

## Presynaptic dopamine D<sub>2</sub> and muscarine M<sub>3</sub> receptors inhibit excitatory and inhibitory transmission to rat subthalamic neurones *in vitro*

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(Received 15 December 1999; accepted after revision 9 March 2000)

1. Whole-cell patch-clamp recordings were made from subthalamic nucleus (STN) neurones in brain slices from rats. Stimulation with bipolar electrodes evoked synaptic currents mediated by glutamate (EPSCs) and GABA<sub>A</sub> (IPSCs) receptors.
2. Dopamine reversibly reduced the amplitude of GABA<sub>A</sub> IPSCs by up to 48% with an IC<sub>50</sub> value of  $3.4 \pm 0.8 \mu\text{M}$ . The dopamine D<sub>2</sub> receptor agonist quinpirole, but not the D<sub>1</sub> receptor agonist SKF 82958, also inhibited GABA<sub>A</sub> IPSCs. This effect was completely reversed by the D<sub>2</sub> receptor antagonist sulpiride but not by SCH 23390, a D<sub>1</sub> antagonist.
3. Muscarine reversibly reduced the amplitude of GABA<sub>A</sub> IPSCs by up to 70% with an IC<sub>50</sub> value of  $0.6 \pm 0.1 \mu\text{M}$ . Inhibition of IPSCs by muscarine was completely blocked by scopolamine (10  $\mu\text{M}$ ), a muscarinic receptor antagonist. The M<sub>3</sub> muscarinic receptor antagonist 4-DAMP effectively reversed muscarine-induced inhibition of IPSCs with an IC<sub>50</sub> of  $0.11 \pm 0.03 \mu\text{M}$ . Although the M<sub>1</sub> receptor antagonist pirenzepine also reversed the inhibition of IPSCs by muscarine, this effect was only observed at relatively high concentrations (IC<sub>50</sub> =  $21.7 \pm 9.4 \mu\text{M}$ ).
4. Dopamine and muscarine both increased the paired-pulse ratio of GABA<sub>A</sub> IPSCs. Neither agent produced sustained changes in postsynaptic holding current.
5. Glutamate EPSCs were also inhibited reversibly by dopamine (by up to 29%; IC<sub>50</sub> =  $16 \pm 3 \mu\text{M}$ ) and muscarine (by up to 41%; IC<sub>50</sub> =  $1.0 \pm 0.4 \mu\text{M}$ ). However, both agents were more potent and efficacious for reducing GABA IPSCs compared with glutamate EPSCs.
6. These results suggest that the most significant effect of dopamine and muscarine in the STN is to reduce inhibitory synaptic input by acting at presynaptic dopamine D<sub>2</sub> and muscarinic M<sub>3</sub> receptors, respectively.

The subthalamic nucleus (STN) is composed of glutamate-containing neurones that project to the major output nuclei of the basal ganglia: substantia nigra reticulata and globus pallidus interna (Albin *et al.* 1995). Due to its excitatory influence on these structures, the STN plays a major role in regulating muscle tone and the dynamics of movement. As an example of the importance of the STN, it has long been known that a stroke to the STN produces hemiballismus. Other hyperkinetic movements such as chorea and athetosis are associated with reductions in STN neuronal activity (Crossman, 1987; Albin, 1995). In contrast, high levels of neuronal activity in the STN are associated with the rigidity, tremor and bradykinesia that are typical of Parkinson's disease. The central role played by the STN in controlling movement is supported further by studies that show that inactivation of the STN, either by surgical lesion

or by high frequency electrical stimulation, is highly effective in obviating most of the signs of parkinsonism in patients with Parkinson's disease and in animals with chemically induced parkinsonism (Bergman *et al.* 1990; Krack *et al.* 1998).

Because Parkinson's disease is known to be produced by loss of dopamine-containing neurones in the substantia nigra, it is not surprising that dopamine replacement therapy (with levodopa) and treatment with dopamine agonist drugs effectively reduce symptoms of this disease. However, before levodopa was used clinically, anticholinergic agents were known to be effective and are still used widely in the treatment of Parkinson's disease. Although the striatum is generally thought to be an important site of action for the antiparkinsonian effects of dopaminergic and anticholinergic drugs, one must also consider the STN – which

is now recognized as having a major role in the regulation of movements – as a possible site of action of antiparkinsonian drugs.

The STN is innervated by dopamine-containing pathways that arise from the substantia nigra zona compacta and the ventral tegmental area (Hassani *et al.* 1997). Moreover, dopamine D<sub>1</sub>- and D<sub>2</sub>-like receptor subtypes are both expressed in the STN (Flores *et al.* 1999). Cholinergic input to the STN arises from neurones in the pedunculopontine nucleus (Bevan & Bolam, 1995), and muscarinic receptors – especially the M<sub>3</sub> receptor subtype – are prominently expressed in the STN (Levey *et al.* 1994). Therefore, the STN receives the anatomic connections and expresses the neurotransmitter receptors that would be required in order for dopamine and acetylcholine to play significant roles in regulating neuronal activity in the STN.

In the present study, we used whole-cell patch-clamp recording to characterize the actions of dopamine and muscarine on synaptic transmission in slices of STN prepared from rat brain. We found that dopamine and muscarine, acting at presynaptic D<sub>2</sub> and M<sub>3</sub> receptors, respectively, are both potent inhibitors of inhibitory transmission mediated by GABA<sub>A</sub> receptors.

## METHODS

### Tissue preparation

Methods used for preparing STN brain slices were virtually identical to those reported previously for preparing slices of midbrain (Shen & Johnson, 1997). Briefly, adult male Sprague-Dawley rats (120–300 g; Bantin & Kingman, Seattle, WA, USA) were anaesthetized with halothane and killed by severing major thoracic vessels, in accordance with national guidelines. The brain was rapidly removed and horizontal slices (300  $\mu\text{m}$ ) containing caudal diencephalon and rostral midbrain were cut in cold physiological saline by a vibratome. After a slice containing the STN was placed on a supporting net in a recording chamber (volume, 500  $\mu\text{l}$ ), it was immersed and superfused (1.5 ml min<sup>-1</sup>) with artificial spinal fluid which contained (mM): NaCl, 126; KCl, 2.5; CaCl<sub>2</sub>, 2.4; MgCl<sub>2</sub>, 1.2; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 19; glucose, 11. This solution was saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and had a pH of 7.35 at 35–37 °C. Using a dissection microscope for visual guidance, the STN was located as grey matter approximately 2.7 mm lateral to the midline and 2 mm rostral to the centre of the substantia nigra reticulata (Paxinos & Watson, 1986).

### Electrophysiological recordings

Whole-cell tight-seal recordings were made with pipettes containing (mM): potassium gluconate, 130; MgCl<sub>2</sub>, 2; CaCl<sub>2</sub>, 1; EGTA, 11; Hepes, 10; ATP, 1.5; GTP, 0.3. The pH of the internal solution was adjusted to 7.3–7.4 with KOH. Membrane currents were recorded under voltage clamp (–70 mV holding potential) and amplified with an Axopatch-1D amplifier. Data were acquired and analysed using pCLAMP software, a Digidata analog/digital interface (Axon Instruments, Foster City, CA, USA) and an IBM-compatible personal computer. Holding currents were recorded continuously using a MacLab analog/digital interface, Chart software (AD Instruments, Castle Hill, Australia) and a Macintosh IIVX

computer. The voltage dependence of membrane currents was measured by delivering a series of hyperpolarizing and depolarizing voltage steps (400 ms duration) from a holding potential of –70 mV. Chord conductance was measured as the linear slope of the current–voltage relationship. Series resistance was electronically compensated by 50–80% to 10–30 M $\Omega$ . Membrane potentials have been corrected for the liquid junction potential (10 mV).

### Synaptic currents

Bipolar stimulation electrodes (tip separation, 300–500  $\mu\text{m}$ ) were made from electrolytically sharpened tungsten wire, and their tips were placed in the slice 300  $\mu\text{m}$  rostral to the STN. A single rectangular pulse (0.1 ms duration) of constant current was used to evoke a synaptic current every 10 s. All synaptic currents shown in the figures are the average of three responses. An IPSC mediated by GABA<sub>A</sub> receptors was isolated pharmacologically by recording in the presence of ( $\pm$ )-2-amino-5-phosphopentanoic acid (AP5; 50  $\mu\text{M}$ ) and 6-cyano-7-nitro-quinoxalone (CNQX; 10  $\mu\text{M}$ ) in order to block NMDA and non-NMDA receptors, respectively. Studies on EPSCs were done in the presence of bicuculline (30  $\mu\text{M}$ ) to block GABA<sub>A</sub> receptors.

### Drugs

All drugs were dissolved in aqueous stock solutions with the exception of CNQX, sulpiride and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), which were dissolved in dimethyl sulfoxide. A stock solution of dopamine hydrochloride was made daily and kept on ice to retard oxidation. Each stock solution was diluted at least 1:1000 in perfusate immediately prior to its use. Dimethyl sulfoxide, diluted 1:1000 in artificial spinal fluid, had no effect on either holding current or synaptic currents. Approximately 30 s was required for the drug solution to enter the recording chamber; this delay was due to passage of the perfusate through a heat exchanger. Bicuculline methiodide, dopamine, muscarine and scopolamine were obtained from Sigma Chemical Co. (St Louis, MO, USA). Sulpiride, SKF 82958, quinpirole, SCH 23390, 4-DAMP, pirenzepine, AP5 and CNQX were obtained from Research Biochemicals, Inc. (Natick, MA, USA).

### Data analysis

Numerical data in the text and error bars in the figures are expressed as means  $\pm$  s.e.m. Two-way analysis of variance (ANOVA) with repeated measures was used to test for significant differences between concentration–response curves (SigmaStat software, Jandel Scientific, San Rafael, CA, USA). Student's two-tailed *t* tests were used to test for significant differences between chord conductances and holding currents. In evaluating concentration-dependent drug effects, an IC<sub>50</sub> was calculated for the responses from each neurone using the KaleidaGraph curve-fitting program (Synergy Software, Reading, PA, USA) on a Macintosh computer. Concentration–response data were fitted to the Hill-Langmuir equation:  $y = ax/(x + b)$ , where *y* is the magnitude of effect, *a* is maximum effect, *x* is the drug concentration and *b* is the concentration that inhibits the effect by 50% (IC<sub>50</sub>). The mean IC<sub>50</sub> and s.e.m. were calculated by averaging all IC<sub>50</sub> values. An apparent dissociation constant for an antagonist (*K<sub>b</sub>*) was calculated for the mean population using the Cheng-Prusoff equation:  $K_b = \text{IC}_{50}/(1 + A/A_{50})$ , where IC<sub>50</sub> is the concentration of the antagonist that causes a 50% maximum reversal of an agonist effect, *A* is the concentration of agonist used and *A*<sub>50</sub> is the concentration of agonist that produces a 50% maximum effect (Cheng & Prusoff, 1973).

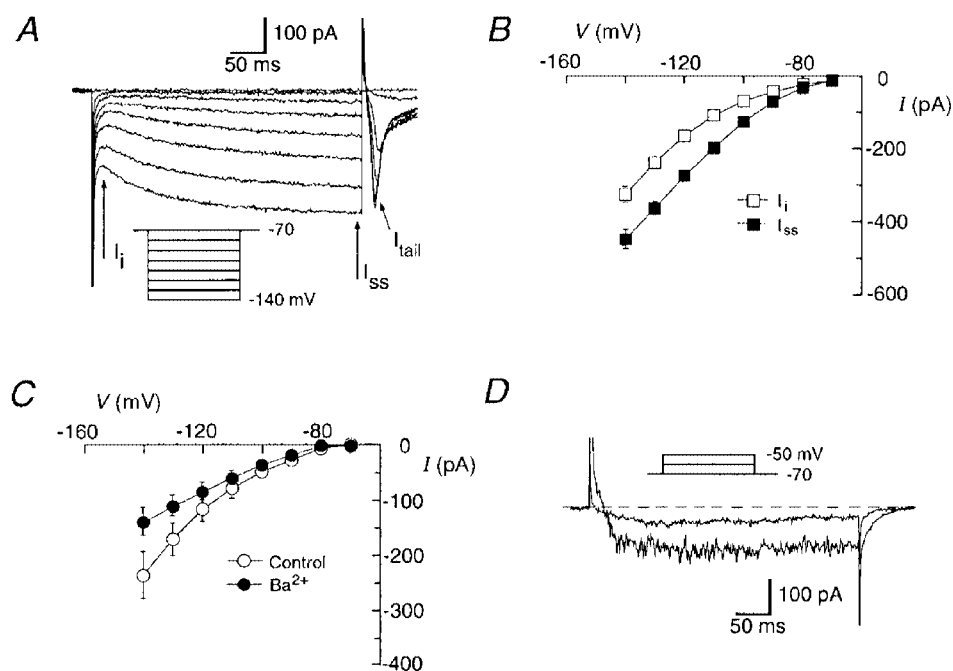
## RESULTS

### Membrane properties and synaptic currents

The vast majority of neurones recorded in the STN fired action potentials (or currents) spontaneously (data not shown). When recording under current clamp (with zero holding current), STN neurones fired action potentials at a rate of  $9.6 \pm 1.9$  Hz (range, 5–18 Hz;  $n = 7$  cells); this rate is consistent with previous reports (Nakanishi *et al.* 1987; Overton & Greenfield, 1995). Each action potential lasted about 1 ms and was followed by significant after-hyperpolarization. When recording under voltage clamp (with zero holding current), the mean membrane potential was  $-59 \pm 1$  mV (range,  $-48$  to  $-74$  mV;  $n = 83$ ). Voltage-clamp recordings made during a series of 400 ms hyperpolarizing voltage steps (0 to  $-70$  mV from a holding potential of  $-70$  mV) showed an ‘instantaneous’ inward current ( $I_i$ ), a time-dependent inward current which slowly reached steady state ( $I_{ss}$ ) and an inward tail current ( $I_{tail}$ ) (see Fig. 1A). Current–voltage plots for  $I_i$  and  $I_{ss}$ , shown in Fig. 1B, demonstrated prominent inward rectification. Chord conductance for  $I_i$  was  $1.2 \pm 0.2$  nS when measured between  $-70$  and  $-80$  mV, but it increased to  $8.7 \pm 0.7$  nS between  $-130$  and  $-140$  mV ( $n = 29$ ). Inward rectification was reduced by barium chloride ( $300 \mu\text{M}$ ,  $n = 5$ ), as shown in Fig. 1C. The difference between  $I_{ss}$  and  $I_i$  was  $-96 \pm 5$  pA (range,  $-26$  to  $-211$  pA) when measured during the voltage

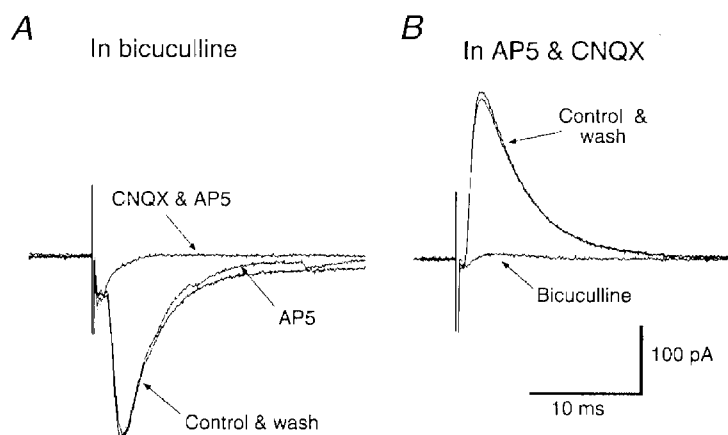
step from  $-70$  to  $-140$  mV ( $n = 81$ ). This time-dependent hyperpolarization-activated inward current was completely blocked by caesium chloride (3 mM,  $n = 5$ ), which is typical for the mixed cationic current  $I_h$  (Halliwell & Adams, 1982). Figure 1D shows that depolarizing voltage steps (to  $-60$  and  $-50$  mV) evoked inward currents that demonstrated little inactivation. These membrane properties of STN neurones are consistent with reports by others (Nakanishi *et al.* 1987; Bevan & Wilson, 1999).

Bipolar stimulation electrodes placed in the rostral region of the slice were used to evoke synaptic currents at a holding potential of  $-70$  mV. As seen in Fig. 2A, a single stimulus evoked an inward synaptic current when recorded in the presence of bicuculline ( $30 \mu\text{M}$ ). Although the late component of the EPSC was slightly reduced by the NMDA receptor antagonist AP5 ( $50 \mu\text{M}$ ), the EPSC was completely blocked by combined application of CNQX ( $10 \mu\text{M}$ ) and AP5 ( $50 \mu\text{M}$ ). This suggests that the EPSC is largely mediated by non-NMDA receptors. When recordings were made in the presence of CNQX ( $10 \mu\text{M}$ ) and AP5 ( $50 \mu\text{M}$ ), focal stimulation evoked a GABA<sub>A</sub> receptor-mediated outward current that was completely blocked by bicuculline ( $30 \mu\text{M}$ ; Fig. 2B). Thus, focal electrical stimulation of the brain slice can evoke synaptic currents mediated by GABA and glutamate.



**Figure 1.** Electrophysiological properties of STN neurones

A, membrane currents recorded under voltage clamp during a series of hyperpolarizing voltage steps. Current traces show instantaneous current ( $I_i$ ), steady-state current ( $I_{ss}$ ) and inward tail current ( $I_{tail}$ ). B, current–voltage plots for  $I_i$  (□) and  $I_{ss}$  (■) measured at times indicated by the arrows in A. Each data point is the mean of 29 cells. C, barium ( $300 \mu\text{M}$ , ●) reduces the inward rectification of  $I_i$  compared with control values (○). Each data point is the mean of 5 cells. D, depolarizing voltage steps evoke inward currents.



**Figure 2. Synaptic currents are mediated by GABA and glutamate**

*A*, superimposed EPSCs recorded in the presence of bicuculline ( $30 \mu\text{M}$ ). Although the late component of the EPSC was slightly reduced by AP5 ( $50 \mu\text{M}$ ), the EPSC was completely blocked by combined application of CNQX ( $10 \mu\text{M}$ ) and AP5 ( $50 \mu\text{M}$ ). *B*, superimposed IPSCs recorded in the presence of AP5 ( $50 \mu\text{M}$ ) and CNQX ( $10 \mu\text{M}$ ). The GABA<sub>A</sub> receptor-mediated outward synaptic current was completely blocked by bicuculline ( $30 \mu\text{M}$ ).

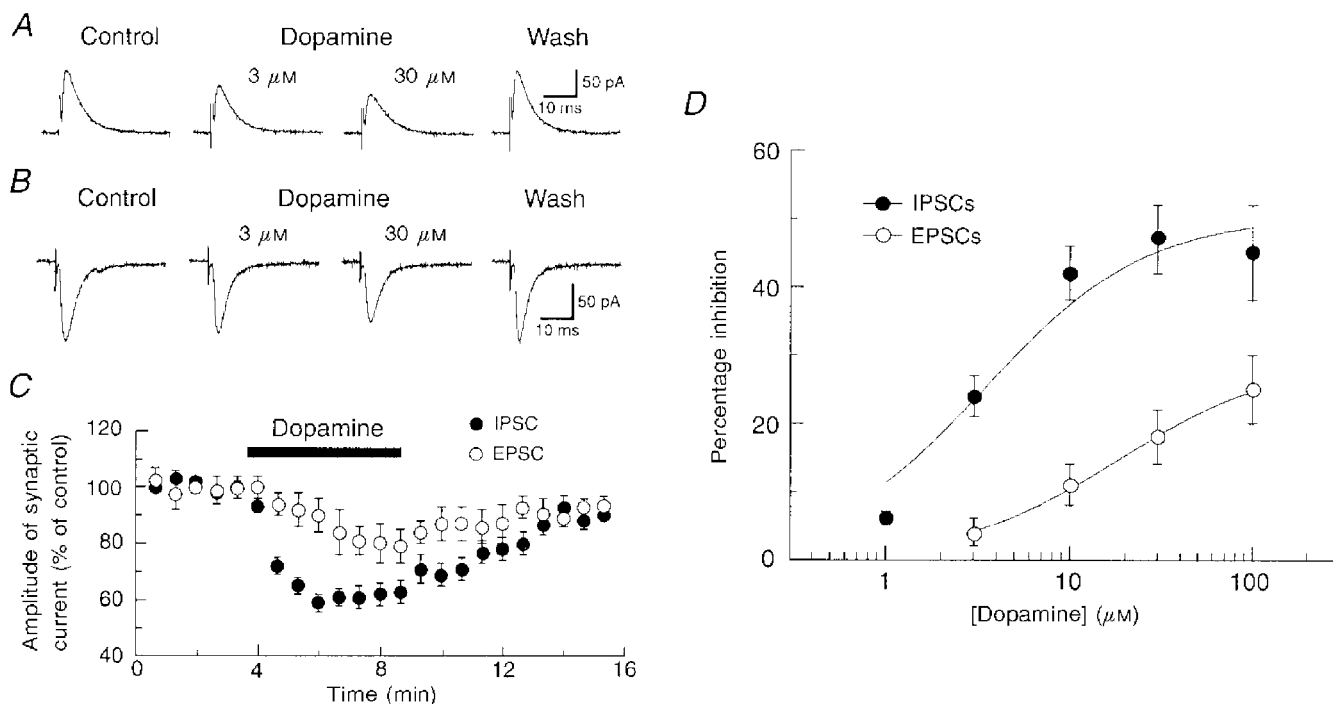
### Inhibition of synaptic currents by dopamine

As seen in Fig. 3*A* and *B*, dopamine produced reversible and concentration-dependent reductions in the amplitude of IPSCs (recorded in  $10 \mu\text{M}$  CNQX and  $50 \mu\text{M}$  AP5) and EPSCs (recorded in  $30 \mu\text{M}$  bicuculline). The actions of dopamine began within 2 min of the start of the perfusion, and its effects reversed 10–15 min after washout (Fig. 3*C*). As seen in the concentration–response curves in Fig. 3*D*, dopamine reduced the amplitude of GABA<sub>A</sub> receptor-mediated IPSCs with an  $\text{IC}_{50}$  of  $3.4 \pm 0.8 \mu\text{M}$  ( $n = 5$ ); the highest concentration of dopamine tested ( $100 \mu\text{M}$ ) reduced

GABA<sub>A</sub> IPSCs by  $48 \pm 5\%$  ( $n = 16$ ). In contrast, dopamine reduced the amplitude of glutamate EPSCs with an  $\text{IC}_{50}$  of  $16 \pm 3 \mu\text{M}$  ( $n = 6$ ), and the highest concentration of dopamine tested ( $100 \mu\text{M}$ ) reduced glutamate EPSCs by  $29 \pm 6\%$  ( $n = 8$ ). Thus, dopamine was significantly more potent for reducing IPSCs compared with EPSCs ( $P < 0.001$ ).

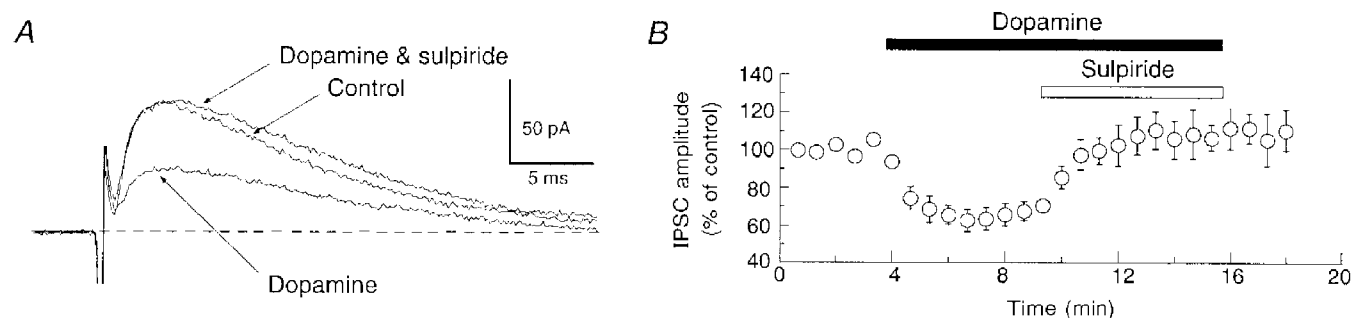
### Dopamine receptor pharmacology

Because dopamine was more potent for inhibiting GABA-mediated IPSCs compared with glutamate-mediated EPSCs, we focused further studies on the effects of dopamine on



**Figure 3. Effects of dopamine on synaptic currents recorded in STN neurons**

*A* and *B*, dopamine produces a reversible and concentration-dependent reduction in amplitude of IPSCs (*A*) and EPSCs (*B*). IPSCs were recorded in AP5 ( $50 \mu\text{M}$ ) and CNQX ( $10 \mu\text{M}$ ), whereas EPSCs were recorded in bicuculline ( $30 \mu\text{M}$ ). *C*, time-dependent reduction of IPSCs (●) and EPSCs (○) by dopamine ( $100 \mu\text{M}$ ). Each data point is either the mean of 8 (EPSCs) or 16 (IPSCs) cells. *D*, concentration–response curves show that dopamine is more potent for reducing the amplitude of IPSCs (●) compared with EPSCs (○). Each point represents the mean of 5–6 cells.



**Figure 4. Antagonism of dopamine-induced inhibition of GABA<sub>A</sub> IPSCs by sulpiride**

*A*, superimposed IPSCs show that the dopamine D<sub>2</sub> receptor antagonist sulpiride (1 μM) blocks the inhibitory effect of dopamine (100 μM) on the GABA<sub>A</sub> receptor-mediated synaptic current. *B*, averaged data from 6 cells showing that sulpiride (1 μM) reverses the reduction in IPSC amplitude produced by dopamine (100 μM). Data were normalized to the amplitude of the first synaptic current evoked in each cell.

IPSCs. As seen in the superimposed current traces in Fig. 4*A*, the reduction in IPSC amplitude caused by dopamine (100 μM) was completely blocked by concurrent treatment with sulpiride (1 μM), which is an antagonist at D<sub>2</sub>-like dopamine receptors. Figure 4*B* shows similar findings obtained from six STN neurones; although dopamine (100 μM) reduced the amplitude of IPSCs to  $63 \pm 6\%$  of control (at  $t = 6.5$  min), addition of sulpiride (1 μM) to the perfusate increased the amplitude of IPSCs to  $105 \pm 10\%$  of control (at  $t = 14$  min). No enhancement of IPSC amplitude was produced by sulpiride alone, nor was there any effect of sulpiride on the holding current ( $n = 6$ ).

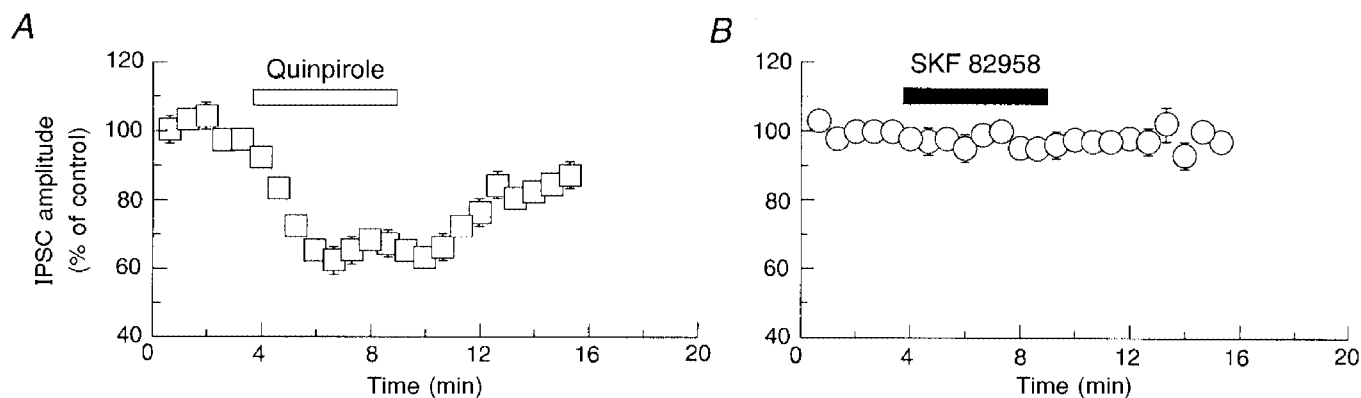
Quinpirole (10 μM), an agonist at D<sub>2</sub>-like receptors, mimicked the effect of dopamine by causing a reversible  $38 \pm 4\%$  reduction in the amplitude of GABA<sub>A</sub> IPSCs ( $n = 8$ ), as shown in Fig. 5*A*. Quinpirole reached its peak effect within 3 min, and its effect reversed 15–20 min after washout. In contrast, an agonist at D<sub>1</sub>-like receptors, SKF 82958 (10 μM), failed to affect the amplitude of GABA<sub>A</sub> IPSCs ( $n = 8$ ; Fig. 5*B*). Furthermore, an antagonist at D<sub>1</sub>-like receptors, SCH 23390 (10 μM), had no effect on

dopamine-induced inhibition of GABA<sub>A</sub> IPSCs ( $n = 4$ ; data not shown). Neither quinpirole (10 μM) nor SKF 82958 (10 μM) had a consistent effect on holding current.

#### Effects of dopamine receptor stimulation on paired IPSCs

In order to examine the site of action, the effects of dopamine were measured on IPSCs evoked by pairs of stimuli. In this paired-pulse protocol (Davies *et al.* 1990), the interval between pulses (30–50 ms) was adjusted such that the second IPSC was smaller than the first (Fig. 6*A*). Under control conditions, the paired-pulse ratio of IPSC amplitudes was  $0.73 \pm 0.05$  ( $n = 5$ ). As illustrated in Fig. 6*B*, dopamine (100 μM) significantly increased the IPSC paired-pulse ratio to  $0.97 \pm 0.03$  ( $P < 0.001$ ). Figure 6*C* shows that the dopamine-induced increase in the paired-pulse ratio occurred within 1 min and was readily reversible after washout. These data are consistent with the hypothesis that dopamine acts presynaptically to inhibit GABA release.

Quinpirole (10 μM) mimicked dopamine by increasing the IPSC paired-pulse ratio from  $0.80 \pm 0.04$  to  $0.99 \pm 0.03$



**Figure 5. The dopamine D<sub>2</sub> receptor agonist quinpirole mimics the inhibitory effect of dopamine on GABA<sub>A</sub> IPSCs**

*A*, quinpirole (10 μM) reversibly reduces the amplitude of GABA<sub>A</sub> IPSCs. Each point represents the mean  $\pm$  s.e.m. of 8 cells. *B*, the dopamine D<sub>1</sub> receptor agonist SKF 82958 (10 μM) has no effect on the amplitude of GABA<sub>A</sub> IPSCs. Each point represents the mean  $\pm$  s.e.m. of 8 cells.

( $n = 6$ ; Fig. 6*D*). In contrast, Fig. 6*E* shows that SKF 82958 ( $10 \mu\text{M}$ ) failed to affect the paired-pulse ratio ( $n = 5$ ). However, sulpiride ( $1 \mu\text{M}$ ) completely blocked the ability of dopamine to increase the IPSC paired-pulse ratio (data not shown). These results suggest that the presynaptic action of dopamine to inhibit GABA release is mediated by a member of the  $D_2$  receptor family.

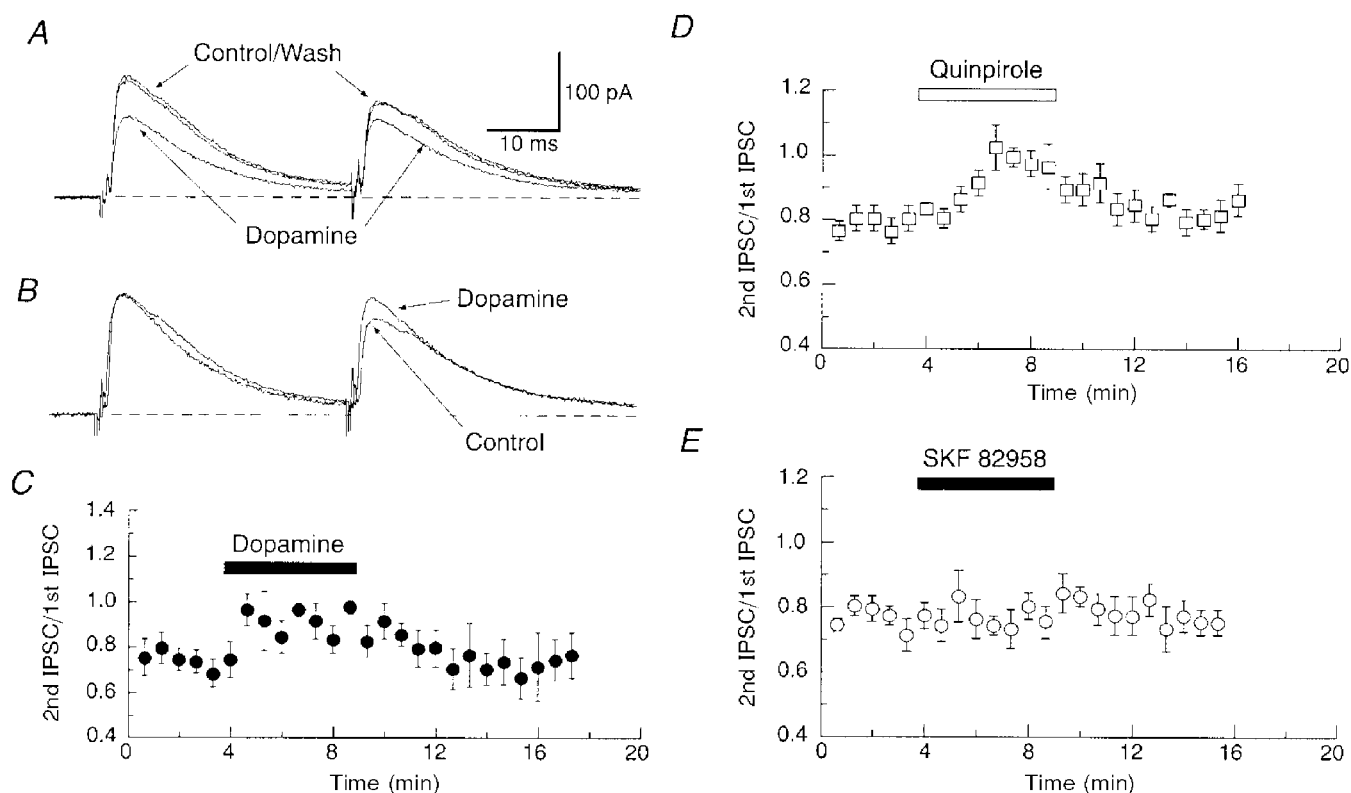
### Muscarine evokes an inward current

Muscarine ( $10 \mu\text{M}$ ) produced a small inward current which reached a peak within 1 min ( $19 \pm 2 \text{ pA}$ ,  $n = 18$ ) and gradually diminished with time despite continued perfusion (data not shown). This inward current reversed polarity at  $-118 \pm 5 \text{ mV}$  (which is near the equilibrium potential for  $\text{K}^+$ ) and was accompanied by a small decrease in membrane conductance ( $0.65 \pm 0.16 \text{ nS}$ ,  $n = 6$ ). Subsequent applications of muscarine produced little or no current. These findings suggest that (1) inward current evoked by muscarine is caused by a reduction in a resting  $\text{K}^+$  conductance, and (2) acute tolerance to this effect of muscarine develops rapidly.

### Inhibition of synaptic currents by muscarine

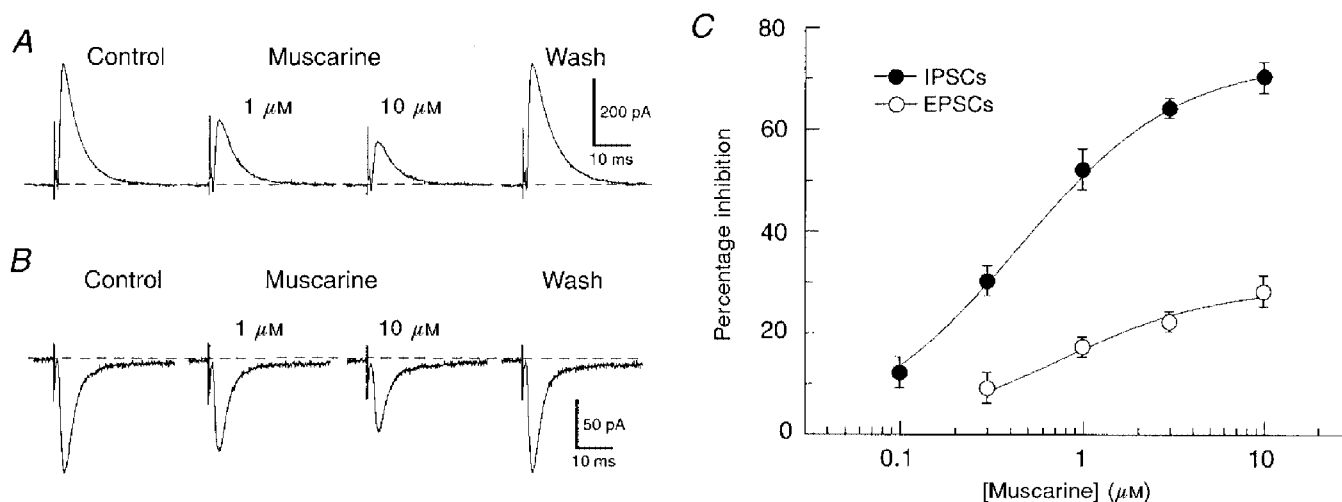
Muscarine reduced the amplitude of synaptic currents within 3 min of the start of perfusion. These effects were sustained during 30 min of continuous drug application even though the inward shift in holding current produced by muscarine returned to control within a few minutes. The effects of muscarine on synaptic currents reversed within 15 min after washout. Although low concentrations of muscarine ( $0.1\text{--}3 \mu\text{M}$ ) produced reproducible reductions in synaptic currents, application of the highest concentrations of muscarine tested ( $10\text{--}30 \mu\text{M}$ ) generally caused less inhibition of synaptic currents when perfused a second time. For example, the first application of  $10 \mu\text{M}$  muscarine reduced the amplitude of IPSCs by  $72 \pm 6\%$  ( $n = 5$ ), whereas the second application reduced IPSCs by only  $43 \pm 2\%$  in the same neurones. Consequently, 10 or  $30 \mu\text{M}$  muscarine was applied only once to each brain slice.

Muscarine produced reversible and concentration-dependent reductions in the amplitudes of GABA<sub>A</sub> receptor-mediated IPSCs (Fig. 7*A*) and glutamate-mediated EPSCs (Fig. 7*B*).



**Figure 6.** Dopamine reduces paired-pulse depression of IPSCs

*A*, superimposed IPSCs evoked by pairs of stimuli (35 ms apart) in the absence (Control and Wash) and presence of dopamine ( $100 \mu\text{M}$ ). *B*, traces are the same as those in *A*, except that the amplitude of the first IPSC recorded in dopamine has been normalized to match the amplitude of the first control IPSC. Note that in the presence of dopamine, the amplitude of the second IPSC is reduced very little compared with the first IPSC. *C*, averaged results from 5 cells show that dopamine ( $100 \mu\text{M}$ ) increases the paired-pulse ratio of IPSCs (ratio of the second IPSC amplitude divided by the first). *D*, quinpirole ( $10 \mu\text{M}$ ) increases the paired-pulse ratio of GABA<sub>A</sub> IPSCs, suggesting a presynaptic site of action. Each data point is the averaged response from 6 cells. *E*, SKF 82958 ( $10 \mu\text{M}$ ), a  $D_1$  receptor agonist, has no effect on the paired-pulse ratio of IPSCs. These results are the means from 5 cells.



**Figure 7. Effects of muscarine on synaptic currents**

*A* and *B*, current traces show that muscarine produces a concentration-dependent and reversible reduction in the amplitude of GABA<sub>A</sub> IPSCs (*A*) and glutamate EPSCs (*B*). IPSCs were recorded in AP5 (50 μM) and CNQX (10 μM), whereas EPSCs were recorded in bicuculline (30 μM). *C*, concentration–response curves for percentage inhibition of synaptic current amplitude by muscarine. Muscarine is significantly more potent for inhibiting GABA<sub>A</sub> IPSCs (●) compared with glutamate EPSCs (○). Each point represents the mean ± s.e.m. of 5–6 cells.

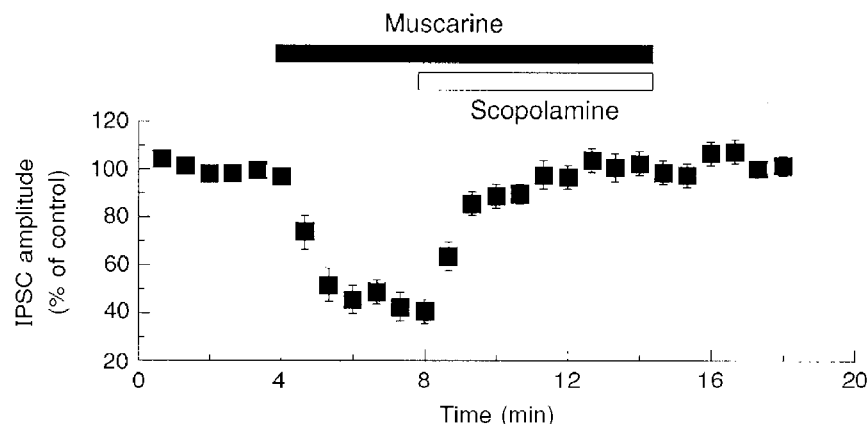
As seen in Fig. 7*C*, muscarine reduced the amplitude of GABA<sub>A</sub> IPSCs with an IC<sub>50</sub> of 0.6 ± 0.1 μM (*n* = 6), and the highest concentration of muscarine tested (10 μM) reduced IPSCs by 70 ± 3% (*n* = 6). In contrast, muscarine reduced glutamate EPSCs with an IC<sub>50</sub> of 1.0 ± 0.4 μM (*n* = 5), and 10 μM muscarine reduced glutamate EPSCs by only 41 ± 5% (*n* = 5). These data show that muscarine was significantly more potent for reducing IPSCs compared with EPSCs (*P* < 0.001).

**Muscarine receptor pharmacology**

Because muscarine was more potent for inhibiting GABA-mediated IPSCs compared with glutamate-mediated EPSCs, further studies were focused on the effects of muscarine on

IPSCs. As seen in Fig. 8, the muscarine-induced inhibition of GABA<sub>A</sub> IPSCs was completely reversed by the non-selective muscarine receptor antagonist scopolamine (10 μM). Although muscarine (10 μM) reduced the amplitude of IPSCs by 60 ± 5%, addition of scopolamine (10 μM) to the superfusate reversed the inhibition and returned the amplitude of the IPSCs to 102 ± 5% of control (*n* = 7).

Receptor pharmacology was investigated further by testing the effects of the muscarinic antagonists 4-DAMP, which binds with high affinity to M<sub>3</sub> and M<sub>1</sub> receptor subtypes (but not to M<sub>2</sub>), and pirenzepine, which is more selective for M<sub>1</sub> and M<sub>4</sub> receptors (Bonner *et al.* 1987; Zubieta & Frey, 1993). In experiments with 4-DAMP and pirenzepine,



**Figure 8. Effects of muscarine on GABA<sub>A</sub> IPSCs**

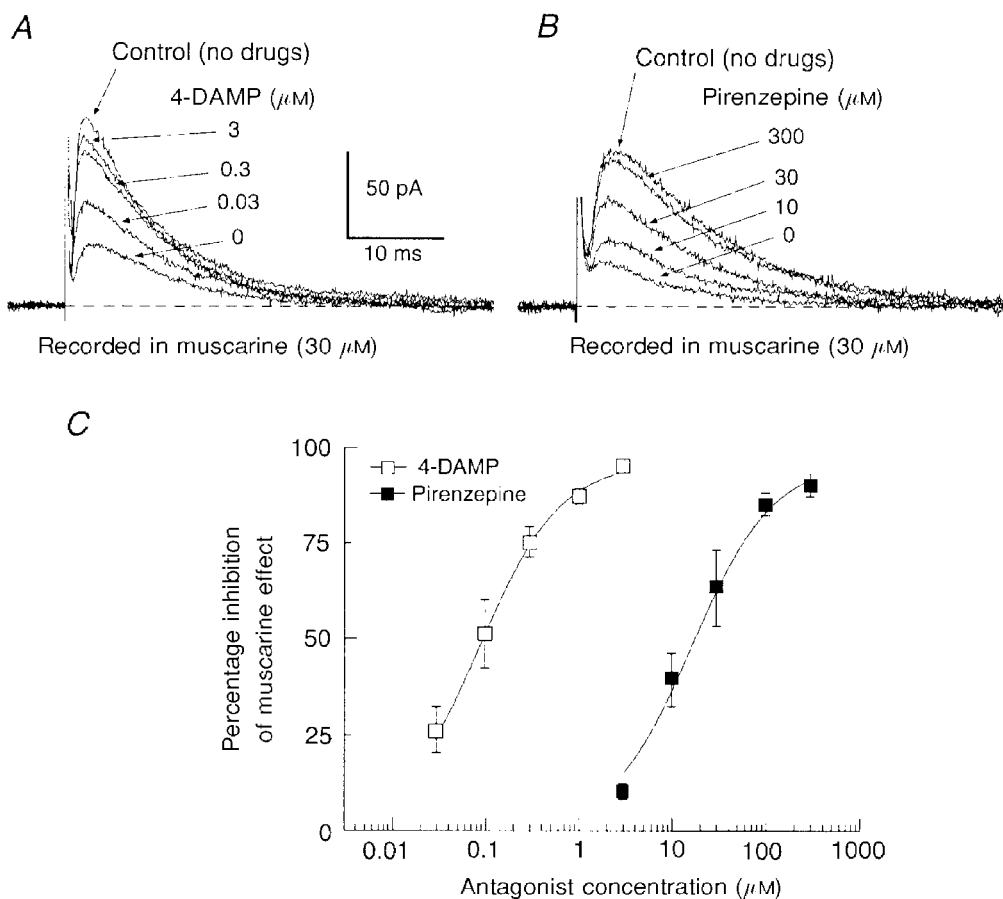
Amplitude of GABA<sub>A</sub> IPSCs recorded over time showing that the reduction in IPSC amplitude by muscarine (10 μM) is reversed by scopolamine (10 μM). Each point represents the mean ± s.e.m. of 7 cells.

concentrations were increased cumulatively because these antagonists were slow to wash out. Although Fig. 9A and B shows that both antagonists could block the inhibition of IPSCs by muscarine (30  $\mu\text{M}$ ), 4-DAMP was much more potent (Fig. 9C). By fitting the data in Fig. 9C to the Hill equation, the  $\text{IC}_{50}$  for 4-DAMP was calculated to be  $110 \pm 30 \text{ nM}$  ( $n = 6$ ), whereas the pirenzepine  $\text{IC}_{50}$  was  $21.7 \pm 9.4 \mu\text{M}$  ( $n = 4$ ). Furthermore, the apparent  $K_b$  for 4-DAMP ( $2.1 \pm 0.5 \text{ nM}$ ,  $n = 6$ ) was much lower than that for pirenzepine ( $411 \pm 178 \text{ nM}$ ,  $n = 4$ ). At the highest concentration tested (3  $\mu\text{M}$ ), 4-DAMP reversed the muscarine-induced inhibition by  $94 \pm 1\%$  ( $n = 6$ ), whereas 300  $\mu\text{M}$  pirenzepine was required to reduce the effect of muscarine by a similar extent ( $90 \pm 3\%$ ,  $n = 4$ ). Although these results show that these antagonists are equally efficacious, 4-DAMP is clearly more potent (by approximately 200-fold) than pirenzepine for blocking the effect of muscarine on GABA<sub>A</sub> IPSCs. Because 4-DAMP has a much higher affinity for the M<sub>3</sub> receptor compared with pirenzepine

(Zubieta & Frey, 1993), these results indicate that inhibition of the GABA<sub>A</sub> IPSC is mediated by the M<sub>3</sub> receptor subtype.

#### Effects of muscarine on paired IPSCs

In order to investigate the site of action of muscarine, we tested the effect of muscarine on IPSCs evoked by pairs of stimuli. Following the same protocol as that used above for studying dopamine, IPSCs were evoked by pairs of stimuli delivered 30–50 ms apart while recording in AP5 (50  $\mu\text{M}$ ) and CNQX (10  $\mu\text{M}$ ) (see Fig. 10A and B). In the absence of muscarine, the ratio of the amplitude of the second IPSC divided by the first was  $0.75 \pm 0.08$  ( $n = 4$ ). However, in the presence of muscarine (10  $\mu\text{M}$ ), this IPSC paired-pulse ratio increased to  $1.11 \pm 0.18$  in the same neurons ( $P < 0.01$ ). Moreover, this increase in the paired-pulse ratio was reversed by the addition of scopolamine (10  $\mu\text{M}$ ) to the superfusate (Fig. 10C). These data support the conclusion that muscarine acts presynaptically to inhibit GABA release.



**Figure 9.** Muscarine acts at the M<sub>3</sub> receptor to inhibit GABA<sub>A</sub> IPSCs

A, superimposed traces show that 4-DAMP reverses muscarine-induced inhibition of a GABA<sub>A</sub> IPSC in a concentration-dependent manner. B, superimposed traces show that pirenzepine also reduces the muscarine-induced inhibition of a GABA<sub>A</sub> IPSC, but that the concentrations are much higher than those required for 4-DAMP. C, concentration–inhibition curves show reversal of muscarine (30  $\mu\text{M}$ )-induced depression of IPSC amplitude by muscarine antagonists. The greater potency of 4-DAMP compared with pirenzepine suggests that M<sub>3</sub> receptors mediate the inhibitory effect of muscarine on GABA<sub>A</sub> IPSCs. Results are means  $\pm$  s.e.m. of 6 cells for 4-DAMP and 4 cells for pirenzepine.



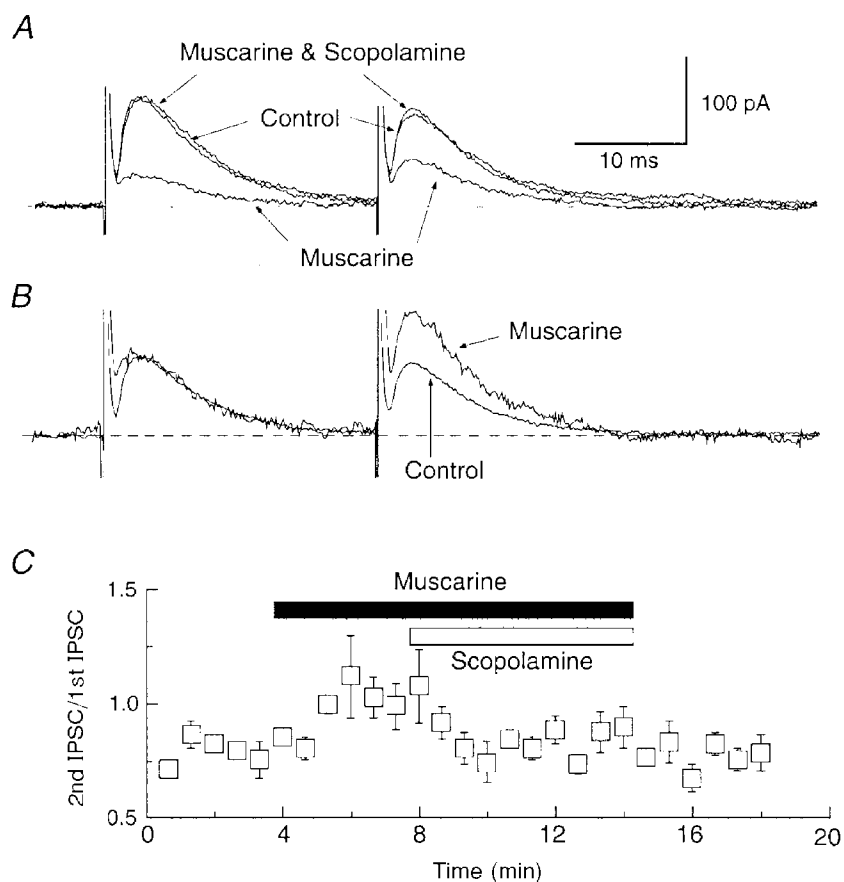
## DISCUSSION

Although our studies show that dopamine and muscarine both inhibit inotropic glutamate and GABA<sub>A</sub> synaptic currents, our most important finding is that both ligands are more potent for inhibiting currents mediated by GABA<sub>A</sub> receptors. Results from paired-pulse studies on IPSCs suggest that dopamine and muscarine both act presynaptically to inhibit GABA release from nerve terminals. Furthermore, our pharmacological studies using relatively selective agonist and antagonist ligands indicate that the presynaptic effect of dopamine is mediated by D<sub>2</sub>-like receptors whereas that of muscarine is mediated by M<sub>3</sub> receptors. Taken together, these data suggest that D<sub>2</sub> and M<sub>3</sub> receptors may regulate the excitability of STN neurones by acting presynaptically to alter GABA release.

By reducing the amplitude of synaptic currents mediated by GABA, one would expect dopamine to disinhibit STN neurones and thereby increase their firing rate. In agreement with this hypothesis, Mintz *et al.* (1986) reported

that dopamine, applied locally to STN neurones by micro-iontophoresis, acted via a member of the D<sub>2</sub> receptor family to increase the spontaneous firing rate of STN neurones recorded extracellularly in rats *in vivo*. However, others reported that this excitatory effect of dopamine was mimicked by the D<sub>1</sub>—rather than the D<sub>2</sub>—family of receptors (Kreiss *et al.* 1996, 1997). In contrast, other studies found an inhibitory effect of dopamine on the spontaneous firing rates of STN neurones (Campbell *et al.* 1985; Hassani & Féger, 1999). An inhibitory role for dopamine is also supported by a report by Parry *et al.* (1994), which showed that microinjection of apomorphine into the STN evoked oral dyskinesia in rats. Thus, there clearly is no consensus in the literature concerning the direct pharmacological actions of dopamine on the excitability of STN neurones.

Despite this lack of consensus, a considerable amount of data from patients with Parkinson's disease and in animals with chemically induced parkinsonism support an



**Figure 10.** Muscarine reduces paired-pulse depression of IPSCs

*A*, superimposed IPSCs evoked by pairs of stimuli in the absence (Control) and presence of muscarine (10  $\mu$ M), and during combined application of muscarine plus scopolamine (10  $\mu$ M). Note that the first IPSC is smaller in the presence of muscarine, but that the second IPSC is larger than the first. Scopolamine reverses the effect of muscarine. *B*, traces are the same as those in *A*, except that the amplitude of the first IPSC recorded in muscarine has been normalized to the first IPSC recorded under control conditions. *C*, the muscarine (10  $\mu$ M)-induced increase in the IPSC paired-pulse ratio is reversed by scopolamine (10  $\mu$ M). These results are the means from 4 cells.

inhibitory role for dopamine on STN neuronal activity. For example, several studies have shown that chronic dopamine depletion increases the firing rate and/or increases the incidence of burst firing in STN neurones in laboratory animals (Hollerman & Grace, 1992; Hassani *et al.* 1996). Experimentally induced parkinsonism also increases neuronal activity in substantia nigra reticulata and globus pallidus interna, which is caused by an increase in excitatory output from the STN (Robledo & Féger, 1990; Burbaud *et al.* 1995). Furthermore, disabling the STN with either high frequency electrical stimuli or surgical lesions is as effective as levodopa in alleviating most symptoms of Parkinson's disease (Limousin *et al.* 1998; Moro *et al.* 1999). Therefore, our finding that dopamine is most potent for inhibiting GABA IPSCs is at odds with the above studies which suggest that dopamine exerts an inhibitory influence on STN neurones. This is because inhibition of GABA-mediated transmission by dopamine, as we report, would be expected to increase the output from STN neurones and thereby worsen symptoms of parkinsonism.

Similar to our observations with dopamine, we found that muscarine was also more potent for inhibiting GABA<sub>A</sub> receptor-mediated IPSCs compared with glutamate-mediated EPSCs. As a consequence, we predict that muscarinic agents should disinhibit STN neurones and thereby increase STN output. This expectation is consistent with results from two studies in which the muscarinic agonist carbachol was shown to excite STN neuronal activity (Flores *et al.* 1996) and increase output from the STN to the substantia nigra reticulata (Rosales *et al.* 1994). Furthermore, the study by Flores *et al.* (1996) found that the carbachol-induced increase in spontaneous firing rate was most consistent with activation of the M<sub>3</sub> receptor subtype. Thus, these results from others are consistent with our hypothesis that muscarinic agonist ligands, which bind to presynaptic M<sub>3</sub> receptors, will stimulate STN neuronal activity by reducing the tonic inhibitory influence of GABA. Moreover, this indirect effect would tend to reinforce a direct depolarizing excitatory influence of muscarinic ligands.

Our results suggest that muscarinic antagonists would prevent endogenous acetylcholine from inhibiting GABA synaptic transmission. In the presence of tonic background stimulation of muscarinic receptors, an anticholinergic agent would be expected to reduce cholinergic-dependent inhibition of GABA release which would thereby reduce the output from the STN. Because a reduction in STN neuronal activity fits with the prevailing theory of basal ganglia function (DeLong, 1990), this action of anticholinergic drugs would be consistent with their clinical efficacy in the treatment of Parkinson's disease. There is one problem, however, in that clinically useful anticholinergic drugs are thought to bind preferentially to M<sub>4</sub> rather than to M<sub>3</sub> receptors (Ciliax *et al.* 1997; Purkerson & Potter, 1998), but the selectivity of these agents used clinically is doubtful. Nevertheless, our data certainly support the conclusion that the STN may be an

important site of action for anticholinergic medication in Parkinson's disease. It may be useful to explore the clinical effectiveness of a selective M<sub>3</sub> receptor antagonist in the treatment of this disease.

It would be useful to know the origin of GABA-containing neurones that express D<sub>2</sub> and/or M<sub>3</sub> receptors and project to the STN. Both of these types of receptor are expressed by STN neurones (Camps *et al.* 1989; Levey *et al.* 1994), but these neurones do not contain GABA. Globus pallidus (externa), which provides the vast majority of GABA input to the STN, reportedly expresses little or no message for the dopamine D<sub>2</sub> receptor (Hurd *et al.* 1994; Ciliax *et al.* 1997), and expression of mRNA for the M<sub>3</sub> receptor is also quite weak (Weiner *et al.* 1990; Levey *et al.* 1994). Because the STN does not receive appreciable GABA-containing inputs from other sources, the globus pallidus is still the most likely source of this input.

In summary, our data clearly show that dopamine and muscarine both alter synaptic transmission in the STN. We suggest that dopamine and cholinergic inputs may play important roles in regulating the output from the STN.

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#### Acknowledgements

This work was supported by USPHS grant NS38715.

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