The Role of Polo-like kinase 2 in Synaptic Function and Plasticity

by

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ABSTRACT

Homeostatic forms of plasticity keep the spiking output of neurons within an optimal range in the face of changing activity levels of the surrounding network, but little is known about the underlying molecular mechanisms, particularly during heightened activity. We report in Chapter 2 that in hippocampal neurons experiencing elevated activity, the activity-inducible protein kinase, Polo-like kinase 2 (Plk2), was required for synaptic scaling in dissociated culture and for reducing membrane excitability in slice culture—two principal compensatory mechanisms underlying homeostatic plasticity. Inhibition of Plk2 activity in slice culture during elevated activity resulted in increased dendritic spine size and density as well as a "run-up" in synaptic strength that prevented subsequent LTP. Thus, the homeostatic functions of Plk2 allow synapses to remain within a modifiable range during prolonged heightened network activity.

In Chapter 3, we show that the homeostatic prevention of run-up during elevated activity also depended on CDK5, which acted as a "priming" kinase for the phospho-dependent binding of Plk2 to its substrate SPAR, a postsynaptic RapGAP. Overexpression of SPAR strengthened synapses, whereas RNAi knockdown of SPAR weakened synapses and disrupted homeostasis. Thus CDK5-dependent recruitment of Plk2 to SPAR, followed by Plk2-mediated degradation of SPAR, constitutes a likely molecular mechanism for Plk2-dependent homeostatic plasticity in neurons.

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Chapter 1—Introduction

Part 1
Polo-like Kinases in the Nervous System

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ABSTRACT

Polo like kinases are key regulators of the cell cycle, but little is known about their functions in post-mitotic cells such as neurons. Recent findings indicate that Plk2 and Plk3 are dynamically regulated in neurons by synaptic activity at the mRNA and protein levels. In COS cells, Plk2 and Plk3 interact with spine-associated Rap guanosine triphosphatase activating protein (SPAR), a regulator of actin dynamics and dendritic spine morphology, leading to its degradation through the ubiquitin-proteasome system. Induction of Plk2 in hippocampal neurons eliminates SPAR protein, depletes a core postsynaptic scaffolding molecule (PSD-95), and causes loss of mature dendritic spines and synapses. These findings implicate neuronal Plks as mediators of activity-dependent change in molecular composition and morphology of synapses. Induction of Plks might provide a homeostatic mechanism for global dampening of synaptic strength following heightened neuronal activity ("synaptic scaling"). Synapse-specific actions of induced Plks are also possible, particularly in light of the discovery of phosphoserine/threonine peptide motifs as binding targets of the polo box domain, which could allow for "priming" phosphorylation by upstream kinases that could "tag" Plk substrates only in specific synapses

INTRODUCTION

The study of Polo-like kinases (Plks) has focused primarily on the critical role of these proteins in the cell cycle. Named after their first member in *Drosophila melanogaster* (Polo) (Sunkel and Glover, 1988; Llamazares et al., 1991), this family of serine/threonine kinases has emerged as a key regulator during all stages of mitosis (Glover et al., 1998; Nigg, 1998; Xie et al., 2002; van de Weerdt and Medema, 2006). Despite their well characterized roles in dividing cells, recent studies suggest that Plks have roles in terminally-differentiated cells of the nervous system. Intriguingly, neuronal Plks are regulated by synaptic activity and can interact with specific synaptic proteins, resulting in loss of synapses. In this review, we briefly summarize the roles of Plks in the cell cycle and then discuss developments on neuronal Plks and their implications for the physiological role of these proteins in the nervous system.

The Plks are characterized by a conserved architecture consisting of an N-terminal kinase domain and a C-terminal Polo box domain (PBD). The latter binds to phosphoserine/threonine motifs and is believed to be important for the functional regulation of the protein by targeting the kinase to specific subcellular locations and substrates (Lee et al., 1998; May et al., 2002; Elia et al., 2003a; Elia et al., 2003b; Ma et al., 2003b; Reynolds and Ohkura, 2003; Lowery et al., 2004). Flies, budding yeast, and fission yeast contain a single Plk, referred to as Polo, Cdc5p, and Plo1, respectively. In mammals, frogs, and worms, there are three Plks, called Plk1, Plk2, and Plk3[1]. Plk1 is thought to be the functional homologue of *Drosophila* Polo and, together with Cdc5p, is the most extensively studied of the Polo-like kinases. Plk1 plays a critical role in the timing of mitotic entry and exit through the activation of Cdc25C and Cdc2-cyclinB at the G2-M transition (Roshak et al., 2000; Toyoshima-Morimoto et al., 2001; Toyoshima-Morimoto et al., 2002) and through activation of the APC in mitosis (Kotani et al., 1998; Golan et al., 2002; but see: Kraft et al., 2003). Plk1 also regulates the mechanics of mitotic processes such as centrosome assembly and separation (Lane and Nigg, 1996; do Carmo Avides et al., 2001; Dai et al., 2002; Blagden and Glover, 2003), chromosome and sister chromatid separation during meiosis I and mitosis, respectively (Sumara et al., 2002; Clyne et al., 2003; Lee and Amon, 2003), fragmentation of Golgi membranes (Carmena et al., 1998; Song and Lee, 2001; Sutterlin et al., 2001; Colanzi et al., 2003), and cytokinesis (Carmena et al., 1998; Song and Lee, 2001; Neef et al., 2003).

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[1]Plk in *Xenopus laevis* is also referred to as Plx. Plk2 and Plk3 are also known as SNK/hSNK and FNK/Prk in mice/humans, respectively.

Plk3 is less well understood, although recent studies have revealed diverse roles, both in the cell cycle and beyond. Like Plk1, Plk3 activates Cdc25C at the onset of mitosis (Ouyang et al., 1997; Ouyang et al., 1999; Bahassi el et al., 2004), participates in breakdown of the Golgi (Ruan et al., 2004; Xie et al., 2004), and is important for cytokinesis (Conn et al., 2000). In addition, Plk3 regulates microtubule dynamics and centrosomal function (Wang et al., 2002), and has been reported to function in cellular adhesion (Holtrich et al., 2000). Following genotoxic stress, Plk3 is activated by Chk2 and helps to mediate the stress response, at least in part by activating p53 (Xie et al., 2001; Bahassi el et al., 2002; Xie et al., 2002). In contrast, Plk1 is inhibited following DNA damage (Smits et al., 2000; van Vugt et al., 2001) and its mRNA expression is repressed by BRCA-1 (Ree et al., 2003) and p53 (Kho et al., 2004).

Plk2 is the least studied of the Plks. Unlike Plk1 and Plk3, Plk2 seems to lack a prominent role in the cell cycle. Plk2 is expressed in the brain, but is notably absent from proliferating tissue such as thymus, liver, or intestine (Simmons et al., 1992). Moreover, Plk2 is not detected in nocodazole-treated cells arrested at M-phase, but is restricted to G1, indicating that Plk2 likely does not function during mitosis (Ma et al., 2003b). Its expression during G1, however, implicates a role for Plk2 in the cell cycle, and $Plk2^{-/-}$ fibroblasts grow slower in culture and show delayed entry into S phase (Ma et al., 2003a). $Plk2^{-/-}$ mice, although smaller at birth, have similar postnatal growth rates compared to controls, consistent with a physiological role in the cell cycle restricted to the embryonic period (Ma et al., 2003a).

Beyond their key role in cell division, Plks are also found in postmitotic cells such as neurons, but the neuronal functions of Plks are poorly understood. Interestingly, several cell cycle regulators are now known to have additional activities in neurons. One example is the anaphase promoting complex (APC), a multi-subunit complex with E3 ubiquitin-ligase activity that coordinates cell cycle transitions, including exit from mitosis (Morgan, 1999; Harper et al., 2002; Peters, 2006). Activity of the APC is stimulated in early mitosis by the regulatory protein Cdc20 and in late mitosis/G1 by Cdh1. Intriguingly, Cdh1 and core components of the APC are also expressed in postmitotic neurons (Gieffers et al., 1999; Peters, 2002), where the Cdh1-APC has recently been shown to play a role in axonal growth and patterning in the developing brain (Konishi et al., 2004; Lasorella et al., 2006; Stegmuller et al., 2006). In addition, Cdh1-APC seems to be involved in cell survival (Almeida et al., 2005), and regulation of synaptic transmission (Juo and Kaplan, 2004; van Roessel et al., 2004). Similarly, Aurora kinases

are involved in chromosome segregation and cytokinesis during mitosis (Glover et al., 1995; Terada et al., 1998; Blagden and Glover, 2003) and have now been shown to phosphorylate and activate cytoplasmic polyadenylation element-binding protein-1 (CPEB-1) in neurons and thereby drive polyadenylation-dependent translation at synaptic sites (Huang et al., 2002; Theis et al., 2003). Taken together, these studies suggest a broad role for cell cycle proteins in differentiated cells of the nervous system. The remainder of this overview will discuss the neuronal functions of Plks.

Activity-induced Expression of Plks in the Brain.

Of the three known Plks, only Plk2 and Plk3 are expressed in the adult brain (Kauselmann et al., 1999). Notably, the mRNA and protein levels of these kinases are highly elevated by synaptic activity. Based on differential screens for activity-regulated genes, it is estimated that somewhere between 15 and 300 of the thousands of genes expressed in the nervous system are rapidly regulated by activity (Hevroni et al., 1998; Lanahan and Worley, 1998; Nedivi, 1999). Activity-induced gene expression is believed to be critical for formation of long-term synaptic changes (synaptic plasticity) in the brain, which might underlie learning and memory. Based on the timescale over which synaptic change persists, several forms of synaptic plasticity can be delineated, each with different underlying molecular mechanisms and dependence on gene expression. Short-term synaptic alterations are mediated largely by posttranslational modification and regulation of existing proteins and are thus independent of new protein synthesis (Izquierdo et al., 2002). However, for long-term synaptic changes to take place, new gene transcription and protein synthesis are required (Frey et al., 1988; Steward and Schuman, 2001; e.g., Bozon et al., 2003). Thus, the expression profile of neuronal Plks would make them well suited to participate in longer lasting forms of synaptic plasticity.

The time course of activity-dependent Plk induction was studied in the brains of rats following drug-induced seizures. After 1 hour, the mRNA levels of Plk2 and Plk3 were increased ~1.6 fold. The fold-induction was similar at 4 hours, and mRNA levels returned to baseline by 10 hours. Similarly, protein levels of Plk2 and -3 were potently induced and enriched in somata and dendrites of activated neurons (Kauselmann et al., 1999). Electrical stimulation sufficient to induce long-term potentiation (LTP) in the hippocampus, a brain region critical for learning and memory, also induced Plk2 and Plk3 mRNAs. On the other hand, low frequency electrical stimulation failed to induce

Plk2 or Plk3 and did not change synaptic strength (Kauselmann et al., 1999). Thus a threshold of synaptic activation is required to upregulate expression of Plk2 and -3.

Based on pharmacological inhibition experiments, a number of signaling proteins are required for the induction of Plk2 in neurons. Thus, antagonists of L-type voltage gated calcium channels (VGCCs), or blockers of postsynaptic glutamate receptors (NMDA receptors and AMPA receptors), are sufficient to prevent activity-induced expression of Plk2 (Pak and Sheng, 2003). In addition, Plk2 induction requires the calcium-calmodulin sensitive protein phosphatase PP2B (also called calcineurin [CaN]) (Pak and Sheng, 2003). These findings are consistent with a model in which calcium entry through NMDA receptors and/or VGCCs, which are activated by synaptic activity, stimulates CaN, leading to transcriptional induction of Plk2 (Pak and Sheng, 2003).

CaN can regulate transcription in neurons by influencing a number of transcription factors. For example, CaN causes inactivation of cAMP response element binding protein (CREB), most likely through protein phosphatase PP1 (Hagiwara et al., 1992; Bito et al., 1996). More pertinently, CaN can activate the transcription factor NFATc4 through dephosphorylation and unmasking of nuclear localization signals (Graef et al., 1999). Recently, mRNA levels of a number of genes were shown to be controlled by CaN by differential expression profiling of cerebellar granule cells in the presence or absence of CaN inhibitors (Kramer et al., 2003), although Plks did not show up in this screen.

Once induced by activity, Plk2 seems to negatively regulate its own expression at the protein level, probably through autophosphorylation and subsequent degradation via the ubiquitin-proteasome system (Figure 1). Thus, proteasome inhibitors strongly elevate Plk2 protein levels, even in unstimulated neurons (Pak and Sheng, 2003). Such a mechanism would shorten the half-life of active Plk2 and limit the duration of Plk2 protein induction following synaptic stimulation.

Kinase activity of Plk2 is inhibited in the presence of the calcium and integrin-binding protein (CIB) (Ma et al., 2003b). Consistent with its possible role as a calcium dependent regulator of Plk2, the sequence of CIB is homologous to several members of the helix-loop-helix "EF-hand" calcium binding protein family, which includes calcineurin B and calmodulin that are known to modulate the activity of calcium-dependent phosphatases and kinases, respectively.

Figure 1

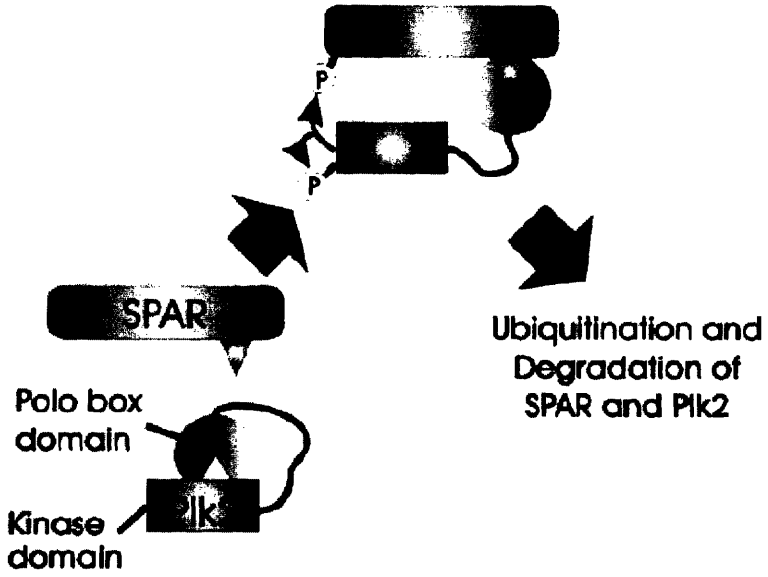

Figure 1. Plk2 phosphorylates SPAR causing its ubiquitination and degradation. Before binding, the polo box and kinase domains of Plk2 interact intramolecularly with mutual inhibition of function (bottom, left). After binding to SPAR via the polo box domain (PBD), the kinase domain is liberated and phosphorylates SPAR and itself via autophosphorylation (top, center). This leads to ubiquitination and degradation of SPAR and Plk2 (bottom, right). P, phosphate group; SPAR, spine-associated RapGAP

Plk2 leads to Degradation of a Spine-associated Protein and Remodeling of Synapses

A breakthrough in the neuronal function of Plk2/3 came with the discovery of a specific interaction between a postsynaptic Rap guanosine triphosphatase activating protein (RapGAP) known as SPAR (Spine Associated RapGAP) and the C-terminal region of Plk2 containing the Polo box domain (Pak and Sheng, 2003). Also an actin-binding protein, SPAR is enriched in dendritic spines, specialized protrusions of dendrites on which most excitatory synapses are formed (Hering and Sheng, 2001). Within spines, SPAR forms a complex with the postsynaptic scaffolding protein PSD-95 and NMDA receptors (Pak et al., 2001; Roy et al., 2002). SPAR seems to function to promote the growth of dendritic spines, at least in part by inhibiting Rap (Pak et al., 2001), a small GTPase involved in the regulation of actin dynamics (McLeod et al., 2004).

In COS cells, co-expression of Plk2 and SPAR results in phosphorylation, ubiquitination, and degradation of SPAR and Plk3 causes SPAR degradation as well. Plk2 kinase activity is necessary for SPAR degradation but not for the interaction between Plk2 and SPAR, which is mediated by the C-terminus containing the PBD (Figure 1) (Pak and Sheng, 2003). Analogously, following phosphorylation by Plk1, somatic Wee-1 is ubiquitinated by the F-box protein β-transducin repeat-containing protein (β-TRCP) and degraded at the onset of mitosis (Watanabe et al., 2004). The E3 ubiquitin ligase responsible for ubiquitination of SPAR is unknown.

What is the effect of Plk2 expression in neurons? After induction by synaptic stimulation in cultured hippocampal neurons, endogenous Plk2 protein accumulates mainly in the cell body and proximal dendrites, where it is enriched in dendritic spines (Pak and Sheng, 2003). Normally, SPAR and PSD-95 are concentrated in synaptic clusters associated with spines along the entire length of dendrites, but after induction of Plk2, these proteins are lost from the same regions where Plk2 is present (Figure 2). Thus there are complementary gradients of distribution of Plk2 and SPAR/PSD-95 immunoreactivity in dendrites of activated neurons. Exogenous Plk2 introduced into neurons by viral infection accumulates to a higher level and is less restricted to the proximal somatodendritic compartments, but nevertheless, the magnitude of effect on loss of SPAR and PSD-95 clusters shows a proximal-to-distal gradient (Pak and Sheng, 2003).

Figure 2

Figure 2. Plk2 induces loss of the spine associated protein PSD-95.
Hippocampal cultures were infected at days in vitro [DIV] 18 to DIV 21 with Sindbis virus expressing Plk2 and double stained for exogenous Plk2 (red) and endogenous PSD-95 (green), as indicated, with merged image shown in color. SPAR shows a similar pattern of immunoreactivity as PSD-95 under the same conditions (data not shown).

The loss of SPAR and PSD-95 clusters correlates with a depletion of mature dendritic spines and reduced density of synapses on affected dendrites (Pak and Sheng, 2003). Interestingly, there is a corresponding increase in thin dendritic protrusions that resemble filopodia, which are generally believed to be the exploratory precursors of spines (Hering and Sheng, 2001).

Dominant negative SPAR constructs that lack RapGAP activity also lead to loss of mature spines and an increase in the number of long, thin dendritic protrusions (Pak et al., 2001). Thus, one mechanism by which activity-induced expression of Plk2 could lead to synapse loss and increased filopodia-like protrusions is through the degradation of SPAR and resulting disinhibition of the actin regulator Rap. Consistent with this idea, overexpression of constitutively active Rap2 mutant in neurons leads to a similar spine phenotype characterized by irregular thin protrusions (Fu et al., 2007).

SPAR is certainly not the only substrate of Plk2 in neurons, and degradation of SPAR is unlikely to be the sole mechanism by which Plk2 induction leads to remodeling of synapses and spines. Although it is likely to be a secondary effect of Plk2 activity, the depletion of PSD-95 that accompanies loss of SPAR could also contribute to elimination of mature spines and synapses. PSD-95 is a key postsynaptic scaffolding molecule important for morphological growth of spines and functional maturation of synapses (El-Husseini et al., 2000, Kim and Sheng, Nature Reviews Neuroscience 2004 submitted). Thus, while the precise mechanism by which Plk2 induction leads to dismantling of synapses and spines is uncertain, the regulation of Plk2 expression in neurons provides a powerful means to remodel synapses following neuronal activity.

Global vs Local Synaptic Modifications

A functional corollary of loss of synapses due to Plk2 should be the weakening of synaptic transmission onto those neurons. Activity-driven induction of Plk2 thus presents an attractive homeostatic mechanism for stabilizing the excitability of neurons following heightened synaptic input (Burrone and Murthy, 2003; Turrigiano and Nelson, 2004). Local changes in synaptic strength, arising from positively correlated activity between pre- and postsynaptic neurons, is thought to be important for long-term memory storage (Bi and Poo, 2001). Without homeostatic mechanisms in place, however, such correlation-based plasticity can lead to unstable excitability of the cell. For example, when a particular synaptic input is potentiated, it becomes easier for that input to drive the postsynaptic cell to fire, which begets further potentiation, eventually causing

"runaway" excitation of the postsynaptic neuron (Miller, 1996). Homeostatic mechanisms are thought to ensure that the excitability of a cell as a whole remains within a certain range, even though a specific subset of synapses may be modified up or down (see also Chapter1-Part2: Review of Homeostatic Mechanisms).

A prominent mechanism of homeostatic plasticity is synaptic scaling, which is thought to occur through multiplicative changes in the strength of all (or most) synapses ("synaptic scaling"), take place over a prolonged time course (hours to days), and retain the relative strengths between synapses (Turrigiano and Nelson, 2000, 2004). Synaptic scaling involves changes in quantal amplitude, but the molecular mechanisms are still unclear (Turrigiano and Nelson, 2004). The activity-dependent expression of Plk2, the timescale of neuronal Plk2 induction (hours), the widespread reach of induced Plk2 protein (i.e. apparently not targeted to small specific subsets of synapses), and the negative effect of activated Plk2 on synapses and spines, together raise the possibility that neuronal Plks contribute to the mechanisms of global synaptic homeostasis (Figure 3 A).

It remains possible, however, that the activity of Plk2, or the accessibility of its substrates, can be regulated in a local synapse-specific manner. In particular, as will be discussed below, the recent discovery of phosphoserine/threonine peptide motifs as the binding targets of the polo box domain (Elia et al., 2003a; Elia et al., 2003b) allows for the possibility of "priming" phosphorylation by upstream kinases that could "tag" Plk substrates only in specific synapses (Figure 3 B). Ultimately, it is still a matter of speculation whether neuronal Plks participate in global homeostatic plasticity mechanisms or in local synaptic change. Electrophysiological studies and specific loss-of-function genetic experiments will be required to address the role of neuronal Plks in synaptic plasticity more directly.

Learning from Mitotic Plks

The multiplicity of Plk substrates in the cell cycle raises the possibility that neuronal Plks could have additional substrates besides SPAR. During mitosis alone, Plks have been shown to interact with and phosphorylate a diverse set of proteins, including BRCA2 (Lee et al., 2004), cyclin B (Toyoshima-Morimoto et al., 2001; Yuan et al., 2002), the mitotic kinesin-like protein MKLP2 (Neef et al., 2003), and the cdk1 phosphatase Cdc25C (Roshak et al., 2000; Bahassi el et al., 2004). Of note, the PBD was found to

Figure 3

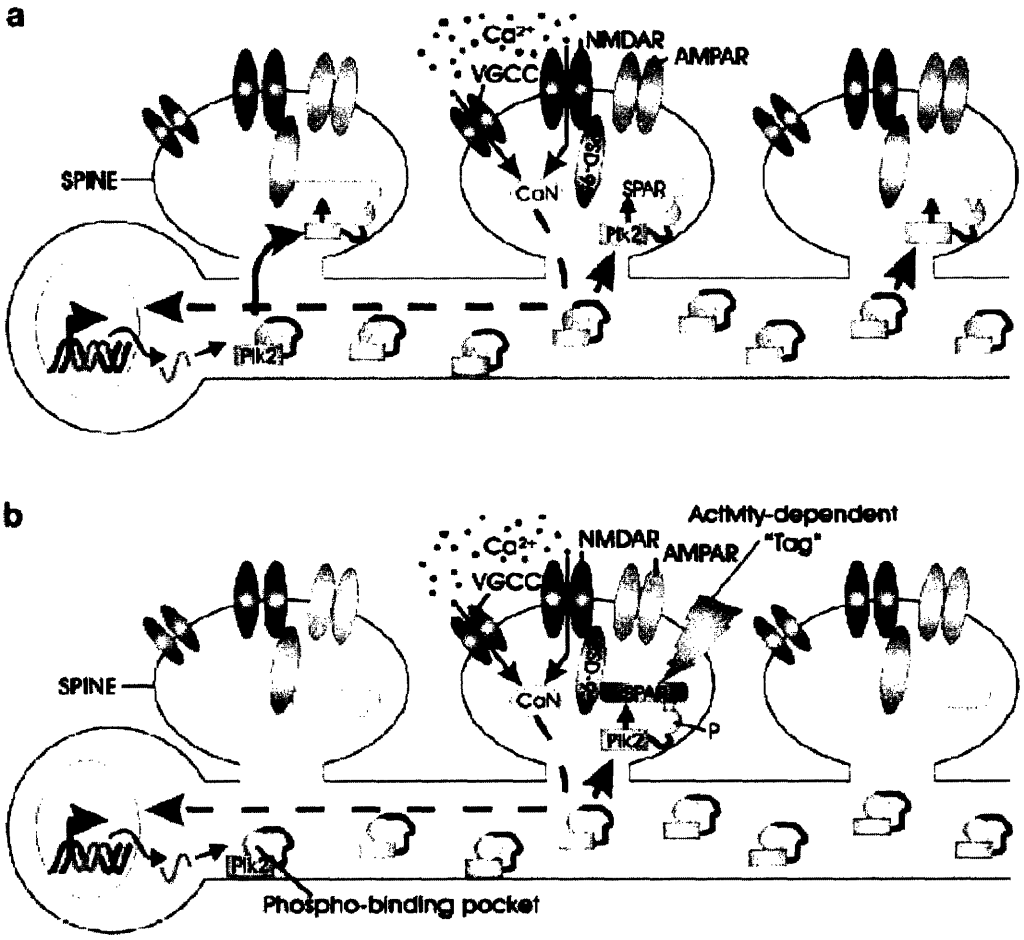

Figure 3. Model for Plk2 induction and global vs synapse-specific function of induced protein.

Calcium influx through NMDA and/or VGCCs after synaptic activity activates CaN, which causes induction of Plk2 mRNA and protein. (a) Induced Plk2 acts globally to bind to and cause degradation of SPAR. This could provide a homeostatic mechanism to broadly depress synaptic strength following heightened activity at a subset of synapses. (b) Induced Plk2 is "captured" specifically at spines "tagged" by activity-induced phosphorylation of SPAR. Such a tag could direct Plk2 to a subset of synapses despite "nonspecific" induction in the cell body. Ca^{2+}, calcium; VGCC, voltage gated calcium channel; CaN, calcineurin; SPAR, spine associated RapGAP; NMDAR, NMDA receptor; AMPAR, AMPA receptor; P, phosphate group.

be critical in mediating the interaction in all cases where it was tested (Lin et al., 2000; Sutterlin et al., 2001; Yarm, 2002; Elia et al., 2003a; Elia et al., 2003b; Neef et al., 2003; Zhou et al., 2003; Litvak et al., 2004; Yoo et al., 2004).

The PBD, which contains two conserved polo boxes, their intervening sequence, and a short stretch of linker sequence between the kinase domain and the first polo box, is critical for correct localization and function of Plks (for review, see Lowery et al., 2004). Mutation of the PBD in mammalian Plk1 prevented its correct localization and its ability to complement a Cdc5 mutant in budding yeast (Lee et al., 1998). Overexpression of the PBD by itself led to a dominant-negative cell cycle arrest in pre-anaphase in mammalian cells (Seong et al., 2002) and prevented completion of cytokinesis in budding yeast (Song and Lee, 2001).

Recently, an optimal consensus binding motif for the PBD was determined with the sequence Ser-[pSer/pThr]-[Pro/X] (Elia et al., 2003a; Elia et al., 2003b). This raises the intriguing possibility that binding of the Plk-PBD might require prior phosphorylation of Plk substrates by a "priming" proline-directed serine-threonine kinase (such as MAP kinases or CDKs). Although such priming events have yet to be investigated in most cases, several recent studies have found results consistent with this idea. In one instance, it was shown that prior phosphorylation of cyclinB by Erk2 greatly enhanced cyclinB phosphorylation by Plk1 (Yuan et al., 2002). More recently, it was found that phosphorylation of the peripheral Golgi protein Nir2 by Cdk1 (Litvak et al., 2004) or of the DNA replication checkpoint mediator Claspin in *Xenopus* egg extracts (Yoo et al., 2004) created docking sites for Plk1 that were required for completion of cytokinesis and further phosphorylation of Claspin by Plk1, respectively.

It will be interesting to see if neuronal Plks behave similarly. Priming phosphorylation of Plk2 targets (e.g. SPAR) within their Plk2 binding motif could allow for synapse-specific recruitment of Plk2 in spite of "non-specific" induction of the mRNA and protein in the cell body. Such a mechanism would be analogous to what has been proposed by the "synaptic-tagging" hypothesis, a model put forward to address the question of how new gene products required for long-term synaptic change are delivered from the nucleus to the few activated synapses within the vast dendritic tree that require them (for review, see Martin, 2002; Martin and Kosik, 2002). According to this model, new protein products are delivered throughout the entire cell, but act specifically only at those synapses that have been "tagged" by synaptic activity. Despite strong evidence that some type of synaptic tagging occurs, both the identity of the "tag" and the

mechanism of subsequent "capture" of newly synthesized proteins have remained elusive (Martin and Kosik, 2002).

CONCLUSION

Recent findings have revealed the regulated expression and a cell biological function of Plks in neurons. Plk2 and Plk3 are induced by neural activity and show promise as possible mediators of synaptic remodeling. Many questions remain to be addressed, however. For example, what are the other substrates of neuronal Plks besides SPAR? And how are they regulated by Plks? Can degradation of SPAR fully account for the spine phenotype seen with induction of Plks? Do Plks have significant roles in the presynaptic terminal or even outside of the synapse? What are the relative contributions of Plk2 and Plk3? Are Plks involved in global or local synaptic plasticity? And what is their significance for brain development, function and behavior? The molecular study of Plks in neurons can benefit from the rich understanding of Plk actions in the cell cycle; but the neurobiological significance of Plks cannot be simply extrapolated from studies of dividing cells.

The study of Plk2/SNK has lagged behind Plk1 and Plk3, primarily because it has a limited role in the cell cycle. Analogous to CDK5 (a cyclin-dependent kinase homolog that is abundant in neurons), Plk2 might turn out to be an "aberrant" family member that has been co-opted by postmitotic cells to function in entirely different contexts. It remains to be seen whether Plks have as broad a role in the nervous system as they do in the cell cycle. Given the tantalizing evidence so far, elucidating the functions of Plks in the nervous system should be richly rewarding.

Chapter 1—Introduction

Part 2
Review of Homeostatic Plasticity in Neurons

INTRODUCTION

An early finding in the study of neuronal homeostasis was that removal of muscle innervation results in compensatory increases in muscle excitability and spontaneous muscle contractions, called "denervation supersensitivity". The increased muscle excitability is due to increased input resistance and a large upregulation of extrajunctional acetylcholine receptors (Axelsson and Thesleff, 1959; Sharpless, 1964; Berg and Hall, 1975). More detailed genetic experiments have since revealed a complex set of homeostatic mechanisms that keep transmission at the neuromuscular junction relatively constant. Reduction in the number of synapses, for example, results in a compensatory increase in the quantal amplitude (the postsynaptic response to release of neurotransmitter from a single vesicle) at the remaining synapses (Davis and Goodman, 1998). Conversely, reduction in quantal amplitude or hyperpolarization of the muscle to make it less active, is compensated by increased presynaptic transmitter release (Sandrock et al., 1997; Davis et al., 1998; Paradis et al., 2001).

Homeostatic plasticity has also been observed in the central nervous system *in vivo*. For example, disruption of spontaneous network activity in the developing chick spinal cord results in a compensatory increase in synaptic strength of motor neuron inputs, which increases excitability in the cord and help maintain appropriate network activity (Gonzalez-Islas and Wenner, 2006). In the developing rodent visual cortex, an increase in frequency of miniature excitatory postsynaptic currents (mEPSCs; the postsynaptic response to a single vesicle of neurotransmitter) is observed concomitantly with a decrease in the amplitude of mEPSCs in the second and third postnatal weeks. The decrease in mEPSC amplitude is dependent on visual activity and is thought to represent a homeostatic mechanism that compensates for the increased afferent activity after eye opening (Desai et al., 2002).

Neurons are remarkable in that they manage to maintain relatively stable activity patterns despite constantly changing numbers and strengths of inputs involved in encoding memories, behavior, and cognition. During development, neurons maintain robust activity patterns despite forming hundreds or thousands of new synapses with other cells, inserting ion channels into their membranes, and growing dramatically in size. This review focuses on the mechanisms that allow a neuron to achieve this stability in the face of such growth, complexity, and plasticity.

Mechanisms of Neuronal Homeostatis

A neuron has a wide variety of homeostatic[2] mechanisms at its disposal. They operate over vastly different time scales (minutes to days), but all have in common that they tend to allow a neuron to meaningfully respond to its environment while ensuring that it remains within a physiologically useful operating range of activity, i.e. that it doesn't undergo "runaway" excitation or quiescence (Burrone and Murthy, 2003). In what follows, I discuss the various known mechanisms in terms of their potential homeostatic contribution, the conditions under which they are most likely active, and the empirical evidence that has been put forward in their support.

Altered Presynaptic Transmitter Release and Altered Quantal Amplitude (Synaptic Scaling) after Long-term Changes in Neuronal Activity

Exposure of neurons to extended periods of heightened or strongly diminished activity can result in compensatory changes in the properties of presynaptic neurotransmitter release and/or changes in quantal amplitude, called "synaptic scaling". Scaling has been observed *in vivo* (Desai et al., 2002; Gonzalez-Islas and Wenner, 2006) and in cultures of neocortical (e.g., Turrigiano et al., 1998), hippocampal (e.g., Stellwagen and Malenka, 2006), and spinal cord neurons (O'Brien et al., 1998). It is characterized by three main features: it is global (all synapses are affected), gradual (it occurs over the course of several hours to days), and multiplicative (all synapses are modified by the same factor) (Turrigiano and Nelson, 2004) (However, see discussion below on global versus local rules of scaling). The multiplicative nature of synaptic scaling is thought to keep the relative weights between synapses intact and thus makes it an attractive homeostatic mechanism to stabilize neuronal activity following Hebbian plasticity mechanisms like long-term potentiation (LTP) and long-term depression (LTD) that arise from correlated pre-and postsynaptic activity.

One way quantal amplitude can be modified in the course of synaptic scaling is through changes in the number or efficacy of postsynaptic receptors. Consistent with such a change, some have found an increased sensitivity to applied neurotransmitter following prolonged inactivity (O'Brien et al., 1998; Turrigiano et al., 1998) as well as an

[2] The word "homeostatic" is used in this review to describe mechanisms that contribute to the stability of a neuron's output. In the strictest sense, a homeostatic mechanism would have to have a well-defined setpoint and precise feedback mechanisms for maintaining that setpoint. At this stage however, stabilizing mechanisms in neurons are not well enough characterized to definitively label them as homeostatic in the stricter sense (Davis GW (2006) Homeostatic control of neural activity: from phenomenology to molecular design. Annu Rev Neurosci 29:307-323.).

increased number of postsynaptic receptors by quantitative immunofluorescence (Lissin et al., 1998; O'Brien et al., 1998; Thiagarajan et al., 2005; Wierenga et al., 2005; Stellwagen and Malenka, 2006).

In addition, quantal amplitude can be modified through changes in neurotransmitter content in individual vesicles (Sulzer and Pothos, 2000; Atwood and Karunanithi, 2002; Liu, 2003). Interestingly, prolonged increases or decreases in activity change levels of vesicular neurotransmitter transporters (De Gois et al., 2005; Wilson et al., 2005; Erickson et al., 2006) and such changes affect the amount of neurotransmitter in individual vesicles (Daniels et al., 2004; Wojcik et al., 2004; Wilson et al., 2005; Daniels et al., 2006). Thus, it appears that both pre-and postsynaptic mechanisms contribute to synaptic scaling, although the relative contributions of each and the conditions under which one might predominate over the other are still unclear (Rich and Wenner, 2007).

While molecular mechanisms underlying synaptic scaling are still largely unknown, a few reports have begun to identify some of them. Release of TNF-α from glia (Stellwagen and Malenka, 2006), BDNF (Rutherford et al., 1998), and the activity-inducible gene Arc/Arg3.1 (Shepherd et al., 2006) are important for upregulation of mEPSC amplitude following prolonged inactivity in neuron cultures. However, hippocampal cultures from TNF and Arc/Arg3.1 knockout mice continued to display significant downwards scaling of their mEPSCs in response to chronic increases in activity with $GABA_A$ receptor antagonists, leading the authors to conclude that molecular mechanisms in addition to TNF-α and Arc/Arg3.1 are likely to be important in the weakening of synapses following long-term increases in activity (Shepherd et al., 2006; Stellwagen and Malenka, 2006). Their identity is, however, still undetermined.

In addition to synaptic scaling, prolonged increases or decreases in neuronal activity can lead to altered regulation of neurotransmitter release that—like scaling—tends to stabilize the overall firing rate of the neuron. This change in neurotransmitter release is generally seen as a change in the frequency mEPSCs recorded from the postsynaptic cell (Thiagarajan et al., 2002; Burrone and Murthy, 2003; Thiagarajan et al., 2005; Wierenga et al., 2006). Although these activity-induced changes in mEPSC frequency could also be due to insertion of AMPA receptors into previously "silent" synapses (Isaac et al., 1995; Liao et al., 1995), it is thought that they more likely reflect changes in presynaptic regulation of neurotransmitter release for a number of reasons.

Using FM 1-43 imaging, it was shown that inactivity leads to increased probability of release (Murthy et al., 2001). Additionally, inactivity increases the size of the recycling pool of vesicles (Murthy et al., 2001; Burrone et al., 2002; Thiagarajan et al., 2005) as well as the vesicle turnover rate (Thiagarajan et al., 2005). Finally, numerous studies have shown that activity deprivation at the neuromuscular junction results in a marked increase in presynaptic neurotransmitter release (Snider and Harris, 1979; Tsujimoto et al., 1990; Plomp et al., 1992, 1994; Hong and Lnenicka, 1995; Wang et al., 2004).

Spike-Timing Dependent Plasticity (STDP)

It has been known for some time that when presynaptic activity precedes postsynaptic spiking or depolarization, LTP is induced (Levy and Steward, 1983), and conversely, that LTD is induced when postsynaptic activity precedes presynaptic activity (Debanne et al., 1994). More recently, many studies have characterized the precise timing requirements of pre- and postsynaptic spiking in the induction of LTP/D, resulting in spike timing "windows" characteristic for different synapses in different parts of the brain (Bell et al., 1997; Markram et al., 1997; Bi and Poo, 1998; Debanne et al., 1998; Egger et al., 1999; Feldman, 2000; Sjostrom et al., 2001; Tzounopoulos et al., 2004). The temporal order of presynaptic followed by postsynaptic spiking in the induction of LTP in spike-timing dependent plasticity (STDP) generally follows the Hebbian rule that synapses are strengthened if presynaptic activity consistently drives postsynaptic acitivity (Bi and Poo, 2001). Conversely, when presynaptic spiking follows postsynaptic spiking, inputs are weakened, consistent with the idea that those inputs are not contributing to postsynaptic activity.

Hebbian plasticity is generally believed to lead to instability in the neuron because strengthening or weakening of a set of synapses facilitates further strengthening or weakening of more synapses in a positive feedback loop, leading to "runaway" excitation or quiescence. But if Hebbian plasticity is prone to instability, how can STDP, which appears to be a Hebbian form of plasticity itself, stabilize neuronal output? In a number of synapses, it has been found that the window for LTD induction is longer than that for induction of LTP (Bi and Poo, 1998; Debanne et al., 1998; Feldman, 2000; Li et al., 2004; Froemke et al., 2005). Consequently, during uncorrelated pre-and postsynaptic activity, LTD would on average predominate over LTP (Abbott and Nelson, 2000; Feldman, 2000). Similarly, during excessively high postsynaptic activity, the precise temporal order between pre-and postsynaptic activity would tend to break down,

as the postsynaptic cell spikes over an envelope of depolarizing presynaptic activity. Because of the uncorrelated activity, LTD would predominate, resulting in weakening of inputs and slowing of postsynaptic spiking, until individual inputs are barely able or unable to elicit postsynaptic spikes. Presynaptic activity would then more likely precede postsynaptic activity, because clusters of inputs would be required to drive the postsynaptic cell, thereby favoring LTP over LTD and providing a balance for the preference of LTD during uncorrelated activity. STDP thus results in neurons that tend to fire irregularly within a dynamic range of frequencies (Song et al., 2000).

STDP can also prevent runaway excitation between reciprocally connected excitatory neurons that would be predicted to occur from purely Hebbian positive feedback rules. Because the temporal order of strengthening and weakening of synapses is essentially antisymmetric in STDP, two neurons that are spiking in a particular order will tend to undergo strengthening of synapses between the leading and lagging neuron and weakening of synapses between the lagging and leading neuron. This will allow recurrent excitatory connections to form without the destabilizing effects of excitatory positive feedback loops (Abbott and Nelson, 2000).

Metaplasticity/ BCM Model for a Sliding Modification Threshold

Metaplasticity describes changes in the ability of a cell to undergo synaptic change over time and is also sometimes referred to as "plasticity of plasticity" (Abraham and Tate, 1997; Bear, 2003). These changes can be elicited by "extrinsic" factors such as circulating hormones or neuromodulatory input, or by "intrinsic" factors such as the history of pre- and postsynaptic activity (Abraham and Tate, 1997). The theoretical framework for metaplasticity was provided by a number of studies describing a model for visual cortical receptive field plasticity. In 1979, Cooper *et al.* suggested the existence of a certain threshold of postsynaptic activity, θ_m, above which active synapses would become strengthened, and below which active synapses would become weakened. In other words, highly correlated pre-and postsynaptic activity would lead to strengthening of those synapses, while weakly correlated activity would lead to weakening of synapses (Cooper et al., 1979). Bienenstock *et al.* then made the additional proposal in what is now known as the *B*ienenstock-*C*ooper-*M*unro (BCM) model that θ_m should be modifiable and should vary as a function of the history of integrated postsynaptic activity (Bienenstock et al., 1982).

The BCM model predicts for example that periods of prolonged increased activity should progressively shift the modification threshold upwards, making it more difficult to induce LTP, and at the same time easier to induce LTD. Such shifts in the modification threshold would have a stabilizing effect on postsynaptic activity over time, as they would tend to keep synapses within a dynamic range of modifiability by preventing saturation of LTP or LTD (Abraham and Tate, 1997).

Empirical evidence has provided support for the existence of such a "sliding" modification threshold for synaptic plasticity *in vivo*. Thus, a period of reduced visual cortical activity due to binocular visual deprivation shifts the LTP threshold to lower stimulation frequencies, which is reversed by restoring normal vision (Kirkwood et al., 1996). The molecular mechanism(s) underlying metaplasticity are still largely unknown, although activity-dependent changes in the NMDA receptor 2A/2B expression ratio seem to contribute to metaplasticity in the visual cortex (Philpot et al., 2007) and *in vitro* (Slutsky et al., 2004). In addition, it has been proposed that mechanisms underlying synaptic scaling could contribute to metaplasticity (Abbott and Nelson, 2000; Thiagarajan et al., 2005; Thiagarajan et al., 2006). Thus, long periods of inactivity *in vitro* lead to scaling up of both calcium permeable GluR1 homomeric AMPA receptors (Thiagarajan et al., 2005) and calcium-permeable NMDA receptors (Watt et al., 2000). The increased resulting synaptic calcium conductance would be predicted to lower the threshold for LTP induction (Abbott and Nelson, 2000). In other words, homeostatic mechanisms like synaptic scaling could "recruit" Hebbian mechanisms to contribute to stabilization of neuronal output by shifting the synaptic modification threshold to promote LTP or LTD.

Heterosynaptic Plasticity

Hebbian forms of plasticity like LTP and LTD are generally thought to be restricted to the synapses that are receiving the correlated pre-and postsynaptic activity. In some cases, however, this "homosynaptic" rule doesn't hold, so that changes in strength from correlated activity at one input result in opposite changes in strength at other inputs, termed heterosynaptic LTP/D. Such heterosynaptic plasticity has been described in the hippocampus (Lynch et al., 1977; Dunwiddie and Lynch, 1978; Abraham and Goddard, 1983; Bradler and Barrioneuvo, 1989; Scanziani et al., 1996b; Scanziani et al., 1996a), amygdala (Royer and Pare, 2003) and neocortex (Hirsch et al., 1992). It has been proposed to contribute to the stabilization of the neuron's output by maintaining the total

synaptic weight onto the neuron relatively constant (Royer and Pare, 2003). The site of induction and expression may occur in different populations of cells through intercellular signaling mechanisms, allowing for a widespread distribution of heterosynaptic plasticity and its potentially stabilizing effects (Scanziani et al., 1996b).

Heterosynaptic plasticity can also sometimes occur with the same polarity as the synapses undergoing homosynaptic change. Thus, LTP (Bonhoeffer et al., 1989; Schuman and Madison, 1994) and LTD (Staubli and Ji, 1996; Nishiyama et al., 2000) have been found to "spread" to other synapses in the same cell as well as neighboring cells, possibly through diffusible messangers, although the spread appears to be fairly limited in distance (~70 μm) (Engert and Bonhoeffer, 1997). Such heterosynaptic plasticity would obviously tend to destabilize the neuron's output, so that the ultimate role of heterosynaptic plasticity in contributing to homeostatic stability is still unclear.

Redistribution of Synaptic Efficacy

Short-term plasticity is a prominent feature of many synapses in the brain and describes changes in the efficacy of synaptic transmission during trains of input stimuli. At many neocortical and hippocampal synapses, short-term depression constitutes the predominant form of short-term plasticity and is at least in part thought to reflect a depletion of the pool of readily releasable vesicles (vesicles that are both docked and primed) at presynaptic release sites during a train of action potentials (Zucker and Regehr, 2002). As a consequence of short-term depression, responses to trains of stimuli become progressively smaller until they reach a steady state level for a particular input frequency, and then recover gradually after cessation of the train. Induction of LTP in some of those synapses has been shown to alter short-term plasticity, such that the response to the first spike in a train becomes significantly larger, while an increase in the rate of depression leads to a more rapid approach to the steady state response that is no different than before induction of LTP (Markram and Tsodyks, 1996b; Volgushev et al., 1997; Buonomano, 1999; Yasui et al., 2005). Thus, the potentiated reponse to the first spike in the train is offset by a more rapid rate of short-term depression. This type of plasticity is called redistribution of synaptic efficacy (RSE) (Markram and Tsodyks, 1996a, b), and because there is no overall gain in the synaptic response to trains of stimuli, it represents a form of plasticity that does not destabilize the network. The changes in short-term plasticity most likely reflect an increase in the probability of transmitter release (Markram and Tsodyks, 1996b).

As a result of RSE, there is an increased efficacy in synaptic transmission of transient input following periods of presynaptic inactivity. Recurrent networks can have occasional bursts of activity and yet remain stable during RSE, because prolonged periods of high activity are suppressed due to the increased short-term depression (Tsodyks et al., 2000).

Homeostatic Regulation of Neuronal Excitability

A neuron's excitability determines its input-output function, i.e. the number of spikes fired for a given strength of inputs, and thus represents an attractive target for homeostatic regulation (Schulz, 2006). By decreasing a neuron's excitability, the spiking output of a neuron can be efficiently reduced, irrespective of how strong its inputs are. Neuronal excitability is determined by passive properties such as resting membrane resistance, size of the neuron, and resting membrane potential. Additionally, excitability depends on voltage-dependent conductances, especially voltage-dependent sodium and potassium conductances.

A number of studies have provided evidence that neuronal excitability is under homeostatic control. For example, prolonged periods of increased or decreased activity bring about compensatory change in membrane excitability in hippocampal slice culture (Aptowicz et al., 2004; Karmarkar and Buonomano, 2006) and dissociated cortical culture (Desai et al., 1999). In addition, brief intense periods of activity rapidly reduce neuronal excitability in acute hippocampal slices (van Welie et al., 2004; Fan et al., 2005). In cerebellar granule cells, genetic disruption of a resting GABA-A conductance doesn't change excitability because of increase in a compensatory "leak" potassium conductance (Brickley et al., 2001). Similarly, genetic increase of an A-type potassium current in neurons of the lobster stomatogastric ganglion fails to affect overall neuron excitability due to increase of a compensatory H-type current (MacLean et al., 2003). In neurons of the crab stomatogastric ganglion, there is large inter-animal variability in several membrane conductances, but remarkably, the firing pattern from animal to animal is highly conserved (Schulz et al., 2006).

Location and Identity of Homeostatic Sensors

In order to prevent a cell from deviating from a certain set point or from falling outside of a target range, homeostatic mechanisms need to be able to sense the neuron's position with respect to the set point or target range and bring about compensatory changes if

the neuron deviates from those. But what is being sensed in neuronal homeostasis? And where are the sensors located? Most evidence supports the idea that homeostatic mechanisms sense and constrain the activity of a neuron within a physiologically useful operating range. Thus, while channel proteins, receptors, second messengers, and membrane components of a neuron turn over on the timescale of minutes, hours or days, neuronal activity is thought to remain relatively unchanged throughout the lifetime of the cell, which can be years or even decades (Marder and Prinz, 2002; Marder and Goaillard, 2006).

A number of studies have provided more direct evidence for the idea that activity is under homeostatic control. For example, when neurons of the crustacean stomatogastric ganglion are dissociated from descending modulatory inputs and plated individually in culture, their usual rhythmic bursting activity is initially silenced. Over the course of several days, however, these neurons resume their usual rhythmic bursting activity (Turrigiano et al., 1994; Thoby-Brisson and Simmers, 2000). Similarly, overexpression of an inwardly rectifying K channel In cultured hippocampal neurons initially causes a significant reduction in spike frequency, but by four days after transfection, the spike frequency is restored to that of control cells in the same culture (Burrone et al., 2002).

A possible sensor of activity in homeostatic plasticity is postsynaptic calcium levels, which are well correlated with neuronal activity (Ross, 1989; Goldberg and Yuste, 2005) and sit atop numerous biochemical signaling cascades affecting gene expression and function/trafficking of ion channels (Burgoyne et al., 2004). Some studies have linked intracellular calcium levels to the homeostatic regulation of neuronal excitability (LeMasson et al., 1993; Turrigiano et al., 1994). In hippocampal neurons, one study showed that KN93 (an inhibitor of the calcium/calmodulin-dependent family of kinases) prevents changes in frequency and amplitude of mEPSCs after activity blockade (Thiagarajan et al., 2002), suggesting that calcium might participate in homeostatic mechanisms through activation of calcium-dependent kinases.

Another question concerns whether homeostatic mechanisms operate cell autonomously, i.e. whether the sensor and the negative feedback response occur in the same cell. In the Burrone *et al.* study, overexpression of an inwardly rectifying K channel to dampen activity occurred in isolated cells that presumably had no effect on overall network activity (Burrone et al., 2002). The finding that these neurons recover their normal spiking output suggests that neurons can sense and regulate their activity level

cell-autonomously. However, there could be a number of different sensors, and different types of homeostatic mechanisms could each have their own sensors in separate locations. Thus, reducing global network activity results in upwards scaling of mEPSC amplitudes in a number of studies (Turrigiano et al., 1998; Burrone et al., 2002; Stellwagen and Malenka, 2006), but reducing activity in an individual cell after synapse formation does not (Burrone et al., 2002). Synaptic scaling thus does not seem to occur cell-autonomously. Rather, scaling in response to activity deprivation appears to require release of TNF-α from surrounding glial cells, possibly in response to changes in ambient glutamate levels from spillover (Stellwagen and Malenka, 2006). It is still unclear, however, whether glia sense and regulate specific synapses, or whether they scale synapses on a broader network level. In any event, these results suggest that different homeostatic mechanisms likely operate in parallel, and that they each utilize different sensors of neuronal activity that may be present in different locations. Finally, it should be noted that some of the mechanisms discussed in the last section (heterosynaptic plasticity, synaptic redistribution, and STDP) don't appear to require activity sensors at all, as their stabilizing properties arise from basic principles of their respective mechanisms.

Global versus Local Homeostatic Regulation

Whether homeostatic mechanisms act on individual synapses or on the neuron as a whole depends on the particular mechanism in question, and for some of the mechanisms, the issue is still unresolved. (For a more detailed discussion about the mechanisms themselves, see the section titled "Mechanisms of Homeostatic Change" above.)

Metaplasticity, as predicted in the BCM sliding threshold model, is thought to stabilize neuronal activity by changing the modification threshold for synaptic plasticity based on the history of the neuron's integrated activity (Abraham and Tate, 1997; Bear, 2003). High activity in the neuron makes it progressively more difficult to strengthen synapses further by LTP, thus preventing further increases in input strength. Changes in the modification threshold are thought to be set globally for the entire neuron.

STDP can stabilize a neuron's activity simply by providing a competitive framework for individual synapses to either be strengthened or weakened based on precise timing constraints of pre-and postsynaptic spike patterns. As such, it can be considered a synapse-specific mechanism. On the other hand, activity patterns in the

postsynaptic neuron globally affect conditions for STDP. Thus, elevated postsynaptic activity makes it less likely that any one input would be responsible for driving the postsynaptic cell (i.e. that a presynaptic spike would directly precede a postsynaptic spike), thereby reducing the chances of synaptic strengthening globally (Abbott and Nelson, 2000). In effect, the properties of STDP provide a real-time sliding global modification threshold for synaptic plasticity. This should be contrasted with the BCM sliding threshold, which integrates past activity over longer time periods.

Heterosynaptic plasticity can stabilize neuronal activity if it occurs with opposite polarity as the synapses undergoing homosynaptic change. Depending on the mechanism, this could occur globally or locally. In one elegant study, heterosynaptic LTD seemed to depend on an extracellular diffusible factor that activated PP1 in its targets. The substance was released after tetanic stimulation and seemed to act globally on all of the synapses in the stimulated cell as well as on synapses in neighboring cells (Scanziani et al., 1996b; Scanziani et al., 1996a).

Redistribution of synaptic efficacy increases early responses to a train of stimuli without increasing the overall response. It occurs only at those synapses undergoing LTP, and should thus be considered a local homeostatic mechanism.

Studies on synaptic scaling and regulation of vesicle release have typically been performed by pharmacologically altering network activity for a period of days in cultured neurons. Because such treatments affect all of the synapses in the culture, such experiments are ill-suited to address whether these homeostatic mechanisms act globally within a neuron or locally at the level of individual syanpses (Turrigiano and Nelson, 2004). Nevertheless, synaptic scaling is presumed to act globally, mostly due to theoretical considerations. Synaptic scaling uniformly changes the weights of all synapses by the same factor (i.e. it is multiplicative), which might be more readily achieved by a global mechanism. In addition, by acting globally and multiplicatively, synaptic scaling would act to preserve relative weights between synapses, making it potentially well suited to stabilize the highly localized synaptic plasticity due to correlated pre- and postsynaptic activity.

The little empirical evidence that bears on this question, however, seems to suggest that local rules may contribute to synaptic scaling. Following activity blockade in one study, GluR1-containing AMPA receptors showed increased insertion into synapses, even though the dendrites exposed to the activity blockade had been transected from

the soma (Ju et al., 2004). Thus, the molecules involved in synaptic scaling seem to be present locally in the dendrites, and can be activated by local activity sensors. Another, more rapid form of scaling of GluR1-containing AMPA receptors was described following blockade of NMDA receptor-mediated mEPSCs (using a cocktail of TTX and APV to block action potentials and NMDA receptors, respectively). This form of scaling could be induced by locally perfusing APV onto dendrites in the background presence of TTX. Intriguingly, the authors found an increase in GluR1 surface expression limited specifically to the region exposed to the perfused APV (spanning about 30-40 μm) (Sutton et al., 2006). Together, these studies suggest that synaptic scaling can exist over different time scales and may involve different induction mechanisms. In addition, at least some forms of synaptic scaling seem to act locally within dendrites to stabilize neuronal activity.

Like scaling, homeostatic regulation of presynaptic release properties has typically been studied in cultures by changing neuronal activity for a few days. Unlike synaptic scaling that manifests itself as a change in quantal amplitude (mEPSC amplitude), changes in presynaptic release are seen as a change in mEPSC frequency (Burrone et al., 2002; Thiagarajan et al., 2002) and are associated with changes in the readily releasable pool of vesicles (Murthy et al., 2001; Moulder et al., 2006). Because the pharmacological and genetic manipulations that have been used to study changes in presynaptic release properties affected the activity of all synapses equally, little is known about whether the rules that govern this type of homeostatic regulation are global or local. One study, however, showed that increasing or decreasing neuronal activity could lead to changes in the percentage of active synapses, which was quantified by the percentage of total synapses (identified by vesicular glutamate transporter immunoreactivity) that took up the dye FM 1-43 (Moulder et al., 2006). Interestingly, increasing activity in the culture by elevating extracellular potassium levels resulted in a decrease in the percentage of active synapses, without affecting the average size of active vesicle pools or the number of docked vesicles. In other words, following increased network activity, some of the synapses appeared to become inactive in an "all or none" fashion—some terminals became inactive, and others remained unchanged. To reconcile an absence of ultrastructural changes in the number of docked vesicles with a decrease in the readily releasable pool of vesicles, the authors suggest that synapses become inactive through unpriming of the docked vesicles (Moulder et al., 2006). This all

or none homeostatic response in presynaptic release following prolonged elevated activity suggests that this form of homeostasis is non-global (Ting et al., 2006). It is not immediately clear that it is local either, however, because the activity manipulation (increased extracellular K) should have affected all synapses equally. Future studies, for example that manipulate the activity of individual synapses, will be required to address the rules governing this type of homeostasis.

CONCLUSION

Homeostatic plasticity allows neurons to respond to their environment without risking detrimental extremes in activity. It is comprised of a host of different mechanisms that function over a range of different time scales to constrain neuronal activity within limits. Despite considerable advances in describing these mechanisms, many unresolved questions remain. The molecules underlying homeostatic plasticity, for example, are still largely unknown. Also still unclear is how the various homeostatic mechanisms that have been described interact with each other to stabilize neuronal activity and under what conditions *in vivo* these mechanisms become relevant.

Studies are now beginning to explore the role of homeostatic mechanisms in the prevention and induction of pathophysiology in the brain. Initial findings suggest that homeostatic plasticity is involved in the development of chronic hyperexcitability and focal epileptogenesis following post-traumatic loss of input to parts of the cortex (Houweling et al., 2005; Bausch et al., 2006; Trasande and Ramirez, 2007). Homeostatic mechanisms have also been implicated in cognitive deficits sometimes observed in infants and children with recurring seizures. These seizures induce homeostatic and neuroprotective processes that reduce glutamatergic neurotransmission including NMDA receptor-mediated transmission. While neuroprotective, decreased NMDA receptor-mediated synaptic transmission also reduces synaptic plasticity, and is thus hypothesized to underlie the observed cognitive deficits (Swann, 2004). From these findings, it is clear that homeostatic mechanisms are critical for normal neuronal function, but can also become maladaptive under special circumstances. To eventually be able to prevent such maladaptive instances, it will be necessary to learn the rules that govern induction, expression, and regulation of homeostatic plasticity in greater detail.

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Chapter 2

Role of Plk2 in Homeostatic Plasticity

ABSTRACT

Little is known about the molecular mechanisms of homeostatic plasticity, which is thought to keep spiking output of neurons within an optimal range in the face of changing ambient activity levels or changes in input strength due to Hebbian plasticity mechanism like LTP or LTD. We report that in hippocampal neurons experiencing heightened activity, the activity-inducible protein kinase, Polo-like kinase 2 (Plk2), was required for reducing quantal amplitude (synaptic scaling) and intrinsic membrane excitability—two principal compensatory mechanisms underlying homeostatic plasticity. In slice cultures exposed to elevated activity, blocking Plk2 function resulted in increased dendritic spine size and density as well as a "run-up" in synaptic strength that prevented subsequent LTP. Together, these results uncover a molecular mechanism for synaptic scaling and indicate that Plk2 may not only stabilize neuronal activity, but may also allow cells to remain plastic during conditions of chronically elevated activity.

INTRODUCTION

Long-term potentiation (LTP) and long-term depression (LTD) are examples of Hebbian-type synaptic plasticity, in which correlated patterns of activity in pre- and postsynaptic neurons lead to long-term changes in synaptic strength. This kind of plasticity is believed to be important for the precise development of neural networks and for memory formation (Malenka and Bear, 2004; Dan and Poo, 2006; Sigurdsson et al., 2006); however, the same mechanisms could also result in runaway excitation or depression of neurons. Homeostatic regulation of synaptic strength, such as "synaptic scaling", is generally invoked to prevent such positive-feedback destabilization. Homeostatic mechanisms are thought to provide compensatory negative feedback through global modulation of synaptic efficacy and membrane excitability to ensure that neurons remain within a suitable operating range of spiking activity (Marder and Prinz, 2002; Burrone and Murthy, 2003; Turrigiano and Nelson, 2004; Davis, 2006). Despite its presumed importance, little is known about the molecular mechanisms involved in either sensing perturbations from the neuron's operating range or in executing the negative feedback control. One likely possibility is that activity-dependent changes in gene expression are important for the homeostatic response. Indeed, long-term changes in neuronal activity induced by drug administration (Nedivi et al., 1993; Hevroni et al., 1998; Bui et al., 2006), genetic manipulation (Guan et al., 2005), or sensory deprivation (Majdan and Shatz, 2006; Tropea et al., 2006) affect transcription of a large number of genes in both mammals and flies.

One of those activity-regulated genes is Polo-like kinase 2 (Plk2; also known as serum-inducible kinase (SNK)), a member of the polo family of serine/threonine protein kinases. Polo-like kinases (Plks) share a common architecture consisting of an N-terminal kinase domain and a C-terminal Polo-box domain (PBD) that mediates autoinhibition of kinase activity as well as phosphorylation-dependent binding to substrates and docking proteins (Elia et al., 2003; Lowery et al., 2004). Although the closely related kinases Plk1 and Plk3 are well known as critical regulators of the cell cycle (for review, see Barr et al., 2004; van de Weerdt and Medema, 2006), Plk2 seems to have a limited role in cell division (Ma et al., 2003). Plk2 mRNA and protein levels are induced in post-mitotic neurons by synaptic activity on the timescale of hours (Kauselmann et al., 1999; Pak and Sheng, 2003). Plk3 (sometimes called FNK) is also expressed in postmitotic neurons and is inducible by activity (Kauselmann et al., 1999).

What is the role of Plk2 in neurons? One seems to be degradation of SPAR (Spine Associated RapGAP), a protein of the postsynaptic density (PSD) that interacts with the scaffolding molecule PSD-95 (Pak et al., 2001; Pak and Sheng, 2003). The physiological function of SPAR is undetermined, but SPAR promotes the growth of dendritic spines, the postsynaptic compartment of excitatory synapses, at least in part by inhibiting postsynaptic Rap signaling (Pak et al., 2001). Plk2 itself seems to be important for the regulation of spines, as its overexpression causes depletion of mature mushroom spines and the overgrowth of thin, filopodia-like spines (Pak and Sheng, 2003). But under what conditions is Plk2 active and what are the functional consequences of Plk2 induction and kinase activity for the physiology of the neuron?

Here, we identify a critical role for Plk2 in homeostatic plasticity. In hippocampal neurons, Plk2 was required for synapses to scale down quantal amplitude and dampen membrane excitability during prolonged periods of heightened activity. In hippocampal slice culture, inhibition of Plk2 function during synchronized bursting activity led to a "runup" in synaptic strength that prevented potentiation through LTP-inducing stimuli. Control cells with unblocked Plk2, in contrast, did not undergo a runup in synaptic strength, and continued to be modifiable by LTP induction protocols. These results suggest that the homeostatic functions of Plk2 can augment and promote Hebbian plasticity mechanisms by keeping synapses within a modifiable range.

EXPERIMENTAL PROCEDURES

DNA Constructs and Statistics

GFP-PBD was generated by PCR of the Plk2-PBD (aa332-682) followed by insertion into a β-actin driven GFP vector. For Plk2 RNAi, the following siRNA sequence was cloned into pSUPER (Brummelkamp et al., 2002): 5'-GCATAAGAGAAGCAAGATA-3'. Site directed mutagenesis was performed using "Quickchange" (Stratagene) and all constructs were verified by DNA sequencing.

Statistical Methods are described in the Figure Legends.

Drugs

The following antibody has been described: polyclonal Plk2 antibody (Pak and Sheng, 2003), The following antibody was purchased from commercial sources: monoclonal α-tubulin (B-5-1-2) antibody (Sigma), polyclonal GFP antibody (MBL).

The drugs used in this study were purchased from Sigma.

Sindbis Virus Preparation and Slice Infection

Plk2 was cloned into a modified pSinRep5 vector containing GFP preceded by an IRES2 internal ribosomal entry site. Sindbis virus was prepared according to the guidelines outlined by the manufacturer (Invitrogen). Briefly, DNA templates were transcribed using an *in vitro* transcription kit (Ambion), and then electroporated into BHK cells. 24-36 hours posttransfection, the supernatant was harvested and used for organotypic hippocampal slice infection by directly injecting virus into the CA1 region of the slice using a picospritzer II (Parker Instrumentation). Recordings were performed ~24 hours post-infection under visual guidance of the GFP signal.

Electrophysiology

Electrophysiological recordings were carried out from organotypic slice cultures as described (Sala et al., 2003). Recordings were performed at 1 day (GFP-PBD overexpression) and 3-4 days (all other constructs) after transfection in solution containing (in mM): 119 NaCl, 2.5 KCl, 4 $CaCl_2$, 4 $MgCl_2$, 26 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 0.1 picrotoxin, 0.002 2-chloroadenosine, gassed with 5% CO_2/95% O_2 at pH 7.4. For I-clamp and mini-EPSC recordings, 2-chloroadenosince was omitted, the $CaCl_2$ and $MgCl_2$ concentration were 2.8 mM and 2 mM, respectively, and recordings were carried out at 30-34°C. TTX (1 μM) was added to mini-EPSC recordings. LTP and

eEPSC recordings were carried out at RT. For I-clamp recordings, excitatory synaptic currents were blocked with APV (50 μM) and NBQX (10 μM); no picrotoxin was added to the perfusion. Whole-cell recordings were made simultaneously from a pair of CA1 pyramidal neurons, one transfected and one untransfected. For eEPSCs, presynaptic fibers were stimulated at 0.2 Hz with a bipolar electrode with single voltage pulse (200 μsec, up to 10 V) placed in stratum radiatum ~200 μm from recorded cells. LTP was induced by pairing 2 Hz stimulation with depolarization of the postsynaptic cell to 0 mV for 100 s.

For voltage clamp recordings, pipettes (2–4 MΩ) were filled with an internal solution containing (in mM): 115 cesium methanesulfonate, 20 CsCl, 10 HEPES, 2. 5 $MgCl_2$, 4 ATP disodium salt, 0.4 GTP trisodium salt, 10 sodium phosphocreatine, and 0.6 EGTA, at pH 7.25. For current clamp recordings, the internal solution contained (in mM): 100 potassium methanesulfonate, 20 KCl, 10 HEPES, 4 ATP magnesium salt, 10 sodium phosphocreatine, 0.03 GTP trisodium salt, pH 7.3.

Current clamp recordings to measure intrinsic excitability was performed as described (Karmarkar and Buonomano, 2006). Briefly, recordings were considered acceptable if the cells showed overshooting action potentials and their resting membrane potential did not vary by more than 5 mV. Additionally, the input resistance was required to be > 80 MΩ and not change by more than 15% during the course of the recording. All cells were held at a membrane potential of −60 mV. Intrinsic excitability of an individual cell was measured as the number of spikes in response to a series of six fixed 250-ms current injection steps (0.05, 0.1, 0.15, 0.2, 0.25, 0.3 nA). The number of spikes was averaged over three sweeps for each current intensity.

Whole-cell current and voltage-clamp recordings were made using a Multiclamp 700A amplifier (Axon Instruments). Voltage and current signals were filtered at 2 kHz and digitized at 10 kHz with a Digidata 1322A (Axon Instruments). Analysis of recordings was performed using Clampfit software (Axon Instruments).

Hippocampal Slice Culture, Transfection, and Immunostaining

Organotypic hippocampal slice cultures were prepared from postnatal 8 day old rats as described previously (Stoppini et al., 1991; Sala et al., 2003). Briefly, following anaesthetization and decapitation, brains were quickly removed and placed into an ice-cold sucrose-based dissection buffer containing (in mM): 238 sucrose, 2.5 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 5 $MgCl_2$, 1 $CaCl_2$. The hippocampi were dissected

out, cut into 350 μm thick slices using a McIlwain tissue chopper, and plated onto tissue plate inserts (Millipore) placed over MEM-based (Cellgro) culture medium supplemented with horse serum (Invitrogen), insulin (2μg/ml), and ascorbic acid (0.0012%), containing (in mM): 26 D-glucose, 5.8 $NaHCO_3$, 30 hepes, 2 $CaCl_2$, 2 $MgCl_2$. Slices were grown in an incubator set at 5% CO_2, 35° C, and transfected on DIV 5-7 (GFP-PBD) or DIV 3-5 (all other constructs) using a biolistic gene gun (Bio-Rad) (Lo et al., 1994) with 1.6 μm gold particles (~0.3 mg per cartridge) coated with cDNAs: GFP-PBD and GFP-CDK5-DN (100 μg), all other constructs (90 μg) together with a GFP-encoding vector (10 μg) for visualization. Recordings and fixation of slices occurred on DIV 6-8. Slices were fixed in 4% PFA, 4% sucrose in PBS over night, then cryoprotected in 30% sucrose in 0.1M phosphate buffer (pH 7.4) for 2 hours at RT, snap frozen on dry ice, thawed in PBS, and stained with GFP antibodies in GDB buffer (0.1% gelatin, 0.3% TX-100, 450 mM NaCl, 32% 0.1M Phosphate buffer [pH 7.4]).

Imaging and Quantitation

Images were acquired using an LSM510 confocal system mounted on an Axiovert 2M (Zeiss). For imaging of slices, a water-immersion 63X objective (NA 1.2) was used. Confocal Z-series image stacks encompassing entire dendrite segments were analyzed using MetaMorph software (Universal Imaging Corporation). For quantitation of spine size and density in slice cultures, measurements were made from the middle third (~70-100 μm in length) of the primary apical dendritic branch and averaged for each neuron.

RESULTS

Plk2 Prevents "Run up" of Synaptic Strength during Heightened Network Activity

Plk2 protein is elevated during periods of high activity in neurons (Pak and Sheng, 2003). To investigate the functional significance of Plk2, we made a dominant interfering construct consisting of the Plk2 PBD fused to GFP as a visual marker. We reasoned that, because the PBD is of critical importance for proper targeting and functioning of Plks during the cell cycle (Lowery et al., 2004), the Plk2-PBD overexpressed in neurons should block endogenous Plk2 function by competing for PBD binding sites on targets and substrates. Both the Plk1-PBD and the Plk3-PBD have been used to dominantly interfere with endogenous Plk1 and Plk3 during mitosis in yeast and mammalian cells (Song et al., 2000; Song and Lee, 2001; Seong et al., 2002; Jiang et al., 2006).

We transfected GFP-tagged Plk2-PBD ("GFP-PBD") into CA1 neurons of organotypic hippocampal slice cultures at 5-7 days in vitro (DIV5-7) and analyzed the effects on spine size and density ~24 hours later. In the presence of the $GABA_A$ receptor antagonist picrotoxin (PTX; 100 μM, 12-18 hours), which increases network activity and induced endogenous Plk2 expression, transfection of GFP-PBD resulted in significantly larger and more numerous spines than in cells transfected with GFP alone (Figure 1A-E and 2A). Under basal conditions (without PTX treatment), GFP-PBD had no effect on spine density or spine size, which is perhaps not surprising since Plk2 is expressed at low levels in unstimulated slice cultures (Figure 2A). By increasing spine size and density, GFP-PBD thus appears to act antagonistically to Plk2 overexpression, which results in thinning and depletion of spines (Pak and Sheng, 2003).

Endogenous Plk2 expression was induced by PTX in DIV 6-8 hippocampal slice cultures over the course of about 6-12 hours (Figure 2A). Such treatment causes synchronized bursting activity in these cultures (Figure 3) and promotes NDMA-dependent strengthening of CA3-to-CA1 synapses (Abegg et al., 2004). Consistent with this report, we found a steady increase in AMPA receptor (AMPAR)-mediated miniature excitatory postsynaptic currents (mEPSCs; measured in the presence of TTX) from CA1 pyramidal cells over the first 6-8 hours of PTX treatment. After that, mEPSC amplitudes failed to strengthen further over the next 6-10 hours, despite the continued presence of PTX (Figure 2B). The temporal profile of mEPSC amplitude suggests that synapses either reach a ceiling of potentiation after 6-8 hours or else homeostatic plasticity mechanisms are activated during ~6-18 hours under these conditions to maintain

Figure 1

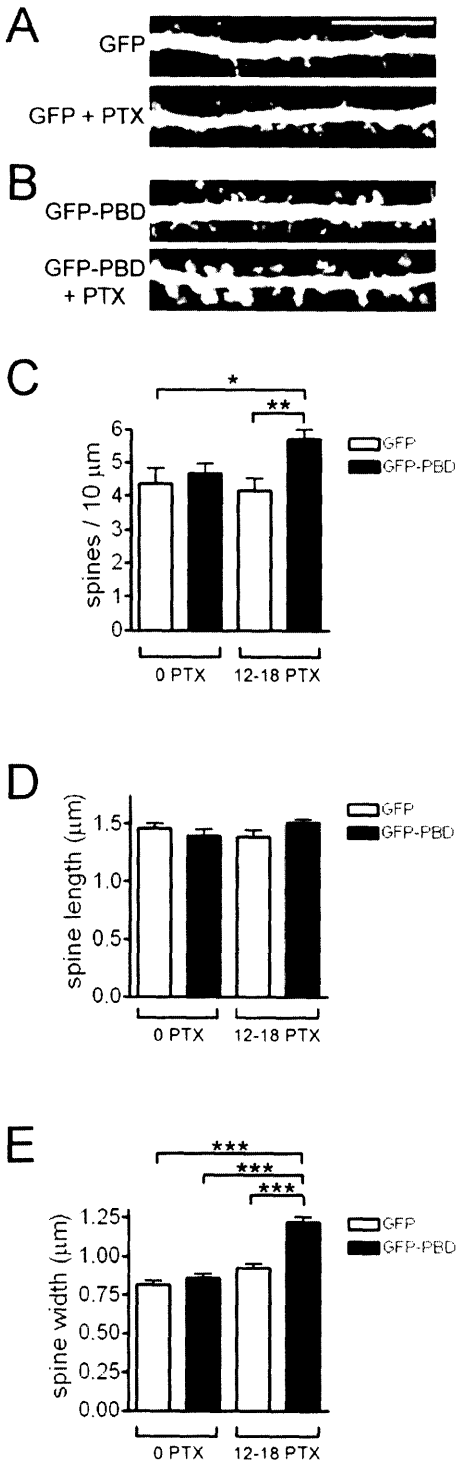
A
GFP
GFP + PTX
B
GFP-PBD
GFP-PBD
+ PTX
C
*
**
spines / 10 µm
0
1
2
3
4
5
6
GFP
GFP-PBD
0 PTX
12-18 PTX
D
spine length (µm)
0.0
0.5
1.0
1.5
GFP
GFP-PBD
0 PTX
12-18 PTX
E
***
***
***
spine width (µm)
0.00
0.25
0.50
0.75
1.00
1.25
GFP
GFP-PBD
0 PTX
12-18 PTX

Figure 1. Increased spine size and density due to GFP-PBD

CA1 pyramidal neurons from P7 organotypic hippocampal slice cultures (DIV5-7) were transfected biolistically with GFP (A) or GFP-PBD (B) and treated with picrotoxin (PTX, 100 μM; 12-18 hours) to increase network activity, as indicated. Slices were fixed ~24 hours after transfection on DIV6-8 and imaged for spine morphology. Histograms (mean ± SEM) of spine density (C), spine length (D), and spine width (E) are shown for the transfected constructs and conditions as indicated. N=6 cells (GFP), N=10 cells (GFP +PTX), N=9 cells (GFP-PBD), N=9 cells (GFP-PBD + PTX), *$p<0.05$, **$p<0.01$, ***$p<0.001$, One-way ANOVA. Scale bar in (A) measures 10 μm.

Figure 2

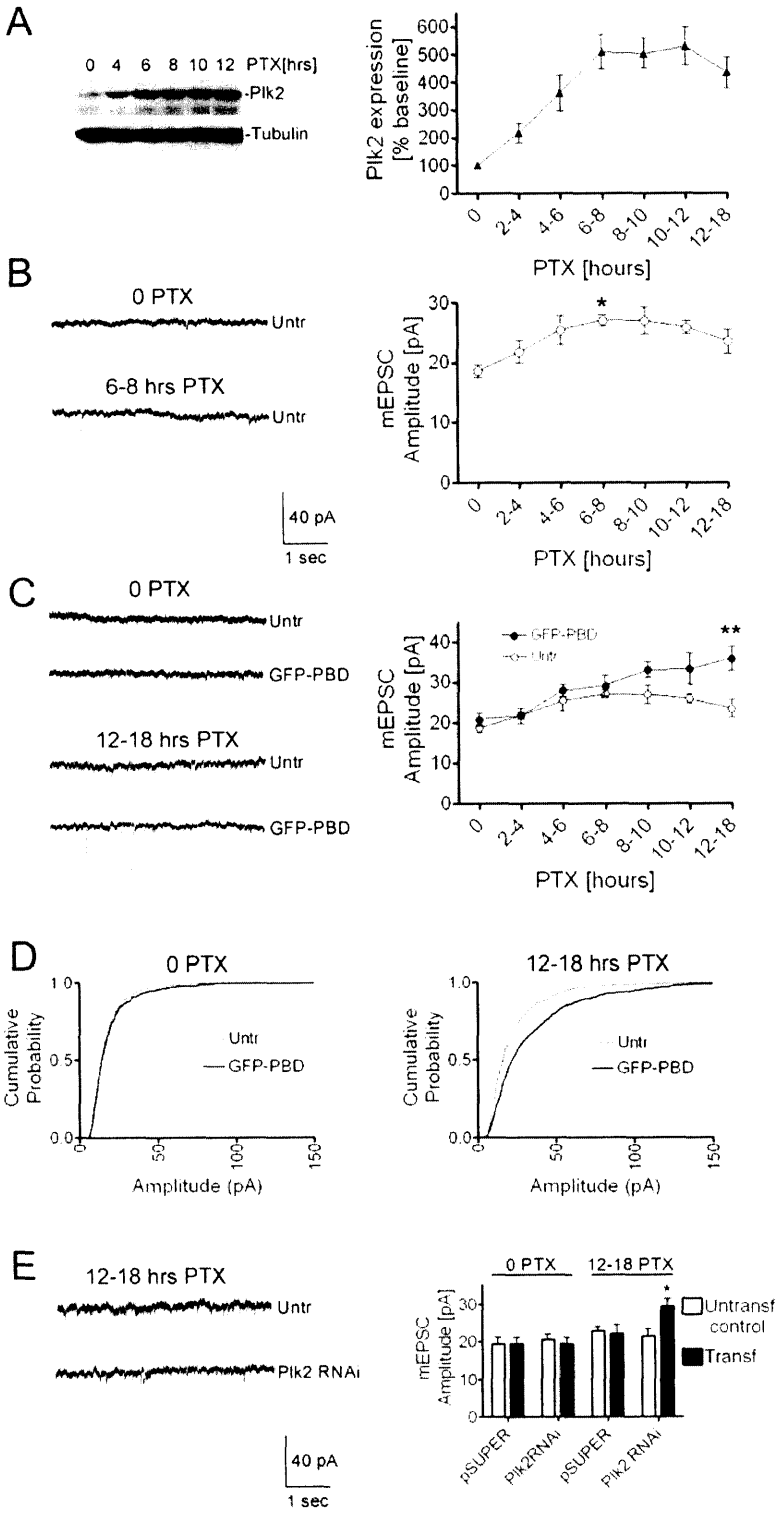
A
0 4 6 8 10 12 PTX[hrs]
-Plk2
-Tubulin
Plk2 expression [% baseline]
PTX [hours]
B
0 PTX
Untr
6-8 hrs PTX
Untr
40 pA
1 sec
mEPSC Amplitude [pA]
PTX [hours]
C
0 PTX
Untr
GFP-PBD
12-18 hrs PTX
Untr
GFP-PBD
GFP-PBD
Untr
mEPSC Amplitude [pA]
PTX [hours]
D
0 PTX
Cumulative Probability
Untr
GFP-PBD
Amplitude (pA)
12-18 hrs PTX
Cumulative Probability
Untr
GFP-PBD
Amplitude (pA)
E
12-18 hrs PTX
Untr
Plk2 RNAi
40 pA
1 sec
0 PTX
12-18 PTX
mEPSC Amplitude [pA]
Untransf control
Transf
pSUPER
Plk2RNAi
pSUPER
Plk2 RNAi

Figure 2. Plk2 prevents runup in synaptic strength during increased network activity

(A) Induction of Plk2 protein expression with increased neuronal activity. Hippocampal slice cultures (DIV6-8) were treated with PTX for indicated times (hours), and then immunoblotted for Plk2 and tubulin (Left). Quantification of Plk2 protein levels was carried out by densitometry (mean ± SEM), normalized to vehicle treated controls (Right). N=7-8 experiments for each time point.

(B) Time course of change in mEPSC amplitude during PTX treatment in hippocampal slice culture. Representative mEPSC traces (Left) from untransfected CA1 pyramidal cells in hippocampal slice cultures (DIV6-8) are shown with or without PTX treatment as indicated. mEPSC amplitudes (mean ± SEM) were plotted against duration of PTX treatment (Right). N=6 cells (0 PTX), N=8-10 cells (all other time points). At 6-8 hours PTX, but not at 12-18 hours PTX, mEPSC amplitude is significantly different from 0 PTX by One-way ANOVA, $p<0.05$.

(C) Time course of change in mEPSC amplitude during PTX treatment in neurons transfected with GFP-PBD for ~24 hours. Representative mEPSC traces (Left) from GFP-PBD transfected and untransfected CA1 pyramidal cells with and without PTX treatment as indicated. mEPSC amplitudes (mean ± SEM) were plotted against duration of PTX treatment (Right) for GFP-PBD transfected and untransfected neurons (replotted from G for comparison). N=5 cells (0 PTX), N=7-10 cells (other time points), **$p<0.001$ for mean amplitude of GFP-PBD vs Untr at 12-18 hours PTX, Two-way ANOVA.

(D) Cumulative frequency distributions of mEPSC amplitudes from GFP-PBD transfected and neighboring untransfected cells without PTX treatment (Left) and with 12-18 hours PTX (100 μM) (Right). The first 100 events from each cell were used to generate the distributions. N=same number of cells for each condition as in H.

(E) Effect of Plk2 RNAi on mEPSC amplitude during prolonged PTX treatment. Representative mEPSC traces (Left) are shown from untransfected CA1 pyramidal cells or cells transfected with a pSUPER based vector for 3-4 days driving expression of small hairpin RNA against Plk2 (Plk2 RNAi) following 12-18 hours of PTX treatment as indicated. mEPSC amplitudes are quantified (mean ± SEM) (Right). N=4 and 5 cells (0 PTX, for pSUPER and neighboring Untransfected, respectively), N= 6-7 cells for each other condition. *$p<0.05$, significantly different than untransfected control condition, Mann Whitney test.

Figure 3

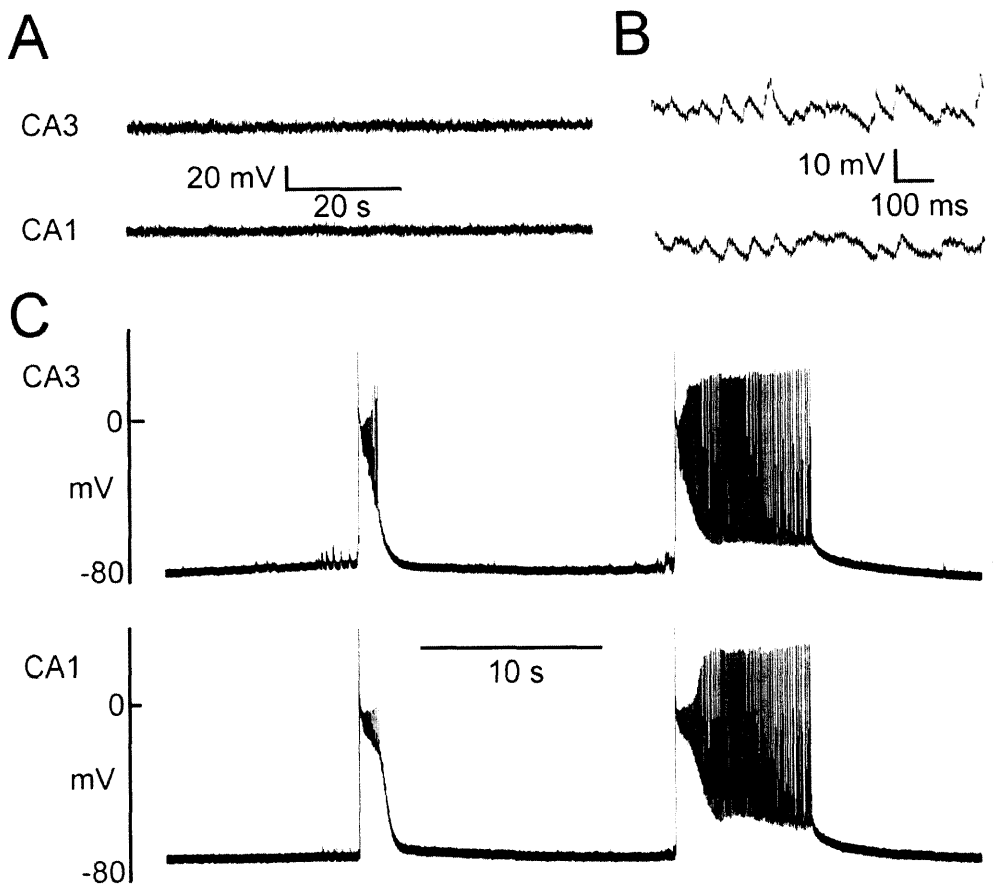

Figure 3. PTX causes Synchronized Bursting in Hippocapal Slice Culture

(A) Simultaneous current clamp recordings from CA3 and CA1 pyramidal cells before PTX treatment. The sample traces illustrate the lack of spiking observed under control conditions.

(B) Expansion of the last second of recording seen in (A). Recordings from CA3 and CA1 cells were expanded to illustrate the spontaneous subthreshold activity observed in these cells.

(C) Simultaneous current clamp recordings from CA3 and CA1 pyramidal cells about 5 minutes after onset of PTX (100 μM) treatment. Bursting was consistently synchronous, as illustrated in these traces.

synaptic strength in the normal range. Interestingly, the time of maximal mEPSC amplitude coincided roughly with the peak of endogenous Plk2 protein levels induced by PTX (at ~6-10 hours) (Figure 2, compare A and B). We recorded mEPSCs because the size of these quantal events are independent of exogenous stimulation of presynaptic fibers and thus can be compared in their absolute values across slices and conditions.

To test whether Plk2 contributed to the plateauing of mEPSC amplitudes during heightened network activity, we transfected the dominant interfering construct GFP-PBD into CA1 pyramidal cells for about ~24 hours. In contrast to neighboring untransfected cells in the same slice, neurons expressing GFP-PBD underwent progressive strengthening of mEPSCs that failed to plateau or decline during 18 hours of PTX treatment (Figure 2C, solid symbols). As a result, the mean mEPSC amplitude was significantly greater after 12-18 hours of PTX treatment in GFP-PBD transfected cells than in untransfected controls (Figure 2C). There was no increase in NMDA receptor (NMDAR)-mediated currents in GFP-PBD transfected cells, resulting in an increased AMPAR/NMDAR current ratio relative to untransfected cells after 12-18 hours of PTX (Figure 4). In addition, the cumulative probability distribution of mEPSC amplitudes was broadly shifted to higher values by GFP-PBD overexpression after 12-18 hours of PTX treatment (Figure 2D). These results show that Plk2 is required to prevent "runup" in synaptic strength during elevated activity and argue against the possibility that synapses in untransfected cells are simply plateauing in strength due to saturation.

To confirm and extend these results, we used a plasmid (pSUPER)-based RNA interference (RNAi) construct (Brummelkamp et al., 2002) to suppress expression of endogenous Plk2. After 12-18 hours of PTX, the mEPSC amplitude of cells transfected with pSUPER vector alone for 3-4 days was not significantly different from untransfected cells (Figure 2E). There was also no significant increase in mEPSC amplitude over unstimulated neurons, consistent with the homeostatic plateauing of synaptic transmission seen after 12-18 hours of PTX (see Figure 2B). RNAi knockdown of Plk2 for 3-4 days, however, resulted in significantly larger mEPSC amplitudes after 12-18 hours PTX, but notably had no effect in the absence of PTX treatment (Figure 2E). A control hairpin directed against the firefly luciferase gene had no effect on mEPSC amplitude relative to untransfected cells with or without PTX treatment (Chapter 3, Figure 8E). The RNAi results corroborate the dominant negative GFP-PBD findings showing that Plk2 is required to prevent "run-up" of synaptic strength during prolonged (>

Figure 4

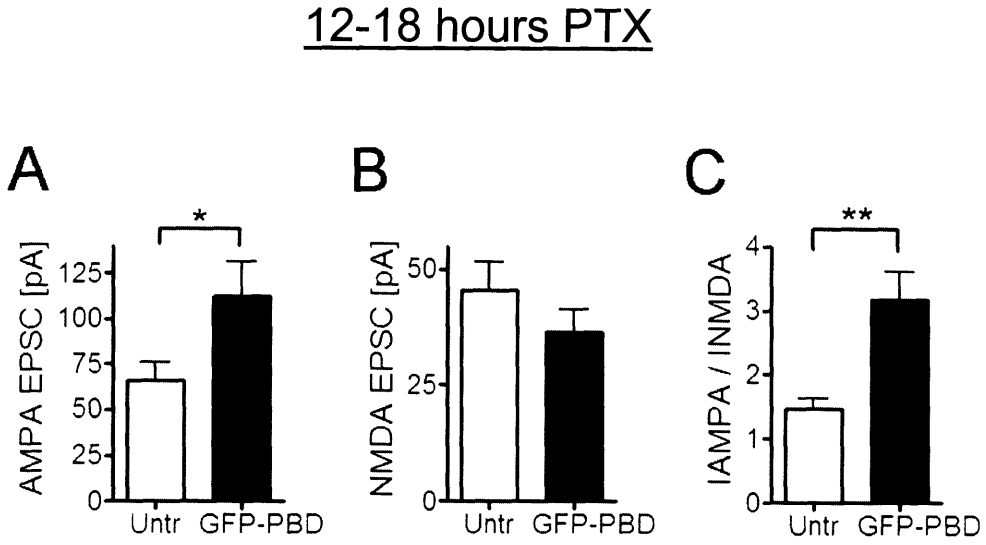
12-18 hours PTX
A
B
C
AMPA EPSC [pA]
125
100
75
50
25
0
*
Untr
GFP-PBD
NMDA EPSC [pA]
50
25
0
Untr
GFP-PBD
IAMPA / INMDA
4
3
2
1
0
**
Untr
GFP-PBD

Figure 4. PTX causes increased AMPA/NMDA ratio in slice culture

(A) Mean evoked AMPAR-mediated EPSC amplitudes from untransfected cells and cells transfected with GFP-PBD recorded from slices treated for 12-18 hours with PTX. Slices were transfected on DIV 4-6 and recordings were performed the next day under voltage clamp at -70 mV to isolate AMPAR-mediated currents. Currents were evoked by stimulating Schaeffer collateral pathway fibers.

(B) Average evoked NMDA receptor (NMDAR)-mediated EPSC amplitudes from the same cells as in (A), but recorded at +40 in the presence of NBQX to isolate NMDAR-mediated currents.

(C) Average ratio of AMPAR to NMDAR-mediated currents in untransfected cells and in cells transfected with GFP-PBD. Mean AMPAR and NMDAR-mediated currents used to calculate the current ratio are shown in (A-B).

8 hours) increased network activity. Thus Plk2 seems to participate in homeostatic plasticity in neurons subjected to high levels of excitation. Interestingly, however, in the absence of PTX treatment (when endogenous Plk2 expression is very low (Figure 2A)), overexpression of Plk2 had no effect on synaptic transmission (Figure 5). mEPSC frequencies were not significantly different between untransfected cells and cells transfected with GFP-PBD when plotted against PTX treatment duration, although cells transfected with GFP-PBD showed a trend toward mEPSCs with higher frequencies (Figure 6).

Plk2 is required for LTP during Increased Network Activity

We next recorded simultaneously from a pair of neighboring transfected and untransfected cells in the CA1 region of hippocampal slice cultures (DIV6-8), while eliciting evoked EPSCs (eEPSCs) by stimulating Schaffer collateral inputs from CA3 (Figure 7). Consistent with our mEPSC recordings, overexpression of the dominant-interfering construct GFP-PBD for ~24 hours had no effect on eEPSC amplitudes in basal conditions (no PTX) (Figure 7A and C). However, GFP-PBD led to significantly enhanced eEPSC amplitude following prolonged increased activity (12-24 hours PTX) (Figure 7B and C), consistent with disruption of synaptic homeostasis.

What are the physiological consequences of loss of Plk2-mediated synaptic homeostasis? In basal conditions, cells transfected with GFP-PBD showed robust LTP in response to a pairing protocol, similar in magnitude to neighboring untransfected cells that were recorded simultaneously (Figure 7D). After 12-24 hours of PTX treatment, untransfected cells in cultured hippocampal slices retained their ability to undergo LTP; however, LTP could no longer be induced in neighboring cells transfected with GFP-PBD (Figure 7E). Thus the function of Plk2 was required for cells to maintain the ability to undergo synaptic plasticity in the face of chronic hyperactivity.

Plk2 is not Required for LTD during Elevated Activity

How does Plk2 allow neurons to remain responsive to LTP-inducing stimuli? One possibility is that cells expressing GFP-PBD can't undergo LTP after increased activity because their synapses are already fully potentiated and thus occlude LTP. A number of observations suggest that this might be the case. Cells transfected with GFP-PBD underwent significant increases in spine size under these conditions (Figure 1 B, E), a change associated with LTP (Matsuzaki et al., 2004). These cells also showed a

Figure 5

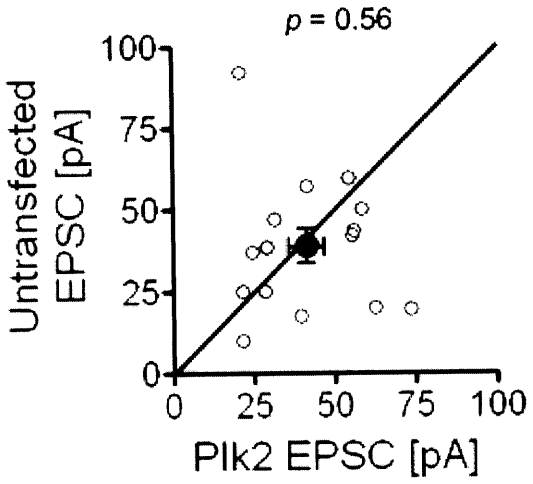
p = 0.56
100
75
50
25
0
0
25
50
75
100
Untransfected EPSC [pA]
Plk2 EPSC [pA]

Figure 5. Overexpression of Plk2 has no effect on synaptic transmission under basal conditions

CA1 neurons of DIV 6-8 hippocampal slice cultures were infected with Sindbis virus expressing Plk2 + GFP for ~24 hours. Evoked EPSC amplitudes (via stimulation of Schaffer collateral inputs) were plotted for individual pairs of transfected versus neighboring untransfected cells (open symbols). Filled circle represents the mean ± SEM. N=15 cell pairs; p value calculated by Mann Whitney test.

Figure 6

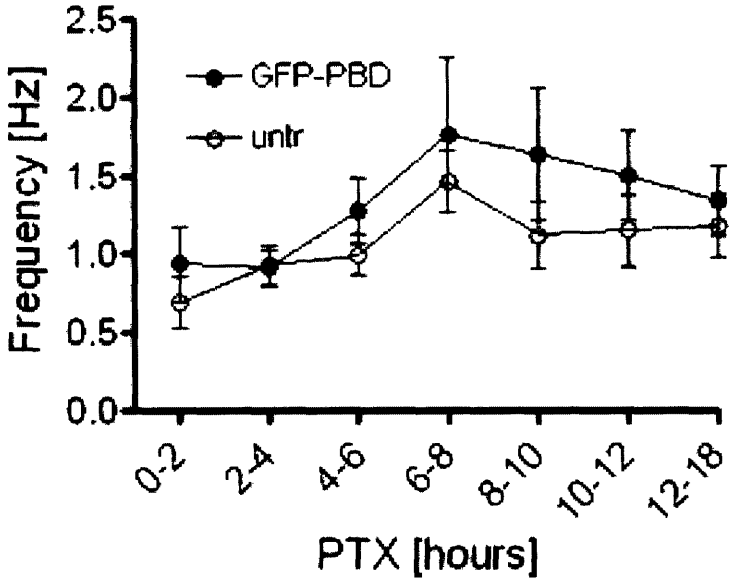
2.5
2.0
1.5
1.0
0.5
0.0
Frequency [Hz]
GFP-PBD
untr
0-2
2-4
4-6
6-8
8-10
10-12
12-18
PTX [hours]

Figure 6. Effect of Plk2 on mEPSC frequency

Time course of change in mEPSC frequency during PTX treatment in CA1 pyramidal cells of DIV6-8 hippocampal slice culture. mEPSC frequencies (mean ± SEM) were plotted against duration of PTX treatment for GFP-PBD transfected (~24 hours) and untransfected neurons. For GFP-PBD: N=5 cells (0 PTX), N=7-10 cells (other time points). For untransfected cells: N=6 cells (0 PTX), N=8-10 cells (all other time points).

Figure 7

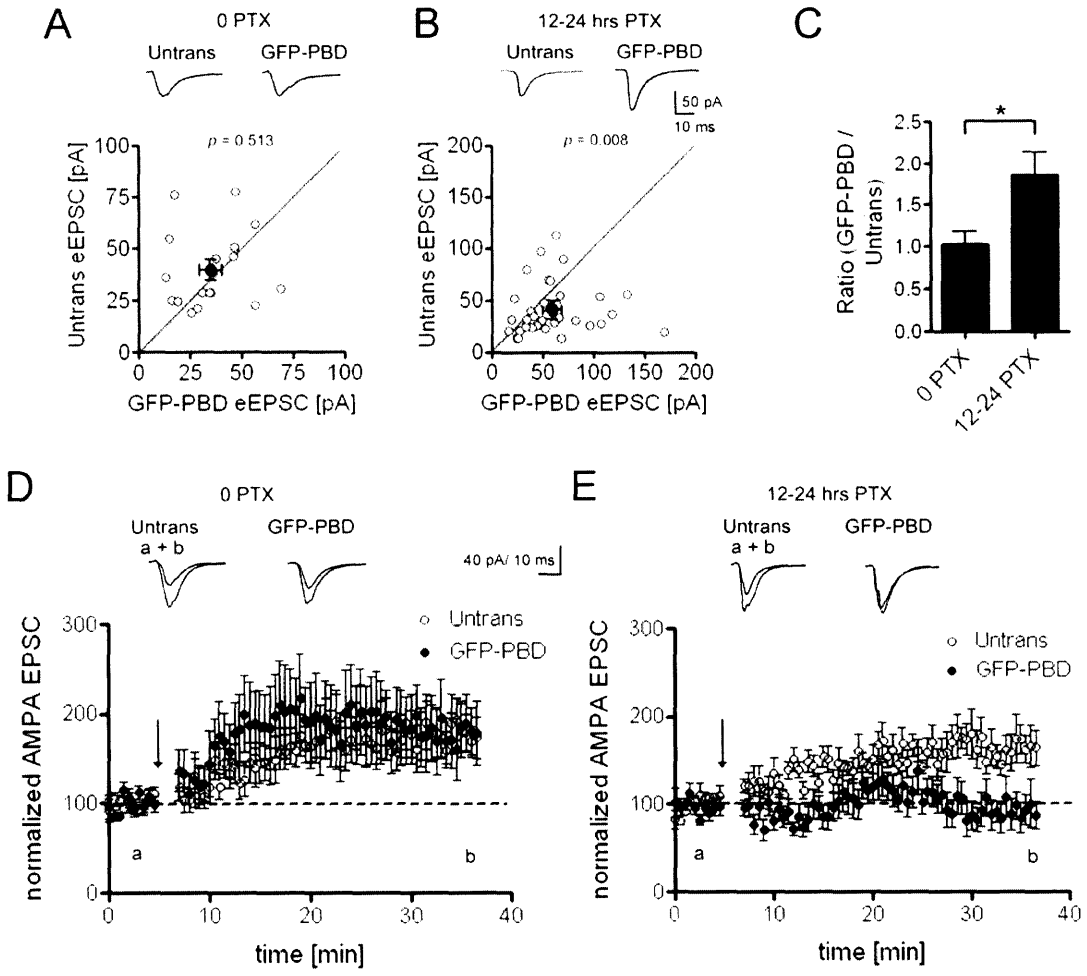
A
0 PTX
Untrans
GFP-PBD
p = 0.513
Untrans eEPSC [pA]
GFP-PBD eEPSC [pA]
B
12-24 hrs PTX
Untrans
GFP-PBD
50 pA
10 ms
p = 0.008
Untrans eEPSC [pA]
GFP-PBD eEPSC [pA]
C
Ratio (GFP-PBD / Untrans)
0 PTX
12-24 PTX
D
0 PTX
Untrans
a + b
GFP-PBD
40 pA/ 10 ms
Untrans
GFP-PBD
normalized AMPA EPSC
time [min]
E
12-24 hrs PTX
Untrans
a + b
GFP-PBD
Untrans
GFP-PBD
normalized AMPA EPSC
time [min]

Figure 7. Plk2 function is required in neurons undergoing increased activity for LTP induction

(A-B) Enhanced synaptic strength in GFP-PBD transfected CA1 pyramidal cells following 12-24 hours of picrotoxin (PTX, 10 μM). Sample evoked AMPA-EPSC traces (Top) are shown from pairs of transfected (GFP-PBD) and neighboring untransfected (Untrans) cells with (B) or without (A) prior treatment of slices with PTX for 12-24 hours. eEPSC amplitudes were plotted for each individual pair of transfected and neighboring untransfected cells in graphs (open circles). Filled circles represent the mean ± SEM. N=17 cell pairs (0 PTX), N=35 cell pairs (12-24 hours PTX), p values calculated by Mann-Whitney test.

(C) Comparison of the eEPSC ratios of GFP-PBD-transfected to neighboring untransfected cells obtained with or without pretreatment of slices with PTX (mean ± SEM). N=17 ratios (0 PTX), N=35 ratios (12-24 PTX), *$p<0.05$, Mann Whitney test.

(D-E) Impaired LTP in GFP-PBD-transfected CA1 pyramidal cells following 12-24 hours of PTX. LTP was induced by pairing depolarization to 0 mV with 2 Hz stimulation for 100 s. Sample AMPAR-mediated eEPSC traces (Top) are shown from transfected (GFP-PBD) and untransfected (Untrans) neurons without (D) or with (E) prior treatment with PTX for 12-24 hours. Graphs show time-course of EPSCs (mean ± SEM), normalized to baseline eEPSC amplitude before delivery of pairing protocol (arrow). Sample traces were obtained at time points indicated by a and b. N=7-8 cell pairs.

significant increase in the ratio of AMPA to NMDA receptor mediated currents after 12-18 hours of PTX relative to untransfected cells (Figure 4), which also occurs during LTP (Kauer et al., 1988; Muller et al., 1988; Liao et al., 1995; Heynen et al., 1996).

Induction of functional Plk2 in untransfected cells might then prevent such saturated potentiation by causing depression of those synapses through an LTD- or depotentiation-like mechanism. We tested this idea by recording from slices treated for 6-8 hours with PTX, a time point when Plk2 expression was already elevated, but before Plk2 had an effect on synaptic strength (see Figure 2). Both untransfected cells and cells transfected with the Plk2 dominant interfering construct GFP-PBD underwent robust depression in response to a pairing protocol that can induce both LTD and depotentiation (Zhu et al., 2002; Zhu et al., 2005) (Figure 8). Plk2 activity was therefore not required for LTD during periods of elevated activity,

Plk2 is Required for Synaptic Scaling

Because Plk2 caused a broad shift in the cumulative distribution of mEPSC amplitudes (Figure 2D), we wondered if Plk2 might be involved in synaptic scaling, which causes a similar shift in the distribution of mEPSC amplitudes (Turrigiano et al., 1998). To test this idea, we turned to dissociated cultures, which have typically been used in the study of synaptic scaling (Turrigiano et al., 1998; Stellwagen and Malenka, 2006). Dissociated hippocampal cultures were transfected with either GFP or GFP-PBD on DIV 19 and treated with PTX (100 μM) on DIV 20 for 48 hours. This shifted the cumulative distribution of mEPSC amplitudes towards lower values in both untransfected cells and cells transfected with GFP (Figure 9 A-B, D), consistent with previous reports (Turrigiano et al., 1998; Shepherd et al., 2006b; Stellwagen and Malenka, 2006). In contrast, overexpression of GFP-PBD to block endogenous Plk2 activity prevented this shift, and even resulted in a small increase in synaptic strength (Figure 9C-D). Neither GFP nor GFP-PBD had an effect on mEPSC amplitude in the absence of PTX treatment (Figure 9E). These results suggest that Plk2 is required for downwards scaling of synapses during heightened activity. Because scaling involves both AMPARs and NMDARs (Watt et al., 2000), Plk2-dependent scaling could contribute to the stabilization of synaptic strength during elevated activity by making it progressively more difficult to induce LTP.

Plk2 Reduces Neuronal Excitability during Increased Network Activity

In addition to regulating global synaptic efficacy, homeostatic mechanisms can stabilize

Figure 8

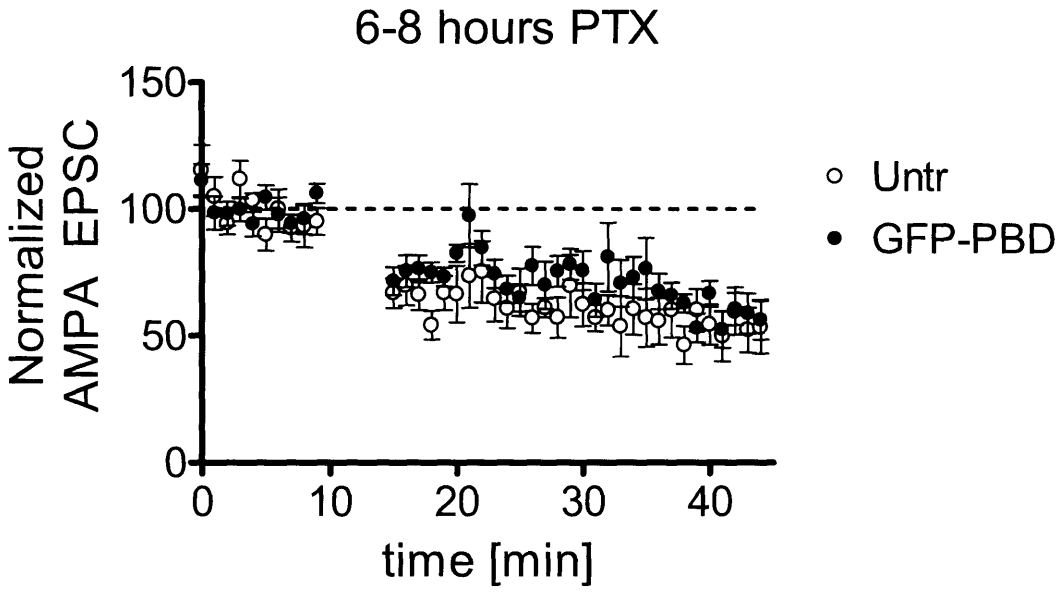
6-8 hours PTX
Normalized AMPA EPSC
150
100
50
0
0
10
20
30
40
time [min]
Untr
GFP-PBD

Figure 8. Plk2 is not required for Depression of Synapses during periods of elevated activity

Depression of synapses using a paired induction protocol. Hippocampal slice cultures (DIV 5-7) were transfected with GFP-PBD for one day and treated with PTX to elevate activity and induce Plk2 expression for 6-8 hours. Synapses were depressed in a pair of neighboring transfected and untransfected cells by pairing postsynaptic depolarization to -45 mV with 1Hz stimulation of presynaptic Schaeffer collateral fibers (300 pulses).

Figure 9

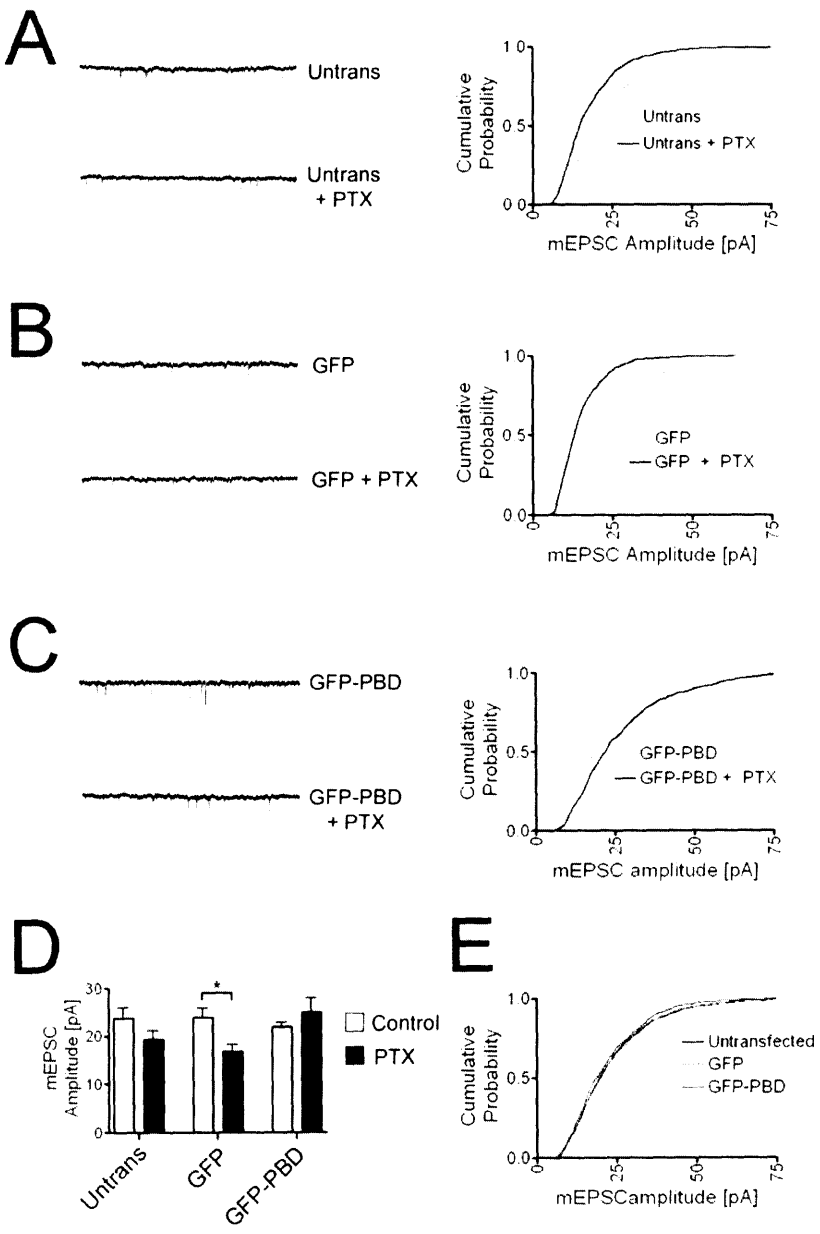
A
Untrans
Untrans + PTX
Cumulative Probability
1.0
0.5
0.0
Untrans
Untrans + PTX
0
25
50
75
mEPSC Amplitude [pA]
B
GFP
GFP + PTX
Cumulative Probability
GFP
GFP + PTX
mEPSC Amplitude [pA]
C
GFP-PBD
GFP-PBD + PTX
Cumulative Probability
GFP-PBD
GFP-PBD + PTX
mEPSC amplitude [pA]
D
mEPSC Amplitude [pA]
30
20
10
0
*
Control
PTX
Untrans
GFP
GFP-PBD
E
Cumulative Probability
Untransfected
GFP
GFP-PBD
mEPSCamplitude [pA]

Figure 9. Activity-dependent synaptic scaling

(A-B) Activity-dependent synaptic scaling in untransfected cells and in cells transfected with GFP. Dissociated hippocampal cultures were transfected on DIV19 and treated on DIV20 for 48 hours with PTX (100 μM). Sample traces (Left) show representative recordings. A cumulative distribution of mEPSC amplitudes (Right) shows the effect of PTX treatment. The first 100 events of each cell were used for the distribution. N=8 cells per condition. Distributions are significantly different between PTX-treated and untreated cultures, $p<0.001$, Kolmogorov-Smirnov test.

(C) Reversed effect of PTX treatment on mEPSC amplitude in cells transfected with GFP-PBD. N=8 cells per condition; Distributions are significantly $p<0.001$, Kolmogorov-Smirnov test.

(D) Summary graph showing average mEPSC amplitude from conditions in A-C. *$p<0.05$, Mann Whitney test. N=8 cells per condition.

(E) Cumulative distribution of mEPSC amplitudes from untreated cultures (no PTX).

neuronal networks by regulating the membrane excitability of cells (Daoudal and Debanne, 2003; Desai, 2003; Saar and Barkai, 2003; Zhang and Linden, 2003; Schulz, 2006). In CA1 pyramidal neurons of hippocampal slice cultures, a prolonged increase or decrease in network activity results in a homeostatic decrease or increase, respectively, of membrane excitability (Karmarkar and Buonomano, 2006). We took the input-output relationship of the number of action potentials elicited by depolarizing injections of increasing current intensities as our measure of membrane excitability (Figure 10). Treatment of DIV 6-8 hippocampal slices with PTX (12-18 hours) resulted in a decrease in membrane excitability (fewer spikes for each current injected) relative to untreated controls (Figure 10A, B, compare PTX and Control). However, neurons overexpressing the dominant-interfering construct GFP-PBD for ~24 hours did not show a decrease in membrane excitability with PTX treatment (Figure 10A, B, compare PTX+GFP-PBD and Control), indicating that Plk2 function is required for the homeostatic decline in membrane excitability during prolonged hyperactivity. As was the case for synaptic transmission, overexpression of the dominant negative GFP-PBD had no effect on membrane excitability in unstimulated slices (Figure 10A, B, compare GFP-PBD and Control). Plk2-dependent decreases in membrane excitability could—like synaptic scaling—contribute to synaptic stabilization by making it more difficult for synapses to become potentiated during chronically elevated activity

Figure 10

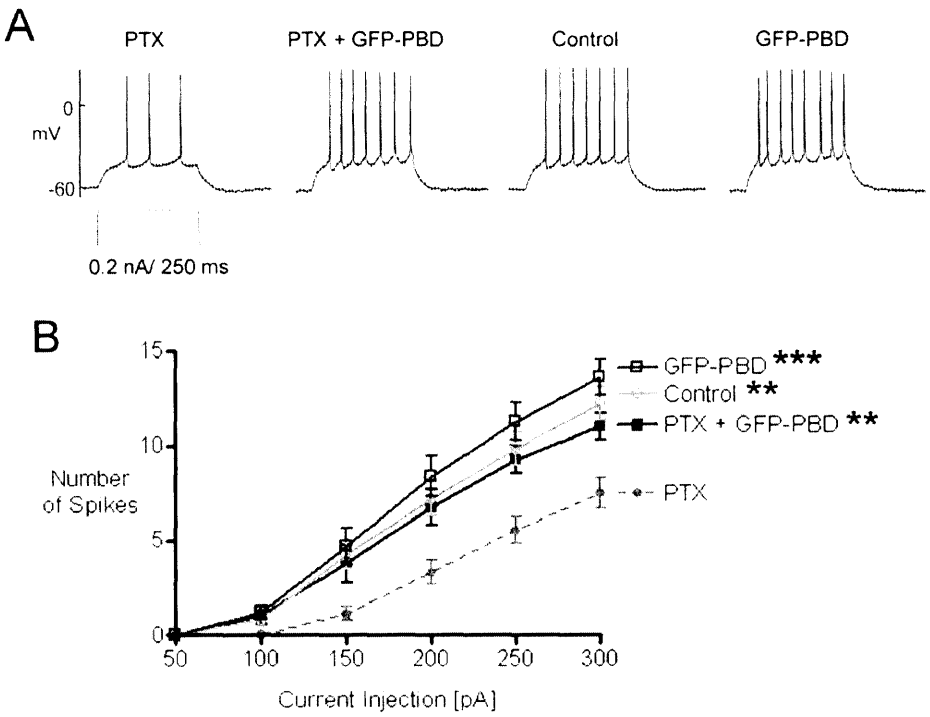
A
PTX
PTX + GFP-PBD
Control
GFP-PBD
0
mV
-60
0.2 nA/ 250 ms
B
15
10
5
0
Number of Spikes
50
100
150
200
250
300
Current Injection [pA]
GFP-PBD ***
Control **
PTX + GFP-PBD **
PTX

Figure 10. Plk2 is required for reduced membrane excitability following increased network activity

(A) Sample traces from CA1 pyramidal cells show spikes elicited by current injections of 200 μA for 250 ms. EPSCs were blocked with APV (50 μM) and NBQX (10 μM). Cells were either untransfected or transfected with GFP-PBD, and treated or not treated with PTX for 12-18 hours, as indicated.

(B) Input-output relationship (current injection versus number of spikes (mean ± SEM)) plotted for neurons transfected with GFP-PBD and/or treated with PTX (12-18 hours), as indicated. Control indicates untransfected neurons, not treated with PTX. PTX-treated cells show a decrease in excitability compared to all other groups, N=10-13 cells, $^{**}p<0.01$, $^{***}p<0.001$, curves significantly different than "PTX" curve, Two-way ANOVA.

DISCUSSION

Homeostatic plasticity has emerged as an important regulatory mechanism for stabilizing the activity of neurons and circuits. Among its proposed functions are prevention of epilepsy, balance of excitation/inhibition, stabilization of neurons during Hebbian plasticity, and regulation of spontaneous network activity during development (Turrigiano and Nelson, 2004; Davis, 2006; Gonzalez-Islas and Wenner, 2006). The molecular mechanisms underlying homeostatic plasticity, particularly in response to elevated activity, are still largely unknown. In this study, we show that the activity-inducible kinase Plk2 is a critical mediator of homeostatic plasticity in hippocampal neurons.

A few reports have addressed molecular mechanisms that contribute to homeostasis following chronic reduction of neuronal activity. Release of TNF-α from glia (Stellwagen and Malenka, 2006), BDNF (Rutherford et al., 1998), regulation of the β to αCaMKII ratio (Thiagarajan et al., 2002), and the activity-inducible gene Arc/Arg3.1 (Shepherd et al., 2006a) were shown to be important for upregulation of mEPSC amplitude and/or frequency following prolonged inactivity in neuron cultures. Hippocampal cultures from TNF and Arc/Arg3.1 knockout mice continued to display significant downwards scaling of mEPSCs in response to chronic increases in activity with $GABA_A$ receptor antagonists, leading the authors to conclude that mechanisms in addition to TNF-α and Arc/Arg3.1 are likely to be important in weakening of synapses following long-term increases in activity (Shepherd et al., 2006a; Stellwagen and Malenka, 2006). Our study points to Plk2 in this role. We suppose Plk2 is unlikely to be involved in homeostasis following hypoactivity, because Plk2 expression is already low in basal conditions and reducing it further by RNAi had no effect on synaptic transmission.

Key questions in homeostatic plasticity include: what are the molecules involved in sensing perturbations from a set point? And how are these transduced into a negative feedback response appropriate for the type and magnitude of the initial perturbation? A likely sensor of activity in homeostatic plasticity is postsynaptic calcium levels, which are well correlated with neuronal activity (Ross, 1989; Goldberg and Yuste, 2005), and sit atop numerous biochemical signaling cascades affecting gene expression and function/trafficking of ion channels (Burgoyne et al., 2004). Induction of Plk2 requires calcium entry through voltage-gated calcium channels and/or NMDA receptors, as well as activation of the calcium-calmodulin-sensitive protein phosphatase PP2B (calcineurin)

(Pak and Sheng, 2003). Thus, intracellular calcium elevation could provide the link between neuronal activity and Plk2.

Overexpressed Plk2 had little effect on synaptic transmission in unstimulated hippocampal slice cultures, but was sufficient to reverse (or prevent) runup in synaptic strength during 48 hours of elevated synchronous activity. One reason for the lack of effect in unstimulated cultures might be the requirement for available Plk2 binding sites in order for Plk2 to become active. The PBD exerts autoinhibition of kinase activity, which is relieved upon binding of the PBD to Plk binding sites (Elia et al., 2003). In Chapter 3, we describe the finding that "priming" phosphorylation of a Plk2 binding site by CDK5 is required for completion of this site, raising the intriguing possibility that Plk2 targeting and function can be controlled by the regulated recruitment of Plk2 to phosphorylated motifs. Plk2 activity would then require the coincidence of two conditions—prolonged elevated neuronal activity to induce Plk2 protein expression, as well as the availability of primed binding sites for the PBD, allowing for spatial and temporal refinement in the control of kinase activity. Another explanation for the ineffectiveness of Plk2 overexpression per se is that Plk2 might require further posttranslational modification (e.g. phosphorylation) to become active. All Plks share a conserved threonine residue within the T-loop region of their kinase domain and phosphorylation of this site increases kinase activity in Plk1 (Lee and Erikson, 1997; Qian et al., 1999; Jang et al., 2002). An upstream kinase for Plk1 has been found in Xenopus (Qian et al., 1998) and mammals (Ellinger-Ziegelbauer et al., 2000), raising the possibility that Plk2 could also require phosphorylation in neurons to become activated. In contrast to slice culture experiments, overexpression of Plk2 in dissociated hippocampal cultures resulted in thinning of spines and loss of synapses (Pak and Sheng, 2003) We speculate that this difference could be due to different basal activity levels in the different culture systems of the priming kinase CDK5 or of a T-loop activating kinase.

Plk2 participated in the homeostatic response to chronic increases in activity not only by dampening synapses, but also by lowering membrane excitability, suggesting it can directly or indirectly regulate intrinsic membrane conductances. It is unlikely that the effect on membrane excitability is entirely secondary to Plk2's synaptic effects, because weakening of synaptic inputs alone would be more likely to increase membrane excitability. Thus, Plk2 appears to orchestrate multiple aspects of the homeostatic

response, although it is still unclear by what mechanism Plk2 affects membrane excitability.

What is the physiological significance of Plk2-mediated homeostatic plasticity? Our data indicate that Plk2 prevents runup in synaptic strength during chronically elevated levels of network activity, thereby keeping cells within a modifiable range with respect to Hebbian plasticity mechanisms like LTP. We believe that the runup in synaptic strength seen in cells with blocked Plk2 activity prevents LTP induction by occluding it, i.e. the runup represents LTP. This is based on the finding that, during elevated activity, these cells show increased spine size and an increased AMPAR to NMDAR current ratio, both of which occur during LTP (Kauer et al., 1988; Liao et al., 1995; Matsuzaki et al., 2004). Thus, we speculate that Plk2 allows synapses to remain plastic by preventing them from becoming fully potentiated during chronically elevated activity.

This idea is consistent with our finding that Plk2 was involved in synaptic scaling and caused decreased membrane excitability during elevated activity. With decreased membrane excitability, inputs would have a harder time driving the postsynaptic cell to fire, which would increase the threshold for inducing LTP and could thus contribute to prevention of LTP. Similarly, synaptic scaling has been proposed to increase the threshold for induction of LTP during increased activity (Abbott and Nelson, 2000). Thus, chronically altered activity levels are known to scale both AMPAR and NMDAR mediated glutamatergic transmission (Watt et al., 2000), and a reduction in NMDA receptor content following elevated activity would be predicted to increase the threshold for LTP induction. Plk2-dependent changes in membrane excitability and synaptic composition could thus constitute a molecular means for ambient activity levels to be translated into a changing synaptic modification threshold, similar to what has been proposed in the BMC sliding threshold model for ocular dominance plasticity (Abraham and Tate, 1997; Bear, 2003).

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Chapter 3

CDK5-Dependent Regulation of Plk2

ABSTRACT

Polo-like kinases (Plks) are induced by elevated neuronal activity in the brain and have homeostatic effects on synaptic strength and plasticity (see chapter 2). The mechanisms by which neuronal Plks are recruited to target sites to ensure spatiotemporal specificity and selectivity of kinase activity are however still poorly understood. We report that binding of Polo-like kinase 2 (Plk2) to its target protein SPAR (Spine-Associated RapGAP) was dependent on upstream "priming" phosphorylation of SPAR by CDK5. Mutation of this phosphorylation site prevented binding by Plk2 and inhibited Plk2-dependent degradation of SPAR in heterologous cells. Inhibition of endogenous CDK5 activity in neurons reduced phosphorylation of the Plk2 binding site in SPAR, increased SPAR expression, and prevented homeostatic plasticity in slices. Overexpression of SPAR increased synaptic strength and knockdown of SPAR by RNAi decreased synaptic strength, suggesting that Plk-2 dependent regulation of SPAR expression could underlie Plk2-mediated control of synaptic strength. Together, these data suggest that "priming" phosphorylation of the Plk2 binding site in SPAR by CDK5 constitutes a mechanism for regulated Plk2 recruitment to synaptic sites that—together with the inducibility of Plk2 by enhanced activity —allows for precise spatial and temporal control of Plk2 kinase activity in neurons.

INTRODUCTION

Polo-like kinase 2 (Plk2) is induced by elevated neuronal activity and leads to depletion of mature spines and synapses and an increase in immature filopodia-like protrusions (Kauselmann et al., 1999; Pak and Sheng, 2003). During periods of elevated activity in hippocampal slice culture, Plk2 function is required to subdue membrane excitability and scale down synaptic strength—two principal means of homeostasis in neurons. Moreover, Plk2 activity is required for chronically stimulated neurons to remain responsive to LTP-inducing stimuli, suggesting that the homeostatic functions of Plk2 allow cells to remain within their useful dynamic range of excitability (see chapter 2). The mechanisms of Plk2 recruitment to target sites and regulation of kinase activity are however still poorly understood.

Polo-like kinases (Plks) share a common architecture consisting of an N-terminal kinase domain and a C-terminal Polo-box domain (PBD) that mediates autoinhibition of kinase activity as well as phosphorylation-dependent binding to substrates and docking proteins (Elia et al., 2003b; Lowery et al., 2004). The molecular basis underlying the function of the C-terminal PBD was recently elucidated, revealing that it acts as a phospho-peptide binding domain with preference for the consensus sequence [Ser]-[phospho-Ser/phospho-Thr]-[Pro] (Elia et al., 2003a; Elia et al., 2003b). Proline-directed kinases like CDKs (cyclin-dependent kinases) and MAP kinases could thus act as "priming" kinases to generate PBD binding sites on substrates and docking proteins. Indeed, several recent reports have found evidence for such priming kinase activity in the regulation of Plk function in the cell cycle. For example, cdc2/CDK1 acts as priming kinase to promote the interaction of Plk1 with several cell cycle related proteins, including the centrosome protein Cep55 (Fabbro et al., 2005), the cytokinesis regulator ECT2 (Niiya et al., 2006), and the kinetochore associated protein Bub1 (Qi et al., 2006).

In this study, we report that CDK5 acted as a priming kinase for the phospho-dependent binding between Plk2 and its neuronal target SPAR (Spine Associated RapGAP), a protein of the postsynaptic density (PSD) that interacts with the scaffolding molecule PSD-95 (Pak et al., 2001; Pak and Sheng, 2003). Phosphorylation of the Plk2 binding site in SPAR was required for Plk2-dependent degradation of SPAR and inhibition of CDK5 kinase activity boosted SPAR expression in neurons and prevented homeostatic plasticity in slice culture. Overexpression of SPAR strengthened synapses and RNAi knockdown of SPAR weakened synapses, consistent with the idea that regulation of SPAR levels is an important mechanism of Plk2-mediated homeostatic

plasticity. Together these findings suggest that phosphorylation of SPAR by CDK5 and subsequent recruitment of activity-induced Plk2 to synapses constitutes a "coincidence" mechanism allowing precise temporal and spatial regulation of Plk2 kinase activity in neurons.

EXPERIMENTAL PROCEDURES

DNA Constructs and Statistics

GFP-PBD was generated by PCR of the Plk2-PBD (aa332-682) followed by insertion into a β-actin driven GFP vector. rtTA-responsive Plk2 was generated by PCR of myc-tagged full length Plk2 from pGW1, followed by insertion into pTRE. For Plk2 RNAi, the following siRNA sequence was cloned into pSUPER (Brummelkamp et al., 2002): 5'-GCATAAGAGAAGCAAGATA-3'. Site directed mutagenesis was performed using "Quickchange" (Stratagene) and all constructs were verified by DNA sequencing. GFP-CDK5, GFP-CDK5-DN, and GFP-p35 were gifts of Li-Huei Tsai. CA-MEK1 was a gift from Myung Jong Kim. CA-JNK (HA-JNKK2-JNK1) was a gift from Natasha Hussain. The following constructs have been described: Kv1.4 C terminus in pBHA, and PSD-95 PDZ 1/2 in pGAD10 (Kim et al., 1995), Act2 in pBHA, Plk2-PBD in pGAD, myc-tagged SPAR in pGW1, GST-tagged Plk2-PBD in pGEX (Pak et al., 2001; Pak and Sheng, 2003), □-actinin-2 (A2.10) in pGAD (Wyszynski et al., 1997). siRNA sequences for SPAR (5'-CCGCCTGAAGTCTCTGATTAA-3') and firefly luciferase (5'-CCGCCTGAAGTCTCTGATTAA-3') in pENTR-Mir U6 vectors were gifts from Wade Harper.

Statistical Methods are described in the Figure Legends.

Antibodies, A-beta(1-40), and Drugs

Phospho-specific α-pS1328 SPAR antibodies were generated by immunizing rabbits (Covance Research Products) with a SPAR peptide corresponding to aa 1323-1335, with substitution of S1328 to a phosphorylated serine. Phospho- antibodies were purified first by negative selection over a column of covalently attached unphosphorylated peptide, followed by positive selection on a column of phosphorylated peptide. Polyclonal GKBD-SPAR antibodies were generated by immunizing rabbits with purified MBP-GKBD fusion protein.

The following antibody has been described: polyclonal C-SPAR antibody (Pak et al., 2001), mouse monoclonal PSD-95 antibody K28/43 was a gift from J. Trimmer (UC Davis). The following antibodies were purchased from commercial sources: mouse monoclonal myc antibody (9E10), myc agarose conjugate, polyclonal GST (Z-5) antybody (Santa Cruz Biotechnology), monoclonal α–tubulin (B-5-1-2) antibody (Sigma), polyclonal GFP antibody (MBL).

A-beta(1-40) was purchased from American Peptide Company and solubilized and used as described (Roselli et al., 2005).
The drugs used in this study were purchased from Sigma.

Yeast Two-Hybrid

Two-hybrid assays were performed using the yeast strain L40 harboring β-gal and HIS3 reporters, as described (Niethammer and Sheng, 1998).

HEK293 Transfection, Doxycycline Timecourse, Immunopreciptation, and GST-"Pulldowns"

HEK293 cells were transfected using Lipofectamine (Gibco BRL). For GST-"pulldown" and immunoprecipitation assays, cells were harvested in RIPA buffer, and lysates centrifuged at >15,000 x g for 30 minutes. For immunopreciptations, myc-antibody (9E10)-coupled agarose, and for "pulldowns", GST, GST-tagged Plk2-PBD, or GST-tagged Plk2-PBD(W504F) coupled to glutathione sepharose were mixed with supernatants for 3 hr at 4°C. After washing 5X in RIPA buffer, immunoprecipitates were analyzed by immunoblotting. For doxycycline timecourse experiments, doxycycline was applied the day after transfection and was staggered so that all treatment periods terminated at the same time. In the case of additional roscovitine or DMSO treatment, all wells were treated at the time point of doxycycline commencement, i.e. 8 hours before lysis.

Peptide precipitation experiments

BL21 *E.coli* expressing GST-PBD were lysed by sonication in ice-cold PBS, followed by addition of TX-100 (1% final concentration) and shaking at 4°C for 20 min. Lysates were centrifuged at > 15,000 x g for 30 min and supernatants were then incubated with cross-linked agarose beads coupled to either the phosphorylated pS1328 peptide or the unphosphorylated S1328 peptide for 3 hr at 4°C under gentle agitation. Peptide coupling was performed using the SulfoLink kit (Pierce). After washing 5X in lysis buffer (PBS, 1% TX-100), precipitated protein was analyzed by coomassie staining and immunoblotting.

Electrophysiology

Electrophysiological recordings were carried out from organotypic slice cultures as described (Sala et al., 2003). Recordings were performed at 3-4 days after transfection

in solution containing (in mM): 119 NaCl, 2.5 KCl, 4 $CaCl_2$, 4 $MgCl_2$, 26 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 0.1 picrotoxin, 0.002 2-chloroadenosine, gassed with 5% CO_2/95% O_2 at pH 7.4. For mini-EPSC recordings, 2-chloroadenosince was omitted, the $CaCl_2$ and $MgCl_2$ concentration were 2.8 mM and 2 mM, respectively, and recordings were carried out at 30-34°C. TTX (1 μM) was added to mini-EPSC recordings. Whole-cell recordings were made simultaneously from a pair of CA1 pyramidal neurons, one transfected and one untransfected. For voltage clamp recordings, pipettes (2–4 MΩ) were filled with an internal solution containing (in mM): 115 cesium methanesulfonate, 20 CsCl, 10 HEPES, 2. 5 $MgCl_2$, 4 ATP disodium salt, 0.4 GTP trisodium salt, 10 sodium phosphocreatine, and 0.6 EGTA, at pH 7.25.

Whole-cell voltage-clamp recordings were made using a Multiclamp 700A amplifier (Axon Instruments). Current signals were filtered at 2 kHz and digitized at 10 kHz with a Digidata 1322A (Axon Instruments). Analysis of recordings was performed using Clampfit software (Axon Instruments).

Hippocampal Slice Culture and Transfection

Organotypic hippocampal slice cultures were prepared from postnatal 8 day old rats as described previously (Stoppini et al., 1991; Sala et al., 2003). Briefly, following anaesthetization and decapitation, brains were quickly removed and placed into an ice-cold sucrose-based dissection buffer containing (in mM): 238 sucrose, 2.5 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 5 $MgCl_2$, 1 $CaCl_2$. The hippocampi were dissected out, cut into 350 μm thick slices using a McIlwain tissue chopper, and plated onto tissue plate inserts (Millipore) placed over MEM-based (Cellgro) culture medium supplemented with horse serum (Invitrogen), insulin (2μg/ml), and ascorbic acid (0.0012%), containing (in mM): 26 D-glucose, 5.8 $NaHCO_3$, 30 hepes, 2 $CaCl_2$, 2 $MgCl_2$. Slices were grown in an incubator set at 5% CO_2, 35° C, and transfected on DIV 3-5 using a biolistic gene gun (Bio-Rad) (Lo et al., 1994) with 1.6 μm gold particles (~0.3 mg per cartridge) coated with cDNAs: GFP-CDK5-DN (100 μg), all other constructs (90 μg) together with a GFP-encoding vector (10 μg) for visualization. Recordings occurred on DIV 6-8.

Dissociated Neuron Culture, Transfection, and Immunostaining

Medium-density dissociated hippocampal or high density cortical cultures were prepared and cultured from E19 Long Evans rat hippocampi as previously described (Pak et al, 2001). Briefly, 150-200 cells mm^{-2} (hippocampal culture) or 1000 cells mm^{-2} (cortical

culture) were plated on 19 mm coverslips coated with 30 µg/mL PDL and 2 µg/mL laminin. Transfections of hippocampal cultures were performed with Lipofectamine 2000 (Invitrogen) at DIV 16, and cells were fixed at DIV 19 in 1% PFA, 4% sucrose in PBS for 2 min at room temperature followed by -20°C MetOH for 10 min for staining of endogenous proteins. Immunostaining was performed in GDB buffer (0.1% gelatin, 0.3% TX-100, 450 mM NaCl, 32% 0.1M Phosphate buffer [pH 7.4]).

Imaging and Quantitation

Images were acquired using an LSM510 confocal system mounted on an Axiovert 2M (Zeiss). For imaging of dissociated cultures, an oil-immersion 63X objective (NA 1.4) was used. Confocal Z-series image stacks encompassing entire dendrite segments were analyzed using MetaMorph software (Universal Imaging Corporation). For measurements of SPAR immunofluorescence intensity as a function of distance from the soma, concentric circles increasing in 10 µm steps were traced around the soma, and three to four dendritic segments of 90 µm were analyzed from thresholded images. For each condition, integrated SPAR immunofluorescence intensity levels from distinct dendrites for each distance (i.e. dendritic segments bound by the same two circles) were first grouped and averaged per neuron. Means from several neurons were then averaged to obtain a population mean and normalized to the 10 µm population mean in vehicle-treated controls (presented as mean ± SEM). SPAR immunofluorescence intensity levels from transfected cells are from thresholded images and represent integrated intensity measurements per unit length of dendrite. SPAR immunofluorescence intensity levels from three to four dendritic segments of 90 µm were collected and averaged per neuron. Average SPAR immunofluorescence intensities for each transfected cell were normalized to values obtained from nearby untransfected cells and then averaged with cells transfected with the same construct to obtain a population mean (presented as mean ± SEM).

RESULTS

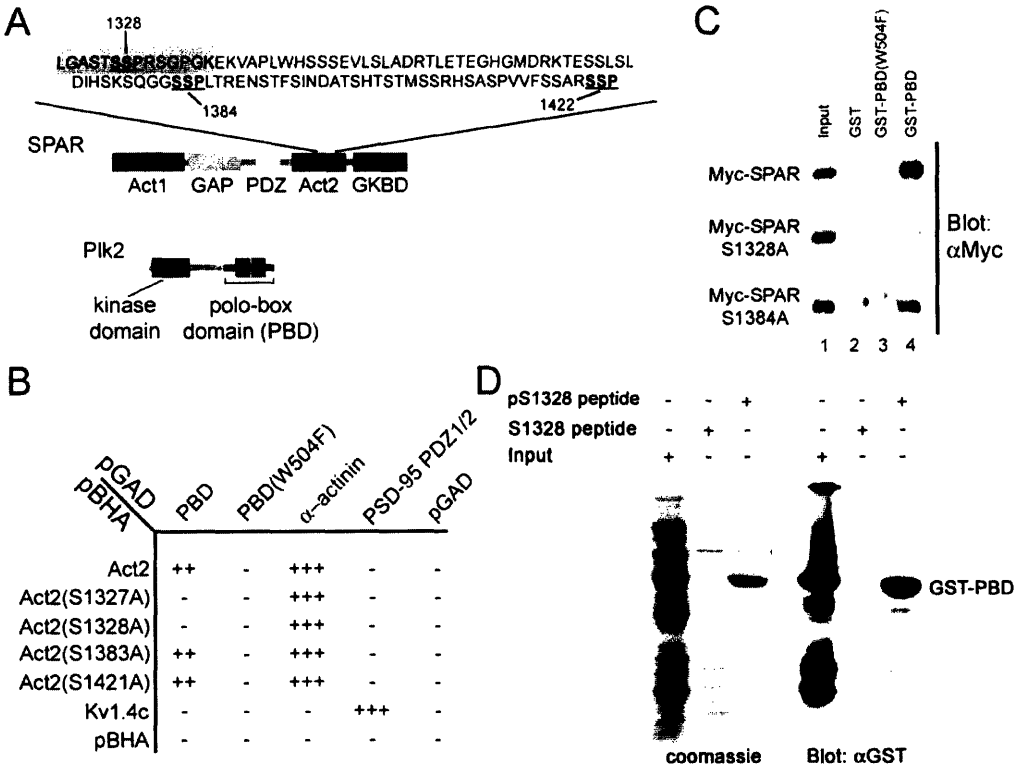
Phosphodependent Binding of Plk2 to SPAR

Precise regulation of subcellular localization is an important mechanism for controlling Plk1 function during cell division (van de Weerdt and Medema, 2006). But how is Plk2 recruited to synaptic sites in neurons and how is this recruitment regulated? Plk2 binds via its PBD to the Act2 domain of SPAR, a PSD-95-binding RapGAP enriched in PSDs and dendritic spines (Pak et al., 2001; Pak and Sheng, 2003). Thus the interaction of Plk2 and SPAR could be one mechanism for targeting Plk2 to postsynaptic sites in neurons. The consensus binding sequence of the PBD contains the core motif -S-S/T-P-, where the central serine or threonine is phosphorylated (Elia et al., 2003b). This sequence is found three times in the Act2 domain of SPAR, with a central serine located at positions 1328, 1384, and 1422 (Figure 1A). Alanine substitution of ser-1328 or its N-terminal neighboring residue (ser-1327) eliminated interaction between the SPAR Act2 domain and Plk2-PBD, as measured in yeast 2-hybrid assays. Alanine substitution of ser-1383 or ser-1421 had no effect (Figure 1B). Conversely, a single point mutation at trp-504 within the PBD of Plk2, a highly conserved residue of the PBD critical for association with Plk binding sites (Lee et al., 1998; Elia et al., 2003b), abolished binding to SPAR-Act2 (Figure 1B). None of these mutations affected binding of SPAR's Act2 domain to α-actinin, another binding partner of SPAR (Figure 1B).

In biochemical "pull-down" experiments, a GST fusion protein of Plk2's PBD (GST-PBD) robustly precipitated full-length wildtype SPAR expressed in HEK-293 cells, but not mutant SPAR in which ser-1328 was changed to alanine [SPAR(S1328A)] (Figure 1C). GST-PBD also precipitated full-length SPAR(S1384A), while GST-PBD(W504F) failed to pull down any of the SPAR constructs (Figure 1C). Thus, the S-S-P motif centered at ser-1328 (within the Act2 domain of SPAR) is necessary for full length SPAR to bind to the PBD of Plk2.

Does the Plk2-PBD interaction with SPAR require phosphorylation of ser-1328? In "pull-down" experiments, agarose beads coupled to a non-phosphorylated Act2 peptide comprising amino acids 1322 to 1335 ("S1328 peptide", highlighted in grey in Figure 1A) failed to precipitate GST-PBD expressed in bacteria. The same peptide sequence phosphorylated on ser-1328 ("pS1328 peptide") efficiently pulled down the PBD fusion protein (Figure 1D). Thus, the PBD of Plk2 binds specifically to a motif in SPAR centered around ser-1328, and only when ser-1328 is phosphorylated. (We presume that phosphorylation of the ser-1328 residue occurs to some extent in yeast

Figure 1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1328
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DIHSKSQGGSSPLTRENSTFSINDATSHTSTMSSRHSASPVVFSSARSSP
1384
1422
SPAR
Act1 GAP PDZ Act2 GKBD
Plk2
kinase domain
polo-box domain (PBD)
B
pGAD
pBHA
PBD
PBD(W504F)
α-actinin
PSD-95 PDZ1/2
pGAD
Act2 ++ - +++ - -
Act2(S1327A) - - +++ - -
Act2(S1328A) - - +++ - -
Act2(S1383A) ++ - +++ - -
Act2(S1421A) ++ - +++ - -
Kv1.4c - - - +++ -
pBHA - - - - -
C
Input
GST
GST-PBD(W504F)
GST-PBD
Myc-SPAR
Myc-SPAR S1328A
Myc-SPAR S1384A
Blot: αMyc
1 2 3 4
D
pS1328 peptide - - + - - +
S1328 peptide - + - - + -
Input + - - + - -
GST-PBD
coomassie
Blot: αGST

Figure 1. Plk2-PBD binds to canonical PBD binding site in SPAR

(A) Domain organization of Plk2 and SPAR proteins. Candidate Plk2-PBD binding sites within Act2 domain of SPAR are underlined. pS1328 and S1328 peptide sequences used in (D) are highlighted in gray.

(B) Interaction of the Plk2-PBD with SPAR-Act2 and Act2 mutants in yeast two-hybrid system. Alpha-actinin, PSD-95, and Kv1.4 are positive controls. pGAD and pBHA are negative controls.

(C) Glutathione *S*-transferase (GST)-PBD pull-down of myc-tagged SPAR and myc-tagged SPAR mutants. Extracts of HEK293 cells transfected with myc-tagged SPAR or SPAR mutants were incubated with Glutathione sepharose beads coupled to GST alone, GST-PBD(W504F), or GST-PBD, as indicated. Bound proteins were immunoblotted for myc. Lane 1 was loaded with 0.5 % of the input.

(D) Binding of GST-PBD to phosphorylated ser-1328 peptide (pS1328). pS1328 and non-phosphorylated S1328 peptides were coupled to agarose beads and incubated with extracts from bacteria expressing GST-PBD. Bound proteins were analyzed by Coomassie blue staining or immunoblotting with GST antibodies. Input lane, 10% of extract loaded onto beads.

and HEK-293 cells, thereby allowing interaction with the PBD to be detected by yeast two-hybrid and GST-pull down assays.)

Phosphorylation of Serine 1328 Important for Plk2-mediated Degradation of SPAR

Overexpression of Plk2 and SPAR in heterologous cells results in Plk2-mediated phosphorylation and degradation of SPAR (Pak and Sheng, 2003). Here, we used a tetracycline-inducible ("Tet On") promoter system to turn on the expression of Plk2 in HEK293 cells expressing SPAR from a constitutive promoter. Following application of doxycycline (100 ng/ml), wildtype SPAR was lost over the course of a few hours (presumably degraded) with rising expression of wildtype Plk2 (Figure 2A, quantified in 2B). Plk2 kinase activity was required for SPAR degradation, because induction of a "kinase-dead" mutant of Plk2 [K.D. Plk2(K108M)] failed to cause degradation of SPAR. Mutations in the PBD of Plk2 at either trp-504 [Plk2(W504F)] or at his-626 and lys-628 [Plk2(H626A K628M)] (two residues responsible for phosphodependent binding of the PBD (Elia et al., 2003b)), largely prevented degradation of SPAR by Plk2. Moreover, the SPAR mutant defective in PBD binding [SPAR(S1328A)] was poorly degraded by wildtype Plk2 (Figure 2A, quantified in 2B). Thus, ser-1328 of SPAR and an intact PBD of Plk2 are critical for Plk2-mediated degradation of SPAR, most likely because interaction of the PBD with phosphorylated ser-1328 is required for Plk2 recruitment to this target.

CDK5 is a "Priming" Kinase for SPAR ser-1328

To study more directly SPAR phosphorylation on ser-1328, we raised phospho-specific antibodies against the pS1328 peptide (see Figure 1A). The affinity-purified antibody ("α-pS1328SPAR") recognized the phosphorylated S1328 peptide on slot blots, but not the unphosphorylated peptide (Figure 3A). On western blots, α-pS1328SPAR antibody detected a signal from wildtype myc-tagged SPAR expressed in HEK293 cells, but not myc-SPAR(S1328A) (Fig 3B, compare lanes 1-6 with lanes 7-12). Collectively, these data indicate that the pS1328SPAR antibody specifically recognizes SPAR phosphorylated on ser-1328.

Because ser-1328 precedes a proline residue (-S-S^{1328}-P-R-S), we supposed that a proline-directed kinase is responsible for phosphorylating this residue in SPAR. We tested drug inhibitors of several proline-directed kinases by applying them to HEK293 cells overexpressing myc-tagged SPAR. Inhibitors of the MAP kinases p38 (SB-202190,

Figure 2

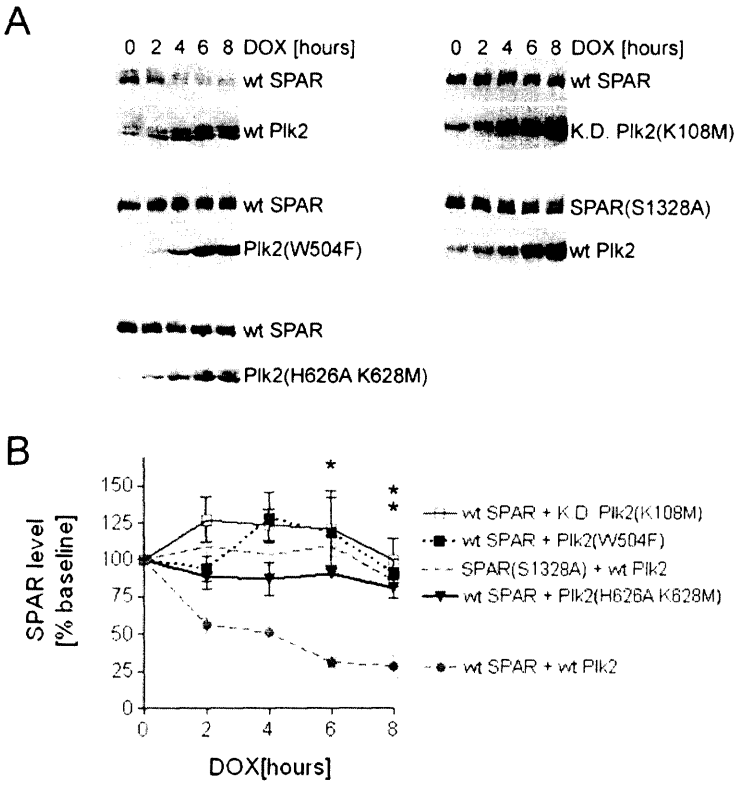

A
0 2 4 6 8 DOX [hours]
wt SPAR
wt Plk2
wt SPAR
Plk2(W504F)
wt SPAR
Plk2(H626A K628M)
0 2 4 6 8 DOX [hours]
wt SPAR
K.D. Plk2(K108M)
SPAR(S1328A)
wt Plk2
B
SPAR level [% baseline]
150
125
100
75
50
25
0
0
2
4
6
8
DOX[hours]
*
**
wt SPAR + K.D. Plk2(K108M)
wt SPAR + Plk2(W504F)
SPAR(S1328A) + wt Plk2
wt SPAR + Plk2(H626A K628M)
wt SPAR + wt Plk2

Figure 2. Intact PBD and ser-1328 required for Plk2-dependent degradation of SPAR

(A) HEK293 cells were triple-transfected with myc-tagged SPAR (wildtype or mutant, as indicated) driven by constitutive CMV promoter, myc-tagged Plk2 (wt or mutant, as indicated) driven by tetracycline-responsive promoter, and pTET-On vector driving reverse tetracycline-responsive transcriptional activator (rtTA). After doxycline treatment (DOX, 100 ng/ml) for indicated time to induce Plk2 expression, cells were immunoblotted for myc.

(B) Quantification of SPAR levels (mean ± SEM) from multiple experiments performed as in A. N=6 (wt SPAR + wt Plk2), N=7 (wt SPAR + Plk2[H626A K628M]), N=5 (SPAR[S1328A] + wt Plk2]), N=8 (wt SPAR + Plk2[W504F]), N=4 (wt SPAR + Plk2[K108M]), *$p<0.05$, **$p<0.01$ for wt SPAR + wt Plk2 compared to all other combinations of SPAR and Plk2 constructs at the indicated DOX treatment time poionts, One-way ANOVA.

Figure 3

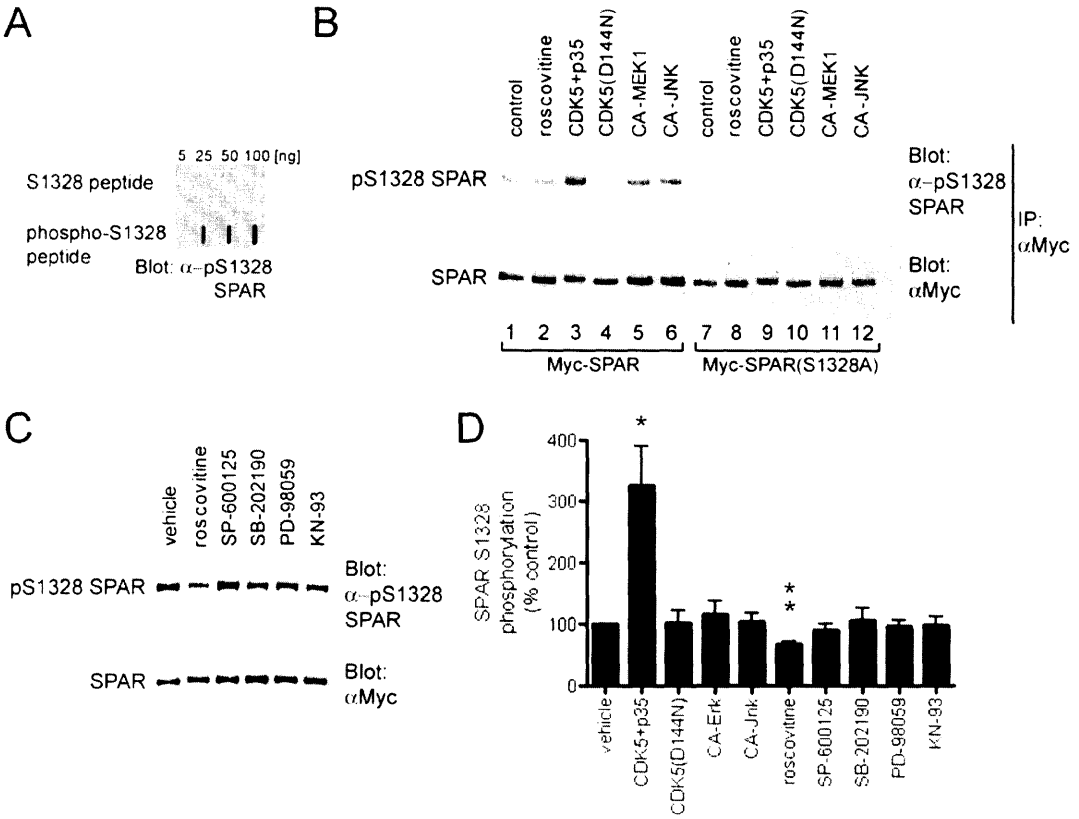

A
5 25 50 100 [ng]
S1328 peptide
phospho-S1328 peptide
Blot: α-pS1328 SPAR
B
control
roscovitine
CDK5+p35
CDK5(D144N)
CA-MEK1
CA-JNK
pS1328 SPAR
SPAR
Blot: α-pS1328 SPAR
Blot: αMyc
IP: αMyc
1 2 3 4 5 6 7 8 9 10 11 12
Myc-SPAR
Myc-SPAR(S1328A)
C
vehicle
roscovitine
SP-600125
SB-202190
PD-98059
KN-93
pS1328 SPAR
SPAR
Blot: α-pS1328 SPAR
Blot: αMyc
D
SPAR S1328 phosphorylation (% control)
400
300
200
100
0
vehicle
CDK5+p35
CDK5(D144N)
CA-Erk
CA-Jnk
roscovitine
SP-600125
SB-202190
PD-98059
KN-93

Figure 3. Phosphorylation of SPAR ser-1328 and regulation by CDK5

(A) "Slot blots" showing specificity of the α-pS328SPAR phospho-antibody for the phosphorylated S1328 peptide. Peptides were bound to nitrocellulose and immunoblotted with α-pS1328SPAR.

(B) CDK5 enhances ser-1328 phosphorylation in heterologous cells. Myc-tagged wildtype SPAR or SPAR(S1328) was transfected into HEK293 cells with the indicated protein kinase constructs, or treated with roscovitine for 2 hours. SPAR was then immunoprecipitated with myc antibody, followed by immunoblotting with α-pS1328SPAR and then stripped and reprobed with myc antibodies.

(C) CDK5 inhibitor reduces SPAR ser-1328 phosphorylation. HEK293 cells were transfected with myc-SPAR and treated for two hours with the indicated drugs, then immunoblotted with α-pS1328SPAR, stripped, and reprobed with myc antibodies.

(D) Quantification of data from (B) and (C), normalized to control conditions from the same blot (mean ± SEM). N=5-6 for each condition, except roscovitine (N=11). *$p<0.05$, **$p<0.001$, compared to control (100%), two tailed T-test.

5 μM), JNK (SP-600125, 20 μM), or Erk, through its upstream activator MEK (PD-98059, 50 μM), had no effect on ser-1328 phosphorylation of SPAR, as measured by immunoblotting with phosphoantibody α-pS1328SPAR (Figure 3C, quantified in D). Roscovitine (10 μM), a CDK5 inhibitor that can also block CDK1 and CDK2 at this concentration (Meijer et al., 1997), significantly decreased the α-pS1328SPAR signal (Figure 3C, D).

To test if neuron-specific CDK5 can phosphorylate ser-1328 of SPAR, we coexpressed SPAR with CDK5 and its activator p35 in HEK cells; ser-1328 phosphorylation was strongly enhanced (Figure 3B, lane 3; quantified in Figure 3D). No effect on SPAR ser-1328 phosphorylation was observed with constitutively active forms of JNK1 or MEK1 (an upstream activator of Erk) (Figure 3B, lanes 5 and 6; quantified in D). KN-93 (5 μM), an inhibitor of CaMKII, had no effect on ser-1328 phophorylation (Figure 3C, D).

Is endogenous SPAR phosphorylated on ser-1328 in neurons? On immunoblots of rat brain homogenates, the α-pS1328SPAR antibody detected a band of molecular weight similar to that of recombinant SPAR (Figure 4, where "R" denotes lanes loaded with recombinant SPAR expressed in HEK293 cells). This band was "quenched" by competition with excess of the phospho-S1328 peptide but not the unphosphorylated peptide (Figure 4, lanes 4 and 3). In addition, treatment of the membranes with calf intestinal phosphatase (CIP) eliminated the α-pS1328SPAR signal, while the signal from a C-terminal SPAR antibody persisted (Figure 4, lanes 7-12). We conclude that α-pS1328SPAR antibody specifically detects phosphorylated SPAR in neurons. We also tried to detect endogenous SPAR ser-1328 phosphorylation by immunocytochemistry of neurons, but the α-pS1328SPAR phosphoantibodies gave no significant signal by staining.

SPAR was also phosphorylated on ser-1328 in cultured cortical neurons (DIV12), as detected by immunoblotting with α-pS1328SPAR (Figure 5A). As in heterologous cells, the α-pS1328SPAR signal in neurons was reduced by application of the CDK5 inhibitor roscovitine for either 2 hours (data not shown), or for 12-18 hours (Figure 5A, compare lanes 2 and 4 to lanes 1 and 3, respectively). Inhibitors of p38 (SB-202190), JNK (SP-600125), MEK (PD-98059), or CaMKII (KN-93) had no effect on phospho-ser-1328 SPAR levels in neurons (Figure 5A, lanes 5-12). Together, these data show that

Figure 4

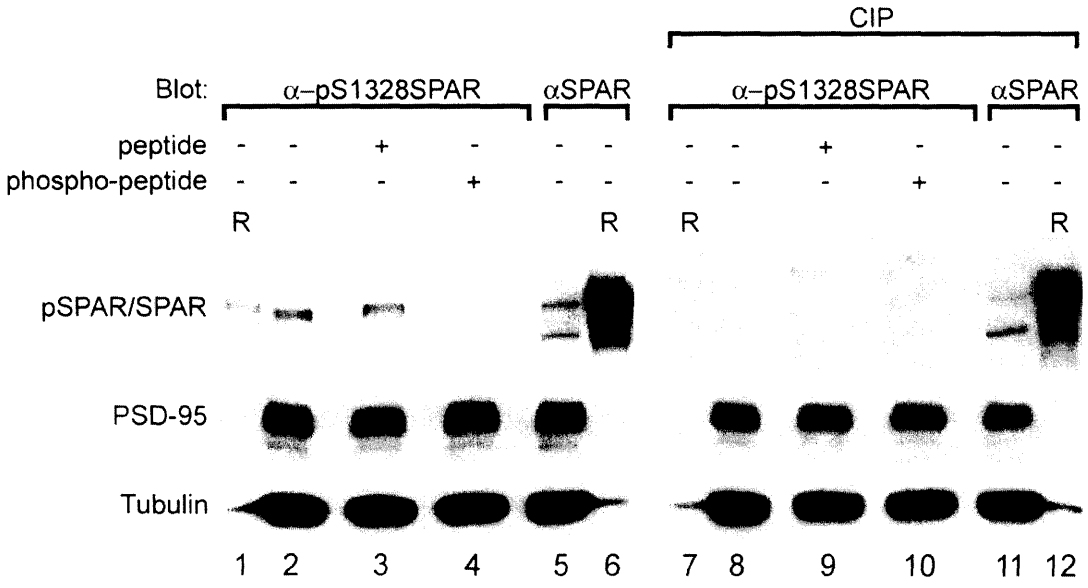
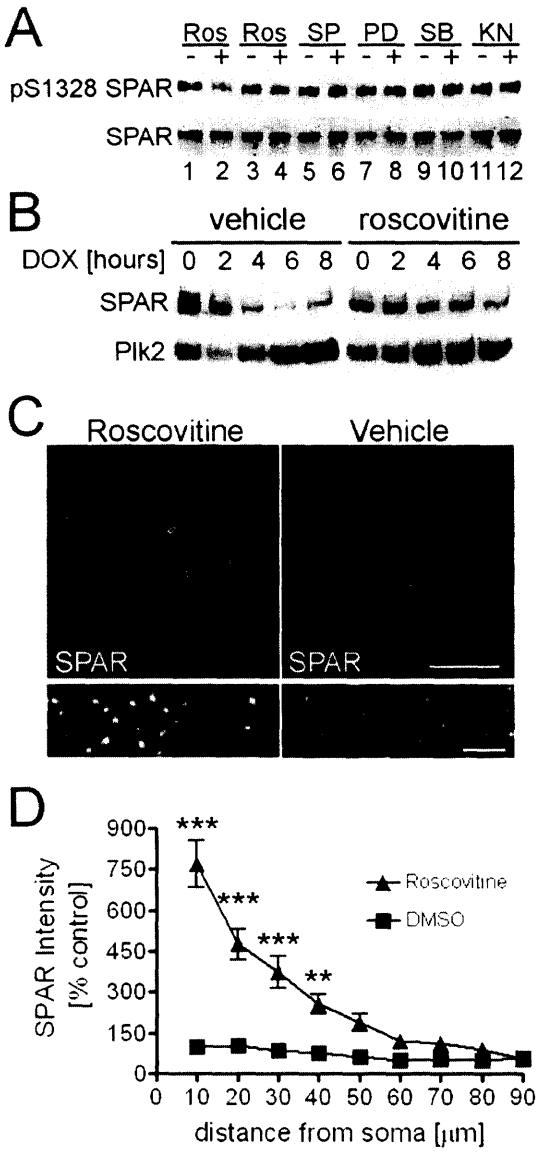

CIP
Blot:
α–pS1328SPAR
αSPAR
α–pS1328SPAR
αSPAR
peptide
phospho-peptide
R
R
R
R
pSPAR/SPAR
PSD-95
Tubulin
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4. Phosphorylation of SPAR ser-1328 in rat hippocampus. Hippocampal extracts were immunoblottted with either α-pS1328SPAR or SPAR antibodies (upper row), and with PSD-95 or Tubulin antibodies. The pS1328SPAR signal was specifically eliminated by the phospho-S1328 peptide (lane 4), but not by the unphosphorylated S1328 peptide (lane 3). Calf intestinal phsophatase (CIP) treatment eliminated the pS1328SPAR signal (lanes 7-10). "R" lanes were loaded with HEK293 cell extract expressing recombinant SPAR protein.

Figure 5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- + - + - + - + - + - +
pS1328 SPAR
SPAR
1 2 3 4 5 6 7 8 9 10 11 12
B
vehicle
roscovitine
DOX [hours] 0 2 4 6 8 0 2 4 6 8
SPAR
Plk2
C
Roscovitine
Vehicle
SPAR
SPAR
D
SPAR Intensity [% control]
900
750
600
450
300
150
0
***
***
***
**
Roscovitine
DMSO
0 10 20 30 40 50 60 70 80 90
distance from soma [μm]

Figure 5. CDK5 promotes degradation of SPAR in neurons

(A) Ser-1328 SPAR phosphorylation in neurons reduced by CDK5 inhibitor. Dissociated cortical cultures (DIV 12) were treated for 12-18 hours with vehicle or roscovitine ("Ros", 10 μM), SP-600125 ("SP", 20 μM), PD-98059 ("PD", 50 μM), SB-202190 ("SB", 5 μM), or KN-93 ("KN", 5 μM), and then immunoblotted with α-pS1328SPAR, stripped, and reprobed with SPAR antibodies.

(B) Roscovitine prevents SPAR degradation by Plk2 in heterologous cells. HEK293 cells triple-transfected with wt SPAR, tet-responsive wt Plk2, and the pTET-On vector were immunoblotted with myc antibodies after doxycycline treatment (as in Figure 5). HEK293 cells were treated with either DMSO (1:1000, "vehicle") or roscovitine (10 μM), as indicated, for the final 8 hours before lysis.

(C) Increased SPAR immunostaining in neurons following pharmacological inhibition of CDK5. Example of mature hippocampal neurons (DIV 21-28) treated for 12-18 hours with either roscovitine or vehicle (DMSO), and stained with SPAR antibodies to visualize endogenous SPAR protein. The outlined region is shown at higher magnification below. Scale bar, 50 μm low magnification, and 5 μm high magnification.

(D) Quantification of SPAR immunofluorescence intensity in hippocampal neurons (DIV 21-18) treated for 12-18 hours with either roscovitine or vehicle (DMSO), plotted as a function of distance from the cell body (see Methods) (mean ± SEM). SPAR immunofluorescence intensity from both treatment conditions was normalized to the 10 μm distance of DMSO-treated neurons. N=18 cells (roscovitine), N=16 cells (DMSO), $^{**}p<0.01$, $^{***}p<0.001$, Two-way ANOVA.

some subpopulation of SPAR is phosphorylated on ser-1328 in neurons, and that this phosphorylation is regulated by CDK5.

CDK5 activity is stimulated by A-beta peptide (Alvarez et al., 2001; Town et al., 2002), which is produced by proteolytic cleavage of the amyloid precursor protein (APP) and is implicated in the early pathogenesis of Alzheimer's disease (Mattson, 2004; Walsh and Selkoe, 2004). We found that overnight treatment with soluble A-beta(1-40) stimulated the phosphorylation of ser-1328 on SPAR in cortical neurons as detected by immunoblotting, consistent with increased activity of CDK5 (Figure 6).

CDK5 Priming Kinase Activity Promotes Degradation of SPAR

We showed that the SPAR(S1328A) mutant is protected from degradation by Plk2 in HEK293 cells, presumably because it cannot be phosphorylated at its PBD binding site and thus cannot interact with the Plk2-PBD (Figure 2A and B). We tested this hypothesis further by repeating the same SPAR degradation assay in the presence of roscovitine, which reduced basal phosphorylation of SPAR ser-1328 in HEK293 cells (see Fig 3B-D). In the absence of roscovitine, application of doxycycline induced expression of Plk2 and concomitant degradation of SPAR (Figure 5B; see also Figure 2). Treatment with roscovitine impeded SPAR degradation in this assay (Figure 5B). Our results support the idea that CDK5 activity promotes SPAR degradation by "priming" phosphorylation of ser-1328 in the Plk2-PBD binding site.

Because roscovitine suppresses ser-1328 phosphorylation in neurons as well (Figure 5A), one would predict that roscovitine would inhibit SPAR degradation, resulting in a higher steady state level of SPAR protein. Indeed, cultured hippocampal neurons (DIV 21-28) treated with roscovitine for 12-18 hours showed markedly increased SPAR levels by immunocytochemistry (Figure 5C, quantified in D). The enhancement in SPAR staining intensity was greatest in dendritic regions close to the cell body, and least in distal dendritic regions (Figure 5D). This proximal-distal gradient is reminiscent of the activity-induced pattern of Plk2 protein expression, which is also highest in proximal dendrites and tapers off with distance from the cell body (Pak and Sheng, 2003). Our result is thus consistent with CDK5 stimulating the degradation of SPAR in neurons via a Plk2-dependent mechanism.

To corroborate the pharmacological data, we transfected DIV16 hippocampal neurons with a GFP-tagged dominant interfering CDK5 construct (GFP-CDK5-DN, in

Figure 6

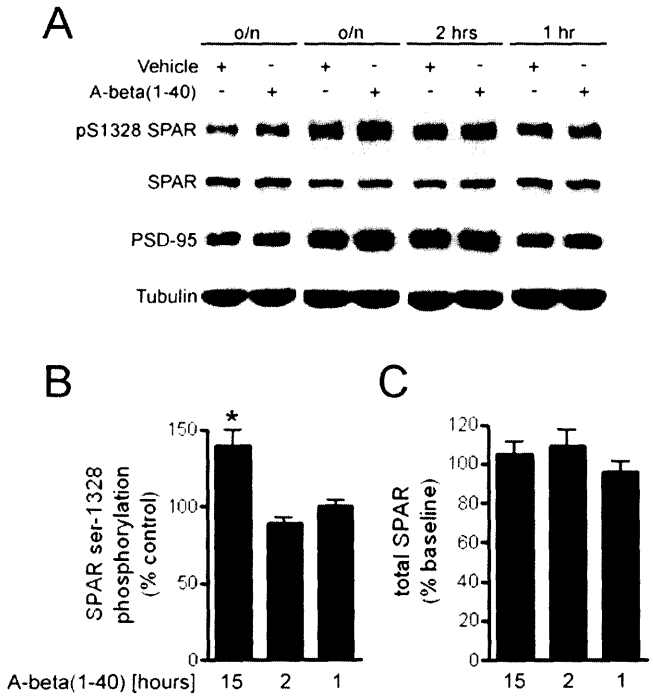
A
o/n
o/n
2 hrs
1 hr
Vehicle + - + - + - + -
A-beta(1-40) - + - + - + - +
pS1328 SPAR
SPAR
PSD-95
Tubulin
B
SPAR ser-1328 phosphorylation (% control)
150
100
50
0
*
A-beta(1-40) [hours] 15 2 1
C
total SPAR (% baseline)
120
100
80
60
40
20
0
15 2 1

Figure 6. Regulation of ser-1328 SPAR phosphorylation in neurons by A-beta peptide

(A) High-density cortical (DIV 12) cultures were treated o/n (12-18 hours), for 2 hours, or for 1 hour with vehicle or A-beta(1-40) peptide (10 μM) and then immunoblotted with the indicated antibodies. Immunoblotting with SPAR antibodies was performed on blots stripped of α-pS1328SPAR.

(B-C) Quantitation of ser-1328 phosphorylation and total SPAR levels from immunoblots in (A), normalized to vehicle-treated cells (mean ± SEM). Values for SPAR ser-1328 phosphorylation (B) were obtained by taking the ratio of the pS1328SPAR signal over the total SPAR signal. N=4 blots (Abeta[1-40] o/n, Abeta[1-40] 2 hours), N=2 blots (Abeta[1-40] 1 hour), *$p<0.05$, compared to control (100%), Two-tailed T-test.

which kinase activity is disrupted by an asp-144 to asn mutation) (Nikolic et al., 1996). GFP-CDK5-DN, but not wildtype GFP-CDK5, strongly increased SPAR expression by DIV19 relative to control cultures transfected with GFP alone (Fig 7A, quantified in B). These results further support that endogenous CDK5 acts as a priming kinase for the degradation of SPAR.

CDK5 and SPAR Participate in Homeostatic Plasticity

If CDK5 priming activity is important for Plk2-dependent functions like degradation of SPAR, blocking CDK5 activity should interfere with Plk2-dependent synaptic homeostasis. To test this idea, we recorded mEPSCs from CA1 pyramidal cells in hippocampal slice culture (see Chapter 2). As expected, 12-18 hours of PTX treatment did not affect mEPSC amplitude in untransfected neurons, reflecting normal homeostasis (Figure 8B; see also Chapter 2-Figure 2B). Overexpression of the dominant negative CDK5 construct GFP-CDK5-DN had no effect on mEPSC amplitude in the absence of PTX treatment (Figure 8B). However, mEPSCs in cells transfected with GFP-CDK5-DN became significantly strengthened after 12-18 hours of PTX treatment compared to neighboring untransfected cells, indicating disruption of homeostasis in these cells (Figure 8A, B). These results show that CDK5 does not significantly regulate synaptic strength under basal conditions in slice culture, but is required under conditions of elevated activity for preventing runup of synaptic strength and thus for homeostatic plasticity. The effect of GFP-CDK5-DN is remarkably similar to that of the Plk2 dominant interfering construct GFP-PBD (see Chapter 2-Figure 2C), consistent with the idea that CDK5 and Plk2 act in the same pathway to mediate homeostasis.

Does degradation of SPAR contribute to Plk2-dependent homeostasis? To address this question, we investigated the role of SPAR in synaptic function during changes in activity levels. RNAi suppression of SPAR expression under basal conditions (no PTX) caused a significant depression of mEPSCs compared to neighboring untransfected cells (Figure 8C, D). These are the first results to show that SPAR promotes synaptic strength. After 12-18 hours of PTX treatment, untransfected neurons again showed no significant increase in mEPSC amplitude, consistent with normal homeostasis (Figure 8D). Neurons transfected with SPAR-RNAi construct, however, showed significantly larger mEPSCs following prolonged hyperactivity than in the absence of PTX (Figure 8D), indicating impaired homeostasis. It is notable that after 12-

Figure 7

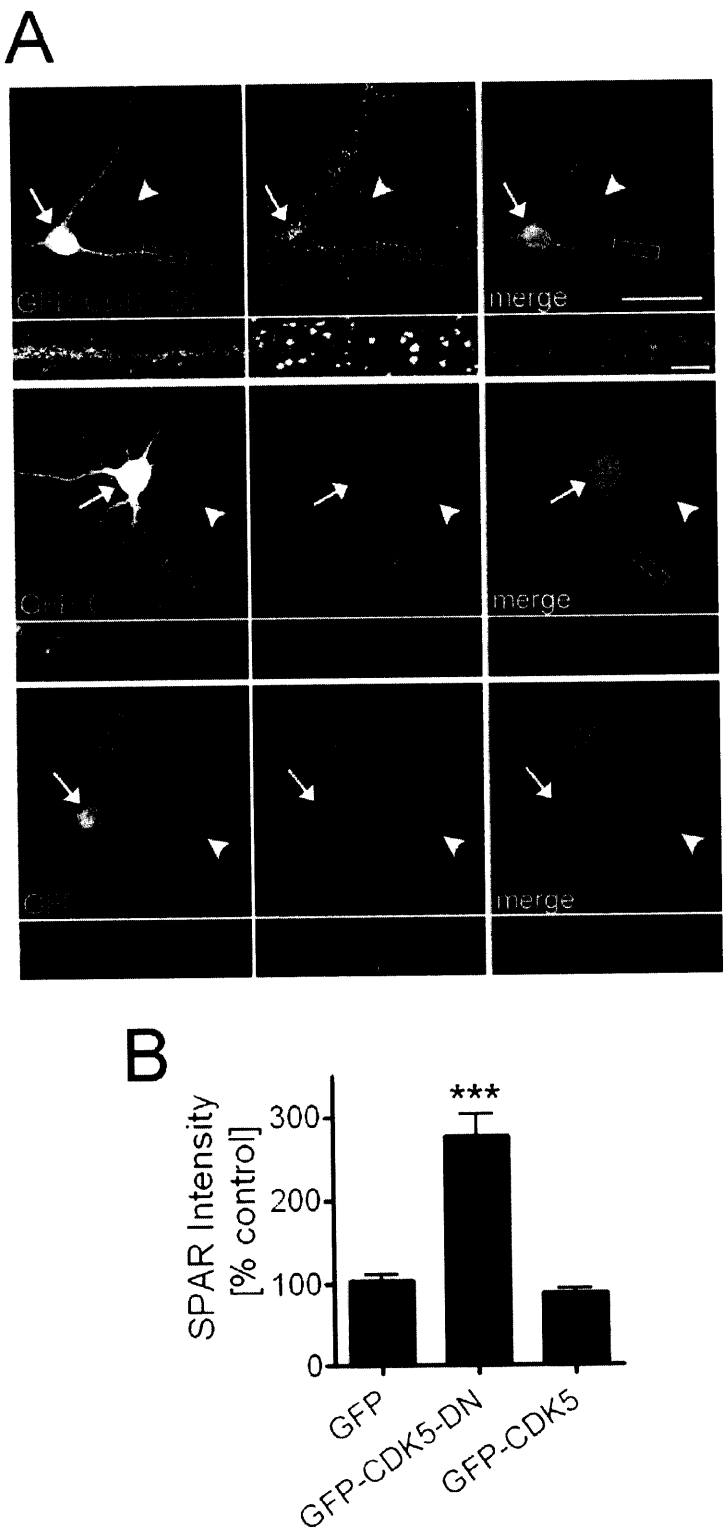
A
merge
merge
merge
B
SPAR Intensity
[% control]
300
200
100
0
***
GFP
GFP-CDK5-DN
GFP-CDK5

Figure 7. SPAR accumulation in neurons overexpressing dominant-negative CDK5 construct.

(A) DIV 16 hippocampal neurons were transfected with GFP-tagged CDK5(D144N) ("GFP-CDK5-DN"), wt GFP-CDK5, or GFP, fixed at DIV 19, and stained with SPAR antibodies to visualize endogenous SPAR protein. The merge of the two signals is shown in color. Scale bar, 50 μm (low magnification), and 5 μm (high magnification).

(B) Quantification of SPAR immunofluorescence intensity in DIV19 hippocampal neurons transfected for three days with GFP-tagged CDK5(D144N) ("GFP-CDK5-DN"), wt GFP-CDK5, or GFP, normalized to neighboring untransfected neurons (see Methods) (mean ± SEM). N=16 cells (all constructs), ***$p<0.001$, One-way ANOVA.

Figure 8

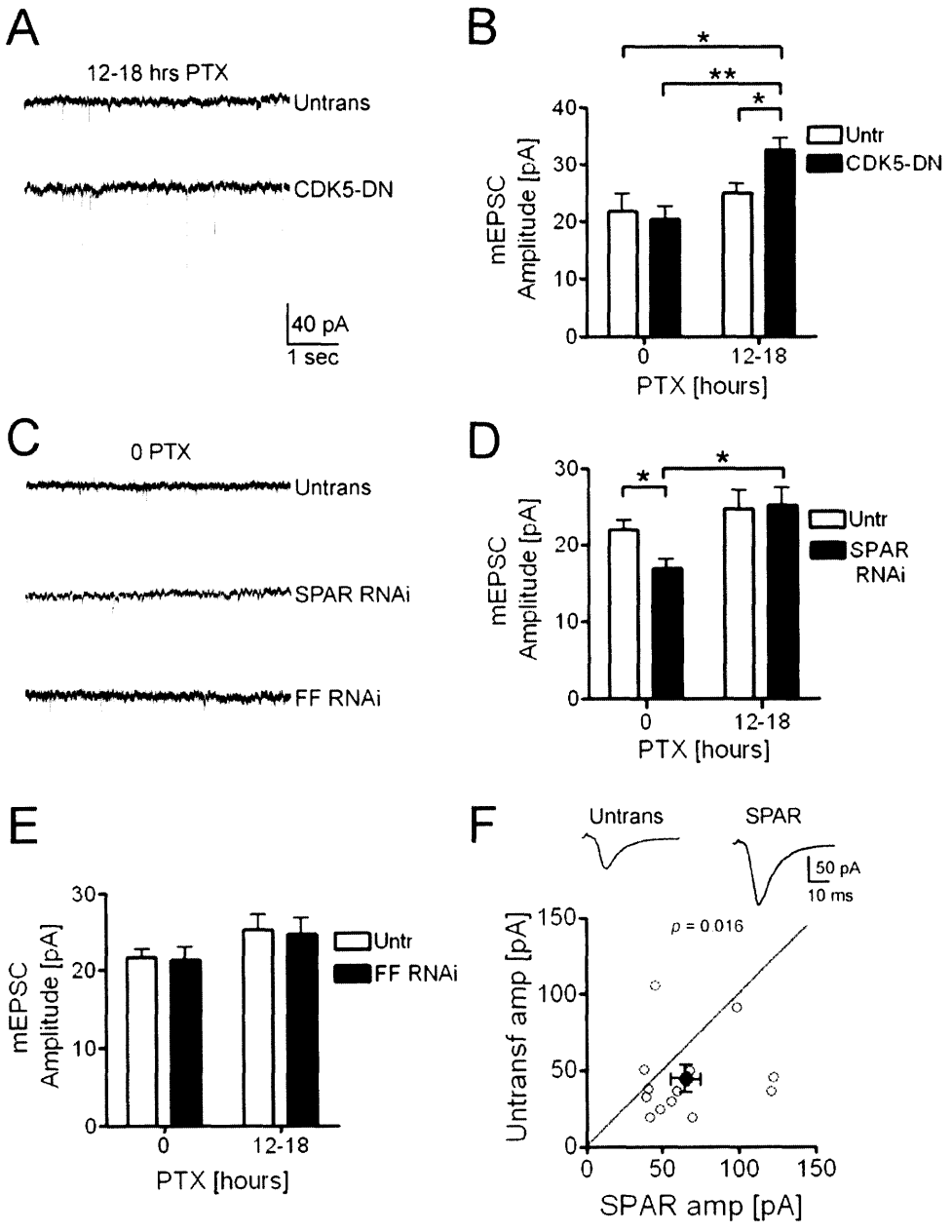
A
12-18 hrs PTX
Untrans
CDK5-DN
40 pA
1 sec
B
mEPSC Amplitude [pA]
Untr
CDK5-DN
PTX [hours]
C
0 PTX
Untrans
SPAR RNAi
FF RNAi
D
mEPSC Amplitude [pA]
Untr
SPAR RNAi
PTX [hours]
E
mEPSC Amplitude [pA]
Untr
FF RNAi
PTX [hours]
F
Untrans
SPAR
50 pA
10 ms
p = 0.016
Untransf amp [pA]
SPAR amp [pA]

Figure 8. CDK5 and SPAR participate in homeostatic plasticity

(A-B) CDK5 inhibitor impairs synaptic homeostasis during heightened activity. Representative mEPSC traces from untransfected CA1 pyramidal cells and cells transfected with GFP-tagged CDK5(D144N) ("CDK5-DN") pretreated for 12-18 hours with PTX (A). Quantitation of mEPSC amplitude (mean ± SEM) in (B). N=6 and 4 cells (0 PTX, for CDK5-DN and neighboring Untransfected, respectively), N=9 and 7 cells (12-18 PTX, for CDK5-DN and neighboring Untransfected, respectively). *$p<0.05$, **$p<0.001$, Mann Whitney test.

(C-E) RNAi knockdown of SPAR impairs basal synaptic transmission and synaptic homeostasis. Representative mEPSC traces from untransfected cells and cells transfected with a pENTR-based vector expressing shRNA against SPAR (SPAR RNAi) or negative control firefly luciferase (FF-RNAi) under basal conditions (no PTX) (C). Quantitation of mEPSC amplitude (mean ± SEM) is shown in D and E. N=7-12 cells for each condition. *$p<0.05$, Mann Whitney test.

(F) SPAR overexpression enhances synaptic transmission in CA1 pyramidal cells of cultured slices. Sample AMPA-EPSC traces (Top) from pairs of transfected (SPAR) and neighboring untransfected cells (Untrans). EPSC amplitudes were plotted for individual pairs of transfected versus neighboring untransfected cells (graph; open symbols). Filled circle represents the mean ± SEM. N=13 cell pairs; p value calculated by Mann Whitney test.

18 hours PTX, the enhanced mEPSCs in SPAR-RNAi neurons merely reached the amplitude shown by neighboring untransfected neurons with intact homeostasis (Figure 8D). These data suggest that degradation of SPAR contributes to Plk2-dependent homeostasis during prolonged hyperactivity. When SPAR is already knocked down by RNAi, it cannot be further suppressed by activity-dependent mechanisms such as Plk2-induced degradation, and therefore synaptic strength runs up, albeit from a lower basal level. The diminished basal EPSC amplitude indicates that SPAR is important for sustaining synaptic transmission even in basal conditions. No effect on mEPSCs in basal or PTX conditions was observed with a control RNAi construct expressing shRNA against firefly luciferase (Figure 8C, E). In CA1 neurons transfected with wildtype SPAR, evoked EPSCs were significantly larger compared to neighboring untransfected cells (Figure 8F), confirming that SPAR acts to boost synaptic transmission.

DISCUSSION

Plk2 is required for cells to undergo homeostatic plasticity in slice cultures exposed to heightened network activity (see chapter 2), but both the molecular targets of Plk2 and the regulatory mechanisms underlying its spatial and temporal control remain poorly understood.

Our findings point to the degradation of SPAR as an important contributor to Plk2-mediated homeostasis. Gain- and loss-of function experiments showed that SPAR is a positive regulator of synaptic strength, and homeostasis was disrupted when SPAR was suppressed by RNAi (thus preventing further downregulation by Plk2-mediated degradation). We showed in chapter 2 that Plk2 is required for activity-dependent synaptic scaling. Because of SPAR's effects on synaptic strength, degradation of SPAR is a plausible mechanism for Plk2-dependent synaptic scaling, although we have not yet directly tested this. Degradation of the postsynaptic RapGAP SPAR by Plk2 would also stimulate the activity of postsynaptic Rap GTPases, which are known to depress synaptic strength (Zhu et al., 2002; Huang et al., 2004; Zhu et al., 2005) and cause thinning of spines (Xie et al., 2005). In addition, degradation of SPAR would be predicted to cause loss of PSD95 and NMDA receptors from synapses (Pak and Sheng, 2003). Thus degradation of SPAR could be an important mechanism of Plk2-dependent homeostasis by scaling down synaptic strengths and contributing to the increase in LTP threshold (increased Rap activity, decreased NMDAR expression), predicted to be important in keeping synapses within a modifiable and physiologically useful range during elevated activity (see chapter 2).

Degradation of SPAR by Plk2 required phosphorylation on ser-1328 of SPAR. We identified CDK5 as the likely proline-directed kinase that "primes" this site, resulting in a phosphomotif that is specifically recognized by the PBD of Plk2. Inhibiting CDK5 reduced ser-1328 phosphorylation and increased SPAR protein levels in neurons. More importantly, CDK5 activity was required to prevent runup in synaptic strength during elevated network activity. These results are consistent with CDK5 playing a critical role in synaptic homeostasis by regulating Plk2-dependent degradation of SPAR.

The fact that CDK5 phosphorylation generates a PBD binding site in SPAR raises the intriguing possibility that Plk2 targeting and function can be controlled by the regulated recruitment of Plk2 to phosphorylated -S-S/T-P- motifs on SPAR and other potential targets. Plk2 activity would then require the coincidence of two conditions—

prolonged elevated neuronal activity to induce Plk2 protein expression, as well as the availability of primed binding sites for the PBD, allowing for spatial and temporal refinement in the control of kinase activity.

In the cell cycle, priming kinases like cdc2/CDK1 strictly regulate the availability of Plk binding sites to ensure that the many functions of mitotic Plks are carried out with required precision in time and subcellular location (Barr et al., 2004; van de Weerdt and Medema, 2006). Similarly precise regulation in neurons could allow the specific recruitment of Plk2 to synapses that have been "tagged" by the phosphorylation of SPAR ser-1328 or other Plk2 binding sites. Although Plk2 participates in synaptic scaling (see chapter 2), which is thought to be a global process (Turrigiano and Nelson, 2004), our study raises the possibility that some of Plk2's functions might operate in a non-global, even synapse-specific, manner. In this context, we note that recent studies have found evidence consistent with "local" homeostatic mechanisms occurring at the level of dendritic segments (Ju et al., 2004; Sutton et al., 2006) (see also discussion of this topic in chapter 1).

CDK5 has been studied most in the context of neural development, but plays a role in a range of physiological and pathological processes, including neuronal migration, dopaminergic function, synaptic transmission, and neurodegeneration (Dhavan and Tsai, 2001; Shelton and Johnson, 2004; Angelo et al., 2006). In addition to our finding that it likely phosphorylates SPAR, CDK5 phosphorylates a number of other postsynaptic proteins, including the NR2A subunit of NMDA receptors, PSD-95, protein phosphatase inhibitor 1, and RasGRF2 (Bibb et al., 1999; Bibb et al., 2001; Li et al., 2001; Kesavapany et al., 2004; Morabito et al., 2004).

Compared with knowledge of its substrates, the regulation of CDK5 activity itself remains relatively unclear. Treatment with excitotoxic levels of glutamate (1 mM) increases CDK5 activity through calpain-mediated cleavage of p35 to p25 (Lee et al., 2000), while treatment with lower levels of glutamate (≤0.1 mM) seems to reduce CDK5 activity by proteasomal degradation of p35 (Wei et al., 2005; Hosokawa et al., 2006). The amyloid beta peptide (A-beta), which is produced by proteolytic cleavage of the amyloid precursor protein (APP), can also induce the conversion of p35 to p25 (Lee et al., 2000) and activate CDK5 (Alvarez et al., 2001; Town et al., 2002). Consistent with these studies, we found that prolonged treatment of neurons with A-beta resulted in an increase in SPAR ser-1328 phosphorylation. Interestingly, soluble forms of A-beta inhibit

synaptic transmission (Kamenetz et al., 2003), and may play a role in the early pathogenesis of Alzheimer's disease (Mattson, 2004; Walsh and Selkoe, 2004), although the mechanism is not yet understood. By showing that CDK5 primes the degradation of SPAR, our work introduces a possible mechanism by which A-beta could cause weakening and loss of synapses.

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Chapter 4

Conclusion

Plk2 is Involved in Neuronal Homeostatic Plasticity

Homeostatic mechanisms regulate neuronal activity (Turrigiano et al., 1994; Burrone et al., 2002; Marder and Prinz, 2002), and are believed to stabilize neuronal output in the face of changing ambient activity and input strength (Turrigiano and Nelson, 2004; Davis, 2006). The molecular mechanisms underlying homeostatic plasticity, however, are still relatively unknown. In this thesis, we have provided evidence that the activity inducible kinase Plk2 participates in neuronal homeostatic plasticity following heightened activity and have delineated a regulatory mechanism involving a "priming" kinase (CDK5) that is needed to recruit Plk2 to substrates and binding sites.

In chapter 2, we showed using hippocampal slice culture that increased neuronal activity led to a small increase in synaptic strength that then plateaued. The plateau phase was dependent on Plk2 function, because blocking Plk2 function led to an unabated runup in synaptic strength during 18 hours of elevated activity. Importantly, Plk2 function was required for cells to undergo LTP, implying that Plk2-dependent homeostatic plasticity allows continuing plasticity in the face of heightened activity.

We then investigated the mechanisms of Plk2-dependent homeostasis during elevated activity. We showed in dissociated hippocampal culture that Plk2 was required for synaptic scaling, a mechanism of homeostatic plasticity that is thought to globally set the gain of all synapses in a multiplicative fashion (Turrigiano and Nelson, 2004). In slice culture, we found that Plk2 was also required for activity-dependent decreases in membrane excitability. Because decreased membrane excitability makes it harder for a given input to drive the postsynaptic cell, and because scaling reduces calcium conductance at synapses (by scaling both AMPA and NMDA receptors (Watt et al., 2000)), we speculate that these homeostatic mechanisms could together significantly increase the threshold for LTP induction and thereby keep neurons within a physiologically useful and modifiable range during elevated ambient activity.

Interdependence of Hebbian Plasticity, Homeostatic Plasticity, and Metaplasticity

It is widely believed that homeostatic mechanisms provide an important negative feedback mechanism in stabilizing circuits modified by positively correlated pre and postsynaptic activity (i.e. Hebbian plasticity) (Abbott and Nelson, 2000; Turrigiano and Nelson, 2004; Davis, 2006). Without such mechanisms in place, neurons would be prone to instability because Hebbian plasticity mechanisms would tend to promote strengthening of effective circuits or weakening of ineffective circuits, resulting eventually

in excessive activity or total quiescence. To promote stability without eroding the information encoded in the signature of Hebbian modifications, it is thought that homeostatic mechanisms are purely passive with respect to Hebbian plasticity, i.e. they neither change the relative weights between synapses, nor change a synapse's capacity to undergo LTP or LTD. This is an attractive hypothesis, but lacks empirical verification or refutation (Turrigiano and Nelson, 2004; Thiagarajan et al., 2007).

Instead of purely promoting stability, our findings add to a number of recent studies suggesting that homeostatic mechanisms may also affect a cell's ability to undergo Hebbian plasticity, a concept sometimes referred to as "metaplasticity" or "plasticity of plasticity" (Abraham, 1999; Bear, 2003). For example, periods of prolonged inactivity result in increased expression of NMDA receptors (Watt et al., 2000) and homomeric GluRI-containing AMPA receptors (Ju et al., 2004; Thiagarajan et al., 2005), both of which are calcium permeable and would be predicted to facilitate the induction of LTP.

These findings indicate that some of the metaplastic effects may themselves be homeostatic in nature, suggesting that the strict distinction between Hebbian and homeostatic plasticity mechanisms may be less pronounced than initially assumed. In other words, neuronal homeostasis might be achieved both directly through changes in the gain of synapses (e.g. through synaptic scaling), as well as indirectly through metaplasticity, i.e. by "recruiting" Hebbian plasticity mechanisms to change synaptic weights in favor of stable neuronal output. In fact, as discussed in chapter 1, such "homeostatic metaplasticity" has been proposed to occur over both short and long timescales. For example, spike timing dependent plasticity (STDP), a prototypical form of Hebbian plasticity (Dan and Poo, 2006), can change its rules depending on the activity state of the neuron, promoting LTP during low activity and LTD during high activity, thereby effectively stabilizing neuronal output. Over longer time scales, sliding modification thresholds for LTP and LTD induction are thought to change based on the history of integrated postsynaptic activity as predicted by the Bienenstock Cooper Munro (BMC) model, for example making it progressively more difficult to potentiate synapses with increasing postsynaptic activity to prevent saturation of LTP (Bear, 2003).

CDK5 and SPAR Participate in Plk2-dependent Homeostasis

Throughout mitosis, precise spatiotemporal regulation of Plk function is required as Plks participate in a vast array of mitotic processes ranging from mitotic entry to cytokinesis

(for review, see van de Weerdt and Medema, 2006). Similarly precise regulation of Plk2 is likely to be important for its homeostatic functions during periods of elevated activity in neurons. In an attempt to better understand how neuronal Plk2 is regulated, we found that Plk2 bound via its C-terminal polo box domain (PBD) to its target SPAR (Spine-Associated RapGAP) in a phospho-dependent manner (see chapter 3), consistent with reports characterizing the PBD as an important phosphomotif binding domain that participates in the regulation of Plk1 during mitosis (Elia et al., 2003a; Elia et al., 2003b; Lowery et al., 2004; Lowery et al., 2007).

We identified CDK5 as the likely "priming" kinase that phosphorylates the PBD binding site in SPAR. Blocking CDK5 activity prevented Plk2-mediated SPAR degradation in heterologous cells, led to increased SPAR expression in neurons, and resulted in a runup in synaptic strength during elevated activity. This runup in synaptic strength was remarkably similar to that observed in cells with blocked Plk2 activity, consistent with the idea that CDK5 and Plk2 function in the same homeostatic pathway. Thus, CDK5-dependent recruitment of Plk2 to SPAR and subsequent degradation of SPAR constitutes a likely molecular mechanism of Plk2-mediated homeostasis (Figure 1).

Consistent with this idea, levels of SPAR expression positively correlated with synaptic strength, so SPAR is a plausible target of Plk2-dependent synaptic scaling. Moreover, degradation of the postsynaptic RapGAP SPAR would stimulate the activity of postsynaptic Rap GTPases, which are known to depress synaptic strength (Zhu et al., 2002; Huang et al., 2004; Zhu et al., 2005) and cause thinning of spines (Xie et al., 2005).

Both Rap1 and Rap2 have been implicated in synaptic weakening. Rap1 is activated during LTD and is thought to cause internalization of AMPARs through activation of the JNK MAPK pathway (Zhu et al., 2002). Rap2, on the other hand, seems to be involved in long-term depotentiation and mediates its effects on synapses through the p38 MAPK pathway (Zhu et al., 2005). Overexpression of activated forms of both Rap1 and Rap2 leads to synaptic weakening as measured by recording of mEPSCs, and is correlated with a reduction in both surface and total GluR2 AMPAR subunit expression level (Fu et al., 2007).

Future Directions and Outstanding Questions

Our work provides exciting and novel insights into some of the molecular mechanisms

Figure 1

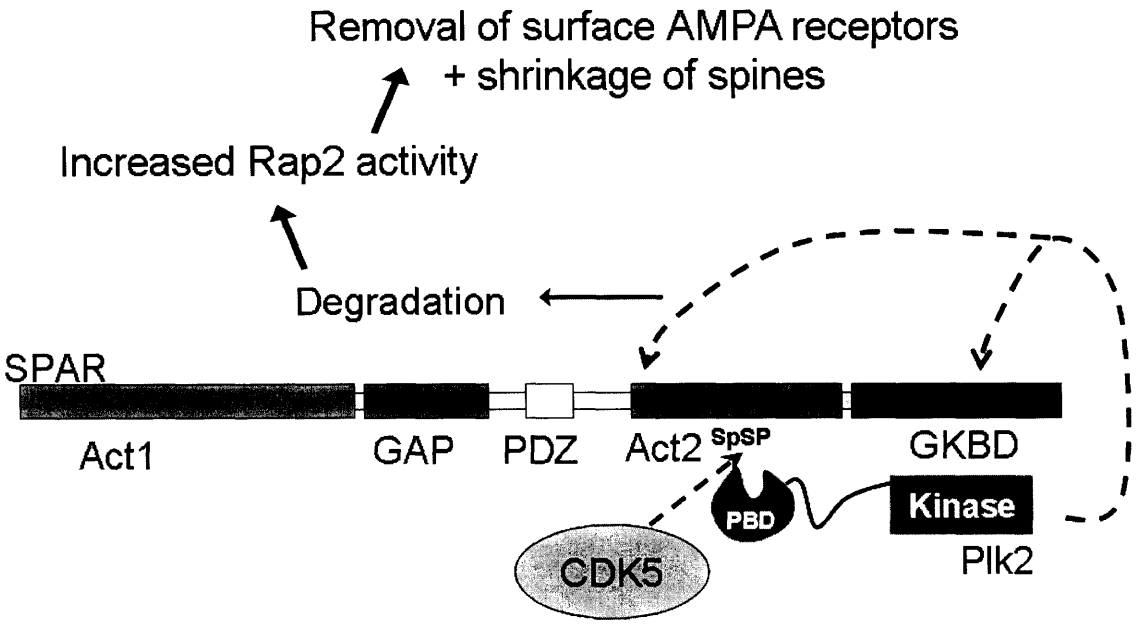
Removal of surface AMPA receptors
+ shrinkage of spines
Increased Rap2 activity
Degradation
SPAR
Act1
GAP
PDZ
Act2
SpSP
GKBD
PBD
Kinase
Plk2
CDK5

Figure 1. CDK5 regulates Plk2-dependent degradation of SPAR

Model of CDK5 dependent regulation of SPAR degradation through Plk2. CDK5 phosphorylates SPAR at ser-1328, which completes the PBD binding site in SPAR and recruits Plk2 to SPAR. This allows Plk2 to phosphorylate SPAR further, resulting in SPAR degradation, activation of Rap2, and removal of surface AMPA receptors, as well as shrinkage of spines.

underlying homeostatic plasticity during chronically elevated activity, but several questions remain unresolved. For example, what is the role of Plk2 *in vivo*? A Plk2 KO animal has been described, but it was only tested for Plk2's role in the cell cycle, and no neurobehavioral or neuroanatomical experiments were performed (Ma et al., 2003). Thus, it remains unclear to what extent Plk2-dependent homeostatic mechanisms govern *in vivo* neurophysiological processes both during development and after completed differentiation of the nervous system. One can envision, for example, that Plk2 expression during development would be critical to prevent excessive spiking activity of neurons as they receive increasing numbers of synaptic inputs and are driven towards increased activity. By causing degradation of SPAR, possibly in a synapse-specific manner, Plk2 could also be involved in activity-dependent synapse elimination that is known to be important in the refinement of connectivity during development. Thus, loss of SPAR would tend to activate Rap GTPases that can in turn cause loss of synapses, spines, and even entire dendritic segments (Fu et al., 2007). After completion of development, Plk2 could continue to be important as a homeostatic regulator that keeps neurons away from harmful extremes of activity. Failure to induce Plk2 expression could thus increase susceptibility towards seizures. In addition, we showed that induction of Plk2 allows cells to remain responsive to LTP inducing stimuli in the face of chronically elevated activity. Failure to induce Plk2 might therefore interfere with the animal's ability to form memories or learn new tasks.

In our studies, we used strong activation protocols to induce expression of Plk2 and to assess its effects on homeostatic plasticity and synaptic transmission. But what is the role of Plk2 under less extreme conditions? Plk2 is expressed at low levels under unstimulated control conditions, and may be involved in fine-tuning synapses even in unstimulated cells. However, because knocking down Plk2 expression or overexpressing the Plk2 dominant negative construct under such "resting" conditions did not have a noticeable effect on synaptic transmission as gauged by mEPSC recordings, we cannot make positive statements about Plk2's roles under those conditions. To become fully active, Plks have to bind a target through their polo box domain (PBD), and have to become phosphorylated in their T-loop within the kinase domain. It may be that these conditions are not met in unstimulated cultures.

In the cell cycle, Plk1 orchestrates several mitotic processes through a large variety of binding and substrate proteins, only some of which are degraded following Plk1 phosphorylation. Similarly, Plk2 probably has many other targets besides SPAR,

not all of which are likely to be degraded by Plk2. For example, regulation of membrane excitability through Plk2 probably involves proteins that directly or indirectly regulate expression or function of voltage gated potassium or sodium channels, as these have been implicated in homeostatic regulation of membrane excitability (Desai et al., 1999). As additional functions of Plk2 are discovered, additional substrates and binding partners will likely surface as well.

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