The Role of Brain Derived Neurotrophic Factor (BDNF) and Colony Stimulating Factor (CSF-1) In the Generation of Central Sensitization and Neuropathic Pain

by

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Abstract

This dissertation addressed two main hypotheses:

1. BDNF acts through different receptors and/or transduction mechanisms to increase release of excitatory transmitter onto excitatory neurons (using the Trk B signaling pathways) and to decrease release of excitatory transmitter onto inhibitory neurons (via the p75 signaling pathway).
2. The release of CSF-1 by primary afferents after nerve injury leading to the generation of neuropathic pain involves an excitatory action on the dorsal horn mediated via the release of BDNF.

Using an organotypic slice preparation of mice spinal cord slices, seven electrophysiological cell types were identified in the *substantia gelatinosa* of GAD67-EGFP Tamaguchi CL-1 white albino Swiss mice using whole-cell recording. Of the seven cell types, delay tonic and delay irregular, which are excitatory in nature almost never expressed GAD67 (GAD67-EGFP-) and are largely glutamatergic.

Long term chronic studies using defined medium organotypic cultures (DMOTC) of mice spinal cords was established and it was determined that all the seven neuronal phenotypes found in acute slices were preserved in culture. The stability of the overall neuronal population decreased but stabilized over the entire duration in culture and slices were intact and healthy during the period of growth. These observations suggested the suitability of using defined medium organotypic cultures of mice spinal cord as long-term disease state models.

Prolonged BDNF treatment caused an increase in synaptic drive in delay neurons in BDNF treated slices and a weaker increase in the frequency of sEPSC was seen in GAD67-EGFP neurons. The effects seen in delay neurons from BDNF treated cultures were both pre and post synaptic and involved the BDNF/TrkB signalling mechanism. Presynaptic effects dominated in GAD67-EGFP neurons recorded from BDNF slices and involved the BDNF/p75NTR signalling pathway.

The debate as to whether CSF-1 activation of resident microglia in the spinal cord is responsible for the release of BDNF leading to neuropathic pain was investigated. Experiments using the BDNF binding protein, Trkb-FC showed that the effects of CSF-1 are largely affected by BDNF mediated pathways on delay neurons and BDNF independent pathways on GAD67-EGFP neurons. Thus, BDNF release from spinal microglia as a result of CSF-1 activation may contribute to the overall increase in excitability by delay excitatory neurons.

Preface

This thesis is an original work by Paul Atta Boakye. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Cellular electrophysiology of Neuropathic Pain”, No. AUP00000338, DATE: July 24th 2015.

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This thesis is dedicated to my wonderful parents, Mr and Mrs Boakye.

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LIST OF ABBREVIATIONS

ACD	Acid citrate dextrose
ACSF	Artificial cerebrospinal fluid
BDNF	Brain derived neurotrophic factor
CCI	Chronic constriction injury
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
CSF	Colony stimulating factor
DH	Dorsal horn
DMOTC	Defined Medium OTCs
DRG	Dorsal root ganglion
EGFP	Enhanced green fluorescent protein
FBS	Fetal bovine serum
GAD	Glutamic Acid Decarboxylase
GBSS	Gey's balanced salt solution
GDNF	Glial cell-line derived neurotrophic factor
GFP	Green Fluorescent Protein
HCN	Hyperpolarization activated cyclic nucleotide

IASP	International Association for the study of Pain
IPSP	Inhibitory post synaptic potentials
KS	Kolmogorov Smirnof
LTP	Long term potentiation
NGF	Nerve growth factor
NRG	Nucleus reticularis gigantocellularis
NRM	Nucleus raphe magnus
NSC	Neural stem cells
OTC	Organotypic cultures
PCD	Programmed cell death
PCR	Polymerase chain Reaction
PNI	Peripheral nerve injured
PSNL	Partial sciatic nerve ligation
PTK	Protein tyrosine kinase
ROI	Regions of interest
SG	Substantia gelatinosa
SNL	Spinal nerve ligation
TNF	Tumor necrosis factor

WHO	World Health Organization

CHAPTER 1

GENERAL INTRODUCTION

1.1 A Brief History of Pain

Over several centuries and even millennia a number of theories have been put forward to describe mechanisms underlying pain perception (Kenins 1988; Perl 2007). Some early researchers described pain alongside other common sensations. However, the emotional character of pain had been highlighted by Aristotle (*Aristotelian concept of Pain, commonly referred to as affective concept of pain*) and by Darwin over 100 years ago. The independent discovery that painful and thermal sensations could be selectively elicited from discrete spots on the skin by Blix, von Frey, Schiff and Donaldson in the 1800s, allowed for a school of consideration that pain from heat or sharp needles emanate from specific sensations (Schiff 1858)(*Specificity theory of pain*).

1.1.1 Specificity Theory of Pain

The specificity theory refers to the presence of dedicated pathways for each somatosensory modality (Moayedi and Davis 2013). A key tenet of the specificity theory is that each modality has a specific receptor and associated sensory fiber (primary afferent) that is sensitive to one specific stimulus (Dubner 1990). Proponents of this theory assume that non-noxious mechanical stimuli are encoded by low-threshold mechanoreceptors, which in turn are associated with specific primary afferents that project to “mechanoreceptive” second-order neurons in the spinal cord or brainstem (Moayedi and Davis 2013) which project in turn to “higher” brain centers. Likewise, noxious stimuli would activate a nociceptor, which would project to spinal cord and higher “pain” centers via a distinct pathway.

René Descartes, the French philosopher noted as one of the first to describe somatosensory pathway in humans described pain as a perception that exists in the brain and went on further to make the distinction between nociception and the perceptual experience of pain (Descartes 1962). A pivot of Descartes' theory was his description of nerves, which he perceived as hollow "**tubules**" that convey both sensory and motor information. In as much as his understanding of pain was beyond his contemporaries. Herophilus, the Greek physician had earlier on in the third century BC demonstrated the existence of sensory and motor nerves, and Erasistratus, the famous Greek anatomist and physician, demonstrated that, the brain influenced motor activity (Rey 1995). Hundreds of years later, Galen, demonstrated that sectioning the spinal cord caused sensory and motor deficits (Ochs 2004), further to this he postulated that three conditions needed to be met for perception:

1) An organ must be able to receive the stimulus

2) There must be a connection from the organ to the brain, and

3) A processing center that converts the sensation to a conscious perception must exist (Rey 1995).

Contributing to Galen's model, Descartes postulated that a gate must exist between the brain and the tubular structures (nerves), which is opened by a sensory cue.

The modern concept of a dedicated pain pathway was developed by Charles Bell (Bell 1987). Investigations that lead to the discovery of specific, cutaneous touch receptors, such as Pacinian corpuscles (Pacini 1835), Meissner's corpuscles (Meissner 1853) Merkel's discs (Merkel

1875) and Ruffini's end-organs (Ruffini 1894), further provided evidence to the specificity theory of pain**.**

In furtherance of the specificity theory of pain, Schiff and Woroschiloff in a series of experiments established the presence of two pathways at different levels of the spinal cord: the anterolateral pathway for pain and temperature and the posterior bundles for tactile sensibility (Dallenbach 1939; Rey 1995). This assertion was largely confirmed by the physician, William Richard Gowers in London, who through a case study on a patient who had a bullet wound to the gray matter of the spinal cord lost the sense of pain and temperature but not touch (Rey 1995). This finding largely led to the conclusion that there were specific pathways for pain and temperature distinct from that of touch. However, in spite of this evidence there were strong opposition to the idea of the specificity theory. A major turning point arose when the Austrian, Goldscheider published his findings on sensory spots on the skin (Dallenbach 1939) in which he defined them as tiny areas of the skin that elicit a specific sensation when touched.

1.1.2 Intensity Theory of Pain

An Intensive (or Summation) Theory of Pain (commonly referred to as the Intensity Theory) was first, put forward in the fourth century BC by Plato (Godderis 1998). The theory defines pain, as an emotion that occurs when a stimulus is stronger than usual. Contributions by (Darwin 1803) and Wilhelm Erb (Dallenbach 1939) highlighted the fact that pain occurred in any sensory system when sufficient intensity was reached rather than being a stimulus modality in its own right. In a series of experiments in 1859, Bernhard Naunyn showed that repeated tactile

stimulation (below the threshold for tactile perception) produced pain in patients with syphilis who had degenerating dorsal columns (Dallenbach 1939). Worthy of note was when this stimulus was presented to patients 60 – 600 times/s and they rapidly developed what they described as unbearable pain. He concluded that there must be some form of summation that occurs for the subthreshold stimuli to become unbearably painful. Goldscheider suggested that repeated subthreshold stimulation or supra-threshold hyper-intensive stimulation could cause pain. This theory gained great prominence and competed strongly with the specificity theory of pain.

However, the revolutionary work of Sherrington on the existence of sensory receptors led to the loss of support for the intensity theory of pain.

1.1.3 Pattern Theory of Pain

The pattern theory proposes that somatic sense organs have an extensive range of responsiveness and that individual afferent neurons respond to stimuli with differing relationships to intensity. The mode and locus of stimulation are indicated by the composite pattern of activity in the population of fibres from a particular body region.

However, this was subsequently challenged by Goldscheider, who argued that pain is the result of intense stimulation, regardless of modality and tissue origin, with a centrally modifiable threshold (Goldscheider 1898) (*Intensity –Summation theory of Pain*). Goldscheider's argument served as the template for explaining the allodynia typically seen in neuropathic pain as a lowering of the central threshold for pain along a mechanical continuum (Craig 2003). The

specificity theory of pain was further strengthened by subsequent findings that clearly showed the spinal dissociation of pain and temperature sensations.

1.1.4 Other Pain Theories

1.1.4.1 The Affective Quality of Pain:

1.1.4.1.1 Pleasure-Pain Theory.

This was strongly supported by many philosophers and in 1895, Marshall proposed the *pleasure-pain theory* (Marshall 1895). Bridging the views held by physiologists, philosophers and psychologists, (Strong 1895) postulated that pain consists of the original sensation and the psychic reaction provoked by the sensation. Sherrington, in his review on sensation in the 1900s (Sherrington 1900), described pain as part of the sense of "the material me," an extension of common sensation, however in the 1940s he rephrased his description as that; pain is a special sensation by introducing nociception, that is, sensory activity evoked selectively by noxious stimuli that cause or threaten tissue damage.

1.1.4.1.2 Peripheral Pattern Theory.

In the twentieth century some researchers believed that pain was helped or supported by specific receptors with fibers projecting to the spinal cord where specific pain pathways in the neuraxis carry the pain information to a pain center (Head 1920). (Sinclair 1955) and (Weddell 1955) proposed the *peripheral pattern theory* suggesting that all fibre endings are alike and the

spatial and temporal pattern of their discharge is produced by intense stimulation of non-specific nociceptors.

1.1.4.1.3. The Central Summation Theory.

This supported the intensity theory suggesting that nerve and tissue damage activate fibres projecting to spinal internuncial neuron pools creating abnormal reverberatory activity that self-excite neural loops (Livingston 1943).

1.1.4.2. Duality concept of pain

This was originally proposed by Strong, and was reintroduced by Hardy et al. (1952) and formed the basis for the fourth Theory of Pain; which primarily suggests that pain includes two components: *the perception of pain and the reaction to it*.

1.1.4.3 Sensory Interaction Theory.

A few years later Noordenbos (1959) proposed the Sensory Interaction Theory which assumes two systems: *the slow unmyelinated and small myelinated afferent fibers system*, which transmits pain, and the *fast myelinated system*, which is responsible for the other somatosensory modalities. The former projects to the cells in the dorsal horn and the summation of their input, once transmitted to the brain, is responsible for pain. The latter inhibits the transmission of

impulses from the small fibers and prevents summation (Carli 2012). This explains why specific diseases and ailments that selectively targets and destroy large myelinated fibers results in the loss of inhibition and thus increases the probability of summation and abnormal neural firing. In the fifties all these observations documented over centuries were collated and documented into the books of (Noordenbos 1959), (Livingston 1943) and (Bonica 1953; Bonica 1991) whose book, The Management of Pain, has been used by generations of doctors interested in pain medicine and treatment. Key findings by Perl, Iggo, and others in the 1960s in identifying electrophysiologically distinct primary afferent nociceptors and mechanoreceptors strengthened the *specificity theory of pain*. As such at there were three experimental theories formulated : – the Specificity Theory, the Intensive-Summation Theory and the Aristotelian concept that pain is an affective quality (Carli 2012).

However, in 1965 Melzack & Wall postulated the convergent gate control theory (Melzack and Wall 1965), which strengthened and extended the pattern/intensity theory into the spinal dorsal horn . Key papers by these two; Melzack & Wall (Melzack and Wall 1965; Wall 1973; Melzack and Wall 1982) strongly sought to argue particularly against the thought that activity in nociceptors ascending “in a straight-through transmission system to a pain center in the brain” is synonymous with the psychological experience of pain. The convergent view of pain as such continues to dominate textbooks and research literature.

1.1.4.4 The Gate Control Theory

The *Gate Control Theory of Pain* formulated by (Melzack and Wall 1965) was one of the most important ideas put forward in the sixties that would revolutionize research in pain. In their assessment of the specificity, intensive and pattern theories, (Melzack and Wall 1962) were very critical of the specificity theory because the theory as it stood did not take into account that information, once coded at the level of the peripheral receptor, could be modulated during transmission. Melzack and Wall also assumed that the threshold to effect pressure stimuli varied from low to high intensity in a continuous distribution thus not taking into cognizance the evidence (Iggo 1959; Hensel, Iggo et al. 1960) adduced that some unmyelinated afferents respond only to high threshold thermal/mechanical stimuli and as behave like nociceptors. (Melzack and Wall 1965) described the intensity theory as been strongly supported by evidence on central summation and input control, but they also ignored peripheral specificity. With this idea they suggested that pain cannot be caused by the neural activity occurring in nociceptive pathways but results from the activity in several interacting neural systems, each with its own specialized function (Carli 2012). With these considerations they proposed the gate control theory for pain (Melzack and Wall 1965) which assumes that :

1) there is a mechanism modulating the transmission from peripheral afferent fibres to spinal cord Transmission cells in the dorsal horn;

2) the activity in large fibers tends to inhibits the transmission of small fibers to Transmission cells (gate closure) by activating the inhibitory effect of SG (substantia gelatinosa) gating mechanism; the activity in small fibres tends to excite the transmission to Transmission

cells (gate opening) by blocking the inhibitory effect of SG gating system; the relative amount of activity in the large and small fibres systems is critical for opening the gate;

3) the SG activity and the spinal gate mechanism are influenced by the descending information from the brain;

4) SG axons inhibit presynaptically both large and small fibres projecting to Transmission cells;

5) The large, fast conducting fibre system projects to the central control system which alerts selective cognitive processes able to influence the descending control system modulating the gating mechanisms. The gate control theory is critical for identifying the sensory-discriminative aspects of the stimulus in order to program an appropriate response (Carli 2011).

The Gate Control Theory of Pain thus provided a neural basis for the findings that supported and reconciled the apparent differences between the Pattern and Specificity Theories of Pain. However, in spite of the pioneering work by (Melzack and Wall 1965), there were major defects to this theory.

1.1.4.4.1. Shortcomings of the Gate Control Theory.

Major defects of this theory stem from the following:

1) The “primary afferent hyperpolarization” (supposed to be produced by SG axon terminals at the level of axon terminals of A fibres synapsing to Transmission neurons) has never

been confirmed and, on the contrary, both A and C fibres evoke primary afferent depolarization (Franz and Iggo 1968; Zimmerma.M 1968; Whitehorn and Burgess 1973).

2) (Melzack and Wall 1965) failed to acknowledge the fact that there was proven research on the functional properties of specific nociceptors (Bessou, Perl et al. 1969; Christen and Perl 1970) (Christen and Perl 1970) and this contradicted some portions of the gate control theory.

In spite of the ambiguity of the gate control theory it must be admitted that although only the concept of convergence between different afferent inputs at spinal level has survived the test of time, we cannot discount the importance of this seminal paper by (Melzack and Wall 1965).

1.1.5 Components of Pain

Over the years, it has become increasingly acceptable that pain is not simply a sensation generated by nociceptors, but also a perceptual phenomenon with particular emotional qualities (Fernandez and Turk 1992). As has been broadly accepted, pain has two vital components: a sensory or nociceptive component, and an emotional or affective component. In the not too distant past the history of pain had been dominated by a view relating pain primarily to tissue damage and such causality been as a result of stimulation of peripheral receptors (nociceptors) at the site of injury and transmitting this information along afferent pathways to a pain center in the brain (Fernandez and Turk 1992). There has also been reports of pain in the absence of identifiable tissue damage commonly observed in psychiatric patients (Chaturvedi 1987), among those motivated by monetary compensation and tertiary gain (Bokan, Ries et al. 1981), and in

conditions like causalgia and phantom limb syndrome where pain can persist despite healing of the primary site of injury, a phenomenon referred to as spontaneous pain (Melzack 1973). Experiencing tissue damage without pain, is also possible, particularly during intense physical activities such as sport and combat (Wall 1979). All these contribute to what is commonly referred to as the sensory component of pain.

Pain as a subjective, perceptual experience, is characteristically different from pure sensation due to its affective quality. Sensation may be external (e.g., pressure, temperature) or internal (e.g., aches, burning), and affect may pertain to level of arousal or qualitatively different emotions (e.g., anger, fear, sadness) (Fernandez and Turk 1992). Some patients of neoplastic disease who do not report pain until they are informed of the diagnosis of cancer (Woodforde and Fielding 1970; Black 1975).

Pain can therefore be characterised as a *sensation* in a part or parts of the body but because of its unpleasantness it is often an *emotional experience*.

1.2 Definitions and Biology of Pain

The International Association for the study of Pain (IASP) defines pain as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". This definition was the culmination of centuries of ideas (as briefly described above) that explored the concept of pain. Pain differs from the classical senses (vision, hearing, touch, taste, and smell) because it is both a discriminative sensation and a graded motivation (or behavioral drive).

In spite of our quest to aptly describe and define pain, it should be noted that 'nociceptive' or good pain is a vital physiological process that signals actual or potential tissue damage (Iadarola and Caudle 1997) and thus has a protective function. By contrast, direct injury to neural tissue can produce 'nerve' or 'neuropathic' pain (neuropathic pain) that lasts for months or years after any injury has healed (Costigan, Scholz et al. 2009; Moulin, Boulanger et al. 2014; Alles and Smith 2018)

1.2.1 Nociception or Pain

It is important to differentiate between nociception and pain, as there is a tendency to use each word interchangeably. By definition, nociception refers to the processing of information by the peripheral and central nervous system (CNS) about the internal or external environment, as generated by the activation of nociceptors (Gebhart, Basbaum et al. 2009). Noxious stimuli would typically activate nociceptors situated in peripheral structures which then transmit information to the spinal cord dorsal horn, where the information continues to the brainstem and ultimately the cerebral cortex, where the perception of pain is generated**.** Pain can therefore be said to be a product of higher brain center processing, whereas nociception can occur in the absence of pain (Gebhart, Basbaum et al. 2009).

1.2.2 Pain as a global-health crisis

Pain is the primary reason why people seek medical care (Dubois, Gallagher et al. 2009; Salter 2014). The incidence of pain as observed by (Nahin 2015) show that among US adults, 25.3 million adults (11.2%) suffer from daily (chronic) pain and 23.4 million adults experience a substantial level of pain, yet other estimates show higher figures. It has been reported that 40% of Americans experience daily pain, including 50 million people with chronic pain and 25 million people with acute pain (Dubois, Gallagher et al. 2009). With a huge health-care burden, the prevalence of pain has a tremendous impact on economies around the world. The high incidence of pain has led to an estimated world market for analgesics of more than 50 billion dollars (Wolkerstorfer, Handler et al. 2016). The subjective nature of pain reflects experiences and circumstances relating to injuries in early life. As individuals the way we receive and react to pain is influenced by social, cultural and psychological factors. More than a third of the world's population suffer from persistent or recurrent pain with a high cost to the American purse, with estimates ranging from $100 billion (Mailis 2003; Dubois, Gallagher et al. 2009) and above $500 billion dollars (Salter 2014) each year in health care, compensation, and litigation.

In Canada, pain is an important and common public health problem (Van Den Kerkhof, Hopman et al. 2003). Chronic pain is estimated to afflict between 15% (Van Den Kerkhof, Hopman et al. 2003) and 29% (Moulin, Clark et al. 2002) of the population thus a huge strain on the Canadian healthcare system. The World Health Organization (WHO) recognizes pain as a significant health-care burden (Who and Consultation 2003). It is associated with many diseases which claim millions of lives each year (Bond and Breivik 2004). (Harstall and Ospina 2003) report

that about 80% of the 67 million people dying from cancer suffer from pain. It must be noted that despite pain being a universal problem, disparities exist globally and nationally in its management (Hunt and Mantyh 2001). In a study by Knaul and colleagues, they noted that in high income countries, which represent less than 15% of the global population, accounted for 94% of morphine use to ease pain (Knaul, Farmer et al. 2015).

1.2.3 Chronic Pain

This type of pain persists beyond the expected course of an acute disease process and is usually defined as pain lasting greater than six months (Russo and Brose 1998) and more difficult to treat. This type of pain is usually persistent, fails to resolve spontaneously and responds poorly to treatments (Russo and Brose 1998). Chronic pain can be further classified into *chronic nociceptive pain*, resulting from ongoing tissue damage, as in the case of cancer or osteoarthritis and *chronic neuropathic pain*, which is an abnormal form of pain that continues to persist long after the resolution of tissue damage or even in the absence of a causative illness or injury (Wolkerstorfer, Handler et al. 2016). Neuropathic pain arises as a result of injury to the somatosensory system and lasts for months or years after the injury has healed (Costigan and Woolf 2000; Treede, Jensen et al. 2008).

1.2.4 Neuropathic Pain

Neuropathic pain is normally defined as “pain caused by a lesion or disease of the somatosensory system” (Jensen, Baron et al. 2011). It is described as a maladaptive disease in the sense that the pain neither protects nor supports healing and repair. It is a disease that can arise from a myriad of conditions such as traumatic nerve, spinal cord or brain injury (including stroke) or can be associated with diabetic, HIV/AIDS, post-herpetic neuropathies or with multiple sclerosis (Treede, Jensen et al. 2008) or cancer and/or the toxic effects of chemotherapeutic agents (Xiao, Boroujerdi et al. 2007; Schmidt, Hamamoto et al. 2010) and as well as complex regional pain syndrome (Taylor 2006).

Globally the exact prevalence of neuropathic pain is a matter of debate and widely unknown, but most studies put estimates at between 1.5% and 8%, equating to between 100 million and 560 million people worldwide (Bouhassira and Attal, 2016;Gilron et al., 2006;Salter, 2014;Torrance et al., 2013;Torrance et al., 2006) such that more than 1·5 billion individuals are said to be afflicted (Gerber and Joe 2005).

Despite numerous technological advances, current treatment strategies remain largely ineffective. Hence, there is the need to better understand the etiology of neuropathic pain as a rational basis for the development of new therapeutic approaches and find new therapeutic strategies to fight this condition (Alles and Smith 2018).

A striking feature of neuropathic pain is that somatosensory signals are misinterpreted by the central nervous system. It is usually characterised by allodynia (pain evoked by a normally innocuous stimulus) (Treede, Jensen et al. 2008), hyperalgesia (an increase in the pain elicited by

a noxious stimulus) (Costigan, Scholz et al. 2009) causalgia (chronic burning pain that persists in the absence of an obvious noxious stimulus) and "spontaneous electric shock" like bursts of stimulus independent pain (Taylor 2006). In the short term, hypersensitivity usually serves a protective function after injury. Typically, a footballer would not continue to use his/her broken foot to play as pain-sensing neural circuits will sense an abnormality and warn the individual if he or she continues to as the pain usually resolves as the injury heals. However in neuropathic pain disorders, allodynia, hyperalgesia, and spontaneous pain are constant features of life (Nickel 2008), and this so called "bad pain" or "disease of pain" is maladaptive and often intractable.

1.2.5 Diagnosis of Neuropathic Pain

As outlined above, pain is a subjective, personal experience that cannot be accurately measured with medical tests or examinations by a health professional. To a physician, making a diagnosis can, therefore, be a very complex undertaking. The clinical diagnosis of neuropathic pain therefore depends on evaluation of the following criteria: 1) pain with a distinct neuroanatomical distribution 2) a medical history that suggests a lesion or disease of the nervous system 3) a confirmatory test to demonstrate neuroanatomical distribution 4) a confirmatory test to demonstrate a lesion or disease of the nervous system (Treede, Jensen et al. 2008).

1.2.6 Current management of Neuropathic Pain

The effectiveness of opioids in the treatment of nociceptive pain has been a huge success, however in stark contrast there is no similar panacea for the treatment of neuropathic pain. It is therefore not far away from the truth to state that any pain that is opioid resistant is likely neuropathic (Alles and Smith 2018). Increasingly it is becoming clear that the effectiveness of a drug towards the treatment of neuropathic pain is dependent on the specific etiology of the underlying disease or injury (Nicholson 2000). This underlines the need for improved understanding of the sensory aberrations that underlie the emergence and persistence of neuropathic pain.

A recent meta-analysis of clinical trial data supported the use of tricyclic antidepressants, serotonin-noradrenaline uptake inhibitors such as duloxetine and the gabapentinoids; pregabalin (PGB) and gabapentin (GBP) as first line treatments (Finnerup, Attal et al. 2015).

The role of opioid analgesics in neuropathic pain remains very controversial (Gilron, Watson et al. 2006). Currently, there is a lack of evidence supporting the long-term efficacy of using opioids in controlling neuropathic pain. Nevertheless, tramadol and controlled-release opioids are recommended as second-line treatments and cannabinoids as third-line treatments.

Methadone, lamotrigine, lacosamide, tapentadol and botulinum toxin are used as fourth-line treatments (Moulin, Boulanger et al. 2014).The limited effectiveness of current treatments, has led physicians to try combining different drugs which has led to improved results at lower doses and with fewer side effects (Gilron, Watson et al. 2006). With limited supporting evidence, many patients with neuropathic pain currently receive varying drug combinations (Gilron and Bailey

2003) with varying success. There is therefore the need to pursue future trials to evaluate optimal drug combinations as well as safety, compliance and cost-effectiveness (Gilron and Max 2005).

1.2.7 Obstacles to development of new therapies

The disappointing history of translation in the field of pain research has undergone significant scrutiny over the years (Dworkin, Backonja et al. 2003; Alles and Smith 2018). The depth of knowledge, understanding and findings related to presumed pain in neuropathic animal models has faired badly as compared to "bench-to bed side" observations. There are a myriad of factors that serve as barriers to the development of effective new treatments. These include the diversity of pathophysiological situations, different pain etiologies, genetic predispositions (Mogil 2012; Zorina-Lichtenwalter, Meloto et al. 2016; Sexton, Cox et al. 2018), and different ethnicities (Hastie, Riley et al. 2012). The role of epigenetics on humans (Stone and Szyf 2013) and the use of rodent models that do not possess the full complement of anatomical substrates is necessary to experience the diversity of symptoms associated with human pain conditions. This is compounded by epigenetic modifications in humans (Stone and Szyf, 2013) and by the limited ability of rodent models to predict clinical efficacy (Mogil 2017; Patel, Montagut-Bordas et al. 2017; Yekkirala, Roberson et al. 2017; Sexton, Cox et al. 2018). Lastly, the differential processing of pain between males and females is now well established (Mogil 2012; Mifflin and Kerr 2013; Sorge, Mapplebeck et al. 2015; Dodds, Beckett et al. 2016; Melchior, Poisbeau et al. 2016; Dickie, McCormick et al. 2017; Sorge and Totsch 2017) yet the majority of preclinical studies have been done on male rodents to avoid possible complications imposed by the estrous cycle.

1.3 The Nociceptive Signaling Pathway

Having a basic understanding of essential pain signaling pathway is mandatory to studying the etiology of nociceptive and neuropathic pain and for developing appropriate treatments.

Nociceptors are pain-sensitive neurons that respond to noxious mechanical, chemical, and/or thermal stimuli. They are located in the skin, vessels, muscles, fascia, joints, and viscera. Nociceptors make up the primary afferent neuron whose cell bodies lie within the dorsal root ganglion (DRG) adjacent to the spinal cord. The grey matter of the spinal cord is divided into 10 layers or laminae, with laminae I to VI making up the dorsal horn of the spinal cord. The dorsal horn of the spinal cord represents the centre where the primary processing of nociceptive information occurs. The pain processing regions of the spinal dorsal horn includes the marginal zone (Rexed lamina I), the *substantia gelatinosa* (Rexed lamina II) (Wang, Kawamata et al. 2005; Costigan, Scholz et al. 2009) as well as the deeper laminae (Pitcher and Henry 2008) are where the primary pain afferent fibers innervate. Excitation of these primary afferent nerve fibers triggers nociceptive pain. Lamina II contains local excitatory and inhibitory neurons. Upon stimulation, nociceptive signals are generated and conducted to the central nervous system via primary afferents which then synapse in the dorsal horn of the spinal cord onto second-order neurons. The axons of these second-order neurons cross the spinal cord to ascend in the spinothalamic tract with their terminal fibers predominantly localized in the thalamus. Once the signal has reached the thalamus, third-order neurons send subsequent axons through the internal capsule to the somatosensory cortex, including the post central gyrus, where discrete localization of the noxious stimulus occurs. In addition to somatosensory localization, fibers from the inter laminar and medial nuclei of the thalamus radiate to the anterior cingulate gyrus and

become involved in the emotional components of pain. During the process of signal transmission to the thalamus, some fibers from the spinothalamic tract arborize into the midbrain and rostral pons synapsing on nuclear complexes, including the nucleus raphe magnus (NRM) and nucleus reticularis gigantocellularis (NRG), both of which appear to be involved in descending regulation of the activity within the second-order neurons.

As described above it can be concluded that the generation of pain involves a complex interplay of nociception, cognition, emotion and behaviour involving various brain regions and as such no distinct pain region can be localized in the brain.

In as much as pain is transmitted upstream (via ascending pain signaling pathway), a descending analgesic pathway serves to suppress excessive or overwhelming pain in stressful situations to allow for proper cognitive functioning for escape and survival. These descending controls, comprise pathways that originate in midbrain and brainstem regions and project onto the spinal cord (Bannister and Dickenson 2017). The key neurotransmitters implicated in descending modulation are noradrenaline (NA) and serotonin (5-HT). Descending inhibition is normally through NA, predominantly via actions at the α_2-adrenoceptor (Jones and Gebhart 1986), whereas 5-HT exerts excitatory influences at specific receptor subtypes thus modulating the development and maintenance of pain by acting alongside central sensitisation in the spinal cord (Ali, Wu et al. 1996; Suzuki, Rahman et al. 2004). In diseased pain states arising from nerve injury and other related nerve damage there is an overall imbalance between descending controls, both excitatory and inhibitory thus resulting in heightened pain sensation. It has therefore been proposed that usually after nerve injury, the descending inhibitory exertion of NA becomes

quiescent and as such the excitatory influence of 5-HT swamps or overwhelms the inhibition (Bannister, Patel et al. 2015).

1.3.1 Primary Afferent Neurons

Primary afferent fibres can be classified by their peripheral targets (such as cutaneous, articular and visceral afferents), conduction velocity (which is related to size and myelination), response properties (including sensory modalities and the intensity of stimulus necessary to activate them) and neurochemical phenotype (for example, peptide expression) (Schulten, Hahn et al. 2001). Most large myelinated cutaneous afferents, referred to as Aβ fibres are low-threshold mechanoreceptors and respond to touch or hair movement. They usually have a large soma diameter of 40 μm or greater and have large heavily myelinated axons. Thinly myelinated afferents with medium sized soma diameters between 30 and 40 μm are known as Aδ fibres. C fibres have small diameter cell bodies less than 30 μm with small axons that lack myelination. Aδ and C afferents are commonly referred to as nociceptors or thermoreceptors. The Aβ, Aδ and C afferents terminate with a specific distribution pattern that is determined by their functional class. Usually, myelinated low-threshold mechanoreceptive afferents arborize in an area extending from lamina IIi–V, whereas nociceptive and thermoreceptive Aδ and C afferents innervate lamina I and much of lamina II, except for its most ventral part (Schulten, Hahn et al. 2001). All primary afferents use glutamate as their principal fast transmitter and thus have an excitatory action on their postsynaptic targets.

Nociceptors are very heterogeneous. They differ in the neuropeptides they contain, the receptors and ion channels they express, their speed of conduction, their response properties to noxious stimuli, and their capacity to be sensitized during inflammation, injury, and disease (Schulten, Hahn et al. 2001).

The Aδ fiber nociceptors conduct action potentials relatively rapidly, and mediate the fast, pricking quality of pain. They are either high threshold mechanoreceptors sensitive to noxious mechanical stimuli such as sharp objects, or mechanothermal nociceptors, which are sensitive to mechanical stimuli and extreme temperatures (>52^{o}C). As a result of their relatively rapid conduction velocity, these nociceptor types are responsible for alerting the brain quickly of very noxious stimuli, which have the potential to cause serious tissue damage usually referred to as "first pain".

C-fibers have unmyelinated axons, conduct action potentials slowly, and have small-diameter cell bodies. C-fibers mediate the slower, burning quality of pain, commonly referred to as "second pain". They are polymodal in nature and respond to a broad spectrum of mechanical or thermal stimuli. They are also sensitive to a wide range of chemical stimuli ranging from bradykinin, eicosanoids and cytokines to increased proton concentration released at the site of injury. C-fibers comprise around 70% of all nociceptors (Schulten, Hahn et al. 2001). Nociceptive C fibres can be divided into two major neurochemical groups: those that contain neuropeptides, such as substance P (Lawson, Crepps et al. 1997) and calcitonin gene-related peptide, and express TrkA receptors (Averill, McMahon et al. 1995), and those that do not (Snider and McMahon 1998). Non-peptidergic C fibres are mainly associated with the skin (Taylor, Peleshok et al. 2009),

they express a surface carbohydrate group that selectively binds to the plant lectin isolectin B4 (IB4). This subpopulation of neurons is supported by glial-derived neurotrophic factor during early postnatal development (Molliver, Wright et al. 1997; Bennett, Michael et al. 1998). The IB4-binding neurons project to a different region of the spinal dorsal horn (inner lamina II) that contains primarily local spinal interneurons. By contrast peptidergic fibres innervate various other tissues, deeper regions of the skin, outermost region of the spinal dorsal horn (lamina I and outer lamina II) and terminate largely on spinal neurons that project to higher-order pain centers in the brain (Plenderleith and Snow 1993; Bennett, Dmietrieva et al. 1996; Perry and Lawson 1998).

1.3.2 Neuronal circuitry for pain processing in the dorsal horn

1.3.2.1 Organization of the spinal dorsal horn

Free nerve endings in the periphery and visceral structures detect pain as are a result of tissue damage, and relay this information through first order sensory neurons (primary afferents) to second order sensory neurons in the dorsal horn of the spinal cord (Alles and Smith 2018). As well as glutamate, neuropeptides such as substance P and calcitonin gene related peptide (CGRP) mediate neurotransmission between primary afferent and second order sensory neurons. Glutamate interacts with Ca^{2+}-permeable and Ca^{2+}-impermeable AMPA and with NMDA receptors but not with kainate receptors (Tong and MacDermott 2006).

Although it is accepted that all primary afferent neurons are glutamatergic (West, Bannister et al. 2015), recent observations seems to suggest that GABA mediated inhibitory

interactions may play a role in sensory processing at the level of the DRG (Du, Hao et al. 2017). If these findings are confirmed, a fundamental principle of sensory physiology would need to be reassessed. There is the possibility that primary afferent neurons release GABA from their cell bodies in DRG but yet release glutamate from their terminals in the spinal cord.

All dorsal horn neurons receive monosynaptic or polysynaptic input from primary afferents and from both excitatory and inhibitory interneurons (Todd 2010). However, the specific types and relative strengths of these inputs differs between neuronal populations. On the basis of the sizes of neuronal cell bodies and their packing density (Rexed 1952) divided the grey matter of the spinal cord into 10 laminae of Rexed (**Figure 1.1 a**). Small interneuronal cells populate Laminae I-III of the dorsal horn although some large cells have been reported in laminae I and III (Todd and Spike 1993). Most cells in lamina II appear to have axons which only arborize within the dorsal horn whereas some cells in laminae I and III long axons project to the brain (Willis and Cogeshall 1991). Lamina II can be identified by its translucent appearance in spinal cord slices (**Figure 1.1 b**). Interneurons laminae I-III play a crucial role in modifying incoming somatosensory information before it is relayed to higher brain centres (Melzack and Wall 1965; Jessell and Iversen 1977; Basbaum and Fields 1978). It must be noted that this interneuronal population has a mix of both inhibitory and excitatory neurons (Ritz and Greenspan 1985; Woolf and King 1989). Nociceptive signals are is received in lamina I, lamina II (*substantia gelatinosa*; (**Figure 1.1 a and b**) and to a lesser extent, lamina V (Todd 2010; Zeilhofer, Wildner et al. 2012; West, Bannister et al. 2015; Peirs and Seal 2016). While lamina I contains interneurons for modulation and projection neurons for transmission of nociceptive information, lamina II contains mainly interneurons which project to lamina I. Despite the plethora of new information

and new insights to our understanding of dorsal horn circuitry, there is still very much that is unknown (Sandkühler 2009; Todd 2010; Prescott, Ma et al. 2014; Peirs and Seal 2016).

1.3.2.1.1 Lamina I / Marginal Zone.

Also referred to as the marginal zone, lamina 1 contains both interneurons and projection neurons and receives inputs from Aβ and C-peptidergic primary afferents fibres (Torsney 2011; Cordero-Erausquin, Inquimbert et al. 2016) (Figure 1c). The immediate "first pain" evoked by tissue injury is conveyed by the Aδ fibres whereas the C- peptidergic afferent fibres, which contain substance P and CGRP, are involved transmitting the "second pain" that results from injury-induced inflammation. Lamina I also receives input from excitatory vertical cells in lamina II. Output neurons to higher centres express neurokinin NK1 receptors (Yu, Da Silva et al. 2005) that are activated by substance P (**Figure 1.1 c**).

1.3.2.1.2 Laminar IIo (outer)

The outer part of lamina II receives glutamatergic nociceptive input from lamina I as well as polysynaptic input from deeper laminae. It contains glutamatergic "stalked" or "vertical" cells (**Figures 1.1 c and 1.2 b**) which project back to lamina I and typically display a delayed firing pattern in response to a depolarizing current command (Yasaka, Tiong et al. 2010). Their dendrites penetrate deeper regions of lamina II and lamina III (Todd 2010; Peirs and Seal 2016).

Another population of excitatory neurons known as central cells express short (<400µm) rostro-caudal projections (Todd 2010; Todd 2017) (**Figure 1.1 c and 1.2 c**).

Inhibitory interneurons in lamina II_o typically display a tonic discharge pattern and "islet cell" morphology and project rostro-caudally for 400µm or more ((Grudt and Perl 2002; Todd 2010) (**Figure 1.2 a**). They receive some of their excitatory synaptic input from non-nociceptive, rapidly conducting Aβ fibres (Daniele and MacDermott 2009) and this may provide a cellular basis for the attenuation of pain by activation of innocuous sensory pathways (Zeilhofer, Wildner et al. 2012). It is likely that islet cell terminals release both glycine and GABA and that both neurotransmitters may be packaged in the same vesicles (Keller, Coull et al. 2001).

1.3.2.1.3 Lamina IIid (inner dorsal).

Neurons in lamina II_{id} receive direct input from C non-peptidergic afferents (Lu and Perl 2005; Cordero-Erausquin, Inquimbert et al. 2016) (**Figure 1.1 c**). These afferents generally do not convey nociceptive information and bind the plant isolectin IB4. They express P2X3 receptors for ATP and the RET – tyrosine kinase-GFR receptor complex for glial cell-line derived neurotrophic factor (GDNF) (Molliver, Wright et al. 1997).

1.3.2.1.4 Lamina II_{iv} (inner ventral)

Contains excitatory interneurons that express calretinin or PKC gamma (Peirs and Seal 2016). Immunohistochemical staining for this layer of PKC gamma expressing neurons has been

used to define the boundary between lamina II and III (Hughes, Hickey et al. 2013; Stebbing, Balasubramanyan et al. 2016; Boyle, Gutierrez-Mecinas et al. 2017).

1.3.2.1.5 Lamina III.

This receives peripheral input from Aβ/Aδ myelinated or lightly myelinated primary afferents (**figure 1.1 c**). Inputs project to parvalbumin containing inhibitory GABA/glycinergic neurons and to excitatory interneurons. NR 1 expressing projection neurons also reside in Lamina III (Todd 2010).

1.3.2.1.6 Laminae IV, V and VI.

These laminae contain wide dynamic range (projection) neurons which receive direct Aβ fibre input from primary afferents as well as polysynaptic inputs that convey nociceptive information from superficial laminae (Peirs and Seal 2016). Their rate of discharge is thus correlated to the type of input, with painful sensation eliciting high frequency discharge and innocuous stimuli generating more modest discharge (Dalal, Tata et al. 1999). These laminae are involved in the complex processing of tactile information such as object size, shape, texture as well as vibration and direction of stimulus movement.

With a diverse functional interneuronal population, for many years several researchers have tried to correlate structural morphology to a specific interneuronal cell type using several techniques including intracellular cell filling, golgi staining (Gobel 1978; Lima and Coimbra 1986)

and whole-cell-patch clamp recording from spinal cord slices in vitro (Grudt and Perl 2002; Heinke, Ruscheweyh et al. 2004; Maxwell, Belle et al. 2007; Yasaka, Kato et al. 2007; Yasaka, Tiong et al. 2010). A key advantage of using whole-cell patch-clamp is that it allows correlation of morphology with electrophysiological properties, for example, action potential firing pattern in response to injected depolarising current can be correlated with morphology determined by intracellular injection of neurobiotin and post-hoc confocal microscopy (Grudt and Perl 2002; Prescott and Koninck 2002; Balasubramanyan, Stemkowski et al. 2006; Lu, Moran et al. 2006; Punnakkal, von Schoultz et al. 2014). Likewise, it is possible to compare morphology with neurotransmitter phenotype, which can be determined by staining for specific vesicular transporters in the axons of recorded neurons (Yasaka, Tiong et al. 2010). For the past decade or so the use of mouse lines in which either inhibitory or excitatory interneurons are selectively labelled with a fluorescent protein has advanced our knowledge on structural morphology and interneuronal type (Heinke, Ruscheweyh et al. 2004; Punnakkal, von Schoultz et al. 2014). However, one major obstacle in using both morphological and electrophysiological properties to classify some of the interneurons in this region is that neither has provided a comprehensive classification scheme (Todd 2010) and thus designing and classifying an all-encompassing scheme for all cells has proven to be a challenge. To a large extent, it is possible to recognise some morphological neuronal classes in each the three laminae (Bennett, Abdelmoumene et al. 1980; Schoenen 1982), however, there is a sizeable number of neurons that are atypical and do not fit into any of the classes (Todd 1988; Réthelyi, Light et al. 1989). The superficial dorsal horn contains an extensive array of neurochemical markers, including neuropeptides and their receptors, calcium-binding proteins and a variety of enzymes (Todd 2017). These neuro-markers

have been shown to exhibit a specific laminar pattern, and are often distinctly distributed between excitatory and inhibitory interneurons (Todd and Spike 1993). As a result several neurochemically distinct populations have been defined (Polgár, Sardella et al. 2013; Gutierrez-Mecinas, Furuta et al. 2016). Using this approach, to define neuronal populations, these can be targeted for electrophysiological recording to provide detailed functional information about the neurons (Heinke, Ruscheweyh et al. 2004; Duan, Cheng et al. 2014; Punnakkal, von Schoultz et al. 2014; Peirs, Williams et al. 2015; Stebbing, Balasubramanyan et al. 2016).

1.3.3 Morphological Classification of Dorsal Horn Neurons in Lamina II

The defining morphological characteristic of neurons in the dorsal horn is their dendritic orientation. The anatomist, (Ramon y Cajal 1909) described three types of neurones in lamina II of the young cat: vertically cells , central cells , and transverse cells. Using Golgi-stained tissue, (Gobel 1975; Gobel 1978) also described four main types of neurones in the cat trigeminal lamina II: islet, stalked, arboreal (stellate-like) and border cells. In the monkey, (Price, Hayashi et al. 1979) observed only stalked and islet cells in the *substantia gelatinosa*. Extensive neuronal morphological variation has been reported by several research groups across all species (Beal and Cooper 1978; Light and Perl 1979; Rethelyi, Light et al. 1989). As a testament to the difficulty in classifying neurons in the *substantia gelatinosa* four morphological types of neurones that only partially fitted categories previously identified in other species were reported by (Schoenen 1982) in the human spinal cord. (Grudt and Perl 2002) using laminar location, geometry, neurite orientation as well as a host of electrophysiological attributes recognised five lamina II

morphological categories: Islet, radial, central, medial-lateral, and vertical neurons. Several neuropeptides are expressed by subsets of interneurons in laminae I-III (Todd 2017). Some of these, for example, neuropeptide Y (NPY), galanin and nociceptin are present in inhibitory interneurons, others, such as somatostatin, neurotensin, GRP, neurokinin B (NKB), substance P and cholecystokinin are found predominantly in excitatory interneurons, while the opioid peptides enkephalin and dynorphin are expressed by both excitatory and inhibitory interneurons (Rowan, Todd et al. 1993; Duan, Cheng et al. 2014; Gutierrez-Mecinas, Watanabe et al. 2014). In recent times the use of neurochemical markers has gained much prominence in classifying interneurons in laminae I-III.

Islet cells are present almost exclusively in lamina II and have dendrites that extend along the rostrocaudal axis of the spinal cord, usually remain within lamina II and often possess characteristic recurrent branches (Gobel 1975; Bennett, Abdelmoumene et al. 1980; Cervero and Iggo 1980; Grudt and Perl 2002). (Gobel 1978) reports that islet cells typically have few dendritic spines and comprise approximately 30% of neurons in laminae II (**figure 1.2 a**). Islet cells respond to step depolarization with a sustained repetition of action potentials and also many showed an I_h-like current (Grudt and Perl 2002).

Vertical neurones are partially a heterogeneous group which have in common a dendritic arbor and possess a distinctive dorso-ventral orientation with sparse dendrites than other lamina II neurones (Grudt and Perl 2002). This cell type have been previously characterised as stalked cells (Gobel 1975; Gobel 1978; Bennett, Abdelmoumene et al. 1980; Cervero and Iggo

1980; Todd and Lewis 1986). They are the most common type of neuron in lamina II (Gobel 1978), (**Figure 1.2 b**).

(Ramon y Cajal 1909) described neurones commonly situated in the mid-zone of the *substantia gelatinosa* central cells due to their location. Central cells have a general configuration similar to islet cells, but with a much smaller dendritic expansion in the rostro-caudal direction. They have small cell bodies with two main dendritic trees emerging from opposite sides of the cell body (**figure 1.2 c**). The axonal arborization of central cells are moderately extensive in the vicinity of the dendritic tree (Grudt and Perl 2002). The dendritic tree of central cells in comparison to islet cells does not extend as far rostrocaudally as islet cells and because of this close similarity, they are sometimes referred to as "small islets" (Todd and Lewis 1986). In view of the fact that both islet and central cells have dendritic trees oriented along the same rostocaudal axis and the difficulty in differentiating their lengths of projections in transverse sections (Lu, Biggs et al. 2009; Stebbing, Balasubramanyan et al. 2016) combined the two naming them as "islet-central" cells. Two major groups of central cell exist (Grudt and Perl 2002) based on their pattern of action potential discharges evoked by a step depolarization: transient central cells (fire a few action potentials and then go silent when depolarized) and tonic central cells (fire action potentials to the depolarizing step in a sustained or tonic fashion).

Radial cells have their dendrites radiating in all directions, hence the name. Spines are present on radial cell dendrites at low to moderate density which form a compact dendritic tree (**figure 1.2 d**). Also (Todd and Lewis 1986) have described a similar set of spiny neurons with

dendrites radiating in all directions. Action potentials of radial neurones are delayed from the onset of a depolarizing step (Grudt and Perl 2002).

Mediolateral neurones are a small group of cells notable for being the sole lamina II neurones with dendrites that extended substantially in the medio-lateral dimension (Grudt and Perl 2002) (**figure 1.2 e**). Mediolateral cells fire action potentials on depolarization in a sustained, repetitive pattern.

It must be said that all the five cell morphological classes primarily receive C fibre inputs with the exception of vertical cells which receive both A-delta and C-fibre inputs (Yasaka, Kato et al. 2007). **Figure 1.2 f** illustrates the orientation of major neuron types in the *substantia gelatinosa* neurons.

1.3.4 Electrophysiological characteristics

Previous studies, mostly done on rats (Grudt and Perl 2002; Prescott and Koninck 2002; Balasubramanyan, Stemkowski et al. 2006) have utilised electrophysiological properties of neurons to classify distinct populations of dorsal neurons. Researchers use action potential firing pattern of neurons in response to depolarising current injections. Using this criteria is a convenient and appropriate way of identifying neuronal populations because the output of neurons determines behaviour and function in neuronal signaling. In the marginal zone of the spinal cord various electrophysiological cell types have been identified. Lamina I neurons in rat have been classified into five different categories based on firing properties. These have been defined as: tonic, phasic, initial burst, delayed onset, and single spike (Prescott and Koninck 2002;

Ruscheweyh and Sandkühler 2002). In rat lamina II, (Balasubramanyan, Stemkowski et al. 2006) identified six electrophysiological cell types ; tonic, phasic, initial burst, delay tonic, delay irregular and irregular firing patterns (**figure 1.3**).

1.3.5 Species Differences

As already mentioned most characterisation of dorsal horn neurons work has been done on rats, surprisingly little is known about dorsal horn circuitry in mice as compared to the extensive reports in rats (Lu and Perl 2005; Balasubramanyan, Stemkowski et al. 2006; Lu, Biggs et al. 2009; Todd 2010; Peirs and Seal 2016; Abraira, Kuehn et al. 2017; Todd 2017). As such, defining these differences represents one of the portions of my dissertation.

1.4 Onset of Neuropathic Pain: Periphery Nerve Injury Studies & Mechanisms

As mentioned already neuropathic pain leads to changes in normal sensory signaling at the level of the periphery, spinal cord and brain (thalamus and cortex) that may occur over the course of weeks or months. Consequently, these changes lead to alterations in genomic expression and differences in cortical structures (Costigan, Scholz et al. 2009; Sandkuhler 2009; Alvarado, Tajerian et al. 2013; Tajerian, Alvarado et al. 2013; Luo, Kuner et al. 2014). These genomic changes that occur prior to its physical and clinical manifestation thus highlight the fact that the pathophysiological changes responsible for the onset of neuropathic pain are distinct from those responsible for its chronic presentation (Alles and Smith 2018). Highlighting this key distinction between the “onset” phase and the “maintenance” phase of neuropathic pain

becomes particularly relevant when attempting to relate findings in animal models with the presentation of pain in the clinic. As pain researchers it behoves on us to recognise this flaw in our research methodologies and design animal studies that seek to address the long-term persistence of pain which is more prevalent in clinical settings but also taking into cognizance the mechanisms that leads to pain onset.

Over the years, several animal models of neuropathic pain have been developed to aid in understanding its pathogenesis. Strong emphasis has been placed on studying traumatic peripheral nerve injury models in rodents, which produce reproducible pain-related behaviour with a defined time course. (Calvo, Dawes et al. 2012; Stemkowski and Smith 2012a). With such outcomes, in experimental animals, peripheral nerve damage, such as chronic constriction injury, induces pain-related behaviours that are widely accepted as a model for human neuropathic pain (Bennett and Xie 1988). To buttress the importance of using peripheral nerve injury models to study neuropathic pain, it has been suggested that injury to the spinal cord *per se* may engage peripheral mechanisms to generate chronic pain (Yang, Wu et al. 2014). Upon injury there is release of inflammatory mediators at the site of injury that triggering alterations in the properties of primary afferent neurons (Watkins and Maier 2002; Scholz and Woolf 2007). This causes an increase in excitability thus leading to the appearance of ectoptic, stimulus-independent activity (Wall and Devor 1983). The result is that there is altered activity arising from changes in the properties and/or expression of various types of voltage-gated Na^+, K^+ and Ca^{2+} channels (Waxman, Cummins et al. 1999; Kocsis and Devor 2000; Abdulla and Smith 2001; Abdulla and Smith 2002; Stemkowski and Smith 2012; Bourinet, Altier et al. 2014; Waxman and Zamponi 2014; Daou, Beaudry et al. 2016). Additional changes occur in the function of Na^+-K^+ ATPases

(Ventéo, Laffray et al. 2016), intracellular Ca^{2+} fluxes (Hogan, Sprick et al. 2014; D'Arco, Margas et al. 2015; Yilmaz and Gold 2016; Yilmaz, Watkins et al. 2017) TRP channels (Basso and Altier 2017) and hyperpolarization-activated cyclic nucleotide–gated (HCN) channels (Hogan and Poroli 2008; Emery, Young et al. 2011; Noh, Kumar et al. 2014; Young, Emery et al. 2014).

The idea of 'central sensitization' was first put forward in the early 1980s to describe the cascade of events attributable to the maladaptive changes in plasticity of sensory processing that occur in neuropathic pain (Woolf 1983; Woolf and Thompson 1991; Woolf and Mannion 1999; Latremoliere and Woolf 2009)(Latremoliere and Woolf, 2009;Woolf, 1983;Woolf and Mannion, 1999;Woolf and Thompson, 1991). Decades of pain research has showed us that injury to sensory axons can bring about changes in the organization of the spinal cord sensory map (Devor and Wall 1978) and that sensory disturbances relating to neuropathic pain can be attributed to changes in excitability of the injured neuron and abnormal ongoing and evoked discharge from "ectopic neural pacemaker sites" (Govrinlippmann and Devor 1978; Wall and Devor 1983). Devor and others (Devor, Wall et al. 1992) showed that lidocaine could silence the ectopic discharge from the neuroma induced by nerve injury or from dorsal root ganglia and reduce pain centralization.

1.4.1 Excitation-Inhibition Balance in Neuropathic Pain.

Continuous maladaptive plasticity within the dorsal horn (DH) of the spinal cord serves as the template or substrate for the development of neuropathic pain following peripheral nerve injury (West, Bannister et al. 2015). As a result of peripheral nerve injury or disease-induced

neuropathies, the peripheral and central neural networks involved in nociception show extensive plasticity (Kuner 2010). As a consequence of changes in synaptic transmission, central sensitization develops within the spinal cord, thus excitatory synaptic processes are enhanced and inhibitory processes attenuated (Kuner 2010; Prescott, Ma et al. 2014; West, Bannister et al. 2015). These plastic changes do not affect the intrinsic properties of dorsal horn neurons like rheobase, threshold, excitability and/or input resistance as a result of peripheral nerve injury (Balasubramanyan, Stemkowski et al. 2006). The increase in ectopic activity in peripheral nerves could be therefore be responsible for driving and maintenance of central sensitization (Devor 2006; Pitcher and Henry 2008; Gold and Gebhart 2010; Vaso, Adahan et al. 2014; Daou, Beaudry et al. 2016; Sexton, Cox et al. 2018).

1.4.1.1 Role of BDNF

The involvement of BDNF in nociceptive processing was fist described almost 20 years (Kerr, Bradbury et al. 1999). As stated earlier , chronic constriction injury (CCI) of the sciatic nerve, is a rodent pain model widely used to investigate the etiology of neuropathic pain (Mosconi and Kruger 1996; Fotis, Gary et al. 2005; Pitcher and Henry 2008; Stemkowski and Smith 2012). Changes in synaptic transmission in the dorsal horn are mediated at least in part by the release of BDNF from microglia. There are striking similarities between the effects produced by CCI *in vivo* and the effect of long term (10d) exposure of neurons in defined medium organotypic culture (DMOTC) to BDNF (Lu, Ballanyi et al. 2007; Lu, Biggs et al. 2009).

Additional evidence supporting a role for BDNF, comes from experiments with activated microglia conditioned medium (aMCM). At least for male rats, activation of dorsal horn microglia is an early consequence of peripheral nerve injury (Weitzman, Zimmerman et al. 1983; Scholz and Woolf 2007; Costigan, Scholz et al. 2009; Latremoliere and Woolf 2009). Based on this reasoning (Biggs, Lu et al. 2012) treated neurons in DMOTC (defined medium organotypic culture) with activated microglia conditioned medium (aMCM) to find a way of "cultivating" the central sensitization process 'in a dish'. Based on the assumption that mediators (cytokines growth factors etc) present in aMCM are similar to those released in the dorsal horn following CCI in vivo, they treated neurons in defined medium organotypic culture (DMOTC) with aMCM from cortical microglial cultures pre-activated with lipopolysaccaride (LPS) (Lai and Todd 2008; Lu, Biggs et al. 2009). Expectedly, exposure of DMOTC to aMCM increased overall dorsal horn excitability as measured by confocal Ca^{2+} imaging (Lu, Ballanyi et al. 2007; Lu, Biggs et al. 2009) and this effect (dorsal horn excitability) was attenuated by sequestering BDNF with the recombinant binding protein, TrkBd5 (Banfield, Naylor et al. 2001; Lu, Biggs et al. 2009). These findings support evidence for the role of microglia-derived BDNF as a major driver of central sensitization (Coull, Beggs et al. 2005; Biggs, Lu et al. 2010; Trang, Beggs et al. 2011; Smith 2014). These and additional effects of BDNF to be described below highlights the fact that this neurotrophin is a key molecule of interest that plays an essential role in pain centralization and justifies the need to study its actions in the onset of neuropathic pain.

1.4.2 Dysfunctional Sensory Processing and Development of Allodynia

Reduction or attenuation of GABAergic and/or glycinergic transmission/signaling leads to aberrant processing of sensory information within the dorsal horn after nerve injury (Baba, Ji et al. 2003; Torsney and MacDermott 2006; Prescott, Ma et al. 2014). Several other factors leading to reduction in inhibitory transmission include changes in chloride gradient (Coull, Boudreau et al. 2003; Coull, Beggs et al. 2005; Coull, Beggs et al. 2005), and decreased excitatory drive to inhibitory neurons (Balasubramanyan, Stemkowski et al. 2006; Lu, Moran et al. 2006; Lu, Colmers et al. 2009; Leitner, Westerholz et al. 2013) as well as the loss of GABAergic terminals (Lorenzo, Magnussen et al. 2014) or reduced glycine release (Imlach, Bhola et al. 2016). As already mentioned, large $A\beta$ fibres which serve as conduits for tactile and innocuous information synapse primarily onto dorsal horn neurons in laminae III and IV (Abraira, Kuehn et al. 2017) whereas noxious information carried by C- and $A\delta$-fibres is transmitted to the superficial laminae I and II (Peirs and Seal 2016) (**Figure 1.1 c**). In a structured manner GABAergic and glycinergic inhibition normally separates these two modalities by suppressing the activity of pre-existing excitatory synaptic circuits (Lu, Dong et al. 2013). However, when this well laid out inhibition structure is compromised, tactile and innocuous information travelling to lamina III and IV crosses over or gains access to the pain processing centres in lamina I and II. Consequently, touch is processed as pain thereby providing a rational explanation for the generation of allodynia (Baba, Ji et al. 2003). In furtherance to this idea, there are reports of the impediment of inhibitory transmission in the spinal cord with bicuculline and/or strychnine produces allodynia and hyperalgesia in uninjured animals (Yaksh 1989; Sherman and Loomis 1994; Loomis, Khandwala et al. 2001). In addition, irregular distribution of tactile information is reinforced by increased

excitatory transmission between deep and superficial laminae through the transient expression of the vesicular glutamate transporter VGLUT3 by a specific population of neurons which receive direct low threshold primary afferent input (Peirs and Seal 2016) (**Figure 1.1 c**).

1.4.3. Other Spinal Mechanisms of Central Sensitization

1.4.3.1. LTP and Memory Processes

The plastic nature of CNS synapses is evident by the fact that repeated activation of presynaptic fibres leads to short term potentiation (lasting for less than half an hour), early phase long term potentiation (LTP, lasting for up to 3h) and late phase LTP which has been shown to depend on protein synthesis and can last indefinitely (Luo, Kuner et al. 2014). It is possible therefore that, after peripheral nerve injury, increasing activity of both primary afferent fibres and dorsal horn neurons would cause these processes to be continuously engaged and serve to reinforce central sensitization (Ji, Kohno et al. 2003; Sandkuhler 2007; Fenselau, Heinke et al. 2011; Ruscheweyh, Wilder-Smith et al. 2011; Luo, Kuner et al. 2014).

There are reports showing that mechanistically spinal LTP involves presynaptic mechanisms (Luo, Kuner et al. 2014) as well as opening of T-type (Ca_v3) calcium channels and activation of NMDA receptors (Ikeda, Heinke et al. 2003; Zhou and Luo 2015). These receptors have long been implicated in the etiology of neuropathic pain (Woolf and Thompson 1991; Kerr, Bradbury et al. 1999; Latremoliere and Woolf 2009; Salter and Pitcher 2012; Hildebrand, Xu et al. 2016). Previously it has been shown that brain-derived neurotrophic factor (BDNF) contributes to spinal long-term potentiation (LTP) and pain hypersensitivity through activation

of GluN2B-containing N-methyl-d-aspartate (GluN2B-NMDA) receptors in rats following spinal nerve ligation (SNL) (Li, Cai et al. 2017).

1.4.3.2. Role of astrocytic glutamate transporter (EAAT2)

Downregulation of the astrocytic excitatory amino acid transporter, EAAT2 correlates with development of signs of neuropathic pain (Cata, Weng et al. 2006; Weng, Chen et al. 2006). It has been reported that both partial sciatic nerve ligation (PSNL) and chronic constriction injury (CCI) significantly reduce expression of EAAT2 in rat dorsal horn (Sung, Lim et al. 2003) whereas spinal EAAT2 gene transfer via recombinant adenovirus significantly decreases mechanical hyperalgesia and allodynia (Maeda, Kawamoto et al. 2008). The signal for downregulation of EAAT2 is unlikely to be BDNF as this neurotrophin upregulates the transporter in other brain regions (Rodriguez-Kern, Gegelashvili et al. 2003).

1.4.3.3. The Pain Matrix

The ‘‘pain matrix’’ including the medial prefrontal cortex, nucleus accumbens, anterior cingulate cortex, insula, amygdala, periaqueductal gray, *locus coeruleus*, and rostroventral medulla (Schweinhardt and Bushnell 2010; Peirs and Seal 2016; Tan, Pelzer et al. 2017; Taylor, Mehrabani et al. 2017) undergo several changes as a result of nerve injury. For instance, synapses in the anterior cingulate cortex are altered after peripheral nerve injury and LTP of glutamatergic transmission appears in the insula (Zhuo 2016). It is worthy of note that brain regions that

comprise the “pain matrix” were identified by functional neuroimaging (Apkarian, Hashmi et al. 2011) and rather interestingly, areas activated by acute pain have been shown not to correspond exactly to those activated in chronic pain (Apkarian, Hashmi et al. 2011; von Hehn, Baron et al. 2012).

1.4.3.4 Changes in Descending Control Mechanisms

Alterations in descending control mechanisms could arise as a result of changes in the pain matrix (Porreca, Ossipov et al. 2002; Ossipov, Dussor et al. 2010). It is well accepted that descending inhibition of nociceptive processing is mediated via α_2 adrenoceptors and $5HT_7$ receptors whereas serotonergic activation of metabotropic $5HT_2$ receptors and ionotropic $5HT_3$ receptors facilitates transmission (Millan 2002; Bannister, Patel et al. 2015; Bannister, Lockwood et al. 2017; Bannister, Qu et al. 2017).

1.4.3.5 Role of Ectopic Activity in Primary Afferent Fibres

As already mentioned in preceding paragraphs, processes involved in the onset of neuropathic pain are different from those involved in its long term persistence and maintenance (Ji and Woolf 2001). As has been reported by several investigators, classical mediators of inflammation such as interleukin 1β (Binshtok, Wang et al. 2008; Stemkowski and Smith 2012; Stemkowski and Smith 2012), interleukin 6 (Ko, Eddinger et al. 2016), prostaglandins (Ma and Eisenach 2002), interleukin 17 and tumor necrosis factor (Leung and Cahill 2010) interact with

or modulate ion channels on primary afferent neurons to instigate ectopic activity that contributes to spontaneous, stimulus independent pain (Pitcher and Henry 2008; Devor 2009; Devor, Vaso et al. 2014). Experiments carried out by (Balasubramanyan, Stemkowski et al. 2006; Devor 2006) have shown that following a peripheral nerve injury there is altered synaptic activity of *substantia gelatinosa* neurons with little effect on their intrinsic electrophysiological properties thus suggesting that changes in the central nervous system are driven by activity of peripheral neurons. This assumption is supported by the observation that application of lidocaine to block nerve conduction and subsequent monitoring of spontaneous baseline discharge that the hyper-excited state of dorsal horn neurons is maintained by ongoing, afferent discharges from the peripheral nerve distal to, and proximal to the site of injury (Pitcher and Henry 2008). One of the many mechanisms that may be involved is that an increase in activity of afferent nerves leads could lead to an upregulation of the $\alpha 2\delta$-1 subunit of voltage gated Ca^{2+} channels (Boroujerdi, Kim et al. 2008) which in turn alters Ca^{2+} channel function in primary afferent terminals and increases transmitter release (Hoppa, Lana et al. 2012).

1.4.3.6. Neuroimmune Interactions in Neuropathic Pain

The role of immunocompetent cell such as astrocytes, endothelial cells, perivascular macrophages, infiltrating T-cells and satellite glial cells of DRG neurons in the etiology and development of neuropathic pain is well established (Scholz and Woolf 2007; Watkins 2007; Costigan, Moss et al. 2009; Beggs, Liu et al. 2010; Calvo, Dawes et al. 2012; Grace, Hutchinson et al. 2014; Vicuna, Strochlic et al. 2015; Dodds, Beckett et al. 2016; Ji, Chamessian et al. 2016; Lim,

Lee et al. 2017; Mikuzuki, Saito et al. 2017). Immune cells express many of the receptors and transduction mechanisms that are activated by injury to neurons (Chiu, von Hehn et al. 2012). The idea of immune system involvement in chronic pain came about from observing classical signs of a systemic sickness response (malaise, lethargy, depression, anxiety) in chronic pain patients (Alles and Smith 2018). This idea was later corroborated by findings showing that elevated levels of interleukin-1β (IL-1β) in peripheral nerves could both mediate and induce hyperalgesia (Grace, Hutchinson et al. 2014). IL-1β, in addition to its proinflammatory actions, can directly activate nociceptors to generate action potential firing, increased membrane availability of $Ca_v3.2$ (T-type) channels and as well as hyperalgesia (Binshtok, Wang et al. 2008; Stemkowski and Smith 2012a; Stemkowski, Noh et al. 2015; Stemkowski, Garcia-Caballero et al. 2017). IL-1β increases expression of colony stimulating factor (CSF-1) in primary afferent neurons (Lim, Lee et al. 2017) which upregulates BDNF gene in spinal microglia and is believed to be necessary and sufficient for the onset of neuropathic pain (Guan, Kuhn et al. 2016; Okubo, Yamanaka et al. 2016).

Papers by (Guan, Kuhn et al. 2016; Okubo, Yamanaka et al. 2016) have recently highlighted the important role of CSF-1 in the onset of neuropathic pain. As a cytokine released by injured primary afferent neurons into the spinal cord after nerve injury, CSF-1 release engages CSF-1 receptors primarily in a DAP12-dependent pathway for microglia gene upregulation and consequent neuropathic pain has been shown (Guan, Kuhn et al. 2016). CSF-1 is involved in the proliferation and differentiation from hematopoietic stem cells to a specific kind of white blood cells, such as macrophages and granulocytes (Jenkins and Hume 2014). Three types of CSFs, macrophage-colony stimulating factor (M-CSF), granulocyte macrophage-colony stimulating

factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) have been shown to exist (Borycki, Lenormand et al. 1993; Han, Ramesh et al. 1996), and these CSFs each has specific receptors; M-CSF receptor (M-CSFr), GM-CSF receptor alpha (GM-CSFr alpha) and G-CSF receptor (G-CSFr). Activation of the M-CSF receptor (M-CSFr) on microglia causes pain (Guan, Kuhn et al. 2016).

Reactive microglia release cytokines and chemokines such as fractalkine (CX3CL1) and other inflammatory mediators which trigger peripheral immune cell infiltration and astrogliosis. Damaged sensory neurons also release chemokine (C-C motif) ligand 2 (CCL2 also known as monocyte chemoattractant protein 1; MCP1) as well as neuregulin 1 and fractalkine. In summary, the net effect of complex interactions between invading and resident immunocompetent cells and numerous signaling molecules is the generation of classical inflammatory mediators such IL-1β, interleukins 6 and 18 and tumor necrosis factor (TNF) which influence neuronal activity by facilitation of spinal LTP (Gruber-Schoffnegger, Drdla-Schutting et al. 2013), direct excitatory actions on neurons (Binshtok, Wang et al. 2008; Gustafson-Vickers, Lu et al. 2008; Kawasaki, Zhang et al. 2008; Stemkowski and Smith 2012a; Stemkowski, Noh et al. 2015), increased release of glutamate from primary afferents (Yan and Weng 2013) and decreased release of GABA from dorsal horn interneurons (Zhang and Dougherty 2011).

1.4.4. Cellular and Molecular Mechanisms of BDNF in Central Sensitization

1.4.4.1 BDNF and a shift in neuronal chloride gradient.

A decrease in transmembrane chloride gradient occurs in rat dorsal horn lamina I neurons following peripheral nerve injury as a result of reduced expression of the potassium-chloride exporter, KCC2 (Coull, Boudreau et al. 2003). This results in the retention of intracellular Cl^- which can causes normally inhibitory GABAergic, anionic, outward synaptic currents to become inward excitatory findings was put forwarded by (Coull, Boudreau et al. 2003; Coull, Beggs et al. 2005) who showed that a knockdown of KCC2 expression a in non-injured rats reduced pain thresholds and resulted in neuropathic pain behaviors. Hence, an injury that causes neuropathic pain results in a marked change in dorsal horn function such that the net excitability of nociceptive lamina I neurons is increased (Prescott, Sejnowski et al. 2006) .

The downregulation of KCC2 and depolarizing shift in anion reversal potential observed in lamina I neurons is thought to result from the release of brain-derived neurotrophic factor (BDNF) from activated spinal microglia (Coull, Beggs et al. 2005). These authors showed that the administration of activated microglia or application of BDNF promoted the cause of the shift in anion gradient seen after nerve injury. Also blocking signaling between BDNF and one of its cognate receptors (TrkB) in nerve injured animals reversed pain behaviors (allodynia) and the shift in anion gradient. Finally, they also showed that allodynia and the shift in anion gradient can be prevented by blocking release of BDNF from microglia by treatment with interfering RNA against BDNF.

It was shown recently that BDNF potentiates excitatory NMDA receptor-mediated currents through activation of TrkB and phosphorylation of the GluN2B subunit by the Src-family kinase Fyn (Hildebrand, Xu et al. 2016). These authors found out that that the ongoing release of BDNF and continuous activation of TrkB-Fyn signaling is required to maintain the basal potentiation of NMDAR responses at lamina I synapses in peripheral nerve injured rats (PNI) and that this signaling pathway requires prior and sustained KCC2-dependent disinhibition. Interestingly, this potentiation appears to require the coincident BDNF mediated Cl^- disinhibition.

1.4.4.2 Role of ATP.

Following peripheral nerve injury, there is an increase in the expression of the ATP-gated ionotropic purinoceptor, P2X4R and in the metabotropic purinoceptor, P2Y12 in microglia and this increase in expression parallels the increase in pain hypersensitivity (Tozaki-Saitoh, Tsuda et al. 2008; Trang, Beggs et al. 2011; Trang, Beggs et al. 2012). The mechanism by which P2X4R upregulation leads to BDNF release from microglia is attributed to influx of Ca^{2+} through P2X4Rs, activation of p38-mitogen-activated-kinase (MAPK) and a consequent increase in synthesis and SNARE-mediated exocytosis of BDNF (Trang, Beggs et al. 2009). It is worthy of note to point out that the mechanism as stated above is specific to male mice as microglia are not required for mechanical sensitivity to pain in female mice in which adaptive immune cells are involved (Sorge, Mapplebeck et al. 2015; Sorge and Totsch 2017).

1.4.4.3 Signaling between injured peripheral nerve and spinal microglia.

The change or transformation of resting microglia into a phenotype that expresses P2X4R has been ascribed to the release of chemical mediators from injured primary afferent fibres. The chemokines, CX3CL1 (fractalkine) (Milligan, Zapata et al. 2004; Grace, Hutchinson et al. 2014), CCL2 and CCL21 (Biber, Tsuda et al. 2011; Toyomitsu, Tsuda et al. 2012) were initially thought to be involved, but, recent work by (Guan, Kuhn et al. 2016; Okubo, Yamanaka et al. 2016) discounts these mediators and instead favors colony stimulating factor (CSF-1) as the primary effector of microglial transformation in neuropathic pain. It has been suggested that the injury-induced release of inflammatory mediators such as interleukin 1β from satellite glial cells in DRG promotes induction of *Csf1* in the cell bodies of primary afferent neurons (Lim, Lee et al. 2017). CSF-1 is released from primary afferents and acts on microglia to induce various genes, including that for the ATP receptor, P2X4R as well as microglial proliferation and self-renewal (Guan, Kuhn et al. 2016). Although CSF-1 is implicated it remains to be determined whether it actually excites dorsal horn neurons and whether this effect is mediated via release of BDNF. This missing link is one issue to be investigate in my thesis. **Figure 1.4 a** presents a summary of cellular processes thought to contribute to central sensitization.

1.4.4.4 Increased Excitatory Drive to Excitatory Neurons and Decreased Excitatory Drive to Inhibitory Neurons

Peripheral nerve damage arising from chronic constriction injury (CCI) or axotomy of the sciatic nerve has also been shown to produce an 'electrophysiological footprint' of neuropathic

pain in the *substantia gelatinosa* (Biggs, Lu et al. 2010; Smith 2014). In this "footprint", (Balasubramanyan, Stemkowski et al. 2006) classified neurons according to their firing pattern as tonic, delay, irregular, phasic or transient and the effects of CCI noted as increases or decreases in the amplitude and frequency of spontaneous or miniature EPSC's. With a clear and established relationship between neuronal firing pattern and its neurotransmitter phenotype (Yasaka, Tiong et al. 2010; Punnakkal, von Schoultz et al. 2014), the footprint reflects an increased excitatory drive to putative excitatory delay-firing and transient firing neurons and a decreased excitatory drive to putative inhibitory tonic-firing neurons (Bailey and Ribeiro-da-Silva 2006; Balasubramanyan, Stemkowski et al. 2006; Chen, Balasubramanyan et al. 2009; Lu, Biggs et al. 2009; Leitner, Westerholz et al. 2013) (**Figure 1.4 b**). Both of these changes would be expected to produce an overall increase in dorsal horn excitability (Alles and Smith 2018)). Decreased excitatory drive to inhibitory neurons involves both pre- and postsynaptic changes including a decreased contribution of Ca^{2+} permeable AMPAR to sEPSC's (Chen, Derkach et al. 2016). Increased excitatory drive to excitatory neurons also involves presynaptic effects and a possible increased contribution of Ca^{2+} permeable AMPAR to postsynaptic events (Chen, Zhou et al. 2013).

1.5 Organotypic Cultures

The study the long-term effects of key mediators such as BDNF after nerve injury is essential to gain a better understanding of the pathophysiology of neuropathic pain. This is not readily accomplished using conventional acutely isolated spinal cord slices as these do not retain viability beyond 1 day, at least not when obtained from mature mammals (Biggs, Lu et al. 2012).

The time frame may be too brief a period for analysing functional changes brought about by processes such as altered protein expression, axonal sprouting, dendritic retraction and synapse formation or elimination (Biggs, Lu et al. 2012). Organotypic slice cultures represent an excellent compromise between single cell cultures and complete animal studies as it replaces and reduces the number of animal experiments (Mewes, Franke et al. 2012). In such cultures, neurons mature and differentiate morphologically, and intricate functional synaptic networks are formed *de novo*. In addition, they represent a long-term model system that, due to particular growth conditions, allow a unique experimental accessibility to cells maintained *in vitro* (Gähwiler 1981). Organotyic cultures from embryonic mouse spinal cords provide an excellent in vitro model to study tissue organization that closely resemble that observed in situ (Gähwiler, Capogna et al. 1997; Avossa, Rosato-Siri et al. 2003). These cultures express GABAergic interneurons that conserve the proper spatiotemporal pattern of development observed in vivo (Avossa, Rosato-Siri et al. 2003).

1.5.1 Defined Medium Organotypic Cultures of Rodent Spinal Cord

Our lab uses defined medium organotypic cultures (DMOTC) to avoid the effects of various undefined growth factors, cytokines and other mediators that may be present in conventional cultures. We find that DMOTC of rat spinal cord slices remain viable for > 6 weeks, thus allowing the investigator to expose intact neurons to mediators or drugs for periods of days or weeks (Lu, Moran et al. 2006; Lu, Colmers et al. 2009). Prior work done in the Smith lab has shown that the synaptic pharmacology and connectivity of cultures obtained from DMOTC spinal

cord slices remain intact and also the various morphological and electrophysiological phenotypes of dorsal horn neurons are preserved (Lu, Moran et al. 2006; Lu, Colmers et al. 2009). Likewise, because individual neurons can easily be visualized, the DMOTC is especially amenable to electrophysiological recordings using infrared differential interference contrast (IR-DIC) optics and to dynamic imaging of the free cytosolic Ca^{2+} concentration which provides the needed information on both the activity and morphology of neuronal (and glial) populations (Lu, Ballanyi et al. 2007; Lu, Biggs et al. 2009). In conclusion we suggest that, spinal cord DMOTC provide an excellent model to study the regulation of neural circuits in the dorsal horn by defined concentrations of putative mediators of chronic pain for extended time period (Gähwiler, Capogna et al. 1997; Lu, Moran et al. 2006; Lu, Ballanyi et al. 2007; Gustafson-Vickers, Lu et al. 2008; Lu, Biggs et al. 2009; Lu, Colmers et al. 2009).

As is described in **chapter 3**, part of my rsearch involved modification of methodology's for rat spinal DMOTC for generation of mouse DMOTC (Boakye, Schmidt et al. 2018).

1.6 Glutamic Acid Decarboxylase (GAD-67) Green Fluorescent Protein (GFP) Expressing Mice

GABA and glycine are the principal inhibitory neurotransmitters in the spinal dorsal horn in the spinal cord. Neurones containing the GABA synthesizing enzyme glutamate decarboxylase (GAD) or immunoreacting with anti-GABA have been described predominantly in the superficial laminae I and II (Kaduri, Magoul et al. 1987; Magoul, Onteniente et al. 1987; Todd and Sullivan 1990; Maquet 2000; Mackie, Hughes et al. 2003). To facilitate the study of changes in excitatory

synaptic drive to GABAergic neurons, which underlies neuropathic pain, transgenic mice that selectively express the "enhanced" derivative of the autofluorescent protein, green fluorescent protein (EGFP) (Morise, Shimomura et al. 1974; Prasher, Eckenrode et al. 1992; Cormack, Valdivia et al. 1996) in subpopulations of GABAergic neurons under the control of a GAD67 regulatory promoter (Jiang, Oliva et al. 2001) will be used to identify spinal GABAergic neurons (Haas and Panula 2003). Several varieties of GAD67-GFP mice are available, (Oliva, Jiang et al. 2000; Punnakkal, von Schoultz et al. 2014) including GIN mice but since not all GABAergic neurons in this strain exhibit GFP, we used the Tamamaki GAD67-GFP mice (Tamamaki, Yanagawa et al. 2003).

Another aspect of my research was to confirm expression of GAD67-EGFP in appropriate cell types as spinal cord slices from "*Tamamaki*" mice were maintained in DMOTC (Boakye, Schmidt et al. 2018) **(Chapter 2)**.

1.7 BDNF and its cognate receptors

BDNF is a 12.4 kDa basic protein and belongs to a family of four structurally and functionally related neurotrophins that regulate the growth, maintenance and apoptosis of neurons in the developing nervous system as well as injured neurons (Davies 2008; Skaper 2012; Ceni, Unsain et al. 2014) and was initially isolated from pig brain (Barde, Edgar et al. 1982; Pezet and McMahon 2006). It is synthesised as a precursor molecule called proBDNF at ~30–34 kDa, and undergoes proteolytic cleavage by furin or other pro-convertases in the endoplasmic reticulum and Golgi apparatus to produce C-terminally mature BDNF at ~13-15 kDa (Teng, Felice

et al. 2010; Richner, Ulrichsen et al. 2014). BDNF binds to its cognate tyrosine receptor kinase receptors; Trk B and to the p75 receptor (Reichardt 2006). BDNF is constitutively synthesized by a subpopulation of dorsal root ganglia (DRG) neurons (Pezet and McMahon 2006) and anterogradely transported into the dorsal horn of the spinal cord where it modulates central sensitisation that underpins maintenance of neuropathic pain (Khan and Smith 2015). These DRG neurons are small and medium-sized peptidergic neurons and represent 10%–40% of the population of DRG neurons (Luo, Rush et al. 2001; Merighi, Carmignoto et al. 2004). However, the pro form of BDNF (proBDNF) binds to to the p75NTR as it's high affinity receptor. A key role of p75 may be to provide more specificity to the interaction of neurotrophins with Trk family members. Benedetti and his group showed that Trk receptor family members respond far more selectively to different neurotrophins when the p75 neurotrophin receptor is co expressed (Benedetti, Levi et al. 1993). By the expression of high levels of the p75 neurotrophin receptor, there is restriction in the ability of $p140^{trk}$ to respond to NGF, and not NT-3. This differential behavior is reflected in the binding of NT-3 to p75, which occurs with much slower kinetics than NGF binding to p75 (Rodríguez-Tébar, Dechant et al. 1992). Hence, other Trk receptor family members may respond far more selectively to different neurotrophins when the p75 neurotrophin receptor is expressed leading to slower kinetics.

Activation of the p75 receptor in some situations can have an inhibitory effect. Although most effects mediated via TrkB receptor activation involve neuronal growth and increases in synaptic transmission (Kerr, Bradbury et al. 1999) retrograde signalling via BDNF (or proBDNF) acting on p75 receptors is implicated on long-term depression in hippocampal neurons (Woo, Teng et al. 2005). Similarly a long-term (5-6d) application of BDNF reduces mEPSC frequency in

tonic firing inhibitory neurons in a similar fashion to peripheral nerve injury (Lu, Biggs et al. 2009; Biggs, Lu et al. 2010), thus raising the intriguing possibility that neuron-derived BDNF or proBDNF may act as retrograde signal via p75NTR to impair excitation of inhibitory dorsal horn neurons. Also of note, is the fact that BDNF enhances the excitability of the cell bodies of small sensory neurons in rat DRG through p75NTR and the sphingosine kinase pathway (Zhang, Chi et al. 2008). Perhaps a similar mechanism operates in their terminals in the dorsal horn. Moreover, p75NTR activation has, under some circumstances, been associated with neuronal degeneration (Singh, Park et al. 2008; Naska, Lin et al. 2010; Park, Grosso et al. 2010), so it is possible that the reduction in mEPSC frequency seen in inhibitory neurons may reflect p75NTR mediated loss of synaptic contacts. In fact, studies of the effect CCI on the morphology of primary afferent terminals seem consistent with this possibility [100]. Investigation of this possibility is a major aim of my dissertation. Interestingly (Leitner, Westerholz et al. 2013) implicated retrograde signalling in decreasing excitatory synaptic drive to inhibitory neurons, but did not address the possible role of BDNF/p75 in this process.

1.7.1 Regulation of tropomyosine receptor kinase B synthesis in models of inflammatory and neuropathic pain.

TrkB is widely expressed throughout the CNS. The TrkB receptor is found in two main forms: the 145 kDa full-length version (also referred to as TrkB-FL) and an ~93 kDa truncated isoform lacking the intracellular tyrosine kinase domain (referred to as TrkB.T1), which results from alternative splicing of the TrkB transcript (Klein, Parada et al. 1989; Klein, Conway et al. 1990) and plays fundamental roles in mediating neurotrophin action. The FL.TrkB contains an extracellular ligand-binding domain and an intracellular catalytic domain, both of which are

associated with the intrinsic protein tyrosine kinase (PTK) activity (Klein, Conway et al. 1990). The binding of BDNF to FL.TrkB has been shown to induce homodimerization of the receptor, leading to autophosphorylates tyrosine residues found within the cytoplasmic domain of FL.TrkB (Yuen and Mobley 1999). Yajima and others, have shown that spinal BDNF/FL.TrkB-mediated nociceptive signaling pathway may be implicated in the development of thermal hyperalgesia induced by nerve injury in mice (Yajima, Narita et al. 2002). In peripheral nerve injury there is an increased expression of the truncated form of TrkB in the DRG and trigeminal neurons CFA (Lee et al. 1999). TrkB is also expressed by post synaptic neurons of the dorsal horn (Pezet, Malcangio et al. 2002) and are upregulated following both increased neuronal activity and peripheral inflammation (Zhou, Parada et al. 1993). Findings affirming a role for augmented spinal BDNF-TrkB signalling in the pathobiology of neuropathic pain as highlighted above were observations that repeated intrathecal administration of anti-BDNF or a BDNF-sequestering TrkB-Fc chimera protein, abolished neuropathic pain behaviours (Yajima, Narita et al. 2002). The augmented signaling of the BDNF-TrkB pathway as a result of injury somehow could be due to the fact that this signalling pathway may be generally excitatory.

Once released in the spinal dorsal horn, BDNF induces a rapid onset and relatively short lasting increase in phosphorylation of its high-affinity receptor trkB (Pezet and McMahon 2006). The downstream effects of TrkB receptor signaling as a result BDNF exerts neuroprotective and growth-promoting effects on a variety of neuronal populations after injury (Keefe, Sheikh et al. 2017).

1.8 Statement of The Problem

From the discussions above the following points emerge:

1. In the generation of neuropathic pain , release of BDNF from microglia increases excitatory drive to excitatory neurons and reduces excitatory drive to inhibitory neuons.
2. In many physiological situations Trk B activation leads to excitatory processes and p75 receptor activation leads to inhibitory signalling.

This leads to my the **first hypothesis** :

BDNF acts through different receptors and/or transduction mechanisms to increase release of excitatory transmitter onto excitatory neurons (using the Trk B signaling pathways) and to decrease release of excitatory transmitter onto inhibitory neurons (via the p75 signaling pathway).

3. CSF-1 has been implicated in microglial activation and in the onset of neuropathic pain following nerve injury. Although its been shown that CSF-1 increases BDNF gene, it is not known whether CSF-1 increases dorsal horn excitability and whether this reflects BDNF release.

This assumption leads to the **second hypothesis**:

The release of CSF-1 by primary afferents after nerve injury leading to the generation of neuropathic pain involves an excitatory action on the dorsal horn mediated via the release of BDNF.

1.9 Approach

These hypothesis were tested using defined medium organotypic cultures (DMOTC) of dorsal horn GAD67-GFP mice.

I first proceeded to determine whether mouse spinal cords can be maintained in DMOTC and whether GAD67-GFP is expressed in the same neurons in culture as in acute slices.

My approach was was based on the assumption that neuronal types in mice dorsal horn would be similar to those in rats. However, this was found not to be the case as I started my studies.

The thesis is therefore structured as follows:

Chapter 1 - General Introduction

Chapter 2 – General Methods

Chapter 3 - Characterization of Spinal Dorsal Horn Neurons in GAD67-EGFP "Tamamaki" Mice

Chapter 4 - Development and Characterization of Spinal Dorsal Horn Neurons From "Tamamaki" Mice And Stability Of Their GAD67-EGFP Phenotype In Defined-Medium Organotypic Culture

Chapter 5 – Investigating The Role Of Spinal Trk B And P75 Receptors In Central Sensitization

Chapter 6 - Spinal Actions of CSF-1

Chapter 7 – General Discussion

Figure 1.1. a. Rexed Laminae of the spinal cord. Laminae I and II form the marginal zone and *substantia gelatinosa* respectively and together these make up the superficial dorsal horn. Within the dorsal horn. Aβ tactile and hair afferents end mainly in laminae III-VI with some extension into lamina IIi with distribution dependent on function. Aδ hair-follicle afferents extend across the lamina II/III border. Aδ nociceptors end mainly in lamina I, occasionally branching to laminae V and X. Peptidergic, nocicpctive C - fibre afferents synapse mainly in lamina I and IIo. Non-peptidergic C-fibres occupy the inner part of lamina II. **b.** Acutely isolated spinal cord slice from a 30 day old rat. The *substantia gelatinosa* is clearly visible as a translucent band under IR-DIC optics. **c.** Diagram to illustrate the principal synaptic connections in the superficial dorsal horn. Modified from Peirs C and Seal RP (2016) Neural Circuits for Pain: Recent Advances and Current Views. *Science* **354**:578-584. http://science.sciencemag.org/content/354/6312/578.full].

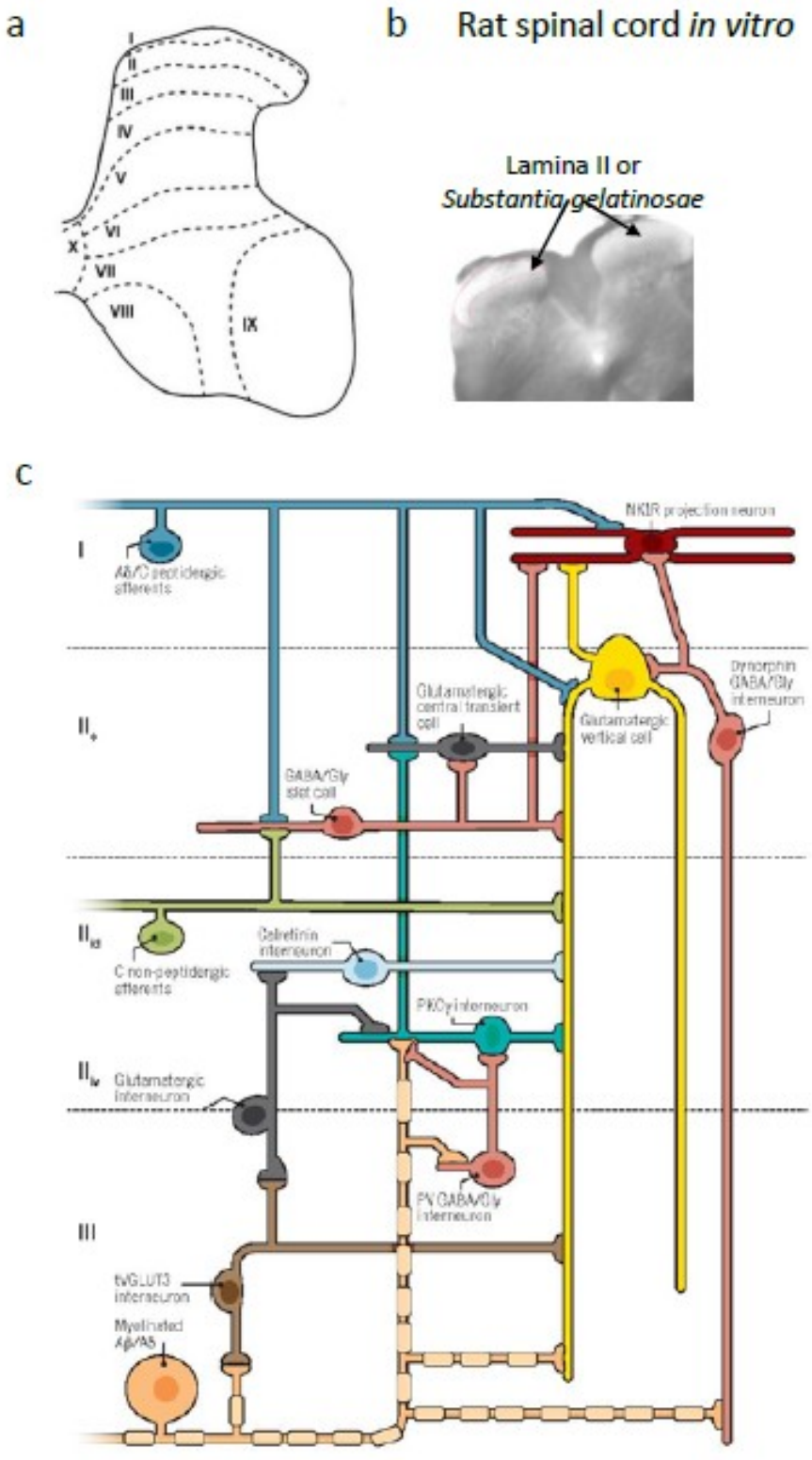
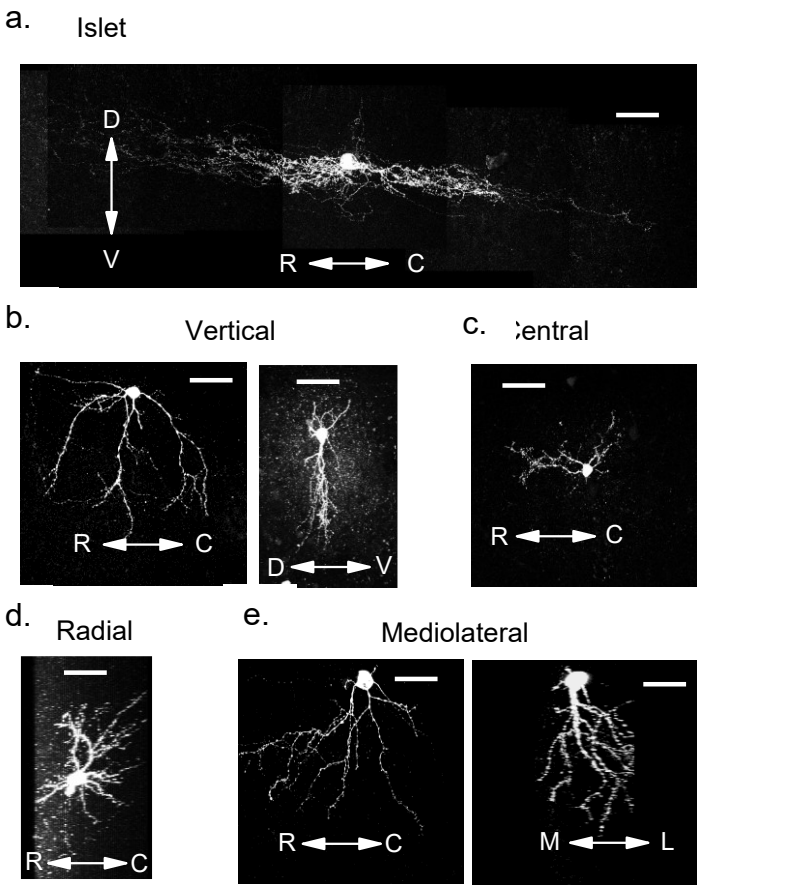

a
I
II
III
IV
V
VI
X
VII
VIII
IX
b
Rat spinal cord *in vitro*
Lamina II or
Substantia gelatinosae
c
NK1R projection neuron
I
Aδ/C peptidergic afferents
II+
Glutamatergic central transient cell
Glutamatergic vertical cell
Dynorphin GABA/Gly interneuron
GABA/Gly islet cell
IIid
Calretinin interneuron
C non-peptidergic afferents
PKCγ interneuron
IIiv
Glutamatergic interneuron
PV GABA/Gly interneuron
III
tdVGLUT3 interneuron
Myelinated Aβ/Aδ

Figure 1.1

Figure 1.2 Images of Z-stacks of rat Lamina II neurons to illustrate morphology. **a-e**, Islet, Vertical, Central, Radial and Mediolateral Neurons respectively. Dorsoventral, Mediolateral and Rostrocaudal planes indicated by D – V, M – L and R – C respectively. Calibration bar in all panels = 50µm. Images modified from (Stebbing et al., 2016) and (Lu et al., 2009). **f.** Scheme to show orientation of various neuronal types in rat *substantia gelatinosa* (Modified from Balasubramanyan, S. PhD thesis, University of Alberta)


a.
Islet
D
V
R
C
b.
Vertical
R
C
D
V
c.
Central
R
C
d.
Radial
R
C
e.
Mediolateral
R
C
M
L

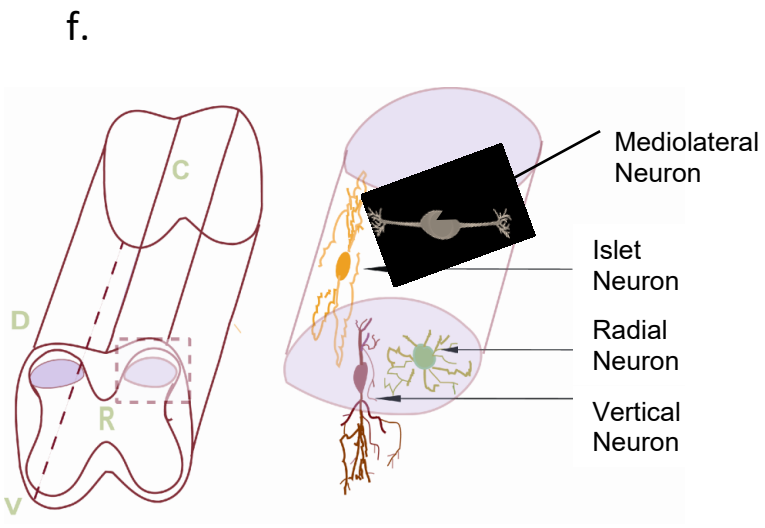

f.
C
D
R
V
Mediolateral
Neuron
Islet
Neuron
Radial
Neuron
Vertical
Neuron

Figure 1.2

Figure 1.3. **a–e**: current-clamp recordings to show discharge patterns evoked in rat *substantia gelatinosa* neurons in response to 3 different intensities of depolarizing current as indicated at the bottom of each panel of records. Membrane potential was set to - 60 mV prior to injection of current pulses. Typical firing characteristics of a tonic neuron **(a)**; discharge persists throughout application of depolarizing current and discharge rate increases with increasing depolarization (**b**, a delay irregular neuron); note long irregular delay prior to onset of spike discharge (c, a delay tonic neuron); exhibits a delay at the beginning and fires tonically (**d**, a phasic neuron); spike discharge ceases after 1–4 spikes (**e**, a transient neuron); only 1 spike is discharged regardless of the applied current intensity (**f**, an irregular neuron).

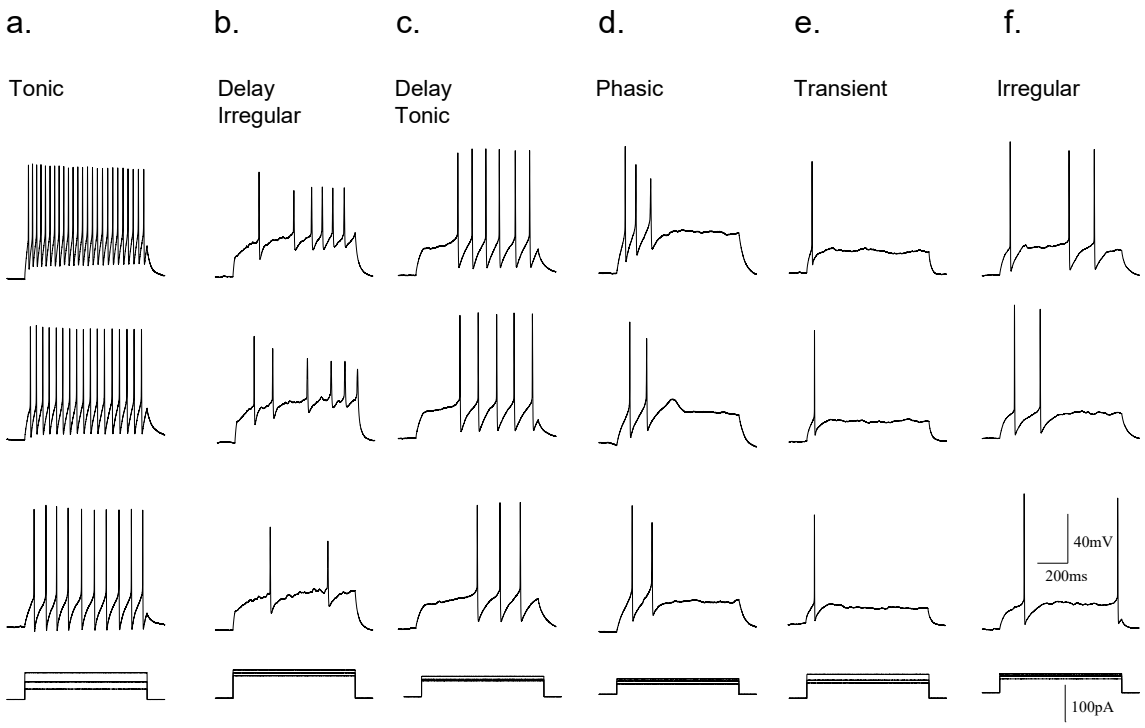
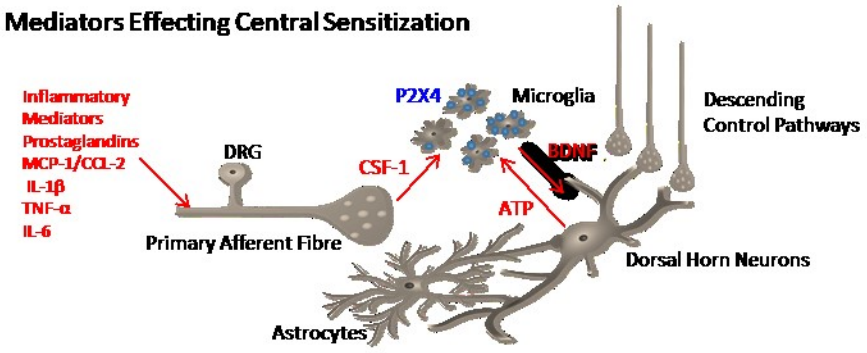

a.
b.
c.
d.
e.
f.
Tonic
Delay
Irregular
Delay
Tonic
Phasic
Transient
Irregular
40mV
200ms
100pA

Figure 1.3

Figure 1.4 (**a**). Diagram to illustrate some of the processes leading to central sensitization. Classical inflammatory mediators released at the site of injury alter the properties of primary afferent fibres such that they become hyper-excitable and/or spontaneously active. CSF-1 released from primary afferents changes the phenotype of microglia that they start to express new receptors including P2X4. ATP released from dorsal horn neurons interacts with P2X4 receptors on microglia to promote release of BDNF which interacts with neurons to increase dorsal horn excitability. (**b**). Primary observation that shows that CCI or BDNF increases excitatory drive to excitatory neurons and reduces excitatory drive to inhibitory neurons.

a.


Mediators Effecting Central Sensitization
Inflammatory
Mediators
Prostaglandins
MCP-1/CCL-2
IL-1β
TNF-α
IL-6
DRG
P2X4
Microglia
CSF-1
BDNF
ATP
Descending
Control Pathways
Primary Afferent Fibre
Dorsal Horn Neurons
Astrocytes

b.

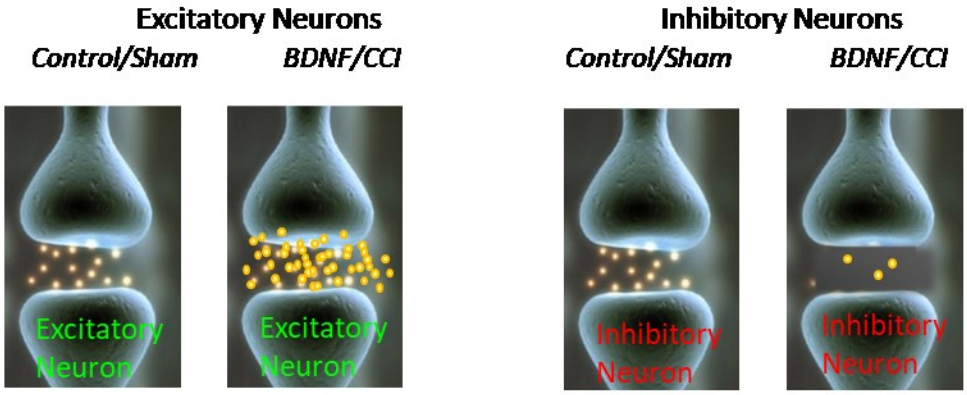

Excitatory Neurons
Control/Sham
BDNF/CCI
Inhibitory Neurons
Control/Sham
BDNF/CCI
Excitatory
Neuron
Excitatory
Neuron
Inhibitory
Neuron
Inhibitory
Neuron

Figure 1.4

CHAPTER 2

GENERAL METHODS

Preparation of Acute Slices, Defined Medium Organotypic Cultures, Calcium Imaging and Whole-Cell Patch Clamp Recordings from Visually Identified Dorsal Horn Neurons.

2.1 Animals

All procedures were carried out in compliance with the guidelines of the Canadian Council for Animal Care and with the approval of the University of Alberta Health Sciences Laboratory Animal Services Welfare Committee (Protocol # AUP 00000338).

Breeding pairs of Tamaguchi CL-1 white albino Swiss mice were obtained from Dr. Jean Claude LaCaille (University of Montreal, Montreal QC Canada) and bred in the Health Sciences Laboratory Animal Services facility at the University of Alberta. Mice were genotyped from ear notch tissue and only those expressing GAD67-EGFP were used in experimental studies.

2.2 Preparation of Acute Spinal Cord Slices

Both male and female mice pups or young adults; p7, p14, p21 and p28, were deeply anesthetized with a large dose of urethane (1.5 g/kg ip). A laminectomy was performed following cessation of respiration and heart beat. Spinal cords were removed and glued with cyanoacrylate glue (Vetbond, WPI, Sarasota, FL) to a trapezoid shaped block cut from 4% agar. This block, with attached spinal cord, was glued to the bottom of a 60-mm diameter glass petri dish, submerged in ice-cold dissection solution containing (in mM) 118 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.3 $MgSO_4$, 1.2 NaH_2PO_4, 1.5 $CaCl_2$, 5 $MgCl_2$, 25 D glucose, and 1 kynurenic acid, and continuously bubbled with 95% O2-5% CO_2. Kynurenic acid is a nonselective acidic amino acid receptor antagonist (Perkins and Stone 1982) that was used to limit acute excitotoxicity during manipulation of the spinal cord. Transverse slices (300 µm) were cut using a HM 650V vibratome (Thermo Scientific). The slices were allowed to recover for at least 1hr at 37 ˚C before use.

2.3 Preparation of Defined Medium Organotypic Cultures

Defined Medium Organotypic Cultures (DMOTC) of spinal cord slices were obtained from e12 Tamaguchi mouse embryos following a method developed by Avossa et al. (2003). Briefly, mouse fetuses were delivered by cesarean section from timed-pregnant GAD67-EGFP mice (Tamamaki, Yanagawa et al. 2003) under isoflurane anesthesia (5% for induction, 2% for maintenance). The adult mice were then euthanized by decapitation under continued isoflurane anesthesia. The entire embryonic sac was placed in chilled, carbonated Gey's balanced salt solution (GBSS), in mM; $CaCl_2$ 1.49; KCl 4.97; KH_2PO_4 0.22; $MgCl_2$; $MgSO_4$ 0.28; NaCl 137.87; $NaHCO_3$ 2.7; Na_2HPO_4 0.84; glucose 5.55 with a pH of 7.40 and osmolarity 296 mOsm. Individual mouse fetuses were removed from their embryonic sacs and rapidly decapitated. The spinal cord from each fetus was isolated in the above solution and sliced into 275-µm transverse slices using a McIlwain tissue chopper. In the e12 animals that were used, the bony vertebral column had not yet formed; this stated to appear in e13-e14 embryos. Slices were chosen from the low thoracic and high lumbar cord slices. Only spinal cord slices with two attached dorsal root ganglia were chosen. These were trimmed of excess ventral tissue and allowed to recover for 1 h at 4 °C. Each slice was then attached to a coverslip with a clot of chicken plasma (sterile) in acid citrate dextrose (ACD, Rockland Immunochemicals, Limerick, PA, USA) and reconstituted thrombin (Sigma–Aldrich Canada, Oakville, ON, Canada) in water Coverslips were placed in 1 ml medium containing 82% Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), B-27 (all from Life Technologies, Burlington ON, Canada) and 8% sterile water in flat-bottomed culture tubes. These were placed into a roller drum rotating at 120 rotations per hour in an

incubator in the presence of humidified atmosphere at 36.5 °C with a concentration of 5.2% CO_2 (Biggs et al., 2012).

2.3.1 Serum-free Media Conditions and Maintenance of Culture

The medium used was supplemented with 20 ng ml^{-1} nerve growth factor (NGF, Alomone Laboratories, Jerusalem, Israel) for the first 4 days, and omitted thereafter. Antibiotic and antimycotic drugs (5 units ml^{-1} penicillin G, 5 units ml^{-1} streptomycin and 12.5 ng ml^{-1} amphotericin B; Gibco) were also included in the media during the first 4 days of culture. After the third day, slices were treated with an antimitotic drug cocktail consisting of uridine, cytosine-β-d-arabino-furanoside (AraC), and 5-fluorodeoxyuridine (all at 10 µM) for 24 h to impair the overgrowth of glial cells.

Fetal bovine serum (FBS) is essential for buffering the acidity of culture medium and also provides nonspecific trophic support for the survival of cells. However, the addition of FBS presents an issue on its own as the undefined factors and mediators present in FBS could confound the interpretation of effects induced by long-term application of trophic factors such as BDNF. So in order to control for these variables, the culture medium was gradually altered to a medium devoid of serum. During antimitotic treatment, the serum medium was progressively switched (first diluted 50: 50 after 3 days, then completely exchanged after 5 days) to a defined, neurotrophin- and serum-free medium consisting of Neurobasal medium with B-27 supplement and Glutamax-1 (all from Gibco). The medium within these tubes was exchanged regularly with freshly prepared medium every 6 days.

In addition, NGF applied at the earlier stages in the medium to support neuronal growth was removed prior to the addition of BDNF. NGF was completely removed from the culture medium after 4 days in culture. Hence the medium applied to the cultures was clearly defined, neurotrophin and serum free.

2.3.2 Treatment of Defined Medium OTCs (DMOTC) with BDNF, TrkB Ligands, TrkB-FC Chimera, p75 Agonist/Antagonist and CSF-1.

2.3.2.1 BDNF

DMOTC slices were maintained *in vitro* 3-4 weeks before experiments to allow cultures to stabilise and recover. Treatment of cultures with BDNF was chosen to parallel previous aged matched animal and *in vitro* studies (Balasubramanyan, Stemkowski et al. 2006; Lu, Biggs et al. 2009). Cultures were treated *in vitro* for a period of 5-6 days with 200ng/ml BDNF (Alomone Laboratories) in the serum-free medium described above. Cultures treated with BDNF was exchanged on the 3rd day of treatment and age-match untreated DMOTCs served as controls.

2.3.2.2 TrkB Ligands

DMOTC slices incubated with BDNF as described above were either withheld or co-incubated with a partial Trk B agonist: 500nM LM22A-4 (Massa, Yang et al. 2010) obtained as a gift from the lab of Dr. Frank Longo (Stanford University, California, USA). Cultures treated with these ligands was exchanged on the 3rd day of treatment and age-match untreated DMOTCs served as controls.

2.3.2.3 p75 Antagonist

The p75 antagonist 100nM LM11A-31(antagonist) (Massa, Xie et al. 2006) received as a gift from the lab of Dr. Frank Longo (Stanford University, California, USA) was incubated with or without BDNF. After the 3rd day of treatment, cultures were exchanged with freshly prepared compound.

2.3.2.4 CSF-1

9 ng/ml CSF-1 was incubated in cultures over a treatment period of 5-6 days. However, on the 3rd day the cultures were exchanged with freshly prepared compound.

2.3.2.4 TrkB-FC Chimera Protein

Cultures incubated with BDNF and CSF-1 as described above were individually treated with 200ng/ml TrkB-FC chimera protein over a 5-6d day incubation period.

2.4 Immunohistochemistry

Acutely isolated spinal cord slices, were fixed with 4% paraformaldehyde overnight at 4^0 C until ready for use. Slices were washed in 0.1% TBS (Tris buffered saline, 6.05g/L Tris base, 9g/L NaCl, pH 7.5 – 7.6) three times for 10 minutes. They were then incubated in 10% normal goat serum, 3% bovine serum albumin, 0.3% Triton X-100 in 1 x TBS (blocking solution), for 1 hr at room temperature, and subsequently incubated with the antibody rabbit polyclonal anti-glial

fibrillary acidic protein (Dako, USA), at a dilution of 1:200. Incubation was done overnight at room temperature in a humid chamber. Acute slices were then washed three times in TBS and incubated with its corresponding secondary antibody Alexa 594 goat anti-rabbit, for GFAP at a dilution of 1:200 for 1.5 hr at room temperature (22^{o}C). Slices were then washed three times in TBS, 10 mins each.

2.5 Electrophysiological recordings

Whole-cell recordings were performed from neurons in acutely-isolated slices or defined medium organotypic cultures (DMOTC). For each experiment, the slice was positioned in a Perspex chamber. Acutely isolated slices were held in place by a "harp" fashioned from strings of nylon affixed to a silver wire frame (Biggs, Lu et al. 2012). Recordings were performed as described by (Balasubramanyan, Stemkowski et al. 2006). Slices were superfused at room temperature (~22°C) with 95% O_2-5% CO_2 saturated artificial cerebrospinal fluid (ACSF) that contained (in mM) 127 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgSO_4$, 2.5 $CaCl_2$, and 25 d-glucose, pH 7.4. S*ubstantia gelatinosa* was identified by its translucent appearance under IR-DIC optics.

Recording pipettes had resistances of 5–7 MΩ when filled with an internal solution containing (in mM) 130 potassium gluconate, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 EGTA, 4 Mg-ATP, and 0.3 Na-GTP, pH 7.2, 290–300 mOsM. Current and voltage clamp recordings were made using an EPC 10 double Patch Clamp Amplifier (Heka Electronik, Lambrecht/Pfalz, Germany).

2.6 Genotyping

Animals were ear notched at the time of weaning (21 day old) for identification and genotyping purposes. The ear tissue collected was digested using protenase K. DNA is extracted using Qiagen DNeasy blood and tissue kit as per manufacturer's instructions. Polymerase chain Reaction (PCR) was carried out using the primers F: 5'-GGCACAGCTCTCCCTTCTGTTTGC-3 ' and R: 5'-CTGCTTGTCGGCCATGATATAGACG-3 '. The PCR conditions used were 94ºC for 3', followed by 30 cycles of 94ºC for 30'' 30 cycles, 68ºC for 3' followed by an extension of 72ºC for 7'. The amplified DNA was run through 1.5% Agarose gel. The samples showing a band at 564bp is positive for GFP knock-in and the corresponding mice are selected for the successive experiments.

2.7 Fluorescence imaging of cytosolic Ca2+

Cytosolic Ca^{2+} was imaged with multiphoton fluorescence microscopy as previously described for rat DMOTC (Lu et al., 2007). Mouse DMOTC slices were placed in a recording chamber (1.5 ml) and perfused with 95% O_2-5% CO_2 saturated artificial cerebrospinal fluid (ACSF) that contained (in mM) 127 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgSO_4$, 2.5 $CaCl_2$, and 25 d-glucose, pH 7.4. (5 ml/min). The membrane-permeable Ca^{2+}-sensitive dye fluo-4-AM (0.5 mM in ACSF) was backfilled into a broken patch pipette (tip diameter, 5–10 µm) and pressure-injected (0.7–1.0 psi) for 10 min (Ruangkittisakul, Schwarzacher et al. 2006). Fluorescence signals were measured using an Olympus MP-Exclusive multiphoton microscope with infrared laser excitation at 810 nm. Within 10–20 min of Fluo-4-AM injection, labeling revealed basic neuronal

morphology and changes in fluorescence. Neurons were typically imaged at tissue depths between 30 and 60 µm. Imaging was done using a 2–3× digital zoom using a full-frame acquisition (512 × 512 pixels).

2.8 Analysis and Statistical Tests

All electrophysiological data, except spontaneous excitatory postsynaptic currents, were analyzed using pCLAMP 10.0 and Origin 2018 software. Data were analyzed using Student's two tail test or one-way ANOVA with post hoc Tukey Kramer test as appropriate. Data from cumulative probability plots were analysed using Kolmogorov Smirnof (KS) statistics. A level of statistical significance of $P < 0.05$ was set.

Data were only collected from neurons that exhibited clear overshooting action potentials of >60 mV in amplitude. All neurons were categorized on the basis of their discharge pattern in response to 800-ms square-wave depolarizing current pulses. Initial resting potential (rmp) was maintained at −60 mV or -85 mV by DC current injection (Balasubramanyan, Stemkowski et al. 2006). Spike width was measured from the first action potential observed in response to a depolarizing current pulse at a voltage midway between the top of the spike and the initial resting potential which was set to -60mV. Spontaneous excitatory postsynaptic currents (sEPSC) were recorded at -70 mV. Mini Analysis Program (Synaptosoft, Decatur, GA, USA) was used to distinguish sEPSC from baseline noise and to generate event lists for cumulative probability plots. Events were detected automatically by setting appropriate amplitude and area threshold for each neuron. All events detected during a 3 min acquisition period were then re-examined and visually accepted or rejected based on subjective visual examination (Moran, Colmers et al.

2004). All detected events were then re-examined and visually accepted or rejected based on visual examination. Acceptable events had a sharp onset and exponential offset, a total duration of < 50ms and an amplitude at least double the baseline noise.

2.9 Drugs

BDNF (Alomone Laboratories) was reconstituted with 0.1% BSA/GBSS and centrifuged for 30 mins at 3500 r.p.m. at room temperature aliquoted and stored at about – 70 $^{\circ}$C.

Both CSF-1 (PeproTech Inc) and TrkB-FC chimera protein (Alomone Laboratories) were initially reconstituted with water and finally dissolved with Neurobasal media.

Fluo-4 AM dye was dissolved in a mixture of DMSO and 20% pluronic acid (Invitrogen, Burlington, Ontario, Canada) to a 0.5 mM stock solution and stored until used. The dye was thawed and sonicated thoroughly before incubating with a DMOTC slice.

CHAPTER 3

CHARACTERIZATION OF SPINAL DORSAL HORN NEURONS IN GAD67-EGFP "TAMAMAKI" MICE

* Portions of this chapter have been published (**Boakye et al., 2018, Neuroscience; 372:126-140**).

ABSTRACT

γ-aminobutyric acid (GABA)ergic neurons in the central nervous play a crucial role in information processing. Using GAD67-EGFP Tamaguchi CL-1 white albino Swiss mice whole-cell recording was performed on neurons located in lamina II in acutely isolated mice spinal cord slices. In these slices, neurons in the *substantia gelatinosa* exhibited 7 different firing patterns in response to 800ms depolarizing current commands; delay irregular, delay tonic, tonic, regular firing, phasic, initial bursting and single spiking. Initial bursting and regular firing neurons were not found previously in rat *substantia gelatinosa.* In acute slices from "Tamamaki" mice engineered to express enhanced green fluorescent protein (EGFP) under the control of the glutamic acid decarboxylase 67 (GAD67) promotor, tonic, phasic and regular firing neurons exhibited the strongest GABAergic (GAD67-EGFP+) phenotype. Delay tonic and delay irregular neurons almost never expressed GAD67 (GAD67-EGFP-). Neurons from acute slices also exhibited oscillatory activity as monitored by examining intracellular Ca^{2+} levels. This activity decreased in older slices compared to younger ones.

Key Words

Neuropathic pain, Lamina II, GABA, Electrophysiology, Synaptic transmission, Genetically modified mice

Highlights

- Seven electrophysiological phenotypes characterize neurons in mouse *substantia gelatinosa.*
- Tonic, regular firing or phasic firing patterns are GABAergic whereas those with delay firing patterns are not.
- GAD67-EGFP cells never exhibit the delay firing pattern.

3.1 Introduction

For the transmission of both sensory and nociceptive signals from the periphery to the central nervous system, the spinal dorsal horn serves as the first and major relay station (Vallbo, Olausson et al. 1999; Punnakkal, von Schoultz et al. 2014). High threshold neurons in the form of nociceptors (Aδ and C-fiber) send their afferent fibers which terminate in the superficial dorsal horn (laminae I and II), whereas low-threshold afferent fibers innervate the deep dorsal horn (laminae III-V) (Todd 2010; Peirs and Seal 2016). A vast majority of neurons in the superficial and deep dorsal horn arborize locally and are referred to as interneurons (Todd 2010; Punnakkal, von Schoultz et al. 2014). These interneurons can be divided into two main classes: excitatory (glutamatergic) and inhibitory, with the inhibitory interneurons using GABA and/or glycine as their main neurotransmitter(s). The balance in the actions of excitatory and inhibitory interneurons in the CNS is therefore key in information processing (Tamamaki, Yanagawa et al. 2003). The proper functioning of these interneurons is required for adequate perception of sensory stimuli (Graham, Brichta et al. 2007; Todd 2010; Zeilhofer, Benke et al. 2012). Dysfunction of dorsal horn interneurons leads to the signs and symptoms of chronic pain (Zeilhofer, Benke et al. 2012) characterized by increased sensitivity to both noxious (hyperalgesia) and non-noxious stimuli (allodynia). A mechanistic understanding of the role these interneurons play in the physiological processing of somatosensory and nociceptive signals and their malfunctioning in pathological pain states depends on a detailed knowledge of their biophysical properties and their integration in dorsal horn neuronal circuits (Punnakkal, von Schoultz et al. 2014; Peirs and Seal 2016). Studying these inhibitory and excitatory interneurons in the dorsal horn would thus lead to a better understanding of pain processing in the spinal cord. In recent

times past, researchers relied on *post hoc* identification of neurons through the use of neurochemical markers (Todd, Hughes et al. 2003; Maxwell, Belle et al. 2007; Schneider and Walker 2007; Yasaka, Tiong et al. 2010; Polgar, Sardella et al. 2013) or on simultaneous recordings of synaptically connected pairs of neurons (Lu and Perl 2003; Lu and Perl 2005). However, to achieve a more precise and accurate understanding of the mechanistic role of the interneuronal population in the spinal dorsal horn, additional new techniques are required. The use of reporter mice over the years has revolutionized the way specific neuronal cell populations in CNS are identified. These mice express fluorescent proteins in defined neuronal populations (Punnakkal, von Schoultz et al. 2014) by introducing the cDNA encoding a green fluorescent protein (GFP) obtained from a jellyfish (*Aequorea victoria*) into the cell or organism of interest (Tamamaki, Yanagawa et al. 2003). The use of the GFP system to identify GABAergic neurons has been demonstrated by the creation of mice in which GFP expression is regulated by GAD67 promoter (Oliva, Jiang et al. 2000). Mice expressing enhanced green fluorescent protein (eGFP) in GABAergic neurons under the transcriptional control of the Gad67 or Gad65 gene, or in glycinergic neurons under the control of the GlyT2 (*Slc6a5*) gene have been used to characterize dorsal horn inhibitory interneurons (Heinke, Ruscheweyh et al. 2004; Zeilhofer, Studler et al. 2005; Cui, Kim et al. 2011). These studies were however somewhat incomplete as they did not encompass a full characterization of all neuronal types in the mouse *substantia gelatinosa*. By contrast with rats (Balasubramanyan, Stemkowski et al. 2006; Peirs and Seal 2016) surprisingly few detailed characterizations of mouse *substantia gelatinosa* have appeared. This chapter seeks to address some of these gaps, thus, the idea here is; 1). Researchers have looked at the characteristics of GAD67-EGFP neurons (Tamamaki, Yanagawa et al. 2003; Punnakkal, von

Schoultz et al. 2014) but are yet to compare or situate it with the whole interneuronal population in the *substantia gelatinosa* 2) Although, a few characterization of mouse *substantia gelatinosa* neurons have been done (Abraira, Kuehn et al. 2017), the authors did not look at GAD67-EGFP and did not pay attention to the pain circuitry. In this study, breeding pairs of Tamaguchi CL-1 white albino Swiss transgenic mice line, which expresses eGFP under the transcriptional control of the glutamic acid decarboxylase enzyme (GAD) were generated and the biophysical properties of both GAD67-EGFP sub population and non GAD67-EGFP interneuronal population was studied.

3.2 Methods

Please refer to **Chapter 2** for all procedures performed. Data for the firing properties of rat neurons were obtained from a database of recordings obtained by previous workers in the laboratory (Drs Yishen Chen and Sridhar Balasubramanyan).

3.3 Results

3.3.1 Electrophysiological properties of substantia gelatinosa neurons in acutely isolated slices of mouse spinal cord.

Primary classification of neurons was based on firing patterns seen when the membrane potential is held at -60mV as this corresponds to the average rmp of neurons in mouse *substantia gelatinosa* (**Table 3.1**). Clear differences were found between the firing patterns encountered in mice compared to rats. Whereas rat neurons fit into six categories (Chen, Balasubramanyan et al. 2009) (**figure 1.3**) mouse neurons were found to fall into seven. Some firing patterns seen in mice were not previously observed in rats and *vice versa*. In mice the following were identified: -

Delay Irregular Neurons. These were defined by the presence of an obvious delay prior to initiation of action potentials in response to 800ms depolarizing current commands delivered from a pre-set holding potential of -60 or -85mV. Discharge was irregular with some variation in interspike interval and spike height. The typical firing pattern of this type of neuron is illustrated in **figure 3.1 a**. Delay irregular neurons accounted for only 4.7% of 211 neurons sampled in acutely isolated slices of mouse *substantia gelatinosa*.

Delay Tonic Neurons represent a larger population of delay firing neurons that exhibit regular firing, constant interspike interval and spike height after an initial delay when depolarized with an 800ms current injection. Delay tonic neurons accounted for 14.7% of the 211 neurons sampled. Their firing pattern is illustrated in **figure 3.1 b**.

Tonic Neurons. These comprised 15.2% of the neuronal population and, as illustrated in **figure 3.1 c**, exhibited robust, high frequency discharge with the smallest current injection used (15pA). Discharge rate was increased with larger current injections and spike height and interspike interval was unchanged throughout the response.

Regular Firing Neurons displayed a similar discharge pattern to tonic neurons except that more current was required to maintain sustained discharge. It was also possible to evoke one or two action potentials with small current injections in regular firing neurons whereas tonic neurons always discharged a train of action potentials once threshold was exceeded. As illustrated in **figure 3.1 d** maximum firing rate was less for regular firing neurons than for tonic firing neurons. Rate of discharge in response to current injection is sometimes described as "gain". Regular firing neurons displayed lower gain than tonic neurons (**figure 3.1 h**). Regular firing neurons comprised 15.2% of the neuronal population in mouse *substantia gelatinosa*.

Phasic Neurons show accommodation of firing rate during the response to an 800ms current command. Spike height remains constant. **Figure 3.1 e** illustrates the firing pattern of a phasic *substantia gelatinosa* neuron. Phasic firing neurons in mice tended to accommodate less completely compared to those from rats (Balasubramanyan et al., 2006; Chen et al., 2009). In mice, phasic neurons comprised 14.7% of the 211 neurons sampled.

Initial Bursting Neurons show a firing pattern that was previously described by Cui *et al* (2011). Initial bursting neurons expressed an initial high discharge rate when depolarizing current was first applied. Spike height declined during this burst and rapidly accommodated so that discharge completely ceased during the application of sustained depolarization. Typical discharge pattern of an initial bursting neuron is illustrated in **figure 3.1 f**, these neurons accounted for 13.3% of those sampled. Initial bursting neurons have not been reported in rat *substantia gelatinosa* (Chen, Balasubramanyan et al. 2009) figure 1.3.

Single Spiking Neurons fired one or at most two spikes in response to a depolarizing current command (**figure 3.1 g**). They accounted for 12.3% of the 211 neurons studied.

10% of neurons encountered did not fit into the above categories and were defined as “unclassified”. Also of note is the observation that a few cells that displayed the ‘gap’ pattern of firing (Heinke, Ruscheweyh et al. 2004). These cells displayed an initial spike followed by a delay and repetitive discharge thereafter. It was also noted that the voltage-sensitive conductances present in delay tonic tonic and/or delay irregular neurons produced an initial small notch or shoulder on the voltage trajectory produced by current injection (**figure 3.1 i**). Sometimes this notch brought cells to threshold for a.p. generation and such that they displayed

the “gap” discharge pattern. At this point I consider “gap” firing neurons to be a subset of delay tonic or delay irregular neurons.

Table 3.1 lists the average rmp of the seven identified neuronal types. All values were close to -60mV and there was no statistical difference between values in the cell types. Table 1 also lists the spike widths of neurons in acute slices. Delay tonic cells exhibited the broadest action potentials (4.4±0.6ms, n=23) and this was significantly greater than that recorded in phasic, regular spiking and tonic neurons. The relatively long spike duration found in single spiking neurons (3.9±0.5ms, n=22) was significantly greater than that of phasic, regular firing or tonic neurons (p values ranged from <0.05 to <0.001, one-way ANOVA with post hoc Tukey Kramer test).

3.3.2 Comparison of neuron types in acute spinal cord slices in rats with those in mice.

In acutely isolated slices of rat *substantia gelatinosa,* the lab has previously characterized neurons into 6 categories according to their firing pattern (**figure 1.3**). These are tonic, delay irregular, delay tonic, phasic, transient and irregular (Chen, Balasubramanyan et al. 2009). The firing patterns of some neurons do not easily fit into these categories and are described as unclassified. This classification of rat neurons is similar to those used by other groups (Grudt and Perl 2002; Yasaka, Tiong et al. 2010) with the exception of the subcategorization of delay firing cells into delay irregular and delay tonic types (Chen, Balasubramanyan et al. 2009).

Figure 3.11 j compares the percentages of neurons of each type found in mouse *substantia gelatinosa* with those found in rat *substantia gelatinosa.* Data are from 211 mouse neurons and 258 rat neurons. The initial bursting and regular firing categories found in mice were

not previously seen in rats (Grudt and Perl 2002; Balasubramanyan, Stemkowski et al. 2006; Chen, Balasubramanyan et al. 2009; Yasaka, Tiong et al. 2010). By contrast, the irregular firing neurons seen in rats were not seen in mice. Delay irregular and tonic neurons were less commonly encountered in mice than in rats. Thus, while delay neurons in rats often displayed the delay irregular phenotype, those in mice often displayed the delay tonic phenotype. The single spiking cells found in mice were somewhat similar to "transient" cells found in rats but these displayed a single, short latency, high threshold, very brief spike at the start of depolarization. Although a few single spiking cells seen in mice had characteristics of transient cells as seen in rats, most discharged a one or occasionally two action potential with small or large current injections **(figure 3.1 g**). For the purpose of comparison, transient and single spiking cells have been combined for mice and compared with transient neurons from rats.

3.3.3 Effect of resting membrane potential on firing pattern of neurons in mouse substantia gelatinosa.

Initial membrane potential can substantially alter the discharge patterns of neurons in response to depolarising current (Prescott and De Koninck 2002; Heinke, Ruscheweyh et al. 2004; Yasaka, Tiong et al. 2010). This may be a reflection of increased availability of voltage-gated Na^+ channels (I_{Na}) and A-type K^+ currents (I_A) as their inactivation is removed at hyperpolarized potentials. Both conductances have been identified in mouse (Graham, Brichta et al. 2007) or rat spinal cord (Safronov, Wolff et al. 1997; Safronov 1999). Activation of hyperpolarization activated cyclic nucleotide gated (HCN) channels at negative voltages may also impact neuronal discharge patterns (Hughes, Boyle et al. 2013). Previous characterizations of

mouse *substantia gelatinosa* neurons have held neurons at either -70mV (Abraira, Kuehn et al. 2017) or from a range of potentials between -50 and -90mV (Cui, Kim et al. 2011). To learn more about firing patterns of mouse *substantia gelatinosa* neurons and to align our findings to published literature, we compared firing patterns evoked at -60mV (close to the average rmp of -62mV, **Table 3.1**) with that evoked from -85mV to remove inactivation of I_A, I_T and I_{Na} and to permit activation of HCN channels. **Figure 3.1 k** illustrates the effect of membrane potential on firing pattern evoked by depolarization. This graph was constructed by determining the firing pattern from a cohort of 113 neurons from both -60 and -85mV. These two points were then linked by a line for each cell. Thus, the thickness of the lines on the graph gives an index of the transitions of firing pattern imposed by altering the initial holding potential. For most cell categories, such as phasic, single spike and delay tonic neurons, thick horizontal lines connect the firing patterns at -60mV with those at -85mV, indicating little effect of membrane potential on firing pattern. However, the thickness of sloping lines indicates the numbers of cells which transition between firing patterns when studied from different holding potentials. Thus, 4 out 15 neurons exhibiting initial bursting and 8 out of 24 exhibiting phasic firing at -85mV showed regular firing at -60mV. Also 4 out 15 neurons showing regular firing from -85mV showed tonic discharge from -60mV.

3.3.4 Relationship between GAD67-EGFP expression and electrophysiological phenotype of mouse substantia gelatinosa neurons in acute slices.

In rat *substantia gelatinosa*, GABAergic neurons frequently display a tonic firing pattern whereas most excitatory neurons display a delay firing pattern (Yasaka, Tiong et al. 2010).

In a previous study of another strain of mice ("GIN" mice as opposed to "Tamamaki" mice), GAD-67 EGFP fluorescence was found to associate with initial bursting cells (the authors terminology, this would correspond to our phasic cells) and not with delay firing cells (Heinke, Ruscheweyh et al. 2004).

Figure 3.1 l shows that GAD67-EGFP in Tamamaki mice primarily associates with tonic, phasic and regular firing neurons and is hardly ever encountered in delay irregular and delay tonic neurons (in 1 out of 74 of GAD67-EGFP+ neurons in each case, Fig 1l). Data were obtained from 74 GAD67-EGFP+ and 146 GAD67-EGFP- neurons in acutely isolated slices. In view of the voltage-dependence of firing patterns, we also examined the relationship for GAD67-EGFP+ neurons. Figure 1m shows data from 47 GAD67-EGFP+ neurons. As for Fig 1k, thickness of horizontal lines illustrates the stability of firing pattern when cell types are assessed from holding potentials of -60 and -85mV. 5 out of 16 neurons that displayed regular firing at -60mV exhibited phasic firing at -85mV.

3.3.5 Spontaneous excitatory synaptic activity in mouse substantia gelatinosa neurons in acutely isolated slices.

The average sEPSC interevent interval (IEI) for the seven neuron types are summarized in **figure 3.2 a**. Delay tonic neurons exhibited the smallest IEI (highest sEPSC frequency); this was significantly smaller than that seen in single spike, phasic, initial burst and regular firing neurons. Regular firing neurons display the longest IEI interval (lowest frequency of sEPSC).

The largest amplitude sEPSC's were seen in tonic neurons (**figure 3.2 b**); amplitude was significantly greater than delay tonic, delay irregular, single spiking and regular firing neurons. Regular firing neurons had the smallest amplitude sEPSC's; significantly smaller than tonic, phasic and initial bursting neurons. Data from **figure 3.2 a and b** for sEPSC characteristics in the seven neuron types are summarized in **figure 3.2 c** where event amplitude for each neuron type is plotted versus IEI.

Spontaneous action potential activity was seen in 6 out of a total of 220 neurons studied.

Regardless of firing pattern, GAD67-EGFP+ neurons had longer IEI (lower frequency) of sEPSC's than GAD- neurons (**figure 3.2 d**, $p<0.001$ KS test) but these events were of greater amplitude (**figure 3.2 e** $p<0.001$ KS test).

3.3.6 Relationship between spontaneous Ca2+ oscillations and age of Slice.

Transient or brief elevations of intracellular calcium levels or calcium oscillations, have been shown to be involved in the regulation of various stages of neuronal development, including proliferation, migration, differentiation, and survival (Rosenberg and Spitzer 2011). In a developing circuitry when sensory systems and functional connections between different regions in the nervous system are still poorly developed, spontaneous forms of correlated neuronal activity seem to control activity-dependent neuronal development (Katz and Shatz 1996). Thus, periodic spontaneous network activity as monitored by Ca^{2+} oscillations is a characteristic feature of the developing nervous system (Blankenship and Feller 2010). Recording in the mice acute slice preparations (**figure 3.3 b**) we see continuous decline in spontaneous activity with age which signals a developing neuronal circuitry and hence fewer oscillations seen with time. This

observation can be attributed to the depolarizing actions of GABA and acetylcholine at early stages of development, whereas at later stages, they become more dependent on glutamatergic neurotransmission (Hanson and Landmesser 2003).

3.3.7 Changes in neuron number in slices.

During embryonic development, programmed cell death (PCD) which is a type of cell death by natural processes or design takes place (Ryu, Hong et al. 2016). Using various animal models with mutations in genes controlling PCD, we now currently know that PCD helps in 1) regulation of the size of progenitor populations, 2) error correction and 3) systems matching (Ryu, Hong et al. 2016). The end-goal of this phenomenon is the efficiency and optimization of systems by the removal of error-ridden or unwanted neural populations in nervous system during development. With the presence of adult neural stem cells (NSCs) which have the potential to self-renew and differentiate into multiple cell types including neurons in the adult nervous system (Gage 2000), adult neurogenesis is the dynamic process that helps to recapitulate the development of the embryonic nervous system. As such cell death and neurogenesis are integral to the development of the nervous system and a delicate balance needs to exist between the two processes. To highlight the immense role played by PCD it is known that about 50 % of sensory neurons or motor neurons innervating their targets (e.g. skin or muscle, respectively) eventually undergo PCD during their provisional synapse formation.

Similarly, our results show a comparable proportion of cell death throughout early postnatal development in acute slices (**figure 3.3 c**). The significant loss of neurons at this stage in nervous system development has been shown by (Sun, Winseck et al. 2004; Kim, Kim et al.

2007; Mouret, Gheusi et al. 2008) and could be due to the triggering of pro death pathways or cues (Pfisterer and Khodosevich 2017). As development continues the loss in neuronal numbers stabilizes as the animal grows into adulthood (**figure 3.3 c**). At this point we see stability in the neuronal population and the presence of pro survival pathways or cues might have been triggered or set into motion and thus might account for the stable neuronal population seen. The triggering of these pro survival cues blocks intrinsic pro-death signaling, thus accounting for neuronal stability and when there is a lack of pro-survival signaling, pro-death pathways are triggered (Pfisterer and Khodosevich 2017). However, it must also be noted that different types of neurons in the CNS might use different pro-survival mechanisms and this could be 'neuron type-specific' pro-survival mechanisms. It should be noted that in as much as there is also a pro-survival mechanism, there could also pro-death pathways and it is the interplay between these two mechanisms based on the presence or absence of specific cues, which might highlight the significant neuronal death and stability in later adulthood seen in **figure 3.3 c**.

3.4 Discussion

The purpose of this study was to identify and characterize the electrophysiological cell types in laminae II of GAD67-GFP positive "*Tamamaki* mice". This characterization serves as a reference for the development and characterization of spinal cord organotypic cultures from these mice in a defined medium **(chapter 4)**. Acute spinal dorsal horn slices were successfully obtained from "*Tamamaki mice*" aged p7, p14, p21 and p28. Seven different electrophysiological cell types: delay tonic, delay irregular, tonic, regular firing, phasic, initial burst and phasic, were

determined by means of whole cell patch clamp recording from laminae 11 of the spinal cord. The tonic, regular and phasic firing phenotype were predominantly associated with GAD67-GFP positive cells and a total absence of the delay excitatory phenotype recorded from these GAD67-GFP positive cells.

3.4.1 Electrophysiological phenotypes in acute slices of mouse spinal cord and relationship to GAD67-EGFP expression.

Although there have been several attempts to classify interneurons in laminae II, there is yet to be a generally accepted scheme that covers all of these cells. We had initially assumed that the electrophysiological phenotypes of mouse *substantia gelatinosa* neurons in acutely isolated slices would be similar to those seen in rats (Balasubramanyan, Stemkowski et al. 2006). Unexpectedly it was found not to be the same, even though they belonged to the same mammalian order *"rodentia".* Interestingly, two neuronal phenotypes found in mice; regular firing and initial bursting (Abraira, Kuehn et al. 2017) (**figures 3.1 d and 3.1 f**) are not found in rats. It is likely that regular firing neurons in mice fulfill a similar function to tonic neurons. Both are frequently GABAergic (**figure 3.1 h**) and regular firing neurons are capable of tonic firing with injection of appropriate amounts of depolarizing current (**figure 3.1 d**). Tonic cells in rats often display a characteristic islet cell morphology (Yasaka et al., 2010) but the usual morphology of mouse regular firing neurons remains to be determined.

Recording from hundreds of acute slices from mice, delay firing (either delay tonic or delay irregular) almost never exhibit the GAD67-EGFP+ phenotype (**figure 3.1 l**). This observation confirms the findings of a previous report using both GIN and Tamamaki mice (Heinke et al., 2004;

Punnakkal et al., 2014) and correspond to observations in rats (Yasaka et al., 2010). Such firm reports from both rat (Yasaka et al., 2010) and mouse (Heinke et al., 2004; Punnakkal et al., 2014) superficial dorsal horn strongly support the idea that delay firing neurons are excitatory and glutamatergic. The excitatory and glutamatergic nature of these delay firing cells have largely been observed to result from an A-type potassium current (I_A) (Ruscheweyh and Sandkühler 2002; Graham, Brichta et al. 2007) involving channels containing the Kv4.2 or Kv4.3 subunits (Huang, Cheng et al. 2005; Hu, Carrasquillo et al. 2006) which suppresses neuronal excitability.

About 50% of initial bursting cells and single spiking cells exhibit the GAD67-EGFP+ phenotype but, in confirmation of the results of Punnakkal et al (2014) this phenotype is seen most frequently in phasic, tonic and regular firing cells (**figure 3.1 l**). It is possible that some GABAergic neurons are not identified by GAD67-EGFP+ expression either because the EGFP signal is too weak to be detected by our system (confocal calcium imaging system) or that the neurons instead express GAD65 (Lorenzo, Magnussen et al. 2014). In acute slices, neurons identified as tonic, phasic and regular firing exhibit the GAD67-EGFP+ phenotype. As stated above, there is a lack of general consensus on the classification of the various interneuronal phenotypes in lamina II, as such, slightly different criteria used by different groups may also account for differences between the findings seen and that obtained with GAD67-EGFP "GIN" mice (Heinke et al., 2004). To illustrate these differences, the authors (Heinke, Ruscheweyh et al. 2004) noted a particularly strong correlation between GAD67-EGFP expression and phasic neurons (termed initial bursting neurons by the authors). One possible explanation for this is our observation that altering holding potential can, in some cases change a phasic firing neuron into a regular firing neuron (**figure 3.1 k**). Perhaps, there is a continuum between these neuronal types and the firing pattern is

determined by slight differences in numbers of ion channels expressed in individual neurons. Alternatively, GIN mice are known to only express fluorescence in a subset of GABAergic neurons (Heinke, Ruscheweyh et al. 2004) and it may be those that express the phasic firing pattern.

3.4.2 Importance of GAD67-EGFP mice for pain research

The development of transgenic mice has allowed researchers to identify GABAergic neurons associated with the GABA-synthesizing enzymes, glutamate decarboxylase (GAD) 65 or GAD67 (Heinke, Ruscheweyh et al. 2004; Lopez-Bendito, Sturgess et al. 2004). Several lines of evidence have suggested that the loss of GABAergic inhibition plays an important role in inflammatory and neuropathic pain (Baba et al. 2003; Malan et al. 2002; Moore et al. 2002; Yaksh 1989). Transgenic mice that coexpress a fluorescent protein such as EGFP with a functional neuronal marker like GAD67 therefore serve as excellent tools allowing for recording from large numbers of neurones with a defined phenotype in a slice or culture preparation by visual identification. Until recently, researchers have been engaged in using arduous and time-consuming post hoc procedures (such as immunohistochemistry) or technically highly demanding paired recordings from monosynaptically connected pairs of spinal neurones (Jonas, Bischofberger et al. 1998; Lu and Perl 2003) were necessary for the targeted investigation of specific neuronal types expressing a specific marker. Thus specifically studying GABAergic inhibitory interneurons may be useful to further understand how the synaptic transmission and intrinsic circuitry of the spinal cord contribute to the perception of pain (Cui, Li et al. 2008). Current approaches in molecular techniques has led to a rapid advancement in our knowledge about the physiological properties of spinal GABAergic neurones by means of genetic

engineering. The loss of tonic GABAergic inhibition leads to abnormal painful conditions such as hyperalgesia and allodynia (Yaksh 1989; Sivilotti and Woolf 1994) which are key symptoms of neuropathic pain. As such, having a better understanding of the GABAergic network in the transmission of pain signals is key to the development of effective strategies by means of medication and management of neuropathic pain states. Using animal models of neuropathic pain and targeting specific subgroup of GABAergic neurones labelled as in the case of GAD67-EGFP mice would help us have a better understanding of the precise role these neurons play in central sensitization leading to the onset of chronic pain.

3.5 Conclusion

This work has shown that mice and rats even though they belong to order *rodentia* do have subtle differences in their dorsal horn electrophysiological properties. It has been shown that there are 7 electrophysiological phenotypes found in the dorsal horn of mice acute slices compared to the 6 electrophysiological phenotypes in the dorsal horn of rat acute slices. Importantly, the GABAergic phenotype identified by the expression of GAD67-EGFP is strongly associated with the tonic, regular and phasic firing phenotype with the total absence of any delay electrophysiological phenotype expressing it. In order to use Tamaguchi CL-1 white albino Swiss mice could be used as a good model to develop defined medium organotypic cultures (DMOTC).

We next had to determine whether the seven neuronal phenotypes found in acute slices were preserved in culture. More importantly we had to determine whether GAD67-EGFP still associated with tonic, regular firing and phasic neurons and not with delay firing neurons in DMOTC as it did in acute slices.

These issues will be addressed in **Chapter 4**.

Table 3.1 Comparison of Electrophysiological Properties of Cell types in Acute slices. Values of rmp for all neuronal types were not significantly different from each other in acute slices (One way ANOVA, Tukey-Kramer post hoc test). Significant differences were however observed in spike width of the various cell types in acute slices as detailed in the main text of the chapter (One way ANOVA, Tukey-Kramer post hoc test).

	Delay irregular	**Delay tonic**	**Initial burst**	**Phasic**	**Regular firing**	**Single spiking**	**Tonic**
rmp (acute)	-64.3±1.7mV (n=7)	-63.9±1.0mV (n=29)	-60.6±1.6mV (n=25)	-62.0±1.0mV (n=33)	-62.1±1.2mV (n=34)	-59.5±0.7 (n=26)	-62.8±1.1mV (n=30)
Spike width (acute)	1.9±0.2ms (n=7)	4.4±0.6 (n=23)	3.1±0.3 (n=22)	2.0±0.3ms (n=22)	2.1±0.3 (n=16)	3.9±0.5 (n=22)	2.0±0.2 (n=22)

Figure. 3.1. a-g Illustrations of typical firing patterns of Delay Irregular, Delay Tonic, Tonic, Regular Firing, Phasic, Initial Bursting and Single Spiking (Transient) neurons in acutely isolated slices of mouse spinal cord. Recordings are displayed on two different time scales. **h.** Relationship between discharge frequency and current injection (gain) for tonic (n=21) and regular firing neurons (n=20). **i.** Illustration of voltage response of a delay neuron on high gain to illustrate notch that was sometimes capable of generating an action potential so that the neuron displayed the "gap" firing phenotype. **j.** Percentage of neurons of each phenotype in rat and mouse acute slices (211 mouse neurons and 258 rat neurons). **k.** Scheme to illustrate effect of holding potential (-60 or -85mV) on neuronal discharge pattern. For each of 113 neurons investigated a line is drawn between its firing pattern at -60 and -85mV. Thus horizontal lines represent numbers of neurons in which firing pattern was unaffected by holding potential. Other lines between categories of firing patterns illustrate numbers of neurons that transitioned between firing patterns as holding potential was altered. **l.** Percentages of 146 GAD- and 74 GAD-EGFP+ neurons showing tonic, delay tonic, delay irregular, initial burst, phasic, regular firing, unclassified and single spike firing patterns in *substantia gelatinosa* region of acutely isolated spinal cord slices. **m.** Scheme as in l to show transitions of firing pattern following alteration of holding potential for 47 GAD67-EGFP+ neurons in acute slices.

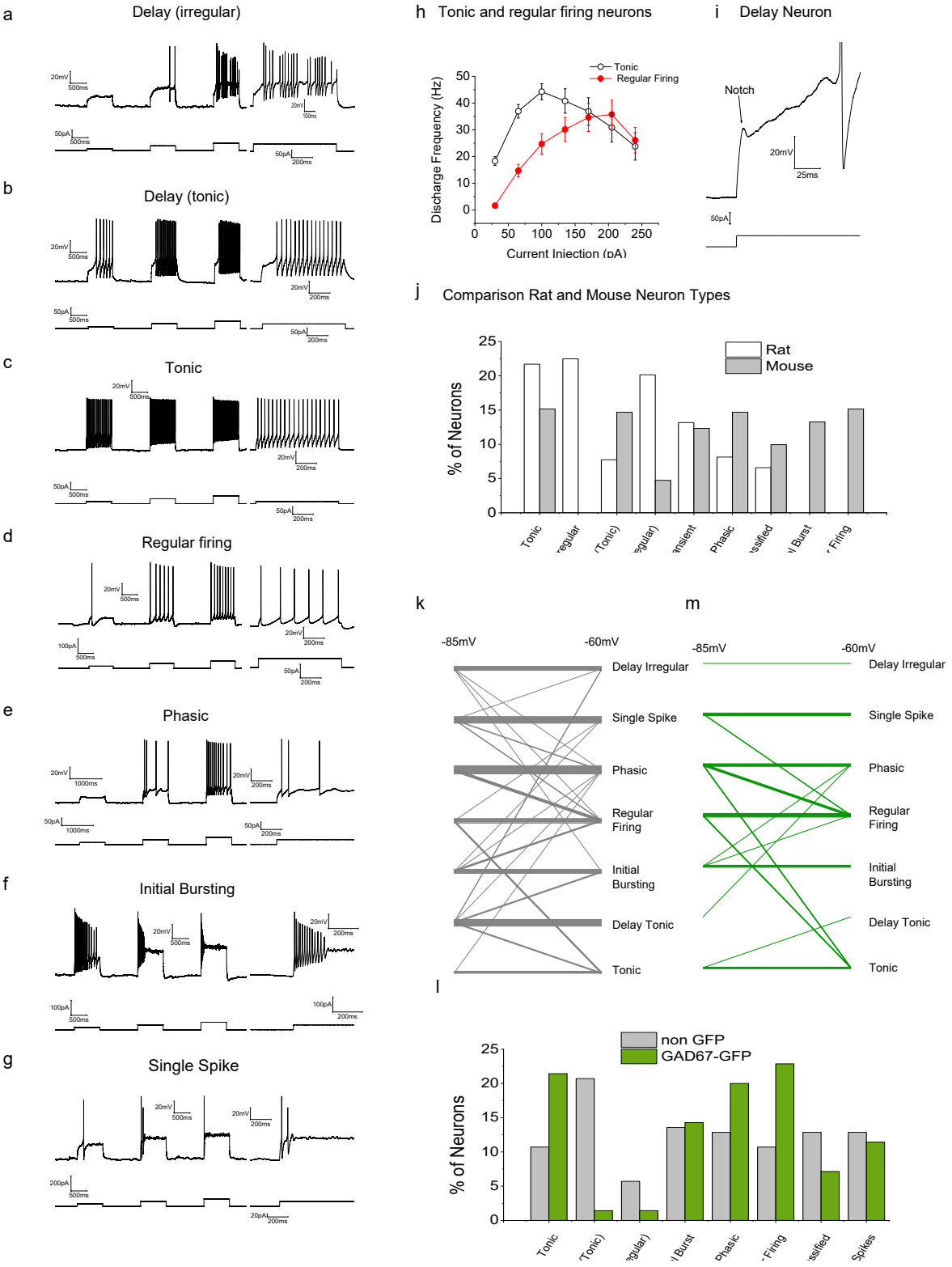

a
Delay (irregular)
b
Delay (tonic)
c
Tonic
d
Regular firing
e
Phasic
f
Initial Bursting
g
Single Spike
h
Tonic and regular firing neurons
Tonic
Regular Firing
Discharge Frequency (Hz)
Current Injection (pA)
i
Delay Neuron
Notch
20mV
25ms
50pA
j
Comparison Rat and Mouse Neuron Types
Rat
Mouse
% of Neurons
k
m
-85mV
-60mV
Delay Irregular
Single Spike
Phasic
Regular Firing
Initial Bursting
Delay Tonic
Tonic
l
non GFP
GAD67-GFP
% of Neurons

Figure 3.1

Figure 3.2. a and b. Summaries of interevent intervals (IEI) and amplitudes of sEPSC's in 7 electrophysiologically defined neuron types in *substantia gelatinosa* region of acutely isolated mouse spinal cord slices. Numbers of neurons in each category indicated on graph. (Differences are not significant unless marked with*** = $p<0.001$ or ** $p<0.01$, one-way ANOVA with post hoc Tukey Kramer test). **c.** IEI data from A plotted against amplitude data from B. **d. and e.** Cumulative probability plots to show differences in IEI and amplitude of sEPSC's in 30 nonGAD neurons and 45 GAD67-EGFP+ neurons. (3066 events from GAD- neurons and 1088 events from GAD67-EGFP+ neurons. For amplitude $p<0.0001$ for IEI $p<0.0001$ (Kolmogorov Smirnof test).

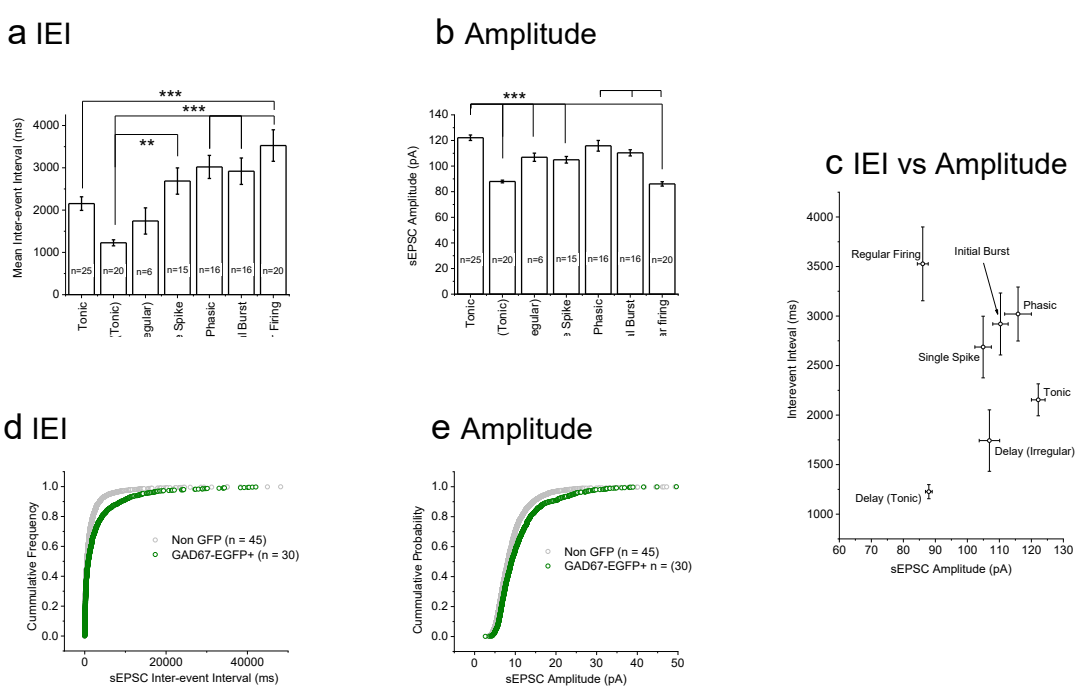
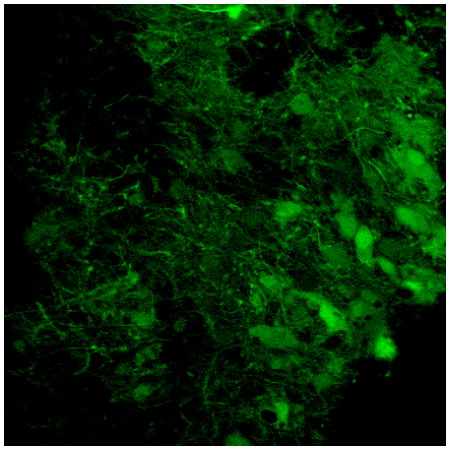

a IEI
b Amplitude
c IEI vs Amplitude
d IEI
e Amplitude
Mean Inter-event Interval (ms)
sEPSC Amplitude (pA)
Interevent Inteval (ms)
Regular Firing
Initial Burst
Phasic
Single Spike
Tonic
Delay (Irregular)
Delay (Tonic)
Cummulative Frequency
Non GFP (n = 45)
GAD67-EGFP+ (n = 30)
sEPSC Inter-event Interval (ms)
Cummulative Probability
GAD67-EGFP+ n = (30)

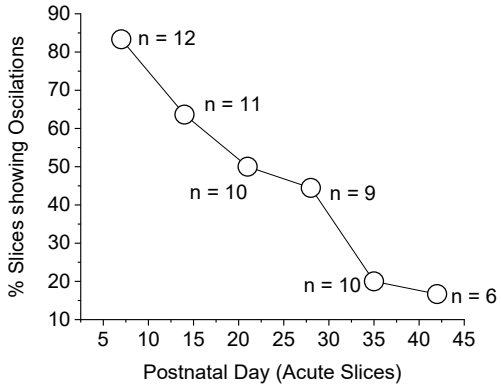
Figure 3.2

Figure 3.3. a*.* Image for video of Ca2+ oscillations in spinal acute slice **b.** Graph showing a decrease in percentage of acute slices exhibiting oscillations as animals mature (n's refer to number of slices examined). **c.** Acute slices prepared from p7, p14, p21 and p28 animals, number of neurons per sq. mm. (* = $p<0.05$, one-way ANOVA with post hoc Tukey Kramer Test).

a. Ca2+ Imaging



b. Age Dependence of Acute Slices



c. Acute slices

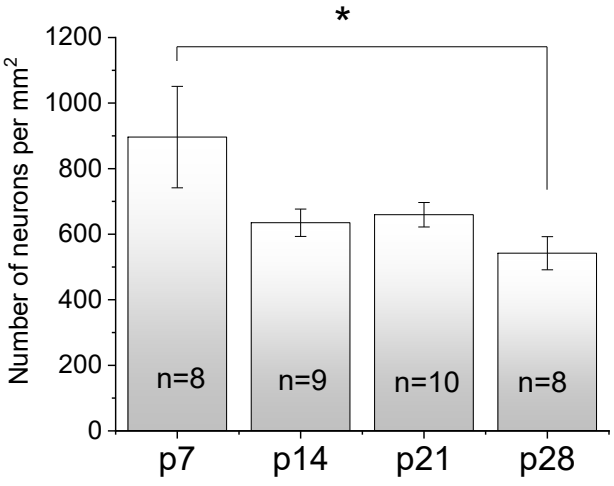


Figure 3.3

CHAPTER 4

CHARACTERIZATION OF SPINAL DORSAL HORN NEURONS FROM "TAMAMAKI" MICE IN DEFINED-MEDIUM ORGANOTYPIC CULTURE AND STABILITY OF THEIR GAD67-EGFP PHENOTYPE

* Portions of this chapter have been published (**Boakye et al., 2018, Neuroscience; 372:126-140**).

ABSTRACT

Previously, I identified and classified 7 different electrophysiological cell types in the *substantia gelatinosa* of GAD67-EGFP Tamaguchi CL-1 white albino Swiss mice (**chapter 3**). For decades organotypic cultures (OTC) or defined medium organotypic cultures (DMOTC) have been used for studying long term chronic disease states. In order to assess and determine whether DMOTC spinal cords from mice are suitable and stable for long term studies I determined whether the seven neuronal phenotypes found in acute slices were preserved in culture. I employed whole-cell patch clamp recording and recorded from cells in lamina II. The same 7 distinct cell types identified in acute slices was seen in DMOTCs of mice spinal cords. Also, GAD67-EGFP neurons identified in DMOTC still associated with tonic, regular firing and phasic neurons and not with delay firing neurons in DMOTC. Stability of the overall neuronal population decreased but stabilized over the entire duration in culture and slices were intact and healthy during the period of growth.

Neurons from DMOTC of mice also exhibited oscillatory activity as monitored by examining intracellular Ca2+ levels similar to that seen in mice acute slices. Similarly, this activity decreased in older slices compared to younger ones. These findings suggest the suitability of using defined medium organotypic cultures of mice spinal cord as long-term disease state models.

Key Words

Lamina II, Defined Medium Organotypic Cultures, Electrophysiology

4.1 Introduction

The superficial dorsal horn, which includes the *substantia gelatinosa* is of paramount importance to the elucidation of pain mechanisms (Peirs and Seal 2016; Yekkirala, Roberson et al. 2017). Understanding the circuitry and processing of such a key pain pathway is fundamental to understanding the processing of afferent information to higher brain centers. Neuropathic pain, as a result of lesion to the nervous system (Dworkin, Backonja et al. 2003) is often intractable and difficult to treat (Turk 2002). Pooled global estimates suggest that neuropathic pain affects 2-3% of the population (Gilron, Watson et al. 2006) and a huge healthcare burden both to the individual and health care system (Tarride, Collet et al. 2006). The etiology and epidemiology of neuropathic pain is still not well understood (Attal 2000; Smith 2004), partly because of the myriad of associated conditions (Gilron, Watson et al. 2006).

As mentioned in chapter 1, peripheral nerve injury, is a common approach used to study the development of neuropathic pain (Kim, Yoon et al. 1997; Decosterd and Woolf 2000; Stemkowski and Smith 2013). Following peripheral nerve injury, peripheral immune cells (resident macrophages and monocytes) rush to the lesion site (Perkins and Tracey 2000; Scholz and Woolf 2007); schwann cell activation and proliferation lead to the release of growth factors such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) (Stoll, Jander et al. 2002; Guertin, Zhang et al. 2005; Scholz and Woolf 2007). These act as potent regulators of gene expression and ion channel properties. Signaling between primary sensory neurons, schwann cells, immune cells, cytokines, chemokines and spinal dorsal horn neurons leads to the onset of mechanical hyperalgesia and allodynia (Scholz and Woolf 2007). As outlined in **figure 1.4 a**, BDNF (Coull, Beggs et al. 2005; Lu, Ballanyi

et al. 2007; Lu, Biggs et al. 2009; Biggs, Lu et al. 2012; Smith 2014; Hildebrand, Xu et al. 2016) and colony stimulating factor 1 (CSF-1) (Guan, Kuhn et al. 2016; Okubo, Yamanaka et al. 2016) play a major role in central sensitization associated with neuropathic pain. I have further investigated their actions in **chapters 4 and 5** of my thesis.

The slowly developing effects of neurotrophins and other biological mediators can be conveniently studied using organotypic cultures (OTC) of neuronal tissue (Gähwiler 1981; Gahwiler, Capogna et al. 1997; Galante, Nistri et al. 2000; Avossa, Rosato-Siri et al. 2003). The use of OTC obtained from spinal cords were first described almost 30 years ago (Braschler, Iannone et al. 1989; Streit, Spenger et al. 1991), however, based on the needs of my thesis, I have modified the methodology to use defined medium cultures (DMOTC), thus, avoiding and removing doubts about data interpretation as the use of undefined factors and mediators present in fetal bovine serum (FBS) can confound the interpretation of effects of slowly acting, exogenously applied neurotrophins such as BDNF or CSF-1 (**chapters 4 & 5**). Previously the lab has used DMOTC of rat spinal cord to study and elucidate the role of BDNF (Lu, Ballanyi et al. 2007; Smith, Biggs et al. 2008; Lu, Biggs et al. 2009) and interleukin 1β (Gustafson-Vickers, Lu et al. 2008) in central sensitization and to study the long-term actions of gabapentenoids. We have found that the properties of rat spinal neurons in DMOTC are reassuringly similar to that of age-matched neurons in acutely-prepared ex vivo slices (Lu, Moran et al. 2006; Biggs, Lu et al. 2012). In the *substantia gelatinosa* of both rats (Prescott and De Koninck 2002; Lu and Perl 2003; Lu and Perl 2005; Balasubramanyan, Stemkowski et al. 2006; Chen, Balasubramanyan et al. 2009; Yasaka, Tiong et al. 2010) and mice (Graham, Brichta et al. 2004; Heinke, Ruscheweyh et al. 2004; Cui, Kim et al. 2011; Tadros, Harris et al. 2012; Punnakkal, von Schoultz et al. 2014; Abraira, Kuehn

et al. 2017), a variety of neuronal phenotypes can be characterised electrophysiologically by their discharge pattern.

Based on our successful characterization of seven electrophysiological phenotypes from acute slices obtained from GAD-67-EGFP mice in **chapter three**, and since mouse models of human disease are far more amenable to genetic manipulation than the corresponding rat models, I modified our existing methodology for the generation of rat spinal DMOTC for use in mice (Boakye, Patterson et al. 2016). I sought to **(1)** investigate whether the electrophysiological phenotypes identified in acute slices were comparable to those in DMOTCs obtained from mouse e12 embryos and **(2)** whether any relationship between firing pattern and GABA expression in acute slices is parallelled as neurons and neighboring astrocytic glial cells developed in DMOTC. This work thus addresses the fundamental question of whether modifications of the mouse genome persist when neurons develop in DMOTC.

4.2 Methods

Please refer to **Chapter 2** for all procedures performed.

4.3 Results

4.3.1 Electrophysiological properties of substantia gelatinosa neurons in DMOTC of mouse spinal cord

Previous work has shown that all neural electrophysiological phenotypes defined in rat neurons are maintained in DMOTC (Lu, Moran et al. 2006; Biggs, Lu et al. 2012), as such it was

important to ascertain the persistence of these electrophysiological phenotypes in mice as changes in action potential characteristics have been reported to occur when mice spinal neurons are maintained in organotypic culture (Avossa, Rosato-Siri et al. 2003).

To maintain consistency and as reported by others, (Grudt and Perl 2002; Hantman, van den Pol et al. 2004) the primary classification of neurons was based on firing patterns seen when the membrane potential is held at -60mV as this corresponds to the average resting membrane potential (rmp) of neurons in mouse *substantia gelatinosa* both in DMOTC (**Table 4.1**) and in acute slices (**Table 3.1**).

Delay Irregular Neurons. As was done for acutely isolated mouse neurons in **chapter 3**, these were defined by the presence of an obvious delay prior to initiation of action potentials in response to 800ms depolarizing current commands delivered from a pre-set holding potential of -60 or -85mV. Discharge was irregular with some variation in interspike interval and spike height. The typical firing pattern of this type of neuron in DMOTC is illustrated in **figure 4.1 a**. Delay irregular neurons accounted for only 2.3% of 131 neurons sampled in DMOTC of mouse *substantia gelatinosa* (**figure 4.2 f**).

Delay Tonic Neurons. As was done in chapter 3, these neurons were identified in DMOTC as exhibiting regular firing, constant interspike interval and spike height after an initial delay when depolarized with an 800ms current injection. Delay tonic neurons accounted for 15.3% of the 131 neurons sampled in DMOTC (**figure 4.2 f**). Their firing pattern is illustrated in **figure 4.1 b**.

Tonic Neurons. These comprised 8.4% of the neuronal population in DMOTC (**figure 4.2 f**) and, as illustrated in **figure 4.1 c**, exhibited robust, high frequency discharge with

the smallest current injection used (15pA). Discharge rate was increased with larger current injections and spike height and interspike interval was unchanged throughout the response. In DMOTC, tonic discharges were often interrupted by inhibitory post synaptic potentials (IPSP's). Since tonic firing neurons are usually inhibitory, the presence of these IPSP's may reflect the generation of **autapsies** in the culture.

Regular Firing Neurons. As in acute cultures (**figure 3.1 d**) these displayed a similar discharge pattern to tonic neurons except that more current was required to maintain sustained discharge. It was also possible to evoke one or two action potentials with small current injections in regular firing neurons whereas tonic neurons always discharged a train of action potentials once threshold was exceeded. As illustrated in **figure 4.1 d** maximum firing rate was less for regular firing neurons than for tonic firing neurons (**figure 4.1 c**). Regular firing neurons comprised 19.9% of the neuronal population in mice DMOTC (**figure 4.2 f**).

Phasic Neurons show accommodation of firing rate during the response to an 800ms current command (as defined in acute slices (**figure 3.1e**). Spike height remains constant. F**igure 4.1 e** illustrates the firing pattern of a phasic *substantia gelatinosa* neuron in DMOTC from a mouse. In mice DMOTC, phasic neurons comprised 15.3% of the 131 neurons sampled (**figure 4.2 f**).

Initial Bursting Neurons. Initial bursting neurons expressed an initial high discharge rate when depolarizing current was first applied. Spike height declined during this burst and rapidly accommodated so that discharge completely ceased during the application of sustained depolarization. Typical discharge pattern of an initial bursting neuron in DMOTC is

illustrated in **figure 4.1 f**, these neurons accounted for 21.37% of those sampled in DMOTC (**figure 4.2 f**).

Single Spiking Neurons in DMOTC fired one or at most two spikes in response to a depolarizing current command (**figure 4.1 g**). They accounted for 6.11% of the 131 neurons studied in DMOTC (**figure 4.2 f**).

In general, the distribution of firing patterns in DMOTC resembles those seen in DMOTC. All cell types seen in acute slices were seen in culture (**figure 4.2 f**)

Table 4.1 lists the average rmp of the seven identified neuronal types in DMOTC. As seen in acute slices (**Table 3.1**) all values were close to -60mV and there was no statistical difference between values in the cell types. **Table 4.1** also lists the spike widths of neurons in DMOTC. Delay tonic cells exhibited the broadest action potentials (6.0±1.7ms, n=17) and this was significantly greater than that recorded in phasic, regular spiking and tonic neurons.

4.3.2 Relationship between GAD67-EGFP expression and electrophysiological phenotype of mouse substantia gelatinosa neurons in DMOTC.

Figure 4.2 g shows that GAD67-EGFP primarily associates with initial bursting, phasic and regular firing neurons in Tamamaki mice spinal cord slices in DMOTC. Only 3 out of 51 GAD67 EGFP+ neurons exhibited a delay (tonic) firing pattern. 5.9% of delay (tonic) cells encountered were GAD-67-EGFP+ in DMOTC compared to 1.4% in acute slices. Thus GAD67-EGFP associated with similar cell types in both acute slices and in DMOTC (**figure 4.2 h**).

4.3.3 Spontaneous excitatory synaptic activity of neurons in DMOTC.

Figure 4.3 a illustrates the IEI of sEPSC's in various neuron types in DMOTC. Tonic and delay tonic neurons exhibited the longest IEI (lowest sEPSC frequency). These were significantly greater than most other neuron types. Single spike and phasic cells exhibited the highest frequency (shortest IEI). **Figure 4.3 b** illustrates mean sEPSC amplitude in the 7 neuron types. No significant difference in sEPSC amplitude was seen between any pairings of neuron types. Data from **figures 4.3 a and 4.3 b** are summarized in **figure 4.3 c**. In DMOTC, delay (tonic) neurons have the smallest and lowest frequency of synaptic events whereas single spike neurons have the largest amplitude and the highest frequency events. These data are replotted in **figure 4.3 f** and superimposed on data from acutely isolated neurons (**from figure 3.2 c, in chapter 3**). Several points emerge from this comparison i) sEPSC's in DMOTC are generally of higher amplitude and higher frequency than those in acute slices. ii) There is greater variation in the amplitude of sEPSC's in each defined neuronal type in DMOTC compared to acute slices. iii) There is greater variation in the inter event interval of sEPSC's in acute slices compared to DMOTC. iv) DMOTC alters the characteristics of synaptic excitation seen in the 7 neuron types. For example, in acute slices, regular firing neurons receive sEPSC's at low frequency (IEI = 3526±373ms, n=20; **figure 3.2 a and c, chapter 3**) and these are of low amplitude (86.6±1.7pA, n=20, **figure 3.2 b and c, chapter 3**). By contrast in DMOTC, sEPSC amplitude (151±18.9pA, n=27; **figure 4.3 b and c**) and frequency (IEI = 461±80.5ms, n=27; **figure 4.3 a and c**) of regular firing cells are among the largest of the 7 neuron types. KS analysis showed that the distribution of IEI of sEPSC's in GAD67-EGFP+ neurons was statistically distinct from that observed in GAD67-EGFP- neurons ($p<0.001$; **figure 4 e**) but these events were of similar amplitude ($0.1>p<0.05$ KS test **figure 4.3 f**).

Eight out of 283 mouse neurons studied in DMOTC exhibited spontaneous action potential discharge that appeared to be driven by synaptic activity (**figure 4.3 g**).

4.3.4 Characteristics of organotypic cultures.

A low power image of a typical slice in DMOTC is as presented in **figure 4.2 a**. Most slices maintain this basic architecture over a six-week period and can still hold this basic architecture over extended periods of time. An interesting feature of the cultures is the migration of the two dorsal root ganglia to the ventral aspect of the slice over time. Also, it was observed that there was a critical time window for culture preparation. Thus, spinal cord slices obtained prior to e11 or after e13 failed to produce viable cultures. Neurons in mouse spinal cord continue to express GAD67-EGFP in DMOTC (**figure 4.2 b**). In **figure 4.2 c**, neurons are labeled with NeuN Alexa 546 (red) in a slice from a GAD67-EGFP^{+} animal and the proportion of GABAergic neurons can be seen as they are indicated by a yellow color. When staining for NeuN or GFAP (see below) controls in which the primary antibody was not present was included.

Neuronal death and the pruning of synaptic connections is characteristic of the normal process of the spinal cord development (Buss et al., 2006; Hildebrand, 1971). Days 9, 16, 23 and 30 of DMOTC closely correspond to p7, p14, p21 and p28 acute slices (**figure 4.2 d**). In DMOTC, there appears to be a large decline in neurons per unit area between 2 and 9 days (**figure 4.2 e**), statistical difference in neuron numbers only appeared when comparing day 2 and day 37 counts.

4.3.5 Oscillations in cytosolic Ca^{2+}level.

Previous studies of rat (Lu et al., 2009) and mouse (Fabbro et al., 2007; Sibilla et al., 2009) spinal neurons in OTC have documented the presence of spontaneous oscillations in cytoplasmic Ca^{2+} levels. In mice, oscillatory activity was observed in both acute slices and DMOTC. Given the morphology of astrocytes in DMOTC (see **figure 4.5 a & c**), these oscillations were clearly of neuronal origin. **Figure 4.4 a** is a still frame from a video which shows the oscillations in DMOTC. Oscillations occurred at 0.07±0.009Hz (n=40). **Figure 4.4 b** shows changes in Fluo 4 fluorescence versus time in selected regions of interest (ROI) from **figure 4.4 a**. Ca^{2+} was elevated for 10-25s during the course of each event. The oscillations are clearly asynchronous. Thus, one neuron in **figure 4.4 b** (blue trace) shows regular oscillations throughout the recording period whereas another neuron (red trace) only shows one spontaneous Ca^{2+} elevation during this time. Other neurons such as that those depicted by the black, purple and brown traces show prolonged periods where no deviation from baseline Ca^{2+} levels occur despite the activity in the neuron depicted by the blue trace. The lack of synchronization between the oscillations is illustrated further in **figure 4.4 c** where some of the data from **figure 4.4 b** is replotted on a faster time scale. Onset of oscillation in one neuron is not temporally coupled to onset in another. **Figure 4.5 d** shows that oscillatory activity was prevalent in younger cultures and in acutely isolated slices from neonatal animals (p7). As neurons aged oscillations were seen less and less frequently.

4.3.6 Properties and proliferation of Astrocytes in DMOTC.

In order to generate DMOTC, E12 spinal cord slices are initially treated with antimitotic agents to curtail the proliferation of astrocytes (**chapter 2 & figure 4.2 d**). The

recovery of the astrocyte population throughout the 6-week time course was visualized by a progressive in increase in GFAP that stabilized after 16d. **Figure 4.5 a** is an image taken from a 30d DMOTC, neurons are labelled red and astrocytes with characteristic stellate morphology and ramifying processes (Avossa et al., 2003) are labelled green. **Figure 4.5 b** shows a significant increase ($p<0.5$) in the percentage area of slices showing GFAP immunoreactivity between 2 and 9d in culture; an increase that becomes highly significant ($p<0.001$) for all points after 16d. For 16d and onwards no further significant increase is seen. The increase in density of with time GFAP was accompanied by a marked expansion in the processes of the cells so that they form a web-like structure that permeated throughout the entire slice. Astrocytes did not however extend processes beyond the initial limit of the culture (**figure 4.5 c and d**).

One characteristic of organotypic cultures is that they spread and thin in vitro to quasi-monolayer thickness (Gahwiler, 1981; Gahwiler et al., 1997). This phenomenon was not seen in DMOTC of mouse spinal cord. Slices as incubated were originally cut to 275 µm and the mean thickness of those in DMOTC was 252.2±7.9 µm (n=6) (**figure 4.5 c & d**).

4.4 Discussion

The purpose of this study was to develop and validate a method for transgenic DMOTC cultures of mouse spinal cord. Cultures were successfully maintained *in vitro* for 6 weeks in a serum-free medium and showed no sign of deterioration. It can be reported that we are the first to successfully develop mice spinal cord DMOTCs in a serum free media. An issue of concern is that the spinal dorsal horn, a highly differentiated component of the spinal cord architecture, containing a diversity of neuronal subtypes and phenotypes (Graham, Brichta et al. 2004; Tadros,

Harris et al. 2012; Peirs and Seal 2016; Abraira, Kuehn et al. 2017); could undergo some form of de-differentiation in culture such that the clear electrophysiological phenotypes seen in acute mouse slices would be lost. Another issue of concern is the fact that the survival of our DMOTCs had a narrow time window, from e11 to e13 embryos, and not from neonatal mouse pups, there is also a possibility that maturation and differentiation of neural phenotypes may be compromised or may not even occur in DMOTC. An additional concern is the fact that developing DMOTCs in vitro using genetically-engineered animals such as GAD67-EGFP mice may alter the relationship between GAD67-EGFP expression and neuron phenotype. A fundamental question to ask is would, for example, the association between GAD67-EGFP expression and tonic, phasic and regular firing neurons in acutely isolated slices (**figure 3.1 j**) be maintained as observed in **chapter 3**? If an answer contrary to what is observed from that obtained from acute slices is the case, any studies of *substantia gelatinosa* neurons in DMOTC would be of questionable physiological significance. With some few exceptions, it was observed that de-differentiation and or failure of differentiation is limited. In almost all scenarios, neurons defined by their biophysical properties (electrophysiological phenotype) in acute slices continue to express this phenotype in DMOTC. Worthy of note is the fact that, GAD67-EGFP expression associates with the same neuronal phenotypes both in acute slices and in DMOTC. With current trends been driven by the widespread use of genetically modified animals in neuroscience research, it is safe based on this study to conclude that such modifications persist when a heterogeneous neuronal population is allowed to develop in organotypic culture.

4.4.1 Electrophysiological phenotypes in DMOTC of mouse spinal cord and relationship to GAD67-EGFP expression.

Based on the findings so far adduced, this study has shown that all seven electrophysiological phenotypes seen in acute slices (chapter 3) are present in DMOTC as well (**figure 4.2 f**). It is therefore likely that differentiation of cell types and firing patterns occurs normally in DMOTC even though the cultures are derived from pre-natal animals. Another key observation is that GAD67 expression in DMOTC is associated with phasic and regular firing neurons in the same way as it is in acute slices (**figure 4.2 h**). A very minor difference observed between DMOTC and acute slices is that GAD67 is seen in less tonic cells in culture. As shown in **figure 3.1 m** (**chapter 3**) however, tonic cells can exhibit a phasic firing pattern or may be regarded as regular firing cells when the membrane potential is changed. Changes in firing pattern as a result of variation in holding potential has been reported (Hubel 1960; Deschenes, Roy et al. 1982; Hirsch, Fourment et al. 1983; Curro Dossi, Pare et al. 1991; Funke and Eysel 2000). Also of note is the fact that GAD- tonic firing cells may correspond to the central cells described in rats (Yasaka, Tiong et al. 2010).

Neurons in DMOTC show no difference in their rmp compared to those in acute slices (**chapter 3**). Most cell types in DMOTC showed a pattern of an increase in spike width, with a pronounced significant increase seen in tonic firing cells (**Table 4.1**). An increase in spike width was also observed by (Avossa, Rosato-Siri et al. 2003) when neurons were maintained in culture. However, it should be mentioned that in that that study neurons were not classified it their firing patterns as was done in this study. In this study, when all spike widths were averaged for all cell

types, DMOTCs had significantly longer spike widths (P<0.05) of 3.6±0.4 (n=96) compared to that found in acute slices 2.9±0.26ms (n=126) (**Chapter 3**).

In general, the frequency and amplitude of sEPSC's are greater in DMOTC, compared to that seen in acute slices. This obvious difference in the excitatory synaptic drive in neurons in DMOTC compared to those in acute slices is summarized in **figure 4.4 f**. This trend has been reported by (Lu, Moran et al. 2006) in DMOTC and in organotypic cultures of rat hippocampus (De Simoni, Griesinger et al. 2003). These observable changes in synaptic connectivity in cultures may be reflective of the establishment of new excitatory connections and perhaps the formation of autapses (Lu, Moran et al. 2006). For each neuron type in DMOTC there is less variation in IEI but more variation in event amplitude (**figure 4.4 d**), whereas, for each cell type in acute slices there is considerable variation in IEI of sEPSC's (**figure 3.2 c, chapter 3**).

Based on these observations, I suggest that DMOTC, including those made from genetically modified animals, may be used to study long term effects of drugs, neurotrophins and other biological agents on the properties of defined cell types of mouse spinal neurons, whereas, studies of the modulation of synaptic connectivity by such agents may produce less meaningful results.

4.4.2 Maturation of cultures over the 6-week period

The roller-tube technique has been used to culture tissue derived from cerebellum, hippocampus, hypothalamus, spinal cord, olfactory bulb, cerebral cortex, brain stem and midbrain and has been shown to yield thin, organotypic cultures (Cui, Kim et al. 2011). However, DMOTC of rat spinal cord do not display this phenomenon (Biggs et al., 2012).

A characteristic feature of developing circuitry networks in all parts of the nervous system is spontaneous activity (O'Donovan, 1999; Whelan et al., 2000) and is manifested as recurrent depolarizing events and oscillatory activity, during which cells within the network are synchronously activated. It is mostly seen at the embryonal and early neonatal stages and quite often disappears there after (Garaschuk, Hanse et al. 1998; Peinado 2001). In organotypic cultures of spinal cord, oscillatory activity is readily discernable as spontaneous, rhythmic fluctuations in intracellular calcium concentration (Lu, Moran et al. 2006; Fabbro, Pastore et al. 2007). Such oscillations were seen in the early weeks of DMOTC of mouse spinal neurons. This observation fits well with our current understanding that synchronous oscillatory activity may be of importance during early development, promoting neuronal maturation, activity-dependent synaptic wiring and network formation (Feller, Wellis et al. 1996; Katz and Shatz 1996; Hanse, Durand et al. 1997; Bregestovski and Spitzer 2005). However, this activity ceases to be so in more mature animals to allow for orderly information processing (Ruscheweyh and Sandkühler 2005). As such, in older DMOTCs these oscillations were less frequent and were absent in slices greater than 35d old (figure 4.4 d). Abatement of oscillatory activity is what might be expected if the maturation processes in DMOTC resemble those seen in acute slices.

4.4.3 Changes in neuron number in culture

Two key developmental processes cell death and neurogenesis are integral to the development of the nervous system and as such a delicate balance needs to exist between the two processes. A common and important feature during embryonic development is programmed

cell death, or apoptosis (Buss et al., 2006; Chu-Wang and Oppenheim, 1978a; Chu-Wang and Oppenheim, 1978b). The large numbers of neurons produced in excess and those that do not make connections usually undergo natural cell death (Baron 2007). Another study on mouse organotypic spinal cords found a 4 to 5-fold decrease in the number of GABAergic cells over the first three weeks in culture (Avossa, Rosato-Siri et al. 2003). Additionally, it has been deduced that programmed cell death in the developing central nervous system could be as high as 50 - 60%, and that this process is preserved in evolution as it serves adaptive functions such as error correction and cell regulation (Buss, Sun et al. 2006). Consistently this study shows a similar proportion of cell death throughout early postnatal development of neurons maintained in DMOTC (**figure 4.2 e**). It is of course possible that the reduction in neuron numbers seen in **figure 4.2 e** simply reflects progressive deterioration of the culture itself. If this were true, we should expect to see a continual decrease in neuronal population. However, this was not the case as the number of neurons stabilized following week 2 in culture (**figure 4.2 e**) in a similar fashion to neurons in vivo (**figure 4.2 d**), suggesting that the loss may be attributed to normal programmed cell death during development. Overall, our finding that DMOTC experience a substantial neuronal cell loss after the first week in vitro reflects other studies of developing embryos and reflects what is already known regarding developmental apoptosis (Coluzzi and Mattia 2005).

4.4.1 Changes in astrocytic coverage in culture

Two main glial cells can be observed in the spinal cord; oligodendrocytes, which myelinate the neuronal processes in the CNS (Noble, Fok-Seang et al. 1984) and astrocytes

responsible for several trophic functions (Lu, Yokoyama et al. 1991). In developing central nervous tissue, astrocytic growth is seen following neuronal development. In this study, an increase and stabilization of astrocyte number (**figure 4.5 b**) is observed in the DMOTC; a finding consistent with activation of normal developmental processes in the cultures. A key observation is that early glial cell appearance is confined to the ventral region of the cord and an even spread of cells is seen across the dorsal and ventral regions of the slice over time. This observation has been reported by (Breckenridge, Sommer et al. 1997; Liu, Wu et al. 2002; Avossa, Rosato-Siri et al. 2003). In interpreting these findings, it is prudent to also acknowledge the fact that GFAP expression is not exclusive to astrocytes, as it has been reported in some specific stem cell populations (Doetsch, Caille et al. 1999) (Doetsch, Caille et al. 1999), as well as within a subset of radial glia (Dupouey, Benjelloun et al. 1985).

4.5 Conclusion

I have developed and validated a genetically engineered mouse organotypic culture in a defined, serum-free medium for six weeks and have found that temporal changes in the numbers of neurons and astrocytes are similar to those observed when neurons mature *in vivo*. It has also been shown that the 7 electrophysiological phenotypes found in acute slices are also found in DMOTC. Importantly, GABAergic phenotype identified by expression of GAD67-EGFP associates with similar electrophysiological phenotypes in both acute slices and DMOTC. Taken together, these results indicate that serum-free defined medium organotypic cultures from genetically

modified mouse spinal cord can be used to address physiological and pathophysiological processes in the dorsal horn, particularly those relating to neuropathic pain.

Table 4.1 Comparison of Electrophysiological Properties of Neurons in DMOTC cultures. Values of rmp for all neuronal types were not significantly different from each other (T-tests). Spike

widths in neurons from DMOTC tend to be wider, the only category that reached significance was tonic cells (* = p<0.05, t-test).

	Delay irregular	**Delay tonic**	**Initial burst**	**Phasic**	**Regular firing**	**Single spiking**	**Tonic**
rmp (DMOTC)	−64.3 ± 1.5 mV (*n* = 3)	−62.1 ± 3.0 mV (*n* = 13)	−61 ± 0.8 mV (*n* = 24)	−61.5 ± 0.7 mV (*n* = 15)	−60.1 ± 0.9 mV (*n* = 16)	−59.0 ± 0.9 (*n* = 6)	−61.4 ± 1.9 mV (*n* = 8)
Spike width (DMOTC)	8.3 ± 3.5 m s (*n* = 3)	6.0 ± 1.7 m s (*n* = 17)	3.2 ± 0.6 m s (*n* = 20)	2.7 ± 0.5 m s (*n* = 18)	2.8 ± 0.4 m s (*n* = 25)	2.3 ± 0.4 m s (*n* = 4)	3.0 ± 0.5 m s (*n* = 8)*

Figure. 4.1. a-g Illustrations of typical firing patterns of Delay Irregular, Delay Tonic, Tonic, Regular Firing, Phasic, Initial Bursting and Single Spiking (Transient) neurons in DMOTC of mouse spinal cord. Recordings are displayed on two different time scales.

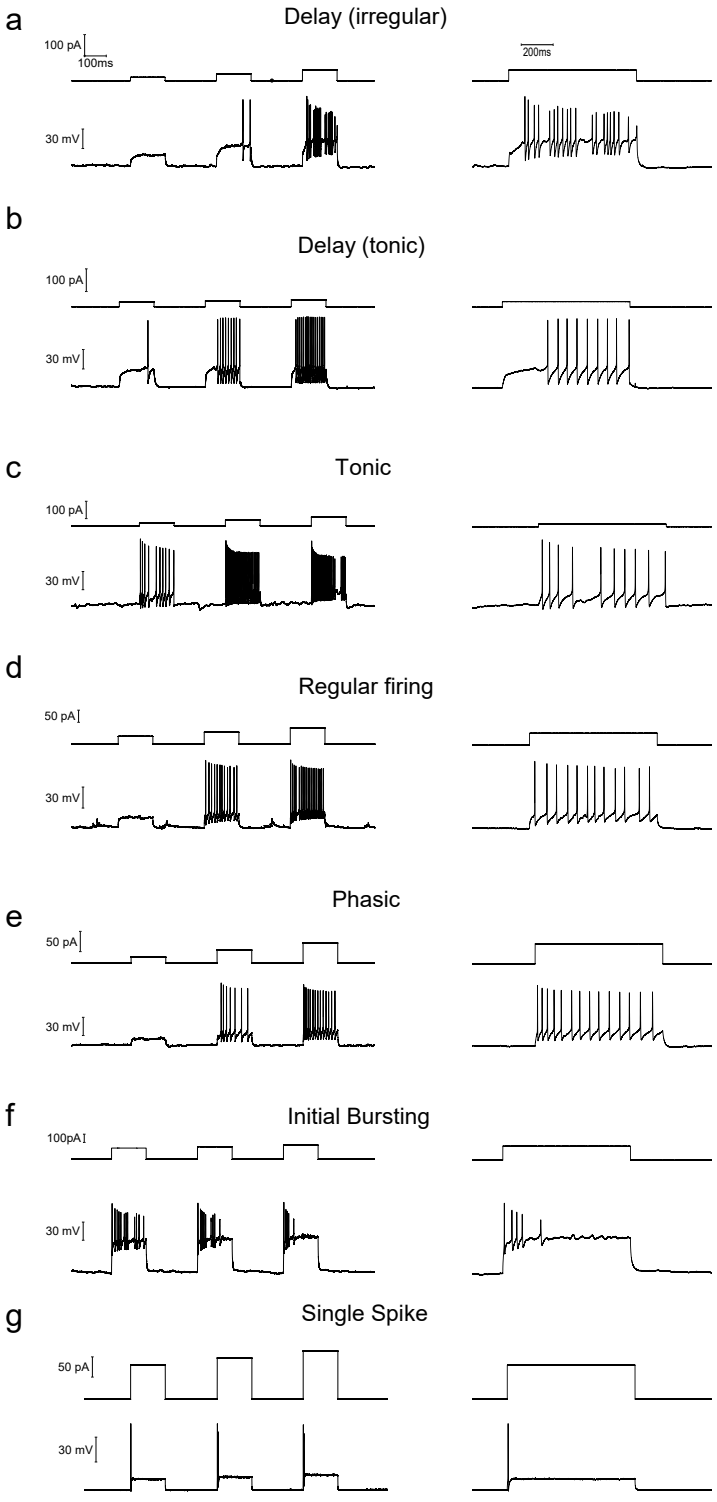

a
Delay (irregular)
100 pA
100ms
200ms
30 mV
b
Delay (tonic)
100 pA
30 mV
c
Tonic
100 pA
30 mV
d
Regular firing
50 pA
30 mV
e
Phasic
50 pA
30 mV
f
Initial Bursting
100pA
30 mV
g
Single Spike
50 pA
30 mV

Figure 4.1

Figure 4.2. a. DMOTC spinal cord slice. Dorsal and ventral sides of slice indicated by D and V, note migration of dorsal root ganglia (DRG) to ventral side of slice. **b.** z-stack image showing green fluorescently labeled GAD67/EGFP+ neurons. A step size of 1 µm was used to acquire z-stack image. **c.** Image of neurons labeled with Alexa 594 (red) in GAD67-EGFP+ slice GABAergic neurons appears yellow. **d.** Time course to show the development of DMOTC compared with age of neurons growing in vivo. **e.** For DMOTC, change in number of neurons per sq. mm. as cultures age (* = $p < 0.05$, a one-way ANOVA with post hoc Tukey–Kramer's test). **f.** Comparison of percentages of neuronal populations n acute slices of mouse spinal cord (from Fig. 1j, n = 211) with those in DMOTC (n = 83). **g.** Comparison of percentages of neuronal populations of GAD- (n = 32) and GAD-EGFP+ (n = 51) neurons in DMOTC. **h.** Comparison of percentages of GAD-EGFP+ (n = 51) neurons in DMOTC with those in acute slices (n = 74 from **figure. 4.1l**).

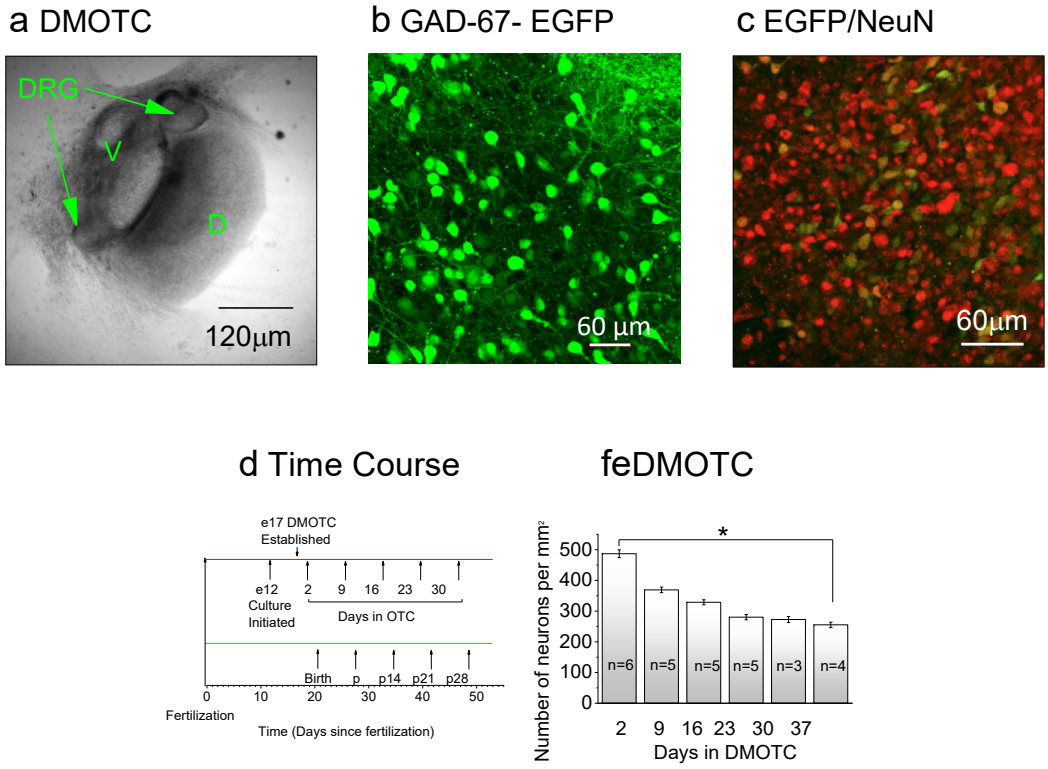

a DMOTC
DRG
V
D
120µm
b GAD-67- EGFP
60 µm
c EGFP/NeuN
60µm
d Time Course
e17 DMOTC Established
e12 Culture Initiated
2 9 16 23 30
Days in OTC
Birth p p14 p21 p28
0 10 20 30 40 50
Fertilization
Time (Days since fertilization)
feDMOTC
Number of neurons per mm²
500 400 300 200 100 0
n=6 n=5 n=5 n=5 n=3 n=4
*
2 9 16 23 30 37
Days in DMOTC
f Acute vs. DMOTC Neuron Types
DMOTC
Acute Slices
Proportion of Neurons (%)
25 20 15 10 5 0
Tonic
Delay Tonic
Delay Irregular
Initial Burst
Phasic
Regular Firing
Unclassified
Single Spikes
g Distribution of GAD67-EGFP+/- in DMOTC
GAD67-GFP+
GAD67-GFP-
Proportion of Neurons (%)
35 30 25 20 15 10 5 0
Tonic
Delay (Tonic)
Delay (Irregular)
Initial Burst
Phasic
Regular Firing
Unclassified
Single Spikes
h Acute vs. DMOTC GAD-67-EGFP Expression
Organotypic Cultures
Acute Slices
Proportion of GAD+ Neurons (%)
35 30 25 20 15 10 5 0
Tonic
Delay Tonic
Delay Irregular
Initial Burst
Phasic
Regular Firing
Unidentified
Single Spikes

Figure 4.2

Figure 4.3 a & b Summaries of interevent intervals (IEI) and amplitudes of sEPSCs in 7 electrophysiologically defined *substantia gelatinosa* neuron types in DMOTC Numbers of neurons in each category indicated on graph. Differences are not significant unless marked with *** = $p < 0.001$ or *$p < 0.05$, a one-way ANOVA with post hoc Tukey–Kramer's test. There are no significant differences in the amplitudes of events. **c.** IEI data from **a** plotted against amplitude data from (**b and d**) Superimposition of plot in c with that from **Figure 2c** to show differences between excitatory synaptic activity in DMOTC and that in acute slices. (**e and f**) Cummulative probability plots to show differences in IEI and amplitude of sEPSCs in 100 GAD67-EGFP− neurons and 70 GAD67-EGFP+ neurons. (6799 events from GAD67-EGFP− neurons and 1388 events from GAD67-EGFP+ neurons.) Difference is significant for IEI ($p < 0.001$ Kolmogorov–Smirnov's test, **e**) but not for amplitude ($0.1 > p > 0.05$; **f**). **g.** Current-clamp recording from neuron in DMOTC, illustrating spontaneous synaptic activity as spontaneous EPSCs and the generation of action potentials

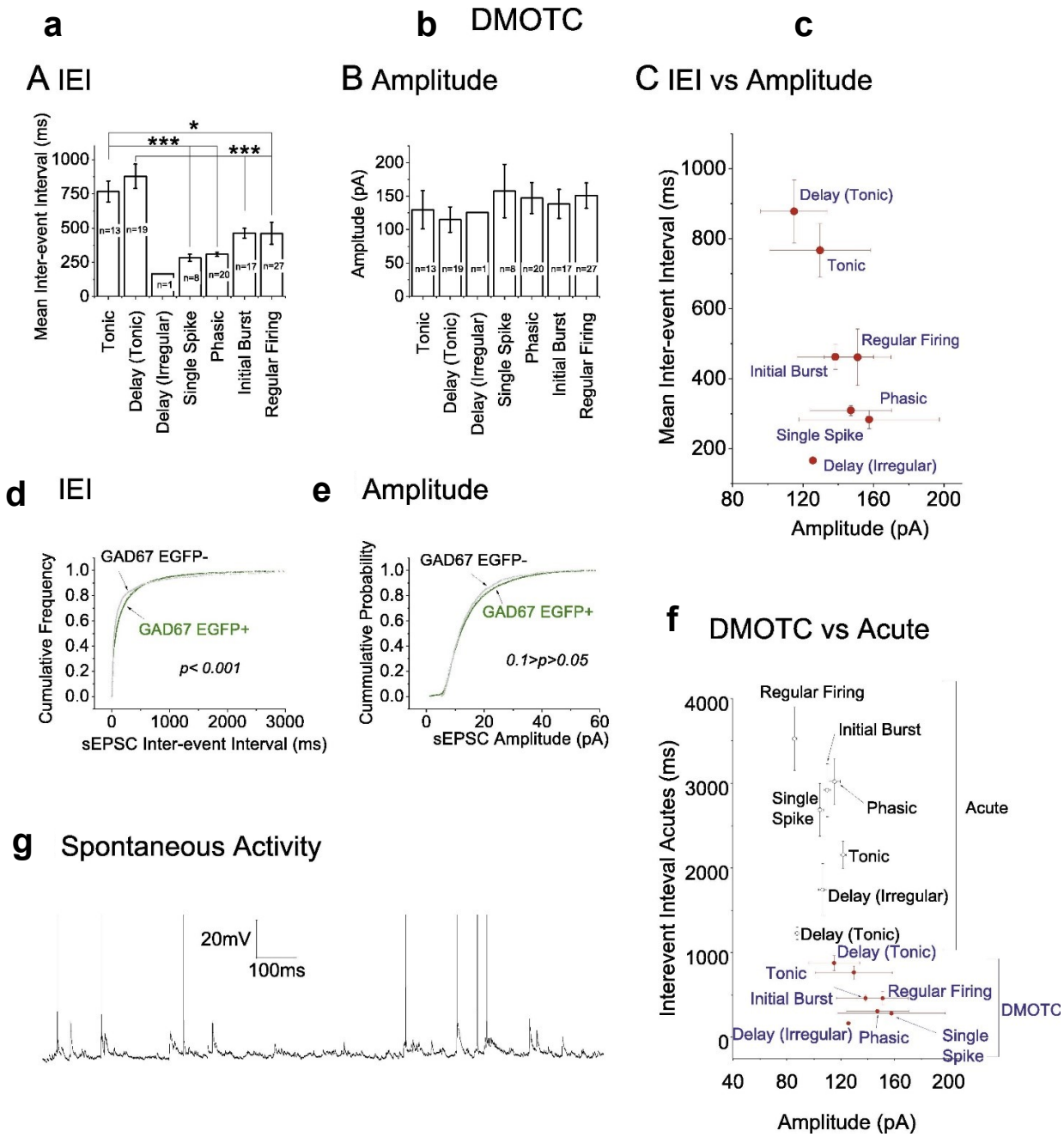

a
b DMOTC
c
A IEI
B Amplitude
C IEI vs Amplitude
Mean Inter-event Interval (ms)
Amplitude (pA)
Tonic
Delay (Tonic)
Delay (Irregular)
Single Spike
Phasic
Initial Burst
Regular Firing
d IEI
e Amplitude
GAD67 EGFP-
GAD67 EGFP+
p< 0.001
0.1>p>0.05
Cumulative Frequency
Cummulative Probability
sEPSC Inter-event Interval (ms)
sEPSC Amplitude (pA)
f DMOTC vs Acute
Interevent Inteval Acutes (ms)
Acute
DMOTC
g Spontaneous Activity
20mV
100ms

Figure 4.3

Figure 4.4 a. Image for video of Ca2+ oscillations in spinal DMOTC **b.** Recordings of changes in Fluo 4 fluorescence over time from selected regions of interest in image from (**a and c**) Sample of data from (**b**) plotted on a faster time scale. Note lack of synchrony between changes in Fluo 4 fluorescence. **d.** Graph to show decrease in percentage of slices in DMOTC exhibiting oscillations as the cultures age (n's refer to number of DMOTCs examined) and decrease in percentage of acute slices exhibiting oscillations as animals mature (n's refer to number of slices examined)

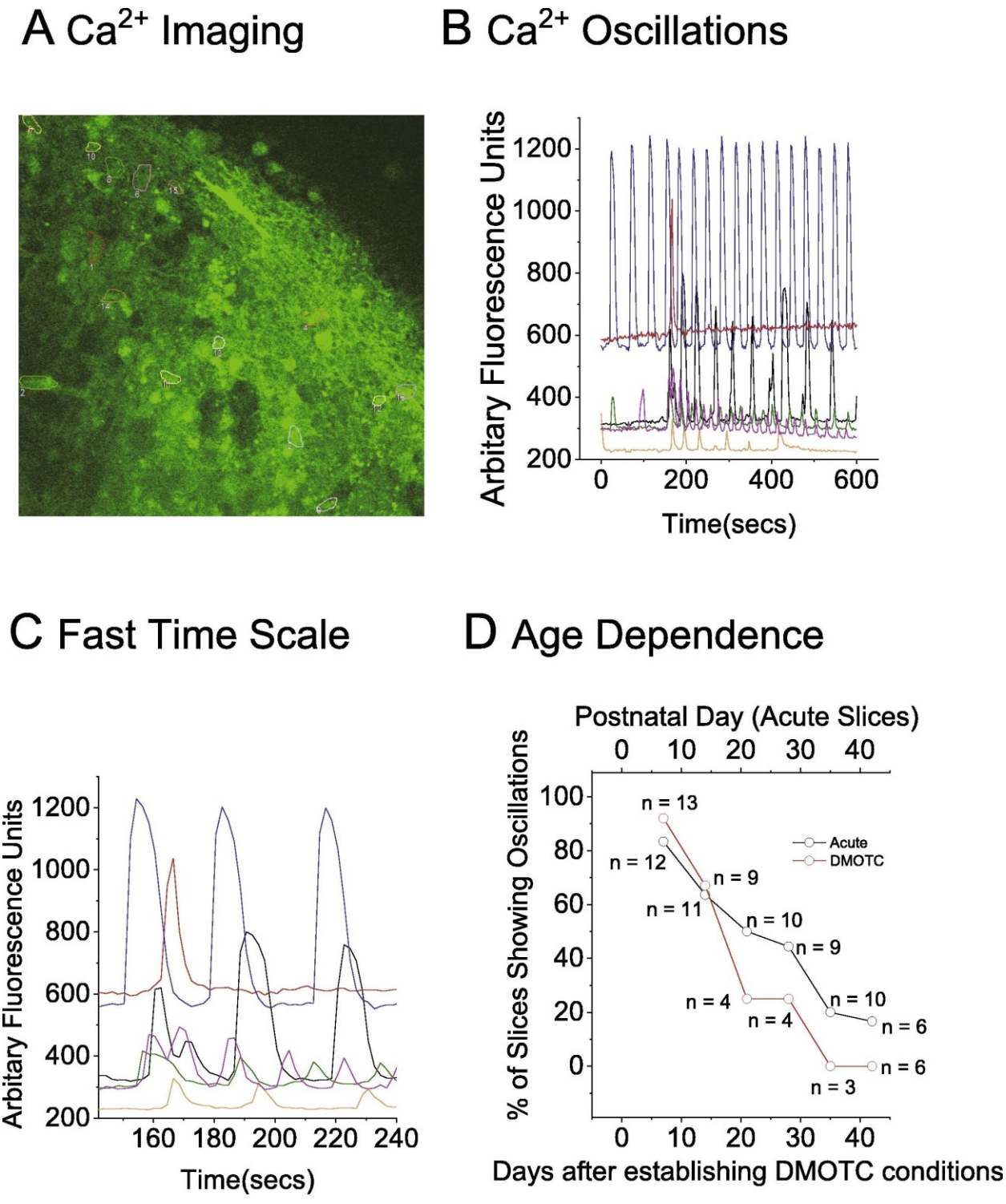

A Ca2+ Imaging
B Ca2+ Oscillations
Arbitary Fluorescence Units
1200
1000
800
600
400
200
0
200
400
600
Time(secs)
C Fast Time Scale
Arbitary Fluorescence Units
1200
1000
800
600
400
200
160
180
200
220
240
Time(secs)
D Age Dependence
Postnatal Day (Acute Slices)
0
10
20
30
40
% of Slices Showing Oscillations
100
80
60
40
20
0
Acute
DMOTC
n = 13
n = 12
n = 9
n = 11
n = 10
n = 9
n = 4
n = 4
n = 10
n = 6
n = 6
n = 3
Days after establishing DMOTC conditions

Figure 4.4

Figure 4.5 a. DMOTC labeled with GFAP-Alexa 488 (green) and NeuN-Alexa 594 (red) for astrocytes and neurons respectively. **b.** Growth of astrocytes in DMOTC. Bars represent% of field of view covered with GFAP immunoreactivity versus time in DMOTC. Error bars = average ± SEM, n's represent numbers of cultures (* = $p < 0.05$, *** = $p < 0.001$ a one-way ANOVA with post hoc Tukey–Kramer's test). **c.** Fluorescent image to show constraint of astrocytes and their processes labeled with GFAP-Alexa 594 within the border of spinal cord slice after 28d in DMOTC. **d**. Bright-field image of stained DMOTC in (C) 100-μm scale bar refers to both (**c**) and (**d**).

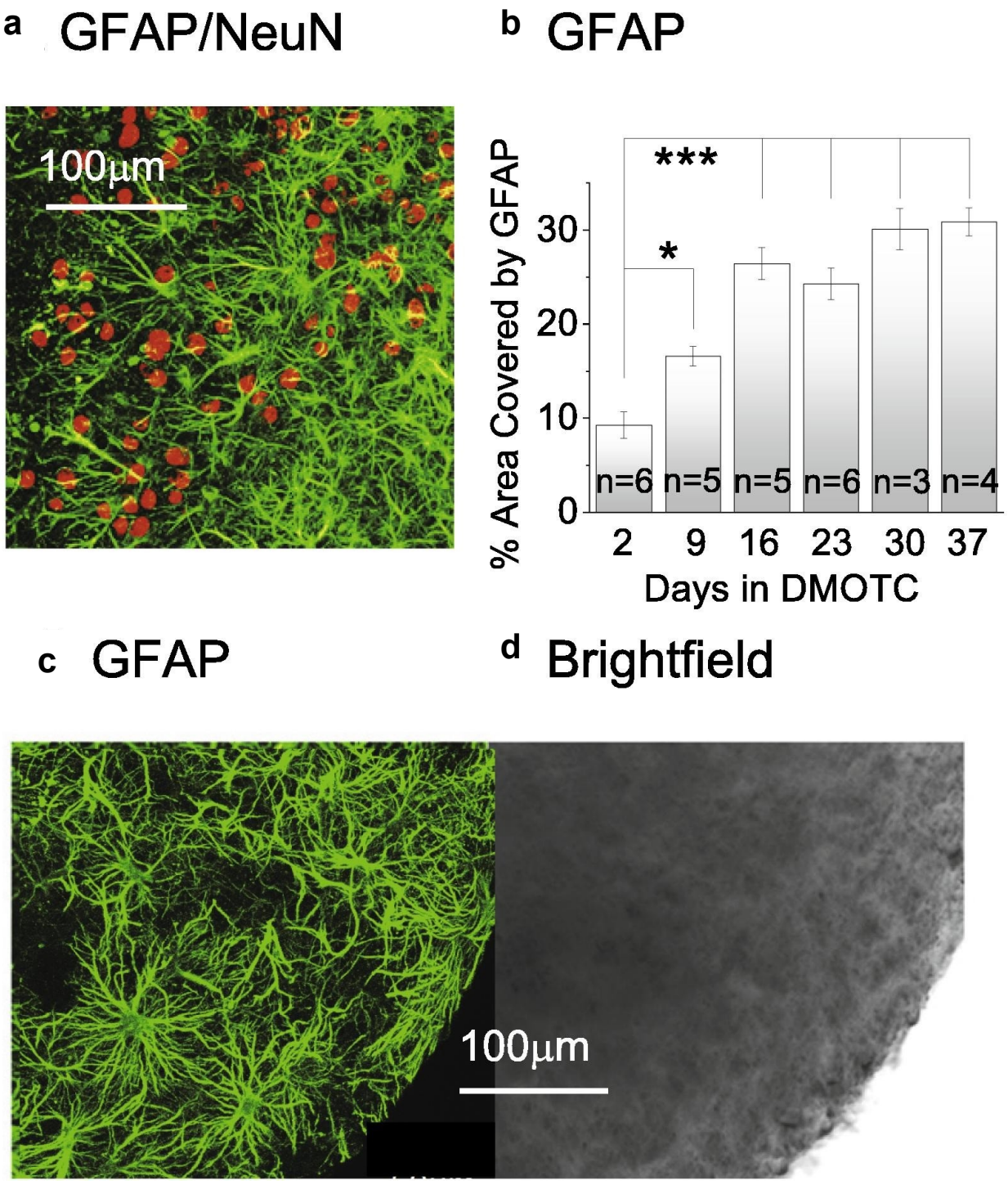

a GFAP/NeuN
100μm
b GFAP
% Area Covered by GFAP
***
*
30
20
10
0
n=6 n=5 n=5 n=6 n=3 n=4
2 9 16 23 30 37
Days in DMOTC
c GFAP
d Brightfield
100μm

Figure 4.5

CHAPTER 5

INVESTIGATING THE ROLE OF SPINAL TRK B AND P75 RECEPTORS IN CENTRAL SENSITIZATION

ABSTRACT

Injury to the Peripheral nervous system such as chronic constriction injury (CCI) of the rat sciatic nerve elicits an enduring increase in the excitability of the spinal dorsal horn. This change, that likely underlies the development of chronic pain, may be a consequence of prolonged exposure of dorsal horn neurons to mediators such as neurotrophins, cytokines, and neurotransmitters. Previous observations from the lab in both CCI models and the long-term effects of such mediators on *substantia gelatinosa* neurons using serum-free, defined-medium organotypic cultures (DMOTC) from E13-14 prenatal rats by means of whole-cell recordings has established that CCI/DMOTC increases excitatory synaptic drive to putative excitatory, 'delay' firing neurons in the *substantia gelatinosa* but attenuates that to putative inhibitory, 'tonic' firing neurons. To further investigate such finding I developed (**chapter 4**) and used a more precise model of DMOTC generated from genetically engineered mice that had their Glutamic Acid decarboxylase enzyme coupled to green fluorescent protein (GAD67-EGFP "*Tamamaki*" mice). Employing whole cell recordings and calcium imaging techniques, similar observations were made. An increase in synaptic drive was observed in delay neurons from BDNF treated slices and and a weaker increase in the frequency of sEPSC was seen in GAD67-EGFP neurons. The effects seen in delay neurons from BDNF treated cultures were both pre and post synaptic and involved the BDNF/TrkB signalling mechanism. Presynaptic effects dominated in GAD67-EGFP neurons and involved the BDNF/p75NTR signalling pathway.

These observations highlight the role of both p75 and TrkB receptors in the cellular mechanisms that may lead to a global downregulation of inhibitory tone leading to the development of neuropathic pain.

Key Words

Lamina II, Defined Medium Organotypic Cultures, Electrophysiology, Pre and post synaptic effects

5.1 Introduction

Previous work in the lab (Lu, Biggs et al. 2009; Lu, Colmers et al. 2009) has shown the important role BDNF plays in the actions of peripheral nerve injury to 1.) **increase** excitatory, glutamatergic synaptic drive to putative **excitatory** neurons in the *substantia gelatinosa* and at the same time 2.) to **decrease** excitatory, glutamatergic synaptic drive to **inhibitory** neurons in the *substantia gelatinosa*. However, it is yet to be determined the receptor mechanism through which BDNF acts through to bring about these effects. Effects of BDNF, as was highlighted in **chapter 1** involves the activation of TrkB and p75 receptor signalling. In many physiological situations, Trk B activation leads to excitatory processes and p75 receptor activation leads to inhibitory signalling. These effects and signalling mechanism led me to the hypothesis of this thesis: BDNF acts through different receptors and/or transduction mechanisms to increase release of excitatory transmitter onto excitatory neurons (using the Trk B signaling pathways) and to decrease release of excitatory transmitter onto inhibitory neurons (via the p75 signaling pathway).

In order to determine the receptor dependence of BDNF actions in the spinal cord I used defined medium organotypic cultures (DMOTC) of genetically engineered mice spinal cords that had their GAD-67 promotor coupled to EGFP and exposed them to BDNF to mimic the duration of time for development of mechanical allodynia *in vivo* (Balasubramanyan, Stemkowski et al. 2006; Lu, Biggs et al. 2009) (see **figure 4.2 d**). Using whole-cell patch- clamp recording and calcium imaging techniques I studied the actions of various TrkB and p75 ligands on spontaneous EPSC's (sEPSC) in both GAD67-GFP+ and GAD67-GFP-, delayed firing neurons.

5.2 Methods

Please refer to ***Chapter 2*** for all procedures performed.

5.3 Results

5.3.1 Ca^{2+} Imaging of Dorsal Horn Excitability after BDNF Incubation

The application of a high K^+ solution as an indirect way of testing dorsal horn excitability via Ca^{2+} imaging is not a physiological stimulus and has been criticized. To address this issue, I used electrical stimulation of the dorsal root entry zone and/or DRGs (**described in chapter 2**) (Biggs, Lu et al. 2012). It must be noted that even though electrical stimulation itself is also non-physiological, the technique makes use of the fact that afferent fibres are activated which are located remote to the Ca^{2+}-imaged cells which causes physiological-like release of neurotransmitter within the network of these cells (Lu, Biggs et al. 2009; de Curtis, Librizzi et al. 2012; Kantor, Panaitescu et al. 2012; Trapp and Ballanyi 2012).

Using the protocol as described in **chapter 2**, where DMOTC are stimulated for 5 s at 50 Hz using a 100 µs single pulse width, a robust Ca^{2+} fluorescence intensity of stimulated cultures was obtained. A series of 8 typical responses (s1 through s8) are shown in f**igure 5.1 a**. As done for rats (Biggs, Lu et al. 2012), the fifth and sixth responses, was chosen for analysis. The reason being that the response amplitude of the fifth and sixth responses changes little during these successive stimuli. In rats, these stimulation-evoked Ca^{2+} responses have been reported to require the activation of kainate/AMPA, NMDA and mGluR5 receptors (Biggs, Lu et al. 2012).

Figures 5.1 b & c, illustrate the effect of 5–6 days treatment of spinal cord DMOTC slices with BDNF with respect to the amplitude and area under the curve response of stimulus evoked

Ca^{2+} responses. At 200 ng/ml, BDNF significantly increases the amplitude of both responses. (*P < 0.001 two*-tailed *T*-test, f**igure 5.1 b** and **c**). This is similar to the results seen in rat DMOTC with electrical stimulation (Biggs, Lu et al. 2012) and with stimulation by high K^+ (Lu, Ballanyi et al. 2007). The import of these findings indicates that the overall excitability of dorsal horn neurons in both mouse and rat DMOTCs is increased after 'chronic' BDNF treatment.

As a preliminary investigation of the receptor dependence of these effects, DMOTC were incubated with 200ng/ml BDNF plus the tyrosine kinase inhibitor, ANA-12 (50μM). Slices incubated with ANA 12 alone served as control. The effect of BDNF on amplitude and area under curve of Ca^{2+} responses was abrogated by ANA-12 (f**igure 5.1 d** and **e**).

A p75 blocking antibody was used to investigate any possible contribution of p75 to the effect of BDNF. Although the effect of BDNF was not blocked by the antibody (**Figure 5.1 f** and **g**), its effectiveness may have been attenuated as the significance for BDNF effectiveness decreased from P<0.001 (**Figure 5.1 b** and **c**) to P<0.05 (Figure **5.1 f** and **g**).

BDNF thus increases overall dorsal horn activity by a mechanism involving tyrosine kinase receptors (presumably TrkB). Its effects may also depend on activation of p75.

5.3.2. Electrophysiological Effects of BDNF on Mice DMOTC

As mentioned above in rat DMOTC, BDNF causes decreased synaptic drive to 'tonic' neurons and increased synaptic drive to 'delay' neurons and that these selective and differential actions of BDNF on inhibitory and excitatory neurons contributed to a global increase in dorsal horn network excitability as shown by the amplitude of depolarization-induced increases in intracellular Ca^{2+} (Lu, Ballanyi et al. 2007). Based on this observation, I wanted to see whether I

could replicate this effect in mice GAD67-EGFP DMOTC. Mice have been shown to be physiologically different from rats (Ellenbroek and Youn 2016; Hok, Poucet et al. 2016) and my data presented in **Chapter 3** show subtle differences in the firing patterns of rat versus mouse *substantia gelatinosa* neurons.

As illustrated, BDNF increased the amplitude of sEPSCs in 'delay' neurons (**figures 5.2 a** & **e.** $P < 0.001$, KS test**; e** & **g.** $P < 0.01$; t-test on mean event amplitudes). In contrast, BDNF had no effect on the amplitude of sEPSCs in 'GAD67-EGFP' neurons (**figure 5.2 b** & **f**. $P > 0.05$; KS test and t-test).

BDNF also increased the frequency (decreased the IEI) of sEPSC's in delay cells and this was statistically significant for KS statistics done on cumulative probability data (**Figure 5.2 c**, $P<0.001$) and from consideration of mean IEI's (f**igure 5.2 g**, $P<0.001$).

Effects of BDNF on GAD67-EGFP neurons were generally much weaker, event amplitude was unchanged (f**igure 5.2 b** and **f**) and event frequency was only slightly increased (**figure 5.2 d** and **h**).

5.3.3 Electrophysiological Effects of proBDNF on Mice DMOTC

I sought to assess the effect of the proneurotrophin, proBDNF on sEPSCs in delay and GAD67-EGFP neurons as it has been reported to preferentially activate p75 neurotrophin receptors (Teng et al 2010) and the data from f**igure 5.1** suggest that these receptors may contribute to the effects of BDNF in spinal dorsal horn. In delay cells, the actions of proBDNF resembled those of BDNF. Amplitude of sEPSC was increased (**figure 5.3 a** & **e.** $P < 0.001$; KS test or one tailed t-test) as was frequency (**figure 5.3 c** & **g.** $P < 0.001$; KS test or one tailed t-test).

By contrast, in GAD67-EGFP neurons actions of proBDNF were very different from BDNF. Both the amplitude and frequency of sEPSCs were markedly reduced (**figures 5.3 b** & **f**. P < 0.001; KS test and one -tailed t-test and **d** & **h**. P < 0.001; KS test and one tailed t-test). Thus, suggestive of the possibility that the p75 signaling pathway could be involved in the spinal actions of BDNF.

5.3.4. Electrophysiological Effects of LM22A-4 on Mice DMOTC

TrkB signaling is not only an important contributor in the induction of heat and mechanical hypersensitivity produced by tissue or nerve injury, but also in the persistence of the pain (Wang, Ratnam et al. 2009). Likewise, the BDNF-trkB system in dorsal root ganglia and spinal cord is upregulated in animal models of pain (Lin, Ro et al. 2011).

To investigate the involvement of the TrkB receptor in BDNF's actions, LM22A-4, a small molecule BDNF loop domain mimetic that acts as a selective TrkB agonist (Massa, Yang et al. 2010) was used. The electrophysiological effects this compound on both delay and GAD67-EGFP cells were assessed.

Effects of LM22A-4 on sEPSC amplitude are illustrated in **figure 5.4. a, b, e & f**, and likewise its effects on interevent interval (the reciprocal of frequency) are illustrated in f**igure 5.4. c, d, g & h**. LM22A-4 significantly increased the amplitude (**figure 5.4 a** & **e.** P < 0.001; KS test and two tailed t-test), and the frequency of sEPSCs in 'delay' neurons (**figure 5.4 c** & **g**. P < 0.001; KS test and one tailed t-test).

LM22A-4 also slightly increased the amplitude of sEPSCs in 'GAD67-EGFP' neurons (**figure 5.4 b** & **f.** P < 0.001) but, unlike BDNF, decreased their frequency (figure **5.4 d** & **h**. P < 0.001).

5.3.5. Electrophysiological Effects of LM11A-31 on BDNF responses in mouse DMOTC

The non-peptide p75NTR ligand; LM11A-31 which blocks p75-mediated signaling (Knowles, Simmons et al. 2013; Massa, Xie et al. 2014) was used to investigate further the involvement of the p75 receptor in the mediating actions of BDNF.

LM11A-31 blocked the BDNF-induced increase in sEPSC amplitude **figure 5.5 a** and **5.5 e** compared to **figure 5.2 a** and **5.2 e** in delay cells**.** BDNF was still able to increase the frequency of sEPSC's in the presence of LM11A-31 (**figure 5.5 c** and **g** $P<0.01$ for both KS test and one tailed t-test) but its effectiveness seemed to be lessened. Compare with **figure 5.2 c** and **g** where p values for BDNF effects are <0.001.

Although BDNF in the presence of LM11A-31 slightly reduced the amplitude of sEPSC's in GAD-EGFP neurons (**figure 5.5 b,** $P<0.01$) this was not reflected in a decrease in mean event amplitude (**figure 5.5 f).** As a t-test may be more rigorous than a KS test, we suggest that BDNF does not affect the amplitude of sEPSC in GAD-EGFP cells either alone (**figure 5.2b** and **f** or in the presence of LM11A-31, **figure 5.5 b** or **5.5 f).** Despite its rather small effect to increase sEPSC frequency in GAD67-EGFP neurons when administered alone (**figure 5.2 d** and **h**), BDNF became much more effective in the presence of LM-11A-31. **Figures 5.5 d** and **h** illustrate the strong effects on sEPSC frequency under these conditions ($P<0.001$ for KS and t-tests).

5.3.6. Electrophysiological Effects of ANA-12 on BDNF responses in mouse DMOTC.

Since BDNF when applied alone had little effect on GAD67-EGFP neurons, **Figure 5.2b, d, f** and **g** the effect of the tyrosine kinase inhibitor, ANA-12 was only studied on BDNF responses in delay neurons. The ability of BDNF to increase sEPSC amplitude was decreased in the presence of ANA-12 in delay neurons. Compare **figure 5.2 a** and **e**, with **5.6 a** and **c**. By contrast, BDNF still produced a robust decrease in IEI (increase in sEPSC frequency) in these neurons. Compare **figure 5.2 c** and **g**, with **5.6 b** and **d.**

5.4 Discussion

Previous work in the lab, (Lu, Ballanyi et al. 2007), has shown that BDNF induces significant changes in excitatory neurotransmission that is reflective of both pre- and postsynaptic mechanism involvement and that the pattern of these changes are specific to putative excitatory delay and putative inhibitory neuronal populations in the dorsal horn. These distinct patterns of changes contribute to an increase in overall excitability in BDNF-treated DMOTC slices. Many of the changes seen in rat DMOTC were also seen in mouse DMOTC and as such are consistent with a role for BDNF in the induction of neuropathic pain.

The main findings of this study are as follows. (1) Similar to its effects in rat DMOTCs, BDNF produces a pattern of neuron type-specific changes in mice DMOTCs as well. As specific and distinct these changes are, this could be suggestive that the actions of BDNF in mice DMOTCs are not a general neurotrophic effect but are instead more relevant to its putative role as a harbinger of neuropathic pain (Coull, Beggs et al. 2005; Yajima, Narita et al. 2005). (2) BDNF increases excitatory synaptic drive to excitatory delay neurons by increasing the amplitude and

frequency of sEPSCs. BDNF has a much weaker effect on inhibitory GAD67-EGFP neurons where it fails to affect sEPSC amplitude and only slightly increases sEPSC frequency. (3) Effects of BDNF are dominated by its interaction with Trk B but a role for p75 cannot be discounted. Thus, in terms of increases in overall excitability, blockade of Trk B with ANA-12 is much more effective than p75 antibodies in attenuating the actions of BDNF (**figure 5.1**).

5.4.1 Differences Between Mice and Rats

Previously it has been shown that in rat DMOTC, BDNF causes an increase in excitatory synaptic drive in putative ``delay`` firing cells by causing an increase in frequency and amplitude of sEPSCs (Lu, Biggs et al. 2009) similar to the effect seen in mice DMOTC (**figure 5.2 & table 5.1**). In contrast the effect of BDNF on putative inhibitory cells by decreasing the frequency and amplitude of sEPSCs (Lu, Biggs et al. 2009) is not replicated in mice DMOTC (**table 5.1**). In mice, its effect on amplitude remains unchanged whereas the frequency of sEPSCs increases slightly (**figure 5.2**). These differences may point to the fact that mice may be very different from rats and hence the need to bear in mind that it is not a straight forward translation of findings in rats to those found in mice. In interpretation of the present data, I have made the assumption that changes in sEPSC amplitude reflect as postsynaptic effects, whereas changes in IEI/frequency reflect presynaptic effects (Moran, Colmers et al. 2004). My findings are illustrated diagrammatically in **figure 5.7**.

5.4.2 Postsynaptic Effect of BDNF on 'Delay' Neurons

Increase in sEPSC amplitude is mediated at least in part by Trk B because:

i. **BDNF** causes **an increase in amplitude** of sEPSCs (**figures 5.2 a & 5.7**)

ii**. LM22A-4** causes an **increase in amplitude** of sEPSCs (**figure 5.4 a & 5.7**)

iii. Effects of **BDNF** are **blocked by ANA-12** (**figure 5.6 a & 5.7**)

It is possible that p75 mechanisms are also involved because:

i. **proBDNF** causes an increase in amplitude of sEPSCs (**figure 5.3 a & 5.7**)

ii. LM11A-31, a p-75NTR receptor blocker, blocks **BDNF effects** (**figure 5.5 a & 5.7**)

This effect seen with LM11A-31 is difficult to interpret, as BDNF should still have access to Trk B.

5.4.3 Presynaptic Effects of BDNF on 'Delay' neurons

Increase in sEPSC frequency in delay neurons also involves TrkB because:

i. **BDNF** causes an **increase in** sEPSCs frequency (**figure 5.2 c**)

ii**. LM22A-4** causes an **increase in** sEPSCs frequency (**figure 5.4 c**)

iii. **BDNF** causes an **increase in** sEPSCs frequency in the presence of **LM11A-31**

(**figure 5.4 a**)

Likewise, the involvement of the p75 NTR is possible because:

i. **BDNF** also causes an **increase in** sEPSCs frequency in the presence of ANA-12

(**figure 5.6)**

ii. **proBDNF** increases frequency of sEPSCs

5.4.4 Postsynaptic Effect of BDNF on 'GAD67-EGFP' Neurons

The presence of post synaptic TrkB receptors on GAD67-GFP neurons is demonstrated by LM22A-4 increasing sEPSC amplitude (**figure 5.4 a)**. In contrast, proBDNF reduces amplitude (**figure 5.3 a**). BDNF has little effect on its own (**figure 5.2 b**). mBDNF alone is known to bind to receptor tyrosine kinase TrkB as a high-affinity receptor and to p75 neuroptophin receptor (p75NTR) as a low-affinity receptor (Rodrigueztebar, Dechant et al. 1990; Klein, Nanduri et al. 1991), perhaps by activating both receptors. Trk B mediated increases in amplitude are balanced by p75 mediated decreases in amplitude such that no obvious effect of BDNF is seen (**figure 5.2 b**).

5.4.5 Presynaptic Effects of BDNF on 'GAD67-EGFP' Neurons

The presence of p75 on excitatory nerve terminals which synapses onto inhibitory neurons is demonstrated by the ability of proBDNF to reduce frequency of sEPSCs (**figure 5.3 & 5.7 b**). The situation with Trk B is complex. Selective activation by LM22A-4 clearly decreases frequency (**figure 5.4 d**). However selective activation of Trk B by BDNF in the presence of LM11A-31 produces the reverse effect; increase in frequency of sEPSC. However, there is no obvious explanation for this difference. Interestingly, BDNF alone has a weak effect to increase frequency (**figure 5.2 b**), and hence may reflect activation of Trk B attenuated by p75 in a similar way fashion to its postsynaptic actions.

5.5 Conclusions

Whereas rat and mouse DMOTC are similar in terms of BDNF effects on delay cells, in mice BDNF fails to produce the decrease in frequency and amplitude seen in rats. It is apparent that p75 dependent and presynaptic TrkB mechanisms are present in mouse 'GAD67-EGFP' neurons. These mechanisms may dominate in rats and accounts for the decrease in amplitude and frequency of sEPSCs, whereas in mice these mechanisms are over-ridden by excitatory effects.

Table 5.1 Comparison of effects of BDNF on spontaneous excitatory post synaptic currents (sEPSCs). In both rats and mice, excitatory neuron sEPSCs amplitude and frequency increase. However, effect of BDNF on inhibitory neurons with reference to sEPSCS amplitude and frequency in both animals differ,

	BDNF Effect in Rats	BDNF Effect in Mice
Excitatory Neurons sEPSC Amplitude	↑	↑
Excitatory Neurons sEPSC Frequency	↑	↑
Inhibitory Neurons sEPSC Amplitude	↓	No effect
Inhibitory Neurons sEPSC Frequency	↓	↑

Figure 5.1 Effect of tetanic electrical stimulation on intracellular Ca^{2+} in *substantia gelatinosa* neurons mice DMOTC. **a.** Based on previous observation in rat DMOTC (Biggs, Lu et al. 2012) and applying the same protocol. Response shown here are to a series of tetanic (5 s train at 50 Hz using a 100 μs pulse width) stimuli (S1-S8) delivered at 5 min intervals. It should be noted that despite the variability, responses S5 and S6 displayed fairly consistent amplitudes. These two responses were used for further comparisons and averaged into one response and used for subsequent responses in each case. **b & c.** Typical S5 and S6 amplitude responses averaged into one response sampled from neurons in control slices and in slices treated with 200 ng/ml BDNF. A summary of data from 30 neurons in control slices, 40 neurons from slices treated with 200 ng/ml BDNF. *** = $P < 0.001$ (One-way Anova with Tukey–Kramer multiple comparisons test). **b.** Averaged amplitude of S5 and S6 responses **c.** Area under curve of s5 and s6 responses (averaged). **c & d**. averaged responses (S5 & S6) in terms of amplitude and area under curve respectively of the effects of ANA-12 on BDNF. A summary of data from 56 neurons in ANA-12 (50μM) only incubated slices, 36 neurons from slices treated with both ANA-12 (50μM) and 200 ng/ml BDNF. ns, $P > 0.05$ (One-way Anova with Tukey–Kramer multiple comparisons test). **e & f**. averaged responses (S5 & S6) in terms of amplitude and area under curve respectively of the effects of p75 antibody on BDNF. A summary of data from 38 neurons in p75 antibody (10ng/ml) only incubated slices, 33 neurons from slices treated with both p75 antibody (10ng/ml) and 200 ng/ml BDNF. ns, $P > 0.05$ (One-way Anova with Tukey–Kramer multiple comparisons test).

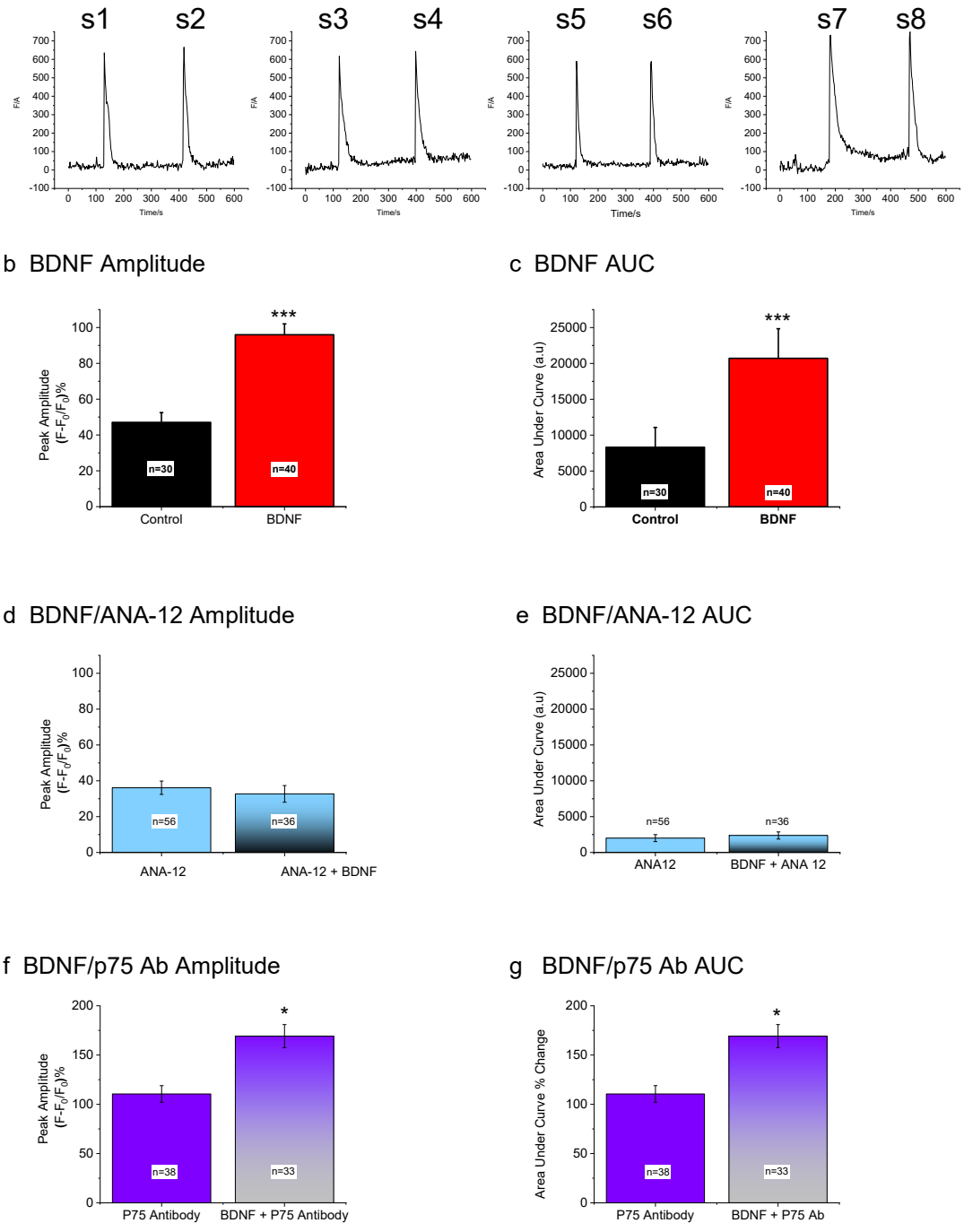

a Stimulation evoked Ca^{2+} responses
s1
s2
s3
s4
s5
s6
s7
s8
F/A
Time/s
b BDNF Amplitude
Peak Amplitude (F-F0/F0)%
n=30
n=40
Control
BDNF
***
c BDNF AUC
Area Under Curve (a.u)
n=30
n=40
Control
BDNF
***
d BDNF/ANA-12 Amplitude
Peak Amplitude (F-F0/F0)%
n=56
n=36
ANA-12
ANA-12 + BDNF
e BDNF/ANA-12 AUC
Area Under Curve (a.u)
n=56
n=36
ANA12
BDNF + ANA 12
f BDNF/p75 Ab Amplitude
Peak Amplitude (F-F0/F0)%
n=38
n=33
P75 Antibody
BDNF + P75 Antibody
*
g BDNF/p75 Ab AUC
Area Under Curve % Change
n=38
n=33
P75 Antibody
BDNF + P75 Ab
*

Figure 5.1

Figure 5.2. Effects of BDNF on delay and GAD67-EGFP neurons in DMOTCs **a** and **b**, In terms of amplitude of sEPSCs, BDNF has an effect and no effect on Delay and GAD67-EGFP neurons, respectively. Excitability was assessed by measuring the amplitude of sEPSCs in both neurons. Cumulative probability plots of amplitudes of sEPSCs obtained from **a.** control neurons (black) are statistically different from those obtained from neurons exposed to BDNF (red) in delay cells ($P < 0.001$; KS test), whereas there is **b.** no significant difference in amplitudes of sEPSCs from GAD67-EGFP neurons. **c** and **d** are cumulative distribution plot of inter-event intervals for delay and GAD67-EGFP neurons in DMOTCs. A decrease in inter interval events (reciprocal of frequency) in sEPSCs is observed in **c.** delay cells exposed to BDNF, which was statistically significant ($P < 0.001$; KS test), likewise **d.** in GAD67-EGFP neurons, IEI of sEPSCs decreases (increase in frequency) and was statistically significant ($P < 0.01$; KS test). **e** and **f** are mean amplitude plots of **a** and **b** above. **g** and **h** are also mean IEI plots of **c** and **d** above. Data for all plots were obtained from the first 100 events following the first minute of recording from neurons both in the absence and presence of BDNF.

Effects of BDNF

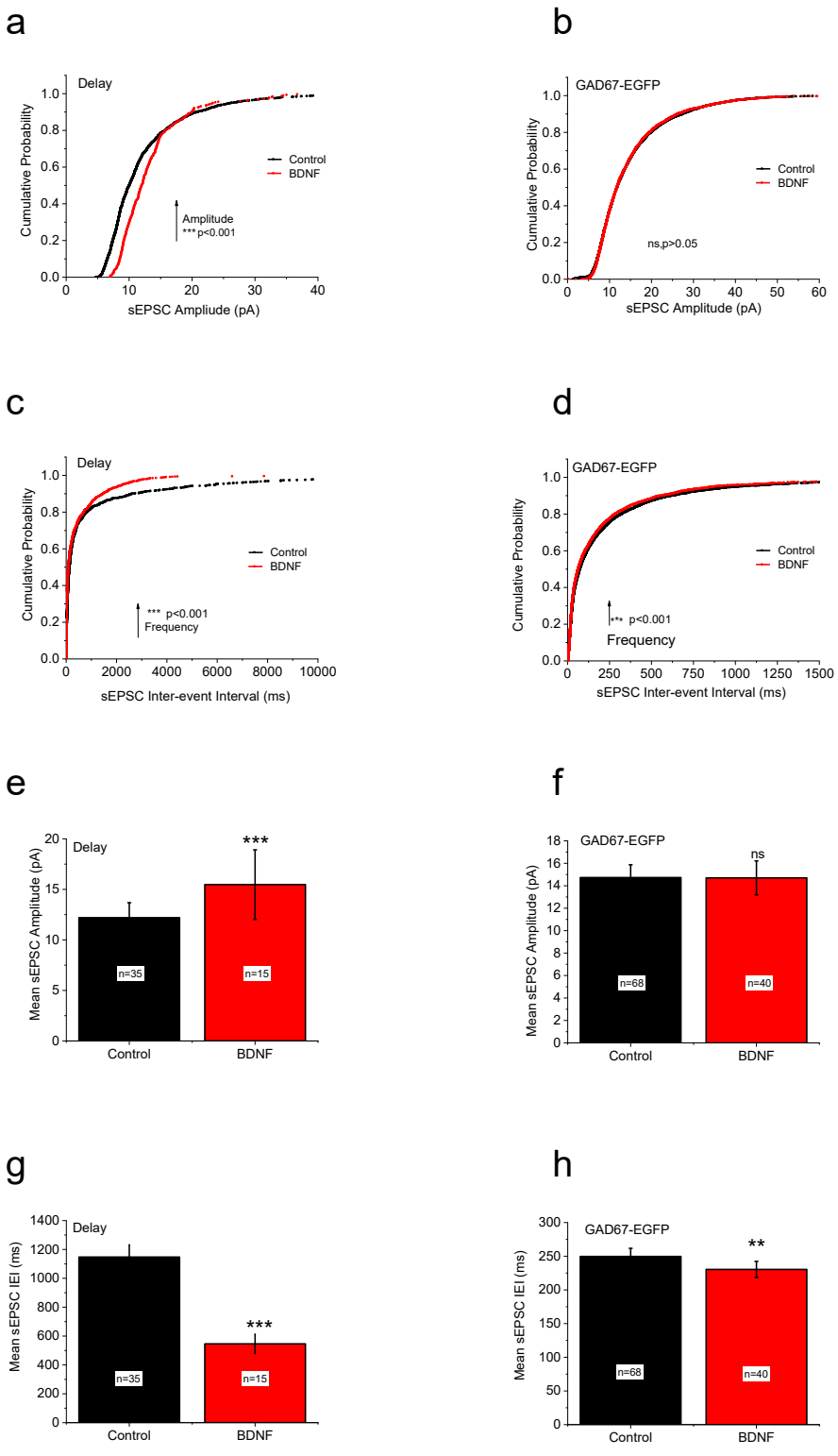


Figure 5.2

Figure 5.3. Effects of proBDNF on delay and GAD67-EGFP neurons in DMOTCs **a** and **b**, proBDNF had contrasting effect on Delay and GAD67-EGFP neurons, respectively. Cumulative probability plots of amplitudes of sEPSCs obtained from **a.** control neurons (black) are statistically different from those obtained from neurons exposed to proBDNF (red) in delay cells ($P < 0.001$; KS test), in an opposite manner **b.** there was a decrease in the amplitude of sEPSCs

obtained from GAD67-EGFP neurons ($P < 0.001$; KS test). **c** and **d** are cumulative distribution plot of inter-event intervals for delay and GAD67-EGFP neurons in DMOTCs. A decrease in inter interval events (an increase in frequency) in sEPSCs is observed in **c.** delay cells exposed to proBDNF, which was statistically significant ($P < 0.001$; KS test) compared to controls, in contrast **d.** in GAD67-EGFP neurons, IEI of sEPSCs increases (decrease in frequency) and was statistically significant ($P < 0.01$; KS test). **e** and **f** are mean amplitude plots of **a** and **b** above. **g** and **h** are also mean IEI plots of **c** and **d** above. Data for all plots were obtained from the first 100 events following the first minute of recording from neurons both in the absence and presence of BDNF.

Effects of pro-BDNF

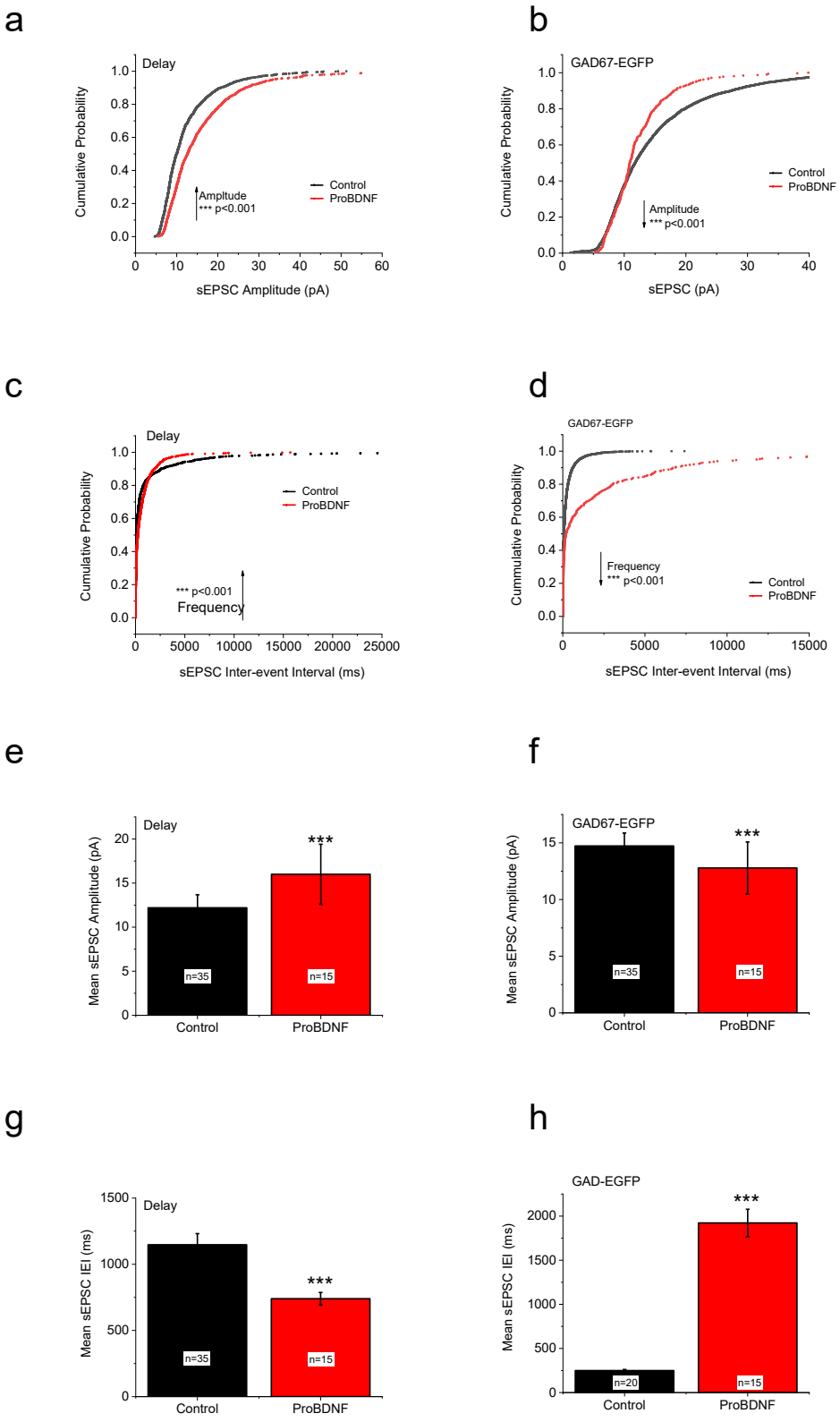


Figure 5.3

Figure 5.4. Effects of LM22A-4 on BDNF responses in delay and GAD67-EGFP neurons in DMOTCs. **a** and **b**, LM22A-4 had contrasting effect on Delay and GAD67-EGFP neurons, respectively. Cumulative probability plots of amplitudes of sEPSCs obtained from **a.** control neurons (black) are statistically different from those obtained from neurons exposed to LM22A-4 (blue) in delay cells ($P < 0.001$; KS test), likewise **b.** there was an increase in the amplitude of sEPSCs obtained from GAD67-EGFP neurons ($P < 0.001$; KS test). **c** and **d** are cumulative distribution plots of inter-event intervals for delay and GAD67-EGFP neurons in DMOTCs. A decrease in inter interval events (an increase in frequency) in sEPSCs is observed in **c.** delay cells exposed to BDNF, which was statistically significant ($P < 0.001$; KS test) compared to controls, in contrast **d.** in GAD67-EGFP neurons, IEI of sEPSCs increases (decrease in frequency) and was statistically significant ($P < 0.01$; KS test). **e** and **f** are mean amplitude plots of **a** and **b** above. **g** and **h** are also mean IEI plots of **c** and **d** above. Data for all plots were obtained from the first 100 events following the first minute of recording from neurons both in the absence and presence of BDNF.

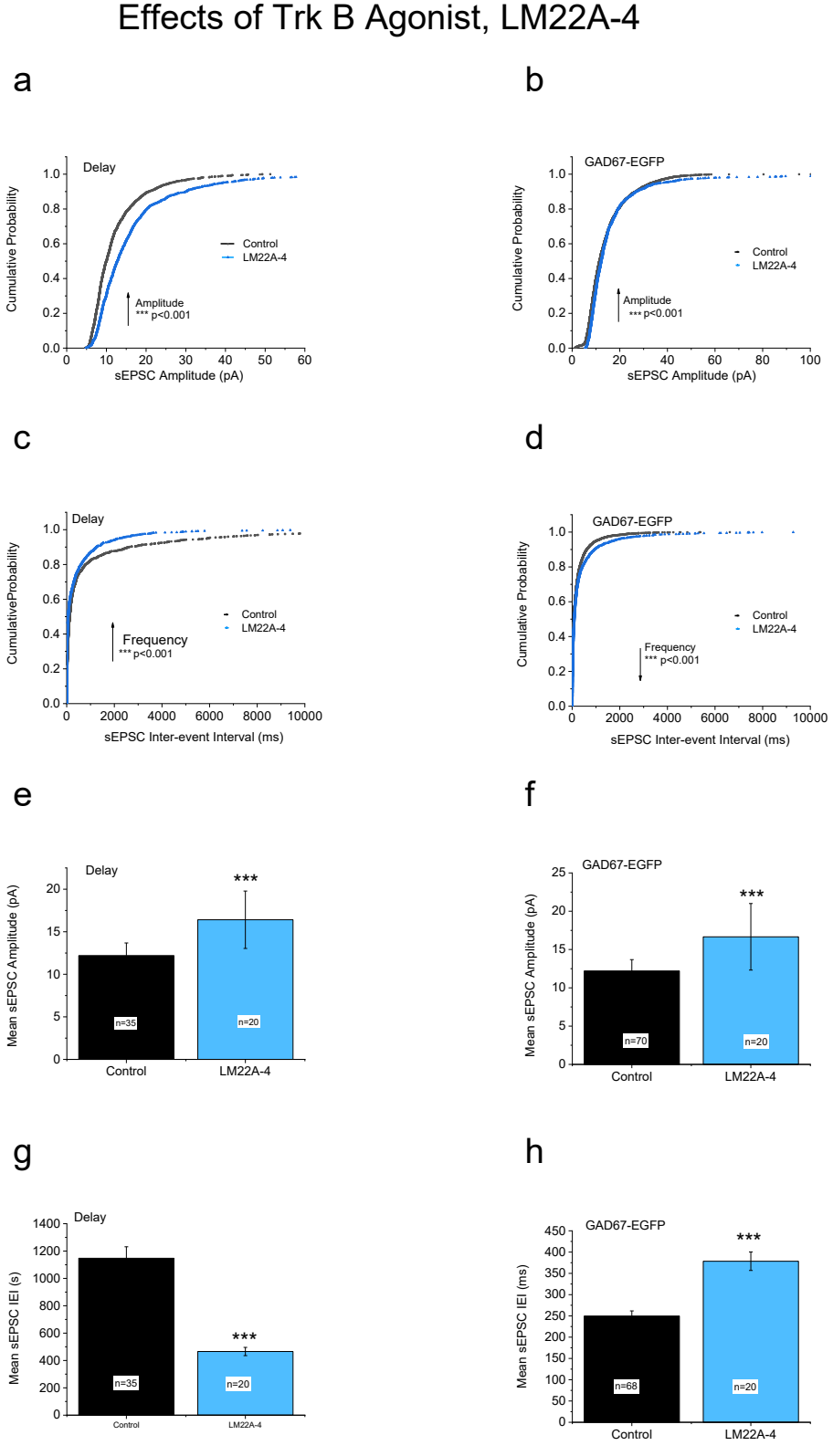

Effects of Trk B Agonist, LM22A-4
a
Delay
Cumulative Probability
sEPSC Amplitude (pA)
Control
LM22A-4
Amplitude
*** p<0.001
b
GAD67-EGFP
Cumulative Probability
sEPSC Amplitude (pA)
Control
LM22A-4
Amplitude
*** p<0.001
c
Delay
CumulativeProbability
sEPSC Inter-event Interval (ms)
Control
LM22A-4
Frequency
*** p<0.001
d
GAD67-EGFP
Cumulative Probability
sEPSC Inter-event Interval (ms)
Control
LM22A-4
Frequency
*** p<0.001
e
Delay
Mean sEPSC Amplitude (pA)
***
n=35
n=20
Control
LM22A-4
f
GAD67-EGFP
Mean sEPSC Amplitude (pA)
***
n=70
n=20
Control
LM22A-4
g
Delay
Mean sEPSC IEI (s)
***
n=35
n=20
Control
LM22A-4
h
GAD67-EGFP
Mean sEPSC IEI (ms)
***
n=68
n=20
Control
LM22A-4

Figure 5.4

Figure 5.5. Effects of LM11-31 on BDNF responses in delay and GAD67-EGFP neurons in DMOTCs. **a** and **b**, Cumulative probability plots of amplitudes of sEPSCs obtained from **a.** LM11A-31 exposed neurons (green) are not statistically different from those obtained from neurons exposed to LM11A-31+BDNF (blue) in delay cells ($P > 0.05$; KS test), however, **b.** there was a slight decrease in amplitude of sEPSCs obtained from GAD67-EGFP neurons exposed to LM11A-31+BDNF ($P < 0.01$; KS test). **c** and **d** are cumulative distribution plot of inter-event intervals for delay and GAD67-EGFP neurons in DMOTCs. A decrease in inter interval events (an increase in frequency) in sEPSCs is observed in **c.** delay cells exposed to BDNF, which was statistically significant ($P < 0.01$; KS test) compared to neurons exposed to only LM11A-31, likewise **d.** in GAD67-EGFP neurons exposed to LM11A-31+BDNF, IEI of sEPSCs decreases (increase in frequency) and was statistically significant ($P < 0.001$; KS test). **e** and **f** are mean amplitude plots of **a** and **b** above. **g** and **h** are also mean IEI plots of **c** and **d** above. Data for all plots were obtained from the first 100 events following the first minute of recording from neurons both in the absence and presence of BDNF.

Effects of LM11A-31 (p75 antagonist) on BDNF responses

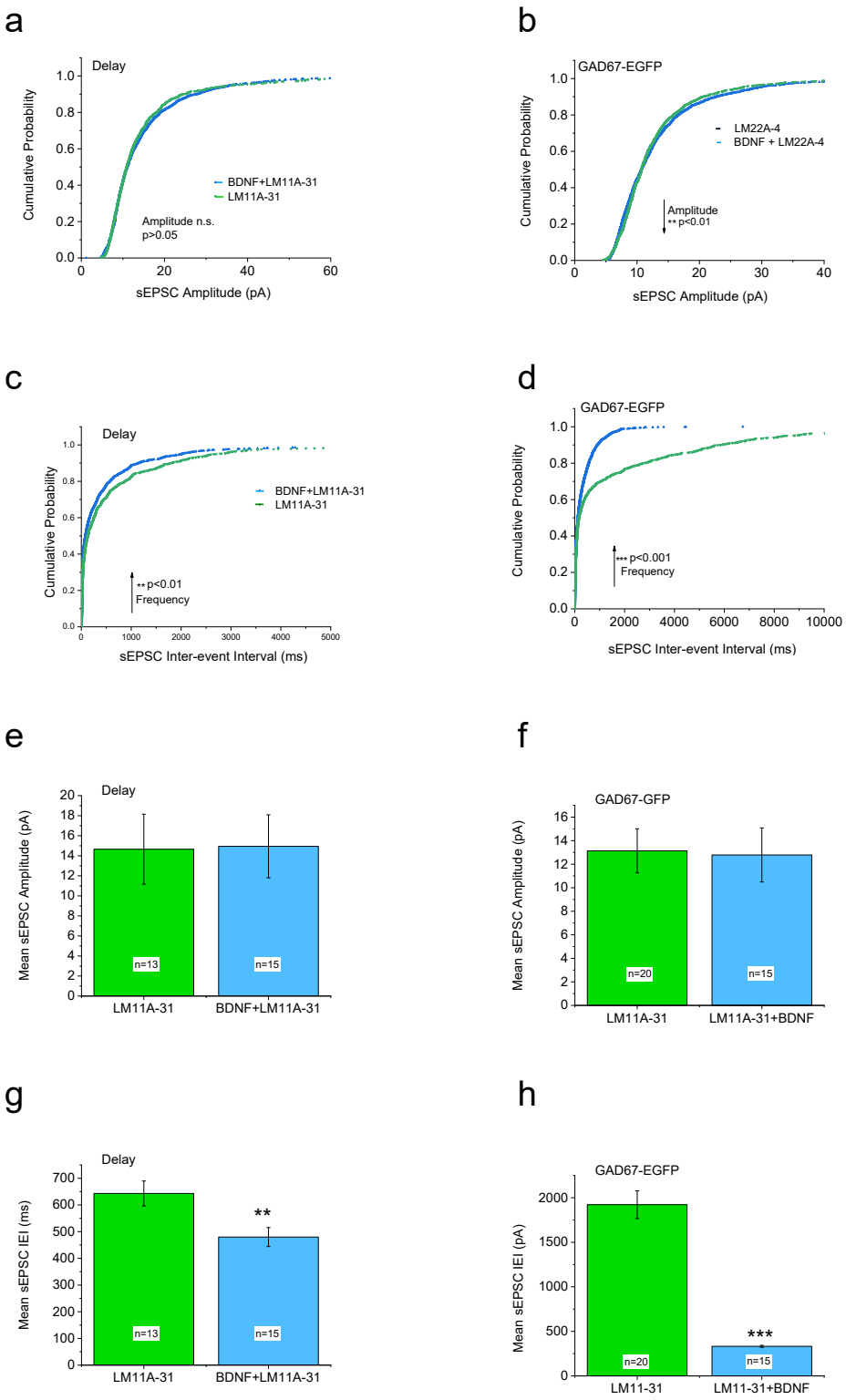
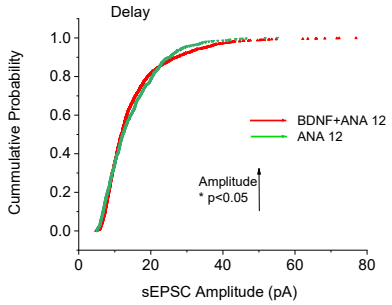


Figure 5.5

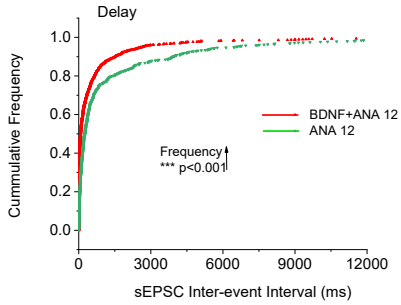
Figure 5.6. Effects of ANA-12 on BDNF responses in delay neurons in DMOTCs. **a** and **b**, Cumulative probability plots of amplitudes and IEI respectively of sEPSCs obtained from **a.** BDNF + ANA-12 exposed neurons (red) are statistically different from those obtained from neurons exposed to ANA-12 only (green) in delay cells ($P < 0.05$; KS test). **b.** cumulative distribution plot of inter-event intervals for delay neurons in DMOTCs. A decrease in inter interval events (an increase in frequency) in sEPSCs is observed in delay cells exposed to ANA-12, which was statistically significant ($P < 0.001$; KS test) compared to neurons exposed to only ANA-12. **c** and **d** are mean amplitude and IEI plots of **a** and **b** respectively.

Effects of ANA-12 (TrkB Antagonist) on BDNF Responses

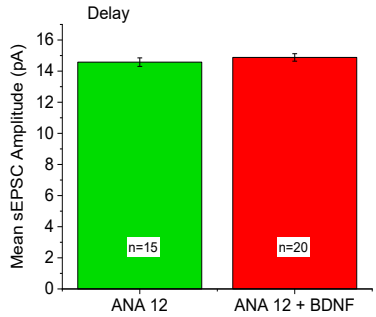
a



b



c



d

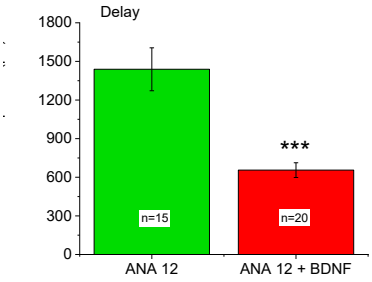


Figure 5.6

Figure 5.7. Schematic diagram showing a summary of spinal Trk B and p75 receptor involvement in central sensitization. In **delay cells a.** BDNF and LM22A-4 bind to Trk B leading to an increase in sEPSC frequency, indicative of a presynaptic mechanism. Further proof of the involvement of Trk B activation is seen when the increase in sEPSC amplitude observed in BDNF exposed delay neurons is blocked by the Trk B antagonist, ANA-12. In addition, the Trk B partial agonist also mimics the effect of BDNF seen on delay neurons by increasing the amplitude of sEPSCs. The involvement of p75 receptor mechanisms can not be discounted as proBDNF causes a similar increase in sEPSC amplitude. In **GAD67-EGFP neurons b.** LM22A-4 reduces sEPSC frequency implying the involvement of TrkB activation, however BDNF on its own has a weak effect on increasing sEPSC frequency. In a similar fashion proBDNF also decreases sEPSC frequency, implying a possible p75 involvement. Similar to its effects on delay neurons, LM22A-4 increases sEPSC amplitude in **GAD67-EGFP neurons** indicative of possible TrkB involvement. Once again, the possible involvement of p75 can not be discounted as proBDNF decreases the amplitude of sEPSCs.

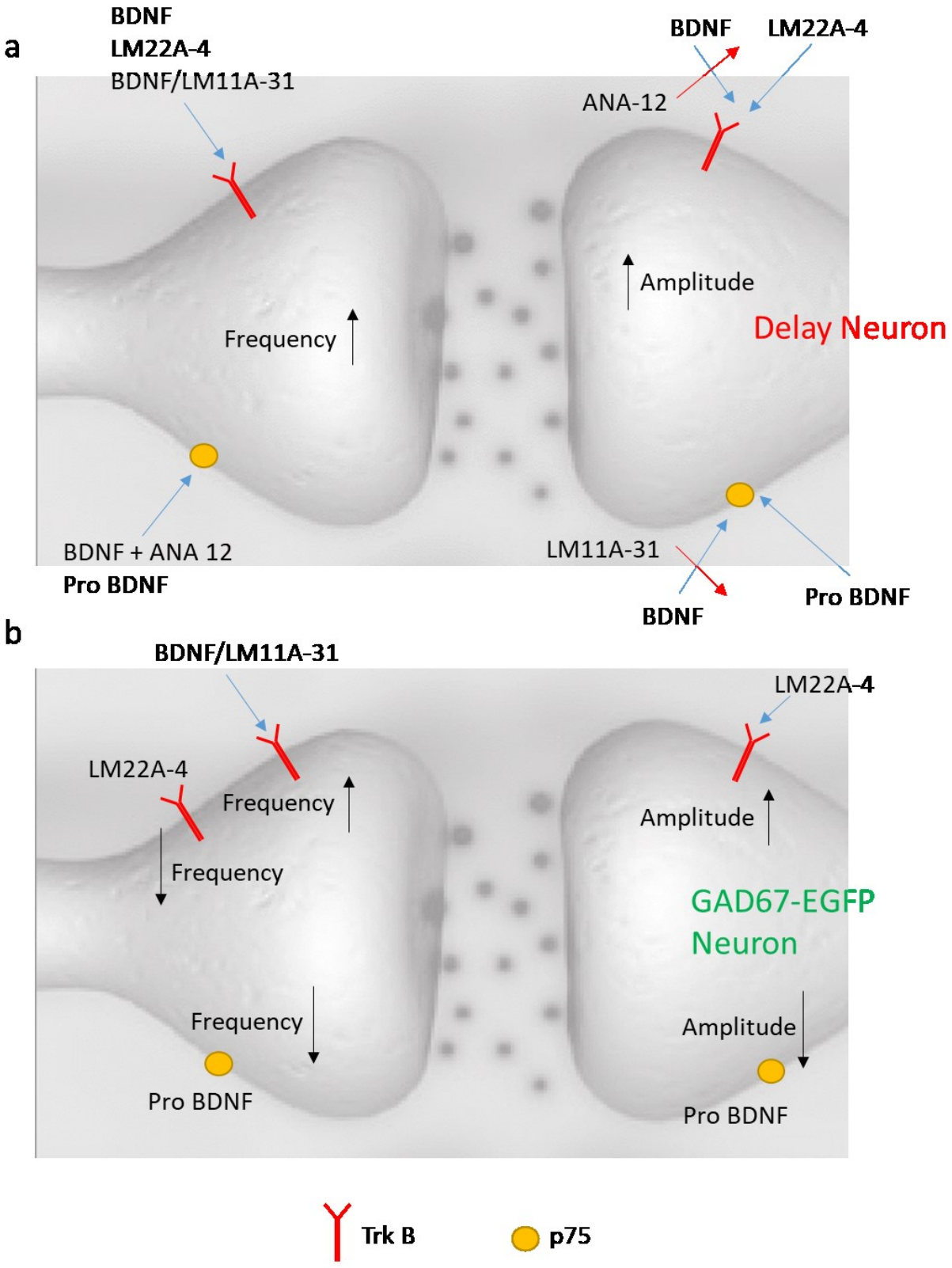

a
BDNF
LM22A-4
BDNF/LM11A-31
BDNF
LM22A-4
ANA-12
Amplitude
Frequency
Delay Neuron
BDNF + ANA 12
Pro BDNF
LM11A-31
BDNF
Pro BDNF
b
BDNF/LM11A-31
LM22A-4
LM22A-4
Frequency
Amplitude
Frequency
GAD67-EGFP
Neuron
Frequency
Amplitude
Pro BDNF
Pro BDNF
Trk B
p75

Figure 5.7

Chapter 6

SPINAL ACTIONS OF CSF-1

ABSTRACT

Injury to the peripheral nervous system induces proliferation of microglia in the spinal cord, which can contribute to neuropathic pain conditions. Recently, it has been shown that CSF-1 released by injured sensory neurons is transported to the spinal cord to activate spinal CSF-1 receptors on microglia. I have previously shown in **chapter 5** that BDNF promotes an overall increase in spinal dorsal horn excitability in mice DMOTCs. However, there has been a debate as to whether CSF-1 activation of resident microglia in the spinal cord is responsible for the release of BDNF leading to neuropathic pain. In this chapter, I have shown using mice DMOTCs (developed in **chapter 4**) that CSF-1 increases synaptic excitation of delay firing excitatory neurons and decreases synaptic excitation of inhibitory GAD67-EGFP cells. Experiments using the BDNF binding protein Trkb-FC showed that the effects of CSF-1 are largely affected by BDNF mediated pathways on delay neurons and BDNF independent pathways on GAD67-EGFP neurons. Thus, BDNF release from spinal microglia as a result of CSF-1 activation may contribute to the overall increase in excitability by delay excitatory neurons.

Key Words

Colony stimulating factor (CSF-1), Neurotrophin binding protein,BDNF

6.1 Introduction

The role of microglia in the spinal cord after nerve injury has been examined in the superficial dorsal horn of the spinal cord by several authors that have studied a variety of nerve injury models that involve ligation or transection of the sciatic nerve (Wall, Devor et al. 1979; Seltzer, Dubner et al. 1990) or the peripheral branches of the sciatic nerve (Decosterd and Woolf 2000; Balasubramanyan, Stemkowski et al. 2006). Significant microglial activation has been observed to contribute directly to hypersensitivity in nociceptive afferent fibers (Keller, Beggs et al. 2007; Tsuda and Inoue 2016) after nerve injury. Originating from hematopoietic stem cells (Kierdorf, Erny et al. 2013), microglia accounts for about 5-20 % of glia in the central nervous system (Yamamoto, Kohsaka et al. 2012) and are often considered as resident macrophages (Nakajima and Kohsaka 2001). They therefore serve important physiological and pathological roles. Physiologically they act as sensors for a range of stimuli that threaten the normal homeostatic milieu such as ischemia, CNS dysfunction and infection (Tsuda, Inoue et al. 2005). Once the normal environment is disturbed in situations such as in pathological conditions, microglia accumulate, activate and proliferate within the affected lesion immediately and begins to surround and repair affected tissues. This proliferation peaks for a couple of days (Gehrmann and Banati 1995). The signal(s) for activating microglia in the models of neuropathic pain has been elusive for sometime, but recent evidence (Guan, Kuhn et al. 2016; Okubo, Yamanaka et al. 2016) indicates its upregulation in the dorsal root ganglia, after nerve injury, raising the strong involvement of the macrophage-colony stimulating factor-1 (MCSF-1) in activating or inducing microglia leading to the generation of neuropathic pain. In addition, they also showed that CSF-1 receptors were exclusive to spinal microglia and that by selectively ablating CSF-1 from sensory

neurons, there was reduced microglia activation. However, intrathecally injecting CSF-1 to such mice resulted in significant microglia activation, hence suggestive of the fact that binding of CSF-1 is necessary and sufficient for microglia activation leading to neuropathic pain.

Macrophage colony-stimulating factor-1 (MCSF-1) is a cytokine that binds to the CSF-1 receptor (CSF-1R) which is a type III receptor tyrosine kinase and involved in stimulating the survival, proliferation and differentiation of mononuclear phagocytes, as well as macrophages (Pixley and Stanley 2004). The existence of three types of CSFs are known; macrophage-colony stimulating factor (M-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) (Borycki, Lenormund et al. 1993; Han, Ramesh et al. 1996) [8–10], and each CSF binds to a specific receptor. In their recent work (Pixley and Stanley 2004; Guan, Kuhn et al. 2016) showed that upon "direct"-nerve injury, afferent neurons in the DRG upregulate the expression of CSF-1, transporting it to the spinal cord, where it activates microglial CSF1R and thus leading to the induction of neuropathic pain after peripheral nerve injury.

Although recent events have strongly implicated CSF-1 as the missing link activating microglia prior to the onset of neuropathic pain, it remains to be determined whether microglia activation by CSF-1 is responsible for the overall dorsal horn excitability reported in **chapter 5** (increase in excitatory synaptic drive to excitatory neurons and decrease drive to GAD67-EGFP neurons) and whether this effect is mediated via release of BDNF. This missing link is one issue I sought to investigate in this chapter of my thesis.

6.2 Methods

Please refer to **Chapter 2** for all procedures performed.

6.3 Results

6.3.1 Electrophysiological Effects of CSF-1 on Mice DMOTC

In their recent work (Guan, Kuhn et al. 2016) showed that injured peripheral dorsal root ganglion neurons (DRG neurons) that were ligated (tied) after nerve injury accumulated CSF-1 at the site of ligation which is suggestive of the fact that CSF-1 is transported along the injured axon and is transported to the spinal cord. This observation clearly indicates a target in the spinal cord to be reached and activated. The presence of CSF-1 receptors on microglia primarily serves as the target. If microglia activation is the instigator of BDNF release then similar effects of BDNF seen in **chapter 5,** effects of BDNF illustrated in **figure 5.2** should be replicated with the treatment of CSF-1. In delay cells, effects of CSF-1 on sEPSC amplitude are illustrated in **figure 6.1 a & c**, and effects on interevent interval (the reciprocal of frequency) are illustrated in f**igure 6.1 e, & g**. As illustrated, CSF-1 increased the amplitude and frequency of sEPSCs in 'delay' neurons (**figure 6.1 a & c, P < 0.001, KS test and one tail T-Test; e. with a P < 0.05, KS test & g. P < 0.001, one tail T test**) in a similar fashion to BDNF (**figures 5.2 a, c,e & g**).

By contrast, CSF-1 slightly decreased the amplitude of sEPSCs in 'GAD67-EGFP' neurons (**figure 6.2 a & c, P < 0.001, KS and one tail T-Test**) but reduced the frequency of these currents (**figure 6.2 e & g; P < 0.001, KS and one tail T-Test**) respectively. These effects are quite different from those of BDNF on GAD67-EGFP neurons where sEPSC amplitude is unchanged (figures **5.2 b & f**) and frequency is slightly increased (**figures 5.2 d & h**)

6.3.2 Effects of TrkB-FC on CSF-1 treated DMOTC`s

A key aspect behind this chapter is the hypothesis that CSF-1 release and activation of spinal microglia is behind BDNF release by microglia. If this is the case, then the mediating effects of BDNF seen in **chapter 5** should be blocked by the BDNF inhibiting protein, TrkB-FC. TrkB-FC prevented an increase in the effect on CSF-1 with reference to the amplitude of sEPSCs in 'delay' neurons (**figures 6.1 b & d, P > 0.05; KS test and one tail T-Test**). However, unexpectedly, in the presence of TrkB-FC, BDNF decreased the frequency of sEPSCs in 'delay' neurons (f**igures 6.1 f & h, P < 0.001, KS test respectively and one tail T-Test**).

In 'GAD67-EGFP' neurons, the presence of TrkB-FC, did not alter the effect of CSF-1. Thus amplitude of sEPSCs (**figure 6.2 b & d, P < 0.01; KS test and one tail T-Test**) were reduced as was their frequency (f**igures 6.2 f, P < 0.001, KS test; & h, P < 0.05, KS test and one tail T-Test**).

6.3.3 Electrophysiological Effects of TrkB-FC responses in DMOTC

To confirm the ability of TrkB-FC to sequester BDNF, its effects on BDNF induced action on delay neurons were examined. As illustrated, TrkB-FC blocked the effects of BDNF on sEPSCs in terms of both amplitude and frequency in delay neurons (**figure 6.3 b. P < 0.001; one tail T-Test and figure 6.3 d. P > 0.05, one tail T-Test respectively**). Note that figures 6.3 a and 6.3 b are illustrations of the same data presented previously in figures 5.2 a & c; BDNF increases both sEPSC amplitude and frequency.

6.4 Discussion

The present study demonstrates that CSF-1 also exerts similar effects to BDNF on synaptic activity on delay neurons but also affects GAD67-EGFP neurons which are relatively insensitive to BDNF.

The findings of the study are presented diagrammatically in **figure 6.4.**

6.4.1 Effect of CSF-1 on Amplitude of Delay Excitatory Neurons

Both BDNF and CSF-1 increased the amplitude of sEPSCs (**figures 5.2 a & 6.1 a)** which may be indicative of similar mechanisms. However, to test whether, CSF-1 works through BDNF mediated mechanisms, the use of Trk B-FC (a BDNF blocker), produced weaker effects of CSF-1 (**figure 6.1 b**). Further statistical analysis on the effects of both BDNF and CSF-1 on sEPSCs using t tests from averaged data (**figure 6.1 c**) further confirmed the effects of CSF-1 increasing the amplitude of sEPSCs in delay cells. Likewise using same statistical test (T tests) from average data (**figure 6.1 d**), the CSF-1 effects seen in (**figure 6.1 c**) was no longer present, hence Trk B-FC blocks CSF-1 increases in sEPSCs amplitude. These results suggest that the effects of CSF-1 on sEPSC amplitude involves the release of BDNF.

6.4.2 Effect of CSF-1 on Frequency of Delay Excitatory Neurons

CSF-1 increased frequency of sEPSCs (**figure 6.1 e**) in a similar fashion to BDNF effects (**figure 5.2 c**). The BDNF blocker, Trk B-FC was once again used to examine whether the effect of CSF-1 is mediated via BDNF dependent mechanisms. The results observed were rather

unexpected as in the presence of Trk B-FC, CSF-1 decreased rather than increase frequency (**figure 6.1 f**). This difference in the effect of CSF-1, in the presence of Trk B-FC is also clearly shown in the T-tests illustrated in **figures 6.1 g & h**. A possible interpretation of this finding is that CSF-1 may function through BDNF to increase sEPSC frequency, but when the BDNF dependent mechanism is blocked by Trk B-FC (**figures 6.1 f & h**), a separate BDNF independent action of CSF-1 is revealed that results in a decrease in sEPSC frequency.

6.4.3 Effect of CSF-1 on Amplitude of GAD67-EGFP Neurons

Although BDNF has little effect on sEPSC amplitude in GAD67-EGFP cells (**figures 5.2 b & f**), CSF-1 produced a clear depression of event amplitude (**figures 6.2 a & c**). However, this effect was not blocked by the presence of Trk B-FC (**figures 6.2 b & d**). These results suggest that CSF-1 reduced amplitude of sEPSCs in inhibitory neurons via a BDNF-independent mechanism.

6.4.4 Effect of CSF-1 on Frequency of GAD67-EGFP Neurons

CSF-1 reduced the frequency of sEPSCs in GAD67-EGFP cells (**figures 6.2 e & g**). However, this effect persisted in the presence of Trk B-FC (**figures 6.2 f & h**), thus indicating a BDNF independent mechanism for the effects of CSF-1 on sEPSC frequency on GAD67-EGFP cells.

6.4.5 Effect of Trk B-FC on BDNF responses in Delay Excitatory Neurons

The effect of BDNF increasing the amplitude and frequency of sEPSCs (**figure 5.2 a & c and re-plotted as figure 6.3 a & c)** are blocked by Trk B-FC (**figure 6.3 b & d**). These results thus reflect the sequestering nature of Trk B-FC in the effects of BDNF.

6.5 Conclusion

CSF-1 after peripheral nerve injury causes activation and proliferation of microglia which leads to the release of BDNF leading to the generation of neuropathic pain and increase in overall dorsal excitability in mice DMOTCs. The data adduced suggests the strong involvement of both BDNF and CSF-1 in causing an increase in dorsal horn excitability, and the likely mediating effects of BDNF release from activated microglia as a result of CSF-1 binding to cause an overall increase in dorsal horn excitability. CSF-1 therefore binds microglia causing the release of BDNF that acts on receptors on delay excitatory cells leading to increase in excitatory effects but may be acting on GAD67-EGFP neurons using a different signalling mechanism. Such independent signaling process might involve proinflammatory cytokines including tumour necrosis factor (TNFα) and interleukin 1 beta (IL1β). Hence, both BDNF dependent and BDNF independent processes may be involved in the actions of CSF-1.

Figure 6.1. Effects of CSF-1 and TrkB-FC on delay neurons in DMOTCs **a.** CSF-1 increases the amplitude of sEPSCs on Delay neurons. Cumulative probability plots of amplitudes of sEPSCs obtained from control neurons (black) are statistically different from those obtained from neurons exposed to CSF-1 (red) in delay cells ($P < 0.001$; KS test). **b.** TrkB-FC caused a weaker effect of CSF-1 on the amplitude of these currents ($P < 0.01$; KS test). c and d are mean amplitude plots of **a** and **b** above (**c.** one-tail T-test, $P < 0.001$, **d.** one-tail T-test, $P > 0.05$). **e** and **f** are cumulative distribution plot of inter-event intervals for delay s in DMOTCs. A decrease in inter interval events (reciprocal of frequency) in sEPSCs is observed in delay cells exposed to CSF-1 (similar to BDNF), which was statistically significant ($P < 0.05$; KS test), unexpectedly, in the presence of TrkB-FC, CSF-1 decreased the sEPSCs frequency ($P < 0.001$; KS test). **g** and **h** are mean IEI plots of **e** and **f** above (**g.** one-tail T-test, $P < 0.001$, **h.** one-tail T-test, $P < 0.001$). Data for all plots were obtained from the first 100 events following the first minute of recording from neurons both in the absence and presence of CSF and TrkB-FC.

CSF-1 on Delay Cells

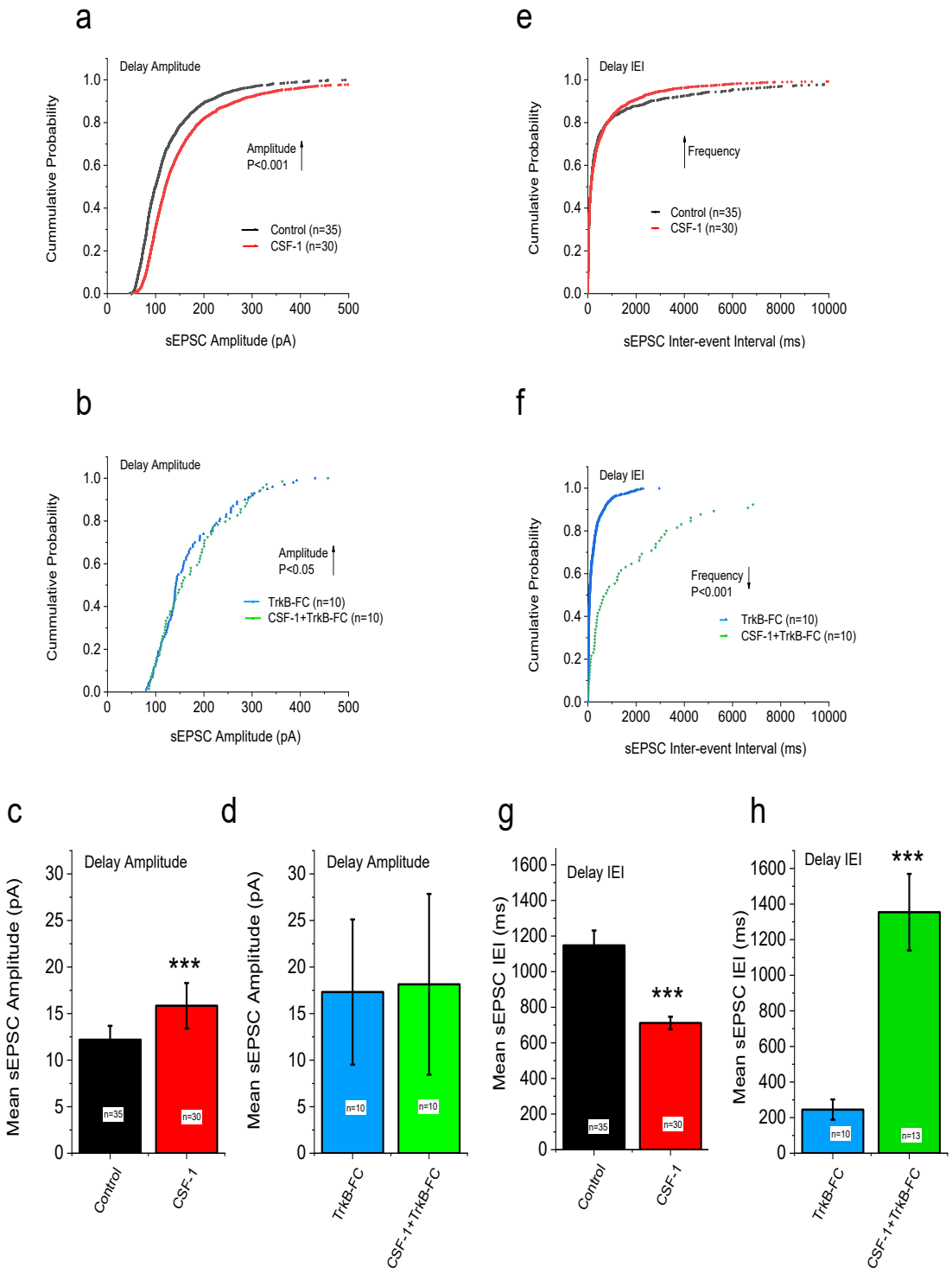


Figure 6.1

Figure 6.2. Effects of CSF-1 and TrkB-FC on GAD67-EGFP neurons in DMOTCs **a.** CSF-1 decreases the amplitude of sEPSCs on GAD67-EGFP neurons. Cumulative probability plots of amplitudes of sEPSCs obtained from control neurons (black) are statistically different from those obtained from neurons exposed to CSF-1 (red) in delay cells ($P < 0.001$; KS test). **b.** TrkB-FC did not block the effect of CSF-1 on the amplitude of these currents ($P < 0.001$; KS test). **c** and **d** are mean amplitude plots of **a** and **b** above (**c.** one-tail T-test, $P < 0.001$, **d.** one-tail T-test, $P < 0.001$). **e** and **f** are cumulative distribution plot of inter-event intervals for GAD67-EGFP neurons in DMOTCs. A clear increase in inter interval events (reciprocal of frequency) in sEPSCs is observed in GAD67-EGFP neurons exposed to CSF-1, which was statistically significant ($P < 0.001$; KS test). This effect was blocked in the presence of TrkB-FC, such that CSF-1 decreased the sEPSCs frequency ($P < 0.001$; KS test). **g** and **h** are mean IEI plots of **e** and **f** above (**g.** one-tail T-test, $P < 0.001$, **h.** one-tail T-test, $P < 0.05$). Data for all plots were obtained from the first 100 events following the first minute of recording from neurons both in the absence and presence of CSF and TrkB-FC.

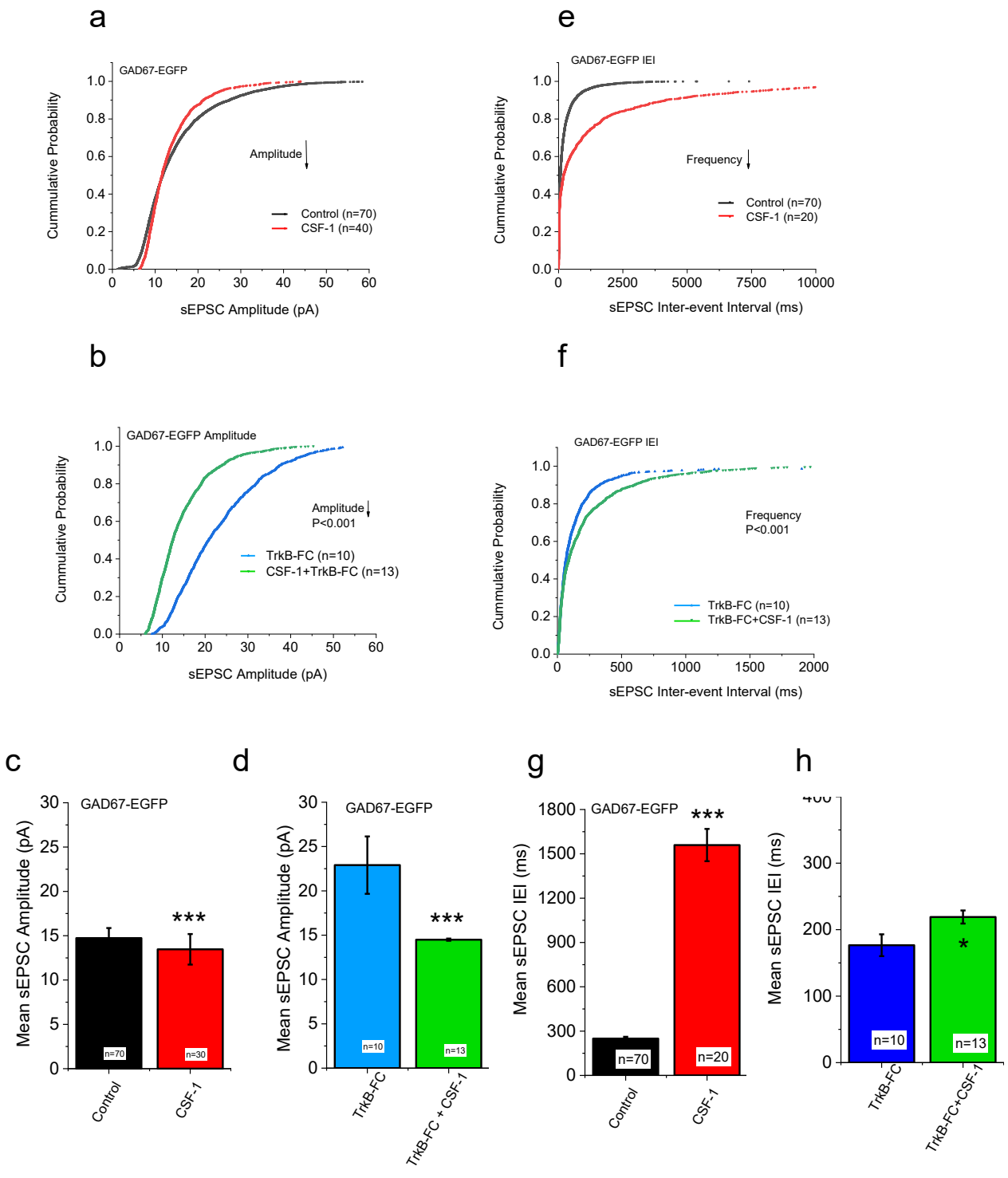

CSF-1 on GAD67-GFP Cells
a
GAD67-EGFP
Cummulative Probability
Amplitude
Control (n=70)
CSF-1 (n=40)
sEPSC Amplitude (pA)
e
GAD67-EGFP IEI
Cummulative Probability
Frequency
Control (n=70)
CSF-1 (n=20)
sEPSC Inter-event Interval (ms)
b
GAD67-EGFP Amplitude
Cummulative Probability
Amplitude
P<0.001
TrkB-FC (n=10)
CSF-1+TrkB-FC (n=13)
sEPSC Amplitude (pA)
f
GAD67-EGFP IEI
Cummulative Probability
Frequency
P<0.001
TrkB-FC (n=10)
TrkB-FC+CSF-1 (n=13)
sEPSC Inter-event Interval (ms)
c
GAD67-EGFP
Mean sEPSC Amplitude (pA)
***
n=70
n=30
Control
CSF-1
d
GAD67-EGFP
Mean sEPSC Amplitude (pA)
***
n=10
n=13
TrkB-FC
TrkB-FC + CSF-1
g
GAD67-EGFP
Mean sEPSC IEI (ms)
***
n=70
n=20
Control
CSF-1
h
Mean sEPSC IEI (ms)
*
n=10
n=13
TrkB-FC
TrkB-FC+CSF-1

Figure 6.2

Figure 6.3. Replot of **figure 5.2 e** as **figure 6.2 a** showing one tail, T-tests in **a**. BDNF increases the amplitude of sEPSCs: $P < 0.001$. This effect is blocked by Trkb-FC **b.** $P < 0.001$. Also a replot of **figure 5.2 g** as **figure 6.2 c** showing one tail, T-test in **c.** BDNF decreases inter event interval sEPSCs : $P < 0.001$. This effect is blocked by Trkb-FC **d.** $P > 0.05$. Data for all plots were obtained from the first 100 events following the first minute of recording from neurons both in the absence and presence of BDNF and TrkB-FC.

Delay Cells Effect of TrkB-FC on BDNF Responses

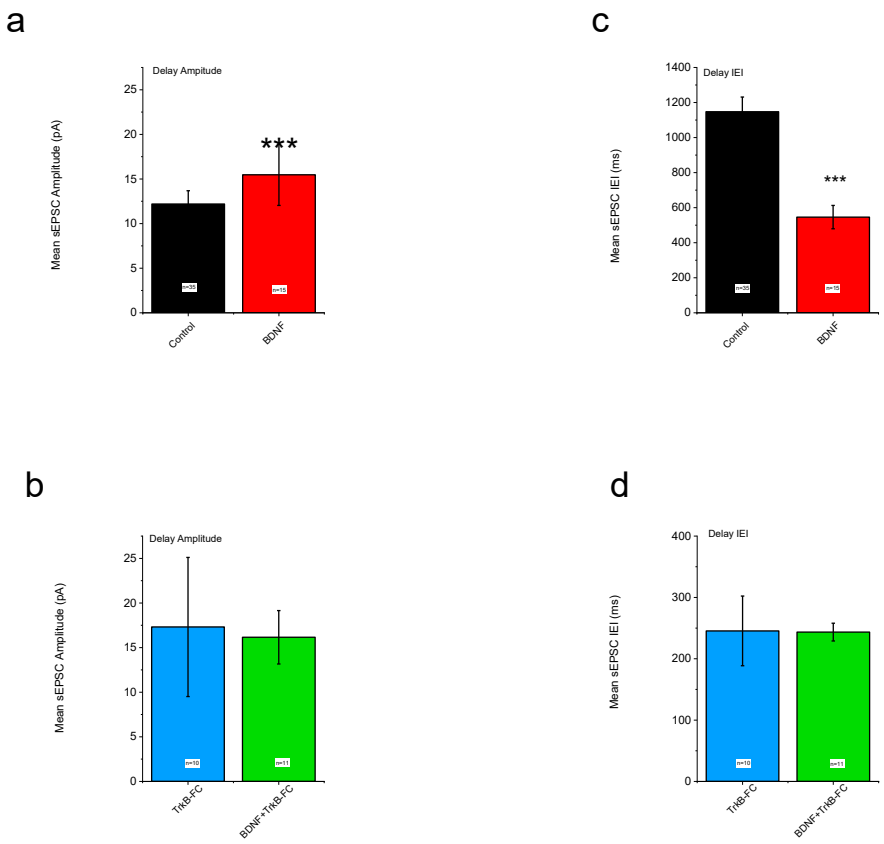


Figure 6.3

Figure 6.4. Schematic illustration showing that CSF-1 release from injured primary afferents acts on spinal microglia causing the release of BDNF from activated microglia. Released BDNF acts on receptors on delay neurons (potentially TrkB receptors) causing an increase in sEPSC amplitude and frequency in these cells. However, on GAD67-EGFP neurons, CSF-1 might be mediating its effects via BDNF independent pathways in decreasing the frequency and amplitude of sEPSC.

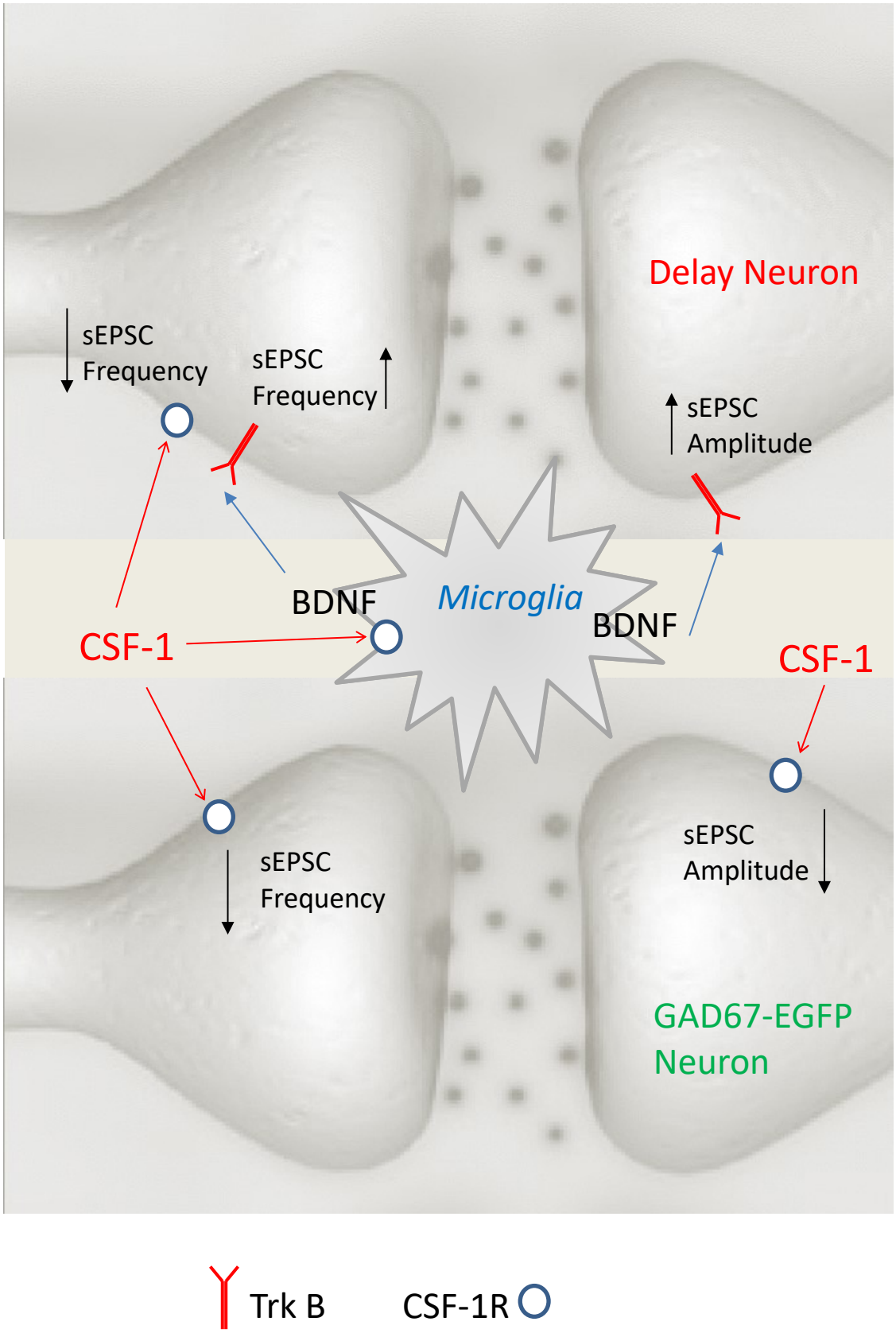

Delay Neuron
sEPSC
Frequency
sEPSC
Frequency
sEPSC
Amplitude
BDNF
Microglia
BDNF
CSF-1
CSF-1
sEPSC
Frequency
sEPSC
Amplitude
GAD67-EGFP
Neuron
Trk B
CSF-1R

Figure 6.4

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

This dissertation addressed two main hypotheses:

1. BDNF acts through different receptors and/or transduction mechanisms to increase release of excitatory transmitter onto excitatory neurons (using the Trk B signaling pathways) and to decrease release of excitatory transmitter onto inhibitory neurons (via the p75 signaling pathway).
2. The release of CSF-1 by primary afferents after nerve injury leading to the generation of neuropathic pain involves an excitatory action on the dorsal horn mediated via the release of BDNF.

In this dissertation I have shown that a key pain mediator, BDNF exerts an overall increase in dorsal horn excitability by engaging specific neurotrophin receptors on neuron specific neurons in the *substantia gelatinosa* of mouse DMOTC. Such an undertaking required long term culture studies of the spinal cord. I first characterised and established seven electrophysiological phenotypes in `Tamamaki` GAD67-EGFP mice from acute spinal cord slices. I proceeded to develop and maintain defined medium organotypic cultures from mice which enabled me to conduct long term BDNF studies. To the best of my understanding this could be the first in establishing and maintaining mice spinal cord DMOTC in a **serum free** medium for six weeks.

Prolonged BDNF release following peripheral nerve injury leads to an overall increase in dorsal horn excitability leading to central sentization and neuropathic pain. The release of BDNF also shows differential and neuron specific effects. Therefore by examining these effects and signalling mechanisms on both delay and GAD67-EGFP neurons in the *substantia gelatinosa*, we

can better understand the mechanistic progression of injury in the peripheral nerves and identify effective therapeutic targets for neuropathic pain treatment.

The main findings from this dissertation are:

1) Seven electrophysiological phenotypes characterize neurons in mouse *substantia Gelatinosa* (**chapter 3**).

2) Tonic, regular firing or phasic firing neurons are GABAergic whereas those with delay firing patterns are not (**chapters 3 & 4**).

3) Spinal cord slices from e12 embryos can be maintained in defined medium organotypic culture (DMOTC) for >6 weeks in a serum free medium (**chapter 4**).

4) The 7 electrophysiological phenotypes and the GABAergic nature of phasic and regular firing neurons persist in DMOTC (**chapter 4**).

5) Genetic modifications thus continue to be expressed when murine neurons develop *in vitro* in DMOTC (**chapter 4**).

6) Effect of BDNF on mice DMOTC are similar but not identical to those previously reported (**mice are different from rats**, **chapters 3 & 4**).

7) Increase in excitatory synaptic drive to delay excitatory neurons is via the TrkB/BDNF signalling cascade and its effects are largely both pre and post synaptic (**chapter 5**).

8) The slight increase in excitatory drive to GAD67-EGFP neurons are largely presynaptic and may involve the p75/BDNF cascade signalling pathway (**chapter 5**).

9) CSF-1 increases dorsal horn excitability both by BDNF dependent and BDNF independent mechanisms (**chapter 6**).

I will discuss **chapters 3 & 4** under the same broad themes because of similarities in their findings.

7.1 Seven Electrophysiological Phenotypes found in mouse acute spinal cord GAD67-EGFP slices are maintained in Mouse DMOTC.

Several researchers have classified spinal dorsal horn neurons in rats (Todd and Lewis 1986; Balasubramanyan, Stemkowski et al. 2006; Lu, Moran et al. 2006) and in mice (Yasaka, Tiong et al. 2010; Punnakkal, von Schoultz et al. 2014). With my long term goal of establishing mice DMOTC for prolonged studies I determined and classified seven electrophysiological phenotypes in the *substantia gelatinosa* (Boakye, Schmidt et al. 2018) of `Tamamaki` GAD67-EGFP mice (**chapter 1**). These seven cell types were not all the seen in both rat acute and DMOTC (Balasubramanyan, Stemkowski et al. 2006; Lu, Moran et al. 2006). My initial assumption was that the electrophysiological phenotypes of mouse *substantia gelatinosa* neurons in acutely isolated slices would be similar to those seen in rats (Balasubramanyan, Stemkowski et al. 2006), but this was not the case. Two neuronal phenotypes found in mice; regular firing and initial bursting (**figure 3.1 d & f**) are not found in rats (**chapters 1 & 2**) . These two neuronal types have been identified by (Abraira, Kuehn et al. 2017) in an extensive characterization of deeper dorsal horn neurons. The regular firing neuron by observation is likely GABAergic (**figure 3.1 l**) and undergo tonic firing after injection of consistent amounts of depolarizing current (**figure 3.1 d**).

It is therefore possible to classify the regular and tonic firing neurons as both GABAergic even though they both exhibit different firing patterns. Thus, the major finding that these two neuronal types (regular firing and initial bursting) were absent in rats highlighted the fact that mouse were clearly different from rats and hence the need for researchers to be mindful of such in their design of experiments in mice after preliminary data has been collected from rats.

Also consistent with previous findings, (Heinke, Ruscheweyh et al. 2004; Punnakkal, von Schoultz et al. 2014) the GAD67-EGFP+ phenotype was never associated with delay firing cells, and this observation was consistent in both acute slices and mice DMOTCs. This finding strengthens a long-held report that the delay firing cells are fundamentally different from the GABAergic GAD67-EGFP+ neurons. The delay cells are fundamentally excitatory and glutamatergic.

The slight disparity in the percentage of GAD67-EGFP+ phenotype seen in my data where this phenotype is seen most frequently in phasic, tonic and regular firing cells (**figure. 1 l**) and that of (Punnakkal, von Schoultz et al. 2014) where about 50% of initial bursting cells and single-spiking cells exhibit the GAD67-EGFP+ phenotype) could be due to the possibility that some GABAergic neurons are not identified by GAD67-EGFP+ expression either because the EGFP signal is too weak to be detected by the imaging system or that the neurons instead express GAD65 (Lorenzo, Magnussen et al. 2014). Such factors may therefore account for these variations.

From data presented in **chapters 3 and 4** respectively (**figures 3.1 & 4.1**) phasic, tonic and regular firing cells may be variations of a single predominantly GABAergic phenotype as regular firing cells can sometimes display a phasic or tonic discharge pattern depending on the initial

holding potential used. This may be one of the plausible explanations for some slight differences between data from acute (**chapter 3**) neurons compared with DMOTC (**chapter 4**). Data from acute slices, indicates that neurons identified as tonic, phasic and regular firing exhibit the GAD67-EGFP+ phenotype whereas in DMOTC, this phenotype associates most strongly with neurons identified as initial burst, phasic and regular firing. In a field where there are subtle differences in classification of spinal dorsal horn neurons (Todd and Lewis 1986; Heinke, Ruscheweyh et al. 2004; Cui, Li et al. 2008; Punnakkal, von Schoultz et al. 2014; Abraira, Kuehn et al. 2017) slightly different criteria used by different groups may also account for differences between my findings and theirs.

7.2 Preservation of electrophysiological phenotypes in DMOTC of mouse spinal cord and relationship to GAD67-EGFP expression

The viability of most of the experiments proposed in this dissertation hinged on the successful replication of results observed in **chapter 3** in **chapter 4**. It was such very refreshing to observe that all seven electrophysiological phenotypes seen in acute slices were present in mouse DMOTC (**chapter 4; figure 4.3 g**). Such a finding thus suggests that differentiation of firing patterns occurs normally in DMOTC even though the cultures are derived from pre-natal animals and grown in the absence of serum and NGF.

Additional comparisons also revealed that GAD67-EGFP expression in DMOTC associated with phasic and regular firing neurons in the same way as it was in acute slices (**figure 4.2 h**). The only slight difference between DMOTC and acute slices is that GAD67-EGFP is seen in fewer tonic

cells in culture. As shown in **figure 3.1 m** however, tonic cells can exhibit a phasic firing pattern or may be regarded as regular firing cells when the membrane potential is changed.

With regards to resting membrane potential, there was no difference between the rmp of neurons in acute slices and those in DMOTC. However, there was a trend toward an increase in spike width in most cell types when they were maintained in culture but this was only significant for tonic cells (**Tables 3.1 and 4.1**). It must be stated that an overall increase in spike width when rat spinal neurons are maintained in culture has been reported (Avossa, Rosato-Siri et al. 2003). It is therefore possible that growth conditions in the culture medium might be responsible but to the best of my observations it has no effect on our results.

It is also quite clear clear that there exists an obvious difference in the excitatory synaptic drive in neurons in DMOTC compared to those in acute slices (**figures 3.2 b & 4.3 b**) . In general, the frequency and amplitude of sEPSCs are greater in DMOTC (**chapter 4**). Previously, (Lu, Moran et al. 2006) have reported of a similar trend when rat spinal cord neurons are maintained in DMOTC and in organotypic cultures of rat hippocampus (De Simoni, Griesinger et al. 2003). Changes in synaptic connectivity in the cultures may reflect the establishment of new excitatory connections and perhaps the formation of autapses (Lu, Moran et al. 2006).

Oscillatory activity in organotypic cultures of spinal cord is clearly visualised as spontaneous, rhythmic fluctuations in intracellular calcium concentration(Lu, Moran et al. 2006) (Lu et al., 2006; Fabbro et al., 2007(Fabbro, Pastore et al. 2007). These clearly distinct oscillations seen in both acutely isolated slices and in DMOTC declined in activity as neurons aged and

matured. It can therefore be argued that the loss of activity is a sign of more established or mature nervous system ad as such characteristic of a developed circuitry.

7.3 Changes in neuron number in culture

The comparable cell death proportions seen in both acute slices (**chapter 3**; **figure 3.3 c**)and mirrored in DMOTC cultures (**chapter 4**; **figure 4.2 e**) could be ascribed to normal apoptotic processes. However, it could also be argued with respect to cultures that reduction in neuron numbers seen (in DMOTCs) is reflective of progressive deterioration of the culture itself. If this assumption were to be true, then, one should expect to see a continual decrease in neuronal population over a period of time. However, this was not the case as the number of neurons stabilized following week 2 in culture (**figure 4.2 e**) in a similar fashion to neurons in vivo (**figure 3.3 c**), suggesting that the loss may be attributed to normal apoptotic processes during development.

7.4 BDNF Promotes An Overall Increase In Dorsal Horn Excitability

Exposure of BDNF to dorsal horn neurons produced differential but distinct synaptic effects in delay and GAD67-EGFP neurons in mice DMOTC. These effects were characteristically comparable to previous observations in rat DMOTCs (Lu, Biggs et al. 2009; Lu, Colmers et al. 2009), albeit minor differences. GAD67-EGFP neurons received significantly less excitatory synaptic drive whereas delay excitatory neurons received increased synaptic drive following BDNF treatment.

It may be argued that these changes in synaptic transmission may have a subtle effect on dorsal horn excitability. However, the additional observation that BDNF treated slices produces an overall increase in intracellular calcium rise (**figure 5.1**) as a result of electrically stimulating the dorsal root entry zone is evident of the role BDNF contributes to the development of central sensitization leading to neuropathic pain.

Since all BDNF treated slices produced alteration in synaptic activity in disinct neuronal populations and thus produce an increase in dorsal horn excitability, it`s critical modulatory role as a mediator of central sensitization leading to neuropathic pain can not be ignored.

The next part of my discussions will be focusing on **chapter 5**. As complicated as this chapter might be, due to the potential interactions of TrkB and p75 NTR with BDNF causing the differential excitatory synaptic effects of BDNF on both GAD67-EGFP and delay neurons, it is prudent to state that the pharmacological interpretations stated here are by and large influenced by whether the drugs used are as specific as advertised in their interactions by the suppliers and distributors of such compound.

7.5 Differential Synaptic Effects of BDNF on delay neurons and GAD67-EGFP involves both TrkB and p75 Neurotrophin Receptors

If the release of BDNF results in a slight increase in excitatory synaptic effect on GAD67-EGFP neurons and significant increase in excitatory drive on delay neurons, then there should be differential mechanisms through which these effects are exerted. It has been suggested that Lamina II ‘delay’ neurons form excitatory connections with Lamina I neurons, which may include

projection neurons that signal to higher pain centres and that this enhanced activation of 'delay' neurons following BDNF treatment could lead to increased transmission of nociceptive information at the spinal level (Lu and Perl 2005). BDNF release arising from noxious stimulation has also been shown to enhance the recruitment and phosphorylation (Galan, Laird et al. 2004; Nagy, Al-Ayyan et al. 2004) of the GluR1 subunit of AMPA receptors (Wu, Len et al. 2004). With our current understanding of GluR1's association with nociceptive primary afferents and its distribution restricted to Lamina II (Lee, Bardoni et al. 2002; Nagy, Al-Ayyan et al. 2004), there is the possibility that if the expression of GluR1 is specific to primary afferent terminals that contact 'delay' neurons, this could represent a possible mechanism by which BDNF can differentially affect one neuron type in the dorsal horn and not another in contrast to it's differential effect on inhibitory GAD67-EGFP neurons.

BDNF exerts its actions both by TrkB and via the p75 neurotrophin receptors. Whereas it is generally accepted that Trk B activation produces positive actions on neuronal growth and sprouting, activation of p75 can sometimes produce effects that are deleterious and can even include apoptosis (Huang and Reichardt 2003). Drawing from this, my hypothesis was that the slight decrease in synaptic excitation seen in rats on GAD67-EGFP neurons was through the apoptotic neurotrophin receptor, p75, whereas the increase in excitatory synaptic drive in delay neurons was through the pro-survival neurotrophin receptor, Trk B.

Whilst the increase in frequecy and amplitude of sEPSCs in delay neurons in rats (Lu, Biggs et al. 2009) was replicated in mice (**figures 5.2 a,b,c & d**), the effects of BDNF on sEPSC amplitude in putative inhibitory neurons were slightly different. Whilst BDNF decreased both frequency and amplitude in rats (Lu, Biggs et al. 2009), it did not affect sEPSC amplitude in mice (**figures 5.2 b &**

h). This difference may in part reflect the difference in criteria used to identify inhibitory cells in rats by their tonic firing and islet morphology and mice by GAD67-EGFP expression. However, given the differences in the electrophysiological properties of rat and mice substantia gelatinosa discussed above, it is likely that the differential effects of BDNF in the two species reflect true physiological differences.

These could reflect differential expression of Trkb and p75 receptors at different pre and post synaptic locations in the two species.

The TrkB partial agonist, LM22A-4, produced similar but even stronger effects on delay neurons (**figures 5.4 a, c & 5.7**) compared to BDNF effects on delay cells (**figure 5.2 a, c & 5.7**). The Trk B partial agonist, LM22A-4 increased both the frequency and amplitude of sEPSCs on delay cells (**figure 5.4 a & c**) similar to the effects of BDNF (**figure 5.2 a & c**) on these cells. It could therefore be argued that since both are ligands of the Trk B receptor and the effects of activation of this receptor leads to similar synaptic excitatory effects, it could be deduced that BDNF binds to Trk B receptors on excitatory delay cells to cause an increase in excitatory synaptic drive. In the brain, BDNF has been shown to confer an excitatory synaptic effect on central cells such as CA1 pyramidal neurons specifically through the activation of Trk B signalling (Kafitz, Rose et al. 1999). Could similar excitatory signalling through Trk B receptors be prevalent in the spinal cord? Similarly, the blockade of the effect of BDNF on sEPSCs amplitude by the potent TrkB antagonist, ANA-12, further strengthens the argument for the involvement of Trk B signaling in the excitatory effects of BDNF on delay neurons. The findings from (Kafitz, Rose et al. 1999) and the present

data adduced clearly suggest the preferential dominance and expression of Trk B receptors on delay cells leading to binding of BDNf.

A slight increase excitatory drive to GAD67-EGFP neurons relative to that seen in delay cells with the effect of BDNF (**figures 5.2 d & h)** would be predictive of decreased release of inhibitory neurotransmitters within the dorsal horn network. In neuropathic pain, there is a global down-regulation in inhibitory tone as a result of impaired excitatory inputs onto inhibitory neurons (Leitner, Westerholz et al. 2013), it is therefore not surprising to see a relatively slight increase in effect on GAD67-EGFP neurons that had been treated with BDNF. The p75NTR is expressed widely in the developing nervous system. Sensory and sympathetic neurons, spinal cord and brainstem motoneurons all express p75NTR at some stage of their development (Friedman, Olson et al. 1991). It should be noted that in most cells, p75NTR expression is switched off at adult stages (Ibanez and Simi 2012), however, some few areas retain p75NTR expression at lower levels, such as the basal forebrain cholinergic neurons, sensory neurons and spinal cord motoneurons (Mobley, Woo et al. 1989; Cragnolini, Huang et al. 2009). During nerve injury p75 is upregulated (Obata, Katsura et al. 2006; Obata, Katsura et al. 2009). The p75 receptor can bind to all neurotrophins with similar affinity. However, under conditions of either reduced or absent Trk signaling, any neurotrophin binding to p75NTR promotes apoptosis signalling leading to cell death (Obata, Katsura et al. 2006). The ability of Trk and p75 receptors to present different binding sites and affinities to particular neurotrophins determines both their responsiveness and specificity (Chao 2003). Likewise the ratio of these receptors is important in dictating the type of interactions between p75 and Trk between different neurotrophins (Chao 2003). Could a similar mechanism exist in the spinal dorsal horn: whereby, there is a mechanistic

upregulation of p75 receptors relative to TrkB receptors on GAD67-EGFP neurons relative to delay neurons leading to the promotion of BDNF/ p75NTR signalling? Even though the p75 antagonist (LM11A-31) fails to block the effects of BDNF, the complex interactions of the receptor due to its relative upregulation in quantity compared to Trk receptors hence BDNF competing for active binding sites may be responsible for the result seen.

7.6 Cellular mechanisms of BDNF action.

Although a major goal of this dissertation was to explore the identity of receptors involved in the two actions of BDNF on delay excitatory neurons and on GAD67-EGFP neurons, the present data gathered could also help address the cellular mechanisms of BDNF'S actions on these two neuronal types. Even though I must concede the absence of mEPSC data, which allows for more precise differentiation between a pre- or postsynaptic site of action, sEPSCs analysis could also give us an idea of the pre and post synaptic effects of BDNF. Thus with an assumption (**chapter 5**) that changes in frequency are reflective of presynaptic effects and changes in amplitude reflecting post synaptic effects, in delay neurons, sEPSC amplitude is increased whereas in GAD67-EGFP neurons, sEPSC amplitude is unaffected. Changes in frequency is significant in both delay and GAD67-EGFP neurons (frequency increases). Thus, BDNF increased the amplitude and frequency of sEPSCs in delay neurons (**chapter 5**). In GAD67-EGFP neurons; sEPSC frequency increased slightly and amplitude stayed unchanged. Kolmogorov–Smirnov (KS) statistical analysis of the two distinct cell types of sEPSCs suggests that the actions of BDNF on synaptic transmission in delay neurons are both pre- and postsynaptic and most likely through BDNF/TrkB signalling, not discounting possible effects of p75 signaling in these effects. By

contrast, its actions on GAD67-EGFP neurons are more complex as 1) there was no change in amplitude reflective of no post synaptic effect and 2) the likely upregulation of p75NTR and possible fewer TrkB receptors present might have dictated the presynaptic effects seen (slight increase in sEPSCs frequency**, chapter 5; figure 5.2 d**). It is therefore likely that actions of BDNF on GAD67-EGFP neurons may be involved or confounded by the presence of both receptors, with a possible upregulation of p75 NTRs. It is quite evident that the differential effects of BDNF and opposing actions on different types of neuron, in this case delay and GAD67-EGFP neurons are mediated exclusively by TrkB and p75NTR and that these receptors are expressed to different extents after nerve injury. The present study demonstrates that per very subtle differences in effects, CSF-1 exerts very similar effects as BDNF release on synaptic activity from dorsal horn neurons in the *substantia gelatinosa*. However, data on whether CSF-1 release causes the up regulation of BDNF leading increase in overall dorsal horn excitability was not too conclusive.

In chapter 6, I sought to investigate and identify the instigator of BDNF upregulation in the spinal dorsal horn after nerve injury. Elegant work done by (Guan, Kuhn et al. 2016; Okubo, Yamanaka et al. 2016) showed that Macrophage-Colony Stimulating Factor Derived from Injured primary afferent induces proliferation of spinal microglia and neuropathic pain in rats. However, it has not been shown whether the release of CSF-1 by these injured neurons is responsible for the overall increase in dorsal horn excitability as a result of BDNF upregulation. Based on my second hypothesis that CSF-1 release is the main instigator for the release of BDNF from activated microglia, I sought to understand the synaptic role of CSF-1 on sEPSCs.

7.7 Effect of CSF-1 on substantia gelatinosa neurons.

Neuropathic pain arising as a result of peripheral nerve injury expands the spinal cord microglia population (Nakajima and Kohsaka 2001; Coull, Beggs et al. 2005; Tsuda, Inoue et al. 2005; Scholz and Woolf 2007; Tsuda and Inoue 2016). In recent times, it has been shown that peripheral nerve injury triggers the proliferation/activation of microglia in the spinal cord at least by day 3 (Jin, Zhuang et al. 2003; Okubo, Yamanaka et al. 2010). The transport of CSF-1 from injured neurons in the peripheral and central terminals of primary afferents leading to proliferation of macrophage in the periphery and microglia in the spinal cord after nerve injury has been recently established (Okubo, Yamanaka et al. 2016). Release of CSF-1 derived from injured-primary afferents binds to CSF-1 receptors on microglia causing the release of many proinflammatory molecules and neurotrophic factors, such as IL-1ß, IL-6, TNF-alpha and brain-derived neurotrophic factor (BDNF) (Trang, Beggs et al. 2011; Ji, Berta et al. 2013). The Smith lab (Lu, Ballanyi et al. 2007; Lu, Biggs et al. 2009; Lu, Colmers et al. 2009) and many others (Yajima, Narita et al. 2002; Garraway, Petruska et al. 2003; Garraway, Anderson et al. 2005; Merighi, Salio et al. 2008; Trang, Beggs et al. 2011) have implicated BDNF as a key modulator in the onset of neuropathic pain. In this study the excitatory effects of BDNF on sEPSCs in delay excitatory neurons are similarly replicated by CSF-1 treatment on mouse delay excitatory neurons. However, the effects of BDNF and CSF-1 on GAD67-EGFP neurons are both opposite to each other. The interpretation of such findings would be discussed below.

7.8 Is the effect of CSF-1 mediated by BDNF release?

The Smith lab (Lu, Biggs et al. 2009) and several others (Yajima, Narita et al. 2002; Garraway, Petruska et al. 2003; Coull, Beggs et al. 2005) have highlighted the immense role BDNF release contributes to overall dorsal horn excitability. The excitatory effects of CSF-1 release on sEPSCs in delay neurons (increases both frequency and amplitude of sEPSCs) if mediated by the neurotrophin BDNF should be attenuated by the application of the TrkB blocker, Trkb-FC. TrkB-FC binds and inactivates all neurotrophins that interact with TrkB receptors (Banfield, Naylor et al. 2001). The blocker weakly attenuated the amplitude of sEPSCS from delay neurons but unexpectedly reduced the frequency of sEPSC. Such an unexpected finding may be explained by the fact that CSF-1 may function through BDNF to increase sEPSC frequency, but when the BDNF dependent mechanism is blocked by Trk B-FC (**figures 6.1 f & h**). However, a separate BDNF independent action of CSF-1 is revealed that results in a decrease in sEPSC frequency. Thus, this observation is in tandem with the observation by (Biggs, Lu et al. 2012) which showed that the overall dorsal horn excitability associated with media from activated microglia is attenuated by the application of Trkb-D5 thus indicating the involvement of Trkb/BDNF signalling. Clearly, the opposite effects of CSF-1 on GAD67-EGFP neurons (**figures 6.2**) relative to the effects of BDNF on GAD67-EGFP neurons (**figures 5.2**) is indicate of an independent BDNF signalling mechanism in the effects of CSF-1 on GAD67-EGFP neurons. The involvement of BDNF signaling mechanism in the excitatory effects of CSF-1 on delay excitatory cells is buttressed by the results seen in **figure 6.3**, where the BDNF binding protein, Trk B-FC attenuates the amplitude and frequency of sEPSC.

To the best of my understanding, no group has reported of the synaptic effects of CSF-1 on *substantia gelatinosa* neurons. This study is the first to do so. The effect of CSF-1 on the amplitude and frequency of sEPSCs (increase) delay neurons and its block by the BDNF binding protein, Trk B-fc is evident of a potential BDNF mediating mechanism by delay neurons after CSF-1 release. However, the decrease in both frequency and amplitude of sEPSCs after CSF-1 release and the absence of effect on sEPSC amplitude and the reduction in frequency of sEPSCs in GAD67-EGFP neurons might be involve non BDNF mediated signalling mechanism. Also activation of microglia and their subsequent release of other inflammatory mediators might be responsible for these discrepancies in results. It can be said that in mice DMOTC with BDNF treatment, only BDNF can be inhibited by TrkB-FC and there aren`t any neurotropin or inflammatory factors to compound results, however, in CSF-1 treated slices with the BDNF blocker, the inhibition of BDNF does not prevent the other inflammatory mediators that were released as a result of microglia activation from exerting their effects on dorsal horn neurons. This is a possible explanation for the slight differences seen between both CSF-1 and BDNF treated slices incubated with the BDNF blocker.

Given that both CSF-1 and BDNF increase the amplitude and frequency of sEPSCs in delay firing neurons (**figure 6.1 & 6.2**), it fits into the idea that both CSF-1 and BDNF use similar signalling mechanisms in delay excitatory cells mediated by BDNF release (**BDNF dependent**).

Lastly, given the differential role of microglia in pain processing in both male and females (Mogil 2012; Sorge, Mapplebeck et al. 2015; Milian, Wong et al. 2017; Sorge and Totsch 2017) it must be noted that the inability to determine the sex of the foetal spinal cord at the point of dissection and culturing plays a limitation on the model used.

7.9 Final Conclusions

Mice and rats are different. Seven electrophysiological phenotypes can be identified in mice *substantia gelatinosa* whereas in rats six *substantia gelatinosa* neurons can be identified, thus indicative of the differences existing in both animals. Hence, the need for researchers to be mindful of such differences in the course of planning experiments. Mice DMOTCs retain all seven cell types identified in acute slices and in furtherance to the suitability and stability of DMOTCs, GAD67-EGFP phenotype is neither present in both DMOTC and acute slices.

The spinal effects of BDNF in rat DMOTC is by and large replicated in mice but with subtle differences and these effects and differences are modulated by complex Trk B and p75 NTR interactions with BDNF. The excitatory effects of BDNF on delay are most likely mediated by TrkB receptor activation with some p75 interactions whereas the slight increases in effect seen in GAD67-EGFP might be mediated by p75 receptor mechanisms.

CSF-1 release by injured neurons mimics the synaptic effects of BDNF in delay neurons, thus engaging BDNF mediated signalling pathways whereas GAD67-EGFP neurons might be engaging BDNF independent signalling such as the release of proinflammatory cytokines including TNFα and IL1β.

The goal of this dissertation has by and large been achieved, hence by establishing and identifying the probable mechanism through which BDNF release leads to neuropathic pain, we can begin to have a better understanding of the aetiology of this disease in the context of BDNF upregulation.

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CHAPTER 1

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