Polysynaptic excitatory postsynaptic potentials that trigger spasms after spinal cord injury in rats are inhibited by $5\text{-HT}_{1B}$ and $5\text{-HT}_{1F}$ receptors.

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ABSTRACT

Sensory afferent transmission and associated spinal reflexes are normally inhibited by serotonin (5-HT) derived from the brainstem. Spinal cord injury (SCI) that eliminates this 5-HT innervation leads to a disinhibition of sensory transmission and a consequent emergence of unusually long polysynaptic excitatory postsynaptic potentials (EPSPs) in motoneurons. These EPSPs play a critical role in triggering long polysynaptic reflexes (LPRs) that initiate muscles spasms. Here we examined which 5-HT receptors modulate the EPSPs and whether these receptors adapt to a loss of 5-HT after chronic spinal transection in rats. The EPSPs and associated LPRs recorded in vitro in spinal cords from chronic spinal rats were consistently inhibited by 5-HT$_{1B}$ or 5-HT$_{1F}$ receptor agonists, including zolmitriptan (5-HT$_{1B/1D/1F}$) and LY344864 (5-HT$_{1F}$), with a sigmoidal dose-response relation, from which we computed the EC50 (50% inhibition) and potency (–log EC50). The potencies of 5-HT receptor agonists were highly correlated with their binding affinity to 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors, and not other 5-HT receptors. Zolmitriptan also inhibited the LPRs and general muscle spasms recorded in vivo in the awake chronic spinal rat. The 5-HT$_{1B}$ receptor antagonists SB216641 and GR127935 and the inverse agonist SB224289 reduced the inhibition of LPRs by 5-HT$_{1B}$ agonists (zolmitriptan). However, when applied alone, SB224289, SB216641 and GR127935 had no effect on the LPRs, indicating that 5-HT$_{1B}$ receptors do not adapt to chronic injury, remaining silent, without constitutive activity. The reduction in EPSPs with zolmitriptan unmasked a large glycine-mediated inhibitory postsynaptic current (IPSC) after SCI. This IPSC and associated chloride current reversed at -73 mV, slightly below the resting membrane potential. Zolmitriptan did not change motoneuron properties. Our results demonstrate that 5-HT$_{1B/1F}$ agonists, like zolmitriptan, can restore inhibition of sensory transmission after SCI without affecting general motoneuron function and thus may serve as a novel class of antispastic drugs.
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 may 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 syndrome 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 a pronounced muscle spasticity syndrome 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). In both rats and humans, these long EPSPs initiate long-lasting muscle contractions (defined here as 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, in part 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. 2010). 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. 2010), due to
constitutive receptor activity (activity in the absence of 5-HT or any other ligand)(Murray et al. 2010). This
spontaneous receptor activity leads to the re-emergence of the large PICs that make the motoneurons
permanently excitable (Harvey et al. 2006b; Murray et al. 2010). 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 postsynaptic inhibitory currents (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.

Thus, three general 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$_2$ (and alpha1 adrenergic) receptor activity, and 3) a loss of postsynaptic inhibition over motoneuron activity. In previous papers, we examined how spasms can be controlled by reducing PICs (Murray et al. 2010; Murray et al. 2011). The present paper examines how we can control spasms by reducing EPSPs by replacing lost 5-HT innervation with 5-HT$_1$ receptor agonists.
METHODS

Recordings were made from motoneurons and associated ventral roots of the sacrocaudal spinal cord of spastic adult rats with chronic spinal cord injury (3.5 – 5 months old). Adult female rats were transected at the S2 sacral level at about 2 months of age (adult rat), and recordings were made at a time after their affected muscles became spastic (1.5–3 months after injury), as detailed previously (Bennett et al. 1999; Bennett et al. 2004). Recordings were made from the whole sacrocaudal spinal cord that was removed from the rat with an S2 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. Control, age matched, normal rats were also studied in vitro. 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 S4 and caudal C1 ventral roots and the C1 dorsal roots. After 1.5 hours in the dissection chamber (at room temperature), the cord was transferred to a recording chamber containing normal ACSF (nACSF) maintained near 23°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 nACSF 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 Ca₁ 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 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 rectified to allow averaging, and then three components of the ventral root reflexes were quantified: 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 15 min intervals, and 5-HT receptor agonists were added immediately after each recording, giving them time to fully act by the next recording session (15 mins later). Cumulative dose-response relations were computed by increasing agonist doses at these 15 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 > 30 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 Cα1 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$_2$PO$_4$, 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, methylsergide, 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, methylsergide, 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 reflex threshold [T]; 6 spasms evoked at 10 s intervals for a trial; trials repeated at 15 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 isoflurane 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, three 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 \(-\log(\text{EC50})\) was used to quantify the drug *potency*: \(\text{pEC50} = -\log(\text{EC50})\).

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 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 \(^3\text{H}\)-5-HT, from the receptor expressed in isolated cells. Binding affinity is computed as \(\text{pKi} = -\log(\text{Ki})\) (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 \(^3\text{H}\)-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).
RESULTS

Polysynaptic reflexes reflect underlying EPSPs.

To study the EPSPs that underlie spasms 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 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 1A, inset), and sometimes also had an earlier monosynaptic reflex component (not present in Fig1A, 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 in intracellularly recorded motoneurons at rest (Fig 1B). The short EPSP was seen without interference from spiking (or the Ca PIC) when the motoneuron was hyperpolarized with a bias current (Fig 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 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 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 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 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 1B) or nearly eliminated by blocking the Ca PICs with isradipine (Fig 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%).

5-HT<sub>1B</sub> and 5-HT<sub>1F</sub> receptor activity inhibits the LPR and associated spasms.

Application of the selective 5-HT<sub>1B/1D/1F</sub> receptor agonist zolmitriptan inhibited the LPR, with increasing doses producing larger responses over about a 100-fold change in dose (Fig 2 and Table 2). This dose-response relation was well approximated by a sigmoidal curve (Figs 2C), from which we computed: 1) the agonist dose to produce 50% maximal inhibition (EC50, Fig 2D), 2) agonist potency (pEC50 = –log EC50) and 3) agonist efficacy (maximal inhibition, reported relative to control LPR size; Fig 2C). For zolmitriptan, the EC50 was about 100 nM with a corresponding potency of about 7 (–log 100x10<sup>-9</sup> M; Table 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 2), suggesting that the associated long EPSP was
also reduced. Zolmitriptan also significantly decreased the LLR (Fig 2D; 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.

Application of agonists with a relatively high affinity for 5-HT\(_1\) receptors, compared to 5-HT\(_2\) receptors (5-CT, EMD386088), likewise significantly inhibited the LPR with a simple sigmoidal dose-response relation (significant efficacy; Table 2). Less selective 5-HT\(_1\) agonists (including alpha-methyl-5-HT, BW723C86, methylergonovine, and 5-HT itself) with relatively high affinity for 5-HT\(_2\) receptors, also inhibited the LPR (Table 2), but this inhibition was partly obscured by their activation of 5-HT\(_2\) receptors (Fig 3), which we have previously shown increases PICs and associated reflexes (Murray et al. 2010; Murray et al. 2011). 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 \textit{biphasic} response. That is, at low doses the agonist increased the long lasting reflexes, including the LPR and LLR (Fig 3A-C). This low-dose response was especially prominent in the entirely PIC-mediated LLR (see sigmoid curve fit to ascending phase in Fig 3B, and low EC50), consistent with 5-HT\(_2\) receptor-mediated facilitation of the PIC, as described previously (Murray et al. 2010; Murray et al. 2011). As successively higher doses were applied, the reflexes eventually reached a peak (peak reflex), after which they decreased with increasing dose (\textit{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 3A,C). As seen in Table 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 \( \mu \)M or the selective 5-HT\(_2\) antagonists like RS127445, 3\( \mu \)M), 5-HT produced a purely
inhibitory action, with similar dose-response relation to that obtained without the block (Fig 3D and Table 2). This also shows that the inhibitory action of these non-selective agonists is mediated by 5-HT1 and not 5-HT2 receptors.

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-HT1B receptors (Ki > 400), whereas it antagonizes/binds most other 5-HT receptors with high affinity (Ki < 500nM; except 5-HT3 and 4 receptors) (Boess and Martin 1994). Thus, the inhibition of the LPR by 5-HT seen after pre-treatment with methysergide (Fig 3D, Table 2) suggests that 5-HT1B receptors specifically inhibit the LPR, though this does not rule out additional involvement of other 5-HT1 receptors blocked by methysergide (5-HT1F).

Prior application of the selective 5-HT1B receptor antagonist SB224289 or the selective 5-HT1B/1D receptor antagonist SB216641 significantly reduced the inhibitory action of both selective (zolmitriptan) and non-selective (5-HT; inhibitory phase) 5-HT1 agonists on the LPR (Table 2 and Fig 2C). 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 2), indicating that the 5-HT1B 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 2C and Table 2). This may be explained by the activation of the 5-HT1F 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-HT1F, compared to the 5-HT1B receptor (Table 1).

Consistent with the possible involvement of 5-HT1F receptors in regulating the LPR, we found that the selective 5-HT1F agonist LY344864 and non-selective 5-HT1F agonists that have negligible affinity for 5-HT1B receptors (e.g. methylergonovine, alpha-methyl-5-HT; Table 1) inhibited the LPR (Table 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 1), also inhibited the LPR (Table 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 1) produced no significant inhibition of the LPR (Table 2 and 3), 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 2 and 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 \( \mu \)M), due to a facilitation of the underlying Ca PIC, as previously reported for the LLR (Murray et al. 2011). 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 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 net inhibitory action of 5-HT_2 receptors on the EPSP underlying the LPR.

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 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 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 unity slope (dashed line; pEC50 = pKi + C) as shown in Fig 4A. Likewise, the potency was also significantly correlated with the agonist affinity for 5-HT_{1F} receptors (Fig 4B), with close to a unity 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 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 correspond to 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 (Murray et al. 2011) required higher drug doses (EC50) to get responses, and thus the potency (pEC50 = −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 (see Discussion). 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\textsubscript{1B} and 5-HT\textsubscript{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, \( n = 6 \) and 5 respectively; dashed unity slope lines in Fig 4A and B).

Also, the difference \( pEC50 – pKi \) (relative potency) was, as hypothesized, highly invariant across all agonists tested at these receptors, on average \(-1.15±0.25\) and \(-1.23 ± 0.27\) for 5-HT\textsubscript{1B} and 5-HT\textsubscript{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 3). Remarkably, this relative potency value of about \(-1\) has been seen for two other functional receptors in our preparation (Murray et al. 2011), and so appears to be an invariant across many or all receptors, in part 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).

In contrast to the invariant relative potency for 5-HT\textsubscript{1B/F} 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 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\textsubscript{1D} receptors was < \(-2\) (Table 3), well outside of the confidence interval, suggesting that its EC50 is too high to be predicted from the Ki for zolmitriptan at the 5-HT\textsubscript{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 3), again suggesting that the 5-HT$_{1D}$ receptor is not involved in modulating the LPR. Sometimes by chance a drug (e.g. EMD386088) had a similar affinity for the 5-HT$_{1B}$ receptor and another receptor (e.g. 5-HT$_{1D}$; Table 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$_{1B/1F}$ receptors, indicating that no receptor, other than the 5-HT$_{1B/1F}$ receptors, was involved in modulating the LPR.

Another way to interpret the relative potency arises from the law of differences of logarithms:

\[ \text{pEC50} – \text{pKi} = –\log(\text{EC50}) – (–\log(\text{Ki})) = –\log(\text{EC50}/\text{Ki}) \]

Thus, the ratio EC50/Ki equals $10^{-(\text{pEC50} – \text{pKi})}$. For the 5-HT$_{1B}$ receptor the relative potency was on average –1.15, and thus the on average EC50/Ki = $10^{0.15} = 14$. 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 5 and Table 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 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 3). The relative potency computed for each agonist relative to its binding affinity at the 5-HT$_{1B}$ receptor (pEC50 – pKi; Table 3) was consistently within 2 SD of –1.0 (our confidence interval), with a mean of –1.03 ± 0.26. Likewise, the relative potency computed for agonists of the 5-HT$_{1F}$ receptor (pEC50-pKi; Table 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 (pEC$_{50}$ = 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 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 3), ruling out a 5-HT$_{1D}$ action of zolmitriptan.

The three 5-HT$_2$ receptor agonists tested that have negligible affinity for 5-HT$_{1B}$ receptors produced no inhibition in the SPR (Table 2 and 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 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.

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 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, $P > 0.05$) or SPR (10.1±34.8%, 6.8 ±31.6% and –3.0 ± 50.0% change, respectively, $P > 0.05$), 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. 2010). 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. 2010; Murray et al. 2011). 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 2), and then applied the antagonists (Fig 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, *P* < 0.05), 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 7B), but we attribute this to the agonist activation of 5-HT_{1F} receptors, which our antagonists did not block.

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 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 8A, –80 mV). At these hyperpolarized
potentials the same dorsal root stimulation evoked an EPSP, typically about 0.5 s long, with two
components: the long EPSP responsible for the LPR and the short EPSP responsible for the SPR (as
described earlier; both polysynaptic EPSPs). 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 \( \mu \)M) 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 8B. This near elimination of the long EPSP, was accompanied by a
loss of activation of PIC-mediated plateaus and LLRs (Fig 8B) measured with the motoneuron at rest, in
all cells tested (\( n = 8 \)). A substantial short EPSP remained in zolmitriptan (Fig 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.

**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 9A), relative to the leak current, as
previously reported (Li and Bennett 2003). This inward current is what produces the large plateau in Fig
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 (\(V_{on}; -0.8 \pm 0.9\%\), Figs 9C). Likewise, zolmitriptan had no significant effect on other motoneuron properties, including input resistance (2.7 ± 17.7% change, \(P > 0.05\)), resting potential (1.5 ± 4.1%, \(P > 0.05\)) and spike threshold (–3.2 ± 6.0%, \(P > 0.05\)).

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 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 an inhibitory postsynaptic current (IPSC; outward current deflection in Fig 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 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 9D, bottom), as expected. Interestingly, once these EPSPs were reduced by zolmitriptan, a long IPSC was revealed (Fig 9D, top), though the peak of this IPSC was not increased (\(n = 5/5\); Fig 10). This long IPSC revealed in zolmitriptan suggests that there is a large inhibitory synaptic input that is normally counterbalanced by a simultaneously activated large excitatory synaptic input. To confirm this, we applied strychnine (2 \(\mu\)M) 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±3.22 mV and 9.70±6.95 mV, respectively, \( n = 5, P < 0.05 \); measured at hyperpolarized potentials, as above, not shown; EPSP latency did not change), 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, \( P < 0.05 \); absolute reduction in EPSP was similar to without strychnine; thus percent change smaller), suggesting that 5-HT1 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.

Reversal potential for inhibitory synaptic currents is at the resting membrane potential after injury. Remarkably, the inhibitory synaptic input always produced negligible potential changes at rest (\( n = 9/9 \)), even when the opposing EPSCs were largely eliminated with zolmitriptan (Fig 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 of −70.9 ± 7.2 mV in chronic spinal rats (\( n = 9, P > 0.05 \)), 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). The reversal potential for this same IPSC in motoneurons of normal rats was significantly lower (−77.6 ± 2.3 mV) than in chronic spinal rats, and significantly lower than the resting potential of 71.8 ± 3.5 mV (\( P < 0.05, n = 5 \) normal rat, recorded as in Li et al. 2004). To independently assess the Cl\(^-\) reversal potential, we measured the reversal potential for chloride-mediated IPSCs produced by antidromic ventral root activation (Renshaw cell mediated). In chronic spinal rats these Renshaw cell IPSCs had a reversal potential at the resting membrane potential (not significantly different from rest, not shown, \( n = 8, P > 0.05 \)), confirming that the reversal potential for Cl\(^-\) was near rest in chronic spinal rats. In contrast, the reversal potential for
the short and the long EPSC in chronic spinal rats were well above rest but below −50 mV (Fig 10), indicative of mixed excitatory and inhibitory underlying currents.

5-HT<sub>2</sub> receptors do not inhibit the EPSPs.

Application of the 5-HT<sub>2A/2B/2C</sub> 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<sub>2</sub> agonists like DOI dramatically facilitate the Ca PICs (Harvey et al. 2006a; Murray et al. 2011), 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 minimize activation of the Na PIC, which is not blocked by isradipine.

**Spasms are reduced in 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 11A). These spasms are the counterpart of the long-lasting reflexes seen *in vitro* (Fig 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 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 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 (\( n = 5, P > 0.05 \)).
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 large transient 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 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 Ca 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, demonstrates 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 may 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 the spasticity syndrome, 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<sub>1B</sub> and 5-HT<sub>1F</sub> receptors agonists is predicted by their binding affinity.

While many agonists bind to both 5-HT<sub>1B</sub> and 5-HT<sub>1F</sub> 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<sub>1B</sub> receptors than 5-HT<sub>1F</sub> receptors, and accordingly its low dose action is likely mediated by 5-HT<sub>1B</sub> receptors. Furthermore, after selectively blocking that action of 5-HT<sub>1B</sub> 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<sub>1F</sub> receptors. In contrast, LY344864 and methylergonovine are relatively selective to 5-HT<sub>1F</sub> receptors, exhibiting negligible affinity at 5-HT<sub>1B</sub> receptors and thus their inhibitory action on the spastic reflexes demonstrates that the 5-HT<sub>1F</sub> receptor also modulates these reflexes and associated EPSPs. Finally, agonists with negligible affinity for 5-HT<sub>1B/1F</sub> receptors, such as the 5-HT<sub>1/5/7</sub> receptor agonist 8-OH-DPAT, exhibit no inhibitory effects on the polysynaptic reflexes (SPR and LPRs), ruling out the involvement of other receptors.

As further evidence for the involvement of just 5-HT<sub>1B</sub> and 5-HT<sub>1F</sub> receptors, we found that the binding affinity of agonists to 5-HT<sub>1B</sub> and 5-HT<sub>1F</sub> receptors is highly correlated with our measured potencies of these agonists at inhibiting the spastic reflexes, whereas the binding affinity at other receptors is not related to the potency. The lack of correlation between affinity and potency for 5-HT<sub>1D</sub> receptors is especially important in ruling out the involvement of the 5-HT<sub>1D</sub> receptor, because this receptor is so similar to the 5-HT<sub>1B</sub> receptor, activated by many of the same agonists (Table 1). We also showed that the reflex potencies can be quantitatively predicted from receptor binding affinity for 5-HT<sub>1B</sub> and 5-HT<sub>1F</sub> receptors, whereas this was not the case for other receptors, including 5-H<sub>1D</sub> and 5-HT<sub>1E</sub> receptors. Specifically we found that
reflex potencies are consistently one log unit less than the agonist affinity for 5-HT\textsubscript{1B} and 5-HT\textsubscript{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\textsubscript{1B} and 5-HT\textsubscript{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\textsubscript{2B} and 5-HT\textsubscript{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 in part reflects diffusion barriers in our whole spinal cord preparation that prevent the full applied dose from reaching the receptors, over the time used between doses (see details in Murray et al. 2011). 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 less likely to be involved in the invariant potency-affinity relation.

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

Considering that we found no action of zolmitriptan on PICs or basic membrane properties of motoneurons, the functional 5-HT\textsubscript{1B} and 5-HT\textsubscript{1F} receptors that inhibit the EPSPs that trigger spasms are not likely to be on motoneurons. There remains a possibility that these receptors could be on motoneurons and only modulate postsynaptic glutamate receptors involved in the EPSPs. However, it is more likely that these receptors are on low threshold group II and I type afferents terminals, which are the main afferents we stimulated (including cutaneous and muscle afferents; see Methods), and consistent with previous reports 5-HT\textsubscript{1B} and 5-HT\textsubscript{1F} receptors on various sensory afferents (Millan 2002). An intriguing possibility is that these 5-HT\textsubscript{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\textsubscript{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 5-HT_{1B} 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. 2010).

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. 2010). 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 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 an inhibitory synaptic input to motoneurons that: 1) is seen at depolarized potentials (Fig 9), 2) peaks shortly after the EPSP onset (interrupting the excitation), 3) is enhanced by blocking opposing EPSCs with zolmitriptan and 4) is 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 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 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 8A)(Li et al. 2004a), with an IPSP interrupting otherwise depolarizing EPSPs and PICs.

Shunting limits EPSPs and spasms.

Functionally, the action of mixed inhibitory and excitatory synaptic inputs is to substantially increase the overall membrane conductance, thus limiting (shunting) the action of 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 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 Ca and Na PICs induced by 5-HT₂ receptor agonists (Harvey et al. 2006a) 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 activated 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 chloride-mediated IPSC is near rest after injury.

Recently, it has been shown that inhibitory chloride currents (IPSCs) are reduced over time after injury (in chronic injury), due to a reduction in the potassium-chloride co-transporter (KCC2, 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 favour 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 KCC2 is so impaired following spinal cord injury that the reversal potential for Cl⁻ is depolarized 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). Boulenguez et al. (2010) also found that the KCC2 was impaired in adult rats after spinal cord injury, though they did not examine the impact on the Cl⁻ reversal potential. We did not find evidence for outright reversed (depolarizing) IPSPs at rest in adult rats after injury. However, our data does suggest that after injury in adult rats the reversal potential for Cl⁻ is depolarized by about 5 mV, from below the resting potential in normal rats to levels similar to the resting potential in chronic spinal rats. Interestingly, 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 could 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). However, improved 5-HT$_{1B/1F}$ agonists would be necessary, as triptans are only clinically used intermittently, and continuous use is not without adverse effects. 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.
ACKNOWLEDGEMENTS

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**FIGURE LEGENDS**

**Figure 1.** Polysynaptic reflexes and their underlying EPSP in chronic spinal rats. (A) Long-lasting reflex triggered by dorsal root stimulation (0.1 ms pulse, 3×T) 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 Ca²⁺ channel with isradipine (15µM). Background root activity, Bkg. (B) PIC-mediated plateau potential and sustained firing (LLR) evoked by dorsal root stimulation (3×T) 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).

**Figure 2.** 5-HT₁B receptor activity inhibits the polysynaptic reflexes in chronic spinal rats. (A) Long lasting polysynaptic reflex triggered by dorsal root stimulation (0.1 ms pulse, 3×T) 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-HT₁B/₁D/₁F agonist, zolmitriptan (300 nM, > 50% reduction). (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-HT₁B antagonist SB224289 (5 µM) or the 5-HT₁B/₁D antagonist SB216641 (5 µM) antagonized the inhibitory action of zolmitriptan (shifting EC₅₀ to the right). Each plot shows the typical response from a single rat, with a different rat for each condition, because agonists are not feasible to wash out and repeat after antagonist application (taking many hours to wash).

**Figure 3.** Mixed 5-HT₁ and 5-HT₂ receptor agonists have a biphasic response, only inhibiting reflexes at high doses. (A–C) Dose-response relation for the 5-HT₁ and 5-HT₂ receptor agonist alpha-methyl-5-HT and 5-HT itself, with increased reflexes (LPR and LLR) at low doses (5-HT₂ mediated) and decreased
reflexes at high doses (5-HT\textsubscript{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\textsubscript{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\textsubscript{2} receptors. (D) Dose response relation for 5-HT affect on the LPR after 5-HT\textsubscript{2} receptor block with
methysergide (10 μM) and 5-HT3 receptor block with granisetron (GR, 0.3 μM), with similar EC50 to that
obtained in C.

**Figure 4.** Potency of 5-HT receptor agonists at inhibiting the LPR is only related to binding to 5-HT\textsubscript{1B} and
5-HT\textsubscript{1F} receptors. (A) 5-HT\textsubscript{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 ($r = 0.96$, $P < 0.05$, $n = 6$). 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. Thin line: significant linear correlation between agonist potency and affinity for
5-HT\textsubscript{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 1, with agonists assumed to
act at a receptor only if Ki < 400 nM. Potencies are from Table 2. Potencies for 5-HT and zolmitriptan
action in the presence of 5-HT\textsubscript{1B} antagonists were used (plotted) for comparison to 5-HT\textsubscript{1D}, 5-HT\textsubscript{1E} and 5-HT\textsubscript{1F} receptor binding affinity, as these antagonists removed confounding affects of 5-HT\textsubscript{1B} receptors.
Table 3 also summarizes agonists/antagonists used for each receptor.

**Figure 5.** 5-HT\textsubscript{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 by zolmitriptan (300 nM).
Figure 6. Potency of 5-HT receptor agonists at inhibiting the SPR is only related to binding to 5-HT\textsubscript{1B} and 5-HT\textsubscript{1F} receptors. (A) 5-HT\textsubscript{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. Thin line: significant linear correlation between potency and affinity ($r = 0.95$, $P < 0.05$, $n = 6$). 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\textsubscript{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 grey zone: no detected effect of agonist on the LPR. Agonists used, potencies and affinities are detailed in Fig 4. Table 3 also summarizes agonists/antagonists used for each receptor.

Figure 7. The 5-HT\textsubscript{1B} receptor is not endogenously active in chronic spinal rats. (A) A block of possible endogenous 5-HT\textsubscript{1B} receptor activity with SB224289 (3 μM, during bar) produced no increase (or change) in the LPR or SPR. Reflexes measured at about 15 min intervals (circles). (B) In contrast, SB224289 (3 μM) increased the LPR and SPR after 5-HT\textsubscript{1B} receptors were exogenously activated by zolmitriptan (1 μM), which initially deceased these reflexes.

Figure 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).
Figure 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 10. Zolmitriptan reduces long EPSC, further revealing IPSCs, with reversal potential at rest. (A) Top plot: Long EPSC (negative currents), measured at 300 ms post stimulation, plotted against the holding potential, for the same motoneuron and stimulation as in Fig 9 (reversing above –60 mV). 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 20–30 ms post stimulation, plotted against holding potential, again during voltage-clamp in Fig 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) inhibited the long EPSC (negative currents reduced), revealing a pure long duration IPSC (positive currents; 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.
Figure 11. Zolmitriptan reduces spasms in the awake chronic spinal rat. (a) Spasm in chronic spinal rat evoked by electrical-cutaneous stimulation of the tail (3xT) and recorded with EMG. (b) Intrathecal application of zolmitriptan (0.1mM in 30 μl saline) reduced the LPR and LLR, quantified at bars.
### Table 1. 5-HT receptor agonists and their receptor bind affinity.

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1989; Hoyer et al. 1985; Ismaiel et al. 1990; 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 abbreviated methylergon. Each agonists is considered to activate a receptor if $K_i < 400$ nM, and listed with that receptor.
Table 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 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 abbreviated 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

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<th>Agonist</th>
<th>Antagonist pre-treatment</th>
<th>Receptors that can be activated (Ki &lt; 400)</th>
<th>Efficacy (% change)</th>
<th>Potency (-logEC50)</th>
<th>Efficacy (% change)</th>
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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.

957

958
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<td></td>
<td></td>
<td>8-OH-DPAT</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LP44</td>
<td>ND</td>
<td>ND</td>
<td>5-HT</td>
<td>$-2.26 \pm 0.17$</td>
<td>$-1.99 \pm 0.28$</td>
</tr>
</tbody>
</table>

**Table 3.** Relative potency of agonists at inhibiting the LPR and SPR. Relative potency computed as the difference between potency (Table 2) and affinity (Table 1): pEC50 – pKi. Pre-treatment with antagonists used to make agonist more selective, indicated in brackets, abbreviated as in Table 2. ND, no detected inhibition of the reflex in Table 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.

REFERENCES.


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


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


Figure 1

A

Bkg

+ 15 uM isradipine

0.5 mV

10 ms

0.1 mV

500 ms

B

-72 mV

Short EPSP

Long EPSP

20 mV

-81 mV

Dorsal root stim (3xT)
Figure 2

A

Dorsal root stimulation

B

+ Zolmitriptan

C

LPR

Zolmitriptan Dose (nM)

0
50
100
Control  10        100      1000        10,000

+ SB216641

D

LLR

Zolmitriptan Dose (nM)

0
50
100
Control  10        100      1000        10,000

+ SB224289

EC50
Figure 5

A  
SPR
0.2 mV
10 ms
Dorsal root stim.

B  
+ Zolmitriptan
Figure 6

A

B

C

D

Binding affinity (-log Ki)

Potency (-log EC50)

Figure 6

A

B

C

D

Binding affinity (-log Ki)

Potency (-log EC50)

Figure 6

A

B

C

D

Binding affinity (-log Ki)

Potency (-log EC50)
Figure 7

A

B

Time (min)

0.0 0.2 0.4 0.6 0.8 1.0

LPR (mV)

0.0 0.5 1.0

0.0 0.4 0.8

SPR (mV)

Time (min)

0 50 100 150

SB224289

Zolmitriptan

SB224289

Zolmitriptan

+ SB224289

+ Zolmitriptan
Figure 8

A

Rest -72 mV

LLR / plateau

Dorsal root stimulation

Short EPSP

Long EPSP

B

+ Zolmitriptan

Rest -72 mV

Short EPSP

LLR / plateau
Figure 9

A

-50 mV

Rest

PIC

B

IPSC

-60 mV

Short Long EPSC

500 ms

Rest, -70 mV

Short Long EPSC

30 ms

C + Zolmitriptan

PIC

D + Zolmitriptan

IPSC

-60 mV

Short EPSC

Rest
Figure 10

A  B

Long EPSC (nA)  Long EPSC (nA)

IPSC (nA)  IPSC (nA)

Short EPSC (nA)  Short EPSC (nA)

Membrane potential (mV)  Membrane potential (mV)

Rest  Rest

+ Zolmitriptan
A

B + Zolmitriptan

Figure 11

Cutaneous stimulation (3 xT)