CMLS, Cell. Mol. Life Sci. 62 (2005) 1571–1578
1420-682X/05/141571-08
DOI 10.1007/s00018-005-5043-1

CMLS Cellular and Molecular Life Sciences

Review

APC/C regulation of axonal growth and synaptic functions in postmitotic neurons: the Liprin-α connection

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Received 31 January 2005; received after revision 23 February 2005; accepted 23 March 2005
Online First 28 May 2005

Abstract. Protein ubiquitination has critical roles in neuronal physiology and defects in protein ubiquitination have been implicated in neurodegenerative pathology. The anaphase-promoting complex/cyclosome (APC/C) is one of two key E3 ubiquitin ligase complexes that functions in regulating cell cycle transitions in proliferating cells by acting on cyclins and components of the mitotic/meiotic apparatus. Documentation of APC/C's action beyond cell division is sparse. In the past year, however, novel and surprising roles for APC/C in postmitotic neurons, particularly in the modulation of axonal growth and synaptic functions, have been revealed. APC/C and its activator Cdh-1 are found in good abundance in neurons, and these seem to function at different cellular locations, modulating apparently diverse processes such as axonal growth and synaptic function. Interestingly, there also appears to be a single link to these apparently divergent actions of APC/C in neurons – the multi-domain, multi-functional scaffolding protein Liprin-α, which is an APC/C substrate.

Key words. Anaphase-promoting complex/cyclosome (APC/C); Cdh-1; Liprin-α, neuron; synapse.

Introduction

The ubiquitin-proteosome system (UPS) provides a major mechanism whereby protein degradation and turnover occur in eukaryotic cells. Ubiquitin-mediated proteolysis is important for basic cellular processes such as the regulation of cell cycle progression, morphogenesis, differentiation and development, and biogenesis of cellular organelles, amongst others. Ubiquitination is an evolutionarily conserved process in eukaryotic cells, whereby the small 76-amino acid peptide ubiquitin is covalently conjugated to lysine residues of targets by a cascade of enzymatic steps. The E1 ubiquitin-activating enzyme activates ubiquitin with ATP, generating a high-energy thiol ester intermediate. The ubiquitin moiety is then transferred by one of several E2 ubiquitin conjugating enzymes to substrates bound to a member of the large E3 family of ubiquitin ligases. E3 ligases come in many forms and are the ones imparting substrate specificity to the system. These processes have been the subject of recent excellent reviews [1–3], and will not be elaborated here.

Studies of the eukaryotic cell cycle have revealed its temporal regulation in fascinating detail. Cyclins, the modulators of cell cycle stage transitions, are synthesized and destroyed in a cyclical manner [4], effected partly by a tight and complex regulation of their ubiquitination and proteolysis. Two ubiquitin ligase complexes, the Skp/Cullin/F-box (SCF) complex and the anaphase-promoting complex/cyclosome (APC/C), are key regulators of the eukaryotic cell cycle stage transitions [5]. The APC/C, first characterized as an E3 ligase specific for B-type cyclins, appears also to act on several mitotic kinases, mitotic spindle proteins and the DNA replication machinery [6–7]. That these ubiquitin ligase complexes have functions in the context of postmitotic cells such as the neu-

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ron is not particularly expected. In the past year, however, major experimental breakthroughs have illuminated novel and intriguing roles for APC/C in postmitotic neurons, particularly in the modulation of axonal growth and synaptic functions. These new roles, as well as a novel APC/C substrate, Liprin-α, which appear to provide the link for the manifestation of APC/C's neuronal function, are the focus of the ensuing paragraphs.

The UPS in neuronal physiology and pathology

The UPS is known to play critical roles in normal neuronal function, and defects in the process have been strongly implicated in various forms of neurodegenerative settings [8–9]. Localized protein synthesis has long been recognized as a requirement for axonal guidance, and localized protein degradation may well be equally important [10]. The retinal growth cone, for example, has served well as a model for understanding molecular changes in terms of protein synthesis and degradation as the axon migrates to the tectum [11–12]. Inhibition of proteasome function blocks retinal growth cone responses to certain guidance cues, such as netrin-1 and lysophosphatidic acid (LPA), but not to others, e.g. Sema3A. In isolated retinal growth cones, netrin-1 and LPA treatment also elicit a rise in ubiquitin-protein conjugates [11].

On the other hand, modulating the levels of surface receptor molecules to guidance cues is also an important process regulating the complicated process of axonal guidance. An example in question is midline crossing in *Drosophila*, which is modulated by the genes Roundabout (Robo) and Commissureless (Comm). Roundabout levels are high on axons that have crossed the midline, and the high levels prevent recrossing. The ability of Commissureless to regulate Robo in the embryo requires interaction with the *Drosophila* ubiquitin-protein ligase DNedd4 by promoting the sorting of Robo into the endocytic pathway [13–14].

Growth cone pruning occurs as axons reach their target areas in order to adjust their synaptic contacts, and is widely used for the refinement of neural circuits in both vertebrates and invertebrates [13]. The pruning of the gamma neurons of *Drosophila* mushroom bodies during metamorphosis is mediated by UPS-mediated protein degradation, as loss-of-function mutations of the ubiquitin activating enzyme (E1) or proteasome subunits in these neurons block axon pruning [15].

The importance of the UPS to the formation and maintenance of the synapse and synaptic plasticity is also well documented [16-17]. Earlier studies in *Aplysia* demonstrated that the UPS has a role in long-term facilitation by mediating the selective degradation of the regulatory subunit for cyclic AMP (cAMP)-dependent protein kinase/protein kinase A [18]. More recent work indicates that proteosome inhibition produces a long-lasting increase in synaptic strength and the number of synaptic contacts between *Aplysia* sensory and motor neurons [19]. Ubiquitin-dependent mechanisms regulate synaptic development at the *Drosophila* neuromuscular junction (NMJ) [16], as antagonizing the ubiquitination pathway in neurons by expression of the fly *fat facets* or the yeast deubiquitinating protease UBP2 results in synaptic overgrowth and dysfunction. In experimental settings of shorter time durations, proteosome inhibitors acutely increase the levels of the synaptic vesicle priming protein DUNC-13 (and a corresponding increase in nerve-muscle synaptic strength) in *Drosophila* [20]. In cultured hippocampal neurons the inhibitors prevented the internalization of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor subunits [21]. In humans, mutations in the E3 ubiquitin ligase gene E6-AP (*Ube3a*) are the underlying cause of Angelman's syndrome, characterized by mental retardation and seizures [22].

Defects in the UPS have been implicated in several neurodegenerative diseases, particularly Parkinson's disease [23–24] and a polyglutamine-based group of diseases manifested by an accumulation in toxic aggregates such as Huntington's disease [25]. Parkin, a gene product responsible for an autosomal recessive juvenile onset form of Parkinsonism, is an E3 ubiquitin ligase whose substrates are potentially neurotoxic when present in abnormally high levels [26]. Simpleminded ways of interpreting the various pathological manifestations associated with these diseases are the following. First, a genetic factor, or age-accumulated genetic damage, results in a reduction in the capacity to degrade accumulating, potentially toxic protein or protein aggregates. Second, it is possible that a massive, anomalous accumulation of toxic protein or protein aggregates may simply overwhelm the capacity of cellular UPS. The latter accumulation could also be genetically predetermined or induced by metabolic and environmental factors. In a related scenario, ubiquitinated proteins have been observed to accumulate in postsynaptic densities after an episode of cerebral ischemia [27]. This accumulation could also be a result of a failure of the UPS to repair and remove damaged proteins, the consequences of which include inhibition of synaptic transmission followed by neurological deficits and delayed neuronal death.

APC/C and the regulation of synaptic size and activity in invertebrate models

With the elaborate list of UPS function in neurons, it would not be unexpected if neurons have a unique set of E3 ubiquitin ligases to serve the specific ubiquitination of neuronal substrates. Mutant analyses in invertebrate systems had in fact revealed the role of the gene encoding the

regulator of presynaptic morphology (RPM-1) in *C. elegans* [28–29] and its *Drosophila* homologue Highwire (HIW) [30] in the regulation of synaptic growth [31]. RPM-1 and HIW contain a RING-H2 finger domain characteristic of a large number of E3 ligases. HIW negatively regulates presynaptic growth, and *hiw* mutants have dramatically larger synaptic size and increased numbers of synaptic boutons at the fly neuromuscular junction. The *Drosophila* deubiquitinating protease gene *fat facets* mentioned above genetically interacts with *hiw*, and a loss-of-function mutation of *fat facets* could suppress some *hiw* phenotype [16]. RPM-1 is localized to the presynaptic periactive zone, and its loss of function results in disorganized presynaptic cytoarchitecture [28–29, 32]. One of its substrates is recently recognized as a component of the p38 MAP kinase signaling pathway [32].

Are the cell cycle-associated ubiquitin-ligase complexes also involved in modulating synaptic function? In *Caenorhabditis elegans*, FSN-1, a novel F-box protein, was recently found to be required in presynaptic neurons for the restriction and/or maturation of synapses [33]. FSN-1 physically associates with the *C. elegans* homologues of SKP1 and Cullin to form a new type of neuronal specific SCF-like complex. This finding is in line with the way some protein complexes that perform a general function in undifferentiated cells work in the specific context of neurons – by co-opting a neuronal specific subunit.

As metioned above, APC/C's known functions are largely associated with cell cycle control. A prominent departure from this theme is its role in the destruction of SnoN, a component of the transforming growth factor-β (TGF-β) signaling network [34]. A more surprising recent set of findings pertain to its important roles in non-proliferating, postmitotic neurons. The obligatory requirement for APC/C in the cell cycle renders the observation of any naturally occurring mutations that might affect postmitotic, differentiated cells highly unlikely. The obvious technical problem in looking specifically for a role for APC/C in neurons in vivo by a loss-of-function type of analysis is the difficulty in bypassing the obligatory requirement of the complex in dividing cells. In *Drosophila*, however, the maternal contribution of *morula* (Mr), the fly orthologue of APC2, a subunit of APC/C [35], enables homozygous loss-of-function mutants to survive until larval or early pupal stages. A recent analysis by Van Roessel et al. [36] of the neuromuscular junction synapses of the Mr mutants (a strong allele designated *APC2/mr3*) revealed a drastic (double that of heterozygote controls) increase in the number of synaptic boutons per synapse (with no significant difference is either bouton size or branching). This increased number of synaptic boutons can be reverted by targeted expression of Mr in all postmitotic neurons of the fly, thus providing a direct association of the observed phenotype with the loss of Mr function. Furthermore, *Drosophila* APC/C appears to regulate muscle synaptic junctions, manifested by an increase in both spontaneous and evoked junction potentials in the Mr mutant. This increase is not explained by any changes in presynaptic properties, but rather corresponds to an up-regulation of postsynaptic glutamate receptors.

Almost simultaneously, Juo and Kaplan reported that APC/C regulates the abundance of glutamate receptors in another invertebrate model system, *C. elegans* [37]. Carefully bypassing gross mitotic defects by shifting the mutants to the restrictive temperature at the fourth larval stage, the authors compared the distribution of green fluorescent protein (GFP)-tagged glutamate receptor GLR-1 in wild-type and five different temperature-sensitive APC/C subunit mutants by quantitative fluorescence microscopy. It was clear that all mutants showed an increase in the abundance of GLR-1 in the ventral nerve cord, and these phenotypes could be reverted by the expression of wild-type subunits driven by the *glr-1* promoter. This suggests that the mutant effects are cell autonomous. Interestingly, mutations that block clathrin-mediated endocytosis block the effects of the mutations – evidence for APC/C regulating some respect of GLR-1 recycling between the post-synaptic plasma membrane and the endosome. In light of the fact that monoubiquitination serves to direct the endocytosis of membrane proteins [38] (contrary to polyubiquitination, which targets a polypeptide for destruction at the proteosome), a role for APC/C in modulating postsynaptic glutamate receptor recycling in both *Drosophila* and *C. elegans* seems to be a fairly logical notion. Lacking the right recognition motifs, GLR-1 is unlikely to be a direct substrate for APC/C. However, scaffolding proteins that associate either directly or indirectly with GLR-1 and regulate its trafficking might be likely substrate candidates.

Cdh1-APC and axonal growth in the mammalian brain

Core components of APC/C and its activator Cdh1 (but not Cdc20) with functional ubiquitination activity are in fact abundant in postmitotic neurons of the mammalian brain [39]. Multiple Cdh1 homologues have also been identified in chick brain [40]. However, a clear demonstration of APC/C function in the growth of postmitotic neurons has been lacking until very recently. Konishi et al. [41] approached the question using a Cdh1 gene knockdown strategy by RNA interference (RNAi). Cdh1 knockdown has a fairly specific effect on the morphology of the neuronal processes of postmitotic granule neurons. In particular, there is an increase in the rate of axonal growth and its final length, while dendritic growth is not affected. Expression of a dominant negative form of an APC/C component, APC11, and an APC/C inhibitor protein, Emi1, had a similar effect.

The authors extended their analysis using a more elaborate ex vivo setup whereby P6 granule neurons (with Cdh1 knockdown or otherwise) were allowed to grow into organotypic cerebellar slices. With this setup some important observations were made. Cdh1 knockdown axons (but not control axons) could extend across different layers of the cerebellar cortex, and a good fraction of these even elaborated long axons over the cerebellar white matter. The latter observation suggests that Cdh1 knockdown may attenuate the inhibitory activity on axonal growth by myelin-associated inhibitors, which was further confirmed in cultured neurons using an axonal growth assay. The action of Cdh1 appears to be cell autonomous, as axonal extension abnormality is only observed in Cdh1 knockdown cells. In the light of the above findings, APC/C thus appears to have a role in both the presynaptic and postsynaptic compartments of the neuron as well as a rather unique role in axonal growth. In dividing cells, APC/C could be activated by Cdc20 or Cdh1 [6–7]. Notably, however, in both mammals and fly, Cdh1 and its orthologues, and not Cdc20, appears to be the activator of APC/C function in neurons.

The Liprin-α connections

What might the critical APC/C substrate(s) in neurons be? Potential candidates should of course bear a destruction box motif [42] or a KEN box [43] that is recognized by the APC/C activator subunits Cdc20 or Cdh-1, and should presumably be present at the synapse. Interestingly, one single molecular candidate appears to fit the bill.

Liprin-α is a member of a family of multidomain scaffolding proteins [44] and was first recognized as the cytosolic binding partner of the leukocyte common antigen-related (LAR) receptor family of receptor protein tyrosine phosphatases. It has three putative destruction box motifs and is localized at the synapse. In *Drosophila*, the receptor protein tyrosine phosphatases, Dlars, are involved in axon guidance [45–46]. Both Dliprin-α and Dlar are required for normal synaptic morphology [47]. Synapse complexity is proportional to the amount of Dlar present, and its activity determines the size of synapses. Both are also required to define the size and shape of the presynaptic active zone, and loss-of-function mutations result in reductions in the number of neuromuscular junction synaptic boutons. Indeed, Van Roessel et al. found that presynaptic Dliprin-α protein levels are significantly greater in Mr mutants than in heterozygote controls, a phenotype that was reversible by expression of Mr. Furthermore, removing one copy of Dliprin-α from the *APC2/mr3* mutant suppresses the increase in neuromuscular synaptic bouton number [36]. In *C. elegans*, the Liprin-α homologue SYD-2 is localized to the presynaptic termini and was also proposed to participate in formation of the active zone [48]. In terms of an increase in the number of synaptic boutons, the loss-of-function of Liprin-α in invertebrates therefore appeared to roughly mirror an effect opposite to that resulting from overexpression of deubiquitinating proteases [16] and APC/C mutation [36], both of which would presumably stabilize Liprin-α.

These results indicate strongly that, at least in *Drosophila*, Liprin-α is the critical substrate for a presynaptic function of APC/C in regulating synaptic bouton number. Recent findings have also implicated other proteins in Liprin-α's presynaptic functions. It directly interacts with the ERC (ELKS-Rab6-interacting protein-CAST) family of proteins [49], members of which are known to bind RIMs, the active zone proteins that regulate neurotransmitter release. ERC2/CAST, an active zone-specific isoform, colocalizes with mammalian Liprin-α1 in cultured neurons and forms a complex with Liprin-α1 in the brain. Interestingly, Liprin-α1 shows a partial synaptic localization when expressed alone in cultured neurons. When coexpressed with ERC2, however, Liprin-α1 was redistributed to synaptic sites. Liprin-α1-ERC2 interaction may therefore be involved in the presynaptic localization and molecular organization of presynaptic active zones [49].

Is Liprin-α also involved in APC/C's modulation of synaptic strength and glutamate receptor levels on the postsynaptic membrane? This connection is also apparent (fig. 1). The movement of the AMPA-type glutamate receptor in mammalian neurons through the dendritic shaft along microtubular tracks requires interaction between the C-terminus of its subunit GluR2 and the PDZ domain of glutamate receptor interacting protein I (GRIP). GRIP is connected to the motor protein KIF1 [50] through Liprin-α, and a dominant-negative Liprin-α mutant that is unable to bind GRIP disrupts the targeting of AMPA receptors to the synapse [51]. It should be further noted that Liprin-α also interacts directly with GIT-1 [52], a multidomain protein with GTPase activating protein (GAP) activity and which interacts with a myriad of receptors and proteins involved in regulating focal adhesion. Dominant negative constructs interfering with Liprin-α-GIT1 association also disrupts AMPA receptor expression on the postsynaptic surface [52]. Interestingly, GIT1 is also present presynaptically, in a complex with the cytomatix protein Picollo, and the Picollo-GIT1 complex also contains Liprin-α [53]. Although the role of Liprin-α in this complex is yet undefined, APC/C's control of Liprin-α levels could therefore be an elegant, multi-faceted regulatory mechanism for modulating both presynaptic and postsynaptic events.

Is Liprin-α also involved in the control of axonal growth by APC/C? As mentioned above, several members of the Liprin-α interacting *Drosophila* DLAR family have been known to control motor axon guidance [45-46]. Further work provided linkages with regards to the tyrosine phos-

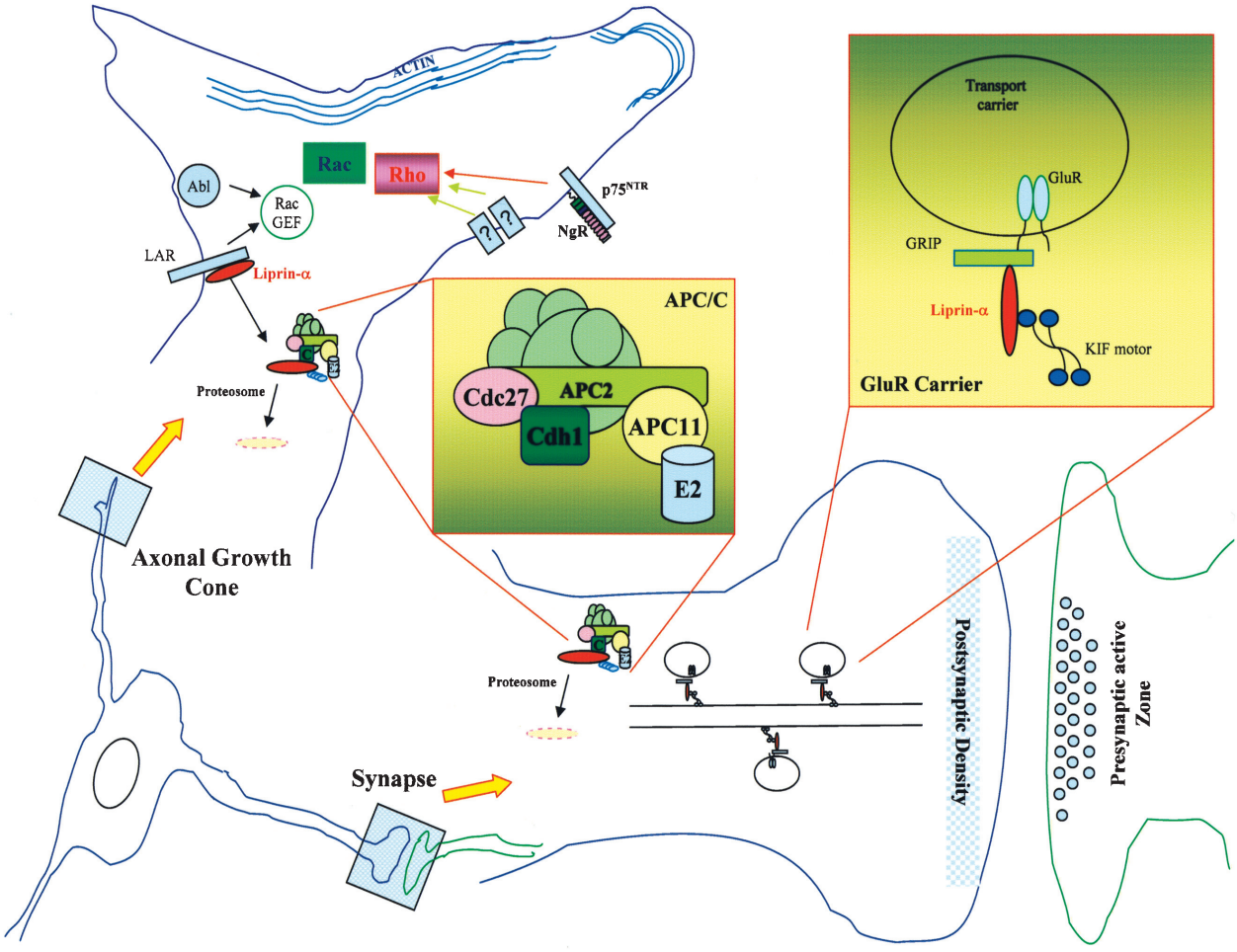

Figure 1. Depicted schematically are the axonal gowth cone (top left) and the dendritic postsynaptic portion (bottom right) of a neuron (with an associated presynaptic compartment). At the dendrite, Liprin-α is involved in intracellular trafficking of AMPA-type glutamate receptors (GluR) (see text), and regulation of its levels by APC/C modulates the expression of glutamate receptors on the postsynaptic plasma membrane. At the axonal growth cone, Liprin-α may, through interactions with the LAR family members of membrane tyrosine phosphatases, modulate the GTPases Rac (or Rho), which are key regulators of actin cytoskeletal changes with growth cone extension and turning. Myelin-associated and other CNS inhibitors of axonal growth and regeneration come in several forms. Some of these, including Nogo and myelin-associated glycoprotein, act through the Nogo-66 receptor (NgR) and its associated neurotrophin receptor $p75^{NTR}$. The receptors for other inhibitors such as the N-terminal domain of Nogo and the chondroitin sulfate proteoglycans are not yet identified (indicated by bars with '?'). Most, if not all, of the inhibitory signals ultimately converge on the activation of Rho and its effector ROCK. A knockdown of APC/C in neurons seem to allow axonal extension on myelinated surfaces. This effect may be exerted through Liprin-α. Liprin-α may have a direct inhibitory effect on Rho activation, or may simply counter Rho activation by activating Rac. Alternatively, APC/C knockdown may serve to elevate the levels of proteins that enhance axonal growth, or transcription factors that regulate the synthesis of these. The insets are more detailed schematic renderings of the APC/C complex and the connections between GluR in a carrier vesicle with the components of its trafficking machinery.

phatase function of DLAR with another growth cone regulator, the Abl tyrosine kinase [54–55]. Both DLAR and the *Drosophila* DAbl are genetically and physically linked to Trio, the guanine nucleotide exchange factor of Rac [56–58] and the Enabled (Ena/VASP) family of proteins, which regulate growth cone actin dynamics. Abl's activation of Cdk5 [59] may also engage proteins downstream of the kinase which modulate microtubule dynamics. The mammalian LAR subfamily of protein tyrosine phosphatase (PTPs) has three members that are homologous to DLAR – LAR, PTPσ and PTPδ. In mice with highly reduced LAR levels, the size of basal forebrain cholinergic neurons was significantly reduced, and cholinergic innervation of the dentate gyrus was markedly decreased [60–61]. Gene disruption of mouse PTPσ resulted in various neuronal defects and neuroendocrine dysplasia [62–64]. Interestingly, there is a noticeable distinction between nerve regeneration in LAR-deficient mice and PTPσ-deficient mice. Regeneration and collateral sprouting after sciatic nerve injury are significantly delayed compared with control in the former [65–66] but significantly enhanced in the latter [67]. In fact, after sciatic

nerve transection with immediate microsurgical repair or allografting, PTPσ-deficient nerve fibers demonstrated errors in directional growth compared with controls, a phenotype that is (albeit remotely) reminiscent of that observed by Konishi et al. when Cdh1 RNAi generating plasmids were injected to the cerebellar cortex of P3 rat pups [41]. The parallel fibers of granule neurons with Cdh RNAi appeared to wander off the main track of parallel fibers and grew abnormally towards the external granule layer. Although there is no direct evidence as yet, specific interactions between Liprin-α and LAR family isoforms may therefore modulate axonal growth, guidance and regeneration (fig. 1).

Finally, a notable and particularly interesting aspect of Cdh1 knockdown in cerebellar granule neurons is the ability of these axons to grow in the presence of myelin. The inability of central nervous system neurons to regenerate upon injury has been attributed to an intrinsic regenerative deficiency [68] and to the presence of myelin-associated inhibitors in the central nervous system (CNS) environment [69]. CNS axons can be coaxed to regenerate to a certain extent in the presence of myelin by strategies that antagonized the inhibitory signaling induced by myelin-associated inhibitors such as Nogo and myelin-associated glycoprotein through the Nogo-66 receptor [70], as well as by removing the chondroitin sulfate proteoglycans enzymatically [71]. Alternatively, CNS axonal growth could be enhanced by inhibition of the functions of endogenous Rho and its effector ROCK and by the elevation of cytosolic cAMP [69]. Arginase 1, a key enzyme in the synthesis of polyamines, is upregulated in response to cAMP, and both overexpression of arginase 1 and exogenous addition of polyamines could overcome the inhibition by some myelin-associated inhibitors [72].

There is no clear connection yet between APC/C, Liprin-α and the ability of neurons to regenerate. It is, however, conceivable that alteration of Liprin-α levels and therefore its modulation of the function of LAR family members could affect Rho and ROCK. It is also worth noting that the immunohistological signals of Cdh1 in neurons are largely nuclear. Whether Cdh1-APC acts on transcription factors that might regulate either the expression of molecules such as arginase 1 or inhibitory signal receptors are among the questions that might be addressed in the near future.

Epilogue

In summary, we have narrated recent findings of important roles of APC/C in postmitotic neurons. We further noted and highlighted, albeit speculatively in some instances, the possible involvement of Liprin-α in all these roles. Although it is clear that general neuronal function involves a myriad of E3 ubiquitin ligases and their respective substrates, one or more of these (such as Liprin-α) may play central roles in the linked coordination of pre- and postsynaptic events. This sort of linked coordination is particularly important for processes involving simultaneous modifications of the presynaptic and postsynaptic compartments, such as the synaptic strengthening processes associated with learning and memory.

Acknowledgements. Work on neuronal regeneration in B.L.T.'s laboratory is supported by research grant number 03/1/21/19/247 from the Agency for Science, Technology and Research (A*STAR)'s Biomedical Research council (BMRC).

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