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

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Murray KC, Stephens MJ, Rank M, D’Amico J, Gorassini MA, Bennett DJ. Polysynaptic excitatory postsynaptic potentials that trigger spasms after spinal cord injury in rats are inhibited by 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors. J Neurophysiol 106: 925–943, 2011. First published June 8, 2011; doi:10.1152/jn.01011.2010.—Sensory afferent transmission and associated spinal reflexes are normally inhibited by serotonin (5-HT) derived from the brain stem. 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. In the present study we examined which 5-HT receptors modulate the EPSPs and whether these receptors adapt to a loss of 5-HT after chronic spinal transaction 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 50% inhibition (EC$_{50}$) and potency (−log EC$_{50}$). 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 to 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 post-synaptic 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, such as 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.

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what receptors are involved, although it is reasonable to suggest that 5-HT$_1$ (or even 5-HT$_2$) receptors could mediate this inhibition (see above). In the current study we addressed 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.

Brain stem-derived 5-HT and NE 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 $\alpha_1$-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 Hounggaard 2003). Thus, with spinal cord injury, motoneurons are often rendered acutely unexcitable, in part due to a lack of brain stem-derived 5-HT and NE 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 reemergence 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 $\alpha_1$-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 reemergence of the large PICs that make the motoneurons permanently excitable (Harvey et al. 2006b; Murray et al. 2010). One goal of the present study was to examine whether 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, although uninjured animals and humans at times have substantial PICs facilitated by brain stem-derived 5-HT and NE (see above and Gorassini et al. 2002; Udina et al. 2010), these PICs do not cause uncontrolled motoneuron firing, because postsynaptic inhibition arising from glycnergic 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 (Boullenguez et al. 2010; Nielsen et al. 2007), in part due to loss of 5-HT and NE (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 $\alpha_1$-adrenergic) receptor activity, and 3) a loss of postsynaptic inhibition over motoneuron activity. In previous articles, we examined how spasms can be controlled by reducing PICs (Murray et al. 2010, 2011). The present article 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 mo old). Adult female rats were transected at the S$_2$ sacral level at about 2 mo of age (adult rat), and recordings were made at a time after their affected muscles became spastic (1.5–3 mo after injury), as detailed previously (Bennett et al. 1999, 2004). 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. 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, 2004b; Murray et al. 2010). Briefly, all the rats were anesthetized 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 C$_9$ ventral roots and the C$_9$ dorsal roots. After 1.5 h 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 1-h period in nACSF was given to wash out the residual anesthetic and mACSF before 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 Ca$_9$ dorsal root stimulation [single pulse, 0.1 ms, 0.02 mA, corresponding to 3 times afferent threshold (3×T); afferent and reflex threshold are similar (Bennett et al. 2004)] using a constant current stimulator (Isolflex, Jerusalem, Israel). The stimulation intensity (3×T) is compatible with activation of low-threshold group I and II (Aβ) afferents. Because the Ca$_9$ 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, although 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-s intervals for each trial. The ventral root recordings were amplified (>2,000), high-pass filtered at 100 Hz, low-pass filtered at 3 kHz, and recorded with a data-acquisition system sampling at 6.7 kHz (Axonoscope 8; Axon Instruments, , Burlingame, CA). Ventral root reflexes were quantified using custom-written software (Matlab, The 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 poststimulus), 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 poststimulus), and the long-latency, long-lasting tonic response associated with the Ca PIC (termed long-lasting reflex, or LLR; 500–4,000 ms poststimulus). Average ventral root activity computed for each trial in a given reflex interval was then averaged for all five stimuli in a trial. Average background ventral root activity before stimulation was measured for the 800 ms preceding 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 min 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 μM doses used). Antagonists took longer to act, and responses reached near steady state typically >30 min after application, at which time responses were averaged. The effect of agonists on the reflexes were reversible on 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 (1.5-mm OD; Warner GC 150F-10) using a Sutter P-87 micropipette puller and filled with either 2 M potassium citrate or a combination of 1 M potassium acetate and 1 M KCl. Electrodes were beveled down from an initial resistance of 40–80 to 26–32 MΩ using a rotary beveler (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) 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 0.8–2.5 nA/mV) modes.

Slow triangular voltage ramps (3.5-mV/s voltage ramp) were applied to the motoneurons to measure their electrical properties (Harvey et al. 2006c). The input resistance (Rᵢ) 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, and the voltage ramps over a 5-mV range near rest and subthreshold to the PIC onset. 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, 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 (Vₒ). The EPSP and associated reflexes were directly measured in motoneurons by stimulating the Ca₄ dorsal roots at 2–3×T, 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 (EPSCs) in response to stimulating the dorsal roots while voltage clamping at various potentials to prevent activation of the PIC or motoneuron spontaneous 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 NaHCO₃, 1.5 CaCl₂, 3 KCl, 5 MgCl₂, 1.4 NaH₂PO₄, 1.3 MgSO₄, 25 n-glucose, and 1 kynurenic acid. The nACSF was composed of (in mM) 122 NaCl, 24 NaHCO₃, 2.5 CaCl₂, 3 KCl, 1 MgCl₂, and 12 n-glucose. Both types of ACSF were saturated with 95% O₂–5% CO₂ and maintained at pH 7.4. Drugs were added to the nACSF as indicated in the text, including 5-HT and (−)-1,2-dimethoxy-4-iodophenyl-2-amino propane hydrochloride (DOI; Sigma-Aldrich), 2-methyl-5-HT, 5-carboxamidotryptamine (5-CT), 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT), α-methyl-5-HT, BW723C86, cisapride, EMD386088, granisetron, isradipine, LP44, LY344864, methylergonovine, methysergide, MK212, RS102221, RS127445, SB216641, SB224829 (all from Tocris), TTX (TTX-citrate; Alomone Labs, Jerusalem, Israel), and zolmitriptan (kindly donated by Astrazeneca, Mississauga, ON, Canada). All drugs were first dissolved as a 10–50 mM stock in water before final dilution in ACSF, with the exception of BW723C86, cisapride, EMD368088, isradipine, LP44, methylergonovine, SR102221, RS127445, and SB224829, 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 electromyogram (EMG). Percutaneous EMG wires (50-μm stainless steel; Cooner Wire) 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 electrical stimulation of the skin at the distal tip of the tail (cutaneous stimulation; 0.2-ms, 10-mA pulse; 3× reflex 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 poststimulus interval of 10–40 ms to quantify the SPR, 40–500 ms to quantify the LPR, and 500–4,000 ms to quantify LLR in vehicle controls).

Zolmitriptan was applied in vivo with intrathecal injections (Mestre et al. 1994). This was done with a direct lumbar puncture under brief isoflurane anesthesia (10–30-μl injections). Rats woke up rapidly (within minutes) after removal of anesthesia, 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 anesthesia and injection volume had negligible effects on the spasms. Also, three control rats were injected with 10 μl of methylene blue solution and euthanized immediately, to verify that the drug spread to the whole sacral area but not up to the brain stem (Mestre et al. 1994).

Data analysis. Data were analyzed using Clampfit 8.0 (Axon Instruments) and SigmaPlot (Jandel Scientific). Data are means ± 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 $-\log EC_{50}$ was used to quantify the drug potency; $pEC_{50} = -\log EC_{50}$. Also, the maximum drug-induced response (efficacy) was computed from the curve (peak of curve). For comparison with our computed potencies ($pEC_{50}$), 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 $K_i$ value (in 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 radiolabeled ligand, such as $[^{3}H]$5-HT, from the receptor expressed in isolated cells. Binding affinity is computed as $pK_i = -\log K_i$ (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-prefering 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 $K_i$ values were not available, human values were used instead, because 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 multiphasic 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; central latency 8–15 ms and lasting 10–30 ms; Fig. 1A, inset) and sometimes also had an earlier monosynaptic reflex component (not present in Fig. 1A, but 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, bottom 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, bottom plot; mean change $-9.7 \pm 41.0\%$, $n = 9$, $P > 0.05$).

After 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...
consistent with a partial involvement of PICs. Thus, under the LPR (bottom to prevent PIC activation, the same stimulation only evoked polysynaptic root activity. At rest (without injected current; T) in motoneuron sustained firing (LLR) evoked by dorsal root stimulation (3/T) and recorded from the ventral roots, with the reflex components long polysynaptic reflex (LPR) and long-lasting reflex (LLR) quantified during periods indicated by horizontal arrows (top trace). Inset: short polysynaptic reflex (SPR) on expanded time scale. Bottom trace shows elimination of LLR, but not LPR, after blocking the L-type Ca\(^{++}\) channel with isradipine (15 \(\mu\)M). Bkg, background root activity. B: persistent inward current (PIC)-mediated plateau potential and sustained firing (LLR) evoked by dorsal root stimulation (3/T) in motoneuron at rest (without injected current; top trace). 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 (bottom trace).

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 \(\mu\)M, \(n = 9, P < 0.05\)), 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 (latency >500 ms) 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 long-lasting reflex (or LLR). The remaining 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), although this effect appeared small (15%).

5-HT\(_{1B}\) and 5-HT\(_{1F}\) 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 (Fig. 2C) from which we computed 1) the agonist dose to produce 50% maximal inhibition (EC\(_{50}\); Fig. 2D), 2) agonist potency (pEC\(_{50}\) = −log EC\(_{50}\), and 3) agonist efficacy (maximal inhibition, reported relative to control LPR size; Fig. 2C). For zolmitriptan, the EC\(_{50}\) value was about 100 nM with a corresponding potency of about 7 (−log 100 × 10\(^{-9}\); Table 2). Overall, the efficacy of zolmitriptan was so large that the LPR was on average reduced to about 3% of predrug 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, \(n = 12, P < 0.05\)), 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 with 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, BW727368, 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, 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 biphasic response. That is, at low doses, the agonist increased the long-lasting reflexes, including the LPR and LLR (Fig. 3, A–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 EC\(_{50}\), consistent with 5-HT\(_{2}\) receptor-mediated facilitation of the PIC, as described previously (Murray et al. 2010, 2011). 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 before 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 EC\textsubscript{50} and efficacy values (Fig. 3, A and C). As shown in Table 2, nonselective agonists with 5-HT\textsubscript{1} and 5-HT\textsubscript{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 EC\textsubscript{50} and efficacy for reflex inhibition from nonselective agonists by showing that after the confounding 5-HT\textsubscript{2} receptor action was blocked with antagonists [methysergide (10 \mu M) or the selective 5-HT\textsubscript{2} antagonists such as RS127445 (3 \mu M)], 5-HT produced a purely inhibitory action, with a similar dose-response relation to that obtained without the block (Fig. 3D and Table 2). This also shows that the inhibitory action of these nonselective agonists is mediated by 5-HT\textsubscript{1} and not 5-HT\textsubscript{2} receptors.

Pretreatment 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\textsubscript{1B} receptors (K\textsubscript{i} \approx 400 nM), whereas it antagonizes/binds most other 5-HT receptors with high affinity (K\textsubscript{i} < 500 nM; except 5-HT\textsubscript{1C} and 5-HT\textsubscript{2A} receptors) (Boess and Martin 1994). Thus the inhibition of the LPR by 5-HT seen after pretreatment with methysergide (Fig. 3D and Table 2) suggests that 5-HT\textsubscript{1B} receptors specifically inhibit the LPR, although this does not rule out additional involvement of other 5-HT\textsubscript{1} receptors blocked by methysergide (5-HT\textsubscript{1F}).

Prior application of the selective 5-HT\textsubscript{1B} receptor antagonist SB224289 or the selective 5-HT\textsubscript{1B/1D} receptor antagonist SB216641 significantly reduced the inhibitory action of both selective (zolmitriptan) and nonselective (5-HT; inhibitory phase) 5-HT\textsubscript{1} 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 (EC\textsubscript{50} significantly increased; Table 2), indicating that the 5-HT\textsubscript{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. 2C and Table 2). This may be explained by the activation of the 5-HT\textsubscript{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\textsubscript{1F} compared with the 5-HT\textsubscript{1B} receptor (Table 1).

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

Application of agonists (or agonist-antagonist combinations) relatively selective for 5-HT\textsubscript{1A/1E}, 5-HT\textsubscript{3}, 5-HT\textsubscript{4}, 5-HT\textsubscript{5}, 5-HT\textsubscript{6}, and 5-HT\textsubscript{7} receptors (Table 1) produced no significant inhibition of the LPR (Tables 2 and 3), suggesting that none of

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**Fig. 2. 5-HT\textsubscript{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, 3\times T) and recorded from the ventral roots, with LPR and LLR components indicated by horizontal bars. B: reduction of LPR and LLR with application of the 5-HT\textsubscript{1B} agonist zolmitriptan (300 nM; > 50\% reduction). C and D: reduction of LPR and LLR, respectively, with increasing zolmitriptan dose (decrease over ~100-fold change in dose; left). Best-fit sigmoidal curves are shown with subsequent estimation of EC\textsubscript{50}. Prior application of a single blocking dose of the selective 5-HT\textsubscript{1B} antagonist SB224289 (5 \mu M) or the 5-HT\textsubscript{1B/1D} antagonist SB216641 (5 \mu M) antagonized the inhibitory action of zolmitriptan (shifting EC\textsubscript{50} 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 washout and repeat after antagonist application (taking many hours to wash).
Inhibition of the polysynaptic reflexes by 5-HT₁B agonists

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Antagonist Pretreatment</th>
<th>Receptors That Can Be Activated (Kᵢ &lt; 400 nM)</th>
<th>Efficacy, %change in LPR</th>
<th>Potency, −log EC₅₀</th>
<th>Efficacy, %change in SPR</th>
<th>Potency, −log EC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-CT</td>
<td>None</td>
<td>5-HT₁,₁B,₁D,₁E,₂B,₅–₇</td>
<td>−96.3 ± 65.4</td>
<td>7.08 ± 0.5</td>
<td>−60.4 ± 77.3</td>
<td>7.16 ± 0.5</td>
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<tr>
<td>5-HT</td>
<td>None</td>
<td>5-HT₁,₁B</td>
<td>−78.5 ± 23.2</td>
<td>6.56 ± 0.17</td>
<td>−63.4 ± 43.7</td>
<td>6.83 ± 0.43</td>
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<tr>
<td>5-HT</td>
<td>Methys + gran</td>
<td>5-HT₁,₁B</td>
<td>−84.6 ± 14.7</td>
<td>6.47 ± 0.38</td>
<td>−29.7 ± 21.3</td>
<td>6.26 ± 0.22†</td>
</tr>
<tr>
<td>5-HT</td>
<td>RSs</td>
<td>5-HT₁,₁B</td>
<td>−108.1 ± 28.7</td>
<td>6.57 ± 0.23</td>
<td>−74.3 ± 38.4</td>
<td>6.36 ± 0.30</td>
</tr>
<tr>
<td>5-HT</td>
<td>RSs + SB216</td>
<td>5-HT₁,₁B,₁F,₂,₅–₇</td>
<td>−52.0 ± 22.0†</td>
<td>5.70 ± 0.36†</td>
<td>−59.2 ± 23.6</td>
<td>5.86 ± 0.32‡</td>
</tr>
<tr>
<td>α-Methyl-5-HT</td>
<td>None</td>
<td>5-HT₁,₁B,₁E,₁F,₂,₄</td>
<td>−57.3 ± 16.5</td>
<td>5.75 ± 0.23</td>
<td>−64.1 ± 29.4</td>
<td>5.80 ± 0.53</td>
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<tr>
<td>BW723C86</td>
<td>None</td>
<td>5-HT₁,₂,₃,₆</td>
<td>−38.0 ± 24.8</td>
<td>5.89 ± 0.48</td>
<td>−49.3 ± 43.2</td>
<td>5.92 ± 0.33</td>
</tr>
<tr>
<td>EMD386088</td>
<td>None</td>
<td>5-HT₁,₁B,₁D,₁E,₁F,₂,₄</td>
<td>−43.6 ± 35.3†</td>
<td>5.53 ± 0.35</td>
<td>−51.2 ± 28.8</td>
<td>5.76 ± 0.36</td>
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<tr>
<td>LY344864</td>
<td>SB216 + gran</td>
<td>5-HT₁,₁B</td>
<td>12.4 ± 110.6†</td>
<td>ND</td>
<td>5.7 ± 43.3</td>
<td>ND</td>
</tr>
<tr>
<td>Metylergonovine</td>
<td>None</td>
<td>5-HT₁,₁B</td>
<td>−57.2 ± 25.4</td>
<td>7.12 ± 0.17</td>
<td>−45.9 ± 26.7</td>
<td>7.14 ± 0.21</td>
</tr>
<tr>
<td>Zolmitriptan</td>
<td>None</td>
<td>5-HT₁,₁B</td>
<td>−40.8 ± 21.3</td>
<td>6.47 ± 0.25</td>
<td>−44.0 ± 25.2</td>
<td>6.51 ± 0.15</td>
</tr>
<tr>
<td>Zolmitriptan</td>
<td>SB216 or GR127</td>
<td>5-HT₁,₁B</td>
<td>−99.5 ± 11.3</td>
<td>7.07 ± 0.40</td>
<td>−55.2 ± 20.6</td>
<td>7.08 ± 0.41</td>
</tr>
<tr>
<td>Zolmitriptan</td>
<td>SB224</td>
<td>5-HT₁,₁B,₁D,₁E,₁F,₂,₄</td>
<td>−41.2 ± 27.6†</td>
<td>5.94 ± 0.25†</td>
<td>−38.2 ± 27.1†</td>
<td>6.15 ± 0.70†</td>
</tr>
<tr>
<td>Zolmitriptan</td>
<td>SB224</td>
<td>5-HT₁,₁B,₁D,₁E,₁F,₂,₄</td>
<td>−26.6 ± 29.0†</td>
<td>5.63 ± 0.53†</td>
<td>−31.5 ± 29.9†</td>
<td>5.92 ± 0.57†</td>
</tr>
</tbody>
</table>

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 (pretreatment). The receptors that can be activated by this agonist-antagonist combination are indicated (Kᵢ < 400 nM; see details in Table 1). The antagonists used, followed by dose and receptors blocked, were as follows: SB224289 (SB224): 5 μM, 5-HT₁B; SB216641 (SB216): 3 μM, 5-HT₁B, GR127935 (GR127): 3 μM, 5-HT₁B, methysgeride (methylsgeride): 10 μM, all but 5-HT₁A/₁F; GRIP (granisetron) (granisetron): 0.3 μM, 5-HT₁A; RS214745: 3 μM, 5-HT₃A, and RS102221: 3 μM, 5-HT₃C (the latter 2 antagonists were applied together and referred to as RSs). The efficacy of the agonists in inhibiting the long (LPR) and short polysynaptic reflex (SPR) are indicated, normalized by the predrug reflex amplitudes (100% indicates complete elimination of the excitatory reflex by agonist). In addition, the agonists 8-OH-DPAT (5-HT₁A/₁B affinity), LP44 (5-HT₁A,₁B affinity), EMD386088 (5-HT₁A,₁B), and MK212 (5-HT₂C) produced no significant inhibition of the LPR or SPR (not shown, doses ≤30 μM; see text). Data are means ± SD; n > 8 per condition. *P < 0.05, significant change in reflex. †P < 0.05, 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 agonist data).

these other receptors inhibit the LPR and associated long EPSP. Furthermore, application of the selective 5-HT₁B receptor agonist DOI or nonselective 5-HT₃ agonists that have negligible affinity for 5-HT₁B and 5-HT₃ receptors also produced no detectable inhibition in the LPR (Tables 2 and 3). However, these 5-HT₁B 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-HT₁B receptors; up to 30 μM), 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-HT₂ receptor 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). 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 3,000 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 (EC\(_{50}\) values and associated potencies, pEC\(_{50}\)) varied by orders of magnitude between the different agonists (Table 2), although this variation was largely accounted for by the differing binding affinity of these drugs to 5-HT\(_{1B}\) and 5-HT\(_{1F}\) receptors (p\(_K_i\); see description of binding affinity in METHODS; Table 1).

That is, we found that for 5-HT\(_{1B}\) agonists, the potency (pEC\(_{50}\)) was significantly correlated with the binding affinity (p\(_K_i\)) of the agonist for 5-HT\(_{1B}\) receptors and, importantly, very close to a line of unity slope (dashed line; pEC\(_{50}\) vs. p\(_K_i\); see 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. 4, B–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 modeling the expected relation between potency and affinity, as described below.

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 (K\(_i\)) should approximately correspond to the agonist dose needed to produce a functional response (e.g., EC\(_{50}\) for LPR), and thus the agonist binding affinity (p\(_K_i\)) should roughly equal its potency (pEC\(_{50}\)) (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 (EC\(_{50}\)) to get responses, and thus the potency (pEC\(_{50}\) = −log EC\(_{50}\)) was higher than the affinity. Furthermore, nonlinearities 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 EC\(_{50}\) dose and potency (see DISCUSSION). Nevertheless, factors such as 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: pEC\(_{50}\) = p\(_K_i\) + 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 pEC\(_{50}\) − p\(_K_i\) = 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 (pEC\(_{50}\) − p\(_K_i\); it reflects all factors that affect potency other than binding affinity). For the 5-HT\(_{1B}\) and 5-HT\(_{1F}\) recep-

Table 3. Relative potency of agonists at inhibiting the LPR and SPR

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Agonist</th>
<th>LPR</th>
<th>SPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT(_{1A})</td>
<td>5-CT</td>
<td>−2.38 ± 0.50</td>
<td>−2.30 ± 0.51</td>
</tr>
<tr>
<td>5-HT</td>
<td>−2.22 ± 0.17</td>
<td>−1.95 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>LP44</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5-HT(_{1B})</td>
<td>5-CT</td>
<td>−2.40 ± 0.50</td>
<td>−2.32 ± 0.51</td>
</tr>
<tr>
<td>5-HT</td>
<td>−2.04 ± 0.17</td>
<td>−1.77 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>LP44</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5-HT(_{1D})</td>
<td>5-CT</td>
<td>−2.35 ± 0.50</td>
<td>−2.27 ± 0.51</td>
</tr>
<tr>
<td>5-HT</td>
<td>−2.06 ± 0.17</td>
<td>−1.77 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>LP44</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5-HT(_{1F})</td>
<td>5-CT</td>
<td>−1.40 ± 0.50</td>
<td>−1.32 ± 0.51</td>
</tr>
<tr>
<td>5-HT</td>
<td>−1.05 ± 0.17</td>
<td>−0.78 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>α-Methyl-5-HT</td>
<td>−1.32 ± 0.23</td>
<td>−1.21 ± 0.53</td>
<td></td>
</tr>
<tr>
<td>BW723C86</td>
<td>−0.71 ± 0.48</td>
<td>−0.68 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>EMD386088</td>
<td>−1.22 ± 0.35</td>
<td>−0.99 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>Zolmitriptan</td>
<td>−1.23 ± 0.40</td>
<td>−1.22 ± 0.41</td>
<td></td>
</tr>
<tr>
<td>5-HT(_{1H})</td>
<td>5-CT</td>
<td>−2.35 ± 0.50</td>
<td>−2.27 ± 0.51</td>
</tr>
<tr>
<td>5-HT</td>
<td>−2.04 ± 0.17</td>
<td>−1.77 ± 0.28</td>
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<tr>
<td>8-OH-DPAT</td>
<td>ND</td>
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<td>LP44</td>
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<td>ND</td>
<td></td>
</tr>
<tr>
<td>5-HT(_{1I})</td>
<td>5-CT</td>
<td>−2.35 ± 0.50</td>
<td>−2.27 ± 0.51</td>
</tr>
<tr>
<td>5-HT</td>
<td>−2.04 ± 0.17</td>
<td>−1.77 ± 0.28</td>
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<tr>
<td>LP44</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>
tors 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. 4, A and B). Also, the difference $p_{EC_{50}} - p_{K_i}$ (relative potency) was, as hypothesized, highly invariant across all agonists tested at these receptors, on average $-1.15 \pm 0.25$ and $-1.23 \pm 0.27$ for 5-HT$_{1B}$ and 5-HT$_{1F}$ receptors, respectively, with each agonist having a relative potency well within two 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 $p_{EC_{50}} - p_{K_i} = -1$ (constant; dashed line in Fig. 4).

In contrast to the invariant relative potency for 5-HT$_{1B/F}$ receptors, we found that for all other receptors, the relative potency computed from the potency of broad-spectrum agonist response ($p_{EC_{50}}$) 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 ($p_{EC_{50}} - p_{K_i}$ not equal to $-1$). For example, the relative potency computed for zolmitriptan’s $p_{EC_{50}}$ compared with its affinity at the 5-HT$_{1D}$ receptors was less than $-2$ (Table 3), well outside of the confidence interval, suggesting that its EC$_{50}$ is too high to be predicted from the 5-HT$_{1D}$ receptor only if $K_i < 400$ nM. Potencies and affinities are from Table 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, because these antagonists removed confounding effects of 5-HT$_{1B}$ receptors. Table 3 also summarizes agonists/antagonists used for each receptor.

Another way to interpret the relative potency arises from the law of differences of logarithms: $p_{EC_{50}} - p_{K_i} = -\log (EC_{50}) - [-\log (K_i)] = -\log (EC_{50}/K_i)$. Thus the ratio $EC_{50}/K_i$ equals $10^{-(p_{EC_{50}} - p_{K_i})}$. For the 5-HT$_{1B}$ receptor, the relative potency was on average $-1.15$, and thus on average, $EC_{50}/K_i = 10^{0.115} \approx 14$. This indicates that the EC$_{50}$ dose needed to affect the LPR in the present whole sacral spinal cord prepa-

Fig. 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 ($p_{EC_{50}} = -\log EC_{50}$) for inhibiting the LPR plotted against the agonist binding affinity to that receptor ($p_{K_i}$). Each agonist is indicated next to its data point: BW, BW723C86; Zolm, zolmitriptan; EMD, EMD386088. Thin line indicates significant linear correlation between potency and affinity ($r = 0.96, P < 0.05, n = 6$). Dashed line represents the 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 indicates significant linear correlation between agonist potency and affinity for 5-HT$_{1F}$ receptors (solid circles; $r = 0.91, P < 0.05, n = 5$). Dashed lines represent the unit slope line. Other receptors had no significant correlation between potency and affinity (open symbols; $P > 0.05$). ND and shaded zone indicate no detected effect of agonist on the LPR. Agonists used and affinities are listed in Table 1, with agonists assumed to act at a receptor only if $K_i < 400$ nM. Potencies are from Table 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, because these antagonists removed confounding effects of 5-HT$_{1B}$ receptors. Table 3 also summarizes agonists/antagonists used for each receptor.
SPR is also inhibited by the 5-HT\textsubscript{1B} and 5-HT\textsubscript{1F} Receptors. Similar to the LPR, the SPR was inhibited by 5-HT\textsubscript{1B} and 5-HT\textsubscript{1F} receptor agonists, including relatively selective agonists (zolmitriptan or agonist-antagonist combinations) and nonselective agonists (5-HT; Fig. 5 and Table 2). Also, the agonist potencies (pEC\textsubscript{50}) were significantly correlated with the agonist binding affinity at 5-HT\textsubscript{1B} and 5-HT\textsubscript{1F} receptors and no other receptor (Fig. 6). In contrast, 5-HT receptor agonists (agonist-antagonist combinations) that have negligible affinity for 5-HT\textsubscript{1B} or 5-HT\textsubscript{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\textsubscript{1B} receptor (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\textsubscript{1F} receptor (Table 3) was consistently within 2 SD of −1.0, with a mean of −1.11 ± 0.18, suggesting that the potency of the agonists on the SPR was well predicted by agonist affinity at the 5-HT\textsubscript{1B} or 5-HT\textsubscript{1F} receptor, with an invariant diffusion factor (pEC\textsubscript{50} = pK\textsubscript{i} − 1), just as 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 (Table 3). An important example is that the relative potency of zolmitriptan for the 5-HT\textsubscript{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\textsubscript{1D} action of zolmitriptan.

The three 5-HT\textsubscript{2} receptor agonists tested that have negligible affinity for 5-HT\textsubscript{1B} receptors produced no inhibition in the SPR (Tables 2 and 3), 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\textsubscript{2} agonists on the LPR, suggesting that the PICs controlled by the 5-HT\textsubscript{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\textsubscript{1B} receptor activity in chronic spinal rats. We next examined whether there was any endogenous 5-HT\textsubscript{1} receptor activity after chronic spinal cord injury. Without prior agonist application, the selective antagonists SB224289 (5-HT\textsubscript{1B} selectivity, 3–5 μM; Fig. 7A), SB216641 (5-HT\textsubscript{1B/1D}, 5 μM), or GR127935 (5-HT\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{1B} receptor activity after injury, unlike what we find with 5-HT\textsubscript{2} receptors (Murray et al. 2010, 2011). As a positive control, we applied 5-HT\textsubscript{1B} agonists (zolmitriptan, 1.0 μM; 5-CT, 1.0 μM; or 5-HT, 0.3 μM) to activate the 5-HT\textsubscript{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\textsubscript{1B} receptor activity. We did find that the antagonists only partially reversed the inhibition of the reflexes by these 5-HT\textsubscript{1} agonists (Fig. 7B), but we attribute this to the agonist activation of 5-HT\textsubscript{1F} receptors, which our antagonists did not block.

Increasing cAMP increases the LPR and SPR. 5-HT\textsubscript{1} receptors are coupled to G\textsubscript{i} proteins that lead to decreased intracellular cAMP levels. Thus our finding that activating 5-HT\textsubscript{1} receptors decreases the LPR and SPR suggests that 5-HT\textsubscript{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\textsubscript{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 (3×T). 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, H11002). 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 poststimulation, n = 10 motoneurons), and the short EPSP was on average larger at 10.85 ± 5.27 mV (peak, at about 5–10 ms), although transient. The 5-HT1B/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 shown 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 \( V_{\text{on}} \) (−0.8 ± 0.9%, Fig. 9C). Likewise, zolmitriptan had no significant effect on other motoneuron properties, including \( R_m \) (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 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; 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), 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), although 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 μM) to block inhibitory glycnergic inputs, which produced synaptic responses that were always net excitatory and doubled both the long and short EPSPs (increasing by 5.77 ± 3.22 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 ± 3.2 and 23.9 ± 7.8% for long and short EPSPs, respectively, P < 0.05; absolute reduction in EPSP was similar to that without strychnine; thus %change was smaller), suggesting that 5-HT₁₃ 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 (n = 5/5; Fig. 9D), suggesting that the reversal potential for these inhibitory glycnergic 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 EPSP 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 rats, recorded as in Li et al. 2004a). 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 potentials 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-HT₂ receptors do not inhibit the EPSPs. Application of the 5-HT₂A/B/C receptor agonist DOI did not significantly affect the EPSPs (short EPSP: 10.9 ± 2.0 mV before and 11.5 ± 2.4 mV after DOI; long EPSP: 5.82 ± 5.0 before and 6.48 ± 4.3 mV after DOI; n = 5, P > 0.05). Considering that 5-HT₂ agonists like DOI dramatically facilitate the Ca PICs (Harvey

Fig. 7. 5-HT₁₃ receptor is not endogenously active in chronic spinal rats. A: a block of possible endogenous 5-HT₁₃ receptor activity with SB224289 (3 μM, horizontal bar) produced no increase (or change) in the LPR or SPR. Reflexes were measured at about 15-min intervals (●). B: in contrast, SB224289 (3 μM) increased the LPR and SPR after 5-HT₁₃ receptors were exogenously activated by zolmitriptan (1 μM), which initially decreased these reflexes.
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, were 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, electrical cutaneous stimulation of the skin on the tip of the tail evoked many-second-long tail muscle spasms that we recorded with EMG (Fig. 1A). 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 of 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 s (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<sub>1B</sub> and 5-HT<sub>1F</sub> 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<sub>1B</sub> and 5-HT<sub>1F</sub> 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<sub>1B</sub> and 5-HT<sub>1F</sub> 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<sub>1B</sub> and 5-HT<sub>1F</sub> receptor agonists such as 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<sub>1</sub> 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 such as baclofen that produce weakness and sedation (Dario and Tomei 2004; Li et al. 2004c). Although 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 et al. 2006; 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.** Although 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-HT1B receptors than 5-HT1F receptors, and accordingly, its low-dose action is likely mediated by 5-HT1B receptors. Furthermore, after selectively blocking that action of 5-HT1B 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-HT1F receptors. In contrast, LY344864 and methylergonovine are relatively selective to 5-HT1F receptors, exhibiting negligible affinity at 5-HT1B receptors, and thus their inhibitory action on the spastic reflexes but does continue to inhibit the reflexes at a high dose, consistent with activation of 5-HT1F receptors. In contrast, LY344864 and methylergonovine are relatively selective to 5-HT1F receptors, exhibiting negligible affinity at 5-HT1B receptors, and thus their inhibitory action on the spastic reflexes demonstrates that the 5-HT1F Receptor also modulates these reflexes and associated EPSPs. Finally, agonists with negligible affinity for 5-HT1B/1F receptors, such as the 5-HT1/5/7 receptor agonist 8-OH-DPAT, exhibit no inhibitory effects on the polysynaptic reflexes (SPR and LPRs), ruling out the involvement of other receptors.

As further evidence for the involvement of just 5-HT1B and 5-HT1F receptors, we found that the binding affinity of agonists to 5-HT1B and 5-HT1F 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-HT1D receptors is especially important in ruling out the involvement of the 5-HT1D receptor because this receptor is so similar to the 5-HT1B 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-HT1B and 5-HT1F receptors, whereas this was not the case for other receptors, including 5-HT1D and 5-HT1E receptors. Specifically, we found that reflex potencies are consistently one log unit less than the agonist affinity for 5-HT1B and 5-HT1F receptors, which means that the 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-HT1B and 5-HT1F receptors, which means that the 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-HT1B and 5-HT1F receptors, which means that the 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-HT1B and 5-HT1F receptors, which means that the 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-HT1B and 5-HT1F receptors, which means that the 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-HT1B and 5-HT1F receptors, which means that the 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-HT1B and 5-HT1F receptors, which means that the 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-HT1B and 5-HT1F receptors, which means that the 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).
including the receptor saturation, intracellular signaling (cAMP), and saturation in EPSPs, although these are specific to each receptor and signaling system and thus are 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 of 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 postsynaptically; 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, 2004; Li et al. 2004b). However, it remains to be resolved why we failed to detect effects of 5-HT\textsubscript{1A}, 5-HT\textsubscript{2A}, 5-HT\textsubscript{3}, and 5-HT\textsubscript{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).

**G\textsubscript{i} protein-coupled receptors that decrease cAMP have antispastic action.** 5-HT\textsubscript{1}-type receptors activate G\textsubscript{i}-coupled proteins that inhibit intracellular cAMP production by inhibiting adenylate cyclase activity (Boess and Martin 1994) and accordingly in many systems are inhibitory, although 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\textsubscript{1B} and 5-HT\textsubscript{1F} receptors (inhibiting EPSPs) is likely mediated by inhibiting cAMP. The involvement of this G\textsubscript{i}-coupled pathway in regulating spasms is also consistent with the known antispastic action of other G\textsubscript{i}-coupled receptors, including \alpha\textsubscript{2a}-adrenergic receptors and GAB\textsubscript{A\textsubscript{R}} 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-HT1B 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-HT1B receptor exhibits less constitutive activity than predicted from the cloned system. As a positive control, we found that SB224289 antagonized 5-HT1B 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-HT1B receptors due to a lack of availability of selective inverse agonists to this receptor. The other 5-HT1B antagonists tested also had no detectable effect by themselves, although 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-HT1B receptor, over time after injury 5-HT2C receptors become constitutively active and help 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-HT1B). A common underlying functional pattern that emerges is that both the adaptation in the 5-HT2 receptor and lack of adaptation in the 5-HT1 receptor lead 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 with 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, which 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 (~15 mV). Although 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 Y 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 s) may prevent the full PIC 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 cotransporter (KCC2; Boulenguez et al. 2010). Such a reduction in the chloride currents might further explain the large net excitatory synaptic inputs we observed by shifting the balance more in favor of EPSPs over IPSPs, although this does not itself explain the large EPSPs seen acutely after injury (Li et al. 2004a). On the basis of data from neonatal rats, Boulenguez et al. (2010) suggested 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 (above −70 mV), and thus at rest, there are net depolarizing responses to synaptically 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, although 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 do 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, blockade of glycine receptors with strychnine reveals a very large EPSP at rest, suggesting that although 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-HT1 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-HT1B and 5-HT1F 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, such as the 5-HT2c 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 EPSPs are of adequate duration (seconds) to trigger the PICs in motoneurons that ultimately cause the sustained motoneuron firing that underlies spasms. Thus reactivating 5-HT1B and 5-HT1F 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-HT1B/1F agonists like the triptans (zolmitriptan), which are currently a first-line treatment for migraines (Millan 2002). However, improved 5-HT1B/1F agonists would be necessary, because triptans are only clinically used intermittently and continuous use is not without adverse effects. In the long run, understanding why 5-HT1B receptors do not become constitutively active after injury, unlike the 5-HT2c 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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5-HT, RECEPTORS INHIBIT SPASM


