

CHAPTER

## 25

# Serine and Threonine Phosphorylation

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## OUTLINE

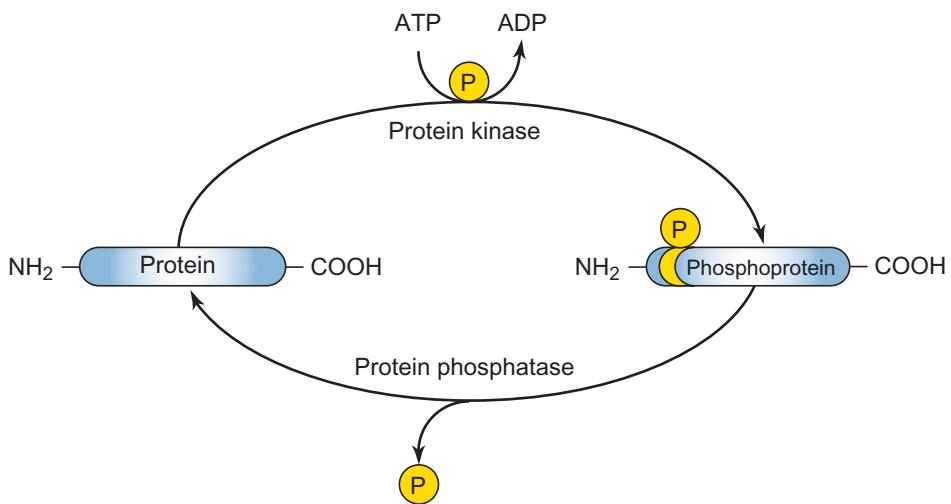
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## PROTEIN PHOSPHORYLATION IS A FUNDAMENTAL MECHANISM REGULATING CELLULAR FUNCTIONS

Protein phosphorylation is the most abundant form of cellular regulation, organizing essentially all cellular functions including metabolism, proliferation, differentiation, motility and survival. From primitive single-cell organisms to complex multicellular organisms, this central regulatory role of protein phosphorylation has been conserved. In fact, in higher eukaryotes, including humans, this role was even further expanded to integrate additional, novel functions that arose at the organ

and whole organism level, including intricate extracellular signaling systems. Consistently, protein phosphorylation is the major molecular mechanism through which protein function is regulated in response to extracellular stimuli. Virtually all types of extracellular signals, including cytokines, hormones, neurotransmitters and neurotrophic factors, as well as physical stimuli such as heat and visible light, produce most of their diverse physiological effects by regulating the phosphorylation state of specific phosphoproteins in their target cells.

Over one-third of all eukaryotic proteins are phosphorylated and virtually every class of protein is regulated by phosphorylation. Protein phosphorylation usually induces



**FIGURE 25-1** Regulatory mechanism of protein phosphorylation. Protein phosphorylation is regulated by antagonistic actions of protein kinases and protein phosphatases. An unphosphorylated protein is converted into a phosphoprotein by a protein kinase and the reversal of this reaction is catalyzed by a protein phosphatase. The phosphorylation of a substrate by a protein kinase is an energy-consuming step that converts ATP into ADP. Dephosphorylation of a phosphoprotein by a protein phosphatase involves hydrolysis of the phosphoester bond, thereby liberating a PO<sub>4</sub><sup>3-</sup> moiety.

conformational changes, thereby affecting protein function. This process is reversible, enabling cells to respond dynamically to a multitude of signals in the environment. Both extra- and intracellular stimuli generally elicit complex patterns of protein phosphorylation to produce their physiological effects. The improper functioning of the machinery regulating protein phosphorylation is often highly disruptive to cellular processes. Consequently, many human diseases, including neuronal disorders, have been linked to dysregulation of protein phosphorylation.

The organization of the nervous system exhibits an outstanding level of complexity. Neuronal functions underlying synaptic plasticity and memory processes rely on highly specialized molecular complexes, which form intracellular signaling networks. The precise organization and proper functioning of this intracellular network requires an extensive degree of high-fidelity regulation, which is largely achieved via protein phosphorylation.

In this chapter, we present the molecular machinery that directs protein phosphorylation. We provide an overview of the crucial role of protein phosphorylation in the regulation of cellular and neuronal functions. Finally, we discuss the consequences of improper functioning of the phosphorylation machinery and its implication in neural disorders.

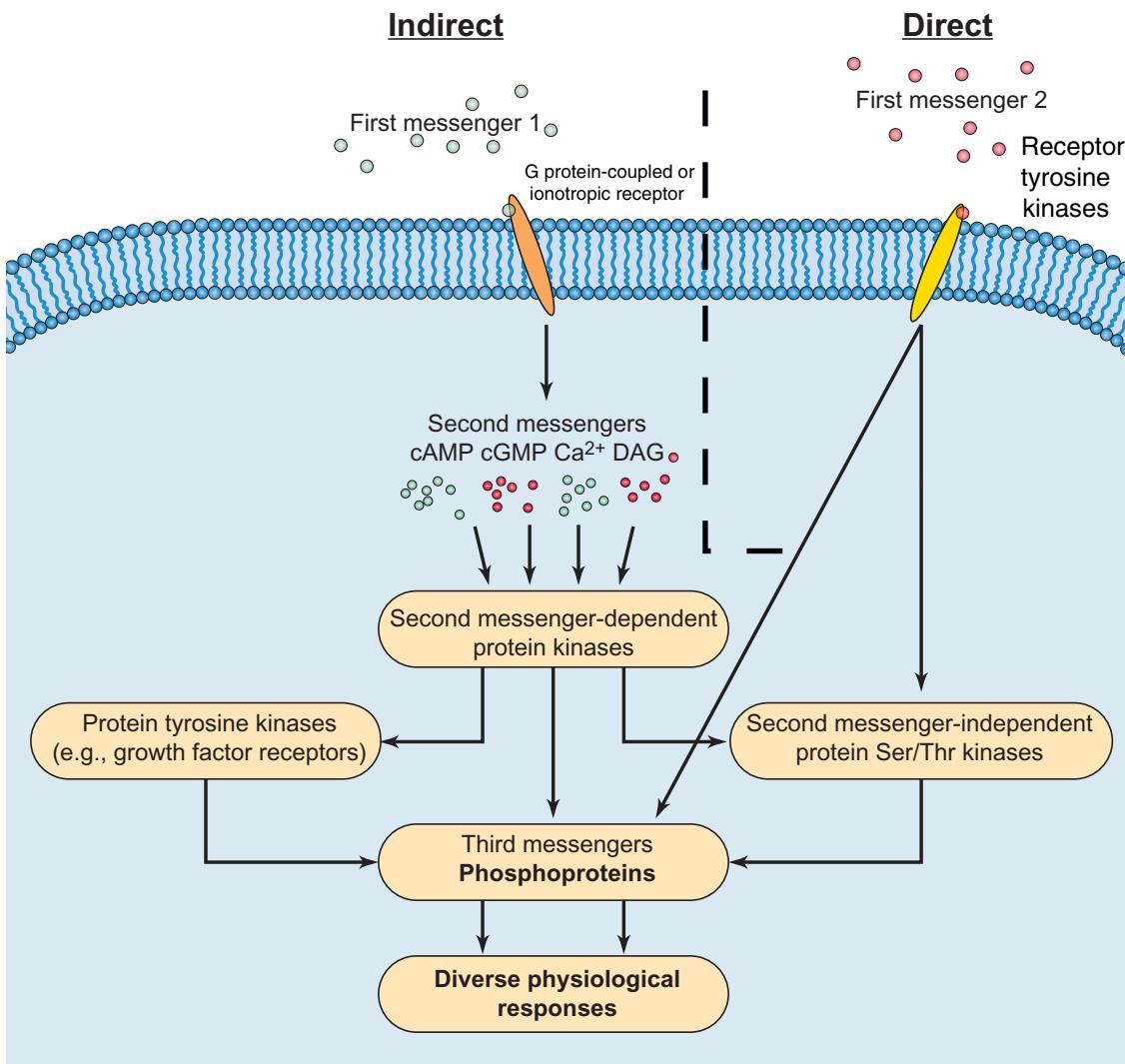
### Phosphorylation levels of substrate proteins are regulated by antagonistic actions of protein kinases and protein phosphatases

Protein phosphorylation is a post-translational modification of proteins, whereby a phosphate group is covalently attached to either a serine (Ser), threonine (Thr) or tyrosine (Tyr) residue. The conversion of a substrate protein from an unphosphorylated state to the phosphorylated form is

catalyzed by a protein kinase (Figure 25-1). The kinase catalyses the transfer of the  $\gamma$ -phosphate group of ATP to the hydroxyl moiety in the respective amino acid residue side chain. To enable this catalytic reaction, all kinases require the presence of a divalent metal ion, such as Mg<sup>2+</sup> or Mn<sup>2+</sup>. The phosphoprotein can be converted back to the unphosphorylated state by a protein phosphatase (Figure 25-1). Protein phosphatases catalyze the cleavage of this phosphoester bond through hydrolysis. This activity-dependent reversible switch, from the unphosphorylated to the phosphorylated form, is the most widely used molecular mechanism, by which physiological signals are transmitted to regulate cellular functions. The addition of phosphate groups to proteins can induce conformational changes that alter biochemical and cellular functions, such as modulation of enzyme activity, cellular location or molecular association.

Under basal conditions, the phosphorylation level of a substrate is determined by an equilibrium in kinase and phosphatase activity. Upon stimulation via extra- and intracellular signals the phosphorylation level can be shifted by increasing or decreasing the activity of either a protein kinase or a protein phosphatase. It may be noted that the transfer of a phosphate group by kinases represents an energy-consuming step. To limit energy expenditure, protein kinases are mostly inactive at a basal cellular state and require activation prior to substrate phosphorylation. Conversely, protein phosphatases generally are active at a basal state. Therefore, under basal conditions most substrates exhibit commonly a low phosphorylation level.

A general scheme of the diverse pathways, through which extracellular signals regulate protein phosphorylation, and hence neuronal functions, is illustrated in Figure 25-2A. Two major mechanisms can be distinguished depending whether the extracellular signals directly or indirectly activate kinases and/or phosphatases. In one mechanism, extracellular



**FIGURE 25-2A General scheme of transmembrane signal transduction cascades.** Extracellular signals, in the form of first messengers, produce specific physiological responses in target cells via the induction of intracellular signaling cascades. Two fundamental pathways can be distinguished, namely, a direct and an indirect pathway. In the **direct pathway**, the first messenger elicits the intracellular signaling cascade directly through the interaction with its specific transmembrane receptor, which in most cases is a protein tyrosine kinase. There are two major classes of protein tyrosine kinases. Some of these enzymes are integral parts of the plasma membrane receptors, such as receptor tyrosine kinases. Others are individual proteins, which can bind to receptors and are regulated indirectly by both second-messenger pathways and receptor protein tyrosine kinases. In the **indirect pathway**, the first messengers trigger, upon binding to transmembrane receptors, the release or production of second messengers. The most common second messengers in the brain include cAMP, cGMP, Ca<sup>2+</sup>, inositol trisphosphate and diacylglycerol (DAG). Each of these second messengers activates specific sets of kinases, termed second messenger-dependent protein kinases, which propagate the intracellular signal. Common protein targets for such second messenger-dependent protein kinases are third-messenger phosphoproteins, second messenger-independent protein Ser/Thr kinases and protein Tyr kinases. Note that in physiological systems there exists virtually every type of molecular cross-talk between the different signaling cascades. Not illustrated in this figure are the actions of protein phosphatases that can antagonize the effect of protein kinases. Indeed, some protein phosphatases can also be regulated directly by second messengers, for example, calcineurin, which is activated by Ca<sup>2+</sup>.

messenger molecules directly regulate protein kinase and phosphatase activities by acting on plasma membrane receptors that possess the enzyme activity within their cytoplasmic domains. Ligand binding induces conformational changes within the receptor, which in turn activates the intrinsic protein kinase or phosphatase properties. Subsequently, this

activation triggers a cascade of phosphorylation state changes of substrate proteins, including kinases and phosphatases, which transmit the signal to evoke specific physiological responses. The direct mechanism is employed by most types of neurotrophic factors as well as many cytokines (Ch. 29) and typically involves protein Tyr kinases (Ch. 26).

In the indirect mechanism, the extracellular messenger molecules act on plasma membrane receptors, which regulate the intracellular concentration of second messengers such as cAMP, cGMP, calcium ( $\text{Ca}^{2+}$ ) ions, diacylglycerol (DAG) and phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>) (Bradshaw & Dennis, 2003). Nomenclature and reactions of phosphoinositides are discussed further in Chapter 23. The second messengers subsequently activate protein kinases and/or protein phosphatases, typically from the Ser/Thr class. These so-called second messenger-dependent kinases and phosphatases in turn regulate the phosphorylation or dephosphorylation of specific substrate proteins, including kinases and phosphatases, triggering a signaling cascade of one or more steps to elicit specific physiological responses. This indirect mechanism is utilized by first messengers that act through G-protein-coupled receptors, including receptors for many neurotransmitters, hormones, cytokines and sensory stimuli such as visible light and odorants (Ch. 21). This mechanism is also used by first messengers that act through receptors containing intrinsic ion channels, such as some glutamate, GABA and acetylcholine receptors (Chs 12, 13). The involvement of second messengers, a particular feature of the indirect mechanism, allows amplification and diversification of a given first messenger's effects through a broader network of transduction cascades.

## PROTEIN SER/THR KINASES

Protein kinases constitute one of the largest and most functionally diverse single enzyme families. The human kinome, a term used for the total entity of protein kinases within the human genome, is composed of 518 protein kinase genes and accounts for about 2% of all human genes (Manning et al., 2002). A dendrogram constructed by genomic sequence comparison demonstrates the complexity of this diverse family of enzymes (Figure 25-2B). According to their substrate specificity protein kinases are grouped into two classes: (1) the protein Ser/Thr kinases, which phosphorylate substrate proteins on Ser and/or Thr residues, and (2) the protein Tyr kinases, which phosphorylate substrate proteins on Tyr residues (Ch. 26). The human kinome comprises 428 protein Ser/Thr kinases and 90 protein Tyr kinases (Manning et al., 2002). A small number of protein kinases are referred to as dual-specificity kinases, because they can phosphorylate substrate proteins on Ser and Thr residues as well as Tyr residues. Over 85% of protein phosphorylation occurs on Ser residues, around 12% on Thr residues, and less than 2% on Tyr residues (Shi, 2009).

### Protein kinases differ in their cellular and subcellular distribution, substrate specificity and regulation

Despite their large number, each of the protein kinases has a specific physiological role. This functional specificity amongst kinases is achieved because each of the kinases features unique transcriptional regulation as well as characteristic structural

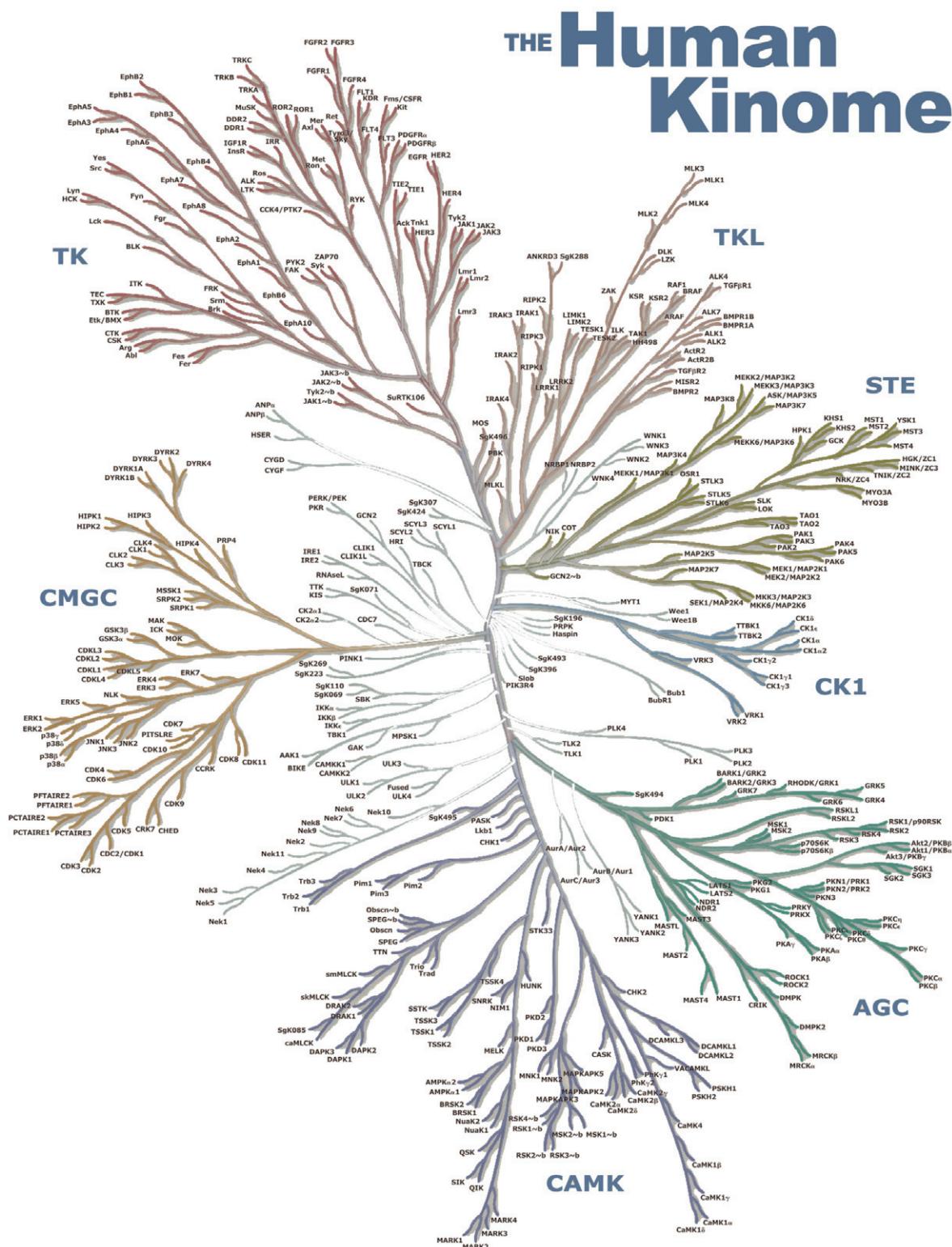
properties. First, protein kinases exhibit distinct spatio-temporal expression patterns and expression levels due to alternative transcriptional and translational regulation. In fact, many protein kinases are themselves regulated by phosphorylation. Every tissue and cell type expresses some subset of kinases. Most of the protein kinases are expressed in brain, albeit with differences amongst cell-types (Hunter, 1995).

Second, protein kinases have distinct substrate specificities, enzymatic regulation and subcellular localization due to molecular and structural differences. The hallmark of protein kinases is the catalytic kinase domain, which harbors a substrate-binding site and an ATP-binding site. The catalytic domain is conserved and shares high levels of homology amongst protein kinases. The structural properties of the substrate-binding pocket determine the sequence specificity and hence the consensus motif for substrates of each kinase (Table 25-1). Sequence variability within the kinase domain accounts for most of the diversity in substrate specificity of kinases. Some of the kinases act specifically on only one or a handful of proteins, while others are multifunctional and have a broad range of substrates. This is highlighted by myosin light chain kinase (MLCK), which has only one physiological substrate, namely, myosin light chain, whereas  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) phosphorylates a wide range of proteins and hence is also termed a multifunctional kinase (Ch. 24). Based on sequence alignments of their catalytic domains the protein kinases have been grouped in defined kinase families as depicted in Figure 25-2B.

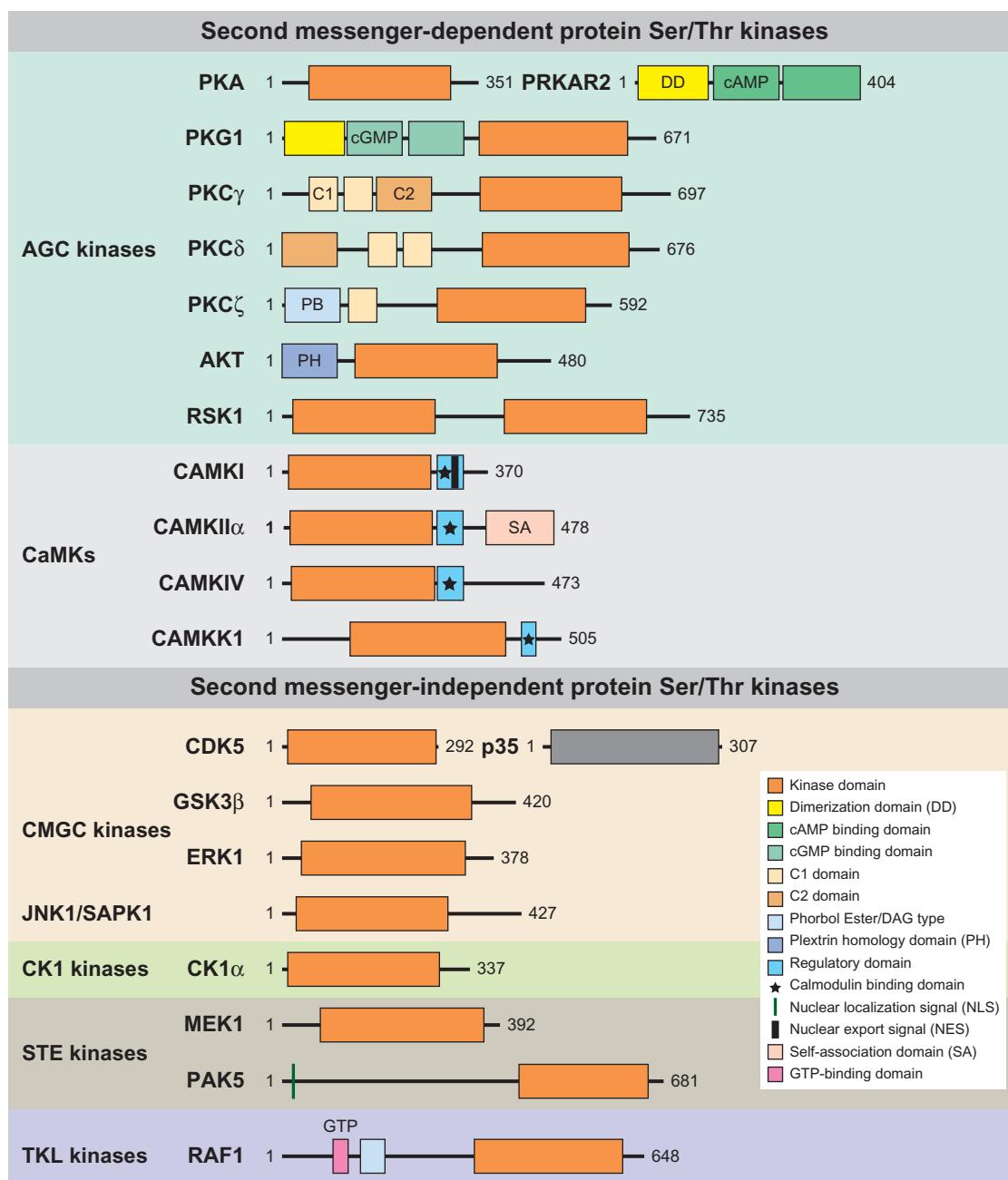
In addition to the kinase core, most of the protein kinases feature additional functional domains, which regulate kinase activity, molecular interactions and intracellular localization. Tight control over kinase activity is imperative for proper functioning of the cell, which is reflected in the multitude of regulatory mechanisms amongst kinases. Control of kinase activity by direct interference with a kinase's catalytic site is termed intrasteric regulation. Many kinases contain inhibitory domains that either directly interact with or sterically hinder access to the catalytic site. Some kinases, such as PKC and CaMKII, contain intrinsic autoinhibitory domains (Figure 25-3). Others, such as PKA, are inactivated through interaction with an inhibitory subunit or protein (Figure 25-3). A special variation of an inhibitory domain is a so-called pseudosubstrate motif, which by mimicking a substrate can bind to the catalytic domain and can thereby block it.

Many kinases are subject to allosteric regulation, whereby the kinase is controlled via the binding of an effector molecule at an interaction motif other than the catalytic kinase domain. Allosteric activation of kinases is commonly triggered by binding of small molecules and cofactors, such as second messengers, as well as by protein–protein interactions.

For example, the function of the kinase Cdk5 is controlled via interaction with its activating subunit p35 (Figure 25-3). Alternatively, activation of kinases can be regulated by phosphorylation and/or dephosphorylation at regulatory motifs. All of these processes induce reversible conformational changes within the kinase, thereby liberating the catalytic kinase domain and rendering it accessible for ATP and substrates. The control of kinase activity via regulatory phosphorylation is of particular importance and widespread application. This mode of activation allows regulatory



**FIGURE 25-2B** The human kinome. This dendrogram or phylogenetic tree delineates the different protein kinase families encoded in the human genome. The proximity between the different kinases reflects the relative degree of homology and relationship between the individual protein kinase sequences. The protein kinases can be classed into seven subgroups: AGC family includes PKA, PKG, and PKC subfamilies; CAMK,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase; CK1, casein kinase 1; CMGC family including CDK, MAPK, GSK-3, CLK family members; STE family including homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases; TK, tyrosine kinase; TKL kinases; tyrosine protein kinase-like kinases. Adapted from the original article (Manning et al., 2002) with permission from Science.



**FIGURE 25-3** Domain structures of selected protein Ser/Thr kinases. In addition to their conserved catalytic kinase domain, protein Ser/Thr kinases contain multiple functional domains that regulate kinase activity, molecular association and subcellular localization. The kinases are grouped according to their subfamilies. Representative examples of protein kinases of the subfamilies are given. An example of each of the PKC subgroups is indicated, including ‘conventional’ (e.g., PKC $\gamma$ ), ‘novel’ (e.g., PKC $\delta$ ) and ‘atypical’ (e.g., PKC $\zeta$ ). AGC kinases includes PKA, PKG, and PKC subfamilies, CaMKs,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases; CaMK1, CaMK kinase 1; CDK5, cyclin-dependent kinase 5; CK1, casein kinase1; CLK, CDC-like kinase; CMGC kinases including CDK, MAPK, GSK-3, CLK family members; DAG, diacylglycerol; ERK1, extracellular signal-regulated kinase 1; GRKs, G-protein receptor kinases; GSK-3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; JNK1, c-Jun N-terminal kinase 1; LRRK2, leucine-rich repeat kinase2; MAP kinase, mitogen activated protein kinase; MEK1, MAPK and ERK kinase 1; MLCK, myosin light chain kinase; PAK5, p21-activated protein kinase 5; PKA, cAMP protein kinase; PKB, protein kinase B (AKT); PKC, protein kinase C; PKG1, protein kinase G 1; PRKAR2, PKA regulatory subunit R2; RSK1, ribosomal S6 kinase 1; SAPK1, stress-activated protein kinase 1; STE, containing the homologs of yeast Sterile 7, Sterile 11, Sterile 20; TKL, tyrosine protein kinase-like.

cross-talk between kinases and/or phosphatases and represents an integral part of many signaling cascades. In fact, regulatory phosphorylation is commonly utilized to filter, specify, diversify and amplify intracellular signaling. A specific variation of regulatory phosphorylation is autophosphorylation, which occurs in many protein Ser/Thr kinases, such as CaMKII.

Finally, kinase activity can be regulated through control of their location in the cell relative to their substrates. Most kinases contain functional domains, which promote molecular interaction with proteins, lipids and other small molecules. Many of these interaction domains serve to localize the kinases to confined subcellular compartments. Prominent examples include (1) nuclear localization signals (NLS) and nuclear export signals (NES); (2) pleckstrin homology domains (PH), which bind to membrane-bound phosphatidylinositol lipids; (3) conserved region 1 (C1, which interacts with DAG; and (4) actin-binding motifs that confer cytoskeletal localization (Figure 23-3). The functionality of the different interaction domains is commonly controlled via regulatory phosphorylation.

## Second messenger-dependent protein Ser/Thr kinases

Second messenger-dependent protein Ser/Thr kinases include members of two major protein kinase families, namely the AGC kinases and the  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases (CaMKs) (Manning et al., 2002). The AGC kinases have been grouped together based on the sequence homologies to the catalytic domains of cAMP-dependent protein kinase (protein kinase A; PKA), cGMP-dependent protein kinase (protein kinase G;PKG) and protein kinase C (PKC). In the next section of this chapter we will introduce important examples of each major class of protein Ser/Thr kinases (listed in Table 25-1). First, second messenger-dependent protein kinases including PKA,PKG,PKC and CaMKs will be presented.

### cAMP-dependent protein kinase

cAMP-dependent protein kinase (PKA) is composed of catalytic and regulatory subunits (Pearce et al., 2010). In an inactive state, the PKA holoenzyme exists as a heterotetrameric complex consisting of two catalytic (C) and two regulatory (R) subunits. In mammals, there are three isoforms of the C subunit, designated  $\text{C}\alpha$ ,  $\text{C}\beta$  and  $\text{C}\gamma$ , which exhibit similar substrate specificity. There exist four R subunits that are further separated in type I ( $\text{RI}\alpha$  and  $\text{RI}\beta$ ) and type II ( $\text{RII}\alpha$  and  $\text{RII}\beta$ ). For its activation, PKA requires the second messenger cAMP, which is synthesized by adenylate cyclase upon stimulation of G-protein-coupled receptors (see Chs. 21 and 22). cAMP binds to the R subunits, thereby inducing a conformational change that causes dissociation of the holoenzyme into a R subunit dimer and free active C subunits. Any change in cAMP level directly impacts on PKA function. Hence, phosphodiesterases that hydrolyze cAMP represent another mechanism controlling PKA activity. PKA also requires regulatory phosphorylation of its activation segment. The RII, but not RI subunits, can undergo autophosphorylation, a prerequisite for PKA activation by enhancing the rate of dissociation

of the RII and C subunits. An alternative mode for controlling PKA function is the regulation of subcellular localization. As PKA lacks localization domains, its cellular distribution is organized via anchoring proteins, termed A kinase anchor proteins (AKAPs) (Skroblin et al., 2010). More than 50 different AKAPs are known; they interact specifically with the  $\text{RII}\alpha$  and  $\text{RII}\beta$  subunits and thereby tether the whole PKA holoenzyme to specific subcellular sites. The different AKAPs exhibit distinct expression profiles and cellular compartmentalization and thereby regulate substrate specificity of PKA. PKA is the most important effector for cAMP mediating numerous physiological responses elicited by G-protein-coupled receptors. PKA subunits show a wide cellular distribution in the brain. They affect many neuronal functions via phosphorylation of a broad range of neuronal substrates. These include integral neurotransmitter receptors, such as AMPA receptor subunits,  $\text{GABA}_A$  receptors and D1 dopamine receptor, and a variety of  $\text{Ca}^{2+}$ - $\text{K}^+$ - and  $\text{Na}^+$ -channels. Furthermore, PKA phosphorylates transcription factors, including CREB and NF $\kappa$ B, which are likely to be crucial for long-lasting synaptic changes underlying memory formation (see Chs. 27, 56 for further discussion of the possible role of CREB in memory).

### cGMP-dependent protein kinase

cGMP-dependent protein kinase (PKG) exists in two isoforms that form homodimers. Each PKG subunit contains a catalytic domain and a regulatory domain, and for its activation requires cGMP, which is synthesized by guanylyl cyclases (Reaume & Sokolowski, 2009) (Ch. 22). Binding of cGMP to PKG induces a conformational change, which removes a pseudosubstrate domain and frees the catalytic site. The activity level of PKG is directly proportional to the cGMP concentration, which is controlled by the activity of guanylyl cyclase and cGMP phosphodiesterases. Guanylyl cyclases are present in membrane-bound forms that are stimulated by natriuretic peptides, and in soluble forms, which are activated by nitric oxide (NO). The implication of NO signaling in synaptic transmission points to an involvement of PKG in neuronal functions (Bliss & Collingridge, 1993). Neuronal substrates of PKG include  $\text{GABA}_A$  receptor  $\beta$ -subunits, G-substrate (an inhibitor of protein phosphatases) and tyrosine hydroxylase. PKG however exhibits a low level of expression in neurons and so far only a few neuronal-specific PKG functions have been characterized (Reaume & Sokolowski, 2009), which might reflect limited second-messenger actions of cGMP in neuronal regulation.

### Protein kinase C

Protein kinase C (PKC) comprises more than 14 isoforms that can be further grouped as follows: conventional PKC (cPKC includes  $\alpha$ ,  $\beta\text{I}$ ,  $\beta\text{II}$ ,  $\gamma$ ); novel PKC (nPKC includes  $\delta\text{I}$ -III,  $\epsilon$ ,  $\eta$ ,  $\theta\text{I}$ -(2); and atypical PKC (aPKC includes  $\lambda$ ,  $\zeta$ ) (Figure 25-3; see also Figure 23-7). All PKC isoforms contain a catalytic domain harboring an ATP binding site and a substrate binding site (Pearce et al., 2010). The regulatory domain is variable amongst the different isoforms. The conventional PKC isoforms require  $\text{Ca}^{2+}$  as well as DAG for activation, whereas novel isoforms depend only on DAG binding for stimulation. The atypical PKC isoforms are considered constitutively active. Additional factors that govern the activity

**TABLE 25-1** Major Protein Ser/Thr Kinase Families

SECOND MESSENGER-DEPENDENT PROTEIN SER/THR KINASES			
Protein Kinase Family	Prominent Member	Consensus Motif for Substrate	Cofactor Required for Activation/Inhibition
AGC kinases	PKA	-x-R-(R/K)-x-S/T-	cAMP
	PKG	-R/K-R/K-x-S/T-	cGMP
	PKC	-R/K-x-S/T-R/K-	Ca <sup>2+</sup> , DAG, lipids
	AKT (PKB)	-(R)-R/(K)-x-S/T-(P)-	PtdIns(3,4,5)P <sub>3</sub>
	RSKs	-R/K-x-R-x-x-S/T-	regulatory phosphorylation
	GRKs	?	PtdIns(4,5)P <sub>2</sub>
	CaMKI	-R-x-x-S/T-	Ca <sup>2+</sup> /CaM
CaM kinases	CaMKII	-R-x-x-S/T-	Ca <sup>2+</sup> /CaM
	CaMKVI	-R-x-x-S/T-	Ca <sup>2+</sup> /CaM
	MLCK	(MLC only substrate)	Ca <sup>2+</sup> /CaM
Other	CaMKK1		Ca <sup>2+</sup> /CaM
SECOND MESSENGER-INDEPENDENT PROTEIN SER/THR KINASES			
Protein Kinase Family	Prominent Member	Consensus Motif for Substrate	Cofactor Required for Activation/Inhibition
CMGC kinases	CDK5	-x-S/T-P-	p35, p39
	ERK (MAPK)	-x-S/T-P-x	regulatory phosphorylation
	GSK3	-x-S/T-P-x or S/T-x-x-S/T(phospho)	regulatory phosphorylation
	CLK		
	JNKs (SAPKs)	(-x-S/T-P-)	regulatory phosphorylation
	CK1	-S/T(phospho)-x-x-S/T-	
STE kinases	MEKs	-T-x-Y-	regulatory phosphorylation
	PAKs		GTPase
TKL kinases	RAF		RAS-GTP
	LRRK2	?	
Other	PINK1	?	

This table lists the major families of the protein Ser/Thr kinases and names some of their most prominent members. Also indicated are the consensus motifs for substrates of some of the kinases and their corresponding regulatory cofactors. Most of the protein kinases presented here are expressed in many cell types, including neurons. These kinases have been included here because of their particular significance in neuronal functions. Many other protein kinases have been omitted that may also play a vital role in many cellular functions including neuronal processes.

AGC, PKA, PKG, PKC subfamily; CaMK, Ca<sup>2+</sup>/calmodulin-dependent protein kinase; CaMKK, CaMK kinase; CDK5, cyclin-dependent kinase 5; CK1, casein kinase1; CLK, CDC-like kinase; CMGC, CDK5, MAPK, GSK-3, CLK subfamily; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; GRKs, G-protein receptor kinases; GSK-3, glycogen synthase kinase-3; JNK, c-Jun N-terminal kinase; LRRK2, leucine-rich repeat kinase2; MAP kinase, mitogen-activated protein kinase; MEKs, MAPK and ERK kinases; MLCK, myosin light chain kinase; PAKs, p21-activated protein kinases; PINK1, PTEN-induced putative kinase; PKA, cAMP protein kinase; PKB, protein kinase B (Akt); PKC, protein kinase C; PKG, protein kinase G; RSKs, ribosomal S6 kinases; SAPKs, stress-activated protein kinases; STE, containing the homologs of yeast Sterile 7, Sterile 11, Sterile 20; TKL, tyrosine protein kinase like.

of PKC are phosphatidylserine (PS) binding and regulatory phosphorylation in the activation loop, which is mediated by phosphoinositide-dependent kinase (PDK1). In the resting state, a pseudosubstrate motif blocks the catalytic domain. This inhibition is relieved when the activating cofactors Ca<sup>2+</sup>, DAG and PS bind to the regulatory domain of PKC. The

second messenger DAG is generated by phospholipase C (PLC), which metabolizes PtdIns(4,5)P<sub>2</sub> (Ch. 23). Under basal conditions, PKC is predominantly a cytoplasmic protein. Upon activation, the enzyme associates with membranes including the plasmalemma or Golgi membranes, or the nuclear envelope, the locations of many of its physiological substrates.

Individual isoforms of PKC exhibit different cellular distribution within the brain and are localized to distinct subcellular compartments. This spatial regulation is controlled via interaction of PKC with various scaffolding proteins. Some scaffolding proteins bind multiple PKC isoforms, while others recruit individual isoforms to unique subcellular locations. There are specific binding proteins that interact with PKC in particular activation states only. For example, activated PKC, but not the inactive form, binds with high affinity to a series of membrane-associated proteins, termed receptors for activated C kinase (RACK) (Pearce et al., 2010). The catalytic domains of the different PKC isoforms have similar substrate specificities. Therefore, the spatial regulation via specific scaffold proteins can confer distinct substrate selectivity to individual isoforms.

PKC phosphorylates a broad range of neuronal substrates. In fact, PKC signaling cascades display an intricate molecular relationship with glutamatergic, GABAergic and cholinergic neurotransmission systems. Most of these signal transduction systems involve the second messenger  $\text{Ca}^{2+}$  and hence activate conventional PKC isoforms that are  $\text{Ca}^{2+}$ -dependent. For example, stimulation of NMDA receptors and subsequent  $\text{Ca}^{2+}$  influx leads to activation of PKC. Active PKC, in turn, phosphorylates target proteins, which ultimately result in enhanced neuronal excitability and potentiation of synaptic responses. One such target of PKC is the AMPA receptor subunit, GluR1. PKC phosphorylates GluR1 at Ser818, thereby controlling synaptic incorporation of AMPA receptors. Such molecular mechanisms are believed to play an important role in synaptic plasticity and memory (see Ch. 56).

A specific role in higher neuronal function has been proposed for the PKC isoform PKM $\zeta$  (Sacktor, 2011). Transcription from an internal promoter within the PKC $\zeta$  gene generates PKM $\zeta$ , which consists of the catalytic site only. Lacking the regulatory domain and hence its autoinhibitory effect, PKM $\zeta$  is constitutively active. In a basal state, the mRNA of PKM $\zeta$  is localized within dendrites in a conformation that cannot be translated. This translational block is relieved upon stimulation of learning-induced forms of synaptic plasticity that trigger molecular changes, including activation of PKA, MAPK, CaMKII and PI3K. Once PKM $\zeta$  is synthesized and phosphorylated by PDK1, it is constitutively active. In this state, PKM $\zeta$  is thought to perpetuate long-term synaptic changes, including enhancement of AMPA receptor responses, and thereby supporting learning and memory. Due to its plasticity-dependent activation mechanism and its long-lasting molecular response, PKM $\zeta$  has been proposed to act as an essential maintenance mechanism required for long-term memory storage.

### **Calcium $^{2+}$ /calmodulin-dependent kinases**

Calcium $^{2+}$ /calmodulin-dependent kinases (CaMKs) are a diverse group of kinases that require  $\text{Ca}^{2+}$  (in the form of  $\text{Ca}^{2+}$ /calmodulin) for activation. Prominent examples with critical neuronal involvement include CaMKI, CaMKII, CaMKIV and MLCK (Lisman et al., 2002; Wayman et al., 2008).

CaMKII exists in four subunits, termed  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ , which are encoded by four different genes. All CaMKII subunits feature three functional parts: a catalytic, a regulatory,

and a self-association domain (Figure 25-3). The regulatory domain contains (1) the autoinhibitory site, which, in the resting state, binds to and inhibits the catalytic domain, (2) the  $\text{Ca}^{2+}$ /calmodulin-binding site and (3) several regulatory phosphorylation sites. The inhibition by the autoinhibitory motif is relieved when  $\text{Ca}^{2+}$ /calmodulin binds to the regulatory domain. Autophosphorylation at Thr286 in CaMKII  $\alpha$  and Thr287 in CaMKII  $\beta$  switches the kinase into an autonomous activity mode that is  $\text{Ca}^{2+}$ -independent (Lisman et al., 2002; Wayman et al., 2008). Because of the stimulation-sensor capability of this autophosphorylation switch, CaMKII, more than any other protein kinase, has been proposed to act as a memory storage mechanism (Lisman et al., 2002) (see Ch. 56). Via self-association, individual CaMKII subunits can tether with each other and form a dodecameric holoenzyme. These CaMKII complexes are commonly found in proximity to  $\text{Ca}^{2+}$  entry sites, such as calcium channels. Each CaMKII subunit displays a distinct pattern of tissue-specific expression, with  $\alpha$  and  $\beta$  being present predominantly in neurons. Expression of CaMKII $\alpha$  is particularly abundant in glutamatergic neurons and constitutes up to 20% of protein within the postsynaptic densities (PSD), which are specialized zones integral for synaptic transmission. Due to its largely forebrain neuronal expression profile, the promoter of CaMKII $\alpha$  has been widely utilized to drive transgene expression in genetically modified mouse models with neuronal-restricted expression. CaMKII is considered to have broad substrate specificity and has been shown to phosphorylate synaptic proteins including AMPA receptors and NMDA receptors. Studies in mice carrying a point mutation that prevents autophosphorylation at Thr286 revealed an important role of CaMKII in synaptic plasticity and memory. Disruption of the autonomous CaMKII activity resulted in severe long-term potentiation (LTP) and spatial learning impairment in these mice. There is, however, still limited knowledge on the physiological substrates of CaMKII and in how far the enzymatic function of CaMKII underlies its fundamental role in neuronal processes.

CaMKI and CaMKIV feature similar domain structures as CaMKII (Fig. 23-3) and also require  $\text{Ca}^{2+}$ /calmodulin for their activation (Wayman et al., 2008). In contrast to CaMKII, they act as monomeric enzymes and exhibit divergent modes of regulation via phosphorylation. In fact, CaMKI and IV depend upon phosphorylation of their activation loop for catalytic activity. This phosphorylation is specifically mediated by a further set of  $\text{Ca}^{2+}$ /calmodulin-dependent kinases, CaMKK $\alpha$  and CaMKK $\beta$ . CaMKK $\alpha$  and  $\beta$  are themselves regulated via phosphorylation by PKA, thereby providing a prominent mechanism by which the cAMP and  $\text{Ca}^{2+}$ -cascades cross-talk. The substrate specificities of CaMKI and IV are mainly regulated by their subcellular localization, with CaMKI being predominantly cytoplasmic and CaMKIV nuclear. In the nervous system, CaMKI and IV play important roles in mediating important second-messenger actions of  $\text{Ca}^{2+}$ , although their physiological substrate range remains only partially known. CaMKI has been found to regulate extracellular signal-regulated protein kinase (ERK), thereby affecting transcription. The nuclear CaMKIV has been directly linked with the control of transcription factors, including CREB, which are crucial for memory function.

MLCK represents a special case in that it phosphorylates physiologically only a single substrate, namely myosin light chain. Through this action MLCK regulates cellular processes, including intracellular transport, cytoskeletal dynamics and cellular motility.

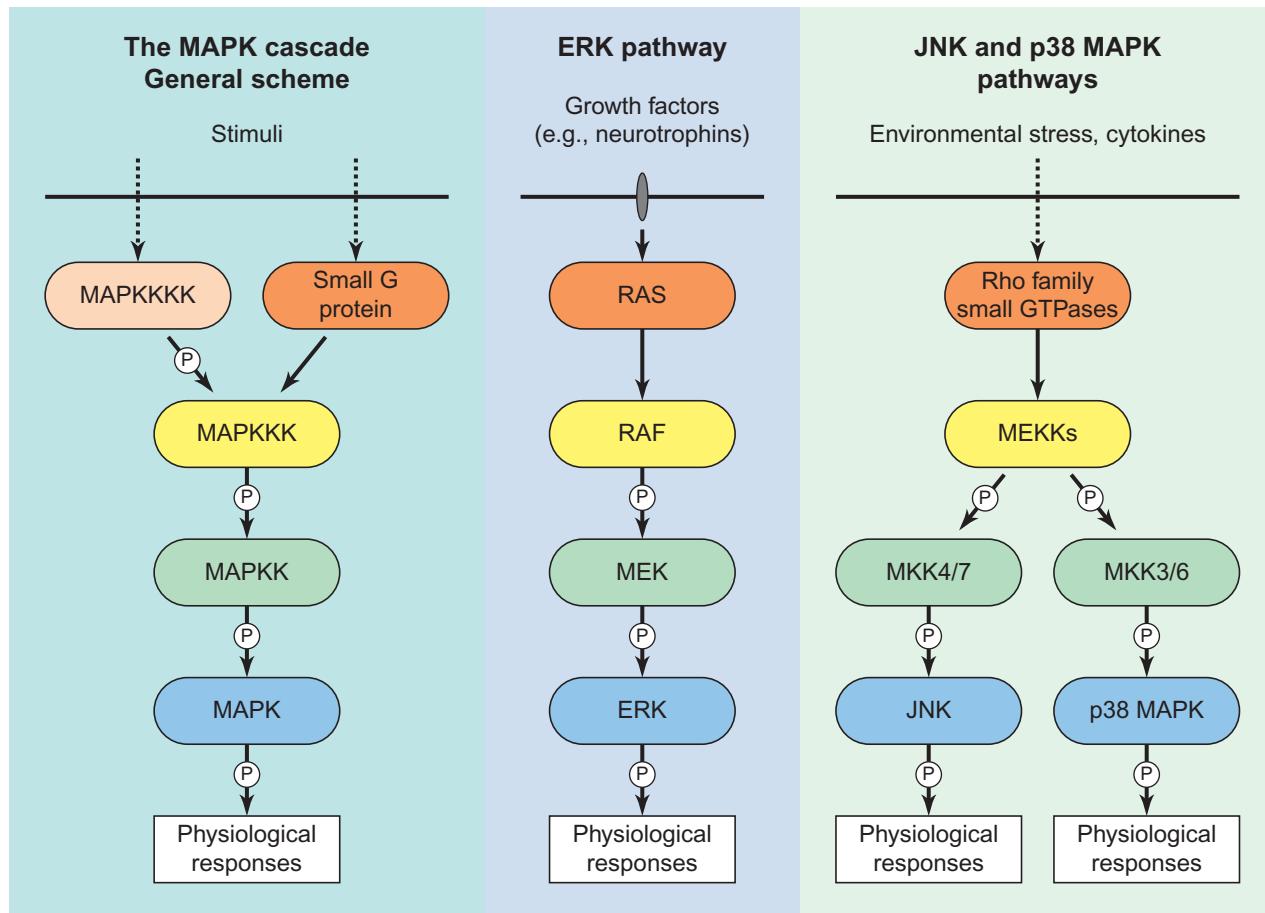
### Second messenger–independent protein Ser/Thr kinases

Although the second messenger–dependent protein kinases were identified first as playing an important role in neuronal function, we now know that many second messenger–independent protein Ser/Thr kinases regulate numerous fundamental neuronal functions. Second messenger–independent protein Ser/Thr kinases generally act downstream of second messenger-induced pathways or protein Tyr kinase signaling (Figure 25-2A). Their activity is commonly controlled by regulatory phosphorylation and/or association with regulatory cofactors. Second messenger–independent protein Ser/Thr

kinases contribute to the transduction of signals via the phosphorylation of specific phosphoproteins and feedback onto upstream protein kinases and phosphatases.

### The MAPK cascade is a classical example of second messenger–independent protein Ser/Thr kinase signaling

Mitogen-activated protein kinases (MAPKs) were first shown to play an important role in cell growth. Diverse extracellular signals can trigger the MAPK cascade, which involves the activation of a series of successive protein kinases, culminating in the activation of MAPKs (Figure 25-4). In brain, numerous members of the MAPK family are expressed and mediate important neuronal functions (Sweatt, 2001). The MAPK members are structurally and functionally related. They are proline-directed protein kinases and hence phosphorylate substrates predominantly at Ser-Pro and Thr-Pro motifs. Importantly, they all share a similar mechanism of



**FIGURE 25-4** The MAPK signaling cascades. The general scheme of the sequential protein kinase activation cascade of mitogen-activated protein kinase (MAPK) is delineated in the left column. The next columns depict the physiological signaling pathways of the three MAPK subfamilies, ERK, JNK and p38 MAPK. The signal transduction steps involving protein phosphorylation are indicated with (p). **ERK**, extracellular signal-regulated kinase; **JNK**, c-Jun N-terminal kinase; **K**, kinase; **MEK** is both MAPK and ERK kinase; **MKK**, mitogen-activated protein kinase kinase; **MEKK**, MKK kinase; **RAF** is a MAP kinase kinase kinase functioning downstream of the **RAS** subfamily of membrane-bound GTPases to which RAF binds directly and once activated, RAF phosphorylates and activates MEK which phosphorylates and activates ERK.

activation, namely regulatory phosphorylation of two residues within a conserved tripeptide motif (Thr-x-Tyr).

In the general scheme, the MAPK cascade is triggered by physiological stimuli that activate a MAPK kinase kinase upon interaction with a member of the small G-protein super family (see Ch. 21). The MAPK kinase kinase then activates a MAPK kinase via regulatory phosphorylation. This MAPK kinase in turn phosphorylates MAPK at the tripeptide motif and thereby activates it. Based on the sequence of the tripeptide motif, the members of the MAPKs family can be further classified into three major groups: extracellular signal-regulated protein kinases (ERKs; Thr-Glu-Tyr); p38 MAPKs (Thr-Gly-Tyr); and c-Jun NH<sub>2</sub>-terminal kinases (JNKs; Thr-Pro-Tyr). Due to their involvement in responses to inflammatory cytokines and environmental stress, p38 MAPKs and JNKs are also collectively named stress-activated protein kinases (SAPKs).

Despite having the same consensus substrate motif (S-P or T-P), individual MAPKs exhibit distinct substrate specificities. This functional divergence within MAPKs is mediated by mechanisms including specific MAPK docking sites on substrates (other than the phosphorylation site) and regulation of subcellular localization via scaffold proteins. In fact, via such molecular interactions specific MAPK cascade signaling complexes can form. These assemblies contain different components of discrete MAPK cascades, thereby establishing dedicated functional units that serve as transmitters and integrators for select physiological signals.

### **Extracellular signal-regulated protein kinases (ERKs)**

Extracellular signal-regulated protein kinases (ERKs) exist in seven isoforms in humans (ERK1, 2, 3, 5, 7, 8 and ERK3-related)(Johnson & Lapadat, 2002; Kim & Choi, 2010). ERKs are activated by regulatory phosphorylation of the tripeptide motif located in the T loop. Dual phosphorylation of the tripeptide motif induces a conformational change within the T loop that liberates the catalytic site. The MAPK kinases responsible for phosphorylation and activation of the ERKs are referred to as MAPK and ERK kinases (MEKs). ERKs are phosphorylated by MEKs on both the Thr and the Tyr residues within the tripeptide motif. MEKs are therefore an example of a dual-specificity protein kinase that can act as a Ser/Thr kinase as well as a Tyr kinase. There are two isoforms, MEK1 and 2, that phosphorylate ERKs only. MEKs are activated through phosphorylation of two Ser residues within an activation segment by RAF, which exists in three isoforms, A-RAF, B-RAF and C-RAF (Roskoski, 2010). The RAF kinases have restricted substrate specificity, with MEKs being their main substrate. The activation of RAF kinases requires dimerization, regulatory phosphorylation and, importantly, association with a small GTPase, RAS. There are three known forms of RAS, whose activation is mediated by the receptor tyrosine kinase-Grb-SOS signaling axis via intricate mechanisms.

Activation of a variety of plasmalemma-associated growth factor–protein tyrosine kinase receptors is known to trigger this so-called RAS-RAF-MEK-ERK signal transduction cascade (Figure 25-4). Neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), and related growth factors, such as epidermal growth factor

bind to these plasma membrane receptors. This binding stimulates the intrinsic protein Tyr kinase activity within the cytoplasmic tail of these receptors, thereby activating the Shc-Grb-SOS complex leading to activation of the RAS-RAF-MEK-ERK cascade (Johnson & Lapadat, 2002) (see Chs. 23, 26 and 29). Alternative modes of activation for the ERK pathway are known, which may have important implications for neuronal functions. These can be RAS-dependent or RAS-independent and involve the stimulation of the ERK pathway via PKC, G-protein–coupled receptors or Ca<sup>2+</sup> stimulated Ras activation.

Given that the major activators of the ERK cascade are growth factors, it is not surprising that these enzymes have been implicated in neuronal proliferation, differentiation and survival. However, it has become apparent that these enzymes also play a role in the adult nervous system regulating neuronal functions underlying synaptic plasticity and memory (Sweatt, 2001). ERKs have a broad substrate range and can phosphorylate many neuronal proteins. Importantly, the ERK pathway has been implicated in transcriptional and translational regulation via phosphorylation and activation of ribosomal protein S6 kinases (RSKs) and mitogen and stress-activated protein kinases (MSKs). RSKs were first identified as regulators of ribosomal proteins, but are now known to phosphorylate other types of substrates, including the transcription factor CREB (Carriere et al., 2008).

### **p38 MAPKs**

p38 MAPKs are present in four subtypes:  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ , all of which contain the regulatory dual phosphorylation motif (Thr-Gly-Tyr). Interestingly, differences in the structure of the kinase site of p38 MAPK $\alpha$  and  $\beta$  allowed the development of isoform-specific small inhibitor molecules. The signaling cascade that regulates p38 MAPKs involves the sequential activation of several protein kinases (Johnson & Lapadat, 2002). Extracellular stimuli such as cytokines stimulate members of the Rho family of small GTPases. This in turn triggers a kinase cascade, including MEKK and MAPK kinases (MKKs) (Figure 25-4). These MKKs are dual-specificity kinases. The MKK isoforms 3, 4 and 6 have been implicated in the activation of p38MAPKs.

P38 MAPKs are activated by stimuli including inflammatory cytokines and environmental stress and thus have been implicated in the pathological changes accompanying inflammatory and apoptotic processes of various cell types including neurons (Kim & Choi, 2010). It is thought that p38 MAPKs impact on these processes via the regulation of gene expression and protein synthesis. Prominent substrates of p38 MAPKs include the protein kinase MAPKAP2 and transcription factors such as ATF2, CREB and myocyte enhancer factor 2 (MEF2).

### **c-Jun NH<sub>2</sub>-terminal kinases**

c-Jun NH<sub>2</sub>-terminal kinases (JNKs) are encoded by three genes, which generate several isoforms via alternative splicing. Expression of JNK1 and 2 is ubiquitous, whereas JNK3 displays limited distribution in tissues including brain. All JNKs contain a dual phosphorylation motif within the T loop, Thr-Pro-Tyr, which regulates activation. Similar to p38 MAPK, JNKs are activated by stimuli including inflammatory cytokines and

environmental stress (Kim & Choi, 2010). These stimuli trigger a signal transduction cascade, which results in the activation of MKKs (Figure 25-4). The MKK isoforms 4 and 7 phosphorylate the tripeptide motif in JNKs and thereby activate it.

JNKs have been implicated in physiological processes including differentiation, survival and apoptosis. They are activated in brain by synaptic activity and are involved in the pathogenesis of Huntington's disease (Morfini et al., 2009) (see Chs 8, 48, 49). Among the best-characterized substrate proteins are transcription factors (Johnson & Lapadat, 2002) (See Ch. 27). JNKs are important regulators of AP-1-dependent gene expression via the phosphorylation of transcription factors, including ATF2 and c-Jun.

### The brain contains many other types of second messenger-independent protein Ser/Thr kinases

Examples of other second messenger-independent protein kinases are listed in Table 25-1. Many of these include enzymes that were identified originally in association with a particular substrate protein but shown later to play a more widespread role in brain signal transduction. Other examples of protein Ser/Thr kinases are the cyclin-dependent kinases (CDKs), identified originally for their role in the control of the cell cycle. Such kinases, again identified originally in yeast, are among a large number of proteins termed cell division cycle (Cdc) proteins. Most CDKs are activated through association with cofactors called cyclins and phosphorylation by upstream protein kinases termed CDK-activating kinases (CAKs).

### Cyclin-dependent kinase 5 (CDK5)

Cyclin-dependent kinase 5 (CDK5) is an atypical member of the CDK family (Dhavan & Tsai, 2001). In contrast to other CDKs, which require cyclins for their activation, CDK5 is activated upon association with the neuron-specific cofactors p35 or p39. The activators p35 and p39 are myristoylated and thus active CDK5 is mainly localized at membranes. CDK5 is highly enriched in neurons and functions during CNS development as well as in adult neurons. CDK5 is a proline-directed protein Ser/Thr kinase and as such phosphorylates Ser-Pro and Thr-Pro motifs. CDK5 has a broad range of substrates and hence is also termed a multifunctional kinase (Angelo et al., 2006). Physiological substrates for CDK5 include many proteins involved in intracellular signaling, such as MEK1, and inhibitors of protein phosphatases, such as inhibitor-1 and DARPP-32. It has been implicated in a large number of CNS functions, including cortical layer formation, synaptic vesicle dynamics, neurotransmission, and memory formation (Angelo et al., 2006). It has been shown that CDK5 modulates synaptic plasticity and memory via the control of NMDA receptors, including NR2B subunits. CDK5 is responsible for regulating the cell surface expression and degradation of NR2B (Hawasli et al., 2007). Consistent with an important function in synaptic plasticity and memory, deregulation of CDK5 activity has been implicated in several human CNS disorders such as Alzheimer's disease (AD),

Parkinson's disease (PD) and drug addiction (Cruz et al., 2006; Qu et al., 2007; Meyer et al., 2008) (see Chs 46, 49, 61).

### Glycogen-synthase kinase-3 (GSK3)

Glycogen-synthase kinase-3 (GSK3) exists in two isoforms, termed  $\alpha$  and  $\beta$ , which are generated from distinct genes (Grimes & Jope, 2001). GSK3 is one of the few kinases that is active in its basal state and is inhibited upon phosphorylation at Ser9 in GSK-3 $\beta$  and at Ser21 in GSK3 $\alpha$ . This inhibitory regulation of GSK3 can be mediated by several kinases including AKT and PKA. Numerous signaling cascades converge onto this inhibitory site, indicating a central role for GSK3 in various physiological functions. The  $\beta$ -isoform additionally features an inhibitory phosphorylation site in its carboxy-terminal region at Thr390, which can be regulated by p38 MAPK. The presence of this additional regulatory site in GSK3 $\beta$ , suggests that the two isoforms might, in fact, have distinct functional properties, despite sharing very similar substrate specificity. An alternative mode of inhibition for GSK3 can occur via protein-protein interactions and complex formation. Association of GSK3 with proteins including FRAT1 and axin inactivate GSK3. Such mechanisms could control GSK3 activity even at a basal state. GSK3 is a proline-directed protein Ser/Thr kinase, but can also phosphorylate so-called "primed" sites, which are Ser or Thr residues in proximity of an already phosphorylated residue.

GSK3 is a multifunctional protein kinase and has a broad range of substrates including signaling and structural proteins, as well as transcription factors (Grimes & Jope, 2001). GSK3 is ubiquitously expressed and is found at high levels specifically in brain. Consistently, many of its substrates are neuronal proteins indicating a fundamental role of GSK3 in neuronal functions. Indeed, GSK3 has been implicated in a variety of processes such as axonal transport, neuronal polarization and postsynaptic signaling.

GSK3 has been implicated in the pathogenesis of neurodegenerative diseases, such as AD (Hooper et al., 2008) (Ch. 46). Deregulation of GSK-3 has also been associated with human psychiatric illnesses including schizophrenia and bipolar disorder (Grimes & Jope, 2001) (Ch. 58 & 60). GSK-3 is involved in the regulation of synaptic plasticity and memory (Hooper et al., 2007; Peineau et al., 2007) (Ch. 56).

### Casein kinase 1 (CK1)

Casein kinase 1 (CK1) represents a unique subgroup within the Ser/Thr protein kinases. It is present as seven isoforms in humans and is termed CK1 $\alpha$ ,  $\beta$  ( $\alpha$ L),  $\gamma$ 1-3,  $\delta$  and  $\epsilon$  (Perez et al., 2010). CK1 acts as a monomeric enzyme and is mostly cofactor independent. The activity of the  $\alpha$ ,  $\delta$  and  $\epsilon$  isoforms is inhibited via autophosphorylation of carboxy-terminal residues. CK1 is ubiquitous in tissues and subcellular distribution and phosphorylates a broad range of substrates including many neuronal proteins. CK1 $\epsilon$  and CK1 $\delta$  have been implicated in the regulation of the circadian clock via the control of nuclear localization and protein stability of their substrates. Consistently, mutations in the CK1 $\delta$  gene have been found to cause a familial sleep-advance cycle syndrome. Via the phosphorylation of tau, CK1 has been implicated in

neurodegenerative diseases. Consistently, expression levels of CK1 $\delta$  are increased in AD.

## PROTEIN SER/THR PHOSPHATASES

Protein dephosphorylation is catalyzed by phosphodrolases called protein phosphatases (PPs). These enzymes function antagonistically to reverse the action of kinases by specifically hydrolyzing amino acid phosphoesters (Figure 25-1). Phosphatases are generally divided into two main families based on substrate specificity: protein Ser/Thr phosphatases and protein Tyr phosphatases (PTPs) (Shi, 2009; Hunter, 1995). PTPs are discussed in Ch. 26. There exist also dual-specificity phosphatases (DUSPs) (Bermudez et al., 2010). In analogy to dual-specificity protein kinases, dual-specificity phosphatases can dephosphorylate Ser and Thr as well as Tyr residues within the same substrate. These DUSPs are structurally more closely related to PTPs.

Based on structural and functional aspects, protein Ser/Thr phosphatases can be further separated into three subfamilies: (1) phosphoprotein phosphatases (PPPs), (2) metal-dependent protein phosphatases (PPMs), and (3) aspartate-based phosphatases, as listed in Table 25-2. Members of the PPPs include PP1, PP2A, PP2B (also known as calcineurin) and PP4-7 (Shi, 2009). One particularity of the PPP family is that the catalytic subunit of most PPP members can associate with a great number of regulatory proteins and thereby control activity.

The PPM subfamily includes PP2C and SCOP/PHLPP (Shimizu et al., 2010). PPMs feature additional functional domains and conserved sequence motifs that may determine substrate specificity, and hence they do not depend on regulatory subunits. PPPs as well as PPMs require divalent metal ions, such as Mg<sup>2+</sup> or Mn<sup>2+</sup>, for activation. In contrast, aspartate-based phosphatases catalyze the reaction via an aspartate-dependent mechanism.

The number of protein Ser/Thr kinases vastly outnumbers the protein Ser/Thr phosphatases, of which about 30 different types are known to exist (Shi, 2009). This numerical underrepresentation may be compensated for by the presence of numerous independent regulators controlling protein Ser/Thr phosphatase. By means of regulatory protein–protein interaction with activator and inhibitor proteins, the phosphatases may achieve a level of functional diversity comparable to protein kinases, imparting upon phosphatases the ability to comprehensively antagonize kinase-dependent phosphorylation, a prerequisite for a functional equilibrium in cells. Although they were initially thought to serve as passive regulators functioning in homeostasis, protein phosphatases are now recognized for their roles in activating and amplifying neuronal signal transduction networks.

In the next section, prominent members of the protein Ser/Thr phosphatase family including PP1, PP2A, PP2B (calcineurin) and PP2C will be introduced (see also Table 25-2). Most of the protein Ser/Thr phosphatases are expressed in brain, but differ in their regional distribution, substrate specificity and regulation by cellular messengers. They play a critical role in the control of neuronal functioning by maintaining a

**TABLE 25-2** Major Protein Ser/Thr Phosphatase Families

Protein Phosphatase Family	Prominent Member	Cofactor Required for Activation/Inhibition
PPP	PP1	Inhibitor-1, inhibitor-2, DARPP-32, NIPP1,
	PP2A	Diverse regulatory (B) subunits, inhibitor1 PP2A, protein SET
	PP2B (calcineurin)	Calmodulin, immunophilins
	PP4	
	PP5	
	PP6	
	PP7	
PPM	PP2C	None
	SCOP/PHLPP	
DUSP	DUSP1 (MKP1)	
	laforin	
	PTEN	
	Cdc14	Inhibitor NET1

This table lists some of the major families of the protein Ser/Thr phosphatase. In addition to the indicated phosphatase families, there is the aspartate-based phosphatase family. The table indicates some of their most prominent members.

Cdc, cell division cycle; DARPP-32, dopamine and cAMP-regulated phosphoprotein of 32 kDa; DUSP, dual-specificity protein phosphatases; MKP, mitogen-activated protein kinase phosphatase; NIPP1, nuclear inhibitor of PP1; PP, protein phosphatase; PPM, metal-dependent protein phosphatase; PPP, phosphoprotein phosphatases; SCOP, suprachiasmatic nucleus circadian oscillatory protein.

critical balance between protein phosphorylation and dephosphorylation. In general, protein phosphatases are less studied than protein kinases, and the vast diversity of regulatory proteins controlling phosphatase activity make this a challenging research field. Much of the insight on phosphatases was derived by studies using pharmacological inhibitors that target the catalytic subunits of phosphatases. Using such inhibitors does not yield insight on the functional role of the regulatory proteins. Inhibitors commonly used are naturally occurring toxins that inhibit cellular protein phosphatase activity. Examples of such toxins are okadaic acid and microcystin, which are potent inhibitors of PP1 and PP2A.

### Protein phosphatase 1 (PP1)

Protein phosphatase 1 (PP1) exists in four subtypes that are derived from three genes. These enzymes are highly homologous and exhibit similar substrate specificities; therefore, they can be considered isoforms. However, the proteins exhibit very distinct tissue expression patterns in the brain. PP1 dephosphorylates a wide array of substrate proteins. Its catalytic subunit can form complexes with over 50 regulatory/targeting proteins in a mutually exclusive manner (Ceulemans & Bollen, 2004; Cohen, 2002). Interactions of these proteins with PP1 affect its activity, substrate specificity and

subcellular localization. The so-called phosphatase inhibitor proteins include: inhibitor-1, inhibitor-2, dopamine- and cAMP-regulated phosphoprotein of 32kDa (DARPP-32) and nuclear inhibitor of PP1 (NIPP1) (see Table 25-2). Importantly, many of these PP1 inhibitors are themselves regulated via phosphorylation. In some cases, this mechanism allows activated kinase pathways to potentiate their signal capacity by shutting down phosphatases. PKA phosphorylation of inhibitor-1 and DARPP-32 turns them into phosphatase inhibitors (Bibb et al., 2001; Bibb et al., 1999). In other instances, phosphorylation of the PP1 inhibitor proteins induces activation of the phosphatase. This mechanism can be used to shunt activity of competing kinase pathways. For example, phosphorylation of NIPP1 by PKA or CK1 inactivates their phosphatase inhibitor function. Inhibitor-2 appears to be functionally different from inhibitor-1, DARPP-32 and NIPP1 that regulate signal transduction. There is evidence that inhibitor-2 associates with PP1 as the phosphatase is newly synthesized and contributes to the proper folding of the enzyme (Wera & Hemmings, 1995). Inhibitor-2 can thus be considered a chaperone protein. The inactive PP1—inhibitor-2 complex is activated upon phosphorylation of inhibitor 2 by GSK3. Inhibitors-1 and -2 and NIPP1 are ubiquitously expressed in mammalian tissues. In contrast, DARPP-32 displays a restricted distribution with enrichment in discrete populations of neurons, most prominently in those with dopamine receptors. Within the hippocampus circuitry, inhibitor-1 is primarily expressed in granular cells of the dentate gyrus. Thus some cell types appear to express unique species of phosphatase inhibitor proteins.

The subcellular localization of PP1 appears to be determined by a series of other proteins, which are now considered to be integral subunits of the enzyme (Ceulemans & Bollen, 2004; Cohen, 2002). For example, the M subunit targets PP1 to myofibrils in smooth muscle. In addition, PP1 isoforms show region-specific expression in the brain. Certain forms, PP1 $\alpha$  and PP1 $\gamma 1$ , are highly enriched in dendritic spines of certain neurons, which presumably reflects the important role played by the enzyme in synaptic transmission. The subunit responsible for this dendritic localization may be a protein called spinophilin (Allen et al., 1997). The cell nucleus also contains high levels of PP1, where it is believed to play an important role in the dephosphorylation of transcription factors and hence transcriptional regulation. One PP1 regulatory protein responsible for nuclear targeting is called PNUTS (phosphatase 1 nuclear targeting subunit) (Allen et al., 1998).

PP1 plays an important role in neuronal functions, as it dephosphorylates many neuronal phosphoproteins and thus antagonizes the actions of many different protein Ser/Thr kinases. For example, protein dephosphorylation catalyzed by PP1 has been demonstrated to be a key aspect of the signaling pathways underlying long-lasting forms of synaptic plasticity. PP1 activity contributes to the induction of long-term depression (LTD), whereas inhibition of PP1 has been shown to promote LTP (Mansuy & Shenolikar, 2006).

### **Protein phosphatase 2A (PP2A)**

Protein phosphatase 2A (PP2A) accounts for as much as 1% of total cellular protein and for the major portion of Ser/Thr phosphatase activity in most cell types (Virshup,

2000). PP2A is a multi-subunit enzyme composed of three subunits termed catalytic (C), scaffolding (A) and regulatory (B) subunits. There are two different C subunits, C $\alpha$  and C $\beta$ , which are structurally and functionally similar. There exist also two A subunits, A $\alpha$  and A $\beta$ , which provide structural support to the C subunit. In contrast, numerous forms of the B subunit occur, which exhibit differential tissue expression. The B subunit in part regulates the functional specificity of the PP2A holoenzyme. Distinct classes of B subunits, termed B, B' and B'', can bind to the AC complex to form a large array of heterotrimeric holoenzyme complexes. The interactions of the various regulatory B subunits with the AC complex are competitive, and these B subunits can differentially exchange, so that PP2A specificity and location may be controlled through their relative abundance and expression. The interaction of the PP2A holoenzyme with regulatory/targeting proteins further controls its functionality, substrate specificity and subcellular localization. There is substantial evidence that PP2A activity is regulated by extracellular signals through phosphorylation and other posttranslational modifications of PP2A holoenzymes. For example, the B56 $\delta$  regulatory subunit imparts cAMP-dependent activation upon PP2A, while the B''/72 subunit confers Ca $^{2+}$ -dependent activation upon PP2A (Ahn et al., 2007a; Ahn et al., 2007b). Moreover, some regulatory B subunits are expressed in specific brain regions, further implicating PP2A regulatory specificity as an important aspect of CNS function. PP2A has been shown to form complexes via AKAP scaffolding with PKA via the B subunit PR130 (Skroblin et al., 2010). Furthermore, PP2A forms complexes with CDK5 and GSK3, suggesting a functional interaction that may be relevant to the phosphorylation state of *tau* and its ability to form neurofibrillary tangles in AD (Plattner et al., 2006).

### **Protein phosphatase 2B (PP2B)**

Protein phosphatase 2B (PP2B), also called calcineurin, is a Ca $^{2+}$ - and calmodulin-activated phosphatase composed of two major subunits: the A subunit, which contains the catalytic activity and is highly homologous to PP1, and the B subunit, which contains two calmodulin-like domains responsible for binding Ca $^{2+}$ /calmodulin (Shi, 2009). The A subunit exists in three gene copies; the B subunit is encoded by two genes. PP2B is highly expressed in brain in a region-specific manner, and enriched particularly in striatum. The activity of PP2B can be regulated by a series of proteins termed immunophilins (Steiner et al., 1996). These proteins, the prototypical example of which is cyclophilin, were first identified as the targets of potent immunosuppressive drugs such as cyclosporin and FK-506. Although first described in the immune system, immunophilins are expressed in the brain in a region-specific manner and have been implicated in the regulation of synaptic transmission.

PP2B, like PP1, exhibits broad substrate specificity. Moreover, based on its regulation by the second messenger Ca $^{2+}$ , PP2B is believed to mediate many physiological responses to neurotransmitters and nerve impulses. The functionality, substrate specificity and subcellular localization of PP2B are additionally controlled by regulatory/targeting proteins. Like PP2A, PP2B binds with high affinity to AKAPs, which localize the enzyme to subcellular sites also enriched in

PKA, such as postsynaptic densities. As will be shown below, this is presumably one molecular basis for the complex interactions found between PP2B and PKA as well as other regulatory proteins. PP2B is involved in the regulation of many molecular mechanisms underlying synaptic plasticity and memory formation. It controls neurotransmitter release, postsynaptic receptors and voltage-gated channels and transcriptional regulation.

### **Protein phosphatase 2C (PP2C)**

Protein phosphatase 2C (PP2C) is a member of the metal-dependent protein phosphatases (PPMs). PP2C is a monomeric enzyme with at least four isoforms in human. Few targeting proteins have yet been identified and, to date, there are no PP2C inhibitors available. As a result, it has been difficult to examine the role of this phosphatase. PP2C is expressed in the brain, and likely neuronal substrates include the autophosphorylation site of CaMKII, the CK1 site on DARPP-32, and the PKA site on tyrosine hydroxylase (see below). PP2C has been implicated in the regulation of the stress-activated MAPK signaling cascade. Further neuronal functions of PP2C include dephosphorylation of metabotropic glutamate receptors (mGluR3) and interaction with the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. Clearly more information is needed on the function and regulation of PP2C and its role in the regulation of receptors and channels in the CNS.

### **Dual-specificity phosphatases (DUSPs)**

Dual-specificity phosphatases (DUSPs) are a large, heterogeneous group of protein phosphatases that can dephosphorylate Ser, Thr as well as Tyr residues (Bermudez et al., 2010; Patterson et al., 2009). However, certain DUSPs can display preference for one type of residue. In terms of their sequence homologies these enzymes are more closely related to PTP than to protein Ser/Thr phosphatases. The active site structures are more variable amongst the DUSPs members. Based on the presence of specific domains and sequence similarity, DUSPs can be divided into six subgroups

The DUSP subgroup of the MAPK phosphatases (MKPs) contains 10 isoforms. MKPs are unique dual-specificity protein phosphatases that can antagonize the activation of the various MAPK cascades (Bermudez et al., 2010; Patterson et al., 2009). MKPs can specifically dephosphorylate MAPKs at both phospho-Thr and phospho-Tyr residues within the tripeptide activation motif. Within the amino-terminal region, the MKPs contain kinase interacting motifs, which confer distinct MAPK substrate specificity. Consequently, individual MKPs display preference for one or more of the MAPKs. For example, DUSP5 is ERK-specific, whereas DUSP-1, -8 and -10 prefer JNKs and p38 MAPKs to ERKs. Most of the MKPs display nuclear localization. DUSP-6, -7 and -16 show cytosolic distribution. MKPs are subjected to intricate regulation at multiple levels, which directly reflects the activation state of the MAPK cascade. For example, many MKPs are products of inducible or early response genes with low expression levels at basal states. Upon stimulation of the MAPK pathway, their expression increases. Moreover, MKPs can be phosphorylated directly by MAPKs, which leads to increased stabilization and prolonged MKP

activation. As major regulators of MAPK signaling, MKPs are thought to play an important role in neuronal processes, but so far there is only limited knowledge on this subject.

The atypical DUSPs form another subgroup including at least 16 different protein phosphatases (Patterson et al., 2009). Their most prominent member is laforin. Missense mutations in the laforin gene cause Lafora disease, also called progressive myoclonus epilepsy (see below and Ch. 43). Lafora disease is a genetic disorder characterized by intracellular polyglucosan inclusions termed Lafora bodies. Even though laforin can dephosphorylate both phospho-Tyr and phospho-Ser/phospho-Thr residues, there is little known about physiological protein substrates.

Other DUSP subgroups include the Cdc14 phosphatases, which dephosphorylate CDKs during cell cycle progression; and PTEN-like phosphatase (phosphatase and tensin homologue deleted on chromosome 10); and myotubularin phosphatases.

## **COMMON STRATEGIES USED FOR THE EVALUATION OF NEURONAL FUNCTIONS OF PROTEIN KINASES AND PHOSPHATASES**

Cellular signaling pathways have been analyzed using diverse independent approaches, including pharmacological, biochemical, cellular and genetic techniques. Protein kinases and phosphatases have long served as targets for drug discovery, and as a result there are many inhibitory compounds available for both therapy and basic investigation. Thus, much of the insight on the physiological roles of protein kinases and phosphatases has been derived from pharmacological studies using small inhibitor molecules. An important advantage of pharmacological inhibitors is their relatively simple applicability in various experimental systems such as *in vitro*, cell culture, brain slices and *in vivo*. Such pharmacological agents have been particularly important in studying the roles of kinases and phosphatases in neurotransmission and synaptic plasticity.

There are drawbacks of pharmacological studies, including the lack of specificity of drugs, the absence of pharmacological reagents for many molecules, and the difficulty in spatial and temporal pharmacological manipulations. Virtually every pharmacological compound can interact with, and thus affect, a number of molecules, resulting in unwanted side effects that can confound the interpretation of results. Many commonly used inhibitors are called ATP-competitive inhibitors because they interact with the ATP binding site of a specific kinase, thereby blocking its catalytic activity. Cross-reactivity of this type of drug is a major concern, because ATP binding domains exhibit a high degree of conservation in their primary and secondary structures. This type of cross-reactivity may be prevented by the use of compounds directed against motifs and domains that exhibit greater sequence variability within kinases or phosphatases, such as substrate-binding sites and regulatory domains or subunits.

An important step in this direction may be the development of small interfering peptides and peptidomimetic synthetic compounds (e.g., peptoids). Such compounds have

already been identified for some targets and show promise of highly specific or regulatory actions. Spatial resolution is another common problem, as systemic administration of a drug will affect every tissue and organ containing drug-sensitive targets. In experimental conditions, local infusion of drugs is often employed to minimize this issue, but this has complications of its own. Finally, temporal resolution, which is a complex interaction between bioavailability, drug clearance and half-life of the drug, may raise complications.

Another important approach is the manipulation of protein kinase and phosphatase expression via recombinant DNA technology. Vectors such as plasmid DNA or modified eukaryotic viral genomes may be used to mediate overexpression of wild-type or mutant forms (e.g., dominant negative, constitutively active, phosphorylation site-mutated, etc.). More recently microRNA technology (siRNA) has been employed to knockdown kinase or phosphatase expression. This approach has moved from use in continuous to primary cultures and is now being employed widely *in vivo* in animal models. A caveat of overexpressing proteins, such as kinases and phosphatases, is that regulatory mechanisms and molecular cross-talk may be influenced by the abnormal levels of expression and thus lead to erroneous conclusions.

Groundbreaking advances in the development of genetically modified animal models enabled the manipulation of kinase and phosphatase activities *in vivo* (Ch. 53). It is now possible to generate knockout (KO), knock-in (KI), targeted point mutation transgenic mouse models to study the role of a specific enzyme in CNS function. These approaches opened up new avenues and helped greatly to elucidate the neuronal processes underlying learning and memory from the molecular and cellular to the systems level.

However as with any technique there are some shortfalls. Mouse models created using gene-targeting techniques, such as KO and KI mice, lack spatio-temporal control of the genetic modification. The mutation is constantly present in every cell of the animal throughout its entire life, which can have severe consequences and may bias or even preclude a functional analysis. Common confounding factors observed in such mutant mice include adaptive and compensatory mechanisms, developmental defects, cell- and tissue-specific side effects and embryonic lethality. To overcome these undesired limitations, more advanced genetic approaches have been developed, which allow controlled gene expression or ablation of gene function in a spatio-temporal fashion. A commonly employed approach is the Cre/loxP system, which allows generation of mouse lines with tissue- and cell-type specific inactivation of genes (i.e. conditional KO mice, cKO) or with temporal control of gene activation. A good example is the CDK5 conditional KO mouse model, which helped to overcome the caveats of pharmacological nonspecificity and the congenital defects of constitutive Cdk5 KO and thus was instrumental in establishing the role of CDK5 in synaptic plasticity and memory (Hawasli et al., 2007).

Another widely applied approach is the generation of transgenic mouse models via random, non-homologous genomic integration, in which tissue specificity and to some degree temporally restricted transgene expression can be controlled. Learning and memory studies commonly use mutant mouse lines in which the transgene is driven by

neuron-specific promoters, including neuron-specific enolase (NSE), prion protein (PrP) and CaMKII $\alpha$  promoter. Confounding factors inherent to the transgenic approach include variable transgene copy numbers and random integration sites. Therefore, transgene expression levels cannot be controlled and expression of endogenous genes may be affected, due to the insertion of the transgene into essential gene loci. Nevertheless, reliable results can be obtained by generating and analyzing multiple transgenic mouse lines with the same transgene. This approach has also been modified to an inducible bi-transgenic system. An example of this is the inducible overexpression of the truncated Cdk5 activator p25 (the product of p35 cleavage by calpain). In this system the promoter controls the expression of the tetracycline transactivator, which is inhibited by providing the antibiotic drug tetracycline in drinking water. Removal of dietary doxycycline induces the overexpression of p25, which then imparts aberrant activity on Cdk5. This approach has been adapted widely in other variations.

As demonstrated in this section, every approach has serious limitations and therefore it is essential that the physiological functions of each kinase and phosphates are established using multiple, independent techniques.

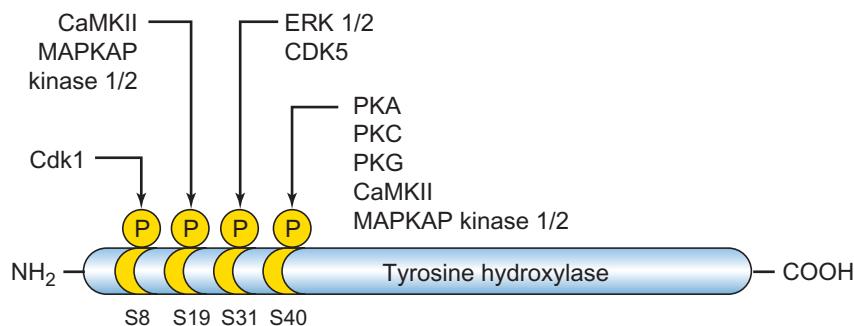
## NEURONAL PHOSPHOPROTEINS

Over one-third of all eukaryotic proteins are phosphorylated. However the fraction of neuronal proteins that are phosphorylated might be substantially higher. In fact, most of the proteins that have been linked to important neuronal processes are regulated by phosphorylation. Hence, virtually every neuronal process fundamentally involves phosphoregulation. Via the regulation of essential presynaptic, postsynaptic and extrasynaptic functions, protein phosphorylation ultimately impinges on synaptic plasticity and higher cognitive function.

In this section, first, general principles governing protein phosphorylation and their impact on phosphoprotein regulation and function are introduced. Thereafter, the importance of Ser/Thr phosphorylation in neuronal functions is illustrated by some select examples of phosphoregulation of integral presynaptic, postsynaptic and extrasynaptic mechanisms.

### Phosphorylation can influence protein function in various ways

As described earlier, protein phosphorylation results in a functional change of the target protein by altering enzyme activity, subcellular location, or association with other proteins. Phosphorylation-induced changes in charge and confirmation can alter the accessibility of enzymatic sites, thereby switching activity on or off, changing the affinity for substrates, or establishing or disrupting protein–protein interactions. Phosphorylation can also change the affinity of proteins for other molecules. For example, phosphorylation alters the affinity of numerous enzymes for their cofactors and end-product inhibitors, and phosphorylation of some transcription factors alters their DNA-binding properties. In neurons, of particular importance is the role of phosphorylation in



**FIGURE 25-5** Regulatory phosphorylation sites on tyrosine hydroxylase. Schematic diagram of tyrosine hydroxylase (TH) indicating the sites of phosphorylation (yellow) and listing the protein kinases that mediate phosphorylation on these sites. Phosphorylation of TH increases the catalytic activity of the enzyme and its affinity for its biopterin cofactor and by decreasing the affinity of the enzyme for its end-product inhibitors.

membrane-spanning proteins, such as ion channels and receptors, which result in dramatic conformational changes of their intracellular, transmembrane or extracellular domains. Such mechanisms have been implicated, for example, in ion channel opening/closing and transmembranal signal transduction of cell adhesion molecules.

### Proteins are often subject to complex phosphoregulation

Phosphoproteins differ considerably in the number and types of amino acid residues phosphorylated. In fact, a large number of proteins can be phosphorylated on more than one amino acid residue by more than one type of protein kinase (e.g., tyrosine hydroxylase; Figure 25-5). Different phosphorylation sites may induce distinct, similar, or opposing functional effects in a protein. A further degree of complexity constitutes the functional interaction between different phosphorylation sites and simultaneous phosphorylation of multiple residues within one substrate. An example of functional interaction is when phosphorylation of one residue influences the ability of other residues to undergo phosphorylation. In the case of simultaneous phosphorylation, the functional changes of the protein can be influenced by each of the phosphorylation sites in an independent, synergistic or competitive manner. An example of synergism is when simultaneous phosphorylation of multiple residues is required to produce a change in the charge of the protein sufficient to induce a functional effect. This appears to be the case for the activation of MAPKs, which requires the phosphorylation of neighboring Thr and Tyr residues (Johnson & Lapadat, 2002; Kim & Choi, 2010).

The complexity, which is inherent to phosphoregulation, constitutes one of the biggest challenges in biological research, including neurosciences. The presence of multiple phosphorylation sites that are highly conserved in numerous proteins, paired with the number of different kinases and phosphatases, result in a virtually indefinite amount of regulatory combinations. It seems obvious that proper cell functioning relies on the precise organization of all of the molecular mechanisms. However, it remains unsolved by which means the necessary degree of regulation can be achieved and maintained.

### Cellular signals converge at the level of protein phosphorylation pathways

For didactic purposes, individual intracellular signaling pathways, such as cAMP,  $\text{Ca}^{2+}$ /CaM and MAPK pathways, are often drawn as distinct linear biochemical cascades that operate in parallel to control cell function (e.g., Figure 25-4). In real biological systems, the various individual pathways interact and influence each other in virtually every conceivable way, often forming complex signaling networks. Different types of pathway interactions are presented here using examples of modulatory phosphoregulation between the cAMP-dependent and  $\text{Ca}^{2+}$ -dependent pathways.

One major mechanism, by which different pathways interact, is regulatory phosphorylation of either upstream or downstream effectors of another kinase. For example, a first messenger that activates the cAMP pathway can inhibit  $\text{Ca}^{2+}$  signaling at many molecular levels via PKA. First,  $\text{Ca}^{2+}$  channels and the IP<sub>3</sub>-receptor can be phosphorylated by PKA to modulate intracellular  $\text{Ca}^{2+}$  concentration (Ch. 24). Second, PKA phosphorylates phospholipase C (PLC) and thereby modulates the generation of IP<sub>3</sub>, as well as DAG, which in turn affects intracellular  $\text{Ca}^{2+}$  concentration and PKC activity, respectively (Ch. 23). Third, PKA phosphorylates and activates inhibitors of PP1, which dephosphorylates numerous substrates for  $\text{Ca}^{2+}$ -dependent protein kinases.

Just as kinases can phosphorylate effectors of a kinase, they can directly phosphorylate the kinase itself. For example inhibitory phosphorylation of CaMKK by PKA at Ser74, Thr108 and Ser458 provides cross-talk between the cAMP and  $\text{Ca}^{2+}$  pathways (Wayman et al., 2008). Thus, activation of PKA leads to suppression of the CaMKK/CaMKI cascade. Furthermore, multiple kinases may regulate the same substrate (either an effector or a downstream kinase). As mentioned above, phosphorylation of the same substrate protein by more than one protein kinase can integrate multiple intracellular pathways to achieve coordinated regulation of cell function. A classical example is the transcription factor, CREB, which is activated via phosphorylation of Ser133, by PKA and CaMKs as well as RSK, which is stimulated by the MAPK cascade (Chs. 12, 27). Lastly, the physical association of target

proteins can be regulated by kinases from distinct pathways either directly or in common complexes. For example, PKA and PP2B, a  $\text{Ca}^{2+}$ /CaM-activated enzyme, are localized in the same complex via AKAPs.

Phosphorylation-independent mechanisms of modulatory regulation, by which individual signaling cascades interact, are also known.  $\text{Ca}^{2+}$ -based signaling can exert potent effects on the cAMP pathways by modulating activity of distinct isoforms of adenylyl cyclase and phosphodiesterases (Ch. 19). These isoforms contain  $\text{Ca}^{2+}$ /CaM-binding domains, and thus  $\text{Ca}^{2+}$ -influx can directly regulate their production of cAMP.

Molecular studies so far have revealed that most physiological responses should be viewed as the complex product of coordinated actions of multiple extra- and intracellular messengers involving numerous signaling proteins and molecular pathways, rather than being generated through a single signaling pathway. In addition, these studies showed that regulation of intracellular messenger pathways does not depend on the actions of a single extracellular signal but, rather, on the integration of multiple signals that impinge on cells at any given point in time. This extraordinary complexity underscores the difficulty in determining the precise molecular basis of a given physiological response. Nevertheless, the role of protein phosphorylation as a central regulatory mechanism can, in fact, be exploited as an experimental framework to study the different molecular layers that underlie and regulate cell function.

### PROTEIN PHOSPHORYLATION IS A FUNDAMENTAL MECHANISM UNDERLYING SYNAPTIC PLASTICITY AND MEMORY FUNCTIONS

Over the last three decades the understanding of the molecular and cellular basis of synaptic plasticity, learning and memory has progressed dramatically. A large body of evidence implicates hundreds of molecules in these phenomena and demonstrates the fundamental importance of protein phosphorylation as a basic mechanism underlying cognition. Integral neuronal functions such as synaptic transmitter release and ion channel modulation are mediated by highly specialized molecular complexes. The precise organization and proper functioning of these complexes requires an extensive degree of regulation, which is established via protein phosphorylation. Consistently, numerous protein kinases and phosphatases contribute crucial functions in synaptic plasticity and memory processes.

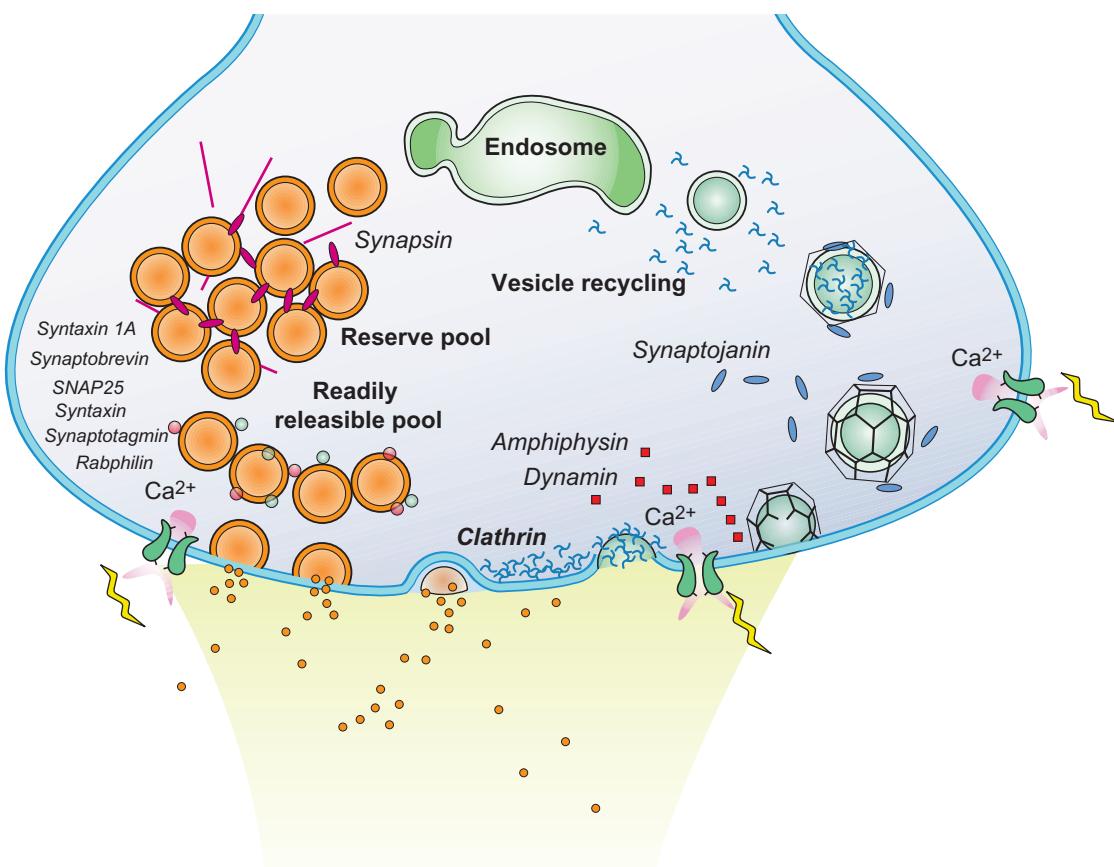
Synaptic plasticity is generally used to describe activity-dependent changes in synaptic transmission that are analyzed using electrophysiological techniques (Bliss & Collingridge, 1993). Such changes may persist from milliseconds to hours or days and based on their temporal characteristics are classified as short or long term. Short-term forms of synaptic plasticity can occur normally in the first 15 minutes and decay within one hour after electrophysiological stimulation. Long-term varieties include LTP and long-term depression (LTD) (Ch. 56). LTP is a long-lasting increase in synaptic strength between two connected neurons that can be divided

**TABLE 25-3** Important Neuronal Phosphoproteins Regulating Synaptic Plasticity and Memory Functions

PRESYNAPTIC	
Phosphoprotein	Cellular Function Affected by Phosphorylation
TH	Neurotransmitter synthesis
Synapsin	Vesicle pool dynamics
Syntaxin	Docking, priming, fusion of vesicle cycles
SNAP25	
Synaptobrevin	
Munc-18	
Dynamin	Endocytosis
Amphiphysin	
POSTSYNAPTIC	
Phosphoprotein	Cellular Function Affected by Phosphorylation
AMPA receptor subunits (e.g., GluR1)	Ion flux
NMDA receptor subunits (e.g., NR2B)	Ion flux
CaMKII	Intracellular signaling
DARPP-32	
PSD-95	Postsynaptic architecture
Spinophilin	
WAVE1	
EXTRASYNAPTIC	
Phosphoprotein	Cellular Function Affected by Phosphorylation
CREB	Transcriptional regulation
CBP	
Histones	Chromatin structure, epigenetics
eIF	Translational regulation
Kinesin	Axonal transport

This table lists some examples of pre-, post- and extrasynaptic phosphoproteins that have been implicated in synaptic plasticity and memory functions. Many other neuronal phosphoproteins have been omitted that are also critically involved in plasticity and memory processes. CaMKII,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II; CBP, CREB binding protein; CREB, cAMP responsive element binding protein; DARPP-32, dopamine and cAMP-regulated phosphoprotein of 32 kDa; eIF, eukaryotic initiation factor; Munc-18, mammalian uncoordinated-18; PSD-95, postsynaptic density protein 95 kDa; SNAP25, synaptosomal-associated protein 25; TH, tyrosine hydroxylase; WAVE1, Wiskott-Aldrich syndrome protein (WASP)-family verprolin homologous protein 1.

into several different phases: (1) a transient phase mainly maintained by protein phosphorylation; (2) another longer phase relying on the synthesis of new proteins from existing



**FIGURE 25-6** The synaptic vesicle cycle. Neurotransmitter-filled vesicles stored in the reserve pool are trafficked to the readily releasable pool, where they are docked, primed and fused with the plasma membrane at the synaptic cleft. Also depicted is clathrin-mediated endocytosis, which is followed by uncoating and recycling via early endosomal fusion and budding of vesicles. Via these actions, the vesicles are returned to the reserve pool. Some of the phosphoproteins that regulate these steps are indicated. For a more detailed description of this process and the phosphoproteins involved the reader is directed to the excellent text by Cowen et al. (2001). See also Figs. 7–9, 12–1.

mRNAs (local protein synthesis); and (3) another even longer-lasting phase dependent upon induction of gene transcription and subsequent *de novo* protein synthesis. LTP is seen as a cellular correlate of memory and thus is extensively used as an experimental model to study molecular and cellular processes underlying synaptic plasticity, learning and memory. In fact, many of the molecular mechanisms integral to synaptic plasticity are also engaged during learning processes and memory formation.

Consistently, recent advances suggest that learning and memory manifested at a behavioral level are established through a multitude of different types of phosphorylation events. These alter the functioning of individual synapses, neurons, and the neural circuits in which they operate. Short-term memory, in analogy to short-term forms of plasticity, may involve the phosphorylation of presynaptic or postsynaptic proteins in response to synaptic activity, which would result in transient facilitation or inhibition of synaptic transmission. Long-term memory, in analogy to LTP, may involve the phosphorylation of proteins that play a part in the regulation of *de novo* protein synthesis. In this case, the

phosphoregulation would induce more permanent changes impinging on the composition of synaptic protein complexes and synaptic structure, thereby lastingly affecting neurotransmission.

To illustrate the importance of protein phosphorylation in neuronal functions underlying synaptic plasticity, learning and memory, some select examples of phosphoregulation of integral presynaptic, postsynaptic and extrasynaptic mechanisms are discussed here.

### Presynaptic mechanisms regulated by protein phosphorylation

As depicted in Figure 25-6, neurotransmitter-filled synaptic vesicles congregate in the presynaptic nerve terminal. The arrival of an action potential at the presynaptic terminal triggers the opening of voltage-gated  $\text{Ca}^{2+}$  channels. The resulting  $\text{Ca}^{2+}$  influx into the presynaptic terminal induces molecular mechanisms that lead to synaptic vesicle exocytosis, fusion of vesicles with the plasma membrane and subsequent neurotransmitter release (Jahn et al., 2003). Subsequently, the

vesicles are recovered by endocytosis and are recycled for subsequent release events. Virtually all of these presynaptic processes that are integral for synaptic plasticity and memory functions depend greatly on phosphoregulation (Table 25-3). The capacity of a neuron for synaptic transmission is partly controlled via the phosphorylation of enzymes that synthesize neurotransmitters. One example is tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of the catecholamine neurotransmitters dopamine, norepinephrine and epinephrine (Ch. 14). Catecholaminergic cells adjust their rate of

neurotransmitter biosynthesis to a host of external stimuli and to changing physiological needs via the phosphorylation of TH by cAMP-dependent and  $\text{Ca}^{2+}$ -dependent protein kinases and by MAPK cascades (Fitzpatrick, 1999; Kumer & Vrana, 1996). Phosphorylation of TH increases the catalytic activity of the enzyme by increasing its  $V_{max}$  and its affinity for its biopterin cofactor and by decreasing the affinity of the enzyme for its end-product inhibitors. At least nine different protein kinases can phosphorylate TH (Fig. 25-5). This astounding degree of regulation of TH provides a striking example

## COGNITIVE ENHANCEMENT STRATEGIES

*Florian Plattner, James A. Bibb*

A decline in memory and cognitive function is a natural aspect of aging. In addition, cognitive deficits are comorbid with many mental disorders and neuronal diseases in humans. Mental retardation, autism, attention deficit disorder and schizophrenia, as well as neurodegenerative diseases including Alzheimer's and Parkinson's diseases all feature prominent deficits in cognitive abilities.

Cognitive enhancement is viewed as a strategy to treat these diseases, or to slow age-related decline of brain function. The development of cognition-enhancing drugs is, therefore, the focus of a considerable research effort. Recent advances in the understanding of the molecular mechanisms underlying learning and memory are providing a significant impetus for the research into cognitive enhancers (Bibb et al., 2010). Most approaches target neuronal processes that have been critically implicated in cognitive functions. Various neuronal processes, including neurotransmission, intracellular signaling, synaptic remodeling, protein transcription and translation, are all essential for cognition. In fact, manipulation of several molecular mechanisms governing these processes has been found to enhance memory functions.

As outlined in this chapter, virtually all of these neuronal processes are fundamentally regulated via protein phosphorylation. Modulators of the phosphorylation machinery therefore represent a valid drug target for cognitive enhancement. In particular, protein kinases and phosphatases involved in the signal transduction of the second messengers  $\text{Ca}^{2+}$  and cAMP appear to be particularly promising targets. For example, postsynaptic increases in  $\text{Ca}^{2+}$  levels induced by NMDA receptor stimulation, and subsequent activation of  $\text{Ca}^{2+}$ -dependent signal pathways have been unequivocally identified as critical for learning and memory (Lee & Silva, 2009). Consistently, molecular modifications that boost expression of the NMDA receptor subunit, NR2B, have been associated with enhanced mnemonic functions in several genetically modified mouse models (Tang et al., 1999). Attenuation of NR2B degradation through knockout of CDK5 enhances synaptic plasticity and cognition in mice (Hawasli et al., 2007).

Comparable to  $\text{Ca}^{2+}$  signaling, production of cAMP and subsequent induction of downstream signaling pathways are integral mechanisms underlying cognition. It is well established that increases in cAMP level are beneficial for memory functions.

Indeed, a classical example of a pharmacological cognitive enhancer, rilopram, exerts its effect by increasing cAMP levels via the inhibition of phosphodiesterase 4 (PDE4), an enzyme that metabolizes cAMP (see Ch. 21). Similarly, overexpression of adenylyl cyclase, the enzyme synthesizing cAMP, results in learning and memory improvement in mice (Wang et al., 2004). Memory enhancement achieved via mechanisms that increase cAMP levels always depend upon the involvement of cAMP-dependent protein kinase (PKA). This central role of PKA in cognitive enhancement is underscored by evidence that modulators of PKA activity themselves have been linked to memory improvements.

$\text{Ca}^{2+}$ - and cAMP-induced activation of protein kinases is counterbalanced via protein phosphatases, and reduction in phosphatase activity has been associated with learning and memory enhancement. For example, inhibition of PP1 via the expression of inhibitor-1 improved memory formation (Genoux et al., 2002). The memory was also strengthened when the inhibition of PP1 was induced after learning, indicating an endogenous role of PP1 in counteracting memory formation. Comparably, decreasing the function of the  $\text{Ca}^{2+}$ -dependent phosphatase PP2B (also called calcineurin) facilitates synaptic plasticity and learning, whereas elevating its activity leads to disruption of these processes (Malleret et al., 2001).

The processes downstream of the  $\text{Ca}^{2+}$  and cAMP signaling cascades are still not well understood. One perspective is that ultimately the protein kinases and phosphatases stimulated by  $\text{Ca}^{2+}$  and cAMP converge onto transcription factors. In this way, they regulate expression of rate-limiting proteins needed to stabilize activity-dependent synaptic changes and thus memory. Consistently, transcription factors, including cyclic-AMP response element binding protein (CREB), are found to be integral for higher mental functions. Increases in levels of CREB activity consistently result in memory enhancements as seen in experiments with CREB overexpression (Matynia et al., 2002). Moreover, learning and memory improvements observed in mice overexpressing CaMKIV, a  $\text{Ca}^{2+}$ -dependent protein kinase, could be associated with increases in CREB phosphorylation and thus CREB activation (Wu et al., 2008; Fukushima et al., 2008).

Even though it may sound utopian, the use of memory-boosting substances to overcome cognitive deficits is in itself not a novel

concept. Psychostimulants, such as caffeine and nicotine, have been used to alter mental states since ancient times. However, only now it is known that they affect cognitive performance via influencing, directly or indirectly, cAMP signaling. Other cognitive drugs, such as methylphenidate and amphetamines are commonly prescribed to treat attention disorders. These compounds modulate the responses of several key neurotransmitters in the brain, thereby affecting cAMP signaling. Nevertheless, most cognitive deficits cannot be addressed by such stimulants and more effective and refined therapeutic strategies are needed. To identify suitable targets for the development of cognition-enhancing drugs, further progress in our understanding of the molecular basis of cognition will be paramount.

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of how multiple intracellular messengers and protein kinases converge functionally through the phosphorylation of a single substrate protein.

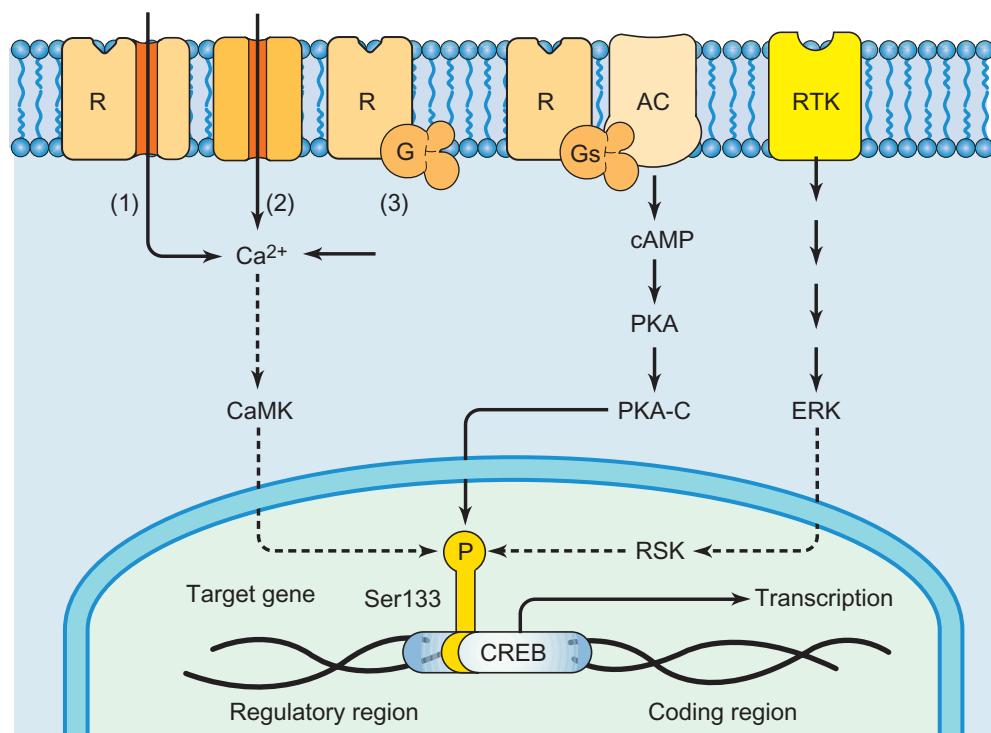
Another regulatory mechanism controlling neurotransmitter release involves the phosphorylation of ion channels (e.g., voltage-gated  $\text{Ca}^{2+}$  channels), which modulates their ability to open or close in response to the propagation of an action potential. A large number of proteins are involved in neurotransmitter release through their role in vesicle cycle dynamics (Jahn et al., 2003) (see Fig. 25-6). Most of these proteins are regulated via phosphorylation by various types of protein kinases (Table 25-3). Phosphorylation of synaptic vesicle-associated proteins, including synapsin and synaptobrevin, regulates vesicle cycling and neurotransmitter release in response to subsequent stimuli (Leenders & Sheng, 2005). Under basal conditions, synapsins retain vesicles in the reserve pool by anchoring them to the cytoskeleton. During synaptic activity, synapsins are phosphorylated by CaMKII, which releases the vesicles, allowing them to move to a readily releasable pool poised for membrane fusion and neurotransmitter release. An integral core of proteins, the so-called SNARE proteins, including synaptobrevin (VAMP2), syntaxin1, and SNAP-25 (25 kDa synaptosomal associated protein), primarily mediate the docking, priming and fusion of synaptic vesicles to the presynaptic plasma membrane (Leenders & Sheng, 2005). The SNARE proteins serve as substrates for various kinases such as CaMKII (syntaxins,

synaptobrevin, SNAP25), PKA (SNAP25), PKC (SNAP25) and CK2 (syntaxin 1A, synaptobrevin). Phosphorylation of the SNARE proteins can affect their ability to interact with their partners and thus modulate exocytosis.

In order to maintain sustained responsiveness to repetitive firing and the structural integrity of the synapse, neurotransmitter release, i.e., exocytosis, must be tightly coupled to vesicle recovery, i.e., endocytosis. Synaptic vesicle endocytosis is mediated by the structurally unrelated dephosphoin family of phosphoproteins, including amphiphysin, dynamin, synaptopjanin and AP180 (Clayton et al., 2007; Clayton et al., 2010). These proteins are involved in clathrin-mediated endocytosis and subsequent uncoating prior to vesicle fusion with synaptic endosomes. All of these proteins are coordinately dephosphorylated by PP2B. Many of the dephosphins serve as substrates for kinases, including CDK5, GSK3 and PKC. The rephosphorylation of the various dephosphins charges the system for dephosphorylation-dependent synaptic activity and thereby prepares the system for subsequent cycles of endocytosis.

## Postsynaptic mechanisms regulated by protein phosphorylation

Postsynaptic processes relevant for synaptic plasticity and memory functions depend greatly on phosphoregulation (Table 25-3). Much of our current understanding of the



**FIGURE 25-7** Signaling pathways regulating CREB phosphorylation. Signals evoked by different types of first messengers are transduced by various specific pathways that converge on cAMP response element-binding protein (CREB). Some first messengers regulate phosphorylation of CREB at Ser133 via the increase of cAMP concentrations by activation of G-protein-coupled receptors (*R*), G<sub>s</sub>, adenyl cyclase (AC) and cAMP-dependent protein kinase (PKA). PKA then phosphorylates CREB on Ser133. Alternatively, other first messengers signal to CREB by increasing Ca<sup>2+</sup> concentrations via (1) the activation of ionotropic receptors (*R*), (2) activation of voltage-gated Ca<sup>2+</sup> channels through membrane depolarization or (3) activation of G-protein-coupled receptors. Increase in Ca<sup>2+</sup> concentration then leads to activation of Ca<sup>2+</sup>/calmodulin-dependent protein kinases (types IV and, possibly, I) (CaMK), which can also phosphorylate CREB on Ser133. Still other first messengers signal to CREB via the extracellular signal-regulated kinase (ERK) pathway (see Figure 25-4). Stimulation of receptor-associated protein tyrosine kinases (RTK) triggers the ERK pathway. Activation of ERK then phosphorylates and activates ribosomal S6 kinase (RSK), which also can phosphorylate CREB on Ser133. The step at which the cytoplasmic signal is transduced to the nucleus is well established for PKA: activation of the kinase leads to its dissociation and generation of the free catalytic subunit (PKA-C), which then translocates to the nucleus and phosphorylates CREB. The process is less well established for the CaMKs and ERK, as indicated by the dashed lines. Phosphorylation of CREB on Ser133 activates its transcriptional activity and leads to transcriptional changes of target genes.

molecular mechanisms underlying learning and memory pertain to excitatory glutamatergic neurotransmission. Presynaptic neurotransmitter release stimulates ionotropic postsynaptic receptors, such as AMPA and NMDA receptors, which are activated by glutamate, and leads to influx of cations, including Ca<sup>2+</sup>, which functions as a second messenger. It is therefore not surprising that the kinases mediating learning and memory are activated either directly (i.e., CaMKII and PKC) or indirectly (i.e., PKA via Ca<sup>2+</sup>-activated adenyl cyclase) by increases in intracellular Ca<sup>2+</sup> induced by excitatory glutamatergic neurotransmission. Once these protein kinases are invoked, they trigger signaling networks and induce changes in diverse postsynaptic and extrasynaptic processes. In the post-synapse, phosphorylation of ion channels and receptors, such as AMPA and NMDA receptors, controls the membrane potential and thus excitability of neurons. Phosphorylation of scaffolding proteins and signaling

proteins regulates protein clustering and activity-dependent molecular rearrangement of postsynaptic structures. Other postsynaptic processes controlled by phosphorylation include protein degradation and local protein synthesis.

An important Ca<sup>2+</sup>-dependent protein kinase that has been strongly implicated in the regulation of activity-dependent postsynaptic processes is CaMKII $\alpha$  (Wayman et al., 2008). Postsynaptic Ca<sup>2+</sup> influx triggers the activation of CaMKII by association with CaM and leads to translocation of the activated CaMKII from the synapse into the postsynaptic densities. There, CaMKII interacts with numerous proteins including the CDK5 activator p35,  $\alpha$ -actinin, densin-180, SynGAP and NMDA receptor subunits. In the active state, CaMKII can associate with the cytoplasmic carboxy terminus of the NMDA receptor subunits NR2B and, with less affinity, NR1 and NR2A. Next to the autophosphorylation of CaMKII at Thr286, these associations represent additional important

mechanism by which CaMKII can persistently exert effects even after  $\text{Ca}^{2+}$  levels drop and CaM dissociates. The association of CaMKII to proteins located within the postsynaptic densities brings the kinase into proximity with numerous substrates. Dephosphorylation of Thr286 by PP1, which is immobilized in the PSD, and PP2A, which is excluded from the PSD, are critical for terminating the autonomous activity of CaMKII. The phosphorylation of specific substrates by CaMKII is thought to modify neuronal function and exert an influence on synaptic plasticity. CaMKII activity has been shown to strengthen synaptic transmission by several mechanisms. One involves the direct phosphorylation of the AMPA receptor subunit GluR1, which in turn increases the single-channel conductance of homomeric GluR1 AMPA receptors. Another mechanism involves the addition of AMPA receptors to synapses, which is thought to depend on changes in receptor trafficking and on the organized addition of anchoring sites for AMPA receptors (Malinow & Malenka, 2002). Based on the molecular properties of CaMKII and its ability to switch from  $\text{Ca}^{2+}$ -dependent to autonomous activity states in response to different degrees of synaptic stimulation, it has been suggested that CaMKII may act as a molecular switch that is capable of storing long-term synaptic memory (Lisman et al., 2002).

### Extrasynaptic mechanisms regulated by protein phosphorylation

Protein phosphorylation also regulates many processes located outside the pre- and post-synapse that are critical for synaptic plasticity and memory function, including cell adhesion, cytoskeletal dynamics, protein trafficking, gene transcription and protein synthesis (Table 25-3). In the extrasynaptic compartment, phosphorylation of cytoskeletal proteins regulates neuronal morphology, axoplasmic transport and dendritic spine formation. Phosphorylation of transcription factors and ribosomal proteins regulates *de novo* protein synthesis in target neurons. Many of these extrasynaptic processes, once triggered, may be promulgated by subsequent synaptic activity, thereby contributing to the formation of stronger synapses with substantially altered constituency and morphology.

Indeed, synaptic plasticity and memory functions are critically dependent on transcription of numerous genes, which are subject to tight transcriptional control. CREB is a prototypical example of a transcription factor, whose activity is regulated by various signal transduction pathways (Ch. 27). Physiological stimulation by a variety of first messengers trigger cAMP,  $\text{Ca}^{2+}$  or MAPK signaling cascades, which induce phosphorylation of CREB at Ser133. Several protein kinases, including PKA, CaMKI, CaMKIV and RSK, have been found to phosphorylate CREB (Figure 25-7). This phosphorylation then promotes the binding of CREB to CREB-binding protein (CBP). Upon binding to CREB, CBP interacts directly with the RNA polymerase II complex, which in turn mediates the initiation of gene transcription. The dephosphorylation of CREB, and thereby its inhibition, are regulated by PP1 and, possibly, PP2A.

Similarly to CREB, virtually all classes of transcription factors are regulated via phosphorylation by numerous types of

protein kinases and phosphatases. Some common regulatory mechanisms observed among transcription factors are the phosphorylation-dependent translocation into the nucleus, export from the nucleus, nuclear retention and DNA interaction. Indeed, phosphoregulation of the transcriptional machinery is fundamental for the expression of specific physiological responses and, thus, cellular functioning.

## PROTEIN PHOSPHORYLATION IN HUMAN NEURONAL DISORDERS

Virtually every physiological process is regulated by protein phosphorylation. Therefore, disruption of the molecular machinery governing protein phosphorylation has, in many cases, serious consequences for cellular integrity. Consequently, many human diseases, including neuronal disorders, have been linked to deregulation of protein phosphorylation. In cancer, the prime example, aberrant signal transduction within cellular growth signal pathways, is the defining characteristic. Consistently, numerous mutations in genes of many kinases and phosphatases have been implicated in cancer or developmental syndromes. This underscores the immense importance of phosphoregulation in integral cellular processes including proliferation, differentiation, survival and death.

### Genetic neuronal disorders due to mutations in genes of protein kinases and phosphatases

Consistent with a crucial role in neuronal functions, some mutations in genes of various protein kinase and phosphatases lead to devastating inherited human neuronal disorders (Table 25-4).

Mutations in several protein kinase genes result in disorders characterized by neurodevelopmental impairment and mental retardation, supporting an important involvement of protein phosphorylation in the development of the CNS. Such mutations have been found in kinases including cyclin-dependent kinase-like 5 (CDKL5), myotonic protein kinase (DMPK1), p21-activated protein kinases 3 (PAK3), and ribosomal protein S6 kinase 2 (RSK2). Loss-of-function mutations in the atypical protein kinase, ataxia telangiectasia mutated (ATM), result in ataxia telangiectasia, a disorder characterized by early onset of progressive loss of motor coordination and balance (ataxia), dilation of superficial blood vessels, and dementia. ATM is involved in the control of DNA damage repair, and thus about 30% of cases develop cancer and immune deficiency. Missense, splice and truncating mutations cause early-onset seizures and severe neurodevelopmental impairment.

Other mutations in protein kinase and phosphatase genes lead to diseases that are characterized by neurodegeneration and movement disorders, such as early-onset Parkinsonism and spinocerebellar ataxia. Missense mutations in the leucine-rich repeat kinase 2 (LRRK2; also called Dardarin) lead to familial forms of Parkinson's disease (PD) (see also Ch. 49). Mutations in LRRK2, a member of the Tyr protein kinase-like subfamily, are the most common cause of autosomal dominant PD. A single mutation in LRRK2, namely Gly2019Ser, accounts for about 5% of all diagnosed inherited forms of

**TABLE 25-4** Hereditary Neuronal Disorders Caused by Mutations in Genes of Protein Kinases and Phosphatases

Mutated Kinase or Phosphatase	Genetic Neuronal Disease	
	Symptoms	
Ataxia telangiectasia mutated (ATM)	Ataxia telangiectasia <i>Progressive loss of motor control, dilation of superficial blood vessels, and dementia.</i>	
Cyclin-dependent kinase-like 5 (CDKL5)	CDKL5 syndrome <i>Early-onset seizures and severe neurodevelopmental impairment (similar to Rett syndrome)</i>	
Myotonic protein kinase (DMPK1)	Myotonic dystrophy Type 1 <i>Progressive muscle weakness and wasting, cataracts, hypogonadism, cardiac arrhythmias, defective endocrine functions, diabetes mellitus and mental retardation</i>	
Laforin	Lafora disease (progressive myoclonus epilepsy) <i>Seizures, muscle spasms and progressive dementia, intracellular polyglucosan inclusions (Lafora bodies)</i>	
Leucine-rich repeat kinase 2 (LRRK2)	Familial form of Parkinson's disease (PD) <i>Tremor, rigidity, slowing of movement, postural instability and dementia</i>	
PTEN-induced putative kinase 1 (PINK1)	Familial form of PD <i>Tremor, rigidity, slowing of movement, postural instability and dementia</i>	
p21-activated protein kinases 3 (PAK3)	Non-syndromic mental retardation type 30 (MRX30) <i>Mental retardation</i>	
Protein kinase C $\gamma$ (PKC $\gamma$ )	Spinocerebellar ataxia type 14 (SCA14) <i>Impaired coordination of movements and balance (ataxia) and cognitive decline</i>	
Ribosomal protein S6 kinase 2 (RSK2)	Coffin-Lowry syndrome <i>Severe mental retardation with facial, digital and progressive skeletal deformations</i>	

PD. This mutation is located within the activation loop of the LRRK2 kinase domain and possibly stimulates catalytic activity. It is currently not known how LRRK2 mutations lead to PD. Another kinase involved in PD is the mitochondrial Ser/Thr-protein kinase PTEN-induced putative kinase 1 (PINK1). Loss-of-function mutations in PINK1 result in autosomal recessive, early-onset Parkinsonism. Heterozygosity for PINK1 mutations might act as a susceptibility factor for PD.

Over 10 point mutations in the gene of protein kinase C-gamma (PKC $\gamma$ ) lead to spinocerebellar ataxia type 14 (SCA14), an autosomal dominant slow-progressive neurodegenerative disease. The symptoms of SCA14 include impaired

coordination of movements and balance (ataxia) and cognitive decline.

More than 30 missense mutations in the gene of the dual-specificity phosphatase laforin cause Lafora disease, also called progressive myoclonus epilepsy. The disease is an autosomal recessive genetic disorder characterized by seizures, muscle spasms and progressive dementia. The pathological hallmarks of Lafora disease are intracellular polyglucosan inclusions; however, the underlying molecular mechanisms are not understood. It is believed that laforin can dephosphorylate glycogen and thus prevent soluble glycogen molecules from becoming insoluble polyglucosan.

### Protein phosphorylation in pathophysiological processes in diseases of the nervous system

In genetic disorders, the initial cause of disease can be traced back to genetic mutations or chromosomal abnormalities. Hence, mutation-bearing molecules can be unequivocally associated with the pathogenesis. Consequently, much insight on various disease mechanisms has been gained through the study of inherited syndromes. In non-genetic pathophysiological processes, in contrast, the initial cause of disease and factors driving the pathogenesis are often not known. In these cases, individual protein kinases and phosphatases may be implicated in pathogenesis via a molecular and functional link to particular pathogenesis-associated proteins. Common examples of mechanistic links include direct interaction with and phosphoregulation of disease-associated proteins, as well as interaction with or regulation of upstream and/or downstream regulatory factors for such proteins. Alternatively, kinases and phosphatases may be implicated in pathogenesis via alterations in their expression level, activity or subcellular localization in diseases. It is often extremely difficult to conclusively determine whether a kinase or phosphatase is contributing to the pathogenic changes or whether the disease is inducing molecular and functional changes in these enzymes. A major issue is determining the temporal sequence of actions, which is further complicated by the astounding complexity of phosphoregulation of physiological functions. Despite these substantial issues, the study of protein kinases and phosphatases has helped greatly to elucidate the molecular machinery involved in the pathophysiology of acute and chronic neuronal diseases, including neuronal injury, sporadic AD and PD (see Chs. 41, 46–49).

### Protein phosphorylation and AD

Sporadic AD is a progressive neurodegenerative disorder and one of the most common causes of dementia (Ch. 46). The neuropathological hallmarks of AD include amyloid plaques, formed mainly from A $\beta$ -peptide; neurofibrillary tangles, composed predominantly of hyperphosphorylated tau protein; and neuronal cell death. Consequently, the molecular and functional properties of A $\beta$ -peptide and tau have been extensively investigated. This research has shown that the amyloid precursor protein (APP), as well as its proteolytic cleavage product, the A $\beta$ -peptide, are subject to regulation by various signaling pathways. Kinases such as CDK5, GSK3 and JNK directly phosphorylate APP intracellularly, thereby modulating its processing, trafficking and physiological functions. In

addition, APP-interacting proteins and proteolytic enzymes that mediate APP processing are regulated via phosphorylation. For example, presenilin1, a protease implicated in AD pathogenesis, is phosphorylated by kinases such as GSK3, CDK5 and PKA. This phosphoregulation is thought to control molecular association, enzymatic activity and degradation of presenilin1.

The hypothesis that aberrant phosphoregulation may contribute to AD pathogenesis is further supported by the finding that neurofibrillary tangles mainly contain hyperphosphorylated tau. This microtubule-associated protein is phosphorylated at numerous sites by various kinases including CDK5, CK1, GSK3 and PKA, and dephosphorylated by phosphatases such as PP1 and PP2A (Ch. 46). Hyperphosphorylation of tau is believed to support the self-assembly of filaments and tangles that are involved in the pathogenesis of AD and other tauopathies. Consistently, numerous mutations in the tau gene, known to dysregulate tau expression and function, cause a neurodegenerative disorder that shares commonalities with AD (see Ch. 47).

Most of the original research associating altered phosphoregulation with disease such as AD has been conducted in human postmortem tissue. A caveat of such studies is that the phosphorylation state of proteins can change rapidly postmortem. The activity of kinases ceases relatively quickly after death occurs. In contrast, phosphatases remain active for prolonged times. Therefore, protein phosphorylation levels normally decrease rapidly postmortem, which can confound the interpretation of results.

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