Embryonic mosaic deletion of APP results in displaced Reelin-expressing cells in the cerebral cortex

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Abstract

It is widely accepted that amyloid precursor protein (APP) plays a central role in the pathogenesis of Alzheimer’s disease. In addition, APP has been proposed to have functions in numerous biological processes including neuronal proliferation, differentiation, migration, axon guidance, and neurite outgrowth, as well as in synapse formation and function. However, germline knockout of APP yields relatively subtle phenotypes, and brain development appears grossly normal. This is thought to be due in part to functional compensation by APP family members and other type I transmembrane proteins. Here, we have generated a conditional mouse knockout for APP that is controlled temporally using CreER and tamoxifen administration. We show that total cortical expression of APP is reduced following tamoxifen administration during embryonic time points critical for cortical lamination, and that this results in displacement of Reelin-positive cells below the cortical plate with a concurrent elevation in Reelin protein levels. These results support a role for APP in cortical lamination and demonstrate the utility of a conditional knockout approach in which APP can be deleted with temporal control in vivo. This new tool should be useful for many different applications in the study of APP function across the mammalian life span.

Keywords

APP; Reelin; migration; Alzheimer’s; cortex; development

Introduction

Amyloid precursor protein (APP) is a highly studied protein, primarily due to its role in Alzheimer’s disease (AD). Dozens of missense mutations have been identified in APP that result in the alteration of the type and amount of amyloid beta (Aβ) generated (reviewed in [1]).
These mutations result in an early-onset form of AD, and they are fully penetrant and dominantly inherited. APP is sequentially cleaved by β-secretase and γ-secretase to generate Aβ. Aβ is the primary component of amyloid plaques, the pathological hallmark of early- and late-onset AD. As Alzheimer’s disease candidate drugs seek to chronically target APP processing, understanding the functions of APP in the healthy brain is essential.

Numerous studies have reported a variety of roles for APP in the central nervous system, but redundancy with other family members has complicated the study of APP. We previously reported that APP mediates cortical cell placement in rats in a Dab1-dependent manner [2]. Displacement of cells below the cortical plate was induced in utero with shRNA constructs directed at APP and rescued with co-expression of full-length APP or its family members. However, mice with germline single knockout (KO) of APP do not show gross abnormalities in cortical cell placement [3]. This discrepancy between phenotypes with loss of APP could be due to a number of factors including: 1) rat versus mouse species differences, 2) compensation by other APP family members upon germline knockout, and/or 3) mosaic deletion of APP resulting in differential competition of wild type and APP mutant cells for extracellular migratory cues.

In support of a role for compensation by family members, deletion of APP and APLP2 results in early postnatal lethality, and triple KO of APP, APLP1, and APLP2 results in death soon after birth [4, 5]. Herms et al. (2004) found that while cortical lamination was grossly normal in APP single KO mouse cerebral cortex, APP/APLP1/APLP2 triple KO mice displayed focal over-migration of subsets of cortical cells, similar to cobblestone (type II) lissencephaly observed in humans [4]. In addition, triple KO resulted in a partial reduction in the number of Reelin-positive, Cajal-Retzius cells present in the marginal zone [4]. A role for APP family members in neuronal placement/migration appears to be evolutionarily conserved, as disruption of APP homologs in Drosophila [6] and Manduca [7] results in disrupted nuclear positioning and inappropriate migration of neuroblasts, respectively.

In order to address the discrepancy between shRNA-induced APP reduction and germline APP knockout and to clarify the role of APP in cortical cell placement in mammalian cells, we have developed a tool for regulated temporal disruption of genomic APP in mice. Here, we report the generation and characterization of APP conditional knockout mice (APPflox), where deletion of APP is tamoxifen-inducible through CreER expression. Tamoxifen administration during an embryonic time window critical for cortical lamination results in reduced APP protein expression, displacement of Reelin-expressing cells, and elevation in Reelin protein expression in the embryonic cerebral cortex. These phenotypes were not observed in our APPflox mice crossed to germline deleter mice, suggesting biologically distinct effects of acute/mosaic versus chronic/complete APP knockout. Taken together, our study supports a role for APP in cell placement, strengthens the link between Reelin and APP signaling, and describes a new resource for the study of APP in developing and adult tissues.

Dev Biol. Author manuscript; available in PMC 2018 April 15.
Materials and Methods

Mice

Mice were housed and cared for under the guidelines established by Harvard University’s Institutional Animal Care and Use Committee in compliance with federal standards. The APP targeting construct was designed and generated at the UNC BAC Engineering Core. Constructs were introduced into J1(129/Sv) ES cells, clones picked and selected, and homologously recombined clones injected into blastocysts at the Partners Healthcare Transgenic Core. F1 animals were genotyped by Southern blot and PCR. Once lines with germline transmission were identified, these animals were crossed to the B6;SJL Tg(ACTFLPe)9205Dym/J mice [8] (Jackson Labs) to delete the FRTneoFRT cassette by Flp-mediated recombination in the germline. To generate the tamoxifen-inducible APP cKO line, the APP\textsuperscript{flox} line was crossed to the Tg(cre/Esr1)5Amc/J line [9] (Jackson Labs). For germline deletion (APP gKO), APP(fl) was crossed to BALB/c-Tg(CMV-cre)1Cgn/J [10] (Jackson Labs). For both APP cKO and APP gKO lines, animals were then backcrossed to C57BL/6 animals for at least five generations.

Genotyping

Floxed APP primers were 5′-CTGCTTCTGGCTTCTGATC-3′ (forward) and 5′-GAATGGGCTAAAGGGTTTG-3′ (reverse), yielding a WT band at 300 bp and the flox mutant band at 400 bp. The PCR protocol was 95°C for 4:00 min, 39 cycles of 94°C for 0:30 min, 58°C for 0:45 min, and 72°C for 1:40 min. Final elongation was 72°C for 5:00 min with a hold at 4°C. Cre\textsuperscript{ER} and CMV-Cre primers were 5′-ACACCAGAGACGGAAATCCAT-3′ (forward) and 5′-GCAGAACCTGAAGATGTTCGC-3′ (reverse). DNA from Cre-positive mice yielded a band at 500 bp. The PCR protocol was 95°C for 3:00 min, 39 cycles of 95°C for 0:30 min, 58°C for 0:30 min, and 72°C for 1:15 min. Final elongation was 72°C for 7:00 min with a hold at 4°C.

Tamoxifen preparation and administration

To activate the latent Cre\textsuperscript{ER} recombinase, cKO mice were given tamoxifen (TAM) via intraperitoneal (IP) injection at one of three time points: embryonic, postnatal, or adult. For all timepoints, tamoxifen was dissolved in a corn oil (Sigma)/10% ethanol solution. For embryonic injections, timed-pregnant female cKO mice (embryonic day 12, E12) were given IP injections of 3 mg TAM (300 uL of 10 mg/mL TAM) daily for 3 days. Brains were harvested 4 d after final TAM treatment on E18. For postnatal injections, mice were injected with 2 mg/mL 4-hydroxy TAM on postnatal days 5 and 7 (P5, P7) and brains were harvested on P14. Adult mice were injected with 6 mg tamoxifen daily for 3 days and brains were harvested 10 days after the final injection (Fig. 1B). Since Cre recombinase is constitutively active in the gKO line, gKO pup brains were harvested at E18 from timed-pregnant gKO females that did not receive TAM injections. For all litters, Cre-negative and Cre-positive mice were mated in order to generate littermate controls (cWT, gWT) within each experiment.
**Western blot analysis**

Immediately after harvest, one cerebral hemisphere per brain was lysed in 1% Nonidet P-40 (NP-40) STEN buffer [150 mM sodium chloride, 50 mM Tris, 2 mM EDTA, and 1.0% (v/v) NP-40]. Lysates were electrophoresed on 4–12% Bis-Tris Nu-Page gels (Invitrogen), transferred to nitrocellulose, and blocked using Odyssey blocking buffer (Licor) or 5% milk in phosphate buffered saline plus Tween. Samples were stored at −80 degrees Celsius with minimal freeze-thaw events. Western blotting was performed with anti-APP, C-terminal antibodies C7 and C9 (Rb; both 1:1,000; Selkoe Laboratory, Brigham and Women’s Hospital), N-terminal antibody APP597 (Rb; 1:1,000; IBL America, Minneapolis, MN) and the N-terminal Reelin monoclonal antibody G10 (Ms, 1:2,000, Abcam). GAPDH (Ms; 1:2,000, Millipore) was used as a protein loading control. IRDye680- and IRDye800-conjugated secondary antibodies (1:10,000; Rockland Immunochemicals) were used prior to analysis on the LiCor detection system. For Reelin, signal was detected using HRP-coupled goat anti-mouse antibodies followed by detection via enhanced chemiluminescence (SuperSignal West Dura Kit, LifeTechnologies).

**Immunofluorescent staining and confocal microscopy**

Immediately after harvest, one cerebral hemisphere per brain was fixed in 4% paraformaldehyde for at least 12 h. Fixed brains were washed in phosphate-buffered saline (PBS), embedded in 2% agarose/PBS, and vibratome sectioned (150–200 um) in the coronal plane. Sections were incubated in blocking buffer (2% donkey serum; 0.1% Triton X-100 in PBS) for 1 h, then incubated in primary antibody [anti-brain lipid-binding protein (BLBP), 1:1000 (Chemicon); anti-CTIP2, 1:500 (Abcam); anti-doublecortin (DCX), 1:100 (Santa Cruz Biotechnology); anti-glial fibrillary acidic protein (GFAP), 1:1000 (Abcam); anti-IBA1, 1:200 (Wako); anti-LIM/homeobox protein (LHX2), 1:500 Santa Cruz Biotechnology; anti-microtubule-associated protein 2 (MAP2), 1:5,000 (Abcam); anti-OCT6, 1:500 (Santa Cruz Biotechnology); anti-Reelin (G10), 1:1,000 (Millipore); anti-TBR1, 1:250 (Abcam) overnight at 4°C, followed by three washes in PBS. Nuclear stains were performed on all sections, using either DAPI or TO-PRO-3 (TOPRO3), (both ThermoFisher, following manufacturer’s instructions). Sections were then incubated with Cy2-, Cy3-, and Cy5-conjugated secondary antibodies (1:2,000; Jackson ImmunoResearch) for 1h followed by three PBS washes. Sections were mounted on glass slides using Fluormount (Sigma). Images were acquired using a Zeiss LSM 510 confocal microscope with Axiovert 100M system. For each pup, the total number of cortical sections with Reelin-positive cells located below layer 1 was quantified by a researcher blinded to the experimental conditions.

**Statistics**

Statistical analyses were performed using GraphPad Prism 7. Two-tailed Student’s t-tests were used to compare two different groups, and, when necessary, unequal variances were corrected using Welch’s correction. A one-way ANOVA was performed to compare means of three or more groups. When homogeneity of variances could not be assumed to perform a one-way ANOVA, a Kruskal Wallis test using Dunn’s multiple comparisons test was used.
Pearson’s correlation tests were used to determine the strength of the relationship between two variables.

Results

Generation of conditional APP\textsuperscript{flox} animals

APP conditional knockout animals were generated by introduction of loxp sites flanking exon 3 of APP (schematic outlined in Fig. 1A). Splicing of exon 2 to exon 4 in the absence of exon 3 is predicted to introduce a frameshift and premature stop codon, which should result in nonsense-mediated decay of the transcript and eliminate expression of APP protein. In order to confirm that deletion of exon 3 was sufficient to eliminate APP expression, APP\textsuperscript{flox} mice were crossed with transgenic mice expressing Cre under transcriptional control of the human cytomegalovirus minimal promoter (CMV-Cre). Since this Cre acts ubiquitously, we refer to these germline deletion mice as APP gKO \cite{10}. In order to temporally control deletion of APP in mice, APP\textsuperscript{flox} mice were crossed with Tg(cre/Esr1)5Amc/J. This line ubiquitously expresses a fusion protein of Cre and a modified form of the estrogen receptor, but Cre protein is only translocated to the nucleus, and therefore able to act on genomic DNA, when bound to 4-hydroxytamoxifen (4OH-TAM) \cite{9}. This allows for the temporal control of APP deletion through introduction of tamoxifen (TAM) at different times in development (Fig. 1B). TAM crosses the blood-placenta and blood-brain barriers, but TAM-induced recombination and excision can be incomplete, often creating a mosaic animal.

The efficiency of APP KO in gKO mice was assessed by measuring APP protein expression in cortical tissue from gKO mice and their Cre-negative littermate controls (gWT). To assess knockout efficiency across litters, each pup’s APP protein level was normalized to GAPDH, then normalized to the wild type average for that litter. Two distinct bands for full-length APP (~100 kDa) were visible in all gWT brains (Fig. 1C). APP protein expression was efficiently eliminated in gKO cortex relative to gWT littermates. Similarly, efficiency of TAM-induced APP knockout in cKO mice was assessed by measuring APP protein expression in cortical tissue from cKO mice and their littermate controls (cWT). Mice were treated with TAM at embryonic, postnatal, or adult time points. APP protein levels were significantly reduced in cKO cortex relative to littermate controls, with an average percent reduction of: (i) embryonic: 44.96% (±5.97; two-tailed t-test, p=0.0001, d.f.=39; cWT N=20 brains, cKO N=21 brains, from 5 litters), (ii) postnatal: 52.1% (±10.8; two-tailed t-test, p=0.0018, d.f. =7), (iii) adult: 86% (±14.9; two-tailed t-test with Welch’s correction for unequal variances, p=0.0013, d.f. =8.5) (Fig. 1C–H). These results demonstrate efficient TAM-induced reduction of APP in cKO mice, although APP reduction did not reach the degree observed in the gKO mice (Fig. 1C, D).

Markers of multiple cell types in the forebrain are qualitatively similar with embryonic conditional deletion of APP

Pregnant APP cKO mice were injected with tamoxifen at E12, 13, and 14 and pups harvested at E18. Immunostaining for markers of radial glia (BLBP), neurons (MAP2) and layer-specific neuronal subtypes (OCT6, LHX2, CTIP2, and TBR1) was performed. Nearly
all markers were expressed in similar patterns between wild type and APP cKO mice (Fig. 2), suggesting that cortical layering was generally intact in spite of reduced APP levels in the developing brain. However, a small number of TBR1+ cells were observed below the cortical plate in a subset of embryos (Fig. 2H). Germline deletion of APP similarly showed no evidence of layering disruption as evidenced by grossly similar expression patterns of MAP2 and layer-specific neuronal subtypes (CUX1, CTIP2, and TBR1) compared to wild type mice (Supp. Fig. 1).

Previous studies of traditional germline APP KO mice showed an elevation in the number of astrocytes in the hippocampus [3, 11]. However, in our APP cKO model, immunostaining for astrocytes using GFAP and for microglia using IBA1 showed grossly similar numbers and morphology of astrocytes and microglia in the hippocampus (Supp. Fig. 2) and cerebral cortex (not shown). GFAP quantification via Western blotting showed no significant differences between wild type and APP KO when APP was deleted embryonically (Supp. Fig. 2D, H) or in the adult (Supp. Fig. 2M). Quantification of astrocyte number showed no significant differences when APP was deleted early postnatally (Supp. Fig. 2I) or through germline deletion (Supp. Fig. 2N).

Deletion of APP at embryonic time points results in displaced Reelin-positive cells

Pregnant APP cKO mice were injected with TAM at E12, 13, and 14 and pups harvested at E18. Cerebral cortices were sectioned and immunostained for Reelin (expressed in Cajal-Retzius cells in the marginal zone), MAP2 (general neuronal marker), and TBR1 (lower layer cortical marker) to assess cortical lamination. TBR1 and MAP2 staining were consistent across brains of all genotypes, with MAP2 immunostaining delineating the entirety of the cortical plate (CP) and TBR1 immunostaining marking the lower layers of the cortical plate (Fig. 3A–H). However, with embryonic deletion of APP, the majority of brains showed displaced Reelin+ cells below the cortical plate (Fig. 3B, D, F, H), and a subset of these brains also showed TBR1+ cells displaced below the cortical plate (Fig. 3H, red arrows). Some brains showed very few Reelin+ cells in the intermediate zone (Fig. 3D, white arrows), with concomitant thinning of the density of Reelin+ cells in the marginal zone (Fig. 3D, white arrowheads). Other brains showed larger numbers of displaced Reelin+ cells in the intermediate zone (Fig. 3F, H). For each brain, the total number of cortical sections with Reelin-positive cells displaced below the cortical plate was quantified and normalized to the total number of cortical sections analyzed per brain. No clear differences were found between the embryonic gKO and littermate gWT brains (Fig. 3I). In the cKO mice, however, a significantly greater percentage of brain sections had Reelin-positive cells displaced from the marginal zone relative to the cWT brains (Fig. 3I; p=0.0005, d.f.=27, cWT N=11 brains; cKO N=12 brains, from 5 different litters). Notably, the percentage of sections with displaced Reelin+ cells varied dramatically among the cKO brains, from 0% up to 100% (Fig. 3I).

Germline and embryonic deletion of APP differentially alter Reelin protein levels in the cerebral cortex

Western blots were performed on E18 cortical tissue lysates to assess protein levels of Reelin. For each pup, Reelin expression was normalized to GAPDH, then normalized to the
wild type average for that litter. Full-length Reelin is large (450 kDa) and is cleaved \textit{in vivo} at two sites, producing multiple fragments named based upon repeats present in the protein (reviewed in [12]). We used an N-terminally directed antibody to detect full-length Reelin, N-R6 (370 kDa), and N-R2 (180 kDa). In the present study, full-length Reelin and the N-R6 fragment (collectively, “upper band” Reelin) were quantified separately from the N-R2 fragment (“lower band” Reelin) (Fig. 4A–D). Acute deletion of APP in the cKO mice yielded a significant, ~50% increase in upper band (full-length/N-R6) Reelin levels (Fig. 4C; \( p=0.0347 \)). In contrast, chronic deletion of APP in the gKO mice did not reveal a significant change in total Reelin levels, although gKO mice showed a significant, ~70% decrease in the N-R2 fragment (Fig. 4D; \( p<0.0001 \)).

**Efficiency of acute embryonic APP disruption may drive changes in Reelin protein levels and Reelin+ cell displacement in the cerebral cortex**

The relationships between degree of APP disruption, Reelin protein levels, and Reelin+ cell displacement following acute embryonic knockdown of APP were assessed (Fig. 5A–C). Increased efficiency of APP knockdown was weakly associated with an increase in the percentage of brain sections with Reelin+ cells displaced below the cortical plate boundary (Fig. 5A) as well as Reelin expression (upper band) (Fig. 5B), although neither correlation was statistically significant. Increased Reelin expression did not appear to be associated with increased Reelin+ cell displacement below the cortical plate (Fig. 5C).

**Discussion**

Numerous studies in a variety of organisms have characterized the role of APP in development and function of the nervous system (reviewed in [13]). The first study of a germline knockout for APP was reported in 1995. These mice showed increased gliosis and decreased locomotor activity and forelimb grip strength [3]. Since then, double and triple knockouts of APP with its family members have been reported. APP/APLP2 and triple KO mice show early postnatal lethality, suggesting functional redundancy of these proteins [4, 5]. More recently, conditional APP knock out animals have been reported [14, 15]. Conditional mice from the Zheng lab were used to examine the role of APP at the neuromuscular junction. In an APLP2 null background, deletion of APP in either the presynaptic motor neuron or postsynaptically in the muscle resulted in an NMJ synapse defect [14].

Here, we have generated a temporally controlled APP cKO model to bypass compensation that may occur with germline knockout, and clarify the developmental functions of APP in the central nervous system \textit{in vivo}. We show in the present study that tamoxifen-mediated knockdown of APP using a Cre recombinase system resulted in an efficient decrease in total cortical expression of APP at multiple developmental time points (Fig. 1C–H). Notably, tamoxifen treatment in the cKO mice yielded more efficient APP knockdown in adult mice compared to embryonic mice. This is most likely due to the differences in the adult and embryonic injection protocols outlined in the methods section. First, the embryonic injection protocol required extensive tamoxifen dosage optimization that yielded efficient APP knockdown without causing excess embryonic mortality. The optimized protocol required a
lower total tamoxifen dose per litter compared to the adult dosing protocol. In addition, tamoxifen must cross both the placenta and the blood-brain barrier in embryonic mice, likely resulting in decreased total bioavailability compared to the adult mice. Further, each pup receives its blood supply via an individual placenta; thus, physiologic variations in placental blood flow may result in variability in tamoxifen delivery within a litter. In spite of expected variations in tamoxifen bioavailability, we demonstrate effective tamoxifen-mediated knockdown of APP at embryonic, postnatal, and adult time points.

Reelin protein plays an essential role in proper cortical lamination in mice and humans. Human subjects with mutation of the Reelin gene (RELN) have a rare form of lissencephaly, and variants in RELN have been implicated in autism and schizophrenia (reviewed in [16]). Mice with RELN mutation show severely disrupted cortical lamination, often described as “inside-out,” or an inversion of the excitatory neurons within different layers of the cerebral cortex [17, 18]. While more recent studies have shown that the lamination defect is much more complex than a simple “inside-out” pattern [19], it is clear that Reelin is an important signaling molecule in the mammalian brain. Reelin is expressed by Cajal-Retzius cells in the marginal zone during embryonic development and in the postnatal brain in subsets of inhibitory neurons. We show here that embryonic reduction of APP expression significantly alters the placement and levels of Reelin-expressing cells in the embryonic cortex. Although it would be ideal to use immunocytochemistry to examine the expression of APP in our model systems, we were unable to do so for both technical and biological reasons. In the wild type cortex, APP protein is present diffusely throughout [2]. When using an N-terminal antibody, both full length APP and secreted, extracellular sAPP are detected. Since sAPP diffuses extracellularly, this cannot give a cell-autonomous readout for APP expression and consequently is not useful for examining if misplaced cells have reduced APP expression. There exist C-terminal antibodies for APP, but via immunostaining show some background signal as assayed in gKO mice (data not shown). This may be due to some level of cross-reactivity with APLP1 and APLP2 since their C-termini are highly identical at the amino acid level. Despite these limitations, future studies will be important for investigating potential effects of APP on Reelin-positive inhibitory neurons in the adult, and our model should be a useful tool for dissociating developmental and adult functions of APP.

In the present study, we observe a defect in the placement of Reelin-expressing cells and levels of Reelin protein at E18, following tamoxifen-induced APP knockdown at E12–14. In the mouse brain, this is an active time of neurogenesis and migration in the cerebral cortex. In the wild type brain, pioneer neurons migrate at E13.5 and undergo radial migration at E14.5, at which time the first cohort of migrating neural precursor cells (NPCs) divides the cortical preplate into the marginal zone and subplate (reviewed in [12]). From the preplate stage (E12.5) through cortical lamination, Reelin-secreting pioneer neurons form the most superficial layer of the cortex, (marginal zone/layer 1), and these cells are conventionally identified as Cajal-Retzius (CR) cells (reviewed in [12]). Lineage studies and birthdating experiments have revealed that these Reelin-expressing CR cells arise from the cortical hem and the rostral pallium [20–22]. A subset of these CR cells express Tbr1 [21], which in the late embryonic and postnatal brain marks lower layers of the cortex. Although we focused our quantitative studies on Reelin, it is worth noting that we did also observe displaced cells expressing Tbr1 (Fig. 2H; Fig. 3D, H).
The origin of the displaced Reelin-positive cells in our model is unclear. These may be late-developing CR cells that fail to migrate correctly, CR cells that are displaced due to focal defects in preplate splitting, and/or subpallially-derived Reelin-expressing cells. In some brains, we observe a reduction in the number of Reelin-positive cells in the marginal zone, perhaps supporting a pallial origin for the displaced cells that could have been displaced from the marginal zone. As mentioned above, triple knockout animals show a defect in cortical migration similar to type II lissencephaly, with an added feature of a reduction in the number of CR cells in the marginal zone [5]. Double knockout of FE65 and FE65L1, adaptor proteins that bind to APP and affect its processing, produces a migration phenotype similar to that observed in APP triple mutant mice [23]. That study suggests that FE65-dependent molecular events may occur downstream of APP to regulate neuronal migration and may provide clues to the molecular mechanisms mediating APP-Reelin interactions.

Having observed displaced Reelin-positive neurons in our cKO mice, we assessed cKO lysates of the cerebral cortex for altered Reelin protein levels. We observed dysregulated Reelin protein levels with APP disruption. Relative to wild type littermates, the embryonic APP cKO mice showed a significant increase in the full length/N-R6(450/370 kDa) Reelin species, with no significant change in the N-R2 (180 kDa) fragment (Fig. 4). Reelin is cleaved in two locations to yield several different fragments. While the full-length and N-R6 fragment retain the domains essential for the action of Reelin on its receptors [24], the N-R2 fragment lacks those critical domains. Previous studies have interrogated the effects of Reelin expression on levels of APP in different model systems [25–29]. In addition, connections between Aβ and Reelin have been shown, including effects of Aβ on Reelin signaling [30] and effects of Reelin on Aβ fibril formation and toxicity [31–32]. While reports differ on the directionality of these effects in different models, there is consensus that these two proteins interact biochemically.

Given our observation of Reelin+ cell displacement and increased “upper band” Reelin expression following APP disruption in the cKO mice, we sought to determine if APP knockdown efficiency was positively correlated with Reelin expression and Reelin+ cell displacement. Specifically, we expected that increased efficiency of APP knockdown would be associated with increased Reelin+ cell displacement and increased Reelin expression (upper band). Although correlations between APP knockdown and Reelin cell displacement and levels were found to be weakly positive, none reached statistical significance. However, efficiency of APP knockdown was assessed on a cortex-wide basis via Western blot, and thus may not precisely reflect the relationship between APP expression and Reelin dysregulation at the local, cellular level. Future studies will be required to confirm at the cellular level if the observed displacement of Reelin+ cells was due to aberrant migration of CR cells, possibly by crossing APP cKO mice with CXCR4-eGFP mice, in which CR cells can be more readily identified [33].

The present study demonstrates the utility of a conditional KO line in which APP can be deleted with temporal control in vivo. Although the present study focused on APP KO during a particular developmental window in order to analyze the effects of APP on cortical lamination, the model presented here can be a valuable tool for studying the effects of APP throughout the lifetime of the animal. Comparing phenotypes between temporally-controlled
conditional and germline APP KO models could allow investigators to study APP-specific contributions to a wide range of neurodevelopmental processes, from neurite outgrowth and synaptogenesis to gliosis and synapse maturation. As the importance of APP in healthy neurodevelopment becomes increasingly apparent and treatments in humans that chronically alter APP processing are advancing, tools that enable in vivo control over the timing of APP expression and function should be valuable resources to clarify APP’s essential roles in the mammalian brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank JrGang Cheng of the UNC BAC transgenic core for the synthesis of the targeting plasmid and members of the Young-Pearse lab and Selkoe lab for helpful advice. This work was supported by R01MH101148 (TYP), NIH R01 AG006173 (DJS), and the Bright Focus Foundation (TYP).

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Significance Statement

Amyloid precursor protein (APP) plays a central role in Alzheimer’s disease pathogenesis. Elucidating the function of APP has been complicated by the presence of partially redundant family members. Here, we develop a mouse model in which APP expression can be eliminated at specific time points. We find that elimination of the protein during an embryonic time window critical for cortical lamination results in the displacement of Reelin-positive cells and an elevation in Reelin protein. Our study supports a role for APP in cortical lamination and provides a new tool for controlled temporal disruption of APP expression.
### Highlights

- Establishment of a temporally controlled APP conditional knock out mouse line
- Efficient reductions in APP protein at embryonic, postnatal, and adult time points.
- Embryonic reduction of APP expression induces displacement of Reelin positive cells
Figure 1. Generation of conditional APP\textsuperscript{flox} animals

(A) Structure of the APP locus before and after introduction of loxp sites. Successful targeting resulted in the introduction of loxp sites flanking exon 3 of APP. Following breeding to a line expressing FLP recombinase, a single FRT site remained in the genome adjacent to the 3\textsuperscript{′}loxp site. B) Schematic of conditional deletion of APP. APP\textsuperscript{flox} mice were crossed either to a line expressing CMV-Cre, which resulted in germline deletion of APP (APP gKO) or crossed to a line expressing Cre\textsuperscript{ER} under a ubiquitous promoter (APP cKO). Tamoxifen injection at embryonic, early postnatal, or adult time points was performed in order to induce deletion of exon 3. C–D) Three tamoxifen injections into pregnant mice at E12, 13 and 14 resulted in a reduction in APP expression at E18, as measured by Western blot. E–F) For early postnatal deletion, injections of 4-OH tamoxifen were performed at P5 and P7, and brains harvested at P14. G–H) For adult deletion, three injections of tamoxifen were performed on three consecutive days, and brains were harvested 10 days later. Western blotting quantification included both bands, which represent immature APP and mature APP with post-transcriptional modifications. Analysis revealed that APP protein levels were significantly reduced in cKO cortex relative to littermate controls with an average percent reduction of: (i) embryonic: 44.96\% (±5.97; two-tailed t-test, p<0.0001, d.f.=39; cWT N=20 brains, cKO N=21 brains, from 5 litters), (ii) postnatal: 52.1\% (±10.8; two-tailed t-test,
p=0.0018, d.f.=7), (iii) adult: 86% (±14.9; two-tailed t-test with Welch’s correction for unequal variances, p=0.0013, d.f.=8.5). ****p<0.0001.
Figure 2. Markers of multiple layers of the cerebral cortex show normal expression patterns following embryonic reduction of APP expression

Pregnant mice were injected with tamoxifen at E12, 13, and 14, and pups harvested at E18. Immunostaining was performed on sections of the cerebral cortex. Immunostaining for markers of neurons (MAP2) and radial glia (BLBP) was performed, as well as markers for particular cortical layers (CTIP2, TBR1, OCT6, DCX, LHX2). Each marker was expressed in both APP WT and APP cKO mouse cortex, and the positioning and numbers of each marker appeared qualitatively similar. Scale bars = 100 μm.

*Dev Biol.* Author manuscript; available in PMC 2018 April 15.
Figure 3. Displacement of Reelin+ cells following acute disruption of embryonic APP expression
Pregnant mice were injected with tamoxifen at E12, 13, and 14. At E18, cerebral cortices were fixed, coronally sectioned, and immunostained for Tbr1 (red), Map2 (purple), and Reelin (green), and counterstained with DAPI (blue). Representative images are shown; scale bars = 100 μm. White boxes highlight approximate region shown in panel directly beneath (G, H). Regions of overlap between Reelin (green) and MAP2 (purple) appear white. Displaced Reelin+ cells were found at the border of the intermediate zone and cortical plate in a subset of APP cKO brains (white arrows) with loss of Reelin+ cells in the marginal zone (white arrows). Some brains showed large amounts of Reelin immunoreactivity below the cortical plate (F, H), and a subset of sections also showed displaced TBR1+ cells (H, red arrows). The percentage of brain sections with Reelin-positive cells displaced below the

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cortical plate boundary relative to the total number of brain sections analyzed was quantified for each pup (I). A Kruskal Wallis testing using Dunn’s multiple comparisons test was performed (**p=0.0008, df=27).
Figure 4. Reelin dysregulation following reduction of APP expression acutely in the embryo

(A, B) Reelin protein levels were assessed by immunoblotting lysates from the cerebral
cortex of E18 embryos. Protein levels of the full-length and N-R6 (450/370 kDa) and N-R2
(180 kDa) forms of Reelin were quantified via densitometry using GAPDH as a loading
control. Reelin protein levels were quantified at E18 in (A) germline APP conditional KO
mice constitutively expressing CMVCre (gKO) and (B) tamoxifen-induced APP knockout in
CreER-expressing mice (cKO). Cre-negative littermates (gWT, cWT) served as controls.
Representative (A, B) and summarized (C, D) Western blot analysis of full-length/N-R6
Reelin (C), and N-R2 Reelin (D) are shown. For statistical comparison across litters, the
Reelin protein level in each brain was normalized to GAPDH, then normalized to the wild
type average for that litter. Two-tailed t-tests were performed between WT/KO pairs with
Welch’s correction (*p=0.0347, df=15.05; ****p<0.0001, df=14.22).
Figure 5. Relationship between Reelin dysregulation and efficiency of APP knockdown on displacement of Reelin+ cells following acute disruption of embryonic APP expression

(A) The relationship between the efficiency of TAM-induced APP knockdown and the percentage of brain sections with Reelin+ cells displaced below the cortical plate boundary was plotted for E18 APP cKO mice. A Pearson’s correlation was performed ($r=0.3877$, $N=12$, $p=0.21$). (B) The relationship between the efficiency of TAM-induced APP knockdown and full-length Reelin expression was plotted for E18 APP cKO mice. A Pearson’s correlation was performed ($r=0.3627$, $N=12$, $p=0.25$). (C) The relationship between full-length Reelin expression and the percentage of brain sections with Reelin+
cells displaced below the cortical plate boundary was plotted for E18 APP cKO mice. A Pearson’s correlation was performed (r=0.2317, N=12, p=0.47).