SUPPLEMENTARY INFORMATION

Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT$_{2C}$ receptors.

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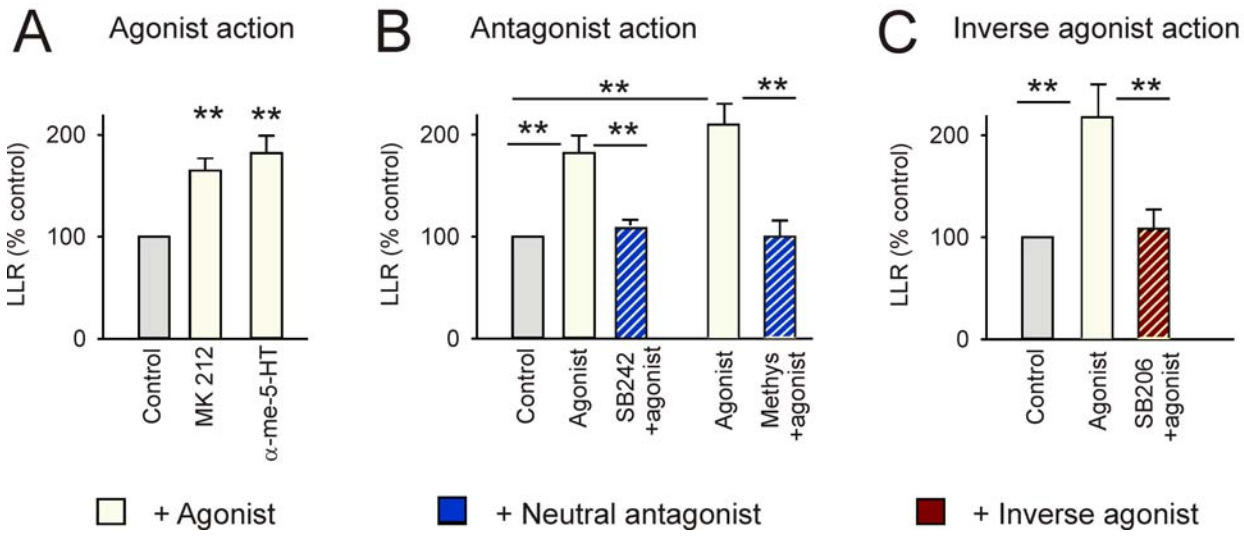

Supplementary Figure 1. 5-HT$_{2C}$ receptor agonists augment spasms and antagonists reverse this. (**A**) Same chronic spinal rat preparation and format as in **Fig. 2**. The selective 5-HT$_{2C}$ agonist MK212[30] (0.3 μM, $n = 8$) and the selective 5-HT$_{2A/B/C}$ receptor agonist α-methyl-5-HT[30] (0.3 μM, $n = 8$) increased the LLR (spasm) recorded *in vitro*, showing that 5-HT$_{2C}$ receptors are involved in spasms. (**B, C**) Application of SB242084 (SB242; 3 μM, $n = 8$), methysergide (Methys; 10μM, $n = 8$) or SB206553 (SB206; 3–5 μM, $n = 8$) antagonized the action of the agonist α-methyl-5-HT (0.3 μM). This demonstrates that both SB242084 and methysergide are effective antagonists, even though they have no effect in the absence of exogenously applied agonists (neutral antagonists, **Fig. 2b,d**). Also, the finding that SB206553 blocked the action of this agonist, confirms its specificity to 5-HT$_2$ receptors, and is consistent with the action of inverse agonists, which block both constitutive *and* ligand-bound receptor activity[31]. $^{**}P < 0.01$. Error bars, s.e.m.

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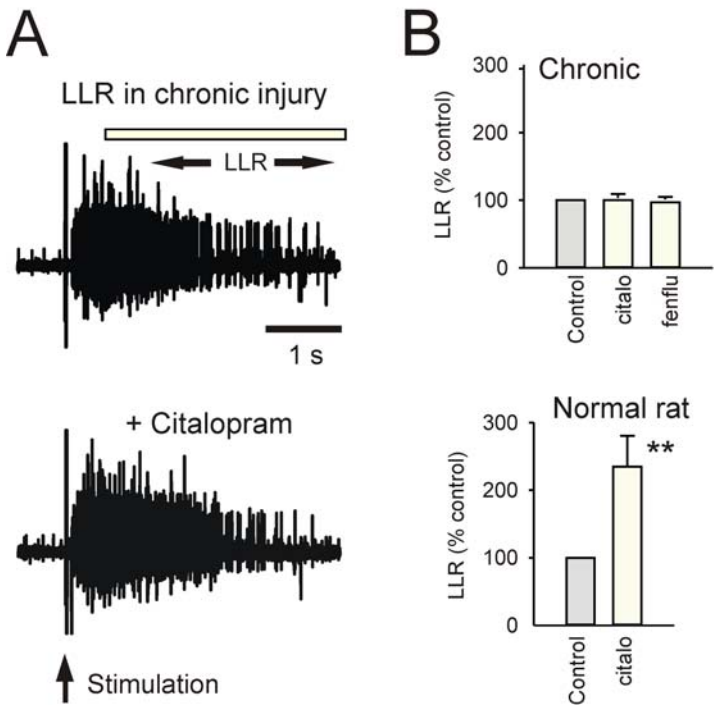

Supplementary Figure 2. Enhancing available residual 5-HT has no effect on spasms. (**A**) In the chronic spinal rat, dorsal root stimulation (3xT) triggered long-lasting reflex responses recorded on the ventral roots *in vitro* (LLRs, quantified as mean rectified activity during horizontal bar). Blocking re-uptake of residual 5-HT with the SERT blocker citalopram[32] (10 μM) did not alter the LLR. (**B**) Group means of LLRs demonstrate no significant change with citalopram (citalo; $n = 8$). Additionally, forcing presynaptic release of 5-HT with the potent 5-HT releaser fenfluramine[33] (fenflu; 10 μM; $n = 12$) had no significant effect on the LLRs. As a positive control, we showed that the LLR was significantly enhanced by citalopram (10 μM; $n = 16$) in sacral spinal cords from normal rats maintained *in vitro* (normal cords have abundant stores of endogenous 5-HT for citalopram to act on[34]; **Fig. 1b**). These normal spinal cords do not have LLRs in control pre-drug conditions[35]. Thus, to match control conditions in the chronic spinal rat, we treated normal cords with a low dose of the glycine receptor blocker strychnine (3 μM) to induce LLRs[36] prior to citalopram application. Citalopram was then tested in the continued presence of strychnine in normal cords. $^{**}P < 0.01$. Error bars, s.e.m.

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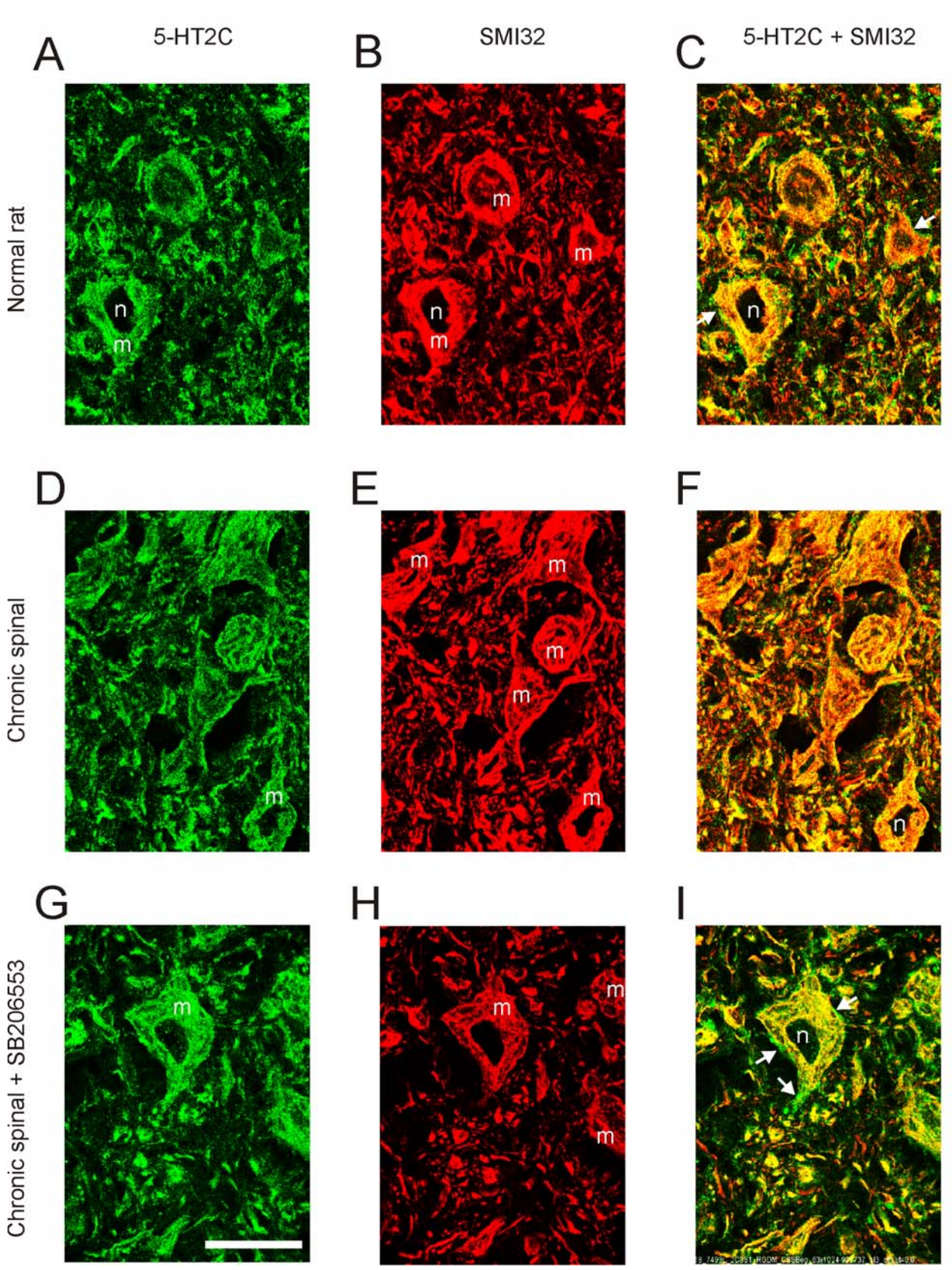

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Supplementary Figure 3. 5-HT_{2C} receptor distribution on motoneurons after SCI. (**A**) Immunofluorescence labeling of 5-HT_{2C} receptors (green, SC-15081 antibody) in the S4 sacral ventral horn of a normal rat, showing extensive receptor distribution, including labeling of the soma of motoneurons (m; nucleus, n), as visualized with 0.5 μm thin optical sections with confocal microscopy ($n = 5$). (**B**) Double-labeling of the same tissue with SMI32 (red), a selective marker of motoneurons in the ventral horn (m, SMI32-positive motoneuron soma), which specifically labels neurofilament in the intracellular space[29]. (**C**) An overlay of 5-HT_{2C} and SMI32-labeling revealed extensive co-localization (yellow and orange), indicating 5-HT_{2C} receptors inside motoneuron soma and dendrites (note perinuclear staining; yellow near nucleus, n). Additionally, there was 5-HT_{2C} receptor labeling (green) in isolation, including a halo of green labeling that surrounded SMI-labeled motoneurons (arrows, putative membrane), suggesting 5-HT_{2C} receptors in (or adjacent to) the motoneuron membrane in normal rats (all rats tested showed a similar receptor distribution, $n = 5$ normal rats). The halo of 5-HT_{2C} receptor labeling *not* co-localized with SMI32 was quantified for individual motoneurons by computing the receptor density in a 0.7 μm wide band around the perimeter of the SMI32-labeled soma. The receptor density in this perimeter band around motoneurons (not co-localized with SMI32) was 59.6 ± 4.4% of the mean receptor density inside the motoneuron (co-localized with SMI32, $n = 5$). Mean ± s.e.m.

(**D–F**) Immunolabeling in the sacral ventral horn (below injury) of a chronic spinal rat also showed 5-HT_{2C} receptors inside motoneurons (yellow/orange co-localization with SMI32), but in this case there was less 5-HT_{2C} labeling in isolation (less green in **F**), suggesting fewer receptors in the membrane (all rats tested showed a similar receptor distribution, $n = 5$ chronic spinal rats). On average, the receptor density not co-localized with SMI32 around the perimeter of motoneurons (in the 0.7 μm band) was 27.3 ± 2.7% of the receptor density inside motoneurons (co-localized with SMI32) in chronic spinal rats, significantly lower than that in normal rats (50% lower, $n = 5$, $P < 0.01$). Thus, after SCI the 5-HT_{2C} receptors appear to be highly internalized in motoneurons, consistent with the presence of constitutively active isoforms of the receptors (like INI isoform), which are characteristically found internalized. That is, the INI isoform has previously been shown to be so constitutively active that as soon as it enters the membrane it couples its G protein and causes intracellular signaling that culminates in receptor phophorylation and internalization[37,38]. This is followed by recycling of the receptor back into the membrane and further constitutive activity, but the internalization rate exceeds the recycling rate, and so the receptor isoform accumulates mostly inside the cell (even though there is a steady G protein activation), unlike in other isoforms[37,38].

(**G–I**) Blocking the INI receptor isoform activity with the inverse agonist SB206553 stops the process of receptor internalization and allows the receptor to accumulate in the membrane[37,38]. Thus, we examined the 5-HT_{2C} receptor distribution in chronic spinal rats treated for 2 hrs with SB206553 (with two IT injections of 10 mM in 30 μL saline, separated by 1 hour, $n = 4$). In these treated rats there was considerable 5-HT_{2C} receptor labeling in isolation (green in **I**), including labeling that surrounded SMI-labeled motoneurons (green halo, shown at arrows), suggesting substantial 5-HT_{2C} receptors in the motoneuron membrane, unlike in untreated chronic spinal rats. On average, the receptor density *not* co-localized with SMI32 around the perimeter of motoneurons (in the 0.7 μm band) was 58.7 ± 3.6 % of the receptor density inside motoneurons (co-localized with SMI32) in treated chronic spinal rats, significantly higher than that in untreated chronic spinal rats (double, $P < 0.01$). This is consistent with the concept that inactive receptors accumulate in the membrane. It also verifies that the present immunolabeling can adequately distinguish membrane receptors from internalized receptors, similar to how internalized μ opiod receptors can be visualized with immunolabeling[39]. Scale bar 50 μm.

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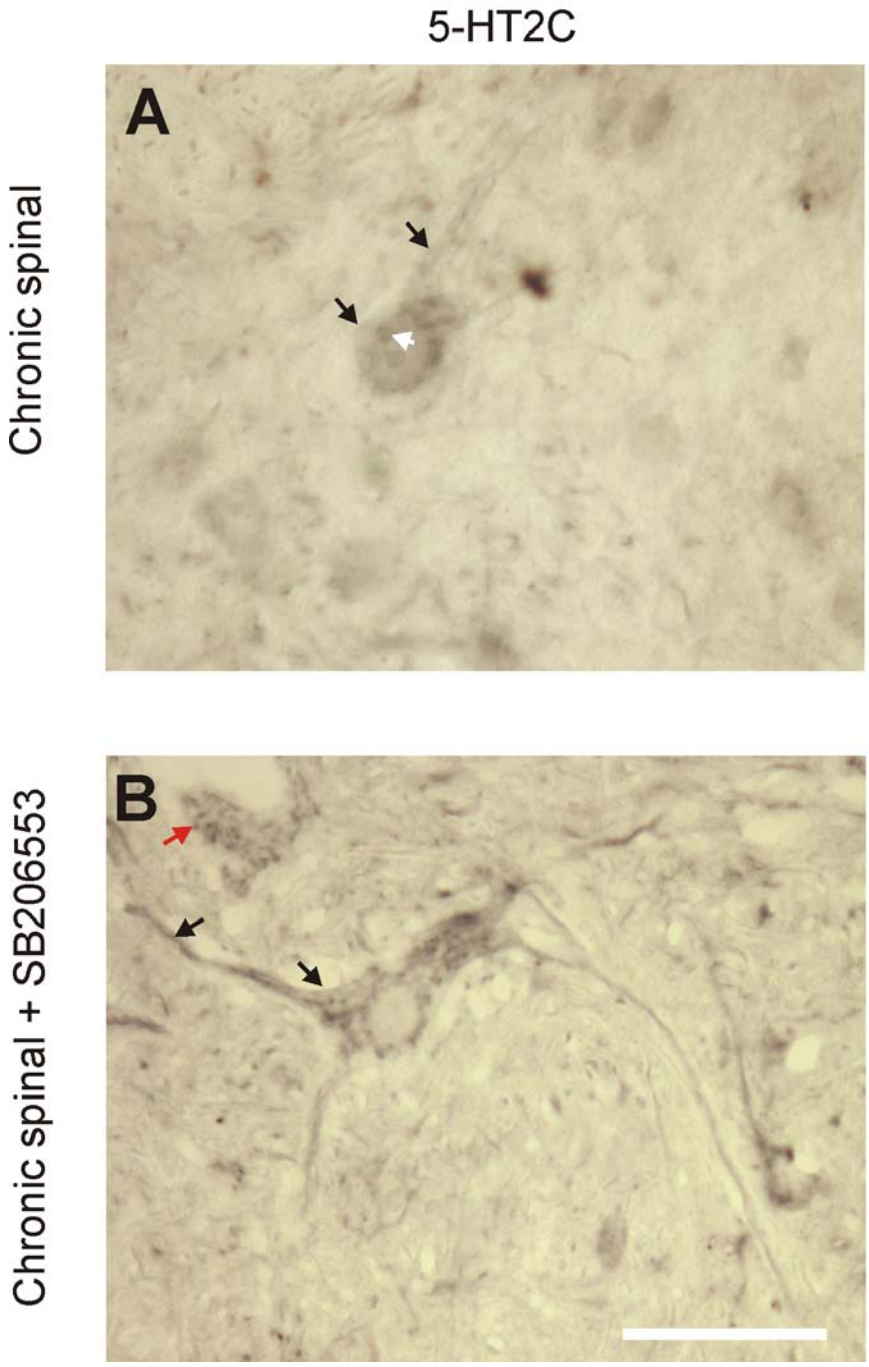

Supplementary Figure 4. 5-HT_{2C} receptor internalization is reduced by SB206553. Similar format to **Supplementary Fig. 3**, but labeling done with a different 5-HT_{2C} receptor antibody (ab32172) and DAB immunohistochemistry. (**A**) Typical 5-HT_{2C} receptor labeling in ventral horn of a chronic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

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(at red arrow). These observations are consistent with the concept that SB206553 enables receptors to accumulate in the membrane by blocking their constitutive activity, whereas in untreated chronic spinal rats the receptors are largely internalized. Scale bar 50 μm.

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

Supplementary Table 1. Human spinal cord injured subjects. The table shows subject number plus sex (M, male; F, female), muscle studied (TA, tibialis anterior), age of subject at the time of experiment, injury level, years post-injury, the AIS – International Standards for Neurological and Functional Classification of Spinal Cord Injury[40], and the MRC manual muscle test score. The latter is a qualitative assessment of the voluntary muscle strength of the examined muscle with 0 = no muscle contraction visible or palpable; 3– = greater than 50% range of motion against gravity; 3 = complete range of motion against gravity; 3+ = complete range of motion against gravity with minimal resistance; 4+ = can contract against moderate to strong pressure.

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Supplementary Methods

Sacral spinal injury model in rat and relation to human spasticity. The S2 sacral spinal cord was transected in rats as described previously[1-3]. Briefly, under general anesthetic (sodium pentobarbital, 58.5 mg·kg^{-1}) and sterile conditions, a laminectomy was performed on the L2 vertebrae to expose the S2 spinal cord. The dura was slit transversely, and 0.1–0.3 ml Xylocaine (1%) was applied topically. Under a surgical microscope, the spinal cord was transected by holding the pia with forceps and sucking under the pia with a fine suction tip. Caution was needed to avoid damaging the anterior artery or posterior/dorsal vein, since the sacrocaudal spinal cord dies without this midline vasculature. The dura was closed with two 8-0 silk sutures, and the muscle layers and skin were tightly sutured over the cord, and the rat allowed to recover. We evaluated spasticity and motoneuron properties 6–12 weeks post-injury (chronic spinal rats). This injury only affects the tail (not bladder or hindlimbs).

Previously, we have shown that the spasticity that emerges in the tail of chronic spinal rats closely mimics the human spasticity syndrome seen in the legs after SCI, with clonus, hypertonus, hyperreflexia, spasms, and general delayed onset of symptoms[3]. Flexor spasms emerge first, followed by extensor spasm (months), as also seen in humans[3]. Finally, we have recently shown that the same ionic mechanism that mediates spasms in the tail of rats also mediates spasms in humans (PICs in motoneuron)[4], thus justifying the use of the sacral spinal rat in the current paper.

Staggered hemisection model of locomotor recovery after SCI and rationale for use. To examine recovery of locomotion after SCI, rats underwent a staggered hemisection[5]. Rats were first hemisected on the right at the T12 spinal vertebrae (L1–L2 spinal level)[6]. Then, four weeks later, they were hemisected on the left at the spinal T6 vertebrae (T6–T7 level). Locomotion was evaluated after another 3 weeks using the BBB score[7]. Bladders were expressed 3 times daily for 3 days, until bladder function recovered. In this staggered hemisection model all direct descending inputs, including 5-HT, are cut (**Fig. 6a**, white and gray neurons), whereas *spared* local propriospinal neurons (**Fig. 6a**, black neurons) relay descending signals (originating from supraspinal neurons; **Fig 6a**, gray neurons) around the lesion site. This allows robust spontaneous recovery of voluntary locomotion (unlike in transected animals) in the absence of most 5-HT[5,8].

Spinal neurons involved in locomotion require neuromodulators like 5-HT to function, setting them into a state of readiness for movement generation[9,10]. The critical importance of these neuromodulators has been demonstrated by the finding in animal models that locomotor-activity can be regained soon after spinal transection with the exogenous application of drugs that activate the neuromodulatory 5-HT, norepinephrine and dopamine receptors (*in vivo* and *in vitro*)[11,12-15], including 5-HT$_2$ and 5-HT$_7$ receptor agonists[16-18]. Notably, over the weeks after injury, locomotor-like movements spontaneously emerge and improve with use[19-21], as if the neuromodulatory receptor systems (e.g. 5-HT$_2$ receptors) are somehow re-activated in the absence of 5-HT. We propose that this spontaneous recovery of locomotion is in part due to constitutive 5-HT receptor activity, and we investigated this with the staggered hemisection model.

Spasms in awake spastic rat. Tail muscle spasms were measured with percutaneous EMG (electromyogram) wires inserted in segmental tail muscles at the midpoint of tail and with kinematic recordings of tail flexion (with video), while the rat was in a Plexiglas tube, as detailed previously (**Fig. 1a,e**)[2]. During EMG recording, muscle spasms were evoked with electrical stimulation of the skin at the distal tip of the tail (cutaneous stimulation; 0.2 ms, 10 mA pulse; 2–

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3x afferent threshold [T]; 6 spasms evoked at 10 s intervals for a trial; trials repeated at 12 min intervals) and the tail was restrained from moving. During kinematic recording, spasms were evoked with mechanical stimulation of the tail skin[2,22], and the tail was free to move, which caused sensory input that extended spasms over many minutes (**Fig. 1e**). EMG was sampled at 5 kHz, rectified and averaged over a 10–40 ms interval post-stimulus to quantify the short latency reflex (SLR), and a 500–4000 ms interval to quantify spasms (long lasting reflex, LLR; using Axoscope, Axon Instr., and Matlab, Mathworks). EMG over 300 ms prior to stimulation was also averaged (background). Tail flexion angle was computed as described previously[22] and averaged over 3 mins post-stimulation.

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

Ventral root recordings of long-lasting reflexes in rats, *in vitro*. Under urethane anesthesia (1.8 $g \cdot kg^{-1}$) the whole spinal cord caudal to the S2 injury level was removed from chronic spinal rats (and age-matched normal rats) and immersed in oxygenated artificial cerebrospinal fluid (ACSF; flowing 8 $ml \cdot min^{-1}$); recordings were made starting 2.5 hr later, as detailed previously[1,23]. Ventral (S4 and Co1) and dorsal (Co1, coccygeal) roots were mounted on silver wires above the ACSF and covered with Vaseline. The dorsal root was stimulated with a single pulse (0.1 ms, 0.02 mA, 3xT; repeated 5 times at 10 s intervals for one trial, trials repeated every 12 mins), and the long-lasting reflex (LLR) response was recorded on the ventral roots, and then analyzed as for the EMG. We quantified the LLR by averaging the rectified ventral root activity over a time-window 500–4000 ms post stimulus, a period previously shown to reflect the motoneuron Ca PIC activity in isolation. We also measured the transient short latency reflex (SLR) by averaging ventral root responses over a window 10–40 ms post stimulus (polysynaptic reflex with about 10 ms central delay). Because of slow diffusion in *whole* spinal cord preparations[24,25], drugs required 10x higher concentrations than in thin slice preparations, and peak effects of even TTX (2 μM; sodium channel blocker) required 10–15 mins. To assure selectivity of drugs, they were titrated to a minimal dose that produced peak effect, and results were reported after 25–45 min of drug application.

Intracellular recordings, *in vitro*. Sharp intracellular electrodes (30 MΩ; filled with 1M K-acetate and 1M KCl) were advanced into the spinal cord with a stepper motor (666, Kopf, Instr.) to penetrate motoneurons (identified by antidromic ventral root stimulation), as detailed before[1,26]. Intracellular recordings were made with an Axoclamp2B amplifier (Axon) running in discontinuous-single-electrode voltage-clamp (gain 0.8–2.5 $nA \cdot mV^{-1}$; for Ca PICs) or discontinuous-current-clamp (switching rate 5 kHz; for EPSPs) mode, filtered at 3 kHz, and sampled at 6.7 kHz (Clampex and Clampfit used; Axon Instr.). In the presence of TTX (2 μM) to block synaptic transmission and sodium currents (Na PIC), slow triangular voltage ramps (3.5 mV s^{-1} voltage-clamp) were applied to the motoneurons to measure the Ca PIC (**Fig. 3b**)[1,26]. During the upward portion of this ramp, the current response initially increased linearly with voltage in response to the passive leak conductance. A linear relation was fit to the current in the region just below the Ca PIC onset (5 mV below) and extrapolated to the whole range of the ramp (leak current, thin line

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in **Fig. 3b**). At depolarized potentials above the Ca PIC onset threshold, the Ca PIC induced a downward deviation from the extrapolated leak current, and this Ca PIC was estimated as the difference between the leak current and the total current (leak-subtracted current). The amplitude of the peak Ca PIC was quantified as the initial peak amplitude of this downward deviation below the leak line (downward arrow **Fig. 3b**). The Ca PIC is thought to be mediated by Cav1.3 L-type calcium channels[1], because it is activated at an unusually low threshold (about 10 mV above rest), blocked by high doses of nimodipine and highly persistent (in contrast, transient currents are inactivated during the slow ramp). There is also a small contribution to the Ca PIC from calcium-activated channels[27].

The excitatory postsynaptic potential (EPSP) and associated reflexes were also measured in motoneurons by stimulating the Co1 dorsal roots (at 2–3xT, as in ventral root reflex recording), while applying hyperpolarizing bias currents to block the PICs (**Fig. 3d**).

mRNA measurements. Following the methods of Nakae et al.[28], RNA was extracted from spinal cords of chronic spinal rats and age-matched normal rats with RNAeasy-Lipid-tissue Mini-Kits (Qiagen, Japan) and used for first-strand cDNA synthesis by Thermoscript (Invitrogen). Quantitative RT-PCR was then performed on the cDNA with ABI-PRISM-7900HT using TaqMan probes (Applied Biosystems) to estimate total 5-HT_{2C} receptor mRNA (and internal control 18SrRNA). To determine the 5-HT_{2C} receptor isoforms in the tissue, rat 5-HT_{2C} receptor fragments were amplified from the cDNA using RT-PCR with the primers ATCATGGCAGTAAGCATGGAGAAGA and ATTGATATTGCCCAAACGATGGCA. The PCR product DNA was cloned into the pCR2.1 vector (Invitrogen), transfected into single E. coli bacterial cells, and > 300 recombinant colonies grown (each corresponding to a *single* 5-HT_{2C} receptor isoform). DNA extracted from 80 randomly picked colonies underwent nucleotide sequencing with an ABI3700 genetic analyzer (Applied Biosystems) to determine the isoform DNA adopted by each colony.

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

Controls in which the primary antibody was pre-absorbed with the antigen that it was raised against were used to verify the selectivity of the antibody labeling. For example, the 5-HT_{2C} receptor antigen (1:180; 5.5 $\mu l \cdot ml^{-1}$, SC-15081P, Santa Cruz) was incubated with the 5-HT_{2C} receptor antibody (SC-15081) for 80 mins at room temperature prior to dilution and application to tissue. Also, controls in which the primary antibody was omitted were used to confirm that the secondary antibody produced no labeling by itself.

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SMI32 labels non-phosphorylated neurofilament H, and does not change with spinal cord injury[29]. We have previously demonstrated that SMI32 labels motoneurons, and *not* interneurons in the ventral horn, though it also labels large cells elsewhere in the brain. Thus, SMI32 provided a fairly selective marker of motoneurons in the ventral horn[29], and in particular labels the intracellular space (neurofilament). Using image analysis software (ImageJ), we defined two regions for 5-HT_{2C} receptor quantification in motoneurons: 1) the internal region inside motoneuron soma, co-localized with SMI32 (region with SMI32 above standardized threshold, as we described in Anelli et al.[29]), and 2) the membrane region, immediately adjacent to the internal SMI32 region. The later was produced by detecting the edge of the SMI32-positive region inside motoneuron soma and expanding this edge to a 0.7 μm wide band that surrounded the perimeter of the soma (not co-localized with SMI32). The mean 5-HT_{2C} immunofluorescence signal density was then computed in these two regions, from which we computed the ratio of the receptor density in the membrane relative to the density inside the cell (x100%) for comparison across normal and injured rats.

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

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