Title: Protein Phosphatases in the brain: regulation, function and disease

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Abstract:

Protein phosphorylation is a critical cellular process regulated by the competing actions of protein kinases (PKs) and phosphatases (PPs). While several hundred PKs exist in mammalian cells, there are only a few dozen PPs. PPs typically target Ser/Thr or Tyr residues but each has different characteristics and specificities and fall into three separate classes. PPs on their own have poor substrate specificity, however, when bound to regulatory proteins their activity and specificity is tightly controlled. Mechanisms of PP regulation range from targeting to subcellular compartments by scaffolding proteins and/or protein complexes which restrict the action of PPs to specific substrates, to the inhibition of PPs by specific inhibitory molecules or inhibitor peptides. In the adult brain, PPs are essential for synaptic functions and are involved in the negative regulation of higher-order brain functions such as learning and memory. Dysregulation of their activity has been linked to several disorders including cognitive ageing and neurodegeneration, but also cancer, diabetes and obesity. Because they are critical for multiple biological functions and could

constitute a new class of drug targets, they deserve to be studied in greater detail. The complexity of their modes of action and of the systems they function in, however, requires the analysis of whole systems rather than of individual proteins in isolation, as has been the case for decades using classical molecular biology. In this review, we discuss PPs in the context of the central nervous system, their role in neuronal functions, and their regulation and dysfunction in disease. Current techniques to investigate PP signalling and associated functional networks, future directions for the development of system-wide proteomic and genomic methods of analysis, and the potential for therapeutic applications are also covered.

Abbreviations:

DSP/DUSP - Dual-specificity phosphatases

PTP - protein Tyr phosphatases

PPP - phosphoprotein phosphatase

NRPTP - non-receptor PTP

RPTP - receptor PTP

LMPTP/LMWPTP - 18 kDa low Mr phosphatase

PPM - protein phosphatase Mg^{2+}- or Mn^{2+}-dependent

PTK - protein tyrosine kinase

PK - protein kinase

PP - protein phosphatases

PTM - posttranslational modification

LTP - long-term potentiation

Introduction

Protein phosphorylation is the most common and important form of reversible protein posttranslational modification (PTM), with up to 30 % of all proteins being phosphorylated at any given time (Hunter, 1998). Protein kinases (PKs) are the effectors of phosphorylation and catalyse the transfer of a γ-phosphate from ATP to specific amino acids on proteins. Several hundred PKs exist in mammals and are classified into distinct super-families (Ubersax and Ferrell, 2007). Proteins are phosphorylated predominantly on Ser, Thr and Tyr residues, which account for 86, 12 and 2 % respectively of the phosphoproteome, at least in mammals (Munton et al., 2007; Olsen et al., 2006). In contrast, protein phosphatases (PPs) are the primary effectors of dephosphorylation and can be grouped into three main classes based on sequence, structure and catalytic function. The largest class of PPs is the phosphoprotein phosphatase (PPP) family comprised of PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7, and the protein phosphatase Mg^{2+}- or Mn^{2+}-dependent (PPM) family, comprised primarily of PP2C. The protein Tyr phosphatase (PTP) super-family forms the second group, and the aspartate-based protein phosphatases the third (Moorhead et al., 2007).

Protein phosphorylation plays a crucial role in biological functions and controls nearly every cellular process, including metabolism, gene transcription and translation, cell-cycle progression, cytoskeletal rearrangement, protein-protein interactions, protein stability, cell movement, and apoptosis (Manning et al., 2002, Ubersax, 2007 #280). These processes depend on the highly regulated and opposing actions of PKs and PPs, through changes in the phosphorylation of key proteins. Histone phosphorylation, along with methylation, ubiquitination, sumoylation and

acetylation, also regulates access to DNA through chromatin reorganisation (Jenuwein and Allis, 2001).

One of the major switches for neuronal activity is the activation of PKs and PPs by elevated intracellular calcium. The degree of activation of the various isoforms of PKs and PPs is controlled by their individual sensitivities to calcium (Lee, 2006). Furthermore, a wide range of specific inhibitors and targeting partners such as scaffolding, anchoring, and adaptor proteins also contribute to the control of PKs and PPs and recruit them into signalling complexes in neuronal cells (Sim and Scott, 1999). Such signalling complexes typically act to bring PKs and PPs in close proximity with target substrates and signalling molecules as well as enhance their selectivity by restricting accessibility to these substrate proteins (Faux and Scott, 1996). Phosphorylation events, therefore, are controlled not only by the balanced activity of PKs and PPs but also by their restricted localisation. Regulatory subunits and domains serve to restrict specific proteins to particular subcellular compartments and to modulate protein specificity. These regulators are essential for maintaining the coordinated action of signalling cascades, which in neuronal cells include short-term (synaptic) and long-term (nuclear) signalling. These functions are, in part, controlled by allosteric modification by secondary messengers and reversible protein phosphorylation.

It is thought that around 30% of known PPs are present in all tissues, with the rest showing some level of tissue restriction (Forrest et al., 2006). While protein phosphorylation is a cell-wide regulatory mechanism, recent quantitative proteomics studies have shown that phosphorylation preferentially targets nuclear proteins (Olsen

et al., 2006). Many PPs that regulate nuclear events, are often enriched in or exclusively present in the nucleus (Moorhead et al., 2007). In neuronal cells, PPs are present in multiple cellular compartments and play a critical role at both pre- and post-synapses, in the cytoplasm and in the nucleus where they regulate gene expression.

Types of PPs

Serine/Threonine PP (PPM/PPP) families

Protein Ser/Thr phosphatases were originally classified using biochemical assays as either, type 1 (PP1) or type 2 (PP2), and were further subdivided based on metal-ion requirement (PP2A, no metal ion; PP2B, Ca^{2+} stimulated; PP2C, Mg^{2+} dependent) (Moorhead et al., 2007). The protein Ser/Thr phosphatases PP1, PP2A and PP2B of the PPP family, together with PP2C of the PPM family, account for the majority of Ser/Thr PP activity *in vivo* (Barford et al., 1998). In the brain, they are present in different subcellular compartments in neuronal and glial cells, and contribute to different neuronal functions.

PP1

The importance of PP1 in neural systems became apparent after the discovery that high levels of several of its catalytic subunits including α, β, γ-1 and γ-2, are expressed in the brain. In nerve cells, they are associated with different binding proteins that compartmentalise them to varying but distinct subcellular compartments. The catalytic subunit PP1c, for example, is in complex with over 100 different regulatory and targeting subunits (Barford et al., 1998; Faux and Scott, 1996; Sim and

Scott, 1999). The α isoform is found predominantly in the soma of neuronal cells, whereas γ-1, in addition to the soma, is present in large amount in dendrites and presynaptic boutons where it co-localises with CaMKII and synaptophysin (Munton et al., 2004; Strack et al., 1999). PP1 is involved in a large range of cellular functions which are regulated by distinct PP1 holoenzymes.

PP2A, PP4 and PP6

The catalytic subunits for the PP2A-type of Ser/Thr PPs (PP2A, PP4 and PP6) are widely expressed and evolutionarily conserved. PP2A has been implicated in the control of cell growth, proliferation, and differentiation (Mumby, 2007). Its catalytic subunit is encoded by two genes which share 97% identity at the protein level in humans (Stone et al., 1987). PP2A has been shown in human cells to function as a component of a large multiprotein complex called the striatin-interacting phosphatase and kinase (STRIPAK) complex where substrate specificity is mediated by several different regulatory subunits (Goudreault et al., 2009). PP4 and PP6 are also thought to function as members of similar multiprotein complexes (Chen and Gingras, 2007). Similar to PP1, the diverse functions of PP2A are largely accounted for by the ensemble of regulatory and targeting proteins it associates with.

PP2B and PP7

Calcineurin (PP2B) is characterised by its dependence on Ca^{2+} for activity. It consists of an A-subunit with an N-terminal catalytic domain, a C-terminal regulatory region containing binding sites for the PP2B B-subunit and calmodulin, and an autoinhibitory sequence at the extreme C-terminus. Optimal Ca^{2+} activation of PP2B requires association of Ca^{2+} to both the B-subunit and calmodulin (Barford et al.,

1998). In the brain, PP2B is highly abundant and regulates multiple neuronal processes depending on its subcellular localisation and regulatory partners (Mansuy, 2003).

PPM

The PPM family, which includes PP2C and pyruvate dehydrogenase phosphatase, are enzymes with Mn^{2+}/Mg^{2+} metal ions that are resistant to classic inhibitors and toxins of the PPP family. Unlike most PPPs, PP2C exists in only one subunit but, like PTPs, it displays a wide variety of structural domains that confer unique functions (Moorhead et al., 2009). In addition, PP2C does not seem to be evolutionarily related to the major family of Ser/Thr PPs and has no sequence homology to ancient PPP enzymes (Moorhead et al., 2009). The current assumption is that PPMs evolved separately from PPPs but converged during evolutionary development (Bellinzoni et al., 2007).

Target specificity and structures of Ser/Thr PPs

The ability of PPs to dephosphorylate a specific residue is largely defined by the association of their catalytic subunits with regulatory or targeting proteins (Mansuy and Shenolikar, 2006). In contrast to PTPs, PPPs generally consist of small catalytic subunits that bind regulatory or targeting subunits encoded by separate genes to form an enormous variety of distinct holoenzymes with different biological functions (Alonso et al., 2004). The metal coordinating residues (Asp, His, and Asn) which interact with the phosphate group of phosphorylated residues, are invariant between all PPPs and occur within five conserved sequence motifs (Barford et al., 1998). Ser/Thr phosphatases are metalloenzymes that dephosphorylate their substrates in a

single reaction step using a metal-activated water molecule or hydroxide group as a nucleophile. The PPM active site is different to that of the PPP family because it contains two Mn^{2+} ions coordinated by four Asp and one Glu residue. Dephosphorylation is catalysed by metal-activated water molecules acting as nucleophiles in a mechanism similar to that of PPPs (Barford et al., 1998). Differences in the active site are reflected in different toxin sensitivities. PP2B is specifically and potently inhibited by cyclclosporin A and FK506 while PP1 and PP2A are inhibited by a variety of naturally occurring toxins such as okadaic acid and microcystin. The PPM phosphatase, PP2C, however, is only poorly inhibited by the toxins that affect PP1 and PP2A (Barford et al., 1998).

Protein Tyrosine Phosphatase (PTPs) family

PTPs are efficient PPs, with constitutive activity causing rapid dephosphorylation of intracellular pTyr residues and disruption of signal transduction pathways. Most PTP family members dephosphorylate Tyr residues and are known as single-specificity PTPs. However, a subset of PTPs can also dephosphorylate Ser and Thr residues (Barford et al., 1998) and are known as dual-specificity phosphatases (DSPs/DUSPs). DSPs may also target messenger RNA, phospholipids and phosphoinositides in addition to phosphoproteins. Tyrosine-specific PTPs are further classified based on their cellular location. Membrane-spanning PTPs are known collectively as receptor-like PTPs (RLPTPs), whereas others lacking membrane-spanning domains are found in the cytoplasm and are known as intracellular or non-receptor PTPs (NRPTPs) (Gee and Mansuy, 2005; Paul and Lombroso, 2003). While most PTPs are ubiquitously expressed several are brain-specific, including striatal enriched phosphatase (STEP) and STEP-like (PTP-SL) PTPs (Boulanger et al., 1995; Paul and Lombroso, 2003). In

general, PTPs are characterised by their sensitivity to vanadate, their ability to hydrolyse p-nitrophenyl phosphate, their insensitivity to okadaic acid and their lack of a requirement for metal ions during catalysis (Paul and Lombroso, 2003). The non-catalytic segments of PTPs also possess amino acid sequences that target them to specific intracellular compartments (Paul and Lombroso, 2003). Based on the amino acid sequences of their catalytic domains, PTPs can be grouped into four separate families, each with a range of substrate specificities (see Table 1) and structures as discussed below (Alonso et al., 2004).

Class I cys-based PTPs

Class I PTPs constitute the largest family. They contain the well-known classical receptor (a) and non-receptor PTPs (b), which are strictly tyrosine-specific, and the DSPs (c) which target Ser/Thr as well as Tyr and are the most diverse in terms of substrate specificity (see Table 1) (Alonso et al., 2004; Andersen et al., 2004).

a) 'Classical' - Receptor-like PTPs

In total, five RPTP sub-types are known (Types I to V) of which all, except type I, are expressed in the CNS (Paul and Lombroso, 2003). RPTPs function as an interface between the extracellular environment of a cell and its intracellular signalling components, and typically possess two intracellular phosphatase domains, D1 and D2 (Tonks, 2006) specific for tyrosine. The extracellular regions of RPTPs are highly variable but all contain motifs implicated in cell adhesion. However, most are orphan receptors and their mode of action and their respective functional ligands remain largely unknown. One RPTP whose ligands are well understood is LAR, which can interact with both surface-bound and soluble ligands. These interactions either favour

(interaction with protein syndecan) or inhibit (interaction with glycosyl-phosphatidylinositol-anchored protein Dallylike) its phsophatase activity. These ligands modulate the activity of downstream pathways and LAR-associated proteins such as Enabled, ultimately resulting in the regulation of the actin cytoskeleton in synapse morphogenesis and synaptic function (Tonks, 2006). Unique to this class is an intracellular helix-turn-helix 'wedge' motif that is thought to mediate dimerisation of two RPTPs for the inhibition of intrinsic phosphatase activity (Majeti et al., 1998; Majeti et al., 2000).

b) 'Classical' - cytoplasmic/non-receptor PTPs

NRPTPs can be either tyrosine- or dual-specific, lack a transmembrane domain, have a single phosphatase domain and multiple variable domains at either the N- and/or C-terminus (Paul and Lombroso, 2003). Members of this class include PTP1B, STEP, PTP-SL and src-homology 2 domain containing phosphatases (SHP1 and SHP2) (Paul and Lombroso, 2003). PTP1B has been shown to negatively regulate two critical metabolic pathways: the insulin signalling pathway, through dephosphorylation of the insulin receptor, and the leptin-signalling pathway in hypothalamic neurons. The non-catalytic sequence of NRPTPs also controls subcellular distribution, thereby indirectly regulating activity by restricting access to particular substrates at defined subcellular locations (Tonks, 2006).

c) Dual-specificity phosphatases

Class I DSPs are highly diverse and share reduced sequence identity with each other than do NRPTPs and RPTPs. While DSPs can dephosphorylate all three residues, several do show specificity for one of the three. KAP (cyclin-dependent kinase

associated phosphatase) dephosphorylates threonine residues on CDKs and VH1-related DSPs (VHR) preferentially desphosphorylate tyrosine residues on MAPKs. Perhaps the best characterised member of this family, the MAPK phosphatases (MKPs), catalyse the inactivation of MAPKs by dephosphorylation of both the Tyr and Thr sites in the kinase activation loop. The MKPs also display distinct subcellular location and specificity for individual MAPKs (Paul and Lombroso, 2003; Tonks, 2006). PTEN (Phosphatase and tensin homolog deleted on chromosome 10) and myotubularins (MTM1) are unique in that they principally target phosphorylation on D3-phosphorylated inositol phospholipids rather than proteins. As lipid phosphatases, PTEN- and MTM1-related (MTMR) proteins dephosphorylate the products of phosphoinositide 3-kinases/AKT signalling, thereby suppressing cell survival and cell proliferation (Wishart and Dixon, 2002; Yin and Shen, 2008).

Class II - Cys-based LMW-PTPs

This class of PTPs is represented by a single gene in humans encoding the 18 kDa low MW phosphatase (LM-PTP/LMW-PTP). Related classes are widely distributed in living organisms and were highly conserved through evolution. The preservation of this class of phosphatases and the involvement of LMPTPs in many common diseases (Bottini et al., 2002) suggest that it is involved in the regulation of many fundamental processes in cellular physiology (Alonso et al., 2004).

Class III - Cys-based PTPs

The third class of PTPs contains three cell cycle regulators, CDC25A, CDC25B and CDC25C, which dephosphorylate CDKs at their N-terminal, a reaction required to drive progression of the cell cycle. They are themselves regulated by phosphorylation

(Honda et al., 1993) and are degraded in response to DNA damage to prevent chromosomal abnormalities.

Class IV - Asp-based DSPs

The haloacid dehalogenase (HAD) superfamily is a further PP group that uses Asp as a nucleophile and was recently shown to have dual-specificity. These PPs can target both Ser and Tyr (Li et al., 2003; Tootle et al., 2003), but are thought to have greater specificity towards Tyr (Jemc and Rebay, 2007b). A subfamily of HADs, the Eyes Absent Family (Eya), are also transcription factors and can therefore regulate their own phosphorylation and that of transcriptional cofactor/s, and contribute to the control of gene transcription. The combination of these two functions in Eya reveals a greater complexity of transcriptional gene control than previously thought (Jemc and Rebay, 2007a, b). A further member of this class is the RNA polymerase II C-terminal domain phosphatase. While this family remains poorly understood, it is known to play important roles in development and nuclear morphology (Alonso et al., 2004; Siniossoglou et al., 1998; Tootle et al., 2003).

Target specificity and structure of Tyr phosphatases

Structurally, most PTPs consist of a combination of modular domains and, in this respect, resemble PTKs but differ markedly from the Ser/Thr PPs (Barford et al., 1998; Manning et al., 2002). In PTKs, protein-protein interactions serve to regulate kinase activity and target the enzyme to substrates or subcellular compartments, which also appears to be the case for PTPs (Alonso et al., 2004). The second significant trait is conservation of the phosphate-binding loop (P- or PTP-loop) in the active site. The conformation of the P-loop is strictly conserved and can be easily

superimposed on different PTPs. This structurally conserved arrangement ensures that the catalytic Cys, the nucleophile, and Arg residues involved in phosphate binding remain in close proximity to form a cradle to hold the phosphate group of the substrate for nucleophillic attack (Tabernero et al., 2008). In contrast to PPPs and PPMs which catalyse dephosphorylation in a single step, PTPs catalyse dephosphorylation in two steps by way of a cysteinyl-phosphate enzyme intermediate (Barford et al., 1998). Substrate specificity for pTyr results from the dimension of the catalytic site cleft in which the pTyr recognition loop is located. The Tyr residue of this motif packs against the phenyl ring of a pTyr substrate and defines the depth of the catalytic site (Barford et al., 1998). Members of the class IV PTP family, such as HADs, use Asp as the nucleophile for catalysis and carry the active-site signature DXDXT/V. This is also identical to that in TFIIF-associating C-terminal domain (CTD) phosphatase-1 (FCP1) and small CTD phosphatase (SCP) enzymes (Moorhead et al., 2007; Zhang et al., 2006). The structure of the dual-specificity active site is slightly different and allows PTPs to accommodate pSer and pThr in addition to pTyr. DSPs also are not as well conserved and share little sequence similarity beyond the Cys-signature motif (Tonks, 2006). *In vitro*, PTPs generally display poor substrate specificity, as they are capable of dephosphorylating most pTyr-containing substrates. However, *in vivo* PTP activity is tightly regulated through compartmentalisation and the binding of inhibitory proteins to ensure appropriate signalling responses (Paul and Lombroso, 2003). The majority of PTPs are also modified posttranslationally, the most common modification being phosphorylation. Such modifications have been shown to regulate activity and further demonstrate the tight interplay between kinases and phosphatases (Birle et al., 2002). While much progress has been made on elucidating the structure of PTPs over recent years, the mode of actions and binding

partners of the PTPs remains undetermined (Barford et al., 1998; Tabernero et al., 2008).

Table 1: Classes of PPs and their target specificity.

Family	Class	Substrate Specificity
Ser/Thr phosphatases		
PPP family	PP1	pSer, pThr
	PP2A	pSer, pThr
	PP4	pSer, pThr
	PP5	pSer, pThr
	PP6	pSer, pThr
	PP2B	pSer, pThr
	PP7	pSer, pThr
PPM family	PP2C	pSer, pThr
PTP superfamily		
Class I Cys-based PTPs		
a/b) Classical	RPTPs	pTyr
	NRPTPs	pTyr
c) DSPs	MPKs	pTyr, pThr
	Atypical DSPs	pTyr, pThr, mRNA
	Slingshots	pSer
	PRLs	pTyr
	CDC14s	pSer, pThr
	PTENS	De-phosphoinositides
	Myotubularins	PI(3)P
Class II Cys-based PTPs	LMPTP	pTyr
Class III Cys-based PTPs	CDC25	pTyr, pThr
Asp-based PTPs	EyA	pTyr, pSer

Regulators of Phosphatases

Regulation of PPPs

The catalytic activity of Ser/Thr PPs and specificity of targeting to their respective substrates is determined by interacting proteins that can regulate and localise PPs to distinct subcellular compartments. With the exception of PP2C, the function of PPs is defined by the regulatory proteins that bind the catalytic subunit or the holoenzyme itself. Many regulators of PP1 have been identified through the presence of a conserved binding motif R/K1-2V/I[X]F/W, where X can be any amino acid except proline, which has allowed for their purification (Moorhead et al., 2007). The diverse localisation of PPs and the various means of their regulation, however, give rise to a

multitude of compartment-specific PPP-complexes that function in different signalling pathways (see Table 2 for overview of regulatory mechanisms). In general, PP2B is regulated by modulatory calcineurin-interacting proteins (MCIPs or calcipressins), Ca^{2+}-sensing calmodulin (CaM), and the regulatory subunit B (CNB), which is related in structure and function to CaM (Oliver et al., 2002). At excitatory synapses of neurons, scaffolding proteins such as the A-kinase anchoring protein (AKAP79/150) and microtubule-associated proteins (MAPs) act to stabilise the catalytic subunit A (CNA) of PP2B at the postsynaptic density (PSD) (Quinlan and Halpain, 1996; Sim et al., 2003). AKAP-PP2B is also in a complex with the regulatory subunit (RII) of protein kinase A (PKA) and protein kinase C (PKC) where they can modulate NMDA and AMPA receptor function (Coghlan et al., 1995; Klauck et al., 1996; Oliveria et al., 2003). At presynaptic sites, phosphoproteins including synapsins, synaptotagmin, rabphilin3A, synaptobrevin, and dephosphins interact with PP2B for the regulation of vesicle endocytosis and exocytosis (Greengard et al., 1993).

PP1 is also anchored to synapses through a member of the AKAP family, yotiao, where it acts antagonistically to PKA to downregulate NMDA receptor gating kinetics (Lin et al., 1998). Spinophilin, a binding protein with a postsynaptic-density-protein/disc-large/zo1 (PDZ) domain, can sequester PP1 to actin in the synapse where it can associate with D2 dopamine receptors, α-adrenergic receptors, and p70S6 kinase (p70S6K) (Burnett et al., 1998). Most regulatory proteins associated with PP1 are inhibitory to PP1 activity and Inhibitor 1 (I1), G-substrate, and DARPP-32 are examples of particularly effective inhibitors. I1 variants specific for the inhibition of PP2A, including PP2AI1 and PP2AI2, however, have the opposite effect on PP1 by

positively increasing catalytic activity (Katayose et al., 2000). Recent studies have shown direct association between some PPs with PKs to create autoregulatory modules. In such modules, the kinase can phosphorylate the phosphatase to suppress its function and the phosphatase can counteract the kinase activity through dephosphorylation of amino acids in the activation loop (Bauman and Scott, 2002; Shenolikar, 2007).

Although PP1 has been studied primarily in the context of cytosolic and synaptic functions, PP1 is also enriched in the nucleus. It has been implicated in gene transcription and, more recently, in chromatin remodelling associated with learning and memory (Canettieri et al., 2003; Miller and Sweatt, 2007). Although over 100 regulatory subunits are known for PP1, only a few nuclear interactors of PP1 have been identified. Two of the most abundant of these regulators are the nuclear inhibitor of PP1 (NIPP1 a.k.a. Ard1) and PP1 nuclear targeting subunit (PNUTS a.k.a. p99). NIPP1 is a small, 39 kDa peptide which can bind to PP1, RNA, splicing factors, and transcriptional regulators to control gene transcription, but can be inhibited by PKA and CK2 phosphorylation (Jagiello et al., 1997; Roy et al., 2007; Vulsteke et al., 1997). Binding of PNUTS to RNA through its interaction with PP1 has been implicated in chromosome decondensation, further aiding transcription (Kim et al., 2003; Landsverk et al., 2005). Repo-Man is another putative nuclear targeting subunit of PP1, shown to help maintain chromosome structure during chromosome segregation (Vagnarelli et al., 2006). Interacting protein ZAP3, a polynucleoside kinase, is also found to sequester PP1 into a complex with various transcriptional factors to modify RNA in the nucleus (Ulke-Lemee et al., 2007).

Table 2: Regulatory mechanisms and their effectors.

Family	Type of Regulation	Example of Effector
Ser/Thr PPPs	inhibitory regulation	I1, I2, G-substrate, DARPP-32
	scaffolding	AKAP-like, PDZ-domain containing
	regulatory subunits	regulatory B, RII, CaM
	targeting	Repo-Man, PNUTS, ZAP3
	phosphorylation	PKA, PKC, CaMKII, Aurora kinase
	substrate binding	Rb1, NFATs
	stress-responsive	heat, osmotic pressure (PPM)
PTP Superfamily	oxidative stress	insulin, PDGF, UV, B/T-cell activation
	redox state	cyclic sulphenamide species
	ligand-specificity	modular domains
	cell-adhesion ligands	pleiotrophin, cadherin
	extracellular matrix ligands	agrin, syndecan, collagen XVIII, dallylike
	active site occlusion	dimerization, wedge motif
	autoinhibition	phosphorylated catalytic site, Eya
	substrate binding	p130cas

Regulation of PTPs

PTP regulation is predominantly dependent on the variable modular domains that define their unique enzymatic activity (Tonks, 2006). The classical membrane-bound RPTPs, which are involved in cell-adhesion, are regulated through extracellular ligand-specific binding to their modular domains. To date, studies of RPTPs have shown that phosphatase activity is restricted to the membrane-proximal domain D1 of PTPs with the exception of catalytically dead RPTPs and PTPα, the latter having catalytic activity in both the D1 and the membrane-distal D2 domains (Tonks, 2006). Importantly, ligand-induced dimerisation of the D1 domain of RPTPα has been shown to result in a wedge motif which occludes the active site of the complementary domain (Bilwes et al., 1996; Majeti et al., 1998). In the case of LAR RPTP, however, the D2 domain prevents wedge-mediated dimerisation of D1 and both active sites are accessible (Nam et al., 1999). RPTP dimerisation is further regulated by extracellular glycosylation which is determined by alternative splicing of three exons, A, B, and C (Xu and Weiss, 2002). A representative cell-adhesion ligand is pleiotrophin which inhibits type V RPTPζ activity and results in alterations in phosphorylation of β-

catenin, β-adducin, n-cadherin, and p190 Rho GAP thereby affecting cellular cytoarchitecture in the brain (Pariser et al., 2005a; Pariser et al., 2005b; Perez-Pinera et al., 2006; Tamura et al., 2006). Type IIb RPTPs such as RPTPμ, a homophilic cell-adhesion molecule highly expressed in neurons and endothelia, are regulated entirely through ectodomain-dependent interactions (Brady-Kalnay et al., 1993; Gebbink et al., 1993). RPTPμ dimers, nearly identical in dimension and length to cadherin, enable the receptor to gauge distance and regulate interactions with cadherin/catenin complexes at synapses (Aricescu et al., 2007; Boggon et al., 2002). Two heparan sulphate proteoglycans have also been identified in neurons as high-affinity ligands for type IIa RPTPs (agrin and collagen XVIII for RPTPσ (Aricescu et al., 2002); syndecan and Dallylike for LAR (Johnson et al., 2006)) that mediate synaptic signalling and morphological changes (Johnson et al., 2006).

The other major mechanism of RPTP regulation is through reversible oxidation by reactive oxygen species (ROS) (Lee et al., 1998). The nucleophilic cysteine residue in the active site of PTPs is susceptible to oxidation, which renders the domain inactive and inhibits PTP function (den Hertog et al., 2005; Tonks, 2005). Oxidative states play a further role in determining whether the modification is reversible, with higher oxidation states of nucleophilic cysteine being irreversible and the PTP permanently inactivated. Moreover, DSPs and LMW-PTPs contain a second cysteine residue which can form disulphide bonds with adjacent cysteines to prevent oxidation of active-site cysteines (Salmeen and Barford, 2005). The phylogenetically distinct D2 domain is also found to have greater sensitivity to oxidation than the D1 domain and can form disulphide bonds to stabilise PTP dimers (van der Wijk et al., 2004). With its redox sensor-like role, and oxidation-induced conformational change of the

extracellular domains, oxidation of RPTPα D2 is suggested to mediate 'inside-out' retrograde signalling (den Hertog et al., 2005). The multiple levels of regulation, from structural changes to ligand-specificity to ROS signalling, suggest that the diverse family of RPTPs are finely regulated in the course of various cellular processes and physiological functions.

The classical cytoplasmic class of NRPTPs, in comparison, is characterised by regulatory domains that interact either directly with the active site to modulate activity, or indirectly through substrate binding specificity. SHP2 is comprised of two N-terminal SH2 domains that, under basal conditions, occlude the active site of the catalytic domain through intramolecular interactions. Interaction with pTyr ligands on the targeting proteins themselves have been shown to remove autoinhibition of the PTP catalytic site (Neel et al., 2003). In the case of PTP-SL regulation, extracellular signal-regulated kinases 1 and 2 (ERK1/2) binding and subsequent phosphorylation of the kinase interaction motif (KIM) on PTP-SL is necessary for its activation (Pulido et al., 1998). The KIM motif is of significant importance in MAP kinase signalling as it targets KIM-containing PTPs to their substrates, ERK2 and p38. Similarly, LMW-PTPs are also regulated by phosphorylation of Tyr131 and Tyr132 by Lck and Fyn tyrosine kinases in T-cells which, when activated, results in auto-dephosphorylation of the regulatory domains (Bucciantini et al., 1999; Tailor et al., 1997). The highly substrate-specific interaction of PTP-PEST for p130cas has also been attributed to the allosteric binding of the SH3 domain of p130cas which greatly increases its affinity for the substrate and further enhances the specificity of the catalytic domain of PTP-PEST for p130cas (Garton et al., 1997).

PPs as regulators of neuronal activity

The multiple mechanisms that control neuronal signalling at synapses and in the nucleus are complex and only partially understood. Although some pathways regulated by phosphorylation have been delineated, a global picture of phosphorylation-dependent changes in the brain is lacking, due in part to the lack of suitable technologies in the past to measure protein phosphorylation. In healthy cells, signal transduction pathways depend on the interplay between several classes of enzymes that work synergistically or in opposition to regulate signal transmission (Westphal et al., 1999). PKs and PPs are critical components of these pathways and are required for the modulation of synaptic efficacy and neuronal transmission (Bradshaw et al., 2003; Wang and Kelly, 1996), and for memory formation (Lisman et al., 2002; Malleret et al., 2001). In general, PKs and PPs act antagonistically to control signalling pathways through phosphorylation and dephosphorylation of specific targets. Some of these targets, in turn, regulate structural modifications at synaptic contacts (Horne and Dell'Acqua, 2007; Oliver et al., 2002; Westphal et al., 1999), and determine whether transcriptional and translational events are initiated to maintain long-term synaptic plasticity and memory (Atkins et al., 2005; Bito et al., 1996; Mansuy and Shenolikar, 2006; Winder and Sweatt, 2001). Prominent PKs, including αCaMKII, PKA, MAPK, act as positive regulators of neuronal signalling. They are necessary for mechanisms promoting long-lasting forms of synaptic plasticity such as long term potentiation (LTP), which reflect a strengthening of synaptic connections (Giovannini et al., 2001; Smolen et al., 2006). Phosphorylation of the AMPA receptor subunit GluR1 by CaMKIIα favours the recruitment of new receptors to the membrane and increases the channel's conductance, resulting in long-lasting functional changes (Malenka and Nicoll, 1999). In contrast, PPs such as PP1

and PP2B act to weaken synaptic plasticity through dephosphorylation of key proteins required for the induction and maintenance of LTP including NMDA receptor subunits, CaMKIIα, I1 (Strack et al., 2000; Yakel, 1997), which are substrates of PKs, and PKs themselves (Colbran, 2004). PPs are primarily involved in synaptic depression or LTD, a form of synaptic plasticity that reflects a weakening of synapses observed in aged neurons (Hedou et al., 2008; Jouvenceau and Dutar, 2006; Luebke et al., 2004; Mulkey et al., 1994; Norman et al., 2000; Thiels et al., 2000). NMDA receptor signalling, however, involves differential regulation of the Ras-ERK pathway which also interacts with downstream targets of PTPs (Kim et al., 2005; Pulido et al., 1998). NR2A and NR2B subunits themselves are phosphorylated by the Src PTKs at multiple Tyr residues, particularly on Tyr1472 (Tezuka et al., 1999; Zhang et al., 2008), and require specific PTPs for their dephosphorylation and endocytosis (Chen et al., 2003; Snyder et al., 2005; Wang and Salter, 1994).

In addition to acting in synaptic terminals to suppress synaptic functions, PPs also contribute to nuclear events and are important regulators of chromatin remodelling. Chromatin remodelling is a complex cellular process regulated by posttranslational modifications of histone proteins, DNA methylation and alterations to chromatin structure. PPs are essential for the regulation of gene expression, and is implicated in multiple brain processes and functions including development (Hsieh and Gage, 2005; Levenson and Sweatt, 2006), neurogenesis (Tsankova et al., 2007), synaptic plasticity (Allis et al., 2007; Hsieh and Gage, 2005), memory (Chwang et al., 2006; Levenson and Sweatt, 2005; Miller and Sweatt, 2007) and behaviour (Chong and Whitelaw, 2004; Richards, 2006). PP1 plays a particularly important role in chromatin remodelling because it can form complexes with chromatin regulatory proteins such

as histone deacetylases (HDACs) and can regulate histone phosphorylation and acetylation. Both PP1 and PP2A have been implicated in dephosphorylation of Ser10 on histone H3, which has previously been shown to affect chromosome condensation and segregation (Hsu et al., 2000; Nowak et al., 2003). *In vitro*, PP1 was shown to decrease histone phosphorylation and acetylation, leading to inhibition of cAMP-response element binding protein (CREB), and repression of immediate gene response elements necessary for transcription (Canettieri et al., 2003; Ceulemans and Bollen, 2004). In contrast to PP1, there is limited evidence for PP2B involvement in chromatin remodeling in the nucleus, however, shared sequence similarity in the Ser/Thr binding motif suggest that PP2B may also be involved in dephosphorylation of histones. Studies of PP2B in the nucleus have primarily focused on its role as a phosphatase acting on transcription factors. A recent finding shows that PP2B-dependent dephosphorylation of retinoblastoma protein (Rb) dissociates Rb from a repressor complex, including HDAC and BRG1, to facilitate gene transcription (Qiu and Ghosh, 2008). There is further evidence that activity-dependent PP2B dephosphorylation of the myocyte enhancer factor 2 (MEF2) in the nucleus results in enhanced MEF2-dependent transcription of a set of genes which prevents dendritic differentiation and suppresses excitatory synapse number in the brain (Flavell et al., 2006; Shalizi et al., 2006). Recent evidence has also shown that PP2B contributes to the regulation of the immediate early gene Zif268 (Baumgartel et al., 2008), and is therefore directly associated with transcriptional events. The potential mechanisms and regulation of PP2B in nuclear chromatin remodelling, however, remain to be determined.

Human diseases and therapeutic targets based on PPs

Neurodegeneration due to ageing and disease is characterised by a loss of spines and a retraction of dendrites, which reduce both the connectivity and structural complexity of neuronal circuits. The compromised and accelerated decay of synaptic connections with age, in turn, functionally perturbs cellular signalling and synaptic transmission, and reduces the brain's capacity for information processing (Foster, 2007; Luebke et al., 2004). On a mechanistic level, multiple molecular processes at both synapses and the nucleus contribute to morphological and functional alterations of neurons in ageing. Since Ser/Thr PP-dependent pathways are negative effectors of neuronal signalling, they are likely to play a significant role in mechanisms associated with age-related cognitive deterioration and neurodegenerative processes. Indeed, the activity and expression of PP1 and PP2B are dysregulated in the aged brain (Foster et al., 2001; Norris et al., 1998; Watson et al., 2002). A shift in the balance of PKs and PPs in favour of PPs has further been associated with defects in synaptic plasticity and memory in rodents (Wang et al., 1996) and restoration of such a shift by inhibition of PP1 through transgenic expression of I1 in aged mice was shown to restore memory (Genoux et al., 2002). PP1 inhibition can also rescue LTP in adult animals when synapses are either depotentiated or pharmacologically depressed (Jouvenceau and Dutar, 2006; Jouvenceau et al., 2006), thus pointing to a general role of PP1 and PP2B in synaptic depression and cognitive deficits. Although a general decrease in phosphatase activity could account for tau pathology in AD, a more complex role of PPs is emerging. Increased activation of PP2B has been observed in AD patients, and PP2B was found to be involved in Aβ-mediated downregulation of NMDA receptors, AMPA receptor internalisation, and spine loss (Knobloch and Mansuy, 2008).

In addition to PPPs, dysregulation of PTPs has also been implicated in the development and pathology of neurodegenerative diseases. Impairments in learning and synaptic plasticity in mice deficient for PTPRZ, an RPTPζ subtype chondroitin sulfate proteoglycan expressed predominantly in the brain, has been attributed to aberrant phosphorylation of p190 Rho GAP which leads to altered Rho-ROCK signalling in hippocampal neurons (Niisato et al., 2005; Tamura et al., 2006). In addition, redox inactivation of MAPK phosphatase 3 (MKP-3) is thought to mediate glutamate excitotoxicity while overexpression of a dominant-negative MKP-3 results in accumulation of phospho-ERK-2 (Levinthal and Defranco, 2005). Knockout of PTPσ is also detrimental to the nervous system and results in enhanced neurodegeneration following sciatic nerve injury (McLean et al., 2002). The DSP phosphatidylinositol phosphatase myotubularins (MTMs) have also been directly linked to human diseases such as X-linked myotubular myopathy (XLMTM) and mytotonic dystrophy (cDM1) (Buj-Bello et al., 2002; Laporte et al., 1996). Charcot-Marie-Tooth disease (CMT4B1) is an inherited demyelinating neuropathy resulting from a mutated human MTMR2 gene (Bolino et al., 2000; Bolis et al., 2005). Knockout of the DSP laforin or mutations in EPM2A have been implicated in progressive myoclonus epilepsy of Lafora type disease, an inherited neurodegenerative disorder with onset in late childhood and characterised by accumulation of highly phosphorylated glucose polymers (Ganesh et al., 2002; Ganesh et al., 2006; Serratosa et al., 1999). Deletion of PTEN, initially uncovered as a tumour suppressor PTP, in the brain of mice was shown to result in abnormal social interaction, macrocephaly, and neuronal hypertrophy, characteristics of human autistic spectrum disorder (Kwon et al., 2006).

In the pharmaceutical industry PKs are still the second main drug target after GPCRs (Cohen, 2002). In contrast, PPs are only just beginning to be recognised as potential therapeutic targets. Because dysregulation of many PPs is implicated in human diseases (Li and Dixon, 2000), there is increasing interest in pursuing them (Bialy and Waldmann, 2005; Hooft van Huijsduijnen et al., 2004; Tabernero et al., 2008; Tautz et al., 2006). A number of PTPs that have been implicated in oncogenesis and tumour progression are current drug targets for cancer chemotherapy. They include PTP1B, a selective negative regulator of insulin and leptin signalling, which may augment signalling downstream of HER2/Neu; SHP2 essential for growth factor-mediated signalling; Cdc25 phosphatases, which are positive regulators of cell cycle progression; and the phosphatase of regenerating liver (PRL) phosphatases, which promote tumour metastasis (Jiang and Zhang, 2008). Ser/Thr phosphatases are also implicated in several diseases and several are already used as drug targets. PP2B, for instance, is a key component of T-cell signalling pathways and is activated by increased Ca^{2+} resulting from antigen presentation. Inhibition of PP2B by immunosuppressant drugs suppresses T-cell activation, and the microbial products cyclosporin A and FK506 that specifically target PP2B are currently the most effective immunosuppressive agents available. Cyclosporin is widely used to prevent rejection after organ transplantation and in inflammatory diseases such as dermatitis (Ehrchen et al., 2008; Faul et al., 2008; Shenolikar, 2007). Specific inhibitors of Ser/Thr phosphatases such as PP5 and PP1γ1 have also been studied as potential anti-tumour drugs. However, developing specific inhibitors has proved difficult (Honkanen and Golden, 2002). As PTPs have emerged as drug targets for cancer, a number of strategies are currently being explored for the identification of various

classes of PTP inhibitors. These efforts have resulted in many potent and, in some cases, selective inhibitors for PTP1B, SHP2, Cdc25 and PRL phosphatases (Jiang and Zhang, 2008). Aspects of PTP biology that may be relevant for cancer research in the future are the regulation of PTPs by oxidation and the possible role of PTPs in angiogeneis (Ostman et al., 2006).

Current and Future approaches to study PPs and their functions

The advent of proteomics has opened a new dimension to genomics, and has provided novel information about gene expression at the protein level. Its importance is underscored by the recognition that the abundance of mRNA transcripts and the corresponding protein products often do not correlate. This is primarily due to the posttranscriptional regulation of mRNA (Griffin et al., 2002). To date, much of the knowledge gathered about phosphorylation in nerve cells was obtained by studies at the level of single molecules. However, novel proteomic methods are beginning to allow more global characterisations of phosphorylation and analyses of its dynamics (Olsen et al., 2006). Mass spectrometry is ideally suited to the analysis of protein posttranslational modifications such as phosphorylation, because modern MS techniques allow site-specific *de novo* assignment of phosphorylation and the investigation of signalling networks in a high-throughput discovery-driven manner. Recent MS studies have increased the number of known phosphorylation sites in the mouse by several orders of magnitude (Collins et al., 2005; Munton et al., 2007; Trinidad et al., 2006; Trinidad et al., 2008; Trinidad et al., 2005). However, for the majority of these sites the PKs and PPs that modulate them remain unknown. Utilising MS to relate PPs and PKs to these sites is a logical next step. One such approach is to exploit the common mechanism of PTP catalysis to create substrate-

trapping mutants that can be applied to all PTP members to pull-out and determine their substrates by MS (Blanchetot et al., 2005). Similarly. phosphorylation dependent protein-protein interactions of signalling molecules and their site/s of phosphorylation have also been analysed using MS techniques (Hinsby et al., 2004; Zhou et al., 2007). Recently, activity-based proteomics has emerged as a powerful method for analysing enzyme activity. This approach applies chemical probes to covalently label active sites of enzymes in an activity-dependent manner, thus providing a direct readout of catalytic activity. The covalently-tagged proteins can then be identified by MS analysis (Schmidinger et al., 2006). Quantitative proteomics has also been applied to identify the components of large multi-protein complexes in the CNS. These large complexes act as signal 'transduction machines' and are typically composed of scaffolding molecules, receptors, kinases and PPs such as PP1 and PP2A (Husi et al., 2000; Ranish et al., 2003). The same workflows have been used to delineate the phosphatase interacting partners of the PP1 (Trinkle-Mulcahy et al., 2006), PP2A (Goudreault et al., 2009) and PP4 (Gingras et al., 2005) complexes. Recent attention has focused on the role of PPs in chromatin remodelling and the use of quantitative proteomics and chromatin immunoprecipitation (ChIP-on-Chip), in particular, has provided unique tools for the analysis of nuclear PPs (Oficjalska-Pham et al., 2006). Recent advances have also allowed genome-wide analyses using techniques such as ChIP-Seq (Schones and Zhao, 2008). While quantitative proteomics has the potential to analyse protein modifications in an unbiased fashion it currently lacks the specificity to analyse these changes at the level of the single gene. It promises, however, to be a method of choice in the coming years (Huang and White, 2008; Trelle and Jensen, 2007; Tweedie-Cullen et al., 2007). Transgenesis and RNAi are complementary methods, which when used in combination with quantitative

proteomic and genomic technologies will provide a powerful tool for the analysis of specific phosphatase pathways. Recent studies have investigated the role of PP1 and PP2B in learning and memory by taking advantage of transgenic methodologies in mice (Baumgartel et al., 2007; Genoux et al., 2002; Mansuy, 2003). The further generation of such models and their combination with proteomic and genomic methodologies will be instrumental for future studies in the nervous system.

Conclusions

PPs are critical regulators of protein function and neuronal activity, with multiple mechanisms regulating their activity and substrate specificity *in vivo*. Their importance in higher-order brain function explains why their dysregulation is involved in cognitive decline, neurodegeneration and multiple psychological disorders. It is increasingly clear that the complex signalling networks in which phosphatases participate, must be studied at a systems level; the current 'one pathway - one drug' approach of current drug research is no long applicable in light of current knowledge. The development and increasing maturity of tools available in the fields of genomics and proteomics has opened up an opportunity to study PP function at the systems biology level. These tools can now be exploited and applied to the various animal models already available to elucidate the underlying mechanisms of neuronal signalling and brain functions as mediated by phosphorylation. Their application to the brain and its pathologies will potentially help uncover the mechanisms of many diseases and lead to the development of novel therapies.

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