APC conditional knock-out mouse is a model of infantile spasms with elevated neuronal β -catenin levels, neonatal spasms, and chronic seizures

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Abstract

Infantile spasms (IS) are a catastrophic childhood epilepsy syndrome characterized by flexion-extension spasms during infancy that progress to chronic seizures and cognitive deficits in later life. The molecular causes of IS are poorly defined. Genetic screens of individuals with IS have identified multiple risk genes, several of which are predicted to alter β -catenin pathways. However, evidence linking malfunction of β -catenin pathways and IS is lacking. Here, we show that conditional deletion in mice of the adenomatous polyposis coli gene (APC cKO), the major negative regulator of β -catenin, leads to excessive β -catenin levels and multiple salient features of human IS. Compared with wild-type littermates, neonatal APC cKO mice exhibit flexion-extension motor spasms and abnormal high-amplitude electroencephalographic discharges. Additionally, the frequency of excitatory postsynaptic currents is increased in layer V pyramidal cells, the major output neurons of the cerebral cortex. At adult ages, APC cKOs display spontaneous electroclinical seizures. These data provide the first evidence that malfunctions of APC/ β -catenin pathways cause pathophysiological changes consistent with IS. Our findings demonstrate that the APC cKO is a new genetic model of IS, provide novel insights into molecular and functional alterations that can lead to IS, and suggest novel targets for therapeutic intervention.

Introduction

Infantile spasms (IS) is a catastrophic childhood epilepsy syndrome (West WJ 1841) characterized by early-onset flexion-extension motor spasms, abnormal electroencephalographic (EEG) activity including hypsarrhythmia (Paciorkowski AR et al. 2011), and progression to chronic epilepsy and intellectual disability (El Achkar CM and SJ Spence 2015). IS occurs in $\approx 1/4000$ live births and is co-morbid with many developmental epilepsy syndromes, yet the underlying molecular causes of IS are poorly defined. The current frontline medications, vigabatrin and adrenocorticotrophic hormone (ACTH), are not efficacious in all individuals with IS and have serious adverse effects. Therefore, identifying novel IS-relevant molecular targets is necessary to lay the foundation for developing new, effective, and more specific therapeutic strategies to attenuate spasms and seizures.

With this goal in mind, we propose a novel molecular model of IS that is centered on malfunction of β -catenin pathways in the brain. β -catenin functions in both cadherin synaptic adhesion complexes and in canonical Wnt signal transduction. Tight regulatory control of these β -catenin pathways is critical for normal brain development and function (Salinas PC 2012; Krumm N et al. 2014; Seong E et al. 2015). Excessive β -catenin levels lead to aberrant dendritic and axonal branching, increased excitatory synapse density, and altered synaptic maturation and function (Yu X and RC Malenka 2004; Tai CY et al. 2007; Salinas PC 2012). These studies were predominantly conducted in cultured neurons. Such changes *in vivo* would be consistent with circuit hyperexcitability and increased seizure susceptibility.

As strong support for the significance of β -catenin pathways to the pathophysiology of IS, the majority (11 out of 15) of the identified human risk genes that link to IS are predicted to function in APC, β -catenin, canonical Wnt signaling networks (Paciorkowski AR *et al.* 2011; Michaud JL *et al.* 2014; Boutry-Kryza N *et al.* 2015) (Table 1). In particular, *MAGI-2/S-SCAM*, *GRIN1*, *GRIN2A*, *STXBP1*, *TSC1/2*, *FOXG1*, *ARX*, *LIS1*, *DCX*, and *NR2F1* gene products all either bind to components of, or function in, these networks. APC has recently been identified to serve as an mRNA-binding protein; its targets include β -catenin and 3 IS-linked gene transcripts (*Foxg1*, *LIS1*, *STXBP1*)(Preitner N *et al.* 2014). APC may regulate the levels and/or localization of these mRNAs. Further, APC binds directly to Lis1 protein, the causative gene in class I lissencephaly, an IS-associated developmental epilepsy. Additionally, individuals with APC heterozygous gene deletions display intellectual impairments, autism and seizures, although IS has not yet been reported (Hockey KA *et al.* 1989; Lindgren V *et al.* 1992; Heald B *et al.* 2007). Intellectual and autistic disabilities are often co-morbid with IS (El Achkar CM and SJ Spence 2015). Taken together, these studies provide compelling evidence suggesting that β -catenin networks may be a convergent target of diverse IS-linked genes and aberrant function of β -catenin pathways may lead to spasms and seizures. Direct evidence, however, is lacking for a link between β -catenin pathway malfunction with spasms and seizures.

To determine whether perturbation of β -catenin pathways can lead to IS-like phenotypes, we generated an experimentally amenable mutant mouse model that expresses excessive β -catenin levels in excitatory forebrain neurons. We conditionally deleted the gene encoding adenomatous polyposis coli protein (APC) in Ca^{2+} /calmodulin-dependent protein kinase II- α (CamKII α) positive neurons. APC is the major negative regulator of β -catenin levels via its role in the multi-molecular β -catenin degradation complex. Consistent with this function, loss of APC leads to excessive β -catenin levels. Our APC conditional knock-out mouse (cKO) is

a useful tool for elucidating the pathophysiological consequences of malfunction of APC/ β -catenin pathways *in vivo*. Our recent studies show that APC cKO mice exhibit elevated β -catenin levels in the brain, learning deficits, and autism-like behaviors, compared with wild-type littermates (Mohn et al., 2014). Here, we show that APC cKO mice recapitulate most features of IS in humans, including neonatal spasms, abnormal EEG activity in neonates and adults, and progression to spontaneous seizures. Our findings provide new insights into molecular perturbations that can cause IS-like phenotypes. We propose that β -catenin/Wnt pathways may be a central signaling axis relevant to multiple IS-inducing genetic insults.

Material and Methods

Animals. APC cKO mice were generated by deletion of floxed APC with CamKII α -Cre-recombinase as previously described (Mohn JL et al. 2014). To assess the cell type Cre expression pattern at early developmental ages, wild-type (WT) APC, Cre-positive mice were crossed with CamKII α -Cre Rosa26^{GFP-L10a} reporter mice (generous gift of Leon Reijmers and Maribel Rios, Tufts University). Cre-dependent GFP-L10a ribosomal subunit expression was analyzed at a range of neonatal and adult ages. Control littermates, either floxed APC, Cre-negative, or WT APC, Cre-positive, were included in all experiments (referred to as WT in text). Both sexes, in approximately equal quantities, were included in all experiments. No differences between the genders were detected. All procedures were approved by the Tufts University Institutional Animal Care and Use Committee in accordance with NIH guidelines. Experimenters were blinded to genotype until data were collected and analyzed, except for EEG experiments where blinding was not possible due to surgical and anesthesia issues.

Immunoblot Analysis. Cortical lysates were prepared and analyzed for quantitative immunoblotting as previously described (Mohn JL *et al.* 2014), using the following antibodies: APC (1:2500, Santa Cruz; Dallas, TX); β -catenin (1:2500, Invitrogen; Grand Island, NY); as a loading control, GAPDH (1:10000, Chemicon; Billerica, MA). The blots were scanned at 300 DPI and quantified using ImageJ.

Immunolabeling. Mouse brains were fixed by transcardial perfusion with 4% paraformaldehyde in phosphate buffered saline (PBS). Immunolabeling was performed on 50 μ m coronal frozen sections with β -catenin antibody (above, 1:400 dilution, in 5% dry milk blocking solution) and counterstained with DAPI (Acros Organics; New Jersey, US) to identify nuclei. WT and APC cKO brains were processed in batch to ensure equivalent access to reagents. All sections were imaged with a Nikon A1R laser confocal scanning microscope with

identical imaging settings within a given batch of processed tissue. β -catenin immunolabeling was quantified by measuring the pixel intensity of the fluorescent immunostaining in layer V pyramidal neurons. Regions of interest were drawn around layer V pyramidal neuron somata based on the presence of immunolabeling and DAPI staining. The intensity of labeling was quantified in individual cells and APC cKO neuron values were normalized to the average intensity in WT layer V pyramidal neurons.

Patch clamp recordings. Cortical brain slices (400 μ M thick in the sensorimotor region) were prepared from P9 APC cKOs or WT littermates and placed in the recording chamber of an Olympus BX51 microscope with continual superfusion of oxygenated artificial cerebrospinal fluid (aCSF) maintained at 32°C. Layer V pyramidal neurons were visually identified with infrared differential interference contrast (DIC) microscopy. Whole cell recordings of spontaneous excitatory postsynaptic currents (sEPSC) were obtained at -70 mV holding potential using patch electrodes (3-5 M Ω). Miniature EPSCs (mEPSC) were isolated with 1 μ M tetrodotoxin (TTX; EMD Millipore). The data were analyzed as previously described (Andresen L et al. 2014).

Behavioral Spasms. Neonatal pups, covering the age range from P5 to P14, were removed from the dam and placed in a warmed chamber for video recording daily for 45 minutes. The behavioral videos were scored for spontaneous high-amplitude spastic movements, low-amplitude movements, and time on side. High-amplitude spastic movements included rapid extension/ flexion movements of 2 to 4 limbs, back arching, whole body curling, and falling over, based on criteria previously described for other mouse models of IS (Price MG et al. 2009). Because the spastic movements often occurred in a series, both the frequency and duration of the movements were scored.

Electroencephalography. EEG data was collected in both neonatal (Zayachkivsky A et al. 2013; Zayachkivsky A et al. 2015) and adult APC cKO and WT littermate mice (Clasadonte J et al. 2013).

Neonatal EEG

Neonatal mice (P8, 9, or 10; based on minimal animal weight required to perform surgery) were anesthetized with isoflurane, surgically implanted with a custom made neonatal EEG radio telemetry head mount (Epitel/Ripple, Salt Lake City, Utah; 100X amplification) with two differential EEG electrodes placed into burr holes made symmetrically (left and right) near the anterior of the parietal bone (over the somatosensory cortex). A common ground electrode was placed in a burr hole near the anterior tip of the interparietal bone. The headmount was affixed with cyanoacrylate and the scalp incision was closed using soft sutures. The pups were returned to the dam for a 24 hour recovery period. One pup per litter was implanted. Daily beginning on P9, implanted pups were placed into a custom made neonatal telemetry EEG receiver within a warmed chamber for a 45 minute EEG recording session with simultaneous video recording. High-amplitude events (>3 standard deviations above the mean EEG amplitude), like those seen in human IS and other animal models of IS (Price MG *et al.* 2009; Scantlebury MH et al. 2010; Frost JD, Jr. et al. 2012), were detected in the EEG signals. Events were identified using an automated MATLAB approach to reduce bias and noise, and telemetry artifacts were excluded.

Adult EEG

Adult EEG was performed using standard protocols (Clasadonte J *et al.* 2013). Briefly, mice were implanted with EEG headmounts (Pinnacle Technology Inc., #8402) which collected 2 channels of EEG and one channel of EMG data. Stainless steel screws were placed in burr holes drilled in the skull (one hole in the left frontal bone for frontal/motor cortex, one hole in the

right parietal bone for somatosensory cortex, one hole directly anterior to the left side of the interparietal bone for a common reference electrode, and one hole directly anterior to the right side of the interparietal bone for a ground electrode). After 5-7 days of post-surgical recovery, animals were placed in an EEG recording chamber, a preamplifier was attached to the EEG headmount (Pinnacle Technology Inc., 8202-SE0) and data was collected for 2 weeks with constant video monitoring. Custom MATLAB scripts were used to detect changes in the spectral components of the EEG, and detected events were then examined in the raw EEG.

Statistics. Paired or two sample t-tests, analysis of variance, Kolmogorov-Smirnov, or Chi-squared tests were used as appropriate. By visual inspection data met normality assumptions. P and D values are reported in text, significance of multiple comparisons was tested using the Holm-Bonferroni method.

Reagents. All salts and glucose for buffers were obtained from Sigma-Aldrich unless otherwise noted.

Results

Conditional deletion of floxed APC leads to β -catenin deregulation in the postnatal mouse cortex.

To test whether disruption of APC/ β -catenin pathways in neurons of the developing mammalian brain can lead to spasms and seizures, we utilized our APC cKO mouse (Mohn JL *et al.* 2014). APC is the major negative regulator of β -catenin levels, via its role as a core component of the β -catenin degradation complex. In the APC cKO mouse, Cre-dependent excision of *loxP* flanked APC exons 11 and 12 leads to a frameshift and premature termination of protein translation, resulting in a 468-amino acid fragment that lacks all identified protein interaction domains (Gounari F *et al.* 2005). The *CamKII α* promoter drives Cre-recombinase expression chiefly in excitatory forebrain neurons during synaptic differentiation (Rios M *et al.* 2001; Mohn JL *et al.* 2014) but Cre-expression also occurs in neurons of the striatum and other regions. *CamKII α* -Cre recombinase activation is progressive during development, relatively sparse at P0, and is fully activated by P21 (Rios M *et al.* 2001). We confirmed this time course of Cre recombinase activity in the neonatal cortex, using the Rosa26^{fl-GFP-L10a} reporter mouse line and detected Cre-recombinase-dependent expression of GFP (Fig. 1D).

Both APC and β -catenin protein levels are altered in the adult APC cKO mouse cortex (Mohn *et al.*, 2014). Here, we set out to determine the developmental changes in β -catenin levels in the APC cKO mouse brain. Quantitative immunoblotting showed progressive reductions in APC and parallel increases in β -catenin protein levels at increasing postnatal ages in APC cKOs, as compared to WT littermates (Fig.1 A,B,C; * $p < 0.05$, ** $p = 0.01$, Student's *t* test; $n = 4$ WT and $n = 5$ cKOs for all ages). At P9, the earliest postnatal age examined, APC and β -catenin protein levels were not significantly different between APC cKOs and WT mice as assayed by immunoblotting (Fig. 1B,C), likely due to the progressive, not yet complete

activation of Cre recombinase expression at this neonatal age. However, semi-quantitative immunofluorescence labeling of β -catenin in P9 APC cKO mice did show significantly increased immunolabeling for β -catenin in cortical layer V pyramidal neurons, relative to WT littermates (Fig. 1E,F; ** $p < 0.01$, Student's t test; $n = 3$ cKO mice, $n = 100$ neurons and $n = 3$ WT mice, $n = 154$ neurons). Similarly, in the cortex of the reporter mice at P9, CamKII α Cre-dependent GFP expression was predominantly identified in a subset of layer V pyramidal neurons, and by young adult ages, all layer V pyramidal neurons expressed Cre-GFP (Fig. 1D). In APC cKOs from P14 on, immunoblotting showed significant reductions in APC and significant increases in β -catenin levels (Fig. 1A,B,C), consistent with the progressive induction of CamKII α -Cre transgene expression during the first three postnatal weeks. In the adult (P60) cortex, β -catenin levels were increased 2-fold in APC cKOs, relative to WT littermates. Other brain regions, such as the hippocampus and striatum, also showed similar progressive reductions in APC and increases in β -catenin protein levels in APC cKOs during this time frame (data not shown). Thus, excision of APC in our mouse model leads to increases in β -catenin levels at least as early as P9, and continues progressively through postnatal brain maturation.

Increased excitatory postsynaptic currents in layer V pyramidal neurons of neonatal APC cKO mice.

Our previous studies of adult APC cKOs showed increased excitatory synaptic activity in hippocampal CA1 pyramidal neurons (Mohn JL *et al.* 2014). Here, we tested whether synaptic activity is altered at early neonatal ages, when we detect increased β -catenin levels in cortical layer V pyramidal neurons. We measured both spontaneous and miniature excitatory postsynaptic currents (EPSCs) in layer V pyramidal neurons in the somatosensory cortex at P9

via whole-cell recordings in acute cortical brain slices (Fig. 2D). sEPSCs from P9 APC cKOs were increased in both frequency and amplitude (Fig. 2C; *** $p < 0.001$ with $D = 0.1843$, Kolmogorov-Smirnov test; $n = 12$ cKO and 12 WT cells; Fig 2d; * $p < 0.05$ with $D = 0.2695$). Similarly, the frequency of mEPSCs was increased in P9 APC cKOs as compared to WT (Fig. 2E; *** $p < 0.001$ with $D = 0.3379$; $n = 9$ cKO and 10 WT cells). The amplitudes of mEPSCs were unchanged (Fig. 2F). Together, these results show that the loss of APC leads to increased excitatory synaptic input onto layer V cortical pyramidal neurons at P9.

Behavioral spasms in neonatal APC cKO mice.

We next examined whether behavioral spasms occur in APC cKO mice during neonatal development. Since the onset of infantile spasms in humans is usually in the first year of life, we quantified behavioral spasms in APC cKO and WT littermate pups at neonatal ages (P5-P14). These ages represent a period of major synapse development and brain growth in the respective species. Spasms were defined as spontaneous high-amplitude, spastic movements, using the criteria developed for assessing IS-like phenotypes in other rodent models (Velisek L et al. 2007; Lee CL et al. 2008; Price MG et al. 2009; Scantlebury MH et al. 2010; Velisek L et al. 2010). P9 APC cKOs exhibited robust spasm behavior (each high amplitude spasm consisted of clusters of spastic movements that included rapid, full flexions and/or extensions of 2 or all 4 limbs, trunk flexion, trunk curling, back arching, as well as failure to right after falling over) (Fig. 3A,B; ** $p = 0.01$, Student's t test; $n = 18$ WT and cKO, Movie 1). The spasms typically occurred in a series and were separated by periods of low amplitude twitches and behavioral arrest, often with the mouse remaining on its side. We quantified the amount of time mice spent on their side and found it to be significantly increased for P9 APC cKO pups compared to WT littermates (Fig. 3C; 17.8 ± 4.5 vs 4.7 ± 2.3 minutes, ** $p = 0.01$, Student's t test; $n = 10$ cKOs and 13 WT). Motor activity was not globally altered, however, as there were no significant

differences in low amplitude movements (slow, low amplitude motor startles, myoclonic twitches, limb and tail movements) between P9 APC cKOs and WT littermates (Fig. 3A). Spasms peaked in intensity and frequency at P9 and decreased with increasing age. By P14, high amplitude spontaneous spasms were not observed, resembling the transient spasms seen in other IS rodent models (Price MG *et al.* 2009; Scantlebury MH *et al.* 2010).

Aberrant cortical activity in neonatal APC cKO mice

To determine whether neonatal APC cKO mice have abnormal brain activity, we performed EEG recordings by implanting pups with miniaturized EEG radio telemetry transponders at P8 (Zayachkivsky A *et al.* 2013; Zayachkivsky A *et al.* 2015). EEG signals were analyzed for the presence of high amplitude EEG spikes, as seen in individuals with IS (Ohtahara S 1984) and other rodent models of IS (Price MG *et al.* 2009; Scantlebury MH *et al.* 2010). High amplitude EEG activity (as defined by >3 standard deviations of the baseline EEG) were commonly seen in APC cKOs, particularly at P9 (Fig. 4B,C; P9, 3.61 ± 0.08 high amplitude EEG events/min). This type of high amplitude EEG activity was significantly less common in WT littermates (Fig. 4A; P9, 0.87 ± 0.82 high amplitude EEG events/min; *** $p < 0.001$, 2-way ANOVA, significant effects of both age and genotype). The peak of aberrant EEG activity at P9 in APC cKOs matches the time frame of the peak in behavioral spasm intensity. Spiking occurred both uni- and bilaterally, similar to human IS. Of note, the high amplitude EEG discharges in APC cKOs were shorter in duration than ictal discharges seen in human IS and in other IS rodent models. Although APC cKOs showed qualitatively disorganized, abnormal EEG, the amplitude of the EEG signal did not reach sufficient amplitude to be categorized as hypersarrhythmia, a characteristic feature of human IS. Additionally, periods of electrodeciment common in human IS were not seen in APC cKOs. Notably, no existing mouse model of IS has exhibited hypersarrhythmia (potentially due to technical or model limitations), although it has been reported in a rat model of IS (Frost JD *et al.* 2012). These studies show that abnormal neonatal

EEG activity occurs commonly in APC cKOs with a similar developmental timeline to behavioral spasms, suggesting an underlying neuronal, versus motor, etiology.

Spontaneous electroclinical seizures occur in adult APC cKO mice

Next, we tested whether adult APC cKO mice (>8 weeks of age) have spontaneous electroclinical seizures using chronic EEG recording and video monitoring. APC cKOs displayed spontaneous electrographic seizures (Fig. 5A,B). During the 2 week period of monitoring, 84.6% of the APC cKOs had >1 and 54.4% had >2 electrographic seizures. No seizures were recorded in WT littermates (n= 13 APC cKOs and 9 WT; >1 seizure, $p<0.001$; >2 seizures, $p<0.05$; chi-squared test). Seizures had an average duration of 86.5 ± 7.1 seconds and displayed a typical progression of increased interictal spiking, high frequency, high amplitude ictal activity, and post-ictal suppression of EEG activity (Fig. 5A). The seizures tended to cluster, with multiple seizures occurring within a 1-2 day period, but the clusters were not frequent (1-2 days within the 2 week recording session). Behavioral phenotypes during seizures included a readily distinguishable behavioral component that included head-bobbing, tail stiffening, forelimb clonus and freezing. Additionally, prominent theta activity, associated with freezing behavior, occurred commonly in all APC cKOs, but not in WT littermates (data not shown). During these frozen behavioral states, the APC cKOs did not respond to gentle handling (Movie 2). Taken together, these data demonstrate that adult APC cKOs have spontaneous electroclinical seizures, consistent with the progression of IS in humans.

Discussion

In this study, we show that APC cKO mice recapitulate multiple salient features of human IS (Swann JW and SL Moshe 2012), including: 1) unprovoked flexor/extensor spasms early in postnatal development that appear in clusters (Fig.3); 2) abnormal neonatal EEG activity (Fig.4), and 3) spontaneous adult electroclinical seizures (Fig. 5). This mimics IS disease progression in humans (Wong M and E Trevathan 2001). Additionally, we find that excitatory synaptic activity in layer V cortical neurons is aberrantly increased at P9, the same neonatal age at which behavioral spasms and EEG abnormalities peak in APC cKOs. Although the mechanism of this increased excitation is not yet known, we favor an increase in the number of excitatory synapses. Our previous work showed that adult APC cKO cortical and hippocampal pyramidal neurons exhibit an increased density of excitatory synaptic spines, with no change in paired pulse facilitation arguing against a change in pre-synaptic function (Mohn JL et al. 2014). Previously, we also demonstrated that adult APC cKO mice display cognitive and autistic disabilities (Mohn JL et al. 2014), which are often co-morbid with IS in humans (Kim EH and TS Ko 2016; Nickels KC et al. 2016). Together, our findings provide novel insights into the consequences of deregulated β -catenin pathways in early brain development and suggest that these pathways may be central to the pathophysiology of IS.

Compelling genetic evidence supports the relevance of APC/ β -catenin/Wnt signaling to human IS. Eleven out of 15 identified IS risk genes (including MAGI-2/S-SCAM, FOXG1, ARX, LIS1, TSC1/2, GRIN1, GRIN2A, DCX, NR2f1 and STXBP1) associate with APC, β -catenin and Wnt signaling pathways (Table 1), (Yanai H et al. 2000; Campos VE et al. 2004; Guerrini R and T Filippi 2005; Khanna R et al. 2007; Hebbar S et al. 2008; Marshall CR et al. 2008; Price MG et al. 2009; Conti V et al. 2011; Paciorkowski AR et al. 2011; Striano P et al. 2011; Go CY et al. 2012; Epi KC et al. 2013). Mutations in ARX, FOXG1, and TSC1/2 are predicted to affect β -

catenin/Wnt signaling levels and to cause changes in neuronal migration and maturation (Mak BC et al. 2003; Seufert DW et al. 2005; Danesin C et al. 2009). MAGI-2/S-SCAM, a synaptic scaffold protein, binds directly to β -catenin and different S-SCAM isoforms are involved in both excitatory and inhibitory synapse assembly (Nishimura W et al. 2002; Hirabayashi S et al. 2004; Sumita K et al. 2007; Rosenberg MM et al. 2010). Lis1 binds directly to APC (Orlova KA and PB Crino 2010). Recently, APC has also been identified as an mRNA-binding protein. Its targets include five IS-linked transcripts, FOXP1, LIS1, DCX, NR2f1 and STXBP1 (Preitner N et al. 2014). APC may regulate the levels, stability, and/or localization of these mRNAs. It will be interesting, in future studies, to explore this possibility in the APC cKO mouse brain.

Our genetic manipulations of the APC/ β -catenin pathway target a critical window of mammalian brain development. Activation of the α CaMKII-Cre-93 driver occurs at a developmental stage at which cortical glutamatergic neurons of both the human and mouse brain display convergent expression of several genes linked to neurodevelopmental disorders (Parikshak NN et al. 2013; Willsey AJ et al. 2013). Further, conditional manipulation of APC gene expression during synaptic differentiation, rather than earlier or later, is necessary to define behavioral and cognitive phenotypes. Global nulls lead to embryonic lethality and deletion or overexpression of APC in neural progenitor cells leads to severe brain malformation (Moser AR et al. 1995; Ivaniutis U et al. 2009; Yokota Y et al. 2009). Our results call for further investigation of the role of APC/ β -catenin signaling in both other IS models and in human IS, in order to elucidate whether disruption of this pathway may be a core pathophysiological mechanism in IS.

Providing a new rodent genetic model that displays IS-like phenotypes is an important advance to understanding the pathophysiology of the disease and allows preclinical testing of potential IS treatments. The ARX (Aristaless-related homeobox) (GCG) 10+7 model of X-linked IS (Price MG et al. 2009) is a currently available genetic model of IS. Pharmacological models

including the TTX infusion, triple hit, and acute NMDA rodent models also exist (Velisek L *et al.* 2007; Lee CL *et al.* 2008; Scantlebury MH *et al.* 2010; Velisek L *et al.* 2010). Additionally, other genetic mouse models with altered IS risk genes manifest some, but not all, aspects of human IS phenotype. These models include conditional deletion of Arx in subpallial- and pallial-derived (Marsh E *et al.* 2009; Simonet JC *et al.* 2015) cortical interneurons, and the CDKL5 knockout model (Wang IT *et al.* 2012). Together, these models suggest roles for altered neuronal maturation, including aberrant interneuron migration and differentiation, impaired excitatory/inhibitory co-ordination, neuroinflammation, and neuroendocrine systems in the etiology of IS. These models underscore the complexity of IS and our limited understanding of the molecular, cellular, and circuit-level basis of this disorder. Most important, several of these models exhibit neonatal spasms and chronic seizures, making them informative in understanding the pathophysiology of IS, and valuable pre-clinical tools for assessing new therapeutic strategies based on specific molecular targets.

The APC cKO mouse provides important new insights into the pathophysiology that can lead to spasms and seizures, and targets with potential for therapeutic intervention. Our findings, and evidence drawn from human IS risk genes (Table 1), suggest a novel role for β -catenin pathway malfunction in the molecular etiology of IS. Interestingly, mTOR pathways, which are implicated in multiple epileptogenic mechanisms, may interact with and modulate β -catenin signaling (Mak BC *et al.* 2003). We propose that greater understanding of the critical roles of β -catenin/Wnt signaling in the formation and function of brain circuits will provide insights into previously unappreciated mechanisms of IS and other epilepsies. A number of compounds which modulate these pathways have been developed for treating cancer, and may also prove useful for treating neurological disease. It will be interesting in future studies to test whether correcting the altered levels of β -catenin/ Wnt pathways in APC cKO mice will remedy spasms and seizures. Such studies will provide essential insights into potential targets for new,

effective therapeutic interventions, with relevance to the multiple IS-linked human genes predicted to alter APC, β -catenin, Wnt pathways.

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Statement of Work

MJ, CGD, AY, and AY conceptualized the project. AP, JA, LAL, AY, AZ, MHJ, and CGD designed the experiments. AP, JA, LAL, DH, AZ, MHJ, and CGD performed and analyzed experiments. AP, MHJ, and CGD wrote the manuscript.

Table 1. Genes linked to human IS and their connection to β -catenin/Wnt signaling.

Fig.1. APC deletion leads to increases in β -catenin protein levels in the developing cortex. (A) Immunoblots of neonatal and adult cortical lysates show a progressive decrease in APC protein levels and parallel increases in β -catenin levels in APC cKO mice (lanes 3-4), compared with WT littermates (lanes 1-2), at the indicated ages. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is included, as a loading control. Average data shows significant changes in both APC (B) and β -catenin (C) protein levels starting from P14 in the APC cKO cortex, after normalizing to GAPDH levels. (* $p < 0.05$, ** $p = 0.01$, Student's t test; $n = 4$ WT and $n = 5$ cKOs for all ages). The extent of change in the CamKII α -Cre expressing neurons is likely greater than that measured by this total cell population-based assay because at each of the ages, not all cells express the CamKII α -Cre recombinase required for APC deletion and subsequent increases in β -catenin. (D) Epifluorescence micrographs of reporter mouse (Rosa26^{fl} GFP,L10A X CamKII α -Cre) demonstrates cells in the cortex with activated Cre recombinase (GFP). At P9, GFP is seen in a relatively small subset of cortical cells, predominantly the layer V pyramidal neurons, and at P60, essentially all layer V pyramidal cells show Cre-mediated GFP expression. Scale bar, 100 μ m. (E) Epifluorescence micrographs of β -catenin immunolabeling (left panels) in brain sections from P9 APC cKOs (right) and WT littermates (left). APC cKOs show increased β -catenin immunoreactivity in the soma of layer 5 pyramidal neurons, identified by their characteristic large soma size, triangular shape, and laminar location (inset, scale bar, 25 μ m), and by staining of their large round nuclei with 4'-6-diamidino-2-phenylindole (DAPI). Scale bar, 50 μ m. (F) Scatter box plot shows quantification of normalized β -catenin immunoreactivity in the soma of individual layer 5 pyramidal neurons in P9 APC cKOs (gray) versus WT littermates (black). ($n = 100$ cKO neurons and $n = 154$ WT neurons; $n = 3$ cKOs and 3 WT mice, ** $p < 0.01$, Student's t test).

Fig. 2. Increased excitatory synaptic currents in P9 APC cKO cortical layer 5 pyramidal neurons. (A) Representative traces of spontaneous EPSC (sEPSC) recordings from a layer V pyramidal neuron in the somatosensory cortex of WT (black, top) and APC cKO (red, bottom) mice at P9. (B) Average sEPSCs from WT (black) and APC cKO (red) animals. (C) Cumulative probability distributions of sEPSC inter-event intervals shows a significant increase in frequency in cKOs as compared to WT, ($***p < 0.001$ with $D = 0.1843$, Kolmogorov-Smirnov test; $n = 12$ cKO and 12 WT cells). (D) Cumulative probability distribution of amplitude of sEPSC show a significant increase in cKOs as compared to WT ($*p < 0.05$ with $D = 0.2695$). (E) Cumulative probability distribution of inter-event intervals of mEPSCs, recorded in the presence of $1 \mu\text{M}$ TTX, shows a significant increase in frequency in cKOs ($***p < 0.001$ with $D = 0.3379$; $n = 9$ cKO and 10 WT cells). (F) Cumulative probability distribution of mEPSC amplitude suggests no significant difference between APC cKOs and WT littermates.

Fig. 3. Behavioral spasms in neonatal APC cKO mice. (A) Representative examples of spontaneous high amplitude spastic movements in P9 APC cKO pups, including falling over; (1) rapid, full extension, (2) and/or contraction of 2 to all 4 limbs; (1,2) trunk curling and (3) back arching (video link). (B) Increased number of high amplitude spastic movements in P9 APC cKOs (white) compared to WT littermates (black), based on scoring of the 45 minute video recording of pup behavior ($**p = 0.01$, Student's t test; $n = 18$ WT and cKOs). Low amplitude movements did not differ between genotypes, suggesting no overall change in locomotor activity. (C) P9 APC cKO spent significantly more time on their sides, indicative of an abnormal behavior state ($**p = 0.01$, Student's t test; $n = 10$ cKOs and 13 WT).

Fig. 4. High amplitude EEG discharges in neonatal APC cKO mice. (A) Example EEG recording from P9 WT mouse showing little to no high amplitude activity. (*Inset*) Increased time scale of (A) at time 1. (B) Example EEG recording from P9 APC cKO animal showing multiple high amplitude EEG events. (*Inset*) Increased time scale of (B) at times 1 and 2. Note: Both (1) simple high amplitude spikes and (2) more complex high amplitude spikes followed by continued disruption of the EEG occur in APC cKO animals. (C) Image of a P12 mouse implanted with a radio-telemetry based EEG head mount. (D) Quantification of the frequency of high amplitude discharges in WT and APC cKO mice ($***p < 0.001$, 2 way ANOVA, WT: P9, $n = 3$, P10, $n = 6$, P11, $n = 6$, P12, $n = 6$; cKO: P9, $n = 3$, P10, $n = 4$, P11, $n = 6$, P12, $n = 6$). The peak of aberrant EEG activity at P9 matches the time frame of the peak in behavioral spasm intensity.

Fig. 5. Spontaneous electroclinical seizures in adult APC cKO mice. (A) Example of an electrographic seizure recorded in an adult APC cKO mouse. Seizures consisted of the following components: (i) buildup of pre-ictal spiking, (ii) high-frequency, high-amplitude ictal activity, and (iii) a post-ictal suppression of the EEG. (B) Approximately 80% of APC cKOs had 1 or more seizures and approximately 50% of APC cKOs had 2 or more seizures during a two-week EEG monitoring period. In contrast, no seizures were recorded in WT littermates during 2 weeks of monitoring. (* $p < 0.05$, *** $p < 0.001$, Chi-squared test, WT: $n=5$; cKO: $n=7$).

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

IS Risk Genes Linked to β -cat/APC

Excitatory Synaptogenesis	S-SCAM/Magi2 - binds directly to β -catenin and NMDARs; recruits PSD-95 that regulates AMPAR NMDARs- GRIN1, GRIN2A, GRIN2B; AMPARs- GRIA1-4 - glutamate receptors STXBP1 - syntaxin-1 binding protein functions in vesicle trafficking & transmitter release
Canonical Wnt Signaling	TSC1/2 - functions in degradation complex that regulates β -catenin levels FoxG1 - inhibitor of Wnt signaling ARX - inhibitor of Wnt signaling
APC Binding Partners (protein)	LIS1 - binds to APC and regulates neuronal cell migration in the developing brain
APC Binding Partners (mRNA)	DCX - microtubule-associated protein NR2F1 - transcriptional regulator