SOCIAL SCIENCES

The authors note that on page 12267, left column, second full paragraph, lines 5–12, “The average gestation length decreases significantly by 0.8 wk ($P < 0.001$) during the first 5 mo of the year and returns to the January level in June, where it remains for the rest of the year. This finding indicates that the seasonal pattern in gestation length is not driven by mothers of different socioeconomic background selecting differently into conception months. The May decrease in gestation length by 0.8 wk leads to an increase in premature births of about 1 percentage point (Fig. S1A).” should instead appear as “The average gestation length decreases significantly by 0.08 wk ($P < 0.001$) during the first 5 mo of the year and returns to the January level in June, where it remains for the rest of the year. This finding indicates that the seasonal pattern in gestation length is not driven by mothers of different socioeconomic background selecting differently into conception months. The May decrease in gestation length by 0.08 wk leads to an increase in premature births of about 1 percentage point (Fig. S1A).” This change does not affect the conclusions of the article.

www.pnas.org/cgi/doi/10.1073/pnas.1313401110

NEUROSCIENCE

The authors note that the following statement should be added to the Acknowledgments: “This material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting the views of the Department of the Army or the Department of Defense.”

www.pnas.org/cgi/doi/10.1073/pnas.1313191110

PLANT BIOLOGY

The authors note that the author name Antione H. P. America should instead appear as Antoine H. P. America. The corrected author line appears below. The online version has been corrected.


www.pnas.org/cgi/doi/10.1073/pnas.1312631110

www.pnas.org
Soluble amyloid precursor protein (APP) regulates transthyretin and Klotho gene expression without rescuing the essential function of APP

Hongmei Li1,a,b,1, Baiping Wang1,a, Zilai Wang1,b,c, Qinxi Guo1,a,d, Katsuhiko Tabuchi1,b,2, Robert E. Hammer3, Thomas C. Südhof4,a,b,1,9, and Hui Zheng1,a,c,d,3

1Huffington Center on Aging, 2Department of Molecular and Human Genetics, and 3Translational Biology and Molecular Medicine Program, Baylor College of Medicine, Houston, TX 77030; Departments of 4Neuroscience and 5Biochemistry and 6Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75390; and 7Department of Cellular and Molecular Physiology, Howard Hughes Medical Institute, Stanford University, Palo Alto, CA 94304

Contributed by Thomas C. Südhof, August 27, 2010 (sent for review May 24, 2010)

Amyloidogenic processing of the amyloid precursor protein (APP) generates a large secreted ectodomain fragment (APPsβ), β-amyloid (Aβ) peptides, and an APP intracellular domain (AICD). Whereas Aβ 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 APPsβ 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 APPsβ protein, we demonstrate that APPsβ 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, APPsβ 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 APPsβ 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 Aβ 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 (APPαs) and β-secretase cleaved soluble APP (APPsβ), respectively; p3 and β-amyloid (Aβ) cleaved peptides, and the APP intracellular domain (AICD) (reviewed in ref. 1). Whereas APPαs has been shown to exert neurotrophic and synaptogenic activities, APPsβ 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 APPsβ (but not APPαs) 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 APPαs (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 APPsβ, we report here that transthyretin (TTR) and Klotho are physiological targets of APP whose expressions are mediated by APPsβ. Despite the positive regulation in gene expression, APPsβ cannot rescue the developmental phenotypes of the APP/APLP2-deficient mice.

Results

Generation of APPsβ ki Mice and Biochemical Characterization of APPsβ Protein. We created an APP ki allele in which a FLAG tag and a stop codon were inserted before the Aβ sequence, resulting in the production of an APPsβ/FLAG fusion protein and depletion of Aβ and intracellular sequences (Fig. 1A and Fig. S1). Similar to the APPαs ki mice (23), homozygous APPsβ 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 APPsβ protein levels (Fig. 1C, 22C11


The authors declare no conflict of interest.

1H.L. and B.W. contributed equally to this work.

2Present address: Department of Cerebral Research, National Institute for Physiological Sciences, 5-1 Higashiyama, Myodaiji, Okazaki 444-8787, Japan.

3To whom correspondence should be addressed. E-mail: huiz@bcm.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1012568107/-/DCSupplemental.
To investigate the processing and secretion of APPsβ, 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 assessed 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. 1F). 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 APPsβ, which undergoes further cleavage by an unknown protease to generate an ∼35-kDa N-APP. The production of only APPsβ protein in our ki animals provided a unique system to investigate the stability and possible cleavage of APPsβ in the absence of APPsα. Western blotting of WT and ki/ki samples using the 22C11 N-APP antibody revealed that APPsβ existed as a nonproteolyzed protein in both the cell lysates and CM (Fig. 1F). The functional nature of the APPsβ protein was further evidenced by analyzing CM taken from WT, APPsβ heterozygous (ki/+), or APPsβ 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 APPsβ 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 APPsβ protein is stable in vivo and does not undergo further proteolytic cleavage under regular cell culture conditions in vitro.

Expression of APPsβ 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 APLI-1 extracellular protein is sufficient to rescue the lethality of C. elegans mutant deleting apl-1. To assess whether APPsβ is able to rescue the lethal phenotype of the APP/APLP2 double-kO mice, we intercrossed double-heterozygous mice harboring one allele each of the APPsβ and APLP2 null mutations (APPK−/− 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 (χ2 = 10.65, P > 0.1). However, genotyping of 218 offspring from the same cross at weaning age (P21) identified very few surviving APPKK−/− APLP2−/−, APPKK−/− APLP2−/−, or APPKK−/− APLP2−/− mice, the number of which significantly deviated from the predicted Mendelian ratio (χ2 = 79.6, P < 0.001). These results demonstrate that contrary to the C. elegans finding, expression and secretion of APPsβ cannot rescue the postnatal lethality of the APLP2−/− double-deficient mice.

We previously reported that APP is targeted to the synaptic sites of the NMJ (26). We performed localization analysis of APPsβ using the anti-FLAG antibody in heterozygous APPsβ 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 APPsβ 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 APPsβ, the synaptic localization of APPsβ is not detectable in the double-deficient mice (ki/ki APP−/− APLP2−/−). This result demonstrates that APPsβ is not sufficient to rescue the APP/APLP2 double-deficient mice.

Fig. 1. Generation and biochemical characterization of APPsβ ki mice. (A) Schematic representation of WT and APPsβ 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 APPsβ ki/ki and APP KO (−/−) mice brains relative to WT controls (+/+). β-Actin was used as an internal control. (C) Representative Western blots of APP in total brain lysate from 2-mo-old homozygous APPsβ ki/ki and APP KO (−/−) primary neurons using the C-terminal anti-FLAG antibody. A γ-tubulin blot was used as a loading control. (D) Determination of the relative ratio of APPsβ to γ-tubulin in cultured neurons because the extra band was not detected in cultured neurons. The ratio of APPsβ/γ-tubulin in ki/ki samples was normalized to 1 in all quantifications. **P < 0.01; ***P < 0.001. N.S., nonsignificant (P > 0.05, t test).

Quantitative immunoblotting of spinal cord total protein lysates and soluble fractions yielded similar results (Fig. S3).
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 expression 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 APPβki mice. However, the early postnatal lethality of the APPβki/−/− 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 APPs 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) APLP2 is expected to be a neuronal protein not expressed in peripheral tissues. Indeed, the absence of APLP2 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 APPβ−/−/− APLP2−/− sample is soluble APPβ. We were able to identify a significant decrease of both TTR (Fig. 4E) and Klotho (Fig. 4F) mRNA levels in the dKO liver. Importantly, this reduction was not seen in APPβ−/−/− APLP2−/−−/− samples (Fig. 4E and F, ki−/−), demonstrating that expression of APPβ is sufficient to support the TTR and Klotho expression. As such, the regulation of TTR and Klotho is mediated by APPβ and likely through a receptor distinct from the APP family proteins.

**Discussion**

We report here that (i) APPβ is highly stable in the central nervous system in vivo and under regular culture conditions in vitro, (ii) expression of APPβ 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 APPβ. Our findings support the notion that secreted APPβ 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 APPβ 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, we believe this is unlikely...
Fig. 3. Analysis of neuromuscular synapse phenotypes of APP± ki mice. (A) Immunofluorescence staining of P0 sternamostoid muscle sections of heterozygous APP± 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 APPki/kiAPLP2−/− mutants (ki/ki) and littermate APP±/APLP2−/− controls (ctrl) with an anti-synaptophysin (Syn) antibody and α-bungarotoxin (AchR) showing diffused pre- and post-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.)

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 APP± 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 APPα and APPβ 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 APPβ 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 APPβ 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
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 APPβ protein precisely and does not address whether the APPβ detected is axonally transported. As such, defective axonal transport of APPβ cannot be formally excluded.

A recent publication by Nikolaev et al. (3) reported that APPβ, 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 APPβ is highly stable and that APPβ 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 APPβ undergoes further cleavage under specific circumstances, such as those used by Nikolaev et al. (3), our data suggest that APPβ exists as a stable protein rather than cleaved fragments and that the neuromuscular synapese defects present in APP+/APLP2− null mice are not caused by the lack of APPβ or, by extension, a defective APPβ/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 APPβ is sufficient to restore the expression of TTR and Klotho in the liver of APPβ−/− APLP2−/− mice in which no APP or any of its family members is present, regulation of TTR and Klotho expression must be mediated by APPβ 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 APPβ 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 LRPs-mediated pathways. Alternatively, APP or APPs has been shown to interact with integrin β1 (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 APPβ makes it a legitimate assumption that the receptor is expressed in the liver. Although DR6 cannot be formally excluded as a potential APPβ 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 Aβ 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 Aβ accumulation may contribute to neuronal dysfunction and Alzheimer’s disease (AD) pathogenesis. In light of the well-documented role of TTR in Aβ 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 Aβ-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 Aβ neurotoxicity during aging. The disruption of this homeostasis, which can be brought about by reduced APPs and/or elevated Aβ, 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 ε-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 APP/−/− null animals (26, 50). The detailed method for constructing the APPβ/ki mice can be found in SI 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 μm 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 Tacon Genesis 200 liquid-handling robot (Manneford). 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−/−APLP2−/− male and female intercrosses was performed using χ2 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 ± SEM.

ACKNOWLEDGMENTS. We thank R. Atkinson of the confocal core, C. Thaller and A. Liang of the RNA in situ core, and the Baylor College of Medicine Eunice Kennedy Shriver Intellectual and Developmental Disabilities Research