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

Jennifer R. Kowalski^{a,*}, Hitesh Dube^a, Denis Touroutine^b, Kristen M. Rush^a, Patricia R. Goodwin^c, Marc Carozza^a, Zachary Didier^a, Michael M. Francis^b, Peter Juo^c

^a Department of Biological Sciences, Butler University, Indianapolis, IN 46208 USA

^b Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA 01605, USA

^c Department of Developmental, Molecular and Chemical Biology, Tufts University School of Medicine, Boston, MA 02111, USA

ARTICLE INFO

Article history:

Received 8 April 2013

Revised 23 November 2013

Accepted 2 December 2013

Available online 7 December 2013

Keywords:

Anaphase-Promoting Complex

Ubiquitin ligase

GABA

NMJ

Synapse

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.

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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 Upadhyay, 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 enzyme, 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, dorsal nerve cord.

* Corresponding author at: Department of Biological Sciences, Butler University, 4600 Sunset Avenue, Indianapolis, IN 46208, USA. Fax: +1 317 940 9519.

E-mail addresses: jrkowals@butler.edu (J.R. Kowalski), hdube@butler.edu (H. Dube), Denis.Touroutine@umassmed.edu (D. Touroutine), kmrush@butler.edu (K.M. Rush), pgoodwin@brandeis.edu (P.R. Goodwin), mcarozza@indiana.edu (M. Carozza), Zpdid01@louisville.edu (Z. Didier), Michael.Francis@umassmed.edu (M.M. Francis), peter.juo@tufts.edu (P. Juo).

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; Stegmüller 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 (Kuczer 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

Fig. 1. APC loss-of-function mutants have increased muscle excitation at the NMJ. (A) Time course of paralysis of wild type (WT), *emb-30(g53)* APC4, *emb-27(g48)* CDC16 and *emb-27(ye143)* CDC16 APC subunit mutants on aldicarb. (B) Time course of paralysis of WT, *emb-30(tn377)* APC4, *mat-2(ax76)* APC1 and *mat-2(ax102)* APC1 APC subunit mutants on aldicarb. For each strain in A and B, approximately 20 worms were assayed in triplicate on plates containing 1 mM aldicarb in at least three independent experiments. Since APC loss-of-function mutations are temperature-sensitive lesions, all strains were grown at the permissive temperature (15 °C) to maintain viability then L4 animals were shifted to the non-permissive temperature (26 °C) for 20 h prior to assay. The average percentage of worms paralyzed at each time point $\pm$ s.e.m. is shown. All strains were tested in at least three independent experiments.

presumably results in misfolding of the mutant APC subunit and loss of activity of the APC complex (Shakes et al., 2003). We found that *emb-30* APC4 (alleles *g53* and *tn377*), *emb-27* CDC16 (alleles *g48* and *ye143*), *mat-2* APC1 (alleles *ax76* and *ax102*), and *mat-3* CDC23 (allele *or180*) mutant animals all exhibit aldicarb-hypersensitivity over a 3-hour period (Fig. 1 and Supplemental Fig. 1), which is indicative of increased muscle activity. These results suggest that the APC functions to inhibit muscle activity at the NMJ.

Because the APC interacts with one of two alternative substrate-binding activator proteins Cdh1 or Cdc20 [reviewed in (Peters, 2006)], we tested if loss-of-function of either Cdh1 or Cdc20 affected normal muscle activity. FZR-1 and FZY-1 are the *C. elegans* homologs of Cdh1 and Cdc20, respectively (Fay et al., 2002; Kitagawa et al., 2002). To test the requirement for either Cdh1 and/or Cdc20 in controlling normal muscle activity at the NMJ, we performed aldicarb assays on *fzy-1* loss-of-function mutants (alleles *ku298* and *ok380*) (Fay et al., 2002) and on animals treated with RNAi targeting *fzy-1*. We found that while the *fzy-1* CDH1 mutants exhibit wild type aldicarb responses, knockdown

of *fzy-1* CDC20 by RNAi between the L1 and young adult stages is sufficient to induce aldicarb-hypersensitivity, indicative of increased muscle contraction (Supplemental Fig. 2). This result is consistent with a large-scale RNAi screen that previously showed knockdown of *fzy-1* CDC20 results in aldicarb-hypersensitivity (Vashlishan et al., 2008). These results suggest that the FZY-1 CDC20 substrate adaptor of the APC, but not FZR-1 CDH1, is required to prevent excessive muscle activity at the NMJ.

The APC functions in GABA motor neurons to control muscle excitation

Since *C. elegans* muscle contraction involves a balance of cholinergic and GABA signaling from separate classes of presynaptic motor neurons onto postsynaptic muscle cells, it is possible that the APC acts in one or more of these cell types to control muscle activity. Postsynaptic muscle responsiveness to ACh can be measured using another paralysis-based assay involving sensitivity to the ACh agonist levamisole. Levamisole binds to and activates heteropentameric nicotinic ACh receptors, which are one of two classes of receptors that promote muscle contraction at the NMJ (Brown et al., 2006; Francis et al., 2005; Richmond and Jorgensen, 1999; Touroutine et al., 2005). Animals with altered muscle activity often exhibit abnormal rates of levamisole-induced paralysis (Brown et al., 2006; Miller et al., 1996). While this is often due to increased postsynaptic sensitivity, mutants with severe defects in GABA transmission, such as *unc-25* GAD and the *unc-47* vesicular GABA transporter are also strongly levamisole-sensitive (Vashlishan et al., 2008) (Supplemental Fig. 3). We found that temperature-sensitive APC loss-of-function mutants exhibit a slight, albeit non-significant, increase in sensitivity to levamisole relative to wild type animals after 60 min of exposure. This mild effect of APC subunit loss-of-function on levamisole-sensitivity compared to *unc-25* GAD mutants (Supplemental Fig. 3) is consistent with the hypomorphic and temperature-sensitive nature of the APC mutant alleles.

We next tested whether the APC acts presynaptically in either cholinergic or GABA motor neurons to control muscle excitation by performing cell type-specific rescue experiments in the *emb-27* CDC16 APC subunit mutant animals. We found that expression of the APC subunit *emb-27* CDC16 cDNA under the promoter for the *unc-30* gene, which is expressed exclusively in the dorsal and ventral cord GABA motor neurons (DD and VD classes) (McIntire et al., 1993), completely rescues the aldicarb-hypersensitivity of *emb-27(g48)* CDC16 mutants (Fig. 2A). No rescue was observed following transgene expression under a cholinergic (*Punc-17*) promoter (data not shown). In addition, to test whether APC catalytic activity is also required in GABA neurons, we blocked APC activity by ectopically expressing human Emi1 (hEmi1), an endogenous pseudosubstrate inhibitor of the APC found in vertebrates (Miller et al., 2006; Reimann et al., 2001), under control of the GABA neuron promoter *Punc-25*. We found that GABA neuron-specific expression of hEmi1 in wild type animals results in aldicarb-hypersensitivity (Fig. 2B), consistent with our APC loss-of-function mutant experiments. If the increased aldicarb sensitivity is due to inhibition of endogenous APC activity by GABAergic expression of hEmi1, we would expect hEmi1 and APC loss-of-function mutants to have non-additive effects; conversely, we would expect an additive aldicarb phenotype if hEmi1 exerts its effects at the NMJ via an APC-independent mechanism. We found that ectopic expression of hEmi1 in the GABA neurons of *emb-30(g53)* APC4 loss-of-function mutants results in non-additive effects on aldicarb-hypersensitivity (Fig. 2B), consistent with the idea that hEmi1 inhibits APC function in GABA neurons. Together, these data suggest that the function of the APC is both necessary and sufficient in GABA motor neurons for normal body wall muscle activity.

We used another behavioral assay based on sensitivity to the drug pentylenetetrazole (PTZ) to support a role for the APC in GABA signaling. PTZ is a GABA_A receptor antagonist and epileptogenic agent in mammals (Huang et al., 2001; Rocha et al., 1996). In *C. elegans*, PTZ

Fig. 2. The APC is necessary and sufficient in GABA neurons for normal NMJ transmission. (A) Rescue of APC function in GABA motor neurons restores normal NMJ transmission. Time course of paralysis of wild type (WT), *emb-27(g48)* CDC16 mutants, and rescued *emb-27(g48)* CDC16 animals (GABA rescue). GABA rescue worms are *emb-27(g48)* CDC16 mutants expressing the *emb-27* CDC16 cDNA under the control of the *unc-30* GABA neuron-specific promoter. (B) Inhibition of the APC in GABA motor neurons results in increased NMJ transmission. Time course of paralysis of WT, *emb-30(g53)* APC4 mutant animals, wild type worms expressing human *EMI1*, an endogenous APC inhibitor under the control of the GABA neuron-specific *unc-25* (GAD) promoter (*Punc-25::hEMI1*) (GABA Emi1), and *emb-30(g53)* APC4 mutant animals expressing *Punc-25::hEMI1*. For all experiments, approximately 20 worms per strain were assayed in triplicate following a 20 h temperature shift at the L4 stage, and the average percentage of worms paralyzed at each time point $\pm$ s.e.m. is shown. All experiments were performed independently at least three times.

induces seizure-like convulsions in animals with defects in GABA neurotransmission. Wild type animals and those with defects only in cholinergic signaling do not exhibit PTZ-induced convulsions (Locke et al., 2006, 2008; Williams et al., 2004). It should be noted, however, that the precise mechanism of action of PTZ in *C. elegans* is not completely clear and cannot only be explained by PTZ acting as a GABA signaling antagonist because GABA signaling mutants alone do not exhibit spontaneous seizures and the effects of PTZ on convulsions in *C. elegans* are only apparent when GABA transmission is also reduced. Nevertheless, because mutations in GABA but not cholinergic transmission sensitize worms to PTZ-induced convulsions, this assay is useful for implicating genes involved in GABA signaling. As expected, we observed no PTZ-induced convulsions in wild type or *unc-4(e120)* mutant animals exposed to 10 mg/ml PTZ for 30 or 60 min (Fig. 3). *unc-4(e120)* mutants are defective in cholinergic signaling due to mutations in the UNC-4 homeodomain protein that is required for cholinergic DA and VA motor neuron development (Esmaeili et al., 2002; Miller et al., 1992). In contrast, 100% of the *unc-25(e156)* mutants, which are

defective in the GABA synthesis enzyme glutamic acid decarboxylase (GAD), and *snb-1(md247)* mutants, which are defective in general synaptic transmission, show robust anterior convulsions after 30 min of PTZ exposure, as has been previously reported (Locke et al., 2006, 2008). Interestingly, we found that nearly 50% of *emb-27(g48)* CDC16 mutant animals exhibit anterior convulsions after 30 min of PTZ exposure and more than 90% of *emb-27(g48)* CDC16 animals and 40% of *emb-30(g53)* APC4 animals were convulsing after 60 min of PTZ exposure (Fig. 3). The stronger effect of the *unc-25* and *snb-1* mutants in this assay compared to the APC subunit mutants is consistent with the temperature-sensitive hypomorphic nature of the APC mutant alleles. These data support the idea that APC mutants have reduced GABA signaling. Because PTZ-induced convulsions are primarily mediated by the GABAergic RME neurons in the head of the animal (Locke et al., 2009), while the activity of the D-type motor neurons (DD and VD neurons) contributes to the body wall muscle activity measured in the aldicarb assay, the presence of both aldicarb-hypersensitivity and PTZ-induced convulsions in the APC mutants suggests that the APC may play a broad role in regulating GABA transmission in *C. elegans*.

The APC regulates synaptic vesicle release in GABA motor neurons

We next investigated the mechanism by which the APC may promote inhibitory transmission at the NMJ. Because the APC regulates cell cycle progression and can promote cell survival in mammalian cortical neurons (Almeida et al., 2005; Peters, 2006), the increased muscle excitation observed in APC loss-of-function mutants could be due to decreased numbers of GABA motor neurons innervating body wall muscles. While we did not expect to see effects on neuronal development because the animals used in all of our experiments were grown at the permissive temperature until the L4 stage, the APC Cdh1 adaptor has been shown to be required for cell survival in primary mammalian cortical neurons and loss of APC function might affect the viability of these differentiated cells (Almeida et al., 2005). We counted the number of GABA and cholinergic motor neuron cell bodies following a shift to the non-permissive temperature in animals expressing GFP::SNB-1 Synaptobrevin under control of the GABA neuron-specific promoter *Punc-25* or the DA/DB cholinergic neuron-specific promoter *Punc-129*, respectively. We found no significant difference between the average number of GABA motor neurons in wild type (17.0 ± 0.25 cell bodies, $n = 16$) and *emb-30(g53)* APC4 mutant animals (17.07 ± 0.25 cell bodies, $n = 15$) ($p = 0.56$, Student's *t* test), whereas we observed a small (~5%) decrease in the average number of cholinergic DA/DB motor neuron cell bodies labeled by the *Punc-129* promoter in the *emb-30(g53)* APC4 mutants (9.85 ± 0.61 cell bodies, $n = 26$), compared to wild type animals (10.4 ± 0.50 cell bodies, $n = 20$) ($p = 0.002$, Student's *t* test). Nevertheless, this decrease in cholinergic motor neurons cannot be responsible for the increased muscle excitation observed in APC mutants. Together, these data suggest that the aldicarb-hypersensitivity of APC loss-of-function animals is not due to decreased GABA or increased cholinergic neuron numbers.

We next examined the distribution of several pre- and postsynaptic markers in GABA and cholinergic motor neurons in APC mutant animals. To do this, we made use of the previously generated strain of worms (*juls1*) in which a green fluorescent protein-tagged version of the synaptic vesicle protein, SNB-1 Synaptobrevin (GFP::SNB-1), is expressed exclusively in GABA motor neurons (Jin et al., 1999) and a second strain (*nuls152*) in which GFP::SNB-1 is expressed in a subset of cholinergic motor neurons (Sieburth et al., 2005) (Fig. 4). In these animals, GFP::SNB-1 localizes to discrete puncta in the dorsal and ventral nerve cords; these puncta correlate with individual NMJs, as determined by colocalization with presynaptic active zone proteins and postsynaptic markers (Jin et al., 1999; McEwen et al., 2006; Zhen and Jin, 1999). Changes in the density of these GFP::SNB-1 puncta likely correlate with changes in the number of synaptic sites, and mutants in several presynaptic proteins that cause an increase in GFP::SNB-1 puncta

Fig. 3. APC loss-of-function mutants have increased anterior convulsions in the GABA transmission-sensitive PTZ seizure assay. Results of a seizure assay using pentylene tetratoze (PTZ), which induces convulsions in animals with GABA transmission defects. Strains tested include APC mutants [*emb-30(g53)* APC4, *emb-27(g48)* CDC16], *unc-25(e156)* (GABA-defective), *unc-4(e120)* (cholinergic neuron-defective), *snb-1(md247)* (transmission-defective), and WT (wild type). 20–30 animals per plate were assayed for convulsions on 10 mg/ml PTZ following a 20 h temperature shift at the L4 stage. Average percentage of convulsing worms per plate $\pm$ s.e.m. is shown (* $p < 0.05$ vs. WT, Student's t test). n = # plates tested per strain.

intensity (Sieburth et al., 2005) also exhibit increased numbers of synaptic vesicles at NMJs as assessed by electron microscopy (Richmond et al., 1999; Weimer et al., 2003). Conversely, changes in the amount of inter-punctal axonal GFP::SNB-1 fluorescence are indicative of alterations in synaptic vesicle endo- and exocytosis (Dittman and Kaplan, 2006; Sieburth et al., 2005). Specifically, increased puncta intensities and decreased axonal fluorescence are observed in several strains that have mutations in genes required for synaptic vesicle exocytosis (e.g., *unc-13* Munc13 and *unc-18* Munc18), while decreased puncta intensity and increased axonal fluorescence are often observed in mutants with endocytic defects (e.g., *unc-11* AP180 and *unc-57* endophilin) (Dittman and Kaplan, 2006; Sieburth et al., 2005).

When we examined the distribution of GFP::SNB-1 in the motor neurons of *emb-30(g53)* APC4-deficient animals following a shift to the non-permissive temperature, we found that there was no change in the density or intensity of GFP::SNB-1 puncta in cholinergic motor neurons (Fig. 4A) in the posterior dorsal nerve cord (DNC) compared to wild type animals. In contrast, we found a 25% increase in GFP::SNB-1 puncta intensity in GABA motor neurons in the DNC of *emb-30(g53)* APC4 mutants compared to wild type animals ($p = 0.008$, Student's t test; Fig. 4B). We also observed an 11.2% increase in the density of GABA synapses in *emb-30(g53)* APC4 mutants ($p = 0.044$, Student's t test, Fig. 4B), indicating that the aldicarb-hypersensitive phenotype of the APC loss-of-function animals is not due to decreased GABA synapse number. There were no significant differences in the width of these synaptic puncta between APC mutant and wild type animals in either cholinergic (wild type, $1.30 \pm 0.05 \mu\text{m}$; *emb-30(g53)*, $1.30 \pm 0.04 \mu\text{m}$, $p = 0.8$, Student's t test) or GABA motor neurons (wild type, $1.41 \pm 0.06 \mu\text{m}$; *emb-30(g53)*, $1.49 \pm 0.05 \mu\text{m}$, $p = 0.4$, Student's t test), suggesting loss of APC function does not impact synapse size as assessed by GFP::SNB-1 fluorescence. APC mutants did exhibit 22% and 26% increases in inter-punctal fluorescence in the DNC axons of cholinergic and GABA motor neurons, respectively ($p = 0.026$ and $p = 0.03$, Student's t test, Fig. 4), indicating that the APC may impact some aspects of synaptic differentiation or function in both motor neuron classes, although specific synaptic accumulations of GFP::SNB-1 are only seen in GABA motor neurons. Further, we found that these

effects of APC loss-of-function in GABA neurons are specific to SNB-1 Synaptobrevin, as there was no significant difference in the density, size, or intensity of another synaptic marker, the mCherry-tagged active zone protein UNC-10 RIM1 (Koushika et al., 2001; Yeh et al., 2005), expressed in GABA motor neurons (*hpls88*) (Supplemental Fig. 4).

To examine potential postsynaptic defects in GABA transmission, we assessed the abundance of muscle-expressed GABA_A receptors, the major subunits of which are encoded by the *unc-49* gene (Bamber et al., 1999, 2005). We quantitatively imaged NMJs in the DNC of wild type and APC mutant adult worms stably expressing UNC-49::GFP under the endogenous *unc-49* promoter (*oxls22*) (Bamber et al., 1999) following temperature shift at the L4 stage. We observed no decrease in the number or fluorescence intensity of UNC-49::GFP postsynaptic puncta in *emb-30(g53)* APC4 mutants compared to wild type animals (Fig. 5). In fact, *emb-30(g53)* APC4 mutants exhibited a 25% increase in inter-punctal UNC-49::GFP fluorescence compared to wild type worms ($p = 0.01$, Student's t test, Fig. 5). These results suggest that increased muscle excitation in the absence of APC function is not due to decreased abundance of postsynaptic GABA receptors at the NMJ.

The genetic rescue data and GFP::SNB-1 imaging results suggest a role for the APC specifically in GABA motor neurons to control synaptic vesicle accumulation and/or release with minimal effects on cholinergic signaling. To directly test the effects of loss of APC function on both GABA and cholinergic transmission at the *C. elegans* NMJ, we performed electrophysiological analyses to record endogenous excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs, respectively) in the body wall muscles of wild type and *emb-27* CDC16 APC subunit mutant animals following temperature shift (Fig. 6). *emb-27(g48)* mutants were chosen for electrophysiological analysis because these conditional APC subunit mutants have the strongest phenotypes based on the aldicarb and PTZ assays. Consistent with the increased accumulation of GFP::SNB-1 at GABA presynapses observed in APC mutants, we found a significant decrease (roughly 40%) in the rate of endogenous IPSCs recorded from APC mutants ($p = 0.0012$, Student's t test) (Fig. 6B). In contrast, the frequency of endogenous EPSCs in APC mutants was not significantly different from the wild type. Thus, APC function is specifically required for normal levels of neurotransmitter release at

Fig. 4. APC loss-of-function mutants have increased synaptobrevin in GABA motor neurons in the dorsal nerve cord. (A) Representative images of GFP::SNB-1 in a subset of DA and DB motor neurons (*nuls152*) in the dorsal nerve cord of wild type and *emb-30(g53)* APC4 mutant animals (upper panel). Quantification of GFP::SNB-1 puncta intensity, inter-punctal fluorescence intensity, and density for the strains in (A) is shown (lower panel). (B) Representative images of GFP::SNB-1 in GABA motor neurons (*juls1*) in the dorsal nerve cord of wild type and *emb-30(g53)* APC4 mutant animals (upper panel). Quantification of GFP::SNB-1 puncta intensity, inter-punctal fluorescence intensity, and density for the strains in (B) is shown (lower panel). Data are averages $\pm$ s.e.m. of 100X images taken from at least 20 worms per genotype after a 20 h temperature shift at the L4 stage. ([#] $p < 0.05$, ^{*} $p < 0.01$, Student's *t* test).

GABAergic, but not cholinergic, synapses. For both synapse classes, we observed more modest effects on the amplitude of endogenous synaptic events (IPSCs: 14% increase in *emb-27(g48)* CDC16 APC subunit mutants; EPSCs: 14.3% decrease in *emb-27(g48)* CDC16APC subunit mutants) (Fig. 6B), suggesting that minor changes in post-synaptic receptor density or number occur either due to a requirement for APC

function in muscles or as a consequence of altered presynaptic release. However, the increased muscle excitation we observed in our pharmacological assays cannot be explained by either increased IPSC amplitude or decreased EPSC amplitude because both of these changes would be predicted to produce aldicarb and levamisole insensitivity, which we did not observe. Thus, our electrophysiology results indicate that the

Fig. 5. Distribution of postsynaptic GABA_A receptors in the muscle of APC mutants. Representative images of UNC-49::GFP in post-synaptic dorsal body wall muscle (*oxIs22*) in wild type and *emb-30(g53)* APC4 mutant animals (upper panel). Quantification of UNC-49::GFP puncta intensity, inter-punctal fluorescence intensity, and density in wild type and *emb-30(g53)* mutants is shown (lower panel). Data are averages $\pm$ s.e.m. from 100 $\times$ images taken of 19 worms per genotype following temperature shift at the L4 stage. (* $p = 0.01$).

major effects of the APC on NMJ signaling occur through APC regulation of GABA release from inhibitory motor neurons.

The APC localizes to presynaptic sites

The APC has been shown to function in both the nucleus and the cytoplasm to control diverse cellular functions. In cerebellar granular neurons, nuclear localization of APC subunits is essential for inhibition of axon outgrowth (Huynh et al., 2009; Konishi et al., 2004; Stegmüller et al., 2006), while their localization to centrosomes is required for dendritogenesis (Kim et al., 2009). The localization of several core APC subunits to the ventral nerve cord in *C. elegans* (Juo and Kaplan, 2004) and to pre- and postsynaptic sites at the *Drosophila* NMJ (Van Roessel et al., 2004), suggests that the APC might also act at synapses to regulate synaptic protein abundance and synaptic transmission. To determine whether the APC might function at GABA synapses, we analyzed the subcellular localization of a GFP-tagged APC subunit EMB-27 CDC16 (GFP::EMB-27) relative to mCherry-tagged SNB-1 Synaptobrevin (mCherry::SNB-1), which marks presynaptic sites, in GABA neurons (Fig. 7A). We found that approximately 90% of the GFP::EMB-27 puncta co-localized with mCherry::SNB-1 puncta ($90.28 \pm 14.11\%$, $n = 20$ animals) and vice versa ($90.11 \pm 15.67\%$, $n = 20$ animals).

Many presynaptic proteins and cargos, including synaptic vesicles, require the kinesin motor UNC-104 KIF1A for their trafficking and localization to synaptic sites (Hall and Hedgecock, 1991; Jacob and Kaplan, 2003; Klopfenstein and Vale, 2004; Nonet, 1999; Ou et al., 2010; Sieburth et al., 2005; Wagner et al., 2009; Zahn et al., 2004). We tested whether APC localization to GABA motor neuron synapses was dependent on *unc-104* KIF1A. Interestingly, we found that while the synaptic localization of SNB-1 is completely lost in *unc-104* KIF1A mutants (Nonet, 1999; Ou et al., 2010; Sieburth et al., 2005), GFP::EMB-27 remains punctate in the DNC in the absence of this motor (Fig. 7B) and

there is no accumulation of GFP::EMB-27 in GABA motor neuron cell bodies in *unc-104* mutants compared to wild type animals (Average GFP::EMB-27 cell body fluorescence intensity (a.u. $\pm$ s.e.m.): wild type: 7286 ± 988 s.e.m.; *unc-104*: 4955 ± 991 , $p = 0.33$, Student's *t* test). These data suggest that localization of APC to puncta in GABA motor neurons does not require UNC-104 KIF1A or synaptic vesicles. Together, these results indicate that APC subunits can localize to GABA motor neuron presynapses, where the APC acts to regulate GABA release.

Studies in flies and mammals suggest that the APC can regulate levels of the scaffolding protein SYD-2 Liprin- α (Hoogenraad et al., 2007; Van Roessel et al., 2004). In *C. elegans* and *Drosophila*, SYD-2 Liprin- α localizes to active zones where it is required for presynaptic differentiation (Astigarraga et al., 2010; Dai et al., 2006; Fouquet et al., 2009; Kaufmann et al., 2002; Patel et al., 2006; Stigloher et al., 2011; Yeh et al., 2005; Zhen and Jin, 1999). SYD-2 Liprin- α contains multiple conserved destruction box motifs (RxxLxxxxN), which are recognized by the APC's substrate-binding activators, Cdc20 and Cdh1 (Van Roessel et al., 2004) [reviewed in (Peters, 2002)]. At the fly NMJ, a glutamatergic synapse, Liprin- α accumulates in presynaptic boutons of mutants lacking APC function (Van Roessel et al., 2004). In mammalian neurons in culture, the APC negatively regulates Liprin- α levels to control dendrite development (Hoogenraad et al., 2007). Additionally, a recent study showed that in rodent neurons, presynaptic Liprin- α levels are regulated by synaptic activity and the ubiquitin-proteasome system (Spangler et al., 2013). To test whether the APC also regulates SYD-2 Liprin- α at GABA motor neuron synapses, we imaged SYD-2::GFP (under control of the *unc-25* promoter) (*hpls3*) (Yeh et al., 2005) in the posterior dorsal nerve cords of wild type and APC mutant animals. We found that while there were no significant changes in the number or size of SYD-2::GFP puncta or the levels of inter-punctal fluorescence, there was a 33% increase in the intensity of SYD-2::GFP puncta in *emb-27(g48)* CDC16 APC mutants compared with wild type animals

Fig. 6. The frequency of IPSCs is specifically reduced in APC mutants. Endogenous inhibitory (A) and excitatory (B) postsynaptic currents (IPSCs and EPSCs, respectively) recorded from the body wall muscles of wild type and *emb-27(g48)* CDC16 mutant animals following a 20 h temperature shift treatment of L4 animals at 25 °C. (A) Representative traces of endogenous IPSCs (upper panel) and EPSCs (lower panel) in wild type (WT) and *emb-27(g48)* CDC16 mutant animals are shown. (B) Average frequency (left) and amplitude (right) of endogenous IPSCs and EPSCs. $n = 8$ animals per strain for IPSCs (upper panels) and $n = 12$ –13 animals per strain for EPSCs (lower panels). (* $p < 0.05$, ** $p = 0.001$, Student's t test).

($p = 0.029$, Student's t test) (Fig. 8). These data suggest that the APC negatively regulates the levels of presynaptic SYD-2 Liprin- α in GABA motor neurons.

Discussion

The ubiquitin signaling system is now well established as a critical regulator of synapse formation and function (Bingol and Sheng, 2011; Ding and Shen, 2008; Tai and Schuman, 2008; Yi and Ehlers, 2007),

and the central role of the APC ubiquitin ligase in controlling diverse neuronal processes (Puram and Bonni, 2011), including glutamatergic synaptic transmission, has been demonstrated across phylogeny (Fu et al., 2011; Juo and Kaplan, 2004; Van Roessel et al., 2004). The need for tight regulation of GABA signaling to maintain proper E:I balance in the nervous system is also now becoming clear; however, the specific molecular mechanisms that govern changes in the amount and timing of GABA transmission have yet to be fully elucidated (Gatto and Broadie, 2010). Here we demonstrate that the APC acts in GABA motor

Fig. 7. GFP::EMB-27 CDC16 localizes to synapses in GABA motor neurons. (A) Colocalization of GFP::EMB-27 CDC16 with mCherry::SNB-1 in GABA neurons. Representative images of the dorsal nerve cords of wild type animals expressing mCherry::snb-1 and GFP::emb-27 CDC16 transgenes under the *unc-25* GABA neuron-specific promoter in wild type animals are shown. On average, $90.1 \pm 15.7\%$ of mCherry::SNB-1 puncta colocalize with a GFP::EMB-27 CDC16 punctum and $90.3 \pm 14.1\%$ of the GFP::EMB-27 puncta colocalize with a mCherry::SNB-1 punctum in each animal (white arrowheads) ($n = 20$). White arrow denotes mCherry::SNB-1 alone; yellow arrows denote GFP::EMB-27 CDC16 alone. (B) GFP::EMB-27 CDC16 localizes independently of the synaptic vesicle motor, UNC-104 KIF1A. Representative images of the dorsal nerve cords of wild type animals expressing Punc-25::GFP::emb-27 CDC16 in wild type (upper panel) or *unc-104* KIF1A mutant animals are shown (lower panel). All images were taken on a 100× objective.

neurons to promote GABA release and to inhibit muscle contraction at the *C. elegans* NMJ.

The APC acts in GABA motor neurons to inhibit muscle excitation

Several lines of evidence support a role for the APC in specifically controlling GABA signaling at the *C. elegans* NMJ. First, loss-of-function mutants in multiple APC subunits, as well as worms treated with RNAi targeting the APC substrate adaptor, FZY-1 CDC20, are hypersensitive to paralysis induced by the acetylcholinesterase inhibitor aldicarb (Figs. 1 and 2; Supplemental Figs. 1 and 2). This aldicarb-hypersensitivity reflects an increase in muscle excitation in the absence of the APC, suggesting that the normal function of the APC is to inhibit muscle excitation. While there was variability in the aldicarb-hypersensitivity induced by loss-of-function of the various APC subunits, and even between alleles of the same subunit gene [e.g., *mat-2(ax76)* and *(ax102)*], this is most likely due to slight fluctuations in timing and/or temperature that result in variable strength of the loss-of-function. In principle, the aldicarb-hypersensitive phenotype could result from the ability of the APC to prevent cholinergic signaling and/or promote GABA signaling by acting either pre- and/or postsynaptically at the NMJ. The results of aldicarb experiments in which the APC was either inhibited or rescued exclusively in GABA neurons demonstrate that APC expression in these cells is both

necessary and sufficient for normal NMJ function, while similar attempts at rescue using a cholinergic motor neuron-specific promoter were unsuccessful (Fig. 2 and data not shown). Second, APC mutants have increased GFP::SNB-1 accumulation at GABA motor neuron presynapses and reductions in endogenous IPSC frequency, suggesting that APC mutants have decreased GABA release (Figs. 4B and 6). Importantly, our data suggest that these decreases in GABA transmission are unlikely to be the result of reduced cholinergic drive on the GABA motor neurons, as we did not observe a concomitant decrease in either the average number of synapses or the amount of synaptically localized GFP::SNB-1 in cholinergic neurons (Fig. 4A); we also found no changes in endogenous EPSC frequency or amplitude that would be consistent with elevated cholinergic signaling (Fig. 6). Third, APC mutants exhibit anterior convulsions in the PTZ-induced seizure assay (Fig. 3), further supporting a role for the APC in GABA signaling. Finally, a GFP-tagged APC subunit localizes to presynaptic sites in GABA motor neurons (Fig. 7) and synaptic levels of the active zone protein SYD-2 Liprin- α are elevated in the GABA neurons of APC mutants (Fig. 8), suggesting that the APC may impact synaptic function through its ability to act at presynapses to control SYD-2 abundance or localization. Taken together, our data strongly support a central role for the APC in controlling muscle excitation through its effects in GABA motor neurons and implicate one potential synaptic substrate of the APC that may contribute to these effects. Interestingly, another recent study showed a GABA neuron-specific function for a putative ubiquitin ligase containing the F-box protein MEC-15 FBXW9 in promoting GABA release at the *C. elegans* NMJ (Sun et al., 2013), underscoring the critical role of the ubiquitin system in controlling inhibitory signaling.

The APC promotes the release of synaptic vesicles in GABA motor neurons

The increase in GFP::SNB-1 puncta intensities in GABA neurons (Fig. 4B) and the decreased IPSC frequency (Fig. 6) indicates that GABA release is decreased in APC mutants relative to wild type animals. This decreased release is not due to altered numbers of GABA or cholinergic synapses, nor can it be explained by changes in GABA motor neuron numbers or in the size of GABA synaptic puncta (Fig. 4 and see Results text). However, while GFP::SNB-1 puncta intensities are increased by 25% in GABA motor neurons, smaller increases in inter-punctal axonal GFP::SNB-1 fluorescence were seen in both GABA and cholinergic motor neurons of *emb-30(g53)* APC4 mutants (Fig. 4). Previous studies showed that GFP::SNB-1 puncta intensity increases and inter-punctal cord fluorescence decreases in animals with defects in SV exocytosis (Dittman and Kaplan, 2006; Sieburth et al., 2005), suggesting that cord fluorescence corresponds to GFP::SNB-1 that reached the plasma membrane via exocytosis and diffused away from the synapse. However, animals with mutations in UNC-2 calcium channels, which are required for the presynaptic release of both acetylcholine and GABA (Mathews et al., 2003), also exhibit increases in both GFP::SNB-1 punctal and inter-punctal fluorescence (Ch'ng et al., 2008). These results suggest that inter-punctal levels of GFP::SNB-1 may also represent internal pools of SNB-1 perhaps from synaptic vesicles that are not well localized to the synapse. Alternatively, because inter-punctal GFP::SNB-1 levels are elevated in both GABA and cholinergic motor neurons, the APC may regulate some aspect of SNB-1 endocytosis in both cell types in addition to its specific effects on SV release in GABA neurons.

Along with the presynaptic accumulation of GFP::SNB-1, we also found that the scaffolding protein SYD-2 Liprin- α accumulates at GABA synapses in APC mutants (Fig. 8). This effect was correlated with decreased GABA transmission but with no change in the active zone protein UNC-10 RIM1 (Figs. 4, 6, and Supplemental Fig. 4). The ability of the APC to negatively regulate SYD-2 Liprin- α levels is consistent with previous studies in flies and rodent neurons (Hoogenraad et al., 2007; Van Roessel et al., 2004). Loss-of-function studies in worms, flies, and cultured rodent neurons showed that *syd-2* Liprin- α is required for the proper localization of active zone components, including ELKS and UNC-10 RIM1, as well as synaptic vesicles, to

Fig. 8. The APC regulates the synaptic abundance of the active zone protein, SYD-2 Liprin- α in GABA motor neurons. Representative images of SYD-2::GFP in GABA motor neurons (*hpls3*) in the dorsal nerve cord of wild type and *emb-27(g48)* CDC16 mutant animals (upper panel). Quantification of SYD-2::GFP puncta intensity, inter-punctal fluorescence, and density for the strains in (A) is shown (lower panel). Data are averages $\pm$ s.e.m. of 100 $\times$ images taken from at least 28 worms per genotype after a 20 h temperature shift at the L4 stage. (* $p < 0.05$, Student's *t* test).

presynaptic sites, which is important for presynaptic differentiation and synaptic transmission (Kaufmann et al., 2002; Patel et al., 2006; Spangler et al., 2013; Stigloher et al., 2011; Zhen and Jin, 1999).

It is not completely clear what effect increased presynaptic abundance of SYD-2, such as we observe in APC subunit mutants, has on active zone components, synaptic vesicle accumulation, and synaptic transmission. Although the effects of specific SYD-2 overexpression on synaptic transmission have not been directly tested, treatment of rodent neurons with proteasome inhibitors results in increased Liprin- α 2 with no effect on the synaptic levels of the active zone protein Bassoon (Spangler et al., 2013). Additionally, Dai and colleagues found that a *syd-2* gain-of-function allele (*ju487*) promotes synaptogenesis in HSN motor neurons specifically through increased presynaptic recruitment of ELKS-1; importantly, these effects occurred in the absence of any increase in SYD-2 protein levels or alterations in synaptic UNC-10 RIM1 localization (Dai et al., 2006). These results are consistent with our finding that UNC-10 RIM1 levels are unchanged in APC mutants despite the accumulation of SYD-2 at presynapses (Supplemental Fig. 4). Finally, in addition to effects on active zone protein recruitment and organization, several studies have shown that SYD-2 Liprin- α can regulate motor and vesicle trafficking (Goodwin and Juo, 2013; Miller et al., 2005; Wagner et al., 2009); thus we cannot exclude a potential role for the APC in regulating synaptic protein transport that influences GABA transmission. Future studies will be needed to test if the APC directly regulates SYD-2 ubiquitination and degradation in GABA neurons and the impact of elevated synaptic SYD-2 levels on GABA transmission at the NMJ.

What is the significance of the finding that, along with increased SNB-1 and SYD-2 accumulation at GABA motor neuron presynapses, APC loss-of-function mutants exhibit decreased endogenous IPSC frequencies in postsynaptic body wall muscles (Fig. 6)? Van Roessel et al. (2004) demonstrated that *liprin- α* is required for the increase in bouton size observed with *apc* loss-of-function at the fly NMJ. These *apc* mutant flies have increased presynaptic bouton size and increased glutamatergic transmission; however the synaptic transmission defect can be completely rescued by APC expression in muscle, suggesting that the

effect of the APC on synaptic transmission is completely due to increased postsynaptic glutamate receptor levels (Van Roessel et al., 2004). Thus, while the functional consequences of the increased bouton size in these *apc* mutant flies are unknown, our observation that synapse size (based on the puncta width of presynaptic markers) is unchanged in either GABA or cholinergic motor neurons at the *C. elegans* NMJ (see Results text) further suggests that APC function may differ in different neuron and synapse types, as well as potentially between organisms. Future work will be required to further elucidate the cell type-specific mechanisms by which the APC exerts its effects on presynaptic structure and function across phylogeny.

Although the levels of GFP::SNB-1 at presynaptic sites in GABA neurons increased in APC mutants relative to wild type animals, levels of muscle-expressed UNC-49 GABA_A receptors also increased moderately (Fig. 5), as did the amplitude of endogenous IPSCs (Fig. 6). These changes are not consistent with the increased muscle excitation and aldicarb-hypersensitivity of the APC mutants. We speculate that the increase in postsynaptic GABA responsiveness could be due to a compensatory up-regulation of GABA signaling under these conditions of low GABA release. In support of this hypothesis, the opposite compensatory response has been observed in response to increased GABA signaling. A previous study showed that reductions in surface levels of GABA_A receptors occurs in *C. elegans* during adaptation after prolonged exposure to elevated levels of the GABA agonist muscimol, and several studies in mammals have demonstrated circuit-level homeostatic responses in interneurons that are mediated by postsynaptic changes in GABA signaling following chronic activity stimulation (Davis et al., 2012; Rannals and Kapur, 2011; Wenner, 2011).

Interestingly, several recent studies have implicated the APC in the control of synaptic plasticity. For example, the Cdh1 APC adaptor is involved in mediating the effects of EphA4 during homeostatic plasticity in mammals (Fu et al., 2011). Conway and colleagues further demonstrated that expression of the Cdc20 substrate adaptor is down-regulated in response to activity in cultured hippocampal neurons, suggesting important connections between the activation of APC function and control of plasticity at diverse synapses (Conway et al., 2007).

Several other studies examined the role of the APC in learning and memory in mice by knocking out various APC subunits. Heterozygous *Cdh1* knock-out mice exhibit defects in late-phase long term potentiation (LTP) and associative fear conditioning (M. Li et al., 2008; W. Li et al., 2008). Conditional *Cdh1* knock-out mice, which lose *Cdh1* expression in neurons at the onset of neuronal differentiation, also exhibit defects in late-phase LTP and in the extinction of fear memories (Pick et al., 2012). Finally, conditional knock-out of the essential APC subunit APC2 from excitatory neurons in the mouse forebrain results in mice with defects in spatial memory formation, associative fear conditioning, and extinction of fear memories (Kuczer et al., 2011). Further studies will be required to determine the specific mechanisms by which the APC regulates synaptic plasticity during learning and memory. While these previous findings define roles for the APC in regulating plasticity at excitatory synapses, our results here suggest that the APC may have parallel functions at GABA synapses in order to establish appropriate balances of excitation and inhibition in the nervous system.

Conclusion

Our data support a novel role for the APC ubiquitin ligase and its FZY-1 CDC20 substrate adaptor in GABA motor neurons where it acts to inhibit muscle contraction in *C. elegans* by promoting GABA release, potentially through effects on the active zone protein, SYD-2 Liprin- α . In the future, it will be intriguing to determine whether SYD-2 or other presynaptic proteins represent APC substrates that mediate its function at the NMJ and to discover the regulators of APC function in this context. In addition, given the importance of the APC in learning and memory, it will be of interest to investigate the role of the APC in controlling inhibitory transmission in the mammalian central nervous system. These studies would provide even greater understanding of the control of inhibitory synaptic transmission, which is essential for maintaining proper E:I balance in the nervous system, and thus may ultimately provide critical insights into the molecular mechanisms behind neurological conditions characterized by GABA signaling defects.

Experimental methods

C. elegans strains

Strains were grown and maintained using standard protocols (Brenner, 1974). For all experiments with temperature-sensitive APC mutants, strains were maintained at 15 °C until the L4 stage, then were shifted to the non-permissive temperature at 25 °C for 20 h prior to assay. Strains used in the following experiments were: N2, *emb-30(g53ts)* APC4, *emb-30(tn377ts)* APC4, *emb-27(g48ts)* CDC16, *emb-27(ye143ts)* CDC16, *mat-2(ax102ts)* APC1, *mat-2(ax76ts)* APC1, *mat-3(or180ts)* CDC23, *unc-25(e156)*, *unc-4(e120)* *snb-1(md247)*, *fzr-1(ku298)* CDH1 *fzr-1(ok380)* CDH1, JRK17 [*kjrEx3* (*Punc-30::emb-27* CDC16; *Pttx-3::gfp*); *emb-27(g48)* CDC16], JRK18 [*kjrEx4* (*Punc-25::mCherry::snb-1*; *Pmyo-2::NLS::mCherry*)], JRK19 [*kjrEx5* (*Punc-25::gfp::emb-27* CDC16; *Pttx-3::gfp*)], JRK20 [*kjrEx5*; *kjrEx4*], JRK38 [*kjrEx5*; *unc-104(e1265)* KIF1A], *juls1* (*Punc-25::gfp::snb-1*), JRK13 [*juls1*; *emb-30(g53ts)* APC4], *nuls152* (*Punc-129::gfp::snb-1*), JRK15 [*nuls152*; *emb-30(g53ts)* APC4]; *oxls22* (*Punc-49::unc-49::gfp*), JRK14 [*oxls22*; *emb-30(g53ts)* APC4]; FJ371 [*pzls9* (*Punc-25::hemi1*); *Pmyo-2::NLS::gfp*]; JRK9 [*pzls9*; *emb-30(g53)* APC4]; *hpls3* (*Punc-25::syd-2::gfp*), JRK36 [*hpls3*; *emb-27(g48)*], *hpls88* (*Punc-25::unc-10::mCherry*), JRK51 [*hpls88*; *emb-27(g48)*].

Constructs, transgenes, and germline transformation

All plasmids were generated via standard techniques. *Punc-30::emb-27* CDC16 (FJ#30) was generated by subcloning the 2.6 kb *Punc-30* promoter from KP#1587 (*Punc-30* in PD49.26) to create plasmid FJ#30 (*Punc-30::emb-27* CDC16). *Punc-25::gfp::emb-27* CDC16 (pJRK#10)

was constructed by first subcloning the *yfp::emb-27* fusion construct from FJ#21 (*Punc-129::yfp::emb-27* CDC16) into FJ#17 (*Punc-25* in PD49.26) using *NheI* and *KpnI* to generate pJRK#8 (*Punc-25::yfp::emb-27* CDC16). The *gfp* cDNA from plasmid FJ#23 (*Punc-129::sad-1::gfp*) was then inserted into the *NotI* sites in place of *yfp* in pJRK#8. *Punc-25::mCherry::snb-1* (pJRK#9) was generated by subcloning the *mCherry::snb-1* fusion gene from pDS#139 in place of *yfp::emb-27* CDC16 in between the *NheI* and *KpnI* sites in pJRK#8. Finally, *Punc-25::hEMI1* (FJ#32) was generated by PCR amplification of the human *EMI1* cDNA from the plasmid PCS2::myc::hEMI1, which carries the mutation DNGYSN in hEMI1 to stabilize it against β -TRCP-mediated degradation (Miller et al., 2006), followed by insertion of the 1.3 kb product into FJ#18 (*Punc-17* in PD49.26) using *NheI* and *KpnI* to generate FJ#31 (*Punc-17::hEMI1*). From there, the stabilized *hEMI1* cDNA was subcloned behind the *Punc-25* promoter in FJ#17 to generate FJ#32.

Transgenic strains expressing these constructs were isolated by using standard techniques following microinjection of the following plasmids into the gonads of adult hermaphrodites: *Punc-30::emb-27* CDC16 (FJ#30) was injected at a concentration of 50 ng/ μ l along with the co-injection marker *Pttx-3::gfp* (50 ng/ μ l), *Punc-25::gfp::emb-27* CDC16 (pJRK#10) was injected at a concentration of 25 ng/ μ l along with the co-injection marker *Pttx-3::gfp* (50 ng/ μ l), *Punc-25::mCherry::snb-1* (pJRK#9) was injected at a concentration of 25 ng/ μ l along with the co-injection marker *Pmyo-2::NLS::mCherry* (5 ng/ μ l), *Punc-25::hEMI1* (FJ#32) was injected at a concentration of 50 ng/ μ l along with the co-injection marker *Pmyo-2::NLS::gfp* (10 ng/ μ l) to generate *pzEx84*. To create the integrated *Punc-25::hEMI1* line *pzls9 X*, *pzEx84* worms were irradiated with UV light at the L4 stage and integrants were selected and mapped by standard procedures (Mitani, 1995).

RNA interference

RNA interference targeting *fzy-1* was performed in the RNAi-enhanced strain, *nre-1(hd20)*; *lin-15b(hd126)* (Schmitz et al., 2007). The RNAi clone targeting the *fzy-1* gene was obtained from a genome-wide library consisting of *Escherichia coli* carrying RNAi constructs in the T7-tailed L4440 vector (Kamath et al., 2003). Bacteria were maintained in the presence of 50 μ g/ml ampicillin and 15 μ g/ml tetracycline for plasmid selection (Kamath et al., 2001). Since *fzy-1* is an embryonic lethal gene (Kitagawa et al., 2002), feeding RNAi was performed on synchronized L1s as follows: 3 ml overnight cultures of bacteria carrying an RNAi plasmid targeting either *fzy-1* or *gfp* (control) were grown in Luria broth (LB) containing 50 μ g/ml ampicillin. 150 μ l of these cultures were spotted onto 35 mm NGM agar plates containing 50 μ g/ml ampicillin and 5 mM IPTG. Plates were dried open for 2 h, then closed overnight at room temperature. Worms were prepared by treatment with a bleaching solution containing 0.5 M NaOH and 25% Na hypochlorite (Sigma-Aldrich), followed by washing and incubation in M9 buffer at a concentration of ≤ 10 eggs/ μ l at 20 °C until hatching (~17 h). Approximately 100 L1s were placed onto each RNAi-containing plate and grown at 20 °C for 3 days (<1-generation RNAi). Young adult worms were then tested in the aldicarb assay as described below.

Aldicarb and levamisole assays

NGM agar plates containing 1 mM aldicarb (Bayer Crop Sciences) or 200 μ M levamisole (Sigma-Aldrich) were prepared and seeded with 150 μ l OP50 *E. coli* 1 day prior to the assay. To begin the experiment, 20–25 adult worms with eggs, which had been previously shifted to the non-permissive temperature (26 °C) for 20 h beginning at the L4 stage, were transferred onto each drug-containing plate. The worms were tested for complete paralysis every 20 min (aldicarb assays) or after 60 min (levamisole assays) of drug exposure. The average percentage of worms of each strain paralyzed $\pm$ s.e.m. was calculated for each

timepoint using Microsoft Excel. Worms were considered paralyzed only if they did not move at all in response to harsh anterior touch with a platinum wire. Three plates were assayed for each strain per experiment with the experimenter double-blinded to genotype. Experiments were performed at least three times and representative experiments are shown.

PTZ assays

PTZ assays were performed as described previously (Locke et al., 2008). Briefly, NGM agar plates containing 10 mg/ml pentylene tetrazole (PTZ, Sigma-Aldrich) assay were made fresh on the day of the experiment, allowed to dry for 1 h, then spotted with 25 μ l OP50 *E. coli* and dried for approximately 2 h. Twenty to twenty-five adult worms with eggs, which had been previously shifted to 25 °C for 20 h beginning at the L4 stage, were transferred onto each PTZ plate to begin the time course. Two plates of each strain were tested at each concentration; wild type worms and either the GABA-deficient mutant *unc-25(e156)* or the general synaptic transmission mutant *snb-1(md247)* were run as controls. Additionally, worms were tested for convulsions at 0 mg/ml PTZ as another control. All worms were assayed at 30 and 60 min and scored as having either a wild type phenotype or exhibiting anterior convulsions. (Full body convulsions and full body paralysis, which are seen in response to PTZ with some strains, were not observed.) (Locke et al., 2006, 2008). The percentage of worms of a given strain displaying each phenotype at 30 and 60 min of exposure was calculated for each plate; results were then pooled for each strain and the average percentages of convulsing worms $\pm$ s.e.m. were computed. Statistically significant differences from wild type were determined by using a Student's *t* test ($p \leq 0.05$).

Fluorescence microscopy

For all quantitative imaging and motor neuron counting experiments, L4 animals were shifted to 26 °C for 20 h and the resulting adult hermaphrodites were immobilized with 30 mg/ml 2,3-butanedione monoxamine (Sigma-Aldrich) for 7–8 min and mounted on 2% agarose pads.

Quantitative imaging experiments were performed using a Zeiss Axiovert M1 microscope (Carl Zeiss) or a Leica DMLB microscope (Leica Microsystems) with a 100 $\times$ Plan Achromat (1.4 NA) objective equipped with GFP and RFP filters. Images were captured using an Orca-ER CCD camera (Hamamatsu) or an Exi Aqua cooled CCD camera (Qimaging) with Metamorph (v7.1 or v7.7) software (Molecular Devices). For quantitative analyses of fluorescent puncta in the dorsal nerve cord, maximum intensity projections of Z series stacks (1 μ m total depth) were made. Exposure settings and gain were set to fill a 12-bit dynamic range without saturation. These settings were identical for all images taken of a given fluorescent marker (i.e., GFP::SNB-1, UNC-49::GFP, SYD-2::GFP, UNC-10::mCherry). All images were taken of the posterior portion of the dorsal nerve cord halfway between the vulva and the tail. At this position in the *nuls152* strain, most of the GFP::SNB-1 signal comes from DB motor neuron axons (Goodwin et al., 2012). Metamorph (v6.0 or v7.1) software was used to generate linescans of dorsal nerve cord puncta, and the linescan data were analyzed with Igor Pro (WaveMetrics) using custom written software as previously described (Burbea et al., 2002). Mercury arc lamp output was normalized by measuring the intensities of 0.5 μ m FluoSphere beads (Invitrogen) for each imaging day. Puncta intensities were calculated by dividing the average maximal peak intensity by the average bead intensity for the corresponding day. Inter-punctal axonal intensities were similarly calculated using the average baseline fluorescence value within the dorsal nerve cord. Puncta densities were determined by quantifying the average number of puncta per 10 μ m of the dorsal nerve cord. For all data, an average of the values for each worm in the data set $\pm$ s.e.m. is reported. Statistical significance of any differences

between wild type and mutant values was determined by using the Student's *t* test ($p \leq 0.05$). Graphs of puncta intensities and inter-punctal axonal fluorescence show data normalized to wild type values.

The numbers of GABA and cholinergic motor neurons were determined in heat-shocked wild type and *emb-30(g53)* APC4 mutant animals expressing either the *juls1* (*Punc-25::gfp::snb-1*) transgene or the *nuls152* (*Punc-129::gfp::snb-1*) transgene. Animals were viewed on a Leica DMLB microscope at 100 $\times$. The 19 DD and VD motor neurons express GFP::SNB-1 in *juls1* (although only 17 were readily visible in the ventral nerve cord in our hands); a subset of DA and a few DB motor neurons (10–11) express GFP::SNB-1 in *nuls152* (Goodwin et al., 2012). Motor neuron cell bodies in the ventral nerve cord were counted and averaged for each strain; motor neuron numbers in wild type and APC mutant strains then were statistically compared using a Student's *t* test ($p \leq 0.05$).

For colocalization and *unc-104* KIF1A studies, the dorsal nerve cords of young adult transgenic worms maintained at 20 °C were imaged halfway between the vulva and the head at 100 $\times$ on a Leica DMLB compound fluorescent microscope (Leica Microsystems). Co-localization images were taken with a SPOT RT color camera (Diagnostic Instruments) and accompanying software (SPOT Imaging Solutions), and images were aligned and overlaid using Adobe Photoshop CS4. The percentages of GFP::EMB-27 puncta overlapping completely with mCherry::SNB-1 puncta and vice versa were calculated for each image and the average percentage overlap per strain computed. *unc-104* KIF1A images were taken with an EXi Aqua cooled CCD camera (Qimaging) and Metamorph v7.7 software (Molecular Devices). To quantify the amount of GFP::EMB-27 in GABA motor neuron cell bodies, images were taken of DD2 cell bodies in wild type and *unc-104* KIF1A mutant animals. The DD2 motor neuron innervates the anterior portion of the dorsal nerve cord where GFP::EMB-27 localization imaging was performed. Maximum intensity projections were generated from Z-series stacks (2 μ m total depth) as described above. The average pixel intensities $\pm$ s.e.m. of three separate regions of each cell body were measured using MetaMorph (v7.7) software.

Electrophysiology

Electrophysiology experiments were performed as previously described (Petrash et al., 2013).

Adult hermaphrodites were immobilized with cyanoacrylic glue and the ventral medial body wall muscles exposed by lateral cuticle excisions. Body wall muscle cell recordings were made in the whole-cell voltage-clamp configuration (holding potential of -60 mV for ACh and 0 mV for GABA mediated miniature currents) using an EPC-10 patch-clamp amplifier and digitized at 2.9 kHz. The extracellular solution consisted of (in mM): NaCl 150; KCl 1; CaCl₂ 1; MgCl₂ 4; glucose 10; sucrose 15; HEPES 15 (pH 7.3, ~ 340 mOsm). The patch pipette (fire-polished 4–6 MOhm resistant borosilicate pipettes) was filled with (in mM): K-gluconate 115; KCl 25; CaCl₂ 0.1; MgCl₂ 5; BAPTA 1; HEPES 10; Na₂ATP 5; Na₂GTP 0.5; cAMP 0.5; cGMP 0.5 (pH 7.2 with KOH, ~ 320 mOsm). Under these ionic conditions the reversal potential for Cl[−] is -46 mV while that of ACh-gated channels (nonspecific cation) is near $+10$ mV. The cholinergic antagonist curare blocks all inward currents at -60 mV under these conditions, indicating ACh-mediated excitatory postsynaptic currents (PSCs) are isolated as inward currents at this holding potential (Francis et al., 2005). In contrast, GABA-mediated inhibitory PSCs are isolated as outward currents at 0 mV under these conditions (Petrash et al., 2013). At least 60–90 s of continuous data were used in the analyses. Data analysis was performed by using Igor Pro (WaveMetrics, Inc.) and Mini Analysis (Synaptosoft, Inc.).

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.mcn.2013.12.001>.

Acknowledgments

The authors would like to thank Josh Kaplan, Derek Sieburth, Peter Jackson, Diane Shakes, Geraldine Seydoux, Mei Zhen, Gary Ruvkun, and Bayer Crop Sciences for generously providing reagents and/or *C. elegans* strains and Richard Nass for providing access to his microinjection set-up. Some strains were also provided by the *Caenorhabditis* Genetics Center (CGC), which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). We would also like to thank Debra Goldsmith for performing preliminary *Punc-25::hEMI1;emb-30(g53)* APC4 experiments and Daniel Lester for the significant work investigating SYD-2 abundance, as well as the members of the Kowalski and Juo labs for their helpful discussions and feedback on this manuscript. This project was funded by the following sources: the Training in Education and Critical Research Skills (TEACRS) postdoctoral program (GMS#5K12GM074869), a Butler University Faculty Research Award, a Research Corporation Cottrell College Science Award, and an NIH R15 award (NS078568) to J.R.K., a 2010 Butler Summer Institute award to H.D., the Synapse Neurobiology Training Program (T32 NS061764) for P.R.G., an NIH R01 grant (NS064263) to M.M.F., and an NIH R01 grant (NS059953) and a Charlton Award to P.J.

References

- Almeida, A., 2012. Regulation of APC/C-Cdh1 and its function in neuronal survival. *Mol. Neurobiol.* 46, 547–554.
- Almeida, A., et al., 2005. Cdh1/Hct1-APC is essential for the survival of postmitotic neurons. *J. Neurosci.* 25, 8115–8121.
- Astigarraga, S., et al., 2010. Three *Drosophila* liprins interact to control synapse formation. *J. Neurosci.* 30, 15358–15368.
- Bamber, B.A., et al., 1999. The *Caenorhabditis elegans* unc-49 locus encodes multiple subunits of a heteromultimeric GABA receptor. *J. Neurosci.* 19, 5348–5359.
- Bamber, B.A., et al., 2005. The composition of the GABA receptor at the *Caenorhabditis elegans* neuromuscular junction. *Br. J. Pharmacol.* 144, 502–509.
- Bingol, B., Sheng, M., 2011. Deconstruction for reconstruction: the role of proteolysis in neural plasticity and disease. *Neuron* 69, 22–32.
- Brenner, S., 1974. The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71–94.
- Brown, L.A., et al., 2006. Contributions from *Caenorhabditis elegans* functional genetics to antiparasitic drug target identification and validation: nicotinic acetylcholine receptors, a case study. *Int. J. Parasitol.* 36, 617–624.
- Burbea, M., et al., 2002. Ubiquitin and AP180 regulate the abundance of GLR-1 glutamate receptors at postsynaptic elements in *C. elegans*. *Neuron* 35, 107–120.
- Chiodi, V., et al., 2012. Unbalance of CB1 receptors expressed in GABAergic and glutamatergic neurons in a transgenic mouse model of Huntington's disease. *Neurobiol. Dis.* 45, 983–991.
- Ch'ng, Q., et al., 2008. Profiling synaptic proteins identifies regulators of insulin secretion and lifespan. *PLoS Genet.* 4, e1000283.
- Ciechanover, A., Brundin, P., 2003. The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. *Neuron* 40, 427–446.
- Clague, M.J., et al., 2012. Cellular functions of the DUBs. *J. Cell Sci.* 125, 277–286.
- Conway, A.M., et al., 2007. Regulation of neuronal cdc20 (p55cdc) expression by the plasticity-related transcription factor zif268. *Synapse* 61, 463–468.
- Dai, Y., et al., 2006. SYD-2 Liprin- α organizes presynaptic active zone formation through ELKS. *Nat. Neurosci.* 9, 1479–1487.
- Davis, E.S., et al., 2002. Multiple subunits of the *Caenorhabditis elegans* anaphase-promoting complex are required for chromosome segregation during meiosis I. *Genetics* 160, 805–813.
- Davis, K.M., et al., 2012. Regulated lysosomal trafficking as a mechanism for regulating GABAA receptor abundance at synapses in *Caenorhabditis elegans*. *Mol. Cell. Neurosci.* 44, 307–317.
- DiAntonio, A., Hicke, L., 2004. Ubiquitin-dependent regulation of the synapse. *Annu. Rev. Neurosci.* 27, 223–246.
- Dimova, N.V., et al., 2012. APC/C-mediated multiple monoubiquitylation provides an alternative degradation signal for cyclin B1. *Nat. Cell Biol.* 14, 168–176.
- Ding, M., Shen, K., 2008. The role of the ubiquitin proteasome system in synapse remodeling and neurodegenerative diseases. *Bioessays* 30, 1075–1083.
- Dittman, J.S., Kaplan, J.M., 2006. Factors regulating the abundance and localization of synaptobrevin in the plasma membrane. *Proc. Natl. Acad. Sci. U. S. A.* 103, 11399–11404.
- Esmaili, B., et al., 2002. The *C. elegans* even-skipped homologue, vab-7, specifies DB motoneurone identity and axon trajectory. *Development* 129, 853–862.
- Fay, D.S., et al., 2002. fzr-1 and lin-35/Rb function redundantly to control cell proliferation in *C. elegans* as revealed by a nonbiased synthetic screen. *Genes Dev.* 16, 503–517.
- Fouquet, W., et al., 2009. Maturation of active zone assembly by *Drosophila* Bruchpilot. *J. Cell Biol.* 186, 129–145.
- Francis, M.M., et al., 2005. The Ror receptor tyrosine kinase CAM-1 is required for ACR-16-mediated synaptic transmission at the *C. elegans* neuromuscular junction. *Neuron* 46, 581–594.
- Fu, A.K., et al., 2011. APC(Cdh1) mediates EphA4-dependent downregulation of AMPA receptors in homeostatic plasticity. *Nat. Neurosci.* 14, 181–189.
- Furuta, T., et al., 2000. EMB-30: an APC4 homologue required for metaphase-to-anaphase transitions during meiosis and mitosis in *Caenorhabditis elegans*. *Mol. Biol. Cell* 11, 1401–1419.
- Gao, S., Zhen, M., 2011. Action potentials drive body wall muscle contractions in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U. S. A.* 108, 2557–2562.
- Gatto, C.L., Broadie, K., 2010. Genetic controls balancing excitatory and inhibitory synaptogenesis in neurodevelopmental disorder models. *Front. Synaptic Neurosci.* 2, 4.
- Golden, A., et al., 2000. Metaphase to anaphase (mat) transition-defective mutants in *Caenorhabditis elegans*. *J. Cell Biol.* 151, 1469–1482.
- Goodwin, P.R., Juo, P., 2013. The scaffolding protein SYD-2/Liprin- α regulates the mobility and polarized distribution of dense-core vesicles in *C. elegans* motor neurons. *PLoS One* 8, e54763.
- Goodwin, P.R., et al., 2012. Cyclin-dependent kinase 5 regulates the polarized trafficking of neuropeptide-containing dense-core vesicles in *Caenorhabditis elegans* motor neurons. *J. Neurosci.* 32, 8158–8172.
- Hall, D.H., Hedgecock, E.M., 1991. Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in *C. elegans*. *Cell* 65, 837–847.
- Harney, D., et al., 2009. The anaphase promoting complex induces substrate degradation during neuronal differentiation. *J. Biol. Chem.* 284, 4317–4323.
- Hegde, A.N., Upadhyay, S.C., 2007. The ubiquitin-proteasome pathway in health and disease of the nervous system. *Trends Neurosci.* 30, 587–595.
- Hershko, A., Ciechanover, A., 1998. The ubiquitin system. *Annu. Rev. Biochem.* 67, 425–479.
- Hicke, L., Dunn, R., 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu. Rev. Cell Dev. Biol.* 19, 141–172.
- Hoogenraad, C.C., et al., 2007. Liprin α 1 degradation by calcium/calmodulin-dependent protein kinase II regulates LAR receptor tyrosine phosphatase distribution and dendrite development. *Dev. Cell* 12, 587–602.
- Huang, R.Q., et al., 2001. Pentylentetrazole-induced inhibition of recombinant gamma-aminobutyric acid type A (GABA(A)) receptors: mechanism and site of action. *J. Pharmacol. Exp. Ther.* 298, 986–995.
- Huynh, M.A., et al., 2009. Regulation of Cdh1-APC function in axon growth by Cdh1 phosphorylation. *J. Neurosci.* 29, 4322–4327.
- Jacob, T.C., Kaplan, J.M., 2003. The EGL-21 carboxypeptidase E facilitates acetylcholine release at *Caenorhabditis elegans* neuromuscular junctions. *J. Neurosci.* 23, 2122–2130.
- Jin, Y., et al., 1999. The *Caenorhabditis elegans* gene unc-25 encodes glutamic acid decarboxylase and is required for synaptic transmission but not synaptic development. *J. Neurosci.* 19, 539–548.
- Juo, P., Kaplan, J.M., 2004. The anaphase-promoting complex regulates the abundance of GLR-1 glutamate receptors in the ventral nerve cord of *C. elegans*. *Curr. Biol.* 14, 2057–2062.
- Kamath, R.S., et al., 2001. Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol.* 2 (RESEARCH0002).
- Kamath, R.S., et al., 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421, 231–237.
- Kannan, M., et al., 2012a. p250GAP is a novel player in the Cdh1-APC/Smurf1 pathway of axon growth regulation. *PLoS One* 7, e50735.
- Kannan, M., et al., 2012b. The E3 ligase Cdh1-anaphase promoting complex operates upstream of the E3 ligase Smurf1 in the control of axon growth. *Development* 139, 3600–3612.
- Kaufmann, N., et al., 2002. *Drosophila* liprin- α and the receptor phosphatase Dlar control synapse morphogenesis. *Neuron* 34, 27–38.
- Kim, A.H., et al., 2009. A centrosomal Cdc20-APC pathway controls dendrite morphogenesis in postmitotic neurons. *Cell* 136, 322–336.
- Kitagawa, R., et al., 2002. The Cdc20 homolog, FZY-1, and its interacting protein, IFY-1, are required for proper chromosome segregation in *Caenorhabditis elegans*. *Curr. Biol.* 12, 2118–2123.
- Klopfenstein, D.R., Vale, R.D., 2004. The lipid binding pleckstrin homology domain in UNC-104 kinesin is necessary for synaptic vesicle transport in *Caenorhabditis elegans*. *Mol. Biol. Cell* 15, 3729–3739.
- Konishi, Y., et al., 2004. Cdh1-APC controls axonal growth and patterning in the mammalian brain. *Science* 303, 1026–1030.
- Koushika, S.P., et al., 2001. A post-docking role for active zone protein rim. *Nat. Neurosci.* 4, 997–1005.
- Kowalski, J.R., Juo, P., 2012. The role of deubiquitinating enzymes in synaptic function and nervous system diseases. *Neural Plast.* 13.
- Kuczera, T., et al., 2011. The anaphase promoting complex is required for memory function in mice. *Learn. Mem.* 18, 49–57.
- Kulathu, Y., Komander, D., 2012. Atypical ubiquitylation – the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. *Nat. Rev. Mol. Cell Biol.* 13, 508–523.
- Lasorella, A., et al., 2006. Degradation of Id2 by the anaphase-promoting complex couples cell cycle exit and axonal growth. *Nature* 442, 471–474.
- Li, M., et al., 2008a. The adaptor protein of the anaphase promoting complex Cdh1 is essential in maintaining replicative lifespan and in learning and memory. *Nat. Cell Biol.* 10, 1083–1089.
- Li, W., et al., 2008b. Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. *PLoS One* 3, e1487.
- Locke, C.J., et al., 2006. Genetic interactions among cortical malformation genes that influence susceptibility to convulsions in *C. elegans*. *Brain Res.* 1120, 23–34.
- Locke, C., et al., 2008. Paradigms for Pharmacological Characterization of *C. elegans* Synaptic Transmission Mutants. *J. Vis. Exp.* 18, e837. <http://dx.doi.org/10.3791/837>.

- Locke, C.J., et al., 2009. Pharmacogenetic analysis reveals a post-developmental role for Rac GTPases in *Caenorhabditis elegans* GABAergic neurotransmission. *Genetics* 183, 1357–1372.
- Love, K.R., et al., 2007. Mechanisms, biology and inhibitors of deubiquitinating enzymes. *Nat. Chem. Biol.* 3, 697–705.
- Mahoney, T.R., et al., 2006. Analysis of synaptic transmission in *Caenorhabditis elegans* using an aldicarb-sensitivity assay. *Nat. Protoc.* 1, 1772–1777.
- Manchado, E., et al., 2012. The anaphase-promoting complex/cyclosome (APC/C): cell-cycle-dependent and -independent functions. *Biochem. Soc. Trans.* 38, 65–71.
- Mathews, E.A., et al., 2003. Critical residues of the *Caenorhabditis elegans* unc-2 voltage-gated calcium channel that affect behavioral and physiological properties. *J. Neurosci.* 23, 6537–6545.
- McEwen, J.M., et al., 2006. Antagonistic regulation of synaptic vesicle priming by Tomosyn and UNC-13. *Neuron* 51, 303–315.
- McIntire, S.L., et al., 1993. Genes required for GABA function in *Caenorhabditis elegans*. *Nature* 364, 334–337.
- Miller, D.M., et al., 1992. *C. elegans* unc-4 gene encodes a homeodomain protein that determines the pattern of synaptic input to specific motor neurons. *Nature* 355, 841–845.
- Miller, K.G., et al., 1996. A genetic selection for *Caenorhabditis elegans* synaptic transmission mutants. *Proc. Natl. Acad. Sci. U. S. A.* 93, 12593–12598.
- Miller, K.E., et al., 2005. Direct observation demonstrates that Liprin-alpha is required for trafficking of synaptic vesicles. *Curr. Biol.* 15, 684–689.
- Miller, J.J., et al., 2006. Emi1 stably binds and inhibits the anaphase-promoting complex/cyclosome as a pseudosubstrate inhibitor. *Genes Dev.* 20, 2410–2420.
- Mitani, S., 1995. Genetic regulation of mec-3 gene expression implicated in the specification of the mechanosensory neuron cell types in *Caenorhabditis elegans*. *Develop. Growth Differ.* 37, 551–557.
- Nguyen, M., et al., 1995. *Caenorhabditis elegans* mutants resistant to inhibitors of acetylcholinesterase. *Genetics* 140, 527–535.
- Nijman, S.M., et al., 2005. A genomic and functional inventory of deubiquitinating enzymes. *Cell* 123, 773–786.
- Nonet, M.L., 1999. Visualization of synaptic specializations in live *C. elegans* with synaptic vesicle protein-GFP fusions. *J. Neurosci. Methods* 89, 33–40.
- Ou, C.Y., et al., 2010. Two cyclin-dependent kinase pathways are essential for polarized trafficking of presynaptic components. *Cell* 141, 846–858.
- Patel, M.R., et al., 2006. Hierarchical assembly of presynaptic components in defined *C. elegans* synapses. *Nat. Neurosci.* 9, 1488–1498.
- Peters, J.M., 2002. The anaphase-promoting complex: proteolysis in mitosis and beyond. *Mol. Cell* 9, 931–943.
- Peters, J.M., 2006. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat. Rev. Mol. Cell Biol.* 7, 644–656.
- Petrash, H.A., et al., 2013. ACR-12 ionotropic acetylcholine receptor complexes regulate inhibitory motor neuron activity in *Caenorhabditis elegans*. *J. Neurosci.* 33, 5524–5532.
- Pick, J.E., et al., 2012. The E3 ligase APC/C-Cdh1 is required for associative fear memory and long-term potentiation in the amygdala of adult mice. *Learn. Mem.* 20, 11–20.
- Prosser, H.M., et al., 2001. Epileptogenesis and enhanced prepulse inhibition in GABA(B1)-deficient mice. *Mol. Cell. Neurosci.* 17, 1059–1070.
- Puram, S.V., Bonni, A., 2011. Novel functions for the anaphase-promoting complex in neurobiology. *Semin. Cell Dev. Biol.* 22, 586–594.
- Rannals, M.D., Kapur, J., 2011. Homeostatic strengthening of inhibitory synapses is mediated by the accumulation of GABA(A) receptors. *J. Neurosci.* 31, 17701–17712.
- Reimann, J.D., et al., 2001. Emi1 regulates the anaphase-promoting complex by a different mechanism than Mad2 proteins. *Genes Dev.* 15, 3278–3285.
- Richmond, J.E., Jorgensen, E.M., 1999. One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. *Nat. Neurosci.* 2, 791–797.
- Richmond, J.E., et al., 1999. UNC-13 is required for synaptic vesicle fusion in *C. elegans*. *Nat. Neurosci.* 2, 959–964.
- Rocha, L., et al., 1996. Pentylentetrazol-induced kindling: early involvement of excitatory and inhibitory systems. *Epilepsy Res.* 26, 105–113.
- Schmitz, C., et al., 2007. Axon guidance genes identified in a large-scale RNAi screen using the RNAi-hypersensitive *Caenorhabditis elegans* strain nre-1(hd20) lin-15b(hd126). *Proc. Natl. Acad. Sci. U. S. A.* 104, 834–839.
- Schuler, A.L., 2001. Autistic syndrome. *Autism* 5, 331–340.
- Shakes, D.C., et al., 2003. Developmental defects observed in hypomorphic anaphase-promoting complex mutants are linked to cell cycle abnormalities. *Development* 130, 1605–1620.
- Sieburth, D., et al., 2005. Systematic analysis of genes required for synapse structure and function. *Nature* 436, 510–517.
- Snodgrass, S.R., 1992. GABA and epilepsy: their complex relationship and the evolution of our understanding. *J. Child Neurol.* 7, 77–86.
- Spangler, S.A., et al., 2013. Liprin-alpha2 promotes the presynaptic recruitment and turnover of RIM1/CASK to facilitate synaptic transmission. *J. Cell Biol.* 201, 915–928.
- Stegmuller, J., et al., 2006. Cell-intrinsic regulation of axonal morphogenesis by the Cdh1-APC target SnoN. *Neuron* 50, 389–400.
- Stegmuller, J., et al., 2008. TGFbeta-Smad2 signaling regulates the Cdh1-APC/SnoN pathway of axonal morphogenesis. *J. Neurosci.* 28, 1961–1969.
- Stigloher, C., et al., 2011. The presynaptic dense projection of the *Caenorhabditis elegans* cholinergic neuromuscular junction localizes synaptic vesicles at the active zone through SYD-2/liprin and UNC-10/RIM-dependent interactions. *J. Neurosci.* 31, 4388–4396.
- Sulston, J.E., 1983. Neuronal cell lineages in the nematode *Caenorhabditis elegans*. *Cold Spring Harb. Symp. Quant. Biol.* 48 (Pt 2), 443–452.
- Sulston, J.E., Horvitz, H.R., 1977. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol.* 56, 110–156.
- Sulston, J.E., et al., 1983. The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* 100, 64–119.
- Sun, Y., et al., 2013. The F-Box Protein MEC-15 (FBXW9) promotes synaptic transmission in GABAergic motor neurons in *C. elegans*. *PLoS One* 8, e59132.
- Tai, H.C., Schuman, E.M., 2008. Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. *Nat. Rev. Neurosci.* 9, 826–838.
- Touroutine, D., et al., 2005. acr-16 encodes an essential subunit of the levamisole-resistant nicotinic receptor at the *Caenorhabditis elegans* neuromuscular junction. *J. Biol. Chem.* 280, 27013–27021.
- Van Roessel, P., et al., 2004. Independent regulation of synaptic size and activity by the anaphase-promoting complex. *Cell* 119, 707–718.
- Vashlishan, A.B., et al., 2008. An RNAi screen identifies genes that regulate GABA synapses. *Neuron* 58, 346–361.
- Wagner, O.I., et al., 2009. Synaptic scaffolding protein SYD-2 clusters and activates kinesin-3 UNC-104 in *C. elegans*. *Proc. Natl. Acad. Sci. U. S. A.* 106, 19605–19610.
- Weimer, R.M., et al., 2003. Defects in synaptic vesicle docking in unc-18 mutants. *Nat. Neurosci.* 6, 1023–1030.
- Wenner, P., 2011. Mechanisms of GABAergic homeostatic plasticity. *Neural Plast.* 2011, 489470.
- White, J.G., et al., 1986. The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 314, 1–340.
- Williams, S.N., et al., 2004. Epileptic-like convulsions associated with LIS-1 in the cytoskeletal control of neurotransmitter signaling in *Caenorhabditis elegans*. *Hum. Mol. Genet.* 13, 2043–2059.
- Yang, Y., et al., 2009. A Cdc20-APC ubiquitin signaling pathway regulates presynaptic differentiation. *Science* 326, 575–578.
- Yao, W., et al., 2010. Cdh1-APC is involved in the differentiation of neural stem cells into neurons. *Neuroreport* 21, 39–44.
- Ye, Y., Rape, M., 2009. Building ubiquitin chains: E2 enzymes at work. *Nat. Rev. Mol. Cell Biol.* 10, 755–764.
- Yeh, E., et al., 2005. Identification of genes involved in synaptogenesis using a fluorescent active zone marker in *Caenorhabditis elegans*. *J. Neurosci.* 25, 3833–3841.
- Yi, J.J., Ehlers, M.D., 2007. Emerging roles for ubiquitin and protein degradation in neuronal function. *Pharmacol. Rev.* 59, 14–39.
- Yuen, E.Y., et al., 2012. Disrupted GABAAR trafficking and synaptic inhibition in a mouse model of Huntington's disease. *Neurobiol. Dis.* 46, 497–502.
- Zahn, T.R., et al., 2004. Dense core vesicle dynamics in *Caenorhabditis elegans* neurons and the role of kinesin UNC-104. *Traffic* 5, 544–559.
- Zhen, M., Jin, Y., 1999. The liprin protein SYD-2 regulates the differentiation of presynaptic termini in *C. elegans*. *Nature* 401, 371–375.