University of Alberta

Changes in the Properties of Substantia Gelatinosa Neurons Induced by Peripheral Nerve Injury

by
Sridhar Balasubramanyan 

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Department of Pharmacology

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Abstract

This thesis examines the effect of chronic constriction injury (CCI) of the sciatic nerve on the properties of different types of *substantia gelatinosa* (SG) neurons. The results from these studies improve our current understanding of the etiology of neuropathic pain at the cellular level.

Whole-cell patch-clamp recording under infrared-differential interference contrast (IR-DIC) microscopy was done to characterize the SG neurons in transverse slices of adult rat lumbar spinal cord. The CCI was performed by applying polyethylene cuffs to the sciatic nerve. Post-hoc morphological analysis of biocytin-filled neurons was done using confocal microscopy.

The main findings are (1) Neurons of the SG could be defined as 'tonic', 'irregular', 'phasic', 'transient' or 'delay' according to their discharge pattern at -60mV. (2) CCI decreased the amplitude and frequency of spontaneous (sEPSC's) and miniature (mEPSC's) excitatory post synaptic currents in 'tonic' neurons yet increased them in most other cell types. This may reflect cell-type specific alterations in the neurotransmitter release machinery as well as possible changes in the postsynaptic effectiveness of glutamate.

Morphological studies suggested that the majority of 'islet' cells showed the tonic firing pattern, while the majority of 'vertical' and 'radial' neurons showed the delayed firing

pattern. CCI decreased the excitatory synaptic drive to the tonic islet neurons, but increased it in vertical and radial delay neurons. The decreased synaptic drive may relate to the putative role of' 'tonic islet cells' as inhibitory GABAergic interneurons whereas increased synaptic drive to 'delay' cells may relate to their putative role as the excitatory output neurons. This specific pattern of changes in the SG neurons after CCI is defined as an 'Injury Footprint'. Such complementary changes in synaptic transmission therefore contribute to the onset of pain centralization.

Nitrendipine (2μM), an L-type Ca^{++} channel blocker significantly suppressed both the evoked and sEPSC's in CCI rats. This suggests an involvement of L-type Ca^{++} channels on primary afferent terminals to the increased excitatory synaptic transmission of the SG neurons after CCI. These studies are especially relevant for the management of neuropathic pain as L-type blockers could be an effective additive therapy along with conventional agents.

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List of Abbreviations

aCSF	Artificial cerebrospinal fluid
AHP	After-hyperpolarization
AIDS	Acquired immune deficiency syndrome
AMH	Aδ-fiber mechano-heat-sensitive
AMPA	α- amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Action potential
ATP	Adenosine triphosphate
Bax	Bcl-2 associated X protein
Bcl-2	B-cell leukemia/lymphoma 2
Calbindin-D28K	Vitamin D-dependent calcium-binding protein
cAMP	Cyclic adenosine monophosphate
CCI	Chronic constriction injury
CDNA	Complementary deoxy ribonucleic acid
CGRP	Calcitonin-gene related peptide
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CTB	Cholera toxin B
CV	Conduction velocity
CVLM	Caudal ventrolateral medulla
DRG	Dorsal root ganglion
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol-bis-(2-aminoethyl)-N,N,N, N-tetra-acetic acid
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
GABA	γ-amino-butyric acid
GABA-IR	GABA-immunoreactive
GAD	Glutamate decarboxylase
GAD67	Glutamate decarboxylase 67kDa isoform
GDNF	Glial derived neurotrophic factor
Glycine-IR	Glycine-immunoreactive
HIV	Human immune deficiency virus
HPC	Polymodal nociceptive
HVA	High voltage activated
I_A	A-type K current
IASP	International association for the study of pain
IB4	Isolectin B4
$I\,Ca^{++}\,p$	Persistent calcium current
I_H	H-current
IL-1	Interleukin 1
$I\,Na^{+}\,p$	Persistent sodium current
IPSC	Inhibitory post synaptic current
IPSP	Inhibitory post synaptic potential
IR	Infrared

IR-DIC	Infrared-differential interference contrast
KCC2	Potassium chloride co-transporter 2
Kdr	Delayed rectifier
Lamina II_o	Lamina II outer
Lamina II_i	Lamina II inner
LIF	Leukemia inhibitory factor
LTP	Long term potentiation
mEPSC	Miniature excitatory postsynaptic current
MHC	Major histocompatability complex
mIPSC	Miniature inhibitory postsynaptic current
mRNA	Messenger ribonucleic acid
NA	Numerical aperture
NGF	Nerve growth factor
NK-1	Neurokinin-1
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NOD	Non obese diabetic
NS	Nociceptive specific
P2X	Ligand-gated purinergic receptor
PAD	Primary afferent depolarization
PAG	Periaqueductal grey
PBA	Parabrachial area
PBS	Phosphate-buffered saline
PKCβ	Protein kinase C beta
PKCγ	Protein kinase C gamma
PNS	Peripheral nerve stimulation
PSL	Partial sciatic nerve injury
RAS	Nuclei of the reticular formation
RVM	Rostral ventromedial medulla
SG	Substantia gelatinosa
SHT	Spinohypothalamic tract
SMT	Spinomescencephalic tract
SNI	Spared nerve injury
SNL	Spinal nerve ligation
SNS	Sensory neuron specific
SRT	Spinoreticular tract
STT	Spinothalamic tract
TNF	Tumor necrosis factor
TrkA	Receprtor tyrosine kinase A
TTX	Tetrodotoxin
VR1	Vanniloid receptor 1
WDR	Wide dynamic range

Chapter 1

General Introduction

Pain in the periphery is a natural consequence of tissue injury. Over one third of the world population will suffer from persistent or recurrent pain at some point in their life (Loeser and Melzack, 1999;Loeser et al., 2001). Because nociceptive pain serves as warning signal against potentially harmful stimuli, it is essential for the survival of the individual. On the contrary, injury to the nervous system induces intractable pain that can persist for years after the initial injury had healed. Because this neuropathic pain serves no useful biological purpose, it is sometimes referred to as 'bad' pain (Iadarola and Caudle, 2002). It contributes to the morbidity associated with post-herpetic neuralgia, diabetic neuropathy, cancer pain, as well as injury to the peripheral nerve, spinal cord or brain. About 25-50% of diabetic patients develop neuropathic pain (Schmader, 2002). Painful neuropathy is also a common complication of HIV/AIDS (Schifitto et al., 2002). The annual incidence of herpes zoster is about 2-4 cases for every 1000 people (Schmader, 2002). One-third to one-half of patients experience peripheral neuropathic pain after surgical procedures such as mastectomy and the reported incidence of phantom limb pain after amputation varies from 50-85% (Macrae, 2001). An internet report suggests that by 2014 there will be over 2.6 million people in the United States alone suffering from neuropathic pain (Global Information, 2004). The estimated market value of drugs used for neuropathic pain is about $450 million (Pullar and Palmer, 2003). Neuropathic pain is refractory to common therapeutic interventions and often requires combination of multiple medications and /or other non-pharmacological methods of pain relief (Sindrup and Jensen, 2000). Hence, it is imperative to develop novel therapeutic strategies. This demands better understanding of the complex etiology of neuropathic pain.

In animal models, peripheral nerve injury invokes spontaneous, persistent activity in primary afferent fibers (Cummins et al., 2000a;Devor and Seltzer, 1999;Govrin-Lippmann and Devor, 1978). With time, this aberrant activity may promote an enduring increase in excitability of the dorsal horn neurons of the spinal cord (Devor and Wall, 1981;Doubell et al., 1999;Ji and Woolf, 2001). This form of spinal cord plasticity, driven by the activity in the primary afferents and their sprouting terminals in the dorsal horn is termed as "Central Sensitization" (Ji and Woolf, 2001;Ji et al., 2003). Because of their resemblance to clinical situations/conditions (Hansson, 2003;Backonja, 2003;Attal and Bouhassira, 2004;Woolf, 2004a), animal models using peripheral nerve injury are widely accepted as models for human neuropathic pain (Bennett, 2005).

The current understanding of the central sensitization after nerve injury is based on anatomical, electrophysiological and molecular biological studies (Costigan and Woolf, 2000;Doubell et al., 1999;Ji and Woolf, 2001;Ji et al., 2003;Woolf and Salter, 2000;Woolf, 2004a). Thus, several mechanisms have been proposed including reorganization of synaptic connections, increased or alterations in excitatory transmission, loss of inhibition involving neuronal apoptosis, and alterations in functions of different ion channels in the dorsal horns neurons (Baba et al., 1999;Kohno et al., 2003;Moore et al., 2002;Scholz et al., 2004;Zhuang et al., 2005;Woolf et al., 1995;Coull et al., 2005). Despite this, many aspects of the relationship between peripheral nerve injury and changes in the dorsal horn excitability are poorly understood.

Thus, the major focus of my thesis is to understand how the recognized cellular and molecular mechanisms conspire to produce an overall increase in the excitability of dorsal horn, particularly the *substantia gelatinosa* neurons. In order to address this question, I have made comprehensive study of the effects of chronic constriction injury on the electrophysiological properties of morphologically and electrophysiologically defined neurons of the *substantia gelatinosa*. In order to put my work into perspective, I will first review the relevant aspects of pain pathways with emphasis on changes in the dorsal horn neurons after peripheral nerve injury.

1.1 Nociception

Nociception, simply refers to the physiological transfer of noxious information along the sensory pathways to higher centers in brain for processing. Nociception thus involves two stages; transduction, where noxious information is converted into an electrical signal and transmission, where the signals are perceived and modulated as they pass through peripheral and central nervous system. Transduction occurs at nociceptors, which are functionally specialized regions of the nerve endings. Peripherally located nociceptors are not merely passive transducers of the nociceptive information, as they can sensitize and thereby alter the body's perceptions and hence the response to injury (Lawson, 2002;Stucky et al., 2001). Nociceptive input is conveyed to the central nervous system predominantly by two classes of primary afferent fibers. Of these, thinly-myelinated Aδ-fibers mediate thermal and mechanical nociception whereas the unmyelinated, polymodal C-fibers are activated by a variety of high-intensity mechanical, chemical, hot and cold stimuli (Djouhri and Lawson, 2004). These signals, in addition to evoking a sensory

response, elicit a withdrawal reflex response at the spinal level. This reflex reaction helps to prevent additional injury and permanent damage. Even though nociception and pain are often used interchangeably, in acute and chronic pain models it is difficult to know what is actually perceived by the animal. Hence, we often study nociception and extrapolate the inference to the perception of pain.

1.2 Pain Definition

No single definition seems to encompass all the aspects of pain which range from a simple reflex response to more complex emotional experiences. The consensus definition set forth by the International Association for the Study of Pain is "an unpleasant sensory and emotional experience which we primarily associate with tissue damage or describe in terms of tissue damage, or both". (Merskey and Bogduk, 1994;Backonja, 2003;Bennett, 2003).

1.3 Nociception and Pain

The severity and extent of a painful response to noxious information is highly dependent on the emotional and psychological state of the individual. Thus, two individuals can receive same intensity of injury and yet differ in response to the perception of pain. The complex interaction of discriminative, motivational and emotional elements on the cognitive processing of sensory information limits our understanding on how neurobiological signals are translated to the perception of pain. The current view is that the pain signaling is a dynamic process involving complex networks of neurons whose functions are regulated by external and internal factors.

1.4 Acute vs Chronic Pain: Pain Paradox

As already mentioned, acute, nociceptive pain can prevent further damage to an injured area or even prevent an injury from occurring. Thus, it warns us against harmful stimuli and activates our defensive fight and flight mechanisms, when threatened by hostile stimuli and hence has useful biological purpose. Acute pain has definable source and is rapid in onset and is relatively of short duration. Thus, acute pain disappears once the harmful stimulus is removed or when the injury has healed. Even though changes occur at different levels of pain pathways, these too are transient. Moreover, the pain responds well to conventional analgesics. On the contrary, neuropathic pain typically develops days or weeks after initial injury, and continues beyond the normal time expected for resolution of the injury (Loeser and Melzack, 1999). This pain has no useful biological purpose. Since it outlasts the healing process, chronic neuropathic pain is considered as a disease in itself (Loeser and Melzack, 1999;Merskey et al., 2005).

1.5 Pain Pathways:

1.5.1 Primary Afferents:

Primary afferent fibers convey sensory information about the external and internal environment and are therefore important for maintenance of homeostasis. Sherrington (1906), first proposed the concept of a distinct class of peripheral nerve fibers for conducting pain signals. Zotterman (1936) recorded the first action potentials from a single nociceptive nerve fiber in response to noxious heat in cat's tongue. Later electrophysiological studies demonstrated the existence of primary sensory neurons that can be excited by noxious heat, intense pressure or irritant chemicals (Burgess and Perl,

1967;Bessou and Perl, 1969). Subsequently, several classes of nerve fibres (Raja et al., 1999) that convey information about the type of painful stimuli have been described. The afferent fibers have specialized sensory receptors including nociceptors at their nerve endings (Raja et al., 1999). Nociceptors can be chemical, thermal or mechanical in nature (Raja et al., 1999) and are present throughout the body except the brain. Nociceptors are numerous in the skin, hence pain detection here is well defined and the source of pain can be easily localized. Visceral organs have fewer nociceptors and hence the pain is poorly localized and is sometimes referred to another area far away from the origin ("referred pain"). One of the most important characteristics that distinguish nociceptors from other types of somatic receptors is sensitization (Perl et al., 1976;Bessou and Perl, 1969;Treede et al., 1992). Unlike other somatic receptors, which fatigue after repeated and strong stimulation, nociceptors become more responsive to previously effective stimuli (LaMotte et al., 1982;LaMotte et al., 1983). Nociceptors and their primary afferents are extremely heterogeneous and differ from each other in several aspects such as the neuropeptides, receptors and ion channels they express, conduction speed, spinal terminations, responses to noxious and non-noxious stimuli, and the plastic changes in response to injury (Abdulla et al., 2001;Lawson, 2002;Millan, 1999). However the most widely used classification of Aα/β, Aδ and C fibers is defined to the basis of conduction velocities and threshold characteristics (Lawson, 2002;Djouhri and Lawson, 2004). The classical view is that the anatomically large, fast-conducting myelinated Aβ axons carry signals about non-noxious stimuli and smaller, slowly conducting, thinly myelinated Aδ and unmyelinated C-fibres carry information about thermal and noxious stimuli (Doubell et al., 1999). It is however important to note that discrepancies have been reported in the

literature over the years (Bessou and Perl, 1969;Light and Perl, 2003;Perl, 1996;Lawson, 2002;Djouhri and Lawson, 2004). Hence, it must be realized that under optimal conditions all of these afferent fibers (Aα/β, Aδ and C) have the capability to carry both noxious and non-noxious information and can be activated by both high and low intensity polymodal stimuli (Doubell et al., 1999). It has also been reported that some primary afferent fibers do not respond to any of the stimuli normally employed to activate sensory receptors (Schaible and Schmidt, 1983). These are termed ``silent nociceptors'', which can however be sensitized by noxious events, after which they become responsive to mechanical and thermal stimuli (McMahon and Koltzenburg, 1990b;McMahon and Koltzenburg, 1990a). The presence of several ion channels which are unique for nociceptors have been demonstrated. These include ATP gated P2X channels, acid sensing ion channels, heat sensitive channels including the vanilloid receptor (VR1), and certain voltage gated Na^+ channels (Stucky et al., 2001;McCleskey and Gold, 1999).

Aα/β fibers are heavily myelinated large diameter fibers with high conduction velocity (>30 m/s) (Lawson, 2002;Waxman, 1980;Treede et al., 1998). These fibers are believed to carry non-noxious cutaneous information and mainly terminate deep in laminae (III-V) (Cervero et al., 1994;Fyffe, 1992). However *in-vivo* studies using intracellular recordings have confirmed the presence of Aβ nociceptors and show that substantial proportions of A-fiber nociceptive neurons conduct in the Aβ-fiber conduction velocity group across various species (Ritter and Mendell, 1992). These Aβ-fiber nociceptive neurons also have similar termination patterns to Aδ-nociceptive fibers in the superficial dorsal horn lamina II (Light and Perl, 1979). Under normal conditions, pain induced by activation of

nociceptive neurons is inhibited by the concurrent activation of low threshold mechanoceptors that promote primary afferent depolarization (Van Hees and Gybels, 1981). Hence, at low intensities of afferent nociceptive inputs, the low level release of GABA probably has anti-nociceptive effects, mediated by presynaptic inhibition (Cervero and Laird, 1996b;Cervero and Laird, 1996a). However after nerve or tissue injury, the nociceptors at the lesion site are sensitized (Raja et al., 1999;Treede et al., 1992). Hence, low threshold mechanoceptor activation produces a much more intense primary afferent depolarization generating spike activity. This activation conducted antidromically results in dorsal root reflex (Willis, Jr., 1999). Such activity in C-fibers could release pain mediators in the periphery leading to enhanced inflammatory response or hyperalgesia (Cervero and Laird, 1996b;Cervero et al., 2003).

Aδ fibers are medium to large diameter fibers which are thinly myelinated and have moderate conduction velocity of 4 to 44 m/sec (median, 9.3 m/sec) (Lawson, 2002;Harper and Lawson, 1985). Aδ-fiber nociceptors have been divided, on the basis of their responses to mechanical and thermal stimuli, into three groups: (i) high threshold mechanoreceptive units responding only to noxious mechanical stimuli, (ii) mechano-heat units that respond to noxious mechanical stimuli and also promptly to a single application of noxious heat and (iii) mechano-cold units responding to both noxious mechanical and noxious cold stimuli. (Burgess and Perl, 1967;Raja et al., 1999).

Aδ fiber nociceptors are thought to evoke sharp pricking or fast pain because the latency of withdrawal response to this pain is too short to be carried by slowly conducting C-

fibers (Campbell and LaMotte, 1983). They also signal very precisely about the localization of the noxious stimulus and are important in triggering rapid reflex withdrawal responses (Lawson, 2002). Aδ-nociceptive neurons project to both superficial (lamina I and IIo) and deeper laminae (lamina V) (Fyffe, 1992).

C–fibers (Bessou and Perl, 1969) carry slow, dull, poorly localized, burning type or 'unbearable' sensation (Raja et al., 1999). They are small diameter fibers, un-myelinated and hence have low conduction velocity (< 2 m/s) (Raja et al., 1999). It is generally believed that C-fiber inputs project to the lamina I and II layers of the dorsal horn (Stucky et al., 2001). C-fibers constitute about 70% of all nociceptors. C-fibers from skin are primarily polymodal nociceptors responding to intense mechanical, thermal and chemical stimuli (Dubner and Bennett, 1983;Perl, 1996). However, low threshold C-fiber mechanoceptors have been described in several species (Bessou et al., 1971;Djouhri et al., 1998), which respond to low intensity mechanical stimuli moving very slowly across the skin (Bessou et al., 1971). Hence it is important to realize that nociception and pain, may not be the sole afferent function of C-fibers (Light and Perl, 2003).

C-fibers can also be differentiated based on the peptide markers they express. One group contains neuropeptides such as calcitonin gene related peptide (CGRP) and substance P, and express high affinity receptor (trkA) for nerve growth factor and project to lamina I and lamina II_0 (Averill et al., 1995;Stucky and Lewin, 1999). The other group lacks these peptides but possesses fluoride-resistant acid phosphatase and thiamine monophosphatase activity and binds the plant lectin, isolectin B_4 (IB_4) (Nagy and Hunt,

1982;Silverman and Kruger, 1990) and expresses purinergic (P2x3) receptors (Vulchanova et al., 1998). These IB4-binding neurons project to inner lamina II (Vulchanova et al., 1998) and are sensitive to glial derived neurotrophic factor in the postnatal period and show over expression of the receptor tyrosine kinase during late embryonic stage (Molliver et al., 1997;Bennett et al., 1998). IB_4-positive nonpeptidergic neurons and the IB_4-negative peptidergic neurons also differ in the electrophysiological properties and their response to noxious heat (Stucky and Lewin, 1999). These differing characteristics suggest that these subclasses of C-fibers may differ in their role in nociceptive and sensory processing.

1.5.2 Superficial Dorsal Horn

The dorsal horn neurons act as the site of termination of primary afferent fibers. The primary afferents communicate with the second order sensory neurons of the dorsal horn by releasing neurotransmitters, mainly glutamate and substance P (Dougherty and Willis, 1992;Yoshimura and Nishi, 1992). The organization and the physiological properties of the spinal cord dorsal horn have striking similarities with sub nucleus caudalis (trigeminal brainstem sensory nucleus) or the medullary dorsal horn (Sessle, 1987). The structural classification of the dorsal horn was first described by Rexed (Rexed, 1952).The dorsal horn of the mammalian spinal cord has been divided into six different laminae (Fitzgerald and Lynn, 1977;Molander et al., 1984). These classifications are based on size of neuronal cell bodies, anatomical and functional organization, termination pattern of sensory fibers, neurotransmitter distribution (Willis, 2001;Lawson, 2002;Djouhri and Lawson, 2004) and intrinsic firing properties. The superficial dorsal horn (laminae I and

II), is the major termination site for thin primary afferent fibers and, are important for processing nociceptive information (Treede et al., 1992). Low-threshold mechanoreceptors, which convey non-nociceptive information, terminate in deeper laminae (III & IV) (Cervero et al., 1994). High-threshold nociceptors, such as Aδ-fibers, terminate in laminae I, II and V (Light and Trevino, 1979;Djouhri et al., 1998) and unmyelinated C-fibers,many of which are nociceptors, terminate predominantly in lamina II (*substantia gelatinosa*) (Lawson, 2002;Djouhri et al., 1998). This difference in the specificity of termination of sensory fibers is important to distinguish between an innocuous sensation and a noxious stimulus (Dubner and Bennett, 1983). Neurons in lamina I project, via the spinothalamic tract to higher centers in the CNS (Shea and Perl, 1985).

Neurons in the *substantia gelatinosa* are thought to be a local neuronal network that is involved in processing nociceptive information (Bennett et al., 1980;Gobel, 1978a). The gate control theory predicts that the transmission of sensory nociceptive information is gated at the level of dorsal horn of the spinal cord, thereby allowing or limiting nociceptive information from reaching the higher centers (Wall, 1978;Melzack and Wall, 1965). These "gates" are controlled by activity in small fibers ('On' switches) and large fibers '(Off' switches). When conditions favor excitation such as acute and chronic pain states, the gates are opened and the signals get through. On the other hand, when conditions favor inhibition, the gate remains closed and the pain sensation is alleviated. Such sophisticated modulation is achieved by the complex interplay of neurons and also by the descending influence from higher centers. The most logical explanation of gate

theory stems from the fact that stimulation of large fibers either in peripheral nerves or in the dorsal column relieves pain (Long, 1976). Spinal cord stimulation peripheral nerve stimulation, and transcutaneous electrical nerve stimulation (Lindblom and Meyerson, 1975;Sweet, 1976) methods used to treat neuropathic pain are based on the neurophysiological predictions of gate theory. Even though some of the underlying concepts have been challenged, (Zimmermann, 1968;Schmidt, 1971), the gate theory, still forms the fundamental basis for modern pain concepts (Merskey et al., 2005). Hence, over the years, it was realized that pain transmission is not mere passage of information but involves dynamic (constantly changing) modulation at different levels of the pain pathway. This paved the way to understanding the plastic changes driven by activity (injury) in the nociceptive pathways which outlast the stimulus resulting in central sensitization.

Numerous studies have addressed the anatomical and functional organization of the dorsal horn (Gobel, 1978a;Bennett et al., 1980;Lu and Perl, 2005;Lu and Perl, 2003;Light and Trevino, 1979;Hantman et al., 2004;Grudt and Perl, 2002;Woolf and Fitzgerald, 1983;Prescott and de Koninck, 2002). Several electrophysiological recording techniques such as extracellular unit recordings, intracellular recording with sharp microelectrodes, and patch-clamp recording from spinal cord slices have helped to understand the intrinsic firing and synaptic properties of the dorsal horn neurons (Laird and Bennett, 1993;Yoshimura and Jessell, 1989a;Lopez-Garcia and King, 1994;Moran et al., 2004;Woolf and Fitzgerald, 1983;Baba et al., 2003). In addition, *in-vivo* recordings have

examined the response characteristics of the dorsal horn neurons to the application of peripheral cutaneuos stimulation (Graham et al., 2004).

Various staining procedures have revealed a tremendous amount of information about the morphological features of the dorsal horn neurons (Gobel, 1978a;Bennett et al., 1980;Okamoto et al., 2001;Heinke et al., 2004). In addition, immuno-histocemical techniques and the use of retrograde tracers have been helpful delineating the functional features of the neurons and their projection sites in the brain (Todd et al., 1996;Spike et al., 1998;Sandkuhler and Liu, 1998;Ruscheweyh et al., 2004;Ikeda et al., 2003). Moreover, recent simultaneous recordings from synaptically connected pairs of neurons and subsequent cell fills have started to provide clues regarding a different connection that exists in the dorsal horn and their projections (Lu and Perl, 2005;Lu and Perl, 2003). Hence, it is possible to perform correlative analysis of the physiological features with morphological and functional profiles that will help in elucidating the complex circuitry of this region. These classifications and correlations were made with a view that one could make assumptions on the role of various types of neurons in mediating sensory transmission in normal conditions and during the onset or presence of chronic pain, based on the differences in their intrinsic properties, transmitter and receptor phenotypes, synaptic properties, inputs from afferent fibers, location, projection characteristics, and response profiles to sensory inputs.

1.5.2 .1 Lamina 1 neurons.

Lamina I is one of the key areas for modulating pain information and transmission to the higher centers (Prescott and de Koninck, 2002;Sandkuhler and Liu, 1998;Ruscheweyh and Sandkuhler, 2002). It is the most superficial region of the sensory dorsal horn and receives "heavy input" from nociceptive primary afferents, mainly small diameter C-fibers and also Aδ fibers (Willis and Coggeshall, 2004). Based on response to cutaneous stimuli, a large proportion of lamina I neurons are considered to be nociceptive. However thermoreceptive, and wide dynamic range type, neurons have also been described (Christensen and Perl, 1970;Willis and Coggeshall, 2004;Bester et al., 2000). Lamina I contains local excitatory and inhibitory interneurons as well as large number of projection neurons (Craig et al., 2001;Nichols et al., 1999;Ikeda et al., 2003;Spike et al., 2003). With the advent of high-resolution anterograde tracers it became possible to label specific nociceptive subregions of the dorsal horn that project to different brain nuclei mainly in medulla and brainstem (Ikeda et al., 2003;Spike et al., 2003;Todd et al., 2000;Ruscheweyh et al., 2004). The axon of lamina I neurons in cats and primates project contralaterally in the lateral spinothalamic tract into brain areas which are critical for the sensations of pain, temperature, itch, and sensual touch in humans (Craig, 2000). There are also direct connections to the medulla and brainstem via the spinoreticular (SRT) and spinomesencephalic (SMT) tracts and to the hypothalamus via the spinohypothalamic tract (SHT) (Craig and Dostrovsky, 1999). The periacqueductal gray matter (PAG), parabrachial area (PBA) and the caudal ventrolateral medulla (CVLM) are the major projecting sites for the lamina I neurons in rat (Bernard et al., 1995;Spike et al., 2003). The projections into variety of regions in brain imply that lamina 1 neurons could

modulate autonomic, endocrine, emotional and cognitive function which could have an impact on pain perception. It is the projection neurons that exhibit activity dependent long tem potentiation (Ikeda et al., 2003;Sandkuhler and Liu, 1998). The PAG and PBA projection neurons but not the unidentified neurons show LTP at their synapses with primary afferent C-fibers (Ikeda et al., 2003). There are three main categories of lamina I projection neurons: nociceptive-specific (NS) cells, innocuous thermoreceptive-specific (COOL or WARM) cells, and polymodal nociceptive (HPC) cells sensitive to noxious heat, pinch, and noxious cold (Christensen and Perl, 1970;Han et al., 1998;Dostrovsky and Craig, 1996;Craig and Bushnell, 1994). These three groups have different response properties, different ascending conduction velocities, and different susceptibility to descending or pharmacological modulation (Craig and Serrano, 1994;Dostrovsky et al., 1983). Current evidence indicates that these three categories are also distinct morphologically and correspond to the three major anatomical types of lamina I neurons that can be distinguished in horizontal sections (Dostrovsky and Craig, 1996;Han et al., 1998). A majority (80%) of lamina I projection neurons express neurokinin 1 (NK1) receptor for substance P and are activated by noxious stimuli (Ding et al., 1995;Todd et al., 2000). Selective ablation (P-saporin) of the NK1 containing neurons reduces pain behaviors in inflammatory and neuropathic pain models, suggesting the essential role of these neurons in development and maintenance of chronic pain states (Khasabov et al., 2002;Nichols et al., 1999). Only a minority (5%) of lamina 1 neurons send projections to brain (Spike et al., 2003). Hence, even though most lamina 1 neurons are responsive to substance P, it is not certain that all project rostrally. So most unidentified neurons are

likely to be interneurons (Dahlhaus et al., 2005), that are essential for modulating sensory information before passing them on to the projection cells.

Traditional views presented lamina I as a layer of horizontally oriented cells of a sole type, the marginal cells of Waldeyer (Ramon y Cajal, 1909). A population of these giant cells receives strong GABAergic inputs (Puskar et al., 2001), suggesting they are under strong inhibitory control. However, later studies have suggested that these giant cells constitute only a small proportion of the cell population (Chung et al., 1984;Willis and Coggeshall, 2004). Gobel described two kinds of multipolar neurons (compact and loose) and two kinds of pyramidal neurons (spiny and smooth) based on Golgi studies in cat laminal layer of medullary dorsal horn (Gobel, 1978b). However, later studies have revealed at least four different morphological types of lamina 1 dorsal horn neurons of the spinal cord based on somatic shape and proximal dendritic branching (Galhardo and Lima, 1999;Lima and Coimbra, 1986;Lima et al., 1993;Prescott and de Koninck, 2002). These are defined as (1) Fusiform neurons, which are characterized by spindle-shaped, smooth surfaced cell bodies oriented along the longitudinal axis, and bipolar dendritic arbors. These cells also had the longest rostro-caudal dendritic length. (2) Multipolar neurons were characterized by the bulging ovoid shape of the cell bodies with numerous primary dendritic branching. These cells had the smallest cell body and thin primary dendrites. Multipolar cells also had significant dorso-ventral extension beyond laminal reaching well into lamina II. (3) Flattened neurons, posses' discoid cell bodies flattened across the dorso-ventral axis. They had scarcely ramified aspiny dendritic arbors expanded in the horizontal plane. (4) Pyramidal Neurons, recognized by the triangular

shape of the cell bodies and dendritic arbors expanding longitudinally and latero-medially inside the marginal zone. It must however be noted that there are further subclasses with each subtype and are some structural differences in the morphological classes between species. In addition to being morphologically different, these cells also have a different projection pattern in the thalamus and differ in their axonal conduction velocities (Craig and Dostrovsky, 2001;Han et al., 1998). In cats, fusiform cells, which are believed to be nociceptive-specific neurons (NS), have unmyelinated axons and respond only to noxious heat and pinch (Han et al., 1998). The multipolar and pyramidal neurons have myelinated axons. While the multipolar neurons are polymodal nociceptive neurons, pyramidal cells are innocuous, thermoreceptive (COLD cells) (Craig and Bushnell, 1994;Dostrovsky and Craig, 1996;Han et al., 1998). Consistent with the fact that only nociceptive dorsal horn neurons respond to substance P in primates most fusiform and multipolar cells show NK1 immunoreactivity, whereas only a small proportion of lamina I pyramidal cells express this receptor (Yu et al., 1999). In rodents however, this relationship does not hold true as most of pyramidal cells also express NK1 immunoreactivity and show c-fos expression to noxious stimuli (Todd et al., 2002;Todd et al., 2005).

Lamina 1 neurons could also be distinguished on the basis of their intrinsic firing properties such as tonic, phasic or initial bursting, delayed onset, single spike or transient. The phasic cells are sometimes termed as initial burst cells (Ruscheweyh and Sandkuhler, 2002;Dougherty et al., 2005). Ruscheweyh et al, observed two more firing patterns termed gap firing and bursting firing (Ruscheweyh et al., 2004). Correlation of morphology with firing properties suggest that tonic cells correspond to fusiform cells,

delay and single spike cells are multipolar. The phasic cells are considered to be pyramidal even though the correlation is not very strong in this case (Prescott and de Koninck, 2002;Dougherty et al., 2005). It must be noted that there is slight disparity among different laboratories in the correlation of morphology with firing properties (Dougherty et al., 2005). This could reflect differences in species and/or age and/or differences in recording conditions. Dougherty et al. have recently shown that the predominant neurons expressing GAD67-EGFP in lamina 1 were fusiform and multipolar cells, suggesting these two cells type could be inhibitory neurons (Dougherty et al., 2005).

Based on studies on cortical cells, central neurons could be of two distinct functional types, coincidence detectors or integrators based on the operational mode by which they process incoming synaptic potential (Konig et al., 1996). This assumption is based on the relationship between the integration time, over which neurons can effectively summate synaptic potentials and the mean interspike interval. If the integration time is short compared to the mean interspike interval, coincidence detection prevails, requires the evaluation of temporal simultaneity on a millisecond timescale. On the other hand, if the integration time is long it results in temporal integration which allows for summation of synaptic events over extended intervals (Konig et al., 1996;Rudolph and Destexhe, 2003). Thus in lamina 1 neurons it is reported that phasic and single spike neurons could act as coincidence detectors whereas the tonic and delay cells behave as integrators (Prescott and de Koninck, 2002). The assumption of tonic neurons as integrators stem from the fact that they exhibit prolonged EPSPs compared to other cell types and hence summate

synaptic events (Prescott and de Koninck, 2002;Prescott and De Koninck, 2005) . It is the synergistic effect of both persistent sodium (I $Na^{+}p$) and calcium current (I $Ca^{++}p$) that contribute to shaping EPSPs in tonic cells (Prescott and De Koninck, 2005).

1.5.2.2 Lamina II neurons:

The substantia gelatinosa of Rolando was described as early as 1824, as a distinctive feature in the spinal cord dorsal horn, (Pearson, 1952). This region is characterized by the paucity of myelinated axons and hence appears as translucent band below the marginal layer of the dorsal horn. This translucent property not only made *substantia gelatinosa*, the most conspicuous layer, but also helps prominently in the delineation of additional laminae. The neuronal composition and synaptic architecture of the Lamina II region seems to originate from multiple lineages of the neuronal progenitor cells (Lee and Jessell, 1999;Muller et al., 2002). The *substantia gelatinosa* is believed to be comprised of complex network of local interneurons (Rexed, 1952). This region is thought to integrate noxious information from different classes of afferent fibers and thus to modify the output projection neurons located in both lamina I and the deeper laminae of the dorsal horn (Kumazawa and Perl, 1978;Light and Trevino, 1979;Willis, 2005;Doubell et al., 1999).

The diversity of sensory modalities is encoded by the type of peripheral afferent fibers that are activated, their synaptic connectivity to the dorsal horn neurons and the intrinsic firing properties the neurons (Cervero, 1987;Willis and Coggeshall, 2004). The afferent input to the *substantia gelatinosa* region includes predominantly fine Aδ-fibers and also

unmyelinated C-fibers (Light and Perl, 1979;Sugiura et al., 1986). However, reports from Golgi studies (Beal et al., 1988), transganglionic transport techniques (Rivero-Melian and Grant, 1990), and single fiber analyses (Light and Perl, 1979;Shortland and Woolf, 1993b;Woolf, 1987) suggest that certain classes of myelinated afferents innervating low-threshold mechanoreceptors extend into the inner *substantia gelatinosa* from deeper laminae. Hence, in addition to well-known modulation of nociceptive input, *substantia gelatinosa* is also involved in signaling temperature, itch and some tactile sensations (Perl, 1984;Sugiura et al., 1986;Vallbo et al., 1999). Lamina II can be further sub-divided into lamina IIo (outer) and lamina 2 IIi (inner) based on expression of several markers (Woodbury et al., 2000;Polgar et al., 1999;Todd and Spike, 1993;Silverman and Kruger, 1990). In rodents, choleragenoid (B), the binding subunit of cholera toxin (CTB), and the B_4 isolectin (IB_4) from *Bandeiraea simplicifolia*, have been shown to preferentially label the central projections of myelinated and unmyelinated cutaneous primary afferents (non peptidergic C-fibers), respectively (Robertson and Grant, 1985;Rivero-Melian and Grant, 1990;Kitchener et al., 1993). Several earlier studies indicate that IB4-positive neurons terminate predominantly in inner lamina II (Silverman and Kruger, 1990;Coimbra et al., 1986;Fitzgerald and Vrbova, 1985), however recent findings from Woodbury et al. in 2000 suggest that IB4 labels putative unmyelinated afferents in the outer half of the *substantia gelatinosa* (Woodbury et al., 2000). The reasons underlying this discrepancy are apparently not clear. Myelinated fibers, labeled with CTB seem to terminate in the inner half of the *substantia gelatinosa* (Robertson and Grant, 1985;Woodbury et al., 2000). Another marker which helps to differentiate the inner from the outer lamina II region is PKCγ. PKCγ-immunoreactivity has been found in a restricted population of

small neurons which are concentrated in the inner ventral part of lamina IIi (Malmberg et al., 1997;Mori et al., 1990).

Activation of primary afferent fibers produces excitatory synaptic events mediated by excitatory amino acids and neuropeptides (Yoshimura and Jessell, 1989b;Yoshimura, 1996;Yoshimura et al., 1993;Yoshimura and Nishi, 1992). These excitatory events mediated by primary afferent stimulation are often followed by a short or long duration inhibitory postsynaptic potential (IPSP) or IPSC (Yoshimura and Nishi, 1993;Yoshimura and Nishi, 1995). These IPSPs/IPSCs have variable latencies and exhibit failures following high frequency stimulation, suggesting they are polysynaptic in origin (Yoshimura and Nishi, 1993). Dense core vesicles are common in lamina II_0 synaptic terminals that store and release neuropepetides such as CGRP, substance P, Neuropeptide Y and opioid peptides (Yoshimura, 1996;Willis and Coggeshall, 2004). Substance P and CGRP serve as long-lasting slow excitatory transmitters (Urban et al., 1985;Yoshimura, 1996) and hence may enhance the efficacy of fast transmission (excitatory amino acid mediated) of *substantia gelatinosa* neurons. Neuropepetide Y and opioid peptides modulate both excitatory and inhibitory synaptic transmission in *substantia gelatinosa* region (Kohno et al., 1999;Moran et al., 2004). Unlike lamina I neurons, the intrinsic *substantia gelatinosa* neurons lack NK1 receptors in their cell bodies and hence do not respond to substance P (Bleazard et al., 1994). However, dendritic fibers from neurons of lamina I or deeper laminae (lamina III and IV) which project into lamina II region contain NK1 receptors contributing to the peptidergic transmission in this region (Naim et al., 1997;Todd et al., 2000;Todd and Spike, 1993).

Morphological, electrophysiological and immuno-histochemical studies have suggested the presence of distinctive types of neurons that differ in their responses to sensory stimuli. In addition, the presence of multiple excitatory and inhibitory mediators in *substantia gelatinosa* suggests the complex synaptic circuitry and functional distinctions. Earlier studies indicate the outflow of information from *substantia gelatinosa* neurons is mainly to the projection neurons of lamina I (Bennett et al., 1980;Bennett et al., 1979;Gobel, 1975) or into the neurons of the deep dorsal horn (Gobel and Falls, 1979;Light and Kavookjian, 1988) possibly contributing to the receptive field of wide dynamic range neurons (Szentagothai, 1964). Although the majority of projection neurons of the spinothalamic tract are present in lamina I, some of them do reside in lamina II-III (Giesler, Jr. et al., 1978). Moreover, studies using retrograde tracers and immuno-histochemistry suggest that brain centers capable of modulating pain and nociception send projections to the *substantia gelatinosa* (Willis and Westlund, 1997;Willis and Coggeshall, 2004). Recently, Braz et al. have identified a voltage gated sodium channel (Nav) 1.8 expressing subset of IB4 sensitive fibers, that are involved in a pathway from periphery to lamina II region and then through deep dorsal horn projection neurons, to the limbic regions in brain (Braz et al., 2005). Hence lamina II can no longer be viewed as just a local network system.

One of the major hindrances in interpreting the function of *substantia gelatinosa* interneurons is the lack of unequivocal structural and functional scheme for classifying the cells. Several studies have attempted to identify and classify multiple different cell types in lamina II based on their morphological and functional characteristics

(Christensen and Perl, 1970;Grudt and Perl, 2002;Hantman et al., 2004;Light and Trevino, 1979;Polgar et al., 1999;Heinke et al., 2004;Ruscheweyh and Sandkuhler, 2002;Bennett et al., 1980;Lopez-Garcia and King, 1994). It must be noted that many neurons do not fit a readily recognizable class and show atypical features (Grudt and Perl, 2002;Rethelyi et al., 1989;Todd, 1988). Moreover, intracellular studies have suggested that there is no obvious relationship between receptive field properties of these cells and the morphological class they belong to (Light and Trevino, 1979;Rethelyi et al., 1989;Bennett et al., 1980;Woolf and Fitzgerald, 1983). The most classical view of the *substantia gelatinosa* suggests just two different cell types, the inhibitory islet cell whose dendrites extend rostro-caudally within lamina II and the excitatory stalked cells, whose dendrites extend dorso-ventrally reaching deeper laminae (Bennett et al., 1980;Bennett et al., 1979). Ramon y Cajal (1909) described three types of neurons in lamina II of young cat: vertical oriented limitroph cells with dendrites directed sagitally, central cells located in middle of lamina II with rostro-caudal dendritic orientation and transverse cells with medio-lateral processes. Gobel, utilizing Golgi staining defined four types of neurons in cat lamina II. (1) Islet neurons with saggitally oriented dendrites, confined largely within lamina II and often posses characteristic recurrent branching. (2) Stalked neurons with vertically oriented dendrites, extending across lamina II into lamina III and sometimes even reaches lamina IV. (3) Arboreal (tree- like) cells whose dendrites burst in tree like fashion into highly focal pattern and confined within lamina II and (4) the border cells, which lie in lamina II-III border. Based on differences of the axonal projections (Gobel, 1978a;Gobel, 1975). Gobel defined islet cells to be inhibitory and stalked as excitatory (Gobel and Falls, 1979;Gobel, 1975). But later studies could not differentiate these two

cell types based on responses to cutaneuous stimulation. In monkey lamina II, only islet and stalked cells were reported (Price et al., 1979). Todd and Lewis found that in rat lamina II even though majority of neurons resembled Gobel's classical islet and stalked cells, there are additional categories of cells (Todd and Lewis, 1986). They went further by subdividing islets cells into small islet cells that do not contain GABA or glycine and have axons that form asymmetrical synapses, indicative of glutametergic interneurons, and the large classical, inhibitory islet cells (Todd et al., 1996).

Several types of *substantia gelatinosa* neurons are distinguished on the basis of their intrinsic firing properties (Grudt and Perl, 2002;Lopez-Garcia and King, 1994;Thomson et al., 1989;Yoshimura and Jessell, 1989a). Thus, lamina II neurons can be classified as tonic, delayed onset, phasic or initial bursting or adapting firing, transient or single spike. The ionic basis for tonic firing was described by Melnick at al (Melnick et al., 2004b). They showed that the voltage gated Na^+ and delayed rectifier K^+ channels (DR) were responsible for generating the basic pattern of tonic firing, while Ca^{++}-dependent slow after–hyperpolarization (AHP) stabilized firing and regulated the discharge frequency (Melnick et al., 2004b). The same authors recently demonstrated the mechanism for spike frequency adaptation in the adapting firing (phasic) neurons. Thus, similar to tonic firing neurons, Na^+ and Kdr channels contribute to adapting firing except their amplitudes were smaller. Unlike the tonic cells, however the Ca^{++} dependent K^+ conductance's do not contribute to the discharge pattern of the phasic neurons and it is the reduction in Na^+ conductance that seems to be the crucial factor for adaptation (Melnick et al., 2004a). Tonic and phasic neurons show lack of transient (I_A) type potassium current (Melnick et

al., 2004b;Melnick et al., 2004a), however the I_A current may play a crucial role in determining the delayed onset firing pattern (Yoshimura and Jessell, 1989a;Grudt and Perl, 2002;Ruscheweyh and Sandkuhler, 2002). A role for a K^+ current with the features of a slowly inactivating 'D' current have been suggested for the single spiking phenotype (Ruscheweyh and Sandkuhler, 2002). Comparison of the relationship between a neuron's biophysical profile (AP firing) and its excitatory or inhibitory response to peripheral cutaneous afferent stimulation implies that most wide dynamic range neurons exhibit tonic firing and the nociceptive specific neurons were initial bursters (Lopez-Garcia and King, 1994). However recent *in-vivo* recordings in mice suggest even though neurons differ in response profiles to current injection, they cannot be differentiated based on processing noxious and innocuous information (Graham et al., 2004). Grudt and Perl (2002) have correlated the morphology with the firing properties of five different neuronal types in hamster lamina II region. (1) Islet cells, had marked rostro-caudal elongation (456μm), exhibit sustained (tonic) AP discharge, express hyperpolarization activated ('H') currents and show monosynaptic excitatory post synaptic currents (EPSC's) mediated by C-fibers. (2) Central cells, which show limited rostro-caudal extension (200μm) and their cell bodies and dendrites restricted mainly within inner lamina II. Central cells exhibit mainly transient (initial few spikes and then remain silent) firing or in few cases tonic firing. Most transient central cells show large GABAergic inhibitory postsynaptic currents (IPSC's) and subgroup exhibit transient I_A type potassium current. The transient central cells received monosynaptic input from C-fibers while the tonic central cells received Aδ-fiber input (3) Radial (stellate) cells, whose dendrites radiate in all directions. These cells exhibit a characteristic delay in AP

discharge following the injection of depolarizing current and most had polysynaptic dorsal root evoked EPSC's and IPSC's. The mean frequency of spontaneous EPSC's was very high in this group. (4) Vertical (stalked) neurons, characterized by distinct vertically oriented sparse, wide dendrites. Vertical cells show tonic or delayed discharge in response to depolarizing current. They exhibit Aδ mediated monosynaptic EPSC's, as well high frequency spontaneous EPSC's. (5) Medio-lateral cells, are a rare category which had large dendritic span in both medio-lateral and dorso-ventral directions. These cells show tonic discharge with presence of I_A current and have relatively high frequency sEPSC's. Figure 1.1 illustrates the major neuronal types of the *substantia gelatinosa* based on their dendritic orientations. Table 1.1 summarizes the different neuronal cell types based on morphological features and firing properties reported in the *substantia gelatinosa.*

About 30-45% of neurons in laminae I-III appear to contain GABA and/ or glycine, and a major proportion of the remaining cells contain the excitatory transmitters, glutamate and aspartate (Todd et al., 1996;Todd and Spike, 1993;Todd and Sullivan, 1990). These include stalked and small islet cells of laminae II (Todd and McKenzie, 1989;Todd and Spike, 1993). Immunoreactivity to glutamate has been demonstrated in Laminae I and II. Immunohistochemistry indicates that glutamate is present in high concentrations in axonal boutons in the dorsal horn and can be studied by post-embedding immunogold method (Spike et al., 2003;Ruscheweyh et al., 2004;Dostrovsky et al., 1983;Dahlhaus et al., 2005). Thus, glutamate and to a lesser extent, aspartate are very likely the major

excitatory transmitters in the superficial dorsal horn (Craig and Bushnell, 1994;Dostrovsky and Craig, 1996;Han et al., 1998;Dougherty et al., 2005).

Approximately one third of neurons in the *substantia gelatinosa* are inhibitory, GABAergic or glycinergic interneurons (Todd and McKenzie, 1989;Todd and Sullivan, 1990). These observations are supported by electrophysiological studies, which indicate that both GABA and glycine mediate fast inhibitory transmission in the *substantia gelatinosa* (Yoshimura and Nishi, 1993;Yoshimura and Nishi, 1995). In addition, studies have also demonstrated co-localization and co-release of GABA and glycine in superficial dorsal horn neurons (Todd et al., 1996;Todd, 1996;Chery and de Koninck, 1999). GABA released from the dorsal horn neurons is involved in post synaptic inhibition (Alvarez et al., 1992;Bernardi et al., 1995). Additionally, a presynaptic mechanism of GABA action in lamina II is supported by the fact that many interneurons in lamina II are GABAergic, form synapses on primary afferent terminals (Rudomin and Schmidt, 1999;Schmidt et al., 1998;Todd, 1996). Behavioral pain manifestations are observed in rodents following blockade of spinal GABA receptors. This suggests a tonic GABAergic inhibition is necessary for maintaining normal sensory responses (Sivilotti and Woolf, 1994;Yaksh, 1989;Malcangio and Bowery, 1996). GABA neurons were initially identified by glutamic acid decarboxylase (GAD) anti-sera, an enzyme that synthesizes GABA (McLaughlin et al., 1975). GABA is found to be in high concentration in lamina I-III of the dorsal horn (Todd and McKenzie, 1989;Todd and Sullivan, 1990;Todd et al., 1996). GAD-immunoreactive cells were large, and found close to the dorsal and ventral borders of lamina II (Hunt et al., 1981) and resembled islet cells (Todd

and Spike, 1993;Todd and McKenzie, 1989). GABA appears to coexist with parvalbumin in islet cells and parvalbumin-immunoreactive neurons resembled islet cells (Antal et al., 1990).

Nowadays, it is becoming popular to label a subset of GABAergic neurons in transgenic mice by the expression of green fluorescent protein (GFP) under the control of a specific promoter sequence (Hantman et al., 2004;Heinke et al., 2004;Dougherty et al., 2005). These subgroups of neurons that express GFP are believed to be homogenous in some aspects. Hantman et al, used one such transgenic mouse that express GFP under control of the mouse prion promoter (van den Pol et al., 2002). They have shown that most GFP expressing cells were located in outer lamina II and fit the category of Grudt and Perl's tonic central cells. These cells exhibit c-fos protein expression in response to noxious stimuli and thus seem to participate in nociceptive processing. As expected, these cells show GABA immunoreactivity suggestive of inhibitory neurons and accounts for about 10% of the GABAergic population in lamina II. They are neurochemically homogenous as they contained PKCβ II but not parvalbumin or PKCγ and also showed only C-fiber evoked EPSC's (Hantman et al., 2004). Heinke et al by using another transgenic mouse model which label subgroup of GAD67-expressing GABAergic cells have shown that most GFP expressing neurons are islet cells (note: Heinke at al did not make a distinction between islet and central cells as they used transverse slices) and accounts for about 35 % of the total GABAergic neurons. These neurons were less homogenous with respect to their characteristics. Even though AP discharge was mainly initial bursting (phasic), other firing patterns were also observed. The GFP-cells also show both Aδ and C-fiber evoked

responses and are neurochemically diverse, thus some cells show parvalbumin, some nNOS, some glycine and some neither (Heinke et al., 2004).

1.5.3 Supra Spinal tracts and Brain Centers involved in Nociceptive Processing:

There are several pathways that carry nociceptive information from the superficial layers of dorsal horn into higher centers in brain. Nociceptive projection neurons in the spinal cord transmit information to a number of regions of the brainstem and diencephalon, including the thalamus, PAG, PBA, and bulbar reticular formation, as well as to limbic structures in the hypothalamus, amgydaloid nucleus, septal nucleus, and other sites (Willis and Coggeshall, 2004;Besson and Chaouch, 1987). In addition, several brain centers exert descending influence on modulation of pain either directly or indirectly (through brainstem) by sending projections to the spinal cord (Ossipo and Porreca, 2005;Fields and Basbaum, 1999).

The anterolateral spinothalamic tract (STT) is the most widely studied and hence well described ascending supra spinal pathway (Willis and Westlund, 1997;Willis and Coggeshall, 2004;Willis, 2005). The majority of the projection neurons that travel in the STT originate in the superficial laminae I and II and deeper lamina V of the spinal dorsal horn (Willis et al., 1979). STT is believed to be the major pathway for mediating sensations of pain, touch as well as thermal information (Willis and Coggeshall, 2004). The STT neurons terminate in multiple nuclei in the thalamus, which modulate emotional, motivational, sensory-discriminative aspects of nociceptive processing (Apkarian and Shi, 1994;Craig and Dostrovsky, 1999;Willis and Westlund, 1997). It is

the thalamus where type, temporal pattern intensity, and topographic localization of the pain is integrated and then transferred to limbic and cortical centers (Craig and Dostrovsky, 1999;Willis and Coggeshall, 2004).

The second prominent ascending pathway that is involved in transmission of nociceptive information is the spinomesencephalic tract (SMT) which terminates in different areas of mid-brain including PAG, nucleus cuneiformis, intercolliculus nucleus, deep layers of the superior colliculus, and Edinger-Westphal nucleus (Willis and Coggeshall, 2004). The SMT tract mainly originates from laminae I, II, and V of the spinal dorsal horn (Millan, 1999;Willis and Coggeshall, 2004;Willis, 2005). Terminations in different areas of the mid-brain could activate different affective components to modulate pain sensation. Thus, the projections to the PAG contribute to the response to aversive stimulation (Millan, 1999). In addition it could contribute to activation of descending pain control system (Craig and Dostrovsky, 1999;Ossipo and Porreca, 2005). PAG is one of the prominent analgesic centers in brain, as electrical stimulation of PAG inhibits activity in the wide range dynamic neurons in response to noxious stimuli in primate spinal cord (Hayes et al., 1979). Moreover, electrical stimulation produces antinociception in rodents and also in humans (Ossipo and Porreca, 2005;Craig and Dostrovsky, 1999). Even though there are direct descending analgesic projections from PAG to the dorsal horn, it can also exert its influence through relay stations like the rostraventral medulla (RVM) and other brain stem areas (Willis and Westlund, 1997;Craig and Dostrovsky, 1999). The RVM is a crucial site for balancing descending modulation (Craig and Dostrovsky, 1999;Fields and Basbaum, 1999). In the RVM, two cell types are thought to be involved in pain

modulation, the 'ON' cells which discharge prior to the occurrence of a nociceptive reflex and are pro-nociceptive and the 'OFF' cells which terminate firing prior to the occurrence of a nociceptive reflex and are anti-nociceptive (Fields and Basbaum, 1999). The RVM and the PAG along with the dorsal horn are the important sites for the action of opioids (Craig and Dostrovsky, 1999;Ossipo and Porreca, 2005;Willis and Westlund, 1997;Fields and Basbaum, 1999).

The third most prominent pathway that conveys nociceptive information is the spinoreticular tract (Willis and Westlund, 1997;Willis and Coggeshall, 2004). SRT originates from the deep layers of the dorsal horn and in laminae VII and VIII of the ventral horn (Willis, 2005). In addition, retrograde and anterograde tracing have identified projections of SRT from lamina 1 neurons to several nuclei of PBA including the locus ceruleus of the brainstem (Craig, 1995;Willis, 2005). These cells terminate in several nuclei of the reticular formation (RAS) of the medulla and pons (Willis and Westlund, 1997;Willis and Coggeshall, 2004). The STT neurons can preferentially be activated by noxious stimuli (Willis and Coggeshall, 2004;Willis, 2005). The major role of this pathway is in mediating arousal responses and activating endogenous analgesic control and also in modulating the homeostatic changes through brainstem autonomic centers (Willis and Coggeshall, 2004;Willis, 2005;Millan, 1999).

Even though the above three pathways are the most studied ones, there are several minor yet significant pathways which carry nociceptive information to brain centers from the spinal cord including the spinolimbic, spinocervicothalamic, spinohypothalamic, and

spinoamygdalar tracts (Willis and Westlund, 1997;Willis and Coggeshall, 2004;Willis, 2005;Millan, 1999).

1.6 Animal models of Neuropathic pain

Neuropathic pain is multifactorial disease with complex etiology (Woolf and Mannion, 1999;Smith, 2004). Depending on the nature of the nerve injury, it can have different consequences and hence be treated differently. For example, neuropathic pain, due to diabetes may be due to excessive glucose (sorbitol) (Kawanishi et al., 2003) and hence is different from neuropathic pain due to trauma (Smith et al., 2002;Woolf, 2004a). Thus neuropathic pain should be viewed as a syndrome with different abnormalities in the pain pathway under different conditions. The underlying commonalities are: no visible signs of injury, paradoxical combinations of sensory loss and hyperalgesia in the affected and nearby areas, spontaneous ongoing pain, an increase in pain response following repetitive stimulation and allodynia (Hansson, 2003;Sindrup and Jensen, 1999). Animal models are the mainstay for development of novel therapeutics. Given the diverse etiology of neuropathic pain, it is not possible to develop one single animal model which encompasses all the attributes with sufficient predictive, and construct validity (Blackburn-Munro, 2004;Kontinen and Meert, 2003). This underlies the importance of development of different animal models which can mimic neuropathic pain in different conditions with different intensities of insults. Despite rapid advancements over the years, an ideal model of neuropathic pain is still a great challenge. Moreover, interpretation of animal's behavioral responses to different pain tests and correlating with human symptoms is a matter of debate (Blackburn-Munro, 2004;Bennett, 2005).

Most neuropathic pain models involve experimentally controlled injuries to the spinal or peripheral nerves. The commonly studied animal models of neuropathic pain are due to direct nerve injury which involves manipulations (cut or ligation) of the peripheral sciatic nerve (main trunk or its branches) or its spinal counterpart the lumbar 5 or 6 spinal nerves (Bennett and Xie, 1988;Kim and Chung, 1992;Wall et al., 1979;Seltzer et al., 1990;Mosconi and Kruger, 1996;Decosterd and Woolf, 2000) (Figure 1.2).

The limitations of animal models are that the pain induced in the laboratory does not exactly mirror the chronic pain situations normally encountered in clinics. Most animal models of neuropathic pain are studies in the laboratory for weeks or months, whereas the clinical course of neuropathic pain is in years or even decades. Moreover, the complex experience of painful sensation in human is reduced to 'Pain like' withdrawal reflexes to mechanical and thermal stimuli in animals (Ekblom and Hansson, 1993;Blackburn-Munro, 2004). As a result, extrapolation of animal results to humans should be made with a degree of caution. Despite these limitations, the animal models have contributed enormously to our current understanding of neuropathic pain etiology.

1.6.1 The Neuroma Model (axotomy)

The neuroma model, developed by Wall et al was the first model of peripheral nerve injury (Wall et al., 1979). This model involves sectioning the sciatic nerve completely at the mid thigh level (Figure 1.2). In some cases, the cut end of the nerve was also encapsulated to prevent possibility of the sprouts invading the surrounding tissue. Following complete nerve transection of the sciatic nerve of rats and mice, a neuroma

develops at the proximal nerve stump, consisting of regenerative nerves sprouting in all directions (Amir and Devor, 1993;Devor and Wall, 1976). Wall et al. observed self-attack and mutilation of the injured limb, and used the term 'autotomy' to describe the behavior that is probably caused by denervation of the hindpaw (Wall et al., 1979;Wall and Gutnick, 1974). This self-mutilatory behavior is interpreted as the animal's response to a spontaneous pain and is viewed as the equivalent of human phantom limb (Coderre and Melzack, 1986;Kauppila, 1998). However, it has also been suggested the autotomy arises due to motor and sensory denervation of the hind paw rather than pain (Rodin and Kruger, 1984;Kauppila, 1998), hence interpretations of autotomy as an index of pain have been a subject of serious debate over the years (Coderre et al., 1986;Kauppila, 1998). The limitation of this model is that the self-mutilation phenomenon is rarely observed in humans (Bennett and Xie, 1988;Mailis, 1996). Even though this model has made enormous contribution in understanding underlying mechanisms of neuropathic pain, it represents only a very special case of nerve damage and also does not exhibit the common manifestations of peripheral neuropathy particularly hyperalgesia and allodynia (Bennett and Xie, 1988).

1.6.2 Chronic Constriction Injury Model (Bennet model or CCI)

This model was developed by Bennet and Xie in 1988, where they reported a peripheral neuropathy model that produces pain symptoms like those seen in man (Bennett and Xie, 1988). This model involved placing loose ligatures around the common sciatic nerve in such a way that the diameter of the nerve was just barely constricted so that the circulation was retarded but not completely arrested (Figure 1.2). The rats showed

behavioral signs such as, guarding (lifting from surface), excessive licking and limping of ipsilateral hind paw, and avoidance of placing weight on the injury side, and symptoms of spontaneous pain (mild to moderate autotomy). Moreoever, the rats also exhibited prominent symptoms of hyperalgesia and allodynia with respect to both thermal and mechanical stimuli (Bennett and Xie, 1988).

1.6.3 Partial sciatic nerve injury (PSL or Seltzer model)

Seltzer et al developed a rat model of neuropathic pain in 1990 to mimic causalgia in humans (Seltzer et al., 1990). The causalgia which is characterized by burning pain combined with hyperalgesia and allodynia is usually observed after partial or incomplete nerve injury, but not complete nerve section. In this model, the sciatic nerve was exposed at high thigh level and ligated with suture in such a way that 1/3–1/2 (33-50%) thickness of the nerve is trapped in the ligature, while leaving the rest 'uninjured'. Rats in this model also exhibited signs of robust allodynia and hyperalgesia.The rats also exhibited signs of spontaneous pain with respect to gait and posture (Seltzer et al., 1990).

1.6.4 Spinal Nerve Ligation Model (SNL) (Kim and Chung)

This model was developed by Kim and Chung in 1992 as another neuropathy model resembling human pain symptoms (Kim and Chung, 1992). In this model either L5 or the L5 and L6 spinal nerves were isolated and tightly ligated distal to the dorsal root ganglia (DRG) with silk thread (Choi et al., 1994;Kim and Chung, 1992) (Figure 1.2). Hyperalgesia and allodynia developed quickly after the ligation. As seen in other models,

symptoms of spontaneous pain such as licking the paw on injured side as well as limping on the operated foot were observed. However, there was no obvious signs of autotomy.

A major problem with Bennet model is the variability in terms of tension around the nerve which in turn will affect the number and type of injured afferent fibers. This is true for even the Seltzer model as the variability may result from failure to damage exactly the same part of the nerve in each animal. Compared to Bennet and Seltzer model the SNL seemed to be more consistent in terms of ligation and also has the advantage of separation of injured and intact spinal segments (Kim et al., 1997;Lee et al., 1998). This permits analysis of the role of injured as well as intact fibers in terms of development and maintenance of pain. In addition, the analgesic effect of sympathectomy was more evident in SNL compared to Bennet or Seltzer model and hence SNL is a preferred model to study mechanisms underlying sympathetically maintained pain (Kim et al., 1997). In terms of behavioral parameters, the sign of touch evoked pain (allodynia) is more robust in SNL whereas the ongoing pain was more evident in Bennet model. The disadvantage of SNL is being technically demanding and requires extensive surgical procedure and hence difficult to perform in smaller rodents.

1.6.5 Chronic Constriction Injury with Polyethylene Cuffs:

This was a variant of the Bennet model. Several labs have utilized the CCI (Bennet) model over the years to study underling mechanisms of neuropathic pain. Even though most labs report evidence of allodynia and hyperalgesia, there is wide variability in the extent of fiber spectrum (myelinated and unmyelinated axons) damage (Attal et al.,

1990;Bennett and Xie, 1988;Coggeshall et al., 1993). In addition, there could be inter-animal variability in terms of pathological change and resulting behavioral manifestations. This makes the comparison of results from different labs in terms of quantitative analyses difficult. These variations could be likely due to in controlling the tightness (Nuytten et al., 1992;Ro and Jacobs, 1993) of the constriction with sutures, and also because of different suture materials (chromic gut, plain gut, silk) (Kupers et al., 1994). Hence, to minimize these shortcomings, Kruger and Mosconi developed a variation in CCI model called the cuff model in 1996, which involves applying small polyethylene cuffs, loosely around the sciatic nerve (Mosconi and Kruger, 1996) (Figure 1.2). As the cuffs were wider, there is no marked constriction or translucency in the constricted region and hence less ischemic damage. The alteration in fiber spectrum damage includes transient decrease unmyelinated and small myelinated axons and a sustained decrease in large myelinated axons. In addition, Wallerian degeneration and inflammatory reactions at near and distal sites of injury were reported. Another significant difference in the cuff model is the non-occurrence of autotomy (Mosconi and Kruger, 1996;Pitcher et al., 1999). The behavioral manifestations of hypersensitivity to mechanical and thermal stimuli were comparable to the Bennet model. Hence, this model has the advantage to being easy, standardized and reproducible constriction injury model (Mosconi and Kruger, 1996;Pitcher et al., 1999;Coull et al., 2003). It was therefore chosen as the neuropathic pain model for the experiments in this thesis.

1.6.6 Spared Nerve Injury (SNI):

This is fairly recent model developed by Decosterd and Woolf in 2000 (Decosterd and Woolf, 2000). The sciatic nerve has three terminal branches namely the tibial, peroneal and the sural nerves which innervate different regions of the dorsal and plantar surfaces of the paw. The earlier models of Bennet and Seltzer consisted of constricting or ligating the main sciatic nerve trunk. In this model however, the tibial and peroneal nerves were ligated and sectioned distally, while leaving the sural nerve intact (Figure 1.2). The aim of such injury is to understand the contribution of both injured and uninjured sensory axons to development and maintenance of pain. This model resulted in substantial and prolonged mechanical and thermal hypersensitivity. Also, compared to Bennett's model where behavioral hypersensitivity is seen in the whole area of the damaged sciatic nerve territory, in SNI it is restricted to the intact sural nerve territory. The author's further claim, by sparing the sural nerve there is minimal co-mingling of intact axons with denervated schwann cells (Decosterd and Woolf, 2000).

There are other less commonly used nerve injury models like sciatic cryoneurolysis model which involves freezing the sciatic nerve to induce damage; sciatic inflammatory neuritis model which involves injection of zymosan around the sciatic nerve (Chacur et al., 2001;DeLeo et al., 1994). There are also central pain models which are based on spinal cord injury like weight drop or contusion model, photochemical model and excitotoxic spinal cord injury (Hao et al., 1991;Yezierski, 1996). In addition, there are neuropathy models induced by diseases such as diabetes (streptozotocin model, insulin deficient BB rats and NOD mice, insulin resistant ob/ob and db/db mice) (Mosseri et al.,

2000;Pieper et al., 2000;Courteix et al., 1994) and post herpetic neuralgia (Rowbotham and Fields, 1996). Moreover, there are also chemotherapy induced neuropathic pain models (Portenoy, 1999).

1.7 Neuropathic Pain and Pain Pathways:

Neuropathic pain is characterized by sensations of burning, electricity, extreme cold, numbness and tingling. It can be triggered by conditions such as trauma, diabetes, post herpetic neuralgia, heavy metal toxicity and some medications. Clinically, it is difficult to define neuropathic pain and set an unequivocal diagnostic criterion (Attal and Bouhassira, 2004;Bennett, 2003;Hansson, 2003;Backonja, 2003). There are certain conditions where identification is relatively straightforward like diabetic neuropathy, herpetic neuralgia and spinal cord or nerve injuries. However, clinical conditions such as fibromyalgia (Moldofsky and Merskey, 2005;Treede et al., 1992) do not have clearly identified lesions but yet may involve some nerve dysfunction (Hansson, 2003). Moreover, in certain conditions such as arthritis or cancer pain, it is difficult to isolate possible neuropathic component from inflammatory component (Backonja, 2003;Portenoy and Lesage, 1999).

Neuropathic pain has been viewed as a consequence of long term plastic changes involving both neuronal and non-neuronal cells. Peripheral nerve damage results in spontaneous, persistent, aberrant activity in the primary afferent fibers (Devor and Seltzer, 1999;Wall and Devor, 1983;Govrin-Lippmann and Devor, 1978) and leads to an increased excitability of the DRG neurons (Amir et al., 2005;Abdulla and Smith,

2001a;Spike and Todd, 1992;Todd and McKenzie, 1989). In addition, there is a sustained increase in excitability of dorsal horn neurons (Woolf and Salter, 2000;McMahon et al., 1993) leading to abnormal pain sensation. Thus the pain, which had a peripheral site of origin, advances into the central nervous system (CNS) and becomes "centralized" and is often irreversible (Devor and Seltzer, 1999;Woolf et al., 1995;Woolf, 1983). The etiology of neuropathic pain is complex so that development of effective therapy is challenging. However, there is a steady increase in the understanding of anatomical, cellular and molecular basis of neuropathic pain. This, along with the advent of many experimental models, has helped us to make considerable progress and has led to the prospect of exploring novel therapeutic targets.

1.7.1 Changes in Primary Afferent Fibers:

Nerve injury induces a phenotypic shift in the properties of primary afferent fibers (Millan, 1999;Xiao et al., 2002). Nerve transection results in the formation of neuroma at the proximal nerve stump which consists of regenerative nerve sprouts (Devor and Wall, 1976;Wall and Gutnick, 1974). The pronociceptive cytokines [e.g., tumor necrosis factor (TNF)α, interleukin (IL)-1 ß], or other inflammatory mediators released from injured site excite the neighboring intact DRG neurons (Li et al., 2000). The increased expression of proinflamatory cytokines affects vascular permeability (Schnell et al., 1999) and leads to increased infiltration of macrophages (Hu and McLachlan, 2002;Watkins et al., 1995). Long term processes initiated by these initial inflammatory events could continue even after the initial injury has healed. These could alter the physiology of the sensory

afferents and their spinal targets, the second order neurons (Watkins and Maier, 2002;Watkins et al., 1995).

Nerve injury promotes aberrant, spontaneous ectopic discharges at various points on the sensory nerve including the injured axonal neuroma, the region of the axonal T junction and in the cell bodies (Liu et al., 2001;Amir and Devor, 1993;Amir et al., 2002;Govrin-Lippmann and Devor, 1978;Wall and Devor, 1983). The increase in the firing discharges in afferent fibers has also been demonstrated in humans with neuropathic pain (Wall and Gutnick, 1974;Nordin et al., 1984) but see (Rice et al., 1994). Moreover, the ectopic activity arises in not only the injured or severed sensory axons but also in their uninjured neighbours (Ma et al., 2003;Li et al., 2000). The spontaneous pain and hyperesthesia that exists after nerve injury is believed to be due to increased excitability of primary afferent nociceptors (Bessou and Perl, 1969;Fitzgerald and Lynn, 1977). Anesthetizing the DRG or performing dorsal rhizotomy alleviates neuropathic pain, suggesting the inputs from cell bodies could play a dynamic role in manifestation of pain symptoms in neuropathic pain (Devor and Seltzer, 1999;Sukhotinsky et al., 2004).

DRG neurons can be classified into three major cell types based on AP firing and cell capacitance as small, medium and large cells (Abdulla and Smith, 1998;Smith et al., 2002). Nerve injury results in alteration in properties of all the cell types and thus nociceptive Aδ and C-fibers as well as high threshold Aβ-fibers are affected (Abdulla and Smith, 2001a;Abdulla and Smith, 2001b;Abdulla and Smith, 2002;Stebbing et al., 1999). It is thus important to realize the pain that arises from nerve injury reflects

changes in both nociceptive and non-nociceptive fibers. Axotomy seems to affect predominantly the excitability of small neurons. In some injury models the appearance of autotomy seems to correlate well with the alterations in the excitability of large (Aβ) cells (Kajander and Bennett, 1992;Abdulla and Smith, 2001a;Abdulla et al., 2003).

Nerve injury seems to affect the properties of different ion channels in the DRG (Lai et al., 2004;McCleskey and Gold, 1999;Chaplan et al., 2003;Abdulla and Smith, 2002;Abdulla and Smith, 2001b). Several mediators that are released from the injured site may enhance the expression of tetrodotoxin (TTX)-resistant Na^+ channels (Nav 1.8) (Gold and Levine, 1996;Khasar et al., 1998;Cardenas et al., 2001;Okamoto et al., 2002). This Nav 1.8 has an important role in AP generation and enables the DRG neurons to fire repetitively and hence has functional significance (Renganathan et al., 2001;Baker et al., 2003;Gold, 1999;Khasar et al., 1998). The alteration with respect to Na^+ currents seems to differ with respect to injury models used (Devor and Seltzer, 1999;Everill et al., 2001;Sleeper et al., 2000;Abdulla and Smith, 2002;Waxman et al., 2000b;Waxman et al., 1999). The nerve injury seems to reduce the amplitude of delayed rectifier K^+ currents and the calcium sensitive K^+ conductance and reduce the expression of Kv1.2, Kv2.1 mRNA in the DRG cell bodies (Abdulla and Smith, 2001b;Yang et al., 2004a;Ishikawa et al., 1999). In addition, there seems to be reduction in high voltage activated (HVA) Ca^{++} channel currents after axotomy (Abdulla and Smith, 2001b;Baccei and Kocsis, 2000). However studies on alteration in gene expression profiles using cDNA array technologies show an increase in the expression of L-type α2 δ1 Ca^{++} channel subunit (Xiao et al., 2002). There is also an upregulation of hyperpolarization activated (Ih) pacemaker

currents in the DRG following nerve injury (Chaplan et al., 2003).The mechanisms responsible for the changes in the channel expression are not yet clear, but current evidence indicates that mediators such as neurotrophins, prostaglandins and serotonin could play a potential role (Cummins et al., 2000b;Leffler et al., 2002;Waxman et al., 2000b;Gold, 1999;Cardenas et al., 2001).

Peripheral nerve injury promotes sprouting of perivascular sympathetic axons to form baskets of noradrenergic terminals around the large cells (Ramer et al., 1999). This leads to noradrenergic excitation of DRG contributing to sympathetically maintained pain (Ramer and Bisby, 1997;Lee et al., 1998). In addition, there is formation of pericellular baskets (axonal sprouts) containing CGRP, substance P, around the large cells which provide an ideal environment for peptidergic neuron-neuron interactions (Wakisaka et al., 1992;Hu and McLachlan, 2001;McLachlan and Hu, 1998). The mechanism responsible for sprouting is not very clear, it is however likely that the neurotropins (NGF or BDNF) and gp130 family of cytokines such as the leukemia inhibitory factor (LIF) (Jones et al., 1999;Ramer et al., 1999;Cafferty et al., 2001) could play a crucial role. In addition, after axotomy, the ability of opioids to suppress Ca^{++} currents in DRG is reduced. This may offer some explanation for reduced effectiveness of opioids in neuropathic pain (Abdulla and Smith, 1998;Abdulla et al., 2003).

Since ectopic foci (spontaneous discharge) in the primary afferents and DRG (Amir et al., 2005;Devor and Seltzer, 1999;Wall and Devor, 1983), may be responsible for initiation of sensitization, therapies have targeted this aberrant activity in an attempt to alleviate

neuropathic pain (Cummins et al., 2000b;Waxman et al., 2000a;Lai et al., 2003). Even though Na^+ channel blockers are valuable in animal models, in the clinical setting they are only of modest efficacy (Sindrup and Jensen, 2000;Sindrup and Jensen, 1999). The majority of reports on the usefulness of Na^+ channel blockers have been performed only in conditions of postherpetic neuralgia, diabetic neuropathy or trigeminal neuralgia (Bach et al., 1990;Sindrup and Jensen, 1999). Moreover, to prevent potentially harmful side effects, blockers specific for sensory neurons should be developed (Lai et al., 2003;Lai et al., 2002). The processes triggering the sensitization phenomenon peripherally may differ from those that maintain it centrally (Cervero et al., 2003;Costigan and Woolf, 2000;Doubell et al., 1999). Hence, inhibition of ectopic foci alone may not be sufficient to counteract the generation of neuropathic pain. This underlies the importance of spinal mechanisms in the etiology of neuropathic pain.

1.7.2 Changes in the Superficial Dorsal Horn

The superficial dorsal horn is the primary site for termination of nociceptive primary afferent fibers (Light and Perl, 1979;Perl, 1984;Sugiura et al., 1986;Stucky et al., 2001;Stucky and Lewin, 1999). The integration of information in this region would have a high impact on the transmission and hence the perception of noxious information in higher centers. Nerve injury induces anatomical and physiological changes in the properties of different cell types on the dorsal horn (Baba et al., 1999;Doubell et al., 1999;Ji et al., 1999;Kohno et al., 2003;Moore et al., 2002;Woolf et al., 1995;Coull et al., 2003). The peripheral events such as sensitization of nociceptors contribute to spontaneous pain and an exaggerated response to noxious stimulation in the areas of

injury (primary hyperalgesia) (Meyer and Campbell, 1981;Treede et al., 1992;Raja et al., 1999). However, the plastic changes in the dorsal horn modify the abnormal or excessive input from the periphery leading to central sensitization. This is a key element underlying development of mechanical allodynia or secondary hyperalgesia observed in neuropathic pain (Costigan and Woolf, 2000;Ji and Woolf, 2001;Ji et al., 2003;Woolf and Salter, 2000;Zhuang et al., 2005).

It has been suggested that peripheral nerve injury results in the appearance of vacant synaptic sites in the superficial dorsal horn (Arvidsson et al., 1986;Kapadia and LaMotte, 1987). This could be due to atrophy of nonmyelinated C-fibers (Castro-Lopes et al., 1990;Coggeshall et al., 1997). Woolf et al. reported anatomical reorganization of the primary afferent fibers after axotomy leading to the misinterpretation of low threshold sensory information as nociceptive (Woolf et al., 1992). Thus, innocuous information conveyed by large myelinated afferents (selectively stained with CTB) (LaMotte et al., 1991;Robertson and Grant, 1985) which normally project to deeper laminae, (Fyffe, 1992;Willis and Coggeshall, 2004) reorganizes into the *substantia gelatinosa* after nerve injury resulting in allodynia (Baba et al., 1999;Shortland and Woolf, 1993a;Woolf et al., 1995). The results from CTB staining should however be interpreted with caution as even C-fibers could take up this stain after nerve injury (Tong et al., 1999;Shehab et al., 2003). Hence, this hypothesis of anatomical rewiring has been seriously questioned (Bao et al., 2002;Tong et al., 1999;Hughes et al., 2003;Shehab et al., 2003). Kohama et al. (2000), using extracellular and intracellular recording techniques, reported that many *substantia gelatinosa* neurones received direct inputs from Aβ afferent fibres following peripheral

nerve neuroma formation (Kohama et al., 2000). Thus, after nerve damage the long latency response of *substantia gelatinosa* neurons to high threshold activation is switched to short latency responses to low threshold stimulation resembling deeper lamina neurons (Baba et al., 1999). The loss of GABAergic inhibition could facilitate the excitatory inputs (Aβ-mediated) from deeper layers to more superficial regions (Baba et al., 1999;Baba et al., 2003;Moore et al., 2002;Okamoto et al., 2001). Even though, the structural rewiring may or may not happen, functionally there is still a possibility of an increase in Aβ-mediated excitatory transmission into lamina II.

Gate theory predicts disruption of balance between inhibition and excitation in the dorsal horn for transmission of nociceptive information (Melzack and Wall, 1965;Wall, 1978). Reports from several studies suggest loss of inhibition as one of the major contributors to the central sensitization in neuropathic pain (Laird and Bennett, 1992;Wall, 1978;Baba et al., 2003;Moore et al., 2002;Woolf and Wall, 1982;Ibuki et al., 1997;Castro-Lopes et al., 1993;Coull et al., 2005). In fact loss of inhibition seems to be a characteristic of neuropathic pain when compared to inflammatory or nociceptive pain (Millan, 1999). GABA is a major inhibitory neurotransmitter in the superficial dorsal horn. About 30% of neurons in lamina 1 and 2 are GABAergic (Todd and Spike, 1993;Todd, 1996). GABA plays an important role in the regulation of nociceptive transmission (Kangrga et al., 1991;Hao et al., 1992). Intrathecal application of a GABA antagonist produces signs of behavioral hypersensitivity (Sivilotti and Woolf, 1994;Malcangio and Bowery, 1996), and tactile allodynia induced by peripheral nerve injury is exacerbated by GABA antagonists (Yamamoto and Yaksh, 1993). GABA has at least two distinct roles in the

superficial dorsal horn. The GABA released from the dorsal horn interneurons could result in primary afferent (where E_{cl} less negative than resting potential) depolarization (PAD) (Rudomin and Schmidt, 1999;Cervero et al., 2003;Laird et al., 2004;Schmidt, 1971). This release can be triggered either due to activity in primary afferent fibers (dorsal root reflex) and/or by descending signals from brain (Willis, Jr., 1999;Cervero and Laird, 1996a;Cervero et al., 2003;Laird et al., 2004) and is mediated through $GABA_A$ receptors (Rees et al., 1995). This forms the basis for the mechanism, whereby activation of A-fibers inhibits C-fiber evoked response (gate theory) (Melzack and Wall, 1965;Wall and Devor, 1981;Fitzgerald and Woolf, 1981). Nerve injury or inflammation results in the alteration of A- fiber mediated inhibition of C- fiber evoked responses in the dorsal horn contributing to loss of inhibition (Wall and Devor, 1981;Laird and Bennett, 1992;Cervero et al., 2003;Laird et al., 2004). The second mechanism of GABA inhibition involves direct hyperpolarization of dorsal horn cells. In a model of chronic constriction injury with polyethylene cuffs, Coull et al. have demonstrated loss of this GABA hyperpolarization (Coull et al., 2003). In this model there is a shift in the Cl^- concentration gradient of the dorsal horn cells due to disruption of anion homeostasis. The underlying mechanism for such change is the reduction in potassium chloride transporter (KCC2) after nerve injury resulting in higher intracellular Cl^- concentrations, and hence more depolarized reversal potential for GABA mediated events (Beggs et al., 2004;Coull et al., 2003). GABA has been shown to be depolarizing in immature neurons of the CNS including the neonatal dorsal horn (Baccei and Fitzgerald, 2004). Thus, after nerve injury there is a phenotypic shift in the GABAergic (Beggs et al., 2004;Coull et al.,

2003;Coull et al., 2005) dorsal horn neurons resembling neonatal characteristics (Cordero-Erausquin et al., 2005).

Immuno-histochemical studies of dorsal horn neurons in several nerve injury models report reduced levels of GABA immunoreactivity or its receptors (Castro-Lopes et al., 1993;Ibuki et al., 1997;Castro-Lopes et al., 1995). GABA immunoreactivity showed some recovery at prolonged survival times after a CCI model suggesting downregulation of GABA synthesis in surviving neurons may play at least a part of the mechanism (Ibuki et al., 1997). However, there are also contrasting reports of GABA levels or function after nerve injury (Cervero and Laird, 1996b). Thus, Somers and Clemente found no significant change in GABA or glycine content in spinal synaptosomes after CCI (Somers and Clemente, 2002). Satoh and Omote report an increase in GABA and glycine in the dorsal half of spinal cord after nerve injury (Satoh and Omote, 1996) and Kontinen et al report enhancement of GABAergic inhibition after spinal nerve ligation (Kontinen et al., 2001).

Central sensitization may also involve neuronal cell death altering the circuitry of dorsal horn (Sugimoto et al., 1990;Azkue et al., 1998;Whiteside and Munglani, 2001;Moore et al., 2002;de, V et al., 2004). Morphological studies suggest an occurrence of cell death in spinal cord following nerve damage (Sugimoto et al., 1990;Coggeshall et al., 2001). Partial nerve injury and chronic constriction injury reduces the primary afferent evoked IPSCs, due to reduced release of GABA from presynaptic terminals of the interneurons. Moreover, the mixture of GABA and glycine IPSC's in control condition was shifted to

pure glycinergic current, as the bicuculline sensitive GABA component was lost after nerve injury (Moore et al., 2002). Coupled with these synaptic changes is the occurrence of neuronal apoptosis suggesting a possible cell death of GABAergic interneurons (Moore et al., 2002;Scholz and Woolf, 2002) Additional report on neuronal apoptosis come from a study which suggests an increase in the expression of the pro-apoptotic bax gene of the *bcl-2* gene family (Yuan and Yankner, 2000) at the lumbar spinal cords of rats after CCI (Maione et al., 2002). Peripheral nerve injury also triggers the induction of immediate early genes, such as *c-jun* and *c-fos* (Zimmermann, 2001;Coggeshall, 2005;Ro et al., 2004). These genes are able to induce cell death by a complex cascade of transcriptional processes (Harper and LoGrasso, 2001). The loss of GABAergic function seems to be dependent on some intact input from the primary afferents or a mixture of injured and uninjured afferent supply, because complete transection of the sciatic nerve (axotomy) alone does not induce neuronal loss or decrease in GABAergic IPSC's (Coggeshall et al., 2001;Moore et al., 2002;Scholz et al., 2004). Hence, these findings imply that neuropathic pain should be viewed as a neurodegenerative disease. Thus, therapies should also focus on preventing the loss of inhibitory neurons (Scholz and Woolf, 2002;Woolf, 2004a;Woolf, 2004b).

There is however some controversy regarding the cell death of inhibitory neurons as a parameter for enhanced pain behaviors in animal models of neuropathic pain. Polgar et al. suggest that there was neither neuronal loss nor reduction in the proportion of neurons that showed GABA or glycine immnuoreactivity in superficial laminae after CCI (Polgar et al., 2003;Polgar et al., 2004). Further, Polgar et al. also performed studies on spared

nerve injury model (Moore et al., 2002), which show robust and long lasting allodynia and also exibit significant neuronal loss. In this model they used two different markers for neuronal apoptosis (tunnel staining and activated caspase3). With their findings they challenged the previous hypothesis, and suggest loss of neurons is not required for tactile hypersensitivity (Polgar et al., 2005). As a further continuation of this controversy, Scholz et al. have showed recently that by blocking apoptosis with a caspase inhibitor (z-VAD), preserves the integrity of synaptic properties and prevents neuronal cell apoptosis. Also, the intrathecal delivery of z-VAD after nerve injury is able to attenuate the neuropathic pain like behavior (Scholz et al., 2005).

Increase in excitatory (glutamatergic) transmission could be another mechanism that contributes to sensitization in dorsal horn (Kohno et al., 2003). Glutamate is the major excitatory transmitter released from primary afferent terminals in the dorsal horn and is a likely mediator of central sensitization in neuropathic pain (Willis, 2001;Yoshimura and Nishi, 1992;Yoshimura and Jessell, 1990). Pain hypersensitivity has been suggested to be a consequence of glutamate induced excitotoxicity and neuronal cell death (Zimmermann, 2001;Sugimoto et al., 1990). CCI and axotomy result in an increase in the incidence of polysynaptic Aβ-mediated EPSC's, at the expense of monosynaptic Aδ-mediated EPSC's in *substantia gelatinosa* neurons (Kohno et al., 2003;Okamoto et al., 2001). Glutamate acts on ionotropic AMPA, NMDA and kainate receptors. In spinal dorsal horn, AMPA and kainate receptors mediate monosynaptic activation of neurons by primary afferent fibers (Yoshimura and Nishi, 1992;Yoshimura and Jessell, 1990), while NMDA receptors are responsible for late and polysynaptic components of the evoked

excitatory post synaptic potentials (Nastrom et al., 1994). Central sensitization involves changes in both AMPA and NMDA receptors mediated components (Garry et al., 2003;Brenner et al., 2004;Ji et al., 2003).

Some aspects of central sensitization in dorsal horn share similarities to early phases of hippocampal long term potentiation as both share similarities in the underlying mechanisms, including involvement of NMDA receptor (NMDAR) mediated component (Ji et al., 2003;Woolf and Thompson, 1991;Woolf and Salter, 2000). This phenomenon is suspected to be triggered by the ongoing ectopic activity in the primary afferents after nerve injury (Koltzenburg et al., 1992). The induction of a LTP-like phenomenon in dorsal horn projection neurons requires activation of NMDA, NK1 receptors along with activation of T-type Ca^{++} currents (Ikeda et al., 2003). NMDARs, by inducing activity dependant plastic changes in the dorsal horn provide positive continual feedback loop for the maintenance of central sensitization (Baranauskas and Nistri, 1998;Woolf and Salter, 2000;Brenner et al., 2004;Ji and Woolf, 2001). Noxious signals induce phosphorylation of NMDAR subunits in the dorsal horn (Brenner et al., 2004;Kawasaki et al., 2004). Application of NMDAR agonists results in enhanced pain behaviors due to increased activity of dorsal horn neurons (Malmberg and Yaksh, 1993). In contrast, NMDAR blockers effectively alleviate pain behaviors and block neuropathic pain (Ren and Dubner, 1993;Suzuki et al., 2001). Activity of NMDARs is regulated by phosphorylation. In particular, the PKC mediated phosporylation increases channel activity (Liao et al., 2001). Spinal PKC activation has been reported to participate in the generation of pain

hypersensitivity (Yajima et al., 2003). An NMDA-dependant loss of spinal opioid analgesia was also observed after nerve injury (Narita et al., 2001).

Because AMPA receptor activation induces nociceptive behavior and AMPA blockers are effective in neuropathic pain, these receptors may also play an important role in central sensitization (Chaplan et al., 1997;Sorkin et al., 2001). GluR1 and GluR2 subunits of AMPA receptors are expressed throughout the superficial layers of dorsal horn (Harris et al., 1996;Spike et al., 1998). The neurons can however be differentiated based on the presence of one or the other subunits (Spike et al., 1998). Based on findings in brain slices, AMPA receptors have diverse properties with respect to different subunit composition, for instance the presence GluR2 imparts Ca^{++} impermeability (Geiger et al., 1995;Jonas et al., 1994). Thus, in superficial dorsal horn, the inhibitory GABAergic (parvalbumin positive) neurons contained the GluR1 subunit (Albuquerque et al., 1999). However, the excitatory (neurotensin and somatostatin positive) interneurons, were associated with GluR2/3 subunits (Spike et al., 1998). Thus GluR1 and GluR2/3 subunits may be differentially altered, and hence are the inhibitory and excitatory cells, under pathological conditions. Ca^{++}-permeable AMPA receptors may be more important in neuropathic pain condition. The CCI causes appearance of dark neurons which undergo degeneration (apoptosis) in the superficial laminae (Sugimoto et al., 1990). Thus nerve injury could cause cytotoxic cell death (Ibuki et al., 1997;Moore et al., 2002) through activation of Ca^{++} permeable subunit resulting in the degeneration of selective neurons (Albuquerque et al., 1999;Antal et al., 1991). There have also been reports on increased GluR 2 immunoreactivity in the ipsilateral dorsal horn CCI (Harris et al., 1996).

Other cellular mechanism that could contribute central sensitization is alteration in properties of various ion channels and currents. Voltage-gated Na^+ channels play a mandatory role in the electrical signaling between the neurons. Recently, Hains et al reported an abnormal expression of Na^+ channel which is normally not expressed in adult spinal cord neurons (Felts et al., 1997;Hains et al., 2002;Hains et al., 2004). This aberrant Nav 1.3 channel is found mainly in the medial dorsal horn cells after peripheral nerve injury resulting in their hyperresponsiveness (Hains et al., 2004). cDNA array analysis also indicates changes in genes of various Na^+ channels in the dorsal horn after nerve injury (Waxman et al., 2000a;Yang et al., 2004b). High voltage-activated Ca^{++} channels L-, N-, P,Q-. and R-types are widely expressed throughout the spinal cord (Kerr et al., 1988). They regulate membrane excitability and neurotransmitter release and hence establish a major link between neuronal excitability and synaptic transmission (Miller, 1987). For these reasons, high voltage activated Ca^{++} channels have been the focus of chronic pain studies. An up-regulation of Ca^{++} channel $\alpha 2\delta$ subunit in the dorsal horn was observed under different nerve injury models (Luo et al., 2002;Field et al., 2000;Snutch et al., 2001). Gabapentin, which has shown efficacy in different neuropathic pain syndromes (Nicholson, 2000) may mediate its analgesic effects through interaction with the $\alpha 2\delta$ subunit (Gee et al., 1996;Field et al., 2000;Luo et al., 2002). In the nerve terminals of primary afferent fibers in the superficial dorsal horn, N-type Ca^{++} channels play an indispensible role in afferent transmission (Catterall, 1998;Westenbroek et al., 1998). After CCI there is upregulation of N-type Ca^{++} channel $\alpha 1B$-subunit in the outer as well as the inner part of lamina II, extending from the medial towards the lateral region (Cizkova et al., 2002). The importance of the spinally expressed N-type Ca^{++} channels in

the development of hypersensitivity following nerve injury is confirmed by the efficacy of intrathecal N-type channel blockers in several models of nerve injury and the clinical use of synthetic ω-conotoxin (ziconotide) (Chaplan, 2000;Matthews and Dickenson, 2001;Snutch et al., 2001). There is also an up regulation of mRNA for L-type Ca^{++} channel in DRG (Xiao et al., 2002) which could result in abnormal appearance of L-channels in the primary afferent terminals. The afferent inputs can also recruit L-type Ca^{++} channel plateau current, which contributes to phenomenon of wind-up, a cellular model of central sensitization (Russo et al., 1997;Russo and Hounsgaard, 1994;Perrier et al., 2002). In chapter 5 of this thesis, I explore the role of L-type Ca^{++} in mediating the synaptic transmission of the *substantia gelatinosa* neurons after CCI.

Classically, glial cells were considered to perform house keeping functions in providing support to the neurons to maintain homeostasis. However, it is now established that glia play a more direct role in modulating neuronal transmission (Haydon, 2001;Watkins and Maier, 2003). Thus, they posses different ion channels and receptors and release several substances which in turn modify the functions of neurons (Bruce-Keller, 1999;Haydon, 2001;Coull et al., 2005;Tsuda et al., 2003;Tsuda et al., 2005). Moreover, they are also involved in the turnover of neurotransmitters and trophic factors. Garrison *et al* first reported the activation of spinal cord glia under conditions of nerve injury (Garrison et al., 1991). The likely role of glial-neuron interactions in mediating dorsal horn plasticity associated with neuropathic pain is a topic of intensive study (Coyle, 1998;Coull et al., 2005;Inoue et al., 2004;Tsuda et al., 2004;Tsuda et al., 2005). Affecting glial activation does not have an impact of normal pain behavior suggesting involvement of glial cells

only under pathological conditions (Milligan et al., 2003;Watkins and Maier, 2003). Glial and microglial hypertrophy in the dorsal horn (Eriksson et al., 1993;Coyle, 1998;Molander et al., 1997) has been reported after peripheral nerve injury. Moreover, spinal delivery of ATP-activated microglia induces allodynia in rats through disruption of anion homeostasis in the dorsal horn neurons, mimicking the peripheral nerve injury (Coull et al., 2003;Coull et al., 2005). The mediator involved has been identified to be BDNF, released from activated microglia and the inhibition of BDNF-TrkB signaling is thus able to reverse the tactile allodynia in rats (Coull et al., 2005). The microglia could be activated and in turn release several pronociceptive substances such as, glutamate, cytokines, NO, prostaglandins and ATP in addition to neurotrophins such as BDNF (Milligan et al., 2003;Watkins and Maier, 2002;Coull et al., 2005;Tsuda et al., 2005). These pain mediators could directly activate the dorsal horn neurons, might induce cell death of inhibitory neurons, and/or could activate abnormal release of neurotransmitters in synaptic sites (Milligan et al., 2003;Watkins et al., 2001;Watkins and Maier, 2003). The activated microglia also expresses several protein molecules including P2X4 receptors, P38 mitogen activated protein kinase, chemotactic cytokine receptor 2, cannabinoid receptor CB2, major histocompatability complex (MHC) class II protein, which have been suggested to be responsible for neuropathic pain behavior (Inoue et al., 2004;Tsuda et al., 2004;Tsuda et al., 2005). Inhibition or activation (CB2) of these proteins has been shown to reverse the evoked pain behaviors in various animal models (Cravatt and Lichtman, 2004;Tsuda et al., 2005). In addition, direct inhibitors of glial activation are effective in blocking allodynia and hyperalgesia in different animal models (Raghavendra et al., 2003;Watkins and Maier, 2003). Hence, inhibiting glia-neuronal

signaling could be exploited for novel therapeutic interventions for neuropathic pain. Many drugs that target neurons are ineffective in managing neuropathic pain, hence It has been suggested that the immune and microglial system may present more appropriate targets (Watkins et al., 1995;Watkins and Maier, 2003;Watkins and Maier, 2002).

1.8 Hypothesis:

Molecular and electrophysiological studies have thus identified several underlying mechanisms for central sensitization in the dorsal horn including attenuation of GABAergic inhibition (Moore et al., 2002;Baba et al., 2003), changes in chloride equilibrium potential (Coull et al., 2003;Baba et al., 2003), upregulation of rapidly repriming Na^+ channels (Hains et al., 2004), changes in protein kinases, amino acid receptors, src, extracelluar regulated kinases, and cAMP response element binding protein (Kawasaki et al., 2004), ATP activation of microglia (Tsuda et al., 2003) and alterations in 'wind-up' mediated via NMDA-receptors (Baranauskas and Nistri, 1998). Although all such cellular changes are conducive to increased excitability, their exact relationship to global, injury-induced changes in dorsal horn activity, as monitored by extracellular recording techniques (Laird and Bennett, 1993;Dalal et al., 1999;Hao et al., 2004), remains to be established. Moreover, the morphological (Gobel, 1978a;Gobel, 1978b), immunohistochemical (Todd and Spike, 1993) and electrophysiological properties of dorsal horn neurons are far from homogeneous (Grudt and Perl, 2002;Thomson et al., 1989;Prescott and de Koninck, 2002;Graham et al., 2004;Lu and Perl, 2005;Dougherty et al., 2005) and it is yet to be determined whether all neuronal types are affected similarly by peripheral nerve injury. Hence, my primary hypothesis is

that Peripheral nerve injury results in cell-type specific changes in the *Substantia Gelatinosa* neurons of the dorsal horn in such a way that the global excitability is enhanced.

To address these issues, first I have characterized multiple cell types in the *substantia gelatinosa* neurons based on their action potential firing properties at -60mV holding potential. Voltage clamp experiments were carried out to determine any underlying potassium currents were responsible for exhibiting a particular firing property. Spontaneous and miniature synaptic currents were recorded for different cell types. These electrophysiological properties were then correlated with the morphology, especially with their dendritic orientations of the different neuronal types.

Secondly, nerve injury was induced in 20 days old rats using the modified chronic constriction injury model developed by Mosconi and Kruger. The mechanical hypersensitivity was evaluated using the Von Frey hair technique. The animals exhibiting enhanced mechanical sensitivity were then subjected to the electrophysiological analysis, 13-25 days after CCI. Any changes in the membrane and synaptic properties of different neuronal types were then analyzed.

Gene array studies have suggested that nerve injury results in an increase in the messages for proteins involved in neurotransmitter relase and ion channels such as L-type Ca^{++} channels (Xiao et al., 2002;Yang et al., 2004b). Hence particular attention is paid to study

any alteration in the spontaneous excitatory transmission in different cells types and any changes in L-type Ca^{++} channels after CCI.

Table 1.1: Morphological Features and the Firing Patterns of *Substantia Gelatinosa* neurons.

Authors	Predominant Dendritic Orientation	Rostro-Caudal			Dorso-ventral	In all direction	Medio-lateral
Grudt, Lu, Hantman and Perl in hamster rat, and GFP mice	Morphological Class	Islet	Central		Vertical	Radial	Medio-lateral
	Major firing pattern	Tonic	Transient (Phasic)		Delay or Tonic	Delay	Tonic
	Minor firing pattern		Tonic		Irregular		
	Functional Phenotype	Inhibitory	Phasic Excitatory? Tonic Inhibitory		Excitatory		
Gobel in cat spinal trigeminal cuadalis	Morphological class	Islet	Arboreal	II-III border	Stalked		
	Inhibitory Vs excitatory Functional Phenotype	Inhibitory			Excitatory		
Todd et al. in rat	Morphological class	Large Islet	Small Islet		Stalked		
	Functional Phenotype	Inhibitory	Excitatory		Excitatory		
Bennet and Dubner in cat	Morphological class	Islet			Stalked		
	Functional Phenotype	Inhibitory			Excitatory		
Schoenen in human	Morphological class	Islet			Filamentous curly	Stellate	
Raymon Cajal in cat	Morphological class	Central			Limitroph or Vertical		Transverse
Heinke et al. in GFP mice Note: No correlation in morphology and firing pattern	Morphological class	Islet/Central			Vertical	Radial	
	Functional Phenotype	Inhibitory					
	Firing pattern	Initial bursting (Phasic)			Delay	Gap firing	

Figure 1.1

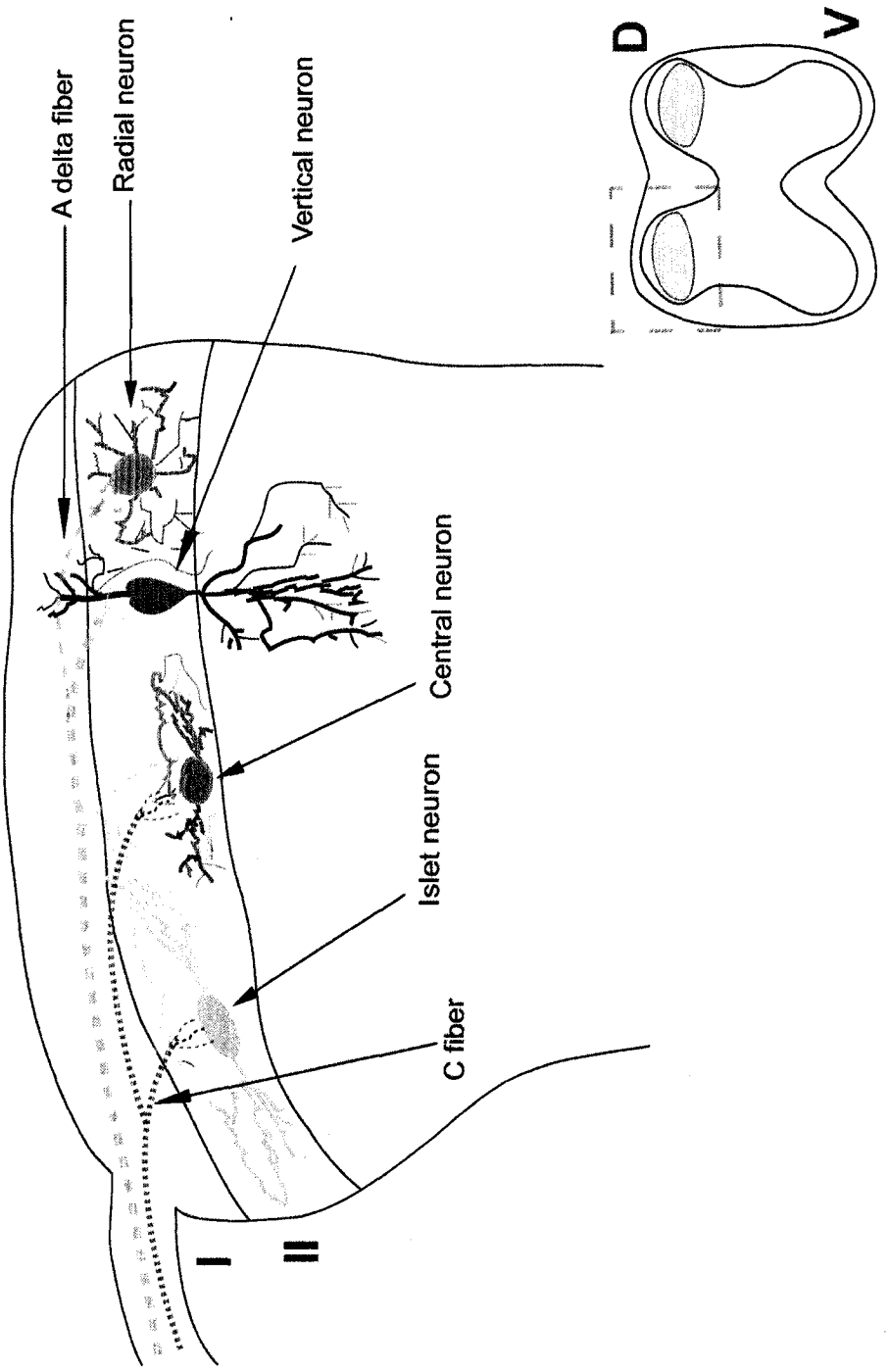
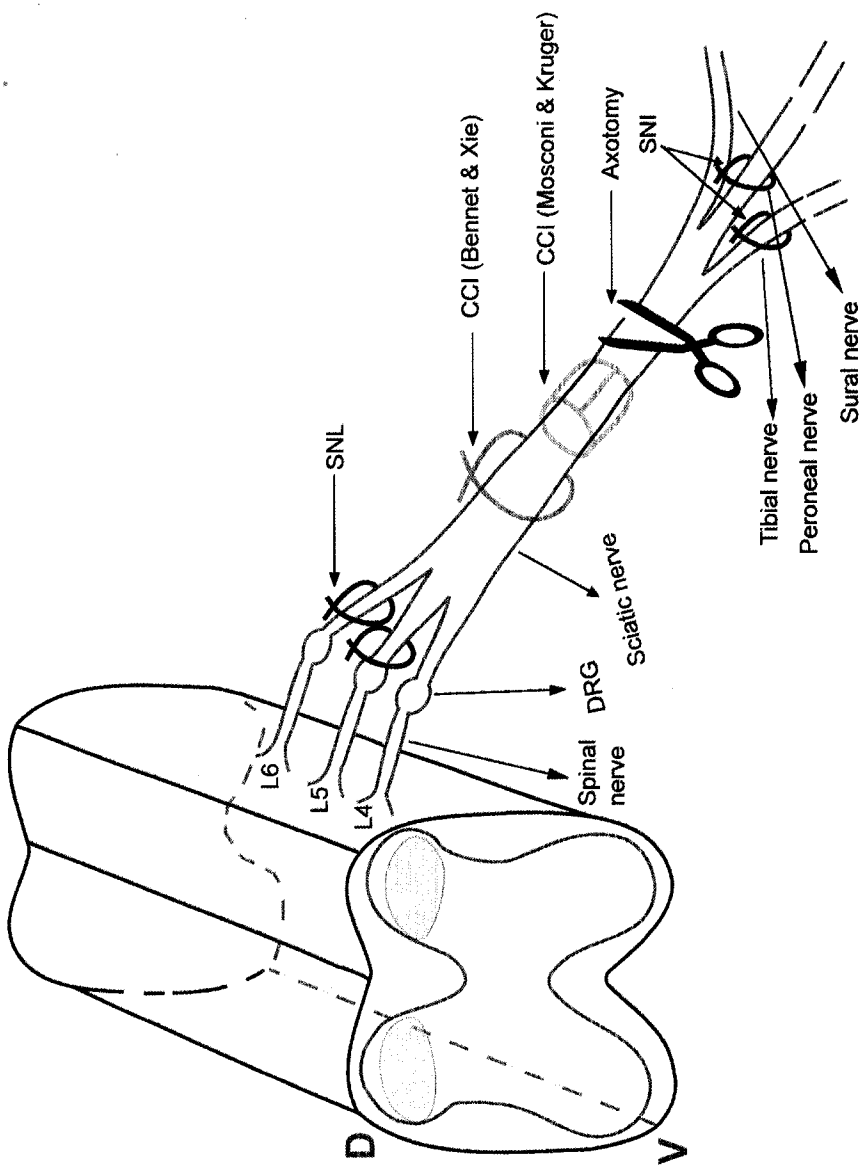


Figure 1.2



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Chapter 2

General Methods

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 Chronic Constriction Nerve Injury (Mosconi and Kruger 1999):

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 which included information on the surgical procedure such as date, rat #, comments (any complication during surgery, time took for completion of the surgery and recovery) on the anesthesia. Male Sprague Dawley rats were employed as they are the commonly used strain for neuropathic pain studies. The polyethylene cuffs were made from PE90 tubing (ID 0.86mm, OD 1.27mm; Intramedic Clay Adams, Becton Dickinson and Co, Sparks MD), on the morning of the day of surgery. They were slit open longitudinally and were soaked in ethanol (95%) and then transferred to sterile water for injection before the start of surgical procedure. The Sprague Dawley rat (20days old, ~40gms) was placed in the induction chamber at 5% isoflurane (O_2 @ 4-5L/min). Once asleep, rat was removed and the mid-thigh region was shaved with a razor blade (Wilkinson Sword Classic, Shick Warner-Lambert Canada, Ontario), and then cleaned with soap and water and then with ethanol. The rat was then transferred to the surgical table and maintained, on mask, at 2% isoflurane (O_2 @ 1-2 L/min). The body temperature was maintained using a hot water pad.

Surgery was commenced once the surgical plane of anesthesia was achieved (determined via loss of pedal reflex). A small incision was made at the mid-thigh level and the underlying muscle layer was slit open using curved forceps. Care was taken not to damage any blood vessels or nerves. The left common sciatic nerve was exposed through biceps femoris muscle before its bifurcation into tibial and peroneal divisions. The sciatic nerve was gently lifted using blunt forceps and, 2mm long polyethylene cuffs were applied to enclose the nerve using fine forceps (Figure 2.1). Each animal received two cuffs. The wound was then closed with 4-O silk sutures (Ethicon INC, Somerville, New Jersey), and an antiseptic lotion (Betadine) was applied to minimize any risk of infection. For sham surgery, animals were anesthetized with isoflurane and the sciatic nerve exposed but not deliberately manipulated. Rats were then allowed to recover and placed in their respective cages. Rats were monitored frequently for any signs of autotomy or other abnormal behavior.

2.2 Behavioral Testing

Measurement of mechanical hypersensitivity was determined using von Frey hairs. Behavioral studies were performed on the respective days during afternoon in specially allocated suites with optimal lighting conditions in the animal house facility. The animals were shifted from holding facility to the allocated suites and allowed to acclimatize to the surroundings before commencing the testing procedures. The testing was carried out using a partitioned (15cm x 15cm x 10cm) plexiglass chamber which can hold 12 animals at a time. Animals were placed in their respective box with a wire grid bottom, through which a series of von Frey hairs were applied to the mid-plantar area of the left paw (injured side), avoiding the foot pads. Hairs were applied only when the animal was standing on its

four paws. Each hair was applied until it buckled, keeping the level of stimulation constant (Figure 2.2). A scoring system was used to evaluate the response of the rats to the stimulation. The responses observed belonged to one of the following categories: Score 0 = no response; Score 1 = withdrawal response (where the animal withdrew its paw away from the stimulus and flinches); Score 2 = the animal avoided further contact with the stimulus by actively moving away and/or licking the stimulated paw. Rats were habituated to the test procedure on pre-operative days. A basal response was taken on the day before surgery. The rats were then tested for mechanical hypersensitivity for a two week period after nerve injury. The von Frey hair kit contains series of hairs in the weight range of 0.008-300g in a logarithmic scale. Some of the earlier studies have used upto 80g for measuring withdrawal threshold (Pitcher et al., 1999). We noticed however, after a certain weight range the hairs passively lifted the paw without bucking. As a result the stimulation intensity could not be kept constant. Hence the range of von Frey hairs used was adjusted so that they remained within 1-15% (0.4-15g) (Gold et al., 2003;Li et al., 2004) of the mean body weight of the rats. Three sets of measurements were obtained with each hair. In each set, three consecutive measurements were made at 5 minute intervals and scores added for each set. Thus, an animal can have a maximum score of 6 for each set of measurements and 18 for each hair. Scores obtained were then converted to percentage of the maximum possible score.

2.3 Preparation of Spinal Cord Slices

Methods for *in-vitro* recording from *substantia gelatinosa* neurons in 30-45 day old Sprague-Dawley rats were similar to those described previously (Moran and Smith, 2002;Moran et al., 2004). The animals were anesthetized with an overdose of intra-

peritoneal urethane (1.5g/kg). After removing the skin and muscle layers, the vertebral column at the mid-thoracic region was grasped using Adson's forceps (11027-12, Fine Science Tools) and a vertically-oriented cut was made through the vertebral column using surgical scissors (14002-13, Fine Science Tools Vancouver BC, Canada). A lumbosacral laminectomy was performed with spring scissors (15012-12, Fine Science Tools) and the spinal cord with attached dorsal and ventral roots was excised. During laminectomy, the cord was constantly irrigated with ice cold dissection solution containing (in mM): 118 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.3 $MgSO_4$, 1.2 NaH_2PO_4, 1.5 $CaCl_2$, 5 $MgCl_2$, 25 D-glucose, 1 kynurenic acid. The spinal cord was then placed in preoxygenated (95%O_2-5% CO_2) dissection solution in a Sylgard (Dow-Corning, USA) coated Petri dish (Corning, USA). After the removal of the dura matter, the dorsal and ventral roots were trimmed. The spinal cord was then removed and glued with cyanoacrylate glue ('Vetbond', WPI, Sarasota, FL, USA), on to the rhomboid-shaped block cut from 4% agar gel. This block, with attached spinal cord, was glued to the bottom of a 60 mm glass petri dish, submerged in ice-cold dissection solution continuously bubbled with 95%O_2-5% CO_2. This set up was then mounted on a Vibratome (TPI, USA) with the dorsal side of the cord facing the blade. Transverse slices (300μm) were cut using a razor blade (Wilkinson). The blade was advanced slowly (1-4 mm/min) to prevent the tissue from being pushed or deformed (Edwards, 1995) (Figure 2.3).The slices were then incubated at 36°C for 1 h in a nylon mesh holding chamber (Sakmann and Stuart, 1995) in oxygenated dissection solution (without kynurenic acid), and then stored at room temperature. All recordings in CCI and sham animals were made from spinal neurons ipsilateral to the sciatic injury. To identify the ipsilateral side of each slice, it was removed from the vibratome with a small piece of agar attached. This piece of agar was cut in a triangular shape using the long side of the

isosceles triangle to mark the side of the cord ipsilateral to the CCI or sham lesion (Figure 2.4).

2.4 Arrangement and Visualization of the Slices:

The following procedure for visualization of the spinal cord slices were adopted from previous studies (Sakmann and Stuart, 1995;Stuart et al., 1993;Moran, 2003). The spinal cord slice was transferred to the recording chamber. This had a diameter of 25 mm and volume of ~1 ml. Only slices with the triangular piece of agar still glued to it were used, for unequivocal identification of the ipsilateral side. The slices were positioned in such a way that the long side of the triangular piece of agar (ipsilateral side) faces the right side and the rostral side is on the top, in the chamber. The slice was held in place with a U-shaped platinum wire (harp) (Goodfellow, Berwyn, PA, USA) frame with attached parallel nylon threads (Refer to Figure 2.4). The distance between the top two threads were wide enough to fit the SG in between them (Figure 2.4). 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, Canada), a 40X 0.75-NA water immersion objective, a 0.9-NA condenser and DIC optics. The *substantia gelatinosa* was identifiable as a translucent band across the dorsal horn with the 4X objective. Under the 4x objective stimulating electrodes were positioned on the dorsal root or the entry zone. Then, using the 40X water immersion objective and with the help of IR sensitive camera (NC-70, Dage-MTI, Michigan City, IN, USA) the healthy cells were identified. The healthy cells were less conspicuous and had smooth appearance in contrast to the more conspicuous unhealthy cells which had high contrast membrane and appeared swollen or wrinkled. Cells were sampled randomly across the medial to the lateral side. Cells could

be visualized to a depth of 100 μm in young rats (35 days old). The success of identifying and patching healthy cells was inversely proportional to the age of the rats.

2.5 Electrophysiological Recordings

For recording, slices were superfused (1-2ml/min) at room temperature (~22°C) with 95% O_2-5% CO_2 saturated aCSF which contained (in mM): 127 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 25 D-glucose, pH 7.4. Whole-cell recordings were made with an npi SEC 05L amplifier (npi Electronic, Tamm, Germany) in discontinuous single-electrode voltage-clamp or bridge-balance current-clamp mode. Switching frequencies were typically between 30-40 kHz. Signals were filtered at 1-2 kHz and digitized between 5 and 10 kHz. The filter and sampling frequencies were based on Nyquist theorem, which states that the sampling frequency should be at least twice the filter frequency. Patch pipettes were pulled from thin-walled borosilicate glass (1.5 mm o.d., 1.12 mm i.d.; TW-150F-4, WPI, Sarasota, FL, USA). Pipettes for recording APs, postsynaptic K^+ currents and spontaneous post synaptic currents had resistances of 5-10 MΩ when filled with an internal solution containing (in mM): 130 potassium gluconate, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 EGTA, 4 Mg-ATP, 0.3 Na-GTP, pH 7.2, 290-300 mOsm. Biocytin was included in the patch pipette solution for subsequent post-hoc cell identification studies in conjunction with their electrophysiological characteristics (Horikawa and Armstrong, 1988). A low concentration of biocytin (0.1-0.2%) was used as higher concentrations (1%) have been reported to affect the membrane properties and post synaptic currents (Eckert, III et al., 2001).

Data were only collected from neurons that exhibited clear overshooting action potentials of >60mV in amplitude. Current-voltage relationships were determined under voltage-clamp using a series of 800ms voltage commands. Current was measured just prior to the termination of each voltage pulse. The peak amplitudes of voltage-gated K^+ currents were measured in response to a series of incremental depolarizing voltage commands. This was done in the presence of 1μM TTX to prevent action potential discharge. DR-type currents were evoked from a holding potential of -80mV and I_A/I_D currents evoked from -110mV and separated by subtraction.

It has been noted that the type of discharge pattern of a given dorsal horn neuron can be affected by the holding potential (Prescott and de Koninck, 2002). To avoid potential misclassification, all neurons were categorized on the basis of their discharge pattern in response to 800ms depolarizing current pulses from -60mV (see Figure 3.2). Despite this, we did encounter a few cells (4.3% of those studied) that did not readily fit into any of the five defined categories. Such unclassified neurons were excluded from the analysis. Membrane excitability was quantified by examining the cumulative latency of action potentials evoked in response to ramp current commands. These were delivered from a set holding voltage of -60mV at 33, 67, 100 or 133 pA/S. Cumulative latencies for the first, second, third and subsequent action potentials were noted.

Spontaneous EPSCs (sEPSC's) and evoked EPSCs (eEPSC's) were recorded in the voltage clamp mode at -70 mV holding potential in the presence bicuculline (10 μM) and strychnine (1 μM) to block inhibitory synaptic inputs. Tetrodotoxin (TTX; 1μM) was

included while recording miniature EPSCs (mEPSC). The effectiveness of the TTX was determined by its ability to block action potentials in current clamp mode.

EPSCs were evoked at 0.05 Hz by placing a custom-made bipolar, teflon-coated nichrome (7620, A-M Systems, Carlsborg, WA, USA) stimulating electrode on the dorsal root or near the dorsal root entry zone to activate primary afferent fibers. The stimulating electrodes were manufactured as follows. Two 20 cm strands of nichrome wire (7620, A-M Systems, Carlsborg, WA, USA) were inserted into a 10 cm thin-walled borosilicate glass tube (WPI, Sarasota, FL, USA) with about 1-2 cm of wire protruding from the ends of the glass tubing. A small amount of epoxy was applied to each end of the glass tubing to seal the wires. They were left for about 5 min to air dry. The strands at one end of the glass tubing were then twisted together and the ends were cut with a scalpel blade to expose the nichrome within the strand. At the other end, after exposing the nichrome wire within the strands they were connected to the stimulus isolation unit (SIU) using alligator clips. While recording eEPSC's, the voltage-gated ion channel blocker QX-314 (5 mM) was included in the internal solution to prevent action potential discharge. Monosynaptic EPSCs were identified by their ability to follow high frequency stimulation (10-20 Hz) with constant latency and the absence of failures (Li et al., 2004). Stimulus intensity for eEPSC's was between 2-40 V and the stimulus duration was 100 or 400 μs.

2.6 Data analysis:

Data were acquired and analyzed using pCLAMP 8.0 (Axon Instruments, Burlingame, CA, USA). Figures were produced with Origin 7.0 (OriginLab, Northampton, MA, USA). Statistical comparisons were made with unpaired t-tests or χ^2 tests as specified and

appropriate, using GraphPad InStat 3.05 (GraphPad Software, San Diego, CA, USA). Statistical significance was taken as $P<0.05$.

Mini Analysis Program (Synaptosoft, Decatur, GA, USA) was used to distinguish spontaneous and miniature excitatory postsynaptic currents (sEPSC and mEPSC) from baseline noise. Details of this program and its implementation may be found at www.synaptosoft.com. Spontaneous or miniature postsynaptic currents were detected automatically by setting appropriate amplitude and area threshold for each neuron. All detected events were then re-examined and visually accepted or rejected based on subjective visual examination. The Mini Analysis Program was used to further analyze the data and to provide spreadsheets for the generation of figures. Cumulative probability plots were generated to compare the amplitude and interevent intervals of sEPSC's and mEPSC's in neurons from sham operated animals and those subject to CCI. Cumulative probability plots ranked individual amplitudes or interevent intervals in order of increasing size and then plotted this rank value against the amplitude or interevent interval. The Kolmogorov-Smirnov two-sample test (KS test) was used to compare distributions of amplitudes and interevent intervals (Van der, 1991;Prescott and de Koninck, 2002;Van der Kloot, 1991). Distributions were considered significantly different if $P<0.05$. For each neuron, sEPSC's were recorded for a total of 3 min. Neurons which failed to generate a detectable event during a 3 min period were classified as 'silent' and excluded from the analysis (see Table 3.3 and associated description). Plots in Figures 3.9 were obtained by analyzing the first 50 events seen after one minute of recording. Data were pooled from 15-34 neurons of each type from sham-operated animals and compared with data from 17-26 neurons of each type in CCI animals (see

legend to Figure 3.9 for additional detail). This approach was not feasible with phasic cells as the frequency of sEPSC's was very low (~ 1 event every 15s). Data were therefore analyzed from the full 3 minutes of recording time.

For plots of mEPSC frequency and amplitude, data were pooled from 6-11 neurons of each type in sham operated animals and 6-11 neurons of each type in CCI animals. Because the mEPSC frequency was considerably less than sEPSC frequency, data were analyzed from the first 30 events in each neuron using the whole 3min standard acquisition period. Because the mEPSC frequency in phasic neurons was so slow, insufficient data were available for the construction of cumulative probability plots for this neuron type (Figure 3.9 K-N, O-R).

2.7 Immuno-histochemical Procedures:

After completion of electrophysiological recording, the slices were transferred to Zamboni's fixative which contains formaldehyde (2%) and picric acid (0.2%) solution in 0.1M sodium phosphate buffer, pH 7.0, (Stefanini et al., 1967) and stored overnight at 4°C. The slices were then placed in DMSO (5x tissue volume), agitated gently and the DMSO was replaced 3 times every 10 minutes. The slices were then rinsed 3 times with PBS and if not processed immediately were transferred to 30% sucrose, 1% sodium azide in PBS and stored in sealed plastic containers at 4°C until further processing.

Slices were then washed in PBS 6 times for 10 minutes each before further processing. To help reduce background staining caused by the secondary antibody, slices were pre-incubated with 20-50µl of 10% Normal Horse Serum (NHS) diluted in PBS with 1% triton for one hour at room temperature. The slices were then transferred to 24-well tissue

culture dish (Corning, Corning, NY, USA). The excess blocking serum was blotted off using clean filter paper with care not to suck up the slices onto the filter paper. Slices were then incubated with 20-50µl of primary antisera, Mouse anti PKC gamma 1:250, [BD Transduction Labs, San Diego, CA]) overnight at 4°C. Care was taken to ensure that tissue floated freely and that it was completely covered by antibody solution. The tissue was then left to incubate in a humidity box for overnight at 4°C. Following 3 x 10 minute washes in PBS, to remove excess primary antibody, the tissues were then incubated with secondary antibodies, Goat anti Mouse Alexa488 1:500, [Molecular Probes – Invitrogen, Mount Waverley, Australia] and Streptavidin Texas Red 1:400 [Amersham Biosciences, Piscataway, NJ]) for 1 hr at room temperature in a humidity box. At the end of the secondary incubation, the slices were once again subjected to 3 further PBS washes to remove excess secondary antibody. They were then mounted onto a glass slide and cover-slipped using buffered glycerol mounting media. They were then labeled and stored in cool place until subjected to confocal imaging.

2.8 Confocal Microscopy:

For confocal imaging, the preparations were viewed on a Biorad MRC1024 confocal scanning laser system installed on a Zeiss Axioplan 2 microscope. The system had a krypton-argon laser for differential visualization of the fluorophores, using 488-nm excitation and 522/535-nm emission for Alexa488 and 568-nm excitation and 605/632-nm emission for Texas Red. The microscope was also fitted with a transmitted light detector which was used to simultaneously obtain dark field images.

Low Power (10 or 20 x objective) images of the biocytin filled cell, PKC immunoreactivity and dark field illumination were captured in order to determine the lamina localization of the cell body. Confocal z stacks of the biocytin and PKC immunoreactivity in and around the cell body were taken at 2 μm intervals using a 40x oil immersion objective to determine the cells content of each antigen and to confirm lamina localization. Z stacks of the entire detectable extent of the biocytin filled cell body and dendritic arbor of each cell were taken at intervals of 1μm. Using these stacks of images a three dimensional reconstructions were also made using the confocal assistant (4.01) software for spatial rotation of each neuron to identify the dendritic orientation in different planes.

For cells with a large dorsoventral or mediolateral extent this sometimes required z stacks to be obtained using several adjacent microscope fields. In most cases the cell's full rostrocaudal extent within the slice was not evidence because the signal was either not detectable deep in the slice or the working distance of the lens was not great enough. Skewing of the rostrocaudal axis either because it had been obliquely cut or because the slice had been squashed by the coverslip was detected by viewing the PKC staining around the cell body and checking that its border was parallel to the z-axis of the microscope.

Analysis of the dendritic orientations was performed by two independent observers. The observations were then combined to derive at a final conclusion on the predominant dendritic orientation of different cell types. Whenever any discrepancies aroused between the observations those cell were considered as unclassified or unclear category.

Figure 2.1: A cartoon illustrating chronic constriction nerve injury by applying polyethylene cuffs. Under anesthesia the sciatic nerve was exposed and the polyethylene cuff was applied to enclose the nerve using fine forceps.

Figure 2.1

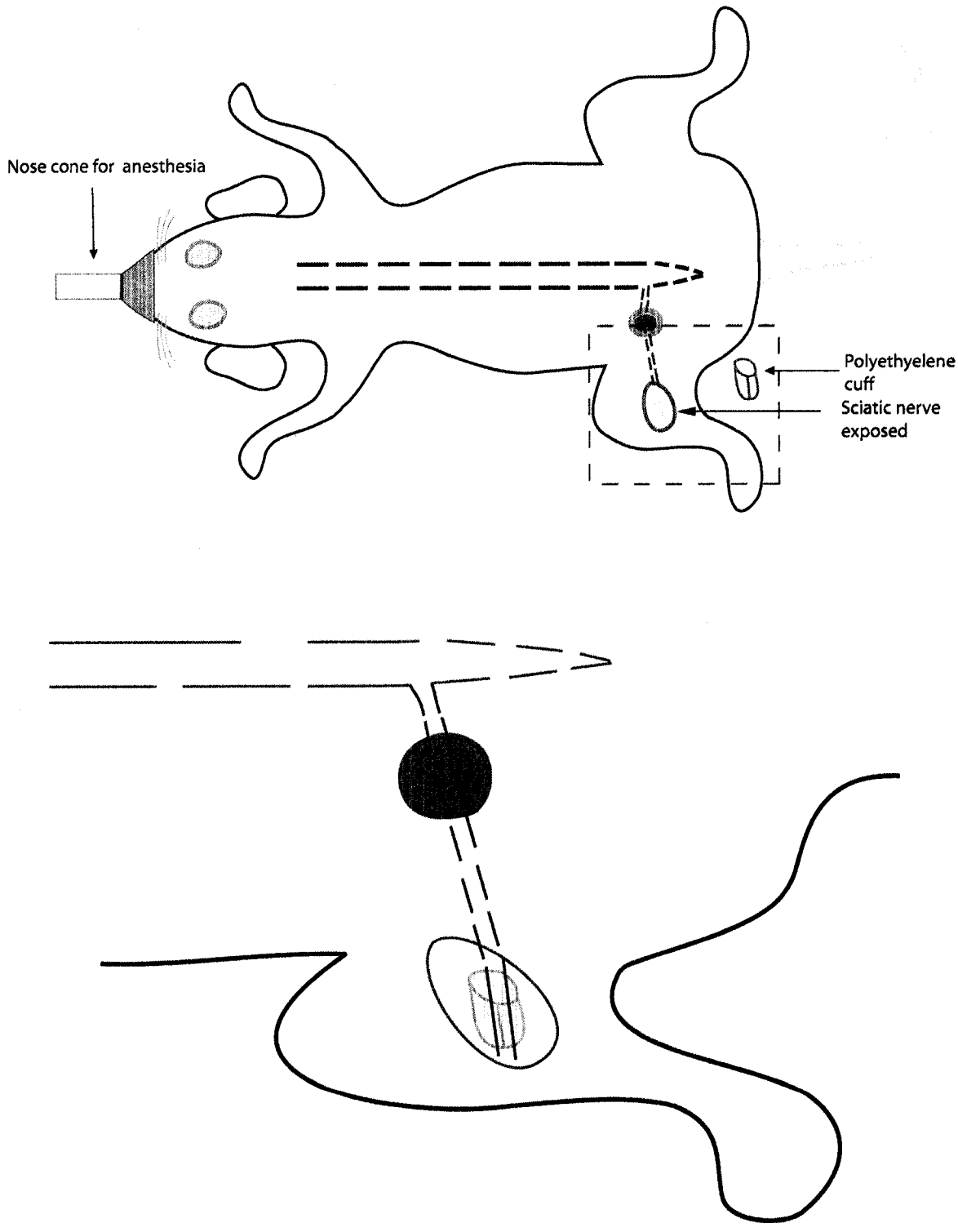
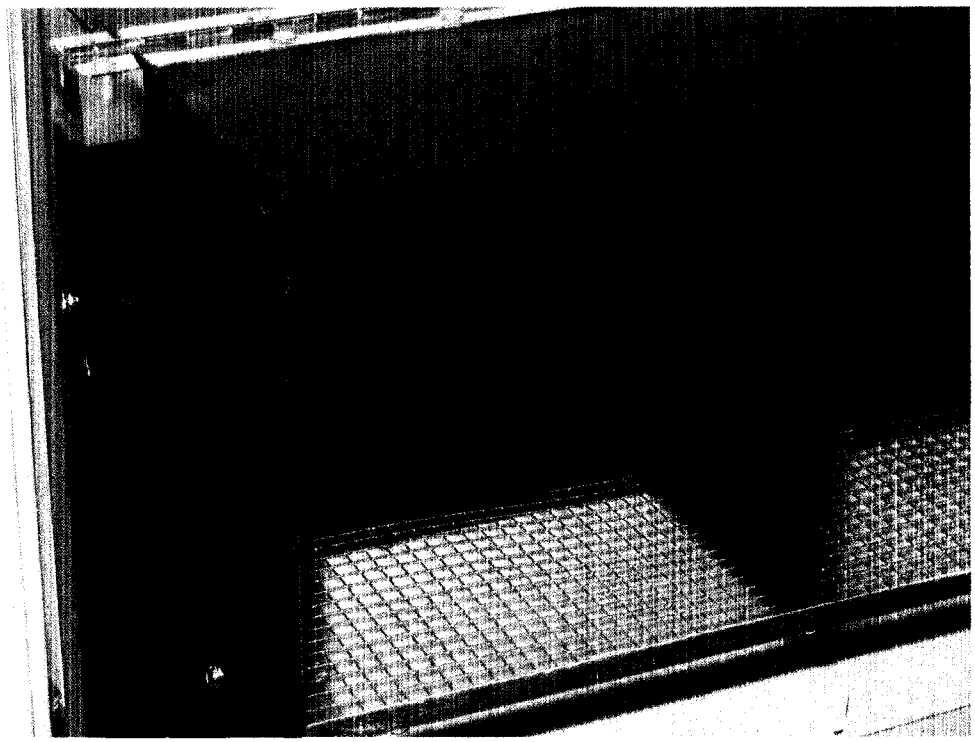


Figure 2.2: Photograph illustrating the measurement of mechanical hypersensitivity by von Frey hair technique. Rats were placed on a chamber with wire mesh bottom. The von Frey hairs were applied to the injured paw until it buckles under pressure.

Figure 2.2



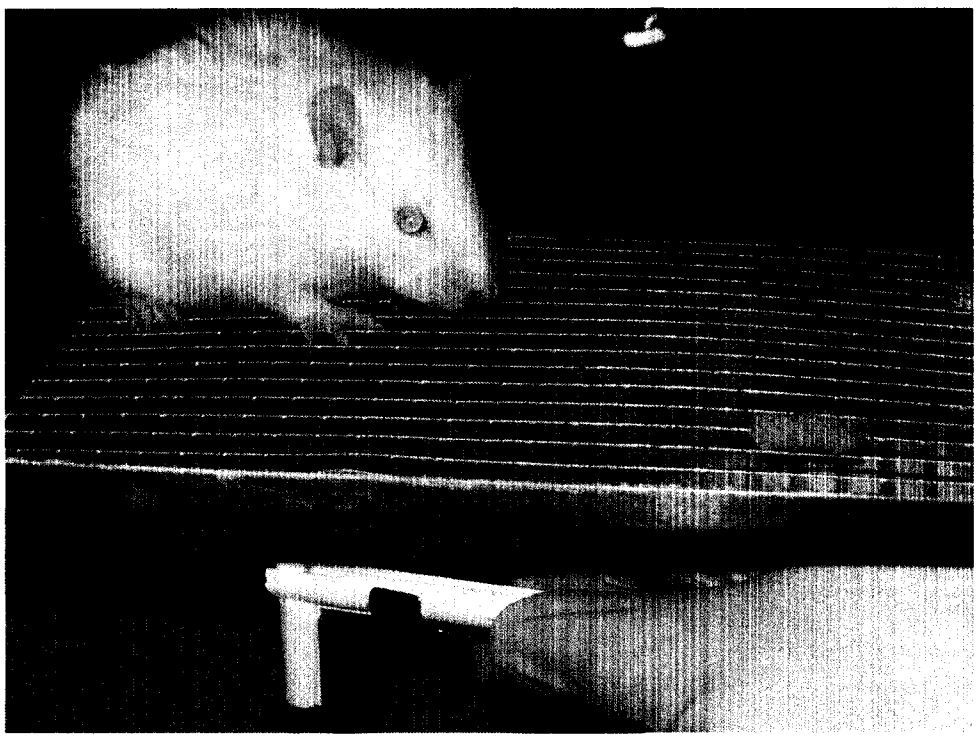


Figure 2.3: A schematic illustration of the preparation of transverse slices of the dorsal horn.The lumbar spinal cord is glued to a 4% agar block. The vibratome blade is advanced slowly at 1-4 mm/min. Approximately 300-350μm slices are cut from the lumbar spinal cord.

Figure 2.3

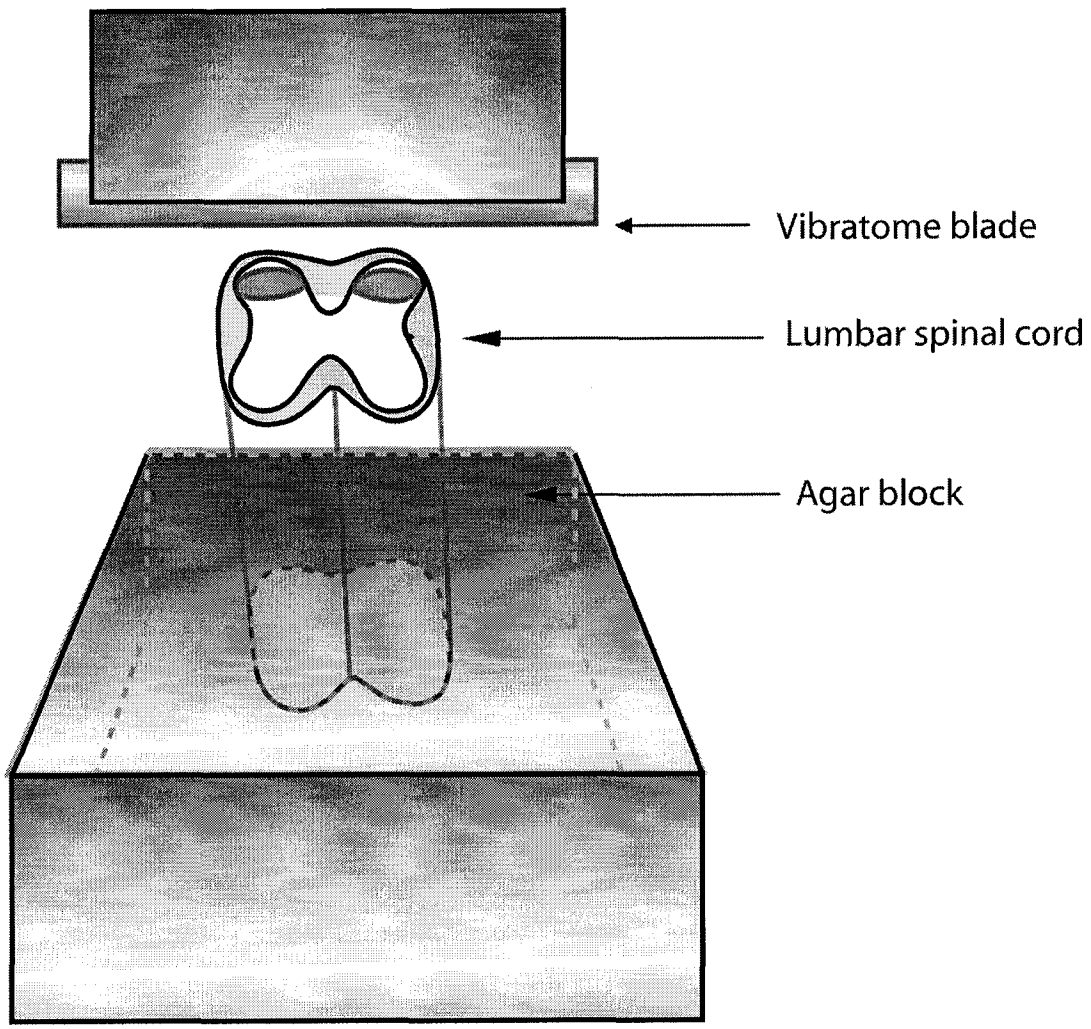
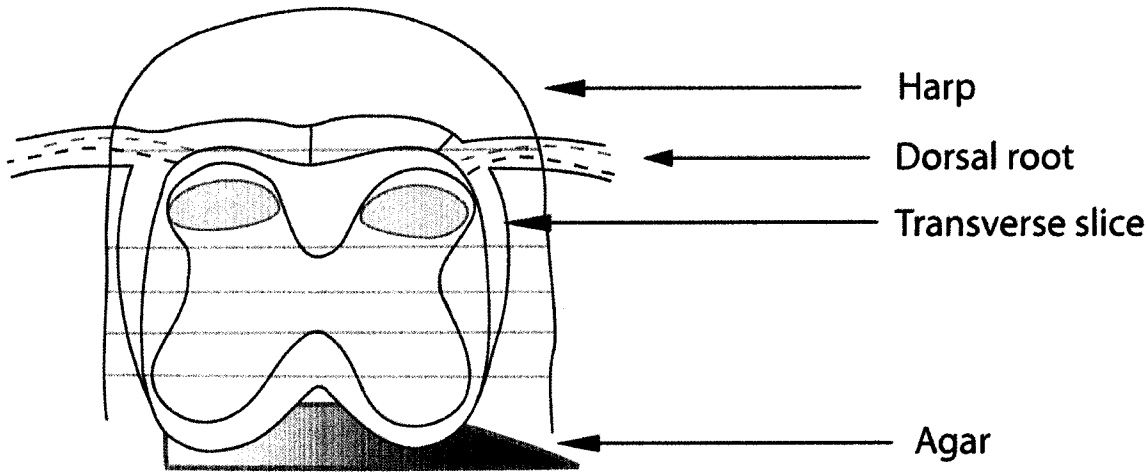


Figure 2.4. A schematic illustration of identifying the ipsilateral side. The transverse slices are cut with a triangular agar piece still attached to it. The long side of the triangle was used to mark the side of the cord ipsilateral to the CCI or sham lesion.

Figure 2.4



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Chapter 3

Sciatic Chronic Constriction Injury Generates a Specific Pattern of Changes in the synaptic excitation and the electrophysiological properties of Rat *Substantia Gelatinosa* Neurons

3.1 Introduction:

In experimental animals, peripheral nerve damage induces pain-related behaviors that are widely accepted as a model for human neuropathic pain (Kim et al., 1997;Mosconi and Kruger, 1996). These behaviors, which include mechanical and thermal hyperalgesia and allodynia, have been attributed to pathophysiological changes at multiple levels of the pain pathway which includes i) Persistent ectopic activity in both damaged and undamaged peripheral nerves and their dorsal root ganglia (Wall et al., 1979;Yoon et al., 1996;Govrin-Lippmann and Devor, 1978;Wall and Devor, 1983;Amir et al., 2005) ii) Enduring changes in the intrinsic properties of neurons and synaptic networks within the dorsal horn (centralization) (Woolf, 1983;Woolf and Mannion, 1999;Moore et al., 2002;Dalal et al., 1999;Coull et al., 2003) and iii) alterations in descending brainstem control of spinal nociceptive pathways (Suzuki et al., 2002). Taken together, these three processes contribute to the phenomenon of 'central sensitization', wherein spontaneous action potential discharge in the dorsal horn is increased and the response of dorsal horn neurons to peripheral stimulation is augmented and prolonged (Laird and Bennett, 1993;Dalal et al., 1999).

As already mentioned in general introduction, even though the role of *substantia gelatinosa* neurons in pain modulation is widely appreciated the organization of this region is still unclear. Hence, this remains a topic of intense investigation (Hantman et al., 2004;Light and Trevino, 1979;Lu and Perl, 2003;Lu and Perl, 2005;Grudt and Perl, 2002;Lu et al., 2006). Several cellular and molecular changes in this region have been attributed to the phenomenon of central sensitization seen after nerve injury (Kohno et al., 2003;Moore et al., 2002;Scholz et al., 2005;Woolf et al., 1992;Zhuang et al., 2005).

Despite this, it still not clear whether nerve injury affects all the cell types in *substantia gelatinosa* region in a similar way. Several different neuronal types have been distinguished in the superficial laminae of rat, mouse, cat and/or hamster spinal cord on the basis of their morphological (Gobel, 1978a;Gobel, 1978b), immunohistochemical (Todd and Spike, 1993) and electrophysiological characteristics (Grudt and Perl, 2002;Thomson et al., 1989;Prescott and de Koninck, 2002;Ruscheweyh and Sandkuhler, 2002;Heinke et al., 2004;Graham et al., 2004;Lu and Perl, 2005;Ruscheweyh and Sandkuhler, 2002). However, there is no general consensus to the precise relationship between neuronal morphology, electrophysiological characteristics, neurotransmitter phenotype and the immunocytochemical properties of *substantia gelatinosa* neurons. Hence, in this chapter I have defined cell types solely on the basis of electrophysiological criteria. This allowed us to ponder upon two basic questions 1) what are the electrophysiological consequences of peripheral nerve damage? and 2) are all cell types affected in the same way?

To address these issues, I have used whole-cell recording to carry out a systematic study of the effect of sciatic nerve injury (polyethylene cuff model) on the electrophysiological properties of various types of neurons in the *substantia gelatinosa* of young adult rats. Because acute rhizotomy occurred during the preparation of the slices, the impact of ectopic activity in the dorsal root ganglia (Wall and Devor, 1983) or from the site of nerve injury (Wall and Gutnick, 1974;Amir et al., 2005) on CCI-induced alterations in *substantia gelatinosa* activity would have been minimized.

Neurons were classified electrophysiologically, according to their discharge pattern in response to sustained depolarizing current (Grudt and Perl, 2002) as 'tonic', 'delay', 'irregular' 'phasic' or 'transient'. Our main finding is that 'tonic' cells respond differently to CCI than all other cell types. The excitatory synaptic drive to 'delay', 'transient' and 'irregular' cells is increased, while that to the 'tonic' cells is diminished. 'Tonic' cells also fired fewer action potentials in response to depolarizing current ramps. These observations relate to the hypothesis that some 'tonic' cells are themselves inhibitory (Hantman et al., 2004; Lu and Perl 2005) whereas many 'delay' cells may be excitatory (Lu and Perl, 2005). These CCI-induced changes are therefore in directions conducive to increased intrinsic activity within the *substantia gelatinosa* and underline the importance of enduring changes in synaptic transmission to the onset of pain centralization.

3.2 Methods

Most of the methods for recording from *substantia gelatinosa* neurons were identical to those described in Chapter 2. In a few experiments however (n=20), neurons were studied using nystatin-perforated patches. During perforated patch experiments, two kinds of internal recording solutions were used. The recording pipettes were first tip-filled with normal internal solution (refer to chapter 2 for description) to facilitate seal formation. They were then back filled with the nystatin containing solution which was prepared as follows. First, nystatin (0.05%), pluronic acid F127 (0.3%), was dissolved (Vortex/sonicate) in DMSO (0.03%), until clear yellow solution was obtained. This was then made up to a volume of 10ml with normal internal solution. The nystatin containing solution was prepared fresh daily. After attaining tight (Giga Ohm) seal, the cell was

monitored until optimal access was achieved. The duration of obtaining perforated access varied from 20-40 minutes.

3.3 Results

3.3.1 Behavioral testing

Since there is some question as to whether 20d old rats, as used in our study, exhibit pain behavior following peripheral nerve injury (Howard et al., 2005), we used standard von Frey hairs to assess mechanical hypersensitivity following CCI. Scores represented in Figure 3.1 were obtained from responses of the left paw that was ipsilateral to the nerve injury. The basal scores prior to surgery indicate that the filament diameters used did not generate withdrawal responses in un-operated animals. The difference in sensitivity between CCI and sham operated animals progressively increased over the 14d testing period. By this time, almost all animals subject to CCI had developed significant mechanical hypersensitivity.

Rats were also assumed to have attained a threshold for displaying mechanical hypersensitivity when they exhibited a withdrawal score of 4 for a total of 9 tests. Since the maximum attainable score was 18, this corresponds to a 22.2% response. This is close to the 20% response used by others (Decosterd and Woolf, 2000). Using this criterion, the baseline threshold prior to surgery threshold was >15g. Two weeks after the injury, the operated rats showed a reduction in mechanical threshold to 5.0±0.5g (n = 46), whereas threshold in sham-operated animals still exceeded 15g (n = 46).

Since both methods of assessing mechanical hypersensitivity revealed clear, statistically significant increases at two weeks, we chose a time frame between 13-25 days post-surgery to examine CCI-induced changes in the electrophysiological properties of *substantia gelatinosa* neurons.

3.3.2 Electrophysiological Characterization of Neuron Types.

The most widely used classification is based on the differences in spiking pattern in response to depolarizing current injection, however there are some discrepancies regarding the classification of firing pattern. Phasic neurons, which fire a short burst of spikes at the beginning of depolarizing pulse were termed adapting firing neurons (Melnick et al., 2004a), initial burst (Heinke et al., 2004) or transient type (Grudt and Perl, 2002). Moreover, Ruscheweyh et al (Ruscheweyh and Sandkuhler, 2002) report absence of tonic firing pattern, while several other studies suggest it is one of the most common type of neuron encountered in the *substantia gelatinosa* (Ruscheweyh and Sandkuhler, 2002;Grudt and Perl, 2002;Lu and Perl, 2003;Lu and Perl, 2005;Melnick et al., 2004b). Some studies suggest variations in holding potential (-60mv to -80mV) could lead to potential misclassification of neurons (Ruscheweyh and Sandkuhler, 2002), while Melnick et al 2004a suggest it is not the case. To clarify these issues I have characterized five different classes of the *substantia gelatinosa* neurons based on their firing characteristics at -60mV. These are as follows.

'Tonic' neurons are defined as those that exhibit continued discharge of action potentials in response to depolarizing current commands and an increased frequency of discharge in response to depolarizing currents of increasing intensity (see Figure 3.2A). Tonic cells

also exhibited lowest rheobase (~24pA) when compared to other cell types (Table 3.1) and hence are easily excitable.

'Delay' neurons exhibited a sustained delay prior to the onset of action potential discharge in response to a depolarizing current. The delay becomes shorter with higher current injections (see Figure 3.2B). Delay cells exhibited high frequency excitatory synaptic activity (Figure 3.10 B).

'Phasic' neurons were those which discharge 3 or more action potentials in response to an appropriate depolarizing stimulus followed by accommodation and cessation of firing (see Figure 3.2C). The rate of adaptation and the level of adapted firing within the range of current steps tested suggest the phasic cells are clearly a distinct and separate class of neurons. Spontaneous excitatory synaptic activity was relatively rare in phasic neurons (Figure 3.10B).

'Transient' neurons were those which fire a single action potential and never more than two spikes no matter how much depolarizing current is injected (see Figure 3.2D). Transient neurons exhibited the highest rheobase (~75pA) of any group of neurons studied (see Table 3.1).

'Irregular' neurons are those in which there is little correspondence between discharge patterns or frequency and intensity of depolarizing current (see Figure 3.2E). We also encountered a few cells (4.3%) that did not readily fit into any of the five categories. Such unclassified neurons were excluded for the analysis.

To confirm that the different firing patterns seen in *substantia gelatinosa* neurons were not an artifact induced by the cellular perfusion during whole-cell recording, we also examined the firing properties of *substantia gelatinosa* neurons (n=20) by means of nystatin-permeabilized patches. The percentage of 'tonic' 'delay' 'phasic', 'irregular' pattern obtained (Figure 3.3, A to E), were comparable with whole-cell recording (Figure 3.2F), with an exception of 'transient' pattern which was not observed in perforated-patch recording conditions. Thus, with the possible exception of 'transient' discharge, the discharge patterns observed can be ascribed to true variations in cellular physiology rather than to differences in recording conditions of *substantia gelatinosa* neurons.

Since the *substantia gelatinosa* is the site of termination of many primary afferent C and Aδ fibers, it is assumed to play an indispensable role in the processing of nociceptive information (Millan, 1999). Recent *in vivo* studies have shown, however, that tonic, phasic, transient, and delay neurons in mouse superficial dorsal horn can participate in the processing of both innocuous and noxious stimulation (Graham et al., 2004). Capsaicin is a useful tool in nociceptive studies as it excites a subpopulation of neurons receiving input from umyelinated C-fibers and also minority of Aδ-fibers (Bevan and Szolcsanyi, 1990;Caterina et al., 1997;Holzer, 1991). Because capsaicin (1μM) increased the frequency or induced sEPSC's in 12/14 tonic cells, 5/6 delay cells, 4/6 phasic cells, 3/7 transient cells and 8/9 irregular cells in rat *substantia gelatinosa,* it is probable that all cell types we studied participate in nociceptive processing. Similar numbers of cells displayed capsaicin sensitivity after CCI; 12/12 tonic cells, 7/9 delay cells, 6/7 phasic cells, 4/5 transient cells and 9/10 irregular cells. The cumulative probability plots demonstrating the

effect of capsaicin on both frequency and amplitude of the sEPSC's in sham and CCI rats are represented in Figures 3.4 and 3.5.

3.3.3 Effects of CCI on Passive Membrane Properties.

Sciatic nerve injury could hypothetically promote a 'phenotypic shift' such that one or more of the aforementioned cell types disappear completely or become more dominant in the neuronal population of the *substantia gelatinosa* as a whole. This did not appear to be the case because tonic, irregular, phasic, transient and delay neurons were found in similar proportions in a sample of 149 neurons from sham operated rats and 179 neurons from CCI rats (Figure 3.2 F and G). Numbers of neurons of different type in the two populations were compared with χ^2 tests and P values ranged from 0.08 to 0.95, indicating no significant change in the contribution of each neuron type to the total population. Analysis of the electrophysiological properties of tonic, delay, phasic, transient and irregular neurons from sham operated and CCI animals was therefore undertaken.

CCI did not affect resting membrane potential (r.m.p), rheobase or input resistance in any neuron type (Table 3.1 and Figure 3.6). It also did not produce a global change in membrane excitability or spontaneous excitatory synaptic activity but rather produced a clear pattern of neuron type-specific changes.

3.3.4 Effects of CCI on Membrane Excitability.

Figure 3.7 illustrates the effect of depolarizing current ramps on action potential discharge in each of the five defined cell types. Although ramp rates of 33, 67, 100 or 133pA/S were used in all cell types, data are only presented from ramp rates that evoked optimal firing in

each cell type. Since 'tonic' cells have a low rheobase they required slower ramp rates than 'transient' cells that have the highest rheobase (Table 3.1). The ramp rates chosen were steep enough to evoke robust firing in most cells tested without producing depolarization block at their most positive excursions. Panels on the left illustrate typical responses of tonic, delay, phasic, transient and irregular cells to current ramps (shown as superimposed dashed lines). Panels on the right of Figure 3.7 illustrate mean cumulative latencies for tonic (Figure 3.7A), delay (Figure 3.7B), phasic (Figure 3.7C), transient (Figure 3.7D) and irregular neurons (Figure 3.7E) from sham operated and CCI animals. Note significantly greater cumulative latencies for 'tonic' cells after CCI indicating decreased excitability (Figure 3.7A) and significantly reduced cumulative latencies for phasic cells (Figure 3.7C) indicating increased excitability. The membrane excitability of delay, transient and irregular cells was unchanged (Figure 3.7B, D and E).

3.3.5 Effect of CCI on Voltage-Sensitive Conductances.

Voltage-gated K^+ currents were recorded from the various cell types in sham operated and CCI animals to examine their possible contribution to changes in excitability. Experiments were done in the presence of 1μM TTX to prevent action potential generation. Neurons were subject to a series of incremental voltage commands from holding potentials of -80 or -110mV (see methods, chapter 2). Surprisingly, some neurons failed to generate voltage-sensitive outward currents under these conditions. Others generated a slowly activating current which resembled a delayed rectifier (DR; Figure Fig 3.8A). Such currents took more than 20ms to activate at -20mV and either did not inactivate or inactivated only slightly during a 100ms command. Other cells exhibited a more transient current that resembled an A or D-current (A/D current). Such currents activated in less

than 20ms at -20mV and displayed > 50% inactivation within 100ms of activation (Figure 3.8B). As shown in Table 3.2, there was no obvious correlation between neuronal type and current exhibited. CCI injury did not significantly alter the expression of current types in the various groups of neurons (χ^2tests on data in Table 3.2). Figures 3.8C and 3.8D illustrate current-voltage plots for DR-type current in phasic and tonic cells. CCI does not produce a significant change in current amplitude. Thus, increased excitability of phasic cells (Figure 3.7C) and decreased excitability of tonic cells (Figure 3.7A) would not appear to reflect changes in voltage-sensitive K^+ currents that can be recorded in the soma.

Injury-induced upregulation of hyperpolarization-activated cation current (I_h) in dorsal root ganglion neurons has been suggested to contribute to generation of neuropathic pain (Chaplan et al., 2003;Yao et al., 2003). We therefore tested whether CCI altered this current in *substantia gelatinosa* neurons. A series of hyperpolarizing voltage commands from a holding potential of -60mV (Figure 3.8E) were used to activate Ih. It was most commonly encountered in tonic neurons (Grudt and Perl, 2002) and was absent from irregular and delay neurons. CCI did not appear to affect the overall chance of observing I_h ($P>0.5$, χ^2 test on all neurons studied). The current was seen in 6/25 tonic neurons from sham operated animals and in 4/24 neurons from CCI animals. It was seen in 1/25 transient neurons from shams and 2/19 after CCI. I_h was seen in 3/13 phasic neurons in the sham group and in 1/13 in the CCI group. Thus, alterations in I_h are unlikely to play a major role in CCI-induced increased activity in the *substantia gelatinosa*.

3.3.6 Effects of CCI on Spontaneous Excitatory Synaptic Currents.

CCI changed excitatory synaptic transmission in different ways in different cell types. Spontaneous excitatory synaptic activity was reduced in tonic cells but increased in delay, transient and irregular cells.

The effects of CCI on sEPSC amplitude are illustrated in Figure 3.9 A-E and its effects on interevent interval (reciprocal frequency) are illustrated in Figure 3.9 F-J. Figures 3.9A and F show that CCI reduced the amplitude and frequency of sEPSC's in 'tonic' cells. By contrast, CCI increased the frequency and amplitude of sEPSC's in delay, transient and irregular cells (Figures 3.9B,D, E, G, I and J). While CCI increased sEPSC frequency in phasic cells (Figure 3.9H), amplitude was reduced (Figure 3.9C). All changes were significant according to the Kolmogorov-Smirnoff test (P values presented in Figure 3.9). Despite this, it is possible that the presence of silent cells in the various populations may have biased the data. Neurons were classified as 'silent' if they failed to display one or more events during the 3 min sampling period and were omitted from the analysis. However, if such cells fired one event, say, every 4 min, they should have been included as very slow firing cells. The inadvertent omission of such cells may have altered the nature of cumulative probability plots. It is unlikely that this type of bias was introduced because comparable numbers of 'silent' cells were found in each cell category in both sham and after CCI animals (χ^2 tests on data in Table 3.3).

We also used a simpler but more rigorous parametric t-test to analyze the same data set as used in Figure 3.9 A-J. This generally identified the same trends as the Komogorov-Smirnoff test. Figure 3.10 E shows that sEPSC amplitude was reduced in tonic neurons

and increased in delay, transient and irregular neurons. These findings correspond to those shown in Figure 3.9A, B, D & E. Phasic cell sEPSC amplitude was unchanged according to the t-test (Figure 3.10E) but decreased according to the KS test (Figure 3.9C). This inconsistency may reflect the limited amount of data available from phasic cells as they displayed the lowest level of spontaneous activity of all the cell types.

Figure 3.10F shows that sEPSC interevent intervals for tonic cells were increased (frequency decreased) whereas those for delay, transient and irregular were reduced (frequency increased). Again, the more rigorous t-test generally supports the conclusions from the KS test (Figure 3.9F, G, I & J). The one exception is phasic cells where no significant change in interevent interval was detected by the t-test. This questions the validity of data presented in Figure 3.9H, where CCI appeared to increase interevent interval of phasic cells.

Spontaneous inhibitory activity (sIPSC) was rarely encountered under our recording conditions that used K^+ as the predominant intracellular cation. It was therefore not feasible to compare sIPSC's between sham and CCI animals. Although sIPSC's can be recorded in rat *substantia gelatinosa* neurons when Cs^+ is used as an intracellular cation (Moran et al., 2004), the use of Cs^+ complicates identification of neuron types on the basis of their action potential discharge pattern.

3.3.7 Effects of CCI on Miniature Excitatory Synaptic Currents

Spontaneous postsynaptic events reflect action potential-dependent as well as action potential-independent release of neurotransmitter. The latter process, which represents

ongoing activity of the release process, can be investigated by examining mEPSC's in the presence of TTX (Edwards et al., 1990). Fig 3.10A compares the mean amplitudes of sEPSC's in 'tonic', 'delay', 'transient' and 'irregular' cells with the mean amplitudes of mEPSC's recorded in 1μM TTX. Insufficient data were available from mEPSC's in 'phasic' cells. TTX had little effect on event amplitude in 'tonic', 'delay' and 'transient' cells but reduced it in 'irregular' cells. It has been argued that each mEPSC represents the release of a single quantum of neurotransmitter (Edwards et al., 1990). If this is so, the lack of effect of TTX on event amplitude may imply that each sEPSC also represents the release of a single quantum so each action-potential dependent sEPSC in 'tonic', 'delay' and 'transient' cells has a low quantal content. Alternatively, if the amount of neurotransmitter released in one mEPSC saturates the postsynaptic (AMPA) binding sites, any increased transmitter release during a sEPSC would not be detected. Figure 3.10B shows that TTX increased interevent interval (decreased frequency) by 100% in 'tonic', 72% in 'delay', 149% in 'transient' and 80% in 'irregular' cells. This implies about half of the sEPSC's recorded in the absence of TTX are actually mEPSC's that reflect the action potential-independent turn-over of the release process (Edwards et al., 1990). (50% of spontaneous events in tonic cells are mEPSC's, 41% in delay cells, 60% in transient cells and 44% in irregular cells). Sample recordings illustrate that mEPSC's (Fig 3.10D) are of comparable amplitude to sEPSC's (Fig 3.10C) but occur less frequently.

There was a marked similarity between CCI-induced changes in mEPSC (Figure 3.9K-Q) and those seen in sEPSC's (Figure 3.9A, B, D-G, and I). Thus, mEPSC amplitude and frequency are decreased in tonic cells (Figure 3.9K and O) and increased in delay and transient cells (Figure 3.9L, M, P and Q). Because the frequency of mEPSCs in phasic

cells was so low, it was not possible to prepare cumulative probability plots from the available data. Also, changes in mEPSC interevent interval were not significant for irregular cells (Figure 3.9R) whereas statistical significance was attained for changes in interevent interval of sEPSC (Figure 3.9J). As with sEPSC's, we had to consider the possibility that the presence of 'silent cells', (*i.e.* those which failed to generate a mEPSC during 3 min recording) could bias the data to yield inaccurate cumulative probability plots. This again did not seem to be the case because similar proportions of all neuron types exhibited mEPSC activity in both sham-operated and CCI animals (Table 3.3).

As was done for sEPSC's, the pool of mEPSC data analyzed with KS statistics (Figure 3.9K-R) was reanalyzed using a parametric t-test (Figure 3.10G and H) and similar changes were found. Thus, CCI significantly reduced mEPSC amplitude of tonic cells, but increased that of delay, transient and irregular cells. It also increased interevent interval (reduced the frequency) of mEPSC's in tonic cells but decreased the interevent interval (increased the frequency) of delay and transient cells. CCI failed to affect the mEPSC frequency of irregular cells (Figure 3.10H), thus confirming the conclusion reached using the KS test (Figure 3.9R).

3.4 Discussion

Because there is, as yet, no standardized classification scheme for *substantia gelatinosa* neurons (Todd and Spike, 1993;Ruscheweyh et al., 2004;Heinke et al., 2004;Hantman et al., 2004;Grudt and Perl, 2002), we used simple electrophysiological criteria to classify the neurons as 'tonic', 'delay', 'phasic', 'transient' and 'irregular'. These five categories are similar to those used by other researchers in the field (Melnick et al., 2004a;Melnick et al., 2004b;Prescott and de Koninck, 2002;Graham et al., 2004;Grudt and Perl,

2002;Hantman et al., 2004;Lu and Perl, 2003;Lu and Perl, 2005). In confirmation of the findings of Grudt and Perl (2002), we found that 'delay' cells had a high level of spontaneous excitatory activity (Fig 3.10B).

CCI altered the synaptic transmission in all cell types studied. Although alterations in sEPSC and mEPSC amplitude likely represent postsynaptic effects, possible presynaptic effects on quantal size or content cannot be discounted. Alterations in mEPSC frequency likely represent alterations in the transmitter release machinery *per se*. This inevitably contributes to the overall CCI-induced alterations in sEPSC frequency as 41 – 60% of sEPSC's represent action-potential independent release (i.e. they are actually mEPSC's; Fig 3.10B). Any additional contribution of CCI-induced alterations in presynaptic action potential frequency to altered sEPSC frequency is difficult to assess. This is because CCI-induced changes in sEPSC frequency paralleled those seen in mEPSC frequency.

Capsaicin excites a subtype of sensory neurons that are involved in nociception. Capsaicin receptors are expressed on sensory neurons that are associated with ummyelinated C fibers and with a minority of Aδ fibers (Bevan and Szolcsanyi, 1990;Caterina et al., 1997;Holzer, 1991). The action of capsaicin in *substantia gelatinosa* neurons has also been reported previously (Yang et al., 1998;Yang et al., 1999). The increase in the frequency of sEPSC's after application of capsaicin suggests that tonic, delay, phasic, irregular and transient cells receive inputs from nociceptive fibers and hence participates in processing of nociceptive information. Moreover, capsaicin experiments also gave us some idea as to the nature of the synaptic inputs in these slices. Since we used thin transverse slices (300μm), the synaptic inputs, in some cases may have been removed

during the preparation of slices. This raises concerns over the impact of CCI on the spontaneous synaptic activity in these cells, where the effects could be due to slicing procedure than nerve injury. However, capsaicin is able to increase the spontaneous excitatory synaptic activity, in both sham and CCI rats, (Figure 3.4 and 3.5). Even in the tonic cells where CCI diminishes the excitatory spontaneous activity, application of capsaicin was still able to increase the sEPSC's. This suggests that in CCI rats the synaptic inputs were comparable to that in control situation and hence the slicing procedure *per se* could not be attributed for changes in synaptic activity.

3.4.1 Effects of CCI on 'Tonic' Cells.

Tonic cells are assumed to function as "integrators". In lamina 1, Tonic neurons exhibit prolonged EPSP's and hence tend to summate synaptic events over prolonged intervals (Prescott and de Koninck, 2002;Prescott and De Koninck, 2005). Moreover, based on studies in deep dorsal horn neurons tonic cells are assumed to signal both static and dynamic components of sensory stimuli and hence function in a "broad band" capacity (Schneider, 2003). Although CCI increased the amplitude and/or frequency of sEPSC's and/or mEPSC's in most cell types, 'tonic' cells are a clear exception. CCI reduced both the amplitude and frequency of both mEPSC's and sEPSC's (Figure 3.9 &3.10). Many GABAergic interneurons in lamina II correspond to 'islet' cells defined morphologically by the rostrocaudal orientation of their dendritic tree (Todd and Spike, 1993). Because islet cells often display a 'tonic' discharge pattern (Lu and Perl, 2003;Lu and Perl, 2005) many 'tonic' cells are likely to be GABAergic. This possibility is supported by the observation that intracellular stimulation of 'islet' cells *in vitro* evoked monosynaptic, bicuculline-sensitive IPSC's in 'central' neurons (Lu and Perl, 2003).

In addition to suppressing the spontaneous excitatory synaptic transmission onto 'tonic' cells (Figure 3.9A, F, K and O, Figure 3.10E-H), CCI also reduced the membrane excitability (Figure 3.7A). If they are predominantly GABAergic, a CCI-induced decrease in excitation and membrane excitability of 'tonic' cells fits with the finding that GABAergic tone is reduced in neuropathic pain states (Laird and Bennett, 1992;Woolf and Mannion, 1999;Moore et al., 2002;Baba et al., 2003). Moreover, depressed synaptic drive to inhibitory neurons may contribute to an overall increase in dorsal horn excitability.

3.4.2 Effects of CCI on 'Delay',' Transient' and 'Irregular' cells.

Delay cells may function as excitatory interneurons corresponding to 'vertical' cells with dorsoventral orientation (Grudt and Perl, 2002). These may serve as output neurons conveying information to lamina I projection neurons (Lu and Perl, 2005). In direct contrast to 'tonic' cells, CCI increased all indices of spontaneous excitatory synaptic activity in 'delay' neurons (Figure 3.9B, G, L and P, Figure 3.10E-H). This coincides with the possibility that an increase in the synaptic drive to 'delay' cells would increase output to projection neurons of lamina I and thereby promote the transfer of nociceptive information.

Increased sEPSC and mEPSC frequency in 'delay' cells presumably reflects presynaptic changes. These may include up-regulation of the transmitter release mechanisms as well as the possible formation of new synapses (Okamoto et al., 2001). These possibilities are supported by microarray analysis following peripheral nerve injury that revealed large increases in message for proteins involved in neurotransmitter release (Xiao et al.,

2002;Yang et al., 2004). There is also evidence for increased expression of the presynaptic vesicle protein, synaptophysin after chronic constriction injury (Chou et al., 2002). Uncertainties regarding the contribution of increased frequency of presynaptic action potentials to the overall CCI-induced increase in sEPSC frequency have already been alluded to. If such an effect does occur, might it reflect increased action potential activity in primary afferent terminals? Although dorsal roots are acutely cut during the preparation of slices, all cell types exhibited increased sEPSC frequency in response to capsaicin. This indicates that primary afferent fibers remain viable and capable of transmitter release within the slices and that increased transmitter release from primary afferents affects all cell types. We cannot therefore exclude the possibility that axonally-generated action potentials in primary afferent fibres subject to CCI (Amir et al., 2005) contribute to the increased sEPSC frequency in 'delay' cells.

Whilst increases in sEPSC and mEPSC amplitude in 'delay' cells may also reflect presynaptic mechanisms, it is likely that postsynaptic mechanisms play a major role. This is because GluR2 adapter proteins, that control the function of specific AMPA receptor subtypes, as well as GluR3 itself, are upregulated in neuropathic pain (Garry et al., 2003) or peripheral nerve injury (Yang et al., 2004). Interestingly, these subunits may be confined to excitatory neurons (Albuquerque et al., 1999;Kerr et al., 1998). Putative inhibitory neurons such as 'tonic'/islet neurons are thought to express the GluR1 subunit (Kerr et al., 1998) and this may not be affected by CCI in the same way as GluR2. It is therefore possible that GluR1 AMPA receptors on putative inhibitory 'tonic' neurons are unchanged or even down-regulated by CCI whereas GluR2 AMPA receptors on putative excitatory, 'delay' cells are up-regulated. This possibility remains to be tested.

Because so little is known about the functional role of 'transient' or 'irregular' cells, it is difficult to attach any significance to the increased synaptic drive that they receive (Figure 3.9D,E, I, J, M, N and Q, Fig 3.10).

3.4.3 Effects of CCI on 'Phasic' Neurons.

Phasic cells might respond well to brief and rapidly adapting stimuli compared to static or slowly changing inputs (Schneider, 2003). These cells are termed as "coincidence detectors" as they tend not to rely on the summation of inputs for generating action potentials (Prescott and de Koninck, 2002). Some 'phasic' cells may be excitatory interneurons, some of which may correspond to morphologically-defined 'central' cells (Lu and Perl, 2005). It should be noted that these authors used the term 'transient cells' to define the population we refer to as 'phasic' cells. In mouse lamina I neurons however, identified GABAergic neurons display both 'tonic' and 'phasic' discharge patterns that can interconvert depending on the current stimulus parameters (Dougherty et al., 2005). Moreover, also in mouse, but in lamina II, it has been suggested that the majority of GABAergic neurons display a 'phasic' rather than a 'tonic' discharge pattern (Heinke et al., 2004). These apparently conflicting findings preclude the characterization of 'phasic' cells as a purely excitatory or inhibitory population. They may instead represent a mixed population of functional phenotypes. Since spontaneous synaptic activity was so rare in 'phasic' cells, and the statistical analyses of sEPSC amplitude and frequency produced different results depending on the method used, (Figure 3.9C and H and 3.10E and F) little can be concluded as to the effect of CCI on synaptic activity in this population. 'Phasic' cells did however exhibit a marked increase in membrane excitability following CCI (Figure 3.7C). Voltage-clamp experiments were therefore done to elucidate the underlying

mechanism. The absence of voltage-gated K^+ currents from the cell bodies of about 30% of *substantia gelatinosa* neurons was unexpected (Table 3.2) as was the lack of correlation between the type of K^+ current observed in a given neuron and its firing pattern. It has been suggested, however, that the soma of *substantia gelatinosa* neurons is not capable of initiating action potentials (Safronov et al., 1997) and that responses recorded in the soma spread passively from the axon hillock where most of the Na^+ channels are localized. Thus, the repetitive firing characteristics of *substantia gelatinosa* may be determined by the Na^+ and K^+ channels located there. Although CCI did not alter the amount of DR-type K^+ current recorded in the cell body (Figure 3.8C and D), it has been estimated that 47% of DR-type K^+ channels reside in the dendrites and 38% reside in the initial segment of *substantia gelatinosa* neurons (Wolff et al., 1998). Thus, variations in the individual geometry of each cell may be a major determinant of how much DR-type K^+ current in seen in the soma and this may override any CCI-induced changes in dendritic and initial segment DR K^+ channels. Thus, we cannot rule out the possibility that changes in K^+ channels in the initial segment exert profound effects on excitability. Changes in excitability may also reflect upregulation of $Na_v1.3$ (Hains et al., 2004) or other Na^+ channel subtypes (Yang et al., 2004) in the initial segments or dendrites.

Several factors likely conspire to instigate increased dorsal horn excitability following CCI. These include release of pro-inflammatory cytokines (Tsuda et al., 2005) and/or growth factors such as BDNF in response to nerve injury (Mannion et al., 1999;Coull et al., 2005). The differential response of 'delay' neurons and 'tonic' neurons to CCI may therefore reflect differential expression of TrkB, perhaps p75 and cytokine receptors on their cell bodies and presynaptic terminals. Moreover, the fact that that CCI produces cell-

type specific changes rather than a ubiquitous increase in excitability of all neuron types provides an 'injury footprint' (a specific pattern of changes) that serves as a 'template' for the identification of putative harbingers of pain centralization. Continuation of these studies may provide further new insights into the process of pain centralization.

Even though our studies suggest the *substantia gelatinosa* neurons could be differentiated based on firing properties alone, correlation of morphological characteristics with firing properties would be very useful. Since no consensus has been reached from other studies (Heinke et al., 2004;Grudt and Perl, 2002) it would be useful for us to establish if a correlation exist between neuronal morphology and firing characteristics. Grudt and Perl have shown that even though a majority of tonic neurons correspond islet cells, a minority of vertical cells also exhibit tonic firing pattern (Grudt and Perl, 2002). Similarly delay neurons could correspond to both radial and vertical neurons (Grudt and Perl, 2002). Hence, more profound effects of CCI could be observed if we categorize neurons based on both morphological and electrophysiological profiles. This topic is dealt in the following chapter.

Table 3.1. Effects of chronic constriction injury (CCI) on rheobase and resting membrane potential (r.m.p.) of various types of *substantia gelatinosa* neurons. No significant differences were seen for any cell type. Values represented as Mean ± SD

	Tonic		**Delay**		**Phasic**	
	Rheobase (pA)	**R.M.P. (mV)**	**Rheobase (pA)**	**R.M.P. (mV)**	**Rheobase (pA)**	**R.M.P. (mV)**
CCI	24.3 ± 8.81 (n=46)	60.1 ± 11.1 (n=46)	53.9 ± 27.37 (n=37)	59.7 ± 8.40 (n=36)	29.4 ± 13.21 (n=33)	60.3 ± 10.91 (n=33)
Sham	20.3 ± 12.60 (n=49)	62.6±13.30 (n=49)	50.1±35.98 (n=28)	55.7±9.52 (n=30)	31.5 ± 14.27 (n=26)	56.6 ± 10.70 (n=26)
	Transient		**Irregular**			
	Rheobase (pA)	**R.M.P. (mV)**	**Rheobase (pA)**	**R.M.P. (mV)**		
CCI	108.8 ± 100.45 (n=26)	55.1 ± 12.9 (n=27)	43.8 ± 35.05 (n=30)	56.2 ± 9.31 (n=30)		
Sham	74.8 ± 63.90 (n=17)	56.2 ± 10.3 (n=17)	32.5 ± 13.41 (n=22)	57.5 ±10.31 (n=22)		

Table 3.2. K^+ currents in various types of neuron from sham operated and CCI animals. *DR* = delayed rectifier type current (see Fig 3.8A), *A/D* = I_A/I_D type inactivating current (see Fig 3.8B) *none* = absence of obvious voltage gated current.

	Sham				**CCI**			
	DR	*A/D*	*none*	Total	*DR*	*A/D*	*none*	Total
Tonic	4	1	7	12	8	1	4	13
Delay	3	5	0	8	4	6	4	14
Phasic	8	0	6	14	12	2	6	20
Transient	1	2	2	5	1	7	5	13
Irregular	3	7	3	13	4	6	6	16
Total	19	15	18	52	29	22	25	76

Table 3.3 Numbers of cells in each category displaying sEPSC and mEPSC in sham operated and CCI animals. P values from χ^2 test.

	Tonic	Delay	Phasic	Transient	Irregular	Total
Sham with sEPSC	35/44	23/26	17/21	18/21	17/20	110/132
CCI with sEPSC	27/39	25/29	19/25	21/26	20/22	112/141
P value χ^2 test	0.41	0.8	0.97	0.95	0.91	0.50
Sham with mEPSC	11/15	7/10	6/7	6/6	7/7	36/44
CCI with mEPSC	7/7	11/14	7/10	10/11	6/6	40/47
P value χ^2 test	0.36	0.63	0.86	0.45	1.0	0.88

Figure. 3.1. Development of mechanical hypersensitivity following chronic constriction injury (CCI). For each panel, abscissa is gauge of von Frey hair used and ordinate is score derived from paw withdrawal measurements (percentage score of maximum, see methods). Baseline data were acquired from 20d animals. Lighter von Frey filaments were used to test these smaller animals to limit the available force to <15% of body weight. Each data point was acquired from 28 to 75 measurements (* P>0.05, ** P>0.01, *** P>0.0001).

Figure. 3.1

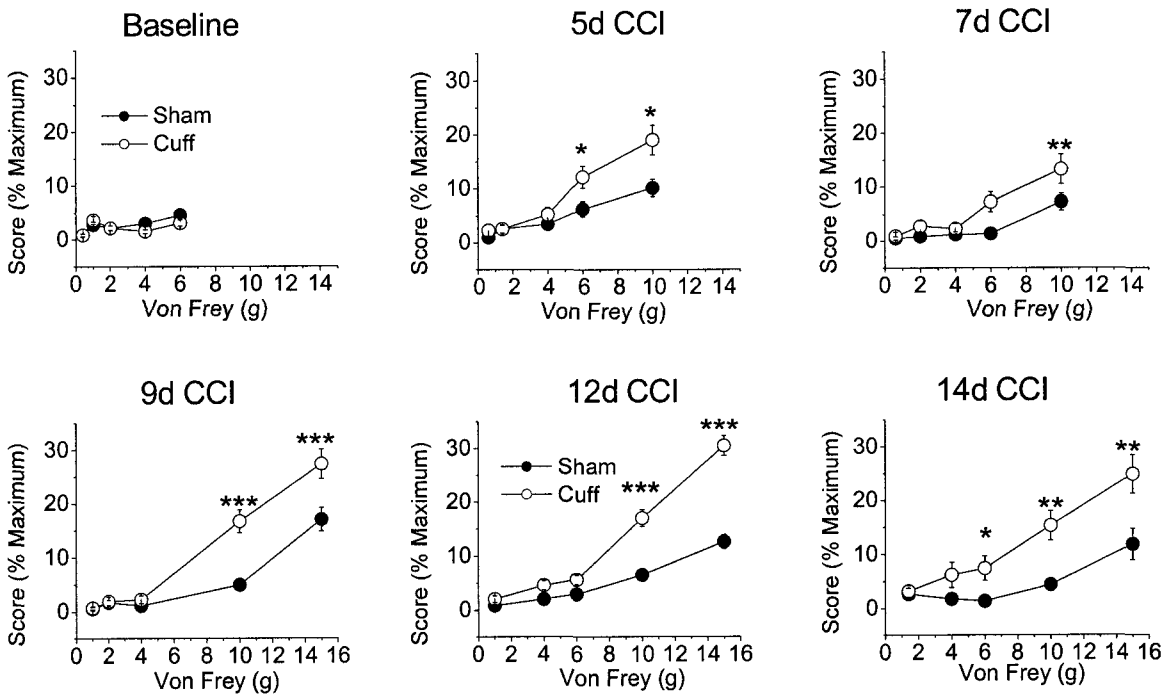


Figure. 3.2. A-E Whole- cell current-clamp recordings to show discharge patterns evoked in *substantia gelatinosa* neurons in response to three different intensities of depolarizing current as indicated at the bottom of each panel of records. Typical firing characteristics of **A.** a tonic neuron; discharge persists throughout application of depolarizing current and discharge rate increases with increasing depolarization, **B.** a delay neuron; note long delay prior to onset of spike discharge, **C.** a phasic neuron; spike discharge accommodates after 1-4 spikes, D. a transient neuron; only one spike is discharged regardless of the applied current intensity, **E.** an irregular neuron; number of spikes discharged is not directly related to intensity of depolarizing current. **F** and **G** Bar graphs to show percentage of each neuron type identified in recordings from sham operated and CCI animals. n=149 neurons from sham operated animals, n= 179 neurons from CCI animals. There is no significant difference in percentage of cells in the two groups, P values range from 0.08 to 0.95, χ^2 test.

Figure. 3.2

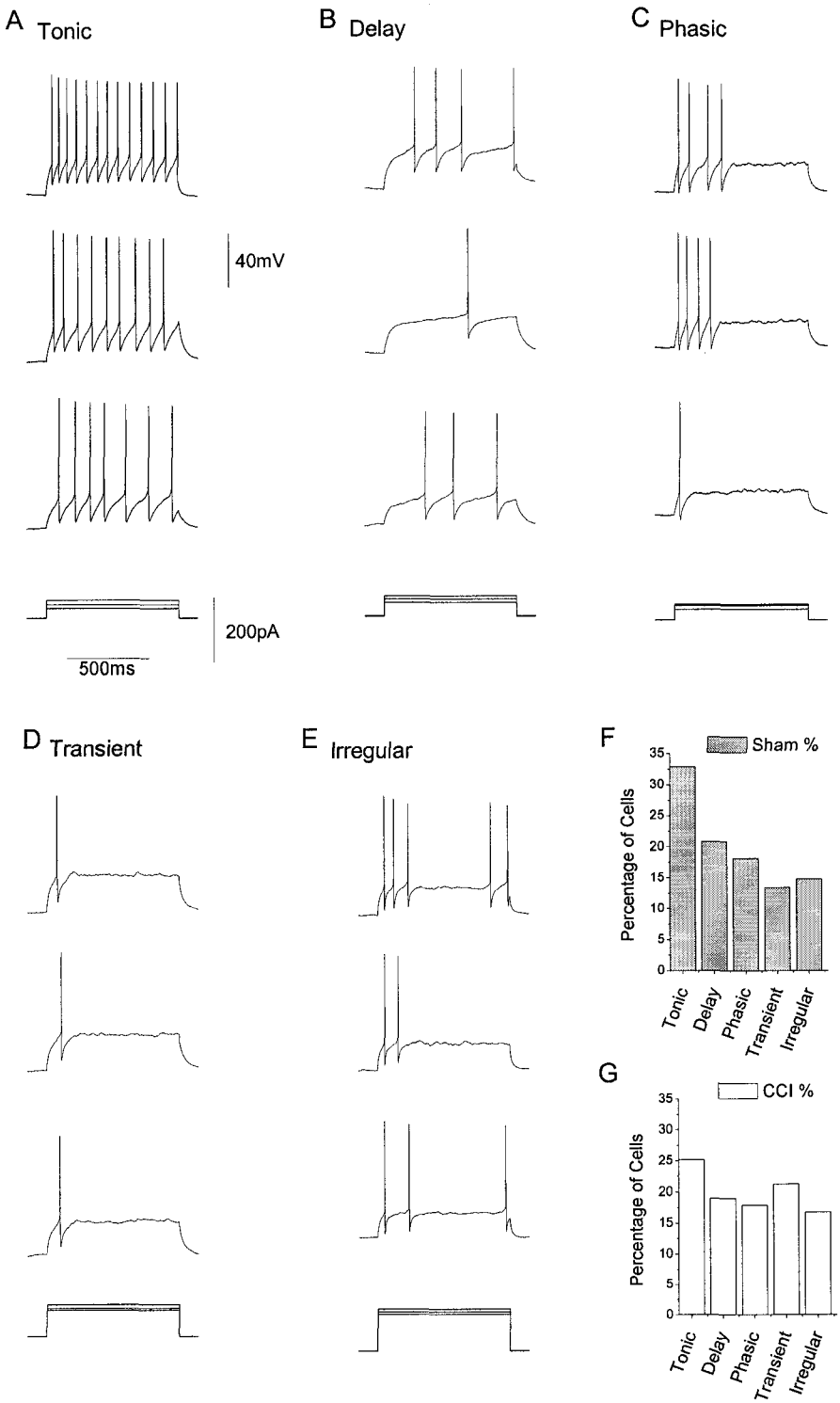


Figure. 3.3. A-E Perforated-patch current-clamp recordings to show discharge patterns evoked in *substantia gelatinosa* neurons in response to three different intensities of depolarizing current as indicated at the bottom of each panel of records. Typical firing characteristics of **A**. a tonic neuron; **B**. a delay neuron; **C**. a phasic neuron **D**. an irregular neuron. **E** Bar graphs to show percentage of each neuron type identified in recordings from control animals. Total no of neurons=20.

Figure. 3.3

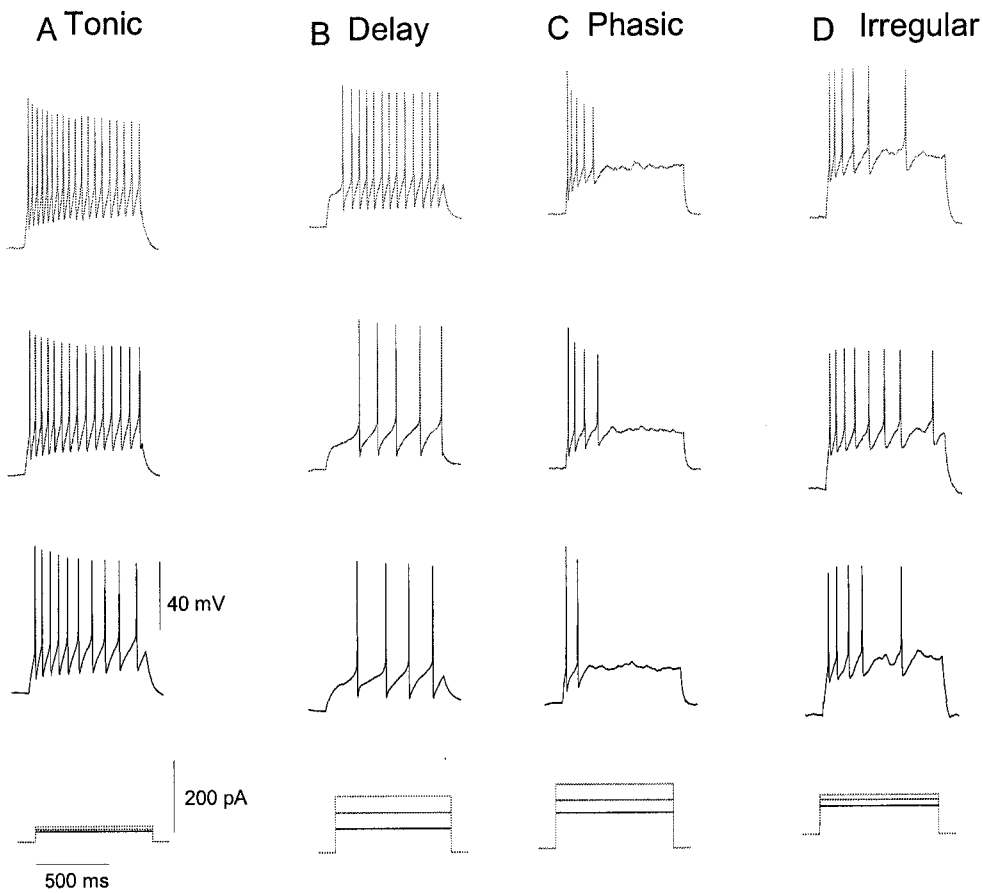


Figure. 3.4. Cumulative probability plots for effects of capsaicin (1μM) on amplitude and interevent interval of s.e.p.s.'c in sham animals. **A-E,** cumulative probability plots for the effect of capsaicin on sEPSC amplitude and **F-J,** interevent interval from tonic, delay, phasic, transient and irregular cells. P values derived from KS test are indicated on the graphs. The following numbers of events were analyzed from the whole 3 min of recording. Tonic neurons; n=12 cells, 1052 events from control, 4502 events after capsaicin treatment. Delay neurons; n=7 cells, 820 (control), 5519 (Capsaicin). Phasic neurons; n= 5 cells, 176 (Control), 1575 (Capsaicin); Transient neurons; n= 5 cells, 220 (Control), 846 (Capsaicin). Irregular neurons; n= 6 cells, 338 (Control), 2174 (Capsaicin).

Figure. 3.4

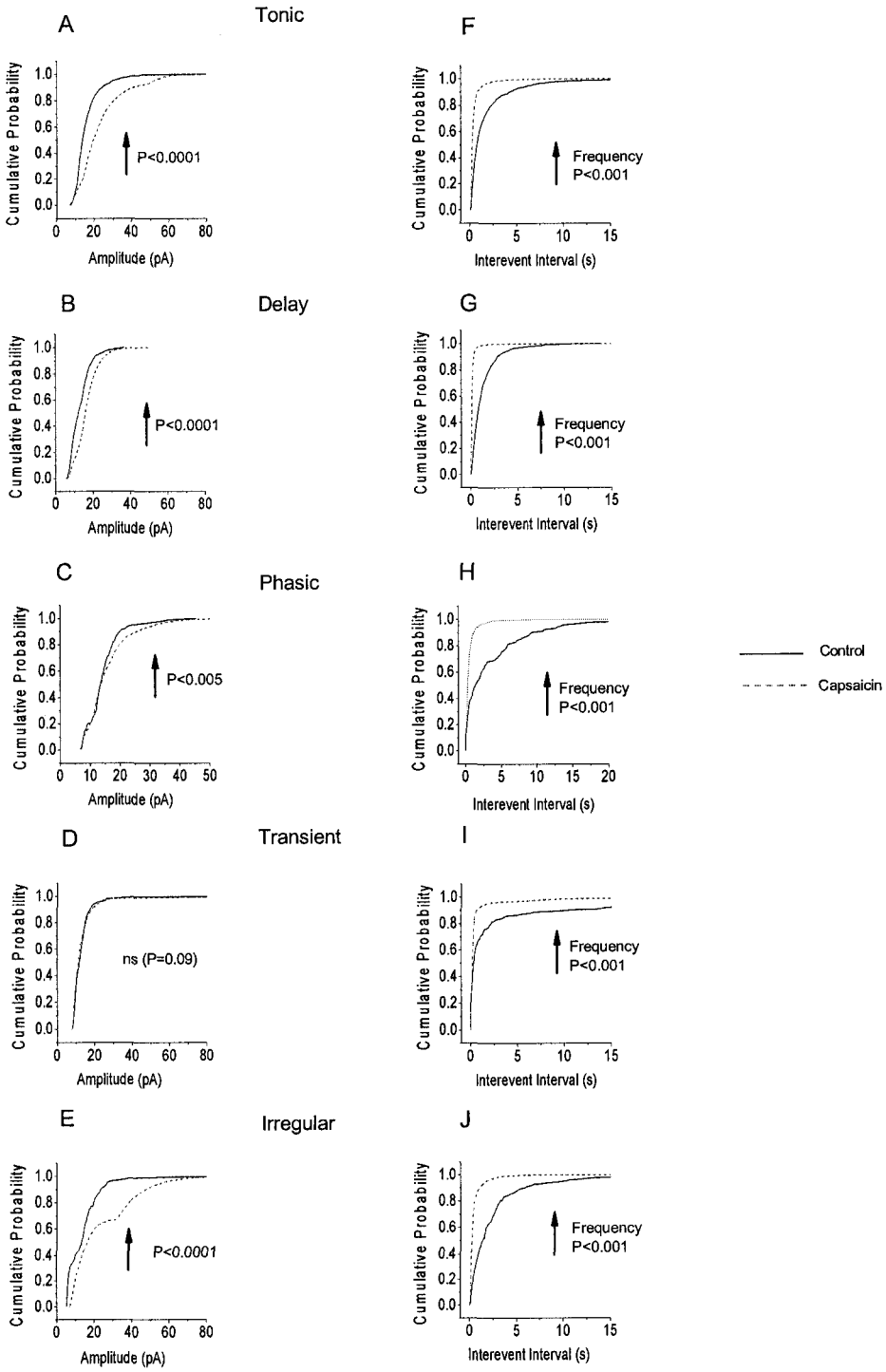


Figure. 3.5. Cumulative probability plots for effects of capsaicin (1μM) on amplitude and interevent interval of s.e.p.s.'c in CCI animals. **A-E,** cumulative probability plots for the effect of capsaicin on sEPSC amplitude and **F-J, interevent** interval from tonic, delay, phasic, transient and irregular cells. P values derived from KS test are indicated on the graphs. The following numbers of events were analyzed from the whole 3 min of recording. Tonic neurons; n=10 cells, 1057 events from control, 3977 events after capsaicin treatment. Delay neurons; n= 9 cells, 826 (control), 5525 (Capsaicin). Phasic neurons; n=7 cells, 420 (Control), 725 (Capsaicin); Transient neurons; n= 5 cells, 603 (Control), 627 (Capsaicin). Irregular neurons; n=8 cells, 714 (Control), 2517 (Capsaicin).

Figure. 3.5

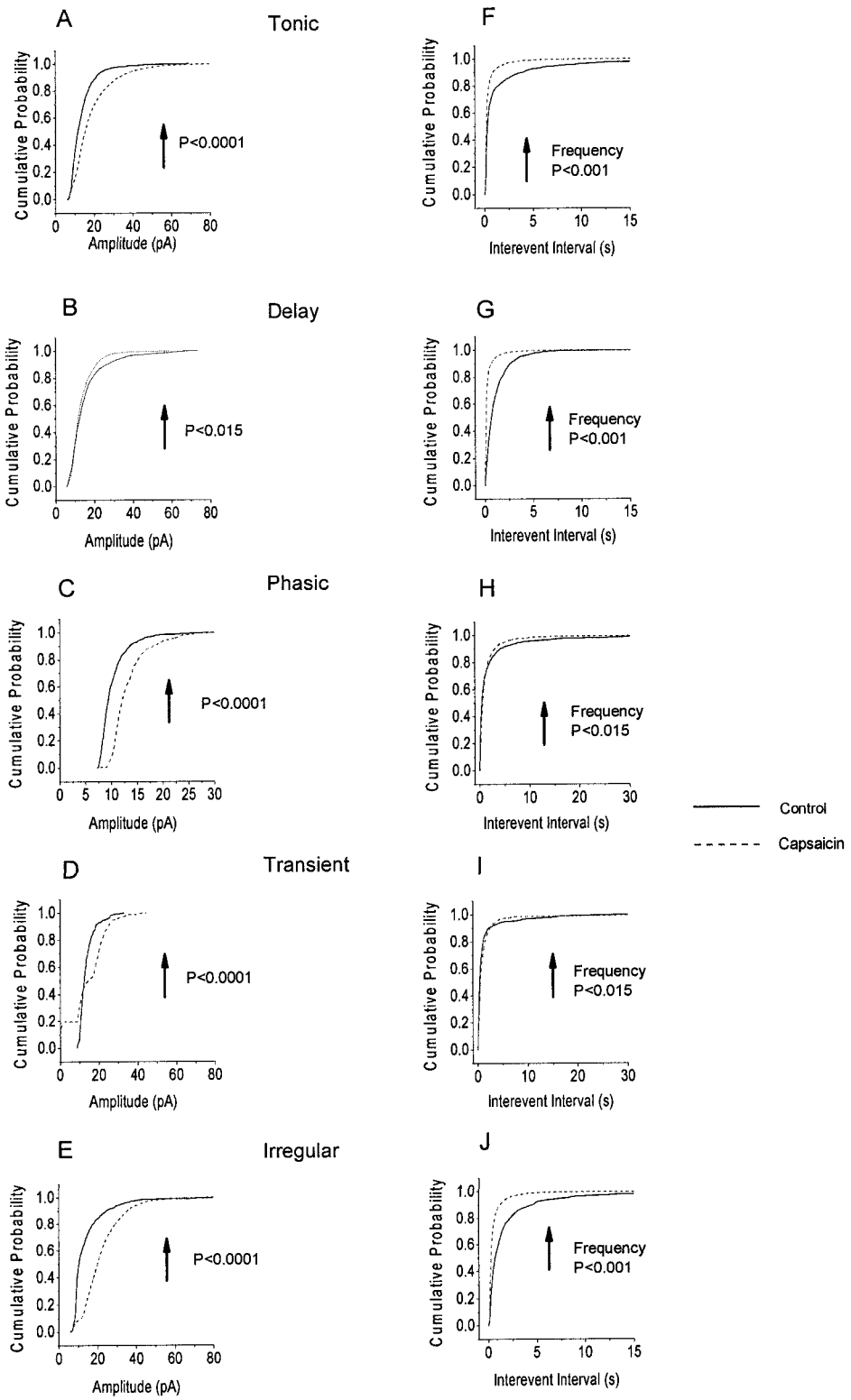


Figure. 3.6. Lack of effect of CCI on current-voltage relationships. **A**. Tonic cells (n = 15 neurons from sham operated animals; n = 17 neurons from CCI animals). **B**. Delay cells (n= 11 neurons from sham operated animals; n=19 neurons from CCI animals). **C**. Phasic cells (n=9 neurons from sham operated animals; n=10 neurons from CCI animals). **D**. Transient cells (n= 9 neurons from sham operated animals; n=13 neurons from CCI animals). **E**. Irregular cells (n= 14 neurons from sham operated animals; n=17 neurons from CCI animals; $P>0.1$ for difference between currents at -140 and 0mV).

Figure. 3.6

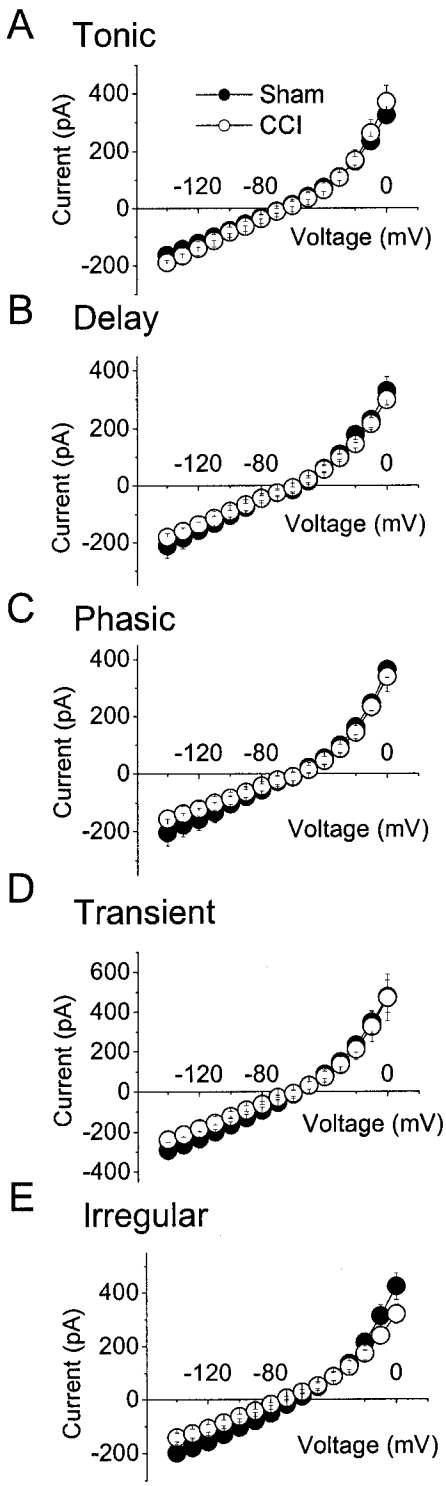


Figure. 3.7. Effect of CCI on membrane excitability measured as cumulative latency of action potential discharge in response to depolarizing current ramps from -60mV. **A-E** left hand panels, sample records of responses of tonic, delay, phasic, transient and irregular cells to depolarizing current ramps. Right hand panels: **A.** Decreased excitability seen in tonic cells after CCI (* $P< 0.05$; ramp rate = 67pA/s; n=33 for sham; n=28-35 for CCI). **B.** Unchanged excitability of Delay cells (ramp rate = 67pA/s; n=13-19 for sham; n=17-25 for CCI) **C.** Increased excitability in phasic cells after CCI (* $P< 0.05$; ramp rate = 67pA/s; n=9-14 for sham; n=14-24 for CCI). **D.** unchanged excitability for transient cells (ramp rate = 100pA/s; n=3 for sham; n=7 for CCI). **E.** Unchanged excitability of irregular cells (ramp rate = 100pA/s; n=8-17 for sham; n=10-22 for CCI). Highest n values are seen for lowest spike numbers, with the exception of transient cells, all other cell types discharged at least 3 spikes in response to the current ramp, but not every cell discharged 8 or 9 spikes. Current, voltage and time calibration in **A** refers to all traces. Solid lines are voltage traces, superimposed dashed lines are corresponding current trace.

Figure. 3.7

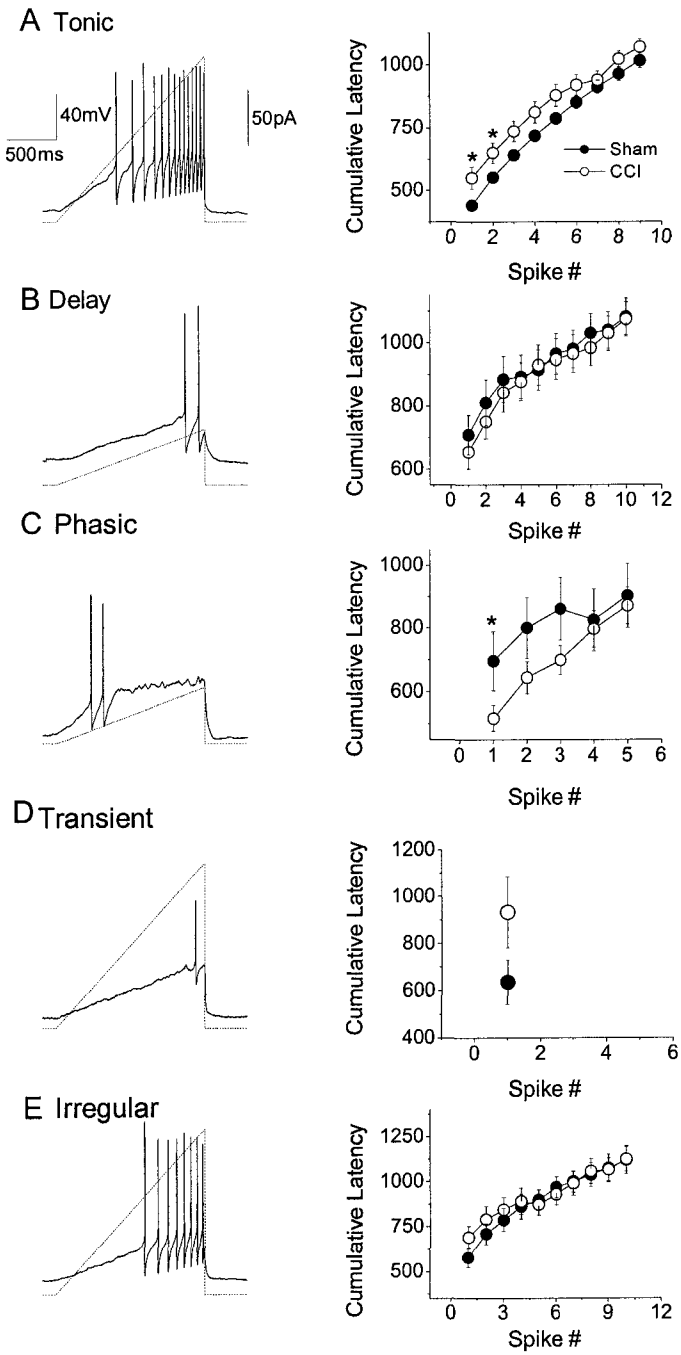


Figure. 3.8. Voltage-dependent conductances activated in *substantia gelatinosa* neurons **A.** Delayed rectifier type K^+ current, **B** Inactivating A- or D-type current **E.** H-current. Relevant voltage recordings are shown in the lower panels of **A, B** and **E.** **C.** Current voltage plots for peak delayed rectifier K^+ current in 8 phasic neurons from sham operated animals and 11 phasic neurons in CCI animals. **D.** Current voltage plots for peak delayed rectifier K^+ current in 4 tonic neurons from sham operated animals and 8 tonic neurons in CCI animals. No significant differences are seen.

Figure. 3.8

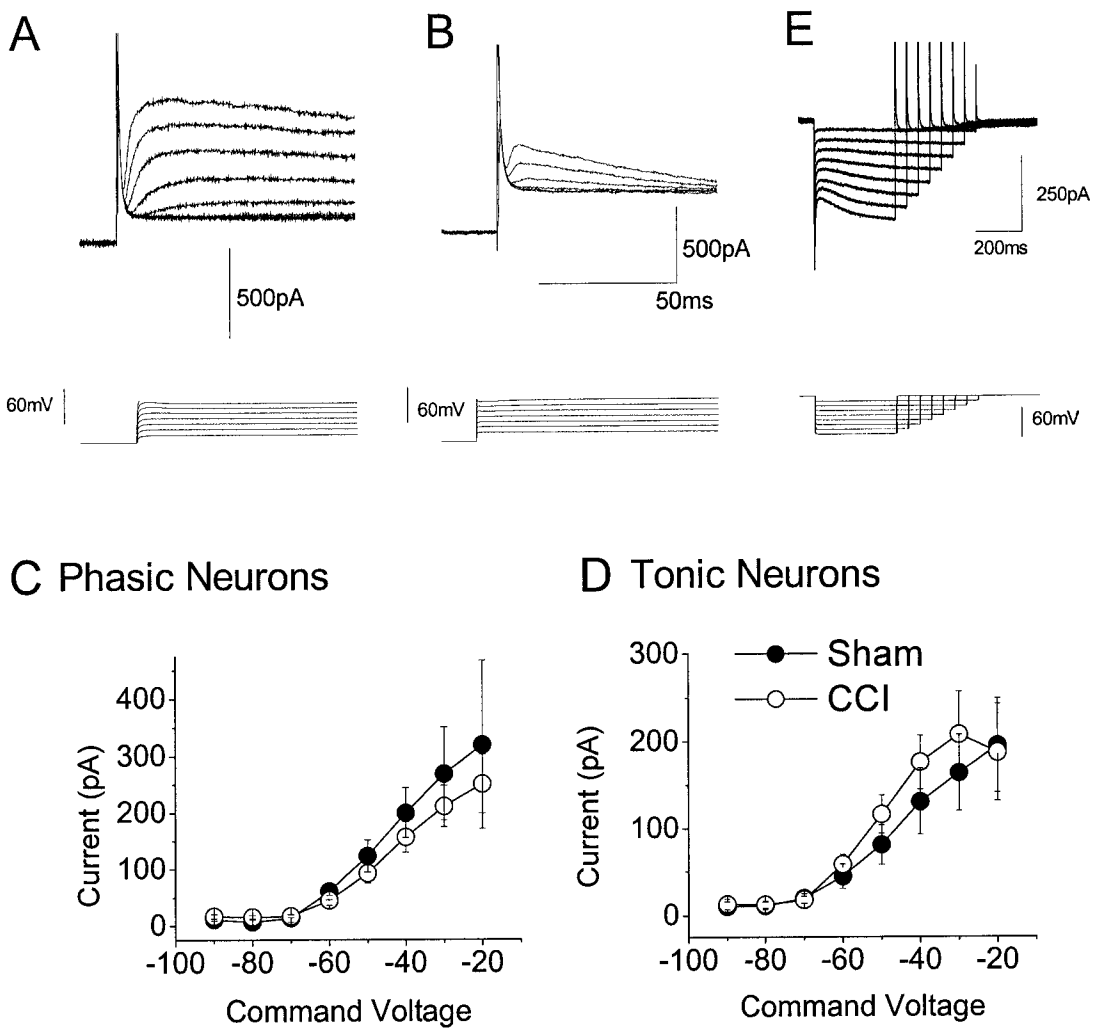


Figure. 3.9. Cumulative probability plots for effects of CCI on amplitude and interevent interval of s.e.p.s.'c and m.e.p.s.c's in *substantia gelatinosa* neurons. The following number of events were analyzed from the first 50 events following 1 min of recording. Tonic neurons; 1700 events from sham operated animals (n=35), 1300 events from CCI animals (n=26). Delay neurons; 1150 (sham) (n=22), 1200 (CCI) (n=23). Phasic neurons; 520 (sham) (n=17), 847 (CCI) (n=15); Transient neurons; 750 (sham) (n=15), 1000 (CCI) (n=20). Irregular neurons; 750 (sham) (n=14), 1000 (CCI) (19). **A-E** and **F-J** cumulative probability plots for sEPSC amplitude and interevent interval from tonic, delay, phasic, transient and irregular cells. P values derived from KS test indicated on graphs. **K-N** and **O-R** cumulative probability plots for mEPSC amplitude and interevent interval from tonic, delay, transient and irregular cells. P values derived from KS test indicated on graphs. Insufficient data were available from phasic cells. The following numbers of mEPSC's were analyzed from the first 30 events at the start of recording. Tonic neurons; 325 events from sham operated animals, 175 events from CCI animals. Delay neurons; 238 (sham), 293 (CCI). Transient neurons; 112 (sham), 256 (neurons CCI). Irregular; 143 (sham), 166 (CCI).

Figure. 3.9

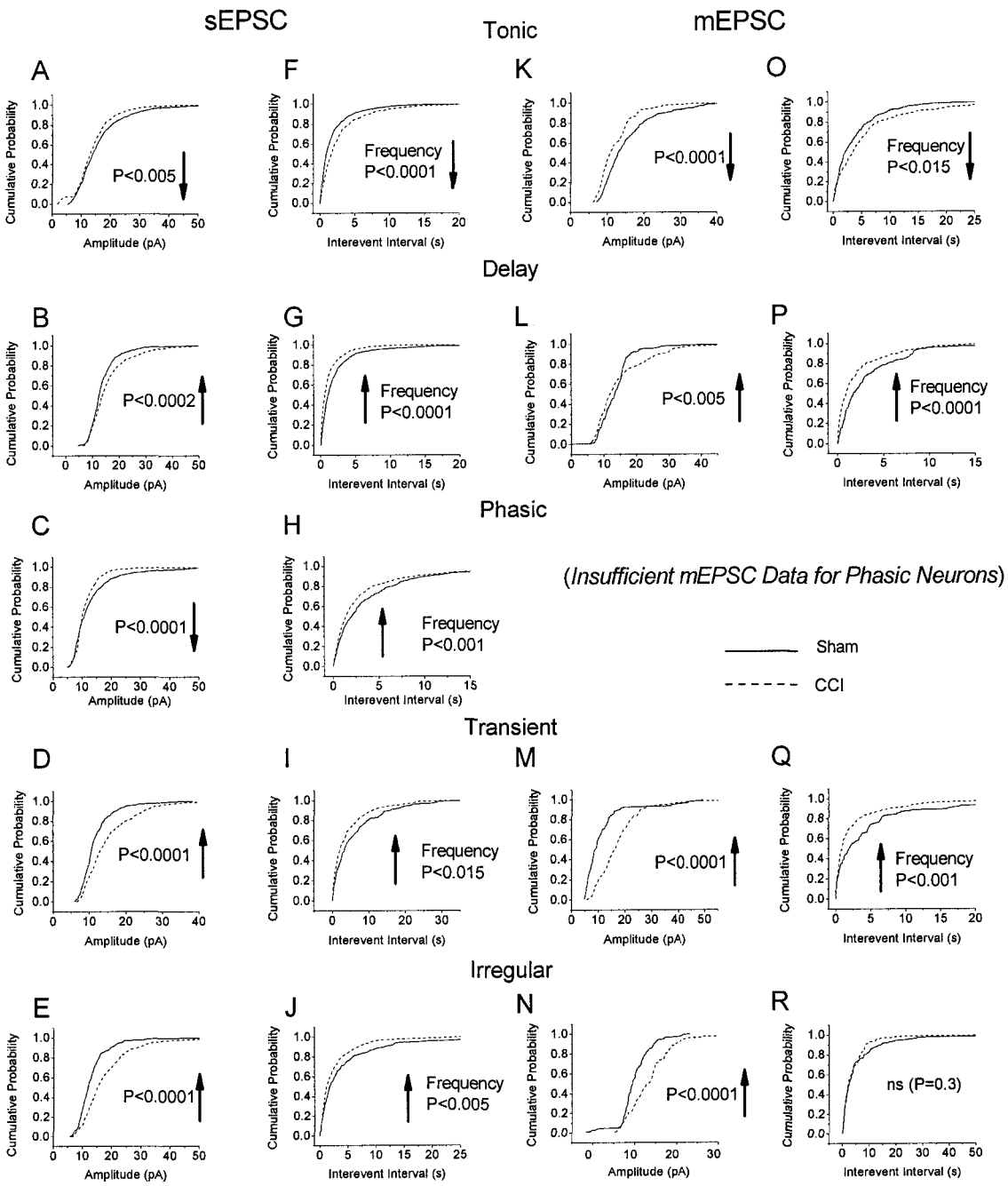
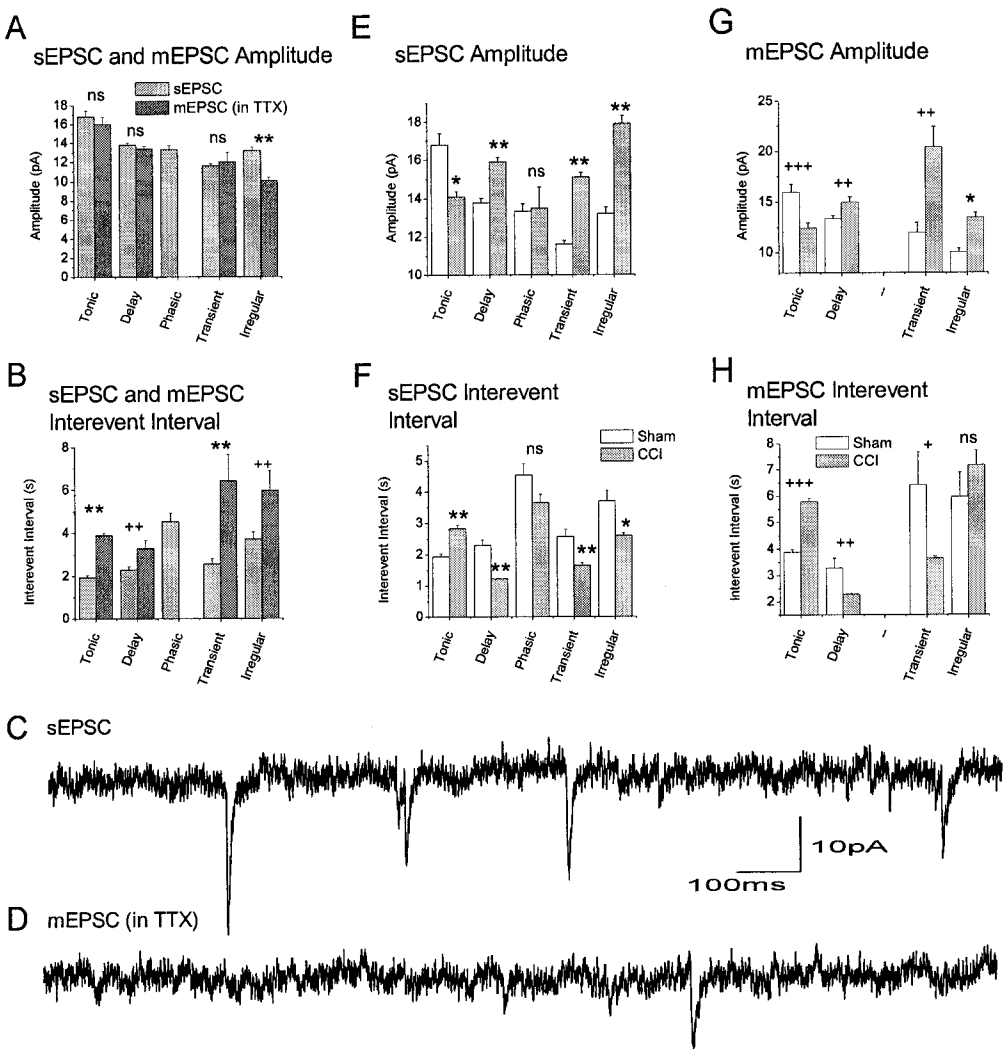


Figure 3.10. A and **B** Comparison of sEPSC and mEPSC amplitude and interevent interval in 'tonic', 'delay', 'transient' and 'irregular' *substantia gelatinosa* neurons from 'sham' operated rats. mEPSC's were recorded in the presence of 1μM TTX and sEPSC's were recorded in its absence. Insufficient data were available for mEPSC's in 'phasic' cells. **(C-D)** Sample traces illustrating the **(C)** sEPSC's and **(D)** mEPSC's**. E-H.** Effects of CCI on average amplitude and interevent interval of (**E** and **F**) sEPSC and (**G** and **H**) mEPSC in rat SG. Insufficient data are available for properties of mEPSC in phasic neurons. For Student's unpaired t-test, **= P<0.0001; *= P<0.001; +++= P<0.002; ++= P<0.02; += P<0.05. Data is a reanalysis of that presented in Figure 3.9 so same n values apply. *i.e.* sEPSC data from 520-1700 events, mEPSC data from 112-325 events.

Figure 3.10



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Chapter 4

Effects of Sciatic Chronic constriction Injury on Morphologically and Electrophysiologically Identified Neurons of Rat *Substantia gelatinosa*

4.1 Introduction

Neurons in the *substantia gelatinosa* comprise a local neuronal network that is involved in processing nociceptive information (Gobel, 1975;Bennett et al., 1980). Several studies have attempted to identify and classify multiple cell types in lamina II based on their morphological and functional characteristics (Gobel, 1975;Grudt and Perl, 2002;Hantman et al., 2004;Kumazawa and Perl, 1978;Light and Trevino, 1979;Light and Trevino, 1979;Lu and Perl, 2005;Prescott and de Koninck, 2002;Graham et al., 2004;Heinke et al., 2004;Ruscheweyh and Sandkuhler, 2002;Ruscheweyh et al., 2004;Todd and Lewis, 1986;Lu and Perl, 2003). Despite this, there is still lack of unequivocal classification scheme for the cells and there are discrepancies in classifying the cells based on their action potential firing properties (Ruscheweyh and Sandkuhler, 2002;Grudt and Perl, 2002;Melnick et al., 2004). Moreover, studies suggest that significant percentage of neurons do not fit readily recognizable class (Todd and Lewis, 1986;Todd, 1988;Grudt and Perl, 2002). Refer to Table 1.1 and Figure 1.1 for different neuronal types in the *substantia gelatinosa.*

Peripheral nerve injury in animal models invokes symptoms that resemble human neuropathic pain. This is often associated with increase in dorsal horn excitability commonly termed 'central sensitization'. Even though several studies have identified multiple mechanisms responsible for such change, it is still uncertain how the peripheral nerve injury alters the properties of morphologically and electrophysiologically identified cells types. In addition to modulating the transfer of noxious information from the periphery, *substantia gelatinosa* neurons also participate in processing of innocuous

tactile, itch and temperature information (Tuckett and Wei, 1987b;Tuckett and Wei, 1987a;Vallbo et al., 1999;Perl, 1984;Sugiura et al., 1986;Light and Perl, 2003). The intracellular studies and *in vivo* recordings in the superficial dorsal horn have suggested that there is no obvious relationship between response of these cells to various sensory modalities and their morphological or firing properties (Woolf and Fitzgerald, 1983;Rethelyi et al., 1989;Bennett et al., 1980;Graham et al., 2004). Several recent studies have pointed out that there are lamina specific differences in the properties of neurons of the superficial dorsal horn (Ruscheweyh and Sandkuhler, 2002;Grudt and Perl, 2002;Light and Perl, 1979a;Woodbury et al., 2000;Todd and Spike, 1993). Moreover, the *substantia gelatinosa* is further subdivided the lamina II into outer and inner divisions. The outer lamina contains mainly the nociceptive neurons and umyelinated C fiber terminals (Light and Perl, 1979a) (Woodbury et al., 2000) while non-noxious mechanoreceptive cells are located in inner lamina II (Light and Willcockson, 1999).Thus, nerve injury may produce different changes in outer lamina II neurons versus the neurons in inner lamina II region. Because *substantia gelatinosa* is a complex network system, any changes in properties of both nociceptive and/or non-nociceptive cells would have an impact on the modulation and hence the output information to higher centers. In the previous chapter I have shown that nerve injury produces a cell type specific pattern of changes in electrophysiologically identified cells types of *substantia gelatinosa*. Earlier observations on the correlation between the neuronal morphology and firing properties of *substantia gelatinosa* neurons have been inconclusive (Grudt and Perl, 2002;Heinke et al., 2004;Melnick et al., 2004). Hence, in this chapter I have further correlate the firing properties of cells with the morphological characteristics. This would

help further to demonstrate that nerve injury decreases the excitability of possible tonic inhibitory neurons whereas increase the excitability of putative excitatory vertical and or radial cells which display a delayed firing pattern.

4.2 Methods

The methods used in this chapter are identical to those described in chapter 2.

4.3 Results

4.3.1 Morphological Characterization of the *Substantia Gelatinosa* Neurons

Earlier studies by Grudt and Perl have shown that there are no significant variations in the soma size between the neurons in lamina II,(Grudt and Perl, 2002) hence the *substantia gelatinosa* neurons were classified according the predominant orientation of the dendrites and their location within the lamina II region. Based on the dendritic features, the neurons were categorized into three morphological types: Islet, vertical and radial neurons. PKCγ-immunoreactivity has been found in a restricted population of small neurons which are concentrated in the inner ventral part of lamina II (IIi) (Malmberg et al., 1997;Martin et al., 1999;Mori et al., 1990). Hence, based on the position of the biocytin stained soma in relation to PKCγ containing cells, I classified the neurons as present in IIo or IIi or on the border (Figure 4.1). It must be noted that I used transverse slices as compared to both transverse and parasagittal slices in earlier studies (Grudt and Perl, 2002;Lu and Perl, 2003). The dendritic orientation of islet cell can be best viewed by parasagital slices. To circumvent this problem, the serial confocal images of each neuron were projected in different planes, to obtain a 3D reconstruction of

different morphological types of biocytin stained neurons. Thus each cell was viewed in frontal (transverse) plane, Left (parasagittal) plane and bottom (horizontal) plane to identify the predominant dendritic orientation. The passive membrane properties such as the resting membrane potential and input resistance were not statistically significant between the three morphologically identified groups of neurons. As reported in Chapter 3, the response features of the cells in response to series of depolarizing steps cells from a holding potential of -60 mV could be of tonic, delay, irregular, transient or phasic type.

4.3.1.1 Islet Neurons

Our islet cells had similar features to the islet cells described by other studies (Grudt and Perl, 2002;Light and Trevino, 1979;Lu and Perl, 2003;Gobel, 1975). The dendrites were dense near the cell body and spread predominantly in the rostrocaudal direction with limited spread in other planes (Figure 4.2). The dendritic arbors were restricted within and rarely reached beyond lamina II. Note that the rostrocaudal projection was clearer in the parasagittal plane than in the transverse plane. In some cases the dendrites after traveling a certain distance in rostrol or caudal direction curved back towards the soma and extend beyond in other direction. I noticed some cells had shorter dendritic extension than others and hence could be a subcategory of islet cells as reported by Grudt and Perl as central neurons. Based on the relative location of the soma in relation to the PKC γ containing cells, islet neurons were found throughout the entire lamina II (Table 4.2).

Most of the islet cells (60.8%) showed sustained or tonic action potential firing pattern in response to series of depolarizing current steps from holding potential of -60mV. The

second most prominent firing pattern exhibited by the islet cells (17.3%) is phasic or intial bursting type. Only a smaller percentage of cells showed delay, transient or irregular pattern of firing (Table 4.1). Islet cells also exhibited spontaneous EPSC's from -70mV with a lower frequency when compared to vertical or radial neurons (Figure 4.7A).

4.3.1.2 Vertical neurons

Vertical neurons had distinctive dorsoventral dendritic orientation with relatively limited spread in mediolateral plane. In most cases, the ventral projections were much longer and dense than the dorsal counterpart. Even though dendrites extended in rostocaudal plane it was less dense and much shorter than islet neurons (Figure 4.3). Some of the neurons also had stalked feature described by Gobel, however it cannot be attributed to all the vertical neurons.

The soma of the vertical neurons was present either on the lamina IIo (8/13) or on the inner-outer border (5/13). In my sample of neurons I never found a vertical neuron with cell body within inner lamina II (Table 4.2). In the neurons whose soma was located on IIo, dendrites could be traced as deep as lamina III or IV, when compared to other neurons which had relatively shorter ventral spread (Figure 4.3).

The majority of vertical cells (77%) fired in a sustained and regular manner after a significant delay at the start of the depolarizing pulse. The delay became shorter with more positive depolarizing steps. About 15.3 % of cells showed tonic firing. I never

observed transient or phasic firing pattern in vertical cells, while very small percentage (7.6%) exhibited irregular pattern of firing (Table 4.1). The vertical neurons exhibit a high frequency sEPSC's at -70mV (Figure 4.7A).

4.3.1.3 Radial Neurons

Radial cells had a round soma and dendrites that radiated in all directions giving a wheel like appearance (Figure 4.4). The radial configuration was clear from the bottom (horizontal) view. These cells were similar to the 'stellate' or star shaped neurons described by other studies (Bicknell, Jr. and Beal, 1984;Grudt and Perl, 2002;Schoenen, 1982). The soma of most of the radial neurons was located in the IIo or on the border (Table 4.2).

A great majority of radial neurons (89.4%) exhibited action potentials after a prominent delay at the onset of the depolarizing current commands (Table 4.1). However unlike vertical neurons, the firing was generally irregular after the delay with variations in spike amplitude. Radial cells also showed high frequency sEPSC's (Figure 4.7A).

4.3.1.4 Other cells:

A total of 32 cells could not be classified into any of the above three categories as they showed atypical morphological features. In some cells, even though the morphology was clear could not be included in the analysis because on unclear firing pattern.

4.3.2 Changes in the Properties of the Cells after Nerve Injury

Correlation of morphological features with the firing properties suggests each morphological class contains a heterogeneous mixture of firing patterns. Hence, to examine any changes in the properties of morphologically defined cell types after nerve injury only the predominant firing pattern within each morphological class was undertaken. Thus the cells were classified as 'islet tonic,' 'vertical delay' and 'radial delay' neurons.

4.3.2.1. Changes in the Membrane Excitability

The resting membrane potential (r.m.p) and the input resistance were not altered in any of the cell types after CCI. While the rheobase of the 'islet tonic cells' was significantly increased after CCI, the 'vertical delay' or the 'radial delay' cells did not show any significant change (Table 4.3). These results suggest a decrease in membrane excitability of 'islet tonic cells' after CCI. Figure 4.5 illustrates the effect of depolarizing current ramps on action potential discharge in each of the three defined cell types. As described in chapter 3 the ramp rates chosen were steep enough to evoke robust firing in most cells tested without producing depolarization block at their most positive excursions. The membrane excitability of islet tonic, vertical delay and radial delay cells were unchanged.

4.3.2 .2. Changes in Spontaneous Excitatory Synaptic Activity

The effects of CCI on the sEPSC amplitude and interevent interval are shown in Figure 4.6. As described in the methods chapter, the figures were obtained for first 50 events after one minute of baseline stabilization. In islet tonic cells, CCI caused a reduction in

both the frequency and the amplitude on the spontaneous EPSC's. On the other hand, in vertical delay and radial delay neurons the frequency and the amplitude of the sEPSC's were increased after CCI. All the results were statistically significant according to Kolmogorov-Smirnoff test. Some neurons failed to display a detectable event during the 3 min sampling period and were hence classified as silent neurons. As shown in our earlier chapter, proportion of silent neurons were not statistically significant between the sham and cuff, hence it is highly unlikely that they would have introduced any bias in our results. I also compared the mean amplitude and frequency of the sEPSC's with more rigorous t test which complemented the conclusions from the KS test (Figure 4.7).

4.4 Discussion

Our morphological studies show that there are at least three distinct categories of neurons in the *substantia gelatinosa*. These are defined as islet, vertical and radial neurons, based on the predominant dendritic orientation. On correlation with previously defined firing pattern suggest, a majority of islet neurons display a tonic firing pattern while vertical and radial neurons show delay firing pattern. Our results are in consensus with previous reports on morphological and firing characteristics of the *substantia gelatinosa* neurons (Grudt and Perl, 2002;Lu and Perl, 2003;Lu and Perl, 2005).

As mentioned in the general introduction (1.5.1), information related to nociception and pain is conveyed into the *substantia gelatinosa* by two different categories of unmyelinated primary afferent fibers (C-fibers) (Hunt and Rossi, 1985). Peptide containing fibers project mainly to the outer lamina II (Averill et al., 1995;Stucky and

Lewin, 1999). The non-peptidergic isolectin B4 binding fibers terminate predominantly in inner lamina II (Alvarez and Fyffe, 2000;Vulchanova et al., 1998). Since islet cells are located throughout the lamina II region they are ideally suited to receive input from both class of C-fibers. The restricted dendritic branching within lamina II would enable these neurons to function as confined local interneurons. Some of these specialized features and their similarities with Golgi type II neurons have implied their role as inhibitory interneurons (Gobel, 1978;Lu and Perl, 2003;Todd and Lewis, 1986;Todd and Spike, 1993). However, limited dorsoventral branching imply their limited ability to communicate with more superficial or deeper layers. The vertical neurons with their extensive dorsoventral projections are ideally suited to convey or receive information across various laminae. These vertical neurons correspond to the 'stalked' cells that are believed to be excitatory interneurons (Todd and Lewis, 1986;Gobel, 1975;Grudt and Perl, 2002;Lu and Perl, 2005). The simultaneous pair recording suggests that these are the output neurons to lamina I being modulated by the islet and central neurons of lamina II (Lu and Perl, 2005). Because the radial cell project in all directions they could receive and convey information through different planes. Their location in predominantly outer lamina II suggests their capability to communicate with lamina I neurons, but limited ventral projection would restrict their access to deeper layers. As pointed out in the earlier section the outer part of lamina II comprise mainly nocicieptive cells while majority of afferent input to inner lamina II is non-nociceptive (Light and Willcockson, 1999;Light and Perl, 1979b). Hence those islet cell on the outer layer could be nociceptive or wide dynamic range neurons (Bennett et al., 1980) while those on the inner lamina II could belong to low threshold mechanoreceptive type (Bennett et al.,

1998;Pan and Pan, 2004;Light and Perl, 1979b). Since vertical and radial neurons are predominantly located on the outer part they could be mainly nociceptive neurons.

The previous findings of Grudt and Perl suggest that the rostrocaudal neurons could be further classified into long islet cells and short central cells in sagittal sections based on the extent and dimensions of the dendrites (Grudt and Perl, 2002). Even though I noticed some cells had short rostrocaudal extension, it was not always possible to trace the whole length of dendritic tree in transverse slices, so I decided to combine both categories of cells. Hence some of my islet cells could infact be the central cells defined by Grudt and Perl. However, Grudt and Perl suggest that the majority of central cells discharge in phasic or transient pattern (Grudt and Perl, 2002;Lu and Perl, 2003;Lu and Perl, 2005). Hence to minimize the contribution from the central cells (may be excitatory) I decided to examine the effect of nerve injury on only the islet tonic neurons. Since the delay neurons could be further classified into vertical and radial neurons based on their morphological features, the effects of CCI were studied separately on these two different populations.

The CCI induced changes in the membrane excitability of morphologically and electrophysiologically defined neurons are at odds with the neurons described solely on firing properties in chapter 3. CCI increased the rheobase of islet tonic neurons indicating a decreased excitability (Table 4.3) without affecting the rheobase of tonic cells (Table 3.1). Also CCI increased the cumulative latency of first few spikes in response to current ramps in tonic neurons (Figure 3.7), but did not show a significant change in islet tonic neurons (Figure 4.5). Because the sample size used for estimation of excitability in

Figure 4.5 is small, at the moment it is difficult to attribute any significance to these findings.

CCI resulted in a reduced excitatory synaptic drive to islet/tonic while an increased drive to vertical/delay and radial/delay neurons. This supports the conclusions of the earlier chapter where neurons where exclusively characterized based on firing properties The decrease in sEPSC frequency seen in 'islet tonic' cells after CCI (Figure 4.6) may reflect alterations in the transmitter release process because, as already mentioned in the previous chapter (Figure 3.10B), mEPSC account for at least 50% of the spontaneous events recorded in these cells. Also, it has recently been shown that 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). If these fibers selectively innervate islet tonic cells, their loss may explain the decrease in their excitatory synaptic drive.

Recently Lu and Perl, have demonstrated functional correlation of neurons through recording from synaptically connected neuronal pairs in *substantia gelatinosa* (Lu and Perl, 2003;Lu and Perl, 2005). Thus, their studies suggest that islet tonic neurons could be inhibitory releasing GABA upon stimulation, and vertical delay neurons being excitatory releasing glutamate upon stimulation (Figure 4.8). By viewing our results, from the perspective of findings from Lu and Perl suggests, after CCI, the putative excitatory interneurons (delay cells, see Lu and Perl, 2005) become more excitable. By contrast, inhibitory neurons (islet tonic cells, see Hantman et al., 2004; Lu and Perl 2005) become

less excitable. Our classification therefore allows us to relate our findings those of others who are actively investigating dorsal horn physiology. However, as suggested by Lu and Perl, this pathway could be just one of the many pathways that exist in complex circuitry of *substantia gelatinosa* (Lu and Perl, 2003;Lu and Perl, 2005). Hence, at this juncture I could only speculate on the impact of CCI on different types neurons based on its functional features.

As mentioned in the general introduction, neurons of *substantia gelatinosa* differ in their immuno-histochemical profiles and expression of different peptides (Spike and Todd, 1992;Todd and Spike, 1993). These neurochemical markers such as calbindin or GAD (65 or 67) are used to identify excitatory and inhibitory neurons respectively (Gamboa-Esteves et al., 2001b;Gamboa-Esteves et al., 2001a;Antal et al., 1991;Heinke et al., 2004). Hence, future studies targeting the co-localization of morphologically and electrophysiologically characterized neurons with immuno-histochemical markers would help to further demonstrate the impact of nerve injury on the properties of *substantia gelatinosa* neurons. These findings would enhance our current understanding on the pathophysiology of neuropathic pain.

Table 4.1: Relationship between the morphology and firing properties of the cells in *substantia gelatinosa.*

Morphological class	**Tonic**	**Phasic**	**Delay**	**Irregular**	**Transient**	**Total**
Islet	**60.8%**	17.3%	8.6%	6.5%	4.3%	46
Vertical	15.3%	-	**77%**	7.6%	-	13
Radial	-	-	**89.4%**	-	5.2%	19
Unclassified or unclear						32

Table 4.2: Relationship between the morphology and the location of the cells in *Substantia Gelatinosa.*

Morphological Class	Location based on PKC γ staining		
	Outer lamina II	**Inner lamina II**	**Border**
Islet	14/46	11/46	21/46
Vertical	8/13	0/13	5/13
Radial	10/19	2/19	7/19

Table 4.3. Effects of chronic constriction injury (CCI) on rheobase and resting membrane potential (r.m.p.) of various types of *substantia gelatinosa* neurons. * Significant ($P<0.02$, Students's unpaired t-test) increase in the rheobase of islet/tonic cells after CCI. Values represented as Mean ± SD

Morphological class	RMP		Rheobase	
	Sham	Cuff	Sham	Cuff
Islet tonic cells	-53.84 ± 10.80 (n=17)	-56.76 ± 10.49 (n=15)	18.6 ± 2.69 (n=17)	29.61 ± 3.23* (n=15)
DV delay cells	-54.22 ± 11.09 (n=10)	-49.66 ± 6.76 (n=8)	40 ± 28.14 (n=10)	45 ± 25.17 (n=8)
Radial delay cells	-52.41 ± 5.16 (n=12)	-57.12 ± 6.54 (n=10)	69.5± 29.44 (n=12)	69.71 ± 20.99 (n=10)

Figure 4.1: PKC γ staining to visualize the location of biocytin filled neurons in the outer or inner lamina II. Arrows indicate the biocytin stained neurons (red) located on **(A)** outer **(B)** inner lamina II relative to PKC γ containing cells (green).

Figure 4.1

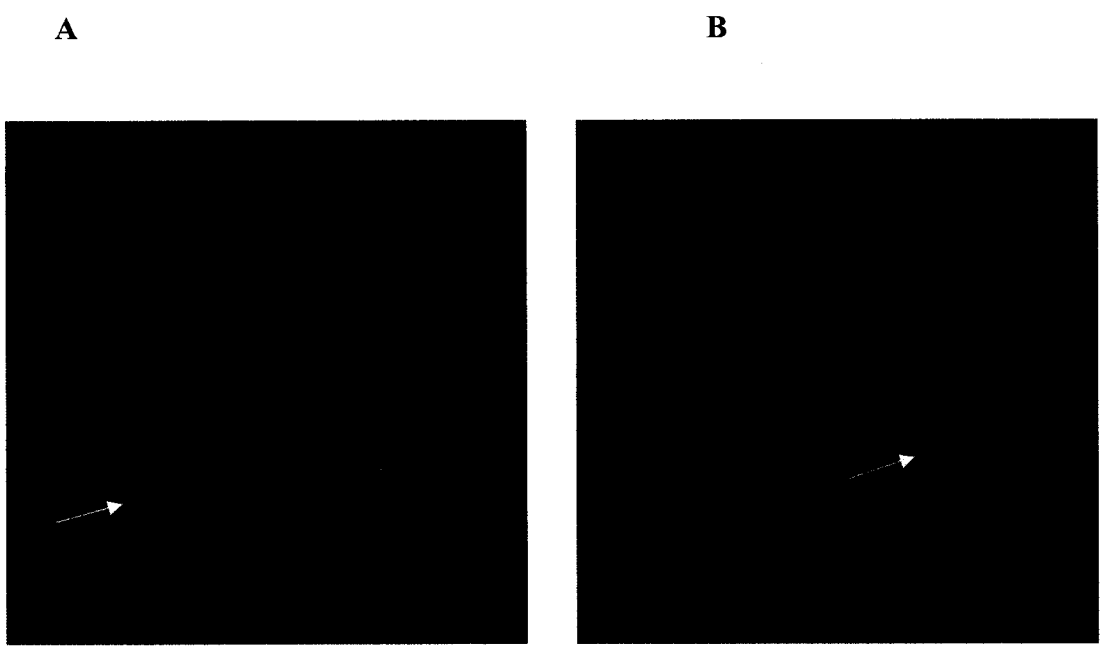
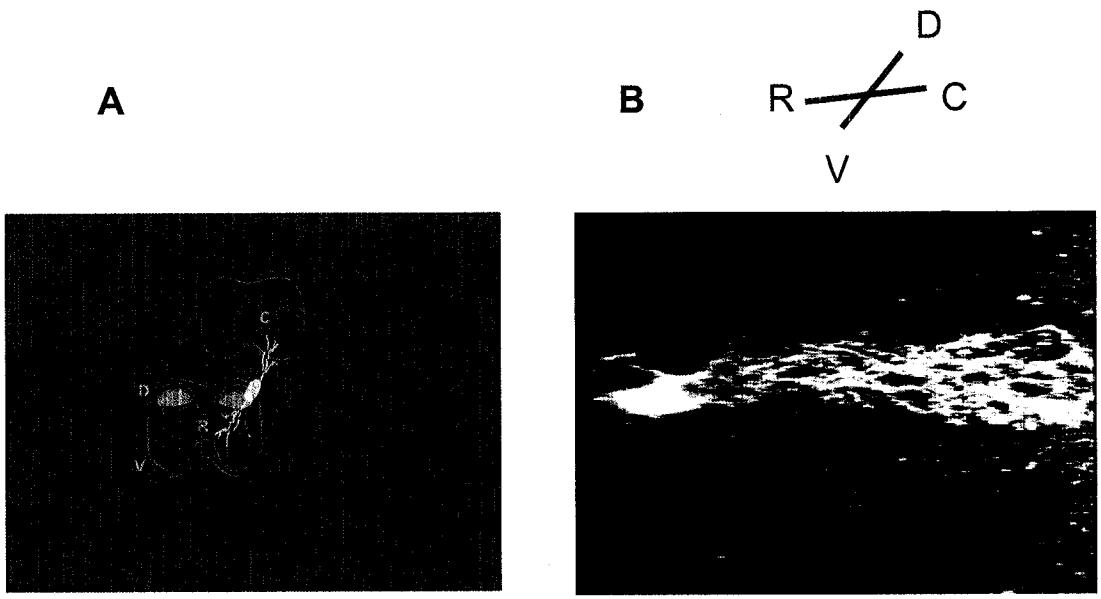


Figure 4.2: Islet cell, **(A)** cartoon representing the predominant dendritic orientation of the islet cell. Maximum projection confocal image of biocytin-labeled islet neuron from **(B)** Parasagittal view and **(C)** Transverse view, showing predominant projection in rostrocaudal plane. The dendritic spread is restricted within the lamina II, a characteristic of islet cell. **(D)** The predominant tonic firing pattern of islet cells in response to depolarizing current steps.

Figure 4.2



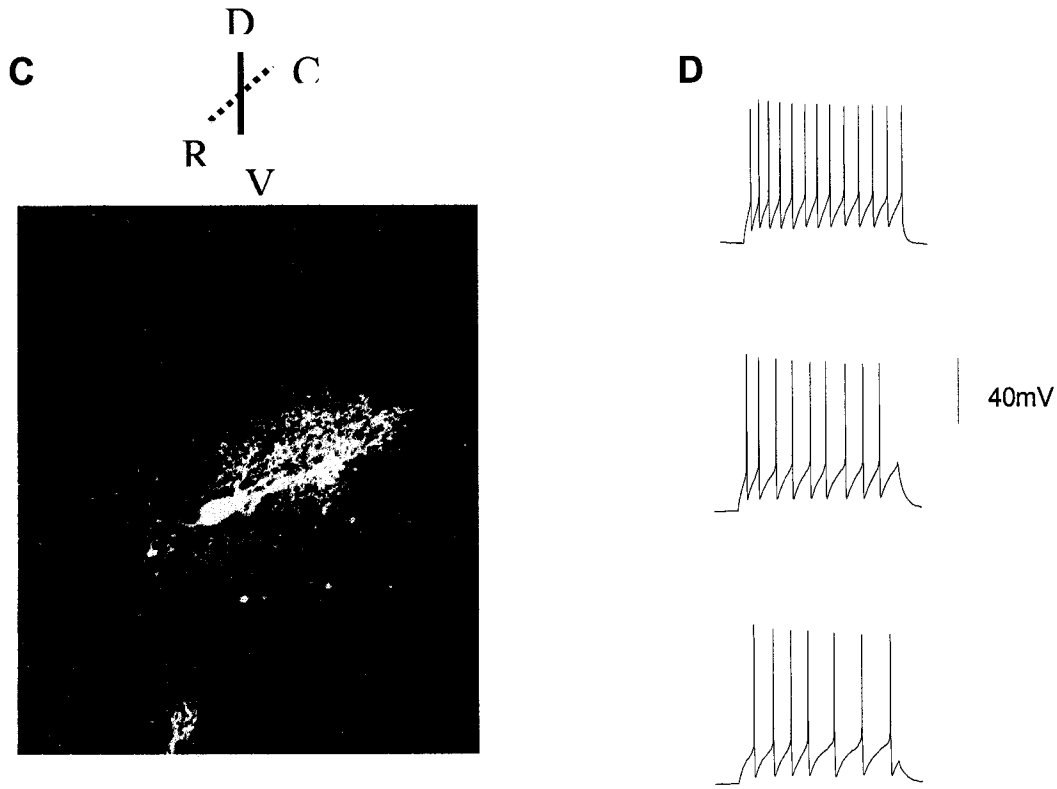


Figure 4.3: Vertical cells, **(A)** Cartoon representing the predominant dendritic orientation of vertical neurons. **(B)** Maximum projection confocal image of biocytin-labeled vertical neuron present on the outer lamina II from transverse view showing predominant projection in dorsoventral plane. Note the long ventral projection reaching into deeper layers **(C)** Maximum projection confocal image of different biocytin-labeled vertical neuron. Note the pyramidal or cone shape of the soma with stubby dendritic branching, but much shorter ventral extension than the previous cell. **(D)** The predominant delay firing pattern of vertical cell in response to depolarizing current steps.

Figure 4.3

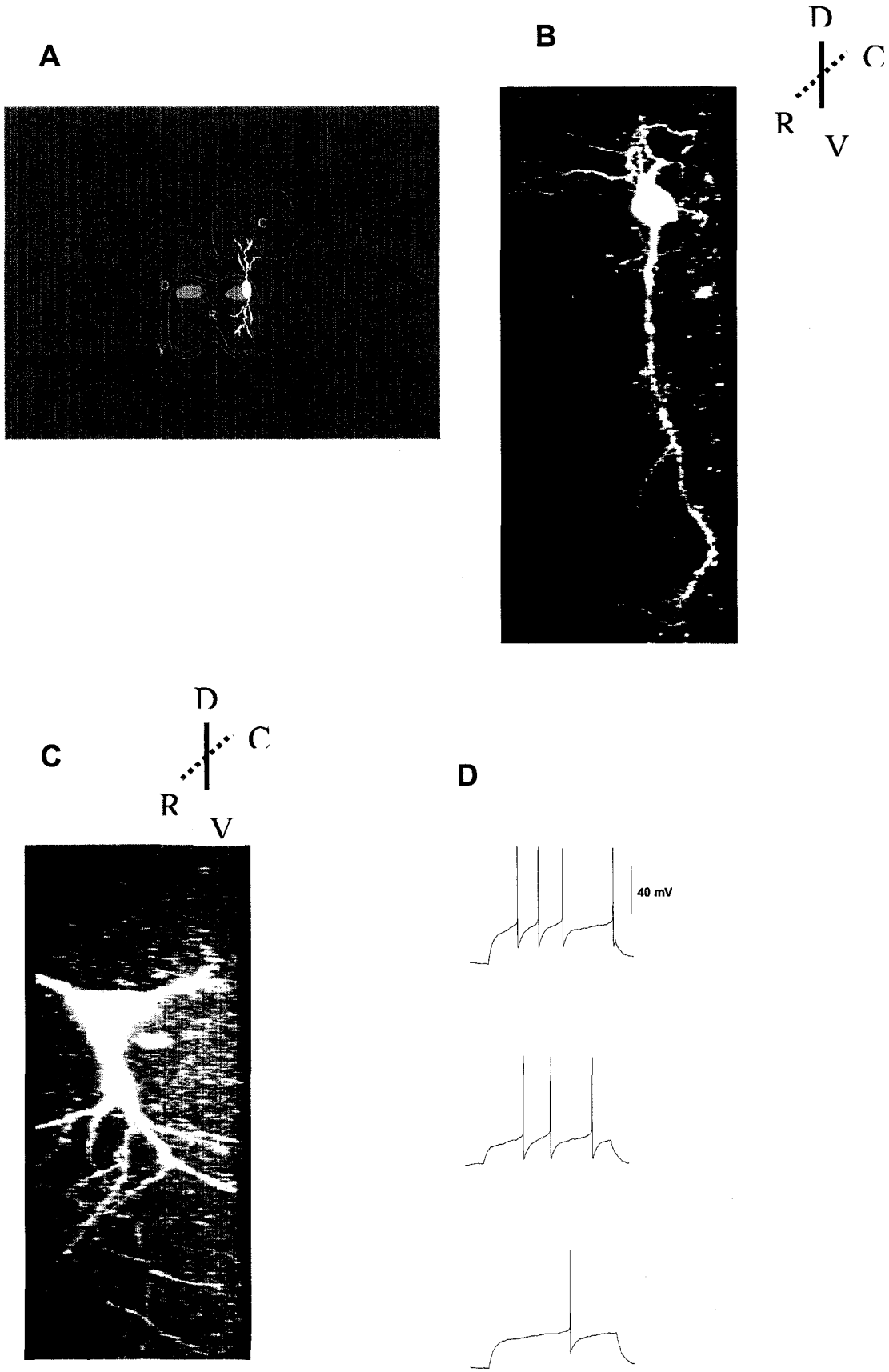


Figure 4.4: Radial cell, **(A)** Cartoon representing the predominant dendritic orientation of radial neuron. Maximum projection confocal image of biocytin-labeled radial neuron from **(B)** Transverse view **(C)** Parasagittal view and **(D)** Horizontal view showing dendritic projections in all directions. **(E)** The predominant delay firing pattern of radial cell in response to depolarizing current steps. Note the irregularity in the firing pattern compared to vertical delay cell.

Figure 4.4

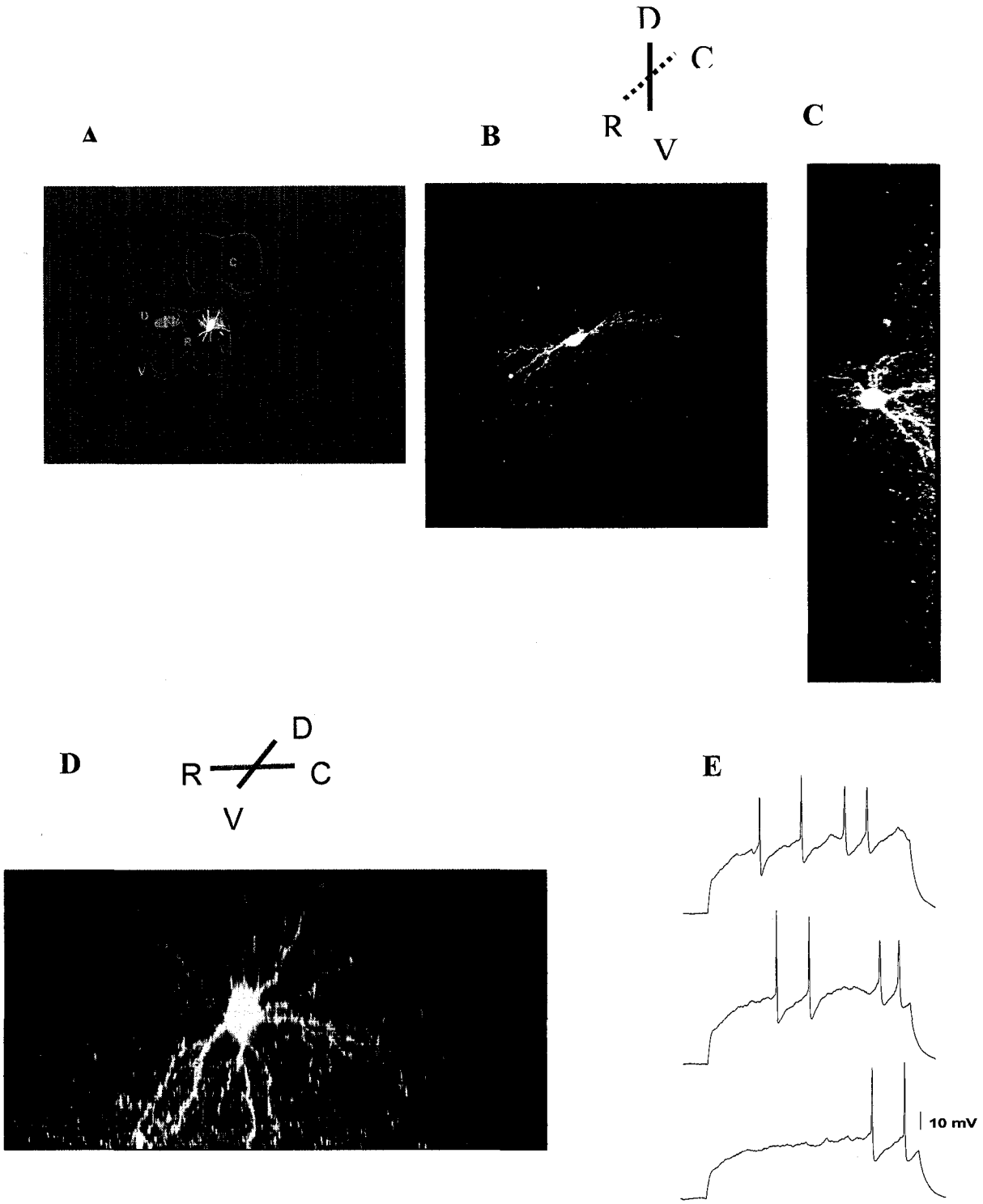
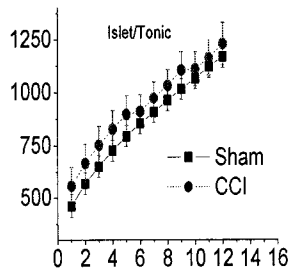


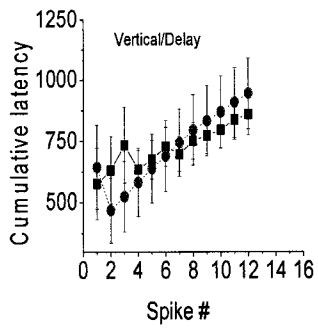
Figure. 4.5. Effect of CCI on membrane excitability measured as cumulative latency of action potential discharge in response to depolarizing current ramps from -60mV. Unchanged excitability seen in **(A)** islet tonic cells (n=8 for sham; n=7 for CCI), **(B)** vertical delay cells (n=7 for sham; n=4 for CCI) and **(C)** radial delay cells (n=7 for sham; n=4 for CCI) after CCI. No significance ($P>0.05$) was found, Student's unpaired t-test.

Figure. 4.5

A



B



C

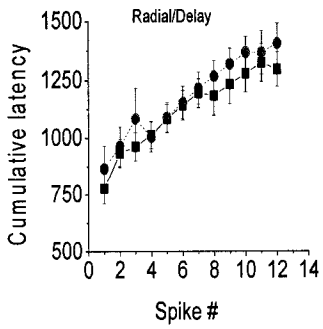


Figure 4.6: Cumulative probability plots for effects of CCI on amplitude and interevent interval of s.e.p.s.'c in morphologically and electrophysiologically identified *substantia gelatinosa* neurons. The following total numbers of events were acquired by analysis of the first 50 events following 1 min of recording. 'Islet/tonic' neurons; 520 events from sham (n=11), 323 events from CCI animals (n=11). 'Vertical/delay'; 309 (sham) (n=6), 407 (CCI) (n=8). 'Radial/delay'; 301 (sham) (n=6) 453 (CCI) (n=9). **A-C** and **D-F** cumulative probability plots for sEPSC amplitude and interevent interval from 'islet/tonic', 'vertical/delay' and 'radial/delay' cells. P values derived from KS test are indicated on graphs.

Figure 4.6

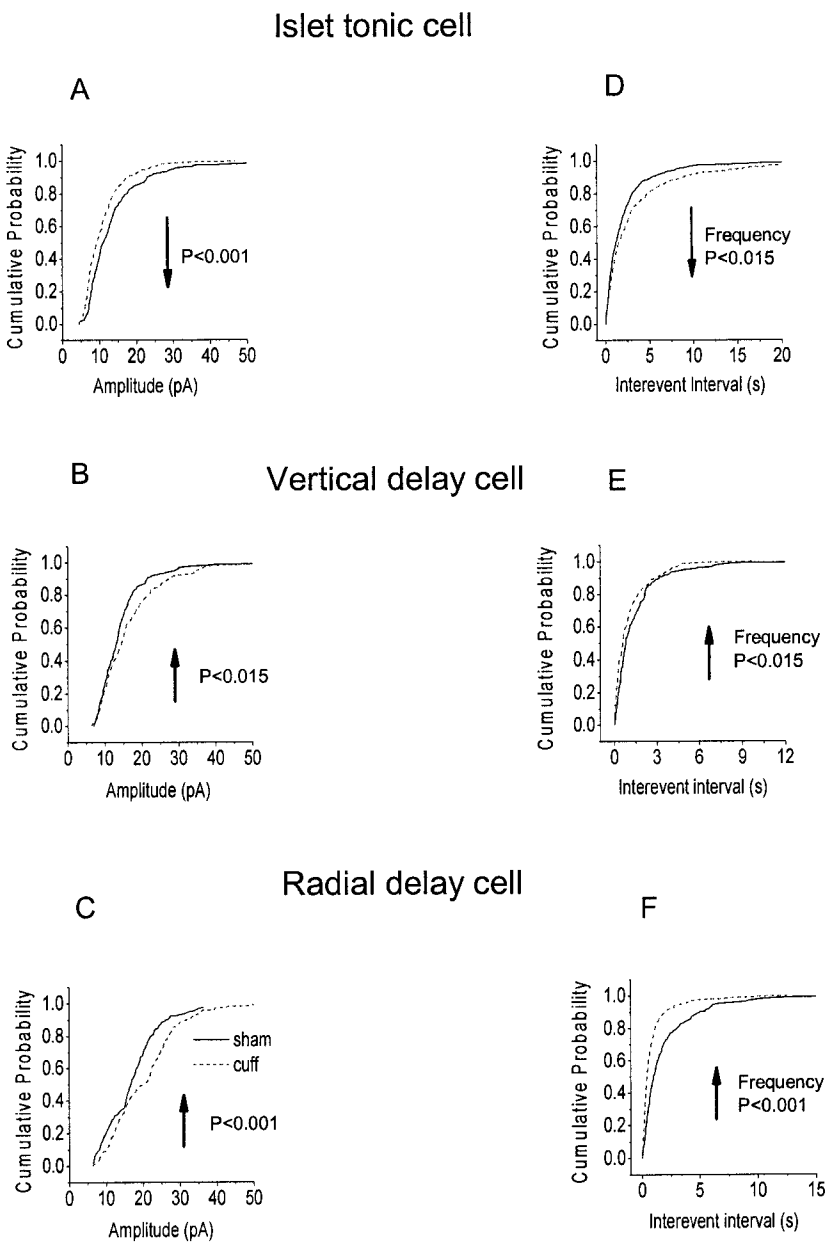


Figure 4.7: A and **B** Effects of CCI on average amplitude and interevent interval of sEPSC in 'islet/tonic', 'vertical/delay' and 'radial/delay' cells of *substantia gelatinosa* . For Student's unpaired t-test, **= P<0.0001; *= P<0.001; ++= P<0.002, += P<0.05. Same n values as figure 4.6.

Figure 4.7

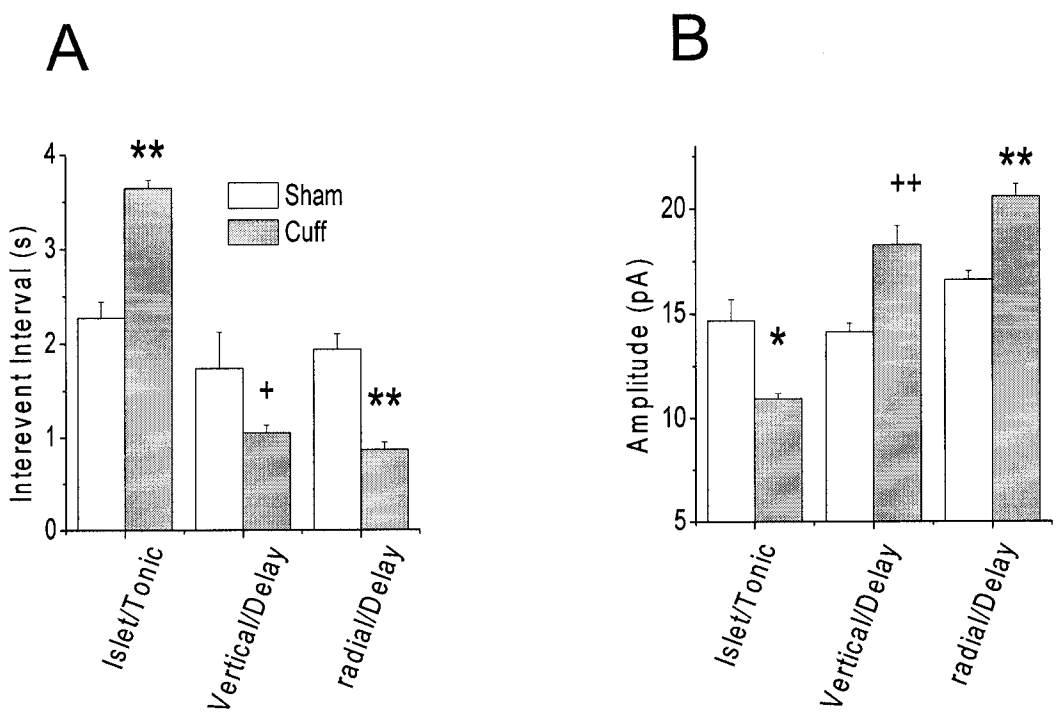
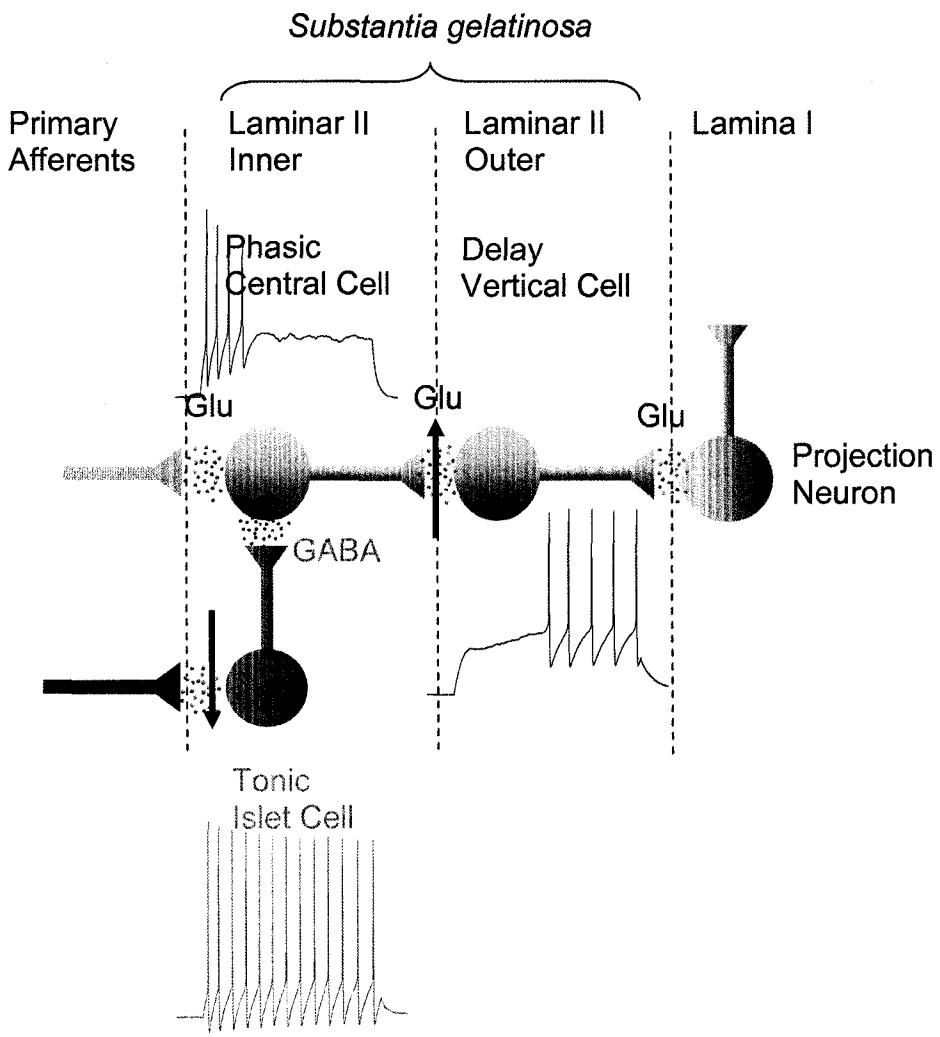


Figure. 4.8: A pathway in the superficial dorsal horn describing the communication between different types of neurons based on the whole-cell recordings from synaptically connected pairs (Lu and Perl, 2003;Lu and Perl, 2005). The arrows indicate decreased excitatory synaptic drive for islet/tonic cell and increased drive for vertical/delay cell after CCI.

Figure. 4.8



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Chapter 5

L-type calcium channels and Neuropathic Pain

5.1 Introduction

Calcium influx through voltage-dependant calcium channels regulates many neuronal processes including neurotransmitter release, neuronal excitability, protein phosphorylation, gene expression and neuronal plasticity such as long term potentiation and depression, (Miller, 1987;Augustine et al., 1987;Llinas, 1988). Different types of Ca^{++} channels have been identified based on their electrophysiological and pharmacological properties such as T,N,L,P/Q and R (Catterall, 2000;Ertel et al., 2000) . All these channels are expressed in the spinal cord including within the superficial dorsal horn (Bao et al., 1998;Ryu and Randic, 1990;Vanegas and Schaible, 2000;Voitenko et al., 2000) and numerous studies support their contribution to nociception. Several types of Ca^{++} channel blockers have shown efficacy in acute and chronic pain conditions including neuropathic pain (Neugebauer et al., 1996;Vanegas and Schaible, 2000;Sluka, 1997;Ryu and Randic, 1990;Dogrul et al., 2001;Chaplan, 2000). Thus, the transmission of pain at the spinal level is undeniably dependant on voltage gated Ca^{++} channels (Altier and Zamponi, 2004).

The high voltage activated (HVA) Ca^{++} channels (L,N,P/Q and R) can be classified based on the pore forming α_1 subunit, that contains the voltage sensor (Catterall, 2000;Hofmann et al., 1999). The HVA Ca^{++} channels are modulated by ancillary subunits such as the $\alpha_2\delta$, β and γ subunits (Catterall, 2000;Ertel et al., 2000). The $\alpha_2\delta$ family consists of three genes $\alpha_2\delta$-(1-3). The $\alpha_2\delta$-1 is ubiquitously expressed, $\alpha_2\delta$-2 is found in several tissues including brain and heart, and $\alpha_2\delta$-3 is brain-specific (Klugbauer et al., 1999). The $\alpha_2\delta$-1 subunit increases the current density of Ca^{++} channels by increasing the

amount of functional channel at the cell surface (Hofmann et al., 1999;Klugbauer et al., 1999). Moreover it also allosterically alters the activation and inactivation of several α_1 subunits (Bangalore et al., 1996;Klugbauer et al., 1999;Felix et al., 1997). $\alpha_2\delta$-1 is also known to enhance dihydropyridine binding to L-type channels and ω-conotoxin GVIA to N-type channels (Brust et al., 1993;Felix et al., 1997), while $\alpha_2\delta$-2 and 3 significantly enhance and modulate the current through a number of HVA and LVA channels (Gao et al., 2000;Klugbauer et al., 1999).

In neuronal synapses, including those in the spinal cord several types of voltage dependent Ca^{++} channels may co-exist and the same neuron may express several channel types (Takahashi and Momiyama, 1993;Dunlap et al., 1995;Dogrul et al., 2001;Nowycky et al., 1985). Inhibitors of a given channel type usually block only a fraction of the voltage dependent Ca^{++} channels present in a complex synapse such as the dorsal horn (Bao et al., 1998), and hence antagonists to different channel types may have additive effects (Dogrul et al., 2001). Furthermore, the relative importance of the various channel types depends on the brain region, the transmitter involved and the functional status of the neuron (Song and Surmeier, 1996;Wheeler et al., 1996).

The L-type Ca^{++} channel activation is involved in the integration of various responses in the dorsal horn of the spinal cord. Two L-type Ca^{++} channel subtypes containing distinct α_1 subunits, Cav1.2 and Cav 1.3 are expressed in the central nervous system including the dorsal horn (Dobremez et al., 2005;Westenbroek et al., 1998b). Neugebauer et al. (1996), through single unit recordings from spinal dorsal horn nociceptive neurons

showed that spinal administration of nimodipine (a L-type Ca^{++} channel blocker) depresses the neuronal responses to innocuous or noxious stimuli (Neugebauer et al., 1996). The interneurons in deep dorsal horn expressing L-type Ca^{++} channels receive input from myelinated and unmyelinated afferents (Russo and Hounsgaard, 1996;Russo and Hounsgaard, 1994). Thus in deep dorsal horn neurons, the generation of plateau potential, a key component of wind-up is dependent of L-type Ca^{++} channels (Morisset and Nagy, 1999;Russo et al., 1997;Voisin and Nagy, 2001). Hence, L-type channels play an important role in mediating dorsal horn plasticity (Perrier et al., 2002). L-type channels have also been suggested to be involved in the neurotransmitter release in midbrain dopaminergic neurons, retinal cells and from the rat sensory neurons in the spinal cord (Bonci et al., 1998;Anwyl, 1991;Perney et al., 1986;Protti and Llano, 1998). However, the role of L-type channels in regulating transmitter release in the superficial dorsal horn under normal conditions remains controversial (Bao et al., 1998). N, P Q and R-type channels are enriched in synaptic sites suggesting their involvement in neurotransmitter release (Dunlap et al., 1995;Miljanich and Ramachandran, 1995;Kerr et al., 1988;Olivera et al., 1994), whereas the L-type channels are mainly observed in the cell bodies and dendrites (Ahlijanian et al., 1990;Westenbroek et al., 1998b;Westenbroek et al., 1990;Dobremez et al., 2005). These suggest under normal conditions L-type channels have very minimal role to play in transmitter release but rather involved in regulating post synaptic membrane excitability (Morisset and Nagy, 1999;Perrier et al., 2002).

Neuropathic pain associated with peripheral nerve injury results in long lasting changes in the excitability of spinal dorsal horn neurons. As mentioned in the general introduction several factors contribute to this increased excitability including increases in local Ca^{++} concentration in the spinal cord (Kawamata and Omote, 1996). Recent studies have demonstrated an altered regulation of L-type channels in conditions of injury (Chung et al., 2001;Westenbroek et al., 2004;Xiao et al., 2002). Dobremez *et al* in 2005, report that sciatic nerve damage results in a differential regulation L-type Ca^{++} channel subunits in deep dorsal horn neurons. Thus after nerve injury there is an up-regulation of Cav1.2 in soma , proximal and distal dendritic segments, while Cav1.3 undergoes down regulation (Dobremez et al., 2005). Analysis of gene expression profiles in the DRG and dorsal horn of the spinal cord suggest an up-regulation L-type Ca^{++} channel $\alpha_2\delta 1$ subunit after peripheral axotomy (Xiao et al., 2002;Yang et al., 2004). However, it remains to be established whether any change occurs in the function of L-type calcium channels in *substantia gelatinosa* neurons after peripheral nerve injury. Studies on CCI rats presented in chapter 3 suggested that after nerve injury there is an increase the spontaneous excitatory synaptic activity in the majority of neurons of the *substantia gelatinosa.* An up-regulation of calcium channels including the L-type channels could contribute to such alterations in transmitter release. Studies so far have indicated an up-regulation of mainly the N-type channels in the superficial dorsal horn after nerve injury (Cizkova et al., 2002). Hence, the focus of the present chapter is to determine if L-type channels could be involved in modulating the increase in excitatory synaptic activity of the *substantia gelatinosa* neurons after nerve injury.

To address this issue I made whole-cell recordings from *substantia gelatinosa* neurons. The effect of Nitrendipine, an L-type Ca^{++} channel blocker was examined on both the evoked and spontaneous post synaptic currents in sham and nerve injured animals. Nitrendipine at 2μM significantly suppressed the spontaneous and the evoked excitatory current in the nerve injured rats, but had very minimal effect in the sham rats. Hence, these results suggest the L-type channels could in fact contribute to the increase in excitatory synaptic transmission after nerve injury possibly due to an up-regulation at the presynaptic sites.

5.2 Methods

The methods for recording from *substantia gelatinosa neurons* in this chapter were identical to those described in chapter 2. The inhibitors of the calcium channels were serial diluted in the external solution from their respective stock solution to achieve the final desired concentration.

5.3 Results

5.3.1 Effects of Nitrendipine on Spontaneous Excitatory Postsynaptic Currents

The effects of Nitrendipine (2μM) on the amplitude and interevent interval of the spontaneous excitatory post synaptic currents in both sham and CCI rats are represented in Figure 5.1. Because Qx 314 (5mM), a voltage gated ion channel blocker was included in the internal solution, it was not always possible to conclusively determine the firing pattern of the different cell types. Hence the effects of nitrendipine on all the cells were pooled together irrespective of their firing pattern.

In sham animals, cumulative probability plots reveal that nitrendipine had a minimal effect ($P<0.05$, KS test) on the sEPSC amplitude (Figure 5.1A), without affecting the interevent interval (Figure 5.1E). However, a simpler but more rigorous parametric t-test performed on the mean amplitude (Figure 5.1B) and interevent interval (Figure 5.1F) suggests no significant change on either the sEPSC amplitude or the interevent interval. These findings are in agreement with earlier reports, suggesting that L-type channels are less likely to be involved in mediating spontaneous excitatory post synaptic currents in the superficial dorsal horn under normal conditions (Bao et al., 1998;Westenbroek et al., 1998b;Heinke et al., 2004).

In the CCI rats, cumulative probability plots suggest that nitrendipine significantly reduced both the amplitude ($P<0.02$, KS test) and the frequency (increase in interevent interval) ($P<0.001$, KS test) of the sEPSC's (Figure 5.1 C&G). The t-test performed on the mean amplitude (Figure 5.1D) and interevent interval (Figure 5.1H), also supports the conclusions of the KS test. These finding imply L-type channels are involved at least partly in mediating the increased excitatory synaptic transmission observed in the superficial dorsal horn neurons after peripheral nerve injury.

5.3.2 Evoked Excitatory Postsynaptic Currents

In the presence of bicuculline (10 μM) and strychnine (1 μM), the stimulation of dorsal root or the entry zone generated EPSC's in *substantia gelatinosa* neurons at a holding potential of -70 mV. Only monosynaptic responses were considered for further analysis to facilitate interpretation of drug responses (Moran, 2003). An example of a

monosynaptic eEPSC is represented in Figure 5.2. Application of AMPA receptor antagonist, CNQX (10μM), and the NMDA receptor antagonist, AP5 (50μM), abolished the evoked EPSC (Figure 5.2). This is in agreement with previous reports suggesting that the fast excitatory transmission in the *substantia gelatinosa* neurons is primarily mediated by AMPA and NMDA receptors (Yoshimura and Nishi, 1992;Yoshimura and Jessell, 1990;Lu and Perl, 2005). In some instances neurons had phases (biphasic or polyphasic) of dorsal root evoked responses, with the first phase having a constant latency of a monosynaptic connection.

5.3.3 Effects of Nitrendipine on evoked Excitatory Postsynaptic Currents

The major effect of nitrendipine on spontaneous excitatory currents in CCI rats was to reduce the frequency of the spontaneous EPSC's. Hence, one possibility is the contribution of the L-type Ca^{++} channels on the presynaptic primary afferent terminals. Hence, to test this possibility the effect of nitrendipine on the primary afferent evoked excitatory post synaptic currents was considered. In sham animals, superfusion of nitrendipine (2 μM) caused a small reduction in the EPSC amplitude by an average of 14.85 ± 5.53 % (n=13, $p<0.05$, paired t-test). This reduction is in agreement with earlier reports on effect of L-type channel blocker on evoked EPSC's in the superficial dorsal horn neurons (Bao et al., 1998;Heinke et al., 2004). In contrast, in the CCI rats nitrendipine caused a much larger reduction of about 40.23 ± 6.77 % (n=12, $p<0.005$, paired t-test), in the amplitude of the evoked EPSC's. Sample traces and a summary histogram of the action of nitrendipine for sham and CCI rats are illustrated in Figure 5.3 A-D. Figure 5.3 E is the time course response of the effect of nitrendipine in sham and

CCI rats. The time course of action of nitrendipine varied from cell to cell. In general, the effect took about 10-15 minutes to develop and the washout time was 30 minutes or longer.

5.3.4 Effects of ω -conotoxin GVIA on Evoked Excitatory Postsynaptic Currents

Figure 5.4 shows the effect of ω-conotoxin GVIA (1μM), an N-type channel blocker on the evoked excitatory currents in sham and CCI rats. In sham rats, ω-Conotoxin GVIA reduced the amplitude of the evoked EPSC by an average of 72.84±10.07 % (n=4) (Figure 5.4A). This in agreement with previous reports on the effect on the effects of ω-conotoxin GVIA on the eEPSC's (Bao et al., 1998;Heinke et al., 2004) suggesting N-type channels are the major contributors of the glutamate release from the afferent terminals in the superficial dorsal horn. In CCI rats, the reduction of EPSC amplitude after ω-conotoxin GVIA was about 68.56 ± 3.94 % (n=4) (Figure 5.4B). Even though the effects of ω-conotoxin GVIA in CCI rats were comparable to the sham controls, our recording conditions do not allow us differentiate the contribution of N-type channels in inhibitory versus excitatory synapses. This in turn could have different physiological consequences depending on the nature of the signaling pathway affected the most.

5.4 Discussion

The most straightforward explanation for our finding is that L-type channels are up-regulated in the primary afferent terminals or their axons following peripheral nerve injury. Such an up-regulation contributes to the enhanced excitatory synaptic transmission observed in the *substantia gelatinosa* neurons after nerve injury. As

demonstrated in Chapter 3, mEPSC contributes to about 50% of spontaneous events, hence the effects of nitrendipine could also include alteration in the miniature synaptic events. An up-regulation just in the primary afferent terminals does not completely account for the increased excitatory synaptic activity seen after injury. Because in addition to primary afferent terminals, synaptic activity may originate from terminals between the local interneurons or from descending inputs (Iyadomi et al., 2000;Yoshimura and Jessell, 1990;Kato et al., 2006;Millan, 2002). Thus, an up-regulation in other potential sites such as the excitatory synapses between the interneurons cannot be excluded.

One of the consequences of peripheral nerve injury is the increase in the excitability of *substantia gelatinosa* neurons (Ji and Woolf, 2001;Kohno et al., 2003). As established in chapter 3, nerve injury causes increase in the excitatory synaptic drive to certain classes of neurons of the *substantia gelatinosa*. Voltage-dependent calcium channels are known to be critical for neuronal excitability and neurotransmission and hence are attractive candidates for the development of analgesics (Dogrul et al., 2001;Dogrul et al., 1997;Vanegas and Schaible, 2000). There are several studies suggesting a possible up-regulation of $\alpha 2\delta$ subunit of high threshold voltage-gated calcium channels in the DRG and dorsal horn after nerve injury (Li et al., 2004;Luo et al., 2002;Field et al., 2000;Xiao et al., 2002;Yang et al., 2004). As mentioned in the general introduction (1.7.2) this gabapentin sensitive subunit is identical to the $\alpha 2\delta$ subunit of L-type calcium channel (Gee et al., 1996). In view of these reports, findings from our study also imply that there could be an abnormal expression or activation of L-type calcium channels in the

substantia gelatinosa neurons contributing to enhanced excitatory transmission after nerve injury.

L-type blockers inhibit calcium currents in the cell bodies of small and medium DRG neurons (Hatakeyama et al., 2001;Kim et al., 2001). This confirms the presence of L-type calcium channels in primary afferent neurons and suggests their presence in the spinal terminals of primary afferents. L-type channels are also involved in neurotransmitter release in other areas of CNS (Bonci et al., 1998;Protti and Llano, 1998), however their contribution in mediating transmitter release in the superficial dorsal horn is debatable (Bao et al., 1998;Heinke et al., 2004). Our results from the control rats also show that the L-type channel has a very minimal role to play in mediating excitatory synaptic transmission in normal conditions. Under conditions of nerve injury, however there is an increase in the contribution of L-type channels towards the excitatory transmission, suggesting a possible up-regulation and or function. The recent gene array studies on DRG and dorsal horn of the spinal cord suggest a possible up-regulation of L-type channels after peripheral nerve damage (Xiao et al., 2002;Yang et al., 2004). Increase in the expression of L-type channel in conditions of injury or chemical treatment in other areas of brain and spinal cord has been demonstrated by other studies (Dobremez et al., 2005;Chung et al., 2001;Westenbroek et al., 2004;Westenbroek et al., 1998a).

Behavioral studies have shown varying effects of calcium channel blockers under different pain models (Malmberg and Yaksh, 1994;Neugebauer et al., 1996;Vanegas and Schaible, 2000). Blockade of N-type and P/Q type but not L-type channels alleviate pain

manifestation in animal models of nerve injury (Xiao and Bennett, 1995;Yamamoto and Sakashita, 1998;Chaplan et al., 1994;Matthews and Dickenson, 2001;Miljanich and Ramachandran, 1995). This suggests L-type channel blockers alone are not very effective in neuropathic pain conditions. However, in complex synapses of the brain and spinal cord several VGCC co-exists and hence a combination of blockers may be more effective than individual ones (Takahashi and Momiyama, 1993;Dunlap et al., 1995;Dogrul et al., 2001;Bao et al., 1998). Hence, our findings suggest that the L-type channels blockers could be used as an add-on therapy along with N-type antagonists in treating resistant neuropathic pain conditions. At the spinal level, combination of L-type blockers, which alone has no antinociceptive activity is able to enhance μ opioid antinociception (Omote et al., 1993;Dogrul et al., 2001;Dogrul et al., 1997). It is well known that there is reduced effectiveness of opioids in neuropathic pain (Arners and Megerson, 1988;Ossipov et al., 1995b;Ossipov et al., 1995a;Dickenson, 1994). In the superficial dorsal horn the efficacy of opioids to suppress the excitatory post synaptic activity is diminished after nerve injury (Kohno et al., 2005). Hence, a possibility for the reduced effectiveness of opioids after nerve injury could be an up-regulation of L-type channel and blocking these could be an effective way to reverse the ineffectiveness of opioids in certain neuropathic pain conditions.

Peripheral nerve injury might result in abnormal sprouting or formation of new synapses the superficial dorsal horn (Coggeshall et al., 1997;Woolf et al., 1992;Woolf et al., 1995;Hughes et al., 2003;Shehab et al., 2003). Consistent with this idea is the up-regulation of synaptophysin, a marker for synaptogenesis in the superficial dorsal horn

after nerve injury (Chou et al., 2002). Hence, there is a possibility that these new synapses could express L-type calcium channels which contribute to the increase in the excitatory transmission onto the second order sensory neurons of the *substantia gelatinosa*. The contribution of L-type channels in mediating glutamate release might happen only in certain synapses or in particular types of neurons of the *substantia gelatinosa* neurons. This is because nerve injury results in cell type specific effects in the *substantia gelatinosa* neurons as opposed to global enhancement of excitatory synaptic activity. Hence, future studies determining specific sites or neurons where such an up-regulation occurs could provide valuable insights into understanding the contribution of L-type channels in mediating the excitability of dorsal horn neurons after nerve injury.

Figure. 5.1. Effects of Nitrendipine (2μm) on the amplitude and interevent interval of s.e.p.s.'c in *substantia gelatinosa* neurons. Analysis was performed from the first 100 events following 1 min of recording. The neurons were voltage clamped at -70mV. 10 μM bicuculline and 1 μM strychnine were included in the external solution to block inhibitory responses. Cumulative probability plots show the effect of Nitrendipine on amplitude (A &C) and Inter-event interval distributions (E & G) for sham (A, &E) and CCI (C, &G) animals. A summary histogram for the effect of Nitrendipine is represented in B, F, D, H. P values were derived from KS test for cumulative probability graphs and paired't' test for the bar graphs. n=24 cells for sham and n=27 cells for cuff.

Figure. 5.1

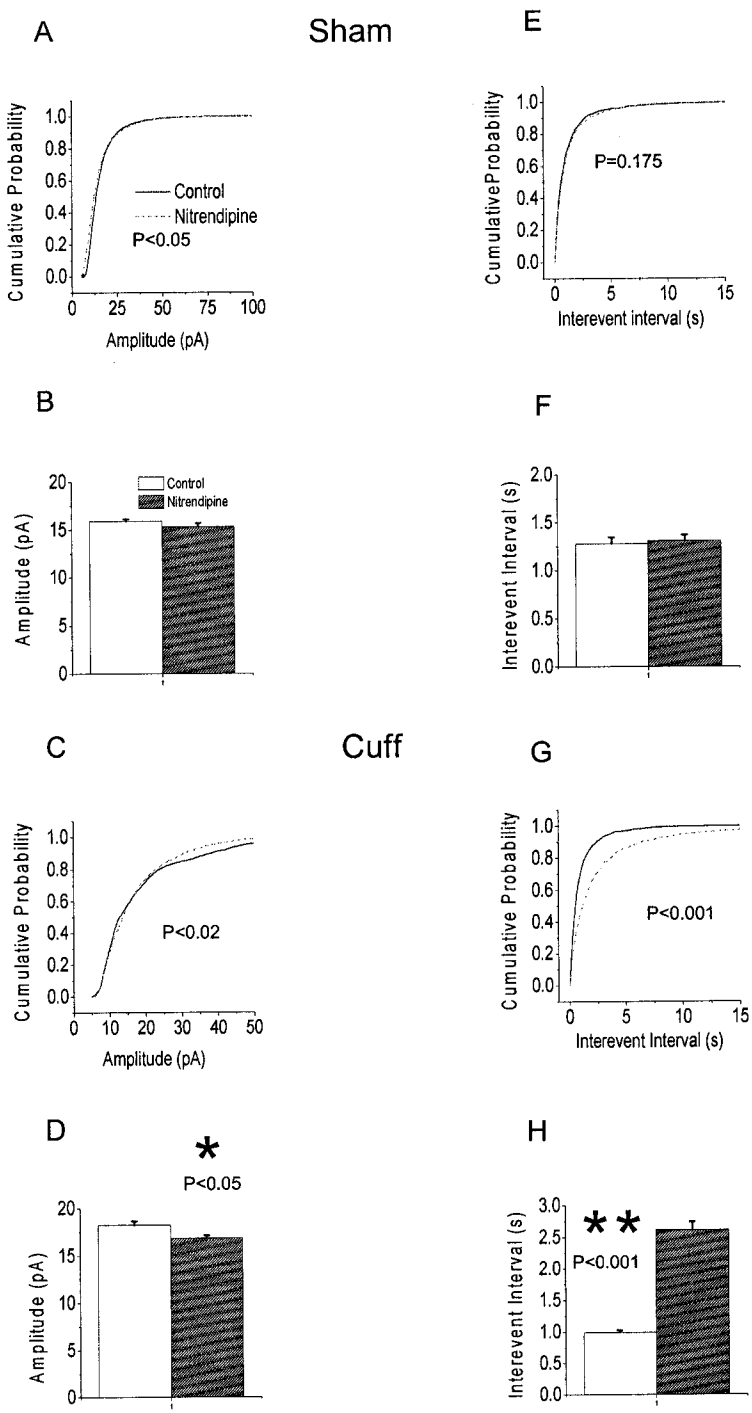


Figure. 5.2. Dorsal root stimulation generates AMPA and NMDA receptor-mediated EPSC's in the *substantia gelatinosa neuron*s. EPSCs were evoked at a holding potential of –70 mV, by dorsal root or dorsal root entry zone stimulation in the presence of 10 μM bicuculline and 1 μM strychnine. Averaged traces (n = 6) of evoked EPSCs before during and after application of the AMPA antagonist, CNQX (10 μM), and the NMDA antagonist, AP5 (50 μM).

Figure. 5.2

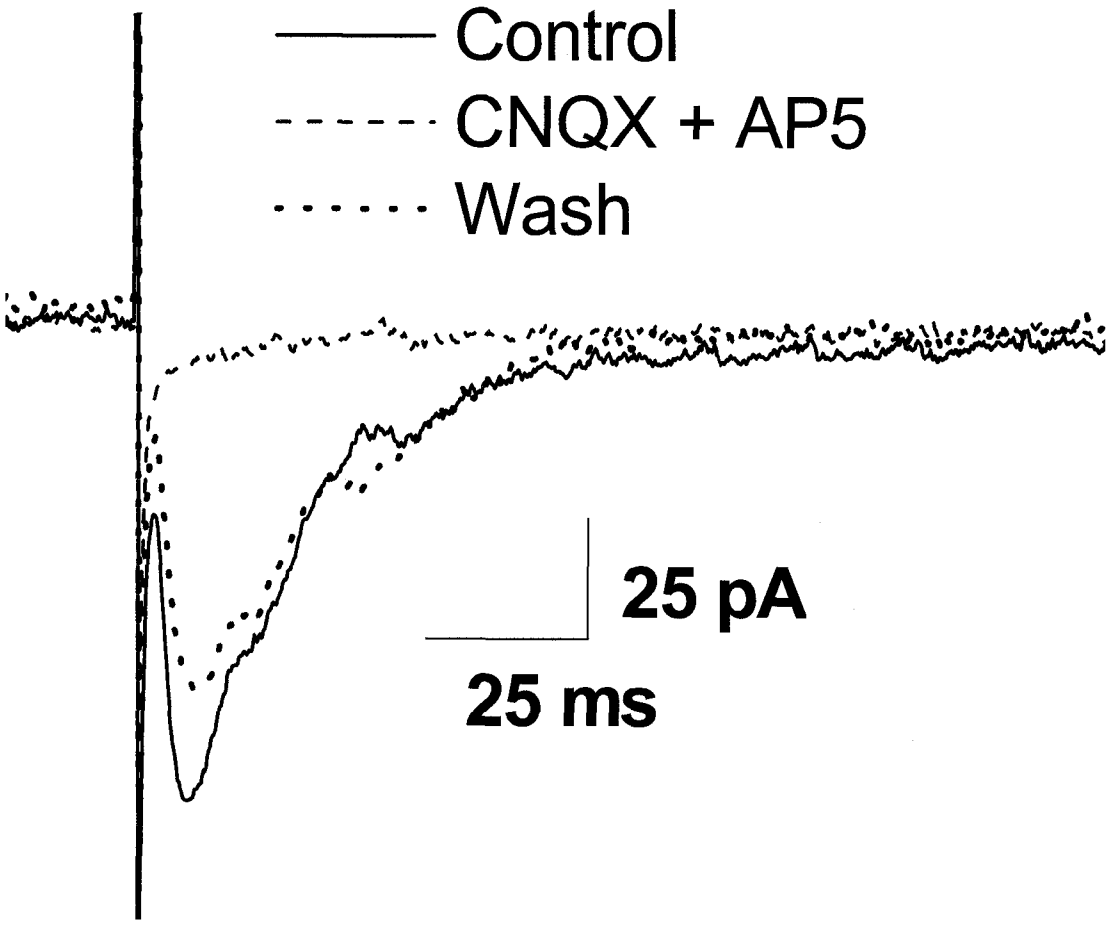


Figure. 5.3. Effects of Nitrendipine (2μM) on the evoked EPSC's in *substantia gelatinosa* neurons of sham and CCI animals. EPSCs were evoked at a holding potential of −70 mV, by dorsal root or dorsal root entry zone stimulation in the presence of 10 μM bicuculline and 1 μM strychnine. Averaged traces (n=6) of the eEPSC's before during and after the application of Nitrendipine for sham (A) and CCI (C) animals. A summary histogram for the effects of nitrendipine on eEPSC's for sham (B) and CCI (D) animals. E, time course of the changes in amplitude of evoked EPSCs during application of Nitrendipine. P values were derived from paired't' test. N=13 for sham and N=12 for CCI rats. * = $P<0.005$, += $P<0.05$

Figure. 5.3

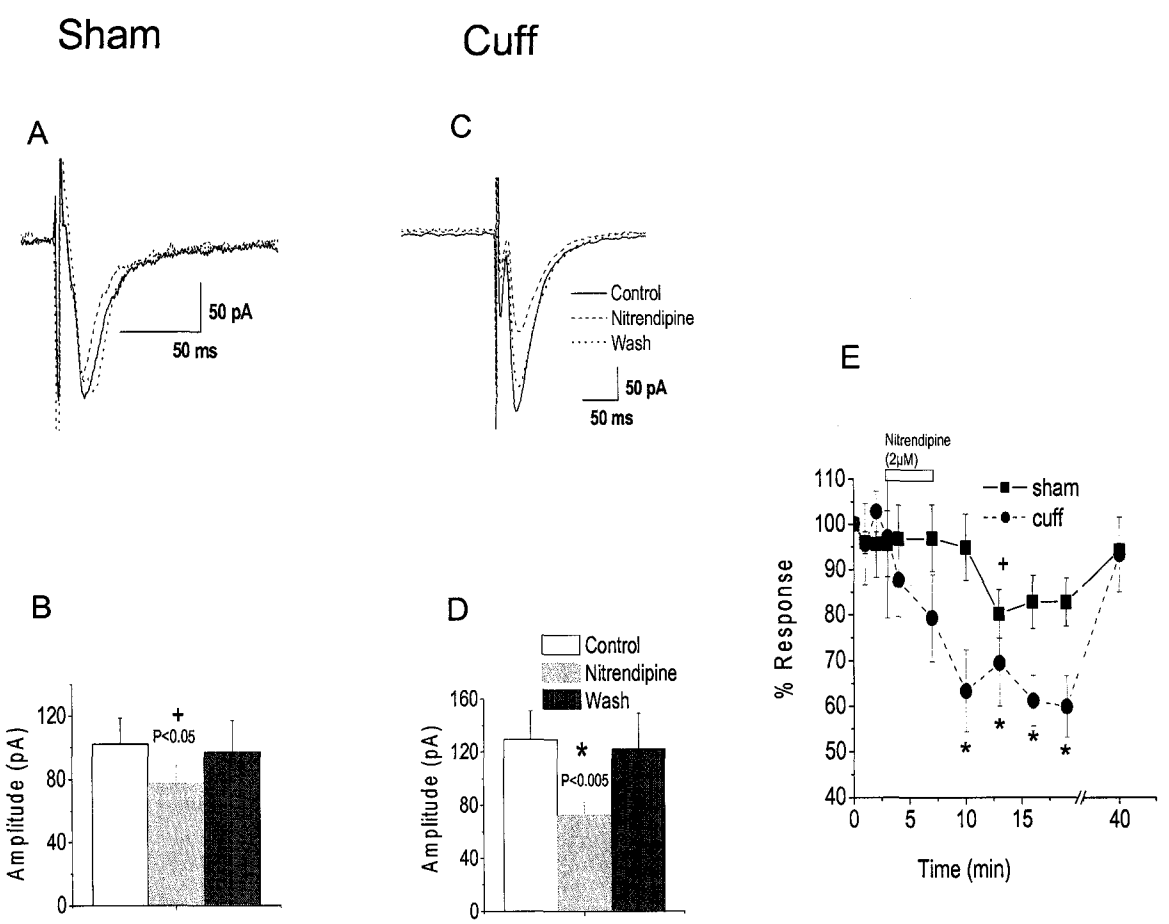
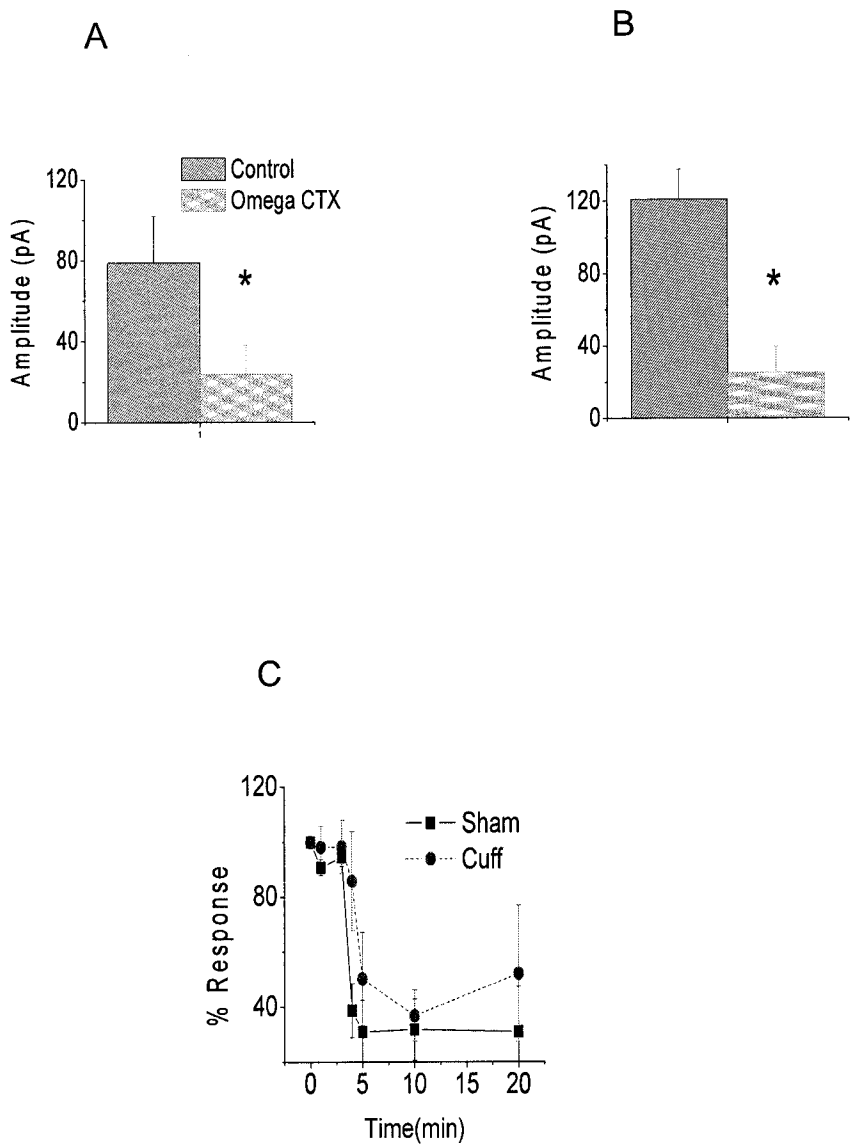


Figure. 5.4. Effects of ω-conotoxin GVIA (1μm) on the evoked EPSC's in *substantia gelatinosa* neurons of sham and CCI animals. EPSCs were evoked at a holding potential of –70 mV, by dorsal root or dorsal root entry zone stimulation in the presence of 10 μM bicuculline and 1 μM strychnine. A summary histogram for the effects of ω-conotoxin GVIA on eEPSC's for sham (A) and CCI (B) animals. C, time course of the changes in amplitude of evoked EPSCs during application of ω-conotoxin GVIA. P values were derived from paired't' test. N=4 for sham and CCI rats. *= $P<0.05$

Figure. 5.4



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Chapter 6

General Discussion

In this thesis, the effects of sciatic chronic constriction injury on the properties of various *substantia gelatinosa* neurons were characterized using an *in vitro* transverse slice preparation. This was the first comprehensive study to examine the effects of peripheral nerve injury on both electrophysiologically and morphologically defined neurons of the *substantia gelatinosa*. Moreover, these experiments provided clues regarding the correlation between neuronal morphology and the firing characteristics of *substantia gelatinosa* cells. This chapter will review the three preceding chapters. Finally it will also propose some general conclusion and suggest future perspectives on exploring possible changes on *substantia gelatinosa* neurons after peripheral nerve injury.

6.1 Chapter summaries

Chapter 3

The peripheral nerve injury induced by polyethylene cuff implantation produced a significant mechanical hypersensitivity compared to the sham controls. This implies that the polyethylene cuff model is simple and reliable model of inducing neuropathic pain like symptoms in rats. The neurons of the *substantia gelatinosa* could be divided into five different types: tonic, delay, phasic, irregular and transient, based on their discharge properties in response to depolarizing current steps. Because the classical studies of the effects of sciatic nerve injury on dorsal horn neurons were done with extracellular unit recording *in vivo* (Laird and Bennett, 1993), it has been difficult to distinguish injury-induced changes in the intrinsic properties of dorsal horn networks and the neurons therein from altered excitation resulting from spontaneous primary afferent activity (but see Sotgiu and Biella, 2000). We therefore used whole-cell recording to carry out a

systematic study of the effect of sciatic nerve injury on the intrinsic electrophysiological properties of neurons in the *substantia gelatinosa* of the juvenile rats. Experiments were done on spinal cord slices, acutely prepared from sham-operated animals or those subject to 13-25d sciatic chronic constriction injury (CCI) (Mosconi and Kruger, 1996).

The most obvious effects of CCI involved neuron type specific alterations in spontaneous synaptic activity. These were attributed, at least in part, to alterations in the action potential-independent neurotransmitter release machinery and to possible alterations in the postsynaptic effectiveness of glutamate. The principal findings were (1) In tonic neurons, CCI reduced the excitatory synaptic drive and membrane excitability, (2) CCI increased the synaptic drive of delay, transient and irregular neurons, (3) In phasic neurons, CCI increased the membrane excitability, while exerting minimal effects on the synaptic drive.

Given the clearly documented increase in the excitability of dorsal horn neurons following peripheral nerve injury *in vivo* (Laird and Bennett, 1993) and the presence of mechanical hypersensitivity in our experimental animals (Figure 3.1), the observed changes in the properties of *substantia gelatinosa* neurons at first seem relatively subtle. It should be noted however, that our experiments were done in thin (300μm) transverse slices of spinal cord. Thus, some of the intrinsic synaptic connections between individual neurons within the *substantia gelatinosa* (Lu and Perl, 2005) may have been compromised by the slice preparation process. Perhaps a more profound effect of CCI would have been seen had this circuitry remained intact. Also, the acute rhizotomy,

which occurred as the slices were prepared, would have removed the contribution of injury-induced spontaneous primary afferent activity. Had this excitatory drive remained intact, it is possible that even more profound changes in sEPSC's would have been seen. It is also possible that CCI affects descending brainstem control of spinal nociceptive circuits (Suzuki et al., 2002). Such changes, which would not be detected by our methodology, could also contribute to CCI-induced hyperexcitability of the dorsal horn *in vivo*. Lastly it should be reiterated that the present studies were exclusively concerned with excitatory synaptic mechanisms. A role for impaired GABAergic function in pain centralization is now quite well established (Coull et al., 2003;Coull et al., 2005). It is therefore suggested, that a combination of changes in intrinsic synaptic (increased excitatory and decreased inhibitory) transmission further driven by alterations in primary afferent and descending inputs leads to the general increase in activity in the intact dorsal horn *in vivo*. The relative contribution of intrinsic, peripheral and descending mechanisms to central hypersensitivity remains to be determined.

Chapter 4

The aim of this chapter is two fold. First, to explore possible correlations between the firing characteristics of the *substantia gelatinosa* neurons with their morphological features. Although the relationships between various neuronal morphologies, their neurotransmitter phenotypes, cytochemical markers and electrophysiological properties are starting to be understood there is, as yet, no universally accepted classification scheme for *substantia gelatinosa* neurons (Heinke et al., 2004;Grudt and Perl, 2002;Hantman et al., 2004;Ruscheweyh and Sandkuhler, 2002;Todd and Spike, 1993).

Hence, I attempted to correlate the firing pattern of the neurons with post-hoc morphological identification. This was accomplished using confocal microscopy which allowed for 3D reconstruction of the biocytin stained neurons to estimate the predominant dendritic orientation. Thus, the main findings were: (1) the majority of islet neurons with predominant rostro-caudal orientation exibited a 'tonic' firing pattern, (2) the majority of vertical neurons with predominant dorso-ventral projection exibited 'delay' regular firing pattern, (3) almost all radial neurons with dendrites projecting equally in all directions exibited a 'delay' but with irregularities in their firing pattern. In addition, based on the relationship to the PKCγ containing cells, the soma of tonic/islet neurons could be found throughout the entire lamina II region, while the vertical and radial cells are preferentially localized on the outer or on the inner/outer border of the lamina II region.

The second aim of this chapter was to understand the changes induced by CCI on the morphologically and electrophysiologically identified neurons. Thus CCI reduced the excitatory synaptic drive to islet tonic neurons, while it increased the synaptic drive to vertical and radial delay neurons. The changes with respect to membrane excitability and rheobase are at odds with the findings of chapter 3. Hence, not much significance could be attributed to this at the moment. If one accepts the hypothesis that many 'tonic/ islet' cells are GABAergic (Grudt and Perl, 2002;Hantman et al., 2004;Lu and Perl, 2003), a CCI-induced decrease in excitatory synaptic drive fits with the finding that GABAergic tone is reduced in neuropathic pain (Laird and Bennett, 1992;Woolf and Mannion, 1999;Moore et al., 2002;Baba et al., 2003). Also, if the vertical/ delay neurons are excitatory (Lu and Perl, 2005), these cells receive an increased excitatory synaptic drive

after nerve injury (Kohno et al., 2003). Hence, the above mechanisms could contribute to an overall increase in dorsal horn excitability.

Further studies in the direction of functional characterization of the different cell types would provide much needed insights to the organization of the *substantia gelatinosa*. Especially it would be interesting to follow up the electrophysiological characterization with post-hoc cell identification with glutamatergic (excitatory aminoacid transporter EAAT3) or GABAergic (GAD 67) markers. Findings from these studies will then help in determining the electrophysiological consequences of peripheral nerve injury on glutamatergic and GABAergic neurons.

Chapter 5

The earlier chapters have demonstrated neuron specific alterations in excitatory transmitter release mechanism after nerve Injury. These findings corroborate the reports on increases in messages for transmitter release proteins after nerve injury (Xiao et al., 2002;Yang et al., 2004). These recent reports analyzing the changes in expression of various genes after nerve injury also suggests an up-regulation calcium channel $\alpha_2\delta 1$ subunits in the DRG and dorsal horn after nerve injury (Xiao et al., 2002;Yang et al., 2004). Interestingly this $\alpha_2\delta 1$ subunit is sensitive to gabapentin which is effective in various neuropathic pain conditions (Snutch et al., 2001;Nicholson, 2000). Moreover this $\alpha_2\delta 1$subunit is thought to be associated with the L-type HVA calcium channel (Xiao et al., 2002;Gee et al., 1996;Field et al., 2000;Nicholson, 2000). However, the electrophysiological studies exploring the changes in L-type calcium currents in the DRG

cell bodies have not reported an increase after nerve injury (Baccei and Kocsis, 2000;Abdulla and Smith, 2001). In fact, after axotomy there is a decrease in HVA calcium currents in the DRG cell bodies (Abdulla and Smith, 2001). Hence this increase expression of L-type channels could have occurred only on the primary afferent terminals or after nerve injury there is a translocation of L-type channels from the cell bodies to the afferent terminals in the dorsal horn (Yang et al., 2004). Hence, this chapter was aimed at determining the possible involvement of L-type calcium channels in mediating excitatory transmitter release after nerve injury. The principal findings were (1) Nitrendipine, an L-type calcium channel blocker significantly reduced both the amplitude and the frequency of spontaneous EPSC in the *substantia gelatinosa* neurons after nerve injury, while having only minimal effects in sham controls (2) Nitrendipine, was also able to reduce the dorsal root evoked EPSC in the nerve injured rats by more than 20% compared to sham controls. Our findings hence suggest at least part of the effects of L-type channels on EPSC's are through those channels present on the primary afferent terminals. However, in complex circuitry such as the *substantia gelatinosa* an up-regulation of L-type calcium channels in other potential sites such as the inter-neuronal synapses cannot be excluded. Moreover, it remains to be determined whether up-regulation or function of L-type channel occurs in both inhibitory and excitatory synapses. Hence future studies should examine neuron specific up-regulation of L-type channels after nerve injury. These studies would further offer insights on the contribution of L-type channel towards mediating excitatory synaptic transmission after nerve injury.

6.2 General Conclusion and Future Perspectives

My results show for the first time that peripheral nerve injury generates a specific pattern of changes in the *substantia gelatinosa* neurons as opposed to a hypothetically global increase in the excitability. The effects are in such a direction that putative inhibitory neurons receive less excitatory synaptic drive while the putative excitatory neurons receive an increased synaptic drive. We refer to such changes as an 'injury foot print'. This pattern of changes could essentially form a template to study whether different pain mediators such as BDNF or IL1β when applied exogenously are able to mimic the responses of peripheral nerve injury. In fact, preliminary data from our lab show that long-term exposure of the organotypic spinal cord cultures to BDNF produces a pattern of electrophysiological changes that resemble the 'injury footprint' seen in acute slices after chronic constriction injury. Specifically, BDNF reduced the exciatatory synaptic drive to tonic cells, while increasing it in all other cell types including the delay neurons, in organotypic spinal cord cultures (Smith et al., 2005).

As mentioned earlier, sciatic nerve injury induced cell-type specific changes in the excitatory synaptic transmission. This may reflect cell-type specific changes in conductance and/or the number of postsynaptic AMPA and/or NMDA receptors, in addition to changes in neurotransmitter release mechanisms. Hence, one interesting future direction is to examine whether AMPA and NMDA receptors are affected differentially between tonic and delay neurons. Based on the effect of CCI on sEPSC amplitude in chapter 3 and 4 (Figures 3.9 and 4.6), I anticipate that AMPA effects will be increased in delay cells and reduced in tonic cells. This may reflect the possibility that tonic cells may

express GluR1 whereas delay cells may express GluR2/3 (Kerr et al., 1998). This could be examined by comparing the Ca^{2+} permeability, rectification properties, jorotoxin sensitivity and desensitization kinetics of AMPA responses in the two cell types (Iino et al., 1996;Albuquerque et al., 1999).

The organization of the superficial dorsal horn has received greater attention recently and several features of it have started to be unraveled. Even within the *substantia gelatinosa* there seems to be region-specific differences in the organization. The outer layer is associated with nociceptive neurons while the inner region is comprised mainly of non-noxious neurons responding to gentle mechanical stimuli (Light and Willcockson, 1999;Light and Perl, 1979). It has recently been shown that 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). The islet/tonic cells receiving input from selective primary afferent C-fibers have been demonstrated (Grudt and Perl, 2002;Lu and Perl, 2003). Moreover, our finding also suggests loss of excitatory synaptic drive to the islet/tonic neurons. It would be hence interesting to determine whether there is a loss of C-fiber evoked EPSC's in the islet/tonic neurons present in inner lamina II after nerve injury. Hence future studies should aim at targeting the differences between the outer versus the inner lamina II neurons after peripheral nerve injury. The sensory afferent input through the sciatic nerve projects to the medial half of the superficial dorsal horn (Coggeshall et al., 2001;Scholz et al., 2005;Swett and Woolf, 1985). Moreover, changes such as abnormal expression of sodium channel (Nav1.3) (Hains et al., 2004), neuronal apoptosis of inhibitory neurons and its associated loss of GABAergic

IPSC's (Moore et al., 2002;Scholz et al., 2005) and alteration in excitatory synaptic transmission (Kohno et al., 2003) seem to occur mainly in the medial part of the superficial dorsal horn. Hence it would be interesting explore whether nerve injury causes differential changes in the properties of medial versus the lateral cells.

The major effect of CCI under the present experimental conditions is to produce alteration in spontaneous and miniature synaptic activity. This possibly reflects changes in the release process including formation of new synaptic connections. Increased expression of the presynaptic vesicle protein, synaptophysin have been reported after nerve injury (Chou et al., 2002). Based on this evidence it would be interesting to determine if there is any alteration in expression of vesicle proteins such as synapsin in the superficial dorsal horn in our injury model. This could possibly provide an explanation for an increased excitatory synaptic drive to the *substantia gelatinosa* neurons after nerve injury. The studies on L-type calcium channel blockers suggest a possible up-regulation or function of L-channels after nerve injury. Future studies using western blot analysis to estimate the expression of L-type channel protein in the superficial dorsal horn neurons in our nerve injury model could further complement our electrophysiological findings. Even though the L-type blockers alone in various neuropathic pain models have been unsuccessful, there is a possibility that they could be used as an add-on therapy under certain condition (Dogrul et al., 2001). This possibly opens up novel therapeutic avenues for the management of neuropathic pain.

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