University of Alberta

Concentration-dependent effects of long-term interleukin-1β treatment on spinal dorsal horn neurons in organotypic slice cultures

by

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Abstract

This thesis investigated the effects of interleukin-1β (IL-1β) on synaptic transmission of dorsal horn neurons in defined-medium organotypic slice cultures (DMOTC). IL-1β has been suggested to play a direct role in the pathophysiology of nerve injury and inflammation, and may be involved in the induction of neuropathic pain.

Experiments were carried out using infrared-differential interference contrast (IR-DIC) microscopy and whole-cell patch-clamp recordings from DMOTC neurons.

The main findings were that treatment with low concentration (1pM) IL-1β increased the amplitudes of spontaneous excitatory post-synaptic currents (sEPSCs) and decreased the frequency of spontaneous inhibitory post-synaptic currents (sIPSCs) in putative inhibitory neurons, while treatment with high concentration (100pM) IL-1β increased sEPSC amplitude and decreased sIPSC frequency in putative excitatory neurons. These data suggest that 1pM IL-1β changes the properties of putative inhibitory neurons to increase inhibitory transmission while 100pM IL-1β affects mainly putative excitatory neurons to increase excitatory transmission in the spinal dorsal horn.

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List of Abbreviations

aCSF – Artificial cerebrospinal fluid

ADP – Adenosine diphosphate

AM – Acetoxymethyl

AMP – Adenosine monophosphate

AMPA – α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

AP – Action potential

AP1 – Activator protein 1

AraC – Cytosine-β-D-arabino-furanoside

ATP – Adenosine triphosphate

BDNF – Brain-derived neurotrophic factor

BSA – Bovine serum albumin

BSS – Balanced salt solution

C_m – Cell capacitance

CAMP – Cathelicidin antimicrobial peptide; also known as LL37

CCI – Chronic constriction injury

CCL-2 – Chemokine (C-C motif) ligand 2; also known as MCP-1

CD4 – Cluster determinant 4

CD14 – Cluster determinant 14

CGRP – Calcitonin gene-related peptide

CNS – Central nervous system

COX-2 – Cyclo-oxygenase 2

CR-3 – Complement type-3 receptor

CRIDs – Cytokine-release inhibitory drugs

CVLM – Caudal ventrolateral medulla

CX3CL1 – Chemokine (C-X3-C motif) ligand 1; also known as fractalkine

CXCL8 – Chemokine (C-X-C motif) ligand 8; also known as IL-8

DMEM – Dulbecco's Modified Eagle Medium

DMOTC – Defined-medium organotypic slice culture

DNA – Deoxyribonucleic acid

DRG – Dorsal root ganglia

E-selectin – Endothelial-cell selectin

EGTA – (ethylenebis(oxyethylenenitrilo)) tetra-; also known as acetic acid

ELISA – Enzyme-linked immunosorbent assay

EPSC – Excitatory post-synaptic current

EPSP – Excitatory post-synaptic potential

GABA – γ-aminobutyric acid

GSTO-1 – Glutathione S-transferase-ω1-1

GTP – Guanosine triphosphate

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPA – Hypothalamic-pituitary-adrenal

ICAM1 – Intracellular adhesion molecule 1

icIL-1RA1 – Intracellular interleukin-1 receptor antagonist isoform 1

icIL-1RA2 – Intracellular interleukin-1 receptor antagonist isoform 2

icIL-1RA3 – Intracellular interleukin-1 receptor antagonist isoform 3

IL-1 – Interleukin-1

IL-1α – Interleukin-1α

IL-1β – Interleukin-1β

IL-1F5 – Interleukin-1 family, member 5

IL-1F6 – Interleukin-1 family, member 6

IL-1F7 – Interleukin-1 family, member 7

IL-1F8 – Interleukin-1 family, member 8

IL-1F9 – Interleukin-1 family, member 9

IL-1F10 – Interleukin-1 family, member 10

IL-1R1 – Type I interleukin-1 receptor

IL-1R2 – Type II interleukin-1 receptor

IL-1RA – Interleukin-1 receptor antagonist

IL-1RAcP – Interleukin-1 receptor accessory protein

IL-6 – Interleukin-6

IL-8 – Interleukin-8; also known as CXCL8

IL-10 – Interleukin-10

IL-18 – Interleukin-18

IL-18R – Interleukin-18 receptor

IPSC – Inhibitory post-synaptic current

IR – Infrared

IR-DIC – Infrared differential interference contrast

K-S test – Kolmogorov-Smirnov two-sample test

LPS – Lipopolysaccharide

LTP – Long-term potentiation

MAPK – Mitogen-activated protein kinase

MCP-1 – Monocyte chemoattractant protein-1; also known as CCL-2

MHC – Major histocompatibility complex

MMP – Matrix metalloproteinase

mRNA – Messenger ribonucleic acid

NF-κB – Nuclear factor-κB

NGF – Nerve growth factor

NMDA – N-methyl-D-aspartate

NO – Nitric oxide

NRM – Nucleus raphe magnus

NSAIDs – Nonsteroidal anti-inflammatory drugs

P2X7 – Purinergic receptor P2X, ligand-gated ion channel, 7

PAG – Periaqueductal gray matter

PBA – Parabrachial area

PBS – Phosphate-buffered saline

PGE_2 – Prostaglandin E_2

RMC – Nucleus reticularis magnocellularis

RMP – Resting membrane potential

RPGL – Nucleus reticularis paragigantocellularis lateralis

S100B – S100 calcium-binding protein; also known as neurite extension factor

SEM – Standard error of the mean

sEPSC – Spontaneous excitatory post-synaptic current

sIL-1R1 – Soluble type I interleukin-1 receptor

sIL-1R2 – Soluble type II interleukin-1 receptor

sIL-1RA – Secreted isoform of interleukin-1 receptor antagonist

sIL-1RAcP – Soluble interleukin-1 receptor accessory protein

sIPSC – Spontaneous inhibitory post-synaptic current

sPSC – Spontaneous post-synaptic current

SSRIs – Selective serotonin reuptake inhibitors

TIR – Toll-interleukin-1 receptor

TGF – Transforming growth factor

TLR – Toll-like receptor

TLR4 – Toll-like receptor 4

TNF – Tumor necrosis factor

TNF-α – Tumor necrosis factor-α

VGAT– Vesicular GABA transporter

VGLUT – Vesicular glutamate transporter

VPI – Ventral posterior inferior nucleus

VPL – Ventral posterior lateral nucleus

CHAPTER 1 GENERAL INTRODUCTION

1.1 Basics of Pain Transmission and Terminology

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Merskey & Bogduk, 1994). Nociception, the signaling of tissue damage which is often perceived as pain (Kandel & Kupfermann, 1995), arises from stimulation of a specific class of sensory neurons called nociceptors (Kunz et al., 2005). The free nerve endings of nociceptors transduce mechanical, thermal, or chemical tissue-damaging stimuli into electrical impulses (action potentials, or APs) that are transmitted along the axons of primary sensory neurons to the spinal cord or to homologous regions of the brain stem (Nestler, Hyman, & Malenka, 2001).

Nociceptive neurons, whose cell bodies are located in dorsal root ganglia, synapse in the dorsal horn of the spinal cord on local interneurons and on projection neurons (Nestler et al., 2001). From there, these projection neurons carry nociceptive information primarily to the brain stem, thalamus, and hypothalamus. These structures integrate the intensity, location, and context of the noxious stimulus and participate in the autonomic, alerting, and cognitive aspects of the pain response. Primary nociceptive neurons are classified according to responsiveness to stimuli, diameter of their axons, conduction velocity, and degree of myelination of their axons. Aδ-fibres are activated by intense mechanical stimuli or by noxious heat, have a diameter of 2-5μm, are thinly myelinated, and have a conduction velocity of 6-30 m/s (Burgess & Perl, 1967), while C-fibres are activated by dull yet noxious thermal, mechanical, and chemical stimuli, have a diameter of 0.2-1.5μm, lack myelination, and have a much slower conduction velocity of 0.5-2 m/s (Nestler et al., 2001; Perl, 1996).

1.1.1 Spinal Nociceptive Pathways

After nociceptive information has been processed and modulated in the dorsal horn, it is transmitted higher centres via several tracts originating from neurons located within the spinal cord grey matter (Willis, 1983; Brodal, 1992). The axons of these neurons travel within the spinal cord white matter and terminate in several regions, including the thalamus and brain stem nuclei (Mehler, Feferman, & Nauta, 1960). Nociceptive pathways in primates ascend from the anterolateral quadrant of the spinal cord to the brain, and the pathways that mediate pain include the spinothalamic tract (traditionally regarded as the most important), the spinoreticular tract, the spinomesencephalic tract, the spinocervical tract, the spinohypothalamic tract, the spinoparabrachial tract, the spinolimbic tract and the postsynaptic dorsal column pathway (Willis, 1983; Willis, 2006). Neurons in these pathways generally project contralaterally, and electrophysiological experiments suggest that the motivational-affective aspects are mediated by activity in spinothalamic tract neurons (Willis, 2006). Brain centres in the rat that receive major projections from the dorsal horn are the periaqueductal gray matter (PAG), parabrachial area (PBA), the caudal ventrolateral medulla (CVLM), and a number of thalamic nuclei such as the ventral posterior lateral nucleus (VPL), the ventral posterior inferior nucleus (VPI), the posterior complex in the lateral thalamus, and the central lateral nucleus in the medial thalamus; thus these ascending projections can modulate autonomic, endocrine, and cognitive functions (Spike, Puskar, Andrew, & Todd, 2003; Willis, 2006). There is evidence that there are plastic changes in the responses of spinothalamic tract neurons following capsaicin injection or peripheral

neuropathy; these enhancements in transmission are similar to long-term potentiation (LTP) in certain brain structures and are a form of central sensitization (Willis, 2006).

In addition to activating brain systems involved in sensing and reacting to noxious stimuli, another role of the ascending nociceptive pathways likely includes engagement of descending control systems that will in turn modify pain transmission (Willis, 1983). The major descending pain pathways include multiple, pharmacologically distinct bulbospinal control systems, originating from projection neurons of the periaqueductal gray (PAG) and various subregions of the rostral ventral medulla, including the nucleus raphe magnus (NRM), the nucleus reticularis magnocellularis (RMC), and the nucleus reticularis paragigantocellularis lateralis (RPGL) (Basbaum & Fields, 1984). These terminate in the spinal dorsal horn, where their inhibitory action may be via direct postsynaptic inhibition, or via indirect mechanisms involving the release of opioid peptides, biogenic amines, and other transmitters (Basbaum & Fields, 1984). It is known that the spinoreticular tract participates in descending pain modulation (Basbaum & Fields, 1984). These control systems may not only be capable of responding to physiological pain transmission, but to cognitive and psychological events signaled by the brain as well, thus these systems may play a strong role in changing the nature of a potentially painful experience (Coren, Ward, & Enns, 2004).

1.1.2 Dorsal Horn Anatomy

The organization of the dorsal horn of the spinal cord was first described in the cat spinal cord by Rexed (1952). According to this model, the dorsal horn is composed of six laminae (laminae I-VI) that in turn consist of neurons of different sizes and densities (Nestler et al., 2001; Rexed, 1952). Primary nociceptive neurons synapse in the dorsal

horn of the spinal cord on neurons in laminae I, II, IV, and V (see Figure 1-1) on both projection neurons and on local circuit interneurons (Nestler et al., 2001). These interneurons process afferent information and convey nociceptive information to motor neurons involved in local withdrawal reflexes and to autonomic neurons involved in spinal autonomic reflexes. They also modulate transmission to higher centres. Consequently, the dorsal horn is not simply a relay station but an important site of integration and modulation of both nociceptive and antinociceptive information (Nestler et al., 2001).

1.1.3 Superficial Dorsal Horn

The two most superficial layers of the dorsal horn, lamina I (also known as the marginal zone) and lamina II (also known as the substantia gelatinosa), receive Aδ- and C-fibre input and are particularly important in the processing of nociceptive information (Figure 1-2) (Snider & McMahon, 1998; Grudt & Perl, 2002). The majority (80%) of lamina I neurons are nociceptive, and a small proportion of these can project to a number of different brain nuclei via the spinothalamic, spinoreticular, and spinomesencephalic tracts (Craig & Dostrovsky, 1999; Todd, McGill, & Shehab, 2000). It is estimated that only 5% of lamina I neurons are projection neurons; the remaining nociceptive neurons are likely local excitatory and inhibitory interneurons (Dahlhaus, Ruscheweyh, & Sandkuhler, 2005; Spike et al., 2003). Of the lamina I neurons that are not nociceptors (20%) (Todd et al., 2000), most have been identified as either thermoreceptive neurons or wide dynamic range type neurons (Christensen & Perl, 1970). Lamina I neurons can be distinguished on the basis of morphology and intrinsic firing patterns; currently, three

different morphological classes and four different firing patterns have been identified (Prescott & de Koninck, 2002).

A recent paper by Prescott & de Koninck (2002) reported that there is a significant correlation between morphological and physiological cell types, thus it is thought that there are three broad classes of neurons in lamina I (Figure 1-3). Tonic cells are neurons which are easily excited to fire action potentials and tend to fire regularly and throughout the duration of the depolarizing pulse; these tend to have fusiform morphology, that is, characterized by two primary dendrites emerging from opposite ends of the soma, elongated in the rostro-caudal axis. The second class, phasic cells, are able to achieve high firing rates in response to stronger stimuli, though they discontinue this firing abruptly before the end of stimulation; these tend to have pyramidal morphology, with three primary dendrites emerging longitudinally from separate points of a triangularly shaped soma. The last class comprised two different physiological types (delayed onset neurons and single spike neurons) with similar multipolar morphology, namely, having at least four primary dendrites with extensive arborization. Delayed onset neurons show a marked delay to the first action potential in response to a current pulse, while single spike neurons fire only a single action potential or a very short burst in response to the strong stimulation required to elicit any response from this cell type. The correlation of morphology with physiological (firing) properties has not been consistent across laboratories, indicating that differences in recording conditions, species, or age may play a role in proper characterization of lamina I neurons; thus, the categories defined above are still under investigation and should be interpreted with caution (Dougherty, Sawchuk, & Hochman, 2005).

While some lamina I neurons can project to a number of different regions in the CNS, it seems as though lamina II neurons are almost exclusively interneurons, whose axons synapse with projection neurons of lamina I or other interneurons in the substantia gelatinosa (Lu & Perl, 2005; Grudt & Perl, 2002). Lamina II neurons have been studied extensively and are known to integrate nociceptive input from afferent fibres via both excitatory and inhibitory interactions made possible by the intricate circuitry of the superficial dorsal horn (Melzack & Wall, 1965).

The morphology, electrophysiology, and connectivity of neurons in lamina II were recently characterized by Lu and Perl (2005), and based on this and other analyses, there are several main classes of neurons found in the substantia gelatinosa (Figure 1-4). Transient central neurons display a prompt but brief AP discharge in response to maintained step depolarization and have primarily rostrocaudal dendritic arborization. Vertical neurons have a delayed AP discharge in response to maintained step depolarization and display dorsoventral and rostrocaudal dendritic arborization. Lastly, islet neurons are characterized by a tonic AP discharge in response to maintained step depolarization and have extended, dense rostrocaudal dendritic arborization. A potential schema of the superficial dorsal horn connectivity is illustrated in Figure 1-5.

1.1.4 Synaptic Transmission in the Superficial Dorsal Horn

Glutamate is the predominant excitatory neurotransmitter in laminae I and II, and is released from Aδ and C-fibre nociceptors (Malcangio & Bowery, 1996). Projection neurons of lamina I and putative excitatory interneurons of lamina II are immunoreactive for the calcium binding protein, calbindin-D28K, which is thought to be a marker for excitatory (glutamatergic) neurons (Gamboa-Esteves, Kaye, McWilliam, Lima, & Batten,

2001; Polgar & Antal, 1995). In the rat lamina II, excitatory postsynaptic potentials (EPSPs) are caused by the release of glutamate from the primary afferents, which activates postsynaptic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kainate receptors (Yoshimura & Nishi, 1993). Kainate receptors can also directly regulate γ-aminobutyric acid (GABA) and glycine release in dorsal horn neurons by a presynaptic mechanism (Kerchner, Wang, Qiu, Huettner, & Zhuo, 2001).

GABA and glycine are the main inhibitory neurotransmitters in laminae I and II (Malcangio & Bowery, 1996). These neurotransmitters most likely act as cotransmitters (they are often released from the same vesicle, and both GABA and glycine receptors coexist at postsynaptic sites) and are thought to originate from local inhibitory interneurons (Malcangio & Bowery, 1996; Todd, 1996). Glycine immunoreactive neurons are present in laminae I and II, typically colocalized in cell bodies that are also GABA immunoreactive (Todd, 1996). Lastly, pharmacological blockade of spinal GABA receptors induces pain behaviours in rodents, suggesting that tonic GABAergic inhibition exists and maintains normal sensory responses (Malcangio & Bowery, 1996).

The complexity of the neuronal networks in the superficial laminae of the dorsal horn implies substantial processing and modulation of incoming activity; this can be accomplished by local interneurons, descending pro- or antinociceptive pathways, and chemical mediators released from neurons and glial cells (Grudt & Perl, 2002; Zeilhofer, 2005). Being the first site of synaptic integration of painful stimuli, the spinal dorsal horn is one of the regions that critically contributes to pathologically exaggerated pain sensations (Zeilhofer, 2005).

1.2 Neuropathic Pain

The ability of an animal to detect and react appropriately to an aversive stimulus is of fundamental importance to its survival (Clatworthy, 1999). Pain is the natural consequence of tissue injury and can serve as a biologically useful defense mechanism that warns against existing or imminent damage (Tsuda, Inoue, & Salter, 2005). In contrast to this nociceptive and acute pain, neuropathic pain (chronic, intractable pain due to nerve injury), serves no biological purpose and can be debilitating and cause extreme physical, psychological, and social distress (DeLeo & Colburn, 1999). This type of pain is no longer a protective signal of disease or injury, since it is chronic and outlasts the healing process; rather, the pain itself becomes the disease process.

Neuropathic pain typically develops after central or peripheral nerves are damaged, such as through trauma, surgery, cancer, diabetes, or infection (Loeser, 1990), and persists long after the initiating event has healed (Woolf & Salter, 2000). Neuropathic pain can also develop after nervous system dysfunction (Merskey & Bogduk, 1994), as in multiple sclerosis (Svendsen, Jensen, Hansen, & Bach, 2005) and amyotrophic lateral sclerosis (Galer et al., 2000). The prevalence of chronic pain in North America, Europe, and Australia in the general population is approximately 11% among adults (Harstall & Ospina, 2003), and neuropathic pain, a subset of chronic pain, is also common, with an estimated prevalence in the general population of 1.5% (Taylor, 2006). Neuropathic pain is among the most costly afflictions: current studies indicate that there is a significant use of health care resources by chronic pain sufferers (Harstall & Ospina, 2003), and in the U.S., annual healthcare charges are three-fold higher for neuropathic patients than for age- and sex-matched controls (Berger, Dukes, & Oster, 2004). Ironically, recent data

suggests that these patients are not receiving the correct pharmacological therapy, and further, that almost one quarter are receiving no treatment for pain (Berger et al., 2004). In a study of 55 686 patients with painful peripheral neuropathies, the largest percentage of patients received a short-acting opioid for treatment (53.2%), and opioids of any type were the most commonly used class (53.9%) (Berger et al., 2004). This is cause for concern, since neuropathic pain is known to be poorly controlled by opioid analgesics (Watkins, Hutchinson, Johnston, & Maier, 2005; Arner & Meyerson, 1988): opioids provide partial relief at best, and are often used inappropriately and in excess (DeLeo & Colburn, 1999). The next largest percentage (39.7%) was being treated with nonsteroidal anti-inflammatory drugs (NSAIDs), which have no effect on neuropathic (i.e. non-inflammatory) pain (Argoff et al., 2006). Two other classes of agents with little or no evidence of efficacy in neuropathic pain, benzodiazepines and selective serotonin reuptake inhibitors (SSRIs), were also widely used, with 21.1% and 14.3% of patients, respectively, receiving them for treatment (Berger et al., 2004). Certain newer antiepileptic drugs, such as gabapentin and pregabalin, are currently the most effective drug therapy for some forms of neuropathic pain (Guay, 2005). The exact mechanism of action is not known, but both gabapentin and pregabalin bind to the $\alpha 2\delta$ subunit of voltage-gated calcium channels (N and P/Q) and may produce analgesia in inflammatory pain states by suppressing release of neuropeptides (e.g. substance P, calcitonin gene-related peptide [CGRP]) (Guay, 2005). Though these drugs have relatively widespread applicability (they are approved for the treatment of diabetic peripheral neuropathy and postherpetic neuralgia), both drugs require multiple daily dosing and can produce undesirable side effects (e.g. dizziness and/or somnolence; 21-26% incidence) (Guay,

2005). Currently, there is no universally effective treatment for neuropathic pain that parallels morphine for nociceptive pain (Woolf & Salter, 2000; Hansson & Dickenson, 2005; Zimmermann, 2001; Woolf, 2004).

Although neuropathic pain can arise from a variety of etiologically different clinical conditions, there are two nociceptive symptoms that are common to this disease: allodynia, which is pain due to a stimulus which does not normally provoke pain (e.g. clothing brushing against the skin being perceived as very painful instead of innocuous) and hyperalgesia, which is defined as an increased response to a stimulus which is normally painful (e.g. a pin prick on skin being perceived as very painful instead of slightly painful) (Merskey & Bogduk, 1994). Hyperalgesia is also a symptom of normal pain conditions that dissipates as tissue healing occurs; an example is the extreme pain sensation that is felt when one has a sunburn and takes a hot shower at a temperature that would normally (i.e. without a sunburn) cause slight discomfort. It must be emphasized that the mechanisms underlying these symptoms are not well understood, and that similar symptoms may not necessarily indicate similar mechanisms (Hansson & Dickenson, 2005).

The main thrust of research on neuropathic pain has been to investigate changes that occur in neurons and neuronal function in the peripheral and central nervous systems after trauma or inflammation (Tsuda et al., 2005). Molecular and cellular alterations are known to occur at various levels of the peripheral and central nervous systems after nerve injury and inflammation, and these result in neuronal plasticity and anatomical reorganization (Tsuda et al., 2005; Woolf & Salter, 2000; Zimmermann, 2001; Woolf, 2004). Although many of these changes are biologically useful and fade away once the

initial insult subsides, some alterations can drive the pathological operation of the pain system if they become long-lasting and somewhat irreversible, such that the system is grossly and aberrantly modified (Woolf & Salter, 2000). The cellular processes that initiate and maintain neuropathic pain are poorly understood, though there are several mechanisms at various levels of the nervous system which have been suggested to be relevant (Hansson & Dickenson, 2005); these are: changes in neuronal behaviour and/or chemical environment leading to increased activity at and around the site of injury (Devor, Lomazov, & Matzner, 1994; Waxman, Dib Hajj, Cummins, & Black, 1999), changes in transmitter release through alterations in various channels (Luo et al., 2001; Matthews & Dickenson, 2001), spinal hyperexcitability, also called central sensitization (Dickenson, Matthews, & Suzuki, 2001), and increases in descending pain facilitation (Porreca, Ossipov, & Gebhart, 2002).

There is much literature to implicate central sensitization in the development of neuropathic pain (Woolf & Salter, 2000; Woolf, 1983; Dalal et al., 1999; Moore et al., 2002). Central sensitization arises when the heightened excitability of damaged axons spreads to higher centres, namely the dorsal horn of the spinal cord and brainstem nuclei (Sommer & Kress, 2004), and is characterized by spontaneous neuronal activity, lowered activation threshold, and increased response to a given stimulus in neurons of the dorsal horn of the spinal cord (Woolf & Salter, 2000; Woolf, 1983; Dalal et al., 1999; Moore et al., 2002). Modulation of the nociceptive pathway, specifically, disinhibition and spontaneous excitation of neurons in the dorsal horn, is a salient possible mechanism not only because it explains why pain persists after the initial stimulus subsides, but also

because the cellular and molecular mechanisms producing this enhancement represent potential therapeutic targets.

To elucidate these cellular and molecular mechanisms, many recent studies have focused on the role of the immune system and glial cells in neuropathy and neuropathic pain (Tsuda et al., 2005; Watkins & Maier, 2003; Watkins, Milligan, & Maier, 2001a; Watkins, Milligan, & Maier, 2001b; McMahon, Cafferty, & Marchand, 2005; Watkins & Maier, 2004). It has been estimated that half of all clinical cases of neuropathic pain are associated with infection or inflammation of peripheral nerves rather than with nerve trauma (Said & Hontebeyrie-Joskowicz, 1992). Evidence has accumulated showing that injury to the nervous system is associated with activation and proliferation of glial cells (Tsuda et al., 2005; Stuesse, Cruce, Lovell, McBurney, & Crisp, 2000; Hashizume, DeLeo, Colburn, & Weinstein, 2000; Ohtori, Takahashi, Moriya, & Myers, 2004), and that stimuli that initiate neuropathic pain also initiate the activation of microglia within the CNS (Tsuda et al., 2005; Watkins, Maier, & Goehler, 1995; Watkins & Maier, 2002; Milligan et al., 2003). Moreover, general inhibitors of glial function attenuate neuropathy- or inflammation-induced hyperalgesia and allodynia (Milligan et al., 2003; Ledeboer et al., 2005; Meller, Dykstra, Grzybycki, Murphy, & Gebhart, 1994; Milligan et al., 2000; Sweitzer, Schubert, & DeLeo, 2001), further underscoring a significant role of glial cells in establishing neuropathic pain. As somewhat of a paradox, it must be remembered that although neuropathic pain is defined clinically as "non-inflammatory" pain, neuro-immune facilitatory cascades that occur at the site of injury and centrally are crucial in the establishment of central sensitization. Thus, although the traditional view is that chronic pain enhancement is mediated solely by neurons, accumulating evidence

suggests that spinal cord glial cells importantly contribute to pain facilitation (Watkins & Maier, 2003; Watkins et al., 2001a; DeLeo & Yezierski, 2001; Ledeboer et al., 2005).

1.3 Role of Microglia in Neuropathic Pain

Neuronal and glial interactions mediate central nervous system (CNS) healing and repair as well as degeneration in response to nervous system injury (Coltman & Ide, 1996). Glial cells account for approximately 90% of the cells in the CNS and 50% of the volume in the brain and spinal cord (Vander, Sherman, & Luciano, 2001). Glial cells physically and metabolically support neurons, and can be divided into three groups: oligodendroglia, which form the myelin covering of CNS axons; astroglia , which metabolically sustain neurons, can stimulate neuronal growth, and may participate in neurotransmitter processing and information signaling in the brain; and microglia, which perform immune functions in the CNS and play a key role in pain hypersensitivity after nerve injury (Tsuda et al., 2005; Vander et al., 2001). The latter, when activated, are analogous to marchophages in the periphery; these two cell types share many phenotypic markers and effector molecules, making it difficult to distinguish between the two in pathological settings (Stoll & Jander, 1999).

Microglia represent 5-10% of glia in the CNS, and act as sensors for a range of stimuli that threaten physiological homeostasis, including CNS trauma, ischemia, and infection (Tsuda et al., 2005). Under normal conditions, microglia assume a resting, ramified form (Brierly & Brown, 1982), yet following nerve injury, inflammation, or chemical insult, microglia become activated and change their morphology to an ameboid, reactive form, morphologically and functionally similar to macrophages and monocytes (Tsuda et al., 2005; Coltman & Ide, 1996; Ledeboer et al., 2005; Piao et al., 2006).

Activation of microglia in the spinal cord dorsal horn, which is a relatively uniform process regardless of the underlying insult, also includes the ability to proliferate rapidly, as well as the upregulation of cell surface molecules such as complement type-3 receptor (CR-3), major histocompatibility complex (MHC) class I and II antigens, cluster determinants 4 and 14 (CD4, CD14), and toll-like receptor 4 (TLR4) (Stoll & Jander, 1999; Raghavendra, Tanga & DeLeo, 2003; Sweitzer, White, Dutta, & DeLeo, 2002; Tanga, Raghavendra, & DeLeo, 2004). Activated microglia synthesize and release a variety of potentially harmful soluble factors. These include reactive oxygen species, nitric oxide, peroxynitrite, proteolytic enzymes, arachidonic acid metabolites, and proinflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), all of which have the potential to contribute to central sensitization in the spinal dorsal horn (McMahon et al., 2005; Stoll & Jander, 1999).

In addition to the positive correlation between spinal microglial activation in vivo and the development or maintenance of pain hypersensitivity (Tsuda et al., 2005; Watkins & Maier, 2003), it has been shown that intrathecal injections of activated microglia simulate signs of neuropathic pain (Tsuda et al., 2003). Further, studies using fluorocitrate, a selective blocker of astrocyte and microglia metabolism (Hassel, Paulsen, Johnsen, & Fonnum, 1992), and the antibiotic minocycline, which inhibits microglial activation without affecting neurons or astrocytes (Zhang, Goetz, & Duncan, 2003), have shown that both compounds can block neuropathic states (Milligan et al., 2003; Ledeboer et al., 2005; Raghavendra et al., 2003). This evidence suggests that activated glia play a role in abnormal sensory processing, though the relative importance of microglia in various forms of pain is currently uncertain (McMahon et al., 2005), as are the precise

mechanisms by which glia become activated (Tsuda et al., 2005). At present, there has not been any direct identification of factors that activate microglia, though many substances have been reported to trigger CNS microglia (McMahon et al., 2005), including ATP (Tsuda et al., 2003; Hide et al., 2000; Ferrari, Chiozzi, Falzoni, Hanau, & Di Virgilio, 1997), cytokines (TNF-α [Chao, Hu, & Peterson, 1995a; Chao, Hu, Sheng, & Peterson, 1995b; Chao, Hu, Sheng, Tsang, & Peterson, 1995c], IL-6 [Sheng, Hu, Kravitz, Peterson, & Chao, 1995], IL-1β [Chao et al., 1995a; Chao et al., 1995b; Chao et al., 1995c]), chemokines (CX3CL1, also known as fractalkine [Verge et al., 2004; Milligan et al., 2004], CCL-2, also known as MCP-1 [Tanaka, Minami, Nakagawa, & Satoh, 2004]), glutamate (Svensson, Hua, Protter, Powell, & Yaksh, 2003a), and peptides (substance P [Svensson et al., 2003b], CGRP [Priller, Haas, Reddington, & Kreutzberg, 1995], galanin [Priller, Hass, Reddington, & Kreutzberg, 1998]).

Many animal models of neuropathic pain, such as compression, ligation, or transection of the sciatic nerve (Bennett & Xie, 1988; Wall et al., 1979; Seltzer, Dubner, & Shir, 1990), are characterized by microglial activation post-injury, though the extent and time course of the changes can vary among the models (Colburn et al., 1997; Colburn, Rickman, & DeLeo, 1999). Spinal microglial activation in response to peripheral nerve injury can be observed within 24 hours, is maximal between days three and seven post-injury, and lasts for approximately four weeks (Tsuda et al., 2005; Coltman & Ide, 1996; Eriksson et al., 1993). There is no definitive marker for microglial cell activation (Guillemin & Brew, 2004), but many studies rely on immunocytochemistry for OX-42, which labels complement receptor type 3 (CR-3) (McMahon et al., 2005). Activated microglia exhibit upregulated OX-42 labeling (Coyle,

1998), and the temporal pattern of OX-42 upregulation in the dorsal horn correlates with the development of tactile allodynia and the development of pain hypersensitivity in a wide variety of nerve injury models (Watkins et al., 2001a; Stuesse et al., 2000; Colburn et al., 1999; Eriksson et al., 1993; Coyle, 1998). There is good evidence that one central mechanism in the manifestation of neuropathic pain is the release of pro-inflammatory cytokines (Watkins & Maier, 2003), and the ensuing upregulation of the enzyme cyclo-oxygenase 2 (COX-2) and subsequent release of prostanoids (McMahon et al., 2005). In particular, IL-1β is responsible, perhaps in conjunction with other cytokines such as IL-6 or IL-18, for central transcriptional activation of COX-2 after peripheral trauma and inflammation, and it is now known that COX-2 induction is the major limiting factor in the production or release of prostaglandins such as PGE_2 (Samad et al., 2001). There are a number of selective COX-2 inhibitors, such as celecoxib (Celebrex) (Penning et al., 1997) and rofecoxib (Vioxx) (Prasit et al., 1999) that are effective at treating pain due to inflammation (Clemett & Goa, 2000), but these have little efficacy in neuropathic pain conditions, most likely due to their poor CNS penetration. In point of fact, a recent study (Allan, Tyrrell, & Rothwell, 2005) using a more potent and more CNS penetrant COX-2 inhibitor, GW406381X, showed that this drug was effective in three models of neuropathic pain and central sensitization (rat chronic constriction injury [CCI], mouse partial ligation, and capsaicin-induced allodynia), further underscoring a major role for COX-2 induction in the onset of neuropathic pain following trauma-induced microglial activation.

To summarize, it is thought that immediately after nerve injury, damaged cells activate molecular pathways via a collection of chemical mediators that ultimately lead to

microglial activation in the dorsal horn. Activated glia then release pro-inflammatory cytokines, among other chemical signals, and it is hypothesized that these cytokines, particularly IL-1β, initiate lasting changes in the properties of the spinal pain-processing network to bring about injury-induced pain hypersensitivity (Tsuda et al., 2005) (Figure 1-6).

1.4 The Interleukin-1 Family

The IL-1 family in mammals contains at least ten ligand proteins and 10 receptor molecules, and plays an important role in inflammation and host defense (DeLeo & Colburn, 1999; Allan et al., 2005; Huising, Stet, Savelkoul, & Verburg-van Kemenade, 2004). IL-1α and IL-1β were the first members of the IL-1 family to be identified (March et al., 1985). Although they are products of different genes, they have high sequence homology and exert similar biological effects, though IL-1β is more potent in activating humoral immune responses (Nakae, Asano, Horai, & Iwakura, 2001) and is predominant in the brain (Rothwell, 1991). They are synthesized as large precursor proteins by various cells of the peripheral and central immune system, such as lymphocytes and monocytes (Allan et al., 2005). Pro-IL-1α is biologically active and is cleaved by calpain to generate the smaller, mature protein; both of these forms remain mostly intracellular (Huising et al., 2004). Pro-IL-1β, on the other hand, is biologically inactive and requires cleavage by caspase-1 to produce the active 17kDa protein (Thornberry et al., 1992).

Both IL-1α and IL-1β bind the membrane-bound type I IL-1 receptor (IL-1R1), which then associates with the IL-1 receptor accessory protein (IL-1RAcP) to form a

complex that allows intracellular signaling (DeLeo & Colburn, 1999; Sims et al., 1988; Korherr, Hofmeister, Wesche, & Falk, 1997). There is also a type II IL-1 receptor (IL-1R2), but it lacks an intracellular signaling domain, thus no downstream signal is initiated when IL-1 binds, and therefore, IL-1R2 is likely a decoy receptor (Subramaniam, Stansberg, & Cunningham, 2004). All three receptor molecules, IL-1R1, IL-1R2, and IL-1RAcP, can be shed from the cell membrane and consequently exist in soluble forms, sIL-1R1, sIL-1R2, sIL-1RAcP, respectively (Allan et al., 2005). sIL-1R2 and sIL-1RAcP both function as inhibitors of IL-1-mediated signal transduction by sequestering pro-IL-1β and IL-1R1, respectively. Additionally, it has also been proposed that signal transduction via IL-1R2 could be initiated if membrane-bound IL-1 were to bind the soluble IL-1R1 (sIL-1R1), and the subsequent association with IL-1RAcP occurred (Allan et al., 2005).

The third ligand member of the IL-1 family that was discovered is a naturally occurring competitive IL-1 receptor antagonist (IL-1RA) (Hannum et al., 1990). IL-1RA is produced by the same cells that express IL-1. There are three intracellular isoforms of IL-1RA: icIL-1RA1, icIL-1RA2, and icIL-1RA3, and there is one secreted isoform: sIL-1RA (Allan et al., 2005). The secreted isoform functions as a competitive antagonist that binds IL-1R1 but does not trigger signal transduction, while the other isoforms currently have poorly defined roles (Malyak, Smith, Abel, Hance, & Arend, 1998).

The fourth member of the IL-1 family to be discovered was IL-18 (formerly identified to be interferon-γ-inducing factor) (Allan et al., 2005). IL-18 is similar to IL-1β in that its pro-form is inactive and requires cleavage by caspase-1 to produce the mature, biologically active protein (Dinarello et al., 1998). IL-18 binds to the IL-18

receptor (IL-18R, previously known as IL-1-receptor-related protein), and both the IL-18 receptor and the IL-1 receptor belong to the IL-1 receptor and Toll-like receptor (TLR) superfamily, which plays a key role in the immune response (Dunne & O'Neill, 2003). These related receptors all have sequence similarity in their cytosolic regions: this conserved region has been termed the Toll-IL-1 receptor (TIR) domain, and it triggers a complex series of signaling events in the target cell that results in the activation of the transcription factor nuclear factor-κB (NF-κB) as well as the stress-related mitogen-activated protein kinases (MAPKs) (Dunne & O'Neill, 2003). This is followed by transcription of multiple inflammation-associated genes, including those that encode the following: chemokines (such as CXC-chemokine ligand 8 [CXCL8; also known as IL-8] and CX3C-chemokine ligand 1 [CX3CL1; also known as fractalkine], cytokines (such as IL-6 and tumor necrosis factor [TNF]), and adhesion molecules (such as endothelial-cell selectin [E-selectin] and intracellular adhesion molecule 1 [ICAM1]) (Subramaniam et al., 2004).

The remaining six putative ligand members of the IL-1 family are known as IL-1F5 (IL-1 family, member 5), IL-1F6, IL-1F7, IL-1F8, IL-1F9, and IL-1F10 (Allan et al., 2005). All members of this family are constitutively expressed in the brain at very low concentrations (Vitkovic, Bockaert, & Jacque, 2000). Since IL-1β is the most potent and well-characterized ligand agonist in the IL-1 family with respect to central nervous system function and injury, it will be the main focus of this section.

1.4.1 Regulation of IL-1β

A diverse range of stimuli can lead to changes in IL-1 expression at the mRNA and/or protein level (Allan et al., 2005). At the transcriptional level, gene expression of

IL-1β can be induced by various proinflammatory stimuli (such as bacterial and viral antigens), other cytokines (such as TNF), cellular injury, and hypoxia (Hsu & Wen, 2002; Perregaux, Bhavsar, Contillo, Shi, & Gabel, 2002). Transcription is also influenced by various factors that recognize and bind DNA regulatory sites, such as the cyclic AMP response element, the activator protein 1 (AP1) binding site, the NF-κB binding site, and the lipopolysaccharide (LPS)-response enhancer site (Watkins, Hansen, Nguyen, Lee, & Maier, 1999). Lastly, several other intracellular and extracellular signals can function to increase or decrease the rate of IL-1 transcription; factors that can increase transcription are complement components, prostaglandin E_2 (PGE_2), the β-chain of the S100 calcium-binding protein (S100B; also known as neurite extension factor), β-amyloid, and excitotoxins; factors that inhibit IL-1 transcription are glucocorticoids and their anti-inflammatory mediator, annexin-1 (also known as lipocortin-1) (Eriksson, Tehranian, Iverfeldt, Winblad, & Schultzberg, 2000; Liu, Li, Van Eldik, Griffin, & Barger, 2005; Relton et al., 1991). Regulation of IL-1 activity can also occur at the post-transcriptional and translational levels; for example, LPS can increase IL-1β protein production by increasing mRNA stability, translation can be increased by epidermal growth factor, corticotropin-releasing hormone, and ICAM1, while dexamethasone, a synthetic glucocorticoid, can inhibit IL-1 translation (Watkins et al., 1999; Kern, Warnock, & McCafferty, 1997).

At the post-translational level, the regulation of cellular release is also a control point for changes in IL-1 levels. The expression and cleavage of pro-IL-1β, and subsequent cellular release of IL-1β, may involve activation of P2X7 (a purinergic ligand-gated ion channel) by ATP in a caspase-1-dependent process that likely depends

on the release of calcium from intracellular stores and on the activation of phospholipase C and phospholipase A_2 (Hazuda, Strickler, Kueppers, Simon, & Young, 1990; Perregaux & Gabel, 1994; Le Feuvre, Brough, Iwakura, Takeda, & Rothwell, 2002; Brough et al., 2003; Andrei et al., 2004). Treatment of microglial cells or macrophages with LPS induces expression of pro-IL-1β, but not cleavage or release. In LPS-primed macrophages, however, activation of P2X7 by ATP or ADP elicits both cleavage and release of IL-1β via caspase-1 (Le Feuvre et al., 2002). Another candidate molecule, the anti-microbial peptide LL37 (also known as CAMP) has been shown to be an inducer of IL-1β release through activation of the P2X7 receptor (Elssner, Duncan, Gavrilin, & Wewers, 2004). Lastly, a novel family of cytokine-release inhibitory drugs (CRIDs) have been shown to block IL-1β release independent of P2X7 activation (Perregaux et al., 2001); one of the main targets of CRIDs is glutathione S-transferase-ω1-1 (GSTO-1), an enzyme which may be a key regulator of IL-1β release (Laliberte et al., 2003).

1.4.2 IL-1β Involvement in CNS Injury and Inflammation

IL-1β that is produced during injury or inflammation to the central nervous system influences not only the local environment, but has actions that modify systemic functions, such as appetite, slow-wave sleep, body temperature, neuroendocrine function, and peripheral immune function (Allan et al., 2005; Vitkovic et al., 2000). These systemic responses, many of them contributing to host defense (i.e. sickness behaviour, fever, and the acute-phase response [Kelley et al., 2003]), are mediated by the effects of IL-1β on neuronal output and on hormonal influences, mainly through the release of corticotropin-releasing hormone and subsequent activation of the hypothalamic-pituitary-adrenal

(HPA) axis (Berkenbosch, van Oers, del Ray, Tilders, & Besedovsky, 1987). Conversely, the local effects of IL-1β on glial cells, neurons, endothelial cells, and immune cells that invade the brain during injury markedly contribute to local inflammation and may be involved in the induction and maintenance of neuropathic pain.

Interleukin-1β is secreted under pathological conditions associated with increased pain and hyperalgesia (Sommer & Kress, 2004; Watkins & Maier, 1999; Watkins et al., 1994; Davis & Perkins, 1994; Perkins & Kelly, 1994). Expression of IL-1β is upregulated rapidly (within hours) at the protein level following experimentally induced neurotoxic stimuli or animal models of neuropathic pain (Rotshenker, Aamar, & Barak, 1992; Allan et al., 2005). In particular, it has been shown that IL-1β production is enhanced in the periphery following crush injury to a peripheral nerve, and in microglia and astrocytes after CNS trauma (Rotshenker et al., 1992; DeLeo, Colburn, & Rickman, 1997; Sweitzer, Colburn, Rutkowski, & DeLeo, 1999). Although all endogenous brain cells (i.e. neurons, glial cells, and vascular endothelial cells) can express IL-1, early expression after injury is seen primarily in monocytes and macrophages, while later expression occurs in astrocytes (Boutin, Kimber, Rothwell, & Pinteaux, 2003). In addition to being associated with pain symptoms and conditions, IL-1β has been suggested to play a direct role in the pathophysiology of nerve injury and inflammation. Research has shown that IL-1β is capable of inducing neuropathic symptoms when delivered centrally or intrathecally (Watkins et al., 1994; Maier, Wiertelak, Martin, & Watkins, 1993; Ferreira, Lorenzetti, Bristow, & Poole, 1988; Fukuota, Kawatani, Hisamitsu, & Takeshige, 1994; DeLeo et al., 1997; Sweitzer et al., 1999; Oka, Oka, Hosoi, Aou, & Hori, 1995), though the most convincing evidence comes from studies

using the IL-1 receptor antagonist or antibodies against IL-1β (Watkins et al., 1994; Maier et al., 1993; Safieh-Garabedian, Poole, Allchorne, Winter, & Woolf, 1995; Cunha, Cunha, Poole, & Ferreira, 2000; Sommer, Petrausch, Lindenlaub, & Tokya, 1999; Relton & Rothwell, 1992; Rothwell, 2003).

Injections or local administration of IL-1β induce mechanical and thermal hyperalgesia in the rat (Watkins et al., 1994, Maier et al., 1993; Ferreira et al., 1988), while IL-1β injected into the hind paw or knee joint has been shown to increase spontaneous firing of sensory nerves, increase neuronal responses to noxious heat and cold stimuli, and decrease the threshold for firing to pressure stimulation (Fukuota et al., 1994). Systemic administration of the IL-1 receptor antagonist, IL-1RA, has been shown to inhibit hyperalgesia mediated by lipopolysaccharide, lithium chloride, complete Freund's adjuvant, or IL-1 (Watkins et al., 1994, Maier et al., Safieh-Garabedian et al., 1995); while overexpression of endogenous IL-1RA markedly inhibits neuronal injury induced by ischaemia, excitotoxins, or trauma (Rothwell, 2003). Studies using antibodies also support the suggestion that IL-1β is neurotoxic and IL-1RA is neuroprotective: neutralizing antibodies to IL-1 receptors have been shown to reduce pain behaviours in mice with experimental neuropathy (Cunha et al., 2000; Sommer et al., 1999), antibodies that neutralize IL-1β or inhibit caspase-1 activity attenuate ischemic brain damage (Yamasaki et al., 1995; Hara et al., 1997), and administration of a neutralizing antibody specific for IL-1RA to rats undergoing occlusion of the middle cerebral artery induced a marked increase in the resultant neuronal injury (Loddick et al., 1997).

Although IL-1β administration has been shown to produce hyperalgesia in rats (Ferreira et al., 1988; Watkins et al., 1994), the nociceptive and anti-nociceptive effects

of central IL-1β are still debated. There is evidence to suggest that IL-1β exerts biphasic responses on thermal and mechanical nociceptive thresholds depending upon the dosage: i.c.v. injection of IL-1β causes hyperalgesia at lower doses, but in contrast, can cause analgesia at higher doses (Coelho, Fioramonti, & Bueno, 2000; Oka, Aou, & Hori, 1993; Oka, Aou, & Hori, 1994; Rady & Fuigmoto, 2001). However, in general, studies have shown that exogenously injected IL-1β is pro-nociceptive in animals, and that blocking the actions of IL-1β can inhibit, prevent, or reduce these pro-nociceptive effects. Thus, there is good evidence for a role of IL-1β in the development and possibly the maintenance of central sensitization and neuropathic pain.

1.5 Organotypic Slice Culture

In vitro techniques are important for studying the physiological and pharmacological properties of neuronal circuits (Gahwiler, Capogna, Debanne, McKinney, & Thompson, 1997). Though acute slices of brain tissue are relatively easy to prepare and retain the cytoarchitechture of the original tissue, they are short-lived, and thus organotypic slice cultures were developed in an attempt to establish in vitro preparations that could be used for long-term studies (Gahwiler et al., 1997; Gahwiler, 1981). The defined-medium organotypic slice culture (DMOTC) technique used in this project is a modified roller tube technique for rat spinal cord slices (Lu et al., 2006), and was adapted from previously published methods (Gahwiler, 1981; Braschler, Iannone, Spenger, Streit, & Luscher, 1989; Ballerini & Galante, 1998). The value of the longevity of organotypic slice cultures in studying mechanisms of neuropathic pain and the effects of IL-1β is immense for two major reasons. First, the phenomenon of neuropathic pain is not quick to develop: many experiments have established that changes in pain

transmission may arise from long-term changes in growth factors such as brain-derived neurotrophic factor (BDNF), inflammatory mediators such as prostaglandins, bradykinin, and cytokines, and neurotransmitters such as glutamate (McMahon et al., 2005; England, Bevan, & Docherty, 1996; Gold, Reichling, Schuster, & Levine, 1996). Secondly, production of IL-1β following injury or inflammation is known to occur over the course of one to two weeks (Coltman & Ide, 1996), thus, long-term applications of IL-1β are a prerequisite for studying its possible role in the development of neuropathic pain.

There are many factors to consider when using organotypic slice cultures in the place of acute slices or in vivo preparations. Age and development are of utmost concern, primarily because slice cultures are usually derived from early postnatal or embryonic animals while acute slices are taken from adult animals (Gahwiler et al., 1997; Howard, Walker, Michael, & Fitzgerald, 2005). With respect to age, organotypic cultures can be left to mature in vitro to allow for age-matched comparisons. Developmentally, although organotypic cultures are subject to differences in environmental cues, it has been recently shown that when matched for age, substantia gelatinosa neurons in DMOTC slices from embryonic rats are electrophysiologically and histologically similar to those in acute slices from young adult rats, suggesting that development in general proceeds similarly in both preparations (Lu et al., 2006). Another potential limitation of organotypic slice cultures is the use of serum-containing medium to maintain neurons (Gahwiler et al., 1997). Medium that contains serum contains undefined trophic components and cytokines, both of which can confound the effects of exogenously applied IL-1β; to minimize this, the culture system (DMOTC) used in this project is maintained with serum-free medium (Gahwiler et al., 1997; Lu et al., 2006).

1.5.1 Comparison of DMOTC Neurons With Neurons in Acute Slice Preparation

In a recent study, Lu et al. (2006) compared substantia gelatinosa neurons of DMOTC slices with those in age-matched acute slices from young adult rats, and found that these are electrophysiologically and histologically similar. In both preparations, neuronal morphology was found to be similar to previously defined sections of rodent dorsal horn (Grudt & Perl, 2002; Heinke, Ruscheweyh, Forsthuber, Wunderbaldinger, & Sandkuhler, 2004), and both displayed similarities in synaptic pharmacology and electrophysiological characteristics (Lu et al., 2006). Specifically, the five electrophysiological phenotypes that were delineated according to their action potential discharge patterns in response to a square pulse of depolarizing current from a holding potential of –60mV were similar to those seen in acute slices (Lu et al., 2006) (Figure 1-7). In DMOTC, "Tonic" neurons displayed a continuous, evenly spaced series of action potentials throughout the duration of the pulse (21.5% of neurons studied). "Irregular" neurons were characterized by an initial accommodation but subsequent action potential, and/or a rate of discharge that was disproportionate to the amount of injected current (27% of neurons studied). "Delay" neurons were defined as those that exhibited a clear delay before the initiation of action potential discharge (28.5% of neurons studied). "Transient" neurons discharged only a single action potential in response to all amounts of injected current (14% of neurons studied). Finally, "Phasic" neurons fired a short burst of action potentials and remained silent throughout the remainder of the current pulse (9% of neurons studied) (Lu et al., 2006). There was one main difference between neurons in DMOTC and those in acute slices: membrane excitability and spontaneous synaptic events were increased in the cultures (Lu et al., 2006), though this has been

attributed to synaptic network properties of the cultures rather than to altered properties of individual neurons (Ballerini & Galante, 1998). The DMOTC culture described in this paper is identical to the one used in the current project.

1.6 Hypothesis

Currently, studies examining the effects of IL-1β on neuropathic pain are limited to acute behavioural and immunohistochemical experiments. Although these studies' findings form the basis of our current model of IL-1β function, they cannot further our understanding of the cellular mechanisms underlying the IL-1β-induced changes that occur after injury or inflammation of central or peripheral nerves. Further, there is evidence that microglia producing and releasing IL-1β continue to change in morphology for up to 10 days post-activation (Coltman & Ide, 1996), indicating that to mimic *in vivo* microglial activation and release of IL-1β, long-term applications of IL-1β are required. The defined-medium organotypic slice culture (DMOTC) technique developed in the Smith lab presents a unique and valuable opportunity to examine the long-term effects of IL-1β on neurons of the dorsal horn.

Using DMOTC, I have examined the concentration-dependence and time course of action of IL-1β on neurons of the dorsal region of the rat spinal cord. I have applied IL-1β for a seven-day period as this mimics the time course of microglial activation and spinal cytokine production in response to nerve injury (Coltman & Ide, 1996). My experiments provide much-needed insights into the long-term effects of IL-1β and the underlying cellular mechanisms.

My primary hypothesis is that long-term (7 days) application of IL-1β produces chronic changes in excitatory and inhibitory synaptic transmission. This was tested by employing two concentration paradigms, high (100pM) and low (1pM), as there is evidence that low, physiological doses and high, septic concentrations of IL-1β may have contrasting effects in the CNS (Desson & Ferguson, 2003). The experimental approach involved whole-cell patch-clamp recordings from dorsal horn neurons in defined-medium, organotypic slice culture (DMOTC) and 1-photon confocal imaging of cytosolic Ca^{2+} concentration.

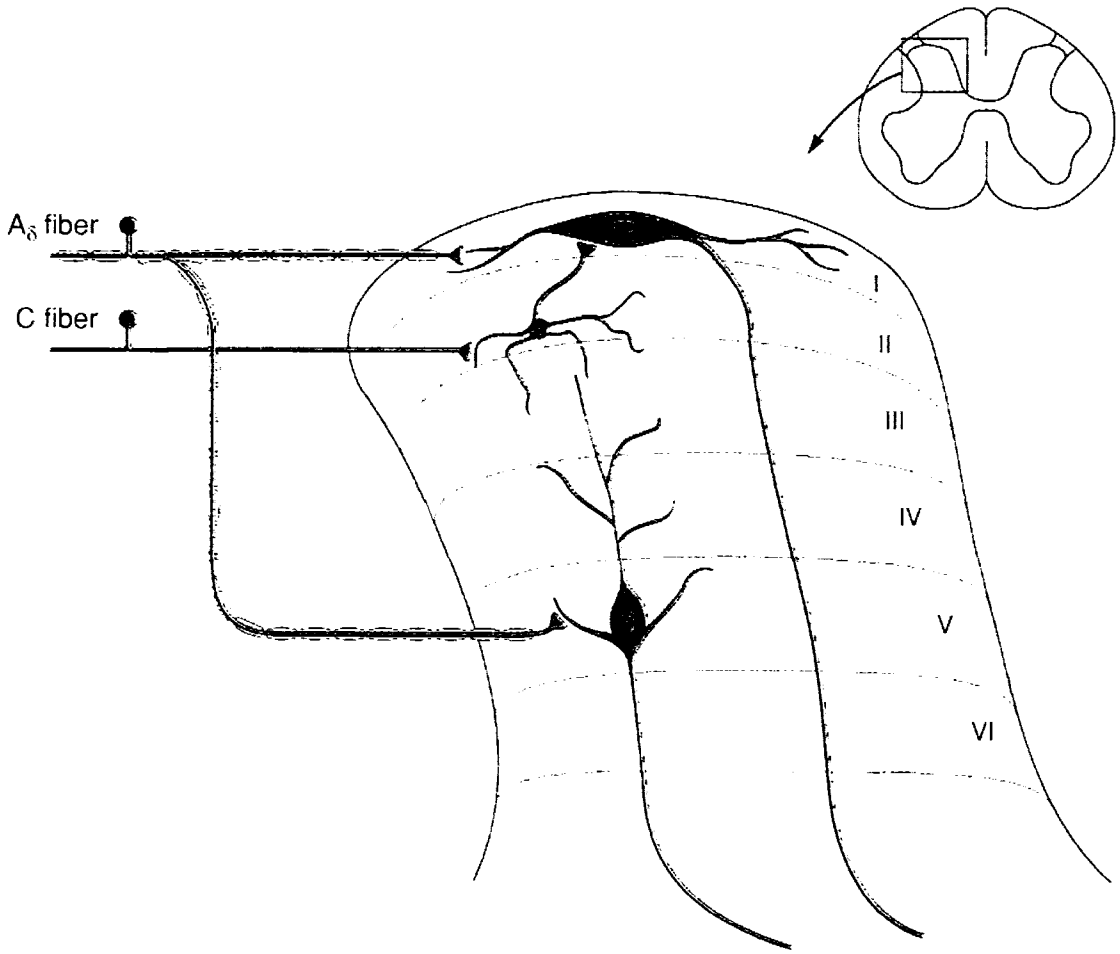


Figure 1-1. Laminae of the dorsal horn of the spinal cord with one of several possible synaptic arrangements of Aδ and C fibres. (Modified from Nestler et al., 2001).

Superficial dorsal horn innervation

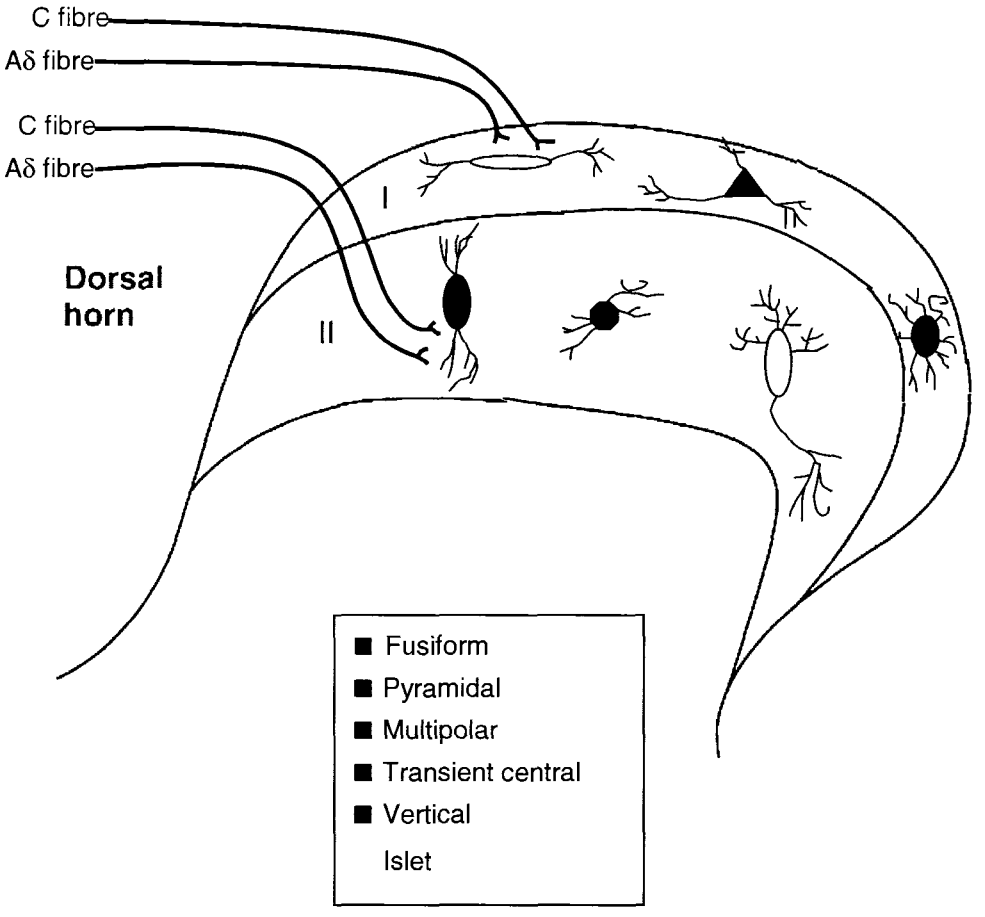


Figure 1-2. Schema of the innervation and cell types found in the superficial dorsal horn.

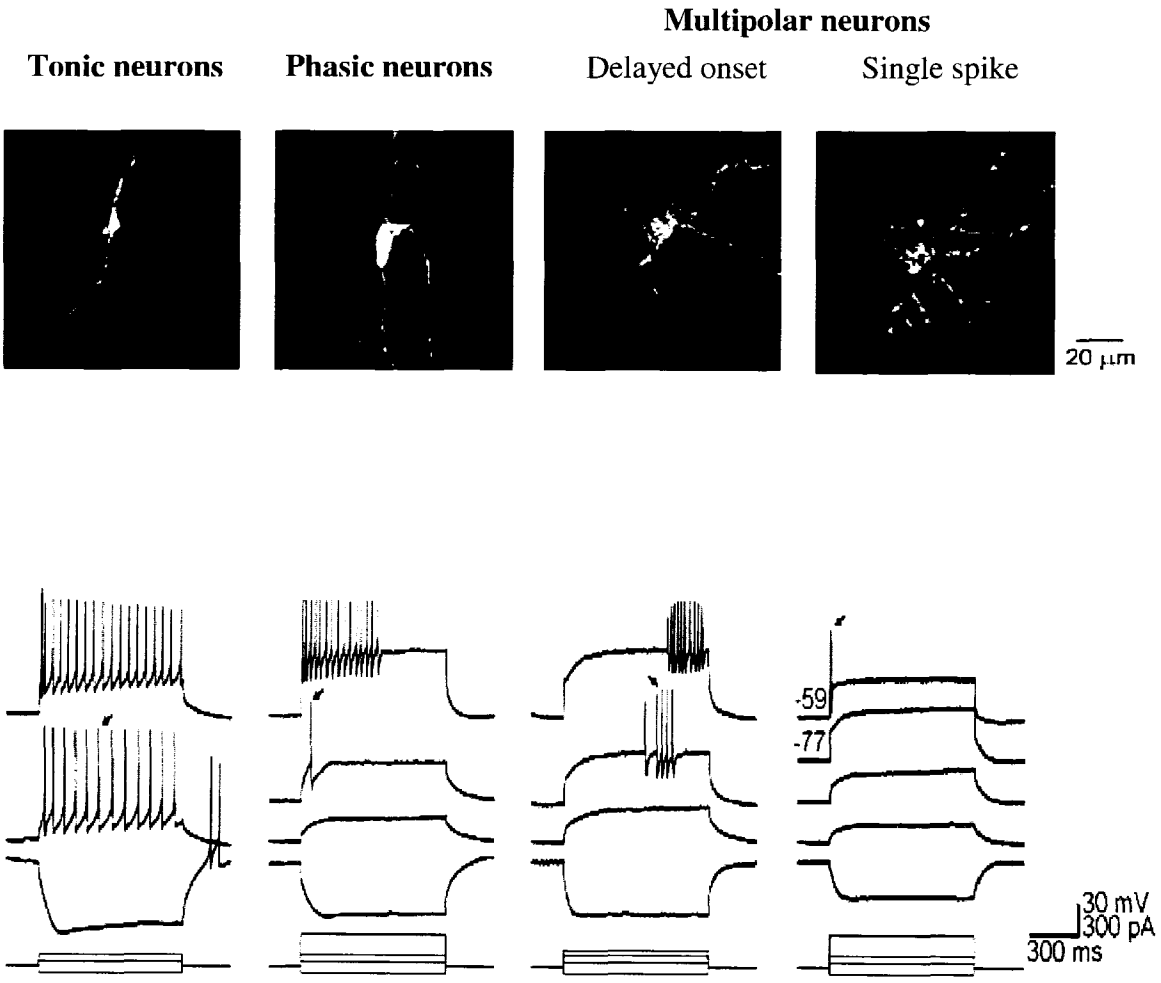


Figure 1-3. Tonic, phasic, and multipolar (delayed onset and single-spike) neurons of Lamina I. (Modified from Prescott & de Koninck, 2002).

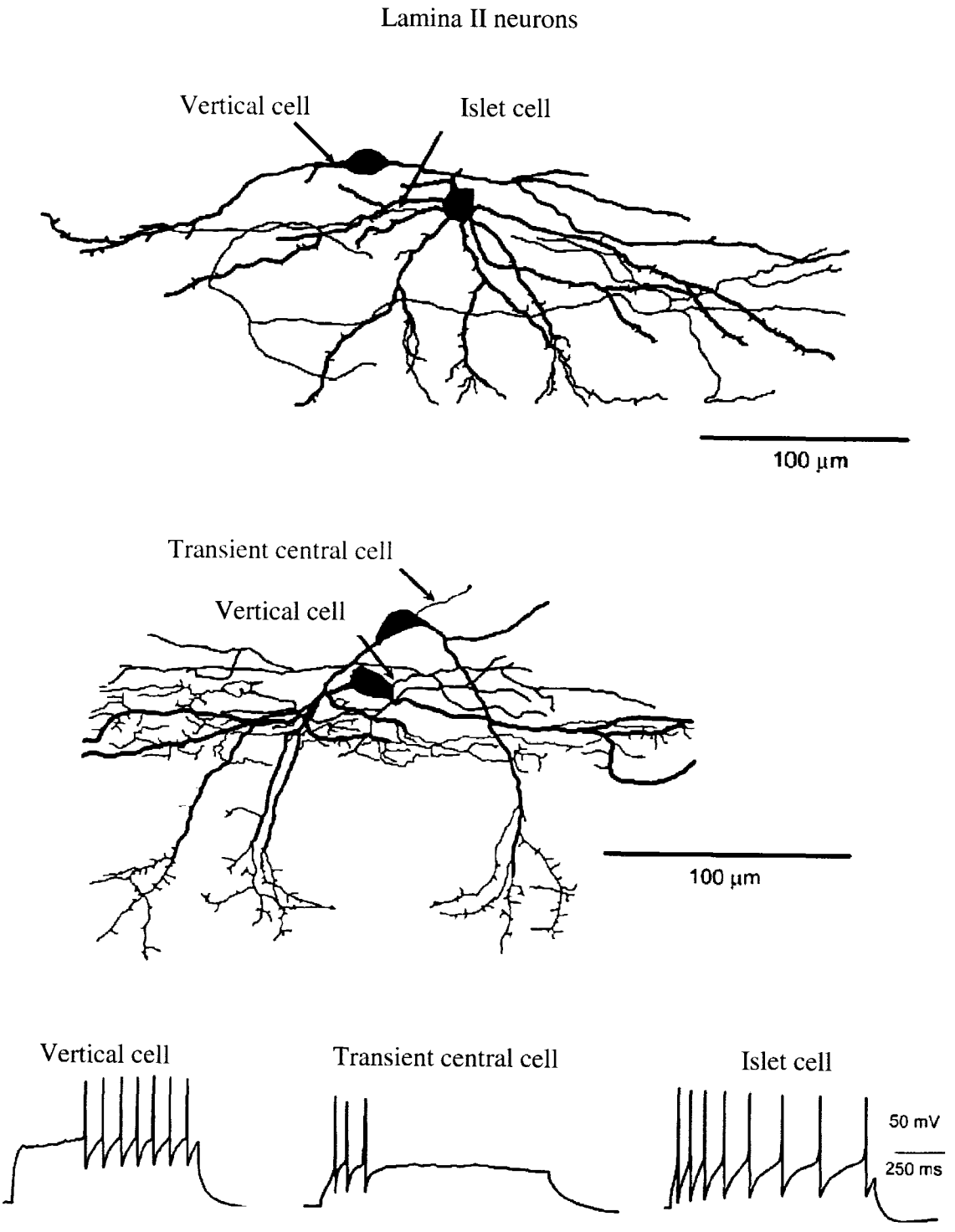


Figure 1-4. Transient central, vertical, and islet neurons of Lamina II. (Modified from Lu & Perl, 2005).

Possible superficial dorsal horn circuitry

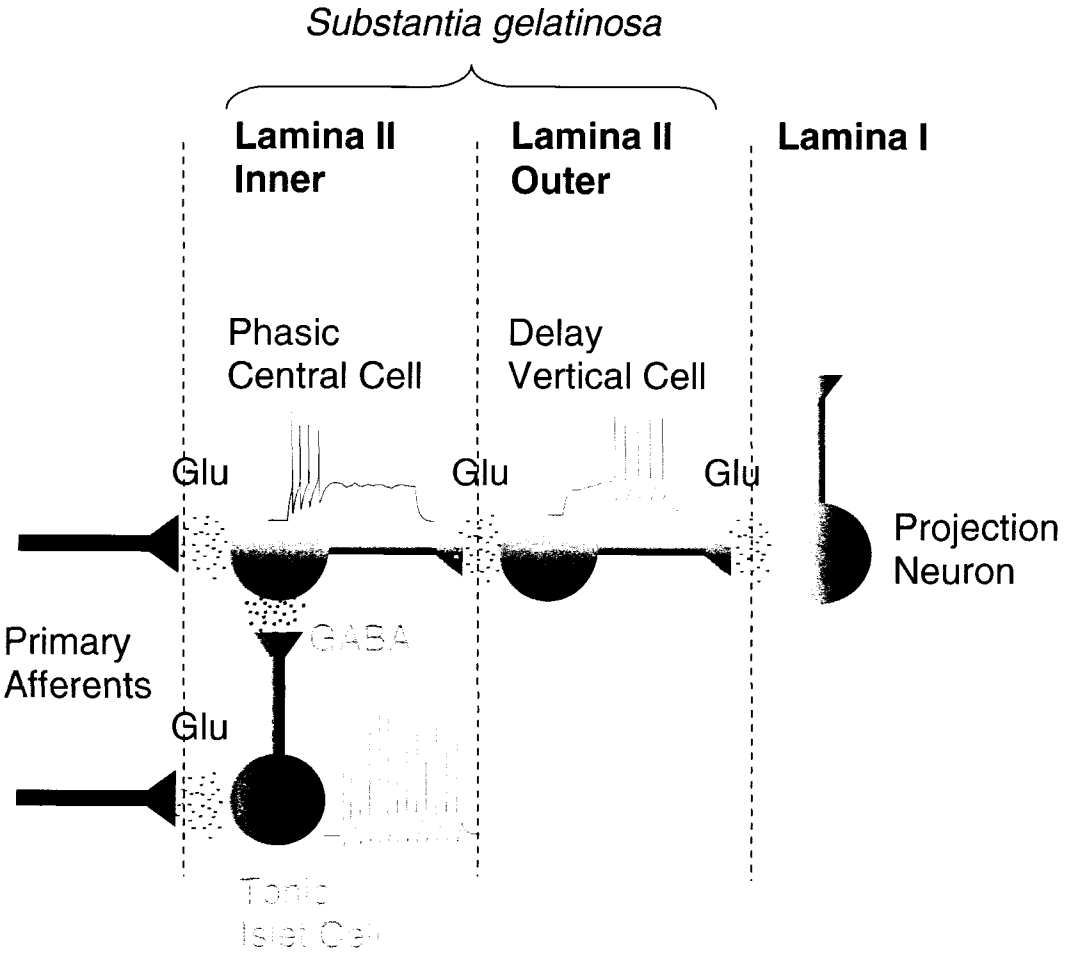


Figure 1-5. Schema of possible arrangement of synaptic circuitry in spinal superficial laminae of dorsal horn.

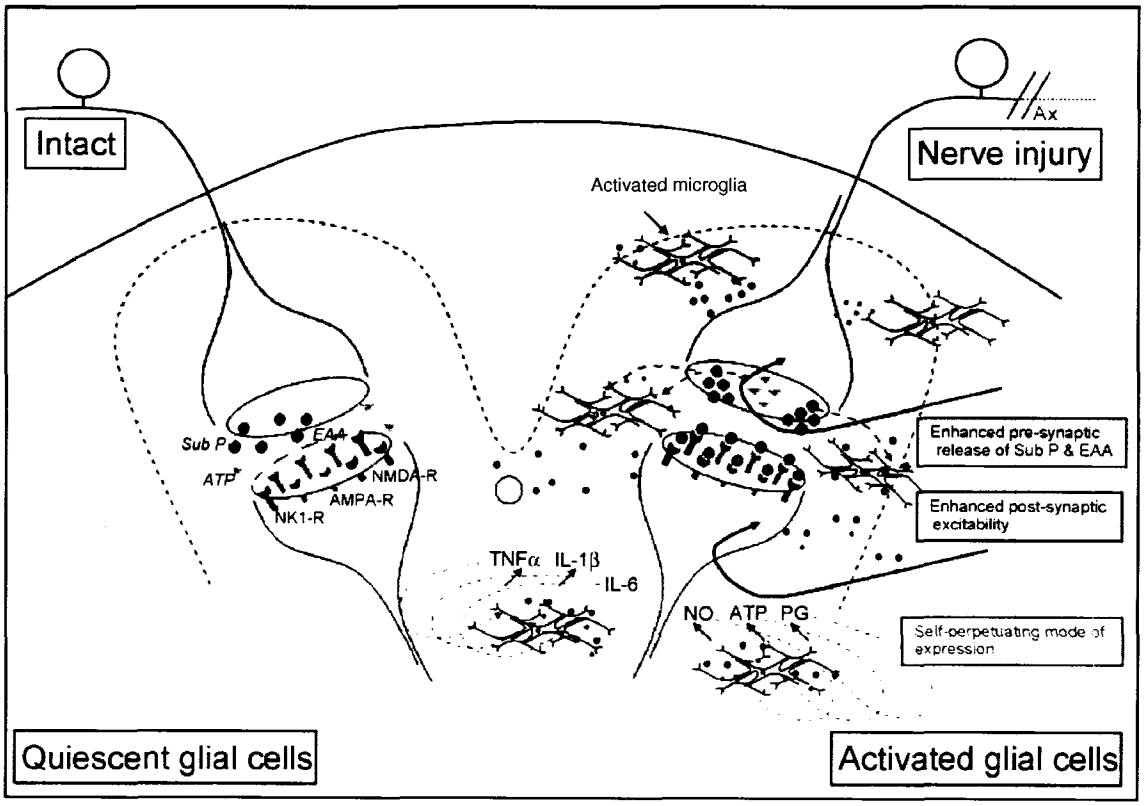


Figure 1-6. Summary of microglial activation and subsequent increase in reactivity following nerve injury or inflammation. (Modified from McMahon et al., 2005).

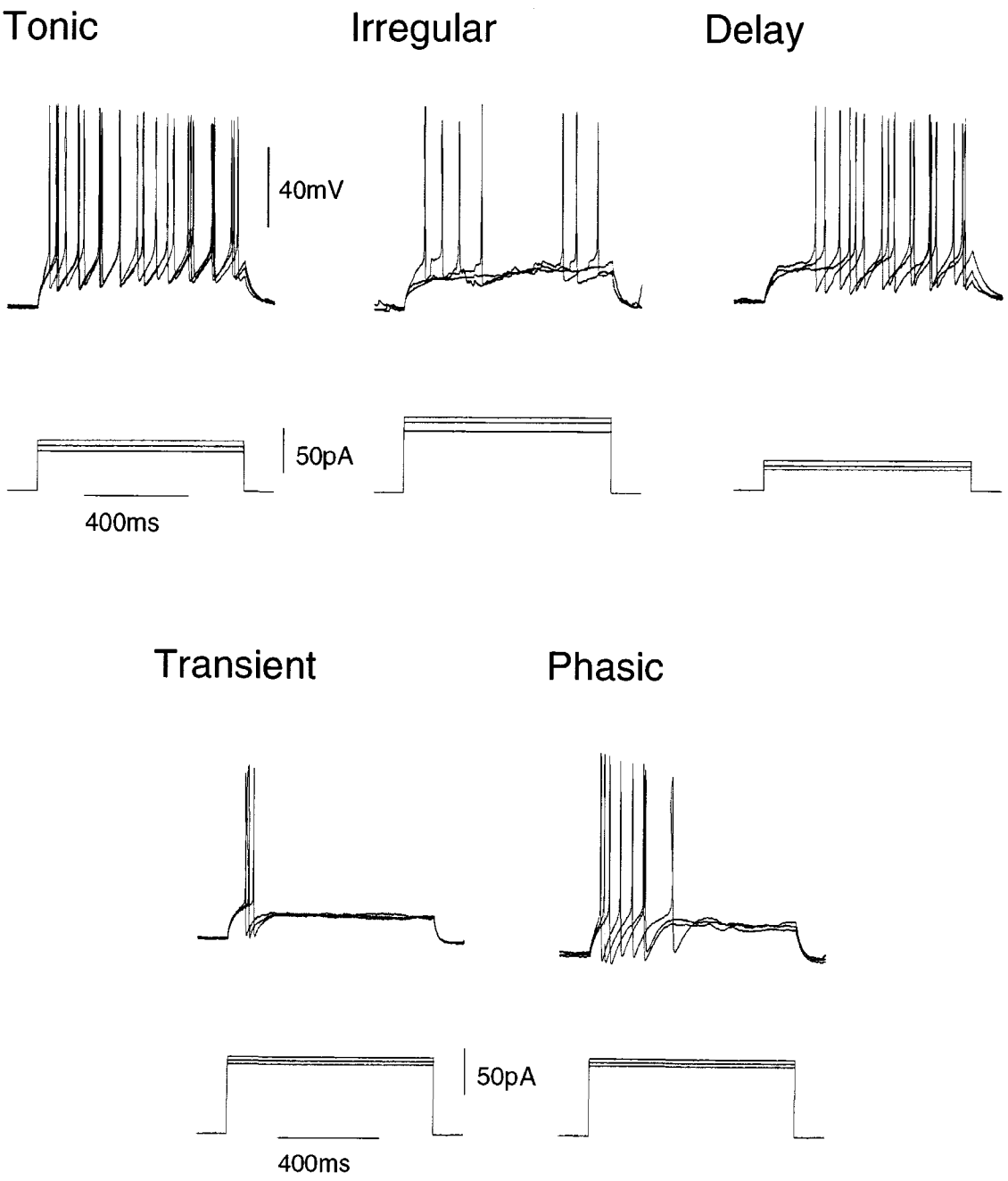


Figure 1-7. Electrophysiological phenotypes in DMOTC characterized by AP discharge patterns in response to a square pulse of depolarizing current. (Modified from Lu et al., 2006).

1.7 References

Allan, S.M., Tyrrell, P.J., & Rothwell, N.J. (2005). Interleukin-1 and neuronal injury. *Nat Rev Immunol, 5*: 629-640.

Andrei, C., Margiocco, P., Poggi, A., Lotti, L.V., Torrisi, M.R., & Rubartelli, A. (2004). Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes. *Proc Natl Acad Sci U.S.A., 101*(26): 9745-9750.

Argoff, C.E., Backonja, M.M., Belgrade, M.J., Bennett, G.J., Clark, M.R., Cole, B.E., et al. (2006). Consensus guidelines: Assessment, diagnosis, and treatment of diabetic peripheral neuropathic pain. *Mayo Clinic Proced, Suppl., 81*(4): S2-S32.

Arner, S. & Meyerson, B.A. (1988). Lack of analgesic effect of opioids on neuropathic and idiopathic forms of pain. *Pain, 33*: 11-23.

Ballerini, L. & Galante, M. (1998). Network bursting by organotypic spinal slice cultures in the presence of bicuculline and/or strychnine is developmentally regulated. *Eur J Neurosci, 10*: 2871-2879.

Basbaum, A.I., & Fields, H.L. (1984). Endogenous pain control systems: Brainstem spinal pathways and endorphin circuitry. *Ann Rev Neurosci, 7*: 309-338.

Bennett, G.J. & Xie, Y.K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain, 33*: 87-107.

Berger, A., Dukes, E.M., & Oster, G. (2004). Clinical characteristics and economic costs of patients with painful neuropathic disorders. *J Pain, 5*: 143-149.

Berkenbosch, F., van Oers, J., del Ray, A., Tilders, F., & Besedovsky, H. (1987). Corticotropin-releasing factor-producing neurons in the rat activated by interleukin-1. *Science, 238*: 524-526.

Boutin, H., Kimber, I., Rothwell, N.J., & Pinteaux, E. (2003). The expanding interleukin-1 family and its receptors: Do alternative IL-1 receptor/signaling pathways exist in the brain? *Mol Neurobiol, 27*: 239-248.

Braschler, U.F., Iannone, A., Spenger, C., Streit, J., & Luscher, H.R. (1989). A modified roller tube technique for organotypic cocultures of embryonic rat spinal cord, sensory ganglia and skeletal muscle. *J Neurosci Methods, 29*: 121-129.

Brierly, J.B. & Brown, A.W. (1982). The origin of lipid phagocytes in the central nervous system: I. The intrinsic microglia. *J Comp Neurol, 211*: 397-406.

Brodal, P. (1992). The somatosensory system. In: *The Central Nervous System: Structure and Function*. pp. 113-154. Oxford University Press: New York.

Brough, D., Le Feuvre, R.A., Wheeler, R.D., Solovyova, N., Hilfiker, S., Rothwell, N.J., et al. (2003). Ca2+ stores and Ca2+ entry differentially contribute to the release of IL-1 beta and IL-1 alpha from murine macrophages. *J Immunol, 170*(6): 3029-3036.

Burgess, P.R. & Perl, E.R. (1967). Myelinated afferent fibres responding specifically to noxious stimulation of the skin. *J Physiol, 190*: 541-562.

Chao, C.C., Hu, S., & Peterson, P.K. (1995a). Modulation of human microglial cell superoxide production by cytokines. *J Leukocyte Biol, 58*(1): 65-70.

Chao, C.C., Hu, S., Sheng, W.S., & Peterson, P.K. (1995b). Tumor necrosis factor-alpha production by human fetal microglial cells: Regulation by other cytokines. *Dev Neurosci, 17*(2): 97-105.

Chao, C.C., Hu, S., Sheng, W.S., Tsang, M., & Peterson, P.K. (1995c). Tumor necrosis factor-alpha mediates the release of bioactive transforming growth factor-beta in murine microglial cell cultures. *Clin Immunol Immunopathol, 77*(3): 358-365.

Christensen, B.N. & Perl, E.R. (1970). Spinal neurons specifically excited by noxious or thermal stimuli: Marginal zone of the dorsal horn. *J Neurophysiol, 33*: 293-307.

Clatworthy, A.L. (1999). Evolutionary perspectives of cytokines in pain. In: L.R. Watkins & S.F. Maier (Eds.) *Cytokines and Pain*, Birkhauser Verlag: Basel, Switzerland.

Clemett, D. & Goa, K.L. (2000). Celecoxib: A review of its use in osteoarthritis, rheumatoid arthritis and acute pain. *Drugs, 59*: 957-980.

Coelho, A., Fioramonti, J., & Bueno, L. (2000). Brain interleukin-1β and tumor necrosis factor-alpha are involved in lipopolysaccharide-induced delayed rectal allodynia in awake rats. *Brain Res Bull, 52*: 223-228.

Colburn, R.W., DeLeo, J.A., Rickman, A.J., Yeager, M.P., Kwon, P., & Hickey, W.F. (1997). Dissociation of microglial activation and neuropathic pain behaviours following peripheral nerve injury in the rat. *J Neuroimmunol, 79*: 163-175.

Colburn, R.W., Rickman, A.J., & DeLeo, J.A. (1999). The effect of site and type of nerve injury on spinal glial activation and neuropathic pain behaviour. *Exp Neurol, 157*: 289-304.

Coltman, B.W. & Ide, C.F. (1996). Temporal characterization of microglia, IL-1β -like immunoreactivity and astrocytes in the dentate gyrus of hippocampal organotypic slice cultures. *Int J Devel Neurosci, 14*: 707-719.

Coren, S., Ward, L.M., & Enns, J.T. (2004). *Sensation and Perception* (6th ed.). John Wiley & Sons: Hoboken, New Jersey.

Coyle, D.E. (1998). Partial peripheral nerve injury leads to activation of astroglia and microglia which parallels the development of allodynic behaviour. *Glia, 23*: 75-83.

Craig, A.D., & Dostrovsky, J. (1999). Medulla to thalamus. In: *Textbook of Pain* (P.D. Wall, R. Melzack, Eds.). pp. 183-214. Churchill Livingstone: Toronto.

Cunha, J.M., Cunha, F.Q., Poole, S., & Ferreira, S.H. (2000). Cytokine-mediated inflammatory hyperalgesia limited by interleukin-1 receptor antagonist. *Brit J Pharmacol, 130*: 1418-1424.

Dahlhaus, A., Ruscheweyh, R., & Sandkuhler, J. (2005). Synaptic input of rat spinal lamina I projection and unidentified neurons in vitro. *J Physiol, 566*: 355-368.

Dalal, A., Tata, M., Allegre, G., Gekiere, F., Bons, N., & Albe-Fessard, D. (1999). Spontaneous activity of rat dorsal horn cells in spinal segments of sciatic

projection following transection of sciatic nerve or of corresponding dorsal roots. *Neurosci, 94*: 217-228.

Davis, A.J. & Perkins, M.N. (1994). The involvement of bradykinin B1 and B2 receptor mechanisms in cytokine-induced mechanical hyperalgesia in the rat. *Brit J Pharmacol, 113*: 63-68.

DeLeo, J.A. & Colburn, R.W. (1999). Proinflammatory cytokines and glial cells: Their role in neuropathic pain. In: L.R. Watkins & S.F. Maier (Eds.) *Cytokines and Pain*, Birkhauser Verlag: Basel, Switzerland.

DeLeo, J.A., Colburn, R.W., & Rickman, A.J. (1997). Cytokine and growth factor immunohistochemical spinal profiles in two animal models of mononeuropathy. *Brain Res, 759*(1): 50-57.

DeLeo, J.A. & Yezierski, R.P. (2001). The role of neuroinflammation and neuroimmune activation in persistent pain. *Pain, 90*: 1-6.

Desson, S.E. & Ferguson, A.V. (2003). Interleukin-1β modulates rat subfornical organ neurons as a result of activation of a non-selective cationic conductance. *J Physiol, 550*: 113-122.

Devor, M., Lomazov, P., & Matzner, O. (1994). Sodium channel accumulation in injured axons as a substrate for neuropathic pain. In: Boivie, J., Hansson, P., & Lindblom, U. (Eds.). *Touch, temperature and pain in health and disease: Mechanisms and assessments. Progress in Pain Research and Management, vol. 3.* Seattle: IASP Press, pp. 207-230.

Dickenson, A., Matthews, E. & Suzuki, R. (2001). Central nervous system mechanisms of pain in peripheral neuropathy. In: Hansson, P., Fields, H., Hill, R. & Marchettini, P. (Eds.) *Neuropathic pain: Pathophysiology and treatment, vol. 21.* Seattle: IASP Press, pp. 85-106.

Dinarello, C.A., Novick, D., Puren, A.J., Fantuzzi, G., Shapiro, L., Muhl, H., et al. (1998). Overview of interleukin-18: more than an interferon-gamma inducing factor. *J Leukoc Biol, 63*: 658-664.

Dougherty, K.J., Sawchuk, M.A., & Hochman, S. (2005). Properties of mouse spinal lamina I GABAergic interneurons. *J Neurophysiol, 94*: 3221-3227.

Dunne, A., & O'Neill, L.A.J. (2003). The interleukin-1/toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci STKE, 2003*(171): re3 (online).

Elssner, A., Duncan, M., Gavrilin, M., & Wewers, M.D. (2004). A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1beta processing and release. *J Immunol, 172*: 4987-4994.

England, S., Bevan, S., & Docherty, R.J. (1996). PGE_2 modulates the tetrodotoxin-resistant sodium current in neonatal rat dorsal root ganglion neurons via the cyclic AMP-protein kinase A cascade. *J Physiol, 495*: 429-440.

Eriksson, C., Tehranian, R., Iverfeldt, K., Winblad, B., & Schultzberg, M. (2000). Increased expression of mRNA-encoding interleukin-1β and caspase-1, and the secreted isoform of interleukin-1 receptor antagonist in the rat brain following systemic kainic acid administration. *J Neurosci Res, 60*: 266-279.

Eriksson, N.P., Persson, J.K., Svensson, M., Arvidsson, J., Molander, C., & Aldskogius, H. (1993). A quantitative analysis of the microglial cell reaction in central primary sensory projection territories following peripheral nerve injury in the adult rat. *Exp Brain Res, 96*: 19-27.

Ferrari, D., Chiozzi, P., Falzoni, S., Hanau, S., & Di Virgilio, F. (1997). Purinergic modulation of interleukin-1 beta release from microglial cells stimulated with bacterial endotoxin. *J Exp Med, 185*(3): 579-582.

Ferreira, S., Lorenzetti, B., Bristow, A., & Poole, S. (1988). Interleukin-1β as a potent hyperalgesic agent antagonized by a tripeptide analogue. *Nature, 334*: 698-700.

Fukuota, H., Kawatani, M., Hisamitsu, T., & Takeshige, C. (1994). Cutaneous hyperalgesia induced by peripheral injection of interleukin-1β in the rat. *Brain Res, 657*: 133-140.

Gahwiler, B.H. (1981). Organotypic monolayer cultures of nervous tissue. *J Neurosci Methods, 4*: 329-342.

Gahwiler, B.H., Capogna, M., Debanne, D., McKinney, R.A., & Thompson, S.M. (1997). Organotypic slice cultures: a technique has come of age. *TINS, 20*(10): 471-477.

Galer, B.S., Twilling, L.L., Harle, J., Cluff, R.S., Friedman, E., & Rowbotham, M.C. (2000). Lack of efficacy of riluzole in the treatment of peripheral neuropathic pain conditions. *Neurol, 55* (7): 971-975.

Gamboa-Esteves, F.O., Kaye, J.C., McWilliam, P.N., Lima, D., & Batten, T.F. (2001). Immunohistochemical profiles of lamina I neurons retrogradely labeled from the nucleus tractus solitarii in rat suggest excitatory projections. *Neurosci, 104*: 523-538.

Gold, M.S., Reichling, D.B., Schuster, M.J., & Levine, J.D. (1996). Hyperalgesic agents increase a tetrodotoxin-resistant Na+ current in nociceptors. *Proc Natl Acad Sci USA, 93*: 1108-1112.

Grudt, T.J. & Perl, E.R. (2002). Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. *J Physiol, 540*(1): 189-207.

Guay, D.R.P. (2005). Pregabalin in neuropathic pain: A more "pharmaceutically elegant" gabapentin? *Am J Geriatr Pharmacother, 3*(4): 274-287.

Guillemin, G.J. & Brew, B.J. (2004). Microglia, macrophages, perivascular macrophages, and pericytes: A review of function and identification. *J Leukocyte Biol, 75*(3): 388-397.

Hannum, C.H., Wilcox, C.J., Arend, W.P., Joslin, F.G., Dripps, D.J., Heimdal, P.L., et al. (1990). Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. *Nature, 343*: 336-340.

Hansson, P.T. & Dickenson, A.H. (2005). Pharmacological treatment of peripheral neuropathic pain conditions based on shared commonalities despite multiple etiologies. *Pain, 113*: 251-254.

Hara, H., Friedlanderdagger-Dagger, R.M., Gagliardinidagger, V., Ayata, C., Fink, K., Huang, Z., et al. (1997). Inhibition of interleukin-1beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. *Proc Natl Acad Sci USA, 94*: 2007-2012.

Harstall, C. & Ospina, M. (2003). How prevalent is chronic pain? *Pain Clin Updates, 9*(2): 1-4.

Hashizume, H., DeLeo, J.A., Colburn, R.W., & Weinstein, J.N. (2000). Spinal glial activation and cytokine expression after lumbar root injury in the rat. *Spine, 25*: 1206-1217.

Hassel, B., Paulsen, R.E., Johnsen, A., & Fonnum, F. (1992) Selective inhibition of glial cell metabolism in vivo by fluorocitrate. *Brain Res, 576*: 120-124.

Hazuda, D.J., Strickler, J., Kueppers, F., Simon, P.L., & Young, P.R. (1990). Processing of precursor interleukin-1β and inflammatory disease. *J Biol Chem, 265*: 6318-6322.

Heinke, B., Ruscheweyh, R., Forsthuber, L., Wunderbaldinger, G., & Sandkuhler, J. (2004). Physiological, neurochemical and morphological properties of a subgroup of GABAergic spinal lamina II neurons identified by expression of green fluorescent protein in mice. *J Physiol, 560*: 249-266.

Hide, I., Tanaka, M., Inoue, A., Nakajima, K., Kohsaka, S., Inoue, K., et al. (2000). Extracellular ATP triggers tumor necrosis factor-alpha release from rat microglia. *J Neurochem, 75*(3): 965-972.

Howard, R.F., Walker, S.M., Michael, M.P., & Fitzgerald, M. (2005). The ontogeny of neuropathic pain: Postnatal onset of mechanical allodynia in rat spared nerve injury (SNI) and chronic constriction injury (CCI) models. *Pain, 115*: 382-389.

Hsu, H.Y. & Wen, M.H. (2002). Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J Biol Chem, 277*: 22131-22139.

Huising, M.O., Stet, R.J.M., Savelkoul, H.F.J., & Verburg-van Kemenade, B.M.L. (2004). The molecular evolution of the interleukin-1 family of cytokines; IL-18 in teleost fish. *Devel Comp Immunol, 28*: 395-413.

Kandel, E. & Kupfermann, I. (1995). From nerve cells to cognition. In: Kandel, E.R., Schwartz, J.H., & Jessell, T.M. (Eds.). *Essentials of neural science and behaviour.* Stamford, CT: Appleton & Lange, pp. 321-346.

Kelley, K.W., Bluthe, R.-M., Dantzer, R., Zhou, J.-H., Shen, W.-H., Johnson, R.W., et al. (2003). Cytokine-induced sickness behavior. *Brain Behav Immun, 17*: S112-S118.

Kerchner, G.A., Wang, G.D., Qiu, C.S., Huettner, J.E., & Zhuo, M. (2001). Direct presynaptic regulation of GABA/glycine release by kainate receptors in the dorsal horn: an ionotropic mechanism. *Neuron, 32*: 477-488.

Kern, J.A., Warnock, L.J., & McCafferty, J.D. (1997). The 3' untranslated region of IL-1beta regulates protein production. *J Immunol, 158*(3): 1187-1193.

Korherr, C., Hofmeister, R., Wesche, H., & Falk, W. (1997). A critical role for interleukin-1 receptor accessory protein in interleukin-1 signaling. *Eur J Immunol, 27*: 262-267.

Kunz, S., Tegeder, I., Coste, O., Marian, C., Pfenninger, A., Corvey, C., et al. (2005). Compartative proteomic analysis of the rat spinal cord in inflammatory and neuropathic pain models. *Neurosci Lett, 381*: 289-293.

Laliberte, R.E., Perregaux, D.G., Hoth, L.R., Rosner, P.J., Jordan, C.K., Peese, K.M., et al. (2003). Glutathione S-transferase omega 1-1 is a target of cytokine release inhibitory drugs and may be responsible for their effect on interleukin-1beta posttranslational processing. *J Biol Chem, 278*: 16567-16578.

Le Feuvre, R.A., Brough, D., Iwakura, Y., Takeda, K., & Rothwell, N.J. (2002). Priming of macrophages with lipopolysaccharide potentiates P2X7-mediated cell death via a caspase-1-dependent mechanism, independently of cytokine production. *J Biol Chem, 277*(5): 3210-3218.

Ledeboer, A., Sloane, E.M., Milligan, E.M., Frank, M.G., Mahony, J.H., Maier, S.F., et al. (2005). Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. *Pain, 115*: 71-83.

Liu, L., Li, Y., Van Eldik, L.J., Griffin, W.S., & Barger, S.W. (2005). S100B-induced microglial and neuronal IL-1 expression is mediated by cell type-specific transcription factors. *J Neurochem, 92*: 546-553.

Loddick, S.A., Wong, M.L., Bongiorno, P.B., Gold, P.W., Licinio, J., & Rothwell, N.J. (1997). Endogenous interleukin-1 receptor antagonist is neuroprotective. *Biochem Biophys Res Commun, 234*: 211-215.

Loeser, J.D. (1990). Peripheral nerve disorders (peripheral neuropathies). In J. Bonica (Ed.) *The Management of Pain* (2nd ed.), Lea and Febiger: Pennsylvania.

Lu, V.B., Moran, T.D., Balasubramanyan, S., Alier, K.A., Dryden, W.F., Colmers, W.F., & Smith, P.A. (2006). Substantia gelatinosa neurons in defined-medium

organotypic slice culture are similar to those in acute slices from young adult rats. *Pain, 121*: 261-275.

Lu, Y. & Perl, E.R. (2005). Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (laminae I and II). *J Neurosci, 25*: 3900-3907.

Luo, Z.D., Chaplan, S.R., Higuera, E.S., Sorkin, L.S., Stauderman, K.A., Williams, M.E., et al. (2001). Upregulation of dorsal root ganglion (alpha)2(delta) calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. *J Neurosci, 21*: 1868-1875.

Maier, S.F., Wiertelak, E.P., Martin, D., & Watkins, L.R. (1993). Interleukin-1 mediates the behavioural hyperalgesia produced by lithium chloride and endotoxin. *Brain Res, 623*: 21-24.

Malcangio, M., & Bowery, N.G. (1996). GABA and its receptors in the spinal cord. *Trends Pharmacol Sci, 17*: 457-462.

Malyak, M., Smith, M.F.Jr., Abel, A.A., Hance, K.R., & Arend, W.P. (1998). The differential production of three forms of IL-1 receptor antagonist by human neutrophils and monocytes. *J Immunol, 161*: 2004-2010.

March, C.J., Mosley, B., Larsen, A., Cerretti, D.P., Braedt, G., Price, V., et al. (1985). Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature, 315*: 641-647.

Matthews, E.A. & Dickenson, A.H. (2001). Effects of spinally-delivered N- and P-type voltage-dependent calcium channel antagonists on dorsal horn neuronal responses in a rat model of neuropathy. *Pain, 92*: 235-246.

McMahon, S.B., Cafferty, W.B.J., & Marchand, F. (2005). Immune and glial cell factors as pain mediators and modulators. *Exp Neurol, 192*: 444-462.

Mehler, W.R., Feferman, M.E., & Nauta, W.J.H. (1960). Ascending axon degeneration following anterolateral cordotomy: An experimental study in the monkey. *Brain, 83*: 718-751.

Meller, S.T., Dykstra, C., Grzybycki, D., Murphy, S., & Gebhart, G.F. (1994). The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. *Neuropharmacol, 33*: 1471-1478.

Melzack, R., & Wall, P.D. (1965). Pain mechanisms: A new theory. *Science, 150*: 971-979.

Merskey, H., & Bogduk, N. (Eds.). (1994). *Classification of Chronic Pain*, (2nd Ed.) IASP Task Force on Taxonomy. Seattle: IASP Press, pp. 209-214.

Milligan, E.D., Mehmert, K.K., Hinde, J.L., Harvey, L.O., Martin, D., Tracey, K.J., et al. (2000). Thermal hyperalgesia and mechanical allodynia produced by intrathecal administration of the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein, gp120. *Brain Res, 861*: 105-116.

Milligan, E.D., Twining, C., Chacur, M., Biedenkapp, J., O'Connor, K., Poole, S., et al. (2003). Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. *J Neurosci, 23*: 1026-1040.

Milligan, E.D., Zapata, V., Chacur, M., Schoeniger, D., Biedenkapp, J., O'Conner, K.A., et al. (2004). Evidence that exogenous and endogenous fractalkine can induce spinal nociceptive facilitation in rats. *Eur J Neurosci, 20*: 2294-2302.

Moore, K.A., Kohno, T., Karchewski, L.A., Scholz, J., Baba, H., Woolf, C.J. (2002). Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *J Neurosci, 22*: 6724-6731.

Nakae, S., Asano, M., Horai, R., & Iwakura, Y. (2001). Interleukin-1 beta, but not interleukin-1 alpha, is required for T-cell-dependent antibody production. *Immunol, 104*(4): 402–409.

Nestler, E.J., Hyman, S.E., & Malenka, R.C. (2001). *Molecular pharmacology: A foundation for clinical neuroscience.* New York: McGraw-Hill, pp. 433-452.

Ohtori, S., Takahashi, K., Moriya, H., & Myers, R.R. (2004). TNF-alpha and TNF-alpha receptor type 1 upregulation in glia and neurons after peripheral nerve injury: Studies in murine DRG and spinal cord. *Spine, 29*: 1082-1088.

Oka, T., Aou, S., & Hori, T. (1993). Intracerebroventricular injection of interleukin-1β induces hyperalgesia in rats. *Brain Res, 624*: 61-68.

Oka, T., Aou, S., & Hori, T. (1994). Intracerebroventricular injection of interleukin-1β enhances nociceptive neuronal responses of the trigeminal nucleus caudalis in rats. *Brain Res, 656*: 236-244.

Oka, T., Oka, K., Hosoi, M., Aou, S., & Hori, T. (1995). The opposing effects of interleukin-1beta microinjected into the preoptic hypothalamus and the ventromedial hypothalamus on nociceptive behaviour in rats. *Brain Res, 700*(1-2): 271-278.

Penning, T.D., Talley, J.J., Bertenshaw, S.R., Carter, J.S., Collins, P.W., Docter, S., et al. (1997). Synthesis and biological evaluation of the 1,5-diarylpyrazole class of cyclooxygenase-2 inhibitors: Identification of 4-[5-(4-methylphenyl)-3-

(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide (SC-58635, celecoxib). *J Med Chem, 40*: 1347-1365.

Perkins, M.N. & Kelly, D. (1994). Interleukin-1β induced-desArg9bradykinin-mediated thermal hyperalgesia in the rat. *Neuropharmacol, 33*: 657-660.

Perl, E.R. (1996). Cutaneous polymodal receptors: Characteristics and plasticity. *Prog Brain Res, 113*: 21-37.

Perregaux, D.G., Bhavsar, K., Contillo, L., Shi, J., & Gabel, C.A. (2002). Antimicrobial peptides initiate IL-1β posttranslational processing: A novel role beyond innate immunity. *J Immunol, 168*: 3024-3032.

Perregaux, D.G., & Gabel, C.A. (1994). Interleukin-1β maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J Biol Chem, 269*: 15195-15203.

Perregaux, D.G., McNiff, P., Laliberte, R., Hawryluk, N., Peurano, H., Stam, E., et al. (2001). Identification and characterization of a novel class of interleukin-1 post-translational processing inhibitors. *J Pharmacol Exp Ther, 299*: 187-197.

Piao, Z.G., Cho, I.-H., Park, C.K., Hong, J.P., Choi, S.-Y., Lee, S.J., et al. (2006). Activation of glia and microglial p38 MAPK in medullary dorsal horn contributes to tactile hypersensitivity following trigeminal sensory nerve injury. *Pain, 121*: 219-231.

Polgar, E., & Antal, M. (1995). The colocalization of parvalbumin and calbindin-D28K with GABA in the subnucleus caudalis of the rat spinal trigeminal nucleus. *Exp Brain Res, 103*: 402-408.

Porreca, F., Ossipov, M. & Gebhart, G. (2002). Chronic pain and medullary descending facilitation. *Trends Neurosci, 25*: 319-325.

Prasit, P., Wang, Z., Brideau, C., Chan, C.C., Charleson, S., et al. (1999). The discovery of rofecoxib, [MK 966, Vioxx (R), 4-(4'-methylsulfonylphenyl)-3-phenyl-2(5H)-furanone], an orally active cyclooxygenase-2 inhibitor. *Bioorg Med Chem Lett, 9*: 1773-1778.

Prescott, S.A. & de Koninck, Y. (2002). Four cell types with distinctive membrane properties and morphologies in lamina I of the spinal dorsal horn of the adult rat. *J Physiol, 539*: 817-836.

Priller, J., Haas, C.A., Reddington, M., & Kreutzberg, G.W. (1995). Calcitonin gene-related peptide and ATP induce immediate early gene expression in cultured rat microglial cells. *Glia, 15*(4): 447-457.

Priller, J., Haas, C.A., Reddington, M., & Kreutzberg, G.W. (1998). Cultured astrocytes express functional receptors for galanin. *Glia, 24*(3): 323-328.

Rady, J.J., & Fujimoto, J.M. (2001). Confluence of antianalgesic action of diverse agents through brain interleukin-1β in mice. *J Pharmacol Exp Ther, 299*: 659-665.

Raghavendra, V., Tanga, F., & DeLeo, J.A. (2003). Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *J Pharmacol Exp Ther, 306*: 624-630.

Relton, J.K. & Rothwell, N.J. (1992). Interleukin-1 receptor antagonist inhibits ischaemic and excitotoxic neuronal damage in the rat. *Brain Res Bull, 29*: 243-246.

Relton, J.K., Strijbos, P.J., O'Shaughnessy, C.T., Carey, F., Forder, R.A., Tilders, F.J., et al. (1991). Lipocortin-1 is an endogenous inhibitor of ischemic damage in the rat brain. *J Exp Med, 174*(2): 305-310.

Rexed, B. (1952). The cytoarchitectonic organization of the spinal cord in the cat. *J Comp Neurol, 96*: 415-495.

Rothwell, N.J. (1991). Functions and mechanisms of interleukin-1 in the brain. *Trends Pharmacol Sci, 12*: 430-436.

Rothwell, N.J. (2003). Interleukin-1 and neuronal injury mechanisms, modification, and therapeutic potential. *Brain Behav Immunol, 17*: 152-157.

Rotshenker, S., Aamar, S., & Barak, V. (1992). Interleukin-1 activity in lesioned peripheral nerve. *J Neuroimmunol, 39*: 75-80.

Safieh-Garabedian, B., Poole, S., Allchorne, A., Winter, J., & Woolf, C.J. (1995). Contribution of interleukin-1β to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. *Brit J Pharmacol, 115*: 1265-1275.

Said, G. & Hontebeyrie-Joskowicz, M. (1992). Nerve lesions induced by macrophage activation. *Res Immunol, 143*(6): 589-599.

Samad, T.A., Moore, K.A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., et al. (2001). Interleukin-1β-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature, 410*: 471-475.

Seltzer, Z., Dubner, R., & Shir, Y. (1990). A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain, 43*: 205-218.

Sheng, W.S., Hu, S., Kravitz, F.H., Peterson, P.K., & Chao, C.C. (1995). Tumor necrosis factor alpha upregulates human microglial cell production of interleukin-10 in vitro. *Clin Diag Lab Immunol, 2*(5): 604-608.

Sims, J.E. March, C.J., Cosman, D., Widmer, M.B., MacDonald, H.R., McMahan, C.J., et al. (1988). cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science, 241*(4865): 585-589.

Snider, W.D. & McMahon, S.B. (1998). Tackling pain at the source: New ideas about nociceptors. *Neuron, 20*: 629-632.

Sommer, C. & Kress, M. (2004). Recent findings on how proinflammatory cytokines cause pain: Peripheral mechanisms in inflammatory and neuropathic hyperalgesia. *Neurosci Lett, 361*: 184-187.

Sommer, C., Petrausch, S., Lindenlaub, T., & Tokya, K.V. (1999). Neutralizing antibodies to interleukin-1 receptor reduce pain associated behaviour in mice with experimental neuropathy. *Neurosci Lett, 270*: 25-28.

Spike, R.C., Puskar, Z., Andrew, D., & Todd, A.J. (2003). A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. *Eur J Neurosci, 18*: 2433-2448.

Stoll, G. & Jander, S. (1999). The role of microglia and macrophages in the pathophysiology of the CNS. *Prog Neurobiol, 58*(3): 233-247.

Stuesse, S.L., Cruce, W.L., Lovell, J.A., McBurney, D.L., & Crisp, T. (2000). Microglial proliferation in the spinal cord of aged rats with a sciatic nerve injury. *Neurosci Lett, 287*: 121-124.

Subramaniam, S., Stansberg, C., & Cunningham, C. (2004). The interleukin 1 receptor family. *Dev Comp Immunol, 28*: 415-428.

Svendsen, K.B., Jensen, T.S., Hansen, H.J., & Bach, F.W. (2005). Sensory function and quality of life in patients with multiple sclerosis and pain. *Pain, 114*(3): 473-481.

Svensson, C.I., Hua, X.Y., Protter, A.A., Powell, H.C., & Yaksh, T.L. (2003a). Spinal p38 MAP kinase is necessary for NMDA-induced spinal PGE2 release and thermal hyperalgesia. *Neuroreport, 14*(8): 1153-1157.

Svensson, C.I., Marsala, M., Westerlund, A., Calcutt, N.A., Campana, W.M., Freshwater, J.D., et al. (2003b). Activation of p38 mitogen-activated protein kinase in spinal

microglia is a critical link in inflammation-induced spinal pain processing. *J Neurochem, 86*(6): 1534-1544.

Sweitzer, S.M., Colburn, R.W., Rutkowski, M., & DeLeo, J.A. (1999). Acute peripheral inflammation induces moderate glial activation and spinal IL-1beta expression that correlates with pain behaviour in the rat. *Brain Res, 829*(1-2): 209-221.

Sweitzer, S.M., Schubert, P., & DeLeo, J.A. (2001). Propentofylline, a glial modulating agent, exhibits antiallodynic properties in a rat model of neuropathic pain. *J Pharmacol Exp Ther, 297*: 1210-1217.

Sweitzer, S.M., White, K.A., Dutta, C., & DeLeo, J.A. (2002). The differential role of spinal MHC class II and cellular adhesion molecules in peripheral inflammatory versus neuropathic pain in rodents. *Neuroimmunol, 125*: 82-93.

Tanaka, T., Minami, M., Nakagawa, T., & Satoh, M. (2004). Enhanced production of monocyte chemoattractant protein-1 in the dorsal root ganglia in a rat model of neuropathic pain: Possible involvement in the development of neuropathic pain. *Neurosci Res, 48*: 463-469.

Tanga, F.Y., Raghavendra, V., & DeLeo, J.A. (2004). Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. *Neurochem Int, 45*: 397-407.

Taylor, R.S. (2006). Epidemiology of refractory neuropathic pain. *Pain Practice, 6*(1): 22-26.

Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., et al. (1992). A novel heterodimeric cysteine protease is required for interleukin-1bold beta processing in monocytes. *Nature, 356*: 768-774.

Todd, A.J. (1996). GABA and glycine in synaptic glomeruli of the rat spinal dorsal horn. *Eur J Neurosci, 8*: 2492-2498.

Todd, A.J., McGill, M.M., & Shehab, S.A. (2000). Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem. *Eur J Neurosci, 12*: 689-700.

Tsuda, M., Inoue, K., & Salter, M.W. (2005). Neuropathic pain and spinal microglia: A big problem from molecules in "small" glia. *Trends Neurosci, 28*(2): 101-107.

Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M.W., et al. (2003). P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature, 424*(6950): 778-783.

Vander, A., Sherman, J., & Luciano, D. (2001). *Human physiology: The mechanisms of body function* (8th ed.). Boston: McGraw-Hill, pp. 175-225.

Verge, G.M., Milligan, E.D., Maier, S.F., Watkins, L.R., Naeve, G.S., & Foster, A.C. (2004). Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. *Eur J Neurosci, 20*: 1150-1160.

Vitkovic, L., Bockaert, J., & Jacque, C. (2000). "Inflammatory" cytokines: Neuromodulators in normal brain? *J Neurochem, 74*: 457-471.

Wall, P.D., Devor, M., Inbal, R., Scadding, J.W., Schonfeld, D., Seltzer, Z., et al. (1979). Autonomy following peripheral nerve lesions: Experimental anaesthesia dolorosa. *Pain, 7*: 103-111.

Watkins, L.R., Hansen, M.K., Nguyen, K.T., Lee, J.E., & Maier, S.F. (1999). Dynamic regulation of the proinflammatory cytokine, interleukin-1β: Molecular biology for non-molecular biologists. *Life Sci, 65*: 449-481.

Watkins, L.R., Hutchinson, M.R., Johnston, I.N., & Maier, S.F. (2005). Glia: Novel counter-regulators of opioid analgesia. *TINS, 28*(12): 661-669.

Watkins, L.R., & Maier, S.F. (1999). Implications of immune-to-brain communication for sickness and pain. *Proc Natl Acad Sci USA, 96*: 7710-7713.

Watkins, L.R. & Maier, S.F. (2002). Beyond neurons: Evidence that immune and glial cells contribute to pathological pain states. *Physiol Rev, 82*: 981-1011.

Watkins, L.R., & Maier, S.F. (2003). Glia: A novel drug discovery target for clinical pain. *Nat Rev Drug Discov, 2*: 973-985.

Watkins, L.R., & Maier, S.F. (2004). Neuropathic pain: The immune connection. *Pain Clin Updates, 7*(1): 1-4.

Watkins, L.R., Maier, S.F., & Goehler, L.E. (1995). Immune activation: The role of pro-inflammatory cytokines in inflammation, illness responses and pathological pain states. *Pain, 63*: 289-302.

Watkins, L.R., Milligan, E.D., & Maier, S.F. (2001a) Glial activation: A driving force for pathological pain. *Trends Neurosci, 24*(8): 450-455.

Watkins, L.R., Milligan, E.D., & Maier, S.F. (2001b). Spinal cord glia: New players in pain. *Pain, 93*: 201-205.

Watkins, L.R., Wiertelak, E.P., Goehler, L.E., Smith, K.P., Martin, D., & Maier, S.F. (1994). Characterization of cytokine-induced hyperalgesia. *Brain Res, 654*: 15-26.

Waxman, S.G., Dib Hajj, S., Cummins, T.R., & Black, J.A. (1999). Sodium channels and pain. *Proc Natl Acad Sci USA, 96*: 7635-7639.

Willis, W.D. (1983). Nociceptive pathways: anatomy and physiology of nociceptive ascending pathways. *Phil Trans R Soc Lond, B 308*: 253-268.

Willis, W.D. (2006). Spinal cord nociceptive pathways. In: *Proceedings of the 11th World Congress on Pain* (H. Flor, E. Kalso, & J.O. Dostrovsky, Eds.). pp. 269-284. IASP Press: Seattle.

Woolf, C.J. (1983). Evidence for a central component of post-injury pain hypersensitivity. *Nature, 306*: 686-688.

Woolf, C.J. (2004). Dissecting out mechanisms responsible for peripheral neuropathic pain: Implications for diagnosis and therapy. *Life Sci, 74*: 2605-2610.

Woolf, C.J. & Salter, M.W. (2000). Neuronal plasticity: Increasing the gain in pain. *Science, 288*: 1765-1769.

Yamasaki, Y., Matsuura, N., Shozuhara, H., Onodera, H., Itoyama, Y., & Kogure, K. (1995). Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats. *Stroke, 26*: 676-680.

Yoshimura, M., & Nishi, S. (1993). Blind patch-clamp recordings from substantia gelatinosa neurons in adult rat spinal cord slices: Pharmacological properties of synaptic currents. *Neurosci, 53*: 519-526.

Zeilhofer, H.U. (2005). Synaptic modulation in pain pathways. *Rev Physiol Biochem Pharmacol, 154*: 73-100.

Zhang, S.C., Goetz, B.D., & Duncan, I.D. (2003). Suppression of activated microglia promotes survival and function of transplanted oligodendroglial progenitors. *Glia, 41*(2): 191-198.

Zimmermann, M. (2001). Pathobiology of neuropathic pain. *Eur J Pharmacol, 429*: 23-37.

CHAPTER 2 METHODOLOGY

All procedures were approved by the University of Alberta Health Sciences Laboratory Animal Policy and Welfare Committee, and thus comply with guidelines of the Canadian Council for Animal Care.

2.1 Defined-Medium Organotypic Cultures

The defined-medium organotypic slice culture (DMOTC) technique was adapted from previously published methods (Gahwiler, 1981; Braschler et al., 1989; Ballerini & Galante, 1998). Embryonic day 13-14 rat fetuses were delivered by caesarean section from timed-pregnant Sprague-Dawley rats (Charles River, Saint-Constant, PQ, Canada) under 2-5% isoflurane anaesthesia. Following the caesarean section, the female rat was euthanized with an overdose of intra-cardiac chloral hydrate (10.5%). Under aseptic conditions, the entire embryonic sac was placed in chilled Hanks' balanced salt solution (BSS) containing (in mM): 138 NaCl, 5.33 KCl, 0.44 KH_2PO_4, 0.5 $MgCl_2$-$6H_2O$, 0.41 $MgSO_4$-$7H_2O$, 4 $NaHCO_3$, 0.3 Na_2HPO_4, 5.6 D-glucose, and 1.26 $CaCl_2$. Individual rat fetuses were removed from their embryonic sacs and rapidly decapitated. Following this, the spine and dorsal tissue of each fetus was cut from the rest of the body and sliced into 275-325 μm transverse slices using a tissue chopper (McIlwain, St. Louis, MO, USA). Only lumbar spinal cord slices with an intact spinal cord, two attached dorsal root ganglia (DRGs) and associated muscle tissue were chosen and transferred to a new dish of Hanks' BSS. These slices were trimmed of excess ventral tissue and chilled for 1 hour at 4°C (Figure 2-1).

Embryonic spinal cord slices, with attached DRGs and some ventral muscle fibres, were plated onto clean glass coverslips (Karl Hecht, Sondheim, Germany) with a clot of reconstituted chicken plasma (lyophilized, 2mg/L heparin; Cocalico Biologicals Inc.,

Stevens, PA, USA) and thrombin (200 units/mL; Sigma, St. Louis, MO, USA). Coverslips were inserted into flat-bottomed tissue culture tubes (Nunc-Nalgene International, Mississauga, ON, Canada) filled with 1mL of culture medium, and placed into a roller drum (Model # TC-8, New Brunswick Scientific, Edison, NJ, USA) rotating at 120 rotations per hour in a dry heat incubator at 36°C (regular air-no CO_2; Figure 2-2). The initial medium in the tubes was composed of 82% Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, and 8% sterile water to adjust osmolarity (all from Invitrogen-Gibco, Grand Island, NY, USA). This medium was supplemented with 20ng/mL nerve growth factor (NGF) (Alomone Laboratories, Jerusalem, Israel) for the first 4 days. Antibiotic and antimycotic (5 units/mL penicillin G, 5 units/mL streptomycin, and 12.5 ng/mL amphotericin B; Invitrogen-Gibco) were also included in the medium during the first 4 days of culture. At the end of this 4 day period, the slices were treated with an anti-mitotic drug cocktail consisting of uridine, cytosine-β-D-arabino-furanoside (AraC), and 5-fluorodeoxyuridine (all from SIGMA, all at 10μM) for 24 hours to prevent the overgrowth of glial cells. Also during this treatment, the serum medium was halved with a serum-free, defined medium consisting of Neurobasal medium, 5mM Glutamax-1 and N2 supplement (all from Invitrogen-Gibco). Washout of the antimitotic drugs and serum medium was carried out on day 5 with the serum-free, defined medium described above. The medium within these tubes was exchanged with freshly prepared serum-free medium every 4-5 days, and DMOTC slices were maintained in serum-free, chemically-defined conditions until recordings were carried out.

2.2 Electrophysiology

For recording, slices were placed in a glass-bottomed chamber (25mm diameter, volume of ~1mL) and held in place with a circular platinum wire frame with attached parallel nylon fibres. Slices were superfused (flow rate ~ 1-2 mL/min) at room temperature (~22°C) with 95%O_2-5% CO_2 saturated artificial cerebrospinal fluid (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 (320-325 mOsm).

DMOTC slices were viewed with a Zeiss Axioskop FS upright microscope equipped with infrared differential interference contrast (IR-DIC) optics and an IR sensitive video camera (NC-70, Dage-MTI, Michigan City, IN, USA). Cells for recording were chosen from within a light band located across the dorsal region of the slice. Whole-cell recordings were made from visually-identified dorsal horn neurons with an npi SEC 05L amplifier (npi Electronic, Tamm, Germany) in discontinuous single-electrode voltage-clamp or current-clamp mode. Switching frequencies were typically between 30-40 kHz. Signals were digitized at 10kHz and filtered at 0.5-2 kHz. 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 action potentials had resistances of 5-10 MΩ when filled with an internal solution containing (in mM): 130 D-gluconic acid, 2 $CaCl_2$, 10 HEPES, 10 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 0.2% Biocytin, adjusted to pH 7.2 with KOH (290-300 mOsm).

Spontaneous EPSCs and IPSCs (sEPSCs and sIPSCs) were digitized at 10kHz and filtered at 2kHz. Recording sEPSCs was done under voltage clamp at a holding potential of –70mV to minimize inhibitory currents. Recording sIPSCs was done under voltage

clamp at a potential of 0mV to minimize excitatory currents. Voltage responses to current ramps were digitized at 10kHz and filtered at 1kHz. These recordings were done under current clamp at a holding potential of –60mV.

2.3 Interleukin-1beta treatment

IL-1β treatments were typically administered to DMOTC spinal cord slices 14 to 28 days after the start of culture, and recordings were typically obtained 21 to 35 days following the start of culture. IL-1β (Calbiochem, Hornby, ON, Canada) stocks, dissolved in bovine serum albumin (BSA; HyClone, Logan, UT, USA), were aliquoted into sterile vials in denominations of 100nM and 1nM; they were diluted using 0.1% BSA. Medium for the IL-1β-treated groups consisted of *n* mL of serum-free medium containing *n* µL of *x* nM IL-1β (*x* = 1 nM for 1 pM IL-1β group; *x* = 100 nM for 100 pM IL-1β group; *n* is the integer representing the volume of treatment media). To account for any unforeseen effects of BSA, control groups consisted of *n* mL of serum-free medium containing *n* µL of BSA. If 'day 1' is considered the treatment day, the DMOTC slices were exchanged on day 4 and recorded from on days 6-8 (Figure 2-3).

An enzyme-linked immunosorbent assay (ELISA) for IL-1β was conducted with our samples of BSA-treated control media, 1pM IL-1β-treated media, and 100pM IL-1β-treated media. As this was a precautionary test which was not part of the current project (i.e. ELISA was done by another laboratory), only one sample was measured after seven days' treatment of either BSA, 1pM, or 100pM IL-1β. As a corollary, the findings should be interpreted with caution as the reliability and validity of this ELISA have the potential

to be low. Future analyses will need to be undertaken to definitively determine the concentrations of IL-1β after long-term treatment.

The reading from the control sample was below the detection limit for IL-1β, indicating that there is a negligible concentration of IL-1β in the BSA-treated control media. Following 1pM IL-1β treatment, it was found that the actual concentration in the media sample as detected by ELISA was 32.2pM. For the 100pM IL-1β treatment condition, it was found that the concentration in the media sample as detected by ELISA was 117.8pM. Not only are these values larger than expected based on the treatment concentrations, but they are not proportionately increased, suggesting that there may be different positive-feedback cascades or pro-inflammatory pathways activated with different concentrations. Additionally, the actual concentrations of each condition when presented on a logarithmic scale are not very different from one another: 32pM and 118pM (i.e. 1.5 and 2, since log 32 = 1.5 and log 100 = 2) are far closer than the intended 1pM and 100pM (i.e. 0 and 2, since log 1 = 0 and log 100 = 2), possibly weakening the attempt to obtain electrophysiological data from a "high" and a "low" concentration of IL-1β.

2.4 Histochemistry

Neurons in DMOTC were routinely filled with biocytin (0.2%) during recordings for post-hoc identification. This low concentration was chosen because biocytin has been reported to interfere with postsynaptic drug responses at higher concentrations (Eckert III, Willcockson, & Light, 2001). At the completion of recording, the patch pipette was slowly withdrawn from the cell and the slice transferred to cold (4°C) 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, and stored overnight at

4°C. Slices were rinsed three times with PBS and transferred to a 24-well tissue culture dish for staining. Slices were incubated with 0.3% Triton X-100 and streptavidin-Texas red conjugate (1:50 dilution, Molecular Probes, Eugene, OR, USA) for 50 minutes on a 3D rotator. Slices were thoroughly rinsed with distilled water, transferred to slides, allowed to dry overnight, and coverslipped with Cytoseal^{TM}-60 (Richard-Allan Scientific, Kalamazoo, MI, USA). A Zeiss LSM 510 confocal laser scanning microscope, equipped with an appropriate laser (HeNe1, wavelength 543 nm) and Texas red filter, was used to examine the tissue. Confocal images and 3D reconstructions were acquired using Zeiss LSM image browser software. Figures were converted to black and white to increase contrast and clarity.

2.5 Confocal Calcium Imaging

Voltage-activated Ca^{2+} channels are present throughout the nervous system, including in spinal dorsal horn neurons. Voltage-gated channels open or close, depending on the transmembrane potential of the cell. Depolarization of neurons occurs when a stimulus causes a critical number of voltage-gated Na^{+} channels to open, allowing Na^{+} ions to flow rapidly down their electrochemical gradient and into the cell. This change in voltage (depolarization) activates Ca^{2+} channels; accordingly, laser scanning confocal imaging of cytosolic Ca^{2+} concentration should be an appropriate tool to visualize neuronal activity and responsiveness to a high K^{+} (depolarizing) pulse.

The membrane permeable acetoxymethyl (AM) ester of the Ca^{2+}-sensitive dye Fluo-4 (TEF Labs Inc. Austin, Texas, USA) was kept in 5 mM stock solution (a prediluted mixture of dimethyl sulfoxide and 20% pluronic acid; Invitrogen, Burlington, Ontario, Canada). Using aseptic techniques, 10μL of the Fluo-4 dye was added to the

DMOTC medium (1mL) of an individual flat-bottomed tissue culture tube, and incubated in the roller drum for 90 minutes. Fluorescence signals were measured using a confocal microscope and software (20x XLUMPlanF1-NA-0.95 objective; Olympus FV300, Carsen group, Markham, Ontario, Canada) as described previously (Nikolenko, Nemet, & Yuste, 2003). Fluorescence was sufficiently intense to reveal basic neuronal morphology and rhythmic changes in fluorescence intensity. The entire slice was fluorescently labeled, and individual cells were labeled at tissue depths up to 150 μm. Cellular activity and morphological features such as soma shape and array of primary dendrites could be resolved in tissue depths up to 60 μm. Neurons were typically imaged at a depth of 30-60μm. Up to 15 cells (typically 3-7) showed rhythmic Ca^{2+} rises in a single confocal x-y-image plane covering the Fluo-4-labelled spot. The spot was assessed using a 2-3x digital zoom at reduced settings for y-axis scanning. Consequently, such "clipped mode" imaging (instead of full frame acquisition of 512 x 512 pixels) was done from an area of about 300 x 100-220 μm. This provided scan rates of 0.7-0.8 seconds, sufficient to detect 70-100% of the peak Ca^{2+} rises. Morphological details of dorsal horn neurons were revealed by combining
"z-stack"-imaging with 3D-animation using the Fluoview software. Since Fluo-4 fluoresces weakly in cells with low cytosolic Ca^{2+} concentrations, fluorescence was measured as slices were exposed to three different concentrations of high K^+ external solution (20 mM, 35mM, and 50mM; normal external solution is 4mM) for 30 seconds.

2.6 Statistical Analyses

Mini Analysis Program software (Synaptosoft, Decatur, GA, USA) was used to distinguish sPSCs from the baseline noise and to prepare cumulative probability plots or

amplitude histograms. The first 100 events after the first minute of recording spontaneous events were used in sPSC analysis. The Kolmogorov-Smirnov two-sample test (K-S test) was used to compare distributions of amplitudes and inter-event intervals in neurons in DMOTC. This method tested the null hypothesis that two independent samples come from populations that are identical with respect to location and distribution. The K-S test was used because it compared the entire distribution of amplitudes or inter-event intervals rather than the means of these data (Van der Kloot, 1991). Distributions were considered statistically significant if $p < 0.05$.

All data collected under voltage clamp and current clamp were acquired using pCLAMP 9.0 (Axon Instruments, Burlingame, CA,USA). Figures were produced with Origin 5.0 (OriginLab, Northampton, MA, USA). Statistical comparisons were made as appropriate using the unpaired t-test, Welsh corrected (GraphPad InStat software, San Diego, CA, USA), Tukey one-way ANOVA with post test (GraphPad InStat software, San Diego, CA, USA), or K-S test as described above. Statistical significance was taken as $p<0.05$ unless otherwise stated.

Embryonic spinal cord slice

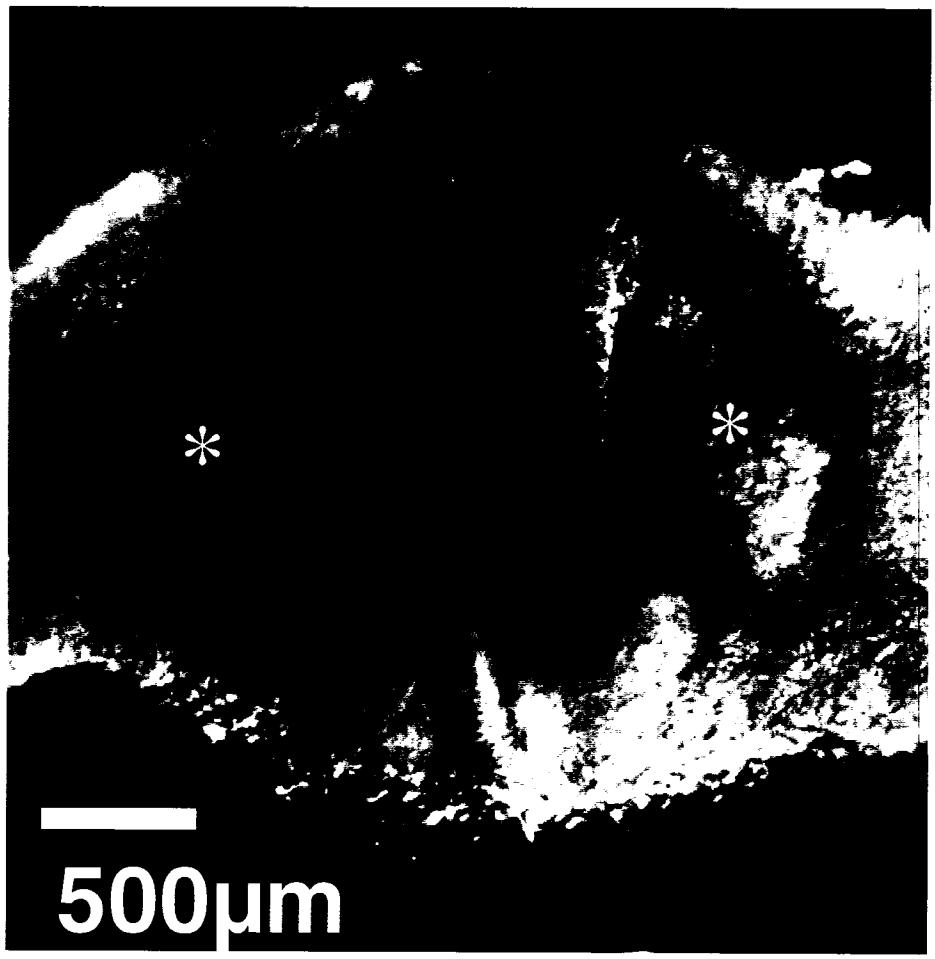


Figure 2-1. Spinal cord slice including DRGs (identified by asterisks) immediately before plating and incubation.

Flat-bottomed tubes and incubator

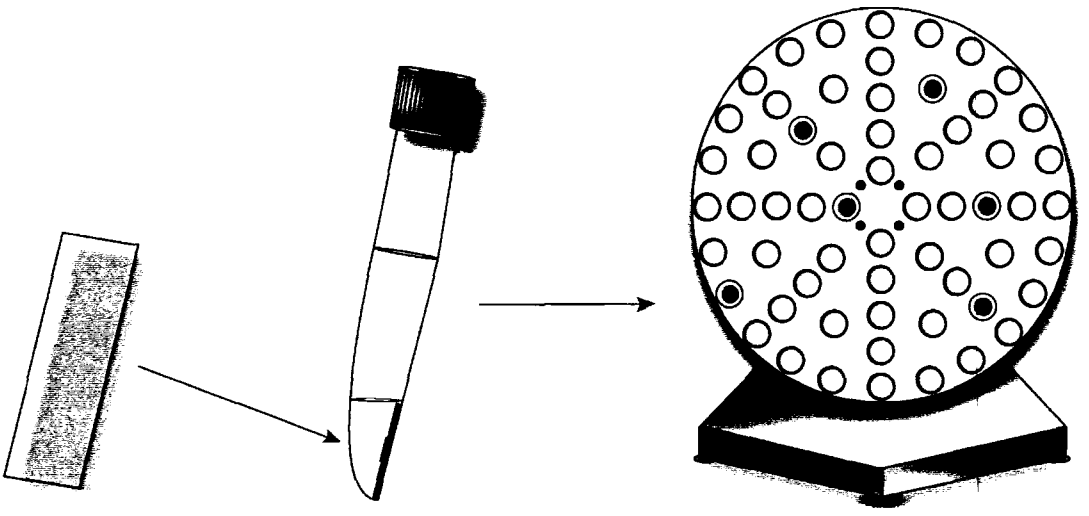


Figure 2-2. Diagram of incubation methods of flat-bottomed tubes with coverslips.

Treatment schedule

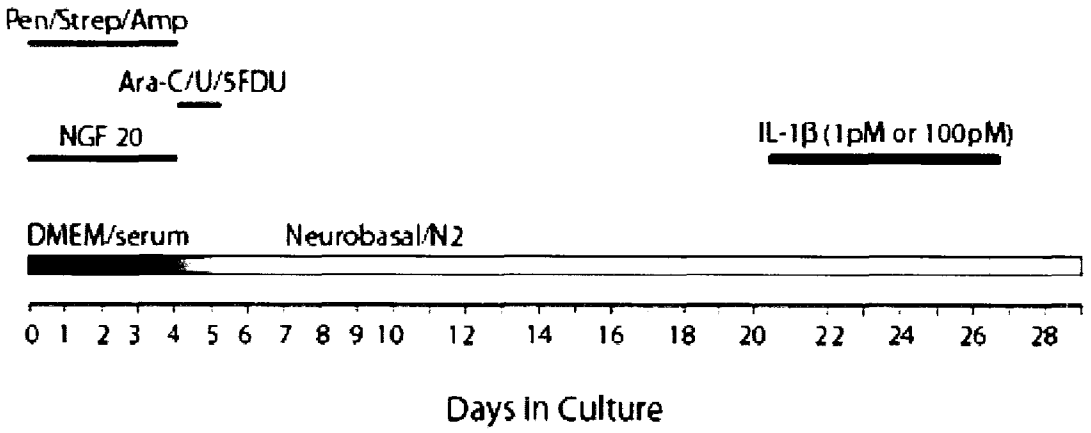


Figure 2-3. Time course of drug application and interleukin treatment in culture medium.

2.7 References

Braschler, U.F., Iannone, A., Spenger, C., Streit, J., & Luscher, H.R. (1989). A modified roller tube technique for organotypic cocultures of embryonic rat spinal cord, sensory ganglia and skeletal muscle. *J Neurosci Methods, 29*: 121-129.

Eckert III, W.A., Willcockson, H.H., & Light, A.R. (2001). Interference of biocytin with opioid-evoked hyperpolarization and membrane properties of rat spinal substantia gelatinosa neurons. *Neurosci Lett, 297*(2): 117-120.

Gahwiler, B.H. (1981). Organotypic monolayer cultures of nervous tissue. *J Neurosci Methods, 4*: 329-342.

Gahwiler, B.H., Capogna, M., Debanne, D., McKinney, R.A., & Thompson, S.M. (1997). Organotypic slice cultures: a technique has come of age. *TINS, 20*(10): 471-477.

Lu, V.B., Moran, T.D., Balasubramanyan, S., Alier, K.A., Dryden, W.F., Colmers, W.F., & Smith, P.A. (2006). Substantia gelatinosa neurons in defined-medium organotypic slice culture are similar to those in acute slices from young adult rats. *Pain, 121*: 261-275.

Nikolenko, V., Nemet, B., & Yuste, R. (2003). A two-photon and second-harmonic microscope. *Methods, 30*: 3-15.

Van der Kloot, W. (1991). The regulation of quantal size. *Prog Neurobiol, 36*: 93-130.

CHAPTER 3 EFFECTS OF 100PM OF INTERLEUKIN-1BETA ON EXCITABILITY OF SPINAL DORSAL HORN NEURONS IN DMOTC

3.1 Effects of a High Concentration (100pM) of IL-1β

In the DMOTC, there were five different neuronal classes that were characterized according to electrophysiological properties, as was discussed in Chapter 1 and as has been previously described (Lu et al., 2006). The relative proportions of each cell type to the entire population of neurons was found for BSA controls, as well as for the IL-β-treated conditions (Figure 3-1).

In controls, it was found that 22.9% of cells were Tonic, 47.9% were Irregular, 10.4% were Delay, 10.4% were Transient, and 8.33% were Phasic. When examining the cells treated with 100pM of IL-1β, it was found that 17.1% were Tonic, 40% were Irregular, 22.9% were Delay, 11.4% were Transient, and 8.57% were Phasic. These proportions are relatively similar to each other, and to the proportions which have been previously reported in DMOTC cultures (Lu et al., 2006).

3.1.1 Resting Membrane Potential, Rheobase, Cell Capacitance

In BSA-treated control cells, there were some differences between the five different neuronal classes with respect to resting membrane potential (RMP), rheobase, input resistance, and cell capacitance (C_m); these are summarized on Table 3-1. Average RMPs ranged from –45mV to –56mV, average rheobase ranged from 25pA to 135pA, and average C_m ranged from 30pF to 57pF. The main trends among the neuronal types were that Tonic cells had the lowest rheobase of all the groups (average = 25 pA), while Transient neurons had the highest rheobase (average = 135 pA) and had the smallest average C_m (average = 30.333 pF). The only statistically significant difference was that rheobase was significantly lower in Tonic neurons when compared to Transient neurons ($p=0.0082$).

The RMP, rheobase, and C_m of the five neuronal classes in the high concentration (100pM) of IL-1β condition were not significantly different than the corresponding neuronal types in the control group (p values ranged from 0.12 to 0.99; Figures 3-2, 3-3, and 3-4). Average RMPs ranged from –44mV to –54mV, average rheobase ranged from 32pA to 174pA, and average C_m ranged from 23pF to 48pF. Also, similar trends were observed: Tonic cells had the lowest average rheobase of all the neuronal types (31.667 pA), while Transient cells had the highest average rheobase (173.75 pA) and the smallest average C_m (23 pF). Statistically significant differences within the 100pM group were that the rheobase of Transient neurons was higher than Tonic, Delay, and Irregular neurons ($p<0.05$).

3.1.2 Spontaneous Post-Synaptic Currents

The amplitudes and frequencies of excitatory and inhibitory spontaneous post-synaptic currents (sEPSCs and sIPSCs, respectively) were measured in both the BSA-treated control cells and the IL-1β-treated cells. The significant differences between sPSCs of IL-1β-treated cells and controls are summarized in Table 3-2.

With respect to sEPSCs, it was found that amplitudes were significantly greater following IL-1β treatment in Tonic cells, Delay cells, and Transient cells (Figures 3-5, 3-7, and 3-11). In contrast, amplitudes were significantly reduced following IL-1β treatment in Irregular cells and Phasic cells (Figures 3-9 and 3-13). sEPSC frequencies were significantly reduced following IL-1β treatment in Delay cells, Irregular cells, and Phasic cells, but significantly greater following IL-1β treatment in Transient cells; lastly, in Tonic cells, there was no significant difference in sEPSC frequency (Figures 3-5, 3-7, 3-9, 3-11, and 3-13).

In terms of sIPSCs, it was found that amplitudes were significantly greater following IL-1β treatment in Irregular cells, Delay cells, and Transient cells, while in Tonic cells and Phasic cells, amplitudes were significantly reduced (Figures 3-6, 3-8, 3-10, 3-12, and 3-14). sIPSC frequencies were significantly reduced following IL-1β treatment in Tonic cells, Irregular cells, and Delay cells, but significantly greater in Transient cells, and not significantly different than controls in Phasic cells (Figures 3-6, 3-8, 3-10, 3-12, and 3-14).

Changes in sPSC amplitude and frequency were quite diverse across cell types and treatment conditions. Both increases and decreases in sIPSC and sEPSC amplitude were seen, but there was a general trend towards a decreased frequency of sEPSCs and sIPSCs in all cell types, with the exception of Transient neurons. The number of cells in the Phasic and Transient groups were low, which is a rationale for excluding these from further analysis.

Table 3-1

Resting Membrane Potential, Rheobase, and Cell Capacitance Means By Neuronal Phenotype and Treatment Condition

BSA Controls

	Tonic	Irregular	Delay	Transient	Phasic
RMP (mV)	$x = -52.455$ SE = 2.626 $n = 11$	$x = -51.261$ SE = 1.775 $n = 23$	$x = -48.600$ SE = 5.913 $n = 5$	$x = -45.000$ SE = 5.244 $n = 5$	$x = -56.250$ SE = 4.090 $n = 4$
Rheobase (pA)	$x = 25.000$ SE = 2.041 $n = 9$	$x = 82.500$ SE = 12.101 $n = 18$	$x = 61.000$ SE = 19.962 $n = 5$	$x = 135.000$ SE = 32.851 $n = 4$	$x = 118.750$ SE = 53.205 $n = 4$
Capacitance (pF)	$x = 51.000$ SE = 8.631 $n = 9$	$x = 48.125$ SE = 6.347 $n = 16$	$x = 38.800$ SE = 3.353 $n = 5$	$x = 30.333$ SE = 9.871 $n = 3$	$x = 57.000$ SE = 22.855 $n = 3$

100pM IL-1β

	Tonic	Irregular	Delay	Transient	Phasic
RMP (mV)	$x = -53.167$ SE = 4.556 $n = 6$	$x = -53.643$ SE = 1.880 $n = 14$	$x = -54.625$ SE = 3.664 $n = 8$	$x = -45.000$ SE = 5.401 $n = 4$	$x = -44.000$ SE = 4.583 $n = 3$
Rheobase (pA)	$x = 31.667$ SE = 10.138 $n = 6$	$x = 70.833$ SE = 11.947 $n = 12$	$x = 72.143$ SE = 8.441 $n = 7$	$x = 173.750$ SE = 65.903 $n = 4$	$x = 105.000$ SE = 20.000 $n = 2$
Capacitance (pF)	$x = 31.400$ SE = 7.711 $n = 5$	$x = 40.333$ SE = 3.793 $n = 12$	$x = 48.286$ SE = 7.318 $n = 7$	$x = 23.000$ SE = 3.782 $n = 5$	$x = 28.500$ SE = 3.500 $n = 2$

1pM IL-1β

	Tonic	Irregular	Delay	Transient	Phasic
RMP (mV)	$x = -57.857$ SE = 3.776 $n = 7$	$x = -48.857$ SE = 2.132 $n = 7$	$x = -50.556$ SE = 2.212 $n = 9$	$x = -70.000$ SE = n/a $n = 1$	$x = -65.000$ SE = n/a $n = 1$
Rheobase (pA)	$x = 33.333$ SE = 6.146 $n = 6$	$x = 45.000$ SE = 9.354 $n = 5$	$x = 40.000$ SE = 12.649 $n = 5$	$x =$ n/a SE = n/a $n =$ n/a	$x = 65.000$ SE = 5.000 $n = 2$
Capacitance (pF)	$x = 47.833$ SE = 8.031 $n = 6$	$x = 34.800$ SE = 6.793 $n = 5$	$x = 40.750$ SE = 7.804 $n = 4$	$x =$ n/a SE = n/a $n =$ n/a	$x = 35.000$ SE = 10.000 $n = 2$

Table 3-2

Summary of Significant Differences in Spontaneous Post-Synaptic Currents (sPSCs) of IL-1β-Treated Cells When Compared With BSA Controls (Statistical Significance Determined with K-S Test, $p<0.05$)

	TONIC		IRREGULAR		DELAY		TRANSIENT		PHASIC	
1pM IL-β sEPSCs	Amplitude ↑	Frequency NS p=0.10	Amplitude ↓	Frequency ↑	Amplitude ↑	Frequency ↓	Amplitude ----	Frequency ----	Amplitude NS p=0.07	Frequency ↓
1pM IL-β sIPSCs	Amplitude ↑	Frequency ↓	Amplitude ↓	Frequency ↑	Amplitude ↑	Frequency ↓	Amplitude ----	Frequency ----	Amplitude ↑	Frequency ↓
100pM IL-β sEPSCs	Amplitude ↑	Frequency NS p=0.40	Amplitude ↓	Frequency ↓	Amplitude ↑	Frequency ↓	Amplitude ↑	Frequency ↑	Amplitude ↓	Frequency ↓
100pM IL-β sIPSCs	Amplitude ↓	Frequency ↓	Amplitude ↑	Frequency ↓	Amplitude ↑	Frequency ↓	Amplitude ↑	Frequency ↑	Amplitude ↓	Frequency NS p=0.37

Neuronal type proportions

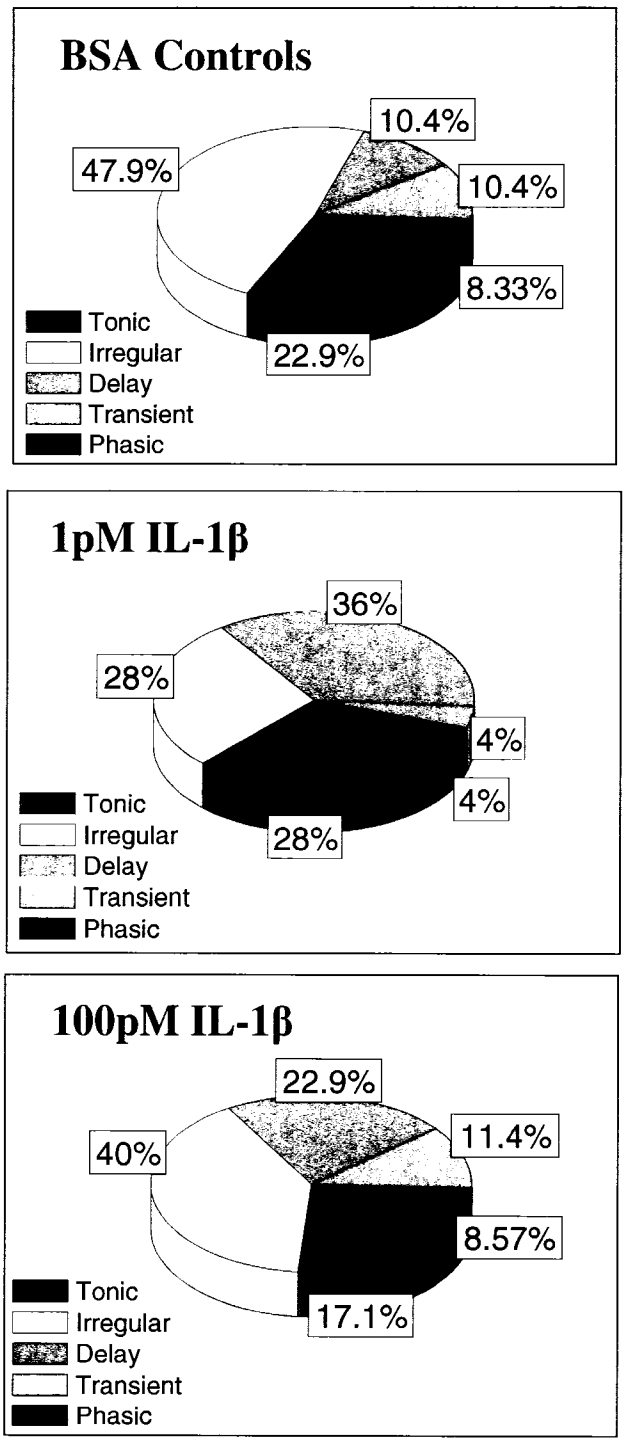


Figure 3-1. Proportions of each neuronal type (by treatment condition)found in the dorsal region of DMOTC slices.

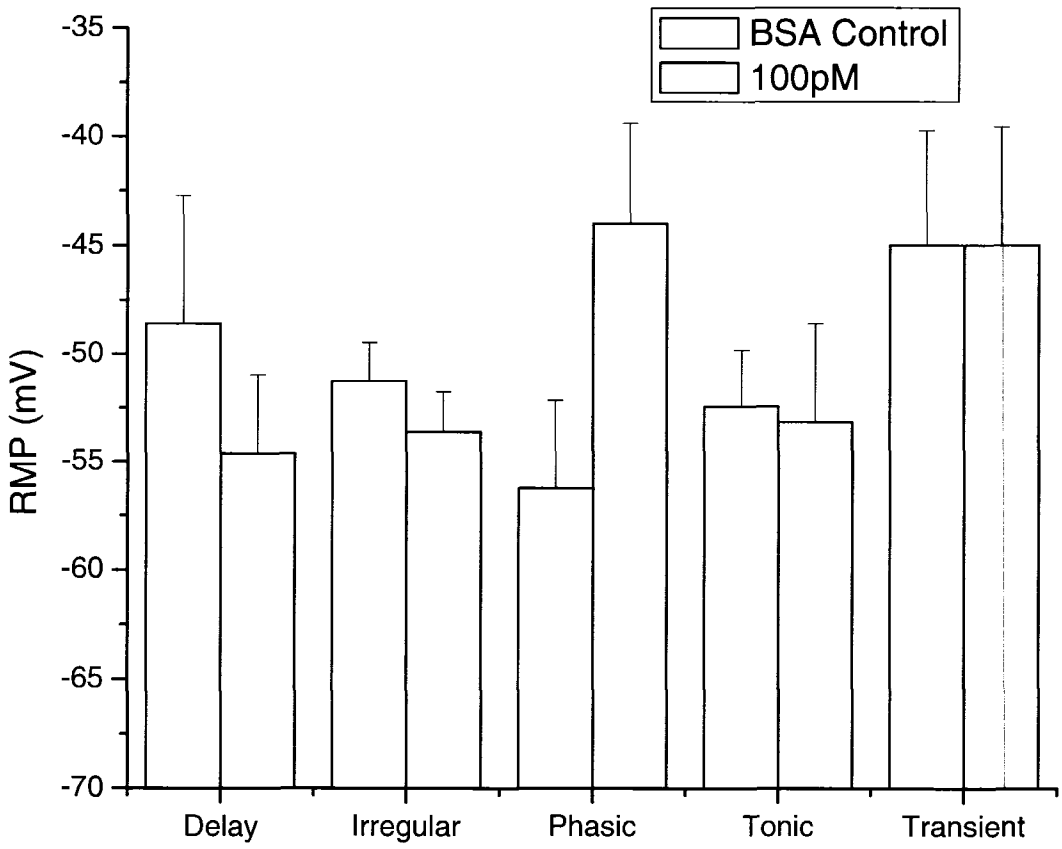


Figure 3-2. Comparison of resting membrane potential of BSA control neurons and neurons treated with 100pM IL-1β. (Error bars = SEM in this and all subsequent figures; for sample sizes '*n*', refer to Table 3-1).

Rheobase – BSA controls and 100pM IL-1β

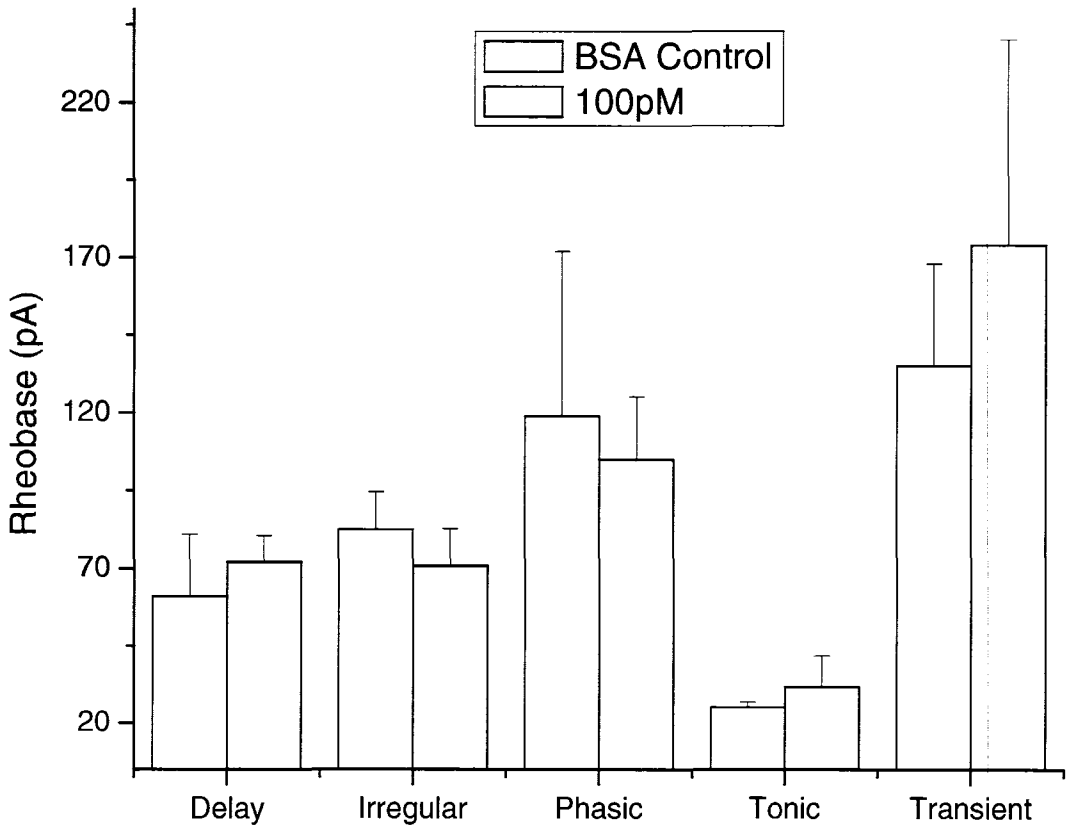


Figure 3-3. Comparison of rheobase of BSA control neurons and neurons treated with 100pM IL-1β.

C_m – BSA controls and 100pM IL-1β

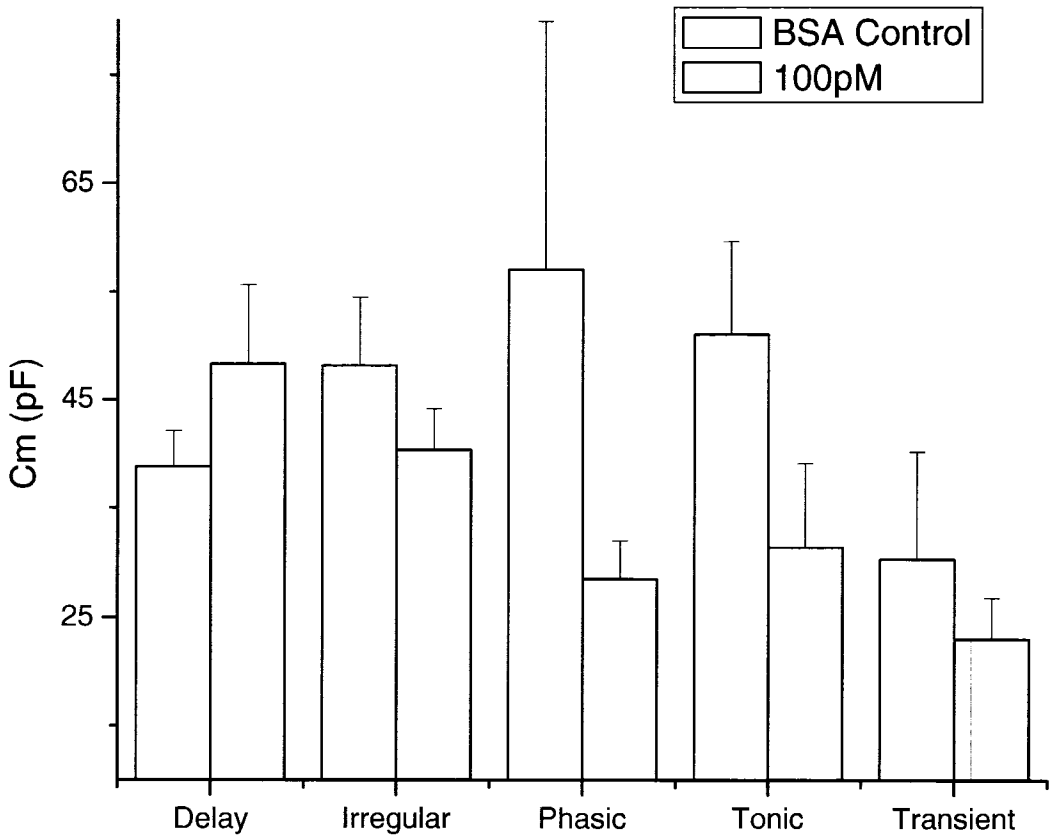


Figure 3-4. Comparison of cell capacitance of BSA control neurons and neurons treated with 100pM IL-1β.

Tonic sEPSCs

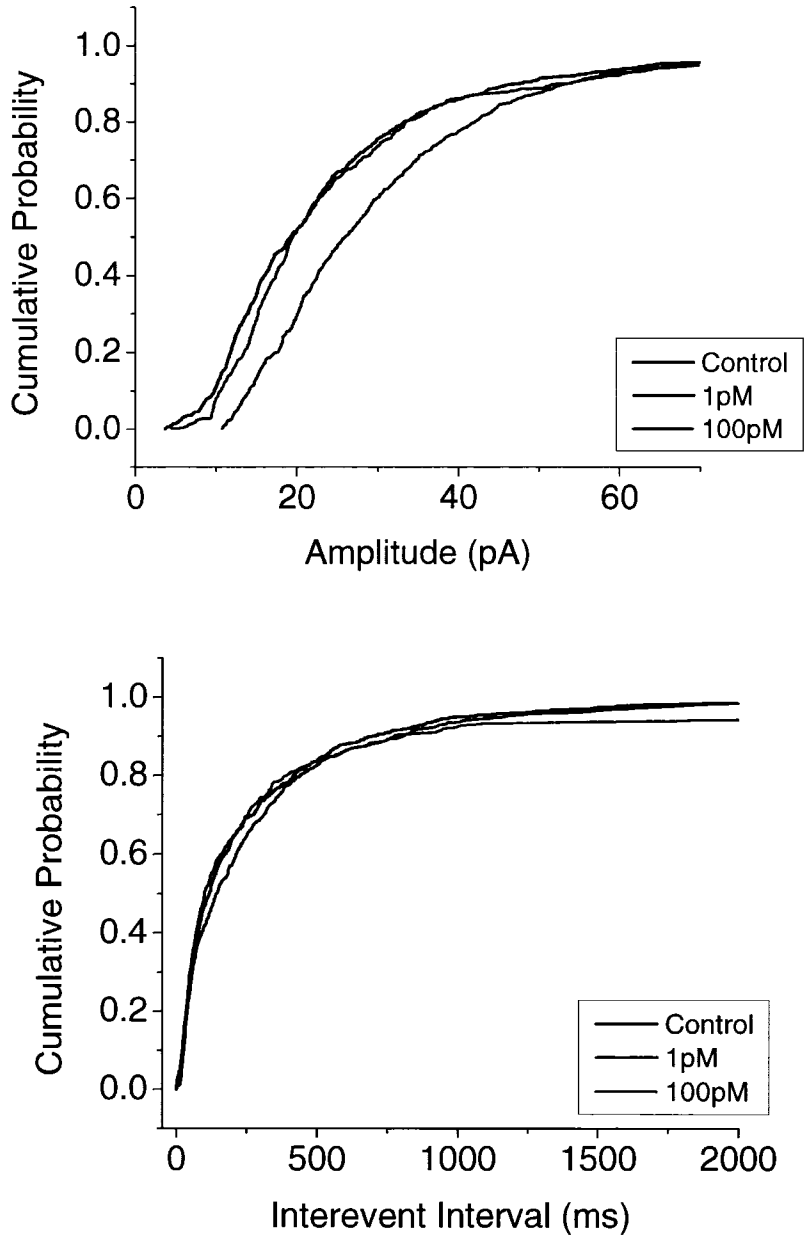


Figure 3-5. Cumulative probability distribution of sEPSC amplitudes and interevent intervals in Tonic neurons treated with BSA or IL-1β. Cumulative probability plots from this point onwards represent the first 100 events after the first minute of recording and comprise between two and 16 cells (see Table 3-1).

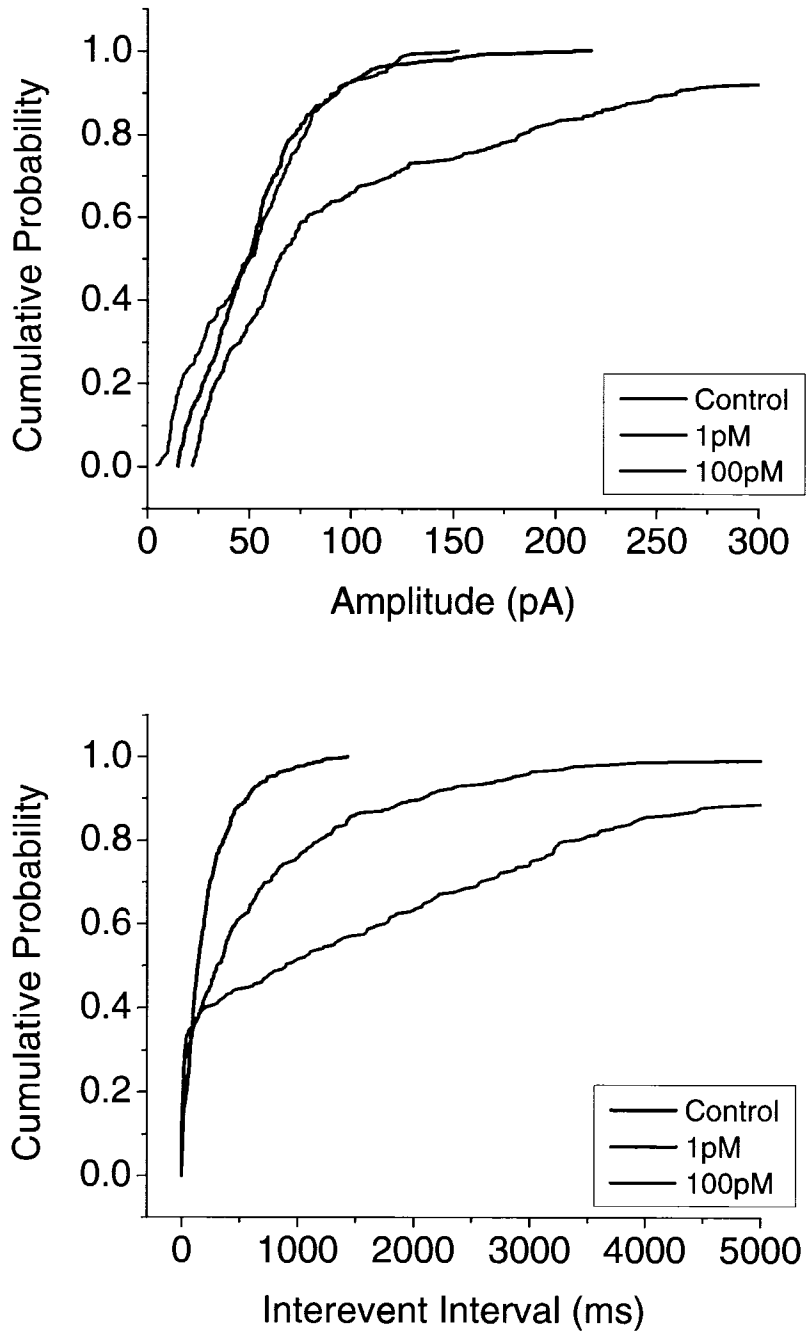


Figure 3-6. Cumulative probability distribution of sIPSC amplitudes and interevent intervals in Tonic neurons treated with BSA or IL-1β.

Delay sEPSCs

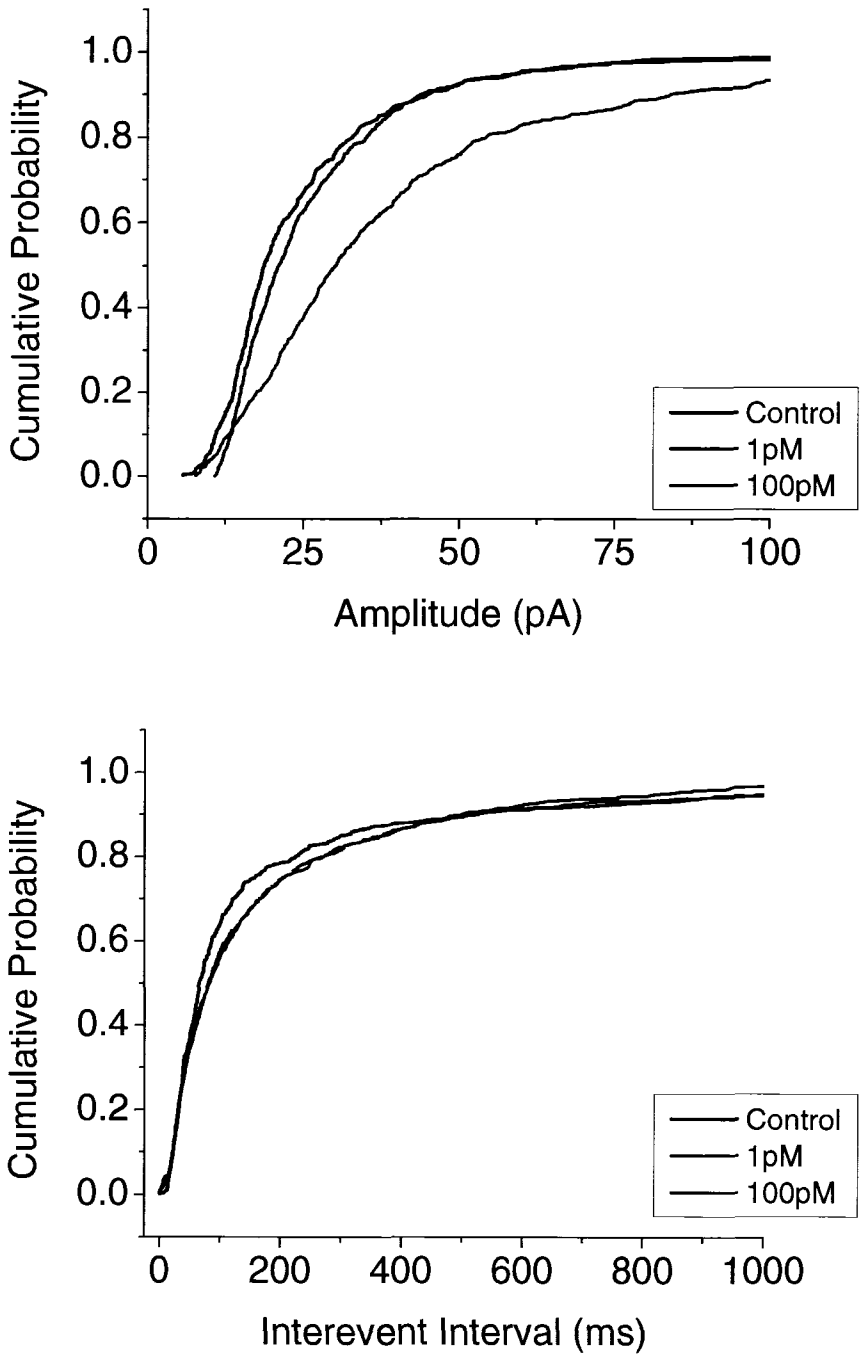


Figure 3-7. Cumulative probability distribution of sEPSC amplitudes and interevent intervals in Delay neurons treated with BSA or IL-1β.

Delay sIPSCs

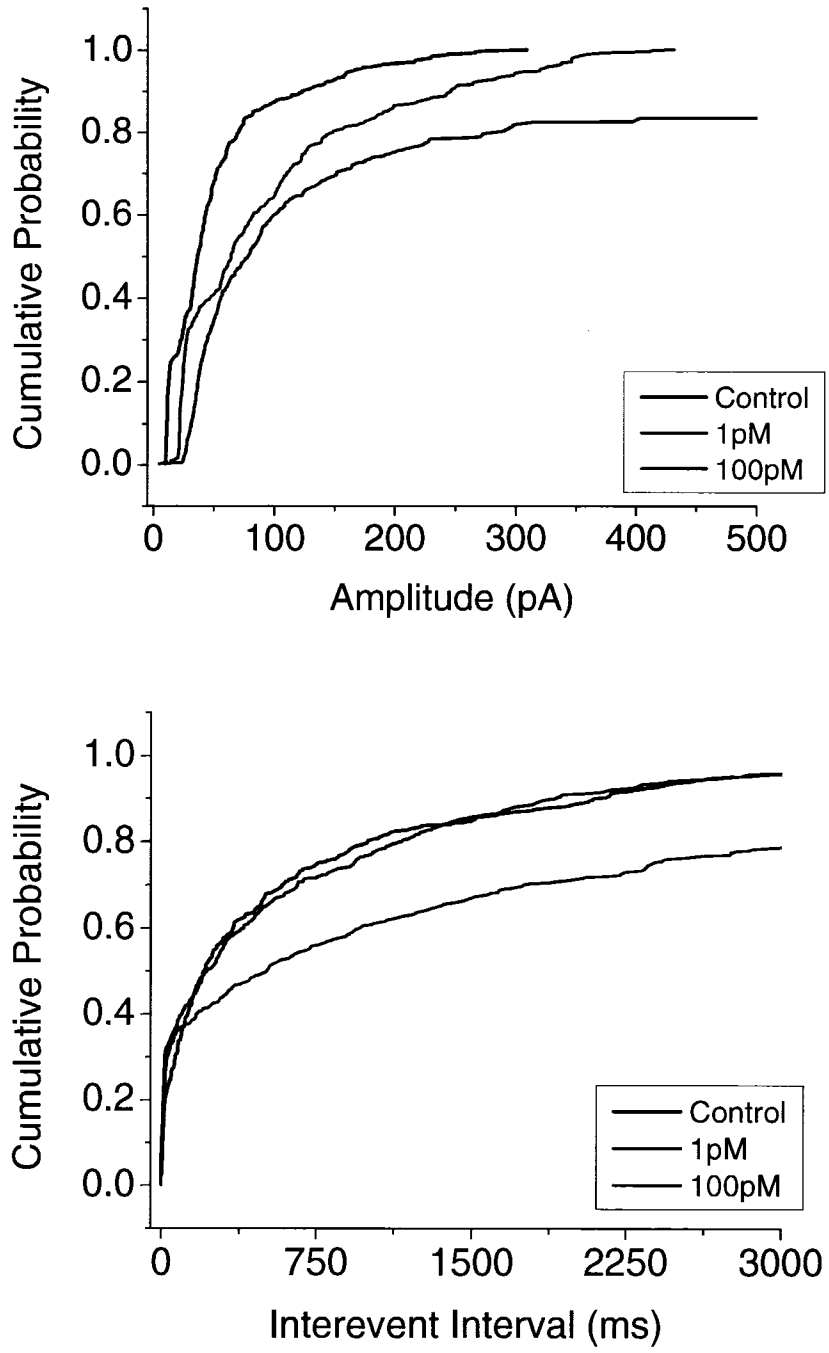


Figure 3-8. Cumulative probability distribution of sIPSC amplitudes and interevent intervals in Delay neurons treated with BSA or IL-1β.

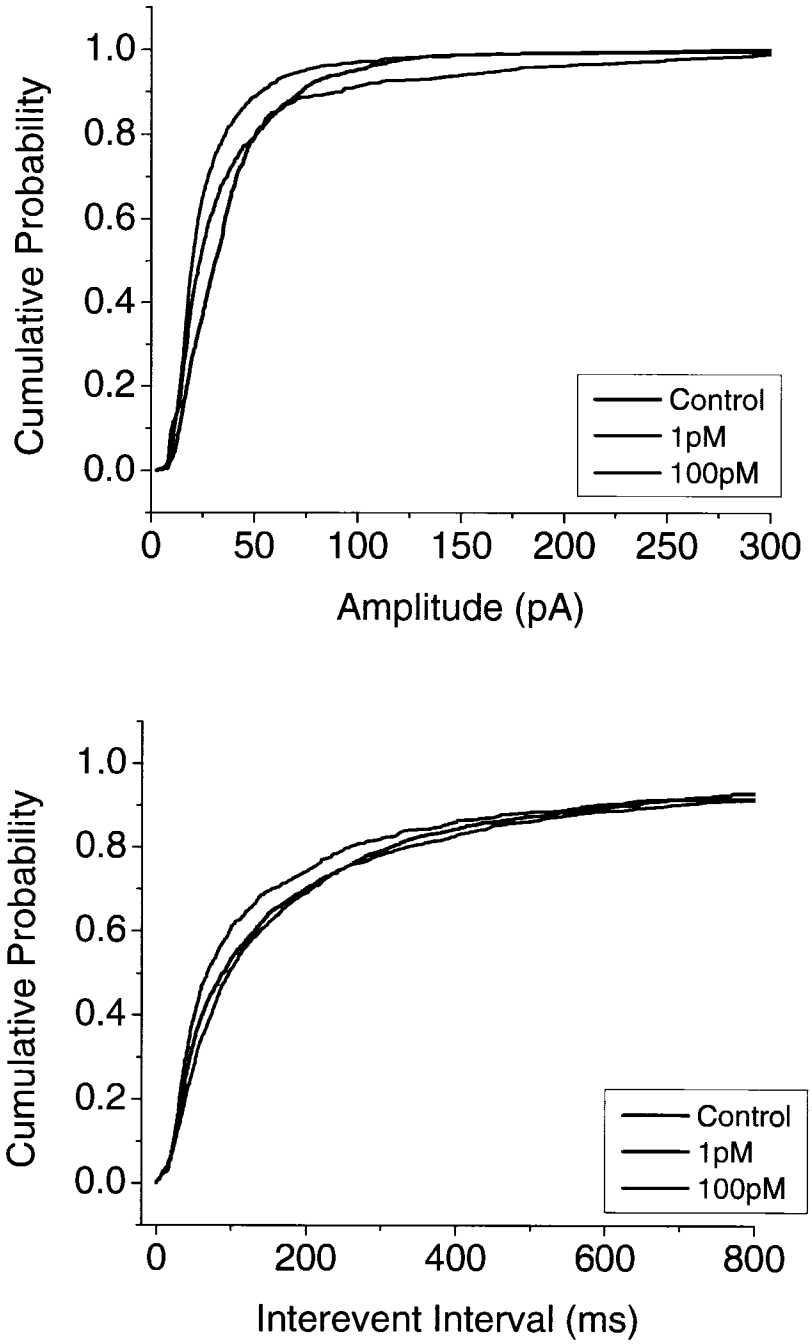


Figure 3-9. Cumulative probability distribution of sEPSC amplitudes and interevent intervals in Irregular neurons treated with BSA or IL-1β.

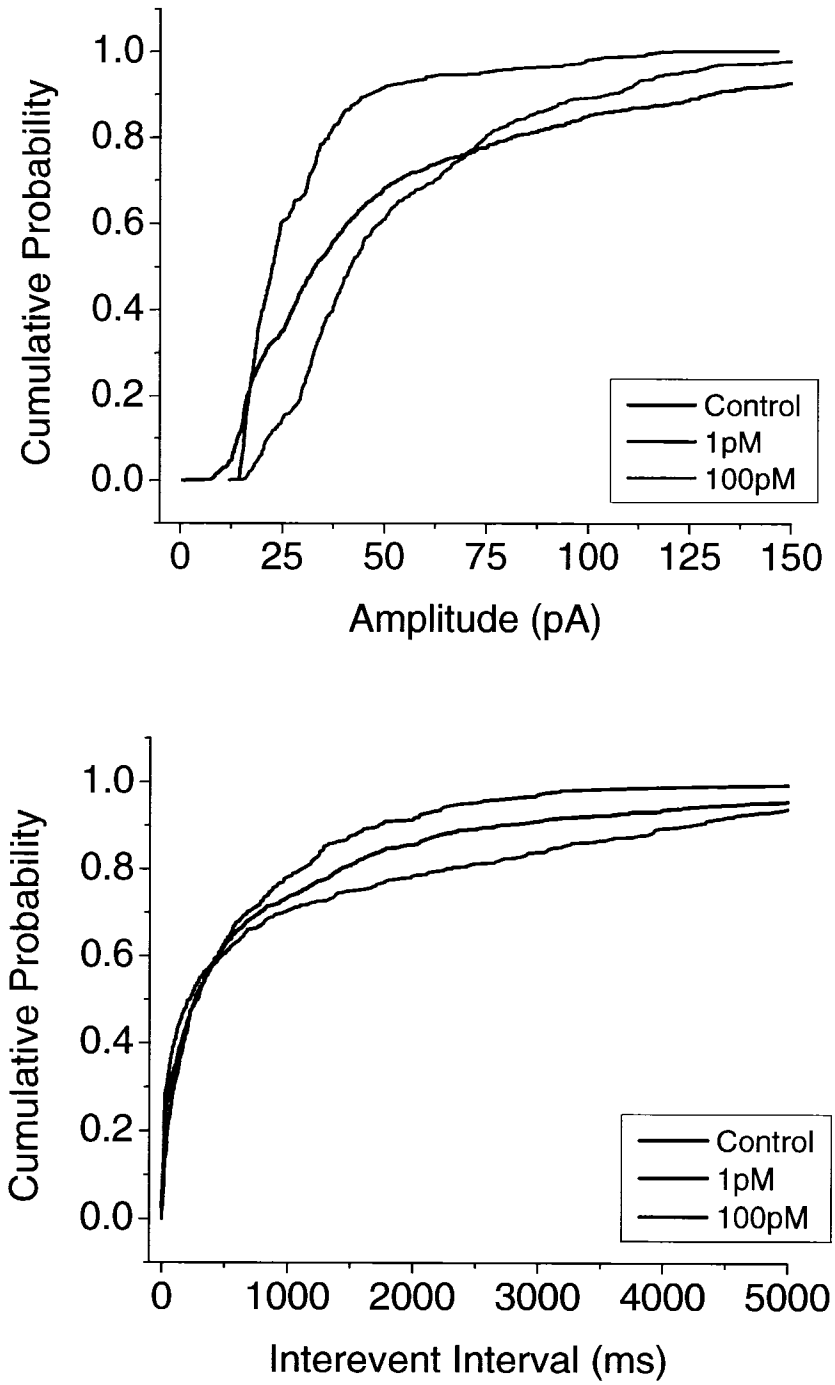


Figure 3-10. Cumulative probability distribution of sIPSC amplitudes and interevent intervals in Irregular neurons treated with BSA or IL-1β.

Transient sEPSCs

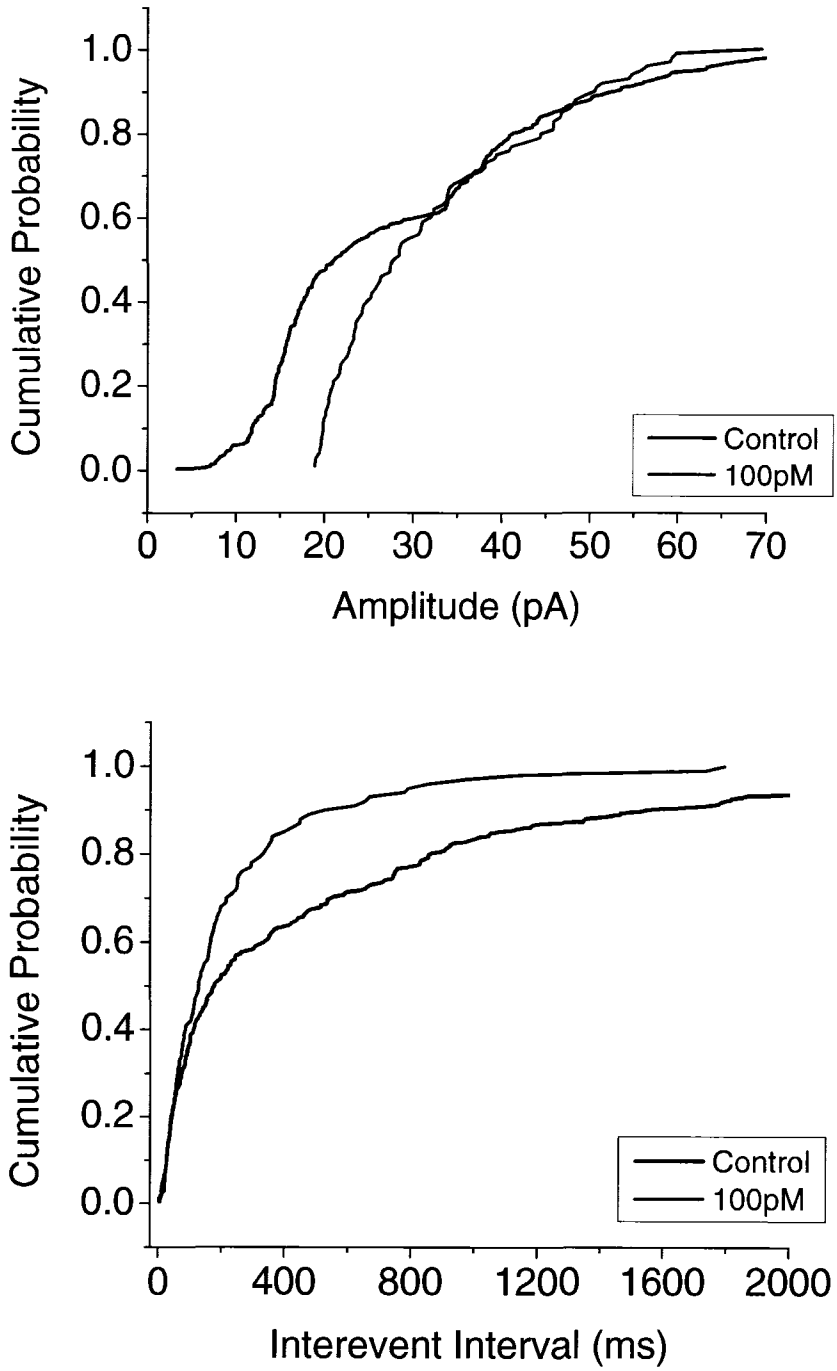


Figure 3-11. Cumulative probability distribution of sEPSC amplitudes and interevent intervals in Transient neurons treated with BSA or IL-1β.

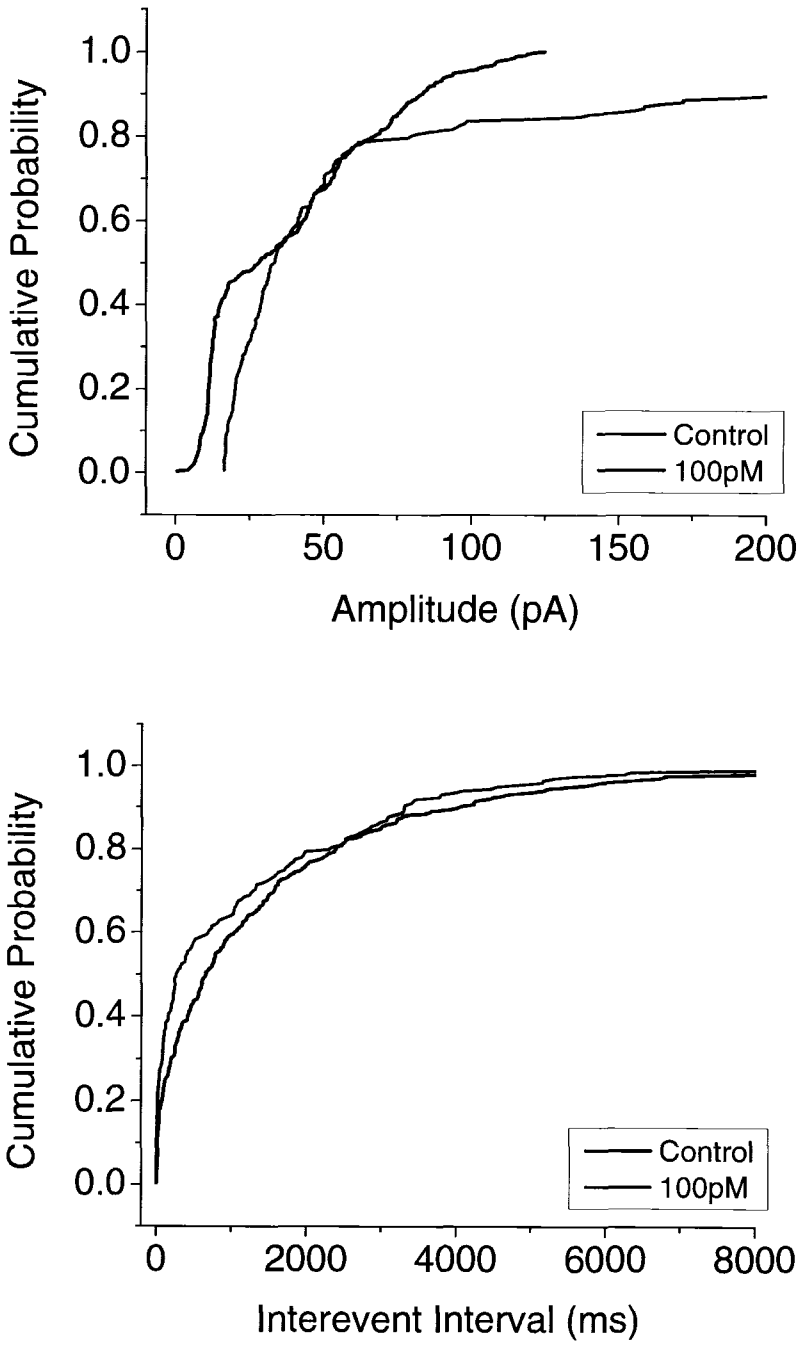


Figure 3-12. Cumulative probability distribution of sIPSC amplitudes and interevent intervals in Transient neurons treated with BSA or IL-1β.

Phasic sEPSCs

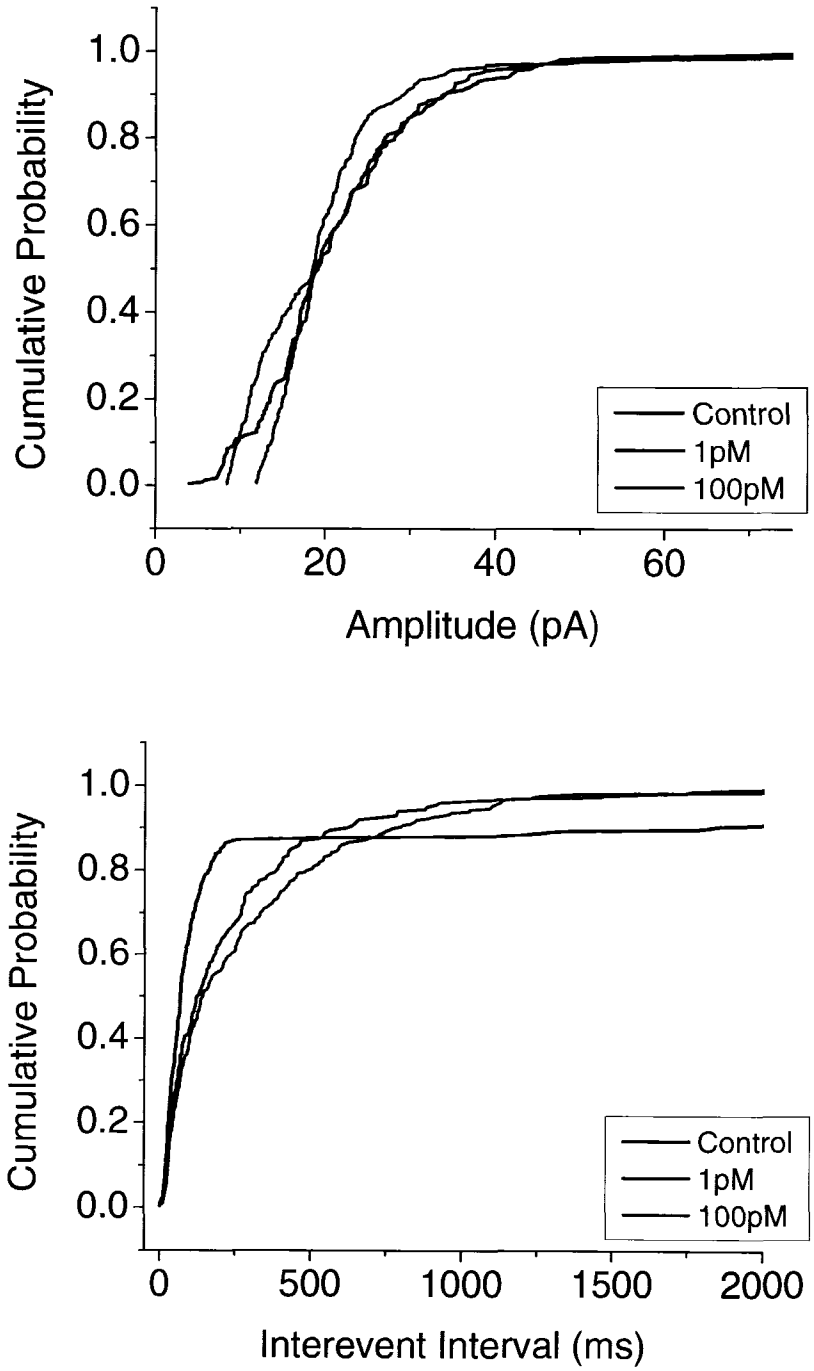


Figure 3-13. Cumulative probability distribution of sEPSC amplitudes and interevent intervals in Phasic neurons treated with BSA or IL-1β.

Phasic sIPSCs

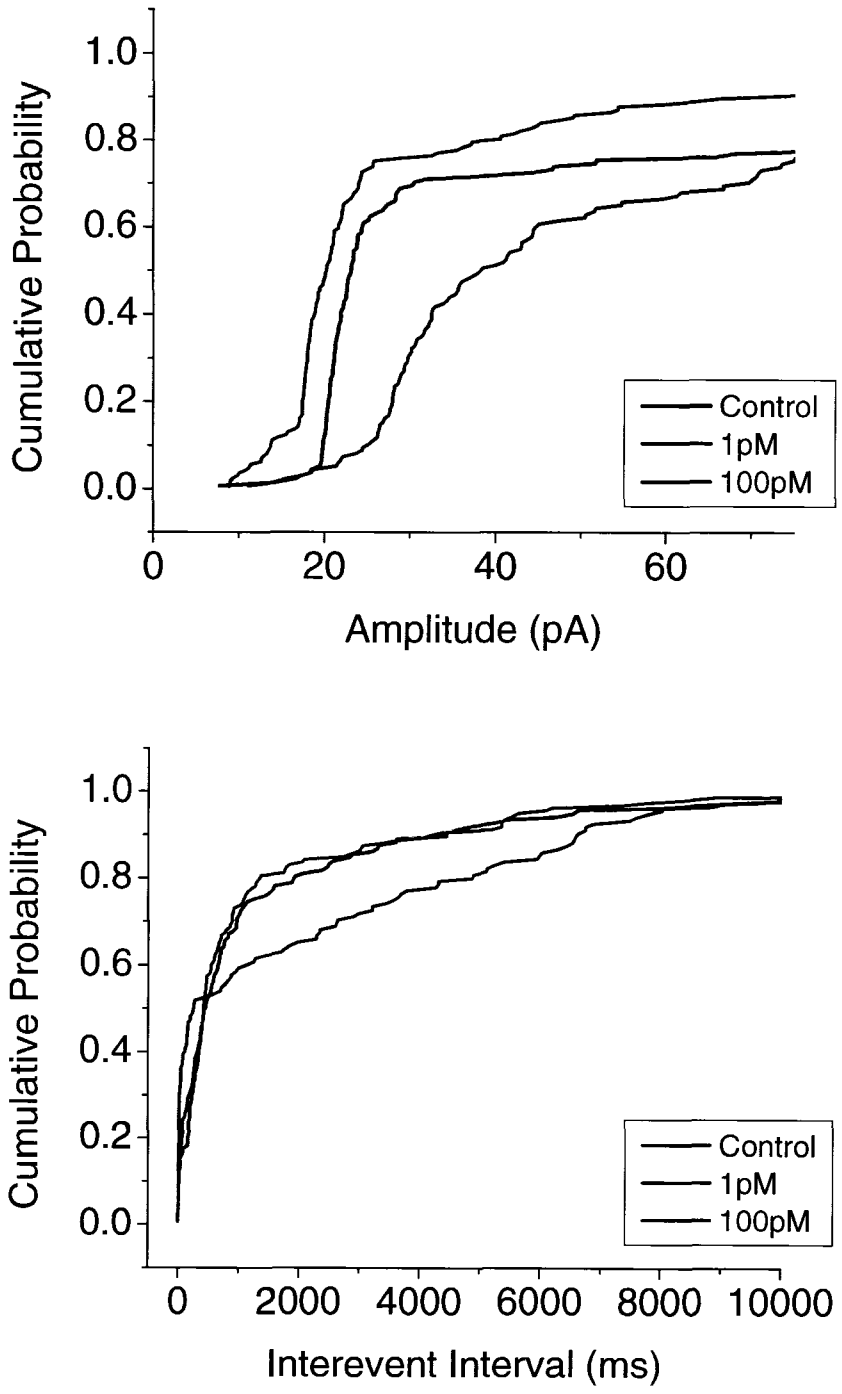


Figure 3-14. Cumulative probability distribution of sIPSC amplitudes and interevent intervals in Phasic neurons treated with BSA or IL-1β.

3.2 References

Lu, V.B., Moran, T.D., Balasubramanyan, S., Alier, K.A., Dryden, W.F., Colmers, W.F., & Smith, P.A. (2006). Substantia gelatinosa neurons in defined-medium organotypic slice culture are similar to those in acute slices from young adult rats. *Pain, 121*: 261-275.

CHAPTER 4 EFFECTS OF 1PM OF INTERLEUKIN-1BETA ON EXCITABILITY OF SPINAL DORSAL HORN NEURONS IN DMOTC

4.1 Effects of a Low Concentration (1pM) of IL-1β

The relative proportions of each cell type to the entire population of neurons was also described for the 1pM IL-β-treated condition (Figure 3-1). It was found that 28.0% were Tonic, 28.0% were Irregular, 36.0% were Delay, 4.0% were Transient, and 4.0% were Phasic. These proportions are relatively similar to those described for BSA controls and the high (100pM) concentration treatment condition, and to the proportions which have been previously reported in DMOTC cultures (Lu et al., 2006).

4.1.1 Resting Membrane Potential, Rheobase, Cell Capacitance

In general, the RMP, rheobase, and C_m of the five neuronal classes in the low concentration (1pM) of IL-1β condition were not significantly different than the corresponding neuronal types in the control group (p values ranged from 0.025 to 0.83; Figures 4-1, 4-2, and 4-3). Average RMPs ranged from –49mV to –70mV, average rheobase ranged from 33pA to 65pA, and average C_m ranged from 35pF to 48pF. Also, as was seen in the BSA controls, Tonic cells had the lowest average rheobase of all the neuronal types (31.667 pA; not statistically significant). All the other differences are summarized in Table 3-1.

The only statistically significant difference on any of the above measures between the neurons in the control condition and the cells treated with 1pM of IL-1β was the rheobase of Irregular cells (p=0.025).

4.1.2 Spontaneous Post-Synaptic Potentials

The amplitudes and frequencies of excitatory and inhibitory spontaneous post-synaptic currents (sEPSCs and sIPSCs, respectively) were measured in both the BSA-

treated control cells and the IL-1β-treated cells. The differences between sPSCs of IL-1β-treated cells and controls are summarized in Table 3-2.

With respect to sEPSCs, it was found that amplitudes were significantly greater following IL-1β treatment in Tonic cells and Delay cells (Figures 3-5 and 3-7). In contrast, amplitudes were significantly reduced following IL-1β treatment in Irregular cells, while sEPSC amplitudes were not significantly different in Phasic cells ($p=0.07$; Figures 3-9 and 3-13). sEPSC frequencies were significantly reduced following IL-1β treatment in Delay cells and Phasic cells, but significantly greater following IL-1β treatment in Irregular cells; lastly, in Tonic cells, there was no significant difference in sEPSC frequency ($p=0.10$; Figures 3-5, 3-7, 3-9, and 3-13).

In terms of sIPSCs, it was found that amplitudes were significantly greater following IL-1β treatment in Tonic cells, Delay cells, and Phasic cells, while in Irregular cells, amplitudes were significantly reduced (Figures 3-6, 3-8, 3-10, and 3-14). sIPSC frequencies were significantly reduced following IL-1β treatment in Tonic cells and Phasic cells, but significantly greater in Irregular cells and Delay cells (Figures 3-6, 3-8, 3-10, and 3-14).

Both increases and decreases in sEPSC and sIPSC amplitude were seen, as well as increases and decreases in sPSC frequency. However, these changes were different from those seen following treatment with 100pM IL-1β.

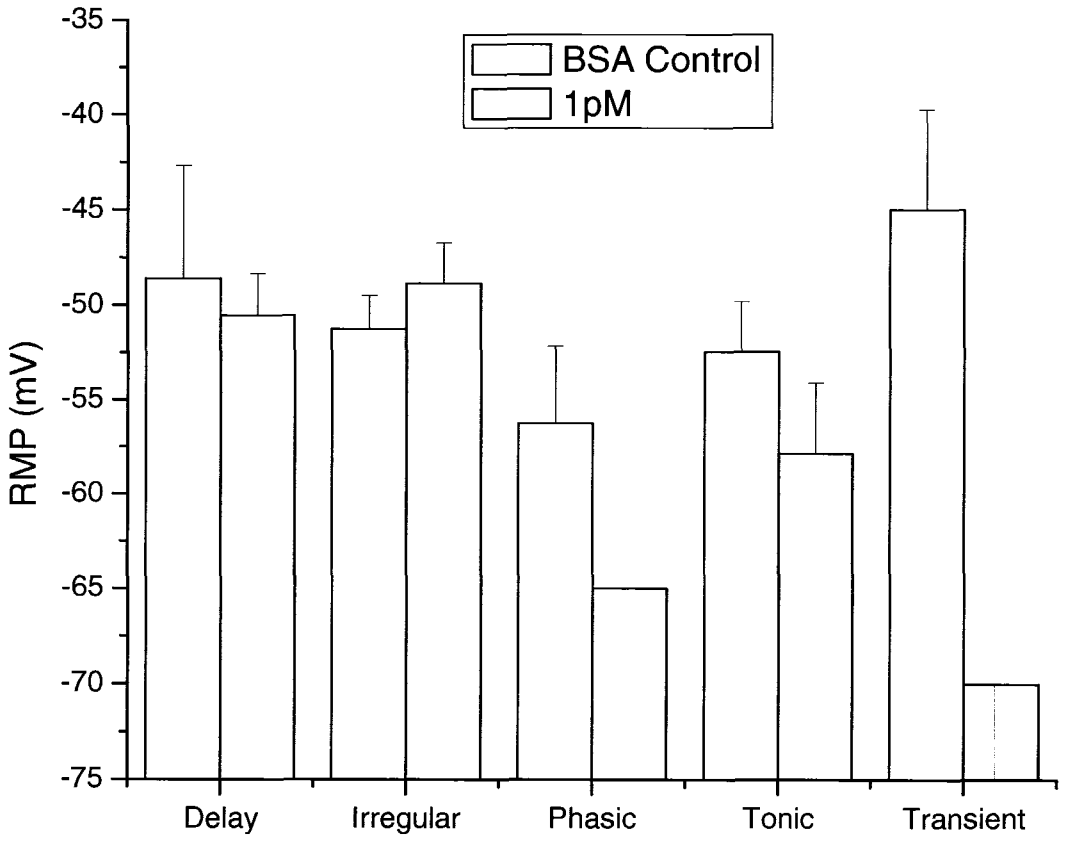


Figure 4-1. Comparison of resting membrane potential of BSA control neurons and neurons treated with 1pM IL-1β.

Rheobase – BSA controls and 1pM IL-1β

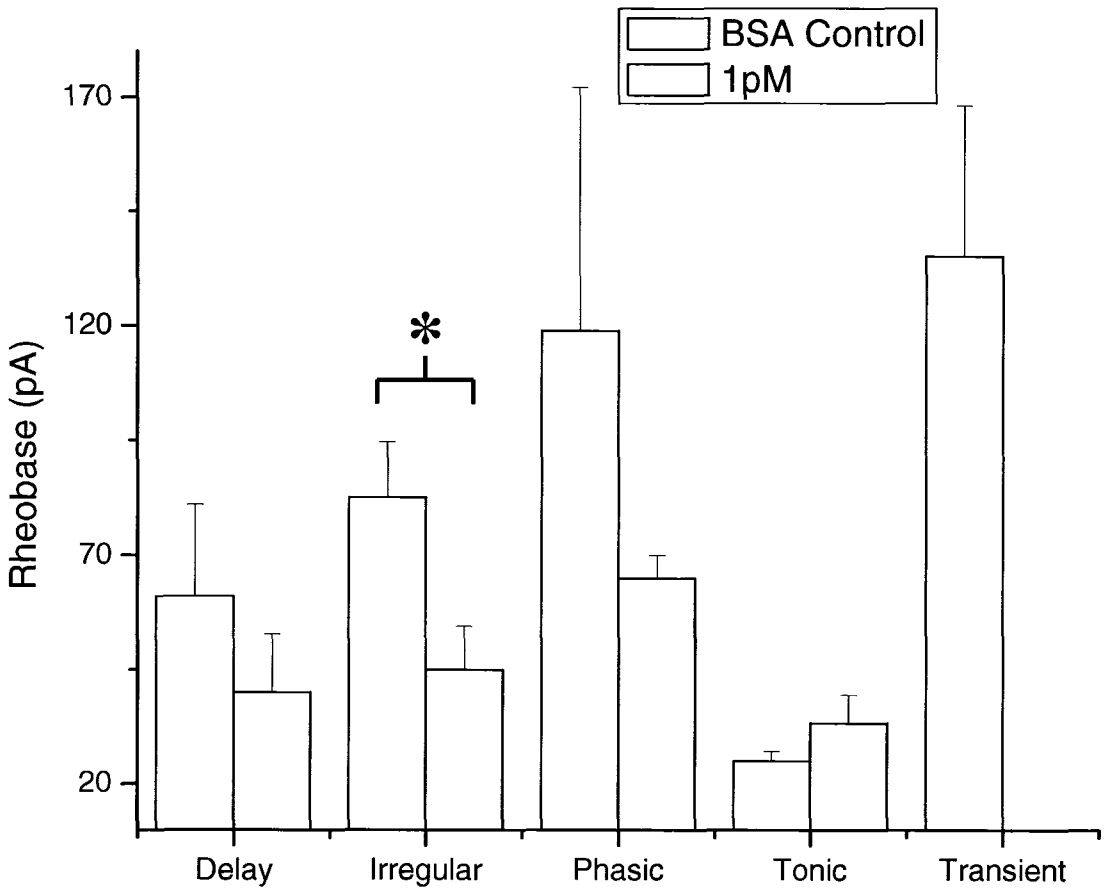


Figure 4-2. Comparison of rheobase of BSA control neurons and neurons treated with 1pM IL-1β.

C_m – BSA controls and 1pM IL-1β

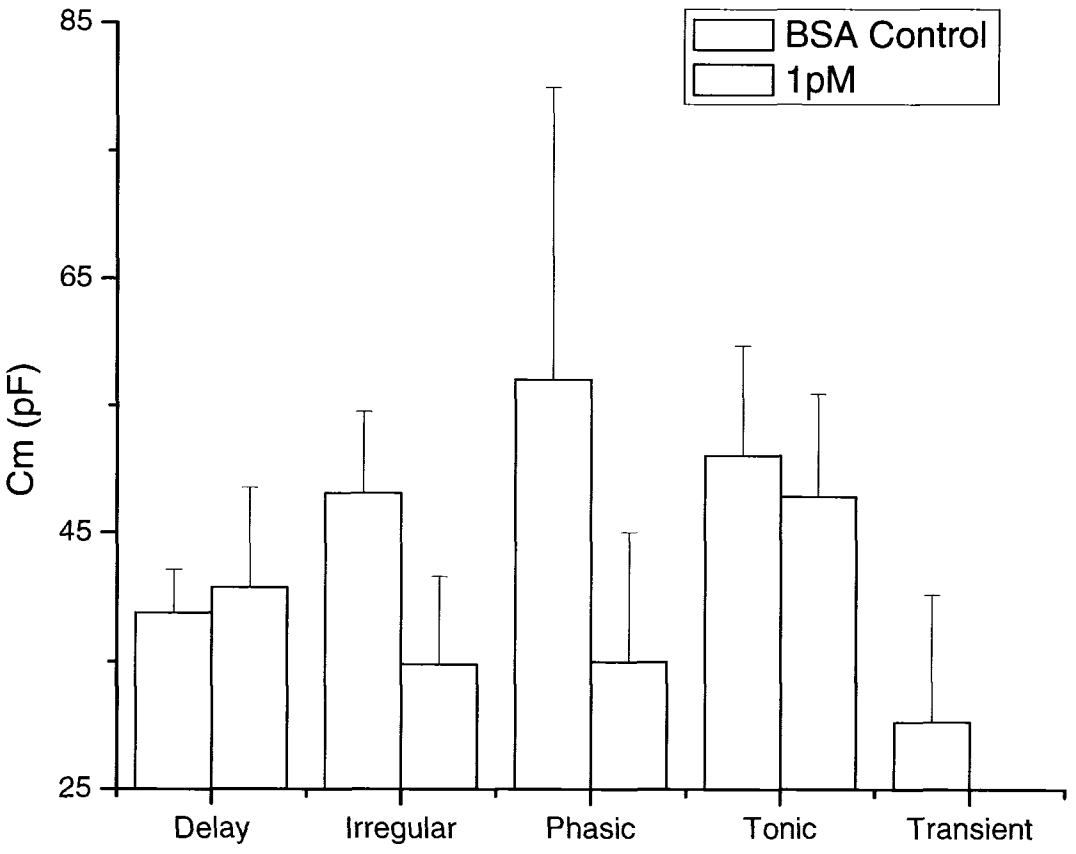


Figure 4-3. Comparison of cell capacitance of BSA control neurons and neurons treated with 1pM IL-1β.

4.2 References

Lu, V.B., Moran, T.D., Balasubramanyan, S., Alier, K.A., Dryden, W.F., Colmers, W.F., & Smith, P.A. (2006). Substantia gelatinosa neurons in defined-medium organotypic slice culture are similar to those in acute slices from young adult rats. *Pain, 121*: 261-275.

CHAPTER 5 ADDITIONAL ANALYSES AND DISCUSSION

5.1 Summary of Relevant Findings

When examining the sPSC results obtained with Kolmogorov-Smirnov statistics (cumulative probability plots), it became apparent that although a majority of the effects were significant, there was no clear direction of results; in fact, almost every possible change was seen. For example, amplitude changes in sPSCs were sometimes opposite in direction to frequency changes, sEPSC changes were not necessarily different from sIPSC changes, and effects on putative excitatory neurons (Delay) were neither the same nor the opposite to effects on putative inhibitory neurons (Tonic). Individually, these observations are not cause for concern nor do they suggest that the K-S test statistics are unfounded; however, collectively, these results did not yield any information regarding global effects of IL-1β or even mechanisms through which IL-1β may act. Thus, further experiments using calcium imaging were undertaken and the sPSC data were analyzed more conservatively in an effort to elucidate the most important effects of IL-1β.

5.2 Calcium Imaging

The presence of the calcium-sensitive dye Fluo-4 is an indicator of cellular Ca^{2+} concentrations, and by measuring cells' fluorescence in response to excitation via a high concentration of external K^+, it is possible to see whether slices treated with IL-1β are more or less excitable than control slices. Twenty-six cells from slices in each condition were analyzed, and both the amplitude and area under the curve of the K^+-induced fluorescence increase were measured for three different concentrations of K^+ (Figures 5-1, 5-2, and 5-3).

With respect to amplitude of the K^+-induced response, it was found that 100pM IL-1β-treated cells' response to 50mM K^+ was significantly greater than the amplitude of

control cells' response (p=0.02). Similarly, the area under the curve of the 100pM IL-1β-treated cells' responses to 35mM and 50mM K^+ was significantly greater than the area of control cells' responses to both these concentrations (p=0.0076 and p=0.0009, respectively). These results suggest that treatment with 100pM IL-1β increases overall excitability.

In cells treated with 1pM IL-1β, it was found that these cells' response to 20mM K^+ was significantly lesser than the amplitude of control cells' response (p=0.008); these responses were not significantly different from control responses after 35mM or 50mM K^+. The area under the curve of the 1pm IL-1β-treated cells' responses was not significantly different from the area of control responses following any K^+ concentration. These results suggest that treatment with 1pM IL-1β has little effect on overall excitability.

5.3 sPSC Analysis - Means and Amplitude Histograms

The sPSC means were analyzed for amplitude and interevent interval of each cell type and each concentration of applied IL-1β. The results are summarized in Figures 5-4, 5-5, 5-6 and 5-7. Statistically comparing the mean changes with an unpaired t-test is generally considered to be more conservative than comparing the cumulative probability distributions with the K-S test; in fact, some would argue that the t-test misses important trends in the data. However, this analysis was done to gain a different perspective on the data and attempt to report effects that may be physiologically significant rather than those that are statistically significant. It must be stated that at this point, the data interpretation became more subjective in that only the effects that were found to be significant with both the K-S test and the unpaired t-test were examined. The assumption was made that

events found to be statistically significant with only the K-S test may not necessarily be physiologically relevant.

5.3.1 sPSC Means

When examining sPSC means of the five neuronal types, it was found that many of the differences between controls and 100pM IL-1β-treated cells were not statistically significant according to a t-test, in spite of significance with the K-S test. The two cell types in which there were significant differences were sIPSC frequency in Delay cells and Irregular cells. Delay cells treated with 100pM IL-1β were found to have significantly lower sIPSC frequency than controls ($p=0.04$); in other words, inhibitory events in these cells were less frequent after treatment with a high concentration of IL-1β. Irregular cells treated with 100pM IL-1β were found to have significantly greater sIPSC frequency than controls ($p<0.01$); in other words, inhibitory events in these cells were more frequent after treatment with a high concentration of IL-1β.

In 1pM IL-1β-treated cells, there were only two cell types in which there were significant differences when compared to BSA controls; these were sIPSC amplitude in Irregular cells and sIPSC frequency in Tonic cells. Irregular cells treated with 1pM IL-1β were found to have significantly reduced sIPSC amplitude than controls ($p=0.05$); in other words, inhibitory events in these cells had a smaller amplitude after treatment with a low concentration of IL-1β. Tonic neurons treated with 1pM IL-1β were found to have significantly reduced sIPSC frequency than controls ($p=0.05$); in other words, inhibitory events in these cells were less frequent after treatment with a low concentration of IL-1β.

From this analysis, four effects were found to be significant. To narrow down which effects were significant at which concentration, the K-S test data was re-examined,

and only the effects that appeared to be extremely significant on the cumulative probability plots ($p<0.0036$) were retained; these eleven events included the four from the sPSC means. From these eleven effects, only the effects on Tonic cells and on Delay cells were considered, since these have been characterized much more extensively than any other cell type: Tonic cells are thought to be inhibitory, while Delay cells are thought to be excitatory (Balasubramanyan, Stemkowski, Stebbing, & Smith, 2006). Thus, there were eight large, quite significant effects observed following application of IL-1β, and these were:

1) an increase in sEPSC amplitude in Tonic cells after treatment with 1pM IL-1β
2) an increase in sIPSC amplitude in Tonic cells after treatment with 1pM IL-1β
3) a decrease in sIPSC frequency in Tonic cells after treatment with 1pM IL-1β
4) an increase in sIPSC amplitude in Delay cells after treatment with 1pM IL-1β
5) an increase in sEPSC amplitude in Delay cells after treatment with 100pM IL-1β
6) a decrease in sEPSC frequency in Delay cells after treatment with 100pM IL-1β
7) a decrease in sIPSC frequency in Tonic cells after treatment with 100pM IL-1β
8) a decrease in sIPSC frequency in Delay cells after treatment with 100pM IL-1β

These results are summarized in Table 5-1 and in Figures 5-8 and 5-9.

5.3.2 sPSC Amplitude Histograms

In an effort to distinguish between populations of individual spontaneous events, amplitude histograms were plotted for each of the big effects. Interevent interval data were not considered, since time interval between events is best fit with a monoexponential equation (Gordon & Bains, 2005), thus it would be impossible to visualize different populations of events. Of the four amplitude changes considered,

three were following treatment with the low concentration (1pM) of IL-1β and one was following treatment with the high concentration (100pM) of IL-1β. All sPSC amplitudes increased following treatment with IL-1β, which might reflect an increase in pre-synaptic quantal content or a change in pre- or post-synaptic neurotransmitter receptors.

After treatment with 100pM IL-1β, sEPSC amplitudes of Delay neurons increased (Figure 5-10). More specifically, there were fewer low-amplitude events (between 1 and 50 pA) and many more high-amplitude events (larger than 100pA). Since Delay cells are putative excitatory neurons, this suggests the cells were more excitable following treatment with 100pM IL-1β.

The major amplitude changes after treatment with 1pM IL-1β were as follows: Tonic cells' sEPSC and sIPSC amplitudes increased (Figures 5-11 and 5-12), and Delay cells' sIPSC amplitudes increased (Figure 5-13). With respect to Tonic sEPSCs, there were fewer low-amplitude events (between 1 and 50 pA) and more high-amplitude events (larger than 100pA), as was seen in Delay cells after 100pM IL-1β. The same trends were seen in sIPSCs of Tonic and Delay cells: after treatment with 1pM IL-1β, there was a rightward shift in the amplitude histograms. In the sIPSCs, it should be noted that events larger than 300pA were extremely rare in the control condition, but after treatment with 1pM IL-1β, more extremely high-amplitude (larger than 300pA) events were seen. Increases in Tonic sEPSC and Delay sIPSC amplitudes suggest that cells were less excitable following treatment with 1pM IL-1β.

Table 5-1

Summary of Very Apparent Significant Differences in Spontaneous Post-Synaptic Currents (sPSCs) of IL-1β-Treated Cells When Compared With BSA Controls

	TONIC		**DELAY**	
1pM IL-β sEPSCs	**Amplitude** ↑	*Frequency* *NS*	*Amplitude* ↑	*Frequency* ↓
1pM IL-β sIPSCs	**Amplitude** ↑	**Frequency** ↓	**Amplitude** ↑	*Frequency* ↓
100pM IL-β sEPSCs	*Amplitude* ↑	*Frequency* *NS*	**Amplitude** ↑	**Frequency** ↓
100pM IL-β sIPSCs	*Amplitude* ↓	**Frequency** ↓	*Amplitude* ↑	**Frequency** ↓

Ca^{2+} fluorescence – Amplitude

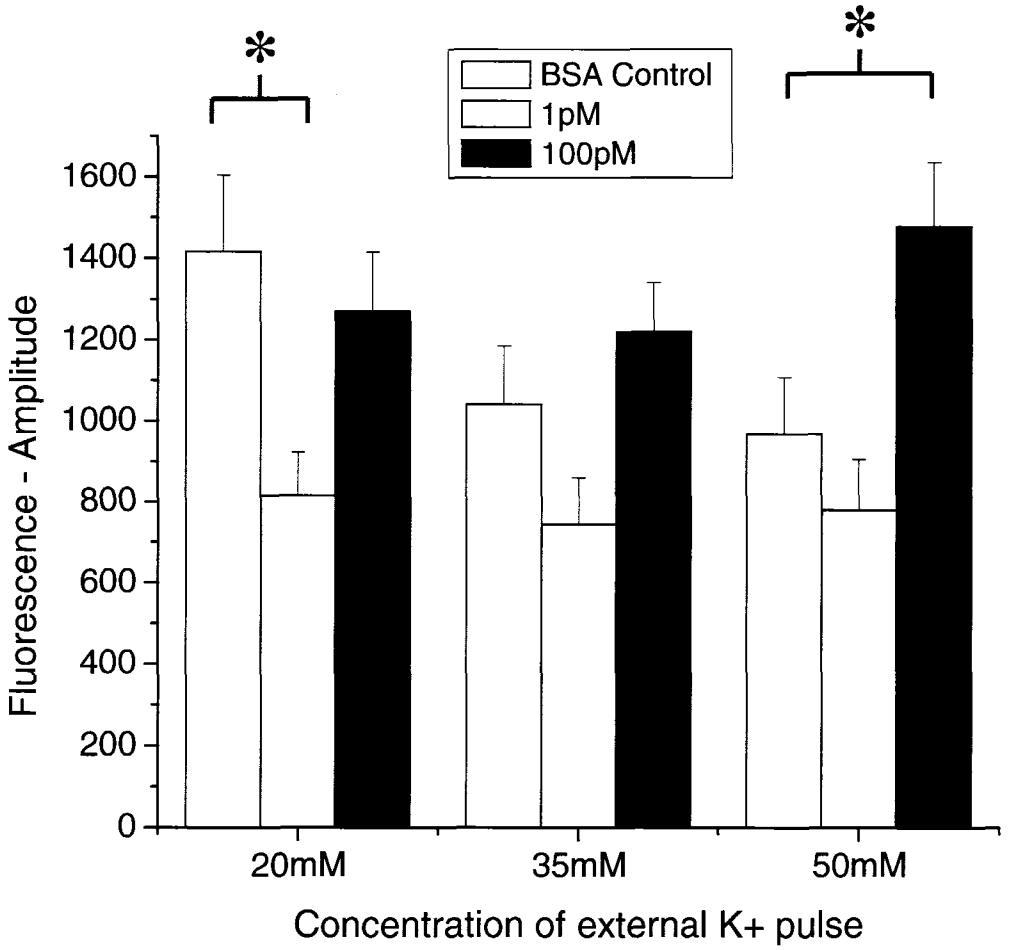


Figure 5-1. Summary of differences by treatment condition in Ca^{2+} fluorescence amplitudes in response to various concentrations of external K^{+}.

Ca^{2+} fluorescence – Area under the response curve

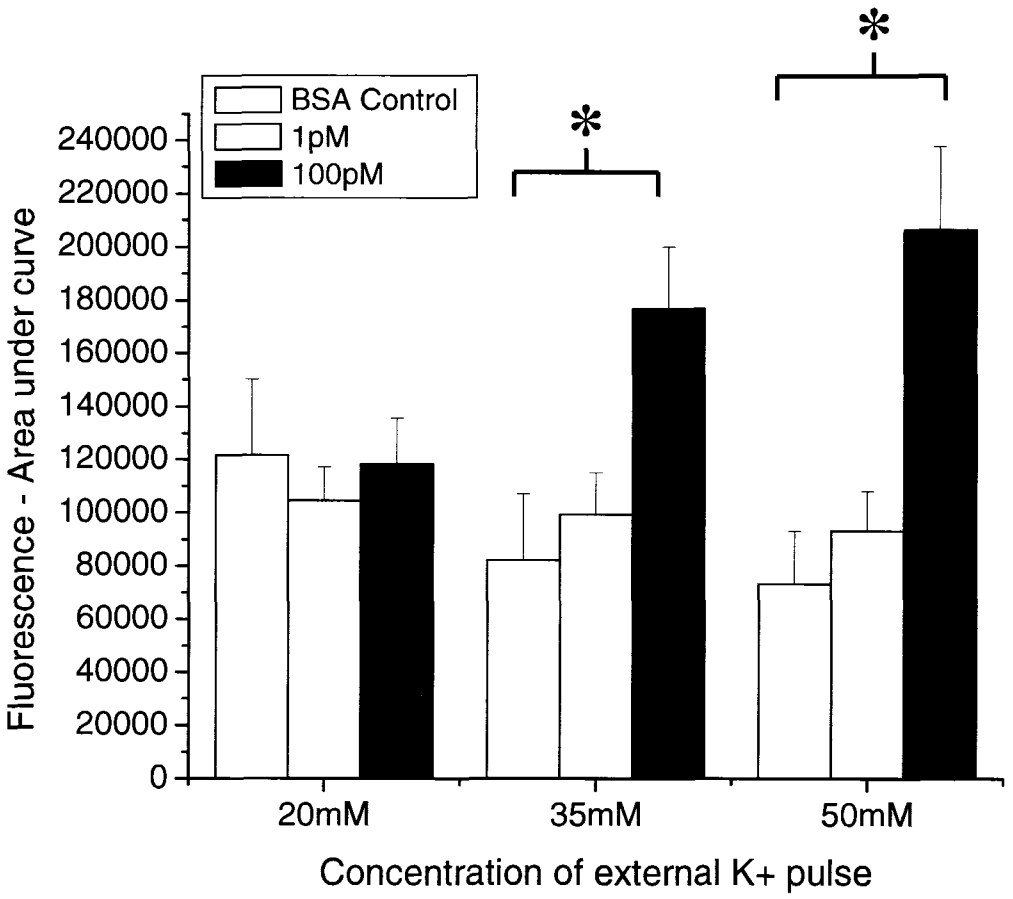


Figure 5-2. Summary of differences by treatment condition in Ca^{2+} fluorescence area under the response curve following various concentrations of external K^+.

Intracellular [Ca^{2+}] fluorescence following application of KCl

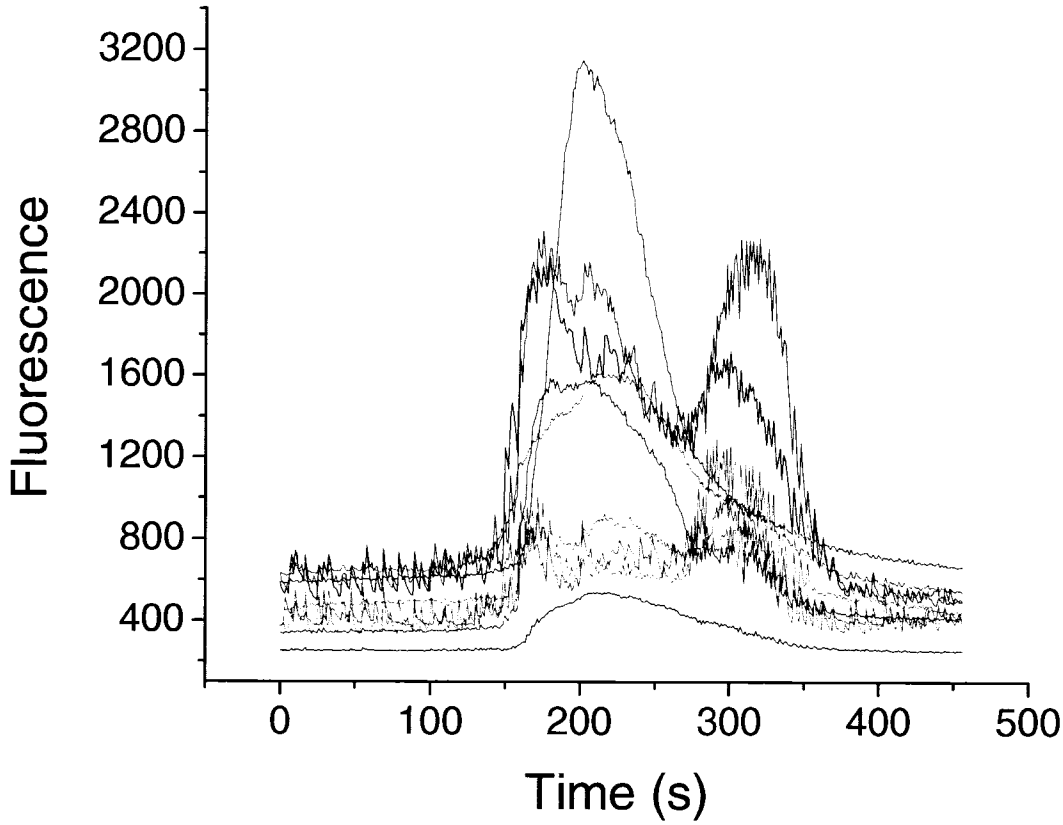


Figure 5-3. Fluorescence of dorsal horn neurons (various regions of interest in different colours) in response to application of 20mM KCl via external bath solution.

sEPSC means – BSA controls and 100pM IL-1β

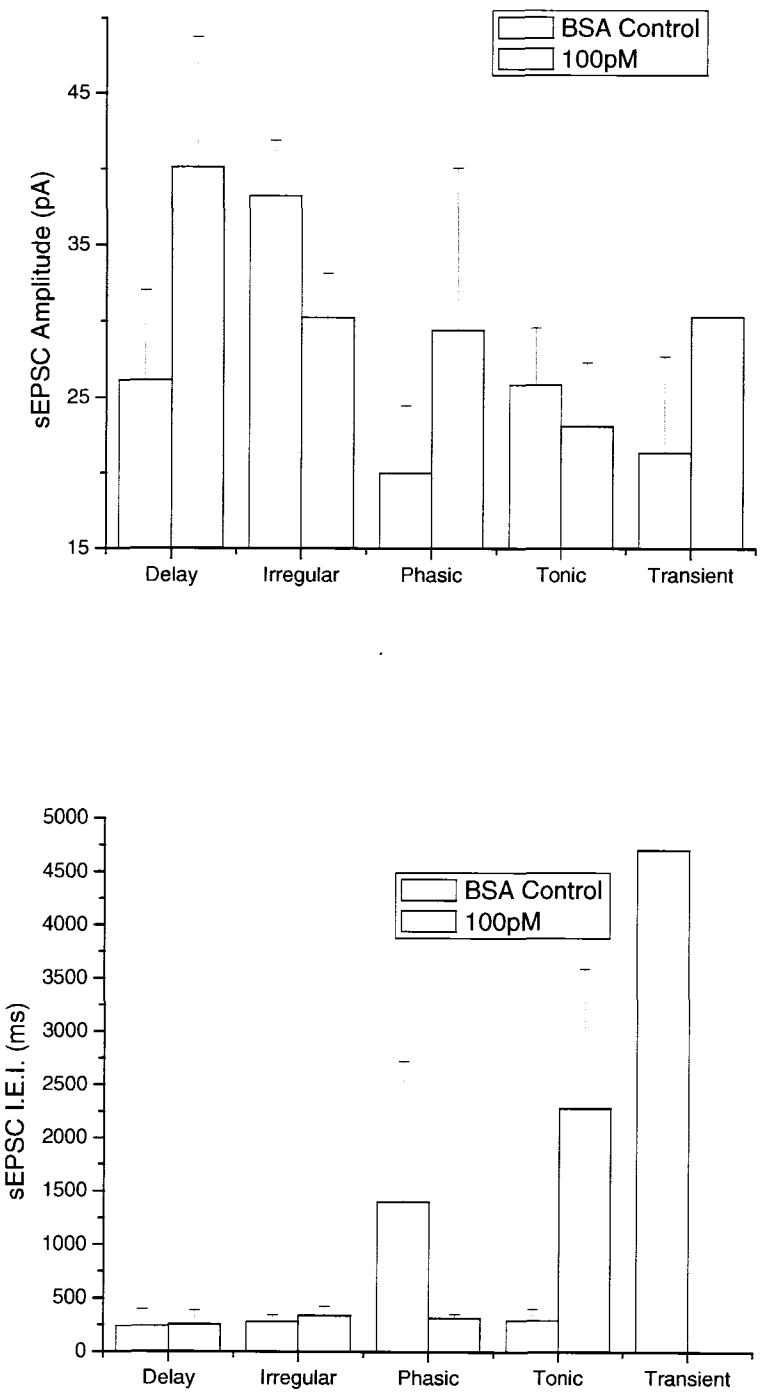


Figure 5-4. Means of sEPSC amplitudes and interevent intervals for BSA control cells and cells treated with 100pM IL-1β.

sIPSC means – BSA controls and 100pM IL-1β

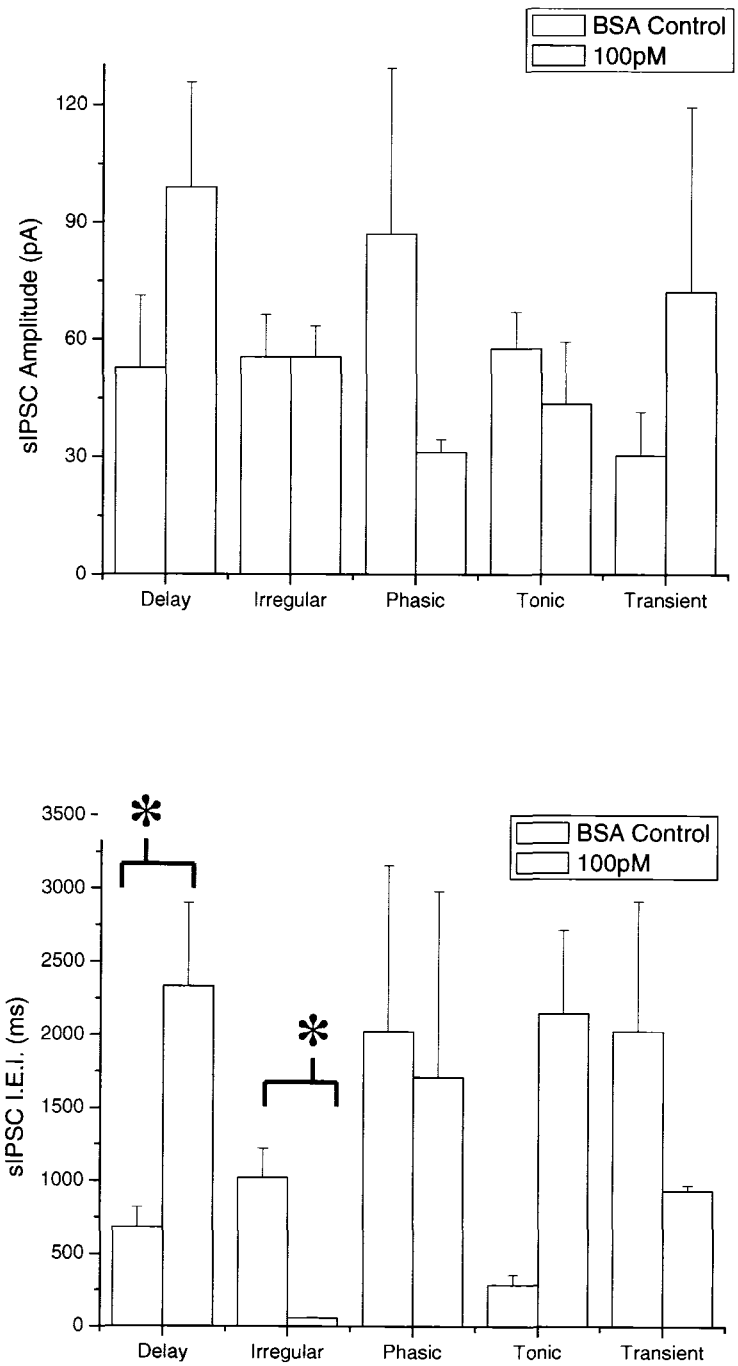


Figure 5-5. Means of sIPSC amplitudes and interevent intervals for BSA control cells and cells treated with 100pM IL-1β.

sEPSC means – BSA controls and 1pM IL-1β

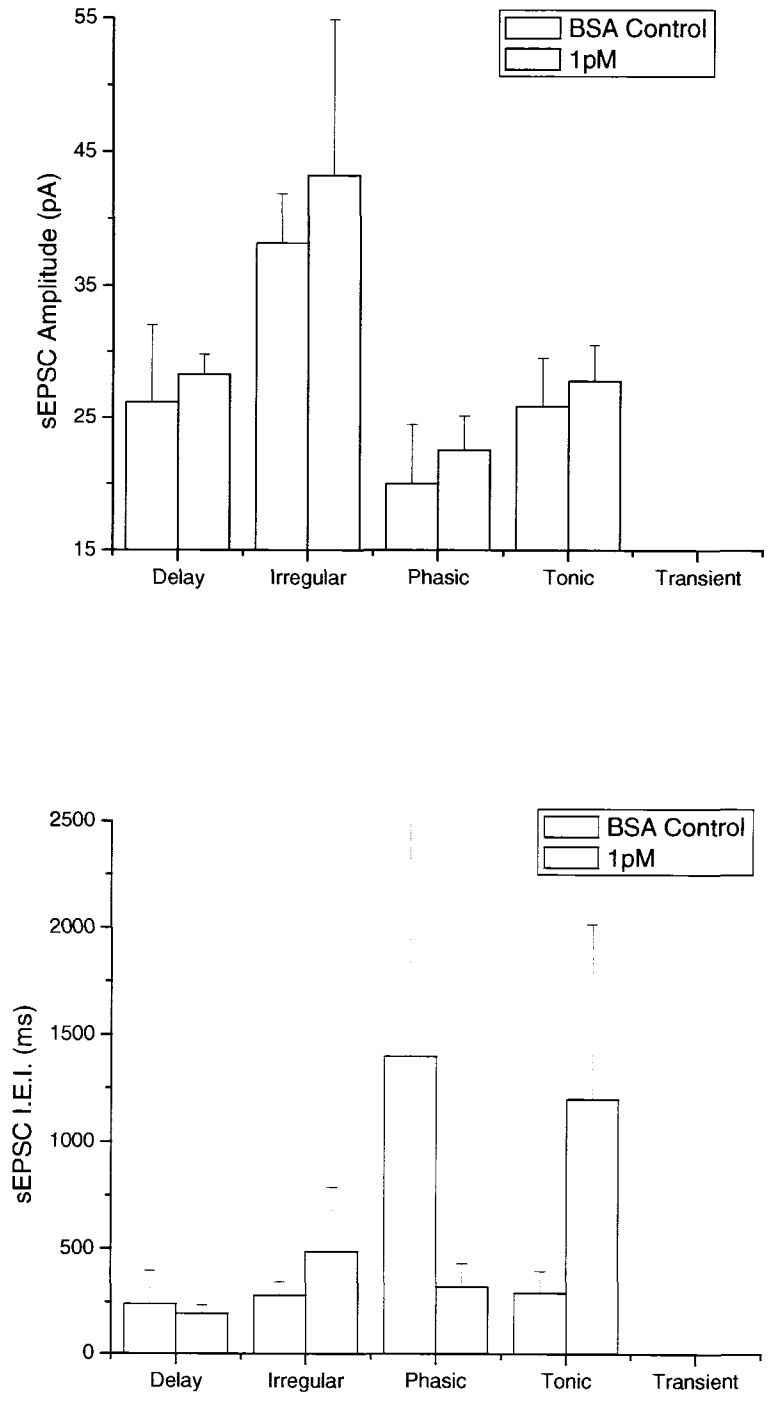


Figure 5-6. Means of sEPSC amplitudes and interevent intervals for BSA control cells and cells treated with 1pM IL-1β.

sIPSC means – BSA controls and 1pM IL-1β

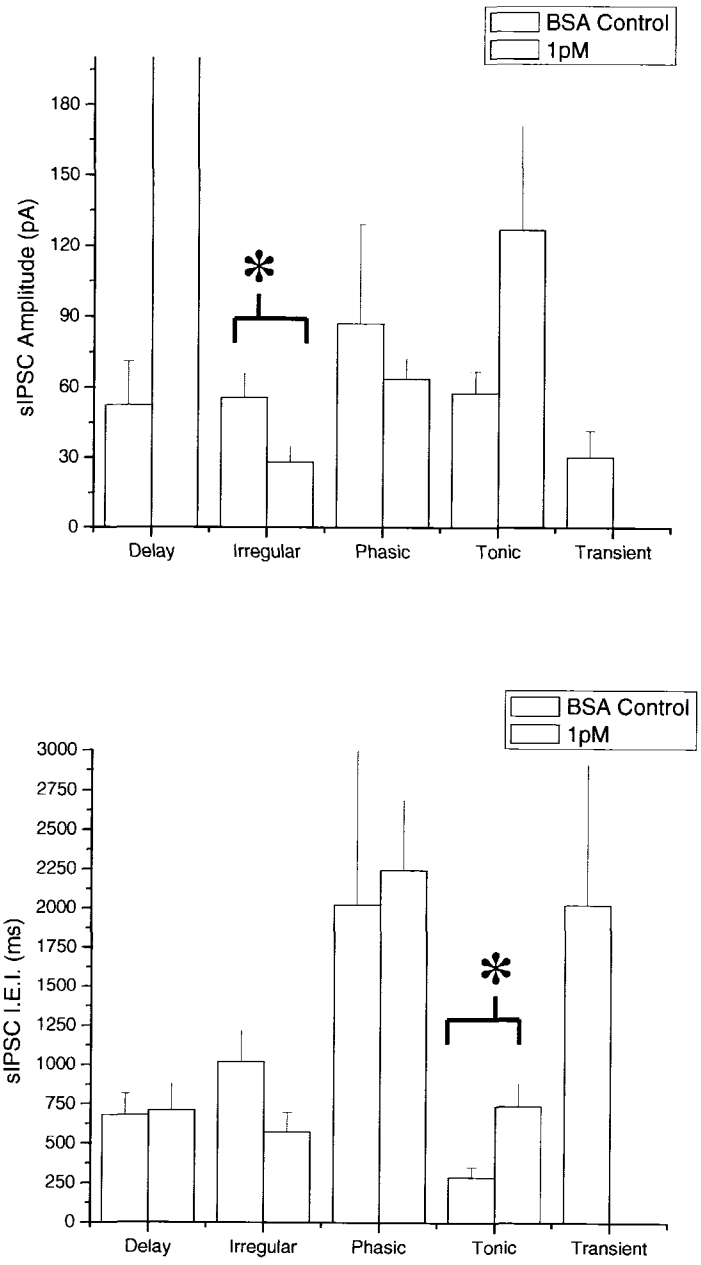


Figure 5-7. Means of sIPSC amplitudes and interevent intervals for BSA control cells and cells treated with 1pM IL-1β.

Very apparent significant differences after 100pM IL-1β

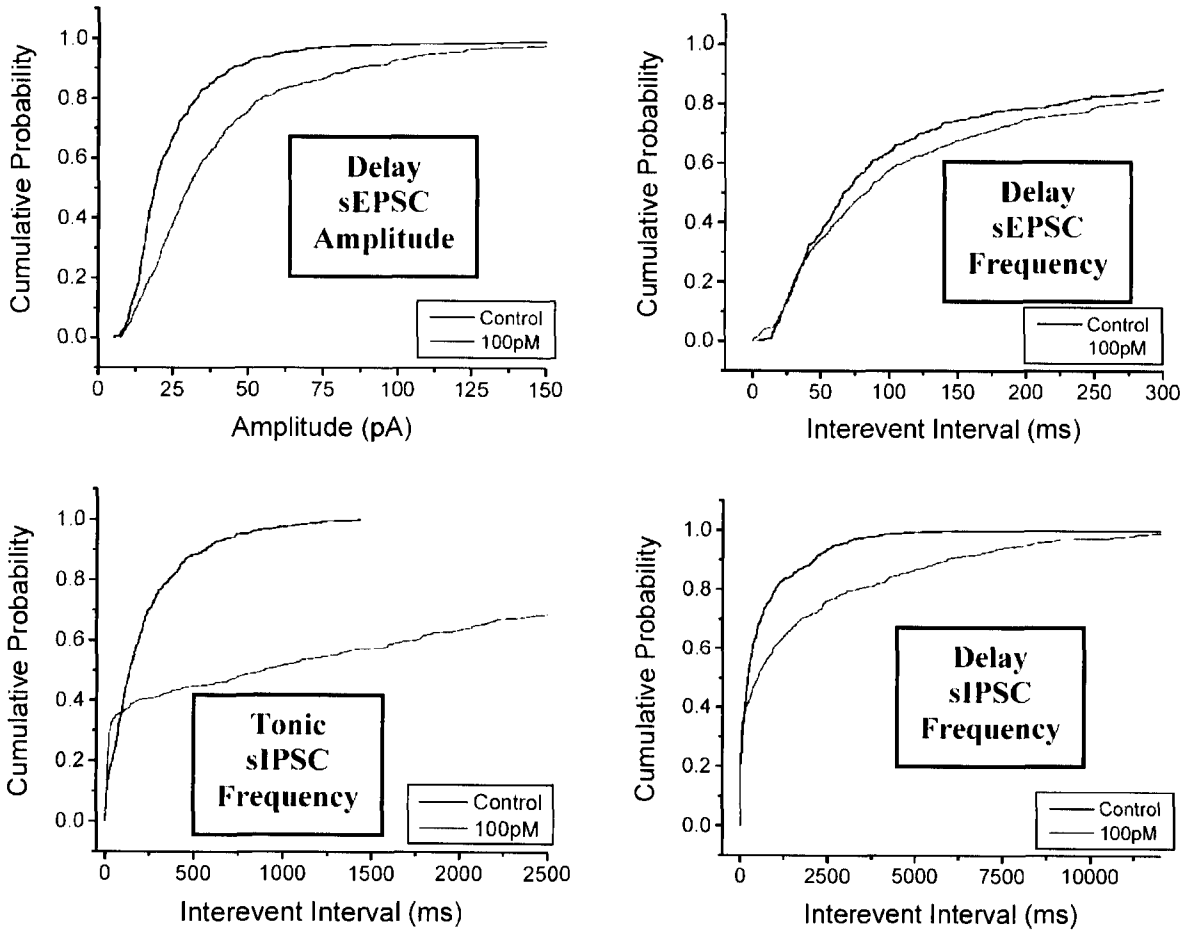


Figure 5-8. Cumulative probability distributions for the most apparent significant differences after treatment with 100pM IL-1β.

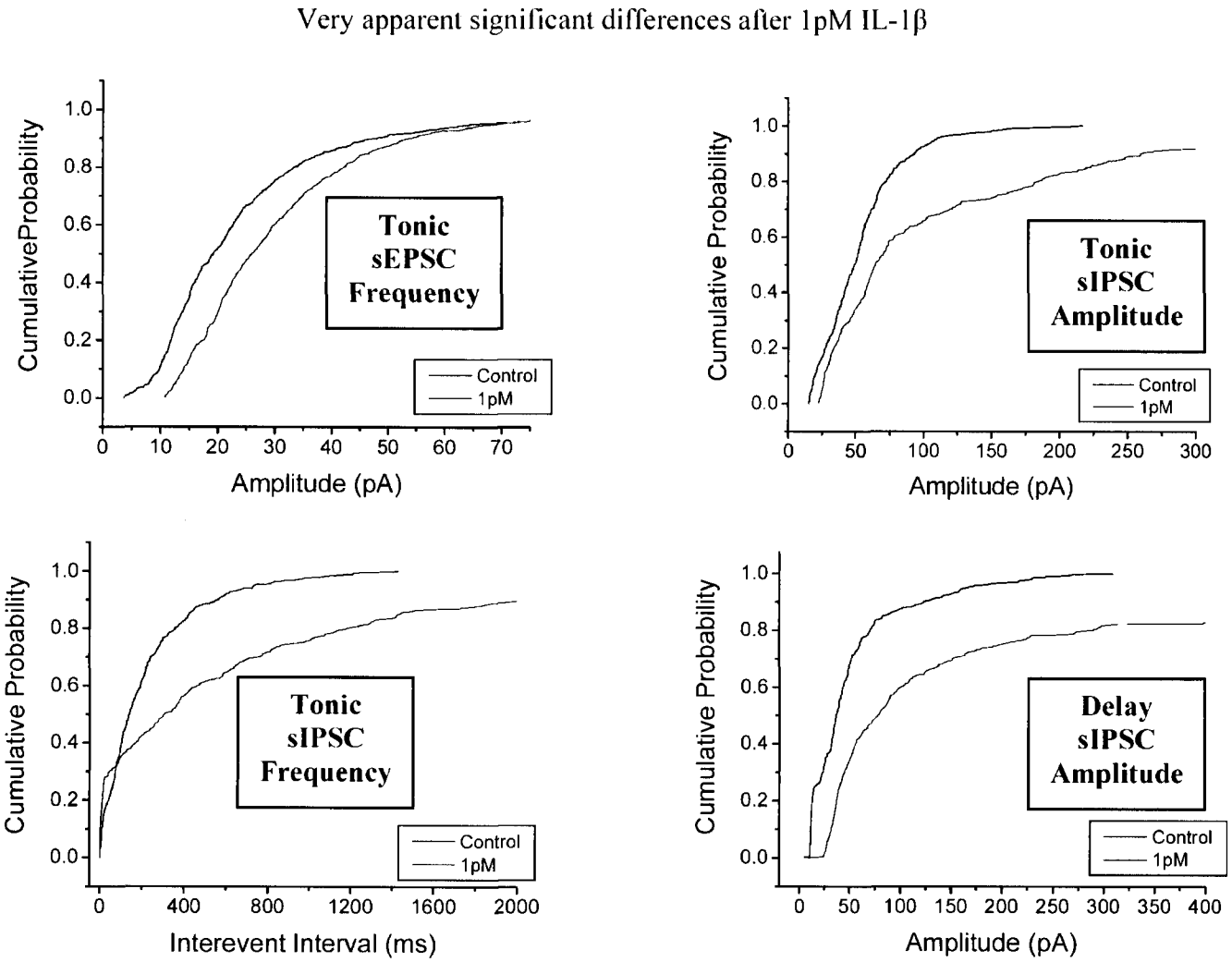


Figure 5-9. Cumulative probability distributions for the most apparent significant differences after treatment with 1pM IL-1β.

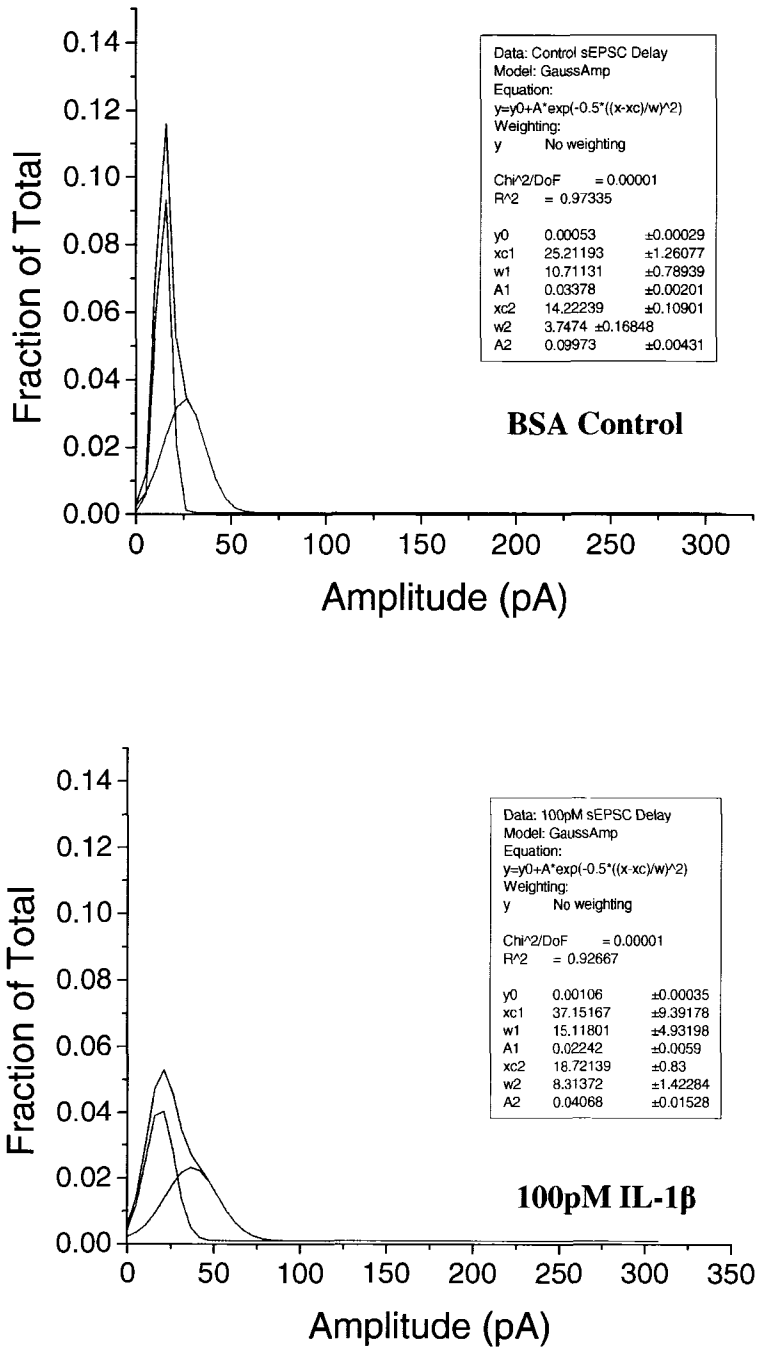
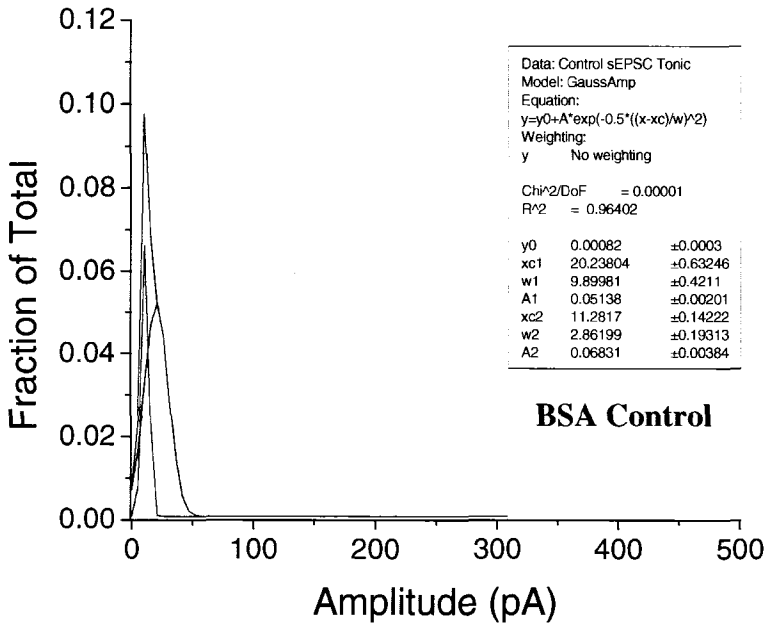


Figure 5-10. Amplitude histogram for sEPSCs in Delay cells following treatment with 100pM IL-1β.



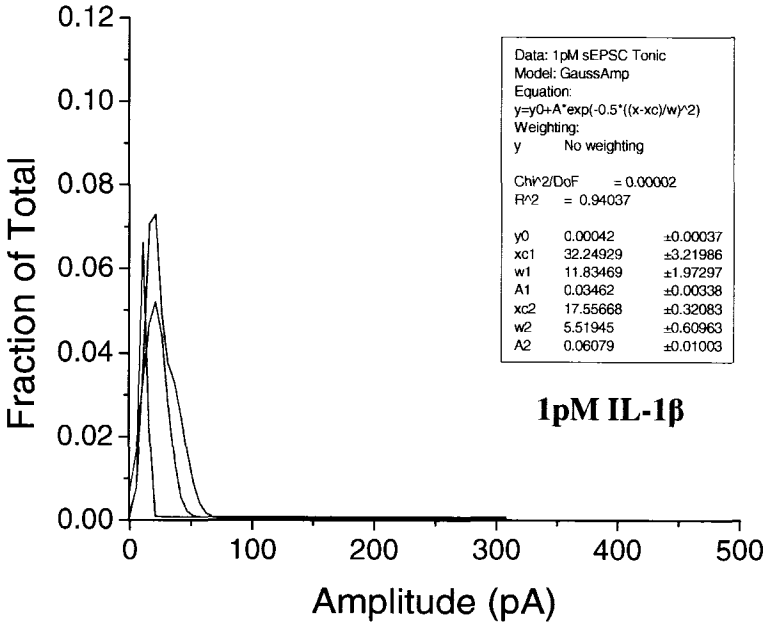


Figure 5-11. Amplitude histogram for sEPSCs in Tonic cells following treatment with 1pM IL-1β.

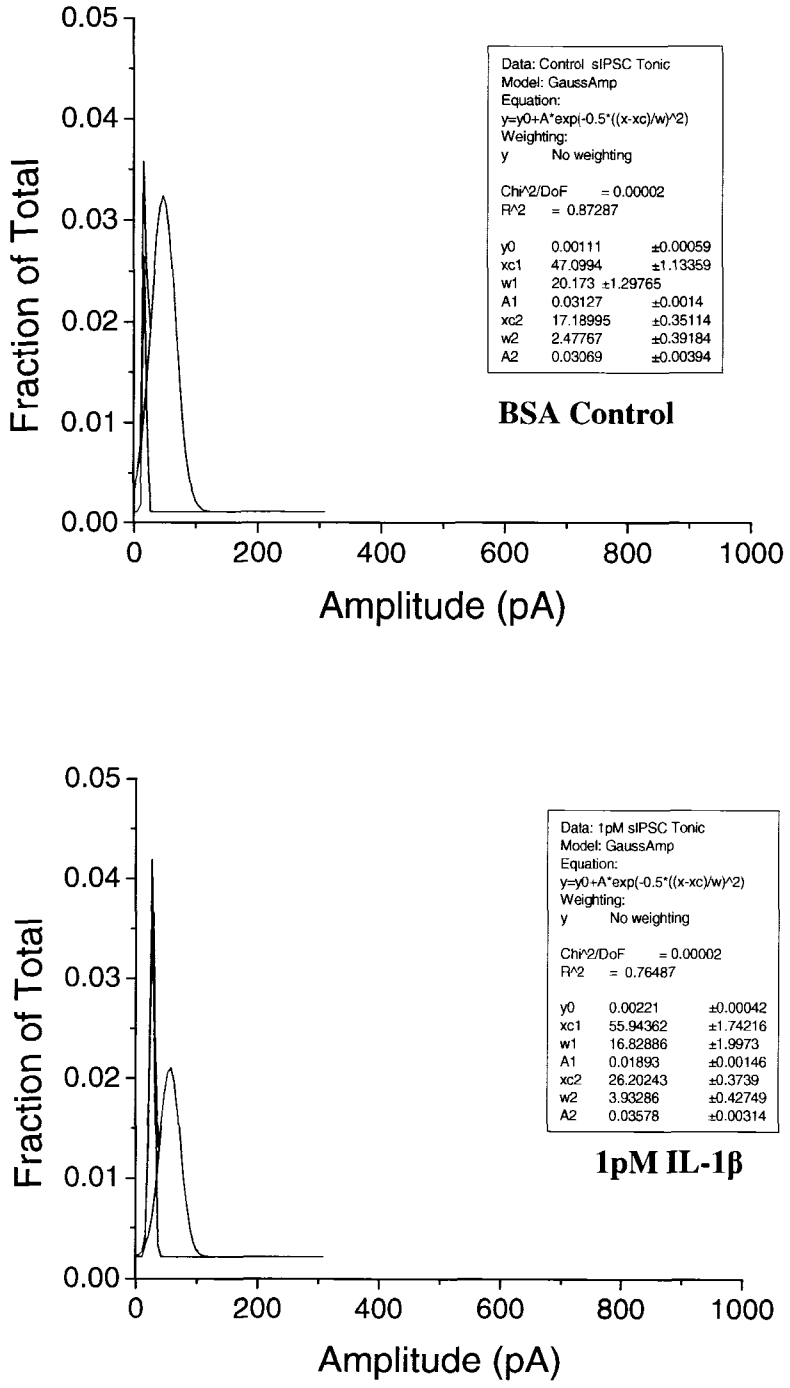


Figure 5-12. Amplitude histogram for sIPSCs in Tonic cells following treatment with 1pM IL-1β.

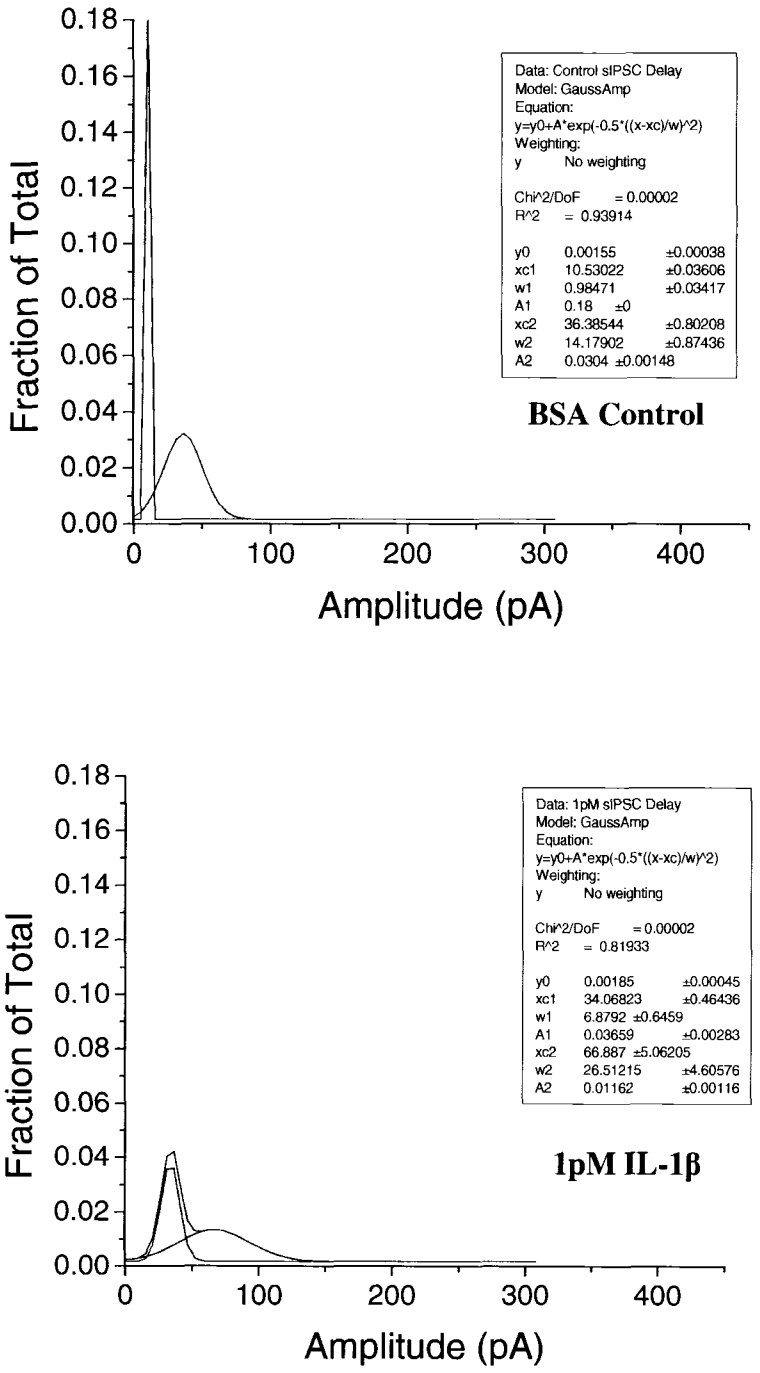


Figure 5-13. Amplitude histogram for sIPSCs in Delay cells following treatment with 1pM IL-1β.

5.4 References

Balasubramanyan, S., Stemkowski, P.L., Stebbing, M.J., & Smith, P.A. (2006). Sciatic chronic constriction injury produces cell-type-specific changes in the electrophysiological properties of rat substantia gelatinosa neurons. *J Neurophysiol, 96*: 579-590.

Gordon, G.R.J., & Bains, J.S. (2005). Noradrenaline triggers multivesicular release at glutamatergic synapses in the hypothalamus. *J Neurosci, 25*: 11385-11395.

CHAPTER 6 GENERAL DISCUSSION AND CONCLUSIONS

6.1 Summary of Results

The main finding of this thesis project was that long-term application of IL-1β produces significant, cell-type-specific changes in neurons of the spinal dorsal horn. Results from calcium-imaging experiments suggest that a low concentration (1pM) of IL-1β has little effect on overall excitability, while a high concentration (100pM) of IL-1β increases excitability in the dorsal horn. Results from the largest eight sPSC changes are similar, but not as definite: it appears as though 1pM IL-1β may act to decrease excitatory transmission and increase inhibitory transmission in the dorsal horn, while 100pM IL-1β may do the opposite: increase excitatory transmission while decreasing inhibitory transmission. This conclusion is based on the observation in acute slices that Tonic neurons tend to be inhibitory neurons, while Delay neurons are most likely excitatory.

Of the largest sPSC effects, there are some other important observations to be made. First of all, 1pM IL-1β had the largest impact on Tonic cells, increasing sEPSC and sIPSC amplitude while decreasing sIPSC frequency. Also, three of the four main effects were changes in amplitude. On the other hand, 100pM seemed to change mainly the properties of Delay cells, increasing sEPSC amplitude and decreasing sEPSC and sIPSC frequency. In this condition, three of the four main effects following 100pM IL-1β treatment were changes in frequency. This may indicate that different mechanisms are at work after treatment with the different concentrations of IL-1β, such that a lower concentration exerts its effects on inhibitory neurons and acts pre- and post-synaptically to alter neuronal properties, while a higher concentration affects excitatory neurons and likely acts pre-synaptically to increase excitability in the dorsal horn.

6.2 Discussion of ELISA Results and Concentrations in Literature

A considerable amount of literature is available concerning the actions of IL-1β, and a variety of concentration-dependent effects have been observed. A study conducted by Desson & Ferguson (2003) showed that in CNS circumventricular organs, IL-1β could produce a variety of dose-dependent effects on neuronal electrophysiological properties. They classified concentrations of 1pM, 500fM, and 100fM as being physiological (subseptic), while concentrations above 1nM were pharmacological (septic) concentrations. Based on these and other findings, the intention of the current project was to employ two concentrations of IL-1β, in hopes that the lower concentration (1pM) would represent an injured state and produce changes characteristic of overall neuronal hyperalgesia, while the higher concentration (100pM) would either result in the same effects on a larger scale, or invoke autoregulatory mechanisms and produce neuronal changes in line with overall analgesia, as there is strong evidence that IL-1β has a bell-shaped dose-response curve (Zelenka, Schäfers, & Sommer, 2005).

The measured concentrations as determined by ELISA of the two treatment conditions in the current project were found to be 32pM (following treatment with 1pM) and 118pM (following treatment with 100pM). This result is not completely unexpected, since the application of IL-1β should induce pro-inflammatory cytokine cascades that result in the amplification of the IL-1β signal (Inoue, 2006), and the ELISA measurements were taken nine days following treatment with IL-1β. The measured concentration of IL-1β after nine days was much greater in the 1pM treatment group (32-fold greater) than in the 100pM group (1.2-fold greater); the latter may reflect the involvement of negative-feedback control mechanisms which have been reported in other

studies following treatment with high concentrations of IL-1β (Bernardino, Xapelli, Silva, Jakobsen, Poulsen, Oliveira et al., 2005; Zelenka et al., 2005). Lastly, it is always important to consider that the effective concentrations of IL-1β at the time of data collection cannot be quantified unequivocally: IL-1β is inevitably degraded by peptidases, there may be local release of IL-1β by microglia, and IL-1β may bind to nonreceptor binding sites (Bernardino et al., 2005).

Thirty-two picomolar and 118pM correspond to 554 pg/mL and 2041 pg/mL, respectively. These concentrations are higher than those which elicited NMDA receptor-mediated changes in intracellular Ca^{2+} concentration in hippocampal neurons in culture: Viviani et al. (2003) showed that 25-100 pg/mL IL-1β increased NMDA-induced intracellular Ca^{2+}, reaching a maximal effect at 50 pg/mL, while higher concentrations of IL-1β (250-500 pg/mL) were ineffective, and 1000 pg/mL IL-1β was slightly inhibitory. On the other hand, another study using hippocampal neurons conducted by Lai et al. (2006) reported no observable changes in surface expression of AMPA receptors at IL-1β concentrations lower than 1000 pg/mL, yet found significant decreases in the surface expression of GluR1 in the presence of 10 000-50 000 pg/mL. These concentrations are much higher than those in the current project, and when compared to other studies in the brain that employ IL-1β, it seems the findings reported by Lai et al. (2006) may reflect pharmacological effects that would never be seen in vivo.

A potential concern in the study of IL-1β is the diversity of treatment concentrations and conditions. Not only is acute administration not comparable to long-term IL-1β production (as one would expect to see in vivo in chronic pain), but even when considering only acute administration in vitro, the modes of delivery and dosages in

each study vary considerably. The lack of consistency on this matter makes the comparison of different experiments and preparations very difficult. The concentrations of IL-1β in vivo in an organism's normal resting state are known to be minuscule (Sommer, Sorkin, & Kress, 2006), yet there is no consensus on IL-1β concentrations following nerve injury or inflammation. Reported values vary depending on mode of delivery (e.g. i.t. injection, cannula perfusion) or stimulus by which IL-1β is produced (e.g. LPS, CFA, gp120), and they are also dependent on the location at which IL-1β levels are measured (e.g. serum, CSF). Thus, it was difficult to come up with treatment conditions in DMOTC that would definitively reproduce the injured or inflamed environment in the dorsal horn.

In summary, extrapolating findings from other studies in an effort to derive "high" and "low" concentrations with which to treat dorsal horn neurons in DMOTC and determining what effects should be expected with 32pM and 118pM under culture conditions is very difficult, as the preparations and dosages vary considerably within each study, and there appears to be a broad range of effective treatment concentrations depending on which neuronal or synaptic effect is being studied. Future research using DMOTC should be focused on establishing dose-response curves with IL-1β in dorsal horn neurons, and considering the ELISA results in the current project, studies of the time course of IL-1β action and peak concentrations in this preparation would also be very beneficial.

6.3 Possible Cellular Mechanisms of IL-1β Action

There is evidence that many cell types can express and release IL-1β in response to neuronal injury; these different cells can produce a wide range of pro-inflammatory and immunoregulatory mediators that can promote cell survival or contribute to cell death (Allan et al., 2005) (Figure 6-1). The classical IL-1β-mediated signaling pathways involve activation of MAPKs and NF-κB (specifically, p38MAPK in neurons and NF-κB in astrocytes [Srinivasan, Yen, Joseph, & Friedman, 2004]) and subsequent release of secondary pro-inflammatory mediators (e.g. PGE_2, nitric oxide, bradykinin), all of which have been shown to contribute to peripheral and central sensitization (Wang, Ehnert, Brenner, & Woolf, 2006; Allan et al., 2005).

In terms of known direct effects of IL-1β, there are a limited number of studies that provide evidence for the role of IL-1β as a neuromodulator. A recent study using primary cultures of rat hippocampal neurons found that IL-1β, but not other pro-inflammatory cytokines, effected two significant changes in neurons: IL-1β decreased the surface expression and Ser831 phosphorylation of the AMPA receptor subunit GluR1, and IL-1β also regulated NMDA-mediated Ca^{2+} influx (Lai, Swayze, El-Husseini, & Song, 2006). These effects were abolished when IL-1β blocking agents were employed. Specifically, IL-1β has been shown to dose-dependently enhance NMDA receptor function through activation of tyrosine kinases and subsequent NR2A/B subunit phosphorylation (Viviani et al., 2003). The application of IL-1β results in an increase in NMDA-induced intracellular Ca^{2+} concentration and this may contribute to glutamate-mediated neurodegeneration (Wang & Salter, 1994; Viviani et al., 2003). Inhibition of NMDA receptor activity or depletion of extracellular Ca^{2+} blocked the effects of IL-1β on

GluR1 phosphorylation and surface expression, suggesting that IL-1β selectively regulates AMPA receptor phosphorylation and surface expression through extracellular Ca^{2+} and an unknown mechanism involving NMDA receptor activity (Lai et al., 2006). Although there is no consensus on which intracellular mechanisms are producing these changes, the stress-activated protein kinase p38 and the NMDA receptor-activated kinases, such as Src, have been implicated (Viviani et al., 2003; Takagi, Nozaki, Sugino, Hattori, & Hashimoto, 2000).

In a study conducted by Bernardino et al. (2005), it was found that at low doses (1000 pg/mL), pre-treatment with IL-1β potentiated AMPA-induced excitotoxicity in hippocampal neurons, while at high doses (10,000 pg/mL), pre-treatment with IL-1β resulted in neuroprotection against AMPA-induced excitotoxicity.

Studies reporting direct effects of IL-1β on AMPA and NMDA receptor function in hippocampal neurons used treatment concentrations ranging from 10 to 50 000 pg/mL (Lai et al., 2006; Viviani et al., 2003; Bernardino et al., 2005). It should be noted that although detectable effects were reported for concentrations as low as 10 pg/mL, the highest treatment concentrations (i.e. 50 000 pg/mL) were typically used in experiments as these 'elicited the strongest response', though they may not be representative of IL-1β concentrations in an injured or inflamed environment. In this vein, it may be that the reported direct neuromodulatory effects of IL-1β are pharmacologic effects that do not occur in vivo following nerve injury or inflammation. As discussed previously, more research is required to determine not only the specific, direct effects of IL-1β in various parts of the CNS and in the periphery, but also the probable concentrations in physiological, in vivo neuropathic pain conditions.

Neuropathic pain studies conducted in the dorsal horn of the spinal cord have shown that injury-induced stimuli can produce profound changes in cellular properties of these neurons. Specifically, peripheral nerve injury has been shown to deplete VGLUT1, which may lead to a decrease in glutamate levels, and thus, a decrease in synaptic efficacy (Hughes, Polgar, Shehab, & Todd, 2004). In terms of glutamate receptors, all four subunits of the AMPA receptor have been shown to exist in the dorsal horn of the spinal cord: GluR2 is almost universally present at AMPA-containing synapses, and following peripheral noxious stimulation, GluR1 subunits in the dorsal horn become rapidly phosphorylated, demonstrating that plastic changes do occur at the synaptic level in the dorsal horn (Nagy, Al-Ayyan, Andrew, Fukaya, Watanabe, & Todd, 2004).

In light of these studies and what has been reported to occur in the brain, it may be that IL-1β has some direct influence on AMPA receptor subunits that would alter neuronal transmission and increase hyperexcitability; however, the concentrations at which this may occur in vivo, and the mechanisms by which this may take place have yet to be clearly established. The results obtained with IL-1β are different from what has been seen in our lab following BDNF treatment in DMOTC, or following CCI in acute slices. The latter two projects have revealed that following injury or administration of BDNF, putative excitatory neurons of the substantia gelatinosa become more excitable, and putative inhibitory neurons are disinhibited, leading to an overall increase in synaptic excitability in this region. Although these specific changes were not seen following IL-1β treatment, it may be that any perturbation of synaptic transmission in the substantia gelatinosa (e.g. a change in AMPA receptor subunit phosphorylation) can lead to pain.

6.4 Future Research

6.4.1 Morphological Analysis

Examining the morphology of DMOTC neurons is a necessary step towards understanding the intricate circuitry and neuronal phenotypes of the superficial dorsal horn. Electrophysiological categorization is merely one aspect of possible neuronal characteristics, and by incorporating morphological analysis, it would become possible to better classify and understand DMOTC dorsal horn neurons. Further, it is possible to conduct immunohistochemical analyses on DMOTC slices, providing the invaluable distinction between excitatory and inhibitory neurons. Currently in the Smith lab, immunohistochemistry is being done using stains for GAD65; it is also possible to stain for calbindin, VGLUT, or other transporters such as VGAT, and these techniques are presently being developed to get a clear picture of what is occurring in both acute slices and DMOTC slices.

Some preliminary work with DMOTC has also been done in regards to morphology; the neurons recorded from in this project were filled with Biocytin (see Chapter 2) as were neurons from another project in the lab (Van B. Lu: application of BDNF to DMOTC neurons). Morphological analysis has resulted in the identification of three distinct morphological phenotypes: Dorsoventral neurons, Islet-central neurons, and Radial neurons. These are similar to morphological types seen in acute slices of rat substantia gelatinosa; however, the DMOTC slices flatten out considerably during the course of the culture, and this does influence the appearance of the neurons. Again, analyses are preliminary and higher sample sizes are required.

6.4.2 Dose-Response Studies and IL-1RA

As has been previously suggested in this chapter, dose-response studies for IL-1β in DMOTC are critical at this stage of research. Employing the natural receptor antagonist IL-1RA in these studies would be an additional safeguard against spurious results, and in conjunction with ELISA analysis, would provide essential information on the effective concentrations and functions of IL-1β. As much of the research surrounding IL-1β in the context of neuropathic pain is behavioural and acute, there is a dire need for these long-term studies. Particularly since the results of this project were confounded by speculative treatment concentrations and unclear effective concentrations of IL-1β in DMOTC, dose-response experiments would provide a much-needed rationale for effects observed in this and other studies of neuropathic pain.

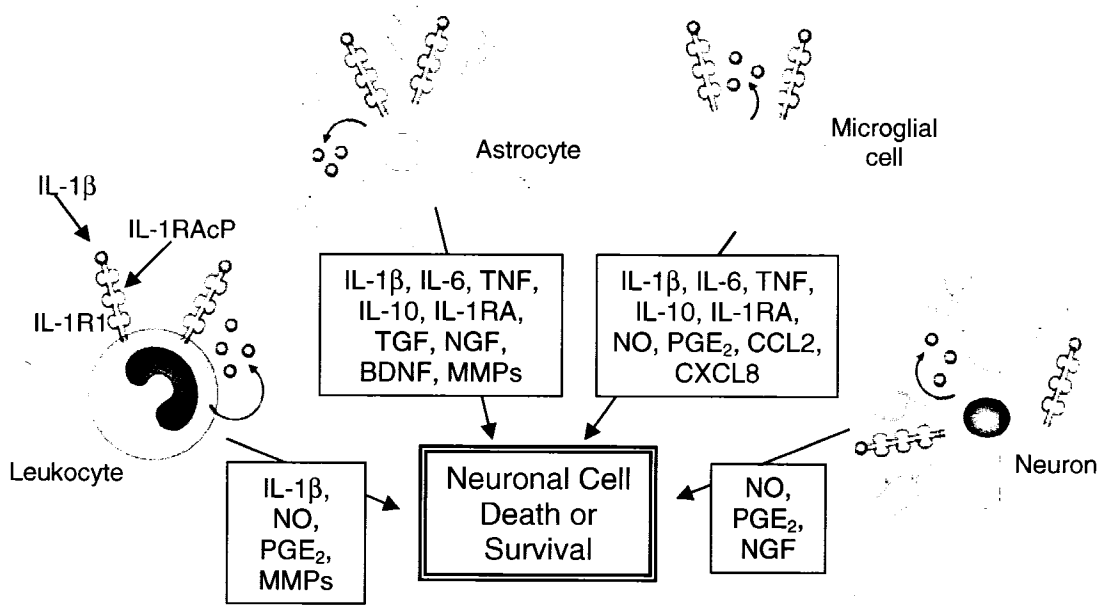


Figure 6-1. Summary of proposed cellular actions of IL-1β in neuronal injury.

6.5 References

Allan, S.M., Tyrrell, P.J., & Rothwell, N.J. (2005). Interleukin-1 and neuronal injury. *Nat Rev Immunol, 5*: 629-640.

Bernardino, L., Xapelli, S., Silva, A.P., Jakobsen, B., Poulsen, F.R., Oliveira, C.R., et al. (2005). Modulator effects of interleukin-1β and tumor necrosis factor-α on AMPA-induced excitotoxicity in mouse organotypic hippocampal slice cultures. *J Neurosci, 25*: 6734-6744.

Desson, S.E., & Ferguson, A.V. (2003). Interleukin 1beta modulates rat subfornical organ neurons as a result of activation of a non-selective cationic conductance. *J Physiol, 550* (Pt. 1): 113-122.

Hughes, D.I., Polgar, E., Shehab, S.A., & Todd, A.J. (2004). Peripheral axotomy induces depletion of the vesicular glutamate transporter VGLUT1 in central terminals of myelinated afferent fibres in the rat spinal cord. *Brain Res, 1017*: 69-76.

Inoue, K. (2006). The function of microglia through purinergic receptors: Neuropathic pain and cytokine release. *Pharmacol Ther, 109*: 210-226.

Lai, A.Y., Swayze, R.D., El-Husseini, A., & Song, C. (2006). Interleukin-1 beta modulates AMPA receptor expression and phosphorylation in hippocampal neurons. *J Neuroimmunol, 175*: 97-106.

Nagy G.G., Al-Ayyan, M., Andrew, D., Fukaya, M., Watanabe, M., & Todd A.J. (2004). Widespread expression of the AMPA receptor GluR2 subunit at glutamatergic synapses in the rat spinal cord and phosphorylation of GluR1 in response to noxious stimulation revealed with an antigen-unmasking method. J Neurosci, 24: 5766-5777.

Sommer, C., Sorkin, L., & Kress, M. (2006). Cytokine-induced pain: From molecular mechanisms to human pain states. In: H. Flor, E. Kalso, & J.O. Dostrovsky (Eds.), *Proceedings of the 11th World Congress on Pain* (pp. 131-145). Seattle: IASP Press.

Srinivasan, D., Yen, J.-H., Joseph, D.J., & Friedman, W. (2004). Cell type-specific interleukin-1β signaling in the CNS. *J Neurosci, 24*: 6482-6488.

Takagi, Y., Nozaki, K., Sugino, T., Hattori, I., Hashimoto, N. (2000). Phosphorylation of *c*-Jun NH(2)-terminal kinase and p38 mitogen-activated protein kinase after transient forebrain ischemia in mice. *Neurosci Lett, 294*: 117-120.

Viviani, B., Bartesaghi, S., Gardoni, F., Vezzani, A., Behrens, M.M., Bartfai, T., et al. (2003). Interleukin-1β enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. *J Neurosci, 23*: 8692-8700.

Wang, H., Ehnert, C., Brenner, G.J., & Woolf, C.J. (2006). Bradykinin and peripheral sensitization. *Biol Chem, 387*: 11-14.

Wang, Y.T., & Salter, M.W. (1994). Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature, 369*: 233-235.

Zelenka, M., Schäfers, M., & Sommer, C. (2005). Intraneural injection of interleukin-1β and tumor necrosis factor-alpha into rat sciatic nerve at physiological doses induces signs of neuropathic pain. *Pain, 116*: 257-263.