Locomotion After Spinal Cord Injury Depends on Constitutive Activity in Serotonin Receptors

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Fouad K, Rank MM, Vavrek R, Murray KC, Sanelli L, Bennett DJ. Locomotion after spinal cord injury depends on constitutive activity in serotonin receptors. J Neurophysiol 104: 2975–2984, 2010. First published September 22, 2010; doi:10.1152/jn.00499.2010. Following spinal cord injury (SCI) neurons caudal to the injury are capable of rhythmic locomotor-related activity that can form the basis for substantial functional recovery of stepping despite the loss of crucial brain stem-derived neuromodulators like serotonin (5-HT). Here we investigated the contribution of constitutive 5-HT2 receptor activity (activity in the absence of 5-HT) to locomotion after SCI. We used a staggered hemisection injury model in rats to study this because these rats showed a robust recovery of locomotor function and yet a loss of most descending axons. Immunolabeling for 5-HT showed little remaining 5-HT below the injury, and locomotor ability was not correlated with the amount of residual 5-HT. Furthermore, blocking 5-HT2 receptors with an intrathecal (IT) application of the neutral antagonist SB242084 did not affect locomotion (locomotor score and kinematics were unaffected), further indicating that residual 5-HT below the injury did not contribute to generation of locomotion. As a positive control, we found that the same application of SB242084 completely antagonized the muscle activity induced by exogenous application of the 5-HT2 receptor agonists alpha-methyl-5-HT (IT). In contrast, blocking constitutive 5-HT2 receptor activity with the potent inverse agonist SB206553 (IT) severely impaired stepping as assessed with kinematic recordings, eliminating most hindlimb weight support and overall reducing the locomotor score in both hind legs. However, even in the most severely impaired animals, rhythmic sweeping movements of the hindlimb feet were still visible during forelimb locomotion, suggesting that SB206553 did not completely eliminate locomotor drive to the motoneurons or motoneuron excitability. The same application of SB206553 had no affect on stepping in normal rats. Thus SCI deprives such spinal neurons caudal to the injury of 5-HT, initially leaving them in a relatively unexcitable state, even though they are not directly injured. The critical importance of these neuromodulators is demonstrated by the repeated finding in animal models that locomotor activity can be regained soon after spinal transection with the exogenous application of drugs that activate the neuromodulatory 5-HT, NA, and dopamine receptors (in vivo and in vitro) (Chau et al. 1998; Cowley and Schmidt 1994; Kiehn and Kjaerulff 1996; Viala and Buser 1971), including 5-HT2 and 5-HT3 receptor agonists (Landry and Guertin 1994; Madriaga et al. 2004; McEwen et al. 1997) or even transplants of 5-HT and NA producing cells into the spinal cord (Gimenez y Ribotta and Privat 1998; Ribotta et al. 2000). Remarkably, over the weeks after injury, rudimentary locomotor-like movements spontaneously emerge and improve with training (Barbeau and Rossignol 1987; de Leon et al. 1999; Kuhn and Macht 1948) as the neuromodulatory systems (e.g., 5-HT2 receptors) are somehow re-activated in the absence of 5-HT. How this compensation for lost brainstem 5-HT occurs is unknown, although a clue is that certain subtypes of the 5-HT2 receptor exhibit a tendency to become constitutively active (Chau et al. 2000; Herrick-Davis et al. 1999; Niswender et al. 1999; Westphal and Sanders-Bush 1994). Such constitutively active 5-HT2 receptors have recently been shown to have functional implications in brain areas including the normal intact striatum and cortex, where 5-HT is present (De Deurwaerdere et al. 2004; Gurevich et al. 2002). Importantly, in cortical neurons, the constitutively active 5-HT2 receptor isoforms are upregulated with reduced 5-HT (Gurevich et al. 2002), suggesting that loss of 5-HT with SCI might have similar effects, and indeed this has been shown for motoneurons after SCI (Murray et al. 2010). In the present paper, we further examine the hypothesis that 5-HT2 receptors denervated by spinal cord injury become constitutively active—spontaneously active in the absence of 5-HT or any other ligand (Chanrion et al. 2008; Herrick-Davis et al. 1999; Niswender et al. 1999; Westphal and Sanders-Bush 1994)—and play an important role in the spontaneously occurring locomotor activity after injury. The spontaneous recovery of locomotor activity is especially important in patients (and animals) with incomplete SCI that spares part of the descending connection from the brain, because patients (and animals) can learn to use these spared connections to produce substantial recovery of functional walking, especially with training (Baller mann and Fouad 2006;
Spared descending axons can sprout and form new connections following SCI, contributing to this recovery; thus understanding and promoting this plasticity in spared connections has been an important focus of spinal cord research. For example, we now know that lesioned corticospinal axons sprout above the injury level and form new connections, including relaying descending inputs around the injury through spared propriospinal pathways (Bareyre et al. 2004; Courtine et al. 2008; Fouad et al. 2001; Vavrek et al. 2006). Furthermore, spared descending fibers sprout below the injury, a process that is correlated well with recovery in animal models (Ballermann and Fouad 2006; Weidner et al. 2001). Ultimately for such spared and new connections to be functional, the locomotor-related neurons below the injury must be ready (primed) to respond to these enhanced or recovered descending signals.

We propose that this requires compensation for lost neuromodulatory (5-HT) innervation, although testing this idea is difficult with most incomplete lesion models because recovery resulting from changes at the spinal level is hard to distinguish from plasticity in spared descending signals, which include neuromodulatory 5-HT inputs. However, the staggered hemisection model of injury (Courtine et al. 2008; Cowley et al. 2008; Jane et al. 1964) is ideal for our purposes, because most of the direct descending inputs, including 5-HT, are ablated (Fig. 1, white cells), whereas spared local propriospinal neurons relay descending signals around the lesion site (A, black cell), unlike in transected animals, allowing robust spontaneous recovery of independent, fully functional locomotion in the absence of most 5-HT (Courtine et al. 2008; Cowley et al. 2008). We thus used this model to examine what primes or sets locomotor-related neurons below the injury into a ready state in the absence of 5-HT. Here we present detailed kinematic, immunohistochemical, and pharmacological data that demonstrate that it is constitutive activity in 5-HT2 receptors that plays this important role in functional locomotor activity following incomplete SCI. A preliminary report suggesting the involvement constitutive 5-HT2 receptor activity in locomotion after SCI appears in our companion paper (Murray et al. 2010), although this suggestion relied only on qualitative assessment of locomotion and lacked definitive evidence that this 5-HT2 receptor activity was not instead due to residual 5-HT in these staggered hemisected rats rather than constitutive activity. The present paper overcomes these limitations, presenting a quantitative analysis of the role of constitutive activity for locomotion after injury. Our companion paper (Murray et al. 2010) focuses on examining the underlying cellular changes, especially in motoneurons, that lead to constitutive 5-HT receptor activity.

**METHODS**

**Spinal lesions**

All animal use was approved by the University of Alberta Animal Care and Use Committee, complying with Canadian Council for Animal Care guidelines. Under fentanyl (Hypnorm 120 μL/200 g; Janssen, Canada) and Midazolam (0.75 mg/200g; Sabex, Belgium) anesthesia, 14 adult female Sprague-Dawley rats were hemisected on the right at the T6, spinal vertebrae (L2 spinal level) (Ballermann and Fouad 2006). Four weeks later, they were hemisected on the left at the spinal T6 vertebrae (T2 level). Locomotion was evaluated after an additional week. Bladders were expressed three times daily for 3 days until urination recovered. Additional control rats (n = 14) received a complete transection at L2 vertebrae (S2 sacral spinal level) (Bennett et al. 2002; Thomas and Gorassini 2005; Wirz et al. 2005). Staggered spinal lesions.

**FIG. 1.** A staggered hemisection eliminates most serotonin (5-HT) input to lumbar spinal cord. A: staggered lesions were carried out in 2 steps: first, a right hemisection was performed at thoracic T12 level; 4 wk later, a left hemisection was performed at T6 with on average 2.1 mm between these 2 hemisectons. Left: schematic illustrates these lesions, showing that, in theory, they ablate all descending neuromodulatory 5-HT fibers (axons with white cell bodies). Also, descending fibers with classical synaptic transmission (gray cell bodies), such as the corticospinal tract, are ablated by this lesion, whereas propriospinal interneurons (black) have been reported to relay descending signals around the injury site, allowing voluntary initiation of locomotion (Courtine et al. 2008; Cowley et al. 2008). Schematic adapted from Murray et al. (2010). Representative reconstruction of the lesion site from 1 animal is shown to the right of each lesion. B: quantification of the percent of the area of the left and right cord lesioned at T6 and T12 levels, mean (±SE) from all animals (n = 8), showing near complete hemisections, with some spread to the contralateral side. C: following lesion, animals recovered locomotor movements as evidenced by the BBB locomotor score; however, recovery did not correlate with the few serotoninergic fibers counted below the level of the injury (r = 0.27, P < 0.05, n = 8). Open symbols left leg, closed right leg. D and E: example of 1 5-HT-immunoreactive fiber in the lumbar spinal cord below the injury site after a staggered hemisection (E: fine fiber with punctate varicosities; typical of n = 8 rats), compared with the dense network of 5-HT fibers in a normal rat at the same lumbar level (D, n = 5 rats), seen as fine dark fibers surrounding motoneurons (neurons lightly counterstained with cresyl violet). Calibration bar indicates 50 μm.
et al. 2004) and were evaluated 7–12 wk post injury. Additional normal rats were also evaluated (n = 5).

Drug injections

For most experiments, drugs were dissolved in sterile saline and administered locally to the spinal cord via transcutaneous intrathecal (IT) injection. This was done under brief isoflurane anesthesia, using a Hamilton-syringe connected to a 25-gauge needle, inserted between the L₅ and L₆ vertebrae (at cauda equina) as detailed by Mestra et al. (1994). Within 2 min of saline control injections (30 μl), animals were fully alert and walking. Rats with staggered hemisections received one IT injection per experimental session, separated by a week each, for three total injections: 1) SB242084 (3 mM concentration in 30 μl sterile saline, 0.9% NaCl; Sigma-Aldrich, Oakville, Ontario, Canada), 2) SB206553 (3 mM, Tocris), and 3) saline control (30 μl). Locomotion was assessed before and at 5–10 min after injection when peak effects of SB206553 appeared and again at 30-min intervals for ≤2 h.

Rats with complete transections received identical IT injections of SB242084 but in combination with the 5-HT₂ receptor agonist alpha-methyl-5-HT (0.01 mM in the 30 μl; Tocris) as follows (n = 5 rats): 1) alpha-methyl-5-HT, 2) SB242084 (3 mM in 30 μl) plus alpha-methyl-5-HT, and 3) saline control. These injections were given sequentially on the same day but separated by >1.5 h to allow for drug washout. In a separate group of spinal rats (n = 5), the preceding three conditions were repeated, but with SB206553 rather than SB242084. Finally, in three of the preceding transected rats and an additional four transected rats (n = 7), alpha-methyl-5-HT was given by itself at varying doses (0.003–10 mM), on a separate day.

In a few additional experiments, SB206553, SB242084, or cyproheptadine (Tocris) were administered systemically with an intraperitoneal (ip) injection. These drugs were dissolved in 1 ml of a vehicle made from distilled water with 0.6% NaCl, 5% polyethylene glycol (P4338, 3350 molecular wt, Sigma) and 8% hydroxypropyl-beta-cyclodextran (Sigma) by weight (325 mosM final osmolarity), which was needed to fully dissolve the drugs at the concentrations used (Kennett et al. 1996). Vehicle control injections (1 ml ip) had no effect on locomotion.

Locomotor assessment

The iliac crest and fifth metatarsal of the foot were marked on the left and right sides with a black permanent spot. Rats were filmed (JVC Digital-Camcorder-GZ-MG50U, 30 frame/s; 1/250 s shutter speed) while walking across a 1.5 m long Plexiglas runway with a mirror underneath, and markers tracked off-line with ClickJoint analysis software (Alea Solutions). Two parameters were measured throughout the step cycle: 1) iliac crest height and 2) leg angle (angle of the line between the 5th metatarsal joint and the iliac crest, relative to vertical; Fig. 2A). Range of movement of the leg was computed (maximum – minimum leg angle; angle modulation). The locomotor abilities were also assessed using the BBB score (Basso et al. 1995), which rates locomotion of rats on a scale of 0 to 21 (21 being no impairment).

During walking, the length of tail dragging on the ground (Lₕ) was measured with ClickJoint. A tail drag index (TDI) was calculated by dividing Lₕ by the total length of the tail (L) minus the iliac crest height (H; the latter to normalize for differences in weight support; TDI = 100*Lₕ/[L – H]), and averaged over the step cycle.

![Fig. 2. Intrathecal (IT) administration of a 5-HT₂ receptor neutral antagonist does not affect hind limb locomotor function following staggered lesion. A and B: representative frames from video sequence of animals walking on a runway before (A) and after (B) injection of the neutral antagonist SB242084 (3 mM in 30 μl; n = 8 rats tested) to block the action of endogenous 5-HT. Hind limb locomotor function was analyzed by measuring iliac crest height (black dot) as indicator of weight support and the leg angle measured as the angle of a line from the iliac crest to a marker on the foot relative to vertical, throughout the step cycle (right leg; shown in A). Video frames order from left to right in top row and then bottom row. C: modulation of the leg angle and iliac crest height (here on the right side) over 3 step cycles before and after injection. D: quantification of the average range of leg movement (angle modulation; maximum – minimum leg angle) of all animals on the left and right side. The leg angle modulation was significantly less in the right vs. left leg when compared before the SB242084 application (P < 0.05 n = 8). However, SB242084 did not significantly change the leg angle modulation in either leg. E: also, when the iliac crest height (maximum and minimum) was compared with assess weight support before and after SB242084 application, no differences were found in either leg. Weight support (height) was not different in left and right legs. F: these kinematic results were confirmed by the use of the BBB locomotor score, showing no difference before and after SB242084 injection. Thus residual endogenous 5-HT has no influence on hind limb locomotor function in rats with staggered lesion. All bar graphs represent group averages (± SE), n = 8. P > 0.05 was considered insignificant.](image-url)
Electromyogram

Tail muscle activity was measured in transected rats with percutaneous electromyography (EMG) in the segmental tail muscles at the midpoint of the tail while the rat was held in a Plexiglas tube (Bennett et al. 2004). Fine wires (AS631; Cooner, Chatsworth,CA) were inserted percutaneously and secured with cyanoacrylate glue at three locations: 1) two wires at the midpoint of tail for EMG, 2) one wire 1 cm rostral to this, and 3) two wires in the distal tip of the tail for skin stimulation (Bennett et al. 2004). The tail was stimulated with a single current pulse (0.2 ms, 10 mA) every 10 s and repeated six times. EMG activity was recorded using the Axoscope system (Axon Instruments, Burlington, CA) at a sampling rate of 5 kHz. Using Matlab software (MathWorks, Natick, MA), the EMG was rectified and averaged over 500–3,500 ms after stimulation and averaged across the six trials. These measurements were repeated at 5-min intervals before and after drug application and peak responses reported, typically at 5–15 min postdrug.

Histology

Rats with staggered hemisection were killed with Euthanyl (Bimeda-MTC; 70 mg/100 mg) and perfused with 4% paraformaldehyde. Spinal cords were postfixed overnight, cryoprotected in 30% sucrose in phosphate-buffered-saline, cut into two tissue blocks evenly spanning each lesion, cut on a cryostat in horizontal 25 μm sections, and mounted on slides. Spinal cord sections were incubated in a 5-HT primary antibody (1:1,000; S5545; Sigma-Aldrich) overnight at 4°C. Sections were then incubated in a biotinylated secondary antibody (1:200; BA9400; Vector Labs) overnight at 4°C, and then in the Vector ABC elite complex, overnight at 4°C. 5-HT fibers were visualized with the Vector DAB kit. A 0.1% cresyl-violet counterstain was then applied. The primary antibody was omitted in control sections. 5-HT fibers crossing a transverse line placed directly caudal to the lower lesion (~500 μm) were counted from every second section. To assess the injury, the spinal cord at the injury was counterstained with cresyl violet, and the cord area at the injury was measured in each horizontal section and transferred onto a schematic transverse section of normal cord, from which lesion size was computed with Scion-Image (National Institutes of Health).

Data were verified to be normally distributed using the Kolmogorov-Smirnov test. Statistical comparisons were performed using a paired t-test with \( P < 0.05 \) and \( P < 0.01 \) (GraphPad, Prism, San Diego, CA). Data are reported as means ± SE.

RESULTS

Robust stepping despite lack of 5-HT in staggered hemisectioned rats

Rat spinal cords were hemisectioned on the right side at the upper lumbar spinal level and 4 wk later hemisectioned on the left side at the mid-thoracic level (Fig. 1A). This staggered hemisection resulted in an average ablation of 95.1 ± 2.7% (mean ± SE) of the area on the right side and 84.6 ± 4.3% on the left side (Fig. 1B; at lesion sites in transverse section); thus most descending supraspinal axons were severed. However, these lesions may still have allowed some residual descending 5-HT fibers to pass the injury site. Using immuno-histochemistry, we specifically found that only a few 5-HT-positive axons (~20/rat) remained in the lumbar spinal cord (L3–L4) below the caudal injury level (Fig. 1, A and D). Despite this loss of most descending innervation, the animals exhibited a robust spontaneous recovery of hind limb locomotor function. As detailed previously (Courtine et al. 2008), after each hemisection, the affected leg was initially paralyzed and then slowly recovered function over 2 wk post injury. Experiments commenced once steady-state recovery of stepping occurred at 3 wk after the second hemisection. Generally, there was good weight support and toe clearance during walking in both limbs. However, there was always a lack of fore- and hind-limb coordination, and accordingly the mean locomotor BBB score was always <12 in both legs (BBB score cannot exceed 12 without forelimb-hindlimb coordination) (BBB defined in Basso et al. 1995). The BBB score was not significantly correlated with the number of spared serotonergic fibers counted in the lumbar spinal cord (L3–L4) below the injury level, suggesting that 5-HT had no influence on the observed locomotion after the staggered lesion (Fig. 1C).

When evaluated with quantitative video kinematics, we found that the right leg was generally more impaired than the left (Fig. 2; the BBB score also showed this in some rats, although changes in leg movement did not always translate into changes in score). To quantify the kinematics, we measured the overall leg angle (to assess range of movement) and iliac crest height (to assess weight support) throughout the step cycle (Fig. 2C). As seen in Fig. 2D, the range of movement was significantly less in right compared with the left leg, although the weight support (Fig. 2E) was not different between legs.

Lack of effect of 5-HT2 receptor neutral antagonist

To specifically prove that residual 5-HT below the injury has no influence on 5-HT2 receptors, we intrathecally injected the highly selective 5-HT2 receptor antagonist SB242084 (3 mM in 30 μl saline) to locally block 5-HT receptors in the spinal cord below the injury. Importantly, SB242084 is a neutral antagonist that blocks the action of 5-HT (or other agonists) on the 5-HT2 receptor but does not inhibit constitutive receptor activity (Channion et al. 2008; Seifert and Wenzel-Seifert 2002). This injection did not affect the locomotor capabilities of the rats, as shown in a representative animal (Fig. 2, A and B) and quantified by leg angle and iliac crest height throughout the step cycle (C). Across all animals, the range of leg movement (leg angle modulation) and weight support were not significantly different after SB242084 (Fig. 2, D and E). These results were confirmed when using the BBB locomotor scores of each leg, where no significant change in the locomotor performance was detected with injection of SB242084 (Fig. 2F) consistent with a lack of effect of residual 5-HT in activation of the 5HT2 receptors involved in locomotion. When given systemically SB242084 (1.5 mg/kg ip), likewise had no significant effect on the BBB score (mean: 9.6 ± 0.6 and 9.8 ± 0.6 pre- and postdrug, respectively, \( P > 0.05, n = 5 \); scores from both legs averaged), suggesting that even 5-HT2 receptors at or above the staggered hemisection are not needed for locomotion.

Elimination of stepping and muscle tone with 5-HT2 receptor inverse agonist

Considering the robust locomotor recovery, we examined whether the loss of 5-HT after injury was compensated for by constitutive activity in 5-HT2 receptors by intrathecally injecting SB206553 (3 mM in 30 μl saline), which selectively binds to 5-HT2 receptors and potently inhibits constitutive activity in...
these receptors (termed inverse agonist) (Chanron et al. 2008; Seifert and Wenzel-Seifert 2002). Within minutes of blocking the constitutively active receptors with the SB206553 injection, there was a dramatic decline in hindlimb locomotor ability and some animals exhibited complete loss of hindlimb weight support over the next 10 min, as illustrated for the animal in Fig. 3, A–C. The quantitative analysis from all animals demonstrates a highly significant drop in the range of leg movement (angle modulation; Fig. 3D) and weight support (E) with SB206553. Similarly, the BBB locomotor score dropped considerably on both sides. The largest effects of SB206553 were on the right leg, with full recovery of locomotor ability by 20–30 min post injection. Control intrathecal (IT) injections of saline (30 μl) or the neutral antagonist SB242084 (see preceding text) had no significant effects on locomotion, ruling out direct effects of the injection procedure itself. In conclusion, these results demonstrate that although 5-HT itself is not available in these animals (sweeping) seen in all animals tested, suggesting that the locomotor input to the motoneurons was not eliminated. This rhythmic activity was quantified from the kinematics recordings as the range of leg/foot movement during locomotion (Fig. 3, C and D) and was always significantly different from zero \( (P < 0.01, n = 8) \). Even when SB206553 halved the range of movement in the most severely impaired limb (right leg), there were still foot movements remaining (~25° range of movement; Fig. 3D).

We noted that the tail was also affected by SB206553, becoming flaccid and dragging on the ground during walking \( (TDI > 90\%) \), whereas prior to the drug substantial tail tone elevated the proximal half of the tail off the ground \( (TDI \) significantly increased by SB206553; Fig. 4; see also Fig. 3A video frames), similar to that in normal rats where the tail is lifted during walking. In contrast, the neutral antagonist SB242084 had no significant effect on tail activity (no change in TDI, Fig. 4). Thus tail muscle activity, like hindlimb activity, relies on constitutively active \( 5HT_2 \) receptors after SCI.

When given systemically, SB206553 (3–15 mg/kg ip) likewise severely impaired locomotion with loss of weight support and BBB scores dropping significantly to \( 5.1 \pm 0.8 \) from \( 10.0 \pm 0.4 \) \( (P < 0.01, n = 5 \) staggered hemisected rats tested), even though control IP injections of SB242084 had no effect (see preceding text). These systemic injections had effects that lasted much longer than the local IT injections, peaking at 20–30 min and locomotion only recovering >60 min post injection.

FIG. 3. Constitutive \( 5HT_2 \) receptor activity is critical for hind limb locomotor function following staggered lesion. A and B: representative frames from the video sequence of 1 step cycle (as in Fig. 2) before and after intrathecal injection of the inverse agonist SB206553 \( (3 \text{ mM in } 30 \mu l, n = 8 \text{ rats tested}) \) to block constitutive receptor activity. C: kinematic analysis over a few step cycles of this animal illustrates a dramatic reduction in the leg angle modulation and weight support (iliac crest height) following injection (solid symbols). D: leg angle of movement (angle modulation) before \( (□) \) and after \( (■) \) injection in all animals shows a highly significant decrease in both the left and right legs with SB206553. Furthermore, the right leg showed a significantly larger decrease, compared with the left \( (P < 0.05, n = 8) \). E: comparison of the iliac crest height shows a highly significant decline in weight support on the left side for both the maximal and minimal value. The horizontal line indicates the height of the iliac crest after complete loss of weight support. As the performance of the right side preinjection was inferior to the left side, the drug effect is not as dramatic here, with a significant reduction of the maximal value only, but still showing a complete loss of weight support. F: the postinjection BBB scores reflect the dramatic decrease in locomotor performance being highly significant in both legs. All bar graphs represent group means \((±SE), n = 8. *P < 0.05, **P < 0.01\).
Positive control for neutral antagonist

To independently verify the efficacy and selectivity of SB242804 and SB206553 at our IT injection dose, we examined whether these drugs could antagonize the action of the selective 5-HT\textsubscript{2} receptor agonist alpha-methyl-5-HT after SCI. We felt that the staggered hemisection animals had such robust locomotion to start with that agonist effects might be difficult to quantify. Thus for this purpose, we assessed IT drug efficacy on tail muscle activity, using EMG measurements (Bennett et al. 2004; Murray et al. 2010). Also we used the simple fully transected chronic spinal rat, rather than the staggered hemisection rat, because the transected rat has pronounced tail muscle activity (spasms evoked by cutaneous stimulation) that we have previously shown increases with 5-HT agonists (Li et al. 2004; Murray et al. 2010). Intrathecal application of the 5-HT\textsubscript{2} receptor agonist alpha-methyl-5-HT significantly increased tonic tail muscle EMG activity (spasms, Fig. 5, A and B) in a dose-dependent fashion with a 50\% effect at a dose of 0.012 ± 0.005 mM (EC\textsubscript{50}) and maximal effects at 0.1–0.3 mM (delivered in 30 \mu l saline; n = 7). Intrathecal application of SB242804 at the dose used during the preceding locomotor experiments (3 mM in 30 \mu l) completely antagonized the effect of alpha-methyl-5-HT applied at 0.01 mM (at EC\textsubscript{50}, Fig. 5, A and B), verifying that the dose we used was appropriate to antagonize the 5-HT\textsubscript{2} receptor even though it has no effect on walking or tail tone by itself (neutral antagonist). Intrathecal application of SB206553 (3 mM, 30 \mu l) also antagonized alpha-methyl-5-HT (Fig. 5C), consistent with its previous classification as a 5-HT\textsubscript{2} receptor inverse agonist (which block both constitutively and agonist-activated receptors) (Herrick-Davis et al. 1999). SB206553 significantly antagonized alpha-methyl-5-HT at lower doses (0.1–1 mM) although the effect was maximal and most rapid at 1–10 mM; thus we used the supramaximal 3 mM dose for the main staggered hemisection experiments in the preceding text.

D I S C U S S I O N

Over the last few years a relation between spontaneous functional recovery after SCI and plasticity in spinal neuronal circuits has been generally recognized (Ballermann and Fouad 2006; Bareyre et al. 2004; Barriere et al. 2008; Courtine et al. 2008; de Leon et al. 1998; Girgis et al. 2007; Weidner et al. 2001). How exactly this plasticity translates into recovery and...
the detailed neuronal mechanisms remain unclear. Suggested mechanisms include axonal sprouting (Ballermann and Fouad 2006; Bareyre et al. 2004; Fouad et al. 2001; Weidner et al. 2001) and changes in neurotransmitter systems intrinsic to the spinal cord pattern generating networks (glucose and glutamate) (Giroux et al. 2003; Tillakaratne et al. 2002). Here we demonstrate a powerful mechanism that contributes to spontaneous locomotor recovery after severe SCI; that is, 5-HT$_2$ receptors become constitutively active, replacing the spinal cord’s dependence on brain-stem-derived 5-HT and ultimately playing an important role in walking and general muscle activity after injury. Such constitutive receptor activity has previously been demonstrated for motoneurons after SCI (Murray et al. 2010), and the present results extend this to the involvement of constitutive receptor activity in the locomotor output. This constitutive 5-HT$_2$ receptor activity must work together in concert with the many other mechanisms needed for functional walking, including propriospinal relays (Courtine et al. 2008; Cowley et al. 2008) and other receptors (Jordan et al. 2008).

Our conclusions are based primarily on our finding that blocking constitutively active 5-HT$_2$ receptors with the selective inverse agonist SB206553 dramatically inhibits hindlimb stepping, weight support, and tail muscle tone. Interestingly many of the classic nonselective 5-HT receptor blockers like ketanserin and cyproheptadine are now recognized as potent 5-HT$_2C$ receptor inverse agonists, which block both constitutive receptor activity and classic 5-HT-mediated receptor activity (Westphal and Sanders-Bush 1994). Thus in retrospect, a number of studies showing effects of these compounds on rhythmic activity after injury (MacLean et al. 1998; Musienko et al. 2008; Wainberg et al. 1990) are perhaps explained by our conclusion, although the possibility of such constitutive activity was not considered before.

However suggestive, the inhibitory action of inverse agonists after SCI is not by itself definitive proof that 5-HT$_2$ receptors are constitutively active because inverse agonists inhibit receptor activity mediated by traditional binding of 5-HT to the receptor in addition to blocking receptor constitutive activity (Kennett et al. 1996; Seifert and Wenzel-Seifert 2002; Westphal and Sanders-Bush 1994). Thus our finding that blocking 5-HT binding to the 5-HT$_2$ receptors with the neutral antagonist SB242084 has no effect on stepping, weight support, or tail muscle tone is crucial to our conclusion because it rules out the possibility that residual 5-HT is activating the 5-HT$_2$ receptors (neutral antagonists do not block constitutive activity). Additionally, we purposely chose models of SCI (staggered hemisection and transection) where there is little or no residual 5-HT below the injury, and this together with the SB242084 data provide definitive proof that SB206553 acts by blocking constitutively active 5-HT$_2$ receptors.

The compounds SB206553 and SB242084 are highly selective to 5-HT$_2$ receptors, binding to these receptors with low nanomolar doses in vitro ($K_i$ values) but have little binding, if at all, to a wide range of other receptors (>100-fold selectivity, $K_i > 1,000$ nM) (Kennett et al. 1996, 1997; Knight et al. 2004). Of the 5-HT$_2$ receptor subtypes, SB206553 and SB242084 bind to both 5-HT$_{2C}$ and 5-HT$_{2B}$ receptors with high affinity but not appreciably to 5-HT$_{2A}$ receptors (Knight et al. 2004). Thus the effects that we report are likely mediated by 5-HT$_{2C}$ or 5-HT$_{2B}$ receptors. Additionally, we find that the dose of SB206553 and SB242084 that we used potently inhibits the action of a selective 5-HT$_{2B/C}$ agonist (alpha-methyl-5-HT; titrated to a minimal dose to assure selectivity; Fig. 5), which confirms that these drugs acted on 5-HT$_2$ receptors and verifies that SB242084 acts in our system as a neutral antagonist.

While the intracellular pathways activated by 5-HT$_2$ receptors are relatively poorly understood in the spinal cord, these receptors are Gq$_i$-protein coupled and thus are likely to activate classic phospholipase-C (PLC) pathways, which involve synthesis of inositol phosphates (IP) and mobilization of intracellular Ca$^{2+}$ stores (Hoyer et al. 2002). Consistent with this, we know that intracellular second messenger signaling by IP and Ca$^{2+}$ are involved in facilitating persistent inward currents and NMDA receptors in spinal motoneurons (Holohan and Hacking 2004; Mejia-Gervacio et al. 2004; Perrier et al. 2000) and have been suggested to be involved in rhythmic locomotor activity (Grob and Guertin 2007). 5-HT$_{2C}$ receptors, like many other receptors (Seifert and Wenzel-Seifert 2002), produce a basal level of intracellular signaling (IP production), in the absence of 5-HT, by constitutive receptor activity. This constitutive activity is inhibited by inverse agonists like SB206553 but not inhibited by neutral antagonists like SB242084 even though both these classes of drugs antagonize normal 5-HT bound receptor activity (Chanrion et al. 2008; Kennett et al. 1996, 1997; Seifert and Wenzel-Seifert 2002). Certain native isoforms of the 5-HT$_{2C}$ receptor exhibit a substantial amount of constitutive activity, spontaneously raising the basal intracellular levels of IP in the absence of 5-HT to a level approaching that achieved with 5-HT (Herrick-Davis et al. 1999; Weiner et al. 2001). In our companion study, we show that one of these isoforms (INI) is up-regulated with SCI (Murray et al. 2010), and this likely causes the increased constitutive receptor activity.

Broadly speaking, two fundamental events are together necessary and sufficient to produce locomotion after injury: 1) the spinal neurons below the injury must be ready or primed for activity and 2) these neurons must be triggered into action by spared connections from the brain (including indirect propriospinal-mediated connections) (Courtine et al. 2008) or peripheral sensory input, such as perineum stimulation (Barriere et al. 2008). In the adult animal, such priming takes weeks because animals cannot step immediately after a transection (Barriere et al. 2008) or two simultaneous staggered hemisections (Courtine et al. 2008). Furthermore, priming of spinal neurons occurs even after partial injuries because weeks after recovery from a hemisection, cats step nearly immediately after a subsequent complete transection (Barriere et al. 2008). Our results show that this priming of spinal neurons fundamentally requires constitutive activity in 5-HT$_2$ receptors; over time this allows the neurons to function independently of supraspinal synaptic input, analogous to how cultured neurons slowly regain their intrinsic oscillatory properties after days in isolation (Turrigiano et al. 1994). Previous work has shown that the function of spinal neurons after injury also depends on NMDA receptor plasticity (Giroux et al. 2003), but this may be ultimately linked back to our finding because NMDA receptor function in locomotion is dependent on 5-HT$_2$ receptor activity (MacLean et al. 1998).

5-HT at or above the injury does not seem to be required for locomotion in our staggered hemisected rats because the neutral antagonist SB242084 did not impair locomotion even when given systemically.
In spinal cord intact animals, 5-HT receptors are also important for locomotion because a block of 5-HT2 receptors with ketanserin or cyproheptadine inhibits locomotion in decerebrate cats (Gerasimenko et al. 2009). However, these receptors are not essential for stepping because unlike with SCI, other transmitter systems can replace/bypass their function, overcoming the effects of a 5-HT2 receptor block with ketanserin (Gerasimenko et al. 2009). This is consistent with our finding that normal rats compensate for a selective block of 5-HT2 receptors with SB206553 with no obvious impairment in function. The finding of some impairment of locomotion with ketanserin and cyproheptadine (Gerasimenko et al. 2009), whereas no impairment with SB206553, may be due to the fact that ketanserin and cyproheptadine block adrenergic receptors as well as 5-HT receptors (Yoshio et al. 2001), whereas SB206553 is highly selective to 5-HT2B and 5-HT2C receptors (Kennett et al. 1996). This is also consistent with our finding that cyproheptadine impairs locomotion in normal rats, probably by its joint action on serotonergic and adrenergic systems (Yoshio et al. 2001). This further underscores the redundancy in the locomotor system in spinal cord intact animals: serotonin and adrenergic systems are involved in locomotion (Rossignol 2006), and blocking both these systems more likely impairs walking in normal animals that just one, but even then other transmitter systems can still compensate. Presumably with incomplete SCI, these other transmitter systems can complement the 5-HT receptor system in recovering function unlike with the severe spinal cord injury that we studied.

The present study provides only indirect evidence for which spinal cord cell types express the constitutively active 5-HT2 receptors involved in locomotion after injury. First, the near complete loss of weight support with SB206553 suggests that motoneuron output is impaired. This is consistent with our companion paper (Murray et al. 2010), which directly demonstrates that constitutive activity in 5-HT2C receptors on motoneurons is increased with SCI. Second, the present study also suggests that while the interneurons involved in locomotion may be affected by 5-HT2 receptors, their activity does not depend entirely on constitutive activity in 5-HT2 receptors because rhythmic foot movements were still seen during locomotion after blocking constitutive activity in 5-HT2 receptors with SB206553. This evidence does not, however, rule out some role for 5-HT2 receptors on interneurons in locomotion. For example, Hochman and colleagues (Shay et al. 2005) demonstrated the presence of 5-HT2C receptors in the deep dorsal horn, intermediate gray matter, and motor nuclei associated with areas with locomotor-related interneurons and motoneurons (Butt et al. 2002). Likewise, propriospinal neurons do not seem to depend much on 5-HT receptors for their activation (Gerasimenko et al. 2009) even though these neurons trigger locomotor activity in the staggered hemisection model (Cowley et al. 2008) and can respond to exogenous 5-HT application (K. C. Cowley, personal communication).

Interestingly, after a complete spinal cord transection, constitutive 5-HT2 receptor activity can become so excessive that it produces uncoordinated muscle spasms (Murray et al. 2010), whereas muscle activity is generally well coordinated following a staggered hemisection, producing functional locomotion (Fig. 2). Thus the amount of constitutive receptor activity may vary with different injuries, and this may well help explain the wide variation in functional recovery and spasticity following SCI. Because 5-HT is absent in both these injury models, it appears that something other than the simple absence of 5-HT may modulate the constitutive receptor activity. Perhaps constitutive receptor activity is regulated to bring neurons to an optimal level of excitability regardless of the injury, and so more constitutive activity occurs in preparations with less residual voluntary activity. Indeed it is telling that the left leg of the rats below our lower T12 hemisection seems to depend less heavily on constitutive receptor activity, because blocking these receptors with SB206553 has a lesser effect, compared with the right leg, and this leg is less affected by the injury in the first place. The left side of the spinal cord below the injury, and not the right, receives propriospinal projections that relay descending commands around the injury, producing excitation not available to the right side of the spinal cord (Fig. 1A), and thus this better innervated portion of the spinal cord may require less constitutive 5-HT receptor activity to maintain its overall excitability.

Understanding naturally occurring repair mechanisms of the CNS provides an opportunity to develop future treatments for injuries and disorders of the entire nervous system. Our results demonstrate that functional benefits are not inevitably based on plasticity or regeneration of descending input but can be caused by changing the activity of receptors and neurons below the level of the injury. Experimental approaches to enhancing functional recovery thus could very well focus on altering 5-HT2 receptor activity, whether by direct 5-HT agonist application or 5-HT cell transplants to increase 5-HT receptor activity (Guerin 2004; Ribotta et al. 2000) or by inverse agonists or genetic manipulations to alter expression of constitutively active 5-HT receptors (or other relevant receptor systems, like 5-HT2 receptors).

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**


Constitutive Receptor Activity and Walking after SCI


