Control of the Subthalamic Innervation of Substantia Nigra Pars Reticulata by D1 and D2 Dopamine Receptors

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INTRODUCTION

Neurons of the substantia nigra pars reticulata (SNr) receive synaptic inputs from the striatum (Chevalier et al. 1985; Smith and Bolam 1991), globus pallidus (Smith and Bolam 1989), and subthalamic nucleus (STN) (Bevan et al. 1994; Iribe et al. 1999; Kita and Kitai 1987; Robledo and Feger 1990). The latter being the best-documented excitatory input, although other sources of excitation have not been discarded. These neurons are also exposed to dopamine released from dendrites of the pars compacta neurons as well as from dopaminergic neurons located inside the pars reticulata (Cheramy et al. 1981; Geffen et al. 1976; Nakarnishi et al. 1987; Richards et al. 1997; Rohrbacher et al. 2000). Dopamine release and a high density of dopamine receptors in the SNr (Barone et al. 1987; Beckstead et al. 1988; Richfield et al. 1987) suggest a role for dopamine in regulating basal ganglia output nuclei (Double and Crocker 1995; Floran et al. 2002; Robertson and Robertson 1989; Trevitt et al. 2001; Waszczak 1990; Weick et al. 1990; Wichmann et al. 2001). However, most studies on the action of dopamine in the reticulata have been directed to understand the role of dopamine D1 receptors present on striatogniral afferents (Barone et al. 1987) that when activated, facilitate GABAergic inhibitory transmission onto SNr neurons (Floran et al. 1990; Radnikow and Misgeld 1998).

In addition to its actions on striatogniral input, D1-class receptors may also facilitate the release of glutamate from subthalamic neuropeptides (Rosales et al. 1997). In support of this hypothesis, STN neurons express both D1 and D2 class receptors (Baufreton et al. 2003; Ciliax et al. 2000; Flores et al. 1999; Hurd et al. 2001; Khan et al. 2000; Svenningsson and Le Moine 2002). Accordingly, in this work, the role of dopamine receptors in the regulation of the subthalamic projection was explored. We found that this regulation is, at least, as important as the regulation of the inhibitory inputs coming from the neostriatum. A preliminary report of these results has been presented in abstract form (Ibañez et al. 2002).

METHODS

Preparation of slices

All procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care Committees of the CINVESTAV and UNAM. The experiments were performed on brain slices obtained from Wistar rats [postnatal day (PD) 14–21]. The rats were anesthetized and decapitated. The brain was rapidly obtained and immersed for 1 min in cold oxygenated saline (4°C; 95% O2-5% CO2) of the following composition (in mM): 124 NaCl, 2.5 KCl, 1.3 MgCl2, 1.2 NaH2PO4, 2.4 CaCl2, and 10 glucose. The same saline but with choline chloride (124 mM) instead of NaCl was used during the slicing procedure. Parasagittal slices (300 μm) containing both STN and SNr (see Fig. 1) were cut on a vibratome (Pelco...
recording a neuron in the posterior STN. When threshold stimulus did not elicit an action potential, no synaptic event was evident (superfused (2–3 ml/min) with oxygenated saline at room temperature. The slices were left for equilibration for 1 h in oxygenated saline at room temperature (25°C). After equilibration, a single slice was transferred to a recording chamber inside the STN boundaries with concentric bipolar tungsten electrodes superfused. Thereafter the pipette was retired, and the slice was superfused (2–3 ml/min) with oxygenated saline at room temperature.

Whole cell recordings

Recordings were made at room temperature (~25°C) from neurons located inside the SNr boundaries as seen in the parasagittal slice. This region contains GABAergic neurons (see following text). Neurons were visualized using infrared differential interference videomicroscopy with an ×40 water-immersion objective. Micropipettes for whole cell recordings were pulled (Sutter Instrument, Novato, CA) from borosilicate glass tubes (1.5 mm OD, WPI, Sarasota, FL) for a final resistance of ~2–5 MΩ when filled with internal saline of the following composition (in mM): 120 KSO3CH4, 10 NaCl, 10 K2EGTA, 10 HEPES, 1 CaCl2, 2 MgCl2, 2 ATP-Mg, and 0.3 GTP-Na (pH 7.3, 290 mosM/l). Voltage-clamp recordings were made with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Liquid junction potentials (~5 mV) were not corrected.

Current-voltage relationships made in current-clamp mode (i.e.: with bridge balance) superimposed tightly with those performed in voltage-clamp mode (i.e.: with 60–80% compensated series resistance; Fig. 3). This coincidence suggested that neither bridge balance nor series resistance (~15 MΩ) represent a problem.

Evoked synaptic currents

Synaptic currents were evoked in SNr neurons by field stimulation inside the STN boundaries with concentric bipolar tungsten electrodes (FHC, Bowdoinham, ME) (50 μm at the tip; 1 kΩ DC resistance) at a frequency of 0.1 Hz (20 μs, 1–10 V or 1–20 μA). To be accepted into the sample, postsynaptic neurons had input resistances >100 or 200 MΩ for the first and second class of recorded neurons, respectively (see Fig. 3).

Single or paired subthreshold stimulation (not enough to evoke action currents) was delivered in the presence of 10 μM bicuculline to block synaptic GABAergic responses. Isolation units (digitimer LTD, Hertfordshire, UK) between the computer and the stimulating electrodes were used to adjust stimulus parameters during the experiment. Distance between recording and stimulating electrode was commonly >500 μm (cf. Fig. 4). When field stimulation electrodes were placed anywhere inside the STN boundaries (Fig. 1), the probability to get a response in a SNr neuron was 1 of 10 trials. If stimulation electrodes were placed outside the STN border, the probability to get a response was null. Thus exploratory anatomical experiments using anterograde biocytin transport were performed to improve the probabilities of finding connections (see Vergara et al. 2003). A sharp micropipette (tip: <0.5 μm) filled with external saline and 5% biocytin (Sigma-Aldrich, St Louis, MO) was placed inside the STN border (near the middle) and left there for 1 h while the slice was constantly superfused. Thereafter the pipette was retired, and the slice was superfused with oxygenated saline for another 6 h. The slice was then fixed overnight in a 0.1 M phosphate-buffered saline (PBS; pH = 7.4; 4°C) with 4% paraformaldehyde and 1% picric acid. Afterward slices were infiltrated with 30% sucrose and cut on a vibratome (Ted Pella, Reading, CA) into 60-μm sections. After washing with Tris-buffered saline (TBS) containing Triton X-100 and avidin-biotin- peroxidase complex (1:100; Vector Laboratories, Burlingham, CA) for 4 h at room temperature, the slices were reacted with 3,3′-diaminobenzidine tetrahydrochloride (DAB; 0.05%) and H2O2 (0.003%) in TBS and mounted on slides to visualize the bound HRP. This enabled resolu-
tion, through trans-illumination microscopy, of labeled neurons inside the STN (Fig. 1A) and, most importantly, of subthalamonomigral fibers projecting to their target. One of such experiments is illustrated in Fig. 1. The inset shows the arrangement of the biocytin-filled electrode, while the photograph shows at left (in negative), the stained STN with numerous subthalamomugal fibers arising from it and going toward both substantia nigra pars compacta (SNC) (Iribe et al. 1999; Kanazawa et al. 1976; Smith and Grace 1992) and SNr (Bevan et al. 1994; Iribe et al. 1999; Kanazawa et al. 1976; Kita and Kitai 1987; Robledo and Feger 1990). The white square inside SNr borders (Fig. 1A) depicts the region expanded in Fig. 1B. Several anterogradely filled fibers can be appreciated. When stimulation electrodes are placed in this region while recording a subthalamic neuron (scheme in Fig. 1C1), antidromic action potentials can be elicited in subthalamic neurons (Fig. 1C, 2 and 3). A dashed circle (S) in the posterior part of the stained STN indicates the region where antidromic action potentials were most probably recorded. It coincides with the origin of many subthalamonomigral fibers. When stimulation electrodes were placed in this region (S), the probability to get an evoked excitory postsynaptic current (EPSC) in a SNr neuron was increased to one of two trials. Therefore most recordings of SNr neurons were done in the anterior part of the SNr (Fig. 2) and field stimulation was given in the posterior part of the STN (Fig. 1A). Holding potential in most experiments was −80 mV.

A paired-pulse protocol was sometimes used for field stimulation with inter-pulse intervals of ~50 ms. It was used to evaluate changes in the paired-pulse ratio (PPR) of EPSC responses (PPR = 2nd EPSC/1st EPSC). PPR values are known to be linearly proportional to the probability of release, and the paired-pulse protocol has been demonstrated to detect presynaptic actions of transmitters (e.g., Baldelli et al. 2005; Dunwiddie and Hass 1985; Guzman et al. 2003; Kamiya and Zucker 1994; Zucker 1999). In addition, 80–120 consecutive 25-Hz trains of subthreshold EPSCs, evoked at a frequency of 0.1 Hz, were used to perform mean-variance analyses in which mean amplitudes of evoked EPSCs (corrected for basal lines from previous EPSCs) were plotted against their peak variance (Clements and Silver 2000; Koos et al. 2004). Then, a parabola of the form (Eq. 1)

\[ y = Ax - Bx^2 \]

was fitted with a Marquart algorithm (Fig. 7); where y represents EPSC variance (ordinates), x represents EPSC mean amplitude (abscissae), and A and B are free parameters. Basically, parameter A indicates the initial slope of the parabola and parameter B depends on the width of the parabola. From this fit, a weighted average of the quantal amplitude, \( Q_w \), was obtained (Eq. 2)

\[ Q_w = A/(1 + CV^2) \]

where CV is the coefficient of variation of EPSCs amplitudes. In addition, the approximate number of release sites (N) and the average probability of release across release sites (assuming a binomial distribution) can be approximated by (Eqs. 3 and 4)

\[ N = 1/B \]

\[ P = x(BA)/(1 + CV^2) \]

Signals were filtered at 5 kHz and either digitized at 10 kHz using a Digidata 1200 interface (Axon Instruments) connected to a PC running Clampex 7.0 software or with an AT-MIO-6040E interface and a DAQ (NI-DAQ) board (National Instruments, Austin, TX) in a PC clone. On-line data acquisition used custom programs made in the LabVIEW environment (National Instruments). The NI-DAQ board was used to save the data on binary files in the computer hard disk for further off-line analysis.

Unless stated otherwise, graphs of time courses averaged six records per point; frequency of stimulation was 0.1 Hz or one trace each 10 s. Thus each point represents 1-min recording. However, illustrated records of PPRs are the average of 25 consecutive events during steady state. All data are given as means ± SE unless stated otherwise. Significance of the effect of drugs as well as the significance of the differences in PPRs was tested with nonparametric statistics: the Wilcoxon t-test or Mann-Whitney’s U test depending on paired or unpaired samples. When the same sample had more than one treatment, Friedman’s statistics with post hoc Student-Newman-Keuls test was employed. Statistical differences of fitted functions were assessed by comparing the obtained parameters and their estimation errors with Student’s t-test.

### Immunocytochemical procedures

To identify the recorded cells, 1% biocytin was included in the pipette solution (Horikawa and Armstrong 1988). To reconstruct biocytin-loaded cells (Fig. 4), a procedure similar to that described in the preceding text to see the trajectory of subthalamonomigral fibers was used except that slices were 40 μm thick. When processed for immunocytochemistry, the slices were incubated in streptavidin conjugated with Cy3 (1:200 dissolved in PBS, Zymed Laboratories, San Francisco CA). This allowed visualize the recorded neuron (1 per slice; Fig. 3). Thereafter slices were incubated 30 min with 1% bovine albumin to block unspecific binding sites. Then incubation for 36 h with a mouse monoclonal antibody against parvalbumin followed (anti-PV; 1:2000, Sigma-Aldrich dissolved in PBS containing 0.25% Triton-X). The slices were then rinsed thrice with PBS and incubated with a goat versus mouse secondary antibody during 1 h. This antibody was conjugated with Cy5 (Jackson Immuno Res Lab, West Grove, PA). Next, slices were mounted on covered slides and observed with different fluorescent filters (vectashield, Vector Laboratories) or in a confocal microscope (Bio-Rad Microscience, London, UK).

### Drugs

Drugs were stored as dry aliquots and stock solutions were prepared just prior to each experiment and added to the perfusion solution in the final concentration indicated. trans-(−)-4aR-4,4a,5,6,7,8,8a,9-octahydro-5-propyl-1H-pyrazolo(3,4-g)quinoline (quinpirole), (RS)-2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine hydrochloride (SKF-38393), R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH 23390), S-(−)-5-amino-sulfonyl-N-[1-ethyl-2-pyrrolidinyl]-methyl-2-methoxybenzamide (sulpiride), 6-cyano-7-nitroquinoxaline-2,3-dione (CNOX), D-(−)-2-amino-5-phosphonopentanoic acid (d-AP-5) and bicuculline methiodide were obtained from Sigma.

### RESULTS

#### Subthalamonomigral connection

As described in METHODS, staining after anterograde transport of biocytin can disclose subthalamonomigral fibers in a parasagittal brain slice that includes both STN and SNr (Fig. 1, A and B). Field stimulation (see METHODS), where subthalamonomigral fibers are abundant inside the SNr can elicit antidromic action potentials in the subthalamic neurons (Fig. 1C3). Most frequently, on neurons located in the posterior part of the STN (Fig. 1A, “S”). Figure 1C1 shows a scheme of the experiment. Field stimulation was of threshold intensity in Fig. 1C, 2 and 3 (i.e.: the strength necessary to evoke an action potential in 50% of cases). Note that an action potential can be evoked in the absence of any underlying synaptic response. Figure 1C4

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illustrates an action potential evoked with an intracellular current injection. These action potentials (Fig. 1C, 3 and 4) can be made to collide (not shown) and subthalomonigral fibers could be seen arising from the location where the antidromic spikes were most probably recorded (Fig. 1A, “S”). The probability to get a response of a postsynaptic SNr neuron after a field stimulus inside the posterior STN was ≈0.5 (see METHODS) when field stimulation was delivered at this site. Thus field stimulation inside the STN borders in a parasagittal slice can evoke synaptic events (EPSCs) on neurons located inside the SNr in the presence of bicuculline (10 μM). Field stimulation inside STN borders but outside this region much lowered the probability to get a response (see METHODS).

Synaptic events evoked by stimulating the subthalomonigral connection are illustrated in Fig. 2. Field stimulation inside STN borders (Fig. 2A) evoked a synaptic inward current in a SNr neuron (Fig. 2B) in the presence of 10 μM bicuculline (Fig. 2B, control). This subthreshold EPSC was greatly, but not completely reduced, by the addition of CNQX (10 μM) to the bath saline at −80 mV (Fig. 2B, +CNQX). Subsequent addition of +d-AP-5 (50 μM) virtually abolished the synaptic response (Fig. 2B, +AP-5). The AMPA receptor antagonist, CNQX, reduced EPSCs by ~61%, from (mean ± SE) 102 ± 12 to 40 ± 2 pA (Fig. 2C; n = 8; *P < 0.001; Friedman’s statistics with post hoc Student-Newman-Keuls test). Posterior addition of d-AP5 to the CNQX-containing medium further reduced the synaptic current to 6 ± 2 pA (Fig. 2C; n = 8; *P < 0.01; Friedman’s statistics with post hoc Student-Newman-Keuls test after a pair-wise comparison with CNQX values), suggesting that about one-third of this current was mediated by NMDA channels at −80 mV (Fig. 2C). It was concluded that EPSCs evoked by field stimulation of the subthalomonigral connections have both AMPA and N-methyl-d-aspartate (NMDA) components as seen from the somatic recording site.

If synaptic components are isolated by digital subtraction (in Fig. 2D, see CNQX- and AP-5-sensitive components), then a current-voltage relationship (I-V plot) of each synaptic component can be built (Fig. 2E). The NMDA component seems to increase during depolarization reaching a maximal amplitude around −40 mV (Fig. 2, D and E, ○) thus exhibiting a voltage-dependent I-V plot. In contrast, AMPA current decreases with depolarization and has a linear I-V plot (Fig. 2, D and E, ⊞). This result suggests that NMDA current may be an important drive to reach firing threshold during physiological conditions.

Figure 3 illustrates the two physiologically distinct classes of neurons that could be recorded in the anterior SNr (Nakanishi et al. 1987; Richards et al. 1997; Rohrbacher et al. 2000). One class (n = 24/114; Fig. 3, A and C) had a strong voltage- and time-dependent inward rectification (Fig. 3A,a and c), could not fire at high frequencies during strong depolarizations (see following text), and had a large outward rectification (Fig. 3d) including an initial, transient, fast-activating outward current, its action potentials lasted longer: 2.3 ± 0.1 ms (n = 24) — measured at half-amplitude (Fig. 3Ca), and its I-V plot did not exhibit a negative slope conductance region (Fig. 3Ad).

The class of neurons most frequently recorded: n = 90/114, could fire at high frequencies on depolarization (Fig. 3Ba), exhibited a slowly activating smaller voltage- and time-depen-
amplitude of the interspike interval during spontaneous firing: −55 ± 5 versus −58 ± 2 mV for the putative dopaminergic and GABAergic samples, respectively. Whole neuron input resistances were $R_N = 169 ± 14 \, \text{MΩ}$ ($n = 5$) for the putative dopaminergic neurons and $365 ± 20 \, \text{MΩ}$ ($n = 10$) for the putative GABAergic neurons, respectively ($P < 0.002$, Mann-Whitney $U$ test). In the present work, we only refer to synaptic responses recorded in the most abundant class of neurons of the SNr, i.e., the putative GABAergic neurons. Some of them were reconstructed with a camera lucida, and three representative neurons are illustrated in Fig. 4. Somata of SNr neurons (Fig. 4A), mainly recorded in the anterior part of the SNr (Fig. 4B, see preceding text), were seen to be parvalbumin immunoreactive cells, had triangular or fusiform somata under infrared videomicroscopy with a major diameter of 18–22 μm. After reconstruction (Fig. 4C), they were observed to have four to six main dendritic trunks that extended rostrocaudally as seen in a parasagittal slice. Dendrites ramify into secondary branches and the axon (Fig. 4C, gray neurites) commonly emerged from a primary dendrite. In some cases, it could be followed out of the borders of the SNr. Together with their immunoreactivity to parvalbumin, these anatomical characteristics supported the view that this class of neuron was the SNr projection neuron: the output of the basal ganglia.
Activation of dopaminergic D1-class receptors facilitates evoked EPSCs

Activation of D1-class receptors with the D1-agonist, SKF-38393 (1 μM; saturating concentration), reversibly increased peak EPSC amplitude by ~30%, from mean 106 ± 17 to 140 ± 23 pA (Fig. 5, A and B; n = 10; P < 0.005; Wilcoxon’s t-test). In the presence of the D1-class receptor antagonist, sulpiride (1 μM), SKF-38393 had no effect (n = 4; not shown but see Fig. 8), indicating a specific action. The effect of the D1-class agonist was accompanied by a 15% decrease in the PPR from 1.3 ± 0.08 to 1.1 ± 0.09 because the first response was enhanced more than the second (Fig. 5, C and D; n = 8; P < 0.02; Wilcoxon’s t-test). Superimposed records in Fig. 5C demonstrate that neither shape nor decay of EPSCs were affected during the action of SKF-38393 (1 μM). *R*<sub>N</sub>, as tested with a hyperpolarizing command, did not change. Therefore the present experiments suggest that activation of D1-class receptors presynaptically located at the subthalamonigral terminals enhances glutamatergic transmission between STN and SNr.

Activation of dopaminergic D2-class receptors depresses evoked EPSCs

Activation of D2-class receptors with the D2-agonist, quinpirole (1 μM; above saturating concentration), reversibly decreased peak EPSC amplitude by ~25%, from mean 89 ± 17 to 67 ± 9 pA (Fig. 6, A and B; n = 9; P < 0.008; Wilcoxon’s t-test). In the presence of the D2-class receptor antagonist, sulpiride (1 μM), quinpirole had no effect (n = 5; not shown but see Fig. 8); indicating a specific action. The effect of the D2-class agonist was accompanied by a 38% increase in PPR because the first response decreased more than the second (Fig. 6, C and D; n = 7; P < 0.02; Wilcoxon’s t-test). Superimposed records in Fig. 6C demonstrate that neither shape nor decay of EPSCs were affected during the action of quinpirole (1 μM). *R*<sub>N</sub>, as tested with a hyperpolarizing command, did not change either. The experiments suggest that the activation of D2-class receptors located presynaptically at subthalamonigral terminals are capable to decrease glutamatergic transmission between STN and SNr.

**FIG. 5.** Activation of dopaminergic D1-class receptors facilitates evoked EPSCs. A: time course of action of 1 μM SKF-38393 (saturating concentration), a D1-receptor agonist, on evoked EPSC amplitude. Black bar indicates the time of agonist application, and top traces are representative records taken at indicated numbers on the time course. B: histogram summarizing the experimental sample: D1-agonist produces a significant increase in EPSC. C: responses to paired-pulse stimulation in control and in the presence of 1 μM SKF-38393 (gray trace at the middle). Bottom trace shows a superimposition of the above traces. There was no change in EPSC time course during agonist application. D: change in the paired-pulse ratio (2nd EPSC/1st EPSC) in a sample of neurons was always in the same direction: enhancement of synaptic depression.
Show a superimposition of the above traces. There was no change in EPSC enhancement of synaptic facilitation. EPSC/1st EPSC) in a sample of neurons was always in the same direction: 2 and in the presence of 1 STN stimulation (see METHODS). Figure 7, Mean-variance analysis of the excitatory subthalamonigral connection is synaptic depression. EPSCs trains were used to perform mean-variance expression. EPSCs trains were used to perform mean-variance analysis. Black bar indicates the time of agonist application, and top traces are representative records taken at indicated numbers on the time course. B: histogram summarizing the experimental sample: D2-agonist produces a significant decrease in EPSC. C: responses to paired-pulse stimulation in control and in the presence of 1 μM quinpirole (gray trace at the middle). Bottom trace shows a superimposition of the above traces. There was no change in EPSC time course during agonist application. D: change in the paired-pulse ratio (2nd EPSC/1st EPSC) in a sample of neurons was always in the same direction: enhancement of synaptic facilitation.

Mean-variance analysis

Trains of evoked EPSCs were recorded from SNr neurons on STN stimulation (see METHODS). Figure 7, A and E, illustrates that at 25 Hz in the control condition, the short-term dynamics of the excitatory subthalamonigral connection is synaptic depression. EPSCs trains were used to perform mean-variance analyses (see METHODS) (see also Koos et al. 2004) to independently confirm if modulatory dopaminergic actions were presynaptic (Clements and Silver 2000). The D1-class receptor agonist, SKF-38393 (1 μM), enhanced synaptic depression by increasing the initial response (cf. Fig. 7, A–C). In contrast, the D2-class receptor agonist, quinpirole (1 μM), reduced short-term depression by decreasing EPSCs (cf., Fig. 7, E–G). Mean-variance plot shows that most control (full circles) and agonists (empty circles) data points cluster together at the initial part of the fitted parabolas (Fig. 7, D and H; controls = black lines; dopamine agonists = gray lines), so that the initial slopes, determined by the A parameter (Eq. 1), were not significantly different. Weighted quantal amplitudes, \( Q_W \) (Clements and Silver 2000), calculated with initial slopes (Eq. 2) had a range of 5–9 pA (n = 4); similar to quantal amplitudes reported for other glutamatergic synapses in the brain (e.g., Bolshakov and Siegelbaum 1995; Paulsen and Heggelund 1994). For example, in the experiment illustrated in Fig. 7D, \( Q_W \) was 6 ± 0.6 and 6 ± 0.5 pA for control and SKF-38393, respectively. In the experiment of Fig. 7H, \( Q_W \) was 9 ± 0.6 for the control and 9 ± 2 pA for quinpirole, respectively. In contrast, the width of the parabolas, determined by parameter B (Eq. 1), was significantly different for the same data as it is seen in Fig. 7, D and H. When B was used to approximate the number of release sites (Eq. 3), the D1-agonist produced an increase in N from 19 ± 3 to 29 ± 4 (Fig. 7D; P < 0.01; Student t for fitted parameter B), and conversely, the D2-agonist produced a decrease in N from 11 ± 3 to 4 ± 1 (Fig. 7H; P < 0.01; Student t for fitted parameter B). Therefore it was concluded that changes were presynaptic because a change in N was detected without a change in \( Q_W \). Dopamine enhances or depresses the activity of release sites.

However, this method could not detect changes in the average probability of release sites because average probability (P), assuming binomial distribution, and approximated with both A and B parameters (Eq. 3) (Clemens and Silver 2000) did not show significant differences. Thus in the presence of the D1-agonist P changed from 0.22 ± 0.1 to 0.17 ± 0.1, and in the presence of the D2-agonist, it changed from 0.22 ± 0.05 to 0.29 ± 0.05. Further quantal analyses have to be performed to discard or show changes in release probabilities, perhaps at the single bouton level. However, for the goals of the present work, mean-variance analysis confirmed that dopaminergic modulation of subthalamonomial transmission occurs presynaptically.

Two questions remained, however, first, if the same SNr neuron could receive terminals with both receptor classes, and second, if the dopaminergic modulation is constitutively active, or else, if it is only responsive to sudden changes in dopamine levels.

Dopamine receptors can be tonically active

STN neurons exhibit tonic activity (Beurrier et al. 1999; Bevan and Wilson 1999). This activity may induce a continuous excitation of SNr and SNC neurons. Continuous activation of dopaminergic neurons in the SNr or SNC (Iribe et al. 1999; Kanazawa et al. 1976) may produce a tonic extracellular level of dopamine in SNr (Falkenburger et al. 2001; Mintz et al. 1986; Richards et al. 1997; Rohrbacher et al. 2000; Rosales et al. 1994, 1997). Accordingly, we explored whether dopamine receptors modulating subthalamonomial connections were partially active in the control (unstimulated) situation. This was explored with dopamine receptor antagonists administered in a sequential manner. Figure 8A shows that the D2 antagonist, sulpiride (1 μM), enhanced peak EPSC amplitude by ~44% from 101 ± 9 to 146 ± 13 pA (Fig. 8, A and B; n = 9; P < 0.01, Friedman’s statistics with post hoc Student-Newman-Keuls test), as if it blocked a tonic D2 action thus leaving a tonic D1-action to predominate. Moreover, the subsequent addition of SCH 23390 (1 μM), in the continuous presence of sulpiride, reduced peak EPSC amplitude by ~15% from 146 ± 13 to 124 ± 12 pA (Fig. 8, A and B; n = 9; P < 0.01, Friedman’s statistics with post hoc Student-Newman-Keuls test for pairwise comparison between sulpiride and sulpiride plus SCH 23390 samples), as though it blocked the D1 action. Note that in the presence of both receptor antagonists, EPSCs
amplitude is above the control level (Fig. 8A3); suggesting that D₂-action may predominate in the control. Control amplitude is recovered on wash out. The experiments suggest that subthalamonaligral transmission is continuously being tuned by endogenous dopamine levels. They also show that terminals making synaptic contacts with single postsynaptic SNr neurons could possess D₁- or D₂-class receptors. Further research is needed to see if both receptor classes can be located at the same terminal.

Dopamine agonists were also added sequentially while observing the PPR (Fig. 9). It was seen that SKF-38393 (1 μM) could turn paired-pulse facilitation seen in the control (Fig. 9A, top) into paired-pulse depression (Fig. 9A, middle). The subsequent addition of quinpirole (1 μM), in the continuous presence of SKF 38393, reduced the synaptic events enlarged by the D₁-agonist and also decreased the amount of D₁-mediated synaptic depression (Fig. 9A, bottom). Superimposed traces can be seen in Fig. 9B. To observe if these actions may affect transmission physiologically, a similar experiment was performed in current-clamp mode. It was evident that these presynaptic effects could influence SNr output: D₁-class receptor action could change a subthreshold EPSP into a threshold EPSP capable to sustain firing, whereas D₂-class receptor actions abolished D₁ actions returning the EPSP into the subthreshold range (Fig. 9C). Thus by controlling subthalamonaligral connections, dopamine may control the firing of basal ganglia output neurons.

**DISCUSSION**

The present results show that dopamine in the substantia nigra pars reticulata presynaptically modulates the glutamatergic inputs coming from the subthalamic nucleus. Both D₁ and D₂ class receptors participate. D₁-class receptors facilitate, whereas D₂-class receptors reduce, the excitatory subthalamonaligral transmission onto SNr neurons. Physiologically, both receptors may be acting simultaneously to dynamically tune, in real time, the output of the basal ganglia. Interestingly, a sizable component of the subthalamonaligral input is mediated by NMDA receptors and SNr neurons display an intrinsic negative slope conductance region in their I-V plot.
Presynaptic control by dopamine

A great body of literature has proven that the paired-pulse protocol is a reliable method to demonstrate presynaptic modulation in synapses (e.g., Dunwiddie and Haas 1985; reviewed in Kamiya and Zucker 1994; Zucker 1999). For example, this protocol has been used to demonstrate a presynaptic site of action for both D1 and D2 receptors in various nuclei of the basal ganglia such as the recurrent axon collaterals that interconnect striatal projection neurons (Guzman et al. 2003; Salgado et al. 2005), the synaptic inputs that target striatal cholinergic interneurons (Pisani et al. 2000; Momiyama and Koga 2001), the excitatory transmission onto dopamine neurons of the ventral tegmental area (Koga and Momiyama 2000), or the inhibitory striatonigral transmission (Radnikow and Misgeld 1998). In many cases, additional procedures were used to further corroborate the findings of the paired-pulse protocol. It was found that both methods coincide in identifying a presynaptic site of action for the actions of dopamine, thus adding to the tests that the paired-pulse protocol has endured.

The present results show that activation of D1-class receptors facilitates, whereas activation of D2-class receptors depresses, subthalamonic excitatory transmission onto SNr neurons. In contrast, glutamatergic cortical terminals only posses D2-class receptors (Bamford et al. 2004; Cepeda et al. 2001; Flores-Hernandez et al. 1997; Tang et al. 2001); showing that dopaminergic receptor distribution is selective: not all excitatory terminals have both receptor classes.

Inhibitory terminals of axon collaterals that interconnect neostriatal neurons may also posses both receptors (Guzman et al. 2003). However, they are probably segregated into different terminals because neurons are separated into the striatopallidal or direct pathway mainly possessing D2-type receptors and the striatonigral or indirect pathway mainly possessing D1-type receptors (Gerfen et al. 1990). Further investigation is necessary to see if this is the case for subthalamonic terminals (e.g., with EPSCs evoked with minimal stimulation). In the present work, we show that both receptor classes could be

FIG. 8. Tonic activation of dopamine receptors. A: time course of the effects of subsequent application of, 1st, S(−)-5-anino-sulfonyl-N-(1-ethyl-2-pyrolidinyl)-methyl]-2-methoxybenzamide (1 μM), and 2nd, SCH 23390 R(−)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetra-hydro-1H-3-benzazepine hydrochloride (1 μM) on the amplitude of evoked EPSCs. Top: recordings obtained at the numbers indicated in the time course. B: histogram summarizing a sample of experiments (*P < 0.01). Note that in this case, each point represents the average of 2 min recordings.

FIG. 9. Subthalamonic terminals with D1 and D2 receptors make synaptic contacts with a single postsynaptic neuron. A1: pair of EPSC in the control condition exhibiting paired-pulse facilitation. A2: addition of 1 μM SKF-38393 enhanced both EPSCs and turned paired-pulse facilitation into mild paired-pulse depression. A3: subsequent addition of quinpirole, in the continuous presence of SKF-38393, reversed both the amplitude increase in EPSCs and paired-pulse depression. B: superimposition of traces in A, 2 and 3. C: a similar experiment was performed with a subthreshold subthalamonic excitatory postsynaptic potential (EPSP). Note that D2-action transformed the subthreshold EPSP into a suprathreshold EPSP, capable to elicit repetitive firing. A subsequent addition of quinpirole returned the suprathreshold EPSP into the subthreshold range again.
detected on terminals making synapses onto the same postsynaptic SNr neuron similarly to the findings in neostriatal neurons (Guzman et al. 2003): inputs onto the same neuron may be enhanced or repressed by dopamine. However, in contrast to neostriatal projection cells, many presynaptic subthalamic neurons possess both D1- and D2-class receptors (Baufreton et al. 2003; Ciliax et al. 2000; Flores et al. 1999; Hurd et al. 2001; Khan et al. 2000; Svenningsson and Le Moine 2002).

**Physiological consequences**

Although the STN is a documented main source of excitation for both dopaminergic SNC neurons (Irie et al. 1999; Kanazawa et al. 1976; Smith and Grace 1992) and GABAergic SNr projection neurons (Bevan et al. 1994; Irie et al. 1999; Kita and Kitai 1987; Robledo and Feger 1990), other excitatory afferents have not been discarded. Nevertheless, stimulation of the subthalamic nucleus has been shown to increase the release of dopamine in the SNr (Falkenburger et al. 2001; Johnson et al. 1992; Mintz et al. 1986; Rosales et al. 1994, 1997). Dopamine released in the SNr may in turn control presynaptic receptors at subthalamonaligral terminals (Floran et al. 1990; Radnikow and Misgeld 1998), thus controlling the activity of SNr neurons. In return, SNr neurons activity may control SNC neurons firing (Tepper et al. 1995). Therefore these interconnections could make the basis for a local circuit that regulates basal ganglia output. Dopamine cells may fire tonically or in bursts (Pucak and Grace 1994), determining dopamine levels. Dopamine levels may rule which receptors are preferentially activated.

At present, it is hard to speculate the physiological significance for such a segregation of presynaptic dopamine receptors: D1-class receptors at both striatonigral (Floran et al. 1990; Hernandez-Lopez et al. 1997; Radnikow and Misgeld 1998) and subthalamonaligral afferents (this work), whereas D2-class receptors only at subthalamonaligral afferents. However, the presence of D1-class receptors on subthalamonaligral afferents may explain some contradictory data in the literature. Thus iontophoretic application of a D1-agonist (SKF-38393) into the SNr increases the firing of pars reticulata neurons (Martin and Waszczak 1994), suggesting that D1-action on the subthalamomaligral pathway predominates in this situation. Nonetheless when given by a systematic route, D1-agonists inhibit SNr neurons firing (Weick et al. 1990), suggesting that the most massive D1-receptor activation of the striatonigral pathway predominates in this situation (Floran et al. 1990; Hernandez-Lopez et al. 1997; Radnikow and Misgeld 1998). Moreover, the inhibitory effects of systemic D1-agonist on the firing of SNr neurons can be potentiated by the co-administration of D2-agonists (Weick and Walters 1987a,b). The present results show that this latter action may also result from inhibition of the subthalamomaligral input by D2 receptors.

Further reinforcing the view of diverse D1-class receptor actions, it has been posited that D1-class receptors present at subthalamomaligral terminals belong perhaps to the D2-type (Baufreton et al. 2003; Ciliax et al. 2000; Khan et al. 2000; Svenningsson and Le Moine 2002). D2-type receptors are reported to have an opposite effect to D1-type receptors in locomotion: they repress not promote movement (Dziewczapolski et al. 1998). And in fact, D1-class actions on STN neurons and terminals lead to burst-firing (Baufreton et al. 2003). These actions would tend to increase SNr neurons firing and thus inhibit movement.

**Contribution of NMDA receptors to the evoked EPSC**

The blockade of AMPA/kainate receptors with CNQX did not completely block subthreshold EPSC evoked at −80-mV holding potential. The blockade of NMDA receptors with d-AP-5 was necessary to block all EPSC. This suggests that in contrast to other synapses (Bonci and Malenka 1999; Kita 1996; Koga and Momiyama 2000; Zhu and Pan 2004), subthalamomaligral transmission has an important contribution of NMDA current even at subthreshold membrane potentials (cf. Maccaferri and Dingledein 2002). In addition, many SNr neurons displayed a negative slope conductance region in their I-V plot. These two characteristics make SNr neurons prone to be activated by tonic excitatory inputs coming from the STN (Beurrier et al. 1999).

In conclusion, dopamine can affect the output from the basal ganglia modulating not only the GABAergic input from the striatum but also the glutamatergic input from the subthalamic nucleus. These effects may help to understand the actions of dopamine in motor control and can be helpful to understand the changes during motor deficits such as Parkinson’s Disease.

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