DARP-32: Regulator of the Efficacy of Dopaminergic Neurotransmission


Dopaminergic neurons exert a major modulatory effect on the forebrain. Dopamine and adenosine 3’,5’-monophosphate–regulated phosphoprotein (32 kilodaltons) (DARP-32), which is enriched in all neurons that receive a dopaminergic input, is converted in response to dopamine into a potent protein phosphatase inhibitor. Mice generated to contain a targeted disruption of the DARP-32 gene showed profound deficits in their molecular, electrophysiological, and behavioral responses to dopamine, drugs of abuse, and antipsychotic medication. The results show that DARP-32 plays a central role in regulating the efficacy of dopaminergic neurotransmission.

Midbrain dopaminergic neurons play a critical role in multiple brain functions (1–3). Abnormal signaling through dopaminergic pathways has been implicated in several major neurological and psychiatric disorders, including Parkinsonism, schizophrenia, and drug abuse (4). The physiological and clinical importance of dopamine pathways in the brain makes it imperative to elucidate the mechanisms by which dopamine, acting on its receptors, produces its biological effects on target neurons.

One well-studied molecular target for the actions of dopamine is DARP-32 (5), which is highly enriched in virtually all medium spiny neurons in the striatum (6). Dopamine, acting on D1-like receptors, causes activation of protein kinase A (PKA) and phosphorylation of DARP-32 on threonine-34 (7). Conversely, dopamine, acting on D2-like receptors, through both inhibition of PKA and activation of calcium/calcmodulin–dependent protein phosphatase (protein phosphatase 2B/calcineurin), causes the dephosphorylation of DARP-32 (8). Several other neurotransmitters that interact with the dopamine system also stimulate either phosphorylation or dephosphorylation of DARP-32 through various direct and indirect mechanisms (9).

DARP-32, in its phosphorylated but not its dephosphorylated form, acts as a potent inhibitor of protein phosphatase-1 (PP-1) (10). PP-1 controls the state of phosphorylation and the physiological activity of a wide array of neuronal phosphoproteins, including neurotransmitter receptors, ion channels, ion pumps, and transcription factors (11).

That numerous pathways regulate, or are regulated by, the DARP-32/PP-1 signaling cascade suggests the central importance of DARP-32 in mediating the biological effects of dopamine. To evaluate this hypothesis, given the absence of any specific pharmacological antagonists for DARP-32, we generated mice that lack this protein (12). The absence of DARP-32 protein from mice homozygous for the mutated DARP-32 gene was demonstrated by immunoblotting striatal extracts. Immunocytochemistry confirmed that the DARP-32 protein was absent from mutant mouse brain (13), although the brains of the DARP-32 mutant mice appeared normal structurally (14, 15).

Phosphorylated DARP-32 inhibits dephosphorylation of numerous other proteins by PP-1. Therefore, we examined the possi-
bility that the DARPP-32 mutant mice might show an aberrant state of phosphorylation of PP-1 substrates in response to stimulation by dopamine. One protein phosphorylated in striatum and nucleus accumbens in response to dopamine is the NR1 subunit of the N-methyl-D-aspartate (NMDA)–type glutamate receptor (16). We tested the effect of mutation of the DARPP-32 gene on dopamine-stimulated phosphorylation of this receptor (Fig. 1A). The total amount of NR1 in slices of nucleus accumbens was unaffected by the loss of DARPP-32. Dopamine increased NR1 phosphorylation by three- to fourfold in wild-type mice, but this increase was abolished in DARPP-32 mutant mice (17). The demonstration that DARPP-32 is involved in dopamine-regulated phosphorylation of the NR1 receptor is consistent with recent electrophysiological studies. Thus, in rat and mouse striatal neurons, dopamine, D1 agonists, and forskolin enhanced responses mediated by activation of NMDA receptors (18, 19). In Xenopus oocytes, DARPP-32 was found to be a critical component of adenosine 3′,5′-monophosphate–dependent regulation of NMDA current (20).

Activation of the dopamine D1 receptor–PKA–DARPP-32 cascade alters the electrophysiological properties of dopaminergic neurons in several ways. One target of D1 receptors in striatal neurons is the electrogenic ion pump Na+- and K+-dependent adenosine triphosphatase (Na+-K+-ATPase) (21), which regulates membrane potential and electrical excitability. The principal role of this transmembrane protein in neurons is to maintain the Na+ and K+ concentration gradients and the membrane potential that underlie electrical excitability. The activity of Na+-K+-ATPase in dissociated mouse striatal neurons was reduced by the D1 receptor

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Fig. 1. Reduced ability of dopaminergic agonists to regulate electrophysiological properties of dopaminergic neurons from DARPP-32 mutant mice. (A) Effect of dopamine (100 μM) on phosphorylation of NR1 subunit of glutamate NMDA receptor in nucleus accumbens slices. Data are expressed as percent radioactivity for the zero time controls (mean ± SEM, n = 5, *P < 0.05, Student’s t test). (B) Na+-K+-ATPase activity. Acutely dissociated striatal neurons prepared from wild-type or mutant mice were incubated in the absence or presence of the D1 receptor agonist SKF 82526 (1 μM) for 10 min (n = 5). Na+-K+-ATPase activity was assayed as described (27). Basal Na+-K+-ATPase activity was similar in wild-type (442 ± 27 nmol of inorganic phosphate per milligram of protein per minute) and mutant (394 ± 56 nmol of inorganic phosphate per milligram of protein per minute) mice. *P < 0.01; paired t test, compared with control. (C) and (D) Plot of peak calcium current versus time in striatal neurons. Application of the D1 receptor agonist SKF 81297 (5 μM) resulted in greater inhibition of the whole-cell current in wild-type neurons (21.4% ± 2.4%, mean ± SEM, n = 10) than in mutant neurons (15% ± 1.1%, n = 12, P < 0.05, Mann-Whitney U test). (Inset) Box-plot summary of the D1 receptor–mediated inhibition of calcium currents in wild-type and mutant neurons. (a and d) Representative current traces from the records used to construct (a) and (c), respectively. (D) Inhibitory efficacy of the D1 receptor agonist SKF 81297 (0.01 M, pipette concentration) on firing rate of nucleus accumbens neurons tested in vivo. Glutamate (0.01 M, pipette concentration) was used to drive the activity of nucleus accumbens neurons. For SKF 81297 delivered at lower iontophoretic currents, glutamate-driven activity was significantly less in neurons recorded from wild-type (n = 7), but not in those from mutant (n = 14) mice. Each data point represents mean ± SEM. **P < 0.01, analysis of variance (ANOVA) followed by Dunnett’s test.
agonist SKF 82526 (Fig. 1B). This inhibition was abolished by the D1 receptor antagonist SCH 23390 (22). In neurons from DARPP-32 mutant mice, the ability of the D1 agonist to inhibit Na⁺,K⁺-ATPase was reduced (Fig. 1B).

D1 receptor stimulation also reduces the responsiveness of medium spiny neurons in the striatum to excitatory input at hyperpolarized membrane potentials through mechanisms that are independent of Na⁺,Ca²⁺ channels—notably, Na⁺ and Ca²⁺ currents that are reduced by D1 receptor–mediated activation of PKA in medium spiny neurons of rats (26). Whole-cell voltage clamp recordings of Ca²⁺ currents revealed that D1 receptor stimulation produces a similar, potent modulation in acutely isolated striatal medium spiny neurons from wild-type mice (Fig. 1C). Although basal current densities were unchanged, the modulation of Ca²⁺ currents by D1 receptor agonists was reduced by about 50% in striatal neurons from DARPP-32 mutant mice (Fig. 1C).

Intracellular recordings from medium spiny neurons in slices also provided evidence for an attenuation of D1 receptor–mediated changes in cellular excitability in DARPP-32 mutant mice. In current-clamp recordings from medium spiny neurons of rats at hyperpolarized membrane potentials, D1 receptor stimulation increased rheobase current (current injection threshold to elicit a single spike) through PKA-mediated reduction in Na⁺ currents (24, 25). In wild-type mice D1 receptor agonists also produced an increase in the current injection threshold of medium spiny neurons. This effect was significantly decreased in neurons from the DARPP-32 mutant mice (27).

D1 receptor stimulation also reduces the responsiveness of medium spiny neurons to exogenous glutamate in vitro (18, 28). In the present experiments, extracellular electrodes were used to record from type I medium spiny neurons in the nucleus accumbens. Glutamate and a dopaminergic ligand were applied near the recorded cell by iontophoresis.

**Table 1. Reduced ability of raclopride to induce catalepsy in DARPP-32 mutant mice.** Catalepsy testing (39) was conducted 30 min after intraperitoneal injection of vehicle or raclopride (n = 12 per dose group). Wild-type and mutant control mice injected with vehicle remained stationary for an average of 17 s. Data represent percentage increase in catalepsy (mean ± SEM) relative to vehicle-injected control animals. Data were analyzed by ANOVA, followed by Student’s t test.

<table>
<thead>
<tr>
<th>Raclopride (mg/kg)</th>
<th>Increase in catalepsy (%)</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>Mutant</td>
</tr>
<tr>
<td>0.25</td>
<td>236.5 ± 48.7</td>
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<tr>
<td>0.5</td>
<td>369.5 ± 62.2</td>
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<tr>
<td>1.0</td>
<td>579.2 ± 65.2</td>
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<tr>
<td>2.0</td>
<td>865.6 ± 49.2</td>
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*P < 0.05 compared with wild-type control; **P < 0.01 compared with wild-type control.
lease from, and methamphetamine-induced toxicity to, dopaminergic neurons demonstrate that the effect of this deletion on the biological properties of the medium spiny neurons is strong enough to alter the characteristics of other neurons in this brain region, which do not contain DARPP-32.

A well-characterized molecular consequence of dopaminergic signaling in the striatum is the regulation of gene expression. Agents that increase dopaminergic neurotransmission—for example, amphetamine and cocaine—have been shown to induce several Fos-like proteins in medium spiny neurons in the striatum, an effect that is mediated largely by activation of D1-like receptors (31). Acute exposure to amphetamine elicited a robust induction of Fos-like immunoreactivity throughout the striatum of wild-type mice. Significant reductions in this response were observed in most regions of the striatum in DARPP-32 mutant mice (Fig. 3A). This deficit in c-Fos induction in the mutant mice was partially overcome by administration of a higher dose of amphetamine (32). Chronic exposure to drugs of abuse leads to the accumulation of distinct Fos-like proteins, isoforms of ΔFosB (33), an effect that also is largely mediated by D1-like receptors (34). Induction of the 35- to 37-kD ΔFosB isoforms, observed in striatum of wild-type mice in response to chronic administration of cocaine, was virtually abolished in the DARPP-32 mutant mice (Fig. 3B). These results indicate that DARPP-32 plays an important role in the short- and long-term changes in gene expression elicited by acute and chronic drug exposure, respectively.

Acute exposure to cocaine stimulates locomotor activity in rodents, an effect largely mediated by increased dopaminergic transmission in the striatum, particularly the nucleus accumbens (see (1)). This effect of cocaine, which is mediated in part via the dopamine D1 receptors (35), was significantly attenuated in DARPP-32 mutant mice at lower, but not higher doses of the drug (Fig. 3C). Acute locomotor responses to D-amphetamine were also reduced in the mutant mice (36). No difference, however, was observed between wild-type and mutant mice in baseline measures of locomotor activity (Fig. 3C) or in the spontaneous locomotor activity measured by 24-hour monitoring in the animals' home cages (36).

Raclopride and other antipsychotic drugs induce catalepsy in rodents by a mechanism involving blockade of striatal D2-like dopamine receptors. Because raclopride increases the basal phosphorylation of DARPP-32 and prevents the D2 receptor-mediated decrease in DARPP-32 phosphorylation in mouse striatal slices (8), we tested the possibility that this behavioral effect of raclopride might be altered in the DARPP-32 mutant mice. Raclopride produced catalepsy in both wild-type and mutant mice; however, its effectiveness at lower concentrations (0.25 and 0.5 mg/kg) was greatly reduced in the mutant mice (Table 1).

This study has revealed that inactivation of the DARPP-32 gene markedly reduced, and in some cases abolished, various responses to dopaminergic agonists and antagonists. In some instances, the impairment of responses could be overcome by increasing the concentration of the test substance used. These observations can be readily explained by the fact that stimulation of dopamine receptors regulates phosphorylation of key substrates by PKA, and the other involves inhibition of their dephosphorylation by PP-1, the activity of which is regulated by DARPP-32. Both pathways are required when the levels of stimulation of dopamine receptors are low (most physiological situations). In contrast, at supraphysiological levels of stimulation, the robust activation of the direct PKA pathway alone appears sufficient to restore responses in the mutant mice, which is why some of the deficits observed in these mice could be overcome by increasing the strength of the stimulus. From these data we conclude that a cascade involving dopamine-mediated receptor activation of DARPP-32, inhibition of PP-1, and potentiation of phosphorylation of neuronal substrates plays a major role in regulating the efficacy of dopaminergic neurotransmission under physiological conditions.

Numerous neurotransmitters besides dopamine have been shown to produce physiological responses and to regulate phosphorylation or dephosphorylation of DARPP-32 in medium spiny neurons (9). The results of this study indicate that such regulation of DARPP-32 is probably a major molecular mechanism by which information received through dopaminergic and other signaling pathways is integrated in these neurons, which constitute the principal efferent pathway from the striatum. Furthermore, the decreased sensitivity of mutant mice to drugs of abuse and antipsychotic agents indicates the involvement of DARPP-32 in mediating the pharmacological effects of both of these classes of compounds. Drugs that mimic or block the inhibitory effects of DARPP-32 on PP-1 might provide useful agents for the treatment of Parkinson's disease, schizophrenia, drug addiction, and other neuropsychiatric disorders involving abnormal dopaminergic function.

References and Notes
Disruption of the DARPP-32 gene was accomplished in the E14 embryonic stem cell line (129/Ola-derived substrate) by using a targeting vector containing 2.2 kb (5') and 6.0 kb (3') of genomic DNA flanking a neomycin-resistance gene (PGK-neo). Homologous recombination at the endogenous locus was designed to result in the replacement of a 400-base pair recombination at the endogenous locus was designed to result in the replacement of a 400-base pair fragment containing the start of translation with PGK-neo. The choice of targeted clones was based on the shift of a genomic fragment from 6.2 to 7.6 kb as determined by Southern (DNA) blot analysis. After C57BL/6J blastocyst injection and embryonic transfer, chimeric offspring were crossed to C57BL/6J females, and those mice carrying the mutation were crossed to generate heterozygous and homozygous mutants. Mice used in this study were selected from the offspring of heterozygous X heterozygous breeding pairs. Mice were selected from three colonies that were maintained at the F3, F4, and N1 levels of backcrossing. Backcrossing was carried out onto the C57BL/6J inbred mouse strain (from the Jackson Laboratory). None of the phenotypes presented appeared to be influenced by the level of backcrossing. All the mice used were males, except for the methamphetamine toxicity experiments, in which females were used. All mice were 3 to 6 months of age, except when we examined the electrophysiology of calcium channels in 3- to 5-week-old mice. DARPP-32 mutant mice showed no obvious differences from wild-type mice in measures of over- and underphysiology of calcium channels in 3- to 5-week-old mice. DARPP-32 mutant mice showed no obvious differences from wild-type mice in measures of over- and underwphysiology of calcium channels in 3- to 5-week-old mice. DARPP-32 mutant mice showed no obvious differences from wild-type mice in measures of over- and under}

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