observed in the absence of microglial erbB3 and erbB4, it could explain the microglial proliferation detected after nerve injury in the CX3CR1CreER^{TM} (+): erbB3$^{-/-}$B4$^{-/-}$ strain.

In order to confirm the locus of action, the level of MEK/ERK1/2 and p38 MAPK (and possibly Akt) phosphorylation could be analysed in the CX3CR1CreER^{TM} (+): erbB3$^{-/-}$B4$^{-/-}$ strain. It is likely that there is some dissociation between NRG1-erbB binding and activation of downstream signalling pathways responsible for microglial proliferation.

It is possible that in the absence of NRG1-erbB signalling, the p38 MAPK pathway (which is not normally implicated in microglial proliferation) compensates for the lack of MEK/ERK1/2 phosphorylation and induces microglial proliferation. Calvo et al. previously demonstrated that whilst the total level of p38 MAPK increases after SNI, the level of p38 MAPK expression is similar with or without interrupting NRG1-erbB signalling (Calvo et al. 2011). There is evidence that the use of p38 inhibitor is

capable of reducing mechanical allodynia after SNL (Jin et al. 2003) and is therefore likely to regulate mechanical allodynia and possibly microglial proliferation.

It would also be interesting to investigate microglial proliferation is seen in animals that lack erbB3 and erbB4 on microglia but are also administered with erbB2 inhibitors. If microglial proliferation is not attenuated after nerve injury in this experimental set up then it is likely that microglial proliferation is not strictly regulated by NRG1-erbB signalling. If microglial proliferation is affected in this set up it is possible that erbB2 plays an important role in microglial proliferation that is as yet undiscovered. However it is difficult to speculate as blockade of erbB2 could give rise to alterations in alternative signalling pathways also dependant on erbB2.

Another possibility is that the CX3CR1 CreER^{TM} transgenic system is measured to be approximately 87% efficient (**Figure 6-2**). It is likely that due to lack of 100% total Cre mediated recombination, the remaining levels of erbB3 and 4 receptors are sufficient to activate MEK/ERK signalling and thus microglial proliferation but insufficient to generate cold allodynia.

6.4.3 Technical limitations

When conducting this study, a few technical issues arose that merit discussion here. When designing behavioural experimental studies, due to limitations in the number of animals with the correct genotype available, the Vehicle Control group could not be included. This group can be used to show that the vehicle (corn oil) does not itself cause any behavioural or histological changes after treatment and after injury. Additionally, for the same reason above, a sham (uninjured) group could not be included either. Whilst the uninjured paw was used for comparison, interpretation of data to fully investigate the role of NRG1-erbB signalling after injury- the optimal

control in behavioural and histological experiments, is preferably a naïve (uninjured) group.

When measuring gene expression changes upon Tamoxifen induced Cre activation, it is ideal to administer Tamoxifen to adult mice and then extract microglia from adult cortex and/or spinal cord. This can allow direct comparisons to be made and the true level of gene expression knockdown to be calculated. Presently, microglial cell lines or primary microglia are isolated from embryonic (Gingras et al. 2007) or neo-natal (Giulian & Baker 1986; Floden & Combs 2007) animals. These are not perfect models as embryonic or neo-natal microglia are not completely mature (Draheim et al. 1999) and behave differently to adult microglia (Sierra et al. 2007). However, extracting microglia from adult brains is notoriously challenging. Some studies have described culture of microglia prepared from adult animals (Sedgwick et al. 1991; Moussaud & Draheim 2010). Such protocols were initially used but failed to generate efficient, confluent, and healthy "looking" cultures. Often, the mixed glial cultures (from which microglia are normally harvested) failed to reach confluence and produced cellular and myelin debris despite taking steps to remove these. This in turn prevented a feeder layer to form. A feeder layer is required to promote microglial survival after the initial stressful isolation procedure and help sustain in vitro microglial proliferation through trophic support (Alliot et al. 1991). Thus, the traditional neo-natal cortex model of microglia culture was used to measure gene expression changes in vitro. Precautions should be taken when interpreting such data.

6.5 Considerations

The CX3CR1 Cre system is highly efficient in selectively ablating genes of interest. The pain behaviour study results presented in this chapter are consistent with those presented in Chapter 4. Upon erbB3 and erbB4 deletion in microglia, behavioural

studies demonstrate the NRG1- erbB signalling is important in specifically regulating cold pain behaviour in the delayed stages of nerve injury and neuropathic pain. It seems NRG1- erbB signalling in microglia may not regulate mechanical allodynia during neuropathic pain. Work in this chapter also highlights that microglial proliferation develops independently of NRG1- erbB signalling and further work is required to dissect the microglial proliferation signalling mechanisms.

7 General Discussion

7.1 Summary of findings

The aim of this thesis was to further investigate the role of NRG1 in regulating microglial induced neuropathic pain after nerve damage. Investigations of Neuregulin gene expression after nerve injury induced neuropathic pain show that NRG1 isoforms are significantly upregulated in the PNS spinal cord after nerve injury. Specifically the type I NRG1 isoform which signals in a paracrine manner showed increased expression in the injured nerve and DRG. I also noted increased NRG1 expression in the spinal cord following nerve injury although I could not determine the exact subtype. Conditional ablation of NRG1 in adulthood did not attenuate mechanical hypersensitivity or microgliosis. With the design and optimisation of a cold pain behaviour assay (called the Place Preference Test) it was determined that conditional NRG1 ablation reduced cold hypersensitivity in the later stages following nerve injury. To confirm NRG1 induced microglial modulation of cold-hypersensitivity, erbB3 and erbB4 receptors were conditionally ablated using a microglial specific Cre Lox P system. In our hands, the CX3CR1 Cre system performed much more efficiently than the Cd11b Cre system to selectively ablate erbB3 and erbB4 receptors on monocytes. Post-injury behavioural studies showed that erbB receptor blockade had no effect on mechanical hypersensitivity and microgliosis whilst cold hypersensitivity was reduced in the later stages following nerve injury.

7.2 NRG1- erbB signaling in microglia modulates cold but not mechanical hypersensitivity following nerve injury

The studies conducted in this thesis suggest that the NRG1- erbB signalling pathway plays quite a selective role in the behavioural consequences of nerve injury. The NRG1 and erbB receptor tissue specific conditional ablation studies display a behavioural phenotype that is very similar. Both studies confirm that the NRG1- erbB signalling does not regulate thermal hypersensitivity. Both studies demonstrate that NRG1-erbB signalling does not regulate mechanical hypersensitivity and both studies demonstrate that cold hypersensitivity is attenuated only at 28 days after spared nerve injury. These findings, somewhat, contradict previous studies.

Previous pharmacological studies conducted in our lab (Calvo et al. 2010; Calvo et al. 2011) demonstrated that intrathecal treatment of NRG1 in naive animals induced microglial proliferation, adoption of an effector morphology and development of cold and mechanical pain-related hypersensitivity. Using the SNT model in rat, erbB receptor blockade resulted in a significant reduction in mechanical hypersensitivity but attenuation of cold allodynia after nerve injury (Calvo et al. 2010; Calvo et al. 2011). Elsewhere, Xiang et al also use a pharmacological approach to demonstrate that NRG1-erbB signaling mediates incision-induced microglial activation and mechanical allodynia. Pharmacological blockade of erbB receptors using PD168393 leads to down-regulation of NRG1 siRNA; decreased erbB2 phosphorylation; decreased p38 phosphorylation; decreased OX42 labeling and alleviation of microglial induced mechanical allodynia after nerve injury (Xiang et al. 2014). Xiang et al. did not investigate cold pain behaviour in their model of nerve injury.

These findings contradict those that are presented in this thesis. This is likely to be

explained by the approaches taken in each study- the different species selected; the different models of nerve injury selected; and the different means of interfering with NRG1-erbB signaling (transgenic approach vs. pharmacological approach). It is well known that there are variations in rodent sciatic nerve anatomy between rat and mice (Rigaud et al. 2008) which is likely to result in some variations in the processing of nociceptive information between rat and mice. It is also worth noting that genetic variability in microglial responses to nerve injury have been observed between various inbred mouse strains *(Suleman et al. 2008, IASP)* (Beggs et al. 2012). It is also well known that the extent of glial activation depends on the axotomy model selected (Jergová & Cízková 2007). Finally, the possibilities of variation between using pharmacological tools and transgenic tools can be significant- given that kinase inhibitors are known to have poor selectivity (Arteaga 2003).

The selectivity of the impact on cold hypersensitivity, presented in our studies, was interesting. A similar example is the role of the C5a receptor in the complement pathway in microglia that has been reported to modulate cold but not mechanical hypersensitivity (Griffin et al. 2007a). Cold hypersensitivity is a common sequelae of nerve injury in humans and is particularly common after certain types of chemotherapy such as Oxaliplatin (Attal et al. 2009; Descoeur et al. 2011). These results would suggest that there are distinct pathways at spinal level mediating cold versus mechanical hypersensitivity following nerve injury. For example Seal et al. demonstrate that a loss of vesicular glutamate transporter 3 ($VGLUT3^{+}$) in primary afferents specifically impairs mechanical pain sensation (in particular the mechanical hypersensitivity to normally innocuous stimuli that accompanies inflammation, nerve injury and trauma) but does not affect normal heat or cold sensation (Seal et al. 2009).

Initially, the hypothesis was that the NRG1 is released by damaged afferents and promotes microglial proliferation to further promote a pro-inflammatory phenotype (and cytokine release) that is responsible for the development of neuropathic pain (as shown by (Calvo et al. 2010; Calvo et al. 2011)). Xiang et al. also present evidence for NRG1 to regulate microglial proliferation (Xiang et al. 2014). However, in our (mouse) model, there is no evidence to suggest that NRG1-erbB signalling modulates microglial proliferation. It is unlikely that there is a simple correlation between a factor altering microglial function to promote pain and that factor then directly regulating microglial numbers and morphology. For example it is known that an increase in P2X4 receptor expression is correlated temporarily with the development of tactile allodynia but it does not regulate expression of microglial proliferation protein markers complement receptor 3 (Tsuda et al. 2003) or IBA1 (Ulmann et al. 2008). Future experiments are required, where experiments conducted in the rat, are replicated in the mouse to determine what aspects of microglial function are modulated by NRG1 (e.g. analysis of microglial proliferation and cytokine release).

7.3 Future Work

Whilst this thesis has provided some useful insights into the behavioural changes associated with NRG1- erbB signalling within microglia in the context of neuropathic pain, many questions remain unanswered. There is evidence for erbB expression in DRG neurons to suggest that the function of at least some nociceptive, proprioceptive and mechanosensitive neurons could be modified by NRG1 (Pearson & Carroll 2004). Most studies have focussed on the idea that NRG1 is expressed by DRG cells and then binds to receptors expressed on glia (Schwann cells and microglia) however potential autocrine effects have been neglected. There are significant changes in

NRG1 gene expression and erbB receptor gene expression within the peripheral nervous system and spinal cord following nerve injury. However there is still much to learn about the changes in NRG1 expression and release after nerve injury. In particular I found that using primers designed to detect all NRG1 isoforms increased expression within the spinal cord following nerve injury. The expression of type I, II or III isoforms did not appear to change however. There are further, recently described, isoforms (IV, V and V1) and future experiments are required to determine whether these isoforms are dysregulated by injury. A further extension would be to look at the protein level.

It is known that there are discrepancies when comparing mRNA expression and protein expression where relative quantification of protein expression is more accurate than measuring mRNA expression ratios (Griffin 2002). Furthermore NRG1 undergoes proteolytic cleavage so that certain isoforms (especially type II) are released from the cell membrane. Therefore, it would be interesting to measure the levels of active NRG1 in mouse CSF. This is likely to be challenging and may require the use of optimised ELISA or a bioassay similar to the one used by Esper et al. to measure NRG1 release from cultured neurons (Esper & Loeb 2004). Using such a bioassay, Esper at al. were able to elucidate the mechanism of this localised, regulated NRG1 release by implicating the delta isoform of protein kinase C (PKC) in the PNS (Esper & Loeb 2009). A similar bioassay could be used to detect NRG1 expression and erbB receptor phosphorylation changes in mouse CSF during neuropathic pain. Current antibodies used to detect NRG1 or erbB protein expression were unable to clearly label the spinal cord. Using techniques that effectively measure protein changes, further information could be generated on the distribution of NRG1 after

injury within the spinal cord and it may identify a predominant NRG1 subtype that is responsible for activating microglial induced pain behaviours.

The work in this thesis demonstrates that conditional NRG1 ablation is capable of alleviating cold hypersensitivity in the delayed stages of neuropathic pain. A similar effect is measured with conditional ablation of the erbB3 and erbB4 receptors. Further investigation on the mechanisms that lead to cold hypersensitivity in the first few weeks is required. It is likely that alternative ligands are capable of activating the MEK/ERK1/2 signalling pathway or other independent pathways in microglia to regulate cold allodynia. For example, Talbot et al. demonstrate that the microglial kinin receptor 1 is responsible for regulating cold allodynia in diabetic pain neuropathy (Talbot et al. 2010b). Additionally, it is necessary to investigate the role of C5a after nerve injury given that the C5a receptor in the complement pathway in microglia has been reported to modulate cold but not mechanical hypersensitivity (Griffin et al. 2007a).

Within our studies, it is also unclear to as what role microglia are immediately playing in response to NRG1- erbB signalling blockade in the first few weeks after injury. When NRG1 or its receptors are conditionally ablated, microglial proliferation, identified by IBA1 labelling, develops and accumulates as normal within the damaged area of the spinal cord with development of mechanical, thermal and cold hypersensitivity. If NRG1- erbB signalling is truly responsible for microglial proliferation (Calvo et al. 2010; Calvo et al. 2011; Xiang et al. 2014), then it is unclear to as how microglial proliferation persists in our injury model in the absence of NRG1 or its ligand bind receptors erbB3 and erbB4.

Additionally, findings in this thesis call into question whether NRG1- erbB signalling truly regulates mechanical hypersensitivity. There is evidence to suggest that mechanical hypersensitivity is regulated via the P2XR4 signalling pathway on microglia but does not impact cold allodynia (Makoto Tsuda et al. 2009). By blocking both the NRG1 signalling pathway and the P2XR4 signalling pathway, it is hypothesised that both mechanical and cold hypersensitivity are attenuated after nerve injury. To test this, the microglial specific CX3CR1 Cre system (Goldmann et al. 2013) could be used to selectively ablate the erbB3/4 and P2XR4 receptors so that pain behaviour and microgliosis could be further investigated.

To further investigate microglial proliferation, *in vitro* culture of microglial cells extracted from conditional erbB3$^{-/-}$B4$^{-/-}$ mice could be tested. Application of NRG1 *in vitro* will allow direct measurement and quantification of microglial proliferation and chemotaxis.

Further work is also required in analysing the various internal signalling pathways within microglia – including the MEK/ERK1/2, p38 MAPK and cJun pathways. If MEK/ERK1 signalling pathways are activated within microglia in the conditional NRG1$^{-/-}$ or conditional erbB3$^{-/-}$B4$^{-/-}$ mice then it is likely that microglial proliferation is indeed dependant on MEK/ERK1/2 but can be activated independently of NRG1-erbB signalling. There is recent evidence to suggest that in the CNS, LPS is capable of inducing microglial activation and proliferation in certain brain specific regions which can then be suppressed using VEGFR associated tyrosine kinase inhibitors (Fukushima et al. 2015). It is then plausible that in the absence of NRG1 or erbB receptors, the Toll Like Receptors found on microglia or the VEGF receptor's are activated by the release of injury signals into the spinal cord to initiate microglial proliferation. Alternatively, it is plausible that in the absence of NRG1 or its receptors

other signalling pathways such as the p38 MAPK or cJun pathways may compensate and initiate microglial proliferation. Understanding the downstream signalling pathways is key. Various experiments could be designed using a combination of pharmacological targets and transgenic tools to study the above. By doing so, we may be one step closer to understanding the true role of NRG1 in microglial induced neuropathic pain and ultimately we may be able to identify novel therapeutic targets.

7.4 The complexities of using transgenic systems to understand signaling mechanisms

The use of pharmacological inhibitors in modulating cell signaling can present a powerful tool and an attractive means to dissect the regulatory features of intracellular signaling pathways. However, the effective inhibition of a signaling pathway in a given cell type is dependent in part on the permeability of the inhibitor; the relative stoichiometry of inhibitor; the intended target kinase; any competing target enzymes; and the relative potencies of the inhibitor for all potential enzyme targets (Penn et al. 1999). The previous use of pharmacological inhibitors to investigate NRG1-erbB signalling in microglia in neuropathic pain gave rise to certain limitations. The lack of specificity to erbB2 receptors raised some uncertainty about the role of NRG1 in neuropathic pain (Calvo et al. 2010; Calvo et al. 2011). Therefore the use of transgenic systems to modulate cell signaling can also present a powerful means to target specific cells, receptors and ligands to dissect the regulatory features of intracellular signaling pathways.

It is worth highlighting that the use of transgenic systems in this thesis gave rise to complications and similar precaution should be taken when using transgenic systems. The Cre-*loxP* site-specific recombination system is widely used for production of tissue-specific and conditional knockout alleles in mice. A reporter mouse model can

be used as a powerful tool to monitor transcriptional activity of a promoter *in vivo* as well as monitoring protein localisation or cell trafficking. When using such tools it transpired that a reporter strain ($Rosa24^{YFP}$) used in this thesis was in fact highly inefficient at measuring Cre recombinase activity (for further information, please refer to chapter 5). Initially it was unclear whether the Cre system was inadequate or whether the reporter system was ineffective in measuring transcriptional activity. Further positive control testing confirmed that the reporter system was incapable of effectively measuring Cre recombinase activity. The use of an alternative reporter system also uncovered some unexpected findings. Initially the Cd11b Cre system was used to target microglial specific gene ablation. However, when a highly efficient reporter system ($ROSA24^{tdtomato}$) was used to measure Cd11b Cre transcriptional activity it became apparent that the Cd11b Cre system is unable to effectively target the genes of interest. Whilst previous studies have used Cd11b Cre to demonstrate a high degree of recombination of the gene of interest (Ferron & Vacher 2005; Boillée et al. 2006; Ferrini et al. 2013; Trang et al. 2011), in our case, it is possible that when the original investigator donated the original strain, there was some form of mutation within the colony that impacted the Cre activity. This lack of transcriptional activity explained why initial mRNA expression studies failed to show effective reduction of erbB3 and erbB4 expression in the presence of Cd11b Cre. These problems all highlight the need for scrutiny of the basic transgenic tools being used to avoid misinterpretation and mis-reporting of effects caused by genetic manipulation.

7.5 Are microglia involved in human chronic pain?

The use of animal models has previously demonstrated that microglia are involved in contributing to neuropathic pain but what evidence is there that these cells also contribute to this disease in humans? Banati et al. used a PET scan in humans to

illustrate the presence of microgliosis in the thalamus of amputee patients suffering from chronic phantom limb pain (Banati et al. 2001).

Some observations suggest that activated glia may contribute to the pathophysiology of chronic pain and support a role for glia in chronic pain in humans. Del Valle et al. conducted a post mortem study of a human suffering from chronic regional pain syndrome to illustrate microglial and astroctytic activation in the spinal cord (Del Valle et al. 2009). Glial mediated pathophysiology was also detected in HIV-related neuropathic pain (Shi et al. 2012), and in CSF sampling of patients with fibromyalgia and chronic low back pain (LBP) (Brisby et al. 1999; Kadetoff et al. 2012). Recently, glial activation was assessed using brain levels of TSPO, formerly called peripheral benzodiazepine receptor. TSPO expression is widely acknowledged as a marker of glial activation in CNS injury and disease (in humans and animals). Using TSPO, Loggia et al. present evidence for significant glial activation in the thalamus of chronic LPB patients (Loggia et al. 2015). Neurodegenerative diseases such as Alzheimer' s Disease, Parkinson Disease, HIV-dementia, amyotrophic lateral sclerosis, multiple sclerosis, Guillain-Barre Syndrome, stroke and spinal cord injury all present some form of neuroinflammation (Rivest 2009; Loggia et al. 2015). There is sufficient evidence to suggest that microglia are involved in the genesis of the neurodegenerative process of some of these aforementioned diseases (Block et al. 2007; Glass et al. 2010; Rivest 2009) .When serum and CSF is extracted from these patients, there is evidence of elevated levels of innate immune system molecules, such as IL-6, IL-1β, TNFα and CCL2 (Forlenza et al. 2009; Galimberti et al. 2008; Wilms et al. 2003; Allan et al. 2005; Nguyen et al. 2002). These cytokines are known to be secreted by CNS glia (microglia and astrocytes) and are known to be potent inducers of neuroinflammation and subsequent neurodegeneration (Block et al. 2007). With

these findings it seems that microglia present a common feature in humans and rodents- they are responsible for neuroinflammation that can lead to neuropathic pain.

It is worth noting that recent animal behaviour studies suggest that immune cell functional differences exist in relation to pain processing. In 2011, Sorge et al. presented evidence that the spinal cord TLR-4 mediates inflammatory and mechanical hypersensitivity in male but not female mice (Sorge et al. 2011). More recently, Sorge et al demonstrated that mechanical allodynia after nerve injury is reversed by microglial inhibition in male but not female mice where mechanical allodynia in female mice is instead mediated by adaptive immune cells (i.e. T Lymphocytes) (Sorge et al. 2015). This gives rise to the possibility that in humans, microglia may present immune cell specific gender differences in the way that immune cell mediated pain processing. It is also possible that differences in pain processing via immune cells do not translate to how neurons function downstream. Therefore, caution should be taken against extrapolating these findings to humans and even to rats. Further work is required to determine if such differences exist in humans, and if so, microglial therapeutic targets may have to be designed to be understand the variations in immune cell mediated pain processing in males versus females.

Finally, the role of microglia can also vary between types of neuropathic pain. For example, microglia hypertrophy and increased microglial proliferation are pronounced in the nerve transection and CCI animal models but there is no microglia hypertrophy or proliferation in the animals treated with paclitaxel, vincristine, oxaliplatin, or 2′,3′-dideoxycytidine (ddC) (Zheng et al. 2011). The response of spinal microglia to chemotherapy-evoked painful peripheral neuropathies is likely to be

distinct from that evoked by traumatic nerve injuries and therefore therapeutic targets need to specifically target traumatic nerve injury in humans.

7.6 Can microglial neurotoxic functions be modulated without disturbing neuroprotective functions?

It is known that microglia can be neurotoxic but in other contexts also neuroprotective. Whilst the neurotoxic roles of microglia after nerve injury have been well described (McMahon & Malcangio 2009; Scholz & Woolf 2007a), the neuroprotective roles of microglia, after nerve injury, have not been fully understood. Microglia may promote healing in the CNS by clearing cellular debris during neuronal death and synaptic elimination (Hickman et al. 2008; Neumann et al. 2009). Microglia may promote neuroprotection through; the release of neurotrophins that regulate normal function and development of CNS immune cells and through the release of anti-inflammatory cytokines (IL-10 and TGF-β1) (Town et al. 2005; Echeverry et al. 2008; Chabot et al. 1999; Hanisch & Kettenmann 2007). The aim of developing therapeutic targets to modulate microglia function would be to inhibit neuropathic pain without impacting the key neuroprotective functions of microglia. As yet, it is unclear whether NRG1 promotes neuroprotection in the CNS after nerve injury. Further work is required to investigate the role, (if any) which the NRG1-erbB signaling pathways could play in regulating neuroprotective functions of microglia.

An ideal therapeutic approach would be to selectively target the subpopulation of microglia that are responsible for the pro-inflammatory response without affecting the subpopulation of microglia that are responsible for the anti-inflammatory response to nerve injury. Alternatively therapeutic targets could be aimed to convert pro-inflammatory responses to anti-inflammatory responses. Ultimately, the aim of microglial therapeutic targets is to prevent neuropathic pain development by reducing

the overall inflammatory reaction elicited by microglia, or to inhibit microglial functions that generate toxic neuroinflammation.

7.7 Conclusion

Microglia are key immune cells within the central nervous system. Microglia are very sensitive to disturbances in the extracellular environment, they play an important pathogenic role in disease progression, including the response to trauma, neuroinflammation and pain. Work in this thesis highlights the complexities in using transgenic systems; animal models of neuropathic pain and; measuring behavioural changes or thresholds in animals. My findings highlight the contribution of NRG1-erbB signalling in microglia to cold hypersensitivity but not mechanical hypersensitivity or microgliosis. Dissecting the complex and multiple mechanisms of neuropathic pain will hopefully lead to the development of novel analgesics targeted to the right groups of patients for the treatment neuropathic pain.

REFERENCES

Abbadie, C. et al., 2003. Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. *Proceedings of the National Academy of Sciences of the United States of America*, 100(13), pp.7947–52. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=164693&tool=pmcentrez&rendertype=abstract [Accessed May 2, 2015].

Abbadie, C., Honoré, P. & Besson, J.M., 1994. Intense cold noxious stimulation of the rat hindpaw induces c-fos expression in lumbar spinal cord neurons. *Neuroscience*, 59(2), pp.457–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8008200 [Accessed June 9, 2015].

Afrah, A.W. et al., 2002. Spinal substance P release in vivo during the induction of long-term potentiation in dorsal horn neurons. *Pain*, 96(1-2), pp.49–55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11932060 [Accessed March 24, 2015].

Ajami, B. et al., 2007. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nature neuroscience*, 10(12), pp.1538–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18026097 [Accessed April 7, 2015].

Akira, S., Uematsu, S. & Takeuchi, O., 2006. Pathogen recognition and innate immunity. *Cell*, 124(4), pp.783–801. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16497588 [Accessed July 9, 2014].

Allan, S.M., Tyrrell, P.J. & Rothwell, N.J., 2005. Interleukin-1 and neuronal injury. *Nature reviews. Immunology*, 5(8), pp.629–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16034365 [Accessed September 20, 2015].

Alliot, F. et al., 1991. Microglial progenitors with a high proliferative potential in the embryonic and adult mouse brain. *Proceedings of the National Academy of Sciences of the United States of America*, 88(4), pp.1541–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=51055&tool=pmcentrez&rendertype=abstract [Accessed June 30, 2015].

Anseloni, V.C.Z. & Gold, M.S., 2008. Inflammation-induced shift in the valence of spinal GABA-A receptor-mediated modulation of nociception in the adult rat. *The journal of pain : official journal of the American Pain Society*, 9(8), pp.732–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2581496&tool=pmcentrez&rendertype=abstract [Accessed March 24, 2015].

Anton, E.S. et al., 1997. Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. *Development (Cambridge, England)*, 124(18), pp.3501–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9342043 [Accessed May 6, 2015].

Argoff, C.E. et al., 2006. Diabetic peripheral neuropathic pain: clinical and quality-of-life issues. *Mayo Clinic proceedings*, 81(4 Suppl), pp.S3–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16608048 [Accessed March 16, 2015].

Arteaga, C.L., 2003. Inhibiting tyrosine kinases: successes and limitations. *Cancer biology & therapy*, 2(4 Suppl 1), pp.S79–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14508084 [Accessed September 27, 2015].

Asensio, V.C. & Campbell, I.L., 1999. Chemokines in the CNS: plurifunctional mediators in diverse states. *Trends in Neurosciences*, 22(11), pp.504–512. Available at: http://www.sciencedirect.com/science/article/pii/S0166223699014538 [Accessed April 15, 2015].

Attal, N. et al., 2010. EFNS guidelines on the pharmacological treatment of neuropathic pain: 2010 revision. *European journal of neurology : the official journal of the European Federation of Neurological Societies*, 17(9), pp.1113–e88. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20402746 [Accessed June 4, 2015].

Attal, N. et al., 2009. Thermal hyperalgesia as a marker of oxaliplatin neurotoxicity: a prospective quantified sensory assessment study. *Pain*, 144(3), pp.245–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19457614 [Accessed September 27, 2015].

Badie, B. et al., 2000. Interferon-gamma induces apoptosis and augments the expression of Fas and Fas ligand by microglia in vitro. *Experimental neurology*, 162(2), pp.290–6. Available at: http://www.sciencedirect.com/science/article/pii/S0014488699973454 [Accessed April 15, 2015].

Balayssac, D. et al., 2014. Assessment of thermal sensitivity in rats using the thermal place preference test: description and application in the study of oxaliplatin-induced acute thermal hypersensitivity and inflammatory pain models. *Behavioural pharmacology*, 25(2), pp.99–111. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24525711 [Accessed January 12, 2015].

Bali, K.K. et al., 2014. Sources of individual variability: miRNAs that predispose to neuropathic pain identified using genome-wide sequencing. *Molecular pain*, 10, p.22. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4113183&tool=pmcentrez&rendertype=abstract [Accessed March 17, 2015].

Banati, R.B. et al., 2001. Long-term trans-synaptic glial responses in the human thalamus after peripheral nerve injury. *Neuroreport*, 12(16), pp.3439–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11733686 [Accessed September 20, 2015].

Bandell, M. et al., 2004. Noxious Cold Ion Channel TRPA1 Is Activated by Pungent Compounds and Bradykinin. *Neuron*, 41(6), pp.849–857. Available at: http://www.sciencedirect.com/science/article/pii/S0896627304001503 [Accessed July 12, 2015].

Bao, J. et al., 2003. Back signaling by the Nrg-1 intracellular domain. *The Journal of cell biology*, 161(6), pp.1133–41. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2172983&tool=pmcentrez&rendertype=abstract [Accessed May 4, 2015].

Bär, M., Bär, D. & Lehmann, B., 2009. Selection and validation of candidate housekeeping genes for studies of human keratinocytes--review and recommendations. *The Journal of investigative dermatology*, 129(3), pp.535–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19209154 [Accessed July 27, 2015].

Barros, C.S. et al., 2009. Impaired maturation of dendritic spines without disorganization of cortical cell layers in mice lacking NRG1/ErbB signaling in the central nervous system. *Proceedings of the National Academy of Sciences of the United States of America*, 106(11), pp.4507–12. Available at: http://www.pnas.org/content/early/2009/02/23/0900355106 [Accessed May 6, 2015].

Le Bars, D., Gozariu, M. & Cadden, S.W., 2001. Animal models of nociception. *Pharmacological reviews*, 53(4), pp.597–652. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11734620 [Accessed March 9, 2015].

Basbaum, A.I. et al., 2009. Cellular and molecular mechanisms of pain. *Cell*, 139(2), pp.267–84. Available at: http://www.sciencedirect.com/science/article/pii/S0092867409012434 [Accessed July 9, 2014].

Bautista, D.M. et al., 2005. Pungent products from garlic activate the sensory ion channel TRPA1. *Proceedings of the National Academy of Sciences*, 102(34), pp.12248–12252. Available at: http://www.pnas.org/content/102/34/12248 [Accessed June 25, 2015].

Beattie, M.S. et al., 2002. ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. *Neuron*, 36(3), pp.375–86. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2681189&tool=pmcentrez&rendertype=abstract [Accessed November 12, 2014].

Beers, D.R. et al., 2006. Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. *Proceedings of the National Academy of Sciences of the United States of America*, 103(43), pp.16021–6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1613228&tool=pmcentrez&rendertype=abstract [Accessed March 30, 2015].

Beggs, S. & Salter, M.W., 2010. Microglia-neuronal signalling in neuropathic pain hypersensitivity 2.0. *Current opinion in neurobiology*, 20(4), pp.474–80.

Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3589562&tool=pmcentrez&rendertype=abstract [Accessed September 8, 2015].

Beggs, S. & Salter, M.W., 2007. Stereological and somatotopic analysis of the spinal microglial response to peripheral nerve injury. *Brain, behavior, and immunity*, 21(5), pp.624–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17267172 [Accessed April 7, 2015].

Beggs, S., Trang, T. & Salter, M.W., 2012. P2X4R+ microglia drive neuropathic pain. *Nature neuroscience*, 15(8), pp.1068–73. Available at: http://www.nature.com/neuro/journal/v15/n8/abs/nn.3155.html#close [Accessed November 12, 2014].

Beller, D.I., Springer, T.A. & Schreiber, R.D., 1982. Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. *The Journal of experimental medicine*, 156(4), pp.1000–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2186827&tool=pmcentrez&rendertype=abstract [Accessed January 19, 2015].

Belteki, G. et al., 2005. Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. *Nucleic acids research*, 33(5), p.e51. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1069131&tool=pmcentrez&rendertype=abstract [Accessed January 20, 2015].

Bennett, G.J. & Xie, Y.K., 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain*, 33(1), pp.87–107. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2837713 [Accessed December 31, 2014].

Bermingham-McDonogh, O. et al., 1997. Neuregulin expression in PNS neurons: isoforms and regulation by target interactions. *Molecular and cellular neurosciences*, 10(3-4), pp.184–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9532580 [Accessed January 6, 2015].

Bian, D. et al., 1998. Tactile allodynia, but not thermal hyperalgesia, of the hindlimbs is blocked by spinal transection in rats with nerve injury. *Neuroscience letters*, 241(2-3), pp.79–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9507925 [Accessed March 24, 2015].

Biber, K. et al., 2011. Neuronal CCL21 up-regulates microglia P2X4 expression and initiates neuropathic pain development. *The EMBO journal*, 30(9), pp.1864–73. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3101996&tool=pmcentrez&rendertype=abstract [Accessed May 18, 2015].

Birchmeier, C., 2009. ErbB receptors and the development of the nervous system. *Experimental cell research*, 315(4), pp.611–8. Available at:

http://www.sciencedirect.com/science/article/pii/S0014482708004461 [Accessed May 7, 2014].

Block, M.L., Zecca, L. & Hong, J.-S., 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nature reviews. Neuroscience*, 8(1), pp.57–69. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17180163 [Accessed July 10, 2014].

Boillée, S. et al., 2006. Onset and progression in inherited ALS determined by motor neurons and microglia. *Science (New York, N.Y.)*, 312(5778), pp.1389–92. Available at: http://www.sciencemag.org/content/312/5778/1389.long [Accessed April 29, 2014].

Bouhassira, D. et al., 2004. Development and validation of the Neuropathic Pain Symptom Inventory. *Pain*, 108(3), pp.248–57. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15030944 [Accessed July 13, 2015].

Bouhassira, D. et al., 2008. Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain*, 136(3), pp.380–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17888574 [Accessed January 11, 2015].

Bourquin, A.-F. et al., 2006. Assessment and analysis of mechanical allodynia-like behavior induced by spared nerve injury (SNI) in the mouse. *Pain*, 122(1-2), pp.14.e1–14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16542774 [Accessed March 7, 2015].

Brenner, D.S., Golden, J.P. & Gereau, R.W., 2012. A novel behavioral assay for measuring cold sensation in mice. M. Sakakibara, ed. *PloS one*, 7(6), p.e39765. Available at: http://dx.plos.org/10.1371/journal.pone.0039765 [Accessed December 11, 2014].

Brinkmann, B.G. et al., 2008. Neuregulin-1/ErbB signaling serves distinct functions in myelination of the peripheral and central nervous system. *Neuron*, 59(4), pp.581–95. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2628490&tool=pmcentrez&rendertype=abstract [Accessed October 31, 2013].

Brisby, H. et al., 1999. Markers of nerve tissue injury in the cerebrospinal fluid in patients with lumbar disc herniation and sciatica. *Spine*, 24(8), pp.742–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10222523 [Accessed September 27, 2015].

Brown, G.C. & Neher, J.J., 2012. Eaten alive! Cell death by primary phagocytosis: "phagoptosis". *Trends in biochemical sciences*, 37(8), pp.325–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22682109 [Accessed February 10, 2015].

Brown, G.C. & Neher, J.J., 2014. Microglial phagocytosis of live neurons. *Nature reviews. Neuroscience*, 15(4), pp.209–16. Available at: http://dx.doi.org/10.1038/nrn3710 [Accessed July 11, 2014].

Brück, W., 1997. The role of macrophages in Wallerian degeneration. *Brain pathology (Zurich, Switzerland)*, 7(2), pp.741–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9161725 [Accessed March 27, 2015].

Buonanno, A. & Fischbach, G.D., 2001. Neuregulin and ErbB receptor signaling pathways in the nervous system. *Current Opinion in Neurobiology*, 11(3), pp.287–296. Available at: http://www.sciencedirect.com/science/article/pii/S0959438800002105 [Accessed September 4, 2014].

Burgess, A.W. et al., 2003. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. *Molecular cell*, 12(3), pp.541–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14527402 [Accessed June 5, 2014].

Burgess, S.E. et al., 2002. Time-dependent descending facilitation from the rostral ventromedial medulla maintains, but does not initiate, neuropathic pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 22(12), pp.5129–36. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12077208 [Accessed March 24, 2015].

Butovsky, O., Hauben, E. & Schwartz, M., 2001. Morphological aspects of spinal cord autoimmune neuroprotection: colocalization of T cells with B7--2 (CD86) and prevention of cyst formation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 15(6), pp.1065–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11292669 [Accessed August 31, 2015].

Calvo, M. et al., 2011. Following nerve injury neuregulin-1 drives microglial proliferation and neuropathic pain via the MEK/ERK pathway. *Glia*, 59(4), pp.554–68. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3222694&tool=pmcentrez&rendertype=abstract [Accessed October 29, 2013].

Calvo, M. et al., 2010. Neuregulin-ErbB signaling promotes microglial proliferation and chemotaxis contributing to microgliosis and pain after peripheral nerve injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 30(15), pp.5437–50. Available at: http://www.jneurosci.org/content/30/15/5437.long [Accessed June 10, 2014].

Calvo, M. & Bennett, D.L.H., 2012. The mechanisms of microgliosis and pain following peripheral nerve injury. *Experimental neurology*, 234(2), pp.271–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21893056 [Accessed April 7, 2015].

Calvo, M., Dawes, J.M. & Bennett, D.L.H., 2012. The role of the immune system in the generation of neuropathic pain. *Lancet neurology*, 11(7), pp.629–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22710756 [Accessed October 29, 2013].

Cao, L. et al., 2009. Critical role of microglial CD40 in the maintenance of mechanical hypersensitivity in a murine model of neuropathic pain. *European journal of immunology*, 39(12), pp.3562–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2810130&tool=pmcentrez&rendertype=abstract [Accessed May 18, 2015].

Cao, L. & DeLeo, J.A., 2008. CNS-infiltrating CD4+ T lymphocytes contribute to murine spinal nerve transection-induced neuropathic pain. *European journal of immunology*, 38(2), pp.448–58. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2963094&tool=pmcentrez&rendertype=abstract [Accessed May 18, 2015].

Cardona, A.E. et al., 2006. Control of microglial neurotoxicity by the fractalkine receptor. *Nature neuroscience*, 9(7), pp.917–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16732273 [Accessed January 29, 2015].

Carlton, S.M. et al., 1994. Behavioral manifestations of an experimental model for peripheral neuropathy produced by spinal nerve ligation in the primate. *Pain*, 56(2), pp.155–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8008406 [Accessed June 9, 2015].

Carpenter, S. & O'Neill, L.A.J., 2007. How important are Toll-like receptors for antimicrobial responses? *Cellular microbiology*, 9(8), pp.1891–901. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17521328 [Accessed April 9, 2015].

Carraway, K.L. et al., 1997. Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. *Nature*, 387(6632), pp.512–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9168115 [Accessed January 5, 2015].

Carraway, K.L. & Cantley, L.C., 1994. A neu acquaintance for ErbB3 and ErbB4: A role for receptor heterodimerization in growth signaling. *Cell*, 78(1), pp.5–8. Available at: http://www.sciencedirect.com/science/article/pii/0092867494905649 [Accessed June 10, 2014].

Carroll, S.L. et al., 1997. Expression of neuregulins and their putative receptors, ErbB2 and ErbB3, is induced during Wallerian degeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 17(5), pp.1642–59. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9030624 [Accessed January 6, 2015].

Castro-Lopes, J., Tavares, I. & Coimbra, A., 1993. GABA decreases in the spinal cord dorsal horn after peripheral neurectomy. *Brain Research*, 620(2), pp.287–291. Available at: http://www.sciencedirect.com/science/article/pii/000689939390167L [Accessed March 24, 2015].

Chabot, S. et al., 1999. Mechanisms of IL-10 production in human microglia-T cell interaction. *Journal of immunology (Baltimore, Md. : 1950)*, 162(11), pp.6819–

28. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10352303 [Accessed September 20, 2015].

Chaplan, S.R. et al., 1994. Quantitative assessment of tactile allodynia in the rat paw. *Journal of neuroscience methods*, 53(1), pp.55–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7990513 [Accessed June 23, 2014].

Chen, B.P., Wolfgang, C.D. & Hai, T., 1996. Analysis of ATF3, a transcription factor induced by physiological stresses and modulated by gadd153/Chop10. *Molecular and cellular biology*, 16(3), pp.1157–68. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=231098&tool=pmcentrez&rendertype=abstract [Accessed July 23, 2015].

Chen, X., Tanner, K. & Levine, J.D., 1999. Mechanical sensitization of cutaneous C-fiber nociceptors by prostaglandin E2 in the rat. *Neuroscience letters*, 267(2), pp.105–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10400223 [Accessed March 23, 2015].

Cheret, C. et al., 2013. Bace1 and Neuregulin-1 cooperate to control formation and maintenance of muscle spindles. *The EMBO journal*, 32(14), pp.2015–28. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3715864&tool=pmcentrez&rendertype=abstract [Accessed January 19, 2015].

Chessell, I.P. et al., 2005. Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain*, 114(3), pp.386–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15777864 [Accessed April 20, 2015].

Chiang, R.P.-Y., Huang, C.-T. & Tsai, Y.-J., 2013. Melatonin reduces median nerve injury-induced mechanical hypersensitivity via inhibition of microglial p38 mitogen-activated protein kinase activation in rat cuneate nucleus. *Journal of Pineal Research*, 54(2), pp.232–244. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23237358 [Accessed April 9, 2015].

Chien, L.-Y. et al., 2007. Reduced expression of A-type potassium channels in primary sensory neurons induces mechanical hypersensitivity. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(37), pp.9855–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17855600 [Accessed March 23, 2015].

Choi, D.C. et al., 2012. Inhibition of ROS-induced p38MAPK and ERK activation in microglia by acupuncture relieves neuropathic pain after spinal cord injury in rats. *Experimental neurology*, 236(2), pp.268–82. Available at: http://www.sciencedirect.com/science/article/pii/S0014488612002129 [Accessed June 29, 2015].

Choi, Y. et al., 1994. Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. *Pain*, 59(3), pp.369–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7708411 [Accessed March 9, 2015].

Chung, K. et al., 1996. Sympathetic sprouting in the dorsal root ganglia of the injured peripheral nerve in a rat neuropathic pain model. *The Journal of comparative neurology*, 376(2), pp.241–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8951640 [Accessed March 23, 2015].

Chung, M.-K. & Caterina, M.J., 2007. TRP channel knockout mice lose their cool. *Neuron*, 54(3), pp.345–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17481385 [Accessed June 9, 2015].

Clark, A.K., Wodarski, R., et al., 2010. Cathepsin S release from primary cultured microglia is regulated by the P2X7 receptor. *Glia*, 58(14), pp.1710–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20629190 [Accessed May 16, 2015].

Clark, A.K. et al., 2007. Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain. *Proceedings of the National Academy of Sciences of the United States of America*, 104(25), pp.10655–60. Available at: http://www.pnas.org/content/104/25/10655.full [Accessed April 16, 2015].

Clark, A.K., Staniland, A.A., et al., 2010. P2X7-dependent release of interleukin-1beta and nociception in the spinal cord following lipopolysaccharide. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 30(2), pp.573–82. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2880485&tool=pmcentrez&rendertype=abstract [Accessed May 16, 2015].

Clark, A.K., Yip, P.K. & Malcangio, M., 2009. The liberation of fractalkine in the dorsal horn requires microglial cathepsin S. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 29(21), pp.6945–54. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2698289&tool=pmcentrez&rendertype=abstract [Accessed March 24, 2015].

Cohen, J.A. et al., 1992. Expression of the neu proto-oncogene by Schwann cells during peripheral nerve development and Wallerian degeneration. *Journal of neuroscience research*, 31(4), pp.622–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1374476 [Accessed January 6, 2015].

Cohen, P., 2002. The origins of protein phosphorylation. *Nature cell biology*, 4(5), pp.E127–30. Available at: http://dx.doi.org/10.1038/ncb0502-e127 [Accessed August 24, 2015].

Colburn, R.W. et al., 2007. Attenuated cold sensitivity in TRPM8 null mice. *Neuron*, 54(3), pp.379–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17481392 [Accessed June 9, 2015].

Colburn, R.W. et al., 1997. Dissociation of microglial activation and neuropathic pain behaviors following peripheral nerve injury in the rat. *Journal of neuroimmunology*, 79(2), pp.163–75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9394789 [Accessed March 27, 2015].

Colburn, R.W., Rickman, A.J. & DeLeo, J.A., 1999. The effect of site and type of nerve injury on spinal glial activation and neuropathic pain behavior. *Experimental neurology*, 157(2), pp.289–304. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10364441 [Accessed March 7, 2015].

Costigan, M., Moss, A., et al., 2009. T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 29(46), pp.14415–22. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2813708&tool=pmcentrez&rendertype=abstract [Accessed May 18, 2015].

Costigan, M., Scholz, J. & Woolf, C.J., 2009. Neuropathic pain: a maladaptive response of the nervous system to damage. *Annual review of neuroscience*, 32, pp.1–32. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2768555&tool=pmcentrez&rendertype=abstract [Accessed August 30, 2014].

Coull, J.A.M. et al., 2005a. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature*, 438(7070), pp.1017–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16355225 [Accessed January 8, 2015].

Coull, J.A.M. et al., 2005b. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature*, 438(7070), pp.1017–21. Available at: http://dx.doi.org/10.1038/nature04223 [Accessed January 8, 2015].

Coull, J.A.M. et al., 2003. Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature*, 424(6951), pp.938–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12931188 [Accessed January 10, 2015].

Coyle, D.E., 1998. Partial peripheral nerve injury leads to activation of astroglia and microglia which parallels the development of allodynic behavior. *Glia*, 23(1), pp.75–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9562186 [Accessed April 7, 2015].

Crowe, M.J. et al., 1997. Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nature medicine*, 3(1), pp.73–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8986744 [Accessed November 12, 2014].

D'Mello, R. & Dickenson, A.H., 2008. Spinal cord mechanisms of pain. *British journal of anaesthesia*, 101(1), pp.8–16. Available at: http://bja.oxfordjournals.org/content/101/1/8.abstract [Accessed November 18, 2014].

Daulhac, L. et al., 2006. Diabetes-induced mechanical hyperalgesia involves spinal mitogen-activated protein kinase activation in neurons and microglia via N-methyl-D-aspartate-dependent mechanisms. *Molecular pharmacology*, 70(4), pp.1246–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16868181 [Accessed April 7, 2015].

Daulhac, L. et al., 2011. Phosphorylation of spinal N-methyl-d-aspartate receptor NR1 subunits by extracellular signal-regulated kinase in dorsal horn neurons and microglia contributes to diabetes-induced painful neuropathy. *European journal of pain (London, England)*, 15(2), pp.169.e1–169.e12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20594879 [Accessed April 7, 2015].

Davis, H.M. et al., 2000. Human granulocyte CD11b expression as a pharmacodynamic biomarker of inflammation. *Journal of immunological methods*, 240(1-2), pp.125–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10854607 [Accessed July 12, 2015].

Decosterd, I. & Woolf, C.J., 2000. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain*, 87(2), pp.149–58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10924808 [Accessed December 31, 2014].

DeLeo, J.A. & Yezierski, R.P., 2001. The role of neuroinflammation and neuroimmune activation in persistent pain. *Pain*, 90(1-2), pp.1–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11166964 [Accessed April 8, 2015].

Descoeur, J. et al., 2011. Oxaliplatin-induced cold hypersensitivity is due to remodelling of ion channel expression in nociceptors. *EMBO molecular medicine*, 3(5), pp.266–78. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3377073&tool=pmcentrez&rendertype=abstract [Accessed July 13, 2015].

Dib-Hajj, S.D. et al., 2007. From genes to pain: Na v 1.7 and human pain disorders. *Trends in neurosciences*, 30(11), pp.555–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17950472 [Accessed March 2, 2015].

Dib-Hajj, S.D. et al., 2005. Gain-of-function mutation in Nav1.7 in familial erythromelalgia induces bursting of sensory neurons. *Brain : a journal of neurology*, 128(Pt 8), pp.1847–54. Available at: http://brain.oxfordjournals.org/content/128/8/1847.abstract [Accessed July 14, 2015].

Dib-Hajj, S.D. et al., 2009. Voltage-gated sodium channels in pain states: role in pathophysiology and targets for treatment. *Brain research reviews*, 60(1), pp.65–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19150627 [Accessed March 2, 2015].

Dickenson, A.H. & Sullivan, A.F., 1987. 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(8), pp.1235–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2821443 [Accessed March 24, 2015].

Diogenes, A. et al., 2011. LPS sensitizes TRPV1 via activation of TLR4 in trigeminal sensory neurons. *Journal of dental research*, 90(6), pp.759–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21393555 [Accessed March 21, 2015].

Dixon, W.J., 1980. Efficient analysis of experimental observations. *Annual review of pharmacology and toxicology*, 20, pp.441–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7387124 [Accessed June 23, 2014].

Dougherty, P.M. & Willis, W.D., 1991. Enhancement of spinothalamic neuron responses to chemical and mechanical stimuli following combined micro-iontophoretic application of N-methyl-D-aspartic acid and substance P. *Pain*, 47(1), pp.85–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1722895 [Accessed March 24, 2015].

Draheim, H.J. et al., 1999. Induction of potassium channels in mouse brain microglia: cells acquire responsiveness to pneumococcal cell wall components during late development. *Neuroscience*, 89(4), pp.1379–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10362322 [Accessed June 29, 2015].

Drysdale, B.E., Howard, D.L. & Johnson, R.J., 1996. Identification of a lipopolysaccharide inducible transcription factor in murine macrophages. *Molecular immunology*, 33(11-12), pp.989–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8960123 [Accessed July 23, 2015].

Dworkin, R.H. et al., 2010. Recommendations for the pharmacological management of neuropathic pain: an overview and literature update. *Mayo Clinic proceedings*, 85(3 Suppl), pp.S3–14. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2844007&tool=pmcentrez&rendertype=abstract [Accessed November 16, 2014].

Eaton, M.J. et al., 1998. Changes in GAD- and GABA- immunoreactivity in the spinal dorsal horn after peripheral nerve injury and promotion of recovery by lumbar transplant of immortalized serotonergic precursors. *Journal of chemical neuroanatomy*, 16(1), pp.57–72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9924973 [Accessed March 24, 2015].

Echeverry, S., Shi, X.Q. & Zhang, J., 2008. Characterization of cell proliferation in rat spinal cord following peripheral nerve injury and the relationship with neuropathic pain. *Pain*, 135(1), pp.37–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17560721 [Accessed April 17, 2015].

EDDY, N.B. & LEIMBACH, D., 1953. Synthetic analgesics. II. Dithienylbutenyl- and dithienylbutylamines. *The Journal of pharmacology and experimental therapeutics*, 107(3), pp.385–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13035677 [Accessed June 26, 2014].

Eder, C., Klee, R. & Heinemann, U., 1998. Involvement of stretch-activated Cl- channels in ramification of murine microglia. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 18(18), pp.7127–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9736636 [Accessed March 30, 2015].

Edwards, J.P. et al., 2006. Biochemical and functional characterization of three activated macrophage populations. *Journal of leukocyte biology*, 80(6), pp.1298–307. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2642590&tool=pmcentrez&rendertype=abstract [Accessed May 6, 2014].

El-Khoury, C. et al., 2002. Attenuation of neuropathic pain by segmental and supraspinal activation of the dorsal column system in awake rats. *Neuroscience*, 112(3), pp.541–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12074897 [Accessed June 9, 2015].

Eliasson, C. et al., 1999. Intermediate filament protein partnership in astrocytes. *The Journal of biological chemistry*, 274(34), pp.23996–4006. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10446168 [Accessed February 6, 2015].

Elward, K. & Gasque, P., 2003. "Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system. *Molecular immunology*, 40(2-4), pp.85–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12914815 [Accessed April 9, 2015].

Erickson, S.L. et al., 1997. ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. *Development (Cambridge, England)*, 124(24), pp.4999–5011. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9362461 [Accessed June 11, 2014].

Esper, R.M. & Loeb, J.A., 2009. Neurotrophins induce neuregulin release through protein kinase Cdelta activation. *The Journal of biological chemistry*, 284(39), pp.26251–60. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2785313&tool=pmcentrez&rendertype=abstract [Accessed September 27, 2015].

Esper, R.M. & Loeb, J.A., 2004. Rapid axoglial signaling mediated by neuregulin and neurotrophic factors. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 24(27), pp.6218–27. Available at: http://www.jneurosci.org/content/24/27/6218.long [Accessed September 27, 2015].

Falls, D., 2003. Neuregulins: functions, forms, and signaling strategies. *Experimental Cell Research*, 284(1), pp.14–30. Available at: http://www.sciencedirect.com/science/article/pii/S0014482702001027 [Accessed August 27, 2014].

Feil, R. et al., 1996. Ligand-activated site-specific recombination in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 93(20), pp.10887–90. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=38252&tool=pmcentrez&rendertype=abstract [Accessed March 13, 2015].

Feil, S., Valtcheva, N. & Feil, R., 2009. Inducible Cre mice. *Methods in molecular biology (Clifton, N.J.)*, 530, pp.343–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19266339 [Accessed February 18, 2015].

Ferrari, D., 1997. Purinergic Modulation of Interleukin-1beta Release from Microglial Cells Stimulated with Bacterial Endotoxin. *Journal of Experimental Medicine*, 185(3), pp.579–582. Available at: http://jem.rupress.org/content/185/3/579.long [Accessed May 10, 2015].

Ferreira, J. et al., 2005. Reduced nerve injury-induced neuropathic pain in kinin B1 receptor knock-out mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 25(9), pp.2405–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15745967 [Accessed May 18, 2015].

Ferrini, F. et al., 2013. Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl^- homeostasis. *Nature neuroscience*, 16(2), pp.183–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23292683 [Accessed October 19, 2013].

Ferron, M. & Vacher, J., 2005. Targeted expression of Cre recombinase in macrophages and osteoclasts in transgenic mice. *Genesis (New York, N.Y. : 2000)*, 41(3), pp.138–45. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15754380 [Accessed January 9, 2015].

Fleck, D. et al., 2013. Dual cleavage of neuregulin 1 type III by BACE1 and ADAM17 liberates its EGF-like domain and allows paracrine signaling. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 33(18), pp.7856–69. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23637177 [Accessed July 19, 2015].

Floden, A.M. & Combs, C.K., 2007. Microglia repetitively isolated from in vitro mixed glial cultures retain their initial phenotype. *Journal of neuroscience methods*, 164(2), pp.218–24. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2041803&tool=pmcentrez&rendertype=abstract [Accessed June 29, 2015].

Fogg, D.K. et al., 2006. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science (New York, N.Y.)*, 311(5757), pp.83–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16322423 [Accessed May 8, 2015].

Forlenza, O.V. et al., 2009. Increased serum IL-1beta level in Alzheimer's disease and mild cognitive impairment. *Dementia and geriatric cognitive disorders*, 28(6), pp.507–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19996595 [Accessed September 20, 2015].

Fricker, F.R. et al., 2013. Axonal neuregulin 1 is a rate limiting but not essential factor for nerve remyelination. *Brain : a journal of neurology*, 136(Pt 7), pp.2279–97. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3692042&tool=pmcentrez&rendertype=abstract [Accessed October 31, 2013].

Fricker, F.R. et al., 2009. Sensory axon-derived neuregulin-1 is required for axoglial signaling and normal sensory function but not for long-term axon maintenance.

The Journal of neuroscience : the official journal of the Society for Neuroscience, 29(24), pp.7667–78. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2875847&tool=pmcentrez&rendertype=abstract [Accessed May 5, 2015].

Fricker, F.R. & Bennett, D.L., 2011. The role of neuregulin-1 in the response to nerve injury. *Future Neurology*, 6(6), pp.809–822. Available at: http://www.futuremedicine.com/doi/full/10.2217/fnl.11.45 [Accessed February 2, 2015].

Fukushima, S. et al., 2015. Robust increase of microglia proliferation in the fornix of hippocampal axonal pathway after a single LPS stimulation. *Journal of neuroimmunology*, 285, pp.31–40. Available at: http://www.sciencedirect.com/science/article/pii/S0165572815001265 [Accessed September 19, 2015].

Funk, K. et al., 2008. Modulation of chloride homeostasis by inflammatory mediators in dorsal root ganglion neurons. *Molecular Pain*, 4(1), p.32. Available at: http://www.molecularpain.com/content/4/1/32 [Accessed February 17, 2015].

Van Furth, R. & Cohn, Z.A., 1968. The origin and kinetics of mononuclear phagocytes. *The Journal of experimental medicine*, 128(3), pp.415–35. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2138527&tool=pmcentrez&rendertype=abstract [Accessed February 11, 2015].

Galimberti, D. et al., 2008. Intrathecal levels of IL-6, IL-11 and LIF in Alzheimer's disease and frontotemporal lobar degeneration. *Journal of neurology*, 255(4), pp.539–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18204920 [Accessed September 20, 2015].

Gálvez, R. et al., 2005. [Prevalence of neuropathic pain in Spain: clinical, working and health care implications]. *Medicina clínica*, 125(6), pp.221–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16022836 [Accessed March 16, 2015].

Gambarotta, G. et al., 2014. Neuregulin 1 isoforms could be an effective therapeutic candidate to promote peripheral nerve regeneration. *Neural regeneration research*, 9(12), pp.1183–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4146285&tool=pmcentrez&rendertype=abstract [Accessed May 6, 2015].

Gambarotta, G. et al., 2013. Neuregulin 1 role in Schwann cell regulation and potential applications to promote peripheral nerve regeneration. *International review of neurobiology*, 108, pp.223–56. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24083437 [Accessed May 6, 2015].

Garcia-Segura, L.M., Chowen, J.A. & Naftolin, F., 1996. Endocrine glia: roles of glial cells in the brain actions of steroid and thyroid hormones and in the regulation of hormone secretion. *Frontiers in neuroendocrinology*, 17(2), pp.180–211.

Available at: http://www.ncbi.nlm.nih.gov/pubmed/8812295 [Accessed March 30, 2015].

Garratt, A.N., Britsch, S. & Birchmeier, C., 2000. Neuregulin, a factor with many functions in the life of a schwann cell. *BioEssays : news and reviews in molecular, cellular and developmental biology*, 22(11), pp.987–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11056475 [Accessed June 10, 2014].

Garrison, C.J. et al., 1991. Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. *Brain research*, 565(1), pp.1–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1723019 [Accessed March 27, 2015].

Gassmann, M. et al., 1995. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature*, 378(6555), pp.390–4. Available at: http://dx.doi.org/10.1038/378390a0 [Accessed May 13, 2014].

Gauriau, C. & Bernard, J.-F., 2002. Pain pathways and parabrachial circuits in the rat. *Experimental physiology*, 87(2), pp.251–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11856971 [Accessed March 24, 2015].

Gauthier, M.-K. et al., 2013. Dysregulation of the neuregulin-1-ErbB network modulates endogenous oligodendrocyte differentiation and preservation after spinal cord injury. *The European journal of neuroscience*, 38(5), pp.2693–715. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23758598 [Accessed November 12, 2014].

Gingras, M. et al., 2007. Optimized protocols for isolation of primary motor neurons, astrocytes and microglia from embryonic mouse spinal cord. *Journal of neuroscience methods*, 163(1), pp.111–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17445905 [Accessed June 29, 2015].

Ginhoux, F. et al., 2013. Origin and differentiation of microglia. *Frontiers in cellular neuroscience*, 7, p.45. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3627983&tool=pmcentrez&rendertype=abstract [Accessed November 6, 2014].

Giulian, D. & Baker, T.J., 1986. Characterization of ameboid microglia isolated from developing mammalian brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 6(8), pp.2163–78. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3018187 [Accessed June 29, 2015].

Glass, C.K. et al., 2010. Mechanisms underlying inflammation in neurodegeneration. *Cell*, 140(6), pp.918–34. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2873093&tool=pmcentrez&rendertype=abstract [Accessed July 18, 2014].

Glezer, I., Lapointe, A. & Rivest, S., 2006. Innate immunity triggers oligodendrocyte progenitor reactivity and confines damages to brain injuries. *FASEB journal : official publication of the Federation of American Societies for Experimental*

Biology, 20(6), pp.750–2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16464958 [Accessed August 31, 2015].

Glezer, I., Simard, A.R. & Rivest, S., 2007. Neuroprotective role of the innate immune system by microglia. *Neuroscience*, 147(4), pp.867–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17459594 [Accessed August 31, 2015].

Goldmann, T. et al., 2013. A new type of microglia gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation. *Nature neuroscience*, 16(11), pp.1618–26. Available at: http://dx.doi.org/10.1038/nn.3531 [Accessed January 27, 2014].

Goldstein, L.B. & Davis, J.N., 1990. Beam-walking in rats: studies towards developing an animal model of functional recovery after brain injury. *Journal of neuroscience methods*, 31(2), pp.101–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2319810 [Accessed February 2, 2015].

Goodearl, A.D. et al., 1993. Purification of multiple forms of glial growth factor. *The Journal of biological chemistry*, 268(24), pp.18095–102. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8394360 [Accessed April 18, 2015].

Gordh, T., Chu, H. & Sharma, H.S., 2006. Spinal nerve lesion alters blood-spinal cord barrier function and activates astrocytes in the rat. *Pain*, 124(1-2), pp.211–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16806707 [Accessed March 30, 2015].

Gordon, S., 2003. Alternative activation of macrophages. *Nature reviews. Immunology*, 3(1), pp.23–35. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12511873 [Accessed July 9, 2014].

Gordon, S. & Taylor, P.R., 2005. Monocyte and macrophage heterogeneity. *Nature reviews. Immunology*, 5(12), pp.953–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16322748 [Accessed July 10, 2014].

Grace, P.M., Rolan, P.E. & Hutchinson, M.R., 2011. Peripheral immune contributions to the maintenance of central glial activation underlying neuropathic pain. *Brain, behavior, and immunity*, 25(7), pp.1322–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21496480 [Accessed May 18, 2015].

Graus-Porta, D. et al., 1997. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *The EMBO journal*, 16(7), pp.1647–55. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1169769&tool=pmcentrez&rendertype=abstract [Accessed May 28, 2014].

Griffin, J.W., George, R. & Ho, T., 1993. Macrophage systems in peripheral nerves. A review. *Journal of neuropathology and experimental neurology*, 52(6), pp.553–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7693878 [Accessed March 11, 2015].

Griffin, R.S. et al., 2007a. Complement induction in spinal cord microglia results in anaphylatoxin C5a-mediated pain hypersensitivity. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(32), pp.8699–708. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17687047 [Accessed August 10, 2015].

Griffin, R.S. et al., 2007b. Complement induction in spinal cord microglia results in anaphylatoxin C5a-mediated pain hypersensitivity. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(32), pp.8699–708. Available at: http://www.jneurosci.org/content/27/32/8699.full [Accessed May 9, 2015].

Griffin, T.J., 2002. Complementary Profiling of Gene Expression at the Transcriptome and Proteome Levels in Saccharomyces cerevisiae. *Molecular & Cellular Proteomics*, 1(4), pp.323–333. Available at: http://www.mcponline.org/content/1/4/323.full [Accessed July 27, 2015].

Grinspan, J.B. et al., 1996. Axonal Interactions Regulate Schwann Cell Apoptosis in Developing Peripheral Nerve: Neuregulin Receptors and the Role of Neuregulins. *J. Neurosci.*, 16(19), pp.6107–6118. Available at: http://www.jneurosci.org/content/16/19/6107.full [Accessed June 18, 2015].

Gritsch, S. et al., 2014. Oligodendrocyte ablation triggers central pain independently of innate or adaptive immune responses in mice. *Nature communications*, 5, p.5472. Available at: http://www.nature.com/ncomms/2014/141201/ncomms6472/full/ncomms6472.html [Accessed February 8, 2015].

Gu, Z. et al., 2005. Regulation of NMDA receptors by neuregulin signaling in prefrontal cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 25(20), pp.4974–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15901778 [Accessed May 4, 2015].

Guenther, S., Reeh, P.W. & Kress, M., 1999. Rises in [Ca2+]i mediate capsaicin- and proton-induced heat sensitization of rat primary nociceptive neurons. *The European journal of neuroscience*, 11(9), pp.3143–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10510178 [Accessed March 23, 2015].

Guo, L.-H. et al., 2006. Combinations of TLR and NOD2 ligands stimulate rat microglial P2X4R expression. *Biochemical and biophysical research communications*, 349(3), pp.1156–62. Available at: http://www.sciencedirect.com/science/article/pii/S0006291X06019784 [Accessed May 16, 2015].

Guo, W. et al., 2007. Glial-cytokine-neuronal interactions underlying the mechanisms of persistent pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(22), pp.6006–18. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2676443&tool=pmcentrez&rendertype=abstract [Accessed March 30, 2015].

Gwak, Y.S. et al., 2006. Activation of spinal GABA receptors attenuates chronic central neuropathic pain after spinal cord injury. *Journal of neurotrauma*, 23(7), pp.1111–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16866624 [Accessed March 24, 2015].

Hanisch, U.-K. & Kettenmann, H., 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nature neuroscience*, 10(11), pp.1387–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17965659 [Accessed July 11, 2014].

Hao, J.X. et al., 1996. Capsaicin-sensitive afferents mediate chronic cold, but not mechanical, allodynia-like behavior in spinally injured rats. *Brain research*, 722(1-2), pp.177–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8813363 [Accessed June 9, 2015].

Harari, D. et al., 1999a. Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. *Oncogene*, 18(17), pp.2681–9. Available at: http://www.nature.com/onc/journal/v18/n17/full/1202631a.html [Accessed April 19, 2015].

Harari, D. et al., 1999b. Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. *Oncogene*, 18(17), pp.2681–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10348342 [Accessed December 22, 2014].

Hargreaves, K. et al., 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain*, 32(1), pp.77–88. Available at: http://www.sciencedirect.com/science/article/pii/0304395988900267 [Accessed June 26, 2014].

Hathway, G.J. et al., 2009. Brief, low frequency stimulation of rat peripheral C-fibres evokes prolonged microglial-induced central sensitization in adults but not in neonates. *Pain*, 144(1-2), pp.110–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2702711&tool=pmcentrez&rendertype=abstract [Accessed March 26, 2015].

Hayashi, S. & McMahon, A.P., 2002. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Developmental biology*, 244(2), pp.305–18. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11944939 [Accessed January 12, 2015].

Haynes, S.E. et al., 2006. The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nature neuroscience*, 9(12), pp.1512–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17115040 [Accessed August 28, 2015].

He, X.-H. et al., 2010. TNF-α contributes to up-regulation of Nav1.3 and Nav1.8 in DRG neurons following motor fiber injury. *Pain*, 151(2), pp.266–79. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20638792 [Accessed March 23, 2015].

Heinricher, M.M. & Neubert, M.J., 2004. Neural basis for the hyperalgesic action of cholecystokinin in the rostral ventromedial medulla. *Journal of neurophysiology*, 92(4), pp.1982–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15152023 [Accessed March 24, 2015].

Herrlich, A. et al., 2008. Ectodomain cleavage of the EGF ligands HB-EGF, neuregulin1-beta, and TGF-alpha is specifically triggered by different stimuli and involves different PKC isoenzymes. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 22(12), pp.4281–95. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2614613&tool=pmcentrez&rendertype=abstract [Accessed April 19, 2015].

Herzberg, U. & Sagen, J., 2001. Peripheral nerve exposure to HIV viral envelope protein gp120 induces neuropathic pain and spinal gliosis. *Journal of neuroimmunology*, 116(1), pp.29–39. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11311327 [Accessed April 7, 2015].

Hickman, S.E., Allison, E.K. & El Khoury, J., 2008. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 28(33), pp.8354–60. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2597474&tool=pmcentrez&rendertype=abstract [Accessed December 20, 2014].

Hickstein, D.D. et al., 1992. Identification of the promoter of the myelomonocytic leukocyte integrin CD11b. *Proceedings of the National Academy of Sciences*, 89(6), pp.2105–2109. Available at: http://www.pnas.org/content/89/6/2105.abstract?ijkey=66ec14dbbb40f5ba18d0848dfc082be705d0c5f7&keytype2=tf_ipsecsha [Accessed May 13, 2014].

Hinwood, M. et al., 2013. Chronic stress induced remodeling of the prefrontal cortex: structural re-organization of microglia and the inhibitory effect of minocycline. *Cerebral cortex (New York, N.Y. : 1991)*, 23(8), pp.1784–97. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22710611 [Accessed March 30, 2015].

Hippenmeyer, S. et al., 2002. A Role for Neuregulin1 Signaling in Muscle Spindle Differentiation. *Neuron*, 36(6), pp.1035–1049. Available at: http://www.sciencedirect.com/science/article/pii/S0896627302011017 [Accessed February 3, 2015].

Hiraoka, Y. et al., 2007. Enhancement of alpha-secretase cleavage of amyloid precursor protein by a metalloendopeptidase nardilysin. *Journal of neurochemistry*, 102(5), pp.1595–605. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17555553 [Accessed April 19, 2015].

Holmes, W.E. et al., 1992. Identification of heregulin, a specific activator of p185erbB2. *Science (New York, N.Y.)*, 256(5060), pp.1205–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1350381 [Accessed April 18, 2015].

Honda, S. et al., 2001. Extracellular ATP or ADP induce chemotaxis of cultured microglia through Gi/o-coupled P2Y receptors. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 21(6), pp.1975–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11245682 [Accessed May 9, 2015].

Honore, P. et al., 2000. Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons. *Neuroscience*, 98(3), pp.585–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10869852 [Accessed February 24, 2015].

Hu, X. et al., 2006. Bace1 modulates myelination in the central and peripheral nervous system. *Nature neuroscience*, 9(12), pp.1520–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17099708 [Accessed February 6, 2015].

Hucho, T. & Levine, J.D., 2007. Signaling pathways in sensitization: toward a nociceptor cell biology. *Neuron*, 55(3), pp.365–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17678851 [Accessed March 23, 2015].

Hughes, P.M. et al., 2002. Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, during acute and chronic inflammation in the rodent CNS. *Glia*, 37(4), pp.314–27. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11870871 [Accessed April 15, 2015].

Hutchinson, M.R. et al., 2008. Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). *European Journal of Neuroscience*, 28(1), pp.20–29. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2588470&tool=pmcentrez&rendertype=abstract [Accessed April 9, 2015].

Ibuki, T. et al., 1997. Loss of GABA-immunoreactivity in the spinal dorsal horn of rats with peripheral nerve injury and promotion of recovery by adrenal medullary grafts. *Neuroscience*, 76(3), pp.845–58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9135056 [Accessed March 24, 2015].

Ifuku, M. et al., 2007. Bradykinin-induced microglial migration mediated by B1-bradykinin receptors depends on Ca2+ influx via reverse-mode activity of the Na+/Ca2+ exchanger. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(48), pp.13065–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18045900 [Accessed April 2, 2015].

Imai, T. et al., 1997. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell*, 91(4), pp.521–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9390561 [Accessed May 18, 2015].

Inoue, K., 2006. ATP receptors of microglia involved in pain. *Novartis Foundation symposium*, 276, pp.263–72; discussion 273–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16805436 [Accessed March 30, 2015].

Iwakura, Y. & Nawa, H., 2013. ErbB1-4-dependent EGF/neuregulin signals and their cross talk in the central nervous system: pathological implications in schizophrenia and Parkinson's disease. *Frontiers in cellular neuroscience*, 7, p.4. Available at: http://journal.frontiersin.org/Journal/10.3389/fncel.2013.00004/abstract [Accessed February 5, 2015].

Jack, C. et al., 2005. Microglia and multiple sclerosis. *Journal of neuroscience research*, 81(3), pp.363–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15948188 [Accessed March 17, 2015].

Jaggi, A.S., Jain, V. & Singh, N., 2011. Animal models of neuropathic pain. *Fundamental & clinical pharmacology*, 25(1), pp.1–28. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20030738 [Accessed February 7, 2015].

Jasmin, L. et al., 1998. The cold plate as a test of nociceptive behaviors: description and application to the study of chronic neuropathic and inflammatory pain models. *Pain*, 75(2-3), pp.367–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9583773 [Accessed January 12, 2015].

Jergová, S. & Cízková, D., 2007. Microglial activation in different models of peripheral nerve injury of the rat. *Journal of molecular histology*, 38(3), pp.245–51. Available at: http://www.researchgate.net/publication/6329530_Microglial_activation_in_different_models_of_peripheral_nerve_injury_of_the_rat [Accessed September 27, 2015].

Jessen, K.R. & Mirsky, R., 2005. The origin and development of glial cells in peripheral nerves. *Nature reviews. Neuroscience*, 6(9), pp.671–82. Available at: http://dx.doi.org/10.1038/nrn1746 [Accessed April 19, 2015].

Jha, S.K. et al., 2015. p38 MAPK and PI3K/AKT Signalling Cascades inParkinson's Disease. *International journal of molecular and cellular medicine*, 4(2), pp.67–86. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4499569&tool=pmcentrez&rendertype=abstract [Accessed August 28, 2015].

Ji, R.-R. et al., 2009. Matrix metalloprotease regulation of neuropathic pain. *Trends in pharmacological sciences*, 30(7), pp.336–40. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2706286&tool=pmcentrez&rendertype=abstract [Accessed April 16, 2015].

Ji, R.-R. et al., 2002. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron*, 36(1), pp.57–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12367506 [Accessed April 9, 2015].

Ji, R.-R. et al., 2006. Possible role of spinal astrocytes in maintaining chronic pain sensitization: review of current evidence with focus on bFGF/JNK pathway. *Neuron glia biology*, 2(4), pp.259–69. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1949390&tool=pmcentrez&rendertype=abstract [Accessed March 26, 2015].

Ji, R.-R. & Suter, M.R., 2007. p38 MAPK, microglial signaling, and neuropathic pain. *Molecular pain*, 3, p.33. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2186318&tool=pmcentrez&rendertype=abstract [Accessed January 4, 2015].

Jin, S.-X. et al., 2003. p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 23(10), pp.4017–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12764087 [Accessed April 9, 2015].

JONES, B.J. & ROBERTS, D.J., 1968. The quantitative measurement of motor inco-ordination in naive mice using an accelerating rotarod. *Journal of Pharmacy and Pharmacology*, 20(4), pp.302–304. Available at: http://doi.wiley.com/10.1111/j.2042-7158.1968.tb09743.x [Accessed June 26, 2014].

Jonsson, S. et al., 2013. Effect of delayed peripheral nerve repair on nerve regeneration, Schwann cell function and target muscle recovery. *PloS one*, 8(2), p.e56484. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3567071&tool=pmcentrez&rendertype=abstract [Accessed June 18, 2015].

Jordt, S.-E. et al., 2004. Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature*, 427(6971), pp.260–5. Available at: http://dx.doi.org/10.1038/nature02282 [Accessed June 2, 2015].

Jourdan, D. et al., 1995. Audible and ultrasonic vocalization elicited by single electrical nociceptive stimuli to the tail in the rat. *Pain*, 63(2), pp.237–49. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8628590 [Accessed March 9, 2015].

Jung, H. et al., 2008. Monocyte chemoattractant protein-1 functions as a neuromodulator in dorsal root ganglia neurons. *Journal of neurochemistry*, 104(1), pp.254–63. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2186066&tool=pmcentrez&rendertype=abstract [Accessed March 27, 2015].

Jung, H. et al., 2009. Visualization of chemokine receptor activation in transgenic mice reveals peripheral activation of CCR2 receptors in states of neuropathic pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 29(25), pp.8051–62. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3097108&tool=pmcentrez&rendertype=abstract [Accessed March 27, 2015].

Jung, S. et al., 2000. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Molecular and cellular biology*, 20(11), pp.4106–14. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=85780&tool=pmcentrez&rendertype=abstract [Accessed January 17, 2015].

Juránek, I. & Lembeck, F., 1996. Evidence for the participation of glutamate in reflexes involving afferent, substance P-containing nerve fibres in the rat. *British journal of pharmacology*, 117(1), pp.71–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1909381&tool=pmcentrez&rendertype=abstract [Accessed March 24, 2015].

Kadetoff, D. et al., 2012. Evidence of central inflammation in fibromyalgia-increased cerebrospinal fluid interleukin-8 levels. *Journal of neuroimmunology*, 242(1-2), pp.33–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22126705 [Accessed September 27, 2015].

Kaisho, T. & Akira, S., 2004. Pleiotropic function of Toll-like receptors. *Microbes and infection / Institut Pasteur*, 6(15), pp.1388–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15596125 [Accessed April 9, 2015].

Kajander, K.C. & Bennett, G.J., 1992. Onset of a painful peripheral neuropathy in rat: a partial and differential deafferentation and spontaneous discharge in A beta and A delta primary afferent neurons. *Journal of neurophysiology*, 68(3), pp.734–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1331353 [Accessed March 23, 2015].

Kane, C.M., Hoskin, P. & Bennett, M.I., 2015. Cancer induced bone pain. *BMJ (Clinical research ed.)*, 350(jan29_7), p.h315. Available at: http://www.bmj.com/content/350/bmj.h315 [Accessed February 18, 2015].

Kanzaki, H. et al., 2012. Expression changes of the neuregulin 1 isoforms in neuropathic pain model rats. *Neuroscience letters*, 508(2), pp.78–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22212401 [Accessed December 17, 2014].

Kao, W.-T. et al., 2010. Common genetic variation in Neuregulin 3 (NRG3) influences risk for schizophrenia and impacts NRG3 expression in human brain. *Proceedings of the National Academy of Sciences of the United States of America*, 107(35), pp.15619–24. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2932571&tool=pmcentrez&rendertype=abstract [Accessed January 12, 2015].

Karimi-Abdolrezaee, S. et al., 2006. Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(13), pp.3377–89. Available at: http://www.jneurosci.org/content/26/13/3377.short [Accessed November 6, 2014].

Karperien, A., Ahammer, H. & Jelinek, H.F., 2013. Quantitating the subtleties of microglial morphology with fractal analysis. *Frontiers in cellular neuroscience*, 7, p.3. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3558688&tool=pmcentrez&rendertype=abstract [Accessed March 30, 2015].

Kawasaki, Y. et al., 2008. Distinct roles of matrix metalloproteases in the early- and late-phase development of neuropathic pain. *Nature medicine*, 14(3), pp.331–6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2279180&tool=pmcentrez&rendertype=abstract [Accessed April 9, 2015].

Kawasaki, Y. et al., 2004. Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 24(38), pp.8310–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15385614 [Accessed March 24, 2015].

Kayser, V. & Christensen, D., 2000. Antinociceptive effect of systemic gabapentin in mononeuropathic rats, depends on stimulus characteristics and level of test integration. *Pain*, 88(1), pp.53–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11098099 [Accessed June 9, 2015].

Kayser, V., Desmeules, J. & Guilbaud, G., 1995. Systemic clonidine differentially modulates the abnormal reactions to mechanical and thermal stimuli in rats with peripheral mononeuropathy. *Pain*, 60(3), pp.275–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7596623 [Accessed June 9, 2015].

Kerber, G. et al., 2003. Neuregulin-1 isoforms are differentially expressed in the intact and regenerating adult rat nervous system. *Journal of molecular neuroscience : MN*, 21(2), pp.149–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14593214 [Accessed January 6, 2015].

Kerr, B.J. et al., 1999. Brain-derived neurotrophic factor modulates nociceptive sensory inputs and NMDA-evoked responses in the rat spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 19(12), pp.5138–48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10366647 [Accessed March 24, 2015].

Khakh, B.S. et al., 2001. International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. *Pharmacological reviews*, 53(1), pp.107–18. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11171941 [Accessed May 16, 2015].

Kim, D. et al., 2007. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. *The Journal of biological chemistry*, 282(20), pp.14975–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17355971 [Accessed April 9, 2015].

Kim, D.S. et al., 2002. Downregulation of voltage-gated potassium channel alpha gene expression in dorsal root ganglia following chronic constriction injury of the rat sciatic nerve. *Brain research. Molecular brain research*, 105(1-2), pp.146–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12399117 [Accessed March 23, 2015].

Kim, K.-W. et al., 2011. In vivo structure/function and expression analysis of the CX3C chemokine fractalkine. *Blood*, 118(22), pp.e156–67. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21951685 [Accessed January 27, 2015].

Kim, K.J., Yoon, Y.W. & Chung, J.M., 1997. Comparison of three rodent neuropathic pain models. *Experimental brain research*, 113(2), pp.200–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9063706 [Accessed January 12, 2015].

Kim, S.-Y. et al., 2002. Activation of p38 MAP kinase in the rat dorsal root ganglia and spinal cord following peripheral inflammation and nerve injury. *Neuroreport*, 13(18), pp.2483–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12499853 [Accessed April 9, 2015].

Kim, S.H. & Chung, J.M., 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain*, 50(3), pp.355–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1333581 [Accessed March 9, 2015].

King, T. et al., 2009. Unmasking the tonic-aversive state in neuropathic pain. *Nature neuroscience*, 12(11), pp.1364–6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3427725&tool=pmcentrez&rendertype=abstract [Accessed September 4, 2015].

Knabl, J. et al., 2008. Reversal of pathological pain through specific spinal GABAA receptor subtypes. *Nature*, 451(7176), pp.330–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18202657 [Accessed January 20, 2015].

Knight, Z.A. & Shokat, K.M., 2007. Chemical genetics: where genetics and pharmacology meet. *Cell*, 128(3), pp.425–30. Available at: http://www.sciencedirect.com/science/article/pii/S0092867407001195 [Accessed June 10, 2014].

Kobayashi, K. et al., 2008. P2Y12 receptor upregulation in activated microglia is a gateway of p38 signaling and neuropathic pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 28(11), pp.2892–902. Available at: http://www.jneurosci.org/content/28/11/2892.full [Accessed April 28, 2015].

Koizumi, S. et al., 2007. UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis. *Nature*, 446(7139), pp.1091–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3464483&tool=pmcentrez&rendertype=abstract [Accessed March 28, 2015].

Komori, K. et al., 2004. Absence of mechanical allodynia and Aβ-fiber sprouting after sciatic nerve injury in mice lacking membrane-type 5 matrix metalloproteinase. *FEBS Letters*, 557(1-3), pp.125–128. Available at: http://www.researchgate.net/publication/8905286_Absence_of_mechanical_allodynia_and_A_beta-fiber_sprouting_after_sciatic_nerve_injury_in_mice_lacking_membrane-type_5_matrix_metalloproteinase [Accessed April 16, 2015].

Kwon, Y.K. et al., 1997. Activation of ErbB2 during Wallerian Degeneration of Sciatic Nerve. *J. Neurosci.*, 17(21), pp.8293–8299. Available at: http://www.jneurosci.org/content/17/21/8293.full [Accessed January 6, 2015].

LaCroix-Fralish, M.L. et al., 2006. Sex differences in lumbar spinal cord gene expression following experimental lumbar radiculopathy. *Journal of molecular neuroscience : MN*, 30(3), pp.283–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17401154 [Accessed January 6, 2015].

Latremoliere, A. & Woolf, C.J., 2009. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *The journal of pain : official journal of the American Pain Society*, 10(9), pp.895–926. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2750819&tool=pmcentrez&rendertype=abstract [Accessed July 10, 2014].

Lawson, L.J., Perry, V.H. & Gordon, S., 1992. Turnover of resident microglia in the normal adult mouse brain. *Neuroscience*, 48(2), pp.405–415. Available at: http://www.sciencedirect.com/science/article/pii/0306452292905002 [Accessed June 24, 2015].

Lee, H.-J. et al., 2002. Presenilin-dependent gamma-secretase-like intramembrane cleavage of ErbB4. *The Journal of biological chemistry*, 277(8), pp.6318–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11741961 [Accessed May 4, 2015].

Lee, I.-H. et al., 2005. Glial and neuronal connexin expression patterns in the rat spinal cord during development and following injury. *The Journal of comparative neurology*, 489(1), pp.1–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15977163 [Accessed March 30, 2015].

Lee, K.F. et al., 1995. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature*, 378(6555), pp.394–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7477377 [Accessed May 18, 2015].

Leger, T. et al., 2011. Glatiramer acetate attenuates neuropathic allodynia through modulation of adaptive immune cells. *Journal of neuroimmunology*, 234(1-2), pp.19–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21295862 [Accessed May 18, 2015].

Lemke, G., 1996. Neuregulins in development. *Molecular and cellular neurosciences*, 7(4), pp.247–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8793861 [Accessed April 19, 2015].

Lemmens, K., Doggen, K. & De Keulenaer, G.W., 2007. Role of neuregulin-1/ErbB signaling in cardiovascular physiology and disease: implications for therapy of heart failure. *Circulation*, 116(8), pp.954–60. Available at: http://circ.ahajournals.org/content/116/8/954.full [Accessed May 9, 2015].

Levi, A. et al., 1995. The influence of heregulins on human Schwann cell proliferation. *J. Neurosci.*, 15(2), pp.1329–1340. Available at: http://www.jneurosci.org/content/15/2/1329.abstract [Accessed June 18, 2015].

Levin, M.E. et al., 2008. Complement activation in the peripheral nervous system following the spinal nerve ligation model of neuropathic pain. *Pain*, 137(1), pp.182–201. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18160218 [Accessed May 18, 2015].

Li, H., Terenghi, G. & Hall, S.M., 1997. Effects of delayed re-innervation on the expression of c-erbB receptors by chronically denervated rat Schwann cells in vivo. *Glia*, 20(4), pp.333–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9262237 [Accessed June 2, 2015].

Li, Q. & Loeb, J.A., 2001. Neuregulin-Heparan-sulfate Proteoglycan Interactions Produce Sustained erbB Receptor Activation Required for the Induction of Acetylcholine Receptors in Muscle. *J. Biol. Chem.*, 276(41), pp.38068–38075. Available at: http://www.jbc.org/content/276/41/38068.full [Accessed May 18, 2015].

Lim, H., Kim, D. & Lee, S.J., 2013. Toll-like receptor 2 mediates peripheral nerve injury-induced NADPH oxidase 2 expression in spinal cord microglia. *The Journal of biological chemistry*, 288(11), pp.7572–9. Available at: http://www.jbc.org/content/288/11/7572.long [Accessed April 9, 2015].

Lindholm, T. et al., 2002. Expression of neuregulin and ErbB3 and ErbB4 after a traumatic lesion in the ventral funiculus of the spinal cord and in the intact primary olfactory system. *Experimental brain research. Experimentelle Hirnforschung. Expérimentation cérébrale*, 142(1), pp.81–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11797086 [Accessed December 16, 2013].

LINDIA, J. et al., 2005. Induction of CX3CL1 Expression in Astrocytes and CX3CR1 in Microglia in the Spinal Cord of a Rat Model of Neuropathic Pain. *The Journal of Pain*, 6(7), pp.434–438. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15993821 [Accessed April 16, 2015].

Lindvall, C. et al., 2003. Molecular characterization of human telomerase reverse transcriptase-immortalized human fibroblasts by gene expression profiling: activation of the epiregulin gene. *Cancer research*, 63(8), pp.1743–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12702554 [Accessed February 6, 2015].

Liu, Y. et al., 2007. Stimulated ErbB4 internalization is necessary for neuregulin signaling in neurons. *Biochemical and biophysical research communications*, 354(2), pp.505–10. Available at:

http://www.sciencedirect.com/science/article/pii/S0006291X07000307 [Accessed May 4, 2015].

Loane, D.J. & Byrnes, K.R., 2010. Role of microglia in neurotrauma. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics*, 7(4), pp.366–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2948548&tool=pmcentrez&rendertype=abstract [Accessed March 2, 2015].

Loeb, J. et al., 1999. Expression patterns of transmembrane and released forms of neuregulin during spinal cord and neuromuscular synapse development. *Development*, 126(4), pp.781–791. Available at: http://dev.biologists.org/content/126/4/781.abstract [Accessed June 9, 2015].

Loeb, J.A. & Fischbach, G.D., 1995. ARIA can be released from extracellular matrix through cleavage of a heparin-binding domain. *The Journal of cell biology*, 130(1), pp.127–35. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2120519&tool=pmcentrez&rendertype=abstract [Accessed May 18, 2015].

Loeb, J.A., Susanto, E.T. & Fischbach, G.D., 1998. The neuregulin precursor proARIA is processed to ARIA after expression on the cell surface by a protein kinase C-enhanced mechanism. *Molecular and cellular neurosciences*, 11(1-2), pp.77–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9608535 [Accessed April 19, 2015].

Loggia, M.L. et al., 2015. Evidence for brain glial activation in chronic pain patients. *Brain : a journal of neurology*, 138(Pt 3), pp.604–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25582579 [Accessed September 27, 2015].

Loonstra, A. et al., 2001. Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, 98(16), pp.9209–14. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=55399&tool=pmcentrez&rendertype=abstract [Accessed February 15, 2015].

López-Bendito, G. et al., 2006. Tangential neuronal migration controls axon guidance: a role for neuregulin-1 in thalamocortical axon navigation. *Cell*, 125(1), pp.127–42. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2365888&tool=pmcentrez&rendertype=abstract [Accessed May 6, 2015].

Lull, M.E. & Block, M.L., 2010. Microglial activation and chronic neurodegeneration. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics*, 7(4), pp.354–65. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2951017&tool=pmcentrez&rendertype=abstract [Accessed March 20, 2015].

Lyons, S.A. & Kettenmann, H., 1998. Oligodendrocytes and microglia are selectively vulnerable to combined hypoxia and hypoglycemia injury in vitro. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, 18(5), pp.521–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9591844 [Accessed March 30, 2015].

Ma, F., Zhang, L. & Westlund, K.N., 2012. Trigeminal nerve injury ErbB3/ErbB2 promotes mechanical hypersensitivity. *Anesthesiology*, 117(2), pp.381–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3406246&tool=pmcentrez&rendertype=abstract [Accessed October 29, 2013].

Ma, W. & Quirion, R., 2002. Partial sciatic nerve ligation induces increase in the phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) in astrocytes in the lumbar spinal dorsal horn and the gracile nucleus. *Pain*, 99(1), pp.175–184. Available at: http://www.sciencedirect.com/science/article/pii/S0304395902000970 [Accessed February 6, 2015].

Maciejewski-Lenoir, D. et al., 1999. Characterization of fractalkine in rat brain cells: migratory and activation signals for CX3CR-1-expressing microglia. *Journal of immunology (Baltimore, Md. : 1950)*, 163(3), pp.1628–35. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10415068 [Accessed May 18, 2015].

Maier, A., 1997. Development and regeneration of muscle spindles in mammals and birds. *The International journal of developmental biology*, 41(1), pp.1–17. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9074933 [Accessed February 8, 2015].

Makinodan, M. et al., 2012. A critical period for social experience-dependent oligodendrocyte maturation and myelination. *Science (New York, N.Y.)*, 337(6100), pp.1357–60. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4165613&tool=pmcentrez&rendertype=abstract [Accessed June 30, 2015].

Mamane, Y. et al., 1999. Interferon regulatory factors: the next generation. *Gene*, 237(1), pp.1–14. Available at: http://www.sciencedirect.com/science/article/pii/S0378111999002620 [Accessed April 15, 2015].

Mantyh, P.W. et al., 2002. Molecular mechanisms of cancer pain. *Nature reviews. Cancer*, 2(3), pp.201–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11990856 [Accessed February 3, 2015].

Marchand, F., Perretti, M. & McMahon, S.B., 2005. Role of the immune system in chronic pain. *Nature reviews. Neuroscience*, 6(7), pp.521–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15995723 [Accessed February 16, 2015].

Marchionni, M.A. et al., 1993a. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature*, 362(6418), pp.312–8.

Available at: http://www.ncbi.nlm.nih.gov/pubmed/8096067 [Accessed April 18, 2015].

Marchionni, M.A. et al., 1993b. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature*, 362(6418), pp.312–8. Available at: http://dx.doi.org/10.1038/362312a0 [Accessed December 14, 2014].

McKercher, S.R. et al., 1996. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *The EMBO journal*, 15(20), pp.5647–58. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=452309&tool=pmcentrez&rendertype=abstract [Accessed March 30, 2015].

McMahon, S.B. & Malcangio, M., 2009. Current challenges in glia-pain biology. *Neuron*, 64(1), pp.46–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19840548 [Accessed September 2, 2015].

Mei, L. & Xiong, W.-C., 2008. Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. *Nature reviews. Neuroscience*, 9(6), pp.437–52. Available at: http://dx.doi.org/10.1038/nrn2392 [Accessed March 2, 2015].

Mei, X.-P. et al., 2011. Ketamine Depresses Toll-Like Receptor 3 Signaling in Spinal Microglia in a Rat Model of Neuropathic Pain. *Neurosignals*, 19(1), pp.44–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21389680 [Accessed April 9, 2015].

De Mello, T.R. et al., 2007. Culture conditions affect proliferative responsiveness of olfactory ensheathing glia to neuregulins. *Glia*, 55(7), pp.734–45. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17348025 [Accessed June 11, 2015].

Mencel, M., Nash, M. & Jacobson, C., 2013. Neuregulin upregulates microglial α7 nicotinic acetylcholine receptor expression in immortalized cell lines: implications for regulating neuroinflammation. *PloS one*, 8(7), p.e70338. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3728089&tool=pmcentrez&rendertype=abstract [Accessed October 29, 2013].

Merighi, S. et al., 2012. Cannabinoid CB2 receptors modulate ERK-1/2 kinase signalling and NO release in microglial cells stimulated with bacterial lipopolysaccharide. *British Journal of Pharmacology*, 165(6), pp.1773–1788. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3372829&tool=pmcentrez&rendertype=abstract [Accessed June 29, 2015].

Meyer, D. & Birchmeier, C., 1995. Multiple essential functions of neuregulin in development. *Nature*, 378(6555), pp.386–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7477375 [Accessed April 19, 2015].

Mifune, M. et al., 2004. Signal transduction of betacellulin in growth and migration of vascular smooth muscle cells. *American journal of physiology. Cell physiology*, 287(3), pp.C807–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15163624 [Accessed February 5, 2015].

Mildner, A. et al., 2007. Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. *Nature Neuroscience*, 10(12), pp.1544–1553. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18026096 [Accessed September 21, 2015].

Milligan, E. et al., 2005. An initial investigation of spinal mechanisms underlying pain enhancement induced by fractalkine, a neuronally released chemokine. *The European journal of neuroscience*, 22(11), pp.2775–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16324111 [Accessed April 16, 2015].

Milligan, E.D. et al., 2004. Evidence that exogenous and endogenous fractalkine can induce spinal nociceptive facilitation in rats. *The European journal of neuroscience*, 20(9), pp.2294–302. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15525271 [Accessed April 15, 2015].

Milligan, E.D. et al., 2001. Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 21(8), pp.2808–19. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11306633 [Accessed April 7, 2015].

Milligan, E.D. et al., 2003. Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 23(3), pp.1026–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12574433 [Accessed April 8, 2015].

Minett, M.S., Eijkelkamp, N. & Wood, J.N., 2014. Significant determinants of mouse pain behaviour. S. Beggs, ed. *PloS one*, 9(8), p.e104458. Available at: http://dx.plos.org/10.1371/journal.pone.0104458 [Accessed January 12, 2015].

Mogil, J.S. & Crager, S.E., 2004. What should we be measuring in behavioral studies of chronic pain in animals? *Pain*, 112(1-2), pp.12–5. Available at: http://www.sciencedirect.com/science/article/pii/S0304395904004579 [Accessed June 26, 2014].

Montero, J.C. et al., 2007. The extracellular linker of pro-neuregulin-alpha2c is required for efficient sorting and juxtacrine function. *Molecular biology of the cell*, 18(2), pp.380–93. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1783780&tool=pmcentrez&rendertype=abstract [Accessed April 19, 2015].

Moore, K.A. et al., 2002. Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience*,

22(15), pp.6724–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12151551 [Accessed March 24, 2015].

Moussaud, S. & Draheim, H.J., 2010. A new method to isolate microglia from adult mice and culture them for an extended period of time. *Journal of neuroscience methods*, 187(2), pp.243–53. Available at: http://www.sciencedirect.com/science/article/pii/S0165027010000397 [Accessed July 16, 2014].

Mueller, M. et al., 2001. Rapid response of identified resident endoneurial macrophages to nerve injury. *The American journal of pathology*, 159(6), pp.2187–97. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1850587&tool=pmcentrez&rendertype=abstract [Accessed March 25, 2015].

Myers, R.R., Heckman, H.M. & Rodriguez, M., 1996. Reduced hyperalgesia in nerve-injured WLD mice: relationship to nerve fiber phagocytosis, axonal degeneration, and regeneration in normal mice. *Experimental neurology*, 141(1), pp.94–101. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8797671 [Accessed March 27, 2015].

Nagy, A., 2000. Cre recombinase: the universal reagent for genome tailoring. *Genesis (New York, N.Y. : 2000)*, 26(2), pp.99–109. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10686599 [Accessed January 29, 2015].

Nagy, J.I., Dudek, F.E. & Rash, J.E., 2004. Update on connexins and gap junctions in neurons and glia in the mammalian nervous system. *Brain research. Brain research reviews*, 47(1-3), pp.191–215. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15572172 [Accessed February 24, 2015].

Nasu-Tada, K. et al., 2006. Possible involvement of increase in spinal fibronectin following peripheral nerve injury in upregulation of microglial P2X4, a key molecule for mechanical allodynia. *Glia*, 53(7), pp.769–75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16534777 [Accessed May 16, 2015].

Nasu-Tada, K., Koizumi, S. & Inoue, K., 2005. Involvement of beta1 integrin in microglial chemotaxis and proliferation on fibronectin: different regulations by ADP through PKA. *Glia*, 52(2), pp.98–107. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15920726 [Accessed April 29, 2015].

Neher, J.J. et al., 2011. Inhibition of microglial phagocytosis is sufficient to prevent inflammatory neuronal death. *Journal of immunology (Baltimore, Md. : 1950)*, 186(8), pp.4973–83. Available at: http://www.jimmunol.org/content/186/8/4973.full [Accessed November 26, 2014].

Neirinckx, V. et al., 2014. Neutrophil contribution to spinal cord injury and repair. *Journal of neuroinflammation*, 11(1), p.150. Available at: http://www.jneuroinflammation.com/content/11/1/150 [Accessed June 24, 2015].

Neniskyte, U. & Brown, G.C., 2013. Lactadherin/MFG-E8 is essential for microglia-mediated neuronal loss and phagoptosis induced by amyloid β. *Journal of neurochemistry*, 126(3), pp.312–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23647050 [Accessed May 10, 2015].

Neniskyte, U., Neher, J.J. & Brown, G.C., 2011. Neuronal death induced by nanomolar amyloid β is mediated by primary phagocytosis of neurons by microglia. *The Journal of biological chemistry*, 286(46), pp.39904–13. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3220594&tool=pmcentrez&rendertype=abstract [Accessed March 12, 2015].

Nesic, O. et al., 2005. Transcriptional profiling of spinal cord injury-induced central neuropathic pain. *Journal of neurochemistry*, 95(4), pp.998–1014. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16219025 [Accessed March 27, 2015].

Neumann, H., Kotter, M.R. & Franklin, R.J.M., 2009. Debris clearance by microglia: an essential link between degeneration and regeneration. *Brain : a journal of neurology*, 132(Pt 2), pp.288–95. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2640215&tool=pmcentrez&rendertype=abstract [Accessed September 20, 2015].

Nguyen, M.D., Julien, J.-P. & Rivest, S., 2002. Innate immunity: the missing link in neuroprotection and neurodegeneration? *Nature reviews. Neuroscience*, 3(3), pp.216–27. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11994753 [Accessed August 31, 2015].

Ni, C.Y. et al., 2001. gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science (New York, N.Y.)*, 294(5549), pp.2179–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11679632 [Accessed May 4, 2015].

Nicol, G.D., Lopshire, J.C. & Pafford, C.M., 1997. Tumor Necrosis Factor Enhances the Capsaicin Sensitivity of Rat Sensory Neurons. *J. Neurosci.*, 17(3), pp.975–982. Available at: http://www.jneurosci.org/content/17/3/975.long [Accessed March 27, 2015].

Nishi, E. et al., 2006. Nardilysin enhances ectodomain shedding of heparin-binding epidermal growth factor-like growth factor through activation of tumor necrosis factor-alpha-converting enzyme. *The Journal of biological chemistry*, 281(41), pp.31164–72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16923819 [Accessed April 19, 2015].

Nissenbaum, J. et al., 2010. Susceptibility to chronic pain following nerve injury is genetically affected by CACNG2. *Genome research*, 20(9), pp.1180–90. Available at: http://genome.cshlp.org/content/early/2010/08/02/gr.104976.110.abstract [Accessed March 17, 2015].

Noda, M. et al., 2011. Fractalkine attenuates excito-neurotoxicity via microglial clearance of damaged neurons and antioxidant enzyme heme oxygenase-1 expression. *The Journal of biological chemistry*, 286(3), pp.2308–19. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3023525&tool=pmcentrez&rendertype=abstract [Accessed May 10, 2015].

Noël, J. et al., 2009. The mechano-activated K+ channels TRAAK and TREK-1 control both warm and cold perception. *The EMBO journal*, 28(9), pp.1308–18. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2683043&tool=pmcentrez&rendertype=abstract [Accessed June 9, 2015].

Nolte, C. et al., 1996. Complement 5a controls motility of murine microglial cells in vitro via activation of an inhibitory G-protein and the rearrangement of the actin cytoskeleton. *Neuroscience*, 73(4), pp.1091–107. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8809827 [Accessed May 18, 2015].

North, R.A., 2002. Molecular physiology of P2X receptors. *Physiological reviews*, 82(4), pp.1013–67. Available at: http://physrev.physiology.org/content/82/4/1013 [Accessed January 5, 2015].

Obata, K. et al., 2008. Toll-like receptor 3 contributes to spinal glial activation and tactile allodynia after nerve injury. *Journal of neurochemistry*, 105(6), pp.2249–59. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18363823 [Accessed April 9, 2015].

Obreja, O. et al., 2002. IL-1 beta potentiates heat-activated currents in rat sensory neurons: involvement of IL-1RI, tyrosine kinase, and protein kinase C. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 16(12), pp.1497–503. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12374772 [Accessed March 27, 2015].

Ohsawa, K. et al., 2007. Involvement of P2X4 and P2Y12 receptors in ATP-induced microglial chemotaxis. *Glia*, 55(6), pp.604–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17299767 [Accessed May 10, 2015].

Orban, P.C., Chui, D. & Marth, J.D., 1992. Tissue- and site-specific DNA recombination in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America*, 89(15), pp.6861–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=49604&tool=pmcentrez&rendertype=abstract [Accessed February 11, 2015].

Orr-Urtreger, A. et al., 1993. Neural expression and chromosomal mapping of Neu differentiation factor to 8p12-p21. *Proceedings of the National Academy of Sciences of the United States of America*, 90(5), pp.1867–71. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=45981&tool=pmcentrez&rendertype=abstract [Accessed April 19, 2015].

Ossipov, M.H. & Porreca, F., 2005. Challenges in the development of novel treatment strategies for neuropathic pain. *NeuroRX*, 2(4), pp.650–661. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1201322&tool=pmcentrez&rendertype=abstract [Accessed March 17, 2015].

Ozaki, M. et al., 2004. Protein processing and releases of neuregulin-1 are regulated in an activity-dependent manner. *Journal of neurochemistry*, 91(1), pp.176–88. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15379898 [Accessed May 6, 2015].

Parkhurst, C.N. et al., 2013. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. *Cell*, 155(7), pp.1596–609. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4033691&tool=pmcentrez&rendertype=abstract [Accessed July 17, 2014].

Parks, W.C., Wilson, C.L. & López-Boado, Y.S., 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nature reviews. Immunology*, 4(8), pp.617–29. Available at: http://dx.doi.org/10.1038/nri1418 [Accessed January 30, 2015].

Parodi, E.M. & Kuhn, B., 2014. Signalling between microvascular endothelium and cardiomyocytes through neuregulin. *Cardiovascular research*, 102(2), pp.194–204. Available at: http://cardiovascres.oxfordjournals.org/content/102/2/194 [Accessed June 16, 2015].

Pearson, R.J. & Carroll, S.L., 2004. ErbB Transmembrane Tyrosine Kinase Receptors Are Expressed by Sensory and Motor Neurons Projecting into Sciatic Nerve. *Journal of Histochemistry & Cytochemistry*, 52(10), pp.1299–311. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15385576 [Accessed October 29, 2013].

Peles, E. et al., 1992. Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell*, 69(1), pp.205–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1348215 [Accessed April 18, 2015].

Penn, R.B. et al., 1999. Pharmacological Inhibition of Protein Kinases in Intact Cells: Antagonism of Beta Adrenergic Receptor Ligand Binding by H-89 Reveals Limitations of Usefulness. *J. Pharmacol. Exp. Ther.*, 288(2), pp.428–437. Available at: http://jpet.aspetjournals.org/content/288/2/428.short [Accessed September 20, 2015].

Peters, C.M. et al., 2007. Intravenous paclitaxel administration in the rat induces a peripheral sensory neuropathy characterized by macrophage infiltration and injury to sensory neurons and their supporting cells. *Experimental neurology*, 203(1), pp.42–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17005179 [Accessed April 7, 2015].

Pethö, G., Derow, A. & Reeh, P.W., 2001. Bradykinin-induced nociceptor sensitization to heat is mediated by cyclooxygenase products in isolated rat skin.

The European journal of neuroscience, 14(2), pp.210–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11553274 [Accessed March 23, 2015].

Pezet, S. et al., 2002. Noxious stimulation induces Trk receptor and downstream ERK phosphorylation in spinal dorsal horn. *Molecular and cellular neurosciences*, 21(4), pp.684–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12504600 [Accessed March 24, 2015].

Pinto-Ribeiro, F. et al., 2004. Chronic unpredictable stress inhibits nociception in male rats. *Neuroscience letters*, 359(1-2), pp.73–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15050715 [Accessed June 9, 2015].

Polazzi, E. & Monti, B., 2010. Microglia and neuroprotection: from in vitro studies to therapeutic applications. *Progress in neurobiology*, 92(3), pp.293–315. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20609379 [Accessed August 31, 2015].

Polito, A. et al., 2011. Hyperglycaemia and apoptosis of microglial cells in human septic shock. *Critical care (London, England)*, 15(3), p.R131. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3218997&tool=pmcentrez&rendertype=abstract [Accessed March 30, 2015].

Qi, J. et al., 2011. Painful pathways induced by TLR stimulation of dorsal root ganglion neurons. *Journal of immunology (Baltimore, Md. : 1950)*, 186(11), pp.6417–26. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3098909&tool=pmcentrez&rendertype=abstract [Accessed March 4, 2015].

Raghavendra, V., Tanga, F.Y. & DeLeo, J.A., 2004. Complete Freunds adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. *The European journal of neuroscience*, 20(2), pp.467–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15233755 [Accessed March 27, 2015].

RANDALL, L.O. & SELITTO, J.J., 1957. A method for measurement of analgesic activity on inflamed tissue. *Archives internationales de pharmacodynamie et de thérapie*, 111(4), pp.409–19. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13471093 [Accessed June 26, 2014].

Ransohoff, R.M., 1997. Chemokines in neurological disease models: correlation between chemokine expression patterns and inflammatory pathology. *Journal of leukocyte biology*, 62(5), pp.645–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9365119 [Accessed March 27, 2015].

Ren, K., 2010. Emerging role of astroglia in pain hypersensitivity. *The Japanese dental science review*, 46(1), p.86. Available at: http://www.sciencedirect.com/science/article/pii/S1882761609000842 [Accessed March 30, 2015].

Rexed, B., 1952. The cytoarchitectonic organization of the spinal cord in the cat. *The Journal of Comparative Neurology*, 96(3), pp.415–495. Available at: http://doi.wiley.com/10.1002/cne.900960303 [Accessed March 24, 2015].

Rezaie, P. & Male, D., 2002. Mesoglia & microglia--a historical review of the concept of mononuclear phagocytes within the central nervous system. *Journal of the history of the neurosciences*, 11(4), pp.325–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12557654 [Accessed March 30, 2015].

Ribeiro, R.A. et al., 2000. Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. *European journal of pharmacology*, 387(1), pp.111–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10633169 [Accessed March 27, 2015].

Riethmacher, D. et al., 1997. Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature*, 389(6652), pp.725–30. Available at: http://dx.doi.org/10.1038/39593 [Accessed May 13, 2014].

Rigaud, M. et al., 2008. Species and strain differences in rodent sciatic nerve anatomy: implications for studies of neuropathic pain. *Pain*, 136(1-2), pp.188–201. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2700063&tool=pmcentrez&rendertype=abstract [Accessed November 12, 2014].

Rio, C. et al., 1997. Neuregulin and erbB receptors play a critical role in neuronal migration. *Neuron*, 19(1), pp.39–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9247262 [Accessed May 6, 2015].

Rivest, S., 2009. Regulation of innate immune responses in the brain. *Nature reviews. Immunology*, 9(6), pp.429–39. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19461673 [Accessed April 19, 2015].

Rohlmann, A. et al., 1993. Facial nerve lesions lead to increased immunostaining of the astrocytic gap junction protein (connexin 43) in the corresponding facial nucleus of rats. *Neuroscience letters*, 154(1-2), pp.206–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8395666 [Accessed March 30, 2015].

Rosenberg, G.A., 2002. Matrix metalloproteinases in neuroinflammation. *Glia*, 39(3), pp.279–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12203394 [Accessed April 16, 2015].

Rosmarin, A.G. et al., 1989. Differential expression of CD11b/CD18 (Mo1) and myeloperoxidase genes during myeloid differentiation. *Blood*, 73(1), pp.131–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2562920 [Accessed January 19, 2015].

Saito, T. et al., 2004. Differential activation of epidermal growth factor (EGF) receptor downstream signaling pathways by betacellulin and EGF. *Endocrinology*, 145(9), pp.4232–43. Available at:

http://press.endocrine.org/doi/full/10.1210/en.2004-0401 [Accessed February 5, 2015].

Sandkühler, J., 2009. Models and mechanisms of hyperalgesia and allodynia. *Physiological reviews*, 89(2), pp.707–58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19342617 [Accessed November 28, 2014].

Sardi, S.P. et al., 2006. Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. *Cell*, 127(1), pp.185–97. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17018285 [Accessed May 4, 2015].

Schaeffer, H.J. & Weber, M.J., 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Molecular and cellular biology*, 19(4), pp.2435–44. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=84036&tool=pmcentrez&rendertype=abstract [Accessed February 17, 2015].

Schäfers, M. et al., 2003. Tumor necrosis factor-alpha induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 23(7), pp.2517–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12684435 [Accessed April 9, 2015].

Schoenborn, J.R. & Wilson, C.B., 2007. Regulation of interferon-gamma during innate and adaptive immune responses. *Advances in immunology*, 96, pp.41–101. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17981204 [Accessed November 4, 2014].

Scholz, J. & Woolf, C.J., 2007a. The neuropathic pain triad: neurons, immune cells and glia. *Nature neuroscience*, 10(11), pp.1361–8. Available at: http://dx.doi.org/10.1038/nn1992 [Accessed May 27, 2014].

Scholz, J. & Woolf, C.J., 2007b. The neuropathic pain triad: neurons, immune cells and glia. *Nature neuroscience*, 10(11), pp.1361–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17965656 [Accessed July 18, 2014].

Schroder, K. et al., 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *Journal of leukocyte biology*, 75(2), pp.163–89. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14525967 [Accessed January 7, 2015].

Schwartz, M. et al., 2006. Microglial phenotype: is the commitment reversible? *Trends in neurosciences*, 29(2), pp.68–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16406093 [Accessed June 25, 2015].

Seal, R.P. et al., 2009. Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. *Nature*, 462(7273), pp.651–5. Available at: http://dx.doi.org/10.1038/nature08505 [Accessed September 13, 2015].

Sedgwick, J.D. et al., 1991. Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. *Proceedings of the National Academy of Sciences of the United States of America*, 88(16), pp.7438–42. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=52311&tool=pmcentrez&rendertype=abstract [Accessed June 29, 2015].

Sheean, M.E. et al., 2014. Activation of MAPK overrides the termination of myelin growth and replaces Nrg1/ErbB3 signals during Schwann cell development and myelination. *Genes & development*, 28(3), pp.290–303. Available at: http://genesdev.cshlp.org/content/28/3/290 [Accessed June 22, 2015].

Shi, Y. et al., 2012. Chronic-pain-associated astrocytic reaction in the spinal cord dorsal horn of human immunodeficiency virus-infected patients. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 32(32), pp.10833–40. Available at: http://www.jneurosci.org/content/32/32/10833.full [Accessed September 27, 2015].

Shields, S.D., Eckert, W.A. & Basbaum, A.I., 2003. Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. *The journal of pain : official journal of the American Pain Society*, 4(8), pp.465–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14622667 [Accessed March 9, 2015].

Shinder, V. et al., 1999. Structural basis of sympathetic-sensory coupling in rat and human dorsal root ganglia following peripheral nerve injury. *Journal of neurocytology*, 28(9), pp.743–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10859576 [Accessed March 6, 2015].

Sierra, A. et al., 2007. Microglia derived from aging mice exhibit an altered inflammatory profile. *Glia*, 55(4), pp.412–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17203473 [Accessed May 18, 2015].

Simone, D.A. & Kajander, K.C., 1996. Excitation of rat cutaneous nociceptors by noxious cold. *Neuroscience letters*, 213(1), pp.53–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8844711 [Accessed June 9, 2015].

Slack, S.E. et al., 2004. Brain-derived neurotrophic factor induces NMDA receptor subunit one phosphorylation via ERK and PKC in the rat spinal cord. *The European journal of neuroscience*, 20(7), pp.1769–78. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15379998 [Accessed March 24, 2015].

Slack, S.E. et al., 2005. TrkB expression and phospho-ERK activation by brain-derived neurotrophic factor in rat spinothalamic tract neurons. *The Journal of comparative neurology*, 489(1), pp.59–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15977164 [Accessed March 24, 2015].

Snider, W.D. & McMahon, S.B., 1998. Tackling pain at the source: new ideas about nociceptors. *Neuron*, 20(4), pp.629–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9581756 [Accessed March 20, 2015].

Sommer, C. & Schäfers, M., 1998. Painful mononeuropathy in C57BL/Wld mice with delayed wallerian degeneration: differential effects of cytokine production and nerve regeneration on thermal and mechanical hypersensitivity. *Brain research*, 784(1-2), pp.154–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9518588 [Accessed March 27, 2015].

Sorge, R.E. et al., 2015. Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nature Neuroscience*, 18(8), pp.1081–1083. Available at: http://dx.doi.org/10.1038/nn.4053 [Accessed June 30, 2015].

Sorge, R.E. et al., 2011. Spinal Cord Toll-Like Receptor 4 Mediates Inflammatory and Neuropathic Hypersensitivity in Male But Not Female Mice. *Journal of Neuroscience*, 31(43), pp.15450–15454. Available at: http://www.jneurosci.org/content/31/43/15450.long [Accessed July 2, 2015].

Spataro, L.E. et al., 2004. Spinal gap junctions: potential involvement in pain facilitation. *The journal of pain : official journal of the American Pain Society*, 5(7), pp.392–405. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15501197 [Accessed March 30, 2015].

Stassart, R.M. et al., 2013. A role for Schwann cell-derived neuregulin-1 in remyelination. *Nature neuroscience*, 16(1), pp.48–54. Available at: http://dx.doi.org/10.1038/nn.3281 [Accessed December 4, 2014].

Staud, R., Robinson, M.E. & Price, D.D., 2007. Temporal summation of second pain and its maintenance are useful for characterizing widespread central sensitization of fibromyalgia patients. *The journal of pain : official journal of the American Pain Society*, 8(11), pp.893–901. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2174917&tool=pmcentrez&rendertype=abstract [Accessed February 17, 2015].

Stevens, B. et al., 2007. The classical complement cascade mediates CNS synapse elimination. *Cell*, 131(6), pp.1164–78. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18083105 [Accessed January 31, 2015].

Streit, W.J. et al., 2005. Role of microglia in the central nervous system's immune response. *Neurological research*, 27(7), pp.685–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16197805 [Accessed April 7, 2015].

Sun, H. et al., 2001. Nerve injury-induced tactile allodynia is mediated via ascending spinal dorsal column projections. *Pain*, 90(1-2), pp.105–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11166976 [Accessed March 24, 2015].

Sung, B. et al., 1998. Supraspinal involvement in the production of mechanical allodynia by spinal nerve injury in rats. *Neuroscience letters*, 246(2), pp.117–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9627194 [Accessed March 24, 2015].

Suter, M.R. et al., 2009. Large A-fiber activity is required for microglial proliferation and p38 MAPK activation in the spinal cord: different effects of resiniferatoxin

and bupivacaine on spinal microglial changes after spared nerve injury. *Molecular pain*, 5(1), p.53. Available at: http://www.molecularpain.com/content/5/1/53 [Accessed March 5, 2015].

Svensson, C.I. et al., 2005. Spinal blockade of TNF blocks spinal nerve ligation-induced increases in spinal P-p38. *Neuroscience Letters*, 379(3), pp.209–213. Available at: http://www.sciencedirect.com/science/article/pii/S0304394005000145 [Accessed April 9, 2015].

Svensson, C.I. & Brodin, E., 2010. Spinal astrocytes in pain processing: non-neuronal cells as therapeutic targets. *Molecular interventions*, 10(1), pp.25–38. Available at: http://triggered.clockss.org/ServeContent?url=http%3A%2F%2Fmolinterv.aspetjournals.org%2Fcontent%2F10%2F1%2F25.full [Accessed February 6, 2015].

Sweitzer, S., Martin, D. & DeLeo, J.A., 2001. Intrathecal interleukin-1 receptor antagonist in combination with soluble tumor necrosis factor receptor exhibits an anti-allodynic action in a rat model of neuropathic pain. *Neuroscience*, 103(2), pp.529–39. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11246166 [Accessed April 8, 2015].

Sweitzer, S.M. & DeLeo, J.A., 2002. The active metabolite of leflunomide, an immunosuppressive agent, reduces mechanical sensitivity in a rat mononeuropathy model. *The journal of pain : official journal of the American Pain Society*, 3(5), pp.360–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14622739 [Accessed May 18, 2015].

Sweitzer, S.M., Pahl, J.L. & DeLeo, J.A., 2006. Propentofylline attenuates vincristine-induced peripheral neuropathy in the rat. *Neuroscience letters*, 400(3), pp.258–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16530953 [Accessed April 7, 2015].

Talbot, S. et al., 2010a. Key role for spinal dorsal horn microglial kinin B1 receptor in early diabetic pain neuropathy. *Journal of Neuroinflammation*, 7(1), p.36. Available at: http://www.jneuroinflammation.com/content/7/1/36 [Accessed April 7, 2015].

Talbot, S. et al., 2010b. Key role for spinal dorsal horn microglial kinin B1 receptor in early diabetic pain neuropathy. *Journal of Neuroinflammation*, 7(1), p.36. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2913947&tool=pmcentrez&rendertype=abstract [Accessed April 7, 2015].

Tan, W. et al., 2007. Molecular cloning of a brain-specific, developmentally regulated neuregulin 1 (NRG1) isoform and identification of a functional promoter variant associated with schizophrenia. *The Journal of biological chemistry*, 282(33), pp.24343–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17565985 [Accessed June 16, 2015].

Tanaka, T. et al., 2004. Enhanced production of monocyte chemoattractant protein-1 in the dorsal root ganglia in a rat model of neuropathic pain: possible involvement in the development of neuropathic pain. *Neuroscience research*, 48(4), pp.463–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15041200 [Accessed May 5, 2015].

Tanga, F.Y. et al., 2006. Role of astrocytic S100beta in behavioral hypersensitivity in rodent models of neuropathic pain. *Neuroscience*, 140(3), pp.1003–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16600520 [Accessed March 27, 2015].

Tanga, F.Y., Nutile-McMenemy, N. & DeLeo, J.A., 2005. The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proceedings of the National Academy of Sciences of the United States of America*, 102(16), pp.5856–61. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=556308&tool=pmcentrez&rendertype=abstract [Accessed April 9, 2015].

Taskinen, H.S. & Röyttä, M., 1997. The dynamics of macrophage recruitment after nerve transection. *Acta neuropathologica*, 93(3), pp.252–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9083556 [Accessed March 27, 2015].

Taveggia, C. et al., 2005. Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron*, 47(5), pp.681–94. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2387056&tool=pmcentrez&rendertype=abstract [Accessed January 13, 2015].

Taveggia, C., Feltri, M.L. & Wrabetz, L., 2010. Signals to promote myelin formation and repair. *Nature reviews. Neurology*, 6(5), pp.276–87. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3062363&tool=pmcentrez&rendertype=abstract [Accessed November 23, 2014].

Terayama, R. et al., 2008. Activation of microglia and p38 mitogen-activated protein kinase in the dorsal column nucleus contributes to tactile allodynia following peripheral nerve injury. *Neuroscience*, 153(4), pp.1245–55. Available at: http://www.sciencedirect.com/science/article/pii/S0306452208004685 [Accessed April 9, 2015].

Thacker, M.A. et al., 2009. CCL2 is a key mediator of microglia activation in neuropathic pain states. *European journal of pain (London, England)*, 13(3), pp.263–72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18554968 [Accessed May 11, 2015].

Thacker, M.A. et al., 2007. Pathophysiology of peripheral neuropathic pain: immune cells and molecules. *Anesthesia and analgesia*, 105(3), pp.838–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17717248 [Accessed March 23, 2015].

Thomazzi, S.M. et al., 1997. Tumor necrosis factor, interleukin-1 and interleukin-8 mediate the nociceptive activity of the supernatant of LPS-stimulated macrophages. *Mediators of inflammation*, 6(3), pp.195–200. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2365829&tool=pmcentrez&rendertype=abstract [Accessed March 27, 2015].

Toulme, E. et al., 2010. P2X4 receptors in activated C8-B4 cells of cerebellar microglial origin. *The Journal of general physiology*, 135(4), pp.333–53. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2847917&tool=pmcentrez&rendertype=abstract [Accessed May 16, 2015].

Town, T., Nikolic, V. & Tan, J., 2005. The microglial "activation" continuum: from innate to adaptive responses. *Journal of neuroinflammation*, 2, p.24. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1298325&tool=pmcentrez&rendertype=abstract [Accessed September 20, 2015].

Toyoda, H. et al., 1997. Distribution of mRNA for human epiregulin, a differentially expressed member of the epidermal growth factor family. *The Biochemical journal*, 326 (Pt 1, pp.69–75. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1218638&tool=pmcentrez&rendertype=abstract [Accessed February 6, 2015].

Tozaki-Saitoh, H. et al., 2008. P2Y12 Receptors in Spinal Microglia Are Required for Neuropathic Pain after Peripheral Nerve Injury. *Journal of Neuroscience*, 28(19), pp.4949–4956. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18463248 [Accessed April 7, 2015].

Trang, T., Beggs, S. & Salter, M.W., 2011. Brain-derived neurotrophic factor from microglia: a molecular substrate for neuropathic pain. *Neuron glia biology*, 7(1), pp.99–108. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3748035&tool=pmcentrez&rendertype=abstract [Accessed October 29, 2013].

Tremblay, M.-È. et al., 2011. The role of microglia in the healthy brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 31(45), pp.16064–9. Available at: http://www.jneurosci.org/content/31/45/16064.short [Accessed July 20, 2014].

Tsantoulas, C. et al., 2012. Sensory neuron downregulation of the Kv9.1 potassium channel subunit mediates neuropathic pain following nerve injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 32(48), pp.17502–13. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3713313&tool=pmcentrez&rendertype=abstract [Accessed March 23, 2015].

Tsuda, M. et al., 2008. Activation of dorsal horn microglia contributes to diabetes-induced tactile allodynia via extracellular signal-regulated protein kinase signaling. *Glia*, 56(4), pp.378–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18186080 [Accessed March 30, 2015].

Tsuda, M. et al., 2004. Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. *Glia*, 45(1), pp.89–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14648549 [Accessed April 9, 2015].

Tsuda, M. et al., 2009. Behavioral phenotypes of mice lacking purinergic P2X4 receptors in acute and chronic pain assays. *Molecular pain*, 5, p.28. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2704200&tool=pmcentrez&rendertype=abstract [Accessed April 20, 2015].

Tsuda, M. et al., 2009. IFN- receptor signaling mediates spinal microglia activation driving neuropathic pain. *Proceedings of the National Academy of Sciences*, 106(19), pp.8032–8037. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2683100&tool=pmcentrez&rendertype=abstract [Accessed April 9, 2015].

Tsuda, M. et al., 2013. Microglial regulation of neuropathic pain. *Journal of pharmacological sciences*, 121(2), pp.89–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23337437 [Accessed March 23, 2015].

Tsuda, M. et al., 2003. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature*, 424(6950), pp.778–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12917686 [Accessed November 12, 2014].

Tsuda, M., Inoue, K. & Salter, M.W., 2005. Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. *Trends in neurosciences*, 28(2), pp.101–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15667933 [Accessed March 30, 2015].

Tsuda, M., Tozaki-Saitoh, H. & Inoue, K., 2010. Pain and purinergic signaling. *Brain research reviews*, 63(1-2), pp.222–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19931560 [Accessed March 30, 2015].

Twining, C.M. et al., 2005. Activation of the spinal cord complement cascade might contribute to mechanical allodynia induced by three animal models of spinal sensitization. *The journal of pain : official journal of the American Pain Society*, 6(3), pp.174–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15772911 [Accessed May 18, 2015].

Ulmann, L. et al., 2008. Up-regulation of P2X4 receptors in spinal microglia after peripheral nerve injury mediates BDNF release and neuropathic pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 28(44), pp.11263–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18971468 [Accessed April 20, 2015].

Vale, M.L. et al., 2003. Antinociceptive effects of interleukin-4, -10, and -13 on the writhing response in mice and zymosan-induced knee joint incapacitation in rats. *The Journal of pharmacology and experimental therapeutics*, 304(1), pp.102–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12490580 [Accessed March 27, 2015].

Del Valle, L., Schwartzman, R.J. & Alexander, G., 2009. Spinal cord histopathological alterations in a patient with longstanding complex regional pain syndrome. *Brain, behavior, and immunity*, 23(1), pp.85–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18786633 [Accessed September 20, 2015].

Velanac, V. et al., 2012. Bace1 processing of NRG1 type III produces a myelin-inducing signal but is not essential for the stimulation of myelination. *Glia*, 60(2), pp.203–17. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3267053&tool=pmcentrez&rendertype=abstract [Accessed June 9, 2015].

Verge, G.M. et al., 2004. Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. *The European journal of neuroscience*, 20(5), pp.1150–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15341587 [Accessed March 25, 2015].

Verma, S., Estanislao, L. & Simpson, D., 2005. HIV-associated neuropathic pain: epidemiology, pathophysiology and management. *CNS drugs*, 19(4), pp.325–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15813646 [Accessed March 16, 2015].

Vial, C., Roberts, J.A. & Evans, R.J., 2004. Molecular properties of ATP-gated P2X receptor ion channels. *Trends in pharmacological sciences*, 25(9), pp.487–93. Available at: http://www.sciencedirect.com/science/article/pii/S0165614704002081 [Accessed May 16, 2015].

Vinet, J. et al., 2012. Neuroprotective function for ramified microglia in hippocampal excitotoxicity. *Journal of neuroinflammation*, 9(1), p.27. Available at: http://www.jneuroinflammation.com/content/9/1/27 [Accessed March 30, 2015].

Wall, P.D. & Gutnick, M., 1974. Properties of afferent nerve impulses originating from a neuroma. *Nature*, 248(5451), pp.740–743. Available at: http://dx.doi.org/10.1038/248740a0 [Accessed March 9, 2015].

Wallace, V.C.J. et al., 2007. Characterization of rodent models of HIV-gp120 and anti-retroviral-associated neuropathic pain. *Brain : a journal of neurology*, 130(Pt 10), pp.2688–702. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2656646&tool=pmcentrez&rendertype=abstract [Accessed April 7, 2015].

Walport, M.J., 2001. Complement. Second of two parts. *The New England journal of medicine*, 344(15), pp.1140–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11297706 [Accessed May 10, 2015].

Wang, G. et al., 2014. Upregulation of neuregulin-1 reverses signs of neuropathic pain in rats. *International journal of clinical and experimental pathology*, 7(9), pp.5916–21. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4203206&tool=pmcentrez&rendertype=abstract [Accessed January 1, 2015].

Wang, J.Y., Miller, S.J. & Falls, D.L., 2001. The N-terminal region of neuregulin isoforms determines the accumulation of cell surface and released neuregulin ectodomain. *The Journal of biological chemistry*, 276(4), pp.2841–51. Available at: http://www.jbc.org/content/276/4/2841.abstract [Accessed May 18, 2015].

Wasserman, J.K., Zhu, X. & Schlichter, L.C., 2007. Evolution of the inflammatory response in the brain following intracerebral hemorrhage and effects of delayed minocycline treatment. *Brain research*, 1180, pp.140–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17919462 [Accessed March 30, 2015].

Watkins, L.R., Milligan, E.D. & Maier, S.F., 2001. Glial activation: a driving force for pathological pain. *Trends in neurosciences*, 24(8), pp.450–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11476884 [Accessed March 24, 2015].

Waxman, S.G. et al., 1999. Sodium channels and pain. *Proceedings of the National Academy of Sciences of the United States of America*, 96(14), pp.7635–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=33593&tool=pmcentrez&rendertype=abstract [Accessed March 23, 2015].

Waxman, S.G. & Zamponi, G.W., 2014. Regulating excitability of peripheral afferents: emerging ion channel targets. *Nature neuroscience*, 17(2), pp.153–63. Available at: http://dx.doi.org/10.1038/nn.3602 [Accessed March 5, 2015].

Wei, F. et al., 2008. Supraspinal glial-neuronal interactions contribute to descending pain facilitation. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 28(42), pp.10482–95. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2660868&tool=pmcentrez&rendertype=abstract [Accessed March 30, 2015].

Weinstein, J.R., Koerner, I.P. & Möller, T., 2010. Microglia in ischemic brain injury. *Future neurology*, 5(2), pp.227–246. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2853969&tool=pmcentrez&rendertype=abstract [Accessed April 7, 2015].

White, F.A. et al., 2005. Excitatory monocyte chemoattractant protein-1 signaling is up-regulated in sensory neurons after chronic compression of the dorsal root ganglion. *Proceedings of the National Academy of Sciences of the United States of America*, 102(39), pp.14092–7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1236537&tool=pmcentrez&rendertype=abstract [Accessed February 25, 2015].

Willem, M. et al., 2006. Control of peripheral nerve myelination by the beta-secretase BACE1. *Science (New York, N.Y.)*, 314(5799), pp.664–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16990514 [Accessed March 31, 2015].

Wilms, H. et al., 2003. Intrathecal synthesis of monocyte chemoattractant protein-1 (MCP-1) in amyotrophic lateral sclerosis: further evidence for microglial activation in neurodegeneration. *Journal of neuroimmunology*, 144(1-2), pp.139–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14597108 [Accessed September 20, 2015].

Winkelstein, B.A. et al., 2001. Nerve injury proximal or distal to the DRG induces similar spinal glial activation and selective cytokine expression but differential behavioral responses to pharmacologic treatment. *The Journal of comparative neurology*, 439(2), pp.127–39. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11596043 [Accessed April 8, 2015].

Wodarski, R. et al., 2009. Gabapentin reverses microglial activation in the spinal cord of streptozotocin-induced diabetic rats. *European journal of pain (London, England)*, 13(8), pp.807–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18977160 [Accessed April 7, 2015].

Wolf, Y. et al., 2013. Microglia, seen from the CX3CR1 angle. *Frontiers in cellular neuroscience*, 7, p.26. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3600435&tool=pmcentrez&rendertype=abstract [Accessed December 12, 2013].

Won, S.J. et al., 2012. Recurrent/moderate hypoglycemia induces hippocampal dendritic injury, microglial activation, and cognitive impairment in diabetic rats. *Journal of neuroinflammation*, 9(1), p.182. Available at: http://www.jneuroinflammation.com/content/9/1/182 [Accessed March 30, 2015].

Woodling, N.S. et al., 2014. Suppression of Alzheimer-associated inflammation by microglial prostaglandin-E2 EP4 receptor signaling. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 34(17), pp.5882–94. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3996215&tool=pmcentrez&rendertype=abstract [Accessed November 20, 2014].

Woodruff, T.M. et al., 2010. The role of the complement system and the activation fragment C5a in the central nervous system. *Neuromolecular medicine*, 12(2), pp.179–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19763906 [Accessed May 10, 2015].

Woolf, C.J., 2011. Central sensitization: implications for the diagnosis and treatment of pain. *Pain*, 152(3 Suppl), pp.S2–15. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3268359&tool=pmcentrez&rendertype=abstract [Accessed July 10, 2014].

Woolf, C.J. & Mannion, R.J., 1999. Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet*, 353(9168), pp.1959–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10371588 [Accessed January 5, 2015].

Woolf, C.J. & Thompson, S.W., 1991. The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartic acid receptor activation; implications for the treatment of post-injury pain hypersensitivity states. *Pain*, 44(3), pp.293–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1828878 [Accessed March 24, 2015].

WOOLFE, G. & MACDONALD, A.D., 1944. THE EVALUATION OF THE ANALGESIC ACTION OF PETHIDINE HYDROCHLORIDE (DEMEROL). *J. Pharmacol. Exp. Ther.*, 80(3), pp.300–307. Available at: http://jpet.aspetjournals.org/content/80/3/300.abstract [Accessed June 26, 2014].

Xiang, Y. et al., 2014. NRG1-ErbB signalling promotes microglia activation contributing to incision-induced mechanical allodynia. *European journal of pain (London, England)*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25159022 [Accessed November 4, 2014].

Xu, J.-T. et al., 2007. Activation of phosphatidylinositol 3-kinase and protein kinase B/Akt in dorsal root ganglia and spinal cord contributes to the neuropathic pain induced by spinal nerve ligation in rats. *Experimental neurology*, 206(2), pp.269–79. Available at: http://www.sciencedirect.com/science/article/pii/S0014488607002002 [Accessed February 5, 2015].

Xu, X.J., Dalsgaard, C.J. & Wiesenfeld-Hallin, Z., 1992. Intrathecal CP-96,345 blocks reflex facilitation induced in rats by substance P and C-fiber-conditioning stimulation. *European journal of pharmacology*, 216(3), pp.337–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1385160 [Accessed March 24, 2015].

Yadav, A. & Collman, R.G., 2009. CNS inflammation and macrophage/microglial biology associated with HIV-1 infection. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology*, 4(4), pp.430–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19768553 [Accessed April 6, 2015].

Yang, X. et al., 2001. Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron*, 30(2), pp.399–410. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11395002 [Accessed February 2, 2015].

Yang, X.-L. et al., 2005. Neuregulin-induced expression of the acetylcholine receptor requires endocytosis of ErbB receptors. *Molecular and cellular neurosciences*, 28(2), pp.335–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15691714 [Accessed May 4, 2015].

Yao, J. et al., 1990. Chemotaxis by a CNS macrophage, the microglia. *Journal of neuroscience research*, 27(1), pp.36–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2254955 [Accessed May 9, 2015].

Yin, T. et al., 1997. Tissue-specific pattern of stress kinase activation in ischemic/reperfused heart and kidney. *The Journal of biological chemistry*,

272(32), pp.19943–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9242662 [Accessed July 23, 2015].

Yokozeki, T. et al., 2007. Meltrin beta (ADAM19) mediates ectodomain shedding of Neuregulin beta1 in the Golgi apparatus: fluorescence correlation spectroscopic observation of the dynamics of ectodomain shedding in living cells. *Genes to cells : devoted to molecular & cellular mechanisms*, 12(3), pp.329–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17352738 [Accessed April 19, 2015].

Young, P. et al., 2008. Single-neuron labeling with inducible Cre-mediated knockout in transgenic mice. *Nature neuroscience*, 11(6), pp.721–8. Available at: http://dx.doi.org/10.1038/nn.2118 [Accessed February 2, 2015].

Zhang, D. et al., 1997. Neuregulin-3 (NRG3): A novel neural tissue-enriched protein that binds and activates ErbB4. *Proceedings of the National Academy of Sciences*, 94(18), pp.9562–9567. Available at: http://www.pnas.org/content/94/18/9562.abstract [Accessed August 31, 2015].

Zhang, F. et al., 2008. Selective activation of microglia in spinal cord but not higher cortical regions following nerve injury in adult mouse. *Molecular pain*, 4, p.15. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2374773&tool=pmcentrez&rendertype=abstract [Accessed April 17, 2015].

Zhang, J. & De Koninck, Y., 2006. Spatial and temporal relationship between monocyte chemoattractant protein-1 expression and spinal glial activation following peripheral nerve injury. *Journal of neurochemistry*, 97(3), pp.772–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16524371 [Accessed May 10, 2015].

Zhang, J.-M., Li, H. & Munir, M.A., 2004. Decreasing sympathetic sprouting in pathologic sensory ganglia: a new mechanism for treating neuropathic pain using lidocaine. *Pain*, 109(1-2), pp.143–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15082136 [Accessed March 23, 2015].

Zhang, R.-X. et al., 2005. Spinal glial activation in a new rat model of bone cancer pain produced by prostate cancer cell inoculation of the tibia. *Pain*, 118(1-2), pp.125–36. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16154703 [Accessed March 30, 2015].

Zhang, X., Goncalves, R. & Mosser, D.M., 2008. The isolation and characterization of murine macrophages. *Current protocols in immunology / edited by John E. Coligan ... [et al.]*, Chapter 14, p.Unit 14.1. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2834554&tool=pmcentrez&rendertype=abstract [Accessed May 12, 2014].

Zhang, Z. et al., 2006. Neuregulin isoforms in dorsal root ganglion neurons: effects of the cytoplasmic domain on localization and membrane shedding of Nrg-1 type I. *Journal of neuroscience research*, 84(1), pp.1–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16615045 [Accessed January 6, 2015].

Zheng, F.Y., Xiao, W.-H. & Bennett, G.J., 2011. The response of spinal microglia to chemotherapy-evoked painful peripheral neuropathies is distinct from that evoked by traumatic nerve injuries. *Neuroscience*, 176, pp.447–54. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3040270&tool=pmcentrez&rendertype=abstract [Accessed April 7, 2015].

Zhu, X. et al., 2012. Mutations in a P-type ATPase gene cause axonal degeneration. *PLoS genetics*, 8(8), p.e1002853. Available at: http://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1002853 [Accessed April 12, 2015].

Zhuang, Z.-Y. et al., 2006a. A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(13), pp.3551–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16571763 [Accessed March 27, 2015].

Zhuang, Z.-Y. et al., 2006b. A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(13), pp.3551–60. Available at: http://www.jneurosci.org/content/26/13/3551.full [Accessed February 8, 2015].

Zhuang, Z.-Y. et al., 2005. ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain*, 114(1-2), pp.149–59. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15733640 [Accessed January 20, 2015].

Appendix

Tables 1 – 8 represent the behavioural studies conducted in **Chapter 4** to measure mechanical, thermal, and cold pain behaviours between NRG1$^{fl/fl}$ CAG Cre (-) Tx. NRG1$^{-/-}$CAG Cre (+) Tx mice and NRG1$^{fl/fl}$ CAG Cre (+) Vh treated mice specific time-points injury.

Table 4: Mechanical withdrawal thresholds do not vary significantly between male and female mice within Vh treated, Cre (-) Tx and Cre (+) Tx groups in the injured left paw ($p>0.05$ repeated measures two way ANOVA post-hoc Bonferroni test conducted on full time course; but only data for day 3 post injury is presented here).

LEFT PAW	**Male**			**Female**			**M+F**		
	Threshold (g)	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**
Vh	0.022	0.008	14	0.016	0.004	14	0.019	0.006	28
Cre (-) Tx	0.089	0.061	9	0.129	0.022	10	0.108	0.041	9
Cre (+) Tx	0.025	0.003	5	0.021	0.002	4	0.023	0.005	9

Table 5: Mechanical withdrawal thresholds do not vary significantly between male and female mice within Vh treated, Cre (-) Tx and Cre (+) Tx groups in the uninjured right paw ($p>0.05$ repeated measures two way ANOVA post-hoc Bonferroni test conducted on full time course; but only data for day 3 post injury is presented here).

RIGHT PAW	**Male**			**Female**			**M+F**		
	Threshold (g)	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**
Vh	1.031	0.360	14	0.691	0.481	14	0.861	0.341	28
Cre (-) Tx	0.617	0.521	9	1.247	0.409	10	0.932	0.63	9
Cre (+) Tx	1.086	0.198	5	0.876	0.112	4	0.981	0.21	9

Table 6: Thermal latency withdrawal thresholds do not vary significantly between male and female mice within Vh treated, Cre (-) Tx and Cre (+) Tx groups in the injured left paw ($p>0.05$ repeated measures two way ANOVA post-hoc Bonferroni test conducted on full time course; but only data for day 3 post injury is presented here).

LEFT PAW	**Male**			**Female**			**M+F**		
	Latency (s)	**SEM**	**n**	**Latency (s)**	**SEM**	**n**	**Latency (s)**	**SEM**	**n**
Vh	5.396	2.112	14	2.884	1.400	14	4.14	2.512	28
Cre (-) Tx	3.875	0.190	9	4.385	0.320	10	4.13	0.510	9
Cre (+) Tx	4.221	2.912	5	2.359	0.980	4	3.29	1.862	9

Table 7: Thermal latency withdrawal thresholds do not vary significantly between male and female mice within Vh treated, Cre (-) Tx and Cre (+) Tx groups in the uninjured right paw ($p>0.05$ repeated measures two way ANOVA post-hoc Bonferroni test conducted on full time course; but only data for day 3 post injury is presented here).

RIGHT PAW	**Male**			**Female**			**M+F**		
	Latency (s)	**SEM**	**n**	**Latency (s)**	**SEM**	**n**	**Latency (s)**	**SEM**	**n**
Vh	11.382	2.002	14	9.299	1.562	14	10.341	2.083	28
Cre (-) Tx	10.788	0.982	9	11.807	1.105	10	11.262	1.019	9
Cre (+) Tx	10.843	0.728	5	10.007	0.915	4	10.425	0.836	9

Table 8: latency to withdraw from cold stimuli (using cold plate) does not vary significantly between male and female mice within Vh treated, Cre (-) Tx and Cre (+) Tx groups in the injured paw ($p>0.05$ repeated measures two way ANOVA post-hoc Bonferroni test conducted on full time course; but only data for day 4 post injury is presented here).

LEFT PAW	**Male**			**Female**			**M+F**		
	Latency (s)	**SEM**	**n**	**Latency (s)**	**SEM**	**n**	**Latency (s)**	**SEM**	**n**
Vh	41.152	3.965	14	36.750	4.458	14	38.951	8.403	28
Cre (-) Tx	25.078	5.625	9	30.947	3.682	10	28.012	5.869	9
Cre (+) Tx	24.454	4.826	5	15.657	4.054	4	20.056	8.797	9

Table 9: latency to withdraw from cold stimuli does not vary significantly between male and female mice within Cre (-) Tx and Cre (+) Tx groups in the uninjured right paw ($p>0.05$ repeated measures two way ANOVA post-hoc Bonferroni test conducted on full time course; but only data for day 29 post injury is presented here).

RIGHT PAW	**Male**			**Female**			**M+F**		
	Latency (s)	**SEM**	**n**	**Latency (s)**	**SEM**	**n**	**Latency (s)**	**SEM**	**n**
Cre (-) Tx	27.279	6.352	9	35.061	7.639	10	31.125	7.872	9
Cre (+) Tx	27.197	9.210	5	38.769	8.363	4	32.983	11.572	9

Table 10: Whilst there is a significant difference in cold hypersensitivity between groups, latency to withdraw from cold stimuli (using cold place preference assay) does not vary significantly between male and female mice within Cre (-) Tx and Cre (+) Tx groups in the injured left paw ($p>0.05$ repeated measures two way ANOVA post-hoc Bonferroni test conducted on full time course; but only data for day 29 post injury is presented here).

LEFT PAW	**Male**			**Female**			**M+F**		
	% occupancy	**SEM**	**n**	**% occupancy**	**SEM**	**n**	**% occupancy**	**SEM**	**n**
Cre (-) Tx	7.983	1.536	9	6.853	0.926	10	7.418	1.130	9
Cre (+) Tx	15.222	2.101	5	17.115	1.812	4	16.217	1.989	9

Tables 9 – 15 represent the behavioural studies conducted in **Chapter 5** to measure mechanical, thermal, heat and cold pain behaviours between erbB3$^{fl/fl}$B4$^{fl/fl}$ Cd11bCre (-) and erbB3$^{fl/fl}$B4$^{fl/fl}$ Cd11bCre (+) mice.

Table 11: Mechanical withdrawal thresholds do not vary significantly between male and female mice within Cre (-) and Cre (+) groups in the injured left paw ($p>0.06$ student t-test).

LEFT PAW	**Male**			**Female**			**M+F**		
	Threshold (g)	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**
Cre (-)	1.121	0.101	6	0.986	0.156	5	1.02	0.089	11
Cre (+)	0.983	0.085	6	0.917	0.109	5	0.931	0.058	11

Table 12: Mechanical withdrawal thresholds do not vary significantly between male and female mice within Cre (-) and Cre (+) groups in the uninjured right paw (p>0.06 student t-test).

RIGHT PAW	**Male**			**Female**			**M+F**		
	Threshold (g)	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**
Cre (-)	0.900	0.120	6	0.931	0.156	5	0.952	0.089	11
Cre (+)	0.869	0.153	6	0.995	0.109	5	0.938	0.058	11

Table 13: Thermal latency withdrawal thresholds do not vary significantly between male and female mice within Cre (-) and Cre (+) groups in the injured left paw (p>0.06 student t-test).

LEFT PAW	**Male**			**Female**			**M+F**		
	Latency (s)	**SEM**	**n**	**Latency (s)**	**SEM**	**n**	**Latency (s)**	**SEM**	**n**
Cre (-)	11.235	0.635	6	11.230	0.369	5	11.511	0.614	11
Cre (+)	10.232	0.586	6	11.214	0.369	5	11.083	0.166	11

Table 14: Thermal latency withdrawal thresholds do not vary significantly between male and female mice within Cre (-) and Cre (+) groups mice in the uninjured right paw (p>0.06 student t-test).

RIGHT PAW	**Male**			**Female**			**M+F**		
	Latency (s)	**SEM**	**n**	**Latency (s)**	**SEM**	**n**	**Latency (s)**	**SEM**	**n**
Cre (-)	11.231	1.263	6	9.686	0.869	5	10.381	1.237	11
Cre (+)	9.068	0.896	6	11.121	0.782	5	10.915	0.250	11

Table 15: latency to withdraw from heat stimuli does not vary significantly between male and female mice within Cre (-) and Cre (+) groups in the injured left paw ($p>0.06$ student t-test).

LEFT PAW	**Male**			**Female**			**M+F**		
	Latency (s)	**SEM**	**n**	**Latency (s)**	**SEM**	**n**	**Latency (s)**	**SEM**	**n**
Cre (-)	7.892	0.865	6	8.112001	0.358	5	8.022	0.230	11
Cre (+)	8.362001	0.612	6	7.275	0.514	5	8.039	0.490	11

Tables 16 – 23 represent the behavioural studies conducted in **Chapter 6** to measure mechanical, thermal, and cold pain behaviours between erbB3$^{fl/fl}$B4$^{fl/fl}$ CX3CR1 Cre (-) Tx treated and erbB3$^{-/-}$B4$^{-/-}$ CX3CR1 Cre (+) Tx treated mice at 28 days post-injury

Table 16: latency to withdraw from heat stimuli does not vary significantly between male and female mice within Cre (-) and Cre (+) groups in the uninjured right paw ($p>0.06$ student t-test).

RIGHT PAW	**Male**			**Female**			**M+F**		
	Latency (s)	**SEM**	**n**	**Latency (s)**	**SEM**	**n**	**Latency (s)**	**SEM**	**n**
Cre (-)	8.807	0.356	6	8.032	0.613	5	8.067	0.289	11
Cre (+)	8.321	0.469	6	7.652	0.695	5	7.984	0.408	11

Table 17: latency to withdraw from cold stimuli does not vary significantly between male and female mice within Cre (-) and Cre (+) groups in the injured left paw ($p>0.06$ student t-test).

LEFT PAW	**Male**			**Female**			**M+F**		
	Latency (s)	**SEM**	**n**	**Latency (s)**	**SEM**	**n**	**Latency (s)**	**SEM**	**n**
Cre (-)	10.912	1.231	6	10.100	0.568	5	10.405	0.718	11
Cre (+)	10.121	0.435	6	9.636	0.362	5	9.719	0.303	11

Table 18: latency to withdraw from cold stimuli does not vary significantly between male and female mice within Cre (-) and Cre (+) groups in the uninjured right paw ($p>0.06$ student t-test).

RIGHT PAW	**Male**			**Female**			**M+F**		
	Latency (s)	**SEM**	**n**	**Latency (s)**	**SEM**	**n**	**Latency (s)**	**SEM**	**n**
Cre (-)	10.912	1.231	6	10.100	0.568	5	10.405	0.718	11
Cre (+)	10.121	0.435	6	9.636	0.362	5	9.719	0.303	11

Table 19: Mechanical withdrawal thresholds do not vary significantly between male and female mice within Cre (-) Tx and Cre (+) Tx groups in the injured left paw ($p>0.05$ repeated measures two way ANOVA post-hoc Bonferroni test conducted on full time course; but only data for day 28 post injury is presented here).

LEFT PAW	**Male**			**Female**			**M+F**		
	Threshold (g)	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**
Cre (-) Tx	0.243	0.231	6	0.097	0.103	5	0.073	0.340	11
Cre (+) Tx	0.059	0.064	4	0.103	0.034	4	0.081	0.045	8

Table 20: Mechanical withdrawal thresholds do not vary significantly between male and female mice within Cre (-) Tx and Cre (+) Tx groups in the uninjured right paw ($p>0.05$ repeated measures two way ANOVA post-hoc Bonferroni test conducted on full time course; but only data for day 28 post injury is presented here).

RIGHT PAW	**Male**			**Female**			**M+F**		
	Threshold (g)	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**	**Threshold (g)**	**SEM**	**n**
Cre (-) Tx	0.696	0.236	6	0.906	0.111	5	0.801	0.211	11
Cre (+) Tx	0.920	0.092	4	0.729	0.142	4	0.825	0.191	8

Table 21: Thermal latency withdrawal thresholds do not vary significantly between male and female mice within Cre (-) Tx and Cre (+) Tx groups in the injured left paw ($p>0.05$ repeated measures two way ANOVA post-hoc Bonferroni test conducted on full time course; but only data for day 28 post injury is presented here).

LEFT PAW	**Male**			**Female**			**M+F**		
	Latency (s)	**SEM**	**n**	**Latency (s)**	**SEM**	**n**	**Latency (s)**	**SEM**	**n**
Cre (-) Tx	6.766	0.726	6	5.924	0.693	5	6.345	0.841	11
Cre (+) Tx	7.195	0.936	4	5.890	1.293	4	6.566	1.324	8

Table 22: Whilst there is a significant difference in cold hypersensitivity between groups, latency to withdraw from cold stimuli (using cold place preference assay) does not vary significantly between male and female mice within Cre (-) Tx and Cre (+) Tx groups in the injured left paw ($p>0.05$ repeated measures two way ANOVA post-hoc Bonferroni test conducted on full time course; but only data for day 29 post injury is presented here).

RIGHT PAW	**Male**			**Female**			**M+F**		
	% occupancy	**SEM**	**n**	**% occupancy**	**SEM**	**n**	**% occupancy**	**SEM**	**n**
Cre (-) Tx	12.887	1.014	6	14.016	0.925	5	13.451	1.129	11
Cre (+) Tx	21.681	0.981	4	22.788	0.568	4	22.234	1.107	8