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STAR METHODS

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RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, George Z. Mentis (gzmentis@columbia.edu).

Material Availability

No new mouse lined were generated in this study. Requests for information for mouse lines used in this study should be directed to and will be fulfilled by George Z. Mentis (gzmentis@columbia.edu).

Data and Code Availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

All surgical procedures were performed on postnatal mice in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and approved by the Columbia Animal Care and Use Committee (IACUC). Animals of both sexes were used in this study.

The original breeder pairs of C57BL6 mice (Jax Stock #000664), *lsl*-Channelrhodopsin2 mice (Ai32; Jax Stock #024109), *lsl*-hM4Di (inhibitory DREADDs) mice (Jax Stock #026219), *lsl*-Archaerhodopsin-3 mice (Ai40D; Jax Stock #021188), and *fsf*-*lsl*-hM4Di (inhibitory DREADDs) mice (Jax Stock #029040) used in this study were obtained from Jackson Laboratories. The Cdx2-

NSE-FlpO mice (Jax Stock #030288) were a kind gift by Dr. David Ginty (Harvard University). The Chx10-CRE mice were a kind gift by Dr. Tom Jessell (Columbia University).

METHOD DETAILS

Genotyping

Genotyping protocols were carried out using standard procedures as described in detail on the Jackson website (www.jax.org). In brief, at P0 (postnatal day 0) tail DNA was extracted from mice and lysed using lysis buffer (100mM Tris pH 8, 5mM EDTA, 0.2% SDS, 200mM NaCl, 100 µg/ml Proteinase K) for 45 minutes at 55°C. PCR primers used to genotype are described on the Jackson website (www.jax.org). A universal PCR reaction consisted of: 12.5 µl of GoTaq Hot Start Green Master Mix (Promega), 0.5 µl of each primer (25 µM; Sigma), and 4 µl of 1:20 diluted lysed tail DNA into a final volume of 25 µl using ddH_2O. For the *lsl*-Channelrhodopsin2 mice, the *lsl*-Archaerhodopsin-3 mice, the *lsl*-hM4Di mice, and the *fsf-lsl*-hM4Di mice the following PCR parameters were used: 95°C for 2 mins, followed by 35 cycles of [95°C for 40 secs, 59°C for 30 secs, 72°C for 1 min], and 72°C for 5 mins. Additional information for oligonucleotides are shown in Table S1.

Immunohistochemistry and confocal analysis

Many of the immunohistochemistry protocols have been previously described (Fletcher et al., 2017; Mentis et al., 2011). In brief, animals were transcardially perfused with 4% PFA, spinal cords dissected and post-fixed in 4% paraformaldehyde (PFA) at 4°C overnight. Transverse 75µm sections were cut on a vibratome after embedding in warm 5% Agar. After blocking in 10% normal donkey serum in 0.01M PBS with 0.3% Triton X-100 (PBS-T; pH 7.4), sections were incubated overnight at room temperature with primary antibodies in the blocking solution. Primary antibodies used in this study included: ChAT, Goat, 1:100 (Millipore); VAChT, Guinea pig, 1:2000 (custom); GFP, Chicken, 1:1000 (Aves Labs, Inc.); GFP, Rabbit, 1:100 (Novus); DsRed, Rabbit, 1:100 (Takara); HA, Rabbit, 1:100 (Abcam); NeuN, Mouse, 1:500 (Millipore); Synaptophysin, Guinea pig, 1:1000 (Synaptic Systems); Parvalbumin, Chicken, 1:20000 (custom); VGluT1, Guinea pig,

1:5000 (custom); VGluT2, Guinea Pig, 1:500 (Millipore); AnkG, 1:500, Rabbit (Santa Cruz). To reveal neurons that were recorded from intracellularly and filled with Neurobiotin, spinal cords were immersion fixed in 4% PFA overnight. Subsequently, streptavidin-Cy3 (Sigma) was used at a dilution of 1:100. Streptavidin was applied to the spinal cord sections and incubated overnight at room temperature along with any primary antibodies in the blocking solution. The following day, sections were washed in PBS-T and incubated with the appropriate species-specific secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted at 1:250 in PBS-T for 3 hours at room temperature. Sections were washed with PBS and mounted on glass slides with 70% glycerol / 30% PBS.

Sections were imaged using either a SP5 or SP8 Leica confocal microscope. Images were scanned with either a 20x air objective, 40x oil objective, or 63x oil objective. All images were analyzed in either LASAF software (Leica) or ImageJ software. For VSCT neuron transduction counts, we analyzed z-stack images (at 3 µm intervals) of each section. VSCT neuronal counts were performed with confocal microscopy in 75µm thick sections. We established that the L1 spinal segment in P45 mice extends in the rostro-caudal axis over 17 transverse sections (75 µm thickness). The number of VSCT was determined by counting all neurons ventral to the medio-lateral line emanating from the central canal, through z-stack scanning at 0.5 µm z-axis intervals. This was repeated in three L1 spinal cord transverse sections. The average number of VSCT from these sections was multiplied by 17 (the total number of sections within the L1 spinal segment). The number of VSCT neurons was calculated from the projection image obtained by all z-stack optical sections for every one of the 3 randomly chosen L1 transverse spinal cord sections. The same method was also applied for DSCTs and VSCTs for neuronal counts at P4-P5. At P4-P5, the number of sections in the L1 segment was 11. To determine the number of DSCTs and VSCTs at P4-P5, the number of VSCTs and DSCTs was calculated from the projection image obtained by all z-stack optical sections for every one of three randomly chosen L1 transverse spinal cord sections and multiplied by 11 (the total number of sections within the

L1 spinal segment at P4-P5). DSCTs were defined as the all neurons positioned dorsal to the medio-lateral line emanating from the central canal.

For dye coupling confocal microscopy analysis, a single VSCT neuron was recorded, filled and analyzed per each mouse (n=12 VSCTs from N=12 mice).

Orthograde and retrograde fills using the *ex vivo* spinal cord preparation

In some experiments, motor neurons and sensory fibers entering the spinal cord were labelled by orthograde and anterograde fills using the *ex vivo* spinal cord. Details have been previously reported (Mentis et al., 2005; Mentis et al., 2011). Briefly, the spinal cord was dissected free under in vitro conditions at ~10°C and continuously superfused with artificial CSF (aCSF). The appropriate ventral and dorsal roots were positioned in suction electrodes and backfilled with either: i) Texas Red Dextran, ii) Cascade Blue Dextran or iii) Fluorescein Dextran [10,000 molecular weight (MW)] for ~24 h (at 10°C) to label motor neurons (from a ventral root) or sensory fibers (from a dorsal root). Following this period, the appropriate lumbar segments immersion-fixed in 4% paraformaldehyde for 24 h. The spinal cord was embedded in 5% warm agar and subsequently sectioned (75μm) using a Vibratome. The sections were collected in wells and processed further for immunohistochemistry against different antibodies by free-floating method (in wells). Finally, sections were mounted on glass slides and coverslipped using an antifading agent (PBS/glycerol, 7:3) until examination with confocal microscopy.

Fluorescence *In Situ* Hybridization (FISH)

Fluorescent *in situ* hybridization protocols were carried out as recently reported (Simon et al., 2017). Fresh spinal cord tissue (L1 and L2 segments) was cryopreserved in sterile 30% sucrose overnight and flash frozen in OCT on dry ice at P4. 18-20μm sections were cut on a cryostat and fixed with 4% PFA for 10 minutes. Washes were carried out as follows: 3x PBS, 1x PBS-T 10 minutes (0.1% Triton X-100), 1x PBS. Acetylation was performed for 15 minutes in 1.17% (v/v) triethanolamine and 0.25% (v/v) acetic anhydride in ddH_2O. 3x PBS washes were followed by overnight hybridization with the RNA probe at 68°C in hybridization solution (50%

formamide, 5X SSC, 1X Denhart's, 1mg/ml baker's yeast RNA in ddH_2O) in a humidifying chamber. The probe was applied at 1ng/µL following denaturation through 5 minutes of warming at 85°C.

The following day, sections were washed with 0.2% SSC 2x for 15 minutes at 68°C, followed by 3x PBS washes. Blocking solution was then applied for 1 hour at room temperature (0.5% Blocking reagent [Roche] in 100 mM Tris-HCl, 150 mM NaCl; pH 7.5). Sections were then incubated with anti-digoxigenin-AP 1:500 (Roche) dissolved in blocking solution for 1 hour at 37°C. Sections were washed for 10 minutes 3x in Washing Buffer (100 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5). Sections were washed for 10 minutes 2x in Detection Buffer (100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 8.0). Development was done with the HNPP Fluorescent Detection Set (Roche) as per the manufacturer's instructions for 1 hour. Sections were washed in dH_2O and mounted with 2% DABCO in 50% glycerol/PBS to preserve fluorescence.

The RNA probe for VGlut2 was obtained through a PCRII-Topo VGlut2 *in situ* probe vector from the laboratory of Dr. Jeffrey Macklis deposited in Addgene as plasmid #45639 (https://www.addgene.org). For probe generation from the linearized plasmid, RNA transcription was conducted in the presence of digoxigenin-labeled nucleotides (Roche). After transcription, probes were precipitated overnight in 410µl precipitation solution (75% EtOH, 0.25 M TE buffer, 100 mM LiCl) at -20°C. The following day, the probes were precipitated through spinning at 13,000 RPM and the supernatant decanted. The probes were washed in 1 ml of 70% ethanol, spun at 13,000 RPM, and the supernatant decanted followed by re-suspension in hybridization solution at 10 ng/µl.

CTb injections and viral gene delivery to VSCTs

VSCTs were retrogradely labeled *in vivo* by intracerebellar injection of cholera toxin B subunit (CTb) conjugated to Alexa-488, Alexa-555, or Alexa-647 (Invitrogen). Newborn P0 mice

were anesthetized by isoflurane inhalation. A small incision and craniotomy over the cerebellum was made to inject ~200-600nl of 0.5-1% fluorescently-conjugated CTb in PBS using a finely-pulled glass microelectrode (P-1000 puller [Sutter Instruments]) under sterile conditions. The CTb was delivered by pressure through an adapted micro-syringe. The incision was closed with sutures. Upon dissection at P3-P5, the injection was determined to be accurate by visualization of fluorescence in the cerebellum under a fluorescence microscope (Leica) without spread to other brain tissue (Fig. S1F,G).

For viral gene delivery, the following viruses were used: CAV2-CMV-GFP (CAV2 = Canine Adenovirus 2), titer = 1.3 x 10^{13} pp/ml [Plateforme de Vectorologie de Montpellier]); CAV2-CMV-Cre, titer = 1.4 x 10^{13} pp/ml; CAV2-CMV-Cre-GFP, titer = 1.3 x 10^{13} pp/ml; Rabies-N2c-ChR2-YFP, titer = 1x10^{9} infectious particles/ml; Rabies-N2c-EnvA-DsRed, titer = ~5x10^{8} infectious particles/ml, AAV2/1-Flex-nGFP-N2c-Gp-TVA; AAV9-ChB-DiO-eGFP, titer = 1.1 x 10^{11} pp/ml (Vector BioLabs). The approximate volume of virus injected into the cerebellum was 0.5µl. WT mice of both sexes were injected intracerebellar at P0 with a 50/50 mixture of the CAV2 virus with 1% CTb-Alexa 555 or CTb-Alexa 647. At P3-P21, the spinal cord was processed for immunohistochemistry. Analysis of the extent of VSCT labeling consisted of comparing the number of VSCT neurons transduced by the virus with the number of VSCT neurons labeled by CTb using confocal images acquired with Leica confocal microscopes and analyzed with the Leica LASAF software. For the *in vivo* adult freely moving mice, mice were injected with the same viruses at P21 and examined at P35-45. For the Chx10-CRE mice experiments, AAV-Flex-nGFP-Gp-TVA was injected in the lumbar spinal cord at P0. Rabies-N2c-EnvA-DsRed was injected in the lumbar spinal cord at P10. To allow for transfection, mice were euthanized for the terminal experiments between P21-P28.

<u>Intracellular recording</u>

Many of the experimental protocols used in this study have been described previously (Fletcher et al., 2017; Mentis et al., 2011). At P3-P5, the animals were decapitated, the spinal

cords dissected and removed under cold (~10°C) artificial cerebrospinal fluid (aCSF) containing (in mM): 128.35 NaCl, 4 KCl, 0.58 $NaH_2PO_4.H_2O$, 21 $NaHCO_3$, 30 D-Glucose, 1.5 $CaCl_2.H_2O$, and 1 $MgSO_4.7H_2O$. The spinal cord was then transferred to a customized recording chamber placed under the objective of an upright confocal microscope (Leica SP5) equipped with a 2-photon laser (MaiTai DeepSee, Spectral Physics).

The intact *ex vivo* spinal cord preparation was perfused continuously with oxygenated (95% O_2 / 5% CO_2) aCSF (~10 ml/min). The physiological solution was kept at room temperature, ranging between 21-25°C, which results in slower conduction velocity for neurons. The ventral root of the L1 or L2 and L5 spinal segments bilaterally were placed into suction electrodes for recording. All potentials were recorded in either DC or AC (0.1 Hz filter) (Cyberamp, Molecular Devices). Recordings were fed to an A/D interface (Digidata 1440A, Molecular Devices) and acquired with Clampex (v10.2, Molecular Devices) at a sampling rate of 10-20 kHz. Data were analyzed offline using Clampfit (v10.2, Molecular Devices). In some preparations, the cerebellum-brainstem-spinal cord was used, while in experiments in which locomotor-like behavior was induced either by electrical stimulation or with a cocktail of drugs, the T4-cauda equina spinal cord was used.

Whole-cell recordings were obtained with patch electrodes advanced through the lateral or ventral aspect of the spinal cord under visual guidance using 2-photon microscopy to visualize individual VSCT neurons. Patch electrodes were pulled from thin-walled borosilicate glass capillary with filament (Sutter Instruments) using a P-1000 puller (Sutter Instruments) to resistances between 5–16 MΩ. The electrodes were filled with intracellular solution containing (in mM): 10 NaCl, 130 K-Gluconate, 10 HEPES, 11 EGTA, 1 $MgCl_2$, 0.1 $CaCl_2$ and 1 Na_2ATP, 0.1 Alexa-555 or Alexa-488 hydrazide (Life Technologies), and 0.5 mg/ml Neurobiotin (Vector Labs). pH was adjusted to 7.2–7.3 with KOH. The final osmolarity of the intracellular solution was 295–305 mOsm. Bridge balance was applied to all recordings. The liquid junction potential was

calculated as −2 mV but was not corrected in any of the recordings. The identity of the recorded neuron as a VSCT neuron was confirmed during the experiment by evoking an antidromic action potential by stimulation of the cerebellum through a concentric electrode applied just under the surface of the vermis of the cerebellum (0.2-0.5 ms, 1.2-3x Threshold). Threshold was determined by the minimum intensity needed to evoke a response in 3 out of 5 consecutive trials at 0.1 Hz. Upon increasing the stimulation intensity, there was no change in the amplitude of the all-or-none action potential induced. In addition, VSCT neurons were also identified by the colocalization of CTb-conjugated fluorochrome injected in the cerebellum at P0, with the intracellular fluorescent dye contained in the intracellular electrode. VSCT neurons were accepted for analysis only if the following criteria were met: (i) stable resting membrane potential of at least −45 mV (ii) an overshooting action potential and (iii) at least 20 mins of recording.

Synaptic potentials were recorded from individual VSCT neurons (DC - 3 kHz, Multiclamp 700B, Molecular Devices) in response to a brief (0.2 ms, 40-100 µA, 0.1-10 Hz) stimulation (A365, current stimulus isolator, WPI, Sarasota, FL) of the ipsilateral homosegmental ventral root (L1 or L2). Recordings contaminated by spontaneous events were discarded. The holding potential of the VSCT was varied from -80 to -40 mV to test for chemical components of synaptic responses. The jitter test was used to test whether the response was monosynaptic *(22, 43)*. The jitter test was conducted by analyzing the coefficient of variation at differing frequencies of ventral root stimulation (0.1, 1, 5, and 10 Hz). As previously published, if the coefficient of variation did not change over increasing stimulation frequency, the synaptic connection was determined to be monosynaptic. Conversely, if the coefficient of variation increased over increasing stimulation frequency, the synaptic connection was determined to be polysynaptic. Sequential bath application of pharmacological antagonists was used to study the components of the ventral root stimulation-mediated response in VSCTs. Drugs were allowed to perfuse into the spinal cord for at least 15 minutes or longer prior to stimulation. Mecamylamine, a nicotinic receptor antagonist, was used at a concentration of 50µM (Tocris). NBQX, an AMPA receptor antagonist, was used at

a concentration of 20µM (Tocris). Carbenoxolone, a gap junction antagonist, was used at a concentration of 100µM (Tocris).

I_h current was tested by negative current injection with examination of the resulting voltage trace for the characteristic sag and post-inhibitory rebound. Sag was calculated as the absolute value of the difference between the most negative voltage reached during negative current injection and the stable plateau voltage potential. Post-inhibitory rebound was calculated as the difference between the least negative voltage reached immediately after negative current injection and the voltage prior to current injection. Time-dependency of the sag and post-inhibitory rebound was tested by injecting steps of negative current (-200 to -500 pA) over different durations (ranging from 250 ms to 5 s with steps of 250-500 ms). Traces were examined for increased sag and post-inhibitory rebound with longer negative current injections. Voltage-dependency of the sag and post-inhibitory rebound was tested by injecting a varying negative current (ranging from -50 to -600 pA with steps of 50 pA) over a set duration (1 s to 2 s). Traces were examined for increased sag and post-inhibitory rebound with larger negative current injections which resulted in increased voltage changes in the recorded neuron. ZD7288, an HCN channel antagonist, was used at a concentration of 100 µM (Tocris).

At the end of the recording session, the electrode was removed from the spinal cord, but kept in the bath to measure any DC offset that might have occurred over the course of the recording. DC offset varied from -2 mV to +1 mV and due its low amplitude was not corrected in any of the recordings. The spinal cord was fixed in 4% PFA overnight and subsequently transferred to PBS and processed for immunohistochemistry.

Somatodendritic labeling of motor neurons

Experimental protocols used in this study have been described before (Fletcher et al., 2017; Mentis et al., 2005). After dissection and intracellular recording, the spinal cord was transferred back to the dissection chamber and the L1 or L2 ventral root was placed inside a suction electrode and backfilled with a Cascade Blue-Dextran (Invitrogen) to label the motor

neurons. The spinal cord was perfused with cold (~10°C), oxygenated (95% O_2, 5% CO_2) aCSF (containing in mM: 128.35 NaCl, 4 KCl, 0.58 $NaH_2PO_4.H_2O$, 21 $NaHCO_3$, 30 D-Glucose, 0.1 $CaCl_2.H_2O$, and 2 $MgSO_4.7H_2O$). After 12 – 20 hours, the cord was immersion-fixed in 4% PFA and washed in 0.01M PBS. Sections were subsequently processed for immunohistochemistry and confocal microscopy.

Neurolucida reconstruction of VSCT neurons and Image J analysis

Spinal cord sections were cut at 75µm and processed for immunohistochemistry to enhance GFP with an anti-GFP antibody. In addition, motor neurons were labelled with ChAT immunoreactivity. In some experiments, the axon identity was verified by AnkG immunoreactivity. Single optical planes were acquired in Z-stacks at 1µm intervals for the entire thickness of the spinal cord section (~75µm). Sections were scanned using an SP5 Leica confocal microscope and analyzed using ImageJ and Neurolucida (MBF Bioscience).

For the colocalization of CTb-488, VGluT2 and synaptophysin signals (Fig. S7) to mark the synapses from SCT axon collaterals, confocal images acquired with LAS (Leica) software were exported to ImageJ software as TIF format files. Then, the different channels were merged and further saved as TIF. In ImageJ software, the channels were split and the threshold adjusted. For each channel we apply the “Subtract 254” math module once. Then two channels were added together and the “threshold” was set to 2-255. This image was then converted-to-mask. For the third channel, was “made binary” and was “added” to the image processed by the addition of the first two channels.

Electroporation and 2-photon calcium imaging

The detailed experimental protocol used in this study has been described before (Bonnot et al., 2005). Briefly, at P4, the spinal cord of wild type mice was dissected and pinned down with the ventral side up, and a broken sharp glass electrode was used to pressure-apply 2µl of calcium-sensitive dye solution under the dura matter in the rostral lumbar area (L1/L2). The calcium sensitive dye calcium green-1 hexapotassium salt (1,147 MW, 55mM; Thermofisher Scientific),

was dissolved in artificial cerebrospinal fluid (aCSF; as above) prior to application. Two gold-plated electrodes (BTX, 3mm in length and 0.5 mm in diameter) were submerged in the aCSF and positioned in parallel on either side of the spinal cord. Square voltage pulses (10-50 V applied voltage, 50-100 ms duration, 4-10 pulses at 1 Hz) were applied between the electrodes within 5min after dye application using the ECM 830 electroporation system (BTX). The dura mater was removed from the lateral sides to allow visualization of the labeled cells. After electroporation, the cord was left for ~1 h to allow washout of the excess of dye from the extracellular space. The spinal cord was subsequently transferred to a customized recording chamber for electrical and optical recordings (as above). The lateral side of the lumbar spinal cord was imaged continuously under two-photon microscopy, while a ventral root was stimulated. Analysis of the fluorescent signal was conducted in Leica LASAF software using the region of interest (ROI) function.

Optogenetic and chemogenetic manipulation

Optogenetic and chemogenetic manipulation experiments were carried out in either *lsl*-Channelrhodopsin2 mice, *lsl*-hM4Di mice, or *lsl*-Archaerhodopsin-3 mice, as appropriate. All mice were homozygotes for the inserted transgene. In some mice, Rabies-N2C-ChR2-YFP was also used to express ChR2 in SCT neurons following cerebellum injection. P0 mice were injected intracerebellar with the CAV2-Cre or CAV2-Cre-GFP (or CAV2-GFP as control) viral vector along with fluorescently-labeled CTb, as described above. At P3-P6, the spinal cord was dissected and the *ex vivo* neonate spinal cord preparations were transferred to the recording chamber for physiological recordings as described above. The ventral roots of the L1 and L5 spinal segments bilaterally were placed into suction electrodes, along with the L5 dorsal root and/or S3 dorsal root ganglion as described above.

For VSCT neuron activation experiments utilizing Channelrhodopsin2, 470nm light was delivered via a LED (CoolLED; pE-100) placed over the L1 and L2 segments bilaterally on the ventral surface of the spinal cord. For DSCT neuron activation, dorsal illumination was used over the L1 and L2 segments bilaterally in the same *ex vivo* spinal cord preparations used for VSCT

activation, by flipping over the spinal cord and replacing the suction electrodes. LED power ranged between 53-106mW. Light was delivered with either continuous illumination for 20s or pulses of light for 100ms at 5Hz or 50ms at 10Hz for 20s. Light was delivered via a customized patch cable built by Thorlabs, Inc. Recording commenced with 1-10 seconds pre-light to establish a baseline, followed by light stimulation. Each preparation was tested for a minimum of three trials. All potentials were recorded in either DC or AC (0.1 Hz filter) (Cyberamp, Molecular Devices). For consistent Channelrhodopsin2 activation, all experiments were conducted in the presence of 1-3 µM all trans-Retinal (Sigma-Aldrich) in the aCSF. Pharmacological antagonism was used to test for the necessity of the h-current and gap junction communication in the production of locomotor-like activity following VSCT neuron photoactivation. Drugs were bath applied into the circulating aCSF and allowed to perfuse into the spinal cord for at least 15mins.

For inhibition experiments utilizing Archaerhodopsin-3, locomotor activity was induced via electrical stimulation of the S3 dorsal root ganglia. After successful induction of locomotion, 585nm light was delivered via a LED (CoolLED; pE-100) placed over the L1 and L2 segments bilaterally on the ventral surface of the spinal cord at the same time of electrically-induced locomotor-like activity. LED power was 47mW. For consistent Archaerhodopsin-3 activation, all experiments were conducted in the presence of 1-3 µM all trans-Retinal (Sigma-Aldrich) in the aCSF. Each condition was tested for a minimum of three trials. Analysis consisted of quantifying the locomotor frequency in each trace under each condition offline on Clampfit (v10.2, Molecular Devices).

For inhibition experiments utilizing inhibitory DREADDs, locomotor activity was induced via electrical stimulation of the L5 dorsal root, S3 dorsal root ganglion, or stimulation of a ventral root or through bath application of the pharmacological cocktail of drugs consisting of NMDA (5µM; Tocris), serotonin (10µM; Tocris), and dopamine (50µM; Tocris). For control experiments, CAV2-GFP was injected at P0 into the cerebellum. After successful induction of locomotion, 10µM CNO (Tocris) was bath applied in the aCSF and allowed to freely circulate for 15-30 minutes.

Induced locomotor activity was then investigated assayed in the pharmacological experiments. CNO was washed out using fresh aCSF and following 30+ minutes of washout, locomotor activity was induced again under the same conditions prior to CNO exposure. Each condition (pre-drug, CNO, washout) was tested for a minimum of three trials. Analysis consisted of quantifying the locomotor frequency in each trace under each condition offline on Clampfit (v10.2, Molecular Devices).

In vivo behavioral experiments

In vivo behavioral experiments were carried out by crossing Cdx2-NSE-FlpO mice (Britz et al., 2015) with *fsf-lsl*-hM4Di (inhibitory DREADDs) mice. The resulting double transgenic pups were heterozygotes for both inserted transgenes. P21 mice were injected intracerebellar with either CAV2-GFP-CRE viral vector, or CAV2-GFP, or CTb-647, as described above. Control mice consisted of mice injected with CAV2-GFP. A few mice (n=3) were co-injected with CAV2-GFP-CRE and CTb-647 for presence of HA in VSCTs labelled with CTb-647.

Mice were allowed to move freely in the open field assay with the Mouse NeuroBehavior Core at Columbia University. At P35-45, each mouse was injected intraperitoneally with 5mg/kg CNO (Tocris Cat. #4936). We opted to utilize the 5mg/kg CNO dose as it has been reported to be the optimal dosage for *in vivo* CNO injections (Manvich et al., 2018). Mice were then placed in the center of a clear Plexiglas arena (40 x 40 x 40 cm, Med Associates ENV-510) lit with dim light (~30 lux). Infrared beams embedded along the X, Y, Z axes of the arena automatically tracked distance moved and horizontal movement. Mice were allowed to ambulate freely for ~30 minutes. Data were collected into 10-minute intervals and total distance traveled for the first 10min after CNO injection and the last 10min for every hour (for the total of 6 hours) after CNO injection was analyzed using SOF-812 Activity Monitor software (Med Associates Inc.). The investigator was blind to the *in vivo* open field assay data set test which was revealed at the end of the analysis.

After the experiment was concluded, mice were euthanized and examined for quality of the injection by cerebellum fluorescence from the CTb under an epifluorescent microscope and from immunohistochemistry and confocal scanning of the L1 segment of the spinal cord.

Videography and analysis of swim test in adult mice

The temperature of the water bath was maintained at ~30C to avoid introducing temperature-bias measurements for all experiments. Mice were naïve in the swim test. Mice were tested once before CNO injection and 3 hours after 10mg/kg CNO injection. The swim test was set for 30sec to comply with IACUC guidelines. A high speed frame acquisition video camera was used for videography (Marshall, USB3.0) equipped with a 2.8mm IR1/2.5" 3MP C lens. The camera was connected to a PC laptop using OBS Studio 27.0.1 (64bit) software. Videos were acquired at 60 frames per second (fps) at 1280x720 resolution. Videos were save in MKV format. For analysis, the software VLC 3.016 Vetinari was used. Videos were saved in MP4 format after analysis.

QUANTIFICATION AND STATISICAL ANALYSIS

Induction of locomotor-like activity and analysis

The L5 dorsal root or S3 dorsal root ganglion was placed into a suction electrode for stimulation to induce locomotor activity (10s, 4Hz, 0.2ms, 1.2-3x Threshold). Threshold was determined by the minimum intensity needed to evoke a response in 3 out of 5 consecutive trials at 0.1Hz. Locomotor-like activity was also induced following L5 ventral root stimulation (0.2 ms, 40-100µA, 0.1-10Hz) as well as through bath application of the pharmacological cocktail of drugs consisting of NMDA (5µM; Tocris), serotonin (10µM; Tocris), and dopamine (50µM; Tocris). Locomotor-like activity was defined by the three major criteria: i) cyclic rhythmic activity, ii) left-right alternation, and iii) flexor-extensor alternation (alternation between ipsilateral L1 and L5 spinal segments).

Quantification of the rhythmic activity in VSCT neurons was done using circular plots and circular statistics (Oriana v4.02). Locomotor cycle length was defined as the time between two

consecutive flexor burst onsets (corresponding to 0 or 0°, for the first, and 1 or 360°, for the second, on a circular scale), for the spinal cord side ipsilateral to the recorded VSCT. Flexor bursts were defined as bursting in the L1 or L2 segment motor neurons, whereas extensor bursts were defined as bursting in the L5 segment motor neurons. Onset of the flexor burst was defined as 50% of the time interval between trough and peak of the filtered (integrated by first low pass filtering at 200 Hz, followed by high pass filtering at 10 Hz, rectification and then low pass filtering at 3Hz) L1 or L2 segment motor neuron activity, as previously reported (Falgairolle et al., 2017).

The first VSCT action potential elicited during each burst of firing with respect to the recording from the homosegmental ventral root was analyzed. The timing of the onset of firing of the VSCT neuron compared to the onset of the flexor burst, normalized to the length of the locomotor cycle, was calculated. For each VSCT, the timing of the onset of action potential firing was plotted in a circular plot corresponding to the locomotor cycle. The mean timing of the first action potential was then calculated and plotted as a vector in the circular plot for each VSCT neuron. The length of the mean vector reflects how concentrated the values are around the mean vector, or the rhythmicity of that VSCT. To determine if the combined population of VSCTs exhibited rhythmic activity, the mean vector value from each of the individual VSCTs was then plotted in a circular plot corresponding to the locomotor cycle (Fig.3B_2). Using the Rayleigh Test, the statistical significance of the clustering of values around the mean vector was computed, reflecting whether that individual VSCT neuron fired in a rhythmic fashion. All recorded VSCT neurons had statistically significant rhythmic firing and were therefore included in further grouped analysis. The mean vector value, length of the mean vector, Raleigh Test Z Score, and *p* value for each of the 6 VSCT neurons were respectively: 1) 252 +/- 9°, 0.94, 3.5, $p < 0.05$; 2) 21 +/- 15°, 0.96, 2.8, $p < 0.05$; 3) 12 +/- 14°, 0.98, 2.9, $p < 0.05$; 4) 315 +/- 8°, 0.84, 3.6, $p < 0.05$; 5) 14 +/- 13°, 0.92, 3.4, $p < 0.05$; 6) 297 +/- 2°, 0.98, 4.75, $p < 0.01$. To determine if the combined population of VSCT neurons exhibited rhythmic activity, the mean vector value from each of the individual VSCT neurons was then plotted in a circular plot corresponding to the locomotor cycle. The mean

timing of the first action potential for the population was calculated and plotted as a vector in a circular plot and the Rayleigh Test was conducted to test for statistical significance.

Statistics

The locomotor-like activity analysis of rhythmic activity in VSCT neurons used circular statistics. For this analysis, statistical analysis was performed using Oriana v4.02. Statistical analysis was carried out with the Rayleigh Test. P values are indicated as follows: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, with $P < 0.05$ considered statistically significant. For all other analysis, statistical analysis was performed using GraphPad Prism 6. All results are expressed as means ± standard error of the mean (SEM). Statistical analysis was carried out with the two-tailed unpaired Student's t-test, the two-tailed paired Student's t-test, or with one-way ANOVA, as appropriate. *Post-hoc* analysis of one-way ANOVA statistical tests was conducted with Tukey's *post hoc* test. P values are indicated as follows: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, with $p < 0.05$ considered statistically significant. No randomization was used.

SUPPLEMENTARY FIGURE LEGENDS:

Fig. S1. (Related to Fig.1 and Fig.2) A set of interneurons located dorsolateral and close to the motor neuron nucleus is activated following motor neuron axon stimulation. Injection of CTb in cerebellum to label SCT neurons. VSCT neurons receive proprioceptive synapses and EPSP responses in a VSCT neuron after ventral root stimulation.

(**A**) Schematic illustrating the experimental approach in which the dye Calcium Green 1, indiscriminately labelled spinal cord neurons by electroporation. 2-photon laser imaging of fluorescence representing calcium entry into the neuron was performed through the lateral side of the spinal cord at P3-P5 following ventral root electrical stimulation. (**B**) Low-magnification view of fluorescent signal of the spinal cord from the lateral side. The ventral and dorsal side of the spinal cord is denoted. Orange dotted box is shown enlarged in (C). (**C**) Change in calcium signal over its baseline fluorescence following ventral root stimulation. Two regions of interest (ROIs) are shown for a motor neuron (black box) and a dorsal interneuron (orange box). These neurons were not Renshaw cells since they are located ventrally to the motor neuron nucleus. In addition, they are not likely to be Sim1+ interneurons since this neuronal type is deep within the intermediate grey matter. (**D**) $\Delta F/F_o$ calcium signal for the ROI over the spinal interneuron [orange box in (C)], and a motor neuron (black ROI in (C)]. Following ventral root stimulation, motor neurons exhibited a large increase in fluorescence change, whereas interneurons exhibited smaller calcium signals (N=4 mice). (**E**) Intracellular whole cell patch clamp traces from an interneuron (targeted visually) revealing graded and short latency excitatory post-synaptic potentials following homosegmental ventral root stimulation. (**F**) Fluorescence image from a P4 mouse that was injected with CTb-555 in the cerebellum at birth (P0). (**G**) Confocal image from a P4 cerebellum section showing the specificity of the injected CTb-555 within cerebellum injected at P0. (**H_{1-5}**) Confocal images with dorsal root orthogradely filled sensory fibers with Cascade Blue Dextran (in blue, H_1), parvalbumin (in white, H_2), VGluT1 (in green, H_3) immunoreactivity and

CTb555 labelled SCT neurons (in red, H_4). Merged image is shown in H_5. The approximate location of the motor neuron nucleus is denoted by yellow dotted oval shape in H_5. Higher magnification images are shown for a VSCT neuron receiving proprioceptive synapses on the soma (I_{1-5}) and on a proximal dendrite (J_{1-5}). Yellow arrows denote synapses that colocalize Cascade Blue, VGluT1 and parvalbumin in apposition to a VSCT neuron. D: dorsal; V: ventral; L: lateral; M: medial. (**K**) EPSPs from a P3 L1 VSCT neuron at different holding potentials following L1 ventral root stimulation. Red arrows indicate the stimulus artifact. (**L**) Normalized EPSP amplitude (with respect to the response at -80mV) at different holding potentials in VSCT neurons (n=4 VSCT, N=4 mice) following ventral root stimulation. (-80 v -70: * $p<0.05$; -80 v -60: ** $p<0.01$; -80 v -50: ** $p<0.01$; -80 v -40: *** $p<0.001$; -70 v -40: * $p<0.05$; One Way ANOVA, Tukey's post hoc test).

Fig. S2. (Related to Fig.2) Minimal spread of current from VSCTs to motor neurons and dye coupling between VSCTs and motor neurons and amongst VSCT neurons.

(**A**) Schematic of experimental setup. A stimulating electrode was place in the cerebellum (vermis) and in the L1 dorsal root, while motor neuron responses were recorded from the L1 ventral root. (**B**) Five superimposed ventral root (L1) responses recorded following L1 dorsal root stimulation at 0.1Hz. Red trace is the average response. (**C**) Five superimposed ventral root (L1) responses following cerebellum stimulation at 0.1Hz. Red trace is the average response. Inset shows the latency of the onset of response in four different experiments (N=4 mice). (**D**) Two superimposed vr-L1 average responses following dorsal root (blue) and cerebellum stimulation (red). (**E**) The area of the ventral root response following cerebellum stimulation is expressed as a percentage of the ventral root response following dorsal root stimulation. The area of the response was measured for the first 250ms after the stimulus (N=3 mice at P4-5). (**F**) Superimposed ventral root responses at three different frequencies (0.1, 1 and 5Hz) of cerebellum stimulation. Red trace is

the average response. Dotted vertical line with arrows indicate the same latency of the onset of response, indicative of monosynapticity. (**G**) Superimposed average responses in control solution (black), 30min after 100µM carbenoxolone (blue) and 15min after addition of 100µM APV and 20µM NBQX (red). (**H**) Percentage expression of ventral root responses under carbenoxolone (blue) and addition of APV/NBQX (red), normalized to control solution (grey) (N=3 mice). * <0.01, *** p<0.001, ANOVA, post hoc: Tukey's multiple comparisons test.

(**I_{1-3}**) Single optical plane confocal images showing Neurobiotin (revealed *post-hoc* with the Avidin-Biotin complex; green) intracellularly filled in a P4 L2 VSCT neuron and diffused into motor neurons (revealed with ChAT immunoreactivity; shown in red). Two motor neurons (white arrows) show co-localization of Neurobiotin and ChAT (yellow). Note that the recorded VSCT is not shown, since it was located in a different spinal cord section (n=4 VSCTs from N=4 mice). (**J_{1-3}**) Enlarged confocal images from the dotted boxes in (I) showing co-localization of Neurobiotin (green) and ChAT (red). (**K**) Confocal image from a z-stack showing the intracellular recorded and filled P4 L2 VSCT neuron with Neurobiotin (red) next to a weakly filled red-labelled VSCT neuron (also labelled with CTb488). Insets show the two VSCT neurons at higher magnification co-localizing with CTb488 (n=4 VSCTs from N=4 mice).

Fig. S3. (Related to Fig.3) Subthreshold rhythmic oscillations in VSCT neurons, analysis of the rhythmic firing of VSCTs during locomotor-like activity and time- and voltage-dependency of sag and post-inhibitory rebound in VSCTs.

(**A**) Intracellular recording from an L1 VSCT neuron (top trace) and extracellular recordings from the right (middle trace) and left (bottom trace) L1 ventral roots, during locomotor-like activity induced by dorsal root stimulation. The resting potential of the VSCT neuron was -65mV. Red box indicates that the VSCT neuron was in phase with the homosegmental ventral root. (**B**) An intracellular response from a P4 L1 VSCT neuron exhibiting rhythmic bursts of action potentials along with a filtered extracellular trace from the ipsilateral homosegmental L1 ventral root during

locomotor-like activity induced by electrical stimulation of an adjacent (L2) ventral root. Time point 0 (1) was the beginning of the locomotor cycle, defined as the point at which the trace reaches 50% of the amplitude between trough and peak in the flexor phase. The timing of the first action potential (AP) of the VSCT neuron during each burst was determined in comparison to the locomotor cycle. Note that the VSCT neuron fires APs before the onset of motor neuron activity. See Methods for further details. (**C**) Superimposed traces from a P4 L2 VSCT neuron exhibiting a time-dependent sag and post-inhibitory rebound to negative current injection. (**D**) Superimposed traces from a P4 L2 VSCT neuron illustrate that the sag and post-inhibitory rebound are also voltage-dependent. (**E**) Current-voltage superimposed responses before and during ZD7288 exposure in a VSCT neuron. Exposure to ZD7288 (data in red) decreased the resting membrane potential (**F**), increased the input resistance (**G**), increased the time constant (**H**), reduced the rheobase (**I**), and did not alter the voltage threshold (**J**) in VSCT neurons (n=3 VSCTs from N=3 mice). ns: not significant ($p>0.05$), * $p<0.05$, *** $p<0.001$, Two-tailed paired t-test.

Fig. S4. (Related to Fig.4) GFP expression in VSCTs, quantification of DSCTs and VSCTs and CAV2 or Retinal does not produce locomotor-like behavior following photo-illumination.

(**A**) Low magnification confocal images from the ventral horn of a mouse in which CAV2-Cre was introduced into VSCTs to express ChR2 in *lsl*-ChR2-GFP mice. CTb-555 (in red) was co-injected into the cerebellum at P0 to retrogradely label VSCT neurons. GFP (in green) was only observed in VSCT neurons labelled with CTb-555. Motor neurons are labeled with ChAT immunoreactivity (in blue). Merged image of all signals is shown on the right side. Dotted rectangular denotes the area shown at higher magnification in (B). (**B**) Higher magnification images from (A) showing VSCT neurons co-localizing CTb-555 (red) and GFP (green). White arrows indicate two VSCT neurons not labelled with GFP (N=4 mice). The GFP signal is weak since the spinal cord was immersion fixed in 4% paraformaldehyde after the completion of the physiological experiment.

Every spinal cord was examined after each physiological experiment. (**C**) Confocal images showing CTb555+ neurons. All neurons dorsal to the medio-lateral line (yellow dotted line) starting at the central canal (cc) were considered as dorsal spino-cerebellar tract (DSCT) neurons, while neurons ventral to the dotted line were considered as ventral spino-cerebellar tract (VSCT) neurons. ChAT immunoreactivity is shown in blue. (**D**) The total number of DSCT neurons in the L1 spinal segment. Each point is the total number of DSCTs in a single mouse (N=6 mice). (**E**) The total number of VSCT neurons. Each point is the total number of VSCTs in the L1 segment from a single mouse (N=6 mice). For the counting details see Methods. (**F**) Schematic illustration of the L1 and L2 segments photo-illuminated from the ventral aspect with a 470nm LED in control preparations in which the cerebellum was injected with CAV2-GFP at P0 in *lsl*-ChR2 mice. (**G**) Retinal was applied in the bath at 1-3 µM and photo-illumination revealed no effects in the corresponding ventral roots. (**H**) Quantification of the responses after photo-illumination in spinal cords in which VSCT neurons were transduced with CAV2-GFP (N=4 mice).

Fig. S5. (Related to Fig.4 and Fig.5) Photo-activation of VSCTs expressing ChR2-GFP introduced by the N2C-Rabies induces locomotor activity and optogenetic silencing of VSCTs via Archaerhodopsin-3 degrades locomotor activity. CNO does not alter synaptic transmission or motor neuron function. CAV2-GFP in VSCTs or exposure to CNO does not have any significant effects in locomotor activity; abolition of locomotor activity induced by photoactivation of VSCTs in L1/L2 after carbenoxolone.

(**A**) Rhythmic activity in a P5 mouse ex vivo preparation following photo-activation (in the L1/L2 segments) of VSCT neurons with a 470nm LED (bottom blue trace). Extracellular traces from the left and right L1 ventral roots and the right vr-L5. Red box illustrates the alternating activity amongst the different roots. (**B**) Quantification of the locomotor frequency following photo-activation of VSCTs (N=3 mice). (**C**) Confocal image from a P5 mouse spinal cord, showing CTb555+ spinocerebellar tract neurons (in red) and GFP (in green) following concomitant

injection with the rabies-N2C expressing ChR2 and GFP. Motor neurons are labelled with ChAT (in blue). (**D_{1-3}**) Confocal images from a P4 mouse showing VSCT neurons expressing GFP (green, D_1) and CTb-555 (red, D_2); motor neurons were revealed by ChAT immunoreactivity (blue, merged in D_3). White arrows show three VSCT neurons which co-express GFP and CTb-555. CTb-555 was injected into the cerebellum at P0 to retrogradely label VSCT neurons along with CAV2 driving Cre expression in a *lsl*-Archaerhodopsin-3-GFP (ArchT) mouse to introduce Archaerhodopsin-3 to VSCT neurons (N=3 mice). (**E,F**) Schematics of the experimental protocol illustrating the bilateral illumination of the L1 and L2 segments with a 585nm LED from the ventral aspect, while motor neuron activity was monitored through bilateral L1s and L5 segment ventral roots with extracellular electrodes. (**G**) Extracellular responses from ventral roots exhibiting locomotor-like activity following sensory fiber electrical stimulation (blue line at the bottom; 4Hz for 10s) in control condition and following photo-illumination with 585nm LED (right) in a P5 *ex vivo* spinal cord preparation. Yellow line represents the duration of photo-illumination. (**H**) Quantification of the locomotor frequency before (red) and after (yellow) photo-silencing of VSCT neurons. * $p<0.05$; Two-tailed student's t-test (N=3 mice).

(**I**) Schematic of the *ex vivo* spinal cord preparation and magnification of the transverse section of the spinal cord to denote the extracellular electrodes for stimulation of the dorsal root and recording from the ventral root. (**J**) Extracellular monosynaptic response in L5 motoneurons following L5 dorsal root stimulation at P5 in *ex vivo* spinal cord preparation in which the inhibitory DREADDs receptor was introduced to VSCTs at P0 by cerebellum injections of CAV2-Cre in a *lsl*-hM4Di (inhibitory DREADDs) mouse. Note that application of CNO elicited no change in the monosynaptic response (N=3 mice). (**K**) Extracellular recordings from control experiments in which CAV2-GFP was injected into the cerebellum at P0 in *lsl*-hM4Di mice. At P4, under control artificial cerebrospinal fluid, electrical stimulation of sensory fibers elicited locomotor-like activity (left side). Bath application of 10μM clozapine-n-oxide (CNO) had no effect on the locomotor frequency induced by sensory fiber stimulation (right side). (**L**) Quantification of the locomotor

frequency before and after CNO application (N=3) (Two-tailed student's t-test). (**M**) Locomotor-like activity evoked from a P4 spinal cord in which VSCT neurons express ChR2 where photo-activated with a 470nm LED (denoted by the blue lines under the traces) under control solution and after 100µM carbenoxolone. The area of photo-illumination was in the L1/L2 spinal segments from the ventral side. Locomotor activity was abolished after bath application of carbenoxolone. (**N**) Quantification of the locomotor frequency following photoactivation of VSCTs before and after bath application of carbenoxolone. *** $p<0.001$; Two-tailed student's t-test (N=5 mice).

Figure S6. (Related to Fig.6) Visualization of VSCT axon collaterals.

(**A-C**) A P3 L1 VSCT neuron filled with Neurobiotin, revealed with the Avidin-Biotin complex (red, B_1). Motor neurons are in green (B_2, labelled with Fluorescein Dextran dye retrogradely applied to the L1 ventral root) and vesicular acetylcholine transporter (VAChT, B_3) immunoreactivity is in blue. Single optical confocal plane images of all fluorochromes in B_1-B_4. Note the abundant arborization of ipsilateral axon collaterals in (C) from a z-stack projection (total z axis: 75µm). (**D**) CTb555 and CAV-Cre was injected in the cerebellum at birth (P0), while AAV9-CBh-DiO-eGFP was injected in the lumbar spinal cord at P5. Spinal cords were examined at P12. (**E**) Confocal image showing a VSCT neuron transfected with AAV9-CBh-DiO-eGFP (in green), CTb555 (in red) and ChAT immunoreactivity (in blue). Insets show at higher magnification the co-localization of CTb555 and GFP. (**F**) The VSCT neuron shown in (B) was reconstructed with Neurolucida (VSCT#1). The somato-dendritic morphology is shown in grey, while the axon and its collaterals are shown in red. Dotted box shows the confocal image of the main axon and two of its axon collaterals. (**G,H**) Two additional VSCT neurons (VSCT#2 and #3) reconstructed with Neurolucida showing their main axon and their collaterals (arrows) (n=7 VSCTs from N=3 mice). Dotted lines denote the grey-white matter border. (**I**) Co-localization of GFP and CTb555 in another VSCT neuron. (**$J_{1,2}$**) The main axon of the VSCT shown in (I) was identified by Ankyrin G (AnkG)

immunoreactivity (in blue; arrow). Insets show that an axon collateral also expressed AnkG (double arrowhead).

Fig. S7. (Related to Fig.6) Arborization of synapses from spinocerebellar tract neurons in the L1/L2 spinal cord. VSCT axon collaterals contact motor neurons and VSCT neurons possess descending axon collateral projections to caudal lumbar segments.

Single optical plane confocal images in the L1 spinal cord from CTb-488 (**A**, in green) injected in the cerebellum at birth, immunoreactivity against VGluT2 (**B**, in red) and synaptophysin (**C**, in blue). (**D**) Points in purple indicate the location of synapses that co-localized CTb488, VGluT2 and synaptophysin. Co-localization was determined through image processing using Image J. Note that the co-localization points are not to scale and have been enlarged to improve visibility. Similar results were observed in N=4 mice. (**E_{1-3}**) Single optical plane confocal images showing a VSCT axon collateral from a P4 L2 VSCT neuron filled with Neurobiotin and revealed with the Avidin-Biotin complex (red, E_1), a motor neuron (MN) dendrite (blue, E_2) labelled with Cascade Blue dextran dye applied retrogradely from the cut ventral root and synaptophysin immunoreactivity (white, E_3). (**E_4**) Merged image showing a putative site of contact (yellow arrow) between a VSCT axon collateral and a motor neuron dendrite (n=5 VSCTs from N=5 mice). (**F_{1-4}**) Single optical plane confocal images showing VGluT2 immunoreactivity (**F_1**), axon collateral(s) from a P4 VSCT neuron, filled with Neurobiotin (**F_2**; red) and ChAT immunoreactivity (**F_3**; blue). Four putative sites (yellow arrows) of synaptic contacts were observed on the motor neuron soma (n=3 VSCTs from N=3 mice). (**G**) CTb-488 was injected into the cerebellum to label VSCT neurons (green) at P0. Concomitantly, CTb-555 was also injected *in vivo* into the L5/6 spinal cord (red). At P5, the L1 and L2 spinal segments were sectioned and examined for co-localization of the two tracers. (**H**) Percentage of VSCT neurons co-expressing CTb-555 and CTb-488 (as shown in I_3), compared to VSCT neurons expressing CTb-488 only. Individual data points denote numbers of VSCTs from 75µm thick transverse sections from three experiments (N=3 mice). (**I_{1-}**

$_4$) Single optical plane confocal images from the L1/2 segments containing a VSCT neuron with both fluorochromes co-localized (yellow in I_3), demonstrating the presence of descending axon collaterals from L1/2 segment VSCT neurons. The motor neuron location was revealed by ChAT immunoreactivity (I_3, blue).

Fig. S8. (Related to Fig.7) No direct connections from the cerebellum to the lumbar spinal cord during early mouse development and in adult mice.

(**A**) CTb-555 was injected *in vivo* in the L2/3 lumbar spinal cord at birth (P0). At P4 (N=6 mice), the entire spinal cord and brain was dissected, sectioned and imaged with a confocal microscope. Montage of confocal images is shown. Higher magnification images (shown in dotted boxes) of: the cervical spinal cord (**B**) which contains propriospinal interneurons, cerebellum (**C**), indicating the absence of neurons with CTb-555, unlike the A11 region (**D**), which contains dopaminergic neurons, as well as and the Raphe pallidus (**E**), which contains serotonergic neurons. These neurons are all known to send axons in the lumbar spinal cord. (**F**) CTb-555 was injected *in vivo* in the L2/3 lumbar spinal cord at P21. At P35 (N=4 mice), the entire spinal cord and brain was dissected, sectioned and imaged with a confocal microscope. Montage of confocal images is shown the entire mouse CNS. Higher magnification images (shown in dotted boxes in (A) of: cerebellum (**G**), indicating the absence of neurons with CTb-555, unlike the dopaminergic neurons of A11 region (**H**), as well as the propriospinal interneurons in the cervical spinal cord (**I**) and the serotonergic neurons in the Raphe pallidus (**J**), which are all known to send axons in the lumbar spinal cord.

Fig. S9. (Related to Fig.7) The inhibitory DREADDs receptor is selectively expressed in VSCT neurons *in vivo*. VSCT neurons are expressed throughout the spinal cord; distance travelled in Cdx2^{FlpO}::*fsf-lsl*-h4MDi mice after CNO following cerebellar injection with CAV2-GFP or CAV2-GFP-CRE.

(**A$_{1-3}$**) *fsf-lsl*-hM4Di mice were crossed with Cdx2^{FlpO} mice and the resulting pups were injected into the cerebellum at P21 with a combination of CTb-555 (to label VSCTs) and CAV2-GFP-Cre (to introduce the inhibitory DREADDs receptor specifically to VSCTs). Immunohistochemistry at P45 against hemagglutinin (HA), co-expressed (yellow arrows) with the inhibitory DREADDs receptor, reveals specific co-localization with CTb555. Asterisk shows a CTb-555+ VSCT neuron that did not express HA (n=3). Absence of HA immunoreactivity in cerebellum (**B**), hippocampus (**C**), cortex (**D**) and brainstem (**E**). NeuN immunoreactivity is shown in red. (**F$_{1-3}$**) Cdx2-mCherry signal amplified with DsRed immunoreactivity in a P45 L2 spinal cord injected with CTb647 (**F$_2$**) at birth to label spinocerebellar tract neurons (**F$_3$** is a merged image) (N=7 mice). VSCT neurons were distinguished from DSCT neurons, as the neurons located ventrally to the white dotted line from the central canal (cc) to the lateral edge of the white-grey matter (annotated by light blue dotted line) as shown in F$_2$. D: Dorsal, V: Ventral, L: Lateral, M: Medial. (**G, H, I**) Confocal images of GFP immunoreactivity (green), ChAT immunoreactivity (red) in the C4 cervical segment (**G**), T7 thoracic segment (**H**) and L5 lumbar segment (**I**) from a Cdx2^{FlpO}::*fsf-lsl*-h4MDi mouse (n=7 mice) that was injected in the cerebellum with CAV2-GFP-CRE. (**J, K**) The distance travelled for 10min at the onset and every hour after 5mg/kg CNO (i.p.) injection in Cdx2^{FlpO}::*fsf-lsl*-h4MDi mice (age: P35-45) which received cerebellar injections at P21 with either CAV2-GFP (**J**) as a control (N=9 mice), or CAV2-GFP-CRE (N=12 mice) (**K**). Each mouse is color-coded in each of the two experimental groups.

SUPPLEMENTAL VIDEOS

Supplemental Video 1. (Related to Fig.7) Swimming of an adult mouse (#A) in which SCT neurons were transduced with CAV2-Cre, before CNO injection (pre-CNO; part 1a) and 3 hours after 10mg/kg CNO (part 1b).

Supplemental Video 2. (Related to Fig.7) Swimming of an adult mouse (#B) in which SCT neurons were transduced with CAV2-Cre, before CNO injection (pre-CNO; part 2a) and 3 hours after 10mg/kg CNO (part 2b).

Supplemental Video 3. (Related to Fig.7) Swimming of an adult mouse (#C) in which SCT neurons were transduced with CAV2-GFP (as a control), before CNO injection (pre-CNO; part 3a) and 3 hours after 10mg/kg CNO (part 3b).

Supplemental Table 1.

Additional oligonucleotides used in the study.