Subthalamic Stimulation-Induced Synaptic Responses in Substantia Nigra Pars Compacta Dopaminergic Neurons In Vitro

YUJI IRIBE, KEVIN MOORE, KEVIN C. H. PANG, AND JAMES M. TEPPER
Center for Molecular and Behavioral Neuroscience, Program in Cellular and Molecular Biodynamics, Rutgers, The State University of New Jersey, Newark, New Jersey 07102

Iribe, Yuri, Kevin Moore, Kevin C. H. Pang, and James M. Tepper. Subthalamic stimulation-induced synaptic responses in substantia nigra pars compacta dopaminergic neurons in vitro. *J. Neurophysiol.* 82: 925–933, 1999. The subthalamic nucleus (STN) is one of the principal sources of excitatory glutamatergic input to dopaminergic neurons of the substantia nigra, yet stimulation of the STN produces both excitatory and inhibitory effects on nigral dopaminergic neurons recorded extracellularly in vivo. The present experiments were designed to determine the sources of the excitatory and inhibitory effects. Synaptic potentials were recorded intracellularly from substantia nigra pars compacta dopaminergic neurons in parasagittal slices in response to stimulation of the STN. Synaptic potentials were analyzed for onset latency, amplitude, duration, and reversal potential in the presence and absence of GABA and glutamate receptor antagonists. STN-evoked depolarizing synaptic responses in dopaminergic neurons reversed at approximately -31 mV, intermediate between the expected reversal potential for an excitatory and an inhibitory postsynaptic potential (EPSP and IPSP). Blockade of GABA$_A$ receptors with bicuculline caused a positive shift in the reversal potential to near 0 mV, suggesting that STN stimulation evoked a near simultaneous EPSP and IPSP. Both synaptic responses were blocked by application of the glutamate receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione. The confounding influence of inhibitory fibers of passage from globus pallidus and/or striatum by STN stimulation was eliminated by unilaterally transecting striatonigral and pallidonigral fibers 3 days before recording. The reversal potential of STN-evoked synaptic responses in dopaminergic neurons in slices from transected animals was approximately -30 mV. Bath application of bicuculline shifted the reversal potential to ~5 mV as it did in intact animals, suggesting that the source of the IPSP was within substantia nigra. These data indicate that electrical stimulation of the STN elicits a mixed EPSP-IPSP in nigral dopaminergic neurons due to the coactivation of an excitatory monosynaptic and an inhibitory polysynaptic connection between the STN and the dopaminergic neurons of substantia nigra pars compacta. The EPSP arises from a direct monosynaptic excitatory glutamatergic input from the STN. The IPSP arises polysynaptically, most likely through STN-evoked excitation of GABAergic neurons in substantia nigra pars reticulata, which produces feed-forward GABA$_A$-mediated inhibition of dopaminergic neurons through inhibitory intranigral axon collaterals.

INTRODUCTION

In vivo dopaminergic neurons fire spontaneously at relatively low rates in a regular or pacemaker-like mode, a random mode or in an irregular pattern punctuated with slow bursts typically comprising two to six action potentials (Bunney et al. 1973; Grace and Bunney 1984a,b; Tepper et al. 1995; Wilson et al. 1977). In vitro, under control conditions, dopaminergic neurons in adults fire only in the pacemaker-like mode (Grace 1987). This difference suggests that afferent input to nigral dopaminergic neurons plays an important role in regulating their neuronal activity.

The burst firing pattern and its afferent control have generated considerable interest, in part because burst firing may alter the dynamics of extracellular dopamine levels in a nonlinear fashion (Gonon 1988). *N*-methyl-D-aspartate (NMDA)-receptor activation has been proposed to contribute to this bursting activity (Chergui et al. 1994; Johnson et al. 1992; Overton and Clark 1992; Seutin et al. 1994). The three principal glutamatergic inputs to substantia nigra arise from the frontal cortex, STN, and the pedunculopontine nucleus (Jackson and Crossman 1983; Kanazawa et al. 1976; Kita and Kitai 1987; Naito and Kita 1994; Van Der Kooy and Hattori 1980).

Although the subthalamic efferents are believed to be predominantly or exclusively glutamatergic and thus excitatory and form asymmetric contacts with nigral and entopeduncular neurons (Bevan et al. 1994; Chang et al. 1984; Hammond et al. 1978; Shink et al. 1996), reports of the effects of STN stimulation on the activity of substantia nigra dopaminergic neurons in vivo are somewhat contradictory. In the earliest report, electrical stimulation of the subthalamic nucleus was found to be excitatory to dopaminergic and nondopaminergic nigral neurons (Hammond et al. 1978). In a subsequent study that used local infusions of bicuculline to stimulate the subthalamic nucleus pharmacologically, approximately equal numbers of excitatory and inhibitory responses were found among dopaminergic neurons, although almost all of the nondopaminergic neurons in pars reticulata were excited (Robledo and Féger 1990). Similarly, when subthalamic neurons were inhibited by local infusions of muscimol, less than one-fourth of the dopaminergic neurons recorded showed the expected decrease in firing rate, whereas approximately half showed excitation and the remainder a biphasic or no effect. In contrast, 8 of 10 nondopaminergic pars reticulata neurons were inhibited. More recently, biphasic effects of electrical stimulation of subthalamic nucleus on electrophysiologically identified dopaminergic neurons were reported, although the initial effect on all dopaminergic neurons as well as on many nondopaminergic neurons was inhibition (Smith and Grace 1992).

The inhibitory responses in dopaminergic neurons are likely an indirect effect, resulting from STN stimulation-induced activation of inhibitory neurons. Early extracellular recording studies showed a reciprocal relationship between the activity of

dopaminergic neurons in pars compacta and nondopaminergic neurons in pars reticulata, suggesting that GABAergic neurons in the substantia nigra pars reticulata may modulate the activity of dopaminergic neurons in pars compacta (Grace and Bunney 1979; Grace et al. 1980). Direct evidence for a functional inhibitory connection between pars reticulata GABAergic neurons and pars compacta dopaminergic neurons was provided by the demonstrations that electrical stimulation of the pars reticulata produced GABAergic inhibitory postsynaptic potentials (IPSPs) in pars compacta dopaminergic neurons in vitro (Hajós and Greenfield 1994), selective activation of pars reticulata GABAergic projection neurons inhibits pars compacta dopaminergic neurons in vivo through activation of $GABA_A$ receptors (Tepper et al. 1995), and intracellularly labeled nigrothalamic projection neurons make synapses onto dopaminergic pars compacta neurons (Damlama 1994). These data suggest that inhibitory responses of pars compacta dopaminergic neurons to STN stimulation could arise disynaptically through activation of pars reticulata GABAergic neurons.

The present study was designed to determine the source of inhibition in substantia nigra pars compacta dopaminergic neurons after electrical stimulation of the STN by recording subthalamic stimulation-induced synaptic responses in electrophysiologically identified pars compacta dopaminergic neurons in an in vitro preparation preserving the connections between the subthalamic nucleus and the substantia nigra.

METHODS

Slice preparation

Young adult male Sprague-Dawley rats (4–8 wk of age, 100–200 g, Zivic-Miller) were used. Animal care and surgical procedures were performed in accordance with the guidelines of the U.S. Public Health Service manual, "Guide for the Care and Use of Laboratory Animals," and were approved by the Rutgers University Institutional Review Board. Animals were anesthetized with ketamine (100 mg/kg ip) and perfused with ice-cold artificial cerebrospinal fluid (ACSF), which contained (in mM) 125 NaCl, 3.0 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.5 $MgCl_2$, 2.5 $CaCl_2$, and 10 glucose. The brain was removed quickly and trimmed to a block containing the midbrain. Parasagittal sections (350–400 μm) were cut with a Campden Instruments Vibroslice and transferred to a holding tank constantly perfused with oxygenated ACSF at 32°C for 2–6 h before being transferred to the recording chamber. Oxygenated ACSF was supplied to both holding and recording chambers (0.3 ml) at a rate of 2–4 ml/min.

Hemitransections

Hemitransections were made 3 days before recording. Three days was selected as a survival time long enough to ensure degeneration of striatonigral and striatopallidal fibers (Nitsch and Riesenberg 1988) but short enough to minimize anterograde degenerative changes in nigral neurons that occur after deafferentation (Saji and Reis 1987). Rats were anesthetized with ketamine (80 mg/kg) and xylazine (12 mg/kg) intraperitoneally and mounted in a stereotaxic frame. Using aseptic surgical techniques a narrow burr hole ~3 mm long was drilled from coordinates L 0.9 to L 3.9 mm overlying the globus pallidus. A knife blade was lowered to 9.0 mm from the cortical surface and moved mediolaterally two or three times to produce a unilateral transection anterior to STN. Successful lesions were accompanied by ipsilateral rotations in the animal that were usually noticeable as soon as the animal awoke and persisted until the animal was killed. All lesions were evaluated under a stereomicroscope before recording. Slices from brains in which the lesion did not completely transect the midbrain and internal capsule throughout their dorsoventral extent just anterior to the subthalamic nucleus were excluded from study.

Intracellular recording

Intracellular recording electrodes were made from 1.2-mm OD capillary tubing on a Sutter Instruments P-97 horizontal pipette puller. They were filled with 1 M potassium acetate containing 1% biocytin and possessed in vitro impedances of 80–120 MΩ. Electrode signals were amplified by a Neurodata IR183 preamplifier and displayed on a Tektronix 5111A oscilloscope. Traces were digitized on a Nicolet 4094C digital oscilloscope and transferred to a Macintosh computer for off-line analysis with custom designed software.

Stimulation

Stimulating electrodes were bipolar stainless wires (100 μm diam) insulated with enamel except for the tips (California Fine Wire). These electrodes were placed on the surface of the slice at the level of the STN near the caudal pole of the nucleus. Stimuli were generated by a Winston A-65 timer and SC-100 constant current stimulus isolation unit and consisted of single square wave pulses (0.1–0.5 ms in duration, 0.1–2.0 mA) delivered at 1 Hz. To test for N-methyl-D-aspartate (NMDA)-receptor-mediated events, in a few cases brief high-frequency trains (interpulse interval, 3 ms; pulse duration, 100 μs) of pulses (10 ms in duration, 0.1–2.0 mA) were applied at 0.25 Hz.

Reversal potential data analysis

Reversal potentials were calculated by recording the synaptic response to STN stimulation at various membrane potentials produced by injection of a steady state current or 300-ms pulses Synaptic responses were measured by subtracting baseline, as measured several milliseconds before simulation, from the response amplitude, measured at the peak of the synaptic response. Reversal potentials were extrapolated from a linear regression of PSP amplitude versus membrane potential over a restricted range of the I-V curve, typically between −50 and −100 mV. The PSP amplitude data were well fit with a linear regression over this range as indicated by the values for the regression coefficients under control conditions which ranged from 0.864 to 0.993 (0.961 ± 0.015; mean ± SE).

Synaptic potentials were examined under control conditions and after bath application of 50 μM bicuculline, a $GABA_A$-receptor antagonist, 50 μM 2-OH saclofen, a $GABA_B$-receptor antagonist, or 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a competitive non-NMDA glutamate receptor antagonist. To investigate the participation of NMDA receptors in the PSP, in some cases brief trains of pulses were used and 10 μM MK-801 was applied. Reversal potentials were measured after 10 min of drug application. Drug applications were followed by 1-h washout period.

The effects of drugs on the reversal potential were tested for significance using a two-tailed Student's t-test. All numerical data are expressed as mean ± SE.

Drugs

Drugs were stored in frozen aliquots that were dissolved in the perfusing solution just prior to each experiment in the concentration indicated. All drugs were applied in the bath. Complete exchange of the bath solutions occurred within 2 min. (−)-Bicuculline methchloride (bicuculline), 2-hydroxysaclofen, CNQX, and (+)-MK-801 hydrogen maleate (MK-801) were purchased from Research Biochemicals International.

Histology

At the end of some experiments, neurons were labeled intracellularly with biocytin by applying depolarizing pulses (1 nA, 300-ms pulses at a 50% duty cycle) for 10 min. Slices were stored in 4% paraformaldehyde in 0.15 M phosphate buffer, pH 7.4, for 2 h at room temperature and rinsed in 0.15 M phosphate buffer overnight at 4°C. Slices were subsequently resectioned into 70-μm sections and processed for visualization of biocytin by using a modification of the procedure originally described by Horikawa and Armstrong (1988) with 3,3′-diaminobenzidine (DAB) as the chromogen.

Some sections were also processed for tyrosine hydroxylase (TH) immunocytochemistry by standard methods previously reported (Tepper et al. 1994). In these cases, biocytin-injected neurons used nickel-enhanced DAB as the chromogen and TH immunostaining used DAB as the chromogen.

RESULTS

Neuronal identification

Dopaminergic neurons were identified based on their morphology, location within the pars compacta and electrophysiological properties previously described from in vitro recordings (Grace 1987; Kita et al. 1986; Lacey et al. 1987; Nakanishi et al. 1987; Yung et al. 1991). Dopaminergic neurons had resting membrane potentials between –50 and –70 mV. Most neurons were spontaneously active and fired regularly on depolarization (Fig. 1A). All cells had long duration action potentials (>2 ms, Fig. 1B) and were followed by pronounced afterhyperpolarizations, as shown in Fig. 1, A and C. Cells also exhibited a slowly activating inward rectification when hyperpolarized as shown in Fig. 1, C and D.

Recordings were made from 75 substantia nigra pars compacta dopaminergic neurons. Fifty of these neurons were recorded from slices taken from intact rats and 25 from slices taken from rats hemitransected between the STN and the globus pallidus. The morphology of intracellularly labeled dopaminergic pars compacta cells has been described previously (Grace and Onn 1989; Kita et al. 1986; Preston et al. 1981; Tepper et al. 1987; Yung et al. 1991). Biocytin-stained dopaminergic neurons displayed a variety of somatic shapes including fusiform, triangular and multipolar. A typical biocytin-stained dopaminergic neuron is illustrated in Fig. 2. Dopaminergic somata gave rise to between two and five thick, smooth principal dendrites that branched infrequently. Higher order dendrites exhibited infrequent spine-like appendages. One population of dendrites remained largely within pars compacta and extended for several hundred microns in a flattened disk around the cell body. Almost all neurons extended one and more rarely two dendrites deep into pars reticulata, perpendicular to the borders of pars compacta. This was invariably the largest dendrite issued by the neuron and extended for distances up to 1,200 μm, even in the 350–400 μm slices used for in vitro recording as illustrated in Fig. 2.

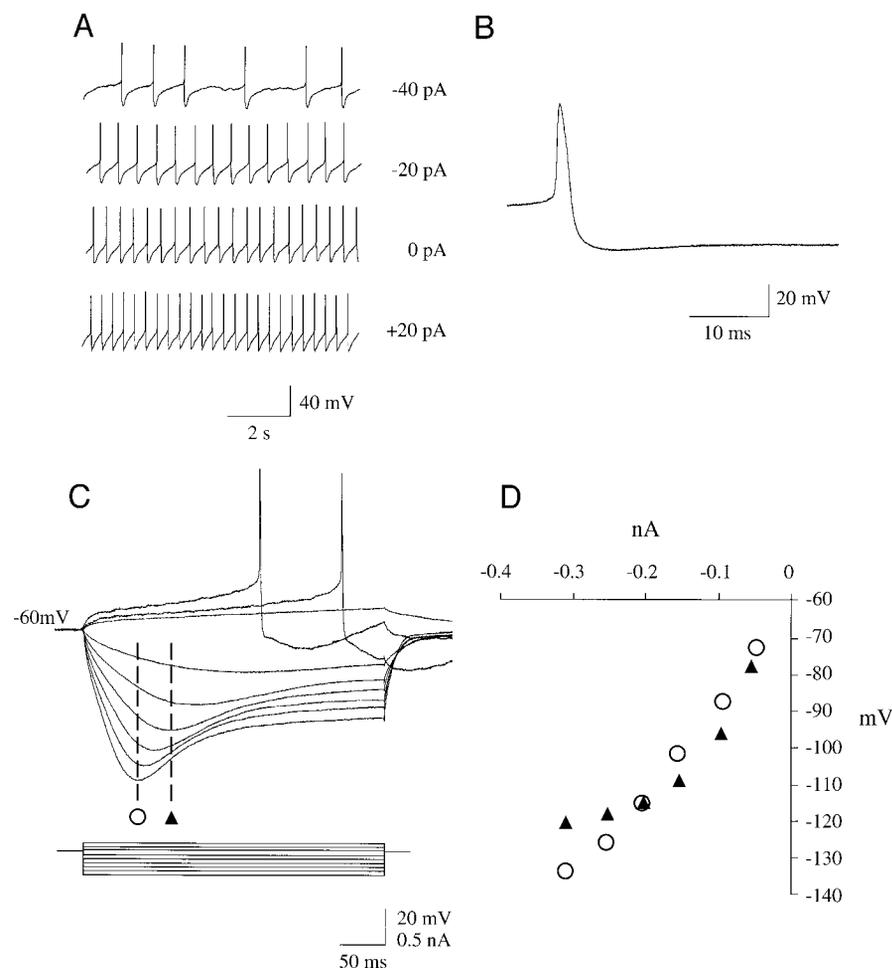The input resistance was calculated from injection of small

FIG. 1. Electrophysiological identification of nigral dopaminergic neurons in vitro. A: spontaneously active dopaminergic neuron firing in the typical pacemaker-like mode seen in vitro. Constant current injection of hyper- and depolarizing pulses manipulated pacemaker-like firing between 0.8 and 4 Hz. Action potential amplitudes are truncated due to aliasing. B: action potentials were of long duration (>2 ms) and exhibited large afterhyperpolarizations. C: intracellular injection of current pulses revealed a slow depolarizing ramp potential in the depolarizing direction and a strong time-dependent inward rectification when the membrane was hyperpolarized. D: current-voltage plots show nearly linear slope and minimal inward rectification at the onset of hyperpolarizing current pulses (open circles) and a much more pronounced inward rectification when I_h begins to activate after about 100 ms (solid triangles).
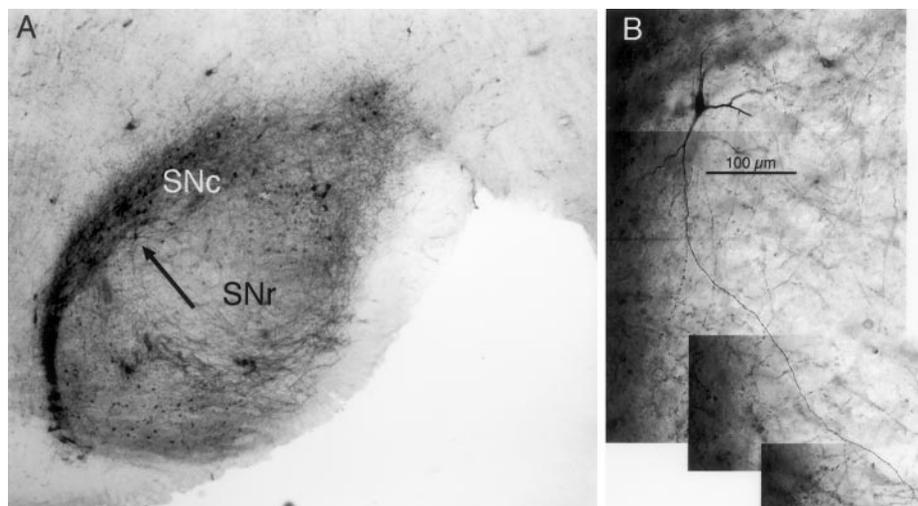
FIG. 2. Dopaminergic neuron intracellularly stained with biocytin in a 400 μm parasagittal slice. *A*: low magnification photomicrograph of an intracellularly labeled dopaminergic neuron (arrow) at the ventral border of pars compacta. The section is counterstained for TH immunocytochemistry. *B*: high magnification photomontage of the dopaminergic neuron shown in *A* illustrating the very long ventral dendrite extending deep into pars reticulata.

hyperpolarizing current pulses (–0.05 to –0.15 nA) (Fig. 1*C*, open circle). The mean input resistance was 156.3 ± 11.3 MΩ ($n = 15$). Spike threshold was –39.9 ± 0.5 mV ($n = 102$ spikes from 12 cells). Resting membrane potential was measured at the point where the membrane potential showed an inflection after the large spike afterhyperpolarization following a spontaneous spike (see Grace and Onn 1989) and averaged –59.4 ± 0.5 mV ($n = 121$ spikes from 12 cells). Most neurons were spontaneously active, with a mean firing rate of 2.41 ± 0.19 Hz ($n = 12$). The mean spike duration was 2.65 ± 0.05 ms ($n = 41$).

Response to STN Stimulation

Stimulation of the STN with a single pulse elicited a depolarizing postsynaptic potential (DPSP) in dopaminergic neurons. The mean stimulus current evoking the maximal amplitude DPSP was 855 ± 58 μA ($n = 56$). The onset latency of the DPSP was 2.71 ± 0.15 ms ($n = 49$) and usually remained constant at different stimulus strengths, suggesting that it was mediated monosynaptically. Under control conditions in slices from intact rats, the reversal potential for the DPSP was –31.6 ± 4.1 mV ($n = 19$), a value intermediate between that expected for an IPSP and EPSP, suggesting that the DPSP was comprised of a near simultaneously occurring EPSP and IPSP. These properties are illustrated for one representative dopaminergic neuron in Fig. 3.

Effects of GABA_A receptor antagonists

After application of bicuculline, a selective GABA$_A$ receptor antagonist, the reversal potential was shifted in the depolarizing direction to –0.8 ± 3.3 mV ($n = 16$) as shown for one representative neuron in Fig. 3. The effects of the GABA$_B$ receptor antagonist, 2-hydroxysaclofen was tested in 5 neurons. 2-hydroxysaclofen failed to shift the reversal potential in the depolarizing direction, rather shifting it slightly but nonsignificantly in the hyperpolarizing direction to –34.8 ± 4.8 mV ($n = 5$). Since GABA$_B$ IPSPs can be elicited in midbrain dopaminergic neurons in vitro even by single pulse stimuli (Cameron and Williams 1993), it was important to determine whether GABA$_B$ receptors might play a role in the IPSP. The fact that bicuculline, but not 2-hydroxysaclofen completely

eliminated the IPSP component indicates that it is mediated by GABA$_A$ receptors.

Effects of glutamate receptor antagonists

The effects of the non-NMDA receptor antagonist, CNQX (20 μM), were tested in 8 additional neurons, all of which had mixed responses in the control condition (control DPSP reversal potential = –38.1 ± 4.3 mV, $n = 8$). In 5 of these neurons, CNQX completely abolished the response to STN stimulation. In these cases, when CNQX was allowed to wash out, bicuculline administration shifted the reversal potential in the positive direction as in controls. These data suggest that in these cases, the IPSP component of the DPSP is polysynaptically mediated. A representative example of a polysynaptically mediated EPSP-IPSP is shown in Fig. 4.

In the 3 remaining neurons CNQX did not abolish the DPSP but instead shifted its reversal potential to –61.1 ± 6.4 mV ($n = 3$), close to the expected reversal potential for a pure GABA$_A$-mediated IPSP. After washout, subsequent administration of bicuculline shifted the DPSP reversal potential to –6.5 ± 3.1 mV ($n = 3$). Thus in these cases, the IPSP component of the DPSP was mediated, at least in part, monosynaptically. An example of a monosynaptically mediated EPSP-IPSP is shown in Fig. 5.

In most cases when the DPSP was elicited by single pulse stimulation of the STN, the excitatory component could be completely blocked by the non-NMDA receptor antagonist, CNQX, as described above. However, in a few neurons, short trains of stimulation were applied to the STN. Under these conditions, a DPSP resulted that could not be completely blocked by 20 μM CNQX alone, but that could be blocked by the addition of 10 μM MK-801 (data not shown).

Effects of hemitransections

Recordings were also obtained from 25 dopaminergic neurons in slices from 21 animals that were given complete unilateral hemitransections between the globus pallidus and the STN 3 days prior to recording to eliminate any confounding effects of stimulation of descending GABAergic axons from striatum and/or globus pallidus. The basic electrophysiological properties of dopaminergic neurons from transected animals
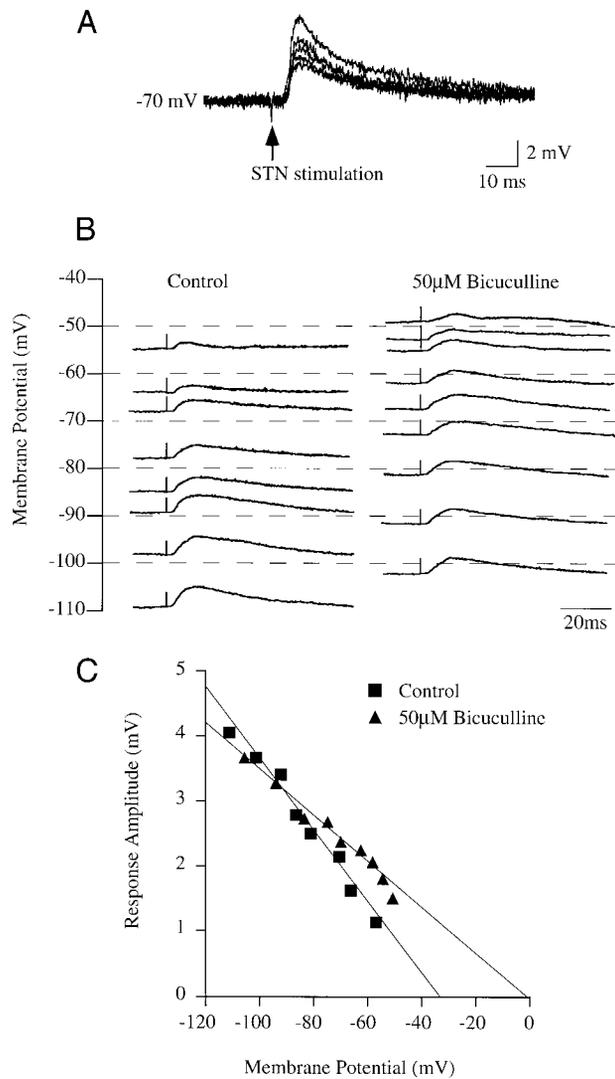
FIG. 3. Synaptic responses to STN stimulation. Stimulation of the STN produced a depolarizing synaptic response (DPSP). *A*: the DPSP had constant onset latency at different stimulus strengths (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mA in this example) indicating that it is monosynaptic. *B, C*: under control conditions the DPSP increases in amplitude with hyperpolarization and decreases in amplitude with depolarization indicating that it is a synaptic response, but exhibits a reversal potential of –33.1 mV, intermediate between that expected for an EPSP and an IPSP. After GABA$_A$ responses are blocked by bath application of bicuculline, the reversal potential of the DPSP shifted to –1.7 mV, the value expected for an EPSP. Traces in *B* are each the average of 4 single sweeps. Stimulus artifacts have been digitally reduced.

did not differ from those in slices from intact rats, although the STN-evoked DPSP appeared to have a greater NMDA component in slices from transected rats. For this reason, some of the experiments in transected rats were conducted with MK-801 (10 μM) present throughout. The mean input resistance was 138.8 $\pm$ 7.5 MΩ ($n = 25$), the spike threshold was –39.8 $\pm$ 0.5 mV ($n = 93$ spikes from 11 neurons), and the resting membrane potential was –59.6 $\pm$ 0.6 mV ($n = 72$ spikes from 11 neurons). The reversal potential of the STN-evoked DPSP in control ACSF was -30.3 ± 5.0 mV ($n = 9$). Addition of bicuculline to the bath removed the IPSP component, leaving a pure EPSP (reversal potential = 5.0 $\pm$ 3.0 mV, $n = 9$). In 7 of these neurons, after bicuculline was washed out for 1 h and the DPSP returned, CNQX was applied and

completely abolished the DPSP, as shown for one representative neuron in Fig. 6*A*. In two additional neurons application of 20 μM CNQX completely abolished the DPSP just as it did following washout of bicuculline. In these two cases the reversal potential of the DPSP under control conditions in the presence of 10 μM MK-801 was –20.5 and –47.1 mV. Following a 1 h washout, the DPSP returned and subsequent application of bicuculline left a pure EPSP in both cells (reversal potential of 8.1 mV and –10.4 mV, respectively). One of these neurons is illustrated in Fig. 6*B*.
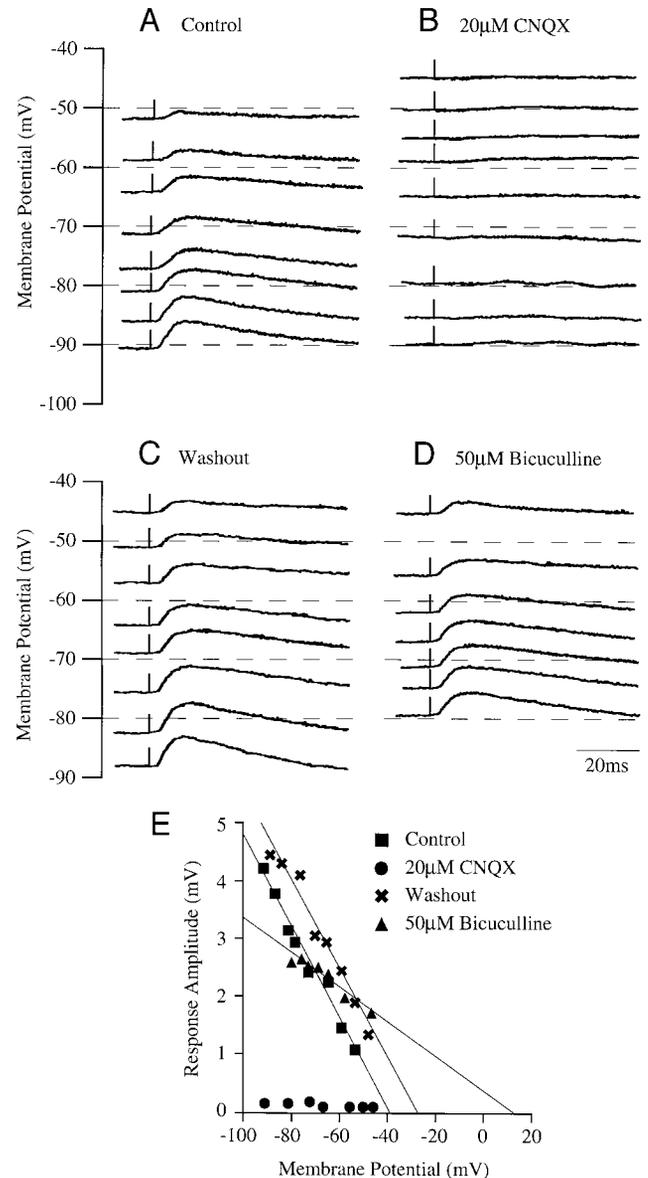

FIG. 4. The IPSP component of the subthalamic nucleus-evoked DPSP in some dopaminergic neurons is polysynaptic. Under control conditions STN stimulation produced a DPSP with a reversal potential of –38.8 mV (*A, E*). Addition of CNQX to the bath completely abolished the DPSP (*B, E*). After a one hour wash, the DPSP returned and still exhibited a hyperpolarized reversal potential as before drug application (*C, E*). Subsequent application of bicuculline shifted the reversal potential in the positive direction to 12.6 mV (*D, E*). Traces in *A–D* are each the average of 4 single sweeps. Stimulus artifacts have been digitally reduced.
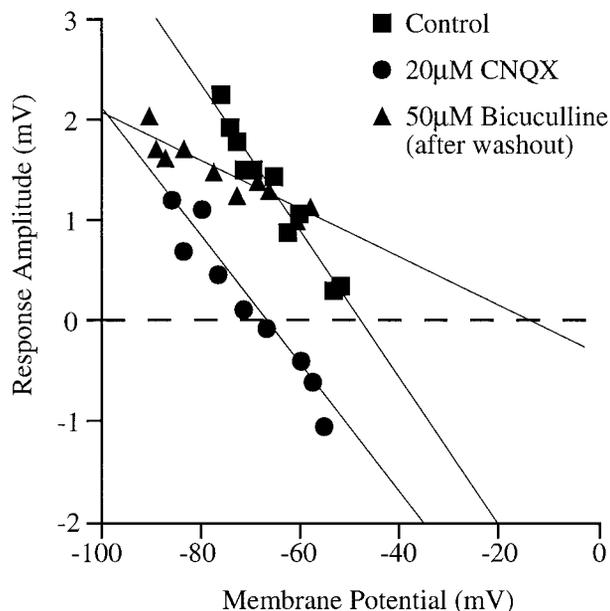
FIG. 5. Monosynaptic IPSP component of the DPSP after STN stimulation. In these cases, application of CNQX did not abolish the DPSP, but instead shifted its reversal potential in the negative direction to around E_{Cl}. Note that in this case, the control reversal potential was even more hyperpolarized than usual for the STN-induced DPSP.

DISCUSSION

The response to STN stimulation is a mixed EPSP-IPSP

Under control conditions STN stimulation resulted in a DPSP in nigral dopaminergic neurons with a mean onset latency of 2.7 ms. This latency is in good agreement with that previously reported for excitatory responses of nigral neurons to subthalamic stimulation in vivo (Hammond et al. 1978 1983; Nakanishi et al. 1987). In most cases the onset latency of the DPSP was invariant with changes in stimulus amplitude suggesting that it was monosynaptic (Park 1987). The DPSP had a mean reversal potential around –30 mV, more depolarized than threshold for dopaminergic neurons (–40 mV), indicating that it is effectively an EPSP. However, –30 mV is considerably more hyperpolarized than the expected reversal potential for glutamate mediated EPSPs in the mammalian CNS (Puil and Benjamin 1988). After application of bicuculline the reversal potential was shifted in the depolarizing direction to the expected value near 0 mV. This indicates that the STN stimulation produced a compound synaptic response consisting of a near simultaneous EPSP and a $GABA_A$-mediated IPSP.

Although it is possible that stimulation of STN produced antidromic excitation in PPN that was conveyed orthodromically to substantia nigra through nigral afferents from the PPN, the short latency of the response is incompatible with the known axonal conduction times of efferents from PPN to the STN and the substantia nigra (Scarnati et al. 1986; Takakusaki et al. 1996, 1997). Furthermore, unlike PPN efferents to substantia nigra which are both cholinergic as well as glutamatergic, the STN-stimulation-induced excitation could be completely blocked by CNQX (see below) whereas some effects of PPN stimulation on dopaminergic neurons are cholinergic (Clarke et al. 1987; Nijima and Yoshida 1988). Thus we conclude that the excitatory portion of the response is mediated by glutamatergic efferents from the STN.

The origin of the IPSP component

Since the efferents from the STN to substantia nigra are exclusively glutamatergic and excitatory (Bevan et al. 1994; Chang et al. 1984; Kita and Kitai. 1987; Shink et al. 1996), the inhibitory component of the DPSP almost certainly does not
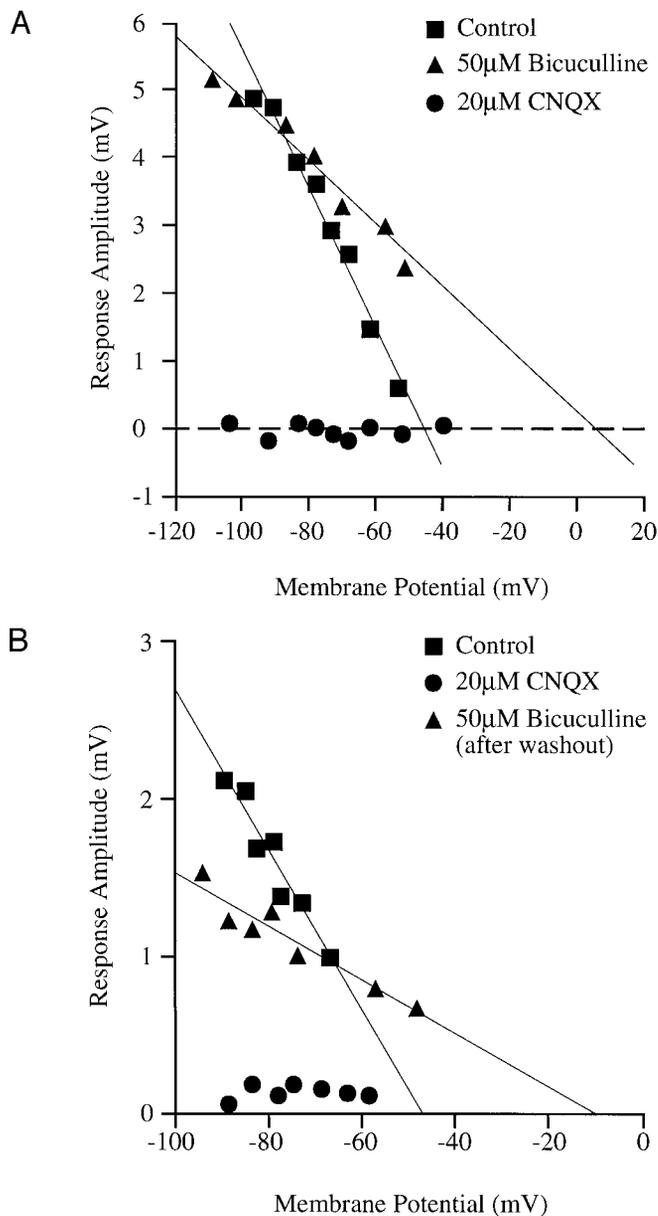

FIG. 6. Dopaminergic neurons still exhibit mixed EPSP/IPSPs after transection of pallidonigral and striatonigral pathways 3 days prior to recording, indicating that the IPSP component was arising disynaptically through glutamatergic excitation of a GABAergic neuron in the slice. *A:* as in slices from intact rats, the DPSP reversal potential was unusually hyperpolarized (-44.6 mV) and was shifted in the depolarizing direction to 5.2 mV by bicuculline. Subsequent CNQX application eliminated the response. After washout of both CNQX and bicuculline restored the DPSP, subsequent administration of CNQX alone abolished both the EPSP and the IPSP. *B:* a different neuron in a slice from a transected rat displays a hyperpolarized reversal potential for the STN-induced DPSP (reversal potential -47.1 mV). Administration of 20 μM CNQX completely abolishes the DPSP. After washing out CNQX for 1 h, subsequent administration of bicuculline shifts the reversal potential in the depolarizing direction to -10.4 mV. MK-801 (10 μM) present throughout for both neurons.

originate monosynaptically from the STN. There are at least two possibilities for the origin of the $GABA_A$ component. The STN stimulation could activate descending GABAergic fibers from the striatum and/or globus pallidus traveling in the fiber bundles surrounding the STN, thereby producing a monosynaptic IPSP as suggested by others (Nakanishi et al. 1987; Smith and Grace 1992). In addition, GABAergic pars reticulata projection neurons and perhaps interneurons may exert feedforward inhibition on pars compacta dopaminergic neurons following subthalamic stimulation by a disynaptic (or polysynaptic) pathway (Robledo and Féger 1990; Smith and Grace 1992; Tepper et al. 1995).

Both striatal and pallidal inputs to dopaminergic neurons have been identified anatomically (Hattori et al. 1975; Somogyi et al. 1981; Smith and Bolam 1990) and in vivo recordings show that striatal or pallidal stimuli result in IPSPs and/or inhibition of firing of electrophysiologically identified dopaminergic neurons (Grace and Bunney 1985; Paladini et al. 1999; Tepper et al. 1987 1990). In vivo studies indicate that the IPSP produced in nigral dopaminergic neurons by stimulation of either striatum or globus pallidus is mediated predominantly or exclusively by $GABA_A$ receptors (Grace and Bunney 1985; Paladini et al. 1999).

When CNQX was administered to block the EPSP component of the response, a pure IPSP with a reversal potential of -61.1 ± 6.4 mV remained in 3 of 8 neurons, indicating that in these cases, the STN stimulation did indeed activate descending inhibitory efferents as well as excitatory subthalamic efferents. This was in fact suggested as the most likely source of mixed EPSP and IPSP responses following electrical stimulation of the subthalamo-nigral pathway in prior in vitro intracellular recording studies (Nakanishi et al. 1987, 1997) and as one of the possibilities for the initial inhibition seen in previous in vivo extracellular experiments (Smith and Grace 1992). However, in 5 of 8 neurons, application of CNQX completely eliminated both the EPSP and the IPSP indicating that in these cases, the IPSP was not caused by stimulation of descending GABAergic afferents but rather by the activation of an excitatory glutamatergic input to a GABAergic neuron within the slice.

This interpretation is supported by the results obtained with slices from rats transected anterior to the STN 3 days prior to recording to eliminate descending GABAergic fibers from striatum and globus pallidus. In many neurons recorded from these slices, stimulation of the STN produced mixed EPSP-IPSPs in which the IPSP could be blocked by either CNQX or bicuculline just as in slices from intact rats. Thus, although subthalamic stimulation can produce a monosynaptic IPSP through inadvertent activation of descending GABAergic nigral afferents as well as a monosynaptic EPSP from the glutamatergic subthalamo-nigral pathway, a polysynaptic IPSP mediated by $GABA_A$ receptors is also evoked by subthalamic stimulation of a GABAergic neuron somewhere in substantia nigra, most likely in pars reticulata, as previously suggested (Nakanishi et al. 1987; Robledo and Féger 1990; Smith and Grace 1992).

In an early extracellular recording study, the firing of pars compacta dopaminergic neurons and GABAergic neurons in pars reticulata was shown to be reciprocally related (Grace and Bunney 1979). More recently, selective stimulation of pars reticulata GABAergic projection neurons achieved by anti-dromic activation from thalamus or tectum was shown to inhibit nigrostriatal dopaminergic neurons (Paladini et al. 1999; Tepper et al. 1995), and the effects of pharmacological excitation and inhibition of globus pallidus on nigral dopaminergic neurons were shown to be mediated indirectly through pars reticulata GABAergic neurons (Celada et al. 1999). Intracellular labeling of pars reticulata projection neurons in vivo shows that these neurons have extensive local collaterals both within pars reticulata and pars compacta (Grofova et al. 1982) and make synapses with somata and proximal dendrites of dopaminergic neurons in pars compacta (Damlama 1994). Stimulation of pars reticulata in slices taken from animals 24–48 h after hemisection through the caudal hypothalamus revealed IPSPs in pars compacta dopaminergic neurons (Hajós and Greenfield 1994). Thus, pars reticulata GABAergic neurons, including but perhaps not restricted to nigrothalamic and nigrotectal projection neurons, inhibit pars compacta dopaminergic neurons via their axon collaterals both in vivo and in vitro.

Although there is clearly a monosynaptic projection from the STN to dopaminergic neurons, there are few boutons in pars compacta. The vast majority of subthalamic nigral efferents terminate in pars reticulata, mostly on the dendrites of nondopaminergic neurons, but also on dopaminergic dendrites (Damlama 1994; Kita and Kitai 1987). Since the pars reticulata GABAergic neurons make functional inhibitory connections with pars compacta dopaminergic neurons, in addition to a monosynaptic EPSP, stimulation of the STN also leads to a disynaptic IPSP that is mediated through the local axon collaterals of substantia nigra pars reticulata GABAergic neurons. A similar role for pars compacta GABAergic interneurons cannot be excluded.

Effects of $GABA_B$ receptor blockade

Blockade of $GABA_B$ receptors by 2-hydroxysaclofen did not shift the reversal potential in the depolarizing direction as did the $GABA_A$ antagonist, bicuculline. This is consistent with other data that demonstrate that although there is a significant postsynaptic GABAergic tone exerted on nigral neurons through $GABA_A$ receptors in vivo and in vitro, there does not appear to be a significant amount of endogenous $GABA_B$ stimulation either in vivo or in vitro (Engberg et al. 1993; Häusser and Yung 1994; Paladini and Tepper 1999; Paladini et al. 1999; Rick and Lacey 1994).

Although it did not reach statistical significance, application of 2-hydroxysaclofen shifted the reversal potential in the opposite direction as bicuculline, toward a more. hyperpolarized potential. This is consistent with the ability of $GABA_B$ receptor agonists to attenuate $GABA_A$-mediated IPSPs in dopaminergic neurons in vitro (Häusser and Yung 1994) and of $GABA_B$ antagonists to augment $GABA_A$-mediated inhibition of dopaminergic neurons in vivo (Paladini et al. 1999), effects that are attributable to stimulation and blockade of presynaptic inhibitory $GABA_B$ autoreceptors.

CONCLUSIONS

Electrical stimulation of the STN gives rise to a mixed response consisting of an EPSP and an IPSP occurring nearly simultaneously in pars compacta dopaminergic neurons in

vitro. The EPSP is mediated monosynaptically by both NMDA and non-NMDA glutamate receptors and the IPSP is mediated mono- and disynaptically by GABA$_A$ receptors. The IPSP arises not only monosynaptically from co-activation of descending GABAergic fibers from striatum and/or globus pallidus, but also disynaptically from glutamatergic synaptic excitation of GABAergic neurons within substantia nigra. It is likely that this is the cause of the initial inhibition frequently reported following stimulation of the STN in vivo (Robledo and Feger 1990; Smith and Grace 1992). Whether activation of the STN by endogenous stimuli in vivo also leads to mixed effects on nigral dopaminergic neurons depends on whether single subthalamic efferent fibers synapse with both dopaminergic and nondopaminergic neurons in substantia nigra, or conversely, on whether different population of subthalamic efferents which synapse selectively on dopaminergic or nondopaminergic neurons are activated simultaneously.

REFERENCES

BEVAN, M. D., BOLAM J. P., AND CROSSMAN, A. R. Convergent synaptic input from the neostriatum and the subthalamus onto identified nigrothalamic neurons in the rat. *Eur. J. Neurosci.* 6: 320–334, 1994.

BUNNEY, B. S., WALTERS, J. R., ROTH, R. H., AND AGHAJANIAN, G. K. Dopaminergic neurons: effect of antipsychotic drugs and amphetamine on single cell activity. *J. Pharmac. Exp. Ther.* 185: 560–571, 1973.

CAMERON, D. L., AND WILLIAMS, J. T. Dopamine D1 receptors facilitate transmitter release. *Nature.* 366: 344–347, 1993.

CELADA, P., PALADINI, C. A., AND TEPPER, J. M. GABAergic control of rat substantia nigra dopaminergic neurons: Role of globus pallidus and substantia nigra pars reticulata. *Neuroscience.* 89: 813–825, 1999.

CHANG, H. T., KITA, H., AND KITAI, S. T. The ultrastructural morphology of the subthalamic-nigral axon terminals intracellularly labeled with horseradish peroxidase. *Brain Res.* 299: 182–185, 1984.

CHERGUI, K., AKAOKA, H., CHARLETY, P. J., SAUNIER, C. F., BUDA, M., AND CHOUVET, G. Subthalamic nucleus modulates burst firing of nigral dopamine neurones via NMDA receptors. *Neuroreport.* 5: 1185–1188, 1994.

CLARKE, P. B., HOMMER, D. W., PERT, A., AND SKIRBOLL, L. R. Innervation of substantia nigra neurons by cholinergic afferents from pedunculopontine nucleus in the rat: neuroanatomical and electrophysiological evidence. *Neuroscience.* 23: 1011–1019, 1987.

DAMLAMA, M. *Subthalamic and Pedunculopontine Inputs to Substantia Nigra. A Light and Electron Microscopic Analysis.* (Doctoral dissertation). Ann Arbor, MI: Rutgers University, UMI Dissertation Services, 1994.

ENGBERG, G., KLING-PETERSEN, T., AND NISSBRANDT, H. GABA$_B$-receptor activation alters the firing pattern of dopamine neurons in the rat substantia nigra. *Synapse.* 15: 229–238, 1993.

GONON, F. G. Nonlinear relationship between impulse flow and dopamine released by rat midbrain dopaminergic neurons as studied by in vitro electrochemistry. *Neuroscience.* 24: 19–28, 1988.

GRACE, A. A. The regulation of dopamine neuron activity as determined by in vivo and in vitro intracellular recordings. In: *Neurophysiology of Dopaminergic Systems—Current Status and Clinical Perspectives,* edited by L. A. Chiodo and A. S. Freeman. Detroit, MI: Lakeshore, 1987, p. 1–66.

GRACE, A. A., AND BUNNEY, B. S. Paradoxical GABA excitation of nigral dopaminergic cells: Indirect mediation through reticulata inhibitory neurons. *Eur. J. Pharmacol.* 59: 211–218, 1979.

GRACE, A. A., AND BUNNEY, B. S. The control of firing pattern in nigral dopamine neurons: single spike firing. *J. Neurosci.* 4: 2866–2876, 1984a.

GRACE, A. A., AND BUNNEY, B. S. The control of firing pattern in nigral dopamine neurons: burst firing. *J. Neurosci.* 4: 2877–2890, 1984b.

GRACE, A. A., AND BUNNEY, B. S. Opposing effects of striatonigral feedback pathways on midbrain dopamine cell activity. *Brain Res.* 333: 271–284, 1985.

GRACE, A. A., HOMMER, D. W., AND BUNNEY, B. S. Peripheral and striatal influences on nigral dopamine cells: Mediation by reticulata neurons. *Brain Res. Bull.* 5(2 Suppl.): 105–109, 1980

GRACE, A. A. AND ONN, S. Morphology and electrophysiological properties of immunocytochemically identified rat dopamine neurons recorded in vitro. *J. Neurosci.* 9: 3463–3481, 1989.

GROFOVA, I., DENIAU, J. M., AND KITAI, S. T. Morphology of the Substantia Nigra Pars Reticulata Projection Neurons Intracellularly labeled with HRP. *J. Comp. Neurol.* 208: 352–368, 1982.

HAJÓS, M. AND GREENFIELD, S. A. Synaptic connections between pars compacta and pars reticulata neurones: electrophysiological evidence for functional modules within the substantia nigra. *Brain Res.* 660: 216–224, 1994.

HAMMOND, C., DENIAU, J. M., RIZK, A., AND FÉGER, J. Electrophysiological demonstration of an excitatory subthalamonigral pathway in the rat. *Brain Res.* 151: 235–244, 1978.

HAMMOND, C., SHIBAZAKI, T., AND ROUZAIRE-DUBOIS, B. Branched output neurons of the rat subthalamic nucleus: electrophysiological study of the synaptic effects on identified cells in the two main target nuclei, the entopeduncular nucleus and the substantia nigra. *Neuroscience* 9: 511–520, 1983.

HATTORI, T., FIBIGER, H. C., AND MCGEER, P. L. Demonstration of a pallidonigral projection innervating dopaminergic neurons. *J. Comp. Neurol.* 162: 487–504, 1975.

HÄUSSER, M. A. AND YUNG, W. H. Inhibitory synaptic potentials in guinea-pig substantia nigra dopamine neurones in vitro. *J. Physiol. (Lond.).* 479: 401–422, 1994.

HORIKAWA, K. AND ARMSTRONG, W. E. A versatile means of intracellular labeling: injection of biocytin and its detection with avidin conjugates. *J. Neurosci. Methods.* 25: 1–11, 1988.

JACKSON, A. AND CROSSMAN, A. R. Nucleus tegmenti pedunculopontinus: efferent connections with special reference to the basal ganglia, studied in the rat by anterograde and retrograde transport of horseradish peroxidase. *Neuroscience.* 10: 725–765, 1983.

JOHNSON, S. W., SEUTIN, V., AND NORTH, R. A. Burst firing in dopamine neurons induced by N-methyl-D-aspartate: role of electrogenic sodium pump. *Science.* 258: 665–667, 1992.

KANAZAWA, I., MARSHALL, G. R., AND KELLY, J. S. Afferents to the rat substantia ngira studied with horseradish peroxidase, with special reference to fibres from the subthalamic nucleus. *Brain Res.* 115: 485–491, 1976.

KITA, H. AND KITAI, S. T. Efferent projections of the subthalamic nucleus in the rat: light and electron microscopic analysis with the PHA-L method. *J. Comp. Neurol.* 260: 435–452, 1987.

KITA, T., KITA, H., AND KITAI, S. T. Electrical membrane properties of rat substantia nigra compacta neurons in an in vitro slice preparation. *Brain Res.* 372: 21–30, 1986.

LACEY, M. G., MERCURI, N. B., AND NORTH, R. A. Dopamine acts on D2 receptors to increase potassium conductance in neurones of the rat substantia nigra zona compacta. *J. Physiol. (Lond.).* 392: 397–416, 1987.

NAITO, A. AND KITA, H. The cortico-nigral projection in the rat: an anterograde tracing study with biotinylated dextran amine. *Brain Res.* 637: 317–322, 1994.

NAKANISHI, H., KITA, H., AND KITAI, S. T. Intracellular study of rat substantia nigra pars reticulata neurons in an in vitro slice preparation: electrical membrane properties and response characteristics to subthalamic stimulation. *Brain Res.* 437: 45–55, 1987.

NAKANISHI, H., TAMURA, A., KAWAI, K., AND YAMAMOTO, K. Electrophysiological studies of rat substantia nigra neurons in an in vitro slice preparation after middle cerebral artery occlusion. *Neuroscience.* 77: 1021–1028, 1997.

NIIJIMA, K. AND YOSHIDA, M. Activation of mesencephalic dopamine neurons by chemical stimulation of the nucleus tegmenti pedunculopontinus pars compacta. *Brain Res.* 451: 163–171, 1988.

NITSCH, C. AND RIESENBERG, R. Immunocytochemical demonstration of GABAergic synaptic connections in rat substantia nigra after different lesions of the striatonigral projection. *Brain Res.* 461: 127–142, 1988.

OVERTON, P. AND CLARK, D. Iontophoretically administered drugs acting at the N-methyl-D-aspartate receptor modulate burst firing in A9 dopamine neurons in the rat. *Synapse.* 10: 131–140, 1992.

PALADINI, C. A., CELADA, P., AND TEPPER, J. M. Striatal, pallidal, and pars reticulata evoked inhibition of nigrostriatal dopaminergic neurons is mediated by GABA$_A$ receptors in vivo. *Neuroscience.* 89: 799–812, 1999

PALADINI, C. A. AND TEPPER, J. M. GABA$_A$ and GABA$_B$ antagonists differentially affect the firing pattern of substantia nigra dopaminergic neurons in vivo. *Synapse* 32: 165–176, 1999.

PARK, M. R. Monosynaptic inhibitory postsynaptic potentials from lateral habenula recorded in dorsal raphe neurons. *Brain Res. Bull.* 19: 581–586, 1987.

PRESTON, R. J., MCCREA, R. A., CHANG, H. T., AND KITAI, S. T. Anatomy and physiology of substantia nigra and retrorubral neurons studied by extra- and intracellular and by horseradish peroxidase labeling. *Neuroscience.* 6: 331–344, 1981.

PUIL, E. AND BENJAMIN, A. M. Functional organization of glutamatergic synapses. In: *Neurotransmitters and Cortical Function,* edited by M. Avoli, T. A. Reader, R. W. Dykes, and P. Gloor. New York: Plenum, 1988, p. 25–37.

RICK, C. E. AND LACEY, M. G. Rat substantia nigra pars reticulata neurones are tonically inhibited via GABA$_A$, but not GABA$_B$, receptors in vitro. *Brain Res.* 659: 133–137, 1994.

ROBLEDO, P. AND FÉGER, J. Excitatory influence of rat subthalamic nucleus to substantia nigra pars reticulata and the pallidal complex: electrophysiological data. *Brain Res.* 518: 47–54, 1990.

SAJI, M. AND REIS, D. J. Delayed transneuronal death of substantia nigra neurons prevented by γ-aminobutyric acid agonist. *Science.* 235: 66–69, 1987.

SCARNATI, E., PROIA, A., CAMPANA, E. AND PACITTI, C. A microiontophoretic study on the nature of the putative synaptic neurotransmitter involved in the pedunculopontine-substantia nigra pars compacta excitatory pathway of the rat. *Exp. Brain Res.* 62: 470–478, 1986.

SEUTIN, V., JOHNSON, S. W., AND NORTH, R. A. Effect of dopamine and baclofen on N-methyl-D-aspartate-induced burst firing in rat ventral tegmental neurons. *Neuroscience.* 58: 201–206, 1994.

SHINK, E., BEVAN M. D., BOLAM, J. P., AND SMITH Y. The subthalamic nucleus and the external pallidum: two tightly interconnected structures that control the output of the basal ganglia in the monkey. *Neuroscience.* 73: 335–357, 1996.

SMITH, Y. AND BOLAM, J. P. The output neurones and the dopaminergic neurones of the substantia nigra receive a GABA-containing input from the globus pallidus in the rat. *J. Comp. Neurol.* 296: 47–64, 1990.

SMITH, I. D. AND GRACE, A. A. Role of the subthalamic nucleus in the regulation of nigral dopamine neuron activity. *Synapse.* 12: 287–303, 1992.

SOMOGYI, P., BOLAM, J. P., TOTTERDELL, S., AND SMITH, A. D. Monosynaptic input from the nucleus accumbens-ventral striatum region to retrogradely labelled nigrostriatal neurones. *Brain Res.* 217: 245–263, 1981.

TAKAKUSAKI, T., SHIROYAMA, T., YAMAMOTO, T., AND KITAI, S. T. Cholinergic and non-cholinergic tegmental pedunculopontine projections neurons in rats revealed by intracellular labeling. *J. Comp. Neurol.* 371: 345–361, 1996.

TAKAKUSAKI, T., SHIROYAMA, T., AND KITAI, S. T. Two types of cholinergic neurons in the rat tegmental pedunculopontine nucleus: electrophysiological and morphological characterization. *Neuroscience* 79: 1089–1109, 1997.

TEPPER, J. M., DAMLAMA, M., AND TRENT, F. Postnatal changes in the distribution and morphology of rat substantia nigra dopaminergic neurons. *Neuroscience* 60: 469–477, 1994.

TEPPER, J. M., MARTIN, L. P., AND ANDERSON, D. R. GABA$_A$ receptor-mediated inhibition of rat substantia nigra dopaminergic neurons by pars reticulata projection neurons. *J. Neurosci.* 15: 3092–3103, 1995.

TEPPER, J. M., SAWYER, S. F., AND GROVES, P. M. Electrophysiologically identified nigral dopaminergic neurons intracellularly labeled with HRP: light microscopic analysis. *J. Neurosci.* 7: 2794–2806, 1987.

VAN DER KOOY, D. AND HATTORI, T. Single subthalamic nucleus neurons project to both the globus pallidus and substantia nigra in rat. *J. Comp. Neurol.* 192: 751–768, 1980.

WILSON, C. J., YOUNG, S. J., AND GROVES, P. M. Statistical properties of neuronal spike trains in the substantia nigra: cell types and their interactions. *Brain Res.* 136: 243–260, 1977.

YUNG, W. H., HÄUSSER, M. A., AND JACK, J. J. Electrophysiology of dopaminergic and non-dopaminergic neurones of the guinea-pig substantia nigra pars compacta in vitro. *J. Physiol. (Lond.).* 436: 643–667, 1991.