METHODS

Experimental subjects

Male and female adult (250-450g) Long Evans (LE) wild type and ChAT::cre+ rats (Witten et al., 2011) were used for all anatomical, *in vivo* electrophysiological and behavioral experiments. For electrophysiology and anatomy experiments, animals were maintained on a 12:12 light cycle (light on 07:00) and had *ad libitum* access to water and food. Animals were group housed except for recovery following surgery and during behavioral experiments in order to avoid infection and post-implantation complications. Following surgery, all animals received i.p. injections of analgesic (ketofen) and antibiotic (Baytril); no experiments were made 7 days following last drugs administration. For behavioral experiments, animals were maintained on a 12:12 inverted light cycle (light on 19:00) and had *ad libitum* access to water and food except during behavioral training. All littermate of the same age were allocated to each group based on their genotype and sex. All procedures were performed in accordance with the Society for Neuroscience policy on the use of animals in neuroscience, with the approval of the Rutgers University Institutional Animal Care and Use Committee and in accordance with the NIH Guide to the Care and Use of Laboratory Animals, and the Animals (Scientific Procedures) Act, 1896 (UK), under the authority of Project License approved by the Home Office. For all experiments, all efforts were made to minimize the number of rats used and any possible discomfort.

Stereotaxic surgery

All stereotaxic injections were performed during deep isoflurane anesthesia (2-4% in O_2; Isoflo, Schering-Plough, Garden City, UK). For all experiments, animals were placed in a stereotaxic frame (Kopf Instruments), the skull surface was exposed and rats were infused with virus (Table 2) at specific coordinates (Table 3) using a nano-syringe (1µl, Hamilton Instrument, product 7001). Injections were made at a rate of 50 nl/min and remained in place for 10 minutes after the end of the infusion. For single unit juxtacellular recordings, four groups of male and female ChAT::cre+

rats were used and received an injection of AAV2-EF1a-DIO-ChR2-eYFP (PPN: n = 11; LDT: n = 13; CIN: n = 16) or AAV2-EF1a-DIO-eYFP (controls, PPN: n = 6, CIN: n = 6). For high-density electrophysiology experiments, male and female ChAT::cre+ rats (n = 6) received an injection of AAV2-EF1a-DIO-ChR2-eYFP in both the PPN and the LDT (two separate injections) and AAV5-EF1a-DIO-NpHr3.0-mCherry in the rostral region of the dorsal striatum. For anatomical mapping, female ChAT::cre+ (n=4) were injected in the brainstem with AAV2-EF1a-DIO-eYFP and in the striatum with AAV5-EF1a-DIO-mCherry. For *in vivo* electrophysiology/pharmacology experiments, male ChAT::cre+ rats (n=7) received an injection of AAV2-EF1a-DIO-ChR2-eYFP in the brainstem (two separate injection in PPN and LDT). For electron microscopy experiments, female ChAT::cre+ rats (n = 4) were injected in the brainstem with AAV2-EF1a-DIO-eYFP. For experiments assessing the expression of a transcription factor, female Chat::cre+ were injected in the brainstem with AAV2-EF1a-DIO-ChR2-eYFP (n = 6) or AAV2-EF1a-DIO-eYFP (n = 4). For behavioral experiments, ChAT::cre+ and WT rats were bilaterally injected with AAV2-hSyn-HA-hM4D(Gi)-DIO-mCherry in the PPN (ChAT::cre+ n = 9; WT n = 5), in the LDT (ChAT::cre+ n= 7; WT n= 5), in the dorsomedial striatum (DMS; ChAT::cre+ n = 6; WT n = 5) and in the dorsolateral striatum (DLS; ChAT::cre+ n = 8; WT n = 5), and bilaterally implanted in the DMS or DLS (see results) with a custom-made 26-gauge stainless steel cannula (Kokare et al., 2011). For retrograde transsynaptic labeling targeting cholinergic interneurons, ChAT::cre+ rats were used (n = 4) and were injected with AAV-DIO-mCherry-TVA and AAV-DIO-RG (ΔG) (helper virus) in the DLS and DMS. Two weeks after injections, animals were injected with pseudotyped rabies virus RVdG-YFP in the same coordinates as the helper virus. For retrograde transsynaptic labeling targeting direct- and indirect-pathway SPNs, animals received an injection of CAV2-Cre in the substantia nigra pars reticulata (SNr; n = 3) or the external globus pallidus (GPE; n = 3), respectively, followed by injection of AAV-DIO-mCherry-TVA and AAV-DIO-RG (ΔG) (helper virus) in the DLS or DMS. A different group of animals were injected with the AAV2-EF1a-IRES-WGA-Cre (Gradinaru et al., 2010) in order to express the wheat germ agglutinin (WGA)-Cre fusion

protein in the SNR or GPE, which will be retrogradely transported to the striatum, or directly into the striatum (to label all striatal neurons, including interneurons). This was followed by helper virus injections into the striatum as above. Two weeks after injections, the pseudotyped rabies virus RVdG-YFP was injected at the same coordinates as the helper viruses. All animals used for rabies virus tracing were kept in a BSL2 facility and were perfused 7 days following last surgery.

Table 1: Coordinates and volumes list

	Coordinates (mm)				
Structure	**AP**	**ML**	**DV**	**volume (nl)**	**rate (nl/min)**
PPN	-7.30	1.80	-7.50	400	40
LDT	-8.50	0.90	-6.00	300	30
DMS	0.50	1.80	-4.5 / -3.5	450	40
DLS	0.50	3.00	-4.5 / -3.5	450	40
DS	0.50	2.60	-4.5 / -3.5	450	40
SNr	-5.80	1.80	-7.50	500	40
GPE	-1.30	3.00	-6.5	300	30

Table 2: Viral constructs

Name	**Construct**	**Titer (vg/ml)**	**Source**
eYFP	AAV2-DIO-EF1a-eYFP	1.10^13	UNC vector core
eYFP-ChR2	AAV2-DIO-EF1a-eYFP-ChR2	1.10^13	UNC vector core
mCherry-NpHR3.0	AAV5-DIO-EF1a-mCherry-NpHr3.0	1.10^13	UNC vector core
mcherry	AAV5-DIO-EF1a-mCherry	1.10^13	UNC vector core
TVA	AAV5-Flex-TVA950-EF1a-mCherry	1.10^12	UNC vector core
ΔG	AAV8-Flex-RG-CAG	1.10^12	UNC vector core
RVdG	SADΔG-GFP(EnvA)	1.10^8	Salk Vector core
CAV2-cre	CAV-Flex(loxP)-cre(Flp)	6.10^12	IGMM vector core
WGA-cre	AAV-EF1A-IRES-WGA-Cre	6.10^12	UNC Vector core
DREADD	AAV2-hSyn-hM4D(Gi)-DIO-mCherry	3.10^12	UNC vector core

Electrophysiology

Juxtacellular recordings

Anesthesia was induced with 4% v/v isoflurane (Schering-Plough) in O_2, and maintained by injection of urethane (1.3 g/kg, i.p.; ethyl carbamate, Sigma, Poole, UK). Supplemental doses of ketamine (35 mg/kg, i.p.; Ketaset, Willows Francis, Crawley, UK) and xylazine (6 mg/kg i.p.; Rompun, Bayer, Germany) were given as required throughout the experiment. Body temperature was maintained at 38°C using a thermistor controlled heating pad. After local skin anesthesia by subcutaneous injection of Marcaine (0.25%), the animals were placed in a stereotaxic frame (Kopf). A subcutaneous incision was made to expose the skull. Then, craniotomies were made for the electrocorticogram (ECoG, bilaterally from bregma: AP +3.0 mm, ML +2.5 corresponding to the somatosensory cortex) and a reference (above the right cerebellum). A small craniotomy was made above the striatum and the dura mater was gently removed to allow the passage of the recording pipette. The exposed brain surface was kept moist with sterile saline (0.9% NaCl) throughout the experiment.

The ECoG was recorded using a 1 mm diameter stainless steel screws and referenced to a steel screw above the cerebellum. The ECoG signals were band-pass filtered at 0.3-1000 Hz (-3 dB limits), amplified 2000 times (DPA-2FS filter/amplifier; Scientifica, Harpenden, UK) and digitized online at 2.5kHz. The ECoG was used to monitor the depth of anesthesia. Extracellular recording of action potentials of individual striatum neurons was made using custom-made glass opto-micropipettes (1.5 mm O.D.; 1.17 mm I.D Harvard Apparatus; 15-25MΩ impedance measured in the cortex, tip diameter ~1.5µm, with a 105µm optic fiber, 0.22NA multimode, Thorlabs) filled with 1.5% w/v Neurobiotin (Vector Laboratories Ltd., Peterborough, UK) in 0.5M NaCl. The optic fiber was inserted and fixed in the glass pipette and the other side was connected to a class IIIb blue laser (473nm; LaserGlow Tech., Canada) in order to provide a minimum power of 5 mW at the tip of the glass pipette. The output power was tested between penetrations and readjusted if needed.

Signals from the glass micropipettes were band-pass filtered at 0.3-5000 Hz (NL125: Digitimer), amplified 10-fold through the active bridge circuitry of an Axoprobe-1-A amplifier (Molecular Devices Corps. Sunnyvale, CA), AC-coupled and amplified a further 100-fold (NL-106 AC-DC Amp: Digitimer Ltd., Welwyn Garden City, UK), and digitized online at 17.5 kHz. Data were acquired and stored using an analog-to-digital converter (Power 1401: Cambridge Electronic Design, Cambridge, UK) connected to a PC running spike2 (ver 7; Cambridge Electronic Design). The whole of the striatum was scanned with the opto-micropipette for spontaneously firing neurons. When action potentials were detected, a minimum of 5 min of basal firing were recorded to establish a mean baseline firing rate and spontaneous discharge pattern. The activity of striatal neurons was recorded during optical stimulation of PPN, LDT or CINs afferents using a train of light stimulation (8 s, 80 pulses, 10Hz, 50ms ON/OFF). Train pulses were repeated at least twice, with a minimum interval of 1 min between each. At the end of the recording, a microiontophoretic current was applied to the neuron to label it with Neurobiotin. To ensure discrimination between neurons during the histological analysis (see below), a maximum of 8 neurons were recorded per animal with a distance of at least 400 µm in all axes (X, Y, Z). Following at least 2 hours of diffusion time, the animals were given a lethal dose of ketamine and intracardially perfused. Brains were stored in PBS at 4°C until sectioning.

High-density extracellular recordings and optogenetics

ChAT::cre+ rats injected with AAV-DIO-ChR2-YFP in the brainstem and AAV-DIO-NpHR-YFP in the striatum were anesthetized, as described above. Anesthesia was maintained by a urethane injection (1.3 g/kg, i.p.). Body temperature was maintained at 38°C. Animals were placed in a stereotaxic frame and the skull exposed. A small craniotomy was made above the striatum and the dura was gently removed to allow passage of the recording electrodes. Extracellular activity was recorded in the striatum using a 16-channels silicon probe with an optical fiber mounted 200µm above the upper-most recording site (Neuronexus, OA16-10mm-100-177). Signals were

amplified, digitized continuously at 20kHz using a head-stage directly attached to the probe (RHD2000, Intan Technology). The fiber attached to the probe was coupled to an optic collimator (CFC-11x-c, thorlabs) focusing a 473-nm laser (15mW, OEM) and a 635-nm laser (6mW, OEM). Laser power was adjusted to minimize optical artifacts. Once one or more units were identified, a 2-to-5 minutes baseline recording was recorded and then neurons received blue stimulation (10Hz, 50ms, 8s duration), yellow stimulation (continuous 8s duration) or both synchronized. All stimulations were repeated at least 3 times, only consistent responses were considered further. Recording files were converted into Spike2 format (CED), band-pass filtered between 300-5000Hz and sorted into single units using principal component analysis. Light responses were determined as variation of the firing rate during laser stimulation (Hz), percentage of variation compared to the 20s prior to stimulation (baseline) or the standard deviation from the mean firing rate during baseline activity (z-score) and compared between stimulation parameters (blue, yellow or both). Following recordings, animals were perfused and their brain processed. In order to locate the recording site, the back of the silicon probes was stained with DiI (Invitrogen, D3911). Only recordings within a region where both ChR2-YFP and NpHR-mCherry fluorescent signals were overlapping were used for analyses. Putative SPNs (pSPNs) were defined by their physiological properties (see below) and their inhibitory response to blue light (as observed during juxtacellular recording); putative cholinergic interneurons were defined by their physiological properties (see below) and their inhibitory response to yellow light (NpHR expression; decrease of at least 80% of their firing rate over a minimum of 2 laser stimulations).

In vivo **pharmacology**

An *in vivo* pressure drug-delivery method combined with optogenetic stimulation was adapted from previous reports (Dautan et al., 2016; Gradinaru et al., 2007) to allow simultaneous recording, laser stimulation and drug delivery to putative spiny projection neurons in anesthetized rats. Custom-made tungsten electrodes (1MΩ, AM-System # 573220) were combined with an

optic fiber (200µm, 0.50NA, Thorlabs) and a stainless-steel tube for drug delivery (87µm O.D., Small Parts Inc.) that was located ~200µm from the recording site. The optic fiber was connected to a blue laser (3mW, LaserGlow Tech.). The cannula was connected to a precision syringe (0.5µl, Hamilton Company) for delivery of 100nl of nicotinic and muscarinic antagonists (methyllycaconitine (MLA) 20 mM, dihydro-β-erythroidine (DHβE) 40 mM, atropine 40 mM and mecamylamine 100 µM), as previously described (Dautan et al., 2016). The recording electrode was connected to an Intan RHD2000 amplifier (20 Ks/S) and the signal was referenced to an anchor screw located above the cerebellum. Signals from the electrodes were band-pass filtered at 500 – 5,000Hz and analyzed using Spike2 software (Cambridge Electronic Design, CED). Only one recording per animal was performed in order to avoid effects of the drugs not washing up. Only neurons that responded to the laser stimulation and that were conserved throughout the entire recording were analyzed.

Identification of neurons from extracellular recordings

Neuron activity was used to determine the putative nature of neurons recorded using extracellular tungsten electrodes and high-density silicon probes. Only neurons with a signal-to-noise ratio higher than 1:3, stable action potential waveform during the entire recording and a minimum inter-spike-interval (ISI) of 2ms were considered as single units. Striatal neurons were categorized based on their basal firing rate, coefficient of variation (CV) and action potential duration as previously described (Sharrott et al., 2012; Lee et al., 2017; Adler et al., 2013). Briefly, putative spiny projection neurons (pSPNs) were defined by their low basal firing rate (<2Hz), a long peak-to-peak action potential duration (>0.5ms) and high coefficient of variation (CV, >1). Putative cholinergic interneurons (pCINs) were defined by a high firing rate (>2Hz), a long peak-to-peak action potential (>0.5ms), a long action potential duration (>1.5ms) and a low CV (<1). Recording and parameters from non-identified extracellular recordings were similar to those from juxtacellularly-labeled neurons.

Electrophysiological data analysis

Spike trains were digitized and converted into a time series of events using in-built Spike2 functions. To determine variations in the firing rate following experimental manipulations, 10-sec segments of baseline activity preceding each stimulus were compared to the firing rate during the laser stimulation. Stimulus-induced response of each neuron was expressed as percentage of change from the mean firing rate of their individual baseline. Neuron responsiveness was determined by computing a time-resolved response to light stimulation as previously described (Dautan et al., 2016). A modified estimation of the mean instantaneous firing rate (iFR) locked to the laser were obtained for all trials and for each neuron. A cumulative distribution function (CDF) was defined by using each spike train j= 1, …, *k* as a sequence of individual event occurring at the time $\{t_{i,j}\}$, I = 1, …, n_j. The number fo events up to $t_{i,j}$ is a strictly increasing function changing at $t_{i,j}$. The mean density of events across the *k* trials is obtained by dividing the slope of the CDF by the number of trials and represents the trial-averaged firing rate. Then a local linear regression based on $N_{i,j}$ = 6 x *k* proximal events is used to estimate the instantaneous firing rate at each reference time $t_{i,j}$ (FR(*t*)). The distribution FR(*t*) of spontaneous activity during baseline (10s before laser stimulation) was then used to compare the activity during the laser period (8s) and post-stimulation period (10s). Percentiles 5 (C_5) and 95 (C_{95}) of FR(*t*) during baseline were selected as thresholds to assess significant responses during laser stimulation. Significance of the response was assessed using cluster-based permutation (n = 200 permutations, $P < 0.05$), if the change occurs during laser stimulation and for at least 2 seconds. If these criteria were not met, then the neuron was considered as non-responding. Z-scored data are represented as mean ± 95% coefficient intervals (CI). The significance level for all tests set to $P < 0.05$. % of changes data are expressed as mean ± SEM.

***In vitro* recordings**

In vitro whole cell recording were performed in adult ChAT::cre+ rats, ChAT-ChR2-YFP mice (Jackson #014546), ChAT-cre mice (Jackson #006410) or wild-type mice (Jackson # 000664) using similar protocols as previously described (Faust et al., 2015). Briefly, adult mice or rats (above 3 months age) were deeply anesthetized using isoflurane and then injected with 100 mg/kg ketamine (i.p.). The animals were then transcardially perfused using ice-cold N-methyl d-glucamine (NMDG)-based solution containing (in mM): 103.0 NMDG, 2.5 KCl, 1.2 NaH2PO4, 30.0 NaHCO3, 20.0 HEPES, 25.0 dextrose, 101.0 HCl, 10.0 MgSO4, 2.0 Thiourea, 3.0 sodium pyruvate, 12.0 N-acetyl cysteine, 0.5 CaCl2 (saturated with 95% O2 and 5% CO2, pH 7.2–7.4). The rodents were then decapitated and their head immediately transferred to a petri dish containing oxygenated, ice cold NMDG-based solution. The brain was extracted and transferred to a Leica vibratome (VT 1200S) containing an oxygenated bath of NMDG-based solution maintained at -4 °C. Sagittal or coronal sections of 250 or 300µm thickness were cut and transferred to an oxygenated bath of NMDG-based solution maintained at 35 °C for recovery for 5 minutes. The slices were transferred to a Ringer's solution bath maintained at 25 °C and allowed to recover for at least an hour. Recordings were performed using glass pipettes (impedance of 3–4 MΩ) containing a regular internal solution composed of (in mM): 130 K-gluconate, 10 KCl, 2 MgCl2, 10 HEPES, 4 Na2ATP, 0.4 Na2GTP, pH 7.3.

Alternatively, CsCl based internal solution (in mM): 125 CsCl, 2 MgCl2, 10 HEPES, 4 Na2ATP, 0.4 GTP was used for some voltage clamp recordings at -70mV. Pipette internal solution was supplemented with biocytin or Alexa 594 to allow for *post hoc* identification of the recorded neurons. Axoclamp 700B amplifier (Molecular Devices) and ITC-1600 digitizer (Instrutech) was used to measure membrane currents and voltages. Data acquisition was done using Axograph (www.axographx.com) software. Slices were visualized using Andor Ixon (Andor Technology Ltd, Belfast, Northern Ireland) camera and software.

For *in vitro* DREADD recordings, ChAT::cre+ rats were injected with AAV-DIO-hM4Di-mCherry virus (500nl) in the striatum and were allowed to recover for 5-6 weeks before recording. mCherry-positive neurons were recorded and confirmed to be CINs by initial identification of their electrophysiological properties and followed by *post hoc* immunolabelling for ChAT. Similar recording in mCherry-negative CINs revealed no changes in their firing rate following exposure to CNO (data not shown).

For optogenetic experiments, ChAT-cre mice were injected with an AAV-DIO-mCherry (to label cholinergic interneurons, 200nl) and an AAV-DIO-ChR2-GFP (to activate transduced CINs, 50nl). When recording YFP+ CINs, optogenetic stimulation produced action potentials. Only CINs neurons not expressing YFP/ChR2 were used for analyses. Optogenetic stimulation consisted of 2-5ms-duration blue-light pulses delivered using high-power (750mW, 450nm) LED. Sweeps were run with an inter-trial interval of 10 seconds. Each trial was 4 seconds long with an optogenetic pulse at the 2 seconds mark.

In vitro carbachol puffs were delivered using a micropipette connected to a Picospritzer (General Valve). Carbamylcholine chloride (Carbachol, 100-250μM) was dissolved in Ringer's solution containing 0.5μM atropine, 10μM 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-(-)-2-Amino-5-phosphonopentanoic acid (AP5). Pressure pulses were applied at 20 psi with a width of 100ms. Pulses were spaced by at least 3 minutes to avoid receptor desensitization (Xiao et al., 2014).

Drugs that were used include: bicuculline (to block GABA-A receptors; 10μM, Sigma), dihydro-b-erythroidine hydrobromide (DhβE; to block type 2 nicotinic receptors; 1μM, Tocris), 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX; to block AMPA receptors; 10μM, Tocris), D-(-)-2-Amino-5-phosphonopentanoic acid (AP5; to block NMDA receptors; 10μM, Tocris) and clozapine-N-oxide (CNO; to activate hM4Di receptors; Sigma, 10μM). All drugs were dissolved in Ringer's solution.

Data were analyzed using paired two-tailed t-test with GraphPad Prism 6 software. Differences were considered to be significant at $P < 0.05$.

Histology

Immunohistochemistry and immunofluorescence

Following behavioral, electrophysiological or anatomical experiments, rats were humanely euthanized with an overdose of pentobarbital (300mg/kg, i.p.) and then intracardially perfused with 0.05 M PBS followed by ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were prepared for either coronal or sagittal sections (50µm) in PBS using a vibrating microtome (VT1200S, Leica). For each experiment, several sections near the injection, drug administration or recording sites were selected and processed for immunostaining as previously described (Dautan et al., 2016). For all experiments, injection sites and implantation sites were confirmed by fluorescent microscopy and only those with on-target injections were processed further.

Briefly, for juxtacellular experiments, recording site sections were incubated in a blocking solution (10% normal donkey serum in 1% Triton X100 in PBS) for 1 h. Sections were then washed and incubated overnight in an antibody against ChAT and GFP. Following several washes, sections were incubated for 4h in CY5-conjugated donkey antibody and Alexa-488-conjugated donkey antibody. All sections in the striatum were processed to reveal neurobiotin using CY3-streptavidin solution in PBS-0.03% Triton. Sections containing CY3-positive neurons were incubated with antibodies against parvalbumin (PV), chicken ovalbumin upstream promoter transcription factor interaction protein 2(Ctip2) or ChAT, based on their relative firing properties, presence of spines and soma size. Following overnight incubation at 4°C and several washes, sections were incubated with their respective fluorophore-conjugated secondary antibody (see table 1). Images of streptavidin-positive neurons were captured using a confocal microscope (LSM-510, Zeiss). If

the neurochemical nature of the neurons was not revealed following the first immunostaining, sections were re-incubated with different antibody (against PV, Ctip2 or ChAT). Neurons that were negative for all 3 antibodies were considered as others (non-PV GABAergic interneurons) and not considered further in this study.

For the histology of brains of animals from behavioral experiments, sections within the striatum and the PPN/LDT were processed for mCherry or HA (Human Influenza) and ChAT with their respective fluorophore-conjugated secondary antibodies. Images of the cannula implantation site and PPN/LDT were acquired using a confocal microscope. Due to the weak labeling of axons from DREADD expressing neurons, a cholinergic neuron expressing mCherry and FG was considered as projecting to the striatum and responsive to CNO administration.

Table 3: Antibodies list

	Target	Raised in	Cat. number	Dilution	Company
Primary antibodies	ChAT	Goat	AB144P	1/500	Abcam
	GFP	Rabbit	A21311	1/1000	Invitrogen
	PV	Guinea Pig	195004	1/500	Synaptic System
	Ctip2	Rabbit	AB28448	1/500	Abcam
	HA	Rabbit	3724	1/1000	Cell Signaling
	mCherry	Rabbit	AB167453	1/1000	Abcam
	pser240-244	Rabbit	2211	1/250	BD Biosciences
Secondary antibodies	anti-Goat CY5	Donkey	705-175-147	1/1000	Jackson Immunoresearch
	anti-Goat CY3	Donkey	705-165-147	1/1000	Jackson Immunoresearch
	anti-Rabbit 488	Donkey	711-545-152	1/1000	Jackson Immunoresearch
	anti-Rabbit CY3	Donkey	711-165-152	1/1000	Jackson Immunoresearch
	anti-Guinea pig 488	Donkey	706-545-148	1/1000	Jackson Immunoresearch

Input neuron analysis

Following immunostaining of SADΔG-eGFP and TVA (mCherry), quantification of starter neurons (mCherry+/GFP+) and SADΔG-eGFP neurons in the PPN/LDT was performed. For starter neurons quantification, low-magnification (10X), high-resolution (1024*1080) confocal images of the injection site in the striatum were acquired. AAV-TVA (mCherry) diffusion volume within the striatum was then determined using in-build freehand tools (ImageJ). In three striatal sections, spaced by 300 µm and containing the injection site, a total of 18 sites of 200µm^2 were randomly selected and the total number of neurons overlapping mCherry and YFP signal were counted offline. For PPN/LDT inputs neurons, all sections containing PPN or LDT that were labeled for ChAT immunostaining and GFP signal were counted. PPN and LDT (defined by ChAT staining) were scanned at high-magnification (20X), high resolution (1024*1080) using mosaic confocal in-build tools (Fluoview FV1260, Olympus). Mosaic files were then transferred to ImageJ software, the contrast was adjusted and borders of the PPN/LDT defined using freehand tools. Positions of the YFP-positive neurons were recorded using multi-point in-build tools and digitized on a representative sagittal section of the PPN/LDT. Counted neurons within the PPN/LDT borders were then expressed as a ratio of inputs neurons within the PPN/LDT per 10,000 starter neurons.

PSer240-244 detection and fluorescence analysis

ChAT::cre+ rats injected in the PPN with AAV-EF1a-DIO-ChR2-YFP (n = 5) or AAV-EF1a-DIO-YFP (n = 5) were deeply anesthetised with 4% v/v isoflurane (Schering-Plough) in O_2, maintained with urethane (1.3 g/kg, i.p.; ethyl carbamate, Sigma, Poole, UK) and placed on a stereotaxic frame. A flat-cut optic fiber (200µm, 0.39NA, thorlabs) was inserted in the DLS. Animals received 10 stimulation trains (10Hz, 50ms pulses, 8s duration, 30 seconds inter-stimulation interval) and were transcardially perfused 30 minutes following stimulations. Following slicing, brain free-floating sections around the stimulation site were collected. Sections were blocked as described above and then incubated overnight for immunostaining of ChAT and the double phosphorylated

S6 ribosomal protein (pSer$^{240-244}$-S6p). Sections were then rinsed several times and incubated with their respective fluorophore-conjugated secondary antibodies.

High-resolution images of ChAT+ neurons in the striatum surrounded by YFP+ projections were acquired (40X, in oil, resolution 1024*1080) and examined under confocal microscopy (Fluoview FV1200, Olympus) using 560 nm and 650nm lasers. Three regions of interest (ROI) were defined using ChAT staining, ROI1: ChAT-immunoreactive area (neuron surface excluding nucleus), ROI2 and ROI3: background signal in the vicinity of the soma. 32-bits pictures were converted to gray scale images using ImageJ (color tools) and the mean gray value (fluorescence intensity: *FI*) of each ROI was obtained for the pSer$^{240-2444}$ channel using ImageJ (mean gray value). For every picture, background fluorescence intensity, ROI2(*FI*) and ROI3(*FI*) were compared and averaged (FI = $\bar{x}$ (ROI2(*FI*)/ROI3(*FI*)), then soma *FI* was compared to the background in order to obtain Δ*Fi* (ROI1(FI)- $\bar{x}$ (Roi2(FI)/Roi3(FI)). We used a two-tailed t-test for two group comparisons (ChR2-YFP and YFP) and compared background FI, soma *FI* and Δ*Fi* (Bertran-Gonzalez et al., 2012). Images scanning, gray-scaling and analyses were done using blind experimenter. Two blinded experimenters performed the quantification of pSer240-244 fluorescence. Background fluorescence and normalized somatic fluorescence were analyzed using one-way ANOVA. The significance level for all tests was set to $P < 0.05$.

Electron microscopy

Sections from ChAT::cre+ rats injected in the brainstem with AAV-DIO-GFP were collected as described above. Sections were incubated in a cryoprotectant solution (0.05 M phosphate buffer, 25% sucrose, 10% glycerol) overnight, then freeze-thawed in order to increase penetration of the reagents. Sections were double-immunolabeled to reveal postsynaptic intrastriatal cholinergic interneurons using an anti-ChAT antibody and presynaptic YFP-containing axons using a biotinylated antibody against GFP. Sections were blocked in 10% normal rabbit serum (NRS,

Vector) in PBS for 2 h prior to incubation of primary antibodies. All sections were incubated for 24h at room temperature in a rat antibody against GFP (1:1000; Nacalai Tesque Inc.) followed by an incubation in a rabbit anti-rat biotinylated antibody (1:500; Vector Labs) overnight at 4°C. After washing thoroughly, sections were incubated in an avidin-biotin-peroxidase complex (ABC Elite, 1:100; Vector) for 3-4 h at room temperature. The sections were incubated in Tris-buffer (0.5M, pH 8; TB containing 0.025% diaminobenzidine solution [DAB wt/vol, Sigma] and 0.5% nickel ammonium sulphate [wt/vol, Sigma] for 20 min). The reaction was initiated by addition of H_2O_2 to a final concentration of 0.01%. To label cholinergic structures, all sections were incubated in a goat antibody against ChAT (1:500 in PBS NRS 1%; Millipore) for 24 h at room temperature. Sections were then incubated in rabbit anti-goat IgG (1:100 in PBS NRS 1%; Jackson Immunoresearch) for 4 h at room temperature, followed by incubation in goat peroxidase-anti peroxidase (1:200 in PBS; Jackson Immunoresearch) for 2h at room temperature. The sections were washed in PBS followed by washes in 0.1M Phosphate Buffer (PB), pH 6.0. Sections were pre-incubated in PB containing tetramethylbenzidine (TMB) as the chromogen. The reaction was initiated by adding glucose oxidase (Sigma G6891) and stopped after 6-8 min with 0.1M PB. The reaction was stabilized with DAB and cobalt chloride and washed thoroughly before being processed for electron microscopy.

The sections were postfixed in osmium tetroxide (1% in PB, Oxkem, Oxford, UK) for 20 min. The sections were dehydrated and contrasted with 1% uranyl acetate and left overnight in resin (Durcupan, ACM, Fluka, UK) before being mounted on slides and cured at 60 °C for at least 48 h. All sections were examined in the light microscope and areas of interest were cut from the slide, embedded in resin blocks and trimmed with a razor blade. Serial sections of 50nm thick were cut using an ultramicrotome (Leica EM UC6), collected in copper grids and lead-stained to be examined on a Philips CM10 electron microscope.

Striatal cholinergic axons of PPN origin were identified by the presence of a black dense material produced by the deposition of the DAB-nickel in axons and terminals. Intrastriatal cholinergic interneurons and their processes could be identified by the presence of TMB crystals within dendrites and cell bodies.

Behavior

Goal-directed test

All animals used for behavioral experiments were handled, and dummy cannula replaced, on a daily basis to avoid stress-related behavior. Both ChAT::Cre and WT male Long Evans rats were used for behavioral testing. Experiments were performed during the dark phase of the light cycle and the room was illuminated with red light to maintain behavioral activity. Four weeks following virus injection and cannula implantation, rats were randomly assigned to an operant box containing one lever (at the right or the left side of the box, randomly attributed) and at the opposite side of a food pellet dispenser. A small LED was placed above the magazine and the box was illuminated by a house light (MED-008-D1, Med Associates). Before training, rats were exposed to sugar pellets (45mg, Bio-Serv, Frenchtown, NJ) in their home cage and then in the operant box magazine. Following two days of habituation, animals were manually shaped for lever pressing until completion of 80 presses within 40 minutes on a fixed-ratio-1 (FR1; one press, one reward) schedule. House light was kept on and the lever was continuously extended during testing. Training occurred at the same time of the day, 7 days a week.

Outcome devaluation test

Following the completion of a successful FR1 session, rats were trained on a random-ratio schedule (RR5; on average one reinforcer every 5 lever presses, minimum 1 press, maximum 9 presses) for 30 minutes during 4 days. Animals received CNO (1µM) delivery through the bilateral

cannulas 30 minutes before daily training. During CNO delivery, animals were gently restrained, dummy cannula removed, and injectors (30 gauges) were lowered. Injections were delivered with a 30 gauge indwelling cannula connected to a high-precision pump (New Pump 11 Elite, Harvard Bioscience, Canada). A total of 250nl of CNO was infused over 5 minutes in each hemisphere. Following infusion, the cannula was kept in place for 2 minutes and the animal returned to the home cage.

Outcome devaluation test occurred in the following two days. Animals were exposed 30 minutes before the behavioral test to sugar pellets (devalued session) or to lab chow pellet (valued session). On the valued session, rats had *ad libitum* access to home-cage outcome (lab chow pellet) while on the devalued session, rats had *ad libitum* access to sugar pellets. Valued and devalued sessions were randomized between animals and consisted of a non-reinforced RR5 session of 15 minutes.

Habit learning test

Following the first outcome devaluation, animals were re-trained on a FR1 schedule until completion of 80 presses within 30-minute sessions (in order to avoid CNO effects). Following successful FR1 completion, animals were tested on a progressive random interval (RI) schedule consisting of 2 days of RI15 (one reinforcer following the first press after an average of 15s, minimum time 1s, maximum time 30s), 3 days of RI30 (average of 30s, minimum time 1s, maximum time 60s) and 3 days of VI60 (average of 60s, minimum time 1s, maximum time 90s). During sessions of RI training, animals received CNO 30 minutes before the behavioral test. Following completion of the RI schedule, rats were tested on two consecutive outcome devaluation tests (as previously described), consisting of two non-reinforced RI60 sessions of 15 minutes each. During all behavioral sessions, head entries, timestamps of reward delivery, and timestamps of lever presses were recorded.

Locomotor activity in open field

Following the last outcome devaluation session, animals were tested for locomotion for two days. Animals received CNO delivery through the cannula (days were randomly distributed). 30 minutes following infusion, animals were placed in a 50cm x 50cm open field for 30 minutes. Horizontal activity and time spent in the center (design of a central square of 20cm x 20cm) were detected using AnyMaze software (StoeltingCo) and an infrared camera (Logitech). Drugs and animal orders were randomized.

Following completion of all behavioral experiments, animals received injections of fluorogold through the cannula (300nl) to retrogradely label projecting neurons in the PPN and confirm cannula location and diffusion. On week after fluorogold injections, animals were perfused and their brains processed as previously described. Animals that did not complete all experiments (training, devaluation and locomotion) or animals that had the cannula misplaced or did not show any fluorogold labeling (cannula blocked) were removed from all analysis (WT: n = 4; PPN: n = 1; DMS: n = 2; LDT: n = 4).

Sugar consumption

Following locomotor activity testing, sugar consumption was tested for 4 days. Animals received CNO (2 days) and vehicle (2 days) delivery through the cannula (days were randomly distributed). 30 minutes following infusion, animals were returned to their home cage and were given access to sugar pellets for 10 minutes (10 grams). Following completion of the 10 minutes, the sugar cup was weighted, and the difference was recorded.

Statistical analyses

No statistical methods were used to predetermine sample size, but all sample sizes were similar to previously reported behavior, cellular or in vivo electrophysiological studies. Animals with injections or implantation sites out of target were excluded using pre-established criteria.

The normality of each data set was assessed using the Shapiro-Wilk test. For behavioral experiments, drugs, assigned boxed, testing order and devaluation order were randomized using unbiased assessment. Number of lever presses, lever-press rate and locomotion data were analyzed using repeated measures ANOVA, using Tukey or Bonferroni *post hoc* analyses when appropriate. Lever presses were normalized using [lever press for Valued or Devalued states/total lever presses Valued + Devalued states)]. For outcome devaluation, data were analyzed using two-way ANOVA comparing number of presses and normalized number of presses during valued and devalued periods for each condition (RR, RI). We examined the strength of the outcome devaluation by testing the devaluation index [(valued lever presses – devalued lever press)/ total lever presses] in the goal-directed (RR) and habitual learning (RI) contexts. Devaluation index data for each group were analyzed using paired *t-tests*. For electrophysiological and anatomical normally distributed data, ANOVAs or t-tests (paired or unpaired) were performed while for data that were not normally distributed or for small samples (i.e. monosynaptic tracing), we used Kruskal-Wallis rank-sum test (one-way ANOVA on rank) and *post hoc* analysis using the Wilcoxon rank-sum test (Mann-Whitney U test). Data analysis was performed using SPSS (IBM) or the statistical toolbox from Matlab. All data are reported as mean ± S.E.M.

Data and software availability

In vivo electrophysiology data were analyzed using custom-written code. Code and data are available under reasonable request to the corresponding author.