

Soluble amyloid precursor protein (APP) regulates *transthyretin* and *Klotho* gene expression without rescuing the essential function of APP

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Amyloidogenic processing of the amyloid precursor protein (APP) generates a large secreted ectodomain fragment (APPs β), β -amyloid (A β) peptides, and an APP intracellular domain (AICD). Whereas A β is viewed as critical for Alzheimer's disease pathogenesis, the role of other APP processing products remains enigmatic. Of interest, the AICD has been implicated in transcriptional regulation, and N-terminal cleavage of APPs β has been suggested to produce an active fragment that may mediate axonal pruning and neuronal cell death. We previously reported that mice deficient in APP and APP-like protein 2 (APLP2) exhibit early postnatal lethality and neuromuscular synapse defects, whereas mice with neuronal conditional deletion of APP and APLP2 are viable. Using transcriptional profiling, we now identify *transthyretin* (*TTR*) and *Klotho* as APP/APLP2-dependent genes whose expression is decreased in loss-of-function states but increased in gain-of-function states. Significantly, by creating an APP knockin allele that expresses only APPs β protein, we demonstrate that APPs β is not normally cleaved *in vivo* and is fully capable of mediating the APP-dependent regulation of *TTR* and *Klotho* gene expression. Despite being an active regulator of gene expression, APPs β did not rescue the lethality and neuromuscular synapse defects of APP and APLP2 double-KO animals. Our studies identify *TTR* and *Klotho* as physiological targets of APP that are regulated by soluble APPs β independent of developmental APP functions. This unexpected APP-mediated signaling pathway may play an important role in maintaining *TTR* and *Klotho* levels and their respective functions in A β sequestration and aging.

Alzheimer's disease | β -secretase | neuromuscular synapse | knockin | conditional knockout

Amyloid precursor protein (APP) is a ubiquitously expressed type I membrane protein existing either as a full-length protein or as various proteolytically cleaved fragments. In particular, extracellular processing by α - or β -secretase, followed by presenilin-dependent γ -secretase cleavage, generates large soluble derivatives termed α -secretase cleaved soluble APP (APPs α) and β -secretase cleaved soluble APP (APPs β), respectively; p3 and β -amyloid (A β) peptides, and the APP intracellular domain (AICD) (reviewed in ref. 1). Whereas APPs α has been shown to exert neurotrophic and synaptogenic activities, APPs β was reported to be much less active in these *in vitro* assays, or may even have inhibitory effects (reviewed in ref. 2). Of interest, a recent publication showed that soluble APPs β (but not APPs α) undergoes further cleavage to produce an N-terminal APP derivative (N-APP), which binds to DR6 receptor and mediates axon pruning and degeneration under trophic withdrawal conditions (3). The AICD has been proposed to function as a transcriptional regulator but not as a transcription factor, by activating Fe65 and the chromatin-remodeling factor Tip60 (4, 5). Follow-up investigations have identified multiple putative downstream

targets (6–11). However, the validity of these proposed targets has been questioned or disputed (12–17).

APP is a member of a conserved protein family that includes APL-1 in *Caenorhabditis elegans*, and APP, amyloid precursor-like protein 1 (APLP1), and APLP2 in mammals. Whereas APP single-KO mice are viable with only subtle cognitive and motor impairments (18, 19), mice doubly deficient in APP and APLP2 [double-KO (dKO) mice] die soon after birth and exhibit profound neuromuscular junction (NMJ) defects (20, 21). Interestingly, although the intracellular sequences are most highly conserved among the APP family of proteins (reviewed in ref. 22), phenotypes reported in APP-deficient mice have been shown to be reversed by expressing only APPs α (23). The dispensable role of the membrane and intracellular sequences is also corroborated by Hornsten et al. (24), who showed that the lethality of *C. elegans* mutant deleting *apl-1* can be rescued by neuronal expression of soluble APL-1 extracellular domain.

By creating two APP alleles in mice (1), a conditional KO allele with specific APP deletion in neurons and (2) a knockin (ki) allele that expresses only APPs β , we report here that *transthyretin* (*TTR*) and *Klotho* are physiological targets of APP whose expressions are mediated by APPs β . Despite the positive regulation in gene expression, APPs β cannot rescue the developmental phenotypes of the APP/APLP2-deficient mice.

Results

Generation of APPs β ki Mice and Biochemical Characterization of APPs β Protein. We created an APP ki allele in which a FLAG tag and a stop codon were inserted before the A β sequence, resulting in the production of an APPs β /FLAG fusion protein and deletion of A β and intracellular sequences (Fig. 1A and Fig. S1). Similar to the APPs α ki mice (23), homozygous APPs β ki (ki/ki) mice are phenotypically normal with respect to overall growth and general brain morphology (Fig. S2). Quantitative real-time PCR (qRT-PCR) analysis showed that compared with their littermate WT controls, there was an ~50% reduction of APP transcript in the ki/ki brain (Fig. 1B), which might result from insufficient poly(A) addition by the human growth hormone poly(A) sequence introduced in the ki construct. This reduction correlated with a similar reduction of total APPs β protein levels (Fig. 1C, 22C11

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The authors declare no conflict of interest.

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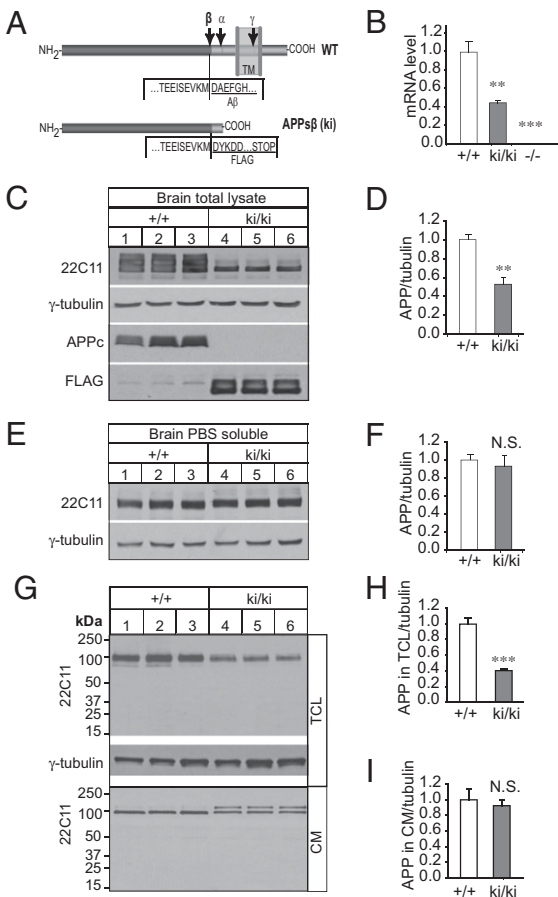


Fig. 1. Generation and biochemical characterization of APPs β ki mice. (A) Schematic representation of WT and APPs β ki alleles. Cleavage sites for α -, β -, and γ -secretase are indicated by α , β , and γ , respectively. In the ki allele, a FLAG sequence followed by a stop codon was inserted immediately downstream of the β -secretase site. TM, transmembrane region. (B) qRT-PCR of APP mRNA in 2-mo-old homozygous APPs β ki/ki and APP KO (-/-) mouse brains relative to WT controls (+/+), showing an ~50% reduction of APP mRNA in ki/ki samples and a negligible amount in APP^{-/-} samples as compared with the WT controls. (C) Western blot analysis of APP expression in total brain lysate from 2-mo-old ki/ki mice and their WT (+/+) littermates using the 22C11, APPc, and anti-FLAG antibodies. A γ -tubulin blot was used as a loading control. (D) Quantification of the relative ratio of 22C11/ γ -tubulin blots. (E) Western blot analysis of PBS-extractable soluble APP in +/+ and ki/ki brains using the 22C11 antibody. A γ -tubulin blot was used as a protein loading control. (F) Quantification of the Western blots in E. (G) Representative Western blots of APP in TCL and CM from cultured WT (+/+) and homozygous APPs β (ki/ki) primary neurons using the 22C11 antibody. A γ -tubulin blot was used as a loading control. (H) Quantification of the ratio of APP/tubulin in TCL of +/+ and ki/ki cultures. (I) Quantification of the ratio of APP/tubulin in CM of +/+ and ki/ki cultures. Note the absence of any cleavage products in the TCL or CM. The ratio of APP/tubulin in +/+ samples was normalized to 1 in all quantifications. ** $P < 0.01$; *** $P < 0.001$. N.S., nonsignificant ($P > 0.05$, t test).

panel, and Fig. 1D, quantified). Blotting with an anti-FLAG antibody confirmed the expression of the FLAG-tagged fusion protein at ~100 kDa only from the ki mice but not from WT littermates (Fig. 1C, FLAG). An anti-APP C-terminal (APPc) antibody showed positive detection only in WT but not ki/ki samples (Fig. 1C, APPc), validating the lack of APP C-terminal sequences in the APPs β ki mice. Even though the total APP expressed in ki/ki mice was reduced, quantitative analysis of PBS-extractable soluble APP revealed no significant differences between the ki/ki mice and their WT littermates (Fig. 1E and F), suggesting that the APPs β produced in the ki mice was more efficiently secreted.

Quantitative immunoblotting of spinal cord total protein lysates and soluble fractions yielded similar results (Fig. S3).

To investigate the processing and secretion of APPs β , we prepared neuronal cultures from postnatal day 0 (P0) ki/ki pups and littermate WT (+/+) controls. Total cell lysates (TCLs) and conditioned medium (CM) were collected at 14 d in vitro (DIV), and APP protein levels were assayed by immunoblotting (Fig. 1G). Consistent with the observations made with brain tissue, cell-associated APP was significantly reduced in the ki/ki neurons (Fig. 1H) but secreted APP was comparable between the two genotypes (Fig. 1I). This result agrees with the finding that deletion of the carboxyl sequences of APP promotes its secretion (25). Of note, we consistently detected two bands in the CM of ki/ki samples (Fig. 1G). Although the exact nature of the two bands is not clear, they appear to be a unique property of the cultured neurons because the extra band was not detected in brain samples or cultured cell lysates.

Nikolaev et al. (3) reported that trophic factor deprivation triggers the release of APPs β , which undergoes further cleavage by an unknown protease to generate an ~35-kDa N-APP. The production of only APPs β protein in our ki animals provided a unique system to investigate the stability and possible cleavage of APPs β in the absence of APPs α . Western blotting of WT and ki/ki samples using the 22C11 N-APP antibody revealed that APPs β existed as a nonproteolyzed protein in both the cell lysates and CM (Fig. 1G). The intact nature of the APPs β protein was further evidenced by analyzing CM taken from WT, APPs β heterozygous (ki/+), or APPs β homozygous (ki/ki) neurons using the C-terminal anti-FLAG antibody at various cultured stages (Fig. S4A) and when expressed at exceedingly high concentrations (Fig. S4B). Finally, analysis of APPs β development in the ki brains during embryonic day (E) 14.5 and E16.5 and P0 and P14 also failed to detect any cleavage products using both the N-terminal 22C11 (Fig. S4C) and C-terminal anti-FLAG antibodies (Fig. S4D). These results strongly argue that the secreted APPs β protein is stable in vivo and does not undergo further proteolytic cleavage under regular cell culture conditions in vitro.

Expression of APPs β on an *APLP2* Null Background Leads to Early Postnatal Lethality and Severe Neuromuscular Synapse Defects.

Hornsten et al. (24) reported that neuronal expression of a soluble APL-1 extracellular protein is sufficient to rescue the lethality of *C. elegans* mutant deleting *apl-1*. To assess whether APPs β is able to rescue the lethal phenotype of the *APP/APLP2* dKO mice, we intercrossed double-heterozygous mice harboring one allele each of the APPs β and *APLP2* null mutations (*APP^{ki/ki}-APLP2^{+/-}*). We then determined the genotypes of the offspring at P1 and P21 and compared the observed and expected numbers (Fig. 2). Genotyping of P1 pups revealed a close to Mendelian distribution of all genotypes, indicating no embryonic lethality as expected ($\chi^2 = 10.65$, $P > 0.1$). However, genotyping of 218 offspring from the same cross at weaning age (P21) identified very few surviving *APP^{ki/ki}APLP2^{-/-}*, *APP^{ki/-}APLP2^{-/-}*, or *APP^{-/-}APLP2^{-/-}* mice, the number of which significantly deviated from the predicted Mendelian ratio ($\chi^2 = 79.6$, $P < 0.001$). These results demonstrate that contrary to the *C. elegans* finding, expression and secretion of APPs β cannot rescue the postnatal lethality of the *APP/APLP2* double-deficient mice.

We previously reported that APP is targeted to the synaptic sites of the NMJ (26). We performed localization analysis of APPs β using the anti-FLAG antibody in heterozygous APPs β ki muscle preparations. Interestingly, the staining pattern was indistinguishable from that of WT full-length APP recognized by the C-terminal APP antibody Y188 (Fig. 3A), suggesting that the APPs β can be targeted to the synaptic terminals without the C-terminal sequences, likely through a mechanism independent of sorting signals (27). Despite the apparent synaptic expression of APPs β , crossing the APPs β ki with *APLP2* KO mice and

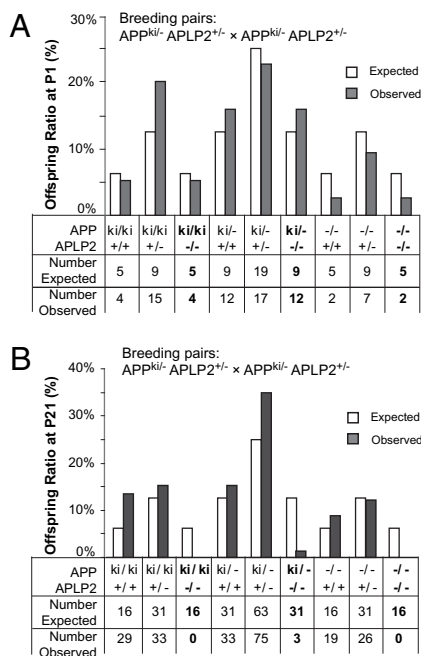


Fig. 2. Survival analysis of APPs β ki mice on *APLP2* null background. (A) Analysis of genotypes of 75 offspring collected at P1 derived from crosses of $APP^{ki/-} APLP2^{+/-}$ male and female mice. All genotypes were recovered at close to a Mendelian ratio (8 df, $\chi^2 = 10.65$, $P > 0.1$). (B) Analysis of genotypes of 218 offspring collected at P21 derived from the same breeding as in A. The number of $APP^{ki/ki} APLP2^{-/-}$, $APP^{ki/-} APLP2^{-/-}$, or $APP^{-/-} APLP2^{-/-}$ animals observed was much lower than expected (highlighted in bold) (8 df, $\chi^2 = 79.6$, $P < 0.001$).

staining of neuromuscular synapses at P0 showed that compared with $APP^{+/+} APLP2^{-/-}$ littermate controls, $APP^{ki/ki} APLP2^{-/-}$ mutants exhibited expanded neuromuscular synapses (Fig. 3 B and D, quantified in the latter), with significant localization of synaptic vesicle proteins in extrasynaptic compartments and a correspondingly reduced apposition of presynaptic markers with postsynaptic receptors (Fig. 3 C and E, quantified in the latter). The degree of the defects was similar to that of the dKO animals characterized previously (21, 26). These results demonstrate that APPs β is inactive in APP-mediated survival and neuromuscular synapse function.

Transcriptional Profiling of Neuronal *APP/APLP2* Double-Conditional KO Mice. APP has been implicated in transcriptional regulation through its intracellular domain. However, our microarray analysis using adult *APP* null hippocampal samples or P0 *APP/APLP2* dKO brains failed to detect appreciable gene expression changes. We reasoned that this might be attributable to the compensatory mechanisms of *APLP2* in the case of *APP* single-KO brains and the mixed cell types collected at P0 in *APP/APLP2* dKO brains, which may dilute any tissue-specific differences. We created an *APP* floxed allele and observed that in contrast to the *APP/APLP2* dKO mice, neuronal-specific *APP/APLP2* double-conditional KO (N-dCKO) mice are viable. This allowed us to bypass the lethality or redundancy issues (26). Indeed, transcriptional profiling of hippocampi dissected from 2-mo-old N-dCKO mice and littermate *APLP2* null controls uncovered ~30 genes that showed significant differences between N-dCKO mutants and the controls, with the vast majority down-regulated in N-dCKO samples (Table S1). We compared our list with published microarray data using *APP*-overexpressing animals before amyloid deposition (28, 29) and chose to focus on *TTR* and *Klotho* because their expression was down-regulated in N-dCKO samples and up-regulated in

APP transgenic brains. The changes in *TTR* and *Klotho* expression as a function of *APP* gene dosage were confirmed by qRT-PCR analysis (Fig. 4A and B). Reduced *TTR* and *Klotho* transcripts in the hippocampus were also confirmed by in situ hybridization (Fig. 4C). In line with the negative microarray results obtained with newborn samples, qRT-PCR of *TTR* and *Klotho* in P0 brains showed no significant differences between the *APLP2* null control and the dKO mutant (Fig. S5). Consistent with the compensatory role of *APLP2*, expression of *TTR* and *Klotho* was comparable between the *APP* single-KO and WT hippocampal samples (Fig. S5).

APP has been proposed to regulate gene transcription by first recruiting Fe65 to the membrane before γ -secretase cleavage, activating Fe65 by an unknown mechanism at the membrane and then releasing Fe65, together with the AICD, by γ -secretase cleavage, thereby allowing Fe65 to enter the nucleus and to interact with the chromatin remodeling factor Tip60 (4, 5). As such, the most straightforward explanation for the altered *TTR* and *Klotho* expression in the *APP/APLP2* double-mutant mice would be that *TTR* and *Klotho* are direct targets of the Fe65-mediated signaling pathway. We tested this possibility by cotransfecting an APPC99 expression vector with or without Fe65 and a luciferase reporter construct driven by the 3-kb *TTR* promoter (30) but failed to detect significant induction of luciferase activities (Fig. S6). These results indicate that other APP regions, such as the APP extracellular motifs, might be involved in modulating *TTR* and *Klotho* expression, a hypothesis that could presumably be tested using the APPs β ki mice. However, the early postnatal lethality of the $APP^{ki/-} APLP2^{-/-}$ mice limited our analysis to the newborn stage, and analysis of *APP/APLP2* dKO brains at P0 failed to establish a reduction of *TTR* or *Klotho* (Fig. S5). We reasoned that this could be attributed to low levels of *TTR* and *Klotho* expression and/or the compensatory activity of *APLP1* in the P0 brain. We chose to examine the expression of these targets in the P0 liver because (i) *TTR* is known to be highly expressed in the liver and (ii) *APLP1* is expected to be a neuronal protein not expressed in peripheral tissues. Indeed, the absence of *APLP1* in the newborn and adult liver tissues was confirmed by qRT-PCR (Fig. 4D). Therefore, at P0, there is no expression of any APP-related proteins in the dKO mouse liver, and the only APP family of proteins present in the $APP^{ki/-} APLP2^{-/-}$ sample is soluble APPs β . We were able to identify a significant decrease of both *TTR* (Fig. 4E) and *Klotho* (Fig. 4F) mRNA levels in the P0 dKO liver. Importantly, this reduction was not seen in $APP^{ki/-} APLP2^{-/-}$ samples (Fig. 4E and F, ki/-), demonstrating that expression of APPs β is sufficient to support the *TTR* and *Klotho* expression. As such, the regulation of *TTR* and *Klotho* is mediated by APPs β and likely through a receptor distinct from the APP family proteins.

Discussion

We report here that (i) APPs β is highly stable in the central nervous system in vivo and under regular culture conditions in vitro, (ii) expression of APPs β is not sufficient to rescue the early postnatal lethality and neuromuscular synapse defects of the *APP/APLP2* null mice, and (iii) *TTR* and *Klotho* are previously undescribed targets of APP whose transcripts are maintained by APPs β . Our findings support the notion that secreted APPs β can elicit an intercellular signal independent of full-length APP but that full-length APP is required for neuromuscular synapse assembly and postnatal survival.

Loss-of-function studies established an essential and redundant role of the APP family of proteins in postnatal survival (20, 31). This essential activity is shared in lower organisms, because *C. elegans* deficient in *apl-1* also dies at the L1 stage (24). Nevertheless, the *apl-1* null lethal phenotype can be rescued by neuronal expression of the APL-1 extracellular domain, whereas expression of APPs β is unable to rescue the lethality of *APP/APLP2* null mice. Although it remains possible that the lethality of *APP/APLP2* null mice could be rescued by APPs α , we believe this is unlikely

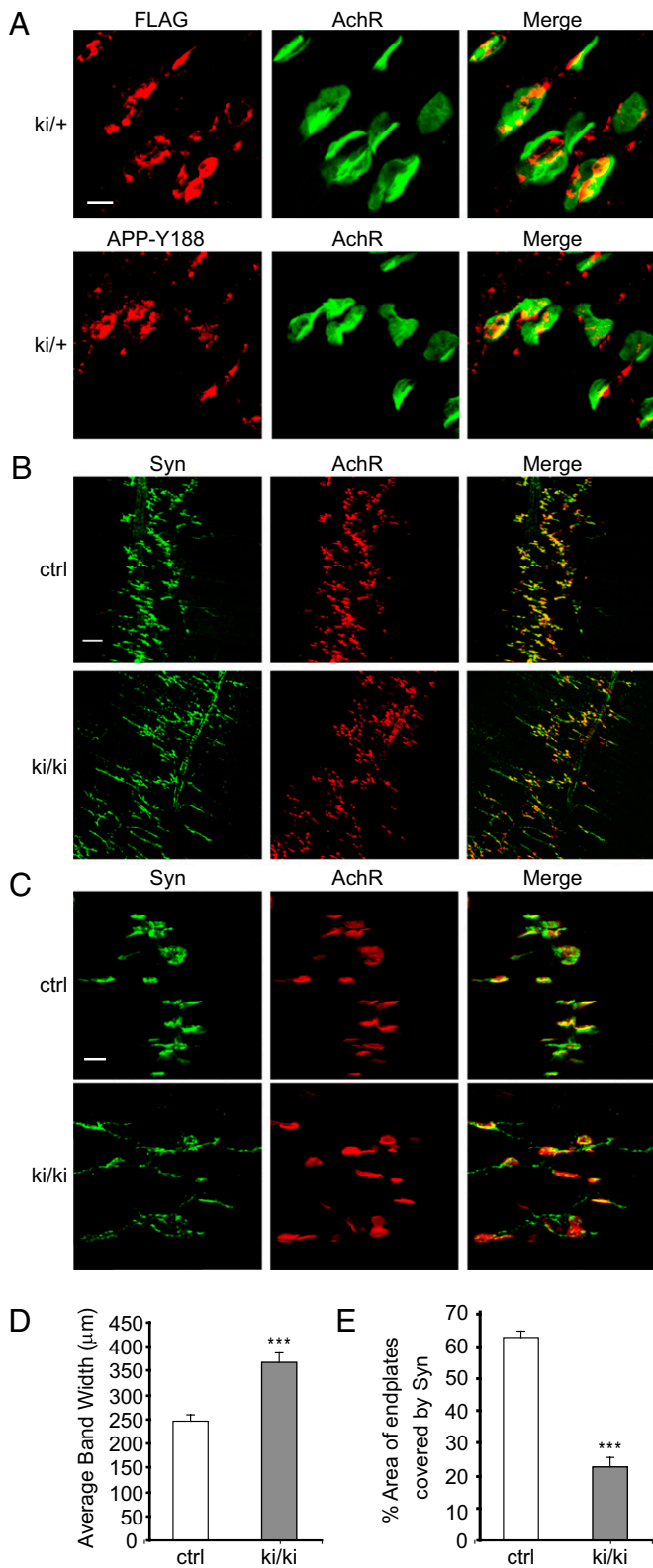


Fig. 3. Analysis of neuromuscular synapse phenotypes of APPsβ ki mice. (A) Immunofluorescence staining of P0 sternomastoid muscle sections of heterozygous APPsβ ki animals (ki/+) using an anti-FLAG antibody and the anti-APP antibody Y188. Staining with α-bungarotoxin was used to mark the postsynaptic AchRs. (Merge) Overlay of APP and AchR images. (B) Whole-mount staining of P0 diaphragm muscles of APP^{ki/ki} APLP2^{-/-} mutants (ki/ki) and littermate APP^{+/+} APLP2^{-/-} controls (ctrl) with an anti-synaptophysin (Syn) antibody and α-bungarotoxin (AchR) showing diffused pre- and post-

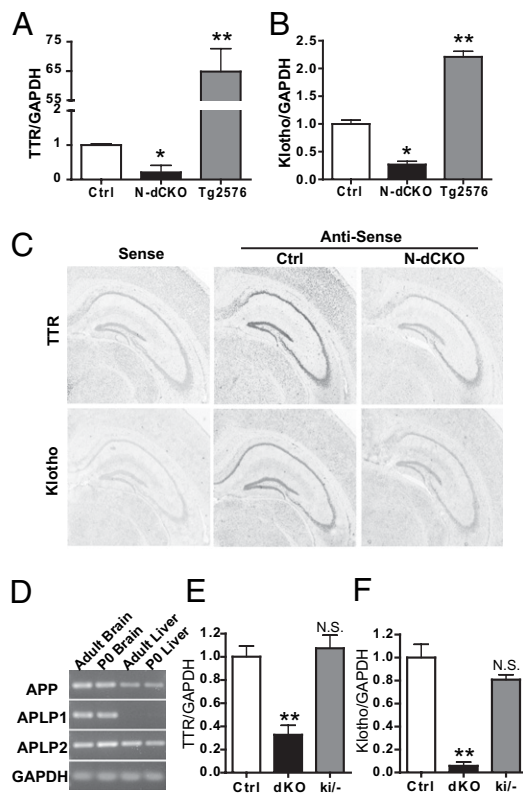


Fig. 4. Analysis of *TTR* and *Klotho* expression. Relative qRT-PCR analysis of *TTR* (A) and *Klotho* (B) mRNA levels from the hippocampi of 2-mo-old control (Ctrl), N-dCKO, and Tg2576 APP transgenic mice ($n = 3$). (C) Representative in situ hybridization images of *TTR* and *Klotho* from littermate APLP2^{-/-} control (Ctrl) and N-dCKO brain sections (sense, hybridization with a sense probe as a negative control; antisense, hybridization using corresponding antisense probes). (D) RT-PCR analysis of *APP*, *APLP1*, and *APLP2* expression in adult and P0 brain and liver. GAPDH was used as an amplification control. Relative qRT-PCR analysis of *TTR* (E) and *Klotho* (F) mRNA levels in P0 livers from control APLP2 null (Ctrl), APP, and APLP2 dKO and APPsβ ki mice on APLP2 null background (ki/-). Data are the mean ± SEM of two independent experiments, each with three samples per genotype. * $P < 0.05$; ** $P < 0.01$. (N.S., nonsignificant, Student's t test).

because we have created another strain of APP ki allele expressing a membrane-anchored APP lacking the last 39 amino acids. Although both APPsα and APPsβ are readily detectable in these mice, these animals display a similar lethality when expressed on an APLP2 KO background (32). The reason for the distinct sequence requirement for *C. elegans* and mouse survival is not known, but it is worth pointing out that the lethality of the *apl-1*-deficient worm is attributable to a molting defect that is not relevant for mammals.

Our finding that APPsβ fails to rescue the NMJ defects of the APP/APLP2 null mice is consistent with our conditional KO studies indicating that full-length APP is necessary for its synaptogenic activity (26). The apparently normal synaptic localization of APPsβ may not be in agreement with various studies showing that kinesin-dependent axonal trafficking of APP requires its intracellular sequences (33), but it is consistent with a recent

synaptic distribution in the ki/ki mutant. (Merge) Overlay of Syn and AchR images. (C) Higher magnification images of synapse structures showing axonal staining of Syn and poorly covered end plates by Syn in the ki/ki mutant. (D) Quantification of the average bandwidth of AchR-positive end plates. (E) Quantification of the percentage of AchR-positive end plates covered by Syn (average ± SEM of 20 end plates per genotype). *** $P < 0.001$ (Student's t test). (Scale bar: A and C, 20 μM; B, 100 μM.)

report that fast anterograde transport of APP does not require the AICD or any known sorting signal (27). It is important to note that the immunostaining method used here does not offer sufficient spatial resolution to define the localization of the APPs β protein precisely and does not address whether the APPs β detected is axonally transported. As such, defective axonal transport of APPs β cannot be formally excluded.

A recent publication by Nikolaev et al. (3) reported that APPs β , via a cleaved N-terminal derivative, binds to the DR6 receptor and mediates axonal pruning and neuronal cell death under conditions of trophic withdrawal. Our results that APPs β is highly stable and that APPs β fails to rescue the nerve-sprouting phenotype of the *APP/APLP2* null NMJ are at odds with these findings. Although we cannot exclude the possibility that APPs β undergoes further cleavage under specific circumstances, such as those used by Nikolaev et al. (3), our data suggest that APPs β exists as a stable protein rather than cleaved fragments and that the neuromuscular synapse defects present in *APP/APLP2* null mice are not caused by the lack of APPs β or, by extension, a defective APPs β /DR6 pathway.

The down-regulation of *TTR* and *Klotho* in *APP/APLP2* loss-of-function mutants and their up-regulation in *APP*-overexpressing mice provide strong support for the notion that they are the direct targets of APP. Because expression of APPs β is sufficient to restore the expression of *TTR* and *Klotho* in the liver of *APP^{ki/-}APLP2^{-/-}* mice in which no APP or any of its family members is present, regulation of *TTR* and *Klotho* expression must be mediated by APPs β independent of full-length APP. Although not conflicting with the putative transcriptional activity of APP (4, 5), our results provide an alternative mechanism whereby the secreted APP, via binding to an unknown receptor(s), activates downstream target genes, including *TTR* and *Klotho*. It should be noted that the link of Fe65 to chromatin remodeling instead of transcription suggests that the AICD probably does not act on specific genes but modulates the overall transcriptional state of a cell (17).

The functional role of APP-mediated *TTR* and *Klotho* expression remains to be established. Because expression of APPs β cannot complement the developmental activities of APP, *TTR* and *Klotho* do not appear to mediate essential functions of APP. With regard to the candidate receptors, secreted APP has been shown (among other receptors) to bind to low density lipoprotein receptor related protein (34) and class A scavenger receptor (35). Caille et al. (36) indicated the presence of binding sites for APPs in EGF-responsive neural stem cells of the adult rodent brain. As such, APPs could presumably stimulate downstream signaling through EGF receptor- or LRP-mediated pathways. Alternatively, APP or APPs has been shown to interact with integrin β 1 (37), netrin-1 (38), and reelin (39). These protein-protein interactions could, in principle, trigger intracellular events leading to *TTR* and *Klotho* expression. The ability to restore liver *TTR* and *Klotho* by APPs β makes it a legitimate assumption that the receptor is expressed in the liver. Although DR6 cannot be formally excluded as a potential APPs β receptor, the absence of N-APP in our system argues against a functional role of this pathway. Nevertheless, identification of the receptor (s) and intracellular pathways leading to *TTR* and *Klotho* regulation awaits further investigation.

Because A β is an integral component of the highly regulated APP proteolytic cleavage cascade, misregulation of APP processing and the APP-dependent signaling pathway as well as A β accumulation may contribute to neuronal dysfunction and Alzheimer's disease (AD) pathogenesis. In light of the well-documented role of *TTR* in A β sequestration and amyloid suppression (40, 41) and of *Klotho* in various aging processes, including oxidative stress and calcium homeostasis (42–44), the identification of these proteins as the APP targets has direct implications for AD pathogenesis. In mice, *TTR* haploinsufficiency leads to enhanced amyloid pathology in an APP/presenilin mouse

model (41), and *TTR* protects A β -induced toxicity and behavioral impairment (45). *TTR* levels are decreased in the cerebrospinal fluid of patients with AD, suggesting a lack of a protective mechanism in the diseased brain (46, 47). We propose a model whereby extracellular APP processing produces APP ectodomain derivatives, which promote *TTR* and *Klotho* expression and protect against A β neurotoxicity during aging. The disruption of this homeostasis, which can be brought about by reduced APPs and/or elevated A β , both associated in individuals with AD (48), may contribute to AD pathogenesis during aging.

Methods

Antibodies and Reagents. 22C11 and Y188 were monoclonal antibodies recognizing the amino (amino acids 66–81) and carboxyl (containing YENPTY) sequences of APP and were obtained from Millipore and Epitomics, respectively. The polyclonal APPc was described previously (49). Anti-FLAG (rabbit polyclonal) and anti-V5 (mouse monoclonal) antibodies were purchased from Sigma and Abcam, respectively, and the antisynaptophysin polyclonal antibody was from DAKO. The α -bungarotoxin was from Molecular Probes.

Animals. Neuronal *APP* and *APLP2* N-dCKO mice were derived by crossing *APP* floxed mice with transgenic mice expressing the Cre-recombinase under the neuronal rat nestin promoter, followed by breeding with *APLP2* null animals (26, 50). The detailed method for constructing the APPs β ki mice can be found in *S1 Text* and *Fig. S1*. All the strains used have been backcrossed onto C57BL/6J background for at least six generations.

qRT-PCR. Total RNA was isolated from brain or liver. Reverse transcription was performed using the SuperScript III first-strand synthesis system for the RT-PCR kit (Invitrogen), and the reaction mix was subjected to qRT-PCR using the ABI PRISM Sequence Detection System 7000 (Applied Biosystems). Primers were designed with Primer Express Version 2.0 software (Applied Biosystems) using sequence data from the National Center for Biotechnology Information. GAPDH primers were used as an internal control for each specific gene amplification. The relative levels of expression were quantified and analyzed using ABI PRISM Sequence Detection System 7000 software. The real-time value for each sample was averaged and compared using the comparative cycle threshold (CT) method. The relative amount of target RNA was calculated relative to the expression of endogenous reference and relative to a calibrator, which was the mean CT of control samples.

In Situ Hybridization. Coronal serial sections (20 μ m in thickness) of heads of 2-mo-old *APLP2^{-/-}* control and N-dCKO mice were cut with a cryostat and placed with adjacent sections on separate slides. After paraformaldehyde fixation and acetylation, the slides were assembled into flow-through hybridization chambers and placed in a Tecan Genesis 200 liquid-handling robot (Mannedorf). Templates for synthesis of digoxigenin-labeled riboprobes for *TTR* and *Klotho* were full-length *TTR* cDNA and the N-terminal 810 bp of *Klotho* cDNA, respectively, in pCR-Blunt II-TOPO vectors (Invitrogen). Antisense and sense probes were transcribed with T7 and Sp6 polymerase, respectively, from linearized vector. Hybridized probes were detected by catalyzed reporter deposition using biotinylated tyramide; this was followed by colorimetric detection of biotin with avidin coupled to alkaline phosphatase. Hybridization with sense control probes did not yield signals above background.

Cell Culture, Western Blotting, and Immunostaining. The primary hippocampal culture was prepared as described (26). CM and TCL (in PBS with complete protease inhibitor mixture) were collected at 14 DIV. For the time-course experiment, 10% of the total volume of neuronal culture CM was taken out at times indicated for Western blotting analysis and replenished with an equal volume of fresh serum-free medium. Western blot analysis and immunofluorescence staining were performed and analyzed as previously described (26).

Statistical Analysis. Genotyping analysis of the offspring from *APP^{ki/-}APLP2^{+/-}* male and female intercrosses was performed using χ^2 analysis. The Student's *t* test was used for all other analyses (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). Data were presented as the average \pm SEM.

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