The role of the adenomatous polyposis coli and beta-catenin proteins in intellectual disabilities and autism

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Abstract

Autism spectrum disorders and intellectual disabilities are neurodevelopmental co-morbidities seen in a growing percentage of the population. Large-scale genetic screens of patients with these disorders have revealed that a large proportion of autism causing genes center on key central pathways. One such pathway is the Wnt/β-catenin pathway, with the highest confidence risk gene for sporadic autism, *CHD8*, being a regulator of Wnt target gene expression. However, direct tests are lacking for a role of β-catenin malfunction, particularly excessive β-catenin signaling, leading to these disorers.

Mutations in adenomatous polyposis coli (APC) and β-catenin, two critical molecules in the Wnt pathway, have been associated with autism and intellectual disabilities. β-catenin functions both at the synapse - in adhesion complexes with the trans-synaptic binding protein N-cadherin, and in the nucleus - as a transcription factor regulating the expression of Wnt target genes. APC is the major negative regulator of β-catenin, forming a destruction complex with Axin and GSK3β that is responsible for targeting β-catenin to the proteasome. APC also is a major regulator of cytoskeletal dynamics by binding microtubules and guiding polarity, and an mRNA binding protein that targets mRNAs critical for neuronal development and function.

This dissertation focuses on how mutations in APC and β-catenin may contribute to the pathophysiology of autism and intellectual disabilities. We did this using three new mutant mouse models: the APC cKO, β-cat cOE, and APC/β-cat cKO mice (cKO: conditional knockout, cOE: conditional overexpression). APC cKO mice display mild cognitive impairments and autistic-like phenotypes (reduced sociability and increased repetitive behaviors). APC cKO mice show increased in β-catenin levels, increased Wnt

target gene expression, modestly enhanced LTP, increased spine density, and increased synaptic association of β-catenin with N-cadherin.

Working on the hypothesis that the observed increase in β-catenin was a major driving force for the cognitive and behavioral phenotypes of the APC cKO, we examined if overexpression of β-catenin was sufficient to replicate the phenotypes. β-cat cOE mice show severe cognitive impairments relative to the APC cKO, with dramatically reduced plasticity (LTP and LTD) and reductions in the membrane levels of the glutamate receptor subunits GluR1 and NR2A/B. We show reductions in protein levels of an APC mRNA target, SynCAM1that plays a role in GluR1 insertion. Interestingly, we see a gel-mobility shift in APC suggesting a post-translational modification of APC that is unique to the β-cat cOE.

As β-cat cOE mice also display the autistic-like phenotypes we observe in the APC cKO model, we tested if preventing the increase in β-catenin in the APC cKO, by conditionally removing the *ctnnb1* gene product as well, would ameliorate the behavioral deficits. APC/β-cat cKO mice demonstrate normal social and repetitive behaviors, suggesting a role for increased β-catenin in the autistic like phenotypes we observed in the other models. Interestingly, these mice display cognitive deficits that are comparable to the APC cKO and an increase in Wnt target gene expression that may be mediated through an increase in the β-catenin family member, γ-catenin.

This dissertation provides new insights into the roles of APC and β-catenin in behaviors associated with autism and intellectual disabilities. We demonstrate that increased β-catenin may contribute to the autism phenotype in mice and that APC may play new and important functions in regulating plasticity. These new models will serve

as important genetic tools for understanding the pathophysiology underlying ASD and ID.

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Common Abbreviations

AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC:	adenomatous polyposis coli
ASD:	autism spectrum disorder
β-cat:	β-catenin
CA1:	cornus ammonis region 1 of the hippocampus
CA3:	cornus ammonis region 3 of the hippocampus
CamKIIα:	calcium/calmodulin kinase 2 alpha
Chd8:	chromodomain helicase DNA binding protein 8
cKO:	conditional knockout
CNS:	central nervous system
CNV:	copy number variant
cOE:	conditional overexpresser
Cre:	cre-recombinase
Ctl:	Control
DHPG:	(S)-3,5-Dihydroxyphenylglycine
EB1:	end-binding protein 1
EPSC:	excitatory post-synaptic current
FAP:	familial adenomatous polyposis
fl(ex3):	floxed exon 3
fl:	Floxed
FMRP:	fragile X mental retardation protein
GABA:	gamma-aminobutyric acid

GFP:	green fluorescent protein
GluR1:	glutamate receptor 1
GluR2:	glutamate receptor 2
GSK3β:	glycogen synthase kinase 3 beta
ID:	intellectual disability
kDa:	Kilodalton
LTD:	long term depression
LTP:	long term potentiation
mEPSC:	miniature excitatory post-synaptic current
mGluR:	metabotropic glutamate receptor
mRNA:	messenger ribonucleic acid
NMDA:	N-methyl-D-aspartate
NR2A:	glutamate [NMDA] receptor subunit epsilon-1
NR2B:	glutamate [NMDA] receptor subunit epsilon-2
one-way ANOVA:	one-way analysis of variance
PDZ:	(P)ost-synaptic density/(D)iscs large/(Z)onula occludens domain
PP2A:	protein phosphatase 2 alpha
PS1:	presenilin 1
PSD95:	post-synaptic density protein 95
RM ANOVA:	repeated measures analysis of variance
RNA:	ribonucleic acid
SAMP:	Ser-Ala-Met-Pro motif

SNP: single nucleotide polymorphism

SynCAM1: synaptic cell adhesion molecule 1

Wnt: wingless-related integration site

Chapter 1:

Introduction

1.1 Autism and Intellectual Disabilities

1.1.1 An Overview of Autism and Intellectual Disability

Autism was first characterized by Leo Kanner in 1943 (Kanner 1943) who described a group of children exhibiting developmental deficits in social behaviors. Today, the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) defines autism by two main diagnostic criteria: 1) deficits in social communication and interactions, and 2) repetitive behaviors or interests (American Psychiatric Association 2013). Based on 2012 data from the Center for Disease Control (CDC), approximately 1 in 68 children will be diagnosed with autism spectrum disorders (ASDs), with boys being four and half times more likely to be identified as girls (Christensen et al. 2016). This rate has increased in recent years although it is unknown if this is due to a higher occurrence rate or better diagnostic criteria. Despite this high prevalence, treatments for autism are lacking.

One striking feature of ASDs is the relatively high prevalence of comorbidities including, intellectual disability (ID), epilepsy, and gastrointestinal disorders. ID is the most commonly reported co-morbidity in ASD, with the CDC listing the co-occurrence rate around 31% (Developmental Disabilities Monitoring Network Surveillance Year Principal, Centers for Disease, and Prevention 2014), although other studies suggest comorbid ID could be as high as 70% (Dawson et al. 2008). Fragile X syndrome is the most common inherited form of both ID and autism (Krueger and Bear 2011) occurring in about 1 in 5000 individuals. Epilepsy is comorbid with an estimated 30% cases of ASD (Clarke et al. 2005) and ID is a characterized as a risk factor for seizures in

individuals with autism (Canitano 2007), suggesting that ID and epilepsy comorbid with autism may share common causes.

Relevant to this dissertation, gastrointestinal dysfunctions are also a common in ASD, with issues being reported in up to 70% of cases (Valicenti-McDermott et al. 2006). Recent studies have indicated potential roles for increased peripheral cytokine signaling (Ferguson et al. 2016), changes in the gut microbiome (Kraneveld et al. 2016), and alterations in both brain and gut epithelial adhesion (with the brain epithelium comprising the blood-brain barrier) (Fiorentino et al. 2016). However it is not possible to rule out a genetic contribution to this highly prevalent comorbidity.

Mutations in the *APC* gene that lead to familial adenomatous polyposis (FAP) - a progressive development of intestinal polyps and eventually colon cancer - are also associated with autism and ID (Heald et al. 2007; Barber et al. 1994; Zhou et al. 2007). The *APC* gene product - the adenomatous polyposis coli protein (APC) - is discussed in more detail below. ASD is also comorbid with obsessive-compulsive disorders, sleep issues, attention deficit hyperactivity disorders, and anxiety (Jacob, Landeros-Weisenberger, and Leckman 2009; Richdale and Schreck 2009; Rommelse et al. 2011; White et al. 2009). Because of this vast array of comorbidities, it is likely that there are a variety of causes underpinning the core behavioral symptoms of ASD.

It is this lack of a distinct cause that makes defining a clinically relevant treatment for ASD a difficulty. Autism is known to have a strong genetic component with reports of monozygotic twins having a concordance rate as high as 90% (Rosenberg et al. 2009; Bailey et al. 1995; Steffenburg et al. 1989). Although these studies highlighted the highly heritable nature of ASDs, it was not until 2003 that Jamain et al. reported that

mutations in the genes encoding for the synaptic adhesion molecules neuroligin-3 and neuroligin-4 were associated with autism that a true genetic link was established. To date, the Simons Foundation Autism Research Initiative has identified 423 autism associated human genes (SFARI). This broad array of genes likely accounts for the wide range in severities and underlying comorbidities seen in individuals with ASD (State 2010) and explains the rationale that no single gene mutation is thought to arise in more than 1-2% of autism cases (Abrahams and Geschwind 2008).

1.1.2 Synaptic deficiencies as the root cause of Autism and Intellectual Disability

Recent studies have shown that autism and ID associated mutations converge on a number of common biological pathways associated with synapse development, synaptic homeostasis, and activity-regulated protein synthesis (Flavell et al. 2008; Gilman et al. 2011; Kelleher and Bear 2008; Sakai et al. 2011; Toro et al. 2010; Sugathan et al. 2014; O'Roak, Vives, Girirajan, et al. 2012; Krumm et al. 2014). Analysis of rare, de novo CNVs in autism revealed that many affected genes are associated with synapse development (Gilman et al., 2011). Sakai et al. (2011) using yeast two-hybrid screening sought to identify the protein interaction network affected by mutations in 35 known autism genes. Remarkably, they were able to demonstrate that these mutations converged on two important synaptic players, Tsc1 and Shank3. Tsc1 is a negative regulator of protein synthesis that acts through inhibition of mTOR (Orlova and Crino 2010). Mutations in either Tsc1 or Tsc2 are the cause of tuberous sclerosis, a disorder often associated with autism and ID (Baker, Piven, and Sato 1998). Shank3 is a postsynaptic scaffolding protein, mutations in which are associated with a non-syndromic form of

autism (Durand et al. 2007; Moessner et al. 2007). Mouse knockout models of Shank3 display repetitive grooming and decreased social interaction – behaviors that are thought to model human ASD – as well as synaptic deficits (Peca et al. 2011).

Another theory that encompasses the synaptic dysfunction seen in autism and ID is that the behavioral deficits are mediated though improper neuronal homeostasis that affects the inhibitory/excitatory balance. This is most directly supported by the evidence that mutations in the primary inhibitory and excitatory receptors (GABA and NMDA receptors) are implicated in autism (Barnby et al. 2005; O'Roak et al. 2011). Analyzing SNPs from 470 families with autism, Ma et. Al (2005) concluded that the GABRA4 receptor was involved in the etiology of autism and may increase autism risk through interactions with GABRB1 (Ma et al. 2005).

The findings that mutations in the genes encoding neuroligins and neurexins are associated with ASD (Feng et al. 2006; Glessner et al. 2009; Jamain et al. 2003; Szatmari et al. 2007) supports the theory that inhibitory/excitatory imbalance may be a root cause of the deficits. The neurexin and neuroligin proteins are extremely varied in their expression pattern at inhibitory versus excitatory synapses, and in their adhesion pairing with each other (Chih, Engelman, and Scheiffele 2005; Craig and Kang 2007). Combined with the existence of a number of splice variants for these genes (Boucard et al. 2005), it is conceivable that mutations in one gene could have a broad effect on the overall homeostatic balance. Supporting this, a mouse model mimicking a point mutation seen in humans with ASD (Neuroligin-3 R451C), shows impaired social interactions concurrent with an increase in inhibitory neuronal transmission, with no change in excitatory signaling (Jamain et al. 2003; Tabuchi et al. 2007).

As additional support that homeostatic balance may be affected in autism, the transcription factor MEF2, necessary for the elimination of spines during development (Flavell et al. 2006), and its target genes have been shown to be mutated in autism and epilepsy (Flavell et al. 2008). This important finding highlights the fact that mutations in genes governing broad neuronal process – such as spine elimination in synaptic development - may be the root cause of ASD and its associated comorbidities.

Another theory for autism and intellectual disabilities involving synaptic homeostatic balance is that aberrant protein synthesis, necessary for synaptic plasticity, can cause phenotypes related to the disorder (Bear, Huber, and Warren 2004; Kelleher and Bear 2008). This theory is largely the result of work done studying the Fragile X Mental Retardation protein (FMRP) – an RNA binding protein, mutations in which are the leading form of inherited ID and ASD (Hatton et al. 2006). Activity-dependent protein synthesis is necessary for synaptic plasticity – the neuronal correlate to learning and memory (Huber, Kayser, and Bear 2000). The basis of this theory is that ongoing abnormal synaptic plasticity results in impaired cognition and behaviors seen in autism. FMRP is thought to act as a brake on local translation (Darnell et al. 2011) and studies have shown that metabotropic glutamate receptor long-term depression (mGluR LTD) related protein synthesis is enhanced in FMRP knockout mice (Huber et al. 2002).

This brief synopsis of synaptic dysfunctions in autism and intellectual disabilities is supported by studies identifying many individual genes in ASD/ID patients. Some of these genes, involved in synaptic development, maturation and function, are part of broader syndromes exhibiting autistic phenotypes and some are non-syndromic. Overall, though, the hope is that mouse lines modeling the genetic causes of ASD and ID will

elucidate the pathology of the disorder and provide a means to test potential therapeutic interventions.

1.1.3 The Wnt signaling pathway in ASD and ID

One pathway that plays a key role in the neuronal functions dysregulated in ASD and ID is the Wnt signaling pathway. Multiple human gene mutations linked to these disorders are predicted to cause malfunction of the Wnt/β-catenin network. These risk genes (Table 1) highlight the importance of maintaining proper β-catenin levels, as mutations that cause either increased or decreased levels of β-catenin can lead to ID and ASD.

Gene	Mutation	ASD/ID phenotype	β-cat/Wnt phenotype
Ctnnb1	Heterozygous point mutation, "batface"	ID; ASD-like increased perseverative behaviors, reduced vocalization	**LOF** adhes; **GOF** Wnt (in mouse studies)[1]
	Haploinsufficiency	ID, ASD, stereotypies, absent or limited speech, postnatal microcephaly	**LOF** adhes; **LOF** Wnt[2]
Chd8	*De novo* nactivating mutations	Sporadic ASD; overlapping ID, Macrocephaly	**GOF** Wnt, **LOF** Wnt[3]
APC	Heterozygous deletions, polymorphisms	ID, ASD, seizures	**GOF** adhes & Wnt (in mouse studies)[4]
Wnt1	Missense variant	ASD	**GOF** Wnt[5]
Ank3	Homo- and heterozygous disruptions	ASD, ID	**GOF** Wnt (in mouse studies)[6]
Tcf3	*De novo* mutation	ASD	**LOF** Wnt[7]
ctnna3	Heterozygous deletion	ASD, moderate ID	**LOF** adhesion and actin binding[8]
Tcf7l2	*De novo* mutation	ASD	**LOF**Wnt[9]
Wnt7a	Gene-disruptive CNVs	ASD	**LOF** Wnt[10]
Syne1	Homozygous missense; *de novo* SNV	ASD, ID	**LOF** Wnt[11]

Table 1. Human ASD/ID genes predicted to cause gain/loss of function in the β-catenin/Wnt network. Table of human genetic mutations found in autism and intellectual disabilities that both positively **(GOF)** and negatively **(LOF)** affect β-catenin function in adhesion and/or Wnt gene

transcription. (References cited for each gene are indicated as a superscript for each gene and are listed separately below). 1 - (Tucci et al. 2014); 2 - (Hormozdiari et al. 2015; O'Roak, Vives, Fu, et al. 2012; O'Roak, Vives, Girirajan, et al. 2012; de Ligt et al. 2012; Dubruc et al. 2014; Kuechler et al. 2015); 3 - (Hormozdiari et al. 2015; De Rubeis et al. 2014; O'Roak, Vives, Fu, et al. 2012; Neale et al. 2012; Talkowski et al. 2012; Durak et al. 2016); 4 – (Barber et al. 1994; Finch et al. 2005; Hockey et al. 1989; Heald et al. 2007; Zhou et al. 2007); 5 - (Martin et al. 2013); 6 - (Talkowski et al. 2012; Bi et al. 2012; Iqbal et al. 2013; Shi et al. 2013; Durak et al. 2014); 7 - (Hormozdiari et al. 2015); 8 - (Bacchelli et al. 2014); 9 - (Iossifov et al. 2014); 10 - (Turner et al. 2016); 11 - (O'Roak et al. 2011; Yu et al. 2013; Neumann et al. 2010; Tulgren et al. 2014).

As an example of a mutation that decreases β-catenin/Wnt function, *ctnnb1* (the gene encoding β-catenin) haploinsufficiency leads to ASD with overlapping ID and reductions in β-catenin levels that lead to reduced Wnt target gene expression and reduced association of β-catenin with N-cadherin synaptic adhesion complex. (Tucci et al. 2014; O'Roak, Vives, Fu, et al. 2012; O'Roak, Vives, Girirajan, et al. 2012; de Ligt et al. 2012; Dubruc et al. 2014; Kuechler et al. 2015). De novo inactivating mutations in *tcf7l2* (*TCF4*), a transcription factor that interacts with β-catenin to mediate Wnt target gene expression, has also been linked to autism (Iossifov et al. 2014).

Mutations that cause gain of function for this pathway have also been associated with ASD and ID. Human genetic data suggest that a monoallelic point mutation (Thr653Lys) in *ctnnb1* that leads to intellectual disabilities, displays excessive β-catenin mediated Wnt pathway activation (Tucci et al. 2014). Similarly, autism links to a rare missense mutation in *wnt1* that is associated with increased canonical Wnt pathway activation (Levy et al. 2011; Chow et al. 2012; Martin et al. 2013).

Of particular importance to this dissertation, patients harboring heterozygous gene deletions of APC, the major negative regulator of β-catenin, display ID, ranging from

severe to mild (Hodgson et al. 1993; Barber et al. 1994; Finch et al. 2005; Raedle et al. 2001; Herrera et al. 1986; Hockey et al. 1989; Kobayashi et al. 1991; Cross et al. 1992; Lindgren et al. 1992; Heald et al. 2007; Casper et al. 2014). APC mutations have also been associated with ASD (Barber et al. 1994; Zhou et al. 2007), although the connection is less prevalent than the link to ID.

Sporadic ASD may also link to Wnt pathway dysregulation, as suggested by de novo inactivating mutations in *chd8*, the highest confidence risk gene for sporadic ASD (O'Roak, Vives, Girirajan, et al. 2012; Thompson et al. 2008; O'Roak et al. 2011; Sanders et al. 2011; Iossifov et al. 2012; Neale et al. 2012; Talkowski et al. 2012; Durak et al. 2016). Although early reports of Chd8 function in non-neuronal cells suggested that it is a negative regulator of β-catenin-mediated Wnt target gene expression (Thompson et al. 2008; Nishiyama, Skoultchi, and Nakayama 2012), Durak et al. (2016) demonstrated that in neurons, phenotypes that occur due to loss of Chd8 can be rescued by increasing expression of β-catenin (Durak et al. 2016).

1.2 Adenomatous polyposis coli

Familial adenomatous polyposis (FAP) was first thought to be identified by H. Luschka in 1861, who described a colon containing thousands of polyps (Luschka 1861). The hereditary nature of this disease came into focus with a second case report in 1882 by Cripps who identified polyposis within two members of the same family (Cripps 1882). Although his findings made allusions to the familial nature of the disease, it wasn't until 1925 when Lockhart-Mummery was able to demonstrate true Mendelian inheritance in the propagation of FAP (Lockhart-Mummery 1925). These results were further refined

by Gardner in 1951, stating that familial polyposis was characteristic of a dominant gene mutation that affected both sexes equally (Gardner 1951).

It wasn't until 1987, however, that the genetic locus for FAP was discovered in three separate studies by Solomon, Leppert and Bodmer (Solomon et al. 1987; Leppert et al. 1987; Bodmer et al. 1987). They were able to show that the long arm of chromosome 5 contained allelic losses that strongly correlated to the presence of the disease. It was not until 1991 that the *APC* gene was identified as being the site of mutations associated with FAP (Kinzler et al. 1991; Nishisho et al. 1991; Spirio et al. 1992). FAP patients predominantly develop colon cancer by their early 40's (Fearnhead, Britton, and Bodmer 2001). To date, over 700 *APC* mutations have been described; the majority of which occur in a central portion of the gene termed the "mutation cluster" that corresponds to the β-catenin binding domain. Mutations in *APC* have also been identified in mice as the cause of multiple intestinal neoplasia, or Min mice (Su et al. 1992). Transgenic mouse models have revealed that APC is critical for early development with loss of APC resulting in embryonic lethality (Moser et al. 1995; Oshima et al. 1995; Ishikawa et al. 2003). APC is ubiquitously expressed throughout the body, including the central nervous system (CNS), where it shows particularly high expression in neurons (Bhat et al. 1994; Senda et al. 1998; Brakeman et al. 1999).

1.2.1 APC Interacting Domains

APC is a large (2843 amino acids), ubiquitously expressed protein that has multiple interaction domains and binding partners (Hanson and Miller 2005)(Figure 1.1). The N-terminus oligomerization domain allows APC to form homodimers which may be

necessary to form an effective β-catenin destruction complex (see below), and although mutations in this region are relatively rare, it has been suggested that dimerization of a mutated, truncated APC protein to a wild-type APC may act in a dominant negative fashion, sequestering the wild-type APC from normal function (Fearnhead, Britton, and Bodmer 2001).

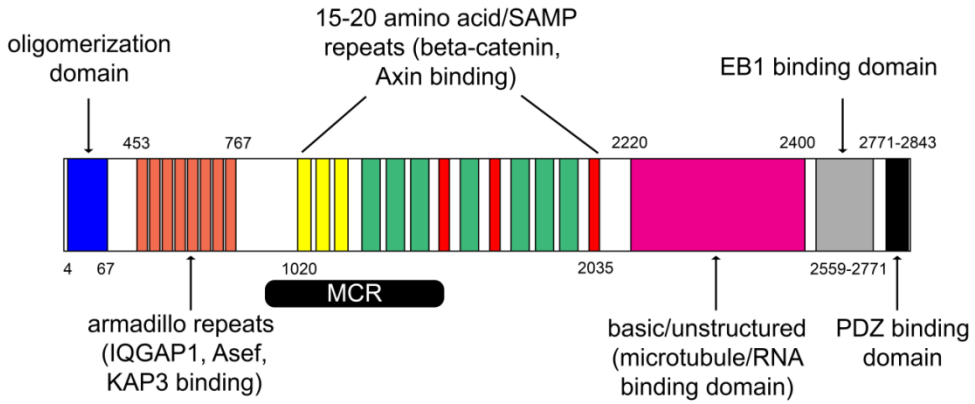

Figure 1.1 - APC structure. APC is a large 312kDa protein containing several functional domains. The N-terminal oligomerization domain forms homodimers, and the armadillo repeats bind a variety of proteins known to regulate many cellular processes. The central 15-20aa and SAMP repeats underlie APC's function in canonical Wnt signaling as an essential component of the β-catenin destruction complex. It is the site of most colorectal cancer causing APC mutations. The C-terminal contains an unstructured basic domain and a microtubule associated plus end-binding protein (EB1) interaction domain that allow APC to regulate cytoskeletal dynamics. The C-terminal end contains a PDZ binding domain that allows association with PDZ containing scaffold proteins. MCR= Mutation Cluster Region.

The seven armadillo repeat domains mediate interactions with a variety of proteins. The IQ Motif Containing GTPase Activating Protein 1 (IQGAP1) regulates the local actin cytoskeleton and recruits key signaling molecules to neuronal synapses (Watanabe et al. 2004). The regulatory B56 subunit of protein phosphatase 2A (PP2A) regulates the activation/ phosphorylation state of multiple targets, such as Glycogen synthase kinase 3

β (GSK3β), and β-catenin, regulating gene expression and FMRP regulated local protein synthesis at active synapses (Yu et al. 2015; Vinyoles et al. 2016; Narayanan et al. 2007; Seeling et al. 1999). APC also binds, the kinesin associated protein 3 (KAP3), which interacts with the kinesin 3A-3B microtubule plus-end directed motor proteins. This complex is required for APC transport along microtubules and clustering at their plus ends in membrane protrusions (Jimbo et al. 2002). Another protein, APC-Stimulated Guanine Nucleotide Exchange Factor 1 (Asef1), is a guanine nucleotide exchange factor (GEF) for the Rac and Rho GTP binding proteins that regulates the actin cytoskeletal network, cell morphology and cell migration (Kawasaki et al. 2000; Kawasaki, Sato, and Akiyama 2003).

The central portion, consisting of three repeats of 15 amino acids, seven repeats of 20 amino acids and three SAMP binding motifs, mediates the interaction of both β-catenin and Axin to form the core of the "β-catenin destruction complex" (Spink, Polakis, and Weis 2000; Polakis 1997). Mutations that lead to colorectal cancers occur most commonly in this region (Miyoshi et al. 1992). APC association with β-catenin and Axin is necessary for directing GSK-3β mediated phosphorylation of β-catenin, and its subsequent ubiquitination and proteosomal degradation. Disruptions in APC/β-catenin association result in β-catenin accumulation within the cytoplasm, translocation to the nucleus, and subsequent activation of canonical Wnt responsive gene expression (Polakis 1995).

The C-terminal region of APC contains an unstructured basic domain, a binding domain for the microtubule associated plus end-binding protein (EB1), and a PDZ binding motif. The large basic domain facilitates APC's interaction with the microtubule

cytoskeleton (Zumbrunn et al. 2001) and, similar to other RNA binding proteins, this "unstructured" region also binds select mRNAs (Preitner et al. 2014). The EB1 binding domain (Barth, Siemers, and Nelson 2002) functions to capture EB1-tagged microtubule plus-ends at specific cell surface sites and thereby regulates the plane of cell division, cell polarity, migration and the trafficking of selected cargo.

The PDZ binding domain mediates the interactions of APC with PDZ domain containing proteins such as Discs large 1 (Dlg1) (Etienne-Manneville and Hall 2003; Zhang et al. 2011), post-synaptic density 95 (PSD95) (Yanai et al. 2000) and PSD-93 (Rosenberg et al. 2008). Interactions with these scaffolding proteins near the plasma membrane are required for normal migration and polarity (through both localization and regulation of APC's cytoskeletal functions) of epithelial cells (Takamori, Shimomura, and Senda 2006) and for the maturation and organization of synaptic specializations in peripheral and central nerve cells (Temburni et al. 2004; Rosenberg et al. 2010; Rosenberg et al. 2008). APC also contains nuclear import and export signals affecting its localization and functions within the cell (Neufeld, Nix, et al. 2000; McCartney and Nathke 2008).

1.2.2 APC Functions

1.2.2.1 *Wnt signaling*

APC is best known as a tumor suppressor and negative regulator of the canonical Wnt signaling pathway. In the absence of the soluble Wnt ligand (Figure 1.2), APC and Axin recruit β-catenin into a complex with GSK3β (Gordon and Nusse 2006), promoting a series of phosphorylation events on β-catenin that increase its affinity for binding to

APC and mark it for ubiquitination by the E3 ligase β-TRCP and subsequent degradation by the 26S proteasome (Aberle et al. 1997).

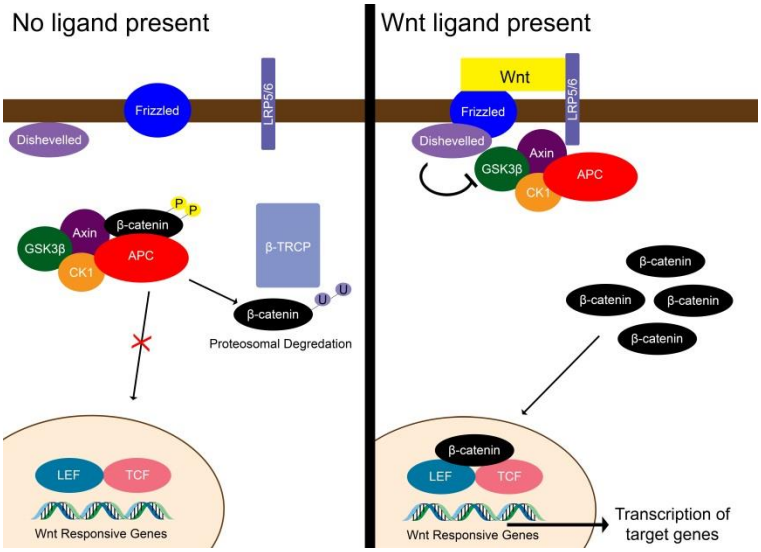

Figure 1.2 - APC's role in the canonical Wnt signaling pathway. APC serves as a core regulator of β-catenin levels and the Wnt signaling pathway. In the absence of intercellular Wnt signaling, APC participates in a multi-molecular degradation complex responsible for phosphorylation of β-catenin and its ultimate proteosomal degradation. Upon soluble Wnt ligand binding to its receptor, GSK3-β is inhibited and de-phosphorylated β-catenin disassociates from APC, accumulates in the cytoplasm, translocates to the nucleus and mediates Wnt target gene expression.

Upon binding of Wnt ligand to the Frizzled receptor and the LRP 5/6 co-receptor (Pinson et al. 2000), Axin is recruited to the plasma membrane and Dishevelled is activated to inhibit GSK3β activity. Inhibition of GSK3β allows de-phosphorylated β-catenin to disassociate from APC. The stable β-catenin accumulates in the cytoplasm (Habas and Dawid 2005), then translocates to the nucleus and activates Wnt target gene expression (Behrens et al. 1996; van de Wetering et al. 1997) via binding to the transcriptional co-factors TCF and LEF (list of Wnt target genes, http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes).

Wnt targets include many genes known to be important for the progression of colon cancer, including *c-myc* (He et al. 1998) and *Cyclin D1* (Tetsu and McCormick 1999). Wnt signaling also seems to regulate a number of genes involved in the Wnt pathway itself, indicating the presence of feedback mechanism to control expression. However, the expression of these genes has been demonstrated to be highly dependent on developmental stage and tissue/cell types so there is no one, definitive Wnt target gene although *Axin2* may be the most commonly expressed (Jho et al. 2002; Clevers 2006).

Wnt signaling plays a role in many aspects of neuronal development. It is essential for axon outgrowth(Salinas and Zou 2008), synapse assemble (Farias et al. 2007; Ahmad-Annuar et al. 2006; Sahores, Gibb, and Salinas 2010; Hall, Lucas, and Salinas 2000; Shimomura et al. 2007), neuronal polarity (Shi et al. 2004), synaptic plasticity (Chen, Park, and Tang 2006; Ma et al. 2011), neurogenesis (Seib et al. 2013), and dorsal-ventral patterning of the brain (Danesin et al. 2009; Parr et al. 1993). Mutations in genes associated with the Wnt pathway have been implicated in many neurological disorders (De Ferrari and Moon 2006) including Alzheimer's Disease and autism.

1.2.2.2 *Cytoskeleton*

APC has been shown to interact, either directly or indirectly, with all members of the cytoskeleton family: microtubules, actin, and intermediate filaments. The unstructured, basic domain of APC interacts directly with microtubules (MTs) and promotes their polymerization and stabilization (Munemitsu et al. 1994; Zumbrunn et al. 2001). Due to it interactions with the plus end-binding protein, EB1, APC localizes to

the plus end of MTs and is located at filipodial-like protrusions in migrating cells (Figure 1.3) (Nathke et al. 1996; Mimori-Kiyosue et al. 2007).

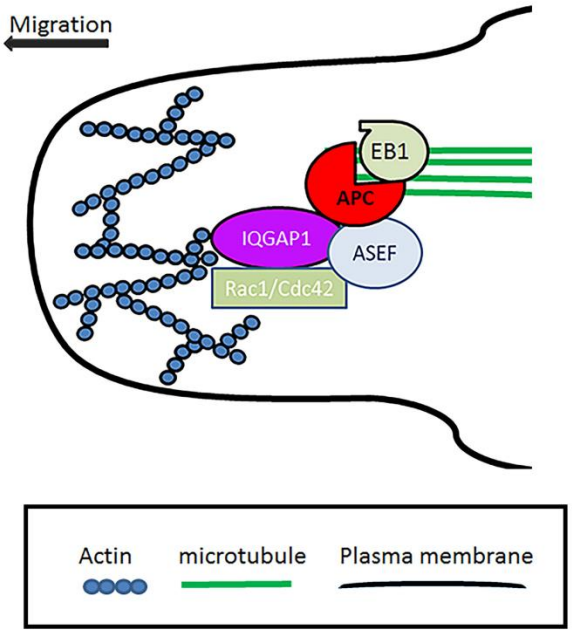

Figure 1.3 APC's role in migration relies on interactions with the MT and actin cytoskeletons. APC binds to microtubules and other proteins that regulate cytoskeletal dynamics at the leading edge of migrating cells. Through its interactions with EB1, APC is localized to the plus-end of MTs in filipodial protrusions. APC helps to regulate actin dynamics that promote migration through its interactions with IQGAP1 and Asef.

Deletion of APC in fibroblasts leads to a loss of cellular migration, reduced protrusions, and decreased MT stability (Kroboth et al. 2007). These migratory functions seem to be dependent on the interaction of APC and EB1, as a dominant negative peptide against EB1 inhibits migration in a cell wound-healing assay (Wen et al. 2004). APC also serves to direct EB1-tagged MTs to adhesion sites rich in β-catenin and the cell adhesion molecule, N-cadherin, and allow for transport of selected cargo to these sites along the captured MTs (Temburni et al. 2004; Gu et al. 2006). APC's ability to bind to and stabilize MTs is inhibited by its phosphorylation by GSK3β, suggesting that Wnt

signaling can influence cytoskeletal dynamics via modulation of APC (Zumbrunn et al. 2001). As this same region binds mRNAs (discussed below), phosphorylation events that affect the basic domain may also affect proper localization and translation of APC mRNA targets, although this regulatory effect is unknown.

APC also serves a hub for actin regulation through interactions with the cytoskeletal effector molecules, IQ motif containing GTPase activating protein 1 (IQGAP1) (Watanabe et al. 2004) and Asef (Kawasaki et al. 2000). IQGAP1 is a scaffolding protein that interacts directly with actin, and also interacts with MTs via the plus end binding-protein CLIP-170 (Fukata et al. 2002; Briggs and Sacks 2003). APC and IQGAP1 interact directly and they are reciprocally dependent on each other for their localization to the leading edge of migrating cells. Loss of either one of these proteins results in a decreased actin framework and a loss of plus-end directed microtubules at the leading edge that inhibits migration (Watanabe et al. 2004).

In neurons, IQGAP1 is likely a key regulator of actin dynamics in synaptic spines, a process thought to underlie activity-dependent changes in spine morphology during plasticity. Loss of IQGAP1 results in a decreased synaptic spine density in neurons (Gao et al. 2011). APC's ability to interact with both EB1 and IQGAP1, suggests a role for APC in neuronal polarization and migration. Studies deleting APC in neuronal precursor cells using Nestin-Cre, resulted in a loss of radial glia polarity and the improper migration of neurons (Yokota et al. 2009).

Asef is a Rac-specific guanine nucleotide exchange factor (GEF) that acts as a link between G protein-coupled receptors and the cytoskeleton to regulate activity-dependent actin dynamics (Kawasaki et al. 2000). Asef promotes actin remodeling, cell

migration, and alters cadherin-containing adhesion complexes in an APC-dependent manner (Kawasaki, Sato, and Akiyama 2003). In combination with IQGAP1 and EB1, APC can likely regulate cell migration through both the MT and actin cytoskeleton.

Much less is known about how APC interacts with intermediate filaments (IF), but Sakamoto et al. (2013) was able to demonstrate that APC interacts with the IF vimentin via its armadillo repeats. APC is responsible for localizing the IF vimentin to microtubules, and promoting IF polymerization and elongation. Truncations of APC correlate with disorganization of IFs and perturb the migration of astrocytes (Sakamoto, Boeda, and Etienne-Manneville 2013).

All together, these studies identify APC as a crucial regulator and integrator of the cytoskeletal architecture involving microtubules, actin and intermediate filaments and as a critical determinant of cellular migration, and polarization, functions that are critical in neuronal functions which will be outlined more in detail below.

1.2.3 Neuronal Specific Functions of APC

Although many of the neuronal functions of APC were touched upon above, I will now give a more thorough examination of APC functions that are more specific to neuronal development and function.

1.2.3.1 *Neurite/axon outgrowth.*

During neuronal polarization, the polarity protein mPar3 is known to accumulate at the tip of the nascent axon (Shi, Jan, and Jan 2003). It is suggested that APC interacts with mPar3, and directs it to the axon tip (Figure 1.4). This is supported by the fact that

either overexpression of APC or expression of a dominant negative APC prevents mPar3 localization and normal polarization (Shi et al. 2004). This interaction and APC function may be mediated by GSK3β as mPar3 localization in the axonal tip is prevented by inhibition of GSK3β. As GSK3β phosphorylation reduces APC mediated MT stability (Zumbrunn et al. 2001), these results show that GSK3β regulation of APC is important for multiple aspects of neuronal polarization.

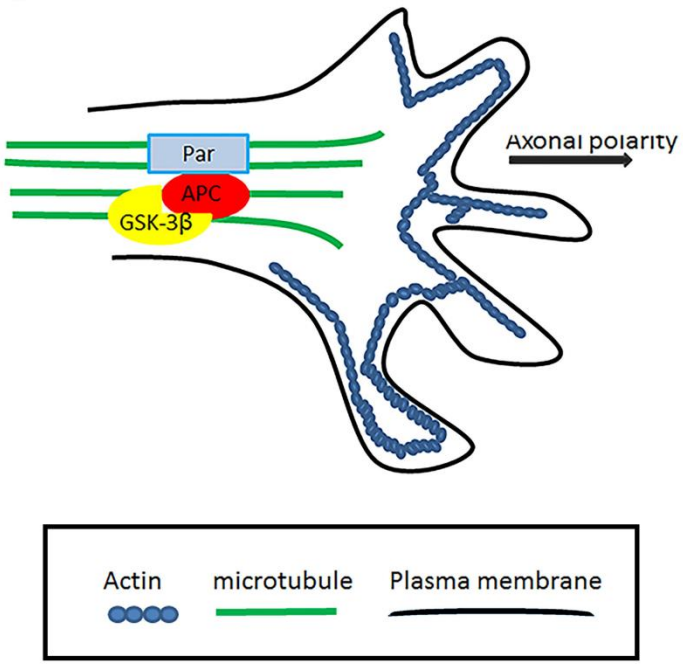

Figure 1.4 – The role APC in Axonal Polarity. Indirect evidence exists that APC associates with the polarity protein, mPar3 to promote axonal polarity in a GSK3β dependent manner (Shi et al. 2004). Inhibition of APC or GSK3β results in reduced mPar3 localization to the axon tip and prevents the emergence of an axon.

Although studies show that GSK3β affects neuronal polarization, it was not clear if this is a Wnt dependent event in normal conditions, as GSK3β is inactivated upon canonical Wnt signaling. Wnt3a stimulation induces enlargement of the growth cone, an event mediated by Dvl1, resulting in APC dissociation from the plus-end of MTs (Purro

et al. 2008). Although it was reported by Zhou et al. (2004) that local GSK3β inhibition by neuronal growth factor (NGF) promotes axon outgrowth through increased APC-mediated MT *stabilization* (Zhou et al. 2004), the study by Purro et al. (2008) suggests GSK3β inhibition leads to increased *disassociation* from the MTs upon Wnt3a stimulation. To account for this incongruity, it has been suggested that different levels of GSK3β inhibitions may be key in mediating the effects on APC's interactions with the MT cytoskeleton (Kim et al. 2006). Therefore, it is likely that tight levels of regulation of GSK3β activation and inhibition is important in mediating APC's function in growth cone dynamics. Similarly, Wnt7a has an effect on growth cone spreading that is mediated through GSK3β inhibition and a loss of stable MTs (Lucas et al. 1998).

1.2.3.2 *Synaptogenesis*

Accompanying APC's role in axonal pathfinding, evidence indicates that the Wnt pathway plays a critical role in the formation of synapses in both the central and peripheral nervous systems (Inestrosa and Arenas 2010; Sahores and Salinas 2011). Wnt 7a stimulation induces presynaptic specialization in both the cerebellum (Hall, Lucas, and Salinas 2000; Ahmad-Annuar et al. 2006) and hippocampus (Davis, Zou, and Ghosh 2008; Sahores, Gibb, and Salinas 2010) and increases mEPSC frequency and amplitude (Ciani et al. 2011). Knockdown of the Frizzled-5 (Fz5) receptor that binds Wnts blocks the synaptogenic effect of Wnt7a stimulation. Furthermore, treatment of neurons with a soluble Fz5 fragment known to inhibit Wnt7a from binding to the endogenous receptor, blocks activity dependent changes in synapse density(Sahores, Gibb, and Salinas 2010). In support of this, knockout of both Wnt7a and Dishevelled 1 (Dvl1) in mice results in

reduced spine density and mEPSC frequency and amplitude in the hippocampus (Ciani et al. 2011).

As Wnt7a increases β-catenin levels in hippocampal neurons, and inhibition of GSK3β mimics the effects of Wnt7a stimulation seen above (Davis, Zou, and Ghosh 2008), this implies the involvement of the canonical Wnt pathway, including APC, in synaptogenesis. In fact, Wnt7a stimulation induces APC clustering in the axons of hippocampal neurons (Farias et al. 2007). In cultured hippocampal neurons, Wnt7a stimulation induces increased association of APC with α7-nicotinic acetylcholine receptors (α7nAChRs) that function presynaptically to modulate vesicle release (Alkondon and Albuquerque 2001; Berg and Conroy 2002). Knockdown of APC reduces α7nAChR clustering (Farias et al. 2007), suggesting a role for APC in presynaptic localization of α7nAChRs and in mediating the synaptogenic effect of canonical Wnt signaling.

Independent of Wnt signaling, APC has been shown to be necessary for proper insertion of α3-nicotinic acetylcholine receptors (α3nAChR) at post-synaptic sites in chick ciliary ganglion neurons (Temburni et al. 2004) where APC is enriched (Onouchi, Takamori, and Senda 2012). Blocking of the EB1 binding domain of APC with a dominant negative peptide reduced surface levels and clustering of α3nAChR *in vivo* and also reduced PSD-93 and EB1 membrane associated clusters. APC also complexes with IQGAP1, the microtubule-associated crosslinking factor (MACF), and the protein 14-3-3 to direct α3nAChR surface delivery and link α3nAChRs to the cytoskeleton at postsynaptic sites (Rosenberg et al. 2008). APC is also necessary for organizing both pre- and post-synaptic specializations by regulating the surface anchoring of neurexins

and neuroligins *in vivo* (Rosenberg et al. 2010) demonstrating that APC is a key scaffolding protein affecting synaptic development.

APC may also promote synaptic maturation through its interactions with postsynaptic scaffolding molecule PSD95. APC interacts directly with PSD95 through APC's C-terminal PDZ binding domain and, *in vivo*, APC forms a complex with NMDA receptors and PSD95 (Yanai et al. 2000). However, this interaction is poorly defined as APC knockdown has no effect on NMDA receptor clustering while reducing synaptic PSD95 accumulation and AMPA receptor clustering in cultured hippocampal neurons (Shimomura et al. 2007). A similar effect was seen upon inhibition of APC/PSD5 interactions using a dominant negative C-terminal fragment of APC (Shimomura et al. 2007). The idea that APC mediates clustering of AMPA receptors, independent of Wnt signaling, is supported by the result that Wnt7a stimulation has no effect on APC/PSD95 interactions (Farias et al. 2007). However, this does not rule out the possible role for Wnt signaling in mediating on AMPA receptor insertion independent of APC/PSD95 interactions.

1.2.3.3 *Synaptic plasticity and behavior*

Despite APC's poorly defined role in synaptic function, APC binding partners such as IQGAP1, PP2A, PSD95, and GSK3β play key roles in regulating synaptic plasticity that underlies learning and memory. Long-term potentiation (LTP) requires the activation of the extracellular signal-regulated kinase (ERK) pathway (Giovannini 2006), and ERK2 is required for long-term memory (Cestari et al. 2006; Satoh et al. 2011; Selcher et al. 2001). Activation of the ERK pathway is dependent on APC binding partner IQGAP1

(Roy, Li, and Sacks 2004; Gao et al. 2011). ERK pathway activation by NMDA treatment is dependent on the presence of IQGAP1, suggesting that learning-mediate phosphorylation of ERK is also dependent on IQGAP1 (Schrick et al. 2007; Gao et al. 2011). As APC is necessary for the proper localization of IQGAP1 (Watanabe et al. 2004), these results suggest a potential role for APC in LTP through activation of the ERK pathway.

LTP is also thought to be dependent on inactivation of the APC binding partner PP2A. LTP depends on the activation of the calmodulin kinase 2 (CamKII) protein (Fukunaga, Muller, and Miyamoto 1995; Barria et al. 1997), which in turn phosphorylates and inactivates PP2A (Fukunaga et al. 2000). PP2A normally exhibits phosphatase activity on the ERK pathway (Kim et al. 2008; Van Kanegan et al. 2005; Zhou et al. 2002), so its inactivation is critical for LTP maintenance (Pi and Lisman 2008). As APC binds the regulatory subunit of PP2A (Seeling et al. 1999), this suggests that APC may serve as a scaffold for regulating kinase and phosphatase activities critical to LTP.

Another APC binding partner, PSD95 (Shimomura et al. 2007), may also provide a mechanism by which APC can regulate LTP. In PSD95 knockout mice, LTP is enhanced but cognition is impaired (Migaud et al. 1998). As PSD95 clustering is reduced in the absence of APC (Shimomura et al. 2007), this suggests that APC and PSD95 may function to coordinate the magnitude of LTP, possibly through regulation of glutamate receptor insertion.

It has also been hypothesized that Wnt signaling is required for the late, protein synthesis-dependent phase of LTP in the hippocampus (Ma et al. 2011). This study

found that activation of the mechanistic target of rapamycin (mTOR), and subsequent activation of LTP, is dependent on the inhibition of GSK3β. GSK3β can inhibit mTOR activation by phosphorylating the tuberous sclerosis complex, an event which requires APC and the β-catenin destruction complex (Inoki et al. 2006; Mak et al. 2003). This conclusion is supported by the result that inhibition of GSK3β was shown to be dependent on Wnt signaling (Ma et al. 2011). Interestingly, this study also found that the effect of Wnt signaling on LTP was independent of Wnt target gene expression, suggesting that inhibition of GSK3β results in phosphorylation dependent changes of the destruction complex that could affect downstream signaling events critical for LTP (Ha et al. 2004). Taken together, these results suggest that APC may play a critical role in integrating Wnt signaling events involved in LTP.

Both PP2A and ERK signaling also play an important role in LTD, particularly the form mediated through mGluRs. As APC interacts with FMRP (Mili, Moissoglu, and Macara 2008), whose translational functions are mediated through these receptors, it suggests that APC may play a role in that form of synaptic plasticity as well. In the study by Mili et al. (2008), they found that APC knockdown in fibroblasts led to a loss of FMRP and its associated mRNAs localized in filipodia. This suggests that APC may be responsible for trafficking FMRP-mRNA complexes to sites of active growth. Although it is not known if APC maintains this function in neurons, if it does, it suggests a potential role for APC in influencing mGluR mediated LTD.

1.2.3.4 *mRNA Binding*

As indicated above, mRNA localization and local translation plays important roles in the establishment and maintenance of polarity in multiple cell types as well as in modulating synaptic function and plasticity in neurons. APC interacts, indirectly or directly with the RNA-binding proteins, FMRP and Fus (Mili, Moissoglu, and Macara 2008; Yasuda et al. 2013). APC associates with FMRP and its target mRNAs in the distal tips of protrusions at the leading edge of migrating fibroblasts, and shRNA mediated reductions in APC disrupt this mRNA localization (Mili, Moissoglu, and Macara 2008). Further, APC association with Fus is required for efficient translation of its associated mRNAs (Yasuda et al. 2013).

Importantly, APC itself has been shown to bind selected mRNAs in neurons (Preitner et al. 2014). Its associated transcripts are highly related to APC functions in Wnt signaling, cytoskeletal regulation, intracellular trafficking, and RNA binding and regulation. Although its functions as an RNA binding protein have not been elucidated completely, APC may regulate the stabilization, localization and translation of its target mRNAs. As an example, morpholino mediated suppression of APC leads to decreases in β2B-tubulin mRNA levels and localization in the axonal growth cone and to impaired neuron migration (Preitner et al. 2014).

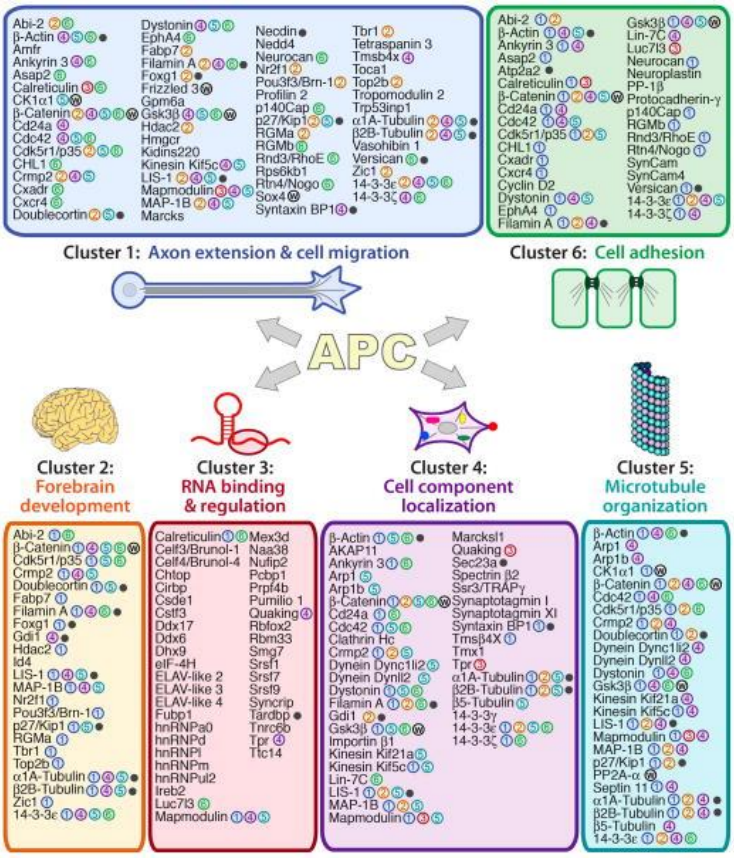

Figure 1.5 – APC mRNA target genes (Figure from Preitner, et al. 2014). APC binds selected mRNA targets that are critical for neuronal functions. Several mRNA targets have been implicated in both ASD and ID. (Reprint License 4013130507368, Elsevier Publishing).

Importantly, APC binds the mRNAs of several genes implicated in human ASD and ID: syntaxin-1 binding protein (Hamdan et al. 2009); T-box Brain1 (Notwell et al. 2016), β-catenin (Tucci et al. 2014; O'Roak, Vives, Fu, et al. 2012; de Ligt et al. 2012; Iossifov et al. 2014), EPH receptor A4 (Marchese et al. 2016), cdc42 (O'Roak, Vives, Fu, et al. 2012; Bhat et al. 2016), ankyrin G (Bi et al. 2012), doublecortin (Vourc'h et al. 2002), lissencephaly-1 (Saillour et al. 2009), β1A- & β2B-Tubulins (Neale et al. 2012; Pinto et al. 2010; Kumar et al. 2010), microtubule associated protein 1B (Liu et al. 2015), and Synaptotagmin I (Baker et al. 2015).

One APC mRNA target, the synaptic cell adhesion molecule 1 (SynCAM1) plays a critical role in cognition. Human mutations in SynCAM1 are associated with both ASD and ID (Zhiling et al. 2008). SynCAM1 interacts directly with the protein 4.1 family of molecules to control membrane insertion of the activity-dependent AMPA receptor subunit GluR1 and the NMDA receptor subunit NR2B (Hoy et al. 2009; Shen et al. 2000; Lin et al. 2009). In mouse models, either overexpression or depletion of SynCAM1 alters synaptic density, plasticity, and cognition (Robbins et al. 2010; Park et al. 2016). Similarly, even small changes in the levels of protein 4.1N or 4.1B have dramatic effects on their regulation of glutamate receptor subunit insertion (Hoy et al. 2009). Taken together, these findings suggest APC mRNA targets may play a fundamental role in the pathophysiology underlying ASD and ID, but further studies are need to assess how APC regulation of these transcripts plays a functional role in the disorders.

Taken together, these studies show that APC plays a fundamental role in regulating a variety of developmental and homeostatic processes in neurons including: Neuronal polarity, axon guidance, synapse maturation, dendritic spine regulation, and synaptic plasticity (Mili, Moissoglu, and Macara 2008; Su, Vogelstein, and Kinzler 1993; Watanabe et al. 2004; Okuda et al. 2007; Murase, Mosser, and Schuman 2002; Shimomura et al. 2007; Zhou et al. 2004; Purro et al. 2008; Inestrosa and Arenas 2010; Sahores and Salinas 2011). APC can modulate both the actin- and MT-based cytoskeleton in Wnt-dependent and -independent manners, either directly, or through interactions with its binding partners (Zumbrunn et al. 2001; Kita et al. 2006; Watanabe et al. 2004). APC interacts with, and directs the localization of a number of proteins

important for synaptic maturation and plasticity, including PSD-95, β-catenin, IQGAP1, PP2A, and FMRP (Seeling et al. 1999; Shimomura et al. 2007; Su, Vogelstein, and Kinzler 1993; Rubinfeld et al. 1993; Watanabe et al. 2004; Mili, Moissoglu, and Macara 2008). *In vivo* disruptions of APC therefore are predicted to have consequences on numerous aspects of neuronal morphology and function, as evidenced by the association of *APC* gene mutations in humans with ASD and ID (Raedle et al. 2001; Barber et al. 1994; Zhou et al. 2007; Hodgson et al. 1993; Herrera et al. 1986; Hockey et al. 1989; Kobayashi et al. 1991; Cross et al. 1992; Lindgren et al. 1992; Heald et al. 2007; Finch et al. 2005), disorders characterized by defects in neuronal connectivity and/or synapse maturation and plasticity (Courchesne and Pierce 2005; Booth, Wallace, and Happe 2011; Zoghbi 2003; Sakai et al. 2011).

1.3 β-catenin and Junction Plakoglobin

Although many of the processes above related to APC functions center on its role in governing β-catenin and Wnt signaling, I will now briefly discuss specific functions of β-catenin itself along with another related family member, junction plakoglobin (γ-catenin) more in depth.

1.3.1 β-catenin structure and localization

The β-catenin protein is a ubiquitously expressed molecule composed of 781 amino acids separated into three distinct domains. The central domain contains 12 Armadillo repeats and acts as a structurally rigid scaffold mediating the binding of β-catenin to a number of molecules, including APC, the cadherins, Axin, and the

transcriptional TCF/LEF complex (Huber, Nelson, and Weis 1997). This binding is mutually exclusive (i.e. β-catenin can bind APC or cadherin, but not both), suggesting that regulation of β-catenin and competition among its binding partners is critical in determining its cellular functions (Huber and Weis 2001).

The N-terminal and C-terminal domains are more flexible in structure, and may serve as the means for how these central domain interactions are regulated (Castano et al. 2002; Solanas et al. 2004). The N-terminal domain is responsible for linking cadherin containing adhesion complexes to the cytoskeleton through binding to α-catenin (Huber and Weis 2001). The C-terminal domain acts as a transcriptional co-activator linking transcription factors (both activators and repressors bound to the central region), to the RNA polymerase II and mediator transcriptional machinery (Mosimann, Hausmann, and Basler 2009).

The intracellular localization of β-catenin is a key determinant of its function. Under normal conditions, the majority of β-catenin is localized to the membrane where it complexes in adherens junctions with the cadherin adhesion complex (Meng and Takeichi 2009). The cytoplasmic tail of cadherins mediates the binding of β-catenin, whereas the cadherin extracellular domain regulates calcium dependent homophilic interactions (Stepniak, Radice, and Vasioukhin 2009). The association of β-catenin and cadherins is constitutive; as newly synthesized cadherins associate with β-catenin while still in the endoplasmic reticulum. This association reciprocally affects both molecules stability: the cadherins contain a motif recognized by a ubiquitin ligase that is shielded when β-catenin is bound and cadherin associated β-catenin cannot complex with the APC/Axin destruction complex (Huber and Weis 2001; Hinck et al. 1994). When bound

to cadherins at the cell surface, the N-terminal domain of β-catenin links to α-catenin, preventing α-catenin homodimerism and regulating actin dynamics(Benjamin et al. 2010). The β-catenin-cadherin complex is very dynamic: loss of cadherin based adhesion can promote β-catenin release and, ultimately, its signaling activity, and cadherins may also serve as a trap for free β-catenin, sequestering it to the membrane (Heuberger and Birchmeier 2010). This relationship has been shown to be mediated by phosphorylation of both cadherins and β-catenin itself (Piedra et al. 2001; Piedra et al. 2003; Huber and Weis 2001; Qi et al. 2006), suggesting that cellular signaling events may also regulate these dynamics.

In the cytoplasm, free β-catenin is quickly recognized by the APC/Axin destruction complex and degraded. The destruction complex serves as a scaffold for associated kinases to phosphorylate β-catenin. Casein kinase 1 α (CK1α) phosphorylates β-catenin at Ser45 and primes β-catenin for subsequent phosphorylation by GSK3β at Thr41, Ser37 and Ser33 (Xing et al. 2003; Liu et al. 2002). β-catenin that is phosphorylated at these sites, is recognized by the beta-transducin repeat containin E3 ubiquitin protein ligase complex (β-TrCP) and degraded by the 26S proteasome (Hart et al. 1999). APC functions to promote β-catenin degradation by stabilizing these phosphorylation events, as disrupting the APC/β-catenin interaction allows the protein phosphatase 2A (PP2A) to rapidly dephosphorylate β-catenin (Su et al. 2008). These phosphorylation sites are located within the third exon of the *ctnnb1* gene, and targeted deletion of this region has been shown to promote β-catenin stabilization in mice (Harada et al. 1999). Activation of Wnt signaling leads to disassembly of the destruction complex and the inhibition of GSK3β.

Free β-catenin accumulates in the cytoplasm and then translocates to the nucleus. Although β-catenin does not contain any classical nuclear import/export sequences, it can bind to nuclear pore complexes which seem to mediate its shuttling in and out of the nucleus (Fagotto, Gluck, and Gumbiner 1998; Sharma et al. 2012). Once in the nucleus, β-catenin is free to bind the TCF/LEF transcription complex and other co-activators to promote Wnt target gene expression. There are three different TCF proteins and a number of splice variants within each, so this may serve as a means for β-catenin mediated Wnt target gene expression to act in a cell-type specific and/or temporal fashion (Arce, Yokoyama, and Waterman 2006; Archbold et al. 2012). Interestingly, the destruction complex components APC and Axin are also able to translocate to the nucleus, sequestering β-catenin from binding to the transcriptional machinery and shuttling it out of the nucleus (Hamada and Bienz 2004; Neufeld, Zhang, et al. 2000; Cong and Varmus 2004).

A very closely related β-catenin family member is the desmosomal associated protein, junction plakoglobin (γ-catenin). γ-catenin shares similar sequence homology and structure to β-catenin (especially in the central domain) (Peifer et al. 1992; Peifer and Wieschaus 1990; Butz et al. 1992), and can not only interact with cadherins and α-catenin to regulate adhesion dynamics (Sacco et al. 1995; Nieset et al. 1997), but also has been shown to play a role in Wnt signaling (although its functional role as a repressor or activator of Wnt target gene expression is somewhat disputed, and may be cell type specific) (Simcha et al. 1996; Simcha et al. 1998; Karnovsky and Klymkowsky 1995; Merriam, Rubenstein, and Klymkowsky 1997; Maeda et al. 2004). APC and Axin are involved in the proteosomal degradation of γ-catenin as well (Hulsken, Birchmeier, and

Behrens 1994; Kodama et al. 1999; Kolligs et al. 2000), and γ-catenin maintains a similar phosphorylation motif for recognition by GSK3β (Rubenstein, Merriam, and Klymkowsky 1997) suggesting that the destruction complex may competitively bind either β-catenin or γ-catenin. In some conditions, it has been shown that upon loss of β-catenin, γ-catenin increases suggesting a potential compensatory overlap between the two proteins (Salomon et al. 1997). Mutations resulting in γ-catenin loss are usually embryonic or perinataly lethal, owing to loss of desmosomal adhesion complexes in cardiac tissue both in humans and mice (Bierkamp et al. 1996; Ruiz et al. 1996; Rampazzo et al. 2002).

1.3.2 Neuronal Functions of β-catenin

Like APC, β-catenin has also been associated with a variety of neuronal functions including promoting normal synapse maturation, and plasticity. Much of this function, however, is dependent on its association with synaptic adhesion molecule, N-cadherin (Arikkath and Reichardt 2008). Either reducing β-catenin levels, or blocking β-catenin binding to cadherins leads to a decrease in mature spine morphology (from mushroom-shaped to filamentous) and a decrease in mEPSC amplitude (Okuda et al. 2007). Conversely, increasing the association of β-catenin to synaptic cadherins leads to increased pre- and post-synaptic association and an increase in mEPSC frequency (Murase, Mosser, and Schuman 2002). Upon neuronal depolarization, soluble canonical Wnts are released and β-catenin accumulates in the dendritic spines concurrent with an increase in dendritic branching. However, this effect does not seem to be dependent on Wnt target gene expression suggesting that Wnt release may affect β-catenin synaptic

localization independent of β-catenin functions in the nucleus regulating TCF/LEF mediated transcription (Yu and Malenka 2003).

β-catenin also interacts with the γ-secretase Presenilin 1 (PS1) (Zhou et al. 1997). PS1 mutations are implicated in the cognitive decline seen in Alzheimer's disease (Sherrington et al. 1995). Upon glutamate stimulation or application of NMDA agonist, PS1 functions to cleave the cytoplasmic domain of N-cadherin (C-terminal fragment 2, CTF2), allowing the nuclear accumulation of β-catenin and subsequent expression of Wnt target genes (Uemura et al. 2006). CTF2 also functions to negatively regulate the cyclic AMP response element binding (CREB) signaling pathway through its interaction with the CREB binding protein (CBP) (Marambaud et al. 2003). Human mutations in CBP are associated with ASD (Barnby et al. 2005). Deletion of the domain on PS1 that mediates the interaction with β-catenin increases endothelial proliferation independent of PS1 mediated CTF2/CBP signaling(Nakajima et al. 2006) indicating that PS1 most likely functions to regulate both Wnt and CREB signaling pathways. GSK3β has also been shown to interact with and phosphorylate PS1, decreasing its association with N-cadherin (Uemura et al. 2007) and mutations in PS1 cause aberrant localization of GSK3β, inhibiting its function in the β-catenin destruction complex (Dobrowolski et al. 2012).

β-catenin also functions pre-synaptically, controlling localization and release of vesicles. Bamji, et al (2003) demonstrated that deletion of β-catenin in the hippocampus reduced the number of reserve vesicle pools and impaired synaptic responses (Bamji et al. 2003). They concluded that this was independent of either cadherin binding or Wnt target gene expression and was instead mediate through a PDZ-binding domain within β-catenin that interacts with presynaptic proteins like Veli and CASK. Interestingly,

postsynaptic beta-catenin also plays a role in regulating presynaptic release through transynaptic N-cadherin signaling (Vitureira et al. 2012).

In addition to β-catenin's role in synaptic maturation, it also plays a role in plasticity. Induction of LTP causes a release of Wnt3a and a subsequent accumulation of β-catenin in the nucleus. This effect can be blocked with an anti-Wnt3a antibody or promoted through inhibition of GSK3β (Chen, Park, and Tang 2006), suggesting that endogenous Wnt signaling is critical for LTP. It has been hypothesized that LTP is mediated through activity-dependent spine enlargement (Matsuzaki et al. 2004; Okamoto et al. 2004; Bourne and Harris 2008), and, as β-catenin, is critical for promoting spine maturation in response (Murase, Mosser, and Schuman 2002; Okuda et al. 2007), it seems likely that proper plasticity requires a Wnt-mediated inhibition of GSK3β and accumulation of synaptic β-catenin.

Interestingly, β-catenin also interacts with the tuberous sclerosis complex, a component of the mTOR signaling pathway involved in plasticity (Hoeffer and Klann 2009). It has been shown that the TSC1/2 complex interacts with both Axin and GSK3β to negatively regulate β-catenin and Wnt target gene transcription (Mak et al. 2003). Mak, et al. (2005) demonstrated that mutations in TSC2 caused upregulation of Wnt target gene expression, and that the TSC/β-catenin complex is dissociated upon Wnt signaling (Mak et al. 2005). As mutations in TSC1 and TSC2 are seen in human ASDs (Smalley 1998), this suggests that increased β-catenin function maybe a component of these diseases.

Given both β-catenin and Wnt function in synaptic homeostasis and plasticity, it is not surprising that they have been implicated in learning and memory. Maguschak and

Ressler (2008, 2011) showed that, during fear conditioning, GSK3β is inhibited and there are changes in β-catenin phosphorylation, inducing a dissociation of β-catenin from the cadherin adhesion complex (Maguschak and Ressler 2008, 2011). This dynamic interaction is known to regulate plasticity and dendritic spine morphology upon neuronal activity (Murase, Mosser, and Schuman 2002; Arikkath and Reichardt 2008). β-catenin also plays a role in memory consolidation, as reducing β-catenin levels in the amygdala blocks long term-memory (Maguschak and Ressler 2008). These learning induced changes in β-catenin are most likely mediated through Wnt, as infusion of the Wnt inhibitor dikkopf-1 (DKK1) blocks both learning mediated dissociation of β-catenin from the cadherins and inhibits long-term memory formation (Maguschak and Ressler 2011). Therefore, either high or low levels of β-catenin may block memory consolidation through it interactions with cadherins.

Very little is known about the neuronal role of γ-catenin. Although it is has been shown to associate with the neuronal cadherins (Tanaka et al. 2012), suggesting a potential role for γ-catenin in the brain, to date, no function for this β-catenin family member in either synaptic maturation, plasticity, or learning has been described.

1.4 Summary

Given the known functions of APC and β-catenin in neuronal development, activity, and learning, it is not surprising that diseases such as ASD and ID may be associated with dysfunction of this critical pathway. Mutations in both APC and β-catenin have been identified in these disorders (Raedle et al. 2001; Hormozdiari et al. 2015; O'Roak, Vives, Fu, et al. 2012; Barber et al. 1994; Zhou et al. 2007; Hodgson et al.

1993; Herrera et al. 1986; Hockey et al. 1989; Kobayashi et al. 1991; Cross et al. 1992; Lindgren et al. 1992; Heald et al. 2007; Finch et al. 2005; Tucci et al. 2014; de Ligt et al. 2012; Iossifov et al. 2012; Iossifov et al. 2014; Dubruc et al. 2014; Kuechler et al. 2015), however there is growing evidence that, regardless of any monogenic "cause" of these disorders, the Wnt pathway plays an essential role (Krumm et al. 2014; Gilman et al. 2011; Kalkman 2012). Recent genome wide association studies have identified *CHD8* as the highest confidence risk gene for sporadic ASD with overlapping ID (O'Roak, Vives, Fu, et al. 2012; O'Roak, Vives, Girirajan, et al. 2012). *CHD8*, has been determined to positively regulate β-catenin and Wnt signaling in neurons (Durak et al. 2016). Therefore, in order to develop effective treatments for ASD and ID moving forward, it will be essential to undertand how the Wnt pathway, and the critical molecules APC and β-catenin, contribute to the pathophysiology of these disorders.

Chapter 2:

Materials and Methods

2.1 Animals

All mouse models were created using CamKIIα-Cre93 mice (a kind gift from Maribel Rios (Rios et al. 2001)) to induce recombination of the various gene products. APC cKO mice were generated using $APC^{lox468/lox468}$ mice (a kind gift from Fotini Gounari (Gounari et al. 2005)), β-cat cOE mice were generated using $ctnnb1^{fl(ex3)/fl(ex3)}$ mice (a kind gift from Naomoto Harada (Harada et al. 1999)), and APC/β-cat cKO mice were created interbreeding the above APC line with $ctnnb1^{fl/fl}$ mice (Jax Mice). Mice were genotyped using the following primer pair sets:

Cre-recombinase	Forward (Fwd) 5'-CGGAAATGGTTTCCCGCAGA-3'
	Reverse (Rev) 5'-CCCGGACCGACGATGAAGCA-3'
APC	Fwd 5'-AAAGCAAGTGGTGGGAGGGTCG-3'
	Rev 5'- AATGGCCTGCAGTCCCCCTA-3'
$Ctnnb1^{fl(ex3)/fl(ex3)}$	E3 5'- CTAAGCTTG GCTGGACGTAAACTC-3'
	E4 5'- ACGTGTGGCAAGTTCCGCGTCATCC-3'
	F1 5'- GGTAGTGGTCCCTGCCCTTGACAC-3'
$Ctnnb1^{fl/fl}$	Fwd 5'-AAAATGGCAGTGCGCCTAGCTG-3'
	Rev 5'- CGGCATCCTCTCACTGCCTCC-3'

Breeding pairs for the mice were as follows: APC cKO - $APC^{fl/fl}$ males x $APC^{fl/+}$:Cre females; β-cat cOE - $Ctnnb1^{fl(ex3)/fl(ex3)}$ males x Cre females; APC/β-cat cKO - $APC^{fl/fl}$:$Ctnnb1^{fl/fl}$ males x $APC^{fl/fl}$:$Ctnnb1^{fl/fl}$:Cre females. For behavior, mice of mixed sexes, 2-4 months of age were used. For electrophysiology, mice 4-8 weeks of age were used. For morphology and immunoblot analysis, mice 2-4 months old were used.

All procedures were approved by the Tufts University Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines.

2.2 Antibodies.

2.2.1 Antibodies for Immunoblots

The antibodies used for immunoblot assays and the relevant information are listed below (antibodies are listed in order of appearance in this dissertation with the same antibodies being used in subsequent experiments): APC (H290) (Santa Cruz) at 1:2,500; GAPDH (Chemicon) at 1:10,000, β-catenin (Invitrogen) at 1:2,500; N-cadherin (Zymed) at 1:1,000; presenilin-1 (Millipore) at 1:1,000, GluR1 (Millipore) at 1:1,1000, GluR2 (Abcam) at 1:1,000; NR2A (Millipore) at 1:1,000; NR2B (Abcam) at 1:1,000; SynCAM1 (MBL International) at 1:5,000; protein 4.1N (Santa Cruz) at 1:500, γ-catenin (R & D systems) at 1:5,000; Histone H-4 (Santa Cruz) 1:500.

Horseradish-peroxidase conjugated secondary antibodies corresponding to the species of the primary antibodies above we obtained from Jackson Immunoresearch and used at a concentration of 1:10,000.

2.2.2 Antibodies for Immunohistochemistry

For immunostaining, β-catenin antibody (Invitrogen) was used at a concentration of 1:500. Myelin basic protein (Chemicon) was used at a concentration of 1:000 . A Cy3-conjugated anti-mouse and anti-rat (respectively) secondary antibody was used at a 1:1,000 dilution. Slices were counterstained with 4′-6-diamidino-2-phenylindole (Acros Organics) at 1:10,000.

2.2.3 Antibodies for immunoprecipitation

For the immunoprecipitation experiments, the following antibody amounts were used: N-cadherin (Zymed) at 2ug per 500ug total protein; LEF1 (Millipore) at 2ug per 500ug total protein; APC C-20 (Santa Cruz) at 4ug per 500ug total protein.

2.3 Immunoblot Analysis of Braine Lysates

Mice were sacrificed by rapid decapitation and hippocampi, cortex were dissected out and solubilized in radioimmunoprecipitation assay lysis buffer (RIPA) supplemented with protease/phosphatase inhibitor tablets (Thermofisher). After homogenization and centrifugation, proteins were quantified using Bio-Rad protein assay (Bio-Rad) and 10ug of protein was combined with LDS buffer/sample reducing agent (Invitrogen) to created gel loading material. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blocked for 1 hr in 5% milk in Tris-buffered Saline supplemented with 0.1% Tween (TBST). All primary antibodies were diluted in blocking buffer and membranes were incubated overnight at 4^{o}C. Membranes were washed three times in TBST for 10 minutes. Secondary antibodies specific to the species of the primary antibody were diluted in blocking buffer, and placed on the membranes for 1 hour at room temperature. Membranes were then again washed three times in TBST for 10 minutes. Membranes were then exposed to enhanced chemiluminescence buffer (GE) for 5 minutes and then exposed to autoradiography film (MidSci). Quantification of protein band intensity was performed on ImageJ relative to the loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.4 Immunohistochemistry

Mouse brains were fixed by transcardial perfusion (4% paraformaldehyde in phosphate-buffered saline). Immunostaining was performed on 50-μm coronal frozen sections after 30 minutes permeablization with 0.1% triton in PBS. Sections were blocked in 5% milk/PBS and placed in primary antibody overnight (described above) at 4^0C. The next day, sections were washed three times in PBS and placed in secondary antibody (described above). Images were obtained with a Zeiss Axioskop epifluorescence microscope (Carl Zeiss Microscopy).

2.5 Immunoprecipitation

Lysates were prepared and quantified as described above. 500ug of total protein lysate in 1.0ml of total RIPA buffer was precleared with 20ul of protein A/G agarose beads for one hour at 4^0C. Precleared lysate was then transferred to a new tube with 20ul beads plus the primary antibody at the dilutions indicated above. Incubation with the antibody was done overnight at 4^0C. The beads were then washed three times with 1mL of RIPA buffer. The beads were pelleted by centrifugation and the supernatant was removed. The beads were resuspended in and equal volume gel loading buffer (20ul) and boiled for 5 minutes. Loading material was resolved on gels as described above. Input represents total lysate before immunoprecipitation and was prepared as described above.

2.6 Subcellular Fractionation

Membrane and nuclear fractions were obtained using the Subcellular Protein Fractionation Kit for Tissues (ThermoFisher) by pooling 4 hippocampi (2 mice) of the same genotype and performing the experiment as per manufacturer's instructions.

2.7 qPCR

RNA preparation from hippocampi, cortices and striata was performed using the RNeasy Plus Mini Kit (Qiagen) as per manufacturer's instructions. Reverse transcription on RNA samples was performed using the cDNA High-Capacity Reverse Transcription Kit (Applied Biosystems) as per manufacturer's instructions. qPCR was performed using Sybr Green (Applied Biosystems) using the following primer sets:

Dkk1	Forward (Fwd) 5'-CTCATCAATTCCAACGCGATCA-3'
	Reverse (Rev) 5'-GCCCTCATAGAGAACTCCCG-3'
SP5	Fwd 5'-TGGGTTCACCCTCCAGACTTT-3'
	Rev 5'-CCGGCGAGAACTCGTAAGG-3'
Neurog1	Fwd 5'-CCAGCGACACTGAGTCCTG-3'
	Rev 5'-CGGGCCATAGGTGAAGTCTT-3'
Syn1	Fwd 5'-GGACAGAGAGAAATCCCTTTCAC-3'
	Rev 5'-CAGGTGGCGGAAGTCTTCATT-3'
SynCAM1	Fwd 5'- AACAGGCAGACCATTTACTTCAG -3'
	Rev 5'- TCCGAGATTGAGACATTCGTCA -3'
APC	Fwd 5'- CTTGTGGCCCAGTTAAAATCTGA -3'
	Rev 5'- CGCTTTTGAGGGTTGATTCCT -3'

GAPDH	Fwd 5'-AGGTCGGTGTGAACGGATTTG-3'
(loading control)	Rev 5'-TGTAGACCATGTAGTTGAGGTCA-3'

2.8 Electrophysiology

2.8.1 Slice Preparation for Electrophysiology

Horizontal hippocampal slices (300 - 330 microns) were prepared as described previously (Pascual et al. 2005). Briefly, the brain was rapidly removed from the skull and chilled with cold (4°C) artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 85 sodium chloride, 2.5 potassium chloride, 1.25 monosodium phosphate, 0.5 calcium chloride, 4 magnesium chloride, 25 sodium bicarbonate, 75 sucrose, 25 glucose and 0.5 ascorbic acid (pH 7.4 adjusted with 95% O_2, 5% CO_2). The brain was then glued on a plate and cut with a Leica VT 1000S vibratome, microdissections of the hippocampus were performed and CA3 to CA1 connections were cut. Resultant slices were incubated at 30-32°C for 1 hr recovery and were recorded at 32°C, unless otherwise indicated.

2.8.2 Miniature Excitatory Postsynaptic Currents

Whole-cell recordings of miniature EPSCs (mEPSCs) from hippocampal CA1 pyramidal neurons were obtained at a holding potential of -60 mV using patch electrodes (3 MΩ) filled with a solution containing (in mM): 100 cesium methanesulonate, 10 tetraethylammonium chloride, 8 sodium chloride, 10 HEPES, 5 QX-314-Cl [N-2(2,6-dimethyl-phenylcarbamoylmethyl) triethylammonium chloride], 2 Mg-ATP, 0.3 Na-GTP,

10 EGTA, with pH 7.25, 290 mOsm. 1 μM TTX, 20 μM bicuculline and 25 μM AP5 were added to ACSF to inhibit spontaneous action potential-mediated synaptic currents and isolate AMPAR-mediated currents. Signals were obtained using MultiClamp 700B amplifier under visual control of Nikon E600FN microscope. All data were acquired using Clampex 9.2, filtered at 1 kHz and digitized at a sampling rate of 1 kHz. Miniature events were detected with a 6 pA threshold level of detection in the Mini Analysis Program.

2.8.3 Extracellular Recordings

Extracellular field recordings were performed using slices obtained from 8-10 weeks old APC cKO and littermate control mice. Slices were perfused with normal ACSF containing (in mM) 124 sodium chloride, 3.1 potassium chloride, 1 magnesium chloride, 2 calcium chloride, 26 sodium bicarbonate, 1 monosodium phosphate, 10 glucose, saturated with 95%O_2 and 5% CO_2 at 32°C. The osmolarity of the normal ACSF was in the range of 280-290 mOsm/kg. Field potentials from the CA1 stratum radiatum were recorded with an extracellular glass pipette (2 - 3 MΩ) filled with ACSF. Schaffer collaterals were stimulated at 0.033 Hz with a bipolar tungsten electrode. Stimulating electrode was 400-500 μm away from the recording electrode. Stimulation intensities were chosen to produce a field EPSP (fEPSP) with an amplitude that was approximately 40 - 50% of that obtained with maximal stimulation. Recordings were performed using Axopatch-1C amplifier. Data were filtered at 1 kHz and sampled 10 kHz with a Digidata 1322A interface and pClamp software.

LTP was electrically induced by theta-burst stimulation. Theta-burst stimulation consisted of 5 trains of theta burst stimulation at 0.033 Hz. A train of theta burst consisted of 50 ms duration 100 Hz trains, repeated 4 times at 200 ms interval. LTD was induced by 10 min application of DHPG (100 μM) or 1 Hz (900 pulses) stimulation. Magnitude of LTP or LTD was measured 1 hour after application of the protocols. LTP and LTD histograms represent the average response over the last 10 minutes of recording.

2.9 Behavioral Testing

2.9.1 General Testing Conditions

All mice were individually housed on a reverse 12 hour light/12 hour dark cycle, and handled 5 minutes daily for a week prior to behavioral testing. All mazes, apparatuses, and objects were cleaned between subjects and trials with 70% ethanol. All behavioral experiments were carried out in the Tufts Center for Neuroscience Research Behavior Core.

2.9.2 Barnes Maze

The Barnes maze was performed using a raised, circular platform measuring 5 feet in diameter. Around the perimeter of the platform are 40 evenly-spaced holes, each measuring 5 cm in diameter. The escape box is a small, black-plexiglass chamber containing a ramp which leads up to the maze. The maze is partitioned from the rest of the testing room by a curtain. Distinct visual cues are posted on the curtain surrounding the maze at 4 points corresponding to quadrants of the maze. Trials were recorded using a ceiling-mounted video camera connected to a computer running ANY-Maze tracking

software. One day before training, mice were given a 180-second habituation period where they were free to roam the platform. Mice were trained on days 1-4. On each day, they were given two 3-minute trials, spaced 20 minutes apart. If mice failed to find the goal after 3 minutes, they were gently guided to the goal and into the escape box. On days 5 and 12, 90-second probe trials were administered in which the goal box was removed. Latency, primary errors, path efficiency, and speed were all calculated by ANY-Maze software. Latency is the time taken before the mouse reaches the goal, primary errors are the number of incorrect holes visited before the goal hole, and path efficiency is the optimal path-to-goal length divided by the traveled path length. Each mouse is given a different goal hole, and the maze itself is rotated a quarter-turn each day while the goal boxes remain in the same position relative to the room.

2.9.3 Y-Maze

The Y-maze is a white plexiglass chamber with equal arms measuring 35cm x 5cm wide. Mice are placed in the center of the apparatus and freely allowed to explore the maze. Their arm entries are observed over the course of a 10 minutes trial. A correct choice is determined by which arm the mouse enters after making its previous two choices. For example, if the mouse enters arm A followed by arm B, the correct choice would be arm C and an incorrect choice would be returning to arm A. This gives the mouse a chance rate of 50%. Each mouse is given a percent correct choice score based on the number of correct choices relative to their total choices.

2.9.4 Contextual Fear Conditioning

For contextual fear conditioning, the total duration of each training trial was 500 seconds. The mouse was placed in the chamber (Coulbourn Instruments) and a mild a foot shock was delivered (2 s, 0.80 mA) at 198 s, 278 s, 358 s, and 438 s. Freezing behavior was measured using a digital camera connected to a computer with Actimetrics FreezeFrame software. Percent freezing was measured within each trial for 2 minutes and 30 seconds, beginning 30 seconds after the mouse was placed in the chamber (before delivery of the first shock). For probe trials, the mouse was placed in the chamber for 3 minutes and percent freezing was measured as above.

2.9.5 Marble Burying

Mice were habituated to standard 7" x 11" mouse cages containing 4 cm-deep bedding for 15 minutes. Mice were removed, bedding was smoothed, and 20 marbles were placed atop the bedding in an evenly-spaced manner. Mice were returned to their respective cages for 15 minutes, after which they were removed, and the number of marbles buried was recorded. Marbles were considered buried if they were at least ¾ covered with bedding.

2.9.6 Repetitive Novel Object Contact

Repetitive novel object contact task was performed in a manner similar to (Pearson et al. 2011). Mice were habituated to a standard, empty 7" x 11" cage for 30 minutes. The following day, 4 novel objects—a jack, a six-sided die, a lego, and a two-pronged outlet cover—were placed one inch from each corner. Mice were video-recorded

for a 10-minute trial. The sequence of objects contacted was manually scored for the entire 10 minute trial. Contact was defined as clear facial or vibrissae contact. Ranked object preference was calculated individually for each mouse. Total contacts to each of the 4 objects were expressed as a percentage of total contacts, and ordered by object rank for each mouse. For sequence preference, the number of times mice contacted objects in each possible sequence of four objects was tallied, and the sequence of objects which was repeated the most times was determined for each mouse. The number of times the preferred sequence was repeated was then divided by total object contacts, for each mouse, and this value was used as frequency index.

2.9.7 Social Olfactory Test

Social olfactory test was performed similarly to (Silverman, Yang, et al. 2010). The test was performed in an empty, standard 7" x 11" mouse cage. Odors were presented on cotton swabs suspended roughly 2.5" above the floor of the cage. For water and non-social odors (vanilla and banana extract, diluted 1:100 in water), swabs were submerged in the liquid before presentation. For social odors, swabs were run along the bottom of cages housing multiple, novel male or female mice. Swabs were prepared for odor presentation immediately before they were used for trials. Mice were exposed, in order, to three consecutive 2-minute trials of water, followed immediately by 2 blocks of three 2-minute trials of both non-social odors, followed immediately by three 2-minute trials of social odors. Trials were scored manually by an observer, measuring time investigating the swab using a stopwatch. Investigation was defined as clear facial or vibrissae contact.

2.9.8 Three Chamber Social Interaction Test

Three-chamber social interaction test was performed similarly to (Moy et al. 2007). Three-chamber apparatus was a rectangular plexiglass cage measuring 60 cm x 40 cm, evenly divided into three chambers with plexiglass partitions. Partitions each contained a small hole allowing mice to move freely between the chambers. Plexiglass slats can be placed to block access through the holes. Mice were given a 10-minute habituation period after which they were confined to the middle chamber. In the center one of the side chambers, an empty, inverted, cylindrical wire-mesh pencil holder was placed. In the other side chamber, a novel, juvenile male mouse was placed within an identical inverted pencil holder. Slats were then removed from the partitions, and the subject mouse was given a 10-minute trial to explore all three chambers. After 10 minutes, the mouse was returned to the center chamber and the apparatus was cleaned. The same juvenile male was then placed in the original social chamber and a novel juvenile male was placed in the chamber that held the empty cage. The slats were again removed and the subject mouse was given 10 minutes to explore all three chambers. Trials were recorded by a video camera connected to a computer running Ethovision software (Noldus). Ethovision video tracking software measured the amount of time mice spent in each chamber, as well as time spent interacting with each cage.

2.9.9 Repetitive Circling Assay

Mice were placed in a novel shoebox cage and recorded from the side for 45 minutes. The number of times a mouse exhibited circling behavior, defined as at least

three tight, consecutive unidirectional rotations of mouse. The data is recorded as a percentage of mice who exhibited the behavior within the first 15 minutes.

2.9.10 Elevated Plus Maze

The elevated plus maze test for anxiety was performed on a fully automated elevated plus maze consisting of two open arms (38 cm x 5 cm) and two closed arms (38 cm x 5 cm) with 15 cm-high black plexiglass walls, and a central intersection zone (5 cm x 5 cm). The maze is elevated 75 cm off the floor. The maze is connected to a computer running Hamilton/Kinder MotorMonitor software, which records photobeam breaks from the maze to determine mouse movements and position. Mice are placed in the central intersection zone and given a single, 5-minute trial.

2.9.11 Hot Plate Test for Pain Sensitivity

To test for pain sensitivity in the mice, we employed a hot plate test on the different genotypes. Mice were placed on an enclosed hot plate with a temperature of 55^0C. An observer with a stopwatch observed the duration of time from when the mouse was placed on the hot plate until a paw lift or paw lick was observed.

2.9.12 Open Field Test

Mice were placed in a clear 40cm x 40cm (x 20cm height) plexiglass chamber with a plexiglass floor. During the course of a 10 minute trial, their velocity and distance traveled was recorded with a camera and analyzed through Ethovision.

Chapter 3:

Adenomatous Polyposis Coli Protein Deletion Leads to Cognitive and Autism-like Disabilities

Contributions: I performed and analyzed some of the behavioral experiments; performed some of the immunoblots, immunoprecipitation, and qPCR; analyzed spine density and shape; assisted with the writing and preparation of the manuscript:

Mohn, J. L., J. Alexander, A. Pirone, C.D. Palka, S. Lee, L. Mebane, P. G. Haydon, and M. H. Jacob. 2014. 'Adenomatous polyposis coli protein deletion leads to cognitive and autism-like disabilities', Mol Psychiatry, 19: 1133-42.

3.1 Overview

Intellectual disabilities and autism spectrum disorders link to human APC inactivating gene mutations. However, little is known about APC's role in the mammalian brain. This study is the first direct test of the impact of APC loss on central synapses, cognition and behavior. Using our newly generated APC conditional knock-out (cKO) mouse, we show that deletion of this single gene in forebrain neurons leads to a multisyndromic neurodevelopmental disorder. APC cKO mice, compared with wild-type littermates, exhibit learning and memory impairments, and autistic-like behaviors (increased repetitive behaviors, reduced social interest). To begin to elucidate neuronal changes caused by APC loss, we focused on the hippocampus, a key brain region for cognitive function. APC cKOs display increased synaptic spine density, and altered synaptic function (increased frequency of miniature excitatory synaptic currents, modestly enhanced LTP). Additionally, we found excessive β-catenin levels and associated changes in canonical Wnt target gene expression and N-cadherin synaptic adhesion complexes. Our findings identify some novel functional and molecular changes not observed previously in other genetic mutant mouse models of co-morbid cognitive and autistic-like disabilities. This work thereby has important implications for potential therapeutic targets and the impact of their modulation. We provide new insights into molecular perturbations and cell types that are relevant to human intellectual disability and autism. Additionally, our data elucidate a novel role for APC in the mammalian brain as a hub that links to and regulates synaptic adhesion and signal transduction pathways critical for normal cognition and behavior.

3.2 Generation of APC cKO Mice

To gain new insights into APC's functions in the mammalian brain, we generated mice with cKO of APC targeted predominantly to excitatory neurons because APC is enriched at these postsynaptic sites (Yanai et al. 2000; Temburni et al. 2004; Rosenberg et al. 2008; Shimomura et al. 2007). Support for the human disease relevance of this approach stems from the recent identification of this particular cell type and developmental stage as central to convergent expression of ASD and overlapping ASD/ID risk genes in human and mouse brains (Parikshak et al. 2013; Willsey et al. 2013). Thus, the APC cKO mouse does not directly model the human genetic disorder but it enables elucidation of ID/ASD relevant biological mechanisms. Further, this cKO approach is necessary because APC global knock-out is embryonic lethal (Moser et al. 1995), and its targeted deletion in all embryonic neural progenitor cells leads to severe defects in radial glia polarity, generation and migration of cortical neurons, and construction of the cerebral cortex (Yokota et al. 2009; Ivaniutsin et al. 2009).

We crossed mice carrying floxed APC alleles with CaMKII-Cre mice expressing Cre recombinase driven by the α-calcium/calmodulin-dependent protein kinase II promoter (Rios et al. 2001). Crosses with the Rosa26R reporter mouse line indicate that the Cre transgene is expressed chiefly in postmitotic excitatory neurons during synapse differentiation, within the first 3 postnatal weeks, throughout the brain, except for the cerebellum (Rios et al. 2001). APC is selectively deleted by Cre-dependent excision of *lox*P flanked exons 11 and 12, leading to out-of-frame splicing of exon 10 to exon 13 and generation of a prematurely terminated, unstable 468−amino acid APC protein lacking all identified protein interaction domains(Gounari et al. 2005). In all experiments APC

cKOs were compared to control littermates consisting of mice expressing either floxed APC alleles alone or Cre-recombinase transgene alone, both show no change in APC protein levels (Figure 3.1A,B).

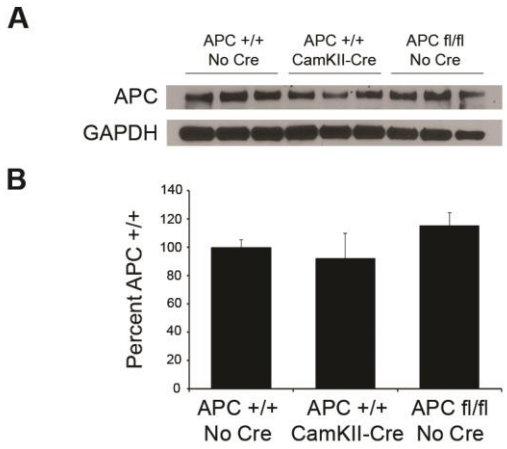

Figure 3.1 - Wild-type APC levels in the forebrain of mice expressing floxed APC alone or Cre-recombinase alone. (A) Western blot showing APC protein levels are unchanged in the brains of mice expressing wild-type APC alleles with Cre-recombinase or floxed APC alleles with no Cre. (B) Histogram showing that the floxed alleles or Cre expression by themselves, do not affect APC levels, with signal normalized to GAPDH control. APC +/+Cre+ = 92.3 ± 17.7% and APC fl/flCre- = 115.4 ± 8.9% compared to mice expressing wild-type APC alleles, APC +/+Cre-, in total forebrain lysate; $p = 0.4255$, one-way ANOVA, $F(2, 6) = 0.9885$; $n = 3$ APC +/+Cre-, 3 APC fl/flCre-, 3 APC +/+Cre+ mice.

We confirmed APC protein depletion in the cKO mouse brain by showing dramatic reductions in the hippocampus and cortex (Figure 3.2A,B; $p = 0.0019$ and $p = 0.022$ respectively, Student's t-test). Small amounts of APC protein remaining likely derives from glial cells and interneurons, as APC is ubiquitously expressed, but α-CaMKII-Cre transgene is not expressed in these cell types (Rios et al. 2001).

However, APC levels are also reduced in the striatum (Figure 3.2A,B; $p = 0.0058$, Student's t-test) which predominantly contains medium-spiny neurons. These inhibitory neurons, in contrast to cortical and hippocampal interneurons, do express the α-CaMKII-

Cre transgene (Rios et al. 2001; Novak and Seeman 2010; Ng et al. 2010). Thus, APC is depleted in these inhibitory neurons, and the pool normally bound to microtubules in corticostriatal excitatory inputs. In comparison, cerebellar APC levels are not altered (Figure 3.2A,B) as this Cre transgene is not expressed in this region (Rios et al. 2001).

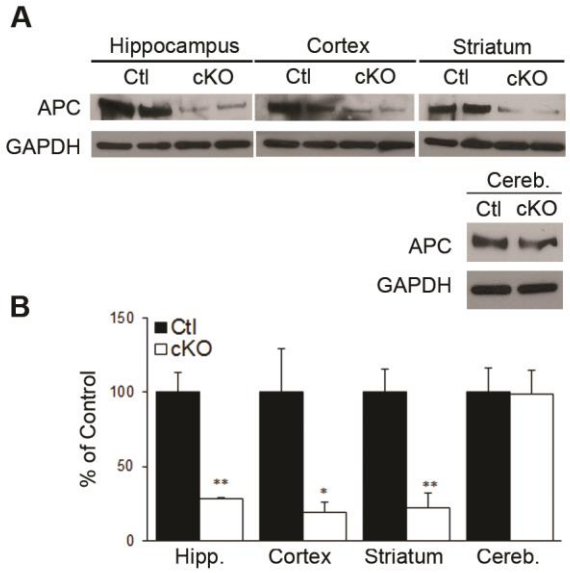

Figure 3.2 APC conditional knockout in mouse forebrain neurons. (A) Western blot showing APC protein levels are dramatically decreased in hippocampal, cortical and striatal, but not cerebellar, lysates of APC cKO mice at 3 months of age. Similar decreases were seen at 1 month (data not shown). APC deletion is driven by CAMKII promoter-dependent expression of Cre recombinase in forebrain postmitotic excitatory neurons and striatal inhibitory interneurons. (B) Histogram of decreased APC levels in the indicated brain region lysates of APC cKO mice, relative to control littermate levels. Signals are normalized to GAPDH as a loading control. (*$p<0.05$, **$p<0.01$, Student's t-test, n = 5 APC cKO mice and 4 control littermates).

3.3 Impaired Learning and Memory in APC cKO Mice

Because patients with *APC* heterozygous gene deletions exhibit ID (Hodgson et al. 1993; Herrera et al. 1986; Hockey et al. 1989; Kobayashi et al. 1991; Cross et al. 1992; Lindgren et al. 1992; Heald et al. 2007; Barber et al. 1994; Raedle et al. 2001; Finch et al. 2005), we tested for altered cognitive function in APC cKO mice, relative to

control littermates. We first employed the Barnes maze task to assess hippocampal-dependent spatial learning and memory (Barnes 1979). APC cKOs exhibited delayed learning. They improved by trial 6, whereas controls improved by trial 3-4 (Figure 3.3A,B). Over the course of training, APC cKOs took more time to reach the goal hole ($p = 0.0075$ repeated measures ANOVA, $F(1, 24) = 8.532$), committed more errors ($p = 0.00017$, $F(1, 24) = 19.78$), and took less efficient routes to the goal ($p = 0.00081$, $F(1, 24) = 16.64$) (Figure 3.3A-D).

To assess memory formation, we performed probe trials on days 5 and 12. On day 5, APC cKOs showed strong preference for the goal (Figure 3.3A-D,F), consistent with their ability to perform the task by day 4 trials. In sharp contrast, APC cKOs performed poorly on day 12, whereas controls maintained strong preference for the goal (Figure 3.3A,G). APC cKOs had double the latency to goal ($p = 0.0064$ Sidak-Bonferroni corrected t-test), committed three times as many errors ($p = 0.000005$), and took less efficient routes to the goal ($p = 0.019$) (Figure 3.3B-D). This poor performance suggests deficits in long-term spatial memory consolidation in APC cKO mice.

As a separate test of hippocampal-dependent working memory and responsiveness to novelty, we used the continuous spontaneous alternation Y-maze task (Hughes 2004). APC cKOs showed reduced rates of alternation between the symmetrical arms (Figure 3.3H; $p = 0.032$ Student's t-test), not significantly different from the chance rate of 50% (cKO $p=0.1078$; control littermate $p= 0.0038$ one-sample t-test).

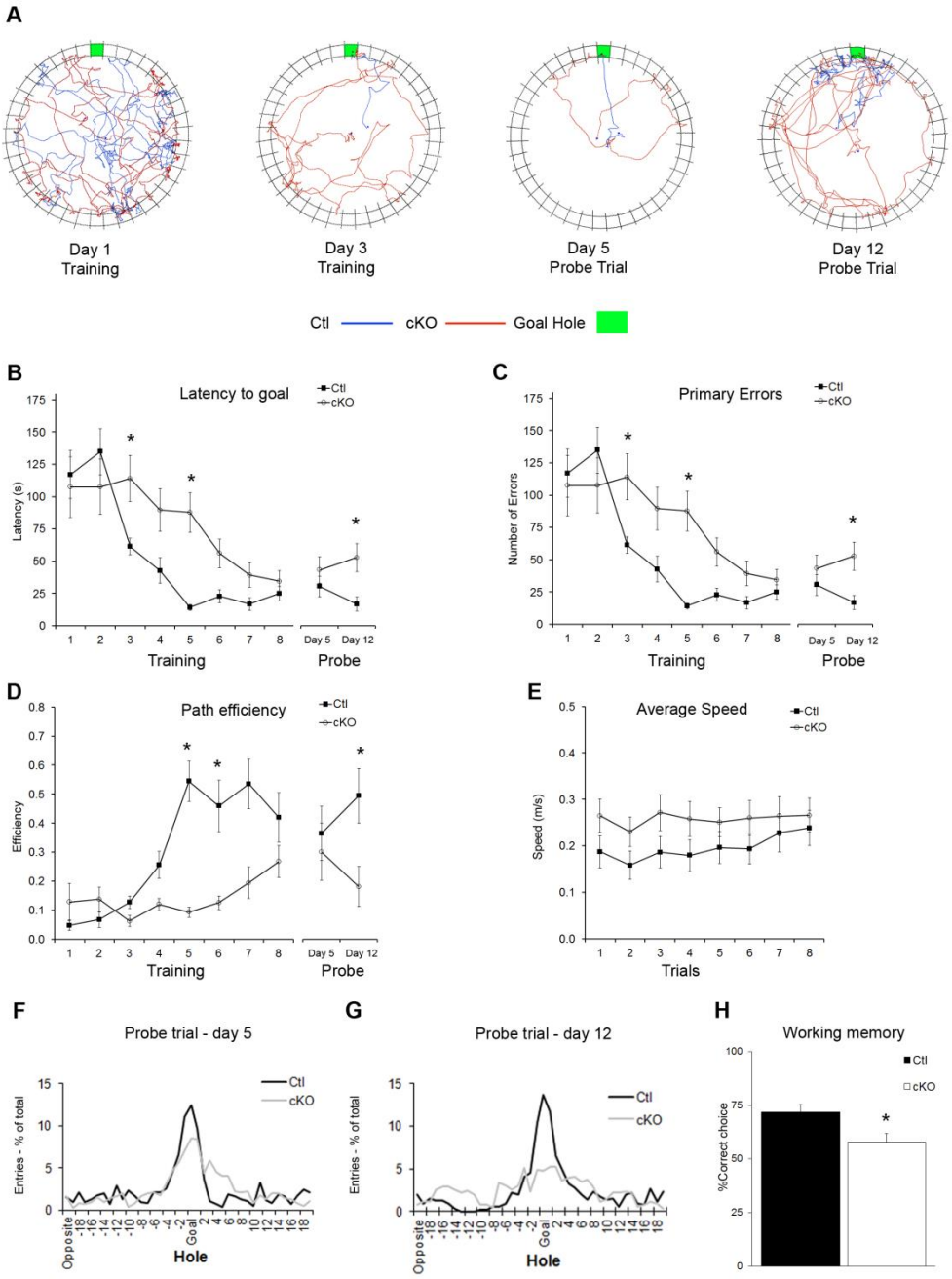

Figure 3.3 Impaired learning and memory in APC cKO mice. (A) Representative traces of APC cKO (red) and control littermate (blue) mice in the Barnes maze task. Control littermates showed rapid improvement and learned the location of the goal hole (green, a), using wall-mounted spatial cues, after 2 days, with 2 trials per day, as measured by (B) latency to find the goal, (C) number of errors committed before reaching the goal, and (D) path efficiency. APC cKOs showed slower improvement, (A,B) requiring 3-4 days to learn the location of the goal hole, (C) committing more errors and (D) taking less efficient paths to the hole throughout most

of the test period. No differences were found in (E) average speed between the cKOs and control littermates during the task. (A,F) Probe trial on day 5 shows that both cKOs and controls display a strong preference for the goal location, as measured by the number of visits to the goal hole. (A,G) Probe trial on day 12 shows that cKOs do not retain preference for the goal location, whereas controls do. One week after training (probe trial day 12), APC cKOs (B) take longer to reach the goal location, (C) commit more errors, and (D) take a less efficient path, relative to control littermates, suggesting impaired long-term memory formation. *$p < 0.05$, Sidak-Bonferroni corrected t-test, n = 12 APC cKOs and 14 control littermates. (H) In the continuous spontaneous alternation task to assess working memory, control littermates showed a preference to alternate, whereas APC cKOs did not; they alternated close to the chance rate of 50%. *$p < 0.05$, Student's t-test, n = 7 APC cKOs and 6 control littermates.

In a related study, APC heterozygous mice (whole body), modeling haploinsufficiency in humans, show age-dependent deficits in working memory, but also anemia and hypoactivity (Koshimizu et al. 2011). As potential confounds that could impact performance in behavior assays, we ruled out impaired locomotion and hypo- or hyper-activity in APC cKO mice (predominantly glutamatergic neuron-specific depletion). APC cKOs, compared with control littermates, displayed no differences in average speed in the Barnes Maze task (Figure 3.3E; $p = 0.20$ repeated measures ANOVA, $F(1, 24) = 1.72$), frequency of arm entries in the Y maze task ($p = 0.432$ Student's t-test), overall activity or distance traveled during home cage monitoring over 30 hours ($p= 0.21$ repeated measures ANOVA, $F (1, 19) = 1.717$), or stride length in gait analysis (right side: $p = 0.48$, left side: $p = 0.81$, Student's t-test). In summary, APC is essential in forebrain neurons for normal cognition.

3.4 Autistic-like Behaviors in APC cKO Mice

Based on ID/ASD in patients with *APC* inactivating mutations (Barber et al. 1994; Zhou et al. 2007), we tested APC cKO mice using diagnostic assays designed to test for autistic-like phenotypes in mice (Silverman, Yang, et al. 2010).

APC cKO mice displayed increased repetitive behavior in the marble-burying assay, burying 1.6 times more marbles than control littermates ($p = 0.0056$, Student's t-test) a result seen in other mouse models of autism (Amodeo et al. 2012; Choi et al. 2016). Increased marble burying reflects repetitive digging behavior and does not correlate with anxiety-related measures (Thomas et al. 2009). Consistent with this, APC cKOs exhibited decreased anxiety-like behavior in the elevated plus maze task, spending four times more time in the open arms ($p = 0.0052$, Student's t-test). The differences are not due to hyperactivity, as the mice travelled similar total distances ($p = 0.407$, Student's t-test).

APC cKOs also showed increased repetitive behavior in the repetitive novel object contact task. This test, used on the BTBR mouse model of autism, measures the number of times the mice investigate each of 4 novel objects, and the sequence in which the objects are visited during the test period (Pearson et al. 2011). Increased repetition of a pattern of sequential object investigations indicates stereotypy. All four objects were of similar interest to cKO and control mice based on their ranked preferences (Figure 3.4A). APC cKOs made a greater number of visits ($p = 0.0035$ repeated measures ANOVA, $F(1,16) = 11.69$), suggesting that controls habituated to the objects, whereas cKOs did not. Most important, APC cKOs showed "preferred" sequences, they repeated a specific sequence of 4-object visits significantly more times ($p = 0.0042$, Student's t-test), after

normalizing for total number of object visits. The "preferred" sequences likely derive from their distinct movement patterns, APC cKOs displayed increased preservation of their direction of movement, traveling clockwise or counterclockwise during object visits (Figure 3.4B).

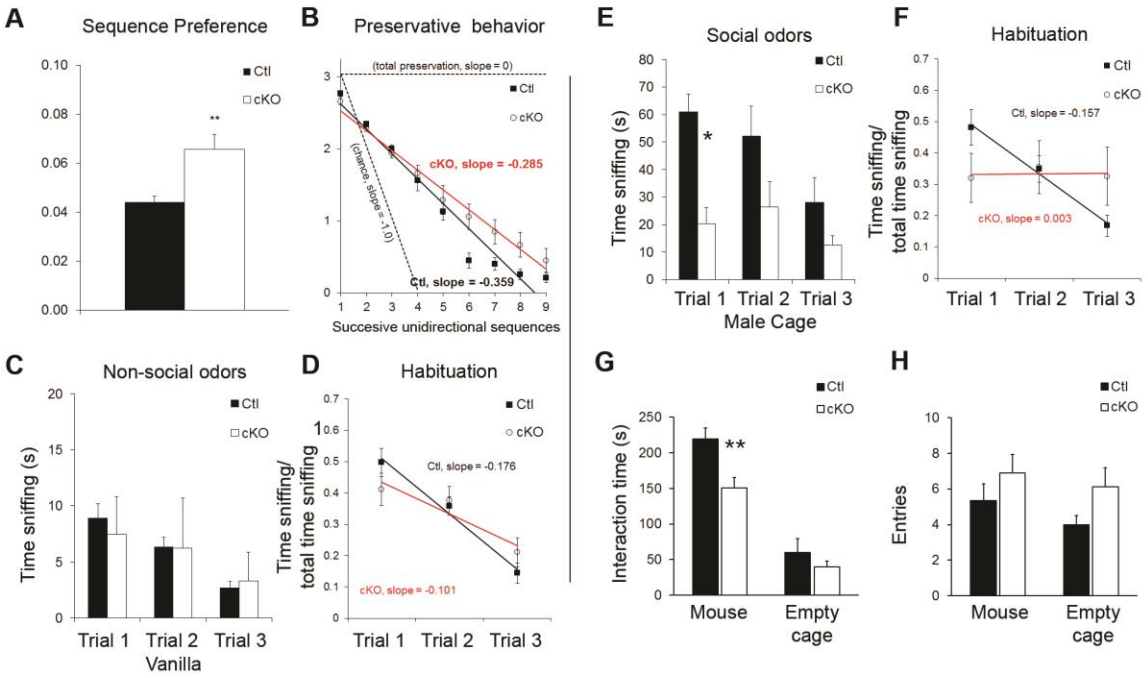

Figure 3.4 Repetitive behaviors and reduced social interest in APC cKO mice. (A-B) In the repetitive novel object contact task, (A) APC cKOs repeated a particular preferred sequence of visits to the four objects more frequently, after normalizing for the total number of object visits. **$p < 0.01$, Student's t-test. (B) The cKOs preserved their direction of movement, traveling clockwise or counterclockwise, significantly more than control littermates, based on differences in the slope of log plots of the directionality of movement, normalized for the total number of object visits $p=0.001345$, linear regression of difference in slopes $F(1,158) = 10.6425$, n = 8 APC cKOs and 10 controls. For comparison, the plot shows the slope for total preservation of movement in one direction (top dotted line) versus totally random directional movements to visit objects (bottom dotted line). (C-F) In the social versus non-social olfaction task, both APC cKOs and control littermates displayed the ability to distinguish odors. (C) They exhibited similar levels of interest in non-social odors, such as vanilla, and showed the typical habituation pattern, spending less time sniffing the same odor over the 3 successive exposures (D), linear regression of differences in the slopes; vanilla, $p = 0.08796$, $F(1,56) = 3.01581$), water (data not shown), $p = 0.3199$ $F(1,56) = 1.00705$; banana, p

= 0.9119, F(1,56) = 0.012352 (E) APC cKOs, compared with control siblings, exhibited significantly less interest in social odors (novel male cage odor, *$p < 0.05$, Student's t-test, n = 11 cKOs and 9 controls). Further, the control mice showed the typical habituation pattern on the successive trials. In contrast, APC cKOs showed less robust habituation, they spent relatively equal amounts of time investigating the social smell in each of the three trials per odor (F, linear regression of the slopes of data in e). (G) In the three-chambered social interaction assay, both cKOs and controls displayed a preference for a caged, novel mouse (ovariectomized wild-type female), versus an empty cage, but APC cKOs spent significantly less time interacting with (sniffing) the novel mouse, suggesting reduced social interest (**$p < 0.01$, Sidak-Bonferroni corrected t-test). (H) APC cKOs and control littermates made a similar number of entries to both side chambers, suggesting no difference in exploration. n = 11 cKOs and 7 controls.

Next, we tested for reduced social interest, another hallmark of ASD. Because social interactions between mice depend on olfactory cues, we first performed a social versus non-social olfaction test(Silverman, Yang, et al. 2010). APC cKOs and control littermates showed a similar level of interest in non-social odors, indicating intact olfactory function (Figure 3.4C,D). In addition, both showed typical habituation, spending less time investigating the same non-social odor over 3 successive exposures, followed by renewed interest for a novel non-social odor.

In contrast, APC cKOs, compared to controls, exhibited significantly less interest in social odors ($p = 0.0033$ repeated measures ANOVA $F(1, 34) = 10.00$), from both male and female cages, suggesting reduced social interest (Figure 3.4E). Further, APC cKOs showed less robust habituation on successive social trials (Figure 3.4F; $p = 0.01564$ linear regression of difference in slopes $F(1,50) = 6.26347$), despite their normal habituation response to non-social odors.

Additionally, we performed the classic three-chambered social interaction test(Silverman, Yang, et al. 2010). As expected, control mice spent more time interacting

with the caged novel mouse, compared to the empty cage (Figure 3.4G). APC cKOs showed a similar trend (p= 0.000001, two-way ANOVA, F(1,24) = 89.23, differences between cages), but spent significantly less time interacting with (sniffing) the novel mouse (p = 0.005, Sidak-Bonferroni corrected t-test; p=0.000001, two-way ANOVA, F(1,24) differences between genotype) (Figure 3.4G). There was no difference in exploration levels between cKOs and controls, as both made a similar number of transitions between chambers (p= 0.1401, two-way ANOVA, F(1,24) = 2.328) (Figure 3.4H). Overall, APC cKO mice display modestly reduced interest social interest, relative to controls, whereas their interest in non-social odors is normal.

3.5 Excessive β-catenin Levels in the APC cKO Forebrain.

To begin to define neuronal changes caused by APC loss, we tested for altered levels of the APC binding partner β-catenin that, in turn, modulates synaptic function and plasticity via its dual roles in cadherin-containing synaptic adhesion complexes and the canonical Wnt signal transduction pathway. Consistent with APC's role as a negative regulator of β-catenin levels in the canonical Wnt pathway (Stamos and Weis 2013), β-catenin protein levels increased two-fold in APC cKO forebrain regions (Figure 3.5A; hippocampus, p = 0.00022; cortex, p = 0.025; striatum, p = 0.012, Student's t-test), whereas *ctnnb1* mRNA levels were not altered (quantitative PCR; cortex, p = 0.285; hippocampus, p = 0.086, Student's t-test).

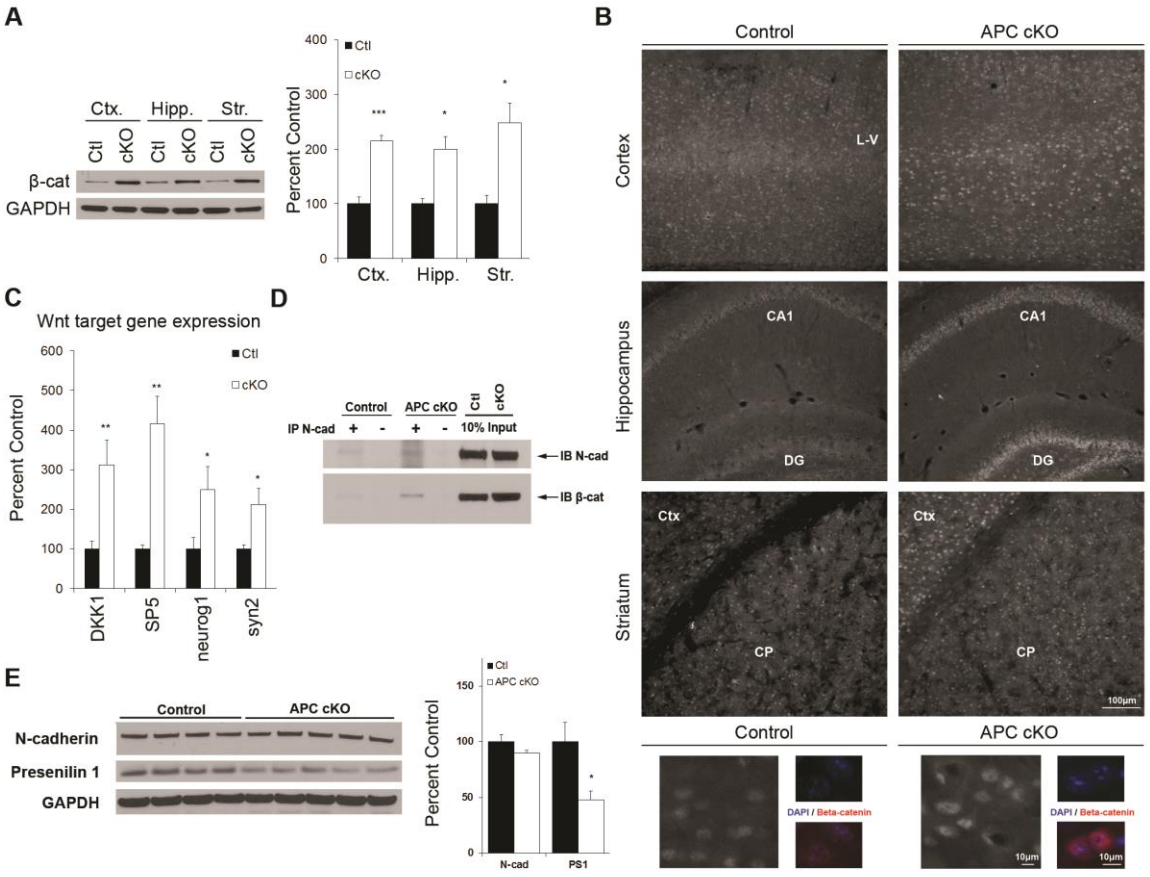

Figure 3.5 – Increased levels of β-catenin and canonical Wnt target gene expression in APC cKO forebrain neurons. Representative immunoblot and quantification (A) of hippocampal, cortical and striatal lysates show two-fold increased levels of β-catenin in APC cKO mice. Signals were normalized to GAPDH as a loading control. n= 5 cKO; 4 ctl littermates. (B) Epifluorescence micrographs show increased β-catenin nuclear immunostaining in forebrain neurons of APC cKO mice, compared with control littermates, processed in parallel, consistent with enhanced canonical Wnt signaling. Neuronal nuclei were identified by their characteristic size and shape, and by DAPI staining. Lower panels: higher magnification views of increased nuclear β-catenin immunostaining in APC cKO cortical layer 5 pyramidal neurons (characteristic triangular shape of soma detectable because of increased cytoplasmic β-cat levels as well) relative to control littermate layer 5 neurons (red, β-catenin staining; blue, DAPI nuclear staining). (C) Histogram showing APC cKOs display increased transcript levels of the Wnt target genes: dkk1, Sp5, neurog1 and syn2 in the hippocampus (*$p < 0.05$, **$p < 0.01$, Student's t-test; n=5 APC cKO, 6 ctl littermate mice). (D) Immunoprecipitation of N-cadherin from the hippocampus showing increased association with β-catenin (n=2 APC cKO, Ctl). (E) Quantitative Immunoblot and histogram showing unchanged levels of N-cadherin and a significant decrease in Presenilin 1 levels (*$p<0.05$, Student's t-test, n=5 APC cKO, 4 Ctl).

APC cKOs displayed dysregulation of the β-catenin/Wnt pathway. β-catenin nuclear localization was increased in APC cKO cortical, hippocampal, and striatal neurons (Figure 3.5B; nuclear fluorescent pixel intensity/um^2; n=4 cKO, 3 ctl mice; n=30 cKO, 33 ctl neurons, p=0.003; 18 cKO, 18 ctl, p=5.0e-7; 49 cKO, 52 ctl, p=0.02 Student's t-test, respective regions). Additionally, several Wnt target genes showed augmented expression levels in the APC cKO hippocampus, including *Sp5*, a transcriptional repressor of Sp1 genes (Fujimura et al. 2007) (p=0.005, Student's t-test), *neurog1*, implicated in cortical neuronal differentiation (Hirabayashi et al. 2004; Luo et al. 2010) (p=0.038), *syn*2, a synaptic vesicle phosphoprotein that functions to maintain the reserve pool of glutamatergic vesicles (Gitler et al. 2004), recently predicted to be a Wnt target (Hodar et al. 2010), (p=0.021) and *dkk1*, a Wnt antagonist (p=0.011) (Figure 3.5C). Increased dkk1 mRNA levels is indicative of a feedback mechanism to restrain enhanced Wnt signaling, as seen in transgenic mice with conditional augmentation of β-catenin in the forebrain (Diep et al. 2004).

N-cadherin adhesion complexes were also altered in the APC cKO hippocampus. β-catenin showed increased co-precipitation with N-cadherin (Figure 3.5D), whereas total levels of N-cadherin were not altered (Figure 3.5E), suggesting their enhanced association at the synapse. Next, we tested for changes in presenilin1 levels, based on its interactions with these synaptic components (Marambaud et al. 2003). Presenilin1 levels were reduced (Figure 3.6F; hippocampus, p = 0.021, Student's t-test). Presenilin1 cKO, using a similar CamkII-Cre line, caused cognitive impairments (Yu et al. 2001). Taken

together, these molecular changes identify APC as a hub that links to and regulates synaptic adhesion and signal transduction networks.

3.6 Increased Spine Density and Electrophysiological Properties in the APC cKO Mouse

Enhanced β-catenin levels lead to increased synaptic spine density in cultured hippocampal neurons (Kundel et al. 2009; Yu and Malenka 2004). APC cKO mice exhibited increased synaptic spine density *in vivo*, on apical dendrites of cortical layer 5 pyramidal neurons ($p=0.014$, Student's t-test) and hippocampal CA1 pyramidal neurons ($p=0.0002$) (Figure 3.6A,B). Further, less mature stub shaped spines predominated in APC cKOs (81.6% to 38.9% ctl, $p=0.00002$, Student's t-test), whereas mushroom spines predominated in control littermates (48.3% to 13.5% cKO, $p=0.00004$).

Next, we assessed synaptic function in hippocampal CA1 pyramidal neurons of freshly isolated brain slices. Whole cell recordings revealed 4-fold increases in the frequency of AMPA-mediated miniature excitatory postsynaptic currents (mEPSCs) in APC cKOs (Figure 3.6C,D; $p = 0.033$, Student's t-test). Increased β-catenin levels may be the underlying cause (Figure 3.6A,B), as augmented β-catenin levels lead to similar changes in mEPSC frequency in cultured hippocampal neurons (Murase, Mosser, and Schuman 2002).

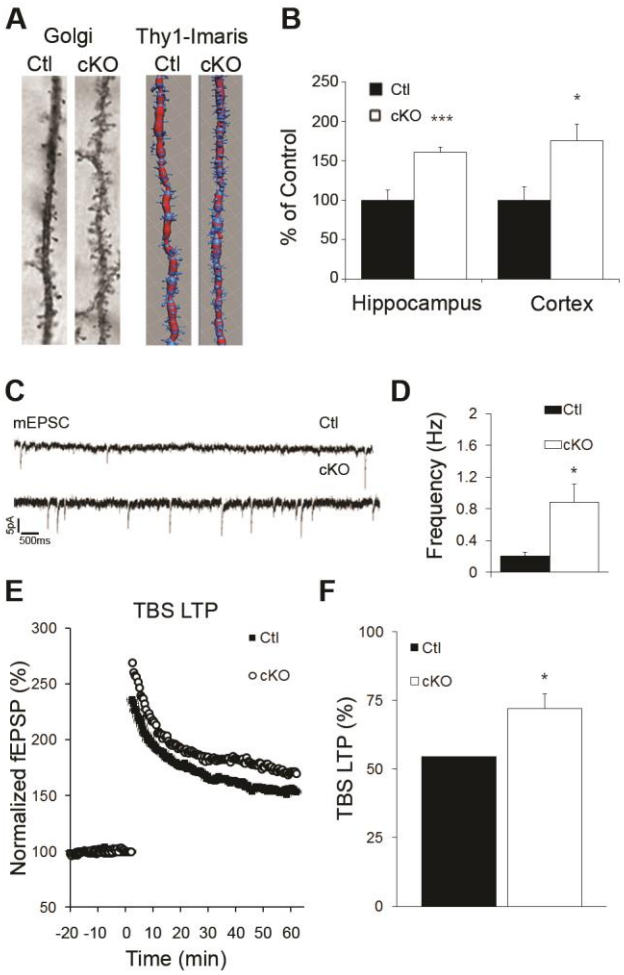

Figure 3.6 - APC loss leads to increased synaptic spine density, greater frequency of mEPSCs, and modestly enhanced synaptic plasticity in pyramidal neurons. (A) Representative images showing increased spines on the apical dendrite of APC cKO cortical layer 5 pyramidal neurons. (left panels) Golgi-Cox stained brightfield images; (right panels) IMARIS reconstructions of confocal stacks of apical dendrite (red) and spines (blue) from APC cKO-Thy-1-YFP and littermate control layer 5 neurons. (B) Histogram shows increased spine density (number of spines per unit length) on the apical dendrite of APC cKO cortical layer 5 pyramidal neurons (175.1 ± 21.7% of ctl littermate levels) and hippocampal CA1 pyramidal neurons in the striatum radiatum (160.7 ± 6.9% of ctl levels) (* $p<0.05$, *** $p < 0.001$, Student's t-test; n = 10 neurons each region, 3 ctl and 3 cKO). (C) Quantification of spine density from golgi stained hippocampal apical dendrites quantified as a percentage of total spines analyzed (*** $p<0.001$, Student's t-test, n=10 APC cKO, 10 Ctl neurons, average of 10 side plane view spines/neuron). (D) Representative traces and histogram of frequency of AMPAR-mediated mEPSCs measured by whole-cell recordings from CA1 neurons of control and APC cKO brain slices. APC cKO CA1 neurons show increased AMPAR mEPSC frequency (cKO = 0.8835 ± 0.22 Hz; ctl = 0.208 ± 0.049

Hz). (E) APC cKO mice show modestly enhanced LTP induced at SC-CA1 synapses by delivering 5 trains of theta burst stimulation in hippocampal slices. *p = 0.0227, repeated measure ANOVA. n = 11 cKOs and 10 ctls.

APC cKO CA1 neurons displayed no change in AMPAR mEPSC amplitude (p = 0.10 Student's t-test) or rise time (p = 0.61), suggesting no alteration in baseline postsynaptic sensitivity. Decay kinetics were more rapid (p = 0.044), however, consistent with AMPARs that lack GRIA2 or contain GRIA4, characteristic of immature synapses (Liu and Savtchouk 2012).

Increased mEPSC frequency in APC cKOs suggests an increase in release probability or synapse number. To assess presynaptic function, paired pulse facilitation (PPF) at Schaffer collateral (SC)-CA1 synapses showed no significant change (p= 0.390, two-way ANOVA, F(1,9) = 0.83) (Figure 3.7A). Increased mEPSC frequency, with no change in PPF, suggests an increase in density of functional presynaptic terminals.

Basal excitatory synaptic transmission was normal, based on extracellular recordings of field excitatory postsynaptic currents (fEPSCs) at SC-CA1 synapses. There was no change in the ratio of stimulus intensity to the slope of fEPSCs (input/output, p= 0.277, two-way ANOVA F(1,9) = 1.34) (Figure 3.7B,C). Taken together, the structural and functional data suggest an increased number of immature excitatory synapses in APC cKOs.

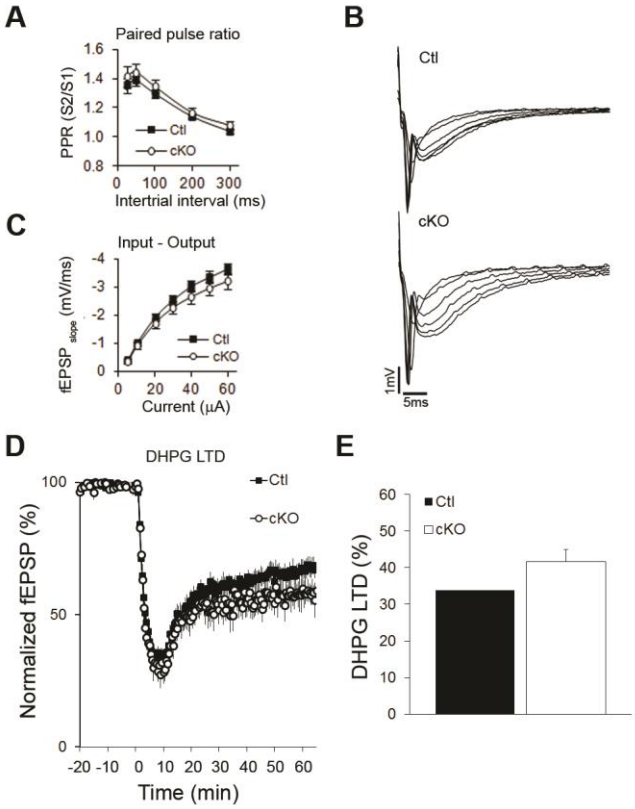

Figure 3.7 - Basal synaptic transmission and mGluR-dependent LTD are not altered in the APC cKO hippocampus. (a) Paired pulse ratio is unaffected at SC-CA1 synapses of APC cKOs. n = 5 cKOs and 6 ctl littermates. (b) Representative traces of fEPSPs measured by extracellular recordings from the CA1 region in response to Schaffer collateral stimulation in freshly isolated brain slices. (c) Quantification of traces (b) indicates normal input-output relationships in APC cKO hippocampal CA1 neurons, suggesting no change in basal synaptic transmission. n = 5 cKOs and 6 ctls. (d) APC cKOs, compared with control littermates, show no significant change in mGluR-dependent LTD induced at Schaffer collateral-CA1 synapses by treating freshly isolated hippocampal slices with DHPG (100 μM) for 10 minutes. p =0.0743 repeated measure ANOVA, F(1,18) = 3.59, n = 11 cKOs and 10 ctls. (e) Histogram for averaged last 10 minutes of each genotype.

APC cKO hippocampal SC-CA1 synapses showed significant, modest increases in long-term potentiation (LTP) induced by five trains of theta burst stimulation (TBS) (Figure 3.6E; p = 0.0315, repeated measure ANOVA, F(1, 19) = 5.392). The modest plasticity change in TBS-induced LTP in APC cKOs resembles that caused by excessive

Wnt signaling in wild-type hippocampal slices, with facilitated induction of LTP via acute and rapid upregulation of synaptic NMDA receptor currents (Chen, Park, and Tang 2006; Cerpa et al. 2011). Metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD) was not significantly altered ($p = 0.0743$ repeated measure ANOVA, $F(1,18) = 3.59$) at SC-CA1 synapses in APC cKO hippocampal slices treated with the group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG) (Figure 3.7D).

Taken together, these findings demonstrate an important role for APC in the mammalian brain in governing the cognitive and behavioral functions associated with ASD and ID.

Chapter 4:

Neuronal Overexpression of β-catenin Results in Severe Intellectual Disabilities

Contributions: I designed the project; I performed and analyzed all behavioral experiments; I performed all of the immunoblots, sub-cellular fractionations, immunoprecipitations, and qPCR experiments

4.1 Overview

Intellectual disabilities (ID) are prevalent in the general population. Treatments are lacking because these disorders are molecularly ill-defined. Our studies are elucidating the role of the Wnt signal transduction pathway in learning and memory, mediated by the two key components, β-catenin and adenomatous APC. β-catenin functions in both cadherin synaptic adhesion complexes and canonical Wnt target gene expression; these pathways modulate synaptic density, maturation, and plasticity, and are essential for proper brain function. APC is the major negative regulator of β-catenin, but also has other neuronal functions - regulating cytoskeletal dynamics and binding selected mRNAs involved in synaptic maturation and function. Human ID-linked gene mutations are predicted to cause either loss- or gain-of-function of the β-cat/canonical Wnt pathway. However, direct tests that β-catenin malfunction can cause comorbid ASD and ID, and knowledge of the underlying pathophysiological changes are limited, particularly for excessive β-cat/Wnt signaling. Chapter 3 studies show that targeted deletion of APC in mouse neurons leads to elevated β-catenin, altered synaptic density and function, and impaired learning and memory. We propose that among the downstream multiple effects of APC loss, excessive β-cat is a major cause of these phenotypes. To test this, we have created a mouse that has targeted deletion of the degradation domain of β-catenin (β-cat cOE), resulting in a stabilized protein product that is not regulated by APC. Our new β-cat cOE mouse shows elevated β-catenin levels in the brain, comparable to that of APC cKOs, but several of their phenotypes differ. The differences include more severe cognitive deficits, drastically reduced synaptic plasticity, reductions in surface levels of AMPA receptor subunit, and an unanticipated increase in levels and phosphorylation of

APC. APC binds the mRNA of SynCAM1, a cell adhesion molecule that interacts with protein 4.1N to regulate normal surface expression of GluR1. β-cat COEs show decreased levels of SynCAM1 protein, but not mRNA, as well as decreases in its binding partner protein 4.1N. In contrast, APC cKOs show increases in SynCAM1 protein, but not mRNA, suggesting that APC regulates its translation. Overall our results define novel molecular changes caused by aberrant β-catenin levels in the developing mammalian brain including a possible role for phosphorylation of APC in the regulation of its target mRNAs. Further, we elucidate molecular etiologies that could cause cognitive deficits of different severities.

4.2 Novel β-catenin Conditional Overexpression Mouse Line

To test our hypothesis that elevated levels of β-catenin are the key molecular change responsible for the cognitive deficits observed in APC cKO mice, we have generated a novel mouse line that targets β-catenin directly. Both the time course of β-catenin overexpression and the phenotypes differ from that of all previous reports of β-catenin malfunction in the brain (Figure 4.1) (Mills et al. 2014). We crossed *ctnnb1*$^{fl(ex3)/+}$ mice (Harada et al. 1999) with mice expressing Cre-recombinase under control of the CamKIIα promoter (Rios et al. 2001), to target the same cell types at the same developmental ages as used for APC cKO (Figure 4.2A) (Mohn et al. 2014). In this new line, loxP sequences flank exon 3 of β-catenin which contains the phosphorylation sites recognized by the APC/GSK3β/Axin β-catenin degradation complex. Cre-recombinase leads to deletion of exon 3 within the one floxed allele, resulting in a stabilized protein product that cannot be degraded (Harada et al. 1999). This particular

CamKIIα-Cre line shows Cre recombinase activity starting at late embryonic stages of brain development (Pirone et al. 2016), progressively increasing to full activation by postnatal day 21 as determined by GFP and Lac-Z expression (Rios et al. 2001). This time frame is similar to the normal developmental time course of CamKIIα expression in the forebrain (Figure 4.1).

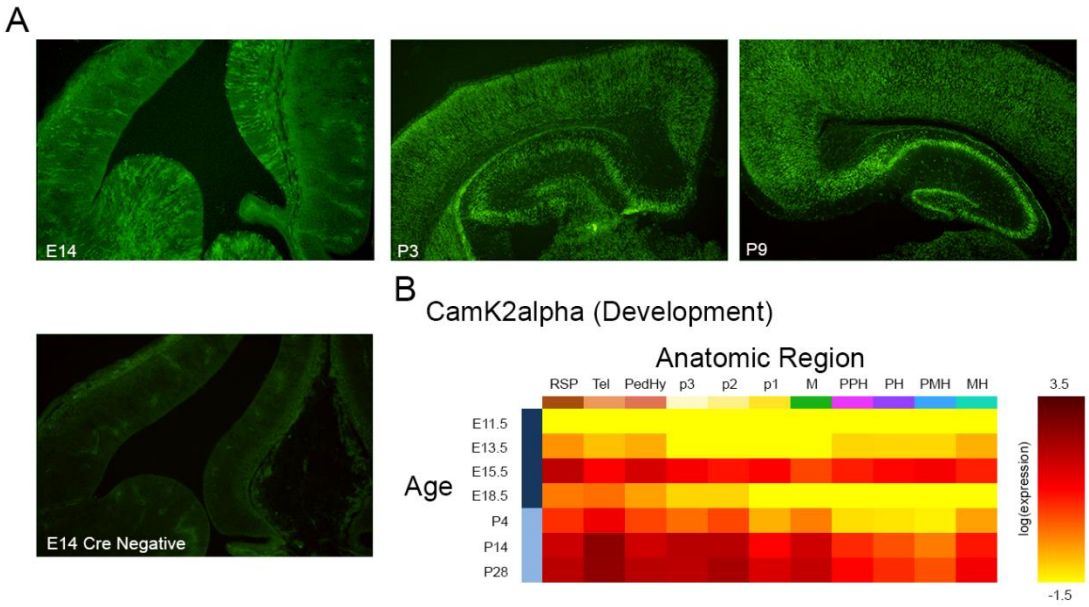

Figure 4.1 Developmental expression of CamKIIα in the mouse. (A) Epifluorescent images of mice expressing a Cre-dependent GFP-tagged ribosomal subunit show early recombination (E14) in the hippocampus when crossed to our CamKIIα-Cre recombinase line. This expression is increased throughout development reaching full activation by P21. (bottom left) GFP expression in Cre negative animals is not observed. (B) This matches the developmental expression pattern of CamKIIα normally seen in the telencephalon which gives rise to the forebrain (Image Credit: Allen Institute).

Quantitative immunoblots of hippocampal lysates show a significant increase in β-catenin levels in the β-cat (fl/+) cOE mice (combined expression of both the full length product from the WT allele and the truncated protein product from the allele missing the exon 3 encoded degradation domain), relative to wild-type littermate levels. Further, the

β-catenin levels are elevated comparably to that of APC cKOs (Control 1.00 ± 0.04, β-cat cOE 1.75 ± 0.12, APC cKO 1.67 ± 0.16, One-way ANOVA F (2, 6) = 12.28, p=0.008; Figure 4.2B). Importantly, the truncated β-catenin protein retains normal function, as it is able to interact with binding partners central to the two key functions, N-cadherin in synaptic adhesion complexes and LEF1, the nuclear transcription co-activator in the canonical Wnt signal transduction pathway (Figure 4.2C). This latter function is confirmed by an increase in the expression of the canonical Wnt target genes *SP5*, *neurog1*, and *syn2* (*SP5*: Control 1.00 ± 0.17, β-cat cOE 1.90 ± 0.15, p=0.028 student's t-test; *neurog1*: Control 1.00 ± 0.13, β-cat cOE 2.28 ± 0.54, p=0.048; *synapsin2*: Control 1.00 ± 0.12, β-cat cOE 1.45 ± 0.13, p=0.040; Figure 4.2D).

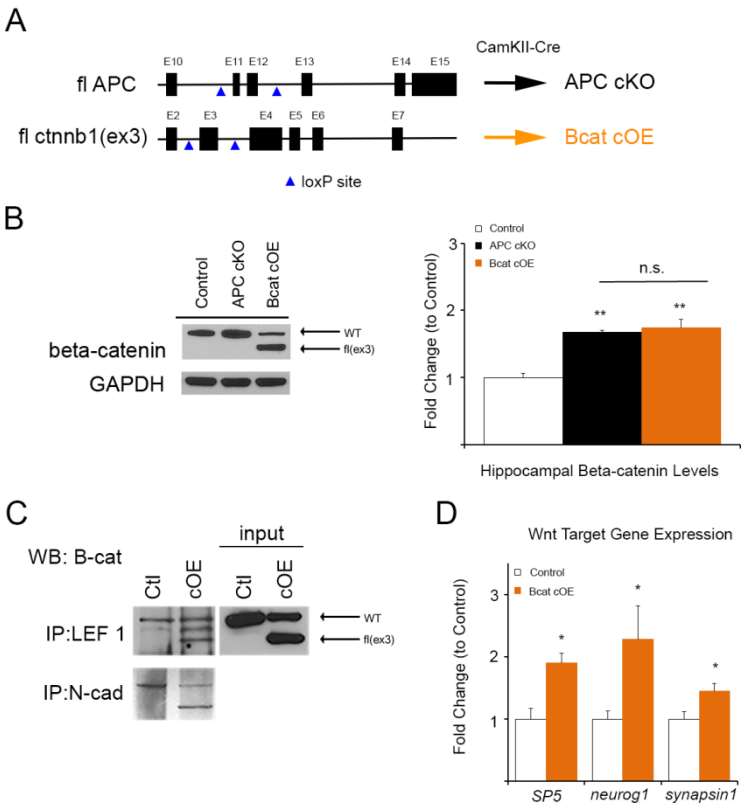

Figure 4.2 A Novel β-catenin overexpressing mouse model. (A) APC cKO and β-cat cOE mice were generated by crossing mice expressing $APC^{fl/fl}$ or $ctnnb1^{+/fl(ex3)}$ to mice expressing Cre-recombinase under control of the CamKIIα promoter. APC cKO are homozygous for the deletion of

APC whereas the β-cat cOE mice are generated through flox-mediated removal of the degradation domain of β-catenin. (B) Immunoblot analysis of hippocampal lysate shows a comparable increase in β-catenin levels in the APC cKO and β-cat cOE mice (n=3 for each genotype, **p<0.01, post-hoc Bonferroni corrected t-test). The truncated protein product in the β-cat cOEs corresponds to the conditionally removed third exon within the ctnnb1 gene. (C) Immunoprecipitation of LEF1 and N-cadherin with β-catenin shows that the mutant β-catenin isoform is able to bind to both synaptic and nuclear components. The middle fragment seen associated with LEF1 may be a calpain mediated cleavage product of β-catenin known to be enhanced through synaptic activity and to have Wnt transcriptional ability (Abe and Takeichi 2007)(D) As expected, there is an increase in expression of three different Wnt target genes, as measured by qPCR (n=5 for β-cat cOE and littermate controls; *p<0.05,**p<0.01, Student's t-test).

4.3 Severe Learning Impairments

Because APC cKO mice demonstrated deficits in hippocampal-dependent learning and memory, we decided to compare the β-cat cOEs to APC cKOs and littermate controls using three separate behavioral tasks for evaluating learning and memory. In the Y-maze test of spontaneous alternating behavior, both β-cat cOE and APC cKO mice showed aberrant memory phenotypes (Figure 4.3A), making a significantly lower percentage of correct choices, compared to control littermates for each line (Control 69.29% ± 1.43, β-cat cOE 54.21% ± 2.09, APC cKO 52.19%. ± 1.36; One-way ANOVA: $F(2,25) = 30.53$, $p<0.001$); both APC cKOs and β-cat cOEs displayed no significant difference from the chance rate of 50% (One-sample t-test: Control $p<0.001$, β-cat cOE $p=0.074$, APC cKO $p=0.153$).

We next tested for spatial learning and memory deficits using the Barnes Maze. As previously demonstrated (Mohn et al. 2014), APC cKO mice took significantly longer to learn the location of the goal hole (2-3 additional trials), and, upon probe trial testing for memory retention, were unable to perform the task (remember the location) after one

week, compared to littermate controls (Figure 4.3B). In sharp contrast, β-cat cOE mice were unable to learn the location of the goal hole over the course of training (up to 8 trials) and were unable to proceed to probe trials due to the lack of learning (Training RM-ANOVA: $F(2,25) = 29.01$, $p<0.001$; Probe RM-ANOVA: $F(1,16) = 53.78$, $p<0.001$). Distance travelled and velocity measured in the Open Field task, showed no differences between genotype, suggesting that the deficits seen in the Barnes Maze were not due to motor defects (data not shown, One-way ANOVA: $F(2, 29) = 0.4997$, $p=0.6118$).

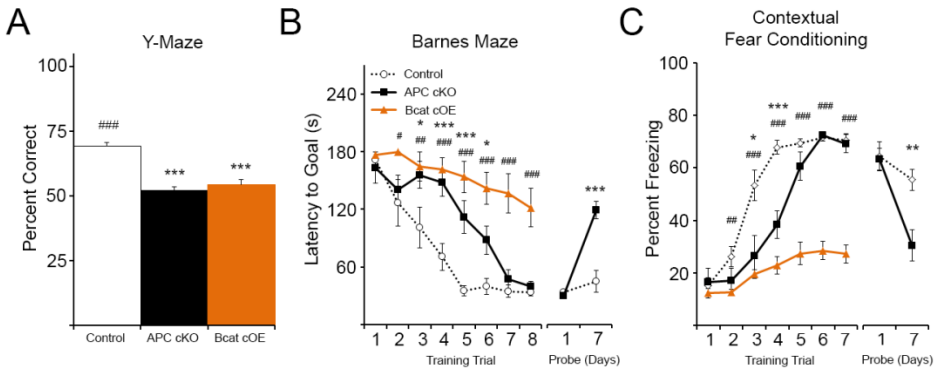

Figure 4.3 Severe cognitive deficits in the β-cat cOE mice. (A) Both β-cat cOE and APC cKO mice show impairments in working spatial memory in the Y-maze (n=10 control, β-cat cOE and 8 APC cKO, ***p<0.001 to control, ###p<0.001 to chance rate of 50%). (B) β-cat cOE show severe spatial learning deficits and were unable to learn the location of the hole over the course of training. By contrast, APC cKO mice show modest learning and memory deficits in the Barnes Maze, taking longer to learn the location of the goal and unable to retain the memory in long-term probe trials after one week. (n=10 control, β-cat cOE and 8 APC cKO; *p<0.05, ***p<0.001 APC cKO to control, #p<0.05, ##p<0.01, ###p<0.001 β-cat cOE to control; post-hoc Bonferroni corrected student t-test). (C) Similar to results seen in the Barnes Maze, β-cat cOE mice show severe deficits in hippocampal dependent contextual fear conditioning, showing a lack of freezing behavior relative to controls over the course of the training trials. APC cKO mice take longer to reach the freezing levels of controls and show decreased long-term memory in probe trials (n=11 control, 9 APC cKO and 8 β-cat cOE; *p<0.05, **p<0.01,***p<0.001 APC cKO to control, ##p<0.01 β-cat cOE to control ###p<0.001; post-hoc Bonferroni corrected student t-test).

To further verify the differences in the severity of cognitive impairments between both mutant models with elevated β-catenin levels, we employed contextual fear-conditioning, another learning and memory assay. Similar to the results observed in the Barnes Maze, APC cKO mice took longer to associate the context with the shock, compared with littermate controls, and showed deficits in long-term retention of the fear memory (Figure 4.3C). The β-cat cOE mice showed little improvement in their performance, never reaching the freezing level of control littermates over the course of training (7 days), and, again, were not able to be tested in probe trials (Training RM-ANOVA: $F(2,25) = 88.9$, $p<0.001$; Probe RM-ANOVA: $F(1,18) = 4.98$, $p=0.039$). Pain threshold, as measured by a latency to paw lick on a heated plate, was no different between genotypes, suggesting the two models exhibit cognitive deficits of different severities, but not sensory defects such as reduced sensitivity to the foot shock (data not shown, One-way ANOVA: $F(2, 21) = 0.4576$, $p=0.6390$).

4.4 Aberrant Synaptic Plasticity

To elucidate the mechanisms that underlie the differences in cognitive skills we observed between APC cKO and β-cat cOE mice, we examined both long-term potentiation and depression (LTP and LTD), thought to be electrophysiological correlates of synaptic plasticity required for learning and memory. We employed 100Hz theta-burst stimulation (TBS-LTP) and 1Hz stimulation (LTD) of the CA3-CA1 Schaffer collateral synapses in acute hippocampal slices. β-cat cOEs exhibited severely reduced LTP, including decreased induction and a very small, although significant increase in fEPSP slope above baseline values. In contrast, APC cKOs showed a slight but significant

enhancement of LTP relative to littermate controls (Figure 4.4A), similar to previous results (Mohn et al. 2014) (RM-ANOVA: $F(2,26) = 49.49$, $p<0.001$; comparison to 100% baseline, One-sample t-test: Control 156.6% ± 5.6, $p<0.001$; β-cat cOE 112.6% ± 2.4, $p=0.0011$; APC cKO 175.4% ± 5.5, $p<0.001$).

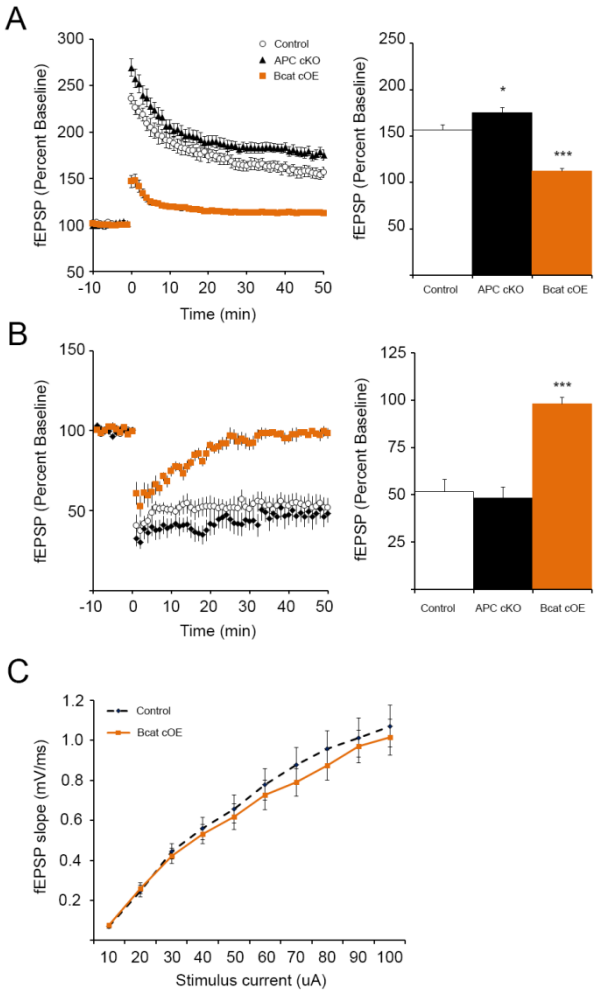

Figure 4.4 β-cat cOEs display drastically reduced synaptic plasticity
(A) Theta-burst stimluation in acute hippocampal slices shows a significant lack of LTP in the β-cat cOE mouse. Conversely, LTP in the APC cKO mice is slightly enhanced (n=10 slices control, 11 APC cKO, 8 βcat cOE; histograms are averaged relative fEPSP slopes over the last 10 minutes of recording from each mouse; *p<0.05,***p<0.001 to control, post-hoc Bonferroni corrected student's t-test). (B) Hippocampal 1Hz LTD shows a severely reduced in the βcat cOE compared to control, returning to baseline levels, while it is normal in APC cKO mice (n=6

slices each genotype; ***p<0.001 to control, post-hoc Bonferroni corrected student's t-test). (C) Input-output curves suggest normal AMPA receptor mediated basal transmission in the βcat cOE mice while it is normal in APC cKOs (Mohn et al. 2014).

LTD stimulation (1 Hz) in β-cat cOE slices displayed LTD induction, but the reduction in fEPSP slope was not maintained and rapidly returned to baseline levels, relative to LTD in control littermates. In comparison, APC cKO hippocampal slices showed no significant difference relative to control littermates (Figure 4.4B), similar to previous results for mGluR5 dependent LTD (Mohn et al. 2014). (RM-ANOVA: $F(2,15) = 33.96$, $p<0.001$; comparison to 100% baseline One-sample t-test: Control 61.2% ± 4.5, $p<0.001$; β-cat cOE 98.2% ± 3.3. $p=0.5992$; APC cKO 48.7% ± 4.8, $p<0.001$). Basal synaptic transmission, measured by input-output curves, was not significantly changed in either β-cat cOE (RM ANOVA: $F(1, 12) = 0.1775$, $p=0.6810$, Figure 4.4C) or the APC cKO (Mohn et al. 2014).

4.5 Abnormal Glutamate Receptor Levels

This led us to question what molecular mechanisms could account for the different electrophysiological and learning phenotypes that we observed in the APC cKO and β-cat cOE mice. LTP and LTD activation is often mediated via NMDA receptor activation modulating the insertion or removal of AMPA receptors containing the activity dependent subunit GluR1. GluR1 levels are significantly decreased in membrane preparations isolated from the hippocampus of β-cat cOEs, whereas GluR1 levels are increased in APC cKOs, compared to controls (Control 1.0 ± 0.02, β-cat cOE 0.59 ± 0.03, APC cKO 1.30 ± 0.06; One-way ANOVA: $F (2, 6) = 223.5$, $p<0.001$ - Figure 4.5). As

hippocampal AMPA receptors are composed of primarily GluR1/GluR2 or GluR2/GluR3 heterodimers, we examined the levels of GluR2, the constitutively trafficked subunit, to see if overall AMPA receptor levels are changed. GluR2 levels are not significantly different in either mutant mouse model relative to controls, suggesting a selective lack of incorporation of the GluR1 subunit, rather than a reduction in total AMPA receptor levels (Control 1.0 ± 0.07, β-cat cOE 1.03 ± 0.01, APC cKO 1.10 ± 0.12; One-way ANOVA: $F(2, 6) = 1.162$, $p=0.375$). The lack of change in GluR2 AMPA receptor levels is consistent with the result that basal synaptic transmission was not significantly altered in either APC cKOs (Mohn et al. 2014) or β-cat cOEs.

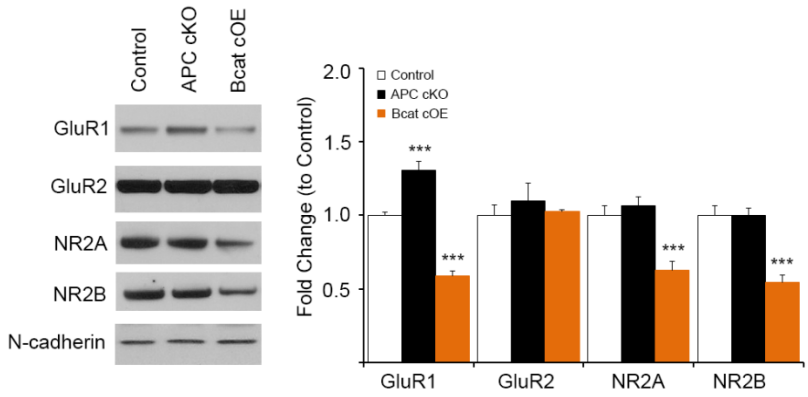

Figure 4.5. Decreased glutamate receptor surface levels in the β-cat cOE hippocampus. Immunoblots from membrane fractions from the hippocampus of β-cat cOE and APC cKO mice show altered levels of glutamate receptor subunit expression. The activity-trafficked GluR1 subunit is significantly reduced in the β-cat cOE whereas it is increased in the APC cKO. GluR2 levels are unchanged in both mutant models. NMDA receptor subunits NR2A and NR2B are both reduced in the β-cat cOE (n=3 sets of membrane fractions from four pooled hippocampi; ***$p<0.001$ to control, post-hoc Bonferroni corrected student's t-test).

Based on the reduced GluR1 levels in β-cat cOEs, we next measured the levels of NMDA receptor NR2A and NR2B subunits in membrane fractions by immunoblotting. We found that both NR2A and NR2B levels are unchanged in APC cKOs. Conversely,

both subunits are in β-cat cOE mice. (NR2A Control 1.00 ± 0.06, β-cat cOE 0.63 ± 0.06, APC cKO 1.09 ± 0.06; One-way ANOVA: F (2, 6) = 42.36, $p<0.001$; NR2B Control 1.00 ± 0.06, β-cat cOE 0.54 ± 0.05, APC cKO 1.00 ± 0.05; One-way ANOVA: F(2, 6) = 68.69, $p<0.001$). This reduction of both the activity-dependent AMPA receptor GluR1 subunit and NMDA receptor NR2A and 2B subunits may be responsible for the severe deficits we observe in LTP induction and the lack of maintenance of both LTP and LTD in the β-cat cOE hippocampus CA1-CA3 synapses.

4.6 Changes in SynCAM1 and protein 4.1N levels

In order to assess these differences in GluR1 levels, we examined proteins that have been reported to affect GluR1 membrane insertion and stability. One of the reported APC mRNA targets encodes the protein SynCAM1, a cell adhesion molecule necessary for proper insertion of both GluR1 and NMDA receptors via interactions with protein 4.1 family members (Hoy et al. 2009).

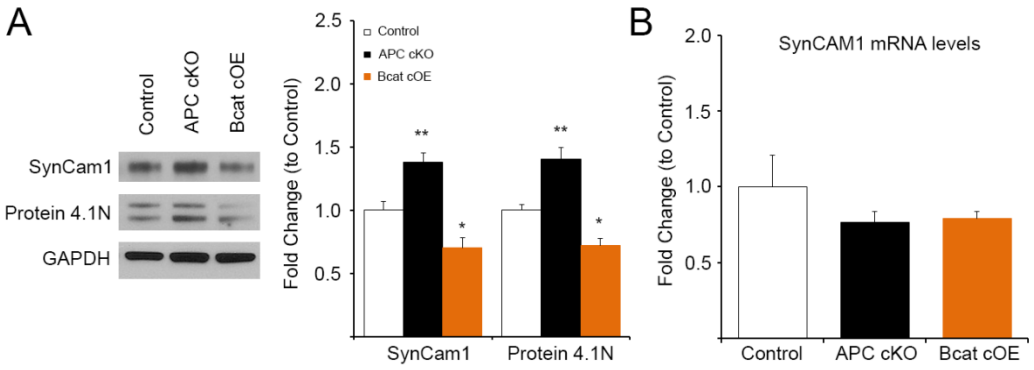

Figure 4.6. Alterations in SynCAM1 and protein 4.1N levels. (A) Immunoblots of an APC mRNA target involved in GluR1 insertion, SynCAM1, shows increased levels in the APC cKO that are decreased in

the β-cat cOE. SynCAM1 has been shown to interact with protein 4.1N to regulate GluR1 clustering. Levels of 4.1N are significantly increased in the APC cKO and decreased in the β-cat cOE (n=6 each genotype; *p<0.05, **p<0.01 to control, post-hoc Bonferroni corrected student's t-test). The doublet observed in protein 4.1N has been also detected in the rat brain with this antibody. (B) *SynCAM1* mRNA levels are unchanged suggesting that the differences in protein levels is not due to differences in transcription.

Immunoblot analysis of β-cat cOE hippocampal lysates show significant decreases in SynCAM and protein 4.1N levels, consistent with the decreases in GluR1 membrane levels. In contrast, SynCAM1 protein levels are significantly increased the hippocampus of the APC cKO, along with a concurrent increase in protein 4.1N (Figure 4.6A), consistent with the increases in GluR1 (SynCam1: Control 1.00 ± 0.04, β-cat cOE 0.72 ± 0.06, APC cKO 1.41 ± 0.09; One-way ANOVA $F(2, 15) = 27.82$, $p<0.001$; protein 4.1N: Control 1.00 ± 0.02, β-cat cOE 0.82 ± 0.02, APC cKO 1.24 ± 0.08; One-way ANOVA $F(2,15) = 26.51$, $p<0.001$). Neither APC cKOs nor β-cat cOEs showed significant changes in SynCAM mRNA levels, compared to control (One-way ANOVA: $F(2, 12) = 0.9853$, $p=0.4016$, Figure 4.6B). Although the extent of change in these protein levels is modest, it has been shown that similarly small alterations in these protein levels can significantly affect plasticity (Hoy et al. 2009) and, therefore, may affect cognitive behaviors. In particular, decreases in protein 4.1N levels have been previously associated with decreases in GluR1 insertion and reduced synaptic strength, whereas increases in levels result in higher GluR1 clustering and increased synaptic strength (Hoy et al. 2009).

4.7 Phosphorylation Shift in APC and increased association of β-catenin

As SynCAM1 is an APC mRNA target (Preitner et al. 2014), the changes in SynCAM1 and protein 4.1N led us to believe that these results may be due to the presence of APC in the β-cat cOE mice. Therefore, we assessed APC levels and functions in the β-cat cOEs. As expected, APC levels were drastically reduced in immunoblots of hippocampal lysate from APC cKOs (the residual levels of APC are most likely due to other cell types (interneurons and glia) that do not express CamKIIα dependent Cre-recombinase) (Figure 4.7A). Surprisingly, we observed what seems to be a size-based mobility shift and increase in levels of APC in the β-cat cOE hippocampus, compared to littermate controls (Control 1.00 ± 0.06, β-cat cOE 1.60 ± 0.12, APC cKO 0.21 ± 0.03, One-way ANOVA $F(2,6) = 73.48$, $p<0.0001$). Size shifts in proteins are often due to post translational modification such as phosphorylation. We do not see changes in APC mRNA levels in the β-cat cOE, suggesting that any increase in APC protein levels is not due to increase transcription.

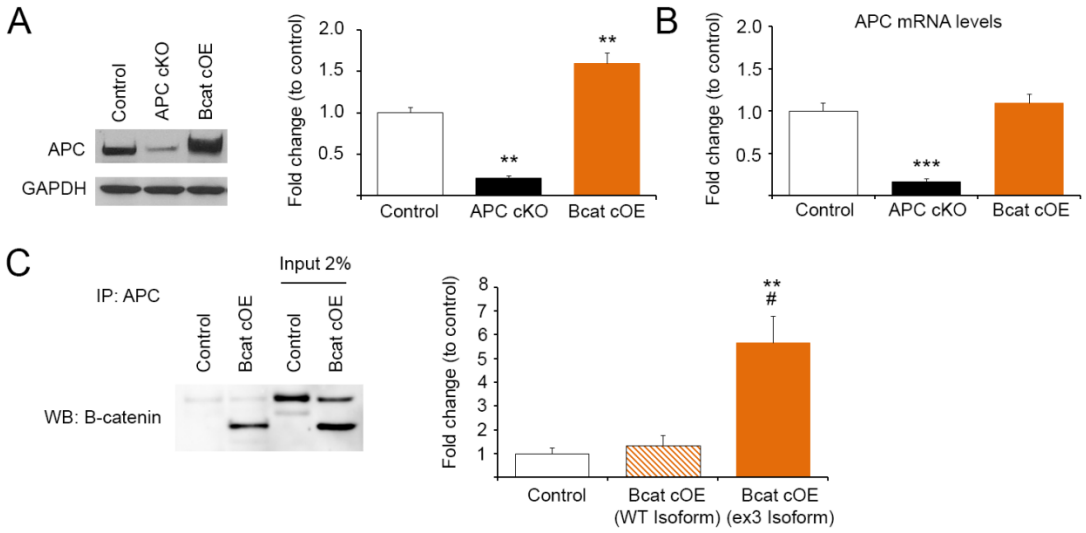

Figure 4.7. Mobility shift in APC and an increased association of APC with β-catenin in the β-cat cOE. (A) Immuno blot of APC shows a size shift, consistent with post-translational modifications, and an increase

in APC levels in the β-cat cOE (n = 3 each genotype, **p<0.01 post-hoc Bonferroni corrected t-test) (B) There is no increase in *APC* mRNA levels in the β-cat cOE hippocampus, suggesting a regulation of the APC protein itself (n = 3 each genotype, **p<0.01 post-hoc Bonferroni corrected t-test) (C) Co-immunoprecipitation of APC shows that the mutant isoform of β-catenin in the β-cat cOE hippocampus associates more strongly with APC (n = 3 each genotype, **p<0.01to control, #p<0.05 to wild-type (WT) isoform, post-hoc Bonferroni corrected t-test)

Previous studies have shown that β-catenin truncations that lack the degradation domain but still retain APC binding function, show increased association with APC (Munemitsu et al. 1996). This is accompanied by a subsequent phosphorylation of APC - potentially by GSK3β which has been shown to phosphorylate residues flanking the 20aa repeats on APC that mediate β-catenin binding (Ikeda et al. 2000), and a shift in APC size similar to what we observe (Munemitsu et al. 1996). Although this modification may stabilize the APC protein, any increases in levels are not due to transcription of the APC gene (Figure 4.7B) as revealed by qPCR (Control 1.00 ± 0.09, β-cat cOE 1.09 ± 0.1098, APC cKO 0.16 ± 0.04, One-way ANOVA $F(2,11) = 28.87$, $p<0.001$).

When we immunoprecipitate APC and probe for β-catenin (Figure 4.7C), we observe an increased association of the truncated β-catenin isoform with APC similar to what was described in the previous report (Munemitsu et al. 1996) (Control 1.00 ± 0.24, β-cat cOE wildtype (WT) isoform 1.36 ± 0.40, βcat cOE degradation resistant (ex3) isoform 5.66 ± 1.09, One-way ANOVA $F(2,6) = 14.3$, $p = 0.0052$). This suggests that the APC gel-mobility shift we observe may be occurring through the same mechanism. It is possible that deletion of exon 3 stabilizes the APC/β-catenin complex and facilitates GSK3β phosphorylation of APC although this remains to be determined.

4.8 A model of APC Regulation of Synaptic Plasticity

Our findings define a novel role for APC by suggesting that it may regulate synaptic plasticity required for learning and memory by functioning to modulate SynCAM levels, which, in turn, alter protein 4.1N and the trafficking of the activity-dependent GluR1 AMPA receptor subunit (Figure 4.8). These results suggest that APC and β-catenin networks play essential and previously unappreciated roles in the mammalian brain.

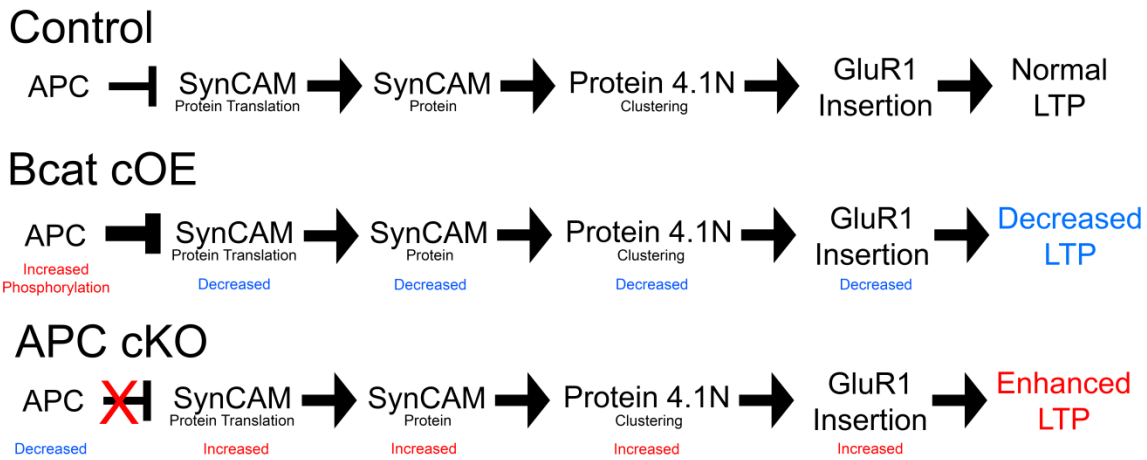

Figure 4.8 Schematic for potential role of APC in the regulation of GluR1. We propose a novel role for APC mayas a negative regulator of SynCAM1 translation. APC binds SynCAM mRNA, and, when APC levels and phosphorylation are increased, as is seen in the β-cat cOE hippocampus, SynCAM1 protein levels are reduced. Conversely, in the absence of APC, as in the APC cKO hippocampus, SynCAM1 levels are increased. SynCAM1 levels positively regulate protein 4.1N levels and GluR1 membrane insertion. Therefore, GluR1 insertion into the surface is dependent on the proper regulation of APC.

In this study we also show that elevating the expression of β-catenin may have implications on APC functions. When APC is hyperphosphorylated, as in β-cat cOEs, cognitive impairments are severe, and both LTP and LTD are drastically reduced. In

contrast, lack of APC (and a subsequent lack of phosphorylation) results in a mild cognitive deficits and a modestly enhanced LTP.

Taken together, our findings are elucidating novel molecular etiologies that can lead to intellectual deficits of different severities.

Chapter 5:

Reduction of β-catenin ameliorates the autistic-like phenotypes of APC cKO mouse

Contributions: I designed the project; I performed and analyzed all behavioral experiments; I performed all of the immunoblots, sub-cellular fractionations, immunoprecipitations, and qPCR experiments

5.1 Overview

Autism spectrum disorders (ASD) and intellectual disabilities (ID) affect a relatively large percentage of children in the United States. Large genome-wide association studies have identified Wnt/β-catenin as one of several key pathways involved in the pathophysiology of the disorder. Our previous studies, using a mouse with conditional knockout (cKO) of the adenomatous polyposis coli (APC) protein, showed that loss of APC in primarily excitatory neurons of the forebrain resulted in autistic-like behaviors including reduced social interaction and increased repetitive behaviors. APC cKO mice also showed learning and memory deficits. Given APC's known functions in regulating β-catenin, APC loss resulted in increases in β-catenin levels in the cortex, hippocampus, and striatum. This resulted in increased binding of β-catenin to synaptic n-cadherin and an increase in Wnt target gene expression. We concluded that the increase in β-catenin might be responsible for the phenotypes we observed. In order to address this we have created two additional mouse models: one with conditional overexpression of β-catenin (β-cat-cOE), and one where we prevent the increase in β-catenin levels in the APC cKO background by conditional removal of the *ctnnb1* gene product as well (APC/β-cat cKO). We report that cOE show autistic-like behaviors, similar (although not identical) to the APC cKO. APC/β-cat cKO, however, do not display any social or repetitive behavioral phenotypes suggesting that the phenotypes we observe in the other mutant models is most-likely due to the increase in β-catenin. Surprisingly, we see an increase in the transcript levels of several Wnt target genes and an increase and apparent functional replacement of β-catenin by the closely related catenin, junction plakoglobin (γ-catenin).

5.2 CamKII Mediated Recombination in the New Mutant Mouse Models

In order to understand the role of β-catenin in the autistic-like phenotypes of the APC cKO we created two new mutant mouse models under the control of the same CamKIIα-Cre driver we have used for the APC cKO mouse (Rios et al. 2001). To test the hypothesis that increased β-catenin results in the behavioral phenotypes we crossed the CamKIIα mice to 1) mice expressing loxP sites flanking exon 3 of the *ctnnb1* gene (see also Chapter 4) (Harada et al. 1999), and 2) mice with loxP sites flanking exon 2 and exon 6 of the *ctnnb1* gene and flanking exon 11 and exon 12 of the *APC* gene (Brault et al. 2001; Gounari et al. 2005) (Figure 5.1A).

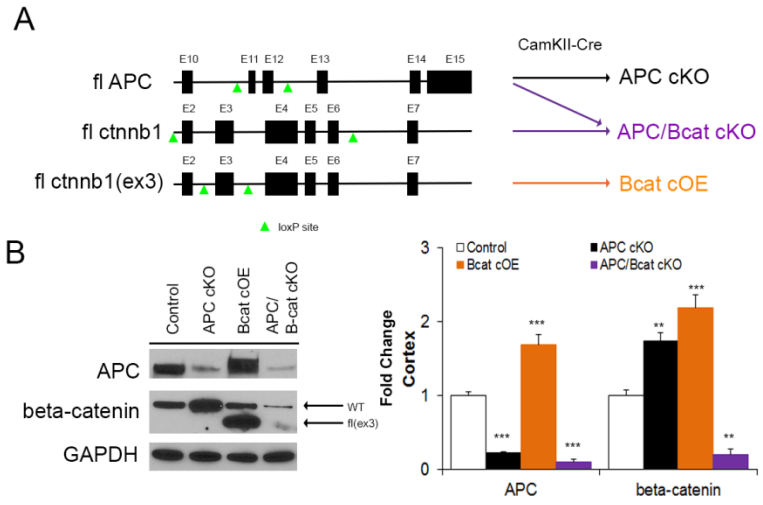

Figure 5.1 Novel mouse models to study the role of β-catenin in ASDs. (A)We created two new mouse conditional mouse models under control of CamKIIα-Cre recombinase to study the role of β-catenin in autistic-like behaviors. The β-cat cOE mouse contains flox sites flanking exon 3 of the *ctnnb1* gene that encodes the degradation domain of β-catenin. Upon Cre mediated recombination, degradation resistant isoform of β-catenin is expressed. The APC/β-cat cKO mice contain flox sites within both alleles of the *APC* and *ctnnb1* genes. CamKIIα-Cre expression results in unstable protein products from both genes that are rapidly degraded. (B) Immunoblot for APC and β-catenin from the cortex of all of the models. Conditional overexpression of β-catenin results in an increase in APC and

β-catenin levels compared to control (n=3 all genotypes. **p<0.01, ***p<0.001 to control, post-hoc Bonferroni corrected t-test).

Exon 3 of *ctnnb1* encodes a domain in the β-catenin protein that contains the phosphorylation sites necessary for degradation by the APC/Axin/GSK3B destruction complex. Cre-mediated recombination in the other mouse model results in unstable protein products for the *ctnnb1* and *APC* genes that result in their rapid degradation. In β-cat cOE mice, heterozygous expression of the degradation resistant isoform of β-catenin is enough to obtain protein levels comparable to what was observed in the APC cKO (Figure 5.1B). Interestingly, we also see an increase in APC levels in this model, suggesting a potential compensation mediated by the cell detecting increase β-catenin levels. APC/β-cat cKO mice show large reductions in both protein levels in the cortex with residual β-catenin and APC levels most likely due to other cell types that do not express CamKIIα (β-catenin: Ctl 1.00 ± 0.08, APC cKO 1.75 ± 0.11, β-cat cOE 2.19 ± 0.17, APC/β-cat cKO 0.20 ± 0.07, One-way ANOVA $F(3,8) = 87.39$, $p<0.001$; APC: Ctl 1.0 ± 0.05, APC cKO 0.23 ± 0.02, β-cat cOE 1.69 ± 0.14, APC/β-cat cKO 0.10 ± 0.03, One-way ANOVA $F(3,8) = 90.13$, $p<0.001$).

Ideally, we would have like to use heterozygous expression of the floxed *ctnnb1* allele, in order to modulate β-catenin levels closer to control (APC cKO/β-cat cHet), but western blots from cortical lysate of this mouse showed no change in β-catenin levels relative to the APC cKO (Figure 5.2) (Control 1.00 ± 0.05, APC cKO 1.59 ± 0.09, APC cKO/β-cat cHet1.61 ± 0.14, One-way ANOVA $F(2,6) = 12.13$, $p = 0.0078$) . This is not all together surprising as these mice still do not have APC, meaning β-catenin protein is

allowed to accumulate even though there is only one allele present. Therefore, these mice were not used in the subsequent experiments.

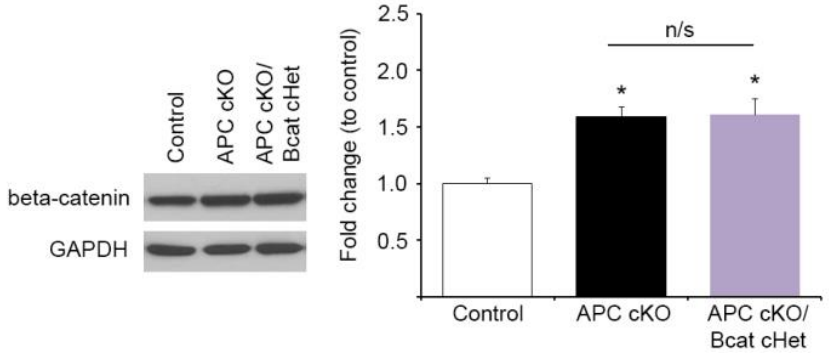

Figure 5.2 Heterozygous deletion of *ctnnb1* in the APC cKO mouse still has elevated β-catenin levels. Cortical lysates from APC cKO/β-cat cHet mice show elevated β-catenin levels comparable to the APC cKO (*$p < 0.05$, post-hoc Bonferroni corrected t-test).

5.3 Autistic-like Phenotypes of the Different Mouse Models

Our previous studies demonstrated that loss of APC resulted in reduced social interaction and increased repetitive behaviors concurrent with an increase in β-catenin levels. To validate our hypothesis that these behavioral phenotypes are mediated by increased β-catenin, we tested the β-cat cOE and APC/β-cat cKO mice in the classic three chamber test compared to the APC cKO and littermate controls. To eliminate motor deficits as a cause for reduced interaction time, distance travelled and velocity were calculated during the habituation phase of this experiment (Figure 5.3) (One-way ANOVA $F(3,38) = 0.4467$, $p = 0.7211$).

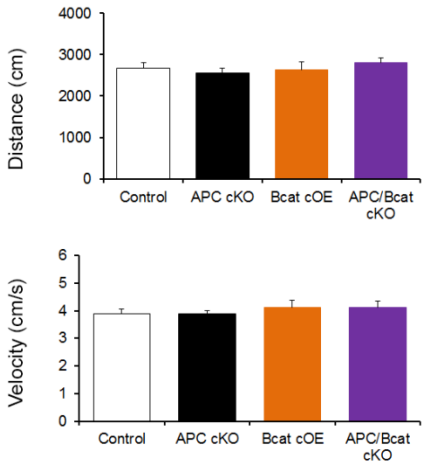

Figure 5.3 Normal locomotor activity. During the habituation phase of three chambered test, no significant differences were seen in both velocity and distance travelled for all of the mouse models.

Similar to previous results, APC cKO mice showed reduced social interaction (Figure 5.4A,B) as measured by the ratio of the time spent interacting with the cage containing the mouse to the empty cage. The β-cat cOE mice also demonstrate this phenotype spending significantly less time interacting with the social cage. Interestingly, APC/β-cat cKO mice demonstrate normal sociability, suggesting that this phenotype can be ameliorated by reducing β-catenin levels (Control 2.03 ± 0.24, APC cKO 1.29 ± 0.18, β-cat cOE 1.15 ± 0.18, APC/β-cat cKO 2.04 ± 0.16; One-way ANOVA, $F(3,38) = 13.17$, $p<0.001$).

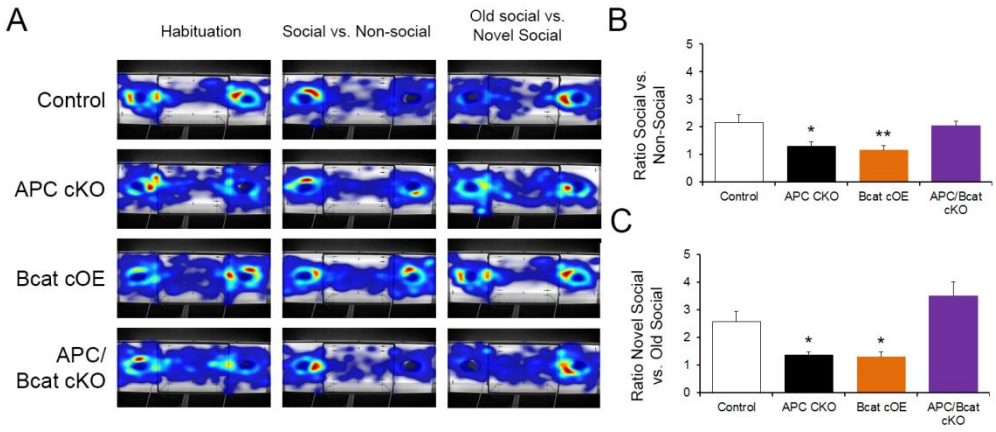

Figure 5.4 Preventing β-catenin increase rescues social behavior. (A) Representative heat-maps of the difference mouse models during the habituation, social interaction, and social memory phases of the three chambered test. (B) Both the APC cKO and β-cat cOE mice spend a significantly reduced ratio of their time interacting with the social cage than with the empty cage compared to controls. APC/β-cat cKO mice show normal sociability (n = 12 control, 10 APC cKO, 10 β-cat cOE, 10 APC/β-cat cOE; *p<0.05, **p<0.01 to control, post-hoc Bonferroni corrected t-test). (C) When presented with a novel mouse, both APC cKO and β-cat cOE mice spend a reduced percent of their time interacting with the novel mouse versus the old social stimuli, suggesting reduced social memory. This phenotype is ameliorated in the APC/β-cat cKO as they interact with the novel mouse much more than the old mouse (*p<0.05 to control, post-hoc Bonferroni corrected t-test).

Our previous studies suggested that APC cKO mice had deficiencies in social memory, as they failed to habituate to social smells on successive trials compared to control (Mohn et al. 2014). To test if the new mutant models had similar deficits we introduced a novel mouse into the three-chambered paradigm above. Both the APC cKO and β-cat cOE mice spent a significantly reduced ratio of time interacting with the novel mouse cage relative to the cage containing the mouse from the previous trial (Figure 5.4 A,C). This is suggestive of reduced social memory. By comparison, the APC/β-cat cKO interacted with the novel-social cage relative to the old-social cage similar to control littermates (Control 2.46 ± 0.32, APC cKO 1.37 ± 0.11, β-cat cOE 1.31 ± 0.17, APC/β-

cat cKO 3.51 ± 0.51, One-way ANOVA F(5,50) = 4.015, p = 0.0039). Although this suggests a lack of social memory, this could also be due to an increased aversion to the new social stimuli, so further experiments are necessary to elucidate this.

As repetitive behaviors are also indicative of an autistic phenotype, we tested the mutant mouse models in the marble burying and repetitive circling assays. Similar to our previous studies (Mohn et al. 2014), APC cKO mice buried significantly more marbles than control (Figure 5.5A). Complementing the results in the three chambered test, APC/β-cat cKO mice buried a comparable number of marbles to control, suggesting that lowering β-catenin ameliorates these phenotypes. Interestingly, the β-cat cOE mice, predicted to harbor similar deficits to the APC cKOs, buried significantly fewer marbles than control (Control 3.20 ± 0.53, APC cKO 8.75 ± 1.00, β-cat cOE 0.82 ± 0.33, APC/β-cat cKO 4.22 ± 0.40, One-way ANOVA F(3,34) = 32.43, p<0.0001).

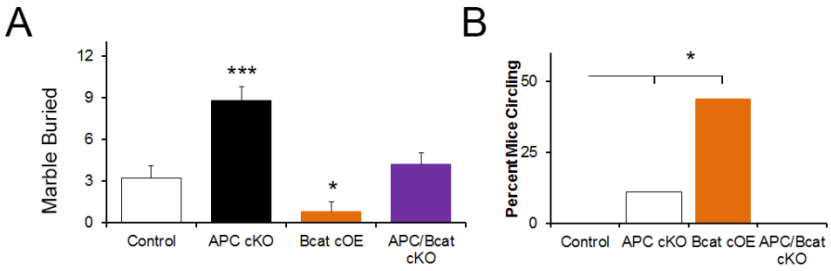

Figure 5.5 Repetitive behavioral phenotypes of the mutant mouse models. (A) APC cKO mice bury significantly more marbles than control in the marble burying assay. This repetitive behavior is rescued in the APC/β-cat cKO model (n = 10 control, 8 APC cKO, 11 β-cat cOE,9 APC/β-cat cKO; *p<0.05,***p<0.001, post-hoc Bonferroni corrected t-test). Although β-cat cOE mice bury significantly less marbles than control, they exhibit (B) circling behaviors (as do the APC cKOs) that are not observed in control or APC/β-cat cKO mice (n = 18 control and APC cKO, 16 β-cat cOE, 11 APC/β-cat cKO; *p<0.05, Chi-squared test).

It was anecdotally observed that the β-cat cOE mice spent much of their time doing unidirectional circling when placed in the marble-containing novel environment, so

this might not only account for the decrease in marble burying we observed in the β-cat cOE mice. When placed in a novel environment, a significant percentage of both APC cKO and β-cat cOE mice displayed the circling behavior (Figure 5.5B). This behavior was not observed in either control or APC/β-cat cKO mice (Chi-squared 14.02, df = 3, p = 0.029). Taken together, these results suggest that elevated β-catenin may be involved in autistic-like behaviors, and that decreased sociability and increased repetitive phenotypes can be ameliorated by reducing β-catenin levels.

5.4 APC/β-cat cKO Mice Show Cognitive Deficits

Give that the autistic-like behaviors of the APC cKO and β-cat cOE were not present in the APC/β-cat cKO mouse model, we sought to determine if the learning and memory phenotypes were ameliorated as well. We tested APC/β-cat cKO mice in the Barnes maze assay to assess their cognitive abilities. Similar to the results seen in the APC cKO mice (see Chapter 3 & 4 for detailed results), APC/β-cat cKO mice took longer to learn the location of the goal hole over the course of training compared to littermate controls (RM ANOVA, F(1,17) = 5.206, p = 0.0357)(Figure 5.6).

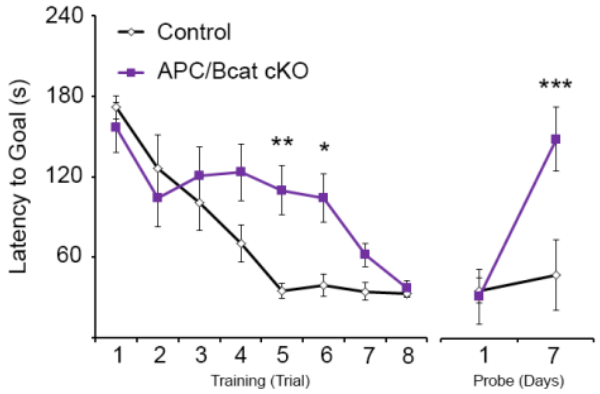

Figure 5.6 Impaired learning and memory in the APC/β-cat cKO mouse. APC/β-cat cKO mice display cognitive phenotypes similar to APC cKO mice (Mohn et al. 2014), taking longer to learn the location of

the goal in the Barnes maze and unable to recall the location at a one week probe trial (n = 10 control, 9 APC/β-cat cKO; *p < 0.05, **p < 0.01, ***p < 0.001, post-hoc Bonferroni corrected t-test).

When we tested for memory retention at one week, the APC/β-cat cKO mice failed to recall the location of the goal (RM ANOVA, $F(1,17) = 95.5$, $p < 0.001$). Thus, although preventing the increase in β-catenin levels that occurs when APC is conditionally removed ameliorates the autistic-like behaviors, this genetic manipulation is unable to ameliorate the cognitive phenotypes. This suggests that the cognitive deficits may be due to lack of APC, as the learning and memory phenotypes of the two models lacking APC (APC cKO and APC/β-cat cKO) show similar results in the Barnes maze independent of β-catenin status.

5.5 Unexpected Wnt Gene Transcription and a Potential Compensatory Role of Plakoglobin in APC/β-cat cKO Mice

Given β-catenin's known role in regulating Wnt target gene expression, we analyzed the expression of several known transcripts to assess levels in the APC/β-cat cKO mouse. We expected to see decreases in expression levels of targets that were elevated in the APC cKO and β-cat cOE models given that we had deleted β-catenin. Unexpectedly, we saw an increase in expression in several Wnt targets across the forebrain including *dkk1*, *sp5*, *neurog1*, and another Wnt target, *c-myc* (n = 5 all genotypes; *dkk1*: Control 1.00 ± 0.49, APC/β-cat cKO 2.4 ± 0.49, p = 0.0434; *sp5*: Control 1.00 ± 0.17, APC/β-cat cKO 2.3 ± 0.42, p = 0.0067; *neurog1*: Control 1.00 ± 0.13, APC/β-cat cKO 2.1 ± 0.45, p = 0.0438; *c-myc*: Control 1.00 ± 0.13, APC/β-cat cKO 2.33 ± 0.51, p = 0.0339, student's t-test for each gene) (Figure 5.7A).

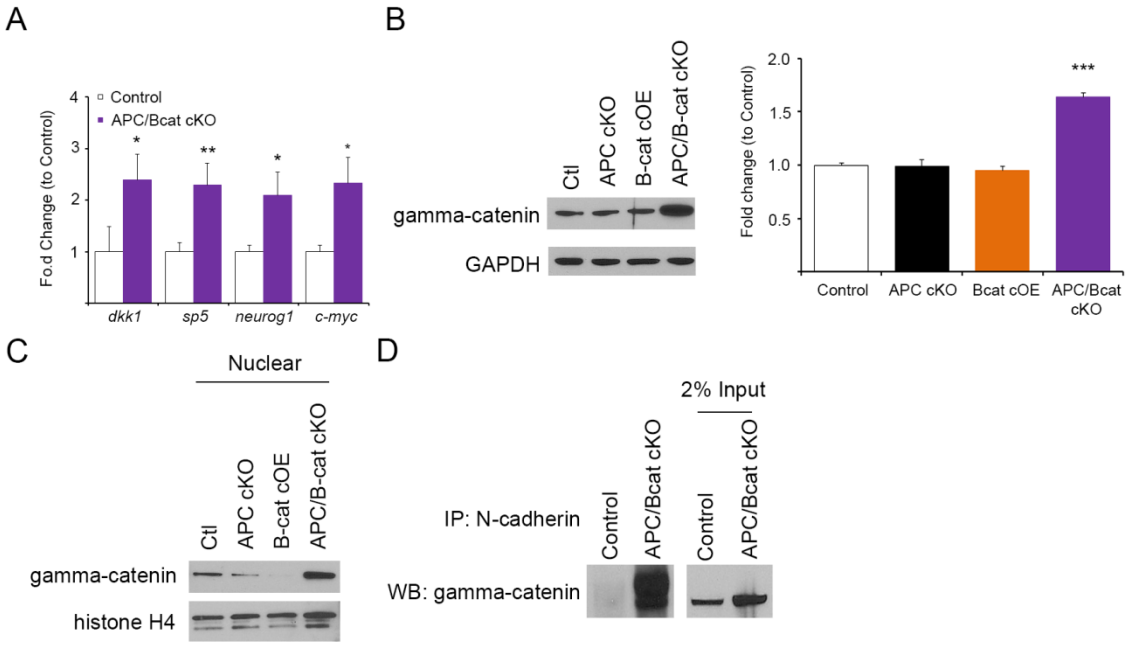

Figure 5.7 Increased Wnt target gene transcription and increased γ-catenin. (A) APC/β-cat cKOs show increased mRNA levels of several Wnt targets, as indicated, in cortex (*$p < 0.05$, **$p < 0.01$, student's t-test). (B) The in the APC/β-cat cKO cortex we observe an increase in the β-catenin family member, γ-catenin (***$p<0.001$, post-hoc Bonferroni corrected t-test). (C) Consistent with the increase in Wnt target expression, we observe an increase in nuclear levels of γ-catenin. (D) γ-catenin may also compensate for β-catenin at the synapse through its increased association with N-cadherin upon β-catenin loss.

β-catenin has a closely related family member, γ-catenin, which has functional transcriptional ability in the Wnt pathway and has been shown to associate with the synaptic adhesion molecule, N-cadherin (Simcha et al. 1996; Simcha et al. 1998; Karnovsky and Klymkowsky 1995; Merriam, Rubenstein, and Klymkowsky 1997; Maeda et al. 2004; Tanaka et al. 2012). Therefore we examined the levels of γ-catenin in the cortex of all of the mutant mouse models. Interestingly, γ-catenin levels were significantly increased in immunoblots of cortical lysate of APC/β-cat cKO mice (Figure

5.7B) (Control 1.00 ± 0.02, APC cKO 0.99 ± 0.06, β-cat cOE 0.95 ± 0.04, APC/β-cat cKO 1.64 ± 0.03, One-way ANOVA $F(3,12) = 57.68$, $p<0.001$). This change suggests that γ-catenin may be functionally compensating for β-catenin in this mutant model.

In order to examine what γ-catenin might be doing functionally, we performed subcellular fractionations to see if γ-catenin localized to the nucleus. Indeed, we observe an increase in γ-catenin in the nuclear fraction of the APC/β-cat cKO suggesting that the Wnt target gene increases we observe may be due to γ-catenin binding to the TCF/LEF transcription machinery (Figure 5.7C) (data not quantified, n = 1 fraction from each genotype). Interestingly, we also observe what looks like a decrease in nuclear γ-catenin levels in the APC cKO and β-cat cOE cortex. As both models express high levels of β-catenin, this may suggest that γ-catenin competes for entry to the nucleus, and, when β-catenin levels are low, γ-catenin is free to enter the nucleus and transcribe Wnt targets. Although γ-catenin has been described as a weak transcriptional activator of Wnt target genes, relative to β-catenin, (Williams et al. 2000; Conacci-Sorrell et al. 2002), it is possible that when APC and β-catenin are both deleted, accumulation of γ-catenin is sufficient to account for the increase in Wnt target gene expression we observe.

Since β-catenin also functions at the synapse to regulate adhesion with the synaptic N-cadherin, we also wanted to determine if γ-catenin could compensate for loss of β-catenin here as well. After immunoprecipitating N-cadherin, we observe an increase in association of γ-catenin compared to control (Figure 5.7D) suggesting that in the absence of β-catenin, γ-catenin may compensate for it loss. Although further studies are necessary to show that γ-catenin retains function in both the nucleus and at the synapse,

these results suggest that γ-catenin levels are increased both in the nucleus and at the synapse when both β-catenin and APC are deleted.

In total, this study highlights the role of β-catenin in governing autistic like behaviors and suggest that β-catenin may be a useful therapeutic target in ASD associated mutations in human that are predicted to increase β-catenin levels.

Chapter 6:

Discussion

6.1 Overview of Dissertation

This dissertation seeks to understand the role of the central signaling molecules, APC and β-catenin, in dysfunctions underlying ASDs and IDs. Our mice are novel, *in vivo* models that elucidate critical roles for these proteins in social, repetitive, and cognitive behaviors – traits that define the core of ASD and ID in humans (Table 6.1). Although each chapter will be looked at in more detail below, the overall results shown throughout the course of this dissertation, combined with the increasing evidence for the Wnt/β-catenin pathway being critical in the human pathophysiology of both ASD and ID, demonstrate that these new models will be critical to our understanding of these diseases.

	APC cKO	β-cat cOE	APC/β-cat cKO
APC levels	Absent	Increased	Absent
β-catenin levels	Increased	Increased	Absent
Wnt target gene expression	Increased	Increased	Increased
Social Behaviors	Impaired	Impaired	Normal
Repetitive Behaviors	Increased	Increased	Normal
Cognition	Impaired	Severely Impaired	Impaired

Table 6.1 Major biochemical and behavioral changes in the APC cKO, β-cat cOE, and APC/β-cat cKO mice. APC cKO mice show increased β-catenin levels and an increase in Wnt target gene expression. They also display autistic-like behaviors and show modestly impaired learning and memory phenotypes. β-cat cOE mice also show increased β-catenin levels and Wnt target gene expression, but also show an increase in APC levels and a mobility shift associated with possible phosphorylation. These mice show reduced social interaction and social memory similar to the APC cKO, and show increased, albeit different, repetitive behaviors. In stark contrast to the modest phenotype in the APC CKO, the β-cat cOE mice display severely impaired cognition. Deletion of both APC and β-catenin in the APC/β-cat cKO remarkably show increased Wnt target gene expression that may be mediated by increased levels of the β-catenin family member, γ-catenin. These mice show normal social and repetitive behaviors suggesting that the increased β-catenin in the other two mutant models may be causing the autistic-like phenotypes we observed. APC/β-cat cKO mice show modest cognitive impairment, comparable to the APC cKOs.

The results in Chapter 3 help to identify the role of APC at central mammalian synapses *in vivo*. The mild cognitive and autistic-like phenotypes seen in these mice are complimented by altered LTP, increased spine-density, altered N-cadherin/β-catenin binding, and reduced PS1 levels. Given APC's role in negatively regulating β-catenin, we observed an expected increase in β-catenin levels across the forebrain and increased Wnt target gene expression. Based on the known functions of β-catenin in neurons, we hypothesized that these increased levels were underlying the autistic like behaviors and cognitive deficits in the APC cKO mouse.

Chapters 4 and 5 focus on two new mouse models we generated using the same CamKIIα-Cre line: the β-cat cOE mice and APC/β-cat cKO mice. My initial hypothesis was that the β-cat cOE mouse would directly phenocopy the APC cKO and we would observe a straight rescue of the behavioral phenotypes in the double knockout model. However, the severe cognitive and electrophysiological phenotypes in the β-cat cOE mouse made us question how two models with similar levels of increased β-catenin could show such dramatically different results. In Chapter 4 we describe a potential new role for APC in regulating translation of an mRNA target, *SynCAM1* and suggest that the severe cognitive phenotype seen could be due to SynCAM1's role in regulating both GluR1 and NR2A/B glutamate receptor subunit insertion at the synapse through the protein 4.1 family. We observe decreases in the membrane levels of these subunits in the β-cat cOE with severely reduced LTP and LTD. Conversely, the APC cKO, which shows slightly enhanced LTP, has increased membrane levels of GluR1. We also observe what looks to be a mobility shift and a potential increase in the levels of APC in the β-cat cOE, which, given the SynCAM1 protein results, led us to think that this

possible modification/stabilization of APC is responsible. Further testing (discussed later) is necessary to demonstrate if APC is being phosphorylated and how or if that event may play a role in translation of APC mRNA targets, but it is an intriguing possibility.

The cognitive deficits seen in the β-cat cOE and the potential new role of APC in regulating learning and memory deserved its own discussion. Therefore, I decided to focus solely on that aspect for Chapter 4, although the results in Chapter 5 are highly complementary. Chapter 5 focuses on the rescue of the autistic-like phenotypes observed in the APC cKO mouse through conditional homozygous deletion of the *ctnnb1* gene in addition to *APC.* Similar to results seen for APC cKOs in Chapter 3, β-cat cOE mice show decreased social behaviors and increased (albeit slightly different) repetitive behaviors, reinforcing the original hypothesis that increased β-catenin was responsible for the phenotypes. The APC/β-cat cKO "rescue" model shows normal levels of social interaction - measured by the three chamber test, and demonstrates no deficit in social memory in the same assay, compared to control. Interestingly, this model does demonstrate an increase in Wnt transcription, an effect that you would expect to see reduced in a model lacking β-catenin. We hypothesize that this effect and maybe some of the rescued phenotypes are due to increases in the levels of the closely related catenin family member, γ-catenin. We see increases in γ-catenin levels in the cortex of the APC/β-cat cKO mouse and an increased association with N-cadherin and nuclear translocation. How γ-catenin differentially regulates not only Wnt gene expression but synaptic adhesion complexes in the brain may be a critical window into studying the undefined neuronal function of γ-catenin. It will also help to identify potential gene

target and direct binding partners where perhaps β-catenin and γ-catenin function do not overlap.

Overall, I believe the work done in this dissertation has validated critical roles for APC and β-catenin in the mammalian brain in governing normal social, repetitive, and cognitive behaviors. These results have also opened up the study of previously poorly defined neuronal functions for APC and β-catenin. It is my hope that the data described here will be useful in defining targets for therapeutic interventions in patients suffering from ASD and ID.

6.2 The APC cKO mouse as a model of ASD and ID

Our data demonstrate that the APC cKO mouse is a new model of cognitive and autistic-like disabilities. This study provides the first direct test, to our knowledge, of APC's role at central synapses *in vivo*. Our findings elucidate the importance of APC in the mammalian brain by showing that it is an essential regulator of both synaptic adhesion complexes and signal transduction networks. This study identifies novel functional and molecular changes (increases in mEPSC frequency, TBS-induced LTP, β-catenin, and canonical Wnt target gene expression, and decreases in presenilin1), not observed previously, to our knowledge, in genetic mutant mouse models with co-morbid cognitive and autistic-like disabilities.

Several lines of evidence support our model of the critical role of APC in the mammalian brain. Patients with APC heterozygous gene deletion, *de novo* and inherited, and polymorphisms display ID and ASD (Finch et al. 2005; Raedle et al. 2001; Herrera et al. 1986; Hockey et al. 1989; Kobayashi et al. 1991; Cross et al. 1992; Lindgren et al.

1992; Heald et al. 2007; Hodgson et al. 1993; Zhou et al. 2007; Barber et al. 1994). APC negatively regulates β-catenin levels in the canonical Wnt pathway (Stamos and Weis 2013). *De novo* mutations in a distinct regulator of β-catenin-mediated gene expression, CHD8, and in β-catenin itself link to sporadic ASD and ID (Krumm et al. ; O'Roak, Vives, Fu, et al. 2012; Sanders et al. 2011; Iossifov et al. 2012; de Ligt et al. ; Neale et al. 2012; Talkowski et al. ; Willsey et al. ; Durak et al. 2016). β-catenin has dual roles in canonical Wnt signaling and cadherin-based synaptic adhesion complexes. Dynamic regulation of both pathways modulates synaptic density, maturation, and plasticity (Salinas 2012; Park and Shen 2012; Rosso and Inestrosa 2013; Yu and Malenka 2004; Murase, Mosser, and Schuman 2002; Brigidi and Bamji 2011). During normal mammalian brain development, Wnt pathway components are expressed at high levels early and down-regulated with maturation (Shimogori et al. 2004). We show here that APC loss leads to excessive β-catenin and canonical Wnt signal transduction in the forebrain as well as cognitive and autistic disabilities.

Augmentation of β-catenin and the Wnt signaling pathway in wild-type hippocampal dissociated neuron and organotypic cultures cause similar functional and structural changes to those seen here in the APC cKO hippocampus *in vivo*, including increases in mEPSC frequency, TBS-induced LTP (including induction), and synaptic spine density (Chen, Park, and Tang 2006; Murase, Mosser, and Schuman 2002; Yu and Malenka 2004; Cerpa et al. 2008; Avila et al. 2010). β-catenin functions presynaptically to enhance mEPSC frequency, with no change in evoked release, via its role in regulating the localization and release of specific synaptic vesicle pools, independent of Wnt-induced transcription (Bamji et al. 2003).

Tight regulation of β-catenin levels also appears to be necessary for memory consolidation. β-catenin levels, experimentally manipulated to be abnormally high or low in the amygdala during fear learning, disrupt fear memory consolidation (Maguschak and Ressler 2011). Our data suggest that high β-catenin levels may impair spatial memory consolidation, based on the poor performance of APC cKOs in Barnes maze probe tests at 12 days. On the other hand, experimentally decreasing Wnt signaling in the dorsal hippocampus immediately after training blocks object recognition memory (Fortress et al. 2013). Thus, constitutively high or low levels of β-catenin and canonical Wnt signaling negatively impact cognitive function. Synaptic activity induces Wnt release and dynamic, transient changes in β-catenin and canonical Wnt signaling levels (Jensen et al. 2012; Maguschak and Ressler 2012; Salinas 2012). We propose that an optimal range and tight regulation of both β-catenin and canonical Wnt target gene expression levels are critical for normal learning and memory formation, highlighting the importance of APC's function as a key regulator of these networks in the mammalian brain.

Via regulation of β-catenin levels, APC also modulates cadherin-catenin synaptic adhesion complexes and thereby regulates other signal transduction pathways critical for normal cognition. APC loss leads to increased association between β-catenin and N-cadherin, and decreased levels of presenilin1, the catalytic subunit of the γ-secretase that cleaves amyloid precursor protein and N-cadherin. The N-cadherin CTF2 fragment translocates to the nucleus and regulates CREB-dependent gene transcription (Marambaud et al. 2003). Cognitive impairments link to presenilin1 cKO in forebrain neurons and human PSEN1 gene mutations link to Alzheimer's disease (Yu et al. 2001).

β-catenin and canonical Wnt signaling levels are aberrantly low in the brains of Alzheimer's disease patients (De Ferrari and Moon 2006; Inestrosa and Varela-Nallar 2014). Studies in aged mice implicate reduced adult neurogenesis as one mechanism of the cognitive deficits (Seib et al. 2013). Spatial learning improved following experimentally induced local increases in Wnt signaling in the dentate gyrus (Seib et al. 2013). In contrast to these positive effects of augmented Wnt pathway activation on cognition, APC cKO mice show high β-catenin/canonical Wnt signaling levels and cognitive impairments. The difference likely stems from APC depleted neurons being unable to rapidly and transiently alter β-catenin/Wnt and presenilin/Creb signal transduction levels, as required for normal memory consolidation (Fortress et al. 2013; Maguschak and Ressler 2011; Yu et al. 2001). Our data have important implications for modulating Wnt pathway levels as a potential therapeutic strategy for cognitive deficits.

Our findings provide new insights into *APC* human gene mutations as a risk factor for ID/ASD. Interestingly, *APC* gene mutations are most commonly clustered in the β-catenin binding domains, resulting in truncated proteins lacking this function. We show here that APC is essential for normal β-catenin, Wnt target gene expression, and presenilin1 levels in the mammalian brain. Our studies provide direct support in an animal model for a critical role of APC in regulating synaptic adhesion complexes and signal transduction pathways that govern synaptic maturation, cognition and behavior.

Consistent with cortical glutamatergic neurons as a site of convergent expression of ASD and overlapping ASD/ID genes in the human and mouse brain (Parikshak et al. 2013; Willsey et al. 2013), we show that loss of APC function in forebrain neurons leads to cognitive and autism-like phenotypes. Future studies are needed to further elucidate

the underlying pathophysiological mechanisms. Selective manipulations of β-catenin and APC (discussed in Chapters 4 and 5 and in the following sections 6.3 and 6.4) will indicate whether these critical Wnt pathway proteins mediate all phenotypes seen with APC loss or whether additional molecular changes may also play a role. Other potential future studies will be discussed below.

6.3 β-catenin overexpression results in severe cognitive deficits

The behavioral differences seen between the APC cKO and β-cat cOE lines highlight that regulation of plasticity in the hippocampus requires a delicate balance of proteins governing essential neuronal functions for normal learning and memory. Compared to controls, β-cat cOE mice show severe cognitive deficits, greatly reduced plasticity and decreases in GluR1 membrane levels. Conversely, the APC cKO mice show mild learning and memory phenotypes and an increase in surface GluR1. We believe these opposite molecular phenotypes may account for the difference in severity of ID and mostly likely occur due to modifications of APC that affects its mRNA binding functions.

Although further studies are needed to conclusively demonstrate this, our findings suggest that increased association between truncated β-catenin lacking the degradation domain and APC causes subsequent phosphorylation on APC that affects translation of its mRNA target SynCAM1. Our findings further suggest that phosphorylation of APC may lead to reduced levels of SynCAM1 protein, but not mRNA, leading to a decrease in protein 4.1N and a subsequent reduction in membrane insertion of GluR1. When APC is absent, as in the APC cKOs, SynCAM1 protein levels (but not mRNA) are elevated.

Together, these opposing actions suggest phosphorylated APC negatively regulates translation of SynCAM1 mRNA. Intriguingly, the phosphorylation state of another mRNA binding protein, FMRP, regulates its ability to repress translation of its mRNA targets (Ceman et al. 2003; Narayanan et al. 2008; Narayanan et al. 2007; Bassell and Warren 2008). As APC associates with kinases like GSK3β and phosphatases like PP2A (Rubinfeld et al. 1996; Seeling et al. 1999; Ikeda et al. 2000), it will be necessary in future studies to define the mechanism that affect APC phosphorylation state and its role in regulating the translation of its mRNA targets. Both GSK3β and PP2A have been shown to positively regulate translation through phosphorylation of the translation machinery including the initiation factor 4E-binding protein 1 (eIF4E-BP1) (Janzen et al. 2011; Shin et al. 2014).

Accumulating evidence supports this idea of APC serving as a regulator of translation. First, APC binds to and positively regulates *tubb2b* mRNA localization and levels *in vivo* (Preitner et al. 2014). Second, APC clusters with the eIF4E translational elongation factor indicating APC is located in proximity to the translational machinery (Preitner et al. 2014) Third, APC deletion reduces the number of ribosomal polysomes and affects mTOR dependent elongation (Faller et al. 2015). Fourth, GSK3β phosphorylation of APC affects the ability of APC to bind microtubules (Zumbrunn et al. 2001), and it is this same domain that is thought to mediate APC mRNA binding (Preitner et al. 2014). Fifth, APC co-immunoprecipitates and localizes with other RNA binding proteins and translational regulators such as FMRP and Fus (Mili, Moissoglu, and Macara 2008; Yasuda et al. 2013), and APC disruption affects the localization of FMRP and its target mRNAs to distal sites (Mili, Moissoglu, and Macara 2008). Taken

together, these data indicate that APC may serve as a translational hub, regulating its own mRNA targets as well as localization of other translational regulators. Combined with our results, these findings provide a strong rationale for this new APC function.

We cannot rule out a role for APC phosphorylation in affecting its stability, protein interactions, or localization which could have secondary effects on SynCAM1 levels. For instance, phosphorylation of APC may change its localization (Caro-Gonzalez et al. 2012), and that of *SynCAM1* mRNA, thereby altering either its proper translation or rendering it incapable of enhancing GluR1 insertion at the synapse. However, our APC cKO data suggests that APC is not involved in the localization of the *SynCAM1* message, as deletion of APC shows increased SynCAM1 protein levels and increased GluR1 surface membrane insertion which we would not expect if APC were not available to regulate the localization of the mRNA. As GluR1 insertion is an activity-dependent event (Heynen et al. 2000; Ehlers 2000; O'Brien et al. 1998), it is intriguing to suggest the potential for activity-dependent changes in APC phosphorylation as a mechanism for regulating synaptic plasticity.

Changes in APC levels and phosphorylation state could be synergistic with elevated β-catenin and the reason why β-cat cOE mice show worse cognitive deficits compared to APC cKOs even though both mutants have comparably increased levels of β-catenin. As evidence for this, mice that are homozygous for a truncating APC mutation that abolishes the C-terminal region including the basic domain responsible for mRNA binding (but maintains normal β-catenin regulation), show mild cognitive phenotypes, similar to our APC cKO mouse (Onouchi et al. 2014). Moreover, these mice show slightly decreased LTP and decreased spine density (compared to increases of both in our

APC cKOs) suggesting that elevated β-catenin plays an additional role in the phenotypes we observe. As further support, aberrant β-catenin levels, either too high or too low, during fear-conditioning training prevents memory consolidation (Maguschak and Ressler 2008, 2011). It is important to note that the isoform of β-catenin lacking the degradation domain may be a part of the reason why β-cat cOEs have more severe deficits, as it does associate more with APC most likely promoting APC's phosphorylation. This would counter-indicate that the function of this β-catenin is "normal".

Altered APC phosphorylation and levels lead to reduced SynCAM1 protein in the β-cat cOE mice and SynCAM1 affects synaptic glutamatergic receptor levels. SynCAM1 is required for proper clustering of protein 4.1N, and even small reductions in protein 4.1N levels have been shown to decrease GluR1 insertion and LTP (Hoy et al. 2009; Lin et al. 2009; Shen et al. 2000). Although the results of the LTD suggest an impaired mechanism of GluR1 internalization that could theoretically be mediated by APC changes, the low steady state GluR1 membrane levels seen in the β-cat cOE suggests the possibility that the absence of LTD-mediated response is due to a lack of surface expression of the activity-dependent AMPA receptor subunit in the first place. Moreover, we observe normal LTD in APC cKO mouse where steady state membrane levels of GluR1 are high.

Our genetic manipulations may target a critical window of brain development based on differences in the time frame and phenotypes between our studies and those of others. We target the dysregulation of APC and β-catenin to early developmental stages that coincide with the period of the largest number of synapses forming in the brain. In

contrast, a study by Mills et al. (2014) showed that overexpressed β-catenin using a CamKII-Cre that is activated much later in development (starting from P17 reaching complete expression by P40), show only had a modest reversal learning deficit and a slight reduction in LTD (Mills et al. 2014). As our CamKIIα-Cre driver turns on late embryonically (Pirone et al. 2016), more in parallel with physiological expression of endogenous CamKIIα (Allen Developing Mouse Brain Atlas), we believe that this may cause the differing results. Further, NMDA receptors are the primary glutamate receptors expressed in the developing brain (Pickard et al. 2000) and AMPA receptor insertion is dependent on the proper functioning of NMDA receptors (Hall, Ripley, and Ghosh 2007). We show that β-cat cOEs display reduced NMDA NR2A and 2B levels. We propose that the altered APC levels and posttranslational modifications may be responsible. As SynCAM1 controls NR2B subunit insertion through protein 4.1B (Hoy et al. 2009), based on our findings of reduced levels of SynCAM1, protein 4.1N and GluR1 at later ages in β-cat cOEs, it will be important to test early perinatal ages for altered levels of NR2B, SynCAM1 and protein 4.1B - all critical components for normal synapse function and maturation.

Consistent with the theory that over-expression of β-catenin in either our APC cKO or β-cat cOE early in development may account for the autistic-like behaviors we observe, the brains of these mice show gross morphological changes (Figure 6.1). Additionally, they display motor spasms at neonatal ages and a progression to seizures, consistent with a childhood epilepsy syndrome, infantile spasms, often co-morbid with autism (Pirone et al. 2016). Both of our high β-catenin mutant mouse models show lack midline crossing of axonal long tracts, including the corpus callosum and the

anterior/posterior commissures, as well as altered structure of the striatum, suggestive of changes in cortico-striatal connections. These long-range neuronal projections are established early in development and changes in this type of connectivity have been suggested to link to autism in humans (Booth, Wallace, and Happe 2011; Hardan et al. 2009; Hughes 2007; Symington et al. 2010). Most intriguing, the autistic-like behaviors and these gross morphological phenotypes are corrected in the APC/β-cat cKO model suggest a role for early increased β-catenin in setting up the abnormal neuronal circuitry of the brain.

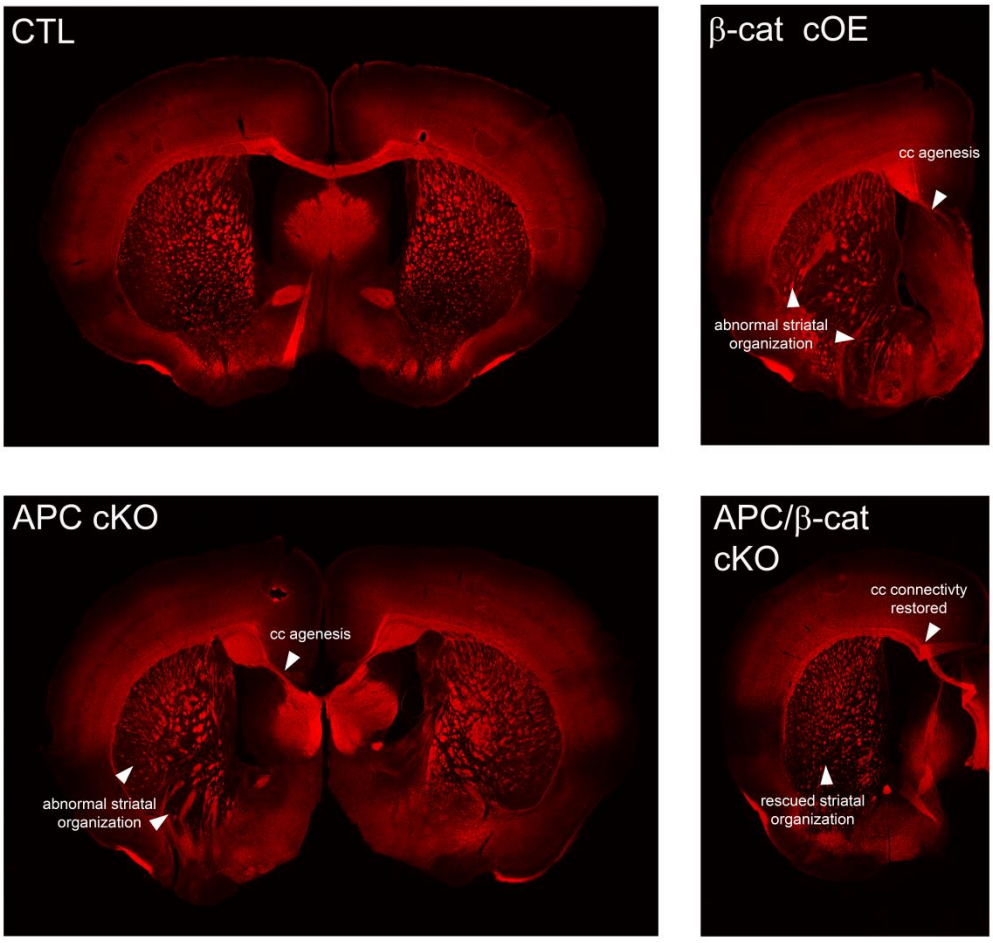

Figure 6.1 Gross morphological changes seen in the APC cKO and β-cat cOE models with increased β-catenin but not in APC/β-cat cKOs. Myelin basic protein staining of coronal sections of the brains the APC

cKO and β-cat cOE mice show corpus callosum (cc) agenesis, reduced or absent anterior and posterior commissures (not shown), and aberrant structure of the striatum. These morphological phenotypes are corrected in the APC/β-cat cKO mouse brain suggesting a role for increased β-catenin in the phenotypes.

Wnt gradients and glial guidepost cells are known to be involved in proper axon guidance and midline crossing of the callosal pioneer neurons (Keeble et al. 2006; Hutchins, Li, and Kalil 2011). However, it is likely that Wnt target gene expression and/or Wnt mediated changes in adhesion dynamics is a key function of these gradients in the guiding projections of the pioneer neurons. Thus, early increased β-catenin in these key cellular subpopulations may be rendered resistant to endogenous Wnt gradients. As callosal pioneer neurons do express CamKII (Hutchins, Li, and Kalil 2011), it is likely that the deficits we observe in these particular long-range connections are due to impaired functions and/or Wnt responses in these neurons. Early structural changes raise key questions about the time frame required for treating individuals with cognitive and autism disorders, and recent studies demonstrate that both genetic and pharmacological treatments can be effective at adult ages (Silverman et al. 2012; Silverman, Tolu, et al. 2010; Han et al. 2014; Guy et al. 2007; Mei et al. 2016; Durak et al. 2016; Auerbach, Osterweil, and Bear 2011). We propose that structural and plasticity changes both play roles in these disorders and highlight the importance of continued studies to identify molecular targets with the potential to ameliorate these disorders.

Gene mutations in APC and β-catenin in humans have been shown to impair cognition function (Raedle et al. 2001; Barber et al. 1994; Zhou et al. 2007; Hodgson et al. 1993; Herrera et al. 1986; Hockey et al. 1989; Kobayashi et al. 1991; Cross et al. 1992; Lindgren et al. 1992; Heald et al. 2007; Finch et al. 2005; Tucci et al. 2014; de Ligt

et al. 2012; Iossifov et al. 2012; Iossifov et al. 2014). Elucidating molecular mechanisms caused by dysregulation of these key synaptic molecules is important in designing effective therapeutic interventions in patients. Our findings identify novel molecular etiologies that lead to learning and memory impairments of different severities. We define a novel role for APC as an mRNA binding protein that regulates synaptic plasticity and cognition.

6.4 The APC/β-cat cKO mouse rescues autistic-like phenotypes associated with increased β-catenin.

Our goal in this chapter was to define the role of increased β-catenin in the autistic-like phenotypes of the APC cKO mouse. To accomplish this, we used our two new mouse lines, the β-cat cOE and APC/β-cat cKO to test if an increase in β-catenin was both necessary and sufficient to cause the aberrant social and repetitive behaviors discussed in Chapter 3.

β-cat cOE mice, similar to APC cKO mice, show reduced social interaction and increased (although different) repetitive behaviors. APC/β-cat cKO mice, on the other hand, do not display any of these ASD related phenotypes. This suggests that a functional reduction in β-catenin may prevent the autistic like phenotypes associated with human mutations that are predicted to cause increased activation of β-catenin associated pathways - cadherin adhesion complexes and Wnt signaling (Barber et al. 1994; Martin et al. 2013; Talkowski et al. 2012; Bi et al. 2012; Iqbal et al. 2013; Shi et al. 2013; Durak et al. 2014; Finch et al. 2005; Hockey et al. 1989; Heald et al. 2007; Zhou et al. 2007).

Interestingly, the APC/β-cat mouse still displays cognitive deficits on a par with what we observed in the APC cKO model in Chapters 3 and 4, and an increase in Wnt target gene expression. We hypothesize that this increase in Wnt activation is mediated through a substantial increase in a closely related β-catenin family member that is also negatively regulated by APC, γ-catenin (Kolligs et al. 2000). We show increases in nuclear localization of γ-catenin and increased association of γ-catenin to the N-cadherin adhesion complex. These results suggest that γ-catenin may be functionally compensating for β-catenin to some degree, however, not in a way that increased levels of it lead to the autistic-like behavioral phenotypes seen in the other models. Studying this functional difference may provide useful new therapeutic approaches for treating human ASDs predicted to affect this pathway.

Because of the unexpected increase in APC levels (or post-translational modifications) seen in the β-cat cOE, it is harder to interpret the results as directly attributable to increased β-catenin levels (as we were expecting APC to look comparable to controls). It is probably prudent to suggest that some of the autistic-like behaviors seen in the β-cat cOE mice can be attributed to their severe cognitive deficits. This may be especially true for social memory, which has been shown to have a hippocampal dependent component (Kogan, Frankland, and Silva 2000; Hitti and Siegelbaum 2014), where we see severe impairments in plasticity and glutamate receptor levels. This caution is also supported by the somewhat different repetitive behavioral phenotypes we observed between the APC cKO and the β-cat cOE mice. It will be important to delineate these functional roles for β-catenin and APC in the behavioral phenotypes going

forward, but the rescue of the autism-associated phenotypes seen in the APC/β-cat model, highly suggests an important role for increased β-catenin in the phenotypes.

Although the increase in γ-catenin was a bit surprising (as it was not observed in the APC cKO mouse and APC is known to negatively regulate γ-catenin in addition to β-catenin (Kolligs et al. 2000)), there is precedent that dose dependent reduction in either β-catenin or γ-catenin can cause an inversely related increase in the other (Salomon et al. 1997). A similar effect is seen in the APC/β-cat cKO, showing increased γ-catenin when β-catenin and APC are absent. Unique to this study, the potential ability of γ-catenin to functionally compensate for β-catenin, although hinted at in the literature (Butz et al. 1992; Kolligs et al. 2000; Maeda et al. 2004; Rubenstein, Merriam, and Klymkowsky 1997; Sacco et al. 1995), has never been described in the CNS *in vivo*. It is important to note that despite the evidence given here, further experiments are needed to describe both γ-catenin's general neuronal functions as well as its potential role in rescuing the behavioral phenotypes seen in the APC cKO model. Potential ways to tease out this function will be discussed below.

Notably, conditional knockout of β-catenin alone (β-cat cKO), does not show autistic-like phenotypes seen in our models with high β-catenin (Figure 6.2) suggesting that deletion of the gene encoding β-catenin may be epistatic to deletion of the *APC* gene. Bcat cKO mice show normal social interactions, comparable to control (Figure 6.2A) in the three chambered test (Control 1.96 ± 0.28, β-cat cKO 2.08 ± 0.44, $p = 0.821$ Student's t-test). These mice also do not display increases in repetitive behaviors (Figure 6.2B) in the marble burying task (Control 3.40 ± 0.58, β-cat cKO 2.10 ± 0.48, $p = 0.102$ Student's t-test). However the epistatic relationship between the APC and β-catenin is difficult to

determine completely because of the cognitive deficits and increases in γ-catenin seen in the APC/β-cat cKO.

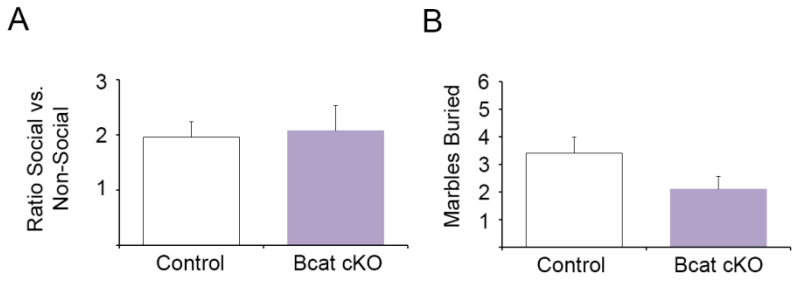

Figure 6.2 β-cat cKO do not show autistic-like behaviors. β-cat cKO show normal (A) social interactions and (B) marble burying behaviors suggesting that decreased β-catenin, regardless of APC status, does not contribute to the autistic-like phenotypes when deleted in CamKII expressing neurons (n=10 Ctl, 9 β-cat cKO).

Overall, the results in this chapter do suggest a role for increased β-catenin in manifesting the ASD related phenotypes described in the APC cKO mouse in Chapter 3. Overexpression of β-catenin in the β-cat cOE mice resulted in social impairments and increased repetitive behaviors. Preventing β-catenin increase in the APC cKO through conditional deletion of *ctnnb1* in the APC/β-cat cKO resulted in normal social interactions, social memory, and repetitive phenotypes.

6.5 Future directions following this dissertation

Several of the proposed studies are ongoing projects in the lab and some are projects that will require more development, but I believe they will be important in following through on outstanding questions raised in this thesis.

The results in this thesis do suggest a potential role for elevated β-catenin in the cognitive and autistic-like phenotypes seen in the APC cKO mouse. One potential way to test this is through pharmacological reduction of β-catenin in the APC cKO mouse.

We are currently using tankyrase inhibitors as a means to do this. The tankyrase proteins act as negative regulators of the β-catenin destruction-complex protein Axin, targeting it for proteosomal destruction. Stabilization of Axin through inhibition of the tankyrase proteins has been shown to reduce the levels of β-catenin even in the absence of APC in xenograft cancer models. If brain bioavailability is sufficient and we see reductions in β-catenin levels concurrent with a rescue of behavioral phenotypes, these experiments could have therapeutic implications for ASD and ID patients where β-catenin levels are predicted to be elevated. As treating humans before the onset of symptoms is not possible and there is precedent for later corrections being effective in the behavioral outcome in mice (Auerbach, Osterweil, and Bear 2011; Mei et al. 2016; Durak et al. 2016; Silverman et al. 2012; Silverman, Tolu, et al. 2010; Han et al. 2014; Guy et al. 2007), we hope that acute treatment in adulthood will be effective in correcting the phenotypes we observe.

Importantly, because of the developmental effects of β-catenin increase suggested by comparing the Mills study (Mills et al. 2014) to our models, it will be necessary to test how these critical windows that we believe we are hitting are relevant to the phenotypes of our mice. This could be done potentially in two different ways. First, we could use an inducible CamKII driver (such as tamoxifen or doxycycline induced) to express Cre (and cause increased β-catenin) at later developmental time points. If we do not observe the same structural changes I noted above, nor the behavioral and plasticity deficits, it suggests that the earlier time windows we are targeting are crucial in the phenotype and may arise from the aberrant connectivity we observe. If we do observe the morphological changes but do not observe the behavioral deficits, it would suggest that

connectivity changes may not play a role in our β-catenin overexpressing mouse models and that there are other earlier changes, not hit by the new inducible approach, that set the stage for malfunction. If the behavioral phenotypes are still present in the absence of any gross structural changes, this suggests that changes in synaptic function are key to the deficits we observe. In any of these outcomes, we will need to go deeper to define the molecular changes that are responsible.

These types of studies might also provide insight as to a critical window for rescue of the autistic-like behaviors, as we achieved in the APC/β-cat cKO mouse. For instance, inducible deletion of β-catenin at a series of later time points in the APC cKO background would tell us when reducing beta-catenin might be necessary for correcting some or all of the fiber tract deficits and the cognitive and autistic-like phenotypes we observe. This will be critical in defining treatment windows for the studies discussed above.

Given the results from Chapter's 3 and 4 from this dissertation it will be important to tease out the role of increased β-catenin and Wnt signaling from APC status in affecting cognitive behaviors. In collaboration with Larry Feig and Shan-Xue Jin, we are currently in the preliminary stages of studying the effect of using RNAi to target APC in the CA1 of the hippocampus of the β-cat cOE. We are using an AAV virus to transduce the RNAi and regulate the expression of both the RNAi and GFP under control of the CamKII promoter. This way we are targeting only cells where β-catenin is conditionally overexpressed. If the mobility shift in APC we see in that mouse model stabilizes APC, it is possible that reducing the amount of APC levels may have an effect on the downstream translation of APC mRNA targets, plasticity, and ultimately cognitive

function. Alternatively, posttranslational modification may be the cause of altered APC function, in which case, lowering levels may not be sufficient. We would need to define and target relevant modifiers, such as kinases and phosphatases, in order to elucidate a mechanism for how they affect APC function.

Although the literature suggests that increased association of a β-catenin fragment lacking the degradation domain bound more tightly to APC and increased APC phosphorylation, it will be important to determine in our *in vivo* β-cat cOE model 1) if APC is being phosphorylated, 2) what kinase is responsible, and 3) what this phosphorylation means functionally. There is support in the literature that suggests that GSK3β can phosphorylate APC and that this modification disrupts APC's microtubule binding function (Zumbrunn et al. 2001), which is the same domain that binds mRNAs (Preitner et al. 2014), so these are useful things to consider when developing experiments down the road.

We are currently performing immunoprecipitations of APC in control versus β-cat cOE hippocampi and probing for pan-serine phosphorylation, GSK3β binding, and PP2A binding to see if there are changes in the β-cat cOE that might indicate this event is occurring and is being mediated by GSK3β. Then using culture models from the β-catenin overexpressing mice, we could test if GSK3β inhibitors reduce the phosphorylation of APC and produce a subsequent increase in levels of SynCAM1 protein or GluR1 surface expression.

If those results show that GSK3β inhibition is successful in reducing the phosphorylation of APC and restoring the levels of SynCAM1 and the affected downstream modulators of plasticity such as GluR1, it would be interesting to see what

role this regulation plays under normal conditions. As Wnts are released upon synaptic activity, and canonical Wnts inhibit GSK3β, if GSK3β functions to regulate the transcription of APC mRNA targets this would suggest an activity dependent role for APC in protein translation. Using the culture model described above, we could stimulate control neurons with both soluble Wnt or induced activity and examine the phosphorylation status of APC and the translation of SynCAM and/or surface expression of GluR1.

The results of these experiments could be translated *in vivo* to test if reducing GSK3β inhibition, using a method such as including lithium in the mouse chow, results in reduced phosphorylation of APC, and subsequent changes in plasticity and behavior. All of this would be useful in delineating a new role for APC in the brain and understanding how mutations that could affect APC function in patients with ASD and/or ID may be successfully targeted for therapeutic intervention.

We are also currently performing other experiments to see what global changes are occurring in the mutant mice that might help to explain the biochemical, electrophysiological, and behavioral phenotypes of the different models. We are using next-gen sequencing to identify changes in mRNA levels from the hippocampus, cortex, and striatum between the APC cKO, β-cat cOE, and APC/β-cat models (in addition to a new mouse model we are characterizing – the β-catenin conditional knockout). This will allow us to identify changes in mRNA expression levels that are unique to the models that show ASD like phenotypes that may be either rescued or compensated for in the APC/β-cat cKO mouse. We will also gain insights into differences in Wnt target gene expression between mutants with high β-catenin (APC cKOs and β-cat cOEs) versus

those with increased γ-catenin (APC/β-cat cKO and β-cat cKO). This will provide new information on the function of γ-catenin in neurons and help to determine how reducing β-catenin may rescue the autistic-like phenotypes in the APC/β-cat cKO mice.

We are also going to use proteomics to see how APC, β-catenin, and γ-catenin complexes change between the two models. This will help us to elucidate what interactions are affected in each of the models and how they contribute to both the ASD and ID phenotypes. This will also allow us to help tease out the roles of γ-catenin that may differ in many respects to β-catenin in regulating neuronal function.

Along these lines, we could also perform chromatin immunoprecipitation of β-catenin vs γ-catenin to help elucidate the different roles of the two proteins in translation of Wnt targets in neurons. Comparing the targets of genes affected by β-catenin or γ-catenin overexpression may provide insights into how Wnt target regulation plays into the pathophysiology of the phenotypes in the different models. These more global approaches will undoubtedly open up further avenues for study and give insight into how human ASD and ID mutations affecting this key pathway (refer to Table 1.1) can be effectively regulated as potential therapeutic targets for these diseases.

6.6 Concluding remarks

The studies outlined in this dissertation provide the foundations for future studies that I hope will help to elucidate the underlying causes of autism and intellectual disabilities, and be used to define potential therapeutic targets for these disorders. The relevance of the Wnt pathway is becoming more and more apparent in patients with the

disease, so developing further understanding of the key components of the pathway, such as APC and β-catenin, will be critical in making this more of a reality.

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