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The Anaphase-Promoting Complex (APC) ubiquitin ligase regulates GABA transmission at the *C. elegans* neuromuscular junction

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ABSTRACT

Regulation of both excitatory and inhibitory synaptic transmission is critical for proper nervous system function. Aberrant synaptic signaling, including altered excitatory to inhibitory balance, is observed in numerous neurological diseases. The ubiquitin enzyme system controls the abundance of many synaptic proteins and thus plays a key role in regulating synaptic transmission. The Anaphase-Promoting Complex (APC) is a multi-subunit ubiquitin ligase that was originally discovered as a key regulator of protein turnover during the cell cycle. More recently, the APC has been shown to function in postmitotic neurons, where it regulates diverse processes such as synapse development and synaptic transmission at glutamatergic synapses. Here we report that the APC regulates synaptic GABA signaling by acting in motor neurons to control the balance of excitatory (acetylcholine) to inhibitory (GABA) transmission at the *Caenorhabditis elegans* neuromuscular junction (NMJ). Loss-of-function mutants in multiple APC subunits have increased muscle excitation at the NMJ; this phenotype is rescued by expression of the missing subunit in GABA neurons. Quantitative imaging and electrophysiological analyses indicate that APC mutants have decreased GABA release but normal cholinergic transmission. Consistent with this, APC mutants exhibit convulsions in a seizure assay sensitive to reductions in GABA signaling. Previous studies in other systems showed that the APC can negatively regulate the levels of the active zone protein SYD-2 Liprin-α. Similarly, we found that SYD-2 accumulates in APC mutants at GABAergic presynaptic sites. Finally, we found that the APC subunit EMB-27 CDC16 can localize to presynapses in GABA neurons. Together, our data suggest a model in which the APC acts at GABAergic presynapses to promote GABA release and inhibit muscle excitation. These findings are the first evidence that the APC regulates transmission at inhibitory synapses and have implications for understanding nervous system pathologies, such as epilepsy, that are characterized by misregulated GABA signaling.

Introduction

Proper nervous system function requires both excitatory and inhibitory synaptic signaling. Disruption of the balance of excitatory to inhibitory transmission (E:I balance) is observed in several neurological disorders, including epilepsy, schizophrenia, autism, and Huntington's Disease, indicating the critical importance of mechanisms controlling this balance (Chiodi et al., 2012; Prosser et al., 2001; Schuler, 2001; Snodgrass, 1992; Yuen et al., 2012). While a number of genes have been implicated in controlling E:I balance, the molecular mechanisms that specifically regulate excitatory versus inhibitory synaptic transmission are largely unknown (Gatto and Broadie, 2010; Sieburth et al., 2005; Vashlishan et al., 2008).

The ubiquitin signaling system is a well-established regulator of diverse neuronal processes (DiAntonio and Hicke, 2004; Ding and Shen, 2008; Tai and Schuman, 2008; Yi and Ehlers, 2007), and loss of ubiquitin system function is observed in several neurological and neurodegenerative disorders, including Angelman's Syndrome and Parkinson's Disease (Bingol and Sheng, 2011; Ciechanover and Brundin, 2003; Ding and Shen, 2008; Hegde and Upadhya, 2007; Tai and Schuman, 2008; Yi and Ehlers, 2007). Ubiquitin is a small 76 amino acid polypeptide that is added post-translationally to lysine residues in target proteins by an enzymatic cascade involving the sequential activity of E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases (Hershko and Ciechanover, 1998). These enzymes conjugate ubiquitin to the ε amino group of lysine residues in target proteins, which may be either mono- or poly-ubiquitinated. Different

Abbreviations: APC, Anaphase-Promoting Complex; NMJ, neuromuscular junction; ACh, acetylcholine; GABA, γ-aminobutyric acid; DNC, dosal nerve cord.

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ubiquitin chain lengths and linkages confer different functional outcomes and/or direct distinct subcellular destinations for target proteins that may impact the activity, localization, or abundance of the ubiquitinated molecules (Kulathu and Komander, 2012). One important consequence of ubiquitination for many proteins is degradation. Mono-ubiquitinated substrates or those with K63 linkages are typically targeted to the multi-vesicular body (MVB)/lysosome pathway for destruction. In contrast, proteins containing other polyubiquitin linkage types are degraded by the 26S proteasome (Hicke and Dunn, 2003; Kulathu and Komander, 2012; Ye and Rape, 2009). In the human genome, there are two E1, approximately 40 E2, and more than 600 E3 ligases, as well as nearly 100 deubiquitinating enzymes (DUBs) that hydrolyze ubiquitin linkages (Love et al., 2007; M. Li et al., 2008; Nijman et al., 2005; W. Li et al., 2008). A number of ubiquitin ligases and several DUBs have been shown to play a role in regulating synaptic transmission at specific synapse types (Bingol and Sheng, 2011; Clague et al., 2012; Kowalski and Juo, 2012); however the detailed molecular mechanisms by which the majority of these enzymes act, as well as their complete cell type specificities and substrate repertoires, have yet to be fully investigated.

The Anaphase-Promoting Complex (APC) is a well characterized RING-finger E3 ubiquitin ligase that plays a critical role in controlling both cell cycle progression and diverse functions in post-mitotic neurons. The APC is well conserved across phylogeny and is one of the largest E3 ligase complexes, composed of 11–13 different subunits, including one of two alternative subunits, Cdh1 or Cdc20. These substrate-binding adaptors have distinct recognition motifs and operate at different times and locations within the cell to target the APC to distinct substrates based on their differential expression and localization (Manchado et al., 2012; Peters, 2006; Puram and Bonni, 2011). The APC, in conjunction with one of several E2 enzymes, generates polyubiquitin chains on its substrates, typically leading to their proteasomal degradation (Peters, 2006). However, a recent study shows that multiple monoubiquitination of the APC substrate cyclin B1 is sufficient to promote its destruction by the proteasome (Dimova et al., 2012). Despite the identification of a growing list of APC functions and substrates in the cell cycle, much remains to be learned about the activities and mechanisms of action of this unique ubiquitin ligase, especially in the nervous system.

In neurons, the APC regulates diverse cellular processes during development including axon (Huynh et al., 2009; Kannan et al., 2012a, 2012b; Konishi et al., 2004; Lasorella et al., 2006; Stegmuller et al., 2006, 2008) and dendrite (Kim et al., 2009) growth and morphogenesis, neuronal precursor differentiation (Harmey et al., 2009; Yao et al., 2010), and neuronal survival (Almeida, 2012; Almeida et al., 2005) [reviewed in (Manchado et al., 2012; Puram and Bonni, 2011)]. In particular, several groups demonstrated roles for the APC in controlling synapse development and function at glutamatergic synapses. In *Caenorhabditis elegans*, the APC prevents excessive glutamatergic signaling by negatively regulating the abundance of GLR-1 glutamate receptors (Juo and Kaplan, 2004). Recent work in cultured mammalian neurons demonstrated the ability of the APC, through its Cdh1 substrate adaptor, to ubiquitinate GluR1 receptors, possibly in the ER, to promote their proteasomal degradation during homeostatic plasticity (Fu et al., 2011). At the fly neuromuscular junction (NMJ), the APC restricts the number of presynaptic boutons via the active zone protein, Liprin-α, and independently limits postsynaptic GluRIIa glutamate receptor abundance (Van Roessel et al., 2004). The APC, via its Cdc20 substrate adaptor, also regulates the presynaptic differentiation of cerebellar granule neurons in rodents (Yang et al., 2009). In addition, recent studies show that mice deficient in specific APC subunits exhibit defects in long-term potentiation, spatial memory, and associative fear memory (Kuczera et al., 2011; M. Li et al., 2008; Pick et al., 2012; W. Li et al., 2008). However, despite the clear roles for the APC in glutamatergic synapse formation and synaptic transmission, its potential functions at other synapse types have not been investigated.

To test whether the APC acts more broadly as a regulator of synaptic transmission in other neuron types, we examined APC function in GABA and cholinergic transmission at the *C. elegans* NMJ. Like the human NMJ, acetylcholine (ACh) released from a subclass of excitatory motor neurons at the NMJ in *C. elegans* induces action potential firing and thus contraction of postsynaptic muscle cells (Gao and Zhen, 2011). *C. elegans* muscles also receive inhibitory GABA signals from a separate class of motor neurons, preventing contraction (Gao and Zhen, 2011; Richmond and Jorgensen, 1999; White et al., 1986). Thus, muscle excitation in these animals is governed by both excitatory and inhibitory synaptic transmission, making it an excellent model in which to investigate mechanisms controlling E:I balance. Here, we used a combination of pharmacological experiments, quantitative imaging, biochemistry, and electrophysiological analyses to show that the APC is required for normal muscle excitation in *C. elegans*. We show that the APC functions specifically in GABA motor neurons where it promotes GABA release and affects the synaptic abundance of the active zone protein SYD-2 Liprin-α. These findings demonstrate that the APC regulates transmission at diverse synapse types and may have important implications for our understanding of neurological disorders which involve defects in GABA signaling.

Results

The APC inhibits muscle excitation at the NMJ

Previously, the APC was shown to regulate synaptic differentiation (Van Roessel et al., 2004; Yang et al., 2009) and transmission at glutamatergic synapses (Fu et al., 2011; Juo and Kaplan, 2004; Van Roessel et al., 2004) in mice, worms, and flies, but the role of the APC in regulating transmission at other synapse types is not known. We tested whether the APC regulates synaptic transmission at the *C. elegans* NMJ. Body wall muscles in *C. elegans* receive both excitatory inputs mediated by cholinergic signaling and inhibitory inputs mediated by GABA signaling (White et al., 1986). Overall muscle activity is the result of a tightly controlled balance between this excitatory and inhibitory signaling and can be measured indirectly using responsiveness to the acetylcholine esterase inhibitor aldicarb (Mahoney et al., 2006; Miller et al., 1996; Nguyen et al., 1995). Exposure of worms to aldicarb results in the accumulation of acetylcholine in the synaptic cleft, which leads to muscle hypercontraction and paralysis. Worms carrying mutations that increase cholinergic or decrease GABA signaling are hypersensitive to aldicarb and thus paralyze faster than wild type animals (Mahoney et al., 2006; Vashlishan et al., 2008). In contrast, animals with mutations that decrease cholinergic or increase GABA transmission are resistant to aldicarb and show slower paralysis in response to the drug (Mahoney et al., 2006; Miller et al., 1996; Nguyen et al., 1995; Sieburth et al., 2005). A large scale RNA interference (RNAi) screen in *C. elegans* identified many genes whose loss-of-function results in hypersensitivity to aldicarb, including two genes that encode subunits of the APC (Vashlishan et al., 2008).

To determine if the APC is required for normal muscle activity in *C. elegans*, we tested several APC subunit mutants for their sensitivity to aldicarb-induced paralysis. Because APC function is essential during the cell cycle and APC null mutants exhibit embryonic lethality at the one-cell stage, we assessed the requirement for the APC in synaptic function using temperature-sensitive alleles in four separate APC subunit mutants (*emb-30* APC4, *emb-27* CDC16, *mat-2* APC1 and *mat-3* CDC23) (Davis et al., 2002; Furuta et al., 2000; Golden et al., 2000). We maintained these strains at the permissive temperature (15 °C) until the fourth larval (L4) stage (at which time cholinergic and GABA neuron cell divisions are complete) (Sulston, 1983; Sulston and Horvitz, 1977; Sulston et al., 1983), and then shifted them to the non-permissive temperature (26 °C) for 20 h prior to measuring NMJ activity in the aldicarb assay. The higher non-permissive temperature