FURTHER STUDIES IN ADENOSINERGIC AND MONOAMINERGIC MECHANISMS OF ANALGESIA BY AMITRIPTYLINE

by

Jean Liu

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "FURTHER STUDIES IN ADENOSINERGIC AND MONOAMINERGIC MECHANISMS OF ANALGESIA BY AMITRIPTYLINE" by Jean Liu in partial fulfilment of the requirements for the degree of Master of Science.

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Supervisor: _____

Readers: _____

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ABSTRACT

In this thesis, rodent models of chronic pain were used to explore analgesic mechanisms that may potentially be engaged in spinal and peripheral compartments by systemically-administered amitriptyline, a tricyclic antidepressant. The first project (Chapter 2) identified the roles of spinal adenosine A_1 and serotonin 5-HT_7 receptors, as well as of peripheral adenosine A_1 receptors, in the acute antinociceptive effects of amitriptyline in mice. The second project (Chapter 3) examined the potential utility of amitriptyline as a preventive analgesic against persistent post-surgical pain, and involved perioperative administration of amitriptyline after peripheral nerve injury in rats. Changes in post-injury behavioural outcomes, as well as spinal noradrenergic sprouting, were assessed. Overall, spinal serotonergic pathways linked to adenosine A_1 receptors, as well as peripheral adenosine A_1 receptors, appear to be important in antinociception by amitriptyline. Preventive analgesia by this drug does not appear to result from anatomical changes in spinal noradrenergic pathways.

LIST OF ABBREVIATIONS USED

5-HT	Serotonin
5-HT_7R	Serotonin 5-HT_7 receptor
6-OHDA	6-hydroxydopamine
A_1R	Adenosine A_1 receptor
A_{2A}R	Adenosine A_{2A} receptor
ABC	Avidin-biotin complex
αβ-MeATP	αβ-methylene-adenosine 5'-triphosphate
AMI	Amitriptyline
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AS-19	(2S)-(+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin
ATP	Adenosine 5'-triphosphate
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CCI	Chronic constriction injury
CNS	Central nervous system
CONTRA	Contralateral
DAB	3,3'-diaminobenzidine
DβH	Dopamine-β-hydroxylase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DRG	Dorsal root ganglion
ENT	Equilibrative nucleoside transporter
FOR	Formalin
GABA	γ-aminobutyric acid
GDNF	Glial-derived neurotrophic factor
G_i	Inhibitory G protein
G_s	Stimulatory G protein
H_2O	Water
H_2O_2	Hydrogen peroxide
i.m.	Intramuscular
i.p.	Intraperitoneal
i.pl.	Intraplantar
IPSI	Ipsilateral
IR	Immunoreactivity
i.t.	Intrathecal
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NGF	Nerve growth factor
NMDA	N-Methyl-D-aspartic acid
NNT	Number needed to treat

NSAID	Non-steroidal anti-inflammatory drug
OCT	Optimum cutting temperature
PAG	Periaqueductal gray
PB	Phosphate buffer
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PBST	1% Triton-X 100 in phosphate buffered saline
PGE_2	Prostaglandin E_2
PNS	Peripheral nervous system
p.o.	Per os
PSL	Partial sciatic nerve ligation
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RVM	Rostral ventromedial medulla
SAL	Saline
SB269970	(2R)-1-[(3-hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine hydrochloride
s.c.	Subcutaneous
SEM	Standard error of the mean
siRNA	Small interfering RNA
SNI	Spared nerve injury
SNK	Student Neuman-Keuls
SNL	Spinal nerve ligation
SNRI	Selective noradrenaline reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TH	Tyrosine hydroxylase
Trk	Tropomyosin-receptor-kinase
TRP	Transient receptor potential
VEH	Vehicle

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Just prior to my arrival at Dalhousie in the summer of 2010, the first thing that my supervisor, Dr. Jana Sawynok, suggested that I read was a book by Marni Jackson, entitled *Pain: The Fifth Vital Sign*. As Dr. Sawynok put it, this book was an exploration of the question "what is this thing we know as pain?" That I was greatly moved and inspired by what I read is no understatement. This book opened my eyes to the profound impact that pain has on the lives of sufferers, and revealed many serious problems that affect the way that pain is treated (and recognized) in our society. Having these broad perspectives of pain kept me focused and motivated in my own research. I am hugely grateful to Dr. Sawynok for showing me the "bigger picture" of pain research, inspiring my future career path, and helping to shape me into a better researcher and writer. I wish to thank her for her unwavering support, and for being an absolutely phenomenal mentor, not just for academic matters, but also for many important life decisions.

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Jean Liu
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CHAPTER 1

INTRODUCTION

1.1 PAIN

1.1.1 Overview of Pain

Pain is an aversive, subjective perceptual experience that is influenced by complex interactions between sensory, affective, and cognitive components (Lee & Tracey, 2010). Pain is critical for survival, serving as a powerful means of alerting an organism to the presence of an injury, thereby promoting avoidance of further tissue damage (Basbaum & Jessell, 2000). The characteristics of particular forms of pain vary depending on their origins, and pain can be classified as nociceptive, inflammatory, or neuropathic, reflecting main mechanisms involved in its generation (Kehlet, Jensen, & Woolf, 2006). **Nociceptive pain** is produced by the activation of nociceptors (high-threshold free nerve endings in the periphery) by noxious mechanical, thermal, and chemical stimuli (Basbaum & Jessell, 2000; Kehlet et al., 2006). In contrast, **inflammatory pain** is generated from the local release of inflammatory mediators in the vicinity of tissue damage, lowering the threshold of nearby nociceptors (peripheral sensitization) and thereby amplifying nociceptive signaling (Costigan, Scholz, & Woolf, 2009; Kehlet et al., 2006).

Both nociceptive and inflammatory aspects of pain become attenuated as the noxious stimulus is removed or the injured tissue heals, indicating that peripheral sensitization is reversible (Kehlet et al., 2006). However, pathological states of pain can develop and persist for extended periods of time, even after the resolution of any obvious

tissue damage (Basbaum & Jessell, 2000; Kehlet et al., 2006). **Neuropathic pain** is a pathological condition that is characterized by a distinctive set of symptoms, including spontaneous pain, allodynia (heightened pain responses to innocuous stimuli), hyperalgesia (exaggerated pain responses to noxious stimuli), and sensory loss (Kehlet et al., 2006). The development of neuropathic pain is typically associated with nerve injury, which may arise from physical trauma to the nerve, or as an outcome of chronic conditions, including metabolic diseases (e.g., diabetic neuropathy), infection (e.g., post-herpetic neuralgia), toxic effects (e.g., chemotherapy-induced neuropathy), and from cancer (Kehlet et al., 2006). The relative lack of effective treatments currently available, coupled with issues of subjectivity in self-reported pain, make neuropathic pain extremely difficult to manage in humans (Kehlet et al., 2006). Chronic neuropathic pain causes widespread suffering, poses a significant socioeconomic burden upon society, and represents an unmet clinical need (Kehlet et al., 2006). Chapter 3 provides a more detailed exploration of neuropathic pain.

The challenge of pharmacological research in pain is not only to develop more effective drugs, but also to better understand the means by which existing drugs exert their analgesic effects. Knowledge of the latter may then contribute to the design of novel therapeutic agents. This thesis will examine the neural mechanisms underlying the analgesic effects of amitriptyline, a tricyclic antidepressant (TCA) drug which is commonly prescribed for the treatment of established chronic pain. Subsequent sections of this chapter will describe the neural processes behind nociceptive transmission and modulation, provide a pharmacological overview of amitriptyline actions, and introduce the two studies that were undertaken during the course of this MSc research.

1.1.2 Distinctions Between Pain and Nociception

As described above, the experience of pain is a top-down process that is governed not only by sensation of noxious stimuli, but also by higher-level affective and cognitive processes (Lee & Tracey, 2010). In terms of the mechanisms and anatomy underlying the sensation of pain, our understanding of ascending nociceptive transmission pathways from the periphery to the brain have primarily been elaborated within preclinical models. Since animals do not possess the ability to communicate their subjective pain experiences, only behavioural correlates can be used as outcome measures of pain (Mogil, 2009). It is, therefore, important to clarify that the word "pain" more precisely refers to a perceptual experience, whereas nociception refers to the pre-conscious, *sensory* component that can occur in the absence of "pain" (Loeser & Treede, 2008). Thus, the term nociception is specifically used to refer to the sensory systems that are responsible for the encoding, transmission, and processing of noxious stimuli (Loeser & Treede, 2008).

1.1.3 Peripheral Mechanisms of Nociception and Sensory Integration

The encoding and processing of nociceptive signals occur at different levels of the peripheral and central nervous systems (Basbaum & Jessell, 2000). Initially, noxious stimuli are detected in the periphery by nociceptors, which are free nerve endings of primary afferent neurons that are present in the skin, muscle, bones, joints, and viscera (Basbaum & Jessell, 2000). Various receptors are present on the extracellular surface of nociceptors, and these transduce specific noxious mechanical, thermal, and chemical

stimuli into membrane excitation (**Table 1.1.1**) (Basbaum & Jessell, 2000; Marchand, Perretti, & McMahon, 2005). Above-threshold membrane depolarization of nociceptors leads to the generation of action potentials along the axons of primary afferent neurons (Aδ and C fibres), which project to the dorsal horn of the spinal cord (Basbaum & Jessell, 2000). Although the dorsal root ganglia (DRG) are made up of the cell bodies of Aδ and C fibres, each primary afferent fibre type possesses distinctive anatomical characteristics that lead to differences in nociceptive signaling. Aδ fibres are large in diameter, thinly-myelinated, and have high conduction velocities, while C fibres are smaller in diameter, unmyelinated, and have low conduction velocities (Basbaum & Jessell, 2000). The differences in conduction velocity via each primary afferent fibre type account for temporal variations in pain perception, such that Aδ fibre activation gives rise to first pain (sharp and immediate), whereas C fibre activation is associated with second pain (slower, prolonged aching) (Basbaum & Jessell, 2000).

Table 1.1.1 Channels and receptors expressed on nociceptors that are involved in the transduction of noxious stimuli

Receptor	Examples of Stimuli	Temperature Sensitivity
TRPV1 channel	heat, capsaicin, H^+, allicin, lipids	43 °C and above
TRPV2 channel	noxious heat, mechanical	52 °C and above
TRPV3 channel	camphor	32-39 °C and above
TRPV4 channel	innocuous heat, mechanical	27-35 °C and above
TRPM4 channel	Ca^{2+}	15-35 °C
TRPM8 channel	cold, menthol	Below 25-28 °C
TRPA1 channel	mechanical	Below 17 °C
Acid-sensing ion channel	H^+	n/a
Adenosine A_{2A} receptor	adenosine	n/a
Bradykinin B2 receptor	bradykinin	n/a
P2X/P2Y receptors	mechanical, ATP	n/a
Prostanoid receptor	prostaglandin E2	n/a
Serotonin 5-HT_3 receptor	5-HT	n/a

Content adapted from Giordano (2005), Marchand et al. (2005), and Tominaga (2007).

1.1.4 Sensory Integration and Ascending Nociceptive Transmission

An important stage of sensory integration occurs within the dorsal horn of the spinal cord, where glutamatergic primary afferent Aδ fibres terminate on transmission neurons in laminae I (superficial layer) and V (deep layer) and C fibres form synapses with interneurons in lamina II and transmission neurons in lamina I (Benarroch, 2008). **Figure 1.1.1** depicts the neuronal organization of primary afferent neurons and their respective targets within the spinal cord. Transmission neurons relay nociceptive signals to the brain via the spinothalamic, spinoreticular, and spinomesencephalic tracts (Basbaum & Jessell, 2000). The spinothalamic tract is the most extensively characterized nociceptive pathway, and consists of transmission neurons in laminae I and V which project axons across the midline into the anterolateral white matter of the contralateral

spinal cord (Basbaum & Jessell, 2000). The axons of these transmission neurons then ascend to the thalamus, where nociceptive inputs are integrated before being relayed to somatosensory areas of the cortex (Basbaum & Jessell, 2000). In addition to undergoing processing within thalamo-cortical circuits, nociceptive inputs are integrated in the reticular formation, amygdala, as well as throughout the cerebral cortex (Lee & Tracey, 2010). The complexity of pain perception can, in part, be attributed to the widespread distribution of activation across different brain regions broadly representing sensory, affective, and cognitive domains.

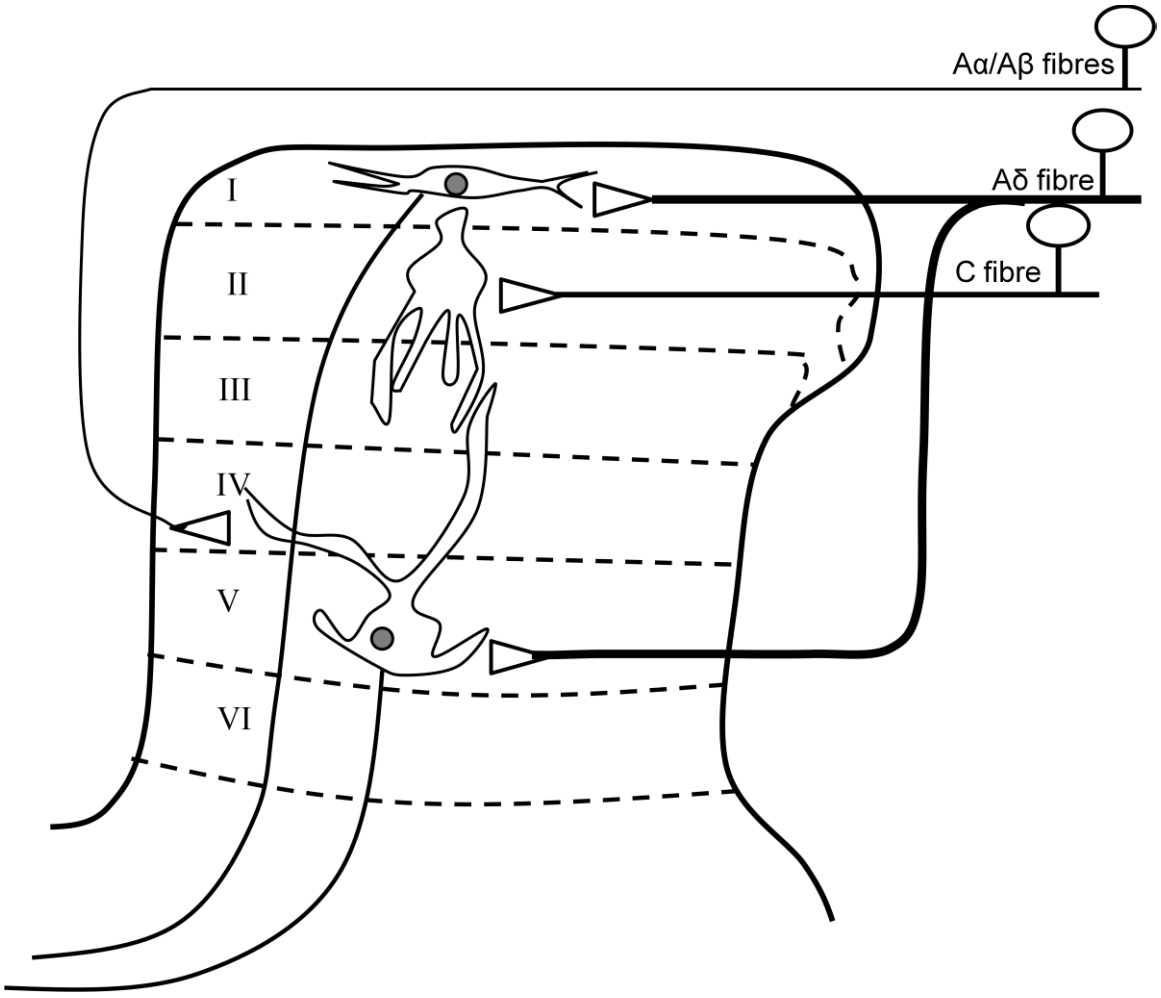

Figure 1.1.1 Termination of primary afferent fibres in the dorsal horn of the spinal cord. The terminals of Aδ fibres synapse with transmission neurons in laminae I and V. C fibre terminals synapse with interneurons in lamina II, and indirectly influence lamina I transmission neurons. Adapted from Basbaum & Jessell (2000).

1.1.5 Descending Modulation of Pain

Along with the ascending nociceptive pathways, descending pathways originating from the brain are involved in shaping the sensory-discriminative aspect of pain. In particular, various brain areas associated with pain (including the periaqueductal gray or PAG, amygdala, as well as the prefrontal, insular, and anterior cingulate cortices) communicate with brainstem regions that subsequently modulate sensory processing within spinal cord circuits (Bee & Dickenson, 2009). These descending brainstem projections synapse with primary afferents, transmission neurons, inhibitory interneurons, and terminals of other descending neurons in the superficial dorsal horn of the spinal cord, and exert both facilitatory or inhibitory effects on incoming nociceptive signaling (Bee & Dickenson, 2009; Benarroch, 2008).

Descending modulatory pathways are primarily monoaminergic (**Figure 1.1.2**), with potential targets such as inhibitory interneurons that release γ-aminobutyric acid (GABA), glycine, and opioids (Benarroch, 2008). Moreover, the bidirectional nature of modulation (facilitatory or inhibitory) depends on which monoamine (serotonin or 5-HT, noradrenaline or NA, and/or dopamine) receptor subtypes are activated (Bee & Dickenson, 2009). Descending noradrenergic projections originating from the locus coeruleus exhibit exclusively inhibitory actions on afferent nociceptive signaling by activating spinal α_2-adrenergic receptors on pre-synaptic primary afferent terminals and post-synaptic transmission neurons (Bee & Dickenson, 2009). In contrast, descending serotonergic projections originating from the rostral ventromedial medulla (RVM) may facilitate via activation of 5-HT_3 receptors, or inhibit nociception via activation of other 5-HT receptor subtypes, such as 5-HT_1 receptors (Bee & Dickenson, 2007, 2009;

Benarroch, 2008). Dopaminergic involvement in the descending regulation of pain has been less well-studied (Millan, 2002), and this system will not be considered further.

Since descending modulatory pathways have the capacity to reduce or amplify incoming nociceptive signaling, a potential endogenous analgesic mechanism recruited by various pharmacotherapeutics for pain could involve the enhancement of descending inhibition and/or the reduction of descending facilitation (Bee & Dickenson, 2009). The involvement of descending inhibitory pathways in the mechanisms of amitriptyline will be examined in subsequent chapters of this thesis. **Figure 1.1.3** summarizes the influences of ascending and descending pathways on nociceptive processing within the spinal cord.

In summary, the resultant convergence of somatosensory, affective-motivational, cognitive, and descending modulatory components ultimately help to shape the complex, polymodal perceptual experience of pain (Basbaum & Jessell, 2000; Kehlet et al., 2006; Lee & Tracey, 2010).

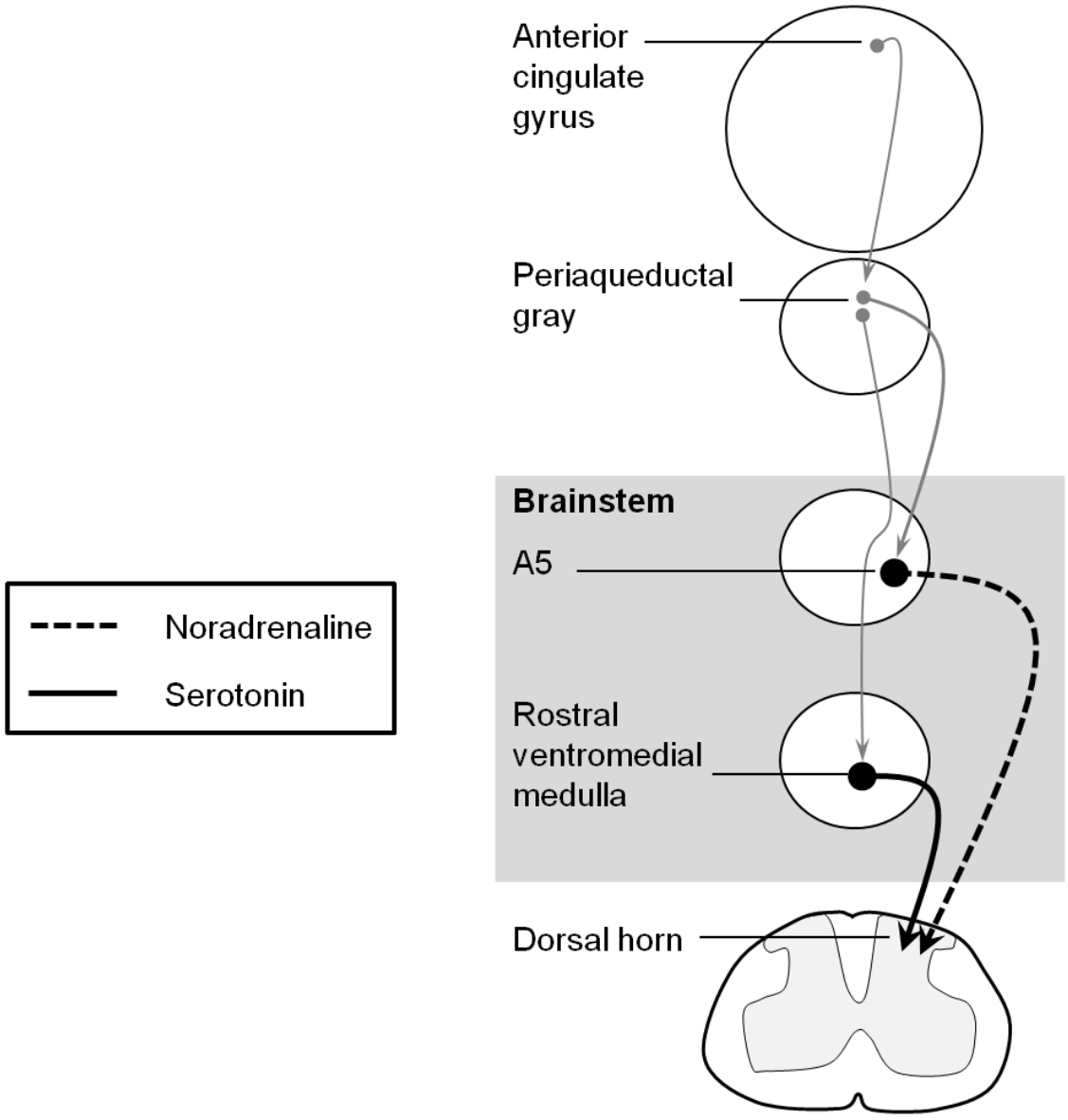

Figure 1.1.2 Descending modulatory pathways projecting from the brainstem to the spinal cord. Higher brain centres (anterior cingulated cortex, periaqueductal gray) signal to monoaminergic nuclei (A5, rostral ventromedial medulla) in the brainstem, leading to the activation of descending modulatory pathways. These descending monoaminergic pathways release NA, 5-HT, and dopamine (not shown) into synapses within the superficial dorsal horn of the spinal cord, producing both facilitatory and inhibitory effects on afferent pain signaling. Adapted from Benarroch (2008).

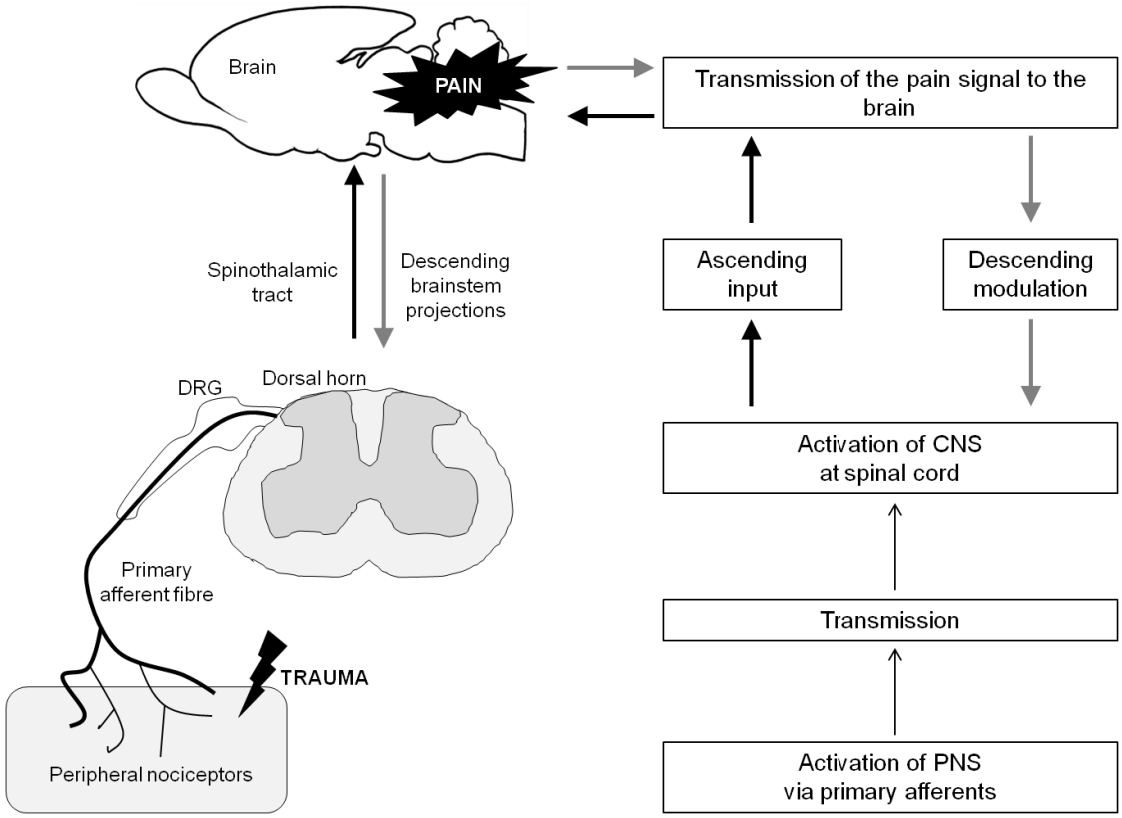

Figure 1.1.3 Summary of ascending and descending pathways in the central nervous system (CNS). These ascending inputs (transmitted from the peripheral nervous system or PNS) and descending modulatory pathways are involved in the processing of nociceptive information within the spinal cord. Adapted from Bingham, Ajit, Blake, & Samad (2009).

1.2 ANTIDEPRESSANTS AND PAIN

1.2.1 Clinical Use of Antidepressants for Chronic Pain

In current medical practice, antidepressants are used to treat chronic pain states, and are among the first-line drugs prescribed for neuropathic pain (Dharmshaktu, Tayal, & Kalra, 2012; Dworkin et al., 2010; Finnerup, Otto, McQuay, Jensen, & Sindrup, 2005; Saarto & Wiffen, 2010). Antidepressants have been traditionally recognized for their effects on mood (hence their primary use in clinical depression), but these drugs also possess considerable efficacy in alleviating pain within non-depressed patients (Micó, Ardid, Berrocoso, & Eschalier, 2006; Saarto & Wiffen, 2010). As pain relief occurs more rapidly than antidepressant actions (Jasmin, Tien, Janni, & Ohara, 2003), and doses required for pain treatment are lower than those necessary to alleviate depression, analgesia appears to be produced by mechanisms distinct from their actions on affective states (Micó et al., 2006). In spite of widespread use of antidepressants for pain, the analgesic mechanisms of these drugs are incompletely understood (Micó et al., 2006).

TCAs are dual reuptake inhibitors of NA and 5-HT and exhibit superior efficacy against neuropathic pain compared to selective noradrenaline reuptake inhibitors (SNRIs) and selective serotonin reuptake inhibitors (SSRIs) (Saarto & Wiffen, 2010). The most extensively-studied TCA is amitriptyline. Although amitriptyline has a number needed to treat (NNT) of 3.1, it produces unpleasant side effects, including dry mouth, blurred vision, constipation, weight gain, and sedation (Saarto & Wiffen, 2010). In comparison, the SNRI venlafaxine (NNT ≈ 4) and SSRIs (NNT ≈ 7) exhibit lower efficacy, but better tolerability than amitriptyline (Finnerup et al., 2005; Saarto & Wiffen, 2010).

1.2.2 Pharmacological Actions of Amitriptyline

The efficacy of amitriptyline and other TCAs against chronic pain has been attributed to a multiplicity of pharmacological actions. Such actions include inhibition of monoamine reuptake, activation of the endogenous opioid system, sodium channel blockade, NMDA receptor antagonism, enhancement of $GABA_B$ receptor activity, modulation of immune factor expression, and recruitment of adenosine systems (Micó et al., 2006). Additionally, amitriptyline also exerts anticholinergic and antihistamine effects, accounting for some of its side effects (Dharmshaktu et al., 2012).

The relative importance of the various pharmacological systems implicated in the analgesic mechanisms of amitriptyline likely depends on which specific sites of action in the body (compartments) are involved. In the central nervous system, amitriptyline is primarily thought to enhance the synaptic availability of noradrenaline and serotonin in the spinal cord, thereby potentially augmenting descending inhibitory tone, which may be weakened in chronic pain states (Bee & Dickenson, 2009; Dharmshaktu et al., 2012). In the periphery, amitriptyline exerts local analgesic effects, and these may reflect actions on sodium channels and adenosine systems (Dualé et al., 2008; Sawynok, Reid, & Esser, 1999).

1.3 OVERVIEW OF PROJECTS

This thesis presents the findings of two projects that were undertaken to examine the effects of amitriptyline on pain. In these independent studies, different analgesic mechanisms potentially engaged by systemically-administered amitriptyline were explored using rodent models of acute and chronic pain. The first project (Chapter 2) examined the involvement of spinal adenosine A_1 and serotonin 5-HT_7 receptors, as well as of peripheral adenosine A_1 receptors, in the acute antinociceptive effects of amitriptyline in mice using a model of ongoing and inflammatory pain. In particular, this study used a compartmental approach to identify a novel spinal cord mechanism, involving adenosine A_1 and 5-HT_7 receptor activation, as well as a peripheral adenosine A_1 receptor action, that appears to be involved in antinociception by systemic amitriptyline.

Since antidepressants are used as first-line treatments for established chronic pain, the second project (Chapter 3) focused on the utility of amitriptyline as a preventive analgesic against chronic post-surgical neuropathic pain. In this study, the effects of a perioperative treatment regime of amitriptyline on certain behavioural and anatomical sequelae accompanying a peripheral nerve injury were explored in rats.

Although the mechanisms of action investigated within each study were different, the overarching concept that unites both studies is that descending pain modulatory (serotonergic and noradrenergic) pathways projecting to the spinal cord are involved in the action of amitriptyline. In Chapter 4, future experimental directions will be addressed in relation to the two studies presented in this thesis.

CHAPTER 2

ADENOSINE INVOLVEMENT IN ANTINOCICEPTION BY AMITRIPTYLINE

2.1 ABSTRACT

This study explored a potential link between spinal serotonin 5-HT_7 receptors (5-HT_7Rs) and adenosine A_1 receptors (A_1Rs) in antinociception by systemic (i.p.) amitriptyline in normal and A_1R knock-out mice using intrathecal (i.t.) delivery of selective receptor antagonists in the 2% formalin test. In normal mice, antinociception by i.p. amitriptyline 3 mg/kg was blocked by i.t. delivery of the A_1R antagonist DPCPX 10 nmol. Blockade was also seen in A_1R +/+, but not in -/- mice. In normal and A_1R +/+ mice, i.t. delivery of the 5-HT_7R antagonist SB269970 3 μg blocked antinociception by i.p. amitriptyline. In contrast, i.t. SB269970 did not prevent antinociception by amitriptyline in A_1R -/- mice. Flinching increased after i.t. co-administration of the 5-HT_7R agonist AS-19 20 μg with SB269970 or with DPCPX. I.t. AS-19 increased flinching in A_1R -/-, but not +/+ mice. From these results, we propose that i.p. amitriptyline reduces nociception by activating spinal A_1Rs, secondarily to spinal 5-HT_7R activation. Spinal actions constitute only one aspect of antinociception by amitriptyline, as intraplantar DPCPX 10 nmol also blocked antinociception by i.p. amitriptyline in normal and A_1R +/+, but not -/- mice. To assess the potential human relevance of these A_1R actions, we examined effects of chronic caffeine, which blocks A_1Rs, as well as A_{2A}Rs, given to normal mice in the drinking water. Oral caffeine (0.1, 0.3 g/L) blocked antinociception by i.p. amitriptyline 3 mg/kg. In conclusion, A_1R-mediated antinociception by systemic amitriptyline occurs both spinally and peripherally, and is reduced by a regime of chronic oral caffeine.

2.2 INTRODUCTION

2.2.1 Pain Modulation by Adenosine

Adenosine is an endogenous signaling and regulatory molecule that is involved in numerous physiological processes and disease states (Dunwiddie & Masino, 2001; Sawynok, 2012). As a metabolite of adenosine-5'-triphosphate (ATP), adenosine is present in the cytoplasm of all cells in the body (Dunwiddie & Masino, 2001). In the central nervous system, adenosine acts as an extracellular neuromodulator in various circuits, including those involved in regulating arousal and sleep, anxiety, cognition, memory, as well as pain (Ribeiro, Sebastião, & de Mendonça, 2002; Sawynok & Liu, 2003a). Adenosine does not behave as a classical neurotransmitter, as it is not exocytosed from synaptic vesicles. Instead, extracellular adenosine levels are determined by the activity of nucleoside transporters, as well as metabolism of extracellular adenine nucleotides (**Figure 2.2.1**, Dunwiddie and Masino, 2001; Wiesner et al., 1999). Since the direction of transport by nucleoside transporters depends on the concentration gradient of adenosine across the membrane, adenosine may either be extruded into the extracellular space or taken up into the cell (Ribeiro et al., 2002). Additionally, adenosine can be synthesized in the extracellular space following the dephosphorylation of adenine nucleotides by ectonucleotidases (Dunwiddie & Masino, 2001). Clearance of adenosine from the extracellular space can occur by the reuptake of adenosine into cells as well as the metabolism of adenosine by adenosine deaminase into inosine (Dunwiddie & Masino, 2001). When present in the extracellular space, adenosine is able to bind to four extracellular G protein-coupled adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3 receptors (encoded by the *ADORA1*, *ADORA2A*, *ADORA2B*, and *ADORA3* genes), thereby

eliciting various cellular effects (Dunwiddie & Masino, 2001; Sawynok, 2012; Zylka, 2011).

Over the past three decades, adenosine A_1 receptors (A_1Rs) have received the most attention with respect to pain. A_1Rs are situated throughout peripheral, spinal, and supraspinal levels of nociceptive transmission pathways, and are highly-expressed in primary afferent neurons, the superficial dorsal horn, and in various brain areas associated with the processing of noxious stimuli (Sawynok, 2012). In conjunction with pharmacological targeting approaches using selective receptor agonists and antagonists, the use of knock-out mice has helped to clarify the involvement of A_1Rs in nociception in preclinical studies (Fredholm, Chen, Masino, & Vaugeois, 2005). Agonists for A_1Rs have been demonstrated to produce peripheral and spinal antinociception in animal models of nociceptive, inflammatory, and neuropathic pain, thereby implicating adenosine as an endogenous analgesic molecule (Fredholm et al., 2005; Sawynok, 2012). Since A_1Rs are coupled to inhibitory G proteins (G_i), the decreased activity of adenylyl cyclase may produce a variety of downstream signaling effects which could account for their inhibitory actions on neuronal activity (Lima et al., 2010; Sawynok, 2012). Such effects include a reduction in excitatory neurotransmitter release from primary afferent terminals in the spinal cord, as well as increased potassium ion conductance into transmission neurons (Li & Perl, 1994; Patel, Pinnock, & Lee, 2001; Sawynok, 2012). Since the activation of A_1Rs has also been implicated in the actions of existing analgesic agents, including acetaminophen and amitriptyline (Sawynok & Reid, 2012; Sawynok, Reid, & Fredholm, 2008, 2010; Ulugol et al., 2002), further elaboration on the precise antinociceptive mechanisms involved are warranted.

Although activation of A_1Rs produces antinociception, adenosine A_{2A} receptors (A_{2A}Rs) appear to be involved in mediating hyperalgesia (reviewed in Sawynok, 2012). Although A_{2A}Rs are expressed in supraspinal regions which are involved in the processing of pain, it is still somewhat unclear if they are present in the DRG or the spinal cord (Bailey et al., 2002; Brooke, Deuchars, & Deuchars, 2004; Kaelin-Lang, Lauterburg, & Burgunder, 1999; Li et al., 2010). As such, the involvement of A_{2A}Rs in the adenosine-mediated effects of amitriptyline will not be specifically examined in the present study.

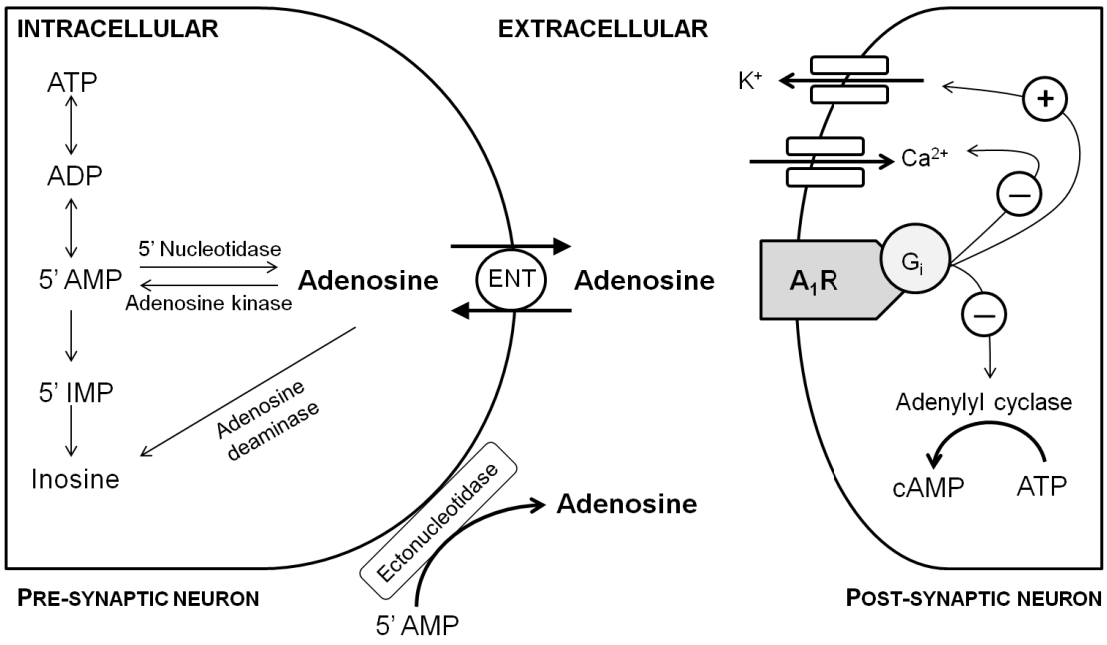

Figure 2.2.1 Extracellular adenosine levels are determined by the activity of the equilibrative nucleoside transporter (ENT), as well as metabolism of extracellular adenine nucleotides by ectonucleotidases. Binding of adenosine to post-synaptic A_1Rs can lead to various inhibitory actions on cellular excitability, including inhibition of Ca^{2+} influx, enhancement of K^+ efflux, and decreased cAMP signaling. Adapted from Wiesner et al. (1999).

2.2.2 Adenosine A_1 Receptor (A_1R) Involvement in Antinociception by Amitriptyline

As mentioned in Chapter 1 (section 1.2.2), amitriptyline is TCA that exhibits a plethora of pharmacological actions, including the modulation of adenosine levels (Micó et al., 2006). Interactions with adenosine systems may be of clinical importance in the analgesic actions of amitriptyline, as rodent studies have indicated that caffeine (a non-specific adenosine receptor antagonist) inhibits amitriptyline antinociception in inflammatory (Sawynok et al., 1999, 2008) and neuropathic pain models (Esser, Chase, Allen, & Sawynok, 2001; Esser & Sawynok, 2000; Ulugol et al., 2002). Despite being an antagonist at A_1Rs, A_{2A}Rs, and A_{2B}Rs (Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999), the ability of caffeine to reverse amitriptyline antinociception is primarily associated with the antagonism of A_1Rs (**Figure 2.2.2**, Sawynok, 2011a). However, there have been few studies which have specifically explored the precise sites where A_1Rs contribute to the action of amitriptyline.

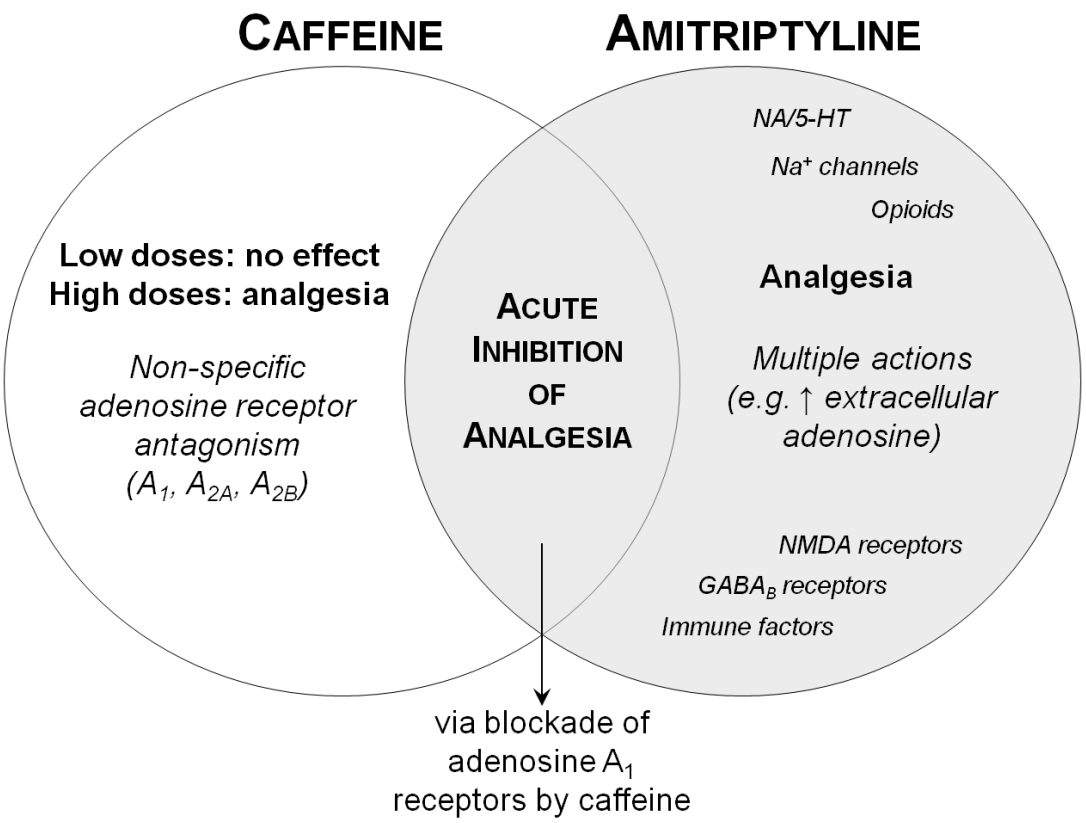

Figure 2.2.2 Acutely-administered caffeine and amitriptyline can interact to inhibit analgesia. In preclinical studies, low doses of caffeine have no intrinsic effect on pain, but high doses produce analgesia. Amitriptyline produces analgesia via a variety of pharmacological actions (reviewed in Micó et al., 2006), but is known to increase extracellular levels of adenosine, which can in turn inhibit nociception by activating adenosine A_1Rs. Blockade of A_1Rs by caffeine can, therefore, inhibit analgesia by amitriptyline.

2.2.3 The Serotonin 5-HT$_7$ Receptor (5-HT$_7$R)

The involvement of serotonergic systems is important for understanding the analgesic actions of amitriptyline, since serotonergic neurons in the brainstem are necessary for the antinociceptive actions of amitriptyline (Zhao et al., 2007) and levels of 5-HT in central synaptic clefts are elevated by TCA treatment (Micó et al., 2006). 5-HT has long been known to play a critical role in nociception, and is either anti- or pronociceptive depending on which of the various serotonin receptor subtypes are being activated (Sommer, 2006). To date, most research concerning the serotonergic control of pain, at supraspinal sites, within the spinal cord, and in the periphery, has focused on members of the 5-HT$_{1-3}$ receptor families, but attention has recently begun to shift towards the emerging role of the 5-HT$_7$ receptor (5-HT$_7$R) (Hedlund & Sutcliffe, 2004). The 5-HT$_7$R was first cloned in 1993, and was characterized as a G protein-coupled receptor that is positively coupled to adenylyl cyclase (Bard et al., 1993; Lovenberg et al., 1993; Ruat et al., 1993). Recent investigations into the functions of 5-HT$_7$Rs have been accelerated due to the increased availability of highly-specific antagonist and agonist drugs, as well as knock-out mice (Hedlund & Sutcliffe, 2004; Leopoldo, Lacivita, Berardi, Perrone, & Hedlund, 2011). In the context of pain, 5-HT$_7$Rs are localized in the superficial laminae (I and II) of the spinal dorsal horn (Doly, Fischer, Brisorgueil, Vergé, & Conrath, 2005) and have been implicated in descending inhibitory serotonergic bulbospinal pathways and in antinociception by several analgesic agents (Dogrul et al., 2012; Dogrul, Ossipov, & Porreca, 2009; Yanarates et al., 2010). However, whether or not 5-HT$_7$Rs are responsible for mediating the antinociceptive effects of amitriptyline has not yet been shown.

2.2.4 Spinal Sites of A_1R and 5-HT Action in Antinociception by Amitriptyline

The spinal cord represents a compartment in which a complex network of central pain inhibitory monoamine pathways may be recruited alongside adenosine mechanisms (Benarroch, 2008; Micó et al., 2006). A previous study showed that superfusion of 5-HT over the spinal cord led to the release of both cyclic adenosine monophosphate (cAMP) and adenosine (Sweeney, White, & Sawynok, 1990). At the time, the 5-HT_7R had not been characterized, and the identity of the 5-HT receptor that was directly (or indirectly) responsible for the release of cAMP and adenosine was not established (Sweeney et al., 1990). Since it is now known that 5-HT_7R activation increases levels of intracellular cAMP by stimulating adenylyl cyclase (Matthys, Haegeman, Van Craenenbroeck, & Vanhoenacker, 2011), and adenosine is formed following metabolism of cAMP (Sawynok & Liu, 2003a), the 5-HT_7R may be the missing link that explains the observations of Sweeney et al. (1990). Since both A_1Rs and 5-HT_7Rs are expressed in the superficial dorsal horn of the spinal cord (Doly et al., 2005; Li & Perl, 1994; Schulte, 2003), 5-HT_7Rs may be uniquely positioned to exert downstream antinociceptive effects via activation of spinal A_1Rs,.

2.2.5 Peripheral Sites of A_1R Action in Antinociception by Amitriptyline

In the periphery, local administration of amitriptyline has been demonstrated to produce antinociception (Dualé et al., 2008; Sawynok et al., 1999). This local effect is blocked by adenosine receptor antagonists including caffeine and 8-cyclopentyl-1,3-dimethylxanthine, implicating the involvement of A_1Rs (Sawynok et al., 1999). Microdialysis experiments have shown that local application of amitriptyline increases extracellular levels of adenosine, possibly through inhibition of its uptake (Sawynok, Reid, Liu, & Parkinson, 2005), and that this likely contributes to peripheral antinociception by amitriptyline (Sawynok et al., 1999). Whether peripheral A_1Rs are important for the action of systemic amitriptyline has yet to be determined.

2.2.6 Project Objectives

The purpose of the present study was to investigate central and peripheral mechanisms of A_1R-mediated antinociception by systemic amitriptyline in normal mice, as well as in mice that either expressed (+/+) or lacked (-/-) the gene for A_1Rs. In all experiments, the formalin test was used as a model of inflammatory pain, and paw flinching behaviours were considered as correlates of pain.

In the first part of this study, we explored a potential interaction between spinal 5-HT_7Rs and A_1Rs in antinociception by systemic amitriptyline. Spinal cord delivery, by acute lumbar puncture, of selective receptor antagonists was used to determine: (1) whether blockade of spinal A_1Rs, with the selective antagonist DPCPX, reverses antinociception by systemic amitriptyline, (2) whether blockade of spinal 5-HT_7Rs, with

the selective antagonist SB269970, reverses antinociception by systemic amitriptyline, and (3) whether 5-HT_7R actions, as revealed by the selective receptor agonist AS-19, are linked to adenosine systems in the spinal cord.

In view of peripheral actions of amitriptyline and adenosine, we also sought to determine: (4) whether peripheral adenosine A_1Rs contribute to antinociception by systemically administered amitriptyline. Finally, since the demonstration of both spinal and peripheral A_1R involvement in the action of amitriptyline may be of clinical relevance, the last experiments in this series determined: (5) whether chronic oral delivery of caffeine, at doses relevant to human intake levels, could prevent antinociception by systemic amitriptyline.

2.3 MATERIALS AND METHODS

2.3.1 Animals

All experiments were approved by the University Committee on Laboratory Animals at Dalhousie University (Halifax, Nova Scotia, Canada) and performed in compliance with the ethical guidelines of the Canadian Council on Animal Care. Experiments were conducted using either adult male C57BL/6 (normal) mice (Charles River Laboratories, Saint-Constant, Québec, Canada) weighing 20–30 g, or both sexes of adult A_1R +/+ (wild-type) and -/- (knock-out) colony mice, weighing 20–30 g. All mice were kept on a 12-hour light/12-hour dark cycle and housed in temperature-controlled rooms (21 ± 1 ºC) in groups of 2 to 5, with free access to food and water.

In experiments involving A_1R colony mice, attempts were made to keep groups gender-balanced. Our laboratory has not previously observed any differences in intrinsic responses to formalin 2% between sexes (n = 8 per group, unpublished data). A_1R colony mice were raised in-house on a C57BL/6 background, and were initially derived from A_1R +/- parents (supplied by Dr. Bertil Fredholm, Karolinska Institutet, Stockholm, Sweden). A_1R -/- mice possessed a disruption in a large protein-coding portion of the *ADORA1* gene (corresponding to exon 6 of the human *ADORA1* gene) (Johansson et al., 2001). Successful knock-out of A_1Rs was previously verified by Southern blot analysis, *in situ* hybridization, and quantitative autoradiography for [^{3}H]DPCPX (Johansson et al., 2001). A_1R -/- mice do not display physical abnormalities and are reproductively viable (Johansson et al., 2001). In order to verify the genotypes of A_1R colony mice, polymerase chain reaction (PCR) was performed on DNA isolated from tail-clips. Genotyping was performed by Dr. Fredholm's laboratory.

2.3.2 Formalin Test

The formalin test is a model of ongoing and inflammatory pain, and involves sensory afferent pathways, ascending transmission, and descending modulatory pathways, as well as peripheral and central sensitization (Dubuisson & Dennis, 1977; reviewed in Sawynok & Liu, 2003b). Each mouse was habituated in a clear plexiglass chamber for approximately 10 minutes before receiving a subcutaneous intraplantar (i.pl.) injection of 2% formalin (20 μL) into the left (ipsilateral) hindpaw. The number of flinches (elevation of the hindpaw, as well as rapid shaking) was counted over 60 minutes, and 2 mice in separate plexiglass chambers were observed in alternating 2 minute bins. Mirrors placed behind the chambers permitted unhindered observation of spontaneous pain behaviours. Since responses to formalin are biphasic, pain behaviours were analyzed separately as the cumulative number of flinches during phase 1 (0–8 minutes) and phase 2 (12–60 minutes). Phase 1 behaviours are associated with the activation of primary afferents, whereas phase 2 behaviours are thought to be reflective of central sensitization processes which occur during the induction of persistent pain (Sawynok & Liu, 2003b). All bar graphs report the cumulative number of phase 2 flinches only. Because 2 mice were observed at a time, numbers represent approximately half of the total number of flinches, and these values were not corrected towards an assumed total. Each mouse was used only once, and was euthanized at the conclusion of the test.

2.3.3 Drugs

Amitriptyline, caffeine, formalin, dimethyl sulfoxide (DMSO), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were purchased from Sigma-Aldrich Ltd. (Oakville, Ontario, Canada). SB269970 hydrochloride ((2R)-1-[(3-hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine hydrochloride) and AS-19 ((2S)-(+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin) were purchased from Tocris Bioscience (Minneapolis, Minnesota, USA). Formalin was diluted to a final concentration of 2% in 0.9% saline. Caffeine was dissolved in the drinking water and replenished with a fresh supply every 2 days. All other drugs were prepared freshly on the day of testing. Amitriptyline was dissolved in 0.9% saline. Drugs administered by intraplantar or intrathecal injection (DPCPX, SB269970, and AS-19) were dissolved at a final concentration of 20% DMSO in saline.

2.3.4 Drug Dosing and Administration

Drugs given by intrathecal (i.t.) (5 μL) injection were administered 20 minutes before formalin (**Figure 2.3.1**), with the exception of AS-19 and its combinations with SB269970 or DPCPX, which were given 15 minutes before formalin (**Figure 2.3.2**). Co-administered drugs were delivered without changing the injection volume. The small injection volume (5 μL) was used to prevent excessive spreading of the drug to non-lumbar regions, as well as to avoid significantly changing the volume of circulating cerebrospinal fluid. Intrathecal delivery of drugs was performed under isoflurane anesthesia by lumbar puncture, which involved the insertion of a 30-gauge needle

between the L5 and L6 vertebrae (Hylden & Wilcox, 1980). Successful entry of the needle into the intrathecal space was verified by a characteristic tail flick (Hylden & Wilcox, 1980). In order to confirm my skill at performing lumbar punctures, correct placement of the needle was initially verified by injecting aniline blue (dissolved in water) instead of drug. Animals that received dye injections were not used in any experiments.

Previous studies guided our determination of spinal and peripheral doses of DPCPX (Sawynok & Reid, 2012) and SB269970 (Dogrul et al., 2012). The spinal dose of AS-19 was inferred from a systemic dose of 10 mg/kg (Brenchat et al., 2009). In the peripheral adenosine experiments, mice were loosely restrained before receiving an injection of DPCPX 10 nmol by subcutaneous i.pl. (10 μL) injection into the ipsilateral (same side as formalin) or contralateral hindpaw. DPCPX was administered 20 minutes before formalin (**Figure 2.3.3**).

For chronic caffeine experiments, mice received caffeine in their drinking water for 8-9 days at concentrations of 0.1, or 0.3 g/L (0.01% or 0.03%) prior to formalin testing (**Figure 2.3.4**). These doses of caffeine in the drinking water are considered to be relevant to human intake levels (Yang et al., 2009). Water consumption was monitored, and on average, all mice consumed a fluid volume of 4.5– 5 mL/day regardless of drug condition. Similar water consumption volumes indicated that taste aversion to caffeine had not developed or that levels of caffeine were low enough such that there was no bitter taste. Assuming that each mouse consumed the same amount of fluid, and that no water spillage had occurred, caffeine concentrations of 0.1 and 0.3 g/L correspond to doses of ~20 and ~55 mg/kg/day.

On each test day, amitriptyline (or saline vehicle) was given 15 minutes before formalin by intraperitoneal (i.p.) injection in a volume of 5 mL/kg. Dosages were selected on the basis of analgesic effects in response to i.pl. formalin 2%. Amitriptyline was administered at a dose of 3 mg/kg in normal mice, and 12 mg/kg in the A_1R colony mice. Doses higher than 3 mg/kg had a sedative effect in normal mice. The differential amitriptyline dosing was attributed to different sensitivities of the two mouse strains to amitriptyline.

2.3.5 Statistical Analyses

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using the Student's t-test, analysis of variance (ANOVA), and the Student Neuman-Keuls (SNK) post-hoc test. Statistical results were taken to be significant at $P < 0.05$.

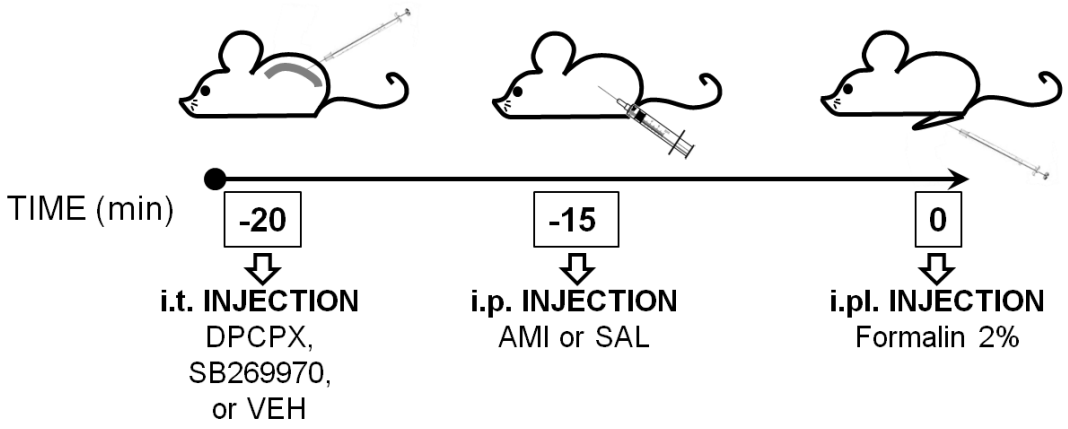

Figure 2.3.1 Injection schedule for intrathecal (i.t.) drug experiments on the day of formalin testing. At 20 minutes before the start of the formalin test, mice received an i.t. injection of DPCPX, SB269970, or 20% DMSO (vehicle or VEH). An intraperitoneal (i.p.) injection of amitriptyline (AMI) or saline (SAL) was given 5 minutes later. Finally, an intraplantar (i.pl.) injection of formalin 2% was delivered to the left hindpaw.

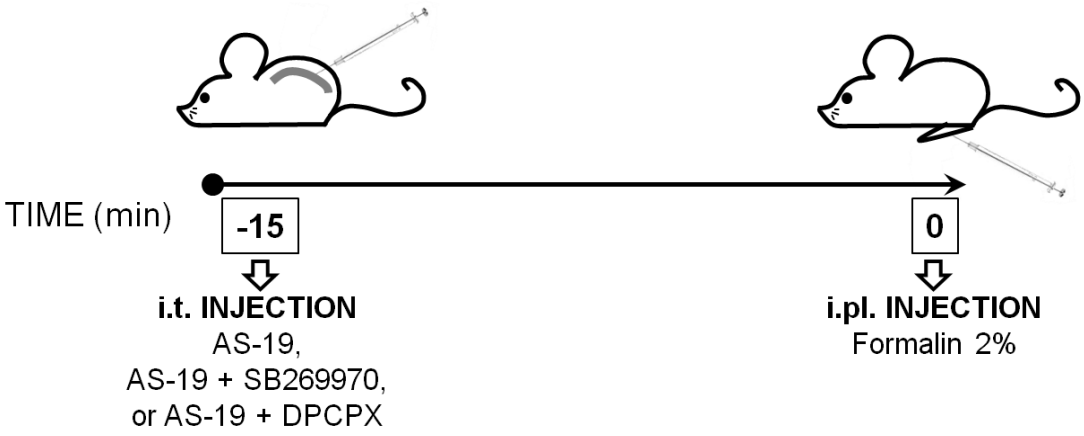

Figure 2.3.2 Injection schedule for AS-19 i.t. drug experiments on the day of formalin testing. Mice received an i.t. injection of AS-19, AS-19 + SB269970, or AS-19 + DPCPX 15 minutes before i.pl. formalin 2% was delivered into the left hindpaw.

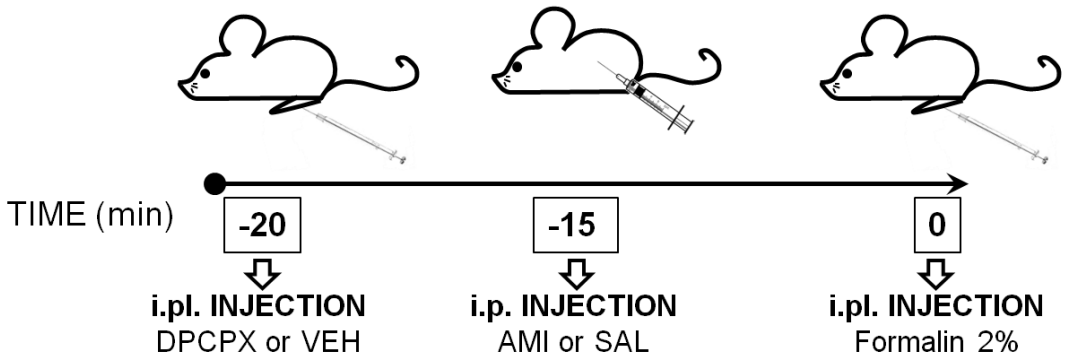

Figure 2.3.3 Injection schedule for peripheral DPCPX experiments on the day of formalin testing. At 20 minutes before the start of the formalin test, mice received an i.pl. injection of DPCPX or 20% DMSO (VEH) into either the ipsilateral (left) or contralateral (right) hindpaw. An i.p. injection of amitriptyline (AMI) or saline (SAL) was given 5 minutes later. Finally, an i.pl. injection of formalin 2% was delivered to the left hindpaw.

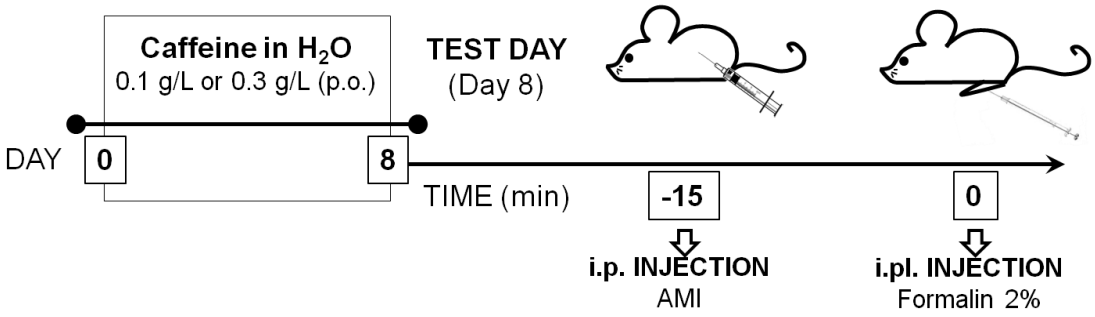

Figure 2.3.4 Experimental timeline and injection schedule for chronic caffeine experiments. Mice were maintained on oral (p.o.) caffeine (dissolved in the drinking water at doses of 0.1 g/L and 0.3 g/L) for 8 days. On the test day, mice received an i.p. injection of amitriptyline (AMI). Fifteen minutes later, an i.pl. injection of formalin 2% was delivered to the left hindpaw.

2.4 RESULTS

2.4.1 Effect of Intrathecal DPCPX on Antinociception by Systemic Amitriptyline

In order to determine whether spinal A_1Rs contribute to antinociception by systemic amitriptyline, DPCPX was given spinally by acute lumbar puncture. In normal mice, systemic amitriptyline 3 mg/kg produced antinociception against phase 2 formalin behaviours, and i.t. pre-treatment with DPCPX inhibited the action of systemic amitriptyline (**Figure 2.4.1**). In a previous study, amitriptyline 12 mg/kg was shown to produce a comparable extent of antinociception in A_1R +/+ and -/- mice (Sawynok et al., 2008). In A_1R +/+ mice, spinal administration of DPCPX followed by systemically administered amitriptyline 12 mg/kg led to increased phase 2 flinching behaviour compared to A_1R -/- mice (**Figure 2.4.2**). It was previously demonstrated that flinching responses to formalin 2% alone do not differ between A_1R +/+, +/-, or -/- mice (Sawynok & Reid, 2012). Spinal administration of DPCPX 10 nmol does not produce intrinsic effects on phase 2 flinching in normal mice (Sawynok & Reid, 2012) or in adenosine A_1R +/+ and -/- mice (**Figure 2.4.2B**, left columns).

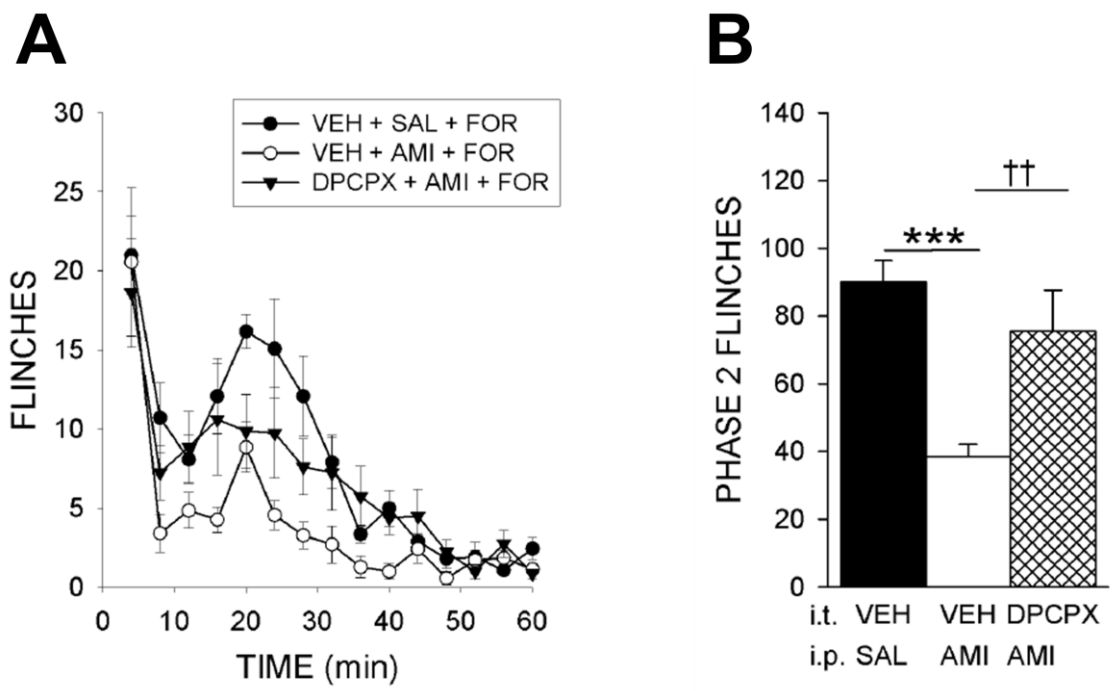

Figure 2.4.1 Antinociception by systemic amitriptyline (AMI) is reduced by blockade of spinal A_1Rs in normal mice. **(A)** Time course of flinching behaviours evoked by formalin 2% (FOR) over 60 minutes following i.t. administration of DPCPX 10 nmol or 20% DMSO (VEH) and i.p. AMI 3 mg/kg or saline (SAL). **(B)** Cumulative phase 2 flinching behaviours showing that DPCPX prevents antinociception by systemic AMI.
(*** $P < 0.001$ vs VEH/SAL, †† $P < 0.01$ vs VEH/AMI, ANOVA and SNK post-hoc test; $n = 11$ in VEH/SAL and DPCPX/AMI groups, $n = 7$ in VEH/AMI group)

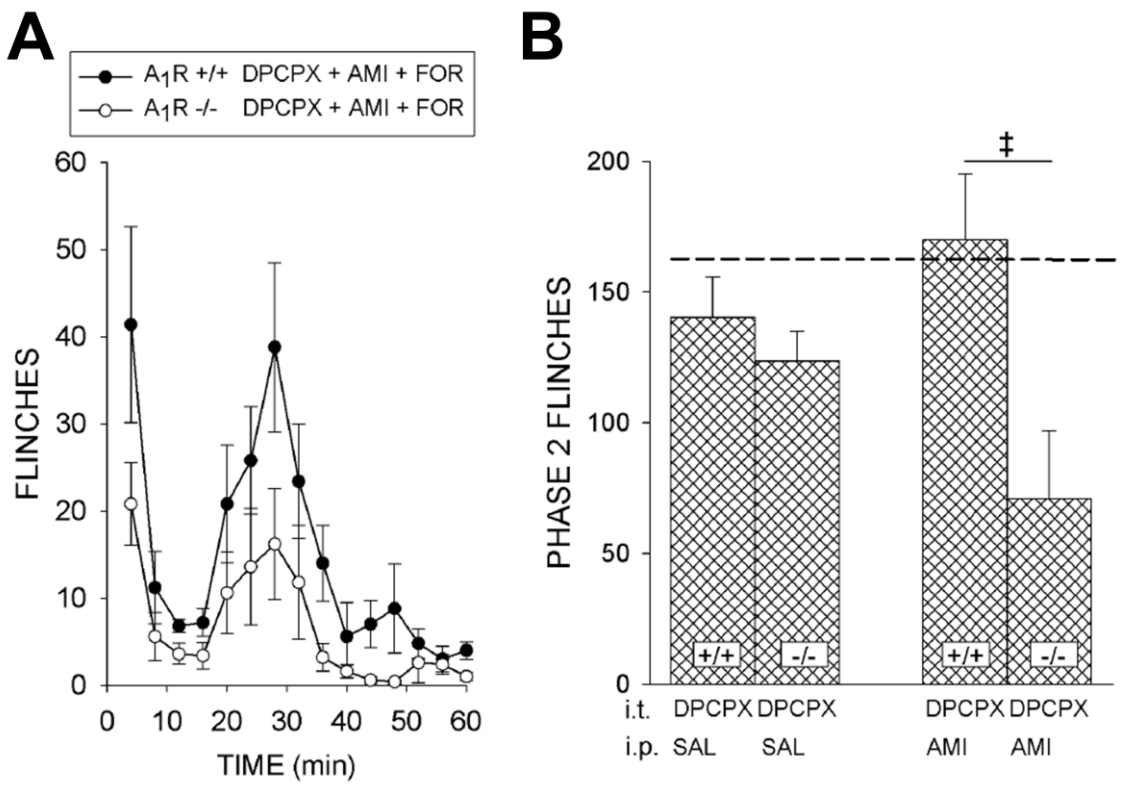

Figure 2.4.2 Antinociception by systemic amitriptyline (AMI) is reduced by blockade of spinal A_1Rs in A_1R wild-type (+/+), but not knock-out (-/-) mice. **(A)** Time course of flinching behaviours evoked by formalin 2% (FOR) over 60 minutes following i.t. administration of DPCPX 10 nmol and i.p. AMI 12 mg/kg in A_1R +/+ and -/- mice.
(B) Cumulative phase 2 flinching behaviours showing that i.t. DPCPX does not have an intrinsic effect on FOR behaviours in A_1R +/+ and -/- mice. Additionally, in A_1R +/+ mice, i.t. DPCPX prevents antinociception by systemic AMI, an effect not observed in A_1R -/- mice. The dotted line indicates the average phase 2 flinches to FOR alone in the two A_1R colony mice genotypes, which did not differ (Sawynok & Reid, 2012).
(‡ $P < 0.05$ vs DPCPX/AMI in A_1R +/+ mice, n = 8-9 in DPCPX/SAL groups; 2-way unpaired Student's t-tests for each treatment condition, n = 5-6 in DPCPX/AMI groups)

2.4.2 Effect of Intrathecal SB269970 on Antinociception by Systemic Amitriptyline

In order to determine the contribution of spinal 5-HT_7Rs to the action of systemic amitriptyline, SB269970, a selective 5-HT_7R antagonist, was administered by intrathecal injection 20 minutes before formalin. In normal mice, a 10 μg dose of SB269970 produced an intrinsic effect on formalin responses, significantly increasing the mean number of phase 2 flinches (**Figure 2.4.3**). A dose of 3 μg SB269970 had no intrinsic effect on formalin responses (**Figure 2.4.3**) but inhibited the antinociceptive action of systemic amitriptyline 3 mg/kg (**Figure 2.4.4**). In A_1R +/+ and -/- mice, SB269970 3 μg had no intrinsic effect on formalin responses (**Figure 2.4.5B**, left columns). SB269970 3 μg inhibited antinociception by systemic amitriptyline 12 mg/kg in A_1R +/+ mice, but did not reverse amitriptyline antinociception in A_1R -/- mice (**Figure 2.4.5B**, right columns). This result indicates that the presence of A_1Rs is required for the reversal of systemic amitriptyline antinociception by the i.t. 5-HT_7R antagonist SB269970.

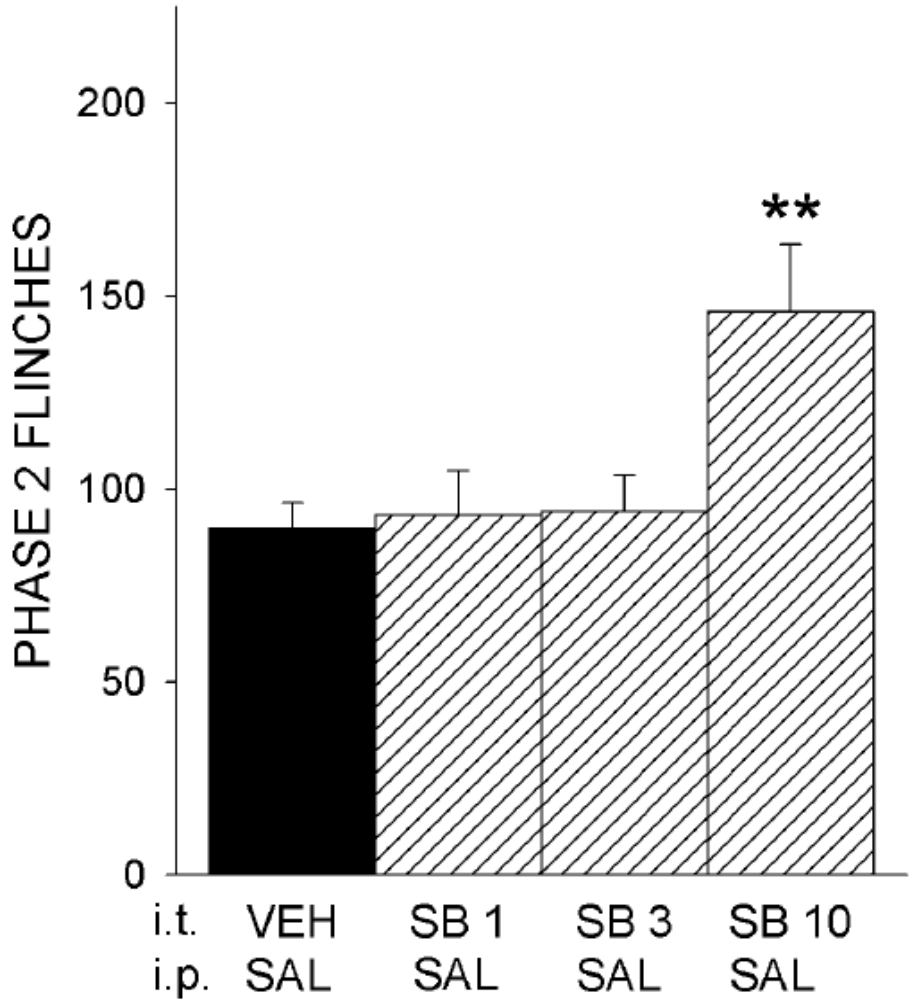

Figure 2.4.3 In normal mice, cumulative phase 2 responses to formalin 2% are increased by a 10 μg dose of SB269970 (SB), but not by doses of 1 or 3 μg compared to 20% DMSO (VEH). (** $P < 0.01$ vs VEH/SAL; ANOVA and SNK post-hoc test; $n = 11$ in VEH/SAL group, $n = 6$-7 for all SB/SAL groups)

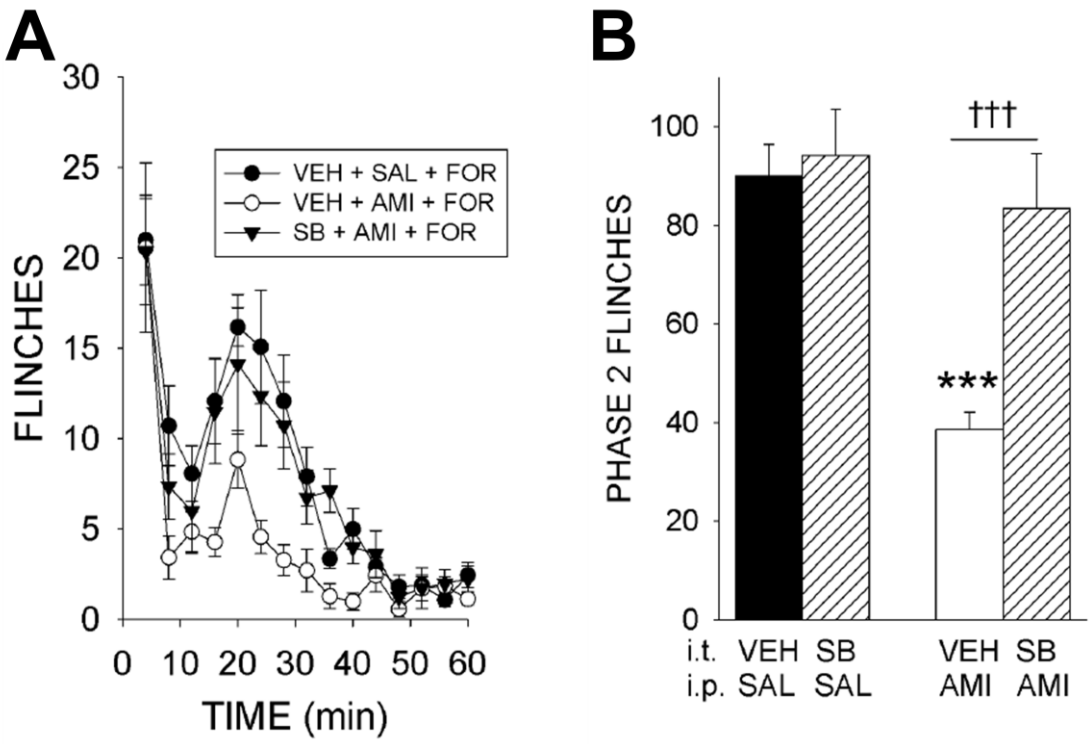

Figure 2.4.4 Antinociception by systemic amitriptyline (AMI) is reduced by blockade of spinal 5-HT_7 receptors in normal mice. **(A)** Time course of flinching behaviours evoked by formalin 2% (FOR) over 60 minutes following i.t. administration of SB269970 (SB) 3 µg or 20% DMSO (VEH) and i.p. AMI 3 mg/kg or saline (SAL). **(B)** Cumulative phase 2 flinching behaviours showing that i.t. SB 3 µg has no intrinsic effect, but that this dose of SB reduces antinociception by systemic AMI. (*** $P < 0.001$ vs VEH/SAL, ††† $P < 0.001$ vs VEH/AMI; ANOVA and SNK post-hoc test; $n = 11$ in VEH/SAL group, $n = 6$-8 in all other groups)

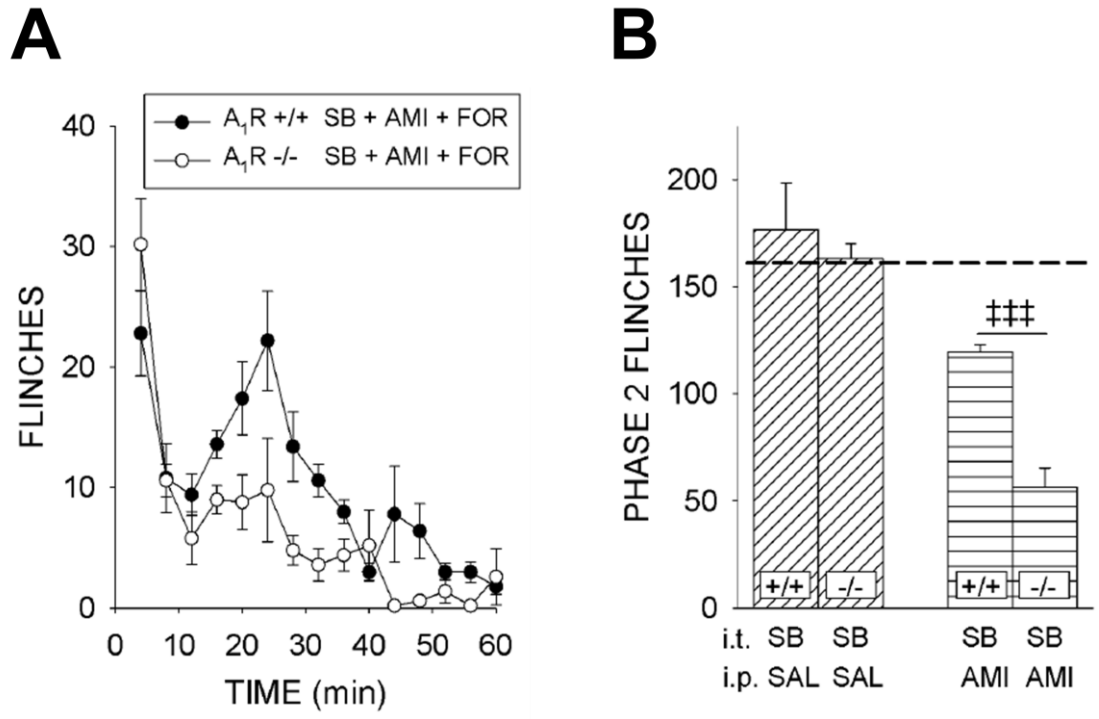

Figure 2.4.5 Antinociception by systemic amitriptyline (AMI) is reduced by blockade of spinal 5-HT_7 receptors in A_1R wild-type (+/+), but not knock-out (-/-) mice.
(A) Time course of flinching behaviours evoked by formalin 2% (FOR) over 60 minutes following i.t. administration of SB269970 3 µg (SB) and i.p. AMI 12 mg/kg in adenosine A_1R +/+ and -/- mice. **(B)** Cumulative phase 2 flinching behaviours showing that in A_1R +/+ and -/- mice, i.t. pre-treatment with SB has no intrinsic effect. However, i.t. SB reduces antinociception by systemic AMI in A_1R +/+, but not -/- mice. The dotted line indicates the average phase 2 flinches to FOR alone in the two A_1R colony mice genotypes (see **Figure 2.4.2**). (‡‡‡ $P < 0.001$ vs SB/AMI in A_1R +/+ mice; 2-way unpaired Student's t-tests for each treatment condition; n = 5-6 per group)

2.4.3 Interactions Between Spinal 5-HT_7R and A_1R Actions in the Spinal Cord

The above results indicated an involvement of spinal A_1R and 5-HT_7Rs in antinociception by systemic amitriptyline. We now sought to determine whether actions at these receptors were linked. In normal mice, spinal administration of a 5-HT_7R agonist, AS-19 20 μg, did not significantly alter the cumulative number of phase 2 formalin-evoked flinches (**Figure 2.4.6B**). However, when AS-19 was intrathecally co-administered with SB269970 3 μg 15 minutes before formalin, there was a significant increase in phase 2 flinching compared to those elicited by formalin/AS-19 (**Figure 2.4.6**), or formalin/SB269970 ($P < 0.05$).

A significant increase in phase 2 formalin-evoked flinches was also observed after intrathecal co-administration of AS-19 20 μg with DPCPX 10 nmol 15 minutes before formalin; this exceeded the number of phase 2 flinches observed after administration of formalin/AS-19 alone (**Figure 2.4.7**). Delivery of i.t. AS-19 20 μg 15 minutes before formalin produced a greater number of phase 2 flinches in A_1R -/- mice compared to A_1R +/+ mice (**Figure 2.4.8**). Collectively, these findings suggest the existence of a direct link between spinal 5-HT_7Rs and A_1Rs within the spinal cord.

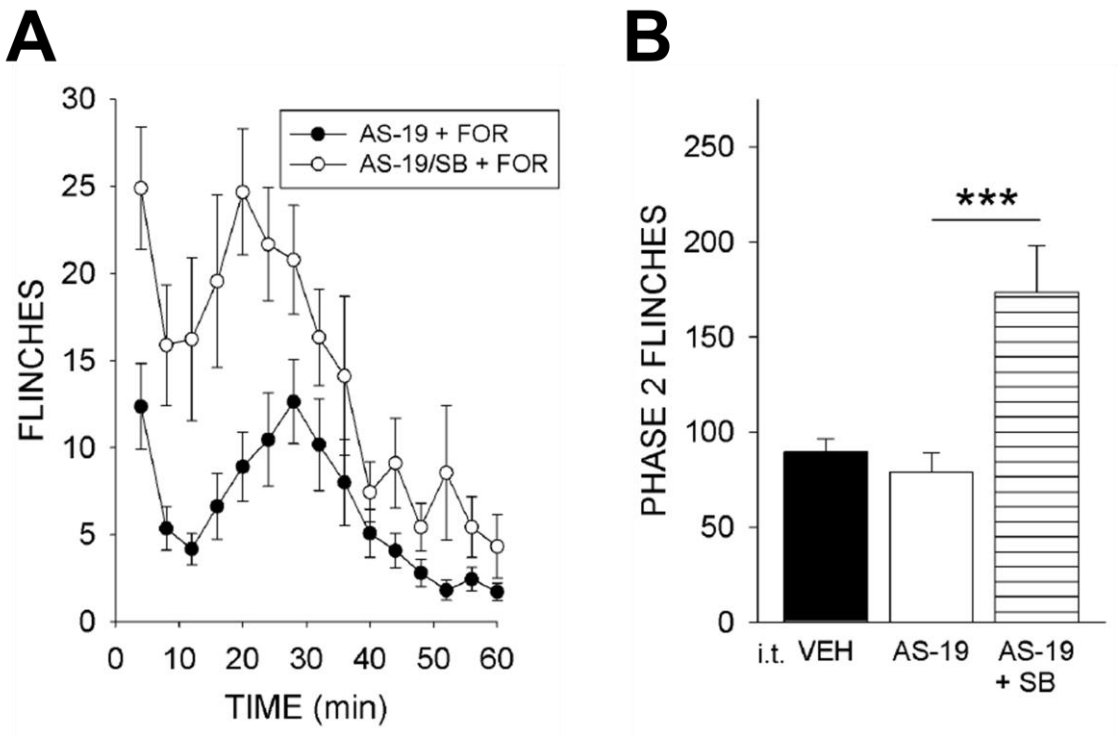

Figure 2.4.6 Co-administration of AS-19 with SB269970 increases formalin-evoked flinching in normal mice. **(A)** Time course of flinching behaviours evoked by formalin 2% (FOR) over 60 minutes following i.t. administration of AS-19 20 µg + SB269970 3 µg (SB) or 20% DMSO (VEH). **(B)** Cumulative phase 2 flinching behaviours showing that i.t. AS-19 has no intrinsic effect alone, but that i.t. co-administration of AS-19 with SB is pronociceptive. (*** $P < 0.001$; ANOVA and SNK post-hoc test; $n = 9$ in AS-19/SB group, $n = 11$ for all other groups)

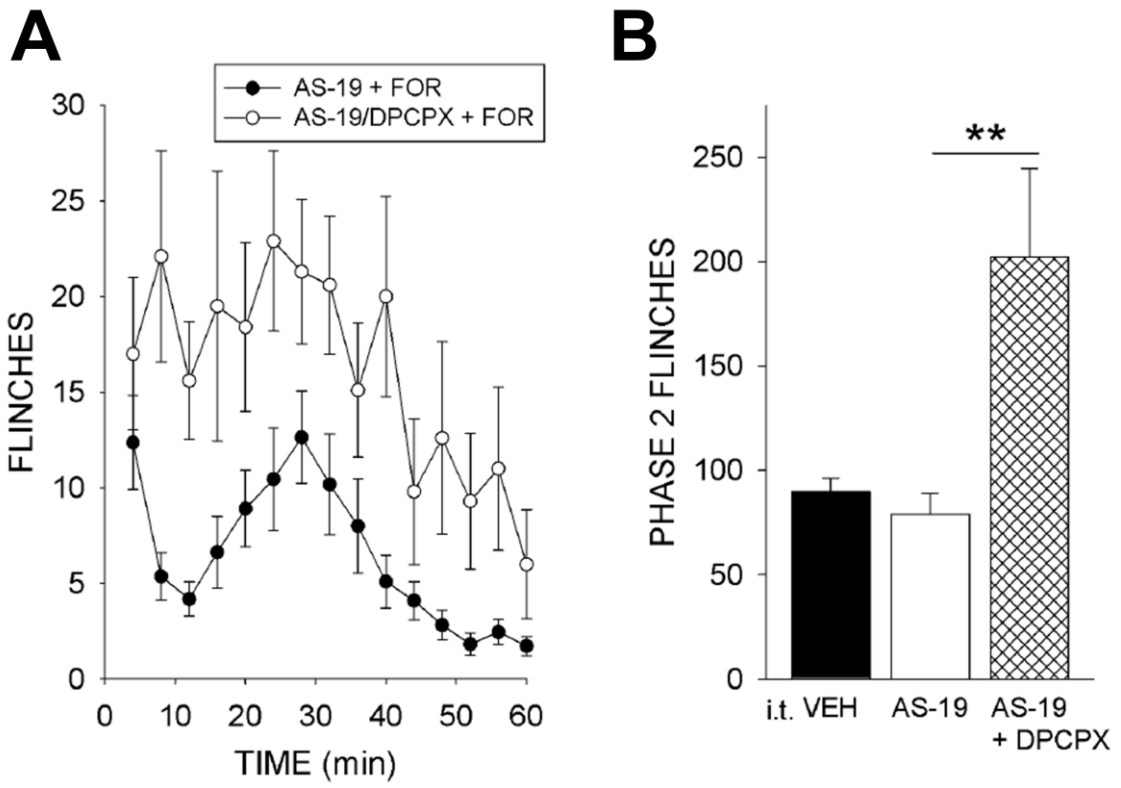

Figure 2.4.7 Co-administration of AS-19 with DPCPX increases formalin-evoked flinching in normal mice. **(A)** Time course of flinching behaviours evoked by formalin 2% (FOR) over 60 minutes following i.t. administration of AS-19 20 μg + DPCPX 10 nmol or 20% DMSO (VEH). **(B)** Cumulative phase 2 flinching behaviours showing that i.t. AS-19 has no intrinsic effect alone, but that i.t. co-administration of AS-19 with DPCPX is pronociceptive. (** $P < 0.01$ vs AS-19; ANOVA and SNK post-hoc test; $n = 10$ for AS-19/DPCPX group, $n = 11$ for all other groups)

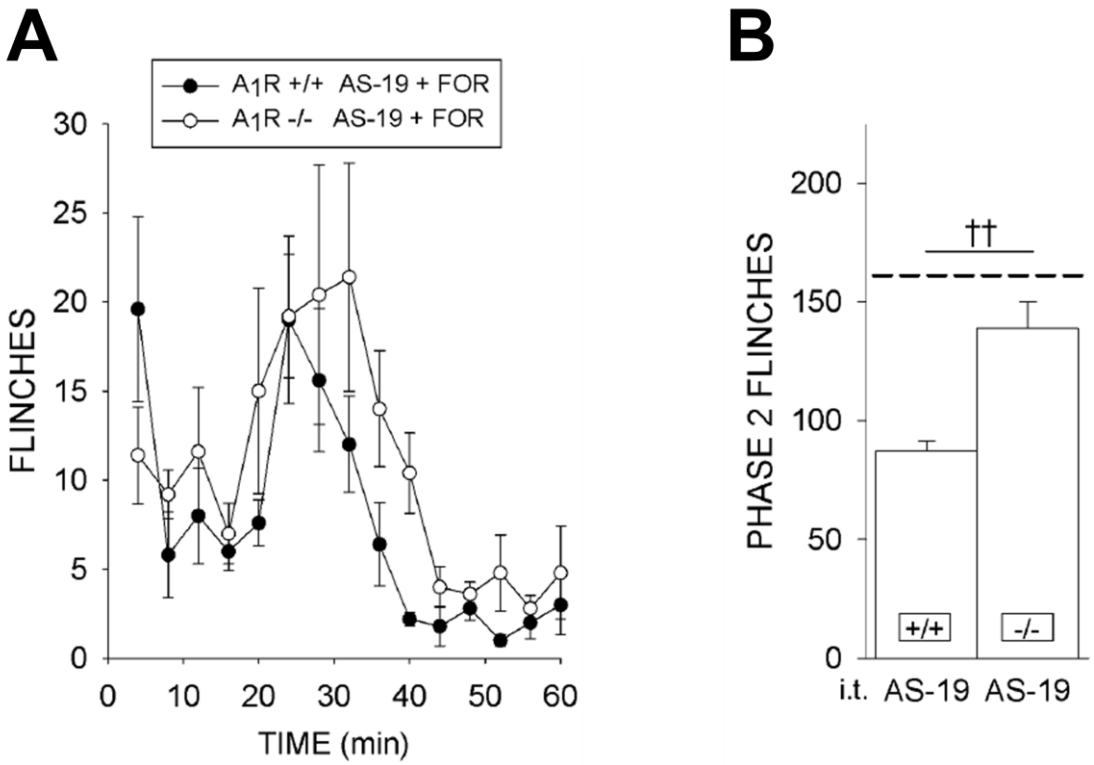

Figure 2.4.8 Interactions between spinal 5-HT_7R and A_1R signaling pathways are involved in modulating nociception**. (A)** Time course of flinching behaviours evoked by formalin 2% (FOR) over 60 minutes following i.t. administration of AS-19 20 µg in A_1R +/+ and -/- mice. **(B)** I.t. AS-19 in A_1R -/- mice produced a greater number of cumulative phase 2 flinches compared to A_1R +/+ mice. The dotted line indicates the average phase 2 flinches to FOR alone in the two A_1R colony mice genotypes (see **Figure 2.4.2**).
(†† P<0.001 vs AS-19 in A_1R +/+ mice; 2-tailed unpaired Student's t-test; n = 5 per group)

2.4.4 Pronociceptive Behaviours Produced by Spinal Administration of AS-19

Our observations that spinal SB269970 could prevent antinociception by systemic amitriptyline in normal mice (**Figure 2.4.4**) led us to ascertain whether activation of 5-HT_7Rs alone (using the highly-selective agonist AS-19) could produce antinociception in the formalin test. Surprisingly, we found that a 20 μg dose of AS-19 did not exhibit a significant antinociceptive effect on formalin-evoked flinching in normal mice (**Figure 2.4.6**). Spinal administration of AS-19 20 μg, both alone and in combination with SB269970 or DPCPX, consistently led to a characteristic set of caudally-directed pronociceptive-like behaviours (**Figure 2.4.9**). AS-19 also produced pronociceptive-like behaviours when given at a dose of 10 μg, but there was no effect on formalin-evoked flinching (data not shown).

Pronociceptive-like behaviours were observed in normal and both groups of A_1R colony mice. Neither i.pl. nor intramuscular (i.m.) injections of AS-19 at this dose elicited any noticeable reactions, suggesting that the behaviours were specifically produced by spinal actions of the drug. An effort was made to minimize the stress of the animals by prolonging isoflurane anesthesia by 3 minutes following lumbar puncture. As mice regained consciousness after anesthesia, they immediately began to display the pronociceptive-like behaviours, which lasted between 10-13 minutes. These behaviours included hindpaw scratching and biting of the lumbar back region and abdomen, rigid extension and flailing of the tail, as well as rapid tapping of the hindpaws. In some cases, mice vocalized and displayed loss of the righting reflex. Since these behaviours would have interfered with responses to formalin, the test was started 15 minutes after i.t. injection of AS-19, when all pronociceptive-like behaviours had ceased.

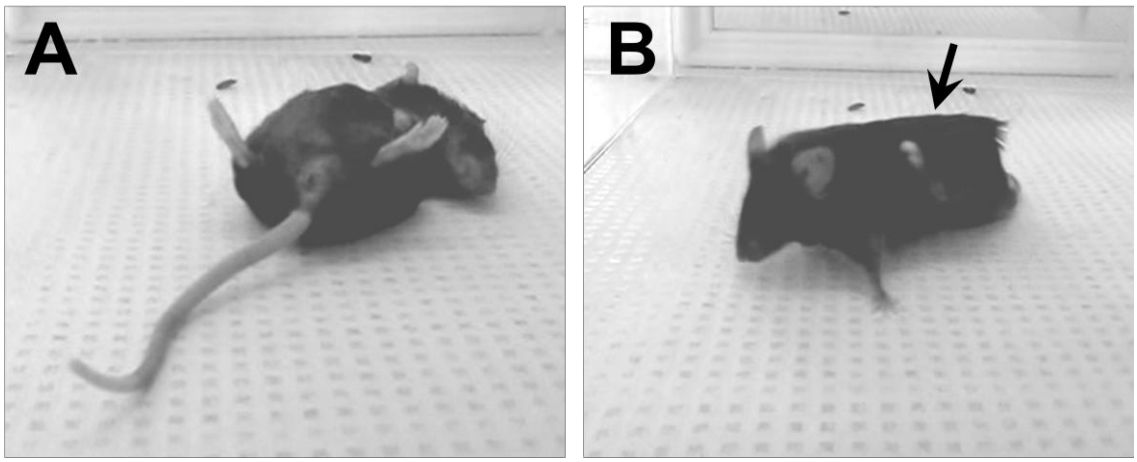

Figure 2.4.9 Spinal administration of AS-19 produces spontaneous pronociceptive-like, caudally-directed biting and licking behaviours, in normal and A_1R colony mice. Behaviours last between 10-15 minutes. **(A)** Soon after awakening from the anesthestic (administered during the lumbar puncture and AS-19 delivery), mice often exhibited "rolling" and loss of the righting reflex. These behaviours were usually accompanied by flailing of the tail in a circular pattern. **(B)** Rapid, caudally-directed scratching of the lumbar back region with the hindpaw was often observed once the righting reflex was regained. The arrow indicates the region at which the scratching was directed.

2.4.5 Effect of Intraplantar DPCPX on Antinociception by Systemic Amitriptyline

Peripheral actions of locally-administered amitriptyline have previously been demonstrated to involve A_1Rs (Sawynok et al., 1999). In the present study, we wished to establish whether peripheral A_1Rs were also involved in mediating antinociception by systemic amitriptyline. In normal mice, i.pl. pre-treatment with DPCPX 10 nmol to the left (ipsilateral) hindpaw led to a significant reversal of phase 2 antinociception by systemic amitriptyline 3 mg/kg (**Figure 2.4.10**). Antinociception by systemic amitriptyline was preserved when DPCPX was delivered to the right (contralateral) hindpaw (**Figure 2.4.10B**), indicating that the effect of DPCPX on A_1Rs was locally mediated. Vehicle administration by the i.pl. and i.p. routes did not alter responses of normal mice to formalin alone (**Figure 2.4.10B**).

In A_1R +/+ mice, i.pl. pre-treatment with DPCPX 10 nmol to the left hindpaw reversed antinociception by systemic amitriptyline 12 mg/kg (**Figure 2.4.11**). However, in A_1R -/- mice, i.pl. DPCPX did not reverse antinociception by systemic amitriptyline (**Figure 2.4.11**).

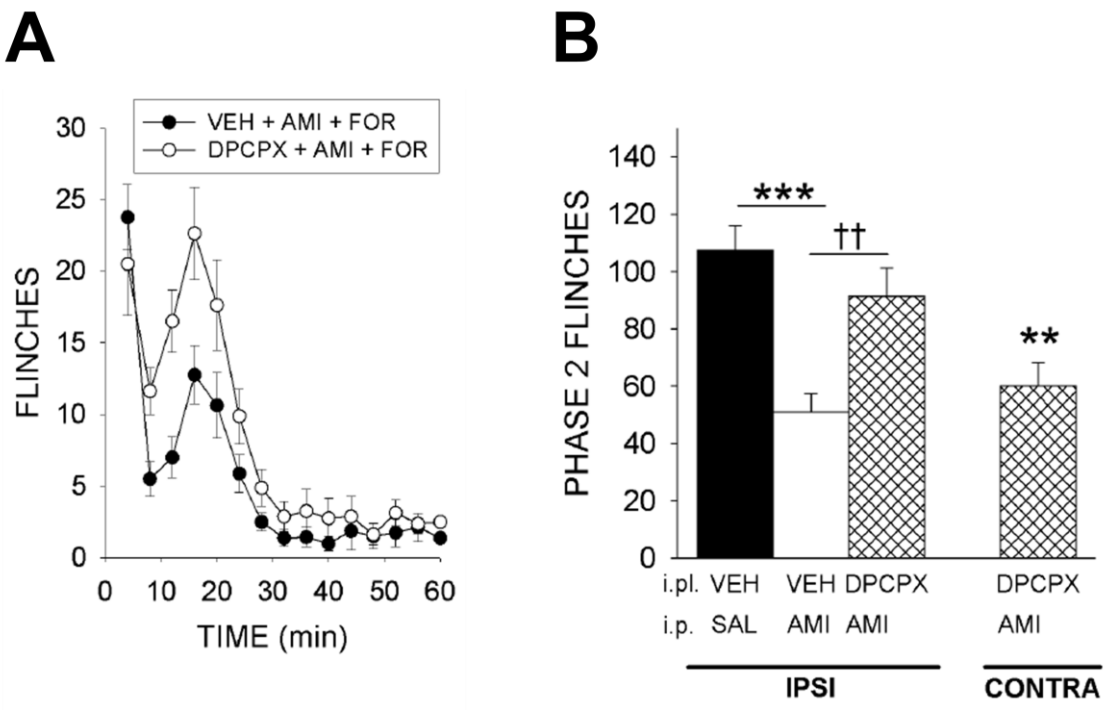

Figure 2.4.10 Antinociception by systemic amitriptyline (AMI) is reduced by blockade of peripheral A_1Rs in normal mice. **(A)** Time course of flinching behaviours evoked by formalin 2% (FOR) over 60 minutes following i.pl. (ipsilateral) administration of DPCPX 10 nmol or 20% DMSO (VEH) and i.p. AMI 3 mg/kg. **(B)** Cumulative phase 2 flinching behaviours showing that i.pl. pre-treatment in the ipsilateral (IPSI) hindpaw with DPCPX prevents antinociception by systemic AMI. I.pl. administration of DPCPX into the contralateral (CONTRA) hindpaw does not reverse antinociception by systemic AMI, indicating that the effects of DPCPX are locally mediated. (** $P < 0.01$, *** $P < 0.001$ vs IPSI VEH/SAL, †† $P<0.01$ vs IPSI VEH/AMI; ANOVA and SNK post-hoc test; n = 7-8 per group)

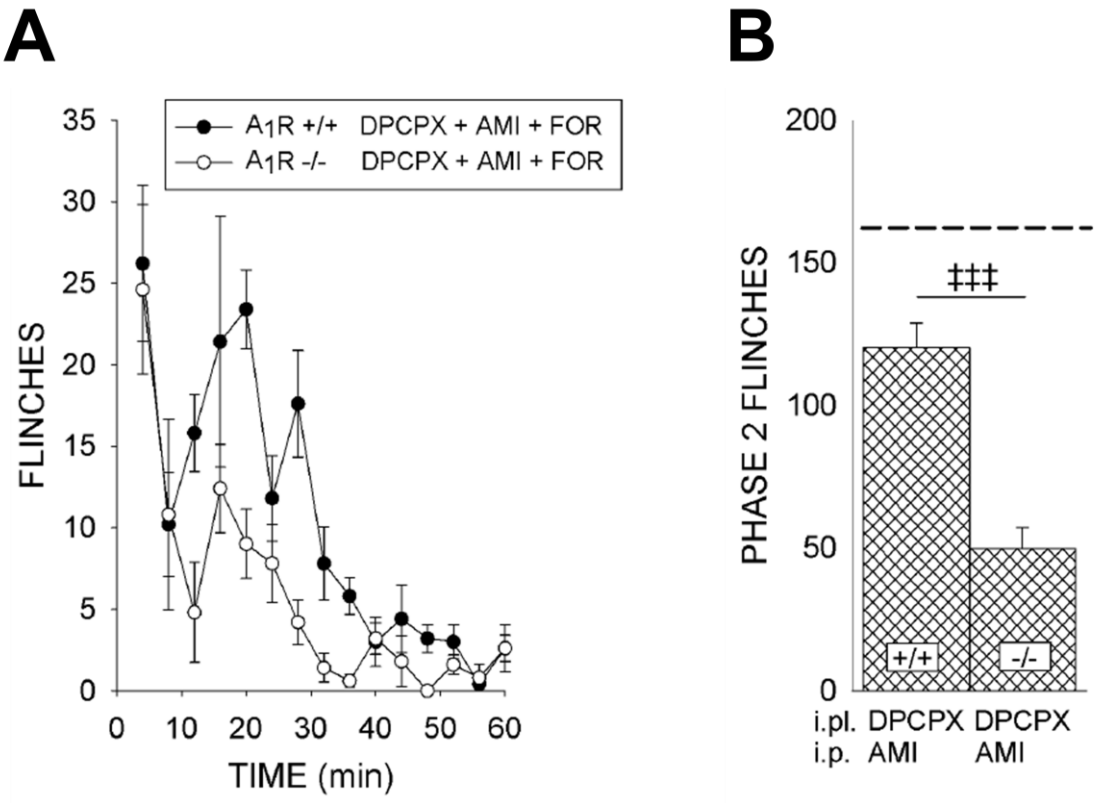

Figure 2.4.11 Antinociception by systemic amitriptyline (AMI) is reduced by blockade of peripheral A_1Rs in A_1R wild-type (+/+), but not knock-out (-/-) mice. **(A)** Time course of flinching behaviours evoked by formalin 2% (FOR) over 60 minutes following i.pl. administration of DPCPX 10 nmol in A_1R +/+ and -/- mice. **(B)** Cumulative phase 2 flinching behaviours showing that in A_1R +/+ mice, i.pl. pre-treatment with DPCPX prevents antinociception by systemic AMI, but that this effect is not observed in A_1R -/- mice. The dotted line indicates the average phase 2 flinches to FOR alone in the two A_1R colony mice genotypes (see **Figure 2.4.2**). (‡‡‡ $P < 0.001$ vs IPSI DPCPX/AMI in A_1R +/+ mice, 2-tailed unpaired Student's t-test; $n = 5$ per group)

2.4.6 Effect of Chronic Oral Caffeine on Antinociception by Systemic Amitriptyline

In the final experiment of this series, we determined whether oral administration of caffeine, at doses which are regarded as relevant to human intake levels, could influence antinociception by acute systemic amitriptyline. Chronic caffeine administration at two dose levels, 0.1 g/L (0.01%) or 0.3 g/L (0.03%), does not produce intrinsic effects on flinching responses to formalin (**Figure 2.4.12A**; Sawynok and Reid, 2012). In normal mice, i.p. amitriptyline 3 mg/kg significantly reduced the number of phase 2 flinches compared to vehicle-treated controls (**Figure 2.4.12B**). However, antinociception by acutely administered amitriptyline was essentially abolished in mice that had received caffeine orally in the drinking water for 8 days prior to formalin testing (**Figure 2.4.12B**).

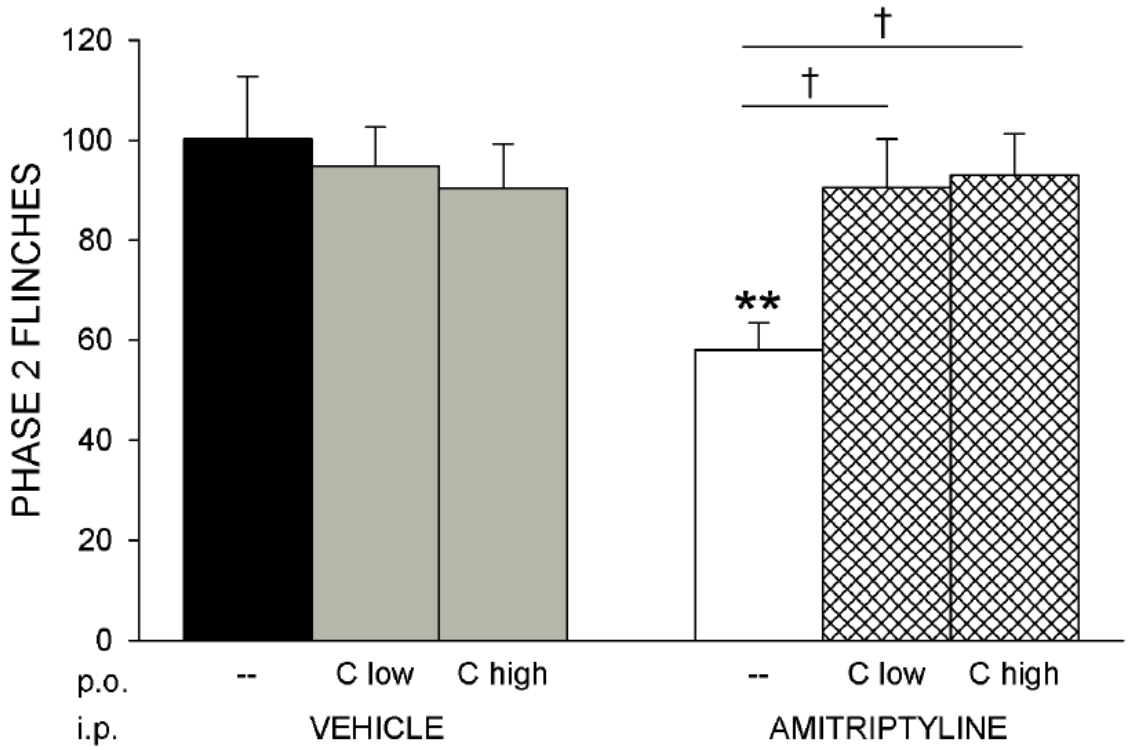

Figure 2.4.12 Chronic caffeine administration in the drinking water at doses of 0.1 g/L (C low) or 0.3 g/L (C high) for 8-9 days prior to testing reduces antinociception by systemic amitriptyline 3 mg/kg in the formalin test. **(A)** Data from a previously published study by our laboratory (Sawynok & Reid, 2012) indicated that chronic caffeine did not affect intrinsic phase 2 flinching responses to formalin 2% with i.p. vehicle (20% DMSO) administration. **(B)** Chronic caffeine administration at two dose levels reversed antinociception by systemic amitriptyline 3 mg/kg (dissolved in saline) given 15 minutes before formalin 2%. (** $P < 0.01$ vs vehicle/no caffeine, † $P < 0.05$ vs amitriptyline/no caffeine, ANOVA and SNK post-hoc test; n = 6-8 per group)

2.5 DISCUSSION

2.5.1 Spinal A_1R Actions in Antinociception by Systemic Amitriptyline

Modulation of the availability and/or activity of adenosine contributes to the antinociceptive effects of amitriptyline and related antidepressants in preclinical studies (Micó et al., 2006). In previous studies, the mechanisms and sites of action involved in A_1R-mediated analgesia by amitriptyline had not been extensively examined. In the present study, we used the site-specific delivery of receptor antagonists to reveal specific compartments in which adenosine systems are modulated by systemically-administered amitriptyline. The spinal compartment was targeted using a lumbar puncture method, allowing for i.t. administration of the highly selective A_1R antagonist DPCPX at a dose of 10 nmol, which blocks antinociception by N^6-cyclopentyladenosine, a highly selective A_1R agonist (Sawynok & Reid, 2012). In the present study, spinal pre-treatment with DPCPX significantly increased formalin-evoked flinching in the presence of systemic amitriptyline (**Figure 2.4.1**), suggesting that activation of spinal A_1Rs was necessary for the antinociceptive effect of systemic amitriptyline. Spinal DPCPX was able to block antinociception by systemic amitriptyline in A_1R +/+ mice, but not in -/- mice (**Figure 2.4.2**). These results demonstrate that A_1Rs participate in antinociception by amitriptyline. Curiously, amitriptyline produces a comparable effect in A_1R +/+ and -/- mice (Sawynok et al., 2008), indicating that while these receptor contribute to, they are not mandatory for the action of amitriptyline. These findings are comparable to those of a previous study which showed that systemic amitriptyline antinociception was reversed by systemic caffeine in A_1R +/+ but not -/- mice, (Sawynok et al., 2008). Thus, the ability of systemic amitriptyline to produce pain relief via A_1R activation may regulate the

analgesic effects which act through other parallel pharmacological mechanisms. This phenomenon, which was also observed in the experiments involving peripheral delivery of DPCPX, will be further discussed in section 2.5.4.

2.5.2 Spinal 5-HT$_7$R Actions in Antinociception by Amitriptyline

Following spinal delivery of the selective 5-HT$_7$R antagonist SB269970 at a dose of 3 μg, we observed an attenuation of the antinociceptive effects of systemic amitriptyline (**Figure 2.4.4**). A previous behavioural study conducted in BALB/c mice reported that a spinal SB269970 dose of 10 μg was inactive against threshold measures of thermal hyperalgesia (phasic noxious stimuli) (Dogrul et al., 2012). However, in our study, a 10 μg dose produced an intrinsic increase in phase 2 of the formalin test (tonic noxious stimuli) when administered with i.p. saline in C57BL/6 mice (**Figure 2.4.3**). We decided to use a 3 μg dose of SB269970, which was inactive against formalin 2% alone, and demonstrated that this was able to block amitriptyline. This result indicated that 5-HT$_7$Rs in the spinal cord were indeed involved in antinociception by systemic amitriptyline. This is consistent with findings conducted using other analgesics, which indicate that spinal 5-HT$_7$Rs mediate antinociception by acetaminophen and tramadol (Dogrul et al., 2012; Yanarates et al., 2010). In order to further explore the effects of 5-HT$_7$R activation on antinociception, the selective 5-HT$_7$R agonist AS-19 was administered into the spinal cord, either alone or in combination with SB269970. Surprisingly, in all animals tested, spinal administration of AS-19, both alone and in combination, evoked *spontaneous* pronociceptive behaviours which lasted for a short

period of time and ceased prior to commencement of the formalin test. The behaviours induced by spinal AS-19 administration are qualitatively similar to those described for i.t. injections of 5-HT, excitatory amino acids, and substance P, which have been attributed to excitatory actions of these ligands on specific receptors on transmission neurons within the dorsal spinal cord (Wilcox, 1988). However, in the presence of formalin, AS-19 did not lead to pronociception unless it was co-administered with SB269970 (**Figure 2.4.6**, also see section 2.5.5).

2.5.3 Interactions Between Spinal 5-HT$_7$R and A$_1$R Actions in Antinociception

Within the spinal cord, A_1Rs are primarily expressed in the substantia gelatinosa of the dorsal horn, where they decrease excitatory afferent transmission (Li & Perl, 1994; Schulte et al., 2003), thereby exerting antinociceptive effects. In the present study, we expanded upon a putative A_1R and 5-HT_7R -mediated spinal mechanism of antinociception by systemic amitriptyline by pharmacologically targeting 5-HT_7Rs in normal and A_1R -/- mice. Spinal SB269970 failed to reverse antinociception by amitriptyline in A_1R -/- mice (**Figure 2.4.5**), indicating that the presence of A_1Rs is required for antinociceptive effects mediated by 5-HT_7Rs to be observed. In congruence with these findings, nociception was potentiated by spinal co-administration of AS-19 and DPCPX (**Figure 2.4.7**), further implicating the involvement of A_1Rs in 5-HT_7R -mediated actions. This result was echoed in a subsequent experiment, in which we observed an augmentation of formalin-evoked flinching responses by spinal AS-19 in A_1R -/- mice compared to +/+ mice (**Figure 2.4.8**). Although the combination of i.t.

AS-19 and DPCPX led to a dramatic "overshoot" in flinching responses of normal mice, a similar increase in response magnitude was not produced by i.t. AS-19 in A_1R -/- mice. Furthermore, in contrast to effects in normal mice (**Figures 2.4.6, 2.4.7**), AS-19 appeared to be potentially antinociceptive in A_1R +/+ mice (**Figure 2.4.8**) relative to previously reported baseline responses to formalin alone (Sawynok & Reid, 2012). Differences in sensitivity have been observed between normal mice and A_1R colony mice for other drugs (see section 2.5.6), and may account for these discrepant observations. In spite of the ambiguity surrounding the potential antinociceptive effects of AS-19, it is clear that its actions also depend, in part, on the presence of A_1Rs.

Intrathecal administration of 5-HT has previously been shown to lead to antinociception, as well as to release of cAMP and adenosine from the spinal cord (Sweeney et al., 1990). Since amitriptyline recruits central serotonergic neurons (Zhao et al., 2007) and enhances the synaptic availability of 5-HT (Micó et al., 2006), spinal A_1Rs may be downstream effectors of a 5-HT link within the spinal cord. Descending serotonergic projections from the RVM terminate in the dorsal horn of the spinal cord, and have been shown to exert both inhibitory and facilitatory actions on nociceptive processing, depending on which spinal 5-HT receptor subtypes are activated (Bee & Dickenson, 2007; Dogrul et al., 2009). Recent rodent studies examining morphine (Dogrul et al., 2009), tramadol (Yanarates et al., 2010), and acetaminophen (Dogrul et al., 2012) have indicated a spinal role for the 5-HT_7R, which is expressed in the substantia gelatinosa (Doly et al., 2005; Matthys et al., 2011), in mediating descending serotonergic pain inhibition and antinociception by those agents. From our results, we infer that antinociception by systemic amitriptyline can occur by enhancement of central

serotonergic tone within the spinal cord by 5-HT_7R activation and a downstream A_1R-mediated mechanism. Our data support the idea that A_1R-mediated antinociception by amitriptyline may be attributed to an increase in cAMP levels due to 5-HT_7R activation (Bard et al., 1993; Lovenberg et al., 1993; Ruat et al., 1993), leading to an enhanced availability of adenosine. This proposed mechanism is depicted in **Figure 2.5.1**.

It is unclear from our experiments whether the 5-HT_7R component of antinociception by amitriptyline in the spinal cord results from activation of descending inhibitory projections or from increased availability of spinal 5-HT due to inhibition of reuptake into nerve terminals. Antinociceptive and anti-inflammatory effects of amitriptyline are at least partially mediated by supraspinal mechanisms, and these may be involved in controlling downstream spinal actions (Galeotti, Ghelardini, & Bartolini, 2001; Hajhashemi, Sadeghi, Minaiyan, Movahedian, & Talebi, 2010). Spinal-supraspinal synergy, as demonstrated for other drugs, such as morphine (He & Lee, 1997) and acetaminophen (Raffa, Stone, & Tallarida, 2000), may be important for antinociception by amitriptyline, and should be explored in future studies.

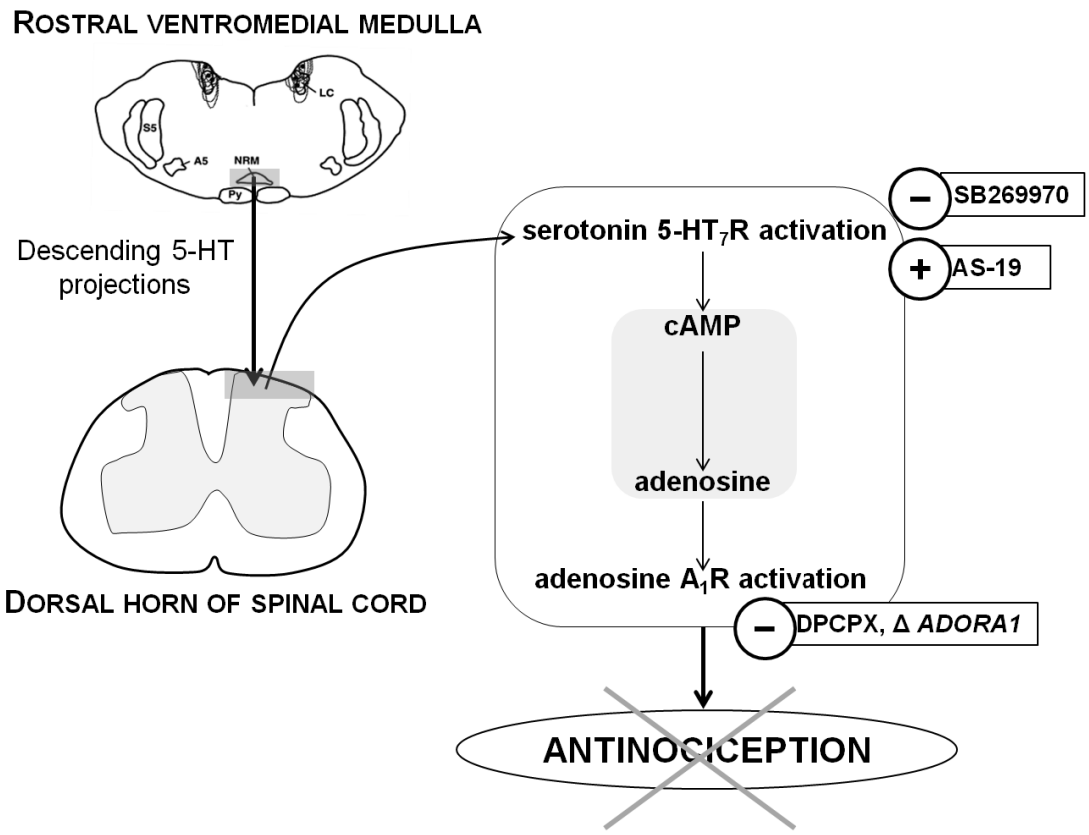

Figure 2.5.1 Proposed spinal cord mechanism for antinociception mediated by a putative interaction between the activation of serotonin 5-HT_7 receptors (5-HT_7Rs) and adenosine A_1 receptors (A_1Rs). Descending inhibitory bulbospinal projections to the spinal cord may activate 5-HT_7Rs in the dorsal horn. The activation of 5-HT_7Rs leads to the production of intracellular cAMP, which may be metabolized to adenosine and transported into the extracellular space. Extracellular adenosine can then bind to A_1Rs expressed pre- and post-synaptically in the dorsal horn, decreasing the excitability of primary afferent and transmission neurons. The selective 5-HT_7R agonist AS-19 was pronociceptive when spinally co-administered with the selective 5-HT_7R antagonist SB269970 or with the selective A_1R antagonist DPCPX. AS-19 increased flinching in mice that lacked the A_1R gene (Δ*ADORA1*) compared to A_1R wild-type mice.

2.5.4 Peripheral A_1R Involvement in Antinociception by Systemic Amitriptyline

The use of site-specific delivery of antagonists allows us to implicate not only spinal sites, but also peripheral sites of A_1R activity in the antinociceptive actions of systemic amitriptyline. Thus, we observed that peripherally-administered DPCPX was able to completely block antinociception by systemic amitriptyline in normal mice (**Figure 2.4.10**). This reversal was also seen in A_1R +/+ mice, but not in -/- mice (**Figure 2.4.11**).

In rat models of inflammatory pain, the activation of peripheral A_1Rs has been observed to reduce hypersensitivity produced by prostaglandin E_2 (PGE_2) (Aley, Green, & Levine, 1995; Lima et al., 2010), formalin (Doak & Sawynok, 1995), and carrageenan (Lima et al., 2010). The activation of peripheral A_1Rs may attenuate hypersensitivity to inflammatory noxious stimuli by suppressing excitatory afferent nociceptive firing via downstream effects of G_i signaling (Lima et al., 2010; Sawynok, 2012). While the precise pathways involved remain poorly understood, a recent study found that recruitment of the nitric oxide/cyclic guanosine monophosphate intracellular signalling pathway was required for a peripherally-administered A_1R agonist to reduce hypersensitivity induced by PGE_2 (Lima et al., 2010).

Although we did not directly characterize the analgesic mechanisms engaged by peripheral A_1Rs in the present study, the peripheral component of antinociception by systemic amitriptyline may be related to the direct enhancement of extracellular tissue levels of endogenous adenosine, rather than to a 5-HT_7R-dependent pathway. Indeed, in threshold and ongoing tests of pain, peripheral administration of 5-HT leads to pronociception, instead of antinociception (Sommer, 2006).

Direct administration of amitriptyline into the rat hindpaw produces antinociception in the formalin test (Sawynok et al., 1999). In addition to sodium channel blockade (Dick et al., 2007), amitriptyline also leads to the local release of purine nucleotides (likely cAMP), which are subsequently converted to adenosine by an ecto-5'-nucleotidase (Sawynok et al., 2005). The release of purine nucleotides and adenosine from sensory afferents (C fibres), as well as endothelial cells, platelets, mast cells, or neutrophils, result in an enhancement of extracellular adenosine levels (Sawynok et al., 2005). Additionally, amitriptyline may act to prevent the uptake of adenosine into these cells by inhibiting the equilibrative nucleoside transporter ENT2 (Sawynok et al., 2005). Since adenosine may also activate adenosine A_{2A}Rs on sensory nerve endings to facilitate pain, it is not clear how A_1Rs become selectively activated by amitriptyline. Moreover, as amitriptyline was given systemically in our experiments, it is unclear whether the drug would be present in the tissue at a sufficient level to elicit a significant release of adenosine, as direct administration into the hindpaw results in higher local levels of the drug than occurs with systemic administration. Nevertheless, the importance of the peripheral site in A_1R-dependent antinociception by systemic amitriptyline is highlighted by our experiments.

While the presence of A_1Rs is required for reversal of antinociception by amitriptyline with acute systemic caffeine (Sawynok et al., 2008) and spinal and peripheral DPCPX (**Figures 2.4.1, 2.4.10**), data obtained using A_1R knock-out animals (**Figures 2.4.2, 2.4.11**) indicate that the activation of these receptors is not obligatory for antinociception by systemic amitriptyline. Consequently, blockade of these receptors, when they are present, appears to override other parallel mechanisms of analgesia by

amitriptyline. These results are consistent with other studies which found that the ability of caffeine to reverse antinociception by various drugs with multiple actions, including amitriptyline (Sawynok et al., 2008), acetaminophen (Sawynok & Reid, 2012), and oxcarbazepine (Sawynok et al., 2010), is dependent on the presence of two copies of the gene encoding the A_1R.

2.5.5 Validity of the Pharmacological Approach

In the present study, both selective pharmacological targeting of A_1Rs and 5-HT_7Rs and genetic ablation of A_1Rs were used to elaborate on the roles of these receptors within particular compartments. It is important to note that our pharmacological approach did not permit us to identify the ligand (or ligands) responsible for A_1R activation. Given that *in vitro* studies have shown that inosine and AMP may bind and activate A_1Rs (Rittiner et al., 2012), the antinociceptive effects of spinal A_1Rs may not necessarily be elicited only by the binding of adenosine. In fact, a recent study showed that spinally-administered inosine, which was previously assumed to be an inactive metabolite of adenosine, also produces antinociception in mice that is dependent on A_1R activation and is blocked by DPCPX (Nascimento et al., 2010).

Since DPCPX (Jacobson & Gao, 2006), SB269970 (Leopoldo et al., 2011), and AS-19 (Brenchat et al., 2010) are all highly selective for their target receptors, it is relatively unlikely that non-specific effects on other receptors could lead to alternative interpretations of our results. However, the specificity of SB269970, which is frequently used in 5-HT_7R research, has recently been called into question by one study, which

reported that the compound antagonized α_2-adrenergic receptors in guinea pig tissues (Foong & Bornstein, 2009). Whether SB269970 can also bind to α_2-adrenergic receptors in mouse tissues is unknown. However, since blockade of α_2-adrenergic receptors would prevent inhibitory neurotransmission, it is possible that the reversal of antinociception by systemic amitriptyline could be attributed to effects on both 5-HT_7Rs and α_2-adrenergic receptors in the spinal dorsal horn. Amitriptyline is known to modulate NA levels (Micó et al., 2006), and therefore could recruit α_2-adrenergic receptors in a separate antinociceptive action, parallel to the effects on A_1Rs and 5-HT_7Rs. Non-specific inhibition of α_2-adrenergic receptors could perhaps account for the intrinsic elevation of formalin-evoked flinching responses by SB269970 10 μg, as well as the observed overshoot in flinching observed after spinal co-adminstration of AS-19 and SB269970 (**Figure 2.4.6**). Nevertheless, the results of delivering spinal AS-19 to A_1R -/- mice (or spinal AS-19 and DPCPX to normal mice) demonstrate that a link exists between A_1Rs and 5-HT_7Rs in nociceptive processing.

2.5.6 Use of the A_1R Knock-Out Mouse Model

The combination of pharmacological targeting and genetic deletion of A_1Rs in the present study enabled us to uncover a signaling link between spinal A_1Rs and 5-HT_7Rs, and also demonstrate the peripheral contribution of A_1Rs to antinociception by amitriptyline. Adenosine receptor gene deletion mice (of the A_1R, A_{2A}R, and A_3R) have been very useful for delineating the involvement of such receptors in several physiological functions, including pain (Fredholm et al., 2005).

As with any experiments involving the use of knock-out mice, the results of this study must be interpreted cautiously, since various functional alterations may have directly or indirectly arisen from the absence of the target gene during development. Additionally, compensatory expression of other genes that are normally related to the A_1R could be a potential confound. In our study, *direct* comparisons between our two mice populations (normal and A_1R colony mice) were not made because of a disparity in their intrinsic responses to formalin 2%, as well as in their differential sensitivities to amitriptyline. Our A_1R colony +/+, +/-, and -/- mice exhibited an elevated flinching response to formalin 2% compared to normal C57BL/6 mice (Sawynok & Reid, 2012). Another indication that the two populations of mice were distinct was the dosage of amitriptyline required to produce antinociception. Amitriptyline was always administered to normal mice at a non-sedating and reliably antinociceptive dose of 3 mg/kg, whereas a dose of 12 mg/kg was required in A_1R colony mice to produce comparable antinociception. Genetic factors could account for this discrepancy in drug dosing. One possibility is that the A_1R colony mice were initially bred on a C57BL/6 background that was a slightly different substrain than that of the normal C57BL/6 mice raised at Charles

River (Zurita et al., 2011). Another factor is that the colony mice could have been subject to genetic drift, which can occur after many generations of mouse inbreeding and potentially lead to the fixation of mutations in genes involved in drug metabolism (Zurita et al., 2011). While we did not measure hepatic enzyme activity, differences in metabolism due to genetic drift may account for the large range of systemic amitriptyline doses used in this study.

2.5.7 Clinical Implications of Interactions Between Caffeine and Amitriptyline

Caffeine is consumed daily through an assortment of beverages and foods by millions of people worldwide (Fredholm et al., 1999). While substantial variation exists in human caffeine intake levels within different countries, North American and European dose levels have been estimated to correspond to 2.5-7.3 mg/kg/day for adults weighing between 55-70 kg (Fredholm et al., 1999; Sawynok & Reid, 2012). Clinical evidence supports the efficacy of caffeine as an adjuvant analgesic, when administered in combination with non-steroidal anti-inflammatory drugs (NSAIDs) and/or acetaminophen (Palmer, Graham, Williams, & Day, 2010; Sawynok, 2011a, 2011b). Preclinical data indicate that caffeine is intrinsically antinociceptive at moderate to high doses of 35-100 mg/kg (Sawynok, 2011a). These adjuvant analgesic effects have mainly been attributed to peripheral blockade of A_{2A}Rs and A_{2B}Rs (Sawynok, 2011a, 2011b). However, recent preclinical studies have indicated that low doses of caffeine (between 1-20 mg/kg) block analgesia by various drugs, including acetaminophen, amitriptyline, and oxcarbazepine (Sawynok, 2011a, 2011b), and these actions involve the inhibition of

A_1Rs. Understanding the involvement of caffeine in amitriptyline actions is clinically important because of the widespread usage of both drugs. Our present findings demonstrate that chronic oral caffeine can inhibit antinociception by amitriptyline (**Figure 2.4.12**). The oral caffeine dosing levels used in the current study (0.1 g/L or 0.01% and 0.3 g/L or 0.03%) are relevant to human intake levels of dietary caffeine (Yang et al., 2009) and have also been demonstrated to block acetaminophen antinociception in the mouse formalin test (Sawynok & Reid, 2012). Attention to caffeine intake may be required in humans in order to determine its effects on the actions of amitriptyline and other commonly used analgesic drugs which rely upon A_1Rs (Sawynok, 2011b). As A_1R activation in the periphery has even been implicated in the analgesic effects of acupuncture (Goldman et al., 2010), blockade of A_1R-mediated antinociception by caffeine also may be relevant in non-pharmacological therapies for pain (Sawynok, 2011b).

To date, there have been few clinical trials examining whether chronic caffeine levels *can reduce* the efficacy of analgesics. Since it would be unrealistic to impose a caffeine restriction on participants in clinical trials, a post-hoc subgroup analysis according to participants' reported caffeine intake levels (separated into low, moderate, or high caffeine consumption categories) could be incorporated (Sawynok, 2011b). This method of categorizing caffeine consumption has already been applied in trials examining the impact of caffeine intake levels on the efficacy of methotrexate in rheumatoid arthritis (Benito-Garcia et al., 2006; Nesher, Mates, & Zevin, 2003; Swanson, Barnes, Mengden Koon, & el-Azhary, 2007) and would be useful in human studies of other analgesic drugs which utilize adenosine systems. It is worthwhile to consider that

differential sensitivities to caffeine have been attributed in part to inter-individual variations in expression levels and genetic sequences of adenosine receptors and enzymes involved in adenosine metabolism (Alsene, Deckert, Sand, & de Wit, 2003; Rétey et al., 2005). Although genetic polymorphisms of the human A_1R gene are not associated with caffeine sensitivity (Alsene et al., 2003), they may potentially influence individual responses to analgesics. Therefore, further investigation into the genetic factors involved in caffeine sensitivity and/or adenosine signaling may indicate whether the preclinical phenomenon of reduced analgesic efficacy by chronic caffeine is also observable in humans.

2.6 SUMMARY AND CONCLUSIONS

The pharmacological and genetic approaches employed in the present study provide evidence that both spinal and peripheral A_1R activation are involved in systemic amitriptyline antinociception in the mouse formalin test. With respect to the spinal compartment, we show for the first time that 5-HT_7Rs are implicated in acute antinociception by systemic amitriptyline. In particular, our results provide evidence for a link between central 5-HT_7R activation and A_1R-mediated antinociception in the spinal mechanisms of action utilized by amitriptyline, and this likely constitutes one component of a multiplicity of pharmacological actions. Evidence for the recruitment of both spinal and peripheral A_1Rs by systemic amitriptyline suggests that a closer examination of, or at least attention to, potential impacts of chronic caffeine consumption on the analgesic efficacy in humans is required.

CHAPTER 3

AMITRIPTYLINE AS A POTENTIAL PREVENTIVE ANALGESIC

3.1 ABSTRACT

This study examined the effects of a perioperative regime of amitriptyline on responses to noxious chemical stimuli following nerve injury in rats. At 7-14 days following spared nerve injury (SNI) surgery, rats developed chemogenic hypersensitivity to an intraplantar co-injection of αβ-methyleneATP (αβ-MeATP) and NA into the injured hindpaw. By days 14-21, SNI-operated rats developed chemogenic hyposensitivity in the injured hindpaw to intraplantar formalin 2.5%. After SNI, perioperative amitriptyline (given systemically at 10 mg/kg before and after surgery, then orally in the drinking water at ~16-20 mg/day for 7 days) prevented hypersensitivity to αβ-MeATP/NA, but had no effect on hyposensitivity to formalin. Since spinal noradrenergic pathways are required for the preventive analgesic effects of perioperative amitriptyline, we determined if noradrenergic fibre sprouting in the superficial spinal dorsal horn occurred after SNI or perioperative amitriptyline treatment. Overall, immunoreactivity of dopamine-β-hydroxylase (DβH) in the lumbar dorsal horn was unchanged by SNI or perioperative amitriptyline. In SNI-operated rats treated with perioperative amitriptyline, DβH-immunoreactivity appeared to be decreased in the lumbar dorsal horn contralateral to injury, but this trend was not significant. Our behavioural findings suggest that perioperative amitriptyline is a potential preventive analgesic against the development of sensory hypersensitivity. However, sprouting of spinal noradrenergic pathways may not constitute a major part of this action.

3.2 INTRODUCTION

3.2.1 The Challenges of Treating Neuropathic Pain

Neuropathic pain is a persistent condition which causes immense suffering in afflicted patients. Damage that occurs to the nerves as a consequence of trauma, metabolic disorders, infection, chemotherapeutics, or cancer, may all lead to the induction of neuropathic pain, which manifests as spontaneous pain, allodynia, hyperalgesia, and sensory hyposensitivity (Costigan et al., 2009; Kehlet et al., 2006). Compared with pains of nociceptive and inflammatory origin, neuropathic pain is pathological, and tends to persist due to the maladaptive changes which have occurred throughout the neuraxis (Costigan et al., 2009; Kehlet et al., 2006). Currently, antidepressants are recommended as first-line treatments (Saarto & Wiffen, 2010) alongside anticonvulsants, including gabapentin and pregabalin (collectively known as the gabapentinoids) (Finnerup et al., 2005). Unfortunately, these drugs are only able to manage symptoms in some patients, and even those who respond to treatment, typically only experience partial pain relief (Costigan et al., 2009). This relative scarcity of effective pharmacotherapies has created an impetus for the development of disease-modifying therapies, which ideally would reverse the maladaptive neuronal sensitization processes which establish the chronicity of neuropathic pain.

3.2.2 Persistent Post-Surgical Pain

Following recovery from various surgical procedures, a large subset of patients may go on to develop long-term pathological pain, which persists for more than 2 months post-operatively (Kehlet & Rathmell, 2010; Kehlet et al., 2006). The incidence and severity of persistent pain varies depending on the procedure, but can be as high as 30-50% after amputation or coronary artery bypass surgery, suggesting that surgical invasiveness may increase the likelihood of pain development (Kehlet et al., 2006). Persistent post-surgical pain is classified as a form of neuropathic pain, since a major factor in its development is inadvertent trauma to the nerves at the surgical site (Kehlet et al., 2006). Although nerve damage is the most important trigger for the development of neuropathic pain, a combination of genetic, demographic, and psychosocial factors may predispose certain individuals to the development of this neuropathological condition (Costigan et al., 2009; Kehlet et al., 2006). While refinements in surgical procedures may reduce the incidence of persistent post-surgical pain, existing symptomatic management of this condition is inadequate, and preventive analgesic strategies should be explored as therapeutic options (Kehlet et al., 2006).

During the surgical procedure, as well as during the acute post-surgical period, nociceptive transmission pathways may undergo sensitization processes which could precipitate the development of persistent neuropathic pain (Dahl & Kehlet, 2011). Therefore, analgesics could be administered during the perioperative period (before and after the procedure), in order to counteract the development of maladaptive, possibly irreversible neuroplastic processes before they occur (Dahl & Kehlet, 2011). This *preventive* analgesic strategy would hopefully lead to disease modification instead of

symptomatic management, and stands in contrast with *pre-emptive analgesia*, which involves administering an analgesic prior to surgery only, with the intent of reducing acute, rather than persistent, post-surgical pain (Dahl & Kehlet, 2011).

3.2.3 Mechanisms Underlying Neuropathic Pain

In the wake of a nerve injury, a reactive series of cellular events occurs throughout various levels of pain signaling pathways, including peripheral sensory nerves, the spinal cord, supraspinal projections, and descending modulatory projections (Costigan et al., 2009; Kehlet et al., 2006). The cellular events that comprise the peripheral and central sensitization processes lead to the amplification of nociceptive transmission, as well as the induction and maintenance of neuropathic pain (Costigan et al., 2009; Kehlet et al., 2006). **Peripheral sensitization** is initiated by the local release of inflammatory mediators from damaged tissue and immune cells in and around the site of injury, and is characterized by local hypersensitivity of nociceptors to noxious and innocuous stimuli, as well as the generation of ectopic impulses (Costigan et al., 2009; Kehlet et al., 2006). Around the site of nerve injury, damaged axons undergo Wallerian degeneration through infiltration by activated macrophages, which promote further inflammation (Costigan et al., 2009). Various inflammatory mediators bind and activate their respective receptors located on nociceptors, triggering downstream intracellular signaling cascades which in turn phosphorylate transducer receptors and voltage-gated ion channels (Costigan et al., 2009; Kehlet et al., 2006). Additionally, intracellular signaling leads to changes in gene transcription within the DRG, resulting in novel gene

expression and upregulation of particular receptors and voltage-gated sodium channel subtypes, as well as increased membrane trafficking of transducer receptors and ion channels to nociceptors (Costigan et al., 2009; Kehlet et al., 2006). Consequently, the threshold of nociceptor activation is lowered, and the increase in voltage-gated sodium channel expression can lead to the generation of ectopic impulses (Costigan et al., 2009). Body areas that are innervated by injured and neighbouring uninjured sensory afferents, therefore, become overly sensitive, and hyperalgesia, allodynia, and spontaneous pain may be experienced (Costigan et al., 2009; Kehlet et al., 2006). Since peripheral sensitization occurs in inflammatory pain, the excitability of nociceptors generally returns to baseline after the inflammation subsides (Kehlet et al., 2006). However, in the case of neuropathic pain, excessive and prolonged activity of the injured nerve, coupled with peripheral sensitization, precipitates another series of events known as central sensitization (Costigan et al., 2009).

Central sensitization is generated by ongoing peripheral sensitization and microglial activation in the spinal cord, culminating in a maladaptive reorganization of spinal neural circuits and abnormal nociceptive transmission (Costigan et al., 2009; Kehlet et al., 2006). It is the occurrence of central sensitization that typically renders neuropathic pain resistant to conventional analgesics. Ectopic firing of both injured and uninjured primary afferent fibres can provoke the abnormal reorganization of neuronal circuitry in the superficial layers of the spinal dorsal horn (Costigan et al., 2009). For instance, Aβ fibre terminals, which normally synapse in deeper dorsal horn laminae and mediate tactile sensation, sprout into the superficial dorsal horn following nerve injury, resulting in intermingling with Aδ and C fibres and augmentation of nociceptive

signaling (Costigan et al., 2009). Additionally, local inhibitory interneurons undergo apoptosis and descending inhibitory influences are weakened, producing disinhibition of afferent nociceptive signaling in the dorsal horn (Costigan et al., 2009). As with peripheral sensitization, the net effect of central sensitization is an increase in the gain of nociceptive transmission to the brain, however aberrant neuroplasticity in the central nervous system is much more difficult, if not impossible, to reverse (Kehlet et al., 2006).

3.2.4 Rodent Models of Neuropathic Pain

Although incisional models of pain have been developed in animals to represent injuries to the skin and muscle which occur during surgery, these are more relevant to acute post-surgical pain, and do not reflect the consequences of nerve trauma that lead to persistent post-surgical pain (Scholz & Yaksh, 2010). Persistent post-surgical pain is typically modeled in animals by surgically inducing an injury to a peripheral sensory nerve, usually the sciatic nerve. The technical advancements in the modelling of chronic pain in animals have improved the processes of analgesic drug screening, providing tools for studying the etiology of neuropathic pain, as well as the mechanisms underlying analgesia. Prior to the development of the first animal models of neuropathic pain, studies examining the efficacy of potential analgesic compounds exclusively relied on behavioural and electrophysiological responses of uninjured animals to acutely-administered noxious mechanical, chemical, and thermal stimuli (Decosterd & Woolf, 2000; Mogil, 2009). By the 1980s, surgically-induced peripheral nerve injuries in animals were recognized to produce symptoms that resembled those seen in clinical neuropathic

pain, such as mechanical allodynia and thermal hyperalgesia (Decosterd & Woolf, 2000). The earliest sciatic nerve injury model, the neuroma model, was developed by Wall et al. (1979), and involved complete transection of the nerve (axotomy), thereby removing all sensory input from the hindlimb. Since thermal or mechanical noxious stimuli could not be administered due to complete sensory denervation, only autotomy (self-mutilation of the toes), could be monitored as a behavioural correlate of pain (Decosterd & Woolf, 2000; Mogil, 2009). This model was most representative of phantom limb pain that is secondary to amputation (Wall et al., 1979), but whether autotomy behaviours in rodents could actually be attributed to pain or simply to hypergrooming (associated with sensory denervation) remains ambiguous to the present day (Decosterd & Woolf, 2000; Mogil, 2009). Moreover, since human neuropathic pain pathophysiology rarely involves complete nerve lesions, adjustments to the nature of the injury were necessary (Decosterd & Woolf, 2000).

Partial lesions of the sciatic nerve were achieved with the introduction of the chronic constriction injury (CCI), involving loose ligation of the sciatic nerve (Bennett & Xie, 1988), as well as the partial sciatic nerve injury (PSL), involving ligation of half of the sciatic nerve (Seltzer, Dubner, & Shir, 1990). A significant advantage conferred by both models was that they permitted sensory changes to noxious stimuli following nerve injury to be assessed through behavioural testing (Decosterd & Woolf, 2000). However, since the distal partial sciatic nerve lesions were not highly reproducible, Kim and Chung (1992) developed a method of ligating the L5 and L6 spinal nerves proximal to the DRG (Decosterd & Woolf, 2000). In spite of the invasiveness of the surgical procedure, the L5/L6 spinal nerve ligation (SNL) model allowed for a more robust induction of

persistent pain behaviours resulting from partial denervation, and has since been widely used. While CCI, PSL, and SNL continue to be widely used in preclinical neuropathic pain studies, none of these models allow for the precise assessment of the relative contributions of injured and uninjured sensory afferents to the pathophysiology of partial denervation. In 2000, Decosterd and Woolf introduced the spared nerve injury (SNI) model, which involves tight ligation and transection of the tibial and common peroneal branches of the sciatic nerve, with "sparing" of the sural branch. The SNI procedure reliably produces an intermingling of injured and uninjured afferents (which project to the L4-L6 DRG) (**Figure 3.2.1**, Decosterd and Woolf, 2000). Since the innervation pattern of the hindpaw is well-defined, the relative contribution of injured versus uninjured afferents to manifestations of pain can be examined by targeting nociceptive stimuli to the lateral and medial aspects of the hindpaw in nociceptive tests (**Figure 3.3.3**, Decosterd & Woolf, 2000). Moreover, the SNI procedure is minimally invasive and simple to perform, leading to greater reproducibility (Decosterd & Woolf, 2000).

In rats, SNI produces abnormal responses in a range of nociceptive behavioural tests, including mechanical allodynia and hyperalgesia, as well as cold allodynia and thermal hyperalgesia (Decosterd & Woolf, 2000). Abnormal responses to noxious chemical stimuli following SNI have also been observed in our laboratory, and these include lateral hindpaw hypersensitivity to αβ-methylene adenosine triphosphate (αβ-MeATP) and NA, lateral hindpaw hyposensitivity to capsaicin and NA (Arsenault & Sawynok, 2009; Meisner, Reid, & Sawynok, 2008), and medial hindpaw hyposensitivity to formalin (Sawynok & Reid, 2011). Preclinical drug development studies have traditionally focused on the resolution of allodynia and hyperalgesia for chronic pain

states. However, in humans with neuropathic pain, sensory loss and hyposensitivity ("negative" symptoms) also accompany symptoms of spontaneous pain, allodynia, and hyperalgesia (Costigan et al., 2009). Ideally, drug therapies would be able to counteract the development of *increased* pain responses, as well as of *decreased* responses to specific sensory endpoints. In other words, preventing the development of abnormal hyposensitive responses to noxious stimuli may be as important as suppressing pathological hypersensitivity.

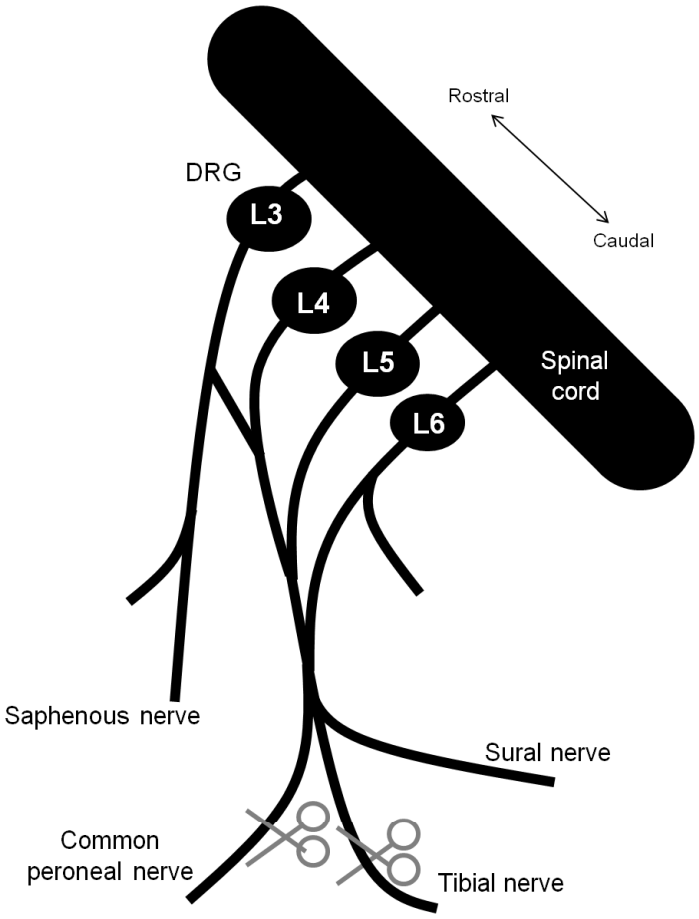

Figure 3.2.1 Schematic diagram of the spared nerve injury (SNI) model of neuropathic pain. In this model, the common peroneal and tibial nerve branches of the sciatic nerve are tightly ligated and then transected. The sural branch of the sciatic nerve is left untouched. Adapted from Decosterd & Woolf (2000).

3.2.5 Antidepressants as Preventive Analgesics

As described earlier, TCAs are currently prescribed to treat established chronic neuropathic pain in humans. The TCA amitriptyline has shown promise as a potential preventive analgesic in a randomized controlled clinical trial for post-herpetic neuralgia in elderly patients (Bowsher, 1997), and also against the development of persistent post-surgical pain in a rodent model (Arsenault & Sawynok, 2009). Using the SNI model, our laboratory showed that a perioperative administration regime of amitriptyline (before and after SNI, and then in the drinking water for 7 days), resulted in a long-term preventive effect on chemogenic hypersensitivity to αβ-MeATP/NA, as well as hyposenstivity to capsaicin/NA (Arsenault & Sawynok, 2009). In order to determine how amitriptyline could exert a preventive analgesic effect, further elaboration of the effects of perioperative amitriptyline treatment on behavioural sequelae accompanying SNI is necessary.

3.2.6 The Role of Noradrenaline (NA) in Neuropathic Pain

Clinical and preclinical data have indicated that TCAs and SNRIs exhibit greater efficacy in chronic pain conditions than do SSRIs, and this may be due in part to the ability of these drugs to modulate central synaptic levels of NA (Nakajima, Obata, Iriuchijima, & Saito, 2012; Saarto & Wiffen, 2010). The release of NA in the spinal cord by descending brainstem noradrenergic projections produces analgesia through the activation of α_2-adrenergic receptors in the superficial dorsal horn (Bee & Dickenson, 2009; Nakajima et al., 2012). Moreover, amitriptyline has been shown to lead to the activation of α_2-adrenergic receptors (Ghlardini, Galeotti, & Bartolini, 2000), and the antinociceptive effects are abolished in α_2-adrenergic receptor knock-out mice (Özdoğan, Lähdesmäki, Mansikka, & Scheinin, 2004). Since antidepressants are effective in chronic pain conditions but are relatively inactive against acutely-induced pain, the "monoamine hypothesis" of neuropathic pain has been proposed to account for this apparent state-dependent efficacy (Bee & Dickenson, 2009; Jasmin et al., 2003). As part of central sensitization processes which lead to the induction and/or maintenance of neuropathic pain, descending inhibition is thought to be diminished while descending facilitatory tone is augmented (Bee & Dickenson, 2009; Jasmin et al., 2003). This shift in the balance between descending inhibition and facilitation may thereby "prime" the nervous system to receive benefit from TCAs, which could act to reinforce descending noradrenergic inhibition.

Several preclinical behavioural studies conducted using nerve injured animals have indicated that spinal noradrenergic pathways are necessary to mediate α_2-adrenergic receptor-mediated analgesia by TCAs (Arsenault & Sawynok, 2009), gabapentinoids

(Tanabe et al., 2005), and α_2-adrenergic receptor agonists (Hayashida, Clayton, & Johnson, 2008), but the anatomical basis of this apparent spinal noradrenergic dependence remains unclear. The sprouting of descending noradrenergic axonal terminal densities in the spinal dorsal horn following nerve injury has been proposed as a compensatory mechanism to increase sensitivity to NA in chronic pain states (Hayashida et al., 2008). According to this hypothesis, reactive central sprouting of noradrenergic fibres could constitute neural substrates upon which analgesics may then act to strengthen inhibitory tone (Hayashida et al., 2008). Noradrenergic fibre sprouting in the lumbar dorsal horn of mice with CCI was observed in one study (Ma & Eisenach, 2003). The same group later reported that SNL in rats was able to induce bilateral noradrenergic sprouting in the dorsal horn of the lumbar spinal cord, and that this sprouting was dependent upon brain-derived neurotrophic factor (BDNF) (Hayashida et al., 2008). However, neither of the aforementioned studies determined if the chronic administration of analgesics would affect this sprouting (Hayashida et al., 2008; Ma & Eisenach, 2003).

It is not currently known whether the sprouting of noradrenergic fibres in the lumbar spinal cord occurs after SNI; however, the preventive analgesic effects of perioperative amitriptyline appear to rely on spinal noradrenergic pathways. In the Arsenault and Sawynok (2009) study, it was observed that ablation of spinal noradrenergic fibres by i.t. treatment with the neurotoxin 6-hydroxydopamine (6-OHDA) abolished the preventive analgesic effect of perioperative amitriptyline against the development of afferent hypersensitivity to αβ-MeATP/NA. The current project examined (a) whether noradrenergic sprouting in the spinal cord occurred following SNI, and (b) whether this was modified by perioperative amitriptyline.

3.2.7 Project Objectives

The objectives of the present study were (1) to further elaborate the effects of perioperative amitriptyline on abnormal pain responses to noxious chemical stimuli (αβ-MeATP/NA and formalin 2.5%) following SNI in rats and (2) to explore a potential spinal cord mechanism for preventive analgesia by perioperative amitriptyline after SNI.

(1) **Behavioural Assessments.** The effects of perioperative amitriptyline on abnormal sensory changes following SNI were assessed behaviourally using intraplantar (i.pl.) injections of αβ-MeATP/NA (at post-surgical day 14) into the lateral hindpaw and formalin 2.5% (at post-surgical day 21) into the medial hindpaw. Since perioperative amitriptyline has been shown to prevent hypersensitivity to αβ-MeATP/NA, we wished to confirm this and determine whether the perioperative regime could prevent the development of hyposensitivity to formalin 2.5%.

(2) **Anatomical Experiments.** Since noradrenergic sprouting in the dorsal horn of the spinal cord has been reported in other models of peripheral nerve injury, we wished to determine if SNI also led to noradrenergic sprouting. We also wished to examine the effects of perioperative amitriptyline on this sprouting. Immunohistochemistry of dopamine-β-hydroxylase (DβH) (a marker for noradrenergic neurons) was performed on the lumbar spinal cords of SNI- and sham-operated rats treated with perioperative amitriptyline or vehicle. Changes in densities of DβH-immunoreactive (DβH-IR) fibres within the dorsal horn of the lumbar spinal cord were evaluated. The main hypothesis was that sprouting of noradrenergic axonal fibres in descending spinal cord pathways was involved in the preventive analgesia by perioperative amitriptyline following SNI.

3.3 MATERIALS AND METHODS

3.3.1 Animals

All experiments were approved by the University Committee on Laboratory Animals at Dalhousie University (Halifax, Nova Scotia, Canada) and performed in compliance with the ethical guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (Charles River Laboratories, Québec, Canada), weighing 150-250 g on the day of surgery, were used for all nerve injury and immunohistochemistry experiments. After their initial arrival at the animal care facility, all rats were allowed to habituate for at least 1 week prior to being subjected to any procedures. Rats were housed in pairs (except for 3 days post-surgery, when they were housed individually) on a 12-hour light/12-hour dark cycle and housed in temperature-controlled rooms (21 ± 1 ºC), with access to food (rat chow) and water *ad libitum*.

3.3.2 Spared Nerve Injury (SNI) Surgery and Post-Surgical Care

Surgical procedures were performed using aseptic technique. Under isoflurane anesthesia (2-3%), rats received a subcutaneous (s.c.) injection (5 mL) of lactated Ringer's Solution or 0.9% saline, as well as an i.m. injection (0.1 mL) of Duplocillin®. The left thigh (surgical area) was shaved with clippers, and the skin was swabbed with 70% ethanol and iodine. Rats then received either a unilateral spared nerve injury (SNI) procedure or a sham surgery. SNI surgery was performed as described by Decosterd and Woolf (2000). As part of the SNI procedure, a small incision was made on the left thigh, and the biceps femoris muscle was separated and retracted such that the sural, common

peroneal, and tibial nerve branches of the sciatic nerve were clearly visible. Individual nerve branches were isolated using a small glass hook, and the common peroneal and tibial nerves were tightly ligated using 6-0 silk suture before the removal of a 2 mm section of the nerves distal to the ligations. The sural nerve was left intact (spared). The wound was closed with cutaneous sutures (3-0 silk). In rats that served as sham controls, the biceps femoris muscle was retracted to expose the sciatic nerve bundle, but the nerves were not manipulated prior to cutaneous closure of the wound. After surgery, rats were placed under a heat lamp for a brief recovery period before being returned to their cages.

After surgery, rats were housed individually for 3 days and then paired with their original cage mate. In rats that had received the SNI, a characteristic hindpaw curl was always observed, and can be attributed to a loss of motor control and proprioception. Sham-operated rats did not display this hindpaw curl. During the 3-day post-surgical period, rats were observed frequently in order to ensure that the wound was healing properly, that walking and standing had not become impaired, and that autotomy (self-mutilation of digits) had not occurred. Rats that showed signs of self-mutilation (usually toenail chewing) were provided with hay; these behaviours typically resolved in the presence of hay or the cage mate. In the rare instance that autotomy worsened or continued, the rat was excluded from experiments and euthanized.

3.3.3 Drugs and Reagents

Amitriptyline, αβ-methylene ATP (αβ-MeATP), NA, formalin, urethane, and Triton-X 100 were purchased from Sigma-Aldrich Ltd. (Oakville, Ontario, Canada). Mouse monoclonal anti-dopamine-β-hydroxylase (anti-DβH), rabbit affinity-purified polyclonal anti-tyrosine hydroxylase (anti-TH), and normal goat serum were purchased from Chemicon (Millipore). The Vectastain ABC Elite Kit was purchased from Vector Laboratories (Burlington, Ontario, Canada).

3.3.4 Drug Dosing

Perioperative Drug Regime: In experiments in which rats were treated with a perioperative amitiptyline regime (**Figure 3.3.1**), amitriptyline was given by i.p. injection 30 minutes before and immediately after surgery, as well as for 7 days post-surgery in the drinking water. Thirty minutes prior to surgery, rats in the amitriptyline group were given i.p. amitriptyline (10 mg/kg, dissolved in 0.9% saline) in an injection volume of 5 mL/kg. Rats in the vehicle (control) group were injected with an equivalent volume of saline. After surgery (which lasted approximately 10 minutes), and immediately following closure of the wound, another i.p. injection of 10 mg/kg amitriptyline or saline was delivered. Animals in the amitriptyline group also received amitriptyline in their drinking water for 7 days post-surgery, whereas the vehicle group was maintained on normal drinking water. Amitriptyline was administered in the drinking water at a dose of 12 mg/100 mL. A fresh drug preparation was made every 2-3 days. In order to prevent photodegradation of amitriptyline, water bottles for each cage were wrapped with

aluminum foil and placed in a plastic tube. Based on average daily water consumption (approximately 50 mL/day), rats in the amitriptyline group received at least 16-20 mg of amitriptyline per day. After the 7-day drug treatment period, rats were maintained on normal drinking water until post-surgical day 21, at which time they were euthanized (immediately following formalin testing) or sacrificed for immunohistochemistry.

Chemogenic Behavioural Testing: Drugs were prepared freshly on each behavioural testing day. αβ-MeATP (50 nmol) and NA (25 nmol) were dissolved in 0.9% saline. Formalin was diluted to a concentration of 2.5% in 0.9% saline.

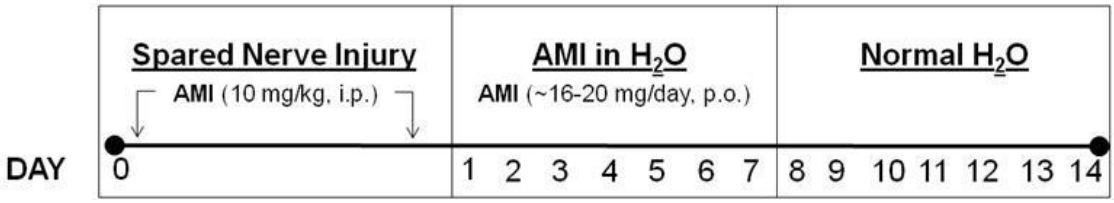

Figure 3.3.1 Timeline of perioperative amitriptyline (AMI) regime. Amitriptyline 10 mg/kg (or an equivalent amount of saline) was administered by intraperitoneal (i.p.) injection at 30 minutes before the surgery and immediately after closure of the wound. Subsequently, amitriptyline was given orally (p.o.) in the drinking water for 7 days after surgery. Rats were maintained on normal drinking water after 7 days. Rats in the vehicle group received normal drinking water throughout the course of the experiment.

3.3.5 Behavioural Assessments

In order to assess nocifensive responses to chemical stimuli (αβ-MeATP/NA and formalin 2.5%), behavioural testing was performed on a subset of the rats used in this study. The timelines for testing used for these experiments are depicted in **Figure 3.3.2**. (Rats that were used in the immunohistochemistry experiments were not subjected to the procedures described below.) Testing was performed during the daytime (between 08:30 and 15:00). For all paradigms, rats were habituated in a plexiglass chamber (30 cm x 30 cm x 30 cm) for at least 20 minutes prior to delivery of the pain stimulus. Two rats were observed at the same time (in alternating 2 minute bins) in separate plexiglass chambers.

αβ-Methylene-ATP and NA: Pain behaviours elicited by local co-administration of αβ-MeATP with NA reflect the activation of primary sensory afferents by $P2X_3$ and α_1-adrenergic receptors (Meisner et al., 2008). Rats were loosely restrained before receiving a 30 μL injection of αβ-MeATP (50 nmol) and NA (25 nmol) into the lateral aspect of the hindpaw (at the junction between the hairy and non-hairy skin), ipsilateral to the surgical site (**Figure 3.3.3**). Flinching behaviours (elevation of the hindpaw, as well as rapid shaking) were recorded over 32 minutes.

Formalin 2.5% Test: The formalin test is widely used in preclinical studies, and reflects ongoing and inflammatory pain (Sawynok & Liu, 2003b). Rats were loosely restrained and formalin 2.5% (30 μL) was injected into the medial aspect of the hindpaw (ipsilateral to the surgical site) (**Figure 3.3.3**). The number of flinches was counted over 60 minutes. Pain behaviours were analyzed separately as the cumulative number of flinches during phase 1 (0–8 minutes) and phase 2 (12–60 minutes). Each rat was euthanized at the conclusion of the formalin test.

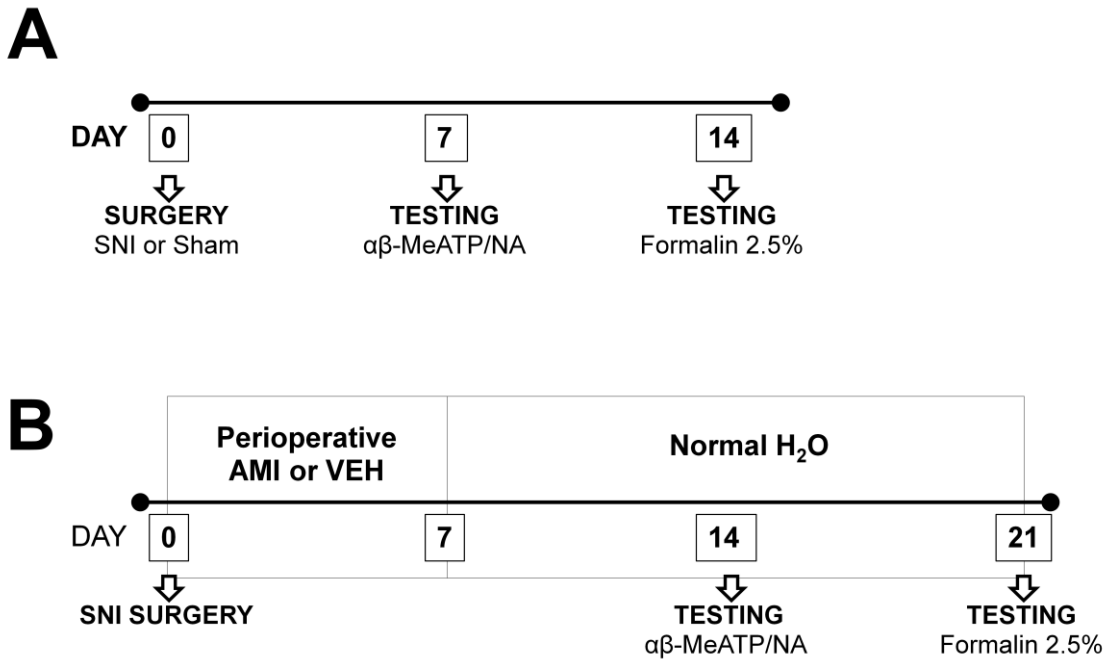

Figure 3.3.2 Testing timelines used for behavioural experiments. **(A)** An initial experiment was conducted using SNI- and sham-operated rats, which were subjected to behavioural tests at post-surgical days 7 and 14. **(B)** An experiment was conducted using SNI-operated rats that had received either perioperative amitriptyline (AMI) or saline/water (VEH) treatment. Behavioural tests were conducted at post-surgical days 14 and 21 (at 7 and 14 days following cessation of drug administration).

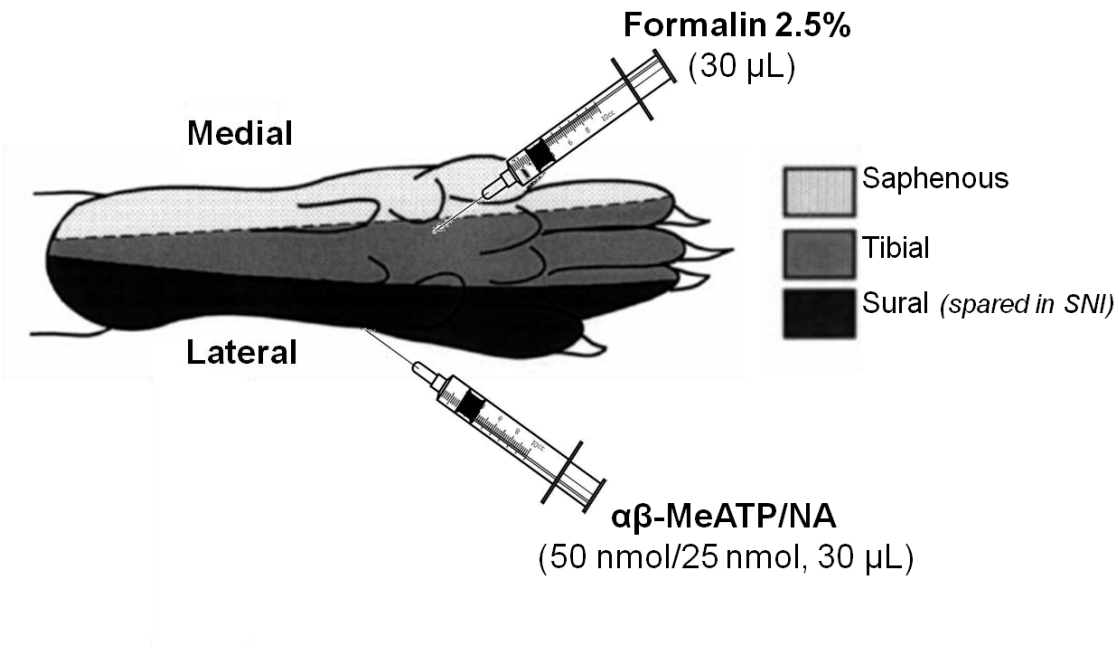

Figure 3.3.3 Hindpaw sites of injection for chemogenic behavioural tests. Responses to chemogenic noxious stimuli were assessed by injections to the hindpaw (ipsilateral to the surgical site). αβ-MeATP/NA (50 nmol/25 nmol) was injected into the lateral aspect of the hindpaw (innervated by the sural nerve), while formalin 2.5% was injected into the medial plantar aspect of the hindpaw (innervated by saphenous and tibial nerves). Figure adapted from Decosterd & Woolf (2000).

3.3.6 Tissue Processing

At 21 days after surgery (**Figure 3.3.4**), rats were deeply anesthetized with an overdose of 40% urethane. Rats were transcardially perfused with 200 mL of 0.1% sodium nitrite in 0.1 M phosphate buffered saline (PBS, pH 7.4) and 400 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The vertebral column enclosing the lower lumbar and sacral spinal cord was removed and post-fixed in 4% paraformaldehyde for 48 hours at 4 ºC. A laminectomy was performed to extract the spinal cord. A 10 mm-long segment of spinal cord (containing segments L4-S1) was removed. To facilitate differentiation between the left (ipsilateral to injury) and right sides of the spinal cord during subsequent analyses, a shallow rostrocaudal incision was made on the right ventral side of the spinal cord. Spinal cord blocks were stored in 0.1 M Millonig's phosphate buffer (pH 7.4) at 4 ºC prior to sectioning. At least 24 hours before cryosectioning, lumbar spinal cord blocks were transferred to 20% sucrose in 0.1 M PB. After the tissue had sunk in sucrose, the block was embedded in a plastic mold covered in Optimal Cutting Temperature (OCT) medium and frozen at -80 ºC. The orientation of the spinal cord was marked on the mold and OCT block. The block was affixed to a cryostat chuck and sectioned into 25 μm-thick sections using a cryostat (Leica Microsystems, USA) set to an internal temperature of -35 ºC. Fine paintbrushes were used to flatten each section onto an anti-roll plate. Sections were thaw-mounted onto Fisherbrand Superfrost Plus or subbed (gelatin/chromium(III) potassium sulfate) glass microscope slides. Care was taken to avoid trapping bubbles under the sections. Slides were allowed to air dry at room temperature, and were stored at -20 ºC until use.

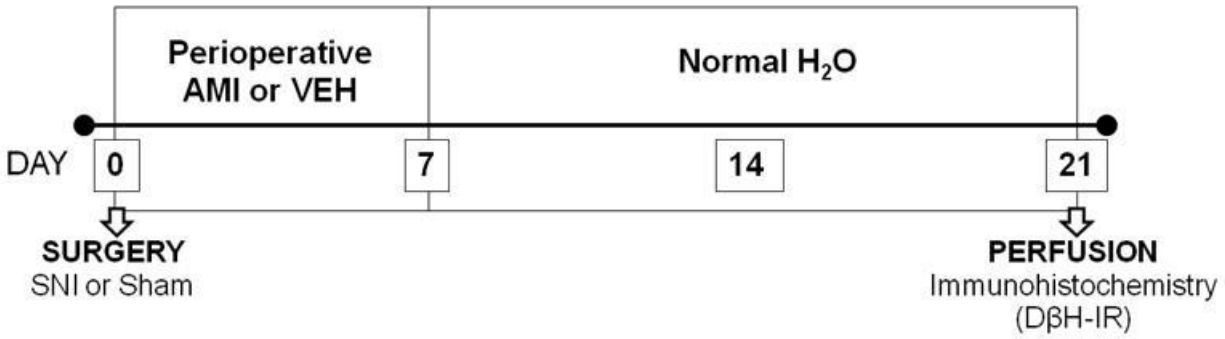

Figure 3.3.4 Timeline used for the immunohistochemistry experiment. Rats were subjected to either SNI or sham surgery, along with perioperative amitriptyline (AMI) or saline/water (VEH) treatment. At post-surgical day 21 (14 days after cessation of drug administration), rats were sacrificed and perfused for immunohistochemistry.

3.3.7 Immunohistochemistry

Immunohistochemical staining was performed directly on tissue that had been thaw-mounted onto slides. Slides were washed in a bath of 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 minutes before being incubated overnight at 4 ºC in a mouse monoclonal anti-DβH (1:250) diluted in 1% Triton-X 100 in PBS (PBST) and 2% normal goat serum (Millipore). Sections were incubated at room temperature in biotinylated goat anti-mouse IgG (1:500) diluted in PBST and 2% normal goat serum, and were subsequently processed using a Vectastain Elite ABC kit (1:500 of ABC solution diluted in PBST) (Vector Laboratories) for 90 minutes. Three types of controls were performed: 1) tissue stained with no primary antibody, 2) no secondary antibody, and 3) no ABC. All PBST-containing solutions (primary and secondary antibodies, as well as the ABC solution) were carefully applied drop-wise to slides (100-200 μL per slide for primary antibody; 200-400 μL per slide for secondary antibody and ABC solution). A small piece of Parafilm was placed over the liquid to seal it to the slide during the incubation step for the primary antibody only. During the antibody and ABC incubation steps, evaporation of the solutions was prevented by storing slides within plastic humidity chambers lined with moist paper towel strips. In between the incubation steps, slides were washed at least three times in a 0.1 M PBS bath, except just prior to the diaminobenzidine reaction, at which time slides were washed in a 0.1 M PB bath. Immunoprecipitates were developed with 3,3'-diaminobenzidine in PB (DAB, 0.5 mg/mL in 0.1 M PB) and 1% H_2O_2 in PB (30 μL/mL of DAB). Slides were dehydrated through ascending alcohols into xylene, and then coverslipped with Cytoseal 60 mounting medium (Thermo Scientific).

3.3.8 Imaging and Densitometric Analysis

Grayscale images of the left and right L4-L6 dorsal horns were captured using a Zeiss Axiocam High-Resolution Colour Camera mounted on an Axioplan II microscope (Carl Zeiss Microscopy, Germany). Raw images were captured at 20x magnification using AxioVision 4.7 software (Carl Zeiss Microscopy, Germany). Between 8 and 10 sections from L4-L6 of each spinal cord were used for the densitometric analyses, which were performed with ImageJ (National Institutes of Health, USA). Each image was labeled with a code in order to blind the experimental condition from the observer. Using ImageJ, a 250 μm x 250 μm square box was superimposed over the centre of each dorsal horn, covering the superficial laminae. The threshold function was applied to each image in order to distinguish DAB-stained neuronal projections against the background. The labeled pixel area was calculated as a percentage of the total area of interest. Mean values for the percentage of immunoreactive axons were calculated for individual rats as well as for each experimental condition (nerve injury, with or without amitriptyline).

3.3.9 Statistical Analyses

Data are presented as mean ± SEM. Statistical analyses were performed using the Student's t-test and ANOVA. Figure captions state which tests were used in each experiment. Statistical results were taken to be significant at $P < 0.05$.

3.4 RESULTS

3.4.1 Responses to Noxious Chemical Stimuli After SNI

Earlier experiments have shown that SNI increases nocifensive behaviours from 7-42 days post-surgery in response to local administration of αβ-MeATP into the lateral hindpaw, and that these responses are enhanced by NA (Arsenault & Sawynok, 2009; Meisner et al., 2008). Additionally, hyposensitivity to formalin 1% and 2.5% in the medial hindpaw has recently been reported following SNI (Sawynok & Reid, 2011). In the present study, an initial experiment involving sham and nerve injury groups was conducted in order to confirm the reproducibility of these responses to αβ-MeATP/NA and formalin 2.5%. Flinching responses to a lateral hindpaw injection of αβ-MeATP/NA (50 nmol/25 nmol) were assessed at 7 days following surgery. The SNI group displayed augmented flinching responses to αβ-MeATP/NA compared to sham-operated counterparts, but this trend only approached significance (**Figures 3.4.1A, 3.4.1B**). At 14 days after surgery, an injection of formalin 2.5% into the medial plantar hindpaw evoked significantly fewer phase 2 flinches in SNI-operated compared to sham-operated rats (**Figures 3.4.1C, 3.4.1D**). These observations essentially recapitulated earlier observations made in this laboratory.

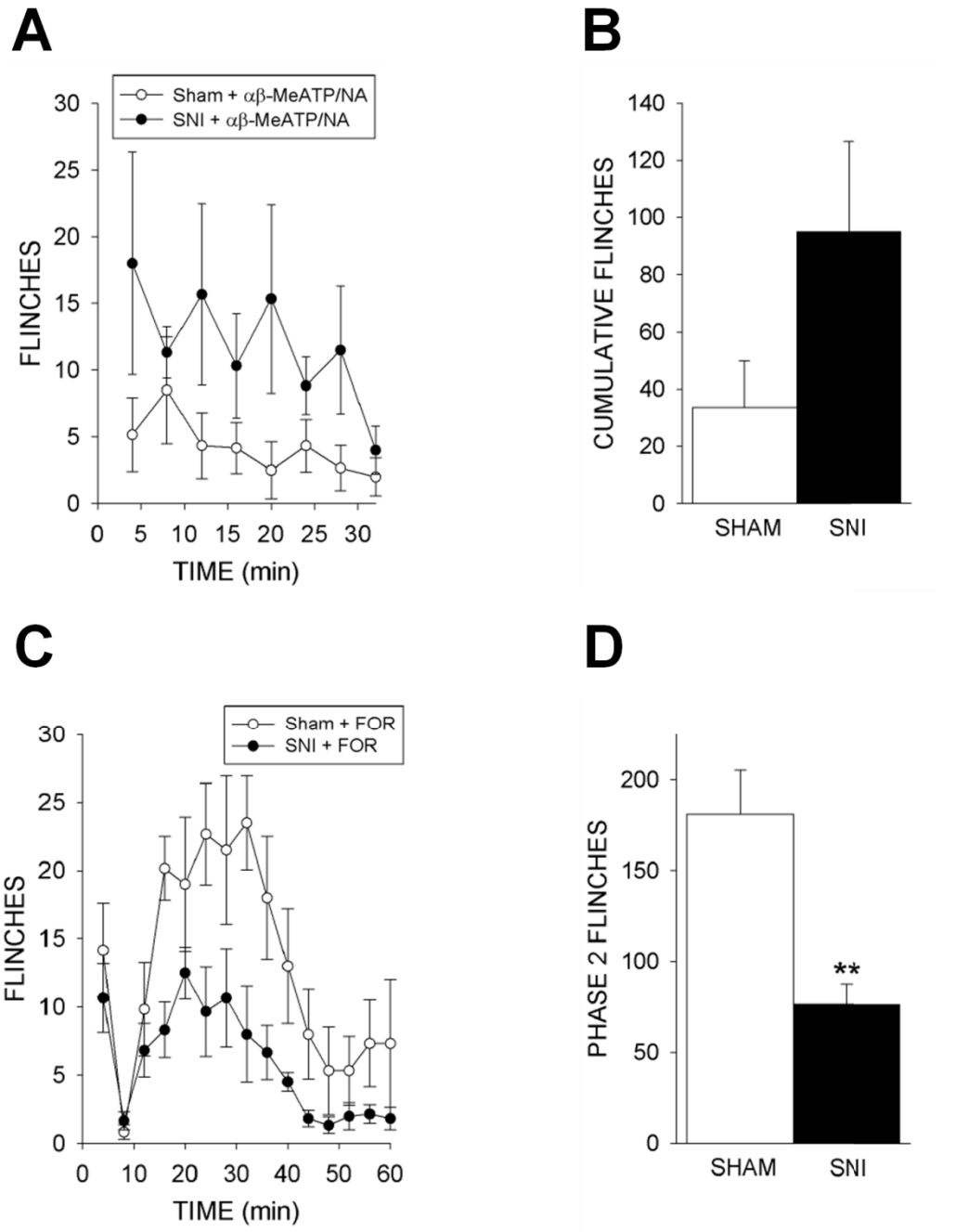

Figure 3.4.1 SNI produces hypersensitivity to αβ-MeATP/NA and hyposensitivity to formalin 2.5%. **(A)** Time course of flinching evoked by a lateral hindpaw injection of αβ-MeATP/NA (50 nmol/25 nmol) over 32 minutes, at post-surgical day 7. **(B)** Cumulative flinching responses evoked by αβ-MeATP/NA increased in SNI- compared to sham-operated rats, but this was not significant. ($P = 0.06$, 1-tailed unpaired Student's t-test; $n = 6$ per group) **(C)** Time course of flinching evoked by a medial plantar injection of formalin 2.5% over 60 minutes, at post-surgical day 14. **(D)** Cumulative phase 2 flinching responses to formalin were reduced in SNI- compared to sham-operated rats. (** $P < 0.01$, 2-tailed unpaired Student's t-test; $n = 6$ per group)

3.4.2 Effects of Perioperative Amitriptyline on Responses to Chemical Stimuli After SNI

In order to determine whether perioperative amitriptyline treatment could act as a preventive analgesic, thereby returning nocifensive responses to baseline levels in these paradigms, a series of experiments was conducted using parallel groups of SNI-operated rats that either received perioperative amitriptyline or vehicle treatment. At 14 days after surgery, flinching responses to a lateral hindpaw injection of αβ-MeATP/NA were significantly reduced in SNI-operated rats treated with perioperative amitriptyline compared to vehicle-treated SNI-operated rats (**Figures 3.4.2A, 3.4.2B**). At 21 days after SNI, phase 2 flinching behaviours evoked by a medial plantar hindpaw injection of formalin 2.5% did not differ significantly between rats that had been treated with perioperative amitriptyline or vehicle (**Figures 3.4.2C, 3.4.2D**).

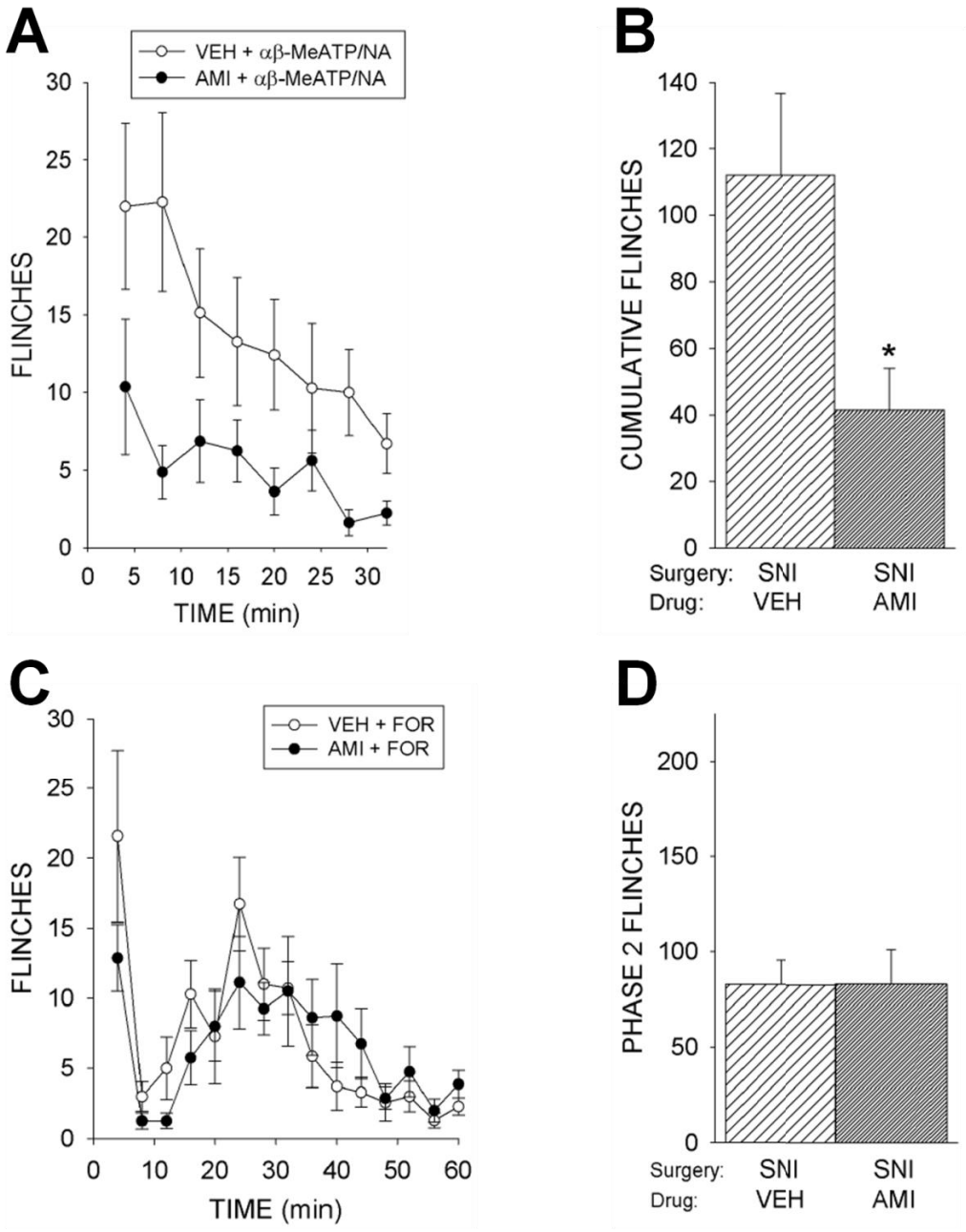

Figure 3.4.2 Perioperative amitriptyline (AMI) after SNI prevents hypersensitivity to αβ-MeATP/NA, but not hyposensitivity to formalin 2.5%. **(A)** Time course of flinching evoked in SNI rats (treated with AMI or vehicle (VEH)) by a lateral hindpaw injection of αβ-MeATP/NA (50 nmol/25 nmol) over 32 minutes, at post-surgical day 14.
(B) Cumulative flinching responses evoked by αβ-MeATP/NA were reduced by AMI (* $P < 0.05$, 2-tailed unpaired Student's t-test; n = 7-8 per group)
(C) Time course of flinching evoked in SNI rats (treated with AMI or VEH) by a medial plantar hindpaw injection of formalin 2.5% over 60 minutes, at post-surgical day 21.
(D) Cumulative phase 2 flinching responses to formalin were unaffected by AMI. ($P = 0.983$, 2-tailed unpaired Student's t-test; n = 7-8 per group)

3.4.3 Noradrenergic Sprouting After SNI and Perioperative Amitriptyline Treatment

The occurrence of noradrenergic axonal fibre sprouting in the dorsal horn of the lumbar spinal cord after peripheral nerve injury has been proposed to increase descending inhibitory tone, thereby potentially explaining the increased efficacy in chronic pain states of analgesics which enhance α_2-adrenergic receptor activity (Hayashida et al., 2008; Ma & Eisenach, 2003). Since amitriptyline recruits central noradrenergic systems in its pharmacological actions, and 6-OHDA reverses the preventive action of amitriptyline on chemogenic hypersensitivity (Arsenault & Sawynok, 2009), we examined DβH-IR in the superficial dorsal horn of the lumbar spinal cord (L4-L6) after SNI and also following perioperative amitriptyline treatment, at post-surgical day 21. Irrespective of experimental condition, we observed numerous DβH-IR axon varicosities and puncta across the entire dorsal horn. Representative photomicrographs taken from each experimental group are shown in **Figure 3.4.3**. In sham-operated rats, no differences in DβH-IR axonal fibre densities were observed between ipsilateral and contralateral superficial dorsal horns, irrespective of whether perioperative amitriptyline or vehicle treatment had been administered. In SNI-operated rats, DβH-IR axonal fibre densities in the ipsilateral and contralateral superficial dorsal horns also did not differ significantly, irrespective of the presence of perioperative amitriptyline (**Figure 3.4.4**) or vehicle treatment. However, in SNI-operated rats that had received perioperative amitriptyline, semi-quantitative analysis indicated that there was non-significant trend towards decreased DβH-IR axonal fibre density in the contralateral dorsal horn (**Figure 3.4.5**). Overall, neither nerve injury nor perioperative amitriptyline had any effect on the densities of DβH-IR axonal fibres in the spinal dorsal horn (L4-L6) (**Figure 3.4.5**).

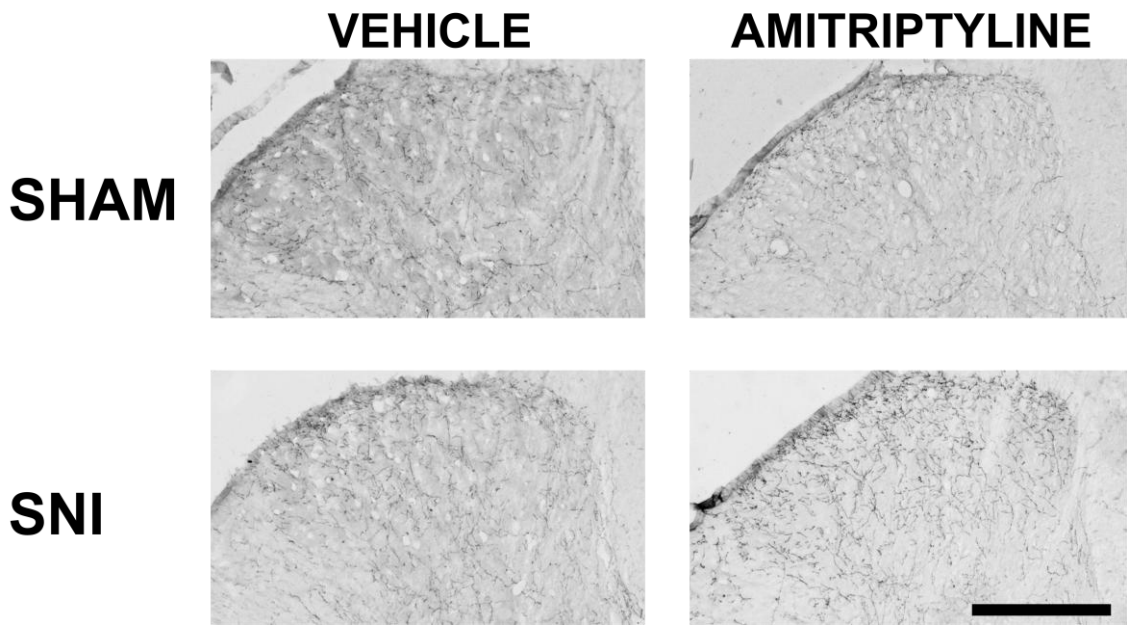

Figure 3.4.3 Representative photomicrographs of DβH immunoreactivity in the ipsilateral L5 dorsal horns of spinal cords taken from SNI- and sham-operated rats treated with perioperative vehicle or amitriptyline. Images were captured at 20x magnification. Scale bar = 200 μm.

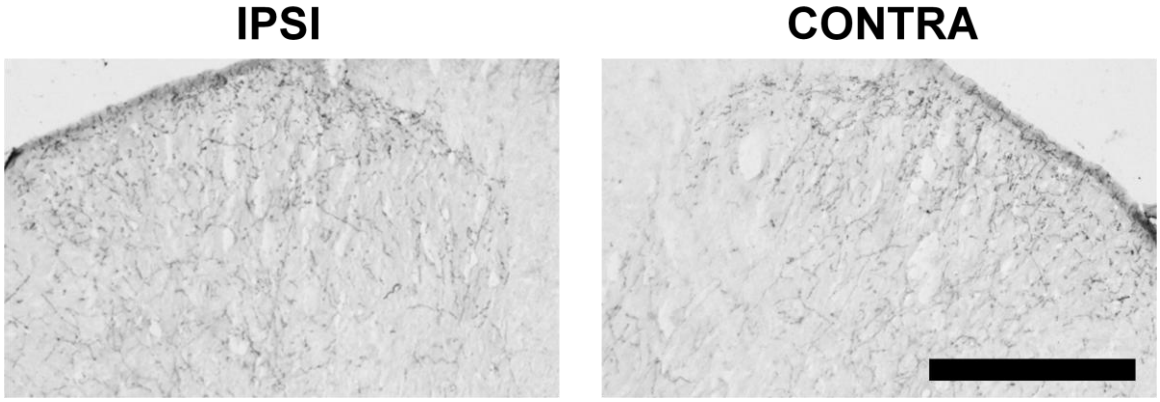

Figure 3.4.4 Representative photomicrographs showing DβH immunoreactivity (DβH-IR) in the L5 spinal dorsal horns of an SNI-operated rat treated with perioperative amitriptyline. Despite an apparent non-significant trend towards decreased DβH-IR in the contralateral (CONTRA) dorsal horns of this group (**Figure 3.4.5**), there were no consistent, visible differences in ipsilateral (IPSI) and CONTRA staining for any of the sections analyzed. For all other groups, DβH-IR in the CONTRA dorsal horns was not significantly different compared to the IPSI dorsal horns (not shown). Images were captured at 20x magnification. Scale bar = 200 μm.

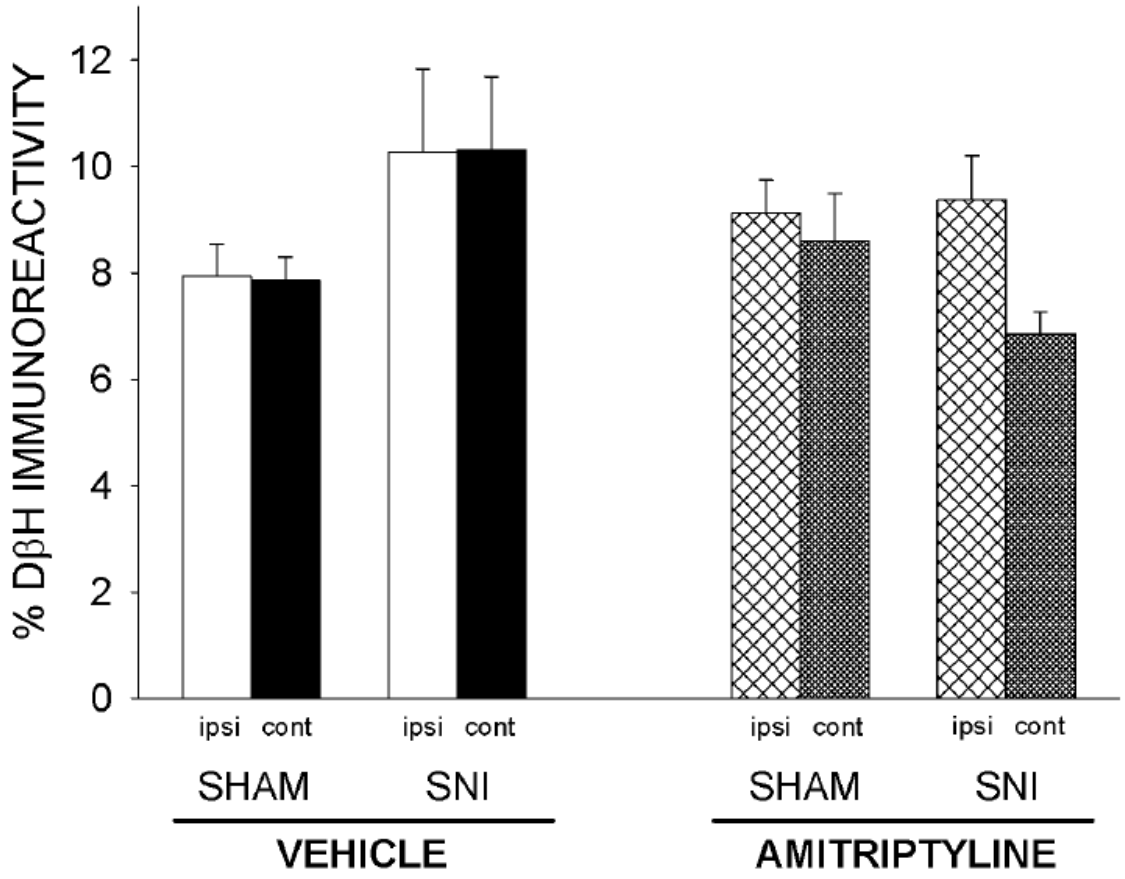

Figure 3.4.5 Sprouting of noradrenergic axonal fibres in the superficial dorsal horn of the lumbar spinal cord is not altered by SNI (left panels) or treatment with perioperative amitriptyline (right panels). Densities of axonal fibres immunoreactive for DβH of SNI-operated rats treated with perioperative vehicle or amitriptyline were not different between the ipsilateral (ipsi) or contralateral (cont) spinal dorsal horns (L4-L6) or between experimental groups. ($P > 0.05$, ANOVA; $n = 4$-5 per group)

3.5 DISCUSSION

SNI is a preclinical model of neuropathic pain which permits the discrete examination of the pathophysiological sensory changes which have occurred within distinct medial and lateral hindpaw sites (Decosterd & Woolf, 2000). In the present study, we used the SNI model to characterize the effects of perioperative amitriptyline treatment against the development of chemogenic hyper- and hyposensitivity in order to assess its potential use as a preventive analgesic. Additionally, we explored a potential anatomical basis for the central noradrenergic dependence of the preventive effects of perioperative amitriptyline after SNI.

3.5.1 Effect of Perioperative Amitriptyline on Responses to αβ-MeATP/NA After SNI

We found that in rats rendered neuropathic by SNI, perioperative amitriptyline treatment was able to prevent chemogenic hypersensitivity to αβ-MeATP/NA. Although the results of an initial experiment showed that SNI only produced a trend towards chemogenic hypersensitivity to αβ-MeATP/NA (**Figure 3.4.1B**), robust hypersensitive responses to this stimulus have been reported in previous literature (Arsenault & Sawynok, 2009; Meisner et al., 2008). Moreover, the initial experiment was subject to a great deal of experimental variability, due to my relative inexperience in performing the surgery and intraplantar injections. However, the subsequent experiment clearly showed that chemogenic hypersensitivity to αβ-MeATP/NA was prevented by perioperative amitriptyline treatment (**Figures 3.4.2A, 3.4.2B**). These results are in agreement with those of Arsenault and Sawynok (2009), who also showed that perioperative amitriptyline

reversed chemogenic hypersensitivity to αβ-MeATP/NA (given at 150 nmol/25 nmol, a higher dose level than that used in the present study). Hypersensitive flinching responses to αβ-MeATP following SNI are mediated by the sensitization of $P2X_3$ receptors expressed on peripheral terminals of primary afferents (Chen, Li, Wang, Gu, & Huang, 2005) and enhanced by the activation of α_1-adrenergic receptors (Meisner et al., 2008). Following SNI, the number of $P2X_3$ receptors expressed at the membranes of primary afferent terminals increases, although no change in the total $P2X_3$ gene expression occurs at the DRG (Chen et al., 2005). Whether perioperative amitriptyline administration affects the expression of genes involved in peripheral nociception was not examined in the present study.

3.5.1 Effect of Perioperative Amitriptyline on Responses to Formalin 2.5% After SNI

Formalin produces nociception by promoting the recruitment of local inflammatory mediators, as well as activating TRPA1 receptors (Bráz & Basbaum, 2010; McNamara et al., 2007; Sawynok & Reid, 2011). Hyposensitivity to formalin has been reported in several partial sciatic denervation models, including SNI (Sawynok & Reid, 2011; Sawynok, Reid, & Meisner, 2006; Vissers, Adriaensen, De Coster, De Deyne, & Meert, 2003). Since the reversal of hyposensitivity would be another useful measure of the efficacy of amitriptyline as a preventive analgesic, we explored the ability of the perioperative regime to resolve medial plantar hindpaw (saphenous and tibial nerve territory) hyposensitivity to formalin following SNI. The current study confirmed that formalin 2.5%-evoked phase 2 flinching behaviours were greatly reduced in SNI-

operated rats (**Figures 3.4.1C, 3.4.1D**), which was consistent with the observations of Sawynok and Reid (2011). However, perioperative amitriptyline did not prevent the development of this hyposensitivity at post-surgical day 21 (**Figures 3.4.2C, 3.4.2D**).

The dissociation of the effects of amitriptyline on various pain modalities and nerve injury models reflect diverse mechanisms which lead to the induction of these behaviours after particular forms of nerve injury. In the SNL and SNI models, amitriptyline may exhibit selective effects on certain behaviours mediated by C fibres, which are involved in thermal hyperalgesia (Esser et al., 2001) and chemogenic responses to αβ-MeATP/NA and capsaicin/NA (Arsenault & Sawynok, 2009). In mice, low concentrations of formalin (<0.5%) activate TRPA1 receptors on C fibres that co-express TRPV1 receptors (Bráz & Basbaum, 2010). However, higher concentrations of formalin (>0.5%) recruit various other mechanisms, and are believed to activate both C and A fibres to produce pain (Bráz & Basbaum, 2010). Following SNI in rats, mRNA transcripts of the TRPA1 gene are downregulated in DRGs (Staaf, Oerther, Lucas, Mattsson, & Ernfors, 2009), and partially may account for the development of chemogenic hyposensitivity after SNI (Sawynok & Reid, 2011). In the present study, we delivered a high concentration of formalin (2.5%) to the medial plantar hindpaw after SNI, but found that perioperative amitriptyline did not reverse hyposensitivity to this stimulus. Since A and C fibres may both become activated by high concentrations of formalin, it is conceivable that hyposensitivity to formalin, along with mechanical allodynia, a primarily A fibre-mediated behaviour (Esser & Sawynok, 1999; Field, McCleary, Hughes, & Singh, 1999), could also be resistant to perioperative amitriptyline treatment. Thus, the preservation of formalin 2.5% hyposensitivity following SNI and

perioperative amitriptyline appears to support the idea that the preventive effects of amitriptyline may be selective for C fibre mediated stimuli. Whether responses to lower concentrations of formalin (0.5%-1%) have greater C fibre dependence, or could be more sensitive to perioperative amitriptyline treatment, remains to be determined.

3.5.2 Modality-Dependent Analgesia by Amitriptyline After Nerve Injury

Although amitriptyline is widely used in the clinical treatment of neuropathic pain, its performance against different manifestations of pain in preclinical models has been somewhat inconsistent. When given acutely, chronically, or perioperatively, amitriptyline exerts heterogeneous analgesic effects against a variety of pain behaviours, which also appear to depend on the nerve injury model used. In animals with SNL and CCI, amitriptyline was shown to relieve thermal hyperalgesia, but not mechanical allodynia (Bomholt, Mikkelsen, & Blackburn-Munro, 2005; Esser et al., 2001; Esser & Sawynok, 1999, 2000). However, amitriptyline has also been reported to be effective against mechanical allodynia in a sciatic nerve crush model, but not in SNI (Decosterd, Allchorne, & Woolf, 2004). Different peripheral and central mechanisms could also be involved in the action of amitriptyline during the perioperative period, when injury-induced peripheral and central sensitization processes are presumably ongoing. When amitriptyline is given perioperatively after SNI, abnormal responses to αβ-MeATP/NA and capsaicin/NA return to baseline levels, whereas the development of mechanical allodynia remains unaffected (Arsenault & Sawynok, 2009). Furthermore, in the present

study, we observed that perioperative amitriptyline treatment after SNI prevented chemogenic hypersensitivity to αβ-MeATP/NA but not hyposensitivity to formalin 2.5%.

The results of the present study and those of Arsenault and Sawynok (2009) indicate that perioperative amitriptyline is a potential preventive analgesic against the development of specific forms of chemogenic afferent hyper- and hyposensitivity. However, perioperative amitriptyline is ineffective at preventing the development of hyposensitivity to formalin 2.5% (the present study), as well as mechanical allodynia (Arsenault & Sawynok, 2009) (see **Table 3.4.1**). Examining the analgesic efficacy profiles of other drugs may be useful in future studies, as it may be possible to enhance preventive analgesia by co-administering analgesics with "complementary" effects as part of a perioperative regime. An optimal combination of specific analgesics could potentially resolve abnormal responses in a greater number of pain modalities, producing a wider pain relief profile. Since the SNI model does not always recapitulate the efficacy of particular analgesics (Decosterd et al., 2004), multiple sensory tests in different neuropathic pain models may be helpful in evaluating the preclinical efficacy of a potential preventive analgesic drug combination (Berge, 2011).

Table 3.4.1 Differential effects of perioperative amitriptyline treatment after SNI on sensory endpoints in several pain behavioural paradigms

Test	Site	Post-SNI	+ perioperative AMI regime	Reference
von Frey	LAT	mechanical allodynia	no effect	Arsenault and Sawynok (2009)
αβ-MeATP/NA	LAT	hypersensitivity	return to baseline	Arsenault and Sawynok (2009), Present study
Capsaicin/NA	LAT	hyposensitivity	return to baseline	Arsenault and Sawynok (2009)
Formalin 2.5%	MED	hyposensitivity	no effect	Present study

LAT = lateral hindpaw testing site (sural nerve territory)
MED = medial plantar hindpaw testing site (saphenous and tibial nerve territory)

3.5.3 Absence of Spinal Noradrenergic Sprouting Following Nerve Injury

In the present study, an anatomical experiment was conducted in order to determine if injury- or analgesic drug-induced plasticity of noradrenergic fibres in the spinal cord could potentially provide the neural substrate which could account for preventive analgesic effects of perioperative amitriptyline after SNI. Although one report in mice (Ma & Eisenach, 2003) and another in rats (Hayashida et al., 2008) suggested that peripheral nerve injury leads to the *intrinsic* sprouting of noradrenergic axonal fibres in the spinal dorsal horn, we found no qualitative (**Figure 3.4.3**) or semi-quantitative evidence for this in the SNI model (**Figure 3.4.5**, left columns). Our results imply that, in the absence of analgesic drug treatment, the induction of SNI alone does not stimulate compensatory changes in the distribution of noradrenergic fibres in the dorsal horn.

Only two studies conducted to date have yielded positive results for noradrenergic sprouting following nerve injury. However, several major issues in methodology could

have seriously confounded the interpretation of the findings in the Ma and Eisenach (2003) study in relation to our results. The Ma and Eisenach (2003) study, which was conducted in CCI-operated mice, may have produced biased results due to inadequate controls, the stress of the behavioural protocol, as well as possible issues in antibody specificity. Normal animals were used as controls, but the immunohistochemical comparisons only examined differences between the ipsilateral and contralateral dorsal horns of CCI-operated mice (Ma & Eisenach, 2003). However, comparisons between the ipsilateral and contralateral sides of the spinal cord may not have been appropriate, since each CCI-operated mouse had also received a sham operation involving exposure of the contralateral sciatic nerve (Ma & Eisenach, 2003). Following the induction of a unilateral nerve injury, sensitization processes in the spinal cord could potentially lead to a spread of neuronal changes to the uninjured, contralateral body side (Kuner, 2010; Miletic & Miletic, 2002; Vissers et al., 2003). Furthermore, all animals in the Ma and Eisenach (2003) study underwent behavioural testing prior to being sacrificed, and thus stress-induced enhancement of TH in the locus coeruleus (Chang, Sved, Zigmond, & Austin, 2000; Watanabe et al., 1995) could have confounded the immunohistochemical results. Lastly, the quality of the DβH immunostaining appeared to be poor, possibly due to non-specific staining obtained through the use of a rabbit polyclonal antibody for DβH. Finally, the use of different nerve injury models, as well as potential species differences, could have accounted for the discrepancies between our results and those of Ma and Eisenach (2003).

In spite of the limitations of the Ma and Eisenach (2003) study, we explored the possibility that the lack of central noradrenergic sprouting in the SNI model could have

been reflective of different consequences arising from this form of injury as compared to others. In another set of anatomical experiments (**Appendix A**), we used immunohistochemistry of both TH (**Figure S1**) and DβH (**Figure S2**) in order to determine whether noradrenergic sprouting in the dorsal horn occurred after three different forms of sciatic nerve injury, namely SNL, PSL, and SNI. These models vary according to the extent of damage to the sciatic nerve, as well as the distance of the injury from the spinal cord. Initially, we had hoped to reproduce the findings of Hayashida et al. (2008), assuming that the SNL model would serve as a positive control for noradrenergic sprouting in the lumbar dorsal horn. Surprisingly, we did not observe any significant qualitative or semi-quantitative changes in the noradrenergic fibre densities of either the ipsilateral or contralateral dorsal horns in SNL-operated rats compared to sham-operated animals (**Appendix A**). Additionally, no qualitative or semi-quantitative changes in noradrenergic sprouting were observed in the PSL or SNI conditions (**Appendix A**).

Several methodological issues could provide clues as to why we could not replicate the findings of Hayashida et al. (2008). Firstly, the control group which served as a comparator for SNL-operated rats for the semi-quantification of spinal noradrenergic sprouting actually consisted of naïve, rather than sham-operated animals (Hayashida et al., 2008), whereas we exclusively used sham-operated animals as controls. Since the SNL procedure itself is highly-invasive, the sham surgery for this model also involves the partial removal of the articular facet and L4 transverse process (Esser & Sawynok, 1999; Kim & Chung, 1992). Thus, because the possibility cannot be ruled out that the sham procedure may potentially produce intrinsic effects on central sprouting, the absence of a sham group in the comparisons made by Hayashida et al. (2008) is surprising. In

addition, the magnitude of the sprouting reported by Hayashida et al. (2008) was small and it was somewhat unclear from the photomicrographs if the increase in DAB-staining intensity was due to increased background or to real axonal fibre sprouting. Moreover, a detailed anatomical comparison using both naïve and sham-operated animals would have potentially yielded information about whether this noradrenergic sprouting involved the formation of aberrant connections due to spinal cord circuit reorganization. Differences in tissue processing techniques, antibody specificity, staining protocols, and quantification methods could also have led to the conflicting results between our study and that of Hayashida et al. (2008).

Based upon our own results, and after careful consideration of the limitations of the two previous studies, we concluded that intrinsic central noradrenergic sprouting following a peripheral nerve injury was not as robust as had originally been assumed. Nevertheless, it was still a possibility that treatment with amitriptyline, if administered during the perioperative period, could lead to spinal noradrenergic sprouting and the reinforcement of descending inhibitory projections.

3.5.4 Absence of Spinal Noradrenergic Sprouting After Perioperative Amitriptyline

Since the ablation of spinal noradrenergic pathways by 6-OHDA was previously found to eliminate the preventive effects of perioperative amitriptyline after SNI (Arsenault & Sawynok, 2009), we hypothesized that perioperative amitriptyline treatment might facilitate and/or directly induce sprouting of descending noradrenergic projections in the lumbar dorsal horn following nerve injury, thereby counteracting effects of central

sensitization by enhancing inhibitory tone. In SNI-operated rats that received perioperative amitriptyline treatment, there appeared to be no obvious qualitative differences in noradrenergic fibre staining (**Figure 3.4.4**), although semi-quantitative analyses indicated that a modest decrease in the sprouting of noradrenergic fibres in the dorsal horn contralateral to nerve injury might have occurred (**Figure 3.4.5**, right columns). However, this trend only approached statistical significance. From these results, we concluded that sprouting of noradrenergic fibres in the dorsal horn does not occur following SNI or perioperative amitriptyline treatment. Therefore, the previously-reported central noradrenergic dependence of perioperative amitriptyline likely operates through alternative mechanisms.

That central noradrenergic sprouting was not found to be involved in the mechanism of perioperative amitriptyline after SNI does not rule out the possibility that descending noradrenergic pathways may still be recruited as part of the multiplicity of amitriptyline actions (Micó et al., 2006). During induction of neuropathic pain, peripheral, spinal, and supraspinal changes lead to an enhancement in pain signaling (Costigan et al., 2009). Simultaneously, this altered sensitivity of the central nervous system may lead to more favourable outcomes to antidepressants and anticonvulsants against neuropathic pain, when compared to responses to acute pain in the uninjured state (Bee & Dickenson, 2009).

One potential spinal mechanism which may explain the analgesic effects of NA release after nerve injury pertains to the enhancement of spinal α_2-adrenergic receptor coupling with the stimulatory G protein (G_s), which has been observed after SNL (Bantel, Eisenach, Duflo, Tobin, & Childers, 2005), and a resultant increase in activation of spinal

cholinergic inhibitory interneurons could account for a state-dependent analgesic effect (Hayashida & Eisenach, 2010). Indeed, antinociceptive effects of gabapentin and clonidine (Hayashida & Eisenach, 2011; Takasu, Honda, Ono, & Tanabe, 2006; Tanabe et al., 2005) have been attributed to actions upon this proposed descending noradrenergic-spinal cholinergic mechanism in the nerve-injured state. Changes in noradrenergic and cholinergic circuits in the spinal cord have been attributed to BDNF (Hayashida & Eisenach, 2011), which was previously implicated in the mechanism of perioperative amitriptyline (Arsenault & Sawynok, 2009). Growth factors are involved in injury-induced plasticity mechanisms at various levels of pain pathways, although the roles that they play on the induction, maintenance, and/or relief of neuropathic pain are still controversial (Bardoni & Merighi, 2009; Merighi et al., 2008). Amitriptyline has also been demonstrated to affect TrkA and TrkB neurotrophin receptors, which bind nerve growth factor (NGF) and BDNF, respectively (Jang et al., 2009; Rantamäki et al., 2011), and may be able to act upon a noradrenergic-spinal cholinergic circuit, or potentially other pathways, such as the descending serotonergic pathways that were discussed in Chapter 2. Future studies should be conducted to determine if, and how, perioperative amitriptyline recruits descending modulatory pathways.

3.5.5 Methodological Issues in the Quantification of Immunohistochemical Stains

Immunohistochemistry is a technique that is widely used for visualizing the levels of specific proteins within tissues. Although the validity of this technique in correlating protein levels with staining intensity has been confirmed through other assays, the

methods used in sample preparation, staining, and quantification are highly variable between different research groups and can hamper the comparison of results (Brey et al., 2003; Taylor & Levenson, 2006). Furthermore, since differences in background staining may occur, distinguishing cells from background staining frequently involves manual, rather than automated, setting of thresholds for densitometric analysis. However, the act of distinguishing signal from noise is highly subjective, and susceptible to observer bias (Taylor & Levenson, 2006). In the present study, efforts were made to blind the observer to the experimental condition during the semi-quantification procedure. However, a more robust method for obtaining consistent thresholds for densitometric analysis would likely have improved the reliability of the results. The lack of standardization in the methods used for immunohistochemistry is a major problem that needs to be addressed (Taylor & Levenson, 2006). Specifically, the issues surrounding the subjective nature of existing immunostain quantification methods have prompted the development of more sophisticated computer-assisted algorithms to reduce bias (Brey et al., 2003). Future experiments could use other biochemical assays, including Western blot analysis, to assist in detecting differences in protein levels.

3.6 SUMMARY AND CONCLUSIONS

Persistent post-surgical pain is a major clinical issue that has the potential to be addressed using a preventive analgesia strategy. If analgesics are administered during the perioperative period, they may be able to prevent peripheral and/or central sensitization processes that could lead to the development of persistent pain. Amitriptyline is currently widely used in the treatment of established neuropathic pain, and has shown some efficacy as a preventive analgesic in preclinical studies. In the present study, perioperative amitriptyline treatment following SNI prevented the development of afferent hypersensitivity to αβ-MeATP/NA. However, perioperative amitriptyline treatment had no effects on the development of hyposensitivity to formalin 2.5% following SNI. Although enhanced sprouting of pain inhibitory descending noradrenergic fibres has been observed in other nerve injury models, we found no evidence for changes in noradrenergic fibre sprouting in the lumbar dorsal horn of the spinal cord following SNI. In addition, perioperative amitriptyline treatment was not found to alter the densities of noradrenergic fibres. Further experiments are necessary to determine the nature of the involvement of central noradrenergic signaling in the context of the preventive effects of perioperative amitriptyline following nerve injury.

CHAPTER 4

CONCLUSION

4.1 ANTINOCICEPTIVE ACTIONS OF A_1RS IN THE MECHANISM OF AMITRIPTYLINE

4.1.1 Supraspinal Involvement in Antinociception by Systemic Amitriptyline

In Chapter 1, a mechanism of antinociception by systemic amitriptyline involving activation of A_1Rs secondarily to activation of 5-HT_7Rs in the spinal cord was proposed. However, it remains unclear whether supraspinal activation of serotonergic neurons is necessary for the subsequent activation of spinal A_1Rs, or if increased synaptic levels of 5-HT due to inhibition of reuptake within the spinal cord are sufficient to activate 5-HT_7Rs. Dissociation of the spinal A_1R and $5HT_7$R mechanism from the influence of the RVM could be one strategy for exploring potential synergism between spinal and supraspinal compartments in antinociception by systemic amitriptyline. Genetic ablation of RVM neurons has been accomplished in conditional knock-out mice (*Lmx1b*$^{f/f/p}$) which lack expression of the *Lmx1b* transcription factor in serotonergic RVM neurons (Zhao et al., 2006, 2007). In *Lmx1b*$^{f/f/p}$ mice, antinociception by i.p. amitriptyline in a threshold test of thermal hyperalgesia was found to be markedly reduced, suggesting that supraspinal control of descending serotonergic inhibition is important in nociception (Zhao et al., 2007). Whether antinociception by systemic amitriptyline in the formalin test is diminished in *Lmx1b*$^{f/f/p}$ mice is unknown. Pharmacological antagonism of spinal A_1Rs and/or 5-HT_7Rs prior to formalin testing could unmask the roles of these receptors in residual spinal antinociception by systemic amitriptyline.

4.1.2 Selective Knock-Down of A_1Rs in Specific Compartments

Antinociception by systemic amitriptyline can be mediated by spinal and peripheral compartments. Both of these actions could potentially be inhibited by caffeine. The results presented in Chapter 2 demonstrate that in normal mice, antinociception by systemic amitriptyline was blocked by spinal and peripheral administration of DPCPX. In both cases, spinal and peripheral DPCPX were unable to block the action of systemic amitriptyline in mice lacking the A_1R gene. These results suggest that amitriptyline is able to recruit mechanisms that are independent of A_1R activation, and that these are sufficient to reduce nociception. However, a major caveat of using a knock-out mouse model is that the absence of a particular gene during development could have caused compensatory changes in the expression of other genes. In order to circumvent the issues of using a developmental A_1R knock-out model, it would be interesting to knock-down the expression of A_1Rs in specific compartments using RNA interference (RNAi).

RNAi is a method of gene downregulation that involves the delivery of small interfering RNA (siRNA) molecules into cells, where specific mRNA transcripts are targeted for degradation through the formation of an RNA induced silencing complex (RISC) (Clark & Miranpuri, 2010; Luo et al., 2005). Direct delivery of siRNA into the central nervous system is challenging, as these molecules do not cross the blood-brain-barrier (Clark & Miranpuri, 2010; Luo et al., 2005). Technical advancements involving once-daily i.t. injections of siRNA using specialized cationic transfection reagents or nanoparticles have led to the robust silencing of several pain-related genes in the spinal cord and DRG (Clark & Miranpuri, 2010). Downregulation of spinal A_1Rs prior to formalin testing in normal adult mice could be achieved by repeated i.t. delivery of

siRNA. Additionally, A_1Rs in the periphery could be silenced prior to formalin testing by i.pl. delivery of siRNA. Together, these experiments could reveal whether the presence of A_1Rs in normal animals is mandatory for antinociception by systemic amitriptyline in the formalin test.

4.1.3 Spinal Adenosine and Serotonin System Changes in Neuropathic Pain States

Although the second phase of the formalin test is thought to be reflective of central sensitization processes that occur during the induction of chronic pain (Sawynok & Liu, 2003b), it would be interesting to confirm whether the antinociceptive mechanism involving A_1Rs and 5-HT_7Rs is recruited in the actions of systemic amitriptyline against nocifensive behaviours following peripheral nerve injury. Since various nerve injury procedures have been adapted for mice, it would be possible to follow the same drug administration protocols as those used in Chapter 2 in order to assess pain-related behaviours of normal and A_1R -/- neuropathic mice. Specifically, DPCPX or SB269970 could be given by i.t. injection before systemic amitriptyline, and then pain-related behaviours could be assessed using threshold nociceptive tests (e.g. for mechanical allodynia, thermal hyperalgesia,), through the delivery of chemogenic stimuli (i.pl. αβ-MeATP/NA, capsaicin/NA), or even using a conditioned place preference test for revealing spontaneous pain (see section 4.2.1). The effect of i.t. AS-19 on pain-related behaviours of neuropathic normal and A_1R -/- mice would also be interesting to observe.

4.2 ANTIDEPRESSANTS AS PREVENTIVE ANALGESICS

4.2.1 Characterization of Spontaneous Pain Following Nerve Injury

In general, animal models of neuropathic pain recapitulate some of the key symptoms that are experienced by patients (allodynia, hyperalgesia, and hyposensitivity), and a battery of behavioural tests are typically used to assess the efficacy of analgesics against *stimulus-evoked pain*. However, a highly clinically relevant measure of pain that has been almost completely overlooked in nerve-injured animals is the manifestation of *spontaneous* or *tonic pain* (King et al., 2009). Unfortunately, the presence of spontaneous pain cannot be detected using traditional tests which measure reflexive behaviours. To circumvent this issue, King and colleagues (2009) developed a conditioned place preference test for spontaneous pain which involves training nerve-injured rats to associate particular contextual cues with analgesic administration. During the test, nerve-injured rats are placed drug-free in a testing area and permitted to choose between two chambers: one that is previously paired with spinal analgesic drug administration, and one that is paired with saline administration (King et al., 2009). Injured animals spend more time in the drug-associated chamber compared to sham-operated animals, but only if analgesics that are able to reduce tonic, but not stimulus-evoked, pain had been administered during the conditioning period (King et al., 2009). The conditioned place preference test is a simple, economical, and useful tool for screening of preventive analgesics, and can be utilized in both rats and mice (He, Tian, Hu, Porreca, & Wang, 2012; King et al., 2009).

From our results and those of Arsenault and Sawynok (2009), it appears that perioperative amitriptyline administration prevents the development of hypersensitivity to

αβ-MeATP/NA and hyposensitivity to capsaicin/NA, but it has no preventive analgesic efficacy against hyposensitivity to formalin 2.5% or mechanical allodynia. However, perioperative amitriptyline may well be effective against other symptoms of neuropathic pain, such as spontaneous pain. Although modifications in the administration of perioperative amitriptyline would be necessary, a more complete efficacy profile of amitriptyline against various outcomes following nerve injury could be obtained by including the conditioned place preference test for spontaneous pain in a battery of behavioural tests.

4.2.2 Contribution of Noradrenergic and Serotonergic Systems to Preventive Analgesia

Clinical and preclinical data have indicated that TCAs and SNRIs are more efficacious against neuropathic pain than SSRIs (Finnerup et al., 2005; Mochizucki, 2004; Nakajima et al., 2012; Saarto & Wiffen, 2010). As such, the inhibition of NA reuptake has been presumed to be a large contributing factor to the efficacy of TCAs and SNRIs (Mochizucki, 2004; Nakajima et al., 2012). The bidirectional effects of 5-HT on pain could be one reason that SSRIs are less effective, especially as descending serotonergic facilitation appears to be enhanced in chronic pain states (Bee & Dickenson, 2009; King et al., 2009). However, 5-HT still may play a critical role in analgesia in parallel with NA, as dual reuptake inhibition of 5-HT and NA produces better pain relief than reuptake inhibition of either monoamine alone (Micó et al., 2006; Mochizucki, 2004). In spite of their lower efficacy, SNRIs and SSRIs produce fewer side effects than

TCAs do, and are, therefore, still valuable drugs for treating chronic pain (Finnerup et al., 2005; Saarto & Wiffen, 2010).

Perioperative regimes of both desipramine and fluoxetine, which are antidepressants that selectively inhibit NA and 5-HT reuptake respectively, have been demonstrated to prevent chemogenic hypersensitivity to αβ-MeATP/NA after SNI in rats (Green & Sawynok, 2010). Since the tolerability of SNRIs and SSRIs is greater compared to TCAs, further exploration of the utility of these drugs in preventive analgesia is certainly warranted.

In order to specify the contribution of each monoamine system to mechanisms of preventive analgesia, it may be useful to further examine the potential molecular effects of desipramine, fluoxetine, as well as amitriptyline, on reversing nerve injury-induced changes in the spinal cord. Specifically, it would be useful to characterize nerve injury-induced changes that may occur in central noradrenergic and serotonergic pathways, while determining the effects of perioperatively administering different antidepressants. Since growth factor inhibition (via i.t. administration of antibodies to BDNF and glial-derived neurotrophic factor or GDNF) during the perioperative period abolishes preventive effects on chemogenic hypersensitivity after SNI (Arsenault & Sawynok, 2009), it will be important to determine whether any injury-induced changes in noradrenergic and serotonergic fibres in the spinal dorsal horn could also be affected by the absence of these growth factors.

Various molecular tools could be utilized to accomplish the objectives listed above. Changes in the levels of NA and 5-HT in the spinal cord could be measured using microdialysis and high-performance liquid chromatography. Anatomical changes could

also be assessed using immunohistochemistry of DβH and 5-HT in the brainstem, as well as DβH, 5-HT, 5-HT_7Rs, 5-HT_3Rs, and/or α_2-adrenergic receptors in the spinal cord. In addition, changes in the expression of various genes relating to NA and 5-HT systems could be determined using *in situ* hybridization or quantitative PCR.

4.3 CONCLUDING REMARKS

In this thesis, two different preclinical models of persistent pain were used to explore different mechanisms involved in the antinociceptive and preventive analgesic effects of amitriptyline. Importantly, actions of systemic amitriptyline at A_1Rs, in both spinal and peripheral compartments, appear to be intimately involved in antinociception. Hopefully, future experiments will provide important mechanistic insights into how amitriptyline and other antidepressants produce acute antinociception, as well as long-term preventive effects on the development of chronic pain. Since antidepressants are already prescribed to manage chronic pain, understanding the specific central and peripheral mechanisms that are recruited might allow healthcare practitioners to efficiently maximize the beneficial effects of these drugs, potentially promoting a longer-lasting method of pain treatment with greater tolerability. Finally, further progress in this research area would aid in the identification of antidepressants and novel compounds that may be considered in clinical trials of new treatments or preventive drug regimes for chronic pain.

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APPENDIX A

LACK OF SPINAL NORADRENERGIC SPROUTING AFTER NERVE INJURY

This Appendix contains supplementary results of a preliminary anatomical experiment which compared the extent of spinal noradrenergic fibre sprouting in three different peripheral nerve injury models (SNI, SNL, and PSL) at post-surgical day 14.

All experimental subjects and general methods (surgery, tissue preparation, imaging, and quantification), used in these preliminary experiments are the same as those outlined in Chapter 3. However, specific variations to the surgical procedure were made depending on which form of nerve injury was being induced. SNL and PSL surgeries were performed by an experienced technician (Allison Reid). Differences in the surgical procedures are summarized in **Table S1**.

Table S1 Surgical procedures of three nerve injury models which were used for the comparative anatomical experiment

Nerve Injury Model	Surgical Procedure
Spinal Nerve Ligation (SNL) *Kim & Chung (1992)*	Tight ligation of L5 and L6 spinal nerves (proximal to the DRG)
Partial Sciatic Nerve Ligation (PSL) *Seltzer et al. (1990)*	Tight ligation of approximately one-half of the sciatic nerve (at the thigh level)
Spared Nerve Injury (SNI) *Decosterd & Woolf (2000)*	Tight ligation and transection of the tibial and common peroneal branches of the sciatic nerve, with "sparing" of the sural branch (at the knee level)

Achieving high-quality histology of the rat spinal cord is challenging, given the small size and delicacy of the tissue. During the course of the study described in Chapter 3, two different tissue processing methods were refined and employed, and these involved the production of floating and frozen sections using a freezing microtome and a cryostat, respectively. Floating sections were produced during all preliminary experiments. To produce 40 μm-thick floating sections, the lumbar spinal cord block was frozen with dry ice and cut transversely using a freezing microtome at -40 ºC. Spinal cord sections were stored at 4 ºC in 0.1 M PBS prior to use. During immunostaining, solutions in each beaker were changed by transferring the spinal cord tissue into small metal strainers. Generally, the intervening buffer washes, H_2O_2 pre-treatment, and DAB steps were the same as outlined in section 3.3.7, except incubations always took place in beakers. Staining from two primary antibodies was examined, and the dilution factors of these were slightly different from those used for frozen sections. Sections were incubated overnight at room temperature in either rabbit polyclonal anti-TH (1:500) or mouse monoclonal anti-DβH (1:500) diluted in PBST and 2% normal goat serum. Later, sections were incubated at room temperature in biotinylated goat anti-rabbit IgG (1:500) for anti-TH staining or biotinylated goat anti-mouse IgG (1:500) for anti-DβH staining. After staining was complete, sections were mounted on gelatin-subbed microscope slides using a fine paintbrush.

However, no quantitative differences existed in the immunoreactivity of TH (**Figure S1**) or DβH (**Figure S2**) within any of the nerve injury conditions. Since the floating sections method may be subject to several major disadvantages (**Table S2**), we decided to switch to the frozen sections method (**Table S3**).

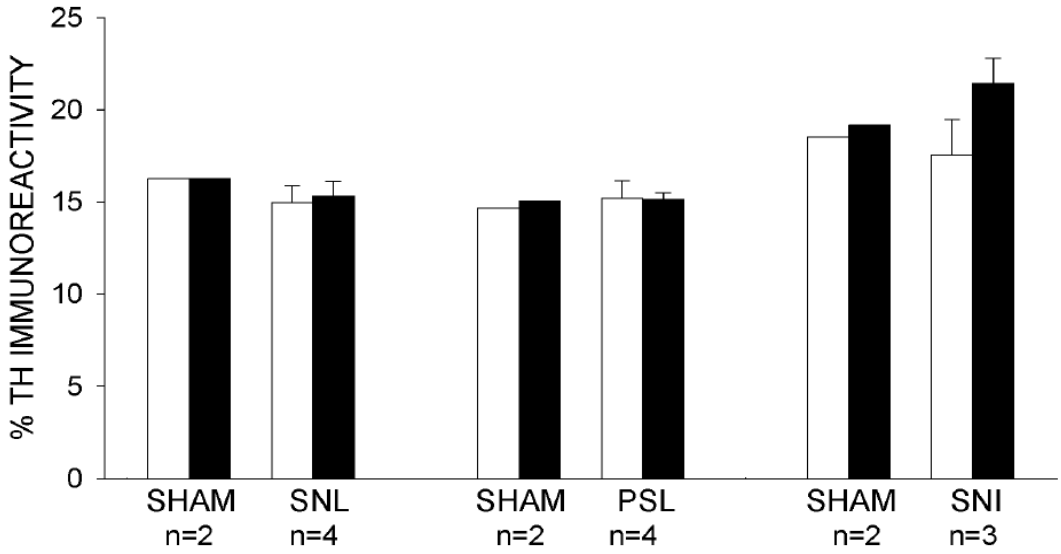

Figure S1 Tyrosine hydroxylase (TH) immunoreactivity (IR) in the L4-L6 dorsal horn of the rat spinal cord following three forms of peripheral nerve injuries (SNL, PSL, and SNI). Sprouting in the region of interest (see section 3.3.8) was not obvious in any of the nerve injury models at post-surgical day 14. White bars depict TH-IR in the ipsilateral dorsal horn, whereas black bars depict TH-IR in the contralateral dorsal horn.

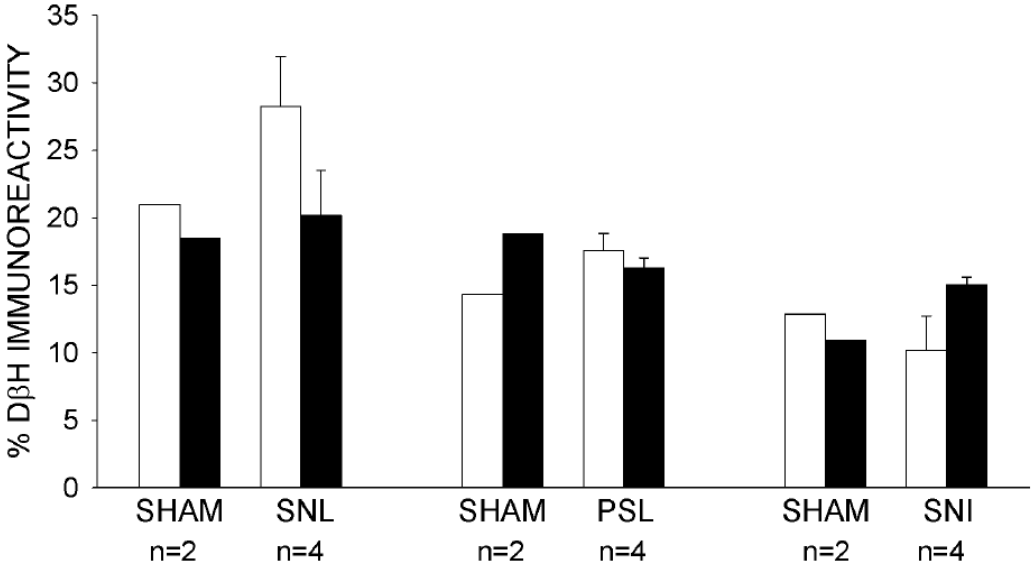

Figure S2 Dopamine-β-hydroxylase (DβH) immunoreactivity (IR) in the lumbar dorsal horn (L4-L6) of the rat spinal cord following three different forms of peripheral nerve injuries (SNL, PSL, and SNI). Sprouting in the region of interest (see section 3.3.8) was not obvious in any of the nerve injury models at post-surgical day 14. White bars depict DβH-IR in the ipsilateral dorsal horn, whereas black bars depict DβH-IR in the contralateral dorsal horn.

Table S2 Comparison of advantages and disadvantages of using floating sections

Advantages	Disadvantages
• Cutting is not labour-intensive and is relatively fast • Immunostaining quality is very good (our protocol was initially optimized for this method)	• Difficult to maintain an optimal cutting temperature with dry ice • Possibility of cross-contamination between beakers or tissue loss (due to small size of spinal cord sections) • Increased likelihood of tissue damage during handling • Sections are not mounted in accurate serial order (difficult to analyze data) • Mounting sections by hand is time consuming • Only thick sections can be cut

Table S3 Comparison of advantages and disadvantages of using frozen sections

Advantages	Disadvantages
• Cryostat maintains optimal cutting temperature automatically (better tissue integrity) • Minimal handling of tissue during cutting and staining (reduced physical damage) • Zero chance of cross-contamination between different spinal cord samples • Sections are mounted in serial order, allowing for more efficient data analysis • Thinner sections can be cut to produce higher resolution of staining	• Slower processing time • Risk of sections coming loose off slides (depends on quality of subbed slides and thickness of sections) • Immunostaining quality may be poorer if primary, secondary, or ABC solutions are not evenly applied to the slide