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The role of the adrenergic system in the recovery of motoneuron excitability and spasms after spinal cord injury

by

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ABSTRACT

Brainstem derived noradrenaline (NA) in the spinal cord functions both to increase motoneuron excitability, by facilitating calcium-mediated persistent inward currents (Ca PICs), and to inhibit sensory afferent transmission to motoneurons (excitatory postsynaptic potentials; EPSPs). Spinal cord injury (SCI) results in a reduction of NA, causing a loss of Ca PICs in motoneurons below the lesion and exaggerated EPSPs to emerge. With time motoneuron Ca PICs gradually recover and are readily triggered by the exaggerated EPSPs, resulting in the development of muscle spasms. The role of the NA in the recovery of Ca PICs and muscle spasms after chronic SCI is examined in this thesis using a rat model of spasticity incorporating both the awake rat (*in vivo*) and the isolated rat spinal cord (*in vitro*). Specific activation of the adrenergic α_1 receptor with agonists facilitated Ca PIC and spasms, whereas activation of the adrenergic α_2 receptor with agonists decreased the EPSPs that trigger spasms. Both receptors were endogenously activated by a ligand *in vivo*, though the α_1 receptor additionally exhibits constitutive activity (activity in the absence of NA), predominantly *in vitro*. The adrenergic α_2 receptor was not found to be endogenously active *in vitro*. Use of amphetamine in rats, which causes a forced efflux of endogenous NA, confirmed the identity of the endogenous ligand as NA and demonstrated that a residual source of NA capable of facilitating the Ca PIC and spasms persists below a chronic transection. Immunohistochemical labelling for an enzyme involved in the synthesis of NA (dopamine-β-hydroxylase) revealed that NA is not synthesized in the spinal cord below a chronic transection,

indicating that the endogenous NA is not intrinsic to the spinal cord. Peripheral injections of NA were used to demonstrate that the residual NA instead originates in the periphery (blood) and is both passively and actively transported across a compromised blood-brain barrier (BBB) after chronic injury. The peripherally derived NA activates central adrenergic receptors to modulate motoneuron excitability, sensory synaptic transmission and muscle spasms after chronic SCI. This novel finding highlights the importance of understanding the adaptations of neurotransmitter systems after injury when developing effective treatment strategies for spasticity.

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LIST OF ABBREVIATIONS USED

5-HT	5-hydroxytryptamine (serotonin)
5-HTP	5-hydroxytryptophan
6-OHDA	6-hydroxydopamine
AADC	aromatic amino acid decarboxylase
ACSF	artificial cerebrospinal fluid
AD	adrenaline
AHP	afterhyperpolarization
ATP	adenosine-5'-triphosphate
BBB	blood-brain barrier
cAMP	3',5'-cyclic adenosine monophosphate
Ca PIC	calcium persistent inward current
Ca_v	voltage-gated calcium channel
CNS	central nervous system
COMT	catechol-O-methyltransferase
CPG	central pattern generator
CSF	cerebrospinal fluid
DA	dopamine
DAG	diaglycerol
DAT	dopamine transporter
DβH	dopamine-β-hydroxylase
DCC	discontinuous current clamp
DOI	2,5-Dimethoxy-4-iodoamphetamine hydrochloride
DOPA decarboxylase	3, 4-dihydroxyphenylalanine decarboxylase
EC50	half maximal effective concentration
EMG	electromyography
EPSP	excitatory post synaptic potential
Fps	frames per second
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein

GIRK channels	G-protein-coupled inwardly-rectifying K^+ channels
G_m	resting membrane conductance
GPCR	G-protein-coupled receptor
I_h	hyperpolarization-activated mixed cation current
I_{on}	onset current of PIC
IP	intraperitoneal (injection)
IP_3	inositol triphosphate
IPSP	inhibitory post synaptic potential
IT	intrathecal (injection)
KCC2	potassium-chloride cotransporter
K_i	binding affinity
L-DOPA	L-3, 4-dihydroxyphenylalanine
LC	locus coeruleus
LLR	long-lasting reflex
MAO	monoamine oxidase
mACSF	modified artificial cerebrospinal fluid
mRNA	messenger ribonucleic acid
NA	noradrenaline
nACSF	normal artificial cerebrospinal fluid
Na PIC	sodium persistent inward current
NET	noradrenaline transporter
PAD	post-activation depression
pAD	peripheral adrenaline
PIC	persistent inward current (both Na PIC and Ca PIC)
PKA	protein kinase A
PKC	protein kinase C
pNA	peripheral noradrenaline
PNMT	phenethanolamine N-methyl transferase
REM	rapid eye movement
R_m	input resistance
RT	reflex threshold

rTAR1	G-protein coupled rat trace amine receptor
SCI	spinal cord injury
SE	standard error
SERT	serotonin transporter
SEVC	discontinuous single electrode voltage clamp
SK	calcium-activated potassium channel
T	threshold
TDI	tail drag index
TH	tyrosine hydroxylase
TTX	tetrodotoxin
V_m	resting membrane potential
VMAT	vesicular monoamine transporter
V_{on}	PIC voltage onset

Chapter 1:

Introduction

1.1 FOREWORD

Following a spinal cord injury (SCI), individuals often develop a debilitating spastic syndrome, which results primarily in the involuntary and sustained contraction of muscles, but also includes increased muscle tone, clonus, and exaggerated reflexes (Ashby et al. 1987; Bennett et al. 2004; Gorassini et al. 2004; Kuhn and Macht 1949; Maynard et al. 1990; Young 1994). It has been estimated that 67-78% of individuals develop spasticity in the months following SCI, making this disorder a common occurrence in patients with SCI (Maynard et al. 1990; Nielsen et al. 2007). The development of spasticity after SCI represents a recovery of neuronal excitability below a chronic injury, and therefore the study of the mechanisms underlying this plasticity is of great scientific interest.

Normal excitability in the spinal cord is heavily influenced by descending monoaminergic input, particularly noradrenaline (NA) and serotonin (5-HT). The supply of these brainstem derived neuromodulators is eliminated below a complete spinal transection, initially resulting in a loss of motoneuron excitability and flaccid paralysis in the affected muscles. However, despite the absence of neuromodulatory input, in the months following SCI motoneuron excitability below the transection gradually returns and is expressed as spasms in the affected muscles. This thesis explores the mechanisms underlying this recovered motoneuron excitability with a focus on how changes in the adrenergic system after chronic SCI contribute to the development of spasticity.

In order to better understand the adaptations in adrenergic input to the spinal cord after SCI, it is helpful to consider the normal physiology of the central adrenergic system, including the biosynthesis cycle of NA, the anatomy of descending adrenergic pathways and the functions of different adrenergic receptors (discussed in detail below). The selective distribution of these receptors in the dorsal, intermediate and ventral areas of the spinal cord influences the balance of inhibition and excitation that descending adrenergic innervation provides in the

normal spinal cord. By first outlining the function of adrenergic innervation in the normal spinal cord, the changes that result from the removal of this input after SCI can be better understood.

Although the primary focus of this thesis is the study of changes in adrenergic input below a chronic transection and the effect on motoneuron excitability and spasms, spinal transection does result in changes directly to various inhibitory and excitatory interneuron pathways as well. Although direct recordings from interneurons are not presented in this thesis, post-synaptic potentials are measured (see Chapter 2) which reflect the activation of motoneurons via sensory pathways, including interneurons. Alterations to the various interneuron pathways additionally contribute to the hyperexcitability of reflexes and lack of inhibitory control over sensory afferent transmission that is associated with spasticity. The effects of spinal transection on motoneuron excitability, interneuron pathways and the blood-brain barrier (BBB) are described in detail below and help to provide a useful context for the results obtained in this thesis.

1.2 THE BIOSYNTHESIS CYCLE OF NORADRENALINE

1.2.1 Synthesis

Noradrenaline, along with dopamine (DA) and adrenaline (AD), are members of a group of neurotransmitters called catecholamines. Catecholamines are synthesized in the CNS tissue as well as in peripheral tissues such as adrenal chromaffin cells and sympathetic nerves. All catecholamines share a common biosynthetic pathway beginning with the amino acid precursor L-tyrosine (Fig 1-1). Tyrosine is present in the bloodstream and is actively transported from the circulation to catecholaminergic cells in the periphery or in the CNS, where it is then concentrated for use in the biosynthetic pathway. The first step in the biosynthesis of NA is the conversion of L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH).

TH is the rate-limiting step in the catecholamine biosynthetic pathway, as inhibition of TH significantly reduces the endogenous concentrations of NA and DA in the brain and in many tissues with sympathetic innervation (Kopin 1968; Moore and Bloom 1979). The enzyme 3, 4-dihydroxyphenylalanine decarboxylase (DOPA decarboxylase), which is also sometimes referred to as aromatic amino acid decarboxylase (AADC), then catalyses the formation of DA from L-DOPA (Kopin 1968). This conversion occurs very rapidly with nearly no time lag between the formation of L-DOPA and its conversion to DA (Kopin 1968). The transformation of DA to NA is completed by the enzyme dopamine-β-hydroxylase (DβH). The expression of the mRNA for DβH is limited to noradrenergic neurons or adrenal chromaffin tissue (Cooper et al. 2003), thereby making DβH expression in tissues an ideal marker to differentiate NA neurons from DA neurons. Experiments described in Chapter 4 of this thesis capitalize on this fact and use DβH labelling to locate any NA synthesizing neurons caudal to chronic spinal transection. As the conversion of DA to NA occurs within the vesicle itself, this is where DβH is concentrated, with much of the DβH bound to the inner vesicular membrane and only a minimal amount present free within the vesicular space (Siegel et al. 2006). In cells that synthesize AD, the final step in the biosynthesis pathway is the conversion of NA to AD by the enzyme phenethanolamine N-methyltransferase (PNMT). This enzyme is primarily restricted to cells in the adrenal medulla, with a negligible amount detectable in a small group of AD releasing brainstem cells (Siegel et al. 2006).

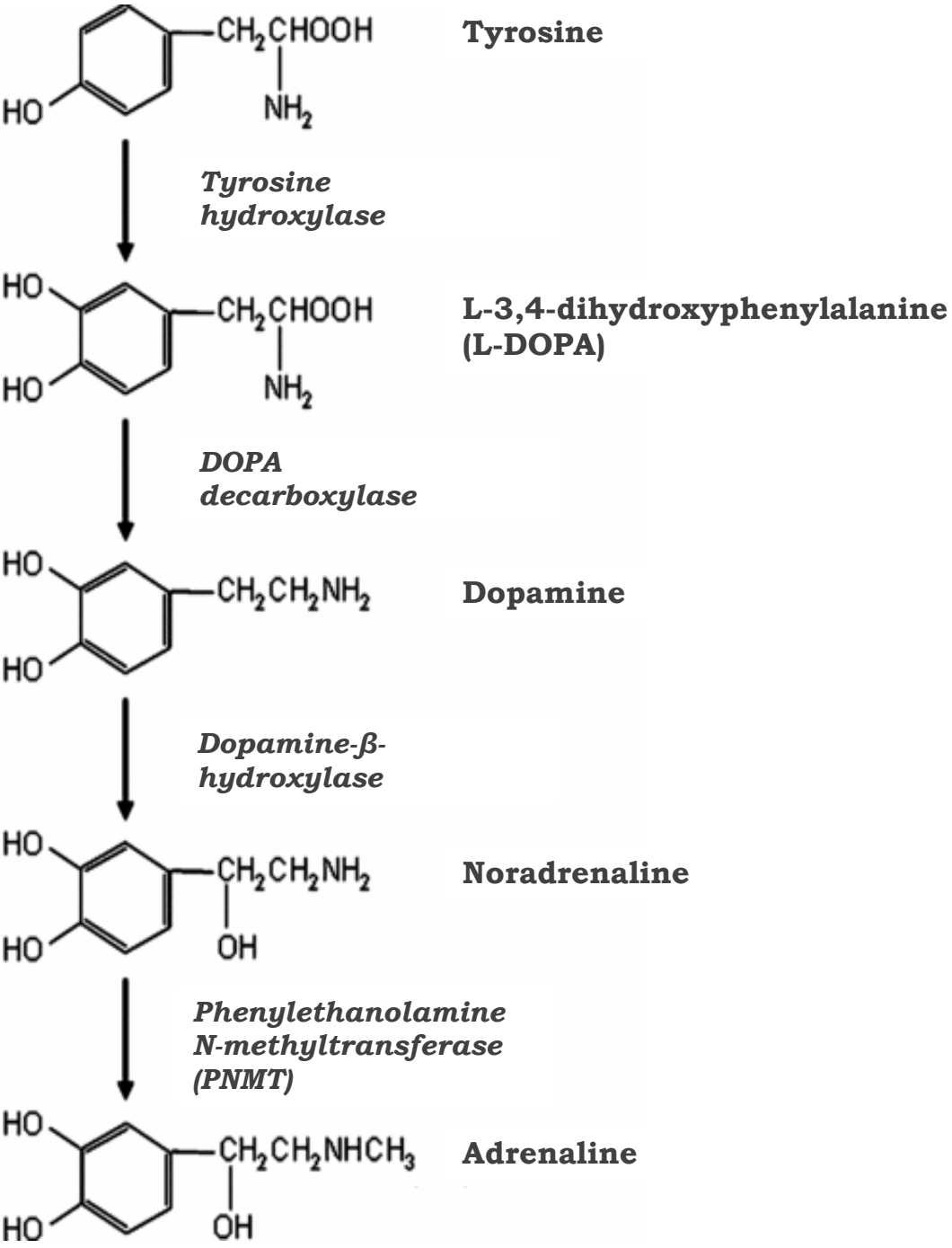

Fig 1-1: The biosynthesis pathway for catecholamines. *Figure adapted from Seigel 2006, pg 212.*

1.2.2 Storage

In neuronal terminals catecholamines are concentrated inside storage vesicles. Only very low concentrations of catecholamines can be found free in the cytosol, where they are quickly metabolised by enzymes such as monoamine oxidase (MAO; described in next section) (Cooper et al. 2003; Kopin 1968; Siegel et al. 2006). Catecholamines are loaded into vesicles via facilitated transport by a vesicular monoamine transporter (VMAT) localized to the membranes of synaptic vesicles and chromaffin granules (Cooper et al. 2003; Headley et al. 2007; Henry et al. 1998). Molecular cloning studies have identified two separate isoforms of the transporter protein: $VMAT_1$ and $VMAT_2$, (Erickson et al. 1992; Hoffman et al. 1998; Liu et al. 1992). The $VMAT_1$ isoform is preferentially expressed in the adrenal medulla by chromaffin cells that synthesize and release monoamines (Cooper et al. 2003) and in the sympathetic ganglia by small, intensely fluorescent cells (Weihe et al. 1994). Conversely, $VMAT_2$ is expressed in adrenergic sympathetic neurons (Headley et al. 2007) and in catecholamine and 5-HT neurons throughout the CNS (Cooper et al. 2003; Hoffman et al. 1998). The $VMAT_2$ isoform shows limited substrate specificity and, in addition to transporting catecholamines, it can also transport indolamines, trace amines and histamine into vesicles (Cooper et al. 2003; Henry et al. 1998).

1.2.3 Metabolism and reuptake

The enzymes primarily responsible for the metabolism and inactivation of catecholamines are MAO and catechol-*O*-methyltransferase (COMT). MAO is localized in the outer membrane of mitochondria, and converts catecholamines to their corresponding aldehydes (Cooper et al. 2003). MAO is present both intra- and extra-neuronally, but the intraneuronal enzyme is of greater importance to NA metabolism. Intracellulary, MAO inactivates any free catecholamines which are not protected within a vesicle inside the nerve terminal (Cooper et al. 2003; Siegel et al. 2006; Youdim and Bakhle 2006). Within the CNS, MAO exists in at least

two separate isoforms, MAO-A and MAO-B, each with different substrate and inhibitor specificities (Cooper et al. 2003; Youdim and Bakhle 2006). While MAO-A preferentially metabolises NA and 5-HT and is inhibited by clorgyline, MAO-B acts on a broad spectrum of phenylethylamines and is selectively inhibited by l-deprenyl (Cooper et al. 2003; Johnston 1968; Siegel et al. 2006; Youdim and Bakhle 2006). The two isoforms also demonstrate differential expression in biogenic amine neurons, where MAO-A is mainly expressed in central and peripheral NA neurons while MAO-B is primarily found in 5-HT neurons of the midbrain raphe nuclei and histaminergic neurons of the hypothalamus (Cooper et al. 2003).

The second enzyme involved in the metabolism of catecholamines, COMT, has a very broad substrate specificity and acts on various catechol compounds (Cooper et al. 2003; Siegel et al. 2006). COMT can be found in the cytoplasm of nearly all cells, with particularly high amounts found in the kidney and liver (Cooper et al. 2003; Siegel et al. 2006). Inside the CNS, COMT is present in high amounts and is also expressed in a variety of sympathetically innervated organs (Cooper et al. 2003). The precise cellular localization of COMT has not been identified, though it is generally thought that the enzyme functions extraneuronally (Cooper et al. 2003; Siegel et al. 2006). Though COMT can play a role in the metabolism of NA, MAO is generally considered the main metabolic inactivating enzyme for NA.

The intracellular localization of MAO prevents this enzyme from inactivating catecholamines once they are released into the synaptic space. For rapid termination of the signal to the postsynaptic cell, NA and other catecholamines must be efficiently removed from the synaptic cleft and taken up into the presynaptic terminal. This reuptake is mediated by a membrane bound noradrenaline transporter (NET) in a saturable process that is Na^+ - and temperature- dependent (Cooper et al. 2003; Eisenhofer 2001; Hoffman et al. 1998; Paton 1973). The NET displays a high affinity for all compounds which

are structurally similar to the catecholamines (Cooper et al. 2003; Eisenhofer 2001), and as such is poorly selective for NA alone, showing a greater affinity for DA than for NA (Cooper et al. 2003). In order for the NET reuptake process to function, the inward transport of NA depends on the co-transport of a single Na^+ and Cl^- ion (Hoffman et al. 1998; Paton 1973). The energy required for the active transport of NA into the presynaptic terminal is related to the inward Na^+ - gradient (Paton 1973). The direction of NA transport by the NET can be altered by reducing extracellular concentrations of Na^+ or exposure to increased concentrations of NA itself (Paton 1973). Under both of these conditions an efflux, rather than uptake, of NA occurs. The ability for the NET to reverse the direction of NA transport under certain conditions is important to consider since after chronic spinal transection the concentrations of NA below the injury are altered and may therefore influence the function of the NET.

1.3 DESCENDING NORADRENERGIC PATHWAYS

Noradrenergic innervation of the spinal cord follows a specific anatomic organization. The innervation patterns of these descending pathways help to illustrate the function of adrenergic input in the spinal cord. The majority of descending noradrenergic pathways originate in and around the locus coeruleus (LC), located in the central gray matter of the caudal pontine nucleus of the brainstem (Cooper et al. 2003; Dahlstrom and Fuxe 1965; 1964; Fritschy and Grzanna 1990; Moore and Bloom 1979). The LC is a very compact group of cells, consisting only of about 3000 neurons bilaterally in the rat brain, and whose collateral axons branch extensively and send projections along well-defined tracts (Cooper et al. 2003). The LC is the most widely projecting CNS nucleus known, with axons projecting to every major region of the brain and densely throughout the spinal cord. This section will focus specifically on the descending projections of the LC and associated cell groups to the rat spinal cord.

The rat spinal cord receives NA projections from the LC, as well as from two additional subcoeruleus cell groups (A5 and A7) located in the ventrolateral reticular formation (Dahlstrom and Fuxe 1964). Although axons from all NA cell groups innervate each spinal cord level, the descending fibres can be organized into two primary columns, a more ventral column consisting of the descending axons from the A5 and A7 regions, and a dorsal column consisting of projections from the LC (Dahlstrom and Fuxe 1965; 1964; Fritschy and Grzanna 1990; Moore and Bloom 1979). LC projections terminate mainly in the dorsal horn and intermediate zone with axons descending nearly the entire length of the spinal cord within superficial dorsal horn laminas I and II (Fritschy and Grzanna 1990; Rajaofetra et al. 1992). The A5 and A7 axons making up the ventral column descend within the ventral and dorsolateral funiculi and terminate in the ventral horn and intermediolateral cell columns on somatic and preganglionic motoneurons (Fritschy and Grzanna 1990). Cells from the A5 and A7 regions innervate motoneurons throughout the entire length of the spinal cord (Fritschy and Grzanna 1990; Rajaofetra et al. 1992). In general, descending NA innervation of the dorsal horn and the commissural gray matter from the ventral column is sparse and scattered, whereas the density of the innervation to the ventral horn and the intermediolateral cell column is much greater (Moore and Bloom 1979; Nygren and Olson 1977).

The differing descending trajectories and pathways of the NA nuclei suggest that descending NA axons from the brainstem consist of at least two different components serving separate physiological functions. As the LC cells project densely to the dorsal horn of the spinal cord, this nucleus may influence a broad range of sensory processes within the spinal cord. Specifically, LC projections terminate in the superficial layers of the dorsal horn and the substantia gelatinosa, suggesting that the LC is likely involved in the control of nociceptive inputs and the modulation of pain (Fritschy and Grzanna 1990; Millan 2002; Segal and Sandberg 1977). In contrast, the neurons of the A5 and A7 areas densely innervate motoneurons at all levels of the spinal cord and are therefore likely

heavily involved in the control of motor functions in the spinal cord (Fritschy and Grzanna 1990; Rekling et al. 2000).

1.4 ADRENERGIC RECEPTORS

Once NA is released from a presynaptic terminal it activates a G-protein-coupled adrenergic receptor on the postsynaptic membrane or on the membrane of another peripheral effector cell. Original classification of adrenergic receptors divided them into two subgroups, namely α and β (Ahlquist 1980; Giroux et al. 1999; Hein 2006; Rekling et al. 2000). Recent pharmacological and molecular studies have expanded this classification system to include three major divisions (α_1, α_2 and β), with each group containing a further three subtypes each (Ahlquist 1980; Giroux et al. 1999; Hein 2006; Rekling et al. 2000). All adrenergic receptors are members of the rhodopsin family of G-protein coupled receptors (GPCRs) (Rekling et al. 2000). Although members of all three major adrenoreceptor subtypes are expressed throughout the CNS, there is little evidence for the expression of the β-adrenoreceptor on motoneurons or in motor nuclei (Rekling et al. 2000). As the focus of this thesis involves the investigation of NA modulation of motoneuron excitability, this section will discuss only the α_1 and α_2 receptor classes and their locations within the spinal cord, rather than on peripheral effector organs.

1.4.1 The α_1 adrenergic receptor and its distribution in the spinal cord

The α_1 adrenergic receptor class consists of three subtypes (α_{1A}, α_{1B}, α_{1D}) which are coupled to $G_{q/11}$ mediated pathways. Activation of the α_1 receptor and its corresponding $G_{q/11}$ coupled pathway leads to an increase in intracellular inositol triphosphate (IP_3) and diaglycerol (DAG), each of which are themselves active second messengers (Hein 2006; Rekling et al. 2000). Intracellular IP_3 increases the concentration of Ca^{2+} within the cell by releasing it from storage sites on the

endoplasmic reticulum, whereas DAG activates protein kinase C (PKC) which then phosphorylates many intracellular target proteins (Cooper et al. 2003; Siegel et al. 2006). Overall, activation of the α_1 adrenergic receptor results in an increased excitability of the cell.

The expression of α_1 adrenergic receptors is widespread and homogenous at all levels of the spinal cord (Giroux et al. 1999; Roudet et al. 1993). The highest densities of α_1 receptors are found in laminae II, IX and X (Giroux et al. 1999), with a high level of expression present on spinal motoneurons (Rekling et al. 2000). This pattern of α_1 receptor distribution correlates well with reports of uniform descending NA terminal distribution throughout the spinal grey matter from the A5 and A7 regions in the brainstem (Fritschy and Grzanna 1990; Giroux et al. 1999; Rajaofetra et al. 1992; Roudet et al. 1993).

Activation of the α_1 adrenergic receptor facilitates excitability and depolarizes the resting membrane potential of neurons (Heckman et al. 2009; Rekling et al. 2000; Russo and Hounsgaard 1999). The ionic mechanism underlying this α_1 receptor mediated depolarization is primarily the reduction in K^+ conductance, most likely mediated by TASK-1 channels (Heckman et al. 2009; Rekling et al. 2000; Russo and Hounsgaard 1999; Talley et al. 2000) and the depolarization of a mixed ion channel responsible for generating the H current (Heckman et al. 2009; Powers and Binder 2001). Increased excitability induced by α_1 receptor activation is associated with a lower current threshold for glutamate-evoked firing, and a decreased AHP (Fung and Barnes 1989; Fung et al. 1991; Parkis et al. 1995; Rekling et al. 2000; White and Neuman 1980).

1.4.2 The α_2 adrenergic receptor and its distribution in the spinal cord

The α_2 adrenergic receptor class contains three subtypes (α_{2A}, α_{2B}, α_{2C},) and is coupled to the inhibitory $G_{i/o}$ protein. The α_2 receptor signalling cascade occurs via activation of both the Gα and the Gβγ subunits of the $G_{i/o}$ protein (Hein 2006).

Activation of the Gα subunit results in the inhibition of adenylyl cyclase and the downstream reduction in cellular 3',5'-cyclic adenosine monophosphate (cAMP) levels (Hein 2006). The outcome of this decrease in cAMP levels is a decline in phosphorylation of proteins by the cAMP dependent protein kinase A (PKA) (Cooper et al. 2003; Hein 2006). Alternately, the Gβγ subunits released from activated $G_{i/o}$ proteins inhibit neuronal Ca^{2+} channels and activate both G-protein-coupled inwardly-rectifying K^+ channels (GIRK K^+) and mitogen-activated (MAP) kinases ERK1/2 (Hein 2006). These secondary actions by Gβγ subunits are thought to be important in the regulation of neuronal function and participate in the inhibition of neurotransmitter release where the α_2 receptor is presynaptic and functions as an autoreceptor on a NA terminal (Hein 2006). Overall, activation of the α_2 adrenergic receptor results in inhibition of cell firing.

All α_2 adrenergic receptor subtypes are expressed in the rat CNS, but the α_{2B} subtype is expressed only in thalamic neurons (Rekling et al. 2000). Expression of the α_2 adrenergic receptor can be found throughout the entire grey matter of the spinal cord, with a somewhat heterogeneous distribution. The highest concentrations of α_2 receptors are in laminae II and X, but they are expressed preferentially in the superficial dorsal horn (Giroux et al. 1999; Roudet et al. 1994). Moderate α_2 receptor densities are be found in lamina III and on motoneurons in lamina IX (Giroux et al. 1999; Roudet et al. 1994), with both the α_{2A} and α_{2C} receptors expressed on motoneurons (Rekling et al. 2000). The pattern of α_2 receptor distribution, predominantly in the superficial layers of the dorsal horn, is well correlated with the distribution of descending NA terminals from the LC (Fritschy and Grzanna 1990; Giroux et al. 1999; Rajaofetra et al. 1992; Roudet et al. 1994).

Activation of the α_2 adrenergic receptor results in inhibitory effects which are mediated by a hyperpolarization of the cell resulting from an increased K^+ conductance. Activation of the α_2 receptor also leads to decreases in the peak amplitude and activation kinetics of the mixed cation hyperpolarization-activated

inward current (I_h), resulting in an overall inhibitory influence on the neuron (Adachi et al. 2005; Rekling et al. 2000).

1.5 FUNCTIONS OF ADRENERGIC INPUT IN THE SPINAL CORD

Noradrenaline is a neuromodulatory neurotransmitter and is thought to function in a way that enhances an organism's reactivity to internal or external stimuli, with activity of brainstem adrenergic neurons changing as a function of the behavioural state of the animal (Jacobs et al. 1991). Understanding not only the pattern of innervation in the spinal cord, but also the functional output of these descending pathways in the normal spinal cord can help to better characterize the effects of the loss of this neuromodulatory input after spinal transection.

Descending NA neurons are tonically active (Ono and Fukuda 1995), but reach peak firing rates during active walking or states of high arousal, with the firing rates gradually declining with reduced levels of activity and arousal, falling silent during REM sleep (Jacobs et al. 1991). Presentation of strong phasic stimuli, such as a sudden noise or painful pinch, elicits a sudden burst of activity in NA neurons in the locus coeruleus, A5 and A7 regions of the brainstem, followed by a brief inhibitory silent period (Jacobs et al. 1991; Abercrombie and Jacobs 1987). Although activation of the widely projecting brainstem NA neurons varies according to the state of arousal of an organism, the precise effects of this NA release are contingent on the composition of the postsynaptic cell. Generally speaking the gradient of adrenergic modulation of activity in the spinal cord progresses from strongly inhibitory in the dorsal horn, to mixed in intermediate portions of the cord, to strongly excitatory in the ventral horn (Heckman et al. 2009). This is in line with the normal distribution of adrenergic receptors, where inhibitory α_2 receptors are more densely distributed in the dorsal horn and excitatory α_1 receptors in the ventral horn.

1.5.1 Adrenergic input in the intermediate spinal cord and the dorsal horn

In the intermediate and dorsal horn the effect of adrenergic input is mixed and strongly inhibitory, respectively. In the intermediate spinal cord NA primarily activates receptors on different populations of interneurons whose end net effects can be inhibitory or excitatory to motor output depending on the type of receptor activated and the type of interneuron involved. For example, activation of excitatory α_1 adrenergic receptors facilitates proprioceptive transmission from group I afferents in the cat (Hammar and Jankowska 2003), but also facilitates the activity of two separate groups of inhibitory interneurons (Ia and Ia/Ib-inhibitory interneurons) (Hammar and Jankowska 2003). The net result of activation of these inhibitory interneurons is an increase in Ia reciprocal inhibition of motoneurons and an increase in non-reciprocal inhibition from group Ia and Ib afferents (Hammar and Jankowska 2003). Conversely, activation of the α_2 adrenergic receptor inhibits transmission from group II afferents to premotor interneurons in the midlumbar spinal cord (Jankowska et al. 1993). Overall, in the intermediate zone of the spinal cord, activation of adrenergic receptors, either α_1 receptors on inhibitory interneurons or α_2 receptors on sensory afferent terminals, results in inhibitory modulation of sensory transmission.

In the dorsal horn activation of adrenergic receptors also strongly inhibits sensory transmission, and this is primarily mediated by α_2 adrenergic receptors, which have a very dense distribution throughout laminae I, II, IV and V (Giroux et al. 1999; Roudet et al. 1994). The expression of α_1 adrenergic receptors in the dorsal horn is sparse compared to the prevalence of α_2 receptors (Fleetwood-Walker et al. 1985; Giroux et al. 1999; Roudet et al. 1994; Roudet et al. 1993). Activation of α_2 adrenergic receptors in the dorsal horn mainly inhibits nociception (Davies and Quinlan 1985; Fleetwood-Walker et al. 1985; Grudt et al. 1995; Millan 2002; Millan and Colpaert 1991; Segal and Sandberg 1977; Yaksh 1985; Yoshimura and Furue 2006). NA applied to neurons in the substantia gelatinosa, a structure primarily involved in pain transmission, has a direct hyperpolarizing effect

mediated by α_2 adrenergic receptors (Davies and Quinlan 1985; Grudt et al. 1995). The α_2 receptor agonists tizanidine and clonidine have both been found to reduce the response to noxious, but not innocuous, stimulation in rodents (Davies and Quinlan 1985; Fleetwood-Walker et al. 1985), thus reinforcing the role of dorsal horn α_2 receptors in anti-nociception. Activation of α_2 receptors has also been shown to reduce the spontaneous activity of dorsal horn neurons in laminae IV and V (Davies and Quinlan 1985), as well as reducing cutaneous reflex excitability (Clarke et al. 2002; Giroux et al. 2001).

1.5.2 Adrenergic input in the ventral horn

The ventral horn contains the highest densities of α_1 receptors in the spinal cord (Giroux et al. 1999), with a particularly high level of expression on spinal motoneurons (Rekling et al. 2000). Not surprisingly then, the α_1 adrenergic receptor in the ventral horn is generally involved in increasing motoneuron excitability and the facilitation of spinal cord reflexes (Barnes et al. 1989; Fung et al. 1991; Heckman et al. 2009; Lai et al. 1989; Ono and Fukuda 1995; Rekling et al. 2000; Russo and Hounsgaard 1999; Tanabe et al. 1990; White and Neuman 1980). Application of the α_1 receptor agonist, methoxamine, enhances overall motoneuron excitability in decerebrate cats, promoting prolonged instances of self-sustained firing and enhancing motoneuron bistability (Lee and Heckman 1999). Electrical activation of the descending coeruleospinal pathways, or exogenous NA application, facilitates lumbar flexor and extensor monosynaptic reflexes in decerebrate cats, and this facilitation can be blocked by blocking the α_1 adrenergic receptor (Barnes et al. 1989; Fung et al. 1991). Monosynaptic and polysynaptic reflex potentials, elicited by the stimulation of flexor reflex afferents in rats, are also facilitated by exogenous application of NA and blocked by the α_1 receptor antagonists (Ono and Fukuda 1995). In rat spinal cord slices, exogenous NA application facilitates motoneuron discharges and this facilitation can also be blocked by α_1 antagonists (Hirayama et al. 1988). Thus, activation of the α_1

adrenergic receptor in the ventral horn facilitates motoneuron excitability and spinal cord reflexes.

While the effects of NA in the ventral horn are predominantly excitatory and mediated by activation of the α_1 adrenergic receptor, there are nonetheless several examples of α_2 receptor mediated effects on spinal cord reflexes. The distribution of α_2 adrenergic receptors in the ventral horn and on motoneurons is moderate (Giroux et al. 1999; Rekling et al. 2000; Roudet et al. 1994). In general, activation of the α_2 adrenergic receptor exerts an inhibitory influence on spinal reflexes. In rat spinal cord slices, application of α_2 receptor agonists reduces motoneuron discharges, and has also been shown in intact rats to markedly decrease both the mono- and polysynaptic reflex potentials elicited by the stimulation of flexor reflex afferents (Ono and Fukuda 1995; Tanabe et al. 1990). Overall, activation of the α_2 adrenergic receptor in the ventral horn primarily results in the inhibition of spinal reflexes.

However, some studies have shown that systemic injection of the α_2 receptor agonist clonidine can actually induce and maintain a locomotor pattern in acute spinal cats (Forssberg and Grillner 1973; Giroux et al. 1999; Jordan et al. 2008). In both intact and acutely spinalized cats, clonidine improves the regularity of walking and results in a more homogeneous and better organized hindlimb EMG pattern (Giroux et al. 2001; Rossignol et al. 1996). Blockade of the α_2 receptor, on the other hand, results in a pronounced asymmetry of stepping (Giroux et al. 2001). Therefore it has been suggested that α_2 receptor activation is required for the initiation of locomotion (Forssberg and Grillner 1973; Jankowska et al. 1967), as well as the coordination of the locomotor pattern. These effects on locomotion likely result from adrenergic modulation of central pattern generator (CPG) interneurons in the lumbar spinal cord.

Overall, the primary effect of adrenergic input in the ventral horn is to facilitate motoneuron excitability, or more accurately, to increase the sensitivity of motoneurons to both excitation and inhibition (Heckman et al. 2005).

1.5.2.1 Adrenergic regulation of normal motoneuron excitability

The adjustment of the sensitivity of motoneurons in the uninjured spinal cord is heavily contingent on the presence of voltage-dependent, slowly inactivating persistent inward currents (PICs), which are considered to be a latent property of spinal motoneurons (Bennett et al. 1999; Gorassini et al. 1999; Heckman et al. 2005; Heckman et al. 2003; Hounsgaard and Kiehn 1989; Hultborn et al. 2003; Powers and Binder 2001). PICs are activated by brief excitatory synaptic input and provide an amplification of this input allowing motoneurons to exhibit sustained depolarization and instances of repetitive firing that can outlast the original excitatory synaptic input (Bennett et al. 1999; Heckman et al. 2005; Heckman et al. 2009; Hounsgaard and Kiehn 1989; Lee and Heckman 1998; Li and Bennett 2003; Powers and Binder 2001). The net PIC consists of two separate currents: a fast-onset, TTX-sensitive persistent sodium component (Na PIC) which is activated near spike threshold and a nimodipine-sensitive persistent calcium component (Ca PIC) mediated by L-type $Ca_V1.3$ channels and activated subthreshold to firing (-44 to -55mV) (Heckman et al. 2005; Hounsgaard and Kiehn 1985; 1989; Li and Bennett 2003; Li et al. 2004a). Activation of the Na PIC facilitates the generation of action potentials by amplifying synaptic input and accelerating the membrane potential toward the spike threshold (Li et al. 2004a). The Na PIC also functions to sustain steady repetitive firing, particularly during slow depolarizations (Lee and Heckman 2001), and enables very slow firing where the inter-spike intervals are much longer than the AHP duration (Li et al. 2004a). Conversely the Ca PIC, which demonstrates slow kinetics and a lack of inactivation, provides a steady depolarizing drive that outlasts the activating stimulus and requires a strong hyperpolarizing input to deactivate

(Kiehn and Eken 1998; Li et al. 2004a). The Ca PIC aids in the recruitment of cells and in sustaining firing (Li et al. 2004a).

The amplitude of the net PIC is a major determinant of motoneuron excitability, and the amplitude of the PIC is in turn contingent on the level of monoaminergic input onto the motoneuron (Heckman et al. 2005; Heckman et al. 2009; Hounsgaard et al. 1988; Lee and Heckman 2000; 1996; Li et al. 2007; Schwindt and Crill 1980). The stronger the monoaminergic input to the spinal cord, the larger the amplitude of the motoneuron PIC. At low levels of monoaminergic drive, as occurs in deeply anesthetised animals, the total PIC is very small and often absent entirely (Heckman et al. 2005; Heckman et al. 2009; Lee and Heckman 2000; Schwindt and Crill 1980). In unanesthetised decerebrate preparations where descending monoaminergic axons are intact and active, corresponding to an intermediate level of monoaminergic input, large motoneuron PICs and bistable behaviour are present (Bennett et al. 1998; Heckman et al. 2005; Heckman et al. 2009; Hounsgaard et al. 1988). In situations approximating high monoaminergic drive to the spinal cord, where exogenous 5-HT or NA agonists are applied to decerebrate preparations, motoneuron PICs are further augmented (Bennett et al. 1998; Heckman et al. 2005; Heckman et al. 2009; Hounsgaard et al. 1988). Conversely, complete removal of monoaminergic input to the spinal cord, via spinal transection, results in a near complete elimination of motoneuron PICs and a corresponding drastic reduction in motoneuron excitability (as discussed below).

1.6 TRANSECTION OF THE SPINAL CORD

Injury to the spinal cord, whether partial or complete, results in significant changes to the tissue caudal to the injury. Considerable plasticity of receptors, neurons and interneuronal networks often occurs after injury to the spinal cord, but differs in severity depending on the extent of injury. For the purpose of this

thesis, the focus will be on changes resulting from complete spinal transection and how this affects adrenergic input and receptor distribution caudal to the injury, as well as the effects on the excitability of motoneurons and on inhibitory and excitatory interneuron pathways below the transection.

1.6.1 Effects of spinal transection on spinal NA content and adrenergic receptors

Transection of the spinal cord results in a gradual degeneration of descending NA fibres. In rats, the decrease in levels of spinal NA after a complete spinal cord transection occurs in two distinct phases: a slow phase in the initial period after transection followed by a rapid phase of decrease which is not completed until 15 days after transection (Dahlstroem and Fuxe 1965; Haggendal and Dahlstrom 1973; Magnusson 1973). In the very early stages after spinal transection (1-3 days), levels of NA in the spinal cord actually remain unchanged (Dahlstroem and Fuxe 1965; Haggendal and Dahlstrom 1973; Magnusson 1973), but by 4 days after transection, NA content caudal to the lesion is decreased by about 15% (Haggendal and Dahlstrom 1973). During this same early period, there is no detectable change in NA fibre fluorescence caudal to the lesion (Carlsson et al. 1964). Following this initial slow phase of decrease, the number of visible NA terminals caudal to the lesion, and consequently the NA content of the cord, decreases rapidly (Carlsson et al. 1964; Dahlstroem and Fuxe 1965; Haggendal and Dahlstrom 1973; Magnusson 1973). Degeneration of NA fibres, and the associated spinal NA content, decreases most rapidly in areas closer to the transection with a slower decrease occurring further away from the injury site (Haggendal and Dahlstrom 1973). By 7 days after the transection, histochemical results suggest that the remaining detectable NA content in the cord is situated outside the degraded NA neurons, possibly taken up into non-aminergic tissues (Haggendal and Dahlstrom 1973). By 15 days post transection the NA content in the spinal cord is reduced to 1-5% of original NA levels and adrenergic terminals

are no longer visible (Carlsson et al. 1964; Dahlstrom and Fuxe 1964; Haggendal and Dahlstrom 1973; Magnusson 1973).

The reduction in adrenergic content of the spinal cord following transection is accompanied by changes to both α_1 and α_2 adrenergic receptor densities which follow a specific time course. In the rat spinal cord the density of both α_1 and α_2 receptors caudal to a transection is increased 15-30 days after injury (Roudet et al. 1994; Roudet et al. 1993). The density of α_1 adrenergic receptors increased uniformly across all grey matter subregions below the transection (Roudet et al. 1993), whereas α_2 adrenergic receptors show significant increases only in the superficial and deep dorsal horn regions (laminae I- IV) in the intermediate zone (laminae VI and VII) (Roudet et al. 1994). In the later stages following spinal transection (30-60 days) densities for both α_1 and α_2 receptors caudal to a transection gradually return back to control levels (Giroux et al. 1999). Densities for both receptor subtypes are indistinguishable from uninjured control animals by 60 days after complete spinal transection (Giroux et al. 1999). The chronic SCI animals used throughout this thesis are generally more than 60 days post-injury, where adrenergic receptor distribution is not different from uninjured animals.

1.6.2 Effects of spinal transection on motoneuron excitability and PICs

As described above, following a complete spinal transection nearly all of the descending monoaminergic input to the spinal cord is eliminated (Dahlstroem and Fuxe 1965; Magnusson 1973). Since motoneuron excitability and PICs depend on monoaminergic input, acute spinalization results in an elimination of PICs and a dramatic reduction in overall excitability below the transection, such that even very strong synaptic inputs result in weak effects (Bennett et al. 1999). Both motoneuron excitability and PICs can be recovered after acute spinal transection by exogenous application of monoamines themselves or of monoaminergic receptor agonists (Bennett et al. 2001a; Bennett et al. 2001b; Conway et al. 1988; Heckman et al. 2005; Hounsgaard et al. 1988) Despite the absence of

monoaminergic input to the spinal cord after transection, motoneurons gradually recover their excitability and redevelop the capacity to generate large PICs, similar in amplitude to those recorded in intact animals, in the weeks following injury (Bennett et al. 2001a; Bennett et al. 2001b; Bennett et al. 2004; Li et al. 2004a; Heckman et al. 2005). However, due to the loss of descending supraspinal inhibitory inputs, activated PICs become very difficult to terminate and this results in uncontrolled motoneuron firing/spasms (reviewed in Heckman et al. 2005). This spontaneous recovery of PICs in chronic SCI underlies the development of a spasticity syndrome, consisting of increased muscle tone, exaggerated reflexes and involuntary muscle spasms (Ashby 1987; Young 1994) often seen after long-term spinal injury. The development of spasticity in chronic SCI corresponds to the re-emergence of motoneuron PICs. The mechanisms of the development of spasticity after chronic injury have been identified in a rat model of SCI (Bennett et al. 2004; Li et al. 2004a; Li et al. 2004b) and verified in human SCI patients (Gorassini et al. 2004; Norton et al. 2008). In the rat model of SCI, spasms can be triggered by the brief activation of low-threshold cutaneous inputs (Bennett et al. 1999), which under normal circumstances would not result in a synaptic input of sufficient amplitude or duration (over 200 ms required) to activate PICs (reviewed in Heckman et al. 2005). After chronic SCI, this innocuous low-threshold stimulation results in unusually long polysynaptic excitatory postsynaptic potentials (pEPSPs; 500-1000ms), which are now long enough to trigger the slowly activating PICs and ultimately result in self-sustained firing and spasms lasting many seconds (Bennett et al. 2004; Li and Bennett 2003; Li et al. 2004a). Importantly, when nimodipine is used to block the L-Type Ca^{2+} channels, the self-sustained firing underlying the spasms seen in chronic SCI animals are eliminated (Li et al. 2004a). This indicates that the Ca PIC is primarily responsible for the production of spasms after chronic SCI.

1.6.3 Effects of spinal transection on inhibitory interneuron pathways

In addition to changes in excitability at the motoneuron itself, significant plasticity of pre-motoneuronal networks, specifically those involving interneurons, have been well described. The changes to inhibitory and excitatory interneuron pathways additionally contribute to the overall increased spinal excitability and resulting spasticity syndrome seen after chronic SCI. Primarily, after SCI, there is an overall weakening of inhibitory actions onto motoneurons and this is generally mediated by a loss of excitatory input onto inhibitory interneurons involved in various spinal pathways (Jankowska and Hammar 2002) as described below.

1.6.3.1 Post-activation depression

Post-activation depression (PAD) is a depression in the size of the monosynaptic Ia EPSP which occurs when Ia afferents have been previously activated by conditioning stimuli delivered with a inter-stimulus interval greater than 1s (Crone and Nielsen 1989; Elbasiouny et al. 2010; Grey et al. 2008; Hultborn et al. 1996). PAD, which can be observed for more than 10s after the conditioning stimulus, results from changes in the probability of transmitter release from the presynaptic terminal (Grey et al. 2008; Lev-Tov and Pinco 1992; Pierrot-Desseilligny and Burke 2005). A reduction in PAD can be observed in humans after SCI as well as in animal models of SCI, where progressive reductions in PAD are well correlated with the development of spasticity (Aymard et al. 2000; Elbasiouny et al. 2010; Grey et al. 2008). The increased size in the H-reflex noted in humans with spasticity after SCI, therefore, is likely related to a reduction in PAD after chronic transection (Nielsen et al. 1993; Nielsen et al. 1995).

1.6.3.2 Presynaptic inhibition

In addition to PAD contributing to increased monosynaptic reflexes after SCI, it is widely reported that a reduction in the presynaptic inhibition of Ia afferents also occurs after SCI. Presynaptic inhibition is mediated by interneurons that synapse onto the terminals of primary afferents. In the uninjured spinal cord, these interneurons depolarize the Ia afferent terminals, leading to a decrease in the probability of transmitter release for each subsequent afferent volley and thereby adjusting the strength of the synaptic inputs (Elbasiouny et al. 2010; Kandel et al. 2000). In human spastic SCI patients the presynaptic inhibition evoked by tendon or muscle vibration is reduced, both at rest and during walking (Faist et al. 1999; Faist et al. 1994; reviewed in Jankowska and Hammar 2002). This indicates that the excitatory input to motoneurons from Ia afferents is increased in chronic SCI by virtue of a decrease in inhibition mediated by interneurons.

1.6.3.3 Disynaptic reciprocal 1a inhibition

The disynaptic reciprocal Ia inhibitory pathway is involved in the regulation of reflexes during voluntary movements by ensuring the maintained relaxation of antagonist muscles during the activation of agonist muscles (Nielsen et al. 2007). Interneurons within the reciprocal Ia inhibitory pathway have been shown to be tonically active in uninjured human subjects, indicating that the pathway is involved in maintaining a low excitability of motoneurons of antagonist muscles at rest (Nielsen et al. 1995). A reduction in reciprocal inhibition has been reported in humans after SCI, where afferent inputs from agonist muscles produce weaker suppression of the H-reflex evoked in antagonist muscles (Crone et al. 2003; Elbasiouny et al. 2010). Furthermore, during isometric contractions spastic SCI patients demonstrated atypical coactivation of antagonist muscles (Elbasiouny et al. 2010). However, no correlation between the degree of spasticity and the reduction of reciprocal inhibition has been shown (Nielsen et al. 2007). It is nonetheless likely that the reduction in the Ia reciprocal inhibition

pathway also contributes to the increased motoneuron excitability seen in chronic SCI.

1.6.4 Effects of spinal transection on excitatory interneuron pathways

Although many of the changes to interneuron pathways after SCI involve a reduction in the influence of inhibitory interneurons, increased activity of excitatory interneuron pathways also contributes to the increased excitability seen after injury. The most prominent group of excitatory interneurons involved are those mediating disynaptic excitatory input to motoneurons from group II afferents. The interneurons in the disynaptic reflex pathway from group II afferents are powerfully affected by descending NA input, and the activation of these interneurons can be blocked using α_2 adrenergic receptor agonists (Jankowska and Hammar 2002). Reflexes mediated by group II afferents, such as stretch reflexes of the quadriceps, have been reported to show significant facilitation in spastic SCI patients when compared to normal individuals (Jankowska and Hammar 2002). Interestingly, these enhanced reflexes in spastic SCI patients can be reduced to approximately normal levels by the α_2 adrenergic receptor agonist clonidine (Remy-Neris et al. 1999). Therefore, after SCI the normal α_2 receptor-mediated inhibition of group II activated interneurons is lost (disinhibition), and this ultimately leads to increased motoneuron excitability.

1.6.5 Effects of spinal transection on the blood-brain barrier

Changes in the blood-brain barrer (BBB) after spinal transection may initially seem unrelated to the overall increases in motoneuron excitability and spasms seen after injury. However, as explored in Chapter 4 of the thesis, the changes to the BBB resulting from CNS injury can significantly influence neurons below the transection. The primary functions of the BBB and how they are changed by spinal transection are explored below.

1.6.5.1 Structure of the blood-brain barrier

The BBB is a functional neurovascular unit encapsulating the CNS that has a range of passive and active features which aid in the regulation of the CNS environment. One of the major roles of the BBB is to assist in supplying the brain and spinal cord with essential nutrients while extruding many of the waste products produced in CNS tissues (Abbott et al. 2010; Abbott et al. 2006; Persidsky et al. 2006). The BBB also regulates ionic traffic, restricting fluid and ionic movement between the blood and the brain, thereby maintaining a brain interstitial fluid that is optimal for neuronal function (Abbott et al. 2010; Abbott et al. 2006; Persidsky et al. 2006). Finally, the BBB also functions to separate neurotransmitter pools that act centrally in the CNS and those that act peripherally (Abbott et al. 2010; Abbott et al. 2006; Persidsky et al. 2006). This allows similar neurotransmitters and other neuroactive agents to be used in the two systems without interfering or causing crosstalk. It is the breakdown of this separation of neurotransmitter pools after CNS injury that critically affects motoneuron excitability after chronic SCI.

The BBB is composed of several components including the microvascular endothelium, astrocytes, basement membrane and pericytes and also includes neurons that are in close proximity to cerebral blood vessels (Persidsky et al. 2006) (see Fig 1-2). The BBB can be divided into three separate types of barriers, based on their functional characteristics: a physical barrier, a transport barrier and a metabolic barrier (Abbott et al. 2010; Abbott et al. 2006; Hardebo et al. 1979; Persidsky et al. 2006). The physical barrier is composed primarily of endothelial cells that line cerebral microvessels and are connected to neighbouring endothelial cells by complex tight junctions (Abbott et al. 2010; Abbott et al. 2006). These tight junctions act as a physical barrier because they form a tight seal between adjacent cells that forces molecular traffic through a highly selective transcellular route, rather than simply diffusing between the cells. This physically excludes all but very small molecules from passing from the blood to the cerebrospinal fluid

(CSF) (Abbott et al. 2010; Abbott et al. 2006). The transport barrier component of the BBB consists of specific active transport systems on both the luminal and abluminal membranes of the microvasculature that regulate transcellular traffic (Abbott et al. 2010; Abbott et al. 2006). The transport barrier is selective for small hydrophilic molecules, such as certain required nutrients, and allows them to pass into the CNS while excluding, or even engaging in the active efflux, of harmful compounds (Abbott et al. 2010; Abbott et al. 2006; Begley and Brightman 2003; Persidsky et al. 2006). The metabolic barrier of the BBB is composed of intracellular and extracellular enzymes which are capable of metabolising peptides and ATP, as well as enzymes such as MAO, AADC or cytochrome P450, whose role is to inactivate a variety of neuroactive or toxic compounds (Abbott et al. 2010; Abbott et al. 2006; Hardebo et al. 1979). This metabolic barrier is capable of excluding most large hydrophilic molecules (Abbott et al. 2010; Abbott et al. 2006; Persidsky et al. 2006).

Of particular importance in the structure of the BBB is the presence of astrocytes, which surround the capillaries of the brain and spinal cord (Abbott et al. 2010), and envelop ~99% of the BBB microvessel endothelium (Abbott et al. 2006; Hawkins and Davis 2005; Persidsky et al. 2006). Many studies have demonstrated that these astrocytes can regulate the function of blood vessels in response to metabolic challenges, increase the tightness of endothelial tight junctions and upregulate the expression of certain transporters and enzymes at the BBB (Abbott et al. 2006; Persidsky et al. 2006). These astrocytes can also secrete a wide range of chemicals which can influence the permeability and function of different components of the BBB (Abbott et al. 2010; Abbott et al. 2006). In short, astrocytes are critical in the active regulation of multiple components of the BBB.

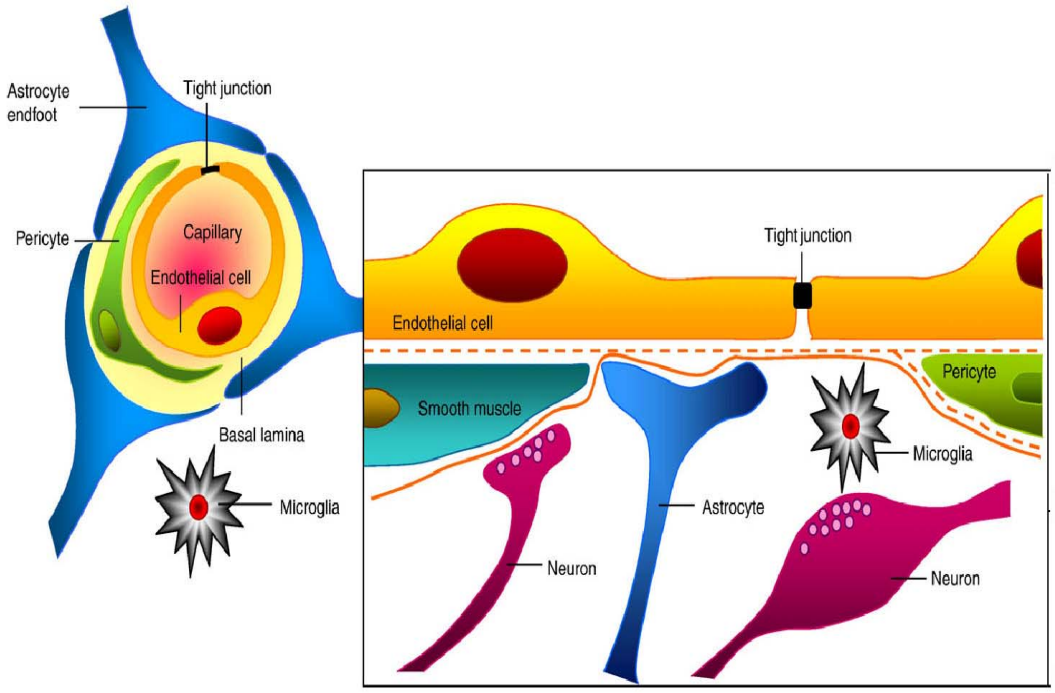

Fig 1-2: The structural components and cell associations of the blood-brain barrier (BBB). Endothelial cells lining cerebral vasculature are joined to neighbouring cells by tight junctions that make up the primary component of the physical barrier properties of the BBB. Pericytes, flat, contractile connective tissue surrounding capillaries, regulate the structural integrity of the BBB. Foot processes from astrocytes surround a majority of the BBB endothelium and are important in the induction and maintenance of barrier properties. Innervation of vascular smooth muscle by axonal projections helps to regulate cerebral blood flow, and vasoactive peptides and neurotransmitters released by axons can influence BBB permeability. Immunocompetent microglia located near the BBB function to counteract infectious agents and reduce resulting inflammation. *Figure adapted from Abbott et al. 2010*

1.6.5.2 Blood-brain barrier permeability changes

Many pathologies of the CNS, particularly those in which cerebral blood flow is reduced or where an inflammatory response is triggered, involve a disruption in the BBB (Abbott et al. 2010; Abbott et al. 2006; Gordh et al. 2006; Palmer 2010; Persidsky et al. 2006). This disruption usually consists of a breakdown in the endothelial tight junction assembly, and can range from a mild and transient opening of the tight junctions to a chronic and progressive breakdown of the barrier (Abbott et al. 2006; Bellavance et al. 2008). Changes to transmembrane transport systems, as well as to the enzymes comprising the enzymatic component of the BBB, have also been observed after trauma to the CNS (Abbott et al. 2006; Hardebo et al. 1979; Persidsky et al. 2006). In particular, the physical trauma of SCI, stroke or peripheral nerve injury can alter the tight junction assembly causing increased permeability of the BBB which remains detectable for up to 56 days following injury (Cohen et al. 2009; Popovich et al. 1996). However, a recent study reported that immune cells, normally confined to the circulation, are detectable within the spinal cord for up to 180 days after a spinal contusion injury (Beck et al. 2010). The increased permeability of the BBB after SCI, therefore, can be persistent and can result in the passage of neuroactive agents, immune cells and various other small molecules from the blood to the CNS. This is important to consider as some neurotransmitters, particularly NA, are present in high quantities in the blood. This thesis explores the possibility of peripheral NA passing through a compromised BBB to affect motoneuron excitability after chronic SCI in Chapter 4.

1.7 ANIMAL MODEL OF SPASTICITY

In humans, the development of a general spasticity syndrome often follows SCI and consists of exaggerated reflexes and muscle tone with especially intense muscle contractions (Ashby 1987; Kuhn and Macht 1949; Young 1994). The

development of this spasticity syndrome in humans follows a general pattern that progresses from a stage of spinal shock and areflexia immediately after injury to heightened reflex and spasm activity in the weeks following injury. After recovery from spinal shock the increased reflex activity and spasms primarily involve flexion movements and increased flexor tone, progressing to mixed flexion and extension in the later stages of injury (Kuhn and Macht 1949). Bennett and colleagues were able to successfully develop a rat model of spasticity which faithfully reproduced the spasticity syndrome as it present in humans (Bennett et al. 1999).

The rat model of spasticity involves a transection of the sacrocaudal spinal cord at S_2 of adult rats. The cord below this level of injury primarily innervates the tail and results in the robust development of a spasticity syndrome in the segmental muscles of the tail that closely parallels that of the classical human spasticity syndrome. The tail muscles progress from spinal shock in the first two weeks of injury, where the tail is flaccid and reflexes are extremely difficult to evoke, to the gradual emergence of tonic muscle activity, and eventually the development of forceful flexion-dominant spasms after 1.5 - 2 months post transection (Bennett et al. 1999). Spasms in the tail can be triggered by brief cutaneous stimulation or muscle stretch, which would be ineffective in evoking a response in uninjured animals. With increasing time after injury, the tone in the muscles of the tail increases while the threshold for cutaneous evoked reflexes decreases. The development of spasticity in the tail muscles of the rat is functionally relevant to the human spasticity syndrome as these segmental tail muscles are similar in anatomy, mechanics and control to those of the axial muscles in the back and neck which often develop muscle spasms after SCI in humans (Li et al. 2004b; Wada et al. 1997).

Spasms can be investigated in the awake spinal rat, at both acute and chronic injury time points, as the animal is able to tolerate prolonged examinations of the spastic tail muscles while being comfortably restrained. Cutaneous or electrical

stimulation of the tail typically results in very strong whole tail, flexion-dominant spasms, often involving coiling behaviour. The kinematics of this spastic coiling can be easily characterised with motion tracking software, resulting in a quantifiable measure of spasms. The amount of muscle activity associated with the spasms can also be quantified using simple percutaneous or surface EMG. A further advantage of the rat sacrocaudal transection model of SCI is the fact that the rat spinal cord below the S_2 transection is small enough to remain viable *in vitro* for prolonged periods of time (Bennett et al. 2001b). With the sacrocaudal cord *in vitro*, full control of the external environment can be exercised and either chronic or acutely lesioned spinal cords can be investigated. Recordings can be made from individual ventral roots in response to dorsal root stimulation, with responses being used as a measure of the output from intact intraspinal networks below the transection. Recordings can also be made directly from individual motoneurons below the transection, using a sharp electrode. This diversity of investigative strategies allows for the examination and characterisation of the mechanisms underlying the development of spasticity after chronic spinal cord transection.

1.8 THESIS OUTLINE

Previous work completed in the Bennett lab has demonstrated that the spontaneous recovery of motoneuron excitability and the re-emergence of PICs, which underlies the development of spasticity in chronic SCI, involves the activation of α_1 adrenergic receptors (Harvey et al. 2006; Li et al. 2004b). These studies successfully demonstrated that α_1 adrenergic receptor activation via agonists, or NA itself, can increase PICs after chronic injury, whereas blocking the α_1 receptor with antagonists can inhibit PICs. However, the precise role of both α_1 and α_2 adrenergic receptor subtypes in the recovery of spasms after SCI has never been investigated. In addition, since α_1 receptor antagonists are effective at reducing the PIC, this indicates that the receptor is spontaneously active. The source of this spontaneous activity at the adrenergic receptor after

chronic SCI has, likewise, never been investigated. The purpose of this thesis, therefore, is to continue this path of investigation and characterise the roles of the α_1 and α_2 adrenergic receptors in the recovery of motoneuron excitability and PICs after chronic SCI, in addition to further exploring the source of spontaneous adrenergic receptor activation after chronic injury. The general hypothesis is that a residual endogenous source of NA capable of activating adrenergic receptors on motoneurons persists below a chronic transection, and this spontaneous receptor activation contributes to the increased motoneuron excitability and spasms seen after SCI.

The second chapter of the thesis examines the specific role of adrenergic receptors in the heightened motoneuron excitability and loss of inhibitory control of sensory afferent transmission that develops in the months following spinal transection. This chapter demonstrates that the α_1 adrenergic receptor is endogenously active in chronic SCI, but the mechanism of the endogenous activity differs *in vitro* and *in vivo*. Whereas *in vitro*, the α_1 receptor is only constitutively active, or active in the absence of a ligand, *in vivo* the α_1 receptor is constitutively active in addition to being activated by a residual endogenous ligand, likely NA. This chapter also demonstrates that activation of the α_1 adrenergic receptor, specifically the α_{1A} receptor, facilitates Ca PIC and spasm activity recorded both *in vivo* and *in vitro*. In contrast, activation of the α_2 adrenergic receptor does not facilitate Ca PICs, but instead decreases spasms by decreasing the excitatory post synaptic potentials (EPSPs). Although the α_2 adrenergic receptor is not spontaneously active *in vitro*, it is activated by an endogenous ligand *in vivo*. The third chapter of the thesis investigates whether an endogenous source of NA persists below a chronic transection and whether this endogenous source of NA is capable of facilitating Ca PIC and spasms. This chapter demonstrates that amphetamine, which causes a forced efflux of endogenous NA, facilitates the Ca PIC and spasms after chronic SCI. This chapter therefore establishes that an endogenous source of NA, capable of facilitating spasms, exists caudal to a chronic transection. Finally, in the fourth chapter of the thesis the source of this endogenous NA that remains, and

facilitates spasms *in vivo* after chronic SCI, is explored. This chapter demonstrates that the residual endogenous NA actually originates in the periphery (blood) and crosses a compromised BBB to act on central α_1 adrenergic receptors to facilitate spasms. This chapter also characterises the mechanisms by which the peripheral NA is able to be transported from the periphery to the spinal cord, and how this NA is then able to reach and activate central α_1 receptors.

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Chapter 2:

Adrenergic receptors modulate motoneuron excitability, sensory synaptic transmission and muscle spasms after chronic spinal cord injury.

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- Completion and analysis of all *in vivo* experiments, completion and analysis of some *in vitro* ventral root reflex experiments, preparation of figures and manuscript.

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- Completion of some *in vitro* ventral root reflex experiments, completion and analysis of some *in vitro* intracellular recording experiments, tissue preparation for all *in vitro* experiments.

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2.1 INTRODUCTION

In the months following a spinal cord injury (SCI) individuals often develop a debilitating spastic syndrome, consisting of increased muscle tone, clonus, exaggerated reflexes and associated widespread muscle spasms (Ashby 1987; Bennett et al. 2004; Gorassini et al. 2004; Kuhn and Macht 1949; Maynard et al. 1990; Young 1994). These involuntary muscle spasms can be triggered by very brief innocuous stimulation, including cutaneous stimulation and can last for many seconds, disrupting residual motor function and compromising rehabilitation efforts. As shown in a rat model of SCI (Bennett et al. 2004; Li et al. 2004a; Li et al. 2004b) and verified in humans with SCI (Gorassini et al. 2004; Norton et al. 2008), spasms result in large part from two factors: 1) a permanently heightened motoneuron excitability, which paradoxically develops in the months following spinal cord transection (Bennett et al. 2004; Button et al. 2008; Hultborn et al. 2004; Li et al. 2004a), and 2) a lack of inhibitory control over sensory afferent transmission leading to exaggerated synaptic inputs to motoneurons (Li et al. 2004a; Norton et al. 2008). The goal of this paper was to understand the role of adrenergic receptors in these two processes.

Normally, the control of both motoneuron excitability and sensory transmission depends on descending monoaminergic drive, including noradrenaline (NA) and serotonin (5-HT), originating primarily from the brainstem and providing the spinal cord with a state-dependent control of excitability (Fung et al. 1991; Hochman et al. 2003; Jacobs et al. 2002; Jankowska et al. 1993; Jordan et al. 2008; Lundberg 1982; Millan 2002; Perrier and Delgado-Lezama 2005; Rekling et al. 2000; Schmidt and Jordan 2000). NA and other monoamines increase motoneuron excitability (Adachi et al. 2005; Elliott and Wallis 1992; Funk et al. 1994; Li et al. 2004b; Rekling et al. 2000) by facilitating persistent inward currents (PICs), consisting of low-voltage activated calcium (Ca PIC) and sodium (Na PIC) currents (Harvey et al. 2006b; Harvey et al. 2006c; Lee and Heckman 1999). These monoamine-dependent PICs are essential for motoneuron function,

amplifying synaptic inputs to motoneurons and providing the basic capacity for sustained depolarization and firing (Harvey et al. 2006b; Hounsgaard et al. 1988; Lee and Heckman 2000). Importantly, adrenergic facilitation of PICs occurs in animals like cats and rats (Harvey et al. 2006b; Lee and Heckman 1999), but also in humans (Udina et al. 2010). The elimination of necessary brainstem-derived monoamines following SCI leads immediately to a dramatic loss of PICs and motoneuron excitability, with some motoneurons incapable of even basic repetitive firing (Harvey et al. 2006c; Hounsgaard et al. 1988).

Paradoxically, in the weeks following the spinal transection, there is a spontaneous recovery of large PICs in motoneurons, across species including rats, cats and humans (Button et al. 2008; Gorassini et al. 2004; Hultborn et al. 2004; Li and Bennett 2003; Li et al. 2004a), despite the continued lack of brainstem-derived monoaminergic input to the spinal cord. However, unlike before injury, these PICs are permanently elevated, without brainstem control, leaving motoneurons in a permanently excitable state. Furthermore, as we discuss later, excitatory sensory-evoked synaptic inputs to motoneurons are augmented after injury, and thus the low-threshold PICs are readily activated by sensory stimulation (Li et al. 2004a). Finally, the powerfully depolarizing actions of these PICs, especially Ca PICs, are difficult to terminate voluntarily, because after SCI the motoneurons have weaker inhibitory inputs (e.g. postsynaptic glycine currents) (Boulenguez et al. 2010; Crone et al. 2003; Li et al. 2004a; Norton et al. 2008), especially from interneurons that are normally regulated by descending systems (Baldissera et al. 1981; Hammar and Jankowska 2003; Jankowska et al. 2000; Lundberg 1982; Shefchyk and Jordan 1985). The ultimate outcome is unchecked motoneuron firing and associated muscle spasms, produced by Ca PICs, which are readily triggered by normally innocuous stimulation and are not easily terminated (lasting many seconds, to minutes) (Bennett et al. 2001; Bennett et al. 2004).

The spontaneous recovery of motoneuron excitability and the re-emergence of PICs, despite of the absence of essential brainstem monoaminergic input, has been somewhat difficult to reconcile. However, by using receptor antagonists to inhibit the PICs, Harvey et al. (2006b) recently demonstrated that this recovery involves the spontaneous activation of both 5-HT_2 and α_1 adrenergic receptors, though the origin of this spontaneous activity was undetermined. Subsequently, Murray et al (2010b) demonstrated that spontaneous activity of the 5-HT_2 receptors occurs in the absence of any residual 5-HT, due to *constitutive receptor activity* (defined as receptor activity in the absence of any neurotransmitter). Similar constitutive activity may account for spontaneous adrenergic receptor activity in SCI, since adrenergic receptors are known to exhibit constitutive activity in reduced single cells systems (Rossier et al. 1999; Seifert and Wenzel-Seifert 2002). Additionally, it is likely that residual NA in the spinal cord may also contribute to adrenergic receptor activity, because increasing endogenous NA release via amphetamine increases reflexes, spasms and Ca PICs after complete spinal cord transection (Nozaki et al. 1980; Rank et al. 2007). We tested these ideas in the present paper, employing both a novel antagonist that blocks only conventional NA-activated receptor activity (neutral antagonist, REC15/2739) (Rossier et al. 1999) and antagonists that block constitutive receptor activity (inverse agonists, WB4101 and prazosin) (Rossier et al. 1999; Seifert and Wenzel-Seifert 2002).

While Harvey et al. (2006b) suggest that α_1 adrenergic receptors contribute to spasms after SCI, the specific receptor subtype is unknown (α_{1A}, α_{1B}, α_{1D}). Furthermore, even in the normal motoneurons it remains uncertain which adrenergic receptors modulate motoneuron PICs, because previous studies of adrenergic modulation of PICs (Lee and Heckman 1999) employed non-selective agonists (e.g. methoxamine) that likely activated both α_1 and α_2 adrenergic receptors (U'Prichard et al. 1977). Thus, prior to examining the origin of the spontaneous adrenergic receptor activity, we first identified the specific receptors

that modulate PICs, EPSPs and spasms using selective activation of receptor subtypes with agonists.

In addition to the facilitatory actions of descending NA on spinal motoneurons, NA also inhibits sensory afferent transmission to motoneurons and ascending sensory systems (Jankowska et al. 1993; Lundberg 1982; Millan 2002; Yoshimura and Furue 2006). For example, NA inhibits afferent transmission from low threshold group I and II muscle and cutaneous afferents, thereby inhibiting polysynaptic flexor reflexes (Clarke et al. 2002; Li et al. 2004b; Lundberg 1982). Thus, with SCI there is an immediate loss of this inhibition (disinhibition), leading to the emergence of unusually long polysynaptic EPSPs evoked by stimulation of group I and II afferents in both rats (Baker and Chandler 1987; Li et al. 2004a) and humans (Norton et al. 2008). Since Ca PICs require depolarizations of about half a second to be fully activated (Li and Bennett 2007; Li et al. 2004a), these long EPSPs are critical for activating the Ca PICs which in turn produce sustained motoneuron firing and uncontrolled muscle contractions.

It remains uncertain though which subclass of adrenergic receptors regulate these unusually long EPSPs responsible for triggering spasms or whether these receptors are constitutively active. The α_2 adrenergic receptor is an ideal candidate for the regulation of the long EPSPs since α_2 receptors are known to play a role in the control of afferent transmission (Clarke et al. 2002; Jankowska and Hammar 2002; Rekling et al. 2000). Furthermore, after SCI inhibition over sensory transmission and reflexes can be restored by application of α_2 receptor agonists (Chau et al. 1998; Clarke et al. 2002; Jankowska and Hammar 2002; Sakitama 1993; Tremblay and Bedard 1995). Thus, another goal of this paper was to determine whether the α_2 receptor inhibits the long EPSP that mediates spasms, and whether loss of ligand-activated receptor activity after SCI is partly compensated for by spontaneous activity in α_2 adrenergic receptors.

2.2 METHODS

Adult female Sprague-Dawley rats with chronic SCI resulting in fully developed spasticity, and normal, previously unlesioned adult rats were utilized in this study. Rats received a complete sacral spinal (S_2) transection at 45 - 55 days old, as previously detailed (Bennett et al. 1999; Bennett et al. 2004). All experiments on chronic spinal rats were conducted after full spasticity had developed in the axial muscles of the tail (2 - 3 months after transection). Experiments on normal rats were conducted at a similar age (3 - 6 months old). Recordings were made from sacrocaudal motoneurons and ventral roots of normal and chronic spinal rats *in vitro* (Li et al. 2004a; Li et al. 2004b). Muscle spasms were also recorded *in vivo* via percutaneous EMG placed in the axial tail muscles of spastic chronic spinal rats (Murray et al. 2010b). All procedures were approved by the University of Alberta Animal Care and Use Committee: Health Sciences.

2.2.1 *In vitro* preparation

Details of the *in vitro* preparation have been previously described in detail (Li et al. 2004a; Li et al. 2004b), and are only briefly summarized here. Rats were deeply anaesthetized with urethane (0.18 g/100 g; with a maximum dose of 0.45 g) and the whole sacrocaudal spinal cord was removed and transferred to a dissection chamber filled with modified artificial cerebrospinal fluid (mACSF) maintained at a constant temperature of 20° C. To remove the cord in chronic spinal rats a transection was made just above the chronic injury (at upper S_2 level). In normal adult rats the cord was cut at the same location (upper S_2) for removal, and they are therefore termed acute spinal rats. All dorsal and ventral spinal roots were removed, with the exception of the sacral S_4 and caudal Ca_1 ventral roots and the Ca_1 dorsal roots. The cord was then allowed to rest in the dissection chamber for 1.5 hrs. Following this rest period, the cord was transferred to a recording chamber containing continuously flowing normal artificial cerebrospinal fluid (nACSF) maintained near 24° C and with a flow rate

> 5 mL/min. Following a 60 min nACSF wash out period to clear any residual anaesthetic and mACSF, the nACSF was recycled with a peristaltic pump.

2.2.2 Intracellular recordings and analysis

Intracellular recordings were made from motoneurons in the sacrocaudal spinal cord of chronic spinal rats, as detailed elsewhere (Li et al. 2004a), and are only briefly summarized here. Sharp intracellular electrodes were made from thick-walled glass capillary tubes (1.5 mm O.D.; Warner GC 150F-10) using a Sutter P-87 micropipette puller. Electrodes were back-filled with a combination of 1M potassium acetate and 1 M KCl. A stepper-motor micromanipulator (660 Kopf) was used to advance into motoneurons. After penetration, motoneuron identification was made with antidromic stimulation of the S_4 and Ca_1 ventral roots, noting ventral horn location, input resistance and time constant (> 6 ms for motoneurons) (Li et al. 2007). Data were collected with an Axoclamp 2b intracellular amplifier (Axon Instruments, Burlingame, CA) running in discontinuous current clamp (DCC, switching rate 4 - 6 kHz, output bandwidth 3.0 kHz, sample rate of 6.7 kHz) or discontinuous single-electrode voltage clamp (SEVC; gain 0.8 to 2.5 nA/mV) modes. To measure the basic electrical properties of motoneurons, slow triangular current ramps (0.4 nA/s) and voltage ramps (ramp speed 3.5 mV/s) were applied. Resting potential (V_m) was recorded 15 mins after cell penetration, allowing time for the cell to stabilize, with a bias current of 0 nA. The input resistance (R_m) was measured during the voltage ramps over a 5 mV range near resting membrane potential and subthreshold to PIC onset. PIC measurements were made during the slow triangular voltage ramps. Firstly, the passive leak current was estimated during the upward portion of the ramp where the current response initially increases linearly with voltage in response to the passive leak conductance. A linear relation was fit to the subthreshold current response 5 - 10 mV below the negative-slope region of the PIC onset, and then extrapolated to more positive voltages. The PIC amplitude was then estimated by subtracting this leak current from the total recorded current

(leak-subtracted current). The onset voltage for the PIC (V_{on}) was measured at the beginning of the first negative slope region in the current (where first zero slope in current response occurred). The peak current of the PIC was measured from the leak subtracted current, where the downward deviation below the leak line reached peak amplitude. EPSPs and associated reflexes were also measured in motoneurons after stimulating the Ca_1 dorsal roots (at 3 x threshold, T) while applying a hyperpolarizing bias current to block the PICs, and peak value quantified at about 200 ms after the stimulation (long polysynaptic EPSP). Data were analysed in Clampfit 8.0 (Axon Instruments).

2.2.3 Ventral root reflex recording and averaging

A detailed description of these procedures can be found in Li et al. (2004b). Briefly, two dorsal roots (left and right Ca_1) and two to four ventral roots (left and right S_4 and/or Ca_1) were mounted on chlorided silver wires suspended above the ACSF of the recording chamber for monopolar stimulation and recording, respectively. The roots were wrapped around the wire above the ACSF and covered with a 1:1 mixture by weight of petroleum jelly/mineral oil. Ventral root reflexes were recorded in response to a single low threshold stimulation pulse (0.1 ms, 0.02 mA; Isoflex stimulator, AMPI) to the dorsal root (3 x reflex threshold, T ~ = 0.007 mA), consistent with stimulation of group I and II afferents, including mainly cutaneous afferents (Bennett et al. 2004; Li et al. 2004a; Li et al. 2004b). Dorsal root stimulation was repeated five times consecutively with an inter-stimulus interval of 10 s to provide multiple ventral root reflexes for averaging. Ventral root reflexes were recorded via a custom built differential preamplifier, with one lead connected to the root and the second to the reference wire in the ACSF [high pass 100 Hz; low pass 3 kHz; amplified by 2000 times; sampling rate 6.7 kHz (Axoscope 8, Axon Instruments)]. Ventral root reflexes were recorded every 10 - 12 mins. When drugs were used, they were added to the bath immediately after a recording so as to ensure the actions of the drug could be recorded at the subsequent 10 - 12 min interval. Dose response curves were

constructed by administering increasing doses of the drug every 10 - 12 mins. Ventral root reflexes were quantified using custom written software (MatLab 7.0.4, MathWorks, Natick, MA). That is, ventral root recordings were high pass filtered (at 800 Hz, using a 1st order Butterworth filter), rectified and then averaged over a time window 500 - 4000 ms post stimulation, which we term the long-lasting reflex (LLR). This reflex period has previously been shown to result mainly from a sustained depolarization of the motoneurons by the Ca PIC (Bennett et al. 2004; Li et al. 2004a), though the activation of the Ca PIC itself depends on a long polysynaptic EPSP evoked by the stimulation (Li et al. 2004a).

2.2.4 Drugs and Solutions

Two kinds of artificial cerebrospinal fluid were used in these experiments; a modified ACSF (mACSF) used during dissection and recovery to minimize neural and metabolic activity and a normal ACSF (nACSF) in the recording chamber. The mACSF was composed of (in mM) 118 NaCl, 24 $NaHCO_3$, 1.5 $CaCl_2$, 3 KCl, 5 $MgCl_2$, 1.4 NaH_2PO_4, 1.3 $MgSO_4$, 25 D-glucose, and 1 kynurenic acid. Normal ACSF was composed of (in mM) 122 NaCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 3 KCl, 1 $MgCl_2$, and 12 D-glucose. Both types of ACSF were saturated with 95% O_2 - 5% CO_2 and maintained at pH 7.4. During intracellular recordings transient and persistent sodium currents were sometimes blocked with tetrodotoxin (TTX Alamone Labs, Isreal) in order to isolate the Ca PIC. Other drugs used include methoxamine, strychnine (Sigma-Aldrich, Oakville, ON), A61603, prazosin, WB4101, clonidine, RX821002, UK14304 (Tocris Biosciences, Ellisville, MO) and Recordati 15/2739 (abbreviated REC 15/2739; generously donated by Recordati S.p.A., Milano, Italy).

2.2.5 Percutaneous electromyogram (EMG) reflex recording

Awake chronic spinal rats were housed inside a clear Plexiglass tube with the tail protruding and held horizontally by taping it to a bar. The tail was kept warm with

a radiant heat lamp. Multi-stranded stainless steel wires (AS631; Cooner Wire Inc., Chatsworth CA) were bared 1 cm at each end and inserted percutaneously into the axial muscles of the mid-tail. EMG electrode placement into the tail muscles was standardized using the 12th coccygeal vertebra as a reference point. Recording electrodes were placed 1- and then 2 cm rostral to this point with the ground electrode placed 1 cm caudal to this point. Long-lasting cutaneous reflexes (termed LLR or spasms) were elicited with two stimulating electrodes inserted percutaneously on the distal tip of the tail, separated from each other by 1.5 cm. As the tip of the tail contains very little muscle, stimulation via the electrodes placed here provides relatively pure cutaneous stimulation (Bennett et al. 1999; Bennett et al. 2004). To prevent movement of the wires, each wire was fixed to the skin using a small amount of cyanoacrylate glue. Spasms were evoked by single pulse stimulation (width 0.2 ms) at 10 mA (Isoflex Stimulator, AMPI; about 3 – 5 x T) every 10 s, and repeated 6 times. LLRs (spasms) were recorded with the EMG wires using a custom built amplifier and Axoscope hardware and software (Digidata 1322A, Axoscope; Axon Instruments, Burlingame CA; amplified 2000 times, low pass filtered at 1000 Hz, high pass filter at 100 Hz and sampled at 5 kHz). LLRs were quantified in a similar manner to that used for ventral root reflexes (see above). To summarize, data were rectified and the long-lasting spastic reflex (LLR) was computed by averaging the rectified EMG over a time-window 500 – 4000 ms post stimulation.

2.2.6 *In vivo* drug injection

Unless otherwise listed, all drugs were administered *in vivo* via transcutaneous intrathecal (IT) injection, under light isofluorane anesthetic. A 1-inch, 25 - guage needle connected to a 100 μL glass Hamilton syringe was inserted into the tissues between the L_5 and L_6 vertebrae on the dorsal side, perpendicular to the spinal column as per (Mestre et al. 1994). This injection site was selected because of easy intervertebral accessibility to the spinal cord as well as a reduced possibility of spinal cord damage, since the injection site is restricted to the area near the

cauda equina. As the needle entered the spinal canal the tail would produce an abrupt lateral twitch, caused by the needle entering the proximity of the ventral roots, and this sign was used to positively confirm the injection site. The drug solution was slowly injected over about 5 s. Drugs injected IT included A616103, prazosin, WB4101 and REC15/2739. All drugs were dissolved in sterile saline at a constant volume of 30μL for each IT injection. Rats woke up within minutes of removal of light anaesthetic, at which point reflex testing resumed. Neither the anaesthetic nor the saline vehicle influenced the reflexes, as tested by control saline IT injections. Chronic spinal animals received multiple intrathecal injections per experimental session, up to a maximum of four injections, with at least 90 mins separating each injection.

2.2.7 Statistics

All data are shown as mean ± standard error throughout the text and figures. Statistical differences were computed at a significance level of $P < 0.05$ with a paired Student's t-test where data were before and after drug applications in the same animals, and otherwise with an unpaired Student's t-test or ANOVA as needed. A Kolmogorov-Smirnov test for normality was applied to each data set, with the level set for significance set to $P = 0.05$, to verify normality, as is required for a t-test. Where dose-response curves are presented, a standard sigmoidal curve (with a Hill slope of unity) was fit to LLR responses with increasing drug doses (in log units). Drug potency, as indicated by the dose at which 50% of the maximal effect was observed (EC50), was measured from the curve. All calculations of EC50 values and accompanying statistics comparing EC50 values were carried out using the logarithm of dose.

2.3 RESULTS

2.3.1 Ca PICs are increased by α_1 receptors

Considering that Ca PICs in motoneurons are a major underling cause of spasms after SCI, we began our study by examining the effect of α_1 receptor activation on Ca PICs. The Ca PICs were quantified *in vitro* during intracellular recordings from motoneurons in the sacral spinal cord of chronic spinal rats (Fig 2-1). TTX (2 μM) was applied to isolate the motoneuron synaptically (blocking spike-mediated transmission) and to block the Na PIC that otherwise can obscure the Ca PIC, as previously described (Li and Bennett 2003). We quantified the Ca PIC using slow voltage ramps to inactivate transient currents, and enable the full voltage-dependence of the Ca PIC to be evaluated. During these slow voltage ramps (under voltage-clamp conditions), the Ca PIC was activated at –57.94 ± 7.63 mV (V_{on}, n = 9 motoneurons) and produced a downward deflection in the recorded current of 1.05 ± 0.26 nA, which we considered an estimate of the Ca PIC amplitude (Fig 2-1A, arrow; previously verified to be mediated by L-type calcium channels; nimodipine-sensitive) (Li and Bennett 2003; Li et al. 2004a). Application of the moderately selective α_1 adrenergic receptor agonist methoxamine significantly increased this Ca PIC amplitude (151.32%; Fig 2-1A, D). In contrast, methoxamine had no effect on the Ca PIC threshold V_{on}, the motoneuron resistance or resting membrane potential (changes with drug: –0.14 ± 1.42 mV, 0.84 ± 0.69 MΩ, –0.13 ± 2.53 mV, respectively; not significant, P > 0.05, n = 9).

2.3.2 Long-lasting reflexes are increased by α_1 receptors

To examine the functional consequences of α_1 adrenergic receptors, we measured the effects of adrenergic receptor agonists on long-lasting reflexes (LLR; quantified 500 - 4000 ms post stimulation) recorded on the ventral roots in response to a brief dorsal root stimuli *in vitro* (single pulse, 3 x T), which have

FIGURE 2-1

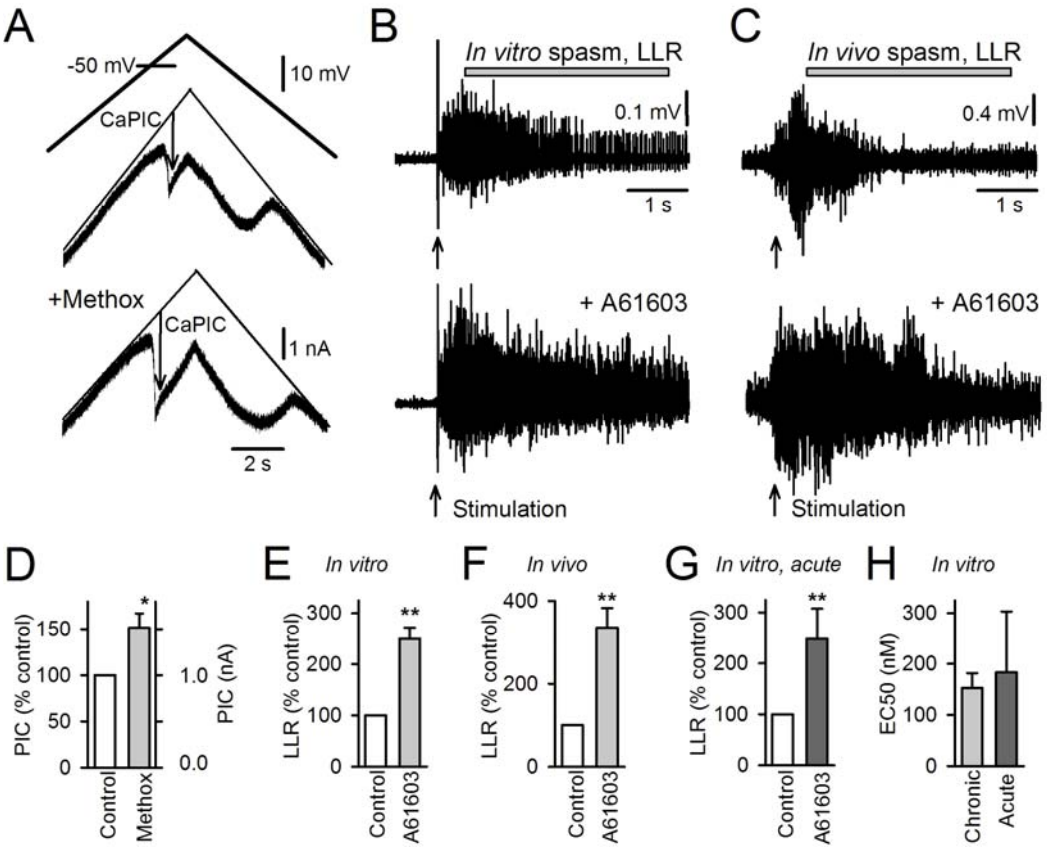
A
-50 mV
10 mV
CaPIC
+Methox
CaPIC
1 nA
2 s
B
In vitro spasm, LLR
0.1 mV
1 s
+ A61603
Stimulation
C
In vivo spasm, LLR
0.4 mV
1 s
+ A61603
Stimulation
D
PIC (% control)
PIC (nA)
Control
Methox
E
In vitro
LLR (% control)
Control
A61603
F
In vivo
LLR (% control)
Control
A61603
G
In vitro, acute
LLR (% control)
Control
A61603
H
In vitro
EC50 (nM)
Chronic
Acute

Figure 2-1

Activation of the α_1 adrenergic receptor increases Ca PICs and spasms. **(A)** Intracellular recording of Ca PIC in motoneuron, recorded in whole sacrocaudal spinal cord below a chronic transection, *in vitro.* Ca PIC measured in isolation by a slowly increasing the membrane potential (top) in presence of 2 μM TTX, and quantified at its initial peak, where it produced a downward deflection in the recorded current (thick black plot, at arrow, Ca PIC) relative to the leak current (thin line in middle plot). Lower plot: increase in Ca PIC with addition of the α_1 adrenergic receptor agonist methoxamine to the bath (10 μM). **(B)** Long-lasting reflex triggered by dorsal root stimulation (single pulse, 3 x T) and recorded from the ventral roots (LLR, quantified during horizontal bar; counterpart of spasms) before and after application of the α_1 receptor agonist A61603 (0.1 μM). **(C)** Long-lasting reflex spasm in awake chronic spinal rat evoked by electrical/cutaneous stimulation of the tail (0.2 ms pulse 10mA) and recorded with tail muscle EMG before and after local intrathecal (IT) injection of A61603 (0.03 mM in 30 μl). Spasm quantified during horizontal bar (LLR). **(D)** Group mean of increase in Ca PIC with methoxamine (abbreviated methox; 10 – 40 μM) in chronic spinal rats ($n = 8$), normalized (left axis) and in absolute current values (right axis). **(E–F)** Normalized group mean of increase in LLR with application of A61603 to the isolated *in vitro* spinal cord of chronic spinal rats (0.03 – 1 μM; $n = 42$) and to the *in vivo* spinal cord in the awake chronic spinal rat (0.03 mM in 30 μl; IT injection, $n = 5$). **(G)** Normalized group mean of increase in LLR with application of A61603 (0.03 – 1 μM; $n = 18$) to the isolated *in vitro* spinal cord removed from normal rats (termed acute spinal). Control values were taken in strychnine (3 μM) to produce a similar LLR to that under control untreated condition in chronic spinal rats. **(H)** Normalized group mean of the dose to produce a 50% increase in the LLR (EC50) with increasing doses of A61603, recorded both in chronic spinal ($n = 30$) and acute spinal ($n = 11$) conditions *in vitro*. $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars, s.e.m. All recordings in B–H were made in the presence of RX821002 (*in vitro*: 0.5 μM; *in vivo*: IP injection, 1mg/Kg) to prevent involvement the α_2 adrenergic receptor.

previously been shown to depend on Ca PICs (Li et al. 2004a). Neither the moderately selective α_1 adrenergic receptor agonist methoxamine (0.1 – 30 μM) (Minneman et al. 1994; Shibata et al. 1995) nor the more selective and potent α_{1A} receptor agonist A61603 (0.03 – 10 μM) (Craig et al. 1997; Knepper et al. 1995; Mehrotra et al. 2007) consistently changed the LLR (non-significant increase of 24.3 ± 31.0% and 10.7 ± 15.5% for methoxamine and A61603 respectively, $P > 0.05$, $n = 14$ each). This was initially unexpected, considering that following the transient dorsal root evoked EPSP (< 500 ms) known to trigger the Ca PICs, the remaining many-second long portion of the LLR that we quantified is almost entirely mediated by the Ca PICs on motoneurons (see Introduction) (Li et al. 2004a; Murray et al. 2010a). However, in retrospect we realized that this was due to a potent inhibition of the EPSP by these agonists, mediated by α_2 receptors, as we describe later, and this counterbalanced the increase in the Ca PICs mediated by α_1 receptors. This occurred because methoxamine and A61603, as well as other available α_1 agonists, have substantial binding affinity for α_2 as well as α_1 receptors (Craig et al. 1997; Mehrotra et al. 2007; Minneman et al. 1994; Shibata et al. 1995; U'Prichard et al. 1977), and the negative effects of α_2 receptors in our preparation were unexpectedly large (see later section). This poor α_1 verses α_2 selectivity had not been anticipated, especially for A61603, because A61603 has otherwise negligible binding at all other receptors previously tested, including α_{1B} and β adrenergic, 5-HT and dopamine receptors (Craig et al. 1997).

To study the effects of the α_1 receptor in isolation, we blocked the α_2 receptors with the selective α_2 receptor antagonist RX821002 (0.3 – 0.5 μM) (Jasper et al. 1998; Sanders et al. 2006) prior to applying the agonist A61603. This effectively makes A61603 highly selective for the α_{1A} adrenergic receptor (Craig et al. 1997). Under these conditions, A61603 significantly increased the LLR (Figs 2-1 and 2-2), more than doubling the LLR amplitude when given at doses above 30 nM. This is consistent with an α_{1A} receptor mediated increase in the PIC. The facilitation of the LLR increased with increasing doses of A61603 (up to 1000 nM), and this dose-response relation was well approximated by a sigmoidal

function, with half-maximal effects at about 150 nM (EC50, Fig 2-1H and 2-2C, sigmoid had Hill slope of 1.0), consistent with the known high affinity of A61603 to the α_{1A} receptor (K_i = 80 nM) (Craig et al. 1997; Mehrotra et al. 2007).

2.3.3 Spasms in awake rat are increased by α_1 receptors

We also examined the effects of α_1 receptor activation on spasms triggered by brief cutaneous stimulation at the tip of the tail (3 x T) and recorded from the axial tail muscles of awake chronic spinal rats with implanted electromyogram (EMG) wires. These spasms are the equivalent of the LLR recorded *in vitro* (Bennett et al. 2004; Li et al. 2004a), lasting many seconds, and were quantified over the same time window (500 – 4000 ms, LLR and spasm used interchangeably; Fig 2-1C). The adrenergic agonists A61603, methoxamine and phenylephrine were applied locally to the spinal cord by intrathecal injection (IT, 0.1 – 1 mM in 30 µl saline), to avoid systemic effects. Again, we found that, by themselves, none of these agonists increased LLRs (spasms) in all rats tested (n = 7/7 rats tested; data not shown), though in two of these animals A61603 induced a regular rhythmic movement of the tail in the absence of spasm-triggering stimulation. In contrast, after a prior application of RX821002 (1 – 3 mg/Kg, IP) to block possible non-selective actions on α_2 receptors, LLRs (tail spasms) were significantly increased by an IT injection of A61603 (Fig 2-1C, F). Control saline injections had no significant effect ($P > 0.05$, $n = 5$; not shown). These results further demonstrate that activation of the α_1 adrenergic receptor increases spasticity and underlying Ca PICs in chronic spinal rats.

2.3.4 Chronic spinal rats are not supersensitive to α_1 receptor activation

The increases in LLRs resulting from α_1 adrenergic receptor activation were not limited to chronic spinal animals. Application of A61603 also lead to increases in LLRs recorded in normal control rats studied *in vitro* (considered acute spinal because of cord removal for *in vitro* recording; Fig 2-1G; in RX821002). For

these acutely spinalized rats, LLRs were initially absent (ie. animals were not spastic). To ensure similar preliminary conditions, a low dose of strychnine was administered *in vitro* which resulted in LLRs that were similar in magnitude to those in chronic spinal rats (only slightly smaller, raw data not shown). The increase in LLRs produced by A61603 in these acutely spinalized control rats, with strychnine, was comparable to that seen in chronic spinal animals (Fig 2-1E vs Fig 2-1G). Moreover, the dose at which A61603 exerted half of its maximal effect on *in vitro* LLRs (EC50) was similar in both chronic and acutely lesioned rats (Fig 2-1H), indicating a lack of supersensitivity to α_1 receptor activation with A61603.

2.3.5 Blocking the α_1 adrenergic receptor reverses agonist-mediated increase in spasms

As mentioned, agonists of α_1 adrenergic receptors generally demonstrate only limited selectivity over other adrenergic receptor subtypes (e.g. α_2 receptors). In contrast, antagonists of α_1 adrenergic receptors are more selective (including REC15/2739, prazosin and WB4101) (Doxey et al. 1983; Ford et al. 1997; Sanders et al. 2006; Schwinn et al. 1995; Shibata et al. 1995), and for that reason we used these drugs to confirm the involvement of the α_1 receptors in facilitating LLRs in chronic spinal rats. We found that the facilitation of the LLR by A61603 (in presence of RX821002, as above) was significantly inhibited by a subsequent application of prazosin or REC15/2739 (Fig 2-2A, B, D, E), *in vitro*. The typical time course of the facilitation of the LLR by the α_1 agonist and subsequent inhibition by the α_1 antagonist is shown in Fig 2-2B, with the antagonist acting relatively slowly, taking > 30 mins to reach peak effect. Part of this antagonist-mediated inhibition might have resulted from a block of endogenously active α_1 receptors (see below). Thus, we also evaluated the action of increasing doses of the agonist A61603 on the LLR after first applying the antagonist and giving time for the intrinsic effects of this antagonist, if any, to reach steady state (agonist

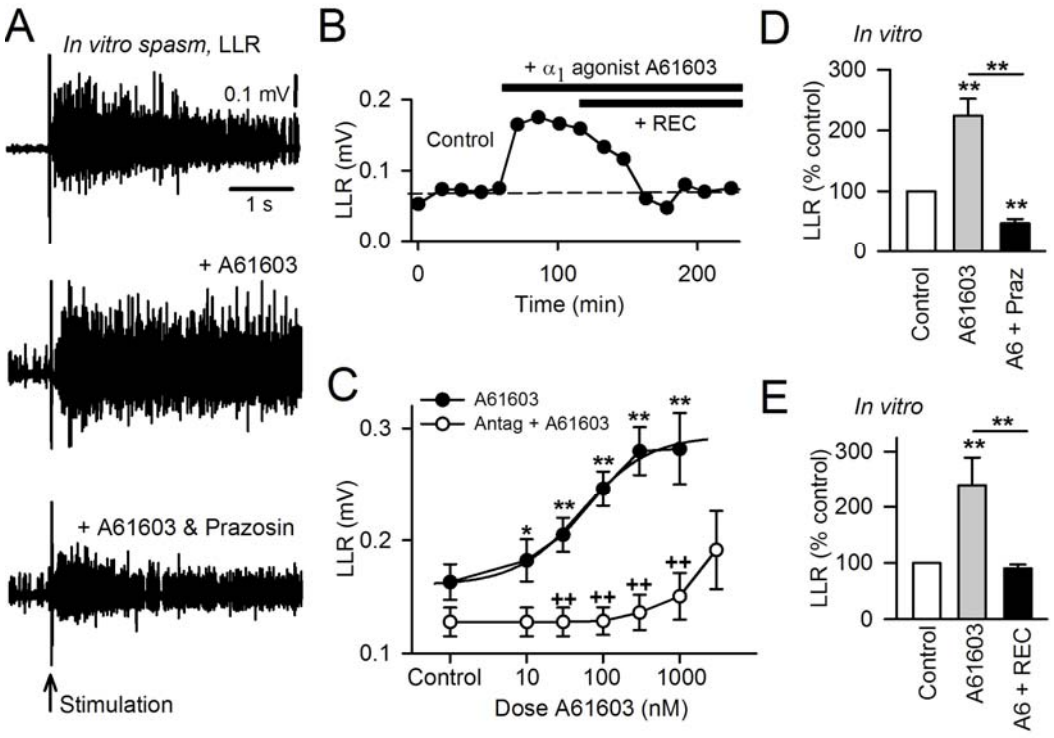

Figure 2-2

The α_{1A} adrenergic receptor agonist A61603 is antagonized by selective α_1 receptor antagonists. **(A)** LLR evoked in the isolated *in vitro* spinal cord of a chronic spinal rat, as described in Fig. 2-1 (top plot), with bath application of the α_{1A} receptor agonist A61603 alone (0.1 μM, middle plot), and with subsequent application of α_1 receptor antagonist prazosin (1 μM, bottom plot). **(B)** Amplitude of LLR (quantified 0.5 – 4 s post stimulus, as in Fig. 2-1) of a chronic spinal rat measured repeatedly over time under control conditions (left), with application of A61603 (upper horizontal black bar; 0.03 μM) and subsequent application of the highly specific α_{1A} receptor neutral antagonist REC15/2739 (abbreviated REC; lower horizontal black bar; 10 μM). **(C)** Mean LLR amplitude in response to increasing doses of A61603 (dose response) recorded in chronic spinal rat *in vitro* (filled circles, upper line; $n > 18$ for each dose) and for A61603 applied after the α_1 receptor antagonists prazosin (1 μM) or WB4101 (3 μM) (open circles, lower line; $n > 8$ for each dose, $++P < 0.01$). **(D)** Normalized group mean of LLR with application of A61603 alone (0.03 – 0.3 μM; grey bar, $n = 42$) and A61603 with subsequent treatment with prazosin (Abbreviated A6 + Praz; black bar, $n = 16$), recorded in chronic spinal rat *in vitro*. **(E)** Same as (D) except treatment with A61603 alone (0.03 μM; $n = 15$), and A61603 with subsequent application of REC15/2739 (abbreviated A6 + REC; 10 μM; $n = 15$). $*P < 0.05$, $**P < 0.01$. Error bars, s.e.m. All recordings were made in the presence of RX821002 (0.5 μM).

given > 30 mins after antagonist). In this situation, increasing doses of the agonist A61603 had no effect until very high doses were reached (1000 nM), whereas without the antagonist A61603 increased the LLR at doses as low as 10 nM, demonstrating that this agonist indeed increased the LLR and associated PICs via α_1 receptors. These experiments were performed in the presence of RX821002 to rule out any non-selective action of A61603 on the α_2 receptor.

2.3.6 Endogenous activity in α_1 receptors *in vivo* and *in vitro*, via two mechanisms.

The drug REC15/2739 is special because it has previously been shown to act as a *neutral* antagonist at α_{1A} adrenergic receptors, meaning that it blocks only the action of a ligand (such as NA) at the α_{1A} receptor, and not constitutive receptor activity (Rossier et al. 1999). REC15/2739 is therefore useful in determining whether the α_{1A} receptors are activated by endogenous NA, or another natural ligand, that somehow persists below a chronic spinal injury. We found that administration of REC15/2739 alone had no effect on *in vitro* ventral root LLRs (Fig 2-3A, C), even though it readily antagonized the α_{1A} agonist A61603 (Fig 2-2E). This suggests that, at least *in vitro*, the α_{1A} receptor is not endogenously activated by residual NA in the spinal cord. In contrast, when we administered REC15/2739 *in vivo* with a localized IT injection, there was a significant decrease in LLRs (spasms Fig 2-3B, D). This demonstrates that the α_{1A} adrenergic receptor is activated by some endogenous ligand, likely NA, *in vivo*, but not *in vitro*, indicating that any residual endogenous NA that affects the spinal cord may originate from the periphery (see Discussion).

Interestingly, application of the α_1 antagonists WB4101 or prazosin significantly decreased LLRs recorded *in vitro* (without prior agonist application, Fig 2-3C and D), even though REC15/2739 did not. WB4101 and prazosin have been previously shown to act as potent *inverse agonists* at α_1 receptors (Noguera et al. 1996; Rossier et al. 1999; Seifert and Wenzel-Seifert 2002), which means that

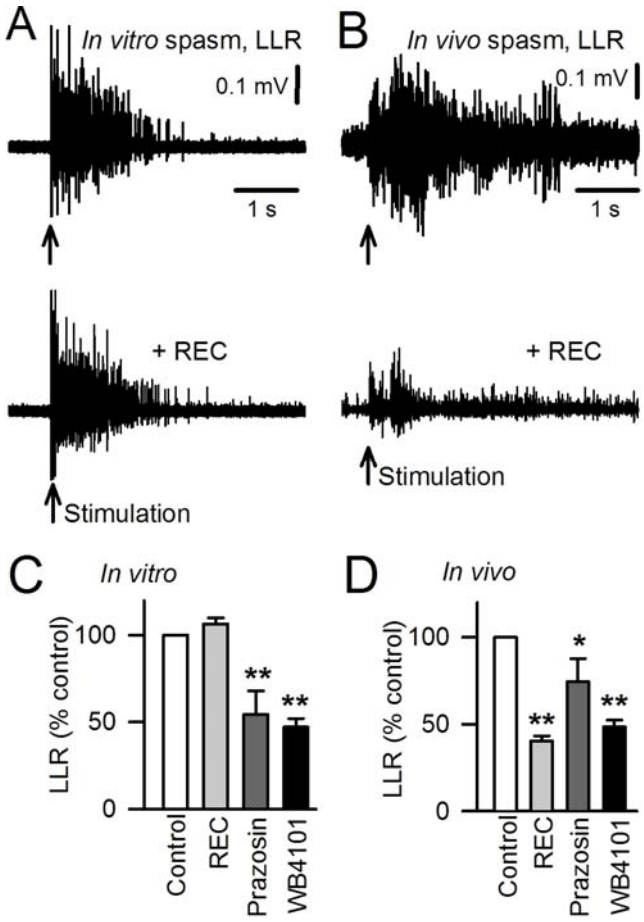

Figure 2-3

Endogenous activation of the α_1 adrenergic receptor is the result of constitutive activity *in vitro*, but a combination of constitutive and ligand activity *in vivo*. **(A)** LLR in chronic spinal rat, evoked in the isolated *in vitro* spinal cord (as described in Fig 2-1, upper plot) and after blocking the action of endogenous NA (or similar ligand) with application of the α_{1A} neutral antagonist REC15/2739 (abbreviated REC; 10 μM, bottom plot). **(B)** Long lasting reflex spasm in awake chronic spinal rat evoked by electrical/cutaneous stimulation of the tail and recorded with tail muscle EMG (LLR computed 0.5 – 4 s post stimulus, as in Fig 2-1) before (top plot) and after blocking endogenous action of NA at the α_{1A} receptor with local intrathecal (IT) injection of REC15/2739 (5 mM in 30 μl). Normalized group mean of chronic spinal rat LLRs recorded *in vitro* (**C**) and *in vivo* (**D**) after application of the α_1 receptor neutral antagonist REC15/2739 (grey bars, *in vitro*: 5 – 10 μM, $n = 24$; *in vivo*: IT injection of 3 - 10mM in 30 μl; $n = 5$), and after application of inverse α_1 receptor agonists prazosin (dark grey bars, *in vitro*: 1 μM, $n = 24$; *in vivo*: IT injection, 1 mM in 30 μl, $n = 9$) and WB4101 (black bars, *in vitro*: 3 – 5 μM, $n = 16$; *in vivo*: IT injection, 1 – 3 mM in 30 μl; $n = 5$). $^*P < 0.05$, $^{**}P < 0.01$. Error bars, s.e.m. All recordings were made in the presence of RX821002 (*in vitro*: 0.5 - 1 μM; *in vivo*: IP injection, 1 mg/Kg).

they block constitutive activity in α_1 receptors, in addition to blocking traditional ligand-mediated activation of the receptor. Thus, the inhibitory action of WB4101 and prazosin on the LLR, together with the lack of action of the neutral antagonist (REC15/2739; no ligand activated receptors), suggests that the α_1 receptors exhibit substantial constitutive activity, at least *in vitro*. When applied *in vivo,* both WB4101 and prazosin (IT) likewise inhibited the LLRs (spasms Fig 2-3D), which is likely due to both a block of ligand-activated receptors (residual NA) and constitutively activated receptors.

2.3.7 The α_2 adrenergic receptor modulates the EPSP, but not the Ca PIC

Considering that we suspected an inhibitory effect of α_2 receptors on the EPSPs that trigger LLRs (spasms), we next measured how the moderately selective α_2 adrenergic (and imidazoline I_1) receptor agonist clonidine, and the highly selective α_{2A} adrenergic receptor agonist UK14304, affected ventral root LLRs *in vitro*. Treatment with both these α_2 agonists significantly decreased LLRs (Fig 2-4A, D), and this decrease was reversed by subsequent treatment with the selective α_2 adrenergic antagonist RX821002 (Fig 2-4A, D). Furthermore the decrease in the LLR with clonidine was dose dependent, with a very low EC50 of 25 ± 7 nM, consistent with the high binding affinity of clonidine to the α_{2A} receptor (K_i = 31 nM) (Millan et al. 2000), and inconsistent with the 10 times lower affinity of clonidine to α_1 receptors (e.g. K_i = 300 nM at α_{1A} receptor) (Millan et al. 2000). These results suggest that α_{2A} adrenergic receptors inhibit LLRs and resulting spasms after chronic SCI.

We next investigated whether this inhibitory effect of α_2 receptors was mediated by a reduction in the dorsal root evoked long polysynaptic EPSP that triggers the PICs, or the PICs themselves that ultimately cause the many seconds of firing during the LLRs (spasms). We recorded EPSPs in motoneurons of chronic spinal rats in response to our standard brief dorsal root stimulation (0.1 ms, 3 x T; Fig 2-4C). The motoneurons were held with a hyperpolarizing bias current in order to

FIGURE 2-4

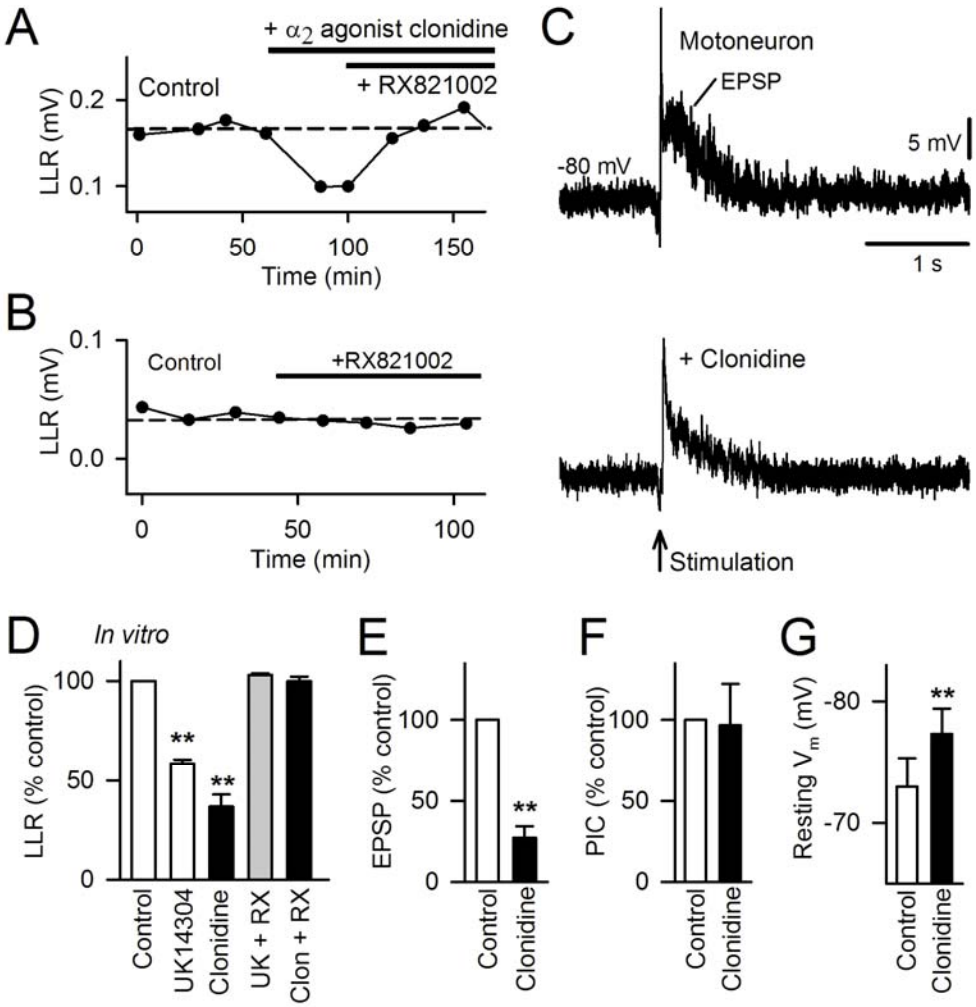
A
+ α2 agonist clonidine
Control
+ RX821002
LLR (mV)
0.2
0.1
0
50
100
150
Time (min)
B
Control
+RX821002
LLR (mV)
0.1
0.0
0
50
100
Time (min)
C
Motoneuron
EPSP
5 mV
-80 mV
1 s
+ Clonidine
Stimulation
D
In vitro
LLR (% control)
100
50
0
**
**
Control
UK14304
Clonidine
UK + RX
Clon + RX
E
EPSP (% control)
100
50
0
**
Control
Clonidine
F
PIC (% control)
100
50
0
Control
Clonidine
G
Resting Vm (mV)
-80
-70
**
Control
Clonidine

Figure 2-4

Activation of the α_2 adrenergic receptor does not directly affect the Ca PIC, but instead inhibits EPSPs **(A)** Amplitude of LLR recorded in the isolated *in vitro* spinal cord of chronic spinal rat and measured repeatedly over time (LLR quantified 0.5–4 s post stimulus, as in Fig. 2-2). Control values are shown on left, followed by activation of α_2 adrenergic receptors with the agonist clonidine (upper horizontal black bar; 0.1 μM) and subsequent application of the α_2 receptor antagonist RX821002 (0.3 μM; lower horizontal black bar). **(B)** Same format as (A) with application of RX821002 alone (0.5 μM). **(C)** Intracellular motoneuron recording of long-latency polysynaptic EPSP (abbreviated EPSP) evoked by dorsal root stimulation (0.1 ms at 3 x T) of chronic spinal rat (quantified at 200 ms post stimulus) during hyperpolarizing bias current before (top plot) and after blocking the α_2 receptor with bath application of clonidine (0.3 μM, bottom plot). **(D)** Normalized group mean for LLR in chronic spinal rats *in vitro* with application of the α_2 receptor agonists UK14304 (0.03 μM; white bar, $n = 8$), and clonidine (0.1 μM; black bar, $n = 5$), and application α_2 antagonist RX821002 (0.3 μM) after UK14304 (abbreviated UK14 + RX; grey bar, $n = 8$) and after clonidine (abbreviated Clon + RX; black bar, $n = 8$). Normalized group mean of intracellulary recorded polysynaptic EPSP (**E**) evoked by 3 x T dorsal root stimulation, PIC (**F**) and resting membrane potential (V_m) (**G**) before and after application of clonidine in chronic spinal rats (0.1 – 1 μM; $n = 5$). *$P < 0.05$, **$P < 0.01$. Error bars, s.e.m.

prevent PIC activation and spiking (holding cell at -80 mV), and thus allow us to investigate the EPSP in isolation (Li et al. 2004a). Under these conditions, a long EPSP was evoked with a 5 – 10 ms latency, lasting about 500 – 1000 ms, and with a mean amplitude of 5.2 ± 2.1 mV measured at 250 ms post-stimulation (at main peak after transient peak at 5 - 10 ms). The α_2 receptor agonist clonidine decreased this long polysynaptic EPSP significantly (Fig 2-4C, E). In contrast, clonidine had no effect on the PIC (Fig 2-4F; recorded under voltage clamp, as described in Fig 2-1A). Interestingly, clonidine significantly hyperpolarized the resting membrane potential by about –4 mV (Fig 2-4). These data suggest that the inhibitory effect of the α_2 receptor on spasms is mediated by a reduction of the long polysynaptic EPSP that trigger the PICs (and associated spasms), rather than by a reduction in the PICs themselves. Additionally, this receptor may act by hyperpolarizing the motoneurons.

2.3.8 Lack of constitutive activity in α_2 receptors

Application of the α_2 adrenergic receptor antagonist RX821002 alone, without agonists, had no effect on the LLRs (Fig 2-4B and Fig 2-5A, C) measured *in vitro*, even though it is a potent α_2 receptor inverse agonist that is capable of blocking constitutively active α_2 receptors (Pauwels et al. 2000). In contrast, RX821002 significantly increased the spasms recorded *in vivo*, both with systemic intraperitoneal (IP) or local spinal intrathecal injection of RX821002 (Fig 2-5D, E). This suggests that, although the α_2 adrenergic receptor is not constitutively active in the isolated *in vitro* spinal cord, it is activated by some endogenous ligand (NA) present below the lesion in the awake rat after chronic SCI, similar to the activation of the α_1 receptor.

FIGURE 2-5

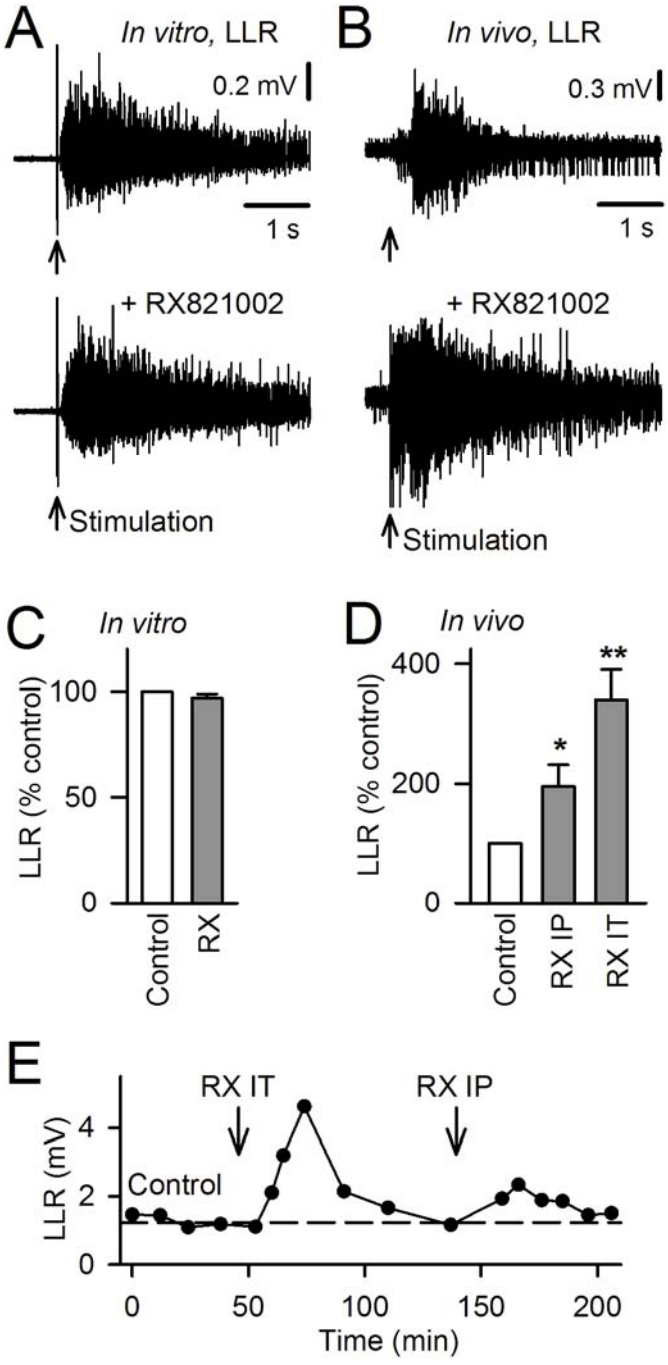
A
In vitro, LLR
0.2 mV
1 s
+ RX821002
Stimulation
B
In vivo, LLR
0.3 mV
1 s
+ RX821002
Stimulation
C
In vitro
LLR (% control)
100
50
0
Control
RX
D
In vivo
LLR (% control)
400
200
0
*
**
Control
RX IP
RX IT
E
RX IT
RX IP
LLR (mV)
4
2
0
Control
0
50
100
150
200
Time (min)

Figure 2-5

The α_2 adrenergic receptor is endogenously active *in vivo*, but not *in vitro.* **(A)** LLR evoked in the isolated *in vitro* spinal cord (as described in Fig. 2-1) of a chronic spinal rat before (top plot) and after blocking possible endogenously activated α_2 adrenergic receptors with the selective α_2 antagonist RX821002 (0.5 μM, bottom plot). **(B)** Long lasting reflex spasm in chronic spinal rat *in vivo* evoked by electrical/cutaneous stimulation of the tail and recorded with tail muscle EMG (LLR computed 0.5 – 4 s post stimulus, as described in Fig 2-1) before (top plot) and after blocking endogenously activated α_2 receptors with a systemic intraperitoneal (IP) injection of RX821002 (1 mg/Kg). **(C)** Normalized group mean for chronic spinal rat LLRs before and after bath application of RX821002 (abbreviated RX) *in vitro* (0.3 - 0.5 μM; $n = 42$) and for (**D**) Systemic IP injection of RX2821001 (abbreviated RX IP, 1 – 3 mg/Kg; $n = 11$) and local intrathecal injection (abbreviated RX IT, 0.3 – 1 mM in 30 μl; $n = 5$) *in vivo*. **(E)** Amplitude of *in vivo* tail spasms of awake chronic spinal rat recorded with EMG (LLR) and measured repeatedly over time under control conditions (left), and after blocking the endogenous activation of the α_2 adrenergic receptor with either local IT or systemic IP injection of RX821002 (abbreviated IT RX, left arrow; 0.3 mM in 30 μl).

2.4 DISCUSSION

The results of our study characterize for the first time the roles of two adrenergic receptor subtypes (α_1 and α_2) in the recovery of motoneuron excitability and spasms after chronic SCI. We find that α_1 receptors increase motoneuron excitability and the α_2 receptors decrease synaptic transmission of sensory inputs to motoneurons, and thus have opposing effects on motor output and spasms after injury, broadly consistent with our understanding of the function of these receptors in normal uninjured animals (Jankowska and Hammar 2002; Jankowska et al. 2000; Jankowska et al. 1993; Lundberg 1982; Millan 2002; Rekling et al. 2000). Notably, we demonstrate a previously undescribed mechanism for compensating for loss of adrenergic innervation with SCI: α_1 receptors become constitutively active (active in absence of NA) and this ultimately contributes to both the recovery of motoneuron excitability (PICs) and emergence of spasms (uncontrolled PICs). Interestingly, we find that a peripheral source of NA, or potentially another ligand, additionally activates the α_1 and α_2 receptors. In contrast, the α_2 receptors do not seem to exhibit constitutive activity, suggesting that these receptors respond differently to injury.

2.4.1 α_{1A} receptor subtype on motoneurons facilitates the Ca PICs and spasms

Our results specifically establish that activation of the α_{1A} subtype of adrenergic receptor facilitates the Ca PIC in motoneurons, thereby increasing excitability, and ultimately increasing the many second long spasms (LLRs), known to be mediated by the Ca PIC. These conclusions are based on α_{1A} receptor agonist-induced increases in the Ca PICs, measured both directly with intracellular recordings and indirectly by assessing the many seconds long ventral root LLRs produced by the Ca PICs, the latter allowing more detailed pharmacological testing not possible during intracellular recordings. We specifically used the highly selective α_{1A} agonist A61603 that has negligible binding affinity for most

other receptors, including other α_1 adrenergic receptor subtypes (α_{1B}, α_{1D}), β adrenergic receptors, dopamine receptors and 5-HT receptors (Craig et al. 1997; Mehrotra et al. 2007). The only non-selective action of A61603 is to bind with high affinity to α_2 receptors (Craig et al. 1997), which initially thwarted our efforts to demonstrate α_{1A} receptor-mediated increases in the LLR, and thus we subsequently applied A61603 in the presence of the α_2 antagonist RX821002 to make it highly selective to α_1 receptors. Under these conditions we found that A61603 consistently increases the LLR, demonstrating the presence of an α_{1A} adrenergic receptor that facilitates the Ca PIC on motoneurons.

Consistent with the involvement of the α_1 receptor in facilitating motoneuron excitability, we found that A61603 increases the LLR at a dose (EC50 of 150 nM) that is remarkably consistent with the binding affinity of A61603 to the α_{1A} receptor measured in isolated cells (K_i = 80 nM), and not other receptors (Craig et al. 1997). We do not know why the EC50 is so close to this K_i value obtained from binding to α_1 receptors in isolated cells, whereas with 5-HT_2 receptor agonists we find that the EC50 for increasing the LLR is consistently about 10 times the agonist binding affinity at the 5-HT_2 receptors (Murray et al. 2010a). One possibility is that the α_{1A} receptors may be located near the surface of the spinal cord, on the distal dendrites of motoneurons where the drug can easily diffuse when applied *in vitro*. In contrast, the 5-HT_2 receptors are located deep in the spinal cord, including on the motoneuron soma (Murray et al. 2010b), where drugs reach less easily, though this needs to be further investigated. We know that the α_{1A} receptors that facilitate Ca PICs and spasms must be located somewhere on motoneurons because the facilitation of the Ca PIC by α_1 agonists occurs in the presence of a sodium channel block with TTX, which renders the motoneurons synaptically silent, essentially isolated from inputs (Li and Bennett 2003). This is consistent with previous reports of widespread expression of the α_1 receptors in the spinal cord, including high levels on motoneurons (Giroux et al. 1999; Rekling et al. 2000; Roudet et al. 1993).

We cannot entirely rule out the possibility that the α_1 adrenergic receptor also increases the LLR and spasms by increasing other motoneuron properties or even the EPSPs that trigger the Ca PICs. The α_1 receptor has been shown to depolarize other motoneurons (Rekling et al. 2000), bringing them closer to threshold and making them more likely to be involved in spastic reflexes (spasms). While we found that the α_1 agonist methoxamine did not depolarize the resting potential of motoneurons, it is still possible that the α_1 receptor depolarizes the sacral motoneurons we studied, but this is masked by the non-selective action of methoxamine on the α_2 receptor, which hyperpolarizes motoneurons (see below). We do not know whether the α_1 receptors facilitate sensory afferent transmission (EPSPs), though if anything they may do the opposite, by facilitation of inhibitory interneurons (Yoshimura and Furue 2006).

2.4.2 α_{1A} receptors act similarly in normal and chronic spinal rats

In spinal cords from normal rats, we also found evidence for the presence of α_{1A} receptor activation on motoneurons that likely act to increase the Ca PICs, because A61603 application (with RX821002) increases sustained motoneuron output in spinal cords of normal rats. Interestingly, when we bring motoneurons of normal and chronic spinal rats to a similar initial level of excitability prior to testing with A61603, by applying a low dose of strychnine in normal rats, the estimated potency of this receptor agonist (EC50) is similar in normal and chronic spinal rats, suggesting that the α_{1A} receptor-mediated responses may not become supersensitive with injury. This is contrary to previous suggestions (Li et al. 2004b) and unlike the supersensitivity of motoneuron PICs to 5-HT receptor activation after chronic injury (Harvey et al. 2006a). However, a lack of supersensitivity in chronic spinal rats (60 - 90 days post injury) is consistent with previous findings that, while the α_1 receptor expression is up regulated transiently after SCI (Giroux et al. 1999; Roudet et al. 1993), it reverts back to normal expression at > 30 days post injury. Caution must be taken in comparing receptor expression to agonist potency in facilitating reflexes though, because increasing

receptor number does not necessarily increase the potency of agonists. Furthermore in normal animals agonists are more likely to be sequestered by the potent NA reuptake transporter (NET) than after SCI, where the NET must be reduced with loss of NA innervation, considering its predominant localization on catecholamine neurons and not glial cells (Blakely et al. 1994). Currently, it is only clear that the α_{1A} receptors appear to act similarly in spinal cords of normal and chronic spinal rats to increase motoneuron excitability.

2.4.3 Constitutive activity in α_{1A} receptors contributes to recovery of motoneuron excitability

Our data demonstrate that when the spinal cord is isolated from peripheral influences (*in vitro*), the Ca PICs are facilitated by endogenous α_{1A} receptor activity that is entirely mediated by constitutive receptor activity. Constitutive activity of wild-type α_{1A} adrenergic receptors has recently been demonstrated in a variety of single cells systems with transfected cloned receptors, and across several species, including rats and humans (Seifert and Wenzel-Seifert 2002). Our findings represent the first time, however, that constitutive activity at the α_{1A} receptor has been shown to play a functional role in the spinal cord. Our conclusions are based on the finding that the LLR (and associated Ca PIC) is reduced by blocking constitutive activity with inverse agonists (WB4101 or prazosin), whereas it is not affected by blocking possible residual NA with the neutral antagonist REC15/2739. In light of these new data (with REC15/2739), we can now re-interpret our previous finding that WB4101 also decreases sodium currents in motoneurons in chronic spinal rats (Harvey et al. 2006b). This now indicates that constitutive α_1 adrenergic receptor activity also facilitates sodium currents, including Na PIC and the fast sodium currents underlying the spike.

Even though the antagonists WB4101, prazosin and REC15/2739 are fairly selective to α_1 receptors compared to other receptors, including α_2 receptors, they are not very selective among the α_1 receptor subtypes and bind potently to α_{1A},

α_{1B}, and α_{1D} receptors (Doxey et al. 1983; Ford et al. 1997; Sanders et al. 2006; Schwinn et al. 1995; Shibata et al. 1995). Therefore, from our WB4101 and prazosin data alone, we only know that one of the α_1 receptor types is constitutively active. However, while REC15/2739 is a neutral antagonist at α_{1A} receptors, it is an inverse agonist at other α_1 receptor subtypes, whereas WB4101 and prazosin are inverse agonists at all α_1 receptor subtypes (Rossier et al. 1999). Thus the inhibition of the LLR by WB4101 and prazosin, and not REC15/2739, indicates that the constitutive activity is mediated by the α_{1A} receptor, further supporting the conclusion that the α_{1A} receptor increases the Ca PIC.

Interestingly, previous reports have shown that the non-selective 5-HT_2 receptor inverse agonists cyproheptadine and ketanserin inhibit the LLR substantially more than can be predicted from blocking constitutively active 5-HT_2 receptors alone (Murray et al. 2010a; Murray et al. 2010b). In light of the present results and considering that these drugs bind to both adrenergic and serotonergic receptors (Yoshio et al. 2001), it now seems likely that these serotonergic drugs also block constitutive activity at α_1 receptors. This helps explain the particular effectiveness of cyproheptadine as an antispastic drug (Barbeau et al. 1982; Murray et al. 2010b; Nance 1994). However, if cyproheptadine also non-selectively blocks the α_2 receptor it may have a paradoxically pro-spastic action, increasing sensory afferent transmission and pain, as we discuss below.

2.4.4 Possible peripheral source of NA after spinal cord injury

The lack of action of REC15/2739 on the LLR in the isolated spinal cord *in vitro*, despite its ability to antagonize exogenously applied α_1 agonists, suggests that in the isolated spinal cord of chronic spinal rats there is no functional source of NA that accounts for activation of the α_1 adrenergic receptors. Interestingly, a forced release of NA with application of amphetamine increases reflexes and motoneuron PICs after chronic SCI (Nozaki et al. 1980; Rank et al. 2007), even in the isolated spinal cord, and so there is a central store of NA, but this store does

not appear to be actively released, at least under our experimental conditions. In contrast, the endogenous α_1 receptor activity seen in the awake rats (*in vivo*) appears to additionally involve α_1 receptors activated by an endogenous ligand (presumably NA), because both the inverse agonists and the neutral antagonist reduce spasms in this case. We do not known where this source of NA arises, but do know that it acts at the spinal level because we applied our antagonists locally to the spinal cord (IT injection).

There are consistent reports of small amounts of residual NA that persists in the spinal cord after SCI (Magnusson 1973; Roudet et al. 1994; Roudet et al. 1993), though the origin of this NA remains a matter of dispute. Based on biochemical methods to visualize catecholamines in the spinal cord, McNicholas (1980) suggested that this residual NA after SCI arises from small sympathetic efferents branching off of blood vessels in the spinal cord. This has more recently been given further support by reports of some residual dopamine β-hydroxylase, the enzyme essential for NA production, after chronic transection (McNicholas et al. 1980; Takeoka et al. 2010). This NA may account for the amphetamine-induced increases in the PICs and spasms that we observe *in vitro* (Rank et al. 2007), but we reiterate that this intrinsic source appears to be functionally inactive in the isolated spinal cord (*in vitro*; lack of effect of REC15/2739). Considering the very sparse distribution of these few residual NA fibres after SCI, and the lack of supersensitivity to α_1 receptors to NA agonists, this sympathetic source of NA seems unlikely to account for the large PICs and spasms we see. Alternatively, since the blood brain barrier (BBB) is chronically compromised after SCI (Popovich et al. 1996), peripheral circulating NA originating in the autonomic system may cross into the spinal cord and activate the α_1 receptors. While unconventional, this peripherally-derived NA seems like a much larger source of NA, and we are currently investigating this possibility.

2.4.5 α_2 receptors inhibit EPSPs, but not PICs

Contrary to the α_1 receptor function, our data demonstrate that the activation of the α_2 adrenergic receptor has no direct effect on the Ca PIC in motoneurons (clonidine-resistant). Rather, activation of the α_2 receptor with clonidine inhibits sensory synaptic transmission to the motoneuron, decreasing the polysynaptic EPSP and thereby preventing activation of the Ca PIC and ultimately reducing the activation of LLRs. We do not know where these α_2 receptors are located, though they are likely on the terminals of the low threshold group I and II afferents that we used to evoke LLRs and EPSPs, or on the interneurons involved in the polysynaptic pathway that produces the EPSPs, consistent with previously reported locations of α_2 receptors (Jankowska and Hammar 2002; Jankowska et al. 2000; Millan 2002; Rekling et al. 2000). Additionally, α_2 receptors may be on motoneurons themselves, because we found that their direct activation with clonidine hyperpolarizes the motoneurons. Indeed, previous studies have shown that α_2 receptors on motoneurons induce a hyperpolarization, by blocking I_h currents (Adachi et al. 2005; Parkis and Berger 1997; Rekling et al. 2000), and such I_h currents contribute +10 mV to the resting potential in our chronic spinal rats (Li et al. 2007). Furthermore, α_2 receptor expression can be detected throughout the the spinal cord, with the highest densities in the superficial dorsal horn, and moderate densities in the portion of the ventral horn containing motoneurons (Giroux et al. 1999; Roudet et al. 1994). All together, α_2 receptor agonists like clonidine or tizanidine are likely to produce their antispastic action primarily by inhibiting afferent transmission to motoneurons, and secondarily by hyperpolarizing motoneurons, making them less likely to be activated during a muscle spasm.

2.4.6 Residual NA after spinal cord injury activates α_2 receptors

Unlike the α_1 receptor, the α_2 receptor does not appear to be constitutively active after SCI, because blocking possible constitutive activity with the α_2 antagonist

RX821002, an inverse agonist, does not affect the reflexes and associated EPSP recorded *in vitro*, even though this same drug readily antagonises the action of exogenously applied α_2 receptor agonists on the reflexes. However, the α_2 receptor does appear to be spontaneously active *in vivo*, providing a tonic inhibition of reflex transmission, because the reflexes and spasms are facilitated by the α_2 antagonists RX821002. We suggest that this spontaneous α_2 receptor activity is due to a peripheral source of NA, which would also activate α_1 receptors as discussed above.

Interestingly, our conclusions might also explain the recent surprising finding that the α_2 receptor antagonist yohimbine markedly facilitates locomotion in transected mice (Lapointe et al. 2008). That is, a peripheral source of NA may tonically inhibit locomotor activity, perhaps by inhibiting reflex transmission, as we have seen, and antagonists may remove this inhibition. In contrast, α_1 receptor activation facilitates rhythmic locomotor activity (Gabbay and Lev-Tov 2004), in addition to its facilitation of motoneuron excitability.

2.4.7 Implications for recovery of motor function

Considering the pronounced opposing effects of α_1 and α_2 receptors that we have uncovered after chronic SCI, the combined functional outcomes of activity in these two receptors remain to be considered. Since the α_1 receptor is both constitutively active and activated by an endogenous source of NA (or other ligand; peripherally-derived), whereas α_2 receptors are only activated by endogenous NA, the former α_1 receptor activity is likely to dominate, at least when there is not much peripheral NA, and thus the net effect of both these receptors is likely increased spasms. However, the levels of endogenous NA are likely to vary, especially as it appears to be peripherally derived, perhaps of autonomic origin, and thus it is interesting to consider what the net effect of this variable peripheral NA should be. Previously, we have shown that very low concentrations of exogenously applied NA can facilitate spasms (LLRs) as well as

increase motoneuron firing, whereas higher concentrations tend to decrease spasms (Li et al. 2004b), suggesting that there should be a similar biphasic action of endogenous NA. The net effect at high levels may therefore be inhibitory, or antispastic in action. This fits with our understanding of the dual action of NA receptors, because regardless of how large the α_1 receptor-mediated PICs, if there are no EPSPs to trigger them, because of α_2 receptor activity, there will not be spasms. Thus, interventions that increase endogenous NA after SCI may well have antispastic benefits, while also increasing overall motor output (PICs) and motor functions (locomotion; via α_1) (Gabbay and Lev-Tov 2004). This is consistent with the positive effects of transplanting brainstem-derived cells that produce NA and 5-HT at a SCI site (Gimenez y Ribotta and Privat 1998).

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Chapter 3:

Role of endogenous release of noradrenaline in muscle spasms after chronic spinal cord injury

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3.0 AUTHOR CONTRIBUTIONS

Rank MM

- Completion and analysis of all *in vivo* experiments, preparation of figures and manuscript.

Li X

- Completion and analysis of all *in vitro* experiments, preparation of some figures.

Bennett DJ

- Running and analysis of all *in vitro* intracellular recording experiments and some ventral root reflex experiments, supervision of students and projects, preparation and editing of manuscript.

Gorassini MA

- Supervision of students and projects, preparation, editing and submission of manuscript.

3.1 INTRODUCTION

Spinal motoneurons contain low-voltage activated persistent inward currents (PICs) that are comprised of both sodium (Na PIC) and calcium (Ca PIC) components (reviewed in Heckman et al. 2005). When activated, PICs can amplify synaptic inputs and importantly, they are essential for normal repetitive firing (Heckman et al. 2003; Lee and Heckman 1999; Li and Bennett 2003; Li et al. 2004a), making motoneuron PICs fundamental in the production and facilitation of movement. Both Na and Ca PICs are enhanced by the presence of serotonin (5HT) (Hounsgaard and Kiehn 1989; Perrier and Hounsgaard 2003) and noradrenaline (NA) (Conway et al. 1988; Hounsgaard et al. 1988; Lee and Heckman 1999) and the presence of PICs is in fact contingent upon these neuromodulators (Harvey et al. 2006b; Perrier and Delgado-Lezama 2005). Because the major source of monoaminergic input to spinal motoneurons comes from descending fibres originating from brainstem neurons (Carlsson et al. 1963; Dahlstrom and Fuxe 1964), and minimal amounts come from terminals of intraspinal neurons and peripheral sympathetic fibres (McNicholas et al. 1980; Newton & Hamill, 1988; Roudet et al., 1994), PICs are greatly reduced immediately after a spinal transection (Bennett et al. 2001a; Bennett et al. 2004; Harvey et al. 2006a; Hounsgaard et al. 1988). However, large motoneuron PICs and the self-sustained depolarizations they produce (plateau potentials) spontaneously recover in the weeks following an injury and their gradual restoration closely parallels the development of debilitating muscle spasms (Harvey et al. 2006c; Li and Bennett 2003). The redevelopment of motoneuron PICs occurs despite the absence of descending monoaminergic drive, possibly because motoneuron PICs develop supersensitivity to the 2-12% of 5HT (Newton and Hamill 1988) and/or 1-5% of NA (Magnusson 1973; Roudet et al. 1994; Roudet et al. 1993) that are supplied by intraspinal and sympathetic neurons described above.

Earlier suggestions that motoneuron PICs develop supersensitivity to residual sources of 5HT come from experiments where very low doses (20-100 mg/Kg) of

the agonist 5-hydroxytryptophan (5-HTP) increased spontaneous and reflex evoked muscle activity in chronically spinalized rats, in contrast to uninjured control animals (Barbeau and Bedard 1981; Tremblay and Bedard 1995). Similar facilitation also occurred in acutely transected animals that had chronic denervation of 5HT fibres, suggesting that enhanced responses to low dose 5HTP in chronic spinal animals were not simply due to general increases in neuronal excitability, but rather, to specific denervation supersensitivity to 5HT (Barbeau and Bedard 1981). Recently, direct evidence of motoneuron PIC supersensitivity to 5HT following chronic spinal injury has been obtained in the rat (Harvey et al. 2006a, b). Facilitation of Na PICs and Ca PICs by 5HT, or from the $5HT_2$ receptor agonist DOI, at doses of 1μM or less was 30 times greater in animals spinalized two to four months previously compared to acutely lesioned animals (Harvey et al. 2006a; Li et al. 2006). Importantly, a combined blockade of $5HT2_a$, $5HT2_c$ and $NA_{\alpha 1}$ receptors completely eliminated the NaPIC and provides evidence that endogenous sources of 5HT and NA below the level of the lesion are necessary for the activation of Na PICs (Harvey et al. 2006b).

The main goal of the present study was to further investigate if endogenous sources of NA, like 5HT, facilitate motoneuron PICs after chronic spinal cord injury. Specifically, we were interested in the CaPIC, which is more involved than the NaPIC in generating long-lasting reflexes, or spasms, characteristic of the spastic syndrome (Bennett et al. 2001b; Li and Bennett 2003). Amphetamine, at low to moderate doses (< 5 mg/Kg), is known to enhance the release of NA from presynaptic nerve terminals and inhibit its reuptake (de la Torre et al. 2004; Munkvad and Randrup 1966). Early evidence using amphetamines has already suggested a role for NA released from intraspinal sources in enhancing flexor reflex activity in chronically lesioned animals (Nozaki et al. 1980). In agreement with this, preserved NA intraspinal neurons can be found caudal to a lesion, albeit in reduced quantities, compared to pre lesion levels in the rat (Cassam et al. 1997).

In the present study, we examined if increasing endogenous release of NA from spinal sources below a lesion increases spinal excitability (spasms) after chronic injury to the sacral spinal cord of adult rats. To do this, we recorded both spasm behaviour and cutaneous-evoked reflex responses in tail muscles of rats receiving a complete injury to the second sacral (S2) level of the spinal cord both before and after amphetamine administration. We then directly measured if amphetamine increases spinal excitability via direct actions on the motoneuron from intracellular recordings in the *in vitro* sacral spinal cord preparation. Reflex responses to dorsal root stimulation in this S2 *in vitro* preparation were also compared to dissociate neural versus potential vascular affects of amphetamine on reflex responses in the awake animal. Lastly, we determined if long-lasting reflexes mediated by motoneuron PICs exhibit supersensitivity to NA by examining if facilitation of reflex activity in chronically injured animals occurs at appreciably lower doses of amphetamine compared to acutely injured animals.

3.2 METHOD

Motor responses to amphetamine administration in transected (S2) female Sprague-Dawley rats were measured under three experimental conditions; 1) kinematic and surface EMG recording of spastic tail reflexes in the awake rat, 2) ventral root recordings from an isolated (S2) spinal cord *in vitro* and 3) intracellular recordings from sacrocaudal motoneurons *in vitro*. Supersensitivity of spasm behavior and long lasting reflex responses to amphetamine was compared between chronic (1.5 to 4 months) and acutely (2-5 days) injured animals in the awake rat only. All procedures were approved by the University of Alberta animal welfare committee.

3.2.1 Tail Reflexes in the Awake Rat

3.2.1.1 Measurement of Spasticity

Reflexes in segmental tail muscles were recorded in chronic spinal and acute spinal rats, ranging in weight from 306 to 342g (chronic) and 196 to 238g (acute). All chronic rats used in the study exhibited clear spasticity in the tail muscles and had a spasticity score of 3 or greater (Bennett et al. 1999). In preparation for surface EMG recordings, the tails were scrubbed with a soap solution and then wiped with a 70% ethanol solution in order to clear any debris and loose scales. Following this, a 2-hour rest period was given to allow any excitation resulting from the cleaning process to subside. To measure the degree of spasticity in the tail in response to mechanical stimuli, the rats were temporarily housed in a Plexiglas tube with the tail protruding and allowed to hang freely. The tail was then stimulated with a standardized stretch/rub maneuver (see Bennett et al., 2004 for details). Briefly, the base of the tail was held with one hand while the other gripped the tail with a damp piece of gauze held between the thumb and finger. The gauze was then quickly slid down the tail, maintaining a firm and consistent grip throughout. This stretch/rub maneuver was repeated three times in rapid succession after which the tail was allowed to move freely, hanging below the tube. The resultant response was video taped for 5 minutes and the degree of

spasticity was evaluated, offline, through kinematic measurements as described below.

3.2.1.2 Kinematic measurements

To quantify movement and spasms of the tail after a stretch/rub maneuver, flexion and extension angles of the tail were calculated as shown in Figure 3-1. When viewing the animal's left side, clockwise movements of the tail were defined as flexion (+'ve numbers), whereas counter-clockwise movements were defined as extension (or -'ve flexion values). The maximum flexion and extension angles, relative to vertical, were measured at the location on the tail that exhibited the greatest degree of flexion or extension, respectively (for details see Fig 3-1*A* and *C*). Most often the tail flexed into a pure clockwise coil, and in these cases the maximum flexion angle was the angle of the tip of the tail (Fig 3-1A). At times, in very spastic animals, flexion and extension both occurred together, due to a co-contraction of flexor and extensor muscles. This caused the tail to form an inverted s-shape (Fig 3-1*B*), with the proximal part of the tail flexing and the tip of the tail extending relative to this. In this case, the maximum flexion angle occurred near the middle of the tail, and was measured relative to vertical, as in Fig 3-1B. The maximum extension of the tip of the tail was measured relative to this maximum flexion angle (max flexion angle - tip angle; Fig 3-1B), and in this case corresponded to the maximum extension angle. Less frequently, the tail extended into a counterclockwise coil, and accordingly, the maximum extension was measured at the point on the tail where maximum extension occurred, analogously to the above treatment of flexion coils (not shown in Fig 3-1, but see small inset in Fig 3-2A).

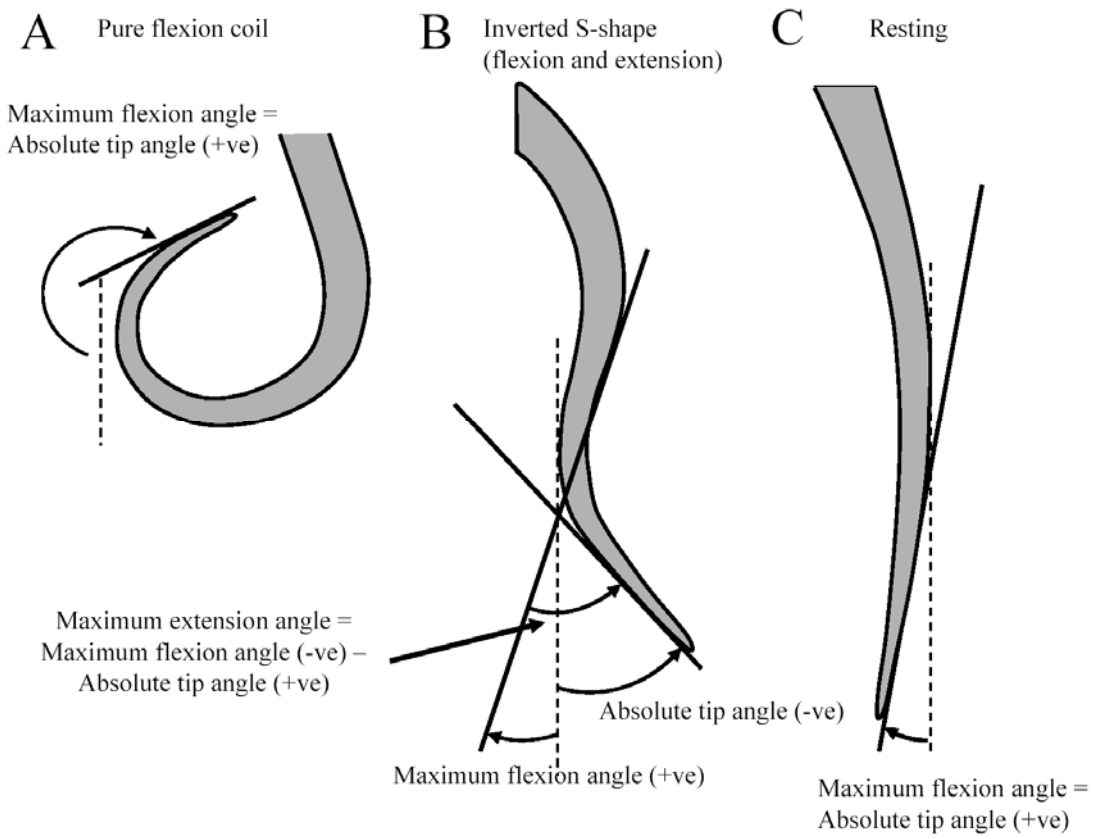

Figure 3-1

Method for measuring maximum flexion and extension angles in spastic rat tail after standardized stretch/rub maneuver is applied. The tail is viewed from the left side of the animal. ***A.*** A pure flexion spasm, where the tail typically forms a coil. The maximum flexion angle is measured relative to vertical from a tangent line at the tip of the tail; the maximum extension angle here is zero as the tail is showing no extension. In a case of pure flexion coiling, the maximum flexion angle occurs at the tip of the tail (absolute tip angle). ***B.*** When flexion and extension both occur, the tail forms an inverted s-shape. To calculate the maximum degree of extension, which occurs at the tip of the tail, the maximum flexion angle is first measured relative to vertical from a tangent drawn from the middle of the tail. The absolute extension angle of the tip of the tail is then measured relative to vertical from a tangent drawn from the tip of the tail. To obtain the maximum extension angle, the maximum flexion angle is then added to the absolute extension tip angle. ***C.*** When the tail is at rest the maximum flexion angle occurs at the tip of the tail and therefore is identical to the absolute tip angle, and the maximum extension value is zero, similar to the full flexion coil in ***A***.

Maximum flexion and extension angles were measured every 5 seconds over the five minutes following the stretch/rub maneuver in chronic and acute spinal rats both before and after amphetamine. In addition to maximum flexion and extension angles, the number of discrete spasms (considered a jump of greater that 50º in flexion or extension angles) was also tallied for the five minutes following the stretch/rub maneuver.

3.2.1.3 EMG - Reflex Testing Protocol

After the stretch/rub maneuver, the rat was moved while still in the tube holder to rest on a temperature controlled water pad that kept the tail at 37ºC for EMG recordings. To record surface EMG of segmental tail muscles, custom built cuff electrodes consisting of Tygon tubing slit open and sewn with silver wire, were filled with conductive electrode gel and placed onto the tail of the rat. Electrode placement on the tail was standardized using the 12^{th} coccygeal vertebra as a reference point. EMG recording electrodes were placed 1 and then 2.5 cm distal to this reference point with the recording ground 3 cm distal to this point. Two small stimulating cuff electrodes were placed on the distal tip of the tail, separated from each other by 1cm. This portion of the tail is abundantly sensitive to touch and contains very little muscle, and thus this stimulation of the tip was used to provide a relatively pure cutaneous activation.

In each animal the reflex threshold (RT) was determined, which was the minimum stimulation intensity required to evoke a small (usually 1.5-2x's larger than noise in signal) short-latency polysynaptic reflex, and varied from 0.05-0.30mA in chronic animals (Isoflex stimulator, AMPI). In many cases, the RT could not be determined in acute animals and thus the average chronic RT (0.07mA) was used, in addition to a maximum 10mA stimulation intensity. To evoke cutaneous reflexes, a single pulse (width 2ms) was delivered every 2 or 10 seconds at 10x's RT. Following this, a pulse train at 1.5 x's RT (width 0.2ms, 100Hz for 500ms) was applied every 30 seconds to more readily evoke long-

lasting reflexes. These recordings and stimuli parameters were repeated while manually eliciting differing background levels of EMG, by touching the tail with a cotton swab, for the purpose of matching background contractions before and after amphetamine administration. EMG activity was recorded using Axoscope hardware and software (Digidata 1322A, Axoscope, Axon Instruments, Burlingame, CA) at a sampling rate of 5 kHz, gain of 2000 and filtering between 100 and 3000 Hz (custom built 4 channel differential amplifier, R&R Designs, Winnipeg, MB).

3.2.1.4 Drug Protocol

Rats were administered 1, 2 or 4 separate doses of D-amphetamine (Sigma-Aldrich, Oakville, ON), with a minimum of 48 hours between doses in chronic animals. Doses in acute animals were administered every 24 hours in order to avoid interference from any early development of spasticity. Following an intra-peritoneal (IP) injection of amphetamine, the rat was allowed to rest for 20 minutes while the drug took effect (Randrup and Munkvad 1966). For each animal, a single pre drug recording took place either 3 hours or the day before a drug injection. Chronic animals received a low dose (0.1-0.2mg/Kg) or a high dose (0.6-1.0mg/Kg) of amphetamine and similarly, acute animals received a low dose (0.2 mg/Kg) or a high dose (0.6-2.0mg/Kg) of amphetamine.

3.2.1.5 Data Analysis: EMG responses

To measure reflex EMG activity in response to single or train stimulation to the tip of the tail, custom written software (The MathWorks, Release 14: Natick, MA) was used for the analysis. After importing data, the program applied an additional high pass filter at 800Hz (1st order Butterworth), and a low pass filter at 1750Hz (1^{st} order Butterworth), followed by rectification. Four specific reflex responses were measured. For single shock trials, the early latency, short-duration reflex

(termed *polysynaptic*) was averaged 15-40 ms after the stimulus; the short latency, longer-lasting reflex (termed *tonic short*) was measured 15-500 ms after the stimulus and the long latency, long-lasting tonic response (termed *tonic long*) was measured 500-5500 ms after the stimulus. For stimulation trains, a 500-5500 ms window was averaged (termed *train*) after the 500 ms stimulus train was applied. An interstimulus interval of 2 s was used for the polysynaptic and tonic short reflexes, 20 s for the tonic long reflexes and 30 s for the train reflexes. Average EMG values were obtained for each reflex response by dividing the rectified EMG activity by the number of samples within the reflex interval for each trial (typically 5 trials were recorded) and then averaging the individual trial values together. Average background levels for each stimulus (single pulse or train) were measured over the 200 ms before the stimulation and were matched across amphetamine doses to maintain comparable background levels of motoneuron excitability. In other measurements, background was not matched to examine effects of amphetamine on general motoneuron excitability. The average noise (i.e., average voltage with no EMG activity) was subtracted from all reflex values. In chronic animals, all post-drug reflex averages were normalized to the pre drug condition (post-drug/pre-drug). In acute animals the size of the reflex responses, especially the long-lasting reflexes, were an order of magnitude smaller than the reflexes recorded in chronic animals and at times, produced large normalization errors. Therefore, the size of acute reflexes was thresholded to a minimum level (0.3 μV; ~10% of average noise) when required. As for chronic animals, post drug reflexes in acute animals were normalized to the pre drug condition (post drug/pre drug).

3.2.2 *In vitro* Ventral Root Reflexes

A detailed description of these procedures can be found in Li et al. (2004). Rats between 1.5 and 4 months post-injury and exhibiting clear spasticity were used. Briefly, ventral and dorsal roots were mounted on chlorided silver wires and were suspended above the ACSF at root entry points for monopolar recording and

stimulation, respectively. The root was wrapped around the wire in the air and then covered with a 1:1 mixture by weight of petroleum jelly/mineral oil to prevent the roots from drying out. Three additional wires were submersed in the ACSF for the stimulation-return, monopolar recording reference and instrumentation ground.

Dorsal roots were stimulated with 0.1ms current pulses for 0.5 s, 100Hz trains usually at 0.05mA which was around 5x's sensory threshold [(5T); Isoflex stimulator, AMPI]. Sensory threshold was determined by transferring an unused dorsal root to the recording chamber and recording at one end while stimulating the other prior to each experiment (T = 0.007-0.01mA; conduction velocity ranged from 16 to 24 m/s at 25ºC). The anode was connected to the dorsal root, and the cathode to the stimulation-return wire in the ACSF. Ventral roots were recorded via a custom built differential preamplifier (QT5, Dean Charles, University of Alberta), with one lead connected to the root and the second to the reference wire in the ACSF [high pass 300 Hz; low pass 3 kHz; amplified by 2000 times; sampling rate 6.7 kHz (Axoscope, Axon Instruments)]. Dorsal root stimulation was repeated more than five times with an inter-stimulation interval set at 20 s to provide multiple ventral root reflexes for averaging.

3.2.2.1 ACSF and Drug Applications

Two kinds of artificial cerebrospinal fluid were used in these experiments; a modified ACSF (mACSF) used during dissection and recovery to minimize neural and metabolic activity and a normal ACSF (nACSF) in the recording chamber. The composition of the mACSF was (in mM) 118 NaCl, 24 $NaHCO_3$, 1.5 $CaCl_2$, 3 KCl, 5 $MgCl_2$, 1.4 NaH_2PO_4, 1.3 $MgSO_4$, 25 D-glucose and 1 kynurenic acid. Ventral root reflex responses were tested in nACSF composed of (in mM) 122 NaCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 3 KCl, 1 $MgSO_4$, and 12 D-glucose mixed in distilled water (osmolarity of 298 mOsm). D-amphetamine (Sigma-Aldrich, Oakville, ON) (low dose 0.1μM or high dose 1-10 μM) was applied while in

nACSF. Both types of ACSF were bubbled with 95% O_2-5% CO_2 to maintain a pH of 7.4.

3.2.2.2 Data Analysis: ventral root responses

Ventral root reflexes (polysynaptic, tonic short, tonic long and train) were quantified similar to that used for surface EMG recordings described above, with minor differences in the times of the reflex window due to the different recording durations used in the root reflex experiments. The polysynaptic reflex was averaged over 10-40 ms after the stimulus; the tonic short reflex was measured 30-600 ms after stimulus; the tonic long reflex was measured 600-3600 ms after stimulation and the train reflex was measured for 1100-4100 ms after an applied train of stimuli. The same custom MatLab software was used to analyze these data, and therefore the same additional filtering procedures were used as in the awake rat data. The average noise signal was also subtracted from all measurements.

3.2.3 *In vitro* Intracellular Recordings

Intracellular recordings were made from motoneurons in the *in vitro* sacrocaudal spinal cord of chronically injured, spastic rats. Details of intracellular recordings can be found elsewhere (Bennett et al. 2001b; Li and Bennett 2003; Li et al. 2004a), so we will only describe details specific to these experiments. Antidromic stimulation of the S_4 and Ca_1 ventral roots, which were mounted on silver chloride wires supported above the recording chamber fluid and sealed with high-vacuum grease, was used to identify motoneurons. Only motoneurons with a stable penetration, resting potential below -60mV, antidromic spike overshoot over 0mV, and reliable repetitive firing were included in the study. Data were collected using an Axoclamp2b intracellular amplifier (Axon Instruments) running in either discontinuous current-clamp modes (DCC, switching rate 7-10

kHz, output bandwidth 3.0 kHz) or discontinuous voltage-clamp modes (gain 1-2.5 nA/mV).

3.2.3.1 Drugs and Solutions

Similar procedures were used to maintain the spinal cord in vitro as were used in the root reflex experiments. Prior to recording, the spinal cords were briefly exposed to nACSF solution containing 0.04% pronase E (Helixx Technologies) for 10 seconds to weaken the pia of the spinal cord and allow for easier penetration. To study the CaPIC in isolation, 2μM tetrodotoxin (TTX; Alamone Labs, Israel) was added to block NaPIC. In some animals (n = 6), 0.15 μM apamin (Alamone Labs, Israel) was added; apamin application was found to have no effect on the amphetamine induced CaPIC, and so data from these animals are included in the overall averages. Only moderately high doses of D-amphetamine were tested (1-10 μM) and responses were similar at all doses and pooled together.

3.2.3.2 Persistent Inward Current in Voltage Clamp Recording

Slow triangular current ramps (0.4 nA/s) and voltage ramps (ramp speed 3.5 mV/s) were applied to the motoneurons in order to measure firing and basic cell properties. In addition to the resting membrane potential (V_m), the resting membrane conductance (G_m) was measured as the slope of the current response during a voltage ramp over a 5mV range near rest and subthreshold to PIC onset. The passive leak current that summed with the PICs to give the total recorded current was estimated using a linear relation fitted to the subthreshold current response in the linear region 10mV below the negative-slope region onset, and then extrapolated to more positive voltages (see Fig. 3-7A). The PIC amplitude was then estimated by subtracting this leak current from the recorded current (leak-subtracted current). The onset voltage for the PIC (V_{on}) was defined as the voltage measured at the beginning of the first negative slope region in the current (where first zero slope in current response occurred). The current value

corresponding to V_{on} was defined by I_{on},, or the onset current. The initial peak current of the PIC was measured from the leak subtracted current, where this current reached its first maximum (at second zero slope in current response; initial PIC, see solid arrow in Fig. 3-7A). The sustained current of the PIC was likewise defined as the leak-subtracted current at the first zero slope point in the current response on the downward current ramp (sustained PIC, see dashed arrow in Fig. 3-7A). Data were analysed in Clampfit 8.0 (Axon Instruments).

3.2.4 Statistics

Data are shown as the mean ± standard error (SE) throughout the text and figures. Where the data were normally distributed, as indicated by the Kolmogorov-Smirnov test for normality, statistical differences were computed with a Student *t*-test at the 95% confidence level. Where the data were not normally distributed according to the Kolmogorov-Smirnov test for normality, statistical differences were computed using a Wilcoxon signed-ranks test at the 95% confidence level.

3.3 RESULTS

3.3.1 Amphetamine induced changes in tail spasm behaviour in the awake chronic spinal rat

Kinematic measurements in response to a rapid stretch/rub of the tail were compared before and after low (0.1 - 0.2 mg/Kg; n = 11) and high (0.6 - 1.0 mg/Kg; n = 11) doses of amphetamine. Prior to administration of amphetamine, the tail of a chronic spinal rat typically showed both flexion (Fig. 3-2*A*) and extension (Fig. 3-2*B*) movements in response to a manual stimulation; sometimes in isolation (e.g., pure flexion coil), and at other times simultaneously (e.g., inverted S-shape, see insets in Fig. 3-2A). The maximum pre drug flexion and extension angles of the tail (see details in Methods and Fig. 3-1) averaged over a 5 min period following stimulation are shown in Fig. 3-2*E* and *F* (open bars; all animals averaged together). Following a low dose of amphetamine, the maximum flexion of the tail increased dramatically (Fig. 3-2*C*), with an average increase of 41.7 ± 12.2º in flexion (n = 11; significant increase), nearly doubling the average flexion prior to amphetamine (Fig. 3-2*E*, grey bar). This increased flexion was necessarily associated with a decrease in extension, with extension eliminated in some animals (Fig. 3-2*D*). On average the maximum extension angle significantly decreased by 17.0 ± 6.9º (n = 11; grey bar Fig. 3-2*F*). High doses of amphetamine produced similar significant increases in flexion and decreases in extension, though these effects were slightly smaller (n = 11; solid bars in Figs. 3-2*E, F*). Overall, with amphetamine application, there was a bias toward more tail flexion, with more forceful flexion contractions overpowering the extensor muscles of the tail.

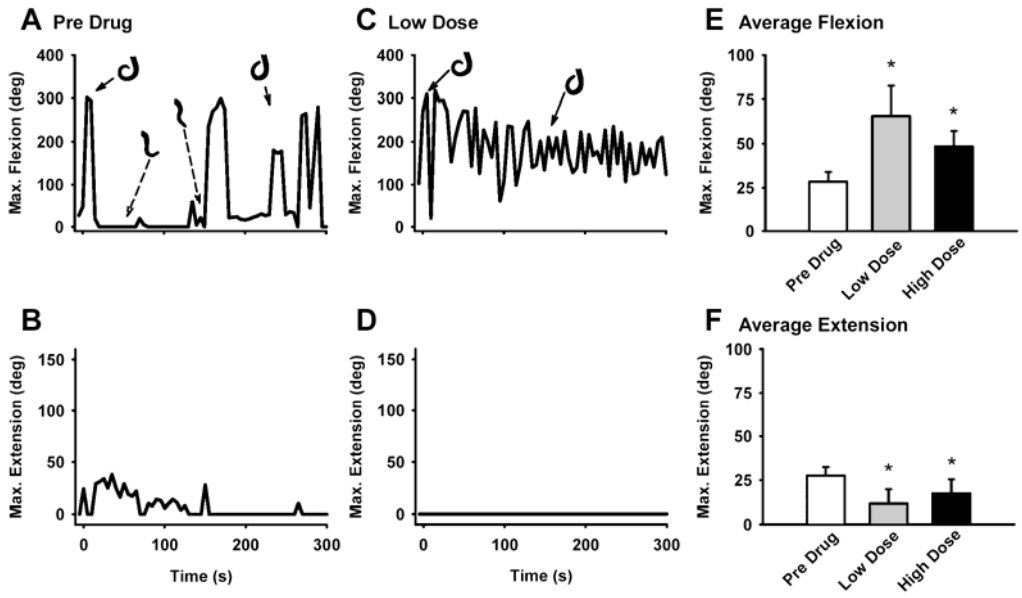

Figure 3-2

Maximum flexion (***A***) and extension angles (***B***) measured in a representative chronic spinal rat following the standardized stretch/rub maneuver applied to the tail before and after amphetamine. Small diagrams in *A* show the shape of the tail at the time indicated by the arrow. Solid arrows define when a spasm (> 50º increase in flexion or extension angle in 5s) was counted. Maximum flexion ***(C)*** and extension ***(D)*** angles measured in the same animal after a low dose (0.1mg/Kg) of amphetamine. Note that after a low dose of amphetamine the animal showed greater maximum flexion and an increase in the number of spasms (from 5 to 20), while the maximum extension values decreased to zero. ***E.*** Group average maximum flexion angles pre drug (open bars), at low (grey bars) and high (solid bars) doses of amphetamine. ***F.*** Same format as *E*, but maximum extension. * = $p<0.05$.

In addition to measuring maximum flexion and extension angles, the number of spasms, defined as sharp increases in flexion and extension angles (greater than 50º increase in 5 seconds as indicated by solid arrows in Fig. 3-2) were counted at low and high doses of amphetamine. Before drug administration the average number of flexion spasms in the 5mins after a stretch/rub was 3.2 ± 0.8 (n = 11). The average number of spasms increased to 5.9 ± 2.6 at low doses of amphetamine (n = 11; not significant) and, significantly, to 5.6 ± 1.2 at high dose of amphetamine (n = 11). Prior to amphetamine the average number of extension spasms was 2.4 ± 0.6, which decreased significantly to 0.8 ± 0.5 at low doses of amphetamine and to 1.6 ± 1.0 at high doses of amphetamine. These data demonstrate that both low and high dose amphetamine administration in chronic spinalized rats leads to an overall increase in number of flexion spasms, and a decrease in the number of extension spasms. This concurs with the kinematic data, which showed an increase in flexor, and as a consequence, a reduction in extensor angles following amphetamine in the chronic spinal rat.

3.3.2 Amphetamine induced changes in tail reflexes in the awake chronic spinal rat

Single shock electrical stimulation to the tip of the tail produced substantial polysynaptic (Fig. 3-3*B*) and long lasting (Fig. 3-3*A*) reflexes in chronic spinal rats as measured by surface EMG over the segmental tail muscles. These reflexes, evoked by the stimulation of cutaneous afferents from the tip of the tail, increased in amplitude following administration of amphetamine. As shown for a representative animal, both low (Fig. 3-3 *C,D*) and high (Fig. 3-3 *E,F*) doses of amphetamine increased long lasting and polysynaptic reflexes. The effects of amphetamine were not limited to stimulus-locked reflex responses, as the amount of tonic background activity occurring prior to the stimulation also increased (compare background EMG before stimulation artifact in Figs. 3-3 *B,D,&F*).

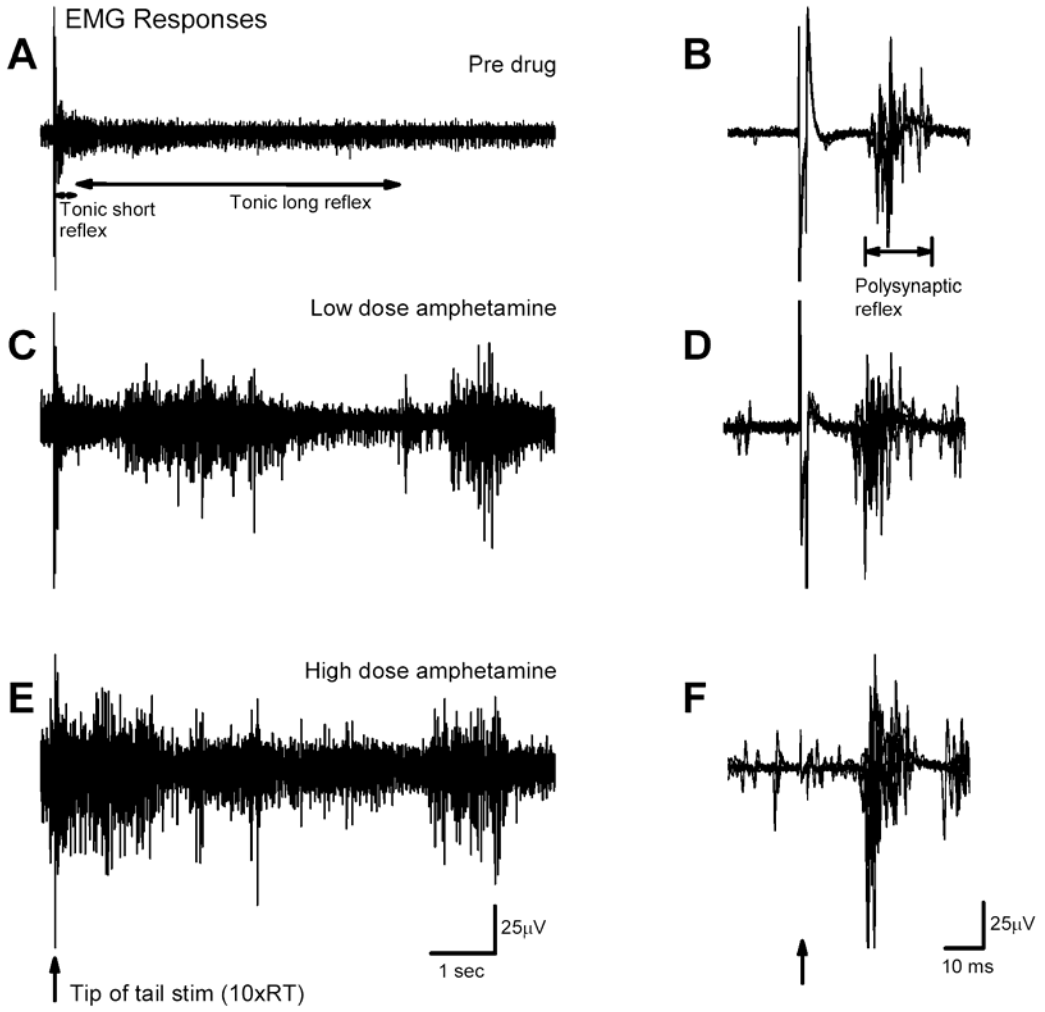

Figure 3-3

Low and high doses of amphetamine increased cutaneous reflexes in representative chronic spinal rat. ***A.*** Segmental tail muscle EMG response to tip of the tail stimulation, single pulse at 10x reflex threshold (RT), before amphetamine. Tonic short reflex measured 15-500ms after stim; tonic long reflex measured 500-5500ms after stim. ***B.*** Magnification of *A* showing greater detail of the polysynaptic reflex (measured 15-40ms after stim). ***C and D.*** Same rat as in as *A* and *B*, but recorded after administration of a low dose (0.2 mg/Kg) of amphetamine. ***E and F.*** Same rat as in *A* and *B,* but recorded after administration of a high (1.0 mg/Kg) dose of amphetamine. Time scales are the same for *A,C,E* and for *B,D,F*. Note background EMG was not controlled for in this example to demonstrate increase in pre-stim EMG after amphetamine.

Averaged group data (Fig. 3-4) demonstrate that amphetamine administration consistently increased reflex responses. The pre drug polysynaptic reflex (Fig. 3-4*A, left*), was relatively large, on average, compared to longer duration reflexes (compare absolute reflex responses, open bars on left of graphs in Fig. 3-4; filled bars on right of graphs show reflex increases after amphetamine) and showed an increase in amplitude with both low (n=11) and high (n=11) doses of amphetamine when normalized to pre-drug values (Fig. 3-4*A, right,* filled bars*;* significant in low dose only). The tonic-short reflex began at a lower average amplitude than the polysynaptic reflex (Fig. 3-4*B*; *left*) but still showed significant increases (≈1.5 times larger) at both low and high doses of amphetamine (Fig. 3-4*B*; right). The tonic long reflex (Fig. 3-4*C*) and the train reflex (Fig. 3-4*D*) increased by three to four fold at both low and high doses of amphetamine. Although the average amplitude of the tonic long and train reflexes was similar compared to the tonic short reflex before amphetamine administration (10 to 13μV), these long-duration reflexes were substantially more sensitive to the drug as they demonstrated larger increases in average reflex amplitudes. The average level of accumulated background EMG activity recorded before single shock stimulation delivered in quick succession (10xRT every 2s) also increased three fold after low and high doses of amphetamine (Fig. 3-4*E*).

3.3.3 Facilitation of ventral root reflexes in the chronic spinal rat

Increases in EMG responses by amphetamine in the awake chronic spinal rat may have been produced, in part, from changes in blood flow to the muscle in response to elevated blood pressure, tachycardia and/or selective vasodilation, known peripheral effects of amphetamine (de la Torre et al. 2004). In order to eliminate the contribution of these peripheral factors and dissociate neural from vascular effects, ventral root reflex responses to dorsal root stimulation in the sacral spinal *in vitro* preparation were also compared. Under *in vitro* recording conditions

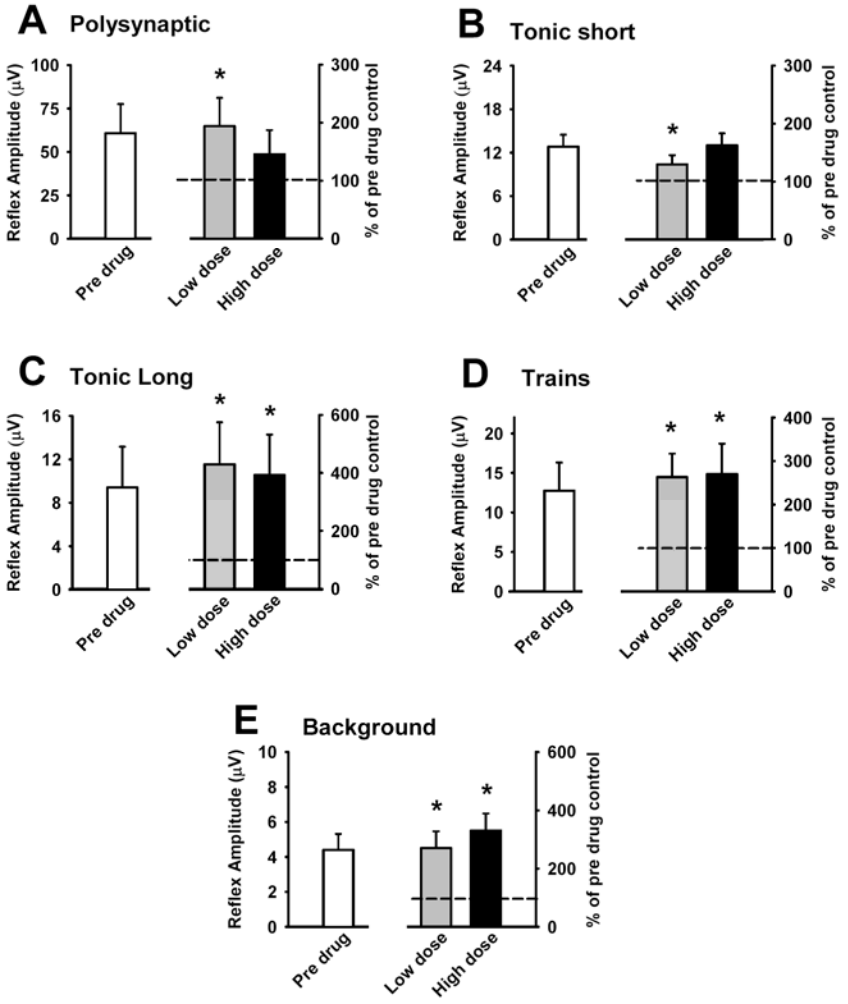

Figure 3-4

Absolute reflex amplitudes (open bars on left of graphs) and respective changes (normalized to pre-drug values) in response to low (grey bars) and high (solid bars, right of graphs) doses of amphetamine for the various reflex responses. Dashed line indicates 100% of pre drug values. ***A.*** Polysynaptic reflex (10-40ms after stim): pre drug reflex is 60.9 ± 16.7 μV and shows a significant increase to 194.1 ± 48.6% at low doses of amphetamine and an increase to 145.7 ± 41.5% at high doses. ***B.*** Tonic short reflex (30-600ms after stim): pre drug reflex is 12.8 ± 1.7μV and shows significant increase at low (129.7 ± 15.9%) but not high (162.5 ± 20.8%) doses of amphetamine. ***C.*** Tonic long reflex (600-3600ms after stim): pre drug reflex is 9.4 ± 3.8μV, and increases to 429.2 ± 145.6% at low and to 392.3 ± 140.2% at high doses of amphetamine. ***D.*** Trains reflex (1100-4100s after 500ms, 100Hz train stim): pre drug reflex is 12.8 ± 3.6μV and increases significantly to 262.8 ± 54.2% at low dose and to 270.0 ± 70.1% at high doses of amphetamine. ***E.*** Background activity: Pre drug levels of activity measure 4.4 ± 1.0μV and increase to 270.4 ± 57.1% at low dose and to 330.2 ± 58.6% at high dose amphetamine. * = $p<0.05$.

large polysynaptic and short and long duration reflexes were evoked from dorsal root stimulation and showed dramatic increases with successively larger doses of amphetamine (Fig. 3-5; n = 12 root pairs tested). These ventral root reflexes were very similar to reflexes evoked in the awake animal (compare Fig. 3-5 with Fig. 3-3). In both cases, amphetamine induced particularly large increases in long duration reflexes in low (Fig. 3-5*C*) and high (Fig. 3-5*E*) doses of amphetamine.

Averaged grouped data quantify these reflex changes and demonstrate significant increases in tonic short (Fig. 3-6*B*), tonic long (Fig. 3-6*C*) and train reflexes (Fig. 3-6*D*) with both low (0.1μM; n = 12) and high (1-10μM; n = 12) doses of amphetamine. High doses were much more effective in increasing reflex responses compared to the low dose, especially in tonic short and long reflexes. The only exception to these substantial reflex amplitude increases is the polysynaptic reflex (Fig. 3-6*A*), which showed a small but significant decrease (24.3%) in amplitude at a low dose of amphetamine, and a small but significant increase (29.1%) in amplitude at a high dose of amphetamine.

3.3.4 Effects of amphetamine on the motoneuron calcium PIC in chronic spinal rat

To examine if increases in reflex responses recorded in the awake animal and *in vitro* preparations were mediated, in part, by amphetamine's action on motoneuron PICs, intracellular recordings were performed in the sacral spinal cord of chronic spinal rats (n = 9 cells from 9 rats). Voltage-clamp recordings of the motoneuron, made after the application of TTX, allow measurement of the CaPIC in isolation, which has been shown to mediate the long lasting reflexes (spasms) recorded in chronic spinal rats (Li and Bennett 2003). The application of a slow depolarizing voltage ramp in TTX (top trace in Fig. 3-7*A*) initially produced a measured current (bottom trace in Fig. 3-7*A*) that increased linearly

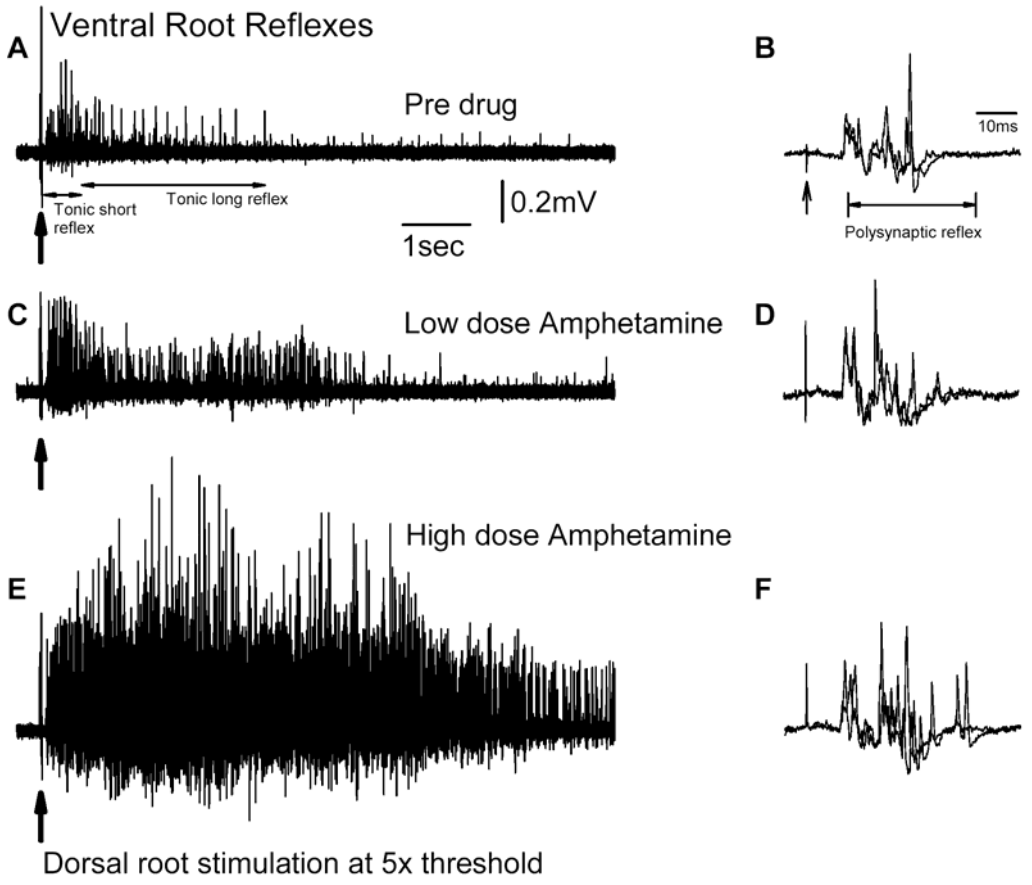

Figure 3-5

Ventral root reflex responses to dorsal root stimulation in the *in vitro* sacrocaudal spinal cord of chronic spinal rats. Low and high doses of amphetamine increase ventral root reflexes of short and long duration. ***A.*** Ventral root response to a single pulse of dorsal root stimulation at 5xT (threshold) before application of amphetamine. Tonic short reflex is measured from 30-600ms after stimulation and the tonic long reflex is measured from 600-3600ms after stimulation. ***B.*** Magnification of *A* showing the polysynaptic reflex (10-40ms) in greater detail. ***C,D and E,F.*** Same as *A* and *B*, but at low (0.1 μM) and high (10 μM) doses of amphetamine respectively.

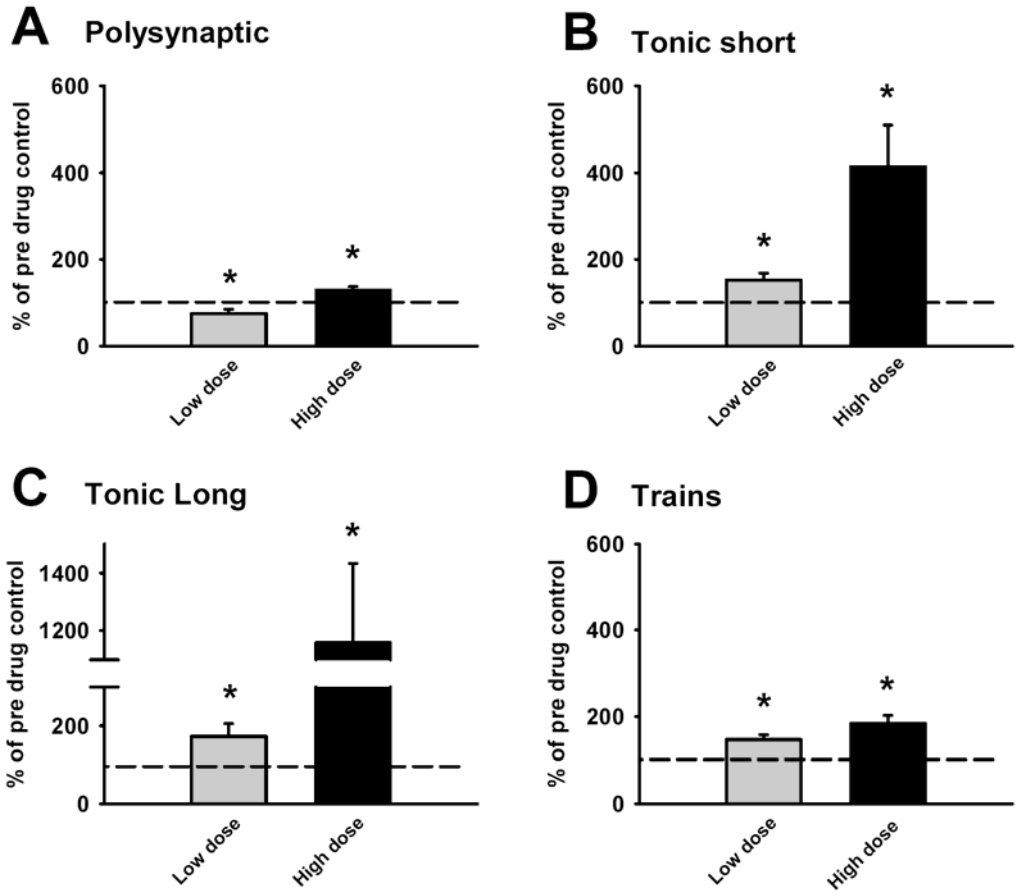

Figure 3-6

Facilitation of ventral root reflexes after administration of low (0.1μM, grey bar) and high (1-10 μM, solid bar) doses of amphetamine. Polysynaptic, tonic short and tonic long reflexes were measured at the ventral root after dorsal root stimulation at 5x threshold (5xT; about 0.05mA). All reflexes are shown normalized to pre drug condition, dashed line in all graphs indicates 100%. ***A.*** Polysynaptic reflex: showed a significant decrease to 75.7 ± 9.4% at low doses of amphetamine, and a significant increase to 129.1 ± 7.7% at high doses of amphetamine. ***B.*** Tonic short reflex: shows significant increases at both low (to 151.9 ± 16.7%) and high (to 415.3 ± 94.1%) doses of amphetamine. ***C.*** Tonic long reflex: low dose of amphetamine caused a reflex increase to 172.3 ± 32.8% and a high dose caused a reflex amplitude increase to 1158.6 ± 274.5%. ***D.*** Trains reflex: shows significant reflex amplitude increases at low (to 146.8 ± 10.9%) and high (to 183.4 ± 18.9%) doses of amphetamine. * $p<0.05$

until the CaPIC was activated, as reflected by a sharp downward deflection from the estimated leak current (solid line). The threshold of the CaPIC (≈ -55mV) is noted by V_{on} in the voltage trace and the size of the *initial* CaPIC (≈ 2.5nA) is indicated by the length of the solid arrow that is measured from the leak current to the point of zero slope in the current trace. This deviation from the leak current persisted for many seconds as reflected by the sustained current on the descending phase of the voltage ramp (*sustained* CaPIC is indicated by dashed arrow in Fig. 3-7*A*). The CaPIC was only turned off when the hyperpolarizing voltage ramp was lower than the voltage at V_{on} (≈ -65mV at V_{off}, Fig. 3-7*A*). In this cell, following the application of amphetamine, the initial and sustained CaPIC both showed substantial increases (Fig. 3-7*B*). In another cell (Fig. 3-7*C*), both the initial and sustained pre drug CaPIC was smaller compared to the cell in Figure 7*A*, but nonetheless, were increased following the application of amphetamine (Fig. 3-7*D*).

Averaged data shows that before amphetamine, the initial peak CaPIC was 2.44 ± 1.20nA and the sustained peak CaPIC was 2.14 ± 0.99nA (n = 9 motoneurons tested; Table 3-1). Following the application of amphetamine, the initial and sustained CaPIC both showed significant increases of 37.3% (to 3.35 ± 1.44nA) and 56.1% (to 3.34 ± 1.23nA) respectively (n = 9, t-test; $p<0.05$; Table 3-1). The effect of amphetamine on increasing the CaPIC was reasonably selective as no significant changes were observed in the onset threshold of the CaPIC (V_{on}), the resting membrane potential (V_m) or on the conductance (G_m) of the cells (Table 3-1).

3.3.5 Induction of very large Ca PIC following amphetamine

In all cells, the time for amphetamine to reach peak effect was 15-20 mins; therefore all measurements (e.g., initial and sustained PIC, etc.) were made during the time when amphetamine had reached its peak effectiveness. Figure 3-8

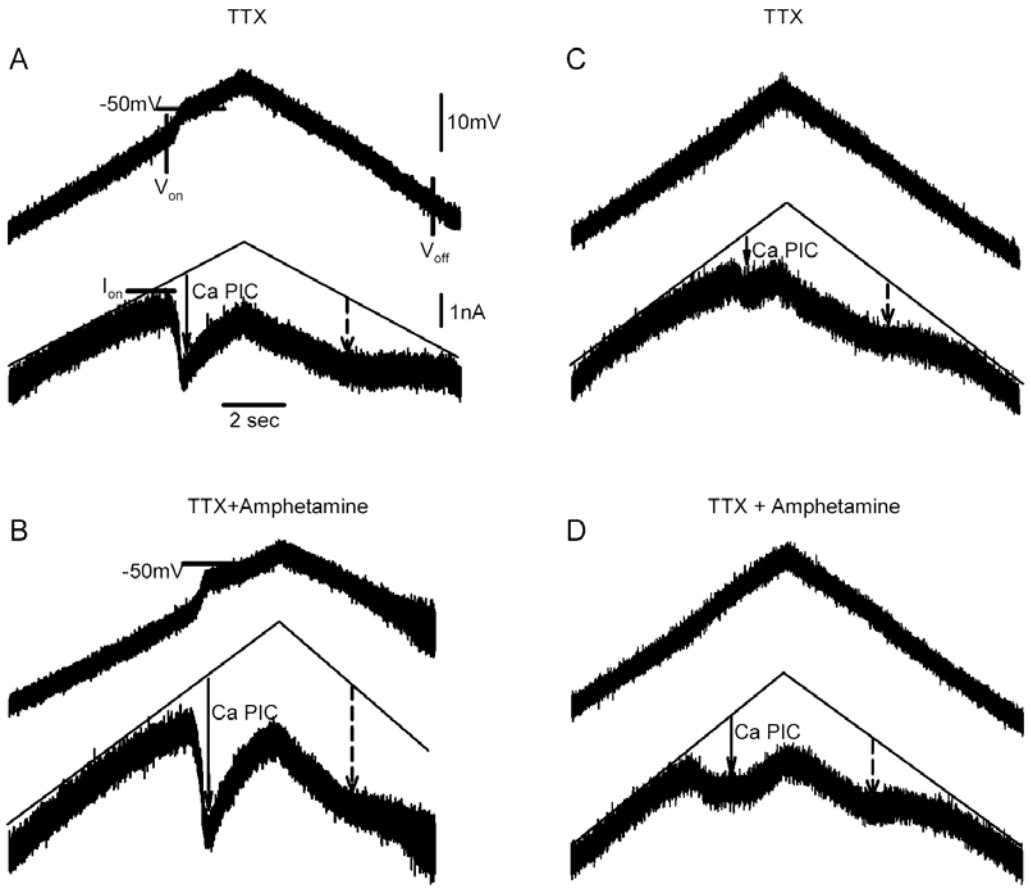

Figure 3-7

Example recordings of CaPIC from two motoneurons of chronic S2 spinal rats measured during a slow triangular voltage ramp. Voltage command in top traces, resulting current in bottom traces with leak current shown as the thin triangular line on the current trace. ***A.*** Response of a motoneuron in TTX, to isolate CaPIC, before amphetamine administration. Solid line on voltage ramp at -50mV indicates spike threshold. V_{on} is the onset voltage of the CaPIC, I_{on} is the corresponding onset current. V_{off} is the offset voltage of the CaPIC. The size of the initial CaPIC, which is measured from the leak current, is indicated by the length of the solid arrow and the sustained CaPIC on the downward voltage ramp is indicated by the dashed arrow. ***B.*** Response of the same motoneuron in *A* after administration of amphetamine. Amphetamine causes significant increases in both the initial CaPIC and sustained CaPIC. ***C.*** A different motoneuron in TTX, which shows a smaller initial and sustained CaPIC than shown in *A*. ***D***. Response of same motoneuron in *C* after amphetamine application. The small CaPIC also shows substantial enhancement following application of amphetamine.

Average N=9	V_m (mV)	G_m (μS)	V_{on} (mV)	Initial PIC (nA)	Sustained PIC (nA)
Control	-79.3 ± 2.8	0.19 ± 0.03	-60.2 ± 2.7	2.44 ± 0.40	2.14 ± 0.33
Amphet **(1-10 μM)**	-74.8 ± 4.1	0.25 ± 0.03	-58.8 ± 3.6	***3.35 ± 0.48***	***3.34 ± 0.41***

Table 3-1

Effects of amphetamine on motoneuron properties and the motoneuron CaPIC. Intracellular recordings made from motoneurons in the sacrocaudal spinal cord of chronically injured, spastic rats (n = 9). V_m = resting membrane voltage (mV), G_m = resting membrane conductance (μS), V_{on} = onset voltage or threshold of the CaPIC (mV), Initial PIC = initial peak current for the CaPIC (nA), Sustained PIC = sustained peak current of the CaPIC (nA). All values are shown as mean ± SE, values in bold are significant (t-test; $p<0.05$).

documents the progression from the time of amphetamine application to when peak effectiveness was observed. An unexpected finding was uncovered in six of the nine motoneurons tested after the application of TTX and in the presence of amphetamine. Before the drug, the CaPIC was controlled throughout the voltage clamp and could be terminated at the hyperpolarizing end of the voltage ramp with the current coming back to control levels near the leak current (Fig. 3-8*A*). Eight minutes after amphetamine application, a moderate increase in CaPIC amplitude was visible (as noted for the sustained CaPIC, see arrow in Fig. 3-8*B*) and the CaPIC could again be terminated by the downward phase of the voltage ramp. A further 15 minutes after amphetamine application, when the drug's full effect was achieved, a very large current was revealed (Fig. 3-8*C*). In this case the CaPIC could not be terminated by the downward voltage ramp, even when a large negative holding potential was reached, suggesting that very large dendritic calcium currents, or possibly a non-dendritic calcium activated cationic current, were activated that could not be terminated by current applied through the electrode in the soma. This uncontrollable CaPIC did not return to control levels and remained activated for many minutes. Any subsequent ramps applied did not reveal any sharp deflections in current indicative of CaPIC activation, as the CaPIC remained fully activated after the original voltage ramp was applied. This condition persisted for up to 10 minutes, even with a large negative bias current, after which a CaPIC could again be evoked (data not shown). In cells where there was a loss of control of the PIC, the amphetamine-induced PIC was taken as the PIC measured just prior to this loss of control. Further, these large, non-inactivating CaPICs were not due to the presence of apamin which was sometimes present in the bath, because they occurred equally in motoneurons with (n =3) and without (n = 3) apamin (n = 6 total with these types of CaPICs).

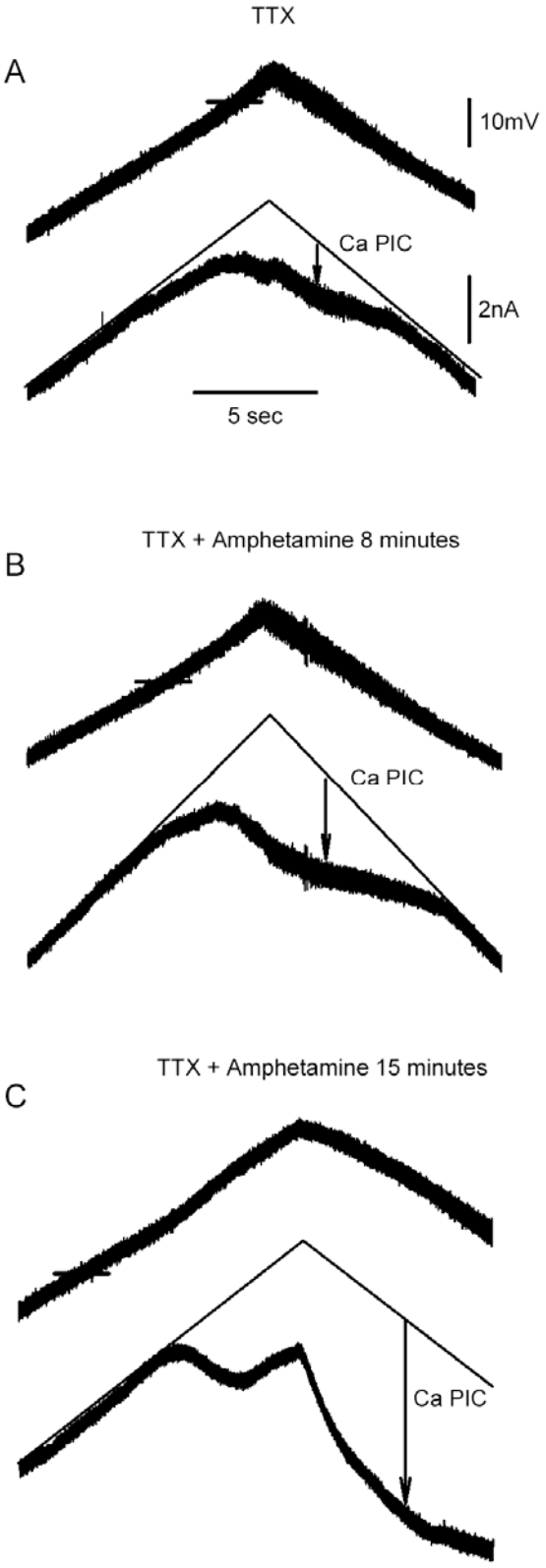

Figure 3-8

Progression of CaPIC from the time of amphetamine application to the time where peak effectiveness is observed (15-20mins); same recording conditions as in Fig. 3-7. Solid line on voltage ramp at -50mV indicates spike threshold. ***A.*** Response of motoneuron in TTX and before amphetamine application. The sustained CaPIC (solid arrow) is modest prior to amphetamine application. ***B.*** Same motoneuron as in *A*, 8 mins after amphetamine application. The sustained CaPIC already shows amplification, but still returns to baseline levels at the end of the voltage ramp. ***C.*** Same motoneuron as in *A* and *B*, 15 mins after amphetamine application. Sustained CaPIC is increased substantially, and could not be terminated by the downward voltage ramp. The current does not return to control levels when the voltage ramp is completed.

3.3.6 Supersensitivity of tail spasms and reflexes to amphetamine in the awake chronic spinal rat

Classic denervation supersensitivity is known to develop in chronic spinal cord injury. To determine if supersensitivity to endogenous NA present below the injury develops, responses to low and high doses of amphetamine were compared in chronic and acute animals, beginning with kinematics. Maximum flexion and extension angles of the tail following the standardized stretch/rub maneuver were measured in the acutely spinalized rat in the same manner as for the chronically spinalized rat (Fig. 3-9). Representative data shows that in acute animals, prior to amphetamine administration, only a small degree of extensor movements were present (Fig. 3-9*B*) and the spasms (if present) elicited by rubbing the tail primarily produced flexion (Fig. 3-9*A*). As acute animals predominantly show flexion spasms, with little to no interference of extension behavior, the maximum flexion angles are consequently larger compared to those in chronic spinalised animals; the balance of activity is flexor biased in acute animals. Following a high dose of amphetamine, an increase in flexion movements were seen (Fig. 3-9*C*), as well as a modest increase in phasic extensor movements (Fig. 3-9*D*). Lower doses of amphetamine had no significant effects.

Averaged group data from the acute animals showed that the amount of maximum flexion before amphetamine was 37.2 ± 3.4º (n = 10) and a significant increase in maximum flexion occurred with high dose amphetamine to 69.4 ± 10.8º (n=10), whereas low dose amphetamine showed no significant change in flexion (53.8 ± 13.2º; Fig. 3-9*E*; n = 10). Extension, conversely, was present in only very small amounts, if at all, prior to amphetamine administration (maximum extension 0.2 ± 0.1º) and did not show any significant change with subsequent doses of amphetamine (Fig. 3-9*F*; 0.0 ± 0.0 at low dose; 0.8 ± 2.0º at high dose). This indicates that when spasms are present in the acute spinal rat, they are comprised mainly of flexor activity and only increase after high, but not low, doses of amphetamine. Unlike chronic animals, acute animals showed negligible

maximum extension movements and extension spasms, even after amphetamine administration. The fact that acute animals showed significant increases in maximum flexion only at high doses of amphetamine, whereas the chronic spinalized animal showed significant increases in maximum flexion even at doses 10 times lower (2.0 mg/Kg in acute vs 0.2 mg/Kg in chronic), is indicative of a development of supersensitivity to NA which develops in chronic animals.

Cutaneous reflex responses in acute, compared to chronic, spinal rats showed similar trends in the development of supersensitivity as those shown in the kinematic data. Following the application of a train of stimuli to the tip of the tail, acute rats (having received a complete S_2 transection less than 5 days prior to testing), showed minimal responses from EMG recorded at the segmental tail muscles (Fig. 3-10*A*) compared to chronically injured rats (Fig. 3-10*D*). Upon the administration of amphetamine at low doses (0.2mg/Kg), acutely injured rats showed no increase in the reflexes (Fig. 3-10*B*), whereas reflexes in chronically injured animals were facilitated at this dose (Fig. 3-10*E*). Only a high dose (2mg/Kg) of amphetamine in acute animals produced an augmentation of both short latency and long lasting reflexes (Fig. 3-10*C*).

Cutaneous reflexes recorded from the segmental tail muscles in the acute spinal rat were much smaller than those recorded from the chronic spinal rat before drug application (Fig. 3-11, compare absolute reflex responses, open bars on left of graphs; acute absolute reflexes significantly smaller). When the data collected from acute animals is grouped, significant increases in all reflex responses only occurred at high doses of amphetamine (0.6-2.0 mg/Kg; n = 10), with the greatest increase seen in the polysynaptic reflex. After a low dose of amphetamine (0.2 mg/Kg, n=10), reflex responses in the acute rat were not significantly different from pre drug values (n = 10), unlike that previously shown for chronic animals. This supersensitivity to amphetamine exhibited by the chronic transected animal is most pronounced in long latency reflexes, as well as in the background activity (compare low dose increases in Fig. 3-4*B*-*E* vs Fig. 3-11*B*-*E*). Parallel to the

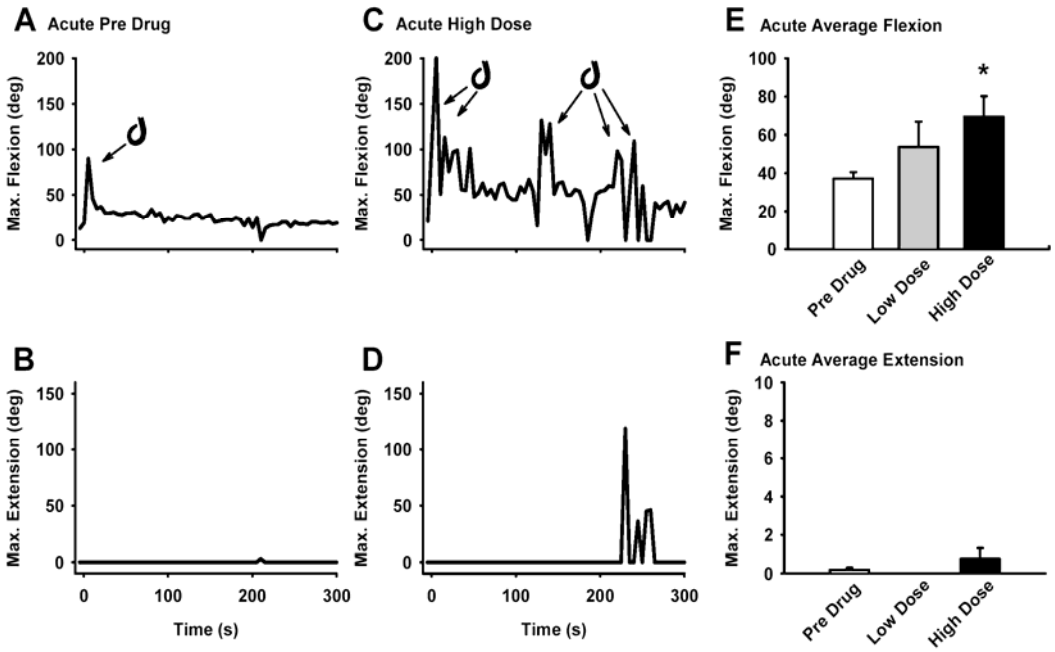

Figure 3-9

Maximum flexion and extension angles measured in a representative acute spinal rat before and after amphetamine . Similar format as Fig 3-2, with example kinematic data from an acute spinal rat for ***A.*** pre drug maximum flexion, ***B.*** pre drug maximum extension, ***C.*** high dose (0.6 mg/Kg) maximum flexion and ***D.*** high dose maximum extension. Note that the amount of maximum flexion and the number of spasms counted increased with a high dose of amphetamine. ***E.*** Group data: average maximum flexion pre drug is 37.2 ± 3.4º and increases significantly by 163.2% at high doses of amphetamine. A trend for increased maximum flexion is evident, but not significant, at low doses (0.2 mg/Kg) of amphetamine. ***F.*** Same format as *E*, but maximum extension. Maximum extension values are negligible prior to amphetamine, and do not show any significant increases with subsequent doses (note the scale). * = $p<0.05$.

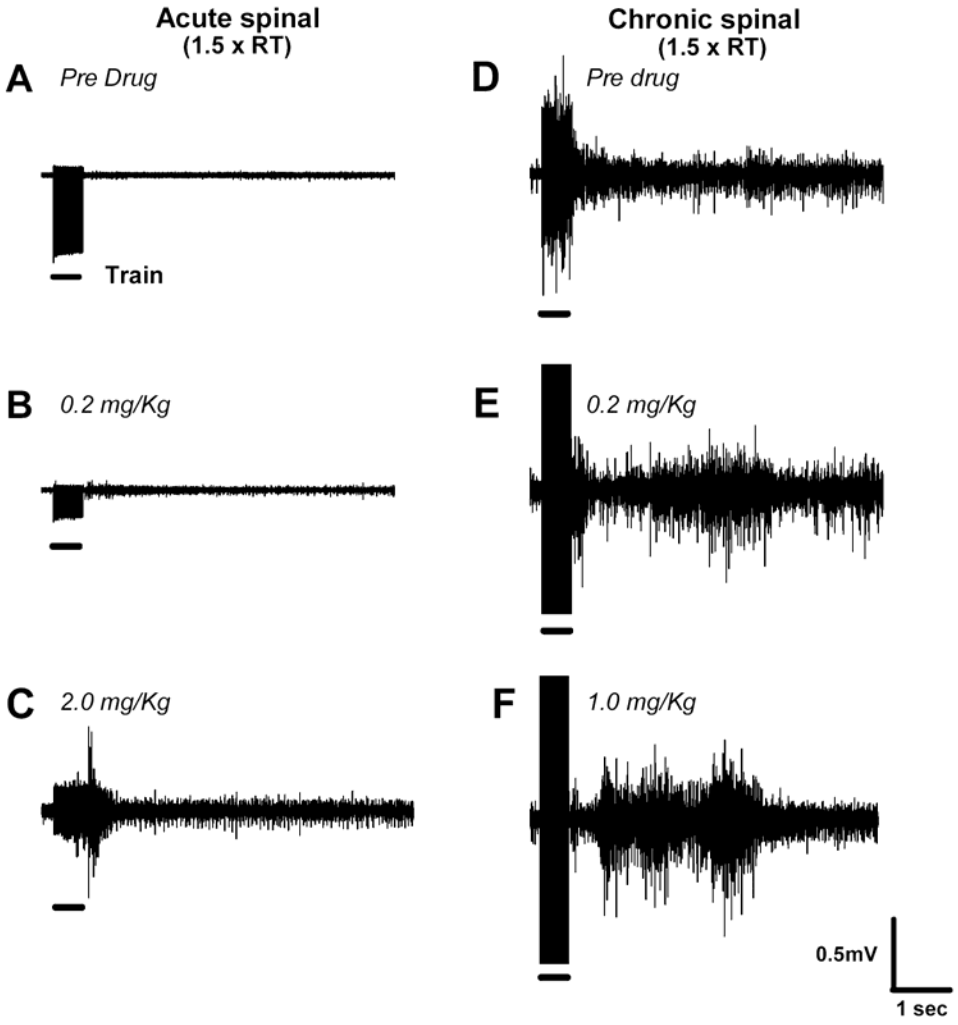

Figure 3-10

Development of amphetamine supersensitivity in chronic S_2 spinal rats, as seen by changes in cutaneous reflexes recorded in the segmental tail muscles evoked by train stimulation (1.5xRT) applied to the tip of the tail. ***A.*** Surface EMG response in acute spinal rat before amphetamine administration. ***B.*** Same rat as in *A*, but recorded after administration of a low dose of amphetamine (0.2 mg/Kg). ***C.*** Same rat as in *A* and *B*, but recorded after a high dose of amphetamine (2.0 mg/Kg). ***D***. Response of a chronic spinal rat to train stimulation prior to amphetamine administration. Reflex response is already much larger than in the acute spinal rat. ***E.*** Same rat as in *D*, but after a low dose of amphetamine (0.2 mg/Kg). ***F.*** Same rat as in *D* and *E*, but after administration of a high dose of amphetamine (1.0 mg/Kg). Despite doses 10x that of the chronic spinal rat, the reflexes recorded in the acute spinal rat never reach similar increases in amplitude, pointing towards the development of a supersensitivity to endogenous NA present below the transection.

kinematic data, this also points toward a development of supersensitivity to NA manifesting in the chronic stages of spinal cord injury.

In acute rat experiments, the stimulation intensity used was similar to those used in the chronic rat (0.075-0.450mA). It is possible that this intensity was too low to activate reflex circuits in the acute rat. To control for this possibility we also compared acute responses to 10mA stimulation and found that the reflex responses at 10mA were equivalent to those recorded at the lower stimulation intensities (not significantly different; data not shown).

FIGURE 3-11

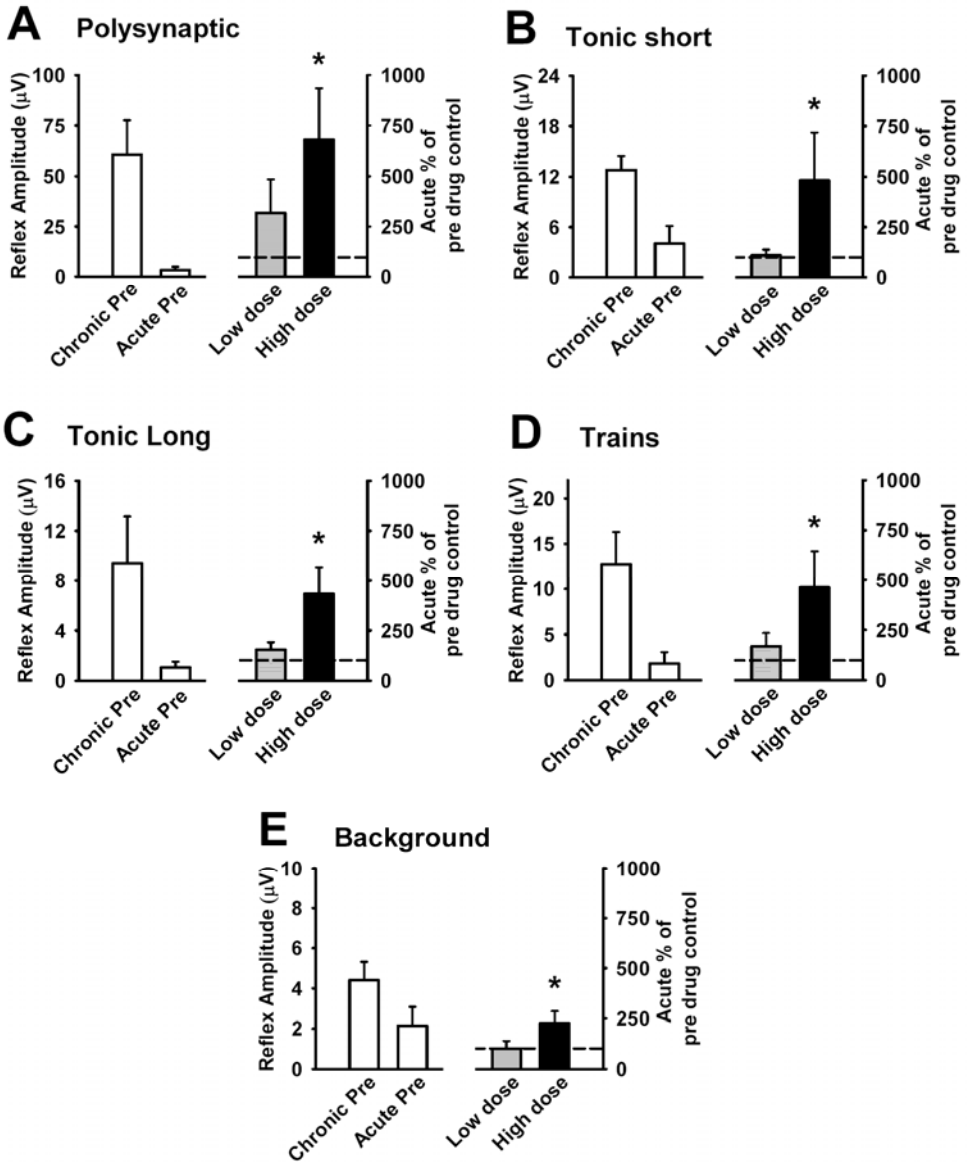
A
Polysynaptic
Reflex Amplitude (μV)
100
75
50
25
0
Chronic Pre
Acute Pre
*
1000
750
500
250
0
Acute % of pre drug control
Low dose
High dose
B
Tonic short
Reflex Amplitude (μV)
24
18
12
6
0
Chronic Pre
Acute Pre
*
1000
750
500
250
0
Acute % of pre drug control
Low dose
High dose
C
Tonic Long
Reflex Amplitude (μV)
16
12
8
4
0
Chronic Pre
Acute Pre
*
1000
750
500
250
0
Acute % of pre drug control
Low dose
High dose
D
Trains
Reflex Amplitude (μV)
20
15
10
5
0
Chronic Pre
Acute Pre
*
1000
750
500
250
0
Acute % of pre drug control
Low dose
High dose
E
Background
Reflex Amplitude (μV)
10
8
6
4
2
0
Chronic Pre
Acute Pre
*
1000
750
500
250
0
Acute % of pre drug control
Low dose
High dose

Figure 3-11

Similar format as Fig. 3-4. Absolute reflex amplitudes and respective changes in acute spinal rats in response to low and high doses of amphetamine for the various reflex responses. ***A***. Polysynaptic reflex: Pre drug reflex in the acute spinal rat is 3.33 ± 1.6 μV compared to the chronic spinal rat 60.9 ± 16.7 μV (left graphs). At low doses of amphetamine (0.2 mg/Kg), the polysynaptic reflex in acute spinal animals increases to 318.0 ± 167.0%, but only significantly at high doses (0.6-2.0 mg/Kg) to 679.0 ± 255.0% (right graphs). ***B.*** Tonic short reflex: Pre drug reflex 4.1 ± 2.1 μV acute, 12.8 ± 1.7 μV chronic. At a low dose of amphetamine, the reflex increases only slightly to 109.7 ± 29.3%, whereas the increase to 481.9 ± 235.8% is significant after a high dose. ***C.*** Tonic long reflex: pre-drug reflex acute: 1.1 ± 0.5 μV, chronic 9.4 ± 3.7 μV in pre drug. A low dose of amphetamine increases the reflex to 154.2 ± 37.1%, and a high dose increases the reflex significantly to 433.0 ± 133.9%. ***D.*** Train reflex: 1.8 ± 1.3 μV acute, 12.7 ± 3.6 μV chronic in pre drug. Low doses of amphetamine increase the reflex to 167.7 ± 67.8% and high doses cause a significant increase to 461.6 ± 182.8%. ***E.*** Background: Pre drug 2.1 ± 0.9 μV acute, 4.4 ± 0.9 μV chronic. The background activity shows a non-significant increase to 101.4 ± 37.2% at low doses of amphetamine and a significant increase to 227.2 ± 60.2 % at high doses of amphetamine. * = $p < 0.05$.

3.4 DISCUSSION

Our findings demonstrate that amphetamine administration dramatically increases reflex and spasm activity in tail muscles and nerves of chronically injured animals, consistent with previous reports that flexor reflexes in the hindlimb of chronic spinal rats are augmented by amphetamine (Nozaki et al. 1980). As discussed below, the mechanism of action of amphetamine at the doses used in our experiments (< 2mg/Kg in vivo; < 10μM in vitro) are primarily to increase the release of NA from presynaptic terminals. Thus, it is very likely that enhancement of reflexes and spasm behaviour following amphetamine administration in completely lesioned animals is mediated by increasing the endogenous release of NA from presynaptic terminals located below the level of the spinal injury. In particular, this study shows that amphetamine increases long-lasting reflexes through facilitated activation of the CaPIC in motoneurons, thus showing an overall increase in intrinsic motoneuron excitability leading to the production of larger and longer spasms in response to brief sensory stimulation. Finally, we have also shown that, similar to 5-HT, long-lasting reflexes can be potentiated by very low doses of amphetamine in chronic, but not acutely, injured animals, suggesting that motoneurons below the level of the injury become supersensitive to endogenous levels of NA. Development of supersensitivity to low levels of NA below the lesion may be responsible for the recovery of motoneuron PICs, known to mediate spasm generation in these animals following spinal cord injury.

3.4.1 Mechanism of amphetamine induced increases in long latency reflexes

Amphetamine has the combined ability to release NA and dopamine from presynaptic terminals and block their reuptake, as well as to inhibit their metabolism by monoamine oxidase (MAO) (Aboul-Enein 1971; de la Torre et al. 2004; Florin et al. 1994; Randrup and Munkvad 1966). While the specific mechanism of action of amphetamine is dependent on the dose utilized (Elliott and Beveridge 2005; Seiden et al. 1993; Sulzer and Rayport 1990; Sulzer et al.

2005), the net effect of amphetamine at the doses used in this study is to increase the presynaptic release of NA via the reversal of the noradrenaline transporter (NET). Although amphetamine can act at both the dopamine (DAT) and serotonin transporters (SERT), producing a similar presynaptic release of these monoamines, amphetamine is 5- to 9- fold less potent at DAT and 200- to 500-fold less potent at SERT (Han and Gu 2006). Therefore, amphetamine likely exerts its main effects through increasing the release of endogenous NA (Carlsson et al. 1965; Randrup and Munkvad 1966).

Based on the high potency of amphetamine for the NA transporter, increases in long-lasting reflexes from amphetamine are likely due to increases in the release of endogenous NA from presynaptic terminals below the spinal lesion. It has already been established that the CaPIC underlies the sustained portion of the PIC as well as long duration (>1s) reflex responses (Bennett et al. 2001b; Li and Bennett 2003). For instance, when a hyperpolarizing current bias is applied to a motoneuron to eliminate the voltage-sensitive PIC, sustained depolarizations of the motoneuron in response to a single shock dorsal root stimulation are dramatically shortened from many seconds before the PIC block to less than one second during the PIC block (Bennett et al. 2001b). Facilitation of the CaPIC is known to be contingent on the monoamines 5HT and NA, as acute spinal transection eradicates self-sustained depolarizations and firing normally associated with the PIC (Hounsgaard et al. 1988), whereas subsequent application of 5HT and NA agonists restores these properties (Bennett et al. 2001b; Conway et al. 1988; Hounsgaard et al. 1988). In contrast, the effect of dopamine agonists on the PIC is minimal (DJ Bennett, personal communication). In this study, amphetamine dramatically increases the dendritic CaPIC, likely via its action of increasing release of endogenous NA. The increase of CaPIC, in turn, likely enhances the amplitude and duration of the long lasting reflexes recorded both in vitro and in vivo.

In addition to facilitating the CaPIC, the increase in monoamines from amphetamine may also influence other post-synaptic currents such as the activation of a non-dendritic calcium activated cationic current. However, the majority of the PICs recorded in tail motoneurons are Na^+ and Ca^{2+} mediated (TTX and nimodipine sensitive currents respectively), with the CaPIC mediating the long-lasting reflexes (Bennett et al. 2001b; Li and Bennett 2003). Alternatively, amphetamine may also activate post-synaptic receptors directly, such as the G-protein coupled rat trace amine receptor, rTAR1 (Bunzow et al. 2001). However, cyproheptadine, which can also activate rTAR1, has been shown to have a depressive effect on motoneuron excitability and spasms (Barbeau et al. 1982; Tremblay and Bedard 1995), which is in direct contrast to the increased motoneuron excitability and spasms seen with amphetamine. Should the primary mechanism of action of cyproheptadine and amphetamine be through rTAR1 activated pathways, it would be expected that these drugs would produce similar effects on motoneuron excitability and spasms, whereas in fact they produce opposing effects. Thus, given the opposing effects of amphetamine and cyproheptadine on spasticity, increases in CaPICs and long lasting reflexes from amphetamine are unlikely to be a result of activation of rTAR1.

Amphetamine may also enhance long-lasting reflexes by directly binding to presynaptic α_2 adrenergic receptors on interneurons or sensory afferent terminals (Ritz and Kuhar 1989). Following spinal transection in rats, it is known that the α_2 adrenergic receptor is present caudal to the lesion, in the distal dorsal horn, and in fact is found to be present in increased density in the chronic spinal rat compared to uninjured controls (Roudet et al. 1994). However, experiments involving the α_2 receptor agonist clonidine, known for its potent anti-spastic action (Anderson et al. 1982), have shown that clonidine has an inhibitory effect on polysynaptic pathways (Chau et al. 1998) and long lasting reflexes (Li et al. 2004b), likely mediated by a blockade of polysynaptic excitatory postsynaptic potentials. Thus, as the dominant effect of α_2 adrenergic receptor activation is inhibitory in respect to reflexes, it is unlikely that there was a marked stimulation of this receptor by

amphetamine given that the overall effect of amphetamine was to increase reflex responses.

3.4.2 Amphetamine biases spasm behaviour to flexion

Following a standardized stretch/rub maneuver in chronic spinal rats, low and high doses of amphetamine increased long lasting flexor reflex spasms exhibited by the tail, and consequently, decreased extension reflexes and spasms. This is consistent with prior research by Nozaki et al. (1980) where an increase in the hindlimb flexor reflex of chronic spinal rats occurred following amphetamine. Our data are also in line with specific differences between 5HT and NA reported by several other groups. L-DOPA, a drug which leads to the synthesis and subsequent release of NA, has been reported to increase flexor reflexes elicited by toe pinching in acute spinal rats (Austin et al. 1976). Conversely, following selective 5HT denervation with 5,6-dihydroxytryptamine, subsequent application of 5-HTP (a serotonin precursor) enhances extensor reflexes elicited by tail pinching in rats (Nygren et al. 1974). Conway et al., (1988) also observed that L-DOPA application to decerebrate acute spinal cats increased flexor activity, while 5-HTP application increased extensor activity. Likewise, 5-HTP injection to acutely spinalized decerebrate cats reveals a bistable behavior in extensor motoneurons due to PIC activation (Hounsgaard et al. 1988), whereas flexor, and to a lesser degree extensor, motoneurons show these bistable properties after L-DOPA administration (Conway et al. 1988). These studies indicate that 5HT mainly affects extensor activity while NA can affect both flexor and extensor activity, but its main effects are observed in flexors. The fact that NA preferentially increases bistable or PIC activation in flexor motoneurons may account for the amphetamine-induced increased in maximum flexion, rather than extension, movements after a stretch/rub stimulus in chronic spinal rats in this study.

3.4.3 Amphetamine causes confounding effects on short duration reflexes

The *in vitro* ventral root reflexes and the reflexes recorded from the awake animal both show consistent increases in long-lasting reflexes at low and high doses of amphetamine, but present more variable results concerning the polysynaptic reflex. The polysynaptic reflex shows a progressive increase with higher doses of amphetamine in the ventral root experiments, whereas in the awake animal a progressive decrease with higher doses is observed. The shorter duration reflexes, such as the polysynaptic reflex, occur within the period of time that motoneurons are being activated by synaptic inputs from interneurons and afferents, as seen in motoneurons under hyperpolarized conditions during PIC block (EPSPs lasting up to 1s) (Bennett et al. 2001b; Conway et al. 1988).

Given that motoneurons are activated by afferent and interneuron inputs during short duration reflexes, compared to the longer duration reflexes (> 1s), the post-drug short duration reflexes may be more variable as a result of the mixed excitatory and inhibitory effects that amphetamine has on interneurons and afferents (Jankowska 1992). It has already been shown that premotor interneurons evoked by group II afferents, as are involved in the cutaneous reflexes recorded in this study, are a heterogeneous population able to exert both inhibitory and excitatory effects on motoneurons (Jankowska 1992). These interneurons are inhibited by NA, which is likely mediated by the α_2 receptor (Maxwell et al. 2000). The increased levels of NA released by amphetamine may cause inhibition of excitatory interneurons and result in reduced polysynaptic reflexes, such as seen during low doses in ventral root reflexes. Likewise, increases in polysynaptic reflexes at high doses in the in vitro preparation may result from a balance of increased excitation of afferents, interneurons and motoneurons in response to increases in NA. The different degrees of facilitation of the polysynaptic reflex at low and high dose in the awake animal may reflect differences in this balance.

3.4.4 Sources of NA caudal to spinal transection

The amphetamine-induced increases in ventral root reflexes, cutaneous reflexes in the awake rat and motoneuron CaPICs observed in this study could not have occurred without an endogenous source of NA present caudal to the transection. The presence of intraspinal noradrenergic neurons below a complete transection has been shown, but in reduced quantities, when compared to pre lesion levels (Cassam et al. 1997). Another source of NA below the transection may originate from peripheral sympathetic fibres as histofluoresence studies in chronic spinal rats show that peripheral sympathetic fibres enter the spinal cord alongside blood vessels, can dissociate from the blood vessels and be seen in the immediate vicinity of neurons (McNicholas et al. 1980). As discussed below, the CaPIC, which mediates long lasting reflexes, may become supersensitive to these residual sources to aid in the development of spasticity in chronic spinal injury.

3.4.5 Supersensitivity of motoneuron PICs to monoamines

It has already been suggested that following chronic spinal cord transection in rats a supersensitivity of motoneurons to intraspinal sources of 5HT develops (Barbeau and Bedard 1981; Harvey et al. 2006a, b; Tremblay and Bedard 1995). Administration of 5HT precursors or agonists immediately following spinal cord transection produces little or no motoneuron activation. However, administration of these drugs in similar concentrations 20-30 days following transection produces a marked increase in motoneuron activation (Barbeau and Bedard 1981; Tremblay and Bedard 1995). More recently, a 30-fold supersensitivity of motoneuron PICs to 5HT was demonstrated in the chronic spinal rat (Harvey et al. 2006a). In this case, application of the $5HT_2$ receptor agonist DOI, at doses of 1μM or less, facilitated both the NaPIC and the CaPIC, and this facilitation occurred at doses 30 times smaller than those required to produce a similar, but smaller, facilitation in acutely spinalized animals.

While the development of denervation supersensitivity to 5HT following spinal transection has been thoroughly investigated (Tremblay and Bedard 1995), the same cannot be said for NA. Our results show that acute spinalized rats require a higher dose of amphetamine than chronic spinalized rats in order to achieve a significant increase in maximum flexion and reflex amplitudes. The reflex responses of the acute rats, with or without amphetamine, were still much smaller than those of chronic spinal rats. These data suggest that denervation supersensitivity to NA is occurring below the level of transection in chronic spinalized rats. Similar effects have been reported previously, where it was shown that amphetamine produced a greater facilitation of the flexor reflex in the hindlimb of the chronic spinal rat than the acute spinal rat (Nozaki et al. 1977) and this facilitation was abolished when catecholaminergic terminals were previously destroyed with 6-hydroxydopamine (6-OHDA) (Nozaki et al. 1980).

The leading theories regarding the mechanism of the development of this noradrenergic denervation supersensitivity are largely concerned with noradrenergic receptors on the post-synaptic membrane rather than with supersensitivity developing at the presynaptic NA transporter resulting in increased presynaptic release of NA at low doses of amphetamine. To support this, spastic reflexes in chronic spinal rats are themselves supersensitive to NA (Li et al. 2004b), indicating postsynaptic receptors likely become supersensitive. Also, the hindlimb flexor reflex of the chronic spinal rat is known to become enhanced following the application of the $NA_{\alpha 1}$ receptor agonist methoxamine, which again suggests a development of supersensitivity on the part of the noradrenergic postsynaptic receptors below the transection (Nozaki et al. 1980). An upregulation of the $NA_{\alpha 1}$ receptor has been found in the spinal cord of chronically transected rats using autoradiographic techniques (Roudet et al. 1994; Roudet et al. 1993). Receptor upregulation following transection is likely to be one mechanism in which denervation supersensitivity develops after spinal cord injury. Another possible mechanism may be that individual receptors located below the transection develop a higher affinity for ligands through a process of

modification of the receptor itself, as is known to occur with the $5HT_{2C}$ receptor (Gurevich et al. 2002). Since both NaPIC and CaPIC are under the control of G protein coupled pathways, development of supersensitivity may also be due to changes or upregulation within these pathways (Alaburda et al. 2002; Cantrell and Catterall 2001). There is currently little research showing the mechanism by which denervation supersensitivity develops in the spinal cord after transection, but there is no reason the above mentioned mechanisms need be mutually exclusive, or cannot function together with an as yet undiscovered mechanism.

3.4.6 Clinical Implications

Amphetamine, which increases the presynaptic release of NA, increases long lasting reflexes and the motoneuron CaPIC underlying them. This strongly implicates the role of NA in the development of spasticity following spinal cord injury. Our data also suggest a development of supersensitivity to NA in the chronic stages of spinal cord injury, while previous research has established that 5HT supersensitivity also develops (Harvey et al. 2006a). Given that a combined blockade of $5HT2_a$, $5HT2_c$ and $NA_{\alpha 1}$ receptors is required to eliminate NaPICs underlying spasticity (Harvey et al., 2006a; see Introduction), it would seem that an ideal clinical approach to spasticity management would include drugs which target both the 5HT and NA systems, especially for modulation of the CaPIC which mediates long-lasting reflexes. Specific control of 5HT or NA release from fibres below the lesion could be targeted pharmacologically, as without these neuromodulators spastic symptoms are unlikely to develop. Alternately, the intraspinal administration of 5HT and NA immediately following injury may prevent the development of supersensitivity to these neurotransmitters in the motoneurons. At this point, it is essential that both 5HT and NA are taken into equal consideration when developing clinical spasticity management treatments.

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Chapter 4:

Peripheral noradrenaline crosses the blood-brain barrier and modulates spasms after chronic spinal cord injury

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A version of this chapter is being prepared for publication.
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4.0 AUTHOR CONTRIBUTIONS

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- Completion and analysis of all *in vivo* experiments, completion and analysis of some *in vitro* ventral root reflex experiments (reboxetine), preparation of figures and manuscript.

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- Running and analysis of some *in vitro* ventral root reflex experiments, supervision of students and projects, preparation and editing of manuscript.

4.1 INTRODUCTION

Patients with injury to the spinal cord often experience symptoms of a spastic syndrome that develop in the months following injury, including readily triggered exaggerated reflexes with associated forceful muscle spasms. Spasms are the result of at least two general processes: 1) an increase in motoneuron excitability (Bennett et al. 2004; Button et al. 2008; Hultborn et al. 2004; Li et al. 2004a) and 2) a loss of inhibitory control over sensory afferent transmission (Li et al. 2004a; Norton et al. 2008). This has been confirmed in animal models of SCI (Bennett et al. 2004; Li et al. 2004a; Li et al. 2004b), and additionally verified in human SCI (Gorassini et al. 2004; Norton et al. 2008). Brainstem derived monoamines serotonin (5-HT), and particularly noradrenaline (NA), have been repeatedly shown to play a critical role in the regulation of these two processes following chronic SCI (Harvey et al. 2006a; b; Li et al. 2007; Murray et al. 2010; Rank et al. 2007). Specifically, selective activation of the α_{1A} adrenergic receptor has been demonstrated to strongly facilitate reflexes and spasms after chronic SCI (Rank et al. 2010). Moreover, it has recently been shown, in awake chronic spinal cord transected rats, that the α_{1A} adrenergic receptor is endogenously active, likely after interaction with the ligand NA (Harvey et al. 2006b; Rank et al. 2010). This is unexpected, considering that spinal cord transection eliminates nearly all descending adrenergic input to the spinal cord (Carlsson et al. 1964; Dahlstrom and Fuxe 1964; Haggendal and Dahlstrom 1973; Magnusson 1973). Nevertheless, treatment with a selective α_{1A} receptor neutral antagonist REC 15/2739, a drug which only blocks a ligand such as NA from activating the receptor, substantially reduces spasms in the awake chronic spinal rat (*in vivo*), indicating that an endogenous source of NA activates the α_{1A} receptor after injury (Rank et al. 2010).

The presence of residual NA below a chronic transection has been previously suggested, given that treatment of chronic spinal rats with amphetamine, which leads to a net efflux of endogenous NA from presynaptic terminals, facilitates

motoneuron excitability and spasms (Rank et al. 2007). However, this residual source of NA is not likely intrinsic to the spinal cord, because use of the neutral antagonist REC 15/2739 has no action when the cord is studied in isolation (*in vitro*) (Rank et al. 2010). It seems possible then, that the residual endogenous NA originates in the periphery (blood), especially since the peripheral nervous system is a strong source of NA. Indeed the blood-brain barrier (BBB), normally responsible for the separation of neurotransmitter pools that act centrally and those that act peripherally (Abbott et al. 2010; Abbott et al. 2006; Persidsky et al. 2006), is known to be chronically compromised after injury (Popovich et al. 1996). In the present paper, we tested the idea that peripheral NA may be crossing a compromised BBB after injury by injecting NA systemically and quantifying the affects of increased peripheral NA on motoneuron excitability (spasms) after chronic SCI. We also explore if the peripheral NA is acting on central α_1 adrenergic receptors by blocking these receptors with antagonists prior to peripheral injections of NA.

Classically, NA originating in the peripheral nervous system does not cross the BBB in uninjured animals (Weil-Malherbe et al. 1959; Weil-Malherbe et al. 1961; Whitby et al. 1961). However, as described above, after chronic SCI endogenous NA, likely originating in the periphery, may somehow cross a permeable BBB to act on central α_1 adrenergic receptors and increase spasms. Therefore another goal of our study was to investigate the mechanism by which peripheral NA is able to accomplish this. The expression of the membrane bound NA-transporter (NET) has been demonstrated at the BBB, where it normally functions to facilitate the efflux of transmitter from the CNS to the blood (Wakayama et al. 2002). However, the direction of the NET-mediated transport is known to be concentration dependent and can reverse, from efflux to uptake, when relative concentrations of NA are altered (Paton 1973), such as below a chronic spinal transection. In this manner, the NET may play a role in the facilitated transport of NA from the periphery to the spinal cord. Thus, our current paper uses the

NET blocker reboxetine to test whether the NET is the mechanism by which NA, originating in the periphery, crosses the BBB to facilitate spasms.

If spasms are facilitated by peripheral NA, which uses the NET-mediated transport to cross the BBB, the NA must somehow navigate the considerable distance from the blood vessels of the spinal cord to central α_1 adrenergic receptors located on, or near, motoneurons. Normally vesicular release of NA from descending adrenergic terminals allows NA to reach and activate central adrenergic receptors. However, given that all descending NA terminals, and therefore the conventional mechanisms by which NA is released onto motoneurons, are degraded below a chronic transection our study addresses the question of how the peripheral NA is handled. Previous experiments have shown that amphetamine, which causes an efflux of NA *from an existing store of vesicles*, increases motoneuron excitability and facilitates spasms in both the awake chronic spinal rat and in the isolated spinal cord (Rank et al. 2007). This suggests that the peripheral NA must be stored inside vesicles somewhere caudal to the transection in the spinal cord. In order to investigate this possibility we targeted the vesicular monoamine transporter ($VMAT_2$), which is responsible for packaging NA into vesicles. By blocking $VMAT_2$ before injecting NA peripherally we examined if peripheral NA is stored in vesicles below a transection and if exocytotic vesicular release is the mechanism by which peripheral NA is able to reach and act on central adrenergic receptors

In addition to using peripheral injection of NA to investigate the origin of residual endogenous NA below a chronic transection, we were also interested in examining how physiological increases in peripheral NA affect spasms after SCI. Plasma levels of NA are known to fluctuate in a state-dependent fashion, with increased levels of plasma NA correlating with a greater degree of physiological stress (Buhler et al. 1978; Kvetnansky et al. 1984). By exposing chronic spinal rats to acute immobilization stress, known to result in significantly increased plasma NA (Buhler et al. 1978; Kvetnansky et al. 1992; Kvetnansky et al. 1984;

Kvetnansky et al. 1978), we assess whether increased endogenous peripheral NA is capable of increasing muscle spasms.

Studying the influence of NA on muscle spasms after chronic SCI is of interest because spasms are an effective indirect measure of changes in motoneuron excitability (Bennett et al. 1998; Heckman et al. 2005; Hounsgaard et al. 1988; Lee and Heckman 2000; 1998; Li et al. 2004a). Motoneuron excitability in the mammalian spinal cord depends on the presence and activation of dendritic persistent inward currents (PICs) (Heckman et al. 2005; Hounsgaard et al. 1988; Lee and Heckman 2000; Li et al. 2004a). These PICs amplify synaptic input and allow motoneurons to produce long-lasting discharges in response to brief excitatory inputs (Heckman et al. 2005). Brainstem derived monoamines, such as 5-HT and NA, strongly influence motoneuron excitability (PICs) and the loss of these monoamines after SCI results in greatly reduced excitability of the motoneurons below the injury (Bennett et al. 2004). Despite the continued absence of descending monoamines, motoneuron excitability gradually returns in the months following injury and combined with a lack of normal descending inhibitory control over sensory afferent transmission, the recovered excitability is unchecked (Bennett et al. 2004; Li et al. 2004a; Li et al. 2004b). This spontaneous and uncontrolled recovery of motoneuron excitability (PICs) has been repeatedly demonstrated to be the mechanism underlying the development of the debilitating muscle spasms seen in chronic SCI (Bennett et al. 2004; Gorassini et al. 2004; Li et al. 2004a; Li et al. 2004b; Norton et al. 2008). Our study, therefore, examines how the NA system is modified after chronic SCI to compensate for a loss of descending NA input and contribute to the recovery of motoneuron excitability and spasms.

4.2 METHODS

Both normal adult female Sprague-Dawley rats (aged 3 - 6 months) and chronic S_2 spinal cord injured rats were utilized in this study. Chronic spinal rats received a complete sacral spinal (S_2) transection at 45 - 55 days old (as described in (Bennett et al. 1999). All experiments on chronic spinal rats were conducted after full spasticity had developed in the axial muscles of the tail (2 - 3 months after transection). Acute spinal rats received a complete sacral spinal transection (S_2) or complete thoracic spinal transection (T_7), (see also Bennett et al. 1999). Experiments on acute spinal rats were conducted 24 to 48 hrs after the transection surgery. Bladders were expressed twice daily in T7 transected rats. Immunohistochemical analysis of chronic injured and uninjured spinal cords was also conducted. Spasticity in the tail of chronic spinal rats was evaluated using kinematic measurements and spastic reflex responses were recorded via percutaneous EMG placed in the axial tail muscles (Murray et al. 2010; Rank et al. 2010). Recordings were also made from ventral roots of isolated normal and chronic injured spinal cords *in vitro* (Li et al. 2004a; Li et al. 2004b). All procedures were approved by the University of Alberta animal welfare committee.

4.2.1 Histology

Sacral spinal cord tissue from chronic spinal rats and age-matched uninjured rats (see above) was labelled for dopamine-beta-hydroxylase (DβH). Horizontal sections of the sacral spinal cord were cut to 20 μM, mounted on slides and incubated at 37° C for 30 mins, followed by PBS wash (Sigma-Aldrich, Oakville, ON). The slides were then immersed for 20 mins in 1% sodium borohydride in 0.1 M Na_3PO_4 (Sigma-Aldrich, Oakville, ON), followed by a thorough washing in PBS. The slides were then incubated in a citrate buffer with a pH of 8.5 at 80° C (Sigma-Aldrich, Oakville, ON) for 30 mins. Following this incubation the tissue was allowed to cool to room temperature, was washed in PBS and then incubated

in 5% rabbit serum + 0.3% Triton-X in PBS for 2hrs at room temperature. Tissue was then washed in 2% rabbit serum + 0.3% Triton-X dissolved in PBS.

The primary Sh anti DβH antibody (1:100; Abcam Inc., Cambridge, MA) in a cocktail of 5% rabbit serum and 0.3% Triton-X in PBS was centrifuged for 10 mins at 4° C. The antibody was applied to the sacral spinal tissue for 48 hrs at 4° C. After incubation with primary antibody the slides were washed with PBS and incubated in Cy3 conjugated secondary antibody for 2 hrs at room temperature (RbxSh-Cy3 in PBS + 0.1% Triton-X 1: 200, centrifuged for 5mins; The Jackson Laboratory, Bar Harbor, ME). Finally, slides were washed in PBS. Serial dehydration of the tissue on the slides was then performed with increasing alcohol concentrations, followed by immersion in xylene for 2 mins. Coverslips were then mounted on the slides with permount.

4.2.2 Spastic tail motion tracking and kinematic measurements

To assess the effect of various drugs on spastic tail behaviour, tail spasms were quantified in chronic spinal rats by tracking the spastic movements and making kinematic measurements. Tails of rats were prepared with high contrast narrow black markings spaced evenly along the length of the tail, separated by 1 - 2 cm. The rats were housed in a Plexiglas tube with the tail protruding and allowed to hang freely. Cutaneous stimulation was then applied to the tail in the form of a standardized stretch/rub manoeuvre (see Bennett et al. 2004 for details). To quantify tail spasms, the high contrast black markings on the tail were tracked using custom written motion tracking and kinematic analysis software (MatLab 7.0.4, MathWorks, Natick, MA). Flexion angles, or clockwise movements of the tail, were quantified between each segment of the tail (defined as the space between each black marking on the tail) relative to the neighbouring segment (termed *relative flexion angle*). Relative flexion angles calculated for each tail segment were then summed to give a measure of total flexion in the tail (termed *spasm angle*), where increased flexion indicates a larger spasm. Spasm angles

were calculated for each frame of the video, at a rate of 60 fps, and averaged over a period of 5 mins beginning immediately after the cutaneous tail stimulation.

To evaluate the effect of increases in endogenous NA and adrenaline (AD) on spasms, tail spasticity was evaluated after exposure to acute immobilization stress. Forced immobilization of an animal is a very effective stressor and causes a massive increase in plasma concentrations of both AD and NA (Buhler et al. 1978; Kvetnansky et al. 1992; Kvetnansky et al. 1984; Kvetnansky et al. 1978). Awake chronic spinal rats were immobilized for 15 - 20 mins by taping all four limbs securely to a piece of cork board. Spasms were then elicited with the standardized stretch-rub manoeuvre and evaluated as described above. After the experiment, rats were lightly anesthetised with isofluorane anaesthetic so the immobilization restraints could be removed.

4.2.3 Percutaneous EMG reflex testing and averaging

To assess the role of various drugs on muscle spasms in vivo, spasms were also quantified in chronic spinal rats via percutaneous electromyography (EMG). The percutaneous EMG reflex testing protocol has been described elsewhere (Murray et al. 2010; Rank et al. 2010). Briefly, rats were housed inside a clear Plexiglass tube with the tail protruding and held horizontally by taping it to a bar. The tail was kept warm with a radiant heat lamp. Multi-stranded stainless steel wires insulated with PVC (AS631; Cooner Wire Inc., Chatsworth CA) were bared 1 cm at each end and inserted percutaneously into the axial muscles of the mid-tail. EMG electrode placement into the tail muscles was standardized using the 12^{th} coccygeal vertebra as a reference point. Recording electrodes were placed 1- and then 2 cm rostral to this point with the ground electrode placed 1 cm caudal to the reference point. Long-lasting spastic reflexes (termed *spasm EMG*) were elicited with two stimulating electrodes inserted percutaneously on the distal tip of the tail, separated from each other by 1.5 cm. Since the tip of the tail contains very little muscle, this method of stimulation provides relatively pure cutaneous

stimulation (Bennett et al. 1999; Bennett et al. 2004). To prevent movement of the wires, each wire was fixed to the skin using a small amount of cyanoacrylate glue. Spasms were evoked by single pulse stimulation (width 0.2 ms) at 10 mA (Isoflex Stimulator, AMPI; about 3 – 5 x T) every 10 s, and repeated 6 times. Spasms were recorded with the EMG wires using a custom built amplifier and Axoscope hardware and software (Digidata 1322A, Axoscope; Axon Instruments, Burlingame CA; amplified 2000 times, low pass filtered at 1000 Hz, high pass filter at 100 Hz and sampled at 5 kHz). The spasm EMG was further processed using custom written software (MatLab 7.0.4, MathWorks, Natick, MA). EMG signalswere high pass filtered (at 800 Hz, using a 1^{st} order Butterworth filter), rectified and then averaged over a time window 500 - 4000 ms post stimulation to quantify the spasm EMG activity. This spastic reflex period has previously been shown to result mainly from a sustained depolarization of the motoneurons by the low-voltage activated Ca^{2+} component of the PIC (Bennett et al. 2004; Li et al. 2004a).

4.2.4 *In vivo* drug injection

All drugs were administered *in vivo* via intraperitoneal (IP) or transcutaneous intrathecal (IT) injection. The transcutaneous intrathecal injection procedure has been previously described elsewhere (Mestre et al. 1994; Rank et al. 2010). To summarize, while under light isoflurane anesthesia, a 25 - guage x 1 - inch needle, connected to a 100 μL glass Hamilton syringe was inserted into the tissues between the L_5 and L_6 vertebrae on the dorsal side, perpendicular to the spinal column of the chronic spinal rat. This injection site was selected because of easy intervertebral accessibility as well as a reduced possibility of spinal cord damage, since the injection site is restricted to the area near the cauda equina. As the needle entered the spinal canal the tail would produce an abrupt lateral twitch, caused by the needle entering the proximity of the ventral roots, and this sign was used to positively confirm the injection site. The drug solution was slowly injected over about 5 s. All drugs were dissolved in sterile saline at a constant

volume of 30 μL for each IT injection. Rats woke up within minutes of removal of light anaesthetic, at which point reflex testing resumed. Neither the anaesthetic nor the saline vehicle influenced the reflexes, as tested by control saline IT injections. Animals received multiple intrathecal injections per experimental session, up to a maximum of four injections, with at least 90 mins separating each injection. Drugs used for *in vivo* experiments included NA, AD, RX821002, amphetamine (Sigma-Aldrich, Oakville, ON), reboxetine, tetrabenazine, and prazosin (Tocris Biosciences, Ellisville, MO).

NA and AD each have affinity for both excitatory α_1 adrenergic receptors, and inhibitory α_2 adrenergic receptors. Activation of the central α_2 adrenergic receptor in chronic spinal rats has previously been shown to inhibit sensory synaptic transmission to the motoneuron by decreasing the polysynaptic EPSP, ultimately reducing motoneuron excitability and causing confounding effects on spasms (Rank et al. 2010). To isolate the effect of NA and AD on central α_1 receptors, central α_2 receptors were first blocked with a peripheral injection of the α_2 receptor antagonist RX821002 (1-3 mg/Kg, IP injection). This dose is known to effectively block only central α_2 receptors, as IP injections of RX821002 reduce spasms *in vivo*, and leave central α_1 receptors unaffected, since local IT injection of the α_1 agonist A61603 facilitates spasms even after injection of RX821002 (Rank et al. 2010).

Importantly, since RX821002 was injected systemically in our experiments, peripheral tissues were exposed to a higer concentration of the drug, and this higher peripheral concentration effectively blocked both peripheral α_1 and α_2 adrenergic receptors. This is fortunate because peripherally injected NA and AD can activate vascular α_1 adrenergic receptors causing vasoconstriction and increased blood pressure (Docherty, 1998), resulting in confounding effects on spasms. The dose of RX821002 used in our experiments was able to block peripheral NA mediated increases in blood pressure (4/4 rats tested). Therefore this dose of RX821002 (1 -3 mg/Kg) blocks both α_1 and α_2 adrenergic receptors

peripherally, but only α_2 receptors centrally, thereby allowing us to investigate the effects of peripherally injected NA on spasms, in the absence of confounding blood pressure changes.

4.2.5 *In vitro* preparation

Details of the *in vitro* preparation have been previously described in detail (Li et al. 2004a; Li et al. 2004b), and are only briefly summarized here. Rats were deeply anaesthetized with urethane (0.18 g/100 g; with a maximum dose of 0.45 g) and the whole sacrocaudal spinal cord was removed and transferred to a dissection chamber filled with modified artificial cerebrospinal fluid (mACSF) maintained at a constant temperature of 20° C. To remove the cord in chronic spinal rats a transection was made just above the chronic injury (at upper S_2 level). In normal adult rats the cord was cut at the same location (upper S_2) for removal, and they are therefore termed acute spinal rats. All dorsal and ventral spinal roots were removed, with the exception of the sacral S_4 and caudal Ca_1 ventral roots and the Ca_1 dorsal roots. The cord was then allowed to rest in the dissection chamber for 1.5 hrs. Following this rest period, the cord was transferred to a recording chamber containing continuously flowing normal artificial cerebrospinal fluid (nACSF) maintained near 24° C and with a flow rate > 5 mL/min. Following a 60 min nACSF wash out period to clear any residual anaesthetic and mACSF, the nACSF was recycled in a closed system with a peristaltic pump.

4.2.6 Ventral root reflex recording and averaging

A detailed description of these procedures can be found in Li et al. (2004b). Chronic spinal rats exhibiting clear spasticity were used as well as normal adult rats. Briefly, two dorsal roots (left and right Ca_1) and two to four ventral roots (left and right S_4 and/or Ca_1) were mounted on chlorided silver wires suspended above the ACSF of the recording chamber for monopolar stimulation and

recording, respectively. The roots were wrapped around the wire in the air and then covered with a 1:1 mixture by weight of petroleum jelly/mineral oil. Leak of the mixture into the bath was prevented by surrounding the oil-covered roots with a bead of high vacuum grease (Dow Corning Corp.). Ventral root reflexes were recorded in response to a single low threshold afferent pulse (0.1 ms, 0.02 mA; Isoflex stimulator, AMPI) to the dorsal root (2 - 3 times threshold, T ~ = 0.007 - 0.01 mA). Dorsal root stimulation was repeated more than five times consecutively with an inter-stimulus interval of 10 s to provide multiple ventral root reflexes for averaging. Ventral root reflexes were recorded via a custom built differential preamplifier, with one lead connected to the root and the second to the reference wire in the ACSF [high pass 100Hz; low pass 3kHz; amplified by 2000 times; sampling rate 6.7 kHz (Axoscope 8, Axon Instruments)].

Ventral root reflexes were quantified using custom written software (MatLab 7.0.4, MathWorks, Natick, MA), identical to the software used for *in vivo* percutaneous EMG analysis. Data were rectified and then filtered in the same manner as for spasm EMG (see above). Only the long lasting tonic response (termed *long-lasting reflex* or *LLR*) was quantified and used as a measure of motoneuron excitability (Bennett et al. 2004; Li et al. 2004a). The LLR was computed by averaging the rectified data in a window beginning 500 ms after the stimulation and lasting 3500 ms (see LLR, Fig 4-2B). This long-lasting reflex period has been shown to result mainly from sustained a depolarization of the motoneurons by just the Ca PIC (Bennett et al. 2004; Li et al. 2004a). Ventral root LLRs were recorded in this manner every 10 - 12mins. Where drugs were used, they were added to the bath immediately after a recording so as to ensure the actions of the drug could be recorded at the subsequent 10 – 12 min interval.

4.2.6.1 Drugs and Solutions

Two kinds of artificial cerebrospinal fluid were used in these experiments; a modified ACSF (mACSF) used during dissection and recovery to minimize neural

and metabolic activity and a normal ACSF (nACSF) in the recording chamber. The mACSF was composed of (in mM) 118 NaCl, 24 $NaHCO_3$, 1.5 $CaCl_2$, 3 KCl, 5 $MgCl_2$, 1.4 NaH_2PO_4, 1.3 $MgSO_4$, 25 D-glucose, and 1 kynurenic acid. Normal ACSF was composed of (in mM) 122 NaCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 3 KCl, 1 $MgCl_2$, and 12 D-glucose. Both types of ACSF were saturated with 95% O_2 - 5% CO_2 and maintained at pH 7.4. Strychnine hydrochloride (Sigma-Aldrich, Oakville, ON) was used for some acute spinal animals in order to reduce glycinergic inhibition thereby increasing motoneuron excitability to near threshold levels. Other drug used include methoxamine hydrochloride (Sigma-Aldrich, Oakville, ON), reboxetine mesylate (Tocris Biosciences, Ellisville, MO), amphetamine and Recordati15/2739 (abbreviated REC15/2739, generously provided by Recordati, Milan, Italy).

4.3 RESULTS

4.3.1 The enzyme responsible for NA synthesis is absent below a chronic spinal transection

Spontaneous activity at the α_1 adrenergic receptor in awake chronic spinal rats is, in part, the result of the activation by an endogenous ligand, likely NA (Harvey et al. 2006b; Rank et al. 2010). In light of this, we began our study with an immunohistochemical analysis of the presence of DβH, the enzyme responsible for the biosynthesis of NA from dopamine (DA), in the spinal cord below a chronic transection. In the normal, uninjured spinal cord DβH positive fibres were visible throughout the tissue in the sacral spinal cord (Fig 4-1A; sacral S_4 level shown; in n = 4/4 rats tested). Particularly dense DβH staining was also visible at blood vessels (Fig 4-1; labelled bv), likely due to sympathetic innervation present at all blood vessels. However, no DβH positive fibres were visible (Fig 4-1B; sacral S_4 level shown) below a chronic transection at sacral level S_2, and there was a reduction in DβH staining at blood vessels compared to uninjured spinal cord tissue (Fig 4-1A; in n = 4/4 chronic spinal rats). The reduced presence and density of DβH fibres appears to be localized to spinal cord tissue below a chronic spinal transection, as DβH positive fibres were still visible above the transection (Fig 1C; sacral S_2 level shown; in n = 4/ 4 rats tested) with a density comparable to that seen in the normal spinal cord (Fig 4-1A). The relative absence of DβH positive fibres indicates that NA is unable to be synthesized below a chronic spinal transection, and therefore the endogenous NA activating the α_1 receptor is unlikely to originate in the spinal cord below a chronic transection.

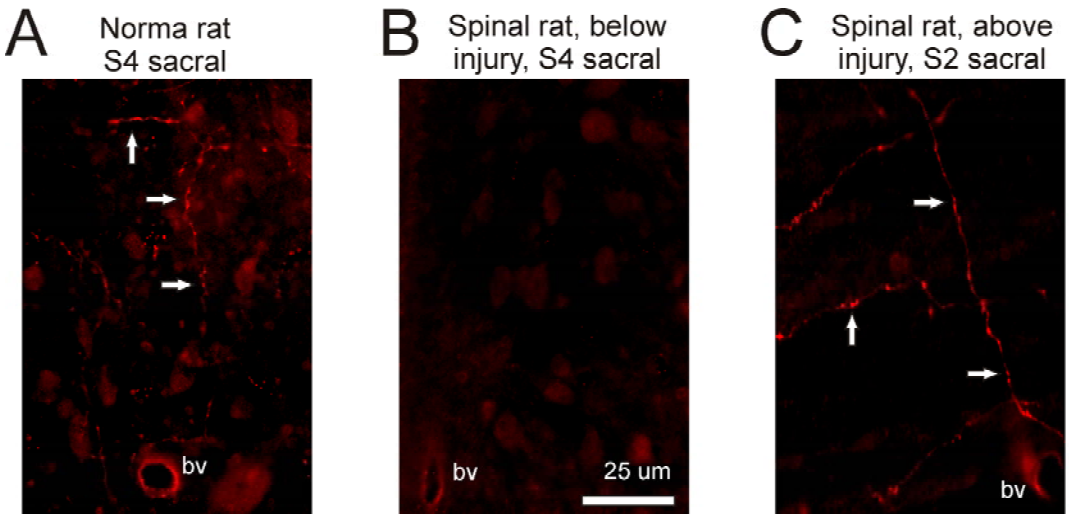

Figure 4-1

Noradrenaline is not synthesized below a chronic spinal transection. Representative immunofluoresence images of the enzyme dopamine-β-hydroxylase (DβH), responsible for the conversion of dopamine to noradrenaline (NA). **(A)** In normal, uninjured rat spinal cord at S_4 level (n = 4 rats) there is a well defined network of DβH positive fibres (beaded, at arrows) where NA synthesis takes place. DβH staining was especially bright at blood vessels (bv). **(B)** Below a chronic sacral spinal transection (S_4 level; n = 4 rats), lack of DβH positive NA synthesising fibres. DβH staining at blood vessels was diminished. **(C)** Above a chronic sacral spinal transection, (S_2 level; n = 4 rats) DβH fibres were well defined (beaded, at arrows), and staining at blood vessels was bright (bv).

4.3.2 Peripheral NA crosses the BBB and acts on central α_1 adrenergic receptors in chronic spinal rats

Previous data have shown that endogenous activation of the α_1 adrenergic receptor by NA (or another ligand) occurs only in the awake chronic spinal animal (*in vivo*), and not in the isolated spinal cord (*in vitro*) (Rank et al. 2010). Since we found no evidence of NA producing fibres below a chronic transection, these data suggest that the ligand (NA) may originate in the periphery of the animal and somehow crosses the BBB to act on central α_1 adrenergic receptors. To investigate this possibility we tested whether a peripheral/systemic injection of NA can facilitate spasms, and if this facilitation can be blocked by blocking central α_1 adrenergic receptors. Since activation of central α_2 adrenergic receptors can have confounding effects on spasms (Rank et al. 2010), experiments were conducted with these receptors blocked by an intraperitoneal (IP) injection of the α_2 receptor antagonist RX821002. We found that a peripheral IP injection of NA (abbreviated pNA) caused a significant increase of more than 400% in the tail spasm angle evoked by cutaneous stimulation and measured by kinematic analysis (Fig 4-2C). Blocking central α_1 adrenergic receptors first with intrathecal (IT) injection of the α_1 adrenergic receptor antagonist prazosin blunted the pNA-mediated facilitation of spasms (Fig 4-2A, C). A similar significant increase in spasms was evoked by brief cutaneous stimulation at the tip of the tail (0.2 ms pulse at 10 mA) and recorded with percutaneous EMG placed in the axial muscles of the midtail after IP injection of NA (Fig 4-2B, D). Blocking central α_1 adrenergic receptors with IT injection of prazosin alone significantly reduced spasm EMG (Fig 4-2D), confirming that the α_1 adrenergic receptor is endogenously activated. However, this reduction with prazosin alone was small (20%), and cannot account for the > 50% reduction seen when pNA mediated spasms are blocked with prazosin. Since, like NA, AD is prevalent in the periphery and also shows a strong affinity for adrenergic receptors (Schwinn et al. 1995; U'Prichard et al. 1977), we tested whether peripherally injected AD (abbreviated pAD) could affect spasms. We found that, similar to pNA, pAD

FIGURE 4-2

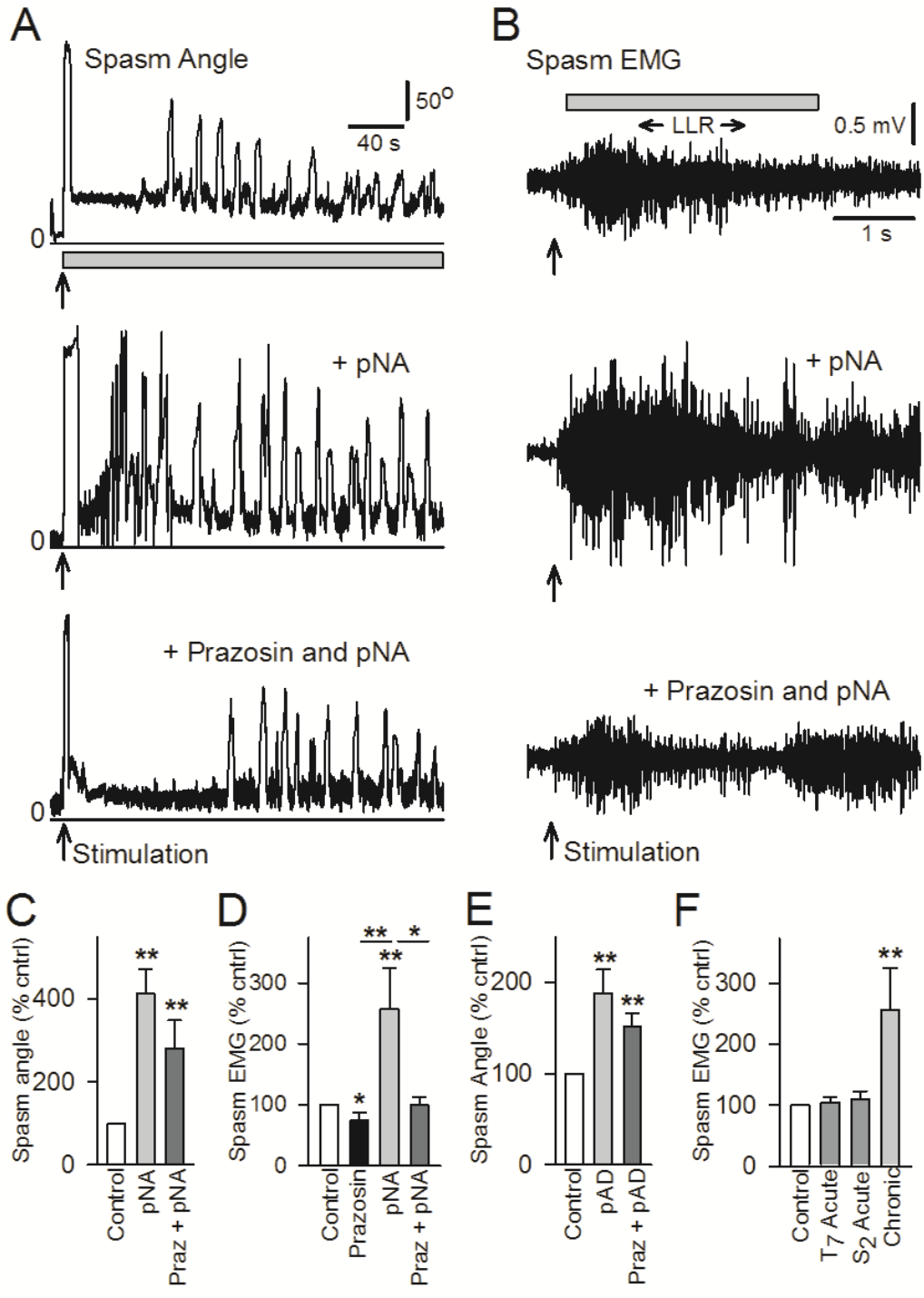
A
Spasm Angle
50°
40 s
0
+ pNA
0
+ Prazosin and pNA
0
Stimulation
B
Spasm EMG
LLR
0.5 mV
1 s
+ pNA
+ Prazosin and pNA
Stimulation
C
Spasm angle (% cntrl)
400
200
0
**
**
Control
pNA
Praz + pNA
D
Spasm EMG (% cntrl)
300
200
100
0
**
**
*
*
Control
Prazosin
pNA
Praz + pNA
E
Spasm Angle (% cntrl)
200
100
0
**
**
Control
pAD
Praz + pAD
F
Spasm EMG (% cntrl)
300
200
100
0
**
Control
T7 Acute
S2 Acute
Chronic

Figure 4-2

Peripheral NA injection facilitates spasms by activating central α_1 adrenergic receptors. **(A)** Flexion spasm in the tail of awake chronic spinal rat elicited by cutaneous stimulation of the tail and quantified by motion tracking (spasm angle, quantified during horizontal grey bar) after stimulation (at arrow) Measured under control conditions (upper plot), after peripheral intraperitoneal (IP) injection of NA (0.03 mg/Kg; middle plot) and after blocking the action of peripheral NA centrally with prior intrathecal (IT) injection of α_1 adrenergic receptor antagonist prazosin (1 mM in 30 µl; lower plot). Increased spasm angle indicates greater spasticity. **(B)** Same as (A) but instead, long-lasting spastic reflex in awake chronic spinal rat evoked by electrical/cutaneous stimulation of the tail (0.2 ms pulse 10 mA) and recorded with tail muscle EMG. Spasm EMG quantified during horizontal grey bar (0.5 – 4 s post stimulus). Spasm EMG epoch is identical to the time period when long-lasting reflexes (LLR) recorded from the ventral roots of in the isolated *in vitro* spinal cord, and triggered by dorsal root stimulation (single pulse, 3 x T) are quantified (LLR, quantified 0.5 – 4 s post stimulus; counterpart of spasms). Normalized group mean for increase in spasm angle **(C)** ($n = 7$) and spasm EMG **(D)** ($n = 6$) in awake chronic spinal rats with peripheral IP injection of NA (abbreviated pNA; 0.03 mg/Kg IP) after blocking the action of peripheral NA centrally with prior IT injection of prazosin (abbreviated Praz + pNA, 1 mM in 30 µl), and after IT injection of prazosin alone (1 mM in 30 µl; $n = 9$). **(E)** Normalised group mean of spasm angle in awake chronic spinal rat after peripheral IP injection of adrenaline (abbreviated pAD, 0.03 - 0.3 mg/Kg IP; $n = 6$) and after blocking the action of peripheral AD centrally with prior IT injection of prazosin (1 mM in 30 µl; $n = 6$). **(F)** Normalized group mean of spasm EMG in acute T_7 spinal rats (abbreviated T_7 acute; $n = 5$), acute S_2 transected rats (abbreviated S_2 acute; $n = 6$) and chronic S_2 transected rats (abbreviated chronic; $n = 6$) with peripheral IP injection of NA (0.03 mg/Kg). All recordings were made in the presence of RX821002 (IP injection; 1 mg/Kg) to prevent involvement the α_2 adrenergic receptor. * $P < 0.05$, ** $P < 0.01$. Error bars, s.e.m.

injection significantly increased spasm EMG in chronic spinal rats and blocking central α_1 receptors first with prazosin blocked the effects of pAD (Fig 4-2E). Overall these results demonstrate that, after chronic SCI, both NA and AD from the periphery are able to cross a compromised BBB and activate central α_1 receptors to facilitate spasms.

4.3.3 Peripheral NA does not cross the BBB in acute spinal rats

The BBB is known to be compromised chronically after SCI (Popovich et al. 1996), but how soon after injury these permeability changes take place is unclear. To test whether the BBB is compromised acutely after injury, and to determine the longitudinal extent of damage to the BBB post-injury we quantified the effects of pNA on cutaneously evoked tail spasms in animals with injuries near the motoneurons innervating the tail (low sacral S_2 injury; termed S_2 Acute), and those with transections farther away from tail motoneurons (mid-thoracic T_7 injury; termed T_7 Acute). Neither rats with acute S_2, nor those with acute T_7 transections showed any changes to spasm EMG in response to the same low dose of pNA used in chronic spinal animals (0.03 mg/Kg of pNA; Fig 4-2F). Acutely lesioned animals only showed significant increases in spasm EMG after a very high dose of pNA (0.3 mg/Kg, T_7 Acute: 243.76 ± 18.06%, S_2 Acute: 235.01 ± 27.57% ; $P > 0.05$, $n = 5$ each, data not shown), which demonstrates that they are at least capable of showing increases in reflexes. Therefore, in the acute stages of injury (1 – 2 days post transection) the BBB is relatively impermeable to peripheral NA, either when the lesion is located near (S_2) or further away (T_7) from the tail motoneurons. This is consistent with previous reports of BBB being impermeable to NA in normal rats (Weil-Malherbe et al. 1959; Weil-Malherbe et al. 1961; Whitby et al. 1961). The facilitation of spasms seen with high doses of NA is likely caused by changes in BBB permeability induced by the extremely high dose of pNA administered (see Discussion).

4.3.4 Blocking NET has inconsistent effects on spasms and blocks pNA mediated effects

Since NA originating in the periphery does not normally cross the BBB, we wanted to understand the mechanism by which peripheral NA is able to reach central α_1 adrenergic receptors in chronic spinal rats. To do this, we tested whether blocking the classical NA transporter (NET) had any effect on the increase in spasms by pNA. In awake chronic spinal rats, blocking the NET with reboxetine (IP) by itself did not significantly change the cutaneously evoked tail spasm angle (Fig 4-3A middle panel, 4-3B). However, when NET was blocked with reboxetine, subsequent peripheral injections of NA (pNA) no longer facilitated these cutaneously evoked spasm angle measurements (Fig 4-3A bottom panel, B), whereas without reboxetine the same pNA injection caused a 400% increase in spasms (detailed above). These results were confirmed with EMG measurement of spasms evoked by cutaneous stimulation of the tip of the tail, with reboxetine again blocking the pNA-induced increase in EMG spasms (0.2 ms pulses at 10 mA; $n = 2$; data not shown). This suggests that the NET is involved in the process by which pNA is actively transported across the compromised BBB below the lesion in chronic spinal rats.

Initially, we found it difficult to reconcile that reboxetine itself did not effect spasms, since the drug should block reuptake and thereby increase endogenous peripheral NA (Eisenhofer 2001; Hoffman et al. 1998; Sacchetti et al. 1999; Wong et al. 2000). However, we now realize that this is because reboxetine has two opposing effects: 1) blocking reuptake and thereby increasing endogenous peripherally derived NA and 2) blocking the transport of endogenous peripheral NA into the spinal cord. These two opposing roles of reboxetine cause conflicting effects, which likely explains the high variability in the effects of reboxetine alone, which generally increases spasms, but not significantly due to the variability (Fig 4-3B).

FIGURE 4-3

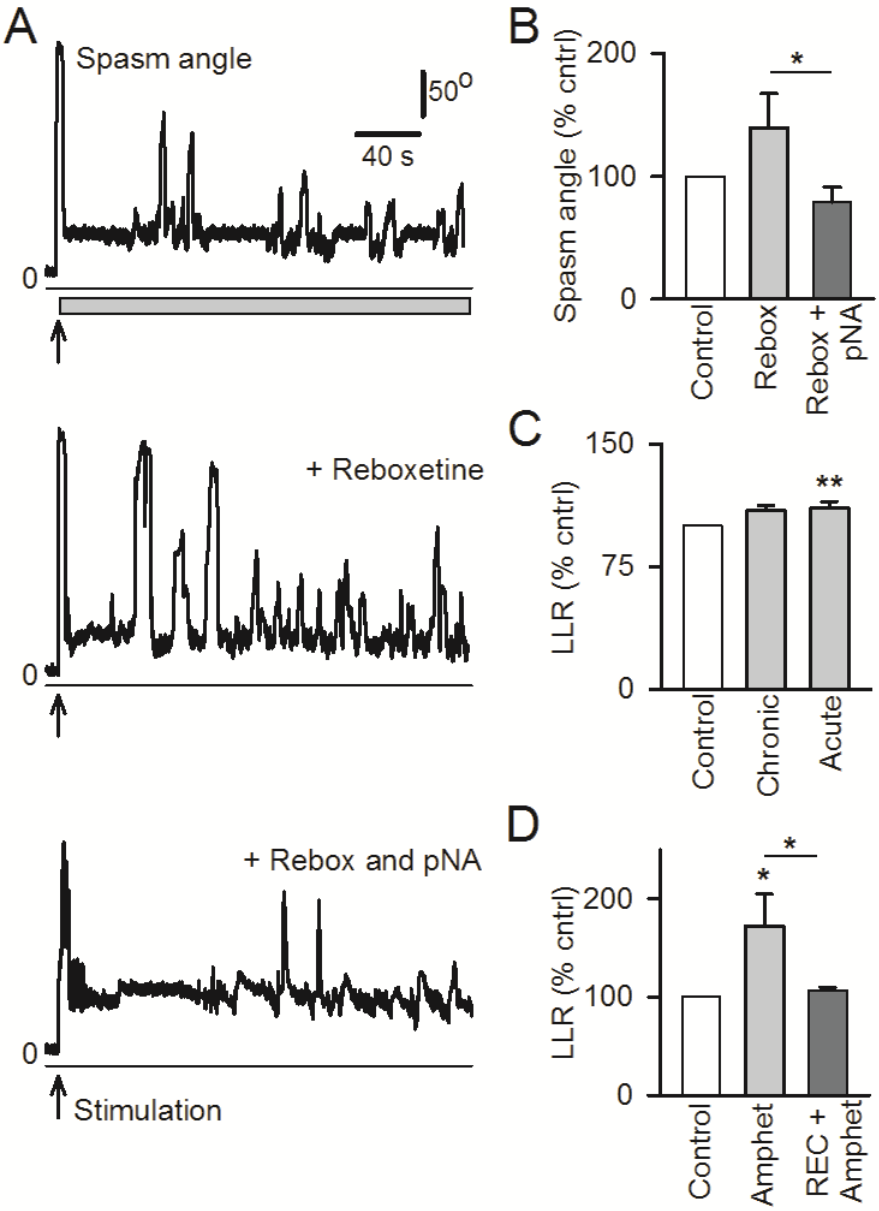
A
Spasm angle
50°
40 s
0
+ Reboxetine
0
+ Rebox and pNA
0
Stimulation
B
Spasm angle (% cntrl)
200
100
0
*
Control
Rebox
Rebox + pNA
C
LLR (% cntrl)
150
75
0
**
Control
Chronic
Acute
D
LLR (% cntrl)
200
100
0
*
*
Control
Amphet
REC + Amphet

Figure 4-3

Blocking the noradrenaline transporter (NET) has an inconsistent effect on spasms, and blocks peripheral NA-mediated facilitation of spasms. **(A)** Spasm angle in awake chronic spinal rat elicited by cutaneous stimulation of the tail (at arrow; quantified for 5.5mins after stimulation, as in Fig 4-2) under control conditions (upper plot), after treatment with the potent NET blocker, reboxetine (IP injection, 10 mg/Kg) (middle plot), and subsequent injection of peripheral NA (IP NA injection, 0.03 mg/Kg) (lower plot). **(B)** Normalised group mean of increased spasms angle in awake chronic spinal rat with IP injection of reboxetine (abbreviated Rebox, 5 – 10 mg/Kg; $n = 11$), and subsequent peripheral IP injection of NA (abbreviated Rebox + pNA; 0.03 mg/Kg, $n = 6$). **(C)** Long-lasting reflex triggered by dorsal root stimulation (single pulse, 3 x T) and recorded from the ventral roots (LLR, quantified 0.5 – 4 s post stimulus) in the isolated *in vitro* spinal cord of chronic ($n = 29$) and acute ($n = 11$) S_2 transected rats before and after application of reboxetine (bath application, 10 μM). Control values for acute rats were taken in strychnine (3 μM) to produce a similar LLR to that under untreated control condition in chronic spinal rats. **(D)** Normalized group mean for LLR in isolated *in vitro* spinal cord of chronic spinal rats after a forced endogenous NA efflux elicited by amphetamine (bath application, 0.1 μM; n = 12), and after blocking the action of endogenous NA with application of the α_{1A} neutral antagonist REC15/2739 (abbreviated REC; 10 μM, $n = 15$). All recordings in A- C were made in the presence of RX821002 (*in vivo*: IP injection, 1 mg/Kg; *in vitro*: 0.5 - 1 μM) $^*P < 0.05$, $^{**}P < 0.01$. Error bars, s.e.m.

We next investigated the presence and function of the NET in the isolated spinal cord *in vitro*, considering that it may be involved in uptake of NA into cells that handle peripheral NA after it crosses the BBB. To do this we examined long-lasting reflexes (LLR) evoked by brief dorsal root stimuli (3 x T), which are the *in vitro* equivalent of tail spasms. Bath application of reboxetine caused a small increase to LLRs that was not significant in chronic spinal rats, and only significant in acute spinal rats (increase of 10%; Fig 4-3C). Since blocking NA reuptake with reboxetine should only enhance the action of any spontaneously released NA, this result demonstrates that only a very small amount of NA is leaked spontaneously in the isolated spinal cord of chronic spinal rats. The positive action of reboxetine in acute spinal rats is consistent with the large stores of NA sequestered in the spinal cord of normal rats which likely remain after acute transection and thus result in an increased spontaneous leak of NA. The efficacy of reboxetine in acute spinal rats also serves as a positive control, indicating that we used reboxetine at an adequate dose.

We also tested the drug amphetamine which has a high potency for the NET (K_i = 59 nM) (Andersen 1989) and, among other actions, reverses the NET and causes an active efflux of endogenous NA stores from cells, regardless of whether or not there is a passive spontaneous leak of the stored NA. We found that bath application of amphetamine caused a significant increase in LLR in chronic spinal rats. Furthermore, blocking α_{1A} adrenergic receptors with the highly specific neutral antagonist REC15/2739, which blocks only NA activation of the α_{1A} adrenergic receptor, prevented the facilitatory effects of amphetamine (Fig 4-3D). This demonstrates that reversing the NET *in vitro*, leads to an increased amount of endogenous NA, which then acts on α_{1A} adrenergic receptors to facilitate spasms. Importantly, this result also suggests that there is some endogenous store of NA caudal to a chronic transection which is able to be released in response to amphetamine, though it is not spontaneously released in the absence of amphetamine (resistant to reboxetine).

4.3.5 Depleting presynaptic NA reduces spasms

If a central store of peripherally-derived NA exists below a chronic transection, it seems likely that the NA is stored and protected inside vesicles within some presynaptic location. The central vesicular monoamine transporter ($VMAT_2$) is responsible for packaging monoamines into vesicles in preparation for exocytotic release, and so we targeted this transporter to investigate its role in the central storage and release of NA. Since all NA not protected inside a vesicle is quickly metabolised by monomine oxidase (MAO) inside the presynaptic terminal, blocking $VMAT_2$ leads to a presynaptic depletion of NA (Jurna et al. 1969; Pettibone et al. 1984; Reches et al. 1983; Tomlinson 1977). When chronic spinal rats were treated with the selective $VMAT_2$ inhibitor tetrabenazine (IP), cutaneously evoked spasm angles were significantly decreased (Fig 4-4A). Moreover, treatment with tetrabenazine prevented the amphetamine-mediated release of endogenous NA and the resulting facilitation of spasms normally seen with amphetamine (Fig 4-4B), presumably by the known depletion of NA stores by tetrabenazine. This demonstrates that in chronic spinal rats, endogenous NA is stored inside vesicles below the transection, likely in some intermediate intraspinal cell.

4.3.6 Blocking $VMAT_2$ blocks the facilitation of spasms by pNA

Although stores of NA exist below a chronic transection, we wanted to assess whether the peripheral NA that crosses the BBB is packaged into vesicles by $VMAT_2$. To do this we first depleted presynaptic terminals of NA by blocking $VMAT_2$ with tetrabenazine alone, then injected NA peripherally and evaluated the effect on tail flexion spasms. Blocking $VMAT_2$ with tetrabenazine in this way prevented the facilitation of spasms normally seen with pNA injection (Fig 4-4C, D). This suggests that peripherally derived NA must first be packaged into vesicles centrally by $VMAT_2$ before the NA can reach and act on central adrenergic receptors. These results indicate that classic exocytotic release of

FIGURE 4-4

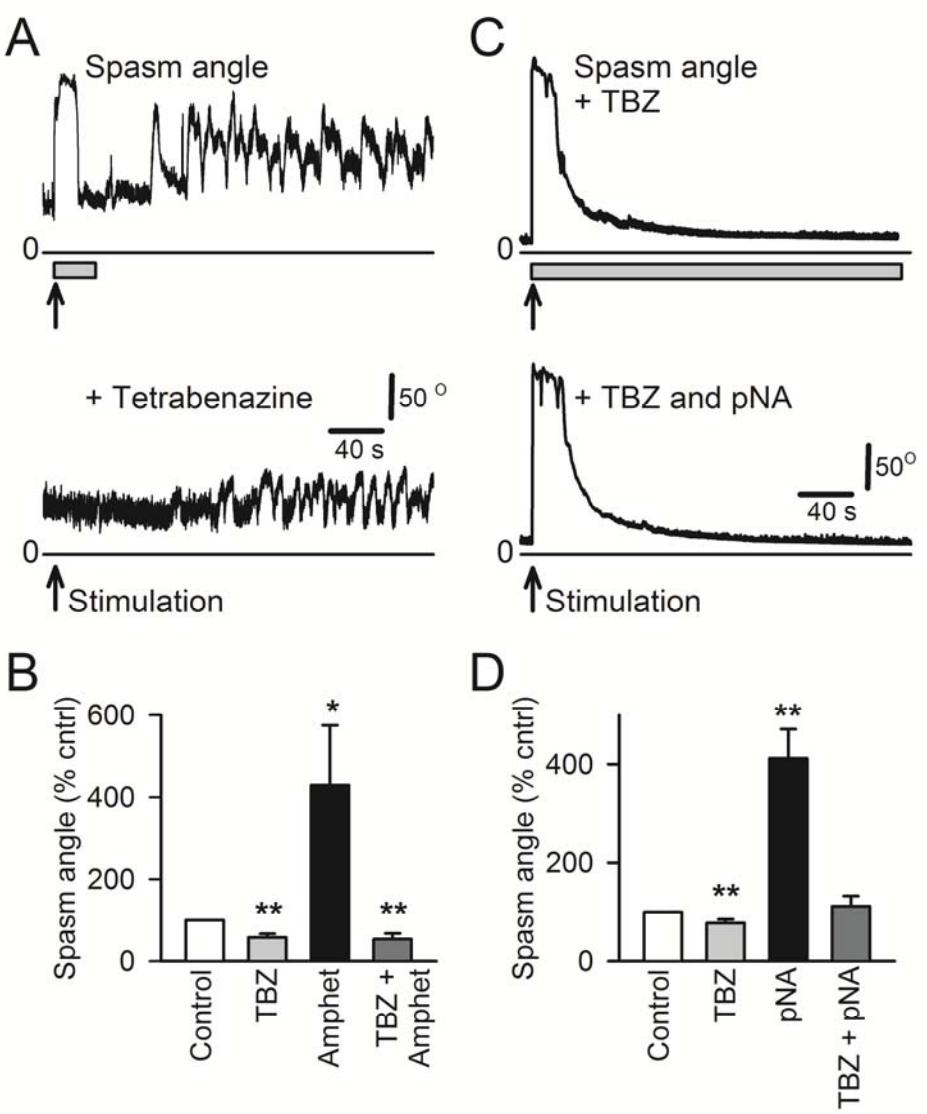
A
Spasm angle
0
+ Tetrabenazine
50 °
40 s
0
Stimulation
C
Spasm angle
+ TBZ
0
+ TBZ and pNA
50°
40 s
0
Stimulation
B
Spasm angle (% cntrl)
600
400
200
0
**
*
**
Control
TBZ
Amphet
TBZ + Amphet
D
Spasm angle (% cntrl)
400
200
0
**
**
Control
TBZ
pNA
TBZ + pNA

Figure 4-4

Blocking the vesicular monoamine transporter ($VMAT_2$) reduces spasms and blocks the peripheral NA-mediated facilitation of spasms. **(A)** Spasm angle in awake chronic spinal rat elicited by cutaneous stimulation of the tail and quantified for 20 – 30 s (horizontal grey bar) after stimulation (at arrow) before (upper plot) and after depletion of monoamines from presynaptic terminals with IP injection of the $VMAT_2$ blocker tetrabenazine (20 mg/Kg; lower plot). **(B)** Normalized group mean of spasm angle in awake chronic spinal rats quantified 20 – 30 s after cutaneous stimulation of the tail after depletion of presynaptic monoamine terminals with IP injection of tetrabenazine (abbreviated TBZ; 20 – 60 mg/Kg, $n = 13$), forced efflux of monoamines from presynaptic terminals by IP injection of amphetamine (abbreviated Amphet; 0.1 - 0.2 mg/Kg, $n = 13$) and depletion of monoamine terminals with tetrabenazine prior to IP injection of amphetamine (abbreviated TBZ + Amphet; $n = 5$). **(C)** Same format as (A), but spasm angle was quantified for 5.5 mins (horizontal grey bar, upper trace) after cutaneous stimulation of the tail (at arrow). Before (upper plot) and after blocking after blocking the action of peripheral NA (IP injection, 0.03 mg/Kg) by first blocking vesicular monoamine packaging with prior IP injection of tetrabenazine (50 mg/Kg). **(D)** Same format as (B) except spasms were quantified for 5.5mins after cutaneous stimulation of the tail after IP injection of tetrabenazine (20 – 60 mg/Kg, $n = 7$), peripheral IP injection of NA (abbreviated pNA; 0.03 mg/Kg, $n = 7$) and after blocking the action of peripheral NA with prior IP injection of tetrabenazine (abbreviated TBZ + pNA; $n = 4$). Recordings for IP injection of pNA alone, and IP injection of pNA after tetrabenazine were made in the presence of RX821002 (IP injection; 1 mg/Kg). $*P < 0.05$, $**P < 0.01$. Error bars, s.e.m.

these vesicles is a requirement for the pNA to facilitate spasms in chronic spinal rats.

4.3.7 Endogenous increase of peripheral NA by acute immobilization stress facilitates spasms

While we showed that peripherally *injected* NA and AD are able to cross the BBB and facilitate spasms, it remained unclear whether *endogenous* peripheral NA and AD has the same facilitating effect on motoneuron excitability and spasms. Accordingly, we investigated this by increasing the release of endogenous peripheral NA and AD, through exposure of chronic spinal rats to acute immobilization stress. This immobilization technique is a potent adrenomedullary activator, resulting in a dramatic increase in the plasma concentration of both NA and AD in rats (Goldstein and Kopin 2008; Kvetnansky et al. 1978). In our experiments, exposure of chronic spinal rats to identical acute immobilization stress to that used by Kvetnansky (1978) (for a period of 15 mins), resulted in a significant increase in spasm angles (Fig 4-5A). Interestingly, the increases in spasms measured after immobilization stress were greater than those seen after either pNA or pAD injections (Fig 4-5B). These results demonstrate that endogenous NA and AD originating in the periphery is able to cross the BBB and facilitate spasms in chronic spinal rats, though stress may have additional effects that make its action stronger than peripherally injected NA or AD.

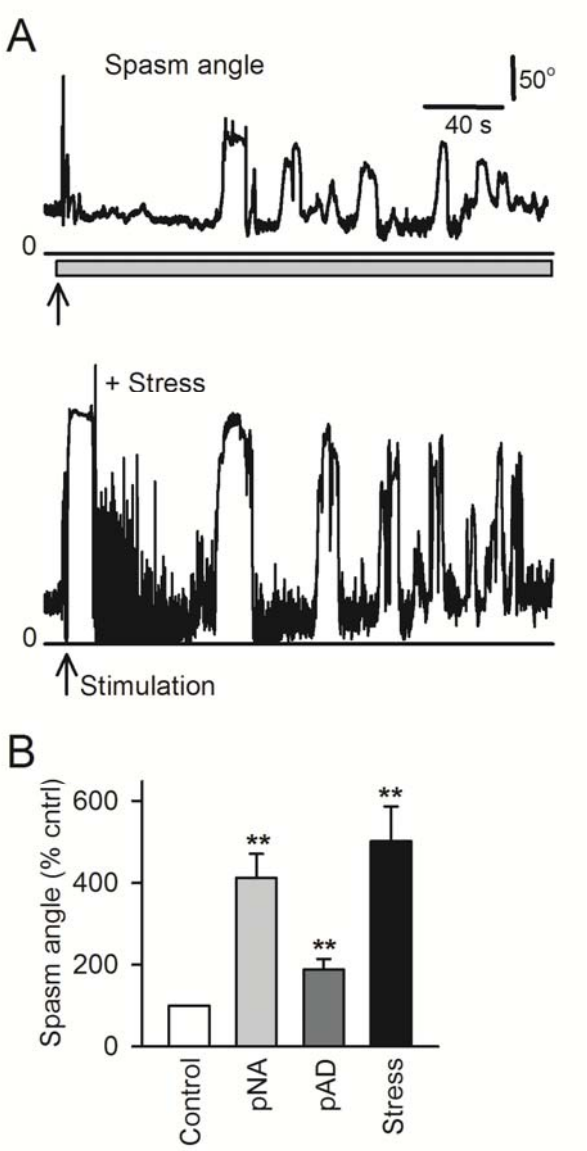

Figure 4-5

Increasing endogenous peripheral NA and AD via acute immobilization stress increases spasms. **(A)** Spasm angle in awake chronic spinal rat elicited by cutaneous stimulation of the tail (at arrow; quantified for 5.5mins after stimulation, horizontal grey bar) before (upper plot) and after increasing the release of endogenous peripheral NA and AD by exposing rats to acute immobilization stress (15 mins, lower plot). **(B)** Normalized group mean for spasm angle in awake chronic spinal rats after exposure to 15 mins of acute immobilization stress (abbreviated stress; $n = 5$), peripheral IP injection of NA (0.03 mg/Kg; $n = 7$) and peripheral IP injection of AD (0.03 - 0.3 mg/Kg, $n = 6$). All recordings were made in the presence of RX821002 (IP injection; 1 mg/Kg) $^{**}P < 0.01$. Error bars, s.e.m.

4.4 DISCUSSION

Although previous studies have suggested the presence of some residual NA below a chronic spinal transection (Rank et al. 2007; Rank et al. 2010), our study establishes for the first time that residual NA in the spinal cord below a chronic transection actually originates in the periphery. It passes from the blood to the spinal cord through a compromised BBB to act on central α_1 adrenergic receptors, thereby increasing motoneuron excitability and facilitating spasms in chronic SCI rats. Importantly, as experiments were conducted while the α_2 adrenergic receptors were blocked with the antagonist RX821002, our study focuses on the effects of pNA on central α_1 adrenergic receptors alone. Our data suggest that the NET and $VMAT_2$ transport proteins are critical for the facilitated transport of NA across the BBB into an instraspinal cell (via the NET) where the peripherally derived NA is packaged into vesicles (via $VMAT_2$) for storage and for exocytotic release, as discussed below and summarized in Fig 4-6.

4.4.1 NA is unable to be synthesised caudal to a chronic sacral transection

The small amount of residual NA that remains below a chronic spinal transection (1-5% of normal) (Cassam et al. 1997; Haggendal and Dahlstrom 1973; Magnusson 1973; Roudet et al. 1994; Roudet et al. 1993) has been reported to originate both from intraspinal neurons and from peripheral sympathetic fibres that enter the spinal cord alongside blood vessels and then become dissociated, but continue through the spinal cord tissue (Cassam et al. 1997; McNicholas et al. 1980). These studies, however, either utilised tissue from animals only up to 14 days after transection (Cassam et al. 1997), or employed a non-specific histofluoresence technique that does not differentiate between NA or DA fibres (McNicholas et al. 1980). This still leaves the origins of the residual NA below a chronic transection (> 60 days post injury) uncertain. A recent study by Takeoka et al. (2010), however, used DβH staining to show that NA axons can be found

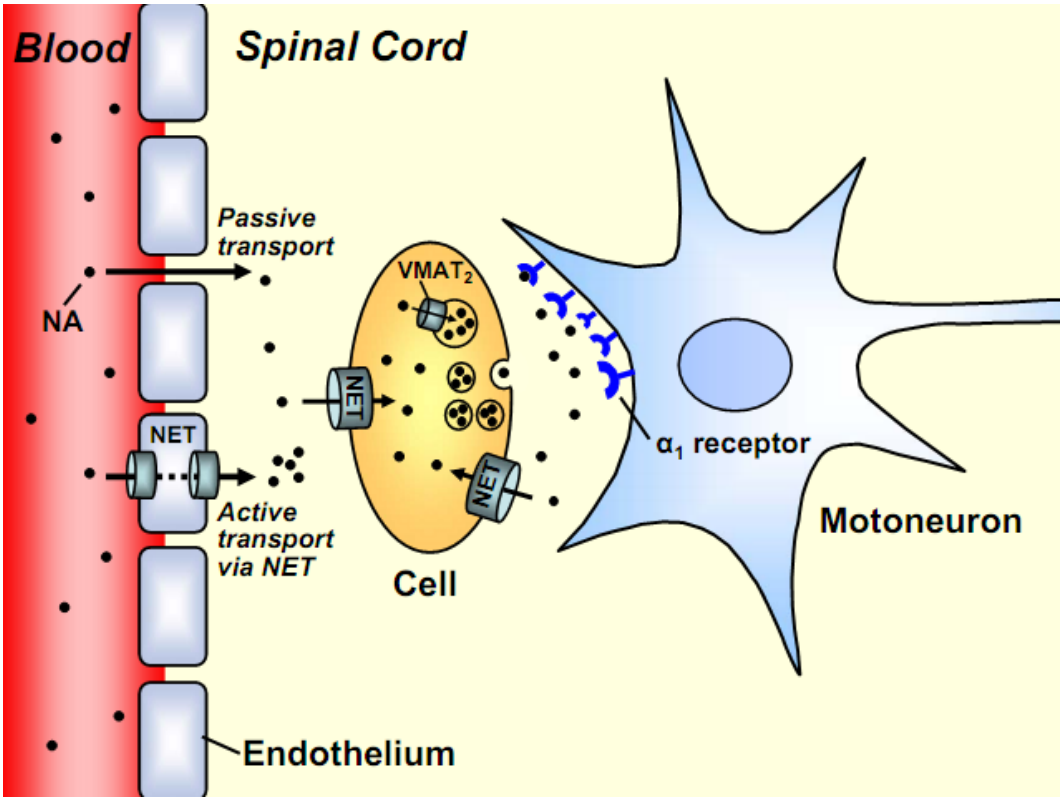

Figure 4-6

After chronic SCI, peripheral NA is transported across a permeable BBB and acts on central α_1 adrenergic receptors to facilitate spasms. A simplified schematic drawing detailing the process by which peripheral NA is transported across a permeable BBB to the spinal cord. NA, originally released by the adrenal glands, circulates peripherally in the bloodstream. Following injury to the spinal cord the tight junctions between the endothelial cells lining the microvessels of the CNS become dysregulated, leaving a physical gap in the BBB, allowing for the passive transport of peripheral NA across the BBB. NA from the periphery is also actively transported across the BBB by the membrane bound NA transporter (NET, blue cylinders on endothelium) expressed on the endothelial cells lining the microvessels. The NA is then taken up into an intraspinal cell, likely an AADC cell, which is known to contain the necessary transporter and packaging proteins. Once taken up into the cell, the NA is packaged into vesicles by the vesicular monoamine transporter ($VMAT_2$), and is then released exocytotically into the extracellular space where it binds to postsynaptic α_1 adrenergic receptors on nearby motoneurons. Any released NA that is not bound to adrenergic receptors is recycled back into the intraspinal cell by the NET, which is additionally located on the membrane of AADC cells. Activation of the α_1 adrenergic receptors by the synaptically released NA leads to increased motoneuron excitability and spasms in chronic SCI.

caudal to a chronic mid-thoracic transection (at T_9, up to 6 months post injury). These fibres enter the spinal cord from the periphery via dorsal and ventral roots, as well as alongside large diameter blood vessels, but the fibres were found only in very small quantities and the amount of NA released by these fibres was insufficient to yield any functional motor effects (Takeoka et al. 2010). In contrast, our study examined tissue from chronic S_2 transected rats and found no evidence of DβH labelling caudal to the transection, therefore indicating that NA is not produced below a chronic transection, at least in our model. We conclude that in our model of SCI, residual NA present caudal to a chronic transection is not intrinsic to the spinal cord and any endogenous NA present must originate from outside the CNS, likely from the periphery. This is in agreement with our previous study which demonstrates that an endogenous ligand, NA, activates central α_1 adrenergic receptors to facilitate spasms in the awake chronic SCI rat, but this endogenous NA is not present in the isolated chronic transected spinal cord maintained *in vitro*, where α_1 receptors are instead constitutively active (Rank et al. 2010).

4.4.2 The BBB is permeable to peripheral NA after injury

Under normal conditions, NA from the peripheral nervous system is prevented from crossing into the CNS by the BBB (Weil-Malherbe et al. 1959; Weil-Malherbe et al. 1961; Whitby et al. 1961), which principally functions to separate neurotransmitter pools that act centrally and those that act peripherally (Abbott et al. 2010; Abbott et al. 2006). The primary mechanism that accomplishes this separation is attributed to the endothelial cells lining all cerebral and spinal microvasculature, which form a physical barrier via their firm coupling with tight junctions to adjoining endothelial cells (reviewed in Abbott et al. 2006). The permeability of this physical barrier can be altered by exposure to very high doses of NA in uninjured animals. That is, high doses of NA injected directly into the cerebral ventricles causes increased pinocytosis in the endothelial cells, thus allowing the passage of substances normally confined to the blood, including NA,

into the CNS (Sarmento et al. 1991), consistent with our data where high doses of peripheral NA induce increased BBB permeability in acute spinal rats. Under normal conditions where NA concentrations are more physiological, such as our standard dose of peripheral NA (0.03 mg/Kg), the permeability of the BBB remains intact. The integrity of the tight junction assembly, however, is known to become compromised after trauma to the CNS, resulting in a physical gap between the endothelial cells lining the wall of CNS microvessels, and an observable permeability in the BBB (reviewed in Abbott et al. 2006). Our results, showing that peripherally injected NA is able to act centrally and increase spasms, are consistent with the presence of a physical gap in the BBB which allows the passive leak of NA from the periphery to the spinal cord.

The changes to the BBB after injury are known to occur as separate early (primary, acute) and delayed (secondary, chronic) events, where the spatial extent of increased permeability differs in each of these separate stages (Cohen et al. 2009; Popovich et al. 1996). In the acute stages of SCI (from 3 to 7 days post transection), increased BBB permeability is limited to the immediate vicinity of the injury (0.2 - 0.6 mm from the epicentre), primarily in the grey matter, and is largely resolved by 7 days post injury (Cohen et al. 2009; Liu et al. 2010; Lotocki et al. 2009; Popovich et al. 1996). This is consistent with our data which shows that acutely spinalized animals (1 – 2 days post transection) do not show increased motoneuron excitability and spasms in response to peripherally injected NA, regardless of whether the injury is within a few segments (at S_2; 5 - 10 mm away) of the S_4 tail motoneurons we recorded or much further away (T_7 transection). In either case, the S_4 motoneurons that generate tail spasms (Li et al. 2004b), are likely well outside of the highly localized area of increased BBB permeability present in the acute stages of injury.

A secondary widely distributed increase in BBB permeability develops beginning 14 days after transection, and remains detectable even 56 days after injury (Cohen et al. 2009; Popovich et al. 1996). In this secondary stage, permeability spreads

progressively from the epicentre of injury, up to 8 mm rostrally and caudally, and is especially high in the superficial white matter of the spinal cord (Cohen et al. 2009; Popovich et al. 1996). Since the chronic increase in BBB permeability extends several spinal segments, the area of increased permeability in our S_2 transected rats easily includes the motoneurons at S_4 involved in the generation of tail spasms. Our results, therefore, are consistent with the presence of a widespread and enduring disruption of the BBB which allows peripherally *injected* catecholamines, as well as *endogenous* peripheral NA and AD, to pass from the circulation to the spinal cord and thus influence motoneuron excitability caudal to a chronic spinal transection. However, as discussed below, peripherally injected NA acts centrally even when given at extremely low doses, suggesting that NA is additionally actively transported across the BBB (via the NET) and accumulated (via $VMAT_2$), likely within cells in the spinal cord which comprise the enzymatic component of the BBB (AADC cells, described below).

4.4.3 Active transport of NA across the BBB

The endothelial cells of the spinal microvasculature, in addition to comprising a physical barrier with tight junctions, also form a transport barrier composed of specific transporter proteins located on the membranes of endothelial cells (Abbott et al. 2010; Abbott et al. 2006; Cornford and Hyman 2005) which allow for transcellular traffic of certain molecules, such as NA via the transporter NET (Wakayama et al. 2002). The expression of the NET on endothelial cells has been described in the brain capillaries of mice (Wakayama et al. 2002), where the active uptake of NA, likely via the NET, has also been observed (Hardebo et al. 1980; Hardebo and Owman 1980; Spatz et al. 1981). This active transport of NA across the BBB is in agreement with our results which show that blocking the NET, with reboxetine, blocks peripheral NA-mediated spasms. It should also be noted that the dose of peripherally injected NA used in our study, which effectively modulates spasms in chronic spinal rats, is quite low (0.03 mg/Kg), especially given that the affinity of α_1 adrenergic receptors for NA itself is not

especially high (Ki = 17nM) (U'Prichard et al. 1977). If the injected peripheral NA were simply passively transported through a physical hole in the BBB (gaps in endothelial tight junctions), this would not likely result in the large α_1 receptor mediated increases to spasms that we report. It is more likely that the peripheral NA is actively transported across the compromised BBB, and concentrated in some central vesicular store which can then be released in a coordinated *en masse* fashion.

The NET localized at the BBB normally mediates the transport of peripheral NA from the brain back into the blood (Wakayama et al. 2002), ensuring that peripheral NA is expelled from the spinal cord. The NET, however, has been reported to operate according to a gradient, facilitating the transport of NA from areas of high concentration to areas of low concentration. For example, exposure to increased concentrations of NA has been shown to lead to a reversal of the NET in rabbit atria, where the transporter facilitates the efflux, rather than the normal uptake, of NA (Paton 1973). Since after chronic SCI there is a dramatic reduction in the concentration of NA in the spinal cord below a complete transection (Cassam et al. 1997; Haggendal and Dahlstrom 1973; Magnusson 1973; Roudet et al. 1994; Roudet et al. 1993), the concentration of NA in the periphery is substantially higher than in the spinal cord. This reversal of the normal concentration gradient below a chronic spinal transection may prompt the reversal of endothelial NET, thereby facilitating the transport of peripheral NA across the BBB, as seen in our study with chronic spinal rats (Fig 4-6). Conversely, in acutely transected rats the levels of NA in the spinal cord below the transection do not decrease until up to 4 days after transection (Dahlstroem and Fuxe 1965; Haggendal and Dahlstrom 1973; Magnusson 1973). This would cause a delay in the concentration dependent reversal of the NET, and therefore peripherally injected NA is unable to reach motoneurons caudal to the transection to facilitate spasms in acute spinal rats, as our results show. In summary, we suggest that after injury the NET, present on the endothelial cells of microvessels, reverses the direction of transport due to changes in the NA concentration

gradients, allowing peripheral NA to be actively transported and concentrated in the spinal cord (Fig 4-6).

4.4.4 Peripheral NA is accumulated and released by intraspinal cells

In addition to the physical and transport barrier properties provided by the endothelial cells, an enzymatic barrier also exists within the spinal cord composed of cells near the microvasculature that contain enzymes and transporters, and which are normally responsible for the uptake and inactivation of compounds (including NA) to further prevent their entry into the spinal cord tissue (Abbott et al. 2010; Abbott et al. 2006; Hardebo et al. 1979). Studies have identified a class of cells comprising part of this enzymatic barrier, located primarily near the central canal and near the capillaries of the spinal cord and brain, whose identifying feature is the presence of the enzyme aromatic amino acid-decarboxylase (AADC) (Hardebo et al. 1977; Hardebo et al. 1979; Hardebo and Owman 1980; Jaeger et al. 1983). This enzyme decarboxylates monoamine precursors and thus converts L-Dopa to DA and 5-HTP to 5-HT. These cells, termed AADC cells, although considered neurons (Jaeger et al. 1983; Karasawa et al. 2007; Mura et al. 2000), do not contain any other monoamine biosynthesis enzymes, such as tyrosine hydroxylase, and therefore cannot themselves synthesize monoamines from their precursor constituents (Jaeger et al. 1983). Studies examining the morphology of AADC cells confirm that they are likely small neurons which feature round, oval or fusiform somata and minimal dedritic processes that lack spines (Jaeger et al. 1983; Mura et al. 2000). In the striatum, and in other parts of the rat brain, the AADC neurons co-localize strongly with GABA, but not with other monoamine synthesis enzymes such as tyrosine hydroxylase (Mura et al. 2000).

AADC cells are known to be capable of the active transport and uptake of amines and precursors (Hardebo et al. 1977; Hardebo et al. 1979; Wade and Katzman 1975), likely including NA uptake via the NET. Furthermore, AADC cells have

also been shown to contain vesicles and form asymmetric synapses onto neighbouring axonal butons (Wade and Katzman 1975). These AADC cells, though themselves unable to synthesize monoamines, appear capable of the packaging of monoamines into vesicles (via $VMAT_2$) for concentrated storage within the cell and for subsequent exocytotic release of these stored monoamines (Jaeger et al. 1983). Our data support this role of the AADC cells, as blocking vesicular packaging of peripherally derived NA by $VMAT_2$, with tetrabenazine, blocks the normally facilitatory effect of peripheral NA on spasms in chronic spinal rats. Blocking vesicular packaging with tetrabenazine alone results in a progressive and significant decrease in spasms, suggesting that the peripheral NA must be packaged into vesicles inside these intraspinal cells before it can be released centrally (Fig 4-6). These cells, therefore, may be involved in the handling and storage of peripherally injected NA that we observe in our experiments.

To summarize, we suggest that our observations of the effects of peripheral NA on motoneuron excitability and spasms after SCI can be explained with the following overall model (Fig 4-6). After chronic transection of the spinal cord, peripheral NA crosses the BBB using two separate mechanisms: increased passive permeability (gaps between endothelial tight junctions) and active transport via the NET located in endothelial cells (as described above). The peripherally derived NA is then accumulated in intraspinal cells, likely AADC cells, with the NET mediating the uptake into the cells, and packaged into vesicles via $VMAT_2$ for concentrated storage and eventual exocytotic release to then activate α_1 adenergic receptors on motoneurons. Our data supports the presence of a separate intraspinal cell involved in the uptake and vesicular release of NA, rather than the microvasculature endothelium alone, since the expression of $VMAT_2$ on BBB endothelium cells has not reported and the endothelium has a low occurrence of vesicles involved in transport and release of substances across the endothelium (Reese and Karnovsky 1967; Sarmento et al. 1991) The endothelial cells likely cannot deliver the peripherally derived NA to the spinal cord to produce the

widespread activation of motoneurons that we see with peripheral NA in our experiments.

In our model the NET has a dual role: the active transport of peripheral NA across the BBB into the spinal cord, and the uptake of NA into intraspinal cells (AADC cells). This dual role of the NET is supported by our data which shows that, although blocking the NET with reboxetine clearly prevents peripheral NA-mediated spasms, reboxetine administered alone causes a general increase in spasms, but with a high degree of variability. This high variability we report likely results from reboxetine having multiple conflicting effects: 1) blocking the NET mediated entry of peripheral NA into the spinal cord (decreasing available NA) and 2) blocking reuptake of spontaneously released extracellular NA into intraspinal AADC cells (increasing available NA) (Fig 4-6). In the awake chronic spinal rat (*in vivo*) reboxetine simultaneously blocks the NET at two different loci (endothelium and intraspinal AADC cell) to both increase and decrease available NA, thus resulting in the inconsistent effects on spasms we see with reboxetine (Fig 4-6). In the isolated spinal cord (*in vitro*), however, reboxetine can only affect the NET located on intraspinal cells to block the reuptake of spontaneously released NA, and thereby increase the amount of synaptically available NA leading to increased spasms. Our finding that reboxetine does not affect spasms suggests that *in vitro* there is no spontaneous release (leak) of NA from the intraspinal AADC cells, or that the leak of NA is too small to detect. This is consistent with our previous finding that the α_1 adrenergic receptor neutral antagonist REC15/2739 does not affect spasms *in vitro*, indicating that there is no spontaneously leaked NA available to activate α_1 receptors below a chronic transection (Rank et al. 2010). Despite the lack of spontaneous release a substantial intracellular store of NA does exist below a chronic transection because a forced release of these stores with amphetmine increases motoneuron excitability and spasms *in vitro* (Rank et al. 2007) (Fig 4-6).

Although the identity of the intraspinal cell capable of the transport, storage and exocytotic release of peripherally derived NA below a chronic transection is unknown, AADC cells, which comprise part of the enzymatic component of the BBB, seem like a reasonable candidate, as we have described. In the uninjured spinal cord AADC cells likely act to take up amines and metabolise them via monoamine oxidase (MAO), thus preventing their entry into the spinal cord (Hardebo et al. 1980; Hardebo et al. 1979; Hardebo and Owman 1980), rather than releasing them. It is uncertain why the role of these cells changes with injury, but it is possible that the reduced quantity of monoamines caudal to the transection prompts an overall downregulation of MAO, including in the AADC cells below the transection, thereby preventing them from effectively metabolising amines such as NA. This may cause the cells to instead release NA exocytotically as the quantity of the transmitter builds up inside the cell. This is consistent with the observation that pargyline, an MAO inhibitor, does not affect spasms in chronic spinal rats (Bennett and Rank, unpublished observation).

4.4.5 Summary

Our study demonstrates for the first time that peripheral catecholamines, specifically NA and AD, either systemically injected or endogenously released by the adrenals into the plasma, are able to cross a compromised BBB and act on central α_1 adrenergic receptors to increase motoneuron excitability and spasms after chronic spinal cord injury. The peripheral NA and AD cross the BBB both passively, through gaps between endothelial tight junctions, and actively via the NET localized on the endothelial membranes. Our study also points to the presence of an intraspinal cell, likely the AADC cell, which expresses both the NET and $VMAT_2$ and which functions to take up, store and release the peripheral catecholamines centrally below a chronic spinal transection (Fig 4-6). The novel discoveries presented here have important implications for the understanding and treatment of spasticity after chronic SCI in patients. Particularly the possibility that other neuroactive substances may also pass through the compromised BBB,

either actively or passively, and thus significantly affect patients after CNS trauma.

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Chapter 5:

Discussion

5.1 The adrenergic system in the recovery of motoneuron excitability, sensory synaptic transmission and spasms after SCI

The projects contained in this thesis characterise the role of the adrenergic system in the recovery of motoneuron excitability and spasms after SCI and specifically describe several adjustments to the system that occur as a consequence of chronic injury. The thesis results firstly clarify the contributions of the α_1 and α_2 adrenergic receptors to spasms in chronic spinal rats. Whereas activation of the α_1 adrenergic receptor increases spasms through direct action on the motoneuron (increasing the Ca PIC), activation of the α_2 receptor modulates sensory transmission to the motoneuron and decreases spasms by decreasing the EPSP that triggers the PIC. Importantly, spontaneous activity at both α_1 and α_2 receptors in chronic SCI rats differs *in vivo* and *in vitro*. The α_1 receptor is endogenously active in both the isolated spinal cord *in vitro* as well as the awake animal *in vivo*, but via different mechanisms in each case. The α_1 receptor is active in the absence of a ligand *in vitro* and *in vivo* (constitutively active), but additional activation of the receptor *in vivo* results from the presence of an endogenous ligand. Conversely, the α_2 receptor is only spontaneously active *in vivo,* where the activity results from the presence of an endogenous ligand. Administration of amphetamine, which causes a forced efflux of endogenous NA and increases motoneuron Ca PIC and spasms, demonstrates that the endogenous ligand activating the adrenergic receptors *in vivo* is in fact NA. The final project of the thesis establishes that the endogenous NA originates in the periphery, but passes through a compromised BBB to act on central adrenergic receptors. After passing through the BBB, the peripheral NA is sequestered in vesicles contained in a central store which can then undergo exocytotic release, as is the case after amphetamine administration.

This work represents the first time that a comprehensive examination of how the adrenergic system contributes to spasticity after chronic SCI has been undertaken. The results stress the importance of the adrenergic system in increased

motoneuron excitability and loss of inhibitory control over sensory afferent transmission that develops in the months following spinal transection. The results of the thesis also provide considerable support for the original hypothesis which, in general, theorized that some residual source of endogenous NA persists below a chronic transection and contributes to spasms by activating central adrenergic receptors. The most substantial, and surprising, finding of the thesis was that this residual endogenous NA originates in the periphery. This, alongside the comprehensive characterization of the function of both α adrenergic receptor subtypes in spasms after chronic injury, presents significant implications for rehabilitative strategies following SCI and for the successful treatment of spasticity.

5. 2 Implications for rehabilitation

Following SCI peripheral NA, likely from the autonomic system, activates both excitatory α_1 adrenergic receptors and inhibitory α_2 receptors below a chronic transection. Since the α_1 receptor is constitutively active in addition to ligand activated *in vivo*, the excitatory effects of α_1 receptor activity dominate, resulting in the increased motoneuron excitability, Ca PIC and spasms seen after injury. Even in cases where peripheral NA, and consequently ligand activation of the α_1 receptor, is reduced the constitutive activity of the α_1 receptor ensures that motoneuron excitability remains elevated. However, although α_1 receptor activity is associated with increased spasms, it has also been shown to facilitate locomotor activity (Gabbay and Lev-Tov 2004). In a staggered hemi-section model of spinal cord injury, blocking the α_1 adrenergic receptor with the antagonist WB4101, alongside a 5-$HT_{2B/2C}$ receptor antagonist, produced a greater deficit in locomotor scores and in functional locomotion than use of just the 5-$HT_{2B/2C}$ antagonist alone (Bennett, Rank and Fouad unpublished data). Some degree of endogenous activation of the α_1 receptor after injury can therefore provide functional recovery benefits, but a balance between excitatory α_1 receptor effects and inhibitory α_2 receptor effects must be achieved. In order to capitalise on the inhibitory effects

of the α_2 adrenergic receptors, whereby the EPSPs triggering the Ca PICs are reduced, the amount of endogenous NA must be increased to a much higher level to allow α_2 receptor activity to dominate, as shown in the isolated spinal cord where high doses of NA inhibit LLRs (Li et al. 2004). With increased activation of α_2 receptors, spasms can be effectively reduced while preserving the beneficial excitatory effects of α_1 receptor activation.

Alternately, blocking only ligand activity at the α_1 receptor with a neutral antagonist, such as REC15/2739, may be another method to reduce spasms while optimising beneficial α_1 receptor mediated increased motoneuron excitability. Blocking ligand activity of the α_1 and the α_2 receptor *in vivo* (with REC15/2739 and RX821002 respectively), reduces spasms significantly. However, the α_1 neutral antagonist was never used in the absence of the α_2 antagonist. It is likely that blocking only ligand activity of the α_1 receptor with REC15/2739, leaving minimal α_1 receptor constitutive activity and some ligand mediated inhibitory α_2 activity unchanged, may be an ideal solution. The use of the neutral antagonist to block only peripheral NA actions on the α_1 receptor may leave just enough motoneuron excitability to benefit motor recovery, while at the same time reducing spasms via ligand activation of the inhibitory α_2 receptor.

5.3 Future Directions

The results of the studies which make up this thesis provide a broad groundwork on the contributions of the adrenergic system to increased motoneuron excitability and spasms after chronic SCI, but do leave several important areas untouched. Firstly, although the results of this thesis confirm that peripheral NA is able to cross the BBB and be sequestered into a central store after chronic injury, the thesis was unable to establish the location of this central store. It is likely that some intermediate cell facilitates the uptake, and vesicular storage and release of the peripheral NA but visualising this cell type would be very helpful in further clarifying the fate of peripheral NA after SCI. To this end, immunohistological

labelling of NA itself below a chronic transection, or alternately the NET which is present on the intermediate cell, should be completed. As the intermediate cell which packages and transports peripheral NA may be a glial cell, labelling for glial fibrillary acidic protein (GFAP) in conjunction with NA would also be beneficial.

In addition to ligand activation of the α_1 adrenergic receptor *in vivo*, the results of this thesis demonstrate that this receptor also exhibits constitutive activity. In the awake chronic spinal animal, this constitutive activity at the α_1 receptor likely provides a tonic support for ligand-mediated α_1 activity. However, the extent of the contribution of α_1 receptor constitutive activity to the recovery of spasms after chronic SCI was unable to be established. One possibility to assess the degree of constitutive α_1 activity after chronic SCI would be to quantify the expression of known constitutively active α_1 adrenergic receptor isoforms caudal to a chronic transection. It would be beneficial to focus on the α_{1A} receptor isoforms, as we have shown activation of this receptor subtype to be particularly important in the development of spasms after chronic SCI. Having a better quantification of the degree of constitutive activity vs ligand activity at the α_1 receptor in *in vivo* models of SCI would be a valuble future pursuit as this would allow a better tailoring of a pharmacological treatment of spasms. If constitutively active α_1 receptor isoforms are present in low quantities, use of neutral antagonists such as REC15/2739 to block ligand activity of the receptor would be of greater benefit. Conversely, if the constitutively active isoforms are present in higher quantities, use of inverse agonists, such as prazosin, would have a more positive outcome for the treatment of spasms. This thesis contributes substantially to the knowledge of the role of the adrenergic system in the recovery of spasms after chronic SCI. Future work should focus on using this knowledge to move toward better pharmacological strategies for the treatment of spasticity.

5.4 REFERENCES FOR CHAPTER 5

Gabbay H, and Lev-Tov A. Alpha-1 adrenoceptor agonists generate a "fast" NMDA receptor-independent motor rhythm in the neonatal rat spinal cord. *J Neurophysiol* 92: 997-1010, 2004.

Li Y, Harvey PJ, Li X, and Bennett DJ. Spastic long-lasting reflexes of the chronic spinal rat studied in vitro. *J Neurophysiol* 91: 2236-2246, 2004.

Appendix:

Additional publications

Harris RL, Putman CT, **Rank MM**, Sanelli L, Bennett DJ. Spastic tail muscles recover from myofiber atrophy and myosin heavy chain transformations in chronic spinal rats. *J Neurophysiol.* Feb;97(2):1040-51, 2007.

- For this project I was involved in the development of a protocol to record EMG from the tail muscles of uninjured, acute spinal, chronic spinal and chronic spinal isolated rats over long periods of time (24 hrs). Additional contributions included analysis and plotting of the EMG data as well as editing of the final manuscript.

Murray K, Nakae A, Stephens M, **Rank MM**, Harvey P, Li X, Harris L, Ballou EW, D'Amico J, Mashimo T, Vavrek R, Sanelli L, Gorassini MA, Bennett DJ, and Fouad K. Recovery of motoneuron and locomotor function after chronic spinal cord injury depends on constitutive activity in 5-HT_{2C} receptors. *Nat Med.* June 16(6):694-700, 2010.

- For this project I completed and analyzed *in vivo* experiments which recorded EMG from the tail muscles of chronic sacral spinal rats before and after intrathecal (IT) injection of the 5-HT_2 neutral antagonist SB242084 and inverse agonist SB206553. In addition, I participated in separate *in vivo* experiments which investigated the effects of these drugs (SB242084 and SB206553) on the recovery of locomotion in dual hemisected rats.

Fouad K, **Rank MM**, Vavrek R, Murray K, Sanelli L, Bennett DJ. Spontaneous recovery of stepping after spinal cord injury depends on constitutive activity in serotonin receptors. *J Neurophysiol.* Sept 22, 2010.

- This project was an expansion of the experiments done in the previously listed publication (Murray et al. 2010). For this project I completed *in vivo* experiments which used IT injection of 5-HT_2 receptor neutral antagonists and inverse agonists to evaluate the contribution of constitutive 5-HT_2 receptor activity to the recovery of locomotion in dual hemisected rats.

Murray K, Stephens M, **Rank MM**, D'Amico J, Gorassini MA, Bennett DJ. Polysynaptic excitatory postsynaptic potentials that trigger spasms after spinal cord injury are inhibited by 5-HT_{1B} and 5-HT_{1F} receptors (*in preparation*)

- For this project I completed and analyzed *in vivo* experiments which recorded EMG in the tail muscles of chronic spinal rats before and after IT injection of the 5-$HT_{1B/1D/1F}$ agonist zolmitriptan.