Distinct roles of dopamine D2L and D2S receptor isoforms in the regulation of protein phosphorylation at presynaptic and postsynaptic sites

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Dopamine D2 receptors are highly expressed in the dorsal striatum where they participate in the regulation of (*i*) tyrosine hydroxylase (TH), in nigrostriatal nerve terminals, and (*ii*) the dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), in medium spiny neurons. Two isoforms of the D2 receptor are generated by differential splicing of the same gene and are referred to as short (D2S) and long (D2L) dopamine receptors. Here we have used wild-type mice, dopamine D2 receptor knockout mice (D2 KO mice; lacking both D2S and D2L receptors) and D2L receptor-selective knockout mice (D2L KO mice) to evaluate the involvement of each isoform in the regulation of the phosphorylation of TH and DARPP-32. Incubation of striatal slices from wild-type mice with quinpirole, a dopamine D2 receptor agonist, decreased the state of phosphorylation of TH at Ser-40 and its enzymatic activity. Both effects were abolished in D2 KO mice but were still present in D2L KO mice. In wild-type mice, quinpirole inhibits the increase in DARPP-32 phosphorylation at Thr-34 induced by SKF81297, a dopamine D1 receptor agonist. This effect is absent in D2 KO as well as D2L KO mice. The inability of quinpirole to regulate DARPP-32 phosphorylation in D2L KO mice cannot be attributed to decreased coupling of D2S receptors to G proteins, because quinpirole produces a similar stimulation of [^{35}S]GTPγS binding in wild-type and D2L KO mice. These results demonstrate that D2S and D2L receptors participate in presynaptic and postsynaptic dopaminergic transmission, respectively.

Dopamine acts by binding to five subtypes of heptahelical G protein-coupled receptors (1–3), which have been divided into two groups: the D1-like receptors, comprising D1 and D5 receptors, both positively coupled to adenylyl cyclase and cAMP production, and the D2-like receptors, comprising D2, D3, and D4 receptors, whose activation results in inhibition of adenylyl cyclase and suppression of cAMP production (4, 5). The D2 subtype of dopamine receptor represents the main autoreceptor of the dopaminergic system (6–11), but is also critical for postsynaptic transmission (3, 10, 12). This receptor has been the subject of extensive studies, which have demonstrated its participation in numerous important physiological functions, such as synthesis and release of pituitary hormones (13–15) and control of motor activity (12, 16, 17). Dopamine D2 receptors represent the major target of antipsychotic drugs and are involved in various neuropathological conditions, including Parkinson's disease, Tourette's syndrome, and drug addiction (3, 12, 18, 19).

Alternative mRNA splicing generates two isoforms of the dopamine D2 receptor, named long (D2L) and short (D2S) isoforms (20), which differ by an insertion of 29 aa in the third intracellular loop of the D2L receptor. Both isoforms function by binding to pertussis toxin-sensitive G proteins, which reduce adenylyl cyclase activity. However, previous studies have shown that D2L and D2S receptors bind to distinct Gi proteins, most likely as a result of their structural differences (21–23). Another important difference emerging from studies of D2L and D2S receptors is related to their involvement in presynaptic and postsynaptic functions. Studies performed by using dopamine D2L receptor-null mice indicate a preferential involvement of D2L receptors in postsynaptic responses, such as the synergistic stimulation of motor activity produced by D1/D2 receptors and haloperidol-induced catalepsy (10, 24). In contrast, the D2S receptor seems to be preferentially expressed by midbrain dopaminergic neurons (21, 25), where it acts as an inhibitory autoreceptor (24, 26).

The dorsal striatum, a major component of the basal ganglia, represents an ideal system in which to study dopaminergic transmission. In this region, dopamine D2 receptors are expressed both postsynaptically, on striatal medium spiny neurons, as well as presynaptically, on dopaminergic nerve terminals originating in the substantia nigra pars compacta. In this study, we have examined the contribution of D2L and D2S receptors to the regulation of two phosphoproteins critically involved in presynaptic and postsynaptic dopaminergic transmission: tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamines, and DARPP-32, a dopamine- and cAMP-regulated phosphoprotein of 32 kDa selectively expressed in striatal medium spiny neurons. These experiments have been performed by using genetically modified mice lacking either the D2L receptor alone [D2L knockout (KO) mice; ref. 10] or both D2L and D2S receptors (D2 KO mice; ref. 16).

Materials and Methods

Chemicals. SKF81297 (a dopamine D1 receptor agonist), quinpirole (a dopamine D2 receptor agonist), dopa, sodium azide and *m*-hydroxybenzylhydrazine were purchased from Sigma. The ECL Plus immunoblotting detection kit was purchased from Amersham Pharmacia Biotech, and the protein measurement kit (bicinchoninic acid) was from Pierce.

Preparation and Incubation of Striatal Slices. Female wild-type mice, D2 KO mice (16), and D2L KO mice (10) (25–30 g) were killed by decapitation, and the brains were rapidly removed. Coronal slices (250 μm) were prepared by using a vibroslice (Campden Instruments, Sileby, U.K.). Dorsal striata were then

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Abbreviations: TH, tyrosine hydroxylase; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; D2S, short dopamine D2 receptor; D2L, long dopamine D2 receptor; KO, knockout.

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dissected out from each slice under a microscope. Two slices were placed in individual 5-ml polypropylene tubes containing 2 ml of Krebs–Ringer's bicarbonate buffer [KRB; 118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.5 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11.7 mM glucose, equilibrated with 95% O_2/5% CO_2 (vol/vol), pH 7]. The samples were equilibrated at 30°C for two 30-min intervals, each followed by replacement of the medium with 2 ml of fresh KRB. Test substances were then added for various intervals. For the determination of dopa, slices were first incubated for 5 min in the presence of test substances and then for 15 min in the presence of test substances plus the L-amino acid decarboxylase inhibitor *m*-hydroxybenzylhydrazine (100 μM). After incubation, the solutions were rapidly removed, and the samples were placed on dry ice until assayed.

Western Blot Assay of Phospho-TH and Phospho-DARPP-32. Frozen tissue samples were sonicated in 75 μl of 1% SDS and boiled for 10 min. Aliquots (5 μl) of the homogenate were used for protein content determination. Equal amounts of protein from each sample were loaded onto 10% polyacrylamide gels, and the proteins were separated by SDS/PAGE and transferred to poly(vinylidine difluoride) membranes (Amersham Pharmacia Biotech), as described (27). The membranes were immunoblotted by using an affinity-purified polyclonal antibody that selectively detects phospho[Ser-40]-TH (THS40p-AB; ref. 28) or a monoclonal antibody to detect phospho[Thr-34]-DARPP-32 (29). Antibody binding was revealed by incubation with goat anti-rabbit horseradish peroxidase-linked IgG (diluted 1:10,000; Pierce) or goat anti-mouse horseradish peroxidase-linked IgG (diluted 1:10,000; Pierce) and the enhanced chemiluminescence ECL Plus immunoblotting detection method. Chemiluminescence was detected by autoradiography. Quantification of the immunoreactivity corresponding to the phospho-TH and phospho-DARPP-32 bands was done by densitometry, using NIH IMAGE (version 1.61) software.

Determination of Dopa. Frozen tissue samples were sonicated in 75 μl of 0.1 mM perchloric acid and centrifuged at 10,000 $\times$ g for 10 min. The pellets were resuspended in 75 μM SDS, and their protein content was determined. The levels of dopa in the supernatant were determined by using HPLC as described (28).

[^{35}S]GTPγS Binding Assay. Mice striata were dissected out, and membranes were prepared with a Teflon glass homogenizer, in 50 vol of 50 mM Tris·HCl buffer (pH 7.4). [^{35}S]GTPγS (>1,000 Ci/mmol, Amersham Pharmacia; 1 Ci = 37 GBq) binding was performed in the presence of increasing concentrations of quinpirole as previously described (30).

Results and Discussion

The medium spiny neurons of the dorsal striatum are innervated by midbrain dopaminergic cells, which originate in the substantia nigra pars compacta (31–33). Release of dopamine from nigrostriatal nerve terminals is subjected to feedback inhibitory control via dopamine D2 autoreceptors (11, 12). The same presynaptic receptors also control synthesis of dopamine by regulating the state of phosphorylation of TH (34, 35). Increases in TH phosphorylation stimulate enzymatic activity and dopamine synthesis; conversely, decreases in the state of phosphorylation reduce TH activity and dopamine synthesis. Previous studies showed that, in rat striatal slices, quinpirole decreases TH phosphorylation at Ser-40 and produces a concomitant inhibition of enzymatic activity (35). Based on these observations, we tested the ability of quinpirole to regulate the state of phosphorylation and activity of TH in striatal slices obtained from wild-type mice, D2 KO mice, and D2L KO mice.

As shown in Fig. 1*A*, incubation of striatal slices for 10 min in the presence of quinpirole produced a concentration-dependent decrease in the state of phosphorylation of TH at Ser-40, in wild-type mice. This inhibition reached statistical significance at a concentration of 100 nM and was 79% at 1 μM. The inhibitory

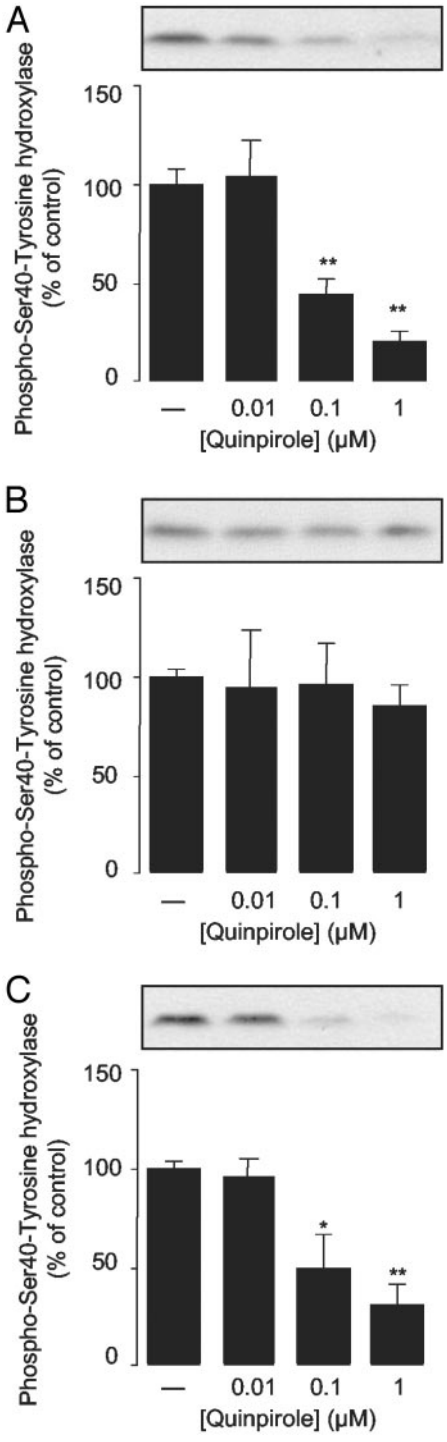

Fig. 1. D2S receptors are specifically involved in the regulation of the state of phosphorylation of TH. Striatal slices from wild-type mice (*A*), D2 KO mice (*B*), and 2DL KO mice (*C*) were incubated for 10 min in the presence of quinpirole (10 nM to 1 μM). The levels of phospho[Ser-40]-TH were determined as described in *Materials and Methods*. (*Upper*) Representative autoradiograms. (*Lower*) Summary of data expressed as means ± SEM ($n = 6$). The amount of phosphorylated TH is expressed as a percentage of that determined in the absence of quinpirole (control). *, $P < 0.01$ and **, $P < 0.001$ vs. respective control group; one-way ANOVA followed by Dunnett's test.

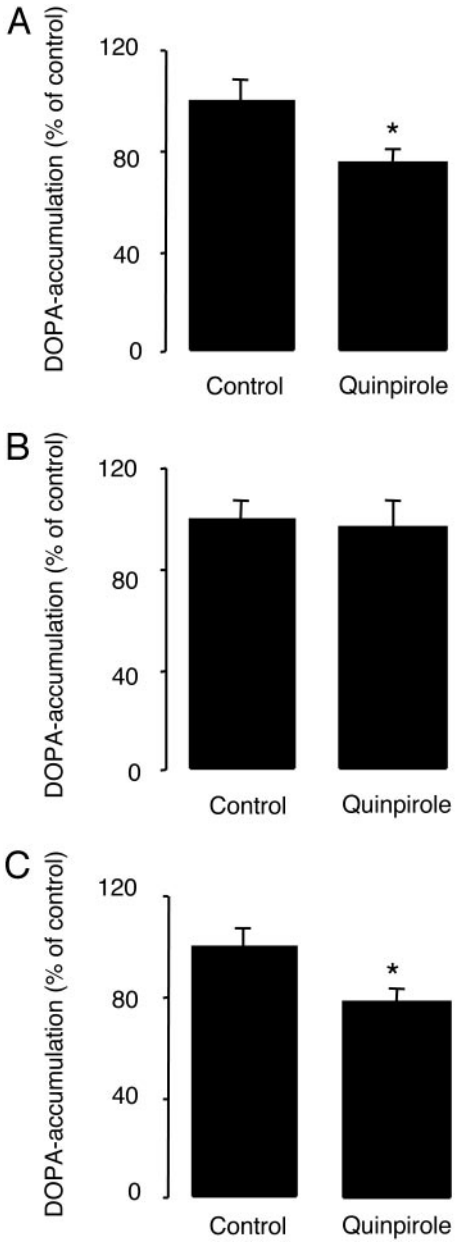

Fig. 2. D2S receptors are specifically involved in the regulation of TH activity. Striatal slices from wild-type mice (*A*), D2 KO mice (*B*), and D2L KO mice (*C*) were incubated for 5 min in the presence of quinpirole (1 μM) and for 15 min in the presence of quinpirole plus *m*-hydroxybenzylhydrazine (100 μM). The samples were then sonicated in 0.1 M perchloric acid and centrifuged. The levels of DOPA recovered in the supernatant were determined by HPLC and normalized according to protein content (see *Materials and Methods*). Data are expressed as means ± SEM ($n = 16$). The amount of dopa is expressed as a percentage of that determined in the absence of quinpirole, and 100% corresponds to 25.4 pmol/min per mg of protein. *, $P < 0.05$ vs. respective control group (Student's *t* test).

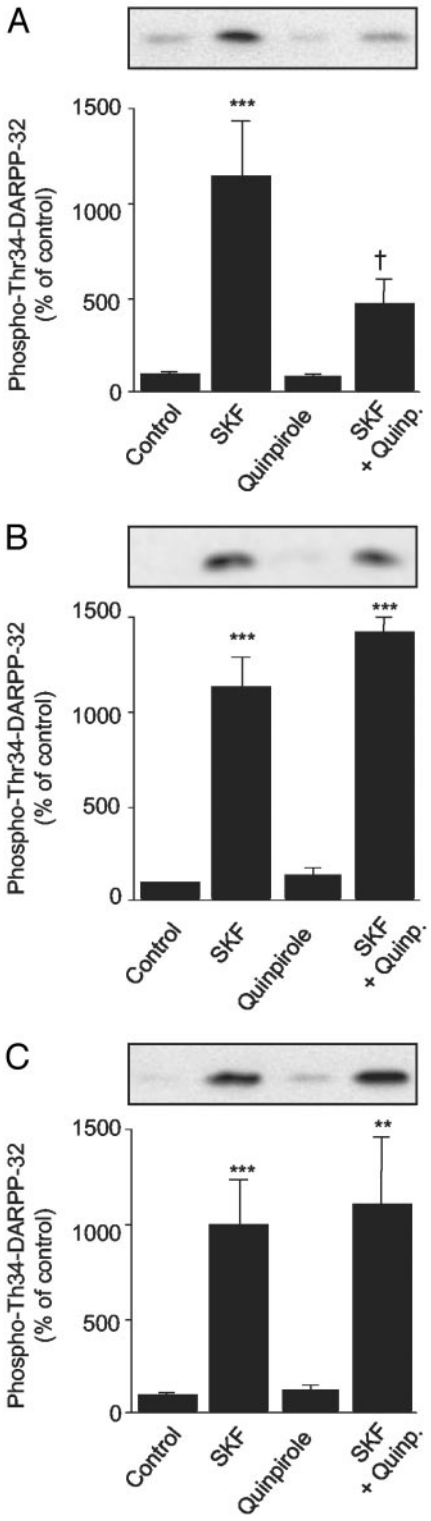

Fig. 3. D2L receptors are specifically involved in the regulation of the state of phosphorylation of DARPP-32. Striatal slices from wild-type mice (*A*), D2 KO mice (*B*), and D2L KO mice (*C*) were incubated for 2 min in the presence of quinpirole (1 μM) and then for 8 min in the presence of quinpirole plus SKF81297 (10 μM). The levels of phospho[Thr-34]-DARPP-32 were determined as described in *Materials and Methods*. (*Upper*) Representative autoradiograms. (*Lower*) Summary of data expressed as means ± SEM ($n = 6$). The amount of phosphorylated DARPP-32 is expressed as a percentage of that determined in the absence of drugs (control). **, $P < 0.01$ and ***, $P < 0.001$ vs. control; one-way ANOVA followed by Newman-Keuls test. †, The effect of SKF81297 was significantly reduced by quinpirole ($P < 0.01$; two-way ANOVA).

effect of quinpirole on TH phosphorylation was abolished in the absence of dopamine D2 receptors (i.e., in D2 KO mice; cf. Fig. 1*B*), further supporting the idea of the specific involvement of this receptor subtype in dopamine autoreceptor function (8, 16). In contrast to the results obtained in D2 KO mice, we found that, in D2L KO mice, quinpirole caused a reduction of TH phosphorylation (Fig. 1*C*) similar to that observed in wild-type mice.

We next examined the effect of quinpirole on TH activity. Striatal slices were incubated for 20 min in the presence of 1 μM

quinpirole, and the levels of dopa were determined. In agreement with previous studies indicating a critical role played by Ser-40 in the regulation of TH activity (28, 35), we found that the dopamine D2 receptor agonist reduced dopa accumulation in both wild-type and D2L KO mice, but not in D2 KO mice (Fig. 2). Taken together with the evidence obtained from the phosphorylation experiments, these results demonstrate the specific involvement of the D2S isoform in the regulation of dopamine biosynthesis. They also extend previous studies implicating the D2S isoform in presynaptic dopaminergic control of dopamine release and dopaminergic midbrain neuron firing (10, 11, 26, 36).

In the striatum, activation of cAMP-dependent protein kinase (PKA) stimulates the phosphorylation of TH at Ser-40 (28, 35, 37). It is therefore possible that the decrease in TH phosphorylation produced by quinpirole is mediated via Gi-dependent inhibition of adenylyl cyclase, reduction of cAMP levels, and inhibition of PKA activity. However, other studies have implicated extracellular signal-regulated kinases 1 and 2 (ERK1/2) in the increase in Ser-40 phosphorylation produced by depolarization (38). In addition, recent evidence demonstrated the existence of a negative regulation exerted by D2 receptors on striatal ERK1/2 (39). D2S receptors may therefore inhibit TH phosphorylation and activity via modulation of the cAMP pathway, the ERK1/2 pathway, or both.

In other experiments, we examined the ability of dopamine D1 and dopamine D2 receptor agonists to regulate DARPP-32 phosphorylation in wild-type mice, dopamine D2 KO mice, and dopamine D2L KO mice (Fig. 3). Activation of dopamine D1 receptors increases Thr-34 phosphorylation via Golf-mediated stimulation of adenylyl cyclase (40), increased production of cAMP (5), and activation of PKA (41, 42). Conversely, activation of dopamine D2 receptors decreases DARPP-32 phosphorylation at Thr-34 via Gi-mediated inhibition of cAMP production (41, 43). In addition, dopamine D2 receptor agonists stimulate protein phosphatase-2B activity, thereby increasing dephosphorylation of DARPP-32 at Thr-34 (41).

We found that incubation of striatal slices for 8 min in the presence of the dopamine D1 receptor agonist, SKF81297 (10 μM), produced a 10- to 11-fold increase in the state of phosphorylation of DARPP-32 at Thr-34, in all genotypes (Fig. 3). Quinpirole, added 2 min before SKF81297, was able to diminish the increase in phosphorylation of DARPP-32 at Thr-34 produced by the dopamine D1 agonist, in wild-type mice (Fig. 3*A*). This effect was absent in D2 KO mice, which lack both isoforms of dopamine D2 receptor (Fig. 3*B*). Interestingly, when tested in D2L KO mice, quinpirole also failed to reduce DARPP-32 phosphorylation (Fig. 3*C*).

Phosphorylation at Thr-34 converts DARPP-32 into an inhibitor of protein phosphatase-1 (44) and represents a positive feedback mechanism able to amplify a large number of dopaminergic and non-dopaminergic responses in the striatum (45, 46). The above results show that the D2L isoform is responsible for D2-like receptor-mediated regulation of DARPP-32 phosphorylation in striatal medium spiny neurons. Thus, the presence of an additional stretch of 29 amino acids in the third intracellular loop confers to the dopamine D2L receptor the ability to interact with specific proteins at postsynaptic level whose activation leads to the control of the state of phosphorylation of DARPP-32.

D2L KO mice express normal levels of D2 receptor binding sites (10). Indeed, lack of exon 6, specific for the D2L isoform, results in the conversion of all dopamine D2 receptor transcripts into D2S receptors. Thus, in D2L KO mice, a higher number of D2S receptors are expressed by medium spiny neurons (10, 21). This abnormal expression might be associated with changes in the coupling of D2S receptors to G proteins and, if so, could explain the inability of quinpirole to regulate DARPP-32 phosphorylation in D2L KO mice. However, the ability of quinpirole to stimulate [^{35}S]GTPγS binding to cell membrane fractions prepared from the striata of wild-type mice and D2L KO mice did not differ (the EC_{50} for quinpirole, calculated by nonlinear regression analysis, was 6.01 $\pm$ 0.2 in wild-type mice, and 6.03 $\pm$ 0.2 in D2L KO mice).

The above results indicate that the absence of quinpirole-mediated regulation of DARPP-32 occurring in D2L KO mice is not attributable to a diminished ability of D2S receptors to activate G proteins and may therefore depend on differential G protein coupling (22, 23) and/or subcellular localization. Whereas D2L receptors seem to be involved in postsynaptic regulation of DARPP-32, the presence in medium spiny neurons of potentially functional D2S receptors points to their possible participation in other types of postsynaptic responses. Further studies will be necessary to identify such responses.

In conclusion, the present study shows distinct roles of D2S and D2L receptor isoforms in presynaptic and postsynaptic signaling. The D2S receptor specifically regulates the state of phosphorylation and activity of TH in nigrostriatal presynaptic terminals, whereas the D2L receptor is mainly involved in the regulation of DARPP-32 phosphorylation in postsynaptic striatal medium spiny neurons. Further work will be necessary to fully understand the differences between D2S and D2L receptor-mediated transmission.

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