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Adenomatous polyposis coli plays a key role, *in vivo* , in coordinating assembly of the neuronal nicotinic postsynaptic complex

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Abstract

The neuronal nicotinic synapse plays a central role in normal cognitive and autonomic function. Molecular mechanisms that direct the assembly of this synapse remain poorly defined, however. We show here that adenomatous polyposis coli (APC) organizes a multi-molecular complex that is essential for targeting $\alpha 3^*$ nAChRs to synapses. APC interaction with microtubule plus-end binding protein EB1 is required for $\alpha 3^*$ nAChR surface membrane insertion and stabilization. APC brings together EB1, the key cytoskeletal regulators macrophin and IQGAP1, and 14-3-3 adapter protein at nicotinic synapses. 14-3-3, in turn, links the $\alpha 3$ -subunit to APC. This multi-molecular APC complex stabilizes the local microtubule and F-actin cytoskeleton and links postsynaptic components to the cytoskeleton—essential functions for controlling the molecular composition and stability of synapses. This work identifies macrophin, IQGAP1 and 14-3-3 as novel nicotinic synapse components and defines a new role for APC as an *in vivo* coordinator of nicotinic postsynaptic assembly in vertebrate neurons.

Introduction

Normal cognitive and autonomic functions require nicotinic synaptic transmission. Malfunction of nicotinic synapses has been implicated in Alzheimer's disease, schizophrenia, nocturnal frontal lobe epilepsy and autoimmune autonomic neuropathies (Dani and Bertrand, 2007). Despite the physiological importance of neuronal nicotinic synapses, little is known about the molecular mechanisms that direct their assembly during development. We recently identified adenomatous polyposis coli (APC) protein as a key synapse organizer by showing that it is essential for localizing nAChRs to postsynaptic sites *in vivo* (Temburni et al., 2004). APC's synaptic function was undefined prior to this work. However, the molecular mechanisms that underlie APC-mediated targeting of nAChRs to synapses are poorly understood. The present study begins to define these mechanisms in an *in vivo* model system. The importance of understanding such mechanisms in the vertebrate nervous system is

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underscored by the association of APC gene mutations with mental retardation, schizophrenia and autism spectrum disorders (Cui et al., 2005; Finch et al., 2005; Zhou et al., 2007).

The APC protein has multiple domains and binding partners, indicating its multi-functional nature (Fearnhead et al., 2001). APC is a known negative regulator in the canonical Wnt gene transcription pathway (Polakis, 1997). In addition, APC organizes and stabilizes the polarized microtubule cytoskeleton in epithelial cells by binding to the microtubule plus-end binding protein EB1, a member of the family of microtubule tracking proteins (Akhmanova and Hoogenraad, 2005; Reilein and Nelson, 2005). Recent studies show that APC also carries out microtubule-organizing functions in neurons. APC plays a role in establishing neuronal polarity, axon specification, and microtubule-mediated axonal targeting of specific proteins (Shi et al., 2004; Zhou et al., 2004; Votin et al., 2005; Farias et al., 2007). New reports extend APC's role to include synapse assembly as well. APC is required for clustering of PSD-95 in cultured hippocampal neurons (Shimomura et al., 2007), muscle-type nAChRs on myotubes (Wang et al., 2003), and $\alpha 3^*$ nAChRs at interneuronal postsynaptic sites *in vivo* (Temburni et al., 2004).

Our work has identified APC as a novel component of neuronal nicotinic synapses (Temburni et al., 2004). Surface clusters of APC selectively co-localizes with synaptic vesicles and $\alpha 3^*$ nAChRs at synapses on chicken ciliary ganglion (CG) neurons. Furthermore, our past studies identified APC's binding partners in the CG as EB1, postsynaptic density protein PSD-93, and β -catenin. Importantly, simultaneous block of APC's interactions with both EB1 and PSD-93 caused specific decreases in $\alpha 3^*$ nAChR clusters (Temburni et al., 2004). Postsynaptic accumulations of EB1 and PSD-93 were also reduced, although the latter is not essential for localizing $\alpha 3^*$ nAChRs at synapses (Conroy et al., 2003; Parker et al., 2004).

The present study tests directly the synapse organizing functions of the APC::EB1 interaction. We provide new insights into APC and EB1 associated proteins and mechanisms that target $\alpha 3^*$ nAChRs to synapses *in vivo*. We show that APC::EB1 interactions are required for both surface delivery and postsynaptic stabilization of $\alpha 3^*$ nAChRs. APC directs these essential aspects of synaptic assembly by organizing a complex of EB1, key cytoskeletal regulators macrophin and IQGAP1, and 14-3-3 adapter proteins at postsynaptic sites. This multi-molecular APC complex stabilizes the microtubule and F-actin cytoskeleton at the synapse and 14-3-3 links $\alpha 3^*$ nAChRs to APC. Our findings identify novel components of neuronal nicotinic synapses and uncover new roles for APC in coordinating assembly of the postsynaptic complex in vertebrate neurons *in vivo*.

Results

Blockade of APC::EB1 interactions specifically decreases $\alpha 3^*$ nAChR surface clusters *in vivo*

To gain new mechanistic insights into neuronal nicotinic synapse assembly, we tested our model of APC as a key organizer of a multi-protein postsynaptic complex that targets $\alpha 3^*$ nAChRs to synapses by regulating the local cytoskeleton. APC interactions with the microtubule plus-end binding protein EB1 are predicted to play a central role in this model. As the first step, we tested directly whether selective blockade of APC::EB1 interactions specifically disrupts $\alpha 3^*$ nAChR localizing at synapses in CG neurons. Single CG neurons express two nAChR types, $\alpha 3^*$ nAChRs and $\alpha 7$ -nAChRs, as well as glycine receptors (GlyRs), each of which segregates to a distinct synapse-associated surface membrane region. This preparation, therefore, provides a valuable opportunity to compare molecular mechanisms that orchestrate the assembly of the different receptor complexes. The $\alpha 3^*$ nAChRs are concentrated in postsynaptic membrane regions that oppose presynaptic terminal active zones (Jacob et al., 1986; Williams et al., 1998). GlyR clusters localize to separate but proximal postsynaptic membrane regions, all under one presynaptic terminal (Tsen et al., 2000). In contrast, $\alpha 7$ -

nAChRs are excluded from the synapse and localize perisynaptically on somatic spines (Jacob and Berg, 1983; Coggan et al., 2005). For the present studies, we developed a new dominant negative construct to selectively block endogenous APC binding to EB1 during synapse formation *in vivo*. We used the dominant negative blocking peptide rather than a knockdown of APC expression (e.g., using RNA interference) because we wished to define the specific functions of APC::EB1 interactions in nicotinic synapse assembly.

We generated a dominant negative (dn) APC C-terminal fragment consisting of the EB1 binding domain in the chicken APC sequence (amino acids 1943–2184), referred to here as APC::EB1-dn. The EB1 binding domain in the chicken APC C-terminus was defined by co-immunoprecipitation assays employing overlapping C-terminal peptide fragments (data not shown). We used retroviral-mediated gene transfer to express hemagglutinin (HA)-tagged APC::EB1-dn, *in vivo*, in chicken CG neurons during the time of synapse formation. Infected cells were identified through HA immunolabeling.

Prior to *in vivo* expression, we tested the specificity and efficacy of the APC::EB1-dn construct in *in vitro* binding assays. We established that HA-tagged APC::EB1-dn peptide specifically interacted with full-length EB1 (Fig. 1A) and blocked the binding of APC recombinant fusion protein to EB1, but not to PSD93 (Fig. 1B,C). We then confirmed the *in vivo* specificity of the APC::EB1-dn by looking for changes in synaptic accumulation of EB1 and other APC binding partners in infected versus uninfected control CG neurons. Typically these proteins are selectively enriched at $\alpha 3^*$ nAChR-containing postsynaptic sites in CG neurons (Temburni et al., 2004). EB1 clusters show close proximity to and partial overlap with $\alpha 3^*$ nAChR surface clusters (Fig. 1D), but the bulk of EB1 staining is cytoplasmic, as expected for a microtubule binding protein. We found specific and significant decreases in EB1 clusters near surface sites in APC::EB1-dn neurons versus uninfected control neurons (26% lower mean pixel intensity levels, $p < 9.3 \times 10^{-7}$; Fig. 1E). In contrast, we found no significant change in the labeling for: PSD-93 or β -catenin, two other APC interacting proteins, N-cadherin, the cell adhesion molecule that recruits β -catenin to specific synapses or APC itself (Supplemental Figure 1 and data not shown). These results suggest that the overexpressed APC::EB1-dn selectively blocks endogenous APC interactions with EB1 in CG neurons *in vivo*. Further, these findings demonstrate that APC interactions are required to localize or selectively retain EB1 at nicotinic postsynaptic sites.

Blockade of APC::EB1 interactions, *in vivo*, led to obvious decreases in $\alpha 3^*$ nAChR surface clusters (Fig. 1F). We found a substantial decrease in fluorescence pixel intensities for $\alpha 3^*$ nAChR surface membrane labeling on APC::EB1-dn infected versus uninfected control CG neurons [uninfected neurons within the same CG (internal control) and uninfected age-matched CGs processed in parallel]. Mean pixel intensity levels were 31% lower for surface $\alpha 3^*$ nAChRs on APC::EB1-dn neurons relative to control neuron values ($p < 10^{-6}$, Student's *t*-test, Fig. 1F). We found a quantitative correlation between the extent of reductions and APC::EB1-dn expression levels (pixel intensity of HA immunolabeling); decreases of 38% in heavily infected neurons and 23% in lightly infected neurons relative to control values. Immunofluorescent labeling, however, likely underestimates the change in receptor number because of limited antibody access to receptor packed at high density accumulations on control neurons. Indeed, direct measurement of functional $\alpha 3^*$ nAChR levels revealed an apparently greater decrease in APC::EB1-dn neurons (see below).

The effects of APC::EB1-dn expression were specific for $\alpha 3^*$ nAChRs, as $\alpha 7$ -nAChR surface clusters and mean pixel intensity levels were not altered ($p > 0.14$, Fig. 2A–C). Moreover, surface GlyR mean pixel intensity levels were significantly increased by 18% on APC::EB1-dn neurons relative to control neuron levels ($p < 0.02$, Fig. 2F). We speculate that the increase in GlyR surface levels may be an indirect effect of changes at the nicotinic postsynaptic sites

caused by the APC::EB1-dn, based on reports of an interplay between nicotinic and GABAergic signaling in developing CG neurons (Temburni et al., 2004; Liu et al., 2006) and the selective co-localization of APC with surface $\alpha 3^*$ nAChRs, but not with GlyRs or $\alpha 7$ -nAChRs (Temburni et al., 2004).

As a further specificity test, we expressed a different dominant negative peptide to block the binding of endogenous APC to PSD-93a, but not to EB1, in CG neurons. PSD-93a is a short isoform that lacks the Src homology 3 and guanylate kinase domains, and it selectively colocalizes with APC and $\alpha 3^*$ nAChR surface clusters in the CG (Conroy et al., 2003; Temburni et al., 2004). The APC::PSD-93-dn consisted of the APC C-terminus-end fragment (amino acids 2185–2232) that contains the consensus PDZ-binding motif (VTSV) at the carboxyl end. *In vitro* binding assays confirmed specificity, as the HA-tagged APC::PSD-93-dn peptide blocked the binding of APC recombinant fusion protein to PSD-93a, but not to EB1 (Fig. 3A,B). In sharp contrast to APC::EB1-dn, expression of APC::PSD-93-dn, *in vivo*, caused no significant change in $\alpha 3^*$ nAChR surface labeling (Fig. 3C–E). Importantly, the two different blocking peptides were expressed at similar levels within CG neurons (pixel intensity of HA immunolabeling). APC interactions with EB1, but not with PSD-93a, are essential for $\alpha 3^*$ nAChR targeting to neuronal synapses *in vivo*.

APC::EB1-dn expression decreases functional $\alpha 3^*$ nAChRs levels

To test whether the decrease in $\alpha 3^*$ nAChR surface clusters reflects reduced clustering or reduced density, we used whole-cell voltage clamp recordings to measure nAChR-mediated currents on acutely dissociated neurons from APC::EB1-dn infected CGs versus control uninfected CGs. The relative contributions of $\alpha 3^*$ nAChRs and $\alpha 7$ -nAChRs to the currents can be readily distinguished because $\alpha 7$ -nAChRs activate and inactivate rapidly, whereas $\alpha 3^*$ nAChRs gate much more slowly (Zhang et al., 1994). In a subset of the experiments, $\alpha 7$ -nAChRs were blocked by adding saturating levels of α -bungarotoxin (100 nM) (Zhang et al., 1994) to the recording solution, and there was no significant change in amplitude or time course of the late phase $\alpha 3^*$ nAChR-mediated current (data not shown).

$\alpha 3^*$ nAChR currents evoked by the agonist nicotine (at 20 μ M) were 2.6-fold lower in peak amplitude in APC::EB1-dn neurons as compared to control neurons (mean current densities = 7.2 ± 1.9 pA/pF versus 18.7 ± 2.1 pA/pF, $p < 2 \times 10^{-5}$, Student's t-test) when measured at a holding potential of -60 mV (Fig. 4), indicating a decrease in the total number of functional $\alpha 3^*$ nAChRs. The APC::EB1-dn-induced reduction in $\alpha 3^*$ nAChRs is not likely to be caused by reduced receptor synthesis, because we found no change in $\alpha 3^*$ nAChR protein levels in the internal biosynthetic pool by quantitative immunolabeling (data not shown). Our immunolabeling and voltage clamp recordings show that the APC::EB1-dn specifically decreased surface $\alpha 3^*$ nAChRs.

Expression of APC::EB1-dn decreases insertion and increases endocytosis of surface $\alpha 3^*$ nAChRs

Next, we began to define the mechanism by which the APC::EB1 interaction directs $\alpha 3^*$ nAChR surface clustering. We tested for changes in $\alpha 3^*$ nAChR insertion and turnover rates in cells expressing APC::EB1-dn protein. For the insertion assay, dissociated neurons were first exposed to saturating concentrations of unlabeled antibody Fab fragments to block existing surface $\alpha 3^*$ nAChRs, and then the appearance of newly-inserted receptor was assessed over time using fluorescently-labeled antibody (Lu et al., 2001; Man et al., 2003). We found 4-fold decreases in the rate of $\alpha 3^*$ nAChR insertion into the surface membrane of APC::EB1-dn expressing neurons relative to control uninfected neurons ($p < 5 \times 10^{-6}$, Fig. 5A,B).

We tested for changes in stable retention of surface $\alpha 3^*$ nAChRs using standard cell surface biotinylation assays of endocytosis (Mammen et al., 1997; Rasmussen et al., 2002). Surface $\alpha 3^*$ nAChRs showed reduced stabilization as indicated by their 1.6-fold faster turnover rates on dissociated APC::EB1-dn neurons relative to control uninfected neurons ($p < 0.04$; Fig. 5C,D). The effects were specific for $\alpha 3^*$ nAChRs, as turnover rates for N-cadherin, another nicotinic synapse surface membrane protein, were not significantly altered in APC::EB1-dn expressing neurons (Fig. 5E,F). Blockade of APC::EB1 interactions specifically decreases $\alpha 3^*$ nAChR surface delivery and stabilization at synapses.

APC organizes a complex of key cytoskeletal regulatory proteins at nicotinic postsynaptic sites

We hypothesize that APC directs $\alpha 3^*$ nAChR surface delivery and stabilization by bringing together key regulators of cytoskeleton dynamics at postsynaptic sites. We have shown that APC is essential for positioning EB1 near surface sites (Fig. 1E). Our model is further based on studies in non-neuronal cells that show IQGAP1 and macrophin (MACF, microtubule-actin cross-linking factor) interact with APC and EB1 (Watanabe et al., 2004; Slep et al., 2005). These proteins bind to and regulate the dynamics of EB1-tagged microtubules and submembranous F-actin at specialized intercellular junctions (Barth et al., 2002; Jefferson et al., 2004; Noritake et al., 2004). However, little is known about the function of these proteins at neuronal synapses.

Consistent with our model, yeast two-hybrid screens of a CG cDNA library using full-length chicken EB1 as bait identified MACF as an EB1 binding partner. We obtained 10 MACF cDNA clones as positives and confirmed the interaction with chicken EB1 by co-precipitation *in vitro* using recombinant peptides (data not shown).

Next we identified MACF and IQGAP1 as novel components of neuronal nicotinic postsynaptic sites. MACF and IQGAP1 exhibited colocalization with $\alpha 3^*$ nAChR surface clusters, and their fluorescence staining intensity profiles co-varied (Fig. 6A,B). Further, APC interactions with EB1 appears to be required for MACF and IQGAP1 accumulation at synaptic sites, as the APC::EB1-dn led to decreases in MACF and IQGAP1 clusters near the neuron surface (Fig. 6C,D). Mean pixel intensity levels were reduced by 31% for MACF and 40% for IQGAP1 synaptic accumulation in APC::EB1-dn neurons relative to control neuron values ($p < 5.3 \times 10^{-7}$ and $p < 2.4 \times 10^{-14}$).

Based on these findings, we looked for changes in the microtubule and submembranous F-actin cytoskeleton. We found 30% reductions in mean intensity levels for neural specific tubulin accumulation near the surface ($p < 0.003$, Fig. 7A), indicative of a change in microtubule organization in APC::EB1-dn neurons. In addition, we found 27% lower mean intensity levels for submembranous F-actin ($p < 0.002$, Fig. 7B). We suggest that APC, EB1, MACF and IQGAP1 function to regulate the local microtubule and F-actin cytoskeleton at nicotinic postsynaptic sites. By bringing these proteins together, APC coordinates their cytoskeletal regulatory roles at neuronal nicotinic synapses *in vivo*.

$\alpha 3^*$ nAChRs link to APC via 14-3-3 adapter protein

We wished to know what molecular interaction might link $\alpha 3^*$ nAChRs to the APC containing scaffold at the synapse. We tested for $\alpha 3^*$ nAChR interactions with APC. APC co-immunoprecipitated with $\alpha 3^*$ nAChRs from CG lysates (Fig. 8A). We speculated that the $\alpha 3$ subunit may mediate the binding to APC because our past studies showed that the $\alpha 3$ long cytoplasmic loop targets chimeric nAChRs to synapses in CG neurons *in vivo* (Williams et al., 1998; Williams et al., 1999). Because native $\alpha 3^*$ nAChRs are heteromers composed of $\alpha 3$, $\alpha 5$, and $\beta 4$ subunits, we tested for APC interactions with the $\alpha 3$ loop by heterologous expression

studies in MDCK cells. Because the $\alpha 3$ subunit alone is not well expressed, we used chimeric $\alpha 7$ -nAChR subunits containing the $\alpha 3$ long cytoplasmic loop (instead of the homologous $\alpha 7$ region; we refer to the chimera as $\alpha 7/\alpha 3$ loop). We found that $\alpha 7/\alpha 3$ loop co-precipitated with endogenous APC from MDCK cell lysates (Fig. 8D). However, the $\alpha 3$ loop did not bind directly to APC in co-immunoprecipitation assays employing recombinant fusion peptides (data not shown), suggesting that $\alpha 3$ interaction with APC likely requires posttranslational modification and/or an adapter protein.

Several lines of evidence, as cataloged below, suggest that 14-3-3 adapter protein is a likely candidate for linking the $\alpha 3$ subunit to APC. 14-3-3 adapter proteins are known to function as dimers that link their binding partners (Aitken et al., 2002; Yaffe, 2002). APC binds to 14-3-3 (Fig. 8B) (Jin et al., 2004; Meek et al., 2004). Sequence analysis of the $\alpha 3$ long cytoplasmic loop indicated a 14-3-3 binding consensus motif (RSSSES, amino acids 412-418 in the $\alpha 3$ loop) of the type RX_1-2SX_2-3S , where the first serine may be phosphorylated (Petosa et al., 1998). In addition, the $\alpha 3$ loop contains a coiled-coil motif with a number of negatively charged and hydrophobic residues that may provide a surface to bind the 14-3-3 amphipathic groove (Wang et al., 1998; Couve et al., 2001). We found a direct interaction between the $\alpha 3$ loop and 14-3-3 using purified fusion peptides in *in vitro* pull down assays (Fig. 8C). Further, the $\alpha 7/\alpha 3$ loop protein co-precipitated with both 14-3-3 and APC from MDCK lysates (Fig. 8D,E). Mutation of the $\alpha 3$ loop [serine-to-alanine substitution (S415A) in the 14-3-3 binding consensus motif] substantially decreased co-precipitation with 14-3-3 and APC (Fig. 8D,E). 14-3-3 also co-precipitated from CG lysates using anti- $\alpha 3$ -subunit antibodies (Fig. 8B). 14-3-3 is enriched at synapses and colocalizes with $\alpha 3^*$ nAChR surface clusters in CG neurons (Fig. 8F). Synaptic accumulations of 14-3-3 were significantly decreased in APC::EB1-dn neurons as compared to uninfected control neurons (23% lower mean intensity levels, $p < 1.3 \times 10^{-8}$, Fig. 8G). All together, these results suggest that 14-3-3 links the $\alpha 3$ subunit to APC at nicotinic postsynaptic sites.

Discussion

Our findings provide new insights into molecular mechanisms *in vivo* that direct the assembly of nicotinic postsynaptic specializations in neurons. We show that APC organizes a multi-molecular postsynaptic complex that directs $\alpha 3^*$ nAChRs localizing at synapses. We demonstrate that APC interaction with EB1 is essential for $\alpha 3^*$ nAChR surface membrane insertion, stabilization, postsynaptic clustering and normal functional levels. APC::EB1 interactions are also required for postsynaptic accumulation of EB1 and the cytoskeletal regulators MACF and IQGAP1. Synaptic localization of EB1, MACF and IQGAP1 is required, in turn, for stabilizing the submembranous F-actin and microtubule cytoskeleton at nicotinic postsynaptic sites. Further, 14-3-3 links $\alpha 3^*$ nAChRs to APC. We show that 14-3-3 directly binds, *in vitro*, to the $\alpha 3$ long cytoplasmic loop and 14-3-3 links the $\alpha 3$ loop to APC in heterologous cell expression studies. This $\alpha 3$ domain targets chimeric nAChRs to synapses in CG neurons *in vivo* (Williams et al., 1998). By bringing EB1, MACF, IQGAP1 and 14-3-3 together, APC organizes a multi-molecular complex that directs $\alpha 3^*$ nAChR surface delivery, stabilizes the local cytoskeleton, and links $\alpha 3^*$ nAChRs to the cytoskeleton at postsynaptic sites. Our work is the first to report that MACF, IQGAP1 and 14-3-3 serve as nicotinic postsynaptic components. Our findings define a new role for APC in coordinating assembly of the nicotinic receptor postsynaptic complex in neurons.

Several lines of evidence suggest that the APC::EB1-dn acts with specificity. First, a different dominant negative, APC::PSD-93-dn, expressed at similar levels to APC::EB1-dn in CG neurons, did not alter $\alpha 3^*$ nAChR surface clusters. Second, APC::EB1-dn specifically decreased $\alpha 3^*$ nAChR surface clusters, but not the neighboring clusters of $\alpha 7$ -nAChRs and GlyRs. The three different receptor types each segregate to a distinct synaptic surface

membrane region and associate with different molecular complexes. $\alpha 3^*$ nAChRs selectively co-localize with APC and its binding partners at postsynaptic sites, GlyRs selectively co-localize with gephyrin at separate postsynaptic sites, and lipid rafts direct $\alpha 7$ -nAChR clustering at perisynaptic regions of the CG neuron surface (Tsen et al., 2000; Bruses et al., 2001; Temburni et al., 2004). Similarly, $\alpha 3^*$ nAChRs and $\alpha 7$ -nAChRs show differences in detergent extractability, indicating that they each associate with different cytoskeletal elements or molecular components (Shoop et al., 2000). Although the different receptor clusters are stabilized by tethering to the cytoskeleton, the selective loss of $\alpha 3^*$ nAChR clusters in APC::EB1-dn neurons suggests that different molecular interactions regulate the local cytoskeleton in the distinct synaptic membrane regions. Similarly, studies at glutamatergic synapses indicate that the receptors are tethered to the cytoskeleton at postsynaptic sites, with more dynamic cytoskeletal organization in surrounding regions where receptor endocytosis and insertion are active (Passafaro et al., 2001; Okamoto et al., 2004; Ehlers et al., 2007; Lu et al., 2007; Tai et al., 2007). Overall, our data indicate that APC interactions organize excitatory nicotinic, but not inhibitory glycinergic, postsynaptic specializations in CG neurons.

We propose a molecular model for the role of APC in localizing $\alpha 3^*$ nAChRs to synapses based on the present findings and studies of the function of APC and its binding partners in non-neuronal cells. In our model, APC accumulates at β -catenin/N-cadherin-marked postsynaptic membrane regions (Temburni et al., 2004). EB1 tags the plus-ends of a subset of microtubules (Mimori-Kiyosue and Tsukita, 2003). APC captures EB1-tagged microtubule plus-ends and thereby positions a microtubule-based transport pathway for delivery of $\alpha 3^*$ nAChRs to the vicinity of the synapse. Microtubule peripheral ends are found in close contact with the postsynaptic density of neurons in ultrastructural studies (Milokhin, 1977). Further, in our model, APC and EB1 interact with IQGAP1 and MACF at nicotinic postsynaptic sites. MACF and IQGAP1 cross-link and stabilize the EB1-tagged microtubule and submembranous F-actin cytoskeletons and link the APC-containing postsynaptic complex to the cytoskeleton. 14-3-3 links $\alpha 3^*$ nAChRs to APC in this complex. These interactions tether $\alpha 3^*$ nAChRs to the local cytoskeleton and thereby promote the stable retention of $\alpha 3^*$ nAChRs at postsynaptic sites. We expect additional as yet unidentified molecular players to be involved in the synaptic localization of $\alpha 3^*$ nAChRs, based on studies showing that multiple binding partners target glutamate receptors to synapses (Li and Sheng, 2003).

Support for our model stems from studies of the function of APC and its interacting proteins at intercellular contact sites in non-neuronal cells. EB1 directs the microtubule-mediated delivery of connexin hemichannels to adherens junctions in epithelial cells (Shaw et al., 2007). APC directs the capture of EB1-tagged microtubules to cadherin/ β -catenin-rich complexes (Lee et al., 2000; McCartney et al., 2001; Barth et al., 2002; Hamada and Bienz, 2002; Mimori-Kiyosue and Tsukita, 2003). Thus, APC positions EB1-tagged microtubules to precise positions of the cell surface. MACF directly binds to EB1, microtubules and F-actin, and is required for tethering EB1-tagged microtubules to the submembranous F-actin network at specialized junctions (Kodama et al., 2003; Subramanian et al., 2003; Slep et al., 2005). Microtubule binding to all three proteins, APC, EB1 and MACF, promotes their stabilization and leads to more frequent and longer lasting attachments (Dikovskaya et al., 2001; Tirnauer et al., 2002; Kodama et al., 2003; Reilein and Nelson, 2005). Further, IQGAP1 plays a key role in regulating actin cytoskeleton dynamics at intercellular contact sites. IQGAP1 directly binds to actin and enhances F-actin polymerization (Noritake et al., 2004; Brown and Sacks, 2006). IQGAP1 also binds to APC and CLIP-170, an EB1 binding partner (Watanabe et al., 2004). Thus, IQGAP1 further links APC and EB1-tagged microtubules to the submembranous F-actin network. Perturbing the function of APC, EB1, MACF or IQGAP1 (by heterozygous mutation, RNAi knockdown or function-blocking antibodies) reduces the accumulation of microtubules and F-actin at intercellular contact sites (Mogensen et al., 2002; Kodama et al., 2003; Noritake et al., 2004; Reilein and Nelson, 2005; Shaw et al., 2007). Similarly, the

interactions of APC, EB1 and shot/kakapo (*Drosophila* ortholog of MACF) are essential for organizing and maintaining a unique microtubule-rich and F-actin-rich domain at the *Drosophila* muscle-tendon junction, and for restricting the localization of specific components to this site (Prokop et al., 1998; Strumpf and Volk, 1998; Subramanian et al., 2003). Accumulating evidence suggests that APC, EB1, MACF and IQGAP1 are evolutionarily conserved proteins that function in targeting proteins to and stabilizing cadherin/ β -catenin-rich intercellular junctions in skin, wings and muscle of *Drosophila* and mammals. (Strumpf and Volk, 1998; Karakesisoglou et al., 2000; Gundersen, 2002; Briggs and Sacks, 2003; Subramanian et al., 2003; Noritake et al., 2004; Shaw et al., 2007). The present study suggests a novel neural role for these proteins as key organizers of the nicotinic receptor postsynaptic complex.

Our model further proposes that 14-3-3 adapter protein links APC and the $\alpha 3$ subunit and that this interaction promotes the stable retention of $\alpha 3^*$ nAChRs at postsynaptic sites. Several lines of evidence support this concept. 14-3-3 isoforms function as homo- and heterodimers that link their binding partners (Yaffe et al., 1997; Aitken et al., 2002). 14-3-3 isoforms (sigma and zeta) bind to APC (Jin et al., 2004; Meek et al., 2004). 14-3-3 is present in postsynaptic densities isolated from rat brain (Martin et al., 1994; Couve et al., 2001; Rajan et al., 2002). Interaction with 14-3-3 promotes the surface expression of selected neurotransmitter receptors and ion channels (Jeanclos et al., 2001; Rajan et al., 2002; Exley et al., 2006). In particular, 14-3-3 binds directly to another neuronal nAChR subunit, the long cytoplasmic loop of $\alpha 4$, a major subtype in the CNS, and this interaction increases surface expression of $\alpha 4^*$ nAChRs in heterologous cell expression studies (Jeanclos et al., 2001; Exley et al., 2006). 14-3-3 binding to the $\alpha 4$ loop overrides retention signals in the endoplasmic reticulum and facilitates forward transport of the assembled receptor (O'Kelly et al., 2002). We propose a related role for 14-3-3 at the synapse: 14-3-3 binding to the $\alpha 3$ long cytoplasmic loop may mask endocytosis motifs in the $\alpha 3^*$ nAChR. Endocytosis is regulated by phosphorylation (Shikano et al., 2006). 14-3-3 binds to specific phosphorylated (pSerine/pThreonine) motifs in its protein targets and occupancy protects the site from phosphatases (Petosa et al., 1998; Tian et al., 2004). It remains to be determined whether 14-3-3 may regulate $\alpha 3^*$ nAChR surface expression by this mechanism. As support, 14-3-3 binding increases the half-life of Kir2.1 fusion proteins on the cell surface (Shikano et al., 2005). Finally, 14-3-3 linking $\alpha 3^*$ nAChRs to APC would tether the receptors to the postsynaptic cytoskeleton and provide an additional mechanism by which 14-3-3 interactions promote the stable retention of $\alpha 3^*$ nAChRs at postsynaptic sites.

Because 14-3-3 binding to its target proteins is often phosphorylation-dependent (Muslin et al., 1996; Aitken et al., 2002), the decreases in 14-3-3 synaptic accumulation in APC::EB1-dn expressing neurons may reflect changes in signaling events at the synapse. The reduced levels of functional $\alpha 3^*$ nAChRs may cause decreases in activity-dependent downstream signaling cascades. Further, a new study shows that IQGAP1 positions Erk kinase at glutamatergic synapses and is required for memory consolidation (Schrack et al., 2007). This IQGAP1 function extends the role of the APC multi-molecular complex to include tethering of signaling cascades that are critical for synaptic plasticity. Our ongoing studies may reveal additional changes in nicotinic synapse composition and function caused by APC::EB1-dn expression.

Our work has identified APC as a key coordinator of nicotinic postsynaptic assembly in neurons. APC organizes a postsynaptic complex consisting of EB1, MACF, IQGAP1 and 14-3-3. This multi-molecular APC complex has key cytoskeletal regulatory functions and is essential for $\alpha 3^*$ nAChR targeting to synapses. Further, the nicotinic synapse organizing functions that we have uncovered for APC may operate at other excitatory synapse types. APC is required for agrin-induced clustering of muscle-type nAChRs in myotubes and localizes to the neuromuscular junction (Wang et al., 2003). APC is also concentrated at neuronal glutamatergic synapses (Matsumine et al., 1996; Yanai et al., 2000) and is required, *in vitro*,

for clustering of PSD-95 that, in turn, clusters AMPA receptors (Shimomura et al., 2007). Interestingly, the APC binding partners IQGAP1 and PSD-93/-95 are also shared between nicotinic and glutamatergic synapses (this study)(Conroy et al., 2003; Parker et al., 2004; Nuriya et al., 2005; Sheng and Hoogenraad, 2007). The emerging concept is that APC is a central organizer of a core postsynaptic complex that directs excitatory synapse assembly in the vertebrate nervous system.

Experimental Methods

Antibodies

Primary antibodies used were: C-20 to APC C-terminus (Santa Cruz Biotechnology, Santa Cruz, CA); ab58 to APC N-terminus (abcam, Cambridge, MA); anti-Chapsyn-110/PSD-93 (Alomone Labs, Jerusalem, Israel); anti-EB1 (Transduction Laboratories, Lexington, KY); anti- β -catenin and anti-N-cadherin (Zymed Laboratories, San Francisco, CA); for α 3-nAChRs: mAb35 (Developmental Studies Hybridoma Bank, Iowa City, IA) which detects an extracellular epitope and mAb313 (Covance, Berkeley, CA) which detects an intracellular epitope; for α 7-nAChRs: biotinylated α -bungarotoxin (Molecular Probes-Invitrogen, Eugene, OR); mAb2b for GlyRs (Synaptic Systems, Göttingen, Germany); anti-SV2 for synaptic vesicles (Developmental Studies Hybridoma Bank); anti-14-3-3 β (K-19)(a pan-subunit 14-3-3 antibody, Santa Cruz); Alexa Fluor 568-phalloidin (Molecular Probes) to detect F-actin; neuronal specific tubulin tuj-1 (Covance); anti-IQGAP1 (gift from David Sacks, Harvard Medical School, Boston, MA, and BD Transduction Laboratories, San Diego, CA); anti-kakapo which detects macrophin (MACF) (gift of Talila Volk, Weizmann Institute of Science, Rehovot, Israel); anti-myc (Cell Signaling, Danvers, MA); anti-MBP antibody (New England Biolabs, Ipswich, MA); anti-GST (GRASP Center, Tufts University) and anti-HA (clone 3F10, Roche Diagnostics, Indianapolis, IN, and Y-11, Santa Cruz Biotechnology). Secondary reagents used were: Alexa-Fluor-488, -555 -594 conjugated secondary antibodies raised in rabbit, rat, mouse, and guinea pig (Molecular Probes), Rhodamine-conjugated avidin DCS (Vector Laboratories, Burlingame, CA) and) goat anti-rat IgG(H+L) Fab fragment (Jackson ImmunoResearch Laboratories, West Grove, PA).

Ciliary ganglion tissue preparations

White Leghorn embryonated chicken eggs, obtained from Charles River Spafas (North Franklin, CT) or University of Connecticut Poultry Farm (Storrs, CT) were maintained at 37°C in a forced air-draft humidified incubator until use. Embryos were staged according to the Hamburger and Hamilton classification scheme; the days of embryonic development refer to the stage (st) rather than the actual days of incubation (Hamburger, 1951). The developmental stages used include: embryonic day (E) 10–13 (st 36–39) and E14–18 (st 40–44).

For light microscopy experiments, CGs from APC::EB1dn-infected and age-matched uninfected control embryos were processed in parallel for frozen sectioning and immunolabeling using previously described methods (Olsen et al., 2007). For confocal microscopy, CGs from APC::EB1dn-infected and age-matched control embryos were acutely dissociated and processed for immunolabeling as previously described (Williams et al., 1998). Controls for specific binding in double-labeling studies included omitting the first or second primary antibody in separate tests; only background labeling was detected (data not shown).

Quantitative light microscopy

Immunolabeled sections were examined with a Zeiss Axioscope microscope (Zeiss, Thornwood, NY), and photographed with a SPOT color CCD camera and software (Diagnostic Instruments, Sterling Heights, MI) for Fig. 1, Fig. 2, and Fig. 5, or with a Q-Imaging Retiga

200R Fast 1394 black and white CCD camera (Surrey, British Columbia, Canada) and Nikon Instruments NIS Elements software (Melville, NY) for Fig. 3 and Fig. 8. For each primary antibody, images were acquired using identical gain and exposure settings such that pixel intensities were below saturation levels. To quantify changes in immunolabeling levels in APC::EB1-dn versus uninfected control neurons, we assessed pixel intensity profiles along ~3 μ m length segments of the brightest labeled surface regions per neuron using either Image J (<http://rsb.info.nih.gov/ij/>) or NIS elements software. For each immunolabel, we analyzed pixel intensities for two or three different line segments per neuron and 10 to 30 dominant negative expressing neurons and uninfected control neurons from 6–12 separate embryos. The pixel intensities of sampled surface segments were binned into incremental groups of 10 pixel intensity steps (eg. 0–9, 10–19 etc., up to 255, saturation) for images acquired with SPOT camera (dynamic range 0–255) or 100 pixel intensity steps for images acquired with Q-imaging camera (dynamic range 0–4095). We calculated the percentage of pixels that belonged to each pixel intensity category and plotted the relative frequency distributions. Bar graphs were generated by calculating the mean pixel intensity for each ~3 μ m line segment analyzed per protein. We calculated the standard error of the means (S.E.M.) and Student t-test values with Microsoft Excel.

Quantitative confocal microscopy

Freshly-dissociated immunolabeled neurons were examined by confocal microscopy with a Leica (Heidelberg, Germany) TCS SP2 microscope using Kr (568nm) and Ar (488nm) laser sources, and a 63 $\times$ 1.32 numerical aperture lens. Optical sections were acquired from the top to the bottom of each neuron at 0.5–1 μ m step intervals. Laser intensity and photomultiplier tube gain were kept constant across experiments. Settings were chosen such that pixel intensities fell below saturation levels. In addition, the wavelengths of light collected in each detection channel were set such that no detectable bleed-through occurred between the different channels. Pixel intensity profiles along ~3 μ m segments of labeled neuronal surface regions were assessed as described above using Leica imaging software.

Electrophysiology

Acutely dissociated E11–13 CG neurons were prepared using minor modifications of methods reported previously (Liu et al., 2006; Olsen et al., 2007). Dissociated cells were plated onto 12 mm German glass coverslips (Fisher Scientific, Pittsburg, PA) coated with 0.1 mg/ml poly-L-lysine (Sigma) and laminin (2 μ g/ml, gift of Dr. Rae Nishi, University of Vermont, Burlington, VT) and maintained at 37°C, 5% CO₂, for 2–6 hours until use. nAChR-mediated currents were recorded from single CG neurons held at –60 mV using standard tight seal, whole-cell recording with a List EPC-7 Patch Clamp Amplifier (Medical System, Greenvale, NY). Cells were continuously perfused with an external recording solution contained in mM: 150 NaCl, 3 KCl, 2 CaCl₂, 1 Mg Cl₂, 10 glucose, 10 HEPES, pH 7.4 (Zhang and Berg, 1995). Starting electrode resistances were 1–2 M Ω when filled with a solution containing in mM: 140 CsCl, 1 MgCl₂, 5 EGTA/CsOH, 5 glucose, 10 Hepes, pH 7.2. For some experiments, α -bungarotoxin (100 nM, Molecular Probes) was bath-applied focally within 100 μ m of the recorded cell in standard external solution. Nicotine (20 μ M, Sigma) was applied for 1 sec with a puffer pipette (~1 μ m pore diameter) close to the recorded cell (~20 μ m) by pressure-driven focal ejection with a Picospritzer (General Valve, Fairfield, NJ). For each cell, nicotine was applied 2–3 times at 1 min intervals to allow recovery between applications. All experiments were performed at room temperature (~23°C). Membrane currents were filtered digitally at 2 KHz and acquired at 5 KHz with a DigiData 1322A A/D interface (Axon Instruments, Foster City, CA) and a Dell computer running pCLAMP 9 software (Axon Instruments). Analyses were performed with pCLAMP 9 software and Microsoft Excel.

Receptor insertion and endocytosis assays

Acutely dissociated E10–12 CG neurons from control and APC::EB1-dn infected embryos were plated onto poly-D-lysine/laminin coated 12 mm glass coverslips (for insertion assays) or chamber slides (BD Labware, for endocytosis assays) as described above, and grown in culture for 1–2 days. The rate of $\alpha 3^*$ nAChR insertion was determined using minor modifications of previously described assays (Lu et al., 2001; Man et al., 2003). Briefly, dissociated neurons were exposed to saturating concentrations of mAb35 (1:50) followed by incubation with saturating amounts of unlabeled rat IgG(H+L) Fab fragment antibody to block existing surface $\alpha 3^*$ nAChRs. The cells were then returned to 37°C to allow for insertion. At $t=0$, 4 or 8 hr after block, the appearance of newly-inserted $\alpha 3^*$ nAChRs was assessed by incubation at 4°C with mAb35 (1:50) followed by incubation with fluorescent-conjugated secondary antibody and fixation. Controls were performed to verify that unlabeled antibodies completely saturated existing surface $\alpha 3^*$ nAChRs throughout the experiment by applying fluorescent-conjugated secondary antibody at either $t=0$ or $t=8$ hrs on living neurons prior to fixation; only background fluorescence was observed. Epifluorescence images were acquired with a Zeiss Axioscope microscope. The number of $\alpha 3^*$ nAChR clusters on the neuronal soma was determined using NIS elements software. We analyzed 25–27 neurons from 3 separate experiments. We quantified the number of clusters per neuron soma, normalized to total surface area and then calculated the rate of insertion using Microsoft Excel.

The turnover rate of surface $\alpha 3^*$ nAChRs was measured using standard cell-surface biotinylation assays of endocytosis (Mammen et al., 1997; Rasmussen et al., 2002). Briefly, dissociated cells from control and APC::EB1-dn-infected embryos were plated at equal density of ~10 000 neurons per well. Surface proteins were biotinylated with 1.0 mg/ml EZ-link sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) for 30 min at 4°C. The reaction was stopped by washes with 2 mg/ml BSA. The cells were returned to 37°C, chased for various lengths of times to allow endocytosis and lysed with (in mM): 100 NaCl, 50 Tris-HCl pH 8.0, 10 EDTA, 50 NaF, 0.1 Na₃VO₄, 10% glycerol, 0.5% Igepal CA-630 (chemically equivalent to NP-40, Sigma-Aldrich), and protease inhibitor cocktail (Roche). Biotinylated proteins were precipitated as described (Rasmussen et al., 2002). Levels of biotinylated surface $\alpha 3^*$ nAChRs (mAb313) and N-cadherin, as another nicotinic synapse surface membrane protein, were assessed by standard immunoblot techniques (Temburni et al., 2004) using ECL plus detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and exposure to X-ray film for 30 sec - 5 min. Band densities were determined using Kodak Image Station 440 (Carestream Molecular Imaging, New Haven, CT) and Kodak 1D software. Samples of total lysate from non-biotinylated sister cultures were loaded onto the same gels to generate standard calibration curves to verify that band densities of biotinylated protein were within the linear range of the film. The experiment was repeated 5 times.

Retroviral vector-mediated gene transfer

APC::EB1-dn cDNA (amino acids 1943–2184; NCB1 accession number XP_001233411; corresponding to the EB1-binding domain of chicken APC sequence) was generated and coupled to the hemagglutinin (HA) tag (YPYDVPDYA) at its N-terminus by PCR. APC::PSD93-dn cDNA [amino acids 2185–2232; corresponding to the APC C-terminus-end fragment that contains the consensus PDZ-binding motif (VTSV) at the 3' end] was similarly generated and HA-tagged. The cDNA constructs were subcloned separately into avian-specific retroviral vector RCASBP (B envelope subgroup type) (Homburger and Fekete, 1996). Viral stocks were prepared in DF1 chicken fibroblast cells (American Type Culture Collection, Manassas, VA). CGs were infected *in ovo* at 36 hrs of development (st 8–9) as previously described (Temburni et al., 2004) and sampled 1–2 weeks later.

***In vitro* binding assays**

Glutathione S-transferase (GST) and maltose binding protein (MBP) fusions of chicken APC, EB1, PSD-93, and $\alpha 3$ nAChR subunit proteins were generated by PCR amplification and cloning in frame into pGex4T-1 and pMalC2 vectors (GE Healthcare and New England Biolabs). The peptides include: chicken APC C-terminus (a.a. 1943–2232; corresponding to the EB1 and PSD93 binding domains), APC::EB1-dn, APC::PSD93-dn, full length EB1, PSD93 (a.a. 282–586; corresponding to PDZ domains 2 and 3 of chicken PSD93a) and $\alpha 3^*$ nAChR subunit long cytoplasmic loop. 14-3-3zeta in pGEX-2TK was the kind gift of Dr. Stephen Moss (University of Pennsylvania). The fusion proteins were expressed in *Escherichia coli* BL21-DE3 cells (Stratagene) and purified using GST Sepharose (Sigma) or amylose resin (New England Biolabs). The affinity-isolated recombinant fusion peptides were used to pull down their purified binding partners in Tris-Triton buffer (in mM: 10 Tris (pH 7.6), 50 NaCl, 30 sodium pyrophosphate, 50 NaF, 5 EDTA, and 0.2 Na₃VO₄, 1% TritonX-100, and protease inhibitor cocktail (Roche). In addition, binding competition assays were performed to test whether the HA-tagged dominant negative peptides prevented the binding of APC with the targeted binding partner. MBP-EB1 (2 μ g) or MBP-PSD93 (2 μ g) was incubated with or without purified APC::EB1-dn (6 μ g) or APC::PSD93-dn peptide (6 μ g) in Tris-Triton buffer for 1 hr at 4°C followed by incubation for 1 hr with GST-APC C-terminal fragment (including both EB1- and PSD93-binding domains; 2 μ g) bound to Sepharose. Bound protein complexes were eluted, and analyzed by SDS-PAGE and immunoblotting. Specific binding was established by using GST-alone (negative control) to precipitate the MBP-tagged protein, and the MBP-tag alone for binding to the recombinant GST-protein.

Co-immunoprecipitation assays

In vivo coimmunoprecipitation of native $\alpha 3^*$ nAChRs with APC and 14-3-3 was performed using CG lysates. Briefly, 50 E14–18 CGs were solubilized with Tris-Triton buffer for 1 hr on ice. Insoluble material was removed by centrifugation at 16,000 $\times$ g at 4°C for 20 min. The supernatant fraction was incubated with $\alpha 3^*$ nAChR (mAb35 or mAb313), APC or 14-3-3 antibodies (1:100). Immune complexes were affinity precipitated with protein-G or protein-A Sepharose beads (for mouse and rabbit antibodies, respectively). The bound protein complexes were eluted, separated by SDS-PAGE, and analyzed by immunoblotting (ECL plus detection system, GE Healthcare)(Temburni et al., 2004). Controls for antibody binding specificity were included.

Co-immunoprecipitation of chimeric nAChR subunits with APC and 14-3-3 was tested in heterologous expression studies in Madin-Darby canine kidney (MDCK) cells (gift of Dr. Larry Feig, Tufts University, Boston, MA). MDCK cells were maintained in DMEM medium (Invitrogen) supplemented with 10% bovine fetal calf serum and antibiotics at 37°C, 5% CO₂. Myc-tagged chimeric $\alpha 7$ -nAChR subunit containing the $\alpha 3$ long cytoplasmic loop (instead of the homologous $\alpha 7$ region; $\alpha 7/\alpha 3$ chimera, previously described in Williams et al., 1998) was generated by PCR and cloned into pcDNA3.1(+) vector (Invitrogen). Similarly, we generated a mutated version containing serine- to- alanine substitution of residue 415 of the $\alpha 3$ long cytoplasmic loop. The chimeric and mutated nAChR constructs were transiently transfected into MDCK cells using Lipfectamine 2000 (Invitrogen) according to manufacturer's instructions. Cells were lysed 48 hrs later with ice-cold Tris-Triton buffer for 30 min. The lysates were centrifuged at 16,000 $\times$ g for 20 min at 4°C. The supernatants were precleared with protein-G agarose beads (Roche) for 3 hours at 4°C and then incubated with anti-myc or anti- $\alpha 3$ -nAChR (mAb313) for 2 hr at 4°C, followed by incubation with protein-G agarose overnight. Immunoprecipitates were washed five times, boiled in Laemmli sample buffer, and analyzed by immunoblotting with the antibody directed against the other protein of the candidate interacting pair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Selective blockade of APC::EB1 interactions led to decreases in $\alpha 3$ * nAChR surface clusters on CG neurons *in vivo*

(A–C) *In vitro* binding assays demonstrate that APC::EB1-dn selectively blocks APC binding to EB1. (A) Purified GST-APC::EB1-dn peptide specifically binds to MBP-EB1 fusion protein, while GST alone does not. (A,B) Input: 15% of MBP-EB1 total input. IB: EB1 antibody. (B,C) APC::EB1-dn peptide specifically prevented the binding of EB1 (B), but not of PSD-93 (C), to GST-APC C-terminal peptide (that contains the EB1- and PSD-93- binding domains of APC) (compare lane 1 and 2 in B and C). (C) IB: MBP antibody. (D) Confocal micrographs of immunostained acutely dissociated E11–13 CG neurons show that EB1 clusters (red) are in close proximity to and partially overlap (yellow) with $\alpha 3$ * nAChR surface clusters (green). (E) Expression of APC::EB1-dn (DN), *in vivo*, causes reductions in EB1 clusters in the vicinity of the neuron surface membrane, marked by a white dashed line. Middle panel, frequency distribution graph shows reductions in the pixel intensity of EB1 labeling near the surface in APC::EB1-dn infected neurons (red boxes) versus uninfected control neurons (blue

triangles) at matched ages (see below). Dashed vertical lines indicate the median intensity values. Right panel, bar graph showing 26% lower mean intensity levels for EB1 labeling near the surface in APC::EB1-dn neurons compared to control neuron values ($*p < 9.3 \times 10^{-7}$, $n = 15$ DN neurons, 11 Ctl neurons). Bars represent the mean $\pm$ SEM. In contrast, APC::EB1-dn expression caused no significant change in labeling for other APC binding partners (Supplemental Fig.1), suggesting that the blocking peptide specifically prevented the targeted interaction. (F) Epifluorescence micrographs of double-labeled E11–13 CG frozen sections showing that $\alpha 3^*$ nAChR surface clusters (red) were decreased in neurons expressing the HA-tagged APC::EB1-dn (DN, green) as compared to a control neuron (Ctl) from an uninfected CG age-matched and processed in parallel (*upper right panel*) and an internal control neuron in the infected CG (DN-, *lower panel*). HA staining (green) shows that retroviral infection was restricted to CG neurons and occasionally a few glial cells (small HA+ cells; not seen here) that surround the neuronal somata. The dark unstained region in the infected neuron cytoplasm is the nucleus. Insets, two-fold magnification views of boxed regions. Middle and right panels, $\alpha 3^*$ nAChR surface labeling shows substantial decreases in pixel intensity levels and 31% lower mean intensity levels in APC::EB1-dn neurons compared to uninfected control neuron values ($*p < 10^{-6}$, Student's t-test, $n = 15$ DN and 15 Ctl neurons). Dashed vertical lines indicate the median intensity values. Bars represent the mean $\pm$ SEM.

For the quantitative assessments, the fluorescence pixel intensities were measured along 3 μ m length segments of the brightest labeled surface regions ($n = 2$ –3 segments per neuron, 10–30 DN and Ctl neurons, and 6–12 embryos for each immunolabeling experiment). The values were binned into incremental groups of 10 pixel intensity steps (from 0–9, 10–19, ..., up to saturation). The percentage of pixels that belonged to each pixel intensity category was calculated and the data plotted as a relative frequency distribution (E, F).

Figure 2. APC::EB1-dn did not cause reductions in neighboring surface clusters of $\alpha 7$ -nAChRs and glycine receptors

(A,D) Epifluorescence micrographs of double-labeled E11–13 CG frozen sections showing no decrease in surface clusters of $\alpha 7$ -nAChRs (A, red) and GlyRs (D, red) in neurons expressing the HA-tagged APC::EB1-dn (A,D, green) as compared to uninfected control neurons. Insets, two-fold magnification views of boxed regions. (B,C) $\alpha 7$ -nAChR surface labeling shows no significant difference in pixel intensity levels in APC::EB1-dn infected neurons versus uninfected control neurons at matched ages ($p > 0.14$, $n = 27$ DN and 21 Ctl neurons). (E,F) In contrast, GlyR surface labeling shows a shift to higher pixel intensity levels (E) and an 18% increase in the mean intensity levels (F) in APC::EB1-dn neurons relative to control neuron values (* $p < 0.02$, $n = 12$ –13 neurons). (B,E) Dashed vertical lines indicate the median intensity values. (C,F) Bars represent the mean $\pm$ SEM.

Figure 3. Expression of a different blocking peptide, APC::PSD-93-dn, does not alter $\alpha 3$ *nAChR surface clusters on CG neurons *in vivo*

(A,B) *In vitro* binding assays show that a different blocking peptide, APC::PSD-93-dn substantially decreased the binding of MBP-PSD-93, but not of MBP-EB1, to APC C-terminal recombinant peptide (compare lane 1 and 2 in A and B) IB: MBP antibody (A) and EB1 antibody (B). (C) Epifluorescence micrographs of immunostained E11–13 CG frozen sections showing no apparent change in $\alpha 3$ *nAChR surface clusters (red) on HA-tagged APC::PSD-93-dn expressing neurons (green, PSD93-DN) as compared to control uninfected neurons (Ctl) age-matched and processed in parallel. Insets, two-fold magnification views of boxed regions. (D,E) $\alpha 3$ *nAChR surface labeling shows no significant difference in pixel intensity levels in APC::PSD-93-dn infected neurons relative to uninfected control neurons ($p > 0.14$, Students *t*-test; D,E, $n = 23$ DN neurons, 30 Ctl neurons). (D) Dashed vertical lines indicate the median intensity values. (E) Bars represent the mean $\pm$ SEM.

Figure 4. APC::EB1-dn expression decreases functional $\alpha 3^*$ nAChR levels

(A) Whole-cell voltage clamp recordings of $\alpha 3^*$ nAChR-mediated currents evoked by the agonist nicotine at 20 μ M from acutely dissociated neurons of APC::EB1-dn infected CGs (gray) and uninfected control CGs (black). Nicotine application time is indicated by the horizontal bar. (B) Bar graph showing 2.6-fold decreases in $\alpha 3^*$ nAChR mean current densities in APC::EB1-dn neurons compared to uninfected control neurons (* $p < 2 \times 10^{-5}$, Student's t-test, $n = 15$ DN neurons, 21 Ctl neurons). Bars represent the mean $\pm$ SEM.

Figure 5. APC::EB1-dn expression altered rates of insertion and endocytosis of surface $\alpha 3^*$ nAChRs (A) Representative epifluorescence micrographs showing fewer newly-inserted $\alpha 3^*$ nAChRs on APC::EB1-dn (DN) neurons relative to age-matched control uninfected neurons (Ctl). Dissociated neuron cultures were first exposed to saturating amounts of anti- $\alpha 3^*$ nAChR antibody (mAb35) and unlabeled antibody Fab fragments to block existing surface $\alpha 3^*$ nAChRs, and then the appearance of newly-inserted receptor was followed over time (after 8 hours in A) using fluorescently-labeled antibody labeling (Lu et al., 2001; Man et al., 2003). (B) Graph showing four-fold decreases in the rate of $\alpha 3^*$ nAChR *de novo* insertion into the surface membrane on APC::EB1-dn infected neurons (white circles) as compared to uninfected control neurons (black circles) (n= 11 neurons for each time point). We quantified

the number of $\alpha 3^*$ nAChR surface clusters per neuron soma and normalized to total surface area. Insertion rates were calculated from the slope of the lines. (C) Representative immunoblot showing lower levels of biotinylated surface $\alpha 3^*$ nAChRs remaining at 5 and 10 hours post-biotinylation on APC::EB1-dn infected neurons as compared to uninfected control neurons in standard cell surface biotinylation assays of endocytosis. (D) Graph of quantitative immunoblot data shows 1.6-fold faster turnover rate for surface $\alpha 3^*$ nAChRs in APC::EB1-dn infected neurons (white circles) as compared to uninfected control neurons (black circles). (E) Representative immunoblot of biotinylated surface N-cadherin, another nicotinic synapse-specific surface membrane protein. In contrast to $\alpha 3^*$ nAChRs, N-cadherin turnover rates are not altered by APC::EB1-dn expression. n= 5 separate experiments.

Figure 6. APC::EB1-dn expression led to decreased accumulation of the cytoskeletal regulatory proteins MACF and IQGAP1 at the neuron surface

(A,B) Confocal micrographs of double-labeled acutely dissociated E11–13 CG neurons showing that MACF (A, red) and IQGAP1 (B, red) clusters at the surface predominantly colocalized with $\alpha 3^*$ nAChR surface clusters (green; overlap indicated by yellow). Right panels, The red and green fluorescence intensity profiles co-varied for MACF and IQGAP1 with $\alpha 3^*$ nAChR surface clusters. (C, D) Confocal micrographs show decreases in MACF (C) and IQGAP1 (D) accumulation near the surface in single dissociated APC::EB1-dn neurons (DN) as compared to control uninfected neurons (Ctl). Insets, two-fold magnification views of boxed regions. Middle and right panels, MACF (C) and IQGAP1 (D) labeling near the neuron surface show shifts to lower pixel intensity levels, as well as 31% and 40% reductions in mean intensity levels in APC::EB1-dn neurons relative to control neuron values (MACF: $*p < 5.3 \times 10^{-7}$, IQGAP1: $*p < 2.4 \times 10^{-14}$, $n = 14$ –19 neurons). Dashed vertical lines indicate the median intensity values. Bars represent the mean $\pm$ SEM.

Figure 7. APC::EB1-dn caused reductions in tubulin and F-actin accumulation near the neuron surface

(A,B) Confocal micrographs show decreases in accumulation near the surface membrane of neuronal-specific tubulin (A, red) and F-actin (B, red) in single dissociated APC::EB1-dn neurons (DN) as compared to control uninfected neurons (Ctl). The reductions in tubulin staining are indicative of a change in microtubule organization in APC::EB1-dn expressing neurons. Insets, two-fold magnification views of boxed regions. Middle and right panels: Tubulin (A) and F-actin (B) labeling near the surface show shifts to lower pixel intensity levels, as well as 30% and 27% decreases in mean intensity levels in APC::EB1-dn neurons relative to control neuron values (tubulin: $*p < 0.003$, F-actin: $*p < 0.002$, $n = 10$ –13 neurons). Dashed vertical lines indicate the median intensity values. Bars represent the mean $\pm$ SEM.

Figure 8. $\alpha 3$ * nAChRs link to APC via 14-3-3 adaptor protein

APC (A) coimmunoprecipitated with $\alpha 3$ -nAChRs and 14-3-3 (B) co-precipitated with $\alpha 3$ -nAChRs and APC from CG lysates. CG homogenates were immunoprecipitated (IP) with: (A,B) $\alpha 3$ -nAChR antibodies mAb313 (A,B) and mAb35 (B) to two different $\alpha 3$ subunit epitopes, (B) APC antibody C20, (A,B) HA antibody as a negative control or (B) pan-14-3-3 antibody as a positive control. The precipitate and 1–2% of total input were separated by SDS-PAGE and immunoblotted (IB) with: (A) APC antibody C-20 or ab58 to two different epitopes or (B) pan-14-3-3 antibody that recognized two bands (~30 and ~33 KDa), as previously reported (Peng et al., 1997); APC ~250 KDa; (B) LC, IgG light chain. (C) The $\alpha 3$ long cytoplasmic loop fusion peptide directly binds to GST-14-3-3, but not to GST alone, in *in vitro* pull down assays. (D,E) APC (D) and 14-3-3 (E) co-precipitated with myc-tagged chimeric $\alpha 7$ -nAChR subunits containing the $\alpha 3$ long cytoplasmic loop ($\alpha 3$ L), but not with myc-tagged chimeric $\alpha 7$ containing the mutated $\alpha 3$ loop (S415A mutation in the 14-3-3 binding consensus motif, $\alpha 3$ L mut) from transfected MDCK cell lysates, suggesting that 14-3-3 links

the $\alpha 3$ long cytoplasmic loop and APC. (D,E) 2% of total lysate shows similar expression levels for $\alpha 3L$ and $\alpha 3L$ mut. Endogenous APC and 14-3-3 expression levels were also similar in MDCK cells transfected with the different $\alpha 3$ -loop constructs (data not shown). $n=3$ or more separate experiments. (F) Epifluorescence micrographs of double-labeled E13 CG frozen sections showing that 14-3-3 (red) is enriched at synapses and co-localizes with $\alpha 3^*nAChR$ surface clusters (green; overlap, yellow). (G) Confocal micrographs show decreases in 14-3-3 accumulation near the surface in APC::EB1-dn neurons (DN) as compared to control uninfected neurons (Ctl). Insets, two-fold magnification views of boxed regions. Middle and right panels, 14-3-3 labeling near the neuron surface shows a shift to lower pixel intensity levels, and 23% lower mean intensity levels in APC::EB1-dn neurons relative to control neuron values ($*p<1.3\times 10^{-8}$, $n=35-42$ neurons). Dashed vertical lines indicate the median intensity values. Bars represent the mean $\pm$ SEM.