Cellular/Molecular

D_2 Autoreceptors Chronically Enhance Dopamine Neuron Pacemaker Activity

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Activation of D_2 autoreceptors on midbrain dopamine neurons has been shown previously to acutely open K^+ channels to inhibit intrinsically generated pacemaker activity. Here we report that D_2 autoreceptors act chronically to produce an opposite action: to increase the speed and regularity of repetitive action potential firing. Voltage-, current-, and dynamic-clamp experiments, using conventional whole-cell and perforated patch-clamp recording, with cultured rat midbrain dopamine neurons show that a change in the number of functional A-type K^+ channels alters firing rate and susceptibility to irregularity produced by other channels. cAMP and protein kinase A mediate the long-term action of D_2 receptors in a manner that counters the short-term effect of this signaling pathway on K^+ channel gating. We conclude that D_2 autoreceptors, in addition to mediating acute negative feedback, are responsible for long-term enhancement of the rate and fidelity of dopamine neuron pacemaker activity.

Key words: **dopamine neuron; K^+ channel; D_2 receptor; channel noise; pacemaker; antipsychotic drug**

Introduction

Midbrain dopamine (DA) neurons are important in normal motor control, cognitive function, and reward behavior, and impairment of their function is involved in neurological and psychiatric diseases, such as Parkinson's disease, schizophrenia, and drug addiction (Egan and Weinberger, 1997; Olanow and Tatton, 1999; Spanagel and Weiss, 1999; Berke and Hyman, 2000; Greengard, 2001; Schultz, 2004). Repetitive DA neuron firing, which serves as the baseline for detection of bursts that encode unexpected reward (Schultz, 2002, 2004), reflects the interplay of voltage-gated ion channels that generate intrinsic pacemaker activity and synaptic inputs. In addition to typical glutamatergic and GABAergic synapses, DA neurons are influenced by D_2 autoreceptors that induce rapid hyperpolarization via activation of K^+ channels (Lacey et al., 1987; Liu et al., 1994; Kim et al., 1995). The significance of this acute negative feedback is revealed by the acute DA neuron excitation induced by D_2 antagonists (Pucak and Grace, 1994, 1996; Werkman et al., 2001).

Recently, it was found that DA neuron intrinsic pacemaker activity is subject to long-term regulation. Specifically, *in vivo* treatment with the antipsychotic drug haloperidol (Hal) for 5 or 6 d persistently reduces the rate and regularity of spontaneous firing of isolated DA neurons (Hahn et al., 2003). The change in rate was attributed to upregulation of A-type K^+ channels, but the basis for more inconsistent pacing, which has also been seen *in vivo* with extracellular recordings (White and Wang, 1983), is not understood. The change in regularity could reflect an increase in channel noise (White et al., 2000), but upregulating A-type K^+ channels would reduce the noise in the macroscopic A-type current. Hence, many channel types could be involved in long-term regulation of DA neurons. Although the biophysical basis of this effect remains unclear, long-term modulation of pacemaking could influence responses to reward and the effects of therapeutic and addictive drugs.

Mechanistically, the remodeling of DA neuron excitability has not seemed approachable because lesion experiments led to the conclusion that long-term effects on DA neurons require intact feedback circuitry (White and Wang, 1983) and so cannot be studied *in vitro*. An alternative interpretation is that lesions perturb conditions that are permissive for more direct effects on DA neurons. With this hypothesis in mind, we studied primary cultures of dissociated midbrain DA neurons that lack native synaptic targets and anatomical cues required for organized circuitry. We show that the delayed and sustained regulation of DA neuron pacemaking activity by haloperidol induced *in vivo* is recapitulated *in vitro*. We then use this experimental system along with the dynamic-clamp technique to determine how pacemaking is controlled on the timescale of days. Unexpectedly, long-term regulation of K^+ channel number is responsible for the changes in both rate and regularity, and D_2 autoreceptors and cAMP alter channel number in a manner that counters their short-term effects on channel gating (Lacey et al., 1987; Liu et al., 1994; Yang et al., 2001). Thus, the timescale of signal integration determines whether the action of D_2 autoreceptors/cAMP signaling is excitatory or inhibitory.

Materials and Methods

Cell preparation and drug treatment. The ventral mesencephalon was dissected from postnatal day 1 Sprague Dawley rats using a previously

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described method (Cardozo and Bean, 1995). Dissected tissue pooled from a litter was incubated with 20 U/ml papain (Worthington, Freehold, NJ) at 37°C for 1 h. The tissue was triturated using a fire-polished Pasteur pipette in trituration solution: Basal Medium Eagle (BME) with 1 mg/ml each of trypsin inhibitor and bovine serum albumin, 20 mM glucose, and 10 mM HEPES, pH 7.3. The suspension of dissociated cells was then layered on top of centrifugation solution (BME with 10 mg/ml each trypsin inhibitor and bovine serum albumin) and spun for 8 min at 200 × *g*. The pellet was resuspended in trituration solution, and the yield was determined by counting cells with a hemocytometer. A total of 35,000 cells were plated on 31 mm poly-lysine-coated glass coverslips. Cultures were maintained in a humid atmosphere of 5% CO_2/95% air at 37°C and were fed twice weekly with feeding medium [BME with N2 supplement (Invitrogen, Carlsbad, CA), penicillin/streptomycin, 2% rat serum, 0.6 mM glutamine, 10 mM glucose, and 10 mM HEPES, pH 7.3] until recordings were performed on 12 to 14-d-old cultures. For chronic treatment experiments, compounds or vehicle were added at 7 or 8 d after generation of cultures and replenished with changes in medium every 3 or 4 d until recordings were performed 5 or 6 d later. For 1 d sulpiride (Sul) treatments, the drug or vehicle was added 1 d before recording from 12- to 14-d-old cultures. When multiple compounds were required, they were added simultaneously. Haloperidol at 100 nM (Research Biochemicals, Natick, MA) was added to medium from 100 μM stock solution, and 1 μM sulpiride (Tocris Cookson, Ballwin, MO) was added to medium from 1 mM stock solution in 70% ethanol and Tyrode's solution (1:10). Quinpirole (Quin), 8-bromo-adenosine 3,5-cyclic monophosphate (8-Br-cAMP), and H-89 (*N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinoline-sulfon-amide 2HCl) (Sigma, St. Louis, MO) were dissolved in water and used at final concentrations of 1 μM, 1 mM, and 1 μM, respectively.

Electrophysiology. DA neurons were identified by fluorescence after loading with 5,7-dihydroxytrpytamine with a 40×/340 UV Olympus Optical (Thornwood, NY) objective using 340 nm light for excitation and a 420 nm long-pass filter for emission fluorescence (Silva et al., 1988; Cardozo and Bean, 1995; Hahn et al., 2003). These cells spontaneously fired long-duration action potentials followed by marked afterhyperpolarizations, as has been shown in acutely dissociated midbrain DA neurons (Hahn et al., 2003). Conventional whole-cell recordings, with 5–6 MΩ of series resistance, were performed in current-clamp and voltage-clamp modes at room temperature. Leak subtraction was performed with p/5 protocols, and 70% series resistance compensation was used with conventional patch-clamp recordings. Electrodes were filled with a solution containing the following (in mM): 130 KCl, 4 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES, and 2 ATP, pH 7.4. The bath solution contained the following (in mM): 150 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.4, and a mixture of synaptic transmission blockers (50 μM AP-5, 10 μM CNQX, and 50 μM picrotoxin). However, for measurements of steady-state A-type channel inactivation and recovery from inactivation, the bath solution was supplemented with 50 mM tetraethylammonium, and the pipette solution contained the following (in mM):140 KCl, 2 $MgCl_2$, 1 EGTA, and 10 HEPES, pH 7.4. Thus, all recordings were performed in the absence of D_2 receptor and cAMP/protein kinase A (PKA) drugs so that only persistent effects of pretreatments were detected.

The dynamic-clamp setup and the virtual dopamine neuron A-type K^+ conductance have been described previously (Kullmann et al., 2004). Notably, to avoid problems with current clamp and series resistance compensation associated with some patch-clamp amplifiers, a true current clamp (Axoclamp 2B; Molecular Devices, Sunnyvale, CA) equipped with a bridge circuit was used in dynamic-clamp experiments (Kullmann et al., 2004). Virtual channels, modeled on previous measurements from DA neurons (Hahn et al., 2003) as described previously (Kullmann et al., 2004), are quantified in terms of g^-, the maximum conductance in the gating model.

In perforated patch-clamp recordings, patch pipettes were tip filled with a solution consisting of 140 mM K-gluconate, 5 mM KCl, 10 mM HEPES, 0.1 mM EGTA, and 2 mM $MgCl_2$, pH 7.35, and backfilled with the same solution containing amphotericin B (0.4 mg/ml). After access resistance had dropped to <20 MΩ, firing activity was recorded in episodes (20–60 s) alternating without and with the dynamic-clamp engaged. Because spontaneous pacemaker activity of perforated patch-clamped cultured dopamine neurons is slow (Ingram et al., 2002), a small direct current (20–70 pA) was injected to increase firing rate to between 0.5 and 2 Hz. Regardless of firing rate, adding or subtracting virtual A-type channels with the dynamic clamp affected rate and regularity of action potential activity in every DA neuron tested.

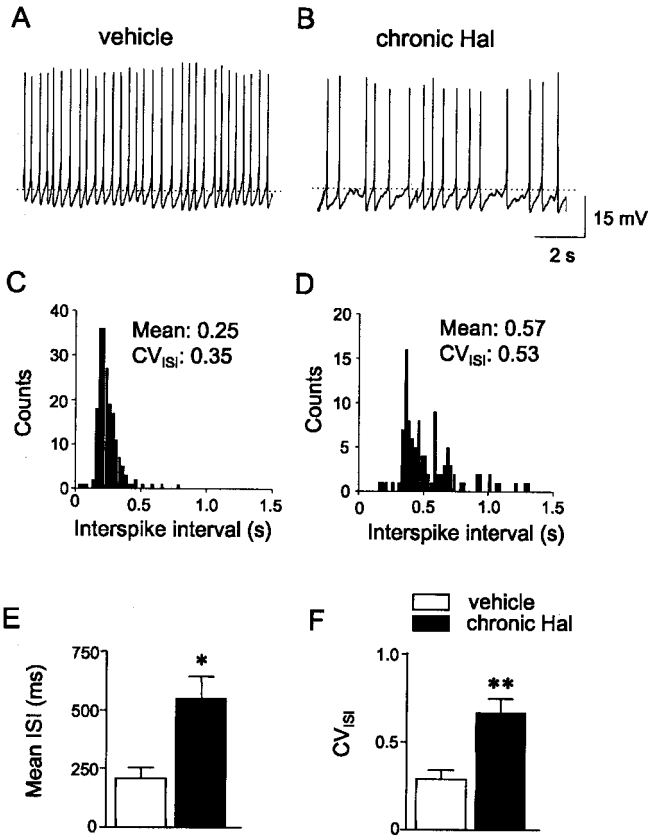

Figure 1. Chronic haloperidol treatment alters DA neuron intrinsic pacemaker activity *in vitro*. Whole-cell current-clamp recordings from cultured DA neurons treated with vehicle (***A***) or chronic Hal (***B***) for 6 d. Spontaneous firing activity was recorded without current injection. Dashed lines indicate −40 mV. ***C***, ***D***, ISI histograms from 60 s of data from the cells shown in ***A*** and ***B***, respectively. Mean ISI (***E***) and CV_{ISI} (***F***) from cultured DA neurons (vehicle, $n = 4$; chronic Hal, $n = 4$). $^*p < 0.05$; $^{**}p < 0.01$.

Data are expressed as mean ± SEM. Statistical significance of paired comparisons was assessed by Student's *t* test unless otherwise indicated.

Results

The long-term *in vivo* effects of haloperidol are recapitulated by DA neurons in culture

To emulate previous *in vivo* experiments (Hahn et al., 2003), spontaneous rhythmic activity was measured from 12- to 14-d-old DA neuron cultures that had been treated with vehicle or 100 nM haloperidol for 5 or 6 d. Whole-cell recordings from identified DA neurons, performed in the absence of haloperidol to reveal persistent effects, showed that the chronic application of haloperidol decreased spontaneous firing (Fig. 1*A*,*B*). Interspike interval (ISI) histograms display a rightward shift in the peak and a widening of the distribution (Fig. 1*C*,*D*). Data from independent experiments show that the induction of slower firing (quantified as the mean ISI; vehicle, 208.7 ± 45 ms; chronic Hal, 550 ± 93 ms; $p < 0.05$) and more irregular spontaneous pacemaker activity [quantified as the coefficient of variation in the ISI (CV_{ISI}); vehicle, 0.29 ± 0.05; chronic Hal, 0.67 ± 0.08; $p < 0.01$] is reproducible (Fig. 1*E*,*F*). Thus, the alteration of DA neuron

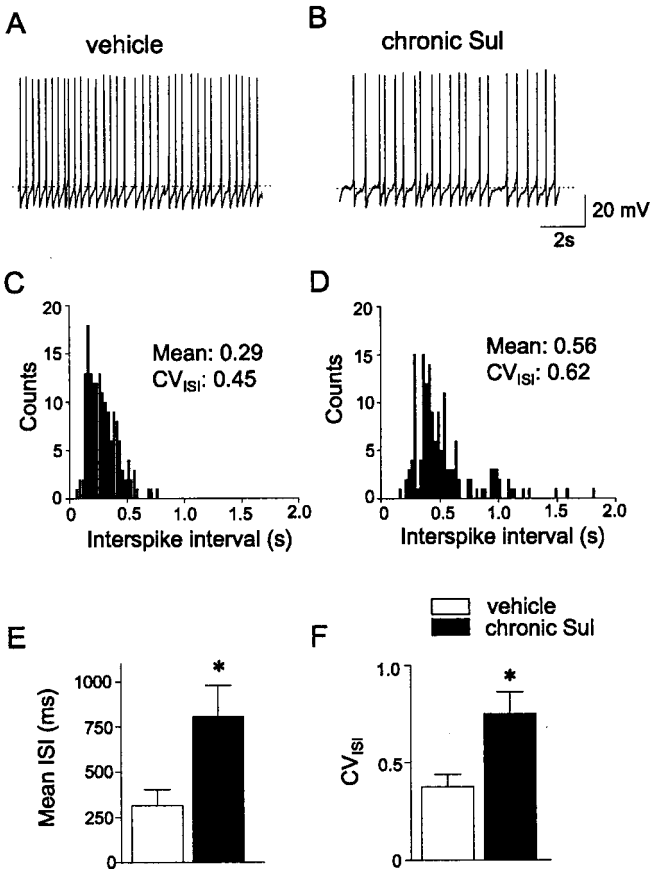

Figure 2. Chronic sulpiride treatment mimics the haloperidol effect on DA neuron intrinsic pacemaker activity. Whole-cell current-clamp recording from cultured DA neurons treated with vehicle (***A***) and chronic Sul (***B***) for 6 d. Spontaneous firing activity was recorded without current injection. Dashed lines indicate −40 mV. ***C***, ***D***, ISI histograms from 60 s of data from the cells shown in ***A*** and ***B***, respectively. Mean pacemaker ISI (***E***) and CV_{ISI} (***F***) from cultured DA neurons (vehicle, $n = 9$; chronic Sul, $n = 10$). *$p < 0.05$.

pacemaker activity originally identified with *in vivo* drug treatment is reproduced *in vitro*, showing that this plasticity does not depend on the organized connectivity found in the brain.

D_2 autoreceptors produce the long-term changes in DA neuron excitability

The mechanism of the long-term effect of haloperidol on DA neuron pacemaker activity was unknown because this drug blocks many types of channels and receptors. However, therapeutic efficacy has been attributed to antagonism of D_2 receptors. D_2 receptors are present on both postsynaptic targets and the DA neurons themselves, but the only neurons that express D_2 receptors in our midbrain cultures are the DA neurons. Because these autoreceptors are acutely inhibitory, one would expect a D_2 antagonist to be excitatory, which contrasts with the effect in Figure 1. Nevertheless, to test for involvement of D_2 autoreceptors, we examined the effect of pretreatment with the selective D_2 antagonist sulpiride.

As can be seen from representative whole-cell current-clamp recordings (Fig. 2*A*,*B*) and the accompanying histograms (Fig. 2*C*,*D*), chronic treatment with 1 μM sulpiride slowed spontaneous pacemaker activity. Quantitative analysis of intrinsic activity showed that the ISI and the CV_{ISI} each changed, again indicating less firing (Fig. 2*E*, ISI) (vehicle, 314.8 ± 89 ms; chronic Sul, 805.3 ± 172 ms; $p < 0.05$) and more irregular activity (Fig. 2*F*, CV_{ISI}) (vehicle, 0.37 ± 0.06; chronic Sul, 0.75 ± 0.11; $p < 0.05$). Thus, these results indicated for the first time that D_2 autoreceptors could be responsible for the long-term effect on intrinsic excitability.

If this conclusion is correct, then sulpiride and haloperidol should share the same molecular targets. Current-clamp recordings from cultured DA neurons treated with sulpiride showed increases in the latency to the first action potential (vehicle, 178 ± 36 ms; chronic Sul, 501 ± 138 ms; $p < 0.05$) and in the first interspike interval (vehicle, 109.2 ± 23 ms; chronic Sul, 201 ± 32 ms; $p < 0.05$) after releasing clamped neurons from a negative potential, −80 mV (Fig. 3*A*). Because these effects are consistent with enhanced A-type K^+ channel activity, we performed whole-cell voltage-clamp recordings to determine whether chronic sulpiride changes A-type K^+ current. Treatment with sulpiride for 1 d did not affect A-type K^+ current density (vehicle, 264 ± 44 pA/pF, $n = 7$; chronic Sul, 224 ± 31 pA/pF, $n = 8$), but the peak amplitude of this transient current increased in DA neurons treated with sulpiride for 5 or 6 d (Fig. 3*B*). Indeed, A-type K^+ current density significantly increased (vehicle, 381.4 ± 39 pA/pF; chronic Sul, 593.9 ± 42 pA/pF; $p < 0.05$), whereas there was no statistically significant change in non-inactivating outward current density with chronic sulpiride treatment (Fig. 3*C*) (vehicle, 248.2 ± 39 pA/pF; chronic Sul, 320.5 ± 33 pA/pF; $p > 0.05$). DA neuron A-type K^+ current density was also increased by chronic haloperidol treatment (Fig. 3*D*) (vehicle, 375.7 ± 40 pA/pF; chronic Hal, 557 ± 67 pA/pF; $p < 0.05$). The regulation of the same channel type by haloperidol and sulpiride further suggests that D_2 autoreceptors induce the long-term change in DA neuron excitability.

Use of a selective D_2 agonist verified the involvement of D_2 receptors. If the long-term control of A-type K^+ channels by sulpiride is mediated by blocking D_2 autoreceptors, then a selective D_2 agonist such as quinpirole should interfere with the effect of this competitive inhibitor. In accordance with this prediction, quinpirole inhibited the increase of A-type K^+ current density caused by sulpiride (Fig. 3*E*) (vehicle, 445.5 ± 108 pA/pF; chronic Sul, 710 ± 66 pA/pF; $p < 0.05$; chronic Quin, 498 ± 58 pA/pF; chronic Sul plus Quin, 431 ± 40 pA/pF; $p > 0.05$). Because two D_2 receptor antagonists increase A-type channel activity and a selective D_2 receptor agonist interferes with this channel regulation, active D_2 autoreceptors must act over days to reduce A-type K^+ channel activity.

cAMP/PKA signaling mediates long-term A-type K^+ channel regulation

D_2 autoreceptors on DA neurons are coupled to G_i and inhibition of adenylate cyclase (Senogles, 1994). Therefore, D_2 antagonists such as sulpiride relieve this inhibition and increase cAMP to stimulate PKA (cAMP-dependent protein kinase). To test for involvement of this pathway, we first examined whether the non-hydrolyzable cAMP analog 8-Br-cAMP alters the long-term action of sulpiride. Chronic treatment of cultured DA neurons with 1 mM 8-Br-cAMP prevented additional upregulation of A-type channels by sulpiride (Fig. 4*A*) (chronic 8-Br-cAMP, 440 ± 96 pA/pF; chronic Sul plus 8-Br-cAMP, 381 ± 59 pA/pF; $p > 0.05$). This occlusion of the sulpiride effect suggests that cAMP is sufficient to produce long-term enhancement of A-type K^+ current density.

We then tested for a role of PKA in long-term upregulation of A-type channels. Chronic treatment with 1 μM H-89, a PKA inhibitor, did not affect A-type K^+ current density (vehicle, 379 ± 28 pA/pF; chronic H-89, 403 ± 15 pA/pF; $p > 0.05$). This suggests

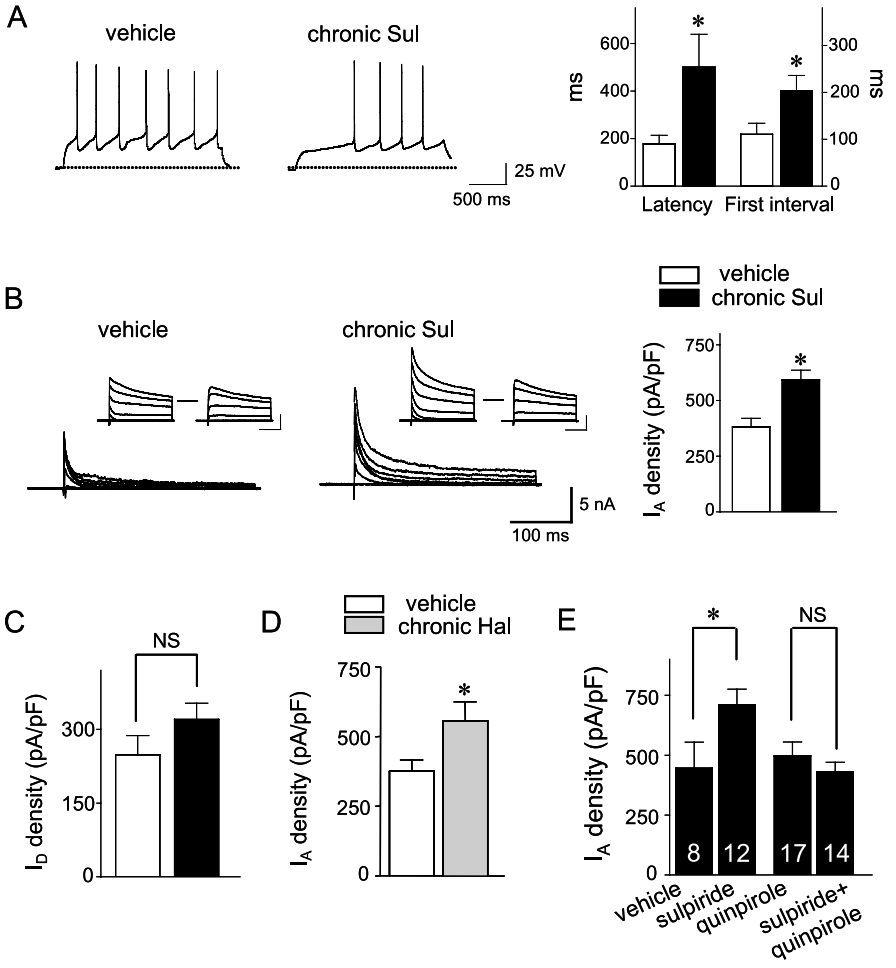

Figure 3. Chronic sulpiride treatment increases A-type K^+ current in DA neurons. ***A***, Left, Whole-cell current-clamp recordings from cultured DA neurons treated with vehicle or chronic Sul for 6 d. Spontaneous activity was recorded when current clamp was released from a holding potential of −80 mV. Right, Mean first spike latency (vehicle, $n = 7$; chronic Sul, $n = 7$) and interspike interval between first and second spikes (vehicle, $n = 8$; chronic Sul, $n = 6$). *$p < 0.05$. ***B***, Left, Whole-cell voltage-clamp recordings from DA neurons treated chronically with vehicle or chronic Sul. Transient currents were obtained by subtracting non-inactivating currents (inset, right traces) from total outward currents (inset, left traces). Currents were evoked by voltage steps in 20 mV increments to −20 to +60 mV from a holding potential of −90 mV for total currents. Calibration (in insets): 100 ms, 5 nA. Non-inactivating currents were evoked with the same protocol after a 500 ms prepulse to −40 mV. Right, Mean current densities of transient K^+ current at 60 mV in vehicle-treated ($n = 26$) and sulpiride-treated ($n = 29$) DA neurons. ***C***, Mean current density of non-inactivating K^+ currents (I_D) at 60 mV is not altered by sulpiride (vehicle, $n = 9$; chronic Sul, $n = 9$). ***D***, Mean transient K^+ current density from cultured DA neurons treated chronically with vehicle ($n = 9$) or chronic Hal ($n = 10$). ***E***, The D_2 agonist quinpirole blocks the increase in A-type channel activity induced by the antagonist sulpiride. The right pair of bars shows that the effect of sulpiride seen in the left pair of bars is abolished in the presence of quinpirole. *$p < 0.05$; NS, not significant.

that there is little baseline activation of PKA in our cultures. However, H-89 inhibited the sulpiride-induced increase in A-type channel activity without causing an effect on its own (Fig. 4*B*) (chronic Sul, 576 ± 60 pA/pF; chronic Sul plus H-89, 441 ± 36 pA/pF; $p < 0.05$, ANOVA analysis). Thus, the cAMP/PKA pathway is necessary and sufficient for the long-term regulation of A-type channel activity by sulpiride. The involvement of this signaling further supports the conclusion that D_2 autoreceptors are responsible for the long-term regulation of A-type channels.

A-type K^+ channels regulate both the rate and the regularity of pacemaker activity

The increase in A-type channel activity produced by blocking D_2 receptors appears to arise from a change in channel number rather than gating. Voltage-clamp experiments showed that the voltage dependence of activation and steady-state inactivation of A-type K^+ current were not affected by chronic sulpiride treatment (Fig. 5*A*,*B*). Likewise, the kinetics of recovery from inactivation did not change (Fig. 5*C*,*D*). Furthermore, the time to reach the peak current at +60 mV, an indicator of rate of activation, and the rate of inactivation of A-type K^+ current were similar in vehicle- and sulpiride-treated neurons (Fig. 5*E*,*F*). Thus, A-type K^+ channel gating was not altered by chronic sulpiride. Because membrane surface area measured as capacitance was not changed by chronic sulpiride treatment (vehicle, 24.4 ± 1.2 pF, $n = 25$; chronic Sul, 23.6 ± 1.5 pF, $n = 25$), the increase in the fast inactivating current with prolonged sulpiride treatment is attributable to an increase in the number of functional A-type K^+ channels.

We then set out to determine whether the change in A-type K^+ channel number alone is responsible for altered spontaneous firing. Because the interspike interval is proportional to A-type channel number in DA neurons (Liss et al., 2001; Hahn et al., 2003), we recorded spontaneous activity and A-type K^+ currents in the same DA neurons after chronic treatment with vehicle or sulpiride. Vehicle-treated DA neurons displayed mean firing frequencies from 1.5 to 4.0 Hz and A-type current densities between 250 and 520 pA/pF at +60 mV ($n = 8$). However, prolonged sulpiride treatment tended to decrease firing frequency (0.3–2.5 Hz) and increase A-type current density (360–1500 pA/pF at +60 mV ($n = 8$). The mean frequency was plotted against the mean A-type current density from the two experimental groups (Fig. 6*A*). The inverse relationship between A-type current density and frequency supports the conclusion that upregulation of A-type channels is responsible for the slowing of intrinsic pacemaking induced by chronic blockade of D_2 autoreceptors.

Dynamic-clamp experiments were performed to directly determine the effect of upregulating A-type K^+ channels on pacemaker activity. Dynamic clamp is a technique in which currents from a computationally modeled conductance can be introduced in real time to living cells (Prinz et al., 2004). It has the advantage of being quickly reversible (i.e., the dynamic clamp can be turned on and off) and does not suffer from the nonspecificity and complexities (i.e., gating and voltage dependence) associated with pharmacological agents. Using this approach, we set out to determine whether manipulation of a virtual A-type K^+ current alone could mimic all or only some of the effects of chronic drug treatment on DA neurons. For this purpose, we used our model A-type current that had been derived using voltage-clamp data from DA neurons (Hahn et al., 2003) and incorporated into the dynamic-clamp system (Kullmann et al., 2004). Whole-cell patch recordings were used to test

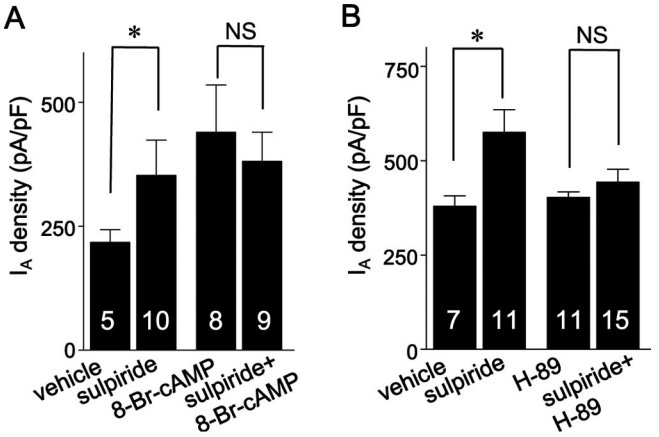

Figure 4. Chronic blockade of D_2 autoreceptors upregulates A-type K^+ currents through cAMP/PKA signaling pathway in cultured DA neurons. ***A***, 8-Br-cAMP (1 mM) prevents additional upregulation of A-type K^+ current density by sulpiride. ***B***, The PKA inhibitor H-89 (1 μM) blocks the effect of sulpiride on A-type K^+ current density. Transient currents density was obtained by the protocol described in Figure 3*B*. *$p < 0.05$; NS, not significant.

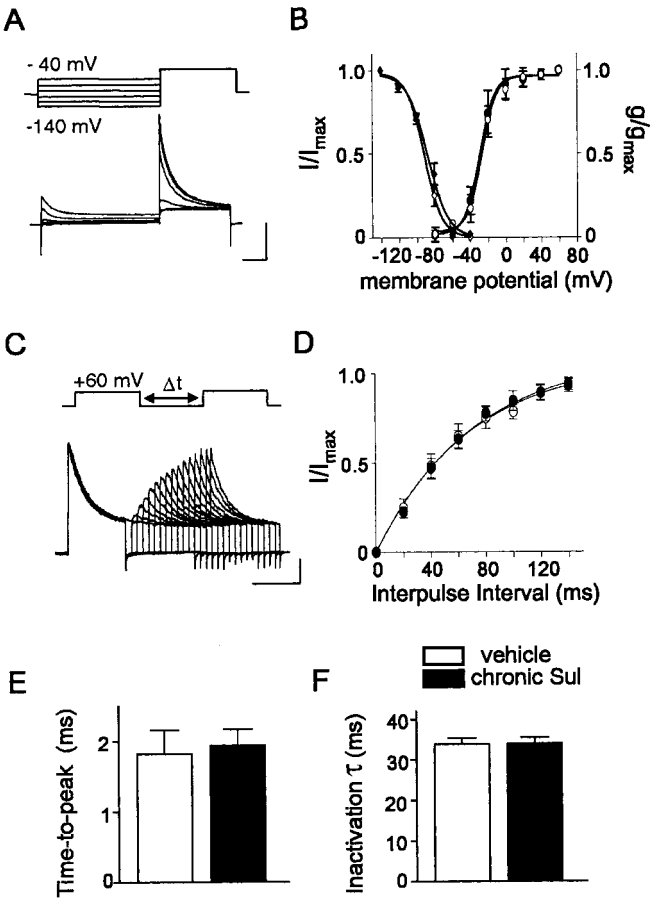

Figure 5. Upregulation of A-type K^+ channel activity occurs without changing gating. ***A*** Example steady-state inactivation current traces. Prepulses were delivered in 20 mV steps from −140 mV, and current was measured at +60 mV. The holding potential was −90 mV. ***B***, Voltage dependence of activation and inactivation of A-type current in DA neurons treated chronically with vehicle or sulpiride. For activation curves, the peak amplitudes were measured from transient K^+ currents generated by the same protocols described in Figure 3*B*. Steady-state inactivation curves were generated by prepulsing to the indicated membrane potential for 0.5 s before the test pulse. Membrane conductances (g) at different voltage levels were obtained by dividing the peak transient K^+ currents by the current driving force and normalized to conductance at +60 mV (g_{max}). The mean data from both groups were fitted with Boltzmann functions, which yielded half-activation potentials of -26.9 ± 1.6 mV for vehicle (○; $n = 6$) and -29.2 ± 1.0 mV for sulpiride (●; $n = 7$). Half-inactivation potentials were -91.1 ± 1.3 for vehicle (◇; $n = 4$) and -87.3 ± 1.8 mV for sulpiride (◆; $n = 4$). ***C***, Recovery from inactivation with a double-pulse protocol. Voltage was stepped to +60 mV with varying interpulse intervals from a holding potential of −90 mV. ***D***, Mean data from vehicle-treated (○; $n = 3$) and sulpiride-treated (●; $n = 5$) DA neurons. ***E***, Time-to-peak for vehicle-treated (open bar; $n = 15$) and sulpiride-treated (filled bar; $n = 15$) DA neurons at +60 mV. ***F***, Inactivation time constant at 60 mV for vehicle-treated (open bar; $n = 15$) and sulpiride-treated (filled bar; $n = 15$) DA neurons.

how virtual A-type K^+ conductance, quantified in terms of the maximum conductance in the gating model ($\bar{g}$), influenced the rate and regularity of DA neuron pacemaking activity.

Dynamic-clamp recordings showed that adding virtual A-type K^+ channels to a cultured DA neuron decreased firing frequency (Fig. 6*B*). Indeed, the mean interspike interval increased in proportion to the added A-type K^+ channel conductance (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Furthermore, the slowing in action potential frequency induced by the addition of dynamic-clamp-mediated A-type K^+ current was reversible (Fig. 6*B*) and reproducible across independent experiments (Fig. 6*C*) (control, 303 ± 50 ms; +150 nS, 577 ± 109 ms; $p < 0.05$). Thus, increasing A-type K^+ channel activity is sufficient to slow DA neuron pacemaker activity.

Additional analysis of dynamic-clamp results showed that adding virtual A-type channels also increased the irregularity of spontaneous pacemaking. Figure 6*B* shows that adding and then removing conductance was sufficient to reversibly broaden and skew the interspike interval distribution. This effect is quantified as a reproducible increase in the coefficient of variation for the interspike interval (Fig. 6*D*) (control, 0.28 ± 0.06; +150 nS, 0.57 ± 0.09; $p < 0.05$).

The computational model of A-type currents implemented in the dynamic clamp was based on differential equations describing macroscopic channel gating, and so noise originating from A-type channels does not change with engagement of the dynamic clamp. Therefore, we can draw two conclusions from these experiments. First, increasing the number of functional A-type channels is sufficient to explain the long-term effects of chronic blockade of D_2 autoreceptors on both frequency and irregularity of DA neuron pacemaker activity. Second, the change in regularity must reflect an indirect effect of A-type K^+ channels that unmasks noise from other channel types that are not regulated by D_2 receptors.

The above analysis relies on dynamic-clamp experiments performed with conventional whole-cell recording. Under these conditions, the cytoplasm is dialyzed with a pipette solution, which could alter the contribution of A-type channels to pacemaking activity. Therefore, we repeated the above dynamic-clamp experiment but used perforated patching to ensure that cells were metabolically intact. Under such conditions, cultured dopamine neuron firing frequency is lower than in our whole-cell recordings (Ingram et al., 2002), possibly because Ca^{2+} chelator in the whole-cell recording solution reduces inactivation of the pacemaker Ca^{2+} channels or activation of Ca^{2+}-activated K^+ channels. Despite this difference, we found that adding virtual A-type channels reversibly increased the ISI and CV_{ISI} in each of the six dopamine neurons studied (Fig. 7*A–C*). Thus, we reproduced the effects originally detected with conventional whole-cell recording with the perforated patch-clamp technique.

We then extended our dynamic-clamp studies by subtracting A-type channel conductance. These independent experiments showed that reducing A-type channel activity lowered both the ISI

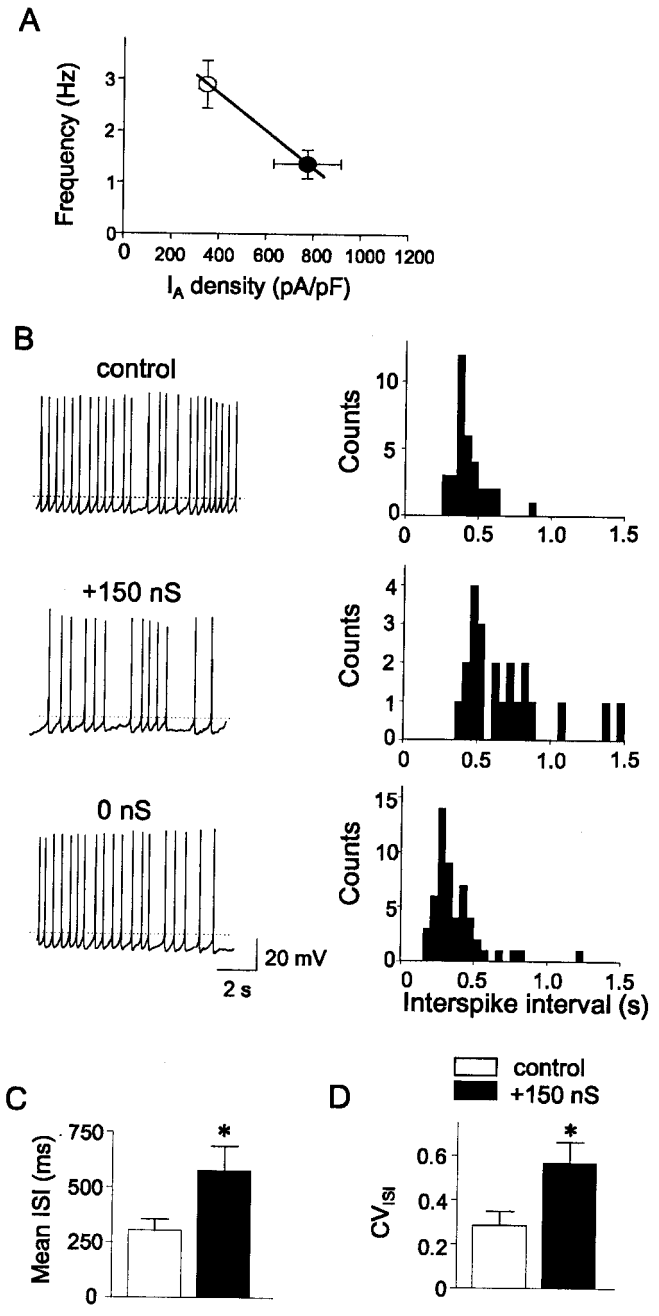

Figure 6. Reversible recapitulation of the effect of chronic autoreceptor blockade on DA pacemaker activity by virtual A-type K^+ channels. ***A***, Pacemaker frequency is inversely proportional to A-type K^+ current density. Spontaneous firing activity and K^+ currents were recorded from the same DA neurons treated chronically with vehicle (○; $n = 8$) or sulpiride (●; $n = 8$). Frequency from each group was plotted against A-type current density from its corresponding group. ***B***, Adding and then removing 150 nS of A-type K^+ channel conductance with the dynamic clamp alters spontaneous firing activity of a cultured DA neuron (left column). Spontaneous firing activity was recorded without current injection. Dashed lines indicate −40 mV. Histograms of interspike interval (right column) from 40 s of data for each condition. The mean interspike interval (***C***) and the coefficient of variation of interspike interval (***D***) from independent cultured DA neurons ($n = 6$). The augmentation of A-type K^+ channel conductance with the dynamic clamp increased the mean value of ISI and CV_{ISI}, respectively. $^*p < 0.05$.

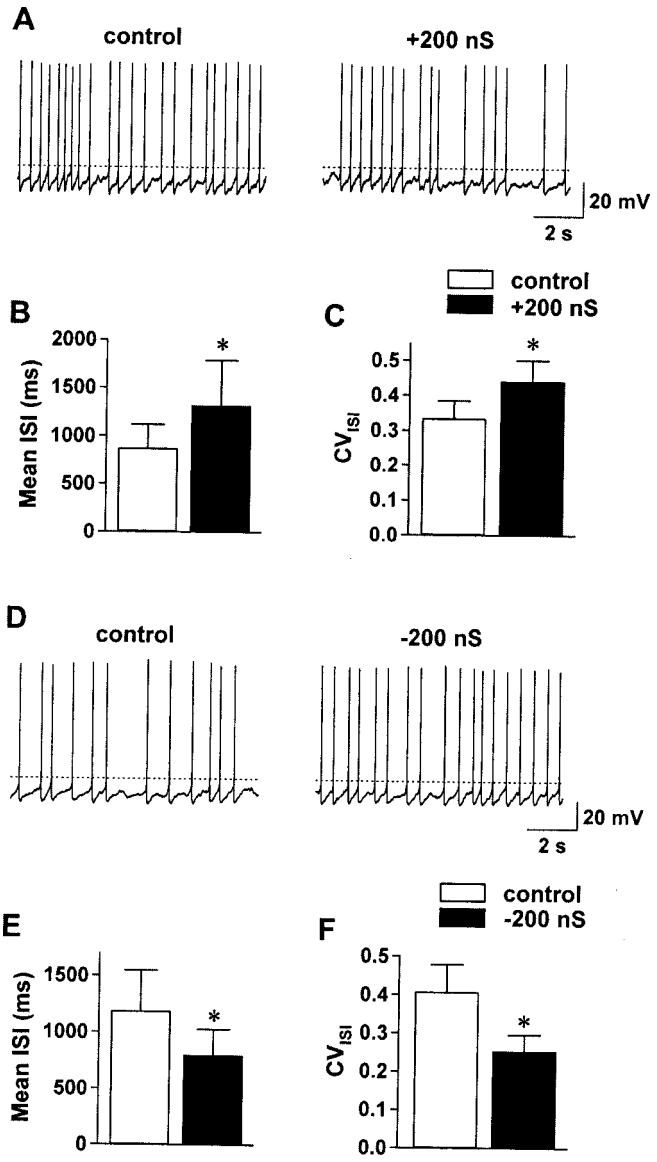

Figure 7. A-type channels control the rate and regularity of pacemaker activity in perforated patch clamped dopamine neurons. ***A***, Action potential activity before (left) and after (right) adding 200 nS of virtual A-type channel conductance. Change in mean ISI (***B***) and CV_{ISI} (***C***) induced by adding virtual A-type channels ($n = 6$). ***D***, Action potential activity before (left) and after (right) subtracting 200 nS of virtual A-type channel conductance. Change in mean ISI (***D***) and CV_{ISI} (***E***) induced by subtracting virtual A-type channels ($n = 6$). Dashed lines indicate −40 mV. $^*p < 0.05$. A Wilcoxon's signed rank test was used in ***B*** because data were not normally distributed.

and CV_{ISI} in every dopamine neuron tested (Fig. 7*D–F*) ($n = 6$). Thus, perforated patch-clamp recordings, along with dynamic clamp, show that the rate and regularity of dopamine neuron pacemaker activity are inversely proportional to A-type channel activity.

Discussion

D_2 autoreceptors acutely inhibit DA neurons and so D_2 antagonists are associated with short-term excitation. However, cultured DA neuron experiments in this study show that D_2 antagonists operate over days to decrease the rate and regularity of DA neuron intrinsic pacemaker activity. This implies that autoreceptors are active in this period and normally have the long-term effect of increasing firing rate and regularity (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). The change in spontaneous activity reflects regulation of A-type channel activity, which also likely affects the impact of synaptic potentials (Schoppa and Westbrook, 1999). Hence, DA neuron spiking, which is driven by intrinsic mechanisms and synaptic inputs, will be more autonomous, rapid, and consistent because of the

long-term regulation of A-type channels by active D_2 autoreceptors. This is relevant because robust, regular activity can serve as a baseline for discrimination of bursts of DA neuron activity that encode unexpected reward (Schultz, 2002, 2004). Furthermore, the long-term A-type channel regulation by active autoreceptors is striking because it opposes the known short-term effects of D_2 receptors and cAMP on channel gating (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Thus, the control of DA neuron excitability changes with the timescale of D_2 autoreceptor activation: over seconds, K^+ channel opening is induced, whereas receptor function over days suppresses K^+ channel number. Finally, our findings indicate that it is the blockade of the latter response that accounts for the long-term dampening of DA neuron pacemaker activity induced by chronic *in vivo* treatment with haloperidol (Hahn et al., 2003).

Our experiments show that A-type K^+ channels control reliability, as well as frequency, of pacemaker activity in cultured DA neurons. Originally, we hypothesized that the modification of pacemaker regularity by chronic haloperidol seen *in vivo* and with acutely dissociated cells (White and Wang, 1983; Hahn et al., 2003) was caused by control of a different channel type. Such a model was consistent with the expectation that an increase in channel noise could produce more irregular firing but that the increase in the number of functional A-type K^+ channels produced by blocking D_2 receptors would reduce the noise in the A-type K^+ current (White et al., 2000). However, because we could acutely and reversibly modify both the rate and regularity of pacemaker activity with the addition and subtraction of dynamic-clamp-generated noiseless A-type channels, we conclude that these channels alone account for the long-term regulation by D_2 autoreceptors.

We suggest that the smaller net inward current below threshold produced by increased outward A-type current has two consequences: pacemaking is slowed and the impact of fluctuations produced by other channels is amplified (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Of course, the impact of the latter fluctuations might depend on experimental conditions (e.g., DA neuron firing is more regular in slices from older animals than in our cultures). However, *in vivo* there are many potential sources of irregularity, including noise in the channels that mediate pacemaker activity, spontaneous hyperpolarizations that are most prevalent in immature animals (Seutin et al., 1998), and synaptic channels. Regardless of the basis of the complexity in DA neuron activity that occurs in the intact brain, our results with cultured neurons suggest that A-type channels serve dual roles as controllers of firing frequency and consistency.

Although long-term changes in DA neuron activity by D_2 antagonists have been attributed to changes in synaptic circuits, the demonstration of autoreceptor-induced remodeling of intrinsic pacemaker activity in cultured DA neurons leads us to conclude that plasticity occurs within DA neurons themselves. Lesions that affected *in vivo* extracellular recordings (White and Wang, 1983) may have acted indirectly to disrupt conditions that are permissive for the control of DA neuron channel expression by autoreceptors. Furthermore, our results show that there must be sufficient DA released by ongoing activity in static cultures to activate D_2 autoreceptors. Nanomolar concentrations of DA acutely hyperpolarize cultured DA neurons (Cardozo, 1993). However, the sensitivity to DA is expected to vary among D_2 receptor-induced effects depending on diverse downstream signaling pathways, the different cellular targets used for controlling channel gating and number, and spare receptors. Therefore, the long-term control of DA neuron excitability could be more sensitive to the transmitter than short-term effects on channel gating. It is also possible that changes in D_2 autoreceptor function induced by unexpected reward or addictive drugs are integrated over long periods to affect channel expression and, hence, future firing activity. Likewise, the regulation described here could be relevant during treatment of schizophrenia because all antipsychotic drugs are D_2 receptor antagonists and take days to exert their therapeutic effects (Kapur, 2004).

Our results, along with previous publications, suggest a mechanism for the long-term D_2 receptor effect on A-type channels. We showed that this effect is mimicked by 8-Br-cAMP and blocked by the PKA inhibitor H-89. Each of these pharmacological agents has limitations in specificity: 8-Br-cAMP activates other cAMP targets (e.g., cyclic nucleotide-gated channels), and H-89 at high concentrations can inhibit kinases in addition to PKA. However, there is no known target that shares sensitivity to this combination of drugs, and the cAMP/PKA pathway is a known target of D_2 receptors. Furthermore, the Kv4.3 gene promoter contains a binding site for the cAMP/PKA-sensitive transcription enhancer cAMP response element-binding protein (CREB) (Patberg et al., 2003). Finally, *in vivo* experiments with haloperidol suggest that a change in Kv4.3 mRNA expression could underlie the long-term A-type channel regulation in DA neurons (Hahn et al., 2003). Therefore, the long-term change in DA neuron pacemaker activity may be mediated by D_2 receptors acting via cAMP/PKA phosphorylation of CREB to control Kv4.3 gene transcription, leading to changes in channel mRNA, protein, and activity.

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