D5 (Not D1) Dopamine Receptors Potentiate Burst-Firing in Neurons of the Subthalamic Nucleus by Modulating an L-Type Calcium Conductance

Jérôme Baufreton,[1] Maurice Garret,[1] Alicia Rivera,[3] Adélaïda de la Calle,[3] François Gonon,[2] Bernard Dufy,[1] Bernard Bioulac,[1] and Anne Taupignon[1]

[1]Signalisation Normale et Pathologique, Unité Mixte de Recherche 5543, and [2]Interactions Neuronales et Comportement, Unité Mixte de Recherche 5541, Université Victor Segalen, 33076 Bordeaux Cedex, France, and [3]Department of Cell Biology, Faculty of Sciences, University of Malaga, Teatinos 29071, Malaga, Spain

Dopamine is a crucial factor in basal ganglia functioning. In current models of basal ganglia, dopamine is postulated to act on striatal neurons. However, it may also act on the subthalamic nucleus (STN), a key nucleus in the basal ganglia circuit. The data presented here were obtained in brain slices using whole-cell patch clamp. They reveal that D5 dopamine receptors strengthen electrical activity in the subset of subthalamic neurons endowed with burst-firing capacity, resulting in longer discharges of spontaneous or evoked bursts.

To distinguish between D1 and D5 subtypes, the action of agonists in the D1/D5 receptor family was first investigated on rat subthalamic neurons. Single-cell reverse transcription-PCR profiling showed that burst-competent neurons only expressed D5 receptors. Accordingly, receptors localized in postsynaptic membranes within the STN were labeled by a D5-specific antibody. Second, agonists in the D1/D5 family were tested in mouse brain slices. It was found that these agonists were active in D1 receptor knock-out mice in a similar way to wild-type mice or rats. This proved that D5 rather than D1 receptors were involved. Pharmacological tools (dihydropyridines, ω-conotoxins, and calciseptine) were used to identify the target of D5 receptors as an L-type channel. This was reached via G-protein and protein kinase A. The action of dopamine on D5 receptors therefore shapes neuronal activity. It contributes to normal information processing in basal ganglia outside striatum. This finding may be useful in drug therapy for various disorders involving changes in STN activity, such as Parkinson's disease and related disorders.

Key words: **dopamine; subthalamic nucleus; Parkinson's disease; burst firing; plateau potential; D5 dopamine receptor**

Introduction

Dopamine is the predominant catecholamine neurotransmitter in the mammalian brain. It acts on five receptor subtypes (D1–D5) whose genes have been characterized. Structural studies show that D1–D5 receptor subtypes fall into two receptor families: D2, D3, and D4 are classified as D2-like, and D1–D5 are classified as D1-like (Missale et al., 1998). The specific function of many of these receptor subtypes is unknown, including in the subthalamic nucleus (STN), a key structure in basal ganglia.

The basal ganglia process information relating to motor function. Tonic dopamine release has a permissive role in movement. It gates the locomotor loop that links the cortex to the thalamus through basal ganglia. It has not been clearly established how dopamine controls the output of basal ganglia. The STN is in a pivotal position because it receives monosynaptic inputs from the cortex and directly excites the basal ganglia output nuclei.

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Correspondence should be addressed to Anne I. Taupignon, Unité Mixte de Recherche 5543, Université Victor Segalen, 146 rue Saignat, 33076 Bordeaux Cedex, France. E-mail: anne.taupignon@umr5543.u-bordeaux2.fr.

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In current models of basal ganglia, dopamine is postulated to act in the striatum, indirectly inhibiting STN firing activity by acting on D2 receptors. However, a growing number of experimental results do not fit with this postulate (Kreiss et al., 1996, 1997; Hassani and Feger, 1999; Mehta et al., 2000; Ni et al., 2001; Svenningsson and Le Moine, 2002). Furthermore, it is well established that dopaminergic terminals and varicosities are found in the STN of rats, monkeys, and humans (Brown et al., 1979; Hassani et al., 1997; Francois et al., 2000). Dopamine receptor expression in the STN is far from being established. mRNAs for various receptor subtypes have been reported, but there is no agreement on which of the five subtypes are expressed in the somatodendritic compartment (Flores et al., 1999; Augood et al., 2000; Svenningsson and Le Moine, 2002). It is clearly necessary to identify and understand the role of the dopamine receptors expressed in the STN.

The current models of basal ganglia represent output and input of the component nuclei as firing rates. However, new experimental studies imply that patterns of firing activity in the component nuclei contribute critically to information processing (Allers et al., 2000; Walters et al., 2000; Boraud et al., 2001; Magill et al., 2001; Raz et al., 2001; Levy et al., 2002). Subthalamic neurons are endowed with intrinsic properties enabling oscillatory activity. These neurons display rhythmic burst-firing *in vivo* and *in vitro* (Beurrier et al., 1999; Plenz and Kital, 1999; Awad et al.,

2000; Magill et al., 2000, 2001; Baufreton et al., 2001), the function of which is unknown.

We investigated the role of receptors in the D1 family. We showed how they control an intrinsic L-type calcium conductance necessary for subthalamic neurons to express bursts of firing *in vitro*. Using reverse transcription (RT)-PCR profiling from single rat neurons and slices from D1 receptor knock-out (KO) mice, we established that only D5 receptors connected by G-proteins to protein kinase A are involved. Our results provide the first direct demonstration that dopamine acting on D5 receptors controls the activity of the STN in a normal state.

Materials and Methods

Slice preparation. Experiments were performed on subthalamic neurons in 400-μm-thick coronal slices obtained from 18- to 22-d-old Wistar rats. After cervical dislocation, the rats were quickly decapitated. Their brains were rapidly removed, and a block of cerebral matter containing the STN was isolated in an ice-cold solution containing (in mM): 250 sucrose, 26 $NaHCO_3$, 7 $MgCl_2$, 2 KCl, 1.15 NaH_2PO_4, 0.5 $CaCl_2$, and 11 glucose, bubbled with 95% O_2 and 5% CO_2, pH 7.4. Three coronal slices containing the STN were prepared from one brain in the same solution using a vibroslicer (Campden Instruments, Loughborrough, UK). They were incubated at room temperature in a Krebs' solution containing (in mM): 124 NaCl, 26 $NaHCO_3$, 3.6 KCl, 1.3 $MgCl_2$, 2.4 $CaCl_2$, 1.25 HEPES, and 10 glucose, pH 7.4, bubbled with 95% O_2 and 5% CO_2, for a 2 hr recovery period.

Electrophysiological recordings. One slice was transferred to an immersion-type recording chamber (Guerineau et al., 1997) and superfused continuously (3.5 ml/min) with the oxygenated Krebs' solution. The slice was examined under a dissecting microscope; the STN was readily identified as ovoid gray matter immediately dorsal to the cerebral peduncle. Recordings were made using the blind patch-clamp technique in the whole-cell configuration and in current-clamp mode. Pipettes were filled with a solution containing (in mM): 140 K-gluconate, 11 EGTA, 10 HEPES, 1 $CaCl_2$, 2 ATP-Mg, and 0.4 Na-GTP. For some experiments, pipettes were filled with 120 K-gluconate, 10 BAPTA, 10 HEPES, 0.3 $CaCl_2$, 2 Mg-ATP, and 0.4 Na-GTP. In both intrapipette solutions, free calcium was 16 nM, as calculated with Chelator software (Stanford, CA). The osmolarity of the intrapipette solutions for whole-cell recordings was between 280 and 300 mOsm, pH adjusted to 7.25. Electrodes were pulled from borosilicate thin-glass capillaries (GC150F-15, Harvard Apparatus, Edenbridge, UK) on a vertical puller (PP-830, Narishige, Tokyo, Japan) and had a resistance of 10–12 MΩ. Signals were recorded using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA) with the amplifier filter set at 5 kHz. The signals were stored on a video tape or digitized at 2.5–20 kHz using a Digidata 1200B. Access resistance (~20 MΩ) was monitored regularly. Junction potentials were measured according to the method described by Neher (1992), and voltage errors were corrected off-line.

APV (40 μM), CNQX (10 μM), and bicuculline (10 μM) were perfused continuously to block rapid synaptic transmission. Care was taken to avoid possible priming effects (Lidow et al., 2001). All of our records came from naive neurons. Once a slice had been perfused with any of the dopaminergic agonists listed below, the slice was discarded, and experiments were performed on another slice. SKF 81297, SKF 82958, and SKF 38393 were used. The two first drugs were used the most often, and the third was used only on a few occasions. We found no qualitative difference in the action of the three drugs. Possible quantitative differences between the three drugs were not investigated. Unless stated otherwise, the term "D1 agonists" refers to these three drugs.

Drugs. Tetraethylammonium chloride (TEA), barium chloride, choline chloride, BAPTA, nifedipine, BayK 8644, APV, GDP-β-S, and GTP-γ-S were purchased from Sigma (Saint Quentin Fallavier, France); CNQX, SKF 81297, SKF 82958, and SCH 23390 were purchased from RBI (Saint Quentin Fallavier, France). Bicuculline and SKF 38393 were obtained from Tocris (Bristol, UK), and TTX was purchased from Latoxan (Valence, FR). Calciseptine and ω-conotoxins GVIA and MVIIC were obtained from Alomone Labs (Jerusalem, Israel). All were prepared as stock solutions and stored at −80°C. When drugs were prepared in DMSO, the final dilution of the solvent was always kept below 0.007. Drugs diluted in the oxygenated Krebs' solution were delivered by means of a multibarrel gravity-feed system (HSSE-2, ALA Scientific Instruments, Sega Electronique, Paris, France) composed of two capillaries positioned just above the patch pipette, allowing up to seven solutions to be tested successively.

Data analysis. Recordings were analyzed using pClamp 6.01 software (Axon Instruments), Origin 5.0 (Microcal, Northampton, MA), and Instat (GraphPad Software, San Diego, CA). The duration and surface of plateau potentials were measured from the end of the electrotonic response. Plateau potentials differed from one neuron to another in rats as well as mice. Each neuron served as both control and test. Plateau potentials were always recorded first in control and then in the presence of a D1 agonist. Percentage changes in duration, number of action potentials, or surface of plateau potentials were calculated. They were compared using the Wilcoxon matched-pairs signed ranks test and the Mann–Whitney *U* test for paired and unpaired values, respectively. In multiple comparisons of plateau potential, the Kruskal–Wallis nonparametric test was used to estimate overall significance. This was followed by two-by-two comparisons using the Wilcoxon matched-pairs signed ranks test. Values of $p < 0.05$ were considered as significant. Box plots were used for graphic presentation of the data because of the small sample sizes. The box plot presents the distribution with the median as a central line. The hinges and edges of the box display the 25th and 75th percentiles, whereas the "whiskers" display the 5th and 95th percentiles. The square represents the mean. In the results, values are given as mean ± SE when means are close to medians. Otherwise, median values are given

Single-cell RT-PCR procedures. In some experiments, the cellular content was harvested for reverse transcription after the neurons were held in a whole-cell configuration for 5–20 min. Reverse transcription and PCR were performed using protocols similar to those described by Maurice et al. (2001). The primers were taken from GenBank rat receptor D1 (accession number M35077) and D5 (NM012768) sequences. Sense primers were D1for1: AAGCAGCCTTCATCCTGATTAGC; D1for2: GCATGGACTCTGTCTGTCCTTATA; D5for1: CCATCCTCATCTCCTTCATCCCG; and D5for2: ACTCAATTGGCACAGAGACAAGG. Antisense primers were D1rev2: ACAGAAGGGCACCATACAGTTCG; D1rev3: GGAGCCAGCAGCACACAAACACC; D5rev1: CAGGATGAAGAAAGGCAACCAGC; and D5rev3: TGCAGAAAGGAACCATACAGTTC. A two-stage amplification strategy was designed to detect low-abundance dopamine receptor mRNAs. In the first step, 10 μl of a single-cell template cDNA was amplified in a 50 μl PCR reaction mixture using 0.25 μM of D1 and D5 for1 and rev2 primers. Twenty cycles were performed with 45 sec at 94°C, 45 sec at 60°C, and 60 sec at 72°C. Then, a 1 μl aliquot of this PCR product was used as a template for a second round of PCR amplification, with each pair of specific nested primers (for2 and rev3). PCR amplification was performed as described above with 35 cycles. PCR products were sequenced and separated on a 2% agarose gel stained with ethidium bromide. Control experiments were run with water instead of cDNA and with cytoplasm samples processed as described above, except that reverse transcriptase was omitted. No amplification products were found in control experiments (data not shown).

Immunohistochemistry. Five 21-d-old Wistar rats (Charles River, L'Arbresle, France) were perfused transcardially with 4% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.4. Their brains were then removed and postfixed in the same fixative for 2 hr at 4°C, cryoprotected with a solution of phosphate-buffered sucrose (30%), and frozen in dry ice. Coronal sections (30 μm thick) were cut with a freezing microtome (CM 1325; Leica, Wetzlar, Germany) and processed for immunochemistry. The antibody used here was raised against a sequence specific to cloned rat D5 receptors. Its specificity was established as described by Khan et al. (2000). Incubation in anti-D5 was performed for 72 hr at 4°C, followed by incubation in biotinylated goat anti-rabbit IgG (1:500; Vector) and peroxidase-conjugated streptavidin (1:2000; Sigma). Reaction was developed with 0.05% DAB, 0.08% nickel ammonium sulfate, and 0.02% H_2O_2. For electron microscopy analysis, animals were perfused with 4% paraformaldehyde and 0.1% glutaraldehyde. Immediately after fixation, coronal sections (40 μm thick) were cut with a Vibratome (VT 1000M;

Leica) and processed for immunochemistry as described above. After DAB reaction, sections were osmicated, dehydrated, and flat embedded in Durpacan resin (Sigma). The resin-embedded sections were cut into ultrathin sections on an ultra-microtome (Reichert) and observed with a Philips CM100 electron microscope.

Transgenic mice. Mice bearing a null mutation for D1 receptor (D1 −/− mice) have been generated by Drago et al. (1994). The D1 −/− mutant mice used here were produced by crossing heterozygous males and females. D1 +/+ mice, referred to as wild-type, were from the same litter as D1 −/− mice. The genotype of all mice was assessed by PCR. Slices from D19–D21 mice were prepared and used in the same way as slices from rats.

Results

Postsynaptic D1 receptors potentiate spontaneous and evoked burst-firing

We used SKF 82958 or SKF 81297 (3–5 μM) to activate receptors in the D1 family. All experiments were performed in the presence of blockers of fast synaptic transmission (APV, 40 μM; CNQX, 10 μM; bicuculline, 10 μM) to focus our study on postsynaptic effects alone, without interference from possible presynaptic modulation of afferent terminals. Whatever the agonist, the most striking effect of D1 receptor activation was potentiation of burst-firing. This was clearly observed in spontaneously burst-firing neurons. Approximately one subthalamic neuron in 10 displays spontaneous burst-firing in brain slices, whether in cell-attached or whole-cell patch-clamp mode (Beurrier et al., 1999; Baufreton et al., 2001). D1 agonists were active on neurons in the whole-cell configuration (Fig. 1*A*) as well as on intact neurons in the cell-attached configuration (Fig. 1*B*). They potentiated burst-firing by increasing the burst duration by 70% (Fig. 1*C*). Mean burst duration was 2.1 ± 0.4 sec in control. This value was significantly lower than in the presence of a D1 agonist (3.9 ± 1.0 sec; $n = 8$). A significant increase in the number of action potentials fired per burst (+106%; 106 ± 38 vs 48 ± 17 in control; $n = 8$) was also found in the same neuron sample. This was caused by the increased burst duration, because the mean firing frequency in the bursts did not change significantly (24 ± 4 vs 22 ± 4 Hz in control; $n = 8$). Conversely, a significant decrease in burst frequency (−36%; 0.09 ± 0.02 vs 0.15 ± 0.03 Hz; $n = 8$) was measured. Overall, the mean firing frequency was barely changed (control: 5.3 ± 1.2; D1 agonist: 5.7 ± 1.8 Hz; $n = 8$). Afterhyperpolarization was often more pronounced, but there was no other effect on cell properties, including input resistance, spike threshold, amplitude, or width ($n = 15$; data not shown).

Although burst-firing is displayed spontaneously by only a small fraction of subthalamic neurons, a larger proportion of subthalamic neurons are nevertheless burst-competent. *In vitro*, persistent burst-firing can be induced by activating group I metabotropic glutamate receptors (Awad et al., 2000). This can also be induced in approximately one neuron in two by continually injecting a small hyperpolarizing current (Fig. 2*A*). Burst-competent neurons give specific responses, called plateau potentials, to short current pulses given at hyperpolarized levels (Nakanishi et al., 1987; Beurrier et al., 1999; Bevan et al., 2000, 2002; Baufreton et al., 2001; Otsuka et al., 2001). These responses always outlast the stimuli and resemble evoked bursts. The term "plateau potential" refers to the long-lasting regenerative depolarization, which maintains the membrane in the voltage range that allows firing of action potentials at high frequency. Plateau potentials were significantly lengthened by D1 agonists, in the same way as bursts. The median increase was +30% for responses evoked by depolarizing (Fig. 2*B*) or hyperpolarizing pulses (Fig. 2*C*). This resulted in a marked increase in the number of spikes

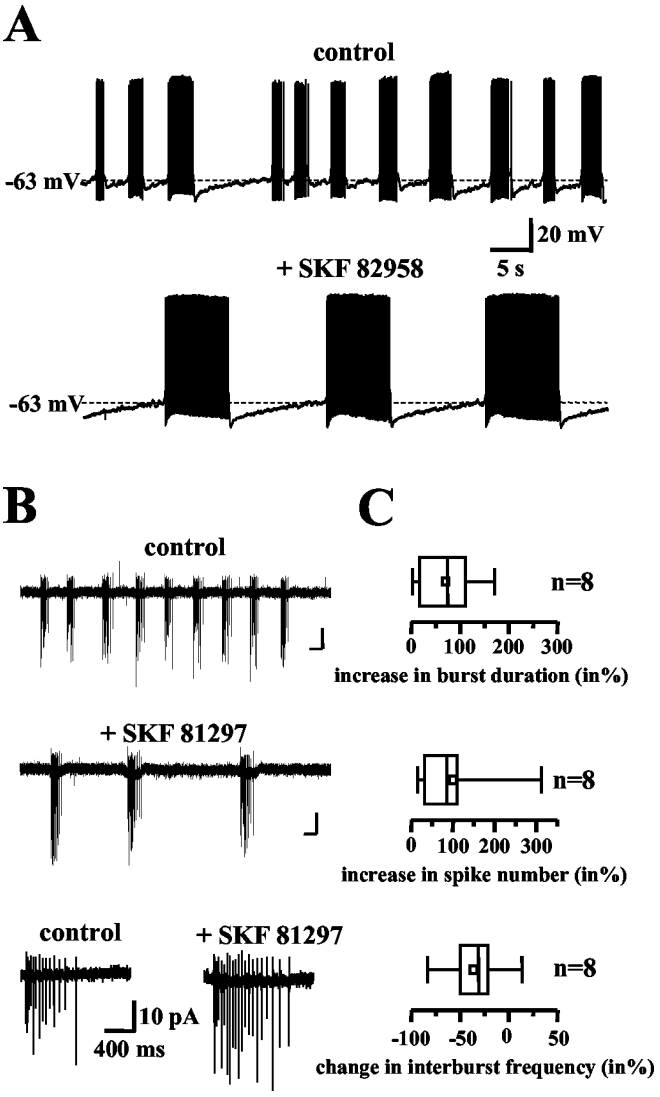

Figure 1. Activation of receptors in the D1 family strengthens spontaneous burst-firing. *A*, Representative examples of spontaneous burst-firing in a subthalamic neuron. The recording was made in the presence of synaptic transmission blockers (APV, 40 μM; CNQX, 10 μM; bicuculline, 10 μM) for this experiment as with all others, and at zero current level. Burst duration was notably increased during perfusion of SKF 82958 (5 μM). *B*, *Top*, The duration of spontaneous bursts recorded in the cell-attached configuration is made longer by SKF 81297 (5 μM). Calibration: 10 pA, 3 sec. *Bottom*, Representative bursts on an expanded time scale. *C*, A *box plot* summary of the changes in typical burst features with SKF 82958 or SKF 81297 (5 μM). The *central line* in the *box* shows the distribution median. The *edges* of the *box* are the interquartiles. The *lines* running from the *edge* of the *box* show the distribution extremes. The *square* displays the mean. *n*, Number of experiments.

(median increase: +50%). Coapplication of an antagonist of D1-like receptors, SCH 23390 (10 μM), reversed these changes, indicating that receptors in the D1 family were specifically activated. Because all of these results were obtained with inhibitors of glutamatergic and GABAergic receptors (Figs. 1, 2) and by direct intracellular stimulation (Fig. 2), it can be concluded that presynaptic D1 receptors were not involved.

Similar results were obtained in the vast majority of burst-competent neurons tested (48 of 53). The other five neurons did not respond to D1-like receptor activation. D1 agonists either were inactive on neurons that only displayed tonic and regular discharge of single spikes ($n = 2$) or induced an increase in firing frequency ($n = 6$) (data not shown). In no case did any of the D1

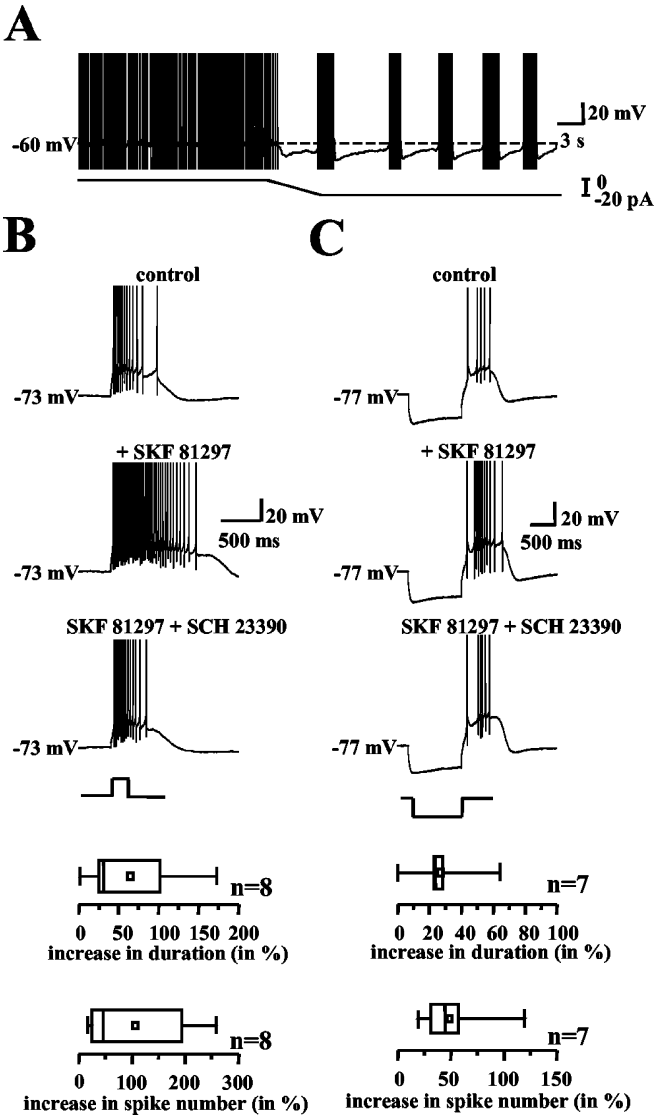

Figure 2. Action of agonists of receptors in the D1 family on burst-competent neurons. *A*, Burst-competent neurons switch from regular, single-spike firing mode at zero current level to burst-firing with negative current injection. *B*, *C*, Depolarizing or hyperpolarizing stimuli trigger plateau potentials. The two regenerative discharges markedly outlast the stimulus. They are potentiated by SKF 81297 (3 μM). Potentiation is reversed by the D1-like receptor-specific antagonist, SCH 23390 (10 μM).

agonists change regular, single-spike firing into irregular or burst-firing.

Plateau potentials are lengthened by D1 agonists

Various mechanisms underlie plateau potentials, depending on the neuronal type (Fraser and MacVicar, 1996; Mattia et al., 1997; Morisset and Nagy, 1999; Brumberg et al., 2000; Alaburda et al., 2002). In the STN, plateau potentials do not rely on fast sodium channels because the underlying long-lasting regenerative depolarizations are TTX resistant (Nakanishi et al., 1987; Beurrier et al., 1999; Otsuka et al., 2001). By contrast, they are facilitated by the replacement of external Ca^{2+} by Ba^{2+}. Indeed, plateau potentials have been shown to involve two types of Ca^{2+}-activated channels: Ca^{2+}-activated K^+ (K_{Ca}) channels and Ca^{2+}-activated nonspecific channels in addition to Ca^{2+} channels (Beurrier et al., 1999; Otsuka et al., 2001). In the presence of TTX (1 μM), and with Ba^{2+} as the divalent charge carrier instead of Ca^{2+}, a short depolarizing pulse induced a large plateau potential (Fig. 3*A*, *inset*). Under these conditions, D1 agonists (SKF 82958, SKF 81297, 3–5 μM) potentiated plateau potentials by increasing their duration, as exemplified by the action of SKF 82958 (Fig. 3*A*). SKF 81297 at 1 μM was active in the same way (data not shown). The values of depolarization sustained in control and in the presence of the D1 agonists were not significantly different. There was no change in resting potential. Full action of D1 agonists, reached in ~3 min, resulted in a dramatic increase of the plateau surface. On average, the surface of plateau potentials increased by 150%. This increase specifically involved receptors in the D1 family because it was fully blocked in all neurons by coapplication of the D1 antagonist, SCH 23390, whereas coapplication of raclopride, a D2-selective antagonist, had no effect (Fig. 3*C*).

Ba^{2+} cannot replace Ca^{2+} for activating calcium-activated channels, whereas it has a greater current-carrying ability than Ca^{2+} itself through Ca^{2+} channels (Tsien et al., 1988; Gola and Crest, 1993; Marrion and Tavalin, 1998). Our data therefore suggest that the primary target of D1 receptors was not calcium-activated channels but Ca^{2+} channels themselves. To further support this view, we assayed the changes in plateau potential induced by activating D1 receptors while we blocked Ca^{2+}-activated channels in two ways. First, we inhibited ionic flux through the channels by using the impermeant cation TEA (20 mM) to block K_{Ca} channels or by replacing Na^+, the main charge carrier trough Ca^{2+}-activated nonspecific channels, by impermeant choline ions. Second, we buffered intracellular Ca^{2+} by chelating intracellular Ca^{2+} ions by using a pipette medium containing 10 mM BAPTA. As described previously (Beurrier et al., 1999; Otsuka et al., 2001), the above procedures affected plateau potentials, as expected from procedures interfering with conductances involved in plateau potentials. However, D1 agonists still increased plateau potential surface under any of these three conditions (Fig. 4*A*,*B*). These results strengthened the conclusion we drew from our experiments with Ba^{2+} instead of Ca^{2+}, i.e., that the primary target of D1 receptors were Ca^{2+} channels.

D1 receptors target on L-type calcium channels

Subthalamic neurons have been shown to express the same wide repertoire of Ca^{2+} channels as many other neuronal preparations (Beurrier et al., 1999; Song et al., 2000). We sought to distinguish which Ca^{2+} channel subtype was involved in plateau potentiation by D1 receptors. Given the time properties of subthalamic plateau potentials, T channels were unlikely to play a role because one of their primary properties is their fast steady-state inactivation. Accordingly, Ni^{2+} (40 μM), which inhibits T channels, was found to have no effect on plateaus (data not shown). By contrast, slowly or noninactivating Ca^{2+} channels such as L, N, P, or Q channels were plausible candidates for generating and maintaining plateau potentials.

To test this assumption, we first recorded plateau potentials while perfusing nifedipine, a dihydropyridine that specifically binds to and inhibits voltage-dependent L-type channels. These experiments were made in the presence of TTX (1 μM) and TEA (20 mM). It must be remembered that D1 agonists augmented plateau potential surface by 19% (median value) in this condition, as shown in Figure 4*B*. In all cases, nifedipine (3 μM) strongly reduced plateau potentials (Fig. 5*A*). Activating D1 receptors by SKF 81297 did not reverse the inhibitory action of nifedipine. In addition, when D1 receptors were first activated by SKF 81297, the plateau potential increase was reversed by perfusing nifedipine (Fig. 5*B*). Plateau potentials were then potently inhibited by 6 μM nifedipine, so that on average, little regenera-

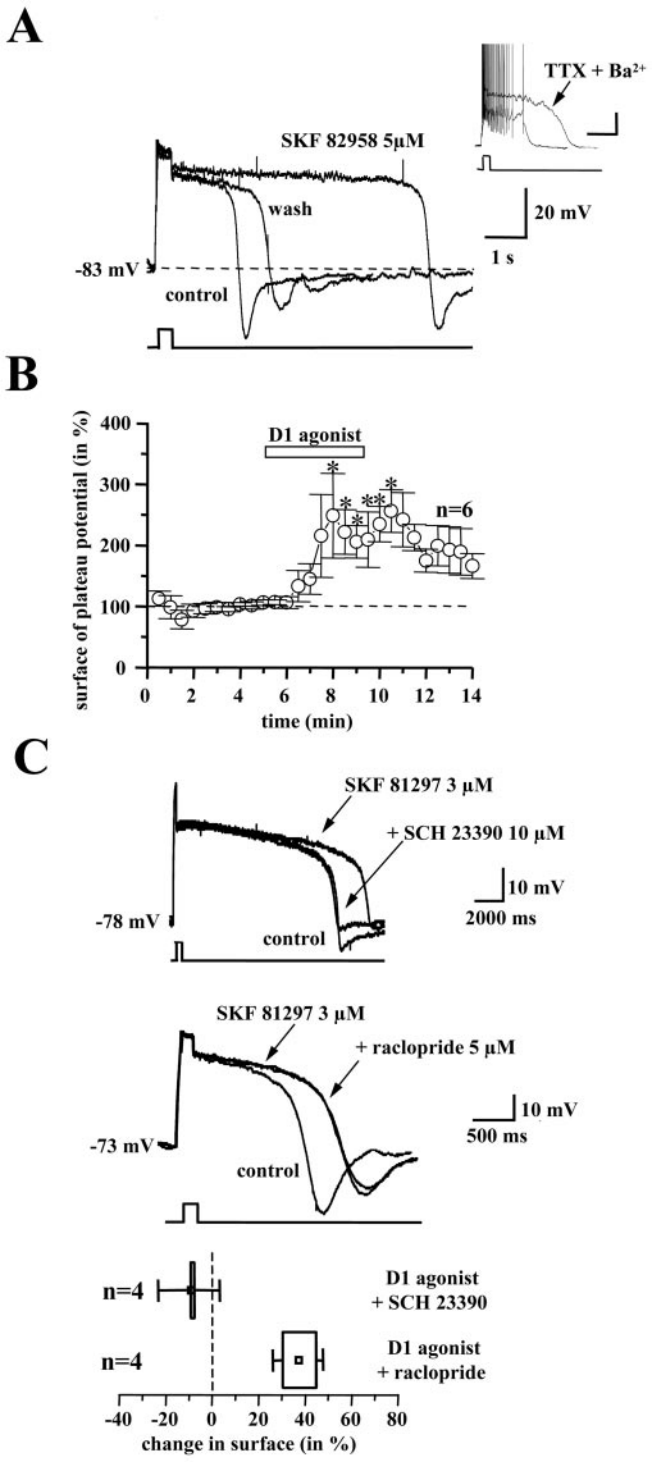

Figure 3. Agonists of receptors in the D1 family increase the duration of the regenerative depolarization. *A*, Superimposed records of plateau potentials in the presence of TTX (1 μM) and with Ba^{2+} replacing Ca^{2+}. Washing off of SKF 82958 partially restored the surface of the plateau. The *inset* shows a plateau potential recorded in normal Ringer's solution, in addition to that recorded during bath perfusion of TTX, with Ba^{2+} instead of Ca^{2+}. *B*, Time course of the action of D1-like agonists (SKF 81297 and 82958; 3–5 μM). The *bar* shows the addition of agonists to bath perfusion. *Significantly different from pretest values. *C*, The D1 receptor antagonist, SCH 23390, reversed the action of SKF 81297, whereas the selective D2 receptor antagonist, raclopride, had no effect, as illustrated by representative traces of plateau potentials. *Box plots* of the changes in the plateau potential surface induced by D1-like agonists (3–5 μM) together with SCH 23390 (10 μM) or raclopride (5 μM) substantiate this finding.

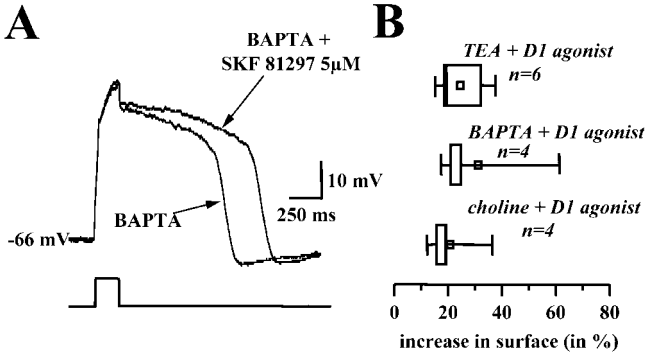

Figure 4. Calcium-activated channels are not involved in potentiation of plateau potential. *A*, Activation of receptors in the D1 family still increases plateau potential when free cytosolic Ca^{2+} is heavily buffered by BAPTA (10 μM) in the intrapipette solution. TEA (20 mM) and TTX (1 μM) were added to the recording solution. *B*, *Box plots* present the changes in plateau potential surface induced by D1 agonists (3–5 μM) in three experimental conditions designed to inhibit Ca^{2+}-activated channels: *top*, with 20 mM TEA in the perfusion solution, to block calcium-activated potassium current; *middle*, with 10 mM BAPTA in the intrapipette medium; *bottom*, with choline replacing Na^{+}, to block Ca^{2+}-activated nonspecific channels, in addition to TEA (20 mM).

tive depolarization outlasted the electrotonic response to the current pulse. Larger plateau potentials could not be evoked, even if the amplitude or duration of stimuli increased. Nifedipine-sensitive Ca^{2+} channels are also sensitive to other dihydropyridines, such as BayK 8644, which stabilize them in an open state. BayK 8644 (5 μM) strongly potentiated plateau potentials, the median surface of which increased by 60% (Fig. 5). Moreover, SKF 81297 failed to increase plateau potential surface in the presence of BayK 8644, as illustrated in Figure 5*C*.

Second, we used two toxins, ω-conotoxin GVIA and ω-conotoxin MCIIV, which bind with high affinity to N (Cav2.2) and P/Q (Cav2.1) channels, respectively, to assess a possible complementary involvement of these two channel subtypes in potentiation of plateau potential. By contrast to nifedipine, ω-conotoxin GVIA (500 nM) and ω-conotoxin MCIIV (250 nM) did not inhibit plateau potentials to a large extent (Fig. 5*D*). The coapplication of the two toxins reduced their median surface by only 45%, indicating that although N and P/Q channels contributed to plateau potentials in addition to L channels, their involvement was not decisive. Accordingly, in the presence of the two ω-conotoxins, activation of D1 receptors was still effective. On average, SKF 81297 induced a significant increase (45%) in plateau potential surface in the presence of the two toxins. This value was not significantly different from that obtained without blocking the N and P channels ($p > 0.05$; median value: +22% with the two ω-conotoxins vs +19% in control).

We finally explored the action of calciseptine, a novel L-type Ca^{2+} channel blocker that specifically binds to L-type Ca^{2+} channels with high specificity (De Weille et al., 1991; Hernandez-Lopez et al., 1997). As with nifedipine, calciseptine (200 nM) not only reversed the action of SKF 81297, but also powerfully inhibited plateau potentials (Fig. 5*D*). In a similar way to nifedipine, calciseptine retained little of the depolarization outlasting the stimulus.

In summary, activating D1 receptors resulted in marked changes in plateau potential surface under all conditions, except with the three drugs that specifically bond voltage-dependent L-type Ca^{2+} channels, i.e., with nifedipine, calciseptine, and BayK 8644. Potentiation of plateau potentials therefore crucially relies on L-type Ca^{2+} channels. BayK 8644 can be seen as defining

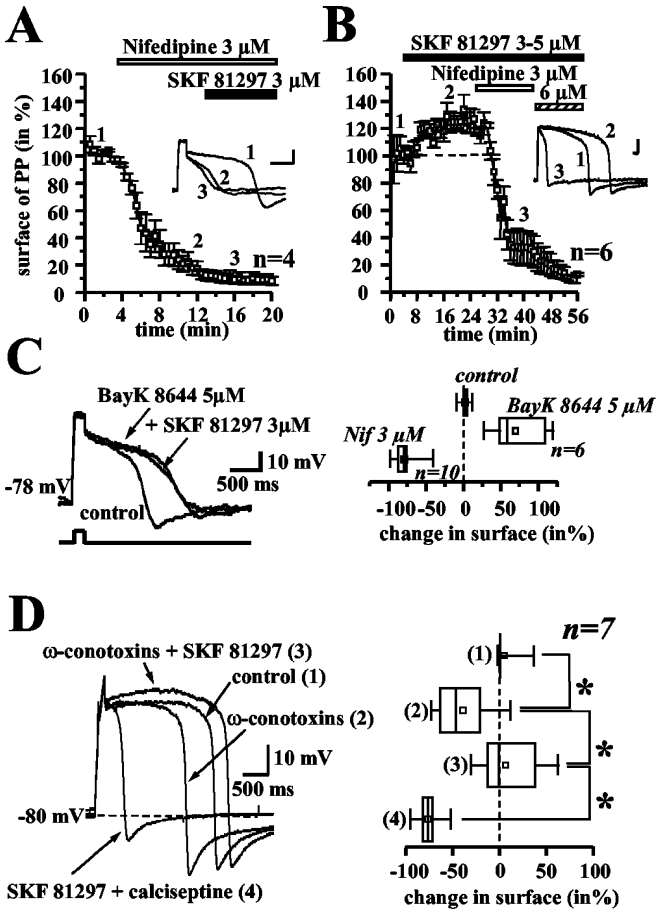

Figure 5. Potentiation of plateau potential involves L-type calcium channels. *A*, Time course of the action of the dihydropyridine, L-type channel antagonist, nifedipine. Nifedipine prevented the action of SKF 81297. The *inset* displays superimposed sample traces taken from positions *1-3*. Calibration: 10 mV, 500 msec. TEA (20 mM) and TTX (1 μM) were present. *B*, The increase in the surface of plateau potential by SKF 81297 (3–5 μM) is reversed by perfusion of nifedipine. Note that nifedipine (6 μM) abolishes the plateau potential. *Inset*, *Superimposed traces* taken from positions *1-3*. Calibration: 10 mV, 500 msec. *C*, The dihydropyridine, L-type channel agonist, BayK 8644, occluded the effect of SKF 81297, as illustrated by representative *traces* of plateau potentials obtained successively in control, during perfusion of BayK 8644 alone, and during perfusion of BayK 8644 with SKF 81297. *Box plots* summarize the changes in the surface of plateau potential induced by BayK 8644 and by nifedipine (data from *A* and *B*). *D*, Coapplication of N- and P/Q-type calcium channel blockers, ω-conotoxin MVIIC (250 nM) and ω-conotoxin GVIA (500 nM), reduced plateau potential. However, addition of 3 μM SKF 81297 in presence of both toxins significantly increased plateau potential (*trace 3*), indicating that N- and P/Q-type Ca^{2+} channels were not involved. Perfusion of calciseptine (200 nM), a specific L-type channel antagonist, occluded the action of SKF 81297 and abolished the plateau potential (*trace 4*). *Box charts* summarize the results from several tests (*significant at $p < 0.05$).

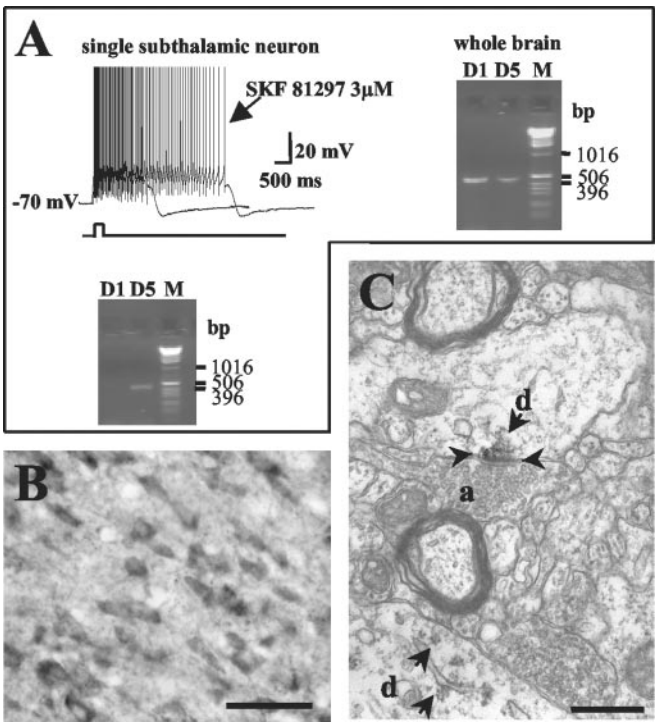

Figure 6. Expression of D5 receptor in subthalamic neurons. *A*, *Left*, Sample records of plateau potentials and PCR amplicons from an SKF 81297-sensitive, burst-competent neuron. *Right*, RT-PCR products obtained from whole-brain RNA extract. D5 receptor mRNA was only detected by single cell-RT PCR amplification, whereas both D1 and D5 receptor mRNAs were detected from whole-brain tissue. Ethidium bromide-stained products of RT-PCR amplifications were resolved by electrophoresis. The positions of standard nucleotide bands of molecular weight markers (*M*) are indicated to the *right* of the gels. *B*, Dopamine D5 receptor immunoperoxidase staining of a coronal section of rat brain shows immunoreactivity in many neuronal cell bodies within the STN. *C*, Electron micrograph revealing labeling of D5 receptors (*top arrow*) in a dendrite (*d*) making asymmetric synapse (*arrowheads*) with an axon (*a*). Labeling (*bottom arrows*) is also associated with microtubules and endoplasmic reticulum. Scale bars: *B*, 200 μm; *C*, 500 nm.

the upper limit of the possible increase in plateau potential by D1 receptors, whereas nifedipine and calciceptine demonstrate that no plateau potential can be produced if L-type Ca^{2+} channels are blocked. Taken together, our results indicate that L-type Ca^{2+} channels are (1) necessary for plateau potential generation, (2) sufficient for its maintenance, and (3) the primary target of D1 receptors.

D5 (not D1) receptors are involved in burst potentiation

D5 receptors have a high homology with D1 receptors. There are currently no drugs to discriminate between D1-class receptors. The response described above could thus be mediated by either of the D1 and D5 receptor subtypes. To determine which of them was responsible for the potentiation of burst-firing, whole-cell recordings were followed by single-cell reverse transcription (scRT)-PCR analysis. D1 and D5 receptor mRNAs were probed by scRT-PCR from subthalamic neurons showing a robust potentiation of plateau potential when challenged with SKF 81297. Figure 6*A* (*left*) shows the response to SKF 81297 and the scRT-PCR amplicons from a subthalamic neuron. When RT-PCR analysis was limited to mRNAs of a single subthalamic neuron, only the D5 isoform was detected. This was true for all of the nine neurons that were tested. However, mRNAs of the two isoforms were detected from whole-brain tissue (Fig. 6*A*, *right panel*).

We then looked for the D5 protein in the STN from rats of the same strain and age as those used for our patch-clamp and scRT-PCR work. We used an antibody raised against a peptide sequence of cloned D5 receptor, the specificity of which was established by Khan et al. (2000). Immunoreactivity was detected in the cell bodies (Fig. 6*B*) using light microscopy. Using electron microscopy (Fig. 6*C*), immunoreactivity was located mainly in postsynaptic structures. Labeling was generally found in dendrites and was associated with endoplasmic reticulum and microtubules. D5 immunolabeling in the inner layer of the plasma membrane was generally associated with asymmetric synapses.

Both sets of data suggested that potentiation of burst-firing was mediated by D5 rather than D1 receptors. To test this possibility directly, we examined whether potentiation of plateau potential was still produced by D1-class agonists in D1 receptor KO

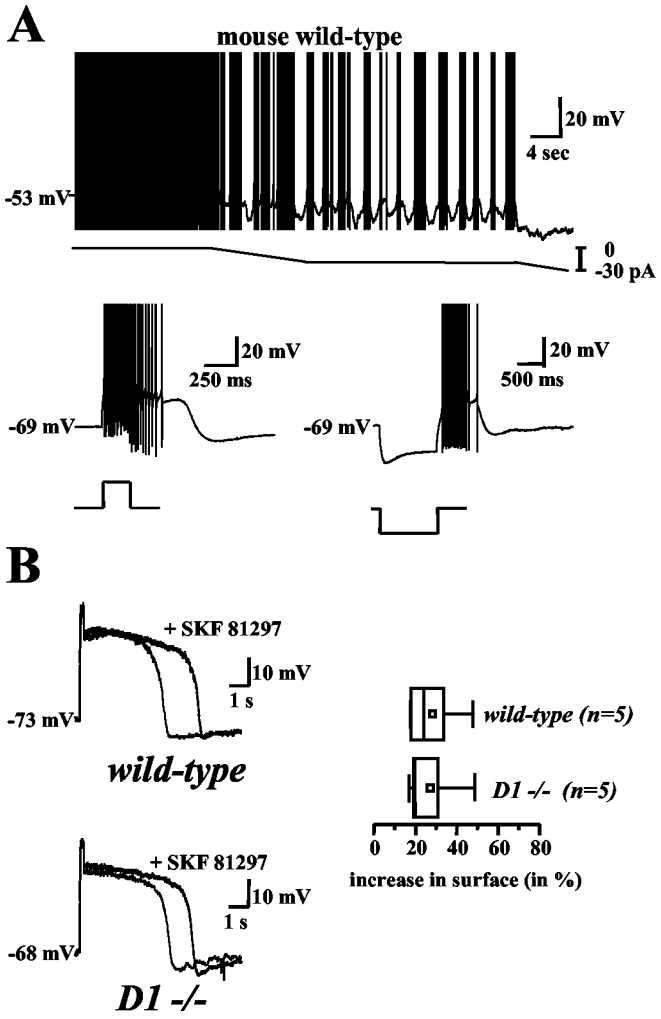

Figure 7. Potentiation of plateau potential is caused by D5, not D1, receptors. *A*, Mouse subthalamic burst-competent neurons display firing properties similar to that of rat neurons. Burst-firing is induced by injecting a negative current. Depolarizing (+80 pA; 200 msec) and hyperpolarizing (−80 pA, 1 sec) stimuli produce long-lasting plateau potential and postinhibitory rebound burst, respectively. *B*, Sample records of plateau potentials from mouse neurons in the presence of TTX (1 μM) and TEA (20 mM). Wild-type and D1 receptor null mutant (D1 −/−) mice displayed strong plateau potentials when stimulated by short current pulses (+80 pA, 200 msec). The agonist of receptors in the D1 family, SKF 81297 (3–5 μM), was active in neurons from the D1 −/− mice as well as in neurons from wild-type mice. The increase in surface of the plateau potential measured in D1 −/− mice was not significantly different ($p = 0.99$; Mann–Whitney *U* test) from that measured in their wild-type counterpart, as summarized by the *box plots*.

mice. First, we checked that subthalamic neurons in slices from wild-type mice had the same specific properties as neurons from rats, i.e., that they showed regular, single-spike, and burst-firing patterns, because there are no data on electrical activity of subthalamic neurons in slices from mice. As shown in Figure 7*A*, we found that most neurons from mice fired single, regular spikes (five of nine). Of these, 11% (one of nine) were able to switch to sustained burst-firing when hyperpolarized by a few millivolts, as described for rat neurons, whereas 62% (13 of 21) displayed plateau potentials. Cell and firing properties of neurons from wild-type mice are summarized in Table 1. Neurons from D1 receptor null mice had essentially the same properties (Table 1). Furthermore, activation of D1 receptors by SKF 81297 (3 μM) in slices from D1 −/− mice did potentiate plateau potentials (Fig. 7B). The median surface of plateau potential was increased by 20% in the D1 KO mice, whereas this increased by 25% in the wild-type mice, the difference being nonsignificant (Mann–Whitney; $n = 5$). This result establishes that potentiation of plateau potential does not rely on D1 receptors but does rely on D5 receptors.

Table 1. Cell and firing properties of mice subthalamic neurons

	C_m (pF)	R_{in} (MΩ)	Burst-competent neurons
Wild type	9.9 ± 0.4 ($n = 15$)	301.2 ± 46 ($n = 15$)	62% (13 of 21)
D1 −/−	10 ± 0.7 ($n = 16$)	283 ± 32 ($n = 16$)	68% (15 of 22)

Basic cell properties (C_m, R_{in}) and proportions of burst-competent neurons in slices from wild-type and D1 receptor null mice (D1 −/−) are not significantly different. C_m, Cell capacity; R_{in}, input resistance.

Protein kinase A as a transduction pathway for D1-like, presumably D5, receptors in the STN

Very little is known regarding the transduction pathways specifically activated by D5 receptors, except those explored in recombinant systems. In such systems, activation of D5 receptors often leads to elevation of cAMP through a cascade initiated by several G-proteins (Sidhu, 1998; Wang et al., 2001). If the potentiation of plateau potentials by D1-like agonists is mediated by the same cascade, analogs of GTP should mimic the receptor-driven potentiation, whereas inhibitors of protein kinase A (PKA), the major cellular target of cAMP, should prevent receptor-driven potentiation. To test this hypothesis, GTP-γ-S was included in the intrapipette solution. As shown in Figure 8*A*, GTP-γ-S (100 μM) always potentiated plateau potentials ($n = 3$). In keeping with this result, GDP-β-S (100 μM) reduced the plateau potentials. The membrane-permeant PKA inhibitor H-89 and the cAMP analog 8-bromo-cAMP were used to determine whether the cellular effects of the D1 agonists were mediated by PKA. Application of H-89 (1–3 μM) per se significantly reduced the surface of plateau potentials. H-89 prevented the response to SKF 81297 (3 μM) (Fig. 8*B*), because there was no significant difference in plateau potential surface with or without SKF 81297. Median values of changes were −26% in both instances. By contrast, application of 8-bromo-cAMP alone increased the plateau potential surface by 40%.

Discussion

Our results show that subthalamic burst-competent neurons have functional postsynaptic receptors in the D1 family. They establish that these receptors control a Ca^{2+} conductance that is necessary for neurons to express burst-firing. Because D5 receptors are expressed in the STN and the Ca^{2+} conductance is controlled in a similar way in D1 receptor null mice, our data provide the first evidence supporting the possibility that dopamine acting on D5 receptors contributes to shaping firing pattern.

Functional D5 receptors in subthalamic neurons

The continued lack of subtype-selective ligands limits our understanding of the respective role of D1 and D5 receptors. There are many discrepancies among autoradiographic studies on the distribution of D1-like receptors in the basal ganglia outside striatum. mRNA encoding D5 receptor has been probed in only one *in situ* hybridization study. This found a high level of D5 receptor mRNA in the STN, whereas mRNA levels for the other dopamine receptors, including D1 receptor, were underneath detection level (Svenningsson and Le Moine, 2002). Accordingly, several *in situ* hybridization studies have failed to detect D1 receptor mRNA in rat or human STN (Boyson et al., 1986; Fremeau et al., 1991; Augood et al., 2000). These results agree with our single-cell

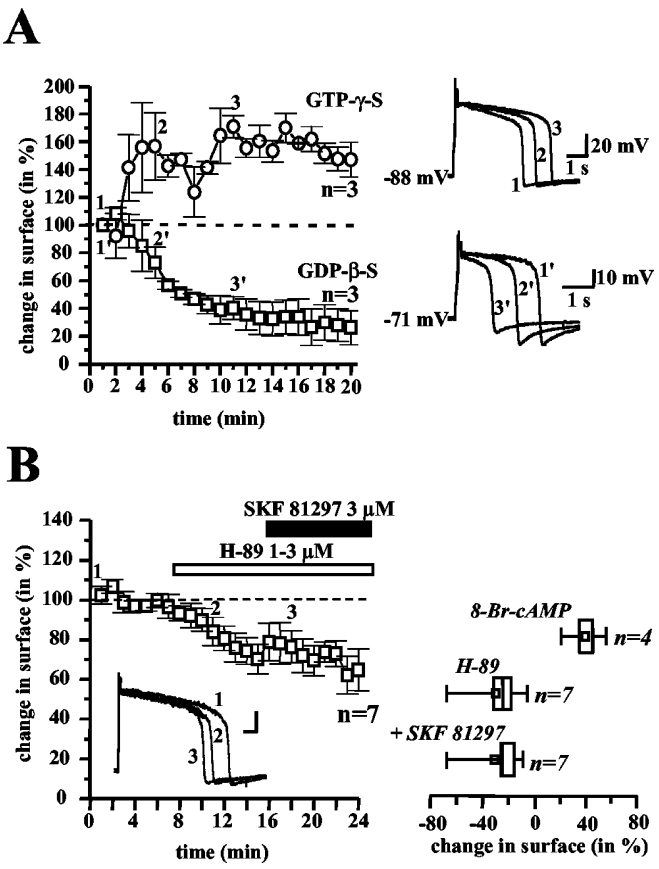

Figure 8. G-protein and protein kinase A are involved in the action of D1-like, presumably D5, receptor. *A*, Time course of changes in plateau potential surface during recording with a pipette medium containing GTP-γ-S and GDP-β-S. The sample records *1*, *2*, and *3* were taken at the beginning and after 6 and 11 min of perfusion, respectively. *B*, Representative examples and time course of changes in plateau potential during perfusion of a membrane-permeant antagonist of protein kinase A, H-89 (1–3 μM). Addition of SKF 81297 (3 μM) failed to increase plateau potential. Perfusion of the membrane-permeant cAMP analog, 8-bromo-cAMP (10 μM), per se potentiated plateau potentials. *Box plots* summarize the results of the trials with H-89 and 8-bromo-cAMP.

profiling. In no case was D1 receptor mRNA amplified from cytoplasm harvested from burst-competent neurons, whereas mRNA of D5 receptor was detected in the same cytoplasm samples. The expression profile of D5 receptor in rat, monkey, and human brains has been reappraised recently using highly selective antibodies raised against a peptide sequence of cloned receptor (Ciliax et al., 2000; Khan et al., 2000). Numerous neurons were markedly labeled within the STN, as confirmed in our study of the STN from rat pups of the same age and strain as that used for our patch-clamp work. In addition, at the subcellular level, D5 receptors were visualized in the soma or dendritic processes of neurons. In the absence of specific agonists able to discriminate between D1 and D5 receptors, D1 receptor null mutant mice offer an alternative approach. We found that burst-firing of subthalamic neurons from D1 −/− mice was potentiated by D1 agonists in the same way as that of neurons from wild-type mice and rats. This establishes that functional D5 (not D1) receptors were being activated in burst-competent neurons. Taken together with the aforementioned studies, our results suggest that subthalamic neurons express D5 but not D1 receptor subtype.

Oscillatory firing pattern is strengthened by D5 receptors

Spontaneous bursts and evoked plateau potentials are caused by the same Ca^{2+} and Ca^{2+}-activated conductances (Beurrier et al., 1999; Otsuka et al., 2001). Activation of D5 receptors led to potentiation of these two types of action potential regenerative discharges. This activation did not affect the membrane potential, contrary to activation of group I metabotropic glutamate receptors (Awad et al., 2000); however, it did increase the duration of the discharge. Using dihydropyridines in addition to specific toxins, we established that the increase in duration was prevented only when L-type channels were blocked but not when N, P, and Q channels were inhibited. It can thus be concluded that D1 agonists increased Ca^{2+} current by L-type channels. The effect of D1 agonists was mimicked by activators of G-proteins. It was also mimicked by a membrane-permeant analog of cAMP, whereas it was blocked when protein kinase A was inhibited, suggesting that it involved the activation of at least adenylate cyclase, as generally found for D1-like receptors. Interestingly, inhibition of adenylate cyclase per se reduced the plateau potentials, suggesting that native D5 receptors have a constitutive action in the absence of ligand as recombinant receptors do (Tiberi and Caron, 1994; Charpentier et al., 1996; Demchyshyn et al., 2000). Gating of L-type channels is sensitive to phosphorylation (Catterall, 2000). It can therefore be assumed that bursts and plateaus were made more robust because of an increased phosphorylation of L-type channels by protein kinase A.

In vivo, burst-firing is found in the STN in the normal state, although it is found much more frequently in the depleted-dopamine state (Bergman et al., 1994; Magill et al., 2001; Ni et al., 2001; Levy et al., 2002). This raises the question of its involvement in the function of the STN. Our data suggest that burst-firing per se is not a marker of function impairment in the STN because it is strengthened by dopaminergic agonists in neurons from normal animals. It has been proposed that burst-firing relies on intrinsic membrane properties (Beurrier et al., 1999). Alternatively, burst-firing may be attributable to synaptic properties in the basal ganglia network (Plenz and Kital, 1999; Magill et al., 2001; Terman et al., 2002). In any case, our results predict significant control of burst-firing by D5 receptors in the normal state because we show that not only persistent burst-firing but also plateau potentials are potentiated. Therefore, the postinhibitory rebound responses evoked by stimulation of GABAergic terminals (Bevan et al., 2002; Terman et al., 2002) and the long-lasting regenerative responses to stimulation of excitatory terminals (Otsuka et al., 2001) will last longer. We suggest that this will markedly affect information processing in the subthalamopallidal network and the impact of inputs from the cortex.

Dopamine acts outside striatum in basal ganglia

Patch-clamp studies in rat brain slices have established the presence of presynaptic and postsynaptic receptors in the D2 family on subthalamic neurons. Activation of presynaptic receptors reduced the impact of the main two inputs of the STN, cortex, and globus pallidus (Shen and Johnson, 2000). The postsynaptic receptors reduced intrinsic K^+ conductance. This resulted in increased frequency of regular, single-spike firing (Zhu et al., 2002). We now present evidence that postsynaptic D5 receptors are expressed in the STN and control burst-firing. Dopamine action in the STN is thus two-faceted. On one hand, its presynaptic receptors reinforce the filter role of the STN. On the other hand, its postsynaptic receptors make the two intrinsic firing modes (single-spike, burst-firing) more distinct; however, another major factor has yet to be defined. No data are available on the modulation of GABA and glutamate receptors in the STN by postsynaptic receptors in the D1 family. Specific modulation of fast synaptic transmission by D1 and D5 receptor subtypes has

been described in many other parts of the brain (Radnikow and Misgeld, 1998; Brunig et al., 1999; Chergui and Lacey, 1999; Flores-Hernandez et al., 2000; Liu et al., 2000; Kerr and Wickens, 2001; Seamans et al., 2001). It also presumably takes place in the STN. Identification and subcellular localization of the dopamine receptor subtypes expressed in the STN are clearly required to shed light on the origin of various motor impairments produced in animals when dopamine agonists or antagonists with various specificities and efficacies are infused locally in the STN. The discovery that D1 agonists locally infused in the STN induce dyskinesia is of particular interest, however, because this action is potentiated in an animal model of Parkinson's disease (Mehta et al., 2000).

Functional implications

The main treatment for symptoms of Parkinson's disease is dopamine replacement therapy. Unfortunately, after the "honeymoon period" of effective response to L-DOPA, long-term side effects develop. These affect ~50% of all patients within 5 years of therapy. Undesirable effects include disabling dyskinesias. It has been proposed that L-DOPA-induced dyskinesias represents a form of pathological learning caused by chronic pulsatile (nonphysiological) stimulation of D1 receptors by short-lived L-DOPA, which activates a cascade of molecular and biochemical events (Calon et al., 2000). On the other hand, deep brain stimulation of the STN also provides anti-Parkinsonian benefits. Interestingly, this is accompanied by a reduction of L-DOPA-related motor complications and an attenuation of the short-duration motor response to an L-DOPA challenge (Limousin et al., 1998; Bejjani et al., 2000; Fraix et al., 2000). This suggests that in addition to the primary defect in dopaminergic tone, downstream mechanisms also contribute to aggravating the disease. The progressive decrease of dopamine during the asymptomatic progression of the disease and the pulsatile L-DOPA levels during treatment entail long-term changes in the expression of crucial proteins in the D1 transduction pathway in the striatum (Andersson et al., 2001; Westin et al., 2001; Gerfen et al., 2002). This may also hold true outside the striatum. Therefore, it may be very useful to look for dopaminergic agents acting on specific D1 receptor subtypes outside the striatum. Given the pivotal position of the STN in basal ganglia, we suggest that selective action on the D5 receptor subtype might lead to better-targeted drug therapy for dopamine–linked disorders.

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