Cellular/Molecular

Dopamine Modulation of GABA Tonic Conductance in Striatal Output Neurons

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We previously reported greater $GABA_A$ receptor-mediated tonic currents in D_2+ striatopallidal than D_1+ striatonigral medium spiny neurons (MSNs) are mediated by α5-subunit-containing receptors. Here, we used whole-cell recordings in slices from bacterial artificial chromosome transgenic mice to investigate the link between subunit composition, phosphorylation, and dopamine receptor activation. Whole-cell recordings in slices from δ-subunit knock-out mice demonstrate that while MSNs in wild-type mice do express δ-subunit-containing receptors, this receptor subtype is not responsible for tonic conductance observed in the acute slice preparation. We assessed the contribution of the β1- and β3-subunits expressed in MSNs by their sensitivity to etomidate, an agonist selective for β2- or β3-subunit-containing $GABA_A$ receptors. Although etomidate produced substantial tonic current in D_2+ neurons, there was no effect in D_1+ neurons. However, with internal PKA application or dopamine modulation, D_1+ neurons expressed tonic conductance and responded to etomidate application. Our results suggest that distinct phosphorylation of β3-subunits may cause larger tonic current in D_2+ striatopallidal MSNs, and proper intracellular conditions can reveal tonic current in D_1+ cells.

Introduction

The majority of neurons in the dorsal striatum, a major nucleus of the basal ganglia, are GABAergic projections called medium spiny neurons (MSNs) that express either dopamine D_1 receptors (D_1+) in the striatonigral pathway or dopamine D_2 receptors (D_2+) in the stratopallidal pathway (Gerfen et al., 1990). Although these two cell types have similar basic physiological properties (Venance and Glowinski, 2003; Day et al., 2006; Taverna et al., 2008), we recently reported that D_2+ MSNs have greater $GABA_A$ receptor-mediated tonic conductance than D_1+ MSNs (Ade et al., 2008). The subunits responsible for tonic conductance are fairly well established in other brain regions (Glykys and Mody, 2007), but those subunits that mediate tonic conductance in the striatum remain elusive.

D_1 and D_2 receptors are G-protein-coupled receptors (GPCRs) and regulate protein kinase A (PKA) phosphorylation via differing second messenger cascade systems. D_1 receptors activate PKA, whereas D_2 agonists inactivate PKA (Chen et al., 2006) through G_s/G_{olf} and $G_{i/o}$ proteins, respectively (Stoof and Kebabian, 1984). PKA activity is essential to the physiological states of $GABA_A$ receptors as β1- and β3-subunits are substrates for PKA-mediated phosphorylation (Moss et al., 1992; McDonald et al., 1998; Flores-Hernandez et al., 2000; Kittler et al., 2005). However, the response to phosphorylation is subunit-specific, and β1-subunit phosphorylation reduces, whereas β3-subunit phosphorylation enhances GABAergic currents (McDonald et al., 1998; Nusser et al., 1999; Flores-Hernandez et al., 2000). Immunostaining studies show that all β-subunits are expressed in the rat striatum (Fritschy and Mohler, 1995; Pirker et al., 2000; Schwarzer et al., 2001), but single-cell PCR results suggest that MSNs do not express the β2-subunit (Flores-Hernandez et al., 2000). Etomidate, a general anesthetic selective for the $\beta2/\beta3$ $GABA_A$ subunits (Hill-Venning et al., 1997), should selectively enhance currents at β3-subunit-containing receptors in MSNs and thus is an ideal tool to detect the presence of this subunit in MSNs. Therefore, all $GABA_A$ receptors in the striatum, both synaptic and extrasynaptic, may potentially be modulated by dopamine and its effects on PKA phosphorylation.

Tonic currents are of particular interest as they control excitability and are differentially expressed in D_1+ and D_2+ neurons. Inhibitory tonic conductance of the dorsal striatum controls the striatonigral and striatopallidal outputs for movement initiation and control. Since Parkinson's disease symptoms result from imbalances in these two pathways (Mallet et al., 2006), striatal tonic conductance may offer a potential therapeutic role in ameliorating physiological manifestations of Parkinsonian symptoms.

In this study, we further investigated the mechanisms underlying tonic conductance in striatal MSNs, and we explored the interactions between dopamine agonists, PKA phosphorylation, and tonic inhibition in MSNs. Our results show that differences in tonic currents are attributable to differential subunit expression patterns and basal phosphorylation rates. Tonic conductance in D_2+ MSNs is attributable to extrasynaptic and basally phosphorylated β3-subunit-containing $GABA_A$ receptors, but D_1+ MSNs also exhibit tonic conductances via phosphorylated

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β3-subunits with internal PKA application or D_1 receptor stimulation. Our studies also suggest that dopamine exhibits influence on cell excitability through these mechanisms.

Materials and Methods

Animals. Bacterial artificial chromosome (BAC) D_2-enhanced green fluorescent protein (EGFP) mice (Gong et al., 2003) (provided by David Lovinger, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD) were crossed with C57BL/6 mice. Slices were prepared from postnatal day 15–23 male and female mice as in Ade et al. (2008), unless otherwise noted. MSNs were classified as being either dopamine D_2 receptor positive (D_2+) or negative by their expression of EGFP. Because previous studies have demonstrated that MSNs express either dopamine D_1 or D_2 receptors (Gerfen et al., 1990; Day et al., 2006), MSNs negative for EGFP in the BAC D_2 EGFP mice were presumed to be D_1+ and those expressing EGFP as D_2+. δ-subunit knock-out mice (provided by Dr. Gregg Homanics, Department of Anesthesiology, University of Pittsburgh, Pittsburgh, PA) were genotyped with Southern blot as described in Mihalek et al. (1999).

Slice preparation. Mice were killed by decapitation in agreement with the guidelines of the American Veterinary Medical Association Panel on Euthanasia and the Georgetown University Animal Care and Use Committee. The whole brain was removed and placed in an ice-cold slicing solution containing (in mM) 85 NaCl, 2.5 KCl, 1 $CaCl_2$, 4 $MgCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, 75 sucrose (all from Sigma). Corticostriatal coronal slices (250 μm) were prepared using a Vibratome 3000 Plus Sectioning System (Vibratome) and were incubated in slicing solution at 32°C for 30 min. Slices recovered for 30 min at 32°C in artificial CSF (aCSF) containing (in mM) 124 NaCl, 4.5 KCl, 1.2 Na_2HPO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, and 10 dextrose at 305 mOsm. During experiments, slices were submerged and continuously perfused (2–3 ml/min) with aCSF at room temperature, 22–24°C, unless otherwise noted. All solutions were maintained at pH 7.4 by continuous bubbling with 95% O_2, 5% CO_2.

Slices were visualized under an upright microscope (E600FN; Nikon) equipped with Nomarski optics and an electrically insulated 60× water-immersion objective with a long working distance (2 mm) and high numerical aperture (1.0). Recording electrodes were pulled on a vertical pipette puller from borosilicate glass capillaries (Wiretrol II; Drummond) and filled with Cs Cl-based internal recording solution (CsCl internal) containing (in mM) 145 CsCl, 10 HEPES, 5 ATP.Mg, 0.2 GTP.NA, and 10 EGTA, adjusted to pH 7.2 with CsOH. In K-gluconate-based internal solutions (K-gluconate internal), CsCl was replaced with equimolar K-gluconate. In some experiments, cell impermeable protein phosphorylation modulators were supplemented in the internal solution. A bovine catalytic subunit PKA (50–75 μg/ml; Sigma) and PKA inhibitory peptide (PKI; 20 μM; Sigma H2N-TYADFIASGRTGRRNAI-amide) were initially prepared in water and maintained at −20°C. The PKA stock solution was used within 3 d of reconstitution according to manufacturer specifications.

Whole-cell recordings. Single and dual voltage-clamp recordings were performed using the whole-cell configuration of the patch-clamp technique at a pipette voltage of −60 mV using the Axopatch 200B and 1D amplifiers (Molecular Devices). Access resistance was monitored during the recordings, and experiments with >20% change were discarded. The baseline membrane potential for current-clamp recordings was set at −70 mV before each series of current step injection protocols. Rheobase current was defined as the first current step, within a series of increasing 20 pA steps, that elicited an action potential.

Stock solutions of bicuculline methobromide (BMR), tetrodotoxin (TTX), 4,5,6,7-tetrahydroisoxazolo{5,4-c}pyridine-3-ol, (THIP), SKF-81297, quinpirole, sulpiride, SCH 23390, and GABA (all from Sigma) were prepared in water. Etomidate (Sigma) was dissolved in dimethylsulfoxide (<0.0001% final concentration). All stock solutions were diluted to the desired concentration in aCSF and applied locally through a Y tube (Murase et al., 1989) modified for optimal solution exchange in brain slices (Hevers and Luddens, 2002).

Currents were filtered at 2 kHz with a low-pass Bessel filter and digitized at 5–10 kHz using a personal computer equipped with Digidata 1322A data acquisition board and pCLAMP9 software (both from Molecular Devices). Off-line data analysis, curve fitting, and figure preparation were performed with Clampfit 9 software (Molecular Devices). Spontaneous and miniature IPSCs (sIPSCs and mIPSCs) were identified using a semiautomated threshold based minidetection software (Mini Analysis; Synaptosoft) and were visually confirmed as in Ade et al. (2008). Briefly, IPSC averages were based on more than 60 events, and

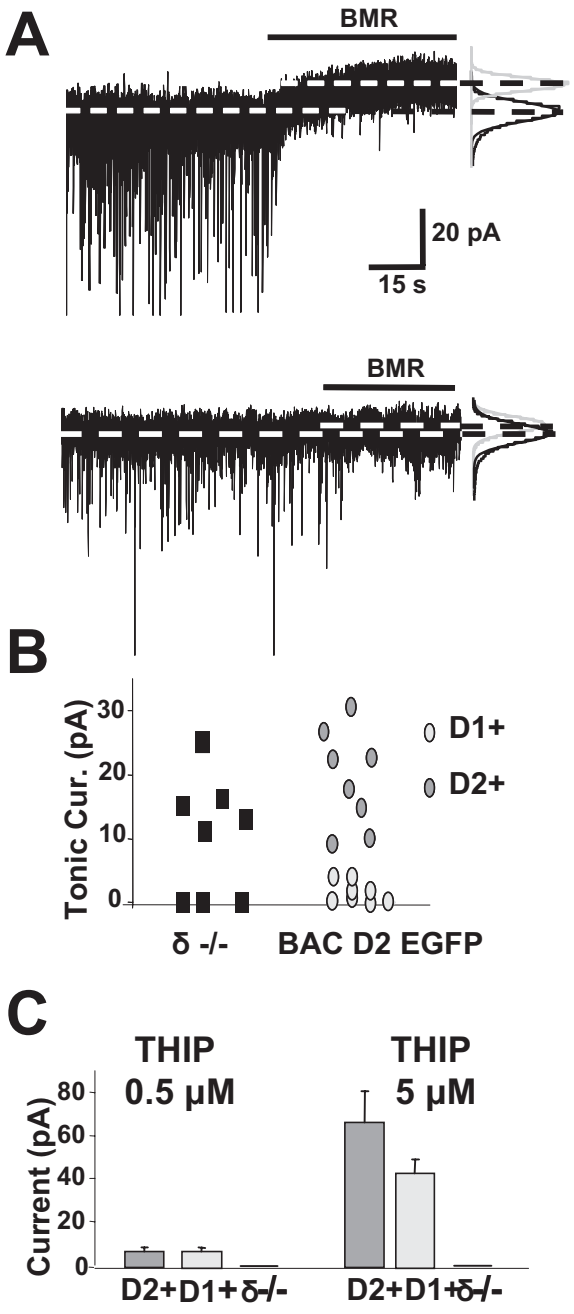

Figure 1. The δ-subunit does not contribute to tonic current. ***A***, Illustrative records from MSNs in a δ−/− mouse showing differential block in tonic current with BMR application. Right, all-points histogram and Gaussian fit from each segment. ***B***, Summary results show that the tonic current expression in a δ−/− mouse resembled the pattern of tonic current expressed in D_2+ and D_1+ MSN from BAC D_2 EGFP mice, suggesting that the δ-subunit is not responsible for the differential tonic currents between D_2+ and D_1+ MSN. ***C***, THIP application in the presence of TTX did not induce tonic current in δ−/− MSNs ($n = 6$), as it did in both D_2+ ($n = 17$) and D_1+ ($n = 6$) MSNs from BAC D_2 EGFP mice, confirming that the δ-subunit is not present.

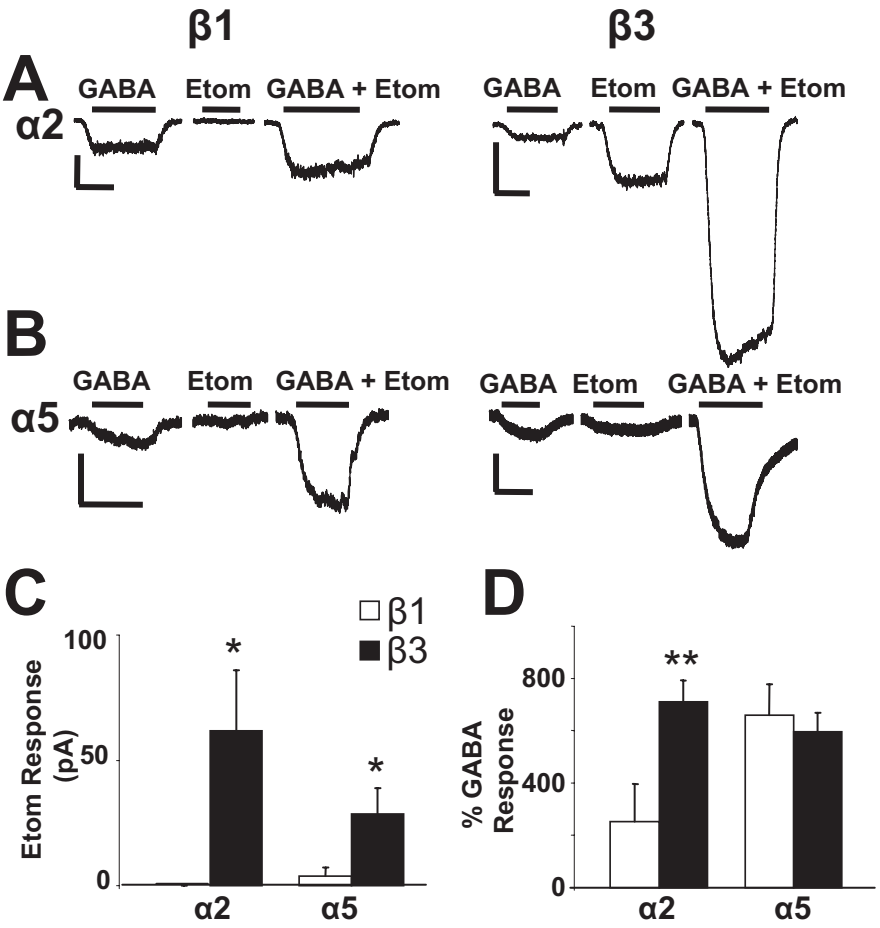

Figure 2. Etomidate's direct effects are selective for β3-containing receptors. ***A***, Representative currents from HEK 293 cells transfected with $\alpha2\beta1\gamma2$ or $\alpha2\beta3\gamma2$ receptors with GABA (3 μM for $\alpha2\beta1\gamma2$, 1 μM for $\alpha2\beta3\gamma2$) and etomidate (3 μM) application, showing β3-subunit selectivity for direct and potentiating effects. Calibration: 200 pA, 10 s. ***B***, Representative currents from HEK 293 cells transfected with $\alpha5\beta1\gamma2$ or $\alpha5\beta3\gamma2$ receptors with GABA (250 nM for $\alpha5\beta1\gamma2$ and $\alpha5\beta3\gamma2$) and etomidate (3 μM) application, showing β3-subunit selectivity for direct effects only. Calibration: 50 pA, 10 s. ***C***, Summary of etomidate's direct effects on striatally relevant $GABA_A$ receptors ($\alpha2\beta1\gamma2$, $n = 6$; $\alpha2\beta3\gamma2$, $n = 10$; $\alpha5\beta1\gamma2$, $n = 5$; $\alpha5\beta3\gamma2$, $n = 7$). ***D***, Summary of etomidate's potentiating effects on GABA currents evoked by EC10 concentrations of GABA on each receptor type ($\alpha2\beta1\gamma2$, $n = 4$; $\alpha2\beta3\gamma2$, $n = 5$; $\alpha5\beta1\gamma2$, $n = 13$; $\alpha5\beta3\gamma2$, $n = 6$).

the decay kinetics were determined using double exponential curve fittings and reported as weighted time constants (tau). All detected events were used for event frequency analysis, but superimposing events were eliminated for the amplitude, rise time, and decay kinetic analysis. Tonic current measurements were made as in Ade et al. (2008). Briefly, an all-points histogram was plotted for a 10 s period immediately before and during BMR application. Tonic currents are represented as the change in baseline amplitude. When PKA or PKI was included in the internal solution, events were analyzed at least 4 min after break-in to allow the peptide to function and equilibrate with the internal components of the cell.

Statistical significance was determined using the two-tailed Student's *t* test (unpaired when comparing two populations of cells and paired when comparing results within the same cell). All values are expressed as mean ± SEM. In all figures, $^*p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$.

Human embryonic kidney 293 cells and transfection. Human embryonic kidney 293 (HEK 293) cells (American Type Culture Collection; CRL1573) were grown in minimal essential medium (Invitrogen), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin (all from Invitrogen) in a 5% CO_2 incubator at 36°C. Growing cells were dispersed with trypsin and seeded at $\sim 2 \times 10^5$ cells/35 mm dish in 2 ml of culture medium on 12 mm glass coverslips coated with poly-D-lysine. The cells were transfected with rat $GABA_A$ receptor subunit cDNAs, because of their high homology to mouse receptors, and EGFP using calcium phosphate precipitation. The following plasmid combinations were used: $\alpha2\beta1\gamma2$, $\alpha2\beta3\gamma2$, $\alpha5\beta1\gamma2$, and $\alpha5\beta3\gamma2$ (all a gift from Peter Seeburg, University of Heidelberg, Heidelberg, Germany) at a ratio of 1:1:4. Mixed plasmids (5 μg total) were added to the dish containing 2 ml culture medium for 8–12 h at which point the media was refreshed. The cells were used for electrophysiological recordings 2–3 d after transfection.

Results

$GABA_A$ receptors in MSNs

Our previous study suggested that the major differences in tonic current between D_1+ and D_2+ MSNs lies in the presence of α5-containing receptors in D_2+ neurons (Ade et al., 2008). Additionally, both D_1+ and D_2+ MSNs had similar sensitivity to low doses of THIP, a δ-subunit-containing $GABA_A$ receptor superagonist (Brown et al., 2002), suggesting that both MSN subtypes express the δ-subunit. To support these results, we performed whole-cell recordings in MSNs in striatal slices prepared from $\delta-/-$ mice. We observed a BMR-sensitive current in a subset of the cells (Fig. 1*A*), and a similar scatter in tonic current expression by distinct MSN subtypes in $\delta-/-$ and BAC D_2 EGFP mice (Fig. 1*B*), supporting the hypothesis that although the δ-subunit is present in MSNs, it is not responsible for tonic current in the age range investigated. However, as shown in Figure 1*C*, MSNs from these mice lost responsiveness to low doses of THIP as previously reported in hippocampal neurons from $\delta-/-$ mice (Glykys et al., 2008).

Because the magnitude of tonic current was not different between BAC D_2 EGFP mice and the $\delta-/-$ mouse, we hypothesized that tonic current in D_2+ cells is mediated by $\alpha5\beta x\gamma2$ receptors. Both β1- and β3-subunits are targets for PKA phosphorylation, and their presence may have robust effects on tonic current. To study the relative β-subunit expression and function in striatal D_2+ and D_1+ cells, we used etomidate (3 μM), a general anesthetic specific for the $\beta2/\beta3$-subunits of the $GABA_A$ receptor (Belelli et al., 1996; Herd et al., 2008). The β-subunit selectivity of etomidate was initially demonstrated in recombinant systems with $\alpha1\beta1\gamma2$- and $\alpha1\beta3\gamma2$-subunit combinations (Slany et al., 1995; Sanna et al., 1997). Subsequently, the differential effects of etomidate between β1- and β2-subunits were shown to be maintained in receptors that contained the α1-, α2-, α3-, or α6-subunits (Hill-Venning et al., 1997). In addition, a study using $\alpha5-/-$ mice demonstrated that etomidate mediates amnestic but not sedative-hypnotic effects by selectively activating the tonic, not phasic, $GABA_A$ currents (Cheng et al., 2006). Because the specificity of this drug has not been tested in recombinant systems that include the α5-subunit, we investigated the efficacy of etomidate in HEK 293 cells transfected with striatally relevant (Fritschy and Mohler, 1995; Pirker et al., 2000; Schwarzer et al., 2001) combinations of α- and β-subunits together with γ2: $\alpha2\beta1\gamma2$, $\alpha2\beta3\gamma2$, $\alpha5\beta1\gamma2$, and $\alpha5\beta3\gamma2$.

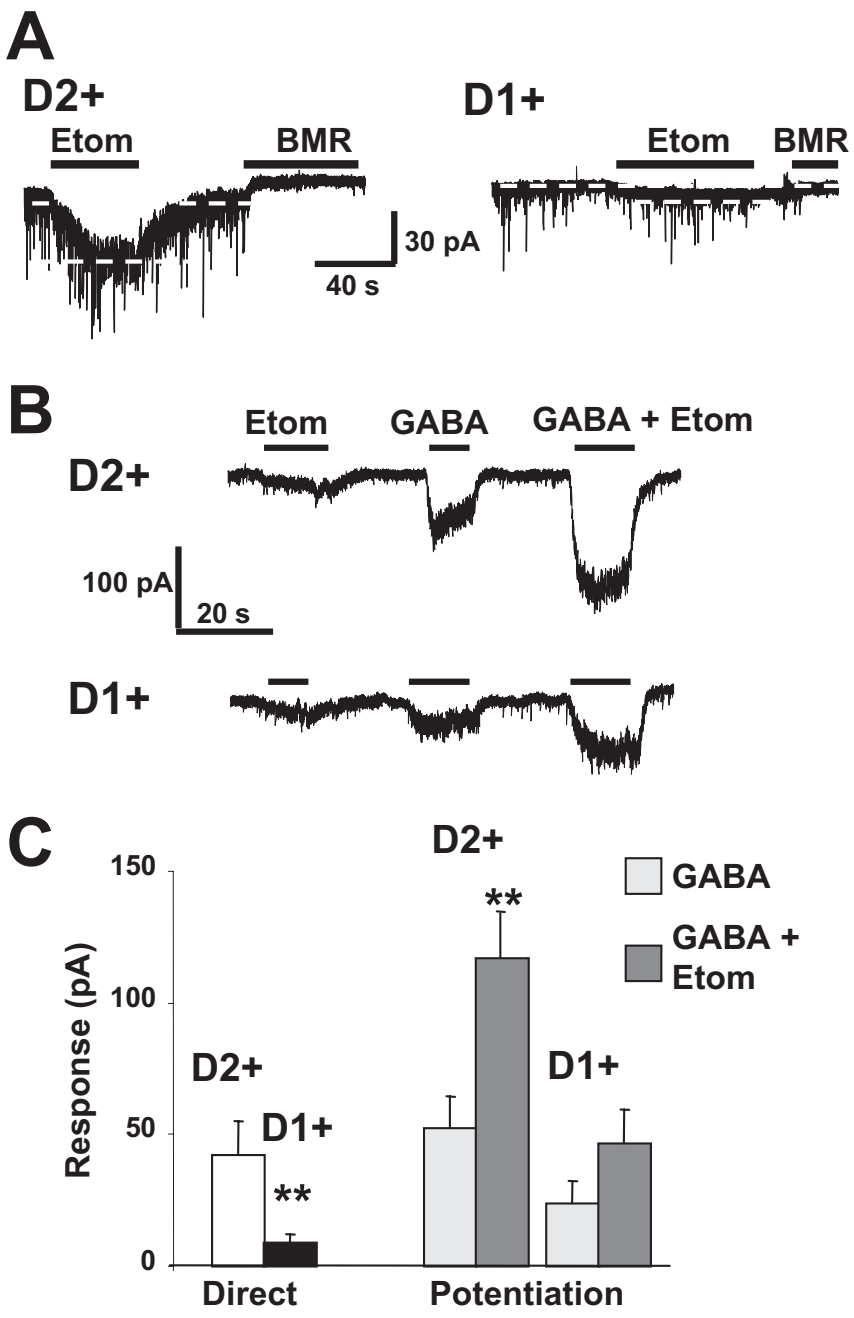

Figure 3. Etomidate selectively activates tonic receptors in D_2+ MSNs. ***A***, Representative current traces of a D_2+ and a D_1+ MSN showing etomidate-elicited currents in the D_2+, but not the D_1+, MSN. ***B***, Representative current traces of two individual MSNs, showing the direct effect of etomidate (3 μM), response to 1 μM GABA, and etomidate's (3 μM) potentiating effects with 1 μM GABA, all in 0.5 μM TTX. ***C***, Summary data of etomidate's direct ($n = 10$, D_2+; $n = 8$, D_1+ MSN) and potentiating ($n = 7$, D_2+; $n = 5$, D_1+ MSN) effects to 1 μM GABA on D_2+ and D_1+ tonic current.

In these cells, we tested both direct activation of the recombinant $GABA_A$ receptors and modulation of GABA responses with etomidate. The general anesthetic produced significant current in cells expressing β3-containing $GABA_A$ receptors but failed to directly activate those cells transfected with the β1-subunit (Fig. 2*A*,*B*), regardless of the α-subunit. In each cell studied, we tested the response to multiple GABA concentrations and compared them to a saturating dose of GABA (3 mM) to select the EC10 used to compare etomidate's direct effects with GABA potentiation. Figure 2, *A* and *B*, shows examples of direct and potentiating effects in transfected cells with $\alpha2\beta1\gamma2$, $\alpha2\beta3\gamma2$, $\alpha5\beta1\gamma2$, and $\alpha5\beta3\gamma2$ receptors. The summary of the results obtained with different α-subunits tested are shown in Figure 2, *C* and *D*. Etomidate robustly potentiated GABA current produced by β3-containing receptors and had a slight potentiating effect on those produced by β1-containing receptors when expressed with the α2-subunit. However, etomidate produced similar potentiating responses in β1- and β3-containing receptors when combined with the α5-subunit. We concluded from these experiments that the direct agonist actions of etomidate are selective for receptors containing β3-subunits but not those containing β1-subunits.

Interestingly, although the maximal GABA response was comparable between the two α2-containing recombinant receptors, the response to 3 μM GABA was $7 \pm 3\%$ ($n = 5$) of the maximal response in $\alpha2\beta1\gamma2$ cells and $31 \pm 7\%$ ($n = 10$) of the maximal response in $\alpha2\beta3\gamma2$ cells ($p < 0.05$). This differential affinity for GABA was not apparent in the α5-containing transfected receptors for any concentrations of GABA (data not shown). However, these receptors had higher sensitivity to GABA than α2-containing receptors (1 μM GABA; $\alpha5\beta1\gamma2$, $25 \pm 4\%$ of maximal response, $n = 18$; $\alpha5\beta3\gamma2$, $17 \pm 2\%$ of maximal response, $n = 23$).

Because striatal MSNs presumably do not express the β2-subunit (Flores-Hernandez et al., 2000), etomidate's effects in these neurons are an indication of the presence of β3-subunit. In Figure 3*A*, we show individual traces from a D_2+ and a D_1+ MSN, and as summarized in Figure 3*C* (left), etomidate produced substantial tonic current in the D_2+ neurons, whereas the response in D_1+ MSNs was significantly smaller and did not differ from baseline. As GABAergic interneurons may express the β2-subunit (Yan and Surmeier, 1997), we repeated these experiments in 0.5 μM TTX to block interneuron activity, given that spontaneous activity of these neurons contributes to tonic current in MSNs (Ade et al., 2008). In TTX, etomidate produced 34 ± 6 pA ($n = 16$) of current in D_2+ MSNs and just 10 ± 2 pA ($n = 13$) in D_1+ MSNs ($p < 0.05$). Tonic current in MSNs is produced by unknown concentrations of ambient GABA. Thus, etomidate effects could be attributable to a combination of direct activation and potentiation of GABA channels. However, ambient GABA is at such low concentrations that any potentiation effect will be minimal. Thus, if the direct activation predominates, it implies that β3-subunit expression is greater in D_2+ than D_1+ MSN.

As D_1+ MSNs do not display endogenous tonic current, we compared the effects of the anesthetic on exogenously applied GABA to determine the potentiating action of etomidate in both cell types. Figure 3*B* shows examples of the etomidate potentia-

tion of current elicited by 1 μM GABA applications in the presence of 0.5 μM TTX in individual D_2+ and D_1+ MSNs. Even in these experimental conditions, the D_2+ MSN exhibited greater potentiating etomidate effects compared with the D_1+ MSN. Similar to etomidate's direct effects, the GABA potentiation with etomidate was significantly larger than the 1 μM GABA response alone in the D_2+, but not the D_1+ MSN (Fig. 3*C*, right).

To assess the etomidate response on synaptic $GABA_A$ receptors, we investigated changes in mIPSCs in the two MSN subtypes. Using CsCl internal solution to enhance detection of mIPSCs from distal locations, basic properties of mIPSCs did not differ between MSN subtypes as reported previously (Ade et al., 2008). Figure 4*A* shows examples of mIPSCs recorded in the presence and absence of 3 μM etomidate together with the overlapping averaged mIPSCs. Figure 4*B* summarizes changes in frequency, amplitude, decay, and rise time of mIPSCs obtained in the two cell types. These data suggest that D_2+ and D_1+ cells have a similar complement of β3-subunits at synaptic locations.

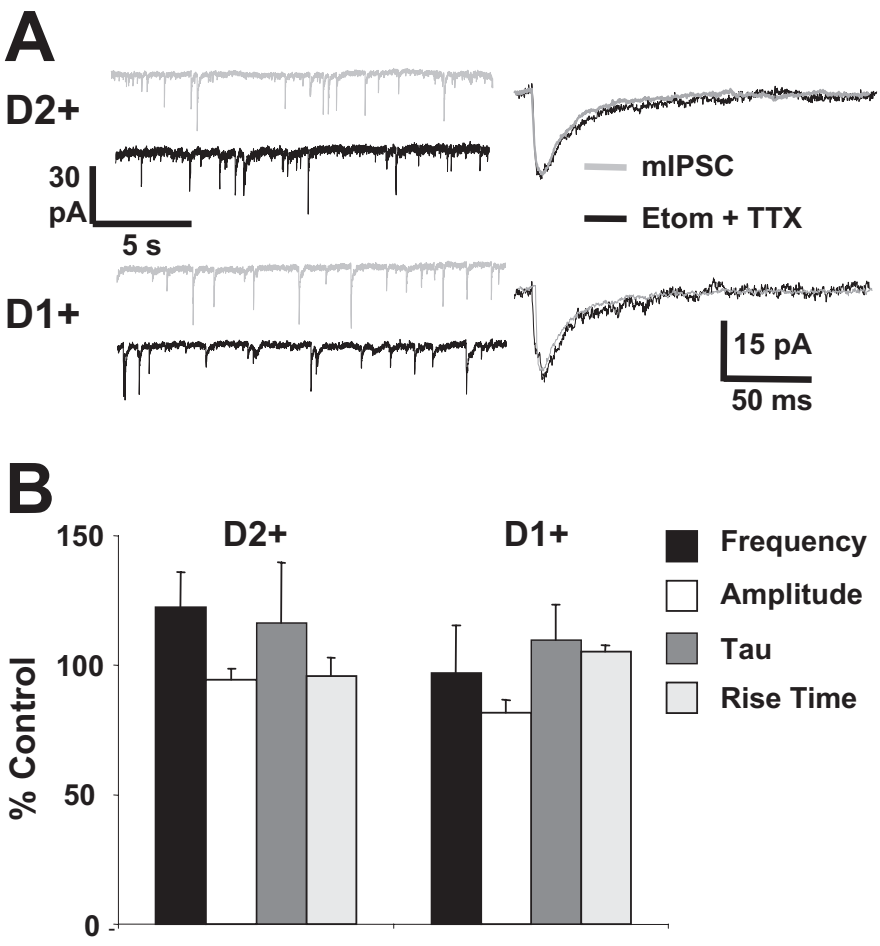

Figure 4. Etomidate does not affect striatal synaptic $GABA_A$ receptors. ***A***, Examples of mIPSCs in D_2+ and D_1+ neurons before (gray) and after (black) etomidate application. Averaged mIPSC traces are normalized and overlaid to demonstrate that etomidate had little effect on current decay. ***B***, Summary of phasic data demonstrating that etomidate had little effect on the frequency (n = 8 and 8), amplitude (n = 8 and 8), weighted tau (n = 8 and 6), or rise time (n = 3 and 3) in both D_2+ and D_1+ MSNs.

PKA regulates MSN tonic current

Although differential extrasynaptic expression of the β3-subunit may yield increased tonic conductance in the D_2+ MSNs, PKA phosphorylation may be an important mediator and increase the number of tonically active GABA channels with distinct subunit combinations. To determine the effect of PKA on D_2+ and D_1+ MSN tonic conductances, we added the catalytic subunit of PKA (50–75 μg/ml) to the CsCl internal solution to measure the effects of postsynaptic PKA modulation without affecting presynaptic release probability. Figure 5*A* shows that with inclusion of the PKA catalytic subunit, a D_1+ neuron had an increased BMR-sensitive tonic current, whereas the tonic current was smaller in a D_2+ MSN. Under conditions that promote PKA phosphorylation, the D_1+ neurons express tonic current, suggesting that phosphorylation is an important regulator of tonic conductance in striatal MSNs. Figure 5*C* shows that internal PKA application did not alter the decay times of mIPSCs for D_2+ or D_1+ neurons.

Based on our hypothesis that tonic current is mediated by β3-containing receptors, we investigated the effect of etomidate on the magnitude of tonic current in D_2+ and D_1+ neurons with internal PKA application. In a simultaneous dual recording of a D_2+ and a D_1+ MSN with internal PKA application (Fig. 5*D*), etomidate uncovered tonic current in the D_1+ MSN, but etomidate responses in D_2+ neurons were slightly smaller compared with control conditions (Fig. 5*D*,*E*). Again, these experiments were repeated in TTX (0.5 μM) to block interneuron activity, and the same observations were made: internal PKA application increased the D_1+ etomidate response (21 $\pm$ 4.4 pA, n = 10, p = 0.07), whereas the D_2+ response decreased (26 $\pm$ 5.1 pA, n = 8, $p < 0.05$). Etomidate responses did not differ between the D_2+ and the D_1+ MSNs with internal PKA application, indicating that the two MSN subtypes have a similar population of β3-containing receptors. Because etomidate revealed a tonic current in D_1+ cells with internal PKA application, it appears that a phosphorylated β3-subunit is responsible, in part, for $GABA_A$ tonic conductance in both MSN subtypes.

To verify that endogenous PKA phosphorylation is involved in mediating D_2+ MSN tonic current, we supplemented the PKI (20 μM) in the internal solution and measured the changes in tonic current compared with control conditions. PKI attenuated the D_2+ MSN tonic current (5.0 $\pm$ 1.0 pA, n = 7 compared with 26 $\pm$ 3.6 pA, n = 14, $p < 0.0005$) while not affecting the tonic current in D_1+ MSNs (4.8 $\pm$ 1.9 pA, n = 4 compared with 3.4 $\pm$ 0.4 pA, n = 11, p = 0.3). PKI had no discernible effects on synaptic receptors as sIPSC decay, amplitude, or frequency did not change (D_2+, n = 13; D_1+, n = 4).

Dopamine modulation of GABA currents

D_1 and D_2 GPCRs contribute to the phosphorylation cascade in MSNs. Although D_1 activation promotes phosphorylation, the D_2 receptor acts to reduce PKA activity and increase activity of protein phosphatase 1 through DARPP-32 (Stoof and Kebabian, 1984). Thus, dopamine release in the striatum should promote PKA activity in D_1+ MSNs while inhibiting PKA activity in D_2+ MSNs. Therefore, we sought to determine basal dopamine levels

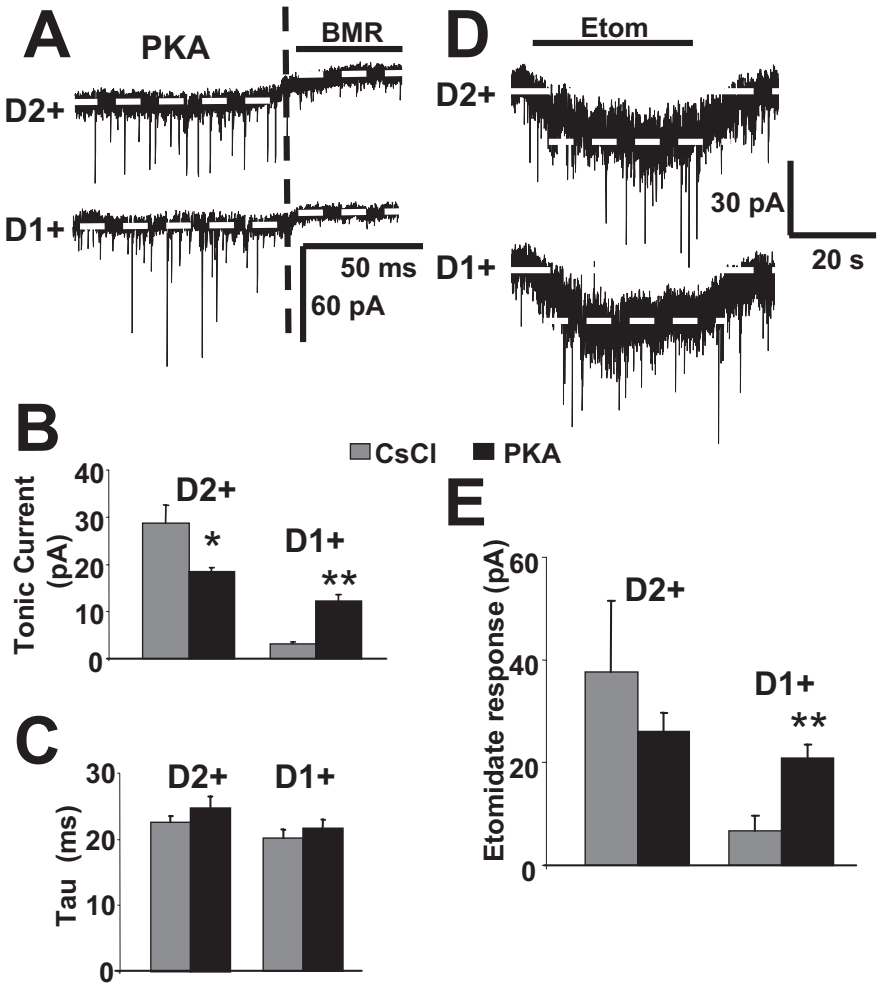

Figure 5. Internal PKA application modulates tonic current. ***A***, Representative traces of two individual MSNs where PKA was supplemented in the internal solution, showing BMR-sensitive tonic current in both cells. ***B***, Summary graph for tonic current in D_2+ and D_1+ MSN in control conditions (D_2+, $n = 12$; D_1+, $n = 10$) and in conditions with internal PKA application (D_2+, $n = 10$; D_1+, $n = 15$). ***C***, Summary graph of the changes in mIPSC decay with internal PKA application (D_2+, $n = 14$; D_1+, $n = 21$) compared with control (D_2+, $n = 31$; D_1+, $n = 26$). ***D***, Representative traces from simultaneous dual recording of D_1+–D_2+ MSNs, showing etomidate responses with internal PKA application. ***E***, Summary graph for current induced by etomidate with internal PKA application. The D_1+ etomidate response increases significantly with internal PKA application, whereas D_2+ responses decrease slightly ($n = 10$ and 8, D_2+ and D_1+, respectively, in CsCl; $n = 9$ and 12 in PKA).

in our slice preparation as free dopamine will affect our system in drastically different ways. In whole-cell recordings from four D_2+ MSNs, the D_2 antagonist sulpiride (2 μM) did not affect tonic or phasic currents (data not shown). Likewise, recordings from D_1+ MSNs in SCH 23390 (10 μM) also did not affect tonic or phasic currents ($n = 5$). These results suggest that dopamine is not present in our recording conditions in young mice or that it is present in such low concentrations that it does not activate D_1 and D_2 receptors.

Because modulating internal phosphorylation altered the tonic GABA currents in MSNs, we sought to determine if dopamine also affects the phosphorylation cascade, yielding altered GABA receptor function in D_2+ and D_1+ neurons. First, we applied the D_2-like agonist quinpirole (10 μM) to both D_2+ and D_1+ MSN followed by application of BMR (25 μM) after recording for ~5 min to allow the full effects of the agonist and GPCR (Price et al., 1999). Figure 6*A* shows recordings from individual D_2+ and D_1+ MSNs with quinpirole (10 μM) application. The D_2-like agonist decreased the D_2+ BMR-sensitive tonic current after 5 min of application without significantly affecting the tonic current in the D_1+ MSN. In a dual recording, SKF-81297 (10 μM) induced a BMR-sensitive tonic current in the D_1+ MSN but also slightly decreased the D_2+ tonic current as well (Fig. 6*B*). As summarized in Figure 6*C*, D_2 receptor stimulation, and probable blockade of PKA phosphorylation, decreased tonic currents in D_2+ MSNs, whereas D_1 receptor stimulation, and promotion of PKA phosphorylation, induced tonic currents in D_1+ MSNs. These drugs were specific for their associated dopamine receptor as application did not alter tonic currents in the opposing cell type (Fig. 6*C*). Therefore, GABA tonic conductance in both D_2+ and D_1+ MSNs is under dopamine control, presumably via a PKA phosphorylation cascade that affects β3-containing $GABA_A$ receptors.

Because temperature may affect phosphorylation function and rates, we repeated these experiments with the D_2 and D_1 receptor agonists at more physiological temperatures (32°C). As reported previously (Ade et al., 2008), tonic current was still significantly larger in D_2+ than D_1+ MSNs in these conditions. At this temperature, application of quinpirole (10 μM) on D_2+ neurons decreased BMR-sensitive tonic current to 38 ± 19% ($n = 3$) of control, whereas BMR sensitive tonic current was increased by SKF-81297 in D_1+ neurons to 184 ± 34% ($n = 3$) of control. At room temperature, quinpirole reduced D_2+ tonic current to 39 ± 10% ($n = 5$) of control, and SKF-81297 increased D_1+ tonic current to 274 ± 32% ($n = 4$) of control. Therefore, phosphorylation cascades remain intact at more physiological temperatures and elicit similar effects as at room temperature.

To confirm that the D_1+ MSN tonic current seen with SKF-81297 application is attributable to extrasynaptic β3-containing $GABA_A$ receptors, we applied etomidate (3 μM) in these conditions. Figure 6*E* shows currents from a D_1+ neuron with etomidate application before and during coapplication with SKF-81297. When coapplied with the D_1 agonist, etomidate produced a significantly greater response than when it was applied alone (etomidate, 8.3 ± 2.2 pA; SKF-81297 plus etomidate, 16 ± 4.2 pA; $n = 4$; $p < 0.05$). These data support the hypothesis that tonic current in D_1+ cells induced by SKF-81297 is mediated through β3-containing receptors.

It has recently been suggested that GABA tonic currents are not present in D_2+ or D_1+ MSNs in older mice (Gertler et al., 2008), suggesting that dopamine and phosphorylation may play different roles in adult mice. We tested for tonic currents in older mice and investigated their modulation through dopamine receptors. We observed BMR-sensitive tonic currents in both D_2+ and D_1+ MSN in animals between p33 and p37, although the magnitude was reversed compared with younger animals. D_2+ MSNs averaged 8.3 ± 3.1 pA tonic current ($n = 4$; $p < 0.05$

compared with younger animals), whereas D_1+ MSNs averaged 18.3 ± 1.2 pA tonic current ($n = 6$; $p < 0.05$ compared with younger animals), suggesting that dopaminergic tone may change through development. Thus, we investigated the effects of specific dopamine receptor antagonists sulpiride (2 μM) and SCH 23390 (10 μM) in D_2+ and D_1+ MSNs from these older animals. With their respective antagonists, tonic current increased to 14.7 ± 1.5 pA in three D_2+ MSNs and 26.4 ± 1.7 pA in four D_1+ MSNs, supporting a change in dopaminergic tone. To determine whether PKA phosphorylation also mediates tonic current in older animals, we supplemented PKI into the CsCl internal and saw a significant reduction in D_1+ tonic current (6 ± 1.8 pA, $n = 4$; $p < 0.0005$) in these older mice compared with normal internal conditions. Internal PKI application did not change the tonic current in four D_2+ cells from older animals.

To determine dopamine's modulatory role on phasic $GABA_A$ receptors, we analyzed sIPSCs before and after agonist application (TTX was not applied so as to not block tonic current). Although both quinpirole and SKF-81297 tended to increase the decay time in the D_2+ and D_1+ MSN, respectively, the results were not significant (Fig. 6*D*). In combination with the etomidate results, these data suggest that D_2+ and D_1+ MSN synaptic receptor populations include both β1- and β3-subunit containing receptors. Because we show here that dopamine modulates GABA tonic currents and our previous study showed that GABA tonic currents control cell excitability (Ade et al., 2008), we tested dopamine's effects on rheobase and firing frequency in MSNs. In a series of current-clamp experiments, we injected increasing depolarizing current steps from a membrane potential of −70 mV before and after D_2 and D_1 agonist application (Fig. 7*A*,*B*). As previously reported, D_1+ MSNs had significantly higher rheobase currents than D_2+ MSNs (Ade et al., 2008; Gertler et al., 2008). Quinpirole (10 μM) significantly increased the rheobase and significantly decreased the firing frequency in D_2+ cells (Fig. 7*D*). In contrast, no differences in rheobase current or firing frequency were observed with SKF-81297 (10 μM) application (Fig. 7*B*,*C*,*E*). These results suggest that D_2+ cells are more excitable than D_1+ cells but that D_2 agonists decrease cell excitability, possibly because of their interactions with GABA tonic currents, as shown by the sensitivity of rheobase to BMR.

Etomidate's direct activation of GABA channels in D_2+ but not D_1+ MSNs suggested that tonic conductance in D_2+ neurons was attributable to the presence of extrasynaptic β3-containing $GABA_A$ receptors. However, internal PKA application and D_1 dopamine receptor stimulation reveals that extrasynaptic β3-containing receptors mediate tonic current in D_1+ cells as well. Therefore, D_1+ and D_2+ MSN both have a population of extrasynaptic β3-containing receptors that mediate tonic current, but the important difference between the cell types is the phosphorylation state which alters the receptors' function. Thus, we speculated that in our experimental conditions, extrasynaptic receptors in D_1+ MSNs are silent and tonic current in striatal D_2+ MSNs is mediated by basally phosphorylated β3-containing receptors. This model is diagrammed with better detail in Figure 8.

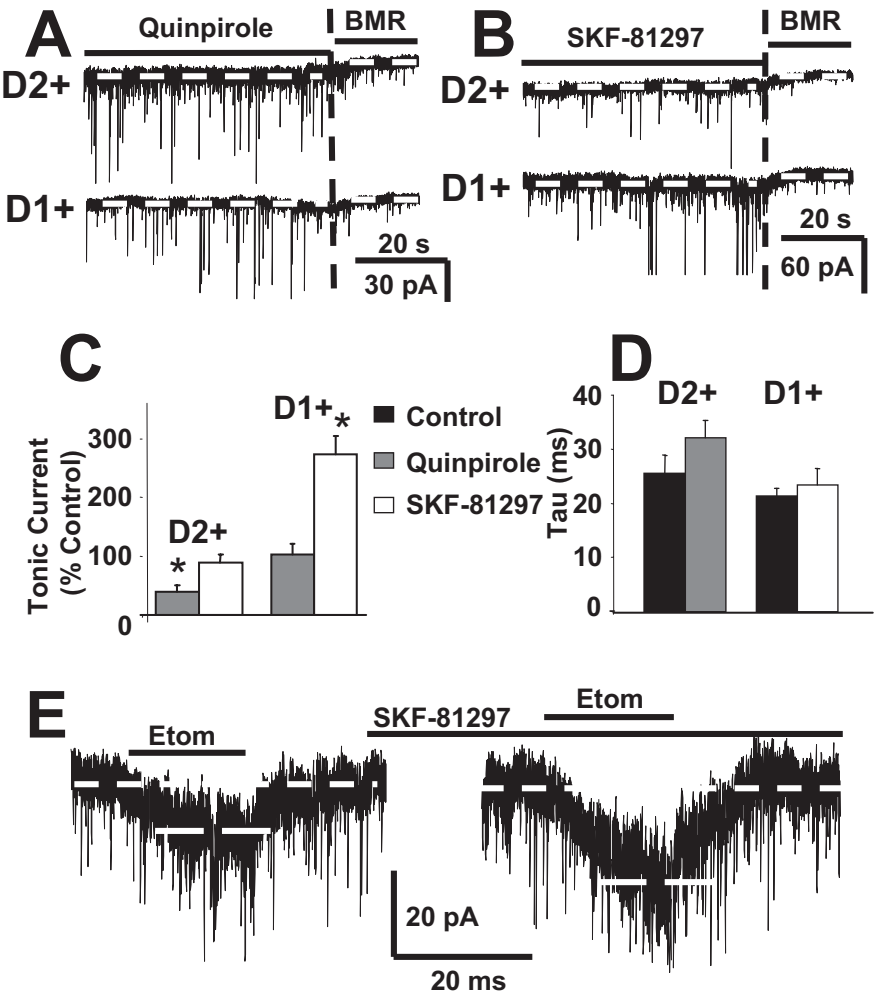

Figure 6. Dopamine agonists alter MSN tonic conductances. ***A***, Representative current traces from individual D_2+ and D_1+ MSN illustrating that the D_2 agonist, quinpirole (10 μM), reduces tonic current in the D_2+ MSN, whereas it does not affect tonic currents in the D_1+ MSN. ***B***, Representative traces of a simultaneous dual recording between a D_2+ and D_1+ MSN illustrating that the D_1 agonist, SKF-81297 (10 μM), induces a tonic current in the D_1+ MSN but also reduces it in the D_2+ MSN. ***C***, Summary graph showing effects on tonic current with quinpirole and SKF-81297 application on D_2+ ($n = 5$ and 3) and D_1+ ($n = 6$ and 4). ***D***, Summary graph of phasic currents of both D_2+ and D_1+ in response to their respective agonists ($n = 6$ and 5 for D_2+, $n = 8$ and 5 for D_1+). ***E***, Representative current trace from a D_1+ neuron where etomidate (3 μM) was given before and during SKF-81297 (10 μM) application. SKF-81297 was given for over 5 min before coapplication with etomidate to allow full drug action.

Discussion

Our previous studies revealed differential tonic conductances in D_2+ and D_1+ MSNs that are likely attributable to differential subunit expression (Ade et al., 2008). We investigated the role of α1-, α5-, and δ-subunits and determined the α5-subunit to be a likely player in tonic conductance (Ade et al., 2008). The other two subunits that make up this functional extrasynaptic receptor remained elusive. We further investigated the δ-subunit because it has been shown to be the primary mediator of tonic GABA current in other brain regions (Farrant and Nusser, 2005; Jia et

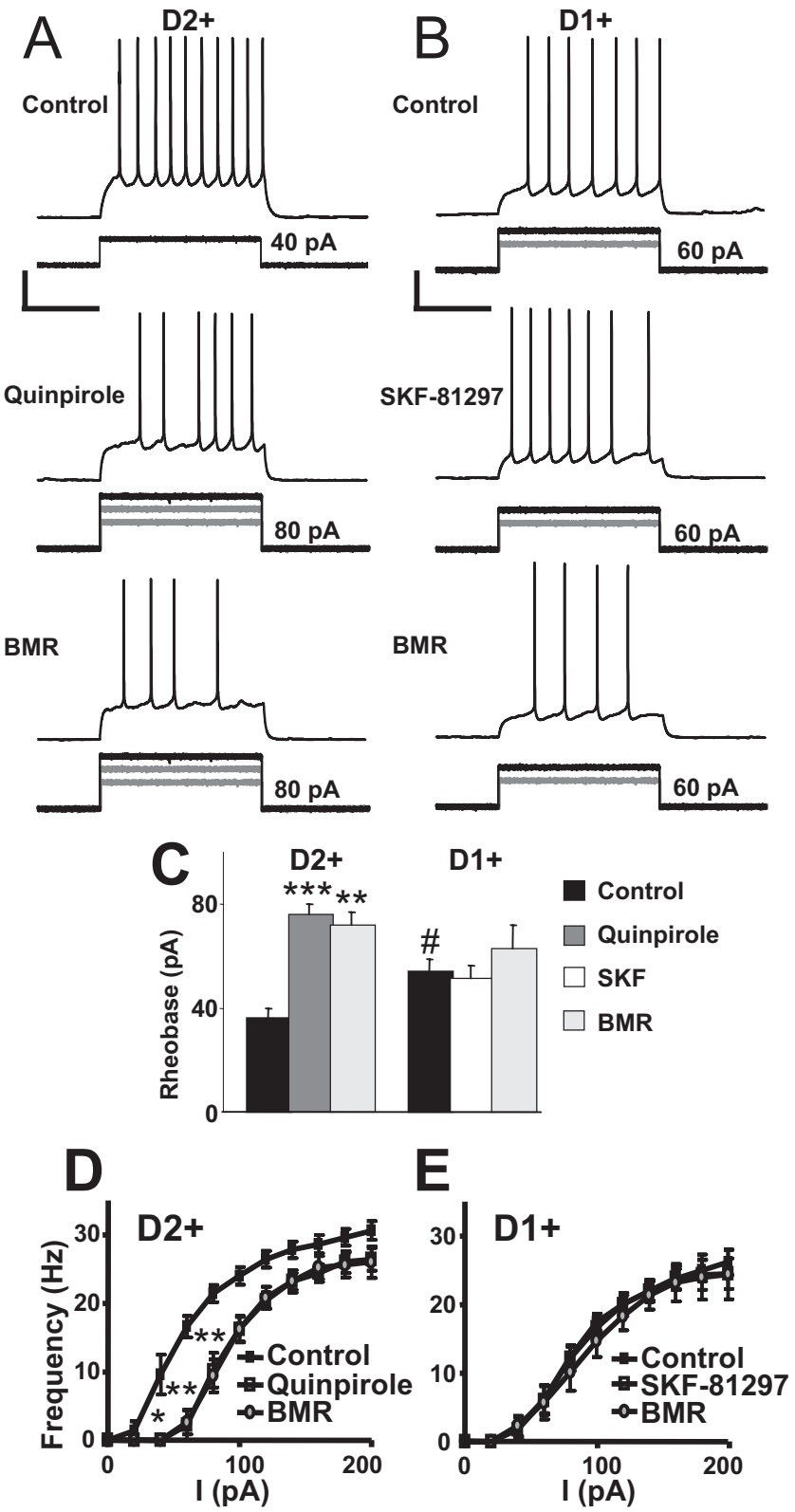

Figure 7. Dopamine modulates MSN cell excitability. ***A***, Representative current-clamp recording from a D_2+ MSN illustrating the responses to a series of depolarizing current injections (20 pA steps) from −70 mV, recorded with K-gluconate internal in the absence and presence of quinpirole (10 μM) and BMR (25 μM). ***B***, Representative example of a D_1+ MSN in the same conditions as ***A***, but with the D_1-like selective agonist, SKF-81297 (10 μM). ***C***, Summary plot showing the averaged rheobase current in D_2+ ($n = 5$) and D_1+ ($n = 7$) MSNs with dopamine agonist and BMR application. ***D***, Summary of action potential firing frequency in response to increasing depolarizing current injections recorded with K-gluconate internal solution in D_2+ MSNs (■) in the absence and presence of 10 μM quinpirole (□) and 25 μM BMR (●). Data derive from the same cells in ***C***. ***E***, Summary of action potential firing frequency in D_1+ MSNs (■) in the absence and presence of 10 μM SKF-81297 (□) and 25 μM BMR (●). *Significance to D_2+ control cells; #significance between D_2+ and D_1+ cells. Calibration: 20 μM, 500 ms.

al., 2005; Glykys et al., 2008). Although our previous study demonstrated that striatal $GABA_A$ receptors contain the δ-subunit in both MSN subtypes (Ade et al., 2008), we discovered that the pattern of tonic conductance in a δ−/− mouse matched that from BAC D_2 EGFP mice. This finding is similar to those obtained with α1−/− mice, suggesting that although α1- and δ-subunits are part of striatal $GABA_A$ receptors, they do not underlie the differences observed between D_1+ and D_2+ MSNs (Ade et al., 2008).

We used the general anesthetic etomidate to better ascertain MSN β-subunit expression but first verified its efficacy as a modulator and activator of GABA channels using striatally relevant recombinant receptors in HEK 293 cells. We confirmed reported selectivity of etomidate on direct activation of recombinant β3-containing $GABA_A$ receptors with all α-subunits tested (Hill-Venning et al., 1997). However, etomidate-mediated GABA potentiation was not significantly different between α5β3- and α5β1-containing receptors. This contrasted with results obtained with α2β3- and α2β1-containing receptors, where etomidate's potentiating role retained β-subunit specificity. These findings were necessary to interpret etomidate's effects on GABA currents in MSNs in striatal slice preparations. The stronger action of etomidate in D_2+ cells indicated that the β3-subunit is more abundant in D_2+ than D_1+ neurons. Low concentrations of exogenous GABA activated current in both D_2+ and D_1+ MSNs, and this current was potentiated by etomidate in both cell types, although the potentiation was significantly greater in D_2+ neurons.

Internal PKA application induced etomidate responses and tonic conductance in D_1+ neurons, suggesting that although D_2+ and D_1+ neurons have similar populations of β3-containing receptors, their difference in tonic conductance is attributable to the β3-subunit phosphorylation state. Together with previous studies that suggest phosphorylation increases currents through β3-subunits (McDonald et al., 1998; Nusser et al., 1999; Flores-Hernandez et al., 2000), these data suggest that tonic current in D_1+ MSNs is mediated, in part, through β3-containing receptors.

Internal PKA application decreased tonic current in D_2+ cells, implying that GABA receptors on D_2+ neurons also include the β1-subunit and/or are basally phosphorylated by an endogenous kinase, either PKA or PKC. Although speculative, too much kinase activity may alter the sta-

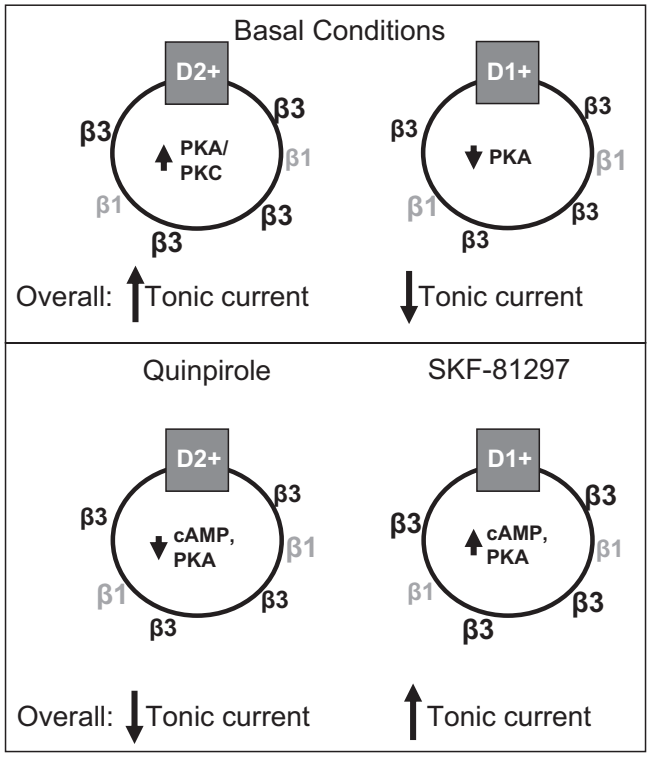

Figure 8. Tonic conductance is mediated through a phosphorylated β3-subunit. Under basal conditions (little to no dopamine), D_2 receptors do not activate the $G_{i/o}$ protein to inhibit PKA phosphorylation, and the β1- and β3-subunits are basally phosphorylated. Because the phosphorylated β3-subunit yields increased currents and may be more plentiful than extrasynaptic β1-subunits, D_2+ MSN display tonic current. Without dopamine, D_1 receptors do not activate the G_s/G_{olf} protein to promote PKA phosphorylation, and the dephosphorylated β3-subunits do not mediate increased conductance, resulting in smaller tonic current in the D_1+ than the D_2+ MSN. More abundant β1-subunit expression in D_1+ MSNs results in increased current only during GABA application. When stimulated, the D_2 receptor activates the $G_{i/o}$ protein to inhibit PKA activity, dephosphorylating the β1/β3-subunits. A dephosphorylated β3-subunit results in smaller tonic currents compared with basal conditions. During D_1 stimulation, the G_s/G_{olf} protein activates cAMP and PKA pathways to phosphorylate the β3-subunit and increase tonic currents.

bility of receptors in the membrane. While we do not know the mechanism that underlies PKA's effect on the decreased D_2+ tonic current, basally phosphorylated β3-containing receptors have been found in the hippocampus (Brandon et al., 2000) and cortex (Kumar et al., 2005), and results from both studies suggested that PKC is responsible for the basal phosphorylation (Kittler and Moss, 2003). Indeed, internal PKI application decreased D_2+ tonic current, suggesting that D_2+ MSN tonic current is under basal PKA modulation. Further studies, including biochemical analysis, are needed to verify a basally phosphorylated β3-subunit in D_2+ neurons.

Our results with dopamine antagonists propose that striatal dopamine is not present in our slice preparation in young mice, and therefore both dopamine receptors remain inactive (Lee et al., 2001). Activation of D_1 receptors in striatonigral neurons stimulates PKA activity, whereas activation of D_2 receptors in striatopallidal MSNs inhibits PKA activity (Stoof and Kebabian, 1984). As modeled in Figure 8, the D_1 receptor agonist SKF-81297 induces tonic currents in D_1+ cells possibly through phosphorylated extrasynaptic β3-containing receptors. On the contrary, D_2+ tonic conductance decreases when the D_2+ agonist quinpirole is applied, suggesting a role for dephosphorylated β3-containing $GABA_A$ receptors. However, quinpirole did not abolish the tonic current, offering a role for β1-containing receptors in D_2+ neurons or suggesting that the remaining current is via the α5-subunit that mediates tonic current, even without enhanced function through phosphorylated β3-subunits. The lack of tonic current in D_1+ cells may also be related to more abundant expression of $\alpha2\beta1$-containing receptors, supported by HEK 293 cells that showed higher sensitivity to GABA in $\alpha2\beta3$-containing receptors than $\alpha2\beta1$-containing receptors. The relative abundance of β1- and β3-subunits, combined with α2- or α5-subunits, together with distinct dopamine receptor mediated phosphorylation/dephosphorylation, regulates tonic GABA conductance in striatal MSNs.

Our results with dopamine agonists on tonic current were obtained without TTX and with an intact striatal network that includes several types of GABAergic interneurons as well as cholinergic interneurons (Tepper et al., 2004), which may contribute to the effects we see with dopamine-selective agonists on the opposing cell type. We cannot exclude that some of the dopamine agonist effects are attributable to a presynaptic mechanism. Because results with dopamine receptor activation and internal PKA application were similar, we suggest that dopamine's effects are primarily postsynaptic and dependent upon PKA activity.

We previously showed that differential extrasynaptic α5-subunit function contributes to D_2+ tonic conductance (Ade et al., 2008). Therefore, D_2+ neurons may have an extrasynaptic population of $\alpha5\beta3$-containing receptors. As D_1+ neurons lack the α5-subunit, tonic conductance in these neurons is most likely mediated via the phosphorylated β3-subunit together with the α2-subunit. Although etomidate's potentiating effect was specific for $\alpha2\beta3$-containing receptors, the effects did not differ between $\alpha5\beta1$- and $\alpha5\beta3$-containing receptors. Tonic current is potentiated by etomidate in D_2+ MSNs because they express $\alpha5\beta3$-containing receptors. In contrast, $\alpha2\beta1$-containing receptors are more abundant than $\alpha2\beta3$-containing receptors in D_1+ MSNs, and the potentiating effect of etomidate is smaller than in D_2+ MSNs.

Although etomidate, internal PKA application, and dopamine agonists induced significant changes to D_2+ and D_1+ MSN tonic currents, these actions failed to significantly alter synaptic currents. These results indicate that D_2+ and D_1+ MSNs have similar synaptic receptor populations that include both β1- and β3-subunits. However, as both β1- and β3-subunits are regulated by PKA and dopamine, it remains to be clarified why these agents fail to alter IPSCs in MSNs. One possible hypothesis suggests that synaptic receptors are composed of complementary amounts of β1- and β3-subunits, and therefore their differential regulation by these agents is countered. Previous studies into dopamine's modulatory role on inhibitory transmission found that dopamine does not modulate IPSCs in the rat dorsal striatum, although it does affect IPSCs in the ventral striatum (Nicola and Malenka, 1998). By exclusively modulating tonic, and not phasic, currents in the dorsal striatum, dopamine may regulate cell excitability.

Dopamine has been shown to modulate cell excitability in several animal models and brain regions through a variety of different mechanisms (Belousov and van den Pol, 1997; Ding and Perkel, 2002; Yasumoto et al., 2002; Perez et al., 2006). We show that dopamine agonists modulate rheobase current and functionally decrease the cell excitability in D_2+ cells without affecting excitability in D_1+ cells. Dopamine modulates many ion channels in MSNs such as Ca^{2+} and inward rectifier K^+ channels (Moyer et al., 2007). One computational model showed that dopamine decreases the excitability of D_2+ MSNs, while increasing

the excitability in D_1+ MSNs independently of GABA tonic conductance (Moyer et al., 2007). Previous results from our lab showed that GABA tonic conductance facilitates MSN cell excitability (Ade et al., 2008). In the present study, BMR did not change rheobase current in D_2+ cells after quinpirole application, suggesting that changes in cell excitability with quinpirole application are mediated through GABA receptors. We suggest that this modulation of GABA tonic currents may support other mechanisms of dopamine modulation for cell excitability.

It has recently been suggested that GABA tonic currents are not present and do not contribute to the different excitabilities between the two types of MSNs in older mice (Gertler et al., 2008). In our study, we observed tonic conductance in both cell types from older mice. These opposing results may be explained by experimental conditions like extracellular K^+ concentrations which may alter ambient GABA concentrations by modulating interneuron activity. Tonic currents in both MSN subtypes from older mice had opposite magnitudes, which were also modulated by PKA. In these mice, the action of specific dopamine receptor antagonists on tonic current suggests a possible role for increased ambient dopamine but will require further investigation.

The results presented here suggest a target for striatal GABAergic tonic conductance in MSNs. We posit that a basally phosphorylated β3-subunit is responsible for the D_2+ tonic conductance but show that internal PKA application or D_1 agonist application reveals extrasynaptic β3-containing receptors that mediate tonic current in D_1+ cells (Fig. 8). Because Parkinson's disease symptoms arise from an imbalance between D_1+ striatonigral projection and D_2+ striatopallidal projection outputs (Mallet et al., 2006), a selective target of tonic conductance in striatonigral or striatopallidal pathways offers potential therapeutic benefits in alleviating debilitating motor control symptoms.

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