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Convergent cortical innervation of striatal projection neurons

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Abstract

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

The basal ganglia are an interconnected collection of sub-cortical nuclei that use cortical information about movement planning to promote contextually appropriate action selection¹. This is accomplished by two parallel basal ganglia networks referred to as the direct and indirect pathways^{2,3}. 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 outcomes⁴.

How activity in cortical networks regulates activity in direct and indirect pathways has been the subject of speculation. The prevailing view is built upon inferences from a detailed analysis of the size of rat cortical axon terminals synapsing upon SPNs^{5–7}. These studies show that 1) cortical terminals on dSPNs tend to be smaller than those on iSPNs and 2) the size of terminals formed by cortical neurons with only intratelencephalic (IT) targets tends to be smaller than those of cortical neurons contributing axons to the descending pyramidal tract (PT)^{5–7}. Taken together, these two observations have led to the conclusion that IT

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neurons project to dSPNs, whereas PT neurons project to iSPNs. If true, this would have fundamental implications for the functions of direct and indirect pathways in reinforcement learning⁸.

Although attractive in its simplicity, there are reasons to question this hypothesis. First, the size distributions of IT and PT terminals overlap, making it difficult to draw firm conclusions about connectivity solely on the basis of terminal diameter^{6,7}. 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 methods⁹.

To directly test this hypothesis, optogenetic approaches were used in transgenic mice in which dSPNs or iSPNs were labeled with fluorescent protein¹⁰. In these mice, a recombinant rabies virus carrying a channelrhodopsin-2-Venus expression construct (rRabies-ChR2-Vn) was used to induce ChR2 expression in either IT or PT 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 both PT and IT neurons^{14,15} (Fig. 1a,b). However, only IT neurons project to the contralateral striatum^{14,15} (Fig. 1b). Therefore, to determine the relative strength of IT connections, a dSPN and a neighboring (~70 μ m) iSPN were simultaneously recorded from 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, like optical path and ChR2 expression (Fig. 1d). SPNs were filled with a cesium-based internal solution to diminish the impact of synaptic position and maximize voltage control. In the presence of antagonists of GABAergic signaling, synaptic currents evoked by optical stimulation were completely antagonized by 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 IT projection to dSPNs and iSPNs (Fig. 1e,f). Contrary to our expectation, maximal IT 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 time, suggesting the IT 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 (Fig. 1g) ($n=22$, $P=0.85$ and $P=0.95$, respectively). Comparing EPSCs evoked by sub-maximal light intensities yielded very similar results (Supplementary Fig. 2). To further verify this result, the motor cortex was injected with an adeno-associated virus carrying a ChR2 expression construct and the responsiveness of contralateral SPNs was examined (Supplementary Figs. 3 and 4). Again the ratio of maximal EPSC amplitude and charge in dSPNs and iSPNs by this IT input were indistinguishable ($n=12$, $P=0.79$ and $P=1.00$, respectively). Together, these results demonstrate that IT neurons do not differentially innervate dSPNs and iSPNs in the dorsolateral striatum.

Next, the connectivity between PT neurons and SPNs was examined. To infect PT neurons, rabies virus was injected into the pons (Fig. 2a, b). These injections selectively labeled cortex layer 5B PT neurons and their axons within 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 IT axon stimulation, the EPSCs evoked by PT axons were mediated by ionotropic glutamate receptors (Supplementary Fig. 5). As with IT stimulation, neighboring dSPNs and iSPNs both responded robustly to PT axon stimulation (Fig. 2d). However, the maximal EPSC produced in dSPNs was about twice that of a neighboring iSPNs on average (Fig. 2d, e) ($n=16$, $P=0.01$). 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 (Fig. 2g) ($n=16$, $P=0.02$ and $P=0.03$, respectively). 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 PT neurons innervate both dSPNs and iSPNs, with the connection to dSPNs being stronger.

Contrary to the inferences drawn from anatomical studies in rats, our experiments provide compelling evidence that IT and PT 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 PT neurons with dSPNs was nearly twice that of iSPNs. The full significance of this overlapping connectivity remains to be determined, but it is of note that both dSPNs and iSPNs are co-activated during movement initiation in rodents¹⁶. 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 action suppression.

Online Methods

Slice Preparation

Acute dorsolateral striatum slices were prepared from P58–P87 hemizygous Bacterial Artificial Chromosome (BAC) D_2 dopamine receptor-eGFP (D_2 -eGFP) or D_1 dopamine receptor-tdTomato (D_1 -tdTomato) mice, males and females in the C57BL/6 background¹⁰. 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) (in mM): 125 NaCl, 7 glucose, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, equilibrated with 95% oxygen, 5% CO₂ plus 0.5 mM CaCl₂, 2 mM MgCl₂, 2 mM ascorbic acid. Coronal slices, 250 μ m, were cut in the above modified ACSF, then incubated at 32°C for 30 min in the above modified ACSF containing 2 mM CaCl₂, 1 mM MgCl₂, 5 mM L-glutathione and 1M pyruvate and subsequently stored at room temperature. Except when noted, drugs were obtained from Sigma, Tocris, or Invitrogen.

Electrophysiology

Slices were perfused at 2–3 ml per minute with oxygenated modified ACSF containing 2 mM CaCl_2 and 1 mM MgCl_2 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 D_1 -tdTomato mice, direct pathway SPNs were identified as containing tdTomato fluorescence in the soma. Indirect pathway SPNs in these mice were identified by the lack of fluorescence in the soma, possessing spines (visualized with the aid of an Alexa Fluor hydrazide fluorescent dye), and passive membrane properties of SPNs. In BAC D_2 -eGFP mice, indirect pathway SPNs were identified as containing GFP fluorescence in the soma. Direct pathway SPNs in these mice were identified by the lack of fluorescence, 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 MOhms. The intracellular pipette solution contained (in mM): 115 cesium methylsulfonate, 5 Hepes, 5 tetraethylammonium-Cl, 2 QX-314, 0.25 EGTA, 2 Mg-ATP, 0.5 Na-GTP, 10 sodium phosphocreatine, and 100–200 μM Alexa Fluor 488 or 568 hydrazide, pH 7.3 and osmolarity 290. After dual somatic recordings were established for a randomly selected dSPN and iSPN pair, no more than 70 μm apart, with similar depths from the slice surface (cell body of each pair had no more than 10 μm depth difference between each other), the cells were allowed to dialyze with the internal solution for 7–10 mins. Cells with dendritic arbors less than 100 μm were discarded. EPSCs were occluded with bath application of 100 μM D-(-)-2-amino-5-phosphonopentanoic acid (APV) and 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or 1 μM tetrodotoxin. Recordings were obtained using a MultiClamp 700A amplifier and pClamp 10 software. Pipette offset and capacitances were adjusted using MultiClamp 700A Commander software. Data were acquired at 100 kHz, filtered at 10 kHz using an 8-pole Bessel filter, and digitized using a DigiData 1440 16-bit A/D converter. Somatic access resistance < 25 MOhms was monitored and cells with unstable access resistance (>30% change) were discarded. Cells were held at –80 mV, not corrected for liquid junction potential (calculated to be approximately –11 mV).

Stereotaxic Injections

Stereotaxic guided surgeries were performed on the above mice anesthetized with ketamine and xylazine mixture or isoflurane. After positioning the head to obtain a flat skull between bregma and lamda, a small hole was bored with a micro drill bit, and a glass pipette was slowly inserted at the following 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 z-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 z-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, over the course of 1

minute the following volumes were slowly injected: 0.2 μ L of a recombinant rabies virus carrying a channelrhodopsin-2-Venus expression construct (rRabies-ChR2-Vn) for pons, 0.1 μ L rRabies-ChR2-Vn for striatum, and 0.1 μ L of an adeno-associated virus carrying a ChR2-Venus expression construct (AAV2/9-ChR2-Vn, supplied by U. Penn Vector Core; Addgene 20071) for motor cortex. Successful targeting produced fluorescence within the pontine nuclei, dorsolateral striatum, or motor cortex, as well as appropriate placement of fluorescent neurons within the cortex. Mice were sacrificed 6–7 days post rabies or 2–3 weeks post AAV introduction.

Optogenetics

To activate channelrhodopsin containing axons within the dorsolateral striatum, 473 nm light was generated with a CrystaLaser (safety protocols for Class IIIB laser), intensity-modulated with a Conoptics Pockels cell, expanded with concave lens (ThorLabs), passed through an adjustable iris, and 5 $\times$ objective lens (0.15 NA) to produce a 700 μ m 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 power was calibrated with a fast photodiode (Thorlabs) at the laser 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 3 trials (up to 10 trials), with an intersweep interval of 1 minute.

Imaging

Coronal or sagittal slices (250–300 μ m) were mounted onto glass slides. Images were acquired on an upright Olympus or Zeiss microscope, QImaging or Photometrics camera and with Ephus¹⁷, Metamorph, or QCapture software. Images were adjusted in NIH ImageJ or Adobe Photoshop for brightness, contrast, and pseudocoloring.

Data Analysis

Axon pClamp data files were 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 within a group using boxplots, illustrating the median (thick line), interquartile range, and minimum/maximum range whiskers. Gray lines indicate data for a pair of dSPN and iSPN collected simultaneously. Responses smaller than 3–4 times the root mean square of the baseline noise average were displayed as 0.1 on the log scale 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 due to 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. Statistical significance ($P < 0.05$) is indicated with asterisk marks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

IT neurons equally innervated dSPNs and iSPNs. **(a)** Schematic of rabies virus containing ChR2-Vn (green) injected into the dorsolateral striatum (DLS), IT axons (green) within contralateral DLS (violet). **(b)** Top, ChR2-Vn expression within the DLS injection site (brightest area). Bottom, contralateral to the injection, cortex layer 5A/B labeled IT neurons and their axons within the DLS (violet). Scale bars: 1.5 mm (top), 500 μ m (left and right). **(c)** Simultaneous dual whole-cell recordings of SPN EPSCs with IT ChR2 activation. dSPN positive for *D1-tdTomato* (yellow) and unlabeled iSPN. Right, activation area (blue) encompassing SPNs dendritic arbors. Scale bars: 20 μ m (right) and 250 μ m (left). **(d)** IT ChR2 activation produced dSPN EPSC and iSPN EPSC (thick line represents the mean from several trials, shading represents the standard deviation). Scale bars represent 50 pA and 10 ms. **(e)** SPNs EPSC peak amplitude. Boxplots represent the median and interquartile range while whiskers represent the minimum/maximum values. Gray lines show simultaneous data pairings. Inset shows how the peak amplitude was measured. **(f)** SPNs 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.

Figure 2.

PT neurons innervated both dSPNs and iSPNs, with stronger connections to dSPNs. (a) Schematics of rRabies-ChR2-Vn injection into the pons (black circle), labeling PT axons (green) within ipsilateral DLS (violet). (b) Top and left, ChR2-Vn injection site within pons (sagittal slice). Right, labeled PT axons in L5B (coronal slice). Scale bars: 1.5 mm (top), 500 μ m (left), and 250 μ m (right). (c) Dual recording of dSPN expressing *D1-tdTomato* (yellow) and unlabeled iSPN. Scale bars 20 μ m (left) and 250 μ m (right). (d) SPNs EPSC after PT ChR2 activation in DLS. Scale bars represent 50 pA and 10 ms. (e) SPNs EPSC peak amplitude (* $P < 0.05$). (f) SPNs EPSC charge. (g) EPSC responses of iSPN normalized to paired dSPN at the maximum activation light intensity.