# Basal Ganglia Circuitry Controlling Action Selection

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Basal Ganglia Circuitry Controlling Action Selection

A dissertation presented

by

Gil Mandelbaum

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Neurobiology

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Cambridge, Massachusetts

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In order to understand the functional consequences of this circuit organization, we designed a behavioral task in which mice were required to base their next choice on their...
previous actions and outcomes associations (AOA) in addition to software and hardware for rapid closed-loop optogenetic manipulations triggered off of specific task and behavioral parameters. Optogenetic stimulation of dorsal lateral STR direct and indirect spiny projection neurons during the AOA formation period caused biases in action choice made several seconds later, contraversive and ipsiversive, respectively. Similarly, stimulation during the delay period after the AOA had been formed but prior to reporting the choice caused the same bias. These data suggest that the execution of actions and reinforcement are linked through the dorsolateral striatum. We now seek to further establish this by silencing and recording activity patterns in the PF, STR, and CTX.
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Chapter 1: Introduction

1.1 Overview of the Thesis

A principal goal of neuroscience is to understand how brain circuits integrate diverse streams of information in order execute in an optimal manner future behavioral choices. A choice is the neural process by which an animal weighs information about its past experiences, current environment, internal state, and prediction about the future to then execute the most beneficial actions. The neural circuits and their activity patterns that underlie this process are currently poorly understood. My long-term research goal is to achieve a thorough understanding, at the neuron and circuit level, of the neural mechanisms of choice, ultimately revealing how these mechanisms go awry in the context of neuropsychiatric diseases. To this end, in my thesis work, I have taken part in developing, and have used technologies that enable, the collection of vast amounts of data, spanning the spectrum from the micro (genes and physiological characters of individual neurons), through the meso (interactions between brain regions) all the way to the macro (control and recording of activity patterns in behaving mice).

Specifically, we focused on the basal ganglia (BG) circuits, a set of phylogenetically old nuclei (Stephenson-Jones et al., 2011) that take part in selecting and generating appropriate motor actions. Indeed, the importance of these nuclei for selecting actions in humans is emphasized by disorders that arise from disrupted components of the basal ganglia circuits, such as Parkinson’s (Wichmann et al., 2011) (Kravitz et al., 2010), Huntington’s (Vonsattel et al., 1985) (Mangiarini et al., 1996), Tourette’s Disorder
(Leckman et al., 2010), (Vinner et al., 2017), Obsessive-Compulsive (Saxena et al., 2001) (Ahmari et al., 2013) and addiction (Everitt and Robbins, 2005) (Hollander et al., 2010).

To understand how activity in the BG circuits leads to motor actions, one must first identify the functional building blocks of such circuits. Exploiting the power of genetics, usage of viruses, and synaptic physiology combined with novel bioinformatic approaches, I collaborated with Julian Taranda from the Osten group at Cold Spring Harbor Laboratories and was able to identify and demonstrate the relationships between macro and micro structures in the BG, discovering previously unknown circuit motifs related to the execution of motor actions (Chapter 2).

Guided by such discoveries, we have recently recorded and controlled simultaneously hundreds of individual neurons in behaving mice. The goal of these experiments is to better understand the novel circuit’s neural correlates of behavior. This has been done using high-density recording site silicon probes or microscopy techniques, combined with manipulations of genetically-defined neuron populations (optogenetics) and high-resolution behavioral analysis. As part of my thesis work, I will present the behavior that I designed in collaboration with Zengcai Guo from the Svoboda Group at the Janelia Research Campus, the analysis of this behavior, in addition to optogenetic experiments that I conducted (Chapter 3).

Several challenges arise in the design of such experiments on behaving mice. For example, advancements in the field of optogenetics promises spatiotemporal precise control of neural processes in the brain, using light. However, the spatial extent of illumination within the brain has been difficult to control and, in the past, could not be adjusted using standard fiber optics. To address this, I worked together with Ferruccio
Pisanello from the De Vittorio group at the Italian Institute of Technology and took part in designing and testing optic fibers that allowed for dynamic illumination of spatially restricted, or large, brain volumes through a single tapered optical fiber. This enabled us and others to achieve focal or broad illumination of light in the brain that could be precisely and dynamically matched to our experimental needs. (Appendix A).

1.2 The Basal Ganglia Circuits

Animals must integrate past experiences, present inputs from the environment, and future predictions, in order to generate appropriate motor actions. Indeed, the mammalian brain dedicates to this task an extensive set of subcortical nuclei, the BG, which are necessary for triggering goal-driven coordinated sequences of motor actions (Jin and Costa, 2010) and promoting the repetition of motor actions that lead to “reward” (Kravitz et al., 2012).

The canonical circuit model (Figure 1), set forward by Deloung et al. (DeLong, 1990) suggests that activity in the BG is initiated through multiple cortical areas that project to the striatum (STR), the input nucleus of the BG (Hintiryan et al., 2016). These cortical-striatal projections excite the two main cell types of the striatum—the direct and indirect striatal projection neurons (dSPNs and iSPNs respectively) (Parker et al., 2016). The dSPNs send direct inhibitory projections to the output nuclei of basal ganglia, the substantia nigra pars reticulate (SNr) and the internal globus pallidus (GPi) (Freeze et al., 2013) (Schmidt et al., 2013). These inhibitory projections from the striatum disinhibit the tonic activity of SNr (Freeze et al., 2013) and GPi to release the inhibitory break and excite
the thalamus (TH), which can then increase cortical activity to close the loop (Oldenburg and Sabatini, 2015).

The iSPNs target the external segment of the globus pallidus, which connects *indirectly* through the subthalamic nucleus (STN) to the SNr and GPi (Schmidt et al., 2013). With the addition of another inhibitory synapse, this circuit is thought to primarily inhibit cortical activity through the TH (Oldenburg and Sabatini, 2015). In addition, the striatum, via SNr and GPi, targets the superior colliculus (among other midbrain nuclei) to directly affect movement (Isoda and Hikosaka, 2008). Importantly, the STR is also influenced by dopaminergic inputs from the substantia nigra compacta (SNc) (Tritsch et al., 2012) and it has been suggested that dopamine cells in the SNc signal reward prediction errors to the striatum (Schultz et al., 1997) most likely by modulating multiple cell types, including iSPNs and dSPNs, and the cholinergic interneurons of the STR (Chuhma et al., 2014). Lastly, the STR is influenced by the thalamo-striatal projections, mostly originating from the PF, an interlaminar nucleus in the caudal TH, which has been a research focus of mine (Smith et al., 2014).

This simplistic presentation above has served as a useful framework on which to model the pathogenesis of Parkinson’s and Huntington’s diseases, as well as the mechanisms of action of drugs of abuse and action selection (DeLong, 1990).
1.3 The Thalamo-Striatal projections—anatomy and nomenclature

In 1956, Cowan and Powell from Oxford published a comprehensive study of 12 macaque monkeys (Cowan and Powell, 1956). They demonstrated the existence of an extensive thalamo-striatal projection system, with inputs to all of the functional territories in the STR. Anatomically, the thalamo-striatal projections may be further separated into two subgroups; the projections originating from the PF, and the centromedian nucleus (CM) which preferentially project to the STR, and all other thalamo-striatal projections, which preferentially project to the cortex (CTX) (Smith et al., 2014). However, the Allen Brain Atlas divides the mouse TH into two main groups, based on their known function, which only to some extent correspond to regions. The first group is called the sensory motor cortex-related nuclei. The second group is the polymodal cortex-
related nuclei. The PF is part of the polymodal cortex-related group and part of the interlaminar nuclei subgroup. In non-human primates and in humans, the TH is divided based on region and not function. As part of the dorsal TH in non-human primates and in humans, there are the intralaminar nuclear complexes which is the analog to the interlaminar groups in the rodent. In the caudal part of the intralaminar nuclear complexes, the PF and CM form one such complex. Importantly, in rodents, the lateral part of PF is considered the homologue of the primate CM, whereas the medial part of PF displays strong similarities with PF proper. However, due to limited delineation between medial and lateral PF in the rodent Nissl stain CM has not been properly defined (Jones, 2007).

**Figure 1.2: The nomenclature of the PF and CM**
1.4 The PF Function In Vivo

The first lesion study in PF, published in 1969, showed that the PF was necessary in order to switch between behaviors when the environment required the rats to do so (Delacour, 1969). Lesioning the PF did not affect learning during a passive avoidance task in which rats avoided a shocked compartment by running to a safe one. However, the rats that sustained lesions could not reverse their motor actions when the compartments were switched. That is, the rats could not learn to cross in the opposite direction to avoid shock. PF necessity for ensuring ongoing motor action flexibility was revisited recently. Recording in CM and PF in the non-human primate showed that cells could generate discrete coherent spiking in response to a wide variety of sensory stimulus. Furthermore, it was shown that the responses of PF neurons had a short latency with brief phasic responses, while CM neurons showed long latency responses that often exhibited an early depressive response as well (Matsumoto et al., 2001). When monkeys received a cue that either preceded a target in the same location or a cue that misdirected attention to a different location relative to the target, PF cells firing patterns corresponded to when the animal was required to shift its orientation to the contralateral side to succeed, suggesting PF cells may be involved in attention processing (Minamimoto and Kimura, 2002). In another task, go actions were rewarded by a large amount of water, whereas performance of the no-go actions were rewarded with a small amount of water. In the second contingency, the action outcomes were switched. In both, during the small reward cue, cells in the CM fired independently of the motor activity that was performed. Next monkeys were trained in a paradigm that after each small reward trial the likelihood of a large reward trial increased; thus the animals’ expectation for such a reward increased.
Firing rates in CM increased as a function of the number of successful sequential small reward trials the animal had. This suggested that CM changes in firing rates might act to abolish the motor response bias to a large reward trial type when presented with a small reward trial (Minamimoto et al., 2005).
1.4 Bibliography


Tritsch, N.X., Ding, J.B., Sabatini, B.L., 2012. Dopaminergic neurons inhibit striatal output through non-canonical release of GABA. Nature 490, 262–266. doi:10.1038/nature11466


Chapter 2: Distinct Cortical-Thalamic-Striatal Circuits Through the Parafascicular Nucleus

2.1 Summary

The PF, an excitatory input to the BG, is targeted with deep-brain-stimulation to alleviate a range of neuropsychiatric symptoms. Furthermore, PF lesions disrupt the execution of correct motor actions in uncertain environments. Nevertheless, the circuitry of the PF and its contribution to action selection are poorly understood. We find that, in mice, PF forms the densest subcortical projection to the STR. This projection arises from transcriptionally- and physiologically-distinct classes of PF neurons that are also reciprocally connected with functionally-distinct cortical regions, differentially innervate STR neurons, and are not synaptically connected in PF. Thus, mouse PF contains heterogeneous neurons that are organized into parallel and independent associative, limbic, and motor circuits. Furthermore, these subcircuits share motifs of cortical-PF-cortical and cortical-PF-striatum organization that allow each PF subregion, via its precise connectivity with cortex, to coordinate diverse inputs to STR. The work presented in this chapter was done in collaboration with the laboratory of Pavel Osten at Cold Spring Harbor Research Campus.

2.2 Introduction

Selecting and generating appropriate motor-actions requires integration of limbic, associative, and sensory information in basal ganglia (BG) circuits (Macpherson et al., 2014) (Hintiryan et al., 2016), a set of phylogenetically old subcortical nuclei (Stephenson-Jones et al., 2011). The importance of these nuclei to action-selection in humans is
emphasized by disorders that arise from disrupted components of the BG, such as Parkinson’s (Wichmann et al., 2011) (Kravitz et al., 2010), Huntington’s (Vonsattel et al., 1985) (Mangiarini et al., 1996), Tourette’s Disorder (Leckman et al., 2010) (Vinner et al., 2017), Obsessive-Compulsive (Saxena et al., 2001) (Ahmari et al., 2013) and addiction (Everitt and Robbins, 2005) (Hollander et al., 2010).

The BG consist of loops formed by projections from cortex (CTX) and thalamus (TH) to the input stage of the BG, the striatum (STR), which than signals via cascading inhibitory nuclei to control cortical-projecting thalamic nuclei (DeLong, 1990) (Nelson and Kreitzer, 2014) (Cowan and Powell, 1956) (Kemp and Powell, 1970) (Hunnicutt et al., 2016) (Hintiryan et al., 2016). Phylogenetically, TH and the STR pre-date the expansion of the CTX (Reiner et al., 1998) and despite the TH being approximately ten times smaller in volume than the CTX in mice (see results), it accounts for approximately a quarter of all glutamatergic synapses in the STR (Huerta-Ocampo et al., 2014). This suggests that these evolutionally conserved projections between TH and STR have a powerful functional impact on BG circuits (Minamimoto et al., 2005) (Bradfield et al., 2013a) (Kato et al., 2011) (Smith et al., 2011) (Bradfield et al., 2013b).

Within TH, the PF and CM nuclei (two separate nuclei that at times are defined as a complex) project heavily to STR (Smith and Parent, 1986) (Berendse and Groenewegen, 1990) (Wall et al., 2013), unlike typical thalamic nuclei that primarily interact with CTX (Sherman and Guillery, 2013). In humans, targeting PF/CM for deep brain stimulation (DBS) has been successful in alleviating a range of symptoms in individuals with BG-related disorders (Testini et al., 2016) (Savica et al., 2012) (Peppe et al., 2008) (Jouve et al., 2010) (Parker et al., 2016) (Picillo et al., 2017). Furthermore,
PF/CM are unique in that they degenerate early in Parkinson’s, unlike other thalamic nuclei that maintain their integrity throughout disease progression (Henderson et al., 2000). However, the PF is omitted from the majority of functional models of the BG in both primate and rodent literature (Penney and A. B. Young, 1983) (DeLong, 1990) (Nelson and Kreitzer, 2014), or grouped together with other thalamostriatal projections, despite evidence that the anatomy and function of PF→STR projections is specialized (Ellender et al., 2013) (Alloway et al., 2014).

In primates, the projections from PF/CM to STR have been proposed to be anatomically organized into multiple functionally distinct output channels (Steiner and Tseng, 2016) (Sadikot and Rymar, 2009), a conclusion that is broadly in agreement with anatomical findings from cats and rats (Giménez-Amaya et al., 2000) (Jones, 2007). However, understanding the polysynaptic nature of circuits across connected regions in genetically-intractable species is challenging. Therefore, although it is known that subregions of PF/CM project to different regions in STR, it has not been possible to link these specialized projections to cell classes within PF/CM or to understand their relationship to the many cortical regions that project to intralaminar (ILM) TH and STR. Thus, it is unknown if cortical-PF-STR and cortical-PF-cortical circuits are organized into conserved motifs. Furthermore, because of the limitations of genetic manipulations and ex-vivo electrophysiology analysis in these species, little is known about the cellular composition and micro-circuitry of PF/CM, the neurons that comprise its input and output channels, and synapses by which PF modulates STR activity. Conversely, in rodents, the lack of clear histological demarcations within PF and between PF and CM as well as the small size and close packing of TH nuclei, has led researchers to treat the PF in
genetically-tractable species such as mice as anatomically uniform and cellularly homogenous (Parker et al., 2016) (Kato et al., 2011) (Aceves Buendia et al., 2017) (Assous et al., 2017) (Choi et al., 2018).

Here we combine anatomical analysis of the circuitry linking cortex, PF and STR, with transcriptional and electrophysiological analyses of PF neurons and their synapses, to deconstruct the mouse PF. Using quantitative whole-brain anatomical approaches, we reveal PF to be the densest sub-cortical input to the STR out of hundreds of brain structures (as annotated in the Allen Institute Common Coordinate Framework). Anatomical, single-cell transcriptional, and physiological analyses reveal distinct neuronal populations in PF that form topographically organized projections to the STR. Based on these results, we target each PF subpopulation to map their cortical inputs and outputs. We find that PF cell classes are targeted by layer 5 of limbic, associate or sensory-motor regions of CTX while also forming topographically organized projections to CTX. Furthermore, these PF cell classes are not synaptically interconnected within PF and differentially innervate striatal neurons, thus forming functionally-distinct and parallel signaling channels.

Our circuit analyses reveal that PF subregions and neuron classes influence distinct regions of STR through independent and parallel channels that carry information principally from limbic, associative, or sensorimotor regions. These channels are organized such that an area of STR receives input from regions of CTX and PF that are themselves interconnected via reciprocal projections. Based on this organization, we propose that PF circuits facilitate and dynamically shape the output of connected and
behaviorally relevant striatal regions to mediate correct action selection in the ongoing sensorimotor context.

2.3 Results

2.3.1 Quantification of the distribution of inputs to striatum across the brain

The input nucleus of the BG, the STR, receives inputs from many parts of the brain, including CTX and TH (Steiner and Tseng, 2016). These inputs were previously mapped using retrograde tracing and manual cell counting to quantify inputs to STR from a few dozen brain regions (Wall et al., 2013). Alternatively, anterograde tracing was combined with image analysis to identify functionally distinct regions in the STR defined by the combination of inputs that they receive from CTX and TH (Hunicutt et al., 2016) (Hintiryan et al., 2016).

We utilized automated image acquisition and analysis to map the distribution of putative STR-projecting neurons across the whole brain (Figure 2.1). We injected 4 locations in the STR of 7 C57BL6/N wild type (WT) mice with a non-pseudotyped rabies virus encoding nuclear localized GFP (RV-nGFP) (Figure 2.1A). The 3D whole brain volume was subsequently imaged, reconstructed, and aligned to the Allen Brain Atlas (ABA) for analysis (Figure 2.1A). As our data is aligned to the ABA, here we use their brain-structure hierarchy and abbreviations defined by the Allen Institute (link: ABA interactive atlas viewer) with the exception of brain-stem which is switched here with sub-cortical (sub-CTX; see Table 1 for all brain structure abbreviations used in figures).

The high signal-to-noise ratio (SNR) of somatic GFP signal versus the neuropil signal was exploited to automatically count RV-nGFP+ cells (Figure 2.1B), permitting an
unbiased estimate of putative inputs to STR across the whole brain and from hundreds of structures. The coefficients of variation (CV) of the volumes of 8 brain-regions of interest across mice were less than 4% (Figure S1A-C) allowing pooling of data across brains. The false positive rate (FPR) for automated detection of RV-nGFP+ cells was estimated from cell counts in the STR and PF contralateral to the injection site as there is no PF→STR or STR→STR connectivity across hemispheres. This yielded an estimate of <1% FPR (ipsilateral counts: STR=23396±2332 cells, PF=6797±81 and contralateral counts: STR=229±42, PF=51±1; n=7 mice; Figure S1D). Furthermore, in a subset of animals. labeled PF neurons were counted manually, yielding numbers very similar to the automated measurements (manual: 3219±80 cells; automated: 3375±48; n=3 mice; P=0.5; Figure 2.1C).

To investigate the distribution of inputs to STR in the sub-CTX main hierarchical divisions, the percentage of the total RV-nGFP+ cells located in each group was calculated. TH had the highest percent of nGFP-labeled cells with the motor region of the midbrain (MBmot) being second (TH=48±1% of cells; MBmot=16±1%) compared to the other 7 sub-CTX groups which together had 35% of putative sub-CTX input to STR (Figure 1D; see Table 1 for full list of abbreviations for this figure and all others). In TH, the majority of nGFP+ cells were in the poly-modal association cortex-related region (DORpm) and not the sensory-motor cortex-related region (DORsm) (DORpm=79±0.6%; DORsm=10±0%; Figure 2.1E-F). Among DORsm nuclei, the ventral anterior lateral complex had the most cells (S1E), similar to previous observations in the squirrel monkey (Smith and Parent, 1986). In DORpm, the intralaminar nuclei group (ILM) had the majority of putative STR inputs (43±3%; Figure 2.1G) with PF having the highest percent of nGFP+
cells compared to all other ILM nuclei (68±1% of cells; Figure 2.1H). Lastly, the density of putative inputs to STR from 706 ABA-defined sub-structures was calculated (defined as % of total cells in a given region divided by its volume) and showed that PF had the densest sub-CTX input to STR, highlighting its potential to exert powerful control of the BG circuits (Figure 2.1H)
Figure 2.1: Serial two photon tomography defines PF as the main sub-cortical input to STR.

A, left, Schematic of the experimental design showing a coronal section at +0.9 mm from a WT mouse with 4 injections of RV-nGFP in the STR (region of injection is highlighted in orange in this and in all subsequent figures). middle, Schematic of the STPT system, which automatically slices and images the whole brain using a microtome (MT) built into a 2-photon laser-scanning fluorescence microscope. right, Image of a brain slices obtained approximately 1 week after virus injection that was aligned to the ABA and 3D reconstructed for further analysis.

B, STPT image of the nucleus of a cell infected with RV-nGFP (white). The boarder of the nGFP and the tissue are marked with a dashed line (green) to highlight the signal to noise ratio obtained with RV-nGFP despite the dense labeling neurons upstream of STR.

C, Number of cells detected in PF by manual (MC) and automated (AC) counting (n=3 mice). P=0.5; Wilcoxon test. Red error bars in this and subsequent panels indicate ±SEM and black bar indicates the mean. In this panel and D, F and G each black dot indicates data from one mouse.

D, Percent RV-nGFP+ cells in subcortical (sub-CTX) regions from the experiment shown in panel (A). Each dot shows the percentage of cells in the indicated brain region measured in one mouse (n=60,857/7; cells/mice). See Table 1 for full list of abbreviations.
Figure 2.1 (Continued)

**E**, Coronal sections of the TH at -1.4 mm (*left*) and -2.1 mm (*right*) showing RV-nGFP infected cells (white). On the left, boundaries of TH nuclei are shown with thin dashed lines contralateral to the injection site in STR to not obscure the nGFP signal in cells across the many nuclei. The thick dash line represents the midline.
Figure 2.1 (Continued)

A. RV-nGFP

B. Image analysis

C. 3D brain cells (x1000)

D. nGFP+ cells (%)

E. ILM nuclei

F. DORpm nuclei groups

G. ATN ILM MED MTN

H. Grey

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2.3.2 Topographic organized of the parafascicular-striatal projections in mice

In cats, primates, and humans, the PF/CM complex is separated into histologically dissimilar PF and CM nuclei which are topographically organized into multiple output channels that target distinct regions of the STR (Jones, 2007) (Steiner and Tseng, 2016). To address whether the PF/CM→STR topography found in larger species exists in mice, WT mice were injected with 3 variants of the retrograde tracer cholera toxin subunit B (CTB) (Conte et al., 2009) into four STR locations (Figure 2.2A). Topographically organized projections were observed in the anterior part of PF (coronal section -2.0mm defined by the ABA as the anterior posterior boarder of PF with the medial dorsal nucleus) between medial PF (mPF) and medial STR (mSTR); central PF (cPF) and dorsal medial STR (dmSTR); and lateral PF (lPF) and dorsal lateral STR (dlSTR) (Figure 2.2B-D). This topography was maintained at coronal section -2.1mm (Figure 2.2E-G) and -2.2mm (Figure 2.2H-J) across animals but becomes less distinct at coronal section -2.3mm, corresponding to the most posterior part of both PF (and the TH) (Figure 2.2K-M). The topography of projections from cPF→dmSTR was also clearly seen in cleared brains (not shown) (Chung et al., 2013). Lastly, the ventral part of mPF (v-mPF) projects to the nucleus accumbens (ACB) (Figure S2A-B). Thus, despite its small size, and no clear boundary to define CM and PF, PF in mice contains distinct and topographically organized PF→STR projections that share similar organizational features with larger species (Jones, 2007) (Giménez-Amaya et al., 2000).
Figure 2.2: PF→STR projections are topographically organized in mice.

A, Schematic of the experimental design showing a coronal section at +0.9 mm from a WT mouse with 4 injections of 3 CTB variants (cyan, magenta, and yellow) in the STR. The region of injection is highlighted in orange.

B, Coronal section from the ABA at -2.0mm with PF highlighted in red and the fasciculus retroflexus (FR) circled with a thick black line inside of PF. The FR was used as a landmark in PF to align images for summary data shown in panels D, G, J, and M.

C, Example image of a coronal section at -2.0mm (left) from the experiment shown in A with the inset indicating the region surrounding the PF enlarged on the right. The distributions of CTB conjugated with different fluorophores are largely not overlapping, highlighting the PF→STR topographical organization.

D, left, Confocal image of PF tissue excited with 488, 594, and 647 nm wavelengths (top to bottom) highlighting the topographical organization of the PF-STR projections. right, Quantification of fluorescence intensity for each imaging channel at coronal section -2.0mm along the medial-lateral axis. Thin lines represent peak-normalized data from individual animals and the thick lines show the means for each channel. The dashed grey region represents the FR location. Scale bar = 250 µm (n=3 mice).

E-G, Atlas schematics, example images, and quantifications as in B-D for coronal sections -2.1 (E), -2.2 (F), and -2.3 (G) mm. The example images are from the same mouse shown in panels (C-D).

Also see related Figure S2.
Figure 2.2 (Continued)
2.3.3 Characterization of PF neuron types using single cell sequencing and electrophysiological interrogation

To examine the neuronal heterogeneity in PF we used a droplet-based single cell RNA sequencing technique (inDrops) (A. M. Klein et al., 2015) (Hrvatin et al., 2018). This technique allowed us to determine whether cells in PFs sub-divisions, defined by the PF→STR projections topography, are transcriptionally distinct. PF and its surrounding areas were manually dissected from acute coronal brain slices and a cell suspension was formed by tissue dissociation (Figure 2.3A). Analysis of transcriptomes of 10,471 cells from 8 mice, revealed 7 main cell classes with distinct transcriptional profiles (Figure 2.3B). The neuronal cell-type class (enriched for Snap25, Syn1) contained 992 cells and expressed markers for glutamatergic (e.g. Slc17a6), but not GABAergic neurotransmission (e.g. Slc32a1). Further sub-clustering of the neuronal cell-type class revealed 3 neuronal subclasses with cell-type enriched gene expression (see Methods) (Figure 2.3C). Examination of the expression patterns of the genes enriched in each subcluster in the ABA in situ hybridization (ISH) database (Link: ABA ISH) (Lein et al., 2007) revealed genes both inside and outside of PF. Genes whose expression is elevated in Cluster 1, including Tnnt1, are expressed outside of PF, primarily in posterior complex and the ventral posteromedial nucleus of TH (Figure 2.3D) (Phillips et al., 2018). Genes defining cluster 2, including Fxyd6, were expressed in mPF, but also ventral and dorsal to the PF (Figure 2.3D). Thus cluster 1 and 2 are enriched for genes and represent cell groups that, within the dissection area, are not unique to the PF. No further analysis of these clusters was carried out.
Genes enriched in cluster 3, such as *Lypd6b*, showed specific expression in PF (Figure 2.3D), including all of its subdivisions. Nevertheless, within cluster 3 genes revealed differential expression along the medial-lateral aspect of PF indicating a heterogeneous neuronal population. For example, *Prodynorphin (Pdyn)*, a marker for the direct striatal projection neurons (dSPNs) in STR (Gerfen and W. S. Young, 1988) was expressed in 117 cells, with a mean 8-fold increase in its expression compared to neuronal clusters 1 and 2. ISH of *Pdyn* mapped the expression specifically to mPF (Figure 2.3E). Furthermore, analysis of gene-gene expression correlation across all cells in cluster 3 revealed those correlated with *Pdyn* expression also mapped to mPF (Figure 2.3F; S3A-C). Conversely, genes whose expression was anti-correlated with that of *Pdyn*, mapped to cPF and lPF (Figure 2.3F; S3D-F). These results indicate that the anatomically-defined subdomains that comprise PF map onto transcriptionally distinct subclasses of neurons.

*In vivo* recordings in primates have revealed different kinetics of activation of PF and CM neurons (Matsumoto et al., 2001). Therefore, we examined if the intrinsic electrophysiological properties of neurons projecting to the STR differ along the mediolateral aspect of the mouse PF. Whole-cell recordings in current-clamp were obtained from CTB labeled PF→STR neurons in acute brain slices from mice injected with two different CTB colors into mSTR and dmSTR or dmSTR and dlSTR (Figure 2.3G). Consistent with our hypothesis, the membrane resistance, capacitance, and resting potential varied across the PF with higher input resistance, lower capacitance, and higher resting potential neurons found in the medial relative to the lateral aspects of the PF (Figure 2.3H). Thus, the same synaptic current would result in a larger synaptic potential.
in the higher input resistance mPF neurons. Coupled with the more depolarized resting potential, this suggests that mPF neurons are likely more excitable than those in the IPF consistent with *in vivo* recordings in the primate (Matsumoto et al., 2001).
**Figure 2.3: Transcriptional and electrophysiological characterization of PF neurons.**

**A,** *left,* Images of an acute coronal slice after microdissection of PF. *right,* Cell suspensions were formed from the dissected tissue and run through the *indrops* platform to reveal transcriptomes of thousands of cells from PF.

**B,** t-SNE plot showing the main identified cell types highlighted by the different colors (n=10471/8 cells/mice). The excitatory neurons are marked by the dashed oval delineates the neuronal cluster.

**C,** t-SNE plot of excitatory glutamatergic neurons (*Slc17a6*-expressing) with the 3 subclusters indicated by different colors (n=992/8; cells/mice).

**D,** Example *in situ hybridization* (ISH) from the ABA with one gene representing each neuronal cluster. *Tnnt1* (Cluster 1; also see Table 3) is expressed in thalamic neurons outside of PF. *Fxyd6* (Cluster 2; also see Table 4) is expressed in mPF neurons and ventral dorsal to PF whereas *Lypd6b* (Cluster 3) is a general PF neuronal marker.

**E,** *Pdyn* is expressed in mPF neurons as shown by ISH.

**F,** Multiple genes show significant correlation or anti-correlation with *Pdyn* expression on a cell-by-cell basis (*left*). This analysis reveals *Spon1* as being anti-correlated (yellow) with *Pdyn* and expressed in IPF whereas other genes, such as *Tnc,* are markers for cPF, as confirmed by ISH (*middle* and *right,* respectively).
Figure 2.3 (Continued)

G, Schematic of a coronal section at +0.9 mm from a WT mouse depicting 2 experimental configurations to use CTB to label neurons from mPF and cPF (top) or from cPF and IPF (bottom) that project to STR. 4 days after injections, whole-cell recordings were made in an acute brain slices of PF (highlighted in green).

H, Summary of intrinsic neuronal parameters (Membrane resistance (Rm), Membrane capacitance (Cm), and resting membrane voltage (Vrest)) as a function of the location and labeling with CTB of the neuron in the medial-lateral axis of the PF. The location of FR is indicated by the gray dashed area.

Also see related Figure S3.
Figure 2.3 (Continued)

A dissection of PF

B dissociation inDrops

C dissociation

D Tnnt1

E Fxyd6

F Lypd6b

G excitation neurons

H Pdyn

I correlation

J distance (μm)

K P<0.0001 P=0.0002 P =0.001
2.3.4 *Prodynorphin* expressing cells are located in the mPF and synaptically target the matrix of STR.

The restricted expression pattern of *Pdyn* in PF and the existence of a well-characterized knock-in mouse that expresses Cre recombinase from the *Pdyn* allele *Pdyn-IRES-Cre* mouse (Krashes et al., 2014) potentially permits specific manipulation of mPF circuitry. Indeed, injection of Cre-dependent AAV (creOn-GFP) in PF of the adult *Pdyn-IRES-Cre* mice (Figure 2.4A) resulted in GFP expression that was restricted to mPF (Figure 2.4B-C, S4A-D), including in the anterior-posterior (An-Po) axis of TH (% of cells: An to PF = 6%; PF = 90%; Po to PF = 3%; n=2 mice; Figure 2.4D). Additionally, ISH for *Pdyn* and *Slt17a6* (vglut2), showed that the *Pdyn*+ cells are glutamatergic (% of cells *Pdyn*/+*/Slt17a6+/ = 98%; n=125/5/2; cells/slices/mice; Figure S4E-F). mPF *Pdyn*+ cells target the medial band of STR (mSTR), from dorsal STR to the ACB, and, as shown by optogenetic activation of synaptic currents, densely innervate STR neurons (Figure 2.4E-H). Indeed, optogenetic stimulation of the Chr2-expressing *Pdyn*+ axons evoked excitatory post synaptic currents (EPSCs) in SPNs in mSTR but not dmSTR or dlSTR SPNs (EPSCs in mSTR neurons: 24/33; dmSTR: 0/7; dlSTR: 0/7. n=4; mice; Figure 2.4G-H), verifying that the *Pdyn*+ cells in mPF target a specific region of the STR and ACB. The fluorophore-labeled axons of mPF *Pdyn*+ neurons were not uniform within the mSTR, suggesting potential differential targeting of patch (striosome) and matrix compartments (Herkenham and Pert, 1981). Indeed, we found little overlap between GFP-labeled mPF axons in STR and regions expressing mu-opioid receptors (MOR), a marker of patches (Pert et al., 1976) (Figure 2.4I). Fluorescence inside of each patch compared to that of a “peri-patch” shape (100 µm wide) surrounding each patch (Figure 2.4I) was consistently
higher for the MOR channel (log fluorescence MOR=0.14±0.01) and lower for the GFP channel (log fluorescence GFP=-0.13±0.00; n=38/9/3; patches, slices, animals), consistent with Pdyn+ mPF fibers avoiding the STR MOR-rich compartments.
**Figure 2.4:** *Prodynorphin* expressing cells are located in the mPF and synaptically target the matrix of STR.

**A,** Schematic of a coronal section at -2.1 mm from a *Pdyn-IRES-cre* mouse depicting an injection of creOn-gfp (cyan) AAV into the PF (injection site in PF highlighted in orange).

**B,** *left,* Example coronal section at -2.1 mm of TH showing expression of GFP (cyan), indicating restricted expression of GFP in mPF. The inset is enlarged on the *right* and shows medially projecting neuronal processes from the GFP-expressing neurons.

**C,** Quantification of fluorescence intensity (Fl) in PF at coronal section -2.1 mm from images such as in panel B. Thin lines represents data from individual animals and the thick lines represent the mean. The dashed grey line represents the FR location relative along the medial-lateral axis (n=3; mice).

**D,** Percent of GFP+ cells anterior (An) and posterior (Po) to PF and in PF from the experiment shown in A. (n=1670/2; cells/mice).

**E,** Image of a coronal section highlighting the STR at +0.9 mm from a mouse manipulated as in panel A. Dorsal STR is separated into sub-regions: Expression of GFP-expressing *Pdyn*+ axons (cyan) from PF are seen in the medial STR (mSTR) but not dorsal-medial STR (dmSTR) and dorsal-lateral STR (dlSTR).

**F,** Quantification of fluroescence in the STR of axons from *Pdyn*+ cells PF at coronal sections between +0.6 mm and +1.2 mm. Thin lines represents data from individual animals and the thick line represent the mean (n=9/3; slices/mice).

**G,** Schematic of a coronal section at -2.1mm (left) depicting injection of AAV encoding Cre-dependent channelrhodopsin (creOn-Chr2) into PF of a *Pdyn-IRES-cre* mouse.
Figure 2.4 (Continued)

Three weeks after virus injection whole-cell recordings were obtained in STR (green) at and around coronal section +0.9 mm.

**H,** EPSC amplitudes evoked by optogenetic stimulation of Pdyn+ PF axons and measured in SPNs as a function of region in STR (mSTR, dmSTR, d1STR). EPSCs were recorded at -70 mV. For each cell the baseline current (open circle) and EPSC following a 5ms light pulse (closed circle) are plotted. Inset shows the mean of 10 light-evoked (blue-line indicates the light pulse) EPSCs from one cell. (n=48/4; cells/mice). Within each striatal region, results are shown ranked from largest to smallest EPSC amplitude.

**I,** Image of a coronal section of the STR at +0.9 mm with mu opioid receptors (MOR) immunolabeled (red, left) with 3 patches in the STR highlighted (white dashed lines). Axons of Pdyn+ PF neurons expressing GFP (center) avoid the MOR-rich patches (overlay, right).

**J,** Quantification of the distribution of fluorescence from GFP labeled PFâmSTR axons in and around the MOR-rich patches. The log of the ratio of the mean MOR and GFP fluorescence in the patch to that in a 100 µm wide ring around the patch (peri-patch) is shown 38 patches (n=9/3 slices/mice).

All data are represented as mean (bar in black) ± SEM (red). Also see related Figure S4.
Figure 2.4 (Continued)

A  
Pdyn-IRES-Cre

B  
CreOn-gfp

C  

t

D  
Po An

E  

F  

G  

H  

I  

J  

2.3.5 No interconnectivity between PF cell classes.

The single cell transcriptional data identified only excitatory neurons within the PF, suggesting that the topographically-organized STR-projecting neurons in PF are not interconnected by GABAergic interneurons. Several lines of analysis indicate that PF→STR projection neurons are also not interconnected by glutamatergic synapses. First, stimulation of ChR2 in *Pdyn-IRES-Cre* mPF neurons (Figure 2.5A) failed to elicit light-evoked EPSCs in CTB-labeled striatum-projecting neurons in cPF (cPF→dmSTR=0/19 EPSC; n=3 mice; Figure 2.5C) despite the triggering suprathreshold currents in the ChR2-expressing *Pdyn* neurons (549pA±136, 9/9 cells; n=3 mice; Figure 2.5B-C). Cell-filling labeling of cPF→dmSTR projection neurons with non-pseudotyped rabies virus expressing GFP (RV-GFP) (Figure 2.5D) showed that axons of these neurons do not overlap with CTB-labeled STR-projecting neurons in the IPF (Figure 2.5D). Moreover, similar experiments using non-pseudotyped rabies expressing ChR2 (RV-ChR2) injected into dmSTR combined with a CTB injection into dlSTR (Figure 2.5E) resulted in light-induced currents large enough to induce action potentials (APs) in cPF neurons (237pA±56, 9/10 cells; n=3 mice; Figure 2.5F) but failed to evoke EPSCs in CTB+ cells in IPF→dlSTR projection neurons (IPF→dlSTR = 0/16 EPSC; Figure 2.5F). In addition, RV-mediated GFP and ChR2 expression in dlSTR-projecting IPF neurons showed no overlap of axons with CTB-labeled dmSTR-projecting neurons in cPF (Figure 2.5G) and no light-evoked EPSCs (cPF→dmSTR=0/13 EPSC; n=2 mice; Figure 2.5H) despite suprathreshold ChR2-currents in IPF neurons (663pA±155, 6/6 cells; n=2 mice; Figure 2.5H-I). Lastly, trans-synaptic retrograde viral labeling with pseudotyped rabies virus (p.RV-GFP) revealed no connectivity across topographical projection zones of the
PF from primary infected starter cells in mPF, or lPF. However, clear labeling in PF-projecting regions such as the Substantia Nigra Reticulata (SNr) and Superior Colliculus (SC) was observed (Figure S5).
**Figure 2.5: The medial, central, and lateral sub-circuits of the PF are not locally interconnected.**

**A,** *left,* Schematic of a coronal section at -2.1 mm depicting a viral injection of creOn-ChR2 into the PF of a *Pdyn-IRES-Cre* mouse. *center,* Coronal section at +0.9mm depicting CTB injection into dmSTR 3 weeks after the creOn-ChR2 injection. *right,* four days later acute brain slices were cut and whole-cell recordings were obtained from ChR2+ or CTB+ PF cells.

**B,** ChR2-mediated excitatory currents in ChR2-expressing mPF neurons (representative example in blue) are activated concurrent with the laser pulse and display shorter latency than cortically-evoked EPSCs (representative example in gray dashed line). The cortically-evoked EPSC shown here for comparison was collected in independent the data set shown in Figure 7.

**C,** EPSC (CTB+ cells) and ChR2-current amplitudes (ChR2+ cells) measured at -70 mV in mPF and cPF evoked by optogenetic stimulation of *Pdyn-Cre+* neurons. For each cell, the baseline (white circle) and light-evoked (colored circles) currents following a 5 ms laser pulse (closed circle) are shown (n=28/3; cells/mice). The dashed grey line represents the FR and its location and circles represent the actual location of cells in the tissue relative to one another and the FR, from medial to lateral. No synaptic currents were detected in CTB+ cells (white circles for baseline and magenta circles following a 5 ms lase pulse).

**D,** Experimental design showing a coronal section at +0.9 mm of a WT mouse depicting injection of RV-GFP and CTB into dmSTR and dlSTR, respectively. Images of resulting retrograde labeling in the PF (-2.1 mm) show expression of GFP (magenta) in the cPF.
Figure 2.5 (Continued)

and CTB (yellow) in the IPF. The overlay (right) shows largely not overlapped cell populations (n=3 mice, example shown from one mouse).

E, As Panel (D) but with an injection of RV-ChR2 and followed by whole cell recordings from ChR2+ or CTB+ cells 4 days after injections.

F, left, As in Panel B showing representative ChR2-mediated currents in ChR- expressing cPF neurons (magenta) compared to an example cortically-evoked EPSC right, As in Panel C, summary of light-evoked ChR2-mediated current (in magenta) and EPSC (yellow) current amplitudes (as in Panel C) (n=26/2; cells/mice). No synaptic currents were detected in CTB+ cells (yellow).

G-I, As in Panels (D-F) but with CTB injected into dmSTR and RV-GFP or RV-ChR2 injected into dISTR (Example images are from one of 3 representative mice. For electrophysiological analysis n=19/2 cells/mice).

Also see related Figure S5.
Figure 2.5 (Continued)

A

B

C

D

E

F

G

H

I

473nm

Pdyn-IRES-Cre

CreOn-Chr2

3w

4d

473nm

EPSC (pA)

EPSC (pA)

EPSC (pA)

EPSC (pA)

EPSC (pA)

EPSC (pA)

EPSC (pA)

EPSC (pA)

rv-gfp

cPF

ctb

ctb

rv-gfp

cPF

IPF

EPSC (pA)

EPSC (pA)

EPSC (pA)

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2.3.6 Subclasses of PF neurons target distinct cortical regions

Many thalamic nuclei project to and receive input from CTX forming circuits that modulate persistent cortical activity (Guo et al., 2017) (Sherman, 2016). In primates, PF neurons innervate prefrontal CTX whereas the histologically distinct CM neurons target motor and premotor areas of CTX (Parent and Parent, 2004). In rats, reconstructed cells in PF project to both the STR and several regions in CTX (Deschenes et al., 1996). However, studies of mouse PF, albeit using manipulations that could not specifically target this small thalamic nucleus, suggest that it does not project to CTX (Oh et al., 2014). To determine if PF projects to CTX in mice, and whether the subclasses of neurons that we identified in PF target distinct regions of CTX, crea-GFP was expressed specifically in each PF cell class and automated image acquisition and analysis were used to map the distribution of GFP-labeled axons in CTX (Figure 2.6A). STR-projection neurons in each subregion of PF in \textit{Pdyn-IRES-Cre} mice were targeted by a different hybrid genetic/viral intersectional strategy (Figure 2.6A). For mPF, Cre-dependent AAV was injected directly into PF to activate expression of GFP in \textit{Pdyn}-expressing mPF neurons. For cPF, axon-infecting AAV encoding Flp recombinase was injected in dmSTR and AAV that expresses GFP in the presence of Flp and absence of Cre was injected into PF. This selects for neurons in cPF that target dmSTR while avoiding the nearby \textit{Pdyn}-expressing neurons in mPF. A similar approach was used for IPF, with injection of axon-infecting Flp-encoding AAV into dlSTR. These strategies succeeded in restricting GFP somatic expression largely to the intended anterior-posterior PF region (% cells in PF for mPF targeting=83%; n=713/15/1; cPF=67%; n=1864/15/1; IPF=66%; n=2171/15/1; cells/slices/mice; Figure S6A). As expected for the PF→STR connectivity described
above, GFP+ axons were observed projecting specifically between mPF→mSTR, cPF→dmSTR, and IPF→dlSTR (Figure 2.6B). To measure the distribution of putative PF→CTX projections GFP+ axons were mapped to the ABA and the relative axon density (RAD) was measured as fraction of all GFP+ pixels that are located in one area divided the fraction of cortical volume contained in the area. This metric gives the relative enrichment of axons in each area compared to a uniform distribution of axons within CTX.

We focused on 2 coronal sections of CTX (from 0.6-1.2 mm and from 2.5-3.1 mm anterior to posterior) and analyzed 11 ABA-labeled cortical subregions (Figure 2.6C, H). The putative output of each PF cell class was not homogenous (Figure 2.6D-E) in the posterior cortical section. For mPF, GFP-labeled axons were relatively enriched in the medial and lateral limbic regions of CTX (RAD from mPF to: ILA=2.8, ACAv=2.0, GU=2.41, AiD=5.65 and Alv=2.42; n=1; mouse; Figure 2.6D-E; refer to Table 1 for abbreviations) and depleted in associative and sensorimotor regions (RAD from mPF to: MOs=0.4, MOp=0.3, SSp=0.2, SSs=0.4; n=1; mouse; Figure 2.6D-E). cPF shared some of these medial and lateral limbic outputs with mPF but also projected heavily to MOs, GU, and AiD (RAD from cPF to: MOs=18.0, GU=59.7, AiD=50.2; n=1; mouse; Figure 2.6D-E). In contrast, IPF projected to SSp, SSs, and GU (RAD for IPF to: SSp=1.5, SSs=5.4, GU=1.6; n=1; mouse; Figure 2.6D-E) with only few axons found elsewhere in CTX. To independently verify the differential projections from PF subregions CTB was injected into posterior MOs or SSp. CTB+ cells were observed in cPF and IPF for the MOp and SSp injections, respectively (Figure 2.6F-G), thus confirming the results obtained with the measurements of RAD.
Similar analyses reveal that PF subregions also differentially target the more anterior section of cortex (Figure 2.6H). mPF projected strongly to ACAd and to PL (RAD from mPF to: ACAd=5.1, PL=3.5; n=1; mouse; Figure 2.6I-J) whereas cPF shared those targets but also projected to Ald (RAD from cPF to: ACAd=3.5, Ald=3.9, PL=1.9; n=1; mouse; Figure 2.6I-J). IPF projected to MOs and MOp of the anterior CTX (RAD from IPF to: MOs=3.5, MOp=3.1; n=1; mouse; Figure 2.6I-J). Thus, striatum-projecting PF neurons differentially innervate cortical regions. mPF and cPF innervate mainly limbic structures while cPF also targets associative areas such as MOs. IPF selectively targets sensorimotor cortical areas in the posterior part of CTX and innervates MOp and MOs regions in the anterior part of CTX. This topography was generally maintained in the cortical sections between these regions (Figure S6B).
**Figure 2.6:** PF→striatum projection neurons send topographically-organized outputs to CTX

**A,** Schematics of the intersectional strategies in *Pdyn-IRES-Cre* mice used to express GFP in subsets of PF→striatum projection neurons. *left,* injection of creON-GFP (cyan) into PF results in expression of GFP in the *Pdyn* neurons which are medially located. *center,* injection of retro-Flp in dmSTR (black) and a creOff-flpOn-GFP (magenta) virus into PF ensures expression specifically in the cPF and avoids leak into *Pdyn*+ neurons in mPF. *right,* injection of retro-Flp (black) in dlSTR and a creOff-flpOn-GFP virus (yellow) into PF ensuring expression of GFP specifically in the lPF.

**B,** Overlay of one brain section of each of three brains targeted with the labeling strategies depicted in Panel A at coronal section -2.1 mm in PF (*left*) and at +0.9 mm in STR (*right*).

**C,** *top,* For the analysis of the distribution of GFP+ axons in CTX, a region spanning from 0.6 to 1.2 mm anterior posterior was taken (red). *bottom,* Regions of interest were chosen spanning the medial lateral portion of CTX as demarcated by dashed lines.

**D,** Representative coronal sections from the posterior region of CTX (0.9mm) for each of the labeling strategies (*left:* mPF→CTX in cyan; *center:* cPF→CTX in magenta; *right:* lPF→CTX in yellow) highlighting the differential projections to medial, central, and lateral parts of CTX, respectively. The ventral and dorsal limits of the CTX were marked with dashes line. CC; corpus callosum.

**E,** Quantification of the relative axon density (RAD) of PF axons arising for each subregion for each of 11 cortical regions. The log(RAD) per region is represented by the gray scale.
within caps set at $\pm 1.5$ log units. A box with an X in it indicates a cortical region not present in the analyzed slice.

**F,** *left,* Experimental design showing a coronal section at $+0.9$ mm depicting an injection of CTB into MOs. *Right,* coronal section at $-2.1$mm showing CTB localized to cPF.

**G,** As in Panel F but targeting SSp with CTB (*left*) resulted in labeling IPF (*right*).

**H-J,** As in Panels C-E but depicting the analysis of axon distribution of an anterior section in CTX (2.5 to 3.1 mm anterior posterior).

The example images shown in panels (B,D,I) are from the same mice. Also see related Figure S6.
Figure 2.6 (Continued)

A. Schematic representation of the retroviral vectors used for Cre and Flp recombination. 

- creOn-GFP or creOff flipOn-GFP
- mPF → CTX or cPF → CTX or IPF → CTX

B. Fluorescence images showing the expression pattern of Cre and Flp recombination.

C. Diagram illustrating the Pdyn-IRES-Cre transgene expression pattern.

D-G. Fluorescence images of different brain regions labeled with Cre and Flp recombination.

H-J. Diagrams showing the relative axon density in various brain regions.

E. Color scale indicating relative axon density.

-1.5 0 1.5
2.3.7 Cortical Layer 5 projections to PF are topographically organized and form feedforward Cortex-PF-Striatum circuits

Some thalamic nuclei modulate sequential processing stages in CTX by receiving input from an upstream cortical region and projecting to its downstream cortical target, thus adding a parallel processing stage linking regions in CTX that are also themselves interconnected (Sherman, 2016) (Stroh et al., 2013) (Theyel et al., 2010). For example, in TH, the Pulvinar nucleus mediates a cortical-thalamic-cortical projection to facilitate transmission of information about attentional priorities between two visual cortical areas that are also directly connected (Saalmann et al., 2012). Since CTX is analogously upstream to the STR, we hypothesized that the cortical-thalamic-cortical circuit organization and function might also be recapitulated in cortical-thalamic-striatal circuit between CTX, PF, and STR (Saalmann, 2014).

Therefore, we examined two potential features of the circuits. First, are regions of PF and CTX reciprocally connected. Second, are regions of CTX and PF that project to the same subregion of STR themselves connected. To examine the first question – i.e. do the regions of CTX that receive input from specific subregions of PF, as defined in Figure 6, project back to those same regions of PF – we virally expressed GFP in Layer 5 projection neurons, including those that project to STR (Gerfen et al., 2013) in Tg(Rbp4-cre)KL100Gsat mice (link: GENSAT resource) (in short, Rbp4cre+/−) and labeled specific PF→STR projection neurons by focal injection of CTB into the STR. Layer 5 neurons were targeted because they give rise the cortical outputs that participate in CTX-TH-CTX circuits described above (Sherman, 2016). Targeting MOs axons and cPF→dmSTR cell bodies in cPF (Figure 7A) (max FI of CTB in: cPF=67±8; in rPF=8±1%; n=14/3;
slices/mice; Figure 2.7B, S7A) revealed that MOs axons in PF preferentially overlap with CTB+ cell bodies in cPF (max fiber FI overlap in: cPF=86%±3; rPF=30%±5; n=14/3; slices /mice; Figure 2.7B-C) across all coronal sections of PF (S7A).

Similarly, analysis of Primary Somatosensory Cortex (SSp) and IPF→dlSTR PF projection neurons (Figure 7D) (max FI of CTB in: IPF=60%±13; rPF=6%±1; N=8/2; slices /mice; Figure 2.7E, S7B) revealed overlap of SSp axons and CTB labeled IPF neurons (log ratio of fiber FI in the IPF/rPF = 0.47±0.07; n=8/2; slices/mice; Figure 2.7E-F; S7B). Conversely, selective targeting of SSp and cPF revealed no overlap between the CTB+ cells and fibers confirming the specificity of SSp→IPF fiber topography (log ratio of fiber FI in the cPF/rPF=-0.76±0.10; N=11/2; slices /mice; Figure 2.7G).

Targeting PFC and mPF→mSTR (Figure 7H) (max FI of CTB in: mPF=75%±7; rPF=18%±1; n=12/2; slices /mice; Figure 2.7I; S7C) revealed overlap of PFC axons and CTB labeled mPF neurons. (log ratio of fiber FI mPF/rPF=0.29±0.03; N=12/2; slices /mice; Figure 2.7I-J; S7C). In contrast, PFC axons had little overlap with cPF→dmSTR PF projection neurons confirming the specific overlap between PFC fibers→mPF (log ratio of fiber FI in the cPF/ rPF=0.02±0.04; n=10/2; slices /mice; Figure 2.7K).

Lastly, fibers from PFC also overlapped with mPF→mSTR PF projection neurons contralateral (con) to the injection site in CTX (Figure 2.7L) (max FI of CTB: con-mPF=64%±9; rPF=12%±2; n=10/2; slices/mice; Figure 2.7M; S7D; For max fiber FI overlap in: con-mPF=67%±9; con-rPF= 27%±2; n=10/2; slices /mice; Figure 2.7M-N; Figure S7D) compared to MOs and SSp which sparsely projected to con-PF in comparison to PF ipsilateral (ipsi) to the injection site (max fiber FI: MOs→ipsi-
Pyramidal tract (PT) cells originating in layer 5 project widely throughout the brain and form excitatory synapses onto many classes of neurons but also course through regions without forming synapses (Harris and Shepherd, 2015) (Levesque et al., 1996) (Shepherd, 2013). To examine the second question and determine whether the topographically organized PT axons observed in PF (Figure 2.7A-N; S7, S8) form functional synapse onto PF→STR projecting cells, whole-cell voltage-clamp recordings were obtained from CTB+ cells in PF and axons of layer 5 Cre+ PT cells expressing ChR2 were stimulated. Excitatory synaptic currents were observed in PF→STR neurons for all the CTX→PF projections tested (Projections: MOs→cPF=11/13 EPSCs; n=2; mice; SSp→IPF=7/21; n=2; mice; PFC→ipsi-mPF=12/23; n=3; mice; PFC→con-mPF=7/15; n=2; mice; Figure 2.7O). Thus, PF striatal projection neurons are a hub for limbic, associative, and sensory-motor information transfer from cortex to STR. Furthermore, each PF subregion targets the same cortical areas from which it receives input, creating circuits organized into cortical-PF-cortical and cortical-PF-striatal motifs. (See discussion for more details).
**Figure 2.7: Cortical Layer 5 projections to PF are topographically organized and form closed Cortex-PF-Striatum circuits**

A, Schematic of coronal sections at +0.9 mm from a *Rbp4-Cre*+/− mouse depicting injection of creOn-gfp (white) into layer 5 of secondary motor CTX (MOs) followed by a CTB injection (magenta) into dmSTR 3 weeks later.

B, Coronal section at -2.1 mm in PF showing the results of the experiment in Panel (A). CTB (left, magenta) and GFP-expressing axons from MOs (center, white) are seen to overlapping in cPF (right, overlay). The ROI in the CTB channel (white dashed line) was manually drawn and applied to the GFP channel (brown dashed line) to measure the fluorescence distribution.

C, Quantification of percentage of the maximal fluorescence intensity (FI) of GFP labeled axons in the cPF ROI, as shown in Panel (B), compared to that in the rest of PF (rPF). Grey filled circles here (and throughout the figure) represent the analyses of coronal section -2.3 mm in PF. (n=12/3; slices/animals). P=0.0001; Wilcoxon test.

D-E, As in (A-B) but with injection of creOn-GFP into primary sensory CTX (SSp) (white) and an injection of CTB (yellow) into dlSTR.

F, left, Quantification of percentage of the maximal FI of GFP labeled axons in the lPF ROI, as shown in panel (E), compared to that in the rest of PF (rPF) in log scale. (n=8/2; slices/animals).

G, Quantification as in panel (F) but for an experiment with an injection of creOn-GFP into primary SSp and an injection of CTB (yellow) into dmSTR and not dlSTR enabling
expression of CTB in the cPF→dmSTR projections. (Experimental design not shown). This confirmed the specificity of SSp→IPF fiber topography (n = 11/2; slices/animals).

**H-J**, As in panel (D-F) but for injection of creOn-GFP into PFC (white) and CTB injection into mSTR (cyan) and ACB (red). (n = 12/2; slices/animals).

**K**, As panel (G) but with an injection of creOn-GFP into PFC and CTB into dmSTR. This confirmed the specificity of PFC→mPF fiber topography (n = 10/2; slices/animals).

**L-N**, As panel (A-C) but for injection of creOn-GFP into PFC and CTB into mSTR and ACB contralateral to injection in PFC. P=0.002; Wilcoxon test. (n = 10/2; slices/animals).

**O**, top, Schematics of four experimental paradigms using *Rbp4-Cre*+/- mice indicating the sites of injection of creOn-ChR2 into CTX and of CTB into STR three weeks later. Acute brain slices were cut (bottom) and ChR2-stimulated corticothalamic EPSCs were measure at -70 mV in CTB+ neurons in the cPF (n=13/2; cells/mice), IPF (n=13/2), mPF ipsilateral (i-mPF n=23/3) and contralateral (c-mPF n=15/2) to the cortical injection. The amplitude of the ESPC (open circles) and equivalent analysis during a baseline period (closed circles) are shown in previous figures.

Grey filled circles in C, F, G, J, K, N represent the analyses of coronal section -2.3mm in PF. Data represented as mean (bar) and s.e.m (red). Also see related figures S7, and S8.
2.3.8 Differential modulation of STR by PF sub-classes

Previous work compared inputs from PF→STR with other thalamic inputs to STR (Ellender et al., 2013) (Alloway et al., 2014). We find that PF has multiple classes of cells that target functionally distinct regions of STR and CTX, and receive disparate inputs from CTX in addition to inputs from the midbrain. There is no connectivity between classes of PF projection neurons, therefore PF forms parallel streams of input to STR, integrating midbrain information with cortical input. However, each channel may have distinct effects on STR by differentially targeting direct and indirect pathway striatal projection neurons (SPNs) and striatal interneurons. To determine if the PF subclasses differently innervate interneurons in STR, whole-cell recordings were made from either cPF→dmSTR or lPF→dlSTR projections in Tg(Lhx6-EGFP)BP221Gsat BAC transgenic mice (link: GENSAT resource) (in short, Lhx6-EGFP) which expresses GFP in low threshold spiking (LTS) and fast spiking (FS) interneurons and leaves SPNs unmarked (Gittis et al., 2010) (Figure 2.8A,C). PF to SPNs connectivity was similarly high for both cPF→dmSTR and lPF→dlSTR projections (cPF→dmSTR=9/15 SPNs; n=3 mice; lPF→dlSTR=9/17; n=4 mice; Figure 2.8B,D). However, in the same animals, PF connectivity to FS cells, identified based on their firing patterns and membrane properties (Saunders et al., 2016), was low between cPF→dmSTR (2 of 23 FS cells innervated) compared to lPF→dlSTR (9 of 14 FS cells innervated) (Figure 2.8B,D). Inputs to LTS cells, also identified based on their firing patterns and membrane properties was low from both cPF (0 of 11 LTS cells innervated) and lPF (3 of 26 LTS cells innervated) (Figure 2.8B,D). Interneuron connectivity between mPF→mSTR was not examined; however, mPF to SPN connectivity was similarly high (24 of 33 SPNs; n=4 mice; Figure 2.4H) compared to
cPF\(\rightarrow\)dmSTR and IPF\(\rightarrow\)dlSTR SPN connectivity. Thus, we find that topographically defined PF\(\rightarrow\)STR projections all robustly target SPNs but differently innervate LTS and FS interneurons of the STR.

The NMDA-receptor (NMDAR) component of the SPN glutamatergic EPSC is thought to mediate induction of plateau potentials (up-states) in SPNs (Plotkin et al., 2011). Previous studies report widely varying NMDAR to AMPA-type (AMPA) glutamate receptor current ratios at PF to SPN synapses: analyses in mice describe that the CTX\(\rightarrow\)STR synapses induce relatively high NMDAR/AMPA current ratios compared to the TH\(\rightarrow\)STR synapses (Ding et al., 2008) whereas the opposite result has been described in rats (Smeal et al., 2007). We reasoned that these differences might have reflected different in the subregions of PF\(\rightarrow\)STR projections studied as opposed to true interspecies differences. Therefore, we measured AMPAR- and NMDAR-mediated synaptic currents (see methods) for the 3 PF\(\rightarrow\)STR projections (Figure 2.8E). The NMDAR/AMPA currents ratio were higher at mPF\(\rightarrow\)mSTR synapses (3.8\(\pm\)0.2; n=17/2; cells/mice; Figure 8E) compared to cPF\(\rightarrow\)dmSTR (1.6\(\pm\)0.1; n=43/4; cells/mice; Figure 2.8F) and IPF\(\rightarrow\)dlSTR (1.9\(\pm\)0.2; n=19/3; cells/mice; Figure 2.8G) which did not differ from one another. Thus, the characteristics of PF\(\rightarrow\)STR excitatory synapses onto SPNs depend on the target region within the STR.
**Figure 2.8: Differential modulation of STR by PF sub-classes**

**A,** Experimental design shown of coronal sections at +0.9 mm from a *Lhx6-EGFP* mouse injected with retro-Cre (white) in dmSTR and creOn-chr2 (magenta) in PF. 3 weeks later acute brain slices were cut and ChR2-stimulated cPF→dmSTR EPSCs were measure at -70 mV.

**B,** EPSC amplitudes from SPNs (*left*), FSs cells (*middle*), and LTSs (*right*) in dmSTR. The amplitude of the ESPC (open circles) and equivalent analysis during a baseline period (closed circles) are shown. (n = 3 mice).

**C-D,** As in panel (A-B) but with injections of retro-Cre (white) in dISTR and creOn-ChR (yellow) in PF. (n = 4 mice).

**E,** Top: Experimental design shown of coronal sections at -2.1mm from a *Pdyn-IRES-cre* mouse injected with creOn-Chr2 in PF. 3 weeks later whole cell recordings were obtained in STR (highlighted in green). EPSCs were recorded at -70mV and at a holding membrane potential of +20mV from the reversal potential of each cell following a light pulse. Bottom left: representative traces of NMDA (red) and AMPA (black) EPSCs. Bottom right: summary data. (n = 17/2; cells/mice).

**F-G,** Same as in panel (E) but for injection of retro-Cre in dmSTR (F) or dISTR (G) followed by injection of Chr2 into PF.
Figure 2.8 (Continued)

A retro-Cre

Lhx6-EGFP

creOn-ChR2 473nm

post light

pre light

B

EPSC (pA)

SPNs (9/15)

FSs (2/23)

LTSs (0/11)

C

retro-Cre

Lhx6-EGFP

creOn-ChR2 473nm

D

EPSC (pA)

SPNs (9/17)

FSs (9/14)

LTSs (3/26)

E

creOn-ChR2

Pdyn-ires-Cre

473nm

+32mV

-70mV

NMDA/AMPA, ratio

mPF

mSTR
dmSTR
dlSTR

+32mV

-70mV

+32mV

-70mV

+32mV

-70mV
2.4 Discussion

Here we present a comprehensive cellular and circuit analysis of the PF, a major subcortical excitatory input to the STR. Based on PF’s anatomical, transcriptional, electrophysiological, and synaptic properties we place its projection-neurons into 3 classes of cells. mPF neurons expressed Pdyn, the precursor protein for the K-opioid receptor agonist, project to matrix compartments of mSTR and to limbic CTX (for example, ILA, Aid, and PL) and receive bilateral input from layer 5 in PFC. mPF→STR projection neurons have higher input resistance, lower capacitance, and higher resting potential relative to those in central and lateral aspects of the PF. In cPF, neurons express Tnc, project to dmSTR and to limbic and associative regions of CTX (for example, to ILA, MOs, and GU), and receive input from layer 5 associative areas (MOs). Lastly, in lPF, neurons express Spon1, project to dlSTR and predominantly sensorimotor regions of CTX (for example, SSp and SSs), and receive input from layer 5 of sensorimotor CTX (SSp). All cell classes in PF have high connectivity to striatal projection neurons but differ in their innervation of striatal interneurons. PF neurons do not interconnect across regions, suggesting that PF subregions do not intermix their incoming cortical and midbrain signals through local inhibitory or excitatory connectivity. Indeed, cells in PF also receive inputs from the SNr, an output structure of the BG, in addition to receiving input from the Superior Colliculus.

2.4.1 Comparing the mouse PF to that of other species

The anatomical organization that we describe for mouse PF→STR appears present in other species (Giménez-Amaya et al., 2000) (Jones, 2007). PF/CM in primates
may also be separated into 3 regions that preferentially innervate motor CTX (lateral CM), sensory motor STR (medial CM), and associative limbic STR (PF) (Sadikot and Rymar, 2009). In primates CM and PF can be distinguished based on cell density and size (Jones, 2007), vulnerability to disease (Henderson et al., 2000), as well as in vivo firing patterns (Matsumoto et al., 2001) and have been proposed to have different functions (Glimcher and Lau, 2005) (Smith et al., 2014). In rats, IPF projects to dlSTR and mPF projects to dmSTR (Berendse and Groenewegen, 1990) which is a similar, albeit a simplified version of the relationship observed between CM, PF and STR in primates. Nevertheless, no region in the rat TH has been defined as CM due to lack of a clear histological boundary.

To our knowledge no previous characterization of PF has been conducted in the mouse. It is widely accepted that the gross nuclear division of mouse TH is similar to that in rats (Jones, 2007), although some thalamic nuclear boundaries are more obscure in mice, likely due to a diffused cytoarchitecture. Here, we define 3 classes of neurons in PF as well as functional, transcriptional, and anatomical differences across the medial-lateral aspect of the PF.

The facility of analysis in mice allowed us to uncover differences between subdivisions of the PF that have not been addressable in traditionally genetically-intractable species such as primates, cats, and rats. Even within mouse studies (Parker et al., 2016) (Kato et al., 2011) (Aceves Buendia et al., 2017) (Assous et al., 2017) (Choi et al., 2018) PF has been treated as cellular homogenous and not having subcircuits nor it being distinct to its neighboring TH nuclei. Our findings reveal transcriptional distinctions that demarcate PF cell-type classes and also separate it from its neighboring medial
dorsal nucleus in the anterior-posterior axis and the posterior nucleus in medial-lateral axis. Thus, these results permit targeted analyses of specific PF subcircuits and neuron classes in normal behavior and disease-models, similar to studies already underway in other brain regions (Svoboda and Li, 2018) (Beyeler et al., 2018) (Wallace et al., 2017) (Saunders et al., 2015) (Girasole et al., 2018) (Mastro et al., 2014) but not previously possible in the ILM of the TH.

### 2.4.2 PF-cortical interactions

Thalamic nuclei typically form reciprocal connections with CTX by receiving input from and projecting to a single cortical region or receiving input from one region and project to another (Sherman, 2016). Thalamic nuclei receive modulatory-inputs from layer 6 while higher-order thalamic nuclei also receive inputs from layer 5 (Harris and Shepherd, 2015) (Sherman, 2016). These CTX-TH-CTX circuits have been proposed to have two functions. First, via recurrent excitation they maintain persistent activity in CTX, as has been shown for projections from motor TH to the anterior lateral motor region of CTX (Guo et al., 2017) and is thought to be necessary for working memory (Bolkan et al., 2017) (Halassa and Kastner, 2017). Second, other CTX-TH-CTX circuits have a triangular motif in which a cortical region targets a second cortical area and a thalamic nucleus that also projects to the second cortical region. This motif, for example, is seen in Pulvinar nucleus outputs to visual cortex, and has been proposed to transmit and synchronize signals about attentional priorities between directly connected cortical regions (Saalmann et al., 2012).

These canonical principals of organization have not been examined fully in ILM TH and its interactions with CTX. It is likely that all subclasses of PF neurons are innervated
by layer 6 cortical neurons (Jeong et al., 2016). We find that PF receives input from neurons in layer 5 of limbic, associative, or sensory-motor regions while also projecting back to those regions. Thus, it seems that the laminar sources of cortical input to PF are similar to those for other nuclei of TH.

PF → CTX projections align with CTX → PF projections, suggesting the existence of a corticothalamic recurrent network through PF. However, unlike typical TH nuclei, the main output of PF is to STR and not to CTX. For this reason, and analogous to the triangle attentional motif described above, we propose that cortical-PF-cortical circuits facilitate and shape striatal output of a behaviorally relevant cortical region. In parallel, PF can integrate information from CTX with that from subcortical nuclei (such as SC and SNr) to facilitate correct action selection in an ongoing sensorimotor context. Since we find that PF neuron classes are not interconnected in PF, these networks of activity can act relatively independently of each other.

We find that PFC projects bilaterally to mPF whereas SSP → IPF or MOs → cPF projections are strictly ipsilateral, highlighting the potential different functions of the PF subclasses characterized here – laterality may be important to maintain for sensory and motor circuits while perhaps a global limbic signal may need to be dispersed across both hemispheres. PT cortical neurons do not project to contralateral STR (Harris and Shepherd, 2015) thus the bilateral PFC → mPF projections may transmit PT related-activity signal near synchronously to both STR without recruitment of bilateral IT-type cortical projections.
2.5 Methods

2.5.1 Mice

This study is based on data from mice postnatal day 50, both males and females. We used the C57BL/6NCrl (Charles River Laboratories, Wilmington, MA, stock #027) and transgenic mice lines: Dyn-IRES-cre (Jackson Laboratories, Bar Harbor, ME, stock #027958), Rbp4-Cre (Gensat project, founder line KL100), and LHX6-EGFP (Gensat project, founder line BP221). Animals were maintained on a C57BL/6 background and kept on a 12:12 light/dark cycle or a reversed cycle under standard housing conditions. Experimental manipulations were performed in accordance with protocols approved by the Harvard Standing Committee on Animal Care following guidelines described in the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. All mice brain coordinates in this study are given with respect to Bregma; anterior–posterior (A/P), medial–lateral (M/L), and dorsal–ventral (D/V).

2.5.2 AAVs

Recombinant adeno-associated viruses (AAVs of serotype 1,2,8 or 9) encoding a double floxed inverted (DFI) gene under the control of CAG, Ef1a, or hSyn promoters were used to express the gene in the Cre-recombinase expression neurons. Additionally, we used intersectional AAVs that expressed the gene only when Flp is present and Cre is absent (FlpOn/CreOff). Retrograde AAVs which efficiently infect axons (Tervo et al., 2016) were used to deliver Flp or Cre recombinase to neurons upstream to the injection site. AAVs were packaged by commercial vector core facilities (UNC Vector Core and
Penn Vector Core) and upon arrival stored at a working concentration 10^{11} to 10^{13} genomic copies per ml at -80C.

2.5.3 Rabies viruses

Rabies viruses carrying the transgene for the H2B:EGFP fusion protein were generated in-house. Synthesized H2B-EGFP vector was cloned into pSPBN-SADΔG-tdTomato plasmid using Smal and Nhel restriction sites, replacing a tdTomato sequence. B19G-SADΔG-H2B:EGFP virions were first generated via cDNA rescue using a procedure based on previously described protocols (Wickersham et al., 2010). Briefly, HEK 293T cells (ATCC CRL-11268) were transfected with pSPBN-SADΔG-H2B:EGFP, pTIT-B19N, pTIT-B19P, pTIT-B19G, pTIT-B19L and pCAGGS-T7 using the Lipofectamine 2000 transfection reagent. 5 to 7 days post-transfection, the supernatant was filtered through a 0.22 µm PES filter and transferred to BHK-B19G cells for amplification. Virions were then serially amplified in three rounds of low-MOI passaging through BHK-B19G cells by transfer of filtered supernatant, with 3 to 4 days between passages. Cells were grown at 35 °C and 5% CO₂ in DMEM with GlutaMAX (Thermo Scientific, #10569010) supplemented with 5% heat-inactivated FBS (Thermo Scientific #10082147) and antibiotic-antimycotic (Thermo Scientific #15240-062). For concentrating the virions, media from dishes containing virion-generating cells was first collected and incubated with benzonase nuclease (1:1000, Millipore #70664) at 37°C for 30 min before filtering through a 0.22 µm PES filter. The filtered supernatant was transferred to ultracentrifuge tubes (Beckman Coulter #344058) with 2 ml of a 20% sucrose in dPBS cushion and ultracentrifugated at 20,000 RPM (Beckman Coulter SW 32 Ti rotor) at 4°C.
for 2 hours. The supernatant was discarded and the pellet was re-suspended in dPBS for 6 hours on an orbital shaker at 4 °C before aliquots were prepared and frozen for long-term storage at -80 °C. Unpseudotyped rabies virus titers were estimated based on a serial dilution method (Osakada & Callaway, 2013) counting infected (H2B:EGFP+) HEK 293T cells, and quantified as infectious units per ml (IU/ml).

B19G-SADΔG-EGFP, EnvA-SADΔG-EGFP, and B19G-SADΔG-ChR2-EYFP viruses were generated by amplification from existing in-house stocks using similar passaging procedures described above. Pseudotyping was performed after the last passaging round of unpseudotyped virion amplification. BHK-EnvA cells were infected with the filtered supernatant containing unpseudotyped virions for 6 hours, followed by two rounds of trypsinization with dPBS washes and re-plating over two consecutive days. Pseudotyped rabies virus titers were estimated as described above counting infected (EGFP+) HEK 293T-TVA800 cells. For quality control, pseudotyped rabies virus stocks were tested in vitro for leak of unpseudotyped virus with a similar titering protocol by infecting HEK 293T cells. Virus batches used had a leak of less than 2 x 10³ IU/ml.

2.5.4 Stereotaxic Intracranial Injection

Mice were anesthetized with 2.5% isoflurane in 80% oxygen and placed in a stereotaxic frame (David Kopf Instruments Model 900). Under aseptic conditions, the skull was exposed and leveled (<100 μm difference between 1.5+A/P and Lambda as a cutoff for proper leveling of the skull). 250 μm craniotomies were made with an electric drill (Foredom Electric Company K.1070) with a ball bur (Busch and Co. S33289) attached to the manipulator. All reagents were injected through a pulled glass pipette (Drummond
Scientific Company pipettes) with a tip of approximate 50 µm (pulled with a P-97 model Sutter Instrument Co. pipette puller). To avoid leak into other brain regions and back spill through the pipette track the injection pipette was lowered 200 µm ventral to the region of injection before being brought up to the point of injection. The pipette was left in place for 3min prior to injection and the reagent of interest was delivered at a rate of 50nl/min using a UMP3 micro-syringe pump (World Precision Instruments). Following injection, we waited 5min at the injection site before raising the pipette 200 µm above the injection site and waited 5 minutes more. We then exited the brain at about 1mm/minute. To minimize their dehydration during surgery mice received a subcutaneous injection of 1ml of sterile saline (Teknova S5819). Additionally, in order to reduce inflammation, mice received an injection of Ketoprofen (Zoetis 07-803-7389) at an amount of 0.01mg per gram of animal mass. Postoperatively, mice were monitored on a heat pad for one hour before being returned to their home cage. Mice were then monitored daily for at least 5 days and received a MediGel Carprofen cup in their home cage (Clear H₂O).

2.5.5 Injection coordinates

All coordinates that were used in this study were relative to Bregma. For PFC; 2.8 mm A/P, 1.2 mm M/L, 0.9 mm D/V, MOs; 0.8, 0.9, both 0.8 and 0.5, SSp; 1.0, 2.2, 1.0, mSTR; 0.8, 1.0, both 3.3 and 2.7, dmSTR; 0.8, 1.6, 2.6, dlSTR; 0.8, 2.4, 2.5, ACB; 0.8, 1.5, 4.6, mPF; -2.1, 0.5, both 3.7 and 3.5, IPF; -2.1, 0.88, both 3.75 and 3.55.
2.5.6 Injection volumes and waiting time for specific anatomical regions and reagents

mSTR: RV-nGFP (150-200nl), CTB (80-200); dmSTR: RV-nGFP (150-200), CTB (80-200), retro-Flp (200), retro-Cre (300), RV-GFP (100), RV-ChR2 (200); dLSTR: RV-nGFP (150-200), CTB (80-200), retro-Flp (200), retro-Cre (300), RV-GFP (100), RV-ChR2 (200); mPF: creOn-GFP (75-150), CreOn-ChR2-mCherry (200-300), CreOn-ChR2-GFP (200-300), FlpOn/CeOff (200), CreOn-TVA (100), CreOn-OG (100), p.RV-GFP (150-200); LPF: FlpOn/CeOff (200), CreOn-ChR2-mCherry (200-400); ACB: CTB (80-140); MOs: creOn-GFP (200), CreOn-ChR2-GFP (250); SSp: creOn-GFP (200), creOn-TdTom (200), CreOn-ChR2-mCherry (100-250); PFC: creOn-GFP (200-250), CreOn-TdTom (200-250), CreOn-ChR2-GFP (100-250). Waiting times for reagents were as followed: CTB: 3-7 days; AAVs: 2.5-5 weeks; RV-nGFP: 5-10 day; RV-EGFP: 7-10 days. RV-ChR2: 3-7 days; p.RV-GFP: 5-10 days;

2.5.7 Histology and Imaging for STPT

Animals were perfused transcardially with ice-cold 0.9 % saline solution followed by 4% paraformaldehyde (PFA) (diluted in 0.2 M phosphate buffer) for 7 min at 7 ml/min, 5 to 10 days after RV-nGFP infection. Brain were fixed in 4% PFA for 24h before being transferred to 0.1 M glycine solution (diluted 0.1 M phosphate buffer), for 48h at 4 °C before being stored in 0.1 M phosphate buffer at 4 °C until imaged. Imaging was done as previously described (Ragan et al., 2012). In short, brains were embedded in 4% agarose in 0.05M PB, cross-linked in 0.2% sodium borohydrate solution (in 0.05 M sodium borate buffer, pH 9.0-9.5). The entire brain (including the olfactory bulb and the cerebellum) was
imaged with a high-speed 2-photon microscope with integrated vibratome at 1μm-1μm x-y resolution for a depth of 50 μm on a TissueCyte 1000 (TissueVision). The 2-photon excitation wavelength was 910 nm, which efficiently excites GFP. A 560 nm dichroic mirror (Chroma, T560LPXR) and band pass filters (Semrock FF01-520/35 an) were used to separate green.

2.5.8 Histology and imaging for all other experiments

mice were anesthetized with isoflurane and perfused transcardially with 4% PFA in 0.1 M sodium phosphate buffer (PBS). Brains were post-fixed for 24-48 hours and transferred to a 0.1M PBS solution until further processing. Coronal slices were made at 50 μm thickness per slices on a vibrating blade microtome (Leica Biosystems VT1000S). Brain sections were mounted on superfrost slides (VWR 48311-703) dried, and coverslipped with ProLong antifade reagent containing DAPI (ThermoFisher P36962). Whole slides were imaged with an Olympus VS120 slide scanning microscope with a 10X objective. Regions of interest were imaged with an Olympus FV1200 confocal microscope using a 10X or 60X objectives at the Harvard Neurobiology Imaging Facility and Harvard Neurodiscovery Imaging Core.

2.5.9 Immunohistochemistry

Images requiring immunohistochemical staining were processed using a protocol previously described in Pisanello and Mandelbaum et al. (Pisanello et al., 2017). In short, slices were incubated in PBS blocking solution containing 0.3% Triton X-100 (PBST) for
1h at RT (20–22 °C). Slices were then incubated over night at 4 °C in the same blocking solution with 1% goat serum with MOR primary antibody (Life Technologies AB5511). The next day, slices were rinsed 3 × 10 min in PBS before being incubated in the blocking solution with secondary antibody (1 mg/mL goat anti-rabbit Alexa Fluor 647 (AB_2535812; Life Technologies) or Alexa Fluor 594 (R37117; Life Technologies) Life Technologies). The slices were then rinsed again, mounted, and imaged as described above.

2.5.10 In situ hybridization

Tissue for in situ hybridization was processed using a protocol previously described in Hvartin and Hochbaum et al. (Hrvatin et al., 2018). In short, animals were euthanized and brains were immediately frozen on dry ice to be sliced via cryostat (Leica CM 1950). Cells in the mPF were marked by *ProDynorphin*, cells in cPF by *TNC*, and cells in lPF by *Spon-1*. Excitatory cells in TH were marked with *Slc17a6* (Vglut-2). For the image presentation of the ISH in Figure 3, nuclei masks were created and each nucleus was pseudo-colored according to the number of puncta contained within the specific mask, as previously described in Hvartin and Hochbaum et al. (Hrvatin et al., 2018).

2.5.11 STPT Image Analysis

Raw images were corrected for non-uniform illumination and light collection, stitched in 2D, and stacked in 3D. **RV-nGFP cells counts.** GFP+ neurons were automatically detected by a convolutional network (ID: 164) trained to recognize nuclear
neuronal cell body labeling. The 3D stack was then registered to a 3D reference brain based on the ABA (Kim et al., 2015) (Sunkin et al., 2013) by 3D affine registration followed by a 3D B-spline registration using the software Elastix (S. Klein et al., 2010). The number of total input neurons in each brain region was normalized by the total number of GFP+ cells detected in the parent region. (For example, in Figure 1D the parent region is sub-CTX).

2.5.12 Brain volume quantification

To measure the volume of anatomical regions, the average reference brain (built using 40 STPT imaged brains) was aligned to the ABA. Segmentation areas were registered onto each brain using the b-spline registration procedure described above (i.e. the ABA segmentation was registered onto each individual brain). The number of voxels that belong to each region in the transformed ABA segmentation were counted and multiplied by 0.02 x 0.02 x 0.05 mm³ (the dimensions of an anatomical voxel unit), resulting in the total volume of each region. Projection mapping data processing. Previously published methods were adopted for quantifying neuronal projections as imaged by STPT (Oh et al., 2014). In brief, filtered images of the original image data were generated by applying a square root transformation, histogram matching to the original image, and median and Gaussian filtering using ImageJ (NIH) software. The original images were then subtracted from the filtered images to generate signal images. These were then converted to binary maps by applying a threshold chosen to maximize signal retention while minimizing background auto-fluorescence. We cannot rule out the possibility that faint and sparse signals were being missed in our automatic detection.
False-positive signals at the injection sites and from bright fluorescence from the dura were removed using manually curated masks for each brain. The method measures fluorescence from all axons, including axons of passage. For this reason, we only analyzed signals in CTX, where fibers of passage are less likely. To calculate the putative output of PF to CTX the relative axon density was measured as fraction of all GFP+ pixels that are located in a given area divided the fraction of cortical volume contained in the area. This metric gives the relative enrichment of axons in each cortical area compared to a uniform distribution of axons within CTX. The scalable brain atlas (link) was used for Figure 6 C-H visualization (Bezgin et al., 2009).

### 2.5.13 Image analysis for all other experiments

Quantification of the fluorescence intensity (FI) of CTB+ cells in PF were done using a custom macro in ImageJ (NIH). For each coronal section in PF, and based on the ABA, the mean pixel FI was calculated across a ventral-dorsal line with an 0.6 µm medial-lateral width. Using Graph-Pad prism (GraphPad Software, La Jolla, CA), a 2nd order smoothing (Savitzky and Golay, 1964) was applied with 200 nearest neighbors prior to normalizing each channel. For quantification of the GFP+ cell bodies in PF, and GFP+ axons in STR (Figure 4) the same analysis was used, with the exception of the ventral dorsal line being 1.0mm wide. For analysis of Pdyn+ PF→STR axons coronal sections of STR at +0.6, +0.9, and +1.2 mm were grouped together. For the analysis of topographical organization of Pdyn+ axons in STR (Figure 4) patches (based on MOR stain) were manually labeled while being blinded to the GFP+ PF→STR fiber location. Using a custom macro in ImageJ each patch label was expanded by 100 µm in all directions and...
the mean FI of the patch and this peri-patch region were calculated for the MOR channel and the fiber channels. For image analysis of the Layer 5 CTX→PF inputs (Figure 7) the DAPI channel was used to mark the location of PF to ensure that the selection was done solely based on anatomical location as defined in Figure 1 and based on the ABA. The CTB channel was used to label the region in PF with all CTB+ cells and mean FI was measured in that region compared to the rest of PF (Figure S7). Next, the areas define as encompassing the CTB+ cells in PF was applied to the CTX→PF fiber channel and FI of fibers were calculated in that region and compared to the rest of PF. Background FI was calculated by taking the mean of 3 random tissue areas of 0.3mm². The comparison of the FI inputs from CTX to PF, ipsilateral to the injection sight vs. contralateral to the injection sight (Figure S8) was done by manually labeling the axons ipsilateral to the injection sight. The axon location contralateral to the injections was at a similar location and shape as ipsilateral to the injection, allowing to apply the same label to the ipsilateral side. Correction for background FI was done as described above.

2.5.14 Whole-cell dissociation and RNA capture

Dissociated whole-cell suspensions were prepared using a protocol adapted from Hrvatin amd Hochbaum et al. (Hrvatin et al., 2018). 8-week old C57BL/6NCrl male mice (Charles River Laboratories, Wilmington, MA, stock #027) were pair-housed for a few days after arrival in a regular light/dark cycle room prior to tissue collection. Mice were transcardially perfused with an ice-cold choline cutting solution containing neuronal activity blockers (110 mM choline chloride, 25 mM sodium bicarbonate, 12 mM D-glucose, 11.6 mM sodium L-ascorbate, 10 mM HEPES, 7.5 mM magnesium chloride, 3.1 mM
sodium pyruvate, 2.5 mM potassium chloride, 1.25 mM sodium phosphate monobasic, 10 µM (R)-CPP, 1 µM tetrodotoxin, saturated with bubbling 95% oxygen/5% carbon dioxide, pH adjusted to 7.4 using sodium hydroxide). Brains were rapidly dissected out and sliced into 250 µm thick coronal sections on a Leica VT1000 vibratome in a chilled cutting chamber filled with choline cutting solution. Coronal slices containing the TH were then transferred to a chilled dissection dish containing choline cutting solution for microdissection of the PF under a stereomicroscope. Dissected tissue chunks were transferred to cold HBSS-based dissociation media (Thermo Fisher Scientific Cat. # 14170112, supplemented to final content concentrations: 138 mM sodium chloride, 11 mM D-glucose, 10 mM HEPES, 5.33 mM potassium chloride, 4.17 mM sodium bicarbonate, 2.12 mM magnesium chloride, 0.9 mM kynurenic acid, 0.441 mM potassium phosphate monobasic, 0.338 mM sodium phosphate monobasic, 10 µM (R)-CPP, 1 µM tetrodotoxin, saturated with bubbling 95% oxygen/5% carbon dioxide, pH adjusted to 7.35 using sodium hydroxide) supplemented with an additional inhibitor cocktail (10 µM triptolide, 5 µg/ml actinomycin D, 30 µg/ml anisomycin) and kept on ice until dissections were completed. The remaining tissue was fixed in 4% PFA in PBS for histological verification. Dissected tissue chunks from 8 mice were pooled into a single sample for the subsequent dissociation steps. Tissue chunks were first mixed with a digestion cocktail (dissociation media, supplemented to working concentrations: 20 U/ml papain, 1 mg/ml pronase, 0.05 mg/mL DNAse I, 10 µM triptolide, 5 µg/ml actinomycin D, 30 µg/ml anisomycin) and incubated at 34 °C for 90 min with gentle rocking. The digestion was quenched by adding dissociation media supplemented with 0.2% BSA and 10 mg/ml ovomucoid inhibitor (Worthington Cat. # LK003128), and samples were kept chilled for
the rest of the dissociation procedure. Digested tissue was collected by brief centrifugation (5 min, 300 g), re-suspended in dissociation media supplemented with 0.2% BSA, 1 mg/ml ovomucoid inhibitor, and 0.05 mg/mL DNAse I. Tissue chunks were then mechanically triturated using fine-tip plastic micropipette tips of progressively decreasing size. The triturated cell suspension was filtered in two stages using a 70 µm cell strainer (Miltenyi Biotec Cat # 130-098-462) and 40 µm pipette tip filter (Bel-Art Cat. # H136800040) and washed in two repeated centrifugations (5 min, 300 g) and re-suspension steps to remove debris before a final re-suspension in dissociation media containing 0.04% BSA and 15% OptiPrep (Sigma D1556). Cell density was calculated based on hemocytometer counts and adjusted to approximately 100,000 cells/ml. Single-cell encapsulation and RNA capture on the inDrop platform was performed at the Harvard Medical School ICCB Single Cell Core using v3 chemistry hydrogels based on previously described protocols (Zilionis et al., 2017). Suspensions were kept chilled until the cells were flowed into the microfluidic device. The encapsulated droplets were broken and cDNA was processed for next-gen sequencing, as previously described (A. M. Klein et al., 2015) generating index libraries that were then pooled and sequenced across 3 runs on the NextSeq500 (Illumina) platform.

2.5.15 Acute Brain Slice Preparation and electrophysiology experiments

Experiments were done as previously described (Wallace et al., 2017). In short, artificial cerebrospinal fluid (ACSF) containing 2mM [Ca] and 1 mM [Mg] was superfused at 3 ml/min. For optogenetics experiments, 3 to 5 ms duration light pulses from a 473 nm laser (at 5-10mW per mm² (measured at the sample plane) were used. Recordings were
performed at 32C using Cs-based internals for voltage-clamp measurements of synaptic currents and K-based internals for current-clamp measurements of firing patterns.

2.5.16 In drops analysis

Transcripts were processed according to a previously published pipeline (A. M. Klein et al., 2015) (Hrvatin et al., 2018). Briefly, a custom transcriptome was assembled from the Ensembl GRCm38 genome and GRCm38.84 annotation using Bowtie 1.1.1, after filtering the annotation gtf file (gencode.v17.annotation.gtf filtered for feature_type="gene", gene_type="protein_coding" and gene_status="KNOWN"). Read quality control and mapping against this transcriptome was performed using default parameters. Unique molecular identifiers (UMIs) were used to reference sequence reads back to individual captured molecules. The output matrix (cells x genes) was then filtered to exclude cells with less than 500 UMIs and used as the input to the Seurat pipeline for further analysis (Satija et al., 2015). Genes were excluded if UMIs were found in 3 cells or less. Cells were excluded if they expressed fewer than 400 genes, or more than 5500 genes. Cells with 15% or more of their transcriptome derived from mitochondrial genes were excluded. Finally, cell doublets were estimated by creating synthetic doublets from the dataset and computing a k-nearest neighbor graph (k = 30) with both cells and synthetic doublets. Cells were ranked according to the percentage of nearest neighbors that were synthetic doublets. Cells in the top 5% of doublet scores were excluded as putative doublets. Cells were then log-normalized and scaled to 10,000 transcripts per cell. Variable genes were identified using the MeanVarPlot() function, which calculates the average expression and dispersion for each gene, then bins genes and calculates a
z-score for dispersion within each bin. The following parameters were used to set the minimum and maximum average expression and the minimum dispersion: x.low.cutoff=0.0125, x.high.cutoff=3, y.cutoff=0.5. Next, the count matrix was regressed against the number of UMIs and percentage of counts comprising mitochondrial genes and scaled. Then PCA was carried out and the top 20 principal components (PCs) were kept. Finally clustering was performed using the FindClusters() routine. Clustering resolution was set to 0.6. This resulted in 13 initial clusters, that were categorized into 7 broad cell-type classes by canonical gene expression patterns (Mrc1/Cd36 for macrophage, Olig1/Pdgfra for oligodendrocytes and oligodendrocyte precursors, Vtn for pericytes, Cldn5/Pecam1 for endothelial and smooth muscle cells, Aqp4 for astrocytes, P2ry12/ Cx3cr1 for microglia, and Snap25/Syn1 for neurons). **Subclustering of neurons.** Cells from neuronal clusters were merged and re-clustered as above with 10 PCA components (estimated as significant by the JackStraw algorithm), yielding 6 initial clusters. Differential gene expression was carried out using Monocle2 (Trapnell et al., 2014). Only 3 clusters had 2-fold enriched genes (Clusters without 2-fold enriched genes were not considered distinct cell types, but instead a result of overclustering). Cells in these 3 clusters were used as a training set to classify the other cells using a random forest classifier (using the Seurat function ClassifyCells()). Bootstrapping by repeating this classification process 1000 times produced a metric for classification. Cells that were classified < 95% of the time to the same cluster were excluded. This final classification was used as input for differential gene expression using Monocle2.
2.5.17 Electrophysiological Analysis.

Electrophysiological properties and ChR2-evoked EPSCs were performed using automated scripts written in MATLAB. Following the electrophysiology analysis white papers of the ABA (link: electrophysiology overview technical whitepaper) we did not make a priori assumptions about the input resistance, resting potential, or minimal firing rate necessary to designate a cell as “healthy”. Therefore, our final data set includes neurons that, for example, do not fire any action potentials to injected current. Selection of neurons for inclusion was based on the series resistance. In short, tables containing the name of the cell, annotations about the position of the cell and conditions of the experiment were noted during the experiment including the start and stop sweeps to be analyzed which were then used to automatically retrieve and analyze data. **Intrinsic properties.** Resting membrane potential was measured as the median of potentials during periods of the sweep that had with no current injection. Membrane capacitance (Cm) and resistance (Rm) as well as series resistance were measured in voltage-clamp mode by fitting a single exponential to the current evoked by a -5 or -10 mV voltage pulse. Rs was estimated from the peak of the exponential fit – i.e. Rs= ΔV/Δt(t=0) with t=0 being the start of the voltage step command. The steady state current was used to calculate Rm=ΔV/ΔI(t=∞) - Rs. Cm is then calculated from the time constant of the fit tau=RsRmCm/(Rs+Rm). **EPSCs.** Resting membrane properties were measured as above. To determine the amplitude of the EPSC and compare it to the amplitudes expected from chance fluctuations of the membrane potential (e.g. due to thermal and seal noise or spontaneous synaptic events), two 15 ms long periods were analyzed in each sweep. The first was a time window after the light pulse in which a genuine ChR2-
evoked EPSC would be expected. In this window, the average current compared to baseline was calculated, as well as its peak deviation (positive for NMDA-receptor mediated currents at positive potentials and negative for AMPA-receptor mediated currents at rest). In addition, a “peri-peak” value was calculated from the average current in a time window (3 ms long) around the time of the peak deviation from rest in the average of all sweeps for the cell. Identical analyses were carried out in a time window occurring 50 or 100 ms before the light pulse to estimate baseline fluctuations for each parameter. All measurements of AMPA-receptor mediated EPSCs were done at -70 mV. For NMDA-receptor mediated EPSCs, the reversal potential of the EPSC was found (typically at nominally +5-10 mV) and the cell was depolarized a further 20 mV, at which the measurement was made. No corrections were made for liquid junction potential (~ 8 mV). FSs, LTSs, and SPNs in the LHX6-GFP were identified based on responses to current injections, membrane resistance, and the presence or absence of dendritic spines as previously described in Saunders et al (Saunders et al., 2016).

2.5.18 Statistical analyses

Data points are stated and plotted as mean values ± SEM. p values are represented by symbols using the following code: * for 0.01<p<0.05, ** for 0.001<p<0.01, and *** for p<0.001. Exact p-values are stated in figure legends and main text. All statistical tests were non-parametric as noted in the text. No a priori power analyses were done.
2.6 Bibliography


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Chapter 3: Basal Ganglia Circuitry Controlling Action Selection In Vivo

3.1 Introduction

Animals must choose appropriate actions based on goals, past experience, and their current sensory environment. This process of action-selection is thought, in vertebrate animals, to depend crucially on the proper function of the basal ganglia (BG). The importance of the BG to normal behavior and action selection in humans is made clear from diseases with components of BG dysfunction, such as Parkinson’s (Wichmann et al., 2011) (Kravitz et al., 2010), Huntington’s (Vonsattel et al., 1985) (Mangiarini et al., 1996), Tourette’s Disorder (Leckman et al., 2010) (Vinner et al., 2017), and Obsessive-Compulsive (Saxena et al., 2001) (Ahmari et al., 2013) disorders. In all these diseases the processes of selecting and initiating appropriate actions are perturbed. On the opposite extreme, in drug addiction, in which pharmacological agents co-opt the reward-reinforcing nature of dopamine in the BG, behavioral repertoires become extremely limited, with an individual fixating on the actions necessary to obtain the addictive drug (Everitt and Robbins, 2005) (Hollander et al., 2010). Despite the importance of BG to normal and perturbed human behavior, how these evolutionarily conserved and phylogenetically old nuclei and circuits carry out action-selection functions is not fully understood.

The circuitry of the motor-related basal ganglia (BG) is generally described as a loop consisting of thalamic and cortical inputs into the dorsal striatum (dSTR), which signals via cascading inhibitory nuclei pathways to cortical projecting thalamic nuclei (DeLong, 1990). Additional outputs from BG to the colliculus and brain stem exist and
likely trigger orienting behaviors and locomotion. Projections to the STR from midbrain dopaminergic centers mediate reward reinforcement and possibly contribute to action initiation in a way not fully understood (Tritsch and Sabatini, 2012).

A dominant model of BG function is as followed: the major input stage of the BG, the dSTR, receives topographically organized glutamatergic drive from cortex (CTX) (Shepherd, 2013) (Hintiryan et al., 2016) and thalamus (TH) (Smith et al., 2014) and sends inhibitory GABAergic projections to downstream nuclei. These projections are dividing into direct and indirect pathways. The cells that give rise to these two striatal projection pathways are the direct and indirect striatal projection neurons (dSPNs and iSPNS, respectively), which are physically intermingled in the dSTR and difficult to distinguish based on intrinsic properties but express different molecular markers (Gerfen et al., 1990).

The dSPNs directly inhibit the substantia nigra pars reticulata (SNr) while the iSPNS inhibit the globus pallidus externus which in turn inhibits the SNr (Parent et al., 1984). The SNr in turn inhibits multiple nuclei involved in motor action such as the TH and the BS including superior colliculus and medial locomotor region. Thus, in terms of motor control, the SNr is thought of as the main output of the BG (Freeze et al., 2013).

This parallel circuit organization model, along with functional evidence that activation of dSPNs promotes movements, excites motor cortex via TH, and is reinforcing whereas activation of iSPNs inhibits movement, inhibits cortex, and is aversive has guided much of BG related research (Kravitz et al., 2010) (Kravitz et al., 2012) (Oldenburg and Sabatini, 2015). However, recent evidence suggests that this functional model of dSPNs and iSPNs requires refinement (Bariselli et al., 2018). Stimulation or
inhibition of the dSPNs and iSPNs, prior to initiating a level press or during an action sequence of level presses showed that both pathways are critical for proper movement (Tecuapetla et al., 2016) and dSPN and iSPN are co-active during movement (Cui et al., 2013) (Klaus et al., 2017) (Markowitz et al., 2018) (Parker et al., 2018). However, the contribution of each pathway specifically to action selection is still not well understood.

To this end we develop a behavioral task using head-restrained mice in which they were required to base their future (or “next”) action choice based on the knowledge of their previous action and the outcome resulting from that action. In our implementation, the action was licking to either one of two ports located to the left or right of the mouse head and the relevant action outcome was delivery or lack of delivery of a water reward through the selected port. The task structure separated motor preparation periods, flexible action choice, and potential reward delivery periods.

We show that the mice learn the task such that the action chosen during the previous trial and the presence or absence of reward, determines future action – thus an action-to-outcome association (AOA) is formed during this period and guides their future choice action. In order to separately examine the contribution of neural activity to action selection during each of the task periods, we designed software and hardware for rapid closed-loop optogenetic manipulations triggered off of specific task and behavioral parameters, such as the action-selection lick in rewarded trials. Optogenetic stimulation of dorsal lateral STR (dlSTR) dSPNs and iSPNs during the AOA period biases action choice made several seconds later. Similarly, stimulation during the delay period after the AOA had been formed but prior to reporting the choice caused the same bias. We now
seek to better understand STR role in this by recordings activity pattern in STR, CTX, and TH alongside inhibition experiments in the of STR. The work presented in this chapter was done in collaboration with the laboratory of Karel Svoboda at Janelia Farms Research Campus.

3.2 Results

3.2.1 A win→stay, lose→switch task with a delay epoch in head-restrained mice

The design of the behavioral task (Figure 3.1) was inspired by operant conditioning behavioral tasks. In such tasks, animals are required to make their “next” choice based on their “previous” actions and the corresponding outcomes of that action (Nonomura et al., 2018) (Tai et al., 2012). Here, mice were head-restrained and indicated one of two choices by symmetrically licking to one of two lick-ports (”lick left/lick right”) after hearing a “go” cue (Figure 3.1A) (Svoboda and Li, 2018) {Guo:2014dsa} {Guo:2014ds}. In this task, the computer selected one port as the “correct” (i.e. rewarded) port and maintained this designation before switching at random to the other port after 4 to 8 trials were rewarded. Importantly, the first lick after the go cue was used to select a port and water was only delivered after the end of this lick, requiring the animal to lick again to test if a reward had been delivered (and subsequently continue licking to collect reward, if present).

The optimal strategy in this task (assuming that mice cannot count – see below), is to implement win→repeat (WR) and lose→switch (LS) behaviors and to avoid win→switch (WS) and lose→stay (LS) behaviors (Figure 3.1B). That is, if mice received a reward at the last port they choose (i.e. a win) they should select that port again on the next trial whereas if they did not receive a reward (i.e. a loss), they should switch ports
on the next trial. Thus, knowledge of the identity of the last action (choosing the right or left port with the first lick) and the reward outcome (as sampled by the second and subsequent licks) uniquely determines the optimal next action choice (stay or switch) (Figure 3.1B).

Each trial can be considered to consists of several distinct periods (Fig 3.1D): 0, a previous action to report choice; 1, outcome evaluation epoch (3 sec) in which the mice either received or did not receive a reward (win or lose) in which there was a formation of an action-to-outcome association (AOA); 2, a random delay epoch (1 to 2 sec) in which mice were required to withhold their licks (i.e., an enforced non lick period – ENLP); 3, an auditory go cue (0.75 sec) which signaled the beginning of the potential response epoch; 4, an action choice in which the mouse reported their “next” choice with their first lick. Phase 4 of the “next” trial corresponds to phase 0 of the “previous” trial.

Data collected from 14 well trained mice over 107 sessions were used for the behavior analysis and compared to 27 sessions from 9 mice in early stages of training (Figure 3.1C, E). Early training was approximately the first 10 days after the start of water deprivation and after the mice had been habituated to the behavioral apparatus. Early in training and trained mice executed on average 193 and 197 trials per session, respectively, (Figure 3.1C). Both sets of mice executed correct strategies on a majority of trials, with, as by definition, trained mice doing so more frequently (early=61±(3.1)%, late=79±(2.3)%; (upper, lower) bound of 95% confidence interval around the median); P=<0.0001; Figure 3.1E). In early stages mice could execute a WR but not a LS strategy which they developed over time (log(%LS/%LR): early=-0.3±0.1; late=0.3±0.1; Figure 3.1E). Thus, mice learned quickly to choose the same port as their previous choice after
they received a reward but required further training to learn to behave in a flexibly and switch actions based on negative AOA. Trained mice suppressed licking until the response epoch (i.e. during the ENL) on a majority of the trials (% of ENL with penalties per session: early=55±(9,6)%, late=22±(6,4)%, P=<0.0001; Figure 3.1E). Reward delivery was triggered by tongue withdrawal from the rewarded port, requiring the mouse to lick a second time to determine if water had been delivered and then continue licking to collect the water reward. Indeed, mice typically licked 2-3 times on no-reward trials and 7-15 times on rewarded trials (Figure 3.1F-G). Thus, head-restrained mice can implement a win→stay, lose→switch strategy, report their choice using licks over a session (Figure 3.1J) and withhold licks in between trials while guiding future actions based on remembered the previous action-to-outcome associations.
Figure 3.1: A win→repeat, lose→switch task with a delay epoch in head-restrained mice

A, Schematic of a head-restrained mouse situated in a tube with two lick-ports positioned in front of its mouth. On each trial, the mouse must lick either the right or left port to indicate its choice and potentially receive a reward.

B, Schematic of task structure. Mice were required to choose their next action based on their knowledge of their previous action (lick left vs. lick right) and the outcome of that action (reward vs. no reward). *left*, The optimal or correct strategy to maximize reward delivery is lick again at a port on the next trial if it received a reward at that port on the previous trial (i.e. win→repeat) and to lick at the other port on the next trial if it failed to receive a reward on the previous trial (i.e. lose→switch). *right*, Win→switch and lose→repeat represent suboptimal or incorrect strategies.

C, Number of trials in each session early and late in training. Red line indicates the S.D. and the bar indicates the mean (early n=9/27 and late n=14/107; mice/sessions).

D, Schematic of the trial structure highlighting the relationship between the “previous” trial and “next” trial. During the delay period (the enforced no-lick period - ENL) mice were not allowed to lick.

E, Comparison of performance on the task early and late in training: *left*, Percentages of trials in which the animal carried out correct strategies (win→repeat and lose→switch). *middle*, Log of the ratio of percentages of lose→repeat to lose→switch. *right*, Percentages of trials with ENL penalties. In each, the red lines indicate the 95% C.I. and the bar the
Figure 3.1 (Continued)

median. Each circle represents one session. ****=P<0.0001; Mann-Whitney test (early: n=9/27; late: n=14/107; mice/sessions).

**F**, Median number of licks on rewarded (filled circles) and non-rewarded (empty circles) trials in trained mice (n=14/107; mice/sessions). The dashed red lines show the mean lick numbers for each trials class.

**G**, Schematic of 50 trials from one representative session late in training. The circle color indicates the computer “selected” correct port (blue=right, pink=left). Filled and open circles indicate if a reward was or was not delivered, respectively. The arrows indicate the directions of each mouse port choice (i.e. the direction of its first lick) and the bars indicate number of licks in each direction (capped at a maximum of 10 for display).
Figure 3.1 (Continued)

A

B

C

D

E

F

G

previous trial

next trial

<table>
<thead>
<tr>
<th>0) previous choice action</th>
<th>1) action outcome association</th>
<th>2) delay non-lick: 1-2 sec random</th>
<th>3) cue: 75 ms</th>
<th>4) response window (&lt;3sec) or the report of choice-action</th>
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![Diagram showing correct and incorrect strategies]

![Bar charts showing correct strategy (%) and ENL penalty (%) for early and late sessions]

![Scatter plot showing number of licks per session]

![Bar charts showing direction of licks (right, left) across sessions]

**Figure 3.1**: Diagram and statistical analysis of mouse behavior during a behavioral task. The figure shows the sequence of events from previous trial to next trial, highlighting the correct and incorrect strategies, latency, and choice-action outcomes. Bar charts and scatter plots illustrate the distribution of correct strategies, ENL penalties, and lick directions across sessions.
3.2.2 The current choice is guided by the previous action-to-outcome association

The task structure was designed to separate between the previous trial in which the AOA is formed from next action choice by a delay period (Figure 3.2A). Licking in the AOA started right after the choice lick and rewarded action evoked prolonged licking during the AOA period whereas licking tapered off earlier following a non-rewarded action (Figure 3.2B). Since, in trained mice, the AOA dictates the optimal and typical next action choice, in the WR condition the majority of licks during the AOA period also corresponded to the direction of next action choice whereas in the LS condition they were mostly to opposite side of the next choice (AOA licks in the direction of next choice: WR: 94%; LS: 29% Figure 3.2B). Thus, although the action outcome must be evaluated during this period and thus the AOA formed, the mouse does not yet adjust its motor action based on this new information gained. Mice were required to wait without licking after the AOA period in order to receive the “go” cue and report their next choice. However, they sometimes did not wait and incurred ENL penalties (Figure 3.1E). When these early licks did occur they were likely to be in the direction that the animal would end up choosing as its next action (ENL penalty in the direction chosen vs. not chosen for the next action: WR: 2.6± 0.2 vs 0.5±0.1 licks, P=<0.0001; LS: 2.1±0.2 vs 0.3±0.1 licks, P = <0.0001; Figure 3.2C). Thus, in the time between the last lick of AOA period and the start of the ENL period the motor plan for the next action was formed, and occasionally erroneously executed, based on the previous AOA. Furthermore, the response time (measured from the end of the cue to the first lick) in trials without early lick-penalties depended on the previous AOA and was slower for the LS action choices (response time in WR vs. LS: 104±(14,8)% vs. 140±(20,8) ms; P<0.0001; Figure 3.2D). This suggests that when mice
switched actions, they executed the action with less confidence compared to when they repeated an action. Additionally, for WR action choices, no difference was observed in the response times of trials with and without early lick penalties in the previous delay period (without or with early licks: 109±(19,14) and 108±(19,18) ms; Figure 3.2E), whereas LS action choices preceded by early licks had faster response times (without or with early licks: 148±(20,18) ms and 129±(21,17) ms; P<0.0001; Figure 3.2E) suggesting that the lick action itself may be reinforcing (Bariselli et al., 2018).
Figure 3.2: The current choice is guided by the previous action-to-outcome association


B, Quantification of lick time and direction during the AOA period: *Left*, Probability of licking in each 100 ms bin as a function of time during the AOA period in win→repeat (brown) and lose→switch (magenta) conditions. Solid and dashed lines correspond to density and cumulative distribution functions, respectively (WR: n=5116/5/37; LS: n=1142/5/37; n=trials/sessions/mice). *Right*, pie charts depicting the percentages of licks during the AOA in the WR (brown) and LS (magenta) conditions. Chosen and not chosen direction on the next trial are in black and green, respectively.

C, Number of ENL penalties during the delay period in the WR (brown) and LS (magenta) conditions that are in the direction of the next chosen (black) or not chosen (green) port. The red line indicates the 95% C.I. and the bar shows the mean. ****=P<0.0001. Wilcoxon test. (WR: n=1032/37/5; LS: n=294/37/5; n=trials/sessions/mice).

D, Response times (from the end of the cue until the first lick) for WR and LS trials (****=P<0.0001, Wilcoxon test). The red line indicates the 95% C.I. and the bar shows the median. Each circle represents a session. (n=107/14; sessions/mice).

E, Response time in the WR or LS condition split based on whether the mice withheld their licks during the delay period (black bars) or erroneously executed a choice during the delay period (white bars). ****=P<0.0001. Wilcoxon test. The red line indicates the
Figure 3.2 (Continued)

95% C.I. and the bar indicated the median. Each circle represents the average response time for each condition in each sessions (n=107/14; sessions/mice).
Figure 3.2 (Continued)

A

<table>
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<th>next trial</th>
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<td></td>
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<tr>
<td>action outcome</td>
<td>delay</td>
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<tr>
<td>association</td>
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| LS             |            |
| action outcome | delay      | change action |
| association    |            |

B

Time vs. P lick

- WR
- LS

P lick vs. time (s)

- chosen
- not chosen

C

Delay

- chosen
- not chosen

D

Action

Response time (ms)

- WR
- LS

E

Delay to action

Response time (ms)

- no ENLp
- ENLp

Significance levels:

- ****
- ****
- n.s.
- ***
3.2.3 Stimulation of the indirect and direct pathway in dorsal lateral STR during action-to-outcome association biases choice of future actions

It has been proposed that beneficial actions and their subsequent selection are reinforced in the STR (Bariselli et al., 2018). However, testing this possibility directly with optogenetics perturbations of STR has been challenging as it requires temporally and action specific manipulations. Instead, optogenetic manipulations have typically consisted of open-loop enhancement or suppression of activity without fidelity to the behavior at the time of stimulation. Indeed, very few experimental designed have been able to preserve the animal’s actions during perturbations of STR activity to be able to cleanly examine subsequent behavior (but see (Yttri and Dudman, 2016) for an example of temporally specific close-loop manipulations which had little impact on the “current” action). To perturb the STR selectively while the mice formed the AOA we triggered light delivery for optogenetic perturbations on the first lick towards a rewarded port, using low light powers (0.2mw to 7.5mw of power). Thus, the perturbation begins when the animal reports its action but before it evaluates the outcome of the action by collection reward (Figure 3.3A). The latency between the lick detection and low power laser activation was ~10 µs which ensured that: (1) the perturbation was triggered in parallel to reward consumption and (2) the previous choice and choice-reporting lick were minimally impacted but importantly the mice always collected the reward (data not shown). We separately analyzed experiments in which ChR2 was expressed specifically in dSPNs and iSPNs (Figure 3.3B-C) and for each compared the probability of expressing a WS action-choice when the stimulus was delivered ipsilateral or contralateral to the direction of the previously rewarded action (Figure 3.3). We found that ChR2 stimulation of dSPNs caused
contraversive bias on the next action choice, dramatically increasing the percentage of WS trials subsequent to the stimulation (WS without stimulation= 5±(2,3)\% and WS with stimulation= 70±30\%; LS=76±(19,14)\%; Figure 3.3D) when previously rewarded port and stimulation were on the same side. Little or no effects were seen for dSPN stimulation on the side contralateral to the previously rewarded actions (WS without stimulation= 4±(4,1)\% and WS with stimulation= 8±(4,8)\%; LS=83±(6,9)\%; Figure 3.3D). Converse effects were seen with ChR2 stimulation of iSPNs. Indeed little or no effects were seen for iSPN stimulation on the side ipsilateral to the previously rewarded action (WS without stimulation= 6±(2,3)\% and with stimulation= 9±(8,9)\%; LS=73±(7,6)\%; Figure 3.3E). However, when the previously rewarded port and stimulation were on opposite sides ipsiversive bias was observed on the next action choice by raising WS action selections (WS without stimulation= 7±(3,2)\% and 45±(26,12)\% with stimulation; LS=63±(3,8)\%; Figure 3.3E).
Figure 3.3: Stimulation of the indirect and direct pathways in dorsal lateral STR during the AOA period biases future action choice.

A, Schematic of task structure to highlight the optogenetic perturbation delivered after the mice reported their action but before they evaluated the action by collected reward.

B, Schematic of the experimental design for dSPN stimulation showing a coronal section at +0.9 mm from a Drd1a-Cre mouse with injections of Chr2 in the STR (region of injection is highlighted in orange in this and in all subsequent figures) followed 1 month later by implantation of a tapered fiber optic.

C, Schematic of the experimental design for iSPN stimulation showing a coronal section at +0.9 mm from a Adora2a-Cre; Ai32 mouse with implantation of a tapered fiber optic.

D, (Left) Percentage of actions chosen by a mice on trials in which the computer had selected the rewarded port on the previous trial to be that ipsilateral (left) or contralateral (right) side of unilateral optogenetic stimulation of dSPNs. Data for win→switch (incorrect strategy) without (white bar) and with (blue bar) optogenetic stimulation and for lose→switch (correct strategy). As the perturbation was delivered only on rewarded trials, there are no lose trials with stimulation. Thus, unilateral stimulation of dSPNs during reward collection after a movement ipsiversive to the stimulation side promotes action choice switching. The red line indicates the 95% C.I. and the bar shows the median. Each circle represents a session and only sessions with more than 8 stimulated trials were used (n =15/3; sessions/animals).
Figure 3.3 (Continued)

E, As in panel D but for optogenetic perturbations of iSPNs in dlSTR (n =15/2; sessions/animals). In this case, unilateral stimulation of iSPNs during reward collection after a movement contraversive to the stimulation side promotes action choice switching.
Figure 3.3 (Continued)

A

previous trial | next trial

| choice | rewarded A-O | delay | action |

B

creOn-ChR2

D1-cre +0.9

C

A2A-ChR2

+0.9

D

rewarded direction:
ipsilateral to stimulation

rewarded direction:
contralateral to stimulation

D1-cre +0.9

E

iSPN-ChR2

WS | LS | WS.stim

WS | LS | WS.stim

WS | LS | WS.stim

WS | LS | WS.stim

WS | LS | WS.stim
3.2.4 Stimulation of the indirect and direct pathway during the delay period biases choice

The region of dorsal lateral STR that was targeted for stimulation during the AOA period receives inputs from layer 5 of the anterior lateral motor cortex (ALM) (Hooks et al., 2018). Preparatory activity for licking direction appears in and remains localized to ALM until movement onset, including in layer 5 neurons (Svoboda and Li, 2018) (Economo et al., 2018). In addition, unilateral inhibition of ALM causes ipsiversive choice bias in future licking direction (Svoboda and Li, 2018) {Guo:2014ds}. We virally expressed ChR2 in upper and lower layer 5 projection neurons in ALM (Gerfen et al., 2013) in Tg(Rbp4-cre)KL100Gsat mice (in short, Rbp4-Cre+/-) and found that we could induce EPSCs in majority of SPNs in the axonal target field (data not shown). Guided by these finding, we asked if ChR2 stimulation in this region in dlSTR during the delay period prior to choice, and after the mice formed a rewarded AOA on the previous trial, could bias choice on the next trial (Figure 3.4A). Indeed, Chr2 stimulation during the delay period of dSPNs and iSPNs caused a contraversive and ipsiversive bias, respectively, on the next action choice in a similar fashion to Chr2 stimulation during the AOA period. For dSPN stimulation when previously rewarded port and stimulation were on the same side: WS without stimulation= 5±(5,2)% and WS with stimulation= 73±(18,7)%; LS=75±(6,16)%; Figure 3.4B) and when stimulation was on the opposing side: WS without stimulation= 8±(4,2)% and WS with stimulation= 0±0%; LS=61±(7,11)%; Figure 3.4B). For iSPN stimulation when previously rewarded port and stimulation were on the same side: WS without stimulation= 5±(6,2)% and WS with stimulation= 0±0%; LS=67±(8,3)%; Figure 3.4C) and when stimulation was on the opposing side: WS without
stimulation = 7±(4,4)% and WS with stimulation = 60±(11,27)%; LS=68±(17,3)%; Figure 3.4C).
Figure 3.4: Stimulation of the indirect and direct pathway in dorsal lateral STR during the delay period biases future action choice.

A, Schematic of task structure highlighting light delivery for optogenetic perturbations after the AOA period and during a delay period prior to mice reporting their next action choice.

B, (Left) Percentage of actions chosen by a mice on trials in which the computer had selected the rewarded port on the previous trial to be that ipsilateral (left) or contralateral (right) side of unilateral optogenetic stimulation. Data for win→switch (incorrect strategy) without (white bar) and with (blue bar) optogenetic stimulation and for lose→switch (correct strategy). As the perturbation was delivered only after rewarded trials, there are no lose trials preceding stimulation. Thus, unilateral stimulation of dSPNs during the delay period while the mice prepare a movement ipsiversive to the stimulation of dSPNs promotes action choice switching. The red line indicates the 95% C.I. and the bar shows the median. Each circle represents a session and only sessions with more than 8 stimulated trials were used (n =21/3; sessions/animals).

C, Similar to B but for stimulation in iSPNs. (n=19/5; sessions/animals).
Figure 3.4 (Continued)

A

###previous trial###
- WiSt
- action outcome
- association (rewarded)

###next trial###
- delay
- action

B

**ipsi target direction**
- $D1$-cre execution (%)

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**contra target direction**
- $D1$-cre execution (%)

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C

**A2A-ChR2 execution (%)**

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3.3 Bibliography


cocaine intake through CREB signalling. Nature 466, 197–202. doi:10.1038/nature09202


Appendix A: Dynamic illumination of spatially restricted or large brain volumes via a single tapered optical fiber

A1 Summary

Optogenetics promises spatiotemporal precise control of neural processes using light. However, the spatial extent of illumination within the brain is difficult to control and cannot be adjusted using standard fiber optics. We demonstrate that optical fibers with tapered tips can be used to illuminate either spatially restricted or large brain volumes. Remotely adjusting the light input angle to the fiber varies the light-emitting portion of the taper over several millimeters without movement of the implant. We use this mode to activate dorsal versus ventral striatum of individual mice and reveal different effects of each manipulation on motor behavior. Conversely, injecting light over the full numerical aperture of the fiber results in light emission from the entire taper surface, achieving broader and more efficient optogenetic activation of neurons when compared to the standard flat-faced fiber stimulation. Thus, tapered fibers permit focal or broad illumination that can be precisely and dynamically matched to experimental needs. The work presented in this chapter was done in collaboration with the laboratory of Massimo De Vittorio and Ferruccio Pisanello at the Italian Institute of Technology.

A2 Introduction

Optogenetic modulation of neuronal activity has become the dominant method of examining the behavioral consequences of activity in specific neuronal populations in vivo. This is due to the synergy of advances in two distinct but well-connected fields: the development of ever-improving light-activated modulators of electrical activity
technologies to deliver light within the brains of free-moving animals (Lee et al., 2015) (Canales et al., 2015) (Jeong et al., 2015) (Kim et al., 2013; Kwon et al., 2015). Nevertheless, attaining the full potential of optical neural control requires new technologies to better control the spatial extent of light delivery and more precisely match illumination to heterogeneous brain structures.

In certain applications, it is necessary to deliver uniform illumination to large brain areas, whereas for others confined illumination of small brain volumes is preferred. Ideally, both modes of illumination could be accomplished via a single, reconfigurable device. To this aim several approaches have been developed, including multiple implanted waveguides (Kwon et al., 2015) (Zorzos et al., 2012) multiple micro light delivery devices (µLEDs) (Jeong et al., 2015) (Kim et al., 2013) (McAlinden et al., 2015) (McCall et al., 2013), holographic illumination via head-mounted objectives (Szabo et al., 2014) and multi-point emitting optical fibers (MPFs) (F. Pisanello et al., 2014) (M. Pisanello et al., 2015). However, these techniques have limitations: implanting multiple waveguides is highly invasive, µLEDs can heat tissue during prolonged illumination, and MPFs require higher input laser power to produce viable optogenetic control. Likely due to the difficulty and cost of building the required devices, these approaches have not been broadly utilized in neuroscience labs. Indeed, the most common light delivery method for optogenetic experiments remains flat-faced optical fibers (FFs), which deliver highly spatially heterogeneous illumination to a relatively small and fixed brain volume near the fiber facet. Furthermore, due to the relatively large and flat area of the cleaved end, these fibers can cause substantial tissue damage during insertion.
Here we describe a tapered optical fiber (TF) whose emission properties can be simply and dynamically reconfigured to switch between relatively homogenous light delivery to a large volume and spatially restricted illumination. Multiple wavelengths of light can be independently modulated and directed to sub-volumes of interest. The device consists of a single, thin and sharp waveguide, thus minimizing invasiveness. To demonstrate the suitability of this approach for more uniform and efficient illumination of extended brain structures, experiments were performed in the primary motor cortex and striatum of awake head-restrained and freely moving mice. We demonstrate that by controlling the angle at which light is injected into the fiber, TFs can emit light from sub-portions of the taper to produce spatiotemporally resolved light patterns that subsample the volume of interest. We use this approach to achieve site specific optogenetic stimulation and demonstrate that activation of indirect pathway striatal projection neurons (iSPNs) in dorsal vs. ventral striatum has different effects on locomotion in freely moving mice while they explore an open arena. Thus TFs provide a simple, inexpensive, and easy to operate multipurpose system for optical control of neural activity.

A3 Results

A3.1 Design principles of tapered optical fibers

TFs are multimode fiber optics that have been engineered to taper gradually from their full width (125-225 µm) to ~500 nm. The taper angle is small (2°<ψ<8°) such that the taper length varies between 1.5-5.6 mm (Fig. A1). This design was chosen to permit smooth insertion into the brain, reduce the implant cross-section, and expose a large area of the fiber for potential light emission. Ray-tracing and geometric models demonstrate the working principle of the device (Fig. A1a-b). A ray injected into the core of the fiber
with an input angle \( \theta \) is guided via total internal reflection (TIR) to the tapered region. At each reflection of the ray its propagation angle with respect to the fiber optical axis increases by an amount equal to the taper angle \( \psi \) (Fig. S9). This occurs until a critical section is met, at which TIR is lost and the ray radiates into the surrounding medium. Increasing \( \theta \) increases the distance between this light-emission point and the taper tip (Fig. A1b) in a manner determined by the fiber numerical aperture (NA) and taper angle (Fig. S10 for optical fibers with NA 0.22 and 0.39 and \( \psi=2.2^\circ \) and \( \psi=2.9^\circ \), respectively). Therefore, the light input angle \( \theta \) selects the output zone along the length of a specific taper.

A consequence of the dependence of the position of light emission on \( \theta \) is that TFs can be used to emit light along the fiber in two fundamentally different ways. First, when light is injected into the TF using its full NA, light is emitted from a broad extent of the taper (Fig. A1c), as desired for illumination of spatially extended brain regions – e.g., the entire cortical thickness or the dorsal-ventral axis of the striatum. For a fiber of a particular NA, the length of the light-emitting segment \( (L) \) depends mainly on the taper angle. Ray tracing simulations indicate that \( L \) can be tailored from a few hundred micrometers up to a few millimeters in the case of 0.22 and 0.39 NA fibers with taper angles \( \psi \) ranging from 2.2\(^\circ\) to 7.4\(^\circ\) (Fig. S11).

We experimentally verified this effect in 0.22 and 0.39 NA fibers by immersing TFs in a fluorescein solution and imaging the resulting fluorescence distribution (Fig. S12). Decreasing the taper angle increases the length of the emitting segment to \( \sim1 \) mm for 0.22 NA 50/125 \( \mu \)m core/cladding fibers and to \( \sim2 \) mm for 0.39 NA 200/225 \( \mu \)m fibers (Fig. A1d). Thus, in this light-injection mode, a TF with the proper NA and taper angle can
be chosen to match the linear extent of light output to the size of many mouse brain structures.

To compare the ray-tracing model with experimental results, we evaluated the emission length, referred to as \( L_{0.5} \), over which the delivered intensity is more than 50% of its peak (Fig. S12). We found good agreement between modeling and experiments for both 0.39 NA and 0.22 NA fibers (Fig. A1d and Fig. S12). The differences observed at low \( \psi \) for 0.39 NA fibers arise because the taper is assumed to be linear in the ray-tracing model, whereas the real structure has a modest parabolic shape (Fig. S13). The diameter at which light starts to outcouple and the total light power delivered are nearly independent of the taper angle (Figs. S14). As a consequence, TFs with lower taper angles spread the available power over a larger taper surface (Fig. S15 and Fig. S16), potentially allowing light delivery to elongated brain regions.
Figure A1. Emission properties of TFs a, Schematic representation of a typical TF geometry (NA=0.39, taper angle $\psi=2.9^\circ$, taper length 4.4 mm, core/cladding diameters 200/225 $\mu$m).

b, Ray tracing simulations of emission from the taper tip resulting from injecting a single ray in the fiber at different angles.

c, Ray distributions resulting from injecting light using the full NA of the fiber.

d, top, Image of fluorescence generated by light emitted from tapered fibers with the specified geometries immersed in a fluorescein solution. bottom, The graphs depict calculated (black) and measured (red) emission lengths (evaluated as the full width at half maximum $L_{0.5}$) for 0.22 NA and 0.39 NA TFs as a function of the taper angle. 0.22 NA TFs have core/cladding diameters of 50/125 $\mu$m, whereas 0.39 NA TFs have core/cladding diameters of 200/225 $\mu$m.
A3.2 Illumination of large brain volumes with TFs

Flat cleaved optical fibers (FFs) are commonly inserted just above a brain volume of interest and the delivered light is strongly attenuated by the tissue, allowing excitation of neurons located only up to a few hundred micrometers from the fiber end (Aravanis et al., 2007) (Yizhar et al., 2011) (Stujenske et al., 2015). Significant excitation of more distant neurons requires large increases in laser power to overcome the approximately exponential decay in power density from the fiber face. In contrast, by virtue of their thin and sharp edge, TFs can be inserted into the volume of interest and light delivered along the length of the taper (Fig. A2). To evaluate the illumination pattern achieved in light-absorbing and scattering brain tissue, we implanted TFs and FFs into fluorescein-impregnated fixed mouse brain slices and imaged the fluorescence generated by light emitted from the fiber (Fig. A2a). As expected, FFs illuminate a small brain volume and fluorescence is strongly attenuated after a few hundred micrometers from the emitting flat-end facet (Fig. A2b). In contrast, TFs emit light along the taper length, resulting in elongated and more homogenous illumination of the tissue (Fig. A2c - d).

The differences in tissue illumination achieved by TF and FF arise from two important differences: (1) TFs emit light from a larger surface, i.e. the cone defined by $L$ and $\psi$; (2) Light emerges from the TFs at a non-zero angle with respect to the taper axis (Fig. A1c). As a consequence, tissue absorption and scattering do not determine light distribution along the fiber axis as in FFs, but along the direction of emitted light, which has a significant component perpendicular to the taper axis. Notably, the depth of the excited volume can be tailored by selecting the fiber taper geometry and NA, instead of by increasing the laser power as commonly done in experiments with FFs. For instance,
TFs with 0.22 NA and $\psi=2.2^\circ$ illuminate the whole cortical depth, whereas TFs with 0.39 NA and $\psi=2.9^\circ$ target most of the depth of the striatum (Fig. A2c-d).
Figure A2. Emission properties of TFs in brain slices

a, Schematic of light delivery in brain tissue through FFs and TFs.

b, left, Image of fluorescence induced by light emission from an FF implanted into cortex in a fluorescein impregnated brain slice. Gray scale represents fluorescence intensity in arbitrary units of a linear scale. right, Normalized fluorescence intensity profile in the tissue starting from the fiber end face.

c and d, Bright field images (left) to identify the position of the TFs in the fluorescein impregnated cortical or striatal brain slice used for acquisition of the fluorescence images (middle). Gray scale is the same as in (b). right, Normalized profiles of fluorescence intensities beside the taper, starting from the first emission point.
A3.3 \textit{In vivo} examination of effective excitation in striatum, a large brain structure

To characterize the efficacy of TFs for \textit{in vivo} optogenetics, we compared the ability of TFs and FFs to activate ChR2-expressing cells in striatum. Either a TF (0.39 NA and $\psi=2.9^\circ$) or a FF (0.22 NA or 0.39 NA) was implanted in the striatum of adult transgenic mice (\textit{Ador2a-Cre; Ai32}) expressing ChR2 in indirect striatal projection neurons (iSPNs) (Fig. A3). This mouse was selected for analysis because iSPNs are GABAergic neurons that locally inhibit other striatal neurons and inhibit recurrent excitatory inputs into striatum, minimizing secondary activation of cells not expressing ChR2 (Oldenburg and Sabatini, 2015). The TF was implanted at a depth of 3.7 mm and the FF at 2.3 mm, respectively. Light (473 nm, 1 mW outputted at fiber exit) was delivered at a 30 sec on/30 sec off cycle for 1 hour to awake animals in their home cages. To compare the spatial distribution of cells activated by light delivered through TFs and FFs, animals were euthanized 2 hours post stimulation and we performed fluorescence immunohistochemistry for c-fos, the protein product of an immediate early gene whose expression is regulated by neuronal activity (Greenberg et al., 1986). C-fos was induced more uniformly across the $\sim$2 mm dorsal-ventral extent of the striatum in TF- compared to FF-implanted animals (Fig. A3a-d). Furthermore, although light delivery through the TF stimulated cells throughout the dorsal-ventral axis (i.e. along the axis of the fiber), differential placement of the fiber permitted selective stimulation of either lateral or medial sub-regions of striatum (Fig. A3b). Thus, as suggested by the simulations and fluorescence excitation \textit{ex vivo} (Figs. A1-2), TFs deliver light \textit{in vivo} across a spatially extended volume of tissue surrounding the thin fiber. Minimal c-fos was induced in animals that expressed ChR2 in iSPNs but were not stimulated and in wild type animals.
without ChR2 that did receive light stimulation (Fig. S17). Lastly, a clear advantage of TFs, likely resulting from their sub-micron sized tips, was the diminished tissue damage and activation of glia and microglia compared to FFs (Fig S18).
Figure A3. *In vivo* examination of effective excitation in striatum

**a,** Schematic of experimental preparation showing a TF inserted into the striatum of a mouse expressing ChR2 in indirect pathway SPNs (iSPNs).

**b,** c-fos expression (red) in the striatum (coronal section, 0.85 mm anterior to Bregma) of an animal expressing ChR2-YFP in iSPNs (green) after light stimulation delivered by a TF in lateral (*left*) or medial (*right*) striatum. DAPI is shown in blue. Representative image of two independent replicates.

**c,** Schematic of experimental preparation as in (a) showing placement of a flat-faced fiber (FF).

**d,** As in (b) showing c-fos induction by light delivery from a FF in dorsal (*left*, 0.22 NA) or ventral (*right*, 0.39 NA) striatum. Representative image of two independent replicates.
A3.4 Optogenetic control of motor cortex with TFs

In order to examine the potential benefits of more uniform light delivery in vivo, TFs were tested in the primary motor cortex of awake head-restrained VGAT-ChR2 BAC adult mice (Zhao et al., 2011) which express ChR2 in GABAergic neurons (Fig. A4a). A TF (NA 0.22, ψ~2.2°) and FF were implanted serially near a 16-contact silicon multi-electrode array (Neuronexus). The FF was placed such that the face was at the same depth as the first emission point (shallow position) or the tip (deep position) of the TF. Light (473 nm) was delivered as 5 x 50 ms pulses at 5 Hz, repeated every 3s for 80 times. Output powers from the TF and FF were matched in order to examine the efficiency of each to inhibit cortex via stimulation of GABAergic interneurons. TFs more effectively suppressed cortical activity at lower power levels (Figs. A4b, S19): Inhibition with TFs was obtained at ~10 µW (~0.20 mW/mm², see Fig. S18 for estimated power density distribution along the taper) whereas FFs (NA=0.22) required ~5-fold higher powers to obtain a comparable effect at both depth (50 µW; ~25 mW/mm², optogenetics light depth calculator). Moreover, even at higher powers, inhibition is more pronounced with TFs, suggesting that tapered fibers stimulate a higher number of ChR2-expressing GABAergic neurons.
Figure A4. Optogenetic manipulation of motor cortex with TFs

a, Schematic of the experimental preparation. A 16 channel multi-electrode array is placed in primary motor cortex of a VGAT-ChR2 BAC transgenic mouse and maintained throughout the experiment. Units are recorded throughout cortical layers. A TF or FF in either a shallow or deep position is placed in cortex. The firing rates of individual units are compared during the basal period or during 50 ms optogenetic excitation cortical GABAergic interneurons.

b, Average normalized firing rates (black line with shaded area showing SEM) across cells with and without light (top) and pseudo-colored representations of normalized across-trial average firing rate of each unit as a function of time (bottom). The color scale (“Rainbow” in Igor Pro) indicates relative firing rates of each cell (0=dark blue to 3=red) normalized to its baseline (0-50 ms). The period of light delivery (50 ms) is shown in the cyan shaded regions. Data (n-values 45, 49, 51, 51, 28, 28, 28, 28, 48, 49, 49, and 49 cells) are shown for each fiber configuration (left: TF, middle: FF shallow, right: FF deep) and at 4 power levels (10, 50, 100, and 200 μW total emission from the fiber before implantation in the brain.)
A3.5 Dynamical selection of illuminated brain regions

A further benefit provided by TFs is the ability to dynamically control the illumination volume by changing the light entry angle $\theta$ at the input end of the fiber. The angle $\theta$ defines the subset of guided modes injected into the fiber (F. Pisanello et al., 2014) (M. Pisanello et al., 2015) which in turn determines the cross-section of the taper at which light emits. The position of the emitting section along the taper depends on $\theta$, as expected from the ray tracing simulations (Fig. S20). In particular, with low input angles light out-couples close to the taper tip, whereas light injected at high input angles is mainly emitted at sections farther from the tip.

To characterize the geometrical emission properties of TFs as a function of $\theta$ (Fig. A5) we implemented a simple optical path in which $\theta$ is changed by translating a mirror (Fig. S21a). Monitoring the fluorescence generated by a TF inserted into a fluorescein solution shows that the emitting segment (~300 µm long) can be moved almost continuously along ~1 mm or ~1.5 mm, respectively, in 0.22 NA/ψ=2.2° or 0.39 NA/ψ=2.9° TFs (Fig. A5a,b). Importantly, total delivered light power is nearly independent of $\theta$, apart for input angles very close to the maximum acceptance angle (Fig. A5c). To rapidly scan the illumination across brain volumes we used a launching system with a scanning galvanometer and relay optics to change $\theta$ (Fig. S21b). This permits rapid switching between different emission segments and near continuous movement of the emitting segment along the taper. Several launching paths can be combined to outcouple multiple wavelengths at the same time from independently addressable emission segments (Fig. S21).
To examine the suitability of this technique for restricted light delivery in brain tissue, site-selective light delivery as a function of $\theta$ was evaluated in fluorescein-stained acute mouse brain slices. Both 0.22 NA/$\psi$ = 2.2° and 0.39 NA/$\psi$ = 2.9° TFs allowed near-continuous tuning of the illuminated brain region in both cortex and striatum (Fig. A5d-g). Tissue absorption and scattering shorten the propagation of emitted light into the tissue, further constraining the spatial geometry of the illuminated area. This leads to spatially separated light delivery volumes, resulting in an easy-to-use and versatile method to direct the light stimulus along a ~2 mm segment by implanting a single fixed fiber.
Figure A5. Site selective light delivery with TFs

a, Light delivery geometry for several values of light injection angle \( \theta \) with a 0.22 NA/\( \psi \)=2.2° TF into a fluorescein solution. Gray scale represents fluorescence intensity in arbitrary units and is the same for all panels.

b, Light delivery geometry for several values of light injection angle \( \theta \) with a 0.39 NA/\( \psi \)=2.9° TF into a fluorescein solution.

c, Total Output power for a fixed input of 2.25 mW for TFs with 0.22 NA/\( \psi \)=2.2° TF (red line) and 0.39 NA/\( \psi \)=2.9° TF (black line)

d, Site selective light delivery with a 0.22 NA/\( \psi \)=2.2° TF implanted into the cortical region of a fluorescently stained mouse brain slice.

e, Normalized fluorescence intensity profiles, measured beside the taper, from the fluorescence images in (d).

f, Site selective light delivery with a 0.39NA/ \( \psi \)=2.9° TF implanted into the striatum of a fluorescently stained mouse brain slice.

g, Normalized fluorescence intensity profiles, measured beside the taper, from the fluorescence images in (f).
A3.6 In vivo multi-site stimulation

Selection of the emitting region of the taper is possible because different input angles generate different sets of guided modes in the fiber (M. Pisanello et al., 2015) which are out-coupled at different taper sections. However, while propagating into the fiber, subsets of guided modes may undergo modal mixing induced by fiber impurities and bends. This could redistribute part of the guided light power to other modes and rearrange light emission along the taper. To evaluate the viability of using TFs for site-selective light delivery in moving mice, we measured the effects of bending and shaking on TF light output (Fig. S22). For a fixed input angle (θ=17°), the patch fiber carrying light to a TF with 0.39 NA/ψ = 2.9° was shaken and bent while emission into a fluorescein droplet was recorded at high frame rate (~100 fps). The fluctuations in fluorescence peak intensity, full-width at half maximum, and center were each less than 5% with fiber shaking and bending (Fig. S22).

To demonstrate the feasibility of multi-site optogenetic stimulation through a TF in an individual animal, TFs were designed (0.39 NA, ψ =2.3°) and implanted spanning the dorsal and ventral medial striatum of adult mice expressing ChR2 in iSPNs (Ador2a-Cre; Ai32). An optical pathway was designed (Fig. A6a) and calibrated to deliver a similar power density from the distal (ventral striatum) and proximal (dorsal striatum) sites (Fig. A6c) using, respectively, 8° (θ₁) and 22.5° (θ₂) launch angles (Fig. A6a; Fig. S23).

Eight days after implant surgery, mouse spontaneous locomotion was monitored in an open circular arena with depth time-of-flight cameras (Wiltschko et al., 2015). Light was delivered to the brain via an optical commutator, a lightweight patch cord (200 µm
core, 0.39 NA, 1 meter long), and two fiber-fiber conjunctions as typically used for unrestrained mouse behavior experiments (Fig. A6a-b). The experimental paradigm consisted of 3 min blocks of either no stimulation (ns), or laser input to the fiber at angle 1 ($\theta_1$) or angle 2 ($\theta_2$) repeated in the following pattern: ns-$\theta_1$-ns-$\theta_2$-ns-$\theta_1$-ns-$\theta_2$-ns, corresponding to alternating stimulation of ventral ($\theta_1$) and dorsal ($\theta_2$) striatum separated by periods of no stimulation (Fig. A6c-d). On the subsequent day the order of ventral ($\theta_1$) and dorsal ($\theta_2$) stimulation were reversed such that 9 blocks per session were recorded in each of 2 days. Basic analyses of locomotion speed and orientation (Fig. A6e) reveal that stimulation at either dorsal or ventral striatum reduced locomotion and triggered contraversive spinning in individual mice (Fig. A6f), as expected for unilateral activation of iSPNs (Kravitz et al., 2010). Ventral stimulation via light injection at angle $\theta_2$ inducing more profound effects (distance traversed/3 min: ns: 3.16±1.45m, n=10 3 min blocks over 2 imaging days for 1 mouse; ventral ($\theta_1$): 0.41±0.27, n=4/2/1 sessions/days/mouse; dorsal ($\theta_2$): 1.18±0.18, n=4/2/1 sessions/days/mouse; $\theta_1$ vs. $\theta_2$: p=0.0286. $\theta_1$ vs. ns: p = 0.002; $\theta_2$ vs. ns: p =0.002 (Fig. A6g).
Figure A6 Selective light delivery with TFs in the open field

a, Schematic of optical setup. The output of a polarized laser was passed through a $\frac{1}{2}$-wave plate (hwp) to rotate the polarization before entering a polarizing beam cube (pbc) that transmits and reflects, respectively, horizontally and vertically polarized light. Rotation of the hwp determines the fraction of laser light entering each path. Each of the laser paths is directed to a 2-inch collection lens via a sliding mirror (sm1 and sm2) that can be moved linearly to determine the launch angle into the first fiber-optic patch cord (fopc1).

b, Fiber optic patch cable (fopc1) was connected from the optical pathway shown in (a) to a commercial optical commutator from which a second fiber optic patch cable (fopc2) led to the TF implanted in the animal. A camera above the arena monitored the location and depth of the mouse.

c, The TF was implanted in the striatum (0.85A, 1.4L, 4.1D) of a transgenic animal expressing ChR2-YFP in iSPNs to allow for stimulation of two regions of striatum. Light input at $\theta_1$ (magenta) and $\theta_2$ (orange) angles resulted in emission that targeted the ventral and dorsal striatum, respectively.

d, Example of stimulation paradigm in the open field arena on day 1. Animals spent a total of 27 min in the open field with 3 min sessions of either no light, light on at angle $\theta_1$, or light on angle at $\theta_2$. On a subsequent day of analysis the order of $\theta_1$ and $\theta_2$ stimulation were reversed.

e, Snapshot of a mouse in the open field with an overlaid vector highlighting the simple feature extraction of position and orientation.
Figure A6 (Continued)

f, Example of the positions of one mouse during 3-minute session of no stimulation (left), ventral stimulation ($\theta_1$, middle), and dorsal stimulation ($\theta_2$, right).

g, Quantification of distance traveled in the 3 conditions for the example mouse shown in (f). Significant differences were observed between the no stimulation (left), ventral stimulation (middle) and the dorsal stimulation (right) conditions. Bars indicate the means of all data points from individual 3 min blocks, which are shown by the circles (not filled: day 1; filled: day 2). *$p = 0.028$, **$p=0.002$, using a two-tailed Mann-Whitney U test.
To understand if dorsal vs. ventral stimulation had qualitatively different behavioral consequences, mouse locomotion and posture (Fig. A7a) were analyzed using a machine learning approach that automatically detects repeated time varying “syllables” corresponding to the animal’s postural dynamics (Wiltschko et al., 2015). This technique produces a hidden Markov model in which each state encapsulates the postural dynamics of the mouse in each expressed behavioral syllable. The model is built using 3-dimensional information derived from video collected at 30 Hz for all mice across all imaging sessions and stimulation conditions. In this case, 14 syllables were sufficient to explain on ~94% of locomotion behavior in all sessions (ns: 87%; ventral (θ1): 98%; dorsal (θ2): 97%) (Fig. A7b).

Consistent with the previously described effects of iSPN stimulation on locomotion (Kravitz et al., 2010) and those revealed by simple video tracking (Fig. A6) the most strongly induced behavior during stimulation was “pausing” (% of time spent in syllables 1 and 2: ns: 1%; ventral (θ1): 74%; dorsal (θ2): 38%) corresponding to a motionless mouse (Fig. A7b). The expression of these pause syllables varied across conditions and was most prominent during ventral stimulation (Fig. A7b).

Movement related syllables (syllables 3-14) were also differentially expressed across stimulation condition (% of time spent in syllables 3-14: ns: 86%; ventral (θ1): 24%; dorsal (θ2): 59%) (Fig. A7b). Furthermore, specific movement-related syllables were more strongly induced by dorsal (e.g. 4,5,6) stimulation whereas others showed the converse pattern (e.g. 3,7) (Fig. A7c). Thus, these results are not consistent with ventral stimulation simply being more effective as discrete motor actions are evoked by stimulation at each site. Each differentially modulated syllable represents a consistent but distinct motor
action: syllable 6, more strongly evoked by dorsal stimulation, involves the animal shaking its body left and right, whereas syllable 7 is a spin to the left (head moves left while tail moves right) (Fig. A7d). Importantly, the head and tail trajectories are similar during epochs of stimulation of the dorsal vs. ventral striatum despite their differences in expression frequency (Fig. A7c-d).
**Figure A7. Mapping sub-second structure of behavior during optogenetic manipulation of ventral or dorsal striatum**

**a,** Snapshot of a mouse in the open field with the height of the body at each pixel indicated by the color scale.

**b,** The percent total behavior data (same mouse as in Figure 6) explained in each condition (ns, θ₁, and θ₂ stimulation) by the major pause related *(black)* syllables (1 and 2), the major movement-related *(green)* syllables (3-14) and all other syllables *(white)*.

**c,** Percent expression of the dominant non-pause, i.e. movement related, syllables (3-14), showing differential expression of syllables across stimulation conditions.

**d,** The trajectories of the head and tail of the mouse relative to its body center for syllable 6 *(left)* and syllable 7 *(right)* in either the ventral (θ₁, *magenta*) and dorsal (θ₂, *orange*) stimulation conditions. The black dots depict the starting point of each of the aligned trajectories of the head and tail. All (limited to 100) instances of the trajectories of the head and tail relative to its body center are shown.
A4 Discussion

We demonstrate that TFs have several advantages for light delivery in the brain that might allow this tool to replace the flat-cleaved optical fibers that are typically used for optogenetic experiments. First, TFs are multipurpose such that the same device allows either large-volume or site-selective light delivery. Second, by virtue of their tapered and smaller average cross section, TFs are minimally invasive and can be implanted directly into the brain region of interest. Third, they are simple to operate and compatible with optical equipment commonly present in neuroscience labs employing optogenetics.

A4.1 Illumination of large brain volumes

When used to deliver light from the entire taper surface, TFs overcome one of the principal difficulties facing experiments in in vivo optogenetics – achieving uniform effective illumination of large brain structures with minimal invasiveness and light power. Although effective stimulation of neuron cell bodies expressing ChR2 can be obtained with power densities in the range 1-5 mW/mm² (Boyden et al., 2005) (Zhang et al., 2007) (and sometimes <1 mW/mm², depending on the sensitivity of the ChR2-expressing cells) (Klapoetke et al., 2014), many published studies utilize orders of magnitude higher power (Aravanis et al., 2007) (Cardin et al., 2010) (Warden et al., 2014) (Eshel et al., 2015). This likely arises because the FF is often positioned above the brain nucleus of interest to minimize damage. Due to the exponential fall off in power density from the FF, this necessitates the use of high light powers in order to stimulate cells throughout the nucleus (Aravanis et al., 2007) (Yizhar et al., 2011) (Al-Juboori et al., 2013) and can result in local tissue heating in the range of several degrees (Stujenske et al., 2015) by both permitting
insertion of the fiber into the nucleus of interest and by delivering light along the linear extent of the taper, TFs achieve efficient light stimulation. This feature may be even more important when considering inhibitory and spectrally shifted opsins that may require more light to achieve effective neuronal perturbation.

Large brain area illumination with TFs is easily achieved with standard fiber-coupled lasers or LEDs, including with inexpensive LED-based commercial fiber launch systems (Fig. S23) that naturally inject light over a large NA and produce emission over the fiber face. Furthermore, the approach is viable for chronic use in freely moving tethered mice and for acute insertion in head-fixed mice.

**A4.2 Spatially restricted and dynamically controlled light delivery**

TFs also solve the problem of achieving on-the-fly adjustable light delivery, even of multiple light wavelengths, to sub-volumes of brain tissue (Fig. A5-7). Site-selective light delivery is achieved using a simple optical setup that injects light into the fiber with a defined and adjustable angle $\theta$. At the minimum, this setup consists of one lens and one translating mirror for slowly adjusting $\theta$ (Fig. S21a). For high-speed control, three lenses and one galvanometric mirror (Fig. S21b) are used to change $\theta$ quickly. With this approach, the emitting region can be modified step-by-step or continuously at various speeds.

**A4.3 Previous use of tapered fibers**

Although the ability of TFs to provide uniform as well as dynamically controlled restricted illumination has not been previously recognized, sharpened fibers have been
sporadically used to increase light delivery angle, to reduce invasiveness, and for biological discovery (Hanks et al., 2015) (Stark et al., 2012) (Gilmartin et al., 2013). This prior work exploits chemical etching of fibers (Lambelet et al., 1998) which can produce steep tapers of short lengths and has not been demonstrated to be able to make long, gradual, and tightly controlled tapers needed to illuminate large brain structures. In contrast, we pull the fibers rapidly after heating by lasers with very small taper angles, and demonstrate reproducible fabrication of fibers with customized taper length and angle. Previous use of chemically etched fibers appears to have been to minimize tissue damage (Hanks et al., 2015) (Gilmartin et al., 2013) and provide a broader illumination angle from the tip (Gilmartin et al., 2013). These studies may draw inspiration from previous work (Stark et al., 2012) in which chemically etched fibers were attached to tetrodes to generate arrays of “optrodes” capable of both photostimulation and recording neural activity. In this study light emission appears to be only from the taper tip and results in a broader illumination angle (their Figure 1B and Figure 3). It is unclear in these studies if light was injected at the fiber back aperture using its full NA, as necessary to achieve light output from the entire fiber face. As far as we know, no previous study demonstrates, as is done here, scanning output along the taper length (Fig. A5) and its application in freely moving animals (Fig. A6-7).

In addition, coating TFs with metal with small openings restricts light delivery. Lambelet et al used TFs covered with aluminum to restrict light emission and collection to a ~100 nm fiber tip, as needed for near-field fluorescence (Lambelet et al., 1998). We previously restricted light emission from a fiber by coating it in gold and making small holes in the metal coat (F. Pisanello et al., 2014). These metalized and milled fibers were
handed-crafted, requiring hours of work by an individual at a highly specialized machine that combines focused ion beam milling within a scanning electron microscope and resulting in ~$1000/fiber production costs. Furthermore, they could not be used for large volume illumination and provided unidirectional illumination at fixed, predefined spots whereas the new technology allows continuous and dynamic selection of illumination zones at a low cost.

A4.4 Novel classes of optogenetic experiment for biological discovery

From the perspective of an experimental neuroscientist, several classes of experiments become possible by exploiting the flexible and controllable nature of light delivery with TFs. For example, subregions of the striatum subserve different functions with specialized contributions to behavior being evident along the dorsal/ventral as well as medial/lateral axes and course topographic mapping to cortex laid out along the anterior/posterior axis. This suborganization is not possible to access in a single experimental animal. As we demonstrate (Fig. A3,A6-7), with a TF it is possible to illuminate an elongated column of striatum that spans the dorsal/ventral axis and compare, in a single animal, the effects of selective optogenetic manipulation of sub-regions along the fiber axis. Given that the striatum spans several millimeters in the mouse, these experiments are impractical with standard FFs as these can neither deliver light to the entire structure nor be repositioned for selective stimulation. Furthermore, with extended TFs a single fiber can be inserted to deliver light to both cortex and striatum. Since light of different wavelengths can be out-coupled from different zones and independently controlled, it will be possible to examine the effects of interactions between...
areas. For example, one can examine if the motor effects of inhibition of motor cortex can be overcome by excitation of the striatum.

In addition, as optogenetics becomes accessible in primates (Afraz et al., 2015), the larger brain structure will require the use of TFs for effective perturbations. For example, visual cortex of macaque is several millimeters in thickness with different cellular and receptive field properties in different layers. In this classic experimental system, current light delivery devices are unable to manipulate cells throughout all layers or, through a single device, test the effects of manipulations of superficial versus deep layer neurons. Furthermore, given the tight topographic organization of primate visual cortex and the use of individual animals for many recording sessions, it is valuable to have a device such as a TF with gentle taper for multiple insertions in each recording sessions.

In summary, we exploit TFs with small taper angles to achieve near uniform illumination of extended brain structures as well as to sample subregions of interest along a taper segment up to 2 mm while causing significantly less damage to the brain compared to FFs. The devices were tested in both mouse motor cortex and striatum, showing ~5 times lower excitation power threshold and larger excitation volume, compared to FF fibers. Furthermore, we demonstrate their effective use through optical commutators and patch cords to compare the effects of stimulation of dorsal vs. ventral striatum in individual unrestrained mice spontaneously exploring an arena. Coupled to the minimum invasiveness of the device, the simplicity of the technique and its intrinsic compatibility with both laser and LED sources, we suggest that this approach can greatly
complement existing methods for light delivery in optogenetics experiments and has the potential to replace commonly used flat-faced fibers for many applications.

A5 METHODS

A5.1 Mice

All mouse handling and manipulations were performed in accordance with protocols approved by the Harvard Standing Committee on Animal Care following guidelines described in the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. In this study we used both male and female mice (P>60). We used the \textit{VGAT-ChR2} mouse from the Jackson Laboratory (B6.Cg-Tg(Slc32a1 COP4*H134R/EYFP) 8GfnJ, stock #014548) , the \textit{Ador2a-Cre} mouse line from GENSAT: (B6.FVB(Cg)-Tg (Adora2acre) KG139Gsat/Mmucd) crossed to \textit{Ai32} mouse line from Jackson Laboratory (RCL1-ChR2(H134R)/EYFP, stock #012569). In addition we used C57BL mice from Charles River (C57BL/6NCrl, stock #027).

A5.2 Ray tracing simulations

The commercial optical ray tracing software Zemax-OpticStudio (http://www.zemax.com/) was used to design and simulate the performances of TFs. The single TF was modeled as a straight core/cladding segment followed by a conical taper. The materials forming all the components of the TFs and the surrounding media were assumed homogenous (refractive index constant in space). The core/cladding diameters are 50/125µm and 200/225µm for fibers with numerical aperture NA=0.22 and NA=0.39,
respectively. core/cladding refractive indexes were set as specified by the fiber producer: 1.464/1.447 for $NA=0.22$ and 1.464/1.411 for $NA=0.39$. Since the tapers were obtained by heat and pull, commonly resulting in a melting of core and cladding materials in the tapered regions, taper refractive index was assumed as the average of core and cladding refractive indexes weighted to the core and cladding cross sectional areas. This resulted in tapers refractive index of 1.450 for fibers with $NA=0.22$ and 1.453 for fibers with $NA=0.39$. The length of the core/cladding block was set to 4mm, whereas the length of the taper is a function of the taper angle.

For results of simulations reported in Fig. A1b and Fig. S10, the source was modeled as a single ray injected into the fiber with a defined angle of incidence with respect to fiber optical axis. For simulations reported in Fig. A1 and Fig. S11 and S12 a monochromatic source ($\lambda=473$ nm) was modeled as a bundle of unpolarized parallel rays having a Gaussian intensity profile. The source was focused into the core/cladding section of the fiber through a Zemax model of the experimentally used aspheric condenser (Rochester Precision Optics www.rpoptics.com). Model A-375-A with focal length $f=7.49$ mm, $NA=0.29$, clear aperture of 4.50 mm was used for TFs with $NA=0.22$ (input Gaussian beam radius 1.66 mm measured at 1/e$^2$, resulting in a focused waist of 1.6 µm RMS spot radius), whereas model A-390-A with $f=4.60$ mm, $NA=0.47$ and clear aperture of 4.90 mm was used for fibers with $NA=0.39$ (input Gaussian beam radius 1.84 mm measured at 1/e$^2$, resulting in a focused spot of 1.9 µm RMS spot radius). The former coupler was used only with the $NA=0.22$ optical fiber, with a Gaussian beam radius of 1.66 mm which focuses on the fiber core with a 1.6 µm RMS spot radius; the latter was used with both fibers, with a Gaussian beam radius of 1.84 mm which produces a 1.9 µm
RMS spot radius. For simulations displayed in Fig. S20 a parallel and unpolarized Gaussian beam with radius 25 μm at 1/e² intensity was injected at different input angles. The irradiance profile of the light outcoupled from the taper was recorded through a rectangular pixelated detector laid along the taper sidewall (Figure S12). Detector length was set as the taper side length and its width to 20 μm. These profiles are then averaged along the short side of the detector and fitted with a Gaussian function. The full-width at half maximum criterion was used to retrieve the emission length ($L_{0.5}$, measured axially from the taper tip) from the irradiance profiles. For each single ray tracing session, 5M rays were launched into the system and each ray was split at the boundary between two different media, according to Fresnel coefficients.

Ray tracing is appropriate for the modeling shown in Figs. A1, 10-14. Mode theory (Helmholtz equation) is typically employed for an accurate analysis of light propagation. This is particularly important when the waveguide size is comparable to the wavelength of light. In our tapered fiber this condition applies only close to the fiber tip. We describe the working principles of TFs using ray optics because the main effects we exploit are not taking place at the tip, but rather over the whole extent of the tapered fiber, and mostly at fiber diameters well above a few micrometers. Ray tracing is used to characterize the full-NA injection method, with good agreement with experimental results (Fig. A1d). In the section “In vivo multisite stimulation” we describe the working principle of the device based on selective modal out-coupling along the taper.
A5.3 TFs stub preparation and optical measurements.

Tapered optical fibers with taper angles in the range $2^\circ < \psi < 8^\circ$ were obtained from OptogeniX (www.optogenix.com) and connected to a ceramic ferrules following the procedure described previously (Sparta et al., 2011). A ceramic ferrule-SMA patch cable was used to connect the fiber stub to the optical setup displayed in Fig. S20 for full NA light injection or to the setups schematized in Fig. S21 for site-selective light delivery. The taper was immersed into a fluorescent PBS:fluorescein solution or inserted in stained brain slices positioned under a 5X objective of a fluorescence Zeiss microscope equipped with a FITC filter. Images were acquired with a Hamamatsu Orca Flash 4.0. sCMOS camera at a resolution 2048x2048 pixel (pixel depth 16 bit). Optical output power was measured in air.

For imaging light delivery geometry in fixed brain slices, slices of thickness ranging from 200 µm to 400 µm were permeabilized with Triton X-100 0.1% for 10 min to allow homogenous cell staining through the whole brain slice thickness and then washed with 1X PBS three times. Slices were then incubated with Sybr Green 1:10.000 (Thermo Fischer Scientific Inc.) for 20 min on an orbital shaker in dark and thoroughly washed with 1X PBS.

A5.4 Analysis of c-fos induction

Transgenic adult \((Ador2a-Cre; Ai32)\) animals (P>60) were anesthetized with isoflurane and placed in a small animal stereotaxic frame (David Kopf instruments). Under aseptic conditions, the skull was exposed and a small hole was drilled. Animals received 0.01mg/gram of sterile Ketofen (Zoetis). For FF implanted animals and the lateral striatum
TF implanted animal the hole was made at 0.85mm anterior, 1.95mm lateral from bregma. For the medial striatum TF implanted animals the hole was made at 0.85mm anterior, 1.65mm lateral from bregma. A TF (3.7mm from pia) or FF (2.3 mm from pia) was inserted manually using a cannula holder (David Kopf instruments). The TF or FF was then glued in place (454 instant adhesive, Loctite). And the skull was covered with dental cement (CandB Metabond, Parkell inc). Regardless of the implant-surgery time animals were under isoflurane anesthesia for a total of 1 hour before stimulation started. Stimulation was delivered in 30-second on/off cycles for a total of 1 hour (473 nm, 1 mW outputted at fiber exit). 2 hours post stimulation animals were euthanized with 0.2 ml of 10% Fatal Plus solution (Vortech pharmaceuticals) in saline before being perfused with 4% paraformaldehyde (PFA) in 0.1M sodium phosphate buffer (PBS). Brains were post fixed for 24 hours in PFA, washed in PBS, and sectioned (50um) coronally.

Immunohistochemistry conditions were the same for all animals. In short, slices were incubated in PBS blocking solution containing 0.3% TritonX (PBST) for 1 hour at RT. Slices were then incubated over night at 4°C in the same blocking solution with 1% goat serum and 1ug/ml c-Fos rabbit polyclonal IgG antibody (H-125, Santa Cruz Biotechnology). The next day slices were rinsed 3x10min in PBS before being incubated in the blocking solution with secondary antibody (1mg/ml goat anti-rabbit Alexa Fluor 647 or Alexa Fluor 594, Life Technologies). The slices were then rinsed again and mounted. After drying, slices were coverslipped with ProLong antifade mounting media containing DAPI (Molecular Probes) and imaged with an Olympus VS 120 slide-scanning microscope using a 10x objective.
A5.5 Analysis of glial response to fiber insertion.

The implant surgery was similar to the one described during the analysis of c-fos induction but both implants were positioned at 0.8mm anterior and 2.0mm lateral to bregma. In addition, the TF was implanted at a depth of 4.0 mm and the FF at 3.5 mm. Adult (C57BL/6NCrl) animals (P>60) were euthanized 48 hours post-surgery and perfused as described above. To compare the spatial distribution of microglia and astrocytes activated near the implant, we performed fluorescence immunohistochemistry (as described in Analysis of c-fos induction section) for GFAP (primary rabbit anti-GFAP, Dako Z0334, 1:1000 dilution; secondary 1:1000 goat anti rabbit 555 ) and CD68 (primary rat anti-CD68, Biorad MCA1957; 1:200 dilution; secondary 1:1000 goat anti rat 647) respectively. 6 coronal sections surrounding the implant were imaged with a VS120 Olympus slide-scanning microscope. Quantification of fluorescence intensity was performed in ImageJ (NIH). ROIs of striatum were determined based on the Allen Brain Atlas 2004. For each section, the total fluorescence intensity summed from the left and right hemispheres was normalized to one such that the fluorescence per hemisphere is expressed as a fraction of the total for that section. No comparisons were made between sections.

A5.6 Multielectrode array recordings

Recordings in primary motor cortex of adult (VGAT-ChR2) animals (P>60) and off-line analysis of spiking rates were accomplished as previously described (Oldenburg and Sabatini, 2015). Mice were habituated to head-restraint prior to the recording session. Only one fiber optic (TF or FF) was inserted at a time and the effects of transient
stimulation of cortical inhibitory neurons on identified units were examined. For each unit, spiking rates were normalized to the pre-illumination rate. Averages were calculated across neurons.

**A5.7 Open field surgery**

The surgery for TF implant was as described above in the analysis of c-fos induction with coordinates for the TF in striatum being 0.85A, 1.4L, 4.1D. Adult (Ador2a-Cre; Ai32) animals (P>60) recovered for 5 days post-surgery and were handled for 3 days before experimentation.

**A5.8 Behavior**

On the day of the experiment animals were positioned in the open field arena. The experimental paradigm consisted of 3 min blocks of either no stimulation (ns), or laser input to the fiber at angle 1 (θ₁) or angle 2 (θ₂) repeated in the following pattern ns θ₁ ns θ₂ ns θ₁ ns θ₂ ns, corresponding to alternating stimulation of ventral (θ₁) and dorsal (θ₂) striatum separated by periods of no stimulation. On the subsequent day of analysis the order of ventral (θ₁) and dorsal (θ₂) stimulation were reversed.

**A5.9 Open field analysis**

Mice were recorded using a Microsoft Kinect (v1), which records depth video data at 30 frames per second. For data in figure A6, scalar features were extracted using previously published methods (Wiltschko et al., 2015). For modeling data presented in Fig. A7, the open field behavior was analyzed using previously published methods.
(Wiltschko et al., 2015). In brief, the data were subjected to machine learning methods that describe the mouse’s behavior as re-usable sub-second modules, or syllables. All free parameters were set to the values described in Wiltschko et al (Wiltschko et al., 2015) with the exception of the stickiness parameter, kappa, which sets the model’s tendency to remain in the same syllable over time (rather than switch between different syllables). This parameter was tuned so that the overall syllable duration distribution qualitatively matched a model-free analysis of behavioral change-points as in Wiltschko et al (Wiltschko et al., 2015). For this analysis we set kappa to 291,600. For head and tail positioning of the mouse in Fig. A7, the head and tail position were computed first by fitting an ellipse to the image of the mouse. The head and tail positions were defined as the two farthest points of the ellipse (i.e. the points of the ellipse that intersect with its principal axis).

**A5.10 Statistics.**

Statistical comparisons were performed in Prism (Graphpad) with non-parametric tests. For the data in Figure A6, a Mann-Whitney two-tailed U test was performed with n-values of 10, 4, and 4 for ns, θ₁ stimulation and θ₂ stimulation. For further details, please see the main text and Figure A6 legend. Each n represents one 3 minute imaging sessions with half the sessions collected on day one and half on day 2. Figure S19. For the data in Figure S19, a Kruskal Wallis ANOVA with Dunn’s multiple comparison correction was performed and the n-values are 45, 49, 51, 51, 28, 28, 28, 28, 48, 49, 49, and 49 cells for 10, 50, 100, and 200 µW power for the TF, shallow FF, and deep FF. Please see Figure S19 legend for further details. No statistical methods were used to
determine sample sizes *a priori*. The sample size was chosen based on prior experience with similar experiments. Normality was not assumed and no per sample calculation of variance was performed. No randomization or blind analysis was used. However, for Figure A6 the analysis was performed with automated routines with no user intervention and the stimuli were delivered in interleaved and counterbalanced orders. No criteria for data exclusion were used.
A6 Bibliography


Appendix B: Supplementary Material

B1 Supplementary material for Chapter 2

B1.1 Supplementary Figures

S1: Related to Figure 2.1

A-C, Volume of regions of interest shown for the 7 mice which were pooled together for subsequent analysis. Coefficient of variation (CV) of each region volume across the 7 mice is noted in red.

Figure S1 (continued)
S1 (Continued)

**D**, Number of RV-nGFP+ cells ipsilateral (ipsi) or contralateral (con) to the injection site in STR (*left*) and PF (*right*). (PF: n=6,848/7; STR: n=165,382/7; cells/mice).

**E**, Percent of total RV-nGFP+ cells distributed across nuclei-groups and nuclei in sensory-motor cortex related TH (DORsm) (n=32,99/7; cells/mice).
**S2: Related to Figure 2.2**

**A,** Experimental design shown by a schematic of a coronal section at +0.9mm from a WT mouse depicting 2 injections of 2 CTB variants in the dlSTR (yellow) and nucleus accumbens (ACB) (white). The injection of the 2 variants of the CTB into two anatomical defined regions allowed to ask whether there is additional topography between PF and STR. The injection site regions (STR and ACB) are highlighted in orange.

**B,** An example of a PF coronal section at -2.1mm from the experiment shown in A. The FR is highlighted in the tissue and labeled to help orient to mPF.
S3: Related to Figure 2.3

Example of *in situ hybridization* from the ABA with genes that are highly correlated (A-C) or anti-correlated (D-F) with *Pdyn* expression on a cell-by-cell basis.
S4: Related to Figure 2.4

A-D, left: Example of a coronal section from a Pdyn-IRES-cre injected with a creOn-gfp into PF and the quantification of the fluorescent intensity in PF (right) at coronal section -2.0mm (A), -2.1mm (B), -2.2mm (C), -2.3 (D). Thin lines represent peak-normalized data from individual animals and the thick lines show the means for each channel. The dashed grey region represents the FR location (n=3 mice).

E, Example of ISH for Pdyn (cyan), and Slc17a6 (white) in the PF of a Pdyn-IRES-cre mouse highlighting the overlap of Pdyn and vglut2.
S5: Related to Figure 2.5

A, Experimental design showing a coronal section at -2.1 mm depicting an injection of creOn-TVA and creOn-OG (left) followed by an injection 3 weeks later of p.RV-GFP in
the PF of a Pdyn-IRES-cre mouse (right). creOn-TVA, creOn-OG are two helper virus that are necessary for rabies infection and its retrograde transfer, respectively.

B, left, example coronal section at -2.1 mm in PF showing expression of TVA in mPF (red) from the experiment in A. Middle, expression of p.RV-GFP (cyan) in IPF. Right, overlay of the two channels highlighting that there is no expression of p.RV-GFP in cPF or IPF. (n=3 mice, example shown from one mouse).

C-D, as in panel (A-B) but expression of creOn-TVA and creOn-OG was induced in IPF using a retrograde traveling AAV with Cre (retro-Cre) injected into dlSTR in a WT mouse. No expression of p.RV-GFP (yellow) was observed in mPF or cPF. (n=3 mice, example shown from one mouse).

E-F, Coronal sections at -3.7 mm from the experiment shown in (A) and (B) respectively highlighting that there is expression of p.RV-GFP+ (cyan or yellow) in the SC and SNr. The SNr is shown in the inset.

G-H, Number of cells counted in the SNr slices from the experiment shown in (A) (n=354/10/2; p.RV-GFP+ cells/slices/animals) and (B) (n=140/8/2; p.RV-GFP+ cells/slices/animals) highlighting medial SNr → mPF and lateral SNr → IPF projections.

I-J, as in panel (A-B) but with no injection of creOn-OG into a WT mouse. This verifies that the movement of the p.RV-GFP movement upstream and across the synapse is dependent on creOn-OG.

K, Coronal section at -3.7 mm from the experiment shown in (I) verifying that there is no expression of p.RV-GFP+ outside of PF. SNr is highlighted in a dashed line.
S6: Related to Figure 2.6

A, Percent GFP+ cells found 200 µm anterior to PF (An), in PF, or 100 µm posterior to PF in the animals that were analyzed for their putative inputs from PF to CTX (for mPF=713/1; cPF=1000/1; IPF=2171/1; cells/mice).

B, Example coronal at +1.4mm from the experimental design and same animals shown in Figure 6 highlighting that the overall topography between PF cell classes and cortex was maintained in between the two regions we analyzed.
S7: Related to Figure 2.7

A, left, Quantification of percentage of the maximal fluorescence intensity of CTB+ in cPF as shown in Figure 7B, compared to that in the rest of PF (rPF). Grey filled circles here (and throughout the figure) represent the analyses of coronal section -2.3mm in PF. P=0.0001; Wilcoxon test. right, Quantification of percentage of the maximal fluorescence
S7 (Continued)

intensity of GFP labeled axons (Fl) in the cPF ROI, as shown in Figure 7B compared to that in the rest of PF (rPF). Each dot represents one section analyzed with its color reflecting its location within PF.

**B-D**, Same as panel (A) but for experiments shown in Figure 7D, Figure 7H, and Figure 7L. For panel B: \( P = 0.0078 \), For C: \( P = 0.0005 \), For D: \( P = 0.0020 \). Wilcoxon test.
S8: related to Figure 2.7

A, Experimental design shown of coronal sections at +0.9 mm from a Rbp4-Cre+/− mouse depicting a viral injection of creOn-GFP (white) into MOs.

B, Example coronal section from experiment in (A) at -2.1 mm in PF, contralateral to the injection site. The arrows highlight the weak fibers in contralateral PF.

C-D, As in panel (A) and (B) but for injection of creOn-GFP into SSp.

E, Quantification of percentage of the maximal FI of GFP labeled axons in PF ipsilateral (IPSI) or PF contralateral (CON) to the injection site in MOs (left), SSp (middle), and PFC (right). This confirmed that MOs→con-PF and SSp→con-PF fibers FI are weak compared to PFC→con-PF fibers.
## B1.2 Table 1: abbreviations lookup table

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S9. Simplified model of ray propagation and outcoupling in TFs.

After total reflection at the core boundary with an angle $\alpha$, a ray entering the taper (blue line) hits the taper sidewalls with an angle of incidence $\gamma_j$ that decreases as the number of hits $j$ increases. The ray can outcouple in the surrounding medium when $\gamma_j < \theta_C$ (i.e. total internal reflection condition on the critical angle $\theta_C$ is not fulfilled). The values for the Figure S9 (continued)
taper and the critical angle ($\Psi = 20^\circ$, $\vartheta_c = 30^\circ$) were selected to improve the readability of the figure. In the picture the cladding is not shown and the refractive index $n$ was assumed to be the same for both the core and the taper. This simplified analysis only applies to meridional rays.
S10: Dependence of the output distance from taper tip on the input angle.

Ray tracing was used to model the propagation of a ray (left) in a TF and determine the position at which it outcouples. Results for ray tracing simulation for TFs with 0.22NA/ψ = 2.2° core diameter 50 µm and cladding diameter 125 µm (red circles) and 0.39NA/ψ = 2.9° core diameter 200 µm and cladding diameter 225 µm (black squares) are shown (right).
S11: Ray tracing simulation of propagation and outcoupling in TFs of different NA and taper angle, as indicated.
S12: Approaches used to model and measure light output from the TFs.

a, Optical setup used to inject light in the TF, filling the whole numerical aperture of the fiber, and to image the fluorescence generated by light output into a fluorescein solution.

b, Schematic representation (left) of light output from a TF, the irradiance profile measured by the linear detector placed alongside the taper (middle), and the Gaussian
Figure S12 (Continued)

fit to the average line profile measured by the detector from which the length to half maximal intensity is calculated \( (L_{0.5}) \) (right).

c, Typical image of fluorescence produce by light emission from the TF into a fluorescein solution (left) and the measured fluorescence intensity profile obtained along the red line (right). \( L_{0.5} \) is calculated from the fiber tip as the width at which the intensity is half of the intensity represented by the dotted vertical line. The latter is the average of all data points exceeding 90% of the intensity detected in the pixel closest to the fiber tip.
S13: Example of a typical low ψ taper with a modest non-linear shape
S14: Characterization of fiber emission as a function of taper angle

a. First emission diameter of 0.39 NA and 0.22 NA TFs as a function of ψ. First emission diameter is evaluated with the fiber stub submerged into a fluorescein solution and measured as the diameter of the taper at the point at which the fluorescence intensity just beside the taper reaches a threshold level ~3 times the noise level.

b. Total output power for 0.39 NA and 0.22 NA TFs as a function of ψ.
S15: Estimated power density along the taper surface outcoupled by 0.39 NA TFs (core diameter 200µm, cladding diameter 125 µm).

A total output power of 1 mW is distributed around the taper following the experimental emission profiles measured in a fluorescent bath as shown in Supp. Fig. 4.
S16: Estimated power density along the taper surface outcoupled by 0.22 NA TFs (core diameter 50 µm, cladding diameter 125 µm).

A total output power of 1 mW is distributed around the taper following the experimental emission profiles measured in a fluorescent bath as shown in Supp. Figure 4.
**S17: Control conditions for c-fos induction experiments.**

Schematic (a) and image (b) of c-fos immunolabeling in an animal that expressed ChR2-YFP in iSPNs but was not exposed to light. Minimal c-fos staining is present.

Schematic (c) and image (d) of c-fos immunolabeling in an animal lacking ChR2-YFP but stimulated through a TF. Minimal c-fos staining is present.
S18: Diminished tissue damage with TF vs. FF.

a, Schematic showing the experimental design: a FF (right side of the brain) and a TF (left side of the brain) were implanted in the striatum of a wildtype animals, at 0.8 mm anterior and 2.0 mm lateral to bregma.
S18 (Continued)

b. Image of a coronal section of the mouse brain showing the damage caused by each fiber. The TF was covered in green fluorescing lipophilic dye to show the track.

c. 6 coronal sections (from top left, starting with 1.3 mm anterior to bottom right, 0.4 mm anterior to bregma) from example mouse immunostained for the astrocyte marker GFAP after being implanted with a FF (right side of the brain) and a TF (left side of the brain) in the striatum.

d. Quantification of fluorescence in tissue immunostained for the astrocyte marker GFAP from two mice (6 coronal sections each). Fluorescence is expressed for the TF and FF hemispheres as a fraction of the total fluorescence per tissue section – thus for each the total in the TF and FF regions sum to 1. The magenta lines indicate the means of the values across tissue sections of the TF and FF implanted hemispheres.

e. Example coronal sections (0.8 mm anterior to bregma) immunostained for the microglia marker CD68.

f. As in panel d but of tissue immunostained for the microglia marker CD68 from two mice (6 coronal sections each).
S19: Complete dataset of SF and TF efficacy in primary motor cortex.

For each recorded unit an index of modulation of firing rate was calculated as:

\[ \text{index} = \frac{f_{\text{on}} - f_{\text{off}}}{f_{\text{on}} + f_{\text{off}}} \]

where \( f_{\text{on}} \) and \( f_{\text{off}} \) are the firing rates of the neuron with the laser light on and off, respectively. In these recordings the vast majority of sampled neurons are excitatory and ChR2 is expressed in all GABAergic neurons. Therefore, the effect of light is expected to be inhibition of most units (\( \text{index} < 0 \)). The small number of units with \( \text{index} > 0 \) may represent directly stimulated GABAergic interneurons. The values of \( \text{index} \) range from -1 to 1, with -1 indicating that all the spikes were with the light off and +1 indicating that all the spikes were with the light on. A value of 0 indicates no change in firing with light. Statistical comparison was done with Anova with Kruskal-Wallis Rank (i.e. non parametric) test and Dunn’s post-hoc multiple comparison test. *** indicates \( p < 0.0001 \) (the limit of reporting of Graphpad). ** indicates \( p = 0.0027 \). Comparisons at 200 \( \mu W \) were not significantly different (\( p = 0.1704 \)). Degrees of freedom are \( n-1 \), where \( n \) is the number of cells. Data for all cells are shown (\( n \)-values are 45, 49, 51, 51, 28, 28, 28, 28, 48, 49, 49, and 49 cells).
S20: Ray tracing simulations for site selective light delivery for a 0.22NA/\(\psi =2.2^\circ\) TF.
S21. Three potential launching system configurations for controlling the zone of light emission from a TF.
Figure S21 (Continued)

a, A laser beam is injected into the fiber by a single lens. Translating one mirror in the direction perpendicular to optical axis of the fiber input facet modifies the input coupling angle. This system was used and described in more detail in Pisanello et al Neuron 2015.

b The input laser beam is focused on the rotation axis of a galvo mirror, which deflects the beam onto a second lens that makes it parallel to optical axis. A third lens can then focus the laser into the optical fiber with a defined input angle.

c, A system comprising two different optical paths similar to the ones displayed in panels a and b but working simultaneously with two different lasers. This configuration allows for delivering different wavelengths at different sections of the taper, also at different scanning rates. In all panels lens diameter is 2".
S22: Effects of fiber movement and bending on site selective light delivery.

a, Optical setup used to inject light at defined angle $\theta$.

b-d, Variability of three metrics of light output (schematized in A) for shaken, bent, and bent and shaken patch fibers connected to a 0.39 NA/$\psi=2.9^\circ$ TF. The percent variability about the mean in peak intensity (red), full-width at half maximum (FWHM, green) and centroid (yellow) of the emission along the taper are graphed. Different colors along the time line depict the movement conditions detailed at the bottom of panels B-D and shown in Supp. Videos 5-7, respectively. $\rho$ is the radius of curvature and the arrows ($\rightarrow$) identify variations from one radius of curvature to another during the measurement.
S23: Characterization of output for the fibers and light input angles used for the experiments described in Figure 6 and 7 and use with a commercial launching system.

a and b, Total power of light output (a) and the distance of the centroid of light output from the fiber tip (b) for light injected at the full NA or each of the two angles used with 1mW input angle (mean ± std shown for n=8 fibers).

C and d, Full NA launching into a TF using a commercial system. Image (left) of a Plexon LED driver and fiber launch system connected to a TF and the resulting fluorescence generated in a fluorescein bath (right).