Convergent cortical innervation of striatal projection neurons

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Anatomical studies have led to the assertion that intratelencephalic and pyramidal tract cortical neurons innervate different striatal projection neurons. To test this hypothesis, we measured the responses of mouse striatal neurons to optogenetic activation of intratelencephalic and pyramidal tract axons. Contrary to expectation, direct and indirect pathway striatal spiny projection neurons responded to both intratelencephalic and pyramidal tract activation, arguing that these cortical networks innervate both striatal projection neurons.

The basal ganglia are an interconnected collection of subcortical nuclei that use cortical information about movement planning to promote contextually appropriate action selection. This is accomplished by two parallel basal ganglia networks referred to as the direct and indirect pathways. These pathways are anchored by principal striatal spiny projection neurons (SPNs). Direct pathway SPNs (dSPNs) project directly to the interface nuclei of the basal ganglia (substantia nigra pars reticulata and the internal segment of the globus pallidus). Indirect pathway SPNs (iSPNs) project to the external segment of the globus pallidus, whose neurons then project to the interface nuclei. It is widely believed that activity in dSPNs promotes actions that have previously led to rewarding outcomes, whereas activity in iSPNs suppresses actions that have led to negative outcomes.

How activity in cortical networks regulates activity in direct and indirect pathways has been the subject of speculation. The prevailing view is built on inferences from a detailed analysis of the size of rat cortical axon terminals synapsing on SPNs. These studies found that cortical terminals on dSPNs tend to be smaller than those on iSPNs and that the size of terminals formed by cortical neurons with only intratelencephalic targets tends to be smaller than those of cortical neurons contributing axons to the descending pyramidal tract. Taken together, these two observations have led to the conclusion that intratelencephalic neurons project to dSPNs, whereas pyramidal tract neuron project to iSPNs. If true, this would have fundamental implications for the functions of direct and indirect pathways in reinforcement learning.

Although attractive in its simplicity, there are reasons to question this hypothesis. First, the size distributions of intratelencephalic and

Image 1

Intratelencephalic neurons equally innervated dSPNs and iSPNs. (a) Schematic of rabies virus containing ChR2-Vn (green) injected into the dorsolateral striatum (DLS), intratelencephalic (IT) axons (green) in contralateral DLS (violet). (b) Top, ChR2-Vn expression in the DLS injection site (brightest area). Bottom, contralateral to the injection, cortex layer 5A/B (L5A and L5B) labeled intratelencephalic neurons and their axons in the DLS (violet outline). Scale bars represent 1.5 mm (top) and 500 µm (bottom left and right). (c) Simultaneous dual whole-cell recordings of SPN EPSCs with intratelencephalic ChR2 activation. A dSPN expressing D1-ttdTomato (yellow) and an unlabeled iSPN are shown. Right, activation area (blue) encompassing SPNs dendritic arbors. Scale bars represent 20 µm (right) and 250 µm (left). (d) Intratelencephalic ChR2 activation produced dSPN EPSCs and iSPN EPSCs (thick line represents the mean from several trials, shading represents the s.d.). Scale bars represent 50 pA and 10 ms. (e) SPN EPSC peak amplitudes. Boxplots represent the median and interquartile range and whiskers represent the minimum and maximum values. Gray lines show simultaneous data pairings. Inset shows how the peak amplitude was measured. (f) SPN EPSC charge. Inset shows the area under the EPSC trace as the calculated value for the EPSC charge. (g) EPSC responses at iSPN normalized to paired dSPN at the maximum activation light intensity.

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BRIEF COMMUNICATIONS

Figure 2  Pyramidal tract neurons innervated both dSPNs and iSPNs, with stronger connections to dSPNs. (a) Schematics of rRabies-ChR2-Vn injections into the pons (black circle), labeling pyramidal tract (PT) axons (green) in ipsilateral DLS (violet). (b) Top and bottom, ChR2-Vn injection site within pons (sagittal slice). Right, labeled pyramidal tract axons in L5b (coronal slice). Scale bars represent 1.5 mm (top), 500 μm (bottom) and 250 μm (right). (c) Dual recording of dSPN expressing D1-tagRatTomato (yellow) and unlabeled iSPN. Scale bars represent 20 μm (left) and 250 μm (right). (d) dSPNs EPSC after pyramidal tract ChR2 activation in DLS. Scale bars represent 50 pA and 10 ms. (e) SPN EPSC peak amplitude (*P < 0.05). (f) SPN EPSC charge. (g) EPSC responses of iSPN normalized to paired dSPN at the maximum activation light intensity. Traces and boxplots presented as in Figure 1.

Pyramidal tract terminals overlap, making it difficult to draw firm conclusions about connectivity solely on the basis of terminal diameter.2,7. This is complicated further by the lack of stereological data on terminal dimensions. Second, electrophysiological studies in vivo have failed to find robust differences in the cortical responsiveness of SPNs identified by antidromic methods.8

To directly test this hypothesis, we used opticogenetic approaches in transgenic mice in which dSPNs or iSPNs are labeled with fluorescent protein.10 In these mice, a recombinant rabies virus carrying a channelrhodopsin–2–Venus expression construct (rRabies-Chr2-Vn) induces channelrhodopsin-2 (ChR2) expression in either intratelencephalic or pyramidal tract cortical neurons.11,12 This rabies virus is taken up by axons, leading to retrograde transport and expression of the construct within days; it is not propagated trans-synaptically.11–13 When the virus was injected into the dorsolateral striatum, cortical pyramidal neurons in both layers 5A and 5B of the motor cortex were labeled ipsilaterally, consistent with the ipsilateral projection of pyramidal tract terminals to pons. However, only intratelencephalic neurons projected to the contralateral striatum.14,15 (Fig. 1a,b). Thus, to determine the relative strength of intratelencephalic connections, we simultaneously recorded from a dSPN and a neighboring (~70 μm) iSPN in the same region of dorsolateral striatum contralateral to the injection site in ex vivo brain slices (Fig. 1a–d). Simultaneous dual recordings from neighboring SPNs at the same depth from slice surface should control for a host of potential sources of variance, such as optical path and ChR2 expression (Fig. 1d). SPNs were filled with a cesium-based internal solution to diminish the effect of synaptic position and maximize voltage control. In the presence of antagonists of GABAergic signaling, synaptic currents evoked by optical stimulation were completely blocked by either ionotropic glutamate receptor antagonists or tetrodotoxin (Supplementary Fig. 1).

The amplitude of the glutamatergic excitatory postsynaptic currents (EPSCs) had a sigmoidal relationship to light intensity (Supplementary Fig. 2). The amplitude and area of the maximal EPSC were used as measures of the strength of the intratelencephalic projection to dSPNs and iSPNs (Fig. 1e,f). Contrary to our expectation, maximal intratelencephalic ChR2-activated EPSC amplitude and charge in dSPNs and iSPNs were indistinguishable (n = 22 pairs, P = 0.24 and P = 0.38, respectively). There were no differences in EPSC rise times, suggesting the intratelencephalic synapses had a similar dendritic distribution in dSPNs and iSPNs (n = 19 pairs, P = 0.61). The ratio of maximal EPSC amplitude in neighboring SPNs was near 1, as was the ratio for charge (n = 22, P = 0.85 and P = 0.95, respectively; Fig. 1g). Comparing EPSCs evoked by submaximal light intensities yielded very similar results (Supplementary Fig. 2). To further verify this result, we injected the motor cortex with an adeno-associated virus carrying a ChR2 expression construct and examined the responsiveness of contralateral SPNs (Supplementary Figs. 3 and 4). Again, the ratio of maximal EPSC amplitude and charge in dSPNs and iSPNs by this intratelencephalic input were indistinguishable (n = 12, P = 0.79 and P = 1.00, respectively). Together, these results indicate that intratelencephalic neurons do not differentially innervate dSPNs and iSPNs in the dorsolateral striatum.

Next, we examined the connectivity between pyramidal tract neurons and SPNs. To infect pyramidal tract neurons, we injected rabies virus into the pons (Fig. 2a,b). These injections selectively labeled cortex layer 5B pyramidal tract neurons and their axons in the dorsolateral striatum (Fig. 2a–c). There are no other projections to the dorsolateral striatum that would be infected by this pontine injection. As with intratelencephalic axon stimulation, the EPSCs evoked by pyramidal tract axons were mediated by ionotropic glutamate receptors (Supplementary Fig. 5). As with intratelencephalic stimulation, neighboring dSPNs and iSPNs both responded robustly to pyramidal tract axon stimulation (Fig. 2d). However, the maximal EPSC produced in dSPNs was about twice that of a neighboring iSPN, on average (n = 16, P = 0.01; Fig. 2d,e). The same relationship was observed when the EPSC charge was compared (n = 16, P = 0.01; Fig. 2f). The ratio of EPSC amplitude and charge in neighboring SPNs (iSPN EPSC/dSPN EPSC) was approximately 0.5 for both measures (n = 16, P = 0.02 and P = 0.03, respectively; Fig. 2g). There was no difference in the EPSC rise time between SPNs, suggesting that the activated synapses had a similar dendritic location (n = 13, P = 0.79). Together, these results demonstrate that pyramidal tract neurons innervate both dSPNs and iSPNs, with the connection to dSPNs being stronger.

Contrary to the inferences drawn from anatomical studies in rats, our results provide compelling evidence that intratelencephalic and pyramidal tract motor cortex neurons are functionally connected to both dSPNs and iSPNs in the dorsolateral striatum of mice (Supplementary Fig. 6). In fact, the functional connectivity of pyramidal tract neurons with dSPNs was nearly twice that of iSPNs. The full relevance of this overlapping connectivity remains to be determined, but it is notable that both dSPNs and iSPNs are coactivated during movement initiation.
in rodents. It is not clear from our studies whether the mapping of non-motor cortical regions also displays the same level of convergence on dSPNs and iSPNs. Nevertheless, our results provide support for the view that cortical information about movement planning and choice are conveyed to both basal ganglia pathways, helping to sculpt not only action selection, but also action suppression.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
G.J.K. conducted the experiments, injections, data analysis and imaging. N.Y. performed injections and imaging. D.L.W. provided technical expertise with all aspects of the photoactivation system. I.R.W. provided rabies virus technical assistance. D.J.S. supervised the project. G.J.K., G.M.G.S., N.Y. and D.J.S. designed the experiments. G.J.K. and D.J.S. prepared the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Electrophysiology. Slices were perfused at 2–3 ml min⁻¹ with oxygenated modified ACSF containing 2 mM CaCl₂ and 1 mM MgCl₂ at ~25 °C. For all experiments, 20 µM gabazine and 2 µM CGP55845 were added to the perfusion to block GABA_A and GABA_B receptors, respectively. SPNs restricted to the dorsolateral striatum were identified using infrared differential interference contrast on an upright Olympus microscope and a cooled CCD camera (CoolSnap HQ) controlled with Metamorph software. In BAC D1 tdTomato mice, direct pathway SPNs were identified as containing tdTomato fluorescence in the soma. iSPNs in these mice were identified by the lack of fluorescence in the soma and by possessing spines (visualized with the aid of an Alexa Fluor hydrazide fluorescent dye) and passive membrane properties of SPNs. In BAC D2 eGFP mice, iSPNs were identified as containing GFP fluorescence in the soma. dSPNs in these mice were identified by the lack of fluorescence and by possessing spines (visualized with the aid of an Alexa Fluor hydrazide fluorescent dye) and passive membrane properties of SPNs. Dual simultaneous somatic whole-cell recordings were made with borosilicate patch pipettes having open tip resistances of 3–4.5 MOhm. The intracellular pipette solution contained 115 mM cesium methylsulfonate, with borosilicate patch pipettes having open tip resistances of 3–4.5 MOhm. Acute dorsolateral striatum slices were prepared from postnatal day 58–87 hemizygous bacterial artificial chromosome (BAC) D2 dopamine receptor-eGFP (D2_eGFP) or D1 dopamine receptor–tdTomato (D1–tdTomato) mice, males and females in the C57BL/6 background. In accordance with Northwestern University Animal Studies committee, mice were deeply anesthetized with ketamine and xylazine, perfused intracardially with ice-cold modified artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 7 mM glucose, 25 mM NaHCO₃, 2.5 mM KCl and 1.25 mM NaH₂PO₄, equilibrated with 95% oxygen and 5% CO₂, as well as with 0.5 mM CaCl₂, 2 mM MgCl₂ and 2 mM ascorbic acid. Coronal slices, 250 µm, were cut in ACSF, then incubated at 32 °C for 30 min in modified ACSF containing 2 mM CaCl₂, 1 mM MgCl₂, 5 mM l-glutathione and 1 mM pyruvate, and subsequently stored at ~25 °C. Except when noted, drugs were obtained from Sigma, Tocris or Invitrogen.

Stereotaxic injections. Stereotaxic guided surgeries were performed on the above mice anesthetized with a ketamine and xylazine mixture or isoflurane. After positioning the head to obtain a flat skull between bregma and lambda, a small hole was bored with a micro drill bit, and a glass pipette was slowly inserted at the coordinates. Dorsolateral striatum injection coordinates (relative to bregma) were 2.0 mm posterior, 3.5 mm lateral, and 3.5 mm ventral, with the manipulator tilted 48° from the x axis and 72° from the y axis. Pons injection coordinates (relative to lambda) were 2.0 and 2.5 mm posterior, 0.5 mm lateral, and 5.5–6.5 mm ventral, with the manipulator tilted 1–2° from the x axis, and the nose bar adjusted to 30° from x-y plane. Motor cortex injection coordinates (relative to bregma) were 1.6 mm anterior, 1.2 mm lateral and 0.75 mm ventral. To minimize diffusion, we slowly injected the following volumes over the course of 1 min: 0.2 µl of a recombinant rabies virus carrying a ChR2-Venus expression construct (rRabies-ChR2-Vn) for pons, 0.1 µl of rRabies-ChR2-Vn for striatum, and 0.1 µl of an adeno-associated virus carrying a ChR2-Venus expression construct (AAV2/9-ChR2-Vn, supplied by University of Pennsylvania Vector Core, Addgene 20071) for motor cortex. Successful targeting produced fluorescence in the pontine nuclei, dorsolateral striatum or motor cortex, as well as appropriate placement of fluorescent neurons in the cortex. Mice were killed 6–7 days post rabies infection or 2–3 weeks post AAV introduction.

Optogenetics. To activate channelrhodopsin containing axons in the dorsolateral striatum, we generated 473-nm light with a CrystaLaser (safety protocols for Class IIIIB laser), which was intensity-modulated with a Conoptics Pockels cell, expanded with convexe lens (ThorLabs), and passed through an adjustable iris and a 5x objective lens (0.15 NA) to produce a 700-µm diameter column of light at the slice surface. Blue light pulses (3 ms) were triggered with a Digidata interface that gated a fast Uniblitz shutter. Light intensity was calibrated with a fast photodiode (Thorlabs) and sample. Light intensity used for maximal activation was approximately 25 mW, and 5 mW for submaximal activation (producing at minimum a 20% decrease in EPSC amplitude compared to the EPSC amplitude at 25 mW). The amplitudes of EPSCs were averaged from at least three trials (up to ten trials), with an intersweep interval of 1 min.

Imaging. Coronal or sagittal slices (250–300 µm) were mounted onto glass slides. Images were acquired on an upright Olympus or Zeiss microscope, QiImaging or Photometrics camera and with Ephus17, Metamorph or QCapture software. Images were acquired in ImageJ (US National Institutes of Health) or Adobe Photoshop for brightness, contrast and pseudocoloring.

Data analysis. Axon pClamp data files was imported into Igor (WaveMetrics) and custom-written routines were used to analyze the data. Graphs were constructed with GraphPad Prism or Igor. Pilot studies to determine the variability of outcome measures and the effective size were used to estimate the number of observations necessary to test the null hypothesis. All graphical representations display the ranks of observations in a group using boxplots, illustrating the median and interquartile range, and minimum/maximum range whiskers. Data points falling outside of the following range were not included in the analysis: the interquartile range multiplied by 3 and then added to the upper quartile value. Responses less than 3–4 times the r.m.s. of the baseline noise average were displayed as 0.1 on the semi-log plots, for graphical reasons. Data points falling outside of the following range were not included in the analysis: the interquartile range multiplied by 3 and then subtracted from the lower quartile value, and the interquartile range multiplied by 3 and then added to the upper quartile value. EPSC peak amplitudes greater than 2 nA were not included in the data set because of changes in voltage control. Distribution-free statistical analysis were performed in GraphPad Prism or OriginPro, using the nonparametric Wilcoxon matched-pairs signed rank two-tailed test or a Wilcoxon signed-rank test comparing the ratio medians to 1.

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