Supplemental Experimental Procedures

Animals and stereotactic surgery

86 adult transgenic or wild-type mice on a C57BL/6 background aged 50 to 100 days were used in the experiments. 13 wild-type mice (8 males, 6 females) were used for electrical stimulation experiments (Fig. 1). 10 vGLUT2-ires-Cre (3 females, 7 males, Jackson Stock# 016963, Fig. 1, 2, S3), 9 vGAT-ires-Cre (4 females, 5 males, Jackson Stock# 016962, Fig. 1, S5), 6 ChAT-ChR2 (2 females, 4 males, Jackson Stock# 014546, Fig. 1) and 6 wild-type C57BL/6 (2 females, 4 males, Jackson Stock# 000664, Fig. 2) mice were used for optogenetic stimulation, inhibition or recording experiments. 6 vGLUT2-Cre and 5 vGAT-Cre (4 and 2 males and 2 and 3 females respectively, same Jackson stock) were used for rabies tracing experiments (Fig. 3). 18 D1-Cre mice (8 females, 10 males, GENSAT #030778-UCD) were used for dMSN stimulation while recording identified responses from from unidentified or identified glutamatergic neurons or inhibiting the MLR (Fig. 4). 12 A2a-Cre mice (8 males, 4 females, GENSAT #031168-UCD) were used for iMSN stimulation while recording responses from unidentified or identified glutamatergic neurons or stimulating the MLR (Fig. 4). 2 male vGLUT2-Cre mice crossed into an Ai14 line (Jackson Stock #007914) were used for confirmation of CAMKIIα expression in vGLUT2-expressing neurons (Fig. S3). 2 male vGAT-Cre mice, 2 vGLUT2-Cre and 2 ChAT-ChR2 mice, all males, were used in slice experiments (Fig. S2 and S5). No randomization of animals was implemented and the researchers were not blinded.

All procedures were in accordance with protocols approved by the UCSF Institutional Animal Care and Use Committee. Mice were maintained on a 12/12 light/dark cycle and fed *ad libitum*. Experiments were carried out during the dark cycle. All surgeries were carried out in aseptic conditions while mice were anaesthetized with isoflurane (5% for induction, 0.5-1.5% afterward) in a manual stereotactic frame (Kopf). Buprenorphine HCl (0.1 mg kg^{-1}, intraperotineal injection) and Ketoprofen (5 mg kg^{-1}, subcutaneous injection) was used for postoperative analgesia. Mice were allowed to recover for five to seven days after surgeries before habituation to the trackball.

Viral injection

For cell-type-specific viral infection of MLR neurons, we injected 300 to 500 nL of adeno-associated virus serotype 5 (AAV5) carrying channelrhodopsin 2.0 (ChR2) fused to enhanced yellow fluorescent protein (eYFP) in a double-floxed inverted open reading frame (DIO) under the control of the EF1α promoter (AAV5-EF1α-DIO-ChR2-eYFP for DIO-ChR2; all viruses obtained from UNC Vector Core or University of Pennsylvania Vector Core), AAV5-EF1α-DIO-eYFP for controls (Fig. 1 & 2). For inhibition of glutamatergic neurons in the MLR, we injected AAV expressing halorhodopsin (eNpHR3.0) expressed under the CAMKIIα promoter (AAV5-CAMKIIα-eNpHR3.0-eYFP) (Fig. 2 & Fig. 4). MLR injections were made bilaterally at -0.80 mm anteroposterior (AP) measured from Lambda, ± 1.2 mm mediolateral (ML) and -3.6 dorsoventral (DV) measured from the skull surface (5 µL NanoFil with 33ga Needles; WPI) mounted to a microsyringe pump (UMP3; WPI) and controller (Micro4; WPI). Injection speed was 100 nL min^{-1} and the injection needle was raised 7 minutes after completion. For activation of the striatal dMSN or iMSN population (Fig. 6 & Fig. S6) we injected 1.000 µL at 200 nL min^{-1} bilaterally at +0.8 mm AP from Bregma, ± 1.5 mm ML and -2.5 DV from the brain surface. MLR infection for these experiments was carried out at the same coordinates for the MLR as above but using ChR2 expressed under the CAMKIIα promoter (AAV5-CAMKIIα-ChR2-eYFP) for stimulation, or halorhodopsin (eNpHR3.0) for inhibition (AAV5-CAMKIIα-eNpHR3.0-eYFP). ChR2 viruses were allowed to express for 3 to 5 weeks before experiments while eNpHR3.0 viruses were allowed to express for 6 to 8 weeks.

For characterization of inputs to specific MLR populations using rabies tracing, vGLUT2-ires-Cre or vGAT-ires-Cre mice were injected in the MLR (coordinates above) with a mixture of DIO-TVA-mcherry (AAV5-EF1a-FLEX-mCherry; “TVA”) and rabies glycoprotein (AAV5-CAG-FLEX-RG, “RG”) (Salk Institute) . On Day 14, mice were injected with EnvA-pseudotyped G-deleted rabies-eYFP, which only infects cells expressing the TVA receptor (Wall et al., 2010) . Tissue was fixed for analysis at Day 23.

Surgical Preparation

For all experiments, mice were implanted with a custom stainless steel headbar for head fixation. The scalp was removed and skull scraped clean and dry using a scalpel. Cyanoacrylate glue (Vetbond, 3M) was lightly dabbed on the skull and a base of dental acrylic (Ortho Jet; Lang Dental) applied in a circle around Lambda to provide the skull/headbar interface. The headbar was further cemented with dental acrylic, and the central hole was then filled with silicone elastomer (Kwik-Cast; WPI).

For activation of striatal MSNs while either recording or stimulating in the MLR (Fig. 6, Fig. S6), 200-µm-diameter optical fibers (Thorlabs #FT200UMT) were: (1) glued into 1.25 mm ferrules (Thorlabs CFLC128-10), (2) polished, and (3) implanted bilaterally 0.5 mm above the striatal viral injection site after headbar mounting by drilling a 0.5 mm hole in the skull, lowering the fiber over 1 minute and gluing into place with Vetbond followed by dental acrylic. For eNpHR3.0 experiments (Fig. 6, Fig. S6), a second set of fibers with ferrules was implanted 0.5 mm over the MLR viral injection site. Mice were allowed 7 to 10 days of recovery from surgery.

For neural recording and some stimulation experiments, a 0.5 to 1 mm burr hole was drilled at ± 1.2 mm mediolateral and -0.8 anterodorsal from lambda. The head plate was filled with silicone elastomer and the animal was allowed to recover for at least 3-4 hours. The animal was then placed on the trackball and the silicone plug removed.

Behavior

Mice were habituated to the trackball for 2 to 3 days prior to the experiment. Movement of the trackball was monitored by 2 optical mice fed into custom MATLAB software as described in Neill and Stryker, 2010 (Lee et al., 2014). For electrical stimulation experiments, a concentric bipolar stimulating electrode (FHC part no CBBBE75) was lowered into the brainstem and 200 µA, 200 µs electric current delivered at 20 Hz for 5 seconds with 20-40 seconds between trials. For optical stimulation experiments, blue light was passed through a 200 µm fiber attached to a recording probe (described below) using a 473 nm laser (OEM, part no 10010351) coupled to an optical multimode fibre (200 µm, 0.39 NA FC/PC, Thorlabs part no M83L01). This allowed these mice to also be used in optical tagging experiments. Light

was delivered in 10 ms pulses at 20 Hz trains of 5 second duration with 20 to 40 seconds between trains at 5 to 10 mW.

For dMSN stimulation followed by MLR inhibition (Fig. 4), 10 seconds of 2.5 mW blue light was delivered unilaterally (side randomized) into a striatal ferrule. At 5 seconds into stimulation, green light (OEM laser, part no G00003) was delivered for 5 seconds continuously at 10 mW bilaterally to the MLR. 5 to 6 minutes were allowed between stimulations. For the open field version of the experiment (Fig. S6), green light in the MLR was delivered 5 seconds before the blue light in the striatum and both were on continuously for 30 seconds, with 5 minutes between stimulations. A 25 second window prior to stimulation was used as the baseline for locomotion and a 25 second window beginning 5 seconds after striatal stimulation onset was used for analyzing locomotion during stimulation. Locomotion was tracked and calculated using ETHOVISION 7.1 hardware and software using parameters described previously (Freeze et al., 2013). For both head-fixed and freely-moving experiments, no inhibition trials (in which the green laser was omitted) were interleaved with inhibition trials.

For iMSN stimulation followed by MLR stimulation (Fig. 4), 10 seconds of 2.5 mW blue light was delivered bilaterally into the striatal ferrules. At 5 seconds after onset of striatal stimulation, 10 ms pulses of blue light were delivered at 20 Hz trains for 5 seconds, with power (at fiber tip) set at 5-10 mW. Trials with no MLR stimulation were interleaved with stimulation trials. If an animal did not run with MLR stimulation alone, it was excluded (3 mice total).

For recording activity during spontaneous locomotion in vGLUT2-Cre::DIO-ChR2 mice (Fig. 2), 2 light-active identification sessions were carried out before and after (“Light Evoked”; Fig. 2) a locomotor session (“Spontaneous”; Fig. 2) in which mice were allowed to run freely on the trackball for 15 to 20 minutes.

For recording MLR activity during iMSN or dMSN stimulation, 2 optical identification sessions were carried out before and after an MSN stimulation session. During the stimulation session, blue light was delivered bilaterally (iMSN) or unilaterally (dMSN) at 2.5 mW for 5 sec with 20 to 60 seconds between stimulations.

In Vivo Electrophysiology

Extracellular spikes were recorded using NeuroNexus silicon probes (part no A1x16-10mm-100-177-A16). 200-µm-diameter optical fibers were manually mounted on the probe, with the upper-most recording site approximately 100 µm below the tip of the fiber and 200 µm lateral from the probe surface. This setup greatly reduced optical artifact. Once the probe had been lowered in the brain, a drop of agarose was used to stabilize it. Voltage signals were band-pass filtered between 150 and 8000 Hz. Each data stream was amplified, processed and digitally captured using commercial hardware and software (Plexon). Typically, only 1 unit was identifiable per recording depth. After each recording, the probe was driven 100 to 200 µm to find other cells. Neurons that appeared to stay on the same channel or appeared on a channel just above a site that had a light-active neuron during the previous recording were excluded.

Optical Identification of ChR2-Expressing Neurons

At the beginning of each experiment involving identified ChR2-expressing neurons (Fig. 2, 4, S5), the probe was lowered to the presumed depth of the MLR through the burr hole and allowed to settle for 10 to 45 minutes. The fiber attached to the probe was coupled to a 473 nm laser (OEM, part no 10010351) using an optical multimode fibre (200 µm, 0.39 NA FC/PC, Thorlabs part no M83L01). Once a stable recording was established, blue light was flashed for 10 ms at 2 to 10 mW through the fiber into the brain at 1-2 Hz for 200 to 300 repetitions. Laser power was adjusted to minimize the latency of activation while also minimizing optical artifact. Once a neuron was identified as possibly being light active, the locomotor/stimulation session would proceed after which a post identification session would be carried out to ensure another unit had not moved into the recording space. Final clustering was performed post hoc.

Analysis of Neural and Behavioral Data

Pre- and post-identification sessions were merged with the associated locomotor or stimulation session to sort single units on the same principle component and peak-to-trough amplitude spaces. Single units were sorted using Offline sorter V2.1 (Plexon). Recorded units in which more than 1% of interspike intervals were shorter than 2 ms were excluded from analysis. MANOVA ($p < 0.01$) and J3 (> 3.0) parameters in 3 dimensions were used to ensure cluster quality.

Light responses were determined using 1 ms bins in NeuroExplorer V4.133. All other analysis of neural and behavioral data was carried out using custom MATLAB software which is available upon request. A neuron was considered identified (ChR2-positive) if: (1) its firing rate cleared the 99% confidence interval (based on a 20 ms baseline period prior to stim) within 5 ms after the onset of the 10 ms pulse, (2) light-evoked spike waveforms (occurring within the 10 ms of the light pulse) were identical to the spontaneous waveform ($R > 0.9$), and (3) light-evoked responses displayed jitter relative to the light onset (indicating spikes were not related to a light artifact). We chose the 5 ms cutoff as there were no light responses prior to 2 ms and therefore di-synaptic activity within a <3 ms time window would be low (Fig. S3). To generate excited/inhibited/non-modulated populations for Figure S5 during GABAergic identification, excited neurons were classified as having multiple bins above the 99% confidence interval during the 10ms pulses (therefore excited neurons could be classified as excited but not identified), inhibited neurons were classified as having multiple bins below the 99% confidence interval and non-modulated fell into neither category. Neural and locomotor data was binned in 100 ms (vGLUT2-Cre::DIO-ChR2, A2a-Cre::CAMKIIα-ChR2, D1-Cre::CAMKIIα-eNpHR3.0) or 50 ms (D1-Cre::DIO-ChR2) windows for ROC analysis which was carried out as described in Freeze et al. 2013 (Freeze et al., 2013). To obtain a p-value, Area Under the Curve (AUC) was calculated with the speed and firing rate data lagged at all time points with greater than 10 seconds of overlapping data. A zero lag AUC outside (two-tailed) 99 % of lagged AUCs was considered significant. To calculate speed versus firing rate correlations, both locomotor and firing rate data was binned in 500 ms windows and z-scored based on firing rates when the mouse was stationary. Linear regression was run on individual cells and $p < 0.01$ using an F-test determined significant correlations. To calculate changes in firing rate aligned to starts,

data was Z-scored based on a 5 sec window of time in which the mouse was stationary before the onset of locomotion or stimulation. To calculate the probability of a start within 1 second given firing rate, data was binned in 100ms windows and z-scored based on stationary firing rates. For spontaneous locomotion aligned to start onset or dMSN stimulation, only trials in which the mouse was stationary (<1 cm s^{-1}) were used. For iMSN stimulation, only trials in which the mouse was moving between 5 and 20 cm s^{-1} were used. For both conditions, data was binned in 100 ms windows and z-scored based on the 5 sec baseline. For MLR stimulation data, trials in which the mouse moved <1 cm s^{-1} were determined to be from a stop while trials in which the mouse was traveling >2 cm s^{-1} were considered to be from a run. To quantify changes in speed from a run, the average speed for the 1 second prior to stimulation was subtracted from the average speed for the final 3 seconds of stimulation. For MLR inhibition, only trials in which the mouse was moving between 2 cm s^{-1} and 40 cm s^{-1} were used. As a control, we determined how locomotion would change spontaneously given the same baseline conditions using all data points outside of the stimulation windows with the same baseline criteria as stimulation trials. For statistics a subset of these points were randomly drawn to match sample size to compare with stimulation periods. To classify CaMKIIα-identified and unidentified neurons were classified as excited, inhibited or unmodulated during dMSN or iMSN stimulation, the 5 s before stimulation onset was compared by Wilcoxon rank sum to the 5 s during stimulation using $p < 0.01$ for significance. Latency to excitation of a CaMKIIα-identified MLR neuron during dMSN or iMSN stimulation (Fig. 4) was determined as the first bin that exceeded 2 standard deviations for 2 consecutive bins. Latency to inhibition was determined as the first bin that fell below 1 s.d. for 2 consecutive bins. Locomotor start onset or latency (Fig. 4) was determined by finding 1 cm s^{-1} threshold crossings and going back to the most recent local minimum within the 1 second preceding the threshold crossing. Latency to deceleration onset (Fig. 4) was defined by the first of 3 consecutive bins after stimulation in which a 5 cm s^{-2} deceleration was observed. Latency results were not significantly different using 2, 5 and 10 cm s^{-2} criteria. Data that were binomial (true or false for a population) were tested using a 1 sided binomial test. χ^2 test was used to detect differences in population

frequencies. All other data were analyzed using a Kruskal-Wallis one-way ANOVA with a Dunn-Sidak post hoc test or a Wilcoxon rank sum as noted negating the need for variance and normality estimates.

Electrophysiology in acute slices

Mice were euthanized with a lethal dose of ketamine and xylazine followed by transcardial perfusion with 8 ml of ice cold artificial corticospinal fluid (aCSF) containing (in mM): glycerol (250), KCl (2.5), MgCl2 (2), CaCl2 (2), NaH2PO4 (1.2), HEPES (10), NaHCO3 (21) and glucose (5). Coronal slices (250uM) containing the MLR were then prepared with a vibratome (Leica) in the same solution, before incubation in 33° C aCSF containing (in mM): NaCl (125), NaHCO3 (26), NaH2PO4 (1.25), KCl (2.5), MgCl2 (1), CaCl2 (2), glucose (12.5), continuously bubbled with 95/5% O_2/CO_2. After 30 minutes of recovery, slices were either kept at room temperature or transferred to a recording chamber superfused with recording aCSF (2.5 ml/min) at 33° C . Whole-cell current-clamp recordings were obtained using an internal solution containing (in mM): KGluconate (130), NaCl (10), MgCl2 (2), CaCl2 (0.16), EGTA (0.5), HEPES (10). Voltage-clamp recordings were obtained using an internal solution containing (in mM): CsMeSO3 (120), CsCl (15), NaCl (8), EGTA (0.5), HEPES (10), Mg-ATP (2), Na-GTP (0.3), TEA-Cl (10), QX-314 (5). Spiking or synaptic release were evoked by flashing 470nm filtered LED (Prizmatix) blue light through the objective (1-10 msec pulse, 1mW/cm^2). EPSCs and IPSCs were measured while holding the membrane potential at -70mV and 0mM, respectively, at 20s intervals. The MLR was identified as the region lateral to the decussation of the superior cerebellar peduncle. Picrotoxin, APV and NBQX were purchased from Tocris, prepared at stock concentration in H_20, then diluted in aCSF for bath application. Data was acquired with custom Igor software.

Histology.

Animals were euthanized with a lethal dose of ketamine and xylazine (400 mg ketamine plus 20 mg xylazine per kilogram of body weight, i.p.) and transcardially perfused with PBS, followed by 4% paraformaldehyde (PFA). Following perfusion, brains were transferred into 4% PFA for 16 –24 h and

then moved to a 30% sucrose solution in PBS for 2–3 d (all at 4 deg C). Brains were then frozen and cut into 30 μm coronal sections with a sliding microtome (Leica Microsystems, model SM2000R) equipped with a freezing stage (Physitemp) and mounted on slides. Slides were blocked for 1 hour in 10% Normal Donkey Serum (NDS) in 0.5% PBST (1% for ChAT staining) then incubated overnight in primary antibody (1:500), 3% NDS in 0.5% PBST (1% for ChAT staining). The following day, they were washed 3 times for 10 minutes each in 0.5% PBST (1% for ChAT staining) and incubated for 1 hour in secondary antibody (1:1000), 3% NDS in 0.5% PBST (1% for ChAT staining) and 1:2000 DAPI. After this, slides were washed for 10 minutes in 0.5% PBST and 2 more 10 min periods with 1:1 PBS. Slides were then washed with 0.05% lithium carbonate and alcohol, rinsed with diH2O, and coverslipped with Cytoseal 60.

Slides were scanned on a VS120 semi-automated fluorescent slide scanner (Olympus Scientific Solutions Americas Corp, USA). Some figure images were acquired using a 6D high throughput microscope (Nikon, USA) or SP5 confocal (Leica, USA), globally gamma-adjusted to reduce background, and pseudocolored using freely available Fiji software. Settings were constant across confocal images in Fig. 1. Confocal and wide view images in Fig. 1, 3 and S1 were made brighter for better print quality using Photoshop function “Vibrance” and “Replace Color” to change LUT. No detail was lost during this manipulation.

Rabies cell counts and injection site verification were manually acquired using open source code Fiji run on the ImageJ platform (NIH, BetheMD). Manual registration of slices was performed by extracting whole slice images using Fiji, then performing scaled rotation in Adobe Illustrator, using the Paxinos mouse atlas (Academic Press, Orlando, FL) panels as a background reference. Animals with less than 100 total neurons labelled with eYFP were excluded (n = 3 exclusions). Areas proximal to (PAG and Inferior colliculus) and within the MLR (CUN, PPTg, MRN) were excluded from fraction of total cell calculations so long range connections could be weighted fairly. Regarding the outlier in Fig. 2d, the data is significant with and without it ($p < 0.05$, Wilcoxon rank sum).

To determine the site of recording or stimulation along the dorsal-ventral axis, the lowest depth of the electrode was noted both post hoc and during the experiment. The difference between these two values was then subtracted from the noted depth during a given recording. For recordings, the recording site distance from the electrode tip was further subtracted to give a more exact position. The electrode track itself was used to locate the ML and AP position. Neurons determined to be outside of the MRN, Cun or PPTg were excluded.

Supplemental Methods References

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