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Slippery Signaling: Palmitoylation-dependent Control of Neuronal Kinase Localization and Activity

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Abstract

Modification of proteins with the lipid palmitate, a process called palmitoylation, is important for the normal function of neuronal cells. However, most attention has focused on how palmitoylation regulates the targeting and trafficking of neurotransmitter receptors and non-enzymatic scaffold proteins. In this review we discuss recent studies that suggest that palmitoylation also plays additional roles in neurons by controlling the localization, interactions and perhaps even the activity of protein kinases that play key roles in physiological neuronal regulation and in neuropathological processes.

Introduction

Proteins can undergo a variety of post-translational modifications that regulate their subcellular localization and function. One such modification is covalent lipid attachment, which increases a protein's hydrophobicity and hence its association with intracellular and/or plasma membranes. Protein-lipid attachment thus has the potential to dramatically affect a protein's localization, trafficking and interactions.

Several protein-lipid attachment mechanisms are known for intracellular proteins, including S-acylation, myristoylation, farnesylation and geranylgeranylation. The first of these, S-acylation, refers to the addition of a fatty acid to protein cysteine residues *via* a thioester bond. When the conjugated fatty acid is the C16 lipid palmitate then this modification is referred to as S-palmitoylation, or simply palmitoylation. For simplicity, the term palmitoylation will be used in this review, although we note that the precise identity of the conjugated lipid has not been determined for many S-acylated proteins.

Palmitoylation occurs even in simple, unicellular organisms such as yeast and is likely important in all eukaryotic cells (Roth et al., 2006). However, control of protein localization and/or function by palmitoylation might be predicted to be especially critical in polarized, morphologically complex cells such as neurons. Consistent with this notion, studies of human patients and genetically modified mice have revealed that loss of function of several

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individual palmitoyl acyltransferases (PATs, the enzymes that catalyze palmitoylation) causes predominantly neuropathological defects (Raymond et al., 2007, Mansouri et al., 2005, Mukai et al., 2008, Li et al., 2010).

These findings raise the question of which PAT substrate(s) account for these phenotypes and, more broadly, which cellular processes in neurons are palmitoylation-dependent. Results from palmitoyl-proteomic studies suggest that these are not simple questions because a large number of neuronal proteins are palmitoylated. These palmitoyl-proteins include numerous neurotransmitter receptors, as well as scaffolding and adhesion proteins (Kang et al., 2008, Wan et al., 2013). In particular, many key pre- and post-synaptic proteins at glutamatergic synapses, the major excitatory synaptic connections in the brain, are palmitoylated. Postsynaptic palmitoyl-proteins include multiple AMPA- and NMDA-type glutamate receptor subunits and several of their scaffolding/auxiliary proteins, while presynaptic palmitoyl-proteins include multiple components of the neurotransmitter release machinery (Kang et al., 2008, Hayashi et al., 2005, Hayashi et al., 2009, Craven et al., 1999, Fukata and Fukata, 2010, Thomas et al., 2012, Thomas et al., 2013, Oku et al., 2013, Prescott et al., 2009). Moreover, palmitoylation-dependent regulation is not just a feature of glutamatergic synapses - specific receptors for the inhibitory neurotransmitter GABA are also palmitoylated, along with gephyrin, a key scaffolding protein at inhibitory synapses, (Dejanovic et al., 2014, Fang et al., 2006, Keller et al., 2004). Key roles for palmitoylation in the targeting and trafficking of many of these receptors and non-enzymatic proteins have been described (reviewed by (Fukata and Fukata, 2010, Thomas and Huganir, 2013, Prescott et al., 2009)). Recently, however, several studies suggest that palmitoylation can more directly regulate neuronal enzymatic signaling, because a select group of protein kinases are modified by palmitoylation. Here, we focus on recent developments in our understanding of how palmitoylation regulates these kinases. Importantly, these new findings not only provide new insights into neuronal regulation but also reveal broader roles for palmitoylation at both the cellular and molecular levels.

Palmitoylation-dependent signaling by Src-family Kinases (SFKs)

The possibility that palmitoylation might be critical for signaling by specific kinases was first raised when several members of the Src family of non-receptor tyrosine kinases (SFKs) were identified as palmitoyl-proteins. In particular, the SFKs Fyn, Lck, Yes, Fgr and Hck were all found to be palmitoylated in non-neuronal cells, although interestingly Src itself was not (Shenoy-Scaria et al., 1994, Koegl et al., 1994, Alland et al., 1994, Paige et al., 1993). A series of key papers revealed that palmitoylation is critical for signaling by both Fyn and Lck SFKs in specific cells of the immune system. In particular, point mutation of Fyn or Lck palmitoylation sites, or pharmacological inhibition of palmitoylation with the compound 2-Bromopalmitate markedly impairs signaling from activated T cell or B cell receptors (Webb et al., 2000, Kabouridis et al., 1997, van't Hof and Resh, 1999). Interestingly, both Fyn and Lck are constitutively modified with the lipid myristate, but myristoylation alone cannot support stable membrane association, so palmitoylation is a critical second lipid modification that is essential for Fyn/Lck membrane targeting and downstream signaling. We highlight several excellent reviews e.g. (Resh, 2006, Bijlmakers,

2009, Yount et al., 2013) for those interested in further details of palmitoylation-dependent SFK signaling in the immune system and in other non-neuronal cells.

Synaptic regulation by the palmitoylated SFK Fyn

In addition to their importance in the immune system and other non-neuronal cells, several palmitoylated SFKs are also expressed in neurons, where they are known to play key roles. In particular, the importance of Fyn for neuronal signaling has long been appreciated because Fyn knockout mice show impairments in hippocampal long-term potentiation (LTP, a long-lasting increase in the strength of transmission at specific synapses that is the best-known cellular correlate of learning and memory). In contrast, genetic deletion of Src or Yes, two other SFKs that are expressed in the hippocampus, does not affect LTP (Grant et al., 1992, Kojima et al., 1997). The importance of palmitoylation for Fyn membrane targeting and signaling in non-neuronal cells strongly suggests that neuronal Fyn signaling likewise requires its palmitoylation-dependent membrane association.

Consistent with the notion that Fyn is active at synaptic membranes, neuronal Fyn substrates include several transmembrane or membrane-associated proteins that are key components of excitatory synapses. One of the best-known neuronal Fyn substrates is the GluN2B subunit of the NMDA-type glutamate receptor. Fyn phosphorylates multiple tyrosine residues in the GluN2B C-terminal tail, one of which (Tyr1472) lies in a motif that binds clathrin adaptor proteins and controls GluN2B internalization. Tyr1472 phosphorylation by Fyn has been proposed to mask this motif, thus stabilizing GluN2B at the neuronal cell surface (Prybylowski et al., 2005, Trepanier et al., 2012). Interestingly, the synaptic scaffolding proteins PSD-95 and PSD-93 are also Fyn binding partners and substrates (Trepanier et al., 2012, Nada et al., 2003). PSD-95/-93 also bind the C-terminal tails of the GluN2A/GluN2B subunits and can thus promote Fyn's ability to regulate NMDA receptors (Trepanier et al., 2012, Tezuka et al., 1999). Like Fyn, GluN2A/2B and PSD-95/-93 are palmitoyl-proteins (Kang et al., 2008, Hayashi et al., 2009, El-Husseini et al., 2002), raising the possibility that palmitoylation targets Fyn and its substrates to similar membrane microdomains and thereby facilitates their phospho-dependent regulation.

Another aspect of palmitoylation-dependent signaling involving Fyn arises from the recent identification of the PAT DHHC5 as a neuronal Fyn substrate. Fyn phosphorylates DHHC5 at a specific residue (Tyr533) which, like the Tyr1472 site on GluN2B, forms part of a clathrin-dependent internalization motif (Brigidi et al., 2015). Again, phosphorylation by Fyn appears to mask this motif, because Tyr533 phosphorylation promotes DHHC5 plasma membrane localization in non-neuronal cells and increases DHHC5 stability on the plasma membrane of neuronal dendritic spines (Brigidi et al., 2015). In an elegant series of experiments, Bamji and colleagues further showed that DHHC5 stability in spines involves a tri-partite complex of Fyn, DHHC5 and PSD-95 (Brigidi et al., 2015). Interestingly, this complex can be rapidly regulated by changes in neuronal activity, with important consequences for synaptic transmission and plasticity. In particular, enhancing neuronal activity leads to rapid dephosphorylation of DHHC5-Tyr533, which triggers rapid DHHC5 internalization. This acute activity-dependent relocalization is necessary for DHHC5 to subsequently palmitoylate the adhesion protein delta-catenin (Brigidi et al., 2015, Brigidi et

al., 2014), which then traffics back into spines with DHHC5. Importantly, previous work from the same group revealed that DHHC5-dependent delta-catenin palmitoylation stabilizes synaptic cadherin-catenin complexes and also plays a key role in activity-dependent increases in the number of synaptic AMPA-type glutamate receptors (Brigidi et al., 2014). Thus, Fyn is not only palmitoylated but is also a key regulator of neuronal palmitoylation events that control excitatory synaptic transmission (Figure 1).

Further questions regarding neuronal roles of palmitoyl-Fyn

Although Fyn plays key roles in the regulation of excitatory synapses, additional Fyn substrates in different subcellular locations have been reported. For example, Fyn also likely phosphorylates GABA receptors, which localize to inhibitory synapses, and the sodium channel NaV1.2 subunit, which preferentially localizes to axons (Ahn et al., 2007, Jurd et al., 2010). In addition, Fyn is implicated in the correct formation of both dendrites and axons during early neurodevelopment, prior to the appearance of mature synapses (Meriane et al., 2004, Sasaki et al., 2002). It will be interesting to determine whether palmitoylation is also critical for Fyn activity towards these additional substrates, and how Fyn targeting to different membrane locations within the neuron is controlled.

The importance of Fyn, and likely its palmitoylation, in postsynaptic regulation raises the additional question of how Fyn palmitoylation is itself regulated. The binding of Fyn to DHHC5 might initially suggest that this PAT palmitoylates Fyn in neurons, but several findings suggest this may not be the case. First, Fyn palmitoylation was not decreased by DHHC5 knockdown in cultured cortical neurons, nor was it decreased in neuronal stem cells from a gene trap mouse line that expresses very low levels of DHHC5 (Thomas et al., 2012, Li et al., 2012). Although other PATs may compensate for the absence of DHHC5 in these situations, it is of note that Fyn palmitoylation in non-neuronal cells is mediated by DHHC21 (Mill et al., 2009). This PAT has not been characterized in the nervous system but is likely expressed in hippocampal neurons and other neuronal cell types (Cajigas et al., 2012, Doyle et al., 2008). It is thus possible that DHHC21 indeed palmitoylates Fyn and perhaps other neuronal proteins. Another possible Fyn PAT is DHHC2, which can palmitoylate the related SFK Lck in non-neuronal cells (Zeidman et al., 2011) and also palmitoylates other key synaptic proteins (Noritake et al., 2009, Woolfrey et al., 2015).

Closer examination of structural differences between these candidate Fyn PATs provides additional interesting, though not definitive, insights into which of them might palmitoylate Fyn in neurons. For example, the assignment of DHHC21 as a Fyn PAT is in part based on Fyn's abnormal localization in hair follicles of *depilated* (*dep*) mice, a mouse line in which a single amino acid in the C-terminal non-catalytic region of DHHC21 is missing (Mill et al., 2009). It is tempting to speculate that this region of DHHC21 (which is poorly conserved in other PATs) is the key factor that accounts for its regulation of Fyn in *dep* hair follicles. However, even if this is the case, whether the same mechanism is important for Fyn palmitoylation in neurons remains to be established. An additional factor arguing against the palmitoylation of Fyn by DHHC5 is that the C-terminus of this PAT forms a PSD-95/*discs large*/ZO-1 (PDZ) domain-binding motif that is essential for DHHC5 to interact with and palmitoylate certain substrates (Thomas et al., 2012). Fyn lacks a PDZ domain and so should

not be recognized by DHHC5 in this way. However, not all DHHC5 substrates contain PDZ domains (Brigidi et al., 2014, Howie et al., 2014) and so DHHC5 cannot be ruled out as a potential Fyn PAT. The identity of the Fyn PAT(s) may thus differ depending on neuron type and/or subcellular location but is an interesting area for future study. Another intriguing question is whether Fyn palmitoylation (and hence its localization and/or kinase activity) is itself regulated by synaptic activity or other stimuli.

Regulation of neuronal cytoskeletal dynamics by palmitoylated kinases

Normal brain function requires not only tight regulation of the number and function of synaptic neurotransmitter receptors, but also precise control of neuronal structure and morphology. This is particularly so for dendritic spines, the actin-rich protrusions that are the site of most excitatory synapses (Hotulainen and Hoogenraad, 2010). Just as receptor number and function can be regulated in a synapse-specific manner, so can the morphology of individual spines (Matsuzaki et al., 2004, Harvey and Svoboda, 2007). Importantly, dynamic changes in the size of individual dendritic spines are frequently observed following LTP and are thus closely associated with higher brain functions such as learning and memory (Fifkova and Van Harreveld, 1977, Bosch and Hayashi, 2012, Murakoshi and Yasuda, 2012). Links between dendritic spine structure and cognitive function are strengthened by findings that spine morphology and/or density is altered in an array of neuropathological conditions, ranging from autism-spectrum disorders to Alzheimer's Disease (Fiala et al., 2002, Penzes et al., 2011).

Rapid changes in spine shape and size largely reflect dynamic modulation of the spine's underlying actin cytoskeleton (Hotulainen and Hoogenraad, 2010), suggesting that neurons possess mechanisms to spatially control spine actin dynamics. However, many actin regulatory proteins are small and thought to be freely diffusible, so how such spatially precise actin regulation might be achieved was unclear. George *et al.* recently hypothesized that modification of actin regulatory proteins by palmitoylation might facilitate precise control of spine morphology. Using bioinformatic tools (Oku et al., 2013, Obenaus et al., 2003) they searched for predicted palmitoyl-motifs in actin regulators and identified such a motif close to the N-terminus of LIM Kinase-1 (LIMK1). LIMK1 regulates actin dynamics by phosphorylating and inactivating the actin severing/disassembly protein cofilin, and LIMK1 knockout mice display specific alterations in dendritic spine morphology (Meng et al., 2002). This raised the possibility that palmitoylation of LIMK1 might be particularly important for the control of spine actin dynamics. Consistent with this notion, LIMK1's N-terminal palmitoyl-motif was found to be necessary and, strikingly, sufficient for its enrichment in dendritic spines (George et al., 2015). The authors then used an shRNA knockdown/rescue approach to replace endogenous LIMK1 in hippocampal neurons with shRNA-resistant wild type or palmitoyl-mutant forms of LIMK1. They found that LIMK1 'knockdown' impairs normal actin turnover in dendritic spines and also attenuates the enlargement of individual spines that normally occurs in response to elevated neuronal activity (George et al., 2015). The deficits in actin turnover and activity-dependent spine enlargement could be 'rescued' by wild type LIMK1 but not by a LIMK1 palmitoyl-mutant (George et al., 2015).

These findings strongly suggested that palmitoylation is critical for LIMK1 dependent regulation of dendritic spines. However, it was initially surprising that the LIMK1 palmitoyl-mutant failed to rescue these phenotypes, despite being detectable in spines, only less enriched than wtLIMK1. The authors first addressed whether the LIMK1 palmitoyl-site mutant is simply misfolded and inactive. However, arguing against this possibility, palmitoyl-site mutation did not affect either LIMK1 activation by the upstream kinase p21-activated kinase-3 (PAK3), or the ability of activated LIMK1 to phosphorylate its downstream substrate cofilin *in vitro* (George et al., 2015). In marked contrast, though, mutation of LIMK1's palmitoylation sites completely prevented LIMK1 phosphorylation at the activatory site, T508, in neurons. The authors suggested that this lack of T508 phosphorylation was because palmitoyl-mutant LIMK1 cannot localize to the spine membrane, where its upstream kinase PAK is located (George et al., 2015). Thus, while exerting a direct effect only on LIMK1 localization, palmitoylation also ensures that only LIMK1 localized to the spine membrane is active in neurons (Figure 2). The authors further suggested that palmitoylation-dependent control of LIMK1 activity might create a “shell-to-core” gradient of actin polymerizing activity at the sub-spine level. This is because palmitoyl-LIMK1 might be predicted to specifically phosphorylate and inactivate cofilin in juxtamembrane regions, thus favoring actin polymerization. In contrast, cofilin in the spine core would remain unphosphorylated and active to ensure that actin filaments are maintained at the appropriate length. However, experimental evidence is needed to test this hypothesis.

These findings not only provide new insights into the spatial control of actin dynamics at the single spine and perhaps even the sub-spine level, but also highlight intriguing areas for future study. For example, the ‘upstream’ proteins that regulate PAK/LIMK1 activity in spines are not known, but it is striking that palmitoylated forms of both Rac and Cdc42, small GTPases that are well known regulators of LIMK1 signaling, are detected in neurons (Kang et al., 2008, Wirth et al., 2013). Moreover, a palmitoylated form of Cdc42 is dominantly expressed in hippocampus (compared to the prenylated form, which is dominant in most other tissues), is preferentially targeted to dendritic spines, and is important for the control of spine morphology (Wirth et al., 2013, Kang et al., 2008). These findings raise the possibility that palmitoylation controls the spine localization and activity of multiple elements of the Rac/Cdc42-PAK-LIMK1 signaling module (Figure 2).

Roles for Palmitoyl-kinases in earlier stages of dendritic development

Interestingly, palmitoylated forms of LIMK1 and cdc42 are also detectable in cultured neurons prior to the formation of the majority of dendritic spines (George et al., 2015, Kang et al., 2008). This finding suggests that palmitoylation-dependent Rac/Cdc42-PAK-LIMK1 signals may also control earlier stages of development of the neuronal cytoskeleton, for example dendritic branching and/or extension. Indeed, another palmitoylated kinase, Calcium/calmodulin-dependent protein kinase-1 gamma (CaMKI-gamma, also known as CLICK-III) may regulate dendritogenesis *via* the Rac/Cdc42-PAK-LIMK1 pathway (Takemoto-Kimura et al., 2007). CaMKI-gamma is part of a family of CaMKs that play key roles in activity-dependent dendritic arborization (Wayman et al., 2006, Redmond et al., 2002). Of the neuronal CaMKs, CAMKI-gamma undergoes two sequential lipid modifications, prenylation followed by palmitoylation, which together recruit CAMKI-

gamma to dendritic lipid rafts (Takemoto-Kimura et al., 2007). CAMKI-gamma knockdown impairs dendritic outgrowth, an effect that is rescued by wild type, but not palmitoyl-deficient, CAMKI-gamma (Takemoto-Kimura et al., 2007), suggesting that palmitoylation of CAMKI-gamma is critical for its dendritogenic role. Interestingly, the impaired dendritic growth caused by CAMKI-gamma knockdown was also rescued by constitutively active Rac, suggesting that raft-localized CAMKI-gamma controls dendritogenesis via Rac (and hence potentially PAK/LIMK1). Taken together these studies on Rac/Cdc42, CAMKI-gamma and LIMK1 suggest that palmitoylation-dependent kinase signaling is critical for multiple stages in the development and regulation of the dendritic cytoskeleton. These findings also raise the possibility that other processes that require precise control of neuronal cytoskeletal dynamics (e.g. rapid, cue-directed steering of axonal growth cones (Gomez and Letourneau, 2014)) may be palmitoylation-dependent.

Synaptic Regulation by Palmitoylated Protein A Kinase Anchoring Proteins (AKAPs)

Thus far we have focused our discussion exclusively on neuronal kinases that are directly palmitoylated. However, recent studies reveal that even signaling by non-palmitoylated kinases can still be palmitoylation-dependent. A good example of this situation is AKAP79/150, a palmitoyl-protein that is important for synaptic regulation by Protein Kinase A (PKA) (Sanderson and Dell'Acqua, 2011, Keith et al., 2012). AKAP79/150 targeting to synapses is palmitoylation-independent under basal conditions, but palmitoylation is required for rapid AKAP79/150 synaptic recruitment following elevated neuronal activity (Keith et al., 2012). This rapid recruitment of AKAP79/150 likely involves its trafficking via dendritic endosomes, where AKAP79/150 is palmitoylated by the endosomal PAT DHHC2 (Woolfrey et al., 2015). Consistent with this model, AKAP79/150 palmitoyl-site mutation, or RNAi-mediated knockdown of DHHC2, prevent activity-dependent increases in both dendritic spine size and synaptic localization of AMPA-type glutamate receptors (Keith et al., 2012, Woolfrey et al., 2015). It will be of interest to determine whether these palmitoylation-dependent effects of AKAP79/150 on synapses solely depend on targeting of PKA activity, or also involve other signaling molecules to which AKAP79/150 binds (Sanderson and Dell'Acqua, 2011). A related question is whether the recently described role of AKAP79/150-PKA signaling in the regulation of specific pools of AMPA-type glutamate receptors also requires AKAP79/150 palmitoylation (Sanderson et al., 2016). More broadly, these findings raise the important possibility that spatial regulation of signaling by other kinases may be controlled by palmitoylation of their specific binding partners.

New Roles for Palmitoyl-kinases in the Peripheral Nervous System (PNS)

Palmitoylation-dependent kinase signaling is thus critical for multiple aspects of Central Nervous System (CNS) function, but recent findings also reveal the importance of palmitoyl-kinases in peripheral neurons. The axons of PNS neurons often project long distances to innervate their target tissues, presenting a great challenge for the relay of both anterograde signals (from soma to axons) and also retrograde signals (from axons to soma). Axonal retrograde signals are critical to stimulate transcriptional programs that control regenerative

responses to peripheral nerve injury (Rishal and Fainzilber, 2014, Abe and Cavalli, 2008). Several key mediators of retrograde injury signaling have recently been identified, of which Dual Leucine-zipper Kinase (DLK) has received considerable attention. DLK is an evolutionarily conserved upstream activator (a MAP3K) of the Mitogen-activated Protein Kinases (MAPKs) c-Jun N-terminal Kinase (JNK) and p38 MAPK (Tedeschi and Bradke, 2013). In mammalian peripheral neurons, DLK-dependent JNK activation is heavily implicated in retrograde signals that result in phosphorylation and activation of the c-Jun transcription factor (Ghosh et al., 2011, Shin et al., 2012). However, it had been unknown how DLK, a predicted soluble kinase, conveys these long-distance signals. Recently, DLK was found to be palmitoylated at a conserved site adjacent to its kinase domain (Holland et al., 2016). ShRNA knockdown/rescue experiments, in which endogenous DLK in sensory neurons was replaced with a palmitoyl-site mutant, revealed that palmitoylation at this site is critical for DLK-dependent retrograde injury signaling (Holland et al., 2016) (Figure 3).

At the cellular level, palmitoylation targets DLK to motile trafficking vesicles, some of which move retrogradely and may thus convey axonal retrograde signals. In addition to this clear effect on trafficking, though, palmitoylation also impacts DLK at the molecular level. In particular, palmitoyl-site mutation prevents DLK's ability to form multiprotein complexes that contain the scaffold protein JNK-interacting Protein-3 (JIP3) and the downstream kinases MAP Kinase Kinase-4 (MKK4) and MKK7 (Holland et al., 2016). Both MKK4 and JIP3 can co-traffic with wild type (i.e. palmitoylated) DLK in axons, suggesting that palmitoylation of DLK may be critical for the assembly and/or stability of axonal complexes that contain multiple JNK pathway signaling components. Finally, palmitoylation is also critical for DLK's ability to phosphorylate its direct substrates MKK4 and MKK7, both *in vitro* and in transfected cells (Holland et al., 2016). These findings strongly suggest that palmitoylation plays an additional role to control DLK-dependent signaling and raise the exciting possibility that palmitoylation directly controls DLK's kinase activity.

Is there any precedent for this unexpected potential role of palmitoylation? The answer would appear to be yes, because another palmitoyl-kinase, G protein-coupled Receptor Kinase-6 (GRK6) was also reported to be markedly more active, even *in vitro*, when palmitoylated (Babu et al., 2004, Loudon and Benovic, 1997). It will be of considerable interest to determine whether roles of GRK6 in regulation of receptors for the neurotransmitter dopamine are palmitoylation-dependent (Gainetdinov et al., 2003). However, it is important to note that the *in vitro* assays that revealed elevated activity of the palmitoylated forms of GRK6 and DLK both used kinases that had been isolated from mammalian cells. Elevated DLK/GRK6 activity may thus have been a secondary effect of a palmitoylation-dependent modification in cells (e.g. activatory phosphorylation by an additional membrane-associated kinase), which persists following isolation of the kinase *in vitro*. Testing the hypothesis that palmitoylation instead acts autonomously to alter DLK and/or GRK6 conformation and hence kinase activity will be challenging and may require high-resolution structures of the palmitoylated and non-palmitoylated forms of these kinases. Nonetheless, regardless of which mechanism is correct, an emerging consensus is that palmitoylation restricts the activity of a given palmitoyl-kinase to a specific subcellular location. Thus, because palmitoylation targets DLK to vesicles, only vesicle-bound DLK is likely active in neurons. Similarly, the activity of LIMK1 is restricted to dendritic spines,

whereas active GRK6 is enriched on membranes that contain its G-protein coupled receptor substrates. It will be of considerable interest to address whether neurons or other cells use ‘multi-functional’ palmitoylation of this type to regulate other kinases and/or other families of signaling enzymes.

Additional Roles for Palmitoylation in DLK-JNK Pathway Signaling

The importance of palmitoylation in DLK-JNK signaling is reinforced by a report that another member of this pathway, the neurally-enriched JNK3, is also palmitoylated (Yang et al., 2012). In peripheral neurons JNK3 plays key roles in retrograde responses to axonal injury and was previously reported to colocalize with JIP3 on axonal vesicles (Keramiris et al., 2005, Cavalli et al., 2005). It is thus tempting to speculate that palmitoylation accounts for this vesicular targeting of JNK3 and that these JNK3-JIP3-positive vesicles are similar, or perhaps identical, to the recently reported DLK-JIP3 positive vesicles (Holland et al., 2016). If so, then palmitoylation would play a key role in modifying multiple DLK-JNK pathway components to ensure the specificity and transport of neuronal injury signals. It is important to note, though, that in CNS neurons, the palmitoylated form of JNK3 was reported to regulate secretory trafficking (Yang et al., 2013) and axonal development (Yang et al., 2012). These different roles of JNK3 may reflect CNS-PNS differences, but multiple functions for JNK3 palmitoylation should perhaps be expected, given that this kinase can play both physiological and pathological roles in neurons (Waetzig and Herdegen, 2003).

Future Questions: additional palmitoyl-kinases, additional roles in neuronal development and function?

Recent studies have thus revealed several novel roles for palmitoyl-kinases, but these roles have largely been elucidated on a case-by-case basis using low throughput approaches. Excitingly, though, high throughput studies have identified several other kinases that are likely palmitoylated, yet for which the roles of their palmitoylation in neurons remain unexplored. For example, members of the Casein Kinase 1 gamma (CK1 gamma) subfamily have been identified in palmitoyl-proteomic studies from several mammalian tissues (Sanders et al., 2015, Blanc et al., 2015). CK1 is highly evolutionarily conserved and roles of palmitoylation in subcellular trafficking and localization have been defined for the yeast CK1 ortholog (e.g. (Babu et al., 2004)). In contrast, much less is known about roles of CK1 palmitoylation in multicellular organisms. Interestingly, though, the *Drosophila* CK1 gamma ortholog, Gilgamesh, is preferentially expressed in neurons of the mushroom bodies (MBs), regions of the fly brain that are critical for olfactory-dependent learning (Tan et al., 2010). Moreover, forced expression of Gilgamesh in adult MBs rescues learning deficits in *gilgamesh* mutants, suggesting that this CK1 gamma ortholog actively participates in signaling events within MB neurons that underlie memory formation (Tan et al., 2010). CK1 gamma is also highly expressed in several mammalian CNS and PNS neuronal populations (Lerch et al., 2012, Cajigas et al., 2012), suggesting that this kinase, and perhaps its palmitoylation, may also be critical for neuronal function in higher organisms.

A second group of kinases identified in palmitoyl-proteomic studies is the Eph (ephrin receptor) family of receptor tyrosine kinases (Kang et al., 2008). Signaling *via* Ephs and

their ephrin ligands regulates a diverse array of processes, including axon guidance, synaptogenesis and the stability and plasticity of mature synapses (Kania and Klein, 2016, McClelland et al., 2010, Klein, 2009). It will be interesting to determine the extent to which the diverse roles of ephrin/Eph signaling in neuronal development and function are palmitoylation-dependent.

Two further kinases that are reported to be palmitoylated are the brain-specific kinases (BRSKs) -1 and -2 (Kang et al., 2008, Rodriguez-Asiain et al., 2011), also known as SAD kinases A and B (Barnes et al., 2007, Barnes et al., 2008, Kishi et al., 2005, Shelly et al., 2007). BRSK-1/-2 play important roles in neuronal polarization, the correct specification of a neuron's axon and dendrites, which is critical for the appropriate transmission and integration of neural information. The process of neuronal polarization likely requires differential activity and/or distribution of intracellular signaling proteins in response to asymmetrically distributed cues and would thus appear to be a prime candidate to be subject to palmitoylation-dependent regulation. Experiments using a recently developed Fluorescence Resonance Energy Transfer (FRET) kinase activity reporter suggest that BRSK activity is indeed spatially regulated, and is elevated in axons compared to other regions of developing hippocampal neurons (Sample et al., 2015). In addition, experiments from non-neuronal cells revealed that BRSK1 associates with lipid rafts and, interestingly, the raft-associated BRSK1 has higher kinase activity than 'non-raft' BRSK1 (Rodriguez-Asiain et al., 2011). It will thus be of interest to determine whether axonal BRSK activity is due to the palmitoylated form of BRSK-1 and/or BRSK-2 and whether palmitoylation is critical for BRSK1/2-dependent control of neuronal polarization. The finding that the BRSK1/2 'upstream' activator Liver Kinase B1 (LKB1) is farnesylated (Collins et al., 2000) raises the possibility that signaling via multiple lipid-modified kinases is critical for axon polarization. This scenario, which would be reminiscent of the Rac/Cdc42/PAK/LIMK1 module, would suggest that lipid modification of multiple signaling proteins in a common pathway represents a frequently used mechanism to ensure spatially precise neuronal signaling.

How many Palmitoyl-kinases are there?

The human genome codes for >500 protein kinases (Manning et al., 2002). So far around 20 of these kinases are reported to be palmitoylated, but several lines of evidence suggest that additional palmitoyl-kinases likely exist. First, palmitoyl-proteomic databases list numerous other kinases that have been identified as palmitoyl-proteins in single reports (Blanc et al., 2015, Sanders et al., 2015). Cognizant that a number of these 'hits' may turn out to be false positives, we thus mainly focused our review on kinases that were identified in multiple palmitoyl-proteomic studies, or those for which targeted studies have been performed. However, palmitoylation is not a constitutive modification, so palmitoylation of a particular kinase may occur only in a specific cell type, at a particular developmental stage, or in response to a specific stimulus. Because palmitoyl-proteomic studies have been performed from a diverse range of cell types and tissues it appears likely that some of these singleton 'hits' will indeed turn out to be *bona fide* palmitoyl-kinases. A related point is that palmitoylation appears to play particularly important roles in neurons, consistent with the morphological complexity of these cells and their consequent need for precise subcellular

control of protein targeting. However, only two palmitoyl-proteomic studies have thus far been carried out using neuronal tissue (Wan et al., 2013, Kang et al., 2008), and none as yet from peripheral neurons. These studies reported an impressive number of palmitoyl-proteins, but did not identify several palmitoylated neuronal proteins, including kinases, that have been identified in targeted studies e.g. (Yang et al., 2012, Holland et al., 2016, George et al., 2015, Levy et al., 2011). This raises the strong possibility that additional studies from different neuronal preparations may identify additional palmitoyl-kinases.

How is Palmitoylation of Neuronal Kinases Regulated?

How palmitoylation is regulated at the cellular level is a key outstanding question for those interested in this regulatory mechanism. Remarkably little is known about this issue even for well-studied palmitoyl-proteins, so the paucity of information regarding regulation of neuronal palmitoyl-kinases, many of which were only recently identified, is unsurprising. Indeed, current knowledge is largely limited to observations that palmitate turnover on LIMK1 in hippocampal neurons is reasonably slow (half-time approximately 6 hours (George et al., 2015)) while palmitate turnover of DLK in cultured sensory neurons is much more rapid (half-time less than one hour) (Holland et al., 2016). Specific stimuli that increase (or decrease) LIMK1 or DLK palmitoylation have not been described. However, an intriguing recent report of rapid palmitoylation of the SFK Lck in response to Fas ligand engagement suggests that palmitoylation of specific kinases may indeed be acutely regulated in response to extracellular stimuli (Akimzhanov and Boehning, 2015).

Perhaps unsurprisingly, even less is known regarding the identity and/or regulation of thioesterase(s) whose activity accounts for palmitate turnover on palmitoyl-kinases. Nonetheless, given the strong links between several palmitoyl-kinases and disease conditions, the identity and regulation of palmitoyl-kinase PATs and thioesterases and the evaluation of these enzymes as potential therapeutic targets are all key areas for future study.

Conclusions

Although initially limited to a few isolated reports, their growing number suggests that palmitoyl-kinases represent a newly defined enzyme family. Several palmitoyl-kinases have been recently described in neurons and so we have focused on this cell type. However, precise subcellular regulation is important in all cells, so findings from neurons may enhance our knowledge of how a wide variety of cell types ensure spatially precise kinase signaling. It will be of great interest to see how these insights shape our understanding of intracellular signaling in health and disease as this field continues to evolve.

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Figure 1. Regulation of Excitatory Synapses by palmitoylated Fyn

Left panel. Several key pre- and post-synaptic proteins are palmitoylated (marked with yellow lipid). These palmitoyl-proteins include the tyrosine kinase Fyn, which is likely localized to the postsynaptic plasma membrane by dual lipid modification (myristate plus palmitate). Under normal conditions, Fyn phosphorylates NMDA receptors and the PAT DHHC5, preventing their internalization. *Right panel:* Elevated synaptic activity leads to dephosphorylation of DHHC5 at the Fyn site, triggering DHHC5 internalization to recycling endosomes (RE). Here, DHHC5 palmitoylates delta-catenin and the two proteins are trafficked back to the spine membrane, where delta-catenin associates with N-cadherin to increase the synaptic pool of AMPA-type Glutamate receptors. Note that several other synaptic palmitoyl-proteins, such as PSD-95, are omitted from the schematic for clarity. Also not pictured is the synaptodendritic PAT DHHC2, which palmitoylates several dendritic/postsynaptic proteins, and may also palmitoylate Fyn.

Figure 2. Control of Spine Morphology by Palmitoylated Cdc42 and LIMK1

Left panel: Palmitoyl-LIMK1 on the dendritic spine membrane is phosphorylated and activated *via* a pathway that involves membrane-bound upstream activators such as Rac, Cdc42 (which is also palmitoylated) and PAK. In contrast, any non-palmitoylated LIMK1 in the spine ‘core’ remains inactive. This palmitoylation-dependent control of LIMK1 localization and activity may facilitate juxtamembrane phosphorylation and inactivation of cofilin, thus allowing spine-specific, and perhaps even sub-spine, control of actin dynamics. *Right panel:* Elevated synaptic activity triggers Rac/Cdc42/PAK activation and recruitment of additional cofilin to spines (Bosch et al., 2014, Murakoshi et al., 2011). Both these events are required for activity-dependent spine enlargement, which also depends on palmitoyl-LIMK1 (George et al., 2015). Control of sub-spine actin polymerization by this signaling pathway may provide the force required for the enlargement of specific spines that is frequently observed during LTP.

Figure 3. Axonal Retrograde Signaling by Palmitoylated DLK-JNK Pathway Kinases

Palmitoylation is essential for DLK's ability to convey retrograde injury signals following distal axonal injury. At the cellular level, palmitoylation targets DLK to trafficking vesicles where DLK forms complexes with its direct substrate MKK4. These complexes also contain the scaffold protein JIP3, which binds dynein/dynactin retrograde motor proteins, and may also involve the downstream palmitoyl-kinase JNK3, which is heavily implicated in axonal retrograde signaling. In addition to this role in DLK localization, palmitoylation is necessary for DLK's ability to activate the JNK pathway. This additional 'security feature' ensures that depalmitoylated DLK does not inappropriately phosphorylate cytosolic MKKs.