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Neuronal Activity in the Subthalamic Nucleus Modulates the Release of Dopamine in the Monkey Striatum

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Abstract

The primate subthalamic nucleus (STN) is commonly seen as a relay nucleus between the external and internal pallidal segments, and as an input station for cortical and thalamic information into the basal ganglia. In rodents, STN activity is also known to influence neuronal activity in the dopaminergic substantia nigra pars compacta (SNc) through inhibitory and excitatory mono- and polysynaptic pathways. Although the anatomical connections between STN and SNc are not entirely the same in primates as in rodents, the electrophysiologic and microdialysis experiments presented here show directly that this functional interaction can also be demonstrated in primates. In three Rhesus monkeys, extracellular recordings from SNc during microinjections into the STN revealed that transient pharmacologic activation of the subthalamic nucleus by the acetylcholine-receptor agonist carbachol substantially increased burst firing of single nigral neurons. Transient inactivation of the STN with microinjections of the GABA-A-receptor agonist muscimol had the opposite effect. While the firing rates of individual SNc neurons changed in response to the activation or inactivation of the STN, these changes were not consistent across the entire population of SNc cells. Permanent lesions of the STN, produced in two animals with the fiber-sparing neurotoxin ibotenic acid, reduced burst firing and firing rates of SNc neurons, and substantially decreased dopamine levels in the primary recipient area of SNc projections, the striatum, as measured with microdialysis. These results suggest that activity in the primate SNc is prominently influenced by neuronal discharge in the STN, which may thus alter dopamine release in the striatum.

Keywords

Substantia Nigra Pars Compacta; Parkinson’s disease; Muscimol; Carbachol; Ibotenic Acid

Introduction

The basal ganglia participate in segregated circuits that also involve thalamus and cortex (Alexander *et al*., 1986; DeLong & Wichmann, 2007). The activity in these circuits is modulated by dopamine, released from terminals of neurons that reside in the substantia nigra pars compata (SNc) and in the ventral tegmental area (see reviews by Smith & Kieval, 2000; DeLong & Wichmann, 2007). In addition to maintaining a tonic level of striatal dopamine, neuronal discharge in the SNc carries information related to the expectation of reward, resulting in transient changes of striatal dopamine release which may alter corticostriatal transmission

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during procedural learning (see reviews by Schultz, 2004; Pisani *et al*., 2005; Grace *et al*., 2007). Recent studies by Redgrave and colleagues have demonstrated that SNc activity may also encode salient stimuli associated with unpredicted events (Redgrave & Gurney, 2006; Redgrave *et al*., 2008).

SNc neurons receive inhibitory input from the neighboring substantia nigra pars reticulata (SNr, reviewed by Tepper & Lee, 2007), and excitatory inputs from prefrontal and other cortical areas (e.g., Meltzer *et al*., 1997; Chiba *et al*., 2001), the subthalamic nucleus (STN, see below), and brainstem areas such as the pedunculopontine nucleus (PPN, e.g., Clarke *et al*., 1987; Futami *et al*., 1995; Charara *et al*., 1996). In addition, recent studies have identified several other sources of inputs that prominently alter SNc activity, such as the superior colliculus (Redgrave & Gurney, 2006; Redgrave *et al*., 2008), and the lateral habenula (Ji & Shepard, 2007; Matsumoto & Hikosaka, 2007).

The interaction between STN and SNc is particularly interesting, because several movement disorders are associated with changes in STN activity, which may thus influence SNc activity. For example, the increased STN activity that accompanies the degeneration of dopaminergic cells in Parkinson's disease may enhance the output from the surviving dopaminergic neurons in a compensatory manner (Bezard *et al*., 1997; 1999; but see Bilbao *et al*., 2006).

A substantial monosynaptic excitatory STN-SNc projection has been demonstrated to exist in rodents (e.g., Hammond *et al*., 1978; Kita & Kitai, 1987; Chergui *et al*., 1994a). In addition, STN also influences SNc in these animals via bi- or polysynaptic routes, including net inhibitory pathways, such as the STN-SNr-SNc pathway or the STN-GPe-SNc pathway, and net excitatory pathways, such as the STN-PPN-SNc pathway, or the STN-GPe-SNr-SNc pathway (Celada *et al*., 1999; Iribe *et al*., 1999; Kitai *et al*., 1999).

The relevance of these findings to monkeys cannot be assumed, because the anatomic relationship between STN and SNc differs substantially between rodents and primates. For example, the monosynaptic STN-SNc connection is less prominent in primates (Smith *et al*., 1990; Francois *et al*., 2000; Sato *et al*., 2000), and may have a higher degree of topographic organization (Matsumura *et al*., 1992; Parent & Hazrati, 1995). On the other hand, the indirect anatomic links between STN and SNc may play a more prominent role in primates. In the experiments described here we examined directly whether changes in STN activity affect SNc activity in awake Rhesus monkeys.

Methods

Animals, surgical preparation, and general outline of experimental procedures

Three adult Rhesus monkeys (*Macaca mulatta*; female monkey V, male monkeys W and C, 4–6 kg) were used. All experiments were carried out in compliance with the NIH Guide for the Care and Use of Laboratory Animals (Anonymous, 1996), the PHS Policy on Humane Care and Use of Laboratory Animals, and the Policies on the Use of Animals and Humans in Neuroscience Research (revised and approved by the Society for Neuroscience in January 1995). The studies were approved by the Animal Care and Use Committee of Emory University.

In preparation for these experiments, the animals were implanted under sterile conditions and gas anesthesia (isoflurane, 1–3%) with head holders and recording chambers (inner diameter, 16 mm). One chamber was aimed stereotactically at the right STN in the parasagittal plane, 36° anterior from the vertical, and the second was aimed at the right SNc in the parasagittal plane, 25° posterior from the vertical. Monkeys V and C received a third recording chamber

at a 50° angle in the coronal plane, giving access to the left STN, SNc and putamen. Chambers and head holders were affixed to the skull with dental acrylic.

During all of the subsequent experimental sessions, the animals were awake and seated in a primate chair, with the head fixed, but the rest of the body free to move. We first carried out electrophysiologic mapping procedures to determine the location of the basal ganglia and other structures in the chambers. In all animals, the posterior chamber was used to record SNc activity, while the anterior chamber was used to inject either the GABA-A receptor agonist muscimol or the acetylcholine receptor agonist carbachol into the STN, to inactivate or activate neurons in this nucleus. Muscimol has been used in many previous studies to transiently inactivate neurons in the STN (e.g., Wichmann *et al*., 1994b; Baron *et al*., 2002; Kita *et al*., 2006). Carbachol injections have been shown in other studies to activate STN neurons (e.g., Mintz *et al*., 1986; Wilson *et al*., 2004), probably through a combination of pre- and postsynaptic effects (e.g., Bevan & Bolam, 1995), which may involve M3-muscarine type and other cholinergic receptors (at least in rodents, see Flores *et al*., 1996; Hernandez-Lopez *et al*., 1996; Shen & Johnson, 2000).

After completion of these experiments, the STN was lesioned in monkeys V and C, using the fiber-sparing neurotoxin ibotenic acid (see, e.g., Bergman *et al*., 1990; Wichmann *et al*., 1994b; Soares *et al*., 2004). SNc activity was then recorded again, and striatal dopamine levels measured with microdialysis via the lateral coronal chamber.

Recording and injection procedures

Single unit recordings were done with tungsten microelectrodes (0.5–2 MΩ @ 1 kHz, Frederick Haer, Bowdoinham, ME). The electrophysiologic signals were amplified (DAM-80 pre-amplifier, WPI, Sarasota, FL, and MDA-2 amplifier, MDA-2, BAK, Mount Airy, MD), filtered (400–6000 Hz, model 3700 filter, Krohn-Hite, Quincey, MA), and stored to tape (model 3000 VCR recording adapter, Vetter, Rebersburg, PA). The signals were also displayed on a digital oscilloscope (DL5200, Yokogawa, Tokyo, Japan), and audio-amplified to facilitate the detection and isolation of single neurons. During the experiments, well-isolated spike potentials that represented single units (identified on the basis of visual inspection of the waveforms) and had a signal/noise ratio of at least 3 were recorded for later analysis. SNc neurons were identified (1) by their anatomic location, specifically their relationship to the neighboring to STN and SNr, i.e., two nuclei that are easily identifiable on the basis of their electrophysiologic activity patterns (e.g., Wichmann *et al*., 2001; Soares *et al*., 2004), and (2) by specifics of their spiking activity, specifically firing rates below 10/s, and spike potential durations longer than 1.8 ms (range 1.8 – 4.2 ms; mean ± SD, 2.9 ± 0.4 ms). The potentials often had an initially positive deflection, followed by a large negative deflection. These criteria were similar to those used by Schultz and coworkers (Aebischer & Schultz, 1984; Schultz, 1986; Romo & Schultz, 1990). SNc cells with the described characteristics and location are in all likelihood dopaminergic, although this can, of course, not be proven with certainty in *in vivo* experiments such as ours (for more discussion of the topic of identification of these neurons, see Ungless *et al*., 2004; Grace *et al*., 2007).

To assess the effects of STN injections of carbachol or muscimol on SNc activity, a recording electrode was first inserted into the SNc through the posterior parasagittal chamber. A combined injection/recording device, loaded with the drug to be tested, was then inserted into the STN through the anterior chamber. The device consisted of a 30-ga steel tube through which a Teflon-coated tungsten recording wire (0.076 mm coated diameter, AM Systems, Sequim, WA) was threaded. The tip of the wire protruded 0.5 mm from the end of the tube, making it possible to identify the injection target on the basis of electrophysiologic multi-unit recordings. Once the injection target location was identified with this system, the injection/recording

system was advanced by a further 0.5 mm so that the tip of the injection cannula is at the desired target position.

We recorded the activity of single SNc cells before, during and after the injections of muscimol (1 μg/μl distilled water, Sigma-Aldrich, St. Louis, MO) or carbachol (4 μg/μl water, Sigma-Aldrich) into the STN. The solutions were pressure-injected by hand with a Hamilton syringe (0.2 μl/30 s to a total of 1–2 μl). Based on previous experience with this technique, the injected solution volume can be expected to affect a sphere of STN tissue approximately 0.5–1.0 mm in diameter (Wichmann *et al*., 1994b). We were able to record stable activity from SNc cells beginning five minutes before these injections and through as much as 20 minutes afterwards. Only one injection was carried out per day. Control injections (solvent only, n = 4) had no effect.

In addition to SNc cells recorded before, during and after drug injections, we also recorded a pool of SNc cells in the control state (before injections), and in the period between 20 and 60 minutes after the intra-STN drug injections. Although a tighter time schedule would have been preferable, such a restriction would have severely reduced statistical power. Previous experience with intra-STN injections has shown that the (behavioral) effects of these injections are stable over this time period.

Some SNc cells were also tested to evaluate their responses to movement. Passive movements were produced by simply holding and moving the arms and legs opposite to the recording site. Active reaching movements were evoked by presenting the animal with small food items. The responses were evaluated by listening to the audio-amplified spiking neural records (as has been done in other studies, e.g., Wichmann *et al*., 1994a).

In a few experiments, the local effects of drug injections on STN neurons were also assessed directly, using an injection-recording system (see, e.g., Kliem & Wichmann, 2004; Kita *et al*., 2007) which permits high-quality recording of neuronal activity within 50–100 μm of the injection site. This device consists of a standard tungsten microelectrode, coupled to a fused silica tube (inner diameter, 42 μm; outer diameter, 110 μm; Polymicro Technologies, Phoenix, AZ). The electrode tip protrudes 50–100 μm beyond the silica tube, with the electrode and the silica tube running together through a polyimide tubing sleeve (outer diameter, 0.5 mm). In this case, the drug solutions were delivered (0.2 μl/min, total of 1–2 μl) through the tube via a microliter pump (Model 22, Harvard, Holliston, MA), and a liquid switch (CMA, Sweden). This device is more difficult to build and handle than the standard injection system mentioned above, and was therefore only used for the few cases in which single-unit recordings were necessary at locations close to the injection site in the STN.

STN Lesioning

Ibotenic acid (Sigma-Aldrich, 10 μg/μl in deionized water, pH 7.2–7.4, 0.3 μl/session) was injected with the standard injection/recording device, consisting of a steel injection cannula with associated wire (see above). Each monkey received four injections in separate sessions (total amount: 9 μg in each animal). The microdialysis experiments were begun six days after the last injection in monkey V, and seven days after the lesion in monkey C.

Microdialysis

Microdialysis probes (custom-modified CMA/11 probes, made by CMA, Solna, Sweden, 2 mm exposed cuprophane membrane) were prepared by placing them into, and flushing them with, deionized water, followed by overnight perfusion with artificial cerebro-spinal fluid (aCSF, pH = 7.2–7.4, 145 nM NaCl, 2.8 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mM Na_2HPO_4) at a rate of 0.5 μl/min.

Each animal underwent multiple microdialysis procedures, separated from one another by at least 48 hours, and at different striatal locations so that fresh tissue was sampled in each experiment. Each experiment was carried out with a new probe.

After initial electrophysiologic mapping of the striatal target, the probes were inserted into the brain and advanced to the target with a microdrive through a 23-ga guide tube. Throughout the experiment, the probes were perfused with aCSF (2 μl/min). After a 120-minute calibration period, five 20-minute microdialysate samples were collected. The perfusion media was changed for the duration of sample #3 to one containing 80 mM K^+ to assess tissue viability. Samples were immediately frozen to −80° C, and later assayed for dopamine, following the HPLC assay protocol (see next section).

Chromatographic analysis of microdialysates for dopamine

The chromatographic analysis of microdialysates for dopamine was adapted from a method originially published by Abercrombie and Finlay (Abercrombie & Finlay, 1991). Immediately upon thawing, the microdialysis samples were made acidic by mixing them with an equal volume of pH 4.0 mobile phase containing dihydroxybenzylamine (DHBA, added as an internal standard) to preserve oxidizable components. Dopamine was then separated from other components by reverse-phase HPLC columns (Alltima C18 3μ, 4.6 × 100 mm PEEK) with a C9-ion pair mobile phase (potassium acetate, 64 mM; citric acid, 48 mM; sodium nonanesulfonate (2.6 mM); EDTA (.025 mM) in 15% aqueous methanol) at 1 ml/min (Model 582 HPLC pump, ESA, Chelmsford, MA). Oxidizable components were detected with a dual coulometric/amperometric electrochemical cell connected to a Coulochem II detector (ESA). Peak areas were estimated by a computerized method (Turbochrom, PENelson, Cupertino, CA) with manual intervention where necessary and compared with the peak area of the internal standard.

Histology

At the end of the study, monkeys were sacrificed by induction of deep pentobarbital anesthesia, followed by transcardial perfusion with saline and aldehydes. Brains were removed, and 50-μm parasagittal slices were stained with cresyl violet, and for tyrosine hydroxylase (TH), and the neuronal marker, microtubule-associated protein 2. Approximate recording sites were reconstructed, based on placement of electrolytic micro-lesions (generated by application of a 5 μA anodal current, 180 s) made during the last experimental session, on the basis of the known depth, as well as anterior-posterior and mediolateral micromanipulator coordinates noted for each recording site during the experiments, and by using information regarding the location of neighboring easily identifiable structures, such as thalamus and SNr, which was noted at recording time for each of the tracks.

Data analysis

Records of SNc neurons were included in the single-cell analysis only if the recording began at least five minutes before the STN injections began, and continued for at least ten minutes after the end of the injections. The taped records were analyzed with a template-matching spike detector (MSD, Alpha-Omega Engineering, Nazareth, Israel). This device samples data at 50 kHz, determines whether the sampled wave crosses a (user-defined) threshold, and attempts to match the data surrounding threshold crossing to a (user-defined) 8-point wave template. If a match is found, the template-matching waveform is identified as a spike, and a time-stamp is generated. Time stamps were later converted to inter-spike-intervals (ISIs).

ISIs were used to calculate discharge rates for each cell before, during and after drug injections (binned in one-second intervals), and to construct ISI distribution histograms to assess the quality of the recordings (1 ms bins, up to a maximal ISI length of 100 ms). To evaluate

responses of individual SNc neurons to drug injections in the STN, comparisons were carried out between the population of ISIs collected in the SNc during the five-minute period before drug injections, and the ISIs collected during the first ten minutes after termination of the drug injection, using the non-parametric Mann-Whitney test.

The drug injections yielded a pair of pre- and post-injection rate values for each neuron. For a summary analysis of the effects of muscimol or carbachol on firing rates, the pre- and post-injection values were compared with the Wilcoxon signed-rank test.

In addition, estimates of the occurrence of burst discharges were generated, using two different algorithms. The first was an algorithm based on the Poisson 'surprise' method (Legendy & Salcman, 1985), applied separately to the pre- and post-injection data. For this, groups of ISIs were compared to the overall distribution of ISIs in the spike train. For initial classification of a burst, two consecutive ISIs (three spikes) had to be each shorter than half of the cell's mean ISI. In addition, the Poisson 'surprise' value for this 'burst', i.e., the negative logarithm of the probability of the occurrence of the sequence of ISIs constituting the burst under the assumption of random (Poisson) firing, had to be ten or greater. In subsequent steps, additional ISIs were added to the beginning or the end of the burst until further extension of the burst did not further increase the resulting burst's surprise value. This burst detection method is frequently used in the primate and human recording literature (Aldridge & Gilman, 1991; Soares *et al*., 2004; Wichmann & Soares, 2006), and yields (with the parameters described above) conservative estimates for the occurrence of bursts.

For better comparison with existing rodent data, a second burst detection algorithm was used in addition, following a procedure that was initially described by Grace and Bunney (Grace & Bunney, 1984). In this procedure, bursts are defined as sequences of spikes starting with ISIs of 80 ms or less, and terminating if the ISI reaches 160 ms. As with the 'surprise' method, the minimal burst length was set to be two ISIs. This method resulted in less conservative estimates of the occurrence of bursts.

In order to assess the possibility that changes in burst indices of individual cells were related to changes in discharge rates, ratios were calculated of the differences between pre- and post-injection burst indices or firing rates and normalized to the sum of pre- and post-injection burst indices or firing rates (i.e., (Xpre − Xpost) / (Xpre + Xpost) with Xpre and Xpost representing pre- and post injection burst indices or firing rates, respectively). We carried out regression analyses of the resulting 'burst index ratios' and 'discharge rate ratios', for the muscimol and carbachol experiments.

The pre- and post-injection burst indices of individual cells were compared with the Wilcoxon signed-rank test.

We also compared firing rates and burst indices for a larger group of SNc cells, each recorded from for more than 10 minutes, either during the pre-injection control period or during the period 20–60 minutes after the end of the drug injection, using Mann-Whitney tests (see above). The chosen post-injection time window represents a compromise between the desire to examine the cellular activity in a relatively stable activity state, and the need to collect a sample size that is sufficiently large to allow meaningful statistical comparisons. Our previous studies of the effects of similarly sized muscimol injections into the STN (Wichmann *et al*., 1994b; Baron *et al*., 2002) had shown that the behavioral state of the animals remains stable during this time period.

From each of the microdialysis experiments, data from samples #1, 2 and 5 were used to calculate baseline dopamine levels. The levels shown in figure 4C are pooled from 5–7 experiments in each state in each of the two monkeys. Between-group comparisons were made

using the non-parametric Mann-Whitney test. Relative increases produced by tissue exposure to high potassium levels were calculated as ratios between the level measured in sample #3 and the average of the pre-stimulation samples (samples #1 and 2).

For all statistical tests, a p-value below 0.05 was accepted as indicative of significance.

Results

Local effects of microinjections of muscimol and carbachol on STN activity

In these experiments, the GABA-A receptor agonist muscimol was used to inactivate portions of the STN, while carbachol was used to activate STN neurons. To verify that these compounds had the predicted effects on the activity of STN neurons, we first examined STN discharge during intra-STN microinjections of muscimol or carbachol. To minimize tissue damage, only a small number of these confirmatory experiments (n = 2 for each compound) were carried out, and only in one of the animals (monkey V). We found that the activity of STN neurons was strongly reduced in response to muscimol injected (2 μg/2 μl, injected at a rate of 0.2 μl/min) into the vicinity of the recorded cells (Figure 1A, see Galvan *et al.*, 2005, for an example of similar injections in the globus pallidus). As expected from previous studies in primates (Hamada & DeLong, 1992; Wichmann *et al.*, 1994b), all of the muscimol injections in our study were accompanied by the development of dyskinetic movements of arm and leg, contralateral to the injection site which typically began within one or two minutes of the start of the injection, and lasted until the end of the experiment (for at least 90 minute after the injection). In contrast, injections of carbachol (4 μg/1 μl) induced significant transient increases in STN activity (Figure 1D). None of the carbachol injections provoked obvious behavioral effects. However, the behavioral examination techniques used here were not sensitive enough to rule out the development of subtle behavioral changes.

Effects of transient changes in STN activity on neuronal discharge rates in SNc

At baseline, SNc neurons fired with an average rate of 5.8 ± 0.2 spikes/s for monkey V (mean ± SEM; n = 66). In this animal, the neurons were sampled for 363.7 ± 160.6 s/neuron (range: 300 – 1200 s), corresponding to 2020.8 ± 1389.8 spikes/neuron (range 934 – 4823 spikes). In monkey W, SNc neurons fired at an average baseline rate of 5.0 ± 0.2 spikes/s for monkey W (n = 32). In this animal, neurons were sampled for 430.7 ± 258.8 s/neuron (range 300 – 1465 s), corresponding to 2970.2 ± 1579.0 spikes/neuron (range 984 – 6426 spikes). In monkey C, the pre-injection baseline firing rate was 5.9 ± 0.3 spikes/s (n = 32). In this animal, neurons were sampled for 554 ± 234.6 s/neuron (range 300 – 1200sec), corresponding to 3190.6 ± 1366.6 spikes /neuron (range 1231 – 5136 spikes). The activity of SNc neurons did not change in response to active or passive movements (similar to other published results, see Schultz, 1998),

In each cell for which pre- and post-injection data were available, the post-injection period was compared to the pre-injection control period (see Methods). Almost all of the recorded SNc neurons showed significant changes in discharge rate after injections of muscimol or carbachol into the STN. Statistical comparisons between the discharge rates before and after the injections showed two distinct response patterns in individual cells (see Figure 1).

Muscimol injections into the STN were carried out in monkeys V (11 injections) and C (7 injections). Firing rates and patterns were compared between five-minute data segments sampled prior to the injections, and a 10 minute data segment sampled thereafter. As shown in Figure 1C, some neurons responded with a decrease in discharge to the muscimol injection. Comparison between the pre-injection ISIs and post-injection ISIs revealed a statistically significant decrease in activity in 9/18 (50%) of the recorded SNc neurons (Mann-Whitney

test, $p < 0.05$ in each cell, see methods). In these cells, the discharge rate fell from a pre-injection average of 5.2 ± 0.1 spikes/s to a post-injection average of 4.0 ± 0.2 spikes/s. Using the same statistical procedure, other neurons (7/18, 37%) showed the opposite type of response, i.e., a statistically significant increase in discharge, as is shown for an example cell in Figure 1B. Two SNc neurons in this sample of cells (11.1%) did not respond while muscimol was injected into the STN. The effects of muscimol on the firing rates of all of the cells tested in this manner are shown in Figure 2A. When all cells were considered, there was no significant effect of muscimol on the cell's firing rate ($p = 0.59$, Wilcoxon's signed rank test)

We also analyzed neurons which were recorded between 20 and 60 minutes after the muscimol injections, and compared their discharge to control recordings which were obtained prior to the injections. Cells recorded before muscimol injections fired at 5.7 ± 0.3 spikes/s ($n = 61$). These cells were sampled for 320.9 ± 154.3 s/neuron (range 300 – 1320 s), corresponding to 1892±655.9 spikes/neuron (range 634 - 332 spikes). The cohort of neurons recorded after muscimol injections fired at 5.1 ± 0.5 spikes/s ($n = 44$). These neurons were sampled for an average of 756.2±299.0 s/neuron (range 300 – 1200 s), corresponding to 3755.4±3109.0 spikes/neuron (range 580 – 9801 spikes). Given that some individual cells increased their firing, and others decreased their firing during muscimol injections (see above), it was not surprising that there was no significant overall change in discharge rates from before to after mucimol ($p = 0.348$, Mann Whitney test).

Injections of carbachol into the STN were carried out in monkeys W (9 injections) and C (8 injections). Activation of STN cells with local carbachol injections also elicited statistically significant increases in discharge in some SNc neurons, and decreases in others. Decreases in discharge were seen in 6/17 (35%) of the neurons recorded before, during, and after the injections (see example in figure 1F), while increases in discharge were seen in 7/17 (41%) of all neurons (see example in figure 1E). Four neurons in SNc did not change their firing rate during carbachol injections into the STN (23.5%). In the aggregate, this change was not significant ($p = 0.352$, Wilcoxon signed rank test). The effects of carbachol on the firing rate of all of the cells tested in this manner are shown in figure 2B.

The group analysis of neurons recorded before and between 20 and 60 minutes after the carbachol injections revealed that cells fired at 4.9 ± 0.4 spikes/s prior to carbachol injections ($n = 58$, 571.8±321.8 s/neuron (range 300 – 1200 s), corresponding to 3122.1±1482.6 spikes/neuron (range 631 – 7221 spikes)), and at a rate of 5.7 ± 0.5 spikes/s after the injections ($n = 44$, sampled for 728.7±237.8 s/neuron (range 300 – 1200sec), corresponding to 4069.2±2475.6 spikes/neuron (range 935 – 8200 spikes)). This change was not significant ($p = 0.1173$, Mann-Whitney test).

Effects of transient changes in STN activity on burst firing in SNc

Pharmacologically induced changes in STN activity had consistent effects on burst activity in the SNc, as detected with burst detection algorithms (see Methods). After injection of muscimol into the STN, firing of SNc neurons became more regular.

As described in the Methods section, two different procedures were used to quantify burst discharges. Using the 'surprise' method, the ratio between the number of discharges in bursts and the total number of action potentials (burst index) decreased. The data from individual cells in which we were able to assess the burst index before, during, and after the muscimol injections are shown in figure 2C. In this set of data, muscimol injection into the STN resulted in a significant reduction of bursting ($p = 0.031$, Wilcoxon signed rank test, $n = 18$).

In the pooled data set (including all cells recorded in the control state [n = 61], and all cells recorded in the post-injection state [n = 44], as defined in the Methods), the burst index

decreased from a control value of 1.7 ± 0.2% to a post-injection value of 1.0 ± 0.1% (p = 0.0146, Mann-Whitney test). According to the method described by Grace and Bunney (Grace & Bunney, 1984), the burst index decreased from a control value of 39.6 ± 2.5% to 30.2 ± 4.0 (p = 0.0298, Mann-Whitney test) after the muscimol injections.

In contrast, after the carbachol injections into the STN, cells in the SNc fired less regularly, and burst activity was increased. The burst index data from all individual cells in which the burst index could be assessed before and after the carbachol injections are shown in figure 2D. Carbachol injections into the STN resulted in a significant increase in bursting (p = 0.0097, Wilcoxon signed-rank test, n = 17). In the pooled data set (including all cells recorded in the control state [n = 58], and all cells recorded in the post-injection state [n = 44], as defined in the Methods), the burst index as detected with the 'surprise' method rose from a pre-injection average of 1.0 ± 0.2 % to a post-injection average of 3.1 ± 0.4 % (p = 0.0004, Mann-Whitney test). According to the method described by Grace and Bunney (Grace & Bunney, 1984), the burst index rose from a control value of 36.0 ± 2.4% to 45.2 ± 3.3% (p = 0.039, Mann-Whitney test).

In order to evaluate whether the changes in burst indices and the changes in firing rate were related, we carried out a regression analysis, using burst index ratios and firing rate ratios (see Methods). As can be seen in Figure 2, parts E and F, there was no consistent relationship between the changes in firing rates and the changes in burst indices (see also, ref. Wichmann & Soares, 2006). Figure 2E shows data from the muscimol injection experiments, while figure 2F shows data from the carbachol injections. The regression coefficient for the muscimol experiments was 0.1904 (p = 0.19, n = 18), and for the carbachol experiments 0.076 (p = 0.28, n = 17).

Effects of STN lesions on neuronal discharge in SNc

In a second series of experiments we examined the effects of lesions of the STN, produced with the fiber-sparing excitotoxin ibotenic acid, on the discharge of SNc neurons, and on dopamine levels in the striatum. These experiments were done in two animals (monkey C and monkey V, see Methods). In both animals, the STN was lesioned without encroachment on the SNc (Figure 3). Both animals showed moderate contralateral dyskinesias after individual ibotenic acid injections, which in each case subsided within 24 hours. Electrophysiologic recordings and microdialysis experiments were carried out starting one week after lesioning, by which time the involuntary movements had completely disappeared.

The STN lesions resulted in significant changes in the activity of SNc neurons. In monkey V, the average discharge rate of SNc neurons fell slightly from 4.8 ± 0.2 discharges/s before the lesion (*n* = 68, recorded for 485.7±221.7 s/neuron (range 300 – 1200 s), corresponding to 1792.4±865.4 spikes/neuron (range 860 – 4230 spikes)) to 4.0 ± 0.4 discharges/s (*n* = 43, 571.2 ±205.0 s/neuron (range 300 – 1220 s), corresponding to 2194.4±1106.8 spikes/neuron (range 718 – 4944 spikes); p = 0.0131; Mann-Whitney test; Figure 4A) thereafter. In monkey C, the discharge rate fell from 5.1 ± 0.3 discharges/s (*n* = 50, recorded for 554.3±234.6 s/neuron (range 300 – 600 s), corresponding to 3190.6±1366.0 spikes/neuron (range 924 – 5136 spikes)) in the control state to 3.6 ± 0.3 discharges/s after the lesion (*n* = 32; recorded for 454.6±156.8 s/neuron (range 300 – 600sec), corresponding to 1622.8±884.1 spikes/neuron (range 698 – 3422 spikes); p = 0.012; Mann-Whitney test; Figure 4A). In both animals, the burst index, calculated by using the 'surprise' method dropped significantly from 1.3 ± 0.2% and 1.2 ± 0.2%, in monkeys V and C, respectively, to 0.4 ± 0.1% in both (p = 0.0011 [monkey V] and p = 0.0016 [monkey C], Mann-Whitney test; Figure 4B). With Grace and Bunney's method (Grace & Bunney, 1984), the burst index rose fell from a pre-lesion control of 31.5 ± 2.5% to 16.5 ± 2.4% in monkey V and from 32.8 ± 2.5% to 24.5 ± 3.0% in monkey C (p = 0.0024 [monkey V] and p = 0.0032 [monkey C], Mann-Whitney test).

Effects of STN lesions on striatal dopamine levels

As a further measure of the functional impact of the STN-SNc interaction, we measured dopamine levels in the striatum, the main target of SNc efferents, before and after lesioning the STN in these animals. The average dopamine concentration prior to lesioning was 37.3 ± 3.4 pg/40μl in monkey C (n = 7 experiments), and 37.6 ± 3.0 pg/40μl in monkey V (n = 10). High potassium exposure during collection of microdialysis sample #3 resulted in an increase of dopamine levels. The ratio between the dopamine level in sample #3 and the pre-potassium exposure baseline level (average of samples #1 and 2) was 7.2 ± 1.2 in monkey C and 3.1 ± 0.5 in monkey V. The increase between baseline and potassium-stimulated values was significant (p = 0.0003 [monkey C] and p = 0.0042 [monkey V], Wilcoxon signed-rank test).

The post-lesioning experiments started seven days after lesioning in monkey C, and six days after lesioning in monkey V. In each case, the individual microdialysis experiments were carried out separated by at least 48 hours. After STN lesioning, the striatal dopamine concentrations were significantly reduced in both cases, to 27.9 ± 2.3 pg/40 μl (n = 5; p = 0.0383, Mann-Whitney test) in monkey C, and 28.4 ± 2.8 pg/40 μl in monkey V (n = 4; p = 0.0394; Mann-Whitney test; Figure 4C). In the post-lesion experiments, the ratio between the dopamine level in sample #3 and the pre-potassium exposure baseline level was 6.0 ± 1.6 in monkey C and 3.7 ± 1.0 in monkey V. The increase between baseline and potassium-stimulated values was significant (p = 0.0004 [monkey C] and p = 0.0036 [monkey V], Wilcoxon signed-rank test).

Discussion

Our results demonstrate that changes in STN activity significantly affect discharge patterns of SNc neurons and striatal dopamine release in primates.

As expected, local injections of muscimol inactivated the STN, while carbachol increased its discharge. Drug spread to the SN was minimized by using small injection volumes which, based on previous experience, affect neuronal activity only within a 0.5–1 mm radius around the injection site (see Wichmann *et al*., 1994b; Baron *et al*., 2002), and by placing the injections in locations which were at least 1 mm away from the STN/SN border. In addition, abnormal eye movements that could be indicative of inadvertent spread of the drug to the SNr (Hikosaka & Wurtz, 1985), were not induced by these injections. Histologic verification in the case of the ibotenic acid lesion experiments showed that the lesions were limited to the STN.

SNc cells normally fire with a slow regular pattern due to intrinsic pacemaker properties that appear to be modulated through synaptic inputs (e.g., Wilson & Callaway, 2000; Redgrave & Gurney, 2006; Grace *et al*., 2007). In our experiments, pharmacologic changes in STN activity significantly altered the spontaneous firing rate of individual SNc neurons. The polarity and magnitude of these responses differed among cells, perhaps because of the existence of STN-SNc pathways of opposing polarity, which may have been inhomogeneously affected by the STN interventions. The observed changes in SNc were most likely the net result of simultaneous changes in activation of excitatory and inhibitory pathways. Increases in discharge after injections of carbachol, and decreases after muscimol, may have resulted from changes in activity along the net excitatory connections between the STN and the SNc. Because the monosynaptic STN-SNc pathway is sparse in primates (Smith *et al*., 1990), it is likely that these responses were primarily mediated via excitatory SNc afferents from the PPN (e.g., Clarke *et al*., 1987; Futami *et al*., 1995; Charara *et al*., 1996). On the other hand, decreases in the discharge rate of SNc neurons in response to STN activation, and increases in response to inactivation, may have involved net inhibitory pathways through SNr, including the glutamatergic projection from STN to SNr, and local inhibitory axon collaterals linking SNr and SNc (Grace & Bunney, 1979; Hajos & Greenfield, 1994; Celada *et al*., 1999).

Changes in the discharge patterns of SNc neurons may be functionally more important than changes in the firing rates. STN inactivation resulted in reduced bursting in SNc, while STN activation had the opposite effect. Similar findings have also been described in rodents (e.g., Iribe *et al*., 1999; Kang & Futami, 1999). These pattern changes may be related to glutamatergic inputs to the SNc. Rodent experiments have provided evidence that changes in the activation of glutamate receptors influence the activity of SNc neurons (Mereu *et al*., 1991; Chergui *et al*., 1994a; Wu *et al*., 1994; Prisco *et al*., 2002). In monkeys, these receptors may be primarily associated with glutamatergic synapses from the abundant PPN input to SNc (see above). In addition to glutamatergic inputs, changes in the activation of GABA receptors may be required for burst firing in the SNc (e.g., Tepper *et al*., 1998; Paladini *et al*., 1999; Grace *et al*., 2007). STN activation may change the activity of SNr or GPe neurons, and may trigger release of GABA from terminals of their projections onto SNc neurons (e.g., Nakamura *et al*., 2003).

STN activation may be involved in the generation of bursts in the SNc under physiologic circumstances. The STN-SNc interaction may be part of the circuitry generating reward-related bursting in SNc (e.g., Schultz, 1998; 2000). This is supported by the fact that reward-related activity in the monkey STN has been described (Matsumura *et al*., 1992; Darbaky *et al*., 2005), and by lesion studies in rats that suggest a role for the STN in reward processing (Baunez *et al*., 2002; Baunez *et al*., 2005). However, given the relatively slow timing of changes in STN activity in response to salient, reward-related stimuli, it seems unlikely that changes in STN activity trigger bursts in the SNc in the time-locked manner which may be needed to alter synaptic strength through dopamine actions (Centonze *et al*., 2003). Recent studies have indicated that (at least in some cases) stimulus-related dopamine-cell bursting may be triggered by inputs from other sources, such as short-latency inputs from the superior colliculus (Redgrave & Gurney, 2006; Redgrave *et al*., 2008). It has been discussed that, rather than directly triggering reward-related bursts, the glutamatergic and GABAergic pathways whose activity is influenced by changes in STN activity may have a permissive role for bursting activity to occur (Goto *et al*., 2007; Grace *et al*., 2007).

In addition to its function in signaling reward-related information, SNc burst firing may act to maintain the background striatal dopamine level. While a direct correlation between physiological dopamine neuron activity and striatal dopamine levels is not established, experiments utilizing electrical stimulation of dopamine fibers have suggested that the rise in tissue dopamine levels per spike may be greater if the spike is part of a burst than when it occurs alone (Gonon, 1988), likely because dopamine uptake is saturated under burst conditions (Chergui *et al*., 1994b; Benoit-Marand *et al*., 2000; see also Cragg & Rice, 2004), so that dopamine cell bursting may translate into an overall greater amount of striatal dopamine. This possibility has been explored in rats in which increased SNc bursting, elicited by bicuculline-induced excitation of the globus pallidus, resulted in substantial increases in striatal dopamine levels (Lee *et al*., 2004). Similarly, the reductions in striatal dopamine level seen in our STN lesion experiments are likely due in large part to reduced bursting in the SNc. Changes in dopamine turnover have also been demonstrated in human patients with hemiballism resulting from STN lesions (Klawans *et al*., 1976; Kostic *et al*., 1988).

The STN-SNc interaction may also have relevance for our understanding of several aspects of Parkinson's disease. Thus, it has been speculated that the STN-SNc interaction may be part of a compensatory response network that prevents the appearance of parkinsonism in early phases of the disease: reduced striatal dopamine levels are known to increase STN activity (e.g., Bergman *et al*., 1994), which may then drive SNc neurons to release greater amounts of dopamine in the striatum, contributing to the prominent biochemical and behavioral compensation in early parkinsonism (see, e.g., Bezard *et al*., 1997; 1999; Zigmond *et al*., 2002; Bezard *et al*., 2003).

A second aspect is that enhanced glutamate release in the SNc due to increased STN activity may enhance oxidative damage to the remaining SNc neurons, accelerating the demise of these cells (Piallat *et al.*, 1996; Carvalho & Nikkhah, 2001; Wright & Arbuthnott, 2007). In support of this notion, STN lesioning afforded protection against the effects of the dopaminergic neurotoxin MPTP in a recent primate study (Wallace *et al*., 2007), contradicting earlier studies (Xu *et al*., 2004; Luquin *et al*., 2006). Neuroprotection through a reduction of the release of glutamate from STN projections is one of the rationales for ongoing trials of glutamic acid dehydrogenase (GAD) gene transfer therapy in the STN (Luo *et al*., 2002; Kaplitt *et al*., 2007).

Finally, the STN-SNc interaction may also be involved in the effects of deep brain stimulation (DBS) of the STN, a highly effective treatment of Parkinson's disease. Rodent experiments have suggested that STN stimulation leads to increased SNc activity, and enhanced striatal dopamine levels (e.g., Mintz *et al.*, 1986; Benazzouz *et al.*, 2000; Bruet *et al.*, 2001; Lacombe *et al*., 2007). Positron emission tomography studies in patients with Parkinson's disease have thus far failed to show an effect of DBS on dopaminergic functions (Abosch *et al*., 2003; Hilker *et al*., 2003; Strafella *et al*., 2003), but these studies were carried out in individuals with advanced dopamine depletion, reducing the likelihood of finding sufficient dopamine release from endogenous sources.

In summary, our results demonstrate that changes in STN activity in primates prominently affect SNc bursting, which may influence the release of dopamine in the striatum. The STN-SNc interaction may have a significant role in the regulation of SNc activity under normal circumstances, and in the compensation for moderate loss of dopamine in the early (preclinical) stages of Parkinson's disease.

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Abbreviations

aCSF, artificial cerebrospinal fluid; DBS, deep brain stimulation; GPe, external segment of the globus pallidus; GABA, γ-amino-butyric acid; GAD, glutamic acid dehydrogenase; ISI, inter-spike interval; PPN, pedunculo-pontine nucleus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus.

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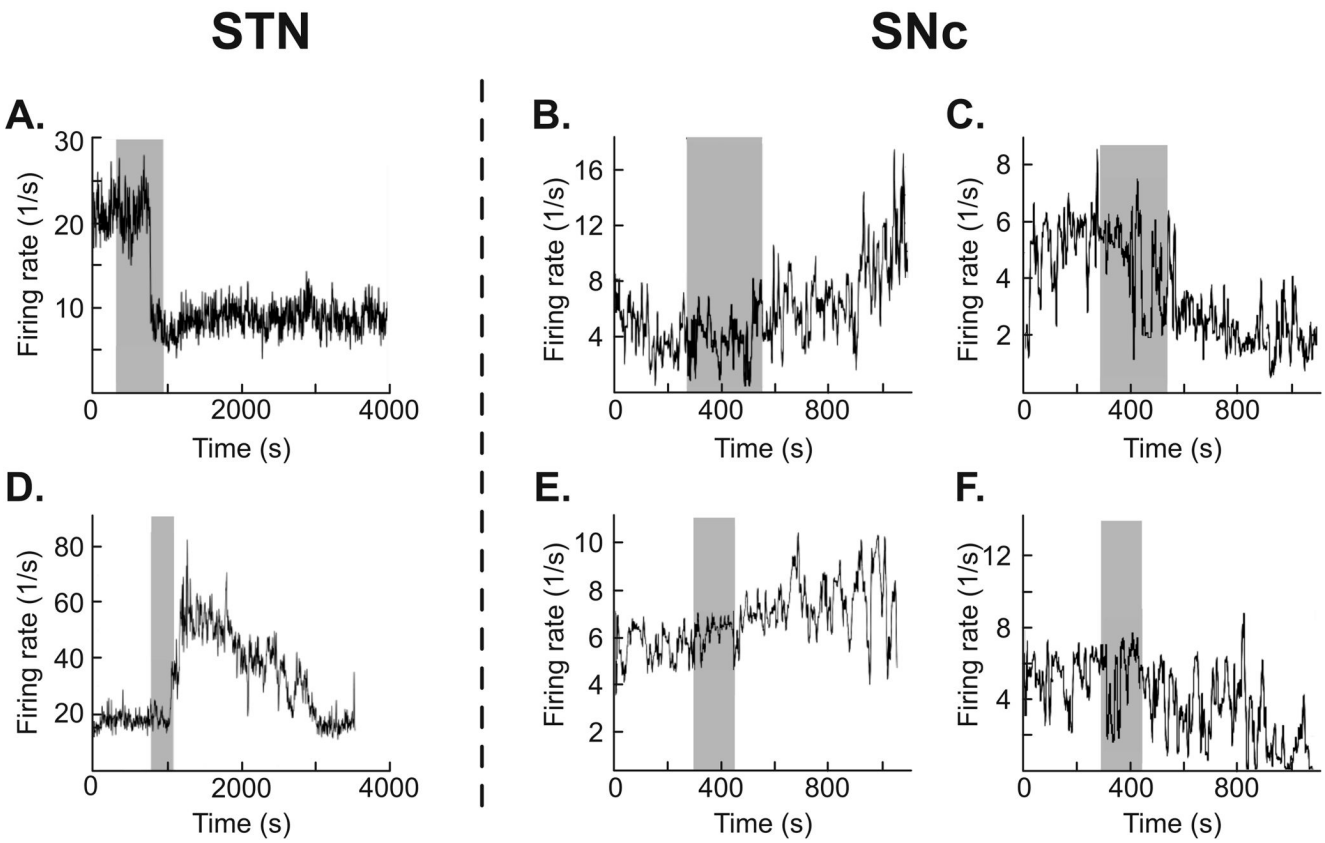

Figure 1.
Example responses of neurons in STN (A and D) and SNc (B, C, E and F) to local microinjections into the STN. The shaded portion of each figure depicts the time during which muscimol (A–C) or carbachol (D–F) was injected into the STN.

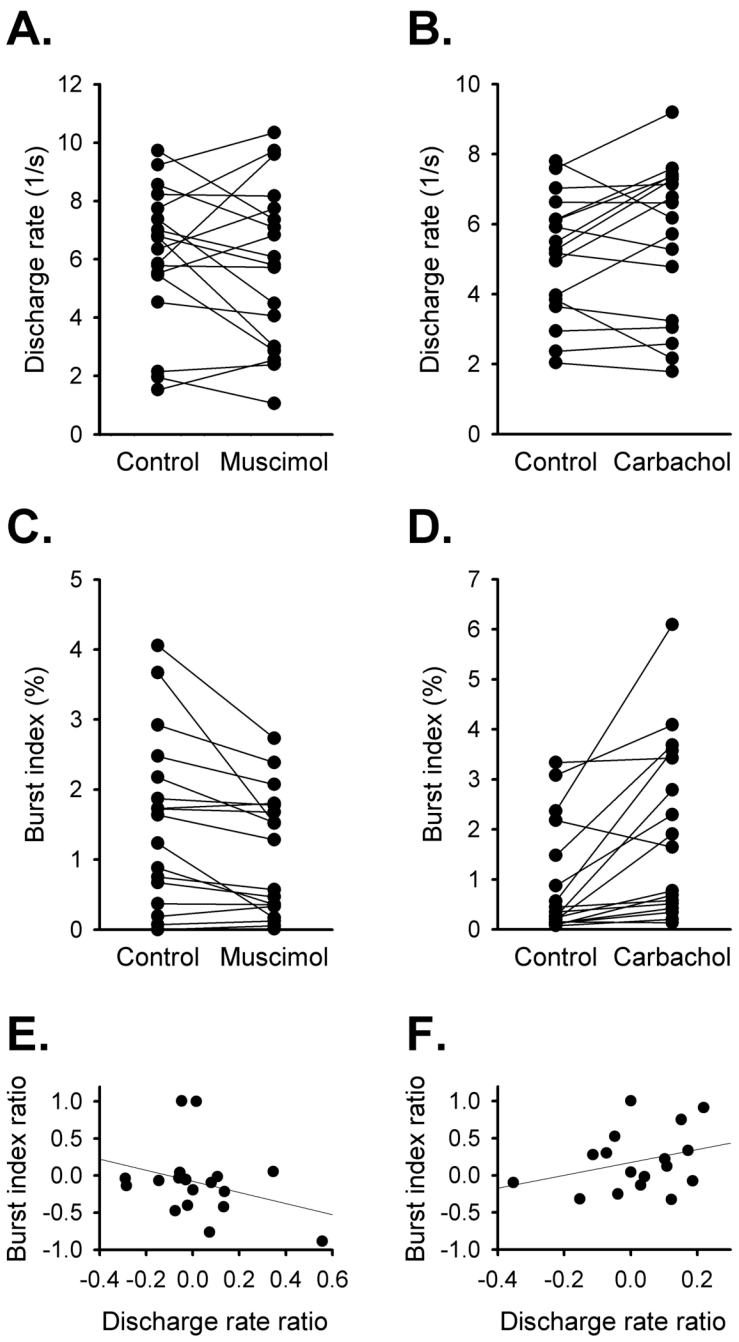

Figure 2.

Changes in the discharge rate (A,B) and burst index calculated with the Poisson 'surprise' method (C, D) of individual cells recorded in the SNc before and after injection of muscimol (A, C, E) or carbachol (B, D, F) into the STN. E and F show regression analyses between the discharge rate ratio and the burst index ratio, calculated for the recorded SNc cells before and after injections of muscimol (E) or carbachol (F) into the STN (see Methods for details). In both cases, there was no significant relationship between the two ratios.

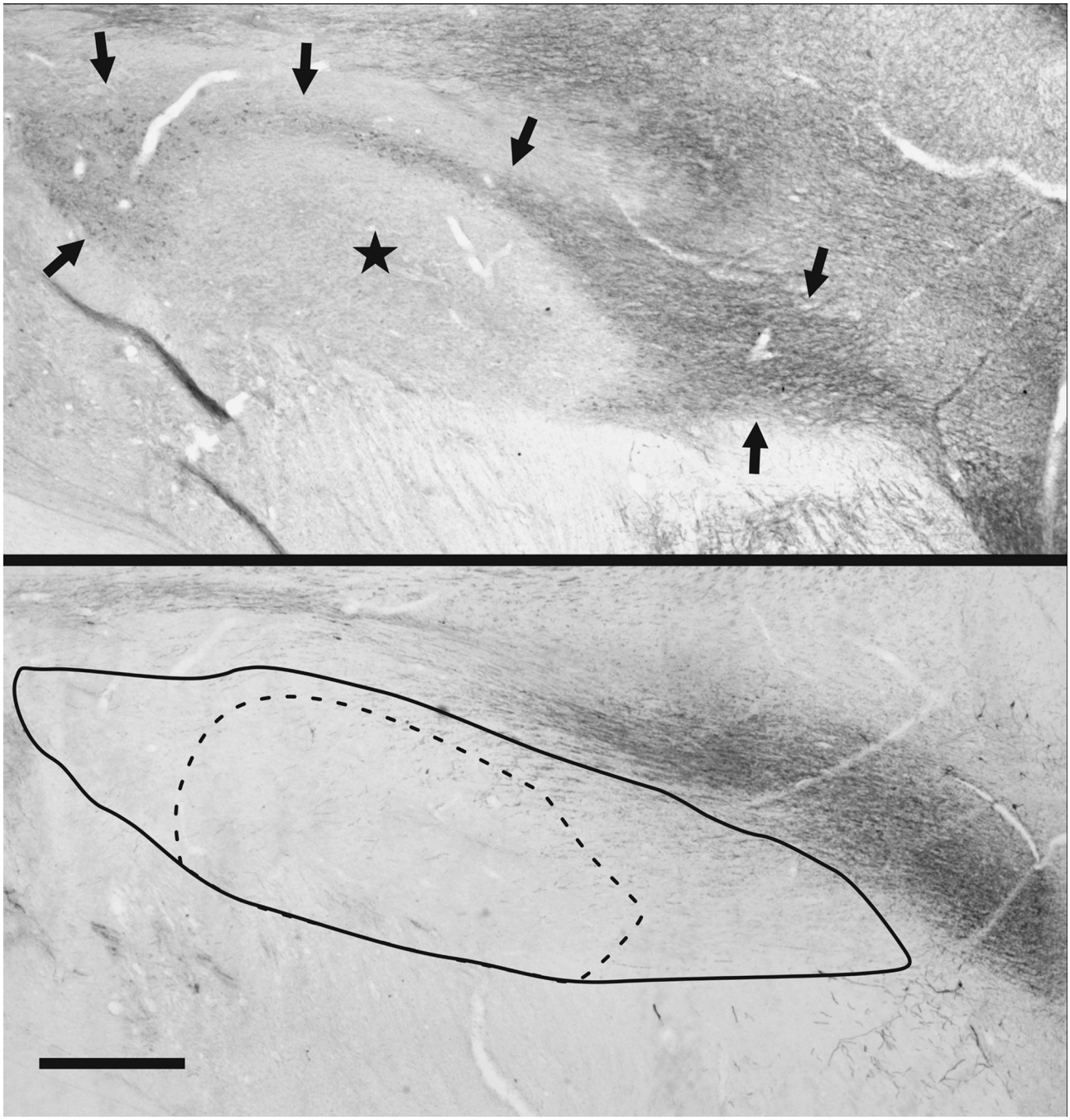

Figure 3.
Histology of ibotenic acid lesions of the STN. Staining with the neuronal marker, microtubulin associated protein 2, (top) shows the lesion (marked *) in the center of the STN (remaining STN tissue is marked by arrows). Tyrosine hydroxylase staining of a neighboring histologic slice (bottom) shows that the SNc was not affected by the lesion. The lines in this image show the approximate borders of the STN (solid lines), and the extent of the lesion (dashed). Scale bar in the bottom image represents 1 mm, and applies to both parts of the figure.

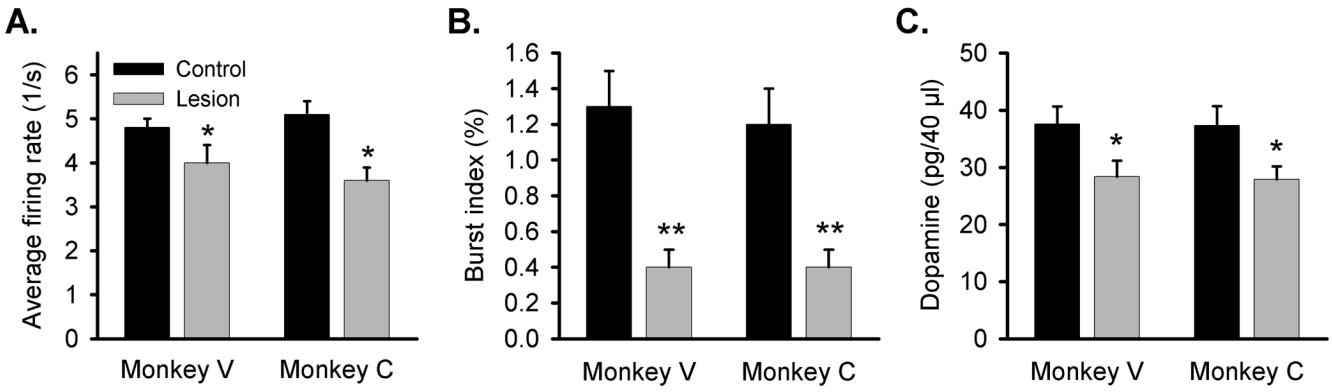

Figure 4.

Summary analysis of the effects of ibotenic acid lesions of the STN on average discharge rates of SNc neurons (A), the proportion of discharges within bursts (burst index, B) of SNc neurons, and the striatal dopamine concentration (C), in monkeys V and C.