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Regulation of striatal signaling by protein phosphatases

Jean-Antoine Girault^{1,2,3}, Paul Greengard⁴, Angus C. Nairn⁵

¹Inserm UMR-S839, 75005 Paris, France

²Université Pierre et Marie Curie (UPMC, Paris 6), Sorbonne Universités, 75005 Paris, France

³Institut du Fer à Moulin, 75005 Paris, France

⁴Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA

⁵Department of Psychiatry, Yale University School of Medicine, 34 Park St, New Haven, CT 06508, USA

^{1,5}**Corresponding Authors**

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Abstract

Striatal projection medium spiny neurons (MSNs) play key roles in basal ganglia circuits by integrating excitatory corticostriatal and thalamostriatal input with modulatory nigrostriatal dopamine (DA) input. MSNs express specific sets of genes involved in intercellular communication and intracellular signaling, some being highly enriched in all MSNs with others specific to either D1-DA receptor-containing striatonigral or D2-DA receptor-containing striatopallidal neurons. Whereas the role of many of them remains to be investigated, much research has focused on the regulation of protein phosphatases. Three small regulatory phosphoproteins, termed DARPP-32 (dopamine- and cAMP- regulated phosphoprotein), RCS (regulator of calmodulin signaling) and ARPP-16, play key roles in DA signaling by regulating the activity of three of the four major classes of serine/threonine protein phosphatases. We provide here an overview of the main characteristics of signaling in MSNs with a focus on the role of protein phosphatase regulation in mediating the modulatory role of DA.

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I. INTRODUCTION

The striatum is a major subcortical structure of the forebrain comprising several regions including the dorsal striatum or caudate-putamen and the ventral striatum or nucleus accumbens (Voorn et al., 2004). As discussed in other chapters of this book, numerous studies have shown that these striatal regions can be further divided into areas that are involved in specific aspects of brain function largely depending on their anatomical connections. For example the dorsal striatum is critical for control of movement and habit formation, whereas the ventral striatum is linked to behavioral orientation and motivation. Alterations of these functions are central to the clinical manifestations of Parkinson's disease and addiction, respectively. In addition to these broad anatomical divisions, the striatum is divided in a patchwork of striosomes and matrix which have distinct inputs and outputs (Gerfen, 1992; Crittenden and Graybiel, 2011).

The focus of this chapter is on molecular aspects of signal transduction in medium spiny neurons (MSNs), which comprise ~95% of striatal neurons in rodents. MSNs are the only striatal efferent neurons and are GABAergic. A notable feature is that their extensive spiny dendrites are contacted by numerous glutamatergic cortical and thalamic terminals, as well as by an abundant innervation by dopamine (DA) terminals coming from the mesencephalon. Although striatal interneurons, including large cholinergic cells and several classes of GABAergic interneurons, play important functional roles through their ability to modulate MSNs (Tepper and Bolam, 2004), little is known about their intracellular signaling mechanisms, and our current knowledge pertains almost exclusively to MSNs.

While having similar morphologies, MSNs are grossly separated into two roughly equal populations, which were initially defined by their anatomical connections (Alexander and Crutcher, 1990). Those which project to the *substantia nigra pars reticulata* and the internal *globus pallidus* form the direct pathway, while those which project to the external *globus pallidus* are the first link of the indirect pathway. It was observed more than 20 years ago that these two neuronal populations differ by the expression of a number of characteristic proteins, including enrichment of substance P and the dopamine (DA) D1 receptor (D1R) in direct pathway MSNs and enrichment of enkephalin and the D2 receptor (D2R) in indirect pathway MSNs (Gerfen et al., 1990). Although these two neuronal populations have been subsequently identified mostly on the basis of the expression of the DA receptor markers, including in the approaches described in this chapter, we refer to them as direct MSNs (dMSNs) and indirect MSNs (iMSNs).

While more focused studies have identified additional proteins that are expressed in one or the other of these two populations, recent sophisticated cell type-specific profiling of mRNAs has greatly expanded our knowledge and has revealed that several hundred genes are highly abundant in MSNs as compared to other types of neurons, with many genes being preferentially expressed in one or the other population (Heiman et al., 2008). While there is a potential for additional heterogeneity in specific subpopulations of MSNs [e.g., (Crittenden and Graybiel, 2011; Gangarossa et al., 2013)], to date most of the studies on intracellular signaling mechanisms are at best only able to distinguish molecular events in dMSNs from those in iMSNs, as well as

between dorsal and ventral regions. It should also be emphasized that while the dichotomy between dMSNs and iMSNs is well documented in mice, cell level molecular profiling data are less abundant or lacking in other species.

II. MSNs EXPRESS SPECIFIC SETS OF SIGNALING PROTEINS

A. Progress in identification of cell type-enriched mRNAs

A major advance in the ability to study MSN function has been the generation of bacterial artificial chromosome (BAC) transgenic mice that express reporter genes (such as fluorescent proteins GFP or dT tomato) under the control of cell type-specific promoters (Gong et al., 2003). The targeting of D1- or iMSNs using the *drd1a* or *drd2* promoters, respectively, has been tremendously useful for the study of striatal anatomy and physiology, indicating distinct properties and patterns of regulation of MSNs within direct and indirect pathways [reviews in (Valjent et al., 2009; Bertran-Gonzalez et al., 2010)]. Although fluorescence-activated cell sorting of the two populations provided some useful information (Lobo et al., 2006), cell dissociation in adult nervous tissue has technical limitations. Building on the use of BAC transgenic mice, a highly efficient method has been developed termed TRAP (translating ribosome affinity purification) (Doyle et al., 2008; Heiman et al., 2014a). The TRAP approach makes use of expression of GFP-tagged ribosomal protein L10a under the control of *drd1a* or *drd2* promoters (Heiman et al., 2008). The fusion protein labels ribosomes selectively in neurons in which it is expressed and polysomes can be immunoprecipitated and quantified by microarray (Heiman et al., 2008; Heiman et al., 2014b). This method therefore provides identification and quantitation of mRNAs undergoing active translation (the “translatome”) and potentially gives a more accurate measure of actual protein abundance than total mRNA measurement (the transcriptome). Combination of BAC-TRAP and improved techniques for RNA quantification, such as high throughput sequencing, allows the identification of additional genes preferentially expressed in one or the other cell population (unpublished).

The results obtained from TRAP profiling give both an in-depth analysis of proteins that are enriched in MSNs in general, as well as those selectively expressed in either D1- or iMSNs. **Table 1 [Table 1 here]** highlights signaling proteins including membrane receptors, G proteins, and intracellular enzymes, that are particularly enriched in MSNs (Heiman et al., 2008). Signaling proteins with mRNAs significantly different between D1 and D2 neurons are listed in **Tables 2 and 3 [Tables 2 and 3 here]**. In most cases these proteins can also be found in other cell types, but not necessarily specifically co-expressed with each other at the high levels found in MSNs. Thus, it is striking that MSNs are characterized by high expression of a specific complement of signaling proteins, whose combination is likely responsible for the functional properties of these neurons. Such particular functional properties of MSNs include a low basal activity and the requirement for strong convergent glutamatergic inputs to trigger their firing. MSNs are also under dual tonic and phasic control by dopamine: ambient dopamine levels exert an inhibitory control on iMSNs, whereas phasic increases in dopamine release facilitate activation of dMSNs (Gerfen and Surmeier, 2011).

B. G Protein-coupled receptors

GPCRs that modulate production of cAMP are notably enriched in MSNs. The classic receptors that stimulate or inhibit cAMP production are D1R and D2R, respectively (Kebabian and Calne, 1979). Other receptors highly enriched in MSNs include serotonin 5HT4R, histamine H2R, which increase cAMP production, and serotonin 5HT1BR, 5HT1DR, alpha-adrenergic 2b and 2c receptors, delta opioid 1 OPRD1, and somatostatin receptor 5 SSTR5, which inhibit cAMP production (**Table 1**). mRNAs for several orphan receptors are enriched in MSNs (GPR6, 12, 83, 88, 149). GPR6 activates adenylyl-cyclase constitutively (Oeckl et al., 2014) and GPR12 is responsive to sphingosine-1P (Frank et al., 2012). GPR88 plays an important role in the control of striatal neuron excitability by controlling GABA_A receptor β 3 and RGS4 (regulator of G protein signaling 4) expression (Quintana et al., 2012). Adenosine A2AR, 5HT4R and GPR6 mRNAs are enriched in iMSNs (**Table 3**). They presumably participate in the basal activation of the cAMP pathway that is counterbalanced by the dopamine tone acting at D2Rs, as evidenced by the effect of D2R antagonists on cAMP-dependent signaling (Bertran-Gonzalez et al., 2009; Valjent et al., 2011; Bonito-Oliva et al., 2013).

C. Heterotrimeric G proteins and adenylyl cyclases

The heterotrimeric G proteins that couple various receptors to adenylyl cyclase are comprised of a number of different α , β , and γ subunits (Herve, 2011). Notably, expression of $G\alpha_s$ is very low in MSNs, whereas these neurons express high levels of $G\alpha_{olf}$ (Herve et al., 1993; Herve et al., 2001), initially identified in olfactory neurons. In the absence of $G\alpha_{olf}$, DA and adenosine lose their ability to activate adenylyl cyclase in the striatum (Corvol et al., 2001). The β and γ subunits associated with $G\alpha_{olf}$ are predominantly β 2 and γ 7, respectively (Schwindinger et al., 2010). Interestingly, mutations in GNAL, the gene coding for $G\alpha_{olf}$, are responsible for autosomal dominant forms of adult-onset cervical and segmental dystonia (Fuchs et al., 2013). The human disease can be induced by heterozygous loss of function, in agreement with the functional phenotype observed in heterozygous mutant mice (Corvol et al., 2007).

The major form of adenylyl cyclase expressed in MSNs is AC5 (Glatt and Snyder, 1993). Recent genetics studies have underlined the role of AC5 deficiency in humans, since its haplo-insufficiency appears responsible for early-onset autosomal dominant chorea with dystonia (Carapito et al., 2014), whereas gain of function mutations give rise to familial dyskinesia with facial myokymia (Chen et al., 2014).

D. G proteins regulators

Several guanine nucleotide exchange factors (GEFs) are enriched in MSNs, including Rasgrp1 and 2, also known as CalDAG-GEFI and CalDAG-GEFII, which stimulate the ERK pathway and are activated by Ca^{2+} and diacylglycerol (Ebinu et al., 1998). CalDAG-GEFI is matrix-enriched and CalDAG-GEFII is striosome-enriched, and they are strongly and inversely dysregulated in proportion to the severity of abnormal movements induced by l-DOPA in a rat model of

parkinsonism (Crittenden et al., 2009). Although not specifically enriched, RasGRF1, a protein that also activates the ERK pathway, and is stimulated by Ca^{2+} , plays an important role in the striatum in response to cocaine (Fasano et al., 2009) and L-DOPA after lesion of DA neurons (Fasano et al., 2010; Cerovic et al., 2015). In addition to GEFs, G protein activating proteins (GAPs) are also enriched in MSNs. Notably, Rap1Gap is phosphorylated by PKA in response to activation of D1Rs, and phosphorylation is associated with inhibition of GAP activity and with morphological changes in dendritic spine head size (McAvoy et al., 2009).

Several regulators of G protein signaling (RGS) proteins are highly expressed in striatal MSNs. RGS2 is induced by haloperidol or cocaine and inhibits GTP hydrolysis by $\text{G}\alpha\text{i}$ and $\text{G}\alpha\text{q}$ and interacts with adenylyl cyclase, reducing cAMP production (Ingi et al., 1998; Kehrl and Sinnarajah, 2002). RGS9 (RGS9-2 splice isoform) interacts with $\text{G}\beta\text{5}$, also enriched in the striatum, to inhibit AC5 activity in basal conditions and in response to stimulation by DA or opiates (Xie et al., 2012).

E. Phosphodiesterases

The regulation of cAMP levels depends on its rate of synthesis by adenylyl cyclases and degradation by phosphodiesterases (PDEs) (Bender and Beavo, 2006; Nishi and Snyder, 2010). mRNAs for several PDEs are enriched in MSN (**Table 1**) in agreement with *in situ* hybridization data (Kelly et al., 2014). PDE1B is a Ca^{2+} -activated enzyme enriched in the striatum (Polli and Kincaid, 1992). The absence of PDE1B or PDE4B increases amphetamine-induced locomotor activity (Reed et al., 2002; Siuciak et al., 2007; Siuciak et al., 2008). In PDE1B knockout mice this phenotype was rescued by the simultaneous absence of DARPP-32 (see below) (Ehrman et al., 2006). PDE7B is highly enriched in the striatum (Reyes-Irisarri et al., 2005) and specifically metabolizes cAMP (Sasaki et al., 2000). Mutation of PDE8B improves motor coordination and memory in mice (Tsai et al., 2012), whereas in humans it has been identified in a family with autosomal-dominant striatal degeneration (Appenzeller et al., 2010). Both mutations apparently result in a virtual absence of the protein (Tsai et al., 2011).

PDE10A is the most investigated of the striatal-enriched PDEs, probably because of its selective expression and of the potential antipsychotic effects of PDE10A inhibitors (Siuciak et al., 2006a; Grauer et al., 2009). Striatal and pallidal loss of PDE10A expression may also be associated with Parkinson's disease duration and severity of motor symptoms and complications (Niccolini et al., 2015). In striatal slices, inhibition of PDE10A by papaverine activates cAMP/PKA signaling in both D1- and iMSNs (Nishi et al., 2008). PDE10A deletion decreases basal locomotor activity but does not modify the response to psychostimulant drugs (Siuciak et al., 2006b). PDE10A knockout also alters aversive and appetitive instrumental reinforcement conditioning and decreases spontaneous firing of MSNs (Piccart et al., 2011; Piccart et al., 2014).

The various PDEs enriched in MSNs are equally expressed in D1 and D2 cell types, but PDE9A that is more abundant in D1-MSNs (**Table 2**). PDE9A has a much higher affinity for cGMP than for cAMP (Bender and Beavo, 2006), suggestive of a link to the specific regulation

of cGMP in dMSNs. However, in contrast, mRNAs for the soluble guanylate cyclase 1 α 3 are enriched in iMSNs (**Table 3**).

F. Protein Kinases

Protein kinases form a very large family of signaling proteins, with more than 500 encoded in the human genome. These enzymes catalyze the transfer of a phosphoryl group (PO_3^{2-}) from ATP to the side chain of an amino acid residue bearing a hydroxyl group, i.e. serine, threonine or tyrosine. This chemical modification is stable, until removed by a protein phosphatase, and dramatically modifies the properties of the substrate protein since the phosphate is bulky and has two negative charges. Depending on the substrate protein, phosphorylation may modify its conformation, its catalytic properties, and its interactions with partners, thereby mediating the effects of signaling pathways on cell function. Most protein kinases comprise a catalytic domain which belongs to the same gene family, the “classical protein kinase family”. About 4/5th of these protein kinases phosphorylate serine or threonine residues, while 1/5th specifically phosphorylate tyrosine residues. In addition, a few protein kinases are able to phosphorylate equally well serine/threonine and tyrosine residues and are referred to as dual-specificity protein kinases. It should be noted that many protein kinases recognize amino acid residues in the vicinity of the phosphorylated residue (i.e. consensus site), providing part of the mechanism of substrate specificity. The kinase domain is found in various combinations with other domains which confer specific properties on each protein kinase. In general protein kinases can be regulated by several types of mechanisms that are combined in various manners. First, many protein kinases need to be phosphorylated on a conserved loop of their catalytic domain, termed the activation loop, to be fully active. This requirement explains the existence of many protein kinases cascades in which a given protein kinase, when activated, phosphorylates and activates a second protein kinase. This can be repeated several times, giving rise to complex phosphorylation cascades. The second regulatory mechanism is linked to the presence of regulatory domain(s) associated with the catalytic domain. Usually such regulatory domains block the activity of the kinase domain and the enzyme is activated by binding of a ligand to the regulatory domain which changes its conformation and relieves its inhibitory effect. This is how second messengers such as cAMP, cGMP, Ca^{2+} , and others, activate their target protein kinases. A third mechanism of regulation is provided by domains which interact specifically with other proteins or cell components, in a constitutive or regulated manner (including by phosphorylation). Targeting domains enrich the protein kinase to a specific cell compartment such as the nucleus or the plasma membrane, while docking domains provide preferential interactions with specific substrates. This short overview of protein kinases properties explains why they can have so many roles and regulations in cells and are ideally suited for intracellular signaling.

Several protein kinases subfamilies play a particularly important role in striatal neurons and are discussed in more details in other chapters or below. These protein kinases include, for example, PKA, which is a key mediator of the actions of cAMP, Ca^{2+} /calmodulin-dependent protein kinases that are activated by Ca^{2+} influx through voltage-gated channels and NMDA

glutamate receptors (NMDAR), protein kinases C (PKC) which are activated by Ca^{2+} and lipid messengers, and extracellular signal-regulated kinases (ERKs) that are typical examples of protein kinases activated by phosphorylation cascades. Although striatal neurons contain many protein kinases found in most neurons, mRNAs for several kinases appear to be enriched in one or the other MSN population (**Tables 2, 3**). For example PKC γ mRNA is preferentially found in dMSNs and PKC μ mRNA in iMSNs. The meaning of these differences is not known. Some regulatory or targeting proteins associated with protein kinases are also enriched in MSNs (**Table 1**), including RII β , one of the isoforms of PKA regulatory subunits (Ventra et al., 1996), and AKAP5, an anchoring protein also referred to as AKAP150 in rodents and AKAP79 in humans. AKAP5 interacts with other signaling proteins including calcineurin and AC5 and participates in PKA localization in striatal neurons (Weisenhaus et al., 2010) and PDE10A (Russwurm et al., 2015).

III. PROTEIN PHOSPHATASES IN MSNs

A. Classification of protein phosphatases

Protein phosphatases are the enzymes that hydrolyze phospho-ester bonds in phosphorylated proteins. They play critical roles in cell regulation, given their ability to reverse the effects of protein kinases. Protein phosphatases are divided between (phospho)serine/threonine (Ser/Thr) and (phospho)tyrosine (Tyr) phosphatases (PTPs). Ser/Thr phosphatases are metalloenzymes belonging to two major gene families (termed PPP and PPM) (Barford et al., 1998; Shi, 2009), whereas PTPs belong to distinct classes of enzymes that utilize a phospho-cysteine enzyme intermediate as part of their catalytic action (Alonso et al., 2004).

Ser/Thr phosphatases were originally classified on the basis of their biochemical properties as type 1 (PP1) that is active towards the β subunit of phosphorylase kinase and inhibited by phospho-inhibitor-1 or inhibitor-2, or type 2 (PP2) that are active towards the α subunit of phosphorylase kinase and insensitive to the protein inhibitors (Ingebritsen and Cohen, 1983). Type 2 enzymes were then further subdivided, on the basis of their sensitivity to divalent cations, into PP2A, independent from divalent cations, PP2B activated by Ca^{2+} , and PP2C, activated by Mg^{2+} . Subsequent protein and gene sequencing revealed that the catalytic subunits of PP1, PP2A, and PP2B belong to the same highly conserved phosphoprotein phosphatase (PPP) family (~ 40-50% amino acid identity) (**Figure 1A**). PP2Cs, in contrast, form a distinct family, based on their amino acid sequence and structure, and are referred to as PPM.

B. Ser/Thr phosphatases

1. Protein phosphatase 1 (PP1)

PP1 is an abundantly expressed, highly conserved Ser/Thr phosphatase, involved in virtually all cell functions including cell cycle, muscle contraction, carbohydrate metabolism, transcription and neuronal signaling (see (Bollen et al., 2010) for a review). Mammalian genomes contain three PP1 catalytic subunit (PP1c) encoding genes, PP1 α , β/δ , and γ , the latter giving rise to two splice variants, PP1 γ 1 and γ 2. As indicated in **Table 1**, PP1 α and PP1 γ 1 are enriched in MSNs.

Within MSNs, both are found in dendritic spines, with PP1 α also being present in perikarya and nuclei (da Cruz e Silva et al., 1995; Ouimet et al., 1995).

The activity of PP1c is controlled by numerous interacting proteins acting as targeting subunits, inhibitors or substrates (Bollen et al., 2010) (**Figure 1B**). As many as 650 different heterodimeric complexes including PP1c likely exist, underlining the importance of PP1 in control of many aspects of cellular regulation. In cases where they are well characterized, PP1 interacting proteins are referred to as PPP1Rx, where x is a number distinguishing each regulatory subunit from the others. Most of these proteins contain a degenerate docking sequence K/R-K/R-V/I-x-F/W (the “RVXF motif”) which is necessary for binding PP1c, but does so without changing PP1 conformation (Egloff et al., 1997; Bollen et al., 2010). X-ray crystallography has revealed that the RVXF motif interacts with a hydrophobic channel located on the opposite side of PP1c relative to the active site (Egloff et al., 1997; Marsh et al., 2010; Ragusa et al., 2010; Choy et al., 2014). Binding to the RVXF motif does not inhibit PP1, implying that in the case of PP1 inhibitors and targeting subunits, other regions of the proteins must interact with the phosphatase to regulate its activity.

The RVXF docking motif is also found in targeting subunits that can compartmentalize PP1, bringing it into proximity to its substrates, including GADD34 that targets it to the eIF2a initiation factor, MYPT1-3, the myosin phosphatase targeting subunits, and PNUTS, one of several nuclear targeting subunits (see (Bollen et al., 2010) for references). Of particular interest in neurons, including striatal neurons, is the neurabin/spinophilin family which is comprised of two proteins that can interact with PP1c and actin filaments (Allen et al., 1997; Nakanishi et al., 1997). Notably, synaptic plasticity is differentially altered in mice lacking neurabin or spinophilin (Allen et al., 2006), consistent with a key role of PP1 in this process.

Several inhibitors of PP1 are more highly expressed in MSNs as compared to other neurons (**Table 1**), including the classic inhibitors, inhibitor-1 (PPP1R1a) and inhibitor-2 (PPP1R2), and DARPP-32 (PPP1R1b), which is highly enriched in the striatum (see below). Interestingly, PPP1R7 (SDS22) and two membrane targeting subunits, PPP1R16A (MYPT3) and PPP1R16B (TIMAP), are also enriched in MSNs but little is known related to their function in striatum or any other cellular context.

2. Protein phosphatase 2A (PP2A)

PP2A enzymes typically exist as heterotrimers comprising catalytic (C), structural (A), and regulatory (B) subunits (Janssens et al., 2008) (**Figure 1C**). PP2Ac and the structural A subunit are each coded by two genes giving rise to two catalytic subunits (PP2Ac α , PP2Ac β) and two scaffolding A subunits. There are at least fifteen genes coding for B subunits, some of them generating multiple isoforms. The combination of these subunits gives rise to at least 70 PP2A holoenzymes, and there are probably many other interacting partners with potential regulatory properties. Some B subunits enriched in striatal neurons confer unique properties to the heterotrimeric PP2A holoenzymes that are generated. B β /PR72 (PPP2R3B) contains two EFX hand domains that bind Ca²⁺, and result in Ca²⁺-induced activation of PP2A (Hendrix et al., 1993;

Janssens et al., 2003; Ahn et al., 2007b). In contrast, B56 δ (also termed PR61 or PPP2R5) subunit isoforms are phosphorylated by PKA, resulting in activation of PP2A (Ahn et al., 2007a). Both the PPP2R3B and PPP2R5 PP2A isoforms are enriched in striatum and influence MSN functions (Ahn et al., 2007a; Ahn et al., 2007b). An interesting partner of PP2A that is highly enriched in MSNs is striatin (also known as PR93/B α or STRN). Striatin, and the related proteins zinedin and SG2NA α/β , contain conserved Ca $^{2+}$ /calmodulin binding sites and interact with caveolin (Benoist et al., 2006), in addition to PP2A. Striatins appear to be scaffolding proteins that can also interact with protein kinases to form kinase/phosphatase STRIPAK complexes (Hwang and Pallas, 2014). Together with cortactin-binding protein 2 (CTTNBP2), striatins might contribute to PP2A targeting to dendritic spines (Gaillard et al., 2006; Chen et al., 2012).

3. Protein phosphatase 2B (PP2B or calcineurin)

PP2B (PPP3C) was initially identified as a major brain calmodulin binding protein and hence termed calcineurin (Klee et al., 1979). It is a ubiquitous enzyme, but is highly expressed in forebrain neurons, including striatal MSNs where it is very highly enriched (Goto et al., 1987). Calcineurin is comprised of two subunits, calcineurin A and B (**Figure 1D**). Calcineurin A includes a catalytic domain, with about 40% sequence identity with PP1 or PP2A, regions for binding calcineurin B and Ca $^{2+}$ -bound calmodulin, and a C-terminal autoinhibitory domain. Calcineurin B is very similar to calmodulin, but is constitutively associated with calcineurin A. Both proteins can bind 4 Ca $^{2+}$ ions. When intracellular cytosolic free Ca $^{2+}$ is low, the calcineurin A/calcineurin B heterodimer is inactive, with the autoinhibitory region obstructing the active site. When its intracellular levels increase, Ca $^{2+}$ binds to calcineurin B and to calmodulin which then becomes able to interact with calcineurin A. Ca $^{2+}$ is able to activate phosphatase activity through calcineurin B binding, but full activity is reached only when the A subunit is bound to Ca $^{2+}$ /calmodulin.

An interesting aspect of calcineurin function has been its role in long-term depression where its relatively high affinity for Ca $^{2+}$ /calmodulin (nM range) compared to CaM kinase II (μ M), is thought to be important (Stefan et al., 2008). Notably, active calcineurin can be inhibited by the immunosuppressant drugs, cyclosporine A and FK506 (tacrolimus) (Schreiber and Crabtree, 1992). These two molecules bind to endogenous proteins, cyclophilin A and FK506-binding protein of 12 kDa (FKBP12) for cyclosporine A and FK506, respectively. These binding proteins are termed immunophilins, which are highly conserved families of proteins with a peptidyl-prolyl cis-trans isomerase function. The immunosuppressant/immunophilin complex binds to calcineurin and inhibits its activity. In striatal neurons calcineurin A expression is decreased by antipsychotic drugs (Rushlow et al., 2005).

4. Protein phosphatase 2C (PP2C / PPM)

PP2C type of phosphatases is a family of at least 16 different genes in the human genome, with additional isoforms generated by alternative splicing (Lammers and Lavi, 2007). They are also referred to as metal-dependent protein phosphatases (PPMs) since their activity requires the

presence of Mg^{2+} or Mn^{2+} . PP2Cs are generally linked to cell growth and to cellular stress signaling. They are found as monomers and their regulation is not as well characterized as the PPPs. These enzymes have received limited attention in the central nervous system. In the striatum PP2C-type activity is involved in the dephosphorylation of DARPP-32 (Desdouits et al., 1998) and of huntingtin (Marion et al., 2014). Interestingly, dephosphorylation of huntingtin appears to be stimulated by dopamine D2 receptors through a Gi-dependent mechanism (Marion et al., 2014). PP2C also interacts with and dephosphorylates metabotropic glutamate receptor 3 (Flajolet et al., 2003).

C. Protein tyrosine phosphatases (PTPs)

1. PTPs enriched in MSNs

Several PTPs are more highly expressed in MSNs than in other neurons, including a non-receptor phosphatase, Ptpn5/STEP (see below), and two receptor-like phosphatases, Ptpn2 and Ptpv (Table 1). Ptpn2 or phogrin is a large dense-core vesicle-associated membrane protein (Wasmeier et al., 2005) that may be a phosphatidylinositol phosphatase (Caromile et al., 2010) rather than a *bona fide* PTP. Ptpv, also known as osteotesticular phosphatase (OST-PTP), has not been studied in neurons. Eya1 mRNA is highly enriched in D1 MSNs as compared to D2 (Table 2). Eya1 belongs to a family of four proteins (Eya1-4) whose function has been well described in development (Li et al., 2003; Tadjuidje and Hegde, 2013). Eya proteins combine several biochemical activities in a single polypeptide: a putative protein tyrosine phosphatase domain, an N-terminal threonine phosphatase domain, and a transcriptional activation domain (Tadjuidje and Hegde, 2013). The PTP domain of Eya proteins does not contain the standard Cys-based catalytic motif that is a hallmark of classical PTPases, but is related to the haloacid dehalogenase (HAD) family of phosphohydrolases (Li et al., 2003). Moreover, the proposed threonine phosphatase domain is not related to other phosphatases. Despite its very selective expression in dMSNs, the function of Eya1 in striatum has not been investigated.

2. Striatal-enriched tyrosine phosphatase (STEP)

STEP (PTP non-receptor type 5, Ptpn5) was originally cloned from a striatal cDNA library (Lombroso et al., 1991) and shown to be enriched in striatal projection neurons, as well as in cerebral cortex, hippocampus and a few other forebrain regions (Lombroso et al., 1993). STEP belongs to a family of non-receptor PTPs that also include HePTP (Ptpn7) and PTP-SL (Ptprr), enriched in hematopoietic cells and cerebellar neurons, respectively. Alternative splicing generates several STEP isoforms that include (STEP61 and STEP46), or not (STEP38 and STEP20), the PTP domain. STEP61 and STEP38 are membrane-associated and enriched in the endoplasmic reticulum, whereas STEP46 and STEP20 are cytosolic.

An important feature of STEP (shared with He-PTP and PTP-SL) is the presence of a kinase interaction motif (KIM) in all isoforms that acts as an accessory binding site for ERK, a physiological target for this PTPase (Pulido et al., 1998). Thus, STEP inactivates ERK1/2 (Pulido et al., 1998) and p38 MAP-kinase (Xu et al., 2009) through dephosphorylation of their regulatory phospho-tyrosine residues. STEP also contributes to the dephosphorylation of Pyk2, Fyn, GluN2B NMDA and GluA2 AMPA glutamate receptors (Goebel-Goody et al., 2012). STEP is

phosphorylated by PKA in the KIM motif, resulting in a loss of affinity for ERK and a decrease of phosphatase activity for this substrate (Paul et al., 2000). The KIM domain phospho-site is dephosphorylated by both calcineurin and PP1. STEP is therefore an important modulator of ERK tyrosine dephosphorylation (Venkitaramani et al., 2009) and its regulation contributes to the control of the ERK pathway in the striatum by D1R (Valjent et al., 2005) and NMDAR (Paul et al., 2003) (see **Figure 4** below).

Through the dephosphorylation of various substrates STEP plays a role in synaptic plasticity (Braithwaite et al., 2006) and its implication in several psychiatric and neurological diseases is supported by observations in humans and in animal models (Goebel-Goody et al., 2012). STEP down-regulation may be a protective factor in Huntington's disease models (Saavedra et al., 2011) and inhibition of STEP improves memory in a mouse model of Alzheimer's disease (Xu et al., 2014). Recent evidence also shows that STEP61 ubiquitinylation is mediated by parkin, although a role for STEP in Parkinson's disease remains to be established (Kurup et al., 2015). Finally, genetic studies in patients and matched controls suggest that STEP may play a role in normal cognitive functioning and contribute to aspects of the neuropathology of schizophrenia (Pelov et al., 2012).

IV. STRIATAL-ENRICHED REGULATORS OF SER/THR PROTEIN PHOSPHATASES

More than thirty years ago, Walaas and colleagues carried out a systematic study of second messenger-regulated protein phosphorylation in an attempt to better characterize the modes of action of neurotransmitters in different regions of the central nervous system (Walaas et al., 1983a; Walaas et al., 2011). Notably, several of the large number of phosphoproteins identified were found to be highly enriched in specific brain regions. For example, two proteins of 32 kDa and 21 kDa were selectively phosphorylated following activation of PKA in striatal extracts. The phosphorylation of the 32 kDa protein was regulated by dopamine (DA) and cAMP and it was therefore termed DARPP-32 (for dopamine and cAMP-regulated phosphoprotein Mr 32,000) (Walaas et al., 1983b). The 21 kDa protein was termed ARPP-21 (Hemmings and Greengard, 1989), as its regulation by DA took longer to be confirmed (Tsou et al., 1993b). Subsequent studies found that when phosphorylated by PKA, ARPP-21 binds to calmodulin and prevents activation of Ca²⁺/calmodulin-dependent enzymes. ARPP-21 was therefore renamed regulator of calmodulin signaling or RCS (Rakhilin et al., 2004). A third PKA substrate, ARPP-16, was also found to be highly enriched in striatum (Girault et al., 1988; Girault et al., 1990; Horiuchi et al., 1990). ARPP-16 belongs to a family of ubiquitous proteins including ARPP-19 and endosulfine, both of which inhibit PP2A when they are phosphorylated by a kinase termed Greatwall (Gwl) in dividing cells (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). A general feature of these three phosphoproteins is that they are cytosolic, of low molecular weight, and intrinsically disordered in terms of structure, properties that enabled their purification and biochemical analysis. Thus, three small proteins enriched in striatal neurons are respectively inhibitors of PP1, PP2B and PP2A, three of the four main classes of Ser/Thr phosphatases.

A. DARPP-32

1. DARPP-32 properties and regulation by phosphorylation

DARPP-32 is very highly expressed in both D1- and iMSNs (**Table 1**), but is also found in other brain regions and non-neuronal cell types where it plays important roles [see (Svenningsson et al., 2004; Walaas et al., 2011; Yger and Girault, 2011) for detailed discussion of DARPP-32 properties and regulation]. DARPP-32 is homologous to PP1 inhibitor-1 (PPP1R1A) (Aitken et al., 1982) and like inhibitor-1, DARPP-32 (PPP1R1B) is a potent inhibitor of PP1 when phosphorylated at Thr34 by PKA (Hemmings et al., 1984b) (**Figure 2**). In addition to phosphorylation of Thr34, a “RVXF” motif (residues 8-11, KIQF) is necessary for binding to and inhibiting PP1 (Hemmings et al., 1984b; Hemmings et al., 1984a) (**Figure 2**). The lack of identifiable secondary structure in DARPP-32 (Neyroz et al., 1993; Dancheck et al., 2008; Marsh et al., 2010) allows for a flexible bidentate interaction with pThr34 likely binding to the active site of PP1c, in a conformation that does not allow for dephosphorylation, coupled with binding of the KIQF sequence to PP1c. Thr34 can also be efficiently phosphorylated by cGMP-dependent protein kinase (PKG), although the regulation of DARPP-32 by cGMP signaling is much less studied (Snyder et al., 1992; Tsou et al., 1993a; Nishi et al., 2005).

In addition to Thr34, DARPP-32 is phosphorylated at several other sites (**Figure 2**). Cdk5 phosphorylates Thr75 (Bibb et al., 1999) and the Cdk5 co-factor, p35 (CDK5R1), is enriched in MSNs (**Table 1**). Notably, phosphorylation of Thr75 was found to result in inhibition of PKA thus providing a negative feedback mechanism that may attenuate the effects of cAMP/PKA acting via DARPP-32 and inhibition of PP1 (Bibb et al., 1999). DARPP-32 is phosphorylated at Ser130 (Ser137 in rat) by CK1 (formally termed casein kinase 1) (Desdouits et al., 1995b). Phosphorylation of Ser130 decreases Thr34 dephosphorylation by calcineurin (Desdouits et al., 1995a). DARPP-32 is also phosphorylated at Ser45 and Ser97 (Ser 102 in rat) by CK2 (Girault et al., 1989). Phosphorylation of DARPP-32 by CK2 facilitates the phosphorylation of Thr34 by PKA, but not by PKG (Girault et al., 1989). Phosphorylation at Ser97 also plays an important role in the nuclear trafficking of DARPP-32 (see below).

Each of the phosphorylation sites in DARPP-32 is subject to additional levels of control through different protein phosphatases (**Figure 2**). Calcineurin and PP2A dephosphorylate phospho-Thr34 (Hemmings et al., 1984b; King et al., 1984; Halpain et al., 1990; Nishi et al., 1999). Thus, cAMP and Ca²⁺ exert opposite effects on Thr34 phosphorylation and PP1 activity, through PKA and calcineurin, respectively (Halpain et al., 1990). PP2A is the main phosphatase that dephosphorylates both pThr75 and pSer97. The B56δ subunit (also known as PR61δ, B'δ or PPP2R5D) is enriched in striatal MSNs (**Table 1**) and mediates dephosphorylation of pThr75 and pSer97 in response to PKA activation (Nishi et al., 2000; Ahn et al., 2007a; Stipanovich et al., 2008). The PR72/B' (PPP2R3A) subunit, which is directly activated by Ca²⁺, mediates Ca²⁺-dependent dephosphorylation of pThr75 (Nishi et al., 2002; Ahn et al., 2007b). Thus, the activity of PP2A appears to be regulated by multiple pathways which depend on the nature of the regulatory subunits expressed in MSNs. Finally, dephosphorylation of Ser-130 is largely mediated by PP2C (Desdouits et al., 1998).

2. Functional roles of DARPP-32 in D1- and iMSNs

Regulation of DARPP-32 phosphorylation has been extensively studied in MSNs and has been shown to be influenced by virtually all neurotransmitter pathways acting on these neurons [see reviews in (Greengard et al., 1999; Greengard, 2001; Nairn et al., 2004; Svenningsson et al., 2004; Walaas et al., 2011; Yger and Girault, 2011)]. The general model developed from these studies was that DARPP-32 functions in striatal MSNs to serve as an essential mediator of DA signaling. DARPP-32, via the phosphorylation of Thr34, primarily acts as a potent inhibitor of PP1 resulting in amplification of the actions of D1R-mediated cAMP/PKA signaling through increased phosphorylation of a variety of critical substrates including ion channels that control cell excitability, various intracellular signaling proteins that control cell function, and proteins in the nucleus that control gene expression. Furthermore, the other phosphorylation sites in DARPP-32 allow integration of additional neurotransmitter inputs to further modulate DARPP-32/PP1 signaling (Svenningsson et al., 2004). Studies in mice in which DARPP-32 was knocked out in all cell types (Fienberg et al., 1998), and in mice in which individual DARPP-32 phosphorylation sites have been replaced by alanine (Svenningsson et al., 2003), largely support this general model. More generally, convergent evidence in mice and humans suggests that DARPP-32 may be associated with general cognitive performance (Meyer-Lindenberg et al., 2007; Kolata et al., 2010).

Most of the earlier studies analyzed DARPP-32 in whole striatum. However, as discussed above, the differential expression pattern of D1 and D2 receptors infers opposing patterns of signal transduction in each of these two classes of MSNs. A study using transgenic mice expressing DARPP-32 with different peptide tags in D1 or D2 MSNs provided strong evidence for such a dichotomy of signaling (Bateup et al., 2008). For example, acute exposure to cocaine resulted in an increase in Thr34 phosphorylation in dMSNs, but a decrease in iMSNs, whereas Thr75 phosphorylation was modified in the opposite direction (Bateup et al., 2008). In contrast, acute exposure to the D2 receptor antagonist, haloperidol, increased Thr34 phosphorylation selectively in iMSNs. Moreover, analysis of mice in which DARPP-32 was conditionally deleted in either MSN population revealed opposite phenotypes. Selective loss of DARPP-32 in dMSNs decreased basal and cocaine-induced locomotion, whereas loss of DARPP-32 in iMSNs increased basal locomotor activity and strongly reduced the cataleptic response to haloperidol (Bateup et al., 2010). These results illustrate how DARPP-32 contributes in specific ways to the function and balance of the two MSNs populations, and highlight the need for selective biochemical and functional analysis at the level of specific types of neurons.

3. Cyto-nuclear trafficking of DARPP-32

Although DARPP-32 is primarily a cytosolic protein, its presence in the cell nucleus of MSNs had been observed (Ouimet and Greengard, 1990), and subsequent analysis found that DARPP-32 undergoes continuous trafficking in and out of the nucleus (Stipanovich et al., 2008). Nuclear export is favored through the high basal phosphorylation of Ser97 by CK2, working in conjunction with an adjacent nuclear export motif (**Figures 2 and 3**). However, in dMSNs PKA-dependent phosphorylation and activation of the heterotrimeric form of PP2A containing the

B56 δ subunit, triggers dephosphorylation of phospho-Ser97, leading to attenuation of DARPP-32 nuclear export and a net increase in nuclear DARPP-32 (Stipanovich et al., 2008). Since activation of PKA simultaneously leads to phosphorylation of Thr34 and inhibition of PKA, this provides a pathway for regulation of nuclear PP1. PP1 is known to have multiple roles in the nucleus, especially in the regulation of phosphorylation of transcription factors, including CREB, and histone modification directly via phosphorylation and indirectly through acetylation/deacetylation (Moorhead et al., 2007). One validated target for nuclear DARPP-32 in dMSNs is the phosphorylation of Ser10 of histone H3 (**Figure 3**). While not yet analyzed directly, presumably the cyto-nuclear trafficking of DARPP-32 also occurs in iMSNs, but with an inverted signaling pattern as a consequence of D2R-mediated suppression of cAMP generation.

A recent study combining experimental measurements and modeling further investigated how cAMP signaling in dendrites of MSNs can lead to increased levels of pDARPP-32 in the nucleus (Li et al., 2015). It shows that the main contributor is the diffusion of the cAMP/PKA pathway components. Surprisingly, the most efficient location for activating PKA-mediated phosphorylation in the nucleus is in the secondary dendrites (Li et al., 2015). The geometry of the dendrites has a profound influence on the efficacy of signaling to the nucleus and counterbalances the effects of distance. These findings on PKA activation in the context of DARPP-32 regulation in MSNs may be more generally applicable.

4. Role of DARPP-32 in the regulation of ERK signaling.

In addition to its contribution to signaling that directly involves the cAMP pathway, DARPP-32 contributes to other types of signaling in MSNs. For example the extracellular signal-regulated kinase (ERK) pathway plays an important role in behavioral and synaptic plasticity induced by drugs of abuse, most studied in the case of cocaine (Valjent et al., 2000; Valjent et al., 2004; Valjent et al., 2006; Pascoli et al., 2012). Activation of ERK by drugs of abuse takes place in D1 MSNs (Bertran-Gonzalez et al., 2008) and requires the activation of both D1R and NMDAR resulting in activation of DARPP-32 (Valjent et al., 2000; Valjent et al., 2005). Inhibition of PP1 by DARPP-32 acts at multiple levels, including via inactivation of the tyrosine phosphatase STEP (**Figure 4**). This elaborate signaling cascade appears likely to be designed to temporally and spatially control long-lasting synaptic plasticity and transcriptional responses to the dMSNs which are simultaneously activated by their glutamate inputs, coding for the functional sensorimotor context, and dopamine released in response to a saliency or reward prediction error signal (Girault et al., 2007). Therefore it was proposed that activation of ERK provides a coincidence detector, or, more appropriately since the timing of the co-activation may not need to be precisely overlapping, a logical AND gate in MSNs, which detects the dual stimulation of glutamate and dopamine receptors (Girault et al., 2007).

The regulation of ERK activation by DARPP-32 may also be involved in disease-related processes. For example, chronic L-DOPA treatment of DA-lesioned rodents triggers abnormal involuntary movements (AIMs), which are thought to model L-DOPA-induced dyskinesia observed in parkinsonian patients (Cenci, 2007). Interestingly, phosphorylation of both DARPP-32 and ERK was correlated with the occurrence of AIMs (Santini et al., 2007) and L-DOPA

selectively induced ERK phosphorylation in D1R-expressing neurons (Santini et al., 2009). D1R and ERK activation are critical for the development of L-DOPA-induced AIMs (Santini et al., 2007; Darmopil et al., 2009; Fasano et al., 2010). A necessary role of DARPP-32 in the generation of L-DOPA-induced AIMs is indicated by their decrease in mice in which DARPP-32 was selectively deleted in dMSNs (Bateup et al., 2010; Santini et al., 2012).

B. ARPP-21/RCS

ARPP-21 (now termed RCS) is a small, heat-stable protein, predicted, like DARPP-32 and ARPP-16, to be an intrinsically disordered protein. RCS is a cytoplasmic protein whose regional distribution is slightly different from that of DARPP-32 (Ouimet et al., 1989) since it is not found in all MSNs and its expression is higher in nucleus accumbens compared to dorsal striatum. A thymocyte-specific protein, termed TARPP (thymus-specific cyclic AMP-regulated phosphoprotein), is produced by alternative splicing from the *Arpp21* gene (Kisielow et al., 2001). Little is known about the function of TARPP, which is expressed at low levels in brain. Interestingly, recent studies showed that the microRNA mir-128-2, which is included in the *Arpp21* gene, plays a critical role in neurons by suppressing the expression of various ion channels and ERK pathway components (Tan et al., 2013).

RCS is phosphorylated by PKA at Ser55 (Hemmings et al., 1989; Williams et al., 1989), and no other phosphorylation sites or other post-translational modifications have been identified. While less extensively studied than DARPP-32, the pattern of phosphorylation of Ser55 is thought to be very similar to that of DARPP-32 Thr34. RCS is phosphorylated at Ser55 by PKA in response to D1 receptor agonists, while activation of D2 receptors leads to decreased phosphorylation (Tsou et al., 1993b; Caporaso et al., 2000). Phosphorylation at Ser55 is also increased in mice in response to either methamphetamine or cocaine (Caporaso et al., 2000). The likely phosphatase responsible for dephosphorylation of Ser55 is PP2A (Hemmings and Greengard, 1989; Caporaso et al., 2000), but this has not been studied in any detail in intact cells or in vivo.

Insight into the function of RCS came from analysis of its protein-protein interactions using the yeast two-hybrid method (Rakhilin et al., 2004). Unexpectedly, the protein was found to interact directly with calmodulin. Notably, the binding affinity of RCS for calmodulin was increased by phosphorylation of Ser55 by PKA. As a consequence of interacting with calmodulin, phospho-RCS was found to be an effective inhibitor of the activities of several calmodulin-dependent enzymes, including calcineurin (**Figure 5**). Accordingly, dephosphorylation of DARPP-32 Thr34 was greater in striatal neurons from RCS knockout mice (Rakhilin et al., 2004). Calcineurin is also known to regulate L-type Ca^{2+} channels in MSNs (Hernandez-Lopez et al., 2000), and this regulation by D2 dopaminergic or by M1 muscarinic receptors was perturbed in RCS knockout mice (Rakhilin et al., 2004). Finally, the regulation of the transcription factor, myocyte enhancer factor 2 (or MEF2), by calcineurin in striatal neurons was found to be regulated by RCS (Pulipparacharuvil et al., 2008). This was suggested to play an important role in the ability of MEF2 to control dendritic spine formation and synaptic plasticity

of MSNs in nucleus accumbens in response to extended exposure to cocaine (Pulipparacharuvi et al., 2008).

Consistent with a role in striatal function, RCS knockout mice exhibit increased anxiety and reduced motivation (Davis et al., 2012). Thus, RCS appears to play an important modulatory role by dampening the effects of Ca^{2+} in striatal neurons.

C. ARPP-16

ARPP-16 and the alternatively spliced ARPP-19 are basic, heat- and acid-stable monomers (Horiuchi et al., 1990) that appear intrinsically unstructured proteins in NMR studies (Huang et al., 2001). ARPP-16 and ARPP-19 are members of an evolutionarily conserved eukaryotic protein family with multiple isoforms (Dulubova et al., 2001). ARPP-19 is identical to ARPP-16 with an additional 16 amino acids at its N-terminus. ARPP-19 is closely related to alpha-endosulfine (or ENSA), a protein originally identified as a putative endogenous ligand of the sulfonyleurea receptor (Heron et al., 1998). ARPP-19/ENSA-related proteins are found in most eukaryotes, including plants and in some archae (Labandera et al., 2015).

ARPP-16 corresponds to the core structure, with the other isoforms containing additional and distinct N- or C-termini and the corresponding ENSA sequence differing only slightly from ARPP-16 (16 out of 96 amino acids are changed) (Dulubova et al., 2001). Two regions are conserved in all species, the first of which contains a motif of eight residues, KYFDSGDY, which includes a novel phosphorylation site (see below) and the second of which encompasses the site phosphorylated by PKA in mammalian species and presumably in other organisms. PKA phosphorylates ARPP-16 at Ser88 (**Figure 5**) (Ser104 in ARPP-19). In mouse striatal neurons in culture, ARPP-16 and ARPP-19 (or presumably ENSA which is much more abundant and cross-reacts with most antibodies) phosphorylation at that position was increased by forskolin and vasoactive intestinal peptide (VIP) (Girault et al., 1988). In striatal slices, the phosphorylation of both proteins at the PKA site was increased by forskolin, dopamine, and a D1R agonist, whereas a D2R-agonist decreased it (Dulubova et al., 2001).

ARPP-16 is found mainly in the dorsal striatum, the nucleus accumbens, amygdala, and frontal cortex (Girault et al., 1990; Brene et al., 1994). In the striatum ARPP-16 levels increase in the first postnatal day, whereas ARPP-19/ENSA are found at their highest level at embryonic day 16 and decline with development (Girault et al., 1990). ARPP-19 may be reduced in the temporal lobe from postmortem samples of individuals with Down's syndrome and in the cerebellum of patients with Alzheimer's disease (Kim et al., 2001).

Several studies have identified possible functions for both ARPP-19 and ENSA. A role for ARPP-19 in nerve growth factor-dependent stabilization of the mRNA for growth-associated protein-43 (GAP-43) was suggested (Irwin et al., 2002) but has not been confirmed by follow-up studies [Andrade and Nairn, unpublished results]. ARPP-19 and ENSA interaction with α -synuclein has also been suggested (Woods et al., 2007), although its physiological relevance remains to be established. ENSA was originally named after studies that identified it as a possible endogenous regulator of the KATP-coupled sulfonyleurea receptor, which is targeted by the sulfonyleurea class of antidiabetic drugs (Virsolvy-Vergine et al., 1992; Heron et al., 1998)

However, it is unlikely that ENSA, a cytosolic protein, could fulfill such a role in vivo (Gros et al., 2002).

The functional importance of the ARPP-19/ENSA family was suggested by the decreased fitness of the null mutation of ARPP-19 orthologue in yeast (Giaever et al., 2002) and the altered meiotic maturation of *Drosophila* oocytes lacking ENSA (Von Stetina et al., 2008). Moreover, mice null for ARPP-16/19 exhibit embryonic lethality [Horiuchi and Nairn, unpublished], suggestive of a critical function for the protein(s). Two recent studies of mitosis in *Xenopus* oocytes unexpectedly revealed a critical functional role for ARPP-19 and ENSA in control of the G2/M phase of the cell cycle (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). Both of these studies were focused on identifying substrates for Gwl kinase that provides a link between Cdk1, the cyclin-dependent controller of mitosis, and PP2A inhibition. Proteomic screens identified ARPP-19 (Gharbi-Ayachi et al., 2010) and ENSA (Mochida et al., 2010) as the major targets for Gwl in oocytes. Gwl phosphorylates the serine residue in the conserved KYFDSGDY domain (Ser62 in ARPP-19, Ser67 in ENSA, Ser46 in ARPP-16), resulting in the binding of ARPP-19 or ENSA to PP2A, and subsequent inhibition of PP2A activity (**Figure 5**). Structural analyses revealed that this region of ENSA containing Ser67 physically interacts with PP2A/B55 at the interface of the B55 and C subunits, where the catalytic center of PP2A is located (Mochida et al., 2010; Mochida, 2014). A similar role for ARPP-19 or ENSA in regulation of mitosis in mammalian cells is implicated by preliminary studies in HeLa cells (Gharbi-Ayachi et al., 2010) and by other studies, which have revealed a critical role for the mammalian homolog of Gwl, termed MASTL (for microtubule-associated serine/threonine kinase-like) in cell cycle control (Voets and Wolthuis, 2010).

Studies in rodent brain have found that ARPP-16 interacts directly with the core AC dimer of PP2A (the A scaffolding and C catalytic subunits) [Andrade et al., in preparation]. Additional studies have found that Ser46 of ARPP-16 is phosphorylated to very high levels under basal conditions in striatal tissue. It is likely that the MAST3 isoform, which is related to MASTL and Gwl, phosphorylates ARPP-16 at Ser46 since this isoform is highly expressed in striatum (Garland et al., 2008). Moreover, in vitro, ARPP-16 phosphorylated by MAST3 inhibits a number of different PP2A heterotrimeric complexes. Finally, phosphorylation of several PP2A substrates is increased in striatal tissue from conditional knockout mice in which ARPP-16 has been deleted in forebrain, including Thr75 of DARPP-32 [Andrade et al., in preparation]. These studies suggest that MAST/Gwl/ARPP-16/19/ENSA signaling regulates PP2A in post-mitotic neurons. This regulation differs from the transient inhibition of PP2A during mitosis, since MAST/ARPP-16 appears to basally inhibit PP2A in MSNs. Although regulation of this protein family by Gwl/MAST is well established, the precise role of phosphorylation of ARPP-16 by PKA in terms of regulation of PP2A remains to be determined.

V. Conclusions: Integrated Regulation of Protein Phosphatases in MSNs

The studies of DARPP-32, RCS and ARPP-16 over the last 3 decades have provided a wealth of understanding of the intracellular signaling pathways that mediate the effects of dopamine in striatal neurons. The most striking feature of the function of these proteins is that they control the

activity of three of the four major sub-classes of serine/threonine protein phosphatases. Protein phosphatases appear to play a particularly important role in MSNs. PP1 provides a basal negative tone on a number of specific signal transduction pathways, which can be transiently overcome only following activation of specific inputs, contributing to a high signal to noise ratio. This may be somewhat analogous to what is observed for the electrical activity of MSNs. During slow-wave sleep or anesthesia, these neurons display step-like membrane potential shifts (from a low to a high state), correlated with cortical field potentials, while during wakefulness these brisk changes are replaced by more subtle variations, which result in an irregular pattern of action potentials (Mahon et al., 2006). Although intracellular signaling cannot be studied with the same time-resolution as membrane potential, the characteristic complement of signaling proteins may provide MSNs with the possibility to undergo similar variations in signaling responsiveness about which we can only speculate at present.

An important element of studies of DARPP-32 has been the appreciation that recruitment of signaling pathways is clearly distinct between D1- and iMSNs. Although an oversimplification, we can propose that in dMSNs, major inputs increase Ca^{2+} (glutamate) or cAMP (dopamine in dMSNs), which trigger a series of parallel signaling responses, involving regulation of protein phosphatases. cAMP activates PKA, which inhibits PP1 through phosphorylation of DARPP-32 on Thr34. In addition, PKA activates B56 δ -containing PP2A which dephosphorylates DARPP-32 on Thr75, thus removing any inhibition of PKA, and on Ser97, facilitating accumulation of phospho-Thr34-DARPP-32 in the nucleus and inhibiting PP1 in this compartment. Ca^{2+} is expected to exert apparently contradictory effects. On the one hand it activates calcineurin, which can switch off DARPP-32-mediated inhibition of PP1 by dephosphorylating Thr34. On the other hand Ca^{2+} can mimic some effects of cAMP on PP2A by activating the PR72-containing isoform. Moreover, D1 and glutamate exert synergistic effects on activation of the ERK pathway at several levels, including receptor-receptor interaction and DARPP-32-mediated inhibition of PP1 and (indirectly) of STEP. This makes ERK activation in dMSNs a logical AND gate, which may be a key threshold for long-term synaptic and transcriptional modifications. The equilibrium between the diverse effects of Ca^{2+} may be finely tuned by RCS, which when phosphorylated by PKA inhibits calcineurin and DARPP-32 Thr34 dephosphorylation. Activation of PP2A is also likely to be tuned by ARPP-16 whose contribution in the context of MSNs requires further investigation (**Figure 5**).

In iMSNs, it appears that tonic stimulation of D2 receptors has a dominant role, since pharmacological blockade of these receptors is sufficient to trigger a number of signaling responses. In these neurons, D2R appears to tonically decrease cAMP levels and reduce DARPP-32 Thr34 phosphorylation, while increasing Thr75 phosphorylation, thereby preventing PP1 inhibition and contributing to PKA inhibition. The observations on the “stimulatory” effects of D2R antagonists on cAMP signaling imply the existence of a constant stimulatory tonus on adenylyl cyclase, which appears to result from A2aR and probably other receptors. A transient decrease in DA levels might be sufficient to stimulate signaling pathways partly similar to those activated by DA and glutamate in dMSNs.

To better understand the complex regulation of signaling in striatal neurons, several computational models have been generated that focus on DARPP-32 (Fernandez et al., 2006; Lindskog et al., 2006; Barbano et al., 2007; Le Novere et al., 2008; Nakano et al., 2010; Qi et al., 2010). Kinetic models have highlighted that DARPP-32 is a robust signal integrator whose functional output depends on the delay between cAMP and Ca²⁺ signals (Fernandez et al., 2006), and that Thr34 phosphorylation is potentiated by the coincidence of these two stimuli (Lindskog et al., 2006). Moreover, DARPP-32 can translate the various strengths of glutamate and DA input into distinct steady states and/or dynamic responses (Qi et al., 2010). Importantly, these models support the conclusion that the positive feedback loop consisting of PKA, PP2A, and Thr75 phosphorylation plays a critical role in signal output (Barbano et al., 2007; Nakano et al., 2010). Large-scale numerical simulations evaluating the resistance of the DARPP-32 signaling network to perturbations found that its complex nature contributed robustness despite variations of the input levels of DA and glutamate (Barbano et al., 2007). Thus, modeling studies support the conclusion that a major role of DARPP-32 is to increase the reliability of signal processing in MSNs. Inclusion of other signaling components including, RCS, ARPP-16 and other signaling proteins selectively enriched in D1- or iMSNs, and of the 3-D geometry of neurons (Li et al., 2015), although technically challenging, should provide a broader understanding of the particular signaling properties of MSNs.

Many outstanding questions remain to be addressed. They include the degree of specificity of the various forms of PP2A for each of the phosphorylation sites it is capable to target. In contrast to DARPP-32, RCS and ARPP-16, few other MSN signaling molecules have been studied in depth in spite of their enrichment in either or both populations of MSNs. Several additional levels of heterogeneity are likely to increase the complexity of striatal signaling (e.g. dorso/ventral and patch/matrix differences). Moreover, persistent changes in expression levels of several of the signaling proteins in response to strong stimuli are likely to tune the responsiveness of MSNs in ways that are expected to contribute to the procedural learning and memory functions of the striatum, but remain largely unexplored.

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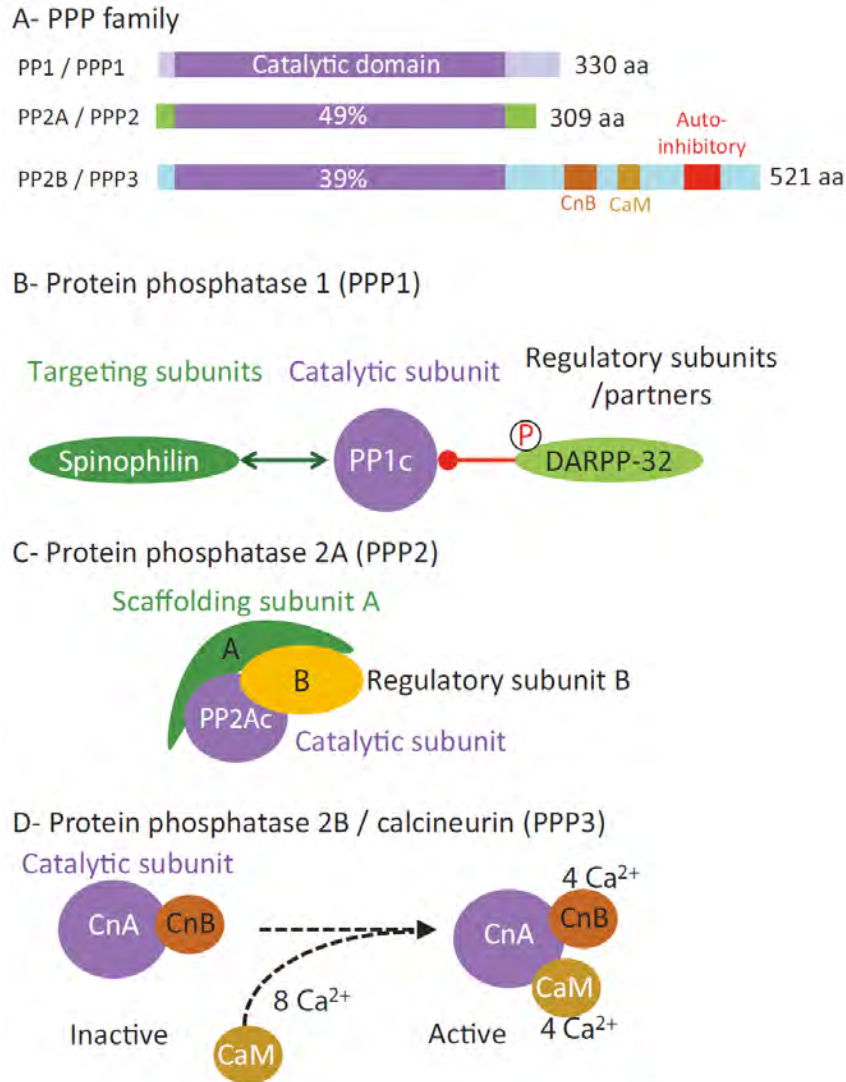


Figure 1. Serine/threonine phosphatases. **A)** The PPP family have a conserved catalytic region (purple) for which the % sequence identity is indicated. In PP2B the position of binding sites for calcineurin B (CnB) and Ca²⁺-bound calmodulin (CaM), and the auto-inhibitory region are shown. The length in amino acid residues (aa) is indicated for each phosphatase. **B)** PP1c interacts with targeting and regulatory subunits. Examples here are spinophilin and phospho-DARPP-32. **C)** PP2A is a heterotrimer comprised of catalytic, scaffolding (A) and regulatory (B) subunits. **D)** PP2B is comprised of the catalytic subunit, calcineurin A (CnA), and a Ca²⁺-binding subunit (CnB). Maximal activation is achieved by binding of 4 Ca²⁺ ions to both CnB and calmodulin (CaM). Red arrow with round end indicates inhibition.

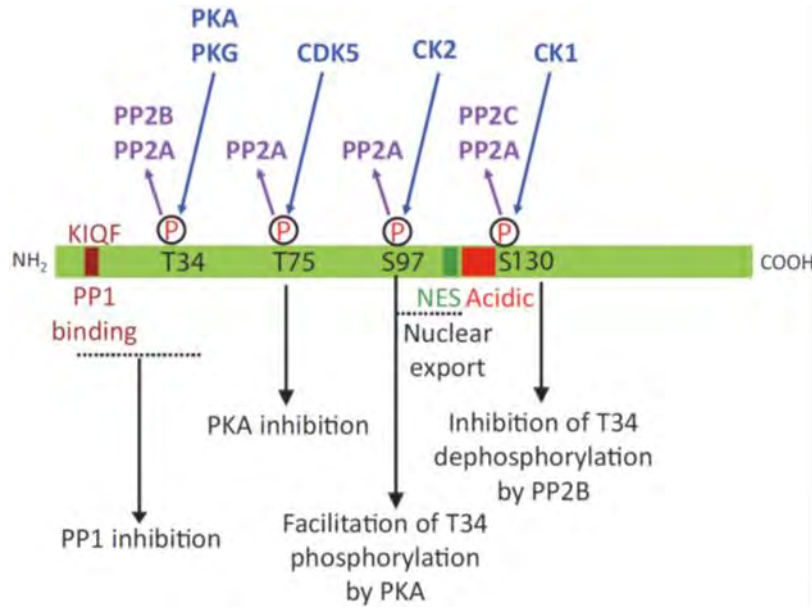


Figure 2. Regulatory phosphorylation sites in DARPP-32. The major phosphorylation sites and their functions are indicated, together with the corresponding protein kinases (blue) and phosphatases (magenta). The position of the PP1 binding motif (KIQF, one letter amino acid code), nuclear export signal (NES), and acidic stretch are indicated. cAMP-dependent kinase, PKA; cGMP-dependent kinase, PKG, cyclin-dependent kinase 5, CDK5.

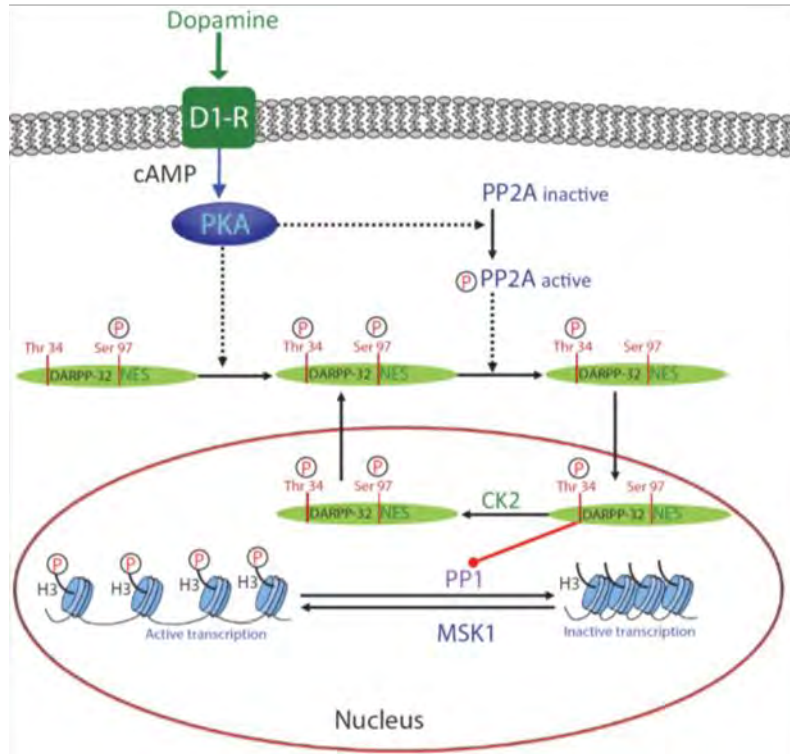


Figure 3. Regulation of DARPP-32 intracellular localization. DARPP-32 can cycle between cytoplasm and nucleus. Nuclear export is facilitated by phosphorylation of Ser97 which is located near a nuclear export signal (NES). PKA activates PP2A comprising the B56 δ B subunit and dephosphorylates Ser97, preventing nuclear export and enhancing DARPP-32 concentration in the nucleus. At the same time PKA can phosphorylate Thr34, turning DARPP-32 into a PP1 inhibitor. In the nucleus PP1 has multiple targets. Its inhibition contributes to phosphorylation of histone 3 (H3), which is a component of active chromatin, and presumably of other proteins. Phosphorylation of Ser97 by CK2, which is present in the nucleus of MSNs, facilitates exit of DARPP-32 to the cytoplasm. Red arrow with round end indicates inhibition.

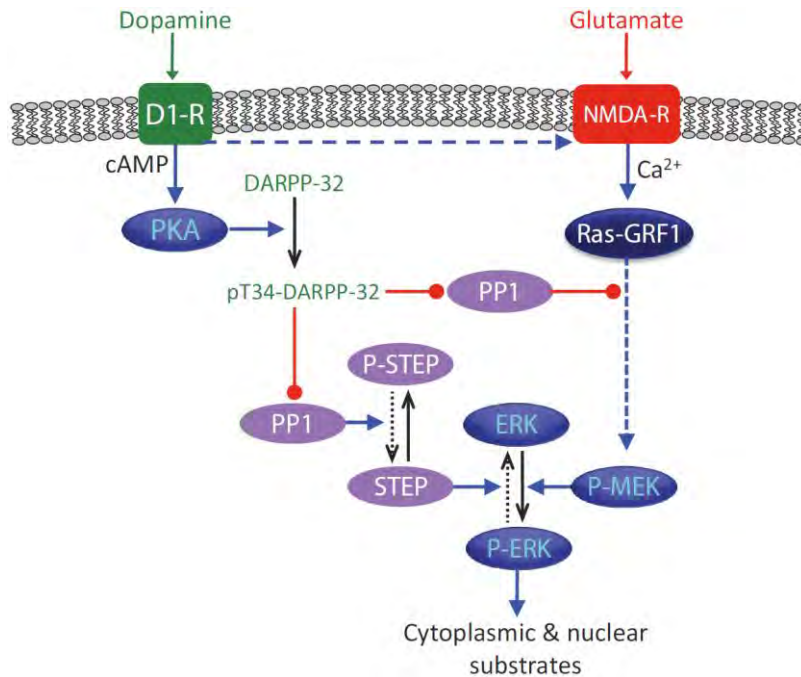


Figure 4. Synergistic activation of ERK by dopamine and glutamate. In response to psychostimulants or other drugs of abuse ERK activation in D1 MSNs requires stimulation of both D1 and NMDA receptors. This results from multiple interactions between the two signaling pathways. Ca^{2+} activates guanyl-nucleotide exchange factors such as Ras-GRF1, which activates the ERK pathway. Dopamine can enhance this response through direct and indirect interactions between D1 and NMDA receptors (dashed arrow). In addition, DARPP-32 has a facilitatory role by inhibiting PP1. PP1 acts at several levels: it acts at an unidentified level upstream from ERK, possibly on MEK, and it maintains the striatal-enriched tyrosine phosphatase (STEP) in the dephospho active form. PKA inactivates STEP by direct phosphorylation (not indicated) and through DARPP-32 inhibition of PP1. These interactions between D1 and NMDA receptors explain why activation of the ERK pathway behaves as a logical AND gate for dopamine and glutamate. Note that dopamine and glutamate can have both antagonistic effects on DARPP-32 signaling pathways (see text). Red arrows with round ends indicate inhibition.

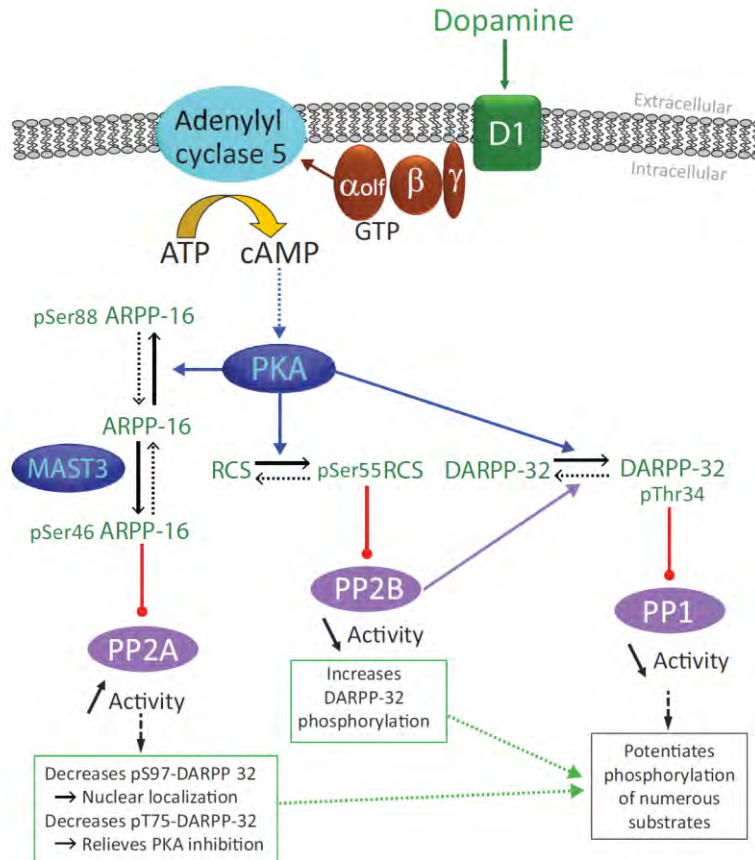


Figure 5. Convergent regulation of phosphatases by cAMP in dMSNs by striatal-enriched regulatory proteins. In response to dopamine, PKA is activated and phosphorylates DARPP-32 at Thr34 to generate a potent PP1 inhibitor. PKA phosphorylates RCS at Ser55, which inhibits Ca²⁺/calmodulin activation of calcineurin (PP2B), thus preventing dephosphorylation of Thr34. PKA also phosphorylates ARPP-16 at Ser88. This appears to prevent the inhibition of PP2A that is achieved by ARPP-16 when it is phosphorylated by MAST3 on Ser46. Each of these signaling events result facilitates inhibition of PP1. Red arrows with round ends indicate inhibition.