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

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ABSTRACT

The brainstem provides most of the noradrenaline (NA) present in the spinal cord, which functions to both increase spinal motoneuron excitability and inhibit sensory afferent transmission to motoneurons (excitatory postsynaptic potentials; EPSPs). NA increases motoneuron excitability by facilitating calcium-mediated persistent inward currents (Ca PICs) that are crucial for sustained motoneuron firing. Spinal cord transection eliminates most NA and accordingly causes an immediate loss of PICs and emergence of exaggerated EPSPs. However, with time PICs recover, and thus the exaggerated EPSPs can then readily trigger these PICs, which in turn produce muscle spasms. Here we examined the contribution of adrenergic receptors to spasms in chronic spinal rats. Selective activation of the \( \alpha_{1A} \) adrenergic receptor with the agonists methoxamine or A61603 facilitated Ca PIC and spasm activity, recorded both in vivo and in vitro. In contrast, the \( \alpha_2 \) receptor agonists clonidine and UK14303 did not facilitate Ca PICs, but did decrease the EPSPs that trigger spasms. Moreover, in the absence of agonists, spasms recorded in vivo were inhibited by the \( \alpha_1 \) receptor antagonists WB4010, prazosin and REC15/2739, and increased by the \( \alpha_2 \) receptor antagonist RX821001, suggesting that both adrenergic receptors were endogenously active. In contrast, spasm activity recorded in the isolated in vitro cord was inhibited only by the \( \alpha_1 \) antagonists that block constitutive receptor activity (activity in the absence of NA; inverse agonists, WB4010 and prazosin), and not by the neutral antagonist REC15/2739, which only blocks conventional NA-mediated receptor activity. RX821001 had no effect in vitro, even though it is an \( \alpha_2 \) receptor inverse agonist. Our results suggest that after chronic spinal cord injury Ca PICs and spasms are facilitated, in part, by constitutive activity in \( \alpha_1 \) adrenergic receptors. Additionally, peripherally-derived NA (or similar ligand) activates both \( \alpha_1 \) and \( \alpha_2 \) adrenergic receptors, controlling PICs and EPSP, respectively.
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-HT₂ and α₁ adrenergic receptors, though the origin of this
spontaneous activity was undetermined. Subsequently, Murray et al (2010b) demonstrated that spontaneous activity of the 5-HT2 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 α1 adrenergic receptors contribute to spasms after SCI, the specific receptor subtype is unknown (α1A, α1B, α1D). 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 α1 and α2 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 $\alpha_2$ adrenergic receptor is an ideal candidate for the regulation of the long EPSPs since $\alpha_2$ 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 $\alpha_2$ 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 $\alpha_2$ 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 $\alpha_2$ adrenergic receptors.
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 (S2) 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.

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 S2 level). In normal adult rats the cord was cut at the same location (upper S2) for removal, and they are therefore termed acute spinal rats. All dorsal and ventral spinal roots were removed, with the exception of the sacral S4 and caudal C(a) ventral roots and the C(a) 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.
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 S4 and Ca1 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 Ca₁ 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).

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 Ca₁) and two to four ventral roots (left and right S₄ and/or Ca₁) 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 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 12 min recording session. Dose response curves were constructed by administering increasing doses of the drug every 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).

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 NaHCO3, 1.5 CaCl2, 3 KCl, 5 MgCl2, 1.4 NaH2PO4, 1.3 MgSO4, 25 D-glucose, and 1 kynurenic acid. Normal ACSF was composed of (in mM) 122 NaCl, 24 NaHCO3, 2.5 CaCl2, 3 KCl, 1 MgCl2, and 12 D-glucose. Both types of ACSF were saturated with 95% O2 - 5% CO2 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).

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.

**In vivo drug injection**

Unless otherwise listed, all drugs were administered *in vivo* via transcutaneous intrathecal (IT) injection, under light isoflurane anesthetic. A 1-inch, 25 - guage needle connected to a 100 μL glass Hamilton syringe was inserted into the tissues between the L5 and L6 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.

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.
RESULTS

Ca PICs are increased by $\alpha_1$ receptors

Considering that Ca PICs in motoneurons are a major underlying cause of spasms after SCI, we began our study by examining the effect of $\alpha_1$ 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 1).

TTX (2 $\mu$M) was applied to synaptically isolate the motoneuron (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 (under voltage-clamp conditions) to inactivate transient currents, and enable the full voltage-dependence of the Ca PIC to be evaluated. During these slow voltage ramps the Ca PIC was activated at $-57.94 \pm 7.63$ mV ($V_{on}$, $n = 9$ motoneurons) and produced a downward deflection in the recorded current of $1.05 \pm 0.26$ nA, which we considered an estimate of the Ca PIC amplitude (Fig 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 $\alpha_{1A}$ adrenergic receptor agonist methoxamine significantly increased this Ca PIC amplitude (151.32%; Fig 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: $-0.14 \pm 1.42$ mV, $0.84 \pm 0.69$ M$\Omega$, $-0.13 \pm 2.53$ mV, respectively; not significant, $P > 0.05$, $n = 9$).

Long-lasting reflexes are increased by $\alpha_{1A}$ receptors.

To examine the functional consequences of $\alpha_1$ 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 previously been shown to depend on Ca PICs (Li et al. 2004a). Neither the moderately selective $\alpha_{1A}$ adrenergic receptor agonist methoxamine (0.1 – 30 $\mu$M) (Minneman et al. 1994; Shibata et al. 1995) nor the more selective and potent $\alpha_{1A}$ receptor agonist A61603 (0.03 – 10 $\mu$M) (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 \( \alpha_2 \) receptors, as we describe later, and this counterbalanced the increase in the Ca PICs mediated by \( \alpha_1 \) receptors. This occurred because methoxamine and A61603, as well as other available \( \alpha_1 \) agonists, have substantial binding affinity for \( \alpha_2 \) as well as \( \alpha_1 \) 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 \( \alpha_2 \) receptors in our preparation were unexpectedly large (see later section). This poor \( \alpha_1 \) verses \( \alpha_2 \) selectivity had not been anticipated, especially for A61603, because A61603 has otherwise negligible binding at all other receptors previously tested, including \( \alpha_{1B} \) and \( \beta \) adrenergic, 5-HT and dopamine receptors (Craig et al. 1997).

To study the effects of the \( \alpha_1 \) receptor in isolation, we next blocked the \( \alpha_2 \) receptors with the selective \( \alpha_2 \) receptor antagonist RX821002 (0.3 – 0.5 \( \mu \)M) (Jasper et al. 1998; Sanders et al. 2006) prior to applying the agonist A61603. This effectively made A61603 highly selective for the \( \alpha_{1A} \) adrenergic receptor (Craig et al. 1997). Under these conditions, A61603 significantly increased the LLR (Figs 1 and 2), more than doubling the LLR amplitude when given at doses above 30 nM. This is consistent with an \( \alpha_{1A} \) 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 1H and 2C, sigmoid had Hill slope of 1.0), consistent with the known high affinity of A61603 to the \( \alpha_{1A} \) receptor (\( K_i = 80 \) nM) (Craig et al. 1997; Mehrotra et al. 2007).
Spasms in awake rat are increased by $\alpha_{1A}$ receptors.

We also examined the effects of $\alpha_1$ receptors 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 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 $\alpha_2$ receptors, LLRs (tail spasms) were significantly increased by an IT injection of A61603 (Fig 1C, F). Control saline injections had no significant effect ($P > 0.05$, $n = 5$; not shown). These results further demonstrate that activation of the $\alpha_{1A}$ adrenergic receptor increases spasticity and underlying Ca PICs in chronic spinal rats.

Chronic spinal rats are not supersensitive to $\alpha_1$ receptor activation.

The increases in LLRs resulting from $\alpha_1$ 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 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; Fig 1E vs Fig 1G). 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 1E vs Fig 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, indicating a lack of supersensitivity to \( \alpha_1 \) receptor activation with A61603.

**Blocking the \( \alpha_1 \) adrenergic receptor reverses agonist-mediated increase in spasms**

As mentioned, agonists of \( \alpha_1 \) adrenergic receptors generally demonstrate only limited selectivity over other adrenergic receptor subtypes (e.g. \( \alpha_2 \) receptors). In contrast, antagonists of \( \alpha_1 \) 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 \( \alpha_1 \) 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 prazosin or REC15/2739 (Fig 2A, B, D, E), in vitro. The typical time course of the facilitation of the LLR by the \( \alpha_1 \) agonist and subsequent inhibition by and the \( \alpha_1 \) antagonist is shown in Fig 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 \( \alpha_1 \) 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 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 \( \alpha_1 \) receptors. These experiments were performed in the presence of RX821002 to rule out any non-selective action of A61603 on the \( \alpha_2 \) receptor.
The drug REC15/2739 is special because it has previously been shown to act as a neutral antagonist at \( \alpha_{1A} \) adrenergic receptors, meaning that it blocks only the action of a ligand (such as NA) at the \( \alpha_{1A} \) receptor, and not constitutive receptor activity (Rossier et al. 1999). REC15/2739 is therefore useful in determining whether the \( \alpha_{1A} \) 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 the ventral root LLRs in the isolated in vitro spinal cord (Fig 3A, C), even though it readily antagonized the \( \alpha_{1A} \) agonist A61603 (Fig 2E). This suggests that, at least in vitro, the \( \alpha_{1A} \) receptor is not endogenously activated by residual NA in the spinal cord. In contrast, when we administered REC15/2739 in awake spastic rats in vivo, with a localized IT injection, there was a significant decrease in LLRs (spasms Fig 3B, D). This demonstrates that the \( \alpha_{1A} \) 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).

Constitutive activity in \( \alpha_{1} \) receptors

Interestingly, application of the \( \alpha_{1} \) antagonists WB4101 or prazosin significantly decreased LLRs recorded in vitro (without prior agonist application, Fig 3C and D), even though REC15/2739 did not. WB4101 and prazosin have been previously shown to act as potent inverse agonists at \( \alpha_{1} \) receptors (Noguera et al. 1996; Rossier et al. 1999; Seifert and Wenzel-Seifert 2002), which means that they block constitutive activity in \( \alpha_{1} \) 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 \( \alpha_{1} \) receptors exhibit substantial constitutive activity, at least in vitro. When applied in vivo, both WB4101 and prazosin (IT) likewise inhibited the LLRs (spasms Fig 3D), which is likely due to both a block of ligand-activated receptors (residual NA) and constitutively activated receptors.
The $\alpha_2$ adrenergic receptor modulates the EPSP, but not the Ca PIC

Considering that we suspected an inhibitory effect of $\alpha_2$ receptors on the EPSPs that trigger LLRs (spasms), we next measured how the moderately selective $\alpha_2$ adrenergic (and imidazoline I1) receptor agonist clonidine, and the highly selective $\alpha_{2A}$ adrenergic receptor agonist UK14304, affected ventral root LLRs in vitro. Treatment with both these $\alpha_2$ agonists significantly decreased LLRs (Fig 4A, D), and this decrease was reversed by subsequent treatment with the selective $\alpha_2$ adrenergic antagonist RX821002 (Fig 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 $\alpha_{2A}$ receptor ($K_i = 31$ nM) (Millan et al. 2000), and inconsistent with the 10 times lower affinity of clonidine to $\alpha_1$ receptors (e.g. $K_i = 300$ nM at $\alpha_{1A}$ receptor) (Millan et al. 2000). These results suggest that $\alpha_{2A}$ adrenergic receptors inhibit LLRs and resulting spasms after chronic SCI.

We next investigated whether this inhibitory effect of $\alpha_2$ 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 4C). The motoneurons were held with a hyperpolarizing bias current in order to 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 $\alpha_2$ receptor agonist clonidine decreased this long polysynaptic EPSP significantly (Fig 4C, E). In contrast, clonidine had no effect on the PIC (Fig 4F; recorded under voltage clamp, as described in Fig 1A). Interestingly, clonidine significantly hyperpolarized the resting membrane potential by about –4 mV (Fig 4). These data suggest that the inhibitory effect of the $\alpha_2$ 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.

Lack of constitutive activity in α2 receptors.

Application of the α2 adrenergic receptor antagonist RX821002 alone, without agonists, had no effect on the LLRs (Fig 4B and Fig 5A, C) measured in the isolated spinal cord in vitro, even though it is a potent α2 receptor inverse agonist that is capable of blocking constitutively active α2 receptors (Pauwels et al. 2000). In contrast, RX821002 significantly increased the spasms recorded in the awake spastic rat in vivo, both with systemic intraperitoneal (IP) or local spinal intrathecal injection of RX821002 (Fig 5D, E). This suggests that, although the α2 adrenergic receptor is not constitutively 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 α1 receptor.
The results of our study characterize for the first time the roles of two adrenergic receptor subtypes (α₁ and α₂) in the recovery of motoneuron excitability and spasms after chronic SCI. We find that α₁ receptors increase motoneuron excitability and the α₂ 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: α₁ 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 α₁ and α₂ receptors. In contrast, the α₂ receptors do not seem to exhibit constitutive activity, suggesting that these receptors respond differently to injury.

α₁A receptor subtype on motoneurons facilitates the Ca PICs and spasms.

Our results specifically establish that activation of the α₁A adrenergic receptor facilitates the Ca PIC in motoneurons, thereby increasing its excitability, and ultimately increasing the many second long spasms (LLRs), known to be mediated by the Ca PIC. These conclusions are based on α₁A 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 α₁A agonist A61603 that has negligible binding affinity for most other receptors, including other α₁ adrenergic receptor subtypes (α₁B, α₁D), 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 α₂ receptors (Craig et al. 1997), which initially thwarted our efforts to demonstrate α₁A
receptor-mediated increases in the LLR, and thus we subsequently applied A61603 in the presence of the 
$\alpha_2$ antagonist RX821002 to make it highly selective to $\alpha_1$ receptors. Under these conditions we found that 
A61603 consistently increases the LLR, demonstrating the presence of an $\alpha_{1A}$ adrenergic receptor that 
facilitates the Ca PIC on motoneurons.

Consistent with the involvement of the $\alpha_1$ 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 $\alpha_{1A}$ 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 $\alpha_1$ 
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 $\alpha_{1A}$ receptors may be located near the surface of the spinal cord, on the 
distal dendrites of motoneurons, where the drug can easy diffuse to, when applied in vitro. In contrast, the 
5-HT$_2$ receptors are located deep in the spinal cord, including on the motoneurons soma (Murray et al. 
2010b), where drugs reach less easily, though this needs to be further investigated. We know that the $\alpha_{1A}$ 
receptors that facilitate Ca PICs and spasms must be located somewhere on motoneurons because the 
facilitation of the Ca PIC by $\alpha_1$ 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 $\alpha_1$ 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 $\alpha_1$ adrenergic receptor also increases the LLR and 
spasms by increasing other motoneuron properties or even the EPSPs that trigger the Ca PICs. The $\alpha_1$ 
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 $\alpha_1$ agonist methoxamine did not depolarize the resting potential of motoneurons, it is still possible that the $\alpha_1$ receptor depolarizes the sacral motoneurons we studied, but this is masked by the non-selective action of methoxamine on the $\alpha_2$ receptor, which hyperpolarizes motoneurons (see below). We do not know whether the $\alpha_1$ receptors facilitate sensory afferent transmission (EPSPs), though if anything they may do the opposite, by facilitation of inhibitory interneurons (Yoshimura and Furue 2006).

$\alpha_{1A}$ receptors act similarly in normal and chronic spinal rats. In spinal cords from normal rats, we also found evidence for the presence of $\alpha_{1A}$ 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 $\alpha_{1A}$ 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 $\alpha_1$ 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 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 $\alpha_{1A}$ receptors appear to act similarly in spinal cords of normal and chronic spinal rats to increase motoneuron excitability.
Constitutive activity in $\alpha_{1A}$ receptors contribute to recovery of motoneurons excitability.

Our data demonstrate that when the spinal cord is isolated from peripheral influences (in vitro), the Ca PICs are facilitated by endogenous $\alpha_{1A}$ receptor activity that is entirely mediated by constitutive receptor activity. Constitutive activity of wild-type $\alpha_{1A}$ adrenergic receptor 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 $\alpha_{1A}$ 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 this 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 $\alpha_1$ 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 $\alpha_1$ receptors compared to other receptors, including $\alpha_2$ receptors, they are not very selective among the $\alpha_1$ receptor subtypes and bind potently to $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$ 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 $\alpha_1$ receptor types is constitutively active. However, while REC15/2739 is a neutral antagonist at $\alpha_{1A}$ receptors, it is an inverse agonist at other $\alpha_1$ receptor subtypes, whereas WB4101 and prazosin are inverse agonist at all $\alpha_1$ 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 $\alpha_{1A}$ receptor, further supporting the conclusion that the $\alpha_{1A}$ 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 $\alpha_1$ receptors. Constitutively active 5-HT$_2$ receptors and $\alpha_1$ adrenergic receptors likely play an equally important role in facilitating spasms, since they contribute equally to PICs and blocking both these receptors essentially eliminates the PICs (Harvey et al. 2006b). This helps explain the particular effectiveness of cyproheptadine as an antispastic drug (Barbeau et al. 1982; Murray et al. 2010b; Nance 1994), by its action in blocking both 5-HT$_2$ receptors and $\alpha_1$ adrenergic receptors. However, broad spectrum drugs like cyproheptadine may also non-selectively block the $\alpha_2$ receptor, which may have a paradoxically pro-spastic action, increasing sensory afferent transmission and pain, as we discuss below.

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 $\alpha_1$ 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 $\alpha_1$ adrenergic receptors. Interestingly, a forced release of NA with application of amphetamine (Rothman et al. 2001) 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 vitro. In contrast, the endogenous $\alpha_1$ receptor activity seen in the awake rats (in vivo) appears to additionally involve $\alpha_1$ 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 know 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 \(\beta\)-hydroxylase, the enzyme essential for NA production, after chronic transection (McNicholas et al. 1980; Takeoka et al. 2010). This NA may partly account for the amphetamine-induced increases in the PICs and spasms that we observe \textit{in vitro} (Rank et al. 2007), but we reiterate that this intrinsic source appears to be functionally inactive in the isolated spinal cord \textit{(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 \(\alpha_1\) 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 crosses into the spinal cord and activate the \(\alpha_1\) receptors. While unconventional, this peripherally-derived NA seems like a much larger source of NA, and we are currently investigating this possibility.

\(\alpha_2\) receptors inhibit EPSPs, but not PICs.

Contrary to the \(\alpha_1\) receptor function, our data demonstrate that the activation of the \(\alpha_2\) adrenergic receptor has no direct effect on the Ca PIC in motoneurons (clonidine-resistant). Rather, activation of the \(\alpha_2\) 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 α₂ receptors are located, though they are likely on the terminals of
the low threshold group I and II sensory 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 α₂ receptors (Jankowska and Hammar 2002; Jankowska et al. 2000; Millan 2002;
Rekling et al. 2000). Additionally, α₂ receptors may be on motoneurons themselves, because we found that
their direct activation with clonidine hyperpolarizes the motoneurons. Indeed, previous studies have shown
that α₂ receptors on motoneurons induce a hyperpolarization, by blocking Iₜ currents (Adachi et al. 2005;
Parkis and Berger 1997; Rekling et al. 2000), and such Iₜ currents contribute +10 mV to the resting
potential in our chronic spinal rats (Li et al. 2007). Furthermore, α₂ receptor expression can be detected
throughout the spinal cord, with the highest densities in the superficial dorsal horn, and moderate densities
in the portion of be ventral horn containing motoneurons (Giroux et al. 1999; Roudet et al. 1994). All
together, α₂ 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.

Residual NA after spinal cord injury activates α₂ receptors.

Unlike the α₁ receptor, the α₂ receptor does not appear to be constitutively active after SCI, because
blocking possible constitutive activity with the α₂ 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 α₂ receptor agonists on the reflexes. However, the α₂ 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 α₂ antagonists RX821002. We suggest that this spontaneous α₂ receptor
activity is due to a peripheral source of NA, which would also activate α₁ receptors as discussed above.
Interestingly, our conclusions might also explain the recent surprising finding that the $\alpha_2$ receptor antagonist yohimbine markedly facilitates locomotion in transected mice (Lapointe et al. 2008). That is, a peripheral source of NA may tonically inhibits locomotor activity, perhaps by inhibiting reflex transmission, as we have seen, and antagonists may remove this inhibition. In contrast, $\alpha_1$ receptor activation facilitates rhythmic locomotor activity (Gabbay and Lev-Tov 2004), in addition to its facilitation of motoneuron excitability.

**Implications for recovery of motor function.**

Considering the pronounced opposing effects of $\alpha_1$ and $\alpha_2$ receptors that we have uncovered after chronic SCI, the combined functional outcomes of activity in these two receptors remains to be considered. Since the $\alpha_1$ receptor is both constitutively active and activated by an endogenous source of NA (or other ligand; peripherally-derived), whereas $\alpha_2$ receptors are only activated by endogenous NA, the former $\alpha_1$ receptor activity is likely to dominate when there is not much endogenous NA, ultimately increasing 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. Interestingly, increasing release of endogenous NA with amphetamine increases spasms even at high doses (Rank et al. 2007), but this may be because amphetamine also binds directly to $\alpha_2$ adrenergic receptors (with similar affinity to NA itself; Boyajian and Leslie 1987), and thus may competitively block the action of released NA on the $\alpha_2$ receptors, making the $\alpha_1$ receptor action of NA dominate. With more natural release of peripheral NA, the net effect of high levels of NA is likely to be inhibitory, or antispastic in action, as we have discussed. This fits with our understanding of the dual action of NA receptors, because regardless of how large the $\alpha_1$ receptor-mediated PICs, if there are no
EPSPs to trigger them, because of $\alpha_2$ 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 $\alpha_1$) (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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**FIGURE LEGENDS**

**Figure 1: Activation of the α₁ 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 α₁ 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 α₁ 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 α₂ adrenergic receptor.
Figure 2: The $\alpha_{1A}$ adrenergic receptor agonist A61603 is antagonized by selective $\alpha_1$ receptor antagonists. (A) LLR evoked in the isolated in vitro spinal cord of a chronic spinal rat, as described in Fig. 1 (top plot), with bath application of the $\alpha_{1A}$ receptor agonist A61603 alone (0.1 $\mu$M, middle plot), and with subsequent application of $\alpha_1$ receptor antagonist prazosin (1 $\mu$M, bottom plot). (B) Amplitude of LLR (quantified 0.5 – 4 s post stimulus, as in Fig. 1) of a chronic spinal rat measured repeatedly over time under control conditions (left), with application of A61603 (upper horizontal black bar; 0.03 $\mu$M) and subsequent application of the highly specific $\alpha_{1A}$ receptor neutral antagonist REC15/2739 (abbreviated REC; lower horizontal black bar; 10 $\mu$M). (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 $\alpha_1$ receptor antagonists prazosin (1 $\mu$M) or WB4101 (3 $\mu$M) (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 $\mu$M; 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 $\mu$M; $n = 15$), and A61603 with subsequent application of REC15/2739 (abbreviated A6 + REC; 10 $\mu$M; $n = 15$). *P < 0.05, **P < 0.01. Error bars, s.e.m. All recordings were made in the presence of RX821002 (0.5 $\mu$M).

Figure 3: Endogenous activation of the $\alpha_{1A}$ 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 1, upper plot) and after blocking the action of endogenous NA (or similar ligand) with application of the $\alpha_{1A}$ neutral antagonist REC15/2739 (abbreviated REC; 10 $\mu$M, 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 1) before (top plot) and after blocking endogenous action of NA at the $\alpha_{1A}$ receptor with local intrathecal (IT) injection of REC15/2739 (5 mM in 30 $\mu$l). Normalized group mean of chronic
spinal rat LLRs recorded *in vitro* (C) and *in vivo* (D) after application of the α₁ 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 α₁ 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, 1mg/Kg).

**Figure 4: Activation of the α₂ 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). Control values are shown on left, followed by activation of α₂ adrenergic receptors with the agonist clonidine (upper horizontal black bar; 0.1 μM) and subsequent application of the α₂ 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 α₂ 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 α₂ receptor agonists UK14304 (0.03 μM; white bar, n = 8), and clonidine (0.1 μM; black bar, n = 5), and application α₂ 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 intracellularly recorded polysynaptic EPSP (E) evoked by 3 x T dorsal root stimulation, PIC (F) and resting membrane potential (Vₘ) (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.
**Figure 5:** The $\alpha_2$ 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. 1) of a chronic spinal rat before (top plot) and after blocking possible endogenously activated $\alpha_2$ adrenergic receptors with the selective $\alpha_2$ antagonist RX821002 (0.5 $\mu$M, 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 1) before (top plot) and after blocking endogenously activated $\alpha_2$ 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 $\mu$M; $n = 42$) 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 $\mu$l; $n = 5$) *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 $\alpha_2$ adrenergic receptor with either local IT injection of RX821002 (abbreviated IT RX, left arrow; 0.3 mM in 30 $\mu$l) or systemic IP injection of RX2821002 (abbreviated RX IP, right arrow; 1 mg/Kg). *P* < 0.05, **P** < 0.01. Error bars, s.e.m.
REFERENCES.


Harvey PJ, Li X, Li Y, and Bennett DJ. Endogenous monoamine receptor activation is essential for enabling persistent sodium currents and repetitive firing in rat spinal motoneurons. *J Neurophysiol* 96: 1171-1186, 2006b.


Hultborn H, Brownstone RB, Toth TI, and Gossard JP. Key mechanisms for setting the input-output gain across the motoneuron pool. *Prog Brain Res* 143: 77-95, 2004.


Figure 1

A

-50 mV
+Methox
CaPIC
+Methox
CaPIC

10 mV
1 nA
2 s

B

In vitro spasm, LLR
In vitro spasm, LLR
Stimulation
Stimulation

0.1 mV
0.4 mV
1 s
1 s

C

In vivo spasm, LLR

+ A61603
+ A61603

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)

Control
A61603

Chronic
Acute
Figure 2

A. In vitro spasm, LLR

B. Graph showing LLR (mV) over time (min) for Control, A61603, and A61603 + REC.

C. Graph showing LLR (mV) vs. Dose A61603 (nM) for Control, A61603, and Antag + A61603.

D. Bar graph showing LLR (% control) for Control, A61603, and A61603 + Praz.

E. Bar graph showing LLR (% control) for Control, A61603, and A61603 + REC.
Figure 3

A. *In vitro* spasm, LLR

B. *In vivo* spasm, LLR

C. *In vitro* LLR (% control)

D. *In vivo* LLR (% control)
Figure 4

A. Time (min)

<table>
<thead>
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B. Time (min)

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<td>0</td>
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C. Motoneuron

-80 mV

D. In vitro

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<td>UK14304</td>
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<td>Clonidine</td>
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<tr>
<td>UK + RX</td>
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<td>Clon + RX</td>
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E. EPSP (% control)

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F. PIC (% control)

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<tr>
<td>Clonidine</td>
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G. Resting Vm (mV)

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<tr>
<td>Clonidine</td>
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Figure 5

A. In vitro, LLR

B. In vivo, LLR

C. In vitro

D. In vivo

E. Control, RX IT, RX IP

** represents statistical significance.