University of Alberta

Electrophysiological Signature of Neuropathic Pain

by

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To my dear husband,

Thank you for loving me, treasuring me and making me the happiest wife.

To my dear little boy,

Thank you for being such an angel and being the sweetest man in my life.

To Daddy and Mommy,

Thank you for loving me, supporting me and making me the luckiest daughter!

ABSTRACT

Neuropathic pain afflicts 1.5-3% of the general population. It can be initiated by traumatic nerve injuries or diseases such as diabetic or post-herpetic neuropathy. Neuropathic pain is often initiated by abnormal spontaneous activity in sensory neurons and this provokes increased excitability of neurons in the dorsal horn of the spinal cord in a process known as “central sensitization”. However, different types of peripheral nerve injury may send different signals to the spinal cord. Therefore, electrophysiological and pharmacological methods were used to examine the properties of interneurons in *substantia gelatinosa* of rat spinal dorsal horn in two common neuropathic pain models: sciatic nerve axotomy and chronic constriction injury (CCI).

Axotomy increased synaptic excitation of putative excitatory neurons and decreased synaptic excitation of putative inhibitory neurons. Axotomy produced similar but weaker changes than CCI in electrophysiological properties of *substantia gelatinosa* neurons.

Axotomy reduced the functional expression of voltage gated Ca^{2+} channels and reduced expression presynaptic Ca^{2+} permeable AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors on primary afferent terminals but CCI did not. Whereas CCI decreased the proportion of Ca^{2+} permeable AMPA receptors on the cell bodies of tonic firing putative inhibitory

neurons, axotomy did not. Whereas CCI increased the proportion of Ca^{2+} permeable AMPA receptors in delay firing putative excitatory neurons, axotomy did not.

The changes in excitatory synaptic transmission produced by both axotomy and CCI likely contribute to the central sensitization that underlies the generation of neuropathic pain. The tendency for CCI to generate more profound changes in the dorsal horn than axotomy may relate to the stronger inflammatory response it produces and the probability that axotomy interrupts peripheral trophic support of presynaptic Ca^{2+} permeable AMPA receptors.

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AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ANOVA: analysis of variance

ATP: adenosine triphosphate

BDNF: brain-derived neurotrophic factor

CaMKII: calcium/calmodulin dependent protein kinase II

CCI: chronic constriction injury

CFA: complete Freund's adjuvant

CGRP: calcitonin-gene related peptide

CNQX: 6-cyano-7nitroquinoxaline-2,3-dione

CNS: central nervous system

CSF : cerebral spinal fluid

CVLM: caudal ventrolateral medulla

D-AP5 : D(-)-2-Amino-5-phosophonopentanoic acid

DRG: dorsal root ganglion

eEPSC: evoked excitatory postsynaptic current

ER: endoplasmic reticulum

GABA: □-aminobutyric acid

GAD: glutamic acid decarboxylase

GLYT: glycine transporter

GRIP: glutamate receptor interacting protein

HCS: home cage scan

IASP: International Association for the Study of Pain

IB4: isolectin B4

IEI: interevent interval

IL-1β : interleukin-1β

IL-6 : interleukin-6

IR-DIC : infrared differential interference contrast

I-V: current-voltage

KA: kainate

KCC2: K^+-Cl^- cotransporter 2

KS: Kolmogorov-Smirnov

LPB: lateral parabrachial area

LTP: long-term potentiation

MCM: microglial-conditioned medium

mEPSC/IPSC: miniature excitatory/inhibitory postsynaptic current

mGluR: metabotropic glutamate receptors

NGF: nerve growth factor

NK1: neurokinin 1

NK1R: neurokinin 1 receptor

NMDA: N-methyl-D-aspartate

NPY: neuropeptide Y

NSAIDS: non-steroidal anti-inflammatory drugs

NSF: N-ethylmaleimide-sensitive fusion protein

NSFA : nonstationary fluctuation analysis

PAG: periaqueductal grey matter

PBS: phosphate buffered saline

PICK1: protein interacting with C kinase 1

PKA: protein kinase A

PKC: protein kinase C

RMP: resting membrane potential

RVM: rostroventral medulla

sEPSC/IPSC: spontaneous excitatory/inhibitory postsynaptic current

SG: substantia gelatinosa

SMT: spinomesencephalic tract

SR95531: 4-[6-(imino-3-(4-methoxyphenyl) pyridazin-1-yl)] butanoic acid

SRT: spinoreticular tract

STT: spinothalamic tract

TARP: transmembrane AMPA receptor regulatory protein

TNF-α: tumor necrosis factor-α

TRPA1: transient receptor potential A1

TRPV1: transient receptor potential vanilloid 1

TTX: tetrodotoxin

VGAT: vesicular GABA transporter

VGLUT: vesicular glutamate transporter

Chapter 1 GENERAL INTRODUCTION

1.1 Definitions and biology of pain

Pain is defined by the International Association for the Study of Pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. Thus, pain is a complex experience involving not only transduction of noxious environmental stimuli but also emotional processing by the brain.

Although it is unpleasant, pain promotes avoidance of actual or impending threat of bodily harm. It also helps prevent further tissue damage as pain persists until the injured tissue has completely healed. In the pain process, peripheral nerves transmit information from the injured tissue to the spinal cord, which relays the location, intensity and quality of the damage to the higher centres. Spinal withdrawal reflexes also remove the endangered part of the body away from the stimulus to avoid further damage.

1.1.1 Acute pain

Acute pain is the immediate pain response or “first pain” carried primarily by Aδ-fibres. The pain sensation during the injury recovery process is referred to as “second pain” and is carried primarily by C-fibres. Because it alerts the brain of tissue damage and is essential for the survival of the individual, acute pain has been described as “good” pain (Iadarola and Caudle, 1997).

1.1.2 Chronic pain

Chronic pain is persistent long-lasting pain that may involve different signalling mechanisms from acute pain. Chronic pain associated with prolonged tissue damage helps to prevent further damage and should abate once the injury recovers. It therefore can also be regarded as “good pain” as it too provides a protective role. Other types of chronic pain persist after the original injury has healed or may be unrelated to any specific injury. If pain results from injury or disease of the somatosensory system, it is defined as neuropathic pain (Dostrovsky, 1999). Neuropathic pain is referred to as “bad pain” as it serves no obvious biological purpose (Iadarola and Caudle, 1997). This maladaptive “disease of pain” has a 1.5 - 3% prevalence within the general population and imposes a significant financial burden on the health care system. Because neuropathic pain can last for months or years, it greatly reduces the quality of life. It can result from direct damage to peripheral nerves (Kim and Chung, 1997), or as a result of diseases such as diabetic neuropathy (Schmader, 2002), HIV/AIDS neuropathy (Schifitto et al., 2002), post herpetic neuralgia and cancer (Vielhaber and Portenoy, 2002) or occasionally channelopathies such as Nav1.7 mutation that causes *paroxysmal extreme pain disorder* (PEPD) (Lampert et al., 2010). Amputation sometimes also induces chronic pain. This so-called

"phantom limb pain" results from activity of damaged nerves originally innervating the lost limb (Flor, 2002).

Symptoms of neuropathic pain include allodynia (pain resulting from innocuous stimuli), hyperalgesia (exaggerated pain response to noxious stimuli) and spontaneous pain.

Normal treatments of pain such as opioids or non-steroidal anti-inflammatory drugs (NSAIDs) are not very effective for chronic neuropathic pain. Gabapentin and pregabalin are commonly used to treat neuropathic pain, but these produce only a moderate benefit (about 30% pain relief) in 43% of the patients and a substantial benefit (about 50% pain relief) in only 31% of the patients (Moore et al., 2011). Opioids are only effective in 1/3 of the neuropathic pain patients (Gilron et al., 2005). Other pharmacological approaches to the management of neuropathic pain include anticonvulsants, antidepressants, topical treatments (lidocaine patch, capsaicin), and ketamine (Vranken, 2009). In view of the limited effectiveness of presently available treatments, there is an urgent need for new therapeutic approaches to neuropathic pain.

1.1.3 Nociception

While pain includes an emotional component, nociception is the physiological processing of noxious stimuli. Nociception involves transduction (noxious

information converted into an electrical signal), and transmission (signal modulated as passing through nervous system) (Millan, 1999). Experiments on pain using human subjects are practically challenging, subjective and ethically limited so laboratory animal models of pain are widely used. The measurements of nociception include reflex responses to heat, cold or mechanical stimuli; spontaneous behaviours like autotomy, guarding, licking or postural change as well as pain-related complex behaviours such as anxiety, disability and sleep disorder (Mogil, 2009). Classic behavioural studies include assessment of evoked withdrawal responses using von Frey hairs (Balasubramanyan et al., 2006;Chaplan et al., 1994), cold or hot plate (Bolcskei et al., 2010) and observation of innate behaviours (Matthies and Franklin, 1992). The reflex responses to noxious stimuli may not require cerebral processing of sensory intensity but result from activation and modulation of spinal and perhaps supraspinal circuits. The cerebral sites that mediate sensory, emotional, and motivational reactions to pain need not be engaged under these circumstances (Vierck et al., 2008). Animal models are therefore a convenient tool to study the nociceptive mechanisms under controlled conditions. However, repeated noxious stimuli, especially in the context of long term behavioral tests, likely activate learning processes which requires the participation of the higher centers and may involve an emotional component. Though the study of the emotional process is technically difficult, if there is a continuous avoidance response to

noxious stimuli, the animals' behaviour may reflect, at least to a certain extent, the experience of pain *per se*.

Recent studies have attempted to evaluate this emotional response to nociceptive stimuli as a true measure of pain *per se*. Mice and rats vocalize in the ultrasonic range in response to acutely painful injuries (Williams et al., 2008), but some investigations concluded that ultrasonic vocalizations could not be used as in index of chronic pain (Wallace et al., 2005;Jourdan et al., 2002). With technological advancements, photocell and video-tracking systems (like the automated behaviour recognition software "HomeCageScan" - HCS) have enabled operant measures. In addition, increasingly sophisticated video-based behavioural algorithms are being assessed for their utility in quantifying spontaneous pain related behaviours (Roughan et al., 2009).

There are also some conditioned behavioural tests that combine animal models with the place conditioning paradigm instead of the traditional administration of unpleasant physical stimuli. Conditioned place avoidance (aversion) and conditioned place preference are two major types of conditional behavioural tests. In conditioned place avoidance, aversive stimulation is given to identify pain-related aversive motivation (LaBuda and Fuchs, 2000). This method combines the aversive and non-aversive stimulation to alternate places to evaluate the pain sensitivity by avoidance of the preferred test location. For example, a sciatic injured rat receives mechanical stimulation of the injured foot

in the dark chamber and of the uninjured foot in the light chamber. In this case, if the stimulus presented to one paw is more aversive than that applied to the other paw, the location that is associated with stimulation of the more aversive paw should be avoided (LaBuda and Fuchs, 2000). Thus an animal that is hypersensitive to painful stimuli will tend to spend more time in the aversive environment of the light chamber.

In conditioned place preference, a pain relief reward is given to identify pain relief related preference (King et al., 2009;Qu et al., 2011). This testing method uses analgesic agents that are not rewarding in the absence of pain as the reward to investigate chronic pain in animals as well as to evaluate the analgesic effects of drugs. The conditioned place tests reflect the affective dimension of pain and is a useful tool to study mechanisms mediating ongoing chronic pain.

Thus, based on the latest research, behavioural nociception could be related to emotional pain processes to some extent and new technology will allow systematic assays for pain translation in animal models.

Another issue to mention is that the measured severity and extent of a response caused by nociceptive stimulation varies between individual animals depending on their emotional and psychological states. Thus, caution should be used when relating nociceptive behaviour to pain. Never the less, in general, pharmacological impairment of nociceptive behaviour in animals may well indicate potential analgesic efficacy

1.1.4 Central sensitization

Electrophysiological recording from the sciatic nerve fibres after axotomy and CCI nerve injury in the rat demonstrated spontaneous ectopic discharges from the region of injury (Chen and Devor, 1998;Wall and Devor, 1983). With time, this aberrant activity promotes an enduring increase in excitability of sensory circuits in the spinal dorsal horn which leads to the phenomenon of "central sensitization"(Woolf, 1983;Sandkuhler, 2009). Central sensitization results from changes in neurons and abnormal responsiveness of the nociceptive system. When neurons in the spinal dorsal horn are subject to central sensitization, they show increased spontaneous activity, reduced activation threshold and increased response to suprathreshold stimulation (Latremoliere and Woolf, 2009;Sandkuhler, 2009). It is generally accepted that central sensitization contributes to the onset and persistence of neuropathic pain (Campbell and Meyer, 2006;Sandkuhler, 2009).

Central sensitization has two major mechanisms contributing to the increased synaptic efficacy: 1) the cell surface expression and trafficking of receptors and channels in primary afferent and dorsal horn neurons and 2) post-translational processing of receptors and regulatory proteins which change the receptors' intrinsic functional properties. Unlike traditional long term potentiation (LTP) which is induced by intense, repeated and sustained noxious stimulus, synaptic

LTP in the pain pathway can be induced by natural, asynchronous and irregular, low-rate discharge patterns in nociceptive C-fibers under physiological conditions and in the presence of tonic pre- and postsynaptic inhibition (Ikeda et al., 2006). Depolarization of the neuronal membrane via activation of AMPA receptors by glutamate triggers release of Mg^{2+} so that NMDA receptors become available for activation. Some authors regard this process as essential for initiating central sensitization (Ma and Woolf, 1995). Entry of Ca^{2+} into the postsynaptic neurons is a key process for synaptic plasticity (Ikeda et al., 2006). Ca^{2+} enters the neuron via NMDA receptors and activates intracellular pathways that are essential for maintenance of central sensitization (South et al., 2003). Besides glutamate, several neuropeptides are released from the primary afferents, for example calcitonin-gene related peptide (CGRP) and substance P which are involved in the process of maintaining central sensitization (Levine et al., 1993). Neurotrophins such as brain-derived neurotrophic factor (BDNF), as well as cytokines such as tumor necrosis factor- α (TNF-α) released from glial cells in the dorsal horn can facilitate synaptic transmission by acting through tyrosine kinase type receptors (Kerr et al., 1999;Wieseler-Frank et al., 2004). The change of the intracellular pathways is also an important process in causing central sensitization. Activation of multiple serine/threonine and tyrosine kinase signalling cascades leads to phosphorylation of ionotropic glutamate receptors which increases synaptic efficacy by altering channel open time, removing Mg^{2+} blockade and promoting the trafficking of receptors to the synaptic membrane (Woolf, 2007).

In addition to central sensitization, peripheral sensitization is also thought to contribute to the etiology of neuropathic pain. This is restricted to the site of tissue injury and represents a reduction in the threshold and amplification in the responsiveness of nociceptors (Hucho and Levine, 2007). Peripheral sensitization requires ongoing peripheral pathology for its maintenance while central sensitization is no longer coupled to peripheral stimuli.

Neurotrophins are also important in mediating and maintaining central sensitization. Nerve growth factor (NGF) and BDNF are two neurotrophins that are especially important mediators and modulators of pain. The activation of neurotrophin trk (tropymyosine receptor kinase) receptors leads to dimerization of the receptor and phosphorylation of different residues so as to promote the activation of signalling pathways (Pezet and McMahon, 2006). The release of neurotrophins into the dorsal horn requires repeated high frequency bursts as may occur following peripheral nerve injury (Lever et al., 2001). Other than its classical role in promoting neuronal growth, NGF can regulate nociceptor gene expression and promote the sensitization and activation of nociceptor terminals in a transcription-independent way (Pezet and McMahon, 2006). BDNF is predominantly expressed in small diameter DRG neurons with trkA receptors and NGF stimulation can increase BDNF synthesis in these neurons (Michael et al., 1997). Other than DRG cell bodies, BDNF can also be produced by spinal microglia in the spinal cord following nerve injury (Ikeda et al., 2001). BDNF is

essential in sciatic nerve CCI induced central sensitization in *substantia gelatinosa* (Lu et al., 2009;Lu et al., 2007).

In addition to the excitatory spinal peptides CGRP and substance P, two other "neurotransmitter" peptides galanin and neuropeptide Y (NPY) are thought to play a role in the generation or modulation of neuropathic pain (Alier et al., 2008;Smith et al., 2007). Galanin is not normally expressed in sensory neurones but is upregulated after damage to sensory neuron axons, for example peripheral nerve axotomy, probably due to the loss of NGF (Pezet and McMahon, 2006). However, in other studies the upregulation of galanin is considered to have an analgesic effect and is different in different injury models (Sten Shi et al., 1999). Thus, the effect of galanin may be related to its effective concentration; the higher dose may have an analgesic effect while insufficient galanin upregulation results in allodynia (Sten Shi et al., 1999). NPY is greatly up-regulated in the DRG cell bodies and the central terminals of these neurons but may actually generate an analgesic effect by different regulation of excitatory and inhibitory neurotransmitter release via the activation of postsynaptic Y1 receptors and presynaptic Y2 receptors (Smith et al., 2007;Landry et al., 2000).

1.2 Pain pathways

Nociceptors are widely distributed in the skin, along vein walls, skeletal fibres and internal organs (Millan, 1999) so pain detection is well defined. Pain signals

start with activation of nociceptors by noxious stimuli, e.g. chemical, thermal or mechanical stimuli. These signals are conducted to primary afferent terminals which synapse on dorsal horn neurons of the spinal cord. Spinal cord neurons integrate the nociceptive information and either directly project to higher brain regions or synapse on to projection neurons whose axons proceed to the brain

1.2.1 Primary afferents

Primary afferent fibres can be divided into 3 broad categories based on their conduction velocities, the extent of myelination and threshold characteristics: Aβ fibres have large myelinated axons and fast conduction velocity and carry low-threshold mechanoreceptor or touch, pressure and proprioceptive information; Aδ fibres are finely myelinated with medium conduction velocity; C fibres have unmyelinated axons and slow conduction velocity. In the dorsal root ganglion (DRG), large diameter neurons are associated with Aβ fibres; medium diameter neurons are associated with Aδ fibres and small diameter neurons are associated with C fibres. Generally Aδ and C fibres are considered to be involved in nociceptive signal conduction. see review: (Julius and Basbaum, 2001).

Large myelinated Aβ axons classically were considered as low-threshold mechanoreceptors that respond to touch or hair movement. However it is now believed that under certain conditions, Aβ fibres can also carry noxious

information and Aβ fibre nociceptive neurons have similar termination patterns to thinly-myelinated nociceptive fibers in lamina II (Light and Perl, 2003).

Aδ fibre nociceptors are further divided into 3 categories based on the responses to mechanical and thermal stimuli: high threshold mechanoreceptive nociceptors which are sensitive to noxious mechanical stimuli; mechano-heat nociceptors which respond to noxious mechanical and heat stimuli and mechano-cold nociceptors which respond to noxious mechanical and cold stimuli (Raja et al., 1999). As mentioned above, due to the relatively fast conduction velocity, Aδ fibres are responsible for the quick response to potential tissue damage stimuli which is also considered as “first pain”. Aδ fibers mainly project to lamina I and V (Light and Perl, 1979).

C fibre nociceptors are polymodal (Bessou and Perl, 1969) and respond to a variety of noxious stimuli including mechanical, thermal and chemical stimuli (Dubner and Bennett, 1983). As already mentioned, they mediate a slower wave of pain referred as “second pain”. C fibres can be differentiated by means of peptide markers they express. One group contains neuropeptides (peptidergic) such as calcitonin-gene related peptide (CGRP) and substance P (Lawson et al., 1997) and the other group lacks peptides (non-peptidergic) but has fluoride-resistant acid phosphatase and thiamine monophosphatase activity. These neurons also bind to isolectin B4 (IB4 positive) (Stucky and Lewin, 1999) and express P2X3 receptors (Vulchanova et al., 1998). C fibres project to lamina I and

lamina II with the primary focus to lamina II (Sugiura et al., 1989). Non-peptidergic fibres innervate the skin (Taylor et al., 2009) and peptidergic fibres innervate various other tissues (Perry and Lawson, 1998).

1.2.2 Superficial dorsal horn

In an early study, the dorsal horn of the spinal cord was divided into a series of six parallel laminae based on differences in the size and packing density of neurons (Rexed, 1952). Laminae I and II are often referred to as the superficial dorsal horn and the main target for nociceptive primary afferents. There are interneurons and projection neurons in the superficial laminae. Interneurons can be divided into two categories: excitatory which use glutamate as neurotransmitter and inhibitory which use GABA or/and glycine as neurotransmitters. Inhibitory neurons can be identified with antibodies against vesicular GABA transporter (VGAT), glutamate decarboxylase (GAD) or the neuronal glycine transporter 2 (GLYT2) and excitatory neurons can be identified by the presence of vesicular glutamate transporters (VGLUTs) (Todd, 2010). Projection neurons are mostly located in lamina I with some in lamina III-VI. They have axons that cross the midline, travel rostrally in the contralateral white matter and terminate in brainstem and thalamic nuclei (Craig and Dostrovsky, 1999).

1.2.2.1 Lamina I neurons

Lamina I is also called the marginal zone. It contains local excitatory and inhibitory interneurons as well as projection neurons which contribute to the spinoreticular and spinothalamic pathways of the spinal cord. Lamina I receives input from mainly Aδ and C fibres (Craig and Dostrovsky, 1999).

Lamina I neurons were first categorized into 3 types, fusiform, pyramidal and multipolar, based on the shape of the somata and the principal dendritic origins (Lima and Coimbra, 1986). Grudt and Perl defined lamina I neurons as ventrolateral tract projection neurons and non-projection neurons based on their axon distribution. This was related to electrophysiological features as the projection neurons had direct Aδ fibre input and non-projection neurons received mono-synaptic C fibre connections (Grudt and Perl, 2002). They could also be classified on the basis of their firing properties, for example initial burst or phasic firing, single spike firing, tonic or bursting and delay onset or gap firing (Salter and Henry, 1991;Dougherty et al., 2005). Cell function can also be used as a criterion to categorize lamina I neurons as nociceptive-specific neurons, innocuous thermoreceptive-specific cells or polymodal nociceptive cells sensitive to noxious heat, pinch and cold (Christensen and Perl, 1970).

The main supraspinal targets for lamina I projection neurons are the caudal ventrolateral medulla (CVLM), the nucleus of the solitary tract (NTS), the lateral parabrachial area (LPB), the periaqueductal grey matter (PAG) and certain nuclei

in the thalamus (Gauriau and Bernard, 2004). Cells with strong or moderate NK1 receptor-immunostaining, which are associated with the development and maintenance of persistant pain (Nichols et al., 1999), were retrogradely labelled from LPB or CVLM and seldom projected to the PAG (Spike et al., 2003). Although most cells project to the contralateral side, some project bilaterally (Spike et al., 2003).

The neurokinin 1 receptor (NK1R) expressed by many lamina I neurons is the main target for substance P. Its expression is restricted to dorsal horn neurons activated by noxious stimuli (Salter and Henry, 1991;Todd et al., 2002). Around 80% of lamina I projection neurons in the rat show NK1R immunoreactivity, which is much higher than its expression level in interneurons (Littlewood et al., 1995).

Although interneurons are the major postsynaptic targets for primary afferents, there are direct synaptic connections between primary afferents and projection neurons. Projection neurons with NK1R expression in lamina I are innervated by peptidergic primary C fibres containing substance P (Todd et al., 2002). Paired recordings showed that glutamatergic vertical cells in lamina II innervated by Aδ fibres have synaptic connection to NK1R expressing lamina I projection neurons (Lu and Perl, 2005). Lamina I projection neurons without NK1R receive little direct synaptic input from primary afferents so their response to noxious stimuli is likely mediated by excitatory interneurons (Polgar et al., 2008a). (see Fig. 1.1)

Loss of inhibitory tone in lamina I neurons is important in the initiation and maintenance of neuropathic pain. BDNF released by activated microglial cells after peripheral nerve injury decreases expression of K^+-Cl^+ cotransporter2 (KCC2) in Lamina I neurons. This decreases Cl^- concentration gradient and reduces inhibition in these cells (Coull et al., 2003;Coull et al., 2005). Moreover, the blockade of glycine or GABA receptors by intrathecal injection of strychnine and bicuculline induces allodynia due to the removal of both inhibitory systems (Loomis et al., 2001). However, the contribution of GABA receptors to fast inhibitory synaptic transmission is greater than glycine receptors in the superficial dorsal horn whereas glycinergic inhibition appears to be more important as almost all deep dorsal horn neurons receive glycinergic drive and glycinergic mEPSCs have much larger amplitude and faster kinetics (Anderson et al., 2009).

1.2.2.2 Lamina II neurons

Lamina II of the spinal dorsal horn, the *substantia gelatinosa* (SG), is characterized by the paucity of myelinated axons and hence appears as translucent band below the marginal layer of the dorsal horn. This region is essential in processing incoming sensory information (Melzack and Wall, 1965). The SG population is dominated by glutamatergic excitatory interneurons. They form multiple synapses on postsynaptic neurons which could increase synaptic

excitability, provide the basis for synaptic plasticity and nociceptive processing (Santos et al., 2009). SG is the target for nociceptive primary afferents and receives primary afferent input predominantly from Aδ and C fibres (Light and Perl, 1979). The outer part of lamina II receives unmyelinated peptidergic C-fibre inputs while the inner part receives unmyelinated nonpeptidergic C- and myelinated Aδ-fibre inputs (Braz and Basbaum, 2009). It integrates noxious information from nociceptive fibres to modify the output projection neurons located in lamina I and deeper laminae of the dorsal horn (Todd and Koerber, 2005). (see Fig. 1.1)

Lamina II also contains inhibitory interneurons which are important in integrating nociceptive information with some making synaptic connections with lamina I projection neurons. GABAergic and glycinergic interneurons in lamina II receive regulatory inputs from the brain and other lamina. These inputs contribute to the modulation of nociceptive information because the sensory information from the periphery is subject to inhibitory modulation in the dorsal horn (Heinke et al., 2004;Todd and Spike, 1993). Removal of inhibition, *i.e.* blocking GABAergic or glycinergic innervation, leads to increased pain behaviour to innocuous stimulation (Yaksh, 1989;Sivilotti and Woolf, 1994) and also increases excitatory transmission between deep and superficial laminae (Baba et al., 2003). The blockade of GABA receptors does not affect fine primary afferent excitatory transmission but only the interneuron transmission (Baba et al., 2003). Thus the attenuation of GABA receptor mediated inhibitory tone in neuropathic pain

situation promotes the spread of excitatory transmission in *substantia gelatinosa* and the other laminae, which may explain allodynia (Baba et al., 2003). Facilitation of inhibition by agonists of $GABA_A$ receptors in the spinal dorsal horn attenuates neuropathic pain triggered by peripheral nerve injury (Di Lio et al., 2011). This again indicates the importance of inhibition in regulating nociceptive transmission.

1.2.2.2.1 Neuron categories

The neuronal organization of the dorsal horn is very complex. One of the major problems with interpreting the function of lamina II interneurons has been the lack of structural and functional classification scheme for the various types of neurons found there. In recent years, however, a considerable amount of progress has been made. Attempts were made to characterize neurons on the basis of their morphology (Bennett et al., 1980;Grudt and Perl, 2002;Heinke et al., 2004;Light et al., 1979;Melzack and Wall, 1965), firing patterns in response to current injection (Grudt and Perl, 2002;Heinke et al., 2004;Santos et al., 2007;Lopez-Garcia and King, 1994;Thomson et al., 1989) and expression of neurochemical markers(Todd and Spike, 1993). Though these studies were conducted in different animal species, similar phenotypes were described.

1.2.2.2.1.1 Morphology

Early studies used Golgi staining to define neuron morphology. This led to the definition of three types of neuron on the basis of dendritic and axonal projections: 1) Islet neurons with dendrites saggitally oriented in the full width of the lamina II and axon extending in the long axis of the layer; 2) stalked neurons with vertically oriented and fine stalk-like branches and dendritic spines, extending to marginal layers; 3) Spiny cells with extensive dendritic tree spanning rostrocaudally and mediolaterally and evenly distributed dendritic spines along their dendrites (Gobel, 1975;Gobel, 1978).

Grudt and Perl later used intracellular biocytin staining and identified five neuronal classes in lamina II by morphology based on dendritic arborizations, which are: islet cells with elongated dendritic trees >400µm in the rostro-caudal direction; vertical cells with vertical orientated wide dendrites spreading from lamina I to III and limited mediolateral spread; radial cells with dendrites extend in all directions; central cells with similar morphological characteristics to the islet cells but a shorter rostrocaudal dentritic spread, approximately 200µm and medial-lateral cells with much larger dendritic span in both the medio-lateral and the dorso-ventral planes than other types, distributing from lamina I to the superficial lamina III (Grudt and Perl, 2002) (Fig. 1.2).

Although different groups have used different criteria to define cell type, there are some overlaps. Islet cells are further divided into small islet cells that do not

contain GABA or glycine and large inhibitory islet cells based on their morphology and neurotransmitter content (Todd, 1988;Todd et al., 1996). Small islet cells with less extended dendritic tree are also called central cells and the definition of islet cells restricted to large inhibitory islet cells (Todd, 1988). Since islet and central neurons have dendritic trees along the same rostrocaudal direction, it is difficult to trace the dendritic tree projection in transverse slices of the spinal cord. Vertical cells have similar morphology description as stalked cells and spiny cells show likeness of dendritic spine extension to radial cells.

1.2.2.2.1.2 Electrophysiological properties

Previous work has shown that membrane properties, especially action potential firing patterns, vary in superficial dorsal horn neurons which could be used as a tool to categorize neurons. Generally the action potential firing patterns could fall into four types: 1) repetitive tonic firing pattern with low threshold; 2) single spike at the beginning or transient firing; 3) phasic firing with an initial burst followed by adaptation; 4) delayed onset of spikes (Thomson et al., 1989;Lopez-Garcia and King, 1994;Balasubramanyan et al., 2006;Grudt and Perl, 2002;Graham et al., 2004). The classification standard used in this thesis divides the firing patterns into 5 groups. The firing pattern that constantly changes with depolarization is classified into the irregular firing pattern group (see Fig 3.2). Delay tonic and delay irregular firing patterns shown in the figure are combined

together as delay group. The “reluctant” firing pattern may be different from the five patterns mentioned above. This refers to neurons which only rarely generate action potentials in response to a depolarizing stimulus (Graham et al., 2007). It is possible that “reluctant” firing cells correspond to transient neurons according to the terminology of Balasubramanyan et al (2006) and/or to non-firing cells which other investigators have excluded from their analysis. One possible explanation for that is reluctant firing cells have high threshold and only respond to the highest-intensity stimulation (Graham et al., 2007).

The tonic firing pattern was found to be generated by voltage-gated Na and delayed-rectifying K channels and stabilized by Ca-sensitive K channels (Melnick et al., 2004b). Single spike firing mechanism is not clear but it is possible that some transient voltage-dependent outward K current with slower activation and inactivation than A-current precludes the neuron from firing more than one action potential (Ruscheweyh and Sandkuhler, 2002). Phasic firing mechanism is similar to tonic firing neurons as Na and delay rectifier K channels contribute to the firing but reduced Na channel conductance determines the adaption phase (Melnick et al., 2004a). Both tonic and phasic firing neurons show very small transient type K (I_A) current (Melnick et al., 2004a;Melnick et al., 2004b). I_A currents control neuronal excitability by delaying the first action potential and reducing discharge frequency, which gives rise to delayed firing pattern (Yasaka et al., 2010;Ruscheweyh and Sandkuhler, 2002).

1.2.2.2.1.3 Summary of classification

Although there are different ways of classifying lamina II neurons, correlations between morphology, electrophysiological properties and neurotransmitter content are starting to emerge.

Islet cells often exhibit sustained repetitive action potentials (*i.e.* tonic firing) (Lu et al., 2009;Todd, 2010;Yasaka et al., 2010). Most tonic firing cells are GABAergic, *i.e.* inhibitory, among which over half of the cells belonged to the islet categary (Yasaka et al., 2010). Islet cells were also found to satisfy the morphology of inhibitory Golgi interneurons (Gobel, 1975) and in later studies they were found to be GABAergic, presumed to inhibitory (Lu and Perl, 2003;Todd and McKenzie, 1989). These cells receive excitatory synaptic inputs from C fibres and polysynaptic inhibitory inputs from Aδ fibres (Yasaka et al., 2007).

Although the majority of radial cells are glycinergic and sometimes GABAergic too (Yasaka et al., 2007), those with A-current induced delayed firing pattern were all found to be glutamatergic (Yasaka et al., 2010). Vertical neurons and radial cells display delayed action potential firing (Todd, 2010;Yasaka et al., 2010;Lu et al., 2009). Vertical delay neurons have only excitatory synaptic projections to postsynaptic neurons and thus classified as excitatory neurons (Lu and Perl, 2005). Central neurons release glutamate when activated so they are also considered excitatory neurons (Lu and Perl, 2005;Lu and Perl, 2003).

Although the distinction is not absolute, there are several cell morphologies and action potential firing patterns that show consistent associations. Thus in all, most excitatory cells have delay firing pattern, while tonic firing combined with islet morphology represents inhibitory neurons (Yasaka et al., 2010;Todd, 2010;Zhang and Dougherty, 2011;Balasubramanyan et al., 2006). However, it must be noted that some neurons do not fit in an already recognized category and show atypical properties (Grudt and Perl, 2002;Todd, 1988;Lu and Perl, 2003;Lu and Perl, 2005;Todd, 2010;Yasaka et al., 2010).

1.2.3 Glial cells

A variety of conditions may generate neuropathic pain, but one common underlying mechanism is the inflammation at the site of the damaged or affected nerves. For example, peripheral nerve injury causes a massive release of immunoactive substances, such as cytokines and neurotrophic factors, at the site of injury, which can initiate a systemic immune response and cause activation of glial cells in the spinal cord and brain (Sandkuhler, 2009).

Neurons and glial cells have close cellular and molecular interactions in the nervous system. Classically glial cells were suggested to provide nutrition, protection and insulation to neurons (Bacci et al., 1999). Now it is known that they are also involved in synthesis and removal of neurotransmitters and the modulation of neurotransmission at the synaptic level (Bradesi, 2010). They also

appear to be involved in the direct regulation of brain blood flow as a result of direct interactions with blood vessels (Moore and Cao, 2008;Attwell et al., 2010).

Glial cells are divided into microglia and macroglia. The latter includes astrocytes and oligodendrocytes. Microglia and astrocytes play an important role in the development and maintenance of neuropathic pain (Coull et al., 2005;Garrison et al., 1991;McMahon et al., 2005). After peripheral injury, peripheral neurons transmit pain signals to the spinal cord by releasing neurotransmitters such as glutamate and ATP, as well as neuropeptides such as substance P. These substances alter the level of activation of microglia and further sensitize postsynaptic neurons (Vallejo et al., 2010) (Fig 1.3A). The extent and duration of changes in microglia and astrocytes is greater after peripheral nerve injury than in peripheral inflammation (Latremoliere and Woolf, 2009).

Normally microglia are in a resting state and have small soma with fine-branched processes. When they are activated, microglia undergo morphological and functional changes including mobilization and proliferation to facilitate isolation of injured cells (Davalos et al., 2005). Activated microglia produce and release trophic factors, neurotransmitters, cytokines and reactive oxygen species (Watkins and Maier, 2002). Although microglia are distributed widely in the CNS, only microglia in the spinal cord are activated after peripheral nerve injury (Zhang et al., 2008) and the activation is limited to predominantly ipsilateral dorsal horn (Jergova and Cizkova, 2007;Chen et al., 2009) (see Fig. 3.1). Activated

microglia also display changes in surface markers and membrane bound proteins including complement receptor3, involved in phagocytosis; toll-like receptor 4, involved in pathogen recognition; CD44, involved with adhesion and migration and up-regulation of MHC I and II, involved in antigen presentation to T cells (Vallejo et al., 2010). The release of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) from activated microglia lead to astrocyte activation (Watkins and Maier, 2003) (Fig. 1.3A). In addition, the release of BDNF and other mediators increases excitatory transmission and decreases inhibitory transmission, which lead to central sensitization (Lu et al., 2009;Coull et al., 2005) (Fig. 1.3A).

Astrocytes make up the majority of glial cells in the CNS. They are phagocytic cells which are important in neuronal development and establishing and maintaining the blood brain barrier (Cahoy et al., 2008). They play a critical role in synaptic transmission as they take up extracellular glutamate and synthesize it from glucose (McMahon et al., 2005). Astrocytes are also involved in the synthesis and catabolism of GABA (Lee et al., 2011). Astrocytes become activated after peripheral nerve injury but with a slower onset and prolonged time course than microglia (Fig. 1.3B). They may therefore be necessary for maintenance of neuropathic pain (Zhuang et al., 2005). There are basal levels of cytokine receptors expressed on resting astrocytes. IL-1β released from activated microglia binds to interleukin-1 receptors and induces the activation of the astrocytes (Watkins and Maier, 2003). Activated astrocytes undergo hypertrophy

and proliferation and increase expression of glial fibrillary acidic protein, an astrocyte-specific activation marker (Garrison et al., 1991). A positive feedback cycle is initiated in which astrocytes release inflammatory mediators in turn to activate other astrocytes (Sama et al., 2008). Astrocyte activation is combined with a decrease in microglia activity level over time (Tanga et al., 2007), which also confirms the idea of microglia responsible for initiation and astrocytes for maintenance of neuropathic pain (Fig. 1.3B).

1.2.4 Supraspinal Tracts and Brain Centers

Nociceptive processing and the initiation of pain do not only include the ascending pathways from the spinal cord to the brainstem, thalamus and hypothalamus but also includes modulation from the higher centers to the spinal cord; the descending pathway.

The major ascending pathways of central nociceptive processing include direct projection to the brain via the spinoreticular tract (SRT) and the spinomesencephalic tract (SMT) which project to the medulla and brainstem and the spinothalamic tract (STT) which projects to the thalamus. The spinothalamic tract is divided into the lateral spinothalamic tract terminating in the ventroposterolateral (VPL) neucleus of the thalamus, and the medial spinothalamic tract terminating in the ventroposteromedial (VPM) nucleus of the thalamus (Craig and Dostrovsky, 1999).

The stimulation of midline brainstem center produced analgesia was the first evidence for the excistence of descending pain-modulating pathways (Mayer et al., 1971). The midbrain periaqueductal grey (PAG) was found to control nociceptive transmission at the level of the spinal cord as part of a CNS circuit (Mayer and Price, 1976;Reynolds, 1969). PAG and the adjacent *nucleus cuneiformis* are the major source of inputs to the rostroventral medulla (RVM) and RVM is the major brainstem souce of axons projecting to the spinal cord dorsal horn so the PAG-RVM connection is critical for pain modulation (Fields and Basbaum, 1999). Stimulation of the RVM can either enhance or inhibit the responses of wide dynamic neurons and nociceptor-specific spinal neurons in responses to noxious stimuli (Urban and Gebhart, 1999). RVM may be involved in the maintenance of chronic pain as pain facilitatory neurons of the RVM are activated by the nociceptive inputs to promote further nociception (Gebhart, 2004). Descending inhibitory influences are tonically active and descend to ipsilateral spinal cord dorsalateral funiculi, whilst descending facilitatory influences come from the RVM to the ipsilateral ventral/ventrolateral spinal cord (Urban and Gebhart, 1997). Though descending influences are activated in the same area of the brainstem, descending signals project to different part of spinal cord on different receptors. Spinal cholinergic and monoaminergic receptors mediate descending inhibition and serotonin receptors mediate descending facilitation (Zhuo and Gebhart, 1990;Zhuo and Gebhart, 1991). Hence, RVM neurons have been physiologically defined as ON, OFF and neutral cells (Fields

and Basbaum, 1999). ON-cells possess μ-opioid receptors and contribute to pro-nociceptive or facilitatory influences on spinal nociceptive processing (Fields and Basbaum, 1999;Heinricher et al., 1989). OFF-cells are correlated with inhibition of nociceptive input and the source of descending facilitation of nociception (Fields and Basbaum, 1999;Heinricher et al., 1989). The OFF-cells are tonically active and stop firing immediately before animal withdraws from the noxious thermal stimulus, whereas the ON-cells accelerate firing immediately before the nociceptive reflex (Ossipov et al., 2005). The activation of descending facilitatory mechanisms from the RVM is critical for the maintenance of the behavioural neuropathic pain state (Porreca et al., 2002). This idea is supported by the observation that injection of lidocaine into the RVM blocks the behavioural signs of neuropathic pain (Pertovaara et al., 1996).

Thus, supraspinal regulation plays an important role in maintaining neuropathic pain status. The changes at supraspinal sites may result in the continuous activation of facilitatory influences on further nociceptive input, which may be one of the many factors causing pathological pain. This also provides new targets for neuropathic pain treatments.

1.3 Animal models

Animal models were developed to investigate the mechanisms involved in neuropathic pain. Chronic constriction injury (CCI) of the rat sciatic nerve has

been a widely used animal model of neuropathic pain (Bennett and Xie, 1988). Other models include polyethylene cuff CCI (Mosconi and Kruger, 1996), partial ligation of the sciatic nerve (Seltzer et al., 1990), ligation of spinal nerves innovating the sciatic (Sun and Jin, 1992), spared nerve injury (Decosterd and Woolf, 2000) and complete ligation or transection of the sciatic nerve, also known as axotomy (Wall et al., 1979). (see Fig. 1.4)

1.3.1 Chronic constriction injury model

The sciatic nerve chronic constriction injury (CCI) model was developed by Bennett and Xie. A set of four chromic gut sutures were placed loosely around the common sciatic nerve (Bennett and Xie, 1988). In this model, rats exhibited thermal and mechanical hyperalgesia, cold allodynia and showed detectable spontaneous pain (Bennett and Xie, 1988). It may model partial nerve lesion or nerve crush in patients who have been subject to traumatic nerve injury.

CCI causes massive demyelination throughout the injury site detected by light and electron microscopy, along with almost total loss of myelinated Aα and Aβ fibres and less loss of thinly myelinated Aδ and unmyelinated C fibres (Munger et al., 1992).

The advantage of CCI model is that both spontaneous and evoked neuropathic pain can be detected indicating the success of mimicking neuropathic pain.

However, it is difficult to produce a consistent extent of damage because of the imprecise looseness control of the ligatures. Unlike the axotomy model in which all the fibres are sectioned, some afferent fibres of the sciatic nerve survive after CCI injury. This model thus allows the use of behavioural tests which involve both sensory and motor nerves. For example, the Von Frey hair test is commonly used to quantify the effects of manipulations of the neuropathic pain state (Chaplan et al., 1994). It is useful in monitoring nociception especially in pharmacological research.

1.3.2 Partial nerve ligation model

The partial nerve ligation model involves tight ligation of half of sciatic nerve, sparing the remaining nerve fibres (Seltzer et al., 1990). In this model, rats display behaviours of spontaneous pain, touch-evoked allodynia, mechanical and thermal hyperalgesia (Seltzer et al., 1990). It may model partial nerve injury due to trauma.

When capsaicin-sensitive C fibre nociceptors of neonatal rats were obliterated by capsaicin, the rats did not develop thermal hyperalgesia after partial sciatic nerve ligation, indicating thermal hyperalgesia was mediated by heat nociceptive C fibres (Shir and Seltzer, 1990).

The partial nerve injury model induces spontaneous pain and like CCI, provides the possibility examining the response to evoked pain by means of behavioural monitoring. But as with CCI, the degree of injury is hard to control and cannot be standardized for each animal.

1.3.3 Spinal nerve ligation model

Spinal nerve ligation was first developed by Kim and Chung. L5 and L6 branches of the sciatic nerve were tightly ligated with 3 silk sutures between the trifurcation of the sciatic nerve and distal to the dorsal root ganglia (Sun and Jin, 1992). L4 branch was left intact because L4 spinal nerve ligation could cause motor deficits due to the denervation of the proximal muscles of the leg (Sun and Jin, 1992). Rats displayed tactile allodynia and thermal hyperalgesia but less cold allodynia than was seen in the CCI model (Sun and Jin, 1992).

The advantage of this model is the separation of intact nerves from injured ones to keep the intact nerve from undergoing Wallerian degeneration and other inflammatory responses. It is also easier to standardize the extent of injury across a population of animals.

1.3.4 Spared nerve injury model

The spared nerve injury model involves tight ligation and subsequent resection of the common peroneal and tibial nerves while leaving the sural nerve intact (Decosterd and Woolf, 2000). Spontaneous pain, thermal hyperalgesia and cold allodynia were detected (Decosterd and Woolf, 2000).

The surgery required for this model is easier than that required for the CCI or nerve ligation. Moreover, a consistent extent of nerve injury can be generated in all treated animals.

1.3.5 Axotomy (neuroma) model

Axotomy was the first reported neuropathic pain model. It was produced by removing 5 mm section of the sciatic nerve at the midthigh level, *i.e.* the total transection of the sciatic nerve (Wall et al., 1979). Following complete nerve transection, a neuroma may develop at the proximal nerve stump, consisting of regenerative nerves sprouting in all directions (Amir and Devor, 1993).Though axotomy precluded direct behavioural tests, it caused significant time-dependent autotomy: a self-attack and mutilation of the denervated paw (Wall et al., 1979). There are arguments about the interpretation of the phenomenon of autotomy. The cause of autotomy may be hyperesthesia (comparable to that seen in humans after peripheral nerve injury) or anesthesia. It has been suggested that deafferrentation reduces the contribution of primary afferent axons to painful pathology and the autotomy was a result of hind paw anesthesia, which might

prompt an animal to shed an insensate appendage (Rodin and Kruger, 1984). But other studies suggest autotomy is a response to spontaneous pain. Increased pain sensitivity of the axotomized rats by prior heat injury or intrathecal administration of substance P could increase the level of autotomy (Coderre and Melzack, 1986). Axotomy was also found to increase small DRG neuron excitability (Zhang et al., 1997). Abdulla and Smith compared the DRG cell excitability and channel properties before and after autotomy and found that the onset of autotomy was coupled by the increase of excitability of large, myelinated sensory neurons (Abdulla and Smith, 2001b;Abdulla and Smith, 2001a), which were likely associated with the onset of chronic pain. Moreover, electrophysiological ectopic activity discovered in primary afferents after the injury was also perceived as on going pain (Devor, 1991;Wall et al., 1979).

1.3.6 Polyethylene cuff chronic constriction injury model

The primary concern of the classical Bennett and Xie suture CCI is the variability of nerve fibre damage which might be due to the controlling of the tightness of the constriction. The principal advantage of the use of polyethylene cuffs is that the level of nerve damage is well controlled and more consistent. It is also easy and reproducible (Mosconi and Kruger, 1996;Coull et al., 2003).

Polyethylene cuff chronic constriction injury model was developed based on sutured CCI to standardize the degree of constriction of the sciatic nerve

(Mosconi and Kruger, 1996). Wallerian degeneration, retrograde degeneration ("die back") and recovery of nerve fibres, mechanical hyperalgesia, tactile and cold allodynia are comparable to suture CCI (Mosconi and Kruger, 1996).

1.3.7 Animal models in the present study

In this thesis, polyethylene cuff chronic constriction injury (CCI) and axotomy were chosen as the neuropathic pain models for the experiments. Although both types of injury can produce chronic pain in humans, CCI and axotomy send quite different signals to the CNS and may therefore promote different types of changes in the spinal dorsal horn (Moore et al., 2002).

CCI is thought to affect primary Aβ and Aδ fibres while leaving C-fibres relatively unscathed (Basbaum et al., 1991). The axons close to the edge of a nerve trunk are likely to be severed or to receive some degree of crush injury. The distal portions undergo Wallerian degeneration as a result of their disconnection from cell bodies in the DRG but axons in the center of the nerve may escape from injury altogether (Fig 1.5). It has been suggested that the release of inflammatory mediators from degenerating axons promotes changes in surviving axons (Shamash et al., 2002). These surviving axons, their terminals, and cell bodies become hyperexcitable and generate ectopic discharges (Amir et al., 2005;Ma et al., 2003). Although there is some debate as to the number and type of fibers that need to be active (Sandkuhler, 2009;Devor, 2006), it is generally held that

the arrival of aberrant and sustained sensory activity in the dorsal horn is the primary trigger for the initiation of central sensitization and pain (Sheen and Chung, 1993). This process involves the release of various mediators, including adenosine 5-triphosphate (ATP), from primary afferent terminals and the activation of spinal microglia (Tsuda et al., 2003;Tsuda et al., 2005;Xie et al., 2009;Trang et al., 2009). These, in turn, release additional mediators such as BDNF, which promote an enduring increase in dorsal horn excitability (Coull et al., 2005;Lu et al., 2007;Lu et al., 2009).

Because virtually all axons in the sciatic nerve are severed by axotomy (Fig 1.5), L4-L5 spinal cord neurons no longer receive input from their normal peripheral receptive field (Devor and Wall, 1981). The proximal segments of the severed axons remain attached to their cell bodies in the DRG. Although these proximal axons "die back" from the site of transaction, degeneration within the first few weeks of injury is minimal (Tandrup et al., 2000). Thus, severed axons are not exposed to an environment where active Wallerian degeneration is taking place. Moreover, because ion channel expression in peripheral neurons is dependent on target contact and neurotrophic support (Petrov et al., 2001;Lei et al., 1997), it is possible that by downregulating Ca^{2+} channels, axotomy may impede the release of neurotransmitters from primary afferent terminals in the dorsal horn (Baccei and Kocsis, 2000). This contrasts with the effects of CCI which is reported to increase the expression of N-type Ca^{2+} channels in the dorsal horn (Cizkova et al., 2002). Despite all these differences, it is well established that axotomy, like

CCI, increases spontaneous activity in both primary afferent fibres (Zhang et al., 1997) and in dorsal horn neurons (Dalal et al., 1999).

As will be discussed in detail in the introduction of chapter 3, axotomy and CCI produce slightly different patterns of ion channel regulation and neuropeptide expression in DRG cell bodies (Abdulla and Smith, 2001b;Abdulla and Smith, 2001a;Abdulla and Smith, 2002;Baccei and Kocsis, 2000;Dib-Hajj et al., 1999;Bauer et al., 2009;Cizkova et al., 2002;Sten Shi et al., 1999), but both are associated with increased excitability of sensory neurons. The first question to be addressed in this thesis is whether these different peripheral signals generated by axotomy produce similar or different effects in dorsal horn neurons compared to CCI.

1.4 Synaptic Signaling in Nociceptive Pathways

The transfer of information at a synapse requires the activation of presynaptic terminals and subsequent release of neurotransmitter and activation of postsynaptic receptors. The information transfer at the synapses is regulated by the nature and amount of neurotransmitter released from presynatic terminal, the density and identity of postsynaptic receptors and the properties of receptor activation. All synapses show evoked and spontaneous neurotransmitter release. Evoked neurotransmitter release is coupled to the presynaptic action potential. Spontaneous synaptic neurotransmitter release was first described at the

neuromuscular junction as a state of continuous secretory activity that produces miniature end-plate potentials (Fatt and Katz, 1950). Later on it was found as spontaneous subthreshold synaptic synaptic potentials or “synaptic noise” in mammalian central neurons (Brock et al., 1952) and was proved to be analogous to miniature end-plate potentials (Katz and Miledi, 1963). Spontaneous excitatory postsynaptic currents (sEPSCs) result from the spontaneous release of small quantities of the excitatory neurotransmitter from presynaptic terminal via vesicle exocytosis and therefore the frequency of sEPSC occurrence is related to factors operating presynaptically (Larkman et al., 1991;Prange and Murphy, 1999).

In the CNS, sEPSCs reflect action potential-dependent as well as action potential-independent release of neurotransmitter. The action potential-independent sEPSC is called a miniature EPSC (mEPSC). The release of neurotransmitter by action potentials in presynaptic nerve terminals depends on the entry of extracellular Ca^{2+} upon membrane depolarizations (Luebke et al., 1993;Katz and Miledi, 1965) whereas the ongoing activity of the release process independent of presynaptic action potentials, which produce mEPSC, is not dependent on Ca^{2+} entry (Edwards et al., 1990). A study in hippocampus neurons found that there were two presynaptic neurotransmitter vesicle pools: one that was released in response to presynaptic activity and another distinct vesicle pool that spontaneously fuses with the plasma membrane (Fredj and Burrone, 2009). The

latter is a 'resting pool' that is responsible for mEPSCs and normally not mobilized by neuronal activity (Fredj and Burrone, 2009).

1.4.1 Presynaptic terminal

Primary afferent terminals are the first site in the ascending pain pathway where peripheral sensory information is transferred to second-order dorsal horn neurons.

Glutamate is the major excitatory neurotransmitter of primary afferent neurons. Its release is determined by membrane depolarization, Ca^{2+} concentration and vesicle storage. Release of glutamate is suppressed presynaptically by some metabotropic G protein-coupled receptors, such as μ-opioid receptors, $GABA_B$ receptors and adenosine receptors (Campbell and Meyer, 2006). Down regulation of μ-opioid receptors was found in the spinal cord both pre and postsynaptically after peripheral nerve injury, which could potentiate glutamate release (Kohno et al., 2005), and may account for the limited effectiveness of opioids in neuropathic pain (Abdulla and Smith, 1998). Glutamate release can be facilitated by positive feedback via presynaptic Kainate receptors, NMDA receptors, P2X3 receptors and mGluRs (Doubell et al., 1999;Lee et al., 1999). However, presynaptic AMPA receptors cause inhibition of glutamate release from primary afferent terminal via primary afferent depolarization (PAD) (Lee et al., 2002b). Anionic ionotropic receptors, for example $GABA_A$ and glycine

receptors, can also depress presynaptic neurotransmitter release as a result of PAD (Engelman and MacDermott, 2004).

Spinal nerve ligation upregulates Ca^{2+} channels in the DRG and spinal cord (Lee et al., 2002a), which might suggest increased Ca^{2+} entry and enhanced release of glutamate. By contrast, sciatic nerve axotomy decreases Ca^{2+} current in DRG cell bodies (Abdulla and Smith, 2001a), which might suggest less Ca^{2+} channels insertion and decreased Ca^{2+} current in primary afferent terminals in the spinal dorsal horn. Although this should reduce synaptic transmission between primary afferents and neurons in the dorsal horn, this possibility has not been directly tested. Experiments to test this possibility are described in chapter 4.

1.4.2 Postsynaptic terminals

Glutamate released from primary afferent terminals binds to several types of receptors on postsynaptic neurons, including ionotropic amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors, kainate (KA) receptors and metabotropic glutamate receptors (mGluR). All glutamatergic postsynaptic membranes in the superficial laminae of the spinal cord express AMPA receptors, and most of them are also immunoreactive for the NR1 subunit of NMDA receptors, indicating co-localization of these two types of receptors (Antal et al., 2008). Besides the primary afferent terminal, glutamatergic neurons also receive excitatory input from other excitatory

neurons. The fast excitatory postsynaptic potential is mediated by activation of AMPA receptors in adult animals, whereas NMDA receptors mediate only "slow" activation due to their tonic blockade by extracellular Mg^{2+}. This block can be removed by membrane depolarization caused by glutamate or by neuropeptides, such as substance P and CGRP. Kainate receptors are involved in unmyelinated and thinly myelinated primary afferent and intrinsic dorsal horn neurons and contribute approximately 30% of the combined AMPA/kainate receptor mediated postsynaptic currents by stimulation at postnatal day 4-21 (Li et al., 1999). However, at postnatal day 21-26, kainate receptors have no major contribution to the C-fiber-evoked EPSC of lamina I neurons (Dahlhaus et al., 2005). In lamina II of adult mice, kainate receptors are not involved in C-fiber evoked EPSCs either (Youn and Randic, 2004). The ratio of expression of kainate receptors to AMPA receptors is very low in adult dorsal horn (Tolle et al., 1993). Hence the animals in my study are in an age group that lacks kainate receptors in superficial dorsal horn and the fast synaptic current is mainly mediated by AMPA receptors.

The inhibitory neurotransmitter GABA is not released from primary afferent terminals but is released from interneurons and from fibres descending from higher brain centers (Reichling and Basbaum, 1990). There are two broad categories of GABA receptors, the ionotropic $GABA_A$ receptors and the metabotropic $GABA_B$ receptors. $GABA_C$ is another type of ionotropic receptor with a novel ρ subunit, which is believed to be a third class of GABA receptor

(Chebib, 2004). Glycine is another inhibitory neurotransmitter widely distributed in the brainstem and spinal cord. $GABA_A$ receptors and ionotropic glycine receptors both open chloride channels. Many inhibitory interneurons co-release GABA and glycine, whereas the others only release GABA (Todd, 2010). In lamina II inhibitory neurons, the inhibitory postsynaptic currents are mediated by both $GABA_A$ and glycine receptors (Labrakakis et al., 2009;Keller et al., 2001). Part of the GABAergic and glycinergic axons are derived from local inhibitory interneurons (Labrakakis et al., 2009), whereas some are supraspinal descending fibres (Todd and Koerber, 2005). Most GABAergic and glycinergic axons form synapses onto dorsal horn neurons, but some form axoaxonic synapses onto primary afferent terminals. The latter are involved in presynaptic inhibition of primary afferents (Todd and Koerber, 2005). GABA receptors are also involved in primary afferent depolarization as the high concentration of Cl^- ions in these fibres displaces the the Cl^- equilibrium potential in the depolarizing direction (Barker and Nicoll, 1972;Rudomin and Schmidt, 1999).

1.5 AMPA receptors

AMPA receptors exhibit fast-opening kinetics and undergo rapid deactivation and desensitization. AMPA receptors are present in the great majority of glutamatergic synapses in the superficial dorsal horn (Yasaka et al., 2009).

1.5.1 Structure of AMPA receptors

There are four main types of AMPA receptor subunits, termed GluR1-4 (also know as GluA1-4). The first Glu receptor subunit was cloned and expressed in *Xenopus oocytes* and named GluR1 (Hollmann et al., 1989). Further cloning studies led to identification of three more subunits, named GluR2-4 (Boulter et al., 1990). Each subunit is composed of an extracellular amino-terminal domain, a ligand binding domain, a transmembrane domain and an intracellular carboxy-terminal domain (Catarzi et al., 2007). The transmembrane domain has three transmembrane helices (M1, M3, M4) with M2 as a semi pore-forming loop entering the membrane from the cytoplasmic side (Fig. 1.6). This re-entrant second membrane loop controls important permeation properties of the ion channel. Two extracellular segments, S1 and S2, constitute the ligand binding domain which is responsible for the binding of neurotransmitter and competitive agonists or antagonists (Mayer and Armstrong, 2004) (Fig. 1.6). Alternative splicing in the extracellular ligand binding domain of the AMPA receptors generates two variants, i.e., flip and flop. These two variants show differences in their desensitization properties, for example, the flop variant of the GluR2 AMPA receptor desensitizes faster than the flip counterpart with the desensitization constant of about 5.9ms in flip and 1.2ms in flop (Koike et al., 2000).

The C-terminal intracellular domain plays an important role in receptor function regulation by presenting multiple protein phosphorylation sites for protein

kinases, such as CaMKII, PKC and PKA. GluR1 has a long C-terminal tail, whereas gluR3 has a short tail, but GluR2 and 4 are expressed with either long or short C-terminal tail. This C-terminal tail is important in regulating receptor trafficking. Receptors with short-tailed subunits are constitutively recycled to and from the postsynaptic membrane, whereas receptors with long-tailed subunits are recruited to synapses in an acitivity-dependent manner (Shepherd and Huganir, 2007).

Each AMPA receptor is composed of 4 subunits, which are arranged as a dimer of dimers. They can be of a variable subunit composition, including homomeric (Traynelis et al., 2010). The GluR2 subunit is particularly important for the functional properties of heteromeric AMPA receptors including low calcium permeability, linear non-inward-rectifying current-voltage relationship, smaller single channel conductance and sensitivity to polyamine block. In the channel pore region (M2 domain), there is one amino acid residue which is arginine (R) in the GluR2 subunit and glutamine (Q) in the other three subunits. Nearly all GluR2 subunits undergo this RNA editing of Q607 to an R607 in the adults, which is an important posttranscriptional event (Larsson, 2009). This difference greatly affects the channel eletrophysiological properties. In particular, arginine in the edited version of GluR2 subunit causes low calcium permeability and linear I-V relationship (Hume et al., 1991). Heteromeric AMPA receptors containing GluR2 also have low single channel conductance because of arginine substitution of glutamine (Swanson et al., 1997). The changes of structure of the S1S2 segment

(the ligand binding site) for GluR2 may also lead to a conformational modifications that regulate ion flow in the receptor channel (Catarzi et al., 2007). The ability of AMPA receptors to admit or exclude Ca^{2+} can have different functional consequences (Vikman et al., 2008). In view of this, AMPA receptors can be divided into Ca^{2+} permeable and Ca^{2+} impermeable subtypes (Derkach et al., 2007). Ca^{2+} permeable AMPA receptors exhibit inward rectification and a single channel conductance of about 12pS, whereas Ca^{2+} impermeable AMPA receptors exhibit linear I-V relationship and a single channel conductance of about 3pS (Guire et al., 2008;Kristensen et al., 2011). Conductance of AMPA receptors can also be regulated by phosphorylation. In particular, if AMPA receptor is GluR1-containing, phosphorylation of Ser831 in GluR1 C-terminus further increases the channel conductance in both Ca2+ permeable GluR1 homomers (Derkach et al., 1999) and Ca2+ impermeable GluR1/GluR2 heteromers (Kristensen et al., 2011). Notably, properties of AMPA receptos can be also controlled by their auxiliary subunits, transmembrane AMPA receptor regulatory proteins (TARPs) and cornichons, greatly adding to functional mosaic of these receptors (Nicoll et al., 2006;Gill et al., 2011).

1.5.2 Distribution of AMPA receptors

AMPA receptors have a widespread distribution in the CNS. They are found in the spinal cord and throughout the brain with high levels of expression in

cerebral cortex, basal ganglia, thalamus and hypothalamus (Catarzi et al., 2007). In the hippocampus, AMPA receptors are expressed more highly in the pyramidal cell layer than stratum radiatum and stratum oriens. Expression Level is also high in the dentate gyrus and the superficial layer of the cerebral cortex (Ozawa et al., 1998). Intermediate levels are found in deeper layer cortex and caudate-putamen and lower levels in the diencephalon, midbrain and brainstem (Ozawa et al., 1998). As for the subunit mRNA, GluR1, GluR2 and GluR3 are abundant in hippocampus but not GluR4 and in the cerebral cortex GluR2 mRNA is found uniformly distributed though all the layers while GluR1, GluR3 and GluR4 is differently distributed (Ozawa et al., 1998).

In the superficial dorsal horn, GluR2 was found at virtually all AMPA receptor containing glutamatergic synapses throughout the spinal grey matter and GluR1 and GluR3 were present at about 60% of the synapses in laminae I and II (Polgar et al., 2008b). Ca^{2+} permeable AMPA receptors lacking GluR2 were found in high intensity in NK1+ receptor expressing lamina I projection neurons of the spinal cord (Engelman et al., 1999), whereas GluR2 subunits were found in the dendritic plasma membranes of NK1+ expressing lamina III and IV projection neurons (Engelman et al., 1999;Todd et al., 2009). Moreover, GluR1 is found at 1/3 of synapses formed by C/Aδ fibers and is preferentially associated with primary afferent synapses (Larsson, 2009). Many presynaptic AMPA receptors in the superficial dorsal horn are Ca-permeable, *i.e.* they lack an edited GluR2 subunit and nociceptors involved primarily in the mediation of neuropathic pain may be

presynaptically modulated by GluR4-containing AMPA receptors (Willcockson and Valtschanoff, 2008). Functional AMPA receptors are present in DRG neurons and most prominently located near central terminals of primary afferent (Lee et al., 2002b). Besides in CNS, there is evidence confirming the presence of the AMPA receptors in peripheral sites such as pancreas, heart and postganglionic sympathetic neurons (Dingledine et al., 1999).

Multiple AMPA receptor subtypes can coexist within the same neurons, supported by both low and high calcium permeability being found in the same neuron (Zhang et al., 1995) and polyamine spider toxins selective blockade of GluR2-lacking receptors (Iino et al., 1996).

1.5.3 AMPA receptor kinetics

Kinetic properties of ligand-gated channel responses provide clues to the mechanisms of fast synaptic transmission. AMPA receptors are rapidly activated by high concentrations of glutamate with high probability of channel opening. They have low-affinity binding sites for endogenous glutamate, whose brief bound time might lead to quick deactivation. As already mentioned, the activation, deactivation and desensitization of AMPA receptors are rapid compared to NMDA receptors. If glutamate stays in the synaptic cleft for a brief duration, *i.e.* < 1ms, the deactivation kinetics of the postsynaptic receptors will dominate the time course of synaptic current delay. However, desensitization

with prolonged exposure to glutamate is often the case for AMPA receptors (Ozawa et al., 1998). The recovery of AMPA receptor from desensitization is almost 10-fold faster than kainate receptors, which could easily make the differentiation of AMPA and kainate currents activated by endogenous glutamate (Dingledine et al., 1999).

The rate of AMPA receptors desensitization is determined by subunit compostion and the RNA splicing at the flip/flop region (Mosbacher et al., 1994). The "flop" splice variants desensitize more rapidly than the flip forms in response to glutamate (Sommer et al., 1990). The editing site upsteam of the flip/flop region can also speed the recovery (Lomeli et al., 1994). Several compounds can relieve desensitization, for example cyclothiazide, aniracetam and AMPAkines, which all work on “flip” receptors (Dingledine et al., 1999). The binding site between M3 and M4 is critical for desensitization and Ser750 of flip GluR subunit is crucial for cyclothiazide sensitivity (Partin et al., 1995).

1.5.4 AMPA receptor trafficking

AMPA receptors are synthesized in the endoplasmic reticulum (ER) and transported through the Golgi apparatus to be packed into vesicles. Vesicles are then transported either to dendrites or to axons. Synthesis of AMPA receptor subunits can also occur in dendrites for a fast incorporation into synapses in activity-dependent manner (Sutton et al., 2006). Delivery into the specialized

membrane is the last step of the transportation journey after synthesis. AMPA receptors are initially inserted at extrasynaptic sites and laterally move and incorporated at synapses in activity and subunit-dependent manner (Henley et al., 2011).

Functional properties and ultimate density of AMPA receptors accumulated at synapses determines synaptic strength. AMPA receptors can only stay on the membrane surface for tens of minutes and undergo constant turnover through endo- and exocytic processes (Man et al., 2000;Bredt and Nicoll, 2003). They can also diffuse rapidly at the neuronal surface and exchange between subcellular compartments such as synaptic and extrasynaptic membrane (Borgdorff and Choquet, 2002). AMPA receptors do not have motor domains and associate with proteins that assist their trafficking. Proteins containing PDZ domains play general roles in scaffolding membrane proteins. PDZ domains are modular protein motifs with about 80 amino acids and bind to the C-terminal of the interacting protein partners (Sheng and Sala, 2001).

The exocytosis of receptors is not only required for the insertion of new receptors but also required for modifying synaptic strength (Park et al., 2004). The receptor for exocytosis comes either from a pool of newly synthesized receptors or from a pool of recycled receptors and the exocytosis site could be directly at the synapse or within the extrasynaptic membrane. Extrasynaptic receptors can undergo diffusion and move to synaptic sites or remain in the

extrasynaptical membrane. AMPA receptors are inserted along dendrites in a subunit-dependent manner followed by their lateral diffusion (Passafaro et al., 2001;Henley et al., 2011). Surface insertion of the GluR1 subunit occurs slowly in basal conditions initially at extrasynaptic sites and is stimulated by NMDA receptor activation and insulin, whereas GluR2 exocytosis is constitutively rapid and more directly at synapses (Passafaro et al., 2001). The rapid accumulation of exocytosed GluR2 subunits depends on the NSF-binding sites in the C-terminal (Beretta et al., 2005). In heteromeric receptors, GluR1 acts dominantly over GluR2 in exocytosis and GluR2/3 dominates the recycling and endocytosis (Passafaro et al., 2001). AMPA receptors undergo rapid constitutive internalization regulated by synaptic activity (Lin et al., 2000). The internalization of AMPA receptors is thought to occur though clathrin-mediated endocytosis (Lin et al., 2000). The synaptic and extrasynaptic AMPA receptors respond differently to endocytosis as the extrasynaptic receptors internalize rapidly and precedes the delayed removal of synaptic receptors (Ashby et al., 2004).

AMPA receptors undergo Brownian diffusion in neuronal membranes. This is marked by receptor number increasing linearly with time at the surface explored, and the diffusion can be regulated by intracellular calcium (Borgdorff and Choquet, 2002). For example, with rising intracellular calcium concentration, the AMPA receptors become immobilized and accumulate locally at the synaptic surface (Groc et al., 2004). Later studies discovered that even within the synaptic

cleft, AMPA receptors are mobile and that the exchange between the synaptic and extrasynaptic domains is permanent (Groc and Choquet, 2006). However, synaptic AMPA receptors are mobile in a different way from extrasynaptic receptors. Synaptic receptors move rapidly but in a confined area with obstacles delimiting membrane compartments. Occasionally they escape from and become extrasynaptic receptors (Choquet, 2010). Extrasynaptic receptors can travel for long distance but have low diffusion coefficient (Choquet, 2010). Peculiarities of the diffusive environment may act like an obstacle controlling the rate of exchange of molecules within the membrane subcompartments and the inhibitory postsynaptic membranes have a higher density of the obstacles than excitatory membranes (Renner et al., 2009).

AMPA receptor trafficking is the key pathway for regulating receptor surface distribution and their cycling between intracellular and plasma membrane so as to regulate synaptic strength. It is therefore one of the mechanisms of synaptic plasiticity.

1.5.5 AMPA receptors in neuropathic pain

As mentioned above, glutamate and AMPA receptors mediate fast excitatory transmission at primary afferent synapses and at other excitatory synapses in the spinal cord. There appears to be a functional relationship between AMPA receptor subunit composition and nociceptive transmission. The basal spinal

nociceptive transmission is not affected by alterations in AMPA receptor subunit expression. However, GluR2 subunit-deficient (ie. increased Ca^{2+} permeable AMPARs) mice showed facilitated nociceptive plasticity and enhanced long-lasting inflammatory hyperalgesia (Hartmann et al., 2004). Moreover the inhibition of spinal Ca^{2+}-permeable AMPA receptors produces thermal antinociception (Sorkin et al., 2001).

Since AMPA receptors are targets for multiple signalling pathways regulating the strength of glutamatergic excitatory synapses, they are implicated in the induction of central sensitization and in hyperalgesia in animal pain models. Synaptic efficacy is dependent on the number and function of synaptic AMPA receptors. The number is associated with receptor trafficking, for example an increase in the number of glutamate receptors was reported at synapses of small primary afferents terminating in lamina II after nerve section (Popratiloff et al., 1998). It has been demonstrated that the AMPA receptor trafficking regulation is essential in the induction of LTP in hippocampal neurons and it has been suggested that spinal cord central sensitization may involve similar mechanisms (Ji et al., 2003). Thus, AMPA receptor trafficking at synapses may be involved in enhanced synaptic strength in neuropathic pain. The function is determined by AMPA receptor subunit composition, modification and interacting proteins. For example, the Ca^{2+}-permeable AMPA receptor antagonists can prevent induction of mechanical allodynia and hyperalgesia induced by burning injury (Jones and Sorkin, 2004).

The regulation of AMPA receptors in the spinal cord varies between different pain models. AMPA receptor research in inflammatory pain mostly supports the idea of increased trafficking of GluR1 subunit to the membrane, both synaptic and extrasynaptic. Intracolonic capsaicin can induce a rapid increase in GluR1, but not GluR2/3 in the synaptosomal membrane fraction and a decrease in GluR1 subunit in the cytosolic fraction in lumbar spinal cord (Galan et al., 2004). In complete Freund's adjuvant (CFA)-induced peripheral inflammation model, the synaptic GluR2 subunit undergoes internalization triggered by NMDA receptor activated PKC in dorsal horn neurons (Park et al., 2009). These data suggest the trafficking of GluR1 subunit from the cytosol to synaptic membrane and the internalization of GluR2 subunit in persistent inflammation. In terms of regulation of AMPA receptor subunit expression, CFA-induced peripheral inflammation increases functional expression of extrasynaptic GluR1-containing AMPARs in tonically firing dorsal horn neurons (Kopach et al., 2011). In a hindpaw capsaicin acute inflammatory model, the hyperalgesia associates with an increased density of GluR1-containing AMPA receptors and an increased synaptic ratio of GluR1 to GluR2/3 subunits at C-fiber synapses (Larsson and Broman, 2008). Reduced GluR2 subunit functional expression was reported in other inflammatory pain model (Hartmann et al., 2004;Katano et al., 2008). If GluR2-containing AMPA receptors are replaced by Ca^{2+}-permeable AMPA receptors, it could be expected that there is an enhanced Ca^{2+} transient which

could in turn lead to increased activation of Ca^{2+}-dependent processes that increase membrane excitability.

However, in neuropathic pain models, the story is different. GluR2 is found to increase at the synaptic membranes. The proteins that mediates GluR2 endocytosis have a reduced rate and resulted in the accumulation of GluR2 at the cell membrane after sciatic nerve CCI (Osten et al., 2000;Garry et al., 2003). GluR2/3 subunits increase significantly at synapses of primary afferent terminals in lamina II after sciatic nerve section (Popratiloff et al., 1998). GluR2 subunits selectively increase in the spinal cord ipsilateral to sciatic nerve CCI (Garry et al., 2003). In other studies, peripheral injury was found to result in the upregulation of GluR1 in the superficial laminae of the dorsal horn (Harris et al., 1996). This contrasts with internalization of Ca^{2+} impermeable (*i.e.* GluR2-containing) receptors in chronic inflammatory pain. Therefore distinct molecular mechanisms of synaptic modifications may underlie different forms of chronic pain.

Thus in my project of comparing two distinct neuropathic pain models, AMPA receptor subunit composition and expression are important and the detailed results will be listed in chapter 5.

1.6 Project outline and hypothesis

Since axotomy and CCI send quite different signals to the CNS, there might be different types of changes happening in the spinal dorsal horn (Moore et al., 2002). Detailed electrophysiological description of *substantia gelatinosa* neurons after sciatic nerve axotomy is considered in chapter 3 of this thesis. The first formal hypothesis to be evaluated is that CCI and axotomy produce different changes in the eletrophysiological properties of *sustantia gelatinosa* neurons.

Since axotomy decreases Ca^{2+} channel current in DRG cell bodies whereas CCI increases it, I will continue to evaluate differences between the response to CCI and that to axotomy by comparing changes in Ca^{2+} channel function in primary afferent terminals. The second formal hypothesis is that axotomy and CCI exert different effects on Ca^{2+} channels in primary afferent terminals. This issue is addressed in chapter 4.

Previous studies in Dr. Smith's laboratory established that CCI of sciatic nerve has cell type specific effect on the properties of different populations of *substantia gelatinosa* neurons: CCI decreased the amplitude and frequency of spontaneous and miniature excitatory post synaptic currents in tonic inhibitory neurons yet increased them in other cell types (Balasubramanyan et al., 2006). My work to be presented in chapter 3 shows that axotomy produces similar effect on *substantia gelatinosa* neurons (Chen et al., 2009). Since the signals sent by the two kinds of injury might be quite different (see introduction), it is possible that the two models differentially affect neurotransmitter release and

postsynaptic efficacy of glutamate. I therefore investigated the detailed sub-cellular mechanisms. Thus, the third formal hypothesis is that postsynaptic AMPA receptors switch to different subtypes after CCI and axotomy.

Experiments to test this and related hypothesis will be described in chapter 5.

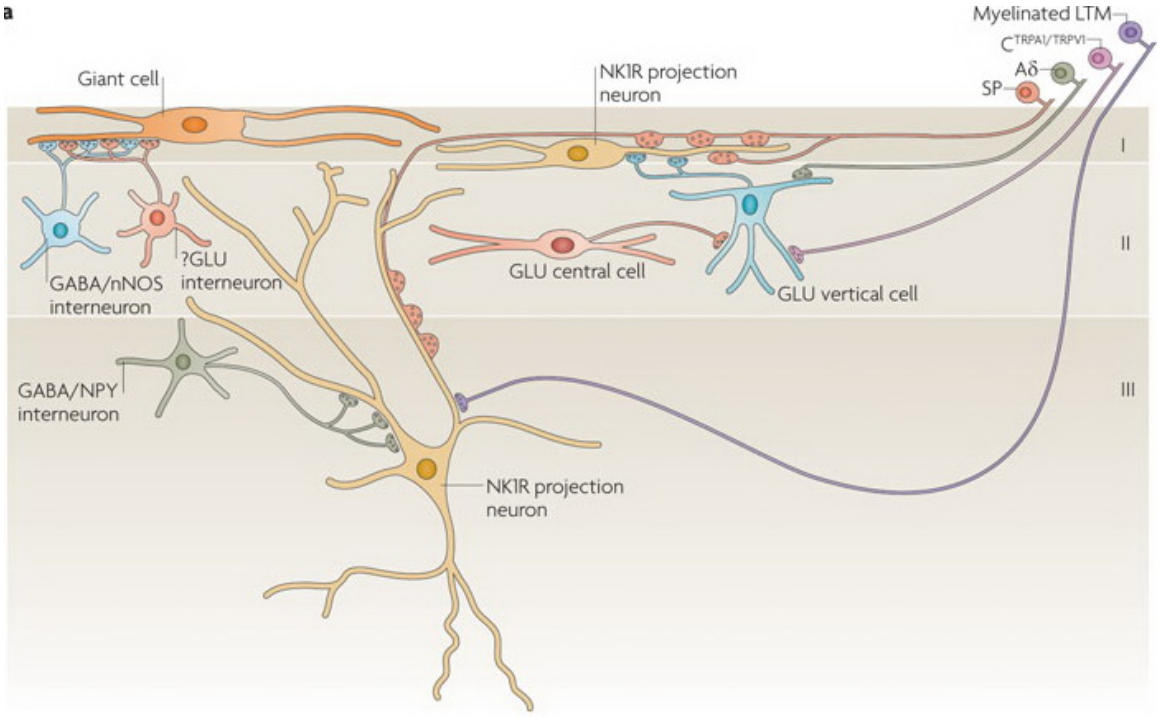

a
Myelinated LTM
C TRPA1/TRPV1
Aδ
SP
Giant cell
NK1R projection neuron
I
II
III
?GLU interneuron
GABA/nNOS interneuron
GLU central cell
GLU vertical cell
GABA/NPY interneuron
NK1R projection neuron

Figure 1.1 A diagram showing some of the synaptic circuits identified in laminae I-III.

Three types of projection neurons are shown: a neurokinin 1 receptor (NK1R)-expressing cell in lamina I, a giant lamina I neuron and an NK1R-expressing cell in lamina III. Two types of interneurons are shown: glutamatergic excitatory interneurons with the morphology of central (GLU central cell) and vertical (GLU vertical cell) styles and GABAergic inhibitory interneurons that contain neuropeptide Y (GABA/NPY interneuron)in lamina II. Both NK1R-expressing neurons are innervated by substance P (SP)-containing primary afferents. Lamina III neurons also receive input from myelinated low threshold mechanoreceptive (LTM) afferents. The lamina III NK1R cells receive a substantial input from GABA/NPY interneuron. NK1R-expressing lamina I projection neurons also receive an input from GLU vertical cell, which are innervated by GLU central cell. The primary afferents that synapse onto vertical cells include Aδ fibres as well as C fibres that express both transient receptor potential A1 (TRPA1) and transient receptor potential vanilloid 1 (TRPV1).

Reproduced from (Todd, 2010).

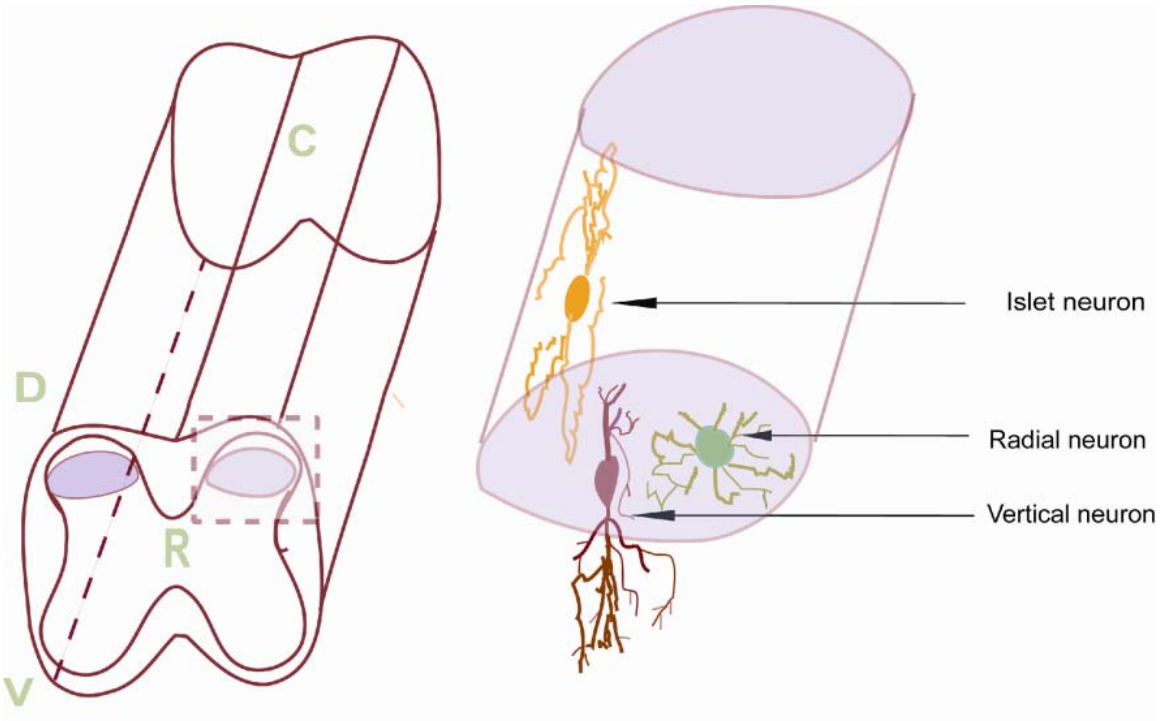
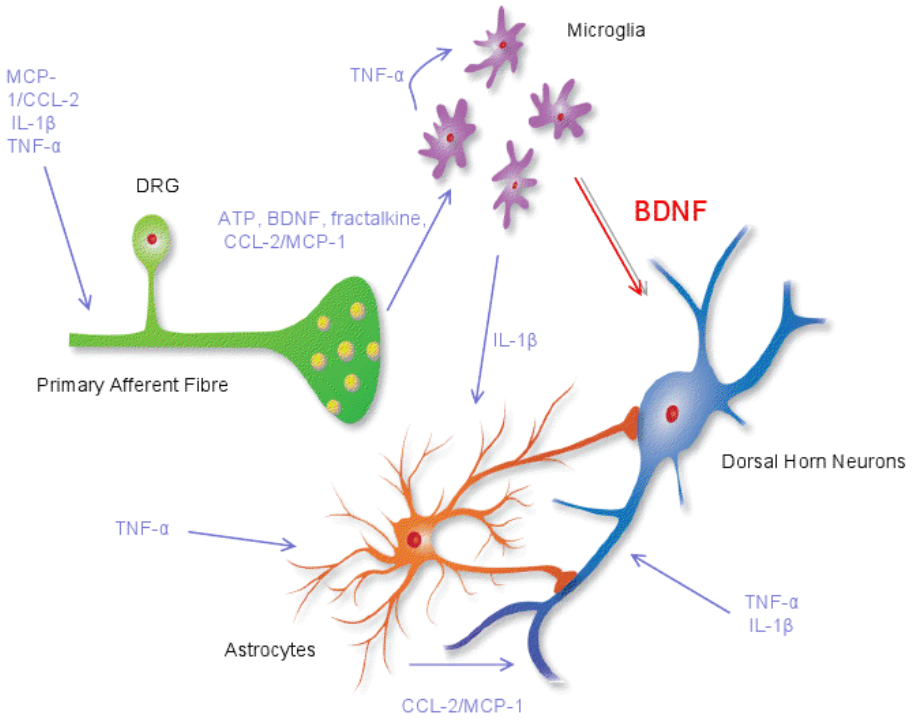

C
D
R
V
Islet neuron
Radial neuron
Vertical neuron

Fig. 1.2 Illustration of morphologies of *substantia gelatinosa* neurons.

Islet neurons have elongated dendrites >400μm in the rostro-caudal direction. Vertical neurons have dendrites going ventrally to the soma with limited mediolateral spread. Radial cells have dendrites extending in all directions.

Reproduced from Balasubramayan, S, PhD thesis, University of Alberta, 2006.

A


Microglia
TNF-α
MCP-
1/CCL-2
IL-1β
TNF-α
DRG
ATP, BDNF, fractalkine,
CCL-2/MCP-1
BDNF
IL-1β
Primary Afferent Fibre
Dorsal Horn Neurons
TNF-α
TNF-α
IL-1β
Astrocytes
CCL-2/MCP-1

B

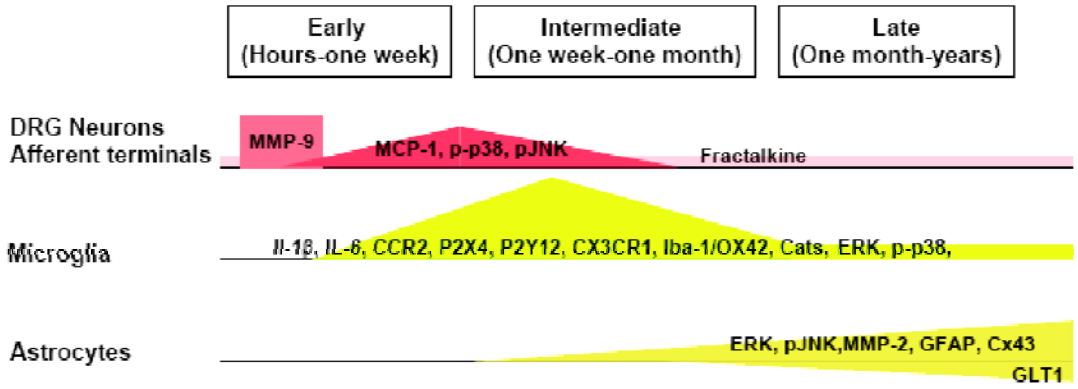

Early
(Hours-one week)
Intermediate
(One week-one month)
Late
(One month-years)
DRG Neurons
Afferent terminals
MMP-9
MCP-1, p-p38, pJNK
Fractalkine
Microglia
Il-1β, IL-6, CCR2, P2X4, P2Y12, CX3CR1, Iba-1/OX42, Cats, ERK, p-p38,
Astrocytes
ERK, pJNK,MMP-2, GFAP, Cx43
GLT1

Fig 1.3 A shows the inflammatory mediators involved in peripheral nerve injury induced activation of microglia and astrocytes and how they interact with neurons. B shows the time scale of activation of DRG neurons, microglia and astrocytes in response to peripheral nerve injury.

Modified from Biggs et.al Molecular pain 6:44 (2010)

Animal models of neuropathic pain

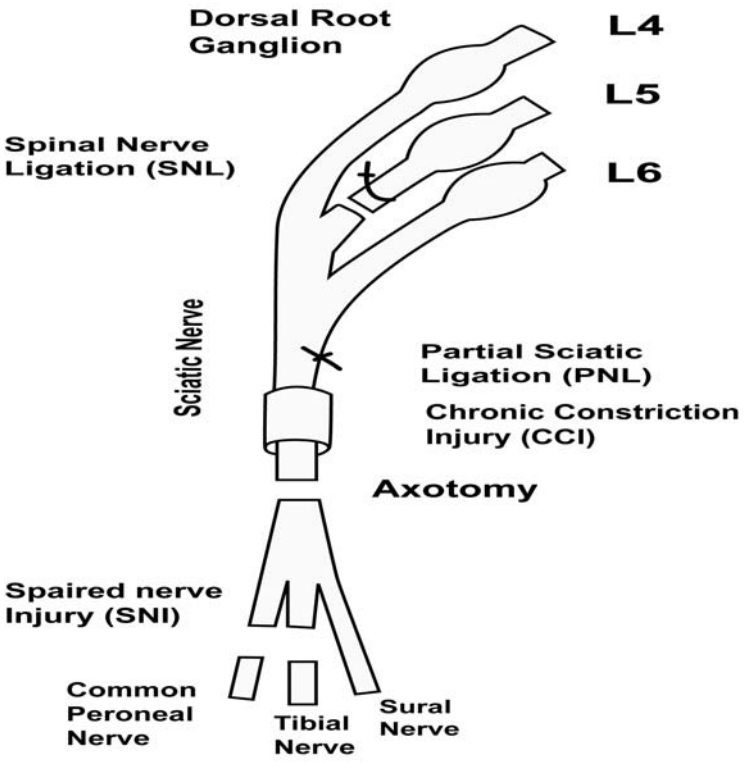


Figure 1.4 The demonstration of various types of peripheral nerve injury.

Modified from (Campbell and Meyer, 2006)

Sham nerve

Axotomized nerve

CCI

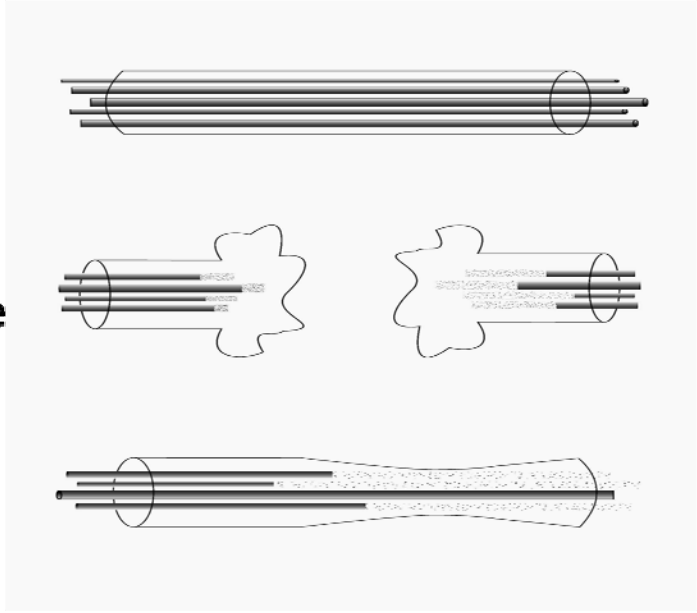


Fig 1.5 A diagram showing sciatic nerve conditions after sham, axotomy and chronic constriction injury (CCI).

After sham manipulation, the nerve is normal and healthy. After axotomy, all the axons are severed and neuroma is formed on both distal and proximal ends. After CCI, the surface axons are crushed and undergo Wallerian degeneration. Surviving axons are exposed to the inflammatory environment produced by Wallerian degeneration.

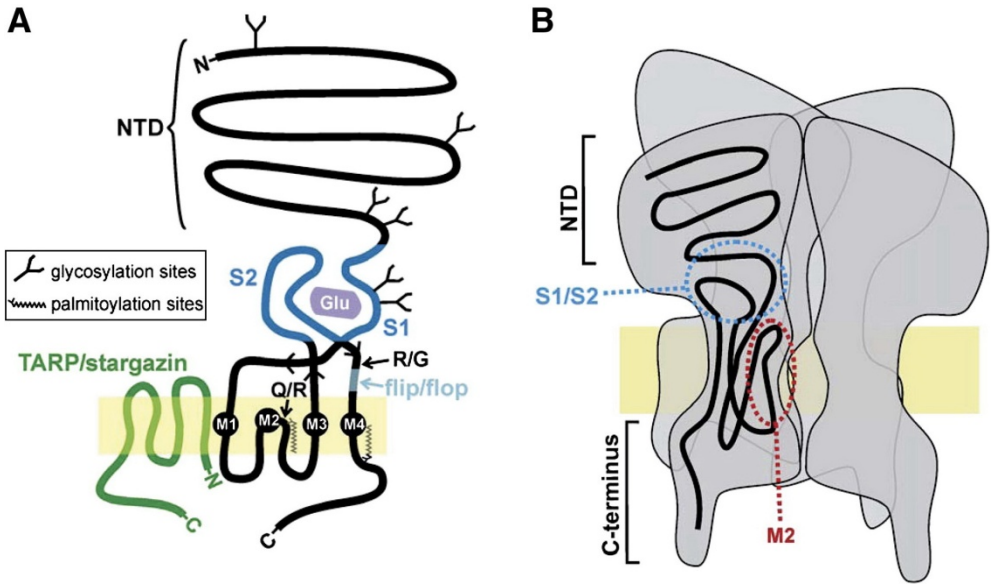

A
N
NTD
glycosylation sites
palmitoylation sites
S2
Glu
S1
R/G
flip/flop
TARP/stargazin
Q/R
M1
M2
M3
M4
N
C
C
B
NTD
S1/S2
C-terminus
M2

Fig 1.6 GluR2 Subunit structure and General Structure of the AMPAR Complex

A. Schematic of an AMPAR subunit in the plasma membrane. N-terminal domain (NTD), S1 and S2 ligand binding domains, membrane spanning domains (M1, M3 and M4), Q/R and R/G RNA editing sites, flip/flop alternatively spliced region, glycosylation and palmitoylation sites are indicated. The associated TARP/stargazing is also shown. B. Schematic of predicted 3D structure of the tetrameric AMPAR complex, with NTD, S1 and S2, M2 and C terminus regions indicated.

Reproduced from (Isaac et al., 2007)

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Chapter 2. GENERAL METHODS

All experimental procedures complied with the guidelines of the Canadian Council for Animal Care and the University of Alberta Health Sciences Laboratory Animal Services Welfare Committee.

2.1 Surgical Procedure for the Sciatic Nerve Injury

All the surgical procedures were conducted under aseptic conditions using the techniques outlined in the health sciences lab animal services "Aseptic techniques in surgery" manual, provided by the University of Alberta Health Sciences Laboratory Animal Services. For each rat, an animal surgery data sheet was completed with information on the surgical procedure such as date, rat I.D. number, weight, time taken for completion of the surgery, surgical treatment (CCI, axotomy or sham) and complications during surgery, if any. 19 day male Sprague Dawley rats were used as this strain is commonly used for neuropathic pain studies. Moreover, we have previously established that 19day old rats routinely develop signs of allodynia and hyperalgesia when subjected to CCI (Balasubramanyan et al., 2006b) (see Fig. 2.1A).

2.1.1 Surgical procedure for chronic constriction nerve injury (CCI)

This surgical procedure was developed based on cuff-induced CCI to standardize the degree of constriction of the sciatic nerve (Balasubramanyan et al.,

2006b;Mosconi and Kruger, 1996). The polyethylene cuffs were made from PE90 tubing (ID 0.86mm, OD 1.27mm; Intramedic Clay Adams, Becton Dickinson and Co, Sparks MD). Tubes were cut approximately 2mm long and slit open longitudinally to make cuffs one day before surgery. They were soaked in ethanol (95%) and then transferred to sterile saline before the start of the surgical procedure. All the instruments were sterilized before surgery.

The rat was put on an inhalation mask with 5% isoflurane (O_2 @ 4-5L/min) to be anesthetised. Following loss of consciousness, the rat's pedal reflex was tested to make sure the surgical plane of anesthesia was achieved and then the isoflurane was reduced to 2% (O_2 @ 1-2L/min) to maintain anaethesia. The left mid-thigh region was shaved with a razor blade (Wilkinson Sword Classic, Shick Warner-Lamberta Canada, Ontario), wiped with diluted antiseptic soap and cleaned with ethanol. The body temperature was maintained using a hot water pad during the procedure.

A small incision was made at the mid-thigh level using a surgical blade (08-918-5C Fisher Healthcare, Hoston TX) and the underlying muscle layers were slit open by the blade and curved forceps with care not to damage nerves. The left sciatic nerve was exposed and gently lifted by blunt forceps. Two polyethylene cuffs were put 2mm apart on each nerve. The wound was then closed with 4-O silk sutures (FS-2, ETHICON Inc., Somerville, New Jersey, USA). Antiseptic Betadine

was applied to the wound and surrounding area to minimize risk of infection. The rat was then returned to its home cage and monitored until fully recovered.

2.1.2 Surgical procedure for sciatic nerve axotomy

The surgical procedure was similar to sciatic nerve CCI except for the treatment to sciatic nerve. After the incision was made on the left mid-thigh level, the sciatic nerve was exposed and gently lifted up. The nerve was then sectioned proximal to its bifurcation into the tibial and the peroneal divisions. A 5 mm segment of nerve was removed to prevent regeneration.

2.1.3 Surgical procedure for sciatic nerve sham

For sham surgery, the sciatic nerve was exposed but not deliberately manipulated.

All animals were monitored at least 3 times a day for any signs of abnormal behaviour such as excessive vocalization, lack of grooming or lack of weight gain after surgery.

2.2 Preparation of spinal cord slices

Methods for *in-vitro* recording from *substantia gelatinosa* (lamina II) neurons in 32-42 day old (13-24days after surgery) Sprague-Dawley rats were similar to those described previously (Balasubramanyan et al., 2006b;Lu et al., 2006) (Fig. 2.1A). The animals were anesthetized with a large overdose of intraperitoneal urethane (1.5g/kg). After cutting open the skin and muscle layers, a transverse cut was made through the vertebral column at mid-thoracic level. A dorsal laminectomy was performed to expose the spinal cord and the spinal column with attached dorsal vertebrae was removed and placed in a dish of ice-cold preoxygenated (95% O_2-5% CO_2) dissection solution containing (mM): 118NaCl, 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, 1 kynurenic acid. Kynurenic acid here is used to block excitatory amino acid receptors and prevent over excitability of the neuron during the preparation process. The lumbar enlargement was isolated and excised with attached dorsal and ventral roots. After the removal of the dura matter, the dorsal and ventral roots were trimmed. Ventral roots were cut at their point of exit from the cord whereas a small stub (about 1mm) of dorsal roots was retained. The spinal cord then was glued onto a rhomboid-shaped block cut from 4% agar gel by cyanoacrylate glue ("vetbond", WPI, Sarasota, FL, USA). This block with attached spinal cord was glued to the bottom of a 60mm glass petri dish, submerged in ice-cold dissection solution continuously bubbled with 95% O_2-5% CO_2.

The whole dish was mounted in an ice cold water bath on a Vibratome (TPI, USA). The dorsal side of the cord was facing the blade. 300µm transverse slices were

cut with a razor blade (Wilkinson). The blade was advanced slowly to prevent the tissue from being crushed. To identify the ipsilateral and contralateral side to the sciatic injury, each slice was removed from the vibratome with a long agar tail attached to the ipsilateral side to mark it. Slices were incubated at 36˚C for 1h in a nylon mesh holding chamber in oxygenated (95% O_2/ 5% CO_2) dissection solution without kynurenic acid and then stored at room temperature.

2.3 Neuron classification

As described in chapter 1, we define neuron types based on the action potential firing patterns in response to 800ms depolarizing current pulses recorded at the holding potential of -60mV. As we saw with previous data from our lab, the neurons can be divided into 5 types: tonic, delay, phasic, transient and irregular (see Fig 3.2). Data from 'delay irregular' and 'delay tonic' neurons were normally combined into the delay neuron category (Balasubramanyan et al., 2006b). Thus in the present study, delay neurons were considered as one whole group so as to enable more meaningful comparison between the present results with axotomy and our previous studies of the effects of CCI (Balasubramanyan et al., 2006b). Data were collected from neurons with action potential amplitude over 40mV.

2.4 Electrophysiological recordings from slices

The slice was placed in the recording chamber with a diameter of 25mm and volume of approximately 1ml. The slice was fixed by a U-shaped platinum wire (Good fellow, Berwyn, PA, USA) with attached parallel nylon threads, the harp (Fig. 2.1B). The spinal cord slices were viewed with a Zeiss Axioskop FS equipped with a 4X 0.10-numerical aperture (NA) objective (Achroplan, Zeiss Canada, Toronto ON, CA), a 40X 0.75-NA water immersion objective, a 0.9-NA condenser and DIC optics. An IR-sensitive camera (MRm AxioCam, Carl Zeiss, Zeiss Canada, Toronto ON) was used to view neurons under a 40X water immersion objective. Healthy neurons had smooth appearance and round cell bodies. Cells were sampled in order from the medial to the lateral side. The electrophysiological recordings were made using a NPI SEC 05L amplifier (npi Electronic Gmbh, Tamm, Germany) in discontinuous single-electrode voltage-clamp or current-clamp mode. Patch pipettes were pulled from thin-walled borosilicate glass (ID 1.12mm, OD 1.5mm; TW-150F-4, WPI, Sarasota, FL, USA) with resistances of 5-10 MΩ when filled with intracellular solutions (see below). Neuron membrane voltage is monitored all through the experiments. Series resistance is not continuously monitored during the recording because the membrane voltage is monitored and displayed in the switching amplifier used in the experiments. If there is any change in series resistance, it will cause the membrane voltage change. Therefore consistent measuring membrane voltage suggests stable electrode sealing and neuron status.

For recording, slices were superfused at room temperature with 95% O_2-5% CO_2 saturated external recording solution containing (mM): 127NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.3 $MgSO_4$, 1.2 NaH_2PO_4, 2.5 $CaCl_2$, 25 D-glucose, with an exchange rate of 1-2ml/min. Pipettes for recording action potentials, sEPSCs, mEPSCs and eEPSCs are filled with internal solution containing (mM): 130 potassium gluconate, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 EGTA, 4 Mg-ATP, 0.3 Na-GTP, pH 7.2, 290-300mOsm. Biocytin (0.2%) was included in the patch pipette solution for future analysis of cell morphology (Horikawa and Armstrong, 1988).

Membrane excitability was quantified by the cumulative latency of action potentials evoked in ramp current commands which were delivered from -60mV at 33, 67, 100 or 133 pA/s (see Fig 3.3D-N).

Current-voltage relationships were measured under voltage-clamp using a series of step voltage commands of 200ms from -50 to 100mV. Current and voltage were measured just prior to the termination of each voltage pulse. Recorded voltage rather than command voltage was used to construct I-V plots.

The sEPSCs and mEPSCs were recorded at the holding potential of -70mV in regular external recording solution. Tetrodotoxin (TTX, 1µM) was used when recording mEPSC's and the effectiveness of TTX was determined by blockade of action potentials in current-clamp mode. For each neuron, sEPSCs or mEPSCs were recorded for a total of 3min. Neurons that failed to generate a detectable event during a 3min period were classified as silent and excluded from the

analysis. Acceptable events had a sharp onset with a 10-90% rise time of < 1ms and an exponential offset, a total duration of <50ms, and an amplitude at least 5 times the baseline noise. An example is shown in Fig. 2.2B.

It was neither necessary nor desirable to pharmacologically isolate the sEPSCs or mEPSCs by using GABA/glycine antagonists. This is because spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs, respectively) would be expected to appear as small outward currents at −70 mV because the estimated E_{Cl} for the Cl− concentrations used in our study was −80 mV. This is illustrated in Fig. 2.2C. As well as sEPSCs, occasional small outward currents, assumed to be sIPSCs, occur at −70 mV. As illustrated in Fig. 2.2D, we also found that CNQX (5 μM) eliminated all spontaneous inward current activity at −70 mV. When the holding potential was changed to 0 mV in the presence of CNQX, occasional spontaneous outward currents were detected (Fig. 2.2F). These were sIPSCs because they were attenuated by the GABA antagonist 4-[6-imino-3-(4-methoxyphenyl) pyridazin-1-yl] butanoic acid (SR95531) (10 μM,Fig. 2.2G). To further demonstrate that the sEPSCs recorded at −70 mV were not contaminated with sIPSC activity, we examined the effect of strychnine (1 μM) plus SR95531 (10 μM). Data are presented as cumulative probability plots for sIPSCs in a delay neuron from a sham-operated animal in Fig. 2.2 H and I. Blockade of GABA and glycine receptors failed to affect the amplitude distribution or IEI of sEPSCs recorded at −70 mV. In some neurons, however, the antagonist mixture actually increased sEPSC frequency. This may perhaps reflect impairment of presynaptic

inhibition or disinhibition of presynaptic excitatory neurons. Since their effects on the frequency of sEPSCs were quite variable, GABA/glycine antagonists were not used in our study. Moreover, the paucity of sIPSCs can be appreciated by comparing the recordings from a sham-operated delay neuron at −70 mV (Fig. 2.2D) with those from the same neuron at 0 mV (Fig. 2.2F); although numerous sEPSCs were seen at −70 mV, only six sIPSCs were seen at 0 mV. Therefore in the present studies, GABA/glycine antagonists were not used to isolate sEPSC or mEPSC at -70mV.

Evoked EPSCs were recorded at -70mV in the presence of SR95531 (10µM), D-AP5 (50µM) and strychnine (1µM) to block GABA, NMDA and glycine receptors. Evoked EPSCs were evoked by placing a customized bipolar stimulating electrode (Stereotrode Tungstem 3' 5M, WPI, Sarasota, FL, USA) on the dorsal root residue or near dorsal root entry zone. Stimulation was given by Master-8 pulse stimulator (ISO-Flex, AMPI, Israel). For recording single evoked EPSCs, the stimulation was given every 20sec and a group of 6 traces was collected and averaged for data analysis. For paired-pulse recording, the stimulation latency between pairs was increased by 15ms from 10ms to 145ms in 10 successive trials with time interval of 20s between each stimulation. A group of 4 series was collected for each recording and the average was used for data analysis (see Fig 4.2).

2.5 Data analysis

All data were collected using pCLAMP 8.0 (Axon Instruments, Burlingame, CA, USA). All data, except sEPSCs and mEPSCs, were analyzed using pCLAMP 8.0. Statistical comparisons were made with paired t-tests and ANOVA (with Bonferroni *post-hoc*) test as specified and appropriate, using GraphPad InStat (GraphPad Software, San Diego, CA, USA). Statistical significance was taken as $p<0.05$.

Mini analysis program (Synaptosoft, Decatur, GA, USA) was used to analyze sEPSC and mEPSC data. Peaks of events were first automatically detected by the software by setting appropriate threshold criteria. The threshold was set to be 5 times the RMS (root mean square) noise level. Then all detected events were visually re-examined and accepted or rejected subjectively. This software was also used to further analyze data. To generate cumulative probability plots to compare the amplitude and interevent intervals, a number of events (≤ 100 acquired within 3 mins of recording) from each neuron was pooled from each neuronal group of different surgical model (see Fig. 2.2A). The Kolmogorov-Smirnov two-sample test (KS test) was used to compare the distribution of events between two populations (Prescott and De Koninck, 2002). Distributions were considered significantly different if $p<0.05$.

Only monosynaptic responses were considered for drug application and analysis. Polysynaptic responses with various latency currents were excluded. An example of monosynaptic response and polysynaptic response were shown in Fig 2.4.

2.6 Nonstationary Fluctuation Analysis (NSFA)

Average single channel conductance for AMPA receptor mediated currents was calculated by nonstationary fluctuation analysis (NSFA) on miniature EPSC (mEPSC) recorded from lamina II interneurons.

Noise analysis was first introduced by Katz and Miledi in 1972 (Katz and Miledi, 1972). It provided an estimation of single-channel current and conductance by analysis of voltage noise regardless to the number of channels recorded and the channel open probability. Variance of fluctuations produced by a homogeneous population of independent channels can be described as

$\sigma^2 = var = i*I*(1-P_o)$ [1],

where i is the single-channel current, I is the macroscopic current, P_o is channel open probability and variance (var) is the square of standard deviation (σ)

Because $I=i*P_o*N$ (N is functional channel number), equation [1] can be converted to

$var=iI-I^2/N$ [2].

This predicts a parabolic relationship between variance and current amplitude (see Fig 2.3C).

Noise analysis was originally applied to equilibrium conditions as would be attained during the continued presence of agonist (Katz and Miledi, 1972). To make it applicable for channels under non-equilibruim conditions, nonstationary fluctuation analysis was introduced (Sigworth, 1980). This could be applied under non-equilibrium conditions and therefore called nonstationary. Since many physiological processes including mEPSCs are non-equilibrium in nature, NSFA is more widely useful.

Several issues need to be considered in applying NSFA to synaptic events. First, synapses can be under different clamp conditions, so proximal and distant synapses can produce current of different amplitude and kinetics. Thus selection of synaptic events is necessary. The mEPSC rise time should be fast, <0.5ms, to allow a precise alignment. There also needs to be stable baseline 10ms before and after mEPSC and no overlapping or fluctuations during the decay phase. Second, although majority of mEPSCs are monoquantal (Bergles et al., 1999), the release of neurotransmitter may still be multivesicular. To make sure the amplitude and shape of mEPSC necessarily reflect channel stochastisity, scaled mEPSCs are used to apply NSFA, the scaled NSFA (Traynelis et al., 1993). The procedure of scaling allows the contributions of other sources of noise to the minimum and leaves only channel stochasticity. The peak scaled average current

is subtracted from individual scaled mEPSC to represent the random receptor fluctuations around the mean.

The procedure for scaled NSFA is as follows: 1) Select mEPSCs suitable for the analysis; 2) Align selected events by their point of maximal rise (Fig. 2.3A) and average to obtain an averaged mEPSC (mean mEPSC); 3) Scale each mEPSC to the averaged event (Fig. 2.3B); 4) Plot the variance of fluctuations of scaled mEPSCs for each normalized event (Fig. 2.3C).

Fig 2.3C shows the analysis of the normalized and aligned data of the example illustrated in Fig 2.3B. The abscissa represents the scaled amplitudes of the mEPSC during its decay phase, the variance in mEPSC amplitude at each time point is represented on the ordinate.

Values for the normalized value for i (in arbitrary units) can be obtained by curve fitting, the exact value of i can be obtained from the equation

i = i(arbitrary units) * I (mean mEPSC amplitude in real units) [3].

Thus single channel conductance γ can be obtained from the equation

$\gamma = i/(V-E)$,

where V is the holding potential and E is the reversal potential (assuming 0mV for reversal potential) (Guire et al., 2008). In this thesis, holding potential is -70 unless stated otherwise.

It should also be noted that when P_0 is small, equation [1] simplifies to

var = i*I.

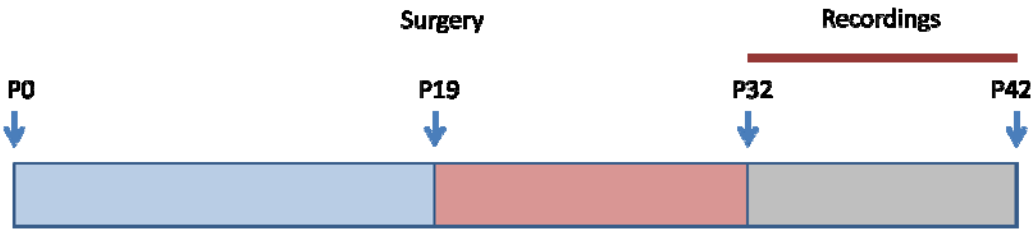
Therefore the gradient of the linear relationship seen when P_0 is small can also be used to calculate the mean single channel current (i).

2.7 Drugs

Unless otherwise stated, all chemicals were purchased from SIGMA (St. Louis, MO, USA).

TTX (Almone Laboratories) was dissolved in HPLC water as a 1mM stock solution and stored at -70˚C till use. Strychnine, D-AP5, CNQX and IEM1460 were from Tocris and made high concentration stock solution in HPLC water. SR95531 (Tocris) was dissolved in DMSO to make a stock solution. All stock solutions were stored at -20˚C unless otherwise stated. Detailed concentration will be stated in the following chapters.

A.



B.

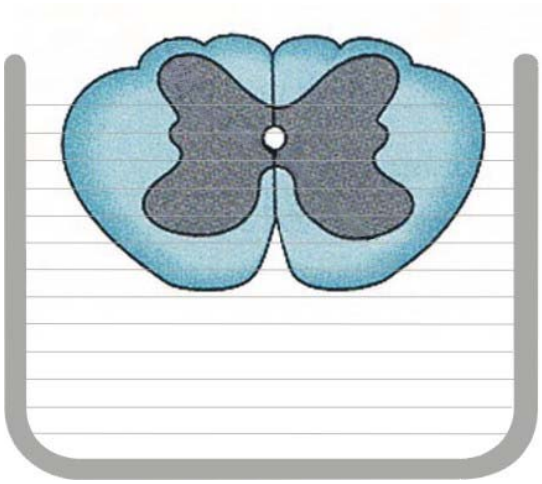


Fig 2.1

A. Time line for surgery and electrophysiological recordings. B. Illustration of harp and the fixation of a spinal cord slice.

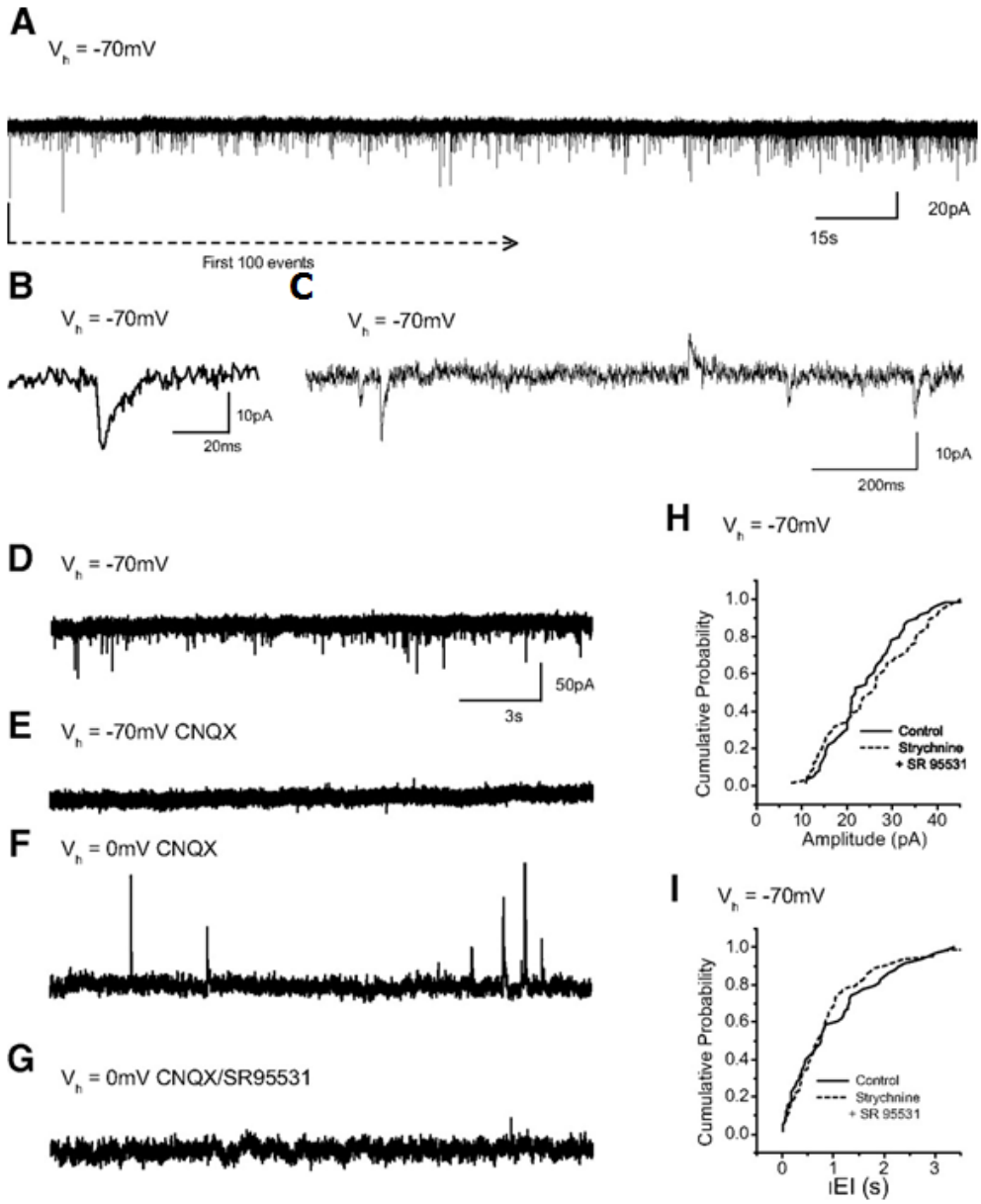
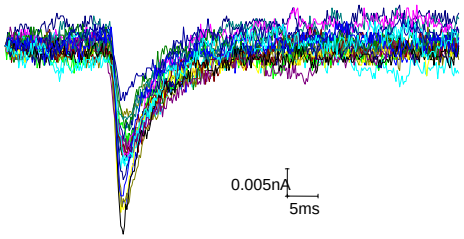

A
V_h = -70mV
20pA
15s
First 100 events
B
V_h = -70mV
10pA
20ms
C
V_h = -70mV
10pA
200ms
D
V_h = -70mV
50pA
3s
E
V_h = -70mV CNQX
F
V_h = 0mV CNQX
G
V_h = 0mV CNQX/SR95531
H
V_h = -70mV
Cumulative Probability
Amplitude (pA)
Control
Strychnine
+ SR 95531
I
V_h = -70mV
Cumulative Probability
IEI (s)
Control
Strychnine
+ SR 95531

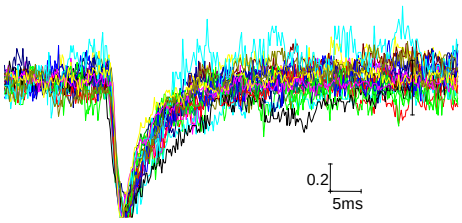
Fig 2.2 Analysis and pharmacology of spontaneous synaptic activity.

A: sample recording of 3-min spontaneous activity at −70 mV. Data were collected from a delay neuron subject to 16-day axotomy. In all neurons studied, data were analyzed from the first 100 or 50 sEPSCs observed during the 3-min recording period. For neurons where <100 events occurred in 3 min, all events were analyzed. *B*: recording of a typical sEPSC on high gain and sweep speed from a phasic neuron 16 days after sciatic nerve axotomy. *C*: another segment of the record from the neuron illustrated in *B* to illustrate the presence of both spontaneous inward and spontaneous outward currents at −70 mV. *D*: recordings of synaptic activity in a delay neuron from a sham-operated animal at −70 mV. Calibration bars in this panel also refer to *E*–*G*. *E*: recording from the same neuron in the presence of 5 μM CNQX. Note that CNQX eliminates all spontaneous inward currents at −70 mV. *F*: further recording from the same neuron at 0 mV. Note that outward currents (sIPSCs) are preserved and that sIPSC frequency is considerably slower than sEPSC frequency. *G*: blockade of outward currents recorded at 0 mV by the GABA antagonist SR95531 (10 μM). *H* and *I*: cumulative probability plots acquired from sEPSCs (at −70 mV) to show lack of effect of the neutral amino acid antagonists strychnine (1 μM) and SR95531 (10 μM). Plots were prepared from 3 min of data acquisition from a delay neuron from sham-operated animals in both the absence and the presence of antagonists. No significant change in amplitude ($P > 0.3$) or IEI were observed ($P > 0.4$, K-S test). sEPSC, spontaneous excitatory postsynaptic current; sIPSC, spontaneous inhibitory postsynaptic current; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; GABA, γ-aminobutyric acid; IEI, interevent interval; K-S, Kolmogorov–Smirnov (2-sample test); SR95531, 4-[6-imino-3-(4-methoxyphenyl)pyridazin- 1-yl]butanoic acid (gabazene).

A


0.005nA
5ms

B


0.2
5ms

C

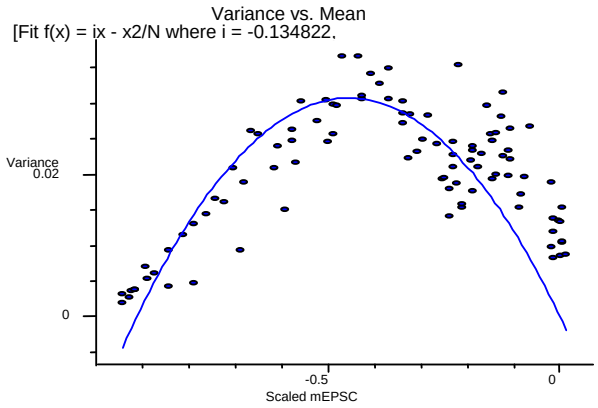
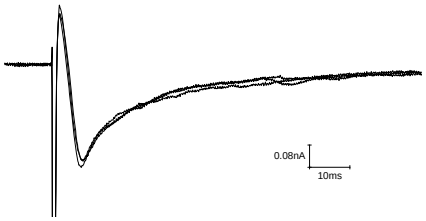

Variance vs. Mean
[Fit f(x) = ix - x2/N where i = -0.134822.
Variance
0.02
0
-0.5
0
Scaled mEPSC

Fig 2.3 An example of NSFA analysis

A is the aligned events detected from one mEPSC recording from a tonic CCI neuron. B is the normalized events from A. C is the NSFA plot made by Clampfit 9.0 from B.

Monosynaptic



Polysynaptic

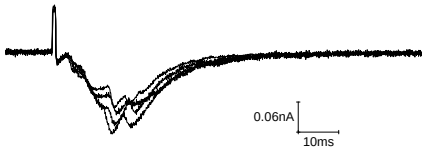


Figure 2.4 Example of monosynaptic and polysynaptic evoked EPSC.

Monosynaptic eEPSC is shown on the left panel, which was recorded at -70mV in the presence of SR95531, D-AP5 and strychnine. Polysynaptic eEPSC is shown on the right panel, which was recorded at -50mV in the presence of SR95531, D-AP5 and strychnine.

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Chapter 3 Effects of Sciatic Nerve Axotomy on Excitatory Synaptic Transmission in Rat Substantia Gelatinosa

A version of this chapter has been published.

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3.1 Introduction

Peripheral nerve injury can promote chronic neuropathic pain. Clinically, a variety of types of traumatic nerve injury are observed, ranging from complete severance of a nerve as in amputation of a limb, to more common constriction injury that accompanies a traumatic insult and/or a disease state. These two situations are modeled in experimental animals by complete section of the sciatic nerve, axotomy (Wall and Gutnick, 1974), or by various types of chronic constriction injury, CCI (Mosconi and Kruger, 1996;Kim and Chung, 1997).

Although both types of injury can produce chronic pain in humans, axotomy and CCI send quite different signals to the CNS and may therefore promote different types of changes in the spinal dorsal horn (Moore et al., 2002). In CCI, axons close to the edge receive crush injury and their distal portions undergo Wallerian degeneration, which makes the survival axons expose to the inflammatory environment and induces ectopic discharges (Ma et al., 2003;Amir et al., 2005). However in axotomy, all axons are severed and the proximal axons "die back" from the site of transaction with minimal degeneration (Tandrup et al., 2000). Thus the severed axons are not exposed to an environment where active degeneration takes place. Therefore it might be argued that axotomy is a weaker stimulus for central sensitization than CCI (see chapter 1).

Studies from our laboratory have shown that 13-24 days of sciatic nerve CCI produces a characteristic "electrophysiological signature" or pattern of changes

in synaptic excitation of five different electrophysiologically defined neuronal phenotypes in the rat substantia gelatinosa. CCI increases excitatory synaptic drive to four of the five neuronal types and diminishes the excitation of one large population of neurons, those exhibiting a tonic discharge pattern (Balasubramanyan et al., 2006b). Since the onset of this "electrophysiological signature" coincides with the appearance of mechanical allodynia and hyperalgesia (Balasubramanyan et al., 2006b), it is likely the basis for the "central sensitization" accompanying CCI.

Because it is impossible to determine whether axotomy produces "central sensitization" by behavioural tests, it is especially instructive to explore the effect of sciatic axotomy from the aspect of electrophysiological changes in spinal dorsal horn. Thus in the present study, the effects of axotomy on *sustantia gelatinosa* neurons were compared with those of CCI. From a clinical perspective, the present work also explores the etiology of total nerve section that may produce "phantom limb pain" as opposed to nerve-trauma-induced neuropathic pain.

3.2 Methods

Surgery for sham, axotomy, CCI and slice preparation were done as described in chapter 2.

3.2.1 Electrophysiology

Solutions, equipment and analysis for whole-cell recordings were described in chapter 2.

All neurons were categorized on the basis of their discharge pattern in resonse to depolarizing current pulses from -60mV (see chapter 2) (Fig 3.2). Current-voltage (I-V) relationships were determined under voltage-clamp using a series of 200ms voltage commands (see chapter 2). Membrane excitability was quantified by examining discharge rates in response to ramp-current commands. Cumulative latencies for the first, second, third and subsequent APs were noted (Fig 3.3). Miniature EPSC and sEPSC were recorded as described in chapter 2. In most cases, analysis was carried out on the first 100 events. For neurons that failed to generate 100 events in 3min, data were analyzed from all events observed. Because they generally had less spontaneous activity than that of other cell types, 50 events were analyzed in phasic and irregular neurons. Details of the numbers of neurons and events analyzed are shown in the legends to Figs. 3.4 and 3.5.

3.2.2 Iba1 immunohistochemistry

As was done for electrophysiological studies, rats were anesthetized with an overdose of urethane (1.5 g/kg, ip) and laminectomy was performed after the

cessation of respiration and loss of ocular and nociceptive reflexes. The slices used for immunohistochemical analysis and for electrophysiological studies were obtained from the same lumbar segments. A few additional slices were prepared from the thoracic region of the cords. All slices were fixed in 10% formalin solution for 5 min. Fixed slices were then incubated for 30 min in a blocking solution containing 10% horse serum, 0.03% hydrogen peroxide, and 2.5% Triton in phosphate buffered saline (PBS). After blocking, the slices were incubated in a primary antibody solution containing 1:1,000 anti–ionized calcium binding adaptor molecule 1 (Iba1, #01-1974; Wako), which labels microglia, 1% horse serum, and 2.5% Triton in PBS at 4°C overnight. The slices were then washed and incubated with the secondary antibody biotinylated immunoglobulin G (1:200, Serotec) for 30 min followed by further washes and incubation with horseradish peroxidase–conjugated strepavidin (1:200, Vector Laboratories, Burlingame, CA) for 30 min (both were diluted in PBS containing 1% horse serum). The labeling was visualized using diaminobenzidine–hydrogen peroxide (Sigma). Incubation with secondary antibody alone failed to generate any noticeable labeling.

3.3 Results

3.3.1 Microglial activation

Activation of spinal microglia is one of the primary consequences of sciatic nerve injury (Coull et al., 2005;Tsuda et al., 2003;Zhang and De Koninck, 2006). To

compare the effects of axotomy and CCI, we used the microglial-specific calcium binding protein Iba1 as a microglial marker. Although this protein is present in resting microglia, it is upregulated when they are activated (Lai and Todd, 2008;Ito et al., 1998). The Iba1 antibody used in our experiments exclusively labels microglia and does not colocalize with microtubule-associated protein 2 in neurons, glial fibrillary acidic protein in astrocytes, or 2',3'-cyclic nucleotide 3'-phosphohydrolase in oligodendrocytes. Photomicrographs of the dorsal horn ipsilateral or contralateral to the CCI, axotomy, or sham lesion are shown in Fig. 3.1, A–F. The pattern of Iba1 staining for microglia produced by axotomy is compared with that produced by CCI. Iba1 staining is especially dense in ipsilateral superficial laminae after CCI compared with sham surgery (Fig. 3.1, A and C) or to the contralateral side (Fig. 3.1, D and F); however, axotomy produces a different pattern because the ipsilateral staining is spread more deeply and is associated with the central canal (Fig. 3.1B). The contralateral control is shown in Fig. 3.1E.

These trends of microglial activation were confirmed by a more quantitative analysis. Data were collected from four sham-operated animals (14–21 days postsurgery), four subject to sciatic CCI (15–30 days postsurgery), and four subject to sciatic axotomy (21 days postsurgery). The dorsal horn was divided into a standard set of six 0.5 × 0.5-mm squares (Fig. 3.1G) and Iba1-positive profiles, such as those illustrated in Fig. 3.1H, counted for each square. Four sections were analyzed per animal for sham, axotomy, and CCI conditions and

cell counts varied <5% between two independent evaluators. Results are plotted in Fig. 3.1I. This confirms that Iba1 staining was increased for both CCI and axotomy in ipsilateral quadrant 2 (ipsi 2), which corresponds to the superficial dorsal horn. Even sham-operated animals exhibited increased staining in this quadrant but not in other parts of the superficial dorsal horn. By contrast, axotomy had a more pronounced effect in ipsilateral quadrants 4 and 5 (ipsi 4 and 5), corresponding to deeper laminae and to the midline portion of the cord. There was no clear effect of CCI or axotomy on Iba1-positive profiles in the ipsilateral ventral horn (ventral) or on the contralateral quadrant of the dorsal horn (contra 4) that mirrored ipsi 4. Although no detailed analysis was made, it was noticed that the nerve-injury–induced pattern of Iba1 staining was also seen in sections from the lumbar spinal cord above the level of the lesion. This may reflect the considerable rostral and caudal projections of primary afferent fibers (Wall and Werman, 1976).

3.3.2 Behavioral consequences of axotomy

Sciatic nerve axotomy frequently induces a self-mutilatory behaviour known as autotomy, which may relate to the onset of neuropathic pain (Coderre and Melzack, 1986;Wall et al., 1979a). In a previous study using 32- to 40-day-old rats, we found that a modest amount of autotomy was present in 45% of animals at 2 week after axotomy (Abdulla and Smith, 2001b). The present study, however,

was constrained by the need to use young animals (19-day-old) for electrophysiological analysis of spinal cord neurons and we found no evidence of autotomy in any of the animals studied. We did find, however, that 2 week of CCI produce mechanical hyperalgesia and allodynia in animals that were 20 days old at the time of surgery (Balasubramanyan et al., 2006b).

3.3.3 Neuron types and effects of axotomy

In our previous studies of the effects of CCI on *substantia gelatinosa*, neurons were classified into five different types, depending on their firing pattern in response to depolarizing current as tonic, delay, phasic, transient, or irregular (Balasubramanyan et al., 2006b). More recently, it was noted that the delay category included two subgroups of neurons: delay irregular neurons and delay tonic neurons (Lu et al., 2009b). Firing patterns of tonic, delay irregular, delay tonic, phasic, transient, and irregular neurons are illustrated in Fig. 3.2, A–F. The continuous row illustrates the firing patterns evoked by increasing current intensities, as indicated in the current traces above each action potential row.

Figure 3.3A illustrates the percentage contribution of each of the six neuronal types to the whole population of neurons studied from sham animals (n = 148) and from animals subject to sciatic nerve axotomy (n = 74). The percentage contribution of each neuron type to the population was unaffected by axotomy ($\chi 2$ test, $P > 0.4$ for all comparisons). With the exception of delay tonic neurons,

that were slightly depolarized, axotomy had no statistically significant effect on the resting membrane potential (RMP) of substantia gelatinosa neurons (Fig 3.3B). Axotomy also failed to significantly affect the rheobase (Fig 3.3C).

Excitability, as measured from the cumulative latency of APs in response to a depolarizing current ramp command (Fig 3.3D), was slightly reduced in phasic neurons but unchanged in all other neuron types. Data for tonic, delay irregular, delay tonic, phasic, and irregular neurons are shown in Fig 3.3 E–I. Excitability of transient neurons was not examined because they did not always fire in response to a depolarizing ramp command.

Figure 3.3, J–N illustrates the effect of axotomy on current–voltage relationships of tonic, delay irregular, transient, phasic, and irregular neurons. Although there appears to be a small reduction in outward current in irregular neurons (Fig 3.3N), I–V relationships of the other four neuron types are scarcely affected. Insufficient data were available to present I–V plots for delay tonic neurons.

3.3.4 Effects of axotomy on sEPSCs

As we saw with CCI (Lu et al., 2009b;Balasubramanyan et al., 2006b), the predominant effect of axotomy was to alter excitatory spontaneous synaptic activity. As in previous studies from Dr. Smith's laboratory, we analyzed changes in sEPSCs by comparisons of average data values using t-tests and by cumulative

probability plots and K-S statistics. As mentioned in chapter 2, data from 'delay irregular' and 'delay tonic' neurons were combined into the delay neuron category.

A clear pattern of axotomy-induced changes emerged with regard to the IEI (Fig. 3.4, A and C–G). The IEI for sEPSCs was increased in tonic neurons, unchanged in irregular cells, and decreased in all other neuron types. These effects were significant according to both *t*-test (Fig. 3.4A) and K-S statistics (Fig. 3.4, C–F). Increased IEI in tonic neurons meant that sEPSC frequency decreased (Fig. 3.4C), whereas the decrease in IEI in delay, phasic, and transient neurons meant that frequency increased (Fig. 3.4, *D*–*G*).

Effects of axotomy on sEPSC amplitude were less obvious and more complex. Although the mean amplitude of sEPSCs in tonic neurons was unchanged (Fig. 3.4B), data from the cumulative probability plot (Fig. 3.4H) suggested that the proportion of large events increased, whereas that of small events decreased, yielding a significant difference on the K-S test. The decrease in sEPSC amplitude seen in delay cells was significant according to both a *t*-test (Fig. 3.4B) and a K-S test (Fig. 3.4I). The K-S test also yielded significant increases in sEPSC amplitude for phasic and transient neurons (Fig. 3.4, J and K) and a significant decrease for irregular neurons (Fig. 3.4L). However, none of these three changes was significant according to a *t*-test (Fig. 3.4B).

3.3.5 Effects of axotomy on TTX-resistant mEPSCs

This analysis was restricted to tonic, delay, phasic and transient neurons because insufficient data were available from irregular neurons. Despite the addition of TTX, the mean amplitudes of mEPSCs were similar to those of sEPSCs (Fig. 3.8). This similarity has been described in several types of central neurons, including hippocampal CA3 pyramidal cells (McQuiston and Colmers, 1996) and in supraoptic neuroendocrine cells of the rat hypothalamus (Wuarin and Dudek, 1993). It likely implies a small quantal content and low release probability of AP-evoked events at central synapses.

Axotomy decreased mEPSC frequency (increased IEI) in tonic neurons (Fig. 3.5, A and C). This effect—which was significant according to both a *t*-test (Fig. 3.5A) and a K-S test (Fig. 3.5C)—contrasted with effects seen in all other cell types. Although data from cumulative probability plots and a K-S test for delay neurons (Fig. 3.5D) were consistent with an increase in mEPSC frequency, analysis of mean frequency using *t*-test (Fig. 3.5A) failed to reveal a significant difference. We therefore suggest that axotomy produced only a weak tendency for mEPSC frequency to increase. Increases in mEPSC frequency for phasic and tonic neurons were significant according to both K-S tests (Fig. 3.5, E and F) and t-test (Fig. 3.5A).

Axotomy decreased mEPSC amplitude in tonic and phasic neurons (Fig. 3.5, B, G, and I), increased it in transient neurons (Fig. 3.5, B and J), but did not affect

mEPSC amplitude in delay neurons (Fig. 3.5, B and I). Differences that were significant according to a K-S test (Fig. 3.5, G, I, and J) were also significant according to a *t*-test (Fig. 3.5B).

3.3.6 Comparison of axotomy effects on sEPSC and mEPSC frequencies

To distinguish possible effects of axotomy on the frequency of presynaptic APs from other effects on presynaptic terminals, we compared its actions on mEPSC frequency with its effect on sEPSC frequency. In tonic cells, axotomy decreased both sEPSC and mEPSC frequencies (increased IEI; Fig. 3.6). By contrast, in delay neurons, axotomy increased sEPSC frequency (decreased IEI; Fig. 3.6) but had little or no effect on mEPSC frequency. This suggests that increases in the frequency of presynaptic APs may explain the axotomy-induced reduction of IEI of sEPSCs in delay neurons. Axotomy reduced the IEI of sEPSCs and mEPSCs in phasic and transient neurons by about the same amount (Fig. 3.6).

3.4 Discussion

The essential finding of this study is that sciatic nerve axotomy produces perturbations in excitatory synaptic transmission in the *substantia gelatinosa* that resemble those seen following sciatic CCI (Balasubramanyan et al., 2006b). In other words, when changes in tonic, delay, phasic, transient and irregular

neurons are considered, the “electrophysiolgocical signature” of axotomy resembles that of CCI. Further analysis of the data is consistent with the possibility that axotomy increases excitatory synaptic drive to excitatory neurons yet decreases that to inhibitory neurons. Both types of change, which are also seen with CCI (Balasubramanyan et al., 2006b), could contribute to the overall increase in dorsal horn excitability that follows peripheral nerve injury (Dalal et al., 1999;Woolf, 1983).

3.4.1 Comparison of the effects of axotomy and CCI

Neither axotomy nor CCI has any major effect on excitability or current–voltage (*I*–*V*) characteristics of substantia gelatinosa neurons (Fig. 3.3 compared with Balasubramanyan et al. 2006). The similar effects of the two manipulations on synaptic transmission are illustrated in Fig. 3.7, which shows the percentage changes in four indices of synaptic transmission in various neuronal types. As seen with CCI (Balasubramanyan et al., 2006b), the effects of axotomy on tonic neurons are qualitatively different from its action on other neuron types. IEIs of both sEPSC (Fig. 3.7A) and mEPSC (Fig. 3.7B) are increased by both axotomy and CCI in tonic neurons. By contrast, both manipulations lead to a decrease of IEIs in delay, phasic, and transient neurons.

There is less correspondence between the effects of axotomy and those of CCI on sEPSC amplitude (Fig. 3.7C). Axotomy-induced changes in sEPSC amplitude

were small (Fig. 3.7C) and those for tonic, phasic, transient, and irregular neurons failed to reach significance on a *t*-test (Fig. 3.4B). I therefore suggest that axotomy exerted only minimal effects on sEPSC amplitude. This contrasts with the more obvious effects of CCI on sEPSC amplitude. This difference may be explicable in terms of differential effects of axotomy and CCI on Ca^{2+} currents in primary afferent terminals. CCI has been reported to increase expression of N-type Ca2+ channels in spinal cord (Cizkova et al., 2002). Axotomy decreases Ca^{2+} channel currents in DRG cell bodies and may exert a similar effect at presynaptic terminals (Abdulla and Smith 2001b; Baccei and Kocsis 2000). Moreover, Spinal nerve ligation (axotomy) could lead to diminished releasable endoplasmic reticulum Ca^{2+} stores in DRG cell bodies (Rigaud et al., 2009), which may suggest diminished Ca^{2+} store in the primary afferent terminals. Alternatively, the relatively small effects on sEPSC amplitude may reflect the probability that axotomy is associated with less peripheral inflammation than that of CCI (see chapter 1) or that it is less effective than CCI in increasing BDNF concentration in substantia gelatinosa (see following text).

For tonic neurons, the axotomy-induced decrease in mEPSC amplitude parallels the effects of CCI (Fig. 3.7D). However, axotomy does not increase mEPSC amplitude in delay neurons, whereas small increases are seen after CCI (Fig. 3.7D). This again may reflect a “weaker” effect of axotomy compared with that of CCI, perhaps as a consequence of the smaller amount of inflammation seen with axotomy compared with that seen with CCI.

Since the "electrophysiological signatures" in the *substantia gelatinosa* are similar in the two models, it is likely that increased primary afferent activity is a major contributor to central sensitization for both types of injury. One small exception to this generalization is the difference between the effects of axotomy and CCI on mEPSC amplitude in transient cells. Insufficient data were available to make comparisons for phasic and irregular neurons.

3.4.2 Changes in inhibition?

It is well established that impediment of inhibition in the dorsal horn contributes to the onset of central sensitization (Coull et al., 2003;Sandkuhler, 2009;Moore et al., 2002;Scholz et al., 2005;Laird and Bennett, 1992). This is thought to involve downregulation of the Cl^- transporter KCC2 and collapse of the Cl^- concentration gradient such that GABA and glycine may produce smaller hyperpolarizing responses or even depolarizing responses (Coull et al., 2005;Keller et al., 2007;Miletic and Miletic, 2008;Prescott et al., 2006). It should be noted, however, that these changes have been studied mostly in lamina I. By contrast, the role of postsynaptic inhibition in the physiology of lamina II is currently a matter of debate. Although some authors (Labrakakis et al., 2009;Santos et al., 2007) argue that inhibition is of minor importance in this region, others have underlined the importance of specific inhibitory pathways (Lu and Perl, 2003). Nevertheless, the apparent paucity of sIPSCs in *substantia*

gelatinosa neurons seen under our experimental conditions led us to concentrate on the effects of axotomy on excitatory transmission. It is of course possible that some of the changes we observed result from nerve-injury-induced alterations in presynaptic inhibition of excitatory primary afferent transmission (Laird and Bennett, 1992). Another possibility is that input to lamina II is altered following changes in inhibition in deeper laminae, where inhibition seems to play a greater role than that of excitation (Schneider, 2008). These possibilities remain to be tested.

Another way in which peripheral nerve injury may impede inhibition involves apoptosis of GABAergic interneurons (Scholz et al., 2005). Since the percentage contribution of tonic neurons to the whole population was not reduced by axotomy (Fig. 3.2A), apoptosis may not have occurred under our experimental conditions. The difference between our observations and those of Scholz et al. (2005) may reflect our use of axotomy rather than various types of constriction injury. These authors also noted that apoptosis of GABAergic interneurons takes about 4 weeks to fully develop, whereas in our experiments animals were subject to axotomy for <22 days.

3.4.3 BDNF, microglia activation and peripheral nerve injuries

Several lines of evidence implicate microglial-derived BDNF in the onset of central sensitization (Coull et al., 2005;Lu et al., 2007;Lu et al., 2009b;Millan, 1999;Tsuda et al., 2003). Thus I have also compared the effects of prolonged BDNF exposure on excitatory synaptic transmission with those of axotomy and CCI (Fig. 3.7). BDNF data were obtained from our previously published work, in which spinal cord organotypic cultures were exposed to BDNF for 5–8 days (Lu et al., 2007;Lu et al., 2009b). In terms of IEI, the effects of BDNF nicely parallel the actions of both axotomy and CCI for both sEPSCs and mEPSCs (Fig. 3.7, A and B). There is, by contrast, much less correspondence between the actions of the three manipulations on event amplitude (Fig. 3.7, C and D). There appears to be more similarity between the actions of BDNF and CCI than that with BDNF and axotomy. In this regard, it is pertinent to mention that the differential patterns of microglial activation produced by axotomy compared with those produced by CCI are paralleled by their differential effects on the pattern of BDNF immunoreactivity (Cho et al., 1998). Thus CCI increases both Iba1 activation (Fig. 3.1A) and BDNF immunoreactivity in superficial laminae (Cho et al., 1998), whereas axotomy is more effective in increasing both neurotrophin content and microglial activation in deeper laminae (Fig 3.1B and Cho et al. 1998). These differences may reflect the different patterns of primary afferent fiber damage seen with axotomy compared with those seen with CCI (Basbaum et al., 1991;Kajander and Bennett, 1992) and the different sites of termination of these

fibers throughout the dorsal horn (Todd and Koerber, 2005;Mirnics and Koerber, 1997).

Because there are now several reports describing actions of BDNF on voltage-gated Na+ and K+ channels, it is perhaps surprising that no marked changes in neuronal excitability were seen in either the present study, in that of Balasubramanyan et al. (2006), or in our previous studies of BDNF effects in the dorsal horn (Lu et al., 2007;Lu et al., 2009b). Although this apparent discrepancy may be conveniently ascribed to the use of expression systems in some studies (Ahn et al., 2007;Colley et al., 2007), and to differential effects of acutely and chronically applied BDNF, a 2-h application of this neurotrophin has been reported to promote a marked increase in excitability of auditory brain stem neurons (Youssoufian and Walmsley, 2007). Long-term application of BDNF, however, does not exert much effect on Na^+ channels in primary afferent neurons (Oyelese et al., 1997). Thus the simplest explanation for the lack of effect of BDNF on excitability in our study is that different neuronal types are affected in different ways by this neurotrophin. Alternatively, BDNF, axotomy, and CCI may affect active conductances in dendrites, which is a change difficult to detect using cell body recordings.

3.4.4 Mechanism of axotomy-induced changes

In delay neurons, axotomy was much more effective in increasing sEPSC frequency than in increasing mEPSC frequency. This suggests that increases in the frequency of presynaptic action potentials, including those in primary afferent fibers, may account for axotomy-induced increased synaptic drive to these neurons. If this is so, how can excitatory drive to tonic neurons decrease? In these neurons, axotomy decreased both mEPSC and sEPSC. This could reflect changes in the presynaptic action potential activity and/or depression of the neurotransmitter release mechanism or perhaps disconnection of afferent axons from postsynaptic dendrites or cell bodies. Additional evidence to support the latter idea comes from ultrastructural studies that show that CCI promotes transient loss of the excitatory synaptic terminals of nonpeptidergic nociceptive fibers in substantia gelatinosa (Bailey and Ribeiro-da-Silva, 2006). These fibers form the synaptic terminals of type 1 synaptic glomeruli (Ribeiro-Da-Silva and Coimbra, 1982) that associate with GABAergic neurons (Todd et al., 1996), many of which display a tonic firing pattern (Labrakakis et al., 2009;Lu and Perl, 2003). In more recent studies, TRPA-1 containing non-peptidergic C-fibres was also found not to make contact with excitatory neurons (Uta et al., 2010;Todd, 2010).

3.4.5 Functional significance of axotomy-induced changes

The axotomy-induced increase in sEPSC frequency in delay neurons may contribute to an overall increase in dorsal horn excitability. The axotomy-induced

decrease in sEPSC/mEPSC frequency in tonic neurons may indicate a decrease in synaptic drive to a population of inhibitory neurons. As already mentioned, axotomy-induced changes in mEPSC/sEPSC amplitude are modest compared with changes in frequency. It is thus unlikely that changes in event amplitude have a major bearing on dorsal horn excitability.

The biological significance of changes in phasic, transient, and irregular neurons is difficult to assess because little is known about the neurotransmitter phenotype of the neuron types. In fact, whereas some phasic neurons have been reported to behave as excitatory interneurons (Lu and Perl, 2005), others may be inhibitory (Heinke et al., 2004;Zhang and Dougherty, 2011).

In general, the effects of axotomy and CCI are more similar than they are different. When all neuron types are considered, they promote a similar "electrophysiological signature" in the *substantia gelatinosa* that may represent a "snapshot" of central sensitization following peripheral nerve injury. Unraveling the mechanism associated with the generation of this "electrophysiological signature" may therefore provide vital information relating to the etiology of the central sensitization that underlies neuropathic pain.

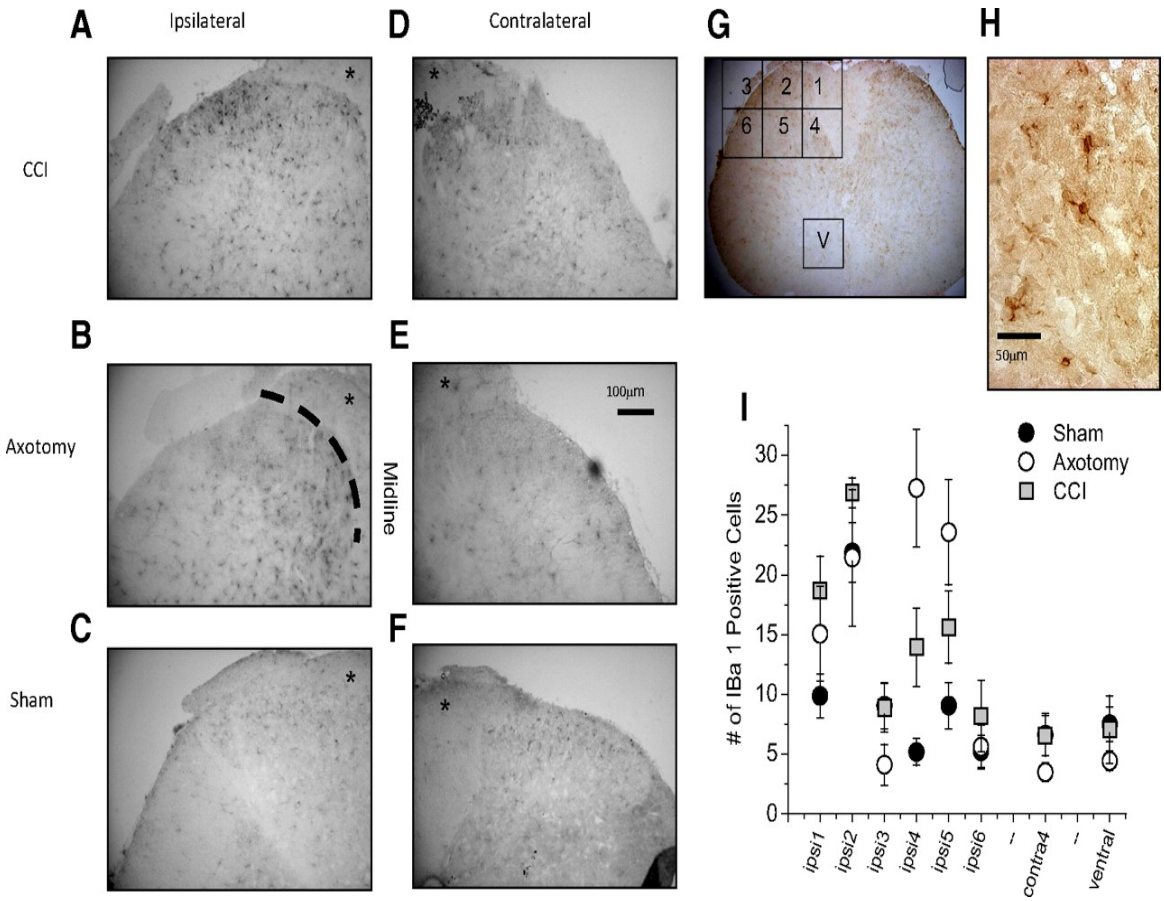
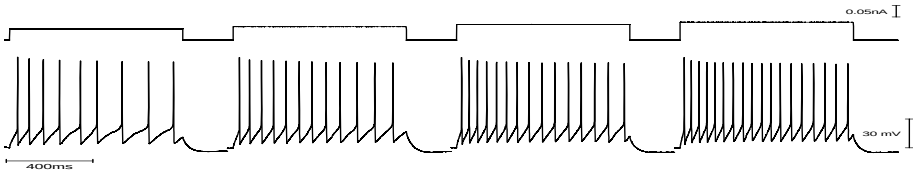

A
Ipsilateral
D
Contralateral
G
H
CCI
3
2
1
6
5
4
V
50μm
B
E
100μm
Axotomy
Midline
I
Sham
Axotomy
CCI
C
F
Sham
# of IBa 1 Positive Cells
0
5
10
15
20
25
30
ipsi1
ipsi2
ipsi3
ipsi4
ipsi5
ipsi6
contra4
ventral

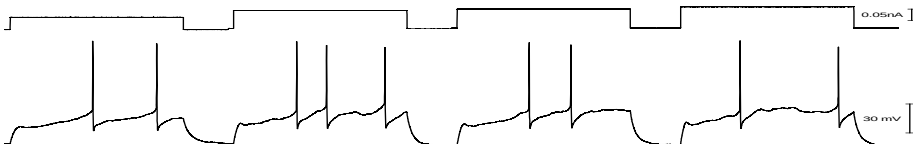
Fig 3.1 Ionized calcium binding adaptor molecule 1 (Iba1) staining of microglial cells in rat spinal cord slices.

A–*C*: staining of dorsal quadrant of slice ipsilateral to chronic constriction injury (CCI, *A*), axotomy (*B*), or sham surgery (*C*). *D*–*F*: staining of dorsal quadrant of slice contralateral to CCI (*D*), axotomy (*E*), or sham surgery (*F*). The 100-µM scale bar in *E* applies to *A*–*F* of the figure. *Denotes fasciculus gracilus. Dotted line represents gracilus/dorsal horn border. *G*: photomicrograph of whole spinal cord slice showing 6 standard locations of 500-µM squares used for cell counting (V, ventral). *H*: high-power image showing Iba1-positive profiles. *I*: graphical representation of distribution of Iba1-positive cells in the various quadrants defined in *G*. Points represent mean counts from a total of 16 slices (4 slices from 4 animals) under each condition. Error bars are standard error (SE). Contra 4 is the contralateral mirror image of quadrant ipsi 4.

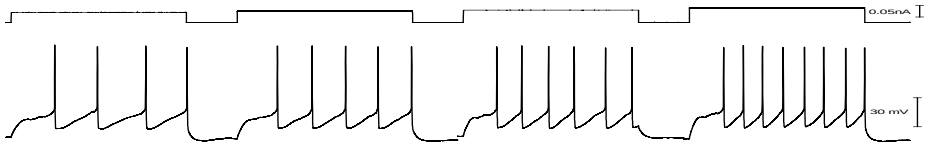
A Tonic



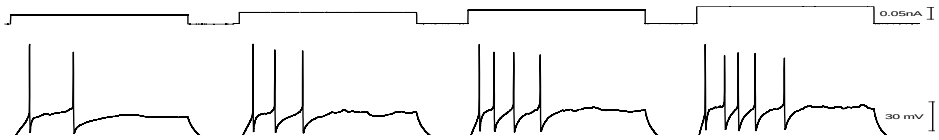
B Delay irregular



C Delay tonic



D Phasic



E Transient

F Irregular

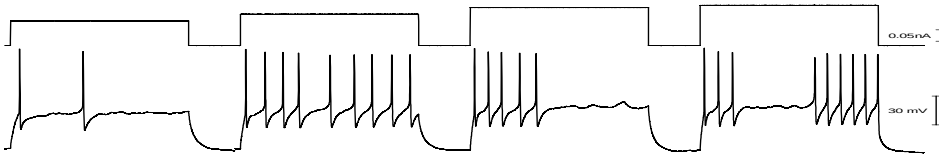


Fig. 3.2 Action potential firing patterns in *substantia gelatinosa* neurons

A–*F*: firing patterns of tonic, delay irregular, delay tonic, phasic, transient, and irregular neurons illustrated by their response to a series of 4 depolarizing current commands from a preset membrane potential of −60 mV. Current records are shown in *top row* of traces and resulting action potential (AP) discharge for 4 different current intensities shown in the *lower rows*.

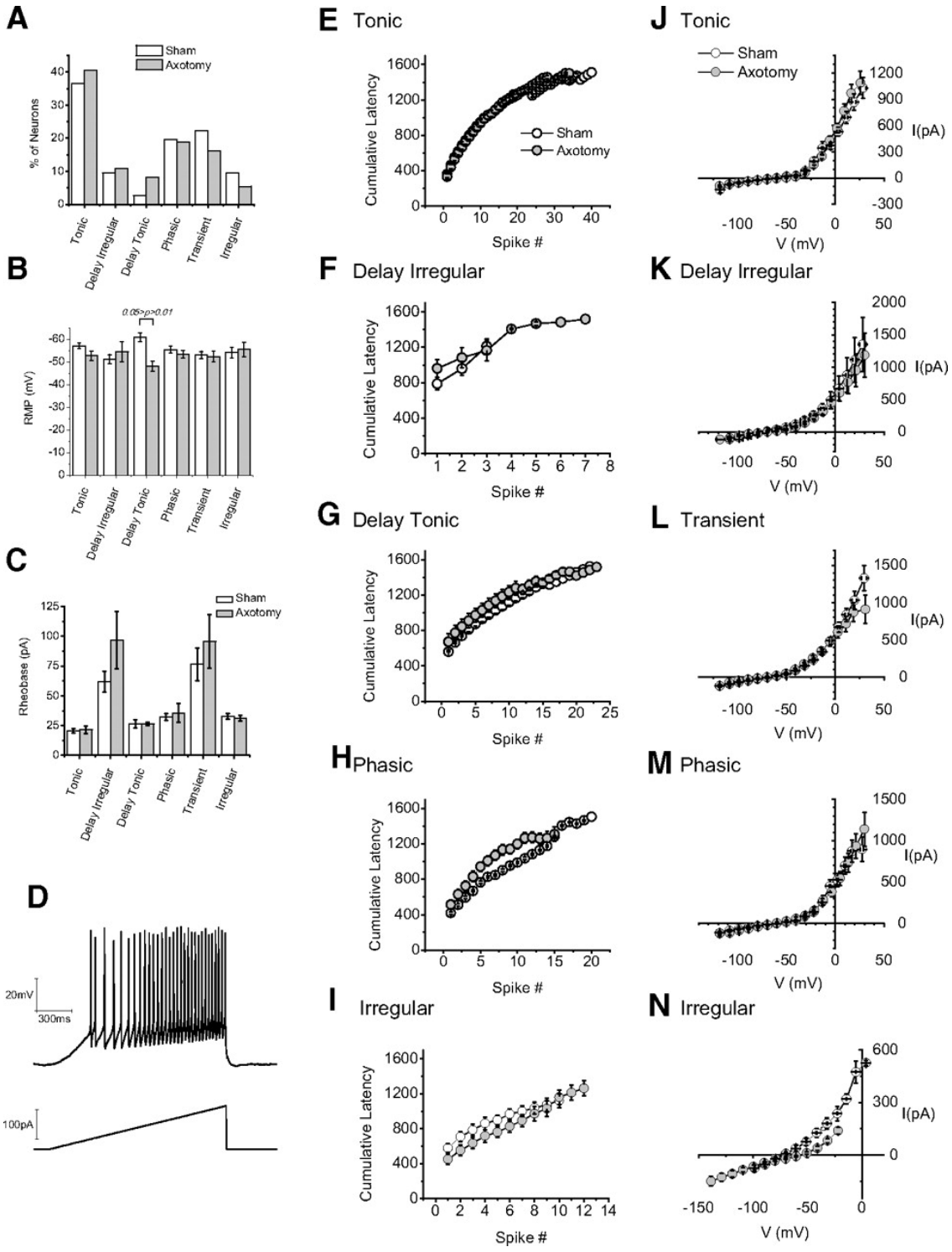

A
Sham
Axotomy
% of Neurons
Tonic
Delay Irregular
Delay Tonic
Phasic
Transient
Irregular
B
0.05>p>0.01
RMP (mV)
C
Rheobase (pA)
D
20mV
300ms
100pA
E Tonic
Cumulative Latency
Spike #
F Delay Irregular
G Delay Tonic
H Phasic
I Irregular
J Tonic
I(pA)
V (mV)
K Delay Irregular
L Transient
M Phasic
N Irregular

Fig. 3.3 Effects of axotomy on the passive and active membrane properties of substantia gelatinosa neurons.

For all bar graphs, clear bars represent data from sham-operated animals; gray bars represent data from animals subject to sciatic nerve axotomy. For line + symbol graphs, open symbols represent data from sham animals and gray symbols represent data from animals subject to axotomy. Error bars indicate SE. *A*: percentages of cell types encountered in slices from sham-operated and axotomized animals. Data from 148 neurons from the sham-operated group and 74 from the axotomy group. No significant change in relative contribution of any of the 6 neuron types to the total population was found after axotomy ($\chi 2 > 0.4$ for all comparisons). *B*: effect of axotomy on membrane potential of substantia gelatinosa neurons. The only significant difference was seen for delay tonic neurons (*n*s, sham/axotomy: tonic 80/22, delay irregular 29/13, delay tonic 11/9, phasic 47/16, transient 40/23, irregular 27/6). *C*: lack of effect of axotomy on rheobase. $P > 0.1$ for all comparisons of each group of neurons in sham and axotomy groups (*n*s, sham/axotomy: tonic 47/17, delay irregular 19/8, delay tonic 8/6, phasic 28/6, transient 19/11, irregular 22/6). *D*: recordings showing a typical experiment to measure the excitability of a tonic neuron. *E*–*I*: effects of axotomy on repetitive discharge characteristics of tonic, delay irregular, delay tonic, phasic, and irregular neurons. Neurons were excited with depolarizing current ramps (100 pA/s for tonic, delay tonic, and irregular neurons; 133 pA/s for delay irregular and phasic neurons; transient neurons not examined) and the cumulative latencies of the 1st, 2nd, 3rd, 4th, and so on spikes noted (*n*s, sham/axotomy: tonic 21/24, delay irregular 3/4, delay tonic 2/5, phasic 8/13, irregular 14/4). The only clear effect appears to be on phasic neurons (*G*) where axotomy increases cumulative latency and thus decreases excitability. *J*–*N*: current–voltage (*I*–*V*) plots for neurons from sham-operated and axotomized animals (*n*s, sham/axotomy: tonic 11/16, delay irregular 5/8, transient 22/13, phasic 12/14, irregular 8/4). Since recorded voltage rather than command voltage was used to construct *I*–*V* plots, the voltage values have a small SE. This was typically <1 mV and thus the voltage error bars are smaller than the symbols used to designate the data points.

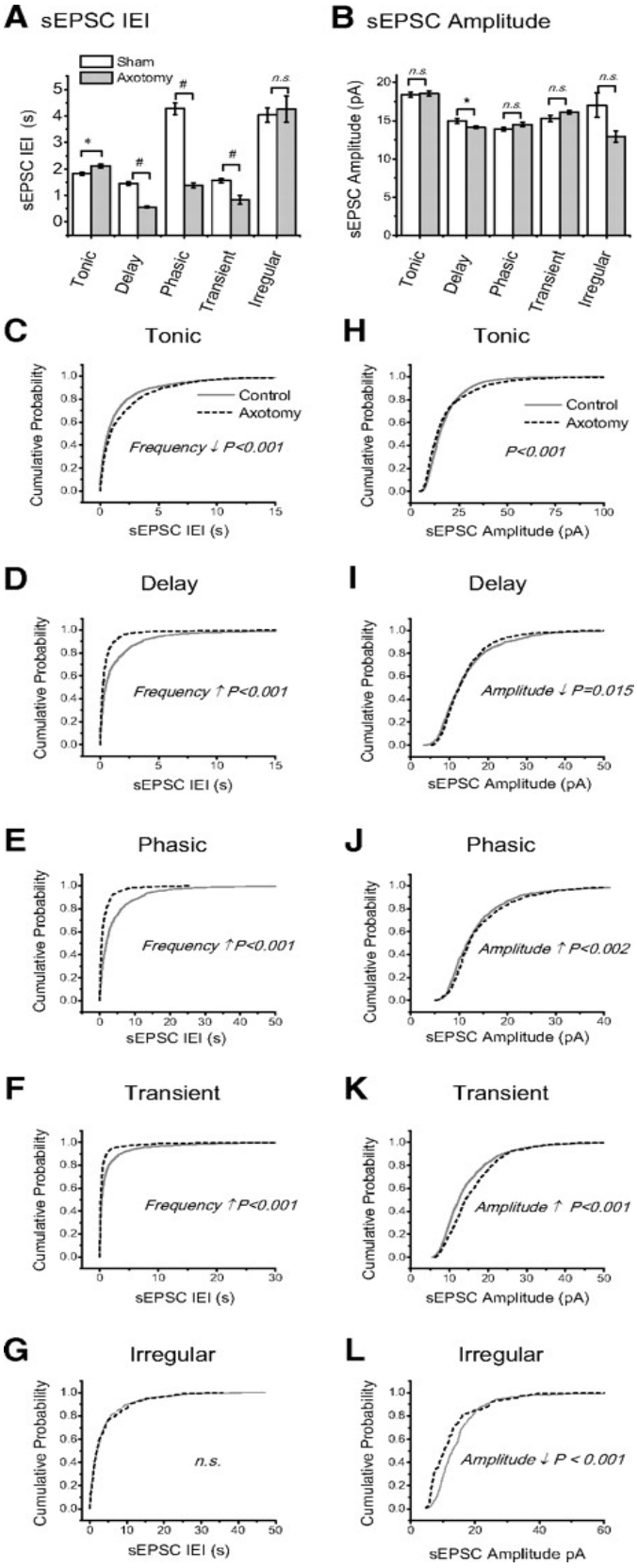

A sEPSC IEI
B sEPSC Amplitude
Sham
Axotomy
sEPSC IEI (s)
sEPSC Amplitude (pA)
Tonic
Delay
Phasic
Transient
Irregular
n.s.
C Tonic
H Tonic
Control
Axotomy
Frequency ↓ P<0.001
P<0.001
Cumulative Probability
sEPSC IEI (s)
sEPSC Amplitude (pA)
D Delay
I Delay
Frequency ↑ P<0.001
Amplitude ↓ P=0.015
E Phasic
J Phasic
Frequency ↑P<0.001
Amplitude ↑ P<0.002
F Transient
K Transient
Frequency ↑P<0.001
Amplitude ↑ P<0.001
G Irregular
L Irregular
n.s.
Amplitude ↓ P < 0.001
sEPSC Amplitude pA

Fig. 3.4 Effects of axotomy on spontaneous synaptic activity (sEPSC) in substantia gelatinosa neurons.

A: effects on mean IEI of 5 defined neuron types; holding potential was −70 mV. *B*: effects on mean sEPSC amplitude of same neuron groups (**P* < 0.05, #*P* < 0.001, Student's two-tail *t*-test). *C*–*L*: cumulative probability plots of sEPSC IEI and amplitude data from tonic, delay, phasic, transient and irregular neurons as indicated. *P* values from K-S tests indicated on graphs. Data in bar graphs (*A* and *B*) obtained from same data sets as cumulative probability plots. For tonic sham neurons, a total of 3,732 events were analyzed from 54 neurons (20–100 events/neuron); for tonic axotomy neurons, 1,770 events were analyzed from 30 neurons (20–100 events/neuron); for sham delay neurons, 1,516 events were analyzed from 18 neurons (30–100 events/neuron); for axotomized delay neurons, 1,495 events were analyzed from 14 neurons (30–100 events/neuron); for sham phasic neurons, 1,001 events were analyzed from 29 neurons (10–50 events/neuron); for axotomized phasic neurons, 667 events were analyzed from 14 neurons (15–50 events/neuron); for sham transient neurons, 2,146 events were analyzed from 33 neurons (10–100 events/neuron); for axotomized transient neurons, 940 events were analyzed from 12 neurons (10–100 events/neuron); for sham irregular neurons, 438 events were analyzed from 14 neurons (12–50 events/neuron); for axotomized irregular neurons, 142 events were analyzed from 4 neurons (20–50 events/neuron).

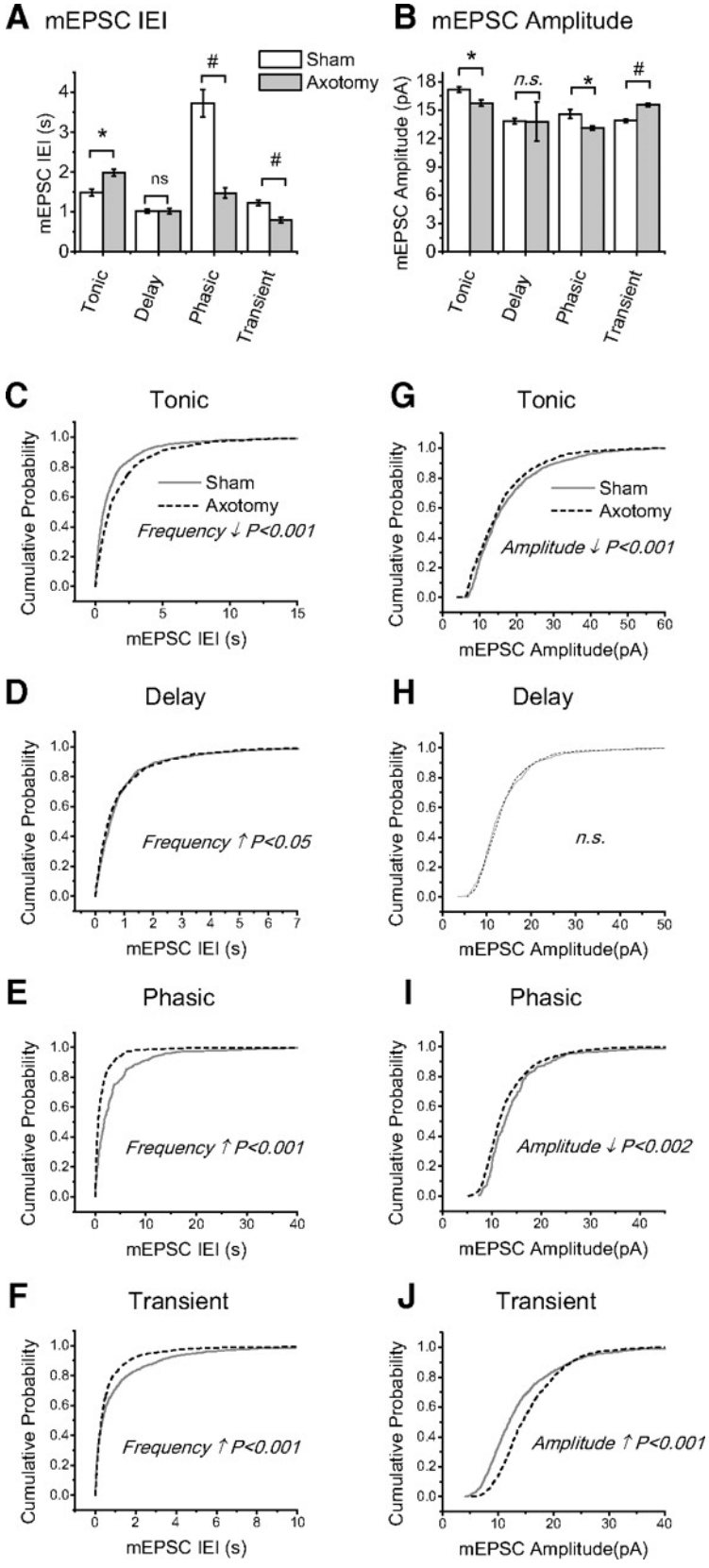

A mEPSC IEI
Sham
Axotomy
mEPSC IEI (s)
Tonic
Delay
Phasic
Transient
B mEPSC Amplitude
mEPSC Amplitude (pA)
C Tonic
Cumulative Probability
Frequency ↓ P<0.001
mEPSC IEI (s)
G Tonic
Amplitude ↓ P<0.001
mEPSC Amplitude(pA)
D Delay
Frequency ↑ P<0.05
H Delay
n.s.
E Phasic
Frequency ↑ P<0.001
I Phasic
Amplitude ↓ P<0.002
F Transient
Frequency ↑ P<0.001
J Transient
Amplitude ↑ P<0.001

Fig. 3.5 Effects of axotomy on miniature synaptic activity (mEPSC) in substantia gelatinosa neurons recorded in the presence of 1 μM tetrodotoxin.

A: effects of axotomy on mean IEI of mEPSCs in tonic, delay, phasic, and transient neurons. Holding potential was −70 mV. *B*: effects on mean sEPSC amplitude of the same 4 groups of neurons (**P* < 0.05, #*P* < 0.001, Student's two-tail *t*-test). *C*–*J*: cumulative probability plots of mEPSC IEI and amplitude data from tonic, delay irregular, phasic, and transient neurons as indicated. *P* values from K-S tests indicated on graphs. Data in bar graphs (*A* and *B*) were obtained from the same data sets as cumulative probability plots. For tonic sham neurons, 1,066 events were analyzed from 16 neurons (17–100 events/neuron); for tonic axotomy neurons, 957 events were analyzed from 13 neurons (16–100 events/neuron); for sham delay neurons, 592 events were analyzed from 6 neurons (50–100 events/neuron); for axotomized delay neurons, 992 events were analyzed from 11 neurons (10–100 events/neuron); for sham phasic neurons, 304 events were analyzed from 9 neurons (8–50 events/neuron); for axotomized phasic neurons, 612 events were analyzed from 13 neurons (12–50 events/neuron); for sham transient neurons, 1,494 events were analyzed from 18 neurons (16–100 events/neuron); for axotomized transient neurons, 1,071 events were analyzed from 12 neurons (23–100 events/neuron).

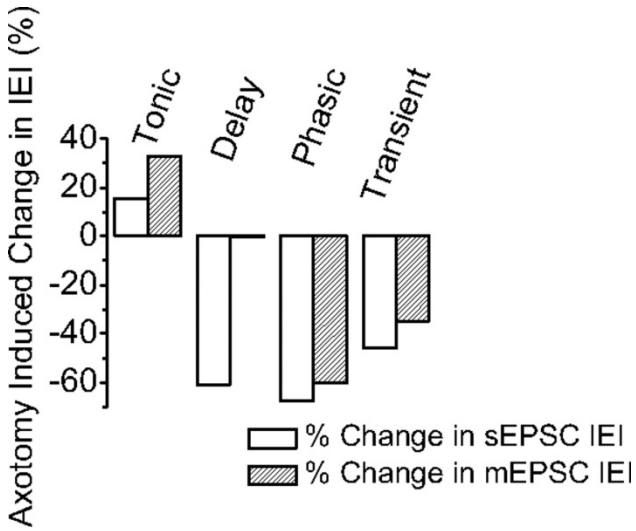
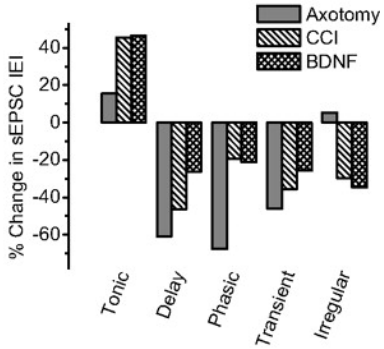
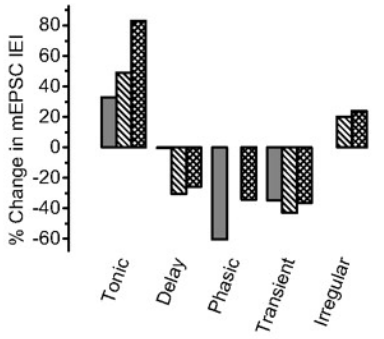

Axotomy Induced Change in IEI (%)
40
20
0
-20
-40
-60
Tonic
Delay
Phasic
Transient
% Change in sEPSC IEI
% Change in mEPSC IEI

Fig 3.6 Comparison of the effects of axotomy on the IEI of mEPSCs with its effect on the IEI of sEPSCs. Percentage changes calculated from the mean values shown in Figs 5A and 6A.

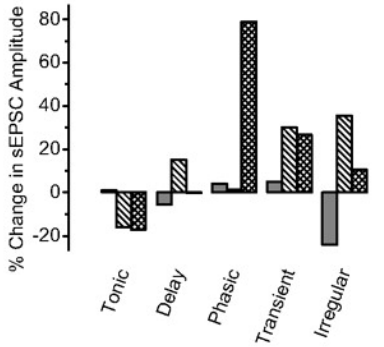
A sEPSC IEI



B mEPSC IEI



C sEPSC Amplitude



D mEPSC Amplitude

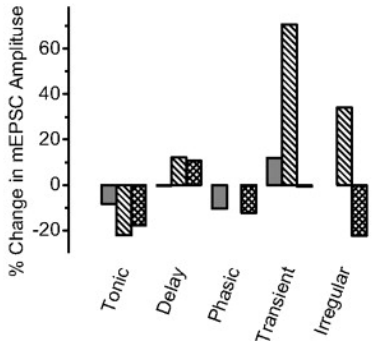


Fig. 3.7 Comparison of the effects of axotomy, CCI, and brain-derived neurotrophic factor (BDNF) on excitatory synaptic transmission in substantia gelatinosa.

For axotomy, percentage changes in sEPSC and mEPSC amplitude and IEI were calculated from data in Figs. 5, *A* and *B* and 6, *A* and *B*. For CCI, percentage changes were calculated from data published in Balasubramanyan et al. (2006) and BDNF percentage changes were calculated from data published in Lu et al. (2007). The BDNF results were obtained by exposing dorsal horn neurons in organotypic culture for 5–8 days (Lu et al. 2007). *A*: comparison of effects of axotomy, CCI, and BDNF on IEI of sEPSCs in 5 cell types. *B*: comparison of effects of the same 3 manipulations on sEPSC amplitude. *C* and *D*: similar comparisons for IEI of mEPSCs and mEPSC amplitude. Note that insufficient data were available to show CCI-induced changes in mESPC in phasic neurons or to show effects of axotomy on mEPSCs in irregular neurons.

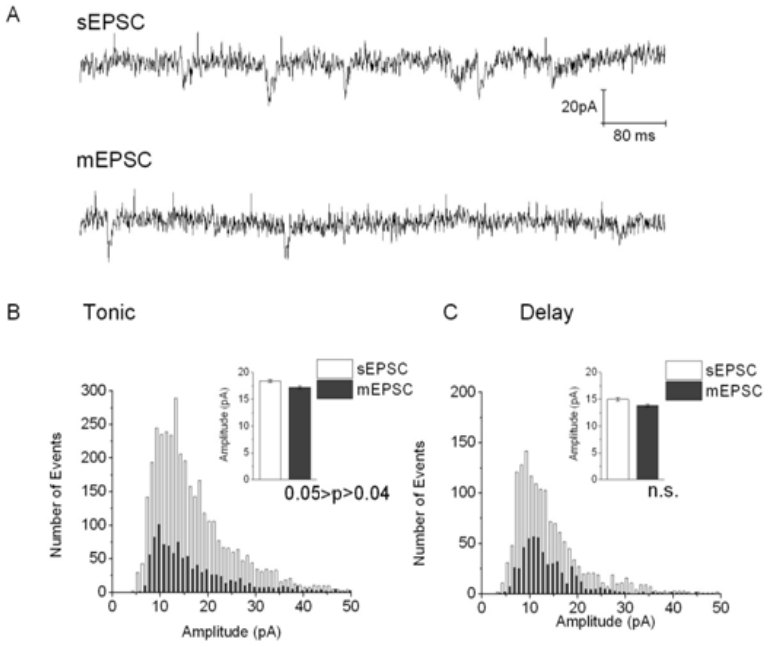

A
sEPSC
20pA
80 ms
mEPSC
B
Tonic
C
Delay
sEPSC
mEPSC
Number of Events
Amplitude (pA)
0.05>p>0.04
n.s.

Figure 3.8 Comparison of the amplitude of sEPSC and mEPSC in delay and tonic neurons.

A. Effect of 1μM TTX on sEPSC's in a transient neuron from a sham operated animal (V = -70mV). Note that amplitude of mEPSC's seen in TTX is similar to that of sEPSC's seen in its absence. B. Comparison of amplitudes of sEPSC and mEPSC's from tonic neurons. Data from Figures 3.4H and 3.5G replotted as a histogram (1pA bin size). Note that the peak of mEPSC amplitudes occurs at ~10pA whereas that for sEPSC amplitude is slightly higher. Inset shows comparison of mean amplitudes. Mean mEPSC amplitude was slightly smaller than sEPSC amplitude in tonic neurons. C. Comparison of amplitudes of sEPSC and mEPSC's from delay neurons. Data from Figures 3.4I and 3.5H replotted as a histogram (1pA bin size). Note that the peak of both mEPSC and sEPSC amplitude occurs at ~10pA. Inset shows comparison of mean amplitudes. There is no significant difference in mean mEPSC amplitude and sEPSC amplitude in delay neurons.

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Chapter 4. Effects of Sciatic Nerve Chronic Constriction Injury and Axotomy on Primary Afferent Terminal Neurotransmitter Release in Substantia Gelatinosa

4.1 Introduction

Chronic neuropathic pain induced by peripheral nerve injury involves an enduring increase in excitability of sensory circuits in the spinal dorsal horn that leads to "central sensitization" (Woolf, 1983).The pathogenic mechanisms are complex and include altered properties and functions at the peripheral tissues, the dorsal horn of the spinal cord and the brain. As mentioned in chapter 1, central sensitization has two major mechanisms that contributes to increased synaptic efficacy: 1) the cell surface expression and trafficking of channels in primary afferent and dorsal horn neurons and 2) post-translational processing of receptors and regulatory proteins which change the receptors' intrinsic functional properties. Similarly, changes in the electrophysiological properties of *substantia gelatinosa* interneurons could also reflect changes from either presynaptic primary afferent terminals or postsynaptic neurons or both. In fact, it is now generally accepted that the increased excitability seen in primary afferent neurons after peripheral nerve injury is responsible for the initiation of the whole process of central sensitization (Zhang et al., 1997b;Sandkuhler, 2009).

Influx of extracellular Ca^{2+} through presynaptic voltage-gated calcium channel (VGCC) triggers synaptic vesicle fusion and neurotransmitter release. Thus changes in VGCC activity at the presynaptic terminals are assumed to affect neurotransmitter release. Different nerve injury models have different impact on primary afferent and DRG cell body calcium channels. Spinal nerve ligation leads

to an upregulation of the α-2-δ subunit of VGCC in the DRG cell body and spinal dorsal horn (Li et al., 2004a). Sciatic chronic constriction results in upregulation of the α-1-β subunit of N-type VGCC in lamina II of spinal dorsal horn (Cizkova et al., 2002). In contrast, sciatic axotomy attenuates high-VGCC currents in L4 and L5 DRG cell bodies (Abdulla and Smith, 2001a) (see Fig 4.1). These data underlie the hypothesis that axotomy and CCI exert different effects on presynaptic Ca^{2+} channels in primary afferent terminals.

To assess the function of presynaptic Ca^{2+} channels, we studied the dynamics of the neurotransmitter release process by measuring the amplitude of evoked EPSCs. When pairs of synaptic currents are evoked with varying latencies, a variety of complex processes associated with paired pulse facilitation and depression determine the relative amplitude of successive responses (Rosenkranz and Johnston, 2007). Reduction in the activity or availability of presynaptic calcium currents is generally associated with increases in paired-pulse ratios. Hence paired-pulse stimulation is used in this chapter to measure the dynamics of neurotransmitter release which directly reflect presynaptic calcium channel function.

4.2 Methods

4.2.1 Slice preparation

Animals had CCI, axotomy or sham surgeries as described in Chapter 2. Transverse spinal cord slices with attached dorsal root residues were prepared on the same day of recording as described in Chapter 2. Recordings were made from lamina II neurons that were ipsilateral to the site of injury.

4.2.2 Electrophysiological recordings

Paired-pulse stimulation was given in a series of successive trails in which latency between the two stimuli was progressively increased by 15ms from 40ms to 130ms per trial. The pulsewidth is 1ms. Only monosynaptic responses were selected for analysis (see chapter 2). Stimulating protocols were written in pclamp 8 and the stimulator was triggered by TTL pulses from the D-A board (DigiData, 1200Series, Axon Instruments Inc.). Pairs of pulses were delivered once every 20s. The whole protocol runs 4 times and the recordings were averaged. Thus the whole stimulation protocol took 540s to execute.

Intracellular recording solution for evoked EPSC recording included (mM): 140 CsCl, 2 $CaCl_2$, 5 HEPES, 10 EGTA, 2 Mg-ATP, 0.3 Na-GTP, 5 QX-314, pH 7.2, 290-300mOsm. Biocytin (0.2%) was added to the intracellular recording solution for future morphological studies. Regular intracellular recording solution (as described in Chapter 2) was used to fill the glass pipette tip for easier patching. Cs solution was filled after pipette tip was filled. After breaking through, the action potential evoked by single depolarizing pulses (1.0nA for 800ms) was

monitored. Loss of the action potential was taken to indicate that the Cs solution (with QX314) had reached the cytosol.

To eliminate synaptic current mediated by receptors other than AMPA receptors, SR95531 (10μM), strychnine (1μM) and D-AP5 (50μM) were included in the extracellular recording solution to block $GABA_A$ receptors, glycine receptors and NMDA receptors, respectively.

4.2.3 Data analysis

Evoked EPSC amplitude was measured at a holding potential of -70mV. The paired pulse ratio was determined by dividing the amplitude of the second EPSC by that of the first EPSC. A ratio less than 1 is defined as depression and a ratio >1 is facilitation. Data were collected from neurons exhibiting EPSCs > 10pA in amplitude.

4.3 Results

4.3.1 Paired-pulse recordings with different time intervals.

In the presence of GABA receptor antagonist SR95531 (10μM), NMDA receptor antagonist D-AP5 (50μM) and glycine receptor antagonist strychnine (1μM), EPSCs mediated by AMPA receptors were generated by the ipsilateral

stimulation at the dorsal root residue or entry zone. Because QX-314 was included in the internal solution to block voltage gated sodium channels, it was not possible to characterise neurons on the basis of their firing pattern (see Fig 3.2). The data from all recorded neurons were pooled together irrespective of their cell types.

Examples of paired-pulse EPSC recordings with a series of 7 different time intervals from 40ms to 130ms are shown in Fig 4.2A-C. The duration of AMPA receptor mediated evoked EPSC's is about 30ms (Diamond and Jahr, 1995) so 40ms is selected to be the first time interval (Fig 4.2D). The measurement of amplitudes of both pulses is shown in Fig 4.2D.

4.3.2 Effects of Axotomy on Paired-pulse Facilitation

In sham animals, paired-pulse recordings of 64 neurons were used and the averaged paired-pulse ratio with different time interval is shown in Fig 4.3A and B. The ratio changed with time interval, from moderate facilitation at the beginning with shorter time intervals to moderate depression with longer time intervals. This initial transient facilitation may represent Ca^{2+} accumulation. With longer intervals between stimuli, Ca^{2+} may return to resting levels.

In animals subject to axotomy, paired-pulse ratios of 46 neurons were averaged and compared to those in sham animals (Fig 4.3A). The ratios had the same trend

of becoming smaller with time interval increase, but all the ratios were >1 which suggested general facilitation in the recording period. Starting from the interval of 40ms, the ratio of axotomized neurons was significantly larger ($P<0.05$, unpaired student t-test) than that of sham neurons. To test if reduced presynaptic Ca^{2+} channel activity could produce a similar effect, $MnCl_2$ (200μM) was applied to the external recording solution on sham cell recordings. Because 1mM Mn^{2+} can block Ca^{2+} channels (Stys et al., 1990) and almost completely eliminates synaptic transmission in peripheral neurons (Smith, 1982), a concentration of 200μM is chosen to partially block Ca^{2+} channel and thus mimics the reduced Ca^{2+} channel activity. The effects of $MnCl_2$ on paired-pulse ratio are variable. In some neurons (7/14), such as illustrated in Fig 4.3C, clear facilitation can be seen, but in other neurons (7/14), paired-pulse ratio is not significantly changed. The facilitation reflects the effect of reduced Ca^{2+} entry on neurotransmitter release from presynaptic terminal. The similarity between the effect of axotomy and $MnCl_2$ on paired-pulse ratio may imply the loss of Ca^{2+} channels or reduction of Ca^{2+} channel activities at the primary afferent terminal. This is in agreement with previous literature which reported attenuated high-voltage-activated Ca^{2+} current in DRG cell body by nerve axotomy (Abdulla and Smith, 2001a;McCallum et al., 2006). More experiments are required to confirm the effect of $MnCl_2$ on paired-pulse ratio but facilitation shown in half of the neurons suggests some relationship between paired-pulse facilitation and loss of voltage gated Ca^{2+} channels at primary afferent terminals.

4.3.3 Paired-pulse Ratio is unchanged by CCI

In animals subject to CCI, paired-pulse ratios of 68 neurons were averaged and compared to those in sham animals (Fig 4.3B). The ratios were not significantly different from those of sham animals. Since CCI was reported to increase Ca^{2+} channel expression in spinal dorsal horn (Cizkova et al., 2002), it might suggest increased expression in primary afferent terminal as well. To mimic the increased expression or potentiated activity of Ca^{2+} channels, BayK8644 (300nM), the L-type Ca^{2+} channel agonist/opener, was used. In the presence of BayK8644, the channels tend to open for a longer time which would cause an increase in the calcium influx. Hence BayK8644 was applied to the external recording solution on the sham slices after control recordings and results were compared. Like CCI, BayK8644 did not change the paired-pulse ratio compared to sham control recordings (Fig 4.3D). These two negative results say little or nothing about effects of CCI effects on primary afferent terminal Ca^{2+} channels, but these might suggest that even if CCI upregulated Ca^{2+} channel expression on presynaptic terminals, the increase would not affect neurotransmitter release.

4.4 Discussion

The finding of this study is that sciatic nerve axotomy produces primary afferent terminal paired-pulse facilitation while CCI produces no change on primary afferent neurotransmitter release properties compared to sham animals. Reduction in the activity or availability of presynaptic calcium channel currents is generally associated with increases in paired pulse ratios while increase of calcium current could decrease the paired pulse ratios. One explanation for this is that the reduced influx of calcium during the first stimulus releases fewer vesicles of neurotransmitter than normal so more vesicles are available for release by the second stimulus, whereas the increased calcium influx releases more vesicles and leaves fewer vesicles available for the second stimulus.

In this experiment, axotomy had similar effect on paired-pulse ratio as $MnCl_{2,}$ which suggested axotomy resulted in reduced primary afferent presynaptic Ca^{2+} current. Decreased Ca^{2+} current could result from reduced expression of channels and/or changed channel properties such as reduced open probability, changed kinetics and reduced single channel conductance. The open probability of VGCC was not altered by axotomy (Abdulla and Smith, 2001a). The activation kinetics of high voltage VGCC were unaffected but the inactivation was increased in axotomized DRG cells (Abdulla and Smith, 2001a;Baccei and Kocsis, 2000), but this could not account for the total reduction in currents. However, axotomy has been shown to reduce N-type VGCC expression on DRG neurons with more reduction on small neurons and less reduction on large neurons (Fuchs et al., 2007). Thus a reduction in calcium channel function in primary afferent terminals

may account for the effect of axotomy on transmitter release and the increase in paired-pulse ratio.

The paired-pulse ratio was not changed by CCI or by BayK8644. This does not necessarily mean that there is no change in presynaptic Ca^{2+} current because there is a possible ceiling effect which is that the neurotransmission is saturated with Ca^{2+} channel expression under normal condition and cannot be increased by overexpression of Ca^{2+} channels at the presynaptic terminals (Cao and Tsien, 2010). There are mainly N-type Ca^{2+} channels mediating neurotransmitter release at the Aδ and C type primary afferent terminal in the superficial dorsal horn with only a small contribution from L-type and P/Q type channels (Heinke et al, 2004). This may also explain why the L-type Ca^{2+} channel opener BayK8644 failed to increase neurotransmitter release. Another study found that there was L-type VGCC expressed in all DRG neurons which contributed predominantly during prolonged activation (Fuchs et al., 2007). Others found that Ca^{2+} current was also diminished in DRG neurons after CCI in a similar pattern as axotomy (Hogan et al., 2000). Since primary afferent terminals directly contact spinal cord neurons, the Ca^{2+} channel expressed in the DRG cell body may not fully represent channels expressed at the terminals though, they may be correlated to a great extent. Synaptophysin is a presynaptic vesicle protein involved in synaptic vesicle exo-endocytosis (Alder et al., 1995). It was increased in the ipsilateral dorsal horn after sciatic nerve CCI and temporally correlated with thermal hyperalgesia (Chou et al., 2002), which suggests increased vesicle transportation and exo-

endocytosis in regulating synaptic transmission. In contrast, the level of synaptophysin in C-fibre DRG neurons projecting to lamina II was not significantly changed after axotomy (Sun et al., 2006).

As mentioned in Chapter 3, axotomy and CCI produce similar changes to interneurons in *substantia gelatinosa,* ie. Increased excitatory synaptic drive to excitatory neurons and decreased synaptic drive to inhibitory neurons, but axotomy has weaker effect than CCI. From what I found from this chapter, I can derive a parallel conclusion that neurotransmitter release from primary afferents seemed to have the same difference between this two injury models though maybe of different mechanisms. Axotomy and CCI both are capable of increasing aberrant spontaneous activity in primary afferent neurons (Wall and Devor, 1983a;Wall and Devor, 1983b), but axotomy produces a weaker effect than CCI in the dorsal horn (Chen et al., 2009) perhaps because of reduced Ca^{2+} channel expression at primary afferent terminals. Some of the DRG neurons including small neurons with unmyelinated fibres would die from peripheral nerve axotomy (Tandrup et al., 2000), which may also reduce the primary afferent input to dorsal horn neurons. Wallerian degeneration at the injury site of CCI would cause release of inflammatory molecules, e.g. TNFα, IL1-α, IL1-β as well as neurotrophic factors like BDNF, synthesized by glial and immune cells, which are involved in the initiation and maintenance of neuropathic pain status (Marchand et al., 2005) so CCI generally produces more severe stimulus for central sensitization than axotomy.

We were unable to identify neuron type (inhibitory or excitatory) because the use of QX314 precluded identification of neurons on the basis of their firing patterns. We were therefore unable to determine whether the effect on primary afferents projecting to different cell types was different. However, together with the data in chapter 3, it is suggested that although axotomy and CCI produce similar “electrophysiological signatures”, axotomy produces weaker effect on the central sensitization process than chronic constriction injury.

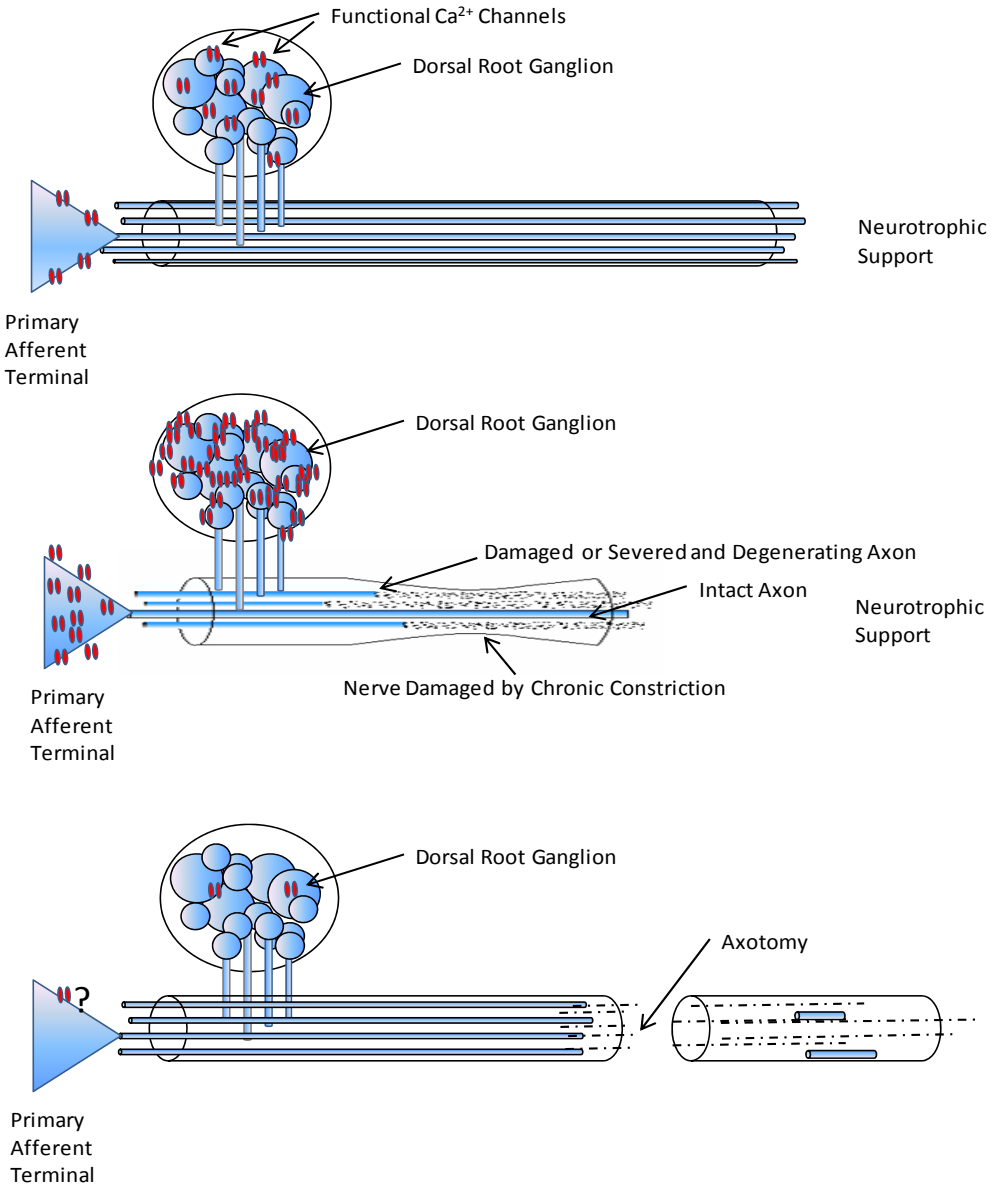
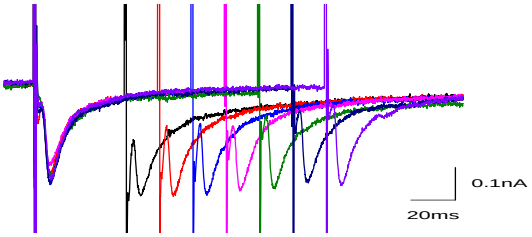

Functional Ca^{2+} Channels
Dorsal Root Ganglion
Neurotrophic Support
Primary Afferent Terminal
Dorsal Root Ganglion
Damaged or Severed and Degenerating Axon
Intact Axon
Neurotrophic Support
Nerve Damaged by Chronic Constriction
Primary Afferent Terminal
Dorsal Root Ganglion
Axotomy
?
Primary Afferent Terminal

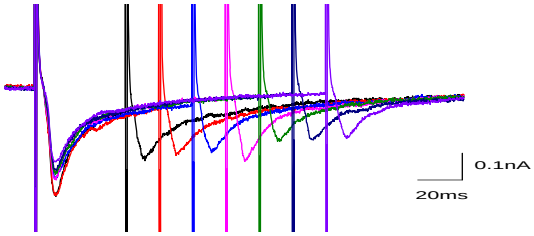
Fig 4.1 Illustration of possible changes to voltage-gated Ca^{2+} channels in DRG neurons and primary afferent terminals after different nerve injury.

The top panel shows Ca^{2+} channel distribution in sham operated animals. The middle panel shows Ca^{2+} channel upregulation in the DRG cell body and possibly in primary afferent terminals. The bottom panel shows Ca^{2+} channel downregulation in the DRG cell body and possibly in primary afferent terminals.

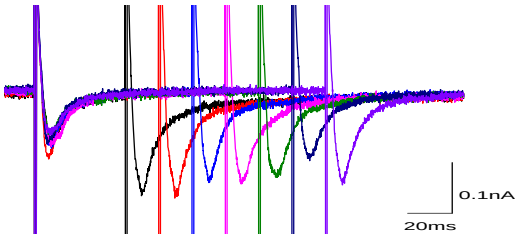
A. Sham



B. CCI



C. Axotomy



D

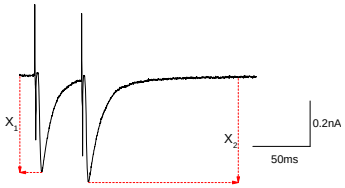
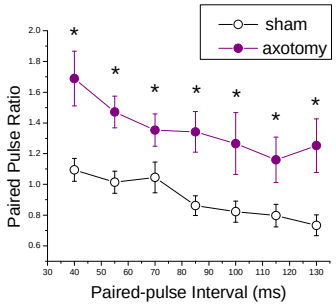


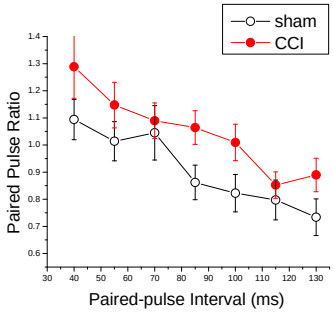
Figure 4.2

A-C. Sample recordings of paired-pulse stimulation in 3 groups of animals. A. Recording from sham animals. B. Recording from CCI animals. C. Recording from axotomy animals. D. Paired-pulse recording from the first stimulation with time interval of 40ms. X_1 represents the measurement of amplitude of the first pulse. X_2 represents the measurement of amplitude of the second pulse.

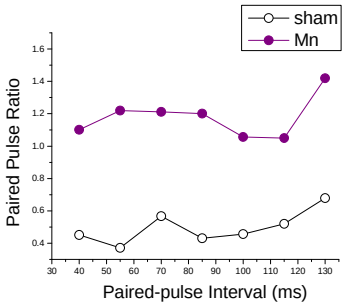
A


sham
axotomy
Paired Pulse Ratio
Paired-pulse Interval (ms)

B


sham
CCI
Paired Pulse Ratio
Paired-pulse Interval (ms)

C


sham
Mn
Paired Pulse Ratio
Paired-pulse Interval (ms)

D

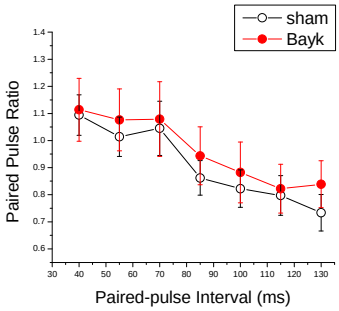

sham
Bayk
Paired Pulse Ratio
Paired-pulse Interval (ms)

Figure 4.3

A. Comparison of averaged paired-pulse ratios of sham (64 neurons) and axotomy (46 neurons) by unpaired t-test with standard error. B. Comparison of averaged paired-pulse ratios of sham and CCI (68 neurons) unpaired t-test with standard error. C. One example of 200μM $MnCl_2$ effect on paired-pulse ratio. D. Average effect of 300nM BayK8644 on paired-pulse ratio (17 neurons). *: $P<0.05$

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Chapter 5. EFFECT OF SCIATIC NERVE CHRONIC CONSTRICTION INJURY ON AMPA RECEPTORS IN SUBSTANTIA GELATINOSA

5.1 Introduction

AMPA receptors are ionotropic receptors responsible for much of the fast excitatory neurotransmission at central synapses. They are also a major target for multiple signalling pathways that regulate the strength of glutamatergic excitatory synapses. The presence of GluR2 subunits critically determines AMPA receptor properties, such as receptor kinetics, single-channel conductance, Ca^{2+} permeability and blockade by endogenous polyamines (Isaac et al., 2007). The GluR2 subunit also regulates AMPA receptor assembly and trafficking which, in turn, affects synaptic strength (Isaac et al., 2007).

Studies have been done on AMPA receptors in the spinal cord after inflammatory pain and mostly support the idea of increased trafficking of GluR1 subunit to the membrane, both synaptically and extrasynaptically (Galan et al., 2004;Kopach et al., 2011;Larsson and Broman, 2008) and internalization or downregulation of GluR2 subunit (Hartmann et al., 2004;Katano et al., 2008;Park et al., 2009). However, in neuropathic pain, the story is different: GluR2/3 subunits significantly increase at synapses of primary afferent terminals in lamina II after sciatic nerve section (Popratiloff et al., 1998). The GluR2 subunit also selectively increases in the spinal cord ipsilateral to sciatic nerve CCI (Garry et al., 2003). It is therefore possible that different mechanisms underly the central sensitization that is involved in inflammatory and neuropathic pain. This prompted me to

investigate changes in AMPA receptor subunit composition after peripheral nerve injury.

Sciatic nerve transection and chronic constriction injury are two major neuropathic pain models (Wall et al., 1979;Mosconi and Kruger, 1996). From the previous chapters, we know that axotomy and CCI produce similar electrophysiological changes in *substantia gelatinosa* neurons but axotomy produced a weaker effect (see chapter 3). It is therefore possible that axotomy and CCI produce different effects on the composition of synaptic AMPA receptors. We therefore compared the effects of these two manipulations.

5.2 Methods

5.2.1 Slice preparation

Animals of 19 days old were subject to CCI, axotomy or sham surgeries as described in Chapter 2. Transverse spinal cord slices with attached dorsal root residues were prepared on the same day of recording as described in Chapter 2. Recordings were made from lamina II on the side ipsilateral to the nerve injury.

5.2.2 Electrophysiological recordings

Intracellular recording solution for action potential recording was used with 0.2% biocytin for future morphological studies.

To eliminate current mediated by receptors other than AMPA, SR95531 (10μM), strychnine (1μM) and D-AP5 (50μM) were included in the extracellular recording solution to block $GABA_A$ receptors, glycine receptors and NMDA receptors respectively. Since KA receptors don't contribute to fast synaptic current in animals at this age (see chapter 1), at the end of each experiment, CNQX (10μM) was used to block AMPA receptors to make sure that all current recorded was mediated by AMPA receptors.

IEM1460 is a voltage-dependent open channel blocker of Ca^{2+} permeable AMPA receptors. It is much more potent in blocking Ca^{2+} permeable AMPA receptors than receptors containing the edited GluR2 subunit with the IC_{50} values of 2.6 and 1102μM respectively (Magazanik et al., 1997). It was used to block Ca^{2+} permeable AMPA receptor mediated postsynaptic excitatory currents. It was made up as a stock solution of 50mM in HPLC water and diluted to a functional concentration of 50μM in aCSF for bath application. IEM1460 was applied for a total of 5min before washing out.

Evoked EPSCs, spontaneous EPSCs and mini EPSCs were recorded at the holding potential of -70mV in voltage clamp. For sEPSC and mEPSC, the first 100 events from each neuron were used for analysis to limit the overwhelming influence of any neurons that had especially high levels of spontaneous activity. Three control

recordings of eEPSC were done before application of IEM1460 to have a better idea of control amplitude and to make sure recording conditions were stable. Membrane potential and holding potential are monitored constantly during the recording (see chapter 2). In each cell, 6 eEPSCs were evoked once every 20sec and the responses averaged. This procedure was carried out at 3min intervals until the amplitude of the control response had stabilized. Cells in which eEPSC amplitude changed by >20% were excluded from analysis. Throughout drug application, sets of 6 eEPSCs were obtained every 5min and averaged. Response amplitudes in the presence of drugs were expressed as percentage of the last control amplitude. Data from 9-18 cells of each group were combined and plotted against time.

5.2.3 Data analysis

The detection of sEPSC and mEPSC and generation of cumulative probability plots of the amplitude and interevent intervals were carried out as previously described in chapter 2.

Scaled NSFA was done by Dr. Victor Derkach using Kaleidagraph 4.1 (Synergy Software). Protocols were described in chapter 2. To ensure an objective analysis, Dr Derkach was not informed of the cell type or the nature of its treatment (i.e. sham, axotomy or CCI). Following Dr. Derkach's analysis, I randomly selected data traces and re-analysed them in a blind fashion and was able to confirm the

numerical values provided by Dr. Derkach. Significance of difference between groups of data was determined using Student's two-tailed t-test or ANOVA (with Bonferroni *post-hoc* test) as appropriate.

5.3 Results

5.3.1 Neuron types

Neurons were classified into five different types depending on the firing pattern of action potentials in response to depolarizing current pulses (Chen et al., 2009;Balasubramanyan et al., 2006). Since there is good correlation between tonic firing pattern and inhibitory neurons and between delay firing pattern and excitatory neurons (Grudt and Perl, 2002;Yasaka et al., 2010), I used only recordings from tonic and delay firing neurons and assumed that they corresponded to inhibitory and excitatory, respectively (see Fig 3.2 in chapter 3).

5.3.2 Changes of Ca^{2+} permeable AMPA receptors on different types of neurons

For clarity, I will first present data on tonic cells and follow that by data on delay cells.

5.3.2.1 Effects of IEM1460 on tonic neurons

5.3.2.1.1 Effects of IEM1460 on primary afferent evoked EPSCs

In the presence of D-AP5, SR95531 and strychnine, EPSCs evoked by primary afferent stimulation were recorded at a holding potential of -70mV. In recordings from sham operated animals, the voltage dependent selective blocker of Ca^{2+} permeable AMPA receptors, IEM1460 (50μM) reduced eEPSC amplitude to 75% ± 2% of control (n=18, P<0.05, ANOVA with Bonferroni post-hoc test) in tonic cells and was not washed out in 20mins (Fig. 5.1B). CNQX nearly fully blocked the eEPSC after 5mins superfusion. The response gradually recovered to about 50% of the control level with 30mins of washing. An example is shown in Fig. 5.1A.

By contrast, in recordings from tonic neurons in animals subject to CCI, IEM1460 had little or no effect on the amplitude of eEPSCs. The amplitude was not significantly changed during the drug perfusion and wash out period (n=14, P>0.05, ANOVA with Bonferroni post-hoc test, Fig. 5.1C). Similar to sham cells, CNQX nearly fully blocked the eEPSC after 5mins application and the amplitude was restored to about 50% of the control level after 30mins washing.

In tonic neurons from animals subject to axotomy, IEM1460 also had no effect on the amplitude of eEPSCs and the amplitude persisted for the 20mins washing period (n=17, P<0.05, ANOVA with Bonferroni post-hoc test, Fig. 5.1D). CNQX also nearly completely blocked the currents and responses recovered to about 50% of their original amplitude in 30mins.

The above result suggested that Ca^{2+} permeable AMPA receptors play a diminished role in primary afferent excitatory synaptic transmission in tonic neurons after both CCI and axotomy.

5.3.2.1.2 Effects of IEM1460 on the amplitude of sEPSCs

Since the effect of IEM1460 on eEPSCs in tonic neurons suggested a reduction in function of Ca^{2+} permeable AMPA receptors after CCI and axotomy, the drug should exert similar effects on the amplitude of sEPSCs. The effect of IEM1460 was therefore tested on sEPSCs. Spontaneous EPSCs were recorded as inward currents at -70mV. Since they could be fully blocked by CNQX (Chen et al., 2009) and kainate receptors are not present in lamina II in rats of this age (Dahlhaus et al., 2005;Tolle et al., 1993;Youn and Randic, 2004), sEPSC's were mediated exclusively by AMPA receptors.

IEM1460 significantly reduced amplitude of sEPSC in sham (n=37) categories according to both t-test (Fig. 5.2A) and K-S test (Fig. 5.2B). By contrast, in tonic neurons from CCI animals (n=31), the sEPSC amplitude was not affected by IEM1460 according to t-test (Fig. 5.2A) and K-S test (Fig. 5.2B). In tonic neurons from axotomy animals (n=19), IEM1460 significantly reduced amplitude of sEPSC according to both t-test (Fig. 5.2A) and K-S test (Fig. 5.2C). These results with sEPSCs are in good agreement with those seen with eEPSCs in CCI animal models but not in axotomy animals (Table 5.1). This may suggest different regulation of

AMPA receptor subtypes at primary afferent synapses and at other synapses after axotomy.

5.3.2.1.3 Effects of IEM1460 on the amplitude of mEPSCs

Spontaneous EPSCs include action potential dependent as well as action potential independent release of neurotransmitter. Miniature EPSCs are action potential independent postsynaptic events which represent ongoing activity of the neurotransmitter release process and can be examined in the presence of TTX (Edwards et al., 1990). 1µM TTX was used to block action potentials. The recording of mEPSC was only started when current injections in current clamp failed to produce an action potential.

Analysis of miniature EPSCs was restricted to recordings from sham and CCI animals as insufficient data were available from animals subject to axotomy. IEM1460 decreased the amplitude of mEPSC in tonic firing cells of sham group (n=16) based on t-test results (Fig. 5.3A) and K-S test (Fig. 5.3B). The amplitude of mEPSCs of tonic CCI neurons (n=17) was not affected by IEM1460 (Fig. 5.3A and C), which is consistent with the results of eEPSC and sEPSC from tonic CCI neurons (Table 5.1).

5.3.2.1.4 Single channel conductance of AMPA receptors

If Ca^{2+} permeable AMPA receptors contribute less to the total synaptic current in animals subject to CCI, there should be a corresponding decrease in the mean single channel conductance (γ) of AMPA receptor channels as estimated by scaled NSFA of mEPSC's. In agreement with this, we found that conductance estimated from NSFA of mEPSCs decreased from 15.9±2.1pS (n=10) in sham animals to 9.2±1.0pS (n=10) in CCI animals. The reduction of conductance is significant according to ANOVA with Bonferroni post-hoc test ($p<0.05$). By contrast, axotomy failed to alter average conductance of AMPA receptors. The mean value of γ for AMPA receptors in neurons subject to axotomy (13.2±1.4pS, n=9) was not significantly different from sham values ($p>0.05$, ANOVA with Bonferroni post-hoc test). Thus γ for AMPA receptors in sham neurons was significantly different from values obtained in CCI neurons but not different from that seen in neurons subject to axotomy.

A further prediction of the above findings is that IEM1460 should have little effect on γ of AMPA receptors in tonic neurons from animals subject to CCI as these would express relatively few Ca^{2+} permeable AMPA receptors. Consistent with this, conductance of tonic neurons subjected to CCI was unaltered by IEM1460. γ from NSFA was 9.8±1.6pS (n=7) in IEM1460, which is not significantly different from control conductance of 9.2±1.0pS (n=10, p=0.74, t-test). It would also be predicted that IEM1460 would reduce the average single channel conductance in tonic neurons from sham operated animals. However,

insufficient data were available to make meaningful comparison. Numerical values for this section are listed in Table 5.2.

5.3.2.1.5 Effect of IEM1460 on the frequency of sEPSCs

AMPA receptors are important in presynaptic regulation of neurotransmitter release (Engelman et al., 1999;Lee et al., 2002). Effects mediated via changes in the properties of presynaptic receptors may be reflected as changes in the frequency (interevent interval, IEI) of sEPSCs and mEPSCs (Larkman et al., 1991). Therefore the effect of IEM1460 on the frequency of sEPSC and mEPSC from sham, CCI and axotomized rats was also examined.

In tonic neurons from sham operated animals, IEM1460 produced a clear increase in the IEI of sEPSC (n=37). This increase was significant according to both t-test (Fig. 5.4A) and K-S statistics (Fig. 5.4B), indicating a decreased frequency. The IEI was also increased significantly according to both tests in tonic CCI neurons (n=31) (Fig. 5.4A and C). this is unlikely to reflect a postsynaptic effect as IEM1460 does not affect sEPSC amplitude in tonic neurons in animals subject to CCI. These results suggested the involvement of Ca^{2+} permeable AMPA receptors in the regulation of tonic neuron presynaptic terminals both in sham operated animals and in those subject to CCI. However, the IEI of tonic cells of the axotomy group (n=19) was not sensitive to the blocker at all according to both tests (Fig. 5.4A and D). A likely explanation for this is that axotomy (but not

CCI) causes down regulation of presynaptic Ca^{2+} permeable AMPA receptors, perhaps implying that their expression requires contact of primary afferents with target tissue.

5.3.2.1.6 Effect of IEM1460 on the frequency of mEPSCs

The effect of IEM1460 on mEPSC IEI is totally different from its effect on sEPSC IEI. Thus the mEPSC IEI of tonic sham neurons (n=16) was not affected by IEM1460 based on both t-test (Fig. 5.5A) and K-S statistics (Fig. 5.5B). This contrasts with the robust effect of IEM1460 on sEPSC IEI of tonic sham neurons (Fig. 5.4A). In tonic neurons from animals subject to CCI, the IEI of mEPSC's was again not affected by IEM1460 based on both tests (n=17) (Fig. 5.5A and C). The lack of effect of IEM1460 on IEI of mEPSC from tonic cells in both groups indicated the change of IEI of sEPSCs by IEM1460 was exerted purely on action potential dependent synaptic events.

5.3.2.2 Effects of IEM1460 on delay neurons

Both the pharmacological data and the NSFA of mEPSC's suggest that CCI reduces the contribution of GluR2-lacking Ca^{2+} permeable AMPA receptors in tonic neurons. Corresponding data from delay neurons are more difficult to interpret, but they do lend some support to the possibility that CCI increases the

contribution of Ca^{2+} permeable AMPA receptors to synaptic transmission in this population.

5.3.2.2.1 Effects of IEM1460 on primary afferent evoked EPSCs

In delay neurons from sham operated animals, the onset of IEM1460 blockade was slower than in sham tonic neurons but eventually 25% ± 7% (n=14, P<0.05, ANOVA with Bonferroni post-hoc test) block was attained (Fig. 5.6A). CNQX rapidly and nearly fully blocked the current after 5mins perfusion and was gradually washed out such that the amplitude was restored to about 50% of the control level in 30mins.

In the CCI treated animal group, the amplitude of delay neuron eEPSC was decreased by IEM1460 but the change was not significant (n=10, P>0.05, ANOVA with Bonferroni post-hoc test) (Fig. 5.6B). Similar to sham cells, CNQX nearly fully blocked the eEPSC after 5mins application and the amplitude was restored after washing. The insignificant decrease of eEPSC amplitude must not be due to the possibility of cells "running down" because after 30mins of washing the amplitude of eEPSCs was restored to its original level in IEM1460 (Fig. 5.6B).

In delay neurons subject to axotomy, the blocker was without effect on sEPSC amplitude (n=9, P>0.05, paired t-test, Fig. 5.6C), whereas CNQX also nearly fully

blocked the current. The amplitude of eEPSCs was restored to about 50% of their initial value in control condition after 30mins washing.

The ineffectiveness of IEM1460 on eEPSCs suggests that the contribution of Ca^{2+} permeable AMPA receptors to synaptic transmission between primary afferents and delay neurons may be reduced by axotomy and CCI, like that for tonic neurons.

5.3.2.2.2 Effects of IEM1460 on the amplitude of sEPSCs

IEM1460 significantly reduced the amplitude of sEPSC of delay sham neurons (n=26) according to both t-test (Fig. 5.7A) and KS-test (Fig. 5.7B). In CCI group (n=28), the amplitude was also significantly reduced by IEM1460 according to both tests (Fig. 5.7A and C). Delay neurons from axotomy animals (n=9) had reduced amplitude in the presence of IEM1460 according to both tests (Fig. 5.7A and D). These clear effects of IEM1460 on sEPSCs suggest that when all input to delay neurons is considered, including that from local circuit interneurons, a more obvious role for Ca^{2+} permeable AMPA receptors can be demonstrated in CCI and axotomy.

5.3.2.2.3 Effects of IEM1460 on the amplitude of mEPSCs

Miniature EPSCs were analyzed from neurons in sham and CCI animals as insufficient data were available from animals subject to axotomy. Delay neurons of sham group (n=12) had IEM1460 sensitive mEPSCs. The amplitude was significantly decreased according to K-S (Fig. 5.8B) and t-test (Fig. 5.8A). The amplitude was also decreased by IEM1460 in delay CCI neurons (n=9) (Fig. 5.8 A and C). The findings with mEPSCs in delay neurons in both sham and CCI groups are in agreement with those with sEPSC (Table 5.1).

5.3.2.2.4 Single channel conductance of AMPA receptors

The single channel conductance (γ) of AMPAR in delay neurons from sham operated animals was 7.6$\pm$1.2pS (n=11). This was increased to 12.2$\pm$1.2pS (n=6, P<0.05, ANOVA with Bonferroni post-hoc test) in animals subject to CCI while not significantly changed in animals subject to axotomy (γ=9.9$\pm$1.0, n=9, P>0.05, ANOVA with Bonferroni post-hoc test).

The increase in average single channel conductance seen with CCI suggests that CCI may upregulate Ca^{2+} permeable AMPA receptors in delay neurons. If this is the case, IEM should reduce average single channel conductance in delay neurons subject to CCI. Under these conditions, the mean value of γ was reduced from 12.2$\pm$1.2pS (n=6) to 8.3$\pm$1.2 pS (n=5). Comparison of data on a neuron by neuron basis yielded p=0.024 (paired t-test). By contrast, IEM1460 did not

reduce the mean single channel conductance (γ=7.7±1.1pS, n=4, P=0.32, paired t-test) in neurons from sham operated animals.

Although the increased single channel conductance of AMPA receptors in delay CCI neurons represents increased proportion of Ca^{2+} permeable AMPA receptors, the suppression of sEPSC amplitude by IEM1460 of sham and CCI neurons is not significantly different. The mean value of sEPSC amplitude before and after IEM1460 application is compared for each neuron. The average of the suppression amount of delay sham and CCI neurons is shown in Fig 5.11. The suppression between those two groups of neurons is not significantly different, which is not consistent with the result of single channel conductance. However, in the data of delay neurons, average single channel conductance was found to be able to divide into two groups: one with conductance above 10pS, which is sensitive to IEM1460 blockade, and the other one with conductance of 4-5pS, which is insensitive to IEM1460 blockade. This may suggest two different types of delay neurons: one with Ca^{2+} permeable AMPA receptors; the other without Ca^{2+} permeable AMPA receptors. Due to the relatively small sample size, the average single channel conductance may not be appropriate enough to represent AMPA receptor changes in delay neurons after nerve injury. Thus, more data of single channel conductance is required to clearly define delay neurons with synapses of different AMPA receptors and compare the changes produced by nerve injury.

5.3.2.2.5 Effect of IEM1460 on the frequency of sEPSCs

As was done for tonic neurons, effects of IEM1460 on IEI were examined to explore possible changes in presynaptic AMPA receptors. Delay sham neurons (n=26) had increased IEI, i.e. decreased frequency, produced by IEM1460 according to both t-test (Fig. 5.9A) and K-S statistics (Fig. 5.9B). The IEI of delay CCI neurons was increased by IEM1460 according to both tests (Fig. 5.9A and C). The increase of IEI in delay axotomy neurons (n=9) was only supported by K-S test (Fig. 5.9D) while t-test result showed no significant difference (Fig. 5.9A).

One interpretation of this result is that Ca^{2+} permeable AMPA receptors are involved in the regulation of delay neuron presynaptic terminals. As was seen in tonic neurons, the role of presynaptic Ca^{2+} permeable AMPA receptors may be diminished by axotomy but not by CCI.

There may however be alternative explanations. The IEM1460 induced decrease in frequency of sEPSCs may also reflect a postsynaptic effect rather than a presynaptic effect. If the total population of sEPSCs represents individual events entirely mediated by Ca^{2+} impermeable AMPA receptors plus others mediated by Ca^{2+} permeable AMPA receptors, the latter may be eliminated by IEM1460 so the total number of events within a given period of record (i.e. the overall frequency of events) would decrease.

5.*3.2.2.6* Effect of IEM1460 on the frequency of mEPSCs

Unlike tonic neurons, IEM1460 reduced the frequency of mEPSC in delay sham neurons (n=12). Results were significant according to both K-S (Fig. 5.10B) and t-test (Fig. 5.10A). The IEI of mEPSC from delay CCI neurons (n=9) was also increased by IEM1460 (Fig. 5.10A and C), indicating decreased frequency. The frequency change of mEPSC of both delay sham and delay CCI neurons is in agreement with the findings for sEPSC (Table 5.1).

5.4 Discussion

The chapter focused on the change of AMPA receptor subunit composition in the spinal cord after different types of peripheral nerve injury. The results suggest that: 1. CCI decreased the proportion of Ca^{2+} permeable AMPA receptors in tonic neurons. 2. CCI did not decrease the proportion of Ca^{2+} permeable AMPA receptors in delay neurons. 3. Axotomy reduced the function of Ca^{2+} permeable AMPA receptors in the presynaptic terminals of tonic and delay neurons.

5.4.1 CCI decreased the proportion of Ca^{2+} permeable AMPA receptors in tonic neurons

Whereas IEM1460 reduced the amplitude of eEPSC, sEPSC and mEPSC in tonic neurons from sham operated animals, the amplitude of all three types of postsynaptic currents was not affected in CCI tonic neurons. This is consistent with removal upon CCI of GluR2-lacking, Ca^{2+} permeable AMPA receptors from synapses and replacement with GluR2-containing, Ca^{2+} impermeable AMPA receptors compared to sham tonic neurons (Figs 5.1, 5.5 and Table 5.1). This is also independently supported by the observed decrease in average single channel conductance in tonic neurons after CCI.

Since tonic firing pattern is well related to inhibitory GABAergic neurons (Grudt and Perl, 2002;Yasaka et al., 2010;Zhang and Dougherty, 2011), our data should correspond with that from inhibitory neurons. Previous studies found that GABAergic neurons express only GluR1 but not GluR2 (Kerr et al., 1998;Engelman et al., 1999). However, other studies showed that GluR1 containing puncta also expressed GluR2 throughout the dorsal horn (Gu et al., 1996;Nagy et al., 2004;Antal et al., 2008). The significant but incomplete attenuation of eEPSCs, sEPSCs and mEPSCs by IEM1460 confirms the presence of Ca^{2+} permeable and impermeable AMPA receptors at synapses onto inhibitory neurons. The latter observation is consistent with the presence of AMPA receptors containing GluR2 subunits.

The frequency of sEPSCs was decreased but that of mEPSC was not affected by IEM1460 in both CCI and sham tonic neurons (Fig 5.4 and 5.5). Normally the

frequency alterations reflected the changes in neurotransmitter release from presynaptic terminals (Heinke et al., 2011). However, in this experiment, it is possible that Ca^{2+} permeable and impermeable AMPA receptors mediate individual postsynaptic events. Therefore elimination of Ca^{2+} permeable AMPA receptors responses can result in loss of a certain population of events which may in turn decrease the frequency of sEPSC's. Thus it may suggest that the change of frequency of sEPSC's following IEM1460 treatment reflected a post synaptic rather than a presynaptic effect. However, in tonic neurons from CCI operated animals, postsynaptic Ca^{2+} permeable AMPA receptors contributed little to postsynaptic currents. Therefore under these conditions, the most likely possibility is that presynaptic Ca^{2+} permeable AMPA receptors participate in the regulation of neurotransmitter release. Presynaptic AMPA receptors in primary afferent terminals may act as autoreceptors facilitating the release of glutamate. This may perhaps occur by depolarization and generation of presynaptic action potential. GluR2-lacking Ca^{2+} permeable AMPA receptors do not appear to directly regulate action-potential independent neurotransmitter release as mEPSC frequency is unaffected (Fig 5.5).

IEM1460 had no effect on the amplitude of sEPSC, mEPSC and eEPSC in tonic CCI neurons. This suggests that the level of Ca^{2+} permeable AMPA receptors expression was downregulated. The decrease in Ca^{2+} permeable AMPA receptors may reflect accumulation of GluR2 subunit at the synapses. The level of GluR2 subunit and GluR2 interacting protein GRIP (Glutamate Receptor Interacting

Protein) is increased selectively in the spinal cord ipsilateral to CCI (Garry et al., 2003). An increased synaptic accumulation of GluR2 may depend on the reduced rate of endocytosis mediated by the GRIP (Osten et al., 2000). Another GluR2 interacting protein PICK1 (Protein Interacting with C Kinase 1) plays a key role in mediating GluR2 endocytosis (Perez et al., 2001). An decrease of GluR2:PICK1 complexes was found after CCI (Garry et al., 2003), which also produced a reduction in GluR2 endocytosis.

Thus our finding of decreased expression of functional Ca^{2+} permeable AMPA receptors at synapses on inhibitory interneurons after CCI is consistent with the increased GluR2 subunit expression described by others (Garry et al., 2003). Although these authors did not specifically examine tonic neurons, this may contribute to the decreased amplitude of sEPSCs and hence the synaptic drive to inhibitory neurons (Balasubramanyan et al., 2006;Chen et al., 2009). This in turn may increase dorsal horn excitability. Alteration of AMPA receptor function on tonic inhibitory neurons thus contributes to the process of CCI induced central sensitization.

5.4.2 CCI did not decrease the proportion of Ca^{2+} permeable AMPA receptors in delay neurons.

Delay firing patterns are well related to excitatory glutamatergic neurons (Grudt and Perl, 2002;Yasaka et al., 2010). Therefore our data from delay neurons can

be interpreted as putative excitatory neurons. Previous studies suggested that putative excitatory interneurons in *substantia gelatinosa* normally do not express Ca^{2+} permeable AMPA receptors (Kerr et al., 1998;Engelman et al., 1999). However, our observation that IEM1460 affects the amplitude of postsynaptic events in delay neurons is inconsistent with this idea (Table 5.1, Figs 5.6-8).

In delay CCI neurons, the insensitivity of eEPSCs to IEM1460 suggests reduced proportion of Ca^{2+} permeable AMPA receptors at synapses making direct contact with primary afferent terminals (Fig. 5.6). However, the amplitude of sEPSCs and mEPSCs, which represent overall synaptic activities in the neurons, is significant decreased by IEM1460 (Fig 5.7 and 5.8), which is similar to delay sham neurons.

The significant increase in average single channel conductance of delay neurons by CCI (Table 5.2) and the observation that mean single channel conductance is reduced by IEM1460 suggests an increase in proportion of Ca^{2+} permeable AMPA receptors. Although it is not consistent with the suppression proportion of sEPSC amplitude, the presence of neurons with single channel conductance over 10pS still suggests the existence of Ca^{2+} permeable AMPA receptors in delay neurons. The bigger average single channel conductance can be caused by increased number of Ca^{2+} permeable AMPA receptors at the synapses or by increased single channel conductance of both Ca^{2+} permeable and impermeable AMPA receptors (Kristensen et al., 2011). However, increase in the number of Ca^{2+}

permeable channels can directly increase the average conductance by raising the proportion of high conductance channels.

Increase of single channel conductance may also result from the phosphorylation of GluR1 subunit. There are two major phosphorylation sites on GluR1, Serine845 by PKA and Serine831 by PKC and CaMKII (Roche et al., 1996;Derkach et al., 1999;Barria et al., 1997). Phosphorylation of Serine845 of GluR1 by PKA increases the channel open probability (Banke et al., 2000), whereas phosphorylation of GluR1 subunit at Ser831 significantly increases the single-channel conductance of homomeric GluR1 AMPA receptors and enhances the glutamate binding and channel opening (Derkach et al., 1999) but the enhancement is absent in the GluR1/GluR2 heteromeric channels (Oh and Derkach, 2005). TARPs in the receptor complex can ensure the increase of channel conductance upon phosphorylation, even in the presence of GluR2 subunit (Kristensen et al., 2011). These receptors will be however insensitive to IEM1460. The enhanced conductance of AMPA receptors can contribute profoundly to synaptic strength and long term potentiation (Luthi et al., 2004;Poncer et al., 2002).

More data of single channel conductance is needed to clarify the change of AMPA receptor subtype in delay neurons. Nevertheless, the existence of Ca^{2+} permeable AMPA receptors in delay neurons can potentially augment

postsynaptic fast excitatory current mediated by AMPA receptors and may thus increase the activity of excitatory pain pathway in central sensitization.

Unlike in inhibitory tonic neurons, both sEPSC and mEPSC frequency were decreased by IEM1460 in excitatory delay neurons and this may indicate the regulatory role of Ca^{2+} permeable AMPA receptors on both action potential dependent and independent neurotransmitter release. An alternative explanation is that the effect of IEM1460 on mEPSC frequency reflects a postsynaptic effect. Delay neurons may receive two different synaptic inputs with quite different composition of AMPA receptors: one with a predominant contribution of Ca^{2+} permeable AMPA receptors (GluR2 –lacking) but another with Ca^{2+} impermeable AMPA receptors (GluR2-containing). Similar examples can also be found in hippocampal neurons (Toth et al., 2000). Thus blocking one type of AMPA receptor is likely blocking one type of synapses, which can lead to decreased frequency.

This possible postsynaptic effect does not exclude the contribution from presynaptic mechanisms. As mentioned above, the frequency of mEPSC represents the machinery of presynaptic neurotransmitter release (Heinke et al., 2011). AMPA receptors in primary afferent terminals can modulate the glutamate transmission and act as autoreceptors (Lee et al., 2002). Other than primary afferent terminals, neurotransmitter release also determines the frequency of sEPSC and mEPSC from local interneurons. Both types of AMPA

receptors were found in the presynaptic terminals in the superficial laminae (Engelman et al., 1999) and it seems presynaptic Ca^{2+} permeable AMPA receptors expressed in nociceptors are most likely GluR1 containing . Therefore, presynaptic Ca^{2+} permeable AMPA receptors of delay neurons may also have specific roles in regulating neurotransmitter release in both sham and CCI conditions. These data suggest that the synapses on inhibitory and excitatory cells are regulated in different ways and AMPA receptors might be importantly involved in the regulation. This issue is considered further in the general discussion.

5.4.3. Axotomy reduced the function of Ca^{2+} permeable AMPA receptors in the presynaptic terminals of tonic and delay neurons.

IEM1460 does not affect the sEPSC frequency of tonic neurons after axotomy (Fig. 5.4A and D, Table 5.1). It also has only a very weak effect on sEPSC frequency in axotomized delay neurons (Fig. 5.9 A and D). This is different from neurons of both sham and CCI groups (Fig. 5.4 A-D and Fig. 5.9 A-D). Thus the loss of effect of IEM1460 after axotomy may reflect loss of presynaptic Ca^{2+} permeable AMPA receptors. This is consistent with the observation that GluR2/3 subunit, primarily GluR2 subunit, was found to selectively increase at the terminals of primary afferents after sciatic nerve axotomy (Popratiloff et al., 1998).

Since axotomy stops the transport of neurotrophic factors from the periphery, the function of Ca^{2+} permeable AMPA receptors may rely on the support of peripheral neurotrophic factors. This concept is corroborated by the observation that sciatic nerve axotomy downregulates AMPA receptor expression on the cell bodies of spinal motorneurons (Popratiloff et al., 1996)

In general, axotomy decreases the function of presynaptic Ca^{2+} permeable AMPA receptors. This effect was not cell type specific, which may contribute to the weaker effect of axotomy than that of CCI in generating central sensitization (see chapter 3). It is possible that loss of positive feedback of neurotransmitter release via presynaptic AMPA receptors after axotomy but not after CCI contributes to this weaker effect.

5.4.4. Effects of inflammatory pain and neuropathic pain on AMPA receptors.

Chronic pain includes a variety of pain states or pain related diseases, each of which generates a unique pattern of neurochemical changes in the spinal cord (Honore et al., 2000). Inflammatory pain and neuropathic pain have been extensively investigated because of the availability of suitable animal models. An example of the neurochemical difference between inflammation and neuropathy is that spinal cord substance P and CGRP are upregulated in inflammation model but downregulated in axotomy model (Honore et al., 2000). There is also some evidence indicating that one group of C-fibres with substance P that are normally insensitive to mechanical and thermal stimulation can be recruited after

peripheral inflammation (Xu et al., 2000). By comparing AMPA receptor subunit change after sciatic nerve injury and after peripheral inflammation, clear differences can also be found.

In the studies of peripheral inflammation, GluR2 subunits were found only to decrease. Some studies found the internalization of synaptic GluR2 subunit via NMDA receptor activated PKC phosphorylation after persistent inflammation (Park et al., 2009;Kopach et al., 2011). In some other studies, GluR1 subunits were found to insert into neuronal membranes but not GluR2 after acute peripheral inflammation, which changed the ratio of GluR1/GluR2 and increased Ca^{2+} permeable AMPA receptors in the plasma membrane (Choi et al., 2010;Larsson and Broman, 2008). Moreover, the GluR1 insertion was found to accompany with pain behavior in animal behavioral test (Choi et al., 2010). Thus, in general, inflammation increases the portion of Ca^{2+} permeable AMPA receptors in the neuronal membrane, which contributes to the increase of neuronal excitability, spinal cord sensitization and pain behavior.

CCI and axotomy are also proved to increase spinal cord sensitization and pain behavior (Balasubramanyan et al., 2006;Chen et al., 2009). However, CCI increase the expression of GluR2 subunit in tonic inhibitory neurons and axotomy increase GluR2 subunit in both inhibitory and excitatory neurons. Increased GluR2 subunit could result in smaller channel conductance, faster kinetics and less Ca^{2+} permeability. Therefore the decrease in Ca^{2+} permeable

AMPA receptor expression in inhibitory neurons may result in the decreased activity of AMPA receptors in the inhibitory pathway which can contribute to the increase of overall excitability of the dorsal horn and the central sensitization.

Increased expression of GluR2 subunit is also found in excitatory neurons after axotomy. This may still be responsible for the central sensitization. GluR2 subunit can bind to a number of intracellular adapter proteins, for example GRIP (glutamate receptor interacting protein), PICK1 (protein interacting with C kinase 1) and NSF (N-ethylmaleimide-sensitive fusion protein), which may link the receptor to proteins with signaling, scaffolding and other roles (Galan et al., 2004).

AMPA receptor changes of CCI and axotomy are different from that of peripheral inflammation. This may reflect part of the mechanisms causing chronic pain.

This study provided new knowledge regarding the effect of peripheral nerve injury on AMPA receptor composition of specific neuron types in *substantia gelatinosa*. This and the findings from previous chapters provide new insights into the mechanism of neuropathic pain at the neuronal and synaptic levels.

Table 5.1 Summary of effects of IEM1460 on tonic and delay neurons.

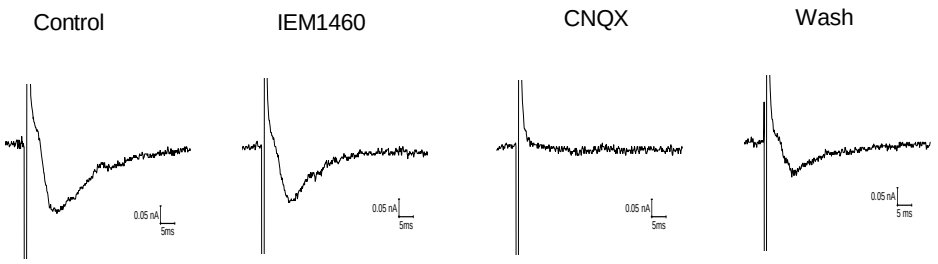
Effects of IEM1460	TONIC NEURONS					DELAY NEURONS				
	sEPSC		mEPSC		eEPSC amp	sEPSC		mEPSC		eEPSC amp
	amp	freq	amp	freq		amp	freq	amp	freq	
Sham	⇩	⇩	⇩	⇔	⇩	⇩	⇩	⇩	⇩	⇩
CCI	⇔	⇩	⇔	⇔	⇔	⇩	⇩	⇩	⇩	⇔
Axotomy	⇩	⇔			⇔	⇩	⇔			⇔

Table 5.2 Values of single channel conductance for AMPA receptors and effects of IEM1460

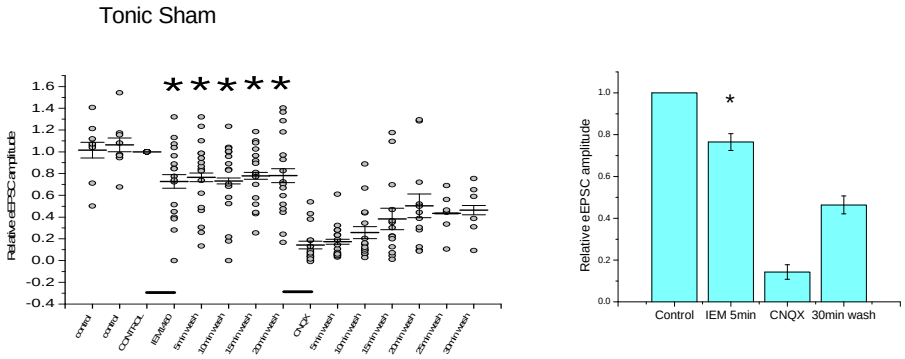
Single channel conductance pS	TONIC NEURONS		DELAY NEURONS	
		With IEM1460		With IEM1460
Sham	15.9±2.1 (n=10)		7.6±1.2 (n=11)	7.7±1.1 (n=4)[5]
CCI	9.2±1.0 (n=10)[1]	9.8±1.6 (n=7)[3]	12.2±1.5 (n=6)[1]	8.3±1.2 (n=5)[4]
Axotomy	13.2±1.4 (n=9)[2]		9.9±1.0 (n=9)[2]	

Data between sham, CCI and axotomy groups were compared by both t-test and ANOVA with Bonferroni correction. Effects of IEM1460 were compared on a neuron by neuron basis using a paired t-test. 1: significant different from sham; 2: not significant different from sham; 3: not significant different from tonic CCI (n=7); 4: significant different from delay CCI (n=5); 5: not significant different from sham (n=4).

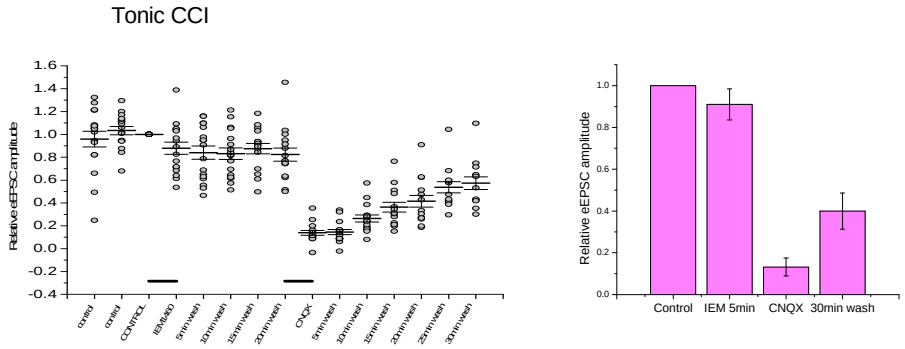
A


Control
IEM1460
CNQX
Wash
0.05 nA
5ms
0.05 nA
5ms
0.05 nA
5ms
0.05 nA
5 ms

B


Tonic Sham
Relative eEPSC amplitude
Control
IEM 5min
CNQX
30min wash

C


Tonic CCI
Relative eEPSC amplitude
Control
IEM 5min
CNQX
30min wash

D

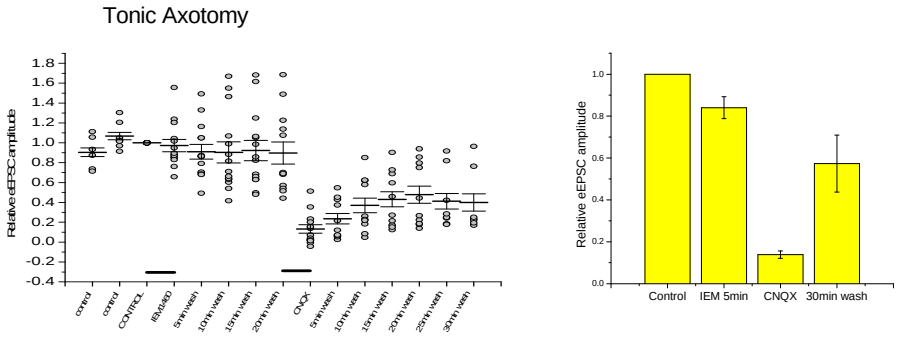
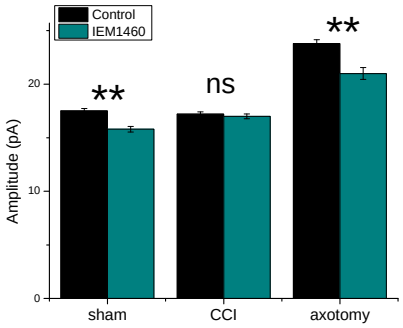

Tonic Axotomy
Relative eEPSC amplitude
Control
IEM 5min
CNQX
30min wash

Figure 5.1 Effects of IEM1460 on evoked EPSCs of tonic neurons

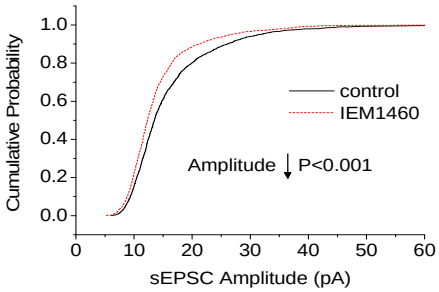
Effects of IEM1460 (50μM) on the evoked EPSC in substantia gelatinosa tonic neurons of sham, CCI and axotomy animals. EPSCs were evoked at the holding potential of -70mV by dorsal root stimulation in the presence of 50μM D-AP5, 10μM SR95531 and 1μM strychnine. A is a sample trace of a sham tonic cell in response to IEM1460, CNQX and the washout of CNQX. Summary histograms for the effect of IEM1460 on eEPSC are shown from B to D. Three control recordings were done before IEM1460 application. IEM1460 was applied for 5mins and washed for 20mins. CNQX was applied for another 5mins to block AMPA receptors and then washed for 30mins. Recordings were done every 5mins. B. Tonic cells from sham animals (n=18); C. Tonic cells from CCI animals (n=14); D. Tonic cells from axotomy animals (n=17). Simplified comparisons between control, 5min wash of IEM1460, CNQX and 30min wash of CNQX are shown beside the original histograms separately. *: $P < 0.05$ on ANOVA test with bonferroni post-hoc test.

Tonic sEPSC amplitude

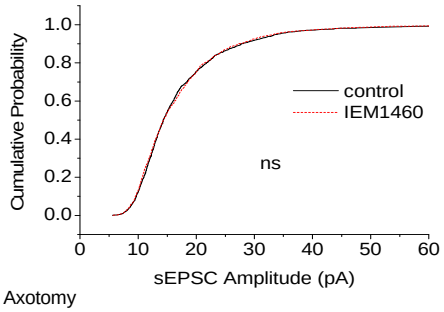
A



B. Sham



C. CCI



D. Axotomy

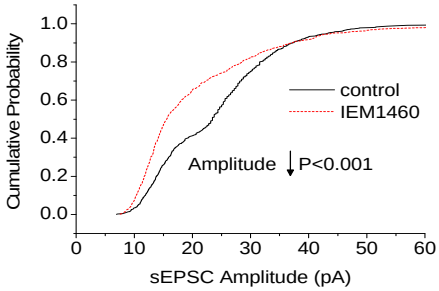
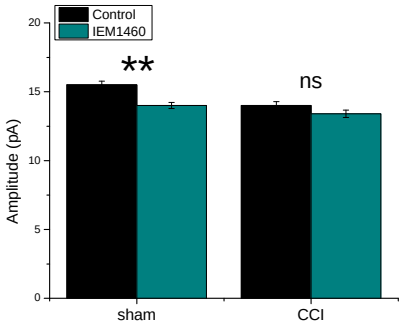


Figure 5.2 Effects of IEM1460 on the amplitude of spontaneous EPSCs of tonic neurons

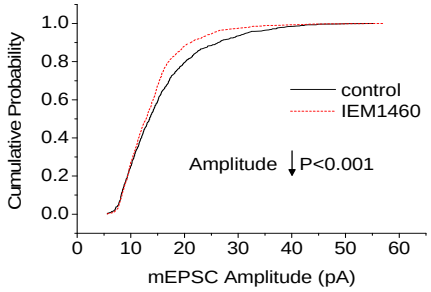
Effects of 50μM IEM1460 on the amplitude of sEPSC in substantia gelatinosa tonic neurons of sham, chronic constriction injury (CCI) and axotomy groups. Holding potential was -70mV. A: effects of IEM1460 on the mean amplitude of tonic neuron sEPSC of sham, CCI and axotomized animals. B: effects of IEM1460 on the cumulative probability of the amplitude from tonic neuron sEPSC of sham animals. C: effects of IEM1460 on the cumulative probability of the amplitude from tonic neuron sEPSC of CCI animals. D: effects of IEm1460 on the cumulative probability of the amplitude from tonic neuron sEPSC of axotomized animals. **: significant different, defined as $P < 0.05$, unpaired t-test. P values from K-S tests indicated on graphs. Data in bar graphs (A) were obtained from the same data sets as cumulative probability plots. For tonic sham neurons, the amplitude of 2302 control events and 1444 IEM1460 events were analyzed from 37 neurons; for tonic CCI neurons, the amplitude of 2187 control events and 1607 IEM1460 events were analyzed from 31 neurons; for tonic axotomy neurons, the amplitude of 854 control events and 672 IEM1460 events were analyzed from 19 neurons.

Tonic mEPSC amplitude

A



B. Sham



C. CCI

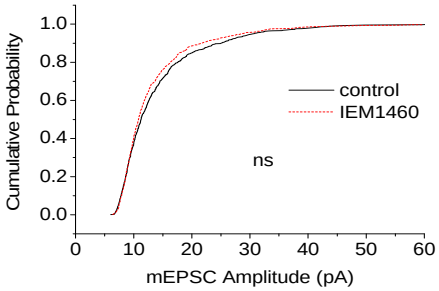
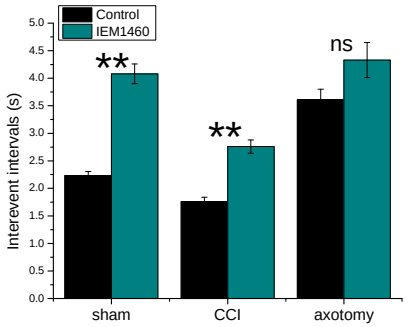


Figure 5.3 Effects of IEM1460 on the amplitude of miniature EPSCs of tonic neurons

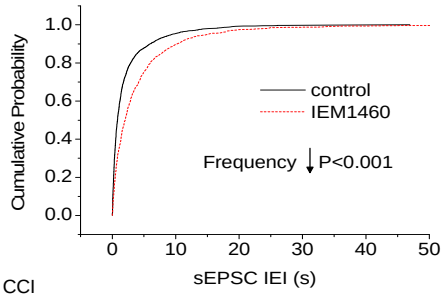
Effects of 50μM IEM1460 on mEPSC in substantia gelatinosa tonic neurons of sham and chronic constriction injury (CCI) groups recorded in the presence of 1μM tetrodotoxin (TTX). Holding potential was -70mV. A: effects of IEM1460 on the mean amplitude of tonic neuron mEPSC of sham and CCI animals. B: effects of IEM1460 on the cumulative probability of amplitude from tonic neuron mEPSC of sham animals. C: effects of IEM1460 on the cumulative probability of amplitude from tonic neuron mEPSC of CCI animals. **: significant difference, defined as $P < 0.05$, unpaired t-test. P values from K-S tests indicated on graphs. Data in bar graphs (A) were obtained from the same data sets as cumulative probability plots. For tonic sham neurons, 814 control events and 779 IEM1460 events were analyzed from 16 neurons; for tonic CCI neurons, 868 control events and 812 IEM1460 events were analyzed from 17 neurons.

Tonic sEPSC IEI

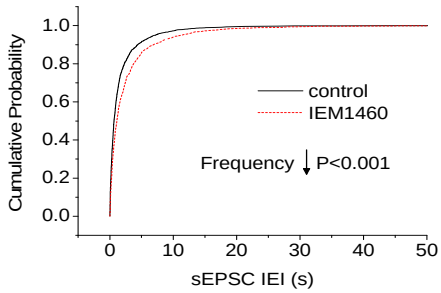
A



B. Sham



C. CCI



D. Axotomy

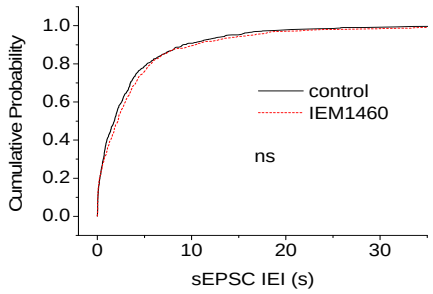
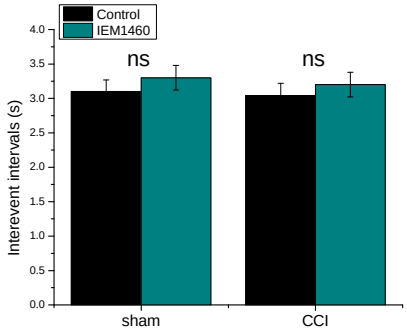


Figure 5.4 Effects of IEM1460 on the frequency of spontaneous EPSCs of tonic neurons

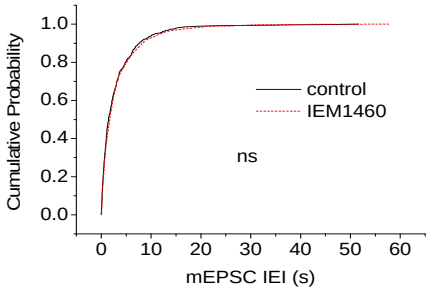
Effects of 50μM IEM1460 on the frequency of sEPSC in substantia gelatinosa tonic neurons of sham, chronic constriction injury (CCI) and axotomy groups. Frequency is represented in the form of interevent interval (IEI). Holding potential was -70mV. A: effects of IEM1460 on the mean IEI of tonic neuron sEPSC of sham, CCI and axotomized animals. B: effects of IEM1460 on the cumulative probability of IEI from tonic neuron sEPSC of sham animals. C: effects of IEM1460 on the cumulative probability of IEI from tonic neuron sEPSC of CCI animals. D: effects of IEM1460 on the cumulative probability of IEI from tonic neuron sEPSC of axotomized animals. **: significant different, defined as $P < 0.05$, unpaired t-test. P values from K-S tests indicated on graphs. . Data in bar graphs (A) were obtained from the same data sets as cumulative probability plots. For tonic sham neurons, IEI of 2302 control events and 1444 IEM1460 events were analyzed from 37 neurons; for tonic CCI neurons, IEI of 2187 control events and 1607 IEM1460 events were analyzed from 31 neurons; for tonic axotomy neurons, IEI of 854 control events and 672 IEM1460 events were analyzed from 19 neurons.

Tonic mEPSC IEI

A.



B. Sham



C. CCI

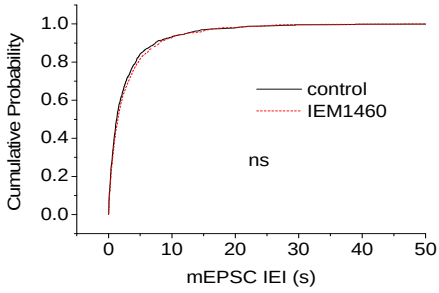
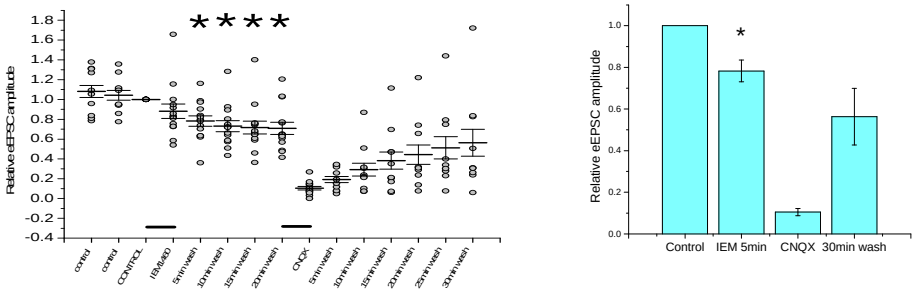


Figure 5.5 Effects of IEM1460 on the frequency of miniature EPSCs of tonic neurons

Effects of 50μM IEM1460 on the frequency of mEPSC in substantia gelatinosa tonic neurons of sham and chronic constriction injury (CCI) groups recorded in the presence of 1μM tetrodotoxin (TTX). Frequency is represented in the form of interevent interval (IEI). Holding potential was -70mV. A: effects of IEM1460 on the mean IEI of tonic neuron mEPSC of sham and CCI animals. B: effects of IEM1460 on the cumulative probability of IEI from tonic neuron mEPSC of sham animals. C: effects of IEM1460 on the cumulative probability of IEI from tonic neuron mEPSC of CCI animals. **: significant difference, defined as $P < 0.05$, unpaired t-test. P values from K-S tests indicated on graphs. Data in bar graphs (A) were obtained from the same data sets as cumulative probability plots. For tonic sham neurons, IEI of 814 control events and 779 IEM1460 events were analyzed from 16 neurons; for tonic CCI neurons, IEI of 868 control events and 812 IEM1460 events were analyzed from 17 neurons.

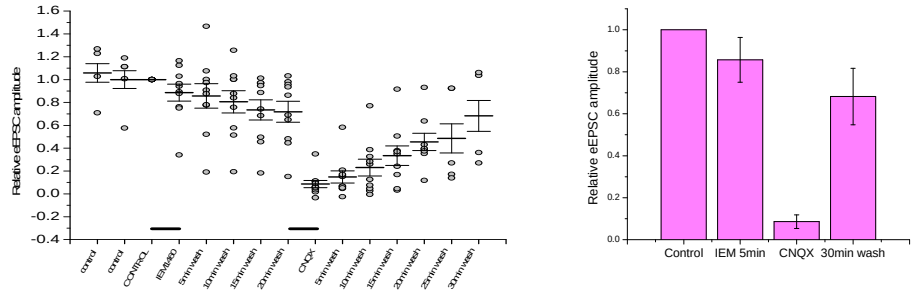
Delay Sham

A



Delay CCI

B



Delay Axotomy

C

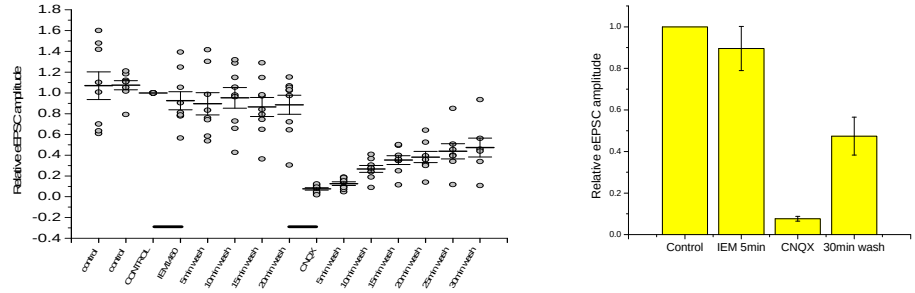
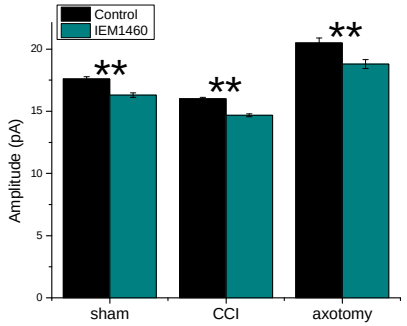


Figure 5.6 Effects of IEM1460 on evoked EPSCs of delay neurons

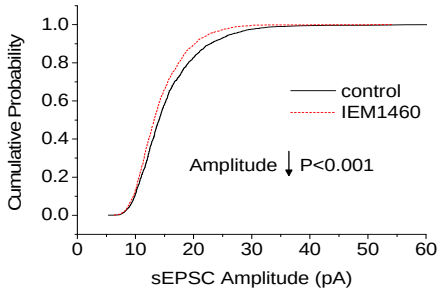
Effects of IEM1460 (50μM) on the evoked EPSC in substantia gelatinosa delay neurons of sham, CCI and axotomy animals. EPSCs were evoked at the holding potential of -70mV by dorsal root stimulation in the presence of 50μM D-AP5, 10μM SR95531 and 1μM strychnine. Summary histograms for the effect of IEM1460 on eEPSC are shown from A to C. Three control recordings were done before IEM1460 application. IEM1460 was applied for 5mins and washed for 20mins. CNQX was applied for another 5mins to block AMPA receptors and then washed for 30mins. Recordings were done every 5mins. B. Delay cells from sham animals (n=14); C. Delay cells from CCI animals (n=10); D. Delay cells from axotomy animals (n=9). Simplified comparisons between control, 5min wash of IEM1460, CNQX and 30min wash of CNQX are shown beside the original histograms separately. *: $P < 0.05$ on ANOVA test with Bonferroni post-hoc test.

Delay sEPSC amplitude

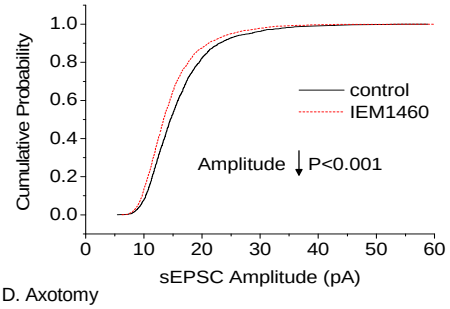
A.



B. Sham



C. CCI



D. Axotomy

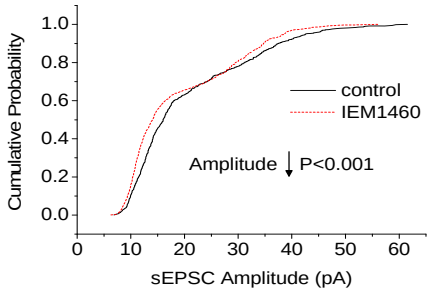
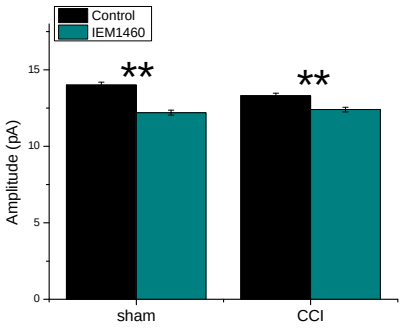


Figure 5.7 Effects of IEM1460 on the amplitude of spontaneous EPSCs of delay neurons

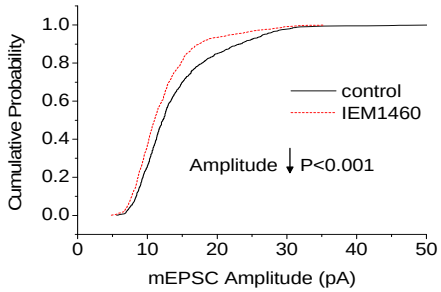
Effects of 50μM IEM1460 on the amplitude of sEPSC in substantia gelatinosa delay neurons of sham, chronic constriction injury (CCI) and axotomy groups. Holding potential was -70mV. A: effects of IEM1460 on the mean amplitude of delay neuron sEPSC of sham, CCI and axotomized animals. B: effects of IEM1460 on the cumulative probability of the amplitude from delay neuron sEPSC of sham animals. C: effects of IEMm1460 on the cumulative probability of the amplitude from delay neuron sEPSC of CCI animals. D: effects of IEm1460 on the cumulative probability of the amplitude from delay neuron sEPSC of axotomized animals. **: significant different, defined as $P < 0.05$, unpaired t-test. P values from K-S tests indicated on graphs. . Data in bar graphs (A) were obtained from the same data sets as cumulative probability plots. For delay sham neurons, the amplitude of 2321 control events and 2058 IEM1460 events were analyzed from 26 neurons; for delay CCI neurons, the amplitude of 2662 control events and 2201 IEM1460 events were analyzed from 28 neurons; for delay axotomy neurons, the amplitude of 721 control events and 811 IEM1460 events were analyzed from 9 neurons.

Delay mEPSC amplitude

A.



B. Sham



C. CCI

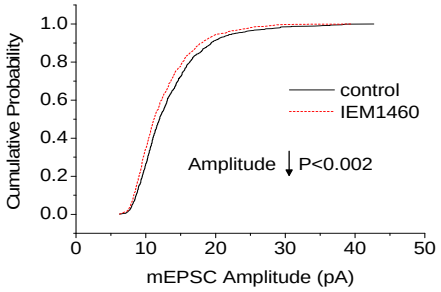
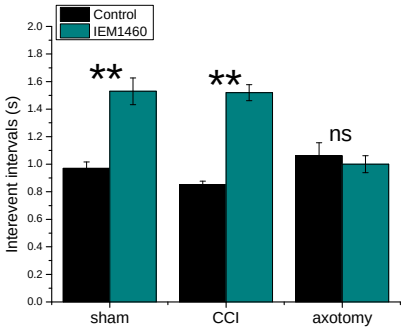


Figure 5.8 Effects of IEM1460 on the amplitude of miniature EPSCs of delay neurons

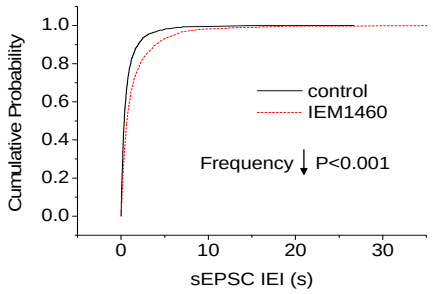
Effects of 50μM IEM1460 on mEPSC in substantia gelatinosa delay neurons of sham and chronic constriction injury (CCI) groups recorded in the presence of 1μM tetrodotoxin (TTX). Holding potential was -70mV. A: effects of IEM1460 on the mean amplitude of delay neuron mEPSC of sham and CCI animals. B: effects of IEM1460 on the cumulative probability of amplitude from delay neuron mEPSC of sham animals. C: effects of IEM1460 on the cumulative probability of amplitude from delay neuron mEPSC of CCI animals. **: significant difference, defined as $P < 0.05$, unpaired t-test. P values from K-S tests indicated on graphs. Data in bar graphs (A) were obtained from the same data sets as cumulative probability plots. For delay sham neurons, the amplitude of 1010 control events and 891 IEM1460 events were analyzed from 12 neurons; for delay CCI neurons, the amplitude of 866 control events and 856 IEM1460 events were analyzed from 9 neurons.

Delay sEPSC IEI

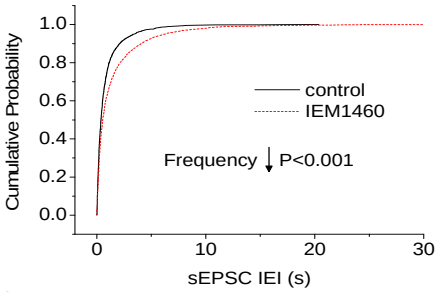
A.



B. Sham



C. CCI



D. Axotomy

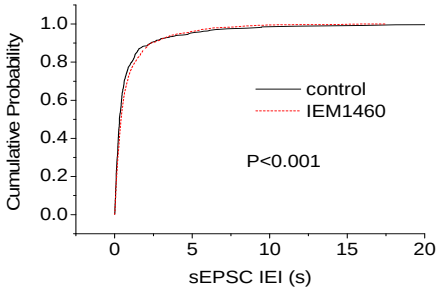
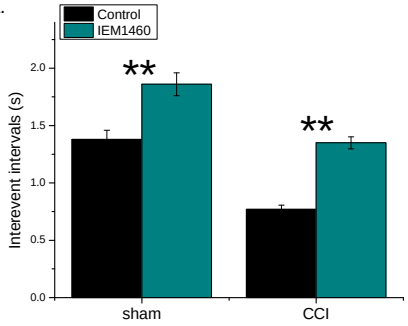


Figure 5.9 Effects of IEM1460 on the frequency of spontaneous EPSCs of delay neurons

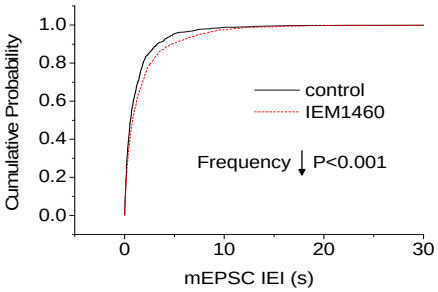
Effects of 50μM IEM1460 on the frequency of sEPSC in substantia gelatinosa delay neurons of sham, chronic constriction injury (CCI) and axotomy groups. Frequency is represented in the form of interevent interval (IEI). Holding potential was -70mV. A: effects of IEM1460 on the mean IEI of delay neuron sEPSC of sham, CCI and axotomized animals. B: effects of IEM1460 on the cumulative probability of IEI from delay neuron sEPSC of sham animals. C: effects of IEM1460 on the cumulative probability of IEI from delay neuron sEPSC of CCI animals. D: effects of IEM1460 on the cumulative probability of IEI from delay neuron sEPSC of axotomized animals. **: significant different, defined as $P < 0.05$, unpaired t-test. P values from K-S tests indicated on graphs. . Data in bar graphs (A) were obtained from the same data sets as cumulative probability plots. For delay sham neurons, IEI of 2321 control events and 2058 IEM1460 events were analyzed from 26 neurons; for delay CCI neurons, IEI of 2662 control events and 2201 IEM1460 events were analyzed from 28 neurons; for delay axotomy neurons, IEI of 721 control events and 811 IEM1460 events were analyzed from 9 neurons.

Delay mEPSC IEI

A.



B. Sham



C. CCI

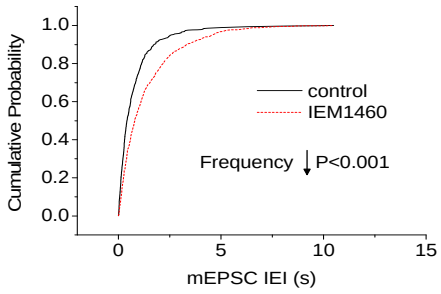


Figure 5.10 Effects of IEM1460 on the frequency of miniature EPSCs of delay neurons

Effects of 50μM IEM1460 on the frequency of mEPSC in substantia gelatinosa delay neurons of sham and chronic constriction injury (CCI) groups recorded in the presence of 1μM tetrodotoxin (TTX). Frequency is represented in the form of interevent interval (IEI). Holding potential was -70mV. A: effects of IEM1460 on the mean IEI of delay neuron mEPSC of sham and CCI animals. B: effects of IEM1460 on the cumulative probability of IEI from delay neuron mEPSC of sham animals. C: effects of IEM1460 on the cumulative probability of IEI from delay neuron mEPSC of CCI animals. **: significant difference, defined as $P < 0.05$, unpaired t-test. P values from K-S tests indicated on graphs. Data in bar graphs (A) were obtained from the same data sets as cumulative probability plots. For delay sham neurons, IEI of 1010 control events and 891 IEM1460 events were analyzed from 12 neurons; for delay CCI neurons, IEI of 866 control events and 856 IEM1460 events were analyzed from 9 neurons.

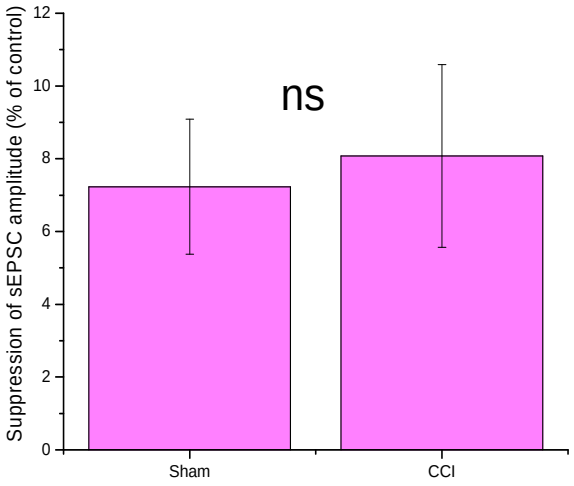

Suppression of sEPSC amplitude (% of control)
12
10
8
6
4
2
0
ns
Sham
CCI

Figure 5.11 Suppression of sEPSC amplitude by IEM1460 in delay sham and CCI neurons.

The mean value of sEPSC amplitude of each neuron before (control) and after IEM1460 application is calculated. The suppression value is calculated as mean control amplitude minus mean IEM1460 amplitude. The suppression value is then divided by the control value to get the suppression percentage. The suppression of sham and CCI group is not significantly different by t-test.

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Antal M, Fukazawa Y, Eordogh M, Muszil D, Molnar E, Itakura M, Takahashi M, Shigemoto R (2008b) Numbers, Densities, and Colocalization of AMPA- and NMDA-Type Glutamate Receptors at Individual Synapses in the Superficial Spinal Dorsal Horn of Rats. J Neurosci 28:9692-9701.

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Chapter 6 GENERAL DISCUSSION

This thesis compares changes in the electrophysiological properties of *substantia gelatinosa* neurons that occur in two models of peripheral nerve injury — axotomy and chronic constriction injury. The main findings are: 1) Sciatic nerve axotomy produces similar yet weaker changes in the electrophysiological properties of *substantia gelatinosa* neurons than CCI. 2) Unlike CCI, sciatic nerve axotomy reduces expression of voltage gated Ca^{2+} channels on primary afferent terminals. 3) CCI decreases expression of Ca^{2+} permeable AMPA receptors and does not decrease the expression of Ca^{2+} permeable AMPA receptors in putative excitatory neurons. 4) Axotomy reduces the function of Ca^{2+} permeable AMPA receptors in the presynaptic terminals of both inhibitory and excitatory neurons. These findings are summarized in Fig. 6.1.

6.1 Dendritic location of synaptic inputs

One issue worthy of mentioning in connection with our analysis is that we have access to synaptic events only as they appear in the cell body. Since the majority of synaptic connections are located on dendrites, altered transmission at these remote sites may not be accurately detected. It is thus possible that very small, slow synaptic events, which would not meet our criteria for bona fide sEPSCs or mEPSCs, may contain additional information about the effects of axotomy on synaptic transmission. It is also possible that alterations in dendritic cable properties following axotomy or CCI may alter the effectiveness of transfer of

remote synaptic events to the cell body. Analysis of such phenomena is not feasible with the technology currently available. This issue may also have a bearing on the paucity of sIPSCs in the somata of lamina II neurons as well as our inability to detect changes in excitability because it is possible that changes in active Na^+, K^+, or Ca^{2+} channel conductances in dendrites may play a role in central sensitization.

6.2 Disinhibition in neuropathic pain

In the present study, we considered delay firing neurons to be excitatory and tonic firing neurons to be inhibitory. This idea is supported by many studies. For delay neurons, first their intracellular stimulation during paired recording experiments produces excitatory events in postsynaptic neurons (Lu and Perl, 2005). Second, studies using a transgenic mouse that coexpresses enhanced green fluorescent protein under the control of the GAD-67 promotor have associated the GABA phenotype with initial burst (phasic), gap (irregular) or tonic neurons and not with delay neurons (Schoffnegger et al., 2006;Zhang and Dougherty, 2011). Third, combined studies of cell morphology, immunohistology and electrophysiology strongly supported the hypothesis that neurons with delay firing pattern are excitatory, while tonically firing neurons are inhibitory (Lu et al., 2009b;Yasaka et al., 2010). Many tonic cells exhibit an islet cell morphology (Lu et al., 2009b) and islet cells are frequently GABAergic (Yasaka et al., 2010;Todd

and Spike, 1993). Moreover, 70% of GAD-expressing neurons in mice exhibit a tonic firing pattern (Labrakakis et al., 2009). Therefore in this thesis, neurons are functionally classified based on their action potential firing pattern.

After sciatic nerve CCI, there is a near complete loss of large myelinated fibers, varieble damage to small myelinated fibres and little damage to unmyelinated fibers (Basbaum et al., 1991;Kajander and Bennett, 1992). In contrast, axotomy produces similar damage in all types of afferents.

The islet/tonic cells were demonstrated to receive input from selective primary afferent C-fibres (Grudt and Perl, 2002;Lu and Perl, 2003) whilst vertical cells can receive monosynaptic inputs from both C and Aδ fibres (Yasaka et al., 2007;Todd, 2010). TRPV1 positive afferents are prominent DR inputs to islet cells (Zheng et al., 2010), whereas TRPA-1 containing C-fibres was found not to make direct contact with islet neurons in *substantia gelatinosa* (Uta et al., 2010), which suggests that different sensory fibres regulate different types of neurons. Sciatic CCI promotes a transient and selective loss of IB4 positive, non-peptidergic primary afferent terminals in inner lamina II (Bailey and Ribeiro-da-Silva, 2006). Axotomy also induces selective loss of IB4 possitive fibers (Reid et al., 2011). Moreover, our findings suggest loss of excitatory synaptic drive to the islet/tonic inhibitory neurons after peripheral nerve injury (Balasubramanyan et al., 2006;Chen et al., 2009). Thus, there is strong possibility that the selective loss of excitatory synaptic drive to inhibitory neurons is caused by the loss of a specific

subpopulation of C fibers. The loss of inhibition in lamina II neurons can increase dorsal horn excitability and nociceptive transmission. In support of this, the pain mediator BDNF decreases sIPSC frequency in some *substantia gelatinosa* inhibitory neurons (Lu et al., 2009a). It was also found that AMPA receptors mediate the excitation spread within dorsal horn in neuropathic pain animals due to the loss of inhibition (Schoffnegger et al., 2008). Presynaptic Ca^{2+} permeable AMPA receptors are primarily distributed on the central terminals of capsaicin-sensitive (TRPV1 channel containing), non-peptidergic afferents (Engelman et al., 1999). Together with the selective loss of non-peptidergic primary afferent terminals (Bailey and Ribeiro-da-Silva, 2006), this may explain the lack of the effect of Ca^{2+} permeable AMPA receptor antagonist on mEPSC frequency of inhibitory neurons after CCI (see chapter 5 and Table 5.1).

The loss of inhibition is not only related to nociceptive C and Aδ fibers. The transmission of polysynaptic inputs from Aβ afferents to neurons in the superficial laminae is increased in neuropathic pain states, which may result from loss of inhibition in deeper laminae (Schoffnegger et al., 2008).

6.3 Axotomy VS. chronic constriction injury

As mentioned before, although sciatic axotomy and CCI produce similar electrophysiological changes in lamina II neurons, they send quite different

signals to the spinal cord. Thus, mechanisms underlying the altered cellular properties may also differ.

6.3.1 BDNF

Depending on the neuropathic pain model, different populations of DRG neurons exhibit differential changes in BDNF expression. Nerve injury models that involve crushing nerves usually promote a marked increase in BDNF expression in DRG neurons of all sizes (Obata et al., 2003). In models involving cutting or ligating nerves, medium and large DRG neurons show an increase in BDNF production, while small DRG neurons show a decrease in BDNF (Zhou et al., 1999). The differences in expression of BDNF with different models of nerve injury could arise from loss of retrograde signaling as a result of severing axons. NGF released during inflammation is a major contributor to BDNF production in afferent C fibres (Michael et al., 1997), and this might be lost following axotomy where augmented NGF synthesis is restricted at sites proximal to the transaction site so the NGF supply is not sufficient for trkA containing DRG cells to produce BDNF(Heumann et al., 1987). Nerve crush models, eg. CCI, damage pheripheral nerves but allow axonal signalling and NGF signalling to continue via surviving axons.

Similarly in the spinal cord, BDNF immunoreactive staining decreases dramatically in the ipsilateral superficial laminae of L5 spinal dorsal horn after

sciatic nerve axotomy while sciatic nerve crush increased BDNF containing axonal fibre expression. This may be related to the difference in trophic support to DRG neurons after the two types of nerve injury (Cho et al., 1998). The synthesis and release of chemical mediators from activated microglia may play a prominent role in neuropathic pain states (Coull et al., 2005;Tsuda et al., 2005). Moreover, the difference in BDNF distribution is also supported by activated microglia distribution in spinal dorsal horn after these two types of injury. CCI increased Iba 1 activation mainly in superficial laminae whereas axotomy induced microglia activation was mainly in deeper laminae (chapter 3). This may reflect the different patterns of primary afferent fiber damage from axotomy and CCI. However, in the recent studies, microglia activation was found at similar location between sciatic nerve axotomized and CCI animals, although CCI produced more activation (Zheng et al., 2011). This is still consistent with our finding that axotomy produced weaker effects than CCI.

BDNF can increase membrane expression of GluR1 subunit while preserving the level of GluR2 and GluR3 in the rodent nucleus accumbens (Li and Wolf, 2011), which suggests BDNF can increase the proportion of Ca^{2+} permeable AMPA receptors. This may be consistent with our observation in putative excitatory delay neurons but does not agree with our findings in tonic inhibitory neurons. There are two possible explanations for this: either BDNF has opposite actions on the two cell types or Ca^{2+} permeable and impermeable receptors are selectively positioned under different primary afferent terminals (Popratiloff et al., 1996),

so in this way, nerve injury-induced retraction of primary afferent fibres (Grelik et al., 2005) may selectively remove terminals onto Ca^{2+} permeable AMPA receptors.

6.3.2 Primary afferent terminals

The Ca^{2+} current in primary afferent terminals was discussed in chapter 4. The paired-pulse stimulation results suggested differential changes in Ca^{2+} current in primary afferent terminals after sciatic nerve axotomy and CCI.

The paired-pulse facilitation in axotomy could result from reduced Ca^{2+} signals in the afferent terminals which may be caused by increased inactivation and decreased single channel conductance of VGCC (Abdulla and Smith, 2001;Baccei and Kocsis, 2000;Jassar et al., 1993;Umemiya et al., 1993), reduced VGCC expression (Fuchs et al., 2007;Baccei and Kocsis, 2000) or reduced intracellular Ca^{2+} store (Rigaud et al., 2009). In the study of AMPA receptors in chapter 5, the lack of effect of IEM1460 on sEPSC frequency in neurons subject to axotomy suggested that Ca^{2+} permeable AMPA receptors do not facilitate neurotransmitter release under this condition. These two factors, plus the retraction of fibres (Grelik et al., 2005) may reduce synaptic input to dorsal horn neurons.

The changes in primary afferent terminal Ca^{2+} signals induced by CCI seemed to differ from those produced by axotomy (chapter 4). Though there are several reports describing the increased VGCC expression in DRG cell bodies (Li et al., 2004) or spinal dorsal horn (Cizkova et al., 2002;Chou et al., 2002), these do not provide direct evidence of the functional Ca^{2+} channel expression in the afferent terminals. Moreover, increased Ca^{2+} signals do not guarantee increased neurotransmission due to the "saturating ceiling effect" (Cao and Tsien, 2010). In my AMPA receptor study, there was no strong evidence supporting the presynaptic AMPA receptor subtype change after CCI compared to sham animals (chapter 5). Though similar to axotomy, there is also loss of selective primary afferent fibers after CCI, the lost fibers are most likely to make contact with inhibitory neurons, which may contribute to increased dorsal horn excitability (see above).

6.3.3 Inflammation in DRG and dorsal horn

In chapter 3, I compared the electrophysiological characteristics of *substantia gelatinosa* neurons and the microglia activation after axotomy and CCI. These properties might be caused by different extent of inflammation induced by the nerve injuries in DRG neurons and spinal dorsal horn.

In DRG cells, there is more neuronal damage after the axotomy and the number of CGRP+ DRG neurons is much more reduced after nerve transection than CCI

(Hu et al., 2007). However, in spinal cord, the aggregation of immunocompetent T-cell lymphocytes in regions of glial activation only occurs after CCI but not axotomy (Hu et al., 2007). This suggests that CCI produce some adaptive immune response that does not occur in axotomy or a retrograde axonal signal is involved in T-cell recruitment into the spinal cord (Hu et al., 2007). The activation of microglia in dorsal horn is also different, with more activation in dorsal side in CCI and more in ventral side in axotomy (Hu et al., 2007). This is consistent with the result of microglia staining (chapter 3).

In all, the inflammation is stronger after nerve CCI because the axons are exposed to the immune cells involved in the inflammatory process of Wallerian degeneration (Basbaum et al., 1991) whereas, after axotomy, the endoneurial environment proximal to the transection site shows only mild inflammation (Hu et al., 2007;Hu and McLachlan, 2003).

6.3.4 Synapses of inhibitory neurons and excitatory neurons

The whole cell recordings performed in this thesis were made in cell bodies and reflected the input information that the neuron received. From previous studies, we know that sciatic nerve CCI and axotomy can both reduce the excitatory synaptic drive to inhibitory neurons and increase that to excitatory neurons in *substantia gelatinosa* (Balasubramanyan et al., 2006;Chen et al., 2009) and BDNF

is probably one of the mediators (Chen et al., 2009;Lu et al., 2007;Lu et al., 2009b).

6.3.4.1 Inhibitory neurons

Downregulation of Ca^{2+} permeable AMPA receptors contributes to inhibitory neuron synaptic events after CCI (chapter 5). Since Ca^{2+} permeable AMPA receptors mediate afferent input onto GABAergic interneurons (Tong and MacDermott, 2006), the downregulated Ca^{2+} permeable AMPA receptors may be linked to the reduced synaptic drive to inhibitory neurons. Reduced excitatory input to inhibitory neurons can result in less inhibitory output which in turn causes the increased dorsal horn excitability. AMPA receptors were found to regulate GABA release from inhibitory neurons by mediting extracellular Ca^{2+} entry (Engelman et al., 2006), which suggested an important role of Ca^{2+} permeabe AMPA receptors. Since we have found reduced Ca^{2+} permeable AMPA receptors after CCI, it is possible that similar regulation of AMPA receptors occurs at the dendrites to reduce inhibitory output.

Sciatic nerve axotomy did not affect the average postsynaptic event amplitude of sEPSC in inhibitory neurons (chapter 3) and there was also not much change in postsynaptic AMPA receptor subtypes (chapter 5). Microglia activation by axotomy is greater in deeper lamina rather than lamina II (chapter 3), which may explain the smaller effect on lamina II neurons. However, the decreased

frequency and its insensitivity of the frequency to IEM1460 may suggest some changes presynaptically (chapter 3 and 5). The activation of presynaptic Ca^{2+} permeable AMPA receptors can increase the frequency of mEPSC, which may reflect the participation of Ca^{2+} permeable AMPA receptors in regulating the neurotransmitter release by controlling Ca^{2+} entry (Engelman et al., 2006).

Moreover, the activation of presynaptic AMPA receptors could inhibit glutamate release from primary afferent terminals onto lamina II neurons via primary afferent depolarization (Lee et al., 2002). This might be another mechanism to regulate glutamate release after nerve injury.

6.3.4.2 Excitatory neurons

The increased amplitude and frequency of postsynaptic events in excitatory neurons suggested increased excitatory synaptic drive after CCI (Balasubramanyan et al., 2006).

Though the amplitude of sEPSCs and mEPSCs in excitatory neurons were unaffected by axotomy, the frequency of both was significantly increased (Chen et al., 2009). The decreased presynaptic Ca^{2+} permeable AMPA receptors (chapter 5) and reduced Ca^{2+} channels (chapter 4) cannot explain the increased frequency of excitatory neurons. It was found that not only the GluR2/3 subunit staining but also the number of glutamate receptors was increased

presynaptically after sciatic nerve transection (Popratiloff et al., 1998). The upregulated GluR2/3 subunits are consistent with our current finding that there is decreased expression of presynaptic Ca^{2+} permeable AMPA receptors. The increased receptor number may explain the increased sEPSC frequency of excitatory neurons. Of course other possible mechanisms may be involved in this process.

6.3.5 AMPA receptors

AMPA receptor antagonists can inhibit thermal hyperalgesia following CCI (Mao et al., 1992). They also appear to have some efficacy in patients with neuropathic pain (Gormsen et al., 2009). Thus both laboratory research and clinical tests suggest the importance of AMPA receptor in the onset and maintenance of neuropathic pain.

The GluR2 subunit of AMPA receptor was reported to increase in the ipsilateral dorsal horn following CCI (Garry et al., 2003;Harris et al., 1996). This is consistent with our findings on inhibitory neurons. Blockade of the interacting protein binding site of GluR2 can attenuate the thermal hyperalgesia induced by CCI (Garry et al., 2003). This is probably due to the disruption of GluR2 endocytosis mediated by protein interacting with C kinase 1 (PICK1) (Perez et al., 2001). Another possible mechanism for synaptic accumulation of GluR2 is that the glutamate receptor interacting protein 2 (GRIP2) reduces the rate of endocytosis

(Osten et al., 2000). This suggested the important role of GluR2 subunit accumulation in mediating the hypersensitivity of pain after nerve constriction injury. GluR2 subunits reduce the single channel conductance of AMPA receptors and reduce Ca^{2+} entry, which may reduce neuronal excitability in inhibitory neurons and contribute to the increase of dorsal horn excitability by decreasing inhibition.

In paired-pulse experiments (chapter 4) I could not define cell types as the use of Cs internal recording solution precluded identification of neurons on the basis of their firing pattern. Our conclusion that axotomy reduces presynaptic calcium currents was therefore based on recordings from all neuron types and we were unable to determine whether axotomy had differential effects on excitatory VS. inhibitory neurons. It was also found in chapter 5 that, in contrast to sham, the frequency of sEPSC of both inhibitory and excitatory neurons was insensitive to the blocker of Ca^{2+} permeable AMPA receptors after axotomy. Therefore reduced presynaptic Ca^{2+} permeable AMPA receptors may contribute to reduced presynaptic Ca^{2+} current after axotomy.

6.3.6 General conclusion

Although axotomy and CCI both increase excitatory synaptic drive to excitatory neurons and decrease synaptic drive to inhibitory neurons (chapter 3), they have different effects on BDNF expression (Cho et al., 1998), inflammation (Basbaum

et al., 1991;Hu et al., 2007), and pre and postsynaptic AMPA receptors (chapter 5). In combination, the factors suggest that axotomy produces similar but weaker effect on dorsal horn excitability than CCI.

6.4 Supraspinal tracts

The acute transverse slice preparation preserves most of the interneuron network in *substantia gelatinosa* but loses the descending control from the supraspinal tracts. The changes in excitability of central neurons that contribute to maintenance of hypersensitivity of pain are not restricted to the spinal cord (Gebhart, 2004). The activation of descending facilitatory mechanisms from the rostroventral medulla (RVM) is critical for the maintenance of the behavioural neuropathic pain state (Porreca et al., 2002). Whole-cell recording on the transverse slices can only reveal the altered impact of surrounding neurons and primary afferent terminal. The descending fibers also make contact with lamina I and II neurons and affect their activities. Thus, one limitation of our approach is that it precluded analysis of changes in supraspinal circuitry in the onset of central sensitization.

6.5 Future perspectives

In the current research, I found out that axotomy and CCI produce a similar "electrophysiological signature" in *substantia gelatinosa* neurons that reflects neuropathic pain. The effects are that putative inhibitory neurons receive less excitatory synaptic drive while putative excitatory neurons receive increased synaptic drive. Different mechanisms underlie neuropathic pain in the two models with different regulation of presynaptic Ca^{2+} and synaptic AMPA receptors. However, more work is needed to confirm the current results and explore more details.

6.5.1 Cell type classification

In the current research, all of the cell type classification is based on the action potential firing pattern at the holding potential of -60mV. As mentioned in chapter 1, cell types also have close relationship with cell morphology. Since in the whole cell recordings biocytin (2%) was added into internal recording solution and the slices were fixed in paraformaldehyde (4%), it is possible to determine the morphology of the recorded cells. Thus, a correlation between electrophysiology and morphology can be made to better define cell types. Moreover, immunostaining of GABA and glutamate transporters can provide direct evidence of inhibitory and excitatory neurons. This will also be used to correlate with electrophysiology and morphology.

Moreover, the Cs internal solution used in paired-pulse recordings made it impossible to determine the cells discharge properties. In this case, cell morphology and immunostaining can be used to identify cell type. Although the average trend for presynaptic Ca^{2+} current change is to decrease after axotomy, there might be different regulation to primary afferents projecting to inhibitory and excitatory neurons. Similar to axotomy induced presynaptic Ca^{2+} current change, CCI may also produce different regulation. Thus this is another point worth of further investigation.

There is also a technique available to label GABAergic neurons directly with enhanced green fluorescent protein (EGFP) under control of the GAD67 promoter in adult mice, which allows direct recording from inhibitory and non-inhibitory neurons (Gassner et al., 2009;Zhang and Dougherty, 2011). This provides a good method of identifying neuron types when other conditions are limited.

6.5.2 AMPA receptors after axotomy

The major effect of CCI and axotomy under the present experimental condition is to produce alteration in spontaneous and miniature synaptic activity. This likely reflects changes in the subunit composition of AMPA receptors. Unfortunately, insufficient data are available to elucidate the effect of axotomy on composition of AMPA receptors. To provide a comprehensive analysis of the different effects

of axotomy and CCI, the sensitivity of miniature EPSCs to IEM1460 in animals subject to axotomy should be done.

6.5.3 Contralateral effects of peripheral nerve injury

As described before, the activation and proliferation of microglia were mostly found on the ipsilateral dorsal horn compared to the contralateral side and sham animals (chapter 3). However, we found a change in mechanical threshold in the behavioural test of the contralateral paw after sciatic nerve CCI (Fig 6.2). The contralateral response is called mirror-image allodynia and arises from the healthy body region contralateral to the site of trauma or inflammation (Milligan et al., 2003). The mirror-image pain is likely resulted from altered spinal processing of incoming sensory information (Watkins and Maier, 2002;Koltzenburg et al., 1999). The pain response in contralateral areas has been found to be less intense and appears later than that experienced on the affected side (Koltzenburg et al., 1999;Arguis et al., 2008).

Thus, the next phase of the project could include an analysis of the electrophysiological properties of neurons on the side contralateral to the nerve injury. Our preliminary data showed that the activities of inhibitory and excitatory neurons were modulated in the same direction on the contralateral side to that on the ipsilateral side. There were studies showing that the spinal glia and proinflammatory cytokines might be responsible for the mediation of

contralateral pain (Milligan et al., 2003;Watkins and Maier, 2002). The widespread glia cell networks provide the means for expansion of pain perceiving region by the gap junctions and propagated calcium waves as well as releasing pain-enhancing substances (Innocenti et al., 2000;Haydon, 2001;Hassinger et al., 1995). Based on these data, cytokines, BDNF and other mediators could presumably diffuse from the ipsilateral to the contralateral side of the spinal cord, but this may not be the case according to our observation that there was no activation of microglia on the contralateral dorsal horn.

As described above, we assume that axotomy produces less inflammation than CCI. The study of sciatic nerve inflammatory neuropathy model showed that low level immune activation produces unilateral allodynia whereas more intense immune activation produces bilateral allodynia (Milligan et al., 2003). Through the examination of electrophysiological properties of neurons and behavioral tests on the contralateral side, we may also be able to relate the electrophysiology and behavior with the extent of inflammation produced by different types of nerve injury.

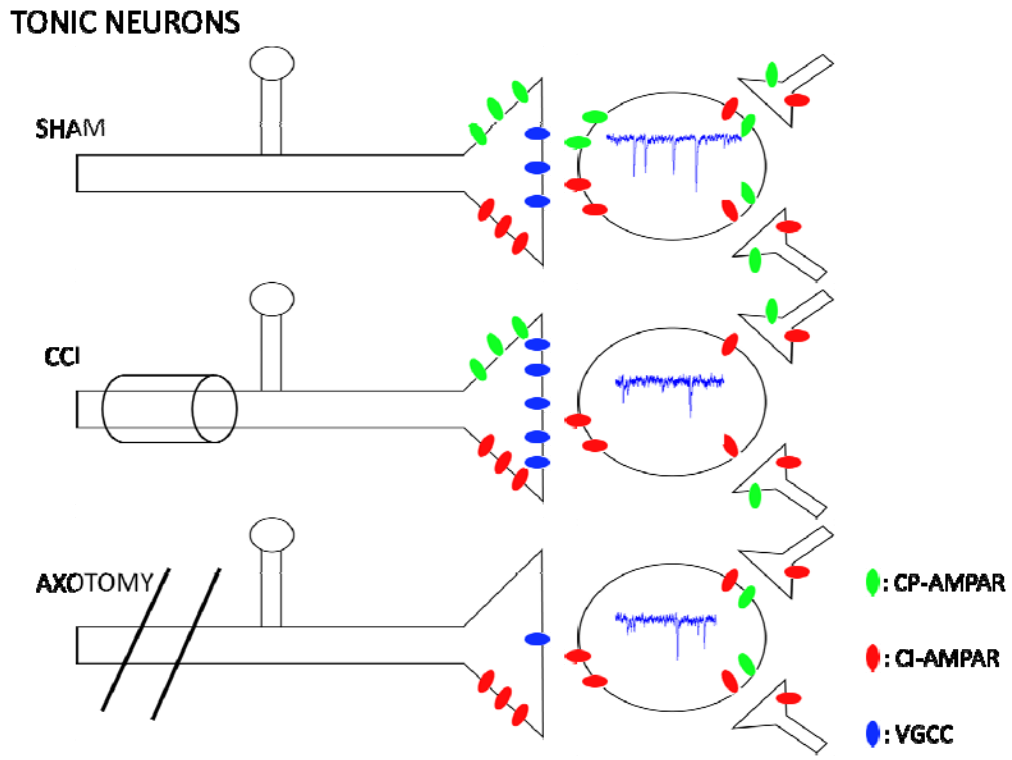
The contralateral thermal hyperalgesia and mechanical allogynia are distinct from each other in terms of the mechanisms involved. The contralateral thermal hyperalgesia is mediated by substance P, glutamate receptors and dynorphin (Coderre and Melzack, 1991;Chen et al., 2000;Malan et al., 2000) whereas contralateral mechanical allodynia is not mediated by NMDA receptors or

dynorphin (Malan et al., 2000). Thus various behavioral tests will also be needed. The results then can be combined with electrophysiological studies and some molecular and pharmacological studies to give better idea of the contralateral effects.

6.6 Final conclusion

Different types of peripheral injury produce similar increases in dorsal horn excitability. They increase the excitatory synaptic transmission to putative excitatory neurons and decrease the excitatory synaptic transmission to putative inhibitory neurons. These changes are caused by different mechanisms. By identifying the underlying molecular mechanisms, we can start to develop treatments and therapies that specifically target different types of neuropathic pain to gain the maximum therapeutic benefit.

A.


TONIC NEURONS
SHAM
CCI
AXOTOMY
: CP-AMPAR
: CI-AMPAR
: VGCC

B.

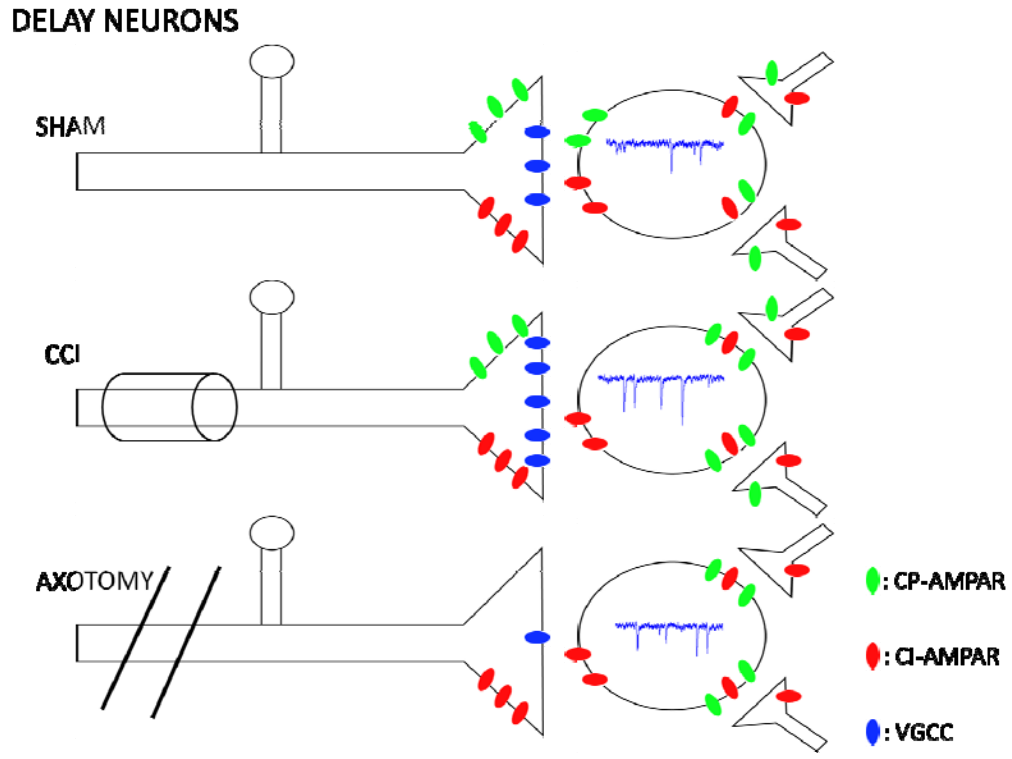

DELAY NEURONS
SHAM
CCI
AXOTOMY
: CP-AMPAR
: CI-AMPAR
: VGCC

Fig. 6.1 Summary of changes in substantia gelatinosa neurons after axotomy and CCI.

A. Summary of changes in tonic neurons. CCI increases VGCCs on primary afferent terminals, decreases Ca^{2+} permeable AMPA receptors and decreases amplitude and frequency of sEPSCs in tonic neurons. Axotomy decreases VGCCs on primary afferent terminals, decreases presynaptic Ca^{2+} permeable AMPA receptors and decreases amplitude and frequency of sEPSCs of tonic neurons. B. Summary of changes in delay neurons. CCI increases VGCCs on primary afferent terminals, decreases Ca^{2+} permeable AMPA receptors on primary afferent synapses and increases amplitude and frequency of sEPSCs of delay neurons. Axotomy decreases VGCCs on primary afferent terminals, decreases presynaptic Ca^{2+} permeable AMPA receptors and increases frequency of sEPSCs of delay neurons.

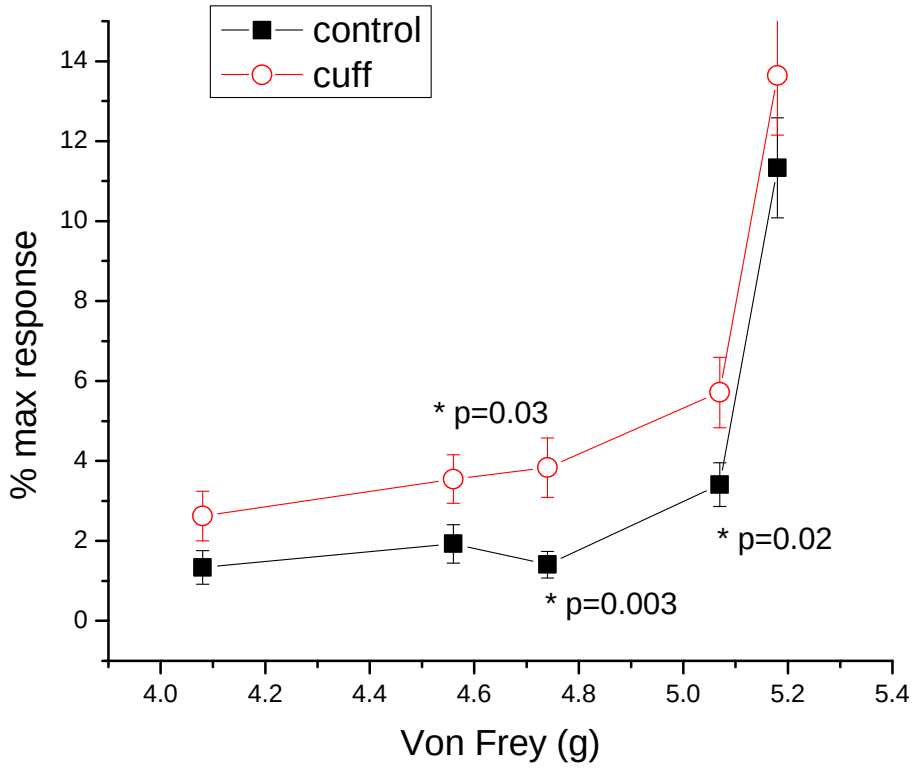

control
cuff
% max response
Von Frey (g)
* p=0.03
* p=0.02
* p=0.003
0
2
4
6
8
10
12
14
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4

Fig 6.2 Comparison of mechanical sensitivity on the contralateral side of sham and CCI rats.

Abscissa is pressure in g exerted by each von Frey hair used and ordinate is response calculated in percentage score of maximum derived from paw withdrawal measurements.

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