Supporting Information

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SI Text

Serotonergic projections in the spinal cord colocalize with different neuropeptides including substance P, dynorphin, galanin, and Thyrotropin releasing hormone (1–6). There is a general agreement that these neuropeptides enhance the excitatory action of serotonin (5-HT) on motoneurons (MNs) through depolarization and increase of their input resistance (7–11). Neuropeptides are stored in large dense core vesicles and are mainly released at extrasynaptic sites during high level of activity (12). It is therefore likely that during high level of activity of the raphe spinal pathway, the neuropeptides will promote the excitatory effect of 5-HT. However, if a spillover of 5-HT occurs and reaches the axon initial segment (AIS), the inhibition of spike initiation will prevail over the facilitation.

SI Materials and Methods

Slice Preparation. The surgical procedures complied with Danish legislation. For voltammetry experiments, animals were killed according to the guidelines of the University of Oxford. Adult turtles (*Chrysemys scripta elegans*) were anesthetized by i.v. injection of Propolipid (0.3 mL/100 g; Frenesius Kabi) and killed by decapitation. After intracardiac perfusion of a high Mg^{2+} solution (120 mM NaCl, 5 mM KCl, 15 mM $NaHCO_3$, 20 mM glucose, 20 mM $MgCl_2$, and 3 mM $CaCl_2$), the spinal cord was removed. The lumbar enlargement (D8-S2) was cut into slices (300 μm or 1,500 μm thick) with a vibratome (MicroM slicer HM 650V equipped with cooling unit CU65) set at 2 °C. Adult turtles were anesthetized by intramuscular injection of ketamine (100 mg/kg; Narketan 10; Vétoquinol) followed by a heart injection of Pentobarbital (50 mg/kg). When the heart had stopped beating, the brain was pithed and the animal was decapitated.

Intracellular Recordings Patch Clamp Recordings. Slices were perfused with Ringer (120 mM NaCl, 5 mM KCl, 15 mM $NaHCO_3$, 20 mM Glucose, 2 mM $MgCl_2$, and 3 mM $CaCl_2$) saturated with 98% O_2 and 2% CO_2 to obtain a pH of 7.6. Experiments were done at room temperature. Neurons were visually identified by means of a BW51WI microscope (Olympus) equipped with an oblique illumination condenser. The patch pipette solution (122 mM K-gluconate, 2.5 mM $MgCl_2$, 5.6 mM Mg-gloconate, 5 mM K-Hepes, 5 mM HEPES, 5 mM Na_2ATP, 1 mM EGTA, and 2.5 mM biocytine, K-OH to adjust the pH to 7.4) contained the fluorescent dye Alexa 488 (250 μM; Sigma) to stain the recorded cells and visualize their dendritic arborization. Electrodes had a resistance ranging from 5 to 18 MΩ that was automatically compensated. Neurons were recorded with a Multiclamp 700B amplifier (Molecular Devices). Recordings were sampled at 10–50 kHz. Voltage clamp recordings were low-pass filtered at a cutoff frequency of 3 KHz (Bessel filter). Data were sampled with a 16-bit analog-to-digital converter (DIGIDATA 1322A, MDS) and displayed by means of the Clampex 9.2 software (Molecular Devices). Fast glutamate and GABA synaptic transmission was blocked by CNQX (20-25 μM; Tocris), AP5 (50 μM, Tocris), Strychnine (10 μM, Sigma), SR 95531 hydrobromide (Gabazine; 10 μM; Tocris). Intracellular recordings were performed with sharp electrodes filled with a mixture of 0.9 mM K-acetate and 0.1 mM KCl.

Microiontophoresis. Microiontophoresis electrodes were made either with a vertical puller PC-10 (Narishige) or a horizontal puller P-87 (Sutter Instrument) from borosilicate capillaries (G150F-3; Warner Instruments). Pipettes were filled with a solution of (±)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide. The pH was adjusted to 4.0–4.2 so that the molecule was positively charged. A −10 to −200 nA retention current was applied to prevent the leakage of 8-OH-DPAT. The pipette was positioned with a motorized micromanipulator (MRE MLE XYR; Luigs & Neumann). The drug was released by passing a positive current (+50 to +200 nA) through the pipette. We performed a series of tests to discard the possibility of direct electrical activation of the recorded neuron: (*i*) We inverted the polarity of the current passed through the microiontophoresis pipette and checked that it induced no response (in case of direct electrical stimulation, the response should be inverted); (*ii*) we verified that the amplitude of the response changed as a function of the distance between the microiontophoresis electrode and the recorded neuron (Fig. S6); and (*iii*) we checked that the effect lasted longer than the duration of the current injection. Only the recordings fitting these criteria were kept for analysis.

Pressure Ejection of 5-HT. Electrodes made from borosilicate capillaries (G150F-3; Warner Instruments) had a tip diameter <1.5 μm. Freshly prepared 5-HT (15 mM in Ringer) was pressure ejected by a homemade device controlled by a TTL (Transistor-Transistor Logic) pulse.

Extracellular Stimulation of the Dorsolateral Funiculus. A 16-stimulation Michigan probe (A1×16-3mm50-413; Neuronexus Technologies) was inserted vertically into the slice at the dorsolateral funiculus (DLF). The most efficient stimulating point was detected by monitoring the change in MN excitability in response to current injection between different sites of the probes. Trains of pulses (0.5–0.8 mA) were applied at a frequency of 10–40 Hz.

Immunohistochemical Labeling. At the end of the recordings, a train of positive current pulses was applied to the cell for at least 15 min to fill the neuron with biocytin. Slices were fixed by incubation in paraformaldehyde (1%; 30 min at 4 °C) and then washed three times in PBS. The slices were incubated in a blocking solution (0.1% Triton X-100, 1% human serum albumin) for 1 h. A staining with streptavidin coupled to Alexa 488 (1:200; Sigma) revealed the recorded neurons. MNs were identified by immunohistochemical staining with a polyclonal antibody directed against choline acetyl transferase (ChAT, 1:50, host goat; Chemicon). The AIS was recognized by labeling the slice with a monoclonal antibody directed against all isoforms of voltage-sensitive sodium channels (Pan Na^+ Channels, PanNaV, 1:50, host mouse; Sigma). The primary antibodies were added to the solution and let for incubation during 6 d at 4 °C. After washout, the slices were incubated with the secondary antibodies (donkey anti-goat 647; 1:500; Molecular Probes) during 4 h at room temperature and then mounted on slides by using VectaShield medium to protect the fluorescence (Vector Laboratories). Large ChAT positive cells present in the lateral part of the ventral horn were considered as MNs (13). To investigate the colocalization of serotonergic boutons at the axon initial segments of MNs, a triple staining was performed by following the same protocol. Slices from three turtles were incubated with the primary antibodies (ChAT, 1:50; PanNaV, 1:250; monoclonal rat anti-5-HT, 1:100; Chemicon) for 6–7 d at 4 °C after fixation and blockage steps. Secondary antibodies (anti-goat Alexa 555, 1:800, Invitrogen; anti-rat Alexa 488, 1:200; Invitrogen; anti-mouse Cy5, 1:500; Chemicon) were added to the bath for 2 h at room temperature. All pictures were ob-

tained with a confocal microscope (Leica TCS SP2). Figures were made by using Adobe Photoshop CS5 software.

Voltammetry. A carbon-fiber electrode was placed in the lateral part of the ventral horn of a slice (1,500 μm thick). Evoked extracellular 5-HT signals were detected by using fast-scan cyclic voltammetry with a Millar voltammeter (Julian Millar, Barts and the London School of Medicine and Dentistry, London, United Kingdom) and 10 μm diameter carbon fiber microelectrodes (tip length 50–100 μm, fabricated in house) as used (14, 15). The scanning voltage was a triangular waveform from −700 mV to +1,300 mV to −700 mV vs. Ag/AgCl at a scan rate of 800 V/sec and scan frequency of 8 Hz. Between scans, the electrode was switched out of comparison of the peak potentials for oxidation and dual reduction currents against those seen in 5-HT calibration solutions (500 nM 5-HT in experimental media). These peak potentials were typically +600 mV for the oxidation current and −60 mV and −560 mV, respectively, for the dual reduction currents (vs. Ag/AgCl).

Data Analysis. ***Measurement of spike characteristics.*** Action potentials (APs) were averaged ($n = 20$). After drug application, measurements were made of the 20 first spikes occurring after the end of the drug (8-OH-DPAT or 5-HT) release. The spike threshold was measured by using the methods described by ref. 16 (illustrated in Fig. S3). The spike amplitude was measured as the difference between the estimated threshold and the peak. The delay to spike corresponded to the delay between the onset of the stimulation and the peak of the spike. The half width and rise slope were estimated by using the software Clampfit 10.2 (Molecular Devices).

Measurement of persistent inward currents. Responses of MNs to voltage ramp depolarizations were low-pass filtered (Bessel filter cutoff setting of 400 kHz; Clampfit 10.1 software; Molecular Devices). The traces recorded after release of drug were subtracted to the corresponding control traces. The difference measured between −45 mV and −25 mV of applied voltage was integrated by using the software Matlab version R2009b (Mathworks). All data were normalized to the corresponding control value before comparison by Wilcoxon test. Means are represented ± SEM.

Estimation of the total charges carried by the inward currents evoked by depolarizing pulses. Inward currents were evoked by 60-mV voltage pulses from a holding potential of −120 mV, before, right after and a few minutes after 8-OH-DPAT release at the hot spot. We used the first derivative of the current plotted as a function of the current to determine a time window of constant current rate of change before the inward current activation. The onset of the inward current was estimated as the time when the rate of change exceeded the mean plus twice the SD measured when the rate of change of the current was constant. The current was integrated during a 25-ms time window starting at the threshold measured for each trace. The areas under the curves were normalized to the maximal value measured in control.

Estimation of the distance between drug releasing site and hotspot. The distance between the hot spot and other releasing site was measured offline on pictures taken during the recording (Adobe Illustrator and Photoshop). The distances corresponded to the distance following the cell membrane and not the point-to-point distances.

Colocalisation of antiserotonin and anti-PanNaV labeling. Pictures were analyzed with the Image J software (National Institutes of Health). After subtracting the background, the antiserotonin labeling was selected as the reference and applied to the images obtained either with anti-ChAT or anti-panNaV antibodies to determine the area of colocalisation. Areas smaller than 0.1 μm^2 were excluded from the study as well as PanNaV stainings that did not correspond to an axon initial segment. For each picture, the density of the colocalisation was calculated by dividing the double stained areas by the motoneuronal area labeled.

Statistical analysis. Data were analyzed by means of Origin 7.5 (Origin Lab) and GraphPad Prism version 5.0 (GraphPad). The normality of distribution of each set of data was tested. When a Gaussian distribution could not be approximated, a nonparametric test was used. For parametric data, one-way ANOVA were followed by Tukey post hoc tests. Data are represented as mean ± SEM. All statistical tests were two-tailed.

Drugs. The following drugs were applied when indicated in the bath: [R]-3-[2-(2-[4-methylpiperidin-1-yl]ethyl)pyrrolidine-1-sulfonyl]phenol (SB269970; 10 μM; Sigma Aldrich); *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide (WAY-100635; 10 μM; Sigma Aldrich); Tetrodotoxin (1 μM; Sigma Aldrich); nifedipine (10 μM; Sigma Aldrich), and nimodipine (10 μM, Sigma Aldrich).

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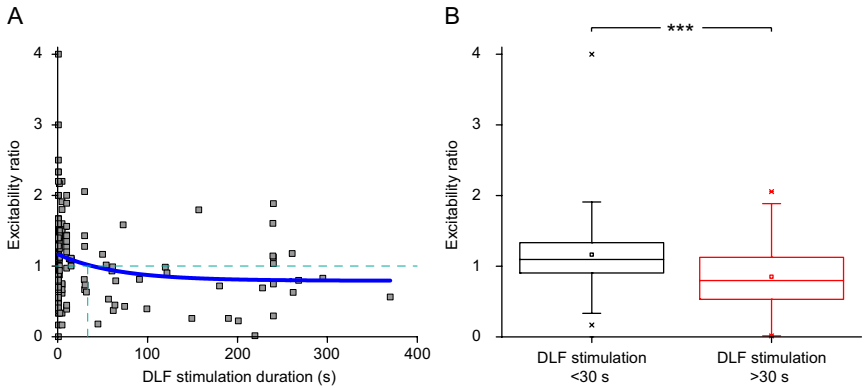

Fig. S1. Switch between excitation and inhibition induced by DLF stimulation. APs were generated by a 2-s depolarizing current pulse before and after stimulation of the DLF. Brief stimulation resulted in an increase in the number of spikes, whereas longer stimulation had an inhibitory effect. The ratio of the number of spikes after and before stimulation was plotted as a function of the duration of the stimulation (*A*). The ratio decreases when the duration of the stimulation increases. A fitted exponential (blue line) indicates that the ratio is equal to 1 for stimulations lasting 30 s. (*B*) Box plot of the ratios for stimulation lasting less than 30 s (black) and more than 30 s (red) (significant difference: Kolmogorov–Smirnov Test; $P = 0.0000094$).

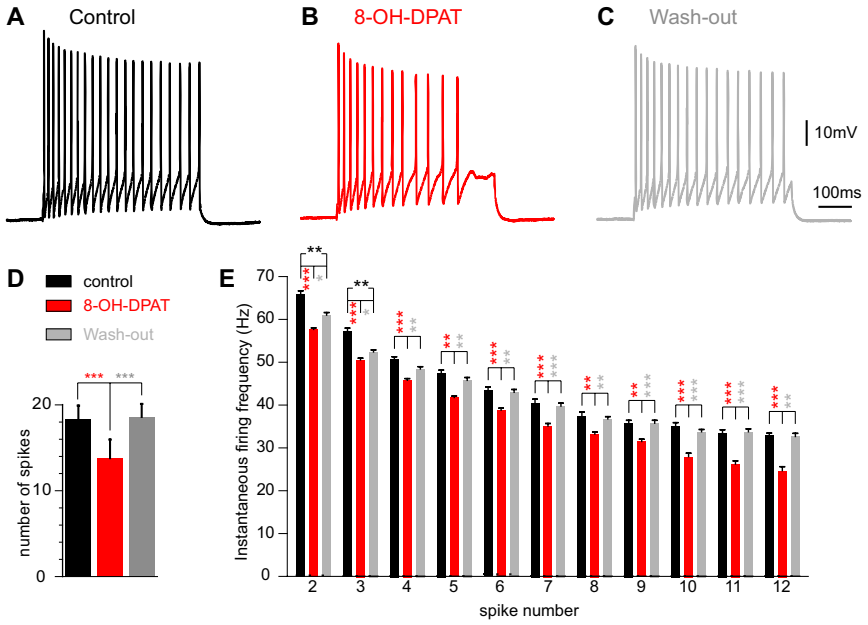

Fig. S2. 8-OH-DPAT decreases the firing frequency of MNs. (*A*) Response of a MN to a depolarizing current pulse. Fast synaptic transmission blocked. (*B*) Iontophoresis of 8-OH-DPAT (40 mM) decreased the number of APs. (*C*) Recovery. (*D*) Mean number of APs during the three conditions. (Control: 18.4 ± 0.7; 8-OH-DPAT: 13.8 ± 1.0; Wash-out: 18.6 ± 0.7); 8-OH-DAPT significantly decreased the number of APs ($P < 0.001$; repeated-measures ANOVA). ***A Tukey post hoc test revealed significant differences after 8-OH-DPAT release vs. control and wash-out, respectively; $q = 11.62$ and $q = 12.13$ ($n = 18$). The difference was nonsignificant between control and wash-out ($q = 0.505$). (*E*) Mean instantaneous frequency of the APs calculated at each spike. A Friedman test followed by a Dunn's post hoc test revealed a significant decrease of firing frequency after 8-OH-DPAT release throughout the 11 interspike intervals represented ($P <$ 0.0001, $n = 165$, mean in Hz: control 44.0 ± 1.45; drug 37.9 ± 1.41; wash-out 42.7 ± 1.23). Significant differences calculated at each spike as indicated on the figure (repeated-measures ANOVA $P < 0.0001$ for all except for spike number 5; $P < 0.01$, followed by Tukey post hoc test *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

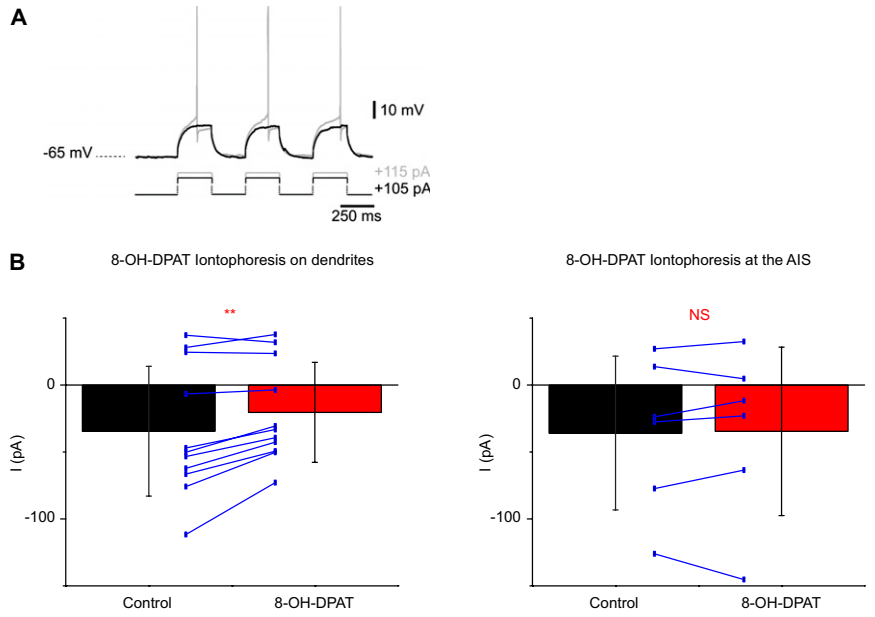

Fig. S3. (*A*) An increase of the amplitude of the depolarizing pulses can rescue the MN firing after 8-OH-DPAT application. The two recordings were performed in the same MN after a 31.25-s release of 8-OH-DPAT (20 mM). The MN was silent after the drug application (black trace). Increasing the depolarizing pulses from +105 pA to +115 pA (gray trace) rescued the firing without changing the membrane potential at rest. When the intensity of the depolarization was set back to the initial value, the MN stopped firing again, demonstrating that the MN had not recovered yet from the effect induced by 8-OH-DPAT. (*B Left*) Iontophoresis of 8-OH-DPAT promotes an outward current when applied on dendrites, but not at the AIS. (*A*) 8-OH-DPAT (20–40 mM) iontophoresed near the somatic or dendritic membrane promoted an outward current. Membrane potential held at −60 mV. Significant difference (Wilcoxon signed-rank test, $P = 0.0068$; $n = 11$). Blue lines, individual measurements. (*B Right*) When iontophoresed at the AIS, 8-OH-DPAT did not induce any significant change (Wilcoxon signed-rank test, $P = 0.84$; $n = 6$). Blue lines, individual measurements.

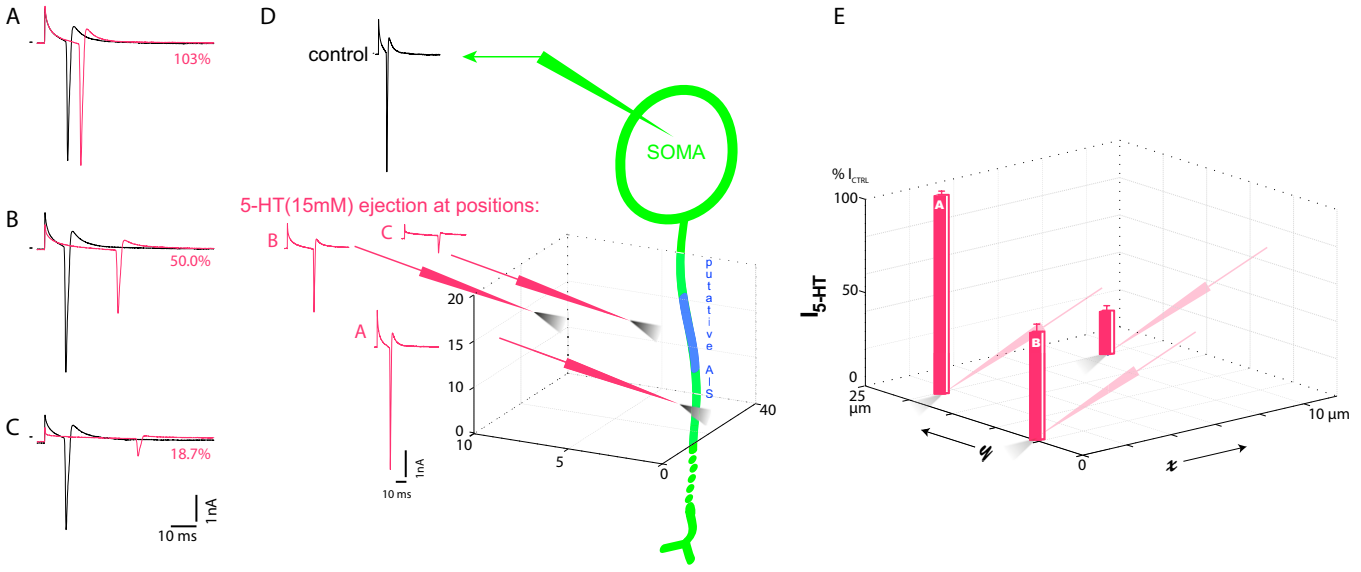

Fig. S4. The effect induced by puffing 5-HT (15 mM) is focal. All recordings were performed in the same MN recorded in voltage-clamp mode. (*A* and *B*) Raw traces of inward currents evoked by a depolarizing step of −120 to −50 mV before (black) and after a 4.5-s puffing 5-HT (15 mM, pink) at three different positions. The amplitude of the inward current evoked after 5-HT release is expressed as a percentage of the amplitude measured in control. (*D*) Spatial representation of the puff pipette positions used in *A*–*C*. The drawing of the MN was made after the pictures taken during the experiment. (*E*) Mean amplitude (+SEM) of the inward current evoked after 5-HT puff (in percentage of the amplitude measured in control) at the three positions tested. The coordinates are calculated in the plane defined by positions A, B, and C. At position C, 5-HT decreased the evoked inward current to 23.06 ± 2.71% of its value in control. Mean amplitude at position B: 57.75 ± 4.40% ($n = 3$). When the pipette was moved 13.9 µm away to reach position A, the current was not affected anymore (106.3 ± 1.85%).

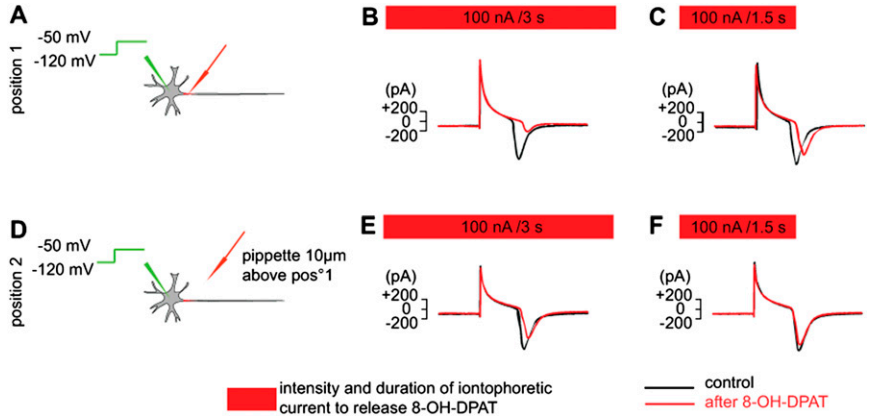

Fig. S5. Inhibition induced by 8-OH-DPAT depends on the distance from the release site to the hot spot and the duration of drug application. All recordings were performed in the same MN recorded in voltage-clamp mode. (*A* and *D*) Experimental procedure. (*B* and *C*, *E* and *F*) Raw traces of inward currents evoked by a depolarizing step of −120 to −50 mV before (black) and after release of 8-OH-DPAT (40 mM, red). 8-OH-DPAT, released during 3 s at the hot pot (*A*), decreased the evoked inward current amplitude strongly (51% of reduction of the amplitude in control). Shortening the release duration to 1.5 s (*C*) decreased the effect to a 7% reduction in amplitude. Moving the iontophoresis pipette 10 µm above its initial position (*E* and *F*) almost abolished the inhibitory effect of the drug (4% of reduction of the amplitude in both cases).

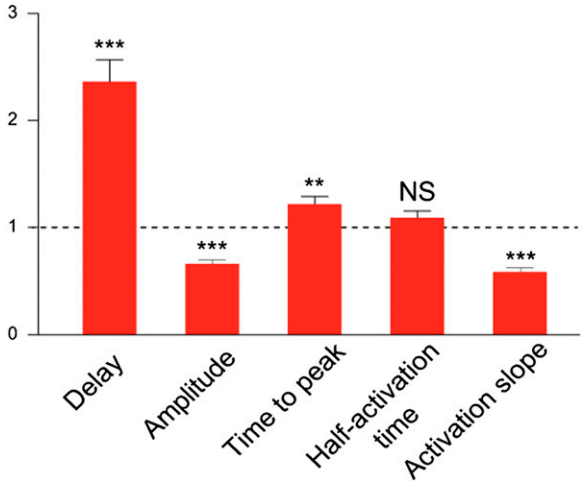

Fig. S6. Histogram of the evoked inward current characteristics in control and after drug application. All data were normalized to the corresponding value in control condition. The drug (8-OH-DPAT, 40 mM; or 5-HT,15 mM) decreased the evoked current amplitude and the activation slope to ratios of 0.66 ± 0.04 and 0.59 ± 0.04, respectively. The delay (defined as the time between the stimulation start and the transient inward current onset) was increased to 2.36 ± 0.20. ***Student's paired *t* tests showed the effect was highly significant for those three characteristics with a *P* value inferior to 0.0001 in each case ($n = 41$). **The time to peak (defined as the time between the transient inward current onset and its peak) was also increased to 1.22 ± 0.07 ($P = 0.0014$, Wilcoxon test, $n = 41$). However, the half activation time was not significantly (NS) affected by the drug (1.09 ± 0.06; $P = 0.5068$, Wilcoxon test, $n = 41$).

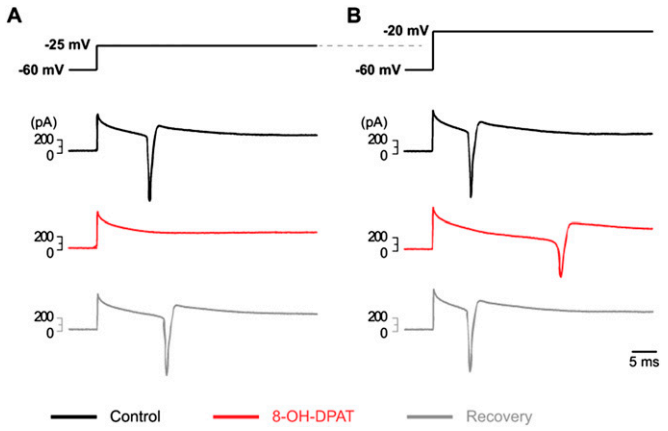

Fig. S7. The voltage threshold for inward currents is increased by 8-OH-DPAT. All recordings were obtained from the same MN in voltage-clamp mode. (*A*) In control (black), a step to −25 mV evoked a transient inward current. (*B*) After application of 8-OH-DPAT (40 mM), a step to −20 mV was necessary for activating the current (observation made after applying 8-OH-DPAT on four cells or 5-HT on 10 cells).

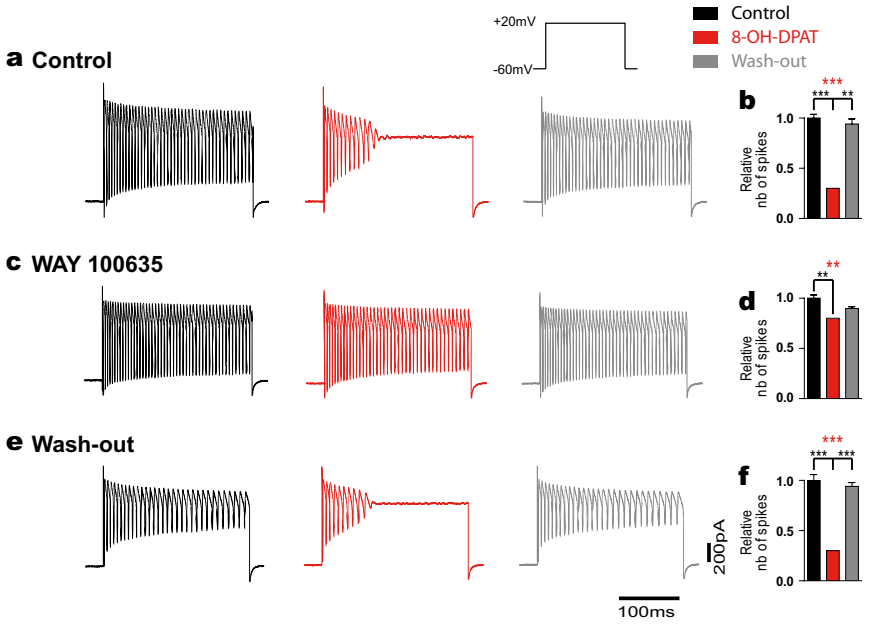

Fig. S8. WAY-100635 blocked the inhibitory effect of 8-OH-DPAT. (*A*, *C*, and *E*) A depolarizing voltage pulse evoked a series of inward currents in a MN. Fast synaptic transmission blocked (*Materials and Methods*). Iontophoresis of 8-OH-DPAT (40 mM) at the hot spot (red) reduced the number of evoked inward currents. (*B*) Relative mean number of evoked inward events. Control, 1 ± 0.04; 8-OH-DPAT, 0.3 ± 0.06; wash-out, 0.94 ± 0.05; ***$P < 0.0001$; one-way ANOVA (in red) followed by Tukey post test (in black) *** control vs. drug, $q = 16.30$, $P < 0.0001$; drug vs. wash-out, $q = 14.90$, **$P < 0.001$). Nonsignificant difference between control and wash-out ($q = 1.397$). (*C*) Recording of the same MN in the presence of the 5-HT1A antagonist WAY-100635 (20 μM). 8-OH-DPAT decreased only marginally the number of evoked inward currents (*D*); **$P = 0.0078$, one-way ANOVA followed by Tukey post test control vs. drug $q = 9.087$, no significant difference between drug vs. wash-out, $q = 4.143$, and control vs. wash-out, $q = 4.944$). (*E*, *F*) Recording of the same MN after washout of WAY-100635. The effect 8-OH-DPAT was restored. Control, 0.9967 ± 0.06; 8-OH-DPAT, 0.3 ± 0.00; Wash-out, 0.94 ± 0.04; $P = 0.0002$, one-way ANOVA followed by Tukey post test ***control vs. drug $q = 22.68$, ***drug vs. wash-out $q = 20.85$, nonsignificant difference between control and wash-out $q = 1.846$).

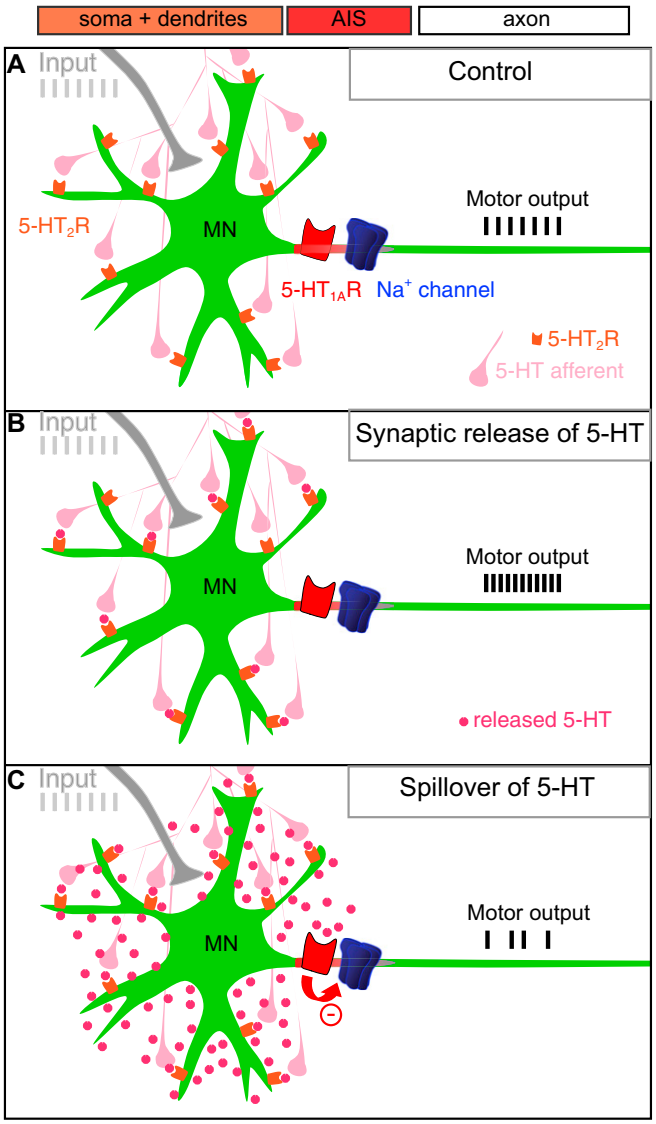

Fig. S9. Spillover of serotonin activates 5-HT_{1A} receptors at the AIS and induces central fatigue. (*A*) Scheme of a MN in control. During a motor exercise, 5-HT boutons located over the somatodendritic compartments (but not at the AIS) release 5-HT. (*B*) The 5-HT_2 receptors that are synaptically activated facilitate the firing. (*C*) When the level of 5-HT released increases, it induces spillover in the MN area. 5-HT activates 5-HT_{1A} receptors at the AIS, which, in turn, inhibit a Na^+ current. The 5-HT_{1A} receptors effect overcomes the excitatory effect induced by 5-HT_2 receptors. The MN output is then decreased.