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Kenneth John Burke, Jr. April 4, 2012

Local NMDA Antagonism Prevents Inversion of Dopamine Responses in Striatal Medium Spiny Neurons in the Parkinsonian Macaque

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Abstract
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Dopaminergic innervation of medium spiny neurons (MSN) of the striatum is essential for normal motor behavior. However, in patients with idiopathic Parkinson's disease (PD), long-term levodopa replacement therapy is associated with debilitating motor complications such as involuntary choreiform movements (levodopa-induced dyskinesias, LID). In non-human primate models of advanced parkinsonism with chronic levodopa therapy, striatal MSNs are profoundly hyperactive and often exhibit reversal of levodopa-induced firing rate changes ("inversions") that is highly correlated with the onset of LID. It was hypothesized that baseline hyperactivity mediated by hyperglutamatergic tone may lead to these abnormal inversions and are ultimately the cause of LID. To address this hypothesis, local microinjections of the subunit non-selective competitive NMDA receptor antagonist LY235959 were performed at the site of extracellular recordings in the striatum in two awake, behaving rhesus monkeys with MPTP-induced advanced parkinsonism with LID using an injectrode apparatus, followed by subcutaneous systemic levodopa administration. We found that local microinjection of the vehicle (artificial cerebral spinal fluid, aCSF) alone had no effect on firing rates and did not alter the pathological heterogeneity of MSN responses to systemic levodopa, whereas reduction of baseline activity via local microinjections of the competitive NMDA antagonist at a 9 mM concentration completely abolished the abnormal inversion responses of MSNs to systemic levodopa. This finding has profound implications for the elucidation of the pathophysiological mechanisms underlying LID, as well as the development of pharmacological agents designed to alleviate dyskinetic side effects of chronic levodopa replacement therapy in idiopathic PD.

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INTRODUCTION

Parkinson's Disease: Clinical Symptoms and Treatment

Parkinson's disease (PD) is a neurodegenerative disease of the central nervous system. Idiopathic PD is relatively common, affecting approximately 4% of the population over the age of 80 (Davie, 2008). It presents with "four cardinal features" (Jankovic, 2012) that can be used to differentiate it from other motor disorders: resting tremor, rigidity, akinesia (or bradykinesia) and postural instability. Together, the exhibition of these motor features is known as the "off state", whereas the state associated with the reversal of these symptoms with treatment is known as the "on state" (Mosley et al., 2010). These "off" symptoms typically worsen with time, especially if left untreated (Clarke, 2007). Patients also present in the early stages of the disease with hyposmia (impaired sense of smell), depression, constipation and other non-motor symptoms, for unknown reasons (Factor & Weiner, 2008, pp. 46).

It is believed that the cause of the motors symptoms of PD is largely due to the progressive degeneration of midbrain dopaminergic projection neurons, originating in the substantia nigra pars compacta (SN_c) and terminating in the nuclei of the basal ganglia (BG; Lee et al., 1995). However, in spite of the great deal of knowledge obtained about physiological mechanisms of the disease, its diagnosis remains primarily clinical. Current estimates suggest that onset of motor symptoms occurs after approximately eighty percent of SN_c neurons have died (Mosley et al., 2010). While neuroprotective therapies or other treatments designed to slow the progression of the disease are not yet available (Jankovic, 2012), there are a number of treatments that can substantially alleviate symptoms. The most effective treatment to date is dopamine replacement with the dopamine precursor, levodopa (l-dopa; Clarke, 2007). Systemic administration of this drug, alongside a peripheral decarboxylase inhibitor "carbidopa" to prevent

peripheral metabolism, has proven highly effective at reversing the motor symptoms of PD (Davie, 2008). This carbidopa-levodopa therapy is thought to act because levodopa can cross the blood-brain barrier, be converted to dopamine in the midbrain, and provide the denervated striatum with excess dopamine, thus compensating for the decreased nigrostriatal dopamine inputs (Nutt et al., 1985; Abrams et al., 1971).

Levodopa-Induced Dyskinesia

In spite of the initial efficacy of levodopa treatment, a number of complications develop with the prolonged employment of this so-called dopamine replacement therapy. These adverse effects, although not fully understood, are though to be related to the "pulsatile" nature of the dopamine exposure (Nutt, 2007). Such complications include a shorter "on state" with a faster reappearance of motor disability ("wearing off"), sudden akinetic episodes, and failures to respond to levodopa ("off" resistant periods) (Clarke 2007; Davie 2008). Of particular interest, both in the clinic and this study, is the development of debilitating choreodystonic involuntary movements known as "levodopa-induced dyskinesia" (LID), present in over half of PD patients after chronic levodopa replacement therapy for five years (Wolf et al., 2010; Mosley et al., 2010; Rascol et al., 2000). Management of these side effects remains one of the most common challenges in late-stage PD, with only limited pharmacological and surgical options available (Wolf et al., 2010; Guridi et al., 2008). Because the side effect is associated with dopamine replacement, patients must often alter their treatment regimen and effectively balance LID with "off" motor disability (National Collaborating Centre for Chronic Conditions [NCC-CC], 2006). This often remains a significant complication leading to surgical intervention for treatment with "deep brain stimulation" (DBS; Elia et al., 2012). In spite of its profound impact on the quality of life of patients with PD, the pathophysiology of LID remains obscure.

MPTP-Induced Parkinsonism

One widely accepted animal model of chronic advanced parkinsonism is the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model in rhesus macaques (Langston et al., 1983; Dauer and Przedborski, 2003). This neurotoxin was discovered when drug addicts attempted to illegally synthesize a structural analog of the narcotic meperidine that yielded MPTP as a byproduct (Dauer and Przedborski, 2003). After injecting MPTP intravenously, these individuals rapidly developed all cardinal motor deficits indicative of idiopathic PD. Interestingly, in both human and nonhuman primate examples of MPTP-induced parkinsonism, many other subtleties of idiopathic PD are reproduced with surprising faithfulness, including the benefits and limitations of levodopa (Papa and Chase, 1996; Rose et al., 1993). In MPTP-induced parkinsonian macaques, chronic levodopa treatment is associated with both a variable "on response" with motor improvement, as well as the eventual development of LID (Guigoni et al., 2005). Thus, the nonhuman primate MPTP model of chronic parkinsonism is considered a valid model of downstream motor complications associated with the natural disease.

While the exact mechanism of action of MPTP remains somewhat controversial, in recent years a number of possibilities have emerged to further confirm the validity of the model. First, MPTP is taken into dopaminergic cells in its actively toxic form 1-methyl-4-phenylpyridinium (MPP^+) through its high affinity for the dopamine transporter (DAT; Przedborski et al., 2000). It then proceeds to interfere with complex I activity of the electron transport chain in the mitochondria, thereby inhibiting ATP production, generating free radicals and ultimately leading to cell death (She et al., 2011). Oxidative stress has been directly implicated in the development of idiopathic PD (NCC-CC, 2006). Second, in addition to the SN_c, other monoamine projection systems may also deteriorate with chronic MPTP administration (Masilamoni et al., 2011;

Mitchell et al., 1985; Nayyar et al., 2009), which may be consistent with neurodegeneration in idiopathic PD (Braak et al., 2003; Halliday et al., 1989). Thus in addition to the observed similarities in behavioral deficits, the MPTP model also shares anatomical and physiological similarities with idiopathic PD.

Basal Ganglia Circuitry

Dopaminergic input to the striatum plays an essential role in the regulation of motor behavior, action selection and associative learning (Balleine et al., 2007; Barto et al., 1995). The primary targets of nigral dopaminergic neurons are the γ-aminobutyric acid (GABA) releasing medium spiny neurons (MSNs) of the striatum, which are segregated in two subpopulations depending on the expression of dopamine D_1 and D_2 receptors that mediate excitatory and inhibitory modulation, respectively (Gerfen, 1992). The classic Albin-DeLong "box and arrow" model of BG function asserts that these D_1- and D_2-bearing cells comprise anatomically and functionally distinct "direct" and "indirect" pathways, respectively (Figure 1; Albin et al., 1989; DeLong, 1990). According to this model, the main input nucleus is the striatum (caudate/putamen), and the main output nuclei are the inhibitory neurons of the internal globus pallidus (GP_i) and substantia nigra pars reticulata (SN_r). The primary inputs driving action potential generation in striatal MSNs include diffuse glutamatergic cortical and thalamic afferents containing motor, sensory and associative information necessary for the aforementioned action selection and associative learning abilities of the circuit (Rosell & Giménez-Amaya, 1999; Smith et al., 2004). The output nuclei of the BG, in turn, inhibit various thalamic nuclei, creating the so-called BG-thalamocortical loops (Sidibé et al., 1997; Alexander et al., 1986).

D_1-bearing direct pathway MSNs inhibit the basal ganglia output nuclei and facilitate movement via disinhibition of thalamic nuclei. Further, D_2-bearing indirect pathway MSNs inhibit movement through removal of disinhibition of these same thalamic nuclei, passing information through the external globus pallidus (GP_e), then the subthalamic nucleus (STN) before ultimately converging onto the output nuclei of the basal ganglia. While a large amount of evidence exists to support the functional distinction of these two subpopulations of striatal MSNs (Kravitz et al., 2010; Surmeier et al., 2007; Graybiel, 2004), recent experimental and computational models have suggested that this circuit-level analysis may overlook finer details of processing events within the BG circuit (DeLong & Wichmann, 2007; Nambu et al., 2002; Sarvestani et al., 2011).

Striatal Neurophysiology in Health and Disease

The electrophysiological and circuit properties of the two subpopulations of MSNs of the striatum are of clear importance in understanding the functional impact of SN_c dopaminergic denervation in PD. Two primary inputs to these cells that determine firing properties are glutamatergic (thalamo- and corticostriatal) and dopaminergic (nigrostriatal) inputs. One important quality of the MSNs is their differential response to dopaminergic input. The D_1-bearing direct pathway MSNs are excited by dopaminergic input, whereas the D_2-bearing indirect pathway MSNs are inhibited by dopaminergic input. The ability of dopamine to facilitate movement in the Albin-DeLong model depends on this differential response (DeLong, 1990), which is likely mediated by the "up-down state" phenomenon (Nicola et al., 2000). In vivo, both subpopulations display characteristic shifts between two membrane potentials, one semi-stable state around -55 mV and the other more stable state around -80 mV, referred to as the "up" and "down" states respectively (Day et al., 2008). The latter is close to the potassium equilibrium

potential of the cell and generates no action potentials, whereas the former state is closer to the threshold of the cell, is associated with an increase in spiking and is thought to be generated by synchronous glutamatergic input (Surmeier et al., 2007; West & Grace, 2002; Day et al., 2008). Activation of D_1 receptors modulates voltage-gated sodium and calcium channels and increases MSN sensitivity to glutamatergic input (and thus the generation of the up-state), whereas activation of D_2 receptors tends to cause opposite effects on downstream ion channel targets, ultimately decreasing the responsiveness of the MSNs to depolarization at the up-state (Surmeier et al., 2007).

Although the removal of excitatory modulation mediated by the D_1 receptor (as in the MPTP model of chronic advanced parkinsonism) might suggest a decrease in spontaneous direct pathway MSN baseline activity, this is in fact increased dramatically across MSN subpopulations. In some neurons this baseline frequency increases from approximately 0-2 Hz ("healthy state") to 23-25 Hz (parkinsonian baseline "off" state) for reasons that are not completely clear (Crutcher & DeLong, 1984; Ingham et al., 1998, Liang et al., 2008). One possible mechanism is through increased glutamatergic drive from cortical and thalamic inputs. This hypothesis is supported by the observation that dopamine denervation is associated with a subsequent loss of dendritic spines in the MSNs that may be caused by increased glutamatergic synaptic activity (Gerfen, 2006; Villalba et al., 2009). Furthermore, alterations in synaptic plasticity at the corticostriatal synapse have been observed with chronic dopamine denervation and levodopa replacement therapy, leading to strengthened corticostriatal transmission (Calabresi et al., 2007; Wilson, 2006). In rodent and nonhuman primate models of parkinsonism with LID, there is evidence of various forms of *N*-Methyl-D-aspartate (NMDA receptor) dysregulation at the corticostriatal synapse that may underlie both LID and this altered synaptic plasticity (Day et

al., 2006; Ahmed et al., 2011; Errico et al., 2011; Paillé et al., 2010; Gardoni et al., 2009; Blanchet et al., 1999). Indeed, NMDA receptor antagonists, when given systemically in conjunction with levodopa, have been demonstrated to reduce LID without compromising the antiparkinsonian efficacy of levodopa in the macaque model (Papa & Chase, 1996; Blanchet et al., 1998). Thus, it has been hypothesized that the increase in spontaneous baseline activity of the MSNs, which may lead to LID, is due to glutamatergic hyperactivity resulting from increased glutamate release (presynaptic mechanism) or MSN hypersensitivity to glutamatergic stimulation (postsynaptic mechanism).

The increase in baseline activity of MSNs in the dyskinetic primate model is accompanied by an altered response of MSNs to dopaminergic stimulation. In the MPTP macaque model of advanced parkinsonism with chronic levodopa therapy, systemic administration of levodopa leads to normalization of motor behavior (i.e. transition from the "off" to the "on" state). If the animal experiences only mild to moderate LID side effects, there is often a time window where the initial "on response" is not accompanied by LID (Porras et al., 2012). This period will be referred to as the "on without dyskinesia" state, or simply the "on state". During this "on without dyskinesia" state, roughly equal increases and decreases of firing rates of MSNs from baseline can be observed due to sampling from to the two subpopulations, with a slight preference for excitation by dopamine (Liang et al., 2008). This window is soon followed by a time period that includes both LID and the peak improvement of parkinsonian motor disability (referred to as the "dyskinetic" state; Porras et al., 2012). With the appearance of dyskinesia, some MSNs exhibit a reversal of the firing frequency changes induced at the beginning of the levodopa response and approach baseline firing frequency again (Liang et al., 2008). The maintenance of firing rate changes through dyskinesia is known as a "unidirectional

response", whereas the inversion of these changes back to pre-levodopa baseline ("off" state) is known as a "bidirectional response". These unidirectional and bidirectional responses create a marked imbalance of activity patterns across MSNs, possibly altering downstream BG circuit function and underlying the pathophysiology of levodopa-induced dyskinesia.

HYPOTHESES

Primary Hypothesis

The aforementioned evidence of hyperglutamatergic tone and altered synaptic plasticity at the corticostriatal synapse in animal models of advanced parkinsonism with LID implicates pathological hyperglutamatergic signaling in the striatum as a possible origin of the development of LID. The observation that various forms of excitatory blockade via NMDA antagonism can reduce LID lends credence to the suggestion that pharmacological intervention in glutamatergic signaling may be key in the normalization of striatal activity. In the current study we hypothesize that reducing MSN activity by local microinjection of NMDA receptor antagonists at the site of extracellular recordings in the striatum of parkinsonian macaques will reduce the bidirectional response of MSNs to systemic levodopa administration, and possibly lead to exclusive unidirectional responses by maintaining the levodopa-induced firing frequency change.

Secondary Hypotheses

This overarching hypothesis entails a number of secondary hypotheses. First, we predict that local microinjection of an NMDA receptor antagonist at sufficient concentrations will reduce firing rates of MSNs, both immediately and sustained over many tens of minutes. We also predict that, given the likely limited spatial extent of diffusion of the microinjection, the microinjection will not diminish the behavioral exhibition of LID appreciably. Finally, because the increase in baseline activity is observed in both MSN subpopulations (Liang et al., 2008), we predict that NMDA antagonism will normalize (i.e. make unidirectional) firing patterns in cells that are both inhibited and excited by subcutaneous levodopa administration.

METHODS

Subjects

Two adult female rhesus monkeys (*Macaca mulatta*, 6 kg) were used for recording experiments, henceforth referred to as "M" and "F". All procedures followed standards set by the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (1996). Chronic, advanced parkinsonism with levodopa-induced dyskinesia was reproduced in M and F as described in Liang et al. (2008). This process consisted of weekly intravenous injections of MPTP until a stable motor disability developed, as measured by the standardized Motor Disability Scale (MDS) adapted for nonhuman primates (Papa & Chase, 1996). Stability of the MDS score was considered vital in discriminating acute toxicity of MPTP from parkinsonian motor disability (Dauer and Przedborski, 2003), and neither animal had been exposed to MPTP for at least five (5) months before recordings began. Following the observation of MDS stability, the monkeys were started on a daily levodopa regimen with oral administration of carbidopa/levodopa as used in human PD patients for management of motor disability (Sinemet, 25/100 mg; Jankovic, 2012). This treatment produced clear and consistent "on" responses and eventually led to the development of severe dyskinesia in both monkeys.

Animals were tested with subcutaneous levodopa methyl ester plus benserazide injections at a range of doses to determine the effective dose to be employed in recording experiments. Doses were chosen based on the following criteria; the injection must elicit a clear "on without dyskinesia" window, followed by a clear "dyskinetic" state. While the exhibition of clear dyskinesia was vital to the experimental design, the severity of the LID exhibited during recording needed to be minimized so as to be compatible with the necessary restraint of the monkey in the primate chair during recording. Ultimately, two doses were chosen: 70 mg

levodopa with 17.5 mg benserazide for M and 150 mg with 37.5 mg benserazide for F.

Both animals were surgically implanted with a recording chamber, angled in the coronal plane to allow the trajectory of the electrode to reach the striatum, GP_e, GP_i, and STN. Basal ganglia nuclei were identified using standard methods of electrophysiological mapping in individual monkeys similar to those used intraoperatively during DBS lead implantation for the treatment of PD (Gross et al., 2006; Figure 2). These data obtained in mapping sessions were used in subsequent recording experiments to determine appropriate coordinates for the electrode track.

Experimental Equipment and Setup

The electrode apparatus used in these experiments, called an "injectrode" (*Alpha Omega*), consisted of a tungsten microelectrode (Z = 100 – 500 KΩ) passed through a microtube for local drug delivery (volume = 200 nl, rate = 1 µl/min) at the site of recording. The tip of the electrode was placed at a fixed distance from the tip of the microtube (d = 300 – 400 µm) that was intermittently measured and did not vary after repeated experiments (data not shown). Injectrodes were lowered through a computer-controlled microdrive (NAN) after penetration of the dura mater with a guide cannula. The data were acquired using a *Plexon* data acquisition system. Signals were amplified and bandpass filtered, and online sorting alongside variable gain settings was used to determine the quality of unit isolation in real time.

Local microinjection was achieved by loading the drug into the injectrode apparatus through a syringe (250 µl, Hamilton Gastight syringe, see Figure 3). The syringe was then loaded into a pump with precise control on the nanoliter range (Harvard PicoPlus Syringe Pump) and placed close to the microdrive but out of arm's reach of the animal. The subcutaneous systemic injection of levodopa was achieved by connecting a butterfly needle to a tube for remote

injection.

The animal was head-fixed during the recording experiments. Maximum freedom of movement was needed to clearly distinguish the various motor states; therefore only the head and the arm ipsilateral to the recording chamber were restrained (all other limbs had complete freedom of movement). The motor behavior of the animal was monitored remotely by video camera during the experiment.

Experimental Design

Using the aforementioned detailed mapping, the posterolateral sensorimotor putamen specifically was targeted, and the globus pallidus and premotor cortex were avoided. Furthermore, signature firing patterns of tonically active cholinergic interneurons, fast-spiking GABAergic interneurons, and fiber bundles were occasionally seen and avoided for recording (Aosaki et al., 1995; Tepper et al., 2010; Kawaguchi, 1993). In each experiment, single units (or occasionally two or three separate units) were isolated and monitored for several minutes before recording the "baseline" file segment to ensure that spontaneous activity was not obviously correlated with observed movements of the animal, and that baseline activity was reasonably stable. After recording the baseline file for approximately four minutes, the local microinjection was performed and the MSN's response to the drug administration was recorded again for four minutes.

For the purposes of this study, we chose to use a competitive, subunit-nonselective NMDA receptor antagonist that has demonstrated antidyskinetic capabilities (LY235959, "LY"; Papa & Chase, 1996) dissolved in artificial cerebrospinal fluid[1] (aCSF, *Tocris Bioscience*). The first set of experiments was designed to determine an appropriate concentration of the antagonist

[1] Highly purified H_2O with ion concentrations (in mM): Na^+ 150, K^+ 3.0, Ca^{2+} 1.4, Mg^{2+} 0.8, P^{3-} 1.0, Cl^- 155

that would reduce the activity of the recorded cells for many tens of minutes. The morning of these experiments, oral maintenance levodopa/carbidopa was withdrawn to avoid confounding effects of plasma levodopa concentrations on unit activity. Because of the presence of mechanosensitive voltage-gated sodium and potassium channels with high expression in the striatum (Fink et al., 1996; Dedman et al., 2009; Wu et al., 2002; Beyder et al., 2010), it was imperative we conduct control experiments with aCSF alone to be sure that the local microinjection itself (due to pressure) did not alter firing properties of the cell. We then tested various concentrations of the antagonist LY to see qualitatively which concentration was sufficient to reduce mean firing frequency consistently over many tens of minutes (see Results section).

Once an effective concentration was determined, the second set of experiments expanded upon this design. The morning of these experiments, the animals would have food withdrawn in addition to oral levodopa, to avoid alteration of levodopa metabolism. In addition to the initial unit isolation and local microinjection described above (9 mM LY or vehicle aCSF for control), this set of experiments incorporated subcutaneous levodopa administration after the response to local microinjection was recorded (Figure 4). Continuous monitoring of the animals revealed that for F and M, onset of the "on without dyskinesia" window began approximately 15-25 minutes after systemic levodopa, depending on the animal and the particular experiment. Wide opening of the eyes, blinking, appearing more alert and aroused, and occasional yawning and limb stretching indicated the beginning of this "on response". At the initial onset of these symptoms, unit activity was recorded for three minutes. The "dyskinetic" stage typically began approximately 20 minutes after onset of "on" symptoms, and was indicated by the presence of dyskinesia unique to each individual animal. For M, typical LID included chroeiform leg

movement and arm movement, whereas for F typical LID included a stereotyped writhing of the left hand with occasional dystonic orolingual behavior. At the onset of these symptoms, unit activity was recorded for three minutes. It is important to emphasize that the timing of recording the "on" and "dyskinetic" stages varied between animals and between experiments, as it was deemed vital to the experimental design to correlate neural activity with the levodopa response exhibited by the animal which itself varied between experiments. After the final "dyskinetic" segment of activity was recorded, the injectrode was raised and removed from the microdrive, and the animal was returned to her home cage with a reward.

Data Analysis

Under half of experiments conducted yielded usable results due to frequent failure to isolate a stable waveform of the unit for the entire recording session (typically 50-60 minutes). Experiment failures were often the result of excessive movement of the animal after levodopa administration that led to either losing the signal or a change in the recorded waveform (and thus the inability to guarantee that it was the same unit). After recording sessions, detailed spike sorting of each recording segment was performed separately offline using the Plexon Offline Sorter. Unit waveforms were separated based on principal component analysis (PCA), often incorporating the total area or peak-valley voltage difference in the distinction of units. Notes taken during the experiment were used to invalidate movement artifacts recognized by the software as spikes. After separate sorting, segments from the same experiment were compared to identify consistent units through all recorded segments. Occasionally, waveforms were found to be inconsistent between the different recording segments; these units were excluded from analysis. Three units in the LY condition were excluded from statistical analysis due to variable baseline activity. A total of thirty units (n = 10, aCSF; n = 20, LY) were ultimately included in

the subsequent statistical analyses.

After offline sorting, each segment was exported to the Neuroexplorer data analysis program. Mean frequencies of MSNs in the pre- and post-local injection conditions were compared by Student's *t*-test for paired samples in SPSS, for the aCSF and the 9 mM LY conditions separately ($\alpha = 0.05$). Spike trains of three minutes or longer were then separated into a series of one-second bins of firing frequencies, and the effects of "group" (aCSF/LY) and "state" (pre-/post-injection) were analyzed with a two-way ANOVA for repeated measures ($\alpha = 0.05$).

The aforementioned frequency bins from the four recorded segments ("PRE", "POST", "ON", "DYS") were then analyzed for each unit by one-way ANOVA for repeated measure, followed by the *post hoc* Bonferroni correction when the *F* value was significant (significance for both *F* and Bonferroni set at $\alpha = 0.05$, the Greenhouse-Geisser correction was used when Mauchly's Sphericity Test was significant at the $p < 0.05$ level). The three post-hoc comparisons that were used were 1) the PRE/POST comparison to see the effect of the local microinjection, 2) the POST/ON comparison to classify the MSN subtype (direct pathway if increased, indirect pathway if decreased), and 3) the ON/DYS comparison to classify the inversion properties of the cell (bidirectional if opposite direction as POST/ON comparison, unidirectional otherwise). Three units (cells 10, 11, & 14; Table 2) in the LY condition were excluded from further analysis due to each of the following: 1) extremely low or variable baseline firing rate (and therefore few significant changes across motor states), 2) failure to demonstrate a firing frequency response to systemic levodopa, or 3) activity patterns that upon offline inspection resembled those of tonically active cholinergic interneurons (Aosaki et al., 1995).

RESULTS

Local Microinjection of aCSF Does Not Change Firing Frequency of MSNs

The successfully isolated MSNs in the control vehicle local injection condition (aCSF, $n = 10$) were analyzed individually by one-way ANOVA for repeated measures across the four experimental stages as described above (Table 1). Each F value was significant at the $p < 0.0001$ level. With an alpha value of 0.05 for the Bonferroni *post hoc* correction, no cells showed significant changes in firing rates with the microinjection of aCSF. The mean frequencies and SEM of individual cells can also be seen in Table 1, as well as Figure 5a (error bars omitted for clarity). To compare the response of all ten analyzed cells, a paired samples t-test was performed and demonstrated no significant difference between the "Pre" and "Post" conditions ($t(9) = -1.018$, $p = 0.335$; Figure 5b).

Local Microinjection of LY235959 at 9 mM Concentration Reduces Firing Frequencies of MSNs

Two-hundred nanoliter microinjections of LY235959 at concentrations less than 9 mM produced only inconsistent, transient responses in firing frequency in MSNs (examples, Figure 6a), whereas injections at 9 mM produced significant ($p < 0.0001$) sustained decreases in firing frequencies over many tens of minutes (examples, Figure 6b). Thus, doses of 9 mM were used for the subsequent experiments analyzing systemic levodopa response.

The successfully isolated MSNs in the experimental condition (LY, $n = 17$) were also analyzed individually across the experimental stages by one-way ANOVA for repeated measures (Table 2). Each F value was significant at the $p < 0.0001$ level. With an alpha value of 0.05 for *post hoc* Bonferroni corrections, most MSNs exhibited a highly significant ($p < 0.0001$) decrease in firing frequency after LY local injection. One MSN showed an increase in firing frequency (cell 12). In order to determine if there was an overall effect of the microinjection in this

condition, the mean frequencies of all seventeen MSNs were included in a paired-samples *t*-test that demonstrated an overall significant decrease in firing frequency between the "Pre" and "Post" conditions ($t(16) = 3.500$, $p = 0.002$; Figure 7b).

To compare the effects of LY versus aCSF local microinjection, a two-way ANOVA for repeated measures was performed as described above. The result indicates a significant interaction effect ($F(1,25) = 6.322$, $p = 0.018$) between "group" (aCSF/LY) and "state" (pre- vs. post-injection), with no significant effect of "group" ($F(1,25) = 0.023$, $p = 0.880$), indicating that the decrease in firing frequency observed was evident only in the LY group, post-injection. There was also a significant effect of the "state" ($F(1,25) = 5.7$, $p = 0.024$), but this is likely due solely to the effect of LY considering the fact that the one-way ANOVAs yielded significant decreases only in the LY neurons and the vehicle injection produced no such change.

Local Microinjection of aCSF Yields a Variety of Striatal MSN Responses to Systemic Levodopa

The *post hoc* results of the one-way ANOVAs for individual neurons in the aCSF condition yielded both highly significant increases and decreases in firing rates after levodopa administration (most with $p < 0.0001$; Table 1) presumably corresponding to D_1- and D_2-receptor activation, respectively. Few cells ($n = 2$) had no significant change in response to levodopa administration (cell 5, $p = 0.068$; cell 8, $p = 0.131$). In the transition from the "on without dyskinesia" ("ON") and the "dyskinetic" ("DYS") states, the MSNs also showed both unidirectional and bidirectional responses (most with $p < 0.0001$). All of the above observations are qualitatively consistent with observations across MSNs of the caudate/putamen made by Liang et al. (2008). Graphs showing the diversity of frequency traces across motor states are shown in Figure 8.

The *post hoc* Bonferroni corrections of the one-way ANOVAs for MSNs in the LY condition showed that for all neurons with a significant decrease in firing rate after local microinjection ($n = 16$), the change in firing frequency between the "on without dyskinesia" ("ON") state and the "dyskinetic" state ("DYS") was either in the same direction as the change observed between the post-injection segment ("POST") and the "ON" state (most with $p < 0.0001$) or was insignificant (Table 2). In other words, for those cells that decreased in firing frequency after LY, increases or decreases with systemic levodopa were maintained through the dyskinetic state (i.e. no bidirectional activity patterns were observed). The cell that showed an increase in firing frequency after local LY microinjection showed bidirectional activity in response to systemic levodopa (cell 12, $p < 0.0001$ for both comparisons). The homogeneity of the responses of cells with decreases in response to local LY injection can be seen in Figure 9.

FIGURES AND TABLES

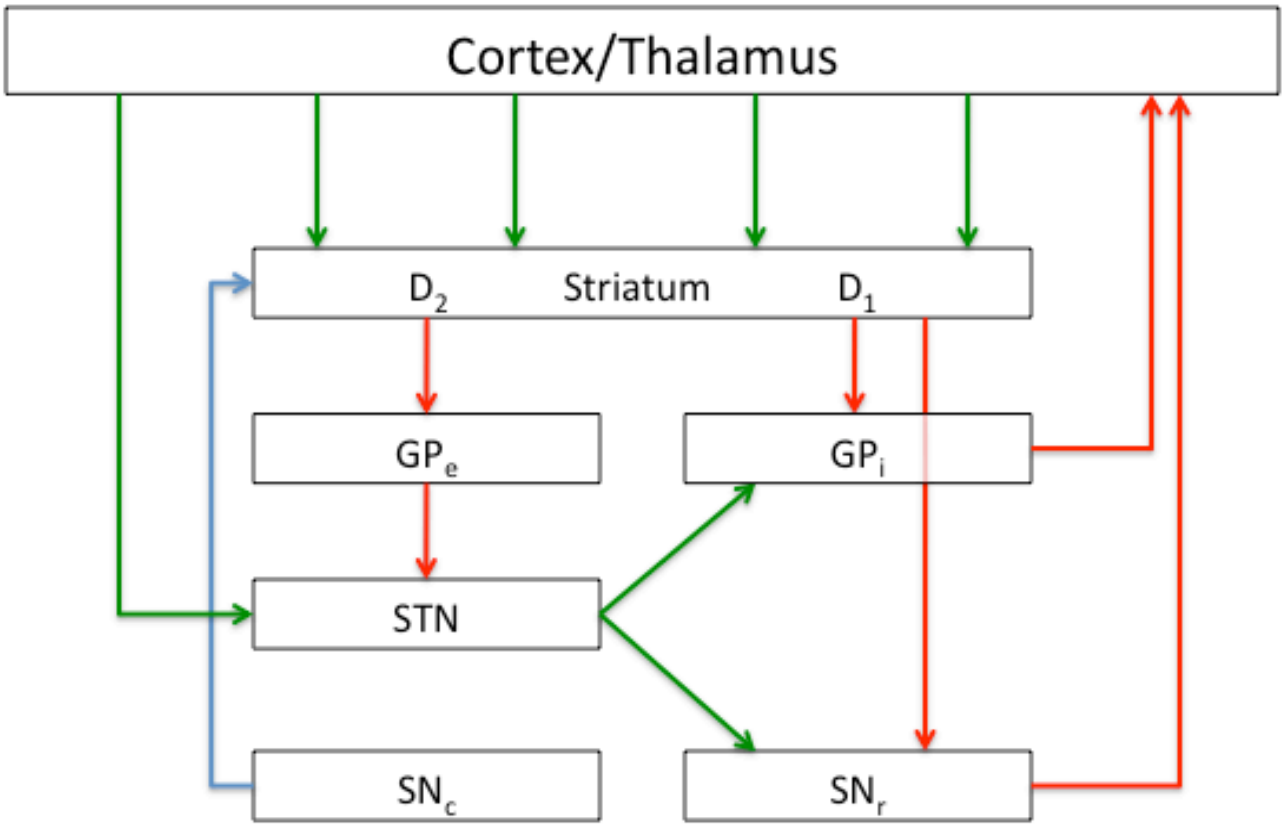

Figure 1: Classic "box-and-arrow" model of basal ganglia functional anatomy. This highly simplified model describes the macroscopic "loops" associated with the "indirect" and "direct" pathways (notice the presence of "hyperdirect" afferents to the STN). Red, green and blue arrows represent GABAergic, glutamatergic and dopaminergic projections respectively. Cortex/Thalamus, sensorimotor and associative cortical and thalamic nuclei; Striatum, caudate/putamen; D_1, D_1-bearing direct pathway neurons; D_2, D_2-bearing indirect pathway neurons; GP_e, external globus pallidus; GP_i, internal globus pallidus; STN, subthalamic nucleus; SN_c, substantia nigra pars compacta; SN_r, substantia nigra pars reticulata.

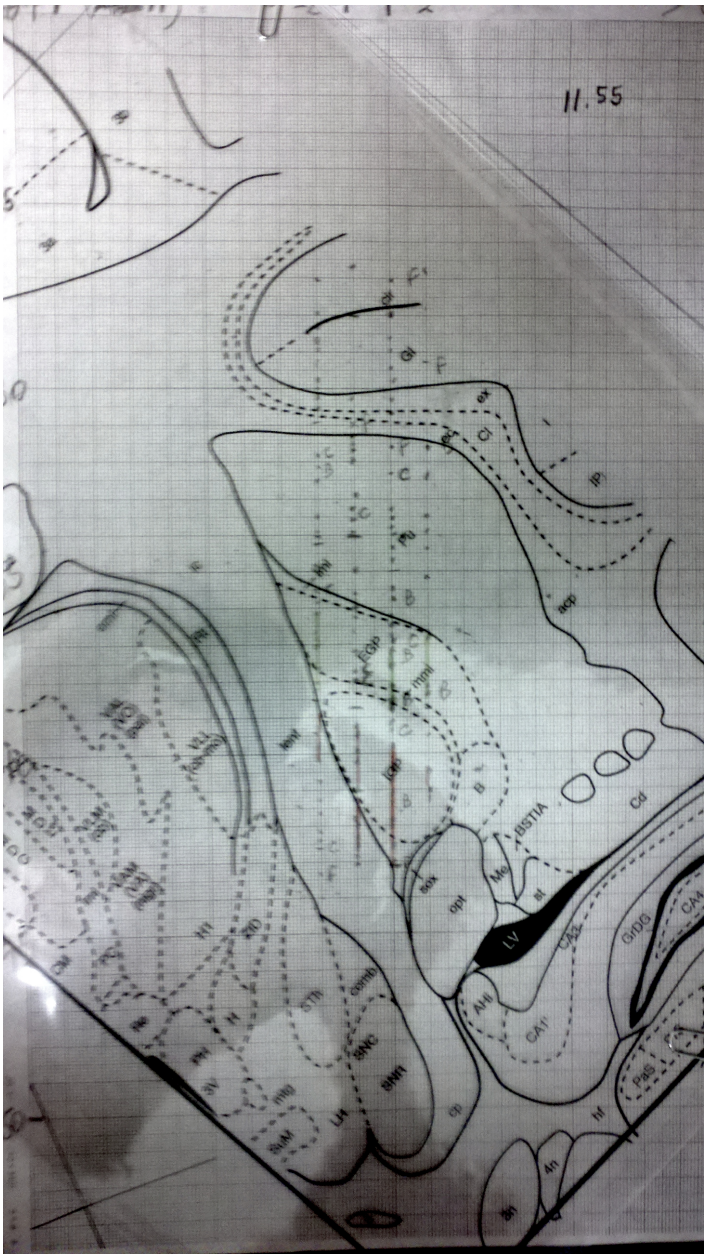

Figure 2: Example of mapping grids. Above is a transparent photocopy of a coronal section from a macaque brain atlas, superimposed at an angle consistent with the chamber posistion upon a grid with 10 μm subdivisions. Mapping procedures lowered "naked electrodes" (no injectrode) down various coordinates in the vertical grid axis, identified unit activity (represented as dots) and classified locations within the brain based on known physiological properties of the various nuclei.

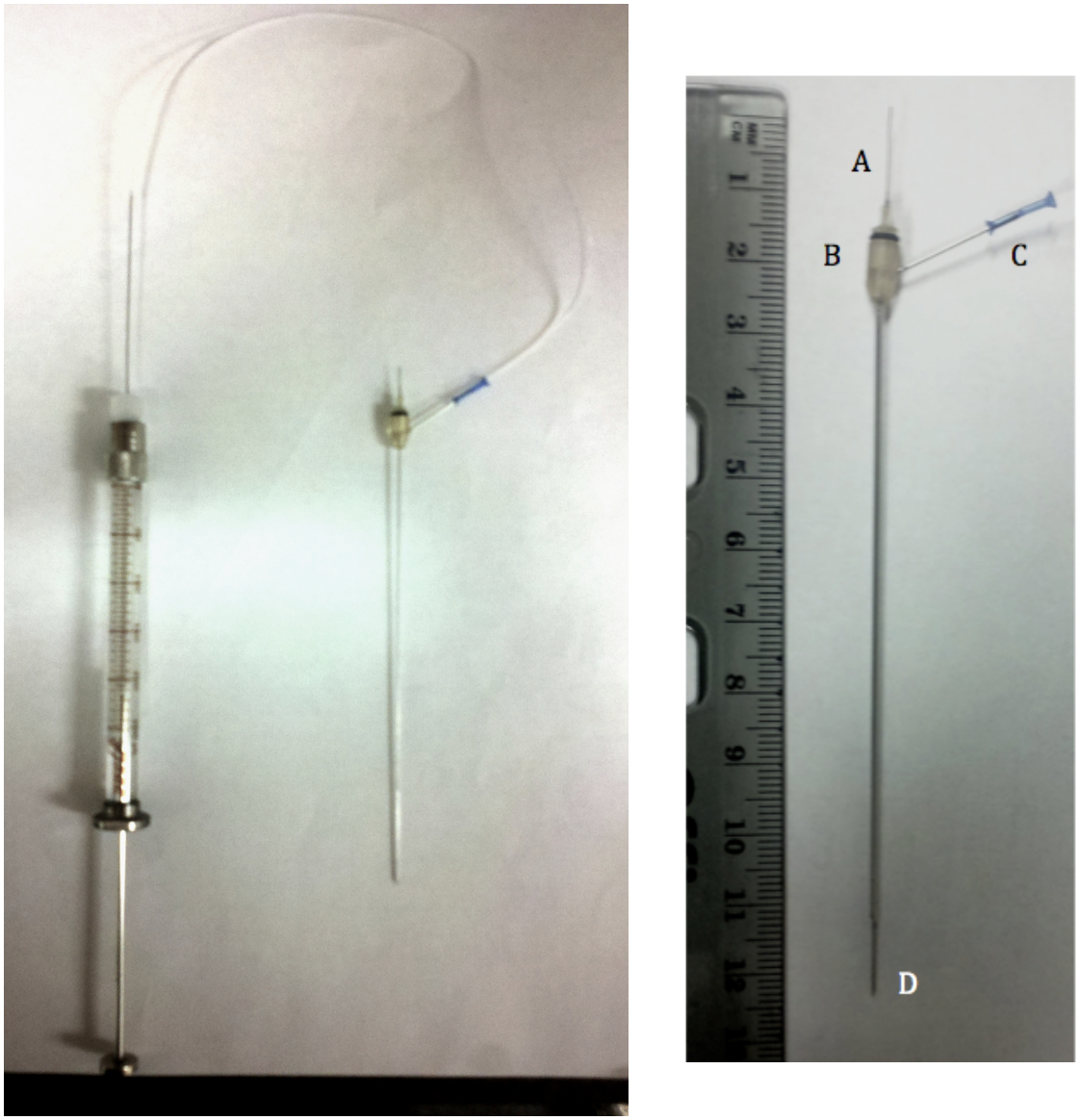

Figure 3: Injectrode Apparatus with Hamilton Syringe. *Left*: A 250 µl Hamilton Gastight syringe loads the drug into the injectrode via airtight tubing. *Right*: Close-up of the injectrode. Top of electrode (A) is visible above the electrode sealer/O-ring complex (B). The airtight tubing is connected to the body of the injectrode by an adaptor (C). At the tip of the injectrode (D), the electrode tip is exposed by approximately 300–400 µm (not visible).

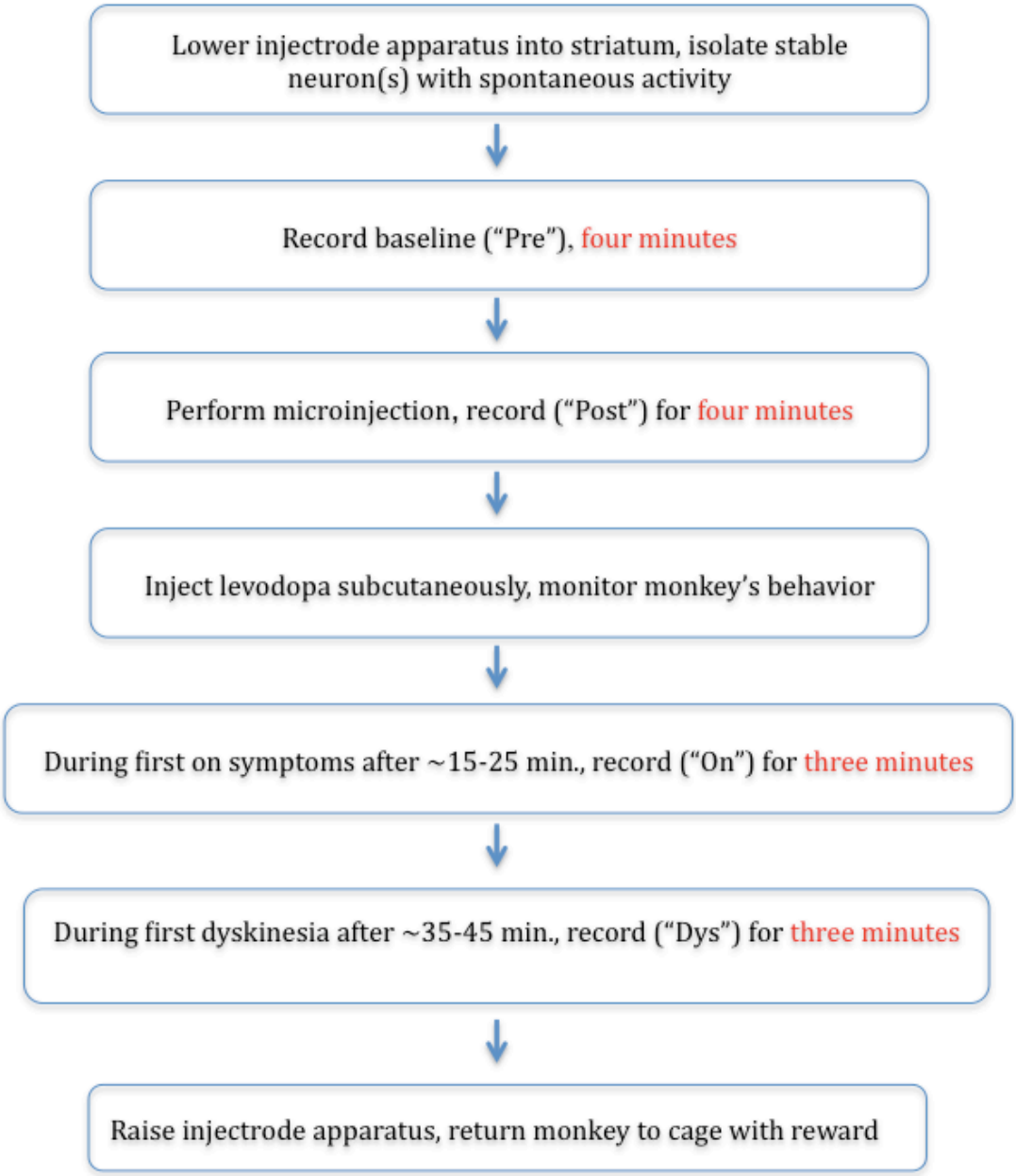

Figure 4: Experimental flow chart. Above is a flow chart describing the course of events and approximate timing of recording events (highlighted in red). Because the onset of "on" symptoms and LID were slightly variable between experiments and animals, the exact timing of recording varied between experiments.

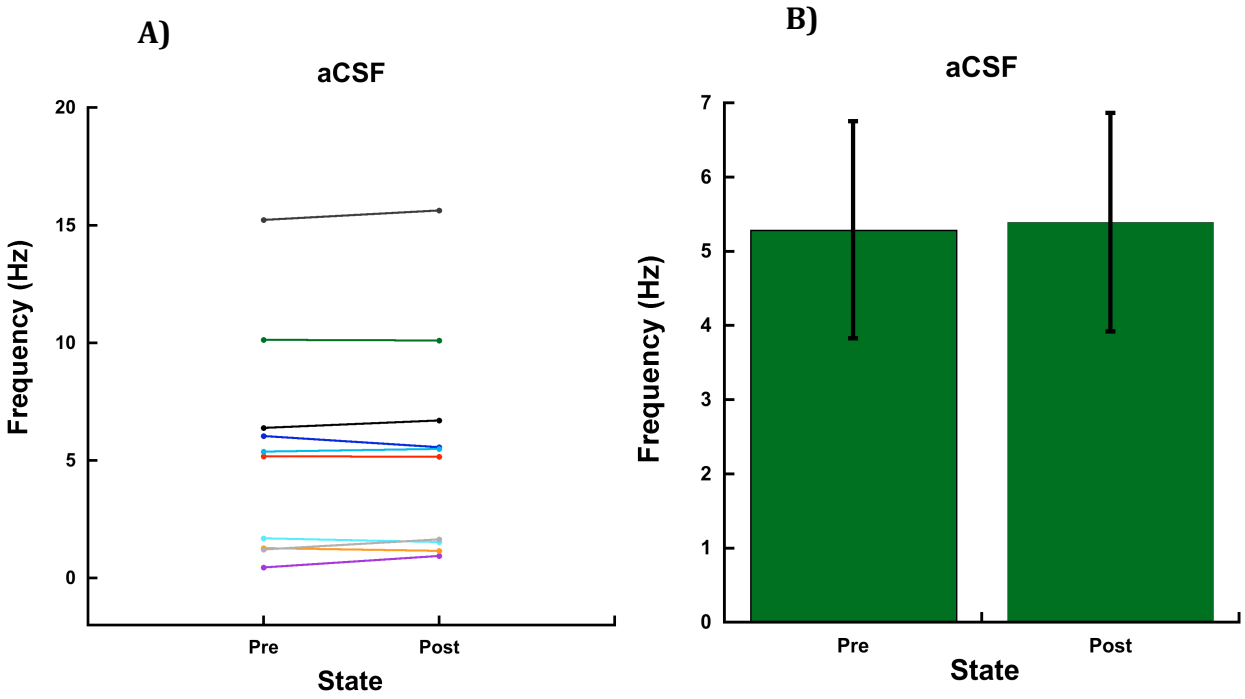

Figure 5: Effect of local microinjection of aCSF on firing frequency. A) Firing frequencies of individual MSNs before ("Pre") and after ("Post") microinjection. Each line represents a different cell. SEM error bars omitted for clarity; see Table 1 for details. B) Average firing frequencies +/- SEM across all aCSF MSNs in the "Pre" and "Post" conditions. There was no significant difference based on the state of the recording (paired samples *t*-test, $t(9) = -1.018$, $p = 0.335$).

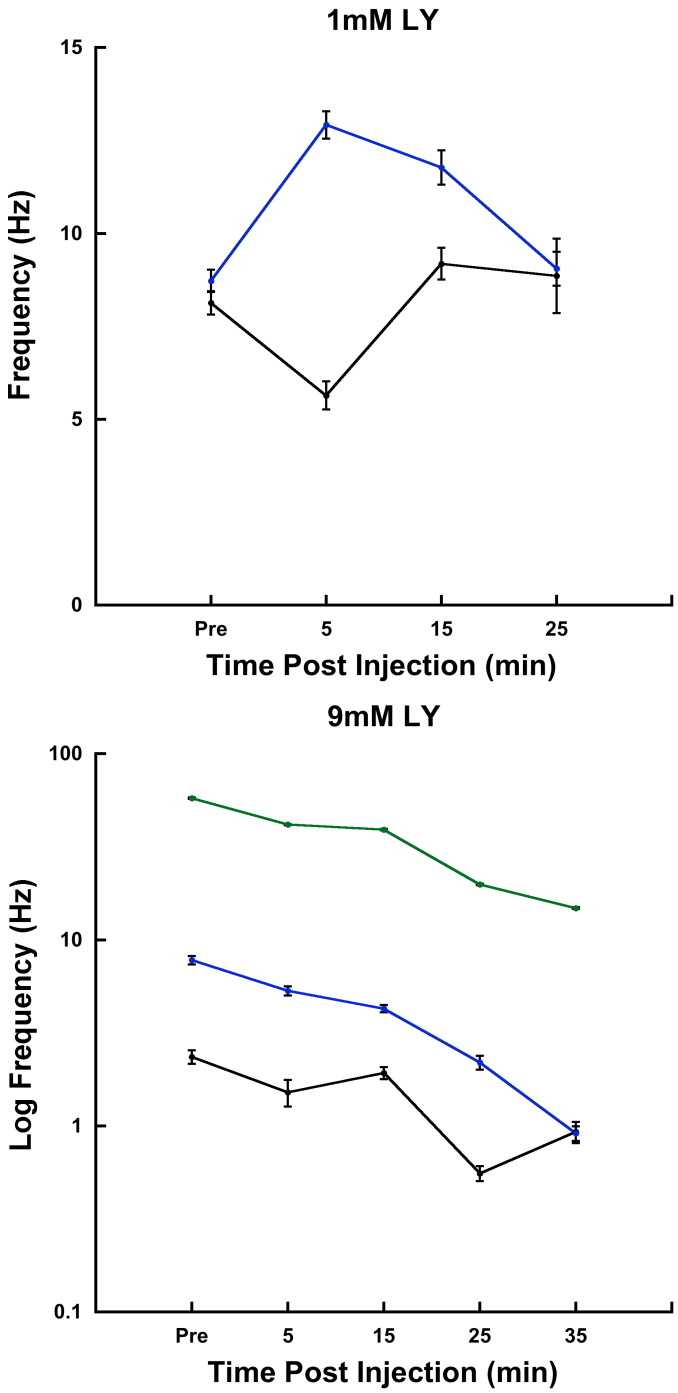

Figure 6: Examples of Effect of NMDA Antagonism Over Time with Varying Doses. *Top*: Two example MSNs in response to local microinjection of 1mM LY in 200 nl of aCSF (mean frequency +/- SEM). Responses were similarly inconsistent and transient for other cells (data not shown). *Bottom*: With 9mM LY at the same volume, firing rate reductions were dramatic, consistent and persisted over many tens of minutes. Note the difference in y-axis scale.

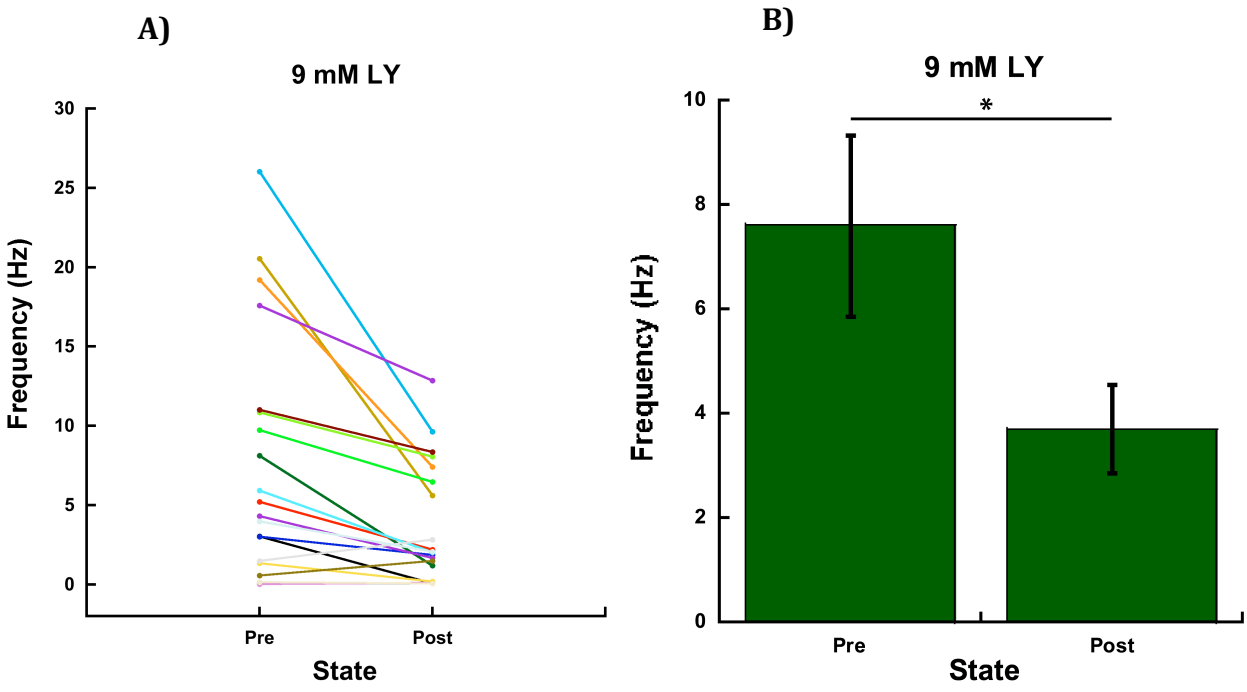

Figure 7: Effect of local microinjection of LY235959 on firing frequency. A) Firing frequencies of individual MSNs before ("Pre") and after ("Post") microinjection. Each line represents a different cell. SEM error bars omitted for clarity; see Table 2 for details. B) Average firing frequencies +/- SEM across all LY MSNs in the "Pre" and "Post" conditions. There was a significant decrease after injection observed (paired samples *t*-test, $t(16) = 3.500$, $p = 0.002$).

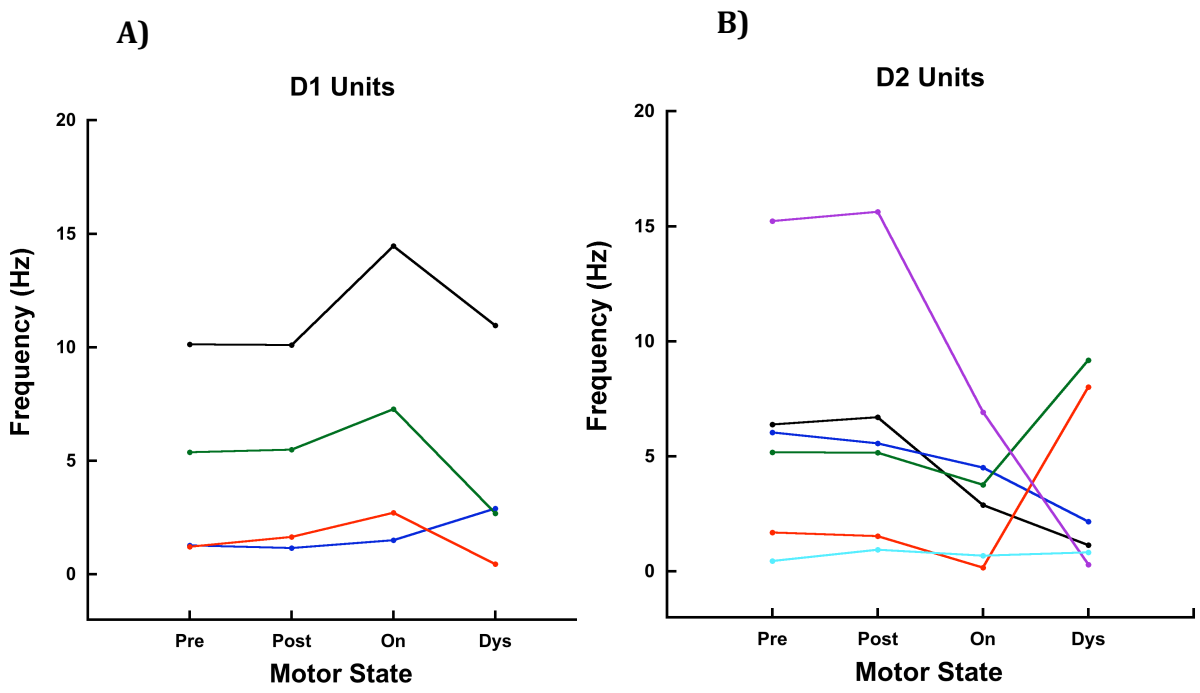

Figure 8: Effect of local microinjection of aCSF on MSN Response to Levodopa. MSNs separated into "D1" (A) and "D2" (B) units based on response to systemic levodopa (difference between "Post" and "On"). Each line represents a different cell, SEM error bars omitted for clarity. Most differences were highly significant ($p < 0.0001$); see Table 1 for details. Note the heterogeneity of responses (unidirectional and bidirectional activity in both D1 and D2 cells).

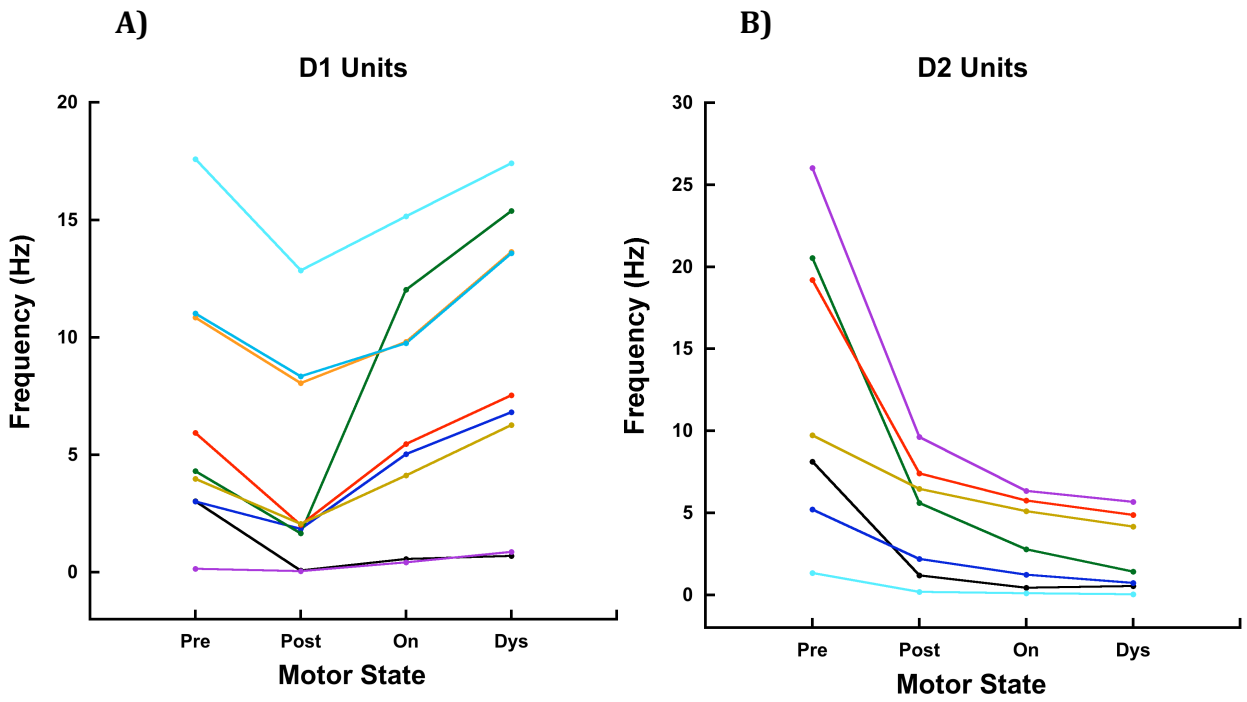

Figure 9: Effect of local microinjection of 9mM LY on MSN Response to Levodopa. MSNs separated into "D1" (A) and "D2" (B) units based on response to systemic levodopa (difference between "Post" and "On"). Only those cells that decreased in firing frequency significantly ($p < 0.05$) after local microinjection are included. Each line represents a different cell, SEM error bars omitted for clarity. Most differences were highly significant ($p < 0.0001$); see Table 2 for details. Note the homogeneity of responses (all unidirectional activity in both D1 and D2 subpopulations).

CONTROL ACSF

Cell#	dfb	dfw	F	p	LI	p	D1/D2	p	Resp	p
1	2.451	681	810.566	<.0001	N	1	2	<.0001	U	<.0001
2	2.13	642	261.217	<.0001	N	0.056	2	<.0001	U	<.0001
3	2.807	675	35.45	<.0001	N	1	1	<.0001	B	<.0001
4	2.435	675	133.844	<.0001	N	1	2	<.0001	B	<.0001
5	2.64	477.792	10.541	<.0001	N	0.12	0	0.068	N/A	0.732
6	2.448	443.05	723.157	<.0001	N	1	2	<.0001	B	<.0001
7	1.785	323.063	669.616	<.0001	N	1	2	<.0001	U	<.0001
8	2.459	590.217	46.579	<.0001	N	1	0	0.131	N/A	<.0001
9	1.599	383.71	65.211	<.0001	N	1	1	0.001	B	<.0001
10	2.739	657.311	82.739	<.0001	N	0.07	1	<.0001	B	<.0001

CONTROL ACSF

Cell#	Pre	Post	On	Dys	SEMr	SEMp	SEMo	SEMd
1	6.374	6.695	2.889	1.142	0.211	0.327	0.074	0.046
2	6.043	5.56	4.505	2.164	0.143	0.103	0.101	0.059
3	10.129	10.105	14.462	10.96	0.239	0.305	0.479	0.354
4	5.17	5.156	3.766	9.176	0.291	0.179	0.101	0.196
5	0.447	0.543	0.677	0.819	0.055	0.08	0.057	0.068
6	1.681	1.536	0.155	8.009	0.125	0.176	0.017	0.159
7	15.216	15.621	6.918	0.288	0.491	0.308	0.138	0.029
8	1.266	1.159	1.498	2.904	0.169	0.099	0.098	0.091
9	5.371	5.495	7.27	2.682	0.149	0.4	0.166	0.083
10	1.212	1.647	2.707	0.444	0.105	0.115	0.083	0.109

Table 1: Summary of unit data from aCSF conditions. *Top*: Summary of one-way ANOVAs for each unit. dfb, between-groups degrees of freedom; dfw, within-group degrees of freedom; F, omnibus F statistic; p, *p* value of the comparison immediately to the left (either *F* or Bonferroni); LI, change after local injection (comparing Pre/Post, N = no change, U = increase, D = decrease); D1/D2, change after systemic levodopa (comparing Post/On, 1 = increase, 2 = decrease, 0 = no significant change); Resp, change with dyskinetic stage (comparing On/Dys, U = unidirectional, B = bidirectional, N/A = no change in Post/On transition). *Bottom*: Summary of mean frequencies and SEM for the different recording segments for each cell. SEMr, SEM of Pre; SEMp, SEM of Post; SEMo, SEM of On; SEMd, SEM of Dys.

9 mM LY235959

Cell#	dfb	dfw	F	p	LI	p	D1/D2	p	Resp	p
1	1.57	281	132	<.0001	D	<.0001	1	<.0001	U	1
2	1.693	304	40	<.0001	D	<.0001	1	<.0001	U	0.041
3	1.419	170	143	<.0001	D	<.0001	2	0.009	U	1
4	1.319	150	19	<.0001	D	0.005	2	0.012	U	0.647
5	2.134	213	348	<.0001	D	<.0001	1	<.0001	U	<.0001
6	2.75	274	170	<.0001	D	<.0001	1	<.0001	U	<.0001
7	1.373	123	797	<.0001	D	<.0001	2	<.0001	U	<.0001
8	1.473	156	609	<.0001	D	<.0001	2	<.0001	U	<.0001
9	1.427	151	495	<.0001	D	<.0001	2	<.0001	U	0.247
10	1.759	316	443	<.0001	N	1	0	0.427	N/A	<.0001
11	1.829	329	45	<.0001	N	0.276	0	0.072	N/A	<.0001
12	1.26	226	192	<.0001	U	<.0001	1	<.0001	B	<.0001
13	1.186	284	272	<.0001	D	<.0001	2	<.0001	U	<.0001
14	2.7	649	200	<.0001	U	<.0001	1	<.0001	U	<.0001
15	2.412	434	550	<.0001	D	<.0001	2	<.0001	U	<.0001
16	2.204	396	111	<.0001	D	0.013	1	<.0001	U	<.0001
17	2.367	445	13	<.0001	D	<.0001	1	0.007	U	0.037
18	1.142	205	37	<.0001	D	0.003	1	<.0001	U	<.0001
19	2.704	543	43	<.0001	D	<.0001	1	0.003	U	<.0001
20	2.723	528	38	<.0001	D	<.0001	1	0.032	U	<.0001

9 mM LY235959

Cell#	Pre	Post	On	Dys	SEMr	SEMp	SEMo	SEMd
1	3.032	0.069	0.561	0.696	0.197	0.013	0.059	0.105
2	3.011	1.848	5.03	6.812	0.148	0.129	0.557	0.31
3	8.112	1.196	0.43	0.545	0.557	0.242	0.095	0.121
4	5.199	2.19	1.22	0.739	0.831	0.208	0.213	0.177
5	4.31	1.662	12.026	15.376	0.255	0.083	0.402	0.49
6	5.931	2.02	5.459	7.541	0.21	0.105	0.171	0.202
7	20.533	5.603	2.777	1.421	0.58	0.181	0.15	0.075
8	19.187	7.408	5.741	4.882	0.429	0.193	0.175	0.122
9	26.028	9.628	6.329	5.681	0.699	0.304	0.249	0.167
10	0.044	0.05	0.414	6.232	0.02	0.01	0.2	0.203
11	0.017	0.088	0	0.309	0.007	0.035	0	0.025
12	0.558	1.491	6.584	0.569	0.045	0.107	0.39	0.082
13	1.337	0.191	0.095	0.03	0.072	0.017	0.012	0.009
14	1.484	2.816	3.775	4.774	0.072	0.093	0.122	0.108
15	9.732	6.47	5.101	4.164	0.122	0.056	0.138	0.08
16	0.144	0.055	0.426	0.869	0.022	0.017	0.05	0.042
17	17.583	12.848	15.152	17.416	0.886	0.344	0.575	0.565
18	3.979	2.065	4.12	6.273	0.554	0.074	0.076	0.129
19	10.849	8.059	9.816	13.644	0.472	0.376	0.278	0.298
20	11.013	8.349	9.746	13.581	0.48	0.374	0.283	0.3

Table 2: Summary of unit data from LY conditions. *See caption for Table 1.* Note that cells 10, 11 and 14 were subsequently excluded from further analysis after initial one-way ANOVA (see Results).

DISCUSSION

The primary objective of this study was to determine the effects of reducing baseline hyperactivity of striatal MSNs on their firing rate response to levodopa. We hypothesized that reducing baseline activity via local NMDA antagonism would normalize (i.e. make unidirectional) their response to levodopa. The results of this study support this hypothesis, noting both considerable heterogeneity of levodopa response in the aCSF condition (Figure 8) and exclusively unidirectional responses in the reduced-baseline 9mM LY condition (Figure 9).

The secondary objectives that were subordinate to the primary hypothesis were also supported. First, the prediction that local microinjections of NMDA receptor antagonists at sufficient concentrations could reduce firing rates, while those of aCSF will not, was supported (Figures 5-7). Second, the prediction that the animals will continue to exhibit LID with small (200 nl) local microinjections was also affirmed (data not shown). Finally, the prediction that both D_1 and D_2 units would maintain unidirectional responses to levodopa in the presence of an NMDA antagonist was supported (Figure 9).

In the current study, statistically significant increases or decreases of MSN firing frequency after the onset of the "on" response are taken as indications of dopamine mediated D_1-dominant or D_2-dominant signaling, respectively. However, with the limitations intrinsic to *in vivo* recordings in awake, behaving macaques, it is not possible to deduce with certainty whether these cells belong to the direct or indirect pathway, particularly in a disease model such as MPTP-induced parkinsonism. Indeed, previous observations indicate that there may be significantly more D_1-receptor mediated excitation of striatal MSNs in response to systemic levodopa in the chronic dyskinetic parkinsonian state than would be anticipated in the healthy state (Liang et al., 2008). Furthermore, there is evidence that striatal upregulation of both

dopamine receptor subtypes may occur after prolonged dopamine depletion (Cai et al., 2002). In spite of these limitations, there exists a large amount of evidence from the literature (described partially in the introduction) that such signaling can produce the predicted differential effects on firing frequencies (West & Grace, 2002; Hernandez-Lopez et al., 2000; Cepeda et al., 2001; Nicola et al., 2000).

A possible future experiment to confirm the presence of D_1/D_2 receptors or association with the direct/indirect pathway could involve optogenetic techniques (Kravitz et al., 2010; Gradinaru et al., 2009; Han et al., 2009). Experiments could be designed that target striatal MSN afferents of each pathway in respective downstream basal ganglia nuclei (e.g. GP_e, GP_i), relying on retrograde transport of the channelrhodopsin-carrying viruses to striatal somata. Thereafter, electrodes coupled to optical fibers could carry out the recordings performed in this study with the added confirmation of afferent location and/or genetic subtype via optical stimulation, after the recording of the dyskinetic state is complete. This could conceivably shed light on the inversion properties of more specific subtypes of striatal MSNs with virally encoded genetic and anatomical rigor.

While the current study successfully demonstrated that reduced firing rates via local NMDA receptor antagonism are associated with unidirectional responses to systemic levodopa, it did not control for the possibility that this is specifically an NMDA-signaling-mediated effect. In other words, it is possible that the blockade of NMDA receptors and thus the potential deprivation of cells in the vicinity of the injection site of calcium, for example, could be the primary cause of the unidirectional activity, and that the reduction in firing rate is secondary and not casually related to any inversion properties of the cell. One way to test this directly would be to use alternative methods of firing rate reduction or blockade of glutamatergic transmission that

do not rely on NMDA receptor blockade. The use of a competitive α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, with appropriate dosing to control for firing rate reduction, would provide highly useful information in making the aforementioned distinction.

While a small volume of drug was intentionally used to avoid complete block of glutamatergic transmission across the striatum, it must be considered that the current study does not address the possibility that the reduction of inversion responses in MSNs after local NMDA antagonism may be primarily an effect of the local striatal microcircuitry instead of the particular electrophysiological properties of the recorded cell. Indeed, in healthy animals dopaminergic stimulation confined to local circuits *in vivo* is associated with an overall decrease in activity of MSNs (Nicola et al., 2000). Thus it is perfectly conceivable, from a basic scientific standpoint, that the global reduction of activity via NMDA antagonism could mimic this state and "normalize" various firing patterns of MSNs, including inversion properties, through unknown circuit mechanisms. However, from a clinical perspective, it bears repeating that the reduction of LID via coadministration of LY235959 with levodopa was successfully performed systemically (Papa & Chase, 1996). As this method of drug administration would likely also be applied for any practical clinical application of the results of the present study, as far as possible future treatments of LID are concerned this may be a moot point.

In addition to refining our understanding of the mechanisms underlying the inversion properties of striatal MSNs, future research will necessarily attempt to decipher the downstream effects on basal ganglia circuit function of these striatal inversions. While striatal hyperglutamatergic tone may prove a viable pharmacological target in LID, circuit-level studies elucidating a mechanistic explanation for the production of LID from altered GP_i/SN_r output, for

example, may produce alternative targets for pharmacological intervention. Revealing both the cellular and systems mechanisms underlying the pathological signaling of striatal MSNs during LID will likely prove crucial in the development of treatments designed to alleviate this debilitating condition, and may ultimately help clinicians provide better care for their patients in the chronic evolution of PD.

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