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DARPP-32: An Integrator of Neurotransmission

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■ **Abstract** Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP-32), was identified initially as a major target for dopamine and protein kinase A (PKA) in striatum. However, recent advances now indicate that regulation of the state of DARPP-32 phosphorylation provides a mechanism for integrating information arriving at dopaminoceptive neurons, in multiple brain regions, via a variety of neurotransmitters, neuromodulators, neuropeptides, and steroid hormones. Activation of PKA or PKG stimulates DARPP-32 phosphorylation at Thr^{34} and thereby converts DARPP-32 into a potent inhibitor of protein phosphatase-1 (PP-1). DARPP-32 is also phosphorylated at Thr^{75} by Cdk5 and this converts DARPP-32 into an inhibitor of PKA. Thus, DARPP-32 has the unique property of being a dual-function protein, acting either as an inhibitor of PP-1 or of PKA. The state of phosphorylation of DARPP-32 at Thr^{34} depends on the phosphorylation state of two serine residues, Ser^{102} and Ser^{137}, which are phosphorylated by CK2 and CK1, respectively. By virtue of its ability to modulate the activity of PP-1 and PKA, DARPP-32 is critically involved in regulating electrophysiological, transcriptional, and behavioral responses to physiological and pharmacological stimuli, including antidepressants, neuroleptics, and drugs of abuse.

BIOCHEMISTRY OF DARPP-32

DARPP-32 was identified as a major target for dopamine-activated adenylyl cyclase in striatum (1, 2). Over the past 20 years, using a variety of molecular, cellular, and functional approaches, DARPP-32 has been established as a crucial mediator

of the biochemical, electrophysiological, transcriptional, and behavioral effects of dopamine. In this review, we summarize recent studies of the biochemical properties of DARPP-32 and highlight the critical integrative role played by DARPP-32 in the actions of various other neurotransmitters, neuromodulators, neuropeptides, and steroid hormones.

DARPP-32 Phosphorylated at Thr^{34} by PKA is a Potent Inhibitor of the Multifunctional Serine/Threonine Phosphatase, PP-1

The amino acid sequence of DARPP-32 (3) revealed that it was similar to inhibitor-1, an inhibitor of PP-1 initially identified in liver and skeletal muscle, but later found to be expressed at low concentrations in various regions of the central nervous system (4). DARPP-32 and inhibitor-1 share a high degree of amino acid sequence identity within the first 40 amino acids and are phosphorylated by protein kinase A at Thr^{34} and Thr^{35}, respectively. Phosphorylation by protein kinase A (PKA) converts each protein into a potent high-affinity inhibitor of PP-1 with an IC_{50} of approximately 10^{-9} M (5). DARPP-32 is expressed in very high concentration ($\sim$50 μM) in virtually all medium spiny neurons (6, 7), including those in both the striatonigral and striatopallidal projection pathways. The total concentration of all PP-1 isoforms in medium spiny neurons is likely less than 20 μM (8), and DARPP-32 can be phosphorylated at Thr^{34} with a stoichiometry of up to 0.2 mol/mol in intact neurons following dopamine D_1 receptor activation. Thus, a substantial proportion of PP-1 activity will be inhibited in response to dopaminergic regulation of medium spiny neurons. Thr^{34} of DARPP-32 is also an excellent substrate for phosphorylation by protein kinase G (PKG) (9).

DARPP-32 Interacts With PP-1 Via a Docking Motif Common to Many PP-1 Binding Proteins

A variety of structure-function studies have indicated that two domains of DARPP-32 (and also inhibitor-1) are involved in its interaction with PP-1 (Figure 1) (10–13). An inhibitory domain, consisting of phospho-Thr^{34} and the surrounding residues, is likely to occupy, or bind close to, the active site of the enzyme in a manner in which access to phosphorylated substrate is prevented. A second domain of DARPP-32, consisting of residues 7–11 (KKIQF), interacts with PP-1 at a site removed from the active site. Studies of a number of PP-1 targeting subunits (11, 14) have revealed that they contain a domain related to the KKIQF sequence and that this constitutes a common structural motif involved in binding of DARPP-32 and the various targeting proteins to PP-1. Identification of the PP-1 docking motif in DARPP-32 and other proteins has allowed the development of peptides that can antagonize the interaction of phospho-DARPP-32 or targeting subunits with PP-1 (15–17). Moreover, the structural basis for the interaction of the docking motif with PP-1 has been elucidated from x-ray crystallography studies (14). The determination of additional details of the interactions of PP-1 with phospho-DARPP-32 may prove useful in the

development of nonpeptide inhibitors for treatment of disorders of dopamine signaling pathways.

The presence of a conserved docking motif in DARPP-32 and many PP-1 targeting subunits predicts that there will be mutually exclusive binding of the inhibitor and the targeting proteins to the catalytic subunit of PP-1. In particular, competition between phospho-Thr^{34}-DARPP-32 and the PP-1 targeting proteins spinophilin and neurabin is important for regulation of PP-1 at postsynaptic sites in medium spiny neurons (18).

DARPP-32 Function is Regulated by Phosphorylation at Multiple Sites

Although the first 40 amino acids at the NH_2 termini of DARPP-32 and inhibitor-1 are very similar, the rest of the proteins are unrelated, and several different types of biochemical approaches have revealed that the remaining COOH-terminal portion of DARPP-32 serves as a substrate for three distinct protein kinases, namely cdk5, CK1, and CK2. In intact neurons, DARPP-32 is highly phosphorylated at Ser^{102} and Ser^{137} under basal conditions. Ser^{102} is phosphorylated by CK2 and Ser^{137} is phosphorylated by CK1 (Figure 1). In vitro, phosphorylation of Ser^{102} of DARPP-32 increases the efficiency of phosphorylation of Thr^{34} by PKA but not PKG (19). In vitro, phosphorylation of Ser^{137} decreases the rate of dephosphorylation of Thr^{34} by PP-2B, and in striatal slices, DARPP-32 phosphorylated at Ser^{137} is phosphorylated to a higher level at Thr^{34} (10, 20). The overall consequence of phosphorylation of DARPP-32 by CK1 or CK2 in intact cells is to increase the state of phosphorylation of Thr^{34}. Thus, the physiological role of these two phosphorylation events is to potentiate D1 dopaminergic signaling through the DARPP-32/PP-1 pathway.

Biochemical studies indicate that Thr^{75} is present within a consensus phosphorylation site for proline-directed kinases, and initial in vitro studies demonstrate that DARPP-32 is an efficient substrate for cdc2 kinase. In postmitotic neurons, cdc2 kinase is not active. However, cdk5, a cyclin-dependent kinase family member, which is activated by the noncyclin cofactor p35, is highly expressed (21). A variety of studies, including the observation that Thr^{75} of DARPP-32 was phosphorylated to a low level in striatal homogenates obtained from p35−/− mice, confirmed that DARPP-32 was a physiological target for cdk5/p35 (22). In vitro, phosphorylation of DARPP-32 at Thr^{75} does not alter the kinetics of phosphorylation by either CK1 or CK2. However, phosphorylation of Thr^{75} has a major inhibitory effect on the phosphorylation of Thr^{34} by PKA. DARPP-32 phosphorylated at Thr^{75} also inhibits the phosphorylation of exogenous substrates, such as inhibitor-1, ARPP-16, and ARPP-21 (two PKA substrates also enriched in the striatum), whereas unphosphorylated DARPP-32 has no effect. The level of phosphorylation of Thr^{75} in intact striatal tissue was found to be ∼0.26 mol/mol, equivalent to a concentration of ∼13 μM, and is at a level that is in excess of the K_i for PKA observed in vitro (K_i of ∼2.7 μM using ARPP-21 as substrate).

These biochemical studies therefore suggested that phosphorylation of DARPP-32 by cdk5 would inhibit PKA-mediated phosphorylation in intact striatal neurons. The resultant decrease in phosphorylation of Thr^{34} of DARPP-32 would inhibit D_1 dopamine signaling through the DARPP-32/PP-1 cascade. As described in more detail below, these possibilities were confirmed in a number of studies in intact neurons. Taken together, the results suggest that DARPP-32 can function either as an inhibitor of PP-1 (when phosphorylated at Thr^{34}) or as an inhibitor of PKA (when phosphorylated at Thr^{75}).

Dephosphorylation of DARPP-32 and Its Role in a Protein Phosphatase Cascade

In vitro, PP-2B (also known as calcineurin) has been shown to be the most effective phosphatase in dephosphorylating phospho-Thr^{34} (23, 24). However, studies both in vitro and in intact neurons indicate that PP-2A can also dephosphorylate this site (see below). In vitro, phospho-Thr^{75} is most effectively dephosphorylated by PP-2A (24), and studies in intact neurons suggest that specific, regulated PP2A isoforms are likely to be involved in the dephosphorylation of this site (24). In vitro and in intact cells, phospho-Ser^{137} is preferentially dephosphorylated by PP-2C (25). In vitro, phospho-Ser^{102} is most efficiently dephosphorylated by PP-2A, although PP-1 also shows significant ability to dephosphorylate this site (19). Interestingly, prior phosphorylation of Thr^{34} inhibits the dephosphorylation of phospho-Ser^{102} by PP-2A (19), further suggesting that dephosphorylation of specific sites in DARPP-32 may be influenced by the state of phosphorylation of other sites. The identity of the phosphatase that dephosphorylates phospho-Ser-^{102} in intact neurons remains to be characterized.

The finding that Ser^{137} phosphorylation influences the ability of PP-2B to act on Thr^{34} (see above) reveals a hierarchical relationship among PP-2C, PP-2B, and PP-1. Thus, PP-2C activity toward Ser^{137} increases the ability of PP-2B to dephosphorylate phospho-Thr^{34}. In turn, dephosphorylation of Thr^{34} by PP-2B leads to generation of active PP-1. Several protein kinase cascades have been described that are critical for signal amplification or for the integration of multiple inputs from distinct extracellular messengers. The hierarchical relationship of the various phosphatases acting on or inhibited by phospho-DARPP-32 is mechanistically distinct from those classical kinase cascades. However, the physiological relationship between PP-2C, PP-2B, and PP-1, which involves DARPP-32, would likely result in a protein phosphatase cascade.

DISTRIBUTION OF DARPP-32 IN THE BRAIN

The distribution of the DARPP-32 protein and mRNA has been studied using various immunological techniques, in situ hybridization, and northern blotting. Anatomical studies have been conducted in several different species, including

mice, rats, monkeys, and humans. In general, the distribution of DARPP-32 is very similar in these species, suggesting that it may be relevant to extrapolate functional data obtained in rodents to man. A prominent aspect of the distribution of DARPP-32 is its high enrichment in dopaminoceptive neurons.

Expression of DARPP-32 During Ontogeny

DARPP-32 is detected in dopaminoceptive regions from gestational day 14–16 in the rat (26). The arrival of tyrosine hydroxylase-immunoreactive axon terminals follows the appearance of DARPP-32, and lesion studies have shown that the development of DARPP-32 is independent of dopaminergic innervation (26, 27). An early appearance of DARPP-32 has also been found in human fetuses. DARPP-32 protein is detectable in the human anlage for striatal neurons as early as seven weeks of gestation (28). DARPP-32 is enriched in striosomes during development both in rats (26) and in humans (28). The functional implications of this pattern of DARPP-32 during ontogeny are not understood. At later postnatal stages, the distribution of DARPP-32 in the striatum becomes homogeneous in rodents and humans. In rodents, the level of DARPP-32 increases at birth and reaches adult levels approximately three weeks postnatally. The early and defined appearance of DARPP-32 suggests that it may influence specific aspects of neuronal differentiation and synaptogenesis. However, the gross morphology of the striatum, as well as other parts of the brain, appear normal in mutant mice lacking DARPP-32 (29).

Distribution of DARPP-32 in the Adult Brain

DARPP-32 is localized, with few exceptions, to regions that receive dopaminergic innervation. A detailed immunohistochemical study in the rat brain demonstrated that the highest levels of DARPP-32 are found in caudatoputamen, nucleus accumbens, olfactory tubercle, bed nucleus of stria terminalis, and portions of the amygdaloid complex (7). These brain regions send projections to various target areas, including globus pallidus, ventral pallidum, the entopeduncular nucleus, and substantia nigra pars reticulata. Within these target areas, high levels of DARPP-32 are found in nerve terminals. Moderate levels of DARPP-32 are found throughout the neocortex, with particular enrichment in layers II, III, and VI. There are also moderate levels of DARPP-32 in several subregions of hypothalamus, including the median eminence, arcuate nucleus, and the medial habenula (7). DARPP-32 is also found in some areas that receive sparse, if any, dopaminergic innervation, of which the Purkinje cells of the cerebellum and the choroid plexus are the most notable. A pattern of distribution of DARPP-32 very similar to that described in the rat brain was found in the primate brain (30). The cloning of the DARPP-32 gene (31, 32) enabled studies on the distribution of DARPP-32 mRNA (33). The results obtained in these studies largely confirmed the immunohistochemical studies.

Striatum as Part of the Basal Ganglia

The basal ganglia are composed of several subcortical nuclei, including the striatum (the caudate putamen and the nucleus accumbens), the globus pallidus (external part of pallidum in primates), the entopeduncular nucleus (internal part of pallidum in primates), the subthalamic nucleus, and the substantia nigra. The basal ganglia play a critical role in the integration of sensorimotor, associative, and limbic information to produce motor behaviors.

The striatum is a central component of the basal ganglia as it integrates excitatory glutamatergic inputs, predominantly from the cortex and thalamus, with dopaminergic and serotonergic inputs from mesencephalon, and sends projections to the output structures of the basal ganglia (Figure 2). The cortical inputs to the striatum originate from glutamatergic pyramidal neurons that arise from most areas of the cortex (34). They project in a topographically well-organized manner so that they define functionally distinct regions of the striatum. Inputs from sensorimotor areas principally innervate the dorsal part of the striatum, whereas inputs from limbic cortical areas terminate in the ventral area (35). At the ultrastructural level, the excitatory cortical projection neurons have been shown to form asymmetric contacts on dendritic spines of striatal neurons.

The striatum is the major target for dopaminergic neurons in the CNS. The dopaminergic input to the striatum is topographically organized so that the ventral tegmental area predominantly innervates the nucleus accumbens, whereas the substantia nigra pars compacta preferentially innervates the caudate putamen (Figure 2) (36). There is a moderate serotonergic innervation of the striatum (Figure 2) (37), which is more dense in the nucleus accumbens than in the caudate-putamen. The serotonergic input to the striatum is less organized than the glutamatergic and dopaminergic inputs.

Neuronal Subpopulations of Striatal Neurons

The medium-sized spiny neurons, which constitute the major cell type (95%) in the striatum, are inhibitory and utilize GABA as their major neurotransmitter (38). These GABAergic neurons are homogeneously distributed in the striatum. Detailed anatomical analyses have demonstrated that they can be divided into two equally large subpopulations based on their peptide content and their projection areas (Figure 2) (39, 40). One subpopulation contains substance P and dynorphin and projects directly to substantia nigra pars reticulata and the entopeduncular nucleus (the direct striatonigral pathway). The other subpopulation contains enkephalin and projects indirectly to these structures via relays in the globus pallidus and subthalamic nucleus (the indirect striatopallidal pathway). Medium-sized spiny projection neurons also send axon collaterals within the striatum (41).

The remaining 5% of the striatal neurons are composed of aspiny interneurons, which are divided in two major classes based on distinct morphological and neurochemical characteristics: the large-sized cholinergic neurons and the medium-sized

GABAergic neurons (42). The GABAergic interneurons can be further subdivided according to their neuropeptide content.

Phenotypical Characterization of Striatal Neurons Expressing DARPP-32

By using morphological criteria, dual labeling methods, and retrograde tracing, it has been demonstrated that DARPP-32 is found in a great majority of the medium-sized spiny neurons in rodents as well as primates (7, 8, 30, 43). DARPP-32 is expressed both in substance P/dynorphin–containing striatonigral neurons and in enkephalin-containing striatopallidal neurons (43). Large-sized cholinergic interneurons do not contain DARPP-32 (43, 44). Likewise, medium-sized GABAergic interneurons are devoid of DARPP-32 immunoreactivity (43, 44).

Ultrastructural Localization of DARPP-32

At the ultrastructural level, DARPP-32 has been found in most subcellular compartments (44). DARPP-32 immunoreactivity is observed throughout the cytoplasm and in dendrites. Some nuclei also contain DARPP-32 immunoreactivity. Although most synaptic contacts on DARPP-32-containing neurons are not immunoreactive, immunolabelled axon terminals are occasionally found in the caudate-putamen. These terminals form symmetric synaptic contacts with DARPP-32-immunolabelled somata or immunolabelled dendritic shafts and most likely represent axon collaterals. In the globus pallidus and substantia nigra pars reticulata, which are the major target areas from striatum, immunoreactivity is confined to axons and axon terminals.

FACTORS THAT REGULATE THE FUNCTION OF DARPP-32 BY ALTERING ITS PHOSPHORYLATION STATE

Biogenic Amines

Dopamine and serotonin are major biogenic amines controlling striatal DARPP-32 phosphorylation.

DOPAMINE The nigrostriatal dopamine system plays a crucial role in the regulation of movements, whereas the mesolimbocortical system mediates the cognitive and rewarding effects of dopamine. Indeed, destruction of nigrostriatal neurons is known to be the major pathology underlying Parkinson's disease (45), whereas depletion of dopamine in nucleus accumbens abolishes the stimulatory and reinforcing actions of the psychostimulants, cocaine and amphetamine (46, 47).

Five different dopamine receptors have been cloned. All of them are metabotropic and alter cAMP signaling; D_1 receptor subtypes (D_1, D_5) stimulate adenylyl cyclase, whereas D_2 receptor subtypes (D_{2S}, D_{2L}, D_3, D_4) inhibit adenylyl cyclase

(48, 48a). In addition, D_1-type receptors have been shown to raise intracellular Ca^{2+} levels (49). This action requires calcyon and priming with an agonist at Gq-coupled receptors. D_2-type receptors have been shown to increase intracellular Ca^{2+} levels and to activate a phospholipase C (PLC)/PP-2B signaling cascade (50).

The cellular distributions of D_1, D_2, and D_3 receptors in striatum have been described in detail. D_2 receptors are found on dopaminergic nerve terminals and postsynaptically on GABAergic medium spiny neurons as well as on cholinergic interneurons. D_1 and D_3 receptors are predominantly expressed postsynaptically on GABAergic medium spiny neurons. Anatomical studies have shown that striatonigral neurons contain high levels of D_1 receptors, whereas striatopallidal neurons predominantly express D_2 receptors (Figure 3) (51). Although the levels of D_1 and D_2 receptors differ between striatal projection neurons, there is biochemical and physiological evidence supporting the idea that many of them possess both D_1 and D_2 receptors (52–54). D_3 receptors are especially enriched in the nucleus accumbens, where they are expressed in both striatonigral and striatopallidal neurons (55).

Dopamine plays an important role in the coordination and regulation of the two output pathways by acting in a bidirectional manner. Many electrophysiological and gene transcriptional data, obtained in vitro and in vivo, suggest that dopamine exerts stimulatory effects via D_1 receptors and inhibitory effects via D_2 receptors (51, 56–59). Similarly, dopamine regulates the state of phosphorylation of DARPP-32 in a bidirectional manner (Table 1) (50). Using striatal slices or whole animals,

TABLE 1 Regulation of DARPP-32 phosphorylation by various factors (for details, see text)

Factor	Receptor	Signaling pathway	Thr^{34}	Thr^{75}	Ser^{137}
Dopamine	D_1	cAMP/PKA/(PP-2A)	↑	↓	
	D_2	cAMP/PKA*	↓	↑	
		Ca^{2+}/PP-2B	↓		
Serotonin	$5HT_2$	PLC/CK1			↑
	$5HT_{4/6}$	cAMP/PKA	↑	↓	
Glutamate	NMDA	Ca^{2+}/PP-2B	↓	↓	
	AMPA	Ca^{2+}/PP-2B	↓	↓	
	$mGlu_{1/5}$	PLC/CK1/cdk5		↑	↑
		A_{2A}/cAMP/PKA	↑		
GABA	$GABA_A$	Ca^{2+}/PP-2B*	↑		
Adenosine	A_{2A}	cAMP/PKA	↑	↓	
NO		cGMP/PKG	↑	↑	
Opioids	μ/δ	cAMP/PKA*	↓		
Neurotensin	$NTR_{1/2}$	D_1/cAMP/PKA	↑		
CCK	CCK_B	NMDA/Ca^{2+}/PP-2B	↓		

All activities are increased, except those with an *, which are decreased.

it has been shown that activation of D_1 receptors, via stimulation of PKA, results in phosphorylation of DARPP-32 at Thr^{34} (50, 60). This effect is counteracted by activation of D_2 receptors and involves inhibition of PKA (61) and stimulation of the PP-2B signaling cascade (50) (Figure 3). Activation of D_1 receptors also decreases the phosphorylation state of DARPP-32 at Thr^{75} by a process that likely involves the PKA-dependent activation of a specific isoform of PP-2A (62) (Figure 4). Thus, enhanced dopaminergic transmission via D_1 receptors leads to a decreased phosphorylation of Thr^{75}-DARPP-32, which reduces inhibition of PKA and thereby facilitates signaling via the PKA/Thr^{34}-DARPP-32/PP-1 cascade.

DOPAMINE SIGNALING IN DARPP-32 KNOCKOUT MICE The generation of DARPP-32 knockout (KO) mice (29) has enabled more detailed studies of the involvement of the protein in the actions of dopamine. As summarized in Table 2 and discussed below, studies using the DARPP-32 KO mice have demonstrated that DARPP-32 mediates many biochemical, electrophysiological, gene transcriptional, and behavioral effects of dopamine (29, 63). In general, the most significant

TABLE 2 Summary of responses that are regulated in DARPP-32 KO mice

Stimulus	Parameter	Example
Dopamine	Neurotransmitter receptor conductance	NMDA, AMPA, and GABA
Dopamine, serotonin, neurotensin	Neurotransmitter receptor phosphorylation	NR1, GluR1, $GABA_A$ $\beta 1/\beta 3$
Dopamine	Ion channel	N/P-type Ca^{2+} channel, Na^+ channel
Dopamine	Ion pump	Na^+/K^+-ATPase inhibition
Corticostriatal stimulation	Synaptic plasticity	LTP, LTD
Dopamine	Transcription factor phosphorylation	CREB
Dopamine, cocaine, amphetamines	Immediate early gene expression	C-fos, ΔfosB induction
Amphetamine	Damage of dopamine neurons	Reactive gliosis
Antipsychotics	Locomotor activity	Catalepsy
Apomorphine	Motor behavior	Cage climbing
Cocaine, ethanol	Reward	Conditioned place preference, self-administration
Caffeine, cocaine, and amphetamine	Locomotor activity	Acute locomotor response
Antidepressants	Helplessness	Tail suspension test
Progesterone	Sexual receptivity	Female lordosis

All actions are attenuated in DARPP-32 KO mice except for ethanol in the conditioned place preference paradigm.

involvement of DARPP-32 is found at physiological concentrations of dopamine, the effects being less pronounced at higher, supraphysiological, concentrations.

PKA and PP-1 regulate the phosphorylation state and activity of many physiological effectors, including neurotransmitter receptors, which regulate the excitability of medium spiny neurons. Using striatal slices and dissociated striatal neurons, it has been shown that treatment with a D_1 receptor agonist causes an increase in the phosphorylation of the NMDA NR1 subunit at Ser^{897}; the AMPA GluR1 subunit at Ser^{845}; and the $GABA_A$ β_1/β_3 subunits, presumably at Ser^{409}, which is paralleled by increased AMPA or NMDA receptor currents and inhibited $GABA_A$ receptor current (16, 29, 64–66). The dopamine-induced phosphorylation and regulation of these ionotropic receptors is strongly attenuated in DARPP-32 KO mice. Moreover, DARPP-32 plays a very important role in the dopamine-mediated regulation of other ion channels and ion pumps. For example, D_1 receptor-mediated inhibition of N/P-type Ca^{2+} channels and of neuronal Na^+,K^+-ATPase is attenuated in DARPP-32 KO mice (29). Several lines of evidence also indicate an important role for DARPP-32 in mediating the effects of dopamine on long-term changes in neuronal excitability. Studies in DARPP-32 KO mice showed that DARPP-32 plays a crucial role in the induction of both long-term depression (LTD) and long-term potentiation (LTP) (67), two opposing forms of synaptic plasticity. The involvement of DARPP-32 in both LTD and LTP may be explained by the fact that these effects were mediated via PKG and PKA, respectively, and involved different subpopulations of striatal neurons.

Long-term changes in synaptic plasticity initiate changes in gene transcription that are important for maintaining an altered synaptic function and for initiating adaptive morphological changes. There is accumulating evidence that regulation of gene transcription by various signal transduction cascades involves altered phosphorylation of transcription factors (47), and hence modulation of their activity. CREB is a functionally very important transcription factor. It is well established that dopamine, via activation of D_1 receptors and PKA, stimulates phosphorylation of CREB at Ser^{133} in striatum (47), and that the dephosphorylation of CREB at Ser^{133} is under the control of PP-1 (68). CREB phosphorylated at Ser^{133} regulates the expression of several immediate early genes, such as different members of the Fos/Jun family. These genes are also under the control of additional transcription factors, such as ELK, which, in turn, is regulated by the MAP kinase signaling cascade (47). Dopamine receptor-mediated activation of MAP kinase, CREB, c-Fos, and ΔFosB is strongly attenuated in DARPP-32 KO mice (16, 29, 69).

SEROTONIN The serotonergic neurotransmitter system, together with the dopaminergic system, regulates emotion, mood, reward, and cognition. Perturbations of these neurotransmitter systems are thought to contribute to the etiology of several common neuropsychiatric disorders, including schizophrenia, bipolar disorder, depression, and drug addiction. Indeed, the serotonergic and the dopaminergic systems appear to be the primary targets for most of the current medications used for the treatment of psychiatric disorders. Moreover, cocaine, as well as amphetamine,

act on both serotonin and dopamine transporters and cause significant increases in the extracellular levels of both dopamine and serotonin in nucleus accumbens (70).

Several serotonin receptors, i.e., 5-HT_{1B}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_4, and 5-HT_6, have been found on medium spiny neurons in nucleus accumbens and caudate-putamen. All of them are metabotropic receptors, with the exception of 5-HT_3 receptors, which are ionotropic. These serotonin receptors act primarily via the following second messenger systems: 5-$HT_{1B/E}$ receptors decrease cAMP formation, 5-$HT_{2A/C}$ receptors increase inositol triphosphate and diacylglycerol formation, 5-HT_3 receptors increase Na^+ and Ca^{2+} influx, and 5-HT_4 and 5-HT_6 receptors increase cAMP formation. Detailed studies in striatal slices and whole animals have shown that serotonin causes an increase in phosphorylation of DARPP-32 at Thr^{34} and Ser^{137} and a decreased phosphorylation at Thr^{75} (71). The actions of serotonin in regulating DARPP-32 phosphorylation at Thr^{34} and Thr^{75} were mediated primarily via activation of 5-HT_4 and 5-HT_6 receptors, whereas the regulation at Ser^{137} was mediated primarily via 5-HT_2 receptors. The three pathways appear to inhibit PP-1 through synergistic mechanisms. Although the pattern of DARPP-32 phosphorylation induced by elevated serotonergic neurotransmission is similar to that induced by elevated dopaminergic neurotransmission, it is largely independent of altered dopaminergic neurotransmission (71). It has been shown that the reinforcing properties of cocaine in the place preference test remain intact in dopamine transporter KO mice or serotonin transporter KO mice, but are severely impaired in mice that lack both transporters (72). These data imply that certain biochemical effects of cocaine can be mediated by either dopamine or serotonin. In support of the likelihood that signaling via DARPP-32 is responsible for this redundancy, the diminished responsiveness to the reinforcing properties of cocaine, observed in a place preference test in double transporter KO mice, is mimicked in DARPP-32 KO mice (73).

Amino Acids

Glutamate and GABA are major amino acids controlling striatal DARPP-32 phosphorylation.

GLUTAMATE Glutamatergic terminals of the corticostriatal and thalamostriatal pathway neurons form asymmetrical synapses on dendritic shafts and spines of medium spiny neurons. Glutamate receptors are subdivided into two categories: ionotropic glutamate receptors and metabotropic glutamate (mGlu) receptors. Glutamatergic regulation of DARPP-32 is very complex (Figure 4). It appears that the immediate and principal glutamate signaling is mediated through NMDA- and AMPA-receptor/PP-2B and that this pathway is modulated by other glutamate-dependent pathways, by both positive and negative feedback mechanisms.

NMDA- and AMPA-type glutamate receptors Glutamate, released at glutamatergic nerve terminals, activates NMDA- and AMPA-type ionotropic glutamate

receptors, leading to a decrease in DARPP-32 Thr^{34} phosphorylation (74, 75). The effects of NMDA and AMPA receptors on Thr^{34} phosphorylation are mediated via PP-2B (74, 75). AMPA receptor channels are permeable to Na^+ but relatively impermeable to Ca^{2+} (76). In contrast, NMDA receptor channels, when depolarized, are highly permeable to Ca^{2+} (77). The increase in PP-2B activity in response to activation of AMPA receptors is mediated through depolarization-induced influx of Ca^{2+} via L-type Ca^{2+} channels (G.L. Snyder & P. Greengard, unpublished data).

Activation of NMDA and AMPA receptors also results in a decrease in DARPP-32 Thr^{75} phosphorylation (75). Surprisingly, the effects of NMDA and AMPA receptors are not mediated through Ca^{2+}-dependent activation of PP-2B, but rather are mediated via a Ca^{2+}-dependent activation of PP-2A, which involves a mechanism that is not yet understood. Glutamate signaling through NMDA and AMPA receptors will reduce the state of phosphorylation of DARPP-32 at Thr^{75} and reduce inhibition of PKA. Thus, under some conditions, glutamate may be able to potentiate dopamine/D_1 receptor/PKA/phospho-Thr^{34} DARPP-32 signaling (Figure 4).

Metabotropic glutamate receptors mGlu receptors are subdivided into three groups: group I ($mGlu_1$ and $mGlu_5$ receptors), group II ($mGlu_2$ and $mGlu_3$ receptors), and group III ($mGlu_4$, $mGlu_6$, $mGlu_7$, and $mGlu_8$ receptors) (78). Individual subtypes of mGlu receptors are assumed to mediate distinct facilitatory (group I) or inhibitory (group II and III) actions on neuronal transmission. In the neostriatum, group I mGlu receptors are expressed in both direct and indirect pathway neostriatal neurons (79), and group II and III mGlu receptors are expressed on the terminals of corticostriatal afferents (80). It has been reported that mGlu receptors participate in the regulation of cAMP formation (81), gene expression (82), locomotor activity (83), and cocaine self-administration (84). Activation of group I $mGlu_5$ receptors stimulates DARPP-32 Thr^{34} phosphorylation in neostriatal neurons (85). The effect of $mGlu_5$ receptors on Thr^{34} phosphorylation is dependent on activation of adenosine A_{2A} receptors by endogenous adenosine, but not of D_1 receptors by endogenous dopamine. Activation of mGlu5 receptors potentiates the effect of an adenosine A_{2A} receptor agonist, CGS21680, and of forskolin, but not that of a cAMP analogue, and it stimulates DARPP-32 Thr^{34} phosphorylation in the presence of a phosphodiesterase inhibitor, suggesting that $mGlu_5$ receptors stimulate the rate of cAMP formation coupled to adenosine A_{2A} receptors. The action of $mGlu_5$ receptors is mediated through MAP kinase signaling, but not through PLC, CK1, or cdk5. Nonreceptor tyrosine kinases, such as pyk2 and src, and/or receptor tyrosine kinases, such as epidermal growth factor receptors, seem to be required for the activation of MAP kinase (86, 87). Selective interaction of $mGlu_5$ and A_{2A} receptors might be explained by the fact that MAP kinase signaling is more active in striatopallidal medium spiny neurons than in striatonigral medium spiny neurons (88). Existence of a heteromeric $mGlu_5/A_{2A}$ receptor complex further supports the selective interaction of $mGlu_5$ and A_{2A} receptors (88a). The molecular mechanisms by which MAP kinase is activated by mGlu5 receptors and by which MAP kinase stimulates cAMP formation coupled to A_{2A} receptors remain to be clarified.

Group I mGlu receptors regulate the phosphorylation of DARPP-32 at Thr^{75} and Ser^{137} by mechanisms different from those described above for the regulation of DARPP-32 at Thr^{34}. Treatment of neostriatal slices with a group I mGlu receptor agonist stimulates the activity of CK1 in a PLC-dependent manner, leading to an increase in the phosphorylation of DARPP-32 at Thr^{75} and Ser^{137} (89, 90). Detailed analysis of the mechanism of CK1 activation revealed that group I mGlu receptors, coupled to Gq, activate PLC and stimulate the generation of IP_3, leading to an increase in intracellular Ca^{2+} (90) (Figure 4). The increased intracellular Ca^{2+} activates PP-2B, most likely causing dephosphorylation of the inhibitory autophosphorylation sites of CK1, which results in the activation of CK1 and the phosphorylation of DARPP-32 at Ser^{137}. Activation of CK1 causes activation of Cdk5 (89). However, the mechanism by which CK1 activates Cdk5 is under investigation. Cdk5, activated by the group I mGlu receptor/PLC/PP-2B/CK1 signaling cascade, stimulates the phosphorylation of DARPP-32 at Thr^{75}.

Interaction of dopamine and glutamate signaling A major antidopaminergic effect of glutamate, through NMDA/AMPA receptor-induced activation of PP-2B, is the dephosphorylation of DARPP-32 at Thr^{34}. In addition to this major effect, glutamate has various other regulatory effects on DARPP-32 phosphorylation (Figure 4). For instance, glutamate, through the group I mGlu receptor/CK1/Cdk5/phospho-Thr^{75}-DARPP-32 pathway, counteracts PKA/phospho-Thr^{34}-DARPP-32/PP-1 signaling. Conversely, glutamate can potentiate PKA/phospho-Thr^{34}-DARPP-32/PP-1 signaling through three pathways: (*a*) by NMDA-AMPA receptor/Ca^{2+}/PP-2A/Thr^{75}-DARPP-32 dephosphorylation, (*b*) by enhancing A_{2A} receptor-coupled cAMP formation mediated through activation of $mGlu_5$ receptors (85), and (*c*) by increasing DARPP-32 Ser^{137} phosphorylation mediated through group I mGlu receptor-induced activation of CK1. Various types of glutamate-mediated regulation of DARPP-32 phosphorylation in striatal neurons are most likely activated with different time courses.

GABA Striatal medium spiny neurons receive most GABAergic inputs from an extensive network of recurrent collaterals (41) and from interneurons (42). Two types of GABA receptors are expressed on medium spiny neurons: bicucullin-sensitive $GABA_A$ receptors (ligand-gated chloride channels) and bicucullin-insensitive, metabotropic $GABA_B$ receptors. Studies performed in striatal slices showed that GABA was able to produce a rapid increase in the state of phosphorylation of Thr^{34} (91). This effect was prevented by bicucullin (91). GABA significantly potentiated the increase in phospho-Thr^{34}-DARPP-32 produced by forskolin, an activator of the cAMP/PKA pathway. This suggested that GABA increased Thr^{34} phosphorylation through inhibition of PP-2B. GABA was also able to stimulate Thr^{34} phosphorylation in slices from the substantia nigra. These effects are most likely mediated via activation of $GABA_A$ ionotropic receptors, increased Cl^- influx, decreased neuronal excitability, decreased Ca^{2+} influx, and inactivation of PP-2B.

Neuromodulators

Adenosine and nitric oxide are major neuromodulators controlling striatal phosphorylation.

ADENOSINE Adenosine is found intra- and extracellularly in all organs of the body. Most adenosine is formed via the breakdown of ATP intracellularly and transported to the extracellular space via equilibrative transporters. Extracellular adenosine acts via G-protein-coupled receptors, two of which, A_1 and A_{2A} receptors, are abundantly expressed in the brain. Adenosine A_1 receptors inhibit, and A_{2A} receptors stimulate, adenylyl cyclase. A_1 receptors have a widespread distribution in the brain, whereas A_{2A} receptors are almost exclusively found in striatum (92, 93). At the cellular level, A_{2A} receptor mRNA is restricted to the GABAergic medium-sized spiny projection neurons that also express enkephalin and dopamine D_2 receptors (92, 93) (Figure 3). A_{2A} receptors are segregated from D_1 receptors. Using striatal slices, the A_{2A} receptor agonist CGS21680 was found to increase the level of DARPP-32 phosphorylated at Thr^{34} in a concentration-dependent manner (94). When CGS21680 was combined with a D_1 agonist, an additive response was observed on cAMP levels and Thr^{34}-DARPP-32 phosphorylation. In whole animals, antagonists at A_{2A} and D_1 receptors had an additive effect in reducing DARPP-32 phosphorylation (94). In accordance with the anatomical colocalization of A_{2A} and D_2 receptors, there is evidence from biochemical, gene transcriptional, and behavioral studies showing interactions between A_{2A} and D_2 receptors (93). Because A_{2A} receptors increase and D_2 receptors decrease the levels of cAMP, the adenosine-dopamine interactions are, in most instances, antagonistic. Indeed, an A_{2A} antagonist significantly counteracted the increase in Thr^{34}-DARPP-32 phosphorylation that was observed following treatment with selective D_2 receptor antagonists (60). Likewise, the ability of D_2 antagonists to increase Thr^{34}-DARPP-32 phosphorylation was dramatically reduced in A_{2A} receptor KO mice (60). These data have provided further support for the notion that adenosine acting on A_{2A} receptors provides a basal tonic activity of the cAMP/PKA/Thr^{34}-DARPP-32 pathway, which is necessary to mediate many of the effects of dopamine acting via D_2 receptors.

Consistent with the biochemical studies, the ability of the A_{2A} agonist CGS-21680 to induce hypolocomotion was attenuated in DARPP-32 KO mice (95). Similarly, the ability of the A_{2A} antagonist SCH 58261 and caffeine (see below) to induce hyperlocomotion was attenuated in DARPP-32 KO mice. In this study (95), an additional effect of A_{2A} receptors on DARPP-32 phosphorylation was shown, namely that A_{2A} agonism via a cAMP-dependent mechanism increases the phosphorylation at Thr^{34}-DARPP-32, but decreases the phosphorylation at Thr^{75}-DARPP-32. Conversely, SCH 58261 increases phosphorylation at Thr^{75}-DARPP-32.

NITRIC OXIDE Nitric oxide (NO) is an intercellular messenger that plays a critical role in the physiology of striatal neurons (67). NO exerts its actions by activating

soluble guanylyl cyclase and hence PKG (96). In agreement with the observation that Thr^{34}-DARPP-32 is an excellent substrate for PKG in vitro (9), it was found that sodium nitroprusside (SNP), a NO donor, stimulates the phosphorylation of DARPP-32 at Thr^{34} in striatonigral nerve terminals (97). The effect of SNP could be inhibited by hemoglobin, a compound that, by complexing with NO, prevents the activation of PKG. Moreover, treatment of striatal slices with SNP or the cGMP selective phosphodiesterase inhibitor zaprinast, according to a protocol known to induce chemical LTD, increased the levels of DARPP-32 at Thr^{34} and Thr^{75} (67). Thus, the NO/cGMP pathway potently regulates the phosphorylation state of DARPP-32. This regulation may play an important role in the modulation of synaptic plasticity in striatum.

Neuropeptides

Opioids, cholecystokinin, and neurotensin control striatal DARPP-32 phosphorylation.

OPIOIDS Three major opioid receptors have been cloned: μ-, δ-, and κ-receptors. Opiates regulate striatal function via an indirect action on mesencephalic dopamine neurons and a direct action on opioid receptors located within striatum. There is a relatively abundant expression of μ-, δ-, and κ-opioid receptors in striatum (98, 99). κ-opioid receptors seem to be expressed primarily on dopaminergic nerve terminals, whereas μ- and δ-receptors are expressed on medium-sized spiny neurons (Figure 3). μ-receptors seem to be enriched in striatonigral neurons where they are colocalized with D_1 receptors (99). δ-receptors are highly expressed in cholinergic interneurons, but there is also some expression in striatopallidal neurons (99). Both μ- and δ-receptors are negatively coupled to adenylyl cyclase (98).

Using striatal slices, activation of opioid receptors was found to modulate the effects of dopamine and adenosine on DARPP-32 phosphorylation at Thr^{34} (100). Thus, the μ-opioid receptor agonist, DAMGO, inhibits the increase in DARPP-32 phosphorylation induced by SKF 81297, a D_1 receptor agonist, but not by CGS 21680, an A_{2A} receptor agonist. Conversely, the δ-opioid receptor agonist, DPDPE, inhibits DARPP-32 phosphorylation induced by activation of A_{2A} receptors, but not by activation of D_1 receptors. These studies are consistent with the colocalization studies mentioned above. Moreover, these studies also suggest the possibility of an involvement of DARPP-32 in mediating effects caused by exposure to opiates.

CHOLECYSTOKININ Cholecystokinin is a 33–amino acid peptide present in the brain mainly in its sulphated carboxy-terminal octapeptide form (CCK-8S) (101). In the striatum, CCK receptors are localized on glutamatergic nerve terminals. CCK-8S acts as an excitatory transmitter (102) and is expressed together with glutamate in cortical projections to the striatum (103). In rat striatal slices, CCK-8S reduced the increase in DARPP-32 phosphorylation at Thr^{34} caused by forskolin.

This effect was abolished by the specific CCK-B antagonist, CI-988, and by MK-801, a noncompetitive antagonist at glutamate NMDA receptors (104). These findings suggested that cholecystokinin regulated DARPP-32 phosphorylation by stimulating release of glutamate from corticostriatal glutamatergic terminals, resulting in activation of NMDA receptors, an increase in Ca^{2+} influx, activation of PP-2B, and dephosphorylation of DARPP-32 at Thr^{34}.

NEUROTENSIN Neurotensin modulates dopaminergic neurotransmission in the nigrostriatal and mesolimbic pathways (105). Neurotensin has been hypothesized to be an endogenous neuroleptic because the behavioral and biochemical effects of centrally administered neurotensin resemble those of systemically administered antipsychotic drugs (106). In the neostriatum, a high proportion of high-affinity NTR_1 neurotensin receptors are located on dopaminergic nerve terminals (107). The low-affinity receptors, NTR_2, are also expressed in the striatum (108). Several studies have demonstrated that neurotensin stimulates the release of dopamine (109) and glutamate (110). Neurotensin increases the state of phosphorylation of DARPP-32 at Thr^{34} in neostriatal neurons (111). The effect of neurotensin is mediated through the release of dopamine from nigrostriatal dopaminergic terminals in an NMDA receptor- and AMPA receptor-dependent manner. The effect of neurotensin is sensitive to a nonselective NTR_1/NTR_2 antagonist, SR142948, but not to an NTR_1 antagonist, SR48692, an NTR_2 antagonist, levocabastine, or the two combined, suggesting the involvement of unidentified neurotensin receptors. Neurotensin also stimulates phosphorylation of the AMPA receptor GluR1 subunit at Ser^{845} (PKA-site). The effect of neurotensin on GluR1 Ser^{845} phosphorylation is lost in DARPP-32 KO mice.

In parallel, neurotensin stimulates the release of glutamate from glutamatergic terminals. Glutamate enhances the neurotensin-mediated release of dopamine through activation of NMDA and AMPA receptors on dopaminergic terminals. In addition, glutamate stimulates the dephosphorylation of DARPP-32 at Thr^{75} by PP-2A through activation of NMDA and AMPA receptors on medium spiny neurons. The decrease in the level of phospho-Thr^{75} DARPP-32 removes the inhibition of PKA. Thus, glutamate, released in response to neurotensin, can potentiate neurotensin/dopamine/D_1-receptor/PKA/phospho-Thr^{34} DARPP-32/PP-1 signaling through two synergistic mechanisms.

Steroids

Studies of a possible role of DARPP-32 in steroid action have been limited to progesterone and estrogen.

PROGESTERONE/ESTROGEN The ovarian steroid hormones estrogen and progesterone play a critical role in the manifestation of sexual behavior during the estrous cycle. In ovariectomized rats, the levels of phospho-Thr^{34}-DARPP-32 in the hypothalamus are increased 48 h following administration of estradiol (112)

or of progesterone (113). Dopamine facilitates sexual receptivity in female rats (113) and vaginal-cervical somatosensory stimulation induces a progesterone-independent facilitation of mating behavior, which appears to involve activation of dopamine D_1 receptors and phosphorylation of DARPP-32 at Thr^{34} (114). In the hypothalamus, progesterone and dopamine, acting on distinct signaling pathways, have been found to stimulate cAMP production, activate PKA, and increase the phosphorylation of DARPP-32 at Thr^{34} (113). In addition, studies performed using antisense oligonucleotides to DARPP-32 as well as DARPP-32 KO mice have demonstrated that DARPP-32 plays an obligatory role in both progesterone- and dopamine-stimulation of sexual receptivity in female rats and mice (113).

Therapeutic Agents

DARPP-32 is involved in the actions of a variety of substances used for the treatment of various psychoactive and neurological disorders.

ANTIPSYCHOTICS Treatment with antipsychotic (or neuroleptic) drugs currently represents the most common therapy for schizophrenia. A common effect of antipsychotic drugs is to act as antagonists at dopamine D_2 receptor subtypes. As mentioned above, activation of dopamine D_2 receptors reduces the state of phosphorylation of DARPP-32 at Thr^{34}. Recent evidence indicates that this effect is mediated via activation of D_{2L}, but not of D_{2S}, receptors (115). Administration of eticlopride or raclopride, selective dopamine D_2 receptor antagonists, increases the levels of phospho-Thr^{34}-DARPP-32 (115a). This effect is due to the blockade of tonic activation by endogenous dopamine of postsynaptic D_{2L} receptors and the consequent removal of the inhibition exerted on the cAMP/PKA pathway. Administration of haloperidol, a typical antipsychotic drug, mimics the effect of eticlopride and increases Thr^{34} phosphorylation. Haloperidol has a relatively high affinity for the D_2 subtype of dopamine receptors (116), and its mechanism of action is most likely similar to that of eticlopride. Interestingly, administration of a low dose (5 mg/kg) of the atypical antipsychotic clozapine, a weak dopamine D_2 receptor antagonist, also stimulates DARPP-32 phosphorylation at Thr^{34}. This effect of clozapine may be due to the blockade of dopamine D_2 receptors and/or clozapine acting on other receptor types, such as serotonergic, muscarinic, or adrenergic receptors. Much higher concentrations of raclopride are required to induce catalepsy in DARPP-32 KO mice as compared to their wild-type littermates (29), providing functional evidence for an involvement of DARPP-32 in the actions of antipsychotic drugs.

ANTIDEPRESSANTS Agents that enhance serotonergic or noradrenergic neurotransmission, such as tricyclic antidepressants (e.g., imipramine), serotonin reuptake inhibitors (e.g., fluoxetine), and monoamine oxidase inhibitors (e.g., moclobemide), are effective as antidepressants. Although these agents immediately

increase the synaptic availability of serotonin and/or noradrenaline, there is a temporal delay in the onset of their beneficial actions. Recent studies in experimental animals have focused on understanding the effects of various antidepressant agents on signal transduction pathways in neurons located in brain regions thought to be implicated in depression. In particular, it has been shown that treatment with various antidepressants, including fluoxetine, enhances the efficacy of the PKA pathway at several different levels in the frontal cortex, hippocampus, and nucleus accumbens (117). In agreement with those data, acute or chronic treatment with fluoxetine caused an increased phosphorylation of DARPP-32 at Thr^{34} and a decreased phosphorylation at Thr^{75} in hippocampus, frontal cortex, and striatum (118). Due to a low signal in the extrastriatal areas, the level of phosphorylation of DARPP-32 at Ser^{137} could be detected only in striatum. In this region, fluoxetine, administered acutely, increased phosphorylation at Ser^{137}. The ability of a challenge with fluoxetine to increase phosphorylation at Ser^{137} was abolished in mice chronically treated with fluoxetine, possibly an important clue to the delay in onset of clinical benefit seen with this compound.

Helplessness models in which experimental animals are exposed to inescapable aversive situations, e.g., tail suspension test, are useful for predicting antidepressant efficacy. It is well established that acute treatment with various clinically effective antidepressant drugs reduces immobility in these tests. The effect of fluoxetine in this test was strongly attenuated in DARPP-32 KO mice (118).

ANTI-PARKINSONIAN AGENTS L-DOPA remains the most effective pharmacological treatment for Parkinson's disease. Unfortunately, as the disease advances, the therapeutic efficacy of L-DOPA declines, such that the minimum dose required to relieve parkinsonism also produces abnormal involuntary movements (AIMs), i.e., dyskinesia (119). Recent evidence points to the involvement of DARPP-32 in the mechanisms underlying the generation of L-DOPA-induced dyskinesia (120). It has been shown that the striatal medium spiny neurons of dyskinetic rats do not show depotentiation in response to low-frequency stimulation of cortical afferents. Lack of depotentiation may reflect a state of altered striatal synaptic transmission, which could produce dyskinesia. Depotentiation is blocked by activation of dopamine D_1 receptors as well as by inhibition of PP-1 (120). Interestingly, dyskinetic animals show abnormally high levels of phospho-Thr^{34} DARPP-32 (120). Based on these findings, it has been proposed that dyskinesia may result from specific changes occurring along the D_1 dopaminergic signaling pathway leading to increased DARPP-32 phosphorylation at Thr^{34}, increased inhibition of PP-1 activity, and loss of depotentiation.

Drugs of Abuse

DARPP-32 is involved in the actions of many categories of drugs of abuse, including opioids (see above), ethanol, caffeine, cocaine, and amphetamine.

ETHANOL DARPP-32 is involved in both acute and long-term responses to ethanol. In striatal slices, 25 mM ethanol increases phosphorylation of Thr^{34} (121). Moreover, DARPP-32 KO mice show a greater sensitivity to the motor stimulant effect produced by a single injection of ethanol. Conditioned place preference studies also indicate a role for DARPP-32 in mediating ethanol reward. This may depend on the ability of DARPP-32 to modulate serotonergic and/or GABAergic transmission (see above) because both serotonin receptors and $GABA_A$ receptors appear to be involved in acquisition of ethanol-induced place preference. DARPP-32 KO mice show a significant decrement in ethanol self-administration (122). Recently, it has been proposed that the involvement of DARPP-32 in ethanol reinforcement depends on the ability of DARPP-32 to regulate the state of phosphorylation of the NMDA receptor (121). It is known that activation of NMDA receptors is critically involved in ethanol reinforcement (121–123). However, in the presence of ethanol, NMDA synaptic currents are dramatically reduced (123). Dopamine, via D_1 receptors, stimulates the PKA-mediated phosphorylation of the NR1 subunit of the NMDA receptor at Ser^{897} and reduces the ethanol sensitivity of the NMDA receptor (121). This, in turn, could promote ethanol reinforcement. The regulation of ethanol sensitivity of NMDA receptors by D_1 receptors is absent in DARPP-32 KO mice (121). Taken together, these observations indicate that DARPP-32 mediates ethanol reinforcement by reducing dephosphorylation of the NMDA receptors and, consequently, by preventing their sensitivity to ethanol.

CAFFEINE Caffeine, the most commonly used psychostimulant, acts as an antagonist at A_1 as well as A_{2A} adenosine receptors (93). It was initially thought that the stimulatory properties of caffeine were primarily due to A_1 receptor antagonism. However, it is now recognized that blockade of A_{2A} receptors is critical for the actions of caffeine (93, 124). Moreover, recent data have provided strong evidence for an involvement of DARPP-32 in the stimulatory actions of caffeine. Systemic administration of caffeine, or of SCH 58261, a selective A_{2A} receptor antagonist, causes an increase in phosphorylation of Thr^{75}-DARPP-32 in wild-type mice (95). The stimulatory effects of caffeine and SCH 58261 on locomotor activity, seen in wild-type mice, were greatly reduced in DARPP-32 KO mice. The blockade of a tonically active A_{2A}/PKA/PP-2A/Thr^{75}-DARPP-32 pathway may, therefore, play a critical role for the stimulatory actions of caffeine.

COCAINE AND AMPHETAMINE It is well established that the dopaminergic system plays an important role in reward-related behaviors, and drugs with reinforcing properties share the ability to increase dopaminergic transmission (47, 125). Depletion of dopamine in nucleus accumbens abolishes the stimulatory and reinforcing actions of the psychostimulants, cocaine and amphetamine (46). Cocaine inhibits reuptake of dopamine, whereas amphetamine promotes release of dopamine from nerve terminals through a weak-base-mediated reverse transport mechanism (47, 125).

Acute treatment with cocaine or amphetamine increases the phosphorylation of Thr^{34}-DARPP-32 and decreases the phosphorylation at Thr^{75}-DARPP-32 (62). Treatment with cocaine and amphetamine are also known to increase the phosphorylation state of CREB, ELK, and multiple immediate early genes (47, 125). CREB, c-Fos, Fras, and many other immediate early genes regulate gene transcription and may coordinate alterations in gene expression, leading to long-term changes in neuronal function. Accumulating evidence has implicated these changes in the development of an addictive state. In particular, the sustained expression of ΔFos B, found following chronic administration of psychostimulants, appears to be important (47, 125). It has recently been found that ΔFos B regulates the transcription of cdk5 and that chronic treatment with cocaine upregulates the levels of cdk5 and p35 in striatum (126). Moreover, chronic treatment with cocaine leads to increased phosphorylation of Thr^{75}-DARPP-32 and decreased phosphorylation of Thr^{34}-DARPP-32 (126). A well-known behavioral consequence of repeated cocaine administration is the development of sensitization. Intraaccumbal application of various cdk5 inhibitors potentiates cocaine-induced behavioral sensitization (126), demonstrating that cdk5 is involved in this as a negative regulation of this phenomenon.

Studies in DARPP-32 KO mice have provided evidence for an involvement of DARPP-32 in the actions of cocaine and amphetamine (summarized in Table 2). One of the most prominent alterations found in the DARPP-32 KO mice is the attenuation of psychostimulant-induced expression of c-Fos and ΔFos B. The acquisition of cocaine self-administration and place preference in DARPP-32 KO mice is also attenuated (73). Moreover, DARPP-32 KO mice show an attenuated locomotor responsiveness to a single injection of 10 mg/kg cocaine (29, 128). In contrast, following chronic treatment with cocaine, DARPP-32 KO mice show increased locomotor sensitization as compared to wild-type mice (128). The different involvement of DARPP-32 in acute and chronic responses to cocaine remains to be understood, but it may be related to the major differences in the relative levels of phosphorylated Thr^{75}-DARPP-32 and phosphorylated Thr^{34}-DARPP-32, which have been found following acute versus chronic treatment with cocaine.

CONCLUSIONS

DARPP-32 acts as an amplifier of PKA- and PKG-mediated signaling when it is phosphorylated at Thr^{34}, which converts it into an inhibitor of PP-1. This amplifying property of DARPP-32 is critical for dopaminergic signaling, but it is also utilized in the actions and interactions of multiple other neurotransmitters, neuromodulators, neuropeptides, and steroid hormones. Under basal conditions, DARPP-32 is phosphorylated at Thr^{75} and inhibits PKA. However, under hyperdopaminergic conditions, the phosphorylation state at Thr^{75} is reduced allowing increased phosphorylation at Thr^{34}. This positive feedback loop acts as a switch to potentiate dopaminergic signaling. Several kinase-phosphatase cascades that are used by dopamine, glutamate, and other factors have been identified. The

complex interactions between these cascades need further investigation, with a particular emphasis on their relative importance in striatonigral and striatopallidal neurons.

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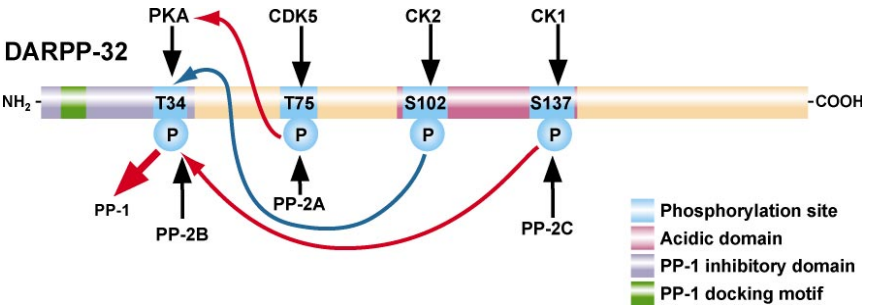

Figure 1 Multisite phosphorylation of DARPP-32. DARPP-32 is phosphorylated at Thr^{34} by protein kinase A (PKA) [and protein kinase G (PKG), not shown], at Thr^{75} by cdk5, at Ser^{102} by CK2, and at Ser^{137} by CK1. Phospho-Thr^{34} is preferentially dephosphorylated by PP-2B (or calcineurin), although PP-2A also can dephosphorylate this site; phospho-Thr^{75} is preferentially dephosphorylated by PP-2A; phospho-Ser^{137} is preferentially dephosphorylated by PP-2C; the phosphatase for phospho-Ser^{102} is not yet fully characterized. Phosphorylation of Ser^{102} of DARPP-32 by CK2 increases the rate of phosphorylation of Thr^{34} by PKA (but not by PKG); phosphorylation of Ser^{137} of DARPP-32 by CK1 decreases the rate of dephosphorylation of phospho-Thr^{34} by PP-2B. Phosphorylation at Thr^{75} converts DARPP-32 into an inhibitor of PKA, reducing its ability to phosphorylate DARPP-32 and other substrates. (*Blue arrow* indicates positive effect; *red arrows* indicate negative effect). Phosphorylation of Thr^{34} converts DARPP-32 into a potent inhibitor of PP-1. The NH_2-terminal domain of DARPP-32, which contains the PP-1 docking motif and phospho-Thr^{34}, is shown. In distinct ways, phosphorylation of Ser^{102} and Ser^{137} acts to increase phosphorylation of Thr^{34} and therefore potentiate dopaminergic signaling via the cAMP/PKA/DARPP-32/PP-1 pathway. In contrast, phosphorylation of Thr^{75} acts to inhibit dopaminergic signaling via this pathway.

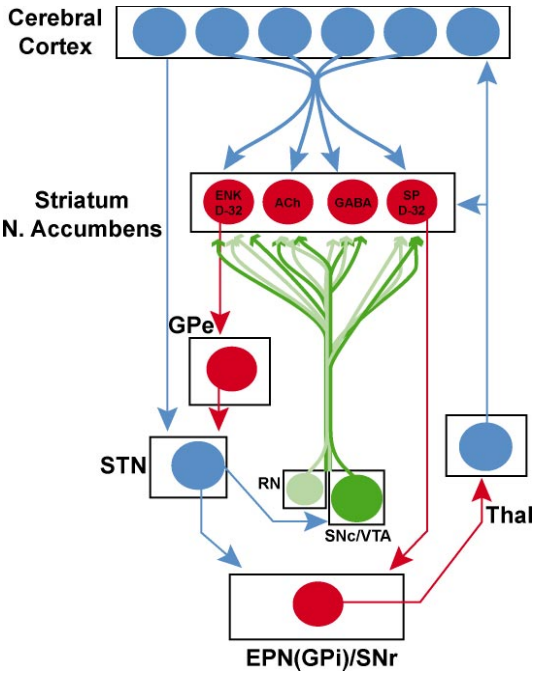

Figure 2 Schematic drawing of the neuronal pathways interconnecting different subnuclei of the basal ganglia. Blue arrows indicate excitatory pathways, red arrows indicate inhibitory pathways, dark green arrows indicate modulatory dopamine pathways, and light green arrows indicate modulatory serotonin pathways. Abbreviations: Ach, acetylcholine; D-32, DARPP-32; ENK, enkephalin; EPN, entopeduncular nucleus; Gpe, globus pallidus external part; GPi, globus pallidus internal part; RN, Raphe nuclei; SP, substance P; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; Thal, thalamus; VTA, ventral tegmental area.

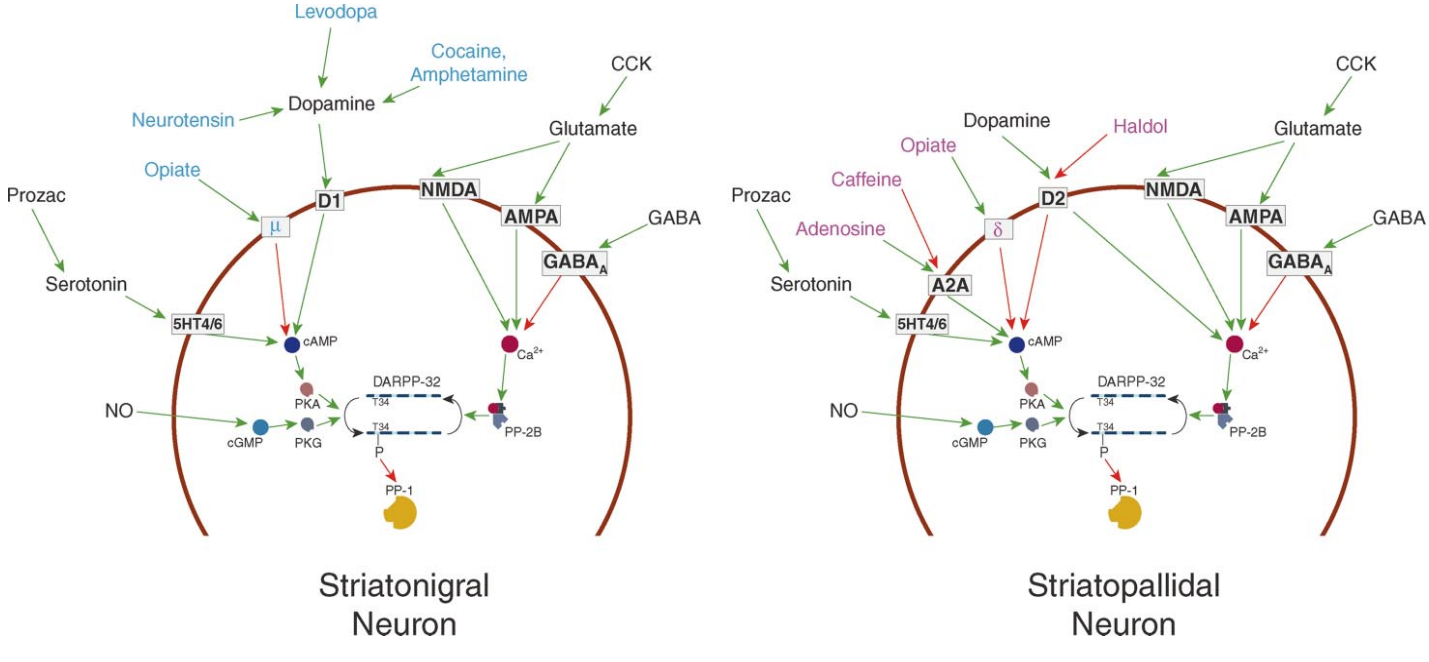

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Figure 3 Signaling pathways mediating the major effects of neurotransmitters and drugs on DARPP-32 phosphorylation at Thr^{34} in the two subtypes of striatal projection neurons. Multiple factors have been shown to regulate DARPP-32 phosphorylation in striatal neurons. Based on the anatomical localization of the receptors mediating the effects of these factors, such regulation occurs predominantly in striatonigral neurons, striatopallidal neurons, or both. In striatonigral neurons, activation by dopamine of D_1 dopamine receptors stimulates the phosphorylation of DARPP-32 at Thr^{34}. This is achieved through a pathway involving the activation of adenylyl cyclase, the formation of cAMP, and the activation of PKA. Activation of this pathway plays an important role in mediating the effects of levodopa, cocaine, and amphetamine on DARPP-32 phosphorylation. In striatopallidal neurons, activation by adenosine of A_{2A} receptors stimulates the cAMP/PKA/Thr^{34}-DARPP-32 pathway. Blockade of this pathway is an important component of the action of caffeine. Activation by dopamine of the D_2 subclass of dopamine receptors causes the dephosphorylation of DARPP-32 through two synergistic mechanisms: (*a*) inhibition of cAMP formation and (*b*) increase in intracellular Ca^{2+}, which activates the Ca^{2+}-dependent protein phosphatase PP-2B (or calcineurin), and, in turn, dephosphorylates DARPP-32 at Thr^{34}. The typical antipsychotic agent, haloperidol (Haldol), exerts some of its effects by antagonizing the D_2 receptor-mediated inhibition of DARPP-32 phosphorylation. Opiates inhibit the cAMP/PKA/Thr^{34}-DARPP-32 pathway. This effect occurs via μ receptors in striatonigral neurons and δ receptors in striatopallidal neurons. In both classes of projection neurons, glutamate functions as a fast-acting and slow-acting neurotransmitter (see Figure 4). GABA, via activation of $GABA_A$ receptors, decreases Ca^{2+} influx, inactivates PP-2B, and causes increased phosphorylation of DARPP-32 on Thr^{34}. Drugs that elevate the synaptic availability of serotonin, including fluoxetine (Prozac), increase phosphorylation of DARPP-32 at Thr^{34}. The intercellular messenger NO activates cGMP/PKG signaling and increases phosphorylation of DARPP-32 at Thr^{34}. Multiple neuropeptides have also been shown to regulate DARPP-32 phosphorylation. Neurotensin increases Thr^{34}-DARPP-32 phosphorylation through stimulating the release of dopamine. Conversely, cholecystokinin (CCK), by stimulating the release of glutamate, decreases Thr^{34}-DARPP-32 phosphorylation.

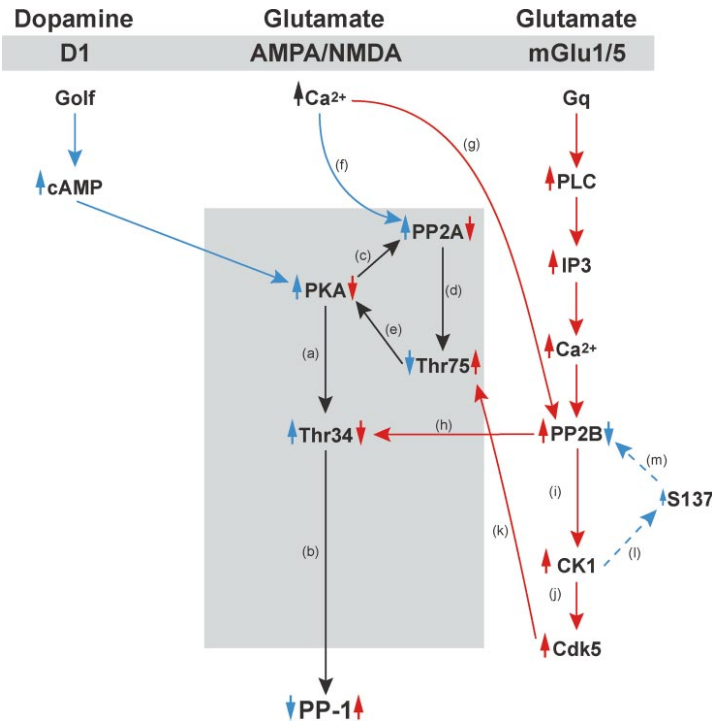

Figure 4 Some of the integration mechanisms involved in dopamine and glutamate signaling via kinase/phosphatase cascades. Dopamine, via D_1 receptors, stimulates Golf, adenylyl cyclase, the formation of cAMP, and the activation of PKA. (*a*) PKA phosphorylates DARPP-32 at Thr^{34}, which (*b*) converts DARPP-32 into a potent inhibitor of PP-1. (*c*) PKA also activates PP-2A, which (*d*) dephosphorylates DARPP-32 at Thr^{75}, thereby (*e*) reducing the inhibitory action of phospho-Thr^{75}-DARPP-32 on PKA activity (prodopaminergic). The PKA/PP-2A/Thr^{75}-DARPP-32/PKA/Thr^{34}-DARPP-32-signaling cascade (*shaded area*) serves as a positive feedback loop in the regulation of the D_1/PKA/Thr^{34}-DARPP-32/PP-1 signaling cascade. Glutamate acts via NMDA/AMPA receptors and $mGlu_{1/5}$ receptors. NMDA/AMPA receptors raise intracellular Ca^{2+} that (*f*) stimulates PP-2A activity through an unknown mechanism (prodopaminergic) and (*g*) activates PP2B, which (*h*) dephosphorylates DARPP-32 at Thr^{34} (antidopaminergic). $mGlu_{1/5}$ receptors, via a Gq/phospholipase C/IP3/Ca^{2+}/PP-2B signaling cascade, regulate dopaminergic signaling through at least three (*h*–*m*) distinct pathways: (*h*) PP-2B dephosphorylation of Thr^{34}DARPP-32 (anti-dopaminergic); (*i*) PP-2B activates CK1, which not only (*j*) activates Cdk5 and (*k*) phosphorylates Thr^{75}-DARPP-32 (antidopaminergic), but (*l*) phosphorylates Ser^{137}-DARPP-32 and (*m*) reduces PP-2B activity toward Thr^{34}-DARPP-32 (prodopaminergic). The fact that NMDA/AMPA and $mGlu_{1/5}$ receptor signaling can each be either pro- or antidopaminergic indicates the complexity of the interaction between dopamine and glutamate signaling. For didactic purposes, steps of regulation of the PKA/PP-2A/Thr^{75}-DARPP-32/PKA/Thr^{34}-DARPP-32 cascade (*shaded area*) that are prodopaminergic are indicated by blue arrows and those that are antidopaminergic by red arrows.