Localization and function of dopamine receptors in the subthalamic nucleus of normal and parkinsonian monkeys

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Galvan A, Hu X, Rommelfanger KS, Pare JF, Khan ZU, Smith Y, Wichmann T. Localization and function of dopamine receptors in the subthalamic nucleus of normal and parkinsonian monkeys. J Neurophysiol 112: 467–479, 2014. First published April 23, 2014; doi:10.1152/jn.00849.2013.—The subthalamic nucleus (STN) receives a dopaminergic innervation from the substantia nigra pars compacta, but the role of this projection remains poorly understood, particularly in primates. To address this issue, we used immunoelectron microscopy to localize D1, D2, and D5 dopamine receptors in the STN of rhesus macaques and studied the electrophysiological effects of activating D1-like or D2-like receptors in normal and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated parkinsonian monkeys. Labeling of D1 and D2 receptors was primarily found presynaptically, on preterminal axons and putative glutamatergic and GABAAergic terminals, while D5 receptors were more significantly expressed postsynaptically, on dendritic shafts of STN neurons. The electrical spiking activity of STN neurons, recorded with standard extracellular recording methods, was studied before, during, and after intra-STN administration of the dopamine D1-like receptor agonist SKF82958, the D2-like receptor agonist quinpirole, or artificial cerebrospinal fluid (control injections). In normal animals, administration of SKF82958 significantly reduced the spontaneous firing but increased the rate of intraburst firing and the proportion of pause-burst sequences of firing. Quinpirole only increased the proportion of such pause-burst sequences in STN neurons of normal monkeys. In MPTP-treated monkeys, the D1-like receptor agonist also reduced the firing rate and increased the proportion of pause-burst sequences, while the D2-like receptor agonist did not change any of the chosen descriptors of the firing pattern of STN neurons. Our data suggest that dopamine receptor activation can directly modulate the electrical activity of STN neurons by pre- and postsynaptic mechanisms in both normal and parkinsonian states, predominantly via activation of D1 receptors.

dopamine; electrophysiology; parkinsonism; subthalamic nucleus; ultrastructural localization

THE SUBTHALAMIC NUCLEUS (STN) is the only glutamatergic structure in the basal ganglia circuitry, and its activity is profoundly altered in Parkinson’s disease (PD) (Galvan and Wichmann 2008). The STN receives GABAAergic projections from the external segment of the globus pallidus (GPe) and glutamatergic inputs from the cerebral cortex, thalamus, and brain stem (Joel and Weiner 1997; Mathai and Smith 2011). It has traditionally been assumed that PD-associated changes of STN activity result from degeneration of the dopaminergic nigrostriatal tract and the resultant loss of striatal dopamine. However, both the primate and rodent STN receive a modest dopaminergic innervation from the substantia nigra compacta (SNc) (Augood et al. 2000; Campbell et al. 1985; Cossette et al. 1999; Francois et al. 2000; Gauthier et al. 1999; Hassani et al. 1997; Hedreen 1999; Lavoie et al. 1989; Rinvik et al. 1979; Smith and Kieval 2000), which may be altered in PD (Rommelfanger and Wichmann 2010). Despite its sparseness, compared with the massive nigrostriatal system (Francois et al. 2000; Lavoie et al. 1989; Pifl et al. 1991), the nigrosubthalamic projection has a significant impact on normal STN activity in rodents (Baufreton and Bevan 2008; Baufreton et al. 2003, 2005) and its degeneration may contribute to altered firing patterns of STN neurons in PD (Francois et al. 2000; Rommelfanger and Wichmann 2010).

Light microscopic immunohistochemical and receptor binding studies have identified dopamine receptors in the STN (Augood et al. 2000; Bouthenet et al. 1987; Boyson et al. 1986; Dawson et al. 1986, 1988; Johnson et al. 1994; Mansour et al. 1992; Smith and Kieval 2000; Smith and Villalba 2008), but apart from reports that examined the ultrastructural localization of D5 receptors in the rat and monkey STN (Baufreton et al. 2003; Ciliax et al. 2000), the extent of the pre- and postsynaptic localization of dopamine receptors in the STN remains unknown. Furthermore, while several reports indicated that the mRNAs for D1, D2, D3, and D5 receptors are present in the rodent STN (Baufreton et al. 2003; Flores et al. 1999; Svenningsson and Le Moine 2002), the pattern of dopamine receptor mRNA expression in primate STN neurons remains unclear (Augood et al. 2000; Hard et al. 2001; Mansour et al. 1992; Quik et al. 2000).

In rats, dopamine is released in the STN in response to local electrical stimulation (Cragg et al. 2004). Although early in vivo studies suggested an inhibitory effect of dopamine on rat STN neurons (Campbell et al. 1985; Hassani and Feger 1999), recent in vitro data from rodent brain slices have indicated that activation of D1-like receptors (specifically D5 receptors) increases burst firing in the STN through a postsynaptic mechanism (Baufreton et al. 2003) while activation of D2-like receptors promotes firing and reduces rebound bursting of STN neurons through presynaptic (Baufreton and Bevan 2008; Zhu et al. 2002) or postsynaptic (Floran et al. 2004b; Shen and
Johnson 2003) mechanisms. Dopaminergic inputs to the STN may be important in regulating movements, as suggested by in vivo studies showing that orofacial movements can be induced by activation of D1-like receptors (Parry et al. 1994) while akinesia results from blockade of dopamine receptors in the rat STN (Hauber 1998).

In light of these rodent studies, we undertook a detailed analysis of the cellular and subcellular localization of dopamine receptors and characterized the electrophysiological effects of D1-like and D2-like agonists on the activity of STN neurons in normal and parkinsonian monkeys. Our findings demonstrate that D1, D2, and D5 receptors are presynaptic and postsynaptically located in the monkey STN and that local activation of these receptors, particularly those of the D1 family, affects the firing rate and bursting activity of STN neurons in normal and 1-methyl-4-phenyl-1,2,3,6-tetrahydro- pyridine (MPTP)-treated parkinsonian monkeys.

MATERIALS AND METHODS

Animals

Five rhesus monkeys (Macaca mulatta; 3 females, 2 males; 4–12 yr old) were used for the electrophysiological studies, while tissue from seven other rhesus monkeys (4 female and 3 males; 3–8 yr old) and one pigtail monkey (Macaca nemestrina; female; 4 yr old) was used for the anatomical studies. All experiments were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Garber et al. 2011) and the US Public Health Service Policy on the Humane Care and Use of Laboratory Animals (amended 2002) and were approved by the Health and Biosafety Committee and the Animal Care and Use Committee of Emory University.

The animals were raised in the breeding colony at the Yerkes National Primate Research Center and were housed under conditions of protected contact or in pairs with other monkeys for most of the study, with free access to chow and water. The animals received vegetables and fruits daily.

Immunohistochemical Localization of D1, D2, and D5 Receptors

These experiments analyzed the ultrastructural localization of D1, D2, and D5 dopamine receptor immunoreactivity in the monkey STN at the electron microscope (EM) level.

Animal perfusion and initial tissue processing. The animals were deeply anesthetized with an overdose of pentobarbital sodium (100 mg/kg iv) before being perfused with a mixture of paraformaldehyde (4%) and glutaraldehyde (0.1%) in phosphate-buffered saline (PBS; 0.01 M, pH 7.4). Brain blocks were cut in 60-μm-thick sections with a vibrating microtome, collected in cold PBS, and processed for immunohistochemistry as described below.

Primary antiseras. We used brain sections from seven monkeys for the immunolocalization of D1, D2, and D5 dopamine receptors. For each receptor subtype, brain tissue from at least three of these seven animals was used (STN tissue from 2 monkeys was used for more than 1 receptor subtype immunolabeling).

To identify D1 and D5 receptors, two affinity-purified highly specific antibodies were used. Although the transmembrane regions of D1 and D5 receptors are highly homologous, these receptors differ significantly at the third intracellular loop and carboxy terminus (Sunahara et al. 1991; Tiberi et al. 1991). We used highly specific monoclonal D1 receptor antibodies (1:500; Sigma-Aldrich, St Louis, MO; D-187), which were raised in rats against a 28-amino acid peptide sequence of the third intracellular loop of the human D2 receptor that is shared by both the long and short forms of the receptor. No significant homology to other dopamine receptors (D1, D3, D4, or D5) has been reported for this peptide. Western blots using human brain tissue identified a specific band at ~50 kDa that has been previously characterized in neuroblastoma cells and with ultrastructural studies in rodent tissue (Lei et al. 2004; Macey et al. 2004; Menguval and Pickel 2002). We have previously reported the use of these antibodies in monkey tissue (Hadjipur-Niktarash et al. 2012; Klim et al. 2009, 2010).

Immunoperoxidase procedure. Before immunohistochemical processing, sections were rinsed in PBS (0.01 M, pH 7.4), incubated in 1% sodium borohydride solution in PBS (20 min), rinsed in PBS, treated with a cryoprotectant solution (PB, 0.05 M, pH 7.4, 25% sucrose, 10% glycerol), frozen at −80°C, thawed, returned to a decreasing gradient of cryoprotectant solutions, and rinsed in PBS. To block nonspecific binding sites, sections were preincubated for 1 h at room temperature in PBS containing 10% normal goat serum and 1% bovine serum albumin. This was followed by incubation with the primary antibody solution containing 1% normal goat serum, 1% bovine serum albumin, and the D1, D2, or D5 receptor antibodies for 48 h at 4°C. The incubation was terminated with several rinses in PBS. Sections were then incubated in secondary biotinylated goat anti-rabbit (to reveal D2 or D5 receptors) or anti-rat (to reveal D1 receptors) IgGs (1:200 dilution; Vector Laboratories, Burlingame, CA) for 90 min. The sections were then rinsed again in PBS and incubated for 90 min with avidin-biotin peroxidase complex at a dilution of 1:100 (Vector Laboratories). Then sections were washed in PBS and Tris buffer (50 mM; pH 7.6) and transferred to a solution containing 0.025% 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich), 1 mM imidazole (Fisher Scientific, Waltham, MA), and 0.005% hydrogen peroxide in Tris buffer for 10 min. Sections were then rinsed in PB (0.1 M, pH 7.4) and treated with 1% OsO4 for 20 min. Thereafter, the tissue was returned to PB and dehydrated with increasing concentrations of ethanol. To increase the contrast of the tissue, 1% uranyl acetate was added to the solution during the 35-min exposure to the 70% ethanol solution. After dehydration, sections were treated with propylene oxide and embedded in epoxy resin for 12 h (Durcupan ACM; Fluka, Buchs, Switzerland), mounted onto slides, and placed in a 60°C oven for 48 h. Tissue samples containing the STN were mounted onto resin blocks with cyanocrylate ester and cut into 60-nm-thick ultrathin sections with an ultramicrotome (Leica Ultracut T2; Nussloch, Germany). These sections were collected on Pioloform-coated copper grids and stained with lead citrate for 5 min to enhance tissue contrast.

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Quantitative ultrastructural analysis. Ultrathin sections were examined with an EM (model 1011 JEOL, Tokyo, Japan, or model EM10C, Zeiss, Oberkochen, Germany). Randomly selected areas containing immunoperoxidase labeling were scanned and photographed at a magnification of ×16,000–25,000. Electron micrographs were taken with a CCD camera (Dual View 300W; Gatan, Pleasanton, CA) controlled by Digital Micrograph software (Gatan) and saved to computer disk. Some micrographs were later adjusted for brightness and contrast with either Digital Micrograph or Adobe Photoshop.

Immunopositive elements were identified based on the presence of the electron-dense amorphous peroxidase reaction product, which was usually associated with the internal surface of the plasma membrane (see, e.g., Fig. 1A) or the external surface of subcellular organelles (see, e.g., Fig. 1B) (Totterdell et al. 1992). From a series of ~100 electron micrographs per animal, immunoreactive elements were categorized as preterminal axons (unmyelinated axons, <0.5 μm in diameter), myelinated axons (recognizable by surrounding layers of electron-dense myelin), dendrites, or glial processes on the basis of ultrastructural criteria (Peters et al. 1991). These micrographs covered a total surface area of 3,640 μm², 3,380 μm², and 4,939 μm² of STN tissue examined for D1, D2, and D5 receptor immunolabeling, respectively. The number of specific immunoreactive structures (such as preterminal axons or dendrites) was expressed as a proportion of the total number of all labeled elements in the STN. Immunolabeled terminals were classified as forming “asymmetric” (Gray’s type I) or “symmetric” (Gray’s type II) (Peters et al. 1991) synapses, and the proportion of each was quantified in relation to the total number of all labeled terminals. As shown by previous studies, the majority of terminals forming symmetric synapses in the STN are likely to be GABAergic boutons from the GPe while most terminals forming asymmetric synapses originate from glutamatergic neurons in the cerebral cortex, thalamus, and brain stem (Smith et al. 1998). Terminals that did not form clear synapses in the examined plane of sections were discarded from this analysis but were included in the calculations of the overall proportion of specific immunoreactive elements.

To calculate the ratio of terminals making asymmetric or symmetric synapses in the STN, we used randomly selected EM micrographs (obtained at ×25,000) of STN neuropil taken from three normal monkeys (2 of these animals also contributed tissue for the dopamine receptor immunolocalization). The total number of terminals forming clearly identifiable symmetric or asymmetric synapses was quantified based on the analysis of 1,875 μm² of STN tissue.

Electrophysiology Recordings and Intra-STN Drug Administration

Animal preparation and surgical procedures. Before the experiments started, the monkeys were acclimated to the laboratory, and positive reinforcement techniques were used to train them to permit handling by the experimenter and to sit in a primate chair (McMillan et al. 2014). In preparation for the chronic electrophysiological recordings, the animals were first implanted under isoflurane anesthesia (1–3%) and aseptic conditions with two stainless steel cylindrical recording chambers (16-mm ID; Cist Instrument, Hagerston, MD). The chambers were stereotactically directed at the STN, either in the parasagittal plane, 36° anterior from the vertical, or in the coronal plane, 36° from the vertical. Along with the recording chambers, a metal bolt (Cist Instrument) for head fixation was implanted. The chambers and head bolt were fixed to the skull with stainless steel screws and dental acrylic. After the surgical procedure, the animals received prophylactic antibiotics and were allowed to recover for at least 7 days before the start of the electrophysiology recordings. The recording chambers were cleaned at least three times every week for the duration of the studies.

MPTP treatment and assessment of parkinsonism. Two of the monkeys used for the electrophysiology studies received weekly injections of MPTP (0.2–0.8 mg/kg im; Natland International, Morrisville, NC) until they developed stable moderate parkinsonism. The degree of motor impairment was assessed every week. We rated motor behavior with a parkinsonism rating scale that comprised gross motor activity, balance, posture, arm bradykinesia, arm hypokinesia, leg bradykinesia, leg hypokinesia, arm tremor, and leg tremor. Each item was scored between 0 and 3 (normal to severe), for a maximum of 27 points. In addition, we used a cage activity monitoring system based on infrared beams and a computer-assisted observation method. A monkey was considered to be parkinsonian if the score in the parkinsonian rating scale was >10 and if the movement counts in the activity monitoring were <60% from baseline (pre-MPTP treatment). We have used these assessment methods extensively in the context of other studies (Galvan et al. 2010; Wichmann et al. 2001).

The total amounts of MPTP administered were 22 mg/kg in one monkey and 32 mg/kg in the other, and the treatment lasted (with interruptions) 16 and 17 mo, respectively. The electrophysiological studies did not begin until the animals were stably parkinsonian for a minimum of 6 wk after the last MPTP injection (see also below).

Electrophysiological recordings. For the electrophysiological recording sessions, the animals were seated in a primate chair with their head restrained but free to move their body and limbs. The animals remained awake during these sessions, as judged by maintained eye opening and occasional movements. The dura was perforated with a 21-gauge guide tube, and a tungsten microelectrode (Z = 0.3–1.0 MΩ at 1 kHz; FHC, Bowdoinham, ME) was lowered into the brain with a microdrive (MO-95; Narishige, Tokyo, Japan). Extracelluar neuronal electrical signals were amplified (DAM-80 amplifier; WPI, Sarasota, FL), filtered (400–6,000 Hz; Krohn-Hite, Brockton, MA), displayed on a digital oscilloscope (DL1540; Yokogawa, Tokyo, Japan), and made audible via an audio amplifier. The neuronal signals were digitized at a sampling rate of 25 kHz and stored on computer disk with a data acquisition interface (Power1401; CED, Cambridge, UK) and commercial software (Spike2, CED).

We identified the STN on the basis of characteristic electrophysiological features (including high cellular density and persistent neuronal activity), its close proximity to fibers of the internal capsule, and the location of the fast-firing neurons of the substantia nigra reticulata (SNr), which are ventral to the STN (Wichmann et al. 1994a).

Intracerebral injections. To examine the effects of activation of D1-like receptors (i.e., D1 or D5 receptors) or D2-like receptors (i.e., D2, D3, or D4 receptors) in the STN, agonists for these receptor subtypes were locally injected into the STN, with concomitant recording of electrophysiological activity in the vicinity of the injection site. To accomplish these injections, we used a device that combines a regular tungsten microelectrode with thin fused silica tubing (as described in detail in Kliem and Wichmann 2004). The drugs were delivered with a microsyringe (CMA Microdialysis, Kista, Sweden) mounted on an infusion pump (model 102, CMA). The pump was remotely controlled by computer. In each experiment, the injection/recording device was positioned in the STN with a microdrive. Once in the STN area the injection/recording device was advanced slowly to the location of the fast-firing neurons of the substantia nigra reticulata (SNr), which are ventral to the STN (Wichmann et al. 1994a).

The D1-like receptor agonist SKF82958 (3 μg/μl) (both drugs from Sigma-Aldrich) was dissolved in artificial cerebrospinal fluid (aCSF; in mM: 143 NaCl, 2.8 KCl, 1.2 CaCl₂, 1.2 MgCl₂, and 1 Na₂HPO₄) at a final pH of 5.0 for SKF82958 and 7.0 for quinpirole. SKF82958 has a much higher affinity to D1-like than D2-like receptors (Neumeyer et al. 2003) but, as other compounds of its kind, does not effectively...
distinguish between D1 and D5 receptors (Sunahara et al. 1991). Quinpirole binds with significantly higher affinity to D2-like than to D1-like receptors (Millan et al. 2002; Seeman and Van Tol 1994) and has a fivefold higher affinity for D2 receptors than for D3 or D4 receptors (Seeman and Van Tol 1994). Injections of aCSF at pH 5 or pH 7 were used as controls. All solutions were filtered through a 0.2-μm-pore size nylon membrane (Fisher Scientific) prior to use.

**Termination of experiments and histological processing.** At the conclusion of the electrophysiology experiments, the animals were deeply anesthetized and perfused and the brains were obtained and sectioned as described above. Sections containing the STN were stained for Nissl substance to visualize electrode and injection system penetrations, while alternate sections were immunolabeled for the neuronal marker microtubule-associated protein 2 (mouse anti-MAP2, 1:1,000; Millipore) to assess the extent of neuronal damage induced by the electrode tracks. The expression of tyrosine hydroxylase (TH) was assessed with immunohistochemistry (monoclonal mouse anti-TH antibody, 1:1,000; Millipore) to evaluate the degree of dopaminergic denervation in the sensorimotor putamen in the two MPTP-treated animals. For comparison, we used brain sections containing the postcommissural putamen from three other normal monkeys from our tissue bank. These were processed simultaneously with the tissue from the MPTP-treated monkeys.

The labeling for MAP2 and TH was visualized by the immunoperoxidase method, as described above, with the difference that the sodium borohydride and freezing steps were omitted and 0.1% Triton X-100 was added to all incubations. After revealing the MAP2 or TH antibodies, the sections were mounted on gelatin-coated slides, dehydrated in alcohol, and immersed in toluene, and a coverslip was applied with Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI). The slides were digitized with an Aperio Scanscope CS system (Aperio Technologies, Vista, CA).

**Assessment of dopaminergic denervation.** With Imagescope viewer software (Aperio), the digital images of the TH-immunolabeled tissue slides were examined and a ×0.7 magnification image containing the postcommissural putamen was obtained. The images were then imported into ImageJ (National Institutes of Health; Rasband 1997–2009), and the extent of the nigrostriatal dopaminergic denervation was determined by measuring the optical density of TH immunolabeling in the postcommissural putamen of MPTP-treated monkeys. The optical density measurement in the internal capsule on the same section was considered to reflect background labeling and was subtracted from that obtained in the putamen. Three sections from each animal were used. These values were compared against measurements made from striatal sections at the same rostrocaudal level in normal monkeys.

**Analysis of electrophysiological data.** We confirmed that neuronal recordings were done in the STN on the basis of the depth reading of the microdrive during the recording sessions and the location of recording tracks visible in the Nissl- and MAP2-immunolabeled tissue sections. The recordings were further analyzed if the signal-to-noise ratio was at least 2. We sorted spikes off-line with a waveform matching algorithm, followed by principal component analysis (Spike2), and constructed interspike interval (ISI) distribution histograms for quality control. Only single-unit recordings were included in the analysis. Custom-written MATLAB (MathWorks, Natick, MA) algorithms were used for the rest of the analysis, which was performed with ISI data.

Several parameters of neuronal firing were considered in our analysis, including the mean firing rate (number of spikes/s), the coefficient of variation of the ISIs (ISI CV, standard deviation/mean), burst characteristics, and power spectra. To detect bursts, we used the method described by Legendy and Salcman (Legendy and Salcman 1985; Wichmann and Soares 2006), with a “surprise” value of 3. In the identified bursts we calculated the mean intraburst firing rate, the maximal increase of firing within a burst (MIFB, i.e., the maximal burst firing rate divided by the mean firing rate of the neuron), the proportion of time that the cell spent in burst discharges, and the proportion of spikes in bursts (compared with the total number of spikes). We also calculated the firing rate during burst and nonburst segments as well as the proportion of time and spikes that were not part of bursts. Nonbursting data segments were generated by removing all ISIs identified as belonging to bursts from the original data stream. Bursts were classified as “pause-burst sequences” if the ISI immediately preceding the burst was at least three times longer than the mean ISI of the neuron’s discharge. Some of these sequences may represent rebound bursts, as described in in vitro experiments (e.g., Bevan et al. 2002; Hallworth and Bevan 2005; Nakashima et al. 1990). In addition, if the duration of ISIs progressively increased throughout the burst, the burst was classified as an “ISI lengthening burst” (ILB).

In extracellular recordings, a finding of gradually lengthening ISIs within a burst has been used as an indicator of T-type calcium channel-dependent “low-threshold-spike” burst (e.g., Guido et al. 1992; Tsoukatos et al. 1997; Zirh et al. 1998). Finally, we calculated the proportion of pause-burst sequences that fulfilled ILB criteria.

The power spectral analysis was done with the Neurospec 2.0 MATLAB functions for frequency domain analyses of neuronal spiking data (Halliday et al. 1995; Nielsen et al. 2005). For each neuron, the raw spectra were integrated in the 1–3 Hz, 3–8 Hz, 8–13 Hz, 13–30 Hz, 30–75 Hz, and 75–100 Hz ranges and the resultant values expressed as a fraction of the power in the entire 1–100 Hz band. Similar methods have been used in our previous publications (Bogenpohl et al. 2013; Galvan et al. 2010, 2011; Sanders et al. 2013). To determine whether individual neurons had significant spectral peaks, we also visually inspected the spectra. A spectral peak was considered to be significant if it was above the mean ± 2 SD line (as based on the spectral components above 100 Hz).

To analyze the effects of the drug infusions, a period of 60 s before the start of the drug infusion was used as a “control” (baseline) segment. The “drug effect” segment was defined as starting with the drug injection and lasting up to 300 s afterwards. The duration of the “effect” segment was 263 ± 58 s (mean ± SD). If either the baseline or the drug effect segment contained fewer than 200 ISIs, the experiment was not further considered.

All descriptors of firing pattern (mean firing rate, CV, burst characteristics, power spectrum) were calculated for the baseline and drug effect segments, and the values for the drug effect segments were normalized to their respective baseline values. For some neurons, the proportion of bursts (total, pause-burst sequences, or ILB) was zero either at baseline or after the drug infusion. For normalization purposes, zero values at baseline were replaced with a value equal to 1/10th of the (nonzero) smallest value obtained for that parameter in other cells. If the value was 0 for both the baseline and the drug effect periods, the “normalized” value was set to “1.”

The normalized values were used for statistical comparisons. Each experimental group (D1- or D2-like receptor agonist treatment in either normal or MPTP-treated conditions) was compared against the respective aCSF injection cases. A drug was considered to have a significant effect if the comparison with the aCSF injection group resulted in a P value < 0.05 in the Mann-Whitney test. The baseline values for each parameter of firing obtained in normal monkeys were compared with those from MPTP-treated parkinsonian monkeys (Mann-Whitney test, P < 0.05 considered statistically significant). All statistical analyses were done with IBM SPSS software.

**RESULTS**

**Immunolocalization of D1, D2, and D5 Receptors**

At the light microscopic level (not shown), there was light to moderate neuropil immunolabeling for D1, D2, and D5 receptors in the STN. Neuronal cell bodies were either lightly immunolabeled or devoid of immunoreactivity. There was no regional patterning of immunolabeling for any of the dopamine receptors.
receptor subtypes under study. The level of dopamine receptor labeling in the STN was below that previously reported with the same antibodies in other monkey basal ganglia nuclei (Hadipour-Niktarash et al. 2012; Kliem et al. 2009).

At the EM level, D1 receptor immunoperoxidase labeling was primarily seen in presynaptic elements, with the greatest expression in preterminal axons (53.7 ± 5.3% of all labeled elements; values are means ± SD) and terminals (16.2 ± 7.1%). A smaller proportion of D1 receptor-immunopositive elements was accounted for by postsynaptic dendrites (7.3 ± 0.9%), myelinated axons (13.6 ± 16%), or glial processes (9.2 ± 4.9%). Micrographs of representative D1-labeled elements are depicted in Fig. 1, A and B, and the summary of quantitative data about their relative distribution is shown in Fig. 2A. Of all D1 receptor-immunoreactive terminals that formed clearly identifiable synaptic contacts (n = 46), 65.4 ± 7.6% formed asymmetric, putatively glutamatergic, synapses while 34.6 ± 7.6% formed symmetric, putatively GABAergic, synapses (Fig. 2B).

Like D1 receptors, D5 receptor immunoperoxidase labeling was also commonly seen in preterminal axons (31.1 ± 17.5%) and terminals (11.6 ± 1.8%; Fig. 2A). Of the terminals whose synapse could be clearly identified (n = 45), 58.7 ± 25.0% formed asymmetric synapses and 41.3 ± 25.0% formed symmetric synapses (Fig. 1C, Fig. 2B). The proportion of D5 receptor-immunopositive dendritic (postsynaptic) elements was larger than that labeled for D1 receptors (32.8 ± 10.6%; Fig. 1D, Fig. 2A). D5 receptor-immunopositive myelinated axons were also found (15.7 ± 15.4%), while glial processes were less abundant (8.8 ± 7.4%).

Fig. 1. Representative micrographs of neuronal elements immunolabeled for D1 receptors (A and B), D5 receptors (C and D), or D2 receptors (E and F) in the subthalamic nucleus (STN) of normal monkeys. Immunoperoxidase labeling is indicated by white arrows. A: a D1 receptor-labeled terminal (Ter) forming an asymmetric synapse (arrowheads) with an unlabeled dendrite (u. Den). B: a D1 receptor-immunopositive dendrite (Den). C: a D5 receptor-labeled terminal forming an asymmetric synapse (arrowheads) with an unlabeled dendrite (u. Den). D: a D5 receptor-labeled dendrite and preterminal axon (ax). E: a D2 receptor-labeled terminal forming a symmetric synapse (black arrows) with an unlabeled dendrite. F: a D2 receptor-labeled terminal forming an asymmetric synapse (arrowheads) with an unlabeled dendrite. Scale bars, 0.5 μm.
D2 receptor immunoperoxidase labeling was also primarily seen in presynaptic elements, but in contrast to D1 and D5 receptors the largest proportion of positive elements were terminals (50.4 ± 15.7%), followed by dendrites (21.5 ± 4.8%). Preterminal axons accounted for 15.8 ± 5.1% of D2 receptor-immunopositive elements, while only scarce labeling was found in myelinated axons (3.2 ± 2.3%) and glial processes (8.9 ± 7%; Fig. 2A). Of the terminals whose synapse could be clearly identified (n = 84), 82.7 ± 15.3% formed asymmetric synapses while fewer (17.3 ± 15.3%) formed symmetric, putatively GABAergic synapses (Fig. 1, E and F, Fig. 2B).

To determine whether the preferential expression of the different dopamine receptor subtypes in terminals forming asymmetric over symmetric synapses (Fig. 2B) was merely due to the relative abundance of these two types of terminals in the monkey STN, we quantified the total number of terminals forming symmetric or asymmetric synapses in the STN of three normal monkeys and found that 76.7 ± 27.7% of terminals in the STN form symmetric synapses while 32.3 ± 7.0% form asymmetric synapses (Fig. 2C). This finding indicates that the abundance of dopamine receptor labeling at terminals forming asymmetric synapses was not due to an overall high incidence of this type of terminals.

**Electrophysiological Studies**

*Database.* We included 74 STN neurons in the analysis. Thirty-three of these neurons were recorded in four normal monkeys and forty-one in two MPTP-treated monkeys (1 monkey contributed cells in both the normal and parkinsonian states). Postmortem histology using Nissl and MAP2-labeled sections confirmed that the recordings were confined to the STN. The two monkeys that received the MPTP treatment...
were considered stably and moderately parkinsonian. During the electrophysiological recordings, their scores on the parkinsonian rating scale were 15 and 11 (of a maximum of 27 points; Wichmann et al. 2001), and their body movements were reduced by 80% (for both monkeys) compared with baseline, as assessed by behavioral observations (see MATERIALS AND METHODS; Galvan et al. 2010; Wichmann et al. 2001). The histological analysis indicated that these animals had a 78% and 73% loss of TH immunolabeling in the postcommissural putamen, compared with normal monkeys, and a near-complete loss of TH-positive axonal processes in the STN (not shown).

The baseline firing characteristics of STN neurons recorded in the normal and parkinsonian states are summarized in Table 1. Compared with STN neurons recorded in normal animals, STN neurons recorded in MPTP-treated animals showed significantly higher MIFB values (P = 0.01, Mann-Whitney test), as well as a higher proportion of ILBs preceded by a pause (P = 0.03, Mann-Whitney test). On the basis of visual inspection, 23% of neurons showed distinct power spectral peaks at frequencies < 20 Hz in the normal state. This proportion was much higher in the STN recordings from MPTP-treated animals (56%).

**Effects of dopamine receptor agonists on firing rates and patterns of STN neurons.** We recorded the activity of STN neurons before, during, and after local administration of the D1-like receptor agonist SKF82958 (3 μg/μl), the D2-like receptor agonist quinpirole (5 μg/μl), or aCSF as control. All injections were delivered at a rate of 0.2 μl/min for a total volume of 0.4 μl.

The final analysis included data from 14 cells that were exposed to control infusions of aCSF (cells from normal and MPTP-treated monkeys were pooled together), 32 cells that were exposed to SKF82958 (13 and 19 neurons from normal and MPTP-treated animals, respectively), and 28 cells that were tested in the presence of quinpirole (17 and 11 from normal and MPTP-treated animals, respectively). The effects of these compounds on the firing rate and other firing parameters of STN neurons were determined by comparing each drug-treated group of cells (D1- and D2-like receptor agonist infusions in normal and in MPTP-treated monkeys) against the aCSF group.

The D1-like receptor agonist SKF82958 significantly reduced the mean firing rate of STN neurons compared with the effects of aCSF, in both normal and MPTP-treated animals (P = 0.029 and 0.026 for normal and MPTP-treated monkeys respectively; Fig. 3A). The reductions in the mean firing rate by SKF82958 were observed not only during the nonbursting segment (P = 0.014 and P = 0.019 for normal and MPTP-treated monkeys, respectively, Mann-Whitney test; Fig. 3B) but also during the bursting episodes of neuronal activity (P = 0.038 and P = 0.011 for normal and MPTP-treated animals, respectively, Fig. 3B). In normal monkeys, SKF82958 also increased the variability of firing of STN neurons (as measured with the CV; P = 0.019; Fig. 3C).

SKF82958 raised the MIFB values of STN neurons in normal monkeys (P = 0.014 compared with aCSF; Fig. 4A). Also, after SKF82958 injections STN neurons in normal and MPTP-treated animals showed an increased proportion of pause-burst sequences, compared with cells recorded after aCSF injections (P = 0.002 for normal and P = 0.016 for MPTP treated; Fig. 4C). Finally, the proportion of pause-burst sequences that also fulfilled the criteria for ILBs was increased after D1-like receptor treatment for normal and MPTP-treated STN neurons (P = 0.003 and P = 0.008 in normal and MPTP-treated cases, respectively; Fig. 4D).

The administration of the D2-like receptor agonist quinpirole did not significantly affect the mean firing rate or CV of STN neurons (Fig. 3). However, quinpirole increased the proportion of pause-burst sequences, including those classified as ILBs (P = 0.036 and P = 0.033, respectively; Fig. 4, C and D). In MPTP-treated monkeys, the effects of quinpirole on the firing rates and CV of STN neurons were highly variable, eliciting either increases or decreases of individual neurons’ activity. In the aggregate, these effects were not significant (Fig. 3). As in normal monkeys, the proportion of pause-burst sequences in STN neurons after D2-like receptor activation in MPTP-treated monkeys was higher than after aCSF, but the difference did not reach statistical significance in this group (P = 0.058; Fig. 4C). In contrast to the effect of quinpirole in normal monkeys, the D2-like receptor agonist did not change the proportion of ILBs preceded by a pause in MPTP-treated monkeys (Fig. 4D).

**DISCUSSION**

Our anatomical study demonstrates that D1, D5, and D2 dopamine receptors are preferentially expressed presynaptically, on preterminal axons and putative glutamatergic terminals in the monkey STN. Although presynaptic labeling predominated for the three receptor subtypes, postsynaptic dendritic expression was also found, most particularly for D5 receptors. We also found that the activation of D1-like recep-
tors in the monkey STN decreased the neuronal firing rate and increased the variability of firing, primarily by increasing the intraburst firing rate and the proportions of bursts that were preceded by a pause. In normal monkeys, the proportions of such pause-burst sequences and specifically that of ILBs preceded by a pause were also increased after activation of D2-like receptors.

**Changes in Firing Characteristics of STN Neurons After MPTP Treatment**

Our data showed that the burst firing pattern of STN neurons changed in MPTP-treated parkinsonian monkeys. Thus there was an increase in the maximal intraburst rate and in the proportion of ILBs preceded by pauses. These specific parameters were previously found to be the best features to discriminate between recordings of STN neurons in normal versus MPTP-treated monkeys (Sanders et al. 2013).

In contrast to other studies (Bezard et al. 1999; Miller and DeLong 1987; Soares et al. 2004), we did not find the average firing rate and distribution of integrated spectral power of the discharge of STN neurons in parkinsonian monkeys to be different from those in normal animals. On the other hand, we found that the proportion of STN neurons with low-frequency oscillations was higher in MPTP-treated monkeys than in normal animals, as reported previously (Bergman et al. 1994).

The differences in these parameters between our study and previously reported studies are likely methodological. For instance, they may have been caused by the relatively small number of STN neurons recorded in our study and the fact that our data in the normal and parkinsonian states were collected in different groups of monkeys, while they were gathered from the same animals in previous studies. Other factors may also have contributed to this discrepancy, such as the specific regimen of MPTP treatment (chronic vs. acute, intracarotid infusion vs. systemic treatment), which may have influenced the extent of lesions, specifically of nondopaminergic brain regions (e.g., thalamus and locus coeruleus; Masilamoni et al. 2011; Villalba et al. 2014).

**Overall Distribution of Dopamine Receptors in STN**

We found most labeling for D1, D2, and D5 receptors on presynaptic elements (preterminal axons and terminals). Similarly, our previous studies in monkeys showed that D1, D2, and D5 receptors in the internal segment of the globus pallidus (GPi), SNr, and GPe were mostly located in preterminal axons (Hadipour-Niktarash et al. 2012; Kliem et al. 2009). Other immunohistochemical studies have shown that dopamine receptors (Caille et al. 1996; Levey et al. 1993; Yung et al. 1995) and other receptors for GABA, serotonin, and adenosine as well as metabotropic and kainate glutamate receptors are also localized in preterminal axons (Hadjipour-Niktarash et al. 2012; Kliem et al. 2009). 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shown that D1 receptors found on small-diameter axons were mainly associated with the plasma membrane, as revealed with immunogold (Caille et al. 1996; Kliem et al. 2010), suggesting that they could be exposed to and activated by extrasynaptic dopamine and modulate transmitter release by altering conduction along the axon. In fact, regulation of transmission via activation of receptors in preterminal axons has been demonstrated for adenosine and nicotinic receptors in the rat hippocampus (Banerjee et al. 2012; Swanson et al. 1998), but it remains to be determined whether dopamine receptors have a similar function. Interestingly, our results showed that the ratio of immunopositive preterminal axons/terminals was higher for D1 than for D2 receptors in the monkey STN, suggesting that the presynaptic regulation of transmission via activation of axonal dopamine receptors could be more prevalent for D1 than for D2 receptors in the monkey STN.

We also found terminal labeling for the different dopamine receptors in the monkey STN, preferentially in putative glutamatergic terminals forming asymmetric synapses. Because our findings indicate that the STN contained at least twice more terminals forming symmetric than asymmetric synapses (Fig. 2C), this differential expression of dopamine receptors between putative excitatory and inhibitory terminals suggests a preferential dopamine-mediated presynaptic regulation of glutamatergic over GABAergic transmission in the primate STN.

The rest of the immunolabeling was found in postsynaptic dendrites (especially prevalent in D5 receptor-immunolabeled material, as discussed below), in some glial processes, and in myelinated axons. The presence of immunolabeling in myelinated axons likely represents receptors being transported along axons of passage through the STN. As in the STN, sparse immunolabeling for dopamine receptors in glia has been observed in other structures (Kliem et al. 2009), but the function of these glial receptors remains to be determined.

**D1-Like Receptors**

The ultrastructural data indicate that D1 receptors are primarily located on presynaptic elements, with greater numbers found on putative glutamatergic terminals than on GABAergic afferents. On the other hand, D5 receptors are more evenly distributed between pre- and postsynaptic elements than D1 receptors, suggesting that the effects of D1 and D5 receptor activation on STN activity may differ.

SKF82958 does not distinguish between D1 and D5 receptors, so that its effects on the firing rate and pattern of STN
neurons could be mediated via either type of receptor (Sunahara et al. 1991). However, experiments using D1-knockout mice have suggested that D5 receptors are the main effective targets of SKF82958 in the (rodent) STN (Baufreton et al. 2003). Our finding of reduced STN firing rates after local activation of D1-like receptors is similar to observations in anesthetized rats (Hassani and Feger 1999; Ni et al. 2001) and could be mediated by activation of presynaptic D1-like receptors on GABAergic terminals. These presynaptic receptors may increase the release of GABA and promote inhibition of (postsynaptic) STN neurons. This effect resembles the actions of SKF82958 in GPi and SNr in monkeys (Kliem et al. 2007).

As shown with in vitro STN recordings from brain slices (Baufreton et al. 2003), application of SKF82958 enhanced several parameters of bursting in the STN neurons in awake monkeys in our study. The increased bursting could be the product of increased GABA release or could be an effect mediated by postsynaptic D5 receptors (Baufreton et al. 2003).

**D2-Like Receptors**

Administration of the D2-like receptor agonist quinpirole in the monkey STN did not significantly affect the firing rate of STN neurons. This observation differs from the results of rodent studies that showed that intra-STN infusions of D2 receptor agonists produce either decreases or increases in firing (Hassani and Feger 1999; Ramanathan et al. 2008; Tofighy et al. 2003; Zhu et al. 2002), likely mediated via postsynaptic D2-like receptors (Ramanathan et al. 2008; Tofighy et al. 2003; Zhu et al. 2002). It is possible that the lack of effects of D2-like receptor agonists on the overall firing rate of monkey STN neurons resulted from the scarcity of postsynaptic D2 receptors in the primate STN, as shown in our EM study.

The lack of effects of D2-like receptor agonists on the firing rate of monkey STN neurons is also different from results obtained with similar approaches in other basal ganglia structures. As we have previously reported, activation of D2-like receptors increases firing in GPe and decreases it in GPi and SNr of normal and parkinsonian monkeys (Hadipour-Niktarash et al. 2012). Differences in D2 receptor density and localization in each structure may account for these differences.

In our study, activation of D2-like receptors increased the proportion of bursts preceded by pauses (which may represent rebound bursts) in STN neurons in normal monkeys. This contrasts with in vitro rodent studies that suggested that D2-like receptor-mediated transmission in the STN reduces rebound burst firing, likely through modulation of GABA release via presynaptic D2-like receptors (Baufreton and Bevan 2008). Other rodent studies showed that GABAergic transmission in the STN is under control of presynaptic D2-like receptors (Floran et al. 2004a; Shen and Johnson 2000). The presynaptic modulation of GABA release in the STN by D2-like receptors may thus be a comparatively minor mechanism in the monkey compared with the rodent STN, as also suggested by our anatomical studies in which D2 receptors on putative GABAergic terminals accounted for only a minority of immunopositive terminals in the primate STN.

Quinpirole may also have acted by activating other members of the D2-like receptor family (D3 and D4 receptors), although quinpirole has higher affinity for D2 than for D3 or D4 (see MATERIALS AND METHODS). Currently, there are no available data regarding the protein expression of D3 and D4 receptors in the monkey STN (Rommelfanger and Wichmann 2010).

**Dopamine Innervation of the STN**

The dopaminergic innervation of the monkey STN is relatively sparse, particularly when compared with the dense and extensive striatal innervation. However, the presence of dopaminergic receptors, as reported in this study and others (Ciliax et al. 2000), sets the stage for dopaminergic modulation of STN activity. Although light microscopy localization studies have described TH- or dopamine transporter (DAT)-immunoreactive axonal processes in the monkey STN (Francois et al. 2000; Lavoie et al. 1989; Smith and Kieval 2000; Smith and Villalba 2008), very few dopaminergic terminals forming conventional synapses were found in the monkey STN (Smith and Kieval 2000). These data differ from the rodent STN, in which dopaminergic synapses are frequently encountered (Cragg et al. 2004). Whether this represents a genuine species difference in the sources of dopamine release between primate and nonprimate STN or a technical limitation in detecting dopaminergic terminals in the monkey STN remains to be established.

In the rat, STN dopamine release evoked by electrical stimulation is modulated by DAT (Cragg et al. 2004), but the expression levels and functions of DAT in the monkey STN have not yet been characterized. Low DAT activity may favor volume transmission of dopamine that may be sufficient to activate nonsynaptic dopamine receptors and elicit dopamine-mediated effects in the primate STN.

In rodents, administration of exogenous dopamine into the STN in vivo or in brain slice preparations most commonly results in increased neuronal firing (Mintz et al. 1986; Ni et al. 2001; Zhu et al. 2002), although some in vivo recording studies found opposite effects (Campbell et al. 1985). Administration of dopamine receptor antagonists increases the firing of STN neurons (Campbell et al. 1985) and induces catalepsy (Hauber 1998) in rodents, thereby suggesting a potential tonic modulation of STN neuron activity in these animals.

It clearly would have been interesting to study the effects of dopamine receptor antagonists in normal monkeys, as this would have informed us about the control of STN firing by endogenously released dopamine. These experiments have not (yet) been done, because we wanted to avoid excessive tissue damage to the STN in our animals. In light of the sparse dopamine innervation (Smith and Kieval 2000), the low concentration of dopamine in the monkey STN (Pifl et al. 1990), and the potential relevance of dopamine receptor antagonists for PD therapeutics, we focused this work entirely on the effects of dopamine receptor activation in the STN.

**Studies in the Parkinsonian State**

The loss of dopamine in the striatum is the most noticeable pathological feature of PD. However, studies in PD patients and in MPTP-treated monkeys have shown that degeneration of dopaminergic cells in the SNc also affects dopamine levels outside of the striatum, including the STN (Francois et al. 2000; Hornykiewicz 1998; Pifl et al. 1990).

Because D5 receptors are constitutively active (Demchyshyn et al. 2000; Tiberi and Caron 1994) and have a higher affinity for dopamine than D1 receptors (Demchyshyn et al. 2000; Missale et al. 1998; Sunahara et al. 1991; Tiberi and Caron...
1994), their function may become particularly relevant in dopamine-depleted states. Activation of D5 receptors in parkinsonism could contribute to the exacerbated bursting pattern that characterizes STN neuronal activity in the parkinsonian state (Baufreton et al. 2005; Galvan and Wichmann 2008). In line with this possibility, blockade of D5 receptors reduced bursting in STN neurons of 6-hydroxodopamine (6-OHDA)-treated rats, presumably by antagonizing their constitutive activity (Cherit et al. 2013).

Although some reports (Cherit et al. 2013; El-Banoua et al. 2004) suggest that the STN may be a target for dopaminergic drug treatment in a rat model of parkinsonism, our results support this notion only partially. In our MPTP-treated monkeys, we found that activation of D1-like receptors decreased the firing rate of STN neurons, a potentially relevant antiparkinsonian effect (Bergman et al. 1990; Wichmann et al. 1994b), but we also found a substantial increase in D1-like receptor-mediated bursting activities (which are already increased in the STN in MPTP-treated animals; see Table 1), thereby suggesting that D1-like receptor agonists may have both pro- and antiparkinsonian effects on STN activity.

Interestingly, the increases in pause-burst sequences and ILBs preceded by pauses seen with D2-like receptor agonists in normal animals were not significant in MPTP-treated monkeys, suggesting that after dopaminergic depletion D2-like receptors may be less sensitive to the pharmacological activation.

Conclusions

We have identified D1, D2, and D5 receptors at pre- and postsynaptic locations in the monkey STN. These receptors provide a physiological substrate for dopamine modulation of STN activity. Our electrophysiological studies suggest that activation of D1-like and D2-like receptors in monkeys modulates the firing rate and pattern of STN neurons.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author Contributions


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