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The Role of Serotonin Receptors in Spasticity After Spinal Cord Injury

by

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ABSTRACT

Brainstem derived serotonin (5-HT) normally facilitates spinal motoneuron excitability and inhibits sensory afferent transmission and associated spinal reflexes. Because the 5-HT innervation of the spinal cord is almost exclusively derived from brainstem neurons, spinal cord injury leads to an immediate and dramatic loss of 5-HT and this in turn leads to the simultaneous loss of motoneuron excitability and increase (disinhibition) of sensory afferent transmission. This thesis examined how spinal cord 5-HT receptors adapt over the months after SCI (chronic injury) to compensate for the loss of 5-HT. We showed that after SCI 5-HT_{2B} and 5-HT_{2C} receptors become *constitutively active* (active in the absence of 5-HT) with chronic injury, and this leads to a recovery of motoneuron excitability and contributes to the recovery of locomotor function. Unfortunately, this also contributes to the development of muscle spasms when combined with the disinhibition of sensory afferent transmission. In contrast, 5-HT_1 receptors that modulate sensory afferent transmission do *not* become constitutively active after chronic SCI, and this contributes to the continued disinhibition of sensory afferent transmission and associated hyperreflexia and muscle spasms after chronic SCI. However, exogenous application of 5-HT_{1B} and 5-HT_{1F} receptor agonists can restore inhibition over sensory afferent transmission and ultimately reduce muscle spasms. In summary, 5-HT_2 receptors exhibit a remarkable adaptation to the loss of 5-HT with SCI, whereas 5-HT_1 receptors do not. Understanding and promoting this natural plasticity may help in the development of better therapeutic interventions for treating SCI.

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Adenylate cyclase	AC
Calcium persistent inward current	Ca PIC
Discontinuous current clamp	DCC
Excitatory post synaptic current	EPSC
Excitatory post synaptic potential	EPSP
Electromyography	EMG
Inhibitory post synaptic current	IPSC
Inhibitory post synaptic potential	IPSP
Inositol phosphate	IP
Long lasting reflex	LLR
Long polysynaptic reflex	LPR
Modified artificial cerebral spinal fluid	mACSF
Normal artificial cerebral spinal fluid	nACSF
Persistent inward current	PIC
Phospholipase C	PLC
Serotonin	5-HT
Serotonin transporter	SERT
Sodium persistent inward current	Na PIC
Short latency reflex	SLR
Tetrodotoxin	TTX

Chapter 1: Introduction

SPINAL CORD INJURY AND MUSCLE SPASMS

Following spinal cord injury (SCI) debilitating spasms frequently develop in the muscles innervated from the spinal cord below the injury. In a study reporting the incidence of spasticity one year after SCI, more than 67% of patients had developed spasticity associated with involuntary muscle spasms (Maynard et al. 1990). Involuntary muscle spasms are one of the many symptoms of a general spasticity syndrome that emerges after SCI, which also includes hyperreflexia, clonus, hypertonus and muscle contractures (Dietz 2000; Skold et al. 1999). Muscle spasms, which can last for 8-10 seconds and occur with a frequency of fifteen times per day on average (Kawamura et al. 1989), can be evoked by brief noxious or innocuous stimuli (Hultborn and Malmsten 1983; Young 1994), including muscle stretch or electrical stimulation of muscle or cutaneous sensory afferents. Spasticity has the potential to severely reduce quality of life by restricting activities of daily living and self-care. It can cause pain and fatigue, and contribute largely to the development of debilitating contractures and pressure ulcers, that may ultimately lead to infections (Burchiel and Hsu 2001; Sheean 2003; Skold et al. 1999; St George 1993; Ward 2003).

The most commonly cited definition for spasticity was published by Lance in 1980, who defined spasticity as ‘a motor disorder characterized by a velocity-dependent increase in tonic stretch reflexes (muscle tone) with exaggerated tendon jerks, resulting from hyperexcitability of the stretch reflex, as one component of the upper motoneuron syndrome’ (Lance 1980). Lance’s definition focuses on stretch reflex pathways, and perhaps pertains better to spasticity that results from a stroke given that in humans, the gain of the stretch reflex is not altered after spinal injury like it is in cerebral trauma (Woolacott and Burne 2006). For spinal cord injury, muscle spasms and exaggerated cutaneous reflexes play a more central role in the spasticity syndrome, and is one of the topics of this thesis (Bennett et al. 2004; Kuhn et al. 1991; Schmidt and Jordan 2000).

Spinal cord injuries reduces or eliminates descending inputs from the cortex and carry voluntary motor commands, ultimately leading to a loss of volitional control. In addition, spinal cord injury interrupts descending inputs from the brainstem that exert both tonic inhibitory and excitatory control over spinal interneurons, afferents and motoneurons (Baldissera et al. 1981; Heckman 1994). Descending brainstem inputs include axons that contain serotonin (5-HT) which originate in the caudal portion of the raphe nucleus and project densely throughout the cord (Alvarez et al. 1998), and axons that contain norepnephrine (NE) (Patel et al. 1997), which originate in the locus coeruleus nucleus and also project densely throughout the spinal cord (Giroux et al. 1999). With spinal cord injury the loss of these monoaminergic axons causes an immediate loss of motoneuronal excitability, and at the same time, a loss of inhibition over many reflex pathways (Bennett et al. 2004; Hounsgaard et al. 1988; Li et al. 2004b). The low motoneuron excitability dominates immediately after injury, leaving the cord in a quiet areflexic state with a complete loss of tendon reflexes and complete muscle paralysis (Dietz 2000; Sherrington 1910). This period was first described as 'spinal shock' (Sherrington 1910) and has been reported to last anywhere from 1 to 3 days (Ditunno et al. 2004) to several weeks after injury at which time the gradual development of exaggerated tendon reflexes, increased muscle tone, and involuntary muscle spasms appear (Dietz 2000). The mechanisms producing the recovery of motoneuron excitability and the persistence of disinhibited reflex pathways in response to the loss of 5-HT (Li et al. 2004b) is another main topic of this thesis.

Pharmacological treatment of spasms

The pharmacological management of spasticity after SCI can be difficult since the aim is to control unwanted muscle spasms and tone while preserving residual motor function. For instance, involuntary muscle activity can increase stability in sitting and standing, facilitate the performance of weight transfers, increase muscle bulk and strength of spastic muscles and increase venous return

(Jozefczyk 2002; Kirshblum 1999; Kita and Goodkin 2000; Parziale et al. 1993). Thus, it may be beneficial to reduce spasms but not to completely eliminate them. Many of the current antispastic drugs are often not well tolerated at clinically useful doses because they produce side effects such as weakness, drowsiness and lethargy (Dario and Tomei 2004). Ideally, a useful antispastic drug regime should have minimal side effects when given systemically (orally). Some of the most commonly used antispastic medications include baclofen, clonidine, tizanidine and dantrolene sodium, all of which have potentially serious side effects. Baclofen ($GABA_B$ agonist) is particularly effective for reducing flexor spasms (Elovic 2001; Gracies et al. 1997; Kita and Goodkin 2000), but can cause weakness that may impair the ability of a patient to walk or stand (Burchiel and Hsu 2001; Kirshblum 1999). Baclofen acts presynaptically, reducing mono- and polysynaptic EPSPs evoked in motoneurons by sensory afferent stimulation (Dario and Tomei 2004; Li et al. 2004e). Clonidine (alpha2 adrenergic agonists), most commonly known for its use in treating hypertension, can also be an effective antispastic agent because it enhances presynaptic inhibition of sensory afferents, thereby suppressing spinal polysynaptic reflexes (Elovic 2001; Kirshblum 1999). However weakness has also been reported to be a side effect to this drug, in addition to significant cardiovascular effects that has limited its usefulness in the treatment of spasticity (Kamen et al. 2008). Tizanidine, like clonidine, is an alpha2 adrenergic agonist that also reduces spasticity by similar presynaptic actions (Elovic 2001; Kita and Goodkin 2000), but is not as potent in decreasing blood pressure or heart rate (Coward 1994). Dantrolene acts peripherally at the muscle, rather than at the spinal cord level, and weakens muscles that are overexcited by inhibiting the release of calcium during muscle contraction (Burchiel and Hsu 2001; Elovic 2001; Gracies et al. 1997; Kirshblum 1999; Kita and Goodkin 2000). However, it also causes the adverse effect of weakening muscles involved in residual function in patients with SCI (Burchiel and Hsu 2001; Elovic 2001; Kita and Goodkin 2000). Overall, there is a need for more effective treatments for spasticity with less adverse side effects, and this is one of the motivations behind the present thesis. A greater understanding of the

neuronal mechanisms underlying spasticity will allow for the continued improvement of management for this debilitating and painful disorder.

Motoneuronal persistent inward currents

The neuronal mechanisms underlying involuntary muscle spasms have recently been identified in a rat model of SCI (Bennett et al. 2004; Li et al. 2004c), and similar mechanisms have been shown to underlie spasms in humans (Gorassini et al. 2004; Norton et al. 2008). In order to understand these mechanisms, we first have to explain normal motoneuron function. Motoneurons in the normal, uninjured spinal cord can produce persistent inward currents (PICs) that amplify and sustain their motor output, and are important for regulating the gain of inputs (Bennett et al. 1998a; b; Brownstone 2006; Heckman 2003; Hounsgaard and Kiehn 1989; Hultborn 2002; Kiehn and Eken 1997; Lee and Heckman 1998b; Schwindt and Crill 1982). PICs are a latent property of normal motoneurons that are regulated by the synaptic input of endogenous neuromodulators, namely 5-HT and NE (Alaburda and Hounsgaard 2003; Delgado-Lezama et al. 1997; Hounsgaard and Kiehn 1989; Hultborn and Kiehn 1992; Lee and Heckman 1999). These brainstem derived monoamines are very important to the facilitation of PICs, because as mentioned earlier, spinal cord transection eliminates this source of 5-HT and NE and consequently largely eliminates PICs (Harvey et al. 2006a; Hounsgaard et al. 1988). Furthermore, subsequent application of monoamine agonists recovers PICs showing their clear involvement in facilitation of PICs (Conway et al. 1988; Hounsgaard et al. 1988).

Net inward persistent currents are generated by both voltage-gated persistent calcium and sodium inward currents (Ca PICs and Na PICs) that are typically activated when the motoneuronal membrane is depolarized beyond a critical threshold (about -50 to -60 mV in sacral rat motoneurons) (Anelli et al. 2007; Bennett et al. 2004; Harvey et al. 2006b; Jiang and Heckman 2006). These currents are mediated by membrane channels located on the dendrites, as evidenced by the hyperpolarized somatic deactivation potential compared to the

activation potential, and delayed current onset in response to a depolarizing voltage-clamp step (Booth et al. 1997; Carlin et al. 2000b; Hounsgaard and Kiehn 1993; Lee and Heckman 1998a; Powers and Binder 2003; Svirskis and Hounsgaard 1997). The Na PICs are mediated by low-threshold, TTX-sensitive sodium channels and are essential for initiating rhythmic firing in motoneurons (Harvey et al. 2006a; Lee and Heckman 2001; Miles et al. 2005). This Na PIC rapidly activates just subthreshold to spike onset voltage, and is critical in ensuring a sufficiently rapid depolarization to securely activate spikes (Crill 1996; Harvey et al. 2006b; Lee and Heckman 2001). However, the NaPIC slowly inactivates after a few seconds of activity (Li and Bennett 2003). The CaPICs are mediated by low-threshold nifedipine-sensitive L-type calcium channels (CaV 1.3; located in distal dendritic regions of spinal motoneurons) (Carlin et al. 2000a; Heckmann et al. 2005; Hounsgaard and Kiehn 1989; Li and Bennett 2003; Zhang et al. 2006). Importantly, the Ca PICs are activated somewhat slowly, requiring depolarizations lasting about 100 ms, but subsequently produce many seconds of sustained firing (Hounsgaard and Kiehn 1989; Lee and Heckman 1998a; Li and Bennett 2003; Schwindt and Crill 1980). In the chronic spinal rat, the Na and CaPIC are of equal amplitude when they are first activated. However, Ca PICs inactive less than the Na PICs and thus, after a few seconds of PIC activation, the Ca PIC dominates the response (two-third of total sustained PIC) (Li and Bennett 2003).

With a brief synaptic input to motoneurons, these PICs are activated due to their unusually low threshold, and they dramatically amplify this synaptic input at motoneuron recruitment (by a factor of 3 or more) (Lee and Heckman 2000). However, after recruitment and PIC activation, the activated PICs paradoxically increase the conductance of the motoneuron, lowering the F-I slope and reducing the effectiveness of additional synaptic input (Li et al. 2004a). Functionally, during motoneuron discharge, PICs can remain active for many seconds, producing a sustained depolarization (plateau) and self-sustained firing that ultimately helps to sustain muscle contractions (Bennett et al. 2004; Brownstone

et al. 1994; Heckmann et al. 2005; Hounsgaard et al. 1988; Hultborn et al. 2003; Lee and Heckman 2000).

Mechanisms underlying spasms

Complete transection of the spinal cord immediately reduces PICs and general motoneuronal excitability due to the massive loss of brainstem monoamine innervation, rendering the spinal cord unexcitable (Hounsgaard et al. 1988; Miller et al. 1996). However, over time after injury (weeks to months), motoneurons spontaneously recover large PICs and accordingly become very excitable (Li and Bennett 2003), despite the continued absence of normal brainstem innervation. However, unlike before injury, the motoneurons are tonically excitable, with no descending control over PIC inhibition. In a normal intact spinal cord there exist numerous inhibitory control mechanisms that help hyperpolarize the motoneurons to terminate PICs and avoid uncontrolled muscle contractions. These include the control of volitional supraspinal inputs to motoneurons and interneurons (Baldissera and Gustafsson 1971), control of brainstem monoaminergic systems (5-HT and NE) (Hounsgaard et al. 1988), and descending inhibition of segmental reflexes (Baker and Chandler 1987; Clarke et al. 2002; Jankowska 1992; Thompson et al. 1992). Spinal injury causes an immediate loss of inhibition over dorsal horn reflex transmission, leading to exaggerated excitatory postsynaptic potentials (EPSPs) on motoneurons in response to brief innocuous sensory stimulation (Baker and Chandler 1987; Bennett et al. 2004; Heckman 1994; Li et al. 2004b). Once the motoneurons recover their excitability (PICs), activated PICs are difficult to terminate, due to the loss of descending inhibitory control, and this ultimately causes sustained motoneuron discharges (muscle spasms) (Hultborn et al. 2003; Rekling et al. 2000).

The long-lasting reflexes (LLRs and associated muscle spasms) present after SCI are produced mostly by Ca PICs as mentioned above; (Li et al. 2007). This is demonstrated by the reduction of self-sustained firing by blocking the L-type calcium channels with nimodipine as well as by hyperpolarizing the motoneuron

(Li et al. 2004b), leaving only a half-second long excitatory polysynaptic potential (EPSP; synaptic input). Thus, spasms result almost entirely from the voltage-dependent Ca PIC activation intrinsic to the motoneurons. The key question that remains is; what causes the recovery of motoneuron excitability (Ca PICs) after injury in the absence of 5-HT? This is the focus of the first two chapters of this thesis.

The 5-HT family of receptors and their signaling pathways

Brainstem derived serotonin (5-HT) is a potent neuromodulator of spinal motoneurons that plays a critical role in regulating normal levels of spinal cord excitability. 5-HT increases motoneuron excitability by facilitating Ca PICs (Li et al. 2007) and also modulates a number of other membrane properties that make PICs more easily activated (i.e. lowers the spike threshold, depolarizes motoneurons, increases input resistance). Each of these properties are mediated by different 5-HT receptors and 5-HT receptor subtypes (Harvey et al. 2006a; Rekling et al. 2000). 5-HT receptors are classified into seven distinct families based on their structure; 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7, several of which have been identified in the spinal cord. In addition, the 5-HT_1 receptor contains the subtypes A, B, D, E and F and the 5-HT_2 receptor has three subtypes A, B and C. The serotonin receptors are a group of G-protein-coupled receptors (GPCRs), with the exception of 5-HT_3 (a directly-gated ion channel), that modulate both excitatory and inhibitory neuronal transmission (Hannon and Hoyer 2008). The 5-HT_1 family of receptors is negatively coupled to adenylate cyclase (AC) via the Gi family of G-proteins; consistent with a general role in inhibiting interneuron and afferent transmission. The 5-HT_2 receptors couple to Gq-proteins that stimulate membrane-bound phospholipase-C (PLC) and are generally excitatory and postsynaptic. The 5-$HT_{4/6/7}$ receptors are positively coupled to cAMP production via the Gs family of G-proteins, and these receptors generally increase neuron excitability. The most recently discovered serotonin receptor is 5-HT_5, appears to resemble the 5-HT_1 receptors in that it acts via Gs-proteins and functionally appears to have inhibitory and presynaptic actions. All

known 5-HT receptors have been cloned, allowing detailed analysis for the development of highly selective drugs, including quantitative information on their binding affinities (Boess and Martin 1994). Many highly selective 5-HT receptor antagonists are now available, however, the current 5-HT receptor agonists are not as selective as antagonists and there can be considerable overlap between binding affinities of a specific ligand for various receptor subtypes. Thus, caution must be taken when ascribing results to a particular receptor. However, by correlating the potency of many agonists in facilitating a response with their published binding affinities at recombinant 5-HT receptor subtypes, it is possible to determine which receptor subtypes are involved in mediating responses, and this is the approach we take in Chapter 3 of this thesis.

The Role of 5-HT_2 receptors in mediating spasm activity

Spinal actions of 5-HT have been strongly related to a facilitation of motor output. The majority of studies performed on spinal motoneurons demonstrate that the 5-HT_2 receptors, which are most abundant in the ventral horn, directly mediate increases in spinal cord excitability (Harvey et al. 2006a; Perrier and Hounsgaard 2003). 5-HT_2 receptor agonists increase the activity of spinal cord motoneurons, facilitate mono- and polysynaptic reflexes, as well as depolarize and increase glutamate-evoked firing in motoneurons (Anderson et al. 1991; Clarke et al. 1996; Jackson and White 1990; Nagano et al. 1988; Yamazaki et al. 1992). Importantly, Perrier and Hounsgaard (2003) and Harvey et al (2006a) have shown that activation of 5-HT_2 receptors on spinal motoneurons facilitates of the Ca PIC. All three 5-HT_2 receptor subtypes (A-C) are Gq-protein coupled receptors that generally activate the classic PLC pathways involved in the synthesis of inositol phosphates (IP) and mobilization of intracellular Ca^{2+} stores. There has been much speculation and uncertainty as to which of the 5-HT_2 receptor subtypes are involved in facilitating motoneuron excitatability (e.g. PICs) in spinal motoneurons (Landry and Guertin 2004; Perrier and Delgado-Lezama 2005; Perrier and Hounsgaard 2003). Determining which 5-HT_2 receptor(s) is involved

after SCI and how this receptor is activated after injury in the absence of brainstem 5-HT are the goals of Chapters 2 and 3 of this thesis.

Possible constitutively active receptors after chronic spinal cord injury?

Even though PICs depend on 5-HT, and nearly all of the 5-HT innervation is lost after spinal cord injury (>90% lost) (Cassam et al. 1997; Newton and Hamill 1988; Schmidt and Jordan 2000), motoneurons somehow recover their PICs as though their monoamine receptor activation is restored. Thus, what causes the recovery of large PICs after chronic injury in the absence of brainstem 5-HT that normally facilitates the Ca PICs via 5-HT_2 receptors? Interestingly, 5-HT_2 receptors exhibit a substantial degree of constitutive activity, in the form of IP production in the absence of 5-HT (or other agonists), producing a basal level of IP (Seifert and Wenzel-Seifert 2002). Furthermore Nakae et al. have recently shown that the constitutively active isoform of the 5-HT_{2C} receptor is upregulated after partial contusion of the spinal cord (Nakae et al. 2008). In constitutive activity, the receptor moves spontaneously from its resting state, to its activated state, which binds to its G-protein (Seifert and Wenzel-Seifert 2002). The 5-HT_2 Gq-coupled receptors have the ability to isomerize from the inactive to the active state spontaneously (independent from agonist binding), increasing basal G-protein- and effector system activity, including increasing basal IP accumulation. The standard assay to determine the presence of constitutive activity in these receptors is the analysis of basal IP accumulation in isolated cells transfected with 5-HT2 receptors (Seifert and Wenzel-Seifert 2002).

In Chapter 2 of this thesis we explore the hypothesis that the development of constitutive activity in the 5-HT_2 receptors is responsible for the recovery of motoneuron function after chronic SCI. To address this question we use a number of antagonists that can block constitutive activity and others that do not. Specifically, a class of antagonists known as inverse agonists (e.g. cyproheptadine) inhibits the basal IP production (i.e. constitutive activity) as well as agonist induced activity (Westphal and Sanders-Bush 1994). In addition, a

class of drugs identified as neutral antagonists (usually termed antagonists, e.g. methysergide) blocks only the action of agonists (Westphal and Sanders-Bush 1994). The action of an inverse agonist alone is not sufficient to prove the presence of constitutive receptor activity, if there is any chance that the receptor is activated by 5-HT (e.g. residual 5-HT in the spinal cord). Definitive proof of constitutive activity only comes by also showing that neutral antagonists have no effect on their own even though they can antagonize exogenously applied 5-HT agonists (Seifert and Wenzel-Seifert 2002). If it does turn out that constitutive 5-HT_2 receptor activity contributes to the emergence of Ca PICs and LLRs/spasm, blocking the 5-HT_2 activity with inverse agonists may prove to be an important focus for pharmacological treatment of the spasticity disorder.

Role of the long EPSP in generating muscle spasms

As mentioned, with spinal cord injury there is a general loss of inhibition over sensory afferent transmission (disinhibition), and this causes unusually large polysynaptic excitatory postsynaptic potentials (EPSPs; > 0.5sec) to emerge immediately after injury. Long EPSPs are generated in motoneurons after injury by activation of low-threshold cutaneous afferents in cats and rats (Baker and Chandler 1987; Li et al. 2004c) and play a critical role in triggering long-lasting reflexes (spasms) because they are long enough to fully activate the slowly activating Ca PICs. This long pronounced EPSP can be observed if the motoneuron is hyperpolarized to block the voltage-dependent PICs (Li et al. 2004b; Li et al. 2004c). If motoneurons do not possess a clear long EPSP then they will not produce LLRs in response to low threshold stimulation even though they still have large Ca PICs (Li et al. 2004b). Interestingly, long EPSPs emerge acutely after injury in the cat (Baker and Chandler 1987) and rat (Li et al. 2004c), and remain unattenuated, suggesting that unlike the PIC, these EPSPs do not adapt to return to their 'normal pre-injury' state after chronic injury. Norton et al (2008) has shown in humans with chronic SCI, evidence for similar large cutaneous-evoked EPSPs using motor unit recordings. In the uninjured rat and human, instead of a pure 1-second long EPSP there appears to be a 300 ms EPSP

that is interrupted by a large IPSP (Bennett et al. 2004). Whereas immediately after spinal cord transection the net synaptic responses are excitatory and the long EPSP emerges, suggesting that the loss of brainstem innervation immediately disinhibits this long polysynaptic EPSP. In the chronic spinal rat, the polysynaptic EPSP remains large, and its effect combined with the voltage-dependent PIC contributes to the first second of firing during long-lasting reflexes, after which only the PIC contributes (Bennett et al. 2004). While the PIC activity underlies the many-second-long spasms seen in awake rats (Bennett et al. 2004) and humans (Gorassini et al. 2004), it is initiated by long duration synaptic inputs like the long EPSP. Thus, inhibiting this long EPSP offers a novel approach to spasticity management, preventing the initiation of PICs and spasms. The role of EPSPs in generating muscle spasms is the focus of Chapter four of this thesis.

Inhibitory actions of 5-HT$_1$ receptors

Along with mediating excitation in spinal motoneurons, 5-HT has a number of inhibitory actions on the processing of afferent input via dorsal horn interneurons, likely including both pre- and post-synaptic effects (Jankowska 1992; 2001; Yoshimura and Furue 2006). High-threshold and low-threshold muscle and skin afferent transmission is inhibited strongly by 5-HT (Schmidt and Jordan 2000). Thus, for example, polysynaptic pathways linked to the withdrawal (flexion) reflex system become hypersensitive following spinal cord transection due to the loss of 5-HT mediated inhibition (Cleland and Rymer 1993). This inhibition of afferent transmission is mediated by 5-HT1 receptors (Lopez-Garcia and King 1996; Schmidt and Jordan 2000; Wang and Dun 1990). Thus, after loss of monoamines with spinal cord injury, reflex transmission is disinhibited. This might explain the emergence of exaggerated and unusually long EPSPs (mentioned above) generated in motoneurons by stimulation of cutaneous nerves (Baker and Chandler 1987; Li et al. 2004d; Norton et al. 2008), and specifically may occur by loss of activation of Gi-coupled 5-HT$_1$ receptors. Thus, in Chapter 4 we test the hypothesis that the long EPSPs that trigger spasms are mediated by the 5-HT$_1$ type receptors, and specifically explore which receptor subtypes are

involved (A-F). We also examine whether there is constitutive activity in these 5-HT_1 receptors, like in the 5-HT_2 receptors, with chronic injury that compensates for loss of brainstem 5-HT. We suspect that because long EPSPs emerge acutely after injury and do not change with chronic injury (see above), compensation via constitutive activity does not occur. If there is a lack of normal 5-HT_1 receptor activity after injury, then reactivating these receptors via drugs offers a novel approach to inhibiting afferent transmission and the treatment of spasticity.

ANIMAL MODELS OF SPASTICITY AND LOCOMOTOR RECOVERY

The animal model used for the study of spasticity was developed by Bennett et al. (1999) where the spinal cords of female adult rats are transected at the S_2 level. This level of injury was chosen for many reasons. The cord below the injury innervates mainly the tail, thus locomotion and bowel/bladder functions are preserved, making animal care much easier. In addition, the tail is convenient to test for spasticity as it is readily accessible to manipulate and record EMG activity. The tail also closely resembles the muscular structure of the axial muscles of the back of human beings, making our findings relevant and important for human studies in the development of spasticity. Histological and electrical studies have shown that motoneuron size and electrical properties (resting membrane potential, input resistance, etc.) below the injury remain nearly identical as pre-injury state (Bennett et al. 2004). Most importantly, and most interestingly, is the ability of the sacrocaudal cord to survive whole when maintained in vitro due to its small size, allowing control over the external environment and viable *in vitro* recordings (Li et al. 2004c).

Immediately and for the first two weeks after injury, the tail does not elicit any responses (flaccid) (Bennett et al. 1999; Li et al. 2004c). Approximately two weeks post injury; tonic muscle activity begins to emerge, usually triggered by cutaneous stimulation or muscle stretch. With increasing time post injury, the muscle tone in the tail markedly increases, and the threshold of the stretch and cutaneous evoked reflexes decreases; that is, muscle spasms develop. At this time, even a brief stimulation is enough to evoke long-lasting reflexes in the chronic spinal rat that would otherwise have no effect on the tail of a normal uninjured animal (Bennett et al. 2001). Brief cutaneous or electrical stimulation of the tail produces whole tail contractions (spasms), the tip of the tail rotates (coils) often more than 360 degrees. These powerful whole-tail contractions only strengthen with time after injury due to the increased involvement of extensor muscles. The development of spasms in the rat tail is similar to the development of spasms in

humans after spinal cord injury (time course similar, with first flexor and then extensor spasms developing), suggesting that muscle spasms in both the rat and human have similar underlying mechanisms (Bennett et al. 1999).

A second animal model was used in order to study the mechanisms of spontaneous locomotor recovery after SCI. A staggered hemisection surgery (L1-L2 hemi-lesion on right side and T6-T7 hemi-lesion on left side four weeks later) was performed, removing most descending supraspinal axons below the injury, but leaving some propriospinal neuron connections intact (Courtine et al. 2008). In these animals, although the cord below the most caudal level of the injury no longer receives descending inputs, the rats are able to recover voluntarily-initiated walking (although forelimb-hindlimb coordination remained affected). This recovered walking is proposed to be mediated by the descending activation of propriospinal interneurons which transmit (bridge) this information to spinal networks and motoneurons below the injury involved in locomtion (Cowley et al. 2008). In Chapter 2, we investigate if the activation of constitutive $5HT_2$ receptors contributes to the spontaneous recovery produced by the staggered lesion.

HYPOTHESIS

The working hypothesis for this thesis was that spinal motoneurons regain their excitability following chronic spinal cord injury due to a change in 5-HT receptor conformation in which they develop constitutively active receptors, and not from a residual source of 5-HT remaining below the injury. In order to test this hypothesis we first needed to determine which 5-HT receptors were involved in spasms and the re-emergence of PICs that underlie the spasticity. Previous work has shown there is a strong relation between 5-HT_2 receptors and the reappearance of PICs, so we began our search there. We used several combinations of agonists and antagonists to isolate the receptors of interest in order to determine their role in generating PICs and spasms/LLRs. In addition we used two classes of antagonists, inverse agonists and neutral antagonists, to determine whether or not the receptor was constitutively active. We also examined whether such putative constitutive activity contributed to recovery of locomotor function after SCI. In addition to the 5-HT_2 receptors, we tested the 5-HT_1 family of receptors, which in a normal intact system play an inhibitory role in sensory afferent transmission to motoneurons (IPSPs and EPSPs). We examined what happened to these receptors following chronic injury, if they were involved in spasticity and if they developed constitutive activity with chronic injury as we suspect of the 5-HT_2 receptors. We used both *in vitro* and *in vivo* animal models, as well as human subjects with chronic SCI to test all hypotheses.

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Chapter 2: Recovery of motoneuron and locomotor function after chronic spinal cord injury depends on constitutive activity in 5-HT_{2C} receptors[1][2]

[1] Revised from Murray K, Nakae A, Stephens MJ, Rank M, D'Amico J, Harvey P, Li X, Harris L, Ballou EW, Anelli R, Heckman CJ, Mashimo T, Vavrek R, Sanelli L, Gorassini MA, Bennett DJ and Fouad K. *Nature Medicine.* 2010 Jun; 16(6): 694-700.

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INTRODUCTION

Severe spinal cord injury (SCI) causes an immediate paralysis of muscles innervated by motoneurons directly caudal to the injury site. This results not only from a loss of supraspinal tracts that subserve voluntary initiation of movement (e.g. corticospinal and reticulospinal tracts that use fast glutamatergic synaptic transmission (Hultborn et al. 2003; Rekling et al. 2000)), but also from a loss of descending brainstem tracts that provide spinal motoneurons with their major source of neuromodulators, like serotonin (5-HT) (Carlsson et al. 1963; Jacobs et al. 2002; Jordan et al. 2008; Rekling et al. 2000). Normally, brainstem-derived 5-HT sets spinal motoneurons and interneurons into an excitable state, ready to respond to fast glutamate synaptic inputs, and cause appropriate muscle contractions (Hounsgaard et al. 1988; Hultborn et al. 2003; Jacobs et al. 2002; Perrier and Delgado-Lezama 2005). 5-HT does this by activating 5-HT_2 receptors that facilitate ionic currents intrinsic to the motoneurons, including voltage-gated *persistent* Ca^{++} and Na^{+} currents (termed persistent inward currents: *PICs;* Ca PICs and Na PICs) (Harvey et al. 2006a; Heckman et al. 2005; Li et al. 2007; Perrier and Hounsgaard 2003; Rekling et al. 2000). These PICs are easily activated by brief synaptic inputs because of their unusually low threshold, and thus, serve a critical role in amplifying and prolonging the action of synaptic inputs, ultimately enabling sustained muscle contractions (Brownstone et al. 1994; Carlin et al. 2000; Heckman et al. 2005; Hounsgaard et al. 1988; Hultborn et al. 2003; Li et al. 2004a). Consequently, when SCI eliminates brainstem-derived 5-HT, motoneurons are left in an unexcitable state with small PICs

(Harvey et al. 2006b; Hounsgaard et al. 1988; Li et al. 2004a; Perrier and Hounsgaard 2003), consistent with the paralysis, areflexia and spinal shock seen early after SCI (Bennett et al. 1999; Bennett et al. 2004; Kuhn and Macht 1948). The importance of lost brainstem-derived 5-HT is demonstrated by the repeated finding that motoneuron excitability (PICs) and associated motor functions (locomotion) can be regained shortly after SCI with exogenous application of 5-HT or selective agonists that activate 5-HT_2 receptors (Harvey et al. 2006a; Heckman et al. 2005; Hounsgaard et al. 1988; Perrier and Hounsgaard 2003; Ung et al. 2008).

Remarkably, over the weeks after SCI (chronic injury), motoneurons spontaneously recover their excitability, with permanently large PICs and associated sustained firing (Button et al. 2008; Li et al. 2004a), despite the continued absence of brainstem-derived 5-HT. However, unlike before injury, the powerful depolarizing actions of PICs are difficult to terminate, because after injury motoneurons have weaker inhibitory inputs (Boulenguez et al.), especially from spinal interneurons that are normally regulated by descending tracts (Baldissera et al. 1981; Bennett et al. 2004; Crone et al. 2003; Li et al. 2004a; Norton et al. 2008; Shefchyk and Jordan 1985; Wallis et al. 1993). Thus, the PICs (especially Ca PICs) can lead to excessive motoneuron activity that produces uncontrolled and debilitating muscle contractions (*spasms*, lasting many seconds), in both humans (Gorassini et al. 2004) and rats (Bennett et al. 2004; Li et al. 2004a). To make matters worse, these PICs and spasms are readily triggered by

synaptic inputs arising from normally innocuous cutaneous stimulation or muscle stretch, because these synaptic inputs are enhanced after SCI (Ashby and McCrea 1987; Baker and Chandler 1987; Bennett et al. 2004; Dietz and Sinkjaer 2007; Li et al. 2004a; Norton et al. 2008).

An important question that remains is: how do motoneurons adapt so profoundly, recovering large PICs in the absence of brainstem-derived 5-HT? Here we consider the novel idea that 5-HT_2 receptors on spinal motoneurons become *constitutively active* (active without 5-HT) to compensate for lost brainstem 5-HT, ultimately helping to produce recovery of motoneuron excitability (PICs) and related motor functions such as locomotion. Constitutively active receptors spontaneously couple to their Gq-proteins and initiate intracellular signaling *without* being bound to 5-HT or any other ligand (Berg et al. 2008; Chanrion et al. 2008; Herrick-Davis et al. 1999; Marion et al. 2004; Niswender et al. 1999; Seifert and Wenzel-Seifert 2002; Westphal and Sanders-Bush 1994), a process well understood in isolated cell culture systems, but not previously considered for motoneurons. The 5-HT_{2C} receptor is an ideal candidate for such constitutive activity because it exhibits a number of native isoforms that have a high degree of constitutive activity in humans and rats (>50% active) (Herrick-Davis et al. 1999; Niswender et al. 1999). Furthermore, expression of these constitutively active isoforms increases in the cortex following depletion of 5-HT (Gurevich et al. 2002), suggesting that a similar change may be possible after SCI. We thus examined whether recovery of motoneuron function after SCI depends on

constitutive 5-HT receptor activity. We initially focused on showing that constitutive receptor activity causes spasms, because the emergence of spasms after SCI is an indirect measure of recovery of motoneuron and general motor function (albeit maladaptive) that is readily studied in rats and humans (motoneuron PICs cause spasms; see above). Following this, we evaluated how constitutive activity contributes to *locomotor* recovery. For studying spasms, we employed a complete spinal transection model (chronic spinal rat, Fig 1), which eliminates brainstem-derived 5-HT, thus minimizing the chance that receptors remain activated by 5-HT. Nevertheless, we still had to consider the role of other 5-HT sources, because even with a complete transection, some *residual* spinal 5-HT remains caudal to the injury (Newton and Hamill 1988), and motoneurons are extremely sensitive to small amounts of 5-HT after SCI (Harvey et al. 2006a; Li et al. 2007).

METHODS

Spinal lesions.

All animal use was approved by the University of Alberta Animal Care and Use Committee: Health Sciences. We completely *transected* spinal cords of adult female Sprague-Dawley rats at the S_2 sacral spinal level, and evaluated spasticity and motoneuron properties 6–12 weeks post-injury (chronic spinal state, see Supplementary Methods) (Bennett et al. 2004; Li et al. 2004a). Also, a separate group of female rats underwent a *staggered-hemisection (Courtine et al. 2008),* which, like a transection, removes most descending supraspinal axons below the injury (including 5-HT axons), but leaves intact some propriospinal neuron connections that enable the animal to voluntarily initiate walking, as detailed in the Supplementary Methods.

All human experiments were carried out with signed, informed consent of subjects and approved by the University of Alberta Health Research Ethics Board. Human subjects had chronic SCI with varied severity (Supplementary Table 2-1)**,** and did not take their antispastic medications on the experiment day.

Spasms in awake chronic spinal rat.

We evoked tail muscle spasms with brief electrical (3xThreshold, T) or manual stimulation of the skin of the tail, and recorded these spasms with tail muscle EMG (electromyogram) and video kinematic analysis, as detailed in the Supplementary Methods. Briefly, EMG was rectified and averaged over 10–40 ms

post stimulus (SLR) and 500–4000 ms post-stimulus (LLR), and tail flexion angle measured.

Spasms in humans with SCI.

We evoked leg spasms with a brief electrical stimulation of the medial arch of the foot (3–5xT) and recorded surface EMG responses over the tibialis anterior (TA) muscle (Fig. 2-5) (Gorassini et al. 2004). We computed the SLR and LLR by averaging EMG over the intervals 50–100 and 500–5000 ms post-stimulation, respectively, and then subtracting background EMG (see Supplementary Methods).

Ventral root and intracellular motoneuron recording in rats, in vitro.

The whole spinal cord caudal to the S_2 injury level was removed from chronic spinal rats and maintained *in vitro* for ventral root and intracellular motoneuron recordings(Li et al. 2004a; Li et al. 2004b), as described in the Supplementary Methods. Briefly, we stimulated a coccygeal dorsal root (Co_1) with a single pulse (0.1 ms, 0.02 mA, 3xT), recorded the reflex response on the S_4 and Co_1 sacrocaudal ventral roots, and computed the mean SLR (over 10–40 ms post-stimulation), LLR (500–4000 ms post-stimulation) and background activity (over 300 ms pre-stimulation). For intracellular recordings, sharp intracellular electrodes were advanced into motoneurons, and the Ca PIC was measured under voltage-clamp. The Ca PIC was quantified as the downward current deflection (Fig 2-3b, thick black line, at arrow) recorded during a slow upward voltage ramp

(Fig 2-3b, top, gray), relative to the leak current (thin line), in TTX. Characteristically, this Ca PIC was activated at low voltages (–56.7 ± 6.0 mV), deactivated at even lower voltages (on downward ramp) and mediated by L-type calcium channels (nimodipine-sensitive), as previously reported (Harvey et al. 2006b; Li et al. 2004a).

Locomotor assessment after SCI in rats.

Locomotion was evaluated 3 weeks after the staggered-hemisection using the BBB score (Basso et al. 1995), as detailed in the Supplementary Methods.

Drugs and solutions.

The drugs used were 5-HT, fenfluramine, SB242084, strychnine, para-chlorophenylalanine-methyl-ester (pCPA) (Sigma-Aldrich), α-methyl-5-HT, citalopram, cyproheptadine, methysergide, MK212, nimodipine, SB206553 (Tocris), and tetrodotoxin citrate (TTX; Alomone). In vitro, the ACSF consisted of (in mM) 122 NaCl, 24 NaHCO3, 2.5 CaCl2, 3 KCl, 1 MgCl2, and 12 D-glucose, saturated with 95% O_2 and 5% CO_2 (pH 7.4) and maintained at 22–24^{o}C. Drugs were dissolved in the ACSF. In vivo, drugs were administered via transcutaneous intrathecal (IT) injection (Mestre et al. 1994), intraperitoneal (IP) injection or oral gavage, and peak effects reported (at 5–20 min post-IT, and 60 min post-oral). SB206553 was used at a dose that produced maximal effects (on spasms) both *in vivo* and *in vitro* (determined by titration), and SB242084 was

used at the same dose, because SB206553 and SB242084 have similar binding affinity at 5-HT_{2C} receptors (Knight et al. 2004).

mRNA measurements.

We extracted RNA from the whole spinal cord below the S_2 injury level, and from this synthesized and amplified cDNA (with RT-PCR) to quantify the mRNA. We quantified RNA editing and 5-HT_{2C} isoforms by sequencing the DNA of bacterial colonies grown from single bacteria cells transfected with DNA fragments synthesized and amplified from spinal cord mRNA/cDNA (using 5-HT_{2C} receptor-related PCR primers; each colony adopts a *single* 5-HT_{2C}-receptor isoform). We computed editing efficiency at each of five sites (A–E in Fig. 2-4a) as the proportion of colonies with editing at that site in their sequence. We computed the proportion of each 5-HT_{2C} receptor isoform in the spinal cord, from the number colonies with that isoform, relative to the total number of colonies. See further details in Supplementary Methods.

Histology.

Immunofluorescence labeling for 5-HT and 5-HT_{2C} receptors was performed as described in the Supplementary Methods.

Statistics.

Statistical comparisons were performed using a paired *t*-test, after verifying normality. Data are reported as mean ± standard error (s.e.m.).

RESULTS

Lack of contribution of residual 5-HT to spasms.

Prior to injury, the spinal cord was densely innervated by 5-HT fibers along its whole length, particularly in the ventral horn (Fig. 2-1b). In contrast, after SCI in the chronic spinal rat only a few short (43.3 ± 25.0 μm) fibers remained (Fig. 2-1b), with on average 18 ± 11 such fibers along the whole length of the spinal cord below the injury.

To examine whether the remaining 5-HT fibers in chronic spinal rats had any functional effect on spasms and associated 5-HT_2 receptors, we blocked the action of 5-HT with an intrathecal injection (IT) of the highly selective 5-HT_{2C} receptor antagonist SB242084 (Fig. 2-1c,f). This injection did *not* significantly change the tail muscle spasms recorded with EMG *in vivo* (Fig. 2-1c; evoked by cutaneous stimulation), indicating that the 5-HT_2 receptors were not activated by residual 5-HT (or other endogenous ligands). Notably, SB242084 is a *neutral antagonist* that blocks only the action of 5-HT (or other agonists) on the 5-HT_2 receptors, and does *not* inhibit constitutive receptor activity (Chanrion et al. 2008; Seifert and Wenzel-Seifert 2002). We also found that depleting residual 5-HT with pCPA (Gurevich et al. 2002) did not significantly influence spasms (Fig 2-1f).

Spasms depend on constitutive 5-HT_2 receptor activity, in vivo and in vitro.

We next examined whether the loss of 5-HT after injury was compensated for by *constitutive activity* in 5-HT_2 receptors by intrathecally injecting SB206553,

which selectively binds to 5-HT_{2C} receptors and potently inhibits their constitutive activity (termed *inverse agonist* (Chanrion et al. 2008; Marion et al. 2004; Seifert and Wenzel-Seifert 2002)). This injection reduced the spasms recorded with either EMG (Fig 2-1d, f) or tail kinematics (Fig 2-1e, f) by well over 50%, whereas control saline injections had no effect. Likewise, oral application of the non-selective 5-HT_2 receptor inverse agonist cyproheptadine (Westphal and Sanders-Bush 1994) significantly reduced spasms (Fig 2-1f).

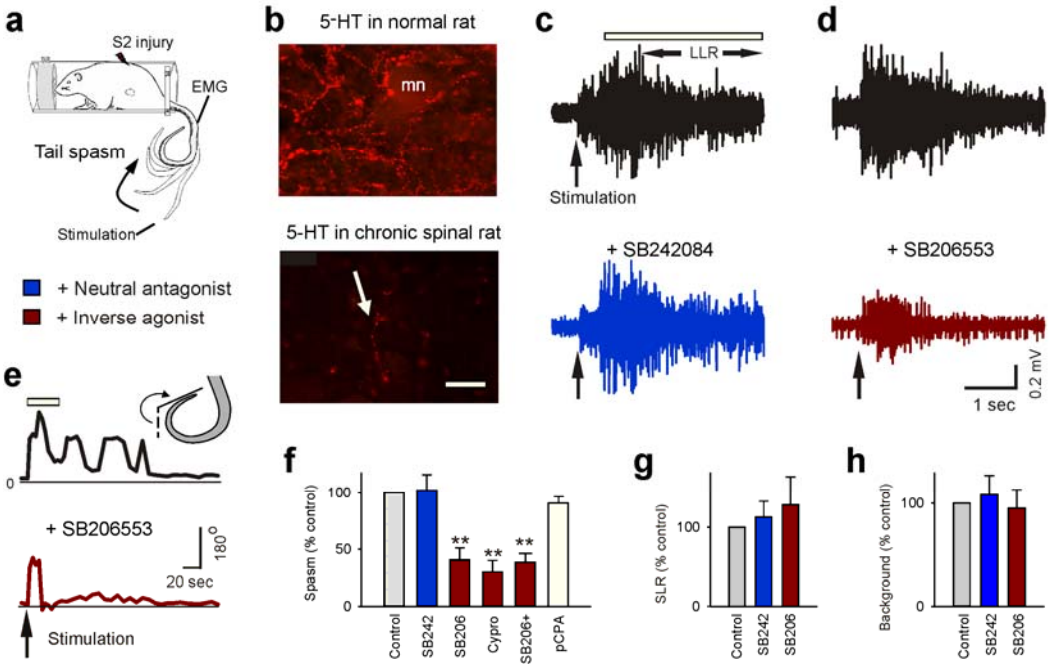

Figure 2-1. Constitutive 5-HT$_2$ receptor activity, but not residual 5-HT, causes spasms. (**a**) Tail spasm in awake chronic spinal rat with S_2 sacral transection. (**b**) Representative immunofluorescence images of 5-HT fibers (beaded) in the S_4 ventral horn of normal rats (top; mn, motoneuron, $n = 5$ rats) and chronic spinal rats (bottom; residual fibre at arrow, $n = 5$; scale-bar: 50 µm). (**c, d**) Spasms in chronic spinal rat evoked by electrical/cutaneous stimulation of the tail (pulse 3xT) and recorded with EMG (quantified during bar, LLR) before and after blocking effects of residual 5-HT with IT injection of the neutral antagonist SB242084 (3 mM in 30 µl saline). (**d**) Lack of spasm/LLR after blocking constitutive receptor activity with the inverse agonist SB206553 (IT, 3 mM in 30 µl saline). (**e**) Tail flexion angle during spasms before and after SB206553 injection, quantified during bar. (**f**) Group means of LLRs/spasms (normalized to pre-drug control) with SB242084 (abbreviated SB242; LLR), SB206553 (SB206 for LLR EMG recording; and SB206+ for tail-angle spasms) and cyproheptadine (cypro; LLR; 10 mg·kg^{-1} orally), and after depletion of residual 5-HT with pCPA (two 300 mg·kg^{-1} IP injections over 48 hours; tail-angle), with $n = 5$ rats per drug. (**g, h**) Normalized group means of SLR and background EMG with SB242084 and SB206553. $^{**}P < 0.01$: significant change relative to pre-drug control, 100%. Error bars, s.e.m.

We next examined the *whole* spinal cord from chronic spinal rats (caudal to the injury) after it was removed and maintained *in vitro,* which eliminated possible peripheral or brain-derived 5-HT influences. We recorded long-lasting reflexes (*LLRs*) from the ventral roots in response to a brief stimulation of dorsal roots (Fig 2-2a-c); these LLRs have previously been shown to underlie muscle spasms recorded *in vivo*(Bennett et al. 2004; Li et al. 2004a). The LLRs were not significantly affected by blocking the possible action of endogenous 5-HT with the 5-HT_{2C} receptor *neutral antagonists* SB242084 or methysergide (Westphal and Sanders-Bush 1994) (Fig. 2-2b, d), even though these antagonists blocked the increase in LLRs induced by exogenous application of selective 5-HT_{2C} agonists (Supplementary Fig 2-1). Furthermore, enhancing available residual endogenous 5-HT with either the 5-HT transport-blocker citalopram or 5-HT releaser fenfluramine did not significantly affect LLRs (Supplementary Fig 2-2). In contrast, the LLRs were markedly inhibited by blocking constitutive 5-HT_2 receptor activity with *inverse agonists* (SB206553 and cyproheptadine; Fig. 2-2c, d). This inhibitory action of the SB206553 was blocked by a prior application of methysergide (Fig 2-2d), which competitively inhibits SB206553 binding to 5-HT_{2C} receptors (Chanrion et al. 2008).

The transient short latency reflexes (SLRs) evoked immediately after the stimulation (Fig 2-2c) were not affected by SB206553, both *in vitro* (Fig 2-2e) and *in vivo* (Fig 2-1g), and not correlated with the LLRs (spasms; $r^2 = 0.10$) (Dietz and Sinkjaer 2007), consistent with a negligible modulation of SLRs by Ca

PICs and associated 5-HT_{2C} receptors. Also, the background activity prior to the LLRs had relatively little (Fig 2-2f, *in vitro*) or no (Fig 2-1h, *in vivo*) change with SB206553.

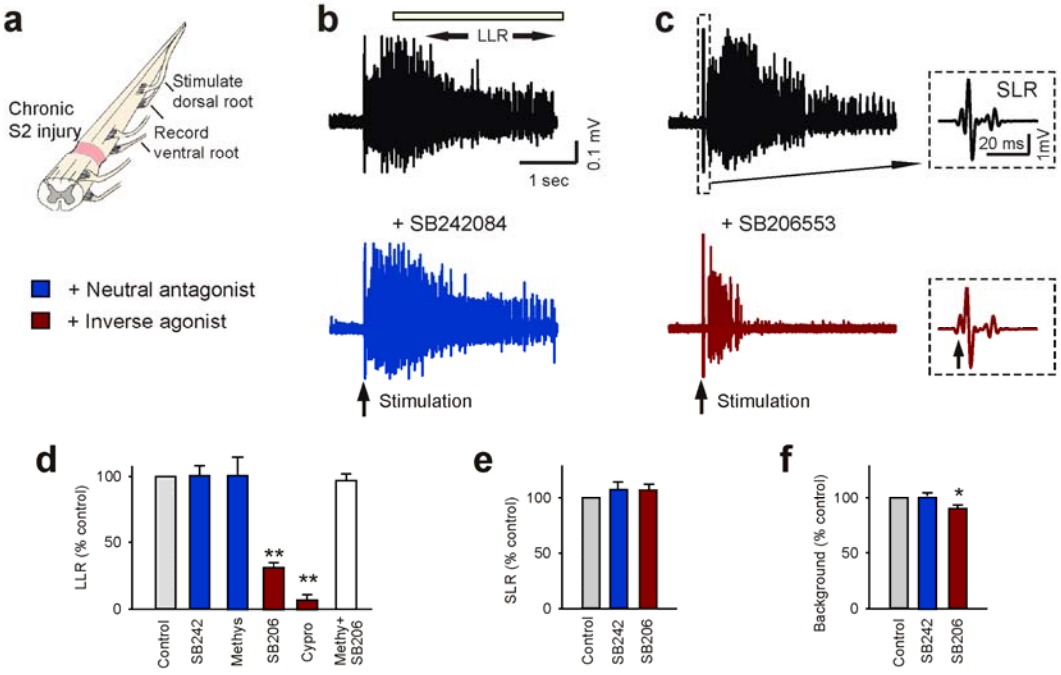

Figure 2-2. Constitutive 5-HT_2 receptor activity contributes to LLRs in the isolated spinal cord, *in vitro*. (**a**) Whole sacrocaudal spinal cord below chronic S2 transection maintained *in vitro*. (**b**) Long-lasting reflex triggered by dorsal root stimulation (single pulse, 3xT) and recorded from the ventral roots (LLR, quantified during horizontal bar; counterpart of spasms in Fig. 2-1) before and after blocking effects of residual 5-HT with the neutral 5-HT_2 receptor antagonist SB242084 (3 - 5 µM). (**c**) Elimination of LLR, but not SLR, after blocking constitutive 5-HT_2 receptor activity with the inverse agonist SB206553 (3–5 µM). Inset: short-latency reflex (SLR, expanded time scale). (**d**) Group means of LLRs (normalized to pre-drug LLRs) with SB242084 (abbreviated SB242, n = 11), methysergide (Methys, 10 µM, n = 12), SB206553 (SB206, n = 24), cyproheptadine (Cypro, 20 µM; n = 6), and SB206553 after prior application of methysergide (30 µM; white bar; Methy+SB206; n = 8). **(e, f)** Normalized group means of the SLR and background ventral root activity with SB206553 and SB242084. *P<0.05, **P<0.01, significance relative to control, 100%. Error bars, s.e.m.

Constitutive 5-HT_2 receptor activity in motoneurons.

Considering that spasms result from persistent calcium currents (Ca PICs) in motoneurons (Gorassini et al. 2004; Li et al. 2004a), we made intracellular recordings from motoneurons after SCI to investigate whether there were constitutively active 5-HT_{2C} receptors on motoneurons that regulate Ca PICs (Fig 2-3). As previously described, the large voltage-dependent Ca PICs in motoneurons were readily observed in isolation as a sharp downward deflection in the current response during an increasing voltage ramp (Fig 2-3b), after sodium currents and synaptic inputs were eliminated with TTX (Li et al. 2004a). A block of constitutively active 5-HT_2 receptors with the inverse agonists SB206553 or cyproheptadine markedly decreased these Ca PICs (Fig 2-3b,f), whereas SB242084 had no effect on Ca PICs (Fig 2-3c,f). The portion of the Ca PICs that resulted from constitutive 5-HT_2 receptor activity (SB206553-sensitive decrease) was 1.99 ± 0.42 nA, which was 42.9 ± 8.9% of the maximum possible Ca PICs produced by activating all 5-HT_2 receptors (with 1 μM 5-HT). The small remaining Ca PICs with inverse agonists in chronic spinal rats was similar to the small Ca PICs observed acutely after spinal transection (Fig. 2-3f, white bar).

When we stimulated the dorsal roots during recording from a motoneuron at rest and in the absence of TTX, the PIC produced a sustained depolarization (plateau) (Li et al. 2004a) that caused many seconds of repetitive firing (LLR; Fig 2-3d). As expected, this LLR and plateau was eliminated by the inverse agonist SB206553 (Fig 2-3e). This LLR and plateau was also eliminated by simply hyperpolarizing

the motoneuron, to prevent activation of the underlying voltage-dependent PIC (Fig 2-3d) (Li et al. 2004a), though there remained a polysynaptic excitatory postsynaptic potential (EPSP), lasting about 0.5 sec. The inverse agonists SB206553 and cyproheptadine had no influence on this EPSP (Fig 2-3e,g).

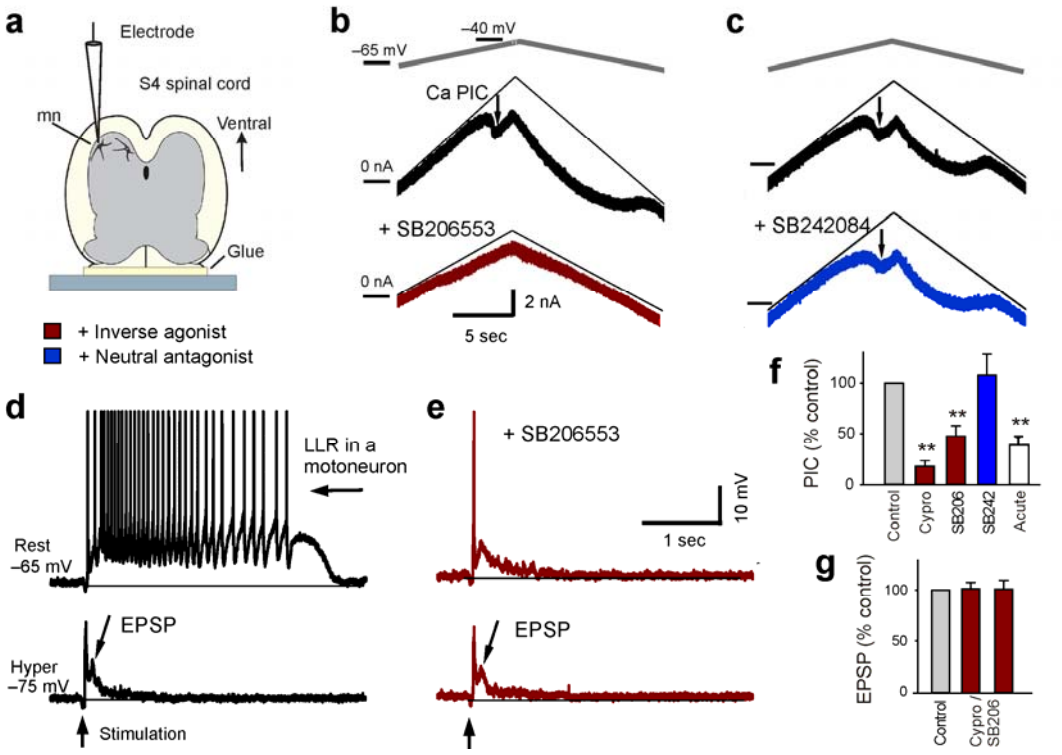

Figure 2-3. Constitutively active 5-HT$_2$ receptors on motoneurons contribute to Ca PICs underlying spasms. (**a, b**) Intracellular recording from motoneuron (mn) in whole spinal cord, *in vitro*. (**b**) Ca PIC in motoneuron of chronic spinal rat, activated by slowly increasing the membrane potential under voltage-clamp 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). Lower plot: small Ca PIC after SB206553 application (5 μM). (**c**) Another motoneuron with Ca PIC (arrow), unaffected by SB242084 application (5 μM). (**d**) PIC-mediated plateau and sustained firing (LLR) evoked by dorsal root stimulation (3xT; without TTX) in a motoneuron at rest (without injected current; top). With a hyperpolarizing bias current to prevent PIC activation, the same stimulation only evoked a polysynaptic EPSP (lower plot). (**e**) Same motoneuron, with SB206553 (5 μM). (**f**) Group means of Ca PIC (normalized to pre-drug Ca PIC in chronic spinal rats, control), with SB206553 (SB206; $n = 7$), cyproheptadine (cypro, 20 μM; $n = 16$), and SB242084 (SB242; $n = 5$) in chronic spinal rats, and in acute spinal rats (white bar, no drugs, $n = 7$). (**g**) Normalized group means of EPSP amplitude (middle bar; control mean 4.4 mV) and duration (right bar, control 480 ms) with inverse agonists cyproheptadine or SB206553 (chronic). $^{**}P<0.01$, relative to control, 100%. Error bars, s.e.m.

Increase in constitutively active 5-HT_{2C} receptor isoform.

The 5-HT_{2C} receptor RNA undergoes post-transcriptional editing at five sites (labeled A to E) that leads to numerous native receptor isoforms in rats and humans (Fig 2-4a) (Chanrion et al. 2008; Herrick-Davis et al. 1999; Nakae et al. 2008; Niswender et al. 1999; Westphal and Sanders-Bush 1994). Functionally, the unedited isoform (INI) exhibits a high degree of constitutive activity, whereas editing reduces this activity, producing isoforms with less constitutive activity, like VNI (with 51% of INI activity) and VSV (32% of INI)(Berg et al. 2008). We thus compared 5-HT_{2C} receptor mRNA from spinal cords of normal (unlesioned) and chronic spinal rats (below S_2 injury level). The total amount of 5-HT_{2C} mRNA did not change with SCI (Fig 2-4b). However, there was a decrease in the amount of RNA editing at the A-site (Fig 2-4c). Corresponding to this, there was also a decrease in the VNI receptor isoform and an increase in the relative proportion of the highly constitutively active INI isoform (Fig 2-4d). The increase in INI isoform expression (400%) was similar to the increase in PIC with chronic injury (Fig 2-4e).

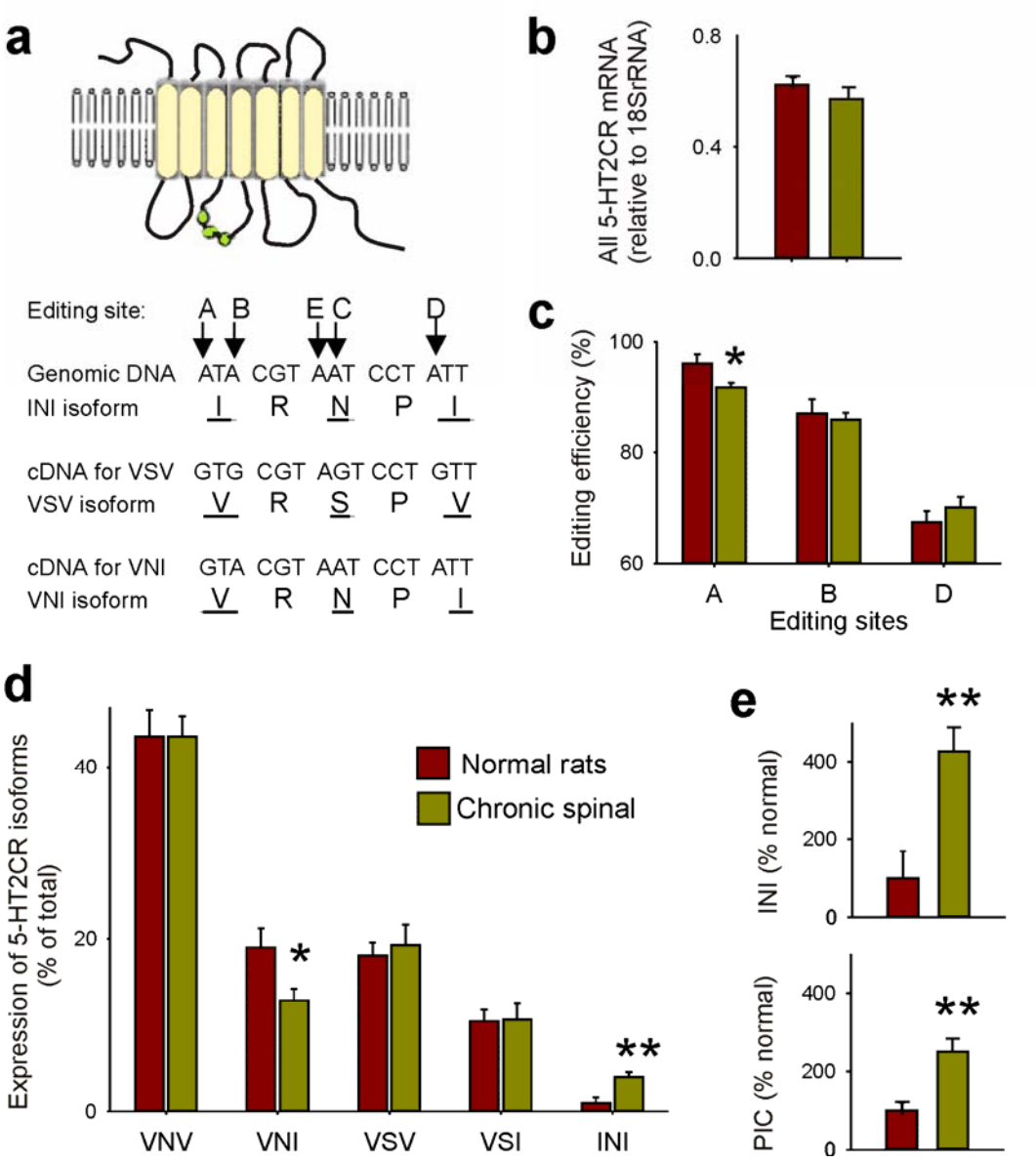

Figure 2-4. A highly constitutively active 5-HT_{2C} receptor isoform is upregulated with injury. (**a**) Three amino acids (underlined) in the 5-HT_{2C} receptor can be changed by post-transcriptional editing of RNA at 5 sites (A–E; adenosine editing), leading to different native receptor isoforms, of which the unedited isoform (INI) is most highly constitutively active. (**b**) Total 5-HT_{2C} receptor mRNA (normalized to an internal control, 18SrRNA) in chronic spinal rats (n = 6, green) and normal uninjured rats (n = 6, red). (**c**) Proportion of 5-HT_{2C} receptor mRNA with editing at sites A, B and D (editing efficiency) in chronic spinal and normal rats (C and E -site editing-efficiency < 30% and not changed, not shown). (**d**) Distribution of the 5-HT_{2C} receptor isoform mRNA in the spinal cord of normal and chronic spinal rats (15 different isoforms detected; 5 most prevalent shown). (**e**) Relative increase in the INI isoform with chronic injury is similar to the increase in Ca PIC with chronic injury (green), compared to normal (red, *in vitro* normal cord). *P<0.05, **P<0.01, significant change with injury. Error bars, s.e.m.

We directly confirmed that the motoneurons of the sacral spinal cord had 5-HT_{2C} receptors after SCI using immunolabeling, as shown in Supplementary Fig 2-3. Furthermore, a large fraction of the 5-HT_{2C} receptor labeling was inside the motoneurons (intracellular) in chronic spinal rats, and this receptor internalization was reduced by SB206553, consistent with the presence of constitutively active isoforms of the receptor on motoneurons, the hallmark of which is a high degree of activity-dependent internalization (INI isoform (Chanrion et al. 2008; Marion et al. 2004); Supplementary Figs 2-3 and 2-4).

Antispastic action of inverse agonists in humans with SCI.

In humans with SCI, we evoked leg muscle spasms with cutaneous stimulation of the foot while recording tibialis anterior (TA) muscle EMG (Fig 2-5a)(Gorassini et al. 2004). Blocking constitutive 5-HT_2 receptor activity with oral administration of the inverse agonist cyproheptadine significantly decreased the muscle spasms (Fig 2-5b). Further, the effect was again selective to the long-lasting portion of the spasm (LLR, Fig 2-5b–d), with no drug-induced change in the SLR (Fig 2-5b,e). Spasms were equally reduced by cyproheptadine in subjects with varying impairment of motor function (AIS B–D; Supplementary Table 2-1).

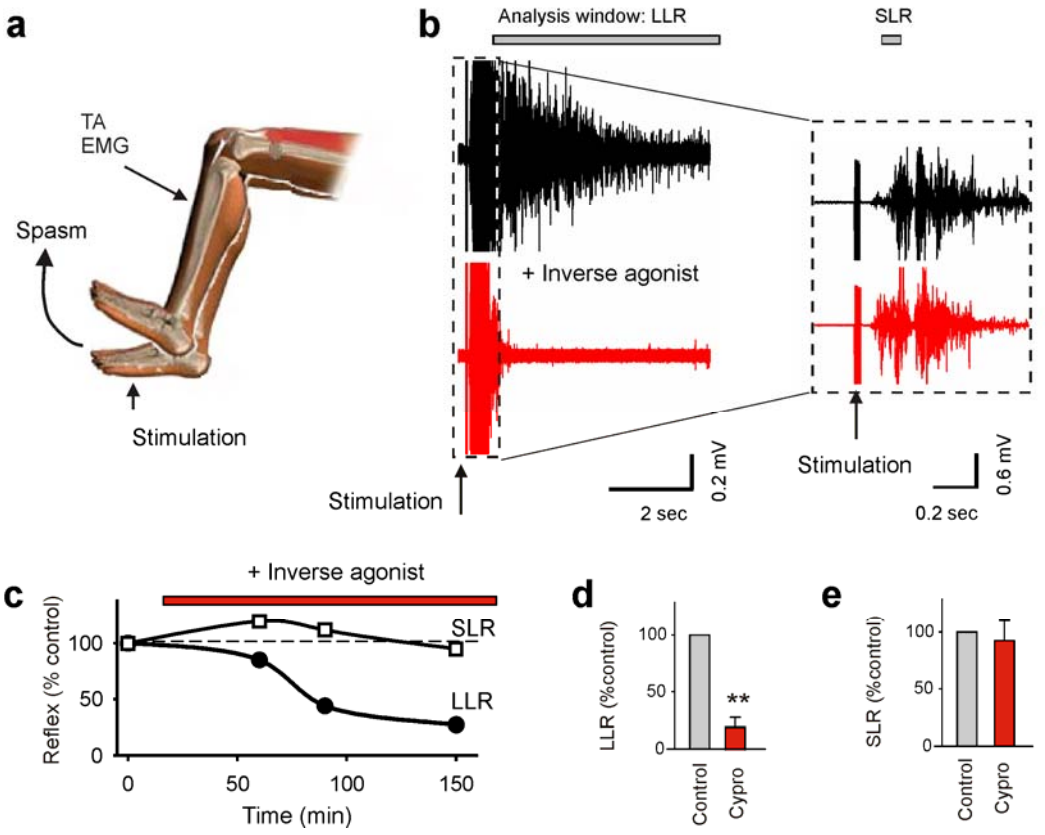

Figure 2-5. 5-HT_2 receptor inverse agonist blocks spasms in spinal cord injured humans. (**a**) Leg spasm triggered by brief electrical stimulation of the medial arch of the foot (3–5xT). (**b**) Spasm recorded with TA muscle surface EMG, and quantified over time windows indicated (LLR and SLR), before and 2 hr after blocking constitutively active 5-HT_2 receptors with cyproheptadine (8 mg administered orally). Inset on different scale shows SLR. (**c**) Gradual reduction in the spasms/LLRs (circles), but not SLRs (squares), over time after inverse agonist application. (**d, e**) Normalized group means for LLRs and SLRs with cyproheptadine (n = 7 subjects). **P<0.01 relative to control, 100%. Error bars, s.e.m.

Dependence of walking on constitutively active 5-HT_2 receptors.

To evaluate if constitutive 5-HT receptor activity contributes to recovery of locomotion after partial SCI, we employed a staggered hemisection injury model (Fig 2-6a) that transects all descending 5-HT axons, but spares enough propriospinal neurons that traverse the injury site to allow the animal to voluntarily initiate functional hindlimb locomotion(Courtine et al. 2008). Three weeks after this injury, rats regained good hindlimb locomotor ability, voluntarily initiating walking with near normal weight support, though they retained a deficit in forelimb-hindlimb coordination (thus BBB score(Basso et al. 1995) < 12; Fig 2-6b). Blocking constitutively active 5-HT_2 receptors with the inverse agonist SB206553 (IT) dramatically reduced weight support (hindlimbs dragged; Fig 2-6c) and overall locomotor ability (BBB score, Fig 2-6c,d). In contrast, blocking possible action of residual 5-HT with the neutral antagonist SB242084 had no significant effect (Fig 2-6d).

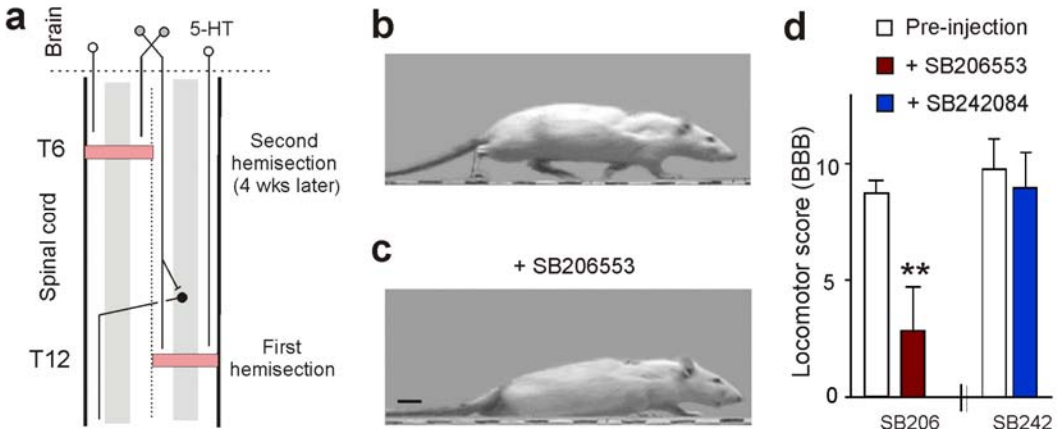

Figure 2-6. Spontaneous recovery of locomotion in staggered-hemisected rats depends on constitutively active 5-HT_2 receptors. (**a**) Schematic of staggered-hemisection SCI, which transects all descending axons from brain, including 5-HT neurons (white circles), but leaves local propriospinal neurons (black) that transverse the injury and help relay descending signals for initiation of locomotion (gray)[41]. (**b**) Rat walking with good weight support and toe clearance three weeks after the staggered-hemisection (after second hemisection). (**c**) Same rat with little hindlimb weight support (just foot paddling motions), while the forelimbs dragged the hind-quarters during walking, after blocking constitutively active 5-HT_2 receptors with SB206553 (3 mM in 30 µl saline, IT; same dose in Fig. 2-1). Scale bar 2 cm. (**d**) Group means of BBB locomotor scores before and after SB206553 injection (n = 8) and control SB242084 injection (3 mM in 30 µl saline, IT; n = 8 rats). $^{**}P<0.01$, relative to pre-injection. Error bars, s.e.m.

DISCUSSION

A loss of brainstem-derived 5-HT after SCI acutely reduces motoneuron excitability(Harvey et al. 2006b; Hounsgaard et al. 1988; Li et al. 2007; Perrier and Delgado-Lezama 2005; Perrier and Hounsgaard 2003) and accordingly depresses all motor functions. Our results demonstrate a novel mechanism for how spinal motoneurons compensate for this lost 5-HT over the months following injury (chronic injury). Decreased editing at a single site on the 5-HT_{2C} receptor RNA (A-site) leads to increased expression of the constitutively active INI isoform of this receptor. Constitutive 5-HT_2 receptor activity in turn leads to large Ca PICs in motoneurons, which ultimately enable motoneurons to recover their excitability, as evidenced by their sensitivity to inverse agonists. Considering that large PICs in motoneurons have been shown to be important in normal motor function in *uninjured* humans and animals(Heckman et al. 2005), these results suggest that constitutive 5-HT receptor activity (with its associated PICs) plays an essential role in recovery of motor function after SCI. Indeed, we show that constitutive 5-HT_2 receptor activity is crucial for spontaneous recovery of hindlimb locomotor function after partial SCI (Fig 2-6), because inverse agonists impair this locomotion.

Considering that inverse agonists inhibit conventional activation of 5-HT_2 receptors by 5-HT, as well as constitutive activity, their action alone is not definitive proof of constitutive activity, without ruling out the influence of endogenous residual 5-HT(Chanrion et al. 2008; Seifert and Wenzel-Seifert

2002). We thus ruled out residual 5-HT by showing a complete lack of effect of: neutral antagonists, 5-HT depletion, *in vitro* spinal cord isolation, SERT blockers, and 5-HT releasers after SCI in rats.

Our results also show that, without normal descending supraspinal control, these constitutively active 5-HT_2 receptors and associated PICs can, unfortunately, lead to uncontrolled motoneuron firing and associated muscle spasms (LLRs), which emerge over the weeks after injury(Bennett et al. 2004). However, blocking this constitutive receptor activity with inverse agonists decreases spasms in rats and humans with SCI, suggesting a new rationale for antispastic drug development, though care must obviously be taken in using a dose that preserves some residual function. For example, the high dose of SB206553 used here to maximally block spasms in the transected rat (Fig 2-1) also eliminates locomotion in the rat after partial SCI (Fig 2-6). In contrast, low doses of the broad-spectrum inverse agonist cyproheptadine have been shown to improve locomotion in humans(Wainberg et al. 1990), presumably by reducing the amplitude (Fig 2-5) and incidence(Barbeau et al. 1982) of spasms that can interfere with stepping, without completely eliminating PICs and muscle strength. The EPSPs that trigger spasms (and associated SLRs) are *not* affected by 5-HT_{2C} receptor inverse agonists (Fig 2-1, 2, 3), whereas they are inhibited by traditional antispastic drugs like baclofen, because they are regulated by other receptors, presynaptically(Davidoff 1985; Li et al. 2004b). Thus, inverse agonists provide an independent and complementary

approach to traditional spasticity management(Ashby and McCrea 1987; Davidoff 1985; Dietz and Sinkjaer 2007).

Taken together, our pharmacological, mRNA and immunolabeling data suggest that the large PICs on motoneurons after SCI are facilitated by constitutive activity in 5-HT_{2C} type receptors on motoneurons (perhaps with additional involvement of 5-HT_{2B} receptors, because SB206553 blocks both 5-HT_{2B} and 5-HT_{2C} receptors(Kennett et al. 1996; Kennett et al. 1997; Knight et al. 2004)). 5-HT_{2C} receptors activate the intracellular phospholipase C (PLC) pathway that leads to inositol phosphate (IP) synthesis and mobilization of intracellular Ca^{2+} stores(Hoyer et al. 2002; Mizuno and Itoh 2009). Constitutive 5-HT_{2C} receptor activity leads to a basal level of activity in this PLC pathway (intracellular IP synthesis), which is inhibited by receptor blockade with inverse agonists like SB206553, but not by neutral antagonists like SB242084(Chanrion et al. 2008; Kennett et al. 1996; Kennett et al. 1997; Seifert and Wenzel-Seifert 2002). Our analogous results with SB206553 and SB242084 suggest that an intracellular PLC pathway in motoneurons is tonically activated after SCI by constitutive activity, especially considering that motoneurons (PICs) are known to be regulated by PLC, IP and intracellular Ca^{2+} levels(Holohean and Hackman 2004; Mejia-Gervacio et al. 2004; Perrier et al. 2000).

The INI 5-HT_{2C} receptor isoform that we find upregulated in the spinal cord following chronic SCI exhibits a substantial amount of constitutive activity, with

basal levels of IP approaching 50% of maximal possible production (Herrick-Davis et al. 1999; Niswender et al. 1999). Other isoforms exhibit substantially less constitutive activity (Berg et al. 2008; Herrick-Davis et al. 1999), and do not increase in expression with injury. However, these isoforms likely contribute to a basal level of constitutive receptor activity in the normal animal, which should persist acutely after injury, contributing to the small PICs measured *in vitro* in the acutely isolated spinal cord of normal rats (Fig 2-4e)(Harvey et al. 2006b). Also, the increase in total 5-HT_{2C} receptor expression reported with severe chronic SCI(Hayashi et al.) should increase the constitutive activity contributed from all isoforms. This might explain why the PIC that is produced by constitutive activity (SB206553-sensitive) after chronic SCI is about 40% of the maximum PIC that can be induced by activating all the 5-HT_2 receptors, even though INI isoform represents only about 4% of all 5-HT_{2C} receptors after injury (Fig 2-4d). This discrepancy might also be explained by the especially effective intracellular signaling capacity of INI receptor isoforms, producing many times more IP than other isoforms(McGrew et al. 2004), and thus perhaps producing a disproportionately large PIC.

We do not know what initiates the remarkable adaptation in 5-HT_{2C} receptors that we see after SCI. Perhaps it is the loss of 5-HT itself (Gurevich et al. 2002). Alternatively, the lack of motoneuron activity and associated intracellular calcium signaling may trigger the adaptation, as in synaptically isolated single neurons(Turrigiano et al. 1994). That is, motoneurons may require an optimal

amount of activity, regardless of where it arises or what form it takes (spasms or walking), and activity-dependent tuning of constitutive activity in 5-HT receptors may help achieve such optimal activity. Perhaps this explains why intense locomotor training activity after SCI in humans not only improves walking but also reduces spastic muscle activity(Gorassini et al. 2009).

Our finding of constitutive 5-HT receptor activity opens up new possibilities for understanding spinal cord plasticity in disease and injury. While the spinal cord is densely innervated by brainstem-derived 5-HT fibers (Fig 2-1b), there are actually relatively few neurons in the brainstem that provide all this innervation (< 10,000; each neuron branches extensively)(Jacobs et al. 2002), leaving motoneurons and spinal functions vulnerable to injury or disease that affects activity in these few 5-HT neurons. Constitutive 5-HT_2 receptor activity provides a safeguard against such loss of 5-HT innervation of the spinal cord, and probably even contributes to basal receptor activity in normal animals. With the loss of 5-HT after SCI, this constitutive activity increases dramatically, replacing the lost 5-HT-mediated activity.

In summary, we have demonstrated that substantial constitutive 5-HT_{2C} receptor activity emerges after SCI, and contributes to recovery of motoneuron function, with both positive (walking) and negative (spasms) outcomes. This constitutive activity must work in concert with the many other factors that contribute to locomotion and spasticity (Ashby and McCrea 1987; Baker and Chandler 1987;

Courtine et al. 2008; Crone et al. 2003; Dietz and Sinkjaer 2007; Jordan et al. 2008; Li et al. 2004b; Norton et al. 2008; Rank et al. 2007).

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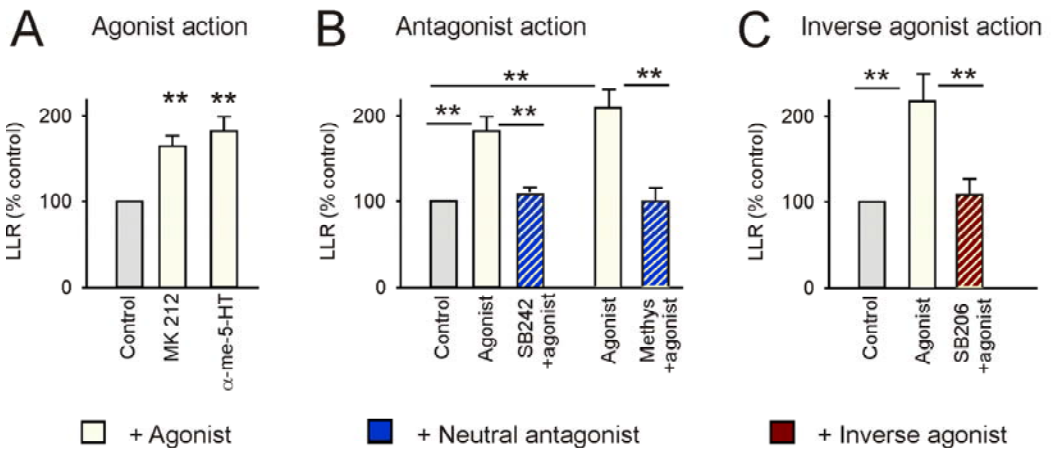
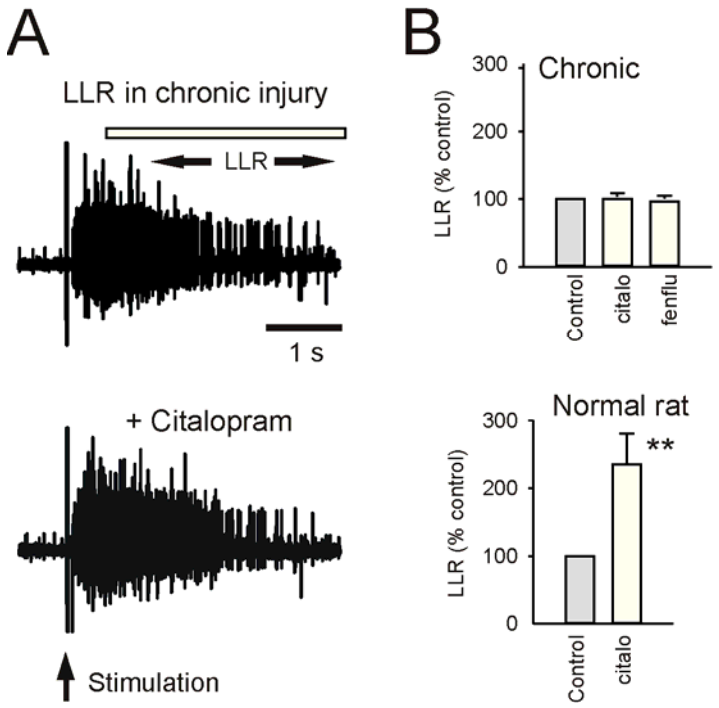
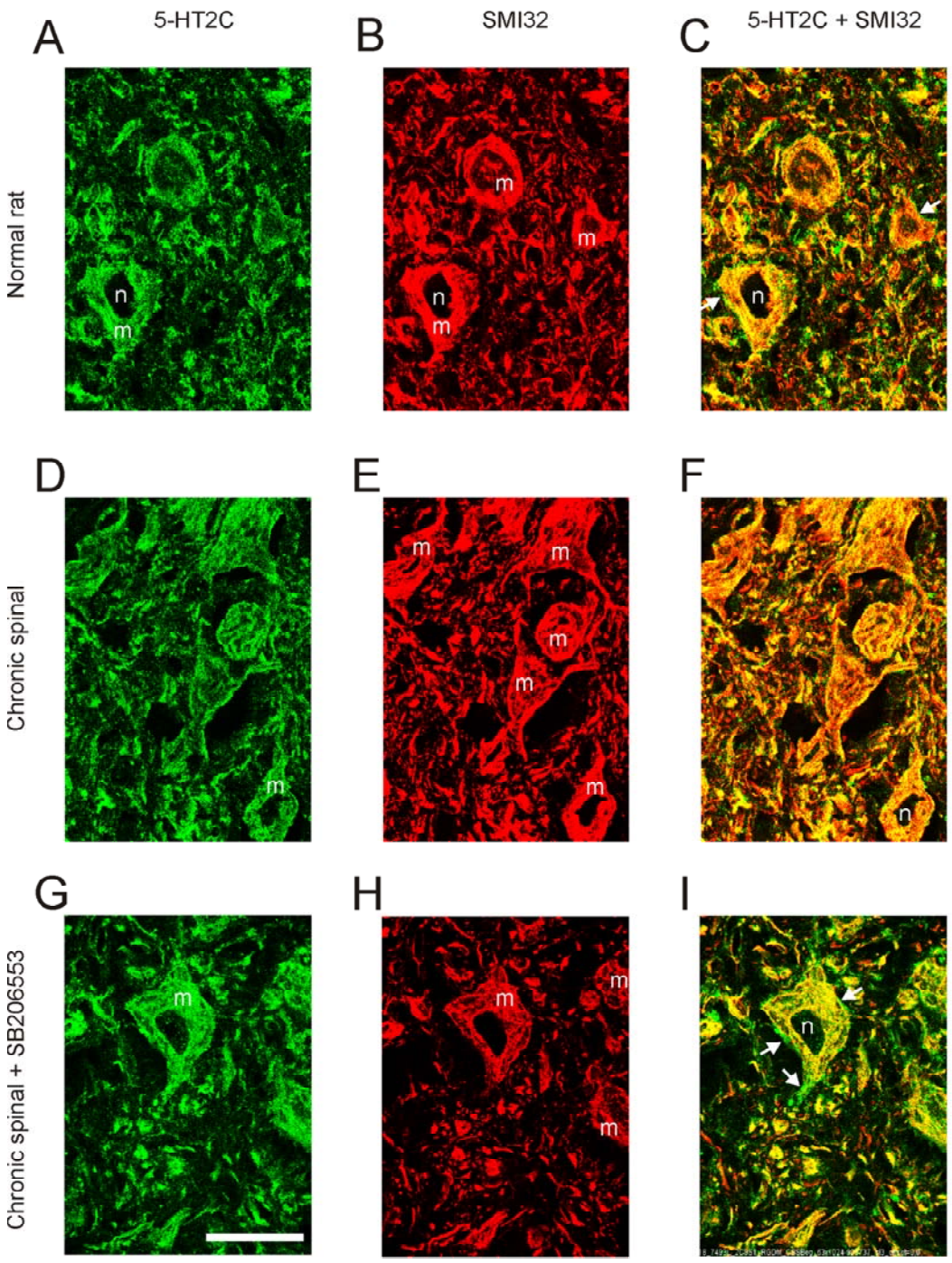


Supplementary Figure 2-1. 5-HT_{2C} receptor agonists augment spasms and antagonists reverse this. (**A**) Same chronic spinal rat preparation and format as in Fig 2-2. The selective 5-HT_{2C} agonist MK212 (Knight et al. 2004) (0.3 μM, $n = 8$) and the selective 5-$HT_{2A/B/C}$ receptor agonist α-methyl-5-HT(Knight et al. 2004) (0.3 μM, $n = 8$) increased the LLR (spasm) recorded *in vitro*, showing that 5-HT_{2C} receptors are involved in spasms. (**B, C**) Application of SB242084 (SB242; 3 μM, $n = 8$), methysergide (Methys; 10μM, $n = 8$) or SB206553 (SB206; 3–5 μM, $n = 8$) antagonized the action of the agonist α-methyl-5-HT (0.3 μM). This demonstrates that both SB242084 and methysergide are effective antagonists, even though they have no effect in the absence of exogenously applied agonists (neutral antagonists, Fig 2-2b,d). Also, the finding that SB206553 blocked the action of this agonist, confirms its specificity to 5-HT_2 receptors, and is consistent with the action of inverse agonists, which block both constitutive *and* ligand-bound receptor activity (Herrick-Davis et al. 1999). $^{**}P < 0.01$. Error bars, s.e.m.



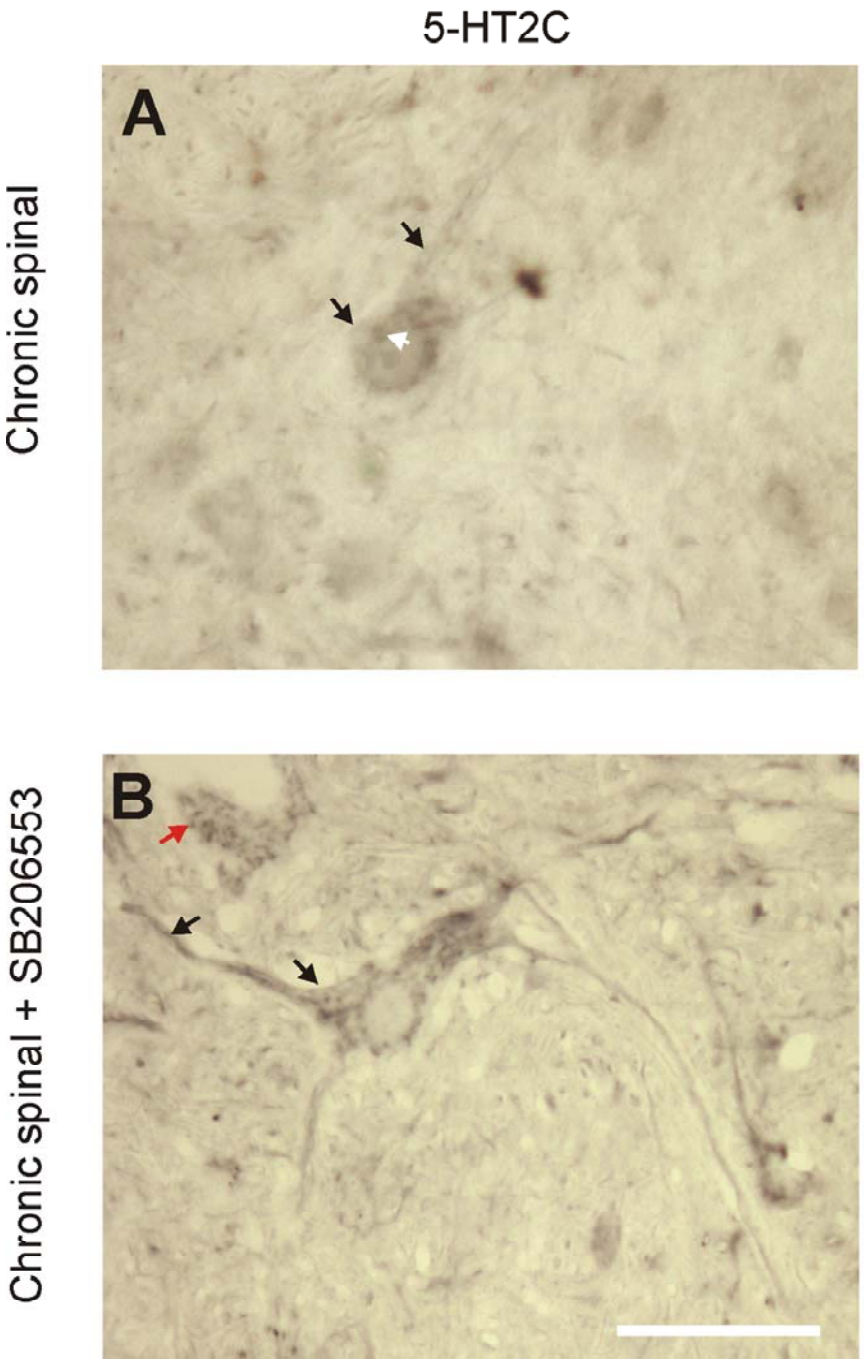
Supplementary Figure 2-2. Enhancing available residual 5-HT has no effect on spasms.(**A**) In the chronic spinal rat, dorsal root stimulation (3xT) triggered long-lasting reflex responses recorded on the ventral roots *in vitro* (LLRs, quantified as mean rectified activity during horizontal bar). Blocking re-uptake of residual 5-HT with the SERT blocker citalopram (Henry et al. 2006) (10 μM) did not alter the LLR. (**B**) Group means of LLRs demonstrate no significant change with citalopram (citalo; $n = 8$). Additionally, forcing presynaptic release of 5-HT with the potent 5-HT releaser fenfluramine (Rothman and Baumann 2002) (fenflu; 10 μM; $n = 12$) had no significant effect on the LLRs. As a positive control, we showed that the LLR was significantly enhanced by citalopram (10 μM; $n = 16$) in sacral spinal cords from normal rats maintained *in vitro* (normal cords have abundant stores of endogenous 5-HT for citalopram to act on (Wallis et al. 1993); Fig. 2-1b). These normal spinal cords do not have LLRs in control pre-drug conditions(Li et al. 2004b). Thus, to match control conditions in the chronic spinal rat, we treated normal cords with a low dose of the glycine receptor blocker strychnine (3 μM) to induce LLRs (Jiang and Heckman 2006) prior to citalopram application. Citalopram was then tested in the continued presence of strychnine in normal cords. $^{**}P < 0.01$. Error bars, s.e.m.


A
5-HT2C
B
SMI32
C
5-HT2C + SMI32
Normal rat
n
m
D
E
F
Chronic spinal
G
H
I
Chronic spinal + SB206553

Supplementary Figure 2-3. 5-HT_{2C} receptor distribution on motoneurons after SCI. (**A**) Immunofluorescence labeling of 5-HT_{2C} receptors (green, SC-15081 antibody) in the S_4 sacral ventral horn of a normal rat, showing extensive receptor distribution, including labeling of the soma of motoneurons (m; nucleus, n), as visualized with 0.5 μm thin optical sections with confocal microscopy ($n = 5$). (**B**) Double-labeling of the same tissue with SMI32 (red), a selective marker of motoneurons in the ventral horn (m, SMI32-positive motoneuron soma), which specifically labels neurofilament in the intracellular space(Anelli et al. 2007). (**C**) An overlay of 5-HT_{2C} and SMI32-labeling revealed extensive co-localization (yellow and orange), indicating 5-HT_{2C} receptors inside motoneuron soma and dendrites (note perinuclear staining; yellow near nucleus, n). Additionally, there was 5-HT_{2C} receptor labeling (green) in isolation, including a halo of green labeling that surrounded SMI-labeled motoneurons (arrows, putative membrane), suggesting 5-HT_{2C} receptors in (or adjacent to) the motoneuron membrane in normal rats (all rats tested showed a similar receptor distribution, $n = 5$ normal rats). The halo of 5-HT_{2C} receptor labeling *not* co-localized with SMI32 was quantified for individual motoneurons by computing the receptor density in a 0.7 μm wide band around the perimeter of the SMI32-labeled soma. The receptor density in this perimeter band around motoneurons (not co-localized with SMI32) was 59.6 ± 4.4% of the mean receptor density inside the motoneuron (co-localized with SMI32, $n = 5$). Mean ± s.e.m.

(**D–F**) Immunolabeling in the sacral ventral horn (below injury) of a chronic spinal rat also showed 5-HT_{2C} receptors inside motoneurons (yellow/orange co-localization with SMI32), but in this case there was less 5-HT_{2C} labeling in isolation (less green in F), suggesting fewer receptors in the membrane (all rats tested showed a similar receptor distribution, $n = 5$ chronic spinal rats). On average, the receptor density not co-localized with SMI32 around the perimeter of motoneurons (in the 0.7 μm band) was 27.3 ± 2.7% of the receptor density inside motoneurons (co-localized with SMI32) in chronic spinal rats, significantly lower than that in normal rats (50% lower, $n = 5$, $P < 0.01$). Thus, after SCI the 5-HT_{2C} receptors appear to be highly internalized in motoneurons, consistent with the presence of constitutively active isoforms of the receptors (like INI isoform), which are characteristically found internalized. That is, the INI isoform has previously been shown to be so constitutively active that as soon as it enters the membrane it couples its G protein and causes intracellular signaling that culminates in receptor phophorylation and internalization (Chanrion et al. 2008; Marion et al. 2004). This is followed by recycling of the receptor back into the membrane and further constitutive activity, but the internalization rate exceeds the recycling rate, and so the receptor isoform accumulates mostly inside the cell (even though there is a steady G protein activation), unlike in other isoforms(Chanrion et al. 2008; Marion et al. 2004).

(**G–I**) Blocking the INI receptor isoform activity with the inverse agonist SB206553 stops the process of receptor internalization and allows the receptor to accumulate in the membrane(Chanrion et al. 2008; Marion et al. 2004). Thus, we examined the 5-HT_{2C} receptor distribution in chronic spinal rats treated for 2 hrs with SB206553 (with two IT injections of 10 mM in 30 μL saline, separated by 1 hour, $n = 4$). In these treated rats there was considerable 5-HT_{2C} receptor labeling in isolation (green in I), including labeling that surrounded SMI-labeled motoneurons (green halo, shown at arrows), suggesting substantial 5-HT_{2C} receptors in the motoneuron membrane, unlike in untreated chronic spinal rats. On average, the receptor density *not* co-localized with SMI32 around the perimeter of motoneurons (in the 0.7 μm band) was 58.7 ± 3.6 % of the receptor density inside motoneurons (co-localized with SMI32) in treated chronic spinal rats, significantly higher than that in untreated chronic spinal rats (double, $P < 0.01$). This is consistent with the concept that inactive receptors accumulate in the membrane. It also verifies that the present immunolabeling can adequately distinguish membrane receptors from internalized receptors, similar to how internalized μ opiod receptors can be visualized with immunolabeling(Sternini et al. 1996). Scale bar 50 μm.


5-HT2C
A
Chronic spinal
B
Chronic spinal + SB206553

Supplementary Figure 2-4. 5-HT_{2C} receptor internalization is reduced by SB206553. Similar format to Supplementary Fig 2-3, but labeling done with a different 5-HT_{2C} receptor antibody (ab32172) and DAB immunohistochemistry. (**A**) Typical 5-HT_{2C} receptor labeling in ventral horn of a chronic spinal rat (black/brown, 10 μm transverse section). Black arrows indicate weakly labeled membrane of a motoneuron and its primary dendrite. Most of the receptor labeling was internalized, in diffuse clumps (e.g., at white arrow near the nucleus). (**B**) Typical 5-HT_{2C} receptor labeling in a chronic spinal rat pre-treated with inverse agonist SB206553 (4 hours incubation in 30 μM SB206553, *in vitro*). In contrast to untreated chronic spinal rats, membranes of motoneuron soma and dendrites were more intensely labeled after treatment (at black arrows). Also, there were punctate, intensely labeled receptor clusters (black dots) that were mostly on or near the cell surface (non-confocal images), as demonstrated by punctate labeling on the surface of another motoneuron that happened to be deeper, and thus mainly the cell surface was in focus (at red arrow). These observations are consistent with the concept that SB206553 enables receptors to accumulate in the membrane by blocking their constitutive activity, whereas in untreated chronic spinal rats the receptors are largely internalized. Scale bar 50 μm.

Subject Code	Muscle examined	Age (years)	Injury level	Age of Injury (years)	AIS	Manual Muscle Test
1M	Right TA	58.7	C3–C6	5.3	C	3+
2M	Left TA	51.7	C5–C6	11.2	B	0
3F	Right TA	46.9	T2–T4	3.8	C	3
4F	Right TA	21.6	C6–C7	0.92	C	3–
5M	Right TA	48.6	C4–C5	11.1	D	4+
6M	Right TA	42.0	C4–C5	2.0	D	4
7M	Right TA	54.0	C3–C4	21.3	D	3

Supplementary Table 2-1. Human spinal cord injured subjects. The table shows subject number plus sex (M, male; F, female), muscle studied (TA, tibialis anterior), age of subject at the time of experiment, injury level, years post-injury, the AIS – International Standards for Neurological and Functional Classification of Spinal Cord Injury (Maynard et al. 1997), and the MRC manual muscle test score. The latter is a qualitative assessment of the voluntary muscle strength of the examined muscle with 0 = no muscle contraction visible or palpable; 3– = greater than 50% range of motion against gravity; 3 = complete range of motion against gravity; 3+ = complete range of motion against gravity with minimal resistance; 4+ = can contract against moderate to strong pressure.

SUPPLEMENTARY METHODS

Sacral spinal injury model in rat and relation to human spasticity.

The S_2 sacral spinal cord was transected in rats as described previously (Bennett et al. 1999; Bennett et al. 2004; Li et al. 2004a). Briefly, under general anesthetic (sodium pentobarbital, 58.5 $mg \cdot kg^{-1}$) and sterile conditions, a laminectomy was performed on the L_2 vertebrae to expose the S_2 spinal cord. The dura was slit transversely, and 0.1–0.3 ml Xylocaine (1%) was applied topically. Under a surgical microscope, the spinal cord was transected by holding the pia with forceps and sucking under the pia with a fine suction tip. Caution was needed to avoid damaging the anterior artery or posterior/dorsal vein, since the sacrocaudal spinal cord dies without this midline vasculature. The dura was closed with two 8-0 silk sutures, and the muscle layers and skin were tightly sutured over the cord, and the rat allowed to recover. We evaluated spasticity and motoneuron properties 6–12 weeks post-injury (chronic spinal rats). This injury only affects the tail (not bladder or hindlimbs).

Previously, we have shown that the spasticity that emerges in the tail of chronic spinal rats closely mimics the human spasticity syndrome seen in the legs after SCI, with clonus, hypertonus, hyperreflexia, spasms, and general delayed onset of symptoms (Bennett et al. 1999). Flexor spasms emerge first, followed by extensor spasm (months), as also seen in humans (Bennett et al. 1999). Finally, we have recently shown that the same ionic mechanism that mediates spasms in the tail of rats also mediates spasms in humans (PICs in motoneuron) (Gorassini et al. 2004), thus justifying the use of the sacral spinal rat in the current paper.

Staggered hemisection model of locomotor recovery after SCI and rationale for use.

To examine recovery of locomotion after SCI, rats underwent a staggered hemisection (Courtine et al. 2008). Rats were first hemisected on the right at the T12 spinal vertebrae (L_1–L_2 spinal level) (Ballermann and Fouad 2006). Then,

four weeks later, they were hemisected on the left at the spinal T_6 vertebrae (T_6–T_7 level). Locomotion was evaluated after another 3 weeks using the BBB score (Basso et al. 1995). Bladders were expressed 3 times daily for 3 days, until bladder function recovered. In this staggered hemisection model all direct descending inputs, including 5-HT, are cut (Fig 2-6a, white and gray neurons), whereas *spared* local propriospinal neurons (Fig 2-6a, black neurons) relay descending signals (originating from supraspinal neurons; Fig 2-6a, gray neurons) around the lesion site. This allows robust spontaneous recovery of voluntary locomotion (unlike in transected animals) in the absence of most 5-HT (Courtine et al. 2008; Cowley et al. 2008).

Spinal neurons involved in locomotion require neuromodulators like 5-HT to function, setting them into a state of readiness for movement generation (Jacobs et al. 2002; Jordan et al. 2008). The critical importance of these neuromodulators has been demonstrated by the finding in animal models that locomotor-activity can be regained soon after spinal transection with the exogenous application of drugs that activate the neuromodulatory 5-HT, norepinephrine and dopamine receptors ((in vivo and in vitro) Chau et al. 1998; Courtine et al. 2009; Cowley and Schmidt 1994; Kiehn and Kjaerulff 1996; Viala and Buser 1971), including 5-HT_2 and 5-HT_7 receptor agonists (Landry and Guertin 2004; Madriaga et al. 2004; McEwen et al. 1997). Notably, over the weeks after injury, locomotor-like movements spontaneously emerge and improve with use (Barbeau and Rossignol 1987; de Leon et al. 1998; Kuhn and Macht 1948), as if the neuromodulatory receptor systems (e.g. 5-HT_2 receptors) are somehow re-activated in the absence of 5-HT. We propose that this spontaneous recovery of locomotion is in part due to constitutive 5-HT receptor activity, and we investigated this with the staggered hemisection model.

Spasms in awake spastic rat.

Tail muscle spasms were measured with percutaneous EMG (electromyogram) wires inserted in segmental tail muscles at the midpoint of tail and with kinematic

recordings of tail flexion (with video), while the rat was in a Plexiglas tube, as detailed previously (Fig. 2-1a,e) (Bennett et al. 2004). During EMG recording, muscle spasms were evoked with electrical stimulation of the skin at the distal tip of the tail (cutaneous stimulation; 0.2 ms, 10 mA pulse; 2–3x afferent threshold [T]; 6 spasms evoked at 10 s intervals for a trial; trials repeated at 12 min intervals) and the tail was restrained from moving. During kinematic recording, spasms were evoked with mechanical stimulation of the tail skin (Bennett et al. 2004; Rank et al. 2007), and the tail was free to move, which caused sensory input that extended spasms over many minutes (Fig. 2-1e). EMG was sampled at 5 kHz, rectified and averaged over a 10–40 ms interval post-stimulus to quantify the short latency reflex (SLR), and a 500–4000 ms interval to quantify spasms (long lasting reflex, LLR; using Axoscope, Axon Instr., and Matlab, Mathworks). EMG over 300 ms prior to stimulation was also averaged (background). Tail flexion angle was computed as described previously (Rank et al. 2007) and averaged over 3 mins post-stimulation.

Human subjects with spinal cord injury and leg spasms.

The human subjects studied had varied severity of SCI, as summarized in Supplementary Table 2-1. The subjects were seated in their wheelchair with limbs free to move, since immobilization interfered with generating muscle spasms. Two disposable surface electrodes (Kendall Soft-E), separated by 1 cm were placed over the tibialis anterior (TA) muscle to record EMG. Spasms were evoked at rest by a brief electrical stimulation of the medial arch of the foot (with a 24 ms train of 7 pulses at 300 Hz, 0.2 ms and 50 mA each pulse; 3–5xT; using a Digitimer DS7A). Stimulation was repeated 8 times at 15 s intervals for each trial, and this was repeated every 30 mins. Spasms were quantified by rectifying and averaging the EMG over the windows in time indicated in Fig 2-5.

Ventral root recordings of long-lasting reflexes in rats, in vitro.

Under urethane anesthesia (1.8 g·kg^{-1}) the whole spinal cord caudal to the S2 injury level was removed from chronic spinal rats (and age-matched normal rats)

and immersed in oxygenated artificial cerebrospinal fluid (ACSF; flowing 8 $ml \cdot min^{-1}$); recordings were made starting 2.5 hr later, as detailed previously(Li et al. 2004a; Li et al. 2004c). Ventral (S_4 and Co_1) and dorsal (Co_1, coccygeal) roots were mounted on silver wires above the ACSF and covered with Vaseline. The dorsal root was stimulated with a single pulse (0.1 ms, 0.02 mA, 3xT; repeated 5 times at 10 s intervals for one trial, trials repeated every 12 mins), and the long-lasting reflex (LLR) response was recorded on the ventral roots, and then analyzed as for the EMG. We quantified the LLR by averaging the rectified ventral root activity over a time-window 500–4000 ms post stimulus, a period previously shown to reflect the motoneuron Ca PIC activity in isolation. We also measured the transient short latency reflex (SLR) by averaging ventral root responses over a window 10–40 ms post stimulus (polysynaptic reflex with about 10 ms central delay). Because of slow diffusion in *whole* spinal cord preparations (Harvey et al. 2006a; Perrier and Hounsgaard 2003), drugs required 10x higher concentrations than in thin slice preparations, and peak effects of even TTX (2 μM; sodium channel blocker) required 10–15 mins. To assure selectivity of drugs, they were titrated to a minimal dose that produced peak effect, and results were reported after 25–45 min of drug application.

Intracellular recordings, in vitro.

Sharp intracellular electrodes (30 MΩ; filled with 1M K-acetate and 1M KCl) were advanced into the spinal cord with a stepper motor (666, Kopf, Instr.) to penetrate motoneurons (identified by antidromic ventral root stimulation), as detailed before(Harvey et al. 2006b; Li et al. 2004a). Intracellular recordings were made with an Axoclamp2B amplifier (Axon) running in discontinuous-single-electrode voltage-clamp (gain 0.8–2.5 $nA \cdot mV^{-1}$; for Ca PICs) or discontinuous-current-clamp (switching rate 5 kHz; for EPSPs) mode, filtered at 3 kHz, and sampled at 6.7 kHz (Clampex and Clampfit used; Axon Instr.). In the presence of TTX (2 μM) to block synaptic transmission and sodium currents (Na PIC), slow triangular voltage ramps (3.5 $mV\ s^{-1}$ voltage-clamp) were applied to the motoneurons to measure the Ca PIC (Fig 2-3b) (Harvey et al. 2006b; Li et al.

2004a). During the upward portion of this ramp, the current response initially increased linearly with voltage in response to the passive leak conductance. A linear relation was fit to the current in the region just below the Ca PIC onset (5 mV below) and extrapolated to the whole range of the ramp (leak current, thin line in Fig 2-3b). At depolarized potentials above the Ca PIC onset threshold, the Ca PIC induced a downward deviation from the extrapolated leak current, and this Ca PIC was estimated as the difference between the leak current and the total current (leak-subtracted current). The amplitude of the peak Ca PIC was quantified as the initial peak amplitude of this downward deviation below the leak line (downward arrow Fig 2-3b). The Ca PIC is thought to be mediated by Cav1.3 L-type calcium channels(Li et al. 2004a), because it is activated at an unusually low threshold (about 10 mV above rest), blocked by high doses of nimodipine and highly persistent (in contrast, transient currents are inactivated during the slow ramp). There is also a small contribution to the Ca PIC from calcium-activated channels(Li and Bennett 2007).

The excitatory postsynaptic potential (EPSP) and associated reflexes were also measured in motoneurons by stimulating the Co_1 dorsal roots (at 2–3xT, as in ventral root reflex recording), while applying hyperpolarizing bias currents to block the PICs (Fig 2-3d).

mRNA measurements.

Following the methods of Nakae et al. (Nakae et al. 2008), RNA was extracted from spinal cords of chronic spinal rats and age-matched normal rats with RNAeasy-Lipid-tissue Mini-Kits (Qiagen, Japan) and used for first-strand cDNA synthesis by Thermoscript (Invitrogen). Quantitative RT-PCR was then performed on the cDNA with ABI-PRISM-7900HT using TaqMan probes (Applied Biosystems) to estimate total 5-HT_{2C} receptor mRNA (and internal control 18SrRNA). To determine the 5-HT_{2C} receptor isoforms in the tissue, rat 5-HT_{2C} receptor fragments were amplified from the cDNA using RT-PCR with the primers ATCATGGCAGTAAGCATGGAGAAGA and

ATTGATATTGCCCAAACGATGGCA. The PCR product DNA was cloned into the pCR2.1 vector (Invitrogen), transfected into single E. coli bacterial cells, and > 300 recombinant colonies grown (each corresponding to a *single* 5-HT_{2C} receptor isoform). DNA extracted from 80 randomly picked colonies underwent nucleotide sequencing with an ABI3700 genetic analyzer (Applied Biosystems) to determine the isoform DNA adopted by each colony.

Immunolabeling.

Rats were euthanized with Euthanyl (Bimeda-MTC; 700 $mg \cdot kg^{-1}$) and perfused intracardially with 100ml saline followed by 400 ml paraformaldehyde (PFA; in phosphate buffer at room temperature; over about 15 mins), with 4% PFA used for 5-HT immunolabeling and 2% PFA for 5-HT_{2C} and SMI32 immunolabeling. Spinal cords were post-fixed in PFA (overnight for 5-HT-labeling and 90 mins for others) at 4 ^{o}C, cryoprotected in 20% sucrose and 2% ethylene-glycol, frozen and cut on a cryostat in horizontal or transverse 25–40 μm sections. Spinal cord sections were incubated overnight at 4 °C with primary antibodies to 5-HT (1:1000 S5545, Sigma, raised in rabbit), 5-HT_{2C} receptors (1:900, SC-15081, epitope near N-terminus, Santa Cruz, goat) or SMI32 (1:000, Sternberger, mouse) in phosphate or Tris buffered saline (PBS or TBS) and 1% normal serum. Antibody labeling was visualized with fluorescent secondary antibodies (5-HT: Texas-Red[TI1000], 1:200, Vector, USA, overnight at 4 °C; 5-HT_{2C}: Alexa488[A11055 or A11078], 1:1000, Invitrogen, 90 min at room-temperature; SMI32: Alexa568[A11008], 1:1000, Invitrogen, 90 min RT) and using a Zeiss-CLSM510 microscope and laser-scanning confocal microscopy.

Controls in which the primary antibody was pre-absorbed with the antigen that it was raised against were used to verify the selectivity of the antibody labeling. For example, the 5-HT_{2C} receptor antigen (1:180; 5.5 $\mu l \cdot ml^{-1}$, SC-15081P, Santa Cruz) was incubated with the 5-HT_{2C} receptor antibody (SC-15081) for 80 mins at room temperature prior to dilution and application to tissue. Also, controls in

which the primary antibody was omitted were used to confirm that the secondary antibody produced no labeling by itself.

SMI32 labels non-phosphorylated neurofilament H, and does not change with spinal cord injury (Anelli et al. 2007). We have previously demonstrated that SMI32 labels motoneurons, and *not* interneurons in the ventral horn, though it also labels large cells elsewhere in the brain. Thus, SMI32 provided a fairly selective marker of motoneurons in the ventral horn (Anelli et al. 2007), and in particular labels the intracellular space (neurofilament). Using image analysis software (ImageJ), we defined two regions for 5-HT_{2C} receptor quantification in motoneurons: 1) the internal region inside motoneuron soma, co-localized with SMI32 (region with SMI32 above standardized threshold, as we described in Anelli et al. (Anelli et al. 2007)), and 2) the membrane region, immediately adjacent to the internal SMI32 region. The later was produced by detecting the edge of the SMI32-positive region inside motoneuron soma and expanding this edge to a 0.7 μm wide band that surrounded the perimeter of the soma (not co-localized with SMI32). The mean 5-HT_{2C} immunofluorescence signal density was then computed in these two regions, from which we computed the ratio of the receptor density in the membrane relative to the density inside the cell (x100%) for comparison across normal and injured rats.

We also labeled 10 μm transverse spinal cord sections from chronic spinal rats with a different 5-HT_{2C} antibody (ab32172, epitope on C-terminus, Abcam), and then visualized the antibody with ABC amplification (Vector-Labs) followed by DAB labeling. For this, spinal cords were initially placed *in vitro* for a 4 hr incubation with and without SB206553 (30 μM) and then fixed in 4% PFA. After cryoprotection, frozen sections were cut and then incubated 15 min in 0.5% triton-x100 in PBS, 5 mins in 1.5% hydrogen peroxide in PBS, 1 hour in 10% normal goat serum in PBS, and then overnight in the primary 5-HT_{2C} antibody in PBS (1:200; ab32172), all at RT. Then, sections were incubated in a biotinylated secondary antibody (1:200; BA9400; Vector-Labs) overnight at 4 °C, followed by

incubation in the Vector ABC elite complex, overnight at 4 °C, and then 5-HT_{2C} receptors were visualized with the Vector DAB kit and viewed with conventional light microscopy.

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Chapter 3: Motoneuron excitability and muscle spasms are regulated by 5-HT_{2B} and 5-HT_{2C} receptor activity

INTRODUCTION

Following spinal cord injury (SCI) debilitating spasms often develop in the muscles innervated from the spinal cord below the injury, and this is the hallmark of the general spasticity syndrome after SCI (Bennett et al. 2004; Gorassini et al. 2004; Kawamura et al. 1989; Kuhn and Macht 1948). Spasms involve simultaneous contractions of many muscles, lasting anywhere from seconds to minutes, and are triggered by brief innocuous sensory stimuli, such as cutaneous inputs. Upwards of 80% of spinal cord injured humans suffer from spasms and general spasticity that can disrupt residual motor function, cause debilitating pain, and interrupt sleep (Maynard et al. 1990). Treatment of spasms with conventional antispastic drugs like baclofen is often not adequate or not tolerated due to adverse side effects such as lethargy and weakness (Dario and Tomei 2004). Here we explore whether activity in one or more of the many 5-HT receptor subtypes facilitated spasms, and examine whether selectively blocking these receptors may serve as a target for novel antispastic treatment.

The neuronal mechanisms underlying spasms have recently been identified in a rat model of SCI (Bennett et al. 2004; Harvey et al. 2006b; Li et al. 2004a; Murray et al. 2010), and similar mechanisms have been shown to underlie spasms in humans (Gorassini et al. 2004; Norton et al. 2008). Briefly, these studies have shown that immediately after spinal cord injury motoneuron excitability plummets; in particular, there is a loss of low voltage-activated persistent calcium and sodium currents (persistent inward currents; Ca and Na PICs) that are normally crucial for enabling motoneurons to produce sustained motoneuron discharges in response to synaptic inputs (Heckman et al. 2005; Hultborn et al. 2004; Li et al. 2004a). Many motoneurons are acutely so impaired that they can only fire transiently and can no longer follow synaptic inputs with appropriate firing (Harvey et al. 2006c). However, over the months after injury (chronic injury) PICs spontaneously return, attaining high levels likely only seen in alert awake animals (Button et al. 2008; Harvey et al. 2006c; Hultborn et al. 2004; Li et al. 2004b). Theses PICs are crucial for the recovery of motoneuron firing ability,

enabling motoneurons to produce sustained firing and associated muscle contractions, in response to transient inputs (Li et al. 2004a). However, these PICs are permanently facilitated and whenever these voltage-dependent currents are activated by a brief synaptic depolarization, they are difficult to terminate due to the loss of normal descending control of inhibitory inputs to motoneurons with SCI (Holstege and Bongers 1991; Jankowska and Hammar 2002; Nielsen et al. 2007; Rekling et al. 2000). Thus, activity in PICs cause uncontrolled motoneuron firing and associated muscle spasms (Bennett et al. 2004; Li et al. 2004a). To make matters worse, after SCI there is a general loss of inhibition over sensory afferent transmission (disinhibition) (Hochman et al. 2003; Lundberg 1982; Schmidt and Jordan 2000), leading to unusually large and prolonged polysynaptic excitatory postsynaptic potentials (EPSPs) on motoneurons (Baker and Chandler 1987; Bennett et al. 2004; Li et al. 2004a). These EPSPs readily trigger the large PICs in chronic SCI, which in turn produce many seconds of uncontrolled motoneuron firing. Thus, spasms are readily triggered by transient sensory stimulation (Bennett et al. 2004).

Brainstem-derived serotonin (5-HT) plays a critical role in regulating normal levels of spinal cord excitability by several receptors (Hochman et al. 2003; Schmidt and Jordan 2000), including facilitating Ca PICs on motoneurons, by activating 5-HT_2 receptors (Harvey et al. 2006a; Heckman et al. 2005; Perrier and Delgado-Lezama 2005) and by inhibiting sensory afferent transmission, by activating 5-HT_1 receptors (Hochman et al. 2003; Millan 2002; Schmidt and Jordan 2000; Yoshimura and Furue 2006). Thus, the immediate loss of motoneuron excitability and emergence of large EPSPs acutely after spinal transection is partly explained by a loss of 5-HT. The spontaneous recovery of motoneuron excitability (PICs) over the months after injury (chronic spinal rat) is more difficult to explain, but recently Harvey et al. (2006b) suggested that it involves re-activation of 5-HT_2 receptors. Furthermore, Murray et al (2010) have recently shown that this activation of the 5-HT_2 receptors occurs in the absence of 5-HT after injury; that is, the receptors become constitutively active, as described

in other systems (Aloyo et al. 2009; Seifert and Wenzel-Seifert 2002). These two studies do not clearly define which specific 5-HT_2 receptor is involved, though both studies suggest it could be the 5-HT_{2C} receptor, and Harvey (2006b) speculated that 5-HT_{2A} receptors may be involved as well. Little is known about the role of other 5-HT receptors in motoneuron function and spasms after injury. Furthermore, since mostly non-selective pharmacological agents (e.g. 5-$HT_{2A/2B/2C}$ receptor agonist DOI) have been used to study 5-HT modulation of normal motoneuron function, it even remains unclear which receptors are specifically involved in normal animals, though these seem to include the Gq-protein coupled 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors (Elliott and Wallis 1992; Harvey et al. 2006a; Holohean and Hackman 2004; Perrier and Hounsgaard 2003; Wang and Dun 1990), Gi coupled 5-HT_{1A} receptors (Holohean et al. 1990; Perrier et al. 2003; Wang and Dun 1990), Gs-coupled 5-HT receptors like 5-HT_7 receptors that increase cAMP (Inoue et al. 2002; Larkman and Kelly 1997) and possibly ionotropic 5-HT_3 receptors (Ziskind-Conhaim et al. 1993). In the present paper we employ numerous 5-HT agonists and highly selective antagonists to determine which receptors facilitate motoneuron function (Ca PICs) and associated long-lasting reflexes (spasms) after SCI, by correlating agonist potency at facilitating the spasms with the published binding affinity of these agonists to all 5-HT receptor types (Barnes and Sharp 1999; Boess and Martin 1994). We then determine whether spontaneous activity in these receptors contributes to recovery of PICs and emergence of spasms, and examine whether this activity results from either residual 5-HT in the caudal spinal cord or constitutive receptor activity (Murray et al. 2010; Seifert and Wenzel-Seifert 2002). As we expected from previous work, we found that 5-HT_{2C} receptors facilitate spasms, and associated motoneuron PICs (Murray et al. 2010). However, we also found that 5-HT_{2B} receptors facilitate spasms and exhibit substantial constitutive activity that contributes equally to 5-HT_{2C} receptors in the large PICs and spasms that emerge after injury. Constitutive activity in 5-HT_{2B} receptors has been reported before in native 5-HT_{2B} receptors of the stomach fundus (Villazon et al. 2003), as well as in cloned receptors transfected into cells (Weiner et al. 2001), but not previously in

the spinal cord or brain.

METHODS

Recordings were made from motoneurons and associated ventral roots of the sacrocaudal spinal cord of spastic adult rats with chronic spinal cord injury (3-8 months old) (Bennett et al. 2004). Adult female rats were transected at the S_2 sacral level at about 2 months of age (adult rat), and recordings were made after their affected muscles became spastic (1.5–3 months after injury), as detailed previously (Bennett et al. 1999; Bennett et al. 2004). All recordings were made from the whole sacrocaudal spinal cord that was removed from the rat with an S_2 sacral transection and maintained *in vitro* (Li et al. 2004b). This transection was made just rostral to the chronic spinal injury, so as not to further damage the sacrocaudal cord. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee: Health Sciences.

In vitro preparation

Details of the *in vitro* experimental procedures have been described in previous publications (Harvey et al. 2006c; Li et al. 2004a; Li et al. 2004b; Murray et al. 2010). Briefly, all the rats were anaesthetized with urethane (0.18 g/100 g; with a maximum dose of 0.45 g) and the sacrocaudal spinal cord was removed and transferred to a dissection chamber containing modified artificial cerebrospinal fluid (mACSF). Spinal roots were removed, except the sacral S_4 and caudal Ca_1 ventral roots and the Ca_1 dorsal roots. After 1.5 hours in the dissection chamber (at 20° C), the cord was transferred to a recording chamber containing normal ACSF (nACSF) maintained near 24°C and with a flow rate > 5 ml/min. A one-hour period in nACSF was given to wash out the residual anaesthetic and mACSF prior to recording, at which time the nACSF was recycled in a closed system with a peristaltic pump (Harvey et al. 2006b).

Ventral root reflex recording and averaging.

Dorsal and ventral roots were mounted on silver-silver-chloride wires above the nASCF of the recording chamber and covered with a 1:1 mixture of petroleum jelly and mineral oil (as for intracellular recording) for monopolar stimulation and

recording (Li et al. 2004b). We evoked ventral root reflexes with a low threshold Ca_1 dorsal root stimulation (single pulse, 0.1 ms, 0.02 mA, corresponding to 3 x afferent threshold, 3xT; afferent and reflex threshold are similar (Bennett et al. 2004)) using a constant current stimulator (Isoflex, Israel). The stimulation intensity (3xT) is compatible with activation of low-threshold group I and II (Aβ) afferents. Because the Ca_1 dorsal root innervates the distal third of the tail which lacks large muscles(Bennett et al. 2004), this stimulation activates largely cutaneous or joint afferents. However there are small intrinsic muscles in the tail with group Ia and II muscle afferents (Steg 1964), and thus to a lesser extent muscle afferents may be activated. The stimulation was repeated 5 times at 10 second intervals for each trial. The ventral root recordings were amplified (x2000), high-pass filtered at 100 Hz, low-pass filtered at 3 kHz, and recorded with a data-acquisition system sampling at 6.7 kHz (Axonscope 8; Axon Instruments). Ventral root reflexes were quantified using custom written software (Matlab, MathWorks, Natick, MA). That is, data were high pass filtered at 800Hz and rectified to allow averaging (Fig 3-1A, lower plot). We quantified the long latency, long-lasting reflex (LLR) by averaging the rectified response 500–4000 ms post stimulus, a period where the response is mainly determined by the motoneuron Ca PIC activity, not by stimulus evoked synaptic inputs, which end by about 500 ms. Average ventral root activity was then averaged for all 5 stimuli in a trial. This recording procedure was repeated at 12 min intervals, and 5-HT receptor agonists were added immediately after each recording, giving them time to fully act by the next 12 min recording session. Cumulative dose-response relations were computed by increasing agonist doses at these 12 min intervals (0.003, 0.01, 0.03. 0.1,..., 30 uM doses used). Antagonists took longer to act and responses reached near steady state typically > 20 mins after application, at which time responses were averaged. The effect of agonists on the reflexes were reversible upon washout of the agonist, but full recovery to baseline only occurred after several hours, likely due to the large size of the whole cord preparation. Thus, washout of agonists was not feasible between doses of the agonists used in the construction of dose-response relations.

Intracellular recording

Sharp intracellular electrodes were made from glass capillary tubes (1.5mm O.D.; Warner GC 150F-10) using a Sutter P-87 micropipette puller and filled with a combination of 1M potassium acetate and 1M KCl. Electrodes were bevelled down from an initial resistance of 40-80 MΩ to 26-32 MΩ using a rotary beveller (Sutter BV-10). A stepper-motor micromanipulator (660, Kopf) was used to advance the electrodes through the ventral cord surface into motoneurons. Large 30 μm forward and backward steps were initially required to breach the white matter (about 100 um thick). After this multiple tracks were made in the grey matter with 2uM steps, without coming completely out of the white matter, by moving the electrode sideways in the white matter before each new tract (to further weaken pia and improve stability). Penetrations were made with capacitance-over-compensation ringing. After penetration, motoneuron identification was made with antidromic ventral root stimulation, and noting ventral horn location, input resistance and time constant (> 6 ms for motoneurons; see detailed before (Li and Bennett 2007; Li et al. 2007)). Cells were always held below rest (< -70mV) with a small continuous bias current, to assure that the Ca PICs were not even partly activated prior to testing, because the Ca PIC slowly deactivates if it is activated for a few minutes, and does not recover from this deactivation for many minutes (unpublished data). 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.

Slow triangular voltage ramps (3.5 mV/s voltage-clamp) were applied to the motoneurons to measure their electrical properties, as detailed previously (Harvey et al. 2006c; Li et al. 2004a). The input resistance (Rm) was measured during the voltage ramps over a 5-mV range near rest and subthreshold to PIC onset. Resting potential (Vm) was recorded with 0 nA bias current, after the cell had been given

about 15 mins to stabilize after penetration. The slow triangular voltage ramps were used to directly measure the PICs as follows (shown in Fig 3-5A, described later). During the upward portion of this ramp, the current response initially increased linearly with voltage in response to the passive leak conductance. A linear relation was fit in the region just below the PIC onset (5 mV below) and extrapolated to the whole range of the ramp (leak current; thin line in Fig 3-5A). At depolarized potentials above the PIC onset threshold, there was a downward deviation from the extrapolated leak current, and the PIC was estimated as the difference between the leak current and the total current (initial peak current at arrow in Fig 3-5A; leak-subtracted current). The onset voltage for the PIC (V_{on}) was defined as the voltage at which the slope of the current response initially reached zero (Li and Bennett 2003). Finally, the excitatory postsynaptic potential (EPSP) and associated reflexes were also measured in motoneurons by stimulating the Ca_1 dorsal roots (at 3xT, as in ventral root reflex recording), while applying hyperpolarizing bias currents to block the PICs (Fig 3-1C, lower plot).

Drugs and solutions

Two kinds of artificial cerebrospinal fluid (ACSF) were used in these experiments: a modified ACSF (mACSF) in the dissection chamber prior to recording 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 0.1- 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. The drugs added to the ACSF were 5-HT, DOI (-) (from Sigma-Aldrich, USA), 2-methyl-5-HT, 8-OH-DPAT, AC90179, alpha-methyl-5-HT, BW723C86, clozapine, cisapride, cyproheptadine, EMD386088, granisetron, isradipine, ketanserin, LP44, LY344864, methylergonovine, methysergide, MK212, prazosin, RS102221, RS127445, SB204741, SB206553, SB224289, SB242084, tryptamine (all from Tocris, USA), TTX (TTX-citrate; Alomone, Israel), and zolmitriptan (kindly donated by Astra Zeneca, Canada). All drugs were first dissolved as a 10 - 50 mM

stock in water before final dilution in ACSF, with the exception of BW723C86, RS102221, RS127445, cisapride, clozapine, SB204741 and prazosin, which were dissolved in minimal amounts of DMSO (final concentration in ACSF < 0.04 %; by itself DMSO had no effect on the LLR, in vehicle controls). During intracellular recording, we usually first blocked the sodium currents (fast and persistent) and synaptic transmission with 2 μM TTX, and then examined the Ca PIC sensitivity to 5-HT ligands. The Ca PIC was verified to be L-type calcium channel mediated by its blockade with dihydropiridines (nimodipine or isradipine; 15 uM).

Data analysis

Data were analyzed in Clampfit 8.0 (Axon Instruments, USA) and Sigmaplot (Jandel Scientific, USA). Data are shown as mean ± standard deviation (s.d.). A Student's *t*-test was used to test for statistical differences before and after drug applications, with a significance level of $P < 0.05$. A Kolmogorov-Smirnov test for normality was applied to each data set, with a $P = 0.05$ level set for significance. Most data sets were found to be normally distributed, as is required for a *t*-test. For those that were not normal a Wilcoxon Signed Rank Test was instead used with $P < 0.05$.

Standard sigmoidal curves were fit to the relation between agonist dose and reflex responses, with doses expressed in log units, and with a Hill slope of unity. The dose that produced 50% effect (EC50) was measured from the curve and –log(EC50) was used to quantify the drug *potency*: pEC50 = –log(EC50). Also, the maximum drug-induced response (*efficacy*) was computed from the curve (peak of curve). For comparison to our computed potencies (pEC50), the binding affinity of each drug at the rat 5-HT receptors was also reported, with values taken from the literature (Table 3-1). The binding of an agonist to a receptor is expressed in terms of its Ki value (nM), which corresponds to the dose that produces 50% binding to that receptor (Knight et al. 2004). This is typically measured by the agonist's ability to displace a standard radiolabelled ligand, like

[^{3}H]-5-HT, from the receptor expressed in isolated cells. Binding affinity is computed as pKi = –log(Ki) (Knight et al. 2004). When possible, binding affinities of different drugs for a given receptor were taken from large studies or summary reviews (Boess and Martin 1994), usually using isolated cloned receptors. Also, high affinity agonist-preferring binding sites were always used, measured with radioactive agonists (usually [^{3}H]-5-HT), rather than radioactive-antagonists that bind to a low affinity site (Egan et al. 2000; Knight et al. 2004). If rat receptor Ki values were not available, human values were used instead, as these are similar for most receptors (Boess and Martin 1994).

DOI is one of the few 5-HT$_2$ agonists that is very selective to only 5-HT$_2$ receptors (it activates just 5-HT$_{2A/2B/2C}$) (Boess and Martin 1994), and accordingly its dose-response relation was a simple sigmoidal relation. In contrast, other agonists like alpha-methyl-5-HT bind non-selectively to other 5-HT receptors, though with much less affinity than to 5-HT$_2$ receptors (Table 3-1). With these drugs the dose-response relation was mostly sigmoidal, until higher doses were reached, at which point the LLR tended to decrease with increasing dose, consistent with a non-selective action on other receptors. To minimize the effect of this non-selective receptor activation, only the main part of the dose-response relation with increasing responses to increasing dose (over 2 decades) was fit to the sigmoidal dose-response relation to obtain an estimate of the EC50. Further, we used a sigmoidal relation with a Hill slope of 1.0, which forced the sigmoid to have a fixed width of about 2 decades (2 orders of magnitude in dose, as is expected for the response to a single receptor). This approach caused the low-dose responses of agonists (e.g. selective 5-HT$_{2B}$ agonists) to dominate the sigmoidal fit where they are most selective, and assured that the EC50 was not affected (e.g. underestimated) by the non-selective action of other 5-HT receptors that may have affected (e.g. inhibited) the LLR at high doses.

Table 3-1

Receptor	Agonist	Ki (nM)	pKi (-logKi)	Receptor	Agonist	Ki (nM)	pKi (-log Ki)
5-HT1A	5-HT	1.65	8.78 [Hoy]	5-HT2B	2-methyl-5-HT	316.23	6.50 [Boe]
	8-OH-DPAT	0.97	9.01 [Boe]		5-HT	10.23	7.99 [Boe]
	LP44	52.48	7.28 [Leo]		α–methyl-5-HT	10.47	7.98 [Boe]
					BW723C86	12.59	7.90 [Bax]
					DOI	27.54	7.56 [Boe]
5-HT1B	5-HT	24.49	7.61 [Hoy]		methylergon	0.50	9.30 [Kni]
	BW723C86	125.89	6.60 [Bax]		tryptamine	112.20	6.95 [Boe]
	zolmitriptan	5.01	8.30 [Mar]				
	EMD386088	179.88	6.75 [Mat]	5-HT2C	5-HT	10.99	7.96 [Ega]
					α-methyl-5-HT	2.69	8.57 [Kni]
					BW723C86	125.89	6.90 [Bax]
5-HT1D	5-HT	2.51	8.60 [Boe]		DOI	9.30	8.03 [Ega]
	BW723C86	125.89	6.30 [Bax]		MK212	97.72	7.01 [Kni]
	zolmitriptan	0.63	9.20 [Mar]		methylergon	4.57	8.34 [Kni]
	EMD386088	109.90	6.96 [Mat]		tryptamine	62.0	7.21 [Ega]
5-HT1E	5-HT	6.16	8.21 [Boe]	5-HT3	2-methyl-5-HT	85.11	7.07 [Mil]
	α-methyl-5-HT	120.22	6.92 [Boe]		BW723C86	316.23	6.50 [Ken]
	methlergon	89.12	7.05 [Boe]		EMD	34.00	7.47 [Mat]
					MK212	29.00	7.54 [Gle]
5-HT1F	5-HT	67.60	7.17 [Boe]				
	α-methyl-5-HT	181.97	6.74 [Boe]	5-HT4	5-HT	6.31	8.20 [Adh]
	LY344864	6.02	8.22 [Phe]		α-methyl-5-HT	263.03	6.58 [Adh]
	methylergon	30.90	7.51 [Boe]		cisapride	25.00	7.60 [Adh]
	zolmitriptan	63.09	7.20 [Mar]				
				5-HT5A	5-HT	7.94	8.10 [Boe]
5-HT2A	5-HT	5.75	8.24 [Boe]		8-OH-DPAT	50.12	7.30 [Boe]
	α-methyl-5-HT	127.05	6.90 [Eng]				
	BW723C86	251.18	6.60 [Bax]	5-HT6	2-methyl-5-HT	52.48	7.28 [B97]
	DOI	0.79	9.10 [Boe]		5-HT	56.23	7.25 [Boe]
	methylergon	0.35	9.45 [Kni]		BW723C86	398.11	6.40 [Ken]
					EMD	7.41	8.13 [Mat]
				5-HT7	5-HT	1.51	8.82 [Boe]
					8-OH-DPAT	34.67	7.46 [Boe]
					LP44	0.22	9.66 [Leo]

Table 3-1. 5-HT receptor agonists and their receptor binding affinity. Agonists Ki and binding affinity (pKi = –log Ki) obtained from high affinity agonist radioligand bindings studies (Adham et al. 1996; Baxter 1996; Boess and Martin 1994; Boess et al. 1997; Egan et al. 2000; Engel et al. 1986; Glennon et al. 1989; Hoyer et al. 1985; Kennett et al. 1997a; Knight et al. 2004; Leopoldo et al. 2007; Martin et al. 1997; Mattsson et al. 2005; Milburn and Peroutka 1989; Phebus et al. 1997), with references abbreviated by the first few letters of authors name, except for Boess et al. 1997 which is abbreviated B97. Methylergonovine abbrevated methylergon. Each agonists is considered to activate a receptor if Ki < 400 nM, and listed with that receptor. Cisapride binds to other receptors, but was only used after blocking all but 5-HT_4 receptors (Table 3-2), and so only listed with this receptor.

RESULTS

Use of LLRs as a measure of Ca PICs and spasms in chronic spinal rats.

When the dorsal roots of the *in vitro* sacrocaudal spinal cord were stimulated with a low-threshold current (3xT), there was a long-lasting reflex (LLR; 2 - 10 s duration) evoked in motoneurons, as recorded both from ventral roots (Fig 1A) and from single motoneurons measured intracellularly (Fig 3-1C, top panel). The many second long portion of this LLR is mediated by a large persistent inward calcium current (Ca PIC) that activates a plateau potential and associated sustained firing in motoneurons of chronic spinal rats (Li et al. 2004a), and we verified this with two methods. First, directly blocking the Ca PIC (with isradipine or nimodipine; 15 μM) largely eliminated the LLR (significantly reduced by 83.9±7.9%, $n = 9$, $P < 0.05$, Fig 3-1B), and second, hyperpolarizing motoneurons to prevent activation of the voltage-dependent Ca PIC eliminated the LLR and associated PIC-mediated plateau (Fig 3-1C, lower panel; $n = 10/10$). In both cases, eliminating the Ca PIC left an approximately 0.5 s long response that was due to a polysynaptic EPSP in the motoneurons (Fig 3-1B and Fig 3-1C, bottom panel), which we discuss in a later paper. For the present study, we focus on the Ca PIC and use the LLR reflex measured at times > 0.5 s as an indirect estimate of this Ca PIC on motoneurons (Fig 3-1A lower panel; rectified response averaged in a 0.5 - 4 s window). This enabled us to efficiently screen many 5-HT drugs and doses with simple ventral root, rather than intracellular motoneuron, recordings. Previous work has shown that the LLR we record *in vitro* also occurs *in vivo* in the awake chronic spinal rat where it produces muscle spasms (Bennett et al. 2004; Murray et al. 2010). Thus, the LLR recorded in *vitro* is also a convenient assay of spasms, and we use it to assess which 5-HT receptors modulate spasms.

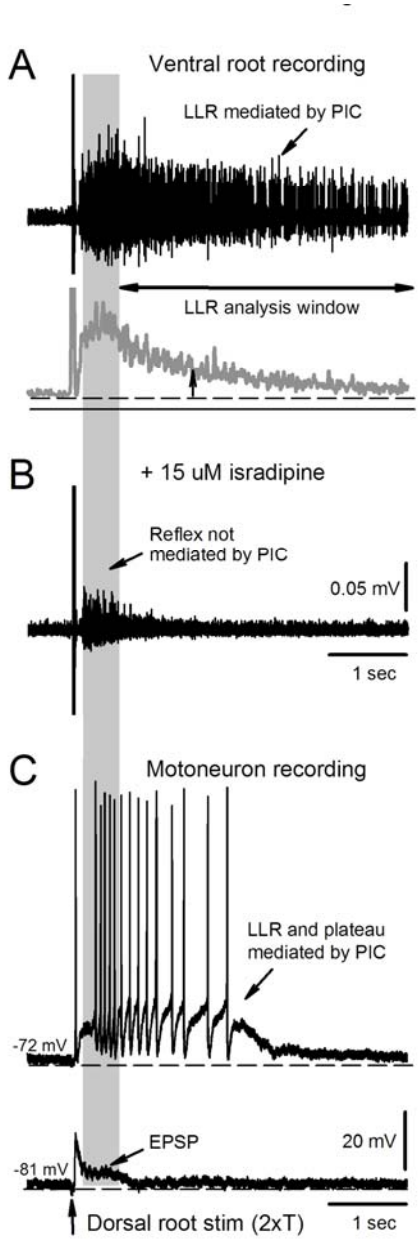

Figure 3-1. Long lasting reflexes, the counterpart of muscle spasms, are mediated by the Ca PIC in motoneurons in chronic spinal rats. (**A**) Long-lasting reflex (LLR) triggered by dorsal root stimulation (0.1 ms pulse, 3xT) and recorded from the ventral roots (top plot). LLR quantified by rectifying the ventral root activity (lower plot) and averaging over window indicated. (**B**) Elimination of LLR after blocking the L-type Ca^{2+} channel with isradipine (15uM). (**C**) PIC-mediated plateau potential and sustained firing (LLR) evoked by dorsal root stimulation (3xT) in motoneuron at rest (without injected current; top). With a hyperpolarizing bias current to prevent PIC activation, the same stimulation only evoked polysynaptic EPSPs that ended within 0.5 sec (in grey shaded region), with no synaptic activity during the subsequent period where the LLR was computed (lower trace).

Only 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors facilitate the LLR

Application of 5-HT$_2$ receptor agonists (alpha-methyl-5-HT, DOI, BW723C86, methylergonovine, MK212, 2-methyl-5-HT, tryptamine and 5-HT) increased the LLR (Fig 3-2), with increasing doses generally producing larger responses over about a 100-fold change in dose (e.g. 10 - 1000 nM for alpha-methyl-5-HT; Fig 3-2D). This agonist dose-response relation was well approximated by a sigmoidal curve (Figs 3-2C and D), from which we computed the agonist dose to produce 50% maximal effect (EC50, Fig 3-2D), agonist potency (-pEC50 = -log(EC50)) and efficacy (maximal response; Fig 3-2C), as detailed in the Methods. All 5-HT$_2$ agonists tested exhibited a significant efficacy in facilitating the LLR (Table 3-2), with efficacy reported as a percent of mean 5-HT-induced LLR, which was 0.393 ± 0.19 mV. This 5-HT induced LLR was 3-fold higher than the pre-drug LLR of 0.11 ± 0.06 mV (100% in Table 3-2), and thus represents a 3-fold increase in LLR. Agonists that are known to bind to both 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors (Ki < 50 nM; alpha, DOI, 5-HT) had consistently the highest efficacies, including 5-HT. Agonists that bind relatively selectively to either 5-HT$_{2B}$ receptors (BW723C86, methylergonovine, 2-methyl-5-HT; i.e., Ki for 5-HT$_{2B}$ about10x higher than 5-HT$_{2C}$; Table 3-2) or 5-HT$_{2C}$ receptors (MK212; or agonists made selective by antagonists, e.g. alpha-methyl-5-HT acts as a selective 5-HT$_{2C}$ agonist when given with the 5-HT$_{2C}$ antagonist RS127445 present; Fig 3-2C; Table 3-2) had significantly lower efficacies. However, the sum of the effects of selective 5-HT$_{2B}$ and 5-HT$_{2C}$ agonists was similar to the effect of broad spectrum 5-HT$_2$ agonists (alpha-methyl-5-HT and 5-HT, Table 3-2), suggesting that both 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors are required to maximally facilitate the LLR.

Prior application of antagonists that blocked *both* 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors (including SB206553, clozapine, cyproheptadine, methysergide or a combination of RS127445 and RS102221) inhibited the 5-HT$_2$ agonist-induced increase in LLR (Fig 3-2C and D; Table 3-2), eliminating responses to all but the highest doses, and significantly reducing the LLR induced by the agonist dose that maximally facilitated the LLR prior to the antagonists (SB206553 reduced LLR to

15.9±25.7, 4.1±13.2, and 1.0±8.2 % of pre-antagonist efficacy for alpha-methyl-5-HT, DOI and 5-HT respectively). The antagonist application shifted the dose-response relation to such high doses that the standard sigmoidal curve with 100-fold dose range could no longer be fit to the few responsive high doses tested and the usual EC50 measurements were thus not detectable (ND; Table 3-2). This thorough inhibitory action of these antagonists suggests that 5-HT_{2B} and/or 5-HT_{2C} receptors facilitate the LLR. Furthermore, the finding that the facilitation of the LLR by 5-HT itself is blocked by these combined 5-HT_{2B} and 5-HT_{2C} antagonists indicates that all other 5-HT receptors (including 5-HT_{2A}) are likely *not* involved in facilitating the LLR or PIC.

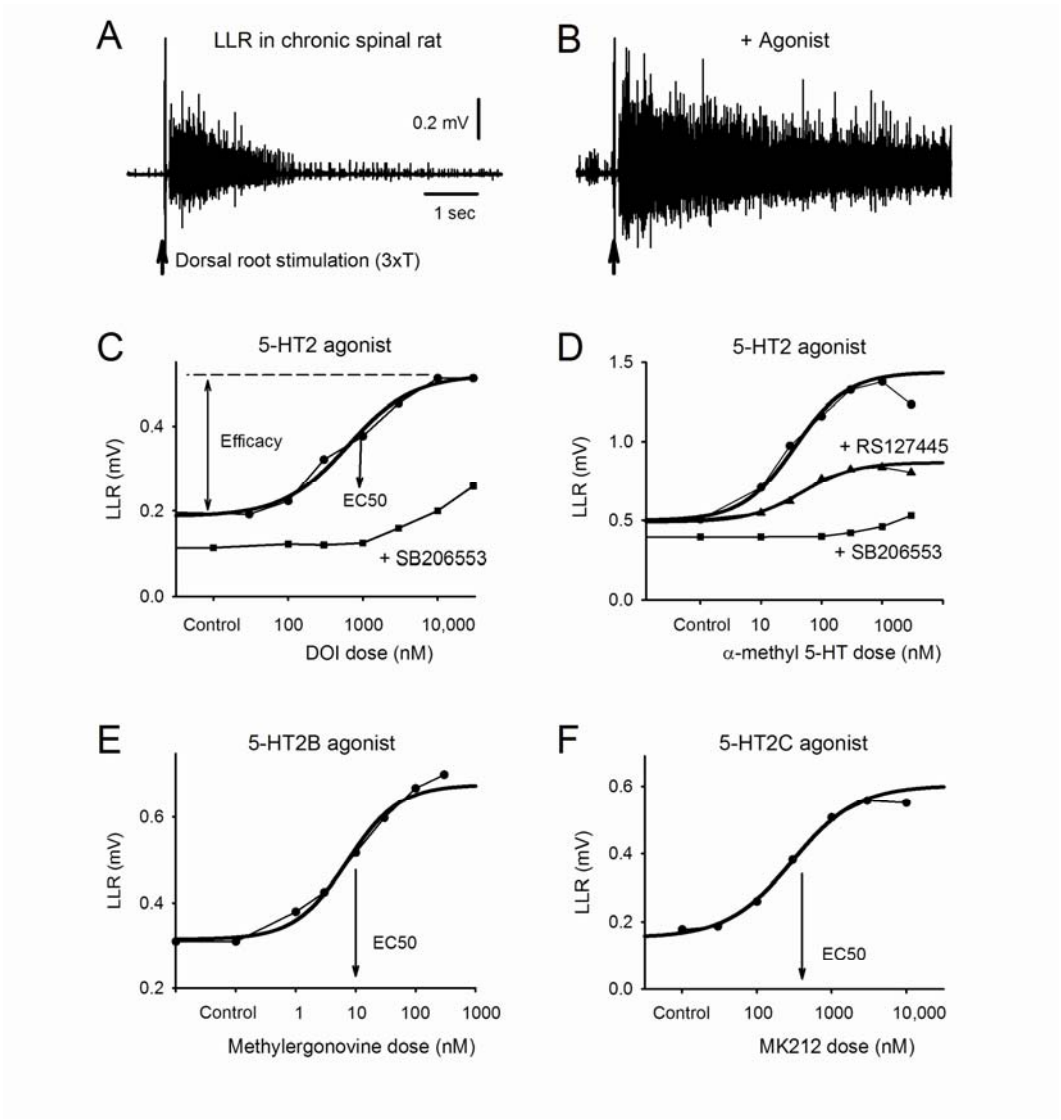
A
LLR in chronic spinal rat
0.2 mV
1 sec
Dorsal root stimulation (3xT)
B
+ Agonist
C
5-HT2 agonist
LLR (mV)
Efficacy
EC50
+ SB206553
Control
100
1000
10,000
DOI dose (nM)
D
5-HT2 agonist
LLR (mV)
+ RS127445
+ SB206553
Control
10
100
1000
α-methyl 5-HT dose (nM)
E
5-HT2B agonist
LLR (mV)
EC50
Control
1
10
100
1000
Methylergonovine dose (nM)
F
5-HT2C agonist
LLR (mV)
EC50
Control
100
1000
10,000
MK212 dose (nM)

Figure 3-2. 5-HT_{2B} and 5-HT_{2C} receptor agonists increase the long lasting reflexes in chronic spinal rats. (**A**) Long lasting reflex (LLR) triggered by dorsal root stimulation (0.1 ms pulse, 3xT) and recorded from the ventral roots. (**B**) Facilitation of LLR with application of the 5-HT_2 receptor agonist, alpha-methyl-5-HT (0.1uM), quantified as indicated in Fig 3-1. (**C–F**) Facilitation of LLR with increasing doses of broad spectrum 5-HT_2 receptor agonists (DOI and alpha-methyl-5-HT; C and D) and relatively selective 5-HT_{2B} (methylergonovine) and 5-HT_{2C} (MK212) receptor agonists (increase over ~100-fold change in dose). Best fit sigmoidal curves and subsequent estimation of EC50 are shown with thick lines. Prior application of a single blocking dose of the selective 5-$HT_{2B/2C}$ receptor antagonist SB206553 (5 μM) or the 5-HT_{2B} receptor antagonist RS127445 (3 μM) inhibited these agonists responses (lower plots in C and D).

Prior application of the selective 5-HT_{2B} receptor antagonist SB204741 significantly inhibited the effect of the selective 5-HT_{2B} agonist BW723C86 (decreasing the potency, doubling the EC50 and decreasing the efficacy, Table 3-2), consistent with the involvement of 5-HT_{2B} receptors. However, the response to BW723C86 was not completely eliminated by SB204741, likely due to the weak action of BW723C86 on the 5-HT_{2C} receptor (we thus consider BW723C86+SB204741 a weak 5-HT_{2C} agonist in Table 3-3, described below). Blocking all 5-HT_2 receptors except the 5-HT_{2B} receptor (with 5-HT_{2C} and 5-HT_{2A} antagonists) did *not* eliminate the facilitation of the LLR by subsequent application of the 5-HT_2 receptor agonist alpha-methyl-5-HT (Table 3-2) and did *not* significantly shift the dose-response curve (EC50 not altered significantly), although the efficacies were lowered (Table 3-2). Similarly, blocking all but the 5-HT_{2C} receptor did not eliminate the alpha-methyl-5-HT-induced LLR or significantly shift the EC50 (Table 3-2). This is consistent with the involvement of both the 5-HT_{2B} and 5-HT_{2C} receptors in facilitating the LLR; the EC50 was not significantly shifted with a block of either 5-HT_{2B} or 5-HT_{2C} receptors, because alpha-methyl binds with similar affinity to 5-HT_{2B} and 5-HT_{2C} receptors (Table 3-1).

Application of selective agonists (or agonist/antagonist combinations) for other 5-HT receptors (5-HT_1, 5-HT_{2A}, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, 5-HT_7) produced no significant facilitation of the LLR even at high doses (3 - 10 μM; Table 3-2), again suggesting that only 5-HT_{2B} and 5-HT_{2C} receptors facilitate the LLR.

Table 3-2

Agonist	Pre-treatment with antagonists	Selectivity/ Receptors activated with Ki < 50nM and (Ki = 50 - 400 nM).	Facilitation of the LLR		
			Potency (-logEC50)	EC50 (nM)	Efficacy (% of 5-HT effect)
2-methyl-5-HT	None	(5-HT2B, 3, 6)	5.41 ± 0.32	3852	70 ± 38*
5-HT	None	5-HT1 – 7	7.48 ± 0.23	33	100 ± 43*
5-HT	SB206553[#]	5-HT1, 2A, 3 – 7	ND	ND	1.0 ± 8.2
α-methyl-5-HT	None	5-HT2A,2B,2C (5-HT1E,1F; 5-HT4)	7.24 ± 0.20	58	116 ± 49*
α-methyl-5-HT	SB206553	5-HT2A (5-HT1E,1F; 5-HT4)	ND	ND	18.4 ± 29.8*
α-methyl-5-HT	RS102+AC901	5-HT2B (5-HT1E,1F; 5-HT4)	7.12 ± 0.31	76	77 ± 30*
α-methyl-5-HT	RS127+AC901	5-HT2C (5-HT1E,1F; 5-HT4)	7.25 ± 0.26	56	74 ± 46*
BW723C86	None	5-HT2B (5-HT1B,1D, 2A,2C,3,6)	6.53 ± 0.31	295	49 ± 48*
BW723C86	SB204741	(5-HT1B,1D, 2A,2C,3,6)	5.84 ± 0.17[†]	1445	31 ± 23*
DOI	None	5-HT2A, 2B, 2C	6.19 ± 0.17	646	119 ± 37*
DOI	SB206553	5-HT2A	ND	ND	5.8±15.7
Methylergonovine	None	5-HT2A, 2B, 2C (5-HT1E, 1F)	7.96 ± 0.25	11	69 ± 28*
MK212	None	(5-HT2C, 3)	6.35 ± 0.24	447	47 ± 29*
Tryptamine	SB224289	5-HT2A (5-HT2B)	6.04 ± 0.39	912	51 ± 24*

Table 3-2. Faciliation of LLR by 5-HT_{2B} and 5-HT_{2C} receptor agonists. Agonists with varying selectivity for the different 5-HT receptors were applied, sometimes after prior application of 5-HT receptor antagonists to effectively make the agonist action more selective (column 2, pre-treatment, starting > 30 mins prior to agonist). The receptors that can be activated by this agonist/antagonist combination are indicated in column 3, with high binding affinity for the agonist listed first (with Ki about 1 - 50nM), followed by moderate affinity (in brackets; Ki = 50 - 400nM; see Table 3-1). Antagonists abbreviations and receptors blocked are: RS127445 (RS127, 5-HT_{2B}); RS102221 (RS102, 5-HT_{2C}); AC90179 (AC901, 5-HT_{2A}); SB206553 (5-$HT_{2B/2C}$); SB204741 (5-HT_{2B}), SB224289 (5-HT_{1B}), prazosin (alpha1 adrenergic); granisitron (5-HT_3); methysergide (5-HT_2) and clozapine (5-HT_2). Antagonist doses are given in Table 3-4, except for prazosin (1 μM), granisetron (0.3 μM) and SB224289 (1 μM). The efficacy (maximal effect) of these agonists in facilitating the LLR is indicated in column 6 (max mean rectified LLR, normalized by 5-HT efficacy). When a significant facilitation (efficacy) was detected the mean agonist potency was computed, from which the EC50 were computed (potency = -log EC50). In addition to the agonists shown, no detectable facilitation (ND, $P > 0.05$) occurred with application of the agonists: 8-OH-DPAT, cisapride(RS), EMD386088, LP44, LY34864 and zolmitriptan when given up to 3 - 10 μM doses, ruling our the involvement of the receptors that these drugs selectively activate (see Table 3-1 for Ki values; cisapride given after pre-treatment with antagonists RS127445, RS1022221, granisetron and prazosin to make it selective to 5-HT_4 receptors, which is abbreviated cisapride(RS)). Also after some antagonist pre-treatments (SB206553) there was no detectable (indicated ND) facilitation of the LLR at all but the highest doses, making it impossible to estimate EC50. Symbols: + between antagonists indicates they were given in combination. # indicates that pre-treatment with methysergide, clozapine or RS127445+RS102221 also eliminated the facilitation of the LLR by 5-HT (ND), like SB206553. † indicates potency significantly lowered by SB204741, $P < 0.05$. * indicates efficacy significantly different from zero (LLR significantly increased), $P < 0.05$. Data shown as mean ± s.d., with $n > 8$ per drug (or combination).

Agonist potency is correlated with receptor binding affinity at 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors.

The effective 5-HT$_2$ agonist doses required to facilitate the LLR (EC50 values and associated potencies, pEC50) varied by orders of magnitude between the different agonists (or agonist/antagonist combinations). However, this variation was largely accounted for by the differing binding affinity of these drugs to 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors (pKi; see description of binding affinity in Methods). That is, we found that the agonist potencies in facilitating the LLR (pEC50 and associated EC50) were significantly correlated with the binding affinity of these agonists (pKi) for 5-HT$_{2B}$ (r = 0.93, slope = 0.95, n = 8 drugs combinations tested, P < 0.05) and 5-HT$_{2C}$ (r = 0.77, slope = 0.89, n = 8, P < 0.05) receptors, as shown in Fig 3B, C. Interestingly, a line with unity slope (pEC50 = pKi + C, where C is a fixed value) was equally well fit to the data, with r = 0.92 and r = 0.77 for 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors, respectively (Fig 3-3A, B), a useful finding that we employ in the next section to model the potency-affinity relation to further predict which receptors modulate the LLR. In contrast, there was no significant correlation with the potency and the binding affinity for 5-HT$_{2A}$ receptors (r = 0.31, n = 8, P > 0.05), or any other receptor (Fig 3-3), and points were scattered far from the unity slope line that fit the 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors.

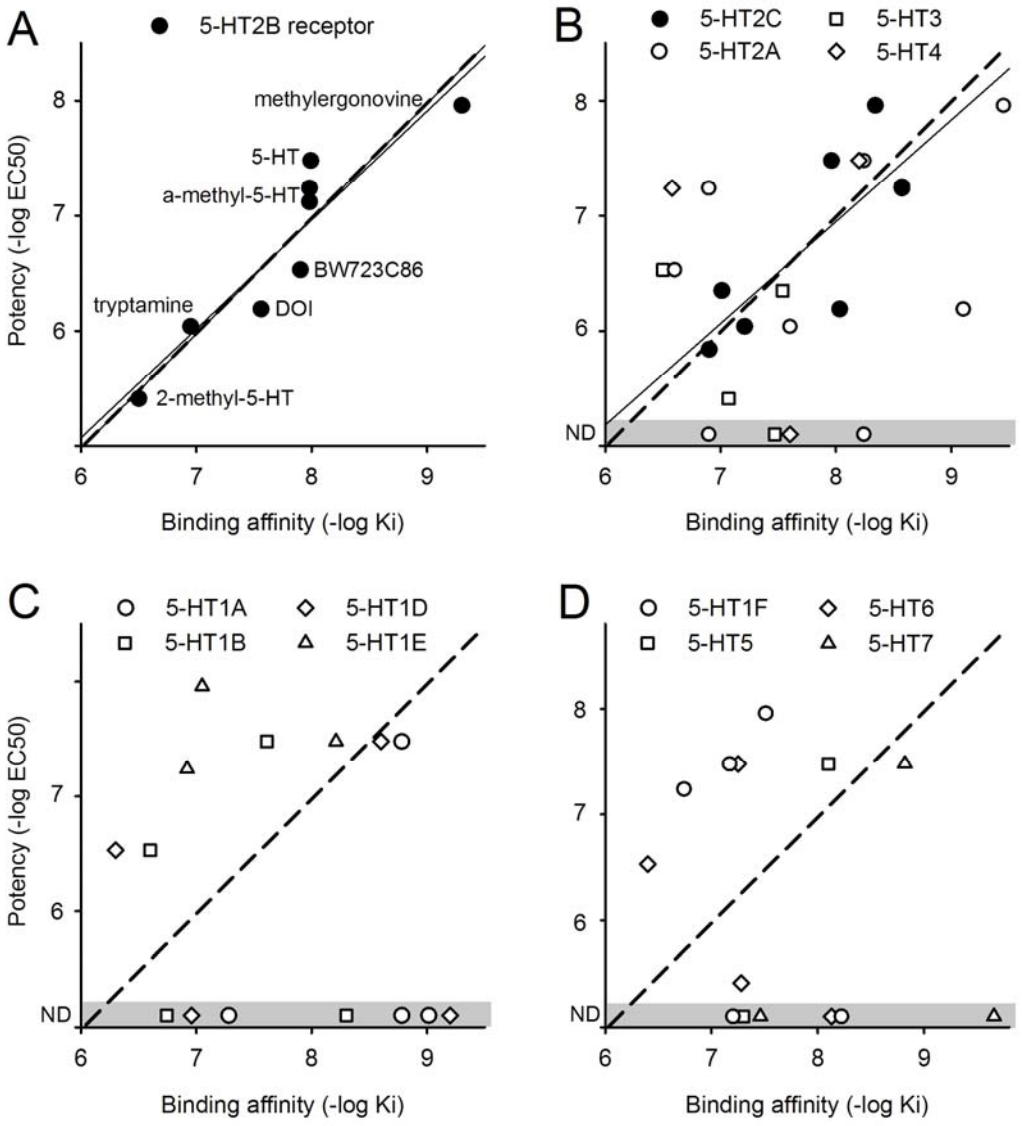
A
5-HT2B receptor
methylergonovine
5-HT
a-methyl-5-HT
BW723C86
tryptamine
DOI
2-methyl-5-HT
Potency (-log EC50)
Binding affinity (-log Ki)
B
5-HT2C
5-HT3
5-HT2A
5-HT4
ND
Binding affinity (-log Ki)
C
5-HT1A
5-HT1D
5-HT1B
5-HT1E
Potency (-log EC50)
ND
Binding affinity (-log Ki)
D
5-HT1F
5-HT6
5-HT5
5-HT7
ND
Binding affinity (-log Ki)

Figure 3-3: Potency of 5-HT receptor agonists at facilitating the LLR is only related to binding to 5-HT_{2B} and 5-HT_{2C} receptors. (**A**) 5-HT_{2B} receptor agonist potency (pEC50 = -log (EC50)) for facilitating the LLR plotted against the agonist binding affinity to that receptor (pKi), with each agonist name indicated next its point. Alpha-methyl-5-HT potency with and without block of 5-HT_{2C} receptor with RS102221 plotted. Thin line: linear correlation between potency and affinity. Dashed line: best fit line with unit slope (potency = binding affinity + C, where C ~ –1). (**B-D**) Similar potency-affinity scatter plots for the remaining 5-HT receptors. Agonists used for each receptor are listed in Table 3-1, with agonists assumed to act at a receptor only if Ki < 400 nM. Potencies are from Table 3-2. Also we used the potency of BW723C86 after treatment with SB204 for the 5-HT_{2C} receptor plot, to avoid dominant 5-HT_{2B} receptor action of this drug. Thin line: correlation between agonist potency and affinity for 5-HT_{2C} receptors. Dashed line: best fit unity slope relation for the 5-HT_{2C} receptor, copied into C and D to show lack of similar relation for other receptors. Other receptors had no significant correlation between potency and affinity (open circles; $P > 0.05$). ND and gray zone: no detected effect of agonist on the LPR.

Potency of each agonist can be quantitatively predicted from its receptor binding affinity.

To directly compensate for the variable receptor binding affinity of different agonists, we computed the potency of each agonist in facilitating the LLR relative to its binding affinity at each receptor, which we term the *relative potency* = pEC50 - pKi. As we just mentioned, this difference is a fixed constant (*C* = relative potency) for the receptors *involved* in modulating the LLR (5-HT_{2B} and 5-HT_{2C}), and thus we expect that the relative potency should be invariant when computed with pKi values for functional receptors in the spinal cord (5-HT_{2B}), and variable for other receptors. This relative potency should account for all factors that affect potency (e.g. drug diffusion, receptor signalling, etc., see below) *other than* receptor binding. As shown in Table 3-3, the relative potency computed for all agonists of the 5-HT_{2B} receptor were indeed very similar, all falling within the 99% confidence interval (± 0.58) about the mean relative potency (–1.02 ± 0.32; mean ± s.d.), and within two standard deviations of this mean, which we set as the similarity criterion. The relative potency of agonists at the 5-HT_{2C} receptor was likewise invariant, clustered remarkably near the mean value of –1.03 ± 0.49, and not significantly different from the relative potency at the 5-HT_{2B} receptor (Table 3-3). Again, most 5-HT_{2C} agonists (only put 5-HT_{2C} agonists in Table 3-3) had relative potencies within the 99% confidence interval about the mean (±0.53), and within two standard deviations of the mean, consistent with the interpretation that 5-HT_{2C} receptors also play a role in modulating the LLR (see also Fig 3-3, squares). The only exception was DOI, which had a response that was dominated by the 5-HT_{2B}, rather than 5-HT_{2C} receptor. These findings suggest that, after taking into account variable binding affinity, all these 5-HT_2 agonists appeared to diffuse equally well to common targets (the 5-HT_{2B} and 5-HT_{2C} receptors) and have similar actions (facilitating the Ca PIC underlying the LLR). Receptor binding affinities (pKi and Ki values) are typically measured in isolated cells (transfected with cloned receptors, Boess and Martin 1994; Knight et al. 2004) and thus they represent absolute minimum agonist doses needed for drug action (Ki dose values). In contrast, we measured

the functional effects of these agonists (the potency or EC50) in a *whole* spinal cord preparation, where there are substantial barriers to drug diffusion to the receptors. Furthermore, downstream actions of receptor binding typically require higher drug doses, even in the absence of diffusion barriers. Thus, the EC50 must exceed the Ki dose value, which means the potency must be less than affinity, and the relative potency (potency-affinity) must be less than zero, and was indeed -1.0 for our whole cord preparation. Another way to interpret relative potency arises from the law of differences of logarithms:

$$C = pEC50 - pKi = -\log(EC50) - (-\log Ki) = -\log(EC50/Ki).$$

Thus, the ratio $EC50/Ki = 10^{-C}$, and again should be a fixed number for a receptor involved in modulating the LLR. For the 5-HT_{2B} receptor the overall EC50/Ki was 10.0[1]. This indicates that the EC50 in the present whole sacral spinal cord preparation is about 10 times the Ki value, which is likely due to common diffusion barriers.

Our consistent findings of fixed relative potencies for 5-HT_{2B} and 5-HT_{2C} receptors suggest that perhaps in general we can predict the EC50 and potency of drugs at a particular receptor from the published Ki values (EC50 = 10 * Ki and potency = affinity – 1), and receptors that do not consistently fit this prediction are unlikely to be functional (assuming similar diffusion to each receptor). The power of this modelling approach is that the lack of involvement of a given receptor in the LLR can be proven by the potency measured from as little as one agonist, if that potency is far from the affinity (relative potency different from –1, and corresponding point far from pEC50 = pKi – 1 line in Fig 3-3; e.g. 5-HT_{2A} cannot be involved because DOI exhibits a relative potency of –3 at this receptor). Indeed, we found that the relative potency at all other 5-HT receptors was highly variable, usually significantly different from the relative potency at the 5-$HT_{2B/2C}$ receptors (> two standard deviations away from –1.0; Table 3-3; and scattered away from pEC50 = pKi – 1 line in Fig 3-3), thus further ruling out the involvement of all but the 5-HT_{2B} and 5-HT_{2C} receptors. Occasionally, the relative potency fell close to –1.0 for a particular agonist, like methylergonovine

at the 5-HT_{2A} receptor, but this was simply by chance, because the relative potency of other agonists at these receptors was not consistently –1.0. For example, MK212 has an impossibly high relative potency of +0.36 at the 5-HT_{2A} receptor, with a corresponding EC50 less than Ki (Table 3-3; MK212 is a poor 5-HT_{2A} agonist). Furthermore, these receptors with inconsistent relative potencies often had substantial binding affinity for agonists (like zolmitriptan at the 5-HT_{1B} receptor) that caused no detectable facilitation of the LLR (ND; Table 3-3). Thus, we again conclude that only the 5-HT_{2B} and 5-HT_{2C} receptors are involved in facilitating the LLR.

Table 3-3

Receptor	Agonist	pEC50 – pKi	Receptor	Agonist	pEC50 – pKi
5-HT1A	5-HT	–1.30±0.33*	5-HT2B	2-methyl-5-HT	**–1.09±0.32***
	5-HT (SB206)	ND		5-HT	**–0.51±0.33***
	8-OH-DPAT	ND		α-me-5-HT	**–0.74±0.20***
	LP44	ND		α-me-5-HT (RS102)	**–0.86±0.31***
				BW723C86	**–1.37±0.31***
5-HT1B	5-HT	–0.13±0.33		DOI	**–1.37±0.17***
	5-HT (SB206)	ND		methylergon	**–1.34±0.25***
	BW723C86	–0.07±0.31		tryptamine (SB224)	**–0.91±0.39***
	EMD386088	ND			
	zolmitriptan	ND	5-HT2C	5-HT	**–0.48±0.33***
				α-me-5-HT	**–1.33±0.20***
5-HT1D	5-HT	–1.12±0.33*		α-me-5-HT (RS127)	**–1.32±0.26***
	5-HT (SB206)	ND		BW723C86 (SB204)	**–1.06±0.17***
	BW723C86	+0.23±0.31		DOI	–1.84±0.17
	EMD386088	ND		MK212	**–0.66±0.24***
	zolmitriptan	ND		tryptamine (SB224)	**–1.17±0.39***
5-HT1E	5-HT	–0.73±0.33*	5-HT3	2-methyl-5-HT	–1.66±0.32
	5-HT (SB206)	ND		BW723C86	+0.03±0.31
	α-me-5-HT	+0.32±0.20		EMD386088	ND
	methylergon	+0.91±0.25		MK212	–1.19±0.24*
5-HT1F	5-HT	+0.31±0.33	5-HT4	5-HT	–0.72±0.33*
	5-HT (SB206)	ND		5-HT (SB206)	ND
	α-me-5-HT	+0.50±0.20		α-me-5-HT	+0.66±0.20
	LY344864	ND		cisapride(RS)	ND
	methylergon	–0.45±0.25			
	zolmitriptan	ND	5-HT5	5-HT	–0.62±0.33*
				5-HT (SB206)	ND
5-HT2A	5-HT	–1.76±0.33		8-OH-DPAT	ND
	5-HT (SB206)	ND			
	α–me-5-HT	+0.34±0.20	5-HT6	2-methyl-5-HT	–1.87±0.32
	α–me-5-HT(SB206)	ND		5-HT	+0.23±0.33
	BW723C86	–0.07±0.31		BW723C86	+0.13±0.31
	DOI	–2.91±0.17		EMD (SB216+)	ND
	methylergon	–1.49±0.25*			
			5-HT7	5-HT	–1.34±0.33*
				5-HT (SB206)	ND
				8-OH-DPAT	ND
				LP44	ND

Table 3-3. Relative potency of agonists at facilitating the LLR. Relative potency computed as the difference between potency (Table 3-2) and affinity (Table 3-1): pEC50 - pKi. Pre-treatment with antagonists used to make agonist more selective, indicated in brackets, abbreviated as in Table 3-2. ND, no detected inhibition of the reflex in Table 3-2. Relative potency not computed for methylergonovine at the 5-HT_{2C} receptor, because this drug has a much higher affinity for 5-HT_{2B} receptors, which dominate its response. *, relative potency within 2 SD of -1.0 (or the mean for 5-HT_{2B} receptors), the confidence interval for similarity. SD, standard deviation of relative potency measurements, indicated. Bold, receptors with relative potency values mostly within confidence interval.

Antagonist dose determination.

The experiments described above partly rely on employing a blocking dose of selective 5-HT_2 receptor antagonists prior to adding agonists, to identify the receptors involved in facilitating the LLR. The antagonist dose that we used was always chosen to be at least 10 times the published Ki value of that antagonist at the target receptor (e.g. 5-HT_2; Table 3-4, column 2), to account for drug diffusion into the whole cord preparation, as described above for agonists (assuming that the EC50 of the antagonist can be predicted from its Ki value, as for agonists: EC50= 10*Ki), and typically set at least 100 times the Ki to maximally block the receptor. Further, to be sure that we did not affect other receptors non-selectively, we tried to employ an antagonist dose that was less than about 10 times the Ki value of that antagonist at other receptors known to bind this antagonist (Table 3-4, column 3 shows the receptor most likely to be non-selectively activated, with the highest published Ki value of all other receptors tested). Satisfying this latter criterion was not always possible for non-selective antagonists like methysergide.

5-HT_{2B} and 5-HT_{2C} receptor antagonists block the action agonists.

To verify that we employed 5-HT_2 receptor antagonists at an appropriate dose to block the 5-$HT_{2B/2C}$ receptors in the experiments described above (e.g. Table 3-2), we directly tested the efficacy of most antagonists in inhibiting a prior dose of the 5-HT_2 receptor agonist alpha-methyl-5-HT (0.3 μM). A submaximal agonist dose (near agonist EC50; Fig dose vs time) was used to assure that the agonist only activated 5-HT_2 receptors. As shown in Figure 3-3 and Table 3-4, all 5-$HT_{2B/2C}$ antagonists tested (selective and non-selective drugs) significantly decreased a prior agonist induced increase in LLR. Importantly, the selective 5-HT_{2B} receptor antagonists (RS127445 and SB204741) and 5-HT_{2C} receptor antagonist (RS102221) each inhibited the LLR induced by the 5-HT agonist (Table 3-4) confirming that both 5-HT_{2B} and 5-HT_{2C} receptors modulate the LLR and associated Ca PIC. In contrast, the 5-HT_{2A} receptor antagonist AC90179 had no effect (Table 3-4).

Table 3-4

Antagonists	Receptors antagonized (Ki, nM)	Non-selective binding to receptor and (Ki, nM)	Refences for Ki	Antagonist dose (nM)	Antagonist inhibition of agonist-induced LLR (% change)	Antagonist inhibition of basal LLR (% change)
RS127445	5-HT2B (1.1)	5-HT2C (470)	Knight04	3,000	-45.2 ± 17.8*	-1.9 ± 5.3
SB242084	5-HT2B (45) 5-HT2C (0.48)	5-HT2A (850)	Cussac02 Knight04	3,000	-89.0 ± 18.2*	0.3 ± 26.9
Methysergide	5-HT2B (0.36) 5-HT2C (4.4)	5-HT1,2A,5,6,7	Knight04 Boess94	10,000	-98.8 ± 13.3*	1.0 ± 56.3
SB204741	5-HT2B (51)	5-HT2C (2100)	Cussac02	30,000	--	-33.1 ± 21.1*
RS102221	5-HT2C (5.0)	5-HT2B (2900)	Knight04	3000 - 10000	-40.9 ± 21.3*	-28.1 ± 8.6*
SB206553	5-HT 2B (5.5) 5-HT2C (3.2)	5-HT2A (2300)	Cussac02 Knight04	3000 - 10000	-79.1 ± 28.2*	-68.6 ± 18.2*
Cyprohept.	5-HT2B (1.5) 5-HT2C (2.2) alpha1 (25)	5-HT2A,5,6,7	Yoshio01 Bonhaus97 Boess94	10,000	--	-92.5 ± 10.0*
Clozapine	5-HT2B (10) 5-HT2C (13)	5-HT2A,3,6,7	Knight04 Boess94	10,000	--	--
Ketanserin	5-HT2A (8.1) 5-HT2C (62) alpha1 (6.3)	5-HT2B (740)	Knight04 Yoshio01	10,000	--	-52.8 ± 9.9*
AC90179	5-HT2A (0.20)	5-HT2C (2.9)	Vanover04	30	0.1 ± 6.6	1.4 ± 9.7

Table 3-4. 5-HT_2 receptor antagonists that inhibit the LLR. The receptors for which the antagonists are selective are shown in column 2, along with Ki values (brackets). Receptors that the antagonists non-selectively bind at higher doses are shown in column 3, along with Ki values. Ki values were obtained from the following references, abbreviated in column 4 (Boess and Martin 1994; Bonhaus et al. 1999; Cussac et al. 2002; Knight et al. 2004; Vanover et al. 2004; Yoshio et al. 2001). Selectivity is defined as the ratio of Ki's for two receptors (e.g. 470/1.1 = 427-fold selectivity of RS127445 at 5-HT_{2B} over 5-HT_{2C} receptors). For the non-selective antagonists methysergide, cyproheptadine and clozapine only Ki values for target receptors are shown, and other receptors affected only listed. Antagonists were employed during LLR reflex testing at the doses indicated in column 5, with choice of dose based on Ki values (see text). Following application of the 5-HT_2 receptor agonist alpha-methyl-5-HT (0.3 μM), antagonists were applied (Fig 3-4A) and the inhibition of the agonists-induced LLR measured (% change in LLR, relative to agonist-induced LLR; column 6; 0% no effect), with significant effects found for all 5-HT_{2B} or 5-HT_{2C} antagonists tested. Antagonists were also tested without agonists present, to examine their negative efficacy (inverse agonist) action (e.g. Fig 3-4F), and the inhibition of the control/basal LLR measured (% change in LLR; column 7), with significant effects found only for inverse agonists. The first 4 antagonists listed are neutral 5-$HT_{2B/2C}$ receptor antagonists (no effect without agonist present, column 7), whereas the next 6 antagonists act as inverse agonists at 5-$HT_{2B/2C}$ receptors (shaded rows; negative efficacy without agonist present, column 7). Data shown as mean ± SD, with $n > 8$ per drug (or combination). -- indicates not tested. * indicates significant inhibition induced by antagonist, $P < 0.05$.

Lack of action of neutral antagonists on the LLR.

Some of the 5-HT_2 receptor antagonists employed had no effect when given by *themselves*, including drugs selective to 5-HT_{2B} receptors (RS127445; Fig 3-4B) and 5-$HT_{2B/2C}$ receptors (SB242084; Fig 3-4D), and the non-selective 5-HT_2 receptor antagonist methysergide (Table 3-4 column 7). All these drugs have previously been classified as *neutral antagonists* (Bonhaus et al. 1999; Chanrion et al. 2008; Westphal and Sanders-Bush 1994). Neutral antagonists, by definition only act to inhibit the action of agonists and have no effect when given by themselves (no intrinsic efficacy), because they do not block constitutive receptor activity (as we see in Table 3-4) (Chanrion et al. 2008; Seifert and Wenzel-Seifert 2002), which would include the action of endogenous 5-HT that may be present in the spinal cord in residual amounts. Thus, the lack of effect of these drugs (when given alone) demonstrates that there is no functional residual 5-HT activating 5-HT_{2B} or 5-HT_{2C} receptors in the spinal cord after chronic spinal injury, consistent with our recent report for 5-HT_{2C} receptors (Murray et al. 2010).

Inverse agonists for 5-HT_{2B} and 5-HT_{2C} receptors directly inhibit the LLR.

The remaining 5-HT_2 receptor antagonists that we employed, including those that bind selectively to 5-HT_{2B} receptors (SB204741), 5-HT_{2C} receptors (RS102221 and ketanserin) or both (SB206553), significantly inhibited the LLR when they were given by *themselves* (without prior agonist application; Table 3-4; Fig 3-4F and H). Thus suggesting that these compounds acted as *inverse agonists*, which by definition can block constitutive receptor activity (receptor activity in the absence of 5-HT), in addition to their conventional ability to antagonize 5-HT-induced receptor activity (Aloyo et al. 2009; Berg et al. 1999; Chanrion et al. 2008; Seifert and Wenzel-Seifert 2002; Weiner et al. 2001; Westphal and Sanders-Bush 1994). Taken together with the lack of functional 5-HT in the chronic spinal rat, this inhibition of the LLR by such inverse agonists suggests that there is substantial constitutive activity in 5-HT_{2B} and 5-HT_{2C} receptors that endogenously facilitates the LLR. The magnitude of the inhibition of the LLR by SB204741 and RS102221 was similar (20-30% reduction in LLR; Table 3-4), and

thus the endogenous facilitation of the LLR is likely equally produced by constitutive activity in both 5-HT_{2B} and 5-HT_{2C} receptors. Furthermore, the inverse agonist SB206553, which selectively binds both 5-HT_{2B} and 5-HT_{2C} receptors (but no other receptors), reduced the PIC by approximately the amount predicted by the sum of the action of the selective 5-HT_{2B} and 5-HT_{2C} inverse agonist SB204741 and RS102221 (Table 3-4), suggesting that the action of 5-HT_{2B} and 5-HT_{2C} receptors is additive in facilitating the endogenous LLR (and associated LLR). The broad spectrum inverse agonists cyproheptadine and ketanserin also inhibited the LLR (Table 4). We have previously reported this action of SB206553 and cyproheptadine, but at the time were only able to conclude that 5-HT_{2B} or 5-HT_{2C} receptors may be constitutively active (Murray et al. 2010). Here we show that both receptors are constitutively active.

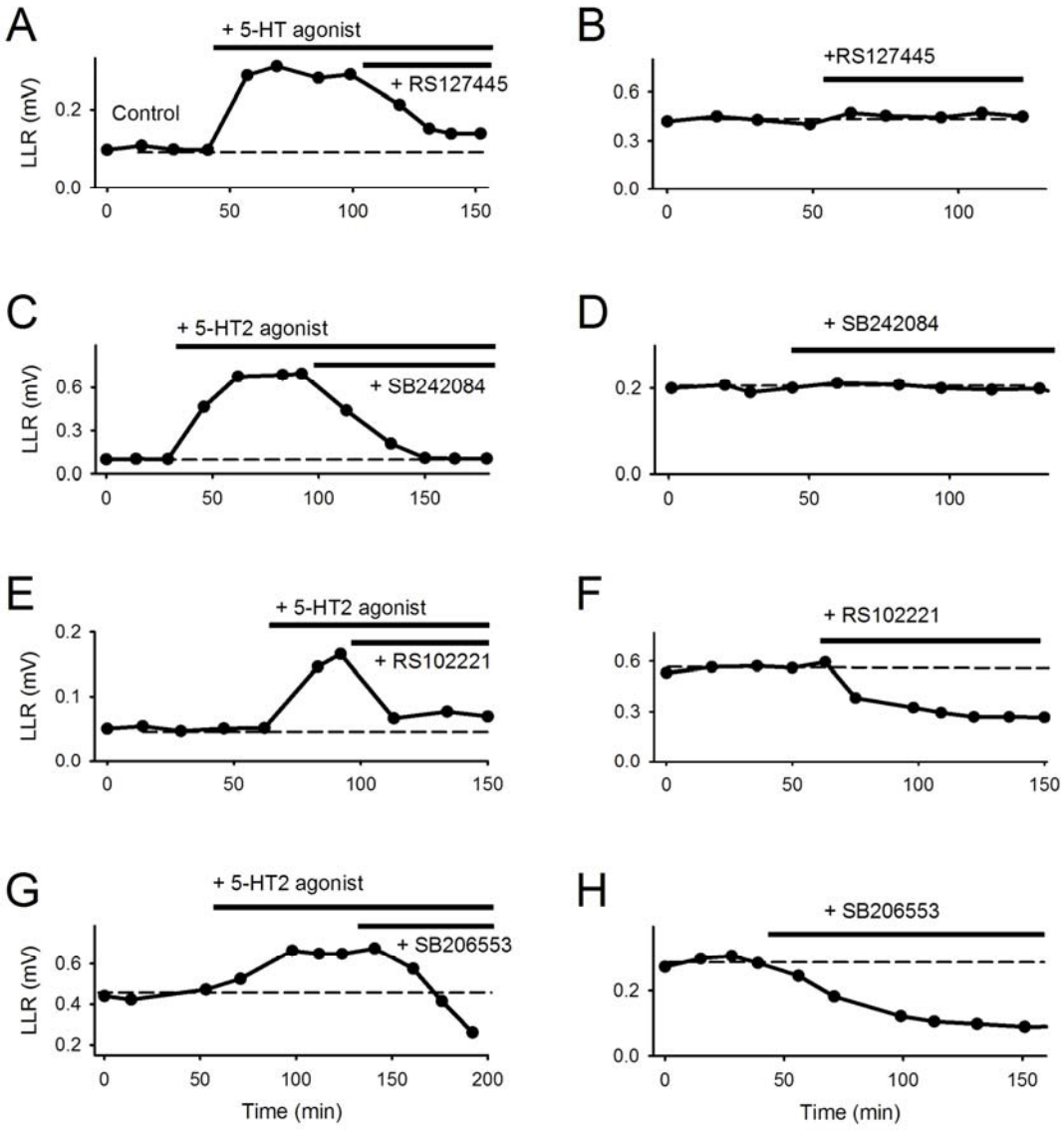
A
+ 5-HT agonist
+ RS127445
Control
LLR (mV)
0.0
0.2
0
50
100
150
B
+RS127445
0.0
0.3
0.6
0
50
100
C
+ 5-HT2 agonist
+ SB242084
LLR (mV)
0.0
0.3
0.6
0
50
100
150
D
+ SB242084
0.0
0.2
0
50
100
E
+ 5-HT2 agonist
+ RS102221
LLR (mV)
0.0
0.1
0.2
0
50
100
150
F
+ RS102221
0.0
0.3
0.6
0
50
100
150
G
+ 5-HT2 agonist
+ SB206553
LLR (mV)
0.2
0.4
0.6
0
50
100
150
200
Time (min)
H
+ SB206553
0.0
0.2
0
50
100
150
Time (min)

Figure 3-4: 5-HT_{2B} and 5-HT_{2C} receptors are constitutively active after spinal cord injury. Plots of mean LLR sampled over time with agonists and antagonist applications. (**A–B**) The neutral antagonist RS127445 (3 μM; selective for 5-HT_{2B} receptors), inhibited the increase in the LLR induced by 5-HT_2 receptor agonist alpha-m-5-HT (0.3 μM; A), whereas when applied alone RS127445 had no effect on the LLR (B). (**C-D**) Likewise, the neutral antagonist SB242084 (3 uM; selective for 5-HT_{2B} and 5-HT_{2C} receptors), inhibited that same agonists induced LLR (C), whereas SB242084 had no effect when applied alone (D). (**E-H**) In contrast, the inverse agonists RS102221 (3 μM, selective for 5-HT_{2C} receptors) and SB206553 (5 μM, selective for 5-HT_{2B} and 5-HT_{2C} receptors), inhibited the agonist induced LLR (E,G) *and* inhibited the spontaneously occurring LLR when given alone (F, H). Considering that inverse agonists block constitutive activity as well as agonist induced activity, whereas neutral antagonists only block agonist induced activity, these results indicate that the 5-HT_{2B} and 5-HT_{2C} receptors exhibit constitutive activity and are not activated by endogenous residual 5-HT. See statistics in Table 3-4.

Notably, cyproheptadine was the most effective of all inverse agonists tested, significantly exceeding the inhibition of the LLR produced by the 5-$HT_{2B/2C}$ inverse agonist SB206553 (Table 3-4). This is likely because cyproheptadine potently inhibits both 5-HT_{2B} and 5-HT_{2C} *and* alpha1 adrenergic receptors (Westphal and Sanders-Bush 1994; Yoshio et al. 2001) (Table 3-4; alpha1 receptors, like 5-HT_2 receptors, are known to facilitate the LLR (Li et al. 2004b)). Also, the 5-HT_{2C} inverse agonist ketanserin produced significantly more inhibition of the LLR than the selective 5-HT_{2C} inverse agonist RS102221 (Table 3-4). Again, this is likely due to the known potent binding of ketanserin to alpha1 receptors (Yoshio et al. 2001), in addition to its 5-HT_{2C} receptors action (Cussac et al. 2002) (Table 3-4; ketanserin also binds potently to 5-HT_{2A} receptors, but these are not involved in modulating the LLR; see above). Together these data indicate that there is endogenous activity in alpha1 receptors, likely mediated by constitutive activity (consistent with Harvey et al. 2006b), as with 5-HT_2 receptors; we address this issue in a separate paper (Rank et al., in preparation).

Intracellular recording of Ca PIC

Considering that the LLR is mediated by the Ca PIC, it is likely that the 5-HT_{2B} and 5-HT_{2C} receptors modulate the Ca PIC, as they do to the LLR. To confirm this, we directly measured the Ca PIC in motoneurons using slow voltage ramps to inactivate transient currents, and in the presence of TTX to synaptically isolate the motoneuron (by stopping spike-mediated transmission) and block the Na PIC, which can otherwise obscure the Ca PIC, as previously described (Harvey et al. 2006c). During this slow voltage ramp (under voltage-clamp conditions), the Ca PIC was activated at on average -49.6 ± 6.41 mV (Von, n = 46 motoneurons), and produced a downward deflection in the recorded current of 1.98 ± 1.07 nA, which we took as an estimate of the Ca PIC amplitude (Fig 5A, arrow; previously verified to be mediated by L-type calcium channels; nimodipine-sensitive (Li et al. 2004c)). Application of the 5-$HT_{2B/2C}$ agonists alpha-methyl-5-HT (0.3μM), DOI (1 μM), BW723C86 (1-2 μM) and 5-HT (1 μM) each significantly increased the Ca PIC amplitude (by -0.71±0.82 nA, n = 11; -0.60±0.75, n = 9; -0.42±0.43, n

= 15; and -0.58±0.68 nA, *n* = 11; respectively; Figs 3-4B and 3-5B), as well as lowered the onset threshold of the PIC, Von (by -7.9±8.0; -5.6±3.8; -3.8±2.2 and -6.3±4.7 mV respectively). The 5-$HT_{2B/2C}$ antagonist SB206553 (5μM) significantly inhibited the facilitation of the Ca PIC by alpha-methyl-5-HT (-1.77±0.48 nA; Fig 3-5C&D). While the selective 5-HT_{2B} agonist BW723C86 increased the Ca PIC, its mean effect was half that of the 5-$HT_{2B/2C}$ agonist alpha-methyl-5-HT (see above), and subsequent application of the 5-$HT_{2B/2C}$ agonist alpha-methyl (0.3 μM) after BW723C86 further significantly facilitated Ca PIC (PIC increased by -0.43±0.55 nA; and Von lowered by -3.3±3.7 mV, *n* = 9; Fig 3-6), suggesting involvement of both 5-HT_{2B} and 5-HT_{2C} receptors. The agonists 8-OH-DPAT (3 μM) and zolmitriptan (1 μM) had no significant effects on motoneuron properties (Ca PIC unaffected; *n* = 5, *P* > 0.05), confirming that 5-$HT_{1A/1B/1D/1F}$, 5-HT_5 and 5-HT_7 receptors have no influence on the Ca PIC. Together these results verify that both 5-HT_{2B} and 5-HT_{2C} receptors are involved in facilitating Ca PIC in motoneurons, whereas other 5-HT receptors are not likely involved.

The 5-HT_2 receptor agonists alpha-methyl-5-HT, DOI and BW723C86 did not significantly change the resting potential (changes 2.4±9.2, -3.2±8.0, and -1.2±6.1 mV, respectively), consistent with previous reports that resting membrane potential is increased by 5-HT via a non-5-HT_2 receptor (Harvey et al. 2006a). The input resistance was significantly increased by these 5-HT_2 receptor agonists (by 2.7±2.4, 0.79±0.89, 0.85±1.15 MΩ; corresponding to 69, 21 and 18% increases, respectively).

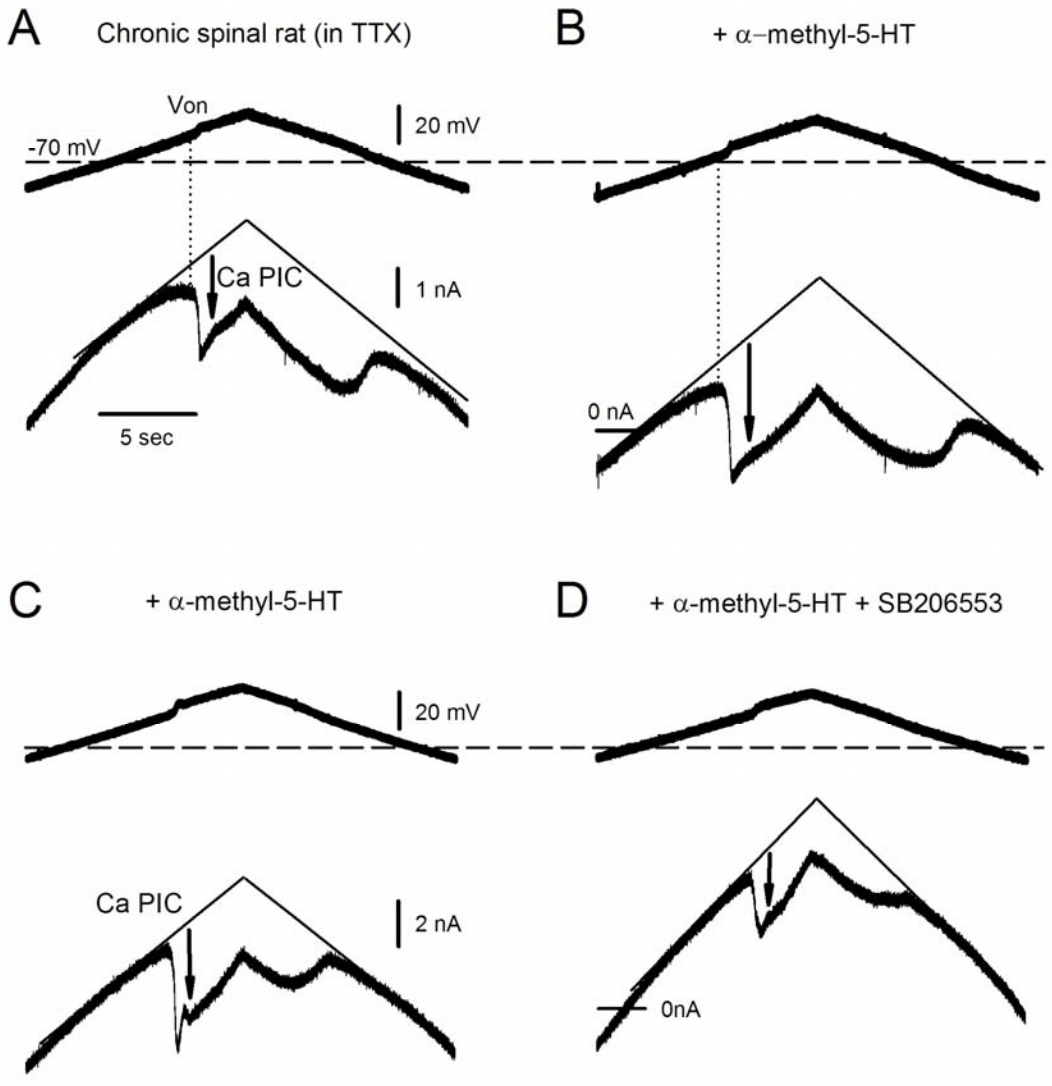
A
Chronic spinal rat (in TTX)
Von
-70 mV
20 mV
Ca PIC
1 nA
5 sec
B
+ α−methyl-5-HT
0 nA
C
+ α-methyl-5-HT
20 mV
Ca PIC
2 nA
D
+ α-methyl-5-HT + SB206553
0nA

Figure 3-5: 5-HT_{2B} and 5-HT_{2C} receptors facilitate motoneuronal Ca PIC, like they do to the LLR. (**A**) Ca PIC in motoneuron of chronic spinal rat, activated by slowly increasing the membrane potential under voltage-clamp, and quantified at its initial peak, where it produced a downward deflection in the recorded current (at arrow) relative to the leak current (thin line). Also, V_{on} represents the voltage at which the Ca PIC was activated. Horizontal dashed line indicates resting potential. Recording in presence of TTX (2 μM) to block the Na PIC and synaptic input, thus isolating the Ca PIC. (**B**) The 5-HT_2 receptor agonist alpha-methyl-5-HT (0.1uM) increased the Ca PIC and reduced V_{on}. (**C-D**) In another motoneuron, the selective 5-$HT_{2B/2C}$ antagonist, SB206553 (5uM), reduced the agonist induced increase in the Ca PIC.

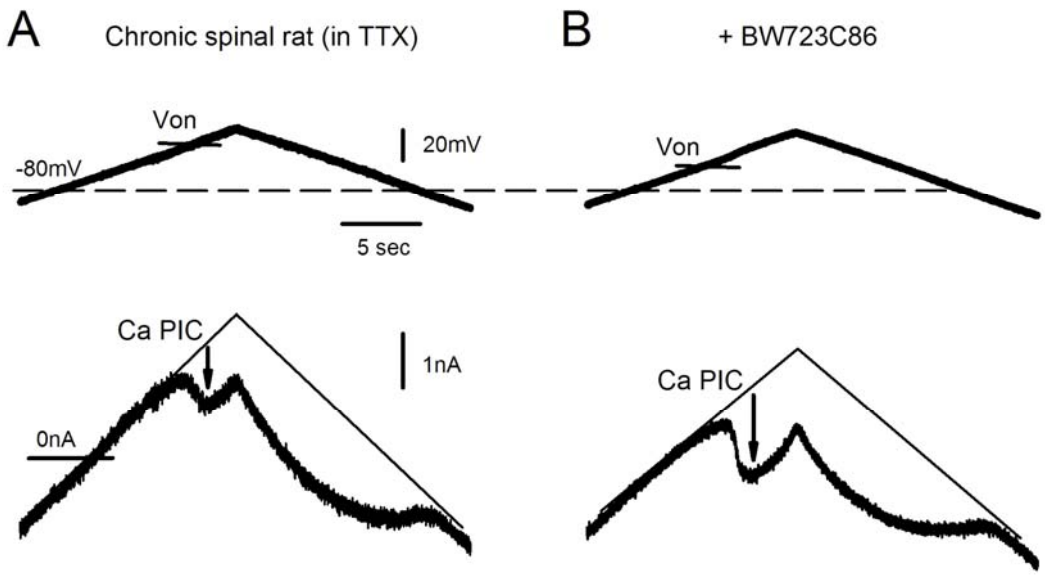

Figure 3-6: 5-HT_{2B} receptor alone facilitates the Ca PIC on motoneurons. (**A**) Ca PIC recorded in motoneuron of chronic spinal rat, quantified at arrow, as detailed in Fig 3-5 (in TTX , 2 μM). (**B**) The moderately selective 5-HT_{2B} receptor agonist, BW723C86 (1 μM) increased the size of the Ca PIC and lowered its onset voltage.

DISCUSSION

Our results demonstrate two novel concepts: first, the 5-HT_{2B} receptor modulates motoneuron excitability (Ca PICs) after SCI, and second, the 5-HT_{2B} receptor plays an essential role in the spontaneous recovery of motoneuron function after SCI, by exhibiting constitutive receptor activity (together with the 5-HT_{2C} receptor). Constitutive 5-HT receptor activity has previously been demonstrated in the central nervous system for 5-HT2A (Harvey et al. 1999) and 5-HT2C (De Deurwaerdere et al. 2004; Murray et al. 2010) receptors, but not previously for 5-HT2B receptors. The 5-HT_{2B} and 5-HT_{2C} receptors we describe are on the motoneurons themselves, because receptor agonists increased the Ca PICs when synaptic transmission was blocked with TTX. In addition immunolabeling shows 5-HT_{2B} (Fig 3-7) and 5-HT_{2C} (Murray et al. 2010) receptors on motoneurons in our preparation.

Considering that the Ca PIC is known to cause long-lasting motoneuron discharges evoked by brief stimuli (LLRs) (Li et al. 2004a; Li et al. 2004b), we used these LLRs as an assay of the Ca PIC, and this allowed us to efficiently estimate the dose-response relations for the Ca PIC/LLR for numerous drugs and perform a thorough analysis of all 5-HT receptors, ruling out the involvement of all but the 5-HT_{2B} and 5-HT_{2C} receptors. We do not know why other receptors, including the 5-HT_{1A}, 5-HT_{2A}, 5-HT_3 and 5-HT_7 do not facilitate the LLR, considering that these receptors are present in the spinal cord (Hochman et al. 2003; Millan 2002; Schmidt and Jordan 2000) and have previously been suggested to have multiple actions on motoneurons, reflexes, or locomotion (Dougherty et al. 2005; Hammar et al. 2007; Holohean et al. 1995; Ziskind-Conhaim et al. 1993) (see Introduction). The relatively selective 5-HT_3 receptor agonist 2-methyl-5-HT (3-fold selectivity in Table 3-1) (Alexander et al. 2004) and 5-HT_{2A} receptor agonist DOI (10-fold selectivity over other 5-HT_2 receptors, Table 3-1) did increase the LLR, but both these drugs worked at doses too low to be comparable with binding to these receptors, and instead acted by their lower affinity binding to the 5-HT_{2B} receptor (Table 3-3; relative potency near –1)

(Wainscott et al. 1993). This underscores the importance of considering the 'non-selective' lower affinity binding of putative agonists, with quantitative comparisons to receptor binding affinity, which requires testing agonists at multiple doses and comparing across multiple agonists. Finally, in the present paper we only examined the 5-HT receptors that facilitate the LLR and associated PIC, and in a separate paper examine the receptors that inhibit sensory afferent transmission in the LLR pathway, which include the 5-HT_{1B} receptor (Murray et al. 2010, in preparation).

We had not expected a prominent role of the 5-HT_{2B} receptor, which is a relatively obscure receptor, first described in the stomach fundus, and not widely expressed in the brain (Barnes and Sharp 1999; Hoyer et al. 2002; Nichols and Nichols 2008; Wainscott et al. 1993). In the brain (and heart) this receptor is mainly associated with developmental plasticity and pathology (knockouts of the 5-HT_{2B} receptor gene is lethal, unlike for other 5-HT receptors) (Barnes and Sharp 1999; Nichols and Nichols 2008). Outside of reports of 5-HT_{2B} receptors modulating frog NMDA receptor function on spinal motoneurons (Hackman and Holohean 2010; Holohean and Hackman 2004), little is known about the role of this receptor in the adult spinal cord, even though it is present in the spinal cord (Hochman et al. 2003; Holohean et al. 1992; Millan 2002). Considering its novelty, we used several approaches to prove the presence and functionality of the 5-HT_{2B} receptor, including showing that: 1) 5-HT_{2B} agonists consistently facilitated motoneuron excitability (LLR and PICs), even when they were made highly selective by a prior blockade of other receptors known to be activated by the agonists (e.g. 5-HT_{2C} and 5-HT_{2A} receptors for DOI), 2) selective 5-HT_{2B} receptor antagonists overcame the agonist action, when given after a low dose of the agonist titrated to just activate 5-HT_2 receptors, 3) agonist action was highly correlated with 5-HT_{2B} binding affinity for the agonists at the 5-HT_{2B} receptor and indeed agonist potency could be quantitatively predicted from binding affinity and 4) 5-HT_{2B} receptors are on motoneurons, using immunolabelling. The same methods were used to show that the 5-HT_{2C} receptor was also present and

functional. In contrast, 5-HT_{2A} receptors were not functional, contrary to our previous erroneous conclusion that they modulated the PICs (Harvey et al. 2006a); this conclusion was primarily based on our finding that DOI enhances motoneuron excitability in normal and chronic spinal rats, which now can be reinterpreted to be consistent with 5-HT_{2B} receptors modulating motoneuron function in normal and chronic spinal rats. Interestingly, both 5-HT_{2B} and 5-HT_{2C} receptors appear to have equally large effects, based on the action of selective agonists and antagonists. The action of both 5-HT_{2B} and 5-HT_{2C} receptors at times complicated matters, because these receptors have fairly similar binding affinities for many of the tested agonists (Table 3-1). For example, this explains why a prior treatment with a selective antagonist to just one of these receptors, does not eliminate the alpha-methyl-5-HT induced facilitation of the LLR, and while it reduces the amplitude of the response (efficacy), it does not affect the EC50 (potency). It takes a block of both receptors to eliminate the action of the agonists on the LLR.

An advantage of studying the action of 5-HT receptor agonists after chronic spinal transection or severe contusion is that 5-HT terminals and associated serotonin re-uptake transporters (SERT) are mostly eliminated in the spinal cord by the injury (Hayashi et al. 2010; Murray et al. 2010; Newton and Hamill 1988), and so cannot interfere with the action of exogenously applied 5-HT or other agonists. SERT is almost exclusively located on 5-HT neurons in the brain (explaining its loss with injury), and regulates the amount of 5-HT in the synaptic cleft (Blakely et al. 1994). Accordingly, SERT dramatically affects the dose of exogenously applied 5-HT needed to activate receptors in the synapse, and serotonin reuptake inhibitors that block SERT (citalopram) decrease the dose of 5-HT or other 5-HT agonists needed to facilitate motoneuron function by a factor of 30 (Elliott and Wallis 1992) (Bennett and Murray, unpublished data). Thus, the lack of synaptic SERT in the chronic spinal rat allows 5-HT to reach receptors unimpeded, and our estimates of drug potency (EC50) therefore are much more accurate. Indeed, our previous work has shown that the *in vitro* dose of 5-HT needed to affect

motoneurons in spinal cords from normal rats is dramatically higher (by a factor of 30; 10 μM vs 0.3 μM) than that in chronic spinal rats (Harvey et al. 2006a). We suggest that this supersensitivity in chronic spinal rats is largely due to the absence of SERT in chronic spinal rats, whereas in normal rats SERT efficiently prevents much of the exogenously applied 5-HT from reaching the 5-HT receptors. SERT is rather promiscuous, transporting any tryptamine-like compound, including probably all 5-HT receptor agonists that we used (e.g. alpha-methyl-5-HT) (Henry et al. 2006). Thus, our quantitative analysis of 5-HT receptor agonist potency and correlation to receptor binding affinity would not have been possible had we not being studying the fully transected rat that lacks SERT, especially because of the variable transport of different agonists by SERT (Henry et al. 2006).

We found a remarkably consistent relation between agonist potency (–logEC50) and receptor binding affinity (–log Ki). Basically, effective agonist doses (EC50) were consistently only about 10 times the binding Ki values of that agonist to 5-HT_{2B} receptors. Receptor binding affinities are typically measured in isolated cell culture systems where agonists are applied directly to the receptors (Boess and Martin 1994), whereas in our whole spinal cord preparations agonists must diffuse substantial distances to reach receptors (see Results). Likely this diffusion difference helps explain the factor of 10 that relates EC50 to Ki values. Assuming that this diffusion affects all 5-HT receptor action equally, this simple relation (factor of 10) between EC50 and Ki ultimately helps us predict doses of agonist and antagonists needed to effectively activate any particular 5-HT receptor. Indeed, we used this to choose doses of antagonists used (Table 4), and then independently verified these doses by their action alone or against an agonist. We also used this simple relation to help rule out the involvement of receptors other than 5-HT_{2B} and 5-HT_{2C}; these other receptors had EC50/Ki ratios far from 10. With this method and direct use of agonists to other receptors (8-OH-DPAT, etc) it is clear that no other 5-HT receptor is involved in modulating the Ca PIC and associated LLR. Different agonists have variable functional efficacy (partial or

full agonists) (Kenakin 1996), but we avoided this issue by focusing on binding affinity and potency, rather than efficacy. However, a caveat that must be noted is that we do not know for certain that all drugs that bind to a receptor act as agonist at that receptor, and indeed, compounds like 8-OH-DPAT have poor efficacy (partial agonists) at some receptors to which they bind (e.g. 5-HT_7 for 8-OH-DPAT) (Eglen et al. 1997); thus we also tested more effective agonist to these receptors (LP44).

Both 5-HT_{2B} and 5-HT_{2C} receptors are Gq-protein-coupled receptors that generally activate the classic PLC pathways involved in the synthesis of inositol phosphates (IP) and mobilization of intracellular Ca^{2+} stores (Hoyer et al. 2002; Kenakin 1996; Lucaites et al. 1996; Mizuno and Itoh 2009). These pathways facilitate PICs (and NMDA receptors) in spinal motoneurons (Holohean and Hackman 2004; Mejia-Gervacio et al. 2004; Perrier et al. 2000). Thus, it is likely that the 5-HT_{2B} and 5-HT_{2C} receptors modulate the PICs via IP pathways in our preparation. Both these receptors types are known to exhibit a substantial degree of constitutive activity (Seifert and Wenzel-Seifert 2002; Villazon et al. 2003; Weiner et al. 2001), usually observed in the form of IP production in the absence of 5-HT (or other agonists), which produces a basal level of IP (Chanrion et al. 2008; Kennett et al. 1997b; Knight et al. 2004). Thus, PICs may in part be facilitated by this basal IP production (without 5-HT), and this may explain the emergence of the large PICs seen after SCI when there is an absence of 5-HT, as we discuss below. Importantly, the subclass of antagonists known as inverse agonists inhibit this basal IP production (or associated functions) in 5-HT_{2B} and 5-HT_{2C} receptors, including classic antagonists like cyproheptadine, clozapine and ketanserin (Chanrion et al. 2008; Villazon et al. 2003; Weiner et al. 2001; Westphal and Sanders-Bush 1994). In contrast, neutral antagonists like methysergide and RS127445 have no effect when given alone (Bonhaus et al. 1999; Chanrion et al. 2008; Westphal and Sanders-Bush 1994). Therefore a combination of these drugs is useful in identifying constitutive activity. Both inverse agonists and neutral antagonists block the action of agonists, and thus the

action of an inverse agonist alone is not sufficient to prove the presence of constitutive receptor activity, if there is any chance that the receptor is activated by 5-HT (e.g residual 5-HT in the spinal cord). Definitive proof of constitutive activity only comes by also showing that neutral antagonists have no effect (as in Table 3-4) (Seifert and Wenzel-Seifert 2002).

Brainstem derived 5-HT normally serves a critical role in tuning the excitability of motoneurons, facilitating Ca PICs that amplify and prolong responses to synaptic input (Heckman et al. 2005). Thus, when SCI eliminates this major source of 5-HT, motoneurons are initially left in a depressed state, not able to produce adequate muscle contractions. However, over the weeks after injury Ca PICs in motoneurons spontaneously recover, to the point where they are permanently large, and not only help with residual motor functions, but also contribute to unwanted contractions (muscle spasms) (Bennett et al. 2004; Li et al. 2004a). Recently, we have shown that a novel form of plasticity in 5-HT_2 receptors causes this recovery of Ca PICs: these receptors become spontaneously active in the absence of 5-HT (constitutively active) (Murray et al. 2010). However, at the time, we were uncertain which particular 5-HT_2 receptor modulates the Ca PIC. The present results resolve this issue, demonstrating that both 5-HT_{2B} and 5-HT_{2C} receptors are constitutively active after SCI, because selective 5-HT_{2B} and 5-HT_{2C} inverse agonists that block this constitutive activity decreases motoneuron excitability (LLRs and associated Ca PICs), whereas neutral antagonists do not. As discussed above, the lack of effect of neutral antagonists shows that there is no residual 5-HT below the chronic injury that activates the 5-HT_2 receptors. Thus, even though inverse agonists we tested can block the action of 5-HT, in addition to blocking constitutive receptor activity, their action can only be attributed to constitutive 5-HT_{2B} and 5-HT_{2C} receptor activity. Considering that most known antagonists can act as inverse agonists at 5-HT_{2B} and 5-HT_{2C} receptors in various model systems, whereas neutral antagonists are rare (Weiner et al. 2001; Westphal and Sanders-Bush 1994), we were not surprised that the selective 5-HT_{2B} and 5-HT_{2C} receptor antagonists (SB204741

and RS102221, respectively) could act as inverse agonists in inhibiting the LLR. However, we had not anticipated such prominent constitutive activity in 5-HT_{2B} receptors, as demonstrated by these compounds, because constitutive activity in this receptor has not previously been demonstrated in the central nervous system, though it has been seen in the stomach fundus (Villazon et al. 2003).

The constitutive 5-HT_2 receptor activity that we observe after SCI arises from at least two mechanisms. First, there is an increase in expression of 5-HT_{2C} receptor isoforms that exhibit a high degree of constitutive activity (Murray et al. 2010). Most 5-HT_{2C} receptor isoforms have some probability of being constitutively active, but certain isoforms, like the INI isoform, have a very high degree of constitutive activity (with activity approaching that produced by maximal activation with 5-HT) (Berg et al. 2008; Herrick-Davis et al. 1999; Marion et al. 2004; Niswender et al. 1999; Weiner et al. 2001), and these isoforms increase in expression with SCI (Murray et al. 2010). Second, the total number of 5-HT_{2C} receptors increases with SCI (Hayashi et al.), which increases overall constitutive activity, because all isoforms exhibit some constitutive activity (Berg et al. 2008; Herrick-Davis et al. 1999). We do not know whether receptor isoform types or total receptor numbers change for the 5-HT_{2B} receptor, though both seem likely.

In summary, we have performed a systematic analysis of the 5-HT receptors that facilitate motoneuron PICs and associated long-lasting reflexes (LLRs/spasms) after SCI, and found that both 5-HT_{2B} and 5-HT_{2C} receptors are involved, whereas 5-HT_1, 5-HT_{2A}, and 5-$HT_{3/4/5/6/7}$ receptors are not. While both 5-HT_{2B} and 5-HT_{2C} receptors are present prior to injury, they exhibit dramatic plasticity following chronic spinal transection, presumably as a compensation for the near complete loss of 5-HT innervation. That is, both receptors become constitutively active, producing large Ca PICs in the absence of 5-HT (whereas acutely after injury PICs are small). These large Ca PICs are essential for the recovery of normal motoneuron firing ability after chronic injury (sustained firing and amplification of synaptic input) (Harvey et al. 2006c; Heckman et al. 2005), and thus must play

an important role in all motor recovery, including locomotion (Murray et al. 2010). However, unlike with normal brainstem control over receptor activation, these receptors are permanently active, leading to permanently large PICs, and ultimately hyperexcitability of motoneurons associated with muscle spasms. Thus, controlling excess constitutive activity in 5-HT_2 receptors, especially 5-HT_{2B} receptors, offers a new approach in managing spasticity after SCI.

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Chapter 4: Polysynaptic excitatory postsynaptic potentials that trigger spasms after spinal cord injury are inhibited by 5-HT_{1B} and 5-HT_{1F} receptors

INTRODUCTION

Descending brainstem systems innervating the spinal cord, especially those releasing serotonin (5-HT) and noradrenaline (NA), potently *inhibit* sensory transmission to spinal motoneurons and ascending tracts, ultimately attenuating both segmental reflexes and sensory perception (reviewed by Lundberg 1982; Millan 2002; Schmidt and Jordan 2000; Yoshimura and Furue 2006). Both 5-HT and NA directly inhibit sensory transmission by activating inhibitory Gi-protein coupled receptors, such as 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and alpha2 adrenergic receptors, on sensory afferent terminals (including low-threshold group I and II muscle and skin afferents and high-threshold pain afferents) and/or excitatory spinal neurons involved in polysynaptic reflexes and ascending sensory transmission (Clarke et al. 2002; Clarke et al. 1996; Di Pasquale et al. 1997; Jankowska et al. 1994; Jankowska et al. 1993; Jordan et al. 2008; Li et al. 2004b; Lundberg 1982; Manuel et al. 1995; Millan 2002; Rekling et al. 2000; Schmidt and Jordan 2000; Singer et al. 1996; Yoshimura and Furue 2006). 5-HT and NA also indirectly inhibit sensory transmission by activating excitatory Gq-coupled 5-HT_2 and alpha1 adrenergic receptors located on inhibitory interneurons (though not on afferents), thus facilitating inhibitory interneurons, such as those involved in group Ia reciprocal and Ib non-reciprocal inhibition (Hammar and Jankowska 2003; Jankowska et al. 2000) and pain transmission (Obata et al. 2004; Yoshimura and Furue 2006).

Within this conceptual framework of brainstem-mediated inhibition, it is likely that the hyperreflexia and general spasticity often seen after spinal cord injury (Ashby and McCrea 1987; Dietz and Sinkjaer 2007; Kuhn and Macht 1948; Maynard et al. 1990; Nielsen et al. 2007; Noth 1991) results partly from a loss of brainstem 5-HT mediated inhibition (disinhibition), especially if the injury includes the dorsal lateral funiculus (DLF) where most of the 5-HT innervation of the dorsal horn arises (Heckman 1994; Lundberg 1982; Schmidt and Jordan 2000; Taylor et al. 1999). We have been investigating this idea with a rat model of spinal cord injury, where pronounced muscle spasticity occurs (Bennett et al.

1999; Bennett et al. 2004), with similar characteristics to that seen in human muscles after injury (Kuhn and Macht 1948; Maynard et al. 1990). In this model, spinal cord injury leads to the emergence of unusually long excitatory postsynaptic potentials (long EPSPs) on motoneurons, lasting up to one second, that are triggered by low threshold cutaneous and muscle afferents (groups I and II, Li et al. 2004a) (see also Baker and Chandler 1987), very similar to the exaggerated synaptic transmission seen in spastic humans with spinal cord injury (using motor unit recordings, Norton et al. 2008). These long EPSPs initiate long-lasting muscle spasms, lasting several seconds, whereas prior to injury the same stimulation mainly evokes inhibition of ongoing muscle activity (Bennett et al. 1999; Bennett et al. 2004; Norton et al. 2008). We know that exogenously applied 5-HT (or NA) can inhibit muscle spasms in these rats (Li et al. 2004b), essentially replacing lost brainstem 5-HT, but do not know where this inhibition occurs (pre or postsynaptic) or what receptors are involved, though it is reasonable to suggest that 5-HT_1 (or even 5-HT_2) receptors could mediate this inhibition (see above). The current paper addresses these questions by applying selective 5-HT receptor agonists while recording spasms and associated EPSPs, as a step toward developing novel antispastic drugs to replace lost 5-HT innervation.

Brainstem derived 5-HT and NA normally *facilitate* motoneuron function (Heckman et al. 2005; Hultborn et al. 2004; Li et al. 2004a; Perrier and Delgado-Lezama 2005; Schmidt and Jordan 2000), in contrast to the inhibition of sensory transmission discussed above. This is mediated by 5-HT_2 and alpha1 adrenergic receptors that lower the sodium spike threshold and facilitate voltage-dependent persistent inward currents (PICs), including both persistent calcium (Ca PIC) and sodium (Na PIC) currents. Together these currents are essential for normal motoneuron function, including sustained firing in response to synaptic inputs (Gilmore and Fedirchuk 2004; Harvey et al. 2006a; Heckman et al. 2005; Perrier and Hounsgaard 2003). Thus, with spinal cord injury motoneurons are often rendered acutely unexcitable, due to a lack of brainstem-derived 5-HT and NA innervation need for normal motoneuron function (Heckman et al. 2005; Li et al.

2004a), especially if the injury includes the ventral and ventrolateral funiculi that contain most of the 5-HT that innervates the ventral horn (Schmidt and Jordan 2000). The functional consequence of this is that the spinal cord becomes areflexic immediately after injury, despite the exaggerated sensory afferent transmission. However, over weeks after injury (chronic spinal state) motoneurons spontaneously regain their excitability, with the re-emergence of large Ca and Na PICs. At this time the exaggerated sensory transmission, especially the long EPSPs, trigger the PICs, which ultimately produce the many second long muscle spasms in humans (Gorassini et al. 2002; Norton et al. 2008) and rats (Bennett et al. 2004; Li et al. 2004a).

Recently, the reasons for the spontaneous recovery of motoneuron function with chronic injury have begun to be understood (Bennett et al. 2004; Button et al. 2008; Gorassini et al. 2004; Harvey et al. 2006b; Hultborn et al. 2004; Murray et al. 2010a). Briefly, 5-HT_2 and alpha1 receptors on motoneurons become spontaneously active in the weeks after spinal transection (Harvey et al. 2006b; Murray et al. 2010a), due to *constitutive* receptor activity (activity in the absence of 5-HT or any other ligand) (Murray et al. 2010a). This spontaneous receptor activity leads to the re-emergence of the large PICs that make the motoneurons permanently highly excitable (Harvey et al. 2006b; Murray et al. 2010a). One goal of the present paper was to examine whether or not similar plasticity (constitutive activity) also occurs in the 5-HT_1 receptors that normally inhibit sensory transmission. This seems plausible, because 5-HT_1 receptors can exhibit constitutive activity in single-cell cloned receptor systems (Selkirk et al. 1998), but functionally may not be important, because general inhibition is not restored in chronic injury, and in particular, the exaggerated long EPSPs that trigger spasms remain even in chronic injury (Baker and Chandler 1987; Li et al. 2004a).

Finally, while uninjured animals and humans at times have substantial PICs facilitated by brainstem derived 5-HT and NA (see above and Gorassini et al. 2002; Udina et al. 2010), these PICs do not cause uncontrolled motoneuron firing,

because postsynaptic inhibition arising from glycinergic and GABAergic neurons in the spinal cord and brain can directly hyperpolarize motoneurons (Holstege and Bongers 1991; Jankowska 1992; Nielsen et al. 2007; Rekling et al. 2000) to appropriately terminate the voltage-dependent PICs (Bennett et al. 1998; Heckman et al. 2005). In contrast, after spinal cord injury, there is a reduction in such inhibitory postsynaptic control (Boulenguez et al. 2010; Nielsen et al. 2007), in part due to loss of 5-HT and NA (Jankowska et al. 2000), making motoneuron PICs and firing difficult to voluntarily terminate.

In summary, three factors contribute to spasms after spinal cord injury: 1) unusually long EPSPs and general disinhibition of afferent transmission, 2) large uncontrollable PICs in motoneurons, mediated by 5-HT_2 receptor activity, and 3) a loss of descending postsynaptic inhibition over motoneuron activity. In previous papers, we examined how spasms can be controlled by reducing PICs (Murray et al. 2010a; Murray et al. 2010b). The present paper examines how we can control spasms by reducing EPSPs by replacing lost 5-HT innervation with 5-HT_1 receptor agonists. We unexpectedly found that reducing EPSPs with 5-HT agonists reveals long lasting inhibitory postsynaptic inputs from cutaneous afferents, suggesting that useful inhibition over motoneurons is by no means lost, but masked by excitation after injury. Thus, we also examined these inhibitory inputs.

METHODS

Recordings were made from motoneurons and associated ventral roots of the sacrocaudal spinal cord of spastic adult rats with chronic spinal cord injury (3-8 months old). Adult female rats were transected at the S_2 sacral level at about 2 months of age (adult rat), and recordings were made after their affected muscles became spastic (1.5–3 months after injury), as detailed previously (Bennett et al. 1999; Bennett et al. 2004). All recordings were made from the whole sacrocaudal spinal cord that was removed from the rat with an S_2 sacral transection and maintained *in vitro*. This transection was made just rostral to the chronic spinal injury, so as not to further damage the sacrocaudal cord. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee: Health Sciences.

In vitro preparation

Details of the *in vitro* experimental procedures have been described in previous publications (Harvey et al. 2006c; Li et al. 2004a; Li et al. 2004b; Murray et al. 2010a). Briefly, all the rats were anaesthetized with urethane (0.18 g/100 g; with a maximum dose of 0.45 g) and the sacrocaudal spinal cord was removed and transferred to a dissection chamber containing modified artificial cerebrospinal fluid (mACSF). Spinal roots were removed, except the sacral S_4 and caudal Ca_1 ventral roots and the Ca_1 dorsal roots. After 1.5 hours in the dissection chamber (at 20° C), the cord was transferred to a recording chamber containing normal ACSF (nACSF) maintained near 24°C and with a flow rate > 5 ml/min. A one-hour period in nACSF was given to wash out the residual anaesthetic and mACSF prior to recording, at which time the nACSF was recycled in a closed system with a peristaltic pump (Harvey et al. 2006b).

Ventral root reflex recording and averaging.

Dorsal and ventral roots were mounted on silver-silver-chloride wires above the nASCF of the recording chamber and covered with a 1:1 mixture of petroleum jelly and mineral oil (as for intracellular recording) for monopolar stimulation and

recording (Li et al. 2004b). We evoked ventral root reflexes with a low threshold Ca1 dorsal root stimulation (single pulse, 0.1 ms, 0.02 mA, corresponding to 3 x afferent threshold, 3xT; afferent and reflex threshold are similar (Bennett et al. 2004)) using a constant current stimulator (Isoflex, Israel). The stimulation intensity (3xT) is compatible with activation of low-threshold group I and II (Aβ) afferents. Because the Ca_1 dorsal root innervates the distal third of the tail which lacks large muscles(Bennett et al. 2004), this stimulation activates largely cutaneous or joint afferents, though there are small intrinsic muscles in the tail with group Ia and II muscle afferents (Steg 1964), and thus to a lesser extend muscle afferents may be activated. The stimulation was repeated 5 times at 10 second intervals for each trial. The ventral root recordings were amplified (x2000), high-pass filtered at 100 Hz, low-pass filtered at 3 kHz, and recorded with a data-acquisition system sampling at 6.7 kHz (Axonscope 8; Axon Instruments). Ventral root reflexes were quantified using custom written software (Matlab, MathWorks, Natick, MA). That is, data was high pass filtered at 800Hz and rectified to allow averaging (Fig 4-1A, lower plot). We quantified three components of the ventral root reflexes: the short lasting, short latency polysynaptic reflex (SPR; averaged 10–40 ms post stimulus), the intermediate latency, longer lasting reflex corresponding to the long EPSP seen in this preparation (termed long-polysynaptic reflex or LPR; averaged 40–500 ms post stimulus), and the long latency, long-lasting tonic response associated with the Ca PIC (termed long-lasting reflex or LLR; 500–4000 post stimulus). Average ventral root activity computed for each trial in a given reflex interval was then averaged for all 5 stimuli in a trial. Average background ventral root activity prior to stimulation was measured over the 800 ms prior to the first stimulus, and subtracted from the reflex averages to give the final reflex responses (SPR and LPR). This recording procedure was repeated at 12 min intervals, and 5-HT receptor agonists were added immediately after each recording, giving them time to fully act by the next 12 min recording session. Cumulative dose-response relations were computed by increasing agonist doses at these 12 min intervals (0.003, 0.01, 0.03. 0.1,..., 30 uM doses used). Antagonists took longer to act and

responses reached near steady state typically > 20 mins after application, at which time responses were averaged. The effect of agonists on the reflexes were reversible upon washout of the agonist, but full recovery to baseline only occurred after several hours, likely due to the large size of the whole cord preparation. Thus, washout of agonists was not feasible between doses of the agonists used in the construction of dose-response relations.

Intracellular recording

Sharp intracellular electrodes were made from glass capillary tubes (1.5mm O.D.; Warner GC 150F-10) using a Sutter P-87 micropipette puller and filled either 2 M potassium citrate or a combination of 1M potassium acetate and 1M KCl. Electrodes were bevelled down from an initial resistance of 40-80 MΩ to 26-32 MΩ using a rotary beveller (Sutter BV-10). A stepper-motor micromanipulator (660, Kopf) was used to advance the electrodes through the ventral cord surface into motoneurons. After penetration, motoneuron identification was made with antidromic ventral root stimulation. 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.

Slow triangular voltage ramps (3.5 mV/s voltage-clamp) were applied to the motoneurons to measure their electrical properties (Harvey 2006). The input resistance (Rm) was measured during the voltage ramps over a 5 mV range near rest and subthreshold to PIC onset. Resting potential was recorded with 0 nA bias current, after the cell had been given about 15 mins to stabilize after penetration. During the upward portion of the slow triangular voltage ramp, the current response initially increased linearly with voltage in response to the passive leak conductance. A linear relation was fit in the region just below the PIC onset (5 mV below) and extrapolated to the whole range of the ramp (leak current). At depolarized potentials

above the PIC onset threshold, there was a downward deviation from the extrapolated leak current, and the PIC was estimated as the difference between the leak current and the total current (leak-subtracted current). The PIC was quantified as the initial peak amplitude of this downward deviation below the leak line (leak-subtracted current). The PIC onset was estimated as the voltage at which the conductance first went to zero during the upward ramp (Von).

The excitatory postsynaptic potential (EPSP) and associated reflexes were directly measured in motoneurons by stimulating the Ca_1 dorsal roots (at 2–3xT, as in ventral root reflex recording), while applying hyperpolarizing bias currents to block the PICs, in current clamp mode. We also measured the corresponding excitatory postsynaptic currents (EPSC) in response to stimulating the dorsal roots while voltage clamping at various potentials to prevent activation of the PIC or motoneuron firing. This allowed synaptic inputs to be assessed at potentials above rest, where EPSPs are normally obscured by firing.

Drugs and solutions

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. The nACSF 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. Drugs were added to the nACSF as indicated in the text, including: 5-HT and DOI (-) (from Sigma-Aldrich, USA), 2-methyl-5-HT, 5-CT, 8-OH-DPAT, alpha-methyl-5-HT, BW723C86, cisapride, EMD386088, granisetron, isradipine, LP44, LY344864, methylergonovine, methysergide, MK212, RS102221, RS127445, SB216641, SB224289 (Tocris, USA), TTX (TTX-citrate; Alomone, Israel), and zolmitriptan (kindly donated by Astra Zeneca, Canada). All drugs were first dissolved as a 10–50mM stock in water before final dilution in ACSF, with the exception of BW723C86, cisapride, EMD386088, isradipine, LP44, methylergonovine, RS102221, RS127445 and SB224289 which were dissolved in minimal amounts of DMSO (final

concentration in ACSF < 0.04%; by itself DMSO had no effect on the LLR in vehicle controls).

Spasms in awake chronic spinal rat.

Tail muscle spasms were evoked with brief electrical stimulation of the skin of the tail, and recorded with tail muscle EMG (electromyogram). Percutaneous EMG wires (50 μm stainless steel, Cooner wires, USA) were inserted in segmental tail muscles at the midpoint of tail and recordings were made while the rat was in a Plexiglas tube, as detailed previously (Bennett et al. 2004). Muscle spasms were evoked with low threshold electrical stimulation of the skin at the distal tip of the tail (cutaneous stimulation; 0.2 ms, 10 mA pulse; 3x afferent threshold [T]; 6 spasms evoked at 10 s intervals for a trial; trials repeated at 12 min intervals) and the tail was restrained from moving. EMG was sampled at 5 kHz, rectified and averaged over a 10–40 ms interval post-stimulus to quantify the short latency polysynaptic reflex (SPR), 40 - 500 ms to quantify the long polysynaptic reflex (LPR) and 500–4000 ms interval to quantify spasms (long lasting reflex, LLR; using Axoscope, Axon Instr., and Matlab, Mathworks). EMG over 300 ms prior to stimulation was also averaged (background), and subtracted from the reflex responses.

Zolmitriptan was applied *in vivo* with intrathecal injections (IT, Mestre et al. 1994). This was done with a direct lumbar puncture under brief isoflourane anaesthesia (10–30 μL injections). Rats woke up rapidly (within minutes) after removal of anaesthesia, and tail spasms were again recorded as detailed above. Control experiments (n = 5) with 30 μl sterile saline injections showed no effect on the spasms, indicating that anaesthesia and injection volume had negligible effects on the spasms. Also, these control rats were injected with 10 μL methylene blue solution and sacrificed immediately, in order to verify that the drug spread to the whole sacral area, but not up to the brainstem (Mestre et al. 1994).

Data analysis

Data were analyzed in Clampfit 8.0 (Axon Instruments, USA) and Sigmaplot (Jandel Scientific, USA). Data are shown as mean ± standard deviation (SD). A Student's *t*-test was used to test for statistical differences before and after drug applications, with a significance level of $P < 0.05$. A Kolmogorov-Smirnov test for normality was applied to each data set, with a $P = 0.05$ level set for significance. Most data sets were found to be normally distributed, as is required for a *t*-test. For those that were not normal a Wilcoxon Signed Rank Test was instead used with $P < 0.05$.

Standard sigmoidal curves were fit to the relation between agonist dose and reflex responses, with doses expressed in log units, and with a Hill slope of unity. The dose that produced 50% effect (EC50) was measured from the curve and –log(EC50) was used to quantify the drug *potency*: pEC50 = –log(EC50). Also, the maximum drug-induced response (*efficacy*) was computed from the curve (peak of curve). For comparison to our computed potencies (pEC50), the binding affinity of each drug at the rat 5-HT receptors was also reported, with values taken from the literature (Table 4-1). The binding of an agonist to a receptor is expressed in terms of its Ki value (nM), which corresponds to the dose that produces 50% binding to that receptor (Knight et al. 2004). This is typically measured by the agonist's ability to displace a standard radiolabelled ligand, like [^{3}H]-5-HT, from the receptor expressed in isolated cells. Binding affinity is computed as pKi = –log(Ki) (Knight et al. 2004). When possible, binding affinities of different drugs for a given receptor were taken from large studies or summary reviews (Boess and Martin 1994), usually using isolated cloned receptors. Also, high affinity agonist-preferring binding sites were always used, measured with radioactive agonists (usually [^{3}H]-5-HT), rather than radioactive-antagonists that bind to a low affinity site (Egan et al. 2000; Knight et al. 2004). If rat receptor Ki values were not available, human values were used instead, as these are similar for most receptors (Boess and Martin 1994).

Table 4-1

Receptor	Agonist	Ki (nM)	pKi (-logKi)	Receptor	Agonist	Ki (nM)	pKi (-log Ki)
5-HT1A	5-CT	0.35	9.46 Boe	5-HT2B	2-methyl-5-HT	316.23	6.50 Boe
	5-HT	1.65	8.78 Hoy		5-CT	151.36	6.82 Boe
	8-OH-DPAT	0.97	9.01 Boe		5-HT	10.23	7.99 Boe
	LP44	52.7	7.28 Leo		α–methyl-5-HT	10.47	7.98 Boe
					BW723C86	12.59	7.90 Bax
5-HT1B	5-CT	3.31	8.48 Boe		DOI	27.54	7.56 Boe
	5-HT	24.49	7.61 Hoy		methylergon	0.50	9.30 Kni
	BW723C86	125.89	6.60 Bax				
	zolmitriptan	5.01	8.30 Mar	5-HT2C	5-HT	10.99	7.96 Ega
	EMD	179.88	6.75 Mat		α-methyl-5-HT	2.69	8.57 Kni
					BW723C86	125.89	6.90 Bax
5-HT1D	5-CT	0.37	9.43 Boe		DOI	9.30	8.03 Ega
	5-HT	2.5	8.60 Boe		MK212	97.72	7.01 Kni
	BW723C86	125.89	6.30 Bax		methylergon	4.57	8.34 Kni
	zolmitriptan	0.63	9.20 Mar				
	EMD	109.90	6.96 Mat	5-HT3	2-methyl-5-HT	85.11	7.07 Mil
					BW723C86	316.23	6.50 Ken
5-HT1E	5-HT	6.16	8.21 Boe		EMD	34.00	7.47 Mat
	α-methyl-5-HT	120.22	6.92 Boe		MK212	29.00	7.54 Gle
	methylergon	89.12	7.05 Boe				
				5-HT4	5-HT	6.31	8.20 Adh
5-HT1F	5-HT	67.60	7.17 Boe		α-methyl-5-HT	263.03	6.58 Adh
	α-methyl-5-HT	181.97	6.74 Boe		cisapride	25.00	7.60 Adh
	LY344864	6.02	8.22 Phe				
	methylergon	30.90	7.51 Boe	5-HT5A	5-CT	0.32	9.50 Boe
	zolmitriptan	63.09	7.20 Mar		5-HT	7.94	8.10 Boe
					8-OH-DPAT	50.12	7.30 Boe
5-HT2A	5-HT	5.75	8.24 Boe				
	α-methyl-5-HT	127.05	6.90 Eng	5-HT6	2-methyl-5-HT	52.48	7.28 B97
	BW723C86	251.18	6.60 Bax		5-CT	252.93	6.60 Mon
	DOI	0.79	9.10 Boe		5-HT	56.23	7.25 Boe
	methylergon	0.35	9.45 Kni		BW723C86	398.11	6.40 Ken
					EMD	7.41	8.13 Mat
				5-HT7	5-CT	0.16	9.80 Boe
					5-HT	1.51	8.82 Boe
					8-OH-DPAT	34.67	7.46 Boe
					LP44	0.22	9.66 Leo

Table 4-1. 5-HT receptor agonists and their receptor binding affinity. Agonists Ki and binding affinity (pKi = –log Ki) obtained from high affinity agonist radioligand bindings studies (Adham et al. 1996; Baxter 1996; Boess and Martin 1994; Boess et al. 1997; Egan et al. 2000; Engel et al. 1986; Glennon et al. 1989; Hoyer et al. 1985; Kennett et al. 1997; Knight et al. 2004; Leopoldo et al. 2007; Martin et al. 1997; Mattsson et al. 2005; Milburn and Peroutka 1989; Monsma et al. 1993; Phebus et al. 1997), with references abbreviated by the first few letters of authors name, except for Boess et al. 1997 which is abbreviated B97. Methylergonovine abbrevated methylergon. Each agonists is considered to activate a receptor if Ki < 400 nM, and listed with that receptor.

RESULTS

Polysynaptic reflexes reflect underlying EPSPs.

To study the EPSPs that underlie spasticity in chronic spinal rats, we first examined the polysynaptic reflexes mediated by these EPSPs, to allow systematic studies of 5-HT receptor pharmacology, not otherwise feasible with direct intracellular recordings of EPSPs (due to limited stability of recordings). When the dorsal roots of chronic spinal rats were stimulated to activate low threshold sensory afferents, there was a multi-phasic reflex response evoked in the motoneurons, as seen both from extracellular ventral root recordings and from single motoneuron intracellular recordings (Fig 4-1, *in vitro*). This reflex response started with a large, but transient short latency reflex that always had a polysynaptic component (short polysynaptic reflex, SPR, 8–15 ms central latency and lasting 10–30 ms; Fig 4-1A, inset), and sometimes also had an earlier monosynaptic reflex component (not present in Fig 4-1A, though see Li et al. 2004b). This transient SPR arose from a large but transient polysynaptic EPSP (short EPSP) that generally produced only one action potential spike in intracellularly recorded motoneurons at rest (Fig 4-1B). The short EPSP was seen without interference from spiking (or the Ca PIC) when the motoneuron was hyperpolarized with a bias current (Fig 4-1B, lower plot). This short EPSP by itself did not trigger Ca PICs or spasms (see later section), consistent with the previous findings that Ca PICs are slowly activated, requiring > 50 ms to substantially activate (Li and Bennett 2007). Nevertheless, we found this SPR useful for studying EPSP modulation in isolation, because it was not affected by Ca PICs; that is, the SPR was not inhibited by a block of Ca PICs with isradipine (Fig 4-1A, lower plot; mean change –9.7 ± 41.0%, $n = 9$, $P > 0.05$).

Following this transient reflex there was a very long lasting reflex (lasting seconds) that underlies muscle spasms (Bennett et al. 2004); we broke this down into two components based on their origin. The first half-second of this long reflex was of polysynaptic reflex origin, and we thus refer to it as the long polysynaptic reflex (LPR; Fig 4-1A). That is, this LPR was initiated by an

unusually long duration polysynaptic EPSP (long EPSP) and further amplified and prolonged by PICs intrinsic to the motoneuron, as previously described (Fig 4-1B) (Li et al. 2004a). The long EPSP underlying this LPR was seen in isolation in motoneurons when the PICs were prevented from activation by hyperpolarizing a motoneuron (PICs are voltage-dependent) (Li et al. 2004a). Also, the effects of the long EPSP on the ventral root reflexes (LPR) were seen in isolation when the Ca PICs were blocked with isradipine (Fig 4-1A) (Li et al. 2004a). On average the LPR was reduced by 52.1±39.5% with isradipine (15 μM, $n = 9$, $P< 0.05$), consistent with a partial involvement of PICs. Thus, under normal resting conditions (without hyperpolarization or isradipine), the long EPSP activated the PICs, which in turn amplified and prolonged the reflex response, thus producing the mixed PIC- and synaptic-mediated LPR. The remaining portion of the long lasting reflex (> 500 ms latency) was entirely mediated by PICs intrinsic to the motoneuron, because it was eliminated by preventing PIC activation (with hyperpolarization; Fig 4-1B) or nearly eliminated by blocking the Ca PICs with isradipine (Fig 4-1B; significant 83.9±13.5% reduction, $n = 9$, $P < 0.05$). Accordingly, it was called the PIC-mediated LLR (or LLR). The remaining long lasting reflex (LLR) in isradipine was likely mediated by the Na PIC, which can produce very slow firing in motoneurons that rest close to threshold (Li et al. 2004a), though this effect appeared small (15%).

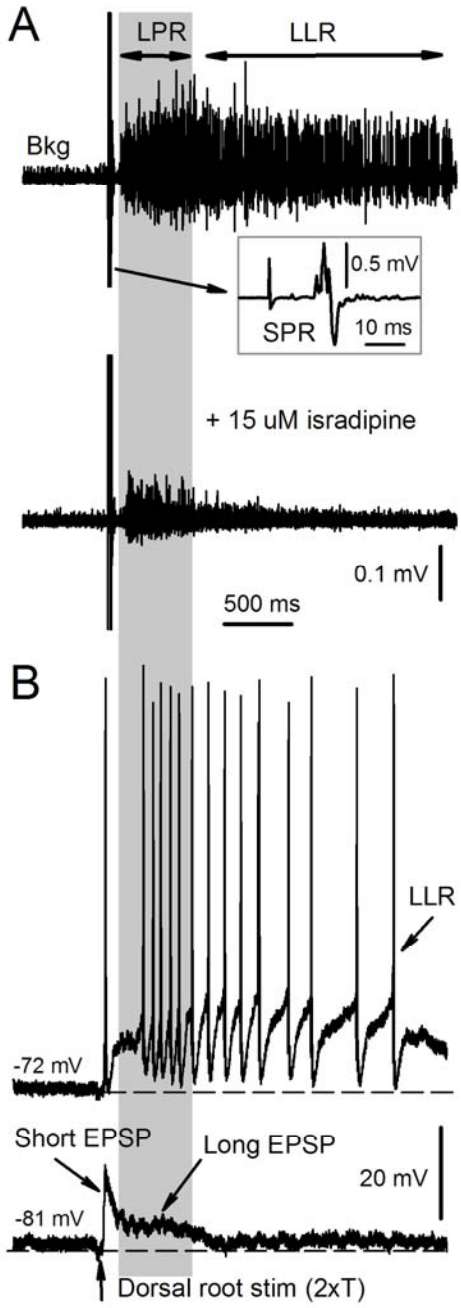

Figure 4-1. Polysynaptic reflexes and their underlying EPSP in chronic spinal rats. (**A**) Long-lasting reflex triggered by dorsal root stimulation (0.1 ms pulse, 3xT) and recorded from the ventral roots, with the reflex components LPR and LLR quantified during horizontal arrows (top plot). Inset: short-polysynaptic reflex (SPR, expanded time scale). Lower plot: elimination of LLR but not LPR, after blocking the L-type Ca^{2+} channel with isradipine (15uM). Background root activity, Bkg. (**B**) PIC-mediated plateau potential and sustained firing (LLR) evoked by dorsal root stimulation (3xT) in motoneuron at rest (without injected current; top). With a hyperpolarizing bias current to prevent PIC activation, the same stimulation only evoked polysynaptic EPSPs, with short and long EPSP components, corresponding to the SPR and the LPR (lower trace).

5-HT$_{1B}$ and 5-HT$_{1F}$ receptor activity inhibits the LPR and associated spasms. Application of the selective 5-HT$_{1B/1D/1F}$ receptor agonist zolmitriptan inhibited the LPR, with increasing doses producing larger responses over about a 100-fold change in dose (Fig 4-2 and Table 4-2). This dose-response relation was well approximated by a sigmoidal curve (Figs 4-2C), from which we computed: 1) the agonist dose to produce 50% maximal inhibition (EC50, Fig 4-2D), 2) agonist potency (pEC50 = –log EC50) and 3) agonist efficacy (maximal inhibition, reported relative to control LPR size; Fig 4-2C). For zolmitriptan, the EC50 was about 100 nM with a corresponding potency of about 7 (–log 100x10^{-9} M; Table 4-2). Overall, the efficacy of zolmitriptan was so large that the LPR was on average reduced to about 3% of pre-drug control LPR (97% inhibition in Table 4-2), suggesting that the associated long EPSP was also reduced. Zolmitriptan also significantly decreased the LLR (Fig 4-1D; to 2.15 ± 10.76% of control, $P < 0.05$, $n = 12$), consistent with an inhibition of the EPSP that triggers this spasm-related reflex.

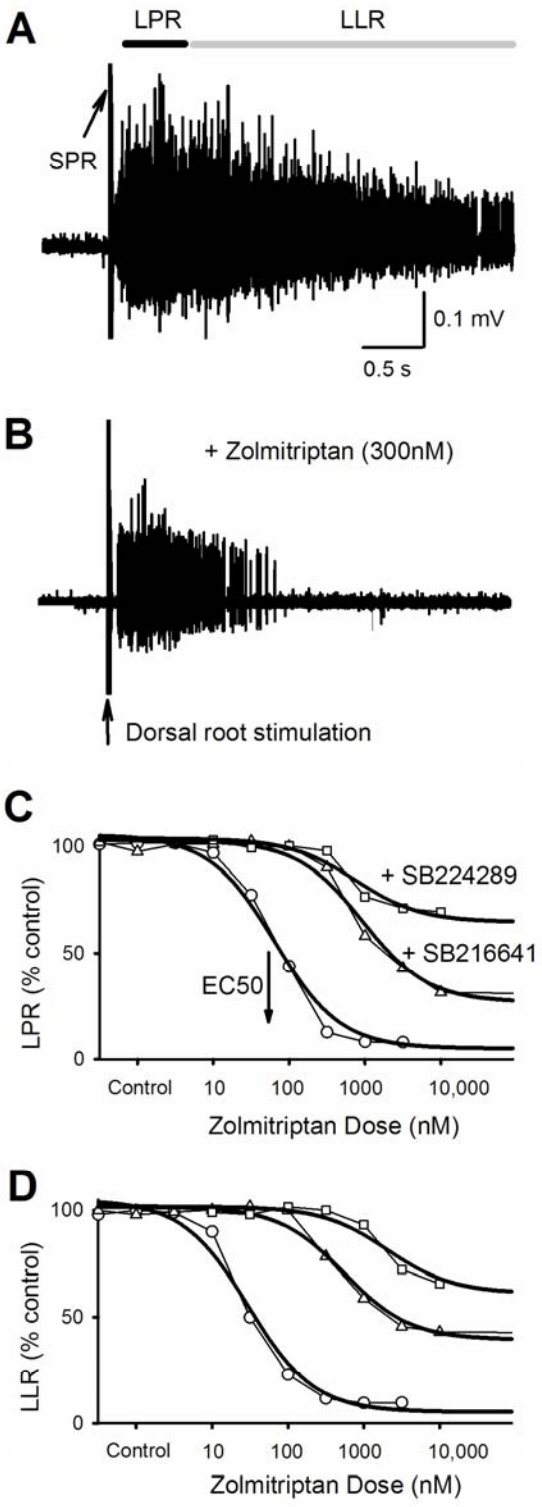
A
LPR
LLR
SPR
0.1 mV
0.5 s
B
+ Zolmitriptan (300nM)
Dorsal root stimulation
C
100
50
0
LPR (% control)
+ SB224289
+ SB216641
EC50
Control
10
100
1000
10,000
Zolmitriptan Dose (nM)
D
100
50
0
LLR (% control)
Control
10
100
1000
10,000
Zolmitriptan Dose (nM)

Figure 4-2. 5-HT_{1B} receptor activity inhibits the polysynaptic reflexes in chronic spinal rats. (**A**) Long lasting polysynaptic reflex triggered by dorsal root stimulation (0.1 ms pulse, 2-3xT) and recorded from the ventral roots, with LPR and LLR components indicated with bars. (**B**) Reduction of LPR and LLR with application of the 5-$HT_{1B/1D/1F}$ agonist, zolmitriptan (300 nM). (**C, D**) Reduction of LPR and LLR with increasing zolmitriptan dose (decrease over ~100-fold change in dose; left plots). Best fit sigmoidal curves and subsequent estimation of EC50. Prior application of a single blocking dose of the selective 5-HT_{1B} antagonist SB224289 (5 μM) or the 5-$HT_{1B/1D}$ antagonist SB216641 (5 μM) antagonized the inhibitory action of zolmitriptan (shifting EC50 to the right).

Application of agonists with a relatively high affinity for 5-HT_1 receptors, compared to 5-HT_2 receptors (5-CT, EMD), likewise significantly inhibited the LPR with a simple sigmoidal dose-response relation (significant efficacy; Table 4-2). Less selective 5-HT_1 agonists (including alpha-me-5-HT, BW723C86, methylergonovine, and 5-HT itself) with relatively high affinity for 5-HT_2 receptors, also inhibited the LPR (Table 4-2), but this inhibition was partly obscured by their activation of 5-HT_2 receptors (Fig 4-3), which we have previously shown increases PICs and associated reflexes (Murray et al. 2010a; Murray et al. 2010b). Fortunately though, the affinity of these agonists for the 5-HT_{2B} and 5-HT_{2C} receptors was substantially higher than the affinity for 5-HT_1 receptors, and thus the effects of each of these receptor types could be observed separately on a dose-response relation, as a *biphasic* response. That is, at low doses the agonist increased the long lasting reflexes, including the LPR and LLR (Fig 4-3A-C). This low-dose response was especially prominent in the entirely PIC-mediated LLR (see sigmoid curve fit to ascending phase in Fig 4-3B, and low EC50), consistent with 5-HT_2 receptor-mediated facilitation of the PIC, as described previously (Murray et al. 2010a; Murray et al. 2010b). As successively higher doses were applied, the reflexes eventually reached a peak (peak reflex), after which they decreased with increasing dose (*inhibitory phase*), often to the point where the reflex fell well below the reflex prior to any drug application (control). We fit a sigmoidal curve to this inhibitory phase of the dose-response curve for these agonist actions on the LPR (from peak reflex dose to maximum dose), and from this computed EC50 and efficacy values (Fig 4-3A, C). As seen in Table 4-2, non-selective agonists with 5-HT_1 and 5-HT_2 receptor action (e.g. 5-HT) produced a significant inhibition of the LPR (efficacy), after the initial excitatory phase. We confirmed the validity of this estimation of the EC50 and efficacy for reflex inhibition from non-selective agonists by showing that after blocking the confounding 5-HT_2 receptor action with antagonists (methysergide, 10 μM or the selective 5-HT_2 antagonists like RS127445, 3μM), 5-HT produced a purely inhibitory action, with similar dose-response relation to that obtained without the block (Fig 4-3D and Table 4-2). This also shows that the inhibitory

action of these non-selective agonists is mediated by 5-HT_1 and not 5-HT_2 receptors.

Table 4-2

Agonist	Antagonist pre-treatment	Receptors that can be activated (Ki < 400)	Inhibition of LPR		Inhibition of SPR	
			Efficacy (% change)	Potency (-logEC50)	Efficacy (% change)	Potency (-logEC50)
5-CT	None	5-HT1A,B,D, 2B, 5-7	-96.3±65.4*	7.08±0.50	-60.4±77.3*	7.16±0.51
5-HT	None	5-HT1-7	-78.5±23.2*	6.56±0.17	-63.4±43.7*	6.83±0.43
5-HT	Methys + gran	5-HT1B, 5-HT4	-84.6±14.7*	6.47±0.38	-29.7±23.1*†	6.26±0.22†
5-HT	RSs	5-HT1,3-7	-108.1±28.7*	6.57±0.23	-74.3±38.4*	6.36±0.30
5-HT	RSs + SB216	5-HT1A,E,F, 3-7	-52.0±22.0†*	5.70±0.36†	-59.2±23.65	5.86±0.32†
α-methyl-5-HT	None	5-HT1B,E,F, 2, 4	-57.3±16.5*	5.75±0.23	-64.1±29.4*	5.86±0.53
BW723C86	None	5-HT1B, 2, 3, 6	-38.0±24.8*	5.89±0.48	-49.3±43.2*	5.92±0.33
EMD386088	None	5-HT1B,D, 3, 6	-43.6±35.3*	5.53±0.35	-51.2±28.8*	5.76±0.36
EMD386088	SB216 + gran	5-HT6	12.4±110.6†	ND	5.7±43.3†	ND
LY344864	None	5-HT1F	-57.2±25.4*	7.12±0.17	-45.9±26.72*	7.14±0.21
methylergon	None	5-HT1E,F, 2	-40.8±21.3*	6.47±0.25	-44.0±25.2*	6.51±0.15
zolmitriptan	None	5-HT1B,D,F	-99.5±11.3*	7.07±0.40	-55.2±20.6*	7.08±0.41
zolmitriptan	SB216 or GR127	5-HT1F	-41.2±27.6†*	5.94±0.25†	-38.2±27.1*†	6.15±0.70†
zolmitriptan	SB224	5-HT1D,F	-26.6±29.0†*	5.63±0.53†	-31.5±29.9*†	5.92±0.57†

Table 4-2. Inhibition of the polysynaptic reflexes by 5-HT_{1B} agonists. Agonists with varying selectivity for the different 5-HT receptors were applied, sometimes after prior application of 5-HT receptor antagonists to effectively make the agonist action more selective (column 2, pre-treatment). The receptors that can be activated by this agonist/antagonist combination are indicated in column 3 (Ki < 400 nM; see details in Table 4-1). The antagonists used were (abbreviations, doses and receptors blocked in brackets): SB224289 (SB224, 5 μM, 5-HT_{1B}), SB216641 (SB216, 3 μM, 5-$HT_{1B/1D}$), GR127935 (GR127, 3 μM, 5-$HT_{1B/1D}$), methysergide (methys, 10μM, blocks all but 5-$HT_{1B/3/4}$), granisetron (gran, 0.3μM, 5-HT_3), RS127445 (3μM, 5-HT_{2B}), RS102221 (3μM, 5-HT_{2C}). The latter two antagonists were applied together and abbreviated RSs. Methylergonovine was abbrevated methylergon. The efficacy of the agonists in inhibiting the LPR and SPR are indicated, normalized by the pre-drug reflex amplitudes (-100% indicates complete elimination of the reflex by agonist). Additionally, the agonists 8-OH-DPAT (5-$HT_{1A/5/7}$ affinity), LP44 (5-$HT_{7/1A}$), 2-methyl-5-HT (5-$HT_{2B/3/1F}$), DOI (5-HT_2), cisapride (5-HT_4) and MK212 (5-$HT_{2C/3}$) produced no significant inhibition of the LPR or SPR (not shown, doses up to 30 μM; see text). * significant change in reflex with $P < 0.05$, $n > 8$ per condition. † significant decrease in efficacy or potency after application of antagonists (SB224, SB216 or GR127), relative to the inhibitory action of agonists alone (e.g. zolmitriptan; row above antagonist data) alone, $P < 0.05$, $n > 8$ per condition.

Pre-treatment with the broad spectrum antagonist methysergide, as just described, also turned out to be particularly useful because methysergide has negligible affinity for rat 5-HT_{1B} receptors (Ki > 400), whereas it antagonizes/binds most other 5-HT receptors with high affinity (Ki < 500nM; except 5-HT_3 and 4 receptors) (Boess and Martin 1994). Thus, the inhibition of the LPR by 5-HT seen after pre-treatment with methysergide (Fig 4-3D, Table 4-2) suggests that 5-HT_{1B} receptors specifically inhibit the LPR, though this does not rule out additional involvement of other 5-HT_1 receptors blocked by methysergide (5-HT_{1F}).

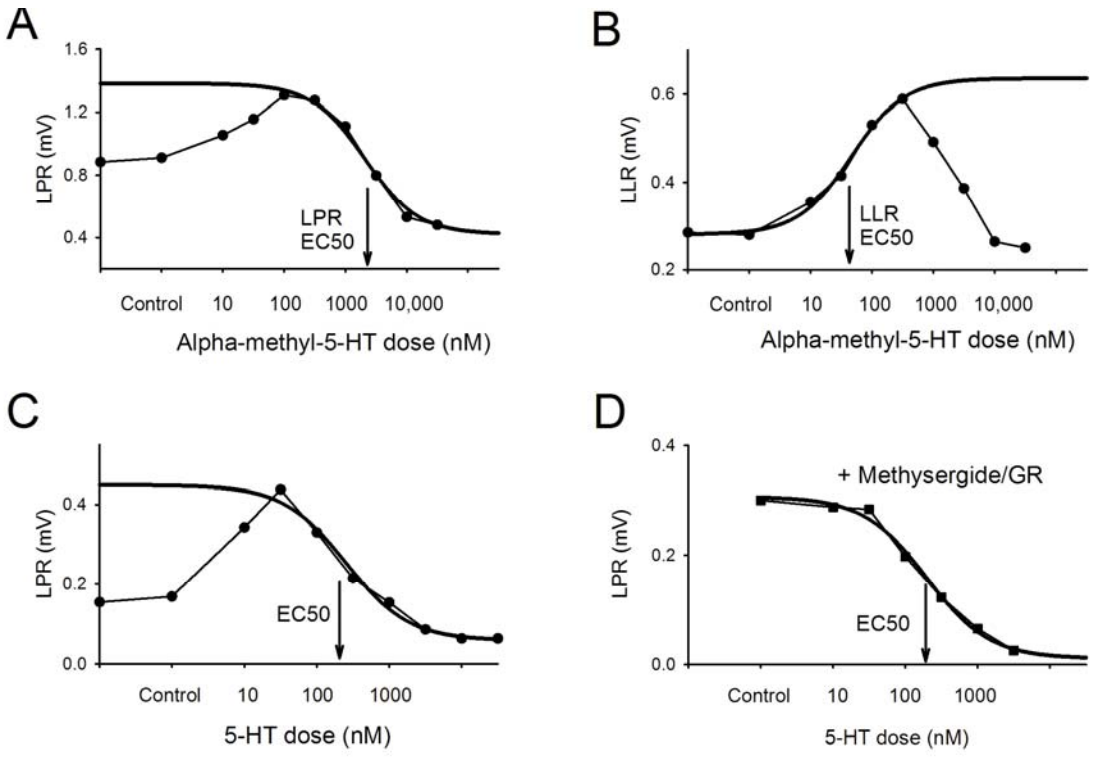

Figure 4-3. Mixed 5-HT_1 and 5-HT_2 receptor agonists have a biphasic response, only inhibiting reflexes at high doses. (**A**–**C**) Dose-response relation for the 5-HT_1 and 5-HT_2 receptor agonist alpha-methyl-5-HT and 5-HT itself, with increased reflexes (LPR and LLR) at low doses (5-HT_2 mediate) and decreased reflexes at high doses (5-HT_1 mediated). In A and C, heavy line is a sigmoidal curve fit to inhibitory phase of dose-response relation and used to estimate the EC50 for the 5-HT_1 receptor mediated inhibitory action. In B the heavy line is a sigmoidal curve fit the excitatory phase of the dose response relation, mediated by 5-HT_2 receptors. (**D**) Dose response relation for 5-HT affect on the LPR after 5-HT_2 receptor block with methysergide (10 μM), with similar EC50 to that obtained in C.

Prior application of the selective 5-HT_{1B} receptor antagonist SB224289 or the selective 5-$HT_{1B/1D}$ receptor antagonist SB216641 significantly reduced the inhibitory action of both selective (zolmitriptan) and non-selective (5-HT; inhibitory phase) 5-HT_1 agonists on the LPR (Table 4-2). These antagonists lowered the efficacy and shifted the agonist dose-response curve by about an order of magnitude to the right (EC50 significantly increased; Table 4-2), indicating that the 5-HT_{1B} receptor is responsible for a large part of the inhibitory action of these agonists. However, in the presence of these antagonists, there was still significant inhibition of the LPR induced by relatively high doses of both zolmitriptan and 5-HT (Fig 4-2 and Table 4-2). This may be explained by the activation of the 5-HT_{1F} receptor, because this receptor is not blocked by SB224289 or SB216641 (Price et al. 1997; Selkirk et al. 1998) and zolmitriptan and 5-HT have a relatively lower affinity for the 5-HT_{1F}, compared to the 5-HT_{1B} receptor (Table 4-1).

Consistent with the possible involvement of 5-HT_{1F} receptors in regulating the LPR, we found that the selective 5-HT_{1F} agonist LY344864 and non-selective 5-HT_{1F} agonists that have negligible affinity for 5-HT_{1B} receptors (e.g. methylergonovine, alpha-methyl-5-HT; Table 4-1) inhibited the LPR (Table 4-2). However, this does not negate the importance of 5-HT_{1B} receptors, because the agonists with substantial affinity for 5-HT_{1B} receptors, but negligible affinity for the 5-HT_{1F} receptors (BW723C86, EMD386088 and 5-CT; Table 4-1), also inhibited the LPR (Table 4-2), indicating that both 5-HT_{1B} and 5-HT_{1F} receptors modulate the LPR.

Application of agonists (or agonist/antagonist combinations) relatively selective for 5-$HT_{1A/1E}$, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7 receptors (Table 4-1) produced no significant inhibition of the LPR (Table 4-2 and 4-3, ND), suggesting that none of these other receptors inhibit the LPR and associated long EPSP. Furthermore, application of the selective 5-HT_2 receptor agonist DOI or non-selective 5-HT_2 agonists that have negligible affinity for 5-HT_{1B} and 5-HT_{1F}

receptors also produced no detectable inhibition in the LPR (Table 4-2 and 4-3). However, these 5-HT_2 agonists did produce a significant facilitation of the LPR (by 417.87 ± 346.5, 172.13 ± 70.22 and 79.66 ± 94.82 %, for 2-methyl-5-HT, DOI, and MK212 respectively, $P < 0.05$, $n > 8$ per condition, over a dose range appropriate to activate 5-HT_2 receptors; up to 30 μM), due to a facilitation of the underlying Ca PIC, as previously reported for the LLR (Murray et al. 2010b). To rule out inhibitory effects of 5-HT_2 receptors on the EPSPs that might be masked by their large facilitation of the Ca PIC, we first blocked the Ca PIC with isradipine, giving us a reflex that reflected the polysynaptic EPSP in isolation (see Fig 4-1 above). With this Ca PIC block present, DOI produced no significant change in the LPR (3.90 ± 10.4% change, $P > 0.05$, $n = 8$, at 3000 nM), suggesting that the excitatory action of DOI is mainly on the Ca PIC, and there is no inhibitory action of 5-HT_2 receptors on the EPSP underlying the LPR.

Table 4-3

		pEC50 – pKi				pEC50 – pKi	
Receptor	**Agonist**	**LPR**	**SPR**	**Receptor**	**Agonist**	**LPR**	**SPR**
5-HT1A	5-CT	–2.38±0.50	–2.30±0.51	5-HT2B	2-methyl-5-HT	ND	ND
	5-HT	–2.22±0.17	–1.95±0.28		5-CT	+0.26±0.50	+0.34±0.51
	8-OH-DPAT	ND	ND		5-HT	–1.43±0.17	–1.16±0.28*
	LP44	ND	ND		α-me-5-HT	–2.23±0.23	–2.12±0.53
					BW723C86	–2.01±0.48	–1.98±0.33
5-HT1B	5-CT	**–1.40±0.50***	**–1.32±0.51***		DOI	ND	ND
	5-HT	**–1.05±0.17***	**–0.78±0.28***		methylergon	–2.83±0.25	–2.79±0.15
	BW723C86	**–0.71±0.48***	**–0.68±0.33***				
	EMD	**–1.22±0.35***	**–0.99±0.36***	5-HT2C	5-HT	–1.40±0.17	–1.13±0.28*
	zolmitriptan	**–1.23±0.40***	**–1.22±0.41***		α-me-5-HT	–2.82±0.23	–2.71±0.53
					BW723C86	–1.01±0.48*	–0.98±0.33*
5-HT1D	5-CT	–2.35±0.50	–2.27±0.51		DOI	ND	ND
	5-HT	–2.04±0.17	–1.77±0.28		MK212	ND	ND
	BW723C86	–0.41±0.48*	–0.38±0.33		methylergon	–1.87±0.25	–1.83±0.15
	EMD	–1.43±0.35*	–1.20±0.36*				
	zolm (SB224)	–2.13±0.53	–3.28±0.57	5-HT3	2-methyl-5-HT	ND	ND
					BW723	–0.61±0.48*	–0.61±0.33*
5-HT1E	5-HT (SB216)	–2.51±0.36	–2.35±0.32		EMD	–1.94±0.35	–1.71±0.36
	α-me-5-HT	–1.17±0.23*	–1.06±0.53*		MK212	ND	ND
	methylergon	–0.58±0.25*	–0.54±0.15				
				5-HT4	5-HT	–1.64±0.17	–1.37±0.28*
5-HT1F	5-HT (SB216)	**–1.47±0.17**	**–1.31±0.32**		α-me-5-HT	–0.83±0.23*	–0.72±0.53*
	α-me-5-HT	**–0.99±0.23***	**–0.88±0.53***		cisapride(RSs)	ND	ND
	LY344864	**–1.10±0.17***	**–1.08±0.21***				
	methylergon	**–1.04±0.25***	**–1.00±0.15***	5-HT5	5-CT	–2.42±0.50	–2.34±0.51
	zolm (SB224)	**–1.57±0.53***	**–1.28±0.57***		5-HT	–1.54±0.17	-1.27±0.28*
					8-OH-DPAT	ND	ND
5-HT2A	5-HT	–1.68±0.17	–1.41±0.28*				
	α–me-5-HT	–1.15±0.23*	–1.04±0.53*	5-HT6	2-methyl-5-HT	ND	ND
	BW723C86	–0.71±0.48*	–0.68±0.33*		5-CT	+0.48±0.50	+0.56±0.51
	DOI	ND	ND		5-HT	–0.69±0.17*	–0.42±0.28
	methylergon	–2.98±0.25	–2.94±0.15		BW723C86	–0.51±0.48*	–0.48±0.33*
					EMD (SB216+)	ND	ND
				5-HT7	5-CT	–2.72±0.50	–2.64±0.51
					5-HT	–2.26±0.17	–1.99±0.28
					8-OH-DPAT	ND	ND
					LP44	ND	ND

Table 4-3. Relative potency of agonists at inhibiting the LPR and SPR. Relative potency computed as the difference between potency (Table 4-2) and affinity (Table 4-1): pEC50 - pKi. Pre-treatment with antagonists used to make agonist more selective, indicated in brackets, abbreviated as in Table 4-2. ND, no detected inhibition of the reflex in Table 4-2. *, relative potency within 2 SD of -1.0, the confidence interval for similarity. SD, standard deviation of relative potency measurements, indicated. Bold, receptors with relative potency values all within confidence interval.

Agonist inhibition potency is correlated with receptor binding affinity at 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors.

The effective 5-HT$_{1B}$ and 5-HT$_{1F}$ agonist doses that inhibit the LPR (EC50 values and associated potencies, pEC50) varied by orders of magnitude between the different agonists (Table 4-2), though this variation was largely accounted for by the differing binding affinity of these drugs to 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors (pKi; see description of binding affinity in Methods; Table 4-1). That is, we found that for 5-HT$_{1B}$ agonists, the potency (pEC50) was significantly correlated with the binding affinity (pKi) of the agonist for 5-HT$_{1B}$ receptors, and importantly, very close to a line of unit slope (dashed line) as shown in Fig 4-4A. Likewise, the potency was also significantly correlated with the agonist affinity for 5-HT$_{1F}$ receptors (Fig 4-4B), with close to a unit slope relation, consistent with an additional involvement of this receptor. The agonist potency was uncorrelated with the agonist binding affinity for other 5-HT receptors (including 5-HT$_{1D}$; Fig 4-4B–D) with potency scattered widely, far from the linear potency-affinity relation found for the 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors. However, for most of these other receptors, only a few broad spectrum agonists with affinity to these other 5-HT receptors produced a response (inhibition of LPR), making the correlation analysis statistically weak ($n < 5$). Thus, we sought an independent method of quantifying whether the agonist response potency was attributed to a given receptor, based on quantitatively modelling the expected relation between potency and affinity, as described in the next section.

Potency of agonist can be quantitatively predicted from its receptor binding affinity.

Ideally, for a receptor to be involved in a particular response, the agonist dose needed to substantially bind to the receptor (Ki) should approximately equal the agonist dose needed to produce a functional response (e.g. EC50 for LPR), and thus the agonist binding affinity (pKi) should roughly equal its potency (pEC50) (Selkirk et al. 1998; Wainscott et al. 1993). However, the substantial barriers to drug diffusion in our whole cord preparation required higher drug doses (EC50) to get responses, and thus the potency (–log EC50) was higher than the affinity. Furthermore, non-linearities in the functional receptor response, such as saturation of the EPSP that underlies the LPR and saturation in receptor responses (receptor reserve, Boess and Martin 1994), may have subtly changed the EC50 dose and potency. Nevertheless, factors like drug diffusion and response saturation do *not* generally depend on the agonist involved (see Discussion). Thus, we hypothesized that the potency could be predicted from affinity by the following simple relation: pEC50 = pKi + C, where C is a constant that is *invariant for all agonist responses at functional receptors that* represents drug diffusion barriers, etc. Rearranging, we have: pEC50 – pKi = C, and thus, determining whether or not a receptor is functional amounts to testing whether the difference between the measured potency and affinity is invariant (C). We call this difference the relative potency (pEC50 – pKi; it reflects all factors that affect potency other than binding affinity). For the 5-HT_{1B} and 5-HT_{1F} receptors that we know are involved in inhibiting the LPR (and associated EPSP), we found that the potency-affinity data significantly fit this simple linear relation (with $r = 0.93$ and 0.91 respectively, $n = 5$ each; dashed unity slope lines in Fig 4-4A and B). Also, the difference pEC50 – pKi (relative potency) was, as hypothesized, highly invariant across all agonists tested at these receptors, on average –1.12±0.26 and –1.23 ± 0.27 for 5-HT_{1B} and 5-HT_{1F} receptors, respectively, with each agonist having a relative potency well within two standard deviations (SD) of the mean (our confidence interval, SD taken from each agonist potency; see Table 4-3). Remarkably, this relative potency value of about –1 has been seen for two other functional receptors in our

preparation (Murray et al. 2010b), and so appears to be an invariant across many or all receptors, mainly reflecting the diffusion barriers to drugs reaching the receptors. Thus, in our preparation if a receptor is functional then pEC50 – pKi = –1 (constant; dashed line in Fig 4-4).

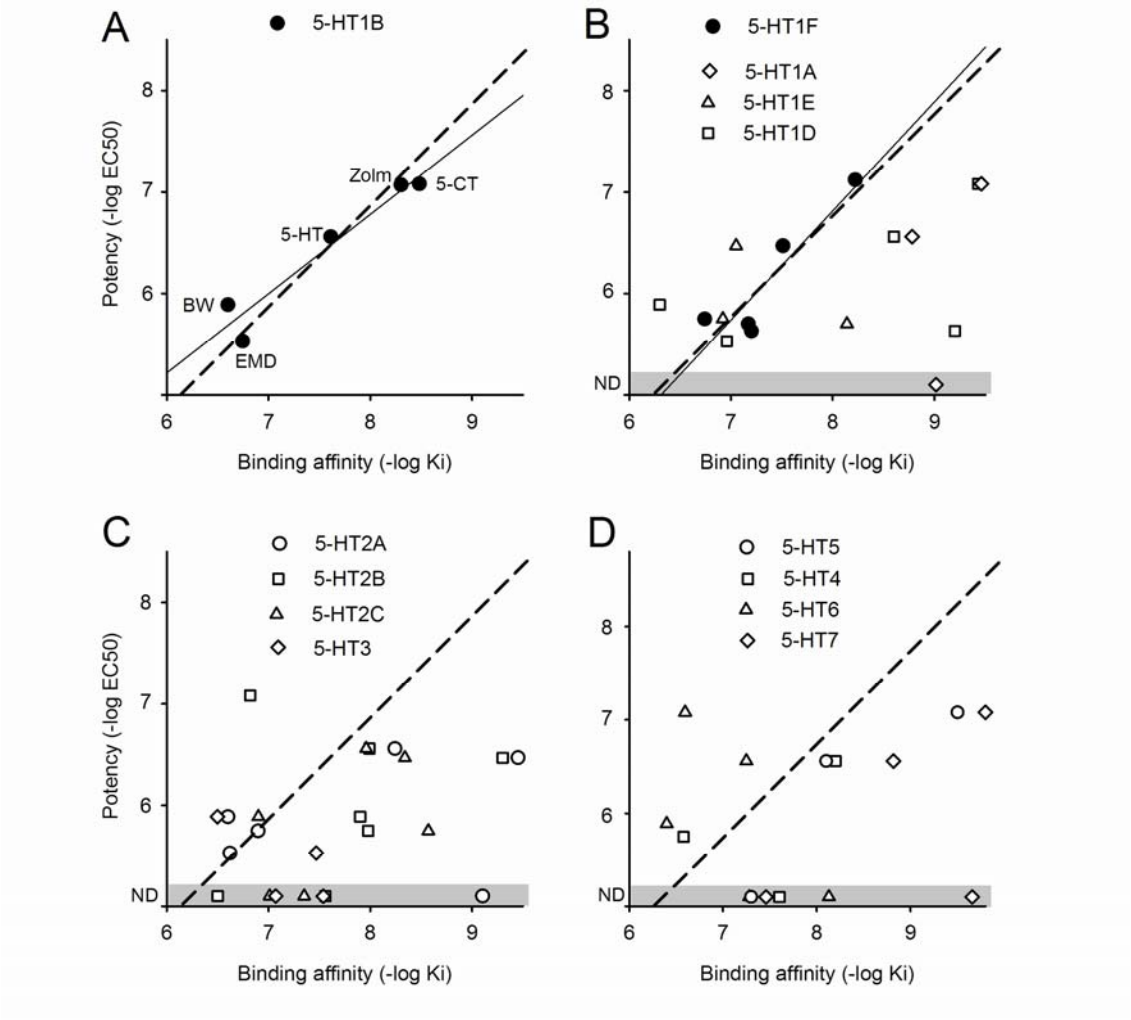
A
5-HT1B
Zolm
5-CT
5-HT
BW
EMD
Potency (-log EC50)
Binding affinity (-log Ki)
B
5-HT1F
5-HT1A
5-HT1E
5-HT1D
ND
Binding affinity (-log Ki)
C
5-HT2A
5-HT2B
5-HT2C
5-HT3
Potency (-log EC50)
ND
Binding affinity (-log Ki)
D
5-HT5
5-HT4
5-HT6
5-HT7
ND
Binding affinity (-log Ki)

Figure 4-4. Potency of 5-HT receptor agonists at inhibiting the LPR is only related to binding to 5-HT_{1B} and 5-HT_{1F} receptors. (**A**) 5-HT_{1B} receptor agonist potency (pEC50 = -log (EC50)) for inhibiting the LPR plotted against the agonist binding affinity to that receptor (pKi). Each agonist indicated next to points, with abbreviations BW, BW723C86; Zolm, Zolmitriptan; EMD, EMD386088. Thin line: significant linear correlation between potency and affinity ($r = 0.97$, $P < 0.05$, $n = 5$). Dashed line: best fit line with unit slope (potency = binding affinity + C, where C ~ –1). (**B-D**) Similar potency-affinity scatter plots for the remaining 5-HT receptors. Agonists used for each receptor are listed in Table 4-1. Also we used the potency of 5-HT and zolmitriptan after pre-treatment with SB216641 and SB224289, to avoid lower dose effects from activation of 5-HT_{1B} receptors by these agonists. Thin line: significant linear correlation between agonist potency and affinity for 5-HT_{1F} receptors (solid circles; $r = 0.91$, $P < 0.05$, $n = 5$). Dashed line: unit slope line. Other receptors had no significant correlation between potency and affinity (open circles; $P > 0.05$). ND and gray zone: no detected effect of agonist on the LPR. Agonists used and affinities are in Table 4-1, with agonists assumed to act at a receptor only if Ki < 400 nM. Potencies are from Table 4-2. Potencies for 5-HT and zolmitriptan action in the presence of 5-HT_{1B} antagonists were used (plotted) for comparison to 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} receptor binding affinity, as these antagonists removed confounding affects of 5-HT_{1B} receptors. Table 4-3 also summarizes agonists/antagonists used for each receptor.

In contrast to the invariant relative potency for 5-HT_{1B} receptors, we found that for all other receptors the relative potency computed from the potency of broad spectrum agonists response (pEC50) varied widely over a range well outside of our confidence interval (2 SD; Table 4-3), suggesting than none of these receptors affect the LPR response (pEC50 – pKi not equal –1). For example, the relative potency computed for zolmitriptan's pEC50 compared to its affinity at the 5-HT_{1D} receptors was < –2 (Table 4-3), well outside of the confidence interval, suggesting that its EC50 is too low to be predicted from the Ki for zolmitriptan at the 5-HT_{1D} receptor, and thus ruling out this receptor for which we otherwise had no selective agonist to directly test. Similarly, the potency of 5-HT and 5-CT could not be predicted from their pKi values at the 5-HT_{1D} receptor (relative potency < –2; Table 4-3), again suggesting that the 5-HT_{1D} receptor is not involved in modulating the LPR. Sometimes by chance a drug (e.g. EMD) had a similar affinity for the 5-HT_{1B} receptor and another receptor (e.g. 5-HT_{1D}; Table 4-3) and in this case the relative potency (pEC50-pKi) was similar for each receptor, and could not be used to distinguish the involvement of these two receptors. Overall, the relative potency varied widely for the action agonists of non-5-HT_{1B} receptors, indicating that no receptor, other than the 5-HT_{1B} receptor, was involved in modulating the LPR.

Another way to interpret the relative potency arises from the law of differences of logarithms:
pEC50 – pKi = –log(EC50) – (–logKi) = –log(EC50/Ki). Thus, the ratio EC50/Ki equals $10^{-(pEC50 - pKi)}$. For the 5-HT_{1B} receptor the relative potency was on average –1.12, and thus the on average EC50/Ki = $10.0^{1.12}$ = 13.2. This indicates that the EC50 dose need to affect the LPR in the present whole sacral spinal cord preparation was about 10 times higher than the Ki value, a factor that is most likely due to drug diffusion.

SPR is also inhibited by the 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors.

Similar to the LPR, the short latency polysynaptic reflex (SPR) was inhibited by 5-HT$_{1B}$ and 5-HT$_{1F}$ receptor agonists, including relatively selective agonists (zolmitriptan or agonist/antagonist combinations) and non-selective agonists (5-HT; Fig 4-5 and Table 4-2). Also, the agonist potencies (pEC50) were significantly correlated with the agonist binding affinity at 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors, and no other receptor (Fig 4-6). In contrast, 5-HT receptor agonists (or agonist/antagonist combos) that have negligible affinity for 5-HT$_{1B}$ or 5-HT$_{1F}$ receptors did not inhibit the SPR (Table 4-3). The relative potency computed for each agonist relative to its binding affinity at the 5-HT$_{1B}$ receptor (pEC50 – pKi; Table 4-3) was consistently within 2 SD of –1.0 (our confidence interval), with a mean of -1.02 ± 0.25. Likewise, the relative potency computed for agonists of the 5-HT$_{1F}$ receptor (pEC50-pKi; Table 4-3) was consistently within 2 SD of –1.0, with a mean of -1.11 ± 018, suggesting that the potency of the agonists on the SPR was well predicted by agonist affinity at the 5-HT$_{1B}$ or 5-HT$_{1F}$ receptor, with an invariant diffusion factor (pEC50 = pKi – 1), just like we found for the LPR. In contrast, the relative potency for other receptors varied widely and for at least one agonist was more than 2 SD from –1 (our confidence interval; Table 4-3). An important example is that the relative potency of zolmitriptan for the 5-HT$_{1D}$ receptor was much too low for this receptor to be involved in the SPR, more than 2 SD below –1.0 (Table 4-3), ruling out a 5-HT$_{1D}$ action of zolmitriptan.

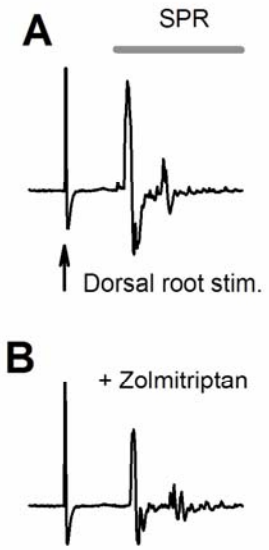

Figure 4-5. 5-$HT_{1B/1D/1F}$ agonist zolmitriptan inhibits the short latency polysynaptic reflex (SPR). (**A**) Short latency polysynaptic reflex (SPR) evoked in ventral root of chronic spinal rat after dorsal root stimulation (0.1 ms, 3xT), quantified during bar. (**B**) Inhibition of the SPR zolmitriptan (300 nM).

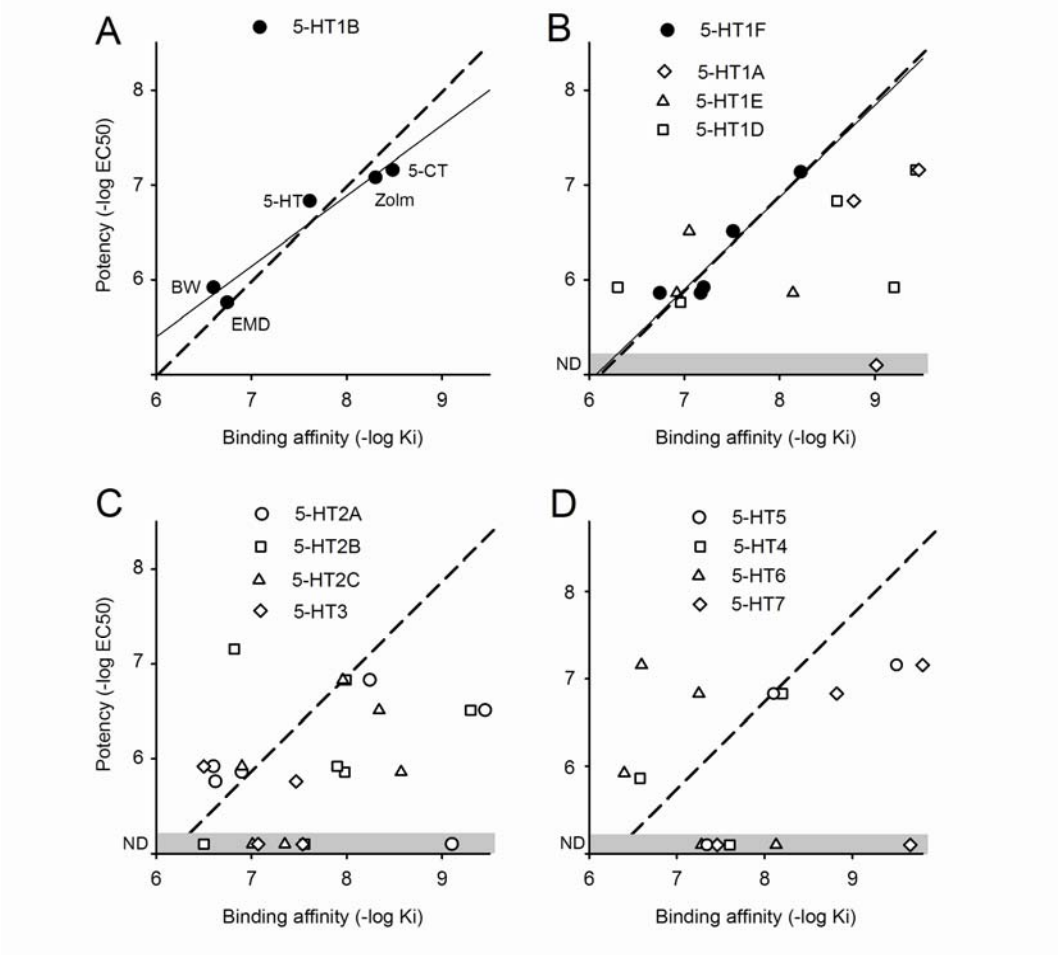

Figure 4-6. Potency of 5-HT receptor agonists at inhibiting the SPR is only related to binding to 5-HT_{1B} and 5-HT_{1F} receptors. (**A**) 5-HT_{1B} receptor agonist potency (pEC50) for inhibiting the SPR plotted against the agonist binding affinity to that receptor (pKi). Identical format and abbreviations as in Fig 4-4. Thin line: significant linear correlation between potency and affinity (r = 0.97, $P <$ 0.05, n = 5). Dashed line: best fit line with unit slope. (**B-C**) Similar potency-affinity scatter plots for the remaining 5-HT receptors. Thin line: significant linear correlation between agonist potency and affinity for 5-HT_{1F} receptors (solid circles; r = 0.94, $P <$ 0.05, n = 5). Dashed line: unit slope line. The remaining receptors had no significant correlation between potency and affinity (open circles; $P >$ 0.05). ND and gray zone: no detected effect of agonist on the LPR. Agonists used, potencies and affinities are detailed in Fig 4-4. Table 4-3 also summarizes agonists/antagonists ued for each receptor.

The three 5-HT_2 receptor agonists that have negligible affinity for 5-HT_{1B} receptors produced no inhibition in the SPR (Table 4-2 and 4-3, ND), and only one of these, MK212, significantly increased the SPR (by 70.15±62.4%; n = 8, P <0.05). The remaining two (DOI and 2-methyl-5-HT) had no effect on the SPR (Table 4-2), unlike the large increase produced by all three of these 5-HT_2 agonists on the LPR, suggesting that the PICs controlled by the 5-HT_2 receptors do not reliably affect this shorter, transient SPR reflex. Also, when we blocked the PICs with isradipine (as above), the SPR remained unaffected by DOI (0.75 ± 14.87 % change, not significant, n = 8, P > 0.05).

Lack of endogenous 5-HT_{1B} receptor activity in chronic spinal rats.

We next examined whether there was any endogenous 5-HT_1 receptor activity after chronic spinal cord injury. Without prior agonist application, the selective antagonists SB224289 (5-HT_{1B} selectivity, 3–5 μM; Fig 4-7A), SB216641 (5-$HT_{1B/1D}$, 5 μM) or GR127935 (5-$HT_{1B/1D}$, 5 μM) produced no significant increase in either the LPR (0.8±13.2%, –4.8 ± 40.2% and –8.5 ± 29.8% change, respectively, *n* = 12 per condition) or SPR (10.1±34.8%, 6.8 ±31.6% and –3.0 ± 50.0% change, respectively), suggesting that there is no endogenous 5-HT_{1B} receptor activity inhibiting the reflexes, and consistent with previous findings that there is little functional 5-HT that remains in chronic spinal rats (Murray et al. 2010a). SB224289 is unique among these three antagonists because it is classified as an inverse agonist (Price et al. 1997; Selkirk et al. 1998), meaning that it not only blocks agonist-induced activity, but also blocks spontaneous activity in the 5-HT_{1B} receptor that occurs in the absence of 5-HT or other agonists (constitutive receptor activity) (Seifert and Wenzel-Seifert 2002). Thus, the lack of action of SB224289, indicates that there is not constitutive 5-HT_{1B} receptor activity after injury, unlike what we find with 5-HT_2 receptors (Murray et al. 2010a; Murray et al. 2010b). As a positive control, we applied 5-HT_{1B} agonists (zolmitriptan, 1.0 μM; 5-CT, 1.0 μM; or 5-HT, 0.3 μM) to activate the 5-HT_1 receptors, which as expected decreased the LPR and SPR (Table 4-2), and then applied the antagonists (Fig 4-7B). In this situation, the antagonists SB224289 (3–10 μM),

SB216641 (5–10 μM) and GR127935 (5 μM) significantly increased the LPR (by 45.8±45.7, 27.7 ± 30.2 and 78.8 ± 87.9% respectively) and the SPR (by 44.0 ± 41.1, 45.4 ± 40.9 and 66.7 ± 60.3% respectively, n = 12 each condition), demonstrating that these antagonists can be used to detect 5-HT_{1B} receptor activity. We did find that the antagonists only partially reversed the inhibition of the reflexes by these 5-HT_1 agonists (Fig 4-7B), but we attribute this to the agonist activation of 5-HT_{1F} receptors, which our antagonists do not block.

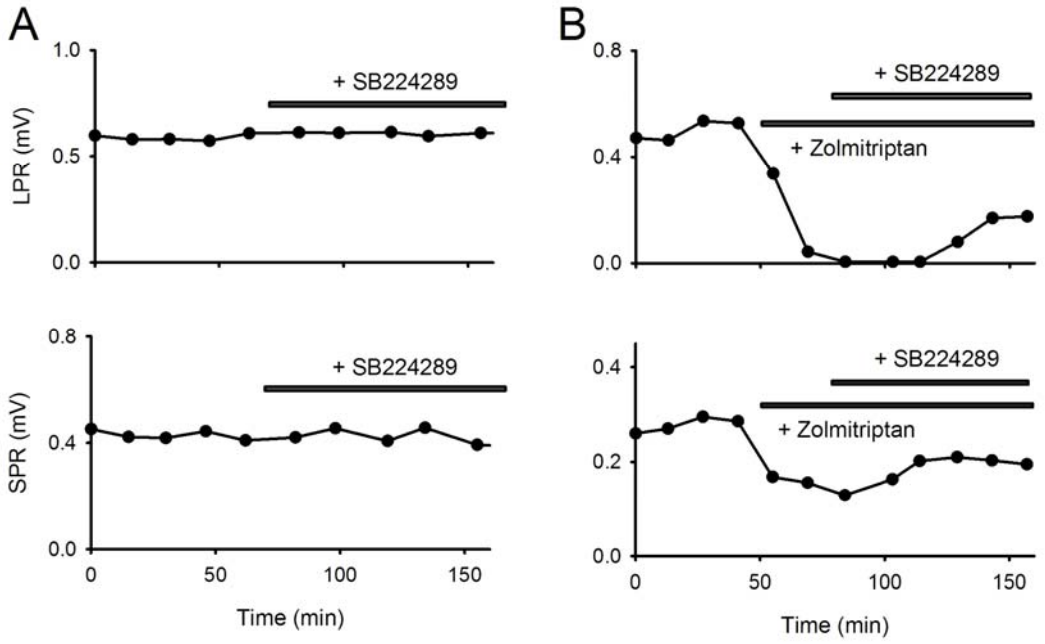

Figure 4-7. The 5-HT_{1B} receptor is not endogenously active in chronic spinal rats. (**A**) A block of possible endogenous 5-HT_{1B} receptor activity with SB224289 (3 μM, during bar) produced no increase (or change) in the LPR or SPR. Reflexes measured at about 12 min intervals (circles). (**B**) In contrast, SB224289 (3 μM) increased the LPR and SPR after 5-HT_{1B} receptors were exogenously activated by zolmitriptan (1 μM), which initially deceased these reflexes.

Increasing cAMP increases the LPR and SPR.

5-HT_1 receptors are coupled to Gi proteins that lead to decreased intracellular cAMP levels. Thus, our finding that activating 5-HT_1 receptors decreases the LPR and SPR, suggests that 5-HT_1 receptors may decrease reflexes by decreasing cAMP, and more generally, these reflexes and associated EPSPs may depend on basal cAMP levels. We tested this idea by applying forskolin (1–10 μM), a membrane permeable drug that increases intracellular cAMP. As expected, forskolin increased both the LPR and SPR (by 116.7±72.0% and 135.7±78.1%, respectively, $n = 8$, $P < 0.05$).

EPSPs in motoneurons are inhibited by zolmitriptan.

To verify that 5-$HT_{1B/1F}$ receptors inhibit the EPSPs underlying the LPR and SPR, we made intracellular recordings from motoneurons in chronic spinal rats (*in vitro*), and measured the EPSPs and associated reflexes (firing) evoked by stimulating the dorsal roots (3xT). When a motoneuron was at rest this stimulation produced a depolarization that activated the large PICs, which in turn produced a many second long plateau potential and associated firing (LLR), as previously described (Fig 4-8A) (Li et al. 2004a). However, distinguishing the depolarization induced by the EPSPs from the PICs (plateau) was not possible at rest. Thus, to observe the EPSP in isolation we hyperpolarized the cell with a steady bias current to prevent the activation of PICs (which are voltage-dependent; Fig 4-8A, –80 mV). At these hyperpolarized potentials the same dorsal root stimulation evoked an EPSP, up to 1 s long, with two components: the long EPSP responsible for the LPR and the short EPSP responsible for the SPR (as described earlier). The long EPSP was on average 2.76±1.74 mV (peak, at 200–500 ms post stimulation, $n = 10$ motoneurons), and the short EPSP was on average larger 10.85±5.27 mV (peak, at about 5-10 ms), though transient. The 5-$HT_{1B/1D/1F}$ agonist zolmitriptan (1 μM) significantly reduced the long EPSP by 89% (changed by –2.47±2.16 mV) and the short EPSP by 44% (by –4.78±2.49 mV, $n = 10$, $P < 0.05$), as seen in Fig 4-8B. This near elimination of the long EPSP, was accompanied by a loss of activation of PIC-mediated plateaus and

LLRs (Fig 4-8B) measured with the motoneuron at rest, in all cells tested (n = 8). A substantial short EPSP remained in zolmitriptan (Fig 4-3B), and yet there was no plateau or LLR evoked, indicating again that the long EPSP is primarily responsible for triggering the PIC and associated LLR.

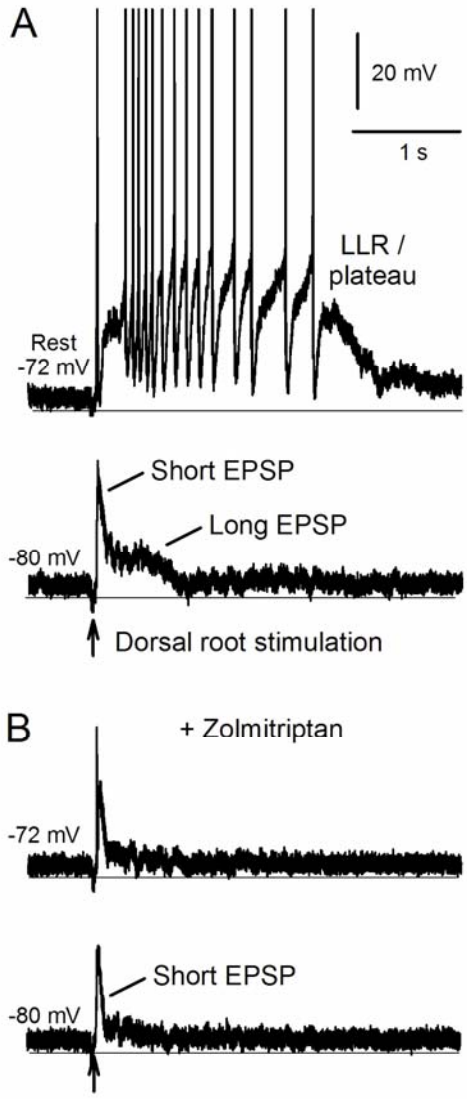

Figure 4-8. Zolmitriptan inhibits polysynaptic EPSPs in motoneurons of chronic spinal rats. (**A**) PIC-mediated plateau potential and sustained firing (LLR) evoked by dorsal root stimulation (0.1 ms pulse, 3xT) in a motoneuron at rest (top plot; -72 mV, without injected current; spikes clipped). With a hyperpolarizing bias current to prevent PIC activation, the same stimulation only evoked a polysynaptic EPSP, with short and long duration components indicated (lower plot; motoneuron at -80 mV). (**B**) In the same motoneuron, zolmitriptan (1 μM) eliminated the plateau and LLR evoked by dorsal root stimulation (top), and inhibited the short and long EPSPs (hyperpolarized, lower plot).

PICs and other motoneuron properties are not affected by zolmitriptan.

When we depolarized a motoneuron with a slow voltage ramp (under voltage-clamp) a large persistent inward current (the PIC) was activated about 10 mV above the resting potential, and produced a marked downward deflection in the recorded current (inward current, Fig 4-9A), relative to the leak current, as previously reported (Li and Bennett 2003). This inward current is what produces the large plateau in Fig 4-8A, when the cell is stimulated at rest (in current clamp), and thus underlies the LLR and spasms (synaptic input activates the dendritic PICs more readily than we can activate the PICs with injected electrode current, and thus the threshold is above rest with intracellular current injection)(Bennett et al. 1998; Li et al. 2004a). Zolmitriptan had no significant effect on the PIC amplitude (9.7±20.5% change, $n = 8$ tested, $P > 0.05$) or onset voltage (Von; Fig 4-9C). Likewise, zolmitriptan had no significant effect on other motoneuron properties, including input resistance (7.6 ± 26.4%, $P > 0.05$), resting potential (-0.9 ± 5.0%) and spike threshold.

Large and long-lasting inhibitory glycinergic synaptic currents are revealed by zolmitriptan.

Because of the large PICs and associated firing that was activated just above rest, it was impossible to evaluate the EPSPs at potentials at or above rest. However, by voltage-clamping at a fixed potential, to prevent firing or PIC activity changes, we were able to evaluate the excitatory postsynaptic currents (EPSCs) at or above rest, as evoked by our standard dorsal root stimulation. At rest there was, as expected, an EPSC (inward, downward current) with short and long duration components, the counterparts of the short and long EPSPs described above (Fig 4-9B, bottom row; seen in $n = 9/9$ motoneurons tested). However, when we voltage-clamped the motoneurons 10 mV above rest (at about the spike and PIC threshold) the same stimulation evoked a large inhibitory postsynaptic current (IPSC; outward current deflection in Fig 4-9B, top row) in addition to EPSCs, in all motoneurons ($n = 9/9$). This IPSC started 2–5 ms after the short EPSC, peaked

at 20–30 ms and then decayed slowly. Thus, this ISPC was positioned between the short and long EPSCs, essentially interrupting them (Fig 4-9B).

Application of zolmitriptan inhibited the EPSCs seen at rest, reducing both the short and long EPSC components in all motoneurons tested (n = 5/5; Fig 4-9D, bottom), as expected. Interestingly, once these EPSPs were reduced by zolmitriptan, a long-lasting IPSC was revealed (Fig 4-9D, top), though the peak of this IPSC was not increased (n = 5/5; Fig 4-10). This long IPSC revealed in zolmitriptan suggests that there is a very large inhibitory synaptic input that is normally counterbalanced by a simultaneously activated large excitatory synaptic input. To confirm this, we applied strychnine (2 μM) to block inhibitory glycinergic inputs, which produced synaptic responses that were always net excitatory, and doubled both the long and short EPSPs (increasing by 5.77±3.22mV and 9.70±6.95, respectively, $n = 5$, $P < 0.05$; not shown), thus producing very large peak EPSPs of about 15 mV. Furthermore, the EPSPs recorded in strychnine were still significantly reduced by zolmitriptan (reduced by 43.7±34.2 and 23.9±7.8% for long and short EPSPs, respectively, $P < 0.05$), suggesting that 5-HT_1 receptor activation (with zolmitriptan) directly reduces the EPSPs, and this action is not secondary to changes in large inhibitory inputs that partially mask the EPSPs.

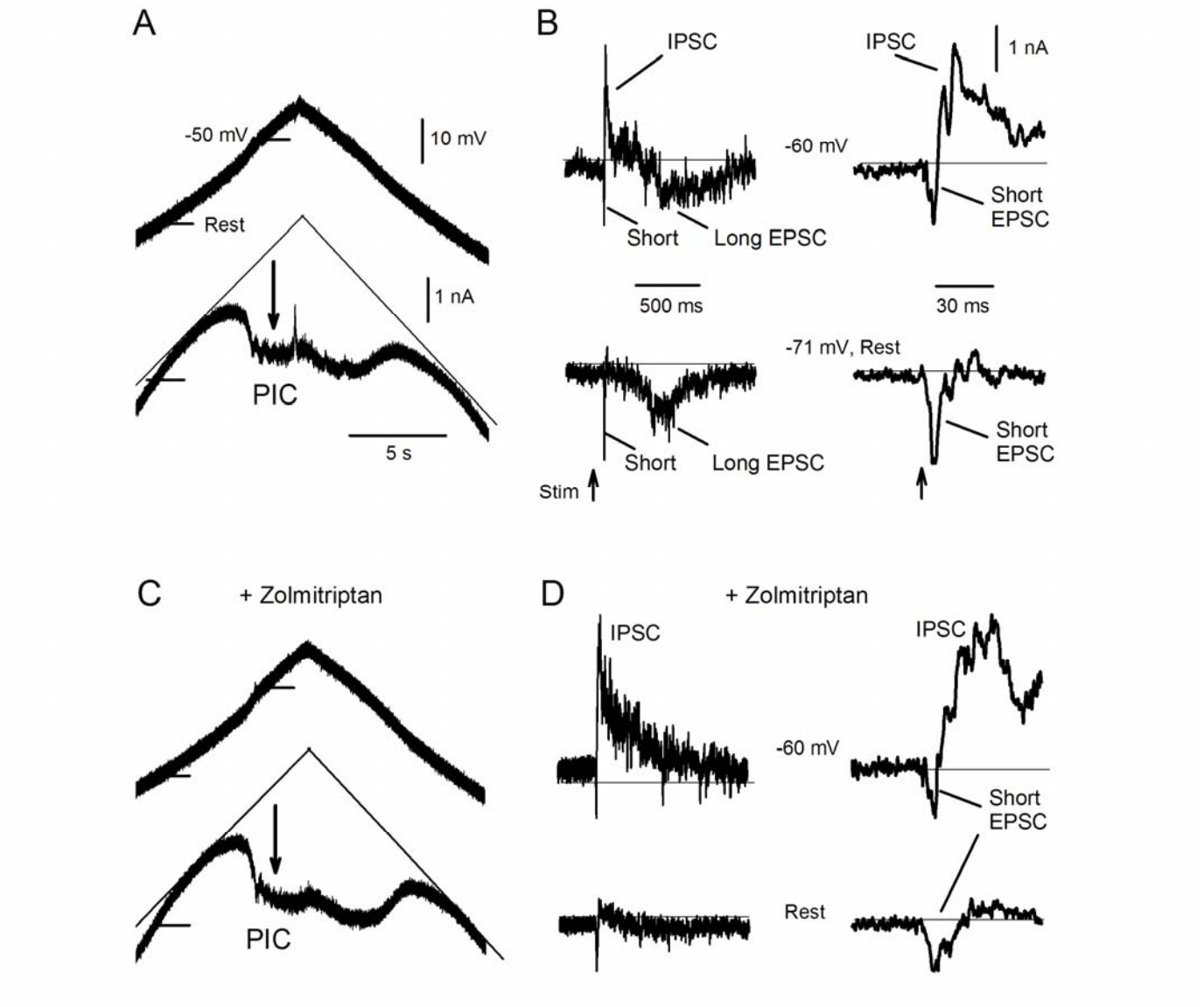

Figure 4-9. Zolmitriptan inhibits excitatory postsynaptic currents but not PICs in motoneurons of chronic spinal rats. (**A, C**) PIC in motoneuron, activated by slowly increasing the membrane potential under voltage-clamp, and quantified at its initial peak, where it produced a downward deflection in the recorded current (at arrow) relative to the leak current (thin line). The PIC was unaffected by zolmitriptan application (1 μM). Dashed marks indicate rest (-71 mV) and -50 mV. (**B**) In the same motoneuron, short and long EPSCs (downward current deflections) and IPSC (upward) evoked by dorsal root stimulation (0.1 ms pulse, 3xT) in voltage-clamp mode at rest (lower trace) and above rest (-60 mV). Expanded time scale shown on right. Note the large IPSC that arises just after the short EPSC at depolarized potentials (-60 mV), which essentially interrupts the EPSCs. (**D**) Zolmitriptan (1 μM) reduced the long and short EPSCs (at rest) and revealed a longer and larger IPSC.

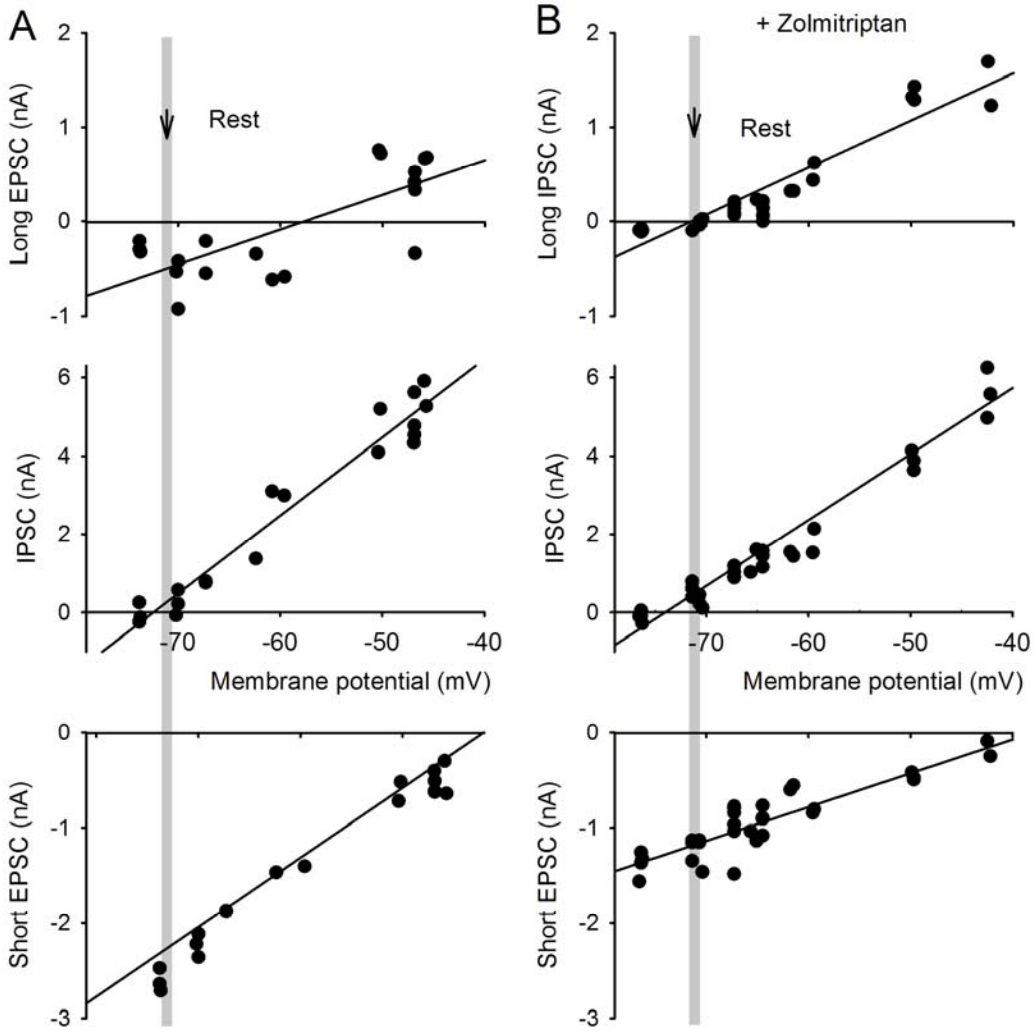

Figure 4-10. Zolmitriptan reverses the long EPSC to a pure IPSC, with reversal potential at rest. (**A**) Top plot: Long EPSC amplitude, measured at 300 ms post stimulation, plotted against the holding potential, for the same motoneuron and stimulation as in Fig 4-9. Linear regression line fit to the data, crosses voltage axis at about –57 mV, the reversal potential for this mixed current. Middle plot: Early peak of IPSC, measured at 20–30ms post stimulation, plotted against holding potential, again during voltage-clamp in Fig 4-9. Linear regression line crosses the voltage axis near rest (gray bar -71 mV), the reversal potential for this pure IPSC. Lower plot: Short latency transient EPSP peak, measured at about 5 ms post stimulus, with reversal potential at about -40 mV. (**B**) Top: Zolmitriptan (1 μM) blocked the long EPSC, revealing a pure long duration IPSC (upward current; measured again 300 ms post stimulation), with a reversal potential near rest (regression line axis crossing). Middle: Zolmitriptan did not affect the early peak of the IPSC measured 20–30 ms post stimulation. Bottom: Zolmitriptan inhibited the short EPSC.

Reversal potential for inhibitory synaptic currents is at the resting membrane potential after injury.

Remarkably, the large inhibitory synaptic input always produced negligible potential changes at rest (n = 9/9), even when the opposing EPSCs were largely eliminated with zolmitriptan (Fig 4-9D; n = 5/5), suggesting that the reversal potential for these inhibitory glycinergic inputs, and their associated chloride currents, was near rest. To verify this, we estimated the Cl^- reversal potential from the reversal potential for the peak of the IPSC, which could generally be measured in isolation because it started abruptly, with a delay relative to short EPSP, and peaked at about 20–30 ms, well after the short EPSC peaked (at 5–10 ms). On average the reversal potential for the peak IPSC was –73.0 ± 3.8 mV, not significantly different from the mean resting potential in chronic spinal rats (–70.9 ± 7.2 mV, $n = 9$, $P > 0.05$), and significantly lower than the spike threshold (by –20.4 ± 4.2 mV, $n = 9$, $P > 0.05$; spike threshold –53.3 ± 3.4 mV). In contrast, the reversal potential for the short EPSC was well above rest (> -50 mV), indicative of a mixed excitatory and inhibitory current.

5-HT_2 receptors do not inhibit the EPSPs.

Application of the 5-$HT_{2A/2B/2C}$ receptor agonist DOI did not significantly affect the EPSPs (short EPSP 10.9±2.0 mV before and 11.5±2.4 mV post DOI; long EPSP 5.82±5.0 before and 6.48±4.3 mV post DOI; $n = 5$; $P > 0.05$). Considering that 5-HT_2 agonists like DOI dramatically facilitate the Ca PICs (Harvey et al. 2006a; Murray et al. 2010b), these EPSPs were recorded in the presence of isradipine to prevent unclamped activation of large dendritic Ca PICs, and as usual recorded at hyperpolarized potentials, in this case to avoid activation of the Na PIC, which is not blocked by isradipine.

Spasms are reduced by zolmitriptan in the awake chronic spinal rat.

In the awake chronic spinal rat, low threshold electrical-cutaneous stimulation of the skin on the tip of the tail evoked many-second long tail muscle spasms that we

record with EMG (Fig 4-11A). These spasms are the counterpart of the long-lasting reflexes seen *in vitro* (Fig 4-1), and accordingly we computed the same short and long polysynaptic reflex components mediated by the EPSPs (SPR and LPR), as well as the long-lasting reflex component mediated by the PIC (LLR). Intrathecal application of zolmitriptan (0.1 mM in 30 μL saline) significantly reduced the SPR and the LPR (by 63.6±8.2% and 63.4±16.0% respectively, $n = 5$, $P < 0.05$), with a clear reduction (notch) in the raw EMG seen during this first half-second period where the EPSPs occur (Fig 4-11B). The reflex over the subsequent 4 seconds (LLR) was also significantly reduced (by 88.2±16.3%, $P < 0.05$), with only transient rather than sustained activity (Fig 4-11B), consistent with a reduction in EPSP, and thus a less effective activation of the PICs that normally produce the spasm. Saline injections had no significant effect on the spasms ($n = 5$, $P > 0.05$).

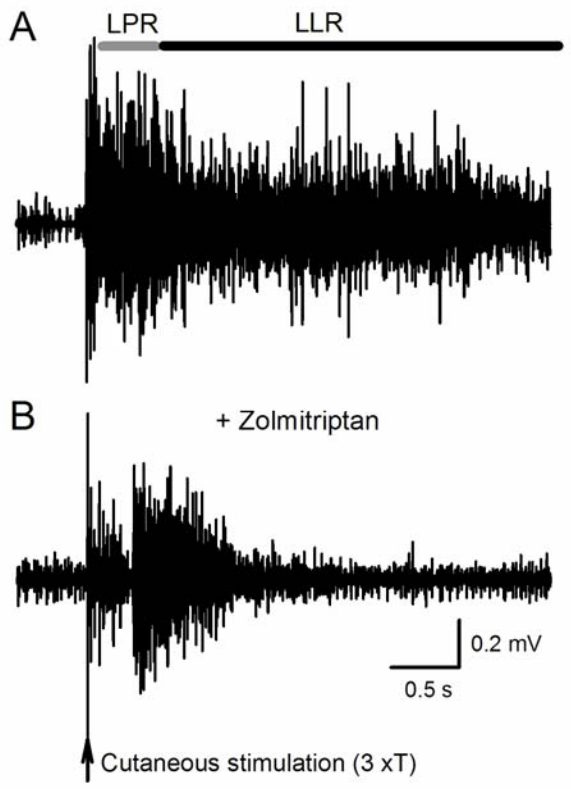

Figure 4-11. Zolmitriptan reduces spasms in the awake chronic spinal rat. (a) Spasm in chronic spinal rat evoked by electrical-cutaneous stimulation of the tail (2xT) and recorded with EMG. (b) Intrathecal application of zolmitriptan (0.1mM in 30 μL saline) reduced the LPR and LLR, quantified at bars.

DISCUSSION

5-HT$_{1B}$ and 5-HT$_{1F}$ receptor agonists have antispastic action.

Our results demonstrate that the long polysynaptic EPSPs (and associated LPR) that trigger spasms after spinal cord injury are inhibited by 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors. Furthermore, the transient but large polysynaptic EPSPs (short EPSPs and associated SPR) are also inhibited by these same receptors. The many second long portion of spasms (LLR) is also inhibited by 5-HT$_{1B}$ and 5-HT$_{1F}$ agonists, both in vitro and in vivo, even though this LLR is ultimately produced by Ca PICs intrinsic to the motoneuron (85% isradipine-sensitive; Fig 4-1A). This is due to an inhibition of the long EPSPs that trigger the Ca PIC, not a reduction in the Ca PIC itself. The short EPSP does not itself trigger the PICs that underlie spasms after spinal cord injury, partly because it is too short to activate Ca PICs (Li and Bennett 2007; Li and Bennett 2003), and partly because it is followed immediately by an inhibitory current that prevents PIC activation (via shunting discussed below). However, the short EPSP may well participate in hyperreflexia and clonus after injury (Ashby and McCrea 1987; Kuhn and Macht 1948), triggered by oscillatory proprioceptive or cutaneous feedback from movement (Bennett et al. 1999). Thus, our finding that 5-HT$_{1B}$ and 5-HT$_{1F}$ receptor agonists like zolmitriptan inhibit short and long EPSPs, demonstrate that such agonists may serve as novel antispastic agents, in controlling the hyperreflexia, clonus and spasms following spinal cord injury. We also found that the 5-HT$_1$ receptor agonist zolmitriptan does not affect overall motoneuron excitability or inhibitory synaptic inputs (IPSCs), both of which are important for general coordinated motor output (Heckman et al. 2005; Hultborn et al. 2004; Schmidt and Jordan 2000). Thus, zolmitriptan has selective antispastic action that should not affect residual motor function, unlike other antispastic agents like baclofen that produce weakness and sedation (Dario and Tomei 2004; Li et al. 2004c). While these conclusions are derived from a sacral spinal rat model of spasticity, they are relevant to humans because the spasticity exhibited in the tail muscles in this rat model (slow onset of hyperreflexia, hypertonus, clonus, muscle

contractures and spasms; Harris; Bennett 1999, 2004) closely mimics the development of the spastic syndrome in humans (Ashby and McCrea 1987; Dietz and Sinkjaer 2007; Kuhn and Macht 1948; Noth 1991). Furthermore, the long EPSPs in this rat model are remarkably similar to those seen in humans with spinal cord injury (Norton et al. 2008).

Antispastic action of 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors agonists is predicted by their binding affinity.

While many agonists bind to both 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors with similar affinity, we found that a number of agonists exhibit sufficient selectivity to definitively demonstrate that both these receptors modulate the spastic reflexes (LPR and SPR) and associated EPSPs. In particular, zolmitriptan has a 10 times greater affinity for 5-HT$_{1B}$ receptors than 5-HT$_{1F}$ receptors, and accordingly its low dose action is likely mediated by 5-HT$_{1B}$ receptors. Furthermore, after selectively blocking that action of 5-HT$_{1B}$ receptors (with SB224289), zolmitriptan no longer has a low dose effect on the spastic reflexes, but does continue to inhibit the reflexes at a high dose, consistent with activation of 5-HT$_{1F}$ receptors. In contrast, LY344864 and methylergonovine are relatively selective to 5-HT$_{1F}$ receptors, exhibiting negligible affinity at 5-HT$_{1B}$ receptors and thus their inhibitory action on the spastic reflexes demonstrates that the 5-HT$_{1F}$ receptor also modulates these reflexes and associated EPSPs. Finally, agonists with negligible affinity for 5-HT$_{1B/1F}$ receptors, such as the 5-HT$_2$ agonist DOI and the 5-HT$_{1/5/7}$ receptor agonist 8-OH-DPAT, exhibit no inhibitory effects on the polysynaptic reflexes (SPR and LPRs), ruling out the involvement of other receptors. 5-HT$_2$ agonists like DOI are somewhat problematic, because they facilitate the PICs and thus increase the LPRs, potentially masking possible 5-HT$_2$ receptor mediated EPSP inhibition. However, we found that DOI continues to lack inhibitory effects on the SPRs or EPSPs after blocking the Ca PICs, ruling out the involvement of 5-HT$_2$ receptors in inhibiting the EPSPs. It is odd that DOI did not increase the SPR or EPSP, via its large facilitation of the fast activated Na PIC (Harvey et al. 2006a), though this might be explained by general synaptic shunting that we

discuss below or by increases in postsynaptic inhibition that can be caused by 5-HT receptor activation (Jankowska et al. 2000).

As further evidence for the involvement of just 5-HT_{1B} and 5-HT_{1F} receptors, we demonstrated that the binding affinity of agonists to 5-HT_{1B} and 5-HT_{1F} receptors is highly correlated with our measured potencies of these agonists at inhibiting the spastic reflexes, whereas the binding affinity at other receptors was not related to the potency. The lack of correlation between affinity and potency for 5-HT_{1D} receptors is especially important in ruling out the involvement of the 5-HT_{1D} receptor, because this receptor is so similar to the 5-HT_{1B} receptor, activated by many of the same agonists (Table 4-1). We also showed that the reflex potencies could be quantitatively predicted from receptor binding affinity for 5-HT_{1B} and 5-HT_{1F} receptors, whereas this was not the case for other receptors, including 5-H_{1D} and 5-HT_{1E} receptors. Specifically we found that reflex potencies are consistently one log unit less than the agonist affinity for 5-HT_{1B} and 5-HT_{1F} receptors, which means that dose (EC50) needed to inhibit the reflexes is 10 times the dose at which agonists bind to these receptors (Ki) in isolated cell systems (see Results). Remarkably, both the 5-HT_{1B} and 5-HT_{1F} receptors exhibit the same factor of 10 (one log unit) greater effective dose (EC50) compared to the Ki value as we have also shown for the 5-HT_{2B} and 5-HT_{2C} receptors in their modulation of the PICs. Thus, this factor of 10 is not only independent of the agonists tested, but also independent of the receptor type, or the response system (EPSPs vs PICs). We therefore suggest that it simply reflects diffusion barriers in our whole spinal cord preparation that prevent the full applied dose from reaching the receptors. Other factors may affect the functional potency (EC50) verses receptor binding affinity relation (Boess and Martin 1994; Egan et al. 2000; Porter et al. 1999), including the receptor saturation, intracellular signalling (cAMP) and saturation in EPSPs, though these are specific to each receptor and signalling system and thus unlikely involved in the invariant potency-affinity relation.

Possible location of 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors on group II afferents and interneurons?

While our results indicate that the EPSPs that trigger spasms are inhibited by 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors that are *not* located postsynaptically on motoneurons (zolmitriptan does not affect motoneurons), the site of this inhibition is uncertain. These receptors could be on low threshold group II type afferents terminals, which are likely the main afferents we stimulated (including cutaneous and muscle afferents; see Methods), and consistent with previous reports 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors on various sensory afferents (Millan 2002). An intriguing possibility is that these 5-HT$_1$ receptors that inhibit EPSPs/spasms may specifically act by inhibiting transmission to spinal interneurons that receive group II afferent input and are modulated by 5-HT$_1$ receptors (pre or post-synaptically Dougherty et al. 2005; Jankowska and Hammar 2002). These interneurons could include commissural interneurons with group II input that have been implicated in coordinated left-right movements, such as rhythmic locomotion (Hammar et al. 2007; Jankowska 2008; Schmidt and Jordan 2000), consistent with the prominent left-right coordination of the spasms we have previously reported in the sacral spinal rat (Bennett et al. 2001; Bennett et al. 2004; Li et al. 2004b). However, it remains to be resolved why we failed to detect effects of 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_3$ and 5-HT$_7$ receptors on reflex transmission in the spastic rat, even though these receptors have previously been suggested to play a prominent role in modulating spinal interneuronal circuits, including interneurons involved in group II sensory transmission (see Introduction and Dougherty et al. 2005; Hammar et al. 2007; Jankowska et al. 1994; Jordan et al. 2008; Schmidt and Jordan 2000).

Gi protein coupled receptors that decrease cAMP have antispastic action.

5-HT$_1$ type receptors activate Gi coupled proteins that inhibit intracellular cAMP production by inhibiting adenylate cyclase activity (Boess and Martin 1994), and accordingly in many systems are inhibitory, though there are some excitatory actions of these receptors, even on motoneurons (Perrier et al. 2003). The EPSPs

in our chronic spinal rats are facilitated by raising cAMP with forskolin, and thus the EPSPs are regulated by cAMP, and our observed antispastic action of 5-HT_{1B} and 5-HT_{1F} receptors (inhibiting EPSPs) is likely mediated by inhibiting cAMP. The involvement of this Gi coupled pathway in regulating spasms is also consistent with the known antispastic action of other Gi coupled receptors, including alpha2 adrenergic receptors and $GABA_B$ receptors that are respectively activated by tizanidine and baclofen, two classic antispastic drugs (Dario and Tomei 2004; Li et al. 2004c).

Not all 5-HT receptors exhibit constitutive activity after spinal cord injury.
The 5-HT_{1B} receptor is known to exhibit substantial activity in the absence of 5-HT (constitutive receptor activity and associated cAMP production), and this is potently inhibited by the inverse agonist SB224289, in isolated cloned receptor cell systems (Selkirk et al. 1998). Thus, our finding that SB224289 has no effect by itself, is somewhat unexpected, and suggests that the native 5-HT_{1B} receptor exhibits less constitutive activity than predicted from the cloned system. As a positive control, we found that SB224289 antagonized 5-HT_{1B} receptor agonist action, and thus we used it at an appropriate dose, and our negative finding is likely due to a genuine lack of constitutive activity. We could not test for constitutive activity in 5-HT_{1F} receptors, due to a lack of availability of selective inverse agonists to this receptor. The other antagonists tested also had no detectable effect by themselves, though these are not inverse agonists (Price et al. 1997), and their lack of action simply confirms our previous finding that there is no functional residual 5-HT in the chronic spinal rat (Murray et al. 2010a).

In contrast to the 5-HT_{1B} receptor, over time after injury 5-HT_{2C} receptors become constitutively active, and helps produce the dramatic increase in the PIC that leads to motor recovery (Murray et al. 2010a). It is intriguing that one 5-HT receptor compensates for lost 5-HT, whereas others do not (5-HT_{1B}). A common underlying functional pattern that emerges is that both the adaptation in the 5-HT_2 receptor and lack of adaptation in the 5-HT_1 receptor leads to increased spinal

cord excitability (larger PICs and EPSP) and associated activity in motoneurons (spasms). Thus, it could be that the receptors are regulated in an activity-dependent manner; although the mechanisms for this remain unknown, it may explain why intensive treadmill training (activity) can reduce spastic activity during walking (Gorassini et al. 2009).

Simultaneous activation of excitatory and inhibitory synaptic inputs.

Our finding that cutaneous reflexes result from the *simultaneous activation of very large excitatory and inhibitory synaptic inputs* may help further explain the changes in these reflexes after injury. We suggest that only subtle changes in the balance of these large synaptic inputs may contribute to the shift to net excitatory reflexes after injury, compared to before; these changes could result from loss of descending inhibition (5-HT, see Introduction), as well as cellular changes in inhibitory currents (Boulenguez et al. 2010). In normal intact humans and rats, cutaneous stimulation, like we used, predominantly evokes long duration inhibitory reflexes and decreases ongoing muscle activity (Bennett et al. 2004; Norton et al. 2008; Schmidt and Jordan 2000). This stimulation does evoke a transient excitation (like SPR), but this is interrupted by a long period of inhibition, that can be followed by a further excitatory reflex, consistent with there being EPSPs that are interrupted by an overriding IPSP (Norton et al. 2008). After spinal cord injury, the net synaptic responses are excitatory (at rest), but there still remains a prominent inhibitory synaptic input to motoneurons that is: 1) seen at depolarized potentials (Fig 4-9), 2) peaks shortly after the EPSP onset (interrupting the excitation), 3) enhanced by blocking opposing EPSCs with zolmitriptan and 4) reduced by eliminating glycine-mediated chloride currents with strychnine, revealing very large net EPSPs (~15mV). While at the most depolarized levels we tested (-50 mV) the long duration synaptic responses are outward (net IPSCs, Fig 4-10), motoneurons do not on average ever reach such depolarized levels, because the potential is limited to being well below the spike

threshold (about –53 mV) by the spike afterhyperpolarization during firing (Li et al. 2007; Li et al. 2004a). Thus, the mixed EPSC and IPSCs seen just above rest are likely most relevant to normal motor function. For example, the slightly delayed onset of the inhibitory synaptic input likely explains the pause in firing often seen after the first spike at the start of spasms (Fig 4-8A)(Li et al. 2004a), with an IPSP interrupting otherwise depolarizing EPSPs and PICs. Our discovery of these large inhibitory inputs opens up the intriguing possibility that we may be able to terminate the PIC and spasms by an appropriate cutaneous stimulation, especially if EPSPs can be even slightly reduced with drugs like zolmitriptan.

Shunting limits EPSPs and spasms.

Functionally, the action of large mixed inhibitory and excitatory synaptic inputs is to substantially increase the overall membrane conductance, thus limiting (*shunting*) the action of all other currents, including intrinsic PICs (Bennett et al. 1998; Berg et al. 2007; Berg and Hounsgaard 2009; Rekling et al. 2000), making them relatively negligible during the synaptic input. Such synaptic shunting may explain why PICs (and spasms) take up to a second to turn on fully when activated by synaptic inputs (Fig 4-9A) (Gorassini et al. 2004; Li et al. 2004a), even though Ca PICs can be turned on much more rapidly with intracellular current injection (Li and Bennett 2007): the shunting from the EPSPs/IPSPs (lasting up to 1 sec) may prevent the full PICs activation until the synaptic input ends. Furthermore, synaptic shunting may explain why the polysynaptic reflex inputs, especially the SPR, are so resistant to the large increases in PICs induced by 5-HT_2 receptor agonists or even calcium channel blockers (isradipine). Possibly, the two distinct short and long EPSPs may actually be mediated by the same synaptic input, but appear separated because they are interrupted by the large inhibitory synaptic input that is activate just after the short EPSP; this inhibitory input must limit the EPSPs by shunting, especially at rest where these inhibitory inputs produce no net hyperpolarization (reversal potential).

Reversal potential for inhibitory synaptic inputs is near rest after injury.
Recently, it has been shown that inhibitory chloride currents (IPSCs) are reduced slowly over time after injury (in chronic injury), due to a reduction in the potassium-chloride co-transporter (KCC, Boulenguez et al. 2010). Such a reduction in the chloride currents might further explain the large net excitatory synaptic inputs we observe, by shifting the balance more in favor of EPSPs over IPSPs, though this does not itself explain the large EPSPs seen acutely after injury (Li et al. 2004a). Based on data from neonatal rats, Boulenguez et al. (2010) suggest that the KCC is so impaired following spinal cord injury that the reversal potential for Cl^- is shifted upward from about –75 mV to well above rest (> -70 mV), and thus at rest, there are net depolarizing responses to synaptic chloride current inputs that normally produce inhibitory hyperpolarizing responses (reversal of IPSPs to depolarizing). In adult animals the KCC is also impaired after spinal cord injury (Boulenguez et al. 2010), though we did not find evidence for outright reversed (depolarizing) IPSPs at rest. However, our data does suggest that the reversal potential for chloride is at rest (no net inhibitory current), and this may be more depolarized than in normal rats, considering that previous reports indicate that the Cl^- reversal potential is negative to rest (Boulenguez et al. 2010). Blockage of glycine receptors with strychnine reveals a very large EPSP at rest, suggesting that while no net change in potential is induced by glycine receptor activation at rest (at reversal potential), the receptor still induces a marked shunting of the EPSP that limits its size, by preventing excitatory current from reaching the soma (or our electrode).

Summary and clinical implications.
In summary, activity in 5-HT_1 receptors inhibits reflex transmission, and thus a loss of 5-HT with injury contributes to an acute loss of inhibition over reflex transmission, and this contributes to the classic disinhibition of reflexes observed acutely after injury. After injury, the 5-HT_{1B} and 5-HT_{1F} receptors are still capable of inhibiting the reflexes when activated by exogenously applied agonists, but these receptors remain inactive because of a lack of endogenous 5-HT in

transected rats and a lack of observed constitutive activity. In contrast, other receptors like the 5-HT_{2C} receptor become constitutively active after injury, leading to a recovery of their normal function (facilitating motoneuron PICs). Thus, reflex transmission remains chronically elevated (disinhibited) after injury, with large net EPSPs. These EPSP are of adequate duration (seconds) to trigger the PICs in motoneurons that ultimately cause the sustained motoneuron firing that underlies spasms. Thus, reactivating 5-HT_{1B} and 5-HT_{1F} receptors after injury offers a new means of selectively controlling spasticity, by reducing EPSPs, without affecting PICs that are critical for motor function. This can be done with clinically available 5-$HT_{1B/1F}$ agonists like the triptans (zolmitriptan), which are currently a first-line treatment for migraines (Millan 2002). In the long run, understanding why 5-HT_{1B} receptors do not become constitutively active after injury, unlike the 5-HT_{2C} receptors, may further help with antispastic therapy and general recovery of motor function after injury, especially if this involves activity-dependent receptor plasticity that can be modulated by intensive rehabilitative training.

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Chapter 5: Discussion

DISCUSSION

5-HT$_{2B}$ and 5-HT$_{2C}$ receptors modulate spasms

The results of this thesis demonstrate that both 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors modulate motoneuron excitability (Ca PICs) and play an essential role in the spontaneous recovery of motoneuron function after SCI. In particular the 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors become constitutively active after chronic spinal cord injury, a powerful mechanism for spinal motoneurons to compensate for the loss of 5-HT. Unfortunately, these constitutively active 5-HT$_2$ receptors and associated PICs also lead to uncontrolled motoneuron firing and associated muscle spasms (LLRs), which emerge over the weeks after injury. What remains unknown; however, are the mechanisms that lead to the up-regulation of the constitutively active isoform of the receptor with chronic injury, and what role they play in uninjured spinal cords. Does constitutive activity develop because 5-HT is no longer present and the receptors are not being activated, as suggested by other systems where 5-HT is lost (Gurevich et al. 2002)? If so, can constitutive activity be suppressed by a chronic administration of 5-HT and 5-HT$_2$ agonists, or by novel therapeutic strategies, such as regeneration of 5HT axons or release of 5-HT from engineered stem cells? A previous study in the brainstem suggests that chronic 5-HT agonist administration will reverse the constitutive activity (Gurevich et al. 2002), suggesting that controlling constitutive activity and associated motor functions may indeed be possible. However, at this point it is not clear how this will help with recovery of function, unless exogenously applied 5-HT levels can be well modulated according to the functional requirements.

Inverse agonists for treatments of spasms

Inverse agonists are a specific class of drugs which target constitutive activity and may be good candidates for antispastic medications. Cyproheptadine, a 5-HT$_2$ receptor inverse agonist, is typically used as an antihistamine, but is also used to promote appetite and to treat migraines via 5-HT effects (Kardinal et al. 1990; Klein and Galant 1980; Mylecharane 1991; Prelusky et al. 1997; Rao et al. 2000).

Cyproheptadine has been used to treat spasticity in the past, but due to adverse side effects, such as drowsiness and weakness, it is not commonly used (Zafonte et al. 2004). Perhaps other inverse agonists that specifically target the 5-HT_{2B} and 5-HT_{2C} constitutively active receptors, such as the 5-$HT_{2B/2C}$ inverse agonist SB206553, will prove to be more effective and can be used to treat this disorder. Although some inverse agonists have been used clinically (cyproheptadine), their long-term effects on constitutively active receptors (like those resulting from chronic spinal cord injury) are unknown. Perhaps chronic use of inverse agonists increases constitutive activity in receptors over time. This might be suggested by the increased expression of the 5-HT_{2C} receptors on the surface of the cell membrane after chronic administration of inverse agonists (Chanrion et al. 2008). An increase in constitutive activity with chronic inverse agonist application could lead to dangerous withdrawal from these drugs, where increased expression of 5-HT_{2C} receptors could lead to increased motoneuron excitability and extremely high spasticity.

Spasms and motor function recovery

It is important to recognize the fine balance between spasticity and motor recovery. The emergence of muscle spasms results from an increase in motoneuron excitability lacking immediately following injury, which may or may not translate into the recovery of motor function (i.e. walking) in all cases. In our staggered hemisection animal model the constitutively active 5-$HT_{2B/2C}$ receptor activity (and associated PICs) plays an essential role in the recovery of motor function after SCI enabling the rat to regain hindlimb control and to voluntarily initiate walking (although fore-limb to hind-limb coordination was not as strong). Blocking the constitutively active 5-HT_2 receptors, will eliminate spasms but will also block motor control and subsequently inhibit walking. Thus, in incomplete injuries where some locomotor function is retained, the more suitable approach may be to eliminate the EPSP (the spasm trigger) directly by activating the inhibitory actions of the 5-$HT_{1B/1F}$ receptors, instead of eliminating the PICs by blocking 5-HT_2 receptors. This approach focuses on blocking the activation of the

Ca PICs and spasms, eliminating the side effects that occur from using drugs that block the 5-HT_2 receptors directly, and consequently effect motor control negatively.

Loss of descending inhibition

With chronic SCI the re-emergence of PICs appears to be a positive adaptation of the spinal motoneurons as it allows the motoneurons to regain excitability. But, the lack of descending inhibition (disinhibition) to terminate the PICs underlying spasms makes their re-emergence non-functional. Perhaps then, the solution is to promote recovery of inhibitory control of spinal motoneurons to work in conjunction with the recovered PICs. Boulenguez et al (2010) recently presented evidence for a previously unknown mechanism underlying disinhibition after SCI, involving a reduction in the potassium-chloride transporter (KCC2) which disrupts the chloride homeostasis (higher neuronal intracellular chloride) and increases excitability of spinal motoneurons (Boulenguez et al. 2010; Boulenguez et al.). Brain-derived neurotrophic factor (BDNF) has been implicated in both down and upregulation of KCC2 after neuronal traumas (Coull et al. 2005; Miletic and Miletic 2008; Rivera et al. 2002) and may also be involved in the disinhibition that is present after spinal cord injury (Boulenguez et al 2010). Thus, increasing the expression of KCC2, whether via manipulating BDNF or by other means may help to recover inhibitory control of spinal neurons. Much success has also come from rehabilitative locomotor training and treadmill training which is based on the hypothesis that use-dependent mechanisms can functionally 'remodel' spinal circuits in the absence of supraspinal inputs (Courtine et al. 2009). In fact, reorganization of propriospinal circuits and spared descending fibers that lead to functional recovery does occur with locomotor training after an incomplete SCI (Cote and Gossard 2004; Courtine et al. 2008; Engesser-Cesar et al. 2007; Gorassini et al. 2009; Petruska et al. 2007), however little evidence for similar plasticity after complete SCI has been shown (Harkema 2008; Ichiyama et al. 2008; Kubasak et al. 2008). Thus, locomotor training is very important in regaining function below an injury and perhaps a combination of training and

pharmacological methods; such as blocking PICs and long EPSPs, or increasing expression of KCC2, would be an additional means of regaining motor function after incomplete and complete SCI.

Determining the ideal combination of selective 5-HT agonists and antagonists

One of the main goals of this thesis was to determine which 5-HT receptors were involved in spasms after SCI via the re-emergence of PICs and the appearance of a long EPSP that activates them. A full block of the 5-$HT_{2B/2C}$ receptors dramatically reduces the PICs so much that synaptic amplification of even short latency reflexes can be impaired, and brief postural corrections (in response to tail perturbations in the rat) are not possible. In addition, in animals that were able to recover partial or total motor function recovery in the absence of 5-HT after chronic injury, motor function was severely impaired after blocking the 5-HT_2 receptors with inverse agonists. Perhaps blocking just the 5-HT_{2B} or just the 5-HT_{2C} receptor activity will provide adequate spasm reduction, and still allow for the preservation of postural tone and short latency reflexes, and ultimately the control of motor function. The long-term effects of using inverse agonists to treat spasms are unknown (see above), and the efficacy may be reduced if receptors are up regulated. In contrast, the lack of constitutive activity in 5-HT_{1B} and likely 5-HT_{1F} receptors, which leads to exaggerated EPSPs can be treated with 5-$HT_{1B/1F}$ agonists. However, again the long-term use of these drugs is uncertain, though we do know that they have good efficacy in chronic management of migraines (Millan 2002).

Thus, the combination of 5-$HT_{1B/1F}$ agonists and 5-HT_{2B} or 5-HT_{2C} blockers (or both) may also prove useful and effective. Finding the ideal combination of selective 5-HT agents that both maximize function and minimize spasticity is something that needs to continue to be explored, as it seems unlikely that blocking the action of only one receptor is sufficient for treating the problem.

Future directions

There are many other 5-HT receptors that likely play a role in spasticity, and thus it is important to study these receptors in the future in order to provide an even better combination of 5-HT agonists and antagonists to treat this disorder. Notably, the 5-HT_6 receptors appear to be important in depolarizing the resting membrane potential and so may well be involved in the maintenance of good postural tone by increasing a basal level of activity (Murray and Bennett unpublished data). After chronic spinal cord injury motoneurons only exhibit subtle changes in their passive resting properties (Button et al. 2008; Harvey et al. 2006) and thus it is unlikely that the Gs-coupled 5-HT_6 receptor becomes constitutively active. Thus, restoring 5-HT_6 receptor activity with agonists may help make motoneurons more excitable, and compensate for any loss of excitability that arises from treating spasms with 5-HT_2 receptor inverse agonists or other antispastic drugs, thus helping to maintain muscle tone and posture while treating spasticity. In general, in order to target and treat spasms via the serotonin system, a comprehensive understanding of the role of all 5-HT receptors is very important in developing the safest and most effective treatment, and is an area of interest for further study.

This thesis focused on spasticity resulting from low sacral spinal transections and axial tail muscle function, with a small focus on rats with thoracic SCI. The sacral spinal rat model used is extremely useful in understanding similar axial muscle spasms of the back muscles in humans (Bennett et al. 1999), in addition the sacral spinal rats develop symptoms of spasticity in the tail muscles that are similar to those seen in limb muscles of humans with spinal cord injury (Bennett et al. 1999). In the future, in addition to exploring the roles of the remaining 5-HT receptors, I would like to evaluate further the higher injuries that affect leg function, spasticity and recovery of locomotion, so as to be able to better understand the needs of humans with higher injury and more accurately determine an effective treatment with little to no side effects. In order to be able to do this, we first need to consider the balance between minimizing or inhibiting spasms

and maintaining motor function.

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