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Optogenetic identification of striatal projection neuron subtypes during *in vivo* recordings

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Abstract

Optogenetics has revolutionized neuroscience over the past several years by allowing researchers to modulate the activity of specific cell types, both *in vitro* and *in vivo*. One promising application of optogenetics is to use channelrhodopsin-2 (ChR2) mediated spiking to identify distinct cell types in electrophysiological recordings from awake behaving animals. In this paper, we apply this approach to *in vivo* recordings of the two major projection cell types in the striatum: the direct- and indirect-pathway medium spiny neurons. We expressed ChR2 in the neurons of the direct or indirect pathways using a cre-dependent viral strategy and performed electrical recordings together with optical stimulation using an implanted microwire array that included an integrated optical fiber. Despite the apparent simplicity of identifying ChR2-expressing neurons as those that respond to light, we encountered multiple potential confounds when applying this approach. Here, we describe and address these confounds and provide a Matlab tool so others can implement our analysis methods.

Keywords

optogenetics; ChR2; striatum; *in vivo* recording

Introduction

Recent advances in optogenetics have provided new approaches for perturbing the brain in a temporally precise, rapidly reversible manner [5, 26]. One of the most fruitful applications of optogenetics has been the ability to activate or inhibit specific cell types and observe the resulting changes at the behavioral level. In recent years, a second application of optogenetics as gained traction, in using light-activation to identify specific neuronal cell types during *in vivo* recordings. Briefly, an opsin such as ChR2 can be expressed in a subset of neurons, which are subsequently identified during a recording by their response to light. This approach has been used to identify and characterize multiple cell types in awake recordings, including fast-spiking interneurons and pyramidal neurons in cortical recordings [1, 2, 18], striatal interneurons and projection neurons [15, 16, 28], dopaminergic and GABAergic neurons in the ventral tegmental area [3], and hippocampal interneurons [22].

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One thing that is clear from examining the methods of each of these studies is that optogenetic identification protocols must be optimized for the unique composition and circuitry associated with each brain structure. We have investigated this approach in the striatum, a basal ganglia nucleus containing primarily GABAergic neurons, to identify direct and indirect pathway medium spiny projection neurons (MSNs) [15, 16]. While this initially appeared to be a straightforward task of identifying cells that responded to the laser stimulation, a number of potential confounds rapidly became apparent. Most importantly, we identify scenarios in which cells might not express ChR2, but nonetheless respond to the laser stimulation with an increase in firing. These include (1) problems with spike sorting in which spikes from a ChR2-positive unit may infiltrate a recorded ChR2-negative unit, (2) rapid synaptic activation or disinhibition through the local network, (3) short-latency visual responses, and (4) photoelectric responses of the electrodes themselves. Here, we provide evidence that these caveats can be addressed and this procedure can be used to reliably identify striatal projection neuron subtypes *in vivo*.

Results

We used a cre-dependent viral strategy to express ChR2 in direct-pathway (using D1-cre mice, see methods) or indirect-pathway (using either D2-cre or A2A-cre mice) MSNs of the striatum [14, 16]. For *in vivo* recording, we first constructed a ferrule-fiber assembly (Figure 1A) [23], and then attached this assembly to a commercially-produced 32-channel microwire array (Innovative Neurophysiology, Durham, NC), such that the fiber tip was positioned about 0.5mm above the wire tips (Figure 1B). These arrays were implanted in the dorsomedial striatum. Following recovery from surgery, mice were placed into an open-field arena and connected to a laser-coupled optical fiber and recording headstage (Figure 1C). The dataset used in this paper consisted of 148 single-unit and 163 multi-unit recordings from 10 mice. Based on the waveform duration of the 143 single units, we classified 15 neurons as putative fast-spiking interneurons (FSIs), and 133 as putative MSNs, as previously described [6, 14]. Histologically verified locations of the recording wires in the dorsomedial striatum are presented in Figure 2. To capture the optimal light-induced responses, we stimulated each cell 100–200 times at each of 4 different laser intensities, ranging from 0.1mW to 3.0mW (~1 to 25 mW/mm⁻²). Different units responded best to different laser intensities, with most neurons responding reliably at the higher laser intensities (Figure 1D, E), although a minority of neurons responded selectively to the lower laser intensities but not to higher intensities. Our stimulation protocol was 1 second on (constant), followed by 3 seconds off. We examined whether longer off periods (29 sec off) improved the strength of ChR2-induced spiking during the laser pulses, but we did not observe any improvement (data not shown).

Optogenetic identification of ChR2-expressing units

To identify neurons that fired significantly above baseline firing rates during the laser stimulation, we first performed a shuffling procedure to obtain a more accurate estimate of baseline firing rates. This procedure was particularly important for low firing neurons. To calculate the spike rate for each 5 ms bin, we shuffled the spikes in a baseline period (250 to 750 ms preceding the laser pulse) 500 times and calculated a histogram of the percent of bins that corresponded to each instantaneous firing rate achieved by that neuron (Figure 1F). We then calculated the instantaneous firing rate of each 5 ms bin in the first 100 ms following the onset of the laser pulse. If any bin during this laser period achieved a higher firing rate than 99.5% of the shuffled baseline bins, that bin was defined as laser-activated (e.g.: blue line at 15 ms in Figure 1F). To minimize false-positive cell identifications, only those neurons with more than one laser-activated bin within the first 100 ms of the laser pulse were categorized as ChR2-expressing neurons. To characterize the timecourse of

ChR2 activation, we generated a cumulative distribution of the fraction of laser-responsive cells within the 100 ms period following laser onset (Figure 1G). Interestingly, this plot revealed a two-component curve, with a population of cells ($n=19$, 13%) that were activated within 15 ms of the laser pulse, and a population ($n=10$, 7%) of cells that were activated with a longer delay (15–100 ms). Rapidly activated cells are consistent with those expressing ChR2 that receive direct illumination of their soma/axon initial segment, whereas cells activated with a longer delay may only receive illumination of some dendrites, or may receive significant lateral inhibition that delays the initial ChR2-driven spikes.

The above procedure differs from that used by prior researchers *in vitro*, and *in vivo* in brain structures where it is possible to drive spikes with high fidelity. Medium spiny neurons have two properties that make them unsuited to identification protocols that require high spike fidelity. First, medium spiny neurons have very low excitability and fire at low spontaneous rates *in vivo*. It is therefore difficult to drive them to spike reliably and at short latencies without using extremely high-powered illumination. As some ChR2 expressing neurons in our study were recorded on the distal edges of our recording arrays (Figure 2D), we reasoned that it would not be possible to deliver sufficient illumination to these cells without reaching a potentially harmful laser power in the tissue closer to the tip of the optical fiber. This specific concern will likely be addressed in future years with advances in recording devices, such as arrays that include multiple light sources, each targeting a subset of recording sites [21]. Second, medium spiny neurons have variable membrane potentials which continuously fluctuate between approximately -50mV and -80mV *in vivo* [12, 13, 19]. As we cannot monitor the membrane potential in our extracellular recordings, we do not know whether the cell is close to spike threshold when we deliver each laser pulse. In fact, on average the laser-responsive neurons that we identified in this study only responded to $\sim 10\text{--}30\%$ of the laser pulses (Figure 1H), likely reflecting the relatively rare conditions in which they were close to threshold when the laser pulse was delivered. We attempted to analyze the jitter in only those trials in which spiking did occur, but this analysis returned high levels of jitter (data not shown), consistent with the fluctuating membrane potential when the pulses were delivered. Nevertheless, the spike fidelity (which overall should not be as dependent as jitter on membrane state) did increase with higher laser powers (Figure 1H). While it would be simpler if identical techniques could be applied for optogenetic identification across multiple brain structures, it appears that optogenetic identification procedures will need to be optimized for each different brain structure, a situation that occurs with nearly all technical approaches in neuroscience.

Challenges of spike sorting during laser stimulation

Examining the activity of individual units recorded during laser stimulation introduces unique challenges for spike sorting. In the ideal case, individual unit clusters remain well separated during the laser identification protocol (e.g. Figure 3A–B). In these cases, it is straightforward to distinguish laser-activated units from non-responsive ones. In many cases, however, the laser identification protocol activates neurons that were quiescent during the baseline period. These newly activated cells may appear as independent units during the spike clustering, or they may appear as an increase in size and change in shape of the multiunit cluster. In several of our recordings, the laser activated multi-unit cluster grew and infiltrated clusters that were well separated during the baseline period (e.g. Figure 3F–G), spuriously making them appear to respond to the laser when in fact the increase in laser-activated spikes likely came from this increase in multiunit activity (e.g. Figure 3I). This infiltration indicates the importance of a conservative approach to spike clustering when performing optogenetic identification procedures. An initial clustering should be performed by including all spikes from the baseline and laser identification periods. The data should then be restricted to show (1) only the spikes from the baseline period (e.g. 3A and F), and

then (2) only the spikes from the laser identification period (e.g. 3B and G), to verify that the individual units are stable and well isolated throughout the laser identification procedure. For seven of the ten recordings, the animals were recorded across a 1 hour baseline period and a ~30 minute laser identification period. We independently characterized several metrics of recording quality in these two periods for these animals. These included waveform amplitude (in mV), correlation between waveform shape between the baseline and laser stimulation periods (R^2), and J3 and Davies-Bouldin indices [4, 20], which provide measures of cluster quality. The waveform amplitude and the correlation between waveform shape both indicated that recorded unit size and shape did not differ between the two periods (Table 1). The cluster quality measures indicated slightly worse clustering during the laser stimulation periods, consistent with the increase in multi-unit activation (Figure 3J), but these differences were not significant, indicating high recording quality in both the baseline and the laser stimulation period (Table 1).

The relatively low yield of identified units (~14%) in this experiment may suggest that our ChR2 stimulation of striatum is weak. However, when we examined our multi-unit recordings we found strong evidence of striatal activation. On about half of our recording channels (163/320 channels) we identified a “multiunit” cluster of neural activity that was larger than the noise-band, but could not be clustered into single units (orange, Figure 3A, F). Many of these multiunit clusters exhibited significant increases in spike frequency within 15 ms of the laser pulse (e.g. Figure 3C, H). This is not surprising, as each multiunit cluster has multiple single units contributing to its spikes, any one of which may express ChR2. In fact, of the 163 recorded multiunits, 76 (47%) exhibited an increase in spiking within 15ms of the laser pulse, and 90 (55%) exhibited an increase in spiking within 100ms following the laser onset (Figure 3J). We conclude that we are strongly activating many cells in the striatum, but the ability to maintain high quality single unit clustering during the laser pulse reduced our single unit identification efficiency.

Potential contributions of synaptic activation or disinhibition

The striatum is an inhibitory structure and is therefore immune to the experimental confound of direct synaptic excitation that plagues optogenetic identification of individual units in electrophysiological recordings from recurrently connected excitatory structures such as cortex and hippocampus [18]. There remains the possibility, however, that by activating one set of ChR2-expressing neurons, we might suppress a second set of neurons, and that this suppression could release a third set of non-ChR2-expressing neurons from a tonic inhibition. Indeed, we observed medium spiny neurons whose firing was markedly suppressed during the laser stimulation (e.g. Figure 4A). However, based on two lines of evidence we conclude that this disynaptic disinhibition is unlikely to contribute to laser-activated increases in spiking in the striatum.

Rapid disinhibition of MSNs would necessarily require there to exist a tonic source of inhibition onto MSNs that gets immediately suppressed within a few milliseconds following our light activation of either the direct- or indirect-pathway MSNs. One potential source of tonic inhibition onto MSNs is the fast-spiking interneurons (FSIs). However, these interneurons do not receive direct synaptic connections from the local MSNs and are therefore unlikely to be rapidly suppressed following the onset of our light pulse. In an empirical test of this, we found that none of the 15 putative FSIs in our recordings were inhibited within 15ms of the laser pulse. We also considered whether other populations of striatal interneurons could be responsible for rapid disinhibition. Persistent low-threshold spiking (PLTS) interneurons form only very weak synapses onto MSNs, and are therefore also a poor candidate for disinhibition of MSNs [8, 10]. The remaining GABAergic interneuron subtypes in striatum are the calretinin-positive interneurons and the newly described neurogliaform (NGF) cells. While it is unknown whether either of these two cell

types might be directly inhibited by MSNs, both cell types are very sparsely distributed in the rodent striatum and are therefore similarly unlikely to be capable of driving disinhibitory responses in a significant number of MSNs [7, 10, 27].

One additional possibility is that the direct lateral connections between MSNs could underlie a potential disinhibitory response. The connection probability between pairs of MSNs is moderately high (~20%), although individual connections are weak [24, 25]. Each individual MSN therefore receives a small amount of inhibitory input from a moderate number of neighboring MSNs. The overall influence of this lateral inhibition onto any one MSN is therefore well described by the average firing rate of the entire MSN population. We calculated the average firing rate across all recorded single units in our dataset (Figure 4B), and found that the average firing rate robustly increased in response to the light stimulation. This indicates that each individual MSN presumably experiences a net *increase* in inhibitory input during the laser stimulation, not the decrease in inhibition necessary for driving a disinhibitory response. We analyzed the time course of the activation or suppression of MSNs over the 1 s light pulse. In order to increase the sensitivity of the analysis to detect decreases in the activity of MSNs with low spontaneous firing rates, we performed this analysis using longer time bins (50 ms for suppression; 5 ms bins for activation in Fig 1). Even with the analysis thus weighted to detect MSNs with even a modest drop in firing rate, we found a larger percentage of the MSNs were significantly activated than suppressed at each time bin over the first 400 ms of the 1 s light pulse. This result is similarly inconsistent with a prominent role for disinhibition in this system.

As an alternative way of addressing this issue, we examined the cell type of all laser-activated cells in each line using a Fos-based immunostaining strategy. We crossed the D2-cre mouse to a D1-tomato (D1-tmt) reporter mouse that expressed a red fluorophore in MSNs of the direct pathway [9]. We then injected a cre-dependent ChR2-YFP virus into the striatum of these mice, so that ChR2-YFP was expressed in MSNs of the indirect pathway, and the red tmt fluorophore was expressed in MSNs of the direct pathway. We stimulated these mice for 60 minutes with a pattern of 30 seconds on, 30 seconds off, to drive Fos expression in activated cells. We immunostained their brains for c-Fos and counted the number of c-Fos positive nuclei that colocalized with tmt fluorescence. Out of 150 c-Fos positive nuclei, 132 (88%) did not express tmt fluorescence (Figure 4D, E). This high percentage supported the conclusion that nearly all activated cells belonged to the indirect pathway. We performed the analogous experiment in the direct pathway, crossing the D1-cre mouse to a D2-GFP reporter mouse that expresses GFP in indirect pathway MSNs [9]. We then injected a cre-dependent ChR2-mCherry virus, so these mice expressed ChR2-mCherry in direct pathway MSNs, and GFP in indirect pathway MSNs. Again, we found that hardly any cell co-expressed both c-Fos and GFP (Figure 4F, G). Out of 269 c-Fos positive nuclei, 248 (92%) were GFP-negative and therefore presumed to be direct pathway MSNs. From these two experiments we concluded that stimulation of one striatal pathway does not result in robust activation of cells in the other pathway. If a large percentage of our laser-activated neurons were the result of disynaptic disinhibition, we would not expect such pathway selectivity to be maintained in this experiment. Instead, this experiment supports the conclusion that disynaptic disinhibition does not play a major role in activating striatal neurons during the light pulse.

Visual responses to the laser

We considered whether using halorhodopsin or archaerhodopsin to *inhibit* activity of direct- or indirect-pathway MSNs might be useful for identifying neurons, as in [22]. However, we encountered a surprising pitfall in these experiments, and we report it here as it is generally relevant to all optogenetics research. In early experiments with archaerhodopsin, we found

that a significant percentage of striatal neurons were excited by yellow laser stimulation, with what appeared to be visual responses to the laser itself.

To investigate this issue systematically, we illuminated ChR2-expressing mice with blue, green, and yellow lasers, either transmitting the light to the brain through the implanted fiber, or shining the laser directly on the mouse's eye (Figure 5A, B, C). When the light was transmitted into the brain, an opaque black paper cone was placed around the ferrule connection to prohibit light from leaking out of the ferrule. We recorded 56 single units from 4 mice in this experiment, and each was tested with 100 laser pulses at each condition (1mW, $\sim 2.5\text{mW/mm}^{-2}$, 1sec laser on, 3 sec off). Several neurons responded only to the blue laser brain stimulation but not to any other condition (e.g. Figure 5D). However, a number of other neurons responded to yellow brain and retinal stimulation, or to both green and yellow brain and retinal stimulation (e.g. Figure 5E, F). The cumulative distributions of neurons responding within a given latency window following the laser pulses are reported in Figures 5G, H, and I. Interestingly, all retinal-driven responses occurred $>25\text{ms}$ following the laser pulse, indicating that our cut-off of 15ms for stringently identifying striatal projection neurons would be unaffected by these visual responses. Fortunately for the use of ChR2, the blue laser was not effective in eliciting visual responses, compared to the yellow and green lasers. It is unclear why this was the case, although it may relate to the cone opsins expressed in the mouse retina, which have peak sensitivity to green and ultraviolet light, but are minimally sensitive to wavelengths in the blue range [11].

This experiment highlights a simple but important confound for experiments involving behavioral optogenetics. Even when pains are taken to prevent light escaping from the fiber optic connections outside of the mouse's head (as in the above experiment), it is possible that the light scattered through brain tissue can reach the back of the retina from the inside of the mouse's head. Depending on the experimental conditions and the structure being studied, it is extremely important to consider whether this might confound results. Mice that express YFP without ChR2 are therefore a necessary control for behavioral experiments where the light might distract the mouse, provide a cue, or otherwise influence the mouse's behavior. When using optogenetics for neural identification, pains should be taken to ensure that increases in firing around the laser pulse are not due to visual responses. In the striatum, the delayed timecourse of visual responses that we observed ($>25\text{ms}$) should allow researchers to work within the sub 15ms realm for identification, but such visual responses may occur more rapidly in other structures such as thalamus, cortex, or superior colliculus. In addition, our data show that opsins that are activated in the green-yellow range (such as halorhodopsin and archaerhodopsin) may be especially susceptible to this experimental confound. When testing optogenetic identification with new opsins, or in new brain regions, it will be important to characterize potential visual responses before concluding that neural activations are truly driven by opsin-mediated depolarization.

Photoelectric artifacts

Finally, we considered whether photoelectric effects could contribute to our results. The photoelectric effect occurs when a material absorbs electromagnetic radiation and emits electrons. In practice, this can cause voltage deflections when an electrode is illuminated by laser light [2]. We looked for evidence of this on our recording electrodes by positioning them in a saline bath and shining the blue laser on their tips with the same 1 sec stimulation protocol we used for identification (Figure 6A). Electrical activity in our system is band-pass filtered into two data-streams: a high-frequency data stream (150–8000Hz) which is analyzed for unit spiking, and a low-frequency data stream (0.7–300Hz) which is analyzed for local field potentials (LFPs). We observed photoelectric deflections in the low-frequency data (Figure 6B, C), but did not observe any events larger than the noise band ($\sim 40\mu\text{V}$) in the high-frequency data stream on any channels of the array. To investigate if the noise band

itself was increased during the laser pulse, we set the spike detection software to identify the noise events on each wire, and quantified whether the frequency of these events increased during the laser pulse. We did not find a significant increase in noise events on any wire of the array. We therefore conclude that the photoelectric effect could not account for increases in single unit or multiunit spiking in our experiments. However, the photoelectric effect was observable on our LFP recordings, indicating that it should be considered when examining LFP recordings during laser stimulation.

Discussion

Neuronal circuits contain a wide structural, molecular, and functional diversity of different cell types that act together in concert. One of the major challenges facing neuroscientists is to develop new approaches to discover how these different cell types operate together as a single neuronal circuit in the awake behaving animal. Unfortunately, existing techniques for distinguishing among different cell types *in vivo* using electrophysiological recordings are generally either limited in their reliability, or so technically difficult that they are only employed by a small number of labs [17]. Optogenetic identification of specific classes of genetically identifiable cells *in vivo* promises to help bridge this gap by allowing researchers to discern the molecular identity of individual electrophysiological units recorded *in vivo*.

In this study, we characterized multiple potential caveats that can arise when using ChR2 to identify striatal projection neurons. These include challenges of spike sorting during laser stimulation, synaptic activation or disinhibition, short-latency visual responses, and photoelectric responses of the electrodes themselves. We were able to investigate and rule out these potential caveats and conservatively identify about 13% of our recorded neurons as striatal projection neurons of one pathway or the other. We conclude that groups employing this technique in the striatum should (1) test cells at a range of laser powers to obtain their optimal spiking response, and (2) identify neurons based on short-latency (<15ms) responses to the laser. Although we tested cells with a protocol that illuminated them for 1 sec, it is likely that 100ms would be sufficient for most identification procedures unless an experimenter wished to characterize laser-evoked activation or suppression of firing that extended beyond this time window. As one final point, we stimulated each cell 100–200 times under the conditions reported in this paper. In a subset of pilot experiments we have tried stimulating cells with many more pulses (1000–5000 pulses). The resulting increase in statistical power from including many more laser pulses allowed a modest increase in identification efficiency. Therefore in practice it may be beneficial to increase the number of laser pulses to increase identification efficiency.

The approach we describe here is tailored to the striatum, a nucleus made up entirely of inhibitory and neuromodulatory cell types. The quantitative methods and logical arguments we use to identify striatal projection neurons should also be effective for identifying certain other cell types such as cerebellar Purkinje cells and hippocampal CA1 pyramidal cells that do not form local excitatory contacts onto one another. However, optogenetic identification of neuronal cell types in recurrently connected excitatory structures, such as cortex or the CA3 region of hippocampus will require a very different approach. In excitatory brain regions, direct optogenetic activation of even a small subset of neurons will result in rapid synaptic activation of a large number of non-ChR2-expressing neurons. The timecourse of this monosynaptic activation will be faster and more potent than the indirect, disinaptic, striatal disinhibition we discuss in this manuscript, and will therefore be significantly more difficult to discriminate from the direct opsin-mediated activation of ChR2-expressing neurons. Additional approaches, such as using high-frequency stimulation may be needed to differentiate opsin-mediated activation from synaptic activation in these structures [18].

Using opsins to identify specific cell types in awake recordings is a relatively new technique which we believe will improve in the coming years. In fact, we hope that this is the case, as our present yield averaged only 2.2 identified cells per 32 wire array, which was somewhat disappointing. Improvements that might increase identification efficiency include restricting opsin expression to a tighter area around the recording array, or using multiple light sources that can better illuminate specific recording sites in a focal manner [22]. Additional advances will also likely come from improved analysis methods for detecting laser activated spikes. To this end, we have published the spiking data from these experiments and our present code for identifying laser responses online (at http://uri.neuinfo.org/nif/nifstd/nlx_151410). It is our hope that this dataset and code will assist other researchers to investigate and improve the utility of optogenetics for identifying specific cell types in awake recordings.

Methods

Subjects

Bacterial artificial chromosome (BAC) transgenic mouse lines that express Cre-recombinase or fluorophores under control of the Dopamine D1 receptor (D1-Cre), D2 receptor (D2-cre) or A2A receptor (A2A-Cre) regulatory elements were obtained from GENSAT. Animals entered the study at ~6 weeks of age, weighing ~20–25 grams. All procedures were approved by the UCSF Institutional Animal Care and Use Committee.

Viral expression of DIO-ChR2-YFP and DIO-YFP

We used double-floxed inverted (DIO) constructs to express ChR2-YFP or ChR2-mCherry (c-Fos experiments only) fusion protein in Cre-expressing neurons. The double-floxed reverse ChR2-YFP or YFP cassette was cloned into a modified version of the pAAV2-MCS vector (Stratagene, La Jolla, CA) carrying the EF-1 α promoter and the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) to enhance expression. The recombinant AAV vectors were serotyped with AAV1 coat proteins and packaged by the viral vector core at the University of North Carolina. The final viral concentration was 4×10^{12} virus molecules/mL (by Dot Blot, UNC vector core).

Stereotaxic viral injections and recording array implants

Anesthesia was induced with a mixture of ketamine and xylazine (100mg ketamine + 5mg xylazine per kg body weight co-injected IP), and maintained with 0.5 – 1.0% isoflurane through a nose cone mounted on a stereotaxic apparatus (Kopf Instruments). The scalp was opened and a craniotomy was drilled large enough to accept the microwire array (centered on +0.8mm AP, $\pm$ 1.5mm ML from Bregma). 1 μ L of DIO ChR2-YFP virus was injected into the center of this craniotomy –2.7mm DV from top of brain) through a 33 gauge steel injector cannula (Plastics1) using a syringe pump (World Precision Instruments) over 10 min. The injection cannula was left in place for 5 min following the injection, and then slowly removed. Two skull screws were then implanted in the opposing hemisphere. Dental adhesive (C&B Metabond, Parkell) was used to fix the skull screws in place and coat the surface of the skull. An array of 32 microwires (35- μ m tungsten wires, 100- μ m spacing between wires, 200- μ m spacing between rows; Innovative Physiology) and one optical fiber (200 μ m core, numerical aperture of 0.37) was lowered into the striatum (2.5mm below the surface of the brain) and cemented in place with dental acrylic (Ortho-Jet, Lang Dental). After the cement dried, the scalp was sutured shut around the implant. To allow time for viral expression and recovery from surgery, animals were housed for at least 2 weeks before any recordings were initiated. Recordings in this study took place between 2.5 and 9 weeks, and we did not notice any systematic change in yield of recorded units, or ChR2-identified

units across this time period. All surgical procedures were performed under aseptic conditions.

***In vivo* electrophysiology**

Voltage signals from each recording site on the microwire array were band-pass-filtered, such that activity between 150 and 8,000 Hz was analyzed as spiking activity, and data between 0.7 and 300 Hz was analyzed as local field potentials. Each data stream was amplified, processed and digitally captured using commercial hardware and software (Plexon). Single units were discriminated with principal component analysis (Offline sorter, Plexon). Multiple criteria were used to ensure quality of recorded units: (1) recorded units smaller than 100 μ V (~3 times the noise band) were excluded from further analysis (2) recorded units in which more than 1% of interspike intervals were shorter than 2 ms were excluded from further analysis, and (3) spikes were observed separately in the baseline period, and the period of laser identification to ensure that clustering was not lost during this identification. During the recording we coupled the array to a laser and pulsed the laser at four intensities (0.1 mW, 0.3 mW, 1 mW, and 3 mW, ~1, 2.5, 10, and 25 mW/mm²). Laser power was calibrated before each recording by measuring the power at the tip of the patch-cord with a PM100D optical power meter with S120C sensor (Thorlabs), and multiplying that power by the transmittance of the ferrule connection on each optrode array (transmittance was measured before implantation). Laser stimulation was run in a cyclical fashion, on for 1 second, and off for 3 seconds. Each neuron received 100–200 pulses at each laser intensity.

Identification of ChR2 expressing units in *in vivo* recordings

We developed a Matlab tool for statistical analysis of whether individual neurons increased their firing in response to laser stimulation. The baseline activity of each neuron was determined by calculating a histogram of the firing rate in 5 ms bins over a range from 250 to 750 ms before each laser pulse, then introducing a randomized offset to the spike train from each pre-pulse baseline window and re-calculating the histogram. This process was repeated 500 times to calculate a comprehensive distribution of firing rates (e.g. Figure 1F). The firing rate of each 5 ms bin during the laser pulse was compared this distribution of possible firing rates (e.g. colored lines in Figure 1F). Bins during the laser pulse were scored as significant if (1) the firing rate was greater than 99.5% of the shuffled baseline bins, and (2) the bin contained at least 3 spikes. A neuron was counted as laser-activated if two or more bins during the laser pulse exceeded these significance criteria. In order to prevent a single burst of activity from an otherwise quiescent cell from counting as statistically significant, the histogram of laser pulse firing was calculated 50 times, excluding 2% of the laser pulses from each histogram. Only bins that passed significance on all 50 trials were counted as significant.

The virtue of this approach is its ability to empirically score the significance of the firing rate at each time point within the laser pulse without making any assumptions about the structure of the baseline activity of the neuron. The Matlab tool we developed reads in a file of comma separated values (*.csv), containing a single column of laser pulse times, followed by columns containing the spike times for each neuron. The user inputs the laser pulse duration and selects the pre-pulse baseline period, the bin width and the significance criteria.

Histology

Animals were sacrificed with a lethal dose of ketamine and xylazine (400 mg ketamine + 20 mg xylazine per kg body weight IP). Animals that had microwire implants received a current injection (10 μ A for 5 sec) through each microwire to lesion the wire tips. All animals were

transcardially perfused with phosphate buffered saline (PBS), followed by 4% paraformaldehyde. Following perfusion, brains were left in 4% paraformaldehyde for 16–24 hours and then moved to a 30% sucrose solution in PBS for 2–3 days. Brains were then frozen and cut into 60 μ m coronal sections with a sliding microtome (Leica Microsystems, Wetzlar, Germany, model SM2000R) equipped with a freezing stage (Physiotemp, Clifton, NJ). To identify fiber locations, relevant sections were identified and mounted on slides. Sections were then photographed in bright field and fluorescence. From these photographs, fiber tip locations were identified and marked on a coronal schematic drawing of the striatum at 0.5 mm anterior to bregma. For the c-Fos experiments, tissue was stained with an anti-c-Fos primary antibody, and a far-red (647nm) secondary antibody. The dorsomedial striatum just under the fiber tip was imaged on a three color Biorad 2000 confocal microscope (488nm, 564nm, and 647nm excitation lasers), and cells that expressed both Fos and *tmt* or GFP fluorophores were counted by eye.

References

1. Cardin JA, Carlen M, Meletis K, Knoblich U, Zhang F, Deisseroth K, Tsai LH, Moore CI. Driving fast-spiking cells induces gamma rhythm and controls sensory responses. *Nature*. 2009; 459(7247): 663–667. [PubMed: 19396156]
2. Cardin JA, Carlen M, Meletis K, Knoblich U, Zhang F, Deisseroth K, Tsai LH, Moore CI. Targeted optogenetic stimulation and recording of neurons in vivo using cell-type-specific expression of Channelrhodopsin-2. *Nat Protoc*. 2010; 5(2):247–254. [PubMed: 20134425]
3. Cohen JY, Haesler S, Vong L, Lowell BB, Uchida N. Neuron-type-specific signals for reward and punishment in the ventral tegmental area. *Nature*. 2012; 482(7383):85–88. [PubMed: 22258508]
4. Davies DL, Bouldin DW. A cluster separation measure. *IEEE Trans Pattern Anal Mach Intell*. 1979; 1(2):224–227. [PubMed: 21868852]
5. Fenno L, Yizhar O, Deisseroth K. The development and application of optogenetics. *Annu Rev Neurosci*. 2011; 34:389–412. [PubMed: 21692661]
6. Gage GJ, Stoetznner CR, Wiltschko AB, Berke JD. Selective activation of striatal fast-spiking interneurons during choice execution. *Neuron*. 2010; 67(3):466–479. [PubMed: 20696383]
7. Gittis AH, Kreitzer AC. Striatal microcircuitry and movement disorders. *Trends Neurosci*. 2012; 35(9):557–564. [PubMed: 22858522]
8. Gittis AH, Nelson AB, Thwin MT, Palop JJ, Kreitzer AC. Distinct roles of GABAergic interneurons in the regulation of striatal output pathways. *J Neurosci*. 2010; 30(6):2223–2234. [PubMed: 20147549]
9. Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A, Leblanc G, Hatten ME, Heintz N. A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature*. 2003; 425(6961):917–925. [PubMed: 14586460]
10. Ibanez-Sandoval O, Tecuapetla F, Unal B, Shah F, Koos T, Tepper JM. A novel functionally distinct subtype of striatal neuropeptide Y interneuron. *J Neurosci*. 2011; 31(46):16757–16769. [PubMed: 22090502]
11. Jacobs GH, Neitz J, Deegan JF 2nd. Retinal receptors in rodents maximally sensitive to ultraviolet light. *Nature*. 1991; 353(6345):655–656. [PubMed: 1922382]
12. Kasanetz F, Riquelme LA, O'Donnell P, Murer MG. Turning off cortical ensembles stops striatal Up states and elicits phase perturbations in cortical and striatal slow oscillations in rat in vivo. *J Physiol*. 2006; 577(Pt 1):97–113. [PubMed: 16931555]
13. Kitano K, Cateau H, Kaneda K, Nambu A, Takada M, Fukai T. Two-state membrane potential transitions of striatal spiny neurons as evidenced by numerical simulations and electrophysiological recordings in awake monkeys. *J Neurosci*. 2002; 22(12):RC230. [PubMed: 12045235]
14. Kravitz AV, Freeze BS, Parker PR, Kay K, Thwin MT, Deisseroth K, Kreitzer AC. Regulation of parkinsonian motor behaviours by optogenetic control of basal ganglia circuitry. *Nature*. 2010; 466(7306):622–626. [PubMed: 20613723]

15. Kravitz AV, Kreitzer AC. Optogenetic manipulation of neural circuitry in vivo. *Curr Opin Neurobiol.* 2011; 21(3):433–439. [PubMed: 21420852]
16. Kravitz AV, Tye LD, Kreitzer AC. Distinct roles for direct and indirect pathway striatal neurons in reinforcement. *Nature Neuroscience.* 2012; 15:816–818.
17. Lapray D, Lasztoczi B, Lagler M, Viney TJ, Katona L, Valenti O, Hartwich K, Borhegyi Z, Somogyi P, Klausberger T. Behavior-dependent specialization of identified hippocampal interneurons. *Nat Neurosci.* 2012; 15(9):1265–1271. [PubMed: 22864613]
18. Lima SQ, Hromadka T, Znamenskiy P, Zador AM. PINP: a new method of tagging neuronal populations for identification during in vivo electrophysiological recording. *PLoS One.* 2009; 4(7):e6099. [PubMed: 19584920]
19. Mahon S, Vautrelle N, Pezard L, Slaght SJ, Deniau JM, Chouvet G, Charpier S. Distinct patterns of striatal medium spiny neuron activity during the natural sleep-wake cycle. *J Neurosci.* 2006; 26(48):12587–12595. [PubMed: 17135420]
20. Nicolelis, M., editor. *Methods for Neural Ensemble Recordings.* 2008. 2011/01/05 ed.
21. Royer S, Zemelman BV, Barbie M, Losonczy A, Buzsaki G, Magee JC. Multi-array silicon probes with integrated optical fibers: light-assisted perturbation and recording of local neural circuits in the behaving animal. *Eur J Neurosci.* 2010; 31(12):2279–2291. [PubMed: 20529127]
22. Royer S, Zemelman BV, Losonczy A, Kim J, Chance F, Magee JC, Buzsaki G. Control of timing, rate and bursts of hippocampal place cells by dendritic and somatic inhibition. *Nat Neurosci.* 2012; 5(5):769–775. [PubMed: 22446878]
23. Sparta DR, Stamatakis AM, Phillips JL, Hovelso N, van Zessen R, Stuber GD. Construction of implantable optical fibers for long-term optogenetic manipulation of neural circuits. *Nat Protoc.* 2012; 7(1):12–23. [PubMed: 22157972]
24. Taverna S, Ilijic E, Surmeier DJ. Recurrent collateral connections of striatal medium spiny neurons are disrupted in models of Parkinson's disease. *J Neurosci.* 2008; 28(21):5504–5512. [PubMed: 18495884]
25. Tunstall MJ, Oorschot DE, Kean A, Wickens JR. Inhibitory interactions between spiny projection neurons in the rat striatum. *J Neurophysiol.* 2002; 88(3):1263–1269. [PubMed: 12205147]
26. Tye KM, Deisseroth K. Optogenetic investigation of neural circuits underlying brain disease in animal models. *Nat Rev Neurosci.* 2012; 13(4):251–266. [PubMed: 22430017]
27. Wu Y, Parent A. Striatal interneurons expressing calretinin, parvalbumin or NADPH-diaphorase: a comparative study in the rat, monkey and human. *Brain Res.* 2000; 863(1–2):182–191. [PubMed: 10773206]
28. Zhao S, Ting JT, Atallah HE, Qiu L, Tan J, Gloss B, Augustine GJ, Deisseroth K, Luo M, Graybiel AM, Feng G. Cell type-specific channelrhodopsin-2 transgenic mice for optogenetic dissection of neural circuitry function. *Nat Methods.* 2011; 8(9):745–752. [PubMed: 21985008]

Figure 1.

A. Ferrule fiber assembly for attaching to microwire array. **B.** Microwire array with ferrule fiber assembly attached. **C.** Mouse with implanted array hooked up to recording system and 473nm laser. **D, E.** Peri-event raster and histogram of a laser-activated unit. **F.** Distribution of shuffled baseline firing rate bins (black bars), plus firing rate in baseline (red bar), 15ms after laser onset (blue bar), and 100ms after laser onset (green bar). **G.** Cumulative percent of recorded single units that were significantly activated at different times following the laser onset. **H.** Percent of trials in which the laser stimulation elicited a spike within 100ms, at the 4 different laser intensities. Spike fidelity for individual mice is shown in gray, and the average fidelity is shown in black.

Figure 2.

A. Photograph of a coronal brain slice showing wire tracts and two lesion sites of electrodes (arrows) within the dorsomedial striatum. Scale bar represents 0.5mm. **B.** Photograph of the same slice as **A** in the green fluorescence channel showing ChR2-YFP fluorescence in the dorsomedial striatum around the electrode lesion site. The large circular area lacking fluorescence was likely caused by the lesion procedure denaturing the fluorescent YFP protein. **C.** Schematic of a coronal brain section (+0.5mm from Bregma), with the estimated centerpoints (blue circles) and medial-lateral spread (blue bars) of the arrays in this experiment. **D.** Schematic showing orientation of the 4x8 electrode array, and the target placement of the 200μm optical fiber. Color scale represents number of ChR2-identified units recorded on each wire of the array (n=19 identified cells across 10 mice).

Figure 3.

A. Waveforms and PCA clustering for 2 single units and 1 multiunit across a 30 minute baseline period devoid of laser stimulation. **B.** Waveforms and PCA clustering for these same units across a 30 minute period during the laser identification protocol. **C–E.** Peri-event histograms showing spiking responses of the multiunit and both single units to the laser pulses. **F–I.** Same graphical conventions as **A–E**, showing a channel that did not maintain unit separation during the laser pulse period. **J.** Cumulative percent of recorded single and multi-units that were significantly activated at different times following the laser onset.

Figure 4.

A. Example PSTH showing a neuron that was inhibited during the laser pulse. **B.** Average PSTH of all recorded cells (n=148). **C.** Percent of cells activated or suppressed at different 50ms bins following the onset of the laser pulse. **D.** Photograph of the dorsomedial striatum of a mouse that expresses ChR2-YFP (green) in D2 cells, *tmt* fluorophore (red) in D1 cells, and was stained for c-Fos (blue). Scale bar represent 50μM. **E.** Quantification of number of c-Fos+ cells that were also *tmt* positive and negative. **F.** Photo of the dorsomedial striatum of a mouse that expresses ChR2-mCherry (red) in D1 cells, GFP fluorophore (green) in D2

cells, and was stained for c-Fos (blue). **G.** Quantification of number of c-Fos+ cells that were also GFP positive and negative.

Figure 5.

A, B, C. Schematics showing brain and retinal stimulation paradigm for different wavelength lasers. **D.** Example of a unit that responded to the blue brain stimulation, but no other condition. **E.** Example of a unit that responded to yellow brain and retinal stimulation. **F.** Example of a unit that responded to green and yellow brain and retinal stimulation. **G.** Percent of recorded units that were significantly activated at different times following the blue laser onset for brain and retinal stimulations. **H, I.** Same conventions as in **G**, except data shown for green and yellow lasers, respectively.

Figure 6.

A. Photograph of experimental setup with 32 microwire array in the saline bath, and laser positioned to illuminate the wire tips. **B.** Photoelectric response of the LFP to the 1s laser pulse (top), as well as raster and histogram showing that the frequency of large-amplitude noise events on that same channel did not increase during the laser pulse (bottom). **C.** Same data as in **B**, zoomed in around the first 100ms of the laser pulse.

Table 1

Comparison of recording quality for between the baseline period and laser stimulation period.

Behavioral period	Waveform Amplitude (mV)	Waveform shape correlation (R^2)	J3 index	Davies-Bouldin index
Baseline	170±240	0.96±0.01	2.73±0.49	0.28±0.01
Laser stim	168±25		2.47±0.52	0.32±0.03
p-value	0.47	NA	0.18	0.07

All values presented as mean ± standard error, and p-values represent t-tests between baseline and laser stimulation periods.