ROLE OF THE NKCC1 CO-TRANSPORTER IN SPINAL NOCICEPTIVE MECHANISMS IN A RODENT MODEL OF PERIPHERAL INFLAMMATION

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Abstract

The Gate Control Theory describes a spinal circuit whereby activity in low threshold mechano-sensitive fibers attenuates activity in nociceptive fibers, such that innocuous touch eases pain. Paradoxically, for many chronic pain sufferers, lightly touching the injured (primary) region or adjacent uninjured (secondary) region may evoke pain rather than ease pain, phenomena referred to as primary and secondary allodynia, respectively. We hypothesize that enhanced activity of the NKCC1 chloride co-transporter in persistent pain states may underlie this effect. NKCC1 is the main chloride accumulator in primary sensory fibers; by setting the intracellular chloride concentration, NKCC1 determines the magnitude of primary afferent depolarization (PAD) and, consequently, whether or not low intensity mechanical input inhibits or produces activity in nociceptive afferent fibers. In this thesis, we use multiple research techniques to determine the role of NKCC1 in capsaicin-induced allodynia. Capsaicin, the active ingredient in hot chili peppers, produces action potentials in nociceptive afferent fibers by activating TRPV1 receptors in the peripheral terminals. First, we demonstrate that spinal NKCC1 blockade as well as spinal TRPV1 blockade reduces intracolonic capsaicin-evoked secondary allodynia as well as TRPV1-mediated allodynia. Next, we determined that AMPA/kainate but not TRPV1 receptor blockade attenuates capsaicin-evoked increases of NKCC1 expression, suggesting a role for pre-synaptic AMPA/kainate receptors as regulators of NKCC1 expression. Spinal NKCC1 blockade also reduces intracolonic capsaicin-induced increase of FOS labeling in the spinal cord. Interestingly, NKCC1 blockade also partially prevents further enhanced FOS labeling following low intensity stimulation of the

region of secondary allodynia. Finally, spinal NKCC1 blockade prevents capsaicin-evoked sensitization of spinal dorsal horn nociceptive neurons, illustrating for the first time that enhanced NKCC1 activity plays a critical role in central nervous system processing of nociceptive input. Together, these studies provide comprehensive support for NKCC1's role in persistent pain as well as evidence that NKCC1 may be a useful therapeutic target.

Résumé

La théorie du portillon décrit un circuit spinal dans lequel l'activation des fibres mécano-sensitives de bas seuil atténue l'activité des fibres nociceptives, de tel sorte que le toucher léger réduit la douleur. Paradoxalement, pour plusieurs personnes souffrant de douleur chronique, la stimulation tactile de la région affectée (région primaire) ou la région adjacente à la lésion (région secondaire) provoque de la douleur au lieu de la réduire, phénomène respectivement nommé allodynie primaire et allodynie secondaire. Nous suggérons que dans les cas de douleur persistante, l'augmentation de l'activité du co-transporteur de chlore, NKCC1, pourait expliquer ce phénomène. NKCC1 est le principal responsable de l'accumulation intracellulaire de chlore au niveau des fibres sensitives primaires; en ajustant la concentration intracellulaire de chlore, NKCC1 détermine la magnitide de la dépolarisation afférente primaire et, par conséquant, déterminera si une stimulation mécanique de basse intensité activera ou inhibera les fibres nociceptives afférentes. Dans cette thèse, plusieurs techniques de recherche sont utilisées pour déterminer le rôle de NKCC1 dans l'allodynie induite par capsaicine. La capsaicine, la molécule active des piments forts, produit des potentiels d'action au niveau des fibres nociceptives afférentes en activant les récepteurs TRPV1 dans les terminaisons périfériques. Premièrement, nous démontrons que lorsque NKCC1 ou TRPV1 sont bloqués au niveau spinal, il y a une réduction de l'allodynie induite par la capsaicine intracolonique ainsi que de l'allodynie induite par l'administration spinal d'un agoniste de TRPV1. Ensuite, nous démontrons que le blocage des récepteurs AMPA/kainate atténuent l'augmentation de l'expression de NKCC1 par la capsaicine, alors que le blocage

des récepteurs TRPV1 n'ont pas d'effet. Cela suggère un rôle des récepteurs pre-synaptiques AMPA/kainate comme régulateur de l'expression d'NKCC1. Dans la moelle épinière, le blocage de NKCC1 réduit aussi l'augmentation de l'expression de FOS induite par la capsaicine intracolonique. Il est aussi intéressant de noté que le blocage de NKCC1 prévient partiellement l'augmentation de l'expression de FOS suivant une stimulation de faible intensité de la région d'allodynie secondaire. Finalement, le blocage de NKCC1 prévient la sensibilisation par la capsaicine des neurones nociceptives de la corne dorsal de la moelle épinière. Cela illustre que l'augmentation de l'activité de NKCC1 joue un rôle modulateur sur les signaux nociceptif afférant. L'ensemble de ces études soutiennent le rôle d'NKCC1 dans la persistance de la douleur et démontre que NKCC1 est une cible thérapeutique prometteuse.

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Alle Ding' sind Gift, und nichts ohn' Gift; allein die Dosis macht, daß ein Ding kein Gift ist.

"All things are poison and nothing is without poison, it is only the dose that makes a thing not be poisonous."

-Paracelsus
(1493-1541)

CHAPTER 1

GENERAL INTRODUCTION

1.1. Pain through the ages

Pain has long been a topic of philosophical and physiological discussion. Many great historical figures contributed to our understanding of pain either through fortuitous discoveries of pain-relieving compounds or through the proposal of pain mechanisms. Surgical techniques were also developed, however, they were not always effective. For example, archeologists studying cave paintings as well as skeletal remains of neolithic humans found evidence for the use of trephanation or the drilling of a hole in the skull, apparently to alleviate various ailments including seizures and migraines (Fig. 1). Interestingly, trephanation is still used today, however, not for the same reasons. Now termed *craniotomy*, the procedure is used to gain access to brain regions for neurosurgery as well as for relieving intracranial pressure.

1.2. Hippocrates

Best known for the oath repeated in his name by newly ordained medical doctors, Hippocrates (460 BC – 370 BC) noted that chewing the bark and leaves of the willow tree relieved pain. Aspirin (acetylsalicylic acid), a derivative of the compound salicin found in willow bark and meadowsweet, was isolated in 1829 by Pierre-Joseph Leroux, a French pharmacist. In fact, Aspirin got its name from the latin name for meadowsweet, *Spiraea ulmaria,* by Bayer AG, the company that first marketed acetylsalicylic acid in 1897.

Hippocrates also was influential in developing the idea that illness, including pain, was due to an imbalance of the four humours, namely blood, phlegm, black bile and yellow bile. Galen continued to develop the idea, popularizing the use of purging and bloodletting to balance the humours.

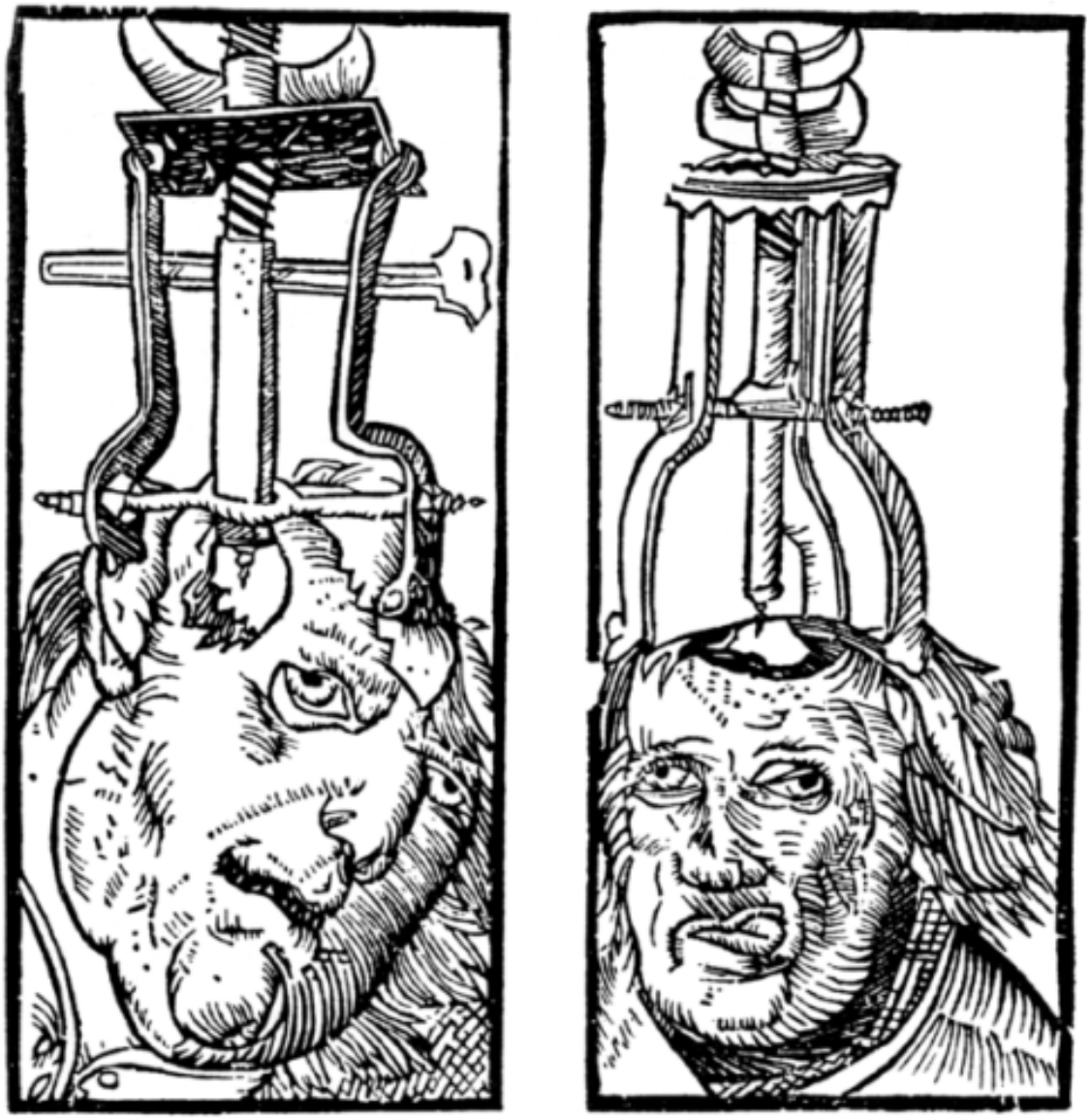

Figure 1: *1525 engraving by Peter Treveris, from the Handywarke of surgeri*

1.3. Galen

Galen, a Greek physician and philosopher who lived between 130 AC - 200 AC, rightly said that *"pain is useless to the pained".* However, evolutionarily speaking, this statement does not seem to make sense. In fact, according to the definition of the International Association for the Study of Pain (IASP), pain is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey & Bogduk, 1994). As such, pain is a danger signal that is both useful and necessary to the survival and wellbeing of the perceiver. However, pain is a double-edged sword: It’s intolerable salience is devastating when co-opted by physiological changes that prevent inactivation of the signal, rendering pain useless, unnecessary and burdensome. Thus, pain that is characterized by persistence and negligible survival value by not signalling impending or actual tissue damage, is indeed “*useless to the pained*”. As a physician who treated the gladiators’ traumatic wounds (Fig. 2), Galen was probably referring to the unrelenting, needless pain that we now refer to as *chronic pain*.

1.4. Paracelsus

According to Paracelsus, the dose makes the poison. Born Phillippus Aureolus Theophrastus Bombastus von Hohenheim (1493–1541) in Salzburg, Austria, Paracelsus reintroduced opium to Western medicine in 1527 after a voyage to the Arabic countries. A solution of opium in ethyl alcohol, laudanum (from Latin laudare, meaning to praise) rapidly became a standard in the physician’s repository for the treatment of pain, insomnia and many other irritations. Opium is derived from the opium poppy (*Papaver somniferum*). Since ancient times, its

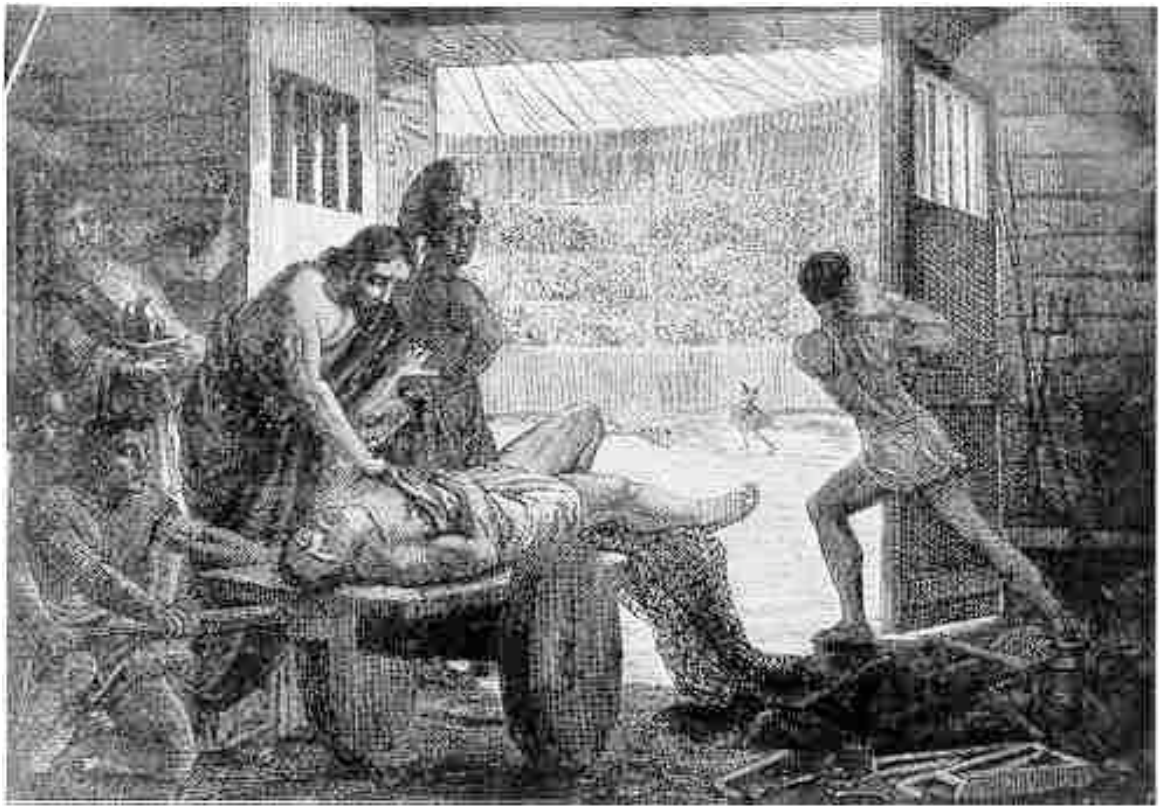

***Figure 2**: Galen, treating a gladiator. From Major, Ralph H., A History of Medicine. Springfield, IL: Charles C. Thomas, 1954.*

latex-like sap has been refined for use as a recreational drug as well as for pain relief. The opium poppy is the source of a number of compounds with medical and recreational uses. Specifically, morphine, codeine, and heroin, among others, are made from the opium poppy. Despite ongoing and intense study of mechanisms and potential therapeutic approaches for chronic pain, morphine continues to be among the most effective tools available.

1.5. Descartes

Rene Descartes (1596-1650), famous for his "*cogito, ergo sum*" as well as his dualism, was the first to describe a specific pain pathway (Fig. 3), where a "thread" pulled in the periphery by heat from a flame travels through the spinal cord to the pineal gland in the brain, where it "opens a valve letting the animal spirits flow from the cavity" in order for the animal to take protective measures. In the case of humans, it also signals to the mind that there is pain. Descartes' pain pathway also was the first explanation that implied that pain occurs in the brain rather than at the location of injury. Interestingly, consistent with the dualist idea that the body is a machine and only humans have "minds", Descartes did not believe that non-human animals felt pain. Instead, their responses to painful stimuli represented a purely mechanical reflex.

1.6. Wilhelm Heinrich Erb

Descartes conceptualization of pain was that pain signals were relayed to the brain by a specific pain system. In contrast, Wilhelm Erb (1840 - 1921), a German neurologist, proposed that a pain signal is produced in any sensory fiber type given sufficient stimulus intensity or pattern. Although this theory has since been

***Figure 3**: Descartes pain pathway, from Treatise of Man (1664).*

disproved with regard to peripheral sensory fibers, to an extent it does apply to a class of spinal nociceptive neurons called wide dynamic range (WDR) neurons; WDR neurons receive input from both peripheral low threshold mechano-receptive fibers as well as nociceptors (nociceptive afferent fibers). As such, they are best characterized by their graded response to graded stimuli. In other words, more intense stimuli are coded by more action potential activity.

1.7. Maximilian Ruppert Franz von Frey

Well known to any modern pain neuroscientist for the development of possibly the most-used pain assay, von Frey (1852 - 1932) was an Austrian-German physiologist who first used calibrated mono-filaments applied to the skin to demonstrate that the skin is composed of an intricate mosaic of sensory spots distinguishing touch and pain. His work contributed to the evidence that Erb's theory (see above) was incorrect such that there exist minute regions of the skin that are specific for responding to a painful stimulus while others respond preferentially to innocuous touch.

1.8. Melzack and Wall's Gate Control Theory

Melzack and Wall's (1965) Gate Control Theory of pain proposed that activity in large, myelinated Aβ primary sensory fibers inhibits activity in nociceptive Aδ/C fibers through interneurons (Fig. 4). In addition, the Gate Control Theory also proposes descending controls, where activity in supraspinal structures influences spinal nociceptive processing. Melzack and Wall's theory has had a profound impact on the study of pain: Firstly, it clearly grounded psychological influences and effects on a solid physiological foundation. Secondly, it generated an intense

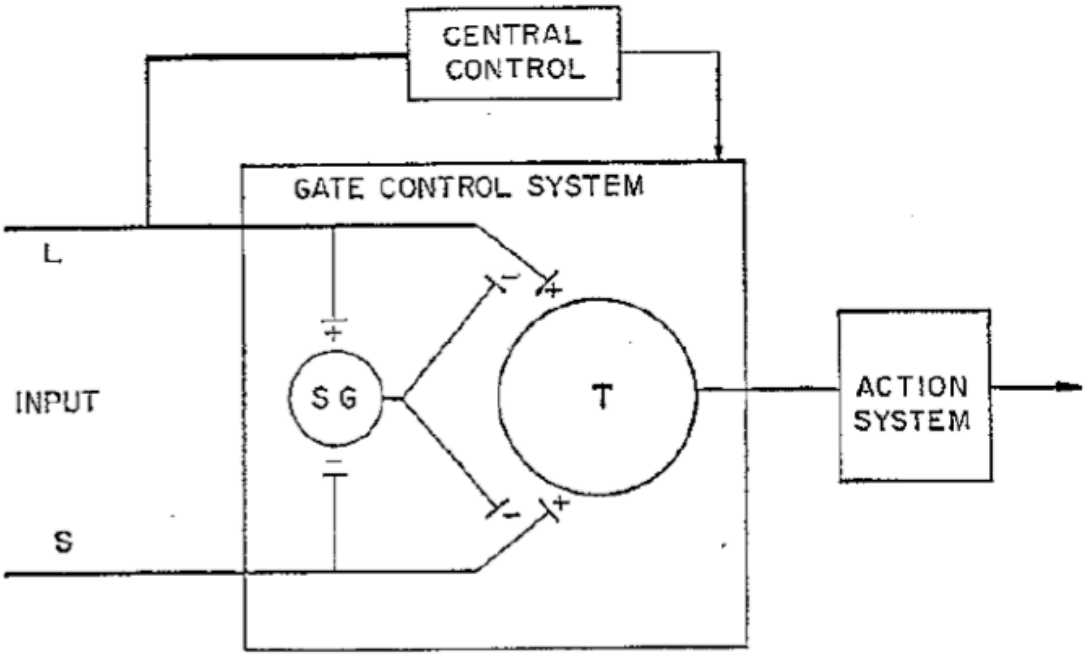

Fig. 4. Schematic diagram of the gate control theory of pain mechanisms: *L*, the large-diameter fibers; *S*, the small-diameter fibers. The fibers project to the substantia gelatinosa (*SG*) and first central transmission (*T*) cells. The inhibitory effect exerted by *SG* on the afferent fiber terminals is increased by activity in *L* fibers and decreased by activity in *S* fibers. The central control trigger is represented by a line running from the large-fiber system to the central control mechanisms; these mechanisms, in turn, project back to the gate control system. The T cells project to the entry cells of the action system. +, Excitation; —, inhibition (see text).

FIGURE 4: *Gate Control Theory diagram from Melzack and Wall (1965)*

interest among anatomists and physiologists to find the elusive gate in the spinal cord. In other words, the Gate Control Theory became a gold rush for pain researchers, producing many important discoveries in the field, some related to the theory and some not. The overall effect was a modernized perspective of pain as well as a renewed interest in explaining it. The physiological substrate for the Gate Control Theory is proposed to be primary afferent depolarization (PAD) in which an impulse in one primary afferent fiber depolarizes the central terminals of another afferent fiber through a pre-synaptic, axo-axonic connection by a GABAergic interneuron. Depolarization of the central terminal by GABA is dependent on the relatively high intracellular chloride $[Cl^-]_i$ concentration, set by the Na^+, K^+, $2Cl^-$ co-transporter type 1 (NKCC1); Activated pre-synaptic $GABA_A$ receptors thus channel chloride out of the neuron producing a sub-threshold depolarization that inactivates a proportion of voltage-gated sodium channels (Willis, 1999), thereby reducing the magnitude of the incoming action potentials and, consequently, decreasing excitatory amino acid release (Fig. 5A/B). Therefore, the Gate Control Theory provides a perceptual correlate of PAD in the context of acute pain, where low threshold mechanical stimulation (e.g. rubbing) of the injured area soothes pain. However, as mentioned above, for those suffering from persistent inflammatory pain, light touch is often excruciatingly painful (Treede et al. 1992). This hypersensitivity is generally referred to as hyperalgesia. Primary hyperalgesia is an abnormal sensory phenomenon that involves an enhanced response to non-noxious (allodynia) and/or noxious stimulation (hyperalgesia) in the region of injury. Secondary (or referred) hyperalgesia refers to an enhanced response to stimulation in a region remote

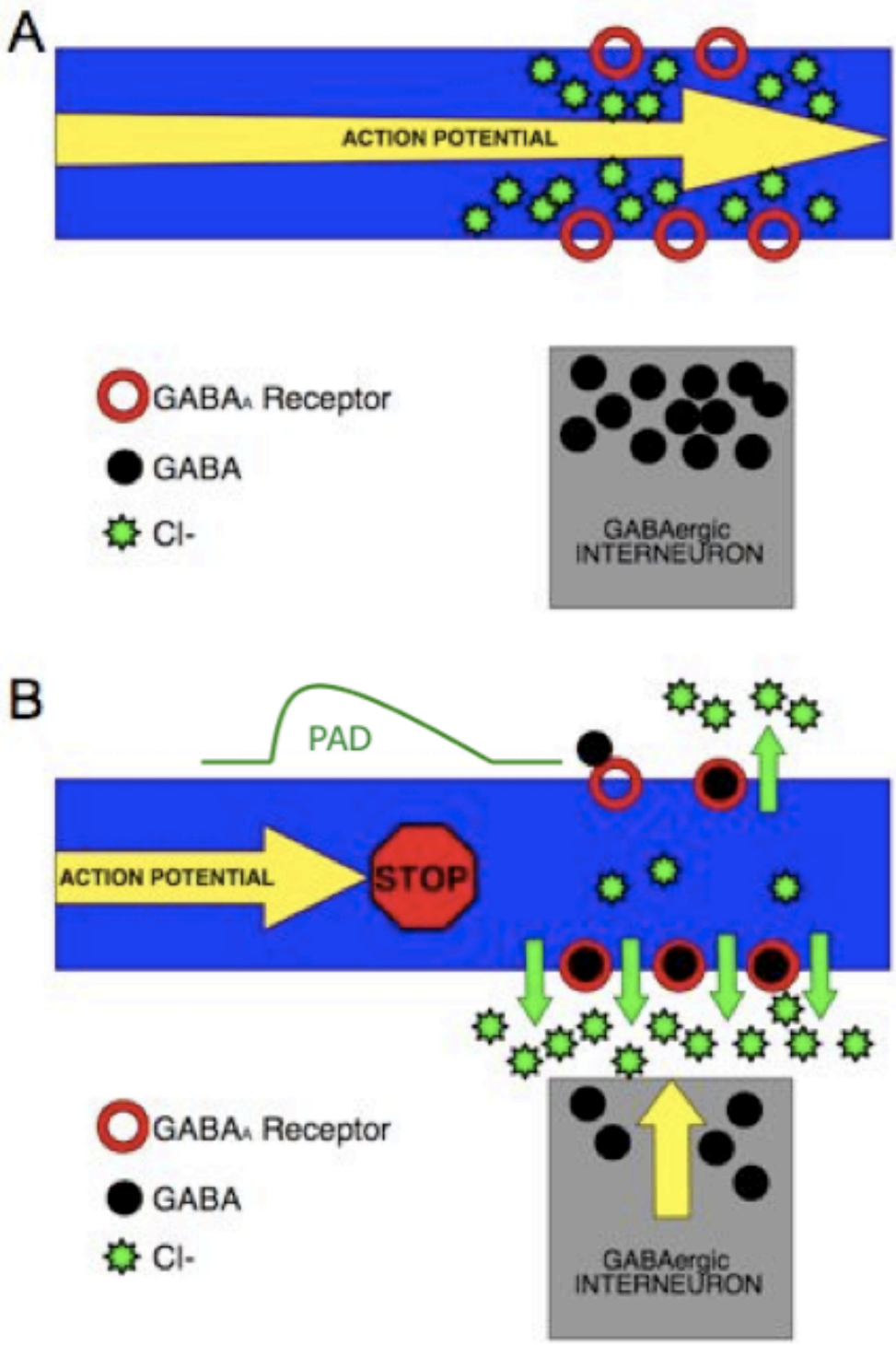

Figure 5: Schematic representation of NKCC1's role in PAD. (A) *Without interneuronal release of GABA, action potentials are transmitted along the afferent fiber.* ***(B)*** *GABA released from interneurons binds to $GABA_A$ receptors on the afferent fiber, allowing outwardly directed Cl- flux and a slight depolarization due to NKCC1-dependent Cl- accumulation. This Cl- shunt reduces neurotransmitter release and subsequent post-synaptic activity.*

from the originating injury. In the present research project, we test the hypothesis that primary and secondary hyperalgesia associated with persistent inflammation results from enhanced NKCC1 activity at the first sensory synapse such that PAD produces increased spike activity of dorsal horn nociceptive neurons (Fig. 6). In other words, in terms of spinal NKCC1 activity, does the dose make the poison?

CHAPTER 2

BACKGROUND

2.1. Role of primary afferent depolarization (PAD), pre-synaptic inhibition, and dorsal root potentials (DRP's) in sensory processing.

Barron and Matthews (1938) provided some of the earliest evidence for PAD; negative depolarizing dorsal root potentials (DRP's) were recorded from the central stumps of cut dorsal roots following electrical stimulation to adjacent roots. DRP's are efferent (antidromic) sub-threshold discharges recorded in afferent fibers. In 1957, Frank and Fuortes elucidated the pre-synaptic nature of PAD; volleys of electrical stimulation to primary afferent fibers reduced intracellularly-recorded, mono-synaptic excitatory post-synaptic potentials (EPSP's) without concomitant changes in membrane conductance, suggesting a pre-synaptic mechanism. In the early 1960's Eccles and colleagues demonstrated that the time course of the negative depolarizing DRP correlated with the positive, hyperpolarizing aspect (P wave) of the cord dorsum field potential recorded from the spinal cord following electrical stimulation of the sciatic nerve (Eccles et al. 1963), suggesting a depolarization-induced inhibition of afferent input to the spinal cord. In terms of somatosensory information processing, PAD has traditionally been conceived to act much like a noise-reducing filter where superfluous afferent information is reduced (Rudomin, 1999), particularly for rythmic movements such as locomotion or scratching (Rudomin and Schmidt, 1999).

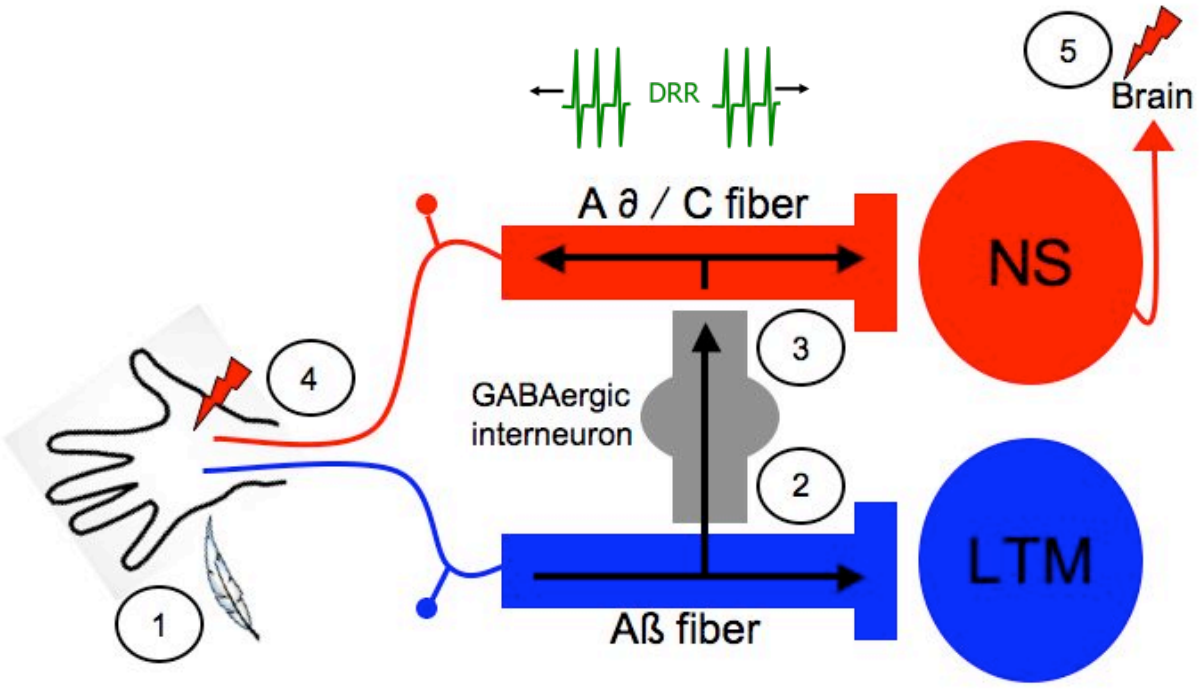

Figure 6: Schematic drawing of the excessive primary afferent depolarization (PAD) hypothesis of touch-evoked pain *(C). Under conditions of inflammation, NKCC1 activity is enhanced and* $[Cl^-]_i$ *is higher than normal. Low threshold input (#1) to the receptive field of an Aß (blue) fiber activates both a low threshold mechano-sensitive dorsal horn neuron (LTM; blue circle) as well as a GABAergic interneuron (#2; grey). Activated pre-synaptic* $GABA_A$ *receptors on the nociceptive Aδ/C (red) fiber shunt Cl- producing PAD of sufficient intensity to generate orthodromic and antidromic action potentials (DRR's; #3). Antidromic action potentials produce neurogenic inflammation in the periphery (#4) while orthodromic action potentials activate dorsal horn nociceptive neurons (NS; red circle) resulting in touch-evoked pain (#5).*

2.1.1. Neurogenic inflammation

Neurogenic inflammation refers to an inflammatory-like reaction (swelling, edema, warmth and pain) in a peripheral tissue that is generated by C-fiber-evoked release of inflammatory mediators such as substance P (SP), CGRP and bradykinin (Holzer, 1998). Neurogenic inflammation is distinct from normal inflammation in that it is evoked by neuronal activity rather than an inflammmatory/immune response. However, that is not to say that inflammatory and/or immune mediators can not elicit neurogenic inflammation through neuronal activation (Holzer, 1998). In peripheral sensory fibers, antidromic action potentials are typically referred to as dorsal root reflexes (DRR's) and are produced by excessive PAD in the central branch of the axon (Willis, 1999). However, another form of antidromic activity may also play a role in neurogenic inflammation: the axon reflex. The axon reflex results from a stimulus-induced impulse traveling orthodromically up one peripheral branch of a nerve to the point of division, where it is produces an antidromic impulse in another branch, releasing inflammatory mediators into the periphery (Yaprak, 2008). However, in light of work showing that intrathecal bumetanide (Valencia-de-Ita et al. 2005), bicuculline, a $GABA_A$ receptor antagonist (Lin et al. 1999), as well as sciatic nerve or dorsal root section (Garcia-Nicas et al. 2001) completely blocks capsaicin-induced antidromic impulses (DRR's) as well as measures of capsaicin-evoked neurogenic inflammation in the periphery (Valencia-de-Ita et al. 2005), it is unlikely that axon reflexes play a dominant role in capsaicin-induced neurogenic inflammation.

2.1.2. Mechanism of PAD

2.1.2.1. $GABA_A$ receptors

While presynaptic G-protein coupled receptors (GCPR's) such as the kappa and mu opioid receptors, expressed on or near presynaptic terminals in primary afferent fibers, exert anti-nociceptive effects through presynaptic inhibition (Tallent, 2008), the pharmacological mechanism underlying DRR-evoked neurogenic inflammation and PAD-evoked pre-synaptic inhibition appears to rely mainly on pre-synaptic $GABA_A$ receptors (Todd 1996; Maxwell et al. 1997) as $GABA_A$ receptor antagonists block PAD (Eccles et al. 1963; Nishi et al. 1974; Gallagher et al. 1978; Mokha et al. 1983; Rees et al. 1995) as well as the depolarizing effect of GABA on pre-synaptic membranes of muscle afferents (Curtis & Lodge, 1982; Stuart & Redman, 1992). Due to the relatively high intracellular chloride concentration ($[Cl^-]_i$) of dorsal root ganglion cells (Alvarez-Leefmans et al. 1988; Sung et al. 2000), $GABA_A$ receptor activation produces chloride efflux and depolarization in DRG cells (de Groat et al 1972; Kudo et al. 1975; Sung et al 2000) that decreases the magnitude of incoming action potentials and reduces the level of excitatory amino acid and neuropeptide release from C-fibres (Go & Yaksh, 1987) and A-fibres (Rudomin & Schmidt, 1999; Willis, 1999). With respect to nociceptive processing, activation of pre-synaptic $GABA_A$ receptors reduces nociceptive input to spinal cord neurons through PAD. In other words, $GABA_A$ receptor activity on the nociceptor inhibits nociceptive signals through a pre-synaptic, depolarizing mechanism. On the other hand, blockade of these $GABA_A$-receptors inhibits this reduction, letting the nociceptive input reach the CNS. For example, bicuculline elicits hyperalgesic states and enhanced spike activity in non-sensitized dorsal horn neurons (Silviotti

and Woolf, 1994; Malan et al. 2002). In sensitized dorsal horn nociceptive neurons, however, the effects of $GABA_A$-receptor agonists and antagonists appear to be reversed: Garcia-Nicas et al. (2006) demonstrated that nociceptive specific (NS) neurons in the superficial laminae of the spinal cord acquire an Aβ-intensity component after intraplantar capsaicin and this response can be inhibited with the $GABA_A$-receptor antagonists bicuculline and picrotoxin. Lao and Marvizon (2005) studied the effects of C-fibre intensity electrical stimulation of the dorsal root on capsaicin-evoked NK1 internalization in the spinal dorsal horn: Picrotoxin reduced spinal NK1 internalization while isoguvacine, a $GABA_A$-receptor agonist, facilitated internalization and, presumably, SP release from nociceptors. Importantly, the isoguvacine-dependent facilitation of NK1 internalization was completely eliminated by spinal application of the NKCC1 blocker bumetanide. Thus, the functional effects, either inhibitory or excitatory, of $GABA_A$-receptor activity seem to depend on the presence or absence of a sensitizing stimulus: A $GABA_A$-receptor agonist is normally inhibitory, reducing nociceptive input to the spinal cord. After capsaicin, however, the agonist exerts excitatory effects, enhancing nociceptive input to the spinal cord.

2.1.2.2. AMPA/Kainate receptors

AMPA/kainate receptors have been implicated in PAD (Rees et al. 1995; Lee et al. 2002; Russo et al. 2000). Using the DRP as a measure of PAD, Both Russo et al. (2000) and Lee et al. (2002) demonstrated that while most of the DRP could be blocked with the $GABA_A$ receptor antagonist bicuculline, a portion was also sensitive to AMPA blockade and kainite receptor blockade. Using the kaolin and carrageenan knee-joint arthritis model, Rees et al. (1995) showed that dorsal

rhizotomy as well as $GABA_A$ and AMPA receptor blockade eliminated DRP's recorded from the proximal medial articular nerve trunk. Also, kainate depolarizes sensory afferents, including C-fibers (Davies et al., 1979; Agrawal and Evans, 1986). While the $GABA_A$ receptor seems to be the main component of DRP generation in primary afferent fibers, these studies indicate that AMPA/kainate receptors also play an important role in PAD. Ultrastructural localization studies agree with physiological findings: AMPA/kainite receptors can be found pre-synaptically, on or near the central terminals of afferent sensory neurons, where, under normal conditions, their activation reduces glutamate release from primary afferent fibers (Kerchner et al. 2001; Lee et al. 2002; Willcockson & Valtschanoff, 2008). These presynaptic non-NMDA ionotropic glutamate receptors may be activated by glutamate spill-over from nearby synapses or by glutamate released by astroglial cells surrounding synaptic terminals (Rustioni, 2005). Interestingly, AMPA/kainite receptors can also be found on GABAergic terminals in the spinal cord (Lu et al. 2005), where their activation presumably increases GABA release either onto intrinsic spinal cord neurons or onto central terminal regions of afferent sensory fibers.

2.1.2.3. Enhanced PAD

In contrast to the inhibitory effects of PAD on nociceptive fibers during acute nociceptive input, persistent nociceptive input may lead to enhanced NKCC1 activity, increased $[Cl^-]_i$ and even greater depolarization due to PAD, surpassing the threshold for action potential generation (Cervero & Laird, 1996; Cervero et al. 2003; Fig.1C). Under these circumstances, low intensity input through $A\beta$ fibers could potentially activate spinal cord nociceptive neurons through the

circuit described in Fig. 6, producing expanded receptive fields as well as primary and secondary hyperalgesia that is inhibited by $GABA_A$-receptor blockade. In other words, the cutaneous receptive field of the spinal nociceptive neuron expands because it no longer reflects input from the nociceptor, but also from a low threshold "touch" afferent that is either overlapping with the nociceptors receptive field or at a remote location. Hence, this mechanism accounts for touch-evoked pain originating in and around the nociceptors receptive field as well as from remote locations.

Capsaicin (CAP) produces persistent primary and secondary hyperalgesia as well as enlarged receptive fields through both peripherally (antidromic) and centrally (orthodromic) directed actions. Peripherally, CAP produces dorsal root reflexes (DRR's), efferent action potentials in afferent fibers that give rise to local neurogenic inflammatory responses (Valencia-de Ita et al. 2006). Centrally, CAP is involved in the sensitization of dorsal horn nociceptive neurons (Lin et al. 1997; Willis, 2002) and NK1-receptor internalization in the superficial dorsal horn (Basbaum, 1999; Marvizon et al. 2003). Accordingly, in spinal cord slices where the attached dorsal roots are immersed in CAP, spinal NK1-receptor internalization was reduced by superfusion with the $GABA_A$-receptor antagonist picrotoxin, while isoguvacine, a $GABA_A$-receptor agonist, increased NK1 internalization (Lao and Marvizon, 2005). Moreover, spinal application of the $GABA_A$-receptor antagonist bicuculline reversed the enlarged cutaneous receptive fields of lamina I nociceptive specific neurons as well as novel low-threshold $A\beta$ fiber input following intraplantar CAP injection (Garcia-Nicas et al. 2006). In terms of antidromic effects including neurogenic inflammation, low

threshold (Aβ) fiber-strength electrical stimulation of the sural nerve in CAP-treated rats increased blood flow in the cutaneous sural nerve territory, an effect usually reserved for higher intensity stimulation (eg. Aδ or C-fiber intensity). Sciatic nerve or dorsal root section attenuated the increased blood flow (Garcia-Nicas et al. 2001). In another study, low intensity mechanical stimulation elicited Aδ/C fiber DRR's following intraplantar capsaicin injection (Lin et al. 2000). Therefore, in light of the critical role played by high $[Cl^-]_i$ in producing these effects, it is crucial to evaluate the extent to which NKCC1, a known chloride accumulator, is involved in PAD under normal circumstances as well as under conditions of peripheral tissue inflammation.

2.2. Cation-Chloride Co-transporters and neuronal chloride balance

Chloride (Cl-) co-transporters are involved in many neuronal processes such as fluid homeostasis and volume regulation (Hoffmann and Dunham 1995). The Cl^- co-transporter family is comprised of four K+/Cl- co-transporters (KCC) and two Na+/K+/Cl^- co-transporters (NKCC). KCC activity results in Cl^- efflux, whereas NKCC activity results in Cl^- influx. Chloride transporters are electro-neutral in that their activity is driven by the Na+/K+ ATPase gradients (Haas and Forbush 1998; Alvarez-Leefmans et al. 1998). KCC2 and NKCC1 largely control the intracellular chloride gradient in neurons (Payne et al. 2003), where $[Cl^-]_i$ is the main determinant of the excitatory/inhibitory tone of $GABA_A$ receptor activity through its effects on the Cl^- reversal potential (Alvarez-Leefmans et al. 1990).

2.2.1. NKCC's

NKCC1 controls Cl^- accumulation in hypothalamic, hippocampal, dorsal root

ganglia (DRG) and sympathetic ganglion neurons (Jang et al. 2001; Alvarez-Leefmans et al. 1998; Hara et al. 1992; Misgeld et al. 1986; Ballanyi and Grafe, 1985). Accordingly, in pre-synaptic hypothalamic neurons, the NKCC1 blocker bumetanide (BTD) reduces GABA-induced depolarization (Jang et al. 2001). In early developmental stages, NKCC1 is highly expressed while KCC2 expression is low (Gilbert et al. 2007), leading to the excitatory effects of GABA at these stages (Hubner et al, 2001; Li et al. 2002; Mikawa et al. 2002; Wang et al. 2002). Later in development, this trend reverses such that KCC2 expression increases in the CNS while NKCC1 decreases, resulting in the switch of GABAergic tone from excitatory to inhibitory. However, this developmental regulation of NKCC1 and KCC2 is currently debated in the literature (Blaesse et al. 2009). In small and medium sized DRG neurons, NKCC1 expression appears to remain high throughout development and into adulthood with concomitant excitatory effects of $GABA_A$ receptor activity in these neurons (Alvarez-Leefmans et al. 1988; Sung et al. 2000; Alvarez-Leefmans et al. 2001; Price et al. 2006). Consequently, the GABA-mediated outward anion current in DRG neurons of wild-type animals is absent in NKCC1 knockout mice (Sung et al. 2000).

In contrast to wide array of tissues in which NKCC1 is expressed, NKCC2 is expressed exclusively in the kidney, specifically in the thick ascending limb (TAL) of the loop of Henle and the macula densa (Castrop and Schnermann, 2008). Functionally, NKCC2 is the main salt transport protein in the TAL and as such is the major determinant of urinary concentration through uptake of Cl^- and K^+. Since its expression is restricted to the kidney and is not involved in sensory systems, it will not be discussed further.

2.2.2. KCC's

The four KCC's, KCC1 – KCC4, transport Cl^- out of the cell by following the K^+ gradient, thereby lowering the intracellular Cl^- concentration below its electrochemical equilibrium. This Cl^- influx, in effect, renders $GABA_A$ activity inhibitory through the hyperpolarization produced by $GABA_A$ receptor activity. Like the NKCC's, KCC's play important roles in neuronal excitability, cell volume regulation and transepithelial movement of salt, potassium and, consequently, water (Gamba, 2005). Of the KCC's, KCC2 has received the most attention. KCC2 is expressed in the retina, cortex, cerebellum and the dorsal horn of the spinal cord (Payne et al. 1996). The other KCC's share much of this expression pattern as well as expression in non-neuronal cell types such as glia and oligodendrocytes (Pearson et al. 2001; Karadsheh et al. 2004). At birth, KCC's are generally expressed at low levels, eventually increasing to adult levels within the first few postnatal week (Stein et al. 2004).

In the spinal dorsal horn, KCC2's involvement in nociceptive processing has transformed our understanding of the role of spinal inhibition in chronic pain states. Coull et al. (2003) showed that a KCC2-mediated shift in the anion gradient in spinal lamina I neurons is involved in the development of neuropathic pain. This shift in anion gradient is due to BDNF released from glial cells that reduces KCC2 expression (Coull et al. 2005). The reduced Cl^--extruding ability of these neurons results in an increased intracellular Cl^- concentration and, consequently, hyperexcitability. To date, there is no evidence that the KCC1, 3, and 4 are involved in nociceptive processing.

2.2.3. Other Cl^- co-transporters and exchangers

Aside from the NKCC and KCC cotransporters of the SLC12 gene family, membrane proteins of the SLC4 family also move anions such as HCO_3^- (bicarbonate). The SLC4 family has been classified into three groups: (1) the Na^+/HCO_3^- (sodium bicarbonate) cotransporters, (2) the Na^+-independent Cl^-/HCO_3^- exchangers and (3) the Na^+-driven Cl^-/HCO_3^- exchangers. As a base, HCO^-_3 is important for buffering the intracellular pH. As such, HCO^-_3 is replenished intracellularly by carbonic anhydrase, which catalyzes the H2O + CO2 reaction, producing H+ and HCO^-_3. (Obara et al. 2008).

Based on the extensive expression patterns of these exchangers/cotransporters (Obara et al. 2008) as well as the fact that the $GABA_A$ receptor can channel both Cl^- and HCO^-_3 (Fatima-Shad & Barry, 1993), it is possible that they play a role in PAD. However, studies utilizing the NKCC1 blocker bumetanide indicate that NKCC1 appears to be the main chloride accumulator in mammalian DRG neurons (Alvarez-Leefmans et al. 1988; Brumback & Staley, 2008).

2.2.4. Na^+/K^+-ATPase

In order for a neuron to maintain its resting membrane potential as well as its ability to produce action potentials, it must keep a low intracellular concentration of Na^+ ions and high levels of K^+ ions. Upon action potential invasion, voltage-gated Na^+ channels open, drawing Na^+ into the neuron and driving the membrane potential beyond threshold such that the action potential is propagated. After reaching its peak, voltage-gated K^+ channels open, releasing K^+ and re-establishing the resting potential for future action potential activity. The ionic gradients required for this vital process are set by the Na^+/K^+-ATPase. The Na^+/K^+-ATPase moves 3 sodium ions out and 2 potassium ions into the neuron

through active transport (hydrolysis of ATP). This establishes the high extracellular Na^+ concentration as well as high intracellular K^+ concentration that favours the influx of Na^+ and the efflux of K^-, gradients critical to the proper function of the NKCC's and KCC's. Since these cotransporters use the energy stored in the Na^+ and K^- gradients, established by the Na^+/K^+-ATPase, they are termed secondarily active transporters. The Na^+/K^+-ATPase is also critical for the regulation of cell volume and osmotic balance. In fact, ouabain, a Na^+/K^+-ATPase inhibitor, elicits cell swelling and even bursting (Alberts et al. 2002; Martinez-Augustin et al. 2009).

2.3. Regulation of NKCC1 expression and activity

Under normal conditions, gene expression of the co-transporters appears to be the main regulator of $[Cl^-]_i$ in neurons (Price et al. 2005a). However, STE20-related protein kinases such as WNK (With-No-lysine Kinase) and SPAK/OSR1 are well known to affect NKCC1 activity through phosphorylation (Liedtke and Cole, 2002; Kurihara et al. 2002; Dowd and Forbush, 2003; Gamba, 2005; Moriguchi et al. 2005; Kahle et al. 2005; Vitari et al. 2006; Garzón-Muvdi et al. 2007; Richardson and Alessi, 2008; Delpire and Austin, 2010). Specifically, NKCC1's Cl^- influx activity can be enhanced by phosphorylation on the Thr^{184} and Thr^{189} sites on the intracellular N-terminal domain (Darman and Forbush 2002), leading to increased ion transport through the cotransporter (Klein et al., 1999; Flemmer et al., 2002). Furthermore, kinases such as JNK (Klein et al., 1999), p38 (Capo-Aponte et al. 2007), ERK (Liedtke and Cole, 2002), PKC∂ (Liedtke and Cole, 2000, 2002) and CaMKIIα (Schomberg et al., 2001) enhance NKCC1-dependent Cl^- accumulation. Both group I metabotropic glutamate receptor

(mGluR) and AMPA agonists alter NKCC1 activity (Schomberg et al., 2001). In addition, intense afferent input sufficient to induce central sensitization increases multiple kinase activity in the dorsal horn (Dai et al., 2002; Fang et al., 2002; Galan et al., 2002; Pezet et al., 2002), possibly enhancing NKCC1 activity and facilitating DRR's.

2.4. Physiological roles of NKCC1

2.4.1. Audition

One of the more striking phenotypes expressed by NKCC1 knockout mice consists of circling behavior and bobbing of the head, indicating inner ear dysfunction (Delpire et al. 1999). Accordingly, startle responses to auditory stimuli are lacking in these mice, indicating deafness. In line with NKCC1's role in fluid homeostasis across secretory epithelia, NKCC1 knockout mice lack NKCC1 in the stria vascularis, a secreting epithelium in the cochlea. In these mice, Reissner's membrane, normally under tension due to the pressure of the NKCC1-mediated fluid secretion, no longer separates the scala media from scala vestibuli, resulting in deafness and vestibular dysfunction (Delpire et al. 1999).

2.4.2. Olfaction

The mammalian olfactory system is exquisitely sensitive to a wide variety of odors even if they are available in only minute quantities. To do this, olfactory neurons use a two-stage amplification mechanism: Initial activation of a cAMP pathway opens Ca^{+}-permeable ion channels that, through Ca^{+} entry, also activate Cl^{-} channels (Kleene, 2008). Since sensory cilia of olfactory receptor neurons accumulate Cl^{-} ions at rest, maintaining a high intracellular Cl^{-} concentration through an NKCC1-dependent mechanism (Kaneko et al. 2004; Reisert et al.

2005), they produce a depolarizing Cl^- current upon odor detection that amplifies the receptor potential and promotes action potential formation, even when the odor signal is weak (Hengl et al. 2010). However, Smith et al. (2008) found no difference between NKCC1 knockouts and wild-type littermates in an operant behavioral assay of olfactory sensitivity, indicating the possibility of an alternative pathway for Cl^- accumulation in olfactory neurons (Nickell et al. 2006; 2007).

2.4.3. Respiration

Loop diuretics such as bumetanide and furosemide can alleviate coughing and other airway defensive mechanisms through an NKCC1-dependent mechanism (Mazzone & McGovern, 2006). Specifically, NKCC1 is expressed on vagal afferents innervating the trachea. Citric acid applied to the trachea produces coughing and respiratory slowing that is exacerbated by removal of extracellular Cl^- and attenuated by furosemide (Mazzone & McGovern, 2006). Ren & Greer (2006) have also shown that respiratory rhythmicity is also mediated by both NKCC1 and KCC2.

2.4.4. Neurite outgrowth

Neurite outgrowth is of fundamental importance to development of the nervous system as well as plasticity, the neural correlate of learning and memory. Neurite growth from PC12 cells is initiated by nerve growth factor (NGF; Levi et al. 1988). NKCC1 is expressed on the plasma membrane of the growth cone during neurite outgrowth. In these cells, knockdown of NKCC1 activity reduced NGF-mediated neurite outgrowth (Nakajima et al. 2007). NKCC1 is also involved in neurite growth after peripheral nerve axotomy (Pieraut et al. 2007): Through the use of bumetanide, spinal application of NKCC1 small interfering RNA (siRNA) and the

use of NKCC1 knockout mice, Pieraut et al. (2007) demonstrated that the neurite growth velocity of axotomized neurons is in part NKCC1-dependent.

2.5. Pathophysiological roles of NKCC1

2.5.1. Epilepsy

2.5.1.1. Neonatal seizures

In neonates, neurological insult such as hypoxia-ischemia, infection, metabolic derangement or other trauma can produce seizure activity (Silverstein & Jensen, 2007). Seizures are more common during the neonatal period than at any other time (Kahle et al. 2008). First-line anti-epileptic drugs efficacious in adults, such as barbiturates and benzodiazepines, are often ineffective in reducing seizures in neinates and may even exacerbate seizures (Painter et al. 1999; Boylan et al. 2002; Castro et al. 2005). In adults, barbiturates and benzodiazepines reduce seizure activity by activating the $GABA_A$ receptor, producing hyperpolarization through Cl^- influx through the $GABA_A$ anion channel (Czapinski et al. 2005). However, in immature neurons where the abundance of NKCC1 and paucity of KCC2 result in a high intracellular Cl^- concentration, benzodiazepines and barbiturates instead produce Cl^- efflux, depolarization and pathological excitatory activity (Kahle et al. 2008). In the neonate rat brain, the NKCC1 blocker bumetanide, when administered systemically in clinically relevant doses, reduces seizure activity (Hannaert et al. 2002; Dzhala et al. 2005; Dzhala et al. 2008). Specifically, Dzhala et al. (2005) showed that the high NKCC1 and low KCC2 expression pattern in neonatal human cortex is similar to that in rodent. Furthermore, blockade of NKCC1 with bumetanide produced a negative shift in E_{GABA} (the GABA reversal potential), blocked GABA-dependent synchronous

excitatory activity as well as interictal activity in the neonatal hippocampus and reduced kainite-evoked seizures in intact neonatal rats. These effects were specific to NKCC1 in that they were evoked by doses of bumetanide specific for NKCC1, they were blocked by $GABA_A$ receptor antagonists, had no effect on NKCC1 knockout mice nor seizure activity in mature cortical tissue (Dzhala et al. 2005).

2.5.1.2. Temporal lobe epilepsy

GABAergic signaling has also been implicated in temporal lobe epilepsy and seizures following ischemia-hypoxia in adults (Staley, 2006; Kahle et al. 2008b). Similar to neonatal seizures, barbiturates and benzodiazepines are less effective in models of temporal lobe epilepsy (Brooks-Kayal et al. 1998), possibly due to an NKCC1-dependent depolarizing shift in the transmembrane Cl- gradients, not unlike gradients found in neonatal neurons (Cohen et al. 2002, Palma et al. 2006; Huberfeld et al. 2007; Payne et al. 2003). A human trial studying the effects of bumetanide on intractable temporal lobe epilepsy is currently underway (Kahle et al. 2008a).

2.5.2. Nociceptive processing

Under normal circumstances, NKCC1 activity in nociceptive sensory fibres provides an adaptive, anti-nociceptive response to nociceptive input. However, NKCC1's role in persistent pain appears to be maladaptive. In 1996, Cervero and Laird proposed a novel hypothesis for touch-evoked pain in inflammatory pain states which accounted for a number of properties of hyperalgesic states, including the rapid transition from touch to pain upon activation of low threshold mechanoreceptors, that were not easily explained by other hypotheses. At the

time, understanding of the structure, function, and regulation of NKCC1 was limited; its involvement in nociception had not yet been put forward. However, NKCC1's key role in Cl^- accumulation in mammalian DRG neurons (Alvarez-Leefmans et al. 1998; 2001) and sensory perception, particularly pain, quickly became apparent (Sung et al. 2000; Delpire, 2000; Cervero et al. 2003). In theory, for NKCC1 to exert anti-nociceptive effects under normal conditions but pro-nociceptive effects in inflammatory states, NKCC1 activity and/or expression must be enhanced after inflammation and its location must be pre-synaptic, on or near the central terminals of nociceptive primary afferent fibers. Accordingly, this hypothesis is generally supported in the literature (Table 1). Specifically, NKCC1 mRNA and protein is expressed in the regions relevant to nociceptive processing, such as spinal cord and dorsal root ganglion (DRG; Kanaka et al. 2001; Toyoda et al. 2005; Price et al. 2006; Gilbert et al. 2007; Gilbert et al. 2007; Chabwine et al. 2009), especially in small- and medium-sized neurons co-expressing TRPV1 and CGRP (Price et al. 2006), consistent with nociceptive-type sensory fibers. Intracellular chloride concentration of DRG neurons (Sung et al. 2000; Pieraut et al. 2007; Gilbert et al. 2007; Funk et al. 2008; Rocha-Gonzalez et al. 2008; Chabwine et al. 2009) and NKCC1 expression, measuring both mRNA and protein, are increased in response to inflammation or nerve injury (Morales-Aza et al. 2004; Galan and Cervero, 2005; Pieraut et al. 2007; Cramer et al. 2008; Funk et al. 2008; Li et al. 2010). Galan et al. (2005) demonstrated that plasma membrane NKCC1 expression in the lumbosacral spinal cord is increased, in

Number of Studies	Approach / technique	Main finding	References *
19	NKCC1 protein and mRNA expression	NKCC1 protein expression increased after injury/inflammation	Morales-Aza et al. 2004 (CFA); Galan and Cervero, 2005 (iCAP); Pieraut et al. 2007 (neuropathy); Cramer et al. 2008 (neuropathy); Funk et al. 2008 (inflammatory mediators)
		NKCC1 mRNA expression increased after injury/inflammation	Morales-Aza et al. 2004 (CFA); Li et al. 2010 (LPS)
		NKCC1 protein expression unchanged after injury/inflammation	Nomura et al. 2006 (formalin); Jolivalt et al. 2008 (diabetic neuropathy); Zhang et al. 2008 (CFA)
		NKCC1 mRNA expression unchanged after injury/inflammation	Funk et al. 2008 (inflammatory mediators); Wu et al. 2009 (formalin)
		NKCC1 protein expression in naïve animals or in tissue cultures without injury/inflammation	Price et al. 2006; Gilbert et al. 2007; Chabwine et al. 2009
		NKCC1 mRNA expression in naïve animals or in tissue cultures without injury/inflammation	Kanaka et al. 2001; Toyoda et al. 2005; Price et al. 2006; Gilbert et al. 2007
5	Behavioral pharmacology	Reduced NKCC1 activity reduces behavioral manifestation of pain	Sung et al. 2000; Laird et al. 2004 (CAP); Granados-Soto et al. 2005 (formalin); Valencia-de Ita et al. 2006 (CAP); Cramer et al. 2008 (neuropathy)
3	2 photon imaging ($[Cl^-]_i$)	Injury increases $[Cl^-]_i$; developmental switch in $[Cl^-]_i$	Gilbert et al. 2007; Funk et al. 2008; Rocha-Gonzalez et al. 2008
3	In vitro electrophysiology ($[Cl^-]_i$)	BTD and/or NKCC1 knockout reduces $[Cl^-]_i$	Sung et al. 2000; Pieraut et al. 2007 (neuropathy); Chabwine et al. 2009
1	In vivo electrophysiology	NKCC1 blockade reduced peripheral effects of capsaicin (DRR's and other measures of neurogenic inflammation)	Valencia-de Ita et al. 2006 (CAP)
1	NK1 internalization (indirect measure of central sensitization)	NKCC1 blockade reduced NK1 internalization	Lao and Marvizon, 2005 (CAP)
1	NKCC1 regulation	SPAK and OSR1 found in relevant regions (DRG and spinal cord)	Geng et al. 2009
1	Intrinsic Optical Signal	C-fiber intensity afferent stimulation increases IOS's in a furosemide-sensitive fashion.	Asai et al. 2002 (electrical stimulation)

** The nociceptive stimulus is also included beside certain references (in brackets)*

***Table 1**: Summary of NKCC1-related literature available in PubMed, using the search terms "NKCC1" or "bumetanide", with relevance to nociceptive processing.*

parallel with a concomitant reduction in the cytosol, following intracolonic capsaicin administration. Moreover, in the same study, intracolonic capsaicin (iCAP) produced a rapid and transient phosphorylation of NKCC1, an effect associated with increased co-transporter activity (Delpire and Austin, 2010). Both acute and chronic experimental arthritis produce increased expression of NKCC1 in rats (Morales-Aza et al. 2004). NKCC1 expression is increased following spinal cord injury in rats (Cramer et al. 2008). On the other hand, diabetic neuropathy and complete Freund's adjuvant (CFA) did not appear to alter spinal NKCC1 expression (Jolivalt et al. 2008; Zhang et al. 2008; Funk et al. 2008). In terms of behavior, thermal hyperalgesia following spinal cord injury is reduced following systemic administration of the NKCC1 blocker BTD (Cramer et al. 2008). Interestingly, while intrathecal BTD, an NKCC1 blocker, attenuated phase II responses to intraplantar formalin (Granados-Soto et al. 2005), it did not appear to affect spinal NKCC1 expression (Nomura et al. 2006; Wu et al. 2009). Valencia-de-Ita et al. (2006) demonstrated that BTD attenuates increased DRR's, hindpaw edema and blood flow associated with neurogenic inflammation, as well as hyperalgesia. Naïve NKCC1 knockout mice have increased thermal nociceptive latencies compared to naïve wild-type mice (Sung et al. 2000) as well as deficits in intraplantar capsaicin-induced mechanical allodynia compared to wild-type mice (Laird et al. 2004). Taken together, these findings clearly implicate NKCC1 in nociceptive processing and provide a potential target for novel therapeutic approaches to the treatment of persistent pain.

2.6. General Aims

Our main goal in designing the experiments described in this thesis was to improve our understanding of NKCC1 function within the context of nociceptive processing. Specifically, we wanted to decipher NKCC1's involvement in the clinically relevant phenomena of primary and secondary hyperalgesia. Our literature review illustrates the need for such work by the relative paucity of functional studies in intact animals (Table 1). Clearly, compared to other molecular targets such as TRPV1 or SP, NKCC1 has received little attention, with only 23 of the over 400 publications found in Pubmed (search terms: "NKCC1" or "bumetanide") that were relevant to nociceptive processing. Nine of these studies used two or three approaches; therefore, a total of 34 different studies examined either NKCC1 expression in regions relevant to nociceptive processing or NKCC1's functional role in nociceptive processing. A total of 19 of the 30 (55.9%) studies looked at NKCC1 expression, while only 5 (14.7%) studied the behavioral effects of NKCC1 modulation on pain. Six (17.6%) studies measured NKCC1-dependent $[Cl^-]_i$ and a single study (2.9%) used in vivo electrophysiology to measure peripheral (anti-dromic) NKCC1-dependent signaling (DRR's; Valencia-de Ita et al. 2006). Only one study attempted to measure the central effects of NKCC1 modulation, albeit indirectly, measuring spinal NK1 internalization (Lao and Marvizon, 2005). Finally, only one study has approached regulation of NKCC1 activity specifically in the pain pathway, looking at SPAK and OSR1 regulation of NKCC1 in the DRG and spinal cord (Geng et al. 2009). Clearly, there is insufficient information available concerning NKCC1's role in central nociceptive processing. Considering the importance of CNS mechanisms in nociceptive processing and the perception of pain, it is surprising how little

attention has been paid to NKCC1's role in spinal nociceptive neuronal activity after injury. Part of this surely stems from the unfortunate lack of pharmacological and immunohistochemical tools targeted specifically to NKCC1, well known among those who study NKCC1. So, with limited tools in hand, novel and relevant experiments must be cleverly designed, providing evidence converging from multiple, well-established techniques. From a clearly linked series of experiments using well-established techniques and a standard model of nociceptive input, we aim to provide a comprehensive, functional and clinically relevant picture of NKCC1's role in processing nociceptive and non-nociceptive input.

2.7. Experimental Rationale

Progressing from a whole-animal approach to the cellular level and finally to molecular determinants of NKCC1 activity, the experiments described herein were designed to demonstrate NKCC1's role in nociceptive processing from a mainly functional standpoint. We begin with a whole-animal approach whereby NKCC1's involvement in nociceptive processing is examined through behavioral characterization of NKCC1 blockade on intracolonic CAP-induced secondary allodynia (Chapter 3). While behavioral pharmacological analyses can provide strong evidence for or against the role of a given molecule, multiple pathways are conceivable. To address these possibilities, it is important to study how NKCC1 activity affects nociceptive processing at the cellular level. Considering that the perception of pain requires ascending activity, a functional measure of CNS nociceptive neuronal responses is required. Therefore, we study NKCC1's influence on *in vivo* spike activity of spinal nociceptive neurons following

sensitization by CAP (Chapter 4). In addition to electrophysiological recording, we also measure functional aspects of NKCC1 using another approach; immunohistochemical labeling of the immediate-early gene FOS permits visualization and quantification of CAP-evoked neuronal activation in spinal cord regions associated with nociceptive processing. Spinal NKCC1 blockade permits us to determine NKCC1's involvement in the activity of these neurons (Chapter 5). Finally, to study through which molecular pathway nociceptive input enhances spinal NKCC1 expression, we conclude with the identification of possible regulators of NKCC1 activity; spinal blockade of target receptors that are hypothesized to alter spinal NKCC1 expression (Chapter 6). Together, these studies provide an integrated view of NKCC1's functional role in persistent pain.

CHAPTER 3

WHOLE-ANIMAL APPROACH – INTRACOLONIC CAPSAICIN-EVOKED SECONDARY ALLODYNIA

3.1. Specific Aim: Effects of spinal NKCC1 blockade on intracolonic capsaicin-induced secondary hyperalgesia

To study the influence of NKCC1 function on secondary hyperalgesia, we employed pharmacological manipulation of NKCC1 in the intracolonic CAP (iCAP) model of referred visceral inflammatory pain, a well-established model of secondary allodynia in mice (Laird et al. 2000, 2002; Galan et al. 2004, 2005; Mansikka et al. 2005; Eijkelkamp et al. 2007, 2008). Since BTD possesses 10 – 100 fold selectivity for NKCC1 vs. KCC2 (Payne, 1997), high doses of BTD would be also expected to act on KCC2, a chloride extruder, presumably producing the opposite effect of NKCC1 on touch-evoked responses. Therefore, we hypothesized that high doses of BTD will not only fail to prevent, but may even augment, iCAP-evoked secondary allodynia and hyperalgesia. In addition, we were interested in identifying potential regulators of NKCC1 activity. While TRPV1's involvement in thermal hyperalgesia is well established (Caterina et al. 2000; Tominaga et al. 1998), recent work indicates that TRPV1 is also involved in mechanical allodynia, possibly due to TRPV1 receptors situated on central terminals of primary afferents in the dorsal horn (Cui et al. 2006; McGaraughty et al. 2008). We hypothesized that spinal administration of n-arachidonoyl-dopamine (NADA), a potent TRPV1 agonist (Huang et al. 2002; Price et al. 2004), may produce allodynia and/or hyperalgesia in the hindpaw. NADA exists in pharmacologically relevant concentrations in the CNS (Huang et al. 2002; Marinelli et al. 2007). In addition, considering that the majority of TRPV1-expressing DRG neurons that project to the superficial laminae of the spinal cord also express NKCC1 (Price et al. 2006), we hypothesized that spinal TRPV1

receptors might be involved in NKCC1 regulation. We studied the effect of spinal TRPV1 blockade on iCAP-induced secondary allodynia as well as the effect of NKCC1 blockade on TRPV1-mediated allodynia. Furthermore, certain intracellular kinases may also regulate NKCC1 activity. As described in Table 1, only one study could be found specifically addressing intracellular kinase regulation of NKCC1 in tissues related to nociceptive processing; the Ste20-related proline-alanine-rich kinase (SPAK) and oxidative-stress response 1 (OSR1) are both found in spinal cord and DRG neurons and both regulate NKCC1 activity (Geng et al. 2009). Two of the major mitogen-activated protein kinase (MAPK) subfamilies known to mediate a significant proportion of the communication between the plasma membrane and intracellular regulatory targets, namely c-Jun N-terminal kinase (JNK; Klein et al. 1999) and p38 (Johnston et al. 2000; Piechotta et al. 2003), also appear to be involved in regulating NKCC1 activity. Of these, only p38 and JNK appear to interact with the SPAK/OSR1-NKCC1 complex (Johnston et al. 2000; Piechotta et al. 2003; Marshall et al. 2005). Capo-Aponte et al. (2007) showed that p38, but not JNK or ERK1/2, stimulate NKCC1 activity. Furthermore, JNK appears to reduce NKCC1 activity through a protein phosphatase pathway, effectively acting as an off-switch (Marshall et al. 2005). Another important MAPK, extracellular-signal related kinase (ERK1/2), is activated by intracolonic CAP and mediates the associated secondary allodynia and hyperalgesia (Galan et al. 2003). However, whether or not ERK1/2 is upstream to NKCC1 (ERK1/2 regulating NKCC1 activity; Liedtke & Cole, 2002) or downstream from NKCC1 (NKCC1 regulating ERK1/2; Lu et al. 2008) is still in dispute. It must be noted that since crosstalk can occur between

different pathways and a given pathway can mediate different responses at different times, not to mention that many of the aforementioned studies focus on systems outside the realm of nociceptive processing, the characterization of specific signaling pathways that regulate NKCC1 activation in afferent nociceptive fibers is complex. Nonetheless, we studied the effect of spinal blockade of p38, ERK1/2 and JNK on iCAP-evoked secondary allodynia.

3.2. Methods

3.2.1. Animals

Male C57BL6 mice between 20–25 g were used for all behavioral experiments. Mice were used for only one experimental procedure and were humanely killed by anesthesia overdose immediately after testing. All testing was performed blind to condition and treatment. Experiments were in accordance with the Canadian Counsel on Animal Care (CCAC) and the International Association for the Study of Pain (IASP) guidelines for the care and use of experimental animals. All protocols were reviewed and approved by the McGill University Animal Care Committee.

3.2.2. Drugs

CAP (0.1%, Tocris, Ellisville, MO) was dissolved in 10% ethanol, 10% Tween 80 and 80% saline. In behavioral experiments with mice, 0.05 ml of 0.1% CAP was delivered intracolonically. BTD (NKCC1 inhibitor; 0.01, 0.1, 1 and 10 nmol in 5 µL; Sigma-Aldrich), AMG 9810 (TRPV1 antagonist; 1 nmol in 5 µL; Tocris) and n-arachidonoyl-dopamine (NADA; TRPV1 agonist; 1 - 10 nmol in 5 µL, Tocris) were dissolved in artificial cerebrospinal fluid (aCSF) vehicle. The aCSF vehicle was comprised of (in mM) 1.3 CaCl2 2H2O, 2.6 KCl, 0.9 MgCl, 21.0 NaHCO3, 2.5

Na2HPO47H2O, 125.0 NaCl, and 3.5 dextrose (pH 7.2–7.4). SB-203580 (p38 antagonist; 26 nmol in 5 µL; Sigma-Aldrich), SP-600125 (JNK antagonist; 45 nmol in 5 µL; Sigma-Aldrich) and U-0126 (ERK1/2 antagonist; 23 nmol in 5 µL; Sigma-Aldrich) were dissolved in 10% DMSO. Intrathecal (IT) injections of BTD, SB-203580, SP-600125, U-0126, AMG 9801 and NADA were made in 5 µl aCSF on lightly anesthetized mice by lumbar puncture at the L4–L5 level with a 30-gauge needle on a 50 µl Hamilton syringe. Compared to other cation Cl^- blockers (eg. furosemide, piretanide), BTD is known to be more specific for NKCC1 (Payne, 1997). NADA was used in these experiments because it is an endogenous TRPV1 ligand found in many CNS regions (Huang et al. 2002) and its synthesis is regulated by neuronal activity (Marinelli et al. 2007).

3.2.3. Intracolonic CAP model of secondary allodynia

Mice were habituated to a Plexiglass testing box (10 cm x 10 cm x 8 cm) for 1–2 hours prior to testing. This box was used to evaluate spontaneous nociceptive behaviors (SNB's; please see section 3.2.4.1.) as well as secondary allodynia and hyperalgesia (please see section 3.2.4.2.). The intracolonic CAP model of referred allodynia has been described previously (Laird et al. 2001). Briefly, just prior to intracolonic application of CAP, petroleum jelly (Vaseline) was applied to the peri-anal area to avoid the stimulation of somatic areas by contact with CAP. CAP was slowly injected via a fine cannula with a rounded tip (external diameter 0.61 mm), gently introduced 4 cm into the colon via the anus.

3.2.4. Behavioral Testing Procedures

3.2.4.1. Spontaneous Nociceptive Behaviors (SNB’s)

We recorded SNB's for the first 15 min post-CAP, as well as the latency to the first SNB. SNB's were comprised of abdominal licking, abdominal retractions, stretching and squashing. Each SNB usually lasted for 1-2 seconds and was counted as one SNB. However, periodically a SNB lasted much longer (eg. 10-15 seconds). Under these circumstances, the number of SNB's was proportional to the duration (eg. 10 SNB's for a 10 second burst of a specific SNB).

3.2.4.2. Secondary Allodynia and Hyperalgesia

Immediately after observations of SNB's, referred visceral allodynia and hyperalgesia was tested on the abdomen with calibrated von Frey filaments (1mN & 32 mN, respectively) at various time-points before and after intracolonic capsaicin treatment. Percent response frequency was calculated from the number of responses to 10 stimulations on the abdomen. Each filament was applied for approximately 1–2 s, with an inter-stimulus interval of 2–5 s. The appearance of any of the following behaviors on application of a filament to the abdomen was considered a withdrawal response: (i) sharp retraction of the abdomen, (ii) immediate licking or scratching of site of application of filament, or (iii) jumping. For experiments with NADA, mice were habituated as above and tested on the hindpaw with von Frey filaments of 1, 4, 8 and 16 mN using the same protocol as for abdominal testing.

3.2.4.3. Stroking Allodynia

A separate group of mice were tested for stroking allodynia by gently brushing the plantar surface of the hindpaw 10 times with a 5 sec inter-stimulus interval with a cotton bud (Q-tip). The following reactions were considered a withdrawal

response to the cotton bud: (i) sharp retraction of hindpaw, (ii) immediate licking or scratching of site of application of the cotton bud, or (iii) jumping.

3.2.4.4. Tail Flick Assay

A separate group of mice were tested for the effects of spinal BTD or BTD-VEH on the tail flick assay. The tail flick assay is based on the time to withdraw the tail in response to a noxious cutaneous thermal stimulation from a light beam aimed at the tail. A cutoff time of 15 sec was set to avoid tissue damage.

3.2.5. Statistical Analyses

Data are shown as mean ± SEM and all analyses involved t-tests, one- or two-way ANOVA with bonferroni post-hoc analyses (indicated in figure captions), where applicable. In all cases $p < 0.05$ was considered significant.

3.3. Results

3.3.1. Spinal NKCC1 blockade has no significant effect on tail-flick latency.

To determine if NKCC1 blockade affects baseline nociceptive processing in naïve mice, two-way ANOVA indicated that intrathecal injection of the NKCC1 blocker BTD did not significantly affect tail flick latency at either 15 or 30 min post-injection (Fig. 7).

3.3.2. Intracolonic capsaicin produces robust secondary allodynia as well as certain spontaneous nociceptive behaviors (SNB's) in C57BL6 mice.

The majority of the results in this and following sections have already been published (Pitcher et al. 2007b). Intracolonic capsaicin (iCAP) produces robust secondary allodynia and hyperalgesia as measured by a significantly increased response frequency to 1mN (Fig. 8C) and 32 mN von Frey stimulation of the abdomen (Fig. 8D). In addition iCAP significantly increases SNB's (Fig. 9).

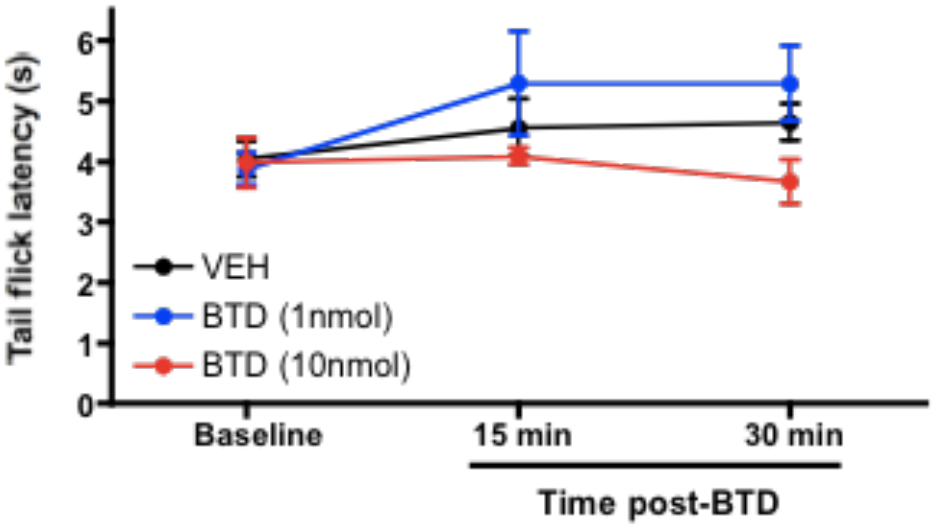

Figure 7: Tail flick latency in naïve mice was not affected by spinal NKCC1 blockade. *Two-way ANOVA indicated that neither BTD dose nor time post-injection affected tail flick latency in naïve mice. $p < 0.05$ was considered significant in all cases ($n = 8 – 9$ per group).*

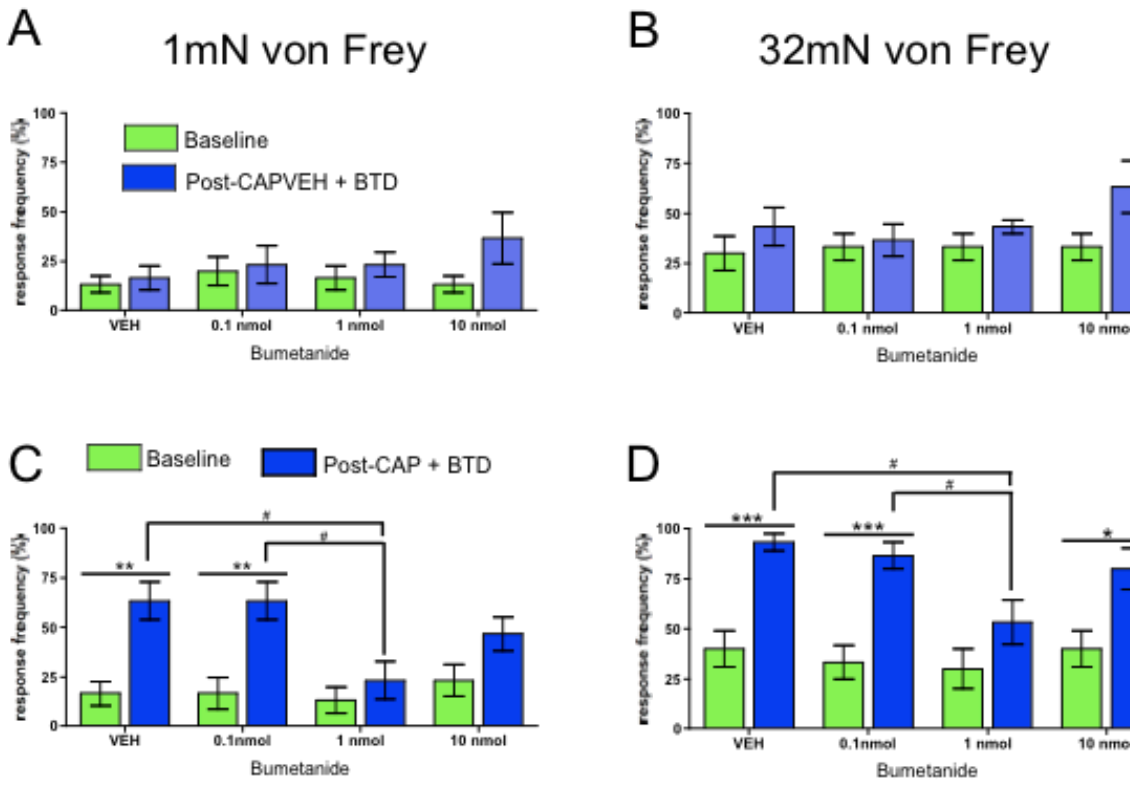

***Figure 8**: **NKCC1 inhibitor BTD blocks intracolonic CAP-evoked referred allodynia and hyperalgesia**. Three doses of BTD or vehicle (VEH) were IT injected and CAP-VEH or CAP was injected into the colon 5 min later. A and B) Baseline response frequencies to abdominal 1 mN and 32 mN von Frey hair application are shown (green bars) as well as 30 min after the intracolonic CAP-VEH injection (light blue bars). Spinal NKCC1 blockade does not affect response frequency in CAP-VEH treated mice. C and D) Response frequencies to abdominal 1 mN (C) and 32 mN (D) von Frey hair application 30 min after the intracolonic CAP injection are shown (dark blue bars) in comparison to baseline von Frey response frequencies (green bars). Spinal NKCC1 (1nmol) reduced response frequency to both 1 and 32mN von Frey stimulation in CAP-treated mice while the highest dose increased responses, possibly reflecting BTD activity at KCC2. Stars (*) indicate significant effects vs. baseline (two-way ANOVA); number signs (#) indicate significant effects of intrathecal bumetanide injections (one-way ANOVA, # or * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 6$ per group). Figure adapted from Pitcher et al. 2007.*

3.3.3. Spinal NKCC1 blockade inhibits iCAP-induced mechanically evoked secondary allodynia and hyperalgesia.

Using spinal application of BTD and intracolonic CAP, we tested the hypothesis that NKCC1 is functionally linked to secondary allodynia and hyperalgesia. We determined the effects of increasing doses of intrathecal BTD without intracolonic CAP (Fig. 8A/B) as well as the effects of BTD on secondary allodynia and hyperalgesia 0.5 hrs following an intracolonic CAP (Fig. 8C/D). In intracolonic saline-treated mice, BTD-vehicle (VEH) as well as BTD at 0.1, 1 and 10 nmol had no effect on response frequencies to 1 (Fig. 8A) or 32 mN (Fig. 8B) stimulation of the abdomen, similar to our findings in the tail flick assay. In contrast, in intracolonic CAP-treated mice, BTD reduced response frequencies to both 1 mN (Fig. 8C) and 32 mN (Fig. 8D) von Frey stimulation of the abdomen at the 1 nmol dose. Since the anti-allodynic/hyperalgesic effect of BTD was limited to the 1 nmol dose (Fig. 8C/D), this dose was used for all subsequent behavioral studies.

3.3.4. Spinal NKCC1 blockade reduces certain spontaneous nociceptive behaviors associated with iCAP.

Spontaneous nociceptive behaviors (SNB's) associated with intracolonic CAP, such as abdominal licking, stretching and abdominal retractions were also evaluated. Firstly, iCAP-treated mice display significantly more spontaneous abdominal licking behavior than mice treated with iCAP-vehicle (Fig. 9). Intrathecal BTD did not significantly influence the latency to first nociceptive behavior (VEH = 77.7 ± 17.4 s, BTD = 57.8 ± 12.9 s). In terms of the total number of SNB’s evoked by intracolonic capsaicin, BTD (1 nmol; 78.5 ± 13.6) had no effect compared either to CAP (64.8 ± 8.6) or to BTD-VEH (88.0 ± 10.7; Fig. 9).

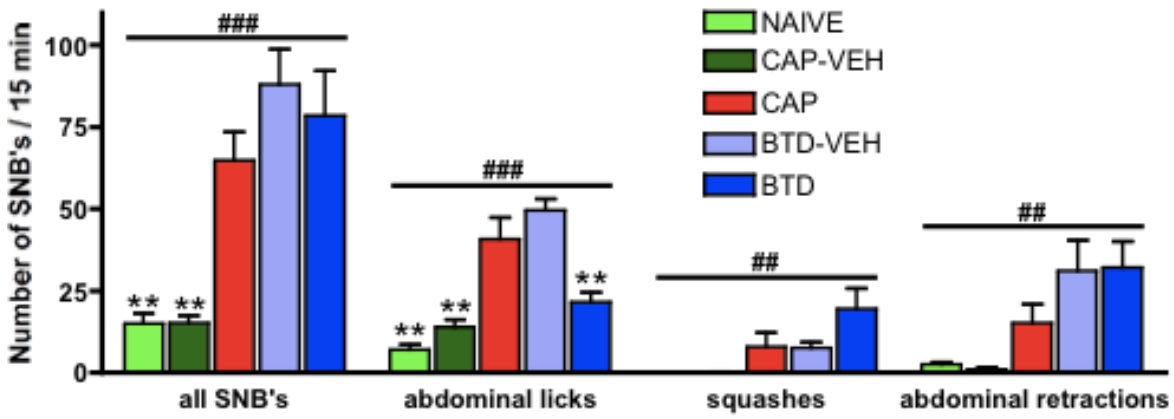

Figure 9: Not all spontaneous nociceptive behaviors (SNB's) associated with iCAP are affected by spinal NKCC1 blockade. *When taken together, BTD does not significantly affect SNB's. However, while BTD prevented abdominal licking, it appeared to increase squashing and abdominal retractions. Number signs (#) indicate significant one-way ANOVA while stars (*) indicate significant Dunnett's post hoc test. All post hoc comparisons are to the iCAP groups (red bars). # or * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 6$ per group*

However, when SNB's were examined individually, BTD exerted significant but differential effects. Specifically, in terms of abdominal licking, BTD significantly reduced abdominal licking (21.5 ± 3.0) compared to CAP (40.7 ± 6.7) and BTD-VEH (49.5 ± 3.6; Fig. 9). In addition, BTD reduced abdominal licking to baseline levels compared to both the naïve (7.0 ± 2.8) and CAP-VEH (14.0 ± 8.4) groups. On the other hand, in terms of both squashing and abdominal retractions, BTD (19.5 ± 3.7; 32.0 ± 8.0, respectively) appears to be increased compared to naïve (0 ± 0; 2.5 ± 0.8, respectively) as well as CAP-VEH (0.2 ± 0.2; 1.0 ± 0.6, respectively) groups (Fig. 9). However, post-hoc analysis yielded no significant differences within these groups.

Next we addressed the time course of the effects of intrathecal BTD on the inhibition of intracolonic CAP-evoked referred allodynia. BTD injected intrathecal 5 min prior to iCAP significantly reduced responses to 1 mN abdominal stimulation at 0.5 hrs (Fig. 10A). However, at 1 and 3 hrs post-CAP, the difference between BTD-injected and VEH-injected mice was no longer evident. BTD injected 20 min following iCAP inhibited secondary allodynia 0.5 and 1 hr post-CAP (Fig 10B). Furthermore, when injected 4 hrs post-CAP, BTD inhibited secondary allodynia at 0.5 and 1 hr post-BTD (Fig 10C).

3.3.5. Blockade of certain MAPK's prevents iCAP-evoked secondary allodynia.

Of the three MAPK's antagonists injected spinally 5 minutes prior to iCAP, only p38 and ERK1/2 blockade reduced iCAP-induced abdominal licking (Fig. 11A) and response frequency to abdominal von Frey stimulation at 0.5 or 1.0 hr post-drug (Fig. 11C/D). Intrathecal blockade of JNK had no effect on either abdominal licking or response frequency (Fig. 11A/B).

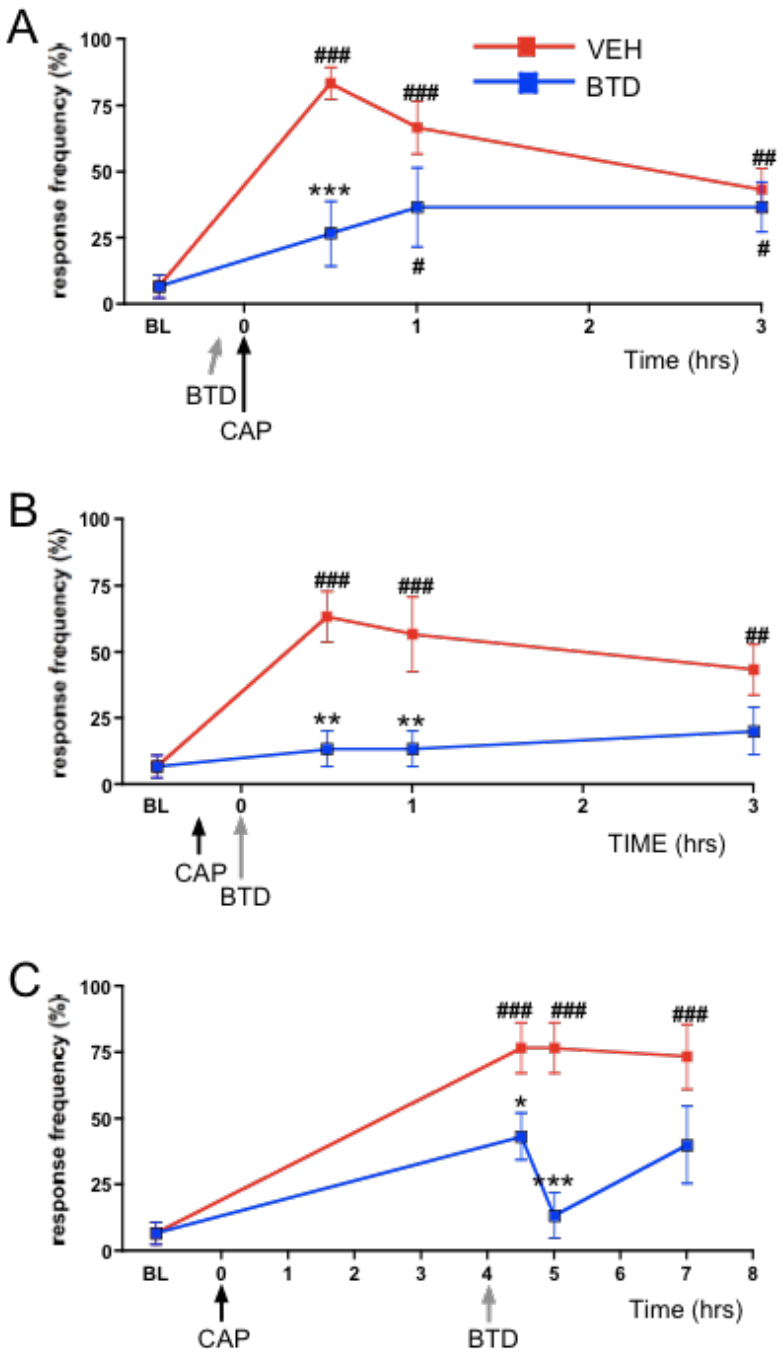

Figure 10: Pre-CAP and post-CAP administration of BTD blocks intracolonic CAP-induced secondary allodynia. *BTD or vehicle (VEH) was injected intrathecally (A) 5 min prior to iCAP, (B) 20 min or (C) 4 hrs after iCAP. Response frequencies to 1 mN von Frey hair applications to the abdomen are shown at the indicated time points after intracolonic CAP. Stars (*) indicate significant effects of BTD (blue sqaures) vs. VEH (red squares; two-way ANOVA) while number signs (#) indicate significant effects vs. baseline (BL) at a given time point (one-way ANOVA, # or * $p < 0.05$, ## or ** $p < 0.01$, ### or *** $p < 0.001$; n = 6 per group). Figure adapted from Pitcher et al. 2007.*

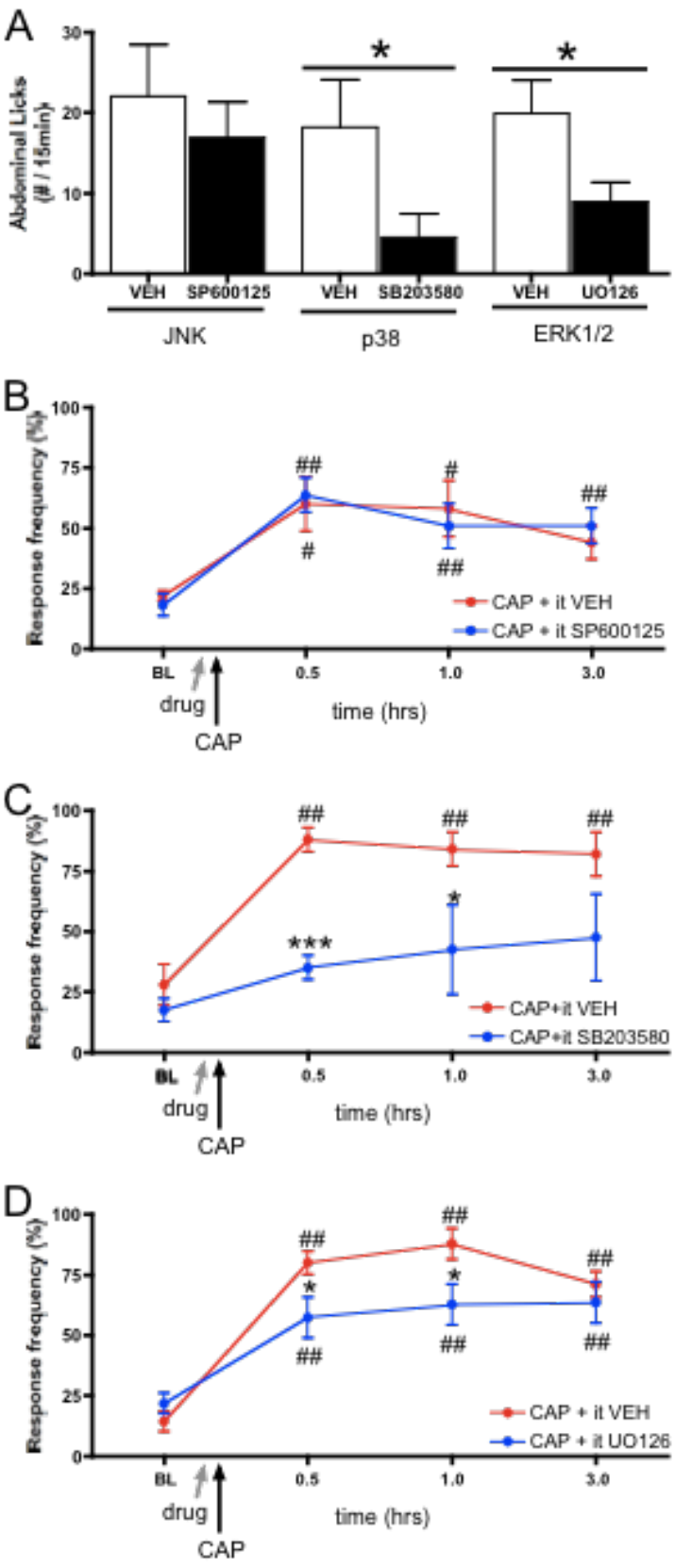

Figure 11: Spinal blockade of p38 and ERK1/2, but not JNK, prevent iCAP-induced secondary allodynia. *A) The p38 antagonist SB203580 and the ERK1/2 antagonist U-0123 prevented iCAP-induced abdominal licking (unpaired t-test). However, the JNK antagonist SP600125 had no effect. B) Spinal blockade of JNK did not affect the response frequency to abdominal stimulation (1 mN von Frey) of iCAP-treated mice. However, both p38 (C) and ERK1/2 blockade (D) did prevent increased response frequency. Stars (*) indicate significant effect of inhibitor group compared to vehicle group (unpaired t-test). Number signs (#) represent significant Dunnett's post hoc tests (all comparisons are to baseline). (# or *p<0.05; ## or **p<0.01; ### or ***p<0.001; n = 4 – 11 per group).*

3.3.6. Spinal TRPV1 receptors are involved in iCAP-evoked secondary allodynia. Intrathecal injection of neither 1 nor 10 nmol NADA, a TRPV1 agonist, produced a change in response frequency to 1 mN von Frey stimulation of the hind paw 15 (Fig 12A) and 60 min (Fig 12B) following the intrathecal injection. Only 10 nmol NADA evoked hyperalgesia to an 8 mN stimulus at the 60 min time point (Fig 12B). In addition, we measured the response frequency to dynamic mechanical stimulation (gently stroking the hindpaw with a cotton bud) in NADA-treated mice. At both 15 (Fig 12C) and 60 min (Fig 12D) following intrathecal NADA (10 nmol), mice displayed stroking allodynia, whereas mice injected with 1 nmol NADA had stroking allodynia only at the 60 min time point (Fig 12D). Furthermore, intrathecal administration of the TRPV1 antagonist AMG 9810 (1 nmol) reversed NADA-evoked stroking allodynia at 15 (Fig 12E) and 60 min (Fig 12F) post intrathecal injection, indicating that the effects of NADA are TRPV1-mediated. Finally, we determined that IT BTD also blocked NADA-evoked stroking allodynia at both 15 (Fig 12G) and 60 min (Fig 12H) following intrathecal injection. Finally, the TRPV1 antagonist AMG 9810 was intrathecally injected 5 min prior to iCAP and the response frequency to 1 mN stimulation of the abdomen was measured at 0.5, 1.0 and 2.0 hrs post-CAP. After iCAP vehicle-treated mice displayed robust secondary allodynia (Fig. 13; red line). However, AMG-9810-treated mice exhibited a significant reduction in secondary allodynia at the 0.5-hour time point (Fig. 13; blue line).

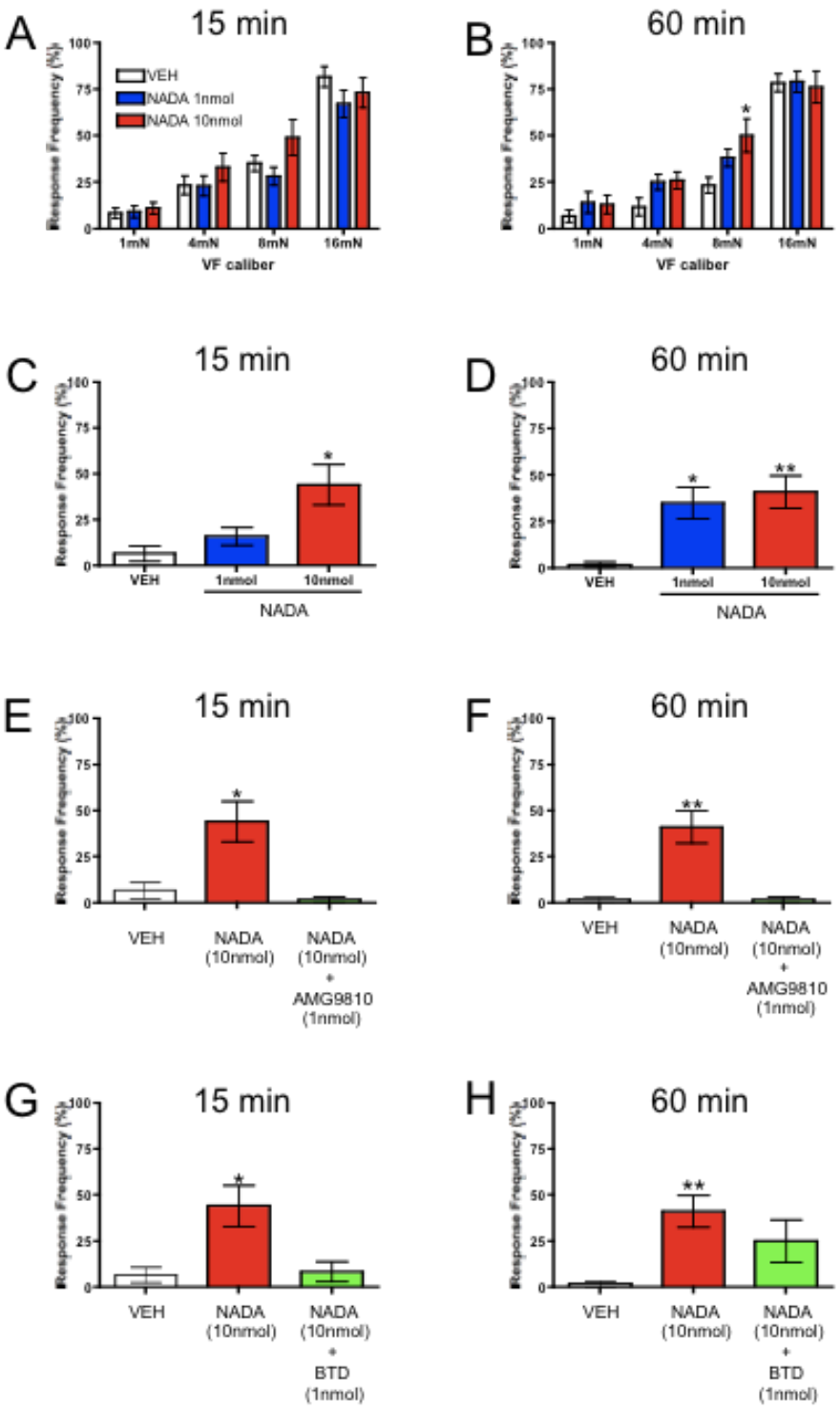

Figure 12: Both TRPV1 and NKCC1 blockade reverse spinal TRPV1-mediated stroking allodynia in the hindpaw. *(A/B) The endogenous TRPV1 agonist NADA (1 or 10 nmol) or vehicle (VEH) was injected intrathecally and the hindpaws were stimulated with von Frey filaments of the indicated calibers at 15 (A) and 60 min (B). In panels C-H, stroking allodynia was measured by gently stroking a cotton bud on the hindpaw 15 (C/E/G) and 60 min (D/F/H) after the intrathecal injection. Stars (*) above columns indicate significant effects of NADA or NADA + AMG or BTD vs. VEH (* $p < 0.05$, ** $p < 0.01$; n = 6 - 10 per group). Panels A-B, two-way ANOVA + Bonferroni post-hoc tests; panels C-H one-way ANOVA + Dunnett's post-hoc tests. NADA = TRPV1 agonist; AMG 9810 = TRPV1 antagonist; BTD = NKCC1 blocker. Figure adapted from Pitcher et al. 2007.*

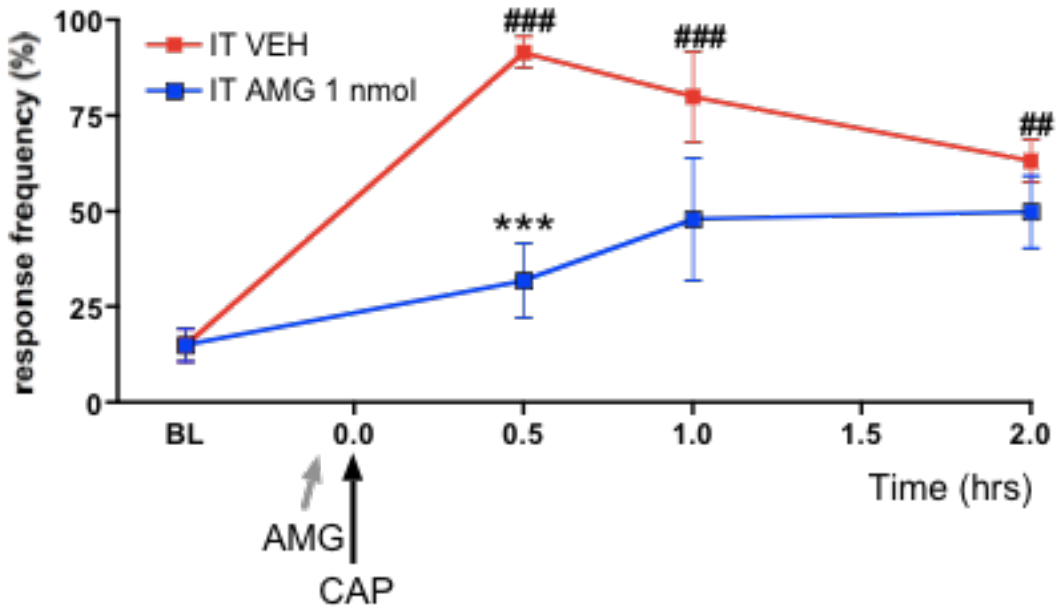

Figure 13: Spinal TRPV1 antagonism inhibits intracolonic CAP-evoked secondary allodynia. *The TRPV1 antagonist AMG 9810 (1nmol) was injected intrathecally 5 min prior to intracolonic CAP. Response frequencies to abdominal von Frey hair (1 mN) stimulation at the indicated time points post-CAP are shown. Stars (*) indicate significant effects of AMG 9810 (blue squares) vs. VEH (red squares; two-way ANOVA) while number signs (#) indicate significant effects vs. baseline (BL, one-way ANOVA) at a given time point (## $p < 0.01$, ### or *** $p < 0.001$; $n = 6$ per group). Figure adapted from Pitcher et al. 2007.*

3.4. Discussion

Our behavioral findings support the hypothesis that modulation of NKCC1 activity on baseline nociceptive processing (naïve mice) has little effect whereas enhanced NKCC1 activity plays an important role in maintaining intracolonic CAP-induced allodynia and hyperalgesia; in naïve mice, spinal blockade of NKCC1 had no effect on either tail flick latency or response frequency to abdominal von Frey stimulation. Specifically, in the absence of iCAP, spinal NKCC1 blockade with BTD at doses lower than 1 nmol had virtually no effect on responses to abdominal von Frey stimulation. However, higher doses began to evoke more behavioral responses to stimulation, likely due to BTD's action at KCC2. For this reason we used 1 nmol BTD for all subsequent behavioral studies. When injected either before or after iCAP, spinal BTD reduced secondary allodynia for up to one hour post-injection, indicating that while spinal NKCC1 blockade transiently inhibits the maintenance of secondary allodynia, it does not appear to prevent its development. This effect may reflect the long-lasting nociceptive stimulus evoked by iCAP (Laird et al. 2001), rapid turnover of BTD-blocked NKCC1 or perhaps a short bioavailability of BTD.

It is evident from our SNB data that while iCAP elicited multiple behaviors (abdominal licking, abdominal retractions and squashing behavior), not all were sensitive to NKCC1 blockade. Interestingly, while BTD reduced the number of iCAP-induced abdominal licks to baseline levels, BTD also appeared to increase both squashing behavior and abdominal retractions. It may be that blockade of abdominal licking with BTD permits subtler, potentially BTD-insensitive behaviors such as squashing and abdominal retractions to become more prominent. It is

also possible that spinal cord sections remote from L4, the region where BTD was injected, mediate these behaviors. Overall, spinal NKCC1 clearly plays an important role in maintaining nociceptive input to the spinal cord.

In terms of the effects of MAPK blockade on iCAP-evoked secondary allodynia, considering that the secondary allodynia produced by iCAP appears to be mediated largely by NKCC1 and if, as suggested by Marshall et al. (2005), JNK were involved in arresting NKCC1 activity, our finding that spinal JNK blockade did not affect iCAP-evoked secondary allodynia would be expected. On the other hand, this result is surprising in light of other findings; spinal JNK inhibition reduced primary mechanical allodynia after complete Freund's adjuvant (CFA; Gao et al. 2010) and neuropathic injury (Obata et al. 2004; Zhuang et al. 2006). Perhaps this discrepancy reflects differences in the pain model, time course or, alternatively, differences in the mechanisms of primary vs. secondary allodynia. Nonetheless, this finding suggests that JNK does not play a role in regulating NKCC1's activity in this model. Further study involving dose-response testing is required. Contrary to our findings with the JNK inhibitor, blockade of both p38 and ERK1/2 reduced CAP-evoked secondary allodynia. Accordingly, CAP rapidly augments phosphorylated ERK1/2 and p38 in small and medium sized DRG neurons (Dai et al. 2002; Mizushima et al. 2005). Consistent with our finding, p38 inhibition with SB203580 reduced primary mechanical allodynia following neuropathic injury (Schafers et al. 2003; Jin et al. 2003; Zhang et al. 2005; Lim et al. 2005; Cheng et al. 2010) and Galan et al. (2003) showed that blockade of ERK1/2 with U-0126 was effective in reducing iCAP-evoked secondary allodynia.

Thus, it is possible that our findings reflect changes in NKCC1 regulation through blockade of p38 and ERK1/2. More work in this area is required.

The effects of spinal TRPV1 blockade were also measured; inhibition of spinal TRPV1 reduced iCAP-induced secondary allodynia while spinal NKCC1 blockade attenuated spinal TRPV1-induced stroking allodynia. Considering that NKCC1 and TRPV1 appear to be co-expressed on nociceptive afferent fibers (Price et al. 2006), our data suggests that in persistent pain states, TRPV1 and NKCC1 activity may be associated. Activation of TRPV1 triggers extracellular regulated kinase 1&2 (ERK; Zhuang et al. 2004) as well as calcium/calmodulin-dependent kinase II α (CaMKIα; Price et al. 2005b), both known to regulate NKCC1 activity (Schomberg et al. 2001; Liedtke and Cole, 2002; but see Lu et al. 2008). Furthermore, both ERK (Galan et al. 2003) and CaMKIIα (Galan et al. 2004) are involved in intracolonic capsaicin-induced referred hyperalgesia. Therefore, intrathecal NADA's effects on TRPV1 receptors may activate ERK and/or CaMKIIα leading to an increase in NKCC1 activity causing touch evoked pain. Another explanation may be that spinal TRPV1 activation mediates allodynia by regulating GABAergic neurotransmission; dorsal horn TRPV1 activation produces SP release in dorsal horn laminae I, III and IV, thus augmenting GABAergic network activity in lamina II of the spinal cord (Ferrini et al. 2007). This enhanced GABA activity may produce excessive PAD on NKCC1-expressing afferent fibers, providing an alternative explanation for NKCC1's role in allodynia mediated by NADA. Our behavioral findings further implicate NKCC1 in inflammatory allodynia and suggest that spinal TRPV1 receptors, as well as p38 and ERK1/2, may be

involved in regulating NKCC1 activity in nociceptive primary afferents. Clearly, more work is needed to elucidate their interaction in secondary allodynia.

CHAPTER 4

CELLULAR LEVEL APPROACH –

IN VIVO ELECTROPHYSIOLOGY

4.1. Specific Aim: Examine the effects of spinal NKCC1 blockade on intraplantar capsaicin-induced sensitization of spinal nociceptive neurons

There are currently no published studies directly measuring the effects of NKCC1 blockade on sensitized CNS nociceptive neurons. Allodynia and hyperalgesia has been linked to the sensitization of dorsal horn wide dynamic range (WDR) and nociceptive specific (NS) neurons (Dougherty and Willis, 1992; Willis 2001; 2002). Immunohistochemistry and molecular biology approaches indicate that NKCC1 is located in tissues and regions relevant to nociceptive processing and is upregulated when challenged by injury/inflammation (Morales-Aza et al. 2004; Galan et al. 2005; Price et al. 2006; Funk et al. 2008; Cramer et al. 2008). Genetic knockout and behavioral studies (Sung et al. 2000; Laird et al. 2004; Granados-Soto et al. 2005; Valencia-de-Ita et al. 2006) indicate NKCC1's involvement in both primary and secondary hyperalgesia. Functional *in vivo* studies of the role of PAD in peripheral nociceptive processes are available (Lin and Fu, 1998; Lin et al. 2000; Garcia-Nicas et al. 2001), however, at the time of writing, only one *in vivo* study concerning NKCC1 involvement in nociceptive processing could be found, and it is focused on peripheral processes (Valencia-de-Ita et al. 2006). Using *in vivo* extracellular electrophysiology techniques on spinal cord neurons of intact rats, we tested the hypothesis that NKCC1 is functionally linked to sensitization of dorsal horn nociceptive neurons. Specifically, we asked whether or not hyperalgesia associated with persistent nociceptive input might result from enhanced NKCC1 activity such that PAD produces spike activity in dorsal horn nociceptive neurons. We wanted to test the

existence of an NKCC1-dependent association between peripheral CAP stimulation and sensitization of spinal dorsal horn nociceptive neurons.

4.2. Methods

4.2.1. Animals

In vivo electrophysiology experiments were conducted on male Sprague Dawley rats weighing 250 - 350g (Charles River; Montreal, Quebec, Canada). Experiments were in accordance with the Canadian Counsel on Animal Care (CCAC) and the International Association for the Study of Pain (IASP) guidelines for the care and use of experimental animals. All protocols were reviewed and approved by the McGill University Animal Care Committee.

4.2.2. Drugs

Capsaicin (0.3ml of 0.1%, Tocris, Ellisville, MO) was dissolved in 10% ethanol, 10% Tween 80 and 80% saline and was used in electrophysiological experiments with rats (intraplantar). The NKCC1 blocker BTD (Sigma, St. Louis, MO) was dissolved in artificial cerebrospinal fluid (aCSF) vehicle. The components of the aCSF vehicle were (in mM) 1.3 CaCl2 2H2O, 2.6 KCl, 0.9 MgCl, 21.0 NaHCO3, 2.5 Na2HPO47H2O, 125.0 NaCl, and 3.5 dextrose (pH 7.2–7.4).

4.2.3. *In Vivo* Electrophysiological Procedures

4.2.3.1 Experimental Protocol

Rats were anesthetized with sodium pentobarbital (initial intraperitoneal (ip) dose of 60 mg / kg; 10 mg / kg / h intravenous (iv) maintenance dose). Reflex motor responses to noxious stimulation (toe pinch) as well as corneal responses to a puff of air were monitored to ensure adequate anesthesia. The right carotid artery and left jugular vein were catheterized for monitoring continuous arterial blood

pressure and for injection of anesthetic, respectively. The trachea was cannulated to allow artificial ventilation and continuous end-tidal CO_2 recordings. Body temperature was kept constant at 38 °C with a rectal temperature feedback-controlled electric blanket. Spinal segments L4 to S1 were exposed by laminectomy of the T13 to L2 vertebra and the animal was mounted on a rigid frame. The animal was paralyzed with pancuronium bromide (0.2 mg / kg initial dose, 0.1 mg / kg / h maintenance dose) and artificially ventilated. The left sciatic nerve was dissected and prepared for stimulation through bipolar silver electrodes. A pool of agar was made around the thoraco-lumbar laminectomy. The exposed spinal cord was protected with 38 °C mineral oil. At the end of each experiment the animal was killed with an overdose of sodium pentobarbital.

4.2.3.2. Recording techniques

Using a monopolar silver electrode the cord dorsum potential recordings defined the spinal cord terminal region of the left (ipsilateral) sciatic nerve. The electrical threshold of the primary afferent volleys in the cord dorsum potential was noted. Single-unit electrical activity was recorded from spinal neurons in the area of sciatic nerve termination previously identified. Glass micropipettes filled with 4M NaCl (resistance measured at 1 kHz was 8 - 15 MΩ) were used. Electrical stimulation of the sciatic nerve at intensity fivefold the threshold of the afferent volley in the cord dorsum potential (supra-maximal for A fibers but sub-threshold for C fibers) was used as the search stimulus. Electrical activity of neurons was amplified and filtered using a 2400A Dagan amplifier (Minneapolis, USA), digitized by a computer interface (CED 1401, CED, Cambridge, UK), and

analyzed with a computer running Spike2 software (CED). All data were stored for off-line analysis.

4.2.3.3. Characterization of the neurons

Mechanical stimulation of the receptive field (RF) of the hind paw (brushing, touch, and light tapping, as well as noxious stimuli such as pin prick and pinching with fine forceps) and electrical stimulation of the sciatic nerve was used to characterize each neuron. Responses to mechanical stimulation were measured with a set of calibrated Von Frey filaments (milliNewtons (mN)) applied to the cutaneous receptive field. Only neurons classified as wide dynamic range (WDR) or nociceptive specific (NS) were selected for further analysis. Prior to intraplantar CAP, WDR neurons responded to both low-intensity and high-intensity stimuli while nociceptive specific (NS) neurons responded only to high-intensity stimuli.

4.2.3.4. Experimental design

After characterization of the neuron, the following experimental protocol was applied. Each experiment began with a 5-min recording of background activity. Then mechanical stimuli of 20 sec duration each were applied (brushing, 49, 196 and 490 mN von Frey). Only after the end of the previous stimulus-evoked response was the next stimuli applied. The same protocol was applied before and 40 min after CAP application. Chemical stimulation of the skin was applied inside the receptive field (RF) of each neuron but distal from the sites of mechanical stimulation. The size of the receptive field, as measured using brush and 49mN von Frey, was also measured before and after CAP application. The effects of the NKCC1 blocker BTD were tested on the responses of the neurons after

sensitization. BTD was applied directly over the spinal cord in a volume of 150 µl. The same stimulus protocol as described above was used 15 min after drug administration. Pre-treatment studies with CAP were not performed because not all neurons sensitize to CAP.

4.2.4. Statistical Analysis

Stimulus-evoked responses were defined as the total number of action potentials (spikes) evoked during the 20 sec of stimulation. NS neurons were considered sensitized if responses to high threshold stimulus increased by at least 20% in addition to the appearance of a novel low threshold component post-CAP. Sensitization of WDR neurons was defined as at least a 20% increase in Post-CAP spike rate averaged across stimuli (brush, 49mN, 196mN, 490mN), compared to baseline. The receptive fields (RF's) were traced on a schematic drawing of the hind paw before and after chemical stimulation, using digits and footpads as landmarks. The areas of the RF's were measured after digitization using specialized measuring software (ImageJ; NIH, USA). RF size was measured at baseline, post-CAP and post-BTD timepoints. The degree of sensitization was operationally defined as the average increase in stimulus-evoked (brush, 49mN, 196mN and 490mN) spike activity after intraplantar CAP (displayed in percent). Effectiveness of BTD was defined as the mean BTD-induced reduction in stimulus-evoked spike rate (displayed in percent), per animal. Data are shown as mean ± SEM. Normality tests were performed on all data sets; non-parametric statistics were used on non-Gaussian distributions. Furthermore, because of the wide variability in spike rate between neurons, parametric statistics such as ANOVA can increase the likelihood of non-

significant mean differences despite potential trends for increased or decreased spike rate. Therefore, non-parametric analysis is preferred because of its non-reliance on variance. In all cases, $p < 0.05$ was considered significant.

4.3. Results

4.3.1. Sample of neurons

The majority of the results in this section have already been published (Pitcher et al. 2010). Results were obtained from a total of 82 neurons. Of these, there were 58 WDR and 24 NS neurons. A total of 34 WDR and 9 NS neurons were fully characterized. Neurons were defined as WDR or NS based on their characteristic responses to low and high threshold stimulation. We measured background (stimulus-independent) spike activity as well as stimulus-evoked activity (49mN, 196mN, 490mN von Frey and brush to the ipsilateral hindpaw). Typically, WDR neurons responded in a characteristic graded fashion to increasing intensity of mechanical stimulation (Fig. 14A) while NS neurons had virtually no response to low threshold input and responded vigorously to higher threshold, noxious input (Fig. 14B). The cutaneous receptive fields (RF's) of both WDR and NS neurons were located mainly in the glabrous skin of the hindpaw, including the digits. Mean RF size of WDR neurons was larger than that of NS neurons ($t(17)=2.05$; $p=0.0281$; Fig. 14C). Mean depth of WDR neurons (789 ± 45μm) was significantly deeper ($t(30)=2.48$; $p=0.0189$) than NS neurons (566 ± 85μm; Fig. 14D).

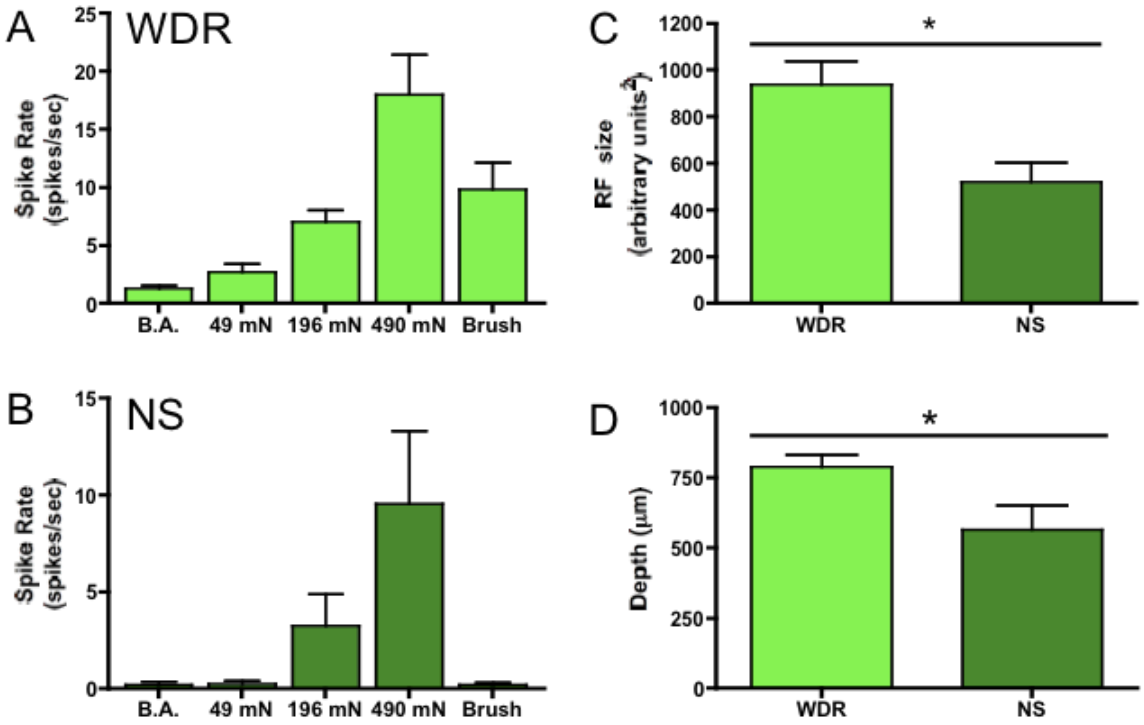

Figure 14: Baseline spontaneous and mechanically evoked spike activity in spinal WDR (A) and NS (B) neurons as well as comparison of RF size (C) and mean depth (D). *Typical of WDR neurons, graded mechanical stimulation produced graded responses, with most intense responses produced by noxious mechanical stimulation within the cutaneous receptive field. NS neurons, however, responded mainly to high intensity stimulation. The RF size of NS neurons was smaller than that of WDR neurons (C). WDR neurons were sampled mainly from deeper regions of the spinal dorsal horn than NS neurons (D). Unpaired t-test (*p<0.05; n = 23 for WDR neurons; n = 10 for NS neurons). BA = background activity.*

4.3.2. Acute responses to intraplantar CAP

A total of 31 WDR and 9 NS neurons were fully tested for sensitization after 0.1% capsaicin (CAP) was injected into the intraplantar region of the ipsilateral hindpaw. Application of CAP produced transient spontaneous activity, referred to as the acute post-CAP response, that was quantified as the number of spikes during the first 15 min post-CAP. The mean number of post-CAP spikes was 3565 ± 670 in WDR neurons and 992 ± 353 in NS neurons.

WDR neurons were considered sensitized if all stimulus-evoked neuronal responses increased by at least 20% post-CAP. NS neurons were considered sensitized if responses to high threshold stimulus increased by at least 20% in addition to the appearance of a novel low threshold component post-CAP. Based on the magnitude of pre-CAP stimulus-evoked responses (Fig. 15A) and development of sensitization after CAP (Fig. 15B), two groups of WDR neurons were defined: i) neurons with higher baseline spike rate that did not sensitize to CAP (Fig. 15B, hatched bars) and ii) neurons with lower baseline spike rate that did sensitize to CAP (FIG 15B, solid bars). In sensitizing WDR neurons, Wilcoxon signed ranks testing revealed that CAP significantly increased background activity (B.A.) from 0.66 ± 0.16 Hz to 3.90 ± 1.02 Hz (W=-53, p=0.002; Fig. 15B). However, CAP had no significant effect on background activity in non-sensitizing WDR neurons, whose background activity was 1.91 Hz ± 0.45 Hz pre-CAP and 2.81 Hz ± 0.73 Hz post-CAP (W=-25, p=0.082). In response to mechanical stimulation, the spike rate of sensitizing neurons increased significantly after CAP (Fig. 15B; solid bars) but did not change in non-sensitizing neurons (Fig. 15B; hatched bars). Of the 31 WDR neurons tested with CAP, 19 (61.3%) sensitized to

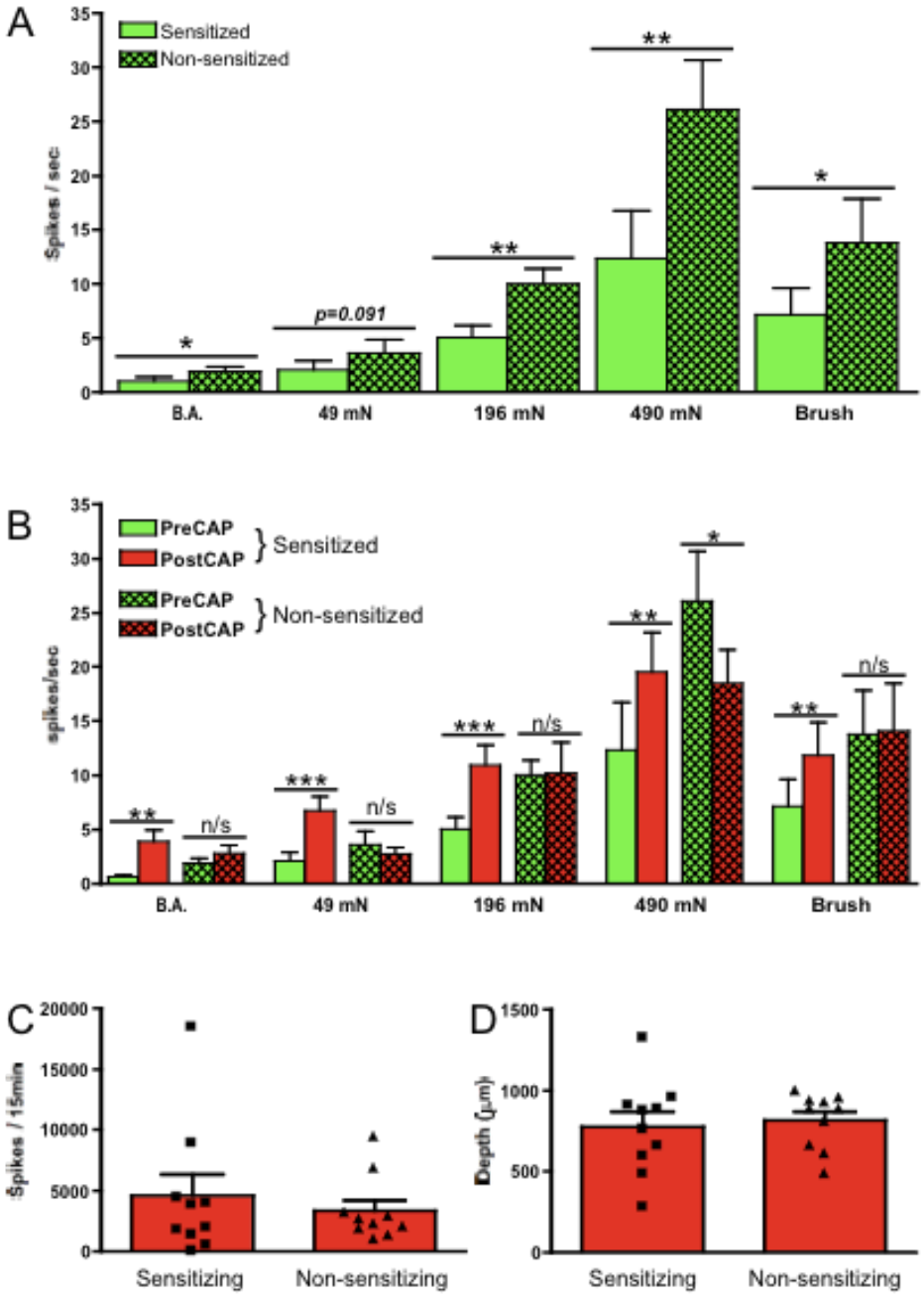

Figure 15: Distinct subsets of spinal WDR neurons based on differences in pre-CAP stimulus-evoked spike activity (A) and responsiveness to CAP (B). *Two subsets of WDR neurons were found in the dorsal horn; neurons with low stimulus-evoked spike rates (panel A, solid bars) that sensitized to CAP (panel B; solid green vs. solid red bars) and neurons with higher stimulus-evoked spike rates (panel A, hatched bars) that did not sensitize to CAP (panel B; green hatched vs. red hatched bars). Sensitizing and non-sensitizing WDR neurons share similar acute responses to CAP (C) and were sampled from similar regions of the dorsal horn (D). Statistical comparisons are either Wilcoxon Signed Ranks tests or t-tests, where applicable. P<0.05 was considered significant in all cases. (*p<0.05; **p<0.01; ***p<0.001; n = 10 or 13 for non-sensitized and sensitized WDR neurons, respectively). BA = background activity.Figure adapted from Pitcher et al. 2010.*

CAP while 12 (38.7%) did not. Sensitizing and non-sensitizing WDR neurons did not differ in terms of the acute post-CAP response (Fig. 15C) or their depth within the spinal cord (Fig. 15D). The acute post-CAP response in sensitized WDR neurons is positively correlated with depth ($r=0.64$, $p=0.02$), with CAP evoking more spikes in deeper WDR neurons. No such correlation was found in non-sensitizing WDR neurons. In non-sensitizing WDR neurons there was no association between the baseline (pre-CAP) spike rate and the number of post-CAP spikes ($r=0.04$, $p=0.92$). However, in sensitizing neurons, pre-CAP spike rate was positively correlated with the number of post-CAP spikes ($r=0.60$, $p=0.02$), such that neurons with more intense baseline responses also generated more post-CAP spikes.

Distinct subsets of NS neurons were also found (Fig. 16A/B). Some neurons sensitized in response to CAP (Fig. 16B; solid bars), and others did not (Fig. 16B; hatched bars). Prior to CAP, NS neurons responded almost exclusively to high threshold stimuli. However, after CAP sensitizing NS neurons responded more intensely to low threshold stimuli (brush, 49mN von Frey; Fig. 16B) indicating a CAP-induced unmasking of low threshold input to NS neurons. Intraplantar CAP also increased background activity from 0.09 ± 0.07 Hz to 2.86 ± 1.57 Hz ($W=-19$, $p=0.031$; Fig. 16B) in sensitizing NS neurons. Both subsets of NS neurons showed similar acute responses to CAP ($t(6)=0.929$, $p=0.389$; Fig. 16C) and were sampled from similar regions of the spinal cord ($t(7)=0.714$, p=o.498; Fig. 16D). In neither group was there a correlation between stimulus-evoked pre-CAP spike rate and the number of post-CAP spikes.

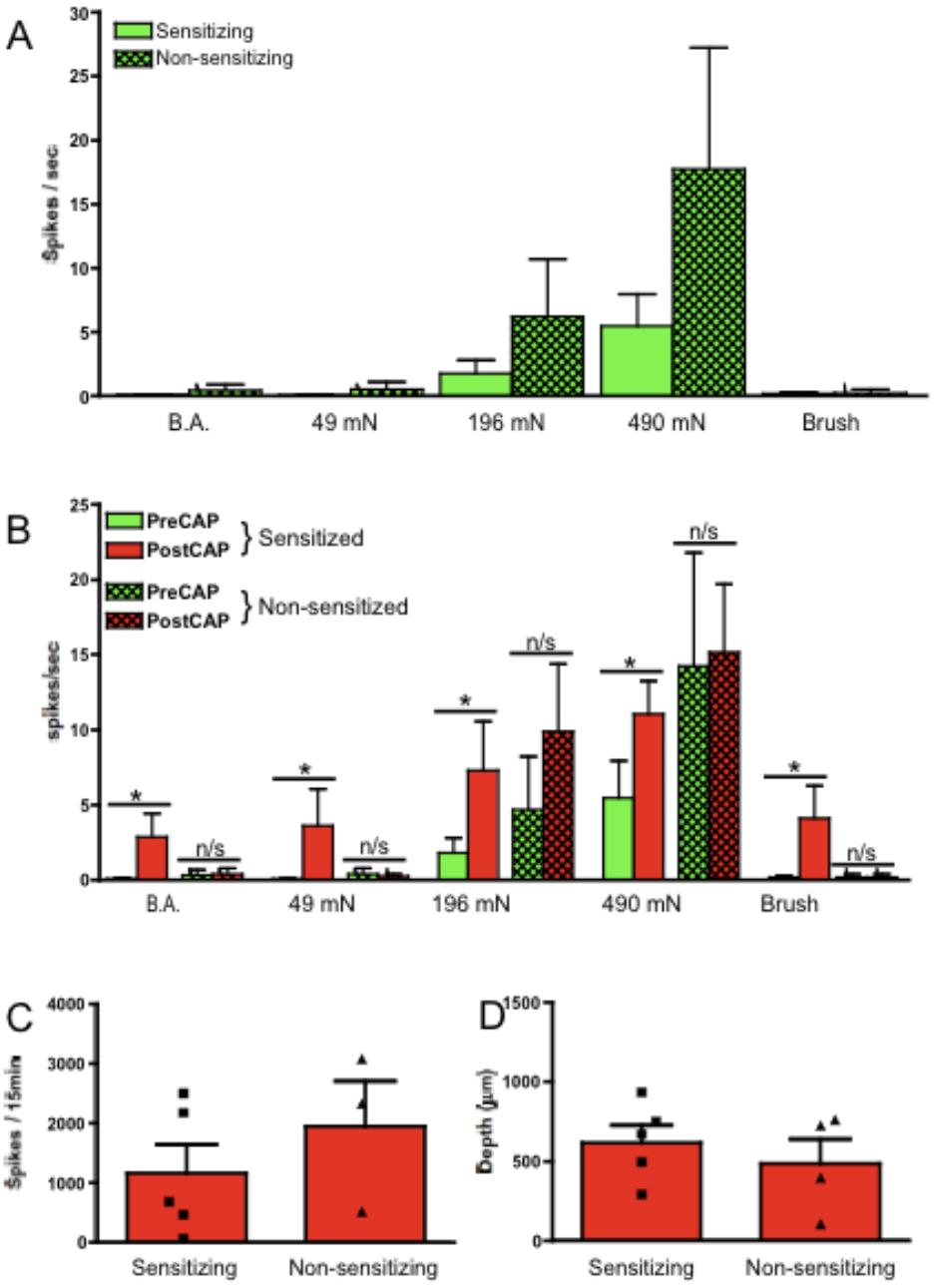

Figure 16: Distinct subsets of spinal NS neurons based on differences in pre-CAP stimulus-evoked spike activity (A) and responsiveness to CAP (B). *Two subsets of NS neurons were found in the dorsal horn; neurons with low stimulus-evoked spike rates (panel A, solid bars) that sensitized to CAP (panel B; solid green vs. solid red bars) and neurons with higher stimulus-evoked spike rates (panel A, hatched bars) that did not sensitize to CAP (panel B; hatched green vs. hatched red bars). In panel A, differences between sensitizing and non-sensitizing neurons were not statistically significant, possibly due to small sample size of non-sensitizing NS neurons. Sensitizing and non-sensitizing NS neurons share similar acute responses to CAP (C) and were sampled from similar regions of the dorsal horn (D). Statistical comparisons are either Wilcoxon Signed Ranks Tests or t-tests, where applicable. P<0.05 was considered significant in all cases. (*p<0.05; **p<0.01; ***p<0.001; n = 3 or 6 for non-sensitizing and sensitizing neurons, respectively). Figure adapted from Pitcher et al. 2010.*

4.3.3. Sensitization after CAP

The magnitude of the sensitization evoked by CAP in each neuron was calculated by dividing the post-CAP spike rate by the pre-CAP spike rate for each stimulus (brush, 49mN, 196mN and 490mN von Frey) and calculating the average. Background activity in WDR neurons was positively correlated with stimulus-evoked spike activity, such that mechanical stimulation elicited more intense responses from WDR neurons with higher background activity (Fig. 17A; $r=0.61$, $p=0.01$).

A significant negative correlation was observed between pre-CAP spike rate and the degree of CAP-induced sensitization ($r=-0.66$; $p=0.003$) in WDR neurons, such that the degree of sensitization decreased as pre-CAP spike rate increased (Fig. 17B). There was no correlation between the degree of sensitization and the acute post-CAP response (Fig. 17C). However, these correlations were not found in NS neurons, perhaps due to the smaller sample size.

4.3.4. CAP-induced alterations in receptive field size

Low threshold stimulation (brush and 49mN von Frey) was used to determine the cutaneous RF size of neurons. Of the 15 sensitized WDR neurons for which we measured RF size, 14 (93.3%) had increased RF sizes after intraplantar CAP. The mean increase was 16.75% ± 3.62%, compared to pre-CAP values. Wilcoxon Signed Ranks testing indicated that RF size increased significantly after CAP (Fig. 18A). The RF size of non-sensitized WDR neurons was unchanged by CAP (Fig. 18B). Of the four NS neurons sensitized by CAP for which we measured the RF, two had increased RF's compared to pre-CAP values (16.30% ± 10.53% compared to pre-CAP RF size). Overall, for all four NS neurons there

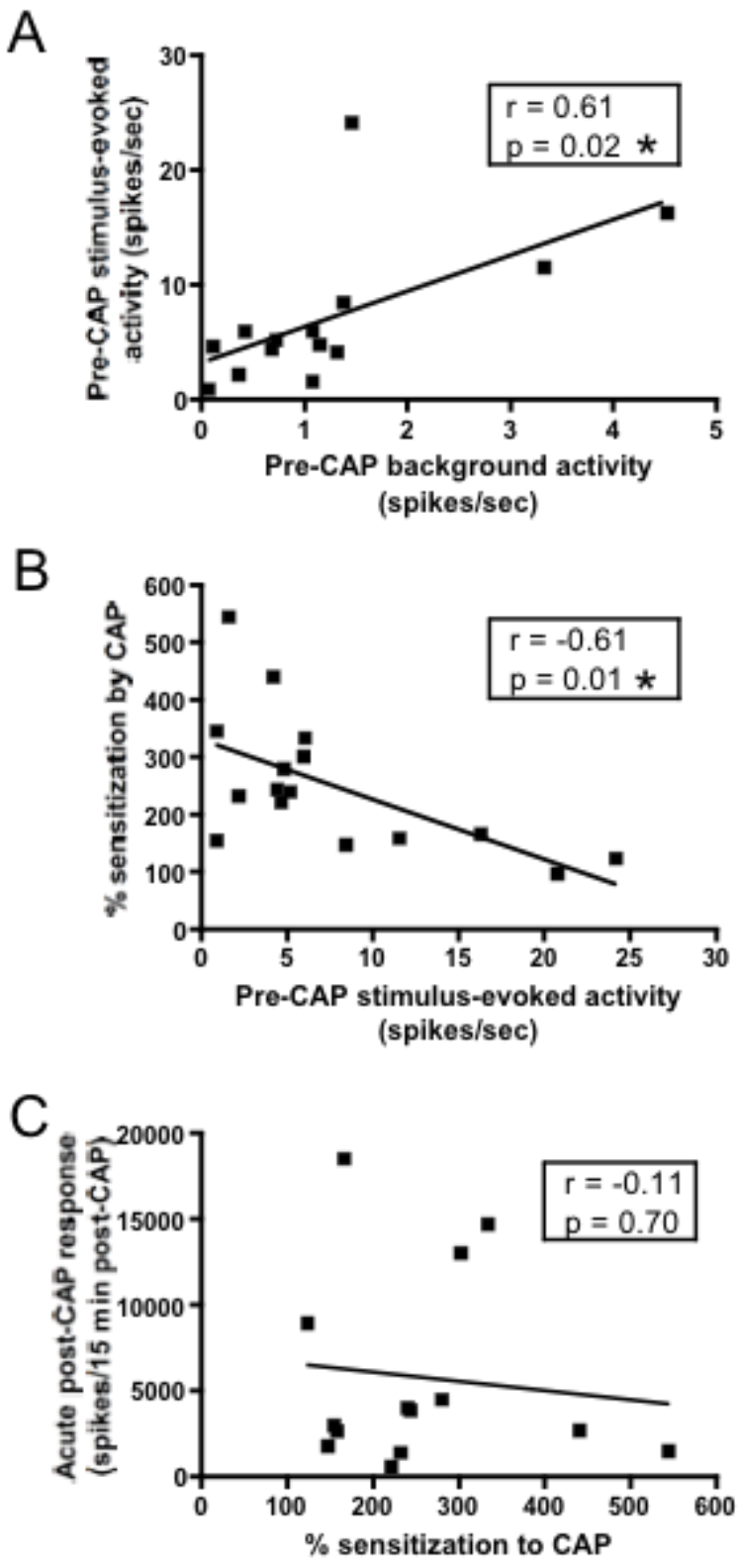

Figure 17: In spinal WDR neurons, pre-CAP stimulus-evoked spike rate is positively correlated with background activity (A) and degree of sensitization after CAP (B). *In panel A, linear regression analysis indicates that background activity and pre-CAP spike rate are positively correlated such that WDR neurons with higher background activity respond more intensely to mechanical stimulation. In panel B, pre-CAP spike rate is predictive of the degree of sensitization after CAP such that neurons that respond more intensely to stimulation prior to CAP tend to become less sensitized after CAP. In panel C, the acute response to CAP, as measured by the number of spikes for the first 15 min post-CAP, is not correlated with the degree of sensitization. P<0.05 was considered significant in all cases.*

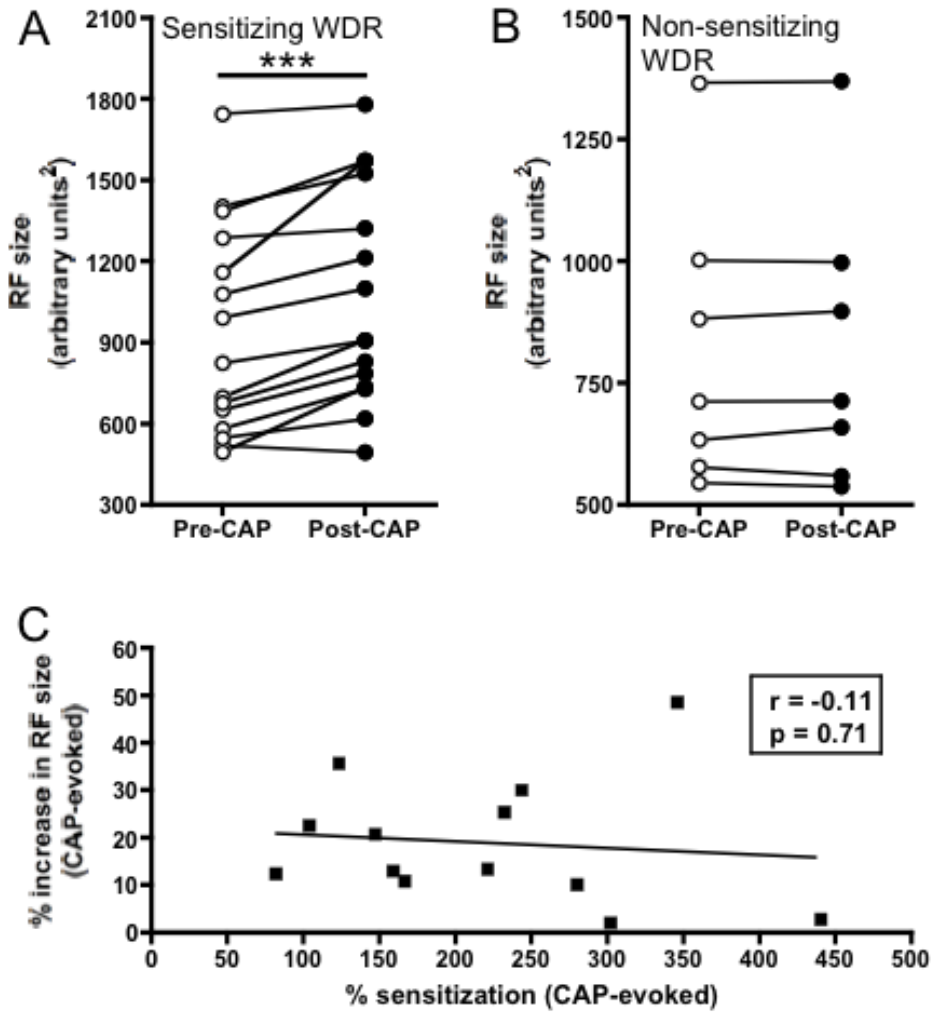

Figure 18: Intraplantar CAP produces increased RF size in WDR (A) but not NS neurons (B). Furthermore, CAP-induced increase in receptive field (RF) size of WDR neurons is unrelated to the degree of CAP-induced sensitization (C). *In panel A, the non-parametric Wilcoxon Signed Rank testing indicated that CAP significantly increased the RF size in WDR neurons. Note the trend for increased RF size in each neuron after CAP. However, CAP did not significantly affect RF size in NS neurons (B). Panel C illustrates that there is no significant correlation between CAP-induced increase in RF size and degree of sensitization; while both increased RF size and sensitization are due to CAP, this finding suggests that they are due to distinct mechanisms. Wilcoxon Signed Rank tests were used in A and B. Pearson correlational analysis and Linear Regression Analyses were used in C. P<0.05 was considered significant in all cases (***p<0.001). Figure adapted from Pitcher et al. 2010.*

was no significant change in Post-CAP RF (W=0, p=0.563). Similar to non-sensitized WDR neurons, the RF size of non-sensitized NS neurons was also unchanged by CAP. CAP-induced changes in RF size were not associated with the level of sensitization for WDR neurons (r=0.11, p=0.714; Fig. 18C) such that the amount of change of RF size after CAP was not related to the level of sensitization.

4.3.5. Blockade of the NKCC1 co-transporter

Spinal NKCC1 blockade by BTD was studied in 31 WDR and 9 NS neurons. A total of 16 (51.6%) WDR neurons sensitized to CAP and 13 (41.9%) of these were treated with 500μM BTD. For NS neurons, 6 (66.7%) neurons sensitized to CAP and were treated with 500μM BTD, while the remaining three neurons did not sensitize to CAP. Spinal application of either BTD-vehicle (aCSF) or 200μM BTD after CAP did not significantly affect mechanically-evoked spike activity in WDR neurons (Fig. 19; light blue and medium blue bars, respectively). However, 500μM BTD significantly reduced spike activity (Fig. 19, dark blue bars). Therefore, 500μM BTD was used for all further electrophysiological experiments.

In sensitized WDR neurons the pre-CAP stimulus-evoked spike rate was strongly correlated with post-CAP stimulus-evoked spike rate, such that neurons that responded more intensely to stimulation prior to CAP also responded more intensely post-CAP (Fig. 20A; r^2=0.88, p<0.0001). In addition, the post-CAP spike rate of WDR neurons (Fig. 20B) was positively correlated with the post-BTD spike rate (r^2=0.87, p<0.0001), so that neurons with higher post-CAP spike rates also had higher post-BTD spike rates. The degree of sensitization of each WDR neuron was not correlated with the effectiveness of BTD in reducing CAP-

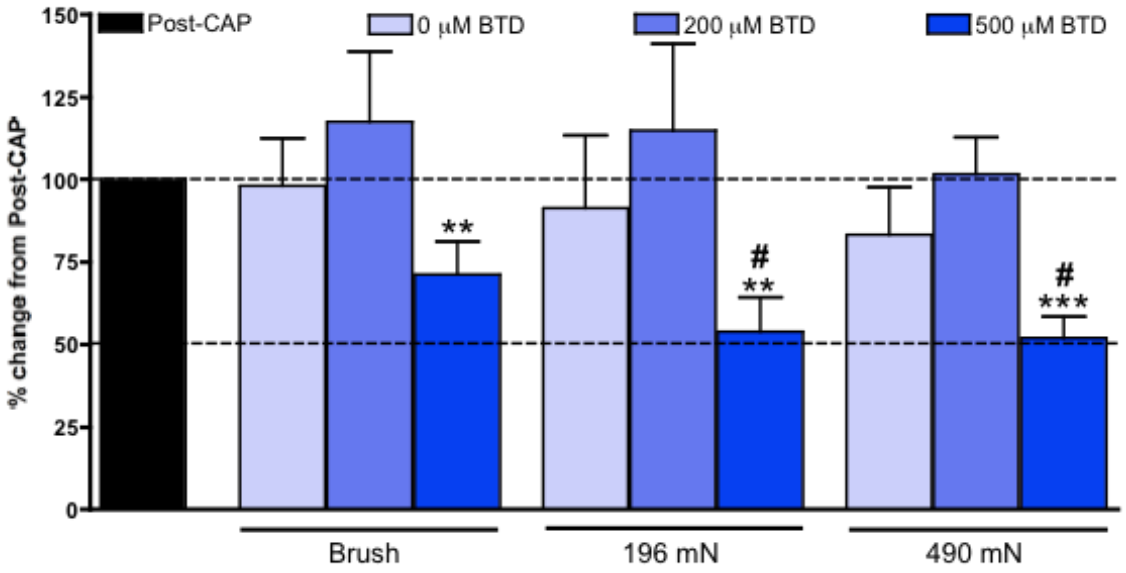

Figure 19: Dose dependency of spinal BTD on low and high intensity mechanical stimulus-evoked WDR neuronal activity. *Compared to post-CAP responses (black bar), stimulus-evoked response rate was neither reduced by BTD vehicle nor 200μM BTD. However, 500μM BTD reduced stimulus-evoked response rate after CAP. One-way ANOVA followed by post-hoc Dunnett's multiple comparison test (n = 5, 3 and 13 for 0μL BTD, 200μL BTD and 500μL BTD, respectively). Stars (*) indicate significant post hoc comparison with post-CAP values (black bar). Number signs (#) indicate significant post hoc comparison with vehicle-treated group (white bars). P<0.05 considered significant in all cases (# p<0.05; **p<0.01; ***p<0.001). Figure adapted from Pitcher et al. 2010.*

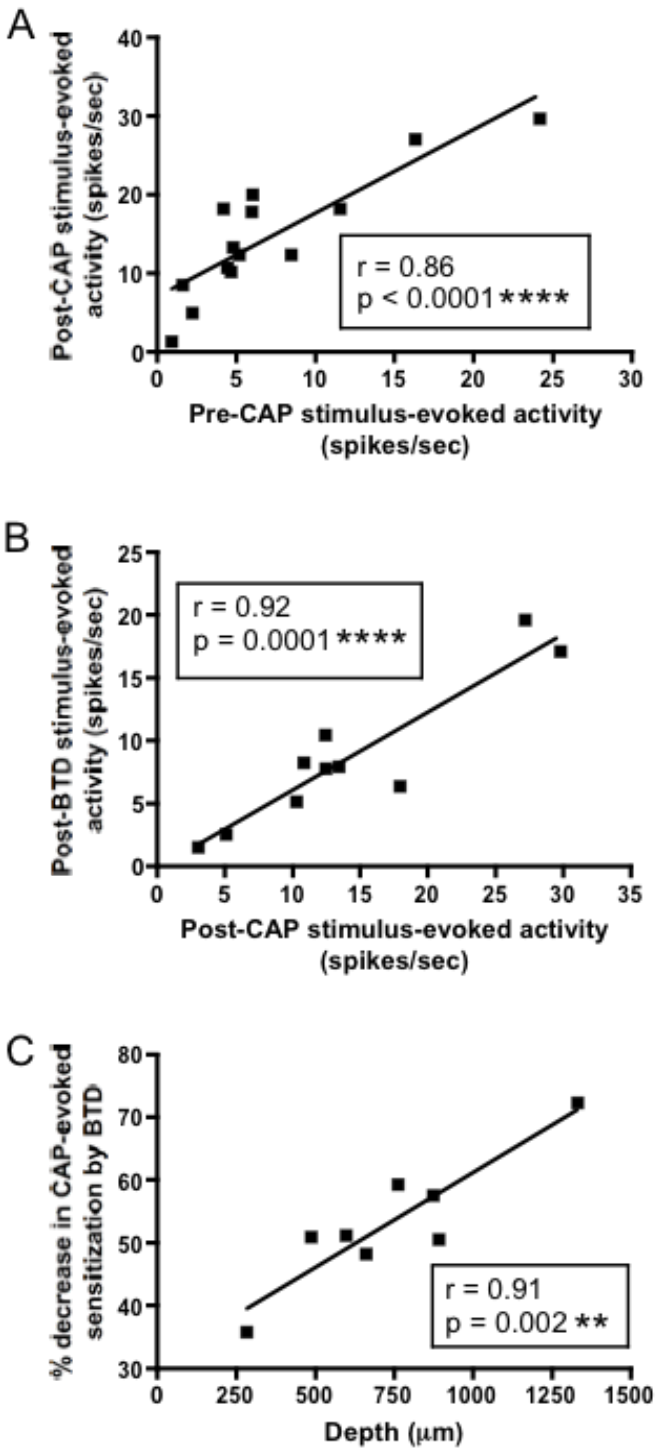

Figure 20: Stimulus-evoked spike rate is predictably altered by both CAP (A) and BTD (B). *Post-CAP stimulus-evoked spike rate is highly predictable from pre-CAP stimulus-evoked spike rate (A). Similarly, post-BTD spike rate is highly predictable from post-CAP spike rate (B). In panel C, effectiveness of BTD in reducing post-CAP spike rate depends on depth of WDR neuron, such that the deeper the WDR neuron, the less effective is BTD at reducing CAP-induced sensitization. P<0.05 considered significant in all cases.*

induced sensitization (r=-0.33; p=0.35). Linear regression analysis of neuronal depth versus BTD's effectiveness (Fig. 20C) indicated that BTD was less effective at reducing sensitization in deeper WDR neurons (r^2=0.82; p=0.002). However, spinal BTD did not significantly affect the RF size of either WDR (W=27, p=0.097) or NS neurons (W=4, p=0.313).

Compared to CAP-induced increases in background activity, spinal BTD reduced background activity from 3.90 ± 1.02 Hz to 2.46 ± 0.69 Hz in sensitized WDR neurons (W=41, p=0.019; Fig. 21A). Furthermore, BTD also returned stimulus-evoked spike rate to baseline levels (Fig. 21A; solid bars) in sensitizing WDR neurons. In non-sensitizing WDR neurons, spinal NKCC1 blockade had no significant effect on either background or stimulus-induced spike rate (Fig. 21A; hatched bars). As described in Fig. 16B, NS neurons acquired a CAP-induced novel low threshold component as well as enhanced responses to high intensity stimulation of the cutaneous RF's. Similar to the effect of spinal NKCC1 blockade on WDR neurons, BTD significantly reduced spike activity in response to high threshold input (196 and 490 mN von Frey; Fig. 21B). However, BTD did not significantly reduce low intensity input-induced responses (Fig. 21B). Moreover, the effect of NKCC1 blockade on background activity, from 2.86 ± 1.57 Hz to 1.13 ± 0.56 Hz, was not statistically significant (W=13, p=0.109). In non-sensitizing NS neurons, NKCC1 blockade had no effect on background or evoked spike rate (Fig. 21B; hatched bars). Figure 22 shows representative spike traces from WDR (Fig. 22A) and NS (Fig. 22B) neurons.

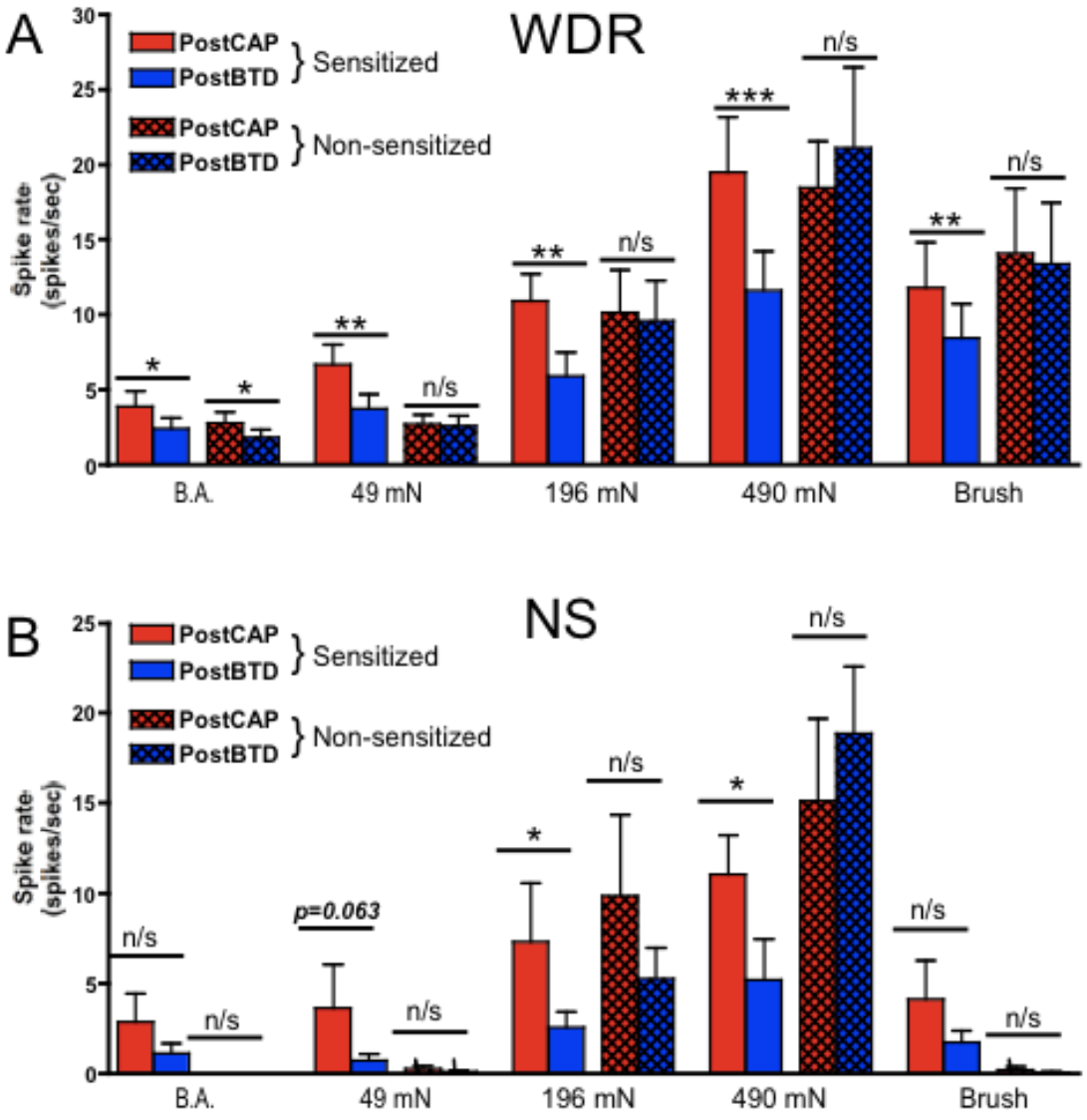

Figure 21: BTD reduced CAP-induced sensitization in sensitizing WDR (A) and NS (B) neurons but had no effect on non-sensitizing WDR and NS neurons. *In panel A, BTD (blue bars) reduced CAP-induced increased spike rate in sensitizing WDR neurons (solid red bars). However, BTD had no effect (except for background activity) on non-sensitizing neurons (hatched bars). In panel B, BTD (blue bars) reduced CAP-induced increased spike rate to high intensity stimulation (196 and 490mN von Frey) in sensitizing NS neurons (solid bars) but not in non-sensitizing neurons (hatched bars). However, BTD did not significantly reduce CAP-induced spike rate after low intensity stimulation (49mN von Frey, brush) in sensitizing NS neurons. All statistical comparisons are Wilcoxon Signed Ranks Tests where P<0.05 was considered significant in all cases. (*p<0.05; **p<0.01; ***p<0.001; n = 10 – 13 for WDR neurons and 4 – 6 for NS neurons). BA = background activity. Figure adapted from Pitcher et al. 2010.*

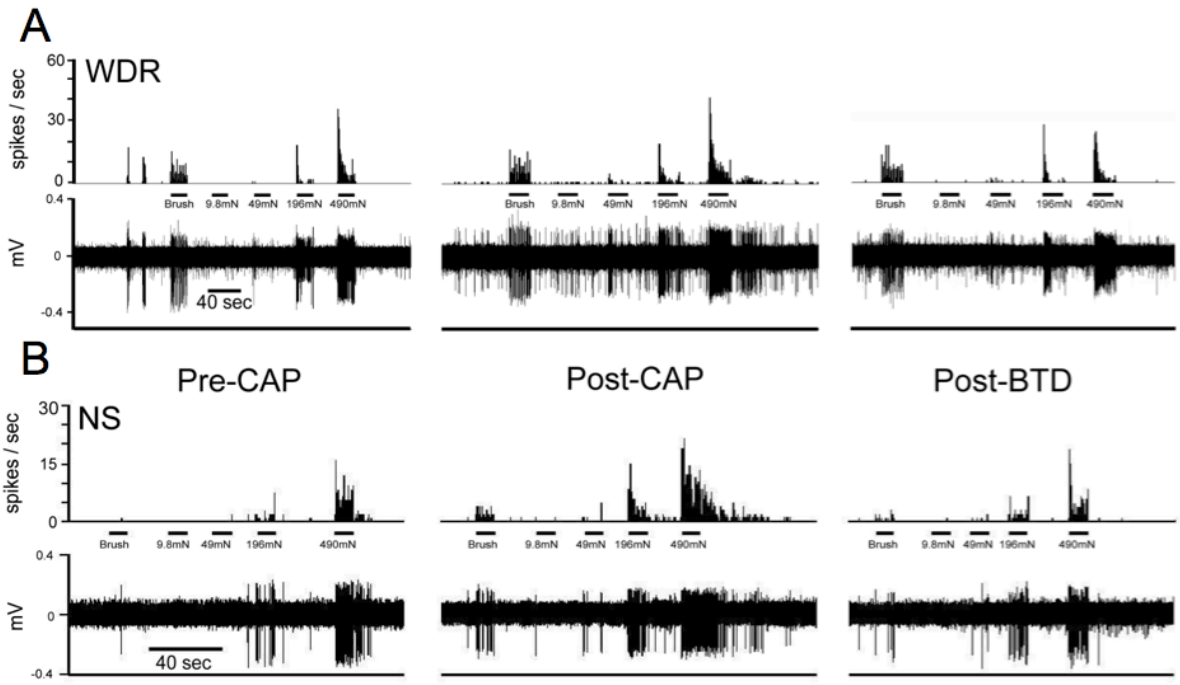

Figure 22: Representative spike activity from a WDR (A) and a NS (B) neuron. *In both the WDR and the NS neuron, the lower trace represents the spikes (action potentials) while the upper trace represents the spike rate (spikes/sec). Note that CAP increased spike rate in a BTD-sensitive manner. Figure from Pitcher et al. 2010.*

4.4. Discussion

There is an increasing amount of literature supporting an important role for NKCC1 in persistent pain states (for review, see Price et al. 2009 and Blaesse et al. 2009). NKCC1 is found in anatomically appropriate regions with respect to nociceptive processing (Kanaka et al. 2001; Toyoda et al. 2005; Price et al. 2006; Gilbert et al. 2007; Chabwine et al. 2009) and its expression levels are increased following intense and persistent noxious stimuli (Morales-Aza et al. 2004; Galan and Cervero, 2005; Pieraut et al. 2007; Cramer et al. 2008; Funk et al. 2008; Li et al. 2010; but see Nomura et al. 2006; Jolivalt et al. 2008; Zhang et al. 2008; Funk et al. 2008; Wu et al. 2009). Both genetic and pharmacological blockade of NKCC1 attenuates behavioral correlates of pain in rodent models (Sung et al. 2000; Laird et al. 2004; Granados-Soto et al. 2005; Valencia de Ita et al. 2006; Cramer et al. 2008). The current study provides *in vivo* electrophysiological evidence that sensitization of nociceptive neurons in the CNS is, at least in part, due to enhanced NKCC1 activity. Specifically, we demonstrate that capsaicin-induced sensitization of spinal WDR and NS neurons can be reduced by spinal NKCC1 blockade. Furthermore, our finding that CAP unmasks low threshold input in spinal nociceptive neurons is in agreement with previous work from our lab (Garcia-Nicas et al. 2006) and is consistent with the hypothesis that enhanced NKCC1 activity unmasks low threshold input to the nociceptive pathway through PAD (Cervero & Laird, 1996; Laird et al. 2004). Importantly, this study is the first to demonstrate functionally the CNS effects of NKCC1 blockade on sensitized CNS nociceptive neurons.

4.4.1. Distinct Groups of Neurons

We found two groups of WDR and NS neurons, distinct in terms of baseline spike rate, response to CAP and response to BTD. In contrast to non-sensitizing WDR and NS neurons, the sensitizing group had lower baseline spike rate, became sensitized to CAP and responded to BTD. In addition, pre-CAP stimulus-evoked spike rate of sensitized WDR neurons is highly correlated with the number post-CAP spikes, where neurons with higher baseline responses to stimulation tended to have more intense acute responses to CAP, an effect not found in non-sensitizing WDR neurons. The higher baseline spike rate observed in non-sensitizing neurons may indicate that these neurons were, in fact, already sensitized. However, this does not appear to be supported by our data. Firstly, since we used a standardized, relatively low intensity stimulation protocol, it is unlikely that this group of neurons would become sensitized. Secondly, BTD's lack of effect on background or evoked spike activity in these neurons, as contrasted to BTD's effect on sensitizing neurons, argues for functional distinctions between these groups. Considering that TRPV1 and NKCC1 are co-expressed on small and medium sized, mainly nociceptive, primary afferents (Price et al. 2006), it would be expected that fibers expressing TRPV1 and NKCC1 would be sensitive to both CAP and BTD, while non-TRPV1/NKCC1 fibers would not. Indeed, C-mechano-heat, A-mechano-heat and C-mechano-insensitive/heat-insensitive fibers preferentially respond to capsaicin, while other types of C- and A- fibers are less sensitive to capsaicin (Szolcsanyi et al. 1988; Ringkamp et al. 2001). Therefore, the sensitizing and non-sensitizing subsets described in the present study likely represent WDR and NS neurons with and without input from TRPV1-expressing fibers.

We calculated the degree of sensitization of each WDR neuron, defined as the mean increase of post-CAP versus pre-CAP stimulus-evoked spike rate, and performed correlational and linear regression analyses in order to reveal potential relationships between this and other variables. Firstly, we found a significant negative correlation between the pre-CAP stimulus-evoked spike rate and the degree of sensitization, such that sensitization decreased as pre-CAP spike rate increased. Considering the larger variability in degree of sensitization among neurons with lower pre-CAP spike rate, this finding may be due, at least in part, to a ceiling effect whereby the level of sensitization is more limited among neurons with higher baseline spike rate. We also studied how sensitization is related to the acute response to CAP; In accordance with Garcia-Nicas et al. (2006) who studied exclusively NS neurons, we found no correlation between the degree of sensitization of WDR neurons and the acute response to CAP (number of post-CAP spikes). In other words, a given neuron may not sensitize to CAP despite intense nociceptive activation (as measured by the acute response to CAP). As discussed by Garcia-Nicas et al. (2006), this finding suggests that the sensitization process of both WDR and NS neurons may involve heterosynaptic connections.

4.4.2. Effects of CAP and BTD on Receptive Field Size

In accordance with Sorkin & Wallace (1999), we found that baseline RF size of WDR neurons is larger than that of NS neurons. Alterations in RF size have often been associated with sensitization of spinal nociceptive neurons, so we were interested in studying the relationship between capsaicin-induced changes in RF size and capsaicin-induced sensitization of WDR neurons. We found that while

post-CAP RF size was increased only in sensitized neurons, the magnitude of RF size increase was not related to the degree of sensitization. Therefore, while an increased RF size accompanies sensitization, the amount of RF size increase does not predict the degree of sensitization. A potential explanation for this is that while both sensitization and increased RF size are consequences of intraplantar CAP, the mechanisms through which they occur are distinct. In support of this theory, BTD effectively reversed CAP-induced sensitization but had no effect on CAP-induced changes in RF size.

4.4.3. Effect of NKCC1 Blockade on Sensitization of WDR and NS Neurons

In the context of NS neurons, our hypothetical circuit predicts the appearance of a novel low threshold component (allodynia) in persistent pain states as well as exaggerated responses to high intensity stimulation (hyperalgesia), as measured by alterations in stimulus-evoked spike activity. Accordingly, our NS data suggests a CAP-induced unmasking of low intensity input as well as exaggerated responses to high intensity input; effects that are generally sensitive to BTD. WDR neurons, on the other hand, normally respond to low threshold input, so *de novo* responses to low threshold input were measured by novel spike activity from regions outside the original RF. Our experiments show that post-CAP RF size was increased in most WDR neurons, indicating a CAP-induced unmasking of low threshold input that was sensitive to BTD. Interestingly, a smaller proportion of NS neurons had increased RF sizes after CAP, compared to WDR neurons, which is in accord with previous studies from our laboratory showing that only a small proportion of NS neurons show RF size increases after CAP (Garcia-Nicas et al. 2006).

We studied the predictability of stimulus-evoked post-CAP spike rate from baseline (pre-CAP) stimulus-evoked responses in WDR neurons. Linear regression analysis revealed that among sensitizing WDR neurons, pre-CAP spike rate was highly predictive of post-CAP spike rate. We found a similar strong correlation between post-CAP spike rate and post-BTD spike rate. Thus, CAP produces a predictable increase in stimulus-evoked spike rate in WDR neurons, regardless of baseline spike rate, that is reduced to an equally predictable degree by BTD.

Using *in vivo* electrophysiological recording of dorsal root reflexes, Valencia-de-Ita et al. (2006) demonstrated that NKCC1 blockade is effective in reducing peripheral antidromic neuronal activity following intraplantar CAP. However, to date, no functional studies examining the effects of spinal NKCC1 blockade on CNS nociceptive neurons in models of persistent pain states can be found in the literature. We have utilized *in vivo* electrophysiological recording to show that spinal NKCC1 blockade virtually reversed CAP-induced sensitization of both WDR and NS neurons. Furthermore, our experiments provide strong evidence that low threshold afferents access the nociceptive channel and, in persistent pain states, activate the nociceptive channel producing touch-evoked pain. These findings are consistent with the view that dynamic (stroking) allodynia in humans may be due to an interaction between Aβ- and C-fibers in the CNS (Lang et al. 2006; 2007). Overall, the data presented here indicate an important role for NKCC1 in spinal mechanisms of persistent pain states, a finding that could potentially be pursued in the clinical setting.

CHAPTER 5

CELLULAR LEVEL APPROACH – SPINAL FOS IMMUNOHISTOCHEMISTRY

5.1. Specific Aim: Determine the role of activation of low threshold afferent fibers after intracolonic CAP on spinal FOS labeling and effect of spinal NKCC1 blockade.

FOS labeling in spinal dorsal horn laminae I-II and IV-V is increased following somatic or visceral application of chemical stimuli such as CAP and CFA, an effect generally understood to reflect activation, and possibly sensitization, of spinal nociceptive neurons following intense noxious input (Coggeshall, 2005). In both rat and mouse, intraplantar CFA produces increased FOS labeling in nociceptive regions of the spinal cord as well as mechanical allodynia (Ma and Woolf, 1996; Wei et al. 1999; Xu et al. 2008). Interestingly, low intensity mechanical stimulation of the inflamed area of CFA-treated rats (Ma and Woolf, 1996; Wei et al. 1999) as well as Aβ-intensity electrical stimulation of the sciatic nerve of neuropathic rats (Molander et al. 1992) exacerbated the increased FOS labeling, possibly providing an immunohistochemical correlate of primary allodynia. In this series of experiments, we studied spinal FOS labeling in intracolonic CAP-treated mice with and without spinal BTD in order to determine whether or not the increased FOS labeling is BTD-sensitive. In another group of mice, low intensity stimulation (LIS) of the abdomen (the area of secondary allodynia) was performed on mice treated with CAP alone or with CAP + BTD. These experiments were designed to determine (1) if stimulus-evoked secondary allodynia expressed in CAP-treated mice translates to changes in FOS labeling in relevant spinal cord regions and (2) if these changes are NKCC1-dependent.

5.2. Methods

5.2.1. Animals

Male C57BL6 mice between 20–25 g were used for all experiments. Mice were used for only one experimental procedure. All testing was performed blind to condition and treatment. Experiments were in accordance with the Canadian Counsel on Animal Care (CCAC) and the International Association for the Study of Pain (IASP) guidelines for the care and use of experimental animals. All protocols were reviewed and approved by the McGill University Animal Care Committee.

5.2.2. Drugs

Capsaicin (CAP; 0.1%, Tocris, Ellisville, MO) was dissolved in 10% ethanol, 10% Tween 80 and 80% saline. In behavioral experiments with mice, 0.05 ml of 0.1% CAP was delivered intracolonically. BTD (Sigma, St. Louis, MO) was dissolved in artificial cerebrospinal fluid (aCSF) vehicle. The aCSF vehicle was comprised of (in mM) 1.3 CaCl2 2H2O, 2.6 KCl, 0.9 MgCl, 21.0 NaHCO3, 2.5 Na2HPO47H2O, 125.0 NaCl, and 3.5 dextrose (pH 7.2–7.4). Intrathecal injections of BTD was made in 5 µl aCSF on lightly anesthetized mice by lumbar puncture at the L4–L5 level with a 30-gauge needle on a 50 µl Hamilton syringe.

5.2.3. Intracolonic Capsaicin

Mice were habituated to a Plexiglass testing box (10 cm x 10 cm x 8 cm) for 1–2 hours prior to testing. This box was used to evaluate referred allodynia and hyperalgesia. Just prior to intracolonic application of CAP, petroleum jelly (Vaseline) was applied to the peri-anal area to avoid the stimulation of somatic areas by contact with CAP. CAP was slowly injected via a fine cannula with a rounded tip (external diameter 0.61 mm), gently introduced 4 cm into the colon via the anus.

5.2.4. Spontaneous Nociceptive Behaviors

In section 3.3.4., we demonstrated that abdominal licking was the most salient of the four SNB's examined. Therefore, in this study we only counted abdominal licking. We observed abdominal licking for the first 15 min post-CAP. Each abdominal lick usually lasted for 1-2 seconds and was counted as one lick. However, periodically the mouse engaged in much longer bouts of licking (eg. 10-15 seconds). Under these circumstances, the number of licks was approximately proportional to the duration (eg. 10 licks per 10 second burst) and so was counted proportional to time.

5.2.5. Low Intensity Abdominal Stimulation

Activation of low threshold Aβ fibers of the abdomen was performed on urethane-anesthetized mice by light stimulation with a small paintbrush. The abdomen was stroked at approximately 0.5-1.0Hz for 30 min post-CAP. At 90 min post-CAP, mice were processed for immunohistochemistry. Mice not receiving low threshold abdominal stimulation were treated identically, minus the low threshold stimulation.

5.2.6. Immunohistochemical Procedures

5.2.6.1. Intracardial Perfusion

Once an adequate depth of anesthesia was verified, as measured by complete absence of blink reflex to a puff of air as well as lack of withdrawal from noxious pinch of the forepaw, the mouse was secured to the perfusion table. The viscera and chest cavity were exposed and a cannula was inserted into the heart and clamped securely. The mouse was perfused with perfusion buffer (1X PBS + 0.01% heparin) for about one minute followed by 250ml of 4% PFA over one

hour. The spinal cord was excised and the lumbosacral region was post-fixed in 4% PFA at 4^{o}C for 3 hours, then transferred to 30% sucrose at 4^{o}C for 2 days.

5.2.6.2. Immunohistochemistry

Using a cryostat (Microm HM 505E, Heidelberg), 30-micron transverse sections were cut from the fourth lumbar region for free floating immunohistochemistry. Sections were washed in 0.01M PBS 4 times for 15 min each, then incubated in 0.3% hydrogen peroxide in PBS+T for 10 min, followed by 5 washes of 15 min each in 0.01M PBS. Sections were then incubated in 10% NGS in PBS+T for 1 hour (250µl/well) followed by incubation in rabbit anti-mouse polyclonal FOS primary antibody (AB7963; Abcam) in a volume of 250ul/well (1:1000 in PBS+T + 5% NGS) for 30min at room temperature then two days at 4^{o}C. Tissue sections were washed 4 times for 15min each in 0.01M PBS followed by incubation in goat anti-Rabbit biotinylated secondary antibody (NB7158; Novus Biologicals) in a volume of 250µl/well (1:300 in PBS+T + 5% NGS) for one hour at room temperature. The sections were then washed 3 times for 15min each in 0.01M PBS, then incubated in ABC for one hour at room temperature (Elite, Vector; 1:400 A + 1:400 B in PBS+T (250µL/well)). Sections were then washed 3 times for 15 min each in 0.01M PBS. Peroxidase activity was revealed using DAB (Vector), where sections were incubated in DAB for exactly 10 min followed by 5µl/well of H_2O_2 (500µl of 30% H_2O_2 in 14.5ml of d$H_2$0) for approximately 30 sec. To stop the reaction, the wells were filled with 0.01M PBS. Sections were then washed 4 times for 15 min each in 0.01M PBS+T. Sections were mounted onto superfrost plus slides (BDH) with a permanent mounting medium (Aqua Poly-Mount).

5.2.6.3. Light microscopy

FOS labeling was visualized with a brightfield Zeiss Axioplan 2 microscope (Germany). FOS-labeled neuronal profiles were counted in lamina I-II, IV-V and X.

5.2.7. Statistical Analysis

Data are shown as means ± SEM. One-way ANOVA with post hoc Dunnett's or t-tests, where applicable. In all cases, $p < 0.05$ was considered significant.

5.3. Results

5.3.1. The L4 spinal region has the highest CAP-evoked FOS labeling

One-way ANOVA of FOS labeling among L3 – L6 regions was statistically significant ($F(3,16) = 4.365$, $p = 0.02$). While Bonferroni's multiple comparison post hoc analysis indicated that only the L4 and L6 region differed significantly, the L4 region had the highest level of intracolonic CAP-evoked FOS labeling (Fig. 23A). Considering that this confirms previous work from another laboratory (Eijkelkamp et al. (2007), for all further studies we sampled only from the L4 region.

5.3.2. Dose-dependency of BTD pretreatment on intracolonic CAP-induced abdominal licking behavior.

One-way ANOVA of the effects of various doses of BTD on CAP-induced abdominal licking was significant ($F(5,68) = 4.98$; $p = 0.0006$). Dunnett's post-hoc analysis indicated that the number of abdominal licking behaviors increased from 1.75 ± 0.52 in naïve mice to 12.56 ± 3.02 in CAP + vehicle (VEH) mice (Fig. 23B). As determined in a previous section, pre-treatment with 1 nmol BTD was the appropriate dose to prevent intracolonic CAP-induced abdominal licking, reducing

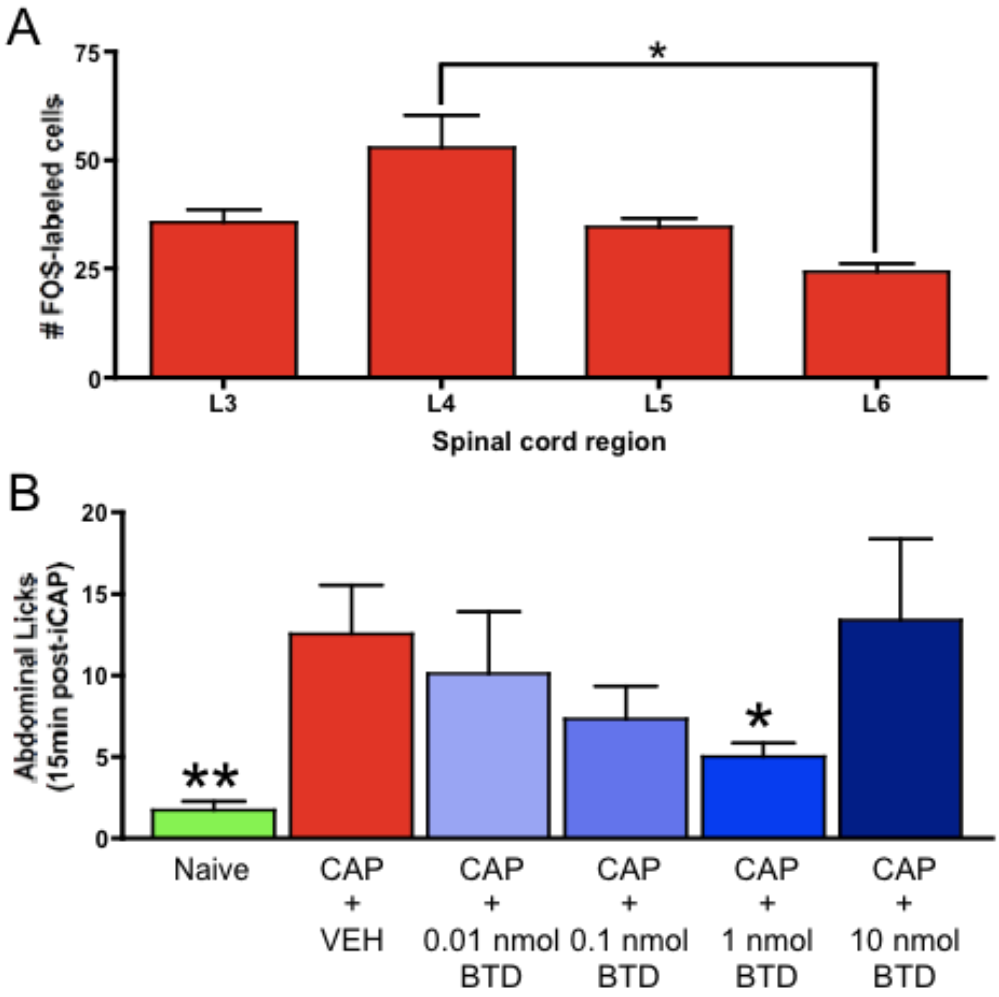

Figure 23: iCAP-induced spinal FOS levels are maximal in the lumbar 4 region of the spinal cord (A). Intrathecal BTD is most effective at reducing iCAP-evoked abdominal licking at the 1 nmol dose (B). *One way ANOVA followed by Dunnetts multiple comparison test were used. Stars (*) indicate significant Bonferroni's (A) or Dunnett's (B) post hoc comparisons. In B, all post hoc comparisons are to the CAP + VEH group. (* $p < 0.05$, ** $p < 0.01$; $n = 3 – 7$ per group).*

it to 5.93 ± 1.27 (Fig. 23B). Neither the lower doses (0.01 and 0.1 nmol) nor the highest dose (10 nmol) had any significant effect compared to VEH. Therefore, we used 1 nmol BTD for all further BTD experiments.

5.3.3. Intracolonic CAP increased FOS labeling in regions of the spinal cord associated with nociceptive processing.

One-way ANOVA comparing naïve, iCAP and iCAP + BTD groups for Laminae I-II ($F(2,14) = 10.18$, $p = 0.0019$) and lamina X ($F(2,13) = 5.01$, $p = 0.0244$) were significant, while results for laminae IV-V bordered on significance ($F(3,13) = 3.62$, $p = 0.056$). Compared to naïve controls, post-hoc Dunnett's analysis indicated that intracolonic CAP produced significant increases in FOS labeling in laminae I-II and lamina X of the L4 spinal cord (Fig. 24A/C, respectively; solid green bars vs. solid red bars). Pretreatment with spinal BTD prevented iCAP-induced increased FOS levels in these regions (Fig. 24A/C; solid red vs. solid blue bars). Linear regression analysis did not reveal a significant relationship between the number of post-CAP abdominal licks and FOS labeling in any laminar region of the spinal cord (Laminae I-II: $r^2 = 0.001$, $p = 0.955$: Lamina IV-V: $r^2 = 0.223$, $p = 0.423$; Lamina X: $r^2 = 0.004$, $p = 0.919$).

5.3.4. Effect of low intensity stimulation of the region of iCAP-induced secondary allodynia on spinal FOS labeling and the effect of BTD pretreatment.

Among mice receiving LIS, one-way ANOVA analyses comparing naïve, iCAP and iCAP + BTD groups were significant for all regions evaluated (laminae I-II: $F(2,12) = 10.98$, $p = 0.0019$; laminae IV-V: $F(2,12) = 20.61$, $p = 0.0001$; lamina X: $F(2,12) = 13.66$, $p = 0.0008$). Post hoc Dunnett's analysis showed that in LIS-treated mice, intracolonic CAP produced higher FOS levels compared to naïve

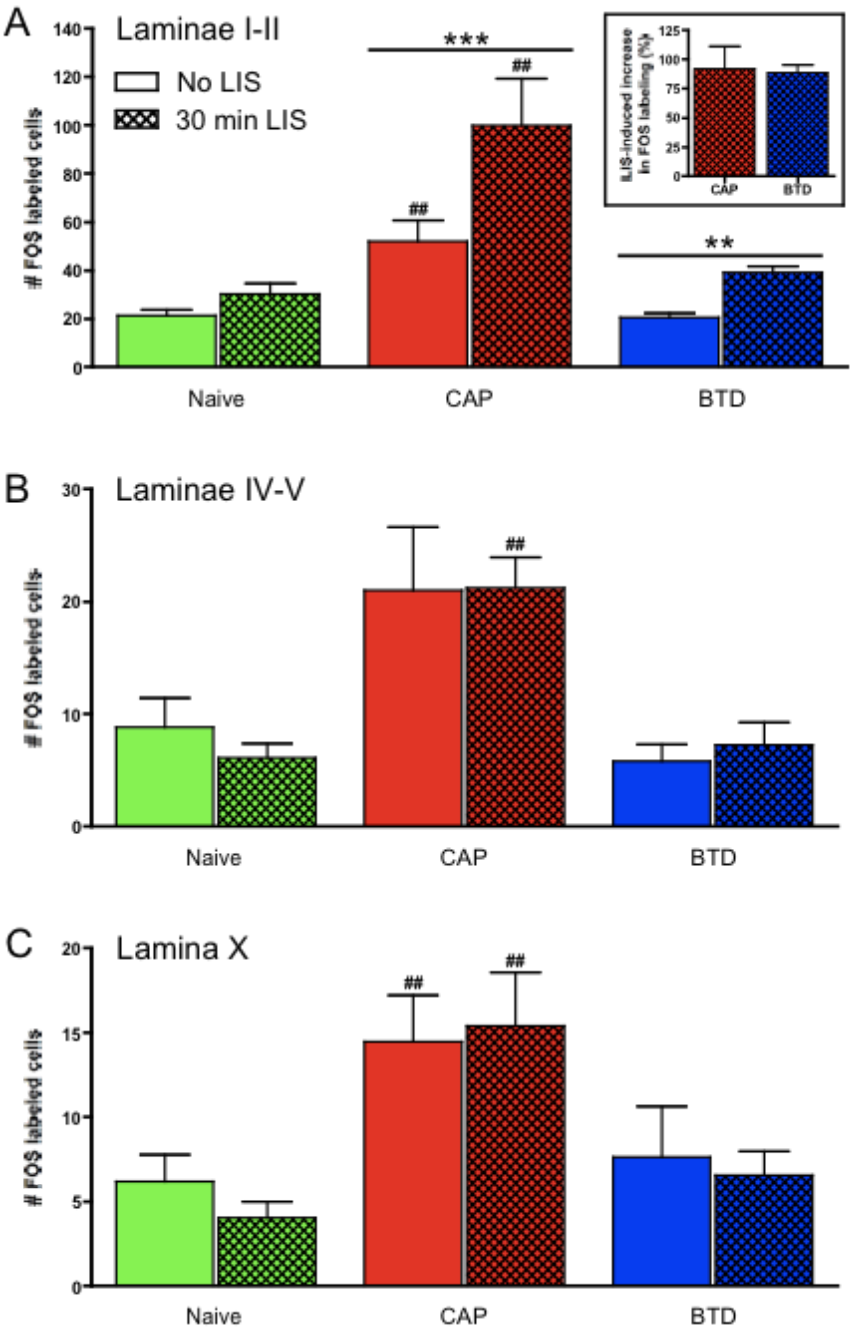

Figure 24: The effects of iCAP, spinal BTD and low intensity stimulation (LIS) on FOS levels in nociceptive regions of the spinal cord. *Spinal FOS levels are increased by iCAP in a BTD-dependent manner in all spinal regions studied (A/B/C; solid bars). However, LIS of the abdomen, the region of iCAP-evoked secondary allodynia, further enhanced FOS in lamina I-II (A; hatched red vs solid red bars) by approximately 90% (A; red bar in inset), but not lamina IV-V (B) or X (C). NKCC1 blockade was effective in preventing increased FOS levels in all regions (A/B/C; hatched blue bars vs hatched red bars). However, in lamina I-II, FOS levels in LIS-treated animals were still almost 90% higher (A; hatched blue bar in inset) than animals not receiving LIS (A; hatched blue bar vs solid blue bar), indicating that a component of the LIS effect is not mediated by NKCC1. Stars (*) indicate significant effects of LIS compared to no LIS group (solid vs hatched bars; unpaired t-tests) while number signs (#) indicate significant Dunnett's post hoc analysis compared to naïve group. (** or ## $p < 0.01$, *** $p < 0.001$; $n = 4 – 6$ per group).*

mice (no iCAP, no LIS) in laminae I-II, IV-V and X (Fig. 24A/B/C, respectively; hatched green bars vs. hatched red bars), an effect that was preventable by BTD pretreatment (Fig. 24A/B/C; hatched red vs. hatched blue bars).

In naïve mice, 30 min of LIS of the abdomen did not significantly affect spinal FOS labeling in any of the spinal cord regions evaluated (Fig. 24A/B/C; solid green bars vs. hatched green bars). In CAP-treated mice, 5 min of LIS (55.40 ± 10.03) did not affect the number of FOS labeled cells compared to the CAP-only group (49.11 ± 3.78; $t(6) = 0.46$; $p = 0.66$). However, again compared to the CAP-only group, FOS levels in the CAP + 30 min LIS group were significantly higher (92.1 ± 19.3 %) in laminae I-II of the spinal cord (Fig. 24A and inset; solid red bars vs. hatched red bars), but not in laminae IV-V or X (Fig. 24B/C, respectively; solid red bars vs. hatched red bars). While LIS did not affect FOS levels in the BTD pretreatment groups in spinal cord laminae IV-V and X (Fig. 24A; solid blue bars vs. hatched blue bars), FOS levels were significantly higher in laminae I-II of LIS-treated mice (88.7 ± 6.7 %; Fig. 24A; solid blue bar vs. hatched blue bar; also see hatched blue bar in inset). Representative micrographs of spinal cords from each group can be seen in Fig. 25.

5.4. Discussion

Our data demonstrates both behaviorally and immunohistochemically that intracolonic CAP unmasks a low threshold component of the nociceptive pathway that is sensitive to spinal NKCC1 blockade. However, care must be taken when using spinal FOS labeling as a marker of nociceptive activity; FOS labeling is a marker of neuronal activity, so its involvement in nociceptive processes can only be evaluated in regions where nociceptive processing is known to occur, namely

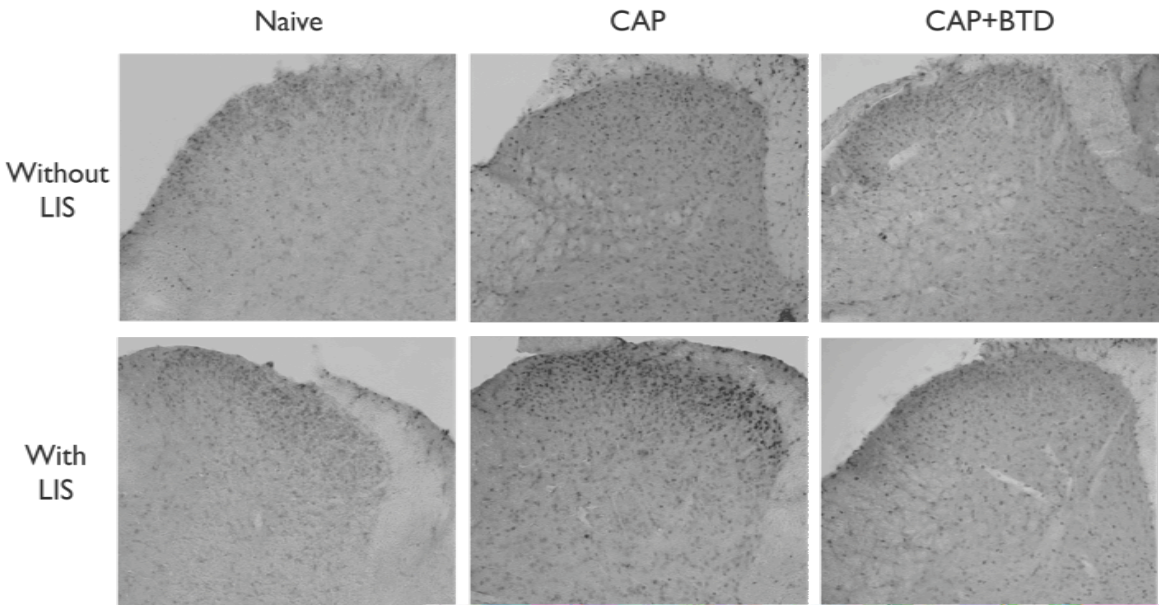

Figure 25: Representative light micrograph of superficial dorsal horn FOS labeling in naïve, iCAP and iCAP + BTD groups with and without LIS of the abdomen. *Note the further increased FOS levels from the iCAP-treated mouse recieving LIS, as well as the effect of BTD.*

lamina I-II, IV-V and X. Moreover, due to the wide variability in levels of spinal FOS labeling as well as the time until maximum labeling, both within and between pain models, cautious interpretation of data is required (Harris, 1998; Coggeshal, 2005).

In agreement with Eijkelkamp et al. (2007), we have confirmed that intracolonic CAP increased both secondary allodynia and FOS labeling in nociceptive regions of the spinal cord, particularly the L4 region. However, our data did not confirm the correlation between intracolonic CAP-induced SNB's and FOS labeling in both laminae I-II and X, such that mice with higher FOS levels show more SNB's, as reported by Eijkelkamp et al. (2007). This discrepancy could be due to methodological and/or statistical differences between the two studies; firstly, in section 3.3.4. of this thesis, we demonstrated that abdominal licking was the only SNB decreased by BTD while, surprisingly, squashing and abdominal retractions both appeared to be exacerbated by BTD. Therefore, in the current section we counted only abdominal licks. Eijkelkamp's group, however, also counted abdominal retractions, squashing and stretching. Secondly, while the FOS data for our regression analysis was drawn exclusively from the L4 spinal cord, the region with maximal post-CAP FOS labeling (see Fig. 23A as well as Eijkelkamp et al. 2007), they derived FOS levels from the entire T12-S1 region. Finally, their data is based on a larger group size than ours. Nonetheless, FOS levels found in our study (L3-L6) are comparable to those in the same regions reported by Eijkelkamp et al. (2007). Therefore, while differences in SNB's, spinal regions and group sizes may explain the contradictory findings, another explanation is possible; perhaps the most salient reason supporting our negative correlational

findings involves the differential time points at which the behavior and FOS levels were measured: In both studies, SNB's were measured early post-CAP while FOS levels were measured much later. Specifically, Eijkelkamp et al. (2007) measured SNB's between 5 and 25 min post-CAP and measured FOS levels at 120 min post-CAP; we opted to measure SNB's during the first 15 min post-CAP and FOS levels were measured at 90 min post-CAP. We chose these time points for a number of reasons; first of all, we required sufficient time post-CAP to perform 30 min of LIS on the abdomen. Secondly, we wanted to study FOS levels at the time point when NKCC1 expression is known to be maximal in the intracolonic CAP model (Galan et al. 2005). While many studies show that nociceptive input produces increased behavioral manifestations of pain as well as enhanced FOS levels, significant correlations on a *per-animal* basis appear to be exceptional, especially when the two parameters are not measured at matching time points (Coggeshall, 2005). Accordingly, SNB's are maximal within the first 10-15 min post intracolonic CAP, virtually disappearing by 20 - 25 min post-CAP. On the other hand, FOS levels usually begin to increase after at least 30 min (Coggeshall, 2005). In order to avoid this confound, both parameters must be measured at relevant, temporally contiguous time points.

Next, we studied the effects of LIS on FOS levels. LIS of the inflamed region increased the spinal FOS labeling in CFA-treated rats beyond the levels of CFA alone (Ma and Woolf, 1996; Wei et al. 1999). Therefore, spinal FOS labeling may be useful as a measure of allodynia as well as a general measure of noxious input. With this in mind, our experiments were designed to test the hypotheses that (1) intracolonic CAP increases both spinal FOS levels and secondary

allodynia in a BTD-sensitive manner, (2) that LIS in the region of secondary allodynia can further increase FOS labeling in relevant spinal cord regions and (3) that these changes are also NKCC1-dependent. Accordingly, we showed that intracolonic CAP increases spinal FOS levels and nociceptive behaviors directed towards the region of secondary allodynia, effects that are sensitive to pretreatment with BTD. Moreover, LIS of CAP-treated mice further increased FOS labeling by approximately 90% in laminae I-II, but had no effect in laminae IV-V or X. We expected that NKCC1 blockade would inhibit the effect of LIS such that BTD + LIS values would be similar to BTD alone, however, this was not the case. Pretreatment with BTD prevented increased FOS levels in all regions. Yet, FOS labeling in laminae I-II of the LIS + BTD group was still approximately 90% higher than the group receiving BTD alone. A possible explanation is that part of the low threshold component unmasked by CAP is resistant to NKCC1 blockade. Without sustained LIS of the region of secondary allodynia, this component would not be activated. A potential candidate mediating this effect could be TRPV1. Largely co-expressed in small and medium-sized afferent fibers (Price et al. 2006), TRPV1 and NKCC1 appear to interact at the spinal level; blockade of either NKCC1 or TRPV1 prevents intracolonic CAP-induced secondary allodynia to a similar extent (sections 3.3.4. and 3.3.6., respectively) and NKCC1 blockade also partially reversed TRPV1-mediated stroking allodynia (section 3.3.6.). In agreement with our finding that a spinal TRPV1 antagonist blocked intracolonic CAP-induced secondary allodynia, spinal TRPV1 blockade in CFA-inflamed rats reduced spinal WDR neuronal responses to low intensity mechanical stimulation (McGaraughty et al. 2008). TRPV1 is found predominantly on C-fibers but also on

Aδ-primary afferent fibers (Caterina et al. 2000; Ma 2002). In the CFA model as well as in other pain models, TRPV1 is preferentially upregulated on Aδ fibers vs. C fibers (Amaya et al. 2003; Hong and Wiley 2005; Hudson et al. 2001; Luo et al. 2004; Rashid et al. 2003a/b). In addition, TRPV1 expression is increased on large, myelinated A fibers, presumably Aβ fibers, in rats with diabetic neuropathy (Hong and Wiley, 2005) as well as in the superficial dorsal horn after intraplantar CFA (Luo et al. 2004). The injury-induced shift in TRPV1 distribution from C- to Aδ- and possibly Aβ-fibers surely affects TRPV1 input to the spinal cord, providing a novel pathway to activate spinal nociceptive neurons. Therefore, reduction of intracolonic CAP-evoked secondary allodynia by spinal blockade of TRPV1 (section 3.3.6.) may have occurred through pathways that are inactive in naïve mice. This normally latent pathway may also underlie the inability of BTD to completely reverse the effects of 30 min of LIS after intracolonic CAP. Behavioral experiments as well as further FOS labeling could be used to study this hypothesis. Specifically, since BTD did not completely prevent LIS-evoked FOS labeling, we could expect LIS to produce exaggerated CAP-evoked secondary allodynia in mice that would not be fully preventable by BTD. In addition, considering that p38 MAPK may be the common pathway underlying both NKCC1 and TRPV1 activity post-CAP, inhibition of p38 as well as co-blockade of NKCC1 and TRPV1 may fully prevent LIS-evoked FOS labeling. Clearly, more work in this area is required.

CHAPTER 6

MOLECULAR LEVEL APPROACH –

SPINAL NKCC1 EXPRESSION

6.1. Specific Aim: Are AMPA/kainite and TRPV1 receptors upstream modulators of spinal NKCC1 expression?

Enhanced NKCC1 activity in rodent peripheral sensory fibers has been linked to touch-evoked pain and neurogenic inflammation through PAD (Laird & Cervero, 2004; Valencia-de-Ita et al. 2006). As discussed in a previous section, various kinase-mediated intracellular pathways underlying increased NKCC1 activity have been proposed (Price et al. 2005; Liedtke and Cole, 2002; Kurihara et al. 2002; Dowd and Forbush, 2003; Gamba, 2005; Moriguchi et al. 2005; Kahle et al. 2005; Vitari et al. 2006; Darman and Forbush 2002; Klein et al., 1999; Liedtke and Cole, 2000, 2002). However, upstream receptor-mediated pathways such as pre-synaptic AMPA/kainate receptors are also known to regulate NKCC1 expression (Schomberg et al. 2001; Chen et al. 2007). AMPA and kainate receptors are found on the central terminals of afferent fibers (Lu et al. 2002; Lee et al. 2002; Lee et al. 2004; Willcockson & Valtschanoff, 2008) and play a role in PAD (Rees et al. 1995; Lee et al. 2002; Russo et al. 2000; Russo et al. 2007), including PAD in C- and Aδ-fibers (Russo et al. 2000; Lee et al. 2002). Specifically, spinal AMPA/kainate blockade reduces glutamate release into the CNS (Sluka and Westlund, 1993a; Lee et al. 2002) as well as reducing peripheral inflammation, as measured behaviorally (heat hyperalgesia) and physiologically (joint swelling) (Sluka & Westlund, 1993b). Spinal CNQX blocks DRR's in rats with intra-articular arthritis (Rees et al. 1995) as well as electrically evoked DRP's in an *in vitro* rat preparation (Evans and Lodge, 1989). Based on these findings, Rees et al. (1995) suggest that the effectiveness of spinal CNQX in limiting inflammation is attributable to a reduction of excessive PAD. Clearly, PAD is

mediated in part by GABA as well as by glutamatergic pathways. Considering that pre-synaptic AMPA/kainate receptor activation increases NKCC1 activity in cortical neurons (Schomberg et al. 2001), NKCC1 regulation in the nociceptive afferent fibers may be downstream of AMPA/kainate activity. Thus, we studied the effects of AMPA/kainate receptor blockade on spinal NKCC1 expression.

We were also interested in determining if TRPV1 activity regulates NKCC1 expression. TRPV1 is co-expressed on afferent fibers with NKCC1 (Price et al. 2006) and, as we showed in a previous section, spinal blockade of NKCC1 reduced TRPV1-dependent allodynia while spinal TRPV1 blockade attenuated intracolonic CAP-induced secondary allodynia. However, another possibility is that an intracellular kinase cascade such as p38 regulates both TRPV1 and NKCC1. Activation of p38 in DRG neurons is involved with the development of heat hyperalgesia by regulating TRPV1 expression (Ji et al. 2002; Obata et al. 2004). Blockade of p38 also reduced TRPV1 expression in CFA-treated rats (Ji et al. 2002) as well as in HEK293T cells challenged with streptozotocin (Pabbidi et al. 2008). In terms of NKCC1, p38 stimulates NKCC1 activity (Capo-Aponte et al. 2007) possibly through an interaction with the SPAK/OSR1-NKCC1 complex (Johnston et al. 2000; Piechotta et al. 2003). Intraplantar CAP rapidly increased phosphorylated-p38 in small- and medium-sized diameter sensory neurons and noxious thermal stimuli produced an increase in size and number of phosphorylated-p38 labeled DRG neurons (Mizushima et al. 2005). Thus, TRPV1 may not regulate NKCC1 activity; instead, p38 may be the common factor, where its activation stimulates both NKCC1 and TRPV1 activity, both of which can be inhibited by their respective blockers. We hypothesized that if TRPV1 does

regulate NKCC1 activity, then TRPV1 blockade prior to iCAP should reduce spinal NKCC1 expression.

6.2. Methods

6.2.1. Animals

Male C57BL6 mice between 20–25 g were used for all experiments. Mice were used for only one experimental procedure. All testing was performed blind to condition and treatment. Experiments were in accordance with the Canadian Counsel on Animal Care (CCAC) and the International Association for the Study of Pain (IASP) guidelines for the care and use of experimental animals. All protocols were reviewed and approved by the McGill University Animal Care Committee.

6.2.2. Drugs

CNQX (AMPA/kainate receptor antagonist; 20nmol in 5uL), AMG 9810 (TRPV1 receptor antagonist; 1nmol in 5uL) were injected intrathecally 5 min prior to intracolonic capsaicin (0.3ml 0.1% capsaicin; Tocris, Ellisville, MO). Capsaicin was dissolved in 10% ethanol, 10% Tween 80 and 80% saline. The competitive AMPA/kainate receptor antagonist CNQX (C239; Sigma, St. Louis, MO) and the TRPV1 antagonist AMG 9810 (2316; Tocris) were dissolved in artificial cerebrospinal fluid (aCSF) vehicle. The aCSF vehicle was comprised of (in mM) 1.3 CaCl2 2H2O, 2.6 KCl, 0.9 MgCl, 21.0 NaHCO3, 2.5 Na2HPO47H2O, 125.0 NaCl, and 3.5 dextrose (pH 7.2–7.4). Intrathecal injections of CNQX and AMG 9801 were made in 5 µl aCSF on lightly anesthetized mice by lumbar puncture at the L4–L5 level with a 30-gauge needle on a 50 µl Hamilton syringe.

6.2.3. Intracolonic Capsaicin

Just prior to intracolonic application of CAP, petroleum jelly (Vaseline) was applied to the peri-anal area to avoid the stimulation of somatic areas by contact with CAP. CAP was slowly injected via a fine cannula with a rounded tip (external diameter 0.61 mm), gently introduced 4 cm into the colon via the anus.

6.2.4. Molecular Biology Procedures

6.2.4.1. Spinal Cord Expulsion

Mice were anesthetized, decapitated and the spinal cord was extracted by pressure expulsion 90 min post-iCAP. Specifically, a 10 ml syringe with an 18-gauge needle was filled with freezing cold saline solution and inserted into the spinal canal at the sacral level. Rapid depression of the plunger forced the intact spinal cord out through the rostral opening. The lumbosacral region was isolated and snap-frozen in liquid nitrogen.

6.2.4.2. Protein Quantification and Western Blot

Tissue samples were homogenized in RIPA buffer and centrifuged at 7400rpm (5,000 x G) for 5 min. Supernatants containing cytosolic and membrane fractions were collected and re-centrifuged at 13000 rpm (16,000 x G) for 1 hr. The supernatant contained cytosolic fraction while the pellet contains membrane fraction. The membrane fraction (pellet) was re-suspended in 40 µl RIPA buffer. Total protein amounts were measured with Bradford assay.

Spinal cord extracts were fractionated by SDS poly-acrylamide gel electrophoresis in 4-10% polyacrylamide gels and transferred to PVDF membranes. Protein membranes were blocked with 1% bovine serum albumin in Tris-Buffered Saline + Tween buffer. Membranes were incubated for 1 hour at room temperature with the T4 anti-NKCC1 antibody (1:1000; Developmental

Studies Hybridoma Bank, Iowa, USA). The membranes were washed with TTBS and incubated with secondary antibody goat anti-mouse peroxidase-conjugated antibody.

6.2.4.3. Image Analysis

The immune complexes were detected by chemiluminiscence and blots were converted into digital images that were transformed to grayscale. Optical density of the bands was evaluated using ImageJ software (NIH, Bethesda, Maryland).

6.2.5. Statistical Analysis

One-way ANOVA followed by post hoc Dunnetts were used for all statistical analyses.

6.3. Results

6.3.1. Spinal AMPA/kainate receptor but not TRPV1 receptor blockade prevents iCAP-evoked increases in spinal NKCC1 levels on the plasma membrane.

Compared to naïve mice, NKCC1 expression on the plasma membrane is increased after iCAP (Fig. 26; green vs. red bar). Pre-treatment with the AMPA/kainate blocker CNQX reduced iCAP-evoked increases in membrane-associated NKCC1 expression to baseline levels (Fig 26; purple vs. green bar). However, mice pretreated with the TRPV1 antagonist AMG9810 had similar levels of NKCC1 expression compared to mice treated only with iCAP (Fig. 26; yellow vs red bar) indicating that spinal TRPV1 does not influence intracolonic CAP-induced increases of NKCC1 expression.

6.4. Discussion

In agreement with Galan and Cervero (2005), we found that intracolonic CAP

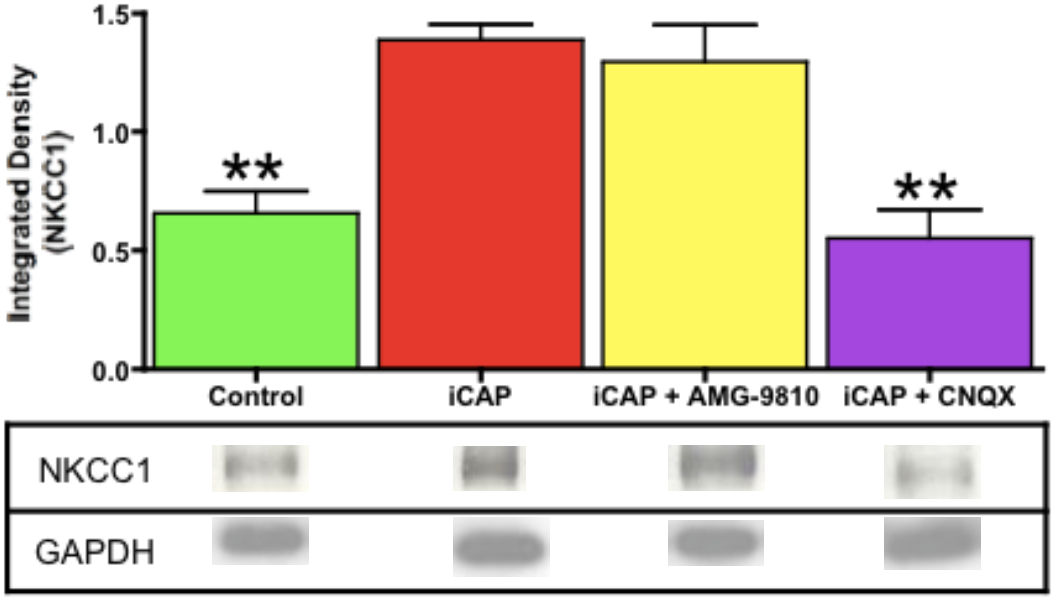

Figure 26: Increased spinal NKCC1 expression by iCAP can be prevented by spinal AMPA/kainate but not TRPV1 receptor blockade. *Intracolonic CAP increased spinal plasma membrane-associated NKCC1 expression (red bar vs green bar). While spinal AMPA/kainate receptor blockade with CNQX prevents iCAP-evoked increase in NKCC1 expression (purple bar vs red bar), spinal TRPV1 receptor blockade with AMG9810 does not (yellow bar vs red bar). One-way ANOVA followed by Dunnett's post hoc tests. **p = 0.01 (n = 3 – 7 per group).*

increased spinal, plasma membrane-associated NKCC1 expression. While some studies show injury-induced increases of NKCC1 protein expression (Morales-Aza et al. 2004; Galan and Cervero, 2005; Pieraut et al. 2007; Cramer et al. 2008; Funk et al. 2008), other studies do not (Nomura et al. 2006; Jolivalt et al. 2008; Zhang et al. 2008). It is possible that certain types of injury may differentially affect NKCC1 expression, however, there does not seem to be any systematic differences among nociceptive models used in these studies (see Table 1). Importantly, the technique used to measure NKCC1 expression could also affect the results; using membrane fractionation methods, Galan et al. (2005) showed an intracolonic CAP-induced increase of plasma membrane NKCC1 expression that was proportional to the decrease of cytosolic NKCC1. Clearly, if fractionation methods had not been used, no change would have been detected. Interestingly, besides Galan et al. (2005), fractionation techniques were not used in the studies mentioned above. It should be noted that the fractionation technique employed in this study does not distinguish between the plasma membrane of the neuron and any intracellular membranes. Considering that prior to being trafficked to the plasma membrane, NKCC1 is membrane-bound intracellularly within the endoplasmic reticulum (Nezu et al. 2009), interpretation of our findings must take into account that levels of membrane-bound NKCC1 include both plasma membrane and intracellular NKCC1. Unfortunately we were unable to find any literature pertaining to the proportion of NKCC1 found in the endoplasmic reticulum compared to the plasma membrane under various conditions. Therefore, while the actual amounts of NKCC1 on the plasma

membrane after CAP may be somewhat lower than our data indicates, we are confident that the overall trend is correct.

Our data is also in agreement with our hypothesis that AMPA/kainate receptors regulate NKCC1 expression in response to nociceptive input. Specifically, we demonstrate that spinal blockade of AMPA/kainate receptors reduced spinal NKCC1 expression after persistent noxious visceral input. Clearly, we cannot state conclusively that these findings are due to pre-synaptic AMPA/kainate receptors. An alternative hypothesis could be that post-synaptic, rather than pre-synaptic, AMPA/kainate receptors are responsible for the effects seen here. However, there are problems with this hypothesis. First of all, both AMPA and kainate receptor activation can promote nitric oxide (NO) release (Radenovic & Selakovic, 2005; Ouardouz et al. 2009) and NO inhibits NKCC1 activity in several tissue types (Akar et al. 1999; Hao et al. 2003; Wangensteen et al. 2006). Since CNQX is an AMPA/kainate blocker, we would expect reduced NO release (Radenovic & Selakovic, 2005), reduced inhibition of NKCC1 activity and, consequently, no change in NKCC1 expression. In addition, Garry et al. (2000) showed instead that increased NO potentiates CAP-evoked central neuropeptide release, which is in contrast to the expected effects if NO reduced NKCC1 activity. Thus, post-synaptic AMPA/kainate receptors are not considered likely candidates for the effects reported here. Another unlikely possibility is that CNQX blocked the $GABA_A$ receptor directly; CNQX, when administered between 20 – 50 μM, is a non-competitive blocker of the $GABA_A$ receptor channel (Jarolimek and Misgeld, 1991). However, considering that the CNQX dose used here was only 4 μM, we do not consider this possibility to be likely. Therefore, based on studies

showing that AMPA/kainate receptors can be found pre-synaptically (Lu et al. 2002; Lee et al. 2002; Lee et al. 2004; Willcockson & Valtschanoff, 2008), are involved in PAD (Rees et al. 1995; Lee et al. 2002; Russo et al. 2000; Russo et al. 2007), and can regulate NKCC1 expression in neuronal tissues (Schomberg et al. 2001; Chen et al. 2007), a pre-synaptic mechanism is certainly plausible. Interestingly, neither AMPA nor kainate receptors appear to activate p38 or JNK (Fuller et al. 2001; Nakatsu et al. 2006), however, they do activate spinal ERK1/2 after intraplantar scorpion venom (Pang et al. 2008). Thus, blockade of the AMPA/kainate receptor-mediated enhancement of NKCC1 expression may involve the ERK1/2 but not JNK or p38 pathways.

In terms of TRPV1, while spinal blockade of TRPV1 and NKCC1 prevented intracolonic CAP-induced secondary allodynia (see Chapter 3), it did not alter NKCC1 expression. Therefore, considering that CAP rapidly increased phosphorylated-p38 in small-to-medium diameter sensory neurons (Mizushima et al. 2005) as well as p38's role in regulating both TRPV1 and NKCC1 activity (Johnston et al. 2000; Ji et al. 2002; Piechotta et al. 2003; Obata et al. 2004; Capo-Aponte et al. 2007), it is possible that p38 is the common factor underlying the expression findings above as well as the behavioral findings described in section 3.3.5. Additional experiments addressing the role of p38 on NKCC1 expression are required.

CHAPTER 7

Conclusions

7.1. Major contributions

The vast majority of evidence available in the literature supports an important role for NKCC1 in persistent pain states (Table 1). As described in previous sections, our data fully supports this, demonstrating that pharmacological blockade of NKCC1 results in reduced behavioral, immunohistochemical and electrophysiological measures of persistent nociceptive input. Specifically, our behavioral, immunohistochemical and electrophysiological findings support the hypothesis that touch-evoked pain results from enhanced spinal NKCC1 activity. Our behavioral results indicate that both TRPV1 and NKCC1 blockade, at the spinal level, attenuate intracolonic CAP-induced referred allodynia as well as TRPV1-mediated stroking allodynia. Our findings that spinal TRPV1 activation produced robust stroking allodynia that was inhibited by pharmacological blockade of spinal NKCC1 are in agreement with previous work from our laboratory showing that genetic deletion of NKCC1 produce mice that do not display stroking allodynia (Laird et al. 2004). Moreover, to our knowledge, we are the first to demonstrate that a spinally administered endogenous TRPV1 agonist causes mechanical allodynia. Interestingly, while intrathecal NADA produced robust stroking allodynia, it is unclear why virtually no punctate hyperalgesia (withdrawal to von Frey stimulation) was evoked. Our FOS experiments showed that CNS neuronal activation by intracolonic CAP could be reduced by NKCC1 blockade. Furthermore, through the administration of LIS of the region of secondary allodynia, even greater FOS labeling was found in the dorsal horn, suggesting that low threshold afferent fibers gained access to the nociceptive channel. While this effect was mostly prevented by spinal NKCC1 blockade, a

component appeared to be resistant to BTD. Based on our NKCC1 expression studies in section 3.4., we suggest that TRPV1 may be involved. Future studies using TRPV1 and p38 MAPK inhibitors would be useful in resolving this issue. Our *in vivo* electrophysiological experiments demonstrate that spinal NKCC1 blockade attenuates intraplantar CAP-induced sensitization of both WDR and NS neurons. Furthermore, these findings further support the hypothesis that under inflamed conditions low threshold touch fibers gain access to the pain neuraxis through pre-synaptic GABAergic interneurons. Taken together, these experiments provide strong evidence that in persistent pain states low threshold afferents, potentially innervating a region remote from the location of injury, gain access to the nociceptive channel and can activate it producing touch-evoked pain. As previously mentioned, this is consistent with the view that touch-evoked pain may be due to an interaction between Aβ- and C-fibers in the human CNS (Lang et al. 2006; 2007). Considering that touch-evoked pain is a common symptom among chronic inflammatory pain sufferers, these experiments are clinically relevant.

7.2. Alternative hypotheses

It should be noted that there exist alternative hypotheses about the site of action of BTD; BTD acts on NKCC1 and to a lesser extent on KCC2, however, it also appears to act directly on $GABA_A$ receptors. BTD reduced GABA-activated currents in whole cell recordings, reflecting direct effects of BTD on $GABA_A$ receptor activity (Sung et al. 2000). Direct effects of high concentrations of furosemide, a loop diuretic closely related to BTD, on $GABA_A$ receptor function have also been observed (Nicoll, 1978; Inomata et al, 1988; Pearce, 1993; Korpi

and Luddens, 1997). Thus, it is possible that the effects reported in the present study could be due to direct action of BTD on $GABA_A$ receptors, where decreased $GABA_A$ receptor activity on primary afferent fibers by BTD would prevent low threshold input from accessing the nociceptive fiber, thus reducing nociceptive input to dorsal horn neurons. However, activity of BTD on $GABA_A$ receptors on intrinsic dorsal horn neurons would also be expected. Blockade of these $GABA_A$ receptors would reduce inhibition resulting in enhanced spike activity in dorsal horn nociceptive neurons, which was not observed in the experiments described here. Therefore, with these considerations in mind as well as the insufficient amount of information currently available on the direct effects of BTD on the $GABA_A$ receptor, this hypothesis has limited support.

As mentioned above, another potential location for BTD activity at a non-NKCC1 site is KCC2. The specificity of BTD for NKCC1 over the KCC's has been previously demonstrated (Gillen et al. 1996; Payne 1997; Holtzman et al. 1998, Dzhala et al. 2005), where KCC's affinity for BTD is approximately 100uM (Aull, 1981; Lauf et al. 1984) while NKCC1's affinity for BTD is approximately 1uM (Payne et al. 1995; Forbush and Palfrey, 1983). Thus, under ideal conditions, KCC activation is possible at the concentration used in the current electrophysiological experiments. Nonetheless, KCC activation by BTD in this study is unlikely for a number of reasons: Firstly, the drug concentration available to the dorsal horn neurons after spinal bath application would be much lower than the original concentration (Brumley et al. 2007), rendering an insufficient concentration necessary to activate KCC's. Secondly, while the expression levels in DRG of KCC1, KCC3 and KCC4 are relatively high, KCC2 expression is very

low (Gilbert et al. 2007). Of the four KCC's, only KCC2 has a clearly demonstrated role in spinal mechanisms of pain (Price et al. 2005a; Vinay et al. 2008; Kahle et al. 2008). Therefore, BTD-induced KCC2 activation would be expected to occur mainly in intrinsic dorsal horn neurons. Thirdly, even if BTD were to act on KCC's, their blockade by BTD would produce effects opposite to the anti-nociceptive effects described here. Specifically, spinal blockade of KCC2 reduced mechanical and thermal nociceptive thresholds in naive rats (Coull et al. 2003). For these reasons, we are confident that the effects described here result from NKCC1 blockade by BTD.

7.3. Future directions

While our laboratory and others have utilized many powerful techniques to study and NKCC1's role in persistent pain states, there remain a number of potential experiments that could provide invaluable information. In general, these experiments have not yet been performed due to technical difficulties.

7.3.1. Improved antibodies for immunohistochemistry

It is widely recognized that NKCC1 antibodies are acceptable for Western blot but yield inconsistent results with immunohistochemistry. If improved NKCC1 antibodies were available, electron microscopic studies would permit clear anatomical identification of the cellular and sub-cellular localization of NKCC1. While there is some debate in the literature as to the proportion of primary afferent neurons expressing NKCC1 (Sung et al. 2000; Alvarez-Leefmans et al. 2001; Kanaka et al. 2001; Price et al. 2006; Gilbert et al. 2007), it is generally agreed that NKCC1 expression is low, compared to KCC2 expression, on intrinsic spinal cord dorsal horn neurons in adult rodents (Price et al. 2006, Kahle

et al. 2008). Hence, in accord with our hypothesis, NKCC1 should be found mainly on pre-synaptic glomerular terminations of primary afferent fibers in the spinal cord. Yet to date no studies have attempted to discriminate NKCC1 expression on intrinsic spinal cord neurons from central terminals of primary afferent fibers or, for that matter, other types of cells (e.g. satellite glial cells). Moreover, as mentioned above, a number of studies have failed to detect significant differences in NKCC1 expression levels in the spinal cord following painful inflammatory stimuli (Nomura et al. 2006; Jolivalt et al. 2008; Zhang et al. 2008). However, using western blot membrane fractionation methods, our laboratory has previously shown that while overall NKCC1 expression changes were not seen after intracolonic CAP, cytosolic NKCC1 was recruited to the membrane (Galan & Cervero, 2005). Hence, electron microscopy could be used to examine (i) the type of cells expressing NKCC1 as well as (ii) the sub-cellular localization of NKCC1 in naïve and CAP-treated mice, permitting evaluation of the effects of peripheral inflammation on the ratio of cytosolic to plasma membrane NKCC1 in both central terminals of primary afferent fibers and intrinsic spinal cord neurons. Furthermore, experiments could be performed utilizing double-labelling of NKCC1 co-transporter and the $GABA_A$ receptor or GABA in order to confirm thier pre- vs post-synaptic apposition. Clearly, electron microscopic localization of NKCC1 would provide a compelling argument for or against the veracity of the hypothetical circuit described in this thesis (Fig. 6).

7.3.2. Nociceptor specific NKCC1 genetic knockout animals

To study the influence of NKCC1 function on nociception, the selective knockout of NKCC1 on NaV1.8-containing nociceptive primary afferent fibers, via the Cre-

Lox knockout paradigm, would be a powerful tool to study NKCC1's role in nociceptive processing without the unwanted effects of NKCC1 knockout in non-target tissues (deafness, locomotor difficulties). This technique is entirely feasible, requiring the breeding of mice expressing CRE-Nav1.8 (CRE expressed only in nociceptors) and LOX-NKCC1 (LoxP flanking NKCC1). Crossing these strains produces a number of possible genotypes, two of which are required for this paradigm: (1) $CRE^{-/-}$ + $LOX^{+/+}$, referred to as NKCC1-LOX (LoxP flanks NKCC1 on both alleles without CRE recombination) and (2) $CRE^{+/-}$ + $LOX^{+/+}$, which is the NKCC1 nociceptor-specific knockout (NKCC1-NSKO) mouse strain, which has NKCC1 knocked out exclusively in Nav1.8 fibers. NKCC1-NSKO mice should have normal Nav1.8 currents in that they retain one normal Nav1.8 allele. Therefore, NKCC1-NSKO mice are experimental mice while the NKCC1-LOX mice are controls.

With these nociceptor-specific NKCC1 knockout mice, we would perform a battery of baseline nociceptive tests (mechanical threshold testing, thermal latency, etc.) as well as established nociceptive models such as intracolonic CAP, intraplantar CFA or formalin, and so on. We could also perform *in vivo* electrophysiological recording of spinal nociceptive neurons (similar to Chapter 4) as well as spinal FOS labeling (similar to Chapter 5). Interestingly, while our hypothesis focuses on NKCC1's role in augmenting orthodromic signaling in nociceptive afferent fibers in persistent pain states such that spinal cord nociceptive neurons become sensitized, antidromic activity resulting in neurogenic inflammation is equally plausible. In support of this hypothesis, recent work from another laboratory demonstrated that spinal BTD attenuates

intraplantar CAP-induced neurogenic inflammation (Valencia-de-Ita et al. 2006). However, a pivotal experiment demonstrating NKCC1's role in antidromic signaling and neurogenic inflammation again involves the use of the nociceptor-specific NKCC1 knockout mouse in an *ex vivo* electrophysiological preparation; in normal rodents, intraplantar CAP increases the number of dorsal root reflexes (DRR's) recorded from central ends of isolated filaments from cut dorsal roots (Valencia-de-Ita et al. 2006). However, under similar conditions, mice lacking NKCC1 in nociceptive primary afferents would not be expected to exhibit increased DRR's. In these same knockout animals, one could also study peripheral alterations in blood flow and edema, both local measures of neurogenic inflammation.

We have undertaken the development and preliminary testing of nociceptor-specific knockout mice, however, due to an error in the development of one of the parent strains by a collaborator, we have not yet been able to complete this study.

7.3.3. Behavioral and immunohistochemical studies in rodents using pharmacological inhibitors

As discussed in previous sections, p38 MAPK may may be a common upstream regulator of both NKCC1 and TRPV1 activity following intracolonic CAP. In fact, p38 is known to regulate both NKCC1 and TRPV1 activity (Johnston et al. 2000; Ji et al. 2002; Piechotta et al. 2003; Obata et al. 2004; Capo-Aponte et al. 2007). NKCC1 and TRPV1 are co-expressed on small- and medium-diameter afferents (Price et al. 2006). Interestingly, phosphorylated p38 expression is increased on these same fibers after CAP (Mizushima et al. 2005). Thus, it is possible that

p38, NKCC1 and TRPV1 activity are functionally related. Behaviorally, we have showed that while spinal NKCC1 blockade reduced TRPV1-mediated allodynia, spinal blockade of NKCC1, TRPV1 and p38 all reduced iCAP-evoked secondary allodynia. However, TRPV1 blockade did not affect NKCC1 expression after iCAP. Thus, while it does not appear that TRPV1 is an upstream regulator of NKCC1, it is unclear whether or not i) NKCC1 is an upstream regulator of TRPV1 and/or ii) p38 is upstream to both NKCC1 and TRPV1. In order to clarify this issue, a few pivotal experiments are required. Specifically, in addition to our findings described above, the effects of spinal p38 and NKCC1 blockade on spinal TRPV1 expression must be studied, as well as the effects of p38 blockade on NKCC1 expression. In addition, following spinal administration of the TRPV1 agonist NADA to provoke stroking allodynia, the effects of spinal p38 blockade must be determined. If p38 is indeed an upstream regulator of both NKCC1 and TRPV1, we expect that p38 blockade would prevent increased expression of both NKCC1 and TRPV1 in the spinal cord. Furthermore, spinal p38 blockade should also reduce TRPV1-dependent stroking allodynia.

In the context of our FOS findings, where NKCC1 blockade only partially reversed LIS-evoked enhancements of FOS labeling, we suggested that increased TRPV1 activity may be involved. Since LIS potentiates spinal FOS labeling after iCAP, it is reasonable to suggest that LIS may also potentiate nociceptive behavioral responses such as response frequency to abdominal stimulation after iCAP. If so, then we would also expect spinal BTD to be only partially effective in reducing this measure of secondary allodynia. If p38 is an upstream regulator of both NKCC1 and TRPV1, then spinal administration of a

p38 antagonist or co-administration of the TRPV1 blocker AMG 9810 and BTD may completely reverse the secondary allodynia as well as FOS levels.

Another potentially useful approach could be *ex vivo* electrophysiological recording of DRR's; the effects of p38 blockade on CAP-evoked DRR's would provide additional support for p38's involvement in regulating NKCC1. Overall, if p38 regulates both NKCC1 and TRPV1 activity in sensory afferent terminals in the spinal cord, the experiments described above would provide strong supportive evidence.

7.3.4. Human studies

Whenever possible, studies on human subjects are preferred because of their clinical relevance. Considering that BTD has long been approved for human use as a diuretic, there is a greater potential for it to be used as an off-label experimental drug. While no published evidence exists of BTD's ability to penetrate the CNS through the blood-brain barrier, the effects of systemic administration of BTD (or the related drug furosemide) on *in vivo* seizure activity in animals (Dzhala et al. 2005) and humans (Haglund and Hochmann, 2005) suggests that this drug can cross the BBB in sufficient amounts to act directly on CNS neurons. Therefore, there is significant clinical interest in NKCC1 blockade for various types of epilepsy (Kahle et al. 2008; Kahle & Staley 2008a). Interestingly, BTD's diuretic effects are reduced by co-administration with NSAID's such as aspirin (Brater, 1985), a fortunate side effect that may add to its clinical potential in treating chronic pain. Consequently, there is a real possibility that pharmacological manipulation of NKCC1 activity in chronic pain populations could become a reality in the near future.

7.4. Final remarks

In the experiments described in this study we have used multiple research techniques to help unravel the role of NKCC1 in spinal mechanisms of persistent pain states. We demonstrated behaviorally that spinal NKCC1 blockade attenuates secondary allodynia and hyperalgesia. Immunohistochemistry for FOS labeling showed that low threshold input gain access to the nociceptive channel in a BTD-sensitive manner. Using *in vivo* electrophysiology we showed that sensitization of spinal nociceptive neurons can be reversed by spinal NKCC1 blockade. Finally, using molecular biological techniques, AMPA/kainate receptors have been identified as a postential upstream regulator of NKCC1 activity. Overall, the data presented here indicate an important role for NKCC1 in spinal mechanisms of persistent pain states.

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