A study of neuronal and glial changes in the rostral ventromedial medulla in an animal model of neuropathic pain

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To my parents, Gary and Jean, and my brothers, Kieran and Brennan.
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Chapter 1
General Introduction

Pain can be an adaptive sensation, an early warning to protect the body from tissue injury. By the introduction of hypersensitivity to normally innocuous stimuli, pain may also aid in repair after tissue damage (Scholz and Woolf, 2002). This nociceptive pain is essential for the survival of organisms in a potentially hostile environment.

Ascending transmission of pain

The process that leads to pain perception is typically initiated by the activation of several different types of peripheral receptors, which selectively detect intense, potentially tissue-damaging stimuli. There are several types of cutaneous nociceptors. Most of these are supplied by A-delta (Aδ) or C-fibers. Aδ mechanical nociceptors are excited best by mechanical stimuli that damage the skin (McIntyre, 1960; Burgess and Perl, 1967; Koltzenburg et al., 1992). Thresholds for these receptors vary; the lowest thresholds are in the innocuous range, although the receptors discharge progressively more as the stimulus intensity extends into the noxious range (Burgess and Perl, 1967; Burgess et al., 1968). It has been thought that these receptors have poor responses to heat or cold; however, Aδ mechano-heat nociceptors do respond well to both noxious
mechanical and heat stimuli (Beck et al., 1974; LaMotte et al., 1982), although the heat threshold of these is higher than that of C-polymodal nociceptors (LaMotte et al., 1983).

C-nociceptors are another abundant receptor type. These receptors are formed by free nerve endings, many of which extend into the epidermis. C-polymodal nociceptors respond well to noxious mechanical, thermal, and chemical stimuli (Bessou and Perl, 1969; Beck et al., 1974; Martin and Murphy, 1995). The effective thermal stimuli are noxious heat (greater than 45°C), (Bessou and Perl, 1969; Handwerker and Neher, 1976) or intense cold (Simone and Kajander, 1996). As already mentioned, C-nociceptors are preferentially activated by low rates of skin heating, whereas Aδ-nociceptors are preferentially activated by high rates of skin heating (Yeomans and Proudfit, 1996; Yeomans et al., 1996). Yeomans and Proudfit (1996) suggest that C mechano-heat nociceptors evoke burning pain, whereas Aδ mechano-heat nociceptors produce pricking pain. C-mechanical nociceptors do not respond to heat (Iggo, 1960; Bessou and Perl, 1969; Beck et al., 1974; Kumazawa and Perl, 1977; Fleischer et al., 1983). It is possible that some of these receptors are also C cold nociceptors, since sufficiently cold stimuli may not always have been used in characterizing them (Bessou and Perl, 1969). Some could thus be C mechano-cold nociceptors (Simone and Kajander, 1996).
Structure of the spinal cord: afferent input

Following a normally painful stimulus, these primary afferent nociceptors (PAN), transmit information to the dorsal horn of the spinal cord. The terminals of the PAN contact neurons in specific laminae of the dorsal horn where they release glutamate and peptides to activate second order neurons (Fields, 2004).

Lamina I is the classical marginal zone, “a thin veil of gray substance, forming the dorsal-most part of the spinal gray matter” (Rexed, 1952). On the basis of differential primary afferent degeneration in lamina I as apposed to lamina II and finding of many ultrafine primary afferent fibers and terminals from individually labeled C fibers in lamina I, it is clear that much primary afferent input into lamina I is from C fibers (Gobel and Binck, 1977; Gobel et al., 1981; Sugiura et al., 1988; 1989). However, relatively large axons also give rise to terminals in lamina I (Beal and Fox, 1976; Beal and Bicknell, 1981), and functional studies and individual filling of high-threshold cutaneous mechanoreceptors conducting in the Aδ range (Light and Perl, 1979a; Hayashi, 1985) show significant Aδ input as well. Thus, lamina I is a major Aδ and C-fiber primary afferent termination site (Mense and Prabhakar, 1986). On the other hand, the primary afferent input into lamina II (substantia gelatinosa) comes primarily from collaterals from fine sensory fibers, which are usually unmyelinated C-fibers (Willis and Coggeshall, 2004).
Laminae III-VI make up the dorsal horn deep to substantial gelatinosa. Lamina III forms a broad band across the dorsal horn. In cytoarchitectonic studies lamina III is distinguished from lamina II by having slightly larger and more widely spaced cells (Rexed, 1952). Both laminae III and IV have a major primary afferent input from primary afferent fibers (thin Aδ and thick Aβ myelinated fibers) that enter from the medial division of the dorsal root, curve ventrally through the medial part of the dorsal horn, and then re-curve to end in large arbors (Scheibel and Scheibel, 1968) in lamina III and form a longitudinal plexus in lamina IV.

Lamina V extends as a thick band across the narrowest part of the dorsal horn and can be distinguished cytoarchitectonically from lamina IV because of the increased variation in cell type, and lamina VI, which consists of a medial zone with small, packed, compact cells (Willis and Coggeshall, 2004). Lamina V receives afferent projections from Aδ mechanical nociceptors and Aβ mechanoreceptors. It is likely that cells in lamina V also receive input from other kinds of afferent fibers that terminate in the superficial layers of the dorsal horn, since many of these cells have dendrites that extend into more superficial layers (Ritz and Greenspan, 1985).

Lamina VI receives proprioceptive afferent fibers that also project to the ventral horn (Willis and Coggeshall, 2004), whereas, lamina X receive projections
from Aβ mechanical nociceptors and unmyelinated visceral afferent fibers (Light and Perl, 1979a; Sugiura et al., 1989).

**Ascending spinal cord pathways**

Beyond the peripheral nociceptor and dorsal horn, pain related information ascends in the contralateral spinothalamic tract (STT) to the thalamus, which in turn has higher order neurons that project to different cortical regions; however, there are also projections to the brainstem (via the spinoreticular tract [SRT] and spinomesencephalic tract [SMT]) (Figure 1-4).

**Spinothalamic Tract**

Although several neurologists of the nineteenth century, including Brown-Sequard (Brown-Sequard, 1860) and Gowers (Gowers, 1877), reported clinical that pain and temperature sensations depend on the activation of tracts that ascend in the anterolateral white matter of the spinal cord, Edinger (1889; 1890) is generally regarded as the first to suggest that there is a direct projection from the spinal cord to the thalamus (Keele, 1957). It is now known that there is a moderate-sized spinothalamic tract (STT) in the cat (Patrick, 1896; Chang and Ruch, 1947; Morin et al., 1951), rat (Lund and Webster, 1967; Zemlan et al., 1978; Peschanski and Mantyh, 1983; Mehler, 2006), and a variety of primates, including humans (Goldstein, 1910; Gardner and Cuneo, 1945; Bowsher, 1957).
The STT in primates arises largely from neurons in the dorsal horn of the spinal cord, although some STT cells are in the intermediate region and ventral horn (Apkarian and Hodge, 1989). In rat, the largest concentration of labeled STT cells is in the upper cervical segments (Granum, 1986). According to Granum (Granum, 1986) and Kemplay and Webster (Kemplay and Webster, 1986), STT cells in the upper cervical segments account for half or more of the population in the entire spinal cord of the rat. In segments below the upper cervical cord, STT cells in rats are found chiefly in the cervical and lumbosacral enlargements, with the greatest concentration in the marginal zone, nucleus proprius, and the medial intermediate gray matter (Burstein et al., 1990). Most STT cells have cutaneous receptive fields and respond to innocuous and noxious mechanical stimulation of the skin (Giesler et al., 1976; Palecek et al., 1992). The axons of STT neurons then decussate at the level near the cell bodies and ascend in the ventrolateral white matter to the thalamus.

Spinoreticular Tract

Spinoreticular neurons in the cat lumbosacral spinal cord have various combinations of ipsilateral or contralateral excitatory or inhibitory receptive fields on the hindlimbs, although most of the cells have excitatory inputs from the ipsilateral hindlimb (Albe-Fessard et al., 1974; Fields et al., 1975). Innocuous stimulation of the skin can excite or inhibit a particular cell, but often noxious stimuli are excitatory (Fields et al., 1975).
Most of the axons of the spinoreticular neurons ascend in the ventrolateral white matter as the spinoreticular tract (SRT). A spino-recticulo-thalamo-cortical pathway is often proposed as an important afferent pathway to the forebrain, especially with reference to pain. However, only a few reticular neurons convey information directly from the reticular formation relay sites of the SRT to the thalamus (Blomqvist and Berkley, 1992).

Spinomesencephalic Tract

The spinomesencephalic tract (SMT) is actually a collection of pathways from the spinal cord to several different midbrain target zones (Willis and Coggeshall, 2004). In rat, wheat germ agglutinin – horseradish peroxidase (WGA-HRP) injected into the midbrain tegmentum showed a significant population of lamina I cells contralateral to the injection site, with the periaqueductal gray (PAG) and nucleus cuneiformis as midbrain sites yielding the largest numbers of retrogradely labeled lamina I cells (Swett et al., 1985). More recent studies have confirmed labeled populations of lamina I, V, VII, and X neurons retrogradely labeled from the PAG and cuneiform nucleus, but also the parabrachial nuclei (Cechetto and Standaert, 1985). Spinomesencephalic axons ascend in the white matter of the ventral half of the spinal cord, in company with the STT and SRT (Mehler et al., 1960; Kerr, 1975) and often terminate contralaterally in the midbrain (Yezierski, 1988).
Rat SMT neurons include cells that respond to innocuous mechanical stimulation (low-threshold cells) in the nucleus proprius. Other cells are activated by innocuous mechanical stimuli but maximally by noxious mechanical stimuli (wide-dynamic-range cells); most such neurons also respond to noxious heat. These cells are in the nucleus proprius (Menetrey et al., 1980). Another group of cells is excited just by noxious mechanical stimuli (high-threshold cells); these cells are located in the nucleus proprius and the marginal zone (Menetrey et al., 1980).

**Descending modulation of pain**

Just as pain signals are important for survival, it is as important to regulate pain signaling in the nervous system. Experimental interest in supraspinal modulation of spinal cord function has a long history. Early investigators established that influences descending from the brain stem modulated activity at the spinal cord dorsal root level (Wall, 1967). These early studies established that descending influences were tonically active and principally inhibitory in function because nociceptive reflexes were exaggerated after transection of the spinal cord (Sherrington, 1906). The motivation for current day investigation of descending modulation of pain came from the work of Reynolds (Reynolds, 1969) and later Liebeskind and colleagues (Mayer et al., 1971; Mayer and Liebeskind, 1974), who reported that focal electrical stimulation in the midbrain periaqueductal gray (PAG) of the awake rat produced profound analgesia. In a dramatic extension of this finding, electrical stimulation of the midbrain PAG in
humans was reported by several neurosurgical groups to produce clinically significant pain relief (for reviews, see (Hosobuchi et al., 1977; Baskin et al., 1986)). Subsequent work using a combination of methods (brain mapping by electrical stimulation, anatomical tract tracing, inhibition of withdrawal reflexes and dorsal horn electrophysiology) rapidly led to detailed knowledge of the anatomy, physiology and pharmacology of this pathway (Fields et al., 2006). It was found that the PAG receives direct inputs from the hypothalamus and from the limbic forebrain, including several regions of the frontal neocortex and the central nucleus of the amygdala (Figure 1-2). The PAG also controls nociceptive transmission indirectly by means of connections through neurons in the rostral ventromedial medulla (RVM) and the dorsolateral pontine tegmentum (DLPT; Figure 1-2). These two regions project through the spinal cord dorsolateral funiculus (DLF) and selectively target the dorsal horn laminae that house the nociceptive relay neurons. The selective control of pain by this circuit is explained by its anatomical selectively for primary afferent nociceptors terminals and somata of dorsal horn neurons that respond to noxious stimulation (Fields, 2004).

**Study of Neuropathic pain**

Pain is divided into two major groups: physiological pain and pathological pain. Physiological pain is an important physiological function for survival. Depending on pain experience, animals and humans gain knowledge of potential dangerous stimuli in the environment, and pain-related unpleasantness help to
form long-term avoidance memory in order to protect themselves (Zhuo, 2007). Unlike physiological pain, pathological pain only happens after injury (e.g. tissue or nerve injury), and is not the result of repetitive application of physiological pain. As mentioned above, long-term changes occur after injury, both peripherally and centrally. Consequently, the injury and injury-related areas undergo long-term plastic changes, and pain sensation is significantly enhanced (hyperalgesia) or non-noxious stimuli cause pain (allodynia). The chronic pain that can occur from these long-term plastic changes is a significant health problem and negatively impacts quality of life for afflicted individuals as well as society in economic terms (approximately $100 billion annually (Anonymous, 1995). Of the chronic pain states, neuropathic pain is perhaps the most perplexing. This chronic pain state generally outlasts the injury triggering its presence, and may arise as a consequence of other disease states or treatments for other conditions (e.g. radiation or chemotherapy) (Ossipov et al., 2006). Individuals afflicted with neuropathic pain often complain of spontaneous ‘burning’ pain, and up to one-third of patients with this pain state do not benefit from currently available pain medications. Those who do see benefits usually gain only partial pain relief while tolerating difficult side effects (Ossipov et al., 2006). Because existing treatments for neuropathic pain have limited effectiveness and produce relatively frequent adverse effects (Wallace, 2007), understanding the basic mechanisms contributing to the generation and/or
maintenance of neuropathic pain in preclinical animal models is key to providing predictable and efficient therapies in patient populations.

Models of Neuropathic Pain

The standard animal models of pain, where behavioral responses to noxious thermal, mechanical or chemical stimuli are measured, have provided important information with regard to the basic mechanisms that underlie pain. Studies employing these models of acute nociception have revealed neuroanatomical pathways that participate in mediating noxious inputs and identified structures that are activated in response to pain. Behavioral assays based on these models have also revealed that the endogenous neuromodulators, the enkephalins and endorphins, may modulate nociception through actions at the opioid receptors and serve as an endogenous pain inhibitory system (Ossipov et al., 2006). These models of acute nociception have become standard tools in drug development, and have predicted the analgesic efficacy and potency of strong analgesics against acute pain states. A shortcoming that became, however, was the fact that models of acute nociception were insufficient to account for neuropathic pain states (Ossipov et al., 2006). Treatments that have been consistently reliable in experimental and clinical acute pain states have been disappointing against neuropathic pain states, highlighting the need for novel models that approximate the features of human neuropathic states (Ossipov et al., 2006). A number of important models
of neuropathic pain have been developed and characterized, all with advantages and disadvantages, and all with limited ability to model precisely the clinical condition. Nevertheless, these models have provided the basis for the mechanistic understanding that we have achieved to date of neuropathic pain syndromes.

**Chronic constriction injury**

The realization that most patients with neuropathic pain due to nerve trauma have partial or incomplete lesions of a nerve led to development of the chronic constriction injury (CCI) model by Bennett & Xie (Bennett and Xie, 1988). A set of four chromic gut sutures are placed loosely around the common sciatic nerve at intervals of 1-2 mm (Figure 1-3) so that they do not completely impede circulation through the epineurium (Bennett and Xie, 1988).

CCI rats exhibit thermal hyperalgesia, indicated by a significant decrease in response latency to noxious radiant heat, within 2 days of CCI and that persists for 2 months. Hypersensitivity to light tactile, but not noxious mechanical, stimuli is observed after CCI (Bennett and Xie, 1988). Cold allodynia after CCI has also been demonstrated by significant increases in number and duration of paw lifts from a plate maintained at 4°C compared to sham-operated and naïve rats (Bennett and Xie, 1988). These behavioral observations may indicate that the CCI model may detect spontaneous pain, revealed by abnormal
postures, guarding, and coincident grooming behaviors, and evoked hyperalgesia and allodynia seen in patients with causalgia (Ossipov et al., 2006).

**Spared nerve injury**

The spared nerve injury model is caused by tight ligation and subsequent resection of the common peroneal and tibial nerves while leaving the sural nerve intact (Decosterd and Woolf, 2000) (Figure 1-3). These nerves comprise the three distal branches of the sciatic nerve. Behavioral signs of neuropathic pain are evident within 1 day after spared nerve injury (SNI) and are maintained for over 9 weeks (Decosterd and Woolf, 2000). Spontaneous pain is suggested by avoidance of weight bearing on the injured hindpaw, with the eversion of the paw and rapid hind paw flexion on contact (Decosterd and Woolf, 2000).

**Spinal nerve ligation**

The model of traumatic nerve injury that arguably has become the most commonly employed and studied is spinal nerve ligation (SNL) (Kim and Chung, 1992). The primary impetus for the development of the SNL model derived from the belief that is not possible to adequately control the numbers and types of primary afferent fibers that are injured with the CCI model. In this model, the L5 and L6 branches of the sciatic nerve are carefully identified and isolated, and tightly ligated with silk suture between the trifurcation of the sciatic nerve and distal to the dorsal root ganglia. A ‘modified’ SNL model is also used. In this model, the L5 spinal nerve is isolated, tightly ligated with silk suture and cut distal
to the suture (Ringkamp et al., 1999; Li et al., 2000) (Figure 1-3). In both versions of the SNL model, robust responses to tactile stimuli are present within 1-2 days after SNL and persist over a period of several weeks (Kim and Chung, 1992; Li et al., 2000). Thermal hyperalgesia is also evident after surgery (Kim and Chung, 1992). However, cold allodynia after SNL is much less robust than that seen after CCI (Kim et al., 1997).

A principal advantage of this model is that the spinal nerves are ligated at specific spinal segments. This allows the manipulation of intact nerves or corresponding segments distinct from the injured ones. Furthermore, both tactile and thermal hyperesthesias are clearly present, whereas signs of serious distress such as autotomy or lack of grooming are absent (Ossipov et al., 2006).

**Mechanisms of Neuropathic pain**

The abovementioned models of neuropathic pain have been important in increasing our understanding of the clinical condition; however, these models rely on behavioral responses to evoked stimuli. The models do not easily predict spontaneous pain, and this is a major limitation as the mechanisms of evoked and spontaneous pain following nerve injury may differ and treat strategies may also be different. Regardless of this important limitation, multiple mechanisms of the neuropathic state (discussed below) with potential importance to the clinical condition have emerged from these animal models (summary see: Figure 1-4).
Peripheral nociceptors

Considerable evidence indicates that dysfunction of injured or adjacent uninjured peripheral nerve fibers is an important mechanism of neuropathic pain. Neuroma formation associated with nerve section results in spontaneous, ectopic discharge at the side of injury (Devor and Wall, 1976; Devor and Govrin-Lippmann, 1979; Devor, 1991). Palpation or mechanical distortion of the neuroma causes sensations ranging from minor dysesthesias to pain that is often referred to the previous innervated region, and the evoke pain is abolished by local anesthetics (Wall and Gutnick, 1974).

Sensitization of peripheral nerves has also been suggested by spontaneous ectopic discharge generated in the dorsal root ganglion of the injured nerve that remains after neuroma excision (Bennett, 1993). Electrophysiological recordings performed on the sciatic nerve bundles after experimental nerve injury in the rat demonstrates spontaneous ectopic discharges from the region of injury (Tal and Eliav, 1996; Chen and Devor, 1998). The uninjured nerve fibers of adjacent nerves also appear to contribute to the pain state. Spontaneous discharges in dorsal root ganglion neurons have been recorded from cells of both intact and injured nerves (Michaelis et al., 2000). Wallerian degeneration of the injured, spontaneously active myelinated fibers may excite unmyelinated fibers through the co-mingling that occurs within the sciatic nerve trunk (Wu et al., 2001; 2002).
**Dorsal horn sensitization**

An important interpretation regarding sustained afferent inputs into the spinal cord is that these cause the spinal dorsal horn to become sensitized to afferent inputs, thus leading to a state of spinal sensitization. Spinal sensitization is related to the demonstrated phenomenon of wind-up, in which noxious stimuli applied to the skin also enhance the excitability of dorsal horn units, such that responses to subsequent stimuli are exaggerated (Mendell, 1966; Chapman et al., 1998; Ziegler et al., 1999). The persistent spontaneous afferent discharges after peripheral nerve injury are believed to produce a sensitized state, leading to enhanced pain (Herrero et al., 2000). In animal models of nerve injury, rats show increased spontaneous activity of spinal dorsal horn units after nerve injury. Furthermore, the stimulus-response function of wide dynamic range (WDR) neurons in response to tactile, but not thermal, stimuli applied within the receptive shifts to the left (Pertovaara et al., 1997).

It has been reported that there is significant enlargement of the receptive field in response to non-noxious, but not to presumably noxious, mechanical stimuli, which may be indicative of spinal sensitization (Suzuki and Dickenson, 2000). Spinal sensitization is also reflected by the induction of Fos expression in the spinal dorsal horn on repeated gentle stroking of the injured paw, with a pattern of distribution that is suggestive of noxious inputs (Catheline et al., 1999). The same stimulus has no effect in normal animals.
Descending modulation from the brainstem: descending facilitation

One area in the brainstem that is thought to play a role in pain modulation, and is of great focus in this dissertation, is the rostral ventromedial medulla (RVM). The RVM is well recognized as an important relay in the modulation of nociceptive inputs at the level of the spinal cord. Along with its long-recognized function as an important source of bulbospinal inhibitory controls, the RVM is also thought to facilitate the input of pain signals (Fields, 2004). Considerable evidence indicates that descending facilitatory influences from the RVM contribute to chronic pain states and may underlie the development of tactile and thermal hyperesthesias (for reviews see (Porreca et al., 2002)). More specific discussion of the RVM’s role in neuropathic pain symptoms continues below.

Rostral ventromedial medulla (RVM): role in descending modulation of nociception

As mentioned above, the RVM plays a role in the descending modulation of nociception. In the rat, the RVM includes the n. raphe magnus, n. reticularis gigantocellularis pars alpha, and n. reticularis paragigantocellularis lateralis. The rostrocaudal extent of the RVM is approximately from the caudal pole of the facial nucleus to the level of the trapezoid body (Basbaum and Fields, 1984).
**Bidirectional nociceptive modulation**

Both inhibition and facilitation of behavioral spinal nociceptive reflexes have been elicited by electrical or chemical stimulation of the RVM (Urban and Gebhart, 1999). In general, low intensities of electrical stimulation or low concentrations of chemical (e.g. glutamate, neurotensin) facilitate spinal nociception, whereas greater intensities of stimulation or concentrations of chemical at the same sites typically inhibit nociception (Zhuo and Gebhart, 1992; Urban and Smith, 1993; Urban and Gebhart, 1997; Zhuo and Gebhart, 1997).

The activation of descending facilitatory mechanisms from the RVM is critical for the maintenance of the behavioral neuropathic pain state (Porreca et al., 2002). Hyperesthetic responses to cold or tactile, but not to noxious heat, stimuli in animals with nerve ligations are abolished by transection of ipsilateral hemisection of the spinal cord (Kauppila et al., 1998); similarly, neuropathic pain after sciatic nerve cut is blocked by spinal transection (Kauppila, 1997; Kauppila et al., 1998). Furthermore, behavioral signs of neuropathic pain are blocked by lidocaine microinjections in the RVM (Pertovaara et al., 1996; Kovelowski et al., 2000; Burgess et al., 2002) and surgical lesions of the ipsilateral DLF, the major conduit of spinopetal projections from the RVM (Burgess et al., 2002).

**Afferents and efferents**

The midbrain periaqueductal gray (PAG) and adjacent n. cuneiformis constitute a major afferent input to the RVM (Beitz, 1982a; Mantyh and
Peschanski, 1982; Marchand and Hagino, 1983) (Figure 1-4). Previous research has suggested that the RVM mediates both stimulation-produced analgesia and opioid-induced analgesia from the PAG: RVM lesions block the antinociceptive responses elicited by stimulating from the PAG (Lovick, 1985) and pre-treatment of the RVM with the opioid antagonist naloxone inhibits the antinociception induced by microinjection of morphine into the PAG (Pan and Fields, 1996). These data suggest that the RVM plays an important role in descending antinociceptive transmission from the PAG.

The RVM also receives significant input from neurons in the dorsally adjacent medullary reticular formation (Abols and Basbaum, 1981; Maciewicz et al., 1984) and the dorsolateral pontine tementum, another area implicated in nociceptive modulation (Holstege, 1988; Haws et al., 1989). Other inputs to the RVM arise from diecephalic and telecephalic structures, including the hypothalamus, the frontal cortex, the amygdala, and the bed nucleus of the stria terminalis (Holstege, 1987).

Although the RVM projects to several brainstem and spinal cord sites, its major descending projections are to the spinal and trigeminal dorsal horns (Bobillier et al., 1976; Basbaum and Fields, 1978; Holstege and Kuypers, 1982; Skagerberg and Bjorklund, 1985; Mason and Fields, 1989). Axons of RVM neurons terminate densely in laminae I, II, and V of the trigeminal nucleus caudalis and project via the spinal dorsolateral funiculus (DLF) to terminate in
laminae I, II, V, VI, and VII of the spinal dorsal horn (Basbaum and Fields, 1978; Holstege and Kuypers, 1982). These laminae are known to contain the terminals of small-diameter nociceptive primary afferents (Light and Perl, 1979b; 1979a; Cervero and Iggo, 1980) as well as neurons that respond to noxious stimuli and project to the brainstem and thalamus (Fields and Basbaum, 1978; Dubner and Bennett, 1983).

**Physiological classification of RVM neurons**

Fields *et al.* have characterized cells in the RVM that may constitute the physiological basis for generation of bidirectional modulation of spinal nociceptive transmission. They have operationally defined three classes of neurons in the RVM: on-cells, off-cells and neutral cells, which are intermixed in the RVM and not anatomically separable (Fields et al., 1983b). Off-cells display an abrupt pause in ongoing activity immediately before nociceptive reflexes and are proposed to contribute to inhibitory influences that descend from the RVM. On-cells display a burst of activity immediately before nociceptive reflexes and are proposed to contribute to facilitatory influences that descend from the RVM. Neutral cells show no nociception-related change in activity. Off-cells, on-cells and neutral cells all project to the spinal dorsal horn (Fields et al., 1995), placing on-cell and off-cell terminals in appropriate laminae (I, II and V) to modulate nociceptive transmission. On-cells and off-cells fire in a reciprocating pattern and tail-flick latency is longer during periods of increase off-cell activity and shorter when on-cells are active. Therefore, it appears that on- and off-cells mediate
descending facilitatory and inhibitory influences from the RVM is supported by several reports demonstrating enhanced on- or off-cell activity during facilitation or inhibition of spinal nociceptive transmission, respectively (Fields et al., 1983b; Bederson et al., 1990).

**Neurotransmitters in the RVM**

In the RVM, there are fibers and terminals containing several kinds of neurotransmitters, including glutamate (Wiklund et al., 1988), neurotensin (Beitz, 1982b), norepinephrine (Takagi et al., 1981), acetylcholine (Fields et al., 1991), substance P (Beitz, 1982b), serotonin (Dahlstrom and Fuxe, 1964; Beitz, 1982a), GABA (Millhorn et al., 1988), and endogenous opioids (Beitz et al., 1983; Fields et al., 1991), that modulate nociception through the PAG-RVM spinal cord circuit. Previous behavioral studies have found that microinjection of an NMDA receptor antagonist into the RVM inhibits antinociception induced by stimulation in the PAG, which suggest the stimulation-induced antinociception is mediated by glutamate in the RVM (Aimone and Gebhart, 1986). It has also been found that microinjection of neurotensin in the RVM can suppress tail-flick (Fang et al., 1987), suggesting that RVM neurotensin plays a role in nociception. Furthermore, pharmacological interventions directed at GABA-mediated synaptic transmission within the RVM have been shown to profoundly alter nociceptive responsiveness – microinjection of GABA<sub>A</sub> receptor antagonists is antinociceptive, whereas application of GABA<sub>A</sub> receptor agonists in the RVM

A significant number of RVM neurons, including many that project to the spinal cord, contain serotonin (5-HT) (Bowker et al., 1981; 1983; Skagerberg and Bjorklund, 1985). In fact, the RVM is the major source of dorsal horn 5-HT (Dahlstrom and Fuxe, 1964; Oliveras et al., 1977). Depletion of spinal cord 5-HT by the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) blocks the antinociceptive effect of morphine microinjected into RVM (Vasko et al., 1984). Supraspinal opioid microinjection evokes release of 5-HT in the spinal cord (Yaksh and Tyce, 1979).

**Rostr al ventromedial medulla (RVM): serotonergic neurons**

**Distribution of serotonergic neurons**

As mentioned above, a significant amount RVM neurons are serotonergic. Current knowledge concerning the distribution of serotonergic neurons and fibers has been obtained by various approaches, including formaldehyde-induced fluorescence histochemistry (Dahlstroem and Fuxe, 1964), immunohistochemistry using antibodies to enzymes, such as tryptophan hydroxylase (Joh et al., 1975), and immunohistochemical methods using antibodies raised against 5-HT itself (Steinbusch, 1981; Takeuchi et al., 1983).
The first histological studies of serotonin revealed the localization of serotonergic neurons near the midline of the brain (Dahlstroem and Fuxe, 1964), and further investigations have suggested that serotonergic neurons are primarily found in the brain stem. The majority of 5-HT neurons are found within the raphe nuclei (i.e. the nucleus raphe dorsalis, nucleus centralis superior, nucleus raphe pontis, nucleus raphe magnus, nucleus raphe obscurus, and nucleus raphe pallidus) (Takeuchi et al., 1983). However, many 5-HT cells are seen outside the raphe system throughout the lower brain stem. These areas are the locus coeruleus complex, including the nucleus parabrachialis medialis and lateralis, the pontine reticular formation, the dorsal pontine tegmentum, and the nucleus reticularis lateralis (Takeuchi et al., 1983).

**Function of serotonergic RVM neurons**

In adult rodents, the amount of RVM neurons that are serotonergic varies but has been estimated to be about 20% (Potrebic et al., 1994). Most of these RVM 5-HT neurons project through the DLF to innervate the dorsal horn (Fuxe, 1965). The function of supraspinal 5-HT on nociception, however, is still unclear. Serotonin has traditionally been associated with descending inhibition of nociception (including opiate analgesia) rather than facilitation. Stimulation of RVM results in increased release of 5-HT in the spinal cord (Hammond et al., 1985; Sorkin et al., 1993); the antinocicpetion resulting from RVM stimulation can be antagonized by intrathecal methysergide, a 5-HT antagonist (Jensen and Yaksh, 1984). Opioid receptors are expressed by 5-HT neurons in the RVM.
(Arvidsson et al., 1995; Kalyuzhny et al., 1996; Kalyuzhny and Wessendorf, 1999; Wang and Wessendorf, 1999; Marinelli et al., 2002) and both RVM stimulation and microinjection of opioids into the RVM (Jensen and Yaksh, 1984; Jones and Gebhart, 1988) results in antinociception. Moreover, the antinociception resulting from microinjection of opioids into the PAG (Lin et al., 1996) or the RVM (Dickenson et al., 1979; Azami et al., 1982; Vasko, 1982; Vasko et al., 1984; Hurley et al., 2003) is reduced by 5-HT antagonists. Intrathecal administration of 5-HT (Yaksh and Wilson, 1979) or of 5-HT agonists (Fasmer et al., 1986; Solomon and Gebhart, 1988; Glaum et al., 1990; Mjellem et al., 1992; Alhaider and Wilcox, 1993; Xu et al., 1994; Peng et al., 1996; Bardin et al., 2000; 2001; Obata et al., 2001; Jeong et al., 2004) has been reported to result in antinociception and direct iontophoretic application of 5-HT onto nociceptive spinal neurons has been reported to frequently inhibit them (Jordan et al., 1978; 1979; Willcockson et al., 1984). Finally, it has been reported that 5-HT terminals innervated nociceptive neurons (Miletic et al., 1984), including spinothalamic tract (STT) neurons (Hylden et al., 1986; Wu and Wessendorf, 1992). Although the role of descending 5-HT neurons in opiate analgesia has been disputed (Gao et al., 1998), it appears likely that RVM 5-HT neurons projecting to the spinal dorsal horn exert antinociceptive effects.

Nevertheless, there is ample evidence that RVM 5-HT neurons also facilitate nociception. Iontophoretically applied 5-HT has been reported to excite some nociceptive neurons in the dorsal horn (Todd and Millar, 1983; 1984),
including some STT neurons (Jordan et al., 1979; Willcockson et al., 1984). The pro-nociceptive effects of stimulation of the RVM have been reported to be inhibited by a 5-HT1 antagonist (Zhuo and Gebhart, 1991). Looking at models of persistent pain, it has been reported that knockout (Zeitz et al., 2002; Kayser et al., 2007) or pharmacological blockade (Abbott et al., 1996; Suzuki et al., 2004; Nishiyama, 2005) of 5-HT3 receptors and/or 5-HT2 receptors dramatically reduce nociception. Activation of 5-HT3 receptors has been reported to mimic the effects of neuropathic pain models on the response properties of spinal neurons (Suzuki et al., 2005) and depletion of spinal 5-HT with the neurotoxin 5,7-DHT reduces both behavioral and electrophysiological responses to stimuli in SNL rats (Rahman et al., 2006). Finally, the 5-HT3 receptor antagonist ondansetron reportedly reduces neuropathic pain in humans (McCleane et al., 2003).

The results in the following chapters of this dissertation shed light on this debate. Specifically, experiments in the following chapters were designed to focus on the relationship of RVM 5-HT neurons and neuropathic pain.
Figure 1-1. Afferent pathway involved in pain sensation. Injury activates the primary afferent nociceptor (PAN), which transmits information to the spinal horn dorsal cord. The terminals of the PAN then contact spinal neurons and release glutamate and peptides to activate the second order neurons. The axons of nociceptive dorsal horn neurons then cross and ascend in the contralateral anterolateral quadrant to form the anterolateral tract (also known as the spinothalamic tract), which terminates in the brainstem and several distinct areas of the thalamus. These areas have higher order neurons that project to cortical regions. SMT, spinomesencephalic tract; SRT, spinoreticular tract.
Figure 1-2. Descending pain-modulatory circuit. Limbic forebrain areas, including the anterior cingulate cortex (ACC), other frontal cortical areas, the hypothalamus, and the central nucleus of the amygdala project to the midbrain periaqueductal grey (PAG). The PAG, indirectly controls pain transmission in the dorsal horn through the rostral ventromedial medulla (RVM). This pathway can exert both an inhibitory (green) and facilitatory (red) control.
Figure 1-3. Schematic of the major animal models of nerve injury. Spinal nerve ligation (SNL) consists of a L5 or L5 and L6 ligation and cut. Chronic constriction injury (CCI) consists of loose ligations of the sciatic nerve, and spared nerve injury (SNI) consists of ligating and cutting sections of the tibial and peroneal nerves and leaving the sural nerve intact.
Figure 1-4. Pathophysiological changes along neural axis after spinal nerve injury. (1) Spinal nerve injury cause spontaneous neural activity and ectopic sensitivity to mechanical stimuli at injury site. (2) The expression of different molecules in the DRG of the injured nerve is up- or down-regulated, reflecting the loss of trophic support from the periphery. Spontaneous neural activity also develops in the DRG. (3) The distal end of the injured nerve undergoes Wallerian degeneration, which exposes non-injured nerve fibers to multiple cytokines and growth factors. (4) Partial denervation of the peripheral tissue leads to an excess of trophic factors, which leads to sensitization of primary afferent nociceptors. (5) The expression of different molecules in the DRG of the uninjured nerve is up- or down-regulated, which reflects increased trophic support. (6) Sensitization of dorsal horn neurons leads to an increase in response to cutaneous stimuli. (7) Activated glial cells contribute to the development of dorsal horn sensitization. (8) Changes in descending modulation may also contribute to enhanced responsiveness of dorsal horn neurons.
Chapter 2

Neuronal loss in the rostral ventromedial medulla in a rat model of neuropathic pain

Introduction

Trauma or disease affecting peripheral nerves frequently results in the development of chronic, sometimes intractable, neuropathic pain. Existing treatments for neuropathic pain have limited effectiveness and produce relatively frequent adverse effects (Wallace, 2007). Therefore, understanding the basic mechanisms contributing to the generation and/or maintenance of neuropathic pain in preclinical animal models is key to providing predictable and efficient therapies in patient populations.

Studies have shown that enhanced pain induced by peripheral nerve injury is associated with increased spontaneous and evoked discharges from injured and/or adjacent nerves (Koltzenburg et al., 1992; 1994; Amir and Devor, 2000; Michaelis et al., 2000; Liu et al., 2001). Although this increased afferent discharge is vital in establishing spinal sensitization in the period immediately following nerve injury, the time course of such abnormal afferent activity is inconsistent with the long duration of heightened pain (Chaplan et al., 1994; Burgess et al., 2002; Porreca et al., 2002). It has been suggested that descending facilitation from the rostral ventromedial medulla (RVM), including its
serotonergic (5-HT) neurons, may play an essential role by maintaining neuropathic pain after its initiation (Burgess et al., 2002; Suzuki et al., 2004; Vera-Portocarrero et al., 2006).

In studies where neuropathic pain is modeled by spinal nerve ligation (SNL) (Kim and Chung, 1992), microinjections of lidocaine into the RVM reduced the hypersensitivity observed after SNL (Pertovaara et al., 1996), as did ablation of populations of RVM neurons (Porreca et al., 2001), and lesions of the dorsolateral funiculus (DLF), in which RVM axons descend (Ossipov et al., 2000). In these studies, reversal of hypersensitivity was not observed immediately after nerve injury, but only several days later, consistent with a role for the RVM in the maintenance (but not initiation) of neuropathic pain.

Previous work has also suggested that apoptosis may play a role in the pathology of neuropathic pain. Apoptosis has been reported in dorsal root ganglia, the spinal dorsal horn and in cerebral cortex in models of neuropathic pain (Whiteside and Munglani, 1998; Coggeshall et al., 2001; Siniscalco et al., 2007; Fuccio et al., 2009; Sekiguchi et al., 2009). However, the loss of neurons has been disputed and its importance in neuropathic pain remains unclear (Polgar, 2004; Polgar et al., 2005).

To help understand the importance of the RVM and its neurons in neuropathic pain, we examined RVM neurons and their spinal projections after SNL. We found that after SNL, the numbers of RVM neurons (including 5-HT
neurons) were significantly reduced. Our findings suggest that the pathology of neuropathic pain may be due in part to death of antinociceptive RVM neurons, leading to decreased descending inhibition of nociception.

Materials and Methods

Animals:

Male Sprague-Dawley rats (150-250 g; Harlan, Madison, WI) were used for these studies; three to ten animals were used for each experimental group. All experiments and procedures were performed using protocols approved by the University of Minnesota Institutional Animal Care and Use Committee.

SNL surgery:

Rats were divided into two experimental groups: a group in which the L5 spinal nerve was ligated and cut (Blenk et al., 1997; Ringkamp et al., 1999; Li et al., 2000), and a sham-operated control group. The left L5 spinal nerve was isolated, tightly ligated and cut distal to the ligation under isoflurane anesthesia (1.5%). The surgical procedure for the sham-operated group was identical to that of the SNL group, except that the L5 spinal nerve was not ligated or cut. General behavior of the rats was monitored before and after the surgery. Any rats showing difficulty elevating a hindpaw were discarded from the study.

Drug treatments:
Rats were treated with 5,7-dihydroxytryptamine (5,7-DHT: 100 µg/10 µl, intracisternal injection) to induce loss of RVM 5-HT neurons. Rats were randomly assigned to one of four groups for these experiments: (1) L5 SNL with 5,7-DHT; (2) L5 SNL with vehicle injection; (3) sham with 5,7-DHT; and (4) sham with vehicle. Injections were given ten days after surgery; animals receiving vehicle injections received sterile saline (10 µl, intracisternal injection). To prevent damage to noradrenergic (NE) neurons, we pre-treated animals 30 minutes before 5,7-DHT (or saline) administration with desipramine (20 mg/kg i.p.), an inhibitor of neuronal NE reuptake (Bjorklund et al., 1975).

For cell proliferation experiments, bromodeoxyuridine (BrdU; 50 mg/kg i.p.) or an equal volume of vehicle was injected once per day into sham and SNL-treated rats 0-3 days, 3-6 days, and 6-9 days after surgery. Rats were sacrificed 10 days after surgery. Naïve rats without any injections were used as additional controls.

**Perfusion:**

Rats were deeply anesthetized with a mixture of ketamine (67.5 mg/kg), xylazine (22.5 mg/kg) and acepromazine (1 mg/kg) and perfused via the ascending aorta with 180 ml oxygenated Ca\(^{2+}\)-free Tyrode’s solution (pH 7.2) followed by 500 ml of 4% formaldehyde (freshly made from paraformaldehyde) in 0.16 M phosphate buffer (pH 6.9). Immediately after fixation, brains were removed and stored in a 5% sucrose solution prior to sectioning.
**Histology and Immunocytochemistry:**

The RVM and spinal cord were sectioned using a freezing microtome (Leica, SM2400) at a nominal thickness of 50 µm. The free-floating sections were washed in phosphate-buffered saline (PBS: 0.8% (w/v) NaCl, 0.02% KCl, 0.144% Na₂HPO₄, 0.024% KH₂PO₄, pH = 7.4) for three 5-minute intervals. RVM sections were incubated overnight at 4° C in solutions containing one of the following antibodies: (1) mouse anti-TPH (T0678, Sigma, Saint Louis, MO, 1:1000), (2) mouse anti-NeuN (MAB377, Millipore, Temecula, CA, 1:500), (3) mouse anti-CD11b (MCA75GA, AbD Serotec, Oxford, UK, 1:1000), or (4) mouse anti-GFAP (GA5, #3670, Cell Signaling Technology, Danvers, MA, 1:1000). Spinal cord sections were incubated with goat anti-5-HT (#20079, ImmunoStar, Hudson, WI, 1:1000). Sections were then washed in PBS and incubated for 4 h with Cy2-conjugated donkey anti-mouse IgG or Cy3-donkey anti-goat IgG (1:500). Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). The fluorescent Nissl stain ethidium bromide (30 nM, Sigma) was used to counterstain RVM tissue (Schmued et al., 1982).

To stain for apoptosis markers, ten micron-thick cryostat sections were incubated with antibodies raised against activated caspase-3 (AF835, R&D Systems, Minneapolis, MN), cleaved caspase-6 (Asp162, Cell Signaling Technology), or stained for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL kit; Roche, Mannheim, Germany). For
caspase staining, RVM sections were incubated overnight at 4° in a solution containing rabbit anti-cleaved caspase-6 (1:500) or mouse anti-active caspase-3 (1:500). Sections were then washed in PBS and incubated for 2 h with Cy3-conjugated donkey anti-rabbit (1:500) or Cy3-conjugated donkey anti-mouse (1:500) and the Nissl counterstain SyBr RNA II Green Gel Stain (S-7564, Invitrogen, Carlsbad, CA, 1:10,000). For TUNEL labeling, sections were rinsed in dH₂O and then permeabilized with tris-buffered saline (TBS; 135 mM NaCl and 25 mM tris-HCl; pH = 7.4) containing 0.2% Triton X-100 and 0.2% Tween-20 for 1 h at room temperature. Following permeabilization, the sections were rinsed in TBS then incubated in TdT reaction buffer (Roche, 1600 U/ml terminal transferase, 40 nM biotin-16-dUTP, 200 mM potassium cacodylate, 5 mM CoCl₂, 250 µg/ml bovine serum albumin, 25 mM tris-HCl; pH 6.6) for 2 h at 37°C. The reaction was terminated in TBS containing 20 mM EDTA; the sections were washed in several changes of TBS and incubated in streptavidin conjugated to Cy3 (Jackson Immunoresearch) and counterstained with SyBr RNA II Green Gel Stain (Invitrogen, 1:10,000) in TBS containing 0.2% Tween-20 and 0.2% casein for 2 h at room temperature.

Tissue was stained for the mu-opioid receptor (MOR) after heat-induced epitope retrieval. Ten micron-thick cryostat sections were exposed to a temperature of 101°C for 30 min in a 10 mM solution of citric acid. Tissue was then washed three times (five minutes each) in TBS and incubated overnight at 4°C in guinea pig anti-MOR and mouse anti-TPH. The guinea pig anti-MOR was
the generous gift of Dr. Robert Elde (University of Minnesota, Minneapolis, MN) and was characterized as specific in MOR knockout mice (Liu et al., 2011). Tissue was washed in TBS and then incubated in Cy3-conjugated donkey antiguinea pig IgG, Cy5-conjugated donkey anti-mouse IgG and counterstained with SYBR Green II (Invitrogen, 1:10,000) for 2 h.

Heat-induced epitope retrieval was also used for BrdU and Ki67 experiments. Tissue (RVM and small intestine, the latter used as a positive control for dividing cells) was washed in TBS and incubated overnight at 4°C in sheep anti-BrdU (#ab1893, Abcam, San Francisco, CA, 1:1000) or rabbit anti-Ki67 (NCL-Ki67p, Novoceastra, Leica Biosystems, Buffalo Grove, IL, 1:1000). Tissue was again washed in TBS and then incubated in Cy-2 conjugated donkey anti-sheep IgG for 2 h and counterstained with ethidium bromide (30 nM) for 15 min.

All tissue was dehydrated in graded alcohols (50-100%) and cleared in xylene. The slides were mounted with coverslips using DPX (Fluka, Ronkonkoma, NY).

**Microscopy and Quantification:**

Conventional microscopy was used to collect images of the RVM for cell counting. An Olympus BX50 fluorescence microscope (Tokyo, Japan) equipped with filter sets designed to allow selective visualization of Cy2 and Cy3 was used. Microscopic images were collected with a Scion 1346 digital camera. Confocal
images were collected using an Olympus FluoView FV1000 microscope (Tokyo, Japan). ImageJ 1.29 (developed at NIH and available at http://rsb.info.nih.gov/ij/) or Photoshop were used to adjust contrast and brightness in images.

The RVM was defined as an isosceles triangle that lies at the level of the facial nucleus with a base having a width equal to that of the combined pyramidal tracts, and its height equal to half the width of the base (Figure 2-1) (Gu and Wessendorf, 2007). The RVM extended from the rostral end of inferior olive to the caudal end of the trapezoid body. It was divided into ipsilateral and contralateral sides by the midline.

In Nissl-stained sections of the RVM, two distinct populations of cells were found: (1) cells with dark cytoplasm, a vacuous nucleus and a single distinct nucleolus (Figure 2-1E1, arrows), and (2) cells with very lightly-stained cytoplasm, a dark nucleus, and nuclear granulations rather than a nucleolus (Figure 2-1E1, arrowheads). The first group was classified as neurons and the second as glia. These distinct Nissl-stained populations were confirmed by immunohistochemistry (e.g., NeuN for neurons and GFAP and CD11B for astrocytes and microglia, respectively). Cell counting was based on cells’ Nissl staining (including counting of TPH-immunoreactive (-ir) neurons, which were Nissl counter-stained); the same sections were used for counting Nissl-stained neurons and TPH-ir neurons. NeuN-ir neurons were counted in a separate population of rats and their counting was not based on Nissl-staining (see below).
The numbers of cells were estimated separately for each half of the RVM (i.e. both ipsilateral and contralateral to the surgery). Systematic random sampling and unbiased stereological methods were used for quantification as described previously (Gu and Wessendorf, 2007). Five RVM sections (each 50 µm thick) per animal were sampled; the first section sampled was selected randomly. The sampling interval thereafter was determined based on the number of sections within the RVM and was usually every sixth section. For each selected section, a low-magnification (2x) image of the RVM was captured. A grid was randomly cast over the image (Figure 2-1, line drawing) and systematic random sampling was again used when choosing the grid intersections to be evaluated (Howard and Reed, 1998). Once an intersection was chosen, a stack of images was made at 40x while focusing through the full thickness of the section (Figure 2-1, C-D). Cells were counted as follows. A counting frame was superimposed on the stack of images (Howard and Reed, 1998). The right boundary and the upper boundary of the counting frame were used as acceptance lines (Figure 2-1, C-D, dotted lines); the other two boundaries were forbidden lines (Figure 2-1, C-D, solid lines). Neurons were counted only if nuclei (for NeuN-labeled neurons) or nucleoli (for Nissl-stained neurons) (Popken and Farel, 1997) either fell entirely inside the counting frame or if they crossed an acceptance line without also crossing a forbidden line. NeuN-labeled neurons were counted when the top of the nucleus was found within the thickness of the tissue section. Nissl-stained neurons were counted when the nucleolus was present within the thickness of
the tissue section and TPH-ir neurons were counted based on their Nissl counterstaining. To estimate the total number of neurons (N) in the RVM per animal, we used the following equation:

\[ N = (D \times V) \]

where \( D \) = the neuronal density of the RVM, \( V \) = the volume of the RVM. To estimate the neuronal density of the RVM in each rat, we used the following equation:

\[ N = (D \times V) \]

where \( N \) = the number of neurons counted per rat, \( a = 2,500 \, \mu m^2 \) (the area of the counting frame sampled), \( t = 50 \, \mu m \) (the thickness of the section sampled), and \( g = 20 \) (the number of counting frames sampled per rat). To estimate the volume of the RVM from each rat, we used the following equation:

\[ V = \Sigma (A \times I \times t) \]

where \( A \) = the cross sectional area of RVM in which the sections were sampled, \( I \) = the interval between adjacent sampled sections, and \( t = 50 \, \mu m \) (the thickness of section sampled).

To determine the effects of SNL on spinal 5-HT innervation, the density of 5-HT labeling was examined in the superficial dorsal horn using a 40x/1.4 NA objective. The number of 5-HT varicosities in the lateral and medial superficial
dorsal horn was determined using the “Finding Maxima” routine of ImageJ. The density was then calculated by dividing those numbers by the corresponding cross-sectional areas of the regions being examined. The same value for noise tolerance was used in all cases.

The intensity of MOR-ir in the RVM was also determined with ImageJ. A region of interest was outlined and the mean intensity determined using the measurement function.

**Statistics:**

Differences among treatment groups were identified either by 1-way or 2-way analyses of variances (ANOVA); post-hoc comparisons were made after 2-way ANOVAs with a Bonferroni test. Chi-square tests were used to detect differences in proportions. P< 0.05 was considered significant. Statistical tests were performed using the GraphPad Prism software (La Jolla, CA) and the statistical tools available on the GraphPad website ([http://www.graphpad.com](http://www.graphpad.com)).

**Von Frey Testing for Tactile Hypersensitivity:**

Mechanical sensitivity was determined by measuring the paw withdrawal threshold in response to the application of von Frey filaments, using the up-down method of Chaplan (Chaplan et al., 1994). In brief, rats were placed on a wire mesh surface, covered by an inverted plastic cage and allowed to habituate for 15 min. Filaments were then applied to the plantar surface of the hindpaw. If
application of the filament evoked no response, the next-larger filament was applied; if application elicited withdrawal, the next-smaller filament was applied. This sequence was repeated until the largest filament was used or until four filaments were applied after the first withdrawal was noted. Withdrawal thresholds were measured prior to surgery and at 2-3 day intervals after surgery. In all cases, thresholds were measured bilaterally.

Results

*SNL induced tactile hypersensitivity ipsilateral to the surgery*

As previously reported (Kim and Chung, 1992; Blenk et al., 1997; Ringkamp et al., 1999; Li et al., 2000), SNL resulted in hypersensitivity to tactile stimuli ipsilateral, but not contralateral, to the lesion. Ten days after surgery the withdrawal threshold for the hindpaw ipsilateral to the surgery was $2.7 \pm 0.5$ g (mean± standard error of the mean, SEM) in rats receiving SNL, which was significantly lower than that in sham-operated rats ($14.0 \pm 2.7$ g; $p<0.05$, $n=8$ animals for each group, total degrees of freedom [d.f] = 31). Thresholds in the ipsilateral paws of rats subjected to SNL were also significantly lower than those in their contralateral hindpaws ($14.5 \pm 1.3$ g; $p<0.05$, Figure 2-2A). Paw withdrawal thresholds determined before surgery were not significantly different between experimental groups.
SNL reduced the number of RVM neurons

SNL resulted in a significant decrease in the number of RVM neurons ipsilateral to the lesion (Figure 2-1A,B; Figure 2-2B). In rats that had received SNL, the number of Nissl-stained neurons in the half of the RVM ipsilateral to the lesion was 11,248±668 (mean±SEM), which was significantly less (by 23%) than the number found in the ipsilateral side of the RVM in sham-operated rats (14,665±800, p<0.05, n=8 animals in each group, total d.f. = 31). The number of neurons ipsilateral to SNL was also significantly less (by 14%) than that on the contralateral side of the same animals (p<0.05, Figure 2-2B) but the number of RVM neurons on the contralateral side was not significantly different between rats receiving SNL and sham-operated rats (SNL: 13,003±471 vs. sham: 13,365±872, p>0.05, Figure 2-2B). No significant differences were observed between the volumes of the RVMs in sham-operated and experimental rats (sham ipsilateral=1.123±0.050 mm³; sham contralateral=1.131±0.057 mm³; SNL ipsilateral=1.075 ±0.079 mm³; SNL contralateral=1.077±0.083 mm³, p>0.05, n=8 animals, in each group, total d.f. = 31, Figure 2-3A).

The loss of neurons after SNL was not non-specific. The volume and the number of neurons in the facial nucleus, which is adjacent to the RVM but not involved in pain modulation, was unchanged (ipsilateral sham: 6,267±219;
ipsilateral SNL 6,204±422; contralateral sham: 6,693±270; contralateral SNL: 6,542±187: p>0.05, n = 8 animals in each group, total d.f. = 31, Figure 2-2C, Figure 2-3B). This suggests that cell loss in the RVM was not due to any indiscriminant brain stem pathology resulting from SNL.

To confirm that SNL did not simply transform RVM neurons to cells with a glia-like morphology, we repeated the experiment in a second group of animals, staining sections for the neuronal marker NeuN (Mullen et al., 1992). SNL resulted in a significant decrease in the number of RVM NeuN-ir neurons ipsilateral to the lesion. In rats subjected to SNL, the number of NeuN-ir neurons in the half of the RVM ipsilateral to the lesion was 11,898±768, which was significantly less (24%) than the number found in the ipsilateral side of sham-operated rats (15,688±945, p<0.05, n=8 animals in each group, total d.f. = 31, Figure 2-2D). The number of NeuN-ir neurons ipsilateral to SNL was also significantly less (by 20%) than that on the contralateral sides of the same animals (14,913±964, p<0.05, Figure 2-2D). Thus, the NeuN results validated those obtained using Nissl staining and the latter method was used as the basis of neuronal counting for the remainder of these experiments.

*RVM TPH-ir neurons were decreased in rats receiving SNL*
TPH, the rate-limiting enzyme in 5-HT synthesis, was used as a marker for 5-HT neurons in the RVM. In both rats receiving SNL and sham-operated rats, TPH-ir neurons were found throughout the RVM. However, the number of RVM TPH-ir neurons ipsilateral to SNL was significantly less (by 35%) than the number of TPH-ir neurons ipsilateral to sham surgery (SNL ipsilateral: 773±134 vs. sham ipsilateral: 1,184±92, p<0.05, n=8 animals in each group, total d.f. = 31, Figure 2-4). Within SNL animals, the number of TPH-ir neurons ipsilateral to the lesion was also significantly less (27%) than the number contralateral to the lesion (SNL contralateral: 1,055±222 p<0.05, Figure 2-4C). The proportion of 5-HT cells lost (35%) was significantly larger than the percent decrease observed among all RVM neurons (23%), suggesting that 5-HT neurons are more sensitive to the effects of peripheral nerve damage than RVM neurons as a whole (p<0.0001; chi-square with Yates correction).

_Fewer 5-HT-ir varicosities found in substantia gelatinosa ipsilateral to SNL_

Since RVM 5-HT neurons project to dorsal spinal cord (Bowker et al., 1981; Skagerberg and Bjorklund, 1985; Kwiat and Basbaum, 1992; Kalyuzhny et al., 1996), we examined whether spinal 5-HT-ir decreased after SNL (Figure 2-5). At spinal levels C2, T2 and L4, the density of 5-HT-ir varicosities in the ipsilateral superficial dorsal horn was significantly lower in rats receiving SNL than in sham-
operated rats. At the cervical level, the density of 5-HT varicosities in rats treated with SNL was 0.033±0.002 varicosities / µm² on the ipsilateral side (where 1 µm² = 10⁻¹² m²), which was significantly lower (by 30%) than in sham-operated rats (0.047±0.004 / µm², p<0.05, n=6 animals receiving SNL, n=8 sham-operated animals, total d.f. = 27, Figure 4A). Densities in animals treated with SNL at the thoracic and lumbar levels (0.039±0.005 / µm² and 0.043±0.004 / µm², respectively) were also significantly lower on the ipsilateral side (decreases of 22% and 15%, respectively) than those in sham-operated rats (Figure 2-5B,C). There were no significant differences on the contralateral sides of the superficial dorsal horn at any spinal level.

*Depletion of 5-HT reversed mechanical hypersensitivity after SNL*

To determine whether loss of the RVM 5-HT neurons remaining after SNL would further exacerbate cutaneous hypersensitivity, we used intracisternal injection of the serotonin neurotoxin 5,7-DHT to deplete 5-HT in rats that had undergone SNL (Figure 2-6). Rather than being lower, mechanical withdrawal thresholds were significantly higher after 5,7-DHT (5.2 ±1.3 g prior to injection vs. 10.2± 1.7 g after injection; p<0.05, n=10 animals in each group, total d.f. = 39, Figure 2-6A). These thresholds were also significantly higher than those in rats that had received SNL and that were subsequently treated with saline rather than
5,7-DHT (10.2±1.7 g vs 4.5±1.3 g, respectively; p<0.05). Treatment with 5,7-DHT markedly reduced 5-HT-ir in the spinal cord (Figure 2-6B).

MOR expression in RVM after SNL

Previous studies have suggested that RVM neurons expressing the mu-opioid receptor (MOR) mediate descending facilitation of nociception after SNL (Porreca et al., 2001). Although RVM neurons were lost after SNL (see above), MOR-ir in the RVM appeared to be unchanged (Figure 2-7A,B). When the intensity of MOR staining was measured there was no significant difference between sham animals (55.5±2.6 arbitrary intensity units) and animals receiving SNL (53.7±3.2 arbitrary intensity units; n=3 animals in each group, t-test, d.f.=4, p>0.05). MOR-ir after SNL was observed in TPH-ir neurons as well as in neurons that were not immunoreactive for TPH (Figure 2-7B).

Glia: activation and increased numbers after SNL

RVM glial activation appears to have a role in descending facilitation of nociception in a model of persistent pain (Wei et al., 2008). To determine whether glial activation might be responsible for the neuronal loss, we stained for
markers of astrocytes (GFAP) and microglia (CD11B) in RVM after SNL. At day three, we found a bilateral increase in activation of astrocytes in animals receiving SNL as compared to sham-operated animals (Figure 2-8A-D, Figure 2-9); GFAP staining was decreased by day 10 although still visible. Conversely, although little labeling was observed at day three, we found markedly stronger labeling for CD11B at day 10 in rats receiving SNL. Again, the labeling increased both ipsilateral and contralateral to SNL (Figure 2-8E-H). Using Nissl-stained tissue, we also found a significant bilateral increase in numbers of glia in the RVM of SNL-treated animals as compared to sham-operated animals (SNL ipsi: 87,277± 10,354 vs. sham ipsi: 60,430± 7,551 or 44% higher, SNL contralateral to SNL: 85,903± 9,091 vs. sham contralateral to SNL: 56,104± 7,912 or 52% higher; p<0.05, n=5 animals in each group, total d.f. = 19, Figure 2-8I). To determine whether this increase in glial activation and number was non-selective and found across the brain stem, we examined the adjacent facial nucleus. In that nucleus we found no significant increase in the number Nissl-stained glia (Figure 2-8J) or in the activation of glia in SNL-treated rats, compared to sham-operated rats.

The mechanism by which the number of glia increases in the RVM may represent proliferation or migration of glia into the brain from other CNS regions. No evidence for glial proliferation was found using either Ki67 or BrdU. When comparing labeling in the RVMs of sham and SNL-treated animals, there appeared to be no difference in the number of Nissl-stained neurons also labeled for BrdU in either experimental group from any of the time groups.
Discussion

Nerve injury and disease can trigger a range of responses in both the peripheral and central nervous systems that contribute to chronic neuropathic pain (Devor, 2006). In the periphery, injured primary sensory neurons develop hyperexcitability and abnormal impulse generation (Blumberg and Janig, 1984). Other pathophysiological changes observed in injured primary sensory neurons include the altered regulation and expression of certain molecules (e.g. neuropeptides, ion channels, enzymes, etc.). Increased spinal cord excitability ipsilateral to the injury (i.e. central sensitization) also appears to contribute crucially to abnormal pain conditions after tissue injury (Ji et al., 2003). N-methyl-D-aspartate (NMDA) receptor activation is one of the principal mechanisms in central sensitization, and its role in neuropathic pain is implied by preclinical studies showing that NMDA receptor antagonists are effective in alleviating experimental neuropathic pain (Wei and Pertovaara, 1999; Woolf and Salter, 2000).

Pathophysiological changes in the RVM also contribute to neuropathic pain. Microinjection of a NMDA receptor antagonist before SNL (Wei and Pertovaara, 1999), and microinjection of lidocaine in the RVM after SNL have both been shown to reduce allodynia (Pertovaara et al., 1996), suggesting that activation of
(and activity in) the RVM contributes to cutaneous hypersensitivity. Consistent with this idea, lesioning the DLF, in which RVM axons descend to the spinal cord, has also been reported to reverse both thermal hyperalgesia and allodynia after SNL (Ossipov et al., 2000). In addition, selective ablation of MOR-expressing RVM neurons has been reported to prevent SNL-induced experimental hypersensitivity (Porreca et al., 2001). However, neither DLF lesions nor microinjection of lidocaine into the RVM reduced cutaneous hypersensitivity within three days after SNL--they did so only 4-6 or more days after SNL (Burgess et al., 2002). Based on these and similar findings, it has been proposed that physiological changes in the RVM contribute to the maintenance of neuropathic pain but not to its initiation.

**Fewer RVM neurons after SNL**

In our present studies, it was found that the number of RVM neurons ipsilateral to SNL was significantly decreased compared to sham-operated rats. Several prior studies have shown that neurons at the spinal level undergo apoptosis ipsilateral to peripheral nerve injury (Sugimoto et al., 1990; Whiteside and Munglani, 2001; Scholz et al., 2005) and apoptosis has been reported in cortex in response to nerve injury (Fuccio et al., 2009). Apoptosis would be expected to result in cell loss, although to date the loss of neurons in those regions has been controversial (Polgar, 2004; Polgar et al., 2005).
Our finding of neuronal loss in the RVM suggests that death of pain modulatory neurons contributes to the pathophysiology of neuropathic pain. Although it is possible that both pro- and anti-nociceptive neurons are lost after SNL, the most parsimonious interpretation of our findings is that SNL selectively kills antinociceptive RVM neurons, thereby facilitating cutaneous hypersensitivity. Glial activation in the RVM may also contribute to cutaneous hypersensitivity (Wei et al., 2008), although since we found that it occurs bilaterally it would not by itself explain the unilateral decrease in withdrawal thresholds; instead, glial activation may be an important factor in the initiation of neuropathic pain (Mei et al., 2011).

Although a significant number of 5-HT neurons were lost after SNL (see below), most cells that were lost (88%) did not express 5-HT. The identity of these neurons is not known but it has been proposed that the antinociceptive neurons of the RVM are physiologically characterized as OFF-cells (Fields et al., 1983a; 1983b; Heinricher et al., 1989; Fields et al., 1991). Many OFF-cells project to the dorsal horn (Fields et al., 1995) and express GABA (Winkler et al., 2006). Previous studies have suggested that changes in ON-cell and OFF-cell firing drive the hypersensitivity observed in SNL (Carlson et al., 2007; Gonçalves et al., 2007), but it has not yet been determined whether or not the number of OFF-cells changes. It has also reported that neuropathic pain is maintained by RVM neurons expressing MOR (Porreca et al., 2001; Burgess et al., 2002). Consistent with this finding, MOR-ir appeared not to decrease after SNL and
RVM neurons (including 5-HT neurons) expressing MOR were still observed after SNL.

**Mechanism of cell loss**

The cause of RVM cell loss is unclear. It has been reported that hypersensitivity after SNL could be blocked by a single microinjection of an NMDA antagonist into the RVM (Wei and Pertovaara, 1999), suggesting that excessive activity among glutamatergic afferents immediately after injury might be sufficient to kill RVM neurons. Although NMDA receptor activation may contribute to cell loss, it is unclear how activation only of ipsilateral glutamatergic afferents could be evoked physiologically. An alternative explanation is that loss of RVM neurons arises from their spinal projections. RVM neurons project predominantly ipsilaterally to the spinal cord dorsal horn (Skagerberg and Bjorklund, 1985) and nerve damage or primary afferent activity have been reported to result in cell loss or apoptosis in the dorsal horn and in dorsal root ganglia (Coggeshall et al., 2001; Whiteside and Munglani, 2001; Maione et al., 2002; de Novellis et al., 2004; Scholz et al., 2005; Hassanzadeh and Ahmadiani, 2006; Jalalvand et al., 2008; Sekiguchi et al., 2009; Meisner et al., 2010). Thus we propose that apoptosis of spinal cells (either neurons, glia or primary afferent fibers) in the terminal fields of bulbospinal neurons triggers retrograde degeneration of RVM neurons.

**Pain facilitation by 5-HT**
In the present study, about 8% of RVM neurons were found to be serotonergic (i.e. they expressed TPH-ir). Of these, about one-third was lost ipsilateral to SNL. The remaining 5-HT neurons appeared to facilitate nociception: administration of 5,7-DHT, a serotonin neurotoxin, significantly improved (i.e. increased) withdrawal thresholds after SNL. This effect might either be direct (e.g., via 5-HT3 receptors (Suzuki et al., 2005) or indirect (e.g., via gating the facilitatory or inhibitory output of the RVM). Since 5,7-DHT was given intracisternally, this finding could be due at least in part to 5,7-DHT acting on 5-HT neurons outside the RVM. However, previous studies have suggested that bulbospinal 5-HT neurons do contribute to persistent pain (McCleane et al., 2003; Suzuki et al., 2004; Rahman et al., 2006; Dogrul et al., 2009; Wei et al., 2010) and our findings are consistent with there being a role for 5-HT in maintaining neuropathic pain.

In summary, our data indicate that almost one-fourth of RVM neurons are lost after SNL, and that preventing this loss reduces the hypersensitivity observed after SNL. We propose that death of antinociceptive neurons in the RVM is responsible, at least in part, for maintaining tactile hypersensitivity after nerve damage and that changes in the RVM and the spinal cord together contribute to the phenomenon of neuropathic pain. Reducing RVM neuronal death and the associated hypersensitivity with anti-apoptotic drugs may prove useful for preventing neuropathic pain in humans.
Figure 2-1. Definition of RVM and description of cell-counting methods. Line Diagram: The RVM was defined as an isosceles triangle, the base of which was the width of the pyramidal tracts. The height of the triangle on the midline was half the width of the base. VII: facial nucleus. Crosses mark examples of randomly selected points used for cell counting. A,B. Nissl-stained sections from sham-treated and SNL-treated rats, respectively. Fewer RVM neurons were observed in rats after SNL. Stars in A and B represent the locations of the counting frames in C and D, respectively. C, D: Examples of high-magnification sequences of images through the thickness of tissue sections used for cell counting. Color of counting frames corresponds to the color of the stars in A and B. Arrows indicate neurons that would be counted. A neuron might appear in multiple images through the thickness of a section but was only counted when its nucleolus was first observed. Neurons were counted if their nucleoli fell entirely within the counting frame, or if they were partially within the counting frame without being in contact with a rejection line (solid line). E1-E3. Comparison of Nissl and NeuN labeling. Arrows indicate Nissl-stained neurons that were also NeuN-ir. Arrowheads indicate Nissl-stained glia that were not NeuN-ir. Cells with dark cytoplasm, a vacuous nucleus and a dark nucleolus (the Nissl criteria we used for a neuron) were always labeled for NeuN. Scale bar in B applies to A and B. Scale bar in D4 applies to C1-4 and D1-4. Scale bar in E3 applies to E1-E3.
Figure 2-2. SNL induced tactile hypersensitivity and neuronal loss in RVM. Paw withdrawal thresholds, measured by von Frey filaments, ipsilateral to the ligation were significantly decreased compared to those in sham-operated rats or to those observed on the contralateral side in SNL rats (*, p<0.05; 2-way ANOVA). B. Significantly fewer Nissl-stained neurons were observed ipsilateral to SNL (*, p<0.05, 2-way ANOVA). C. SNL resulted in no significant differences in the number of neurons in the facial nucleus (p>0.05, 2-way ANOVA). D. SNL resulted in a significant decrease in the number of NeuN-labeled neurons ipsilateral to the surgery (*, p<0.05, 2-way ANOVA).
Figure 2-3. No significant differences in the volumes of RVM and Facial nucleus after SNL. Comparisons between the ipsilateral and contralateral sides of both regions yielded no differences after SNL. The same was true when comparing the ipsilateral sides of sham and SNL animals (p>0.05; 2-way ANOVA).
Figure 2-4. SNL induced loss of tryptophan hydroxylase-immunoreactive (TPH-ir) neurons in the RVM. A-B. Confocal images showing TPH-ir in the RVM in sham-operated (A) and SNL-treated (B) rats. Parameters for image acquisition and display (brightness, contrast, filtering) were identical. “Ipsilateral” and “contralateral” are relative to the nerve ligation. C. The number of RVM TPH-ir neurons ipsilateral to SNL was significantly less than contralateral to SNL. In addition, the number was significantly less than that found ipsilateral to sham-surgery (*, p<0.05; 2-way ANOVA). TPH-ir neurons were counted based on their Nissl counter-staining, as described in the Methods section. Scale bar in B applies to A and B.
Figure 2-5. Effect of SNL as serotonin-immunoreactive (5-HT-ir) in the superficial dorsal horn of the spinal cord. A-C. SNL significantly decreased the density of 5HT-ir varicosities at all spinal levels examined. (A) Segment C2, (B) Segment T2, (C) Segment L4 (*, p<0.05; 2-way ANOVA). D. Images of 5-HT-ir in superficial dorsal horn of rats receiving sham surgery or SNL. Note reduced 5-HT-ir in SNL image. Box in inset denotes area from which images were obtained.
Figure 2-6. Intracisternal injection of the serotonin neurotoxin 5,7 dihydroxytryptamine (5,7-DHT) partially reversed mechanical hypersensitivity after SNL. A. Withdrawal thresholds became significantly higher after rats receiving SNL were injected with 5,7-DHT. Also, thresholds were significantly higher in rats receiving SNL that were treated with 5,7-DHT than in rats receiving SNL that were given saline (*, p<0.05; 2-way ANOVA). B. Image of 5-HT-ir in superficial dorsal horn of a rat that had received SNL and that was treated with 5,7-DHT (compare to Figure 4D). Note lack of 5-HT-ir. Box in inset denotes area from which image was obtained.
Figure 2-7. MOR-immunoreactivity in the RVM appears to be unchanged after SNL. A. MOR-ir in RVM ipsilateral to sham surgery. B. MOR-ir in RVM ipsilateral to SNL. RVM neurons, including TPH-ir neurons expressed MOR-ir after SNL. Section was double-stained; insets show expression of MOR-ir by TPH-ir RVM neurons. Insets were taken from the region outlined by the square. C. Absorption control. Section of RVM serially adjacent to that in B; note marked reduction in labeling. Section was also double-stained and the same TPH-ir neurons visible in B can be seen in the insets. Again, the square indicates the region from which the insets were taken. Scale bar in insets = 30 µm. The 300 µm scale bar in C applies to A-C.
Figure 2-8. SNL induced a bilateral activation of astrocytes and microglia and an increase in glial number. A-D. At day 3, astrocytes were activated (as shown by increased GFAP labeling) in the ipsilateral and contralateral RVM after SNL, compared to sham. E-H. At day 10, microglia were activated after SNL compared to sham, as shown by increased labeling for the microglial marker, CD11b. I. We observed significantly more Nissl-stained glia in the RVM after SNL and the increase was bilateral. J. No significant increases in glial number were found in Facial nucleus after SNL. Scale bar in H applies to A-H.
Figure 2-9. Low magnification images of RVM show increased activation of astrocytes at day 3 and day 10 post-SNL. Scale bar = 125 µm.
Chapter 3

Prevention of SNL-induced RVM cell loss and mechanical hypersensitivity by tauroursodeoxycholic acid

Introduction

As mentioned in the previous chapter, we have found that after L5 spinal nerve ligation (SNL), a rat model of neuropathic pain, that the number of neurons in the RVM ipsilateral to the ligation decreases by almost one-fourth and the number of 5-HT in the same region decreases by over one-third. The cause and the mechanism of RVM cell loss are unclear. Although several prior studies have shown that neurons in the cortex (Fuccio et al., 2009) and at the spinal level undergo apoptosis after peripheral nerve injury (Sugimoto et al., 1990; Whiteside and Munglani, 2001; Scholz et al., 2005), there has no been no reliable evidence of traditional markers (activated caspase-3, cleaved caspase-6, and TUNEL staining) of apoptosis at the level of the RVM (Wei et al., 2008; Leong et al., 2011). However, this may be in part because apoptotic events are brief (Kerr and Wyllie, 1972; Sanchez et al., 1992) and may result in few or no apoptotic
cells being detected (Lawen, 2003). Therefore, we investigated the role of apoptosis in RVM cell loss.

Various investigators have found that neurons undergo apoptosis and that blocking this process with intrathecal zVAD, an inhibitor of the caspases that mediate apoptosis, reduces the tactile hypersensitivity that rats display after nerve damage. Unfortunately, zVAD is a peptide, which makes this drug less practical for treating potential CNS apoptosis. However, previous studies have reported that the bile acid ursodeoxycholic acid (UDCA) and its taurine conjugate tauroursodeoxycholic acid (TUDCA) inhibit apoptosis and that they have been shown to be useful for reducing brain damage in a rat model of stroke (Rodrigues et al., 2002; 2003). UDCA can be given orally or parenterally and is approved in the United States for use in humans for cholestatic liver disease, but because TUDCA has greater solubility in water, we decided to use this bile acid to investigate the mechanism of RVM neuronal loss and whether reversal can improve neuropathic symptoms (i.e. mechanical hypersensitivity).

Materials and Methods

The majority of the protocols for the following experiments are identical to those mentioned in the previous chapter; therefore, the latter will be briefly described and additional materials and methods will be detailed further.
**Animals:**

Again, 3-10 male Sprague-Dawley rats (150-250 g; Harlan, Madison, WI) were used for each experiment in these studies. All experiments and procedures were performed using protocols approved by the University of Minnesota Institutional Animal Care and Use Committee.

**SNL surgery:**

Rats were divided into two experimental groups: a group in which the L5 spinal nerve was ligated and cut (Blenk et al., 1997; Ringkamp et al., 1999; Li et al., 2000), and a sham-operated control group.

**Perfusion:**

Rats were deeply anesthetized and perfused via the ascending aorta with oxygenated Ca\(^{2+}\)-free Tyrode’s solution (pH 7.2) followed by 500 ml of 4% formaldehyde in 0.16 M phosphate buffer (pH 6.9). Immediately after fixation, brains were removed and stored in a 5% sucrose solution prior to sectioning.

**Drug treatments:**

We tested the efficacy of TUDCA as an inhibitor of apoptosis by examining its effects on dexamethasone-induced apoptosis in thymus. Two TUDCA injections (300 mg/kg i.p.) were given: one two days prior to dexamethasone (1 mg/kg i.p.) administration and one the day of dexamethasone
administration. Control animals were given saline injections instead of TUDCA solution. Rats were killed one day after dexamethasone administration.

To test the effects of inhibiting apoptosis on SNL, we administered tauroursodeoxycholic acid (TUDCA: 300 mg/kg i.p.) or saline prior to and after surgery. TUDCA has been reported to inhibit apoptosis and to be active systemically (Rodrigues et al., 2003); systemic administration avoids the potential for damage associated with microinjections made directly into the brain parenchyma. For each group, injections were given every-other day, starting 3 days prior to surgery and continuing until sacrifice at day 10.

For another experimental group, at day 10, animals were not sacrificed but instead TUDCA administration (300 mg/kg i.p.) was ceased. To examine the long-term effects of TUDCA, mechanical withdrawal thresholds were monitored every-other day after TUDCA cessation until day 20 post-SNL when animals were killed.

To test whether TUDCA administration raises mechanical withdrawal thresholds if started after SNL, rats received either TUDCA (300 mg/kg i.p.) or vehicle starting either on day 2 or day 10 post-surgery. Once started, injections were given every-other day and continued for 8 days for those animals that first received TUDCA or vehicle on day 2 post-surgery and 14 days for those animals that first received TUDCA on day 10 post-surgery. Mechanical withdrawal
thresholds were monitored on days without injections, and animals were sacrificed either on Day 10 or Day 24 post-SNL.

**Histology and Immunocytochemistry:**

The RVM and spinal cord were sectioned using a freezing microtome (Leica, SM2400) at a nominal thickness of 50 µm. The free-floating sections were washed in phosphate-buffered saline (PBS) for three 5-minute intervals. RVM sections were incubated overnight at 4°C in a solution containing mouse anti-tryptophan hydroxylase (TPH; T0678, Sigma, Saint Louis, MO, 1:1000). Sections were then washed in PBS and incubated for 4 h with Cy2-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, 1:500). The fluorescent Niss stain ethidium bromide (30 nM, Sigma) was used to counterstain RVM tissue (Schmued et al., 1982).

To stain for apoptosis markers, ten micron-thick cryostat sections were stained for activated caspase-3 (AF835, R&D Systems, Minneapolis, MN), cleaved caspase-6 (Asp162, Cell Signaling Technology), or stained for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL kit; Roche, Mannheim, Germany) after heat-induced epitope retrieval. Sections were exposed to a temperature of 101°C for 30 min in a 10 mM solution of citric acid. Tissue was then washed three times (5 minutes each) in tris-buffered saline (TBS; 135 mM NaCl and 25 mM tris-HCl; pH = 7.4) and permeabilized with TBS containing 0.2% Triton X-100 and 0.2% Tween-20 for 1 h at room
temperature. For caspase staining, after rinsing with TBS, tissue was incubated overnight at 4°C in mouse anti-activated caspase-3 (1:500) or cleaved caspase-6 (1:500). Tissue was then again washed in TBS and incubated for 2 h with Cy3-conjugated donkey anti-mouse and the Nissl counterstain SyBr RNA II Green Gel Stain (S-7564, Invitrogen, Carlsbad, CA, 1:10,000). For TUNEL labeling, following permeabilization (again with TBS containing 0.2% Triton X-100 and 0.2% Tween for 1 h at room temperature), the sections were rinsed in TBS then incubated in TdT reaction buffer (Roche, 1600 U/ml terminal transferase, 40 nM biotin-16-dUTP, 200 mM potassium cacodylate, 5 mM CoCl₂, 250 µg/ml bovine serum albumin, 25 mM tris-HCl; pH 6.6) for 2 h at 37°C. The reaction was terminated in TBS containing 20 mM EDTA; the sections were washed in several changes of TBS and incubated in streptavidin conjugated to Cy3 (Jackson Immunoresearch) and counterstained with SyBr RNA II Green Gel Stain (Invitrogen, 1:10,000) in TBS containing 0.2% Tween-20 and 0.2% casein for 2 h at room temperature.

**Microscopy and Quantification:**

Conventional microscopy was used to collect images of the RVM for cell counting.

The RVM was again defined as an isosceles triangle that lies at the level of the facial nucleus with a base having a width equal to that of the combined pyramidal tracts, and its height equal to half the width of the base (Figure 2-1).
The RVM extended from the rostral end of inferior olive to the caudal end of the trapezoid body. It was divided into ipsilateral and contralateral sides by the midline.

Nissl-stained sections were used to count neurons and glia. Neurons had dark cytoplasm, a vacuous nucleus, and single distinct nucleolus. On the other hand, glia had very lightly-stained cytoplasm, a dark nucleus, and nuclear granulations rather than a nucleolus. Cell counting was based on cells’ Nissl staining, including counting of TPH-ir neurons, which were Nissl counter-stained.

Systematic random sampling was used to choose the sections and fields to be evaluated (Howard and Reed, 1998); the entire RVM was sampled. Unbiased stereological methods (i.e. a counting frame and the optical disector method) were used to count cells within those fields.

Statistics:

Differences among treatment groups were identified by 2-way analyses of variances (ANOVA); post-hoc comparisons were made after 2-way ANOVAs with a Bonferroni test. Chi-square tests were used to detect differences in proportions. P< 0.05 was considered significant. Statistical tests were performed using the GraphPad Prism software and website (La Jolla, CA, http://www.graphpad.com).

Von Frey Testing for Tactile Hypersensitivity:
Mechanical sensitivity was determined by measuring the paw withdrawal threshold in response to the application of von Frey filaments, using the up-down method of Chaplan (Chaplan et al., 1994). Withdrawal thresholds were measured prior to surgery and at 2-3 day intervals after surgery. In all cases, thresholds were measured bilaterally.

Results

Administration of tauroursodeoxycholic acid (TUDCA) reduced both the loss of RVM neurons and cutaneous hypersensitivity after SNL

Previous studies have reported that neuronal apoptosis occurs in the spinal cord and cerebral cortex after nerve injury and that inhibiting caspases reduces cell loss (Coggeshall et al., 2001; Whiteside and Munglani, 2001; Scholz et al., 2005; Siniscalco et al., 2007; Fuccio et al., 2009). In our previous experiments, we have been unable to show reliable apoptosis labeling; therefore, to further test whether apoptosis might mediate the cell loss we observed in the RVM, we administered an apoptosis inhibitor. To avoid the local damage that could result from a direct microinjection of a caspase inhibitor into the RVM, we gave rats a long-acting inhibitor of apoptosis that can be administered systemically. (Ward et al., 1984; Rodrigues et al., 1998; Rodrigues and Steer,
The taurine conjugate of UDCA (TUDCA) has been reported to inhibit apoptosis (Rodrigues et al., 2003; Ramalho et al., 2008), promote cell survival (Schoemaker et al., 2004), and has been shown to have beneficial effects in a number of in vivo and in vitro models of neurological damage that result in apoptosis, including hemorrhagic and ischemic stroke, Huntington's disease, and Alzheimer's disease (Sola et al., 2007).

We first tested the actions of TUDCA by examining its effects on apoptosis in the thymus. We induced apoptosis in the thymus by treatment with 1 mg/kg dexamethasone, an immunosuppressant glucocorticoid (Compton et al., 1987; Quaglino and Ronchetti, 2001), which greatly increased staining for caspase-3. However, in rats treated with both TUDCA and dexamethasone, we found a marked decrease in caspase-3 labeling compared to treatment with dexamethasone alone (Figure 3-1).

In the brain stem, we found that administration of TUDCA prevented loss of RVM neurons after SNL (Figure 3-2A). In rats receiving SNL and treated with TUDCA, the number of RVM neurons ipsilateral to the lesion was 17,976±1,202, which was significantly higher (by 43%) than the number found in the ipsilateral sides of vehicle-treated rats that had received SNL (12,540±930; p<0.05, n=8 animals in each group, total d.f. = 31, Figure 3-2A). As expected, in vehicle-treated rats, the number of RVM neurons in the ipsilateral side (12,540±929) was significantly less (by 26%) than the number found in the contralateral side.
There was no significant difference between the number of neurons in the ipsilateral and contralateral sides of TUDCA-treated rats receiving SNL (TUDCA ipsi: 17,976±1,202, TUDCA contra: 16,675±828, Figure 3-2A), nor between the numbers of neurons found in the contralateral sides of the two treatment groups (SNL with TUDCA: 16,675±828, SNL with saline: 16,949±1,074, p>0.05, Figure 3-2A).

TUDCA also prevented the loss of 5-HT neurons in the RVM. The number of RVM 5-HT neurons ipsilateral to SNL in TUDCA-treated animals (1,353±96) was significantly higher (by 30%) than the number in vehicle-treated rats (1,043±118, p<0.05, n=8 animals in each group, total d.f. = 31, Figure 3-2B). Again, as expected, the number of 5-HT-ir RVM neurons in the ipsilateral side of vehicle-treated rats (1,043±118) was significantly less (by 27%) than that in the contralateral side (1,427±211, p<0.05, Figure 3-2B). In TUDCA-treated rats receiving SNL, there was no significant difference between the number of neurons in the ipsilateral and contralateral sides (TUDCA ipsi: 1,353±96, TUDCA contra: 1,251±99, p>0.05, Figure 3-2B).

TUDCA administration also reduced SNL-induced mechanical hypersensitivity. Beginning at day 4 post-SNL, the withdrawal thresholds of the ipsilateral hindpaws of TUDCA-treated rats (Day 4: 3.5±0.5 g; Day 6: 4.0±0.5 g; Day 8: 4.6±1.1 g; Day 10: 5.2±1.3 g) were significantly higher than those animals treated with vehicle (Day 4: 1.1±0.3 g; Day 6: 1.3±0.2 g; Day 8: 1.2±0.3g; Day
10: 1.2±0.2 g; p<0.05, n=10 animals treated with TUDCA, n=9 animals treated with vehicle, d.f. interaction = 7, d.f. treatment = 1, d.f. time = 7, d.f. subjects = 17, d.f. residual = 119, total d.f. = 151, Figure 3-2C).

Administration of TUDCA reduced the increase in glia observed after SNL

After SNL, we observed a bilateral increase in the number of glia in the RVM. Treatment with TUDCA significantly reduced the increase in RVM glia observed after SNL (TUDCA ipsi: 62,306±3,736 vs. vehicle ipsi: 85,541±2,972, or 27% fewer; TUDCA contra: 60,437±4,315 vs. vehicle contra: 80,086±2,899, or 25% fewer; p<0.05 in both cases, n=7 animals treated with TUDCA, n=6 animals treated with vehicle, total d.f. = 25). These findings suggest that the increase in glia was in response to cell death.

Treatment with TUDCA exerts significant long-last effects on withdrawal thresholds

We found that pretreatment with TUDCA reduces hypersensitivity after nerve injury. To address whether it would be necessary to continue TUDCA administration indefinitely to be able to continue to observe this reduction, we
monitored withdrawal thresholds before cessation of TUDCA administration (administration stopped at day 10 post-SNL) and afterward until sacrifice at day 20 post-SNL. Again, we found that there was a significant difference between ipsilateral paw withdrawal thresholds at day 10 between vehicle-treated and TUDCA-treated animals (TUDCA: 12.5±0.6 g, vehicle: 2.8±0.5 g, p<0.05, Figure 3-3), but we also found that there was still a significant effect 10 days after cessation of treatment (TUDCA: 6.6±1.9 g, vehicle: 2.1±0.4 g, p<0.05, n=8 for each group, total d.f. = 31, Figure 3-3).

*TUDCA administration raises mechanical withdrawal thresholds if started 2 days after SNL but not if started 10 days after SNL*

To determine whether treatment with TUDCA is still effective if begun after nerve injury, TUDCA administration was begun either on day 2 or day 10 after SNL surgery. In the first group, SNL-treated animals were given either TUDCA or saline beginning 2 days after surgery; animals treated with TUDCA showed a significant increase in mechanical withdrawal thresholds compared to animals given saline at day 9 post-SNL (TUDCA: 8.0±2.8 g, vehicle: 2.8±0.5 g, p<0.05, n=5 animals for each group, total d.f. =59, Figure 3-4). In the group that received TUDCA for 10 days beginning at day 10 post-SNL, there was no significant
difference in ipsilateral hindpaw withdrawal thresholds when comparing them before and after TUDCA administration (p>0.05, n=3 animals, Figure 3-4).

Discussion

Prevention of neuronal loss and mechanical hypersensitivity by apoptosis inhibitor

Nerve damage or primary afferent activity have been reported to result in cell loss or apoptosis in the dorsal horn and in dorsal root ganglia (Coggeshall et al., 2001; Whiteside and Munglani, 2001; Maione et al., 2002; de Novellis et al., 2004; Polgar et al., 2005; Scholz et al., 2005; Hassanzadeh and Ahmadiani, 2006; Jalalvand et al., 2008; Sekiguchi et al., 2009; Meisner et al., 2010). In our experiments we were unable to demonstrate caspase-3, caspase-6, or TUNEL in the RVM after SNL; however, we did show that the antiapoptotic drug, TUDCA, blocked SNL-induced RVM neuronal loss (Figure 3-2A,B) and prevented hypersensitivity. TUDCA significantly decreased tactile hypersensitivity four or more days after SNL but did not reduce hypersensitivity one-to-three days after SNL (Figure 3-2C), suggesting that cell loss underlies the RVM’s contribution to the maintenance phase of neuropathic pain and not the initiation phase (Vera-Portocarrero et al., 2006). As we proposed in the previous chapter, glial activation in the RVM may also contribute to the observed hypersensitivity but
only to its initiation (Mei et al., 2011). In our studies, we found that the number of glia increased bilaterally after SNL, and that this increase was blocked by TUDCA, suggesting it may represent migration of glia and not proliferation (see lack of BrdU labeling in previous chapter) in response to RVM neuronal death.

Our data also showed that TUDCA reduced mechanical hypersensitivity long after it had been stopped being administered (Figure 3-3). There was still a significant effect of TUDCA administration 10 days after cessation of treatment, which suggests that by preventing apoptosis or promoting cell survival in the RVM that it would not be necessary to continue TUDCA administration indefinitely to enjoy the benefits. It is unclear, nonetheless, how long this effect continues. Further experiments where longer survival times are used are needed to determine the extent of TUDCA’s long-lasting beneficial effects on pain thresholds. It should also be noted that when comparing mechanical hypersensitivity during the period of TUDCA treatment prior to cessation to that in our previous experiments (see Figure 3-2C), that there appears to be a significant amount of variation in withdrawal thresholds. During comparable timelines in both experiments, TUDCA-treated rats had significantly higher withdrawal thresholds compared to their sham-operated counterparts but also showed a high degree of variation in the increased threshold values between individual TUDCA-treated rats. We are unsure of the reasons for this phenomenon; however, one may hypothesize that individual variation in RVM cell
death, as well as different levels of glial activation may contribute to the different responses to TUDCA administration.

**Attenuation of mechanical hypersensitivity**

We have shown that administration of TUDCA prior to nerve injury prevents the full development of mechanical hypersensitivity after SNL. Our data also suggest that TUDCA administration can begin 2 days after nerve injury and still have a beneficial effect. However, when we delayed TUDCA treatment until 10 days after SNL there was no significant difference in mechanical withdrawal thresholds before and after treatment. These results suggest that there is an early time window after nerve injury where TUDCA can prevent hypersensitivity. To narrow down the timeframe in which the drug is effective and to determine the maximum delay possible prior to initiation of treatment, additional behavioral experiments are needed. Furthermore, to confirm whether TUDCA acts by stopping RVM cell loss after nerve injury, cell-counting experiments are required for SNL-treated rats receiving TUDCA or vehicle 2 days after surgery.

**Mechanism of cell loss**

Because TUDCA was given systemically, there is a possibility that it acted indirectly (i.e. on spinal cord or dorsal root ganglia; see above) rather than directly on the RVM to prevent apoptosis. The latter would agree with our inability to demonstrate TUNEL or caspase labeling in the RVM after SNL. However, apoptotic events are brief (Kerr and Wyllie, 1972; Bursch et al., 1990;
Sanchez et al., 1992), which can result in few or no apoptotic cells being detected (Lawen, 2003). TUDCA can also act to promote cell survival by other means than inhibiting apoptosis (Schoemaker et al., 2004), and it is possible that RVM neurons are lost via some mechanism other than the classical apoptotic pathway.

**Clinical implications**

Despite the testing of many compounds, to date there has been relatively little progress toward prevention of neuropathic pain in the clinic (Bordet and Pruss, 2009; Albers et al., 2011). In part, this may be due to the fact that many compounds that appear the most promising in pre-clinical trials, such as NMDA-receptor antagonists (Mao et al., 1992; Smith et al., 1994; Munglani et al., 1999; Wei and Pertovaara, 1999; Wilson et al., 2005) have side effects that interfere with their clinical utility. In contrast, UDCA is already clinically used for other purposes, such as for treatment for cholestatic liver disease, and is well tolerated (Festi et al., 2007). This gives our findings a clinical appeal. We have shown that UDCA’s taurine conjugate, TUDCA, reduces mechanical hypersensitivity and blocks loss of brain stem neurons after nerve injury, if it is administered starting three days prior to the injury (Figure 3-2). This regimen would be useful for preventing post-surgical pain, where the date of surgery is known ahead of time but would be impractical to use for accidental trauma. However, our results also show early administration of the bile acid after nerve injury decrease mechanical
hypersensitivity. These results suggest that TUDCA administration will be useful not only to prevent development of neuropathic pain, but also to prevent development of neuropathic pain following traumatic injury. Consequently, our results from these studies may help with the development of relevant clinical therapies.
Figure 3-1. TUDCA inhibited dexamethasone-induced apoptosis in thymus. A. Dexamethasone (1 mg/kg) increased staining for activated caspase-3 in thymus. B. Treatment with TUDCA decreased caspase-3-ir in dexamethasone–treated rats. C. TUDCA administration alone did not cause increased caspase-3 staining. D. Thymus tissue from naïve rats showed little apoptosis.
Figure 3-2. TUDCA prevented neuronal loss after SNL, including serotonergic cell loss, and partially reversed SNL-induced mechanical hypersensitivity. (A) Compared to vehicle-treated rats, TUDCA-treated rats had significantly more Nissl-stained neurons ipsilateral to SNL (*, p<0.05, 2-way ANOVA). (B) TUDCA-treated rats also had significantly more TPH-ir neurons after SNL (*, p<0.05, 2-way ANOVA). In contrast to saline-treated rats, there were no significant differences between the numbers of RVM neurons ipsilateral and contralateral to SNL in TUDCA-treated animals. C. Beginning four days post-SNL, the withdrawal thresholds of the ipsilateral hindpaws of TUDCA-treated rats were significantly higher than those in animals treated with saline (p<.05; 2-way ANOVA). There were no significant differences between the withdrawal thresholds of the contralateral hindpaws at any time point. Inverted triangles = contralateral saline; squares = contralateral TUDCA; circles = ipsilateral TUDCA; triangles = ipsilateral saline.
Figure 3-3. Duration of TUDCA effect. TUDCA was begun 3 days prior to SNL and continued to 10 days after SNL. The TUDCA group (red) still showed significantly higher von Frey thresholds ipsilateral to SNL than vehicle controls (black triangles) 10 days after cessation. N=8.
Figure 3-4. Effects of post-surgical administration of TUDCA on withdrawal threshold. A: TUDCA was started 2 days after SNL and administered every-other day until sacrifice at day 10. Day 9 withdrawal threshold of SNL-treated animals given TUDCA were significantly higher than SNL-treated animals given vehicle (*, p<0.05, 2-way ANOVA, n=5 for each group). B: Results from early preliminary studies suggested no significant effects on withdrawal thresholds if TUDCA was started 10 days after SNL (p>0.05, n=3). Saline-treated animals were not included.
Chapter 4

Do chronic constriction injury and spared nerve injury models result in neuronal loss in rostral ventromedial medulla?

Introduction

We have found that SNL results in a significant decrease in the total number of RVM neurons, including 5-HT neurons (Figure 2-2, Figure 2-4), suggesting that the increased nociception observed after SNL may result from loss of inhibitory neurons in the RVM that project to the superficial dorsal horn (Figure 2-5). This decrease appears to be due to apoptosis because administration of TUDCA, an inhibitor of apoptosis, prevents neuronal loss (Figure 3-2). We find no cell loss in the facial nucleus, just lateral to the RVM (Figure 2-2), suggesting that cell loss is specific to the RVM. We also found that an intracisternal injection of the serotonin neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), can partially reverse SNL-induced mechanical hypersensitivity. On the other hand, sham animals that received 5,7-DHT had significantly increased mechanical hypersensitivity, which suggests that tactile allodynia observed in neuropathic pain may be mediated, at least in part, by bulbospinal 5-HT neurons. Given our findings, the evidence for RVM 5-HT neurons evoking antinociception, and reports of the prominence of 5-HT’s role in neuropathic pain in humans and in animal models (McCleane et al., 2003; Suzuki et al., 2004; 2005; Donovan-Rodriguez et al., 2006; Rahman et al., 2006), we hypothesize that nerve injury induces the selective loss of antinociceptive RVM 5-HT neurons, leaving behind
RVM 5-HT neurons that facilitate pain. To further test this hypothesis, we decided to investigate neuronal numbers in other animal models of neuropathic pain.

There are many animal models of neuropathic pain but additional models used in rats include: chronic constriction injury (CCI), partial sciatic nerve ligation, and spared nerve injury (SNI). All involve injury to a nerve innervating the hindpaw and share the characteristics of tactile and thermal hypersensitivity, although they differ qualitatively with regard to amounts of ongoing pain behavior and degree of tactile hypersensitivity (Kim et al., 1997; Decosterd and Woolf, 2000). However, behavioral responses in the partial sciatic nerve ligation model have been reported to vary depending on diet (Shir et al., 1998). Thus, we propose not to use partial sciatic nerve ligation but instead to examine CCI and SNI models in these studies. If neuropathic pain results in part from the death of RVM 5-HT neurons, we would expect that in CCI and SNI models of neuropathic pain would also provoke loss of these neurons. Furthermore, it would suggest that loss of RVM 5-HT neurons is required for chronic expression of neuropathic pain.

**Materials and Methods**

**Animals:**
Male Sprague-Dawley rats (150-250 g; Harlan, Madison, WI) were used for these studies; six to nine animals were used for each experimental group. All experiments and procedures were performed using protocols approved by the University of Minnesota Institutional Animal Care and Use Committee.

**Animal surgeries:**

Chronic constriction injury (CCI): The common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through bicep femoris. Proximal to the sciatic’s trifurcation about 1 cm of nerve was freed of adhering tissue and 4 ligatures (4.0 chromic gut) were tied loosely around it with about 1 mm spacing (Bennett and Xie, 1988). The length of nerve thus affected was 4-5 mm long. Great care was taken to tie the ligatures such that the diameter of the nerve was seen to be just barely constricted when viewed with magnification. In sham animals, there was sciatic exposure without ligation.

Spared nerve injury (SNI): As previously described (Decosterd and Woolf, 2000), under 1.5% isoflurane anesthesia, the skin on the lateral surface of the thigh was incised and a section made directly through the biceps femoris muscle exposing the sciatic nerve and its three terminal branches: the sural, common peroneal and tibial nerves. The SNI procedure comprised an axotomy and ligation of tibial and common peroneal nerves leaving the sural nerve intact. The common peroneal and the tibial nerves were ligated with 5.0 silk and sectioned.
distal to the ligation. Great care was taken to avoid any contact with or stretching of the intact sural nerve. Sham controls involved exposure of the sciatic nerve and its branches without any lesion.

**Perfusion:**

All animals were perfused 10 days after surgery. Rats were deeply anesthetized with a mixture of ketamine (67.5 mg/kg), xylazine (22.5 mg/kg) and acepromazine (1 mg/kg) and perfused via the ascending aorta with 180 ml oxygenated Ca\(^{2+}\)-free Tyrode’s solution (pH 7.2) followed by 500 ml of 4% formaldehyde (freshly made from paraformaldehyde) in 0.16 M phosphate buffer (pH 6.9). Immediately after fixation, brains were removed and stored in a 5% sucrose solution prior to sectioning.

**Histology and Immunocytochemistry:**

The RVM was sectioned using a freezing microtome (Leica, SM2400) at a nominal thickness of 50 \(\mu\)m. The free-floating sections were washed in phosphate-buffered saline (PBS) for three 5-minute intervals. RVM sections were incubated overnight at 4°C in a solution containing mouse anti-tryptophan hydroxylase (TPH; T0678, Sigma, Saint Louis, MO, 1:1000). Sections were then washed in PBS and incubated for 4 h with Cy2-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, 1:500). The fluorescent Niss
stain ethidium bromide (30 nM, Sigma) was used to counterstain RVM tissue (Schmued et al., 1982).

**Microscopy and Quantification:**

Conventional microscopy was used to collect images of the RVM for cell counting. An Olympus BX50 fluorescence microscope (Tokyo, Japan) equipped with filter sets designed to allow selective visualization of Cy2 and Cy3 was used. Microscopic images were collected with a Scion 1346 digital camera.

The RVM was defined as an isosceles triangle that lies at the level of the facial nucleus with a base having a width equal to that of the combined pyramidal tracts, and its height equal to half the width of the base (Figure 2-1). The RVM extended from the rostral end of inferior olive to the caudal end of the trapezoid body. It was divided into ipsilateral and contralateral sides by the midline.

Nissl-stained sections were used to count neurons and glia. Neurons had dark cytoplasm, a vacuous nucleus, and single distinct nucleolus. On the other hand, glia had very lightly-stained cytoplasm, a dark nucleus, and nuclear granulations rather than a nucleolus. Cell counting was based on cells’ Nissl staining, including counting of TPH-ir neurons, which were Nissl counter-stained.

Systematic random sampling was used to choose the sections and fields to be evaluated (Howard and Reed, 1998); the entire RVM was sampled.
Unbiased stereological methods (i.e. a counting frame and the optical disector method) were used to count cells within those fields.

**Statistics:**

Differences among treatment groups were identified by 2-way analyses of variances (ANOVA); post-hoc comparisons were made after 2-way ANOVAs with a Bonferroni test. Statistical tests were performed using the GraphPad Prism software and website (La Jolla, CA, [http://www.graphpad.com](http://www.graphpad.com)).

**Von Frey Testing for Tactile Hypersensitivity:**

Mechanical sensitivity was determined by measuring the paw withdrawal threshold in response to the application of von Frey filaments, using the up-down method of Chaplan (Chaplan et al., 1994). Withdrawal thresholds were measured prior to surgery and at 2-3 day intervals after surgery. In all cases, thresholds were measured bilaterally.

**Results**

*CCI and SNI both induced tactile hypersensitivity ipsilateral to the surgery*
As previously reported (Bennett and Xie, 1988; Decosterd and Woolf, 2000), CCI resulted in hypersensitivity to tactile stimuli ipsilateral, but not contralateral, to the surgery. Ten days after surgery, the withdrawal threshold for the hindpaw ipsilateral to the surgery was 3.8±1.5 g (mean± standard error of the mean, SEM) in rats receiving CCI, which was significantly lower than that in sham-operated rats (13.9±1.1 g; p<0.05, n=9 animals receiving CCI, n=8 sham-operated rats, total degrees of freedom [d.f.] = 33, Figure 4-1). Thresholds in the ipsilateral paws of rats subjected to CCI were also significantly lower than those in their contralateral hindpaws (14.0±0.7 g; p<0.05, Figure 4-1).

Similar results were found when comparing SNI experimental groups. At day 10 post-surgery, the withdrawal threshold for the hindpaw ipsilateral to the lesion had significantly decreased (0.7±0.1 g) in SNI-treated rats when comparing to sham-operated rats (14.2±0.8 g; p<0.05, n=8 in both groups, total d.f. =31, Figure 4-3). In addition, ipsilateral paw thresholds of animals receiving SNI were significantly lower than those in their contralateral hindpaws (15.0±0.0 g; p<0.05, Figure 4-3).

In all cases, paw withdrawal thresholds determined before surgery were not significantly different between experimental groups.
No decrease in the number of RVM neurons, including 5-HT neurons, in CCI-treated rats

At 10 days after surgery, unlike our SNL experiments (Chapter 2), CCI resulted in no significant decrease in the total number of RVM neurons ipsilateral to the lesion (Figure 4-2A). In rats that had received CCI, the number of Nissl-stained neurons in the half of the RVM ipsilateral to the lesion was 12,622±1,885 (mean±SEM), which was not significantly less than the number found in the ipsilateral side of the RVM in sham-operated rats (14,412±1,270, p>0.05, n=8 animals in each group, total d.f. = 31). Additionally, there was no significant difference between the number of neurons in the ipsilateral and contralateral sides (p>0.05, Figure 4-2) of the same CCI-treated animals and when comparing the number of RVM neurons on the contralateral side of rats receiving CCI and sham-operated rats (CCI: 14,785±2,604 vs. sham: 13,920±1,007, p>0.05, Figure 4-2A).

When making comparisons of 5-HT neuronal counts between experimental groups, we found that the number of 5-HT neurons in the ipsilateral RVM also did not change between CCI-treated and sham-operated rats (CCI: 1,213±163 vs. sham: 1,1422±127, p>0.05, Figure 4-2B). Nor was there a significant difference when comparing the ipsilateral and contralateral sides of the animals that received CCI (contra: 1342±167, p>0.05, Figure 4-2B).
No significant difference in RVM neuronal number in rats receiving SNI

As with our CCI results, we did not observe any significant decrease in the total number of RVM neurons, including RVM 5-HT neurons, after SNI (Figure 4-4A,B). There were no differences when comparing the ipsilateral sides of SNI-treated and sham-operated rats (SNI ipsi: 20,550±1,937 vs. sham ipsi: 18,943±1,297, SNI ipsi 5-HT: 2,454±447 vs. sham ipsi 5-HT: 1,861±213, p>0.05, n=8 SNI-treated animals, n=6 sham-operated animals, total d.f. = 27, Figure 4-4A,B). Furthermore, there were no significant differences when comparing the ipsilateral and contralateral sides of the same SNI animals SNI contra: 18,549±1,287, SNI contra 5-HT: 2,201±279, p>0.05, Figure 4-4A,B).

Glial number unchanged after CCI and SNI

In a model of trigeminal neuropathic pain, where chronic constriction injury was made to the infraorbial nerve, RVM glial activation appears to have a role in the descending facilitation of nociception (Wei et al., 2008). Our results from SNL experiments confirm that there was increased glial activation and glial number in the RVM after nerve injury. To determine whether glial number increases in other models of neuropathic pain, we used systematic random
sampling and unbiased stereological methods to count glial number after CCI and SNI. Using Nissl-stained tissue, we did not find an increase in numbers of glia in the RVM of CCI-treated animals as compared to sham-operated animals in either side of the RVM (CCI ipsi: 72,859±4,207 vs. sham ipsi: 82,057±7,395 and CCI contra vs. sham contra: 77,357±6,969, p>0.05, n=8 animals in each group, total d.f. = 31, Figure 4-2C). In addition, no significant differences were found when comparing numbers in animals receiving SNI and those receiving a sham operation (SNI ipsi: 56,471±8,221 vs. sham ipsi: 55,321±1,596 and SNI contra: 56,604±8,210 vs. sham contra: 53,612±3,865, p>0.05, n=8 animals receiving SNI, n=6 sham-operated animals, total d.f.=27, Figure 4-4C). This finding suggests that cell death after SNL precedes the increase of glia and that glia are responding to damaged neurons.

Discussion

Peripheral nerve damage induces various degrees of hypersensitivity, which may be accompanied by sensory and motor deficits (Sanoja et al., 2008). Several phenomena, both at the periphery and at the spinal cord, are responsible for the increased excitability of spinal nociceptive neurons as well as for the abnormalities in axonal connectivity and in expression of neuromediators that underlie neuropathic pain (Campbell and Meyer, 2006). Another important player in neuropathic pain is a descending pain-control system. Both facilitatory and inhibitory influences descend from the nucleus raphe magnus and other
structures of the RVM on the spinal dorsal horn (Campbell and Meyer, 2006). In our previous SNL experiments, we found that the number of RVM neurons (including 5-HT neurons) ipsilateral to SNL was significantly decreased compared to sham-operated rats. It appears that this loss is due to apoptosis. Our finding of neuronal loss in the RVM suggests that death of pain modulatory neurons contributes to the pathophysiology of neuropathic pain. Glial activation in the RVM may also contribute to cutaneous hypersensitivity, although since we found that it occurs bilaterally it would not itself explain the unilateral decrease in withdrawal thresholds; instead, glial activation may be an important factor in the initiation of neuropathic pain (Mei et al., 2011).

In chapter 2 we proposed that neuronal loss in the RVM was due to neuronal loss in the spinal cord. Interestingly, RVM neuronal loss and increase in glial number after spinal nerve injury was not observed in CCI and SNI models ten days after injury. The reason for this discrepancy is unclear. All three neuropathic pain models exhibit functional and structural alterations of spinal networks, however we hypothesize that there might be greater cell death at 10 days post- SNL than at the same time point after SNI and CCI because of the shorter physical distance between the site of nerve injury and the CNS in the SNL model, and therefore, a shorter time required for transport of tumor necrosis factors (TNF) and neurotrophins (see Discussion below).

**Axonal transport of proteins and cell death**
Nerve injury can initiate a series of inflammatory responses that contribute to neuropathic pain symptoms (Myers et al., 2006). Previous investigations have revealed that the increase in retrograde axonal transport of proteins to dorsal root ganglia (DRG) (Redshaw and Bisby, 1984), including neurotrophins (DiStefano and Curtis, 1994; Curtis et al., 1998) and tumor necrosis factor-alpha (TNF-α); (Shubayev and Myers, 2001), represents a response to sensory nerve injury. Within a week of mammalian peripheral nerve injury, dynamic reorganization within the damaged sensory nerve fibers (e.g. axonal and myelin reorganization) causes an increase in retrograde axonal transport of small proteins, which provides a way of introducing an array of factors into neuronal cell bodies of DRG (Myers et al., 2006). One explanation for the increased amount of TNF transported may be the increased availability of the cytokine (i.e. from activated immune cells) (Schäfers et al., 2002). Alternatively, as shown for neurotrophins (Tonra et al., 1998), increased TNF transport may be related to upregulation of its cell surface receptors or a redistribution of preexisting receptors within the injured DRG or nerve fibers. Furthermore, neuronal transport may be upregulated because of the increase of transport capacity of injured neurons, which is possibly related to increased expression of axonal transport motor proteins (Su et al., 1997). These mechanisms allow local TNF-α and its receptors to be retrogradely transported to DRG neurons, which is thought to be a stimulus for upregulation of TNF-α in CNS neurons and glia (Myers et al., 2006). Furthermore, when TNF-α tracer is injected at a nerve injury site it
undergoes retrograde transport, which is followed by anterograde transport to the spinal cord from the DRG (Shubayev and Myers, 2001; 2002). Similar transport of neurotrophins from the periphery to the CNS has been reported; significant retrograde transport of nerve growth factor (NGF, a type of neurotrophin) to the DRG after CCI (Schäfers et al., 2002) and nerve crush (Curtis et al., 1998) has been observed, along with increased transport the spinal cord (Curtis et al., 1998).

The increased transport and expression of neurotrophins and TNF-α after nerve injury may be responsible for the observed cell death at the levels of the DRG and dorsal spinal cord after nerve injury. Neurotrophins are thought to act as survival signals to suppress the cell death. However, the interaction of neurotrophins with the neurotrophic receptor, p75NTR, can induce cell death under certain conditions, suggesting that neurotrophins might as death ligands (Yuan and Yankner, 2000). The p75 neurotrophin receptor (p75NTR) is a member of the TNF receptor superfamily that can bind all neurotrophins. Its intracellular domain contains a region that bears similarity with the 'death domain,' which mediates protein-protein interactions and is present in other members of the TNF family (Yuan and Yankner, 2000). TNF-α is also known to lead to apoptosis; specifically through the TNF-receptor-1 (TNFR1). TNF-α binds to TNFR1 and results in trimerization and activation of the death receptor (Naismith and Sprang, 1998) and the caspase signaling pathway (Micheau and
Indeed, one study has shown that the amount of TNF-alpha in the DRG is related to the rate of apoptosis in DRG neurons (Sekiguchi et al., 2009).

If nerve injury and dysfunction can trigger transport of neurotrophins and TNF-α and ultimately DRG cell death, then it is conceivable that DRG apoptosis can initiate cell death at the level of the spinal cord through a series of similar events. Apoptosis of spinal cord neurons has been shown to be triggered by afferent activity (Scholz et al., 2005). Therefore, by examining the different timelines of increased DRG TNF-α (Shubayev and Myers, 2001) and DRG and spinal cord apoptosis after nerve injury (Scholz et al., 2005; Sekiguchi et al., 2009), it is feasible that one event triggers a cascade that can ultimately affect supraspinal structures (i.e. spinal dorsal horn cell loss may initiate RVM neuronal loss through retrograde degeneration and transport of apoptosis-inducing factors). Thus, delaying any of the abovementioned events by inducing injuries further away from the CNS might delay changes in the RVM. Additionally, at the level of the spinal cord, apoptotic profiles have been observed 7 days after SNI (Polgar et al., 2005) and CCI (Scholz and Woolf, 2007), which may not be sufficient time to allow significant retrograde RVM cell loss at 10 days after surgery. To confirm this hypothesis, our next experiments would be to perform RVM cell counts at later time points after SNI and CCI.
Currently, our understanding of the neuroinflammatory mechanisms after different nerve injuries is incomplete. Therefore, by focusing on and understanding the link between peripheral nerve injury, abnormal ectopic electrophysiological activity in nociceptive fibers, and cell activation and loss in DRG and the spinal cord, this knowledge will provide rationale for new therapies targeted at predictable temporal events of nerve degeneration.
Figure 4-1. Chronic constriction injury (CCI) induced tactile hypersensitivity. Paw withdrawal thresholds, measured by von Frey filaments, ipsilateral to the injury were significantly decreased compared to those in sham-operated rats or to those observed on the contralateral side in CCI rats (*, p<0.05; 2-way ANOVA).
Figure 4-2. No significant differences in RVM neuronal and glial number after CCI. A. CCI did not induce any changes in the number of Nissl-stained or (B) 5-HT neurons (p>0.05; 2-way ANOVA). C. Additionally, CCI resulted in no significant difference in the number of glia in the RVM (p>0.05; 2-way ANOVA).
Figure 4-3. Spared nerve injury (SNI) induces mechanical hypersensitivity. As measured by von Frey filaments, animals receiving SNI displayed decreased withdrawal thresholds and increased mechanical hypersensitivity (*, p<0.05, 2-way ANOVA).
Figure 4-4. No significant differences in RVM neuronal and glial number after SNI. A. SNI did not significantly change the number of Nissl-stained neurons or (B) 5-HT neurons ($p>0.05$; 2-way ANOVA). Furthermore, the number of glia (C) did not change after SNI ($p>0.05$; 2-way ANOVA).
Chapter 5
Conclusions and Therapeutic Opportunities

There is ample evidence that suggests that manifestations of chronic pain require active participation of supraspinal sites. The heightened responses to mechanical or cold, but not to noxious thermal, stimulation, in rats with peripheral nerve injury or hindpaw inflammation is abolished by transection of the thoracic spinal cord (Porreca et al., 2002). Microinjection of a NMDA receptor antagonist before spinal nerve ligation (SNL) (Wei and Pertovaara, 1999) and microinjection of lidocaine in the rostral ventromedial medulla (RVM) after SNL (Pertovaara et al., 1996) have also been shown to reduce allodynia, which suggests that activation of (and activity in) the RVM contributes to cutaneous hypersensitivity. Consistent with these observations, selective disruption of the dorsolateral funiculus (DLF) ipsilateral, but not contralateral, to SNL abolishes tactile and thermal hypersensitivity (Ossipov et al., 2000; Porreca et al., 2002). However, neither DLF lesions nor microinjection of lidocaine into the RVM reduces cutaneous hypersensitivity within three days after SNL--they did so only 4-6 or more days after SNL (Burgess et al., 2002). These results are consistent with the hypothesis that the descending facilitation of spinal nociceptive input from the RVM is important for the maintenance (not initiation) of neuropathic pain.

Our data showing a significant decrease in RVM neurons, including serotonergic neurons, in the ipsilateral side of SNL-treated rats suggest that
death of supraspinal pain modulatory neurons contributes to the pathophysiology of neuropathic pain. The identity of the non-serotonergic neurons that are lost after SNL is not known but it has been proposed that the antinociceptive neurons of the RVM are physiologically characterized as OFF-cells (Fields et al., 1983a; 1983b; Heinricher et al., 1989; Fields et al., 1991). Many OFF-cells project to the dorsal horn (Fields et al., 1995) and express GABA (Winkler et al., 2006). Previous studies have suggested that changes in ON-cell and OFF-cell firing drive the hypersensitivity observed in SNL (Budai et al., 2007; Carlson et al., 2007; Gonçalves et al., 2007), but it has not yet been determined whether or not the number of OFF-cells changes. It is possible that both pro- and antinociceptive neurons are lost after SNL; however, the most parsimonious interpretation of our findings is that SNL selectively kills antinociceptive RVM neurons, thereby facilitating cutaneous hypersensitivity (for a summary see: Figure 5-1).

Our data also show that treatment with the apoptosis inhibitor, tauroursodeoxycholic acid (TUDCA), blocked RVM cell loss and significantly decreased tactile hypersensitivity four or more days after SNL but did not reduce hypersensitivity one-to-three days after SNL. Therefore, our findings suggest that cell loss underlies the RVM’s contribution to the maintenance phase of neuropathic pain (see above). Glial activation in the RVM may also contribute to cutaneous hypersensitivity (Wei et al., 2008); however, it is thought to be a factor in only the initiation of neuropathic pain (Mei et al., 2011). In our studies, we
found that the number of glia increased bilaterally after SNL. This increase was blocked by TUDCA, suggesting it may represent glial migration in response to RVM neuronal death.

**Clinical implications**

The abovementioned TUDCA findings are particularly exciting because they suggest that by preventing RVM neuronal loss we can reverse the symptoms of neuropathic pain. To date, there has been relatively little progress toward prevention of neuropathic pain in the clinic; furthermore, the majority of the patient population suffering from neuropathic pain does not benefit from currently available pain medications. If patients do find relief they often tolerate a plethora of difficult side effects. The pharmacological treatments with the best evidence for efficacy in the management of neuropathic pain are gabapentin, tricyclic antidepressants (TCAs), alpha-2-delta (\(\alpha_2\delta\)) ligands, serotonin and norepinephrine reuptake inhibitors (SNRIs), and opioids (Dworkin et al., 2003). However, their associated side effects can be quite severe and include dizziness, gastrointestinal and cardiovascular symptoms, sedation, nausea, and even cognitive impairment (Dworkin et al., 2003). On the other hand, TUDCA’s parent compound, UDCA, is approved in the United States for cholestatic liver disease and is well tolerated (Paumgartner and Beuers, 2004); therefore, few impediments would remain to development of TUDCA or UDCA as clinical therapies.
Another piece of evidence for TUDCA’s potential usefulness in treating neuropathic pain comes from our data that show that treatment with the bile acid is still effective if begun 2 days after nerve injury. This suggests that TUDCA administration will be useful not only to prevent development of post-surgical pain, but also to prevent development of neuropathic pain following traumatic injury.

We believe, based on our current TUDCA experiments, that TUDCA can reduce but not entirely block neuropathic pain symptoms by preventing RVM cell loss. TUDCA reportedly inhibits apoptosis and has been shown to have beneficial effects in in vivo and in vitro models of neurological damage that result in apoptosis, including hemorrhagic and ischemic stroke (Rodrigues et al., 2002; 2003). However, to date we have been unable to demonstrate caspase-3, caspase-6 or TUNEL in the RVM after SNL, and in a study of another model of neuropathic pain, chronic constriction injury, apoptosis was not observed in the RVM (Wei et al., 2008). Thus, since TUDCA was given systemically, we conclude that it may have acted indirectly (i.e. on spinal cord or dorsal root ganglia: see above) rather than directly on the RVM. Nevertheless, apoptotic events are brief (Kerr and Wyllie, 1972; Bursch et al., 1990; Sanchez et al., 1992), and therefore, it remains possible that TUDCA blocks RVM cell loss by acting directly on RVM neurons.
If we discover that chronic constriction injury (CCI) or spared nerve injury (SNI) do not cause RVM cell loss even after extending survival times (see Chapter 4), we may be able to further address the mechanism by which TUDCA works. If these animals display significantly higher withdrawal thresholds (i.e. decreased mechanical hypersensitivity) after TUDCA administration, we may hypothesize that TUDCA offers benefits by acting primarily at sites other than the RVM. While these experiments would again be useful for understanding the mechanism, the most intriguing component would be whether TUDCA has beneficial effects in other animal models of neuropathic pain, particularly in those that are highly clinically relevant (i.e. streptozotocin model of diabetic neuropathy). Diabetes is a growing challenge with an aging population and diabetic neuropathy is a serious clinical problem. If TUDCA does show promising results in other models, than I believe that there is a strong argument for its suitability for translation to human therapy.
Figure 5-1. Summary diagram. The loss of antinociceptive neurons underlies, in part, the hypersensitivity observed in neuropathic pain. Gray triangle indicates the borders of the RVM. We propose that after SNL, antinociceptive RVM neurons (filled circles) retrogradely degenerate after apoptosis of intrinsic spinal cells (squares) and primary afferent fibers. The resulting loss of descending inhibitory tone contributes to cutaneous hypersensitivity.


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