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DARPP-32 40 years later

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Abstract

DARPP-32 (dopamine- and cAMP-regulated phosphoprotein with an apparent Mr of 32 000), now also known as phosphoprotein phosphatase 1 regulatory subunit 1B (PPP1R1B), is a potent inhibitor of PP1 (also known as PPP1) when phosphorylated at Thr34 by cAMP-dependent protein kinase (PKA). DARPP-32 exhibits a remarkable regional distribution in brain, roughly similar to that of dopamine innervation. Its discovery was a culmination of the long-standing effort of Paul Greengard to understand the mechanisms through which neurotransmitters such as dopamine exert their effects on target neurons. DARPP-32 is particularly enriched in striatal projection neurons where it is regulated by numerous signals through which it integrates and amplifies responses to many stimuli. Molecular studies of DARPP-32 have revealed that its regulation and function are more complex than anticipated. It is phosphorylated on multiple sites by different protein kinases that modulate DARPP-32 properties. Primarily, when phosphorylated at Thr34 DARPP-32 is a potent inhibitor of PP1, whereas when phosphorylated at Thr75 by Cdk5 it inhibits PKA. Phosphorylation at serine residues by CK1 and CK2 modulates its intracellular localization and its sensitivity to kinases or phosphatases. Modeling studies provide evidence that the signaling pathways including DARPP-32 are endowed of strong robustness and bistable properties favoring switch-like responses. Thus DARPP-32 combined with a set of other distinct signaling molecules enriched in striatal projection neurons plays a key role in the characteristic properties and physiological function of these neurons.

Keywords:

dopamine, striatum, phosphorylation, protein kinase, protein phosphatase, cAMP, drugs of abuse, L-DOPA-induced dyskinesia, schizophrenia

Abbreviations:

Cdk5 cyclin-dependent protein kinase 5

CK1, casein kinase 1

CK2, casein kinase 2

D1, D1 dopamine receptor (a.k.a. Drd1)

D2, D2 dopamine receptor (a.k.a. Drd2)

DARPP-32, dopamine- and cAMP-regulated phosphoprotein with an apparent Mr of 32 000 (a.k.a. PPP1R1B)

ERK, extracellular signal-regulated kinase

GWAS, genome-wide association study

I1, inhibitor 1 (a.k.a. PPP1R1A)

KIQF, Lys-Ile-Gln-Phe consensus motif binding

L-DOPA, L-dihydroxyphenylalanine

LID, L-DOPA-induced dyskinesia

LTD, long-term depression

LTP, long-term potentiation

MSN, medium-size spiny neuron (a.k.a. SPN)

NES nuclear export signal

NMDA, N-methyl-D-aspartate

PKA, cAMP-dependent protein kinase

PKG, cGMP-dependent protein kinase

PP1, protein phosphatase 1 (a.k.a. PPP1)

PP2A, protein phosphatase 2A (a.k.a. PPP2)

PP2B, protein phosphatase 2B (a.k.a. PPP3, calcineurin)

PPP1, phosphoprotein phosphatase 1 (a.k.a. PP1)

PPP1R1A, phosphoprotein phosphatase 1 regulatory subunit 1A (a.k.a. inhibitor 1, I1)

PPP1R1B, phosphoprotein phosphatase 1 regulatory subunit 1B (a.k.a. DARPP-32)

PPP1R1C, phosphoprotein phosphatase 1 regulatory subunit 1C

PPP2, phosphoprotein phosphatase 2 (a.k.a. PP2A)

PPP2R3A, phosphoprotein phosphatase 2 regulatory subunit (a.k.a. PR72, Ca²⁺-sensitive regulatory subunit)

PPP2R5D, phosphoprotein phosphatase 2 regulatory subunit 5D (a.k.a. B56δ)

PPP3, calcineurin (a.k.a. protein phosphatase 2B, PP2B)

PPM, phosphoprotein phosphatase requiring Mg²⁺/Mn²⁺ (a.k.a. PP2C)

PR72, 72-kDa regulatory protein of PP2A (a.k.a. PPP2R3A)

SPN, striatal projection neuron (a.k.a. MSN)

tDARPP, truncated DARPP-32

Introduction

DARPP-32 stands for dopamine- and cAMP-regulated phosphoprotein with an apparent Mr of 32 000 on polyacrylamide gel electrophoresis. Now, it is also known as PPP1R1B, phosphoprotein phosphatase 1 regulatory subunit 1B, based on its major biochemical property, which is to inhibit phosphoprotein phosphatase 1 (PPP1, previously known as PP1) when DARPP-32 is phosphorylated on Thr-34 (see below). DARPP-32 is arguably one of the most prototypical proteins discovered in Paul Greengard's laboratory and certainly one of the most iconic (its name appears in more than 1200 Pubmed references and 236 000 hits in Google). Since its discovery forty years ago, it has been the subject of intense study and has been key to understanding the complexity of signal transduction in neurons, especially in striatal projection neurons in which DARPP-32 is particularly abundant. Three related proteins with sequence similarities to DARPP-32 are found in vertebrates encoded by the *PPP1R1A*, *PPP1R1B*, and *PPP1R1C* genes, but only the first two are highly expressed in neurons. PPP1R1B/DARPP-32 has a number of unique features that distinguish it from the two other paralogues. This review summarizes what we have discovered about DARPP-32 over the past 40 years, focusing on the contributions of the laboratories of Paul Greengard and his close collaborators and placing them in the perspective of current knowledge of neuronal signaling mechanisms.

The discovery of DARPP-32

In the '70s, Greengard and his colleagues identified mechanisms by which neurotransmitters exert many of their effects, through second messenger systems and activation of protein kinases (Greengard, 1978). In doing so they extended and generalized findings initially done in the study of glycogen metabolism in the liver and skeletal muscle (Krebs, 1992). These studies provided a basis for elucidating the molecular mechanisms of complex synaptic properties such as neuromodulation and synaptic plasticity. In the

course of this work several novel second messenger-regulated protein kinases were identified and their actions investigated in neurons. In the late '70s and early '80s a major question was the identity of the substrates of these protein kinases, the "third messengers" that were ultimately responsible for the action of neurotransmitters and other intercellular messengers (Nestler & Greengard, 1983). In an attempt to identify these substrates and to determine whether they were the same in different cells, new approaches were explored to compare potential substrates for various protein kinases in different brain regions. Notably, this was more than ten years before the word proteome was coined and tools became available to explore it. Walaas, Nairn, and Greengard in 1983 published 2 papers describing many proteins whose phosphorylation was enhanced by activation of diverse second messengers (Walaas, Nairn, & Greengard, 1983a, 1983b).

A few of these proteins attracted particular attention because they were detected in only one or a few of the brain regions investigated. Among these, a protein in the "soluble fraction" phosphorylated in response to cAMP stimulation was rapidly scrutinized because of its enrichment in the striatum, a region densely innervated by dopamine, which was known from previous work to act, at least in part, through stimulation of cAMP production (Kebabian & Greengard, 1971). Walaas and colleagues showed the phosphorylation of this protein was increased by cAMP and dopamine in striatal slices and named it "DARPP-32" for that reason (Walaas, Aswad, & Greengard, 1983). Attempts to purify DARPP-32 were rapidly successful and the first information obtained about its properties showed it resembled a molecule known as protein phosphatase 1 inhibitor-1 (I1) (Hemmings, Nairn, Aswad, & Greengard, 1984). I1 had been discovered in the lab of Philip Cohen in Dundee, a leader in the description of phosphatases, and shown to inhibit one of them, PP1. Sensitivity to phosphorylated I1 was in fact one of the key operational criteria to classify protein phosphatases, at a time when their sequences were not known (Ingebritsen & Cohen, 1983). Hugh Hemmings Jr., the graduate student who had purified DARPP-32 in Paul Greengard's laboratory, went for a few weeks to Philip Cohen's laboratory and showed that

DARPP-32 was a very potent PP1 inhibitor when it was phosphorylated by cAMP-dependent protein kinase (PKA) (Hemmings, Greengard, Tung, & Cohen, 1984). These discoveries were major steps in the early understanding of how dopamine could act in the striatum. It opened up a broad perspective for understanding of the mechanisms of neuromodulation by dopamine since phosphatase inhibition was potentially capable to have numerous modulatory effects on target cells by reinforcing the effects of diverse signaling pathways, including, but not limited to, cAMP.

Detailed immunohistofluorescence studies showed that DARPP-32 was highly enriched in all striatal projection neurons (SPNs, a.k.a. medium-size spiny neurons or MSNs), as well as in a number of other cells in dopaminergic regions such as the cerebral cortex and beyond, for instance in Purkinje cells in the cerebellum or in non-neuronal cells in the hypothalamus (Ouimet, Miller, Hemmings, Walaas, & Greengard, 1984). Further studies of DARPP-32 revealed that it had a wider distribution and more complex properties and functions than initially thought. First, DARPP-32 is expressed in many neurons and a few other cell types including some that do not receive dopamine innervation. For example DARPP-32 is present at high levels in choroid plexus where it is regulated by serotonin (Snyder et al., 1992). Outside of the CNS, DARPP-32 is found, for example, in kidney (Aperia et al., 1990) or parathyroid (Meister, Askergren, Tunevall, Hemmings, & Greengard, 1991). Even in the main target of dopamine neurons, the striatum, it was apparent that the SPNs are divided in two populations, striatonigral and striatopallidal, with only the first being enriched in D1 dopamine receptors which activate adenylyl cyclase (Gerfen et al., 1990). Therefore the link between DARPP-32 and dopamine was not obligatory and turned out to be more complex than originally thought (Svenningsson et al., 2004). Part of this complexity is related to the multiple mechanisms of regulation of DARPP-32 by phosphorylation which we briefly discuss [for in-depth reviews see (Svenningsson et al., 2004; Walaas, Hemmings, Greengard, & Nairn, 2011; Yger & Girault, 2011)].

Regulation of DARPP-32 by phosphorylation of Thr34

DARPP-32 was discovered as a protein phosphorylated by PKA with a notable distribution in brain regions that generally overlapped with dopamine innervation (Walaas, Aswad, et al., 1983). The first biochemical studies showed that DARPP-32 is a protein with little or no stable 3-D structure, making it resistant to acidic pH and heat (Hemmings, Nairn, Aswad, et al., 1984), corresponding to a typical intrinsically unstructured protein (Dancheck, Nairn, & Peti, 2008). The key sequences for PPP1 inhibition include the phosphorylated Thr34 and a conserved motif found in many proteins interacting with PPP1 (Lys-Ile-Gln-Phe or KIQF) (Kwon et al., 1997). It is likely that the combination of these two binding sites is responsible for the very low IC₅₀ of pThr34-DARPP-32 on PPP1 activity (Desdoutis, Cheetham, et al., 1995; Kwon et al., 1997).

Thr34 is phosphorylated by PKA and also by cGMP-dependent protein kinase, *in vitro* (Hemmings, Nairn, & Greengard, 1984) and in cells (Snyder et al., 1992; Tsou, Snyder, & Greengard, 1993). It is dephosphorylated by protein phosphatases 2A (PP2A, now PPP2) (Hemmings, Greengard, et al., 1984) and 2B (PP2B, now PPP3, a.k.a. calcineurin), a Ca²⁺-activated phosphatase (King et al., 1984). This latter property provides, among others, an interesting cross-talk between cAMP and stimuli that raise intracellular free Ca²⁺, including glutamate acting on NMDA receptors which can have opposing effects on DARPP-32 Thr34 phosphorylation (Halpain, Girault, & Greengard, 1990). Over the years a large number of extracellular signals increasing cAMP, or sometimes cGMP, levels have been shown to enhance DARPP-32 Thr34 phosphorylation (Svenningsson et al., 2004). Conversely various stimuli increasing intracellular free Ca²⁺ trigger its dephosphorylation, thereby finely tuning PPP1 activity (Nishi, Snyder, Nairn, & Greengard, 1999). Importantly the regulation and function of Thr34 in DARPP-32 is similar to that of the homologous threonine residue in I1 (PPP1R1A), which is also abundant in the striatum but has a much broader distribution throughout brain (Hemmings, Girault, Nairn, Bertuzzi, & Greengard, 1992). This raises the question of why DARPP-32 was selected throughout evolution to

exhibit its unique expression pattern. The answer may come from additional properties of DARPP-32, which were discovered through the thorough investigation of its other phosphorylation sites (see **Figure 1**).

[Insert Fig. 1 about here]

Regulation of DARPP-32 by phosphorylation of multiple residues

When labeled with ^{32}P in living striatal slices, DARPP-32 is mostly phosphorylated on serine (Girault, Hemmings, Williams, Nairn, & Greengard, 1989; Halpain et al., 1990). Study of these site(s) first led to the discovery that DARPP-32 is a substrate for “casein kinase II”, now referred to as protein kinase CK2, which is broadly expressed and highly active in the brain (Girault, Hemmings, Zorn, Gustafson, & Greengard, 1990). Mutant mice lacking CK2 α in D1-expressing neurons have a strong phenotype including novelty-induced hyperlocomotion and exploratory behavior as well as defective motor control and motor learning, presumably resulting from enhanced D1 signaling (Rebholz, Zhou, Nairn, Greengard, & Flajolet, 2013). CK2 phosphorylates Ser45 and Ser97 (in mouse sequence numbering, Ser102 in rat) (Girault, Hemmings, et al., 1989). Although phosphorylation at these site(s) appears to facilitate DARPP-32 phosphorylation by PKA (Girault, Hemmings, et al., 1989), the phosphorylation of Thr34 was increased in CK2 α KO mice (Rebholz et al., 2013), suggesting that this direct effect was overcome by indirect mechanisms *in vivo*. In agreement with this hypothesis, CK2 has a strong negative effect on G α_s and G α_{off} (Rebholz et al., 2009), the latter being the G protein that activates adenylyl cyclase in SPNs (Corvol, Studler, Schonn, Girault, & Herve, 2001).

Importantly, phosphorylation of Ser97 regulates intracellular localization of DARPP-32, facilitating its nuclear export (Stipanovich et al., 2008). Dephosphorylation of Ser97 is catalyzed by PPP2 (Girault, Hemmings, et al., 1989). This dephosphorylation can be stimulated by phosphorylation of PPP2

regulatory subunit B56 δ (PPP2R5D), triggering nuclear accumulation of pThr34-DARPP-32, thus facilitating inhibition of nuclear PPP1 and enhancing phosphorylation of histone H3 (Stipanovich et al., 2008). Conversely, dephosphorylation of Ser97 by PPP2 associated with a Ca²⁺-sensitive regulatory subunit, PR72 (PPP2R3A) in response to glutamate stimulation favors nuclear accumulation of dephospho-Thr34-DARPP-32 and reduces PKA-dependent DARPP-32 signaling (Nishi et al., 2017).

DARPP-32 is also phosphorylated by casein kinase I (protein kinase CK1) on Ser130 (in mouse sequence, Ser137 in rat sequence) (Desdouits, Cohen, Nairn, Greengard, & Girault, 1995). This phosphorylation takes place at the C-terminus of a long stretch of acidic residue present in DARPP-32 from many species. It partly corrects the aberrant mobility of DARPP-32 in SDS/PAGE, which has a mobility of a 30-32-kDa protein whereas its real molecular mass is about 22 kDa, an observation which led to the identification of this phosphorylation site (Desdouits, Cohen, et al., 1995). Phosphorylation of DARPP-32 by CK1 renders phosphoThr34 resistant to the action of PPP3 (Desdouits, Siciliano, Greengard, & Girault, 1995) and is enhanced by stimulation of metabotropic glutamate receptors (Liu et al., 2001). PhosphoSer130 is dephosphorylated by PPP2 and, especially *in vivo*, by PP2C (phosphoprotein phosphatase requiring Mg²⁺/Mn²⁺, PPM) (Desdouits, Siciliano, Nairn, Greengard, & Girault, 1998).

Finally, DARPP-32 Thr75 is a substrate of Cdk5 and when DARPP-32 is phosphorylated on that site it becomes an inhibitor of PKA catalytic subunit (Bibb et al., 1999). Thus, DARPP-32 can switch from mediating cAMP-induced PPP1 inhibition to inhibiting PKA in response to phosphorylation by Cdk5. The switch-like property is enhanced by the fact that PKA can activate PPP2 associated with B56 δ (PPP2R5D), which dephosphorylates Thr-75 and amplifies cAMP signaling (Nishi et al., 2000).

Recent work has identified protein partners of DARPP-32 and emphasizes the importance of localized regulation of PKA activity by DARPP-32. DARPP-32 can interact with adducins (Engmann et al., 2015), which are important linkers between the actin/spectrin cytoskeleton and the plasma membrane.

Phospho-Thr75-DARPP-32 facilitates phosphorylation of β -adducin at Ser713 through inhibition of a cAMP-dependent protein kinase/phosphatase-2A cascade thereby facilitating the depolymerization of the cortical cytoskeleton (Engmann et al., 2015). Behavioral evidence suggests that this regulatory mechanism is implicated in the effects of enriched environment on the properties of SPNs and their responsiveness to cocaine (Engmann et al., 2015). Dysregulation of this mechanism may be implicated in the depressive phenotype of a mouse model of Huntington's disease (Brito et al., 2019). Thr75 phosphorylation is enhanced by caffeine and may contribute to its psychostimulatory effects (Lindskog et al., 2002). Thr75 is also implicated in the interaction of DARPP-32 with the CLOCK protein, which prevents the binding of CLOCK to BMAL and its transcriptional effects, suggesting a possible link with caffeine-induced effects on mood (Trautmann, Burek, Hubner, Girault, & Engmann, 2020).

These regulations by multiple phosphorylation sites distinguish DARPP-32 from the other related inhibitors of PPP1, PPP1R1A and PPP1R1C. Experimental evidence suggests these sites are implicated in the fine regulation of DARPP-32 function. However, the resulting imbrication of these various regulatory mechanisms and the multiple potential positive and negative feedback interactions make it challenging to make simple predictions about their contribution to regulatory processes. Therefore it has been of great interest to use the power of computer modeling to dissect the potential regulation and roles of DARPP-32 in a more integrated context.

Computational modeling of DARPP-32 phosphorylation and activity

The positioning of DARPP-32 at the crossroad of signaling by important neurotransmitters with a strong importance in the control of basal ganglia functions and in several pathological conditions has attracted the attention of several computationally-oriented groups. A first attempt showed that DARPP-32 is a robust signal integrator, whose response depends on the delay between cAMP and Ca^{2+} signals

(Fernandez, Schiappa, Girault, & Le Novere, 2006), providing a mechanism for tuning physiological responses to the timing of inputs to SPNs. Moreover, a non-intuitive convergent synergism of cAMP- and Ca^{2+} -dependent pathways on DARPP-32 phosphorylation was disclosed by a different study (Lindskog, Kim, Wikstrom, Blackwell, & Kotaleski, 2006). A collaborative effort between Paul Greengard's lab and his son, who is mathematician, used an approach of dimensionality reduction to provide evidence that the network in which DARPP-32 participates has particularly robust features in the presence of noise (Barbano et al., 2007). A model including additional steps identified a positive feedback loop consisting of PKA, PPP2, and pThr-75 DARPP-32 serving as a major switch for inducing LTD and LTP (Nakano, Doi, Yoshimoto, & Doya, 2010). Calcium input modulates this loop through the PPP3-CK1-Cdk5-Thr75 pathway and PPP2, whereas Ca^{2+} and dopamine input activate the loop via PKA. Interestingly this positive feedback loop displayed robust bistable responses. A recent study also provided experimental and computational evidence for a switch-like PKA response in the nucleus of SPNs that requires a positive feedback through a phosphatase inhibitor like DARPP-32 (Yapo, Nair, Hellgren Kotaleski, Vincent, & Castro, 2018).

A different approach investigating DARPP-32 as an index of signaling from synapses to the nucleus, revealed the importance of dendrite diameter in the cellular responses (Li, Gervasi, & Girault, 2015). Model predictions of nuclear DARPP-32 phosphorylation as a readout of cAMP signaling pointed to the particular efficiency of synapses on secondary dendrites to activate nuclear PKA, which was confirmed by live imaging (Li et al., 2015). Taken together these modelling approaches have shown that the complex regulatory steps in which DARPP-32 plays a central role have important properties including robustness to noise and bistability. They also led to the discovery of the importance of dendritic tree geometry on synapse-to-nucleus signaling. It is important to emphasize that the regulation of protein phosphatases by DARPP-32 is only one aspect of regulatory signaling networks in SPNs which also

include several other regulatory proteins and phosphatase inhibitors (see paper by Cunningham and Nairn in this issue) that shape the particular responses of SPNs.

Role of DARPP-32 in the physiology of the basal ganglia

Since DARPP-32 is a phosphorylation-controlled phosphatase/kinase inhibitor, with the complex regulations emphasized above, it has been difficult to ascribe to it a simple function. Depending on its state of phosphorylation, its intracellular localization, and its interaction with partners, it can modulate a variety of targets. To try and address the functional implications of this signaling regulations, mutant mice lacking DARPP-32 either completely (Fienberg et al., 1998) or in specific cell types (Bateup et al., 2010) have been used. In addition, the consequences of point mutation of the various phosphorylation sites have been investigated in some detail (Svenningsson et al., 2003). The first conclusion from these studies was that, in laboratory housing conditions, mice do perfectly well without DARPP-32 (Fienberg et al., 1998). They breed and reproduce as their wild type littermates. However, the consequences of the absence of the protein become apparent when mice are challenged. The initial studies revealed that a variety of direct or indirect responses to dopamine or other neurotransmitters are altered (Fienberg et al., 1998; Hiroi et al., 1999). In most instances these effects are not completely lost but require stronger stimuli to occur; in other words the dose-response curves are shifted to the right. However, in particular experimental conditions, the responses can be lost. For example it is striking that in striatal slices, in the absence of DARPP-32, specific forms of long-term potentiation and depression (LTP and LTD) at corticostriatal synapses disappeared and were restored by bath application of a cell-permeant phosphatase inhibitor (Calabresi et al., 2000). An elegant study compared the consequences of a conditional loss of DARPP-32 in either of the two populations of SPNs (Bateup et al., 2010). The loss of DARPP-32 in striatonigral neurons decreased basal and cocaine-induced locomotion. Conversely, the loss

of DARPP-32 in striatopallidal neurons produced a robust increase in locomotor activity and strongly reduced cataleptic response to the antipsychotic drug haloperidol. Point mutation of the main phosphorylation sites in knock-in mice (Svenningsson et al., 2003) allowed a dissection of their contribution *in vivo* and was helpful to address the role of DARPP-32 in pathology.

What is the role of DARPP-32 in the brain in physiological conditions? It is probably over simplistic to look for a straightforward correlation between a signaling molecule with complex regulatory functions and a “simple” functional output at the behavioral level. Several studies, however, provide evidence for an implication of DARPP-32 in the processing of natural rewards. In rats it was shown that the phosphorylation pattern of DARPP-32 undergoes specific modifications in the nucleus accumbens in response to exposure to food reward, in line with the important role of dopamine in this context (see Scheggi, De Montis, & Gambarana, 2018) for a review). Mutation of DARPP-32 does not grossly impair food intake and DARPP-32 KO mice learn to nose-poke for food as wild type controls (Heyser, Fienberg, Greengard, & Gold, 2000). However, these mice lacking DARPP-32 are impaired when the rewarded hole is reversed (Heyser et al., 2000) and motivation for food reward is decreased in Ser97Ala mutant mice (Stipanovich et al., 2008), providing evidence of the implication of this molecule in tuning food related behaviors. Evidence also suggests a role of DARPP-32 in some aspects of sexual behavior both in female (Mani et al., 2000; Meredith et al., 1998) and male (McHenry, Bell, Parrish, & Hull, 2012) rodents. These effects appear to indicate a role of DARPP-32 in hypothalamic nuclei, and, at least in females, its contribution to actions of progesterone. These results suggest that DARPP-32 is implicated at various levels in the correct processing of natural rewards and their consequences on behavior, possibly improving complex adaptive responses.

DARPP-32 in pathological conditions

The possible role of DARPP-32 in various pathological conditions has been investigated both in humans, using post-mortem tissues and genetic approaches, and in animal models. Notably, besides its role in neurons, DARPP-32 and its shorter splice variant that lacks the N-terminal moiety including Thr34 and termed “truncated DARPP-32” (tDarpp), have been implicated in several cancers (see (Avanes, Lenz, & Momand, 2019) for a recent review). These important findings are not discussed here and we focus on neurological and psychiatric conditions.

Drugs of abuse and addiction

Given the enrichment of DARPP-32 in SPNs, which are the major target of dopamine, and since drugs of abuse all share the ability to increase extracellular dopamine in the striatum, particularly in its ventral component, the nucleus accumbens (Di Chiara, 1999), it is not surprising that many drugs of abuse act via DARPP-32. Many drugs of abuse alter DARPP-32 phosphorylation and their effects are modified in DARPP-32 KO mice (see (Nairn et al., 2004) for a review). DARPP-32 participates in the signaling mechanisms that promote ERK phosphorylation in response to cocaine (Valjent et al., 2005), an important step in the long-term responses of striatal neurons (Girault, Valjent, Caboche, & Herve, 2007). The use of point mutant mice pinpointed the importance of diverse components of DARPP-32 signaling. Mice bearing the Thr34Ala point mutation display a decreased locomotor sensitization in response to cocaine (Valjent et al., 2005) or morphine (Valjent et al., 2010) administration in a 2-injection protocol. Cocaine-induced conditioned place preference was also decreased in DARPP-32 KO (Zachariou et al., 2002) and Thr34Ala mutant mice (Zachariou et al., 2006). Acquisition of stable cocaine self-administration was delayed in Thr34Ala mice but both Thr34Ala and Ser130Ala DARPP-32 mutant mice self-administered more cocaine than their respective wild-type controls (Zhang et al., 2006). Interestingly it appears that acute injection and chronic self-administration of cocaine have different effects on Thr34 phosphorylation, which is increased in the first case (Bateup et al., 2008; Nishi et al., 2000) and decreased in the second (Zhang et al., 2006). Accordingly, induction of Cdk5 by chronic cocaine and the

resulting phosphorylation of DARPP-32 at Thr75, may act as a negative feedback homeostatic mechanism with respect to the behavioral effects of cocaine (Bibb et al., 2001). In conclusion, although DARPP-32 is not necessary for the appearance of addiction-like behavior, it plays a dual modulatory role in animal models of cocaine addiction, with positive and negative effects on different aspects.

Movement disorders

The treatment of Parkinsonian patients with L-DOPA corresponds to another condition in which there is a strong pharmacological stimulation of dopamine signaling in SPNs. Converging evidence shows that this intermittent stimulation, beyond its initial symptomatic efficacy, contributes to the appearance of the abnormal movements triggered by L-DOPA that complicate treatment of Parkinson's disease after several years. These movements are designated as L-DOPA-induced dyskinesia or LID. Intermittent L-DOPA treatment alternates with periods of lack of dopamine stimulation, a situation that presents some similarity with what is observed in addiction when the self-administration of drugs triggers irregular and intense stimulation of dopaminergic neurotransmission. Although striatal levels are not altered in Parkinsonian patients (Girault, Raisman-Vozari, Agid, & Greengard, 1989; Raisman-Vozari et al., 1990), DARPP-32 contributes to the generation of LID. In rats with lesioned dopamine neurons that display LID following L-DOPA treatment, the lack of synaptic depotentiation is accompanied by high levels of pThr34 DARPP-32 (Picconi et al., 2003). LID are attenuated in DARPP-32 KO mice as well as following treatment with a compound that prevents activation of the ERK pathway (Santini et al., 2007). Thr34Ala mutation diminishes LID and ERK activation (Santini et al., 2012) suggesting some similarities in the consequences of L-DOPA treatment and cocaine administration (Valjent et al., 2005). In the case of L-DOPA, however, ERK activation is not completely dependent on DARPP-32 (Alcacer et al., 2012; Gerfen, Paletzki, & Worley, 2008). Strikingly, conditional KO of DARPP-32 in striatonigral neurons abolishes LID (Bateup et al., 2010). These results indicate that DARPP-32 is an important component of the signaling pathways

that result in LID, and may be involved in the induction or in the expression of these abnormal movements or in both.

Psychiatric disorders

Because of DARPP-32's role in mediating dopamine actions and the implication of dopamine in schizophrenia, several studies have been carried out in animal models and in humans to address the possible involvement of DARPP-32 in this disease or group of diseases. In mice various psychotomimetic drugs change DARPP-32 phosphorylation and their effects are altered by selective mutation of DARPP-32 phosphorylation sites (Svenningsson et al., 2003). These results supported the role of DARPP-32 in the signaling mechanisms activated by these drugs, but evidence for a possible role in human patients required the investigation of human brain samples. A first post-mortem study found that DARPP-32 protein levels were decreased in the prefrontal cortex of schizophrenic patients as compared with matched controls (Albert et al., 2002). Similar observations were then reported in patients with schizophrenia or bipolar disorder (Ishikawa, Mizukami, Iwakiri, & Asada, 2007), in schizophrenic patients dead from suicide (Feldcamp, Souza, Romano-Silva, Kennedy, & Wong, 2008) and in schizophrenic patients (Kunii et al., 2019). A frequent 7-single nucleotide polymorphism (7-SNP) haplotype in DARPP-32 gene (*PPP1R1B*) predicting the expression of its isoforms in postmortem human brain, was associated with enhanced performance on cognitive tests depending on frontostriatal function (Meyer-Lindenberg et al., 2007). Although preliminary evidence suggested that this haplotype could be associated with risk of schizophrenia (Meyer-Lindenberg et al., 2007), the association of *PPP1R1B* locus with increased risk for major psychiatric was not found in other small scale studies or in major GWAS. However, relationships between genetic variants and expression levels have been confirmed. In a study including a large number of cases, tDarpp was found to be increased in the dorsolateral prefrontal cortex of patients with schizophrenia and bipolar disorder, and was strongly associated with *PPP1R1B* genotypes (Kunii et al., 2014). Some evidence even suggested that genetic variants associated with higher tDarpp expression

predict worse cognitive performance (Kunii et al., 2014). Taken together these studies support the existence of a decrease in DARPP-32 associated with an increased expression of tDarpp in the prefrontal cortex of patients with schizophrenia and possibly bipolar disorder. Since tDarpp may oppose cAMP-regulated functions of DARPP-32, this combination suggests an overall decreased function of DARPP-32 in these conditions. How these observations are related with the disease mechanisms remains to be determined. One obvious link could be through the consequences of chronic treatment with antipsychotics, but, in rats, prolonged administration of dopamine antagonists or antipsychotics does not alter DARPP-32 or tDARPP levels (Grebb, Girault, Ehrlich, & Greengard, 1990; Kunii et al., 2014). It is therefore possible that the observed changes reflect primary cellular alterations in patients which warrant further exploration. Given the reported alterations of DARPP-32 in both bipolar disorder and schizophrenia and the heterogeneity of the latter condition, it is possible that they are more pronounced in specific subgroups of patients. Whether this corresponds to particular clinical features or response to treatment is an intriguing question. However, the current impossibility to non-invasively evaluate DARPP-32 levels in living subjects makes it very difficult to address.

Conclusion

Throughout his academic career Paul Greengard provided major discoveries related to the mechanisms of action of neurotransmitters in general, but especially to the details of dopamine signaling. After the initial discovery that dopamine activated the production of cAMP in the striatum in the early '70s, he pioneered the elucidation of the mechanisms by which cAMP acts in striatal neurons. This demonstrated the paramount importance of intracellular protein phosphorylation pathways, an idea that Greengard put forward early on. In-depth exploration of the regulation and properties of DARPP-32 in striatal neurons led to the discovery of a variety of mechanisms whose importance, or even existence, in brain

was previously not known. They include the role of several protein kinases such as CK1, CK2, and Cdk5 as well as the importance and intricacy in neurons of the regulation of major protein phosphatases, PPP1, PPP2, PPP3, and PPM. Given that DARPP-32 is a regulatory protein with multiple functions at the crossroads of several signaling pathways, it is not surprising that its role cannot be restricted to a simple functional output. The use of computer modeling revealed that it provides robustness to intracellular signaling pathways and endows them with a bistable activity, switching between states of strong and weak cAMP signaling. As a result we can hypothesize that DARPP-32 enhances the contrast in the responses of the striatal network to stimulation, and increases the overall signal to noise ratio. A characteristic of the regulatory networks in which DARPP-32 participates is their high degree of redundancy, which makes them difficult to fully evaluate *in vivo*, since altering any single component has only relatively mild consequences. However the properties of these multiple intricate signaling networks, including their redundancy, are likely to be essential for the fine and adapted control of action and movement as well as its adaptation over time through reward-controlled procedural learning that characterizes the integrated function of striatal projection neurons.

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Figure legend

Figure 1: Schematic representation of DARPP-32 main features.

The main phosphorylated amino acid residues are indicated with the kinases that phosphorylate them (green) and the phosphatases that remove phosphates from these residues (red). Some additional features are indicated: KIQF, consensus motif (Lys-Ile-Gln-Phe) that binds PPP1 and contributes to its inhibition with phosphoThr34, the nuclear export sequence (NES) and the acidic stretch characteristic of DARPP-32. Other abbreviations: CK1, casein kinase 1, CK2, casein kinase 2, Cdk5, cyclin-dependent protein kinase 5, PKA, cAMP-dependent protein kinase, PKG, cGMP-dependent protein kinase, PPP1, protein phosphatase 1 (a.k.a. PP1), PPP2, protein phosphatase 2A (PP2A), PPP3, calcineurin (a.k.a. protein phosphatase 2B, PP2B, calcineurin), PPM, phosphoprotein phosphatase requiring Mg^{2+}/Mn^{2+} (a.k.a. PP2C).