

Cooperative Activation of Dopamine D₁ and D₂ Receptors Increases Spike Firing of Nucleus Accumbens Neurons via G-Protein $\beta\gamma$ Subunits

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Dopamine in the nucleus accumbens modulates both motivational and addictive behaviors. Dopamine D₁ and D₂ receptors are generally considered to exert opposite effects at the cellular level, but many behavioral studies find an apparent cooperative effect of D₁ and D₂ receptors in the nucleus accumbens. Here, we show that a dopamine-induced enhancement of spike firing in nucleus accumbens neurons in brain slices required both D₁ and D₂ receptors. One intracellular mechanism that might underlie cooperativity of D₁ and D₂ receptors is activation of specific subtypes of adenylyl cyclases by G-protein $\beta\gamma$ subunits ($G_{\beta\gamma}$) released from the $G_{i/o}$ -linked D₂ receptor in combination with $G_{\alpha s}$ -like subunits from the D₁ receptor. In this regard, dopaminergic enhancement of spike firing was prevented by inhibitors of protein kinase A or $G_{\beta\gamma}$. Furthermore, intracellular perfusion with $G_{\beta\gamma}$ enabled D₁ receptor activation but not D₂ receptor activation to enhance spike firing. Finally, our data suggest that these pathways may increase spike firing by inhibition of a slow A-type potassium current. These results provide evidence for a novel cellular mechanism through which cooperative action of D₁ and D₂ receptors in the nucleus accumbens could mediate dopamine-dependent behaviors.

Key words: nucleus accumbens; dopamine; G-protein $\beta\gamma$ subunits; PKA; K⁺ channels; spike firing

Introduction

Dopamine (DA) in the nucleus accumbens (NAcb) has long been considered an important modulator of addiction and goal-directed behaviors (Spanagel and Weiss, 1999). The shell region of the NAcb in particular is implicated in a number of cellular and behavioral phenomena, especially in relation to addictive drugs (Zahm, 1999). Although a diversity of functional effects and ionic targets can be modulated by DA, the exact role of DA receptor activation in the NAcb is only partially understood (Greengard et al., 1999; Nicola et al., 2000). DA receptors are generally grouped into two subfamilies, the D₁-like receptors and the D₂-like receptors (Missale et al., 1998). Opposing influences of D₁ and D₂ receptor activation on cAMP-dependent signaling have been reported in many studies (Stoof and Kebabian, 1981; Missale et al., 1998), with D₁ receptors acting through the stimulatory G_s -like $G_{\alpha s}$ and D₂ receptors acting through the inhibitory $G_{i/o}$ proteins.

In contrast, results from a number of behavioral studies suggest a cooperative interaction of D₁ and D₂ receptors in the NAcb (Plaznik et al., 1989; Chu and Kelley, 1992; Wolterink et al., 1993; Phillips et al., 1994; Hodge et al., 1997; Ikemoto et al., 1997; Gong et al., 1999; Koch et al., 2000; Nowend et al., 2001). For example,

animals will self-administer D₁ and D₂ agonists directly into the NAcb in combination but not alone (Ikemoto et al., 1997). Furthermore, self-administration of amphetamine (Phillips et al., 1994) and ethanol (Hodge et al., 1997), lever pressing for a conditioned reinforcer (Chu and Kelley, 1992; Wolterink et al., 1993), and evaluating the relative cost of obtaining a reward (Koch et al., 2000; Nowend et al., 2001) all may involve coactivation of D₁ and D₂ receptors in the NAcb. Dose-dependent modulation of firing rate and TTX-independent Fos induction by cooperative D₁ and D₂ receptor activation have also been reported (Chiodo and Berger, 1986; Wachtel et al., 1989; Williams and Millar, 1990; Hu and White, 1997; LaHoste et al., 2000).

Despite the implication of D₁ and D₂ receptor cooperativity in several behaviors, the specific cellular and biochemical pathways that mediate the interaction between D₁ and D₂ receptors are uncertain. The present study was designed to examine the effects of DAergic agonists on spike firing in medium spiny neurons (MSNs) in the NAcb shell and the cellular mechanisms that might mediate these effects.

Materials and Methods

Slice preparation and electrophysiology. Coronal slices (300 μ m) were prepared from male postnatal day 22–28 Sprague Dawley rats (50–80 gm). After cutting, slices recovered at 32°C in carbogen-bubbled artificial CSF (ACSF) (in mM: 126 NaCl, 1.6 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.4 CaCl₂, 18 NaHCO₃, and 11 glucose, pH 7.2–7.4, 301–305 Osm) for 30 min to 5 hr. During experiments, slices were submerged and continuously perfused (using a peristaltic pump, ~2 ml/min) with carbogen-bubbled ACSF warmed to 31–32°C and supplemented with CNQX (10 μ M, to block AMPA-type glutamate miniature EPSPs), picrotoxin (50 μ M, to block GABA_A receptors), and sodium metabisulfite (50 μ M), an antioxidant to preserve DAergic reagents (Nicola and Malenka, 1997).

Received Jan 16, 2003; revised April 4, 2003; accepted April 7, 2003.

This work was supported by funds provided by the State of California for medical research on alcohol and substance abuse through the University of California, San Francisco (F.W.H. and A.B.), by the Alcoholic Beverage Medical Research Foundation (M.G.C.), and by Department of the Army Grant DAMD17-0110803 (F.W.H. and I.D.). The United States Army Medical Research Acquisition Activity (Fort Detrick, MD) was the awarding and administering acquisition office. We thank H. Fields, M. Melis, S. Nicola, J. Pierce-Shimomura, B. Schilstrom, and M. Ungless for assistance during preparation of this manuscript.

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CNQX and picrotoxin were added to isolate the cell from several major sources of neurotransmitter input whose release is known to be inhibited by dopamine (Pennartz et al., 1992a; Nicola and Malenka, 1997). In preliminary experiments, DA-induced increases in spike firing were observed in the absence of these three reagents (data not shown). All reagents were bath-applied.

All experiments were performed using whole-cell recording, except where specifically indicated that an amphotericin perforated patch was used. Patch clamping was performed using visualized infrared differential interference contrast with 2.5–3.5 M Ω electrodes. Current pulses were applied using Clampex 8.0 and an Axo-1D patch amplifier in current-clamp mode (Axon Instruments, Foster City, CA). On breaking into neurons, the resting membrane potentials were between –95 and –80 mV. In most experiments, the membrane potential for each neuron was set to approximately –85 mV using the patch amplifier ~5 min after breaking into a cell, except for experiments involving intracellular perfusion with okadaic acid, norokadaone, or G $\beta\gamma$ subunits, in which the resting membrane potential of cells was maintained at given potential (either –90 or –80 mV) throughout experiments to compensate for small drifts in membrane potential that can occur after break-in. Series resistance correction was 15–20 M Ω . To record EPSPs, a bipolar stimulating electrode was placed ~100 μ m lateral to the recording electrode. Afferents were stimulated with 10 pulses at 20 Hz (20 μ sec) every 30 sec using a Master 8 (AMPI, Jerusalem, Israel), and EPSPs were recorded using Clampex in current-clamp mode.

For voltage-clamp experiments, ACSF was modified to contain 3.6 mM MgCl₂, 0.2 mM CaCl₂, 0.1 mM CdCl₂, and 1 μ M TTX to block calcium and sodium current. Neurons were held at –80 mV with the patch amplifier. Our voltage protocol for determining effects of DA and 4-aminopyridine (4-AP) on potassium currents involved a series of 11 steps. In each step, a neuron was hyperpolarized to –100 mV for 700 msec and then depolarized for 300 msec. The depolarization ranged from –80 to +20 mV across the 11 steps. This protocol was applied every 30 sec. For analysis, magnitude of the total evoked potassium current was determined 280 msec into the current response after depolarization to 0 mV.

All data are shown as mean \pm SEM. Unless otherwise indicated, all statistics were performed using a two-tailed, unpaired *t* test.

Analysis of spike firing. All such data were analyzed using Axograph (Axon Instruments, Foster City, CA). To calculate percent change in spiking, a current pulse was selected that exhibited approximately seven or eight spikes at baseline. The same current pulse was used for all time points of a given cell. Spike firing rates during the 3 min before addition of the reagent were averaged, and this value was normalized to 100%. Statistical significance was determined for the average spike firing change during the last 2 min of exposure to reagents. In most cases, statistical significance of changes in spike rate for a particular experimental condition was determined in comparison with an appropriate control condition. For experiments in which we systematically varied resting membrane voltage (V_{m-rest}) and determined the relationship between change in V_{m-rest} and change in spike firing, we first determined, for each cell, the change in number of spikes per millivolt change in V_{m-rest} . Then, to make these data comparable with the percent change in spike firing measured with DAergic agonists, we chose a baseline number of spikes of 7.2 (the mean number of spikes before addition of DAergic agonists in the cells shown in Fig. 5C) and determined the percent increase in spike firing per millivolt change in V_{m-rest} for each cell.

We attempted to estimate the proportion of neurons that responded to a given treatment, and, as is commonly observed (Uchimura et al., 1986; Surmeier et al., 1995), we found that most but not all MSNs responded to DAergic agonists. Approximately 70% of cells responded under whole-cell conditions, and ~80% of cells responded under perforated patch conditions (with a threshold of 15% increase in spiking to be considered responding), suggesting that a small washout of some signaling molecules might have occurred under whole-cell conditions. However, to avoid the arbitrary designation required to delineate cells as responders or not, we included all neurons exposed to a given condition in all of our analyses.

Reagents. Whole-cell experiments were performed with potassium methanesulfonate- or potassium chloride-based solutions. KMeS/KOH,

0.95% (v/v); methanesulfonic acid, 0.76% (v/v); 20 mM HEPES; 0.2 mM EGTA; 2.8 mM NaCl; 2.5 mg/ml Mg-ATP; 0.25 mg/ml GTP, pH 7.2–7.4, 275–285 Osm; and KCl/KCl, 144 mM replacing KOH and methanesulfonic acid. Amphotericin experiments used the methanesulfonate-based solution, except without ATP and GTP. Amphotericin was made fresh as a 30 mg/ml stock in DMSO, sonicated, added at 0.2% (v/v) to a pipette solution containing 0.25 mg/ml pluronic acid, and sonicated again. Peptide sequences were as follows: FVIII, YEDSYEDISAYLLSKNNAIPR; and SP $\beta\gamma$, DALRIQMEERFMASNP SKVSYPIT (Ma et al., 1997; synthesized by Synpep, Dublin, CA). Peptides were prepared as a 500 \times stock in DMSO and kept at –80°C. Peptides were used within 2 weeks of dilution in DMSO. In half of the peptide experiments, the experimenter did not know the identity of the peptide. Purified bovine whole-brain G $\beta\gamma$ subunits (Calbiochem, Lucerne, Switzerland; Huang et al., 1998) were aliquoted and kept at –80°C. Maltose-binding protein (MBP; a generous gift from Dr. Dorit Ron and Alicia Vagts, Ernest Gallo Clinic and Research Center), which is approximately the same molecular weight as G $\beta\gamma$ (~50 kDa for MBP and ~46 kDa for G $\beta\gamma$), was prepared in the same vehicle as G $\beta\gamma$ subunits, with a final concentration of 20 μ M DTT, 20 μ M EGTA, and 0.002% Lubrol.

Most reagents were prepared fresh each day, including 4-aminopyridine, DA, sodium metabisulfite (all in Ringer's solution), ω -conotoxin-GVIA and ω -agatoxin (in water), eticlopride, propylnorapomorphine (NPA), okadaic acid, quinpirole, SCH23390, and U0126 (all in DMSO), and nifedipine (in 95% ethanol; Bargas et al., 1994). Dendrotoxin (Alomone Labs, Jerusalem, Israel), CNQX, Rp isomer of cAMPs (Rp-cAMPs) (Biolog, La Jolla, CA), and amphetamine were dissolved in water and kept at –20°C. SKF81297, SKF82957, forskolin, and dideoxy-forskolin were dissolved in DMSO and kept at –20°C. More than 85% of D₁ agonist experiments used SKF81297, and, when tested, we saw similar results with both. Picrotoxin was dissolved in water and kept as a room temperature stock. Unless otherwise indicated, all reagents were made at 1:1000 stock and were purchased from Sigma (St. Louis, MO) or Research Biochemicals (Natick, MA).

Results

Coactivation of D₁ and D₂ receptors increases spike firing

Coherent excitatory synaptic inputs *in vivo* drive MSNs from a strongly hyperpolarized state, the “down state,” to a depolarized state, the “up state,” which is close to the threshold for action potential generation (Plenz and Kitai, 1998; Wickens and Wilson, 1998; Nicola et al., 2000). Although dopamine can have a number of effects within the basal ganglia (Greengard et al., 1999; Nicola et al., 2000), including modulation of release of several transmitters (McGinty, 1999), we focused on the postsynaptic effects of dopamine receptor activation on spike firing. Understanding how dopamine could modulate spike firing is critical, because spike firing is a major mechanism by which neurons process information. In addition, there is considerable interest in modulation of spike firing of NAc MSNs in relation to behavioral events (Schultz et al., 1992; Bowman et al., 1996).

We used two criteria to restrict our investigation to MSNs. First, we only recorded from medium-sized cells to exclude the much larger cholinergic interneurons. The majority of neurons showed the slow, repetitive spike-firing pattern typically reported for MSNs (Nisenbaum et al., 1994; Plenz and Kitai, 1998; Wickens and Wilson, 1998; Mahon et al., 2000), and all such neurons were included for study. A small proportion of cells (~5%) exhibited a clearly different firing pattern, with higher rates of firing, a larger fast afterhyperpolarization, and a more depolarized resting membrane potential. These properties are typical of the fast-spiking GABAergic interneurons (Plenz and Kitai, 1998; Bracci et al., 2002), and these cells were not analyzed further.

To test the firing properties of MSNs during continuous depolarization, a series of 300 msec current pulses was delivered to an MSN every 30 sec. The current pulses ranged from –100 pA

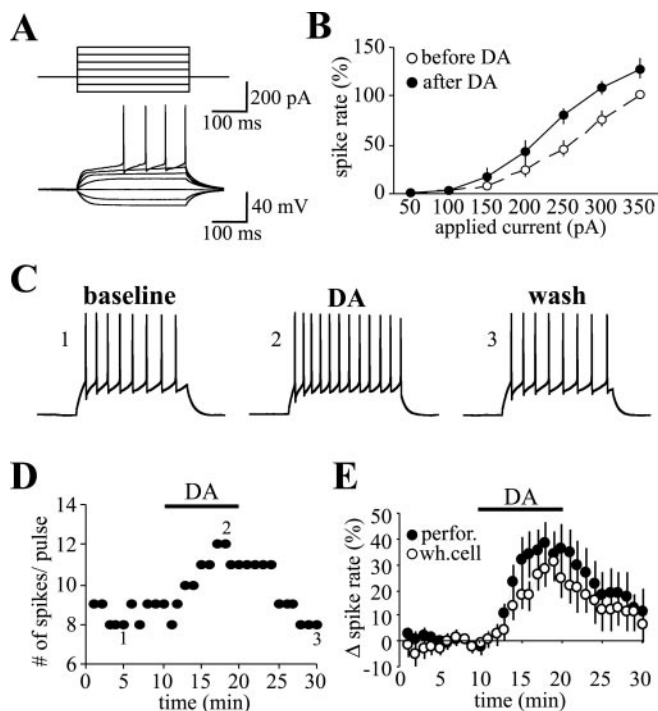


Figure 1. Dopamine (75 μ M) increased spike firing in MSNs from the NAc shell. *A*, Example of applied current pulses and voltage responses. The traces represent subthreshold current pulses and the first pulse able to elicit spikes. Current pulses eliciting a greater number of spikes are not shown. V_{m-rest} was -79 mV. *B*, Input–output relationship showing significant enhancement of spike firing by DA for all current pulses >150 pA in magnitude ($p < 0.05$, paired t test). The spike rate for each cell was normalized to the number of spikes elicited by a 350 pA current pulse before addition of DA (10.0 ± 1.4 spikes). *C*, Example traces showing reversible increase in spike firing in the presence of DA (250 pA current pulse). V_{m-rest} values of the example traces shown were -83.2 , -80.8 , and -83.0 mV. *D*, Time course experiment demonstrating an enhancement of spike firing after application of DA. Data correspond to examples of spike firing shown in *B*. All data shown in *A–D* are from perforated patch recordings. *E*, Compiled data from neurons exposed to DA during whole-cell (wh.cell; open circles) and perforated patch (perfor.; filled circles) recordings.

(hyperpolarizing) to $+350$ pA (depolarizing, both subthreshold and suprathreshold for action potentials) in 50 pA steps (Fig. 1*A*). Bath application of either 75 or 30 μ M DA significantly and reversibly elevated spike firing (75 μ M, Fig. 1*B–E*; $n = 10$ and 27 for perforated patch and whole-cell experiments, respectively; 30 μ M, $17.3 \pm 6.6\%$; $n = 7$; both $p < 0.05$, paired t test), but 10 μ M DA did not ($1.9 \pm 4.7\%$; $n = 5$). Enhancement of spike firing was relatively slow to develop, reaching a peak ~ 5 –7 min after application of DA. Spike firing was also significantly enhanced by amphetamine (10 μ M, $18.5 \pm 5.4\%$; $n = 5$; $p < 0.05$, paired t test), which causes release of DA by reversal of the DA transporter (Seiden et al., 1993). However, spike firing was not altered by a selective D₁ agonist alone (SKF81297 or SKF82957, 1–10 μ M, Fig. 2*A1,B,D*) or by a selective D₂ agonist alone (quinpirole, 1–10 μ M, Fig. 2*A1,B,D*; NPA, 3 μ M, Fig. 2*D*). Instead, spike firing was significantly increased after exposure to a D₁ agonist and the D₂ agonist quinpirole in combination with 3 μ M of each (Fig. 2*D*; $p < 0.05$, paired t test) or 10 μ M of each (Fig. 2*A2,B,D*; $p < 0.05$ vs D₁ or D₂ agonist alone) but not with 1 μ M of each (Fig. 2*D*). Enhancement of spike firing was also observed using a D₁ agonist in combination with NPA, a D₂ receptor agonist structurally unrelated to quinpirole (Fig. 2*D*). Thus, only D₁ and D₂ agonists in combination produced an enhancement in spike firing similar to that observed with DA.

If the DA-mediated increase in spike firing required coopera-

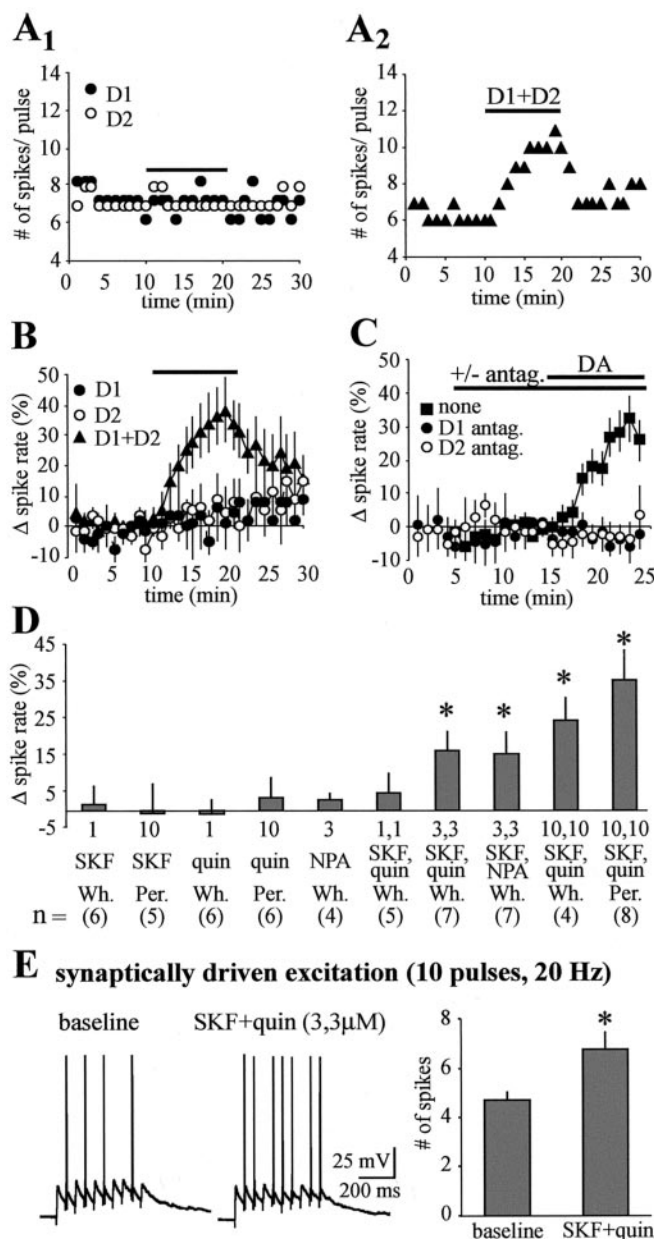


Figure 2. Coactivation of both D₁ and D₂ receptors was required to increase spike firing. *A*, Examples showing (*A1*) no effect of a D₁ agonist (SKF82957, 10 μ M) or a D₂ agonist (quinpirole, 10 μ M) alone on spike rate (*A1*), but (*A2*) an increase in spike rate with a combination of 10 μ M concentrations of a D₁ receptor agonist (SKF82957) and a D₂ receptor agonist (quinpirole) (*A2*). *B*, Compiled data from neurons exposed to a D₁ agonist (SKF82957 or SKF81297, 10 μ M) or a D₂ agonist (quinpirole, 10 μ M) alone or in combination. *C*, Preexposure to a selective D₁ antagonist (SCH23390, 10 μ M) or a selective D₂ antagonist (antag.; eticlopride, 3 μ M) prevented DA-mediated increases in spike firing. DA data are the same whole-cell results shown in Figure 1*D*, to assist in comparison of the effects of DA with and without receptor antagonists. *D*, Compiled data showing dose response of spike firing enhancement by dopamine receptor agonists. Per., Wh., Experiments performed using perforated patch or whole-cell recording, respectively. *E*, Enhancement of synaptically evoked spike firing by a combination of 3 μ M SKF81297 and quinpirole (quin). V_{m-rest} was -81.6 mV before agonists and -82.3 mV after. *Significant increase in spike firing ($p < 0.05$, paired t test).

tive activation of D₁ and D₂ receptors, then either a D₁ or a D₂ antagonist should block this activation. DA-mediated enhancement of spike firing was prevented by preexposure to either the D₁ antagonist SCH23990 (1 μ M, $-0.5 \pm 4.6\%$; $n = 5$; 10 μ M, Fig. 2*C*; $n = 6$; both concentrations $p < 0.05$ vs DA without antago-

nists) or the D₂ antagonist eticlopride (300 nM, $2.5 \pm 5.5\%$; $n = 6$; $3 \mu\text{M}$, Fig. 2C; $n = 11$; both concentrations $p < 0.05$ vs DA without antagonists). Therefore, DA-mediated increases in spike firing required activation of both D₁ and D₂ receptors.

To address whether D₁ and D₂ receptor signaling might involve a synaptically released factor, slices were preincubated for 15–60 min with irreversible antagonists of the N-type (ω -conotoxin GVIA, 500 nM) and P/Q-type (ω -agatoxin IVA, 250 nM) calcium channels, as well as continuous exposure to the L-type calcium channel antagonist nifedipine (30 μM). This treatment completely inhibited evoked glutamatergic EPSCs even 1 hr after exposure to toxins (data not shown) but did not prevent the enhancement in spike firing by DA ($24.0 \pm 5.0\%$; $n = 6$; $p < 0.05$, paired t test). These data suggest that the DA-mediated signaling events did not require a synaptically released factor.

Because firing of NAc neurons *in vivo* usually requires glutamatergic excitation to elicit action potentials (Plenz and Kitai, 1998; Wickens and Wilson, 1998; Nicola et al., 2000), we determined whether activation of DA receptors would increase the number of spikes evoked during synaptically driven spike firing. Thus, using 10 pulses at 20 Hz (with stimulation current set to evoke four or five spikes in the basal condition), we found that exposure to a combination of 3 μM SKF81297 and quinpirole significantly enhanced the number of spikes elicited by synaptically driven excitation (Fig. 2E; from 4.7 ± 0.3 to 6.8 ± 0.7 spikes; $n = 5$; $p < 0.05$). These results suggest that DA receptor activation can enhance spike firing under conditions that more closely mimic the *in vivo* situation.

DA receptor-mediated increase in spike firing requires cAMP and G-protein $\beta\gamma$ subunits

Several studies suggest that protein kinase A (PKA) plays a major role in DA signaling (Greengard et al., 1999). Addition of 100 μM Rp-cAMPS (Rp), an inhibitor of cAMP-dependent processes, during the DA response significantly reduced spike firing to pre-DA levels (Fig. 3A; $n = 4$ with Rp; $n = 5$ without Rp; $p < 0.01$). However, Rp alone did not affect basal firing activity ($-3.8 \pm 2.6\%$; $n = 4$). These data suggest that the cAMP system did not regulate the spike rate under basal conditions but was required for expression of the increased firing rate observed during exposure to DA. In support of this possibility, forskolin (5 μM ; $n = 12$), an activator of adenylyl cyclases, increased spike firing to a similar degree as DAergic agonists, whereas dideoxy-forskolin (5 μM ; $n = 6$), an inactive analog of forskolin, had no effect (Fig. 3B).

One intracellular mechanism that might underlie cooperativity of D₁ and D₂ receptors is activation of specific subtypes of adenylyl cyclases by G-protein β -gamma subunits ($G_{\beta\gamma}$) released from the $G_{i/o}$ -linked D₂ receptor in combination with $G_{\alpha s}$ -like subunit signaling from the D₁ receptor. This $G_{\alpha s}/G_{\beta\gamma}$ interaction allows $G_{i/o}$ -linked receptors to contribute to, rather than oppose, activation of the PKA system (Fig. 3D) (Sunahara et al., 1996; Watts and Neve, 1997). In this regard, dialysis of neurons with 200 μM SP $\beta\gamma$ ($n = 9$), an inhibitory peptide that interferes with binding of $G_{\beta\gamma}$ to several targets (Ma et al., 1997), prevented the increase in spike firing elicited by D₁ and D₂ coactivation, whereas a 200 μM concentration of the inactive peptide FVIII ($n = 10$) had no effect (Fig. 4A,B; $p < 0.05$). Activation of spike firing by forskolin was not prevented by either SP $\beta\gamma$ or FVIII (Fig. 4C; $n = 4$ and 6, respectively), indicating that the inhibition mediated by SP $\beta\gamma$ was upstream of adenylyl cyclase.

If D₁ and D₂ receptor cooperativity occurred via the $G_{\beta\gamma}$ -

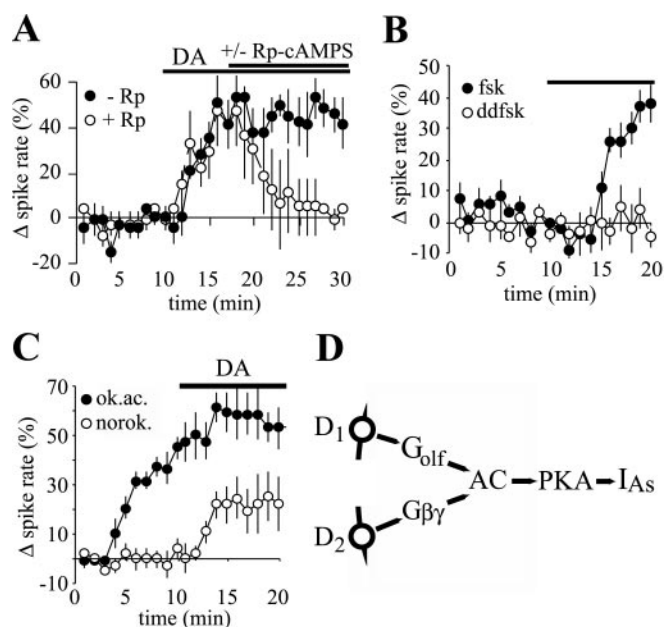


Figure 3. PKA activation was required for DA-mediated increases in spike firing. *A*, Application of 100 μM Rp-cAMPS during the plateau DA response reduced spike firing to baseline levels. Only neurons showing a change in spike firing with DA were tested for effects of Rp-cAMPS. *B*, Forskolin (fsk), an activator of the PKA system, led to a similar increase in spike rate as DA, whereas dideoxy-forskolin (ddfsk), the inactive control, had no effect. *C*, Inhibition of protein phosphatases 1 and 2A via intracellular perfusion with okadaic acid (1 μM) enhanced spike firing and occluded DAergic effects, whereas the inactive analog norokadaone (1 μM) had no effect alone and did not occlude DA-mediated enhancement of spike firing. ok.ac., Intracellular perfusion with okadaic acid; norok., intracellular perfusion with norokadaone. *D*, Model showing a possible intracellular pathway by which a combination of D₁ and D₂ receptor agonists can activate PKA. AC, Adenylyl cyclase. We should note that there may be intervening molecules between PKA and I_{As}, and also that D₁ and D₂ receptors might be on the same cell or different cells.

dependent mechanism described above, we would predict that, after intracellular perfusion with $G_{\beta\gamma}$ subunits, D₁ receptor agonists should enhance spike firing, whereas D₂ receptor agonists should not. Intracellular perfusion with purified bovine brain $G_{\beta\gamma}$ subunits (20 nM) had no effect alone ($3.7 \pm 3.6\%$ change in spiking 10 min after break-in; $n = 9$). However, a D₁ agonist (SKF81297, 10 μM ; $n = 5$) but not a D₂ agonist (quinpirole, 10 μM ; $n = 4$) significantly enhanced spike firing in cells perfused with $G_{\beta\gamma}$ (Fig. 4D; $p < 0.05$). The D₁ agonist did not enhance spike firing in neurons perfused with the $G_{\beta\gamma}$ vehicle plus maltose-binding protein, which is similar in size to $G_{\beta\gamma}$ ($-2.5 \pm 2.5\%$; $n = 5$). Although we cannot completely rule out effector sites for $G_{\beta\gamma}$ other than adenylyl cyclases, taken together, these data strongly suggest that $G_{\beta\gamma}$ and the cAMP system are required for the D₁- and D₂-mediated enhancement of spike firing.

PKA signaling can involve other downstream signaling molecules such as MAP kinase (Impey et al., 1998) and protein phosphatase 1 (PP1) (Surmeier et al., 1995; Schiffmann et al., 1998; Greengard et al., 1999). The MAP kinase inhibitor U0126 (10 μM), which blocks long-term potentiation in cortical neurons in a slice (Di Cristo et al., 2001), did not prevent DA-induced enhancement of spike firing ($22.9 \pm 5.8\%$; $n = 4$; $p < 0.05$, paired t test). However, spike firing was significantly enhanced by intracellular perfusion of okadaic acid (1 μM ; Fig. 3C; $n = 5$; $p < 0.01$, paired t test), an inhibitor of PP1 and PP2A, and this enhancement occluded DA-mediated changes in spike firing ($p > 0.1$, paired t test). Norokadaone (1 μM), an inactive analog of okadaic

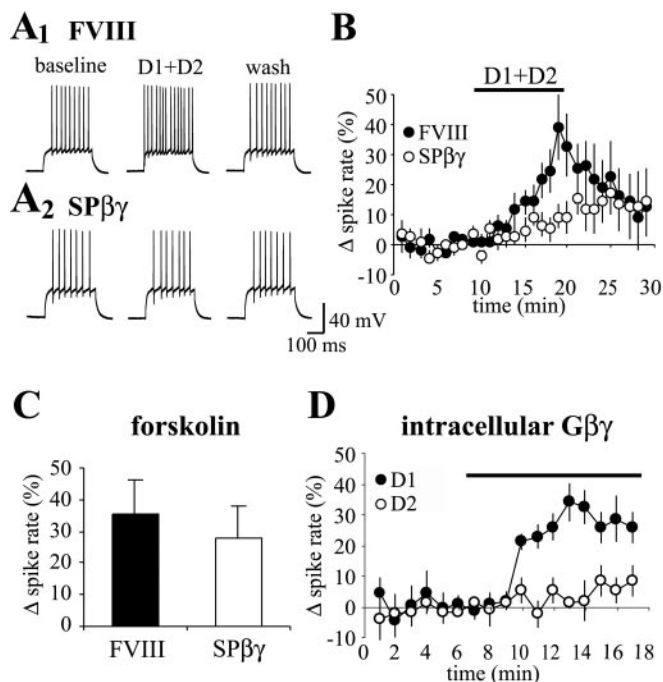


Figure 4. $G_{\beta\gamma}$ was required for the increased spike firing produced by coapplication of D₁ and D₂ receptor agonists. *A*, Example traces showing increased spike firing during dialysis with FVIII, the inactive control (A1; 250 pA current pulse) but not when SPβγ, the $G_{\beta\gamma}$ -inhibitory peptide, was present in the intracellular solution (A2; 250 pA current pulse). For traces in A1, V_{m-rest} values were -78.4 , -80.4 , and -78.5 mV. For traces in A2, V_{m-rest} values were -79.6 , -81.1 , and -82.5 mV. *B*, Time course experiment demonstrating that SPβγ but not FVIII prevented the enhancement of spike firing after coapplication of D₁ and D₂ receptor agonists. *C*, Forskolin-mediated increases in spike rate were not affected by either FVIII or SPβγ. *D*, Intracellular perfusion with $G_{\beta\gamma}$ subunits enabled enhancement of spike firing by D₁ but not D₂ receptor agonists.

acid, had no effect by itself and did not prevent the effects of DA (Fig. 3C; $n = 5$). In several studies, okadaic acid mimics the inhibition of PP1 by DA and cAMP-regulated phosphoprotein of M_r 32,000 (DARPP-32) (Surmeier et al., 1995; Schiffmann et al., 1998), which is normally PKA-dependent (Greengard et al., 1999), suggesting that the DA- and PKA-mediated enhancement of spike firing observed here may involve signaling through DARPP-32.

Role of slow A-type potassium current in spike firing enhancement

A number of ionic mechanisms have been reported to be modulated by dopamine (for review, see Greengard et al., 1999; Nicola et al., 2000). Therefore, we first analyzed several baseline electrophysiological parameters that might be altered on exposure to DA or the combination of a D₁ and a D₂ receptor agonist. However, we observed no significant changes in resting membrane voltage (Fig. 5A1, V_{m-rest}) or input resistance (Fig. 5A2, R_{input} ; both $p > 0.25$, each parameter tested by one-way ANOVA across all groups shown in Fig. 5A1). We also performed a within-cell comparison of the spike firing change and the change in R_{input} or V_{m-rest} . For this analysis, we grouped cells exposed to 75 μ M DA and a 10 μ M concentration of each of the D₁ and D₂ receptor agonists ($n = 51$ cells). Although change in R_{input} did not significantly correlate with change in spike firing ($p > 0.2$; data not shown; Spearman rank order correlation), the change in V_{m-rest} nearly did ($p > 0.07$; Fig. 5B). To further address this issue, we systematically varied V_{m-rest} and determined the relationship be-

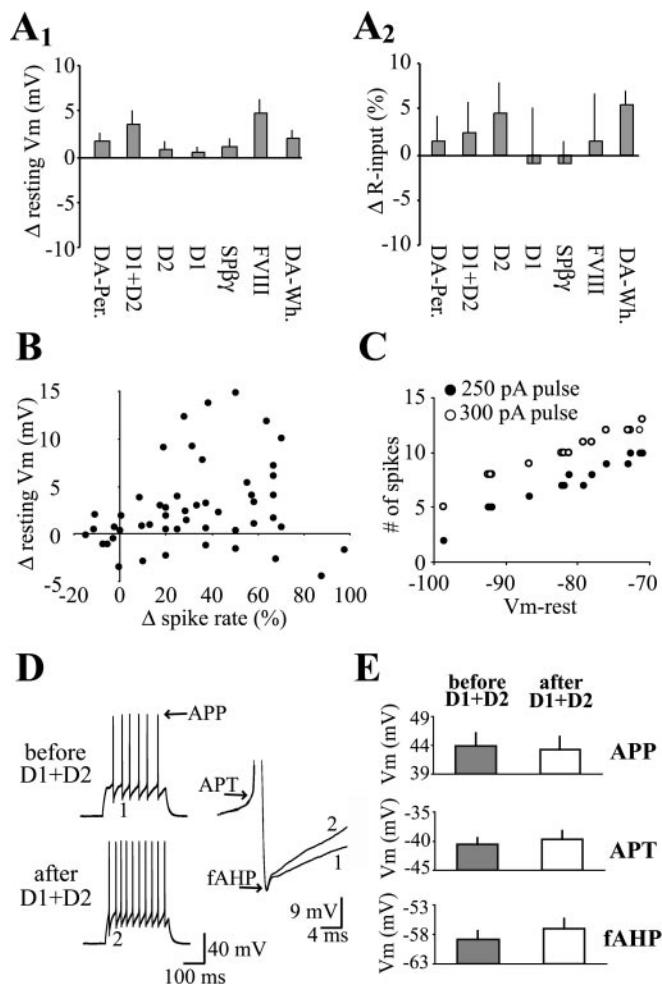


Figure 5. DAergic agonists enhanced spike firing without altering several electrophysiological parameters. *A*, Exposure to DA or D₁ and D₂ agonists (10 μ M), either alone or in combination, did not significantly change resting membrane potential (V_{m-rest} ; A1) or input resistance (A2) of MSNs. DA-Per., DA-Wh., Neurons exposed to DA during perforated patch and whole-cell recording, respectively. *B*, Distribution of percent change in spiking versus change in V_{m-rest} for cells exposed to DAergic agonists. *C*, Example trace showing 3.2% change in spike firing per millivolt increase (calculated for the 250 pA pulse). *D*, Example traces of spike firing before and after exposure to a combination of D₁ and D₂ receptor agonists (250 pA current pulse). Inset, Action potential threshold and width and the relative hyperpolarization peak were unchanged. V_{m-rest} values for the traces shown were -83.7 and -80.1 mV. *E*, Averaged data for APP, APT, and fAHP before (dark column) and after (light column) exposure to D₁ and D₂ receptor agonists in combination.

tween change in V_{m-rest} and change in spike firing ($3.5 \pm 0.4\%$ increase in spike firing per millivolt depolarization; $n = 5$; Fig. 5C; for details of analysis, see Materials and Methods). Thus, for the majority of neurons shown in Figure 5B, the contribution of the change in V_{m-rest} to change in spike firing is negligible. Finally, in several experiments (intracellular perfusion with $G_{\beta\gamma}$, okadaic acid, or norokadaone), we maintained the V_{m-rest} of a neuron at a given potential (either -80 or -90 mV) throughout the experiment by injecting current through the patch amplifier while in current-clamp mode. Of course, in current-clamp mode, the membrane potential would be free to deviate from this V_{m-rest} in response to hyperpolarizing or depolarizing current pulses. Under these conditions, we still could observe an enhancement of spike firing with DAergic agonists, further supporting the possibility that DAergic enhancement of spike firing did not require, and could occur independently from, changes in V_{m-rest} . Thus,

a mechanism other than changes in V_{m-rest} primarily accounts for the elevation in spike firing observed here, although DA-dependent depolarization of V_{m-rest} can occur in a minority of cells and can account for the observed changes in spike firing in these cells.

We also measured a number of features of the action potential and found that DAergic activation did not significantly change action potential threshold (APT), action potential width (APW), action potential peak (APP), fast afterhyperpolarization (fAHP), and magnitude of depolarization by subthreshold current pulses (data not shown; all $p > 0.25$, each parameter tested by one way ANOVA across all groups shown in Fig. 5A1). Figure 5D shows an example cell with an overlay of the action potential before and after exposure to D₁ and D₂ agonists in combination, whereas averaged data for APP, APT, and fAHP are shown in Figure 5E. In particular, several changes that would be predicted by alterations in the function of sodium channels (changes in APT, APW, and APP; Calabresi et al., 1987; Schiffmann et al., 1998) and the delayed rectifier potassium channel (changes in APW and fAHP; Rudy and McBain, 2001) were not observed (Fig. 5D,E).

Thus, DA or the combination of D₁ and D₂ receptor agonists enhanced spike firing without altering baseline parameters or several features of the action potential. Changes in calcium channel function (Surmeier et al., 1995; Cepeda et al., 1998) might underlie the observed pattern, but the experiments described above using calcium channel antagonists suggested that L-, N-, and P/Q-type calcium channels were not required for the DA-related enhancement of spike firing. Instead, our data indicate that the DAergic enhancement of spike firing was mediated by inhibition of the slow A-type potassium channel (I_{As} ; Surmeier et al., 1991; Surmeier and Kitai, 1993; Nisenbaum et al., 1994; Gabel and Nisenbaum, 1998; Mahon et al., 2000).

We tested pharmacological inhibitors of I_{As} , including α -dendrotoxin (α -dtx), which is highly selective for I_{As} , and 4-AP, which is relatively selective for I_{As} at a concentration range of 5–60 μ M (Surmeier et al., 1991; Nisenbaum et al., 1994). All these compounds significantly enhanced spike firing (α -dtx, 0.5 μ M, Fig. 6A,B; $n = 4$; 4-AP, 5–60 μ M, Fig. 6C,D; all $p < 0.01$, paired t test). The enhancement of firing observed with 10 μ M 4-AP persisted in the presence of the combination of calcium channel inhibitors described above ($25.7 \pm 4.2\%$; $n = 4$), suggesting an action via a postsynaptic mechanism. Furthermore, α -dtx and 4-AP significantly occluded the effects of DA (α -dtx, Fig. 6B; $n = 4$; 10 μ M 4-AP, $11.4 \pm 5.1\%$; $n = 5$; 60 μ M 4-AP, Fig. 6D; $n = 6$; all $p > 0.05$, paired t test testing the effect of DA). Occlusion was not simply attributable to a limitation on the number of spikes a neuron could fire after exposure to 60 μ M 4-AP, because application of glutamate (200 μ M) further increased spike rate (Fig. 6D; $p < 0.01$, paired t test). In addition, the enhancement of spike firing by okadaic acid was occluded by ~ 13 min preexposure to 60 μ M 4-AP ($16 \pm 4.3\%$ firing change with okadaic acid in the presence of 4-AP; $n = 4$; $p < 0.05$ vs okadaic acid without preexposure to 4-AP). Taken together, these occlusion experiments suggest that DA, $G_{\beta\gamma}$, okadaic acid, α -dtx, and 4-AP all enhance spike firing by a common mechanism, inhibition of I_{As} .

We also examined the effects of DA and 4-AP on total potassium currents, comprising I_{As} , delayed rectifier, and noninactivating potassium currents (Surmeier et al., 1991; Surmeier and Kitai, 1993; Nisenbaum et al., 1994; Gabel and Nisenbaum, 1998; Mahon et al., 2000; Rudy and McBain, 2001), by performing voltage-clamp experiments in which sodium and calcium currents were blocked (for details, see Materials and Methods). DA

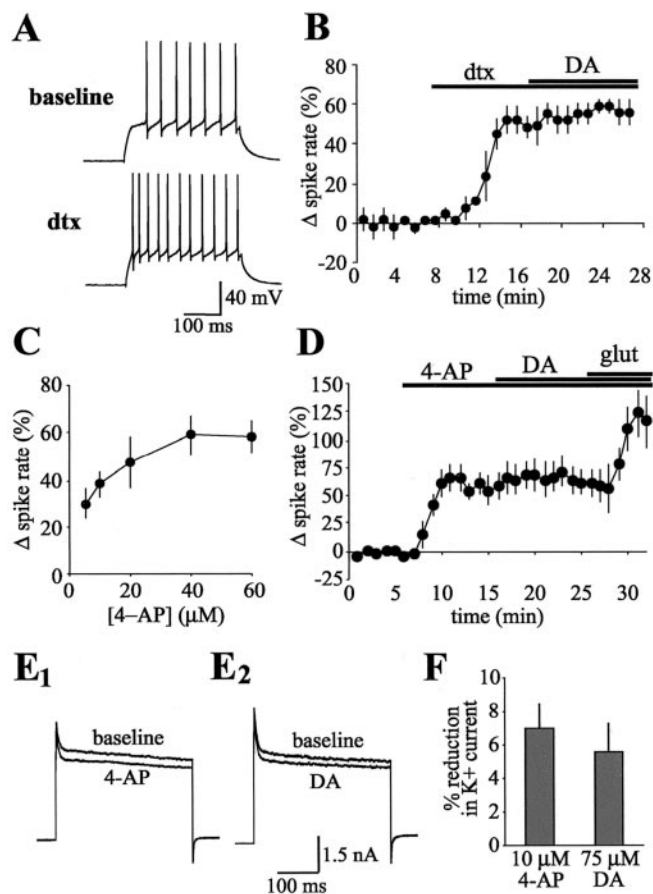


Figure 6. Inhibition of I_{As} increases spike firing. *A*, α -Dendrotoxin (0.5 μ M) increased spike firing. *B*, Preexposure to α -dendrotoxin occluded the effects of DA. *C*, 4-AP increased spike firing in a dose-dependent manner. Data for 5, 10, 20, 40, and 60 μ M 4-AP were collected from 9, 11, 4, 7, and 6 neurons, respectively. *D*, Preexposure to 60 μ M 4-AP occluded the DA-mediated enhancement of spike firing. However, glutamate (glut; 200 μ M) was able to further increase the spike rate significantly. *E*, Example showing that exposure to 4-AP (10 μ M; *E1*) or DA (75 μ M; *E2*) led to a small but significant reduction in the total potassium current evoked during voltage-clamp experiments in which cells were depolarized from -80 to 0 mV. *F*, Compiled data showing reduction of total potassium current by DA or 4-AP.

(75 μ M; $n = 8$) and 4-AP (10 μ M; $n = 6$) produced similar small but significant inhibition in the potassium current evoked by a 300 msec pulse to 0 mV (Fig. 6E,F; DA and 4-AP, both $p < 0.05$ change in current, paired t test), consistent with previous reports showing that I_{As} contributes a minor amount to the total evoked potassium current (Surmeier and Kitai, 1993; Bekkers and Delaney, 2001). However, Bekkers and Delaney (2001) showed that, despite the modest contribution of I_{As} to the total potassium current, inhibition of I_{As} produces a significant enhancement in spike firing, emphasizing the critical role that I_{As} plays in regulation of action potential firing (Nisenbaum et al., 1994; Wickens and Wilson, 1998; Mahon et al., 2000).

Current-clamp experiments of I_{As} in striatal MSNs also suggest that I_{As} is a key regulator of the latency to firing the first action potential during prolonged depolarization (see Fig. 1A) (Nisenbaum et al., 1994; Mahon et al., 2000); thus any condition that inhibits I_{As} should shorten the latency to firing. As shown in Table 1, a significant reduction in latency to fire was observed after exposure to DAergic agonists, forskolin, or antagonists of I_{As} . These data further support the contention that DA, okadaic acid, α -dendrotoxin, and 4-AP enhanced spike firing through inhibition of I_{As} .

Table 1. Changes in latency to firing first action potential with drug exposure, determined at the lowest current step at which spike firing was evoked

Condition	Baseline (msec)	After drug exposure (msec)	Change in latency (msec)*
SKF81297 + quinpirole	160 ± 35	95 ± 18	−66 ± 27
DA	162 ± 28	92 ± 14	−70 ± 25
Forskolin	179 ± 22	66 ± 17	−113 ± 24
4-AP (10 μM)	166 ± 20	77 ± 17	−89 ± 16
4-AP (60 μM)	193 ± 21	53 ± 11	−140 ± 15
α-dtx	174 ± 13	68 ± 26	−106 ± 16

SKF81297 and quinpirole (10 and 10 μM) and DA (75 μM) are from perforated patch experiments.

**p* < 0.05 for all conditions; paired *t* test.

Discussion

The present study shows that DA increases spike firing in MSNs from the NAc shell. This enhancement of spike firing requires coactivation of D₁ and D₂ receptors, because neither agonist alone modifies spike firing, and the effect of DA is inhibited by either a D₁ or a D₂ receptor antagonist. The increased spike firing after coactivation of D₁ and D₂ receptors is mediated intracellularly by G_{βγ} and cAMP-dependent processes. Finally, our biophysical and pharmacological studies suggest that enhancement of spike firing occurs primarily through inhibition of a slow A-type potassium current (*I_A*).

Our results may provide a cellular mechanism to explain observations from behavioral studies suggesting a cooperative action of D₁ and D₂ receptors in the NAc. For example, rats will self-administer D₁ and D₂ agonists into the NAc in combination but will not self-administer either alone (Ikemoto et al., 1997). Both synergistic and additive effects of D₁ and D₂ receptor activation in the NAc have been reported by studies of conditioned reinforcement (Chu and Kelley, 1992; Wolterink et al., 1993). Also, results from studies of amphetamine self-administration (Phillips et al., 1994) and evaluating the relative cost of obtaining a reward (Koch et al., 2000; Nowend et al., 2001) are suggestive of a cooperative role for D₁ and D₂ receptors in the NAc in behavioral expression. Although D₁–D₂ interaction is not observed for all behaviors involving NAc DA (Coccurello et al., 2000), these studies suggest that D₁ and D₂ receptors interact cooperatively in the expression of a number of reward- and motivation-related behaviors mediated by the NAc.

These behavioral observations are quite intriguing, given that D₁ and D₂ receptors are traditionally thought to oppositely couple to the G-protein–PKA system (Missale et al., 1998). G_{βγ} provides a mechanism by which G_s- and G_{i/o}-coupled receptors, such as D₁ and D₂, respectively, can act cooperatively to activate PKA (Sunahara et al., 1996; Watts and Neve, 1997), especially perhaps for behaviors involving NAc PKA signaling (Self et al., 1998; Sutton et al., 2000). Interestingly, a recent paper demonstrated that self-administration of ethanol is significantly reduced after inhibition of G_{βγ} function in the NAc (Yao et al., 2002), in agreement with decreased ethanol consumption observed after block of D₁ or D₂ receptors within the NAc (Hodge et al., 1997). Here, enhancement of spike firing after coactivation of D₁ and D₂ receptors required both G_{βγ}- and cAMP-dependent processes. In particular, intracellular perfusion with G_{βγ} enabled D₁ but not D₂ enhancement of spike firing, indicating that G_{βγ} derived from D₂ was required for a spike firing increase. These data raise the interesting possibility that the DAergic signaling pathway we have identified mediates self-administration of ethanol and perhaps other behaviors.

Our results are also consistent with studies suggesting that

PKA plays a major role in DA signaling in MSNs (Greengard et al., 1999). We should note that there are likely to be multiple forms of DA receptor and G_{βγ} signaling, including presynaptic modulation (McGinty, 1999) and interaction with signaling pathways other than PKA (Seiden et al., 1993; Sunahara et al., 1996; Missale et al., 1998; Hernandez-Lopez et al., 2000). Also, although we used relatively high concentrations of DA (Pennartz et al., 1992a; Nicola and Malenka, 1997), it is likely that the high density of dopamine transporters and strong dopamine transporter activity around MSNs (Uchimura and North, 1990; Jones et al., 1995; Hersch et al., 1997) greatly reduce the extracellular concentration of DA. Finally, the relatively slow onset of response after application of agonists, which has been observed in other studies (Nicola et al., 1996), likely reflects both the time required to superfuse the slice with a reagent and the time for intracellular signaling events to occur. For example, enhancement of spike firing after bath application of direct I_{As} antagonists (e.g., 4-AP) still takes several minutes, suggesting that some delay in onset of drug action is to be expected in brain slice experiments.

Our data also indicate that DA might increase the firing rate of MSNs in the NAc *in vivo*. However, *in vivo* studies of DAergic modulation of MSN spike firing have produced mixed results, with observations of both excitation and inhibition (for review, see Siggins, 1978; Nicola et al., 2000). Several factors might contribute to these discrepancies. First, DAergic reduction of firing might be attributable to inhibition of glutamate release (Pennartz et al., 1992a; Nicola et al., 1996; Nicola and Malenka, 1997). Second, several studies have observed dose-dependent effects of DA, with lower doses activating and higher doses inhibiting firing (Chiodo and Berger, 1986; Wachtel et al., 1989; Williams and Millar, 1990; Hu and White, 1997). In this regard, DA release after stimulation of the ventral tegmental area or the median forebrain bundle, which might result in more moderate DA levels compared with direct application, can strongly enhance spike firing in MSNs (Chiodo and Berger, 1986; Gonon and Sundstrom, 1996). Thus, DA likely has multiple effects, perhaps depending on dose or signaling context, but there is a strong precedent for DAergic activation of MSNs. Of particular interest are recent studies of NAc firing in response to cues that indicate food reward (S. M. Nicola, I. A. Yun, K. T. Wakabayashi, and H. L. Fields, unpublished observations). In some NAc cells, firing rates increase during presentation of the cue, and this enhancement of firing is greatly reduced by VTA inactivation. VTA inactivation or infusion of dopamine receptor antagonists into the NAc also greatly inhibits behavioral responding to the cue. Taken together, these data suggest that DA enhances firing in a set of NAc neurons, and that this change in firing may be important for proper task performance after the cue is observed.

These electrophysiological studies, taken together with the behavioral results described above, suggest that the D₁ and D₂ cooperative interaction we have characterized in brain slices may have particular relevance *in vivo*. However, we should note that, as *in vivo*, *in vitro* studies of DAergic effects on MSN function have produced mixed results (Nicola et al., 2000). Some studies from NAc slices found no DA-related changes in postsynaptic properties (Pennartz et al., 1992a; Nicola et al., 1996; Nicola and Malenka, 1997), whereas others observed significant DA-dependent changes in input resistance or resting membrane potential (Uchimura et al., 1986; Uchimura and North, 1990; O'Donnell and Grace, 1996). The latter may reflect action of DA on cell types other than MSNs (Yasumoto et al., 2002), and such influences were negated here by studying DAergic signaling in relative pharmacological isolation. Some discrepancies may also

relate to differences among MSNs from the dorsal striatum, NAcb core, and NAcb shell (Kelly and Nahorski, 1987; Calabresi et al., 1992; Pennartz et al., 1992b; O'Donnell and Grace, 1993; Paxinos, 1995; Thomas et al., 2000). In particular, D₁ and D₂ receptors in the dorsal striatum are more clearly segregated in the so-called "patch" and "matrix" compartments, whereas such distinction is much less clear in the NAcb shell (Paxinos, 1995). Because slice studies from the dorsal striatum have generally not addressed the compartmental localization of the neurons under investigation, differential signaling among compartments could contribute to the variety of DAergic effects observed *in vitro* (for review, see Nicola et al., 2000).

Pharmacological and biophysical analyses suggest that the DA-mediated enhancement of spike firing observed here was mediated by inhibition of I_{As} and was not associated with a change in function of several other channel types, including sodium and L-, N-, and P/Q-type calcium channels, although we should note that other groups have observed modulation of sodium channels by DA in the NAcb (Zhang et al., 1998). Pharmacological inhibitors of I_{As} , such as α -dendrotoxin and 4-AP (5–60 μ M), enhanced spike firing and occluded the effects of DA on spike firing. Also, using voltage-clamp methods, we observed a small but significant inhibition of potassium currents by 4-AP and DA (Surmeier and Kitai, 1993). In this regard, Bekkers and Delaney (2001) found that inhibition of I_{As} produced only a small decrease in total potassium current but led to a significant enhancement in spike firing, consistent with the critical role I_{As} contributes to action potential firing (Nisenbaum et al., 1994; Wickens and Wilson, 1998; Mahon et al., 2000). Although these results, particularly occlusion of DAergic effects after inhibition of I_{As} function, suggest that DA enhances spike firing by inhibiting I_{As} , we should acknowledge that additional effects beyond those on I_{As} cannot be definitively excluded, and that changes in V_{m-rest} in particular can account for the changes in spike firing in a small percentage of cells.

On the basis of analyses of the up and down state transitions and biophysical properties, it has been suggested that potassium channels are key regulators of excitability of MSNs (Wickens and Wilson, 1998). I_{As} activates at voltages around spike threshold, and inhibition of I_{As} may allow previously subthreshold synaptic input to elicit action potential firing (Nisenbaum et al., 1994; Wickens and Wilson, 1998; Mahon et al., 2000). In agreement, we found that DAergic activation increased the number of spikes fired during synaptic stimulation. Our data predict that DA will enhance the number of spikes fired *in vivo* during an up-state transition or during any other coherent glutamate excitation, with little effect on the hyperpolarized down state. This is consistent with the idea that DA is modulatory and normally requires glutamate receptor activation for DAergic effects to be observed (Chiodo and Berger, 1986; Nicola et al., 2000). Thus, by inhibition of I_{As} , coactivation of D₁ and D₂ receptors in the NAcb shell could enhance glutamate-mediated cellular excitation and thereby contribute to the expression of goal-directed behaviors.

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