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A

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By

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Houston, Texas
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Abstract

TRPV1 Channels Contribute to Behavioral Hypersensitivity and Spontaneous Activity in Nociceptors After Spinal Cord Injury

Publication No._____
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A majority of persons who have sustained spinal cord injury (SCI) develop chronic pain. While most investigators have assumed that the critical mechanisms underlying neuropathic pain after SCI are restricted to the central nervous system (CNS), recent studies showed that contusive SCI results in a large increase in spontaneous activity in primary nociceptors, which is correlated significantly with mechanical allodynia and thermal hyperalgesia. Upregulation of ion channel transient receptor vanilloid 1 (TRPV1) has been observed in the dorsal horn of the spinal cord after SCI, and reduction of SCI-induced hyperalgesia by a TRPV1 antagonist has been claimed. However, the possibility that SCI enhances TRPV1 expression and function in nociceptors has not been tested. I produced contusive SCI at thoracic level T10 in adult, male rats and harvested lumbar (L4/L5) dorsal root ganglia (DRG) from sham-treated and SCI rats 3 days and 1 month after injury, as well as from age-matched naive control rats. Whole-cell patch clamp recordings were made from small (soma diameter <30 μm) DRG neurons 18 hours after dissociation. Capsaicin-induced currents were significantly increased 1 month, but not 3 days, after SCI compared to neurons from control animals. In addition, Ca^{2}+ transients imaged during capsaicin application were significantly

greater 1 month after SCI. Western blot experiments indicated that expression of TRPV1 protein in DRG is also increased 1 month after SCI. A major role for TRPV1 channels in pain-related behavior was indicated by the ability of a specific TRPV1 antagonist, AMG9810, to reverse SCI-induced hypersensitivity of hindlimb withdrawal responses to heat and mechanical stimuli. Similar reversal of behavioral hypersensitivity was induced by intrathecal delivery of oligodeoxynucleotides antisense to TRPV1, which knocked down TRPV1 protein and reduced capsaicin-evoked currents. TRPV1 knockdown also decreased the incidence of spontaneous activity in dissociated nociceptors after SCI. Limited activation of TRPV1 was found to induce prolonged repetitive firing without accommodation or desensitization, and this effect was enhanced by SCI. These data suggest that SCI enhances TRPV1 expression and function in primary nociceptors, increasing the excitability and spontaneous activity of these neurons, thus contributing to chronic pain after SCI.

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Abbreviations

AA, arachidonic acid

A-fiber, A group, rapidly conducting, myelinated, peripheral sensory axon

ASO, antisense oligodeoxynucleotide

C-fiber, C group, slowly conducting, unmyelinated, peripheral sensory axon

DAG, diacylglycerol

DMEM, Dulbecco's modified eagle medium

DRG, dorsal root ganglion

GABA, γ-aminobutyric acid

i.p., intraperitoneal;

MMO, mismatch oligodeoxynucleotide

NGF, nerve growth factor

ODN, oligodeoxynucleotide

PAR2, protease-activated receptor 2

PIP2, phosphatidylinositol 4,5-bisphosphate

PKA, protein kinase A

PKC, protein kinase C

SA, spontaneous activity

SCI, spinal cord injury

TRP, transient receptor potential (a large family of ion channels)

TRPV1, transient receptor potential vanilloid receptor 1

TRPV4, transient receptor potential vanilloid receptor 4

TTX, tetrodotoxin

CHAPTER 1: *Introduction*

1.1. Spinal cord injury (SCI) induces chronic pain

The spinal cord includes neurons and major bundles of axons that carry impulses to and from the brain and the rest of the body. Although the spinal cord is surrounded and protected by rings of bone (vertebrae), it can be damaged, most commonly by vehicle accidents, war injuries, sports-related accidents, and tumors. In the US, acute SCI affects 12,000 individuals every year, and approximately 1 million people in the US have sustained traumatic spinal cord injuries. Traumatic injury to the spinal cord is usually characterized pathologically by a central neuronal necrosis surrounded by a region of demyelinated white matter with a centrifugal distribution, resulting in sensory, motor, and autonomic dysfunction.

In addition to motor and low-threshold mechanosensory dysfunction, approximately two-thirds of patients who have sustained SCI experience slowly developing chronic pain after injury (Siddall and Loeser, 2001; Defrin et al., 2002). In most cases, SCI-induced chronic pain is termed ‘neuropathic’ since it usually results from nervous system injury. Neuropathic pain after SCI can be divided into three forms based on the location of pain: most commonly pain is at-level (including two segments above and below the lesion level) and below-level (at least three segments below the level of injury) relative to the injury site, but above-level pain is also reported by some patients (Siddall and Loeser, 2001). Patients report pain in response to normally innocuous stimuli (allodynia) and in

exaggerated form to noxious stimuli (hyperalgesia). This neuropathic pain can be devastating, and conventional treatment approaches are often inadequate (Eide, 1998). Hence, it is urgent to find out the cellular and molecular mechanisms underlying post-SCI pain.

1.2. Neurobiology of neuropathic pain

Noxious stimulation evokes a wide range of physiological changes in the organism, resulting in a series of motivational and affective activities designed to avoid injury. Specialized primary sensory neurons that detect noxious stimuli are termed ***nociceptors***, which usually have high thresholds and activate pathways within the central nervous system (CNS, spinal cord and brain) that produce acute nociceptive pain, helping to protect individuals from dangerous input. In addition to exciting pain pathways, noxious stimulation also activates ascending and descending modulatory systems that can increase or decrease the gain of pain pathways (Woolf and Salter, 2000).

Dorsal root ganglion (DRG) neurons and the associated primary afferent fibers in peripheral nerves comprise the first stage of the pain pathway, carrying information in multiple sensory modalities (chemical, thermal, mechanical) from the periphery to the spinal dorsal horn. DRG neurons differ in soma size, excitability characteristics, and central projections. Certain types of DRG neurons and their afferent fibers, primarily thinly myelinated Aδ- and unmyelinated C-fiber afferents, generally are considered to be nociceptors and are capable of initiating

nociceptive reflexes and pain sensation upon noxious stimulation (Harper and Lawson, 1985; Slugg et al., 2000; Dirajlal et al., 2003).

Nociceptive pain is usually transient. However, under various pathological conditions, such as peripheral inflammation and nerve injury, pain can be long lasting. An important contribution to pain in these cases is made by nociceptors, which can be sensitized to respond to innocuous stimuli and fire spontaneously, thereby contributing to the development and maintenance of persistent pain, including neuropathic pain (Baron, 2006; Djouhri et al., 2006; Hucho and Levine, 2007; Gold and Gebhart, 2010). Electrophysiologically, nociceptor sensitization is characterized by hyperexcitability (including spontaneous activity), and hypersensitivity (decreased threshold for activation by thermal, mechanical, and chemical stimuli, and increased response to supra-threshold stimuli). These abnormal electrophysiological activities may drive spontaneous pain, allodynia, and hyperalgesia.

Neuropathic pain is currently defined by the International Association for the study of Pain as "pain arising as direct consequence of a lesion or disease affecting the somatosensory system" (Treede et al., 2008). It has been reported that the same disease entity (e.g. peripheral nerve injury) can cause completely different pain symptoms in patients (Fields et al., 1998). Moreover, patients suffering from different neuropathic pain syndromes may present similar sensory signs (Baron et al., 2009; Scholz et al., 2009), indicating that multiple mechanisms

are probably involved in the generation of neuropathic pain (Price et al., 1989; Westermann et al., 2012). The mechanisms underlying neuropathic pain have been extensively studied in peripheral nerve injury models. Nerve fibers signaling nociceptive information that are axotomized by peripheral nerve injury (and sometimes by SCI) have compromised nociceptive function. Thus, the induction of chronic pain after neural injury suggests the occurrence of qualitative changes in some pain pathways. However, in pain pathways that have not been axotomized during bodily injury, nociceptive function often increases (Amaya et al., 2003; Gold and Gebhart, 2010).

Several mechanisms have been proposed for the initiation and maintenance of chronic pain induced by peripheral nerve injury. Enhanced neuronal activity occurring in primary afferents is widely assumed to be at the core of neuropathic pain (Gold and Gebhart, 2010; von Hehn et al., 2012). Specific environmental stimuli (including thermal, mechanical, and chemical stimuli) are encoded into membrane potential changes by activating ion channels expressed in sensory neuron terminals. It has been demonstrated that nerve injury-induced hyperexcitability of primary sensory neurons could result from upregulation of several ion channels enriched in nociceptors. For example, transient receptor potential vanilloid 1 (TRPV1) channels, which are expressed most abundantly in subtypes of small primary sensory neurons and are activated physiologically by various noxious stimuli, are upregulated in DRG and uninjured C fibers after a nerve injury, contributing to nerve injury-induced heat hyperalgesia and

mechanical allodynia (Hudson et al., 2001; Kanai et al., 2005; Kim et al., 2012). In addition, the expression of TRPV1 may be induced in large, low-threshold sensory neurons, which normally do not express TRPV1 (Hong and Wiley, 2005; Pabbidi et al., 2008). Nerve injury also induces upregulation of fast sodium channel Nav1.3, and dysregulation of the function or synthesis of slowly inactivating voltage-gated Na^+ channels (especially tetrodotoxin (TTX)-resistant channels) (Dib-Hajj et al., 1996; Dib-Hajj et al., 1998; Kral et al., 1999; Bongenhielm et al., 2000; Sleeper et al., 2000), as well as potassium channels (Everill and Kocsis, 1999; Yang et al., 2004). In addition, peripheral nerve injury induces pathological alterations within the central nervous system at spinal and supraspinal levels by: 1) sensitization of central neurons involving pathological cellular alterations (Latremoliere and Woolf, 2009); 2) microgliosis and astrogliosis in the spinal cord (Scholz and Woolf, 2007; Gao and Ji, 2010); 3) alteration of inhibitory and excitatory transmission of nociceptive signals (Coull et al., 2005; Aira et al., 2012); 4) anatomic reorganization in central neuronal networks (Lekan et al., 1996).

1.3. Multiple sites are altered after SCI, including DRG

Spinal cord injury also causes long-lasting alterations at multiple sites along the pain pathway (Hains et al., 2003; Hains et al., 2005; Hains and Waxman, 2006; Zhao et al., 2007; Carlton et al., 2009; Bedi et al., 2010). Most research into the mechanisms underlying SCI-induced chronic pain has focused on injury-induced plasticity occurring in both glial cells and dorsal horn neurons (see below). The GABAergic system is an essential component in spinal nociceptive processing.

The predominant inhibitory neurotransmitter, γ-aminobutyric acid (GABA), in the adult CNS generally hyperpolarizes the membrane potential through $GABA_A$ receptors, and thus decreases neuronal activity. However, SCI disrupts this pathway in two ways: 1) SCI induces an apparent loss of GABAergic interneurons in the superficial dorsal horn, resulting in reduced inhibitory GABAergic tone and chronic pain (Meisner et al., 2010); 2) GABA excites rather than inhibits superficial dorsal horn neurons in SCI animals because of a depolarized shift of the Cl^- equilibrium potential (Lu et al., 2008). Also, a descending antinociceptive serotonergic pathway is disrupted in animals displaying behaviorally expressed hypersensitivity (Bruce et al., 2002). Morphological evidence indicates that SCI enhances dendritic spine length and density (Tan and Waxman, 2012), suggesting enhancement of synaptic functions. Furthermore, elevated expression of voltage-gated sodium channel Nav1.3 has been implicated in SCI-induced hyperexcitability of dorsal horn nociceptive neurons as well as pain-related behaviors (Hains et al., 2003). In addition, SCI induces activation of microglial cells in the CNS (Hains and Waxman, 2006), which along with activation of astroglia spreads beyond the injury site in the spinal cord and thalamus (Zhao et al., 2007; Detloff et al., 2008; Carlton et al., 2009), resulting in greatly enhanced activity of dorsal horn neurons (Hains and Waxman, 2006) and ventral posterolateral neurons in the thalamus (Zhao et al., 2007). Intrathecal infusion of minocycline, a non-specific microglia inhibitor, prevents the SCI-induced hyperexcitability of lumbar dorsal horn neurons (Hains and Waxman, 2006) and relieves (Hains and

Waxman, 2006; Marchand et al., 2009) or preemptively reduces (Tan et al., 2009) reflexive signs of SCI pain in rodent models.

The possible contribution of primary sensory neurons to SCI-induced chronic pain has been neglected until recently. Importantly, all of the alterations described in the spinal cord and brain might be driven, at least partly, by enhanced activity in primary afferent neurons. Cytokines and inflammatory factors released from activated microglia and astrocytes in the spinal cord will reach not only lamina I and II neurons that are the postsynaptic targets of DRG neurons, but also presynaptic terminals of the DRG neurons in the dorsal horn. In addition, SCI can cause inflitration of macrophages and T-cells into widespread DRG (McKay and McLachlan, 2004). Thus, SCI-induced pathologic effects may not be limited to the CNS, and may directly influence primary sensory neurons. This possibility is supported by both morphological and physiological evidence. For example, cultured small and medium-sized neurons dissociated from lumbar and thoracic DRG show enhanced elongating growth in vitro 3 days after T10 contusion (Bedi et al., 2012). Sprouting of central terminals of primary sensory neurons (especially calcitonin gene-related peptide-positive nociceptors) in the spinal cord is observed in some SCI models (Helgren and Goldberger, 1993; Krenz and Weaver, 1998; Weaver et al., 2001; Ondarza et al., 2003; Zinck et al., 2007; Hou et al., 2009). Electrophysiological evidence indicates that the function of TTX-sensitive Na^+ channels is enhanced after SCI in dissociated sensory neurons innervating the bladder (Yoshimura and de Groat, 1997). Also, contusive SCI at T10 results in

increased spontaneous activity (SA) of nociceptors recorded from rat forelimb median nerves in an isolated skin-nerve preparation, and the recorded units show sensitized responses to mechanical and thermal stimulation of the skin (Carlton et al., 2009). Furthermore, recent work from our laboratory indicates that chronic SA intrinsic to the cell bodies of primary sensory neurons (both *in vivo* and *in vitro*) occurs in widespread DRG of rats with SCI. Most of the isolated DRG neurons displaying SA are small, capsaicin sensitive, and bind isolectin B4 (IB4), indicating that these neurons are nociceptors (Bedi et al., 2010). However, the molecular mechanisms of this persistent nociceptor SA and hyperexcitability following SCI are unknown.

1.4. Hyperexcitability of primary nociceptors appears to be an important mechanism underlying chronic pain after SCI

It has been demonstrated that the generation of SA in large diameter DRG neurons depends on spontaneous subthreshold oscillations of membrane potential (Devor, 2009). Only DRG neurons with spontaneous oscillations of 5 mV or more at normal resting membrane potentials can generate SA. In contrast, DRG neurons without subthreshold oscillations may also generate action potentials during depolarizing stimulation, but the spiking is not sustained during continued depolarization. Moreover, DRG neurons with subthreshold oscillations often have a depolarized resting potential as compared to the neurons without oscillations (Amir et al., 1999). Thus, the oscillations repeatedly draw the resting membrane potential toward threshold and cause DRG neurons to fire

spontaneously. It is not known if small DRG neurons also exhibit spontaneous subthreshold oscillations of membrane potential that drive spontaneous firing.

Under neuropathic conditions, the expression and kinetics of ion channels may be changed and background oscillations enhanced, increasing the proportion of DRG neurons with spontaneous subthreshold (and suprathreshold) oscillations of membrane potentials, elevating SA in these neurons. For example, peripheral nerve injury greatly increases the proportion of Aβ neurons that display subthreshold oscillations and SA (Study and Kral, 1996; Amir et al., 1999). As discussed above, contusive SCI at T10 caused increased SA in small diameter nociceptors recorded from the forelimb median nerve, lumbar dorsal roots, and dissociated small DRG neurons (Carlton et al., 2009; Bedi et al., 2010). We observed that SA in nociceptors was associated with membrane depolarization (about 5 mV) as compared to silent neurons (Bedi et al., 2010). Similar to what was observed in large DRG neurons in a peripheral nerve injury model (Study and Kral, 1996), small DRG neurons with SA after SCI exhibited more negative thresholds for triggering action potentials, and displayed more repetitive firing when depolarizing pulses were applied (Bedi et al., 2010). This suggests that any additional small inward currents (such as currents produced by opening of transient receptor potential [TRP] channels) should enhance subthreshold oscillations and increase the probability of triggering action potentials, thus enhancing SA and increasing the excitability of nociceptors.

The increased SA incidence of DRG neurons was significantly correlated with above-, at- and below-level behavioral hypersensitivity (Bedi et al., 2010). Both mechanical and thermal hypersensitivity for hindlimb responses was significantly correlated with SA incidence in neurons sampled from L4/L5 DRG. Furthermore, SA in the neurons dissociated from above-level (T8, T9, C6, and C7) DRG was correlated with behaviorally expressed forelimb hyperreflexia. Significant correlations between the enhanced vocalization and incidence of SA in sensory neurons dissociated from at- and above-level DRG was also observed during responses to at-level and above-level stimuli. The correlation with vocalization suggests that SA in DRG neurons might also enhance supraspinally mediated responses (including emotional and cognitive components of pain) after SCI. Strong evidence for the behavioral importance of SA in nociceptors has come from a recent, unpublished study (Yang et al., 2012). We found that eliminating SA in most nociceptors in the lumbar region can potently reduce SCI-induced hypersensitivity of hindlimb withdrawal responses. This was accomplished by intrathecal injection of oligodeoxynucleotides (ODN) antisense to Nav1.8 (Lai et al., 2002). Nav1.8 is a voltage-gated Na^+ channel that is widely expressed in primary sensory neurons, and preferentially (although not exclusively) expressed in nociceptors (Dib-Hajj et al., 1998; Shields et al., 2012). Furhermore, Nav1.8 is necessary for SA generated in neuromas after peripheral nerve transection (Roza et al., 2003). We found that injection of ODN antisense to Nav1.8 selectively knocked down Nav1.8 protein expression, reduced corresponding inward currents, and eliminated SA in dissociated nociceptors while reversing behavioral signs of

hindlimb hypersensitivity following SCI. Mismatched control ODNs had no effect (Yang et al., 2012). These exciting findings strongly suggest that **nociceptor SA and hyperexcitability make major contributions to chronic pain after SCI**.

1.5. TRPV1 in nociceptors is potentially involved in SCI chronic pain

SCI-induced chronic pain includes both spontaneous and evoked pain. Interestingly, SCI patients describe their spontaneous pain most commonly as "burning" (Cruz-Almeida et al., 2009). Moreover, both noxious heat and innocuous cold evoke enhanced pain behaviors in rodent models of SCI (von Heijne et al., 1999; Carlton et al., 2009; Bedi et al., 2010), as well as in SCI patients (Dykes et al., 2006; Felix and Widerstrom-Noga, 2009). This suggests that temperature-sensitive ion channels may contribute to SCI-induced chronic pain. Particularly important in this regard are the TRP channels, a family of cation-conducting channels characterized by six-transmembrane domains with cytoplasmic N- and C-termini (Tominaga, 2007). Several members of this family respond to moderate to noxious temperature changes, including TRPV1, transient receptor potential vanilloid-4 (TRPV4) and transient receptor potential ankyrin-1 (TRPA1) (Tominaga, 2007).

TRPV1 was the first mammalian TRP channel to be cloned (Caterina et al., 1997). It is a nonselective cation channel, highly permeable to Ca^{2+}, that is opened by capsaicin, noxious heat, protons, and numerous lipid mediators, including anandamide and lipoxygenase products (Tominaga, 2007). Although TRPV1

channels are expressed in other parts of the nervous system as well (Cui et al., 2006; Cavanaugh et al., 2011; Kim et al., 2012), their expression levels are by far the highest in primary polymodal nociceptors (Tominaga, 2007). TRPV1 appears to play an important role in controlling the excitability of nociceptors under some pathologic conditions. It has been demonstrated that a proportion of primary afferents from C57BL6 mice exhibits peripherally generated SA, and the incidence of SA is inversely correlated with threshold for activation by heat (Banik and Brennan, 2009). Primary afferents are sensitized and exhibit elevated SA following plantar incision injury, which can be dramatically attenuated by genetic deletion of TRPV1 (Banik and Brennan, 2009). Also, we found that, after SCI, 80% of dissociated small DRG neurons exhibiting SA after dissociation were responsive to capsaicin (Bedi et al., 2010). The importance of TRPV1 in various forms of chronic pain has been strongly suggested by several lines of evidence: 1) while acute withdrawal responses to heat stimulation were still present in TRPV1-/- mice, these mice failed to develop thermal hyperalgesia upon inflammatory treatment (Davis et al., 2000); 2) systemic administration of TRPV1 antagonists reversed signs of heat hyperalgesia in an inflammatory pain model by actions primarily on peripheral fibers of nociceptors (Cui et al., 2006); and 3) TRPV1 channels were upregulated in DRG neurons from rats showing heat hyperalgesia after peripheral inflammation or nerve injury (Hudson et al., 2001; Fukuoka et al., 2002; Ji et al., 2002; Breese et al., 2005; Kanai et al., 2005).

1.6. Several factors modulating TRPV1 channels are elevated after spinal cord injury

The endogenous activators of TRPV1 are uncertain. However, both native and cloned TRPV1 channels have been shown to be activated by endogenous cannabinoid receptor agonist, anandamide, and metabolites of arachidonic acid (AA) (Zygmunt et al., 1999; Hwang et al., 2000; Watanabe et al., 2003). The primary sequences of TRPV1 predict many putative phosphorylation sites for a variety of protein kinases, including protein kinase C (PKC) (Numazaki et al., 2002; Peng et al.), which is activated by a lipid product diacylglycerol (DAG). TRPV1 channel activity can be enhanced by PKC (Cesare et al., 1999; Numazaki et al., 2002; Mandadi et al., 2006; Cao et al., 2009). Moreover, both PKC and DAG have been shown to activate TRPV1 directly (Premkumar and Ahern, 2000). As TRPV1 is generally inhibited by phosphatidylinositol 4,5-bisphosphate (PIP2), reduction of PIP2 during its conversion to DAG by activated phospholipase C may also promote TRPV1 channel activity (Amadesi et al., 2004; Dai et al., 2004; Dai et al., 2007).

Such lipid species are signaling molecules playing important roles in multiple physiological responses. Accumulation of these lipid products in pathological conditions, however, results in extensive stimulation/modulation of their targets that can change the excitability of nociceptors and induce pain. It has been demonstrated that injection of complete Freund's adjuvant induces DAG accumulation in the spinal cord (Fuchs et al., 2004). Also, activated PKC increases

in the spinal cord dorsal horn following nerve injury (Mao et al., 1992), and PKC manipulation modifies thermal hyperalgesia and mechanical allodynia (Coderre, 1992; Hua et al., 1999). Inflammatory proteases cleave and activate protease-activated receptor 2 (PAR2) in a subset of primary afferent neurons. TRPV1 can be activated downstream of PAR2 via the PLC/PIP2/PKC signaling pathway and cause inflammatory pain (Amadesi et al., 2004; Dai et al., 2004; Dai et al., 2007; Grant et al., 2007). Also, it has been demonstrated that SCI induces excessive accumulation of AA in the spinal cord in a phospholipase A2-dependent manner (Liu and Xu, 2010). Lipid signals generated in the spinal cord might activate TRPV1 channels in the central processes of DRG neurons, as well as TRPV1 channels in dorsal horn neurons (see Chapter 7). Such lipid products can be produced in response to nerve growth factor (NGF), cytokines and chemokines, which are elevated in the spinal cord and/or DRG after SCI (Bakhit et al., 1991; Bartholdi and Schwab, 1997; Murakami et al., 2002; Brown et al., 2004).

TRPV1 channel currents induced by endogenous lipid products may be too small to induce spontaneous activity in DRG neurons (Shin et al., 2002) under physiological conditions. However, as discussed above, nociceptors with SA are relatively depolarized and exhibit reduced thresholds for triggering action potentials (Study and Kral, 1996; Bedi et al., 2010). Under these conditions, any additional small inward currents may convert the subthreshold oscillations to action potentials, thus significantly enhancing SA and hyperexcitability of nociceptors. In contrast, larger inward currents may lead to membrane

depolarization sufficient to inactivate sodium channels, reducing electrical activity of nociceptors (Wu and Pan, 2007).

1.7. TRPV1 antagonists may block SCI chronic pain

As discussed above, TRPV1 channel upregulation, resulting from increased synthesis, and sensitization by lipid products is likely to contribute to intrinsic SA/hyperexcitability of nociceptors and subsequent pain behaviors. TRPV1 channels are Ca^{2+} permeable (Tominaga, 2007). As a second messenger, Ca^{2+} plays an essential role in controlling many neuronal processes, including neurotransmitter release, excitability, gene expression, development, repair, and apoptosis. Thus, activity of TRPV1 channels can result in at least four consequences: 1) direct depolarization of DRG neurons to elicit SA/hyperexcitability; 2) direct and/or indirect increase in intracellular Ca^{2+} concentration, which will enhance neurotransmitter release and amplify peripheral input to pain projection neurons in the dorsal horn; 3) synergism with other pain-related signals, such as those promoting translocation of PKC to the plasma membrane (Oancea and Meyer, 1998); and 4) enhancement of pain-related protein expression. Thus, enhanced activity of TRPV1 channels may contribute to both the development and maintenance of post-SCI pain.

In addition to their extensive expression in primary sensory neurons TRPV1 channels have also been reported in spinal dorsal horn neurons where they are thought to contribute to the maintenance of pain (Zhou et al., 2009; Wei et al.,

2010; Kim et al., 2012), although the occurrence and significance of TRPV1 expression in the spinal cord is controversial (Caterina et al., 1997; Cui et al., 2006; Cavanaugh et al., 2011; Kim et al., 2012) (see Chapter 7). In this regard, systemic application of A-784168 (Abbott Laboratories, Abbott park, IL), a TRPV1 antagonist with good blood-brain barrier penetration, demonstrates a more potent reduction of mechanical allodynia than A-795614 (Abbott laboratories), a peripherally restricted TRPV1 antagonist, although both antagonists show similar potency on thermal hyperalgesia in a peripheral inflammatory pain model (Cui et al., 2006). This difference could be explained by effects of the antagonist on central synaptic terminals of primary nociceptors as well as effects on dorsal horn neurons. Increased expression of TRPV1 has been reported in the spinal cord following SCI using both immunohistochemistry and RT-PCR methods (Zhou et al., 2002; DomBourian et al., 2006). Thus, targeting TRPV1 channels expressed both peripherally and centrally may optimize analgesic effects.

Anti-hyperalgesia effects induced by antagonists of TRPV1 have been observed in various rodent pain models (Gavva et al., 2005; Cui et al., 2006; Yu et al., 2008). For example, AMG9810 (i.p. and intrathecal), a specific TRPV1 antagonist, significantly relieves behaviorally expressed mechanical and heat hypersensitivity in an inflammatory pain model (Gavva et al., 2005; Yu et al., 2008). These findings indicate that the TRPV1 channel is involved not only in the maintenance of thermal hyperalgesia as discussed above, but also mechanical allodynia. SCI produces both mechanical allodynia and thermal hyperalgesia.

Thus, targeting TRPV1 may reduce nocifensive behaviors and pain following SCI. Indeed, the TRPV1 antagonist AMG9810 has been reported to reduce thermal hyperalgesia following SCI (Rajpal et al., 2007). Thus, TRPV1 channels could be attractive targets for treating post-SCI pain.

1.8 Hypotheses

The observations reviewed above lead to my general hypothesis: **chronic pain induced by SCI is maintained by SA in nociceptors, and this nociceptor SA is promoted by increased TRPV1 function.** This general hypothesis contains two specific hypotheses that were tested in this dissertation:

Hypothesis 1. SCI increases the expression and function of TRPV1 in primary nociceptors.

Hypothesis 2. Enhanced TRPV1 function contributes to intrinsic SA and hyperexcitability of primary nociceptors.

Hypothesis 2. Behaviorally expressed hypersensitivity to heat and mechanical stimuli following SCI can be reduced by attenuating TRPV1 activity.

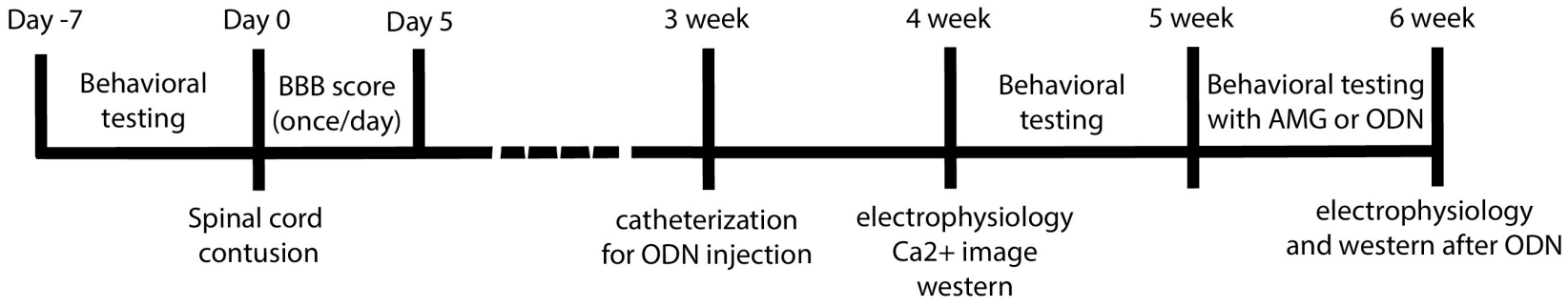

Figure 1. Timeline of experiment design. Behavioral tests were only performed on rats that received AMG or ODN treatments. ODN, oligodeoxynucleotide; AMG, AMG9810; BBB score, Basso, Beattie, Bresnahan locomotor rating scale.

CHAPTER 2: *Material & Methods*

2.1. Animals

A total of 112 rats (62 SCI, 22 sham-treated, 28 naïve) were used in these studies. All procedures conformed to the guidelines of the International Association for the study of Pain, and were approved by the Animal Care and Use Committee of the University of Texas Medical School at Houston. Male, adult, Sprague-Dawley rats (200-300g) were used in this study. Animals were housed 2 per cage in a controlled environment (12 hour reversed light/dark cycle, 21 ± 1°C) with standard food and water. The rats were allowed to adjust to their environment for a week before tests began (**Fig.1**).

2.2. Spinal cord injury

Rats were anesthetized with ketamine (80 mg/kg), xylazine (20 mg/kg) and acepromazine (0.75 mg/kg). Animals received a laminectomy at T10 (**Fig. 2**) followed by a spinal contusion using the Infinite Horizons impactor (150 kdyne, 1 s dwell time) using procedures based on described methods (Dulin et al., 2013). Sham-treated animals received the laminectomy and identical treatment except for the spinal impact. The surgical site was flushed with 1 ml of normal saline. The overlying muscles were replaced over the spine and the skin incision was then stapled with wound clips. Cages containing rats were then placed on a heating pad to maintain body temperature at ~37 °C for 24 hours. Food and water

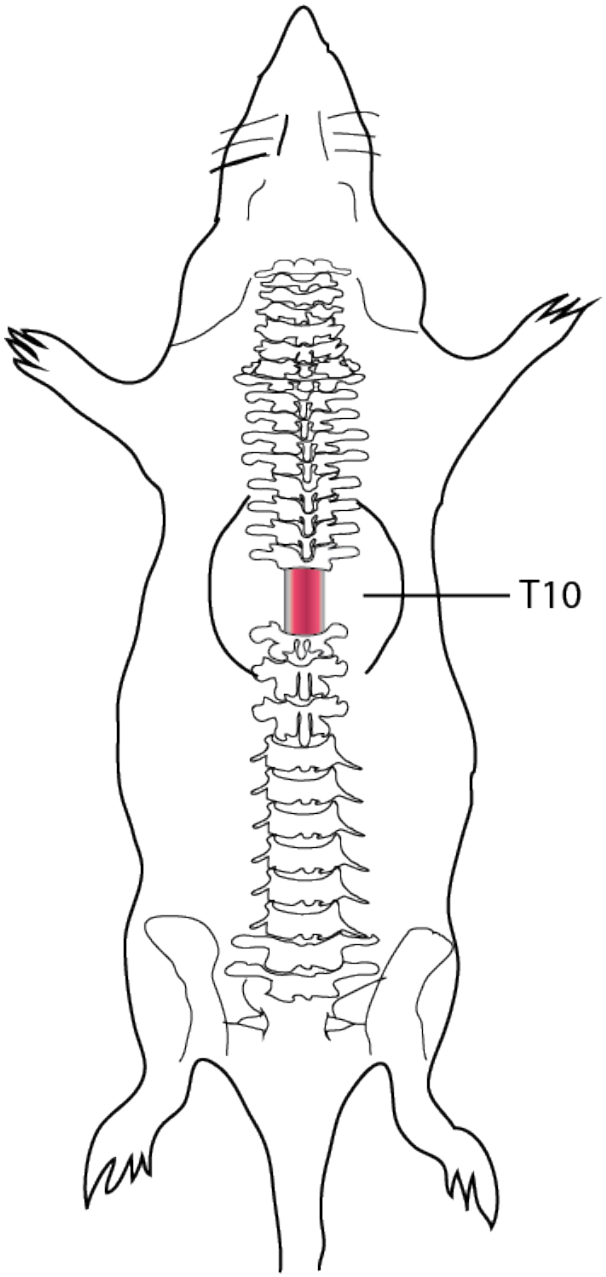

Figure 2. Schematic diagram illustrating the contusion site at T10.

were placed within easy reach to permit eating and drinking easily without assistance. Animals then received twice daily injection (i.p.) of lactated Ringers solution (2 ml) and analgesic (buprenorphine; 0.02 mg/kg, i.p.) for 5 days post-injury and prophylactic antibiotics (Baytril, 2.5 mg/kg) for 10 days. Manual bladder evacuations were performed twice daily until neurogenic bladder voiding returned. Possible signs of spontaneous pain, including marked inactivity, excessive grooming, and autotomy were noted. Autotomy is rare in the spinal contusion model, and the very few animals exhibiting severe autotomy (n = 3) were euthanized immediately.

2.3. Dissociation and culture of DRG neurons

After rats were intracardially perfused with cold PBS under deep anesthesia (Beuthanasia, 75 mg/kg, i.p.), the thoracic and lumbar segments of the vertebral column were removed. A laminectomy was performed to expose lumbar DRG, and L4 and L5 DRG were then removed. The excised ganglion was minced, and the fragments transferred into Dulbecco's modified eagle medium (DMEM, Invitrogen, Grand Island, NY) containing collagenase (0.6 mg/ml, Roche, Mannheim, Germany) and trypsin (0.4 mg/ml, Worthington, Lakewood, NJ), and incubated 40 min at 34^{o}C. The cells were spun down and transferred to 35 mm Petri dishes containing poly-L-lysine (50 mg/ml)-precoated cover glass (8 mm, Warner Instruments, Hamden, CT) and incubated with DMEM (without serum) at 37^{o}C in 5% CO_2 overnight. After 18-24 h incubation, living cells were digitally imaged and recorded.

2.4. *In vitro* recording of DRG neurons

Electrodes with a resistance of ~2 MΩ were pulled from BF150-86-10 glass capillaries (inner diameter, 0.86 mm; outer diameter, 1.5 mm; Sutter Instrument Co, Novato, CA) using a micropipette puller (P-2000, Sutter Instrument Co, Novato, CA). Neurons were visualized using differential interference contrast (20 x) optics on an inverted microscope (Axiovert 200M, Zeiss Oberkochen, Germany). Images of cells were taken with a CCD camera. Neurons (soma diameter < 30 μm) were recorded in the whole-cell configuration using an EPC-10 amplifier (HEKA Instruments, Lambrecht, Germany). After forming a tight seal (> 1 GΩ) the membrane was ruptured. After the whole-cell configuration was established, the cell membrane capacitance and series resistance were electronically compensated. In current-clamp mode resting membrane potential (RMP) and any SA were recorded. Under whole-cell voltage-clamp capsaicin responses were recorded. All experiments were performed at room temperature (~23 °C). Signals were digitized at 2-20 kHz (depending on the duration of recording), and acquired using the Pulse software program (HEKA). The pipette solution contained (in mM) 134 KCl, 1.6 $MgCl_2$, 13.2 NaCl, 3 EGTA, 9 HEPES, 1 Mg-ATP, and 0.3 Na-GTP (pH 7.2 adjusted with KOH, osmolarity 300 mOsM). The bath solution contained (in mM) 140 NaCl, 3 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 Glucose (pH 7.4 adjusted with NaOH, osmolarity 320 mOsM). For perforated patch recordings, the pipette was initially filled with regular pipette solution by brief immersion, and the remainder of the pipette was back-filled with

pipette solution containing gramicidin. A 50 mg/ml gramicidin solution was prepared in DMSO, and then diluted to a final concentration of 100 μg/ml in the pipette.

2.5. Calcium imaging

Intracellular free Ca^{2+} concentration was measured using Fura-2 fluorescent indicator as described previously (Wu et al., 2007). Dissociated DRG neurons were incubated in 5 μM Fura 2-AM (Calbiochem, Darmstadt, Germany) in extracellular solution for 20 min and then washed 4 times in the dark at room temperature. Fluorescence images were acquired with a Hamamatsu C2400 iCCD. Quantitative measurements of $[Ca^{2+}]_i$ in DRG neuron somata were made with an InCyt Im2 Fluorescence Imaging System (Intracellular Imaging) equipped with a PixelFly CCD camera (Cooke). R, the ratio of the fluorescence intensity (511 nm), was obtained upon excitation at 340 nm and 380 nm for each cell of interest.

2.6. Drug application:

Drugs were dissolved in distilled water at 1,000 times their final concentration and kept frozen in aliquots except for capsaicin, AMG9810 (Amgen, Thousand Oaks, CA), and Fura 2-AM, which were prepared as stock solutions dissolved in ethanol and DMSO, respectively. The stock solutions were diluted in extracellular or intracellular solution just before use and held in a series of independent syringes connected to an array of corresponding fused silica columns (inner

diameter, 200 μm). The exchange of solutions was achieved rapidly by shifting the tubes horizontally with a micromanipulator. The distance from the column mouth to the cell examined was about 100 μm. Cells in the recording chamber were continuously bathed in the extracelluar solution. Each drug solution was delivered to the recording chamber by gravity. In vivo delivery of AMG9810 was achieved by a single i.p. injection (30 mg/kg, 2 ml). Drugs and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except Fura 2-AM (Calbiochem).

2.7. Antisense oligodeoxynucleotide (ODN) delivery

The sequence of antisense oligodeoxynucleotide (ASO) targeting TRPV1 was previously reported (Christoph et al., 2007), which was 5'-CATGTCATGACGGTTAGG-3'. The sequence for mismatch oligodeoxynucleotide (MMO) was 5'-CATGCTATGAGCGTTGAG-3'. This ASO induces nearly complete degradation of TRPV1 mRNA, and can be taken up well in vivo by DRG neurons after intrathecal delivery (Christoph et al., 2007). Oligonucleotides (ODN) were purchased from Sigma-Aldrich. 1 month after SCI, rats were anesthetized using an isoflurane/oxygen mixture. A chronically indwelling intrathecal catheter (PE-10, Becton Dickinson, Sparks, MD) was implanted through a dural slit over the atlantooccipital joint (Yaksh and Rudy, 1976). The catheter terminated at the lumbar enlargement (**Fig. 3**). After catheterization, rats were housed individually and allowed to recover for at least 5 days before the injection. ODN were injected during brief isoflurane sedation twice daily for 3 days, and then once per day for 2 days, with 45 μg ODN in 5 μl saline injected each time, followed by a 10 μl saline

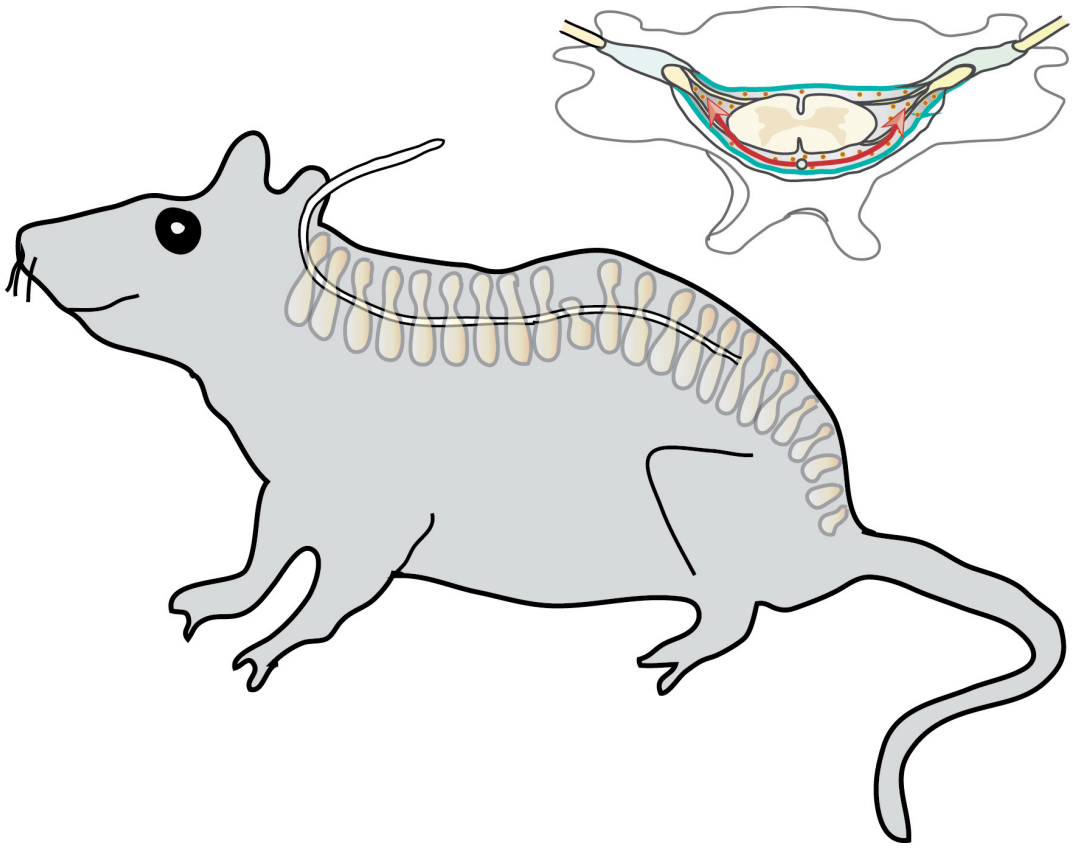

Figure 3. An intrathecal catheter was surgically implanted in contusive rats to permit intrathecal ODN administration. The PE-10 14-cm long catheter was inserted into the subarachnoid space and passed along the posterior aspect of the spinal cord to reach the lumbar area for ODN delivery.

flush. Tip location and patency were verified post-mortem.

2.8. Western blotting

1 month after surgery or 5 days after the knockdown procedure, animals were anesthetized with Beuthanasia and perfused with ice-cold PBS. Four DRG (bilateral L4, L5) from each rat were removed and immediately placed on dry ice. DRG were homogenized in 300 µl lysis buffer (RIPA, Teknova) containing protease inhibitor cocktail (Sigma). After homogenization, samples were sonicated 3 times (10 s pulse), and centrifuged at 14,000 rpm for 10 min at 4^{o}C. Protein concentration of lysates was determined by the BCA method (Pierce BCA Protein Assay Kit). Samples were prepared for SDS-PAGE (Bio-Rad, 4-20% Tris-HCl) by 1:1 dilution with Laemmli buffer and 30 µg proteins were loaded in each well. After electrophoresis, the gel was transferred to a PVDF membrane and blocked with 10% nonfat dry milk prior to incubation with antibody against TRPV1 overnight at 4^{o}C. The membrane was incubated with anti-rabbit or anti-mouse IgG for 1h at room temperature, developed using the ECL kit (Pierce), and scanned. Protein expression was quantified by optical density using Image J software (NIH). Color molecular weight standards were run on each gel. β-actin was used as a loading control.

2.9. Behavioral tests

To monitor effects of SCI on hindlimb motor function, animals were placed in an open field (child's pool) and their spontaneous behavior videotaped in white

light and scored on the 22-point Basso, Beattie, Bresnahan (BBB) locomotor rating scale (Basso et al., 1996). Animals accepted for this study exhibited BBB scores in the range of 0-1 one day after SCI. Behavioral sensitivity data were collected during the animals' active phase in red light and analyzed using blind procedures. Animals received a standard 5 day sequence of tests for behaviorally expressed hyperreflexia prior to impact and then before and after AMG9810 or ODN delivery, as we described previously (Bedi et al., 2010). Prior to each test, the animals were habituated for 20 min in each of the testing chambers on day1, and received the mechanical and thermal test stimuli on days 2 and 3 to habituate to the test procedures. Data were collected during the same tests on days 4 and 5. Below-level signs of heat hypersensitivity were tested with the Hargreaves radiant heat method using an IITC Plantar Analgesia Meter (Woodland Hills, CA) to measure the latency to hindpaw withdrawal. The heat stimulus ended after 20 s stimulus if no response occurred to prevent possible tissue injury. Both left rear and right rear hindpaws were tested at 5 min intervals. This test sequence was repeated 2 times, at 20 min intervals. Below-level signs of mechanical hypersensitivity (decreased threshold) were tested with a single series of calibrated von Frey filaments (Stoelting, Wood Dale, IL) delivered to the glabrous surface of the hindpaws (Hulsebosch et al., 2000) in a sequence determined by the "up-down" method (Chaplan et al., 1994). Only one test series was applied to each hindpaw.

2.10. Data analysis

Analyses were performed with Sigmaplot 11 (Systat software, San Jose, CA) and Prism 5.0 (Graphpad, La Jolla, CA). Statistical data are presented as means ± S.E.M. All comparisons among different group of animals were tested for significance using the Student's unpaired t-test or one-way ANOVA with repeated measures followed by Bonferroni's *post-hoc* tests. Paired t-tests were utilized to analyze treatment effects in within-animal comparisons. SA incidence among different group of animals was compared using Fisher's exact tests. For all statistical analyses, $P < 0.05$ was considered to be statistically significant. Statistically significant differences were indicated in each figure (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). The n in all experiments indicates the numbers of DRG neurons (electrophysiology) or rats (western blot or behavior) tested in each condition.

CHAPTER 3: *SCI enhances TRPV1 channel function in nociceptors*

3.1. Introduction

SCI patients describe their spontaneous pain most commonly as “burning” (Cruz-Almeida et al., 2009). Moreover, noxious heat evokes enhanced pain behaviors in rodent models of SCI (von Heijne et al., 1999; Carlton et al., 2009; Bedi et al., 2010), as well as SCI patients (Dykes et al., 2006; Felix and Widerstrom-Noga, 2009). This suggests that temperature-sensitive ion channels may contribute to SCI-induced chronic pain. Particularly interesting in this regard are TRPV1 channels. TRPV1 is a nonselective cation channel that is highly permeable to Ca^{2+} and is opened by capsaicin, noxious heat, protons, and lipid mediators, including anandamide and lipoxygenase products (Tominaga, 2007). TRPV1 channel expression levels are by far the highest in primary polymodal nociceptors (Caterina et al., 1997; Tominaga et al., 1998; Caterina et al., 2000; Lauria et al., 2006; Hoffman et al., 2010). After SCI, various factors, including cytokines, chemokines, and NGF are elevated in the spinal cord (Bakhit et al., 1991; Bartholdi and Schwab, 1997; Murakami et al., 2002; Brown et al., 2004). In addition to sensitizing spinal dorsal horn neurons and promoting the activation of glial cells (Miller et al., 2009), these factors communicate also with central terminals of primary sensory neurons, thus potentially regulating the function of various ion channels in DRG neurons, including TRPV1. It has been demonstrated that CC chemokine ligand 2 (CCL2) increases the current density of TRPV1

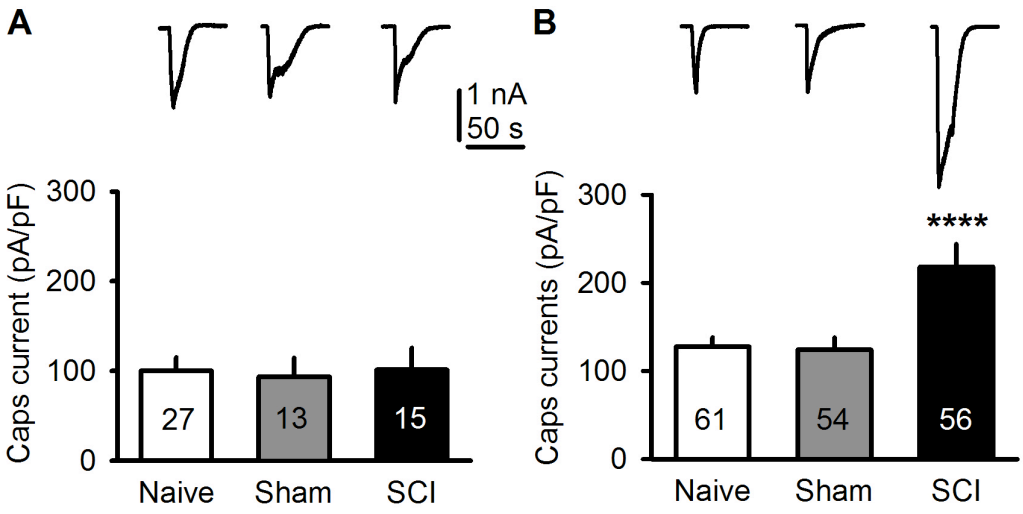

Figure 4. Comparison of capsaicin-sensitive currents among adult DRG neurons dissociated from naïve, sham and SCI rats. A, original current traces and summary data show that SCI had no obvious effect on 3 μM capsaicin-induced currents as compared to sham treatment 3 days after surgery. B, original recordings and summary data show that capsaicin-induced currents in DRG neurons were increased 1 month after SCI, as compared to age-matched naïve and sham rats.

channels in DRG neurons (Kao et al., 2012). Also, TRPV1 channels are upregulated in DRG neurons from rats showing thermal hyperalgesia after peripheral inflammation or nerve injury (Hudson et al., 2001; Fukuoka et al., 2002; Ji et al., 2002; Breese et al., 2005; Kanai et al., 2005). SCI is accompanied by persistent inflammatory effects within the spinal cord (Alexander and Popovich, 2009; Dulin et al., 2013) and within DRG (McKay and McLachlan, 2004; Carlton et al., 2009). Little is known about potential TRPV1 alterations after SCI (DomBourian et al., 2006; Rajpal et al., 2007), but immunohistochemical evidence suggests that SCI increases TRPV1 expression in DRG (Ramer et al., 2012). I thus hypothesize that TRPV1 channel function is enhanced after SCI.

3.2. Results

3.2.1. SCI enhances currents evoked by capsaicin 1 month but not 3 days after SCI

Because maximal activity of TRPV1 in DRG neurons dissociated from rats can be achieved by 3 μM capsaicin (McIntyre et al., 2001; McLatchie and Bevan, 2001), I used 3 μM capsaicin to determine the difference in TRPV1 channel function among nociceptors dissociated from naïve, sham and SCI animals. The diameters for recorded neurons dissociated from naïve, sham and SCI rats were 23.21 ± 0.33 (n = 88), 21.65 ± 0.51 (n = 67), 22.34 ± 0.43 μm (n = 71), respectively. Cells were held at -60 mV under voltage clamp. A 5 s, gravity-fed stream of capsaicin (3 μM) was applied ~100 μm from the soma through a fused silica column (i.d. 200 μm), and inward currents recorded. A resulting inward

current > 40 pA was considered a capsaicin response (Dirajlal et al., 2003). Capsaicin-induced currents were significantly increased 1 month, but not 3 days, after SCI compared to neurons from sham and naïve animals. At 3 days post-injury in the SCI, sham and naïve groups, the capsaicin-induced current densities were 101.5 ± 24.3 (n = 15), 93.7 ± 20.9 (n = 13), and 100.4 ± 15.0 (n = 27) pA/pF, respectively ($F_{2,52}$ = 0.037; one-way ANOVA, **Fig. 4A**). In contrast, in DRG neurons dissociated from animals 1-2 months after SCI the capsaicin-induced current density was significantly higher (218.4 ± 25.6 pA/pF, n = 56) than that recorded from age-matched sham (124.1 ± 13.9 pA/pF, n = 54) and naïve (127.7 ± 10.4 pA/pF, n = 61) groups ($F_{2,168}$ = 9.091; P < 0.0001, one-way ANOVA, **Fig. 4B**).

To determine whether SCI alters the distribution of TRPV1 in DRG neurons, I compared the proportions of dissociated neurons responding with small but reliably detectable inward currents (> 40 pA) among these groups. No differences were found among these groups in the percentage of capsaicin sensitive neurons (~80%) (**Fig. 5A and B**). To further investigate the differences of capsaicin-induced responses in DRG neurons among 1 month SCI, sham, and age-matched naïve groups, DRG neurons were grouped according to soma diameters. A histogram was plotted to show the capsaicin currents in different diameters among these three groups of cells. The largest number of small cells had soma diameters of 20-25 μm (**Fig. 5C**). Most importantly, capsaicin-induced currents were higher in DRG neurons from the 1 month SCI group compared to DRG neurons dissociated from sham or naïve animals in each diameter range (**Fig. 5C**). The occurrence of

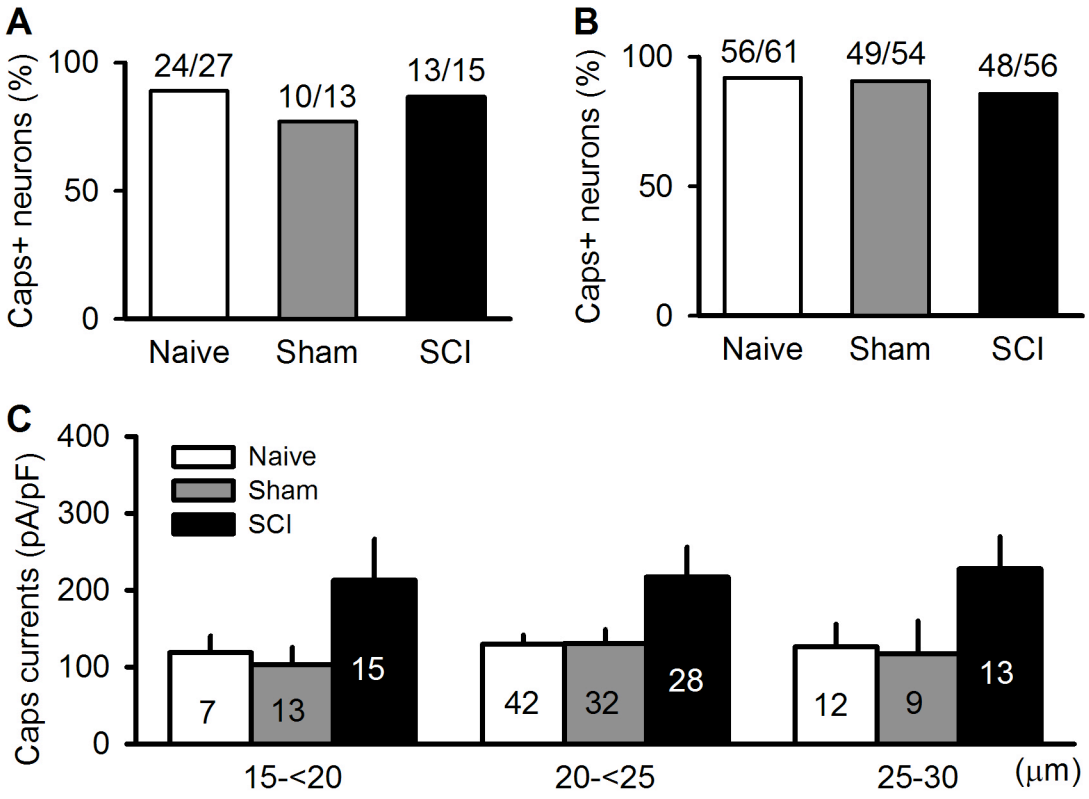

Figure 5. Capsaicin-sensitive currents are not redistributed after SCI. A, summary data indicates no differences among 3 day SCI, sham and naïve groups in the percentage of small DRG neurons that were sensitive to capsaicin. B, summary data indicates that percentage of small DRG neurons that were sensitive to capsaicin were not changed following 1 month SCI. C, Increased responses after SCI occur similarly in very small neurons (probably exclusively C-fiber neurons) and cells that are almost medium-sized (probably containing Aδ neurons).

larger capsaicin-induced currents in neurons with diameters in the 26-30 μm range suggests that this effect occurs in some Aδ-fiber neurons as well as C-fiber neurons (Harper and Lawson, 1985). These results show that currents evoked by activation of TRPV1 channels are enhanced by SCI long after the injury.

3.2.2. SCI enhances Ca^{2+} responses evoked by capsaicin 1 month but not 3 days after SCI

The TRPV1 channel is highly permeable to Ca^{2+} (Caterina et al., 1997), thus, alterations in TRPV1 channel function can be reflected by alterations in capsaicin-induced intracellular Ca^{2+} responses. By measuring changes in the Fura-2 emission ratio, intracellular calcium concentrations can be monitored. Similar to what was observed in the electrophysiological recordings, DRG neurons dissociated from animals 3 days after SCI showed little alteration in intracellular Ca^{2+} responses to capsaicin delivery (0.31 ± 0.04, n = 13) as compared to responses in neurons from sham (0.34 ± 0.04, n = 14) and naïve (0.34 ± 0.05, n = 23) animals ($F_{2,37}$ = 0.8824; **Fig. 6A**). However, the change in intracellular free calcium concentration evoked by capsaicin (3 μM) was significantly higher in DRG neurons dissociated from 1 month SCI (0.69 ± 0.12, n = 19) versus sham (0.41 ± 0.06, n = 17) and naïve (0.33 ± 0.05, n = 25) animals ($F_{2,51}$ = 5.606; *P* = 0.0063, one-way ANOVA, **Fig. 6B**). These results show that Ca^{2+} entry through TRPV1 channels is enhanced by SCI long after the injury.

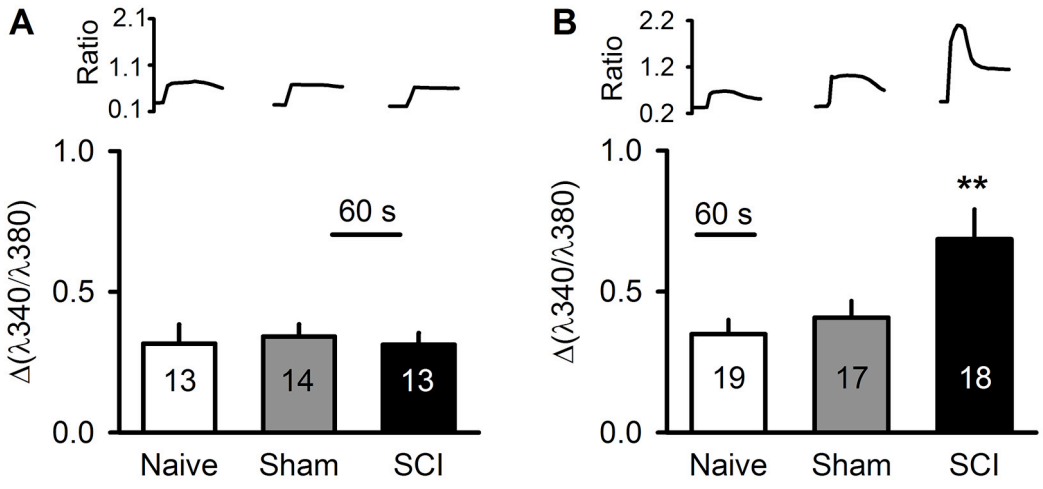

Figure 6. Comparison of capsaicin-induced intracellular [Ca2+] change among adult DRG neurons dissociated from naïve, sham and SCI rats using Fura-2 ratiometric calcium dye. A, representative traces and summary data show similar response in DRG neurons isolated from 3 day SCI, age-matched naïve and sham rats, following capsaicin (3 μM) administration. B, representative traces and summary data shown 3 μM capsaicin-induced response was significantly enhanced 1 month after SCI.

3.3. Conclusions

3.3.1. TRPV1 channel function is enhanced 1 month following SCI

These studies show that capsaicin-induced responses are enhanced in small and medium-sized DRG neurons 1 month, but not 3 days, after SCI. Capsaicin is a selective agonist of TRPV1 channels, and induces a maximal response at 3 μM in rat DRG neurons (McIntyre et al., 2001; McLatchie and Bevan, 2001). In my studies, TRPV1 channel function was measured by both whole-cell voltage-clamp and Ca^{2+} imaging methods. When TRPV1 channels are opened upon capsaicin exposure, the cellular response is largely determined by four factors: 1) the number of TRPV1 channels, 2) the activity of individual TRPV1 channels, 3) membrane potential, and 4) the Ca^{2+} and Na^{+} gradients across the plasma membrane. In my voltage-clamp recordings, DRG neurons were hold at -60 mV, and the recording solutions were invariant during each experiment and the same for all groups. Thus, changes in these currents evoked by maximal doses of capsaicin could be caused by changes in the maximal number of available TRPV1 channels and the responsiveness of each channel in each neuron tested. However, SCI also causes chronic depolarization of small DRG neurons that is present after dissociation (Bedi et al., 2010). Thus, an interesting question is how the capsaicin responses may change after SCI in dissociated neurons that are not all clamped at the same holding potential. Thus, I also performed Ca^{2+} imaging experiments to examine TRPV1 channel function at the native resting membrane potentials 1 day after dissociation and found that both patch-clamp and Ca^{2+}

imaging experiments yielded similar results: TRPV1 channel function is enhanced in small DRG neurons 1 month SCI.

3.3.2. The distribution of TRPV1 in small and medium-sized DRG neurons is unchanged 1 month after SCI

I focused on relatively small DRG neurons with soma diameters ≤30 μm. The diameters of sampled neurons were not significantly different among naïve, sham, and SCI groups. Alteration of TRPV1 distribution across different sizes of DRG neurons has been reported in several chronic pain models. The proportion of TRPV1-positive cells among small and medium-sized DRG neuron subpopulations was increased in a peripheral inflammatory model, but decreased after axotomizing nerve injury (Amaya et al., 2003; Ma et al., 2005; Shinoda et al., 2008). In addition, SCI can produce hypertrophy of TRPV1-expressing DRG neurons measured in situ (Ramer et al., 2012). However, I found that the percentage of relatively small DRG neurons responding to capsaicin was unchanged after T10 contusion. Furthermore, when I grouped these relatively small DRG neurons according to soma diameter, the capsaicin-induced currents were consistently higher after SCI than sham treatment or in the naïve group in neurons with soma diameters in all parts of this size range. I cannot exclude the possibility that TRPV1 is induced in larger DRG neurons by SCI because I did not sample DRG neurons with soma diameters > 30 μm. Large DRG neurons are reported to become TRPV1 positive in some peripheral chronic pain models (Amaya et al., 2003; Ma et al., 2005; Shinoda et al., 2008). Although larger DRG

neurons (including non-nociceptors) might show similar effects after SCI, my data support the hypothesis that long-term enhancement of TRPV1 function in small DRG neurons (probable nociceptors) is produced by SCI.

CHAPTER 4: *SCI enhances the expression of TRPV1 protein*

4.1. Introduction

One mechanism that could produce the increases in capsaicin-evoked inward currents and Ca^{2+} transients observed after SCI is increased expression of TRPV1 channels. TRPV1 channel expression is upregulated in DRG neurons from rats showing thermal hyperalgesia after peripheral inflammation or nerve injury (Hudson et al., 2001; Fukuoka et al., 2002; Ji et al., 2002; Breese et al., 2005; Kanai et al., 2005). SCI is accompanied by persistent inflammatory effects within the spinal cord (Alexander and Popovich, 2009; Dulin et al., 2013) and within DRG (McKay and McLachlan, 2004; Carlton et al., 2009). Thus, TRPV1 channel activity might be enhanced in nociceptors following SCI by increased expression of the protein. TRPV1 synthesis and expression are enhanced by NGF through activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway (Ji et al., 2002; Puntambekar et al., 2005; Stein et al., 2006). SCI elevates NGF levels in the spinal cord (Bakhit et al., 1991; Murakami et al., 2002; Brown et al., 2004), which has been suggested to contribute to increased TRPV1 staining in the superficial dorsal horn (below the injury level) 3-14 days after spinal cord transection as compared to sham treatment (Zhou et al., 2002). Elevated NGF after SCI may influence primary sensory neurons by binding to receptors on their central terminals, thereby stimulating upregulation of TRPV1 in the DRG, and contributing to the development of abnormal behavior after SCI (Gwak et al., 2003). Here I test the hypothesis that SCI increases the expression of TRPV1

protein in DRG below the injury level.

4.2. Results

If DRG neurons respond to the central inflammation caused by SCI in the same way that they respond to peripheral inflammation, then TRPV1 channels should show increased expression in nociceptor somata in the DRG and in their terminals within the dorsal horn. I thus performed Western blot analysis to determine the expression levels of TRPV1 protein in DRG harvested bilaterally from segments L4 and L5 in each group of rats. A representative result is shown in **Fig 7A** and the relative amounts of TRPV1 protein (normalized to β-actin) obtained 1 month after injury in SCI, sham, and naïve groups are plotted in **Fig 7B**. The amount of TRPV1 protein was significantly different among DRG excised from naive (n = 6), sham-treated (n = 5), and SCI (n = 6) animals ($F_{2,14} = 22.93$; $P < 0.0001$), with the levels being higher in ganglia from SCI than sham-treated or naive animals. When protein levels in DRG from sham-treated and SCI animals were further normalized to those of DRG from naive animals run on the same gels, levels from SCI animals were significantly greater than those from sham animals (**Fig. 7B**; $P = 0.006$, t-test). This indicates that SCI stimulates an increase in TRPV1 protein in DRG neurons 1 month after injury.

4.3. Conclusions

These results support the hypothesis that at least part of the enhanced sensitivity of small DRG neurons to capsaicin after SCI is caused by increased

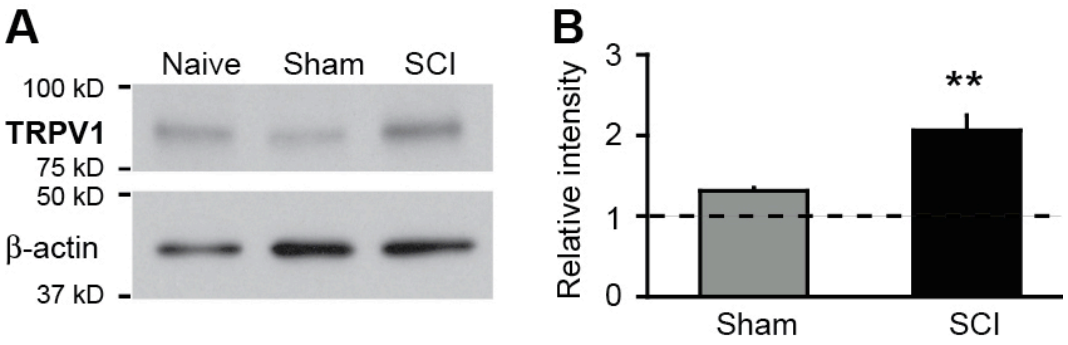

Figure 7. Expression of TRPV1 in rat DRG neurons was upregulated 1 month after SCI. Left panel (A) shows western blot bands of TRPV1 and β-actin probed with their specific antibodies. β-actin was used as the internal control. A total of 5 experiments performed on DRG indicate the relative intensity of TRPV1 band increased significantly after 1 month of SCI (B). Intensities of TRPV1 bands were first normalized to that of β-actin in each sample and then to that value from the paired naïve sample.

expression of TRPV1 channels. In this study, TRPV1 expression was measured at the protein level using western blotting, and thus extends immunohistochemical evidence for increased expression of TRPV1 in the DRG after SCI (Ramer et al., 2012). Immunohistochemical results (Ramer et al, 2012; Yang, Wu, and Walters, unpublished observations) indicate that the increased expression occurs within neurons rather than other cell types in the DRG. The Western blot and immunohistochemical studies don't distinguish between channels located in the neuronal membrane and in the cytoplasm, but in combination with the results in Chapter 3 showing increased TRPV1 activity, the Western blot findings strongly suggest that at least some of the increased activity occurs because of a larger number of functional channels in the membrane after SCI. My functional data (Chapter 3) indicate that the increased expression after SCI occurs, at least in part, in small DRG neurons that are probably nociceptors. In naive animals TRPV1 channels are rarely expressed in large DRG neurons, but a shift of TRPV1 expression towards large DRG neurons has been reported after peripheral nerve injury (Hudson et al., 2001; Ma et al., 2005). After another form of SCI, complete low thoracic (T10) spinal cord transection, a shift in expression towards larger DRG neurons was not observed (Ramer et al., 2012), suggesting that upregulation of TRPV1 channels is largely restricted to smaller DRG neurons. As described in Chapter 3, SCI altered capsaicin-induced currents to a similar degree across all DRG neurons with soma diameters less than 30 μm. Taken together, these observations suggest that enhanced TRPV1 expression in DRG after SCI occurs

in a large fraction of C-fiber nociceptors and probably in some Aδ-fiber nociceptors.

CHAPTER 5: *Inhibiting TRPV1 activation reduces SCI-induced pain behaviors*

5.1. Introduction

Increased activity of TRPV1 channels, resulting from increased channel expression, ongoing activation by endogenous ligands, or modulation by intracellular regulators, appears likely to contribute to intrinsic SA in nociceptors and consequent pain. TRPV1 channels are Ca^{2+} permeable (Tominaga, 2007), and Ca^{2+} plays an essential role in controlling many neuronal activities, including neurotransmitter release, gene expression, and development. Activity in TRPV1 channels could have at least four consequences that promote pain after SCI: 1) direct depolarization of DRG neurons, bringing membrane potential into a range that enhances SA and excitability; 2) direct and/or indirect increase in intracellular Ca^{2+} concentration, which can enhance neurotransmitter release and amplify primary afferent input to pain projection neurons; 3) synergism with other signals, e.g., by promoting translocation of PKC to the plasma membrane (Oancea and Meyer, 1998); and 4) enhancement of pain-related protein expression within nociceptors.

Application of TRPV1 channel antagonists, including AMG9810, effectively reduces behaviorally expressed thermal and mechanical hypersensitivity in various rodent pain models (Gavva et al., 2005; Cui et al., 2006; Yu et al., 2008). Although most TRPV1 antagonists produce significant hyperthermia as a side

effect, limiting their clinical utility (Gavva et al., 2007; Gavva et al., 2008), recent discoveries of antagonists that do not alter body temperature are revitalizing this approach for treating chonic pain (Reilly et al., 2012).

Both mechanical allodynia and heat hyperalgesia develop after SCI, and mechanical allodynia is a particularly debilitating problem for many SCI patients (Sjolund, 2002). Weak support for TRPV1 channel involvement in SCI-induced heat hypersensitivity came from a very small preliminary study (lacking control groups) claiming that AMG9810 reduces thermal hypersensitivity following SCI (Rajpal et al., 2007). Although differences were not found between “hyperalgesic” and “non-hyperalgesic” animals in that study, less than 50% of the animals exhibited SCI-induced decreases in withdrawal latency during heat stimulation (possibly because systematic habituation to the context and stimuli were not performed before data collection), which is much less than typically found in our laboratory (Bedi et al., 2010), Here, I will rigorously test the hypothesis that continuing TRPV1 activity is important for chronic behavioral hypersensitivity after SCI.

5.2. Results:

5.2.1 Systemic injection of AMG9810 ameliorates SCI-induced heat and mechanical hypersensitivity of hindlimb withrawal responses

In principle, enhancement of TRPV1 channel function in nociceptors after SCI would be expected to contribute to SCI-induced chronic pain. I have tested the effects of in vivo application of AMG9810, a specific TRPV1 antagonist (Gavva et

al., 2005), on behaviorally expressed hindlimb hypersensitivity (**Fig. 8A, B**). Before contusion, each animal received behavioral pretests (thermal and von Frey stimuli applied to plantar surface). One month after SCI, the same tests were performed 2 days before injection, at which time behavioral sensitivity increased to both heat (from 14.17 ± 0.77 to 7.67 ± 0.69 s, n = 6) and mechanical (8.59 ± 1.90 to 3.02 ± 0.79 g, n = 7) test stimuli. Animals then received either AMG9810 (30 mg/kg, i.p.) or vehicle (saline with 0.1% DMSO, 2 ml, i.p.). Posttests were performed 30 min after each injection to see if injections reduce SCI-induced enhancement of paw withdrawal responses. AMG9810 application 1 month after SCI significantly reversed both heat hypersensitivity (12.99 ± 0.60 s) and mechanical hypersensitivity (11.70 ± 1.88 g) (**Fig. 8A, B**), while vehicle injection (n = 6) failed to reduce the hypersensitivity (**Fig. 8A, B**). For heat sensitivity (**Fig. 8A**) two-way ANOVA with repeated measures revealed significant effects of the test sequence (pretest, post-SCI, post-injection) ($F_{2,17}$ = 11.37; P = 0.007) and drug treatment ($F_{1,11}$ = 10.70; P = 0.008), with latencies for withdrawal to noxious heat significantly longer after injection of AMG9810 than vehicle (P = 0.0003; n = 6 animals in each group). For mechanical sensitivity (**Fig. 8B**) two-way ANOVA with repeated measures revealed significant effects of the test sequence (injury and recovery) ($F_{2,17}$ = 6.98; P = 0.006) and drug treatment ($F_{1,11}$ = 19.09; P = 0.001), with mechanical thresholds significantly greater after injection of AMG9810 than vehicle (P = 0.015; n = 6 animals in each group). These results indicate that ongoing activation of TRPV1 channels contributes substantially to chronic heat and mechanical hypersensitivity after SCI.

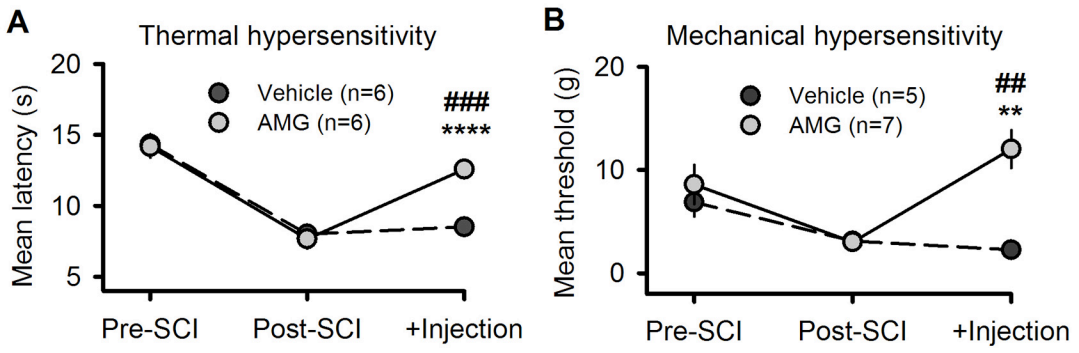

Figure 8. AMG9810, a specific TRPV1 channel blocker, ameliorates SCI-induced mechanical and thermal hypersensitivity. A, summary data indicates that AMG9810 (30 mg/kg, i.p.), but not vehicle (saline), reversed established thermal hypersensitivity (decreased hindpaw withdrawal latency) induced by SCI 30 min after injections. B, summary data indicates that AMG9810 (30 mg/kg, i.p.), but not vehicle (DMSO), reversed established mechanical hypersensitivity (decreased hindpaw withdrawal threshold) induced by SCI 30 min after injections. *, comparing to postcontusion before AMG9810. #, comparing to postcontusion with vehicle. AMG, AMG9810.

5.2.2. Reversal of SCI-induced heat and mechanical hypersensitivity by knockdown of TRPV1.

It has been demonstrated that knocking down TRPV1 channels is sufficient to reverse behavioral signs of pain induced by nerve injury and inflammation (Christoph et al., 2006; Christoph et al., 2007; Christoph et al., 2008). Thus, I used ODN targeted to mRNA of TRPV1 channels (Christoph et al., 2007) to further evaluate the role of TRPV1 channels in SCI-induced chronic pain. This ASO is reported to induce nearly complete degradation of TRPV1 mRNA, and can be taken up well in vivo by DRG neurons after intrathecal delivery (Christoph et al., 2007). To confirm that expression of TRPV1 protein in L4/L5 DRG neurons was knocked down and to estimate the degree of knockdown, protein abundance was assessed by western blotting, and the degree of functional knockdown was assessed by electrophysiological recording of capsaicin-evoked currents. Western blot analysis showed that ASO treatment was effective in knocking down TRPV1 protein expression in L4 and L5 DRG from animals treated for 5 days with ASO injections 6 weeks after contusive SCI (**Fig. 9A, B**). The intensity of the band representing TRPV1 was significantly decreased (by 63%) in samples taken from rats treated with ASO targeting TRPV1, as compared to samples taken from rats treated with a MMO (n = 6; $P = 0.0058$) (**Fig. 9B**). No significant difference was found in the intensity of the band representing TRPV4, another member of the TRPV family (Guler et al., 2002; Tominaga, 2007) when comparing DRG from the TRPV1 ASO group and MMO group (**Fig. 9B**). This suggests that the ASO treatment was specific to TRPV1. Finally, the ASO effect on TRPV1 was

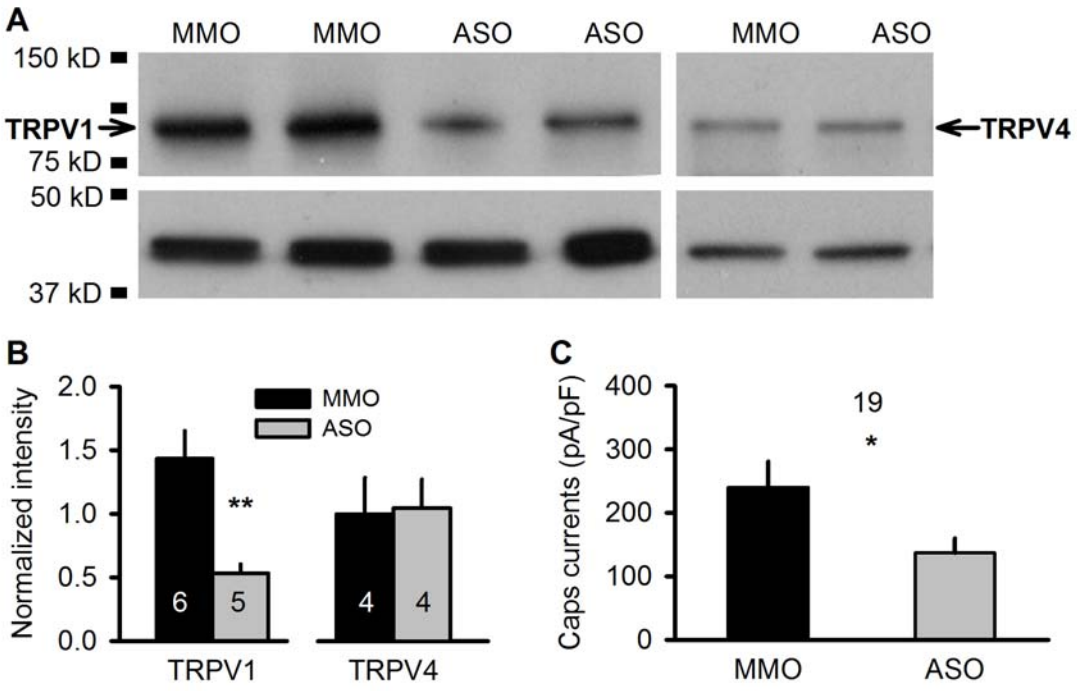

Figure 9. Intrathecal delivery of antisense oligodeoxynucleotide targeting TRPV1 specifically knocked down TRPV1 in DRG. Representative band (A) and summary data (B) from western blots indicate that ASO, but not MMO, knocked down TRPV1 protein expression by about 63% in lumbar DRG. Furthermore, TRPV4 protein levels were almost the same between tissues dissociated from ASO and MMO treated SCI rats. Intensities of TRPV1/TRPV4 bands were normalized to that of β-actin. C, Comparison of capsaicin-sensitive currents among adult DRG neurons dissociated from ASO and MMO pretreated SCI rats. 3 μM capsaicin-induced currents were significantly decreased in ASO group as compared to MMO group (36 hours after the last ODN injection). MMO, mismatch ODN; ASO, antisense ODN.

confirmed functionally. Capsaicin-induced inward current measured at a holding potential of -60 mV in DRG neurons dissociated from the MMO group (240 pA/pF, n = 20 neurons) was similar to that recorded from the untreated SCI group (**Fig. 4B**). However, capsaicin-induced inward current was significantly reduced in DRG neurons dissociated from ASO rats (137 pA/pF, n = 19 neurons; $P = 0.0358$) (**Fig. 9C**).). This 43% decrease brought the mean amplitude of capsaicin-evoked currents in ASO-treated SCI animals (**Fig. 9C**) close to the levels exhibited by neurons taken from naive (128 pA/pF) and sham-treated animals (124 pA/pF) (**Fig. 4B**).

One month after SCI, the mechanical and heat thresholds for withdrawal of hindpaws in response to stimulation of the plantar surface with von Frey filaments or heat were measured and compared to the threshold measured in the same animal just before spinal injury (day -1). The thresholds for mechanical and heat stimulation were lowered to 46.91% and 62.37% of the control in the operated animals, respectively. Some of the SCI animals (n = 6) then received intrathecal administration of TRPV1 ASO molecules at the L4/L5 level twice daily for 3 days, and then once per day for 2 days. MMO treatment served as control in the remaining SCI animals (n = 5). The heat and mechanical thresholds were measured again ~15 hours after the last ODN injection (prior to harvesting the DRG). As shown in **Figure 10A and B**, the specific knockdown of TRPV1 significantly attenuated SCI-induced mechanical (from 3.36 ± 0.55 to 14.03 ± 1.46 g) and heat (from 7.15 ± 0.43 to 11.17 ± 0.75 s) hypersensitivity, whereas the

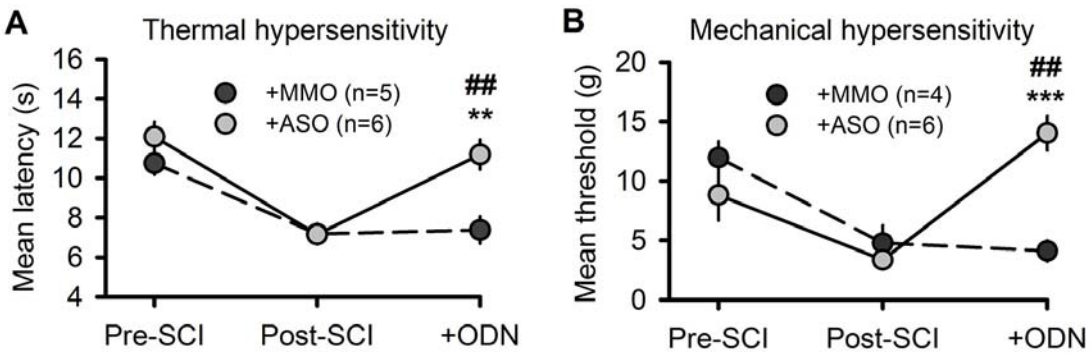

Figure 10. TRPV1 knocking down significantly decreases reflex sensitization after SCI. Hindlimb withdrawal responses to mechanical and radiant heat stimuli were tested with standard methods before SCI, after SCI, and after ODN injections (during the 4th and 5th d of a 5-d knockdown sequence). The last behavioral test was followed by harvesting of DRG and dissociation of neurons for cellular tests. Both thermal (A) and mechanical (B) sensitivity were increased 1 mo after spinal contusion. Subsequent intrathecal injection of ASO but not MMO, reversed at least part of the mechanical and thermal hypersensitivity. *, comparing to postcontusion before ASO. #, comparing to postcontusion with MMO. MMO, mismatch ODN; ASO, antisense ODN.

responses did not change in the MMO group for both mechanical (from 5.99 ± 1.85 to 5.87 ± 0.89 g, n = 6) and thermal (from 7.15 ± 0.52 to 7.38 ± 0.68 s, n = 6) tests. For heat sensitivity (**Fig. 10A**) two-way ANOVA with repeated measures revealed a significant effect of the test sequence (pretest, post-SCI, post-ODN) ($F_{2,18}$ = 22.78; P < 0.0001) and ODN treatment ($F_{1,9}$ = 11.03; P = 0.0090), with latencies for withdrawal to moderately noxious heat significantly longer after injection of ASO (n = 6) than MMO (n = 5; P = 0.0051). For mechanical sensitivity (**Fig. 10B**) two-way ANOVA with repeated measures revealed significant effects of the test sequence (injury and recovery) ($F_{2,1}$ = 6.98; P = 0.006). A partitioned analysis on each factor using one-way ANOVAs and pairwise comparisons at each test revealed a significant effect of ODN treatment in the post-ODN test, with the threshold for withdrawal higher in ASO-treated than MMO-treated rats (P = 0.007). These results show that TRPV1 ASO treatment can effectively reverse chronic behavioral hypersensitivity to heat and mechanical stimuli after SCI.

5.2.3. TRPV1 channel knockdown, but not acute block, decreases enhanced spontaneous activity in dissociated nociceptors after SCI

We also measured the incidence of SA in DRG neurons dissociated from SCI rats treated with ODN. Unexpectedly, in the near absence of nearby cells (potential sources of TRPV1 ligands) SA incidence in ASO DRG neurons (6 out of 19) was lower than that from MMO DRG neurons (15 out of 20) (P = 0.010, Fisher's exact test, **Fig. 11A and B**). The firing rate of the DRG neurons that did display SA was unchanged after previous ASO treatment compared to MMO

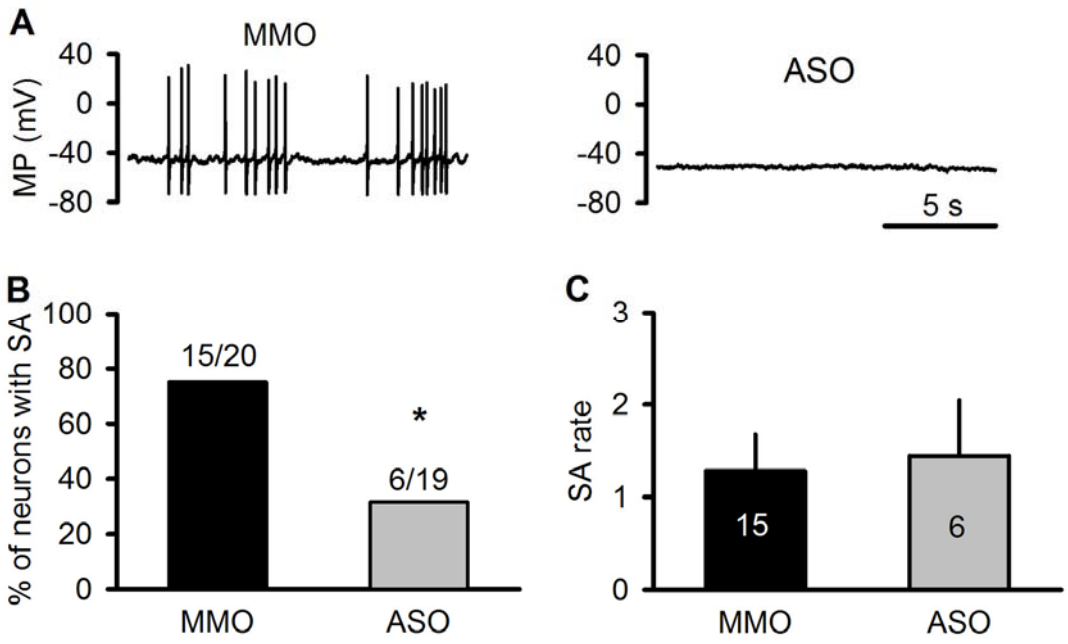

Figure 11. Knocking down TRPV1 channel *in vivo* decreases the excitability of dissociated nociceptors. A, example of lack of SA in a small DRG neuron from an ASO-treated SCI animal (right trace). A similar neuron from an MMO-treated animal displayed typical SCI-induced SA (left trace). This and other prolonged recordings were digitized at a low sampling rate (2 KHz), so the action potentials are clipped. B, summary data indicates incidence of SA in DRG neurons dissociated from MMO or ASO pretreated SCI animals. C, Lack of effect of TRPV1 ASO treatment on SA firing rate in the neurons (n = 6) that still fired spontaneously after the treatment. ASO, antisense ODN; MMO, mismatch ODN.

treatment (**Fig. 11C**). Tests performed after SA measurement showed that all of these cells were capsaicin-sensitive.

I then tested whether intrinsic TRPV1 channel activity contributes to SCI-induced SA in dissociated DRG neurons after SCI via endogenous ligands binding to the capsaicin-binding site (Jara-Oseguera et al., 2008) by testing the effects of AMG9810 on SA in vitro. DRG neurons were dissociated from rats 1 month post-SCI. Only neurons exhibiting SA were tested. The threshold for SA generation was determined by applying a series of descending hyperpolarizing pulses (5 s each). Multiple ion channels are probably involved in intrinsic SA generation, and blocking one might not be enough to hyperpolarize cells below action potential threshold and thus reveal the involvement of TRPV1 channels in SA generation. Thus, I hyperpolarized neurons displaying SA to a potential just above SA threshold and recorded stable SA. Cells were then exposed to 10 μM AMG9810 for another 15 s followed by washout. AMG9810 failed to decrease SA in this controlled recording condition (**Fig. 12A**), although it blocked currents evoked by application of 1 μM capsaicin (**Fig. 12B**). The failure of AMG9810 to reduce SA in vitro after SCI suggests that the SA under this condition is not promoted by ongoing activation of TRPV1 via the capsaicin-binding site on the channel.

5.3. Conclusions

5.3.1. TRPV1 channels, especially those expressed in primary sensory neurons, play an important role in SCI-induced chronic pain

In these studies, I used both pharmacological and molecular methods to provide strong evidence supporting my hypothesis that activation of TRPV1 channels contributes significantly to SCI-induced chronic pain. First of all, SCI rats developed both thermal and mechanical hyperreflexia, since the thresholds were decreased to both radiant heat and von Frey filament stimuli 1 month after SCI. This behaviorally expressed hypersensitivity after SCI was largely reversed by systemic delivery of AMG9810, which suggests that TRPV1 channels play an important role in SCI-induced chronic pain. In addition to their high expression in primary nociceptors, TRPV1 channels have also been reported in brain tissue, and in dorsal horn neurons (Caterina et al., 1997; Mezey et al., 2000; Cui et al., 2006; Kim et al., 2012). Because AMG9810 penetrates the blood brain barrier, AMG9810-induced effects may result not only from actions on its target in primary nociceptors, but also on TRPV1 channels in the CNS. After intrathecal injection of antisense ODN targeting TRPV1, both capsaicin-induced currents in DRG neurons and TRPV1 band intensity in DRG were significantly decreased in the ASO group as compared to the MMO group. A stronger ASO-induced knockdown compared to the decrease in capsaicin-induced currents may be due to the fact that the capsaicin currents were measured ~36 hours after the last ODN injection, a time when significant recovery of TRPV1 protein expression should have occurred compared to when samples were taken for western blot assays (~18 hours after the last ODN injection). Furthermore, ASO targeting TRPV1 failed to alter the band intensity of TRPV4. These data strongly suggest that intrathecal injection of antisense ODN targeting TRPV1 specifically knocked down TRPV1 channels in

DRG, and possibly the dorsal horn. The extent to which TRPV1 channels are expressed in the spinal cord is controversial (Caterina et al., 1997; Cui et al., 2006; Cavanaugh et al., 2011; Kim et al., 2012; Zhang et al., 2012). TRPV1 knockdown was accompanied by decreased heat and mechanical hypersensitivity. Given that TRPV1 channel expression is highest by far in primary sensory neurons (Tominaga, 2007), and its expression in dorsal horn neurons may be very low, it seems likely that much of the effects induced by AMG9810 and by antisense ODN treatment result from reducing the activity of TRPV1 channels expressed in primary sensory neurons.

5.3.2. TRPV1 channel activity in vivo but not in vitro is important for maintaining intrinsic SA of nociceptors after SCI.

It was surprising to find that ASO significantly decreased SA incidence in dissociated nociceptors. Because of the very low cell density in this in vitro preparation, it is unlikely that this effect results from disrupted *in vitro* basal activity maintained by ongoing exposure to endogenous TRPV1 activators, such as lipid products, released by nearby cells. During current clamp recording, DRG neurons were constantly perfused by external solution, which would wash away any secreted activators. Also, AMG9810 failed to block or reduce the firing rate in those DRG neurons that did display SA, whereas the same concentration of AMG9810 was enough to block 1 μM capsaicin-induced currents. These observations indicate that SA in vitro is not maintained by endogenous ligands binding to the common AMG9810- and capsaicin-binding site on TRPV1. An

alternative hypothesis that should be tested in the future is that knockdown of TRPV1 expression may impair a positive-feedback loop in vivo in which endogenous activators of TRPV1 (in the dorsal horn and/or the DRG) promote an intrinsic hyperexcitable-spontaneously active state in nociceptors that can maintain SA in a nociceptor for at least 1 day after dissociation (Bedi et al., 2010; Walters, 2012). For example, evidence exists for neuron-glia interactions where extensive sensory excitation activates glial cells, which in turn cause plasticity of DRG neurons (Miller et al., 2009; Xie et al., 2009).

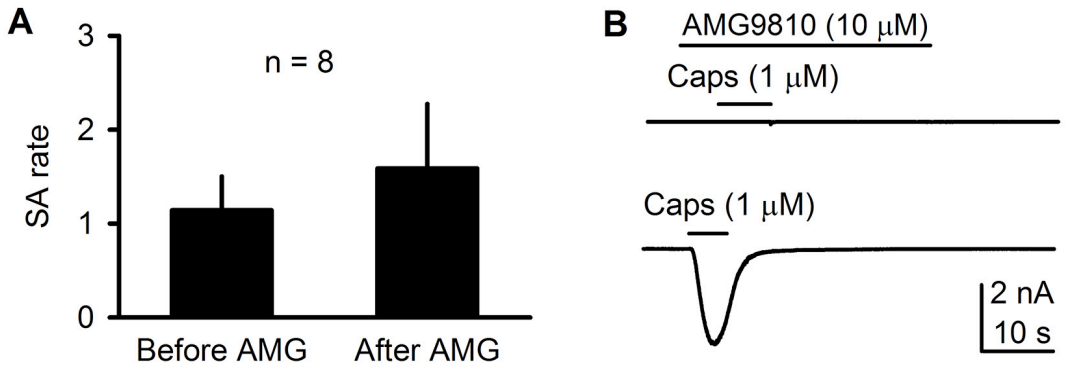

Figure 12. Lack of effect of AMG9810 on SA firing rates. A, summary data indicates AMG9810 failed to alter SA rate in a controlled condition where DRG neurons displaying SA were hyperpolarized to just above SA threshold. After current-clamp recording, cells in A and B were switched to voltage-clamp model, capsaicin was then applied to confirm that they had TRPV1 channels. B, 10 μM AMG9810 effectively blocked capsaicin (1μM)-induced current (upper trace) in capsaicin-sensitive neurons (lower trace). AMG, AMG9810; Caps, capsaicin.

CHAPTER 6: *TRPV1 channels are involved in SCI-induced spontaneous activity in nociceptors*

6.1. Introduction

SA in DRG neurons has been implicated as a potentially important contributor to chronic pain in several different models, including SCI. SA has been investigated most extensively in large DRG neurons after peripheral nerve injury or inflammation (Wall and Devor, 1983; Xie et al., 2005). In large DRG neurons SA has been shown to depend on subthreshold spontaneous oscillations of membrane potential, the amplitude of which increases with depolarization (Devor, 2009). Peripherally generated SA also occurs in peripheral inflammation and injury models in nociceptive C-fiber neurons (Wu et al., 2001; Djouhri et al., 2006). Contusive SCI at T10 causes an increase in the incidence of peripherally generated SA in **small diameter nociceptors** recorded from a forelimb nerve (Carlton et al., 2009), and a dramatic increase in SA recorded from lumbar dorsal roots and dissociated small DRG neurons that is probably generated in the nociceptor soma (Bedi et al., 2010). The SA in dissociated neurons was associated with depolarization of the cell body (Bedi et al., 2010), as had been described in large DRG neurons exhibiting SA after nerve injury (Amir et al., 1999). Also, some DRG neurons with SA exhibit more negative thresholds for triggering action potentials (Study and Kral, 1996; Bedi et al., 2010). Under these conditions, any small inward currents, including currents through TRPV1 channels, should promote action potential generation. TRPV1 channels can be activated by various

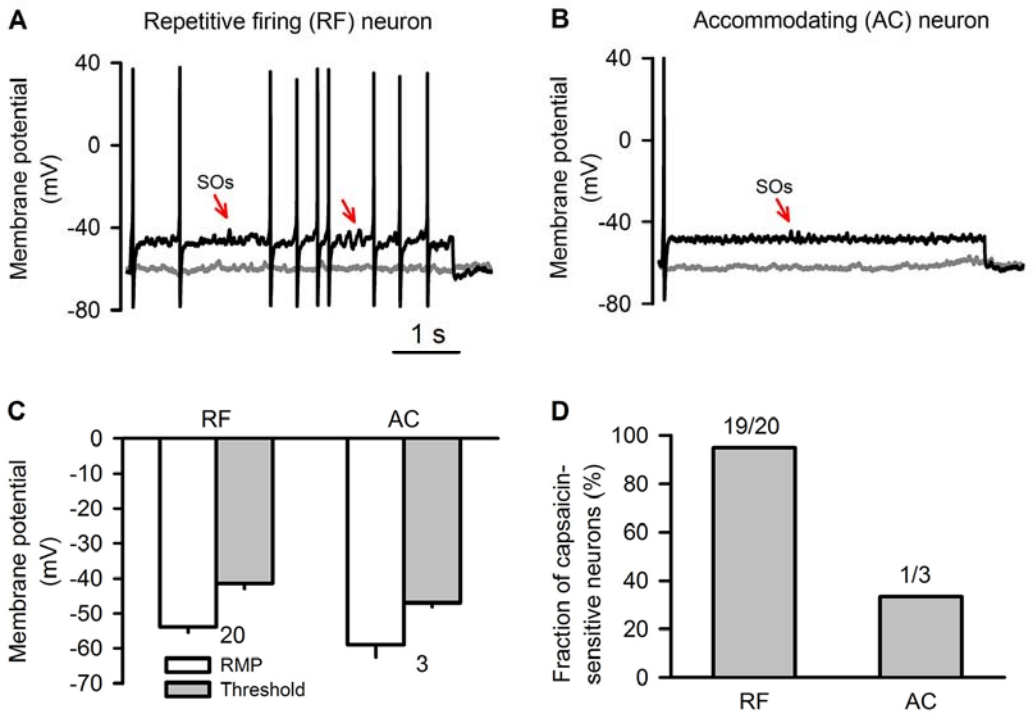

Figure 13. Depolarization induces repetitive firing (RF) in previous silent DRG neurons. A, depolarization increased subthreshold oscillations (SOs, red arrows) and induced repetitive firing (RF) in majority of DRG neurons (left trace), while a few of cells showed accommodation (Ac, right trace). B, depolarization required to reach threshold was similar in cells displaying repetitive firing and accommodation. C, sustained, low-frequency firing occurred in > 90% of capsaicin-sensitive nociceptors. In contrast, capsaicin-insensitive DRG neurons often accommodated during prolonged depolarization. In current clamp mode, a series of depolarizing currents (from 0 to 100 pA for 5 s with 5 pA increments) were injected into small silent DRG neurons dissociated from naïve rats. RMP, resting membrane potential.

lipid products (see Chapter 1), and many of these lipid products are elevated in the spinal cord after SCI (Liu and Xu, 2010). Also, I have shown that SCI enhances TRPV1 channel function. Thus, I tested the hypothesis that activation of TRPV1 channels can contribute to the chronic SA observed in nociceptors after SCI.

6.2. Results

6.2.1. Depolarization induces repetitive firing in most nociceptors

Since DRG neurons displaying SA are usually depolarized as compared to silent neurons, and activation of TRPV1 channels depolarizes DRG neurons, I tested whether membrane depolarization enhances repetitive firing in silent DRG neurons. DRG neurons were dissociated from naïve rats. In current clamp mode a series of currents (from 0 to 100 pA for 5 s with 5 pA increments at 10 s intervals) was injected into silent DRG neurons. A majority of the tested DRG neurons showed increased amplitudes of subthreshold spontaneous oscillations during depolarizing pulses (**Fig. 13A**). When membrane potentials were depolarized (to about -43 mV in this example), repetitive firing occurred in most of the neurons, and continued without accommodation for as long as the injected currents were applied (**Fig. 13A and C**). In contrast, a small proportion of tested DRG neurons failed to increase the amplitude or frequency of subthreshold spontaneous oscillations when currents injected (**Fig. 13B**). These cells generated only one or two action potentials at the beginning of the pulse when membrane potential was depolarized (-46 mV in the example in **Fig. 13B**); however, the spiking was not sustained even though currents equal to twice the rheobase were injected (**Fig**

13B and **C**). Interestingly, cells displaying repetitive firing during depolarizing stimulation usually responded to capsaicin under voltage-clamp (holding the neuron at -60 mV), while 2 out of the 3 DRG neurons showing accommodation failed to exhibit any currents in response to capsaicin application.

6.2.2. Spontaneous activity requires extracellular Ca^{2+}

It has been demonstrated that SO and SA in large Aβ DRG neurons are blocked by the voltage-gated Na^{+} channel blocker, TTX, but not voltage-gated Ca^{2+} channel blockers (Matzner and Devor, 1994; Amir et al., 1999). However, nothing is known about the ionic dependence of subthreshold spontaneous oscillations and SA in C-fiber neurons. I tested whether the same ionic mechanisms reported to be important for SA in large DRG neurons also underlie SA generation in small DRG neurons. 18-24 hours after dissociation from SCI rats, L4/L5 DRG neurons displaying SA were recorded under whole-cell current clamp. SA was first recorded for 15 s in normal external solution. External solution containing TTX (300 nM) or lacking Ca^{2+} (with Ca^{2+} replaced by Mg^{2+} in the external solution) was applied for another 30 s followed by washout. In contrast to what has been reported in large DRG neurons (Amir et al., 1999), 300 nM TTX failed to inhibit SA in small DRG neurons (**Fig. 14**). However, when these cells were exposed to Ca^{2+}-free external solution, SA firing rate was greatly decreased (from 1.84 ± 0.59 to 0.14 ± 0.09, P = 0.02, paired t-test, **Fig 15A** and **B**) while membrane potential was not significantly changed (**Fig. 15C**). These results indicate that extracellular Ca^{2+} is important for SA generation.

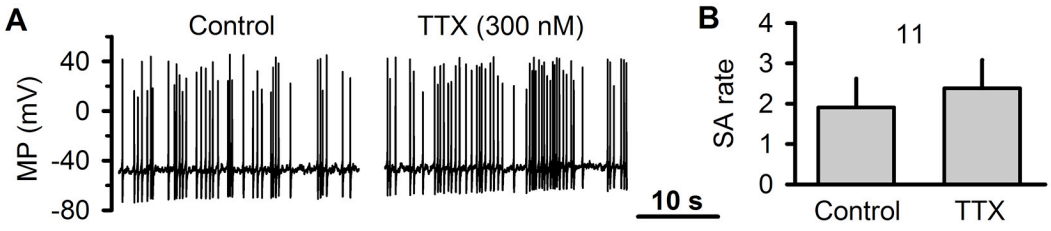

Figure 14. TTX fails to inhibit SA in small DRG neurons. A, Representative traces showing that bath application of TTX (300 nM) had no effect on SA in a DRG neuron dissociated from a SCI rat. B, Summary data showed that TTX failed to inhibit SA. TTX, tetrodotoxin.

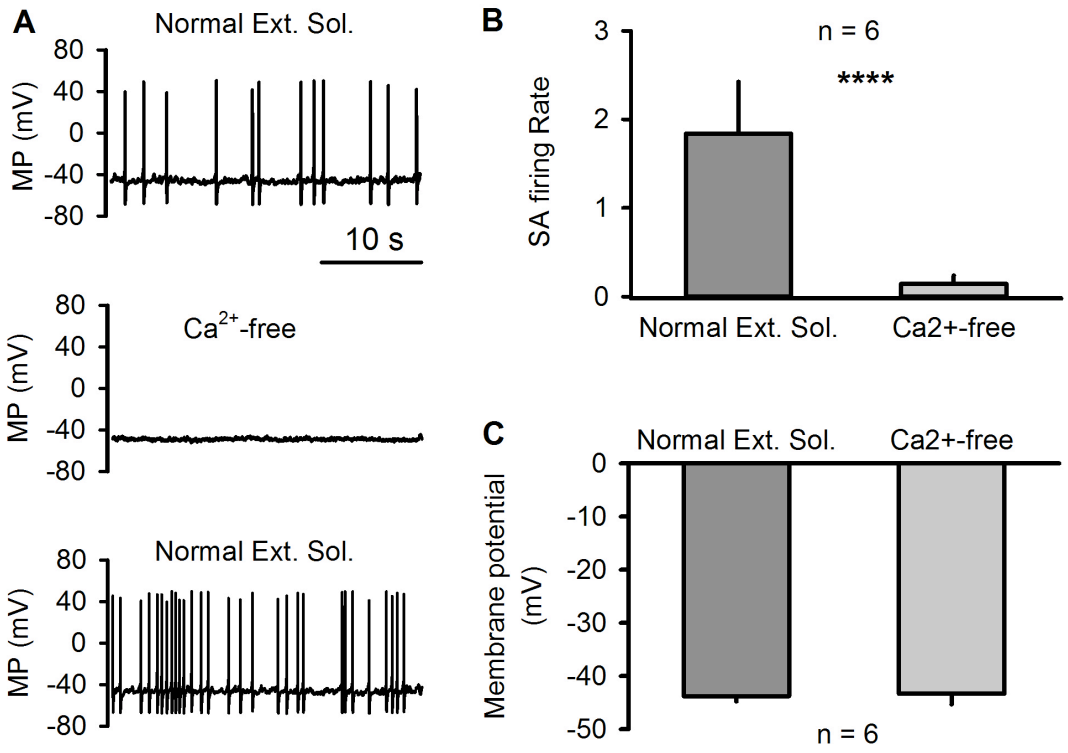

Figure 15. Removing Ca^{2+} from extracellular solution decreases SA. A, Representative recordings showing that Ca^{2+}-free solution reduced the incidence of spontaneous firing in a neuron examined 1 month after SCI. B, summary data showing that removing extracellular Ca^{2+} significantly decreased the firing rate (comparing to its baseline) of DRG neurons (paired t-test). C, summary data showing that removal of extracellular Ca2+ did not change resting membrane potentials.

6.2.3. Low concentrations of capsaicin induce repetitive firing in nociceptors

When TRPV1 channels in DRG neurons are activated, extracellular Ca^{2+} and Na^{+} diffuse through the channels into the cell, causing depolarization. Because depolarization and extracellular Ca^{2+} are required for generation of both SA and non-accommodating repetitive firing in small DRG neurons, I tested whether limited activation of TRPV1 is sufficient to induce repetitive firing in nociceptors. Silent DRG neurons dissociated from naïve rats were recorded under current clamp at the resting membrane potential for 15 s. Cells were then exposed to 10 nM or 50 nM capsaicin for 30 s followed by washout. Most of the DRG neurons were gradually depolarized during capsaicin application (**Fig. 16A**). The depolarizing response was concentration dependent; 10 nM and 50 nM capsaicin depolarized membrane potential by 3.22 ± 1.05 and 6.53 ± 1.22 mV, respectively (**Fig. 16B**). Some of the cells generated repetitive firing during the capsaicin-induced depolarization (**Fig. 16A**), with 6 out of 10 silent DRG neurons showing repetitive firing during 50 nM capsaicin application, and 3 of 8 neurons showing repetitive firing during 10 nM capsaicin application (**Fig. 16C**). All these cells became silent when membrane potential recovered after washout of the capsaicin. The recording configuration was then switched to voltage-clamp mode, holding at -60 mV, and sensitivity to a saturating concentration of capsaicin (3 μM) was tested. Capsaicin-insensitive cells were excluded from the analysis. These results show that low concentrations of capsaicin can induce repetitive firing in nociceptors from naive animals.

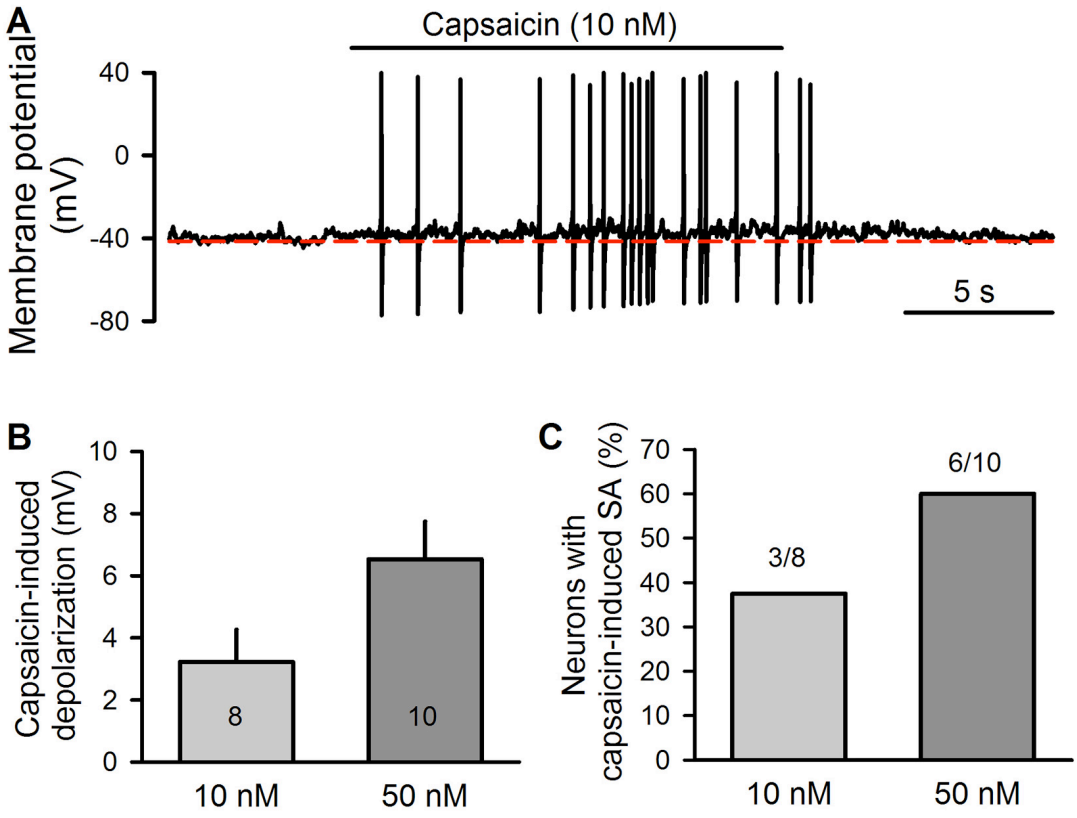

Figure 16. Low concentrations of capsaicin depolarize nociceptors and produce repetitive firing in previously silent neurons. A, representative traces showing that addition of capsaicin (10 nM) induces repetitive firing in a previously silent neuron. Low concentrations of capsaicin induced membrane depolarization (B) and continuous firing (C) in a concentration dependent manner.

6.2.4. Prolonged application of low concentrations of capsaicin induces repetitive firing without accommodation

It been suggested that endogenous TRPV1 activators may accumulate and continuously activate TRPV1 channels under various pathological conditions (Shin et al., 2002; Starowicz et al., 2007), which could produce sustained repetitive firing of primary nociceptors. It is well known that relatively high concentrations of capsaicin induce strong desensitization of TRPV1 channels (Cholewinski et al., 1993; Koplas et al., 1997). Endogenous activators of TRPV1 probably exist in very low concentration in vivo (Huang et al., 2002). I therefore examined whether prolonged application of a very low concentration of capsaicin can induce non-accommodating repetitive firing in silent nociceptors, and, if so, whether this effect is enhanced by SCI. DRG neurons were dissociated from rats 1-2 months post-SCI, as well as from age-matched naïve animals. Silent DRG neurons were recorded using the perforated patch current-clamp method at the neuron's resting membrane potential for 30 s. Each neuron was then exposed to 10 nM capsaicin for 10 min or longer followed by washout. Similar to what was observed with the 30-s capsaicin applications, the much longer application of 10 nM capsaicin gradually depolarized membrane potential and triggered repetitive firing. The firing rate was low initially, but gradually increased with further membrane depolarization and become stable within 2 min. Sustained depolarization during the capsaicin exposure was always accompanied by an increase in amplitude of subthreshold spontaneous oscillations of membrane potential. Importantly, both the firing rate

and depolarized membrane potential showed no apparent recovery for as long as the cells were exposed to capsaicin (**Fig 17**).

The increases in TRPV1 expression and function I demonstrated in DRG neurons after SCI predicted that SCI should also increase the responsiveness of dissociated DRG neurons to very low concentrations of capsaicin. Therefore, I compared the degree of membrane depolarization and the percentage of silent cells that fired repetitively during 10-min exposure to 10 nM capsaicin between SCI and naïve groups. Silent DRG neurons (n = 16) from SCI animals showed significantly greater depolarization of resting membrane potential during this capsaicin exposure than neurons (n = 16) dissociated from naive animals (**Fig. 18A** and **B**; $P < 0.001$, paired t-test). Correspondingly, 10 nM capsaicin induced repetitive firing in 81.25% (13/16) silent neurons dissociated from SCI rats, whereas only 5 out of 14 neurons from naive rats showed repetitive firing during 10 nM capsaicin application (**Fig. 18C**, $P = 0.024$, Fisher's exact test). After switching to voltage-clamp mode, currents evoked by 3 μM capsaicin were recorded at -60 mV. Capsaicin-insensitive cells were excluded from analysis. These results show that SCI increases the likelihood that very low concentrations of a TRPV1 activator will cause persistent, non-accommodating firing of previously silent nociceptors.

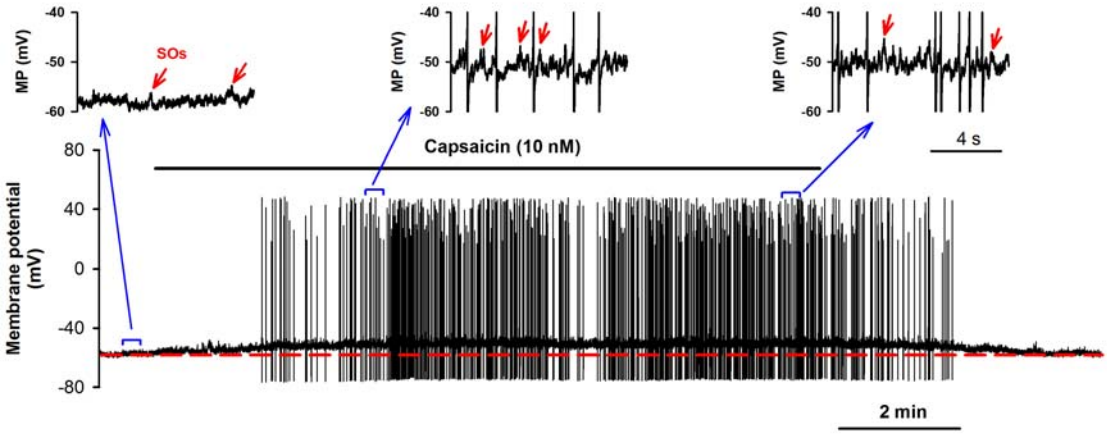

Figure 17. Prolonged application of low concentrations of capsaicin promotes SA without accommodation in subsequently dissociated nociceptors. Perforated patch recording showing non-desensitizing excitation of a dissociated DRG neuron by 11 min exposure to 10 nM capsaicin. Inserted traces from the same record show the increased amplitude of spontaneous membrane oscillations during the capsaicin-evoked depolarization. Action potentials and afterhyperpolarizations are clipped in the insert. Red arrows indicate subthreshold oscillations (SOs).

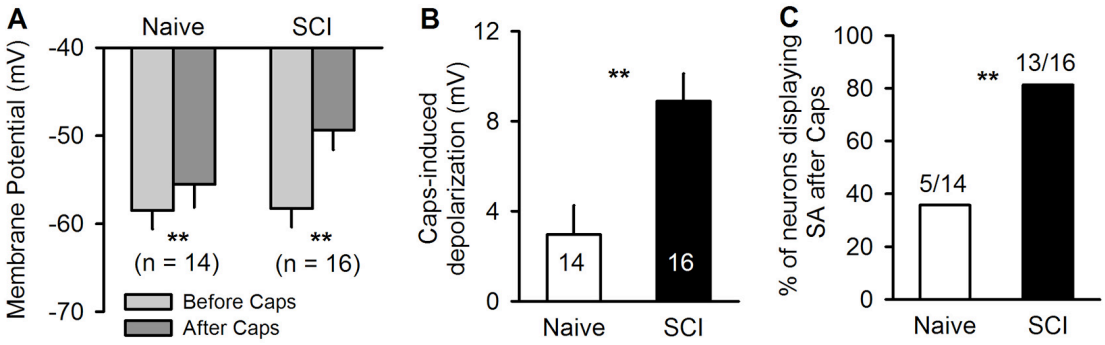

Figure 18. SCI enhances the sensitivity of DRG neurons upon limited TRPV1 activation. A and B, SCI increased capsaicin-induced depolarization in DRG neurons. C, increased incidence of firing evoked by prolonged application of 10 nM capsaicin in neurons dissociated from SCI animals. Caps, capsaicin.

6.3. Conclusions

6.3.1. Membrane depolarization and extracellular Ca^{2+} are important for generation of SA and repetitive firing in small DRG neurons

Large diameter DRG neurons fire repetitively upon depolarization, and the repetitive firing is sensitive to TTX and insensitive to Ca^{2+} channel blockers (Amir et al., 1999). Nociceptors are usually small and sometimes medium-sized DRG neurons, which express many differences in molecular and electrophysiological properties as compared to large DRG neurons. For example, small nociceptive DRG preferentially express TTX-resistant Na^{+} channels and TRPV1 channels (Akopian et al., 1996; Caterina et al., 1997). Thus, the molecular mechanisms underlying the generation of SA and repetitive firing may be different in nociceptors. Similar to what was described in large DRG neurons (Amir et al., 1999), depolarizing current injection induced non-accommodating repetitive firing in most capsaicin-sensitive DRG neurons. The few neurons that showed accommodation (little or no repetitive firing) were usually insensitive to capsaicin. These studies indicate that a function of TRPV1-expressing nociceptors is to fire repetitively upon modest depolarization. Voltage-gated Na^{+} channels are critical for action potential generation. In this study, 300 nM TTX had no effect on SA, suggesting that TTX-resistant Na^{+} channels (Nav1.8 and/or Nav1.9), but not TTX-sensitive Na^{+} channels are required for SA generation in dissociated nociceptors. In addition, I found that extracellular Ca^{2+} is important for SA generation. In this study, I simply substituted Ca^{2+} with Mg^{2+} in the extracellular solution, so there was still probably a physiologically significant amount of Ca^{2+} in the recording chamber

while the cells exposed to the nominally "Ca^{2+}-free" solution. Nevertheless, this result indicates that a sufficient amount of extracellular Ca^{2+} is also required for SA generation.

6.3.2. Limited activation of TRPV1 channels in DRG neurons is sufficient to generate repetitive firing without accommodation

Consistent with a requirement for depolarization and extracellular Ca^{2+} for SA generation, I found that very low concentrations of capsaicin induced membrane depolarization and repetitive firing similar to SA. I chose a low concentration of capsaicin because: 1) the endogenous activators of TRPV1 are probably very low in physiological (and pathological) conditions *in vivo* (Huang et al., 2002); 2) higher concentrations of capsaicin cause larger amounts of Ca^{2+} and Na^{+} to enter neurons, strongly depolarizing the neurons and inactivating voltage-gated Na^{+} channels, thus inhibiting the excitability of DRG neurons, as well as desensitizing the TRPV1 channels (Cholewinski et al., 1993; Koplas et al., 1997; Wu and Pan, 2007). In my studies, low concentrations of capsaicin induced modest membrane depolarization, and caused repetitive firing in some of the cells. An important feature of TRPV1 channel is desensitization upon prolonged or repeated exposure of activators (Cholewinski et al., 1993; Koplas et al., 1997; Touska et al.), which attenuates capsaicin-induced effects. However, my study indicated that little or no desensitization occurred with prolonged application (10 min) of 10 nM capsaicin, which produced constant depolarization of the cells before washout in perforated patch recordings. This experimental condition may better reflect the actions of

endogenous activators of TRPV1 channels in vivo during chronic pain conditions than the more common experimental conditions where high, desensitizing concentrations of capsaicin are applied.

6.3.3. SCI enhances the responsiveness of DRG neurons to limited activation of TRPV1 channels

As compared to DRG neurons dissociated from naïve rats, SCI enhanced not only the currents evoked by a saturating concentration of capsaicin, but also more physiological responses (depolarization and repetitive firing) to very low concentrations of capsaicin. This confirmed predictions based on the observed increases in TRPV1 expression and function after SCI. In vivo, SCI may cause an elevation of endogenous TRPV1 channel activators in the DRG and spinal cord, where they might produce the same effects as I found with prolonged application of a very low concentration of capsaicin, helping to depolarize and drive SA in nociceptors. Thus, after SCI, continuing activation of TRPV1 channels by low concentrations of endogenous ligands *in vivo* might contribute to the maintenance of nociceptor SA, and thereby to SCI-induced chronic pain.

CHAPTER 7: *Discussion*

The goal of the studies described in this dissertation was to test two hypotheses: 1) that SCI increases the expression and function of TRPV1 in primary nociceptors, contributing to intrinsic SA and hyperexcitability in these neurons, and 2) that behaviorally expressed hypersensitivity to heat and mechanical stimuli following SCI can be reduced by attenuating TRPV1 activity. As discussed below, these hypotheses were supported by my findings that SCI leads to upregulation of TRPV1 channels in DRG neurons, and enhances the sensitivity of isolated nociceptor somata to high and low concentrations of a selective activator of TRPV1, capsaicin. Unexpected support for these hypotheses also came from my discovery that very low concentrations of capsaicin can promote SA without producing obvious desensitization, and that this effect is enhanced by SCI. Most important, interventions that reduced TRPV1 function provided evidence that TRPV1 channels play an important role in maintaining chronic behavioral hypersensitivity after SCI.

7.1. SCI enhances capsaicin responses in small DRG neurons by upregulating TRPV1 channels.

My first hypothesis is strongly supported by the findings that upregulation of TRPV1 after SCI was associated with increased responses to a saturating concentration of capsaicin, as measured by voltage clamp (**Fig. 4**) and Ca^{2+} imaging (**Fig. 6**). These findings suggest that SCI produces a persistent increase

in the number of functional TRPV1 channels in the plasma membrane of primary nociceptors. I observed increases of capsaicin-induced responses in the cell bodies of DRG neurons 1 month, but not 3 days, after contusion, suggesting a delayed increase in TRPV1 function after SCI. However, TRPV1 upregulation in the spinal cord (L6/S1) was reported to be detectable immunohistochemically as early as 3 days after T9 transection (Zhou et al., 2002). This difference might reflect earlier increases of TRPV1 protein after SCI in central terminals of DRG neurons than in cell bodies, or differences in the type of spinal injury (transection versus contusion) or the TRPV1 measure (immunohistochemistry versus capsaicin sensitivity). A delayed effect on capsaicin sensitivity in DRG neuron somata after SCI might reflect a requirement for transport of SCI-induced factors from central terminals to the somata of DRG neurons (Walters, 2012).

The TRPV1-dependent effects I observed are unlikely to be direct results of axonal injury produced in the tested DRG neurons by SCI. Most C and Aδ fibers only project 1-2 segments from their segment of entry (Chung et al., 1979). In this study, DRG neurons were taken from L4/L5, which is far below the contusion site, so they were unlikely to be axotomized or directly damaged by the contusion (Bedi et al., 2010). However, descending and ascending fibers passing through the injured level are likely to be directly affected by SCI. Destruction or injury to these fibers could remove inhibitory and/or excitatory influences on dorsal horn neurons (and glia) that might in turn affect central terminals of primary afferents after SCI (Hubscher et al., 2008; Densmore et al., 2012; Walters, 2012).

A shift toward larger DRG neurons in TRPV1 channel distribution has been reported in nerve injury and cancer chronic pain models, although this effect might be triggered by axotomy (Ma et al., 2005; Shinoda et al., 2008). On the other hand, peripheral inflammation increases the proportion of TRPV1-positive cells in small and medium-sized DRG neurons (Amaya et al., 2003). Although I found that the fraction small DRG neurons responding to capsaicin was unchanged after SCI, I cannot exclude the possibility that TRPV1 also increases in larger neurons, because large DRG neurons were not sampled in my experiments. Indeed, an increase in soma diameter in TRPV1-positive DRG neurons (L4/L5) has been reported after high thoracic (T3), but not low thoracic (T10) spinal cord transaction (Ramer et al., 2012).

TRPV1 channel function might be enhanced in nociceptors following SCI due to increased expression of the channels. Increases in TRPV1 protein expression in the dorsal horn of the spinal cord have been found after SCI, as detected by immunohistochemistry and RT-PCR, (Zhou et al., 2002; DomBourian et al., 2006). Similar to a recent study in which high thoracic (T3) transection increased staining intensity of TRPV1 in L4/L5 DRG (Ramer et al., 2012), I found that TRPV1 expression was increased in L4/L5 DRG 1 month after T10 contusion. Upregulation of TRPV1 in primary afferent neurons has been described in various inflammatory situations and in undamaged neurons (likely exposed to inflammatory signals) in neuropathic pain models (Hudson et al., 2001; Fukuoka et

al., 2002; Ji et al., 2002; Breese et al., 2005; Kanai et al., 2005). A greater role of inflammation than axon injury in the upregulation of TRPV1 in L4/L5 nociceptors is suggested by the distance of these DRG from the T10 contusion site in my studies. Few primary nociceptor axons project that far, so few would be axotomized. In addition, widespread inflammation occurs in the spinal cord after SCI (Alexander and Popovich, 2009; Dulin et al., 2013) (affecting central processes of nociceptors), and inflammatory cells can inflitrate into DRG several segments away from an SCI site (McKay and McLachlan, 2004).

TRPV1 expression is regulated by NGF. In rats made hyperalgesic by peripheral inflammation, NGF is elevated in the inflamed skin (Woolf et al., 1994). This peripherally produced NGF is believed to be involved in TRPV1 upregulation in the DRG by activating the p38 signaling pathway (Ji et al., 2002). It is not known whether SCI increases NGF in DRG, but NGF levels are elevated in the spinal cord after SCI (Bakhit et al., 1991; Murakami et al., 2002; Brown et al., 2004). Elevated NGF may be communicated to primary sensory neurons via their central terminals, and this signaling might participate in the upregulation of TRPV1 channels in DRG neurons, thus increasing nociceptor excitability and contributing to the development of abnormal somatosensory behaviors after SCI (Gwak et al., 2003).

7.2. TRPV1 activation is important for nociceptor SA after SCI

We have previously demonstrated that SCI promotes SA in the cell bodies of small DRG neurons that usually respond to capsaicin (Bedi et al., 2010). Thus an important question is whether upregulated TRPV1 channels can enhance SA generation in nociceptors. Although the endogenous agonists of TRPV1 are uncertain, various lipid products are able to activate TRPV1 ion channels (Bang et al., 2010). For example, the endogenous cannabinoid receptor agonist anandamide and 12-lipoxygenase metabolites of arachidonic acid (AA) activate both native and cloned TRPV1 channels (Zygmunt et al., 1999; Hwang et al., 2000; Watanabe et al., 2003). Opening TRPV1 channels by PKC activation was also reported (Premkumar and Ahern, 2000). TRPV1 channel currents induced by endogenous activators may be very small or only detectable in a small proportion of DRG neurons (Shin et al., 2002). However, currents produced by endogenous activators may be enhanced after SCI, which could increase intrinsic SA and hyperexcitability of nociceptors since: 1) SCI induces a large accumulation of lipid products (Liu and Xu, 2010); 2) I have shown that the responsiveness of TRPV1 channels is enhanced following SCI; 3) and nociceptors with SA are depolarized and exhibit reduced thresholds for the triggering of action potentials (Study and Kral, 1996; Bedi et al., 2010). This possibility is strongly suggested by my finding of sustained repetitive firing in response to prolonged application of a very low concentration of capsaicin to dissociated DRG neurons. Many endogenous activators act through the capsaicin-binding site of TRPV1 (Sutton et al., 2005; Jara-Oseguera et al., 2008; Wen et al., 2012). Thus, I mimicked ongoing actions of this general type of activator by prolonged application of capsaicin at a dose (10

nM), which is far lower than the EC_{50} values reported for dissociated DRG neurons in rats (200-500 nM) (Wood et al., 1988; McLatchie and Bevan, 2001). Remarkably, the low dose of capsaicin caused prolonged firing that showed no desensitization or accommodation for as long as the drug was present (> 10 min). Importantly, significantly more nociceptors dissociated from SCI animals showed repetitive firing under this condition compared to neurons dissociated from naïve rats. This suggests that upregulation of TRPV1 channels after SCI may enhance the ability of endogenous TRPV1 activators to promote SA generation in nociceptors in vivo.

Capsaicin-induced depolarization was always associated with increased amplitude of spontaneous subthreshold oscillations of membrane potential (**Fig. 17**), which are thought to underlie SA in larger DRG neurons (Amir et al., 1999). Thus, it is interesting to know whether enhanced TRPV1 channel function participates in SA generation in small DRG neurons. As compared to mismatch ODN treatment, DRG neurons dissociated from TRPV1 antisense ODN-treated rats showed decreased SA incidence. Although TRPV1 channel activity is likely to be important for nociceptor sensitization after SCI in vivo, AMG9810 failed to decrease SA in dissociated DRG neurons, which suggests that intracellular activators of TRPV1 are not involved in the maintenance of nociceptor SA after isolation of the neurons. Nonetheless, SCI-induced intrinsic SA may be promoted in vivo by increased responsiveness to extracellular TRPV1 activators that may be released chronically after SCI. An additional possibility is that elevated activity of

DRG neurons after injury helps to sustain astrogliosis and microgliosis (Xie et al., 2009). Activated glial cells release cytokines, growth factors and other factors that enhance excitability of primary sensory neurons (Milligan and Watkins, 2009; Gao and Ji, 2010; Parpura and Zorec, 2010; Wang et al., 2012), which could produce a positive feedback loop, potentially contributing to the initiation and maintenance of nociceptor hyperexcitability (Miller et al., 2009; Walters, 2012). Breaking the positive feedback loop by knocking down TRPV1 might reduce or terminate intrinsic hyperexcitability in DRG neurons after SCI.

7.3. TRPV1 activation is important for behavioral hypersensitivity after SCI

The mechanisms just discussed suggest that TRPV1 activation may contribute to pain-related behavior, and these considerations led to my second hypothesis -- that reducing TRPV1 activity may reduce pain-related behavior after SCI. This hypothesis received strong support from my demonstration that behavioral hypersensitivity expressed by hindlimb withdrawal responses to heat and mechanical test stimuli after SCI was reversed by two interventions that reduce TRPV1 activity: acutely by delivery of the TRPV1 antagonist, AMG9810, and for longer periods by selective antisense knockdown of TRPV1 channels in lumbar DRG and spinal cord.

The contribution of TRPV1 channels to peripherally generated neuropathic pain has been controversial (Caterina et al., 2000; Pomonis et al., 2003; Maione et al., 2006). As heat-sensitive receptors, TRPV1 channels are expected to be important

for mediating heat hyperalgesia produced by inflammation or nerve injury. This expectation has been supported by the finding that peripheral delivery of a TRPV1 antagonist, iodoresiniferatoxin, attenuates noxious heat (45°C)-induced excitation of spinal wide dynamic range (WDR) neurons in vivo in anaesthetized rats after carrageenan-induced inflammation or L5/6 spinal nerve ligation (Jhaveri et al., 2005). On the other hand, behaviorally expressed thermal hypersensitivity still developed in TRPV1 knockout mice after partial nerve injury (Caterina et al., 2000). Likewise, the involvement of TRPV1 channels in mechanical hypersensitivity is unclear. It has been reported that TRPV1 knockout does not prevent the development of behaviorally expressed mechanical hypersensitivity after nerve ligation (Caterina et al., 2000; Christoph et al., 2008). Furthermore, peripheral delivery of iodoresiniferatoxin failed to attenuate responses of WDR neurons to mechanical stimuli in anaesthetized rats after spinal nerve ligation (Jhaveri et al., 2005). Moreover, the expression level of TRPV1 channel is reported to be downregulated in nociceptors in at least one peripheral nerve injury model (Michael and Priestley, 1999). It should be noted, however, that the downregulation only occurs in axotomized DRG neurons; peripheral nerve injury increases TRPV1 expression in undamaged DRG neurons (Hudson et al., 2001), including previously TRPV1-negative A-fibers (Rashid et al., 2003). Also, peripheral inflammation enhances TRPV1 expression in small and medium-sized DRG neurons (Ji et al., 2002; Breese et al., 2005). Therefore, it is widely accepted that the TRPV1 channel is important for behaviorally expressed hypersensitivity in various pathologic situations. Indeed, selective TRPV1 antagonists have been

reported to attenuate both thermal and mechanical hypersensitivity in several neuropathic pain models (Pomonis et al., 2003; Honore et al., 2005; Kanai et al., 2005; Drizin et al., 2006). Consistent with these pharmacological studies, knocking down TRPV1 in transgenic mice expressing shRNA against TRPV1 attenuated not only the development of thermal hyperlgesia, but also mechanical allodynia after spinal nerve ligation (Christoph et al., 2008).

Although TRPV1 channels are expressed most abundantly in primary afferent neurons (Caterina et al., 1997; Tominaga, 2007) they are also reported to be present in spinal dorsal horn neurons and to contribute at that locus to the maintenance of pain (Cui et al., 2006; Kim et al., 2012). A recent study described TRPV1 channels located in spinal cord GABAergic interneurons, and provided evidence that these spinal TRPV1-expressing neurons are important for intrathecal capsaicin-induced mechanical hypersensitivity by showing reduction of hypersensitivity in TRPV1-/- mice and in wild-type mice in which TRPV1 expressing neurons were desensitized by intrathecal spinal injection but not systemic injection of resiniferatoxin (Kim et al., 2012), an ultrapotent TRPV1 agonist (Szallasi and Blumberg, 1999). However, this study does not exclude the involvement of TRPV1-expressing primary afferent neurons in mechanical hypersensitivity. For example, selectively silencing TRPV1-expressing axons in the sciatic nerve by introducing QX-314 (a membrane-impermeable Na^+ channel blocker) into neurons via opening of TRPV1 channels abolished inflammation-induced thermal and mechanical hyperalgesia, but not mechanical allodynia

(Brenneis et al., 2013). This suggests that TRPV1-expressing neurons may be more important for responses to stronger mechanical stimuli than those typically used in mechanical allodynia studies (Brenneis et al., 2013). Pharmacological evidence also indicates that in addition to its role in mediating thermal hypersensitivity, TRPV1 channels expressed in primary afferent neurons also play an important role in mechanical allodynia (Cui et al., 2006). Systemic application of A-795614, a peripherally restricted TRPV1 antagonist, significantly reduced mechanical allodynia in a peripheral inflammatory pain model, although less effective than A-784168, a TRPV1 antagonist with good blood-brain barrier penetration. Both antagonists showed similar potency on thermal hyperalgesia (Cui et al., 2006). This study did not distinguish between effects of the centrally acting blocker on the central terminals of primary afferents and on TRPV1-expressing dorsal horn neurons. Importantly, a recent study using an ultra-sensitive genetically expressed TRPV1 reporter in mice failed to detect any TRPV1 expression in the spinal cord (Cavanaugh et al., 2011), and it has been pointed out that none of the claims for TRPV1-expressing neurons in the CNS (with the exception of a population of neurons in the supramammilary nucleus of the rostral midbrain) have identified or cloned TRPV1 transcripts in the CNS (Zhang et al., 2012). Thus, the analgesic effect I showed to be induced by intrathecal injection of TRPV1 antisense ODN may mainly come from its effect on primary sensory neurons, and include effects on presynaptic TRPV1 channels in nociceptor terminals in the dorsal horn.

TRPV1 channel agonists are used for pain therapy based on their ability to desensitize TRPV1 channel-containing neurons (Knotkova et al., 2008). However, the initial discomfort caused by agonist administration limits their use in clinical settings. Anti-hyperalgesic effects induced by antagonists of TRPV1 have also been observed in various rodent pain models (Gavva et al., 2005; Cui et al., 2006; Yu et al., 2008). In this regard, administration of AMG9810 (i.p.), a brain permeable antagonist that blocks all the major modes of TRPV1 activation (Gavva et al., 2005), dramatically attenuates mechanical allodynia and thermal hyperalgesia caused by Freund's adjuvant injection (Gavva et al., 2005; Yu et al., 2008). Knocking down the expression of TRPV1 channels is also sufficient to reverse behaviorally expressed hyperreflexia (mechanical and thermal) induced by nerve injury and inflammation (Christoph et al., 2006; Christoph et al., 2007; Christoph et al., 2008). These findings indicate that TRPV1 channel is involved not only in the maintenance of thermal hyperalgesia as discussed above, but also mechanical allodynia. This is important because SCI produces both mechanical allodynia and thermal hyperalgesia. Indeed, I found that the TRPV1 antagonist AMG9810 reverses thermal and mechanical hyperalgesia following SCI. I further confirmed the contribution of enhanced TRPV1 channel function to SCI-induced chronic pain-related behavior by knocking down TRPV1 channels (in DRG and possibly spinal cord neurons) and showing that behaviorally expressed heat and mechanical hypersensitivity were both relieved.

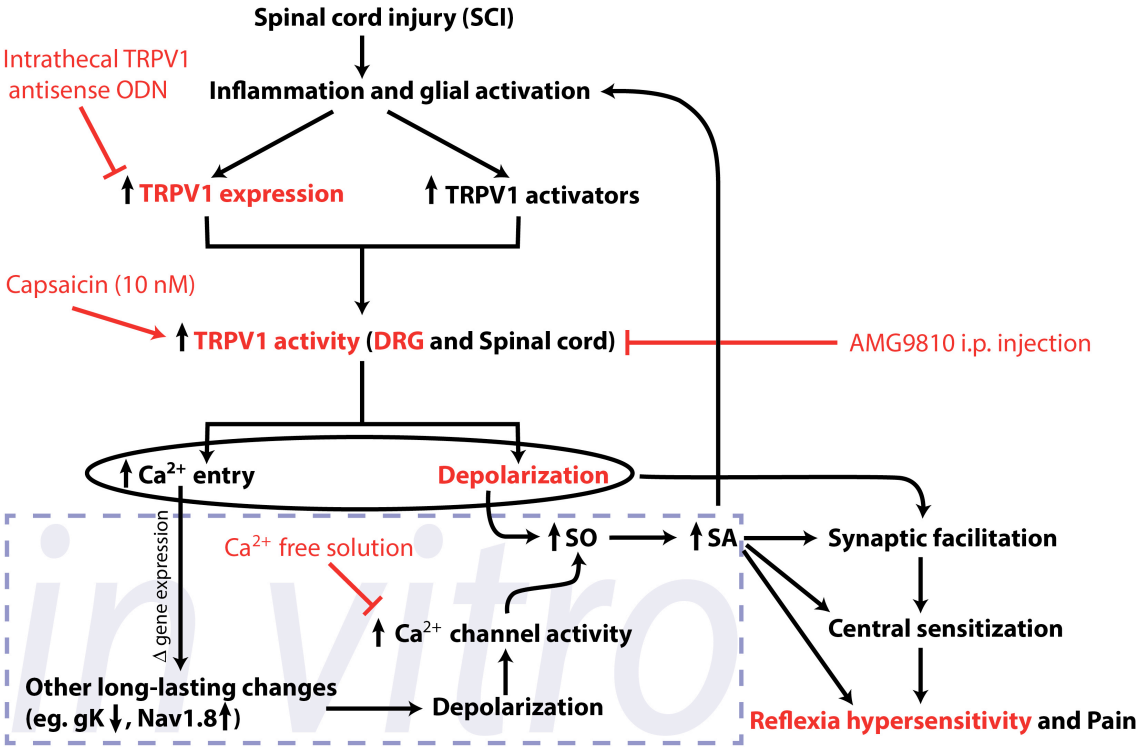

Figure 19. Potential general mechanisms involved in SCI-induced enhancement of TRPV1 function that promotes nociceptor SA and pain. The red color indicates the experiments performed in this study. The blue box indicates the intrinsic, transcriptionally-dependent alterations necessary for intrinsic SA (revealed after nociceptor dissociation). This intrinsic SA component together with ongoing activation of TRPV1 channels in the CNS and periphery in vivo may be necessary for chronic behavioral hypersensitivity and pain. SO, subthreshold oscillations; SA, spontaneous activity; gK, K^+ channel.

Although my studies show that TRPV1 function is persistently enhanced by SCI and is important for both nociceptor sensitization and behavioral hypersensitivity after SCI, recent findings have raised questions about the clinical utility of TRPV1 antagonists. Specifically, blockade of TRPV1 channels by commonly used TRPV1 blockers, including AMG9810, produces a significant and undesirable side effect of hyperthermia (Gavva et al., 2008; Othman et al., 2012). Fortunately, a group of TRPV1 blockers has now been discovered that lack this problematic side effect and these new drugs appear to offer considerable promise for targeting TRPV1 channels to treat chronic pain (Reilly et al., 2012). My results suggest that these drugs may be useful for treating the chronic, intractable pain that often follows SCI.

7.4. Model for TRPV1 channels' contributions to chronic behavioral hypersensitivity and pain after SCI

The results reported in this thesis support the following model for how TRPV1 channels contribute to SCI-induced chronic pain (**Fig. 19**). As discussed above, it has been well established that glial cell activation and inflammatory factors play an important role in chronic pain following SCI. SCI-induced inflammation is not limited to the injury site; it spreads to both the brain, caudal spinal cord, and other organs (Zhao et al., 2007; Detloff et al., 2008; Carlton et al., 2009; Herrera et al., 2010; Dulin et al., 2011; Dulin et al., 2013). Thus, inflammatory mediators may directly interact with primary sensory neuronal terminals in both the CNS and

periphery. In addition, inflammatory factors infiltrate into the dorsal root ganglia that contain the cell bodies of primary sensory neurons (McKay and McLachlan, 2004). Similar to several inflammatory pain models (Ji et al., 2002; Breese et al., 2005; Obata et al., 2005; Alessandri-Haber et al., 2008), SCI upregulates TRPV1 in primary sensory neurons, as well as in superficial dorsal horn where nociceptor synaptic terminals are (Zhou et al., 2002). The endogenous agonists of TRPV1 are uncertain. However, it has been shown that various lipid products and other signals are able to sensitize or activate TRPV1 ion channels (Bang et al., 2010), including the endogenous cannabinoid receptor agonist anandamide, metabolites of arachidonic acid (AA), (Zygmunt et al., 1999; Hwang et al., 2000). It is unknown whether lipid products are elevated in DRG after SCI, but it has been demonstrated that SCI induces marked accumulation of AA and its cyclooxygenase and 5-lipooxygenase products, including prostaglandin E2 (PGE2) and leukotrienes B4 (LTB4), in the spinal cord (Liu and Xu, 2010; Dulin et al., 2013). Moreover, both LTB4 and PGE2 have been shown to directly activate or sensitize TRPV1 channels (Lopshire and Nicol, 1998; Hwang et al., 2000; Moriyama et al., 2005). Together with TRPV1 upregulation, lipid signals accumulating in the spinal cord may increase the activity of TRPV1 channels in the central processes of DRG neurons, as well as TRPV1 channels in dorsal horn neurons, thus facilitating synaptic transmission and amplifying peripheral input to pain projection neurons in the dorsal horn. TRPV1 channels are Ca^{2+} and Na^{+} permeable (Tominaga, 2007). Thus, the activity of TRPV1 channels will result in direct depolarization of DRG neurons to increase SOs and elicit SA. It has been

reported that elevated activity of DRG neurons after injury promotes astrogliosis and microgliosis (Xie et al., 2009), which release cytokines, growth factors and other factors that enhance excitability of primary sensory neurons (Milligan and Watkins, 2009; Gao and Ji, 2010; Parpura and Zorec, 2010; Wang et al., 2012), This positive feedback loop potentially contributes to the initiation and maintenance of nociceptor hyperexcitability (Miller et al., 2009; Walters, 2012). Breaking the positive feedback loop by blocking TRPV1 might reduce intrinsic SA/hyperexcitability in DRG neurons after SCI. Interrupting this feedback loop to reduce intrinsic SA might require changes in nociceptor gene transcription (see below) and thus might be evident after knockdown of TRPV1 over several days in vivo (**Fig. 7, 9B**) but not after brief application of a TRPV1 antagonist in vitro (**Fig. 9C**).

The Ca^{2+} entering through TRPV1 channels is likely to play an important role in controlling gene expression in nociceptors. In addition to enhancing TRPV1 channel expression (**Fig. 7**), SCI elevates the function of TTX-sensitive Na^{+} channels and the expression of TTX-resistant Nav1.8 channels in DRG neurons (Yoshimura and de Groat, 1997; Yang et al., 2012). We also observed that SA in nociceptors is associated with chronic membrane depolarization and increased membrane resistance in the contusive SCI model (Bedi et al., 2010). This observation strongly suggests that intrinsic SA is also promoted by chronic closing or downregulation of K^{+} channels, which would not only increase membrane resistance but depolarize the cell soma. The membrane depolarization may

activate voltage-gated Ca^{2+} channels (and/or other Ca^{2+}-sensitive proteins, see Fig. 15) to increase the amplitude of SOs and enhance the generation of SA (**Fig. 19**). Thus, SCI produces multiple intrinsic alterations in nociceptors (perhaps representing a discrete hyperexcitable-SA state; Bedi et al., 2010; Walters, 2012) that probably depend upon altered gene expression and that can persist for at least a day after isolation of the nociceptors.

An important question is why a TRPV1 channel blocker, AMG9810, rapidly reverses behavioral hypersensitivity after SCI when injected *in vivo* (**Fig. 8**), but the same blocker fails to reduce nociceptor SA when applied 1 day after dissociation **(Fig. 11C**). Part of the answer is that SA after SCI can occur in isolated noiceptors without the need for continuing activation of TRPV1 channels because of the intrinsic, transcriptionally-dependent alterations just described. However, while nociceptor SA also occurs in vivo (Carlton et al., 2009; Bedi et al., 2010), and this SA may be necessary for chronic behavioral hypersensitivity and pain, either the nociceptor SA itself, or the degree of SA supported by the intrinsic alterations, may not be sufficient to drive the behavioral alterations, and thus additional TRPV1 actions may be required for behaviorally expressed effects in vivo. In our previous study, we demonstrated that SCI induces intrinsic SA in small DRG neurons that is correlated with hypersensitivity to various stimuli (Bedi et al., 2010). However, it is not known whether this low frequency intrinsic SA alone is enough to induce and maintain SCI-induced pain behaviors. Our *in vivo* recording from teased dorsal root filaments indicates that both SA incidence and

frequency in primary sensory neurons are higher when the neurons are connected to the periphery than when the DRG and dorsal roots are disconnected from the periphery (Bedi et al., 2010). Thus, SA incidence and frequency in nociceptors connected to both the periphery and dorsal horn should be greater than that observed *in vitro*, and TRPV1 channels may enhance peripherally generated SA and evoked activity in vivo after SCI. These additional TRPV1 components may also play an important role in central sensitization and behavioral hypersensitivity. Yet another TRPV1 component of the pain pathway that would be blocked by AMG9810 *in vivo* but not *in vitro* are the TRPV1 channels on the presynaptic terminals of nociceptors, which enhance transmission at the first synaptic relay of the pain pathway (Kim et al., 2009). In addition, AMG9810 would be expected to block any functionally important TRPV1 channels on other pain-related neurons in the spinal cord and brain (Starowicz et al., 2008; Zhou et al., 2009; Kim et al., 2012). Future studies of these additional TRPV1 channel contributions to *in vivo* behavioral alterations should further define the roles of this important channel in SCI-induced chronic pain.

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Vita

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