Epigenetic control of aquaporin 1 expression by the amyloid precursor protein

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ABSTRACT **Cellular processing of the amyloid precursor protein (APP) has been extensively studied, but its precise function remains elusive. The intracellular domain of APP has been proposed to regulate expression of several genes by mechanisms that are largely unknown. We report that APP regulates expression of the aquaporin 1 (AQP1) gene in mouse embryonic fibroblasts and in transgenic mice. AQP1 mRNA and protein were down-regulated in fibroblasts lacking APP or presenilin 2 in which AQP1 expression was restored by stable expression of full-length APP or presenilin 2 but not by APP deleted from its carboxy-terminal domain. The transcriptional activity of the AQP1 gene promoter and the stability of AQP1 mRNA were identical in fibroblasts expressing or not expressing APP. Control of AQP1 expression by APP was sensitive to trichostatin A, an histone deacetylase inhibitor, and histone deacetylase activity coimmunoprecipitated with APP. Altogether, these data show that a presenilin-2-dependent γ-secretase activity releases the intracellular domain of APP involved in the epigenetic control of AQP1 expression. Since AQP1 is found in astrocytes surrounding senile plaques, this epigenetic control of AQP1 expression could have important implications in Alzheimer disease.—Huysseune, S., Kienlen-Campard, P., Hébert, S., Tasiaux, B., Leroy, K., Devuyst, O., Brion, J.-P., De Strooper, B., Octave, J.-N. Epigenetic control of aquaporin 1 expression by the amyloid precursor protein. *FASEB J.* 23, 4158–4167 (2009). www.fasebj.org**

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Amyloid precursor protein (APP) is a type I protein with a single transmembrane domain, which is expressed in all cell types, including neurons (1). Different secretase activities are involved in APP processing. The α- and β-secretases (2, 3) cleave the ectodomain of APP to generate membrane-anchored C-terminal fragments (α- or β-CTF), which are further processed within their transmembrane domain by the γ-secretase. The γ-secretase is a multiprotein complex containing ≥4 proteins: a presenilin (PS1 or PS2), nicastrin (Nct), Pen2, and Aph-1 (4, 5). At least 3 variants of Aph-1 are expressed in mammalian cells, which can associate to either PS1 or PS2 to generate 6 different γ-secretase complexes that produce similar cleavage of APP (6–8). Different isoforms of β-amyloid peptide (Aβ), the major constituent of senile plaques found in the brain of patients with Alzheimer disease (AD), are produced by the cleavage of β-CTFs at their γ-site. The cleavage of both α- and β-CTFs at the ε-site, a few residues downstream (9), releases the APP intracellular domain (AICD). The structural properties of APP transmembrane α-helices and their ability to self-associate are key regulators in the switch between γ- and ε-cleavage (10, 11).

The secretase activities involved in APP processing are also involved in Notch processing and release the Notch intracellular domain (NICD), which is a transcription factor (12, 13). AICD, contrary to NICD, is a 50-aa peptide that does not contain any of the typical domains of transcription or transactivation factors. However, the nuclear localization of AICD (14) suggested that it could be involved in the regulation of gene transcription, when associated with the adaptor protein Fe65 and with the histone acetyl transferase Tip60. This trimeric complex is able to promote the transcriptional activity of several gene promoters in luciferase reporter assays (15, 16). APP target genes have been recently identified, including tetraspanin KAI1/CD82, APP, glycogen synthase kinase-3β, and neprilysin (17–21). However, some of these genes might be regulated by Fe65 in an AICD-independent manner (22). An emerging hypothesis is that AICD, in

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association with Fe65, recruits factors like Tip60, SET, and Teashirt proteins that are involved in histone acetylation/deacetylation and modify gene expression by chromatin remodeling and epigenetic mechanisms (23).

In this study, we demonstrate that a PS2-mediated cleavage of APP releases AICD capable of interacting with histone deacetylase (HDAC) activities in order to stimulate the expression of AQP1, a water channel of the aquaporin family (24). This epigenetic control of AQP1 expression by APP occurs not only in cultured cells but also *in vivo*, since a significant decrease of AQP1 was observed in kidneys of APP-knockout mice. An up-regulation of AQP1 expression was demonstrated in AD brains, where AQP1 was located in astrocytes surrounding amyloid deposits of senile plaques.

Materials and Methods

Antibodies and reagents

The human specific anti-APP WO-2 monoclonal antibody was from the Genetics Company (Schlieren, Switzerland). The anti-C-terminus APP rabbit polyclonal antibody was kindly provided by N. Sergeant (INSERM U422, Lille, France). The anti-actin and anti-tubulin rabbit polyclonal antibodies were from Sigma (St. Louis, MO, USA), and the anti-AQP1 rabbit polyclonal antibody was from Chemicon (Temecula, CA, USA). The anti-PS1 CTF and anti-PS2 rabbit polyclonal antibodies were described elsewhere (22, 25). Secondary antibodies were from Amersham Bioscience (Uppsala, Sweden). Mouse monoclonal antibodies to Aβ (Dako, Glostrup, Denmark) and to glial fibrillary acidic protein (Sigma) were used for immunohistochemistry.

Cell culture reagents were from Invitrogen (Carlsbad, CA, USA). Trichostatin A, actinomycin D, and cycloheximide were from Sigma.

Cell culture and treatment

Mouse embryonic fibroblasts (MEFs) were cultured in DMEM/Ham's F-12 medium supplemented with 10% serum and antibiotics. MEFs stably expressing human APP695 in an $APP^{-/-}$ background were described previously (22). The recombinant lentiviruses expressing human APP695 or human APP695 truncated in its C-terminal region from residue 652 to residue 695 (APP Δ-C) were generated by subcloning the respective APP cDNA sequences in the AgeI and *Xba*I restriction sites of a lentiviral backbone vector (26). Virus production and cell infection were carried out as described previously (27). Cells were treated 1 d after plating during 24 h with 5, 7.5, or 10 ng/ml of trichostatin A with or without 10 μg/ml of cycloheximide (6 h), or during 6 h with 20 μg/ml of actinomycin D.

Tissue samples

Postmortem human control (n=3) or AD brains (n=6) were recovered with the approval of the Ethical Committee of the Medical School of the Free University of Brussels. One hemisphere was frozen in liquid nitrogen and stored at −80°C for biochemical analysis. The other hemisphere was fixed by immersion in 10% formalin (v/v) for neuropathological analysis. The neuropathological staging of AD cases was performed according to Braak and Braak (28). The neuropathological examination indicated the presence of numerous amyloid plaques and a severe neurofibrillary pathology in 3 AD patients (stage VI) and numerous plaques and less important neurofibrillary pathology in 3 other AD patients (stage III). In the 3 control subjects, no or only a moderate neurofibrillary pathology (limited to the entorhinal cortex) was present (stages I and II). No significant differences were observed for age (P=0.11) and postmortem delays (P=0.27) between control and AD subjects.

Kidneys from 3-mo-old $APP^{-/-}$ and $APP^{+/+}$ mice were recovered, frozen in liquid nitrogen, and stored at −80°C until analysis.

Protein analysis in immunoblotting

Cells or frozen powdered tissues were recovered in lysis buffer (deoxycholate 1%, pH 11.3 and complete protease inhibitor cocktail), and 30 μg of proteins was analyzed by immunoblotting following electrophoresis on 10–20% Tricine or 4–12% Bis-Tris gels (Invitrogen). Nitrocellulose membranes were incubated overnight at 4°C with the primary antibody (0.5 μg/ml WO-2; 1:2500 anti-APP C-ter; 1:1000 anti-AQP1; 1:10,000 anti-PS1; 1:5000 anti-PS2; 1:2000 anti-actin; and 1:4000 anti-tubulin), washed, and incubated with 1:10,000 secondary antibody (anti-mouse or anti-rabbit Ig) conjugated to horseradish peroxidase and revealed by ECL. Autoradiograms (Amersham) were digitalized with a ChemiDoc imaging device coupled to the Quantity One analysis software (Bio-Rad, Hercules, CA, USA) for quantification. Actin or tubulin was used as an internal standard to normalize protein load in gels.

Luciferase assays and transfections

MEF cells were transfected with 0.8 μg/well of the following constructs: pGL3-Luc (control), pGL3-AQP1-Luc (29), and phRG-TK (0.02 μg/well) with Lipofectamine according to the manufacturer's instruction (Invitrogen). Cells were harvested 48 h post-transfection in 0.1 ml/well of reporter lysis buffer. Firefly and *Renilla* luciferase activities were measured with the Dual Glo luciferase assay system (Promega, Madison, WI, USA). Firefly luciferase activity was standardized by the *Renilla* luciferase activity to correct for transfection efficiency.

RNA preparation and quantitative (q)PCR

RNAs were extracted in TRIzol reagent and reverse-transcribed using an iScript cDNA synthesis kit (Bio-Rad). Real-time qPCR analyses were performed on 2 ng of cDNA template by using iQ SYBR Green Supermix (Bio-Rad) in an iCycler IQ multicolor Real-Time PCR detection system. PCR conditions were typically 94°C for 3 min, followed by 40 cycles of 30 s at 95°C, 45 s at 60°C, and 15 s at 79°C. PCR primers were as follows: AQP-1 forward: TGAGATCATTGGCACTCTGC; AQP-1 reverse: TGATACCGCAGCCAGTGTAG; SREBF1 forward: GAGGGCTTGGCACAGA; SREBF1 reverse: AGCTGTGGCCTCATGTAGGAA; GAPDH forward: CCCCCAATGTATCCGTTGTG; and GAPDH reverse: TAGCCCAGGATGCCCTTTAGT. The relative changes in the target gene-to-GAPDH mRNA ratio were determined by the $2^{\Delta\Delta Ct}$ calculation.

Coimmunoprecipitation and HDAC activity

Cells were washed in ice-cold PBS and solubilized in immunoprecipitation buffer (1 ml/well of 25 mM Tris, pH 7.6; 0.5% Triton X-100; and 0.5% Nonidet P-40 containing a protease inhibitor mix). Cellular extracts were cleared by

centrifugation (20,000 *g*, 5 min, 4°C). Coimmunoprecipitations were carried out with 35 μl/ml of anti-C-terminal antibody. Immunoprecipitates were analyzed by the HDAC assay kit (Sigma) according to the manufacturer's instructions.

Immunohistochemistry

The immunolabeling was performed on 10-μm-thick tissue sections from paraffin-embedded tissue blocks of hippocampus and frontal cortex. Tissue sections were treated with H_2O_2 to inhibit endogenous peroxidase and incubated with a blocking solution (10% v/v horse serum in TBS containing 10 mM Tris and 150 mM NaCl, pH 7.4). After overnight incubation with the anti-AQP1 antibody, sections were sequentially incubated with a goat anti-rabbit antibody conjugated to biotin followed by the ABC complex (Vector Labs, Peterborough, UK). Peroxidase activity was revealed using diaminobenzidine as a chromogen.

For double fluorescent immunolabeling, the primary antibodies were incubated together; the rabbit antibody was detected by a biotin-cojugated goat anti-rabbit antibody (Vector) followed by streptavidin conjugated to Alexa594 (Molecular Probes, Eugene, OR, USA). The mouse antibody was detected by a goat anti-mouse antibody conjugated to Alexa488 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Sections were counterstained by DAPI staining. For immunolabeling with the Aβ antibodies, rehydrated tissue sections were pretreated with 100% formic acid for 10 min. Immunocytochemical controls were performed by omitting the primary antibodies. Tissue sections were examined with an Axioplan microscope (Zeiss, Oberkochen, Germany), and digital images were acquired with an Axiocam HRc camera (Zeiss).

Statistical analysis

The number of samples (n) in each experimental condition is indicated in figure legends. Statistical analysis was performed by 1-way ANOVA followed by Bonferroni's multiple comparison posttest or by an unpaired t test (2 experimental conditions).

RESULTS

APP increases AQP 1 gene expression

Regulation of gene transcription by APP was investigated using gene-chip microarrays (Affymetrix, Santa Clara, CA, USA) on MEFs expressing or not expressing APP ($APP^{+/+}$ *vs.* $APP^{-/-}$). Since AICD, which is thought to mediate APP-dependent gene transcription, is released from APP by a presenilin-dependent γ-secretase activity, gene expression patterns in MEFs expressing or not expressing presenilin 1 and 2 ($PS^{+/+}$ *vs.* $PS^{-/-}$) were also studied (Supplemental Fig. 1). Comparison of the results allowed the identification of 8 genes differentially expressed in both cellular models that meet stringent criteria (fold change >3 or <3, difference between mean intensity >100). Four of these genes are involved in regulation of development and differentiation, 2 are related to metabolic and inflammatory pathways, and 2 genes are coding for either ion or water channels. AQP1 is a water channel that is up-regulated in AD brains (30). Therefore, we studied the mechanisms involved in the regulation of AQP1 gene expression. To validate the microarray data, qRT-PCR was performed on mRNA extracted from $APP^{+/+}$ and $APP^{-/-}$ MEFs after 24 h of culture. Using AQP1-specific primers, we confirmed a 6-fold decrease of the AQP1 mRNA levels in $APP^{-/-}$ MEFs (**Fig. 1*A***). The expression of APP and AQP1 was also analyzed in cells lysates by immunoblotting using specific AQP1 and APP antibodies (Fig. 1*B*). Quantification of several immunoblots indicated a 10-fold decrease of AQP1 in $APP^{-/-}$ MEFs (Fig. 1*C*). To confirm that APP specifically regulates AQP1 expression, rescue experiments were performed in $APP^{-/-}$ MEFs using lentiviral and retroviral stable expression of human APP695. These cells expressed high levels of human APP695 detected

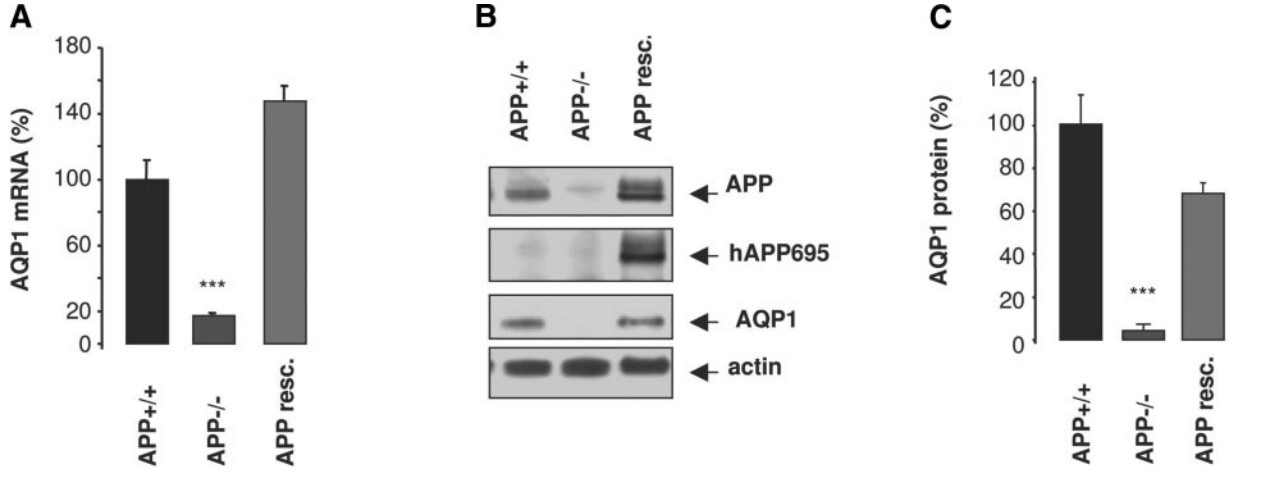

Figure 1. APP-dependent expression of AQP1. *A*) Comparative qRT-PCR analysis of AQP-1 mRNA in $APP^{+/+}$, $APP^{-/-}$, and APP MEFs rescue expressing hAPP695. Results are normalized by GAPDH mRNA and expressed as mean ± SE percentage of $APP^{+/+}$ (n>4). *B)* Expression of cellular AQP-1, endogenous APP, and human APP 695 was analyzed by Western blotting (30 μg/lane) revealed by anti-AQP1, anti-C-terminal, and WO-2 antibodies, respectively. Actin was used as a protein loading control. *C*) AQP1 expression was quantified from Western blots in different cell lines and expressed as mean ± SE percentage of expression in $APP^{+/+}$ MEFs (n=4). ***$P < 0.001$.

by the human specific anti-APP WO-2 antibody (Fig. 1*B*), and this expression restored the AQP1 mRNA (Fig. 1*A*) and protein levels (Fig. 1*B*, *C*). Interestingly, when different levels of human APP695 were present in different rescue cell lines, AQP1 expression correlated with human APP expression (Supplemental Fig. 2*A*). APLP2$^{-/-}$ MEFs were also analyzed in immunoblotting using anti-AQP1 antibodies. Results presented in Supplemental Fig. 2*B* indicate that APLP2 is not involved in the regulation of AQP1 expression. Since APLP1 is not expressed in MEFs (31), we can conclude that AQP1 expression is specifically regulated by APP in these cells. Other genes, which were previously described to be transcriptionally regulated by APP (17–20), were also analyzed in APP$^{+/+}$ and APP$^{-/-}$ MEFs by Affymetrix microarrays (Supplemental Fig. 1) and the corresponding proteins were analyzed by immunoblotting (Supplemental Fig. 2*B*). Our results confirm that APP does not regulate the transcription of these genes, as previously demonstrated (22).

APP does not increase either transcriptional activity of the AQP1 gene promoter or the AQP1 mRNA stability

To study whether APP increased the transcriptional activity of the AQP1 gene promoter, the transactivation properties of APP were measured in a luciferase-based reporter assay using a 1.7-kb fragment of the AQP1 promoter fused to the luciferase reporter gene (AQP1-P-luc; ref. 29). APP$^{+/+}$ and APP$^{-/-}$ MEFs were transfected with the AQP1-P-luc or the PGL3-basic plasmids, and the luciferase activity was measured 48 h after transfection. As shown in **Fig. 2*A***, the AQP1 gene promoter was able to strongly induce luciferase activity, but this induction was similar in APP$^{+/+}$ and APP$^{-/-}$ MEFs. Similar results were obtained with a luciferase reporter gene harboring a 4.4-kb fragment of the mouse AQP1 gene promoter (not shown). We can therefore conclude that APP does not stimulate the transcriptional activity of the AQP1 gene promoter when measured with reporter genes.

A 6-fold decrease in AQP1 mRNA in APP$^{-/-}$ MEFs could result from the stabilization of AQP1 mRNA by APP. We measured AQP1 mRNA stability in APP$^{+/+}$ and APP$^{-/-}$ MEFs in the presence of actinomycin D, an inhibitor of transcription. A 6-h treatment of MEFs with 20 μg/ml of actinomycin D showed a 60% decrease in SREBF1 mRNA in both APP$^{+/+}$ and APP$^{-/-}$ cells (Fig. 2*B*). On the contrary, the same treatment did not significantly decrease AQP1 mRNA, indicating its high stability in both APP$^{+/+}$ and APP$^{-/-}$ MEFs (Fig. 2*B*). AQP1 mRNA levels were similar in APP$^{+/+}$ and APP$^{-/-}$ after actinomycin D treatment. Therefore, the differences in AQP1 mRNA levels in APP$^{+/+}$ and APP$^{-/-}$ MEFs do not result from an APP-mediated stabilization of AQP1 mRNA.

Epigenetic control of AQP1 expression by APP

Since APP does neither activate the transcriptional activity of the AQP1 gene promoter in a luciferase reporter assay nor stabilize AQP1 mRNA, the 6-fold increase of AQP1 mRNA observed in APP$^{+/+}$ MEFs could result from an increased accessibility of the AQP1 gene for transcription, due to chromatin decondensation. These effects are hardly measurable with reporter genes that are extrachromosomic and do not adopt the chromatin structure of the corresponding endogenous promoters (32). The epigenetic control of gene expression is partly regulated by histones acetylation, resulting from a dynamic balance between histone acetyltransferase (HAT) and HDAC activities (33). We studied whether AQP1 gene transcription was affected by trichostastin A (TSA), an HDAC inhibitor. In APP$^{+/+}$ MEFs, a 24 h treatment in the presence of 5 or 7.5 ng/ml of TSA very significantly decreased AQP1 mRNA, while TSA did not significantly affect AQP1 mRNA in APP$^{-/-}$ MEFs (**Fig. 3*A***). Since inhibition of HDAC increases acetylation of histones leading to

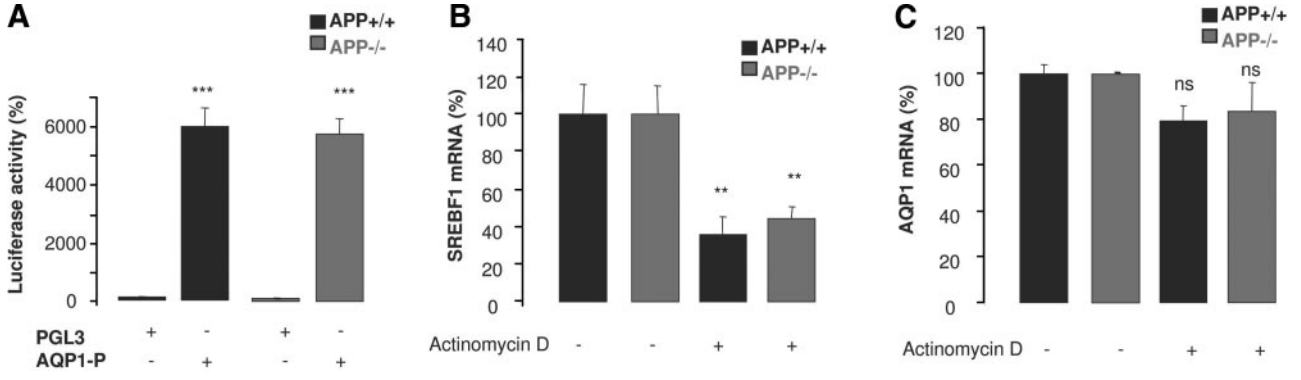

Figure 2. APP does not control either transcriptional activity of the AQP1 gene promoter or the AQP1 mRNA stability. *A*) Relative luminescence from APP$^{+/+}$ or APP$^{-/-}$ MEFs transfected with either the PGL3- or the AQP1-P-luciferase construct, 48 h after transfection. Results are expressed as mean ± SE percentage of PGL3 luminescence ($n \geq 3$). *B*, *C*) Comparative analysis of SREBF1 (*B*) or AQP1 (*C*) mRNA in APP$^{+/+}$ and APP$^{-/-}$ MEFs by qRT-PCR after 6 h of treatment with actinomycin D. Results are normalized by GAPDH mRNA and expressed as percentage of expression in APP$^{+/+}$ as 100% (mean±SE; $n>4$). $^{**}P < 0.01$; $^{***}P < 0.001$. ns, not significant.

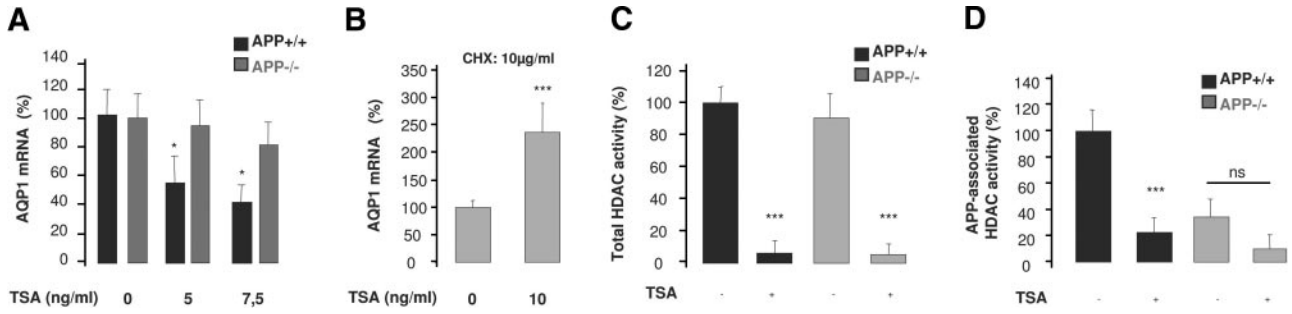

Figure 3. Epigenetic control of AQP1 expression by APP. *A*) Comparative analysis of AQP1 mRNA in $APP^{+/+}$ and $APP^{-/-}$ MEFs by qRT-PCR after 24 h of treatment with different concentrations of TSA. Results are normalized by GAPDH mRNA and expressed as mean ± SE percentage of untreated MEFs (n>4). *B*) Quantitative RT-PCR analysis of $APP^{+/+}$ MEFs treated during 6 h with different concentrations of TSA in the presence of 10 µg/ml cycloheximide. Results are normalized by GAPDH mRNA and expressed as mean ± SE percentage of untreated MEFs (n>4). *C*) TSA-sensitive HDAC activities measured in $APP^{+/+}$ and $APP^{-/-}$ MEFs before immunoprecipitation. Results are normalized by the protein content and expressed as mean ± SE percentage of $APP^{+/+}$ untreated MEFs (n>4). *D*) TSA-sensitive HDAC activities associated with APP measured in $APP^{+/+}$ and $APP^{-/-}$ MEFs after coimmunoprecipitation with the anti-C-terminal antibody. Results are expressed as mean ± SE percentage of $APP^{+/+}$ untreated MEFs (n>4). $^{*}P < 0.05$; $^{***}P < 0.001$.

chromatin decondensation, TSA was expected to increase AQP1 mRNA. However, a TSA-mediated decrease in gene transcription has been observed for several genes (34, 35) and could result from an increased expression of a transcription repressor. In that case, the effect of TSA can be reversed in the absence of protein synthesis (36). $APP^{+/+}$ MEFs were exposed during 6 h to TSA (10 ng/ml) in combination with 10 µg/ml of the protein synthesis inhibitor cycloheximide. In these conditions, TSA induced an increase in AQP1 mRNA (Fig. 3*B*). Altogether, our results indicate that inhibition of HDAC activities using TSA is able to decrease AQP1 mRNA in $APP^{+/+}$ MEFs. This epigenetic regulation needs *de novo* protein synthesis.

Since APP and TSA have opposite effects on AQP1 mRNA, we wondered whether APP would be able to recruit HDAC activities. HDAC activities inhibited by TSA were very similar in $APP^{-/-}$ and $APP^{+/+}$ MEFs (Fig. 3*C*). Following immunoprecipitation of APP using an anti-C-terminal antibody, HDAC activities inhibited by TSA were coimmunoprecipitated (Fig. 3*D*). $APP^{-/-}$ MEFs were used as negative controls (Fig. 3*D*). These results demonstrate a direct interaction between APP and HDACs.

The C-terminal domain of APP is involved in the epigenetic control of AQP1 expression

To study whether the C-terminal domain of APP was involved in the regulation of AQP1 expression, rescue experiments were performed in $APP^{-/-}$ MEFs using lentiviral stable expression of human APP695 lacking the intracellular C-terminal fragment from residue 652 to residue 695 (APP Δ-C). Several cell lines with a high level of APP Δ-C expression were not able to restore the AQP1 protein levels (**Fig. 4*A***). Lentiviral expression of

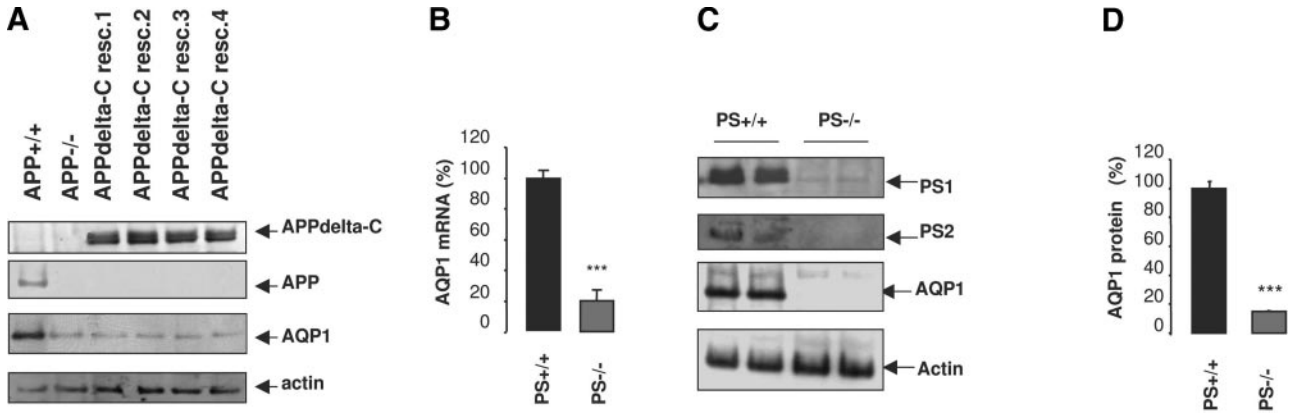

Figure 4. The C-terminal domain of APP is involved in the epigenetic control of AQP1. *A*) Expression of AQP1 in various $APP^{-/-}$ cell lines expressing hAPP695 lacking the C-terminal fragment (APPdelta-C). Western blots (30 µg /lane) were revealed by the WO-2, anti-C-terminal, and anti-AQP1 antibodies. Actin was used as a protein loading control. *B*) Comparative analysis of AQP-1 mRNA in $PS^{+/+}$ and $PS^{-/-}$ MEFs by qRT-PCR. Results are normalized by GAPDH mRNA and expressed as mean ± SE percentage of $PS^{+/+}$ (n>4). *C*) Expression of cellular AQP-1 and endogenous PS1 and PS2 was analyzed by Western blotting (30 µg/lane) revealed by the anti-PS1, anti-PS2, and anti-AQP1 antibodies. Actin was used as a protein loading control. *D*) AQP1 expression was quantified from Western blots in the different cell lines and expressed as mean ± SE percentage of expression in $PS^{+/+}$ MEFs (n=4). $^{***}P < 0.001$.

AICD in $APP^{-/-}$ MEFs allowed both detection of low amounts of the APP fragment, resulting from its rapid degradation, and partial rescue of AQP1 expression (not shown). These results clearly demonstrate that the C-terminal domain of APP is needed for the epigenetic control of AQP1 expression.

Presenilins are catalytic components of the γ-secretase complex that releases the C-terminal domain of APP involved in the epigenetic control of AQP1 expression. AQP1 expression was therefore studied in $PS^{+/+}$ and $PS^{-/-}$ MEFs. A 5-fold decrease of the AQP1 mRNA was measured in $PS^{-/-}$ MEFs (Fig. 4*B*). The expression of PS1, PS2, and AQP1 was also analyzed in cells lysates by immunoblotting using specific PS1, PS2, and AQP1 antibodies (Fig. 4*C*). Quantification of several immunoblots indicated a 7-fold decrease in AQP1 in $PS^{-/-}$ MEFs (Fig. 4*D*). These data are very similar to those obtained in $APP^{+/+}$ and $APP^{-/-}$ MEFs. We can therefore conclude that presenilins are involved in the regulation of AQP1 expression.

PS2, but not PS1, is involved in APP-mediated epigenetic control of AQP1 expression

The γ-secretase complexes containing either PS1 or PS2 might have different biological properties and different subcellular localizations. To further characterize the role of PS1 and PS2 in the control of AQP1 expression, $PS^{+/+}$, $PS^{-/-}$, $PS1^{-/-}$, and $PS2^{-/-}$ MEFs were analyzed for their content in AQP1 mRNA and protein. Results of **Fig. 5*A*** confirm a 5-fold decrease in AQP1 mRNA in $PS^{-/-}$ MEFs. The same decrease was observed in $PS2^{-/-}$ MEFs. In strong contrast, $PS1^{-/-}$ cells produced AQP1 mRNA in amounts very similar to those found in $PS^{+/+}$ cells (Fig. 5*A*). PS1, PS2, and AQP-1 were detected in the different cells lines by immunoblotting. Similar amounts of AQP1 were detected in $PS^{+/+}$ and $PS1^{-/-}$ MEFs, while the protein was not detected in $PS2^{-/-}$ and in $PS^{-/-}$ MEFs (Fig. 5*B*). These results clearly demonstrate that PS2, but not PS1, is needed for the control of AQP1 expression.

To confirm that PS2 is specifically involved in the control of AQP1 expression, rescue experiments were performed using retroviral stable expression of PS2 in $PS2^{-/-}$ MEFs. This expression restored both AQP1 mRNA (Fig. 5*C*) and protein (Fig. 5*D*).

Altogether, our results suggest that the C-terminal domain of APP that is associated with HDACs has to be released by a PS2-dependent γ-secretase activity to control AQP1 expression.

$APP^{-/-}$ mice express lower levels of AQP1

To analyze whether APP would be able to control AQP1 expression *in vivo*, different tissues from $APP^{+/+}$ and $APP^{-/-}$ mice were analyzed in immunoblotting using anti-AQP1 antibodies. The pattern of expression of AQP1 in the different tissues from 3-mo-old $APP^{+/+}$ mice indicated the highest levels of AQP1 expression in kidneys, as expected (not shown). Therefore, expression of AQP1 was analyzed in kidneys recovered from $APP^{+/+}$ and $APP^{-/-}$ mice. As shown in **Fig. 6*A***, $APP^{-/-}$ mice expressed lower levels of AQP1. Quantification of several experiments (Fig. 6*B*) indicated a 35% decrease of AQP1 in $APP^{-/-}$. These results indicate that APP is involved in the regulation of AQP1 expression not only in cultured cells but also *in vivo*.

AQP1 expression in AD

AQP1 was previously demonstrated to be up-regulated in some neurodegenerative diseases, including AD (30, 37). Human brain samples from nondemented age-matched controls and from AD patients with intermediary (stage III) or severe (stage VI) neuropathological staging were analyzed both by immunoblotting and immunohistochemistry. In immunoblots, AQP1 was significantly increased in stages III and VI of AD (**Fig. 7*A*, *B***). Immunohistological stainings were performed on control and AD brains at stage III and stage VI (28) with anti-Aβ (Fig. 7*C1–3*) or anti-AQP 1 antibodies (Fig. 7*C4–6*) antibodies. AQP1 staining was

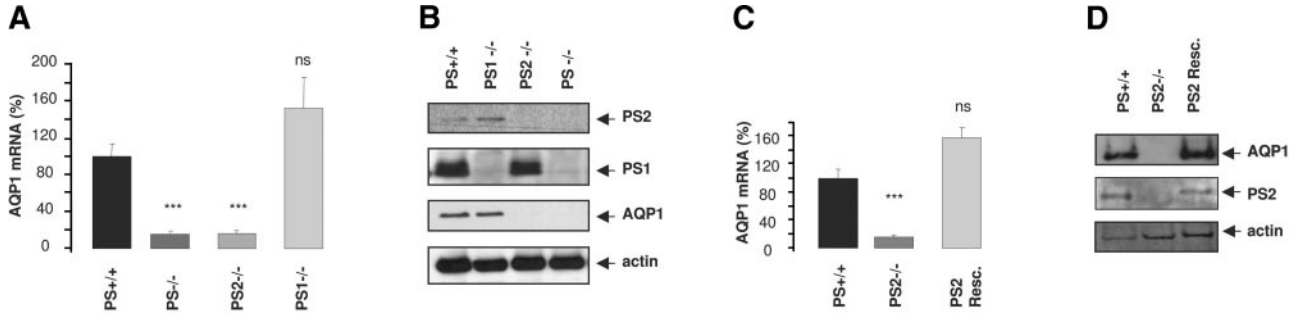

Figure 5. PS2-dependent control of AQP1 expression. *A*) Comparative analysis of AQP-1 mRNA in $PS^{+/+}$, $PS^{-/-}$, $PS1^{-/-}$, and $PS2^{-/-}$ MEFs by qRT-PCR. Results are normalized by GAPDH mRNA and expressed as mean ± SE percentage of $PS^{+/+}$ ($n>4$). *B*) Expression of cellular AQP-1 and endogenous PS1 and PS2 was analyzed by Western blotting (30 μg/lane). Actin was used as a protein loading control. *C*) qRT-PCR analysis of $PS^{+/+}$, $PS2^{-/-}$, and $PS2^{-/-}$ rescued with human PS2. Results are normalized by GAPDH mRNA and expressed as mean ± SE percentage of $PS^{+/+}$ MEFs ($n>4$). *D*) Expression of AQP1 and PS2 was analyzed by Western blots of total $PS^{+/+}$, $PS2^{-/-}$, and PS2 rescued with human PS2 MEF extracts (30 μg/lane) using specific antibodies. $^{***}P < 0.001$.

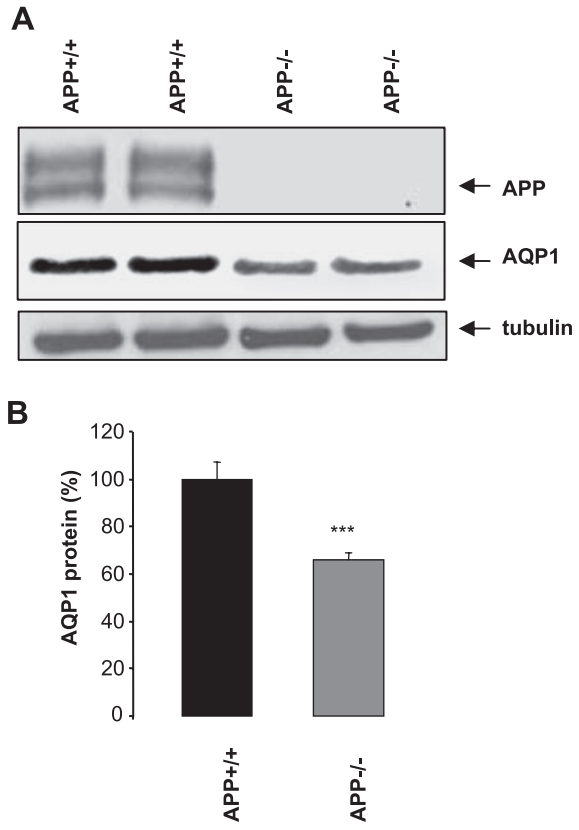

Figure 6. $APP^{-/-}$ mice express lower levels of AQP1 in kidneys. *A*) Tissue extracts from kidneys recovered from $APP^{+/+}$ and $APP^{-/-}$ mice at 3 mo of age were analyzed by Western blotting (10 μg/lane). Expression of AQP-1 was analyzed using AQP-1 antibody. Tubulin was used as a protein loading control. *B*) AQP1 expression was quantified from Western blots in the $APP^{+/+}$ and $APP^{-/-}$ mice and expressed as mean ± SE percentage of expression in $APP^{+/+}$ mice (n=4). ***$P < 0.001$.

strongly increased at stages III and VI in the gray matter of the hippocampus and of the frontal cortex where the load of amyloid plaques was also increased, as expected. Costainings showed that the AQP1 signal found in AD brains was exclusively present in astrocytes and that the AQP1-reactive astrocytes were mainly located near amyloid deposits (Fig. 7*D*). AQP1 expression by astrocytes could be needed for motility of these cells to migrate near amyloid deposits to tentatively remove these deposits by phagocytosis (38, 39).

DISCUSSION

In the Notch canonical signaling pathway, the last cleavage of Notch by γ-secretase releases the NICD, an 80-kDa transcription factor regulating expression of several genes (13). The cleavage of APP by γ-secretase releases the AICD, a 50-aa fragment. By analogy with the Notch processing, it has been proposed that AICD, in association with additional cofactors like Fe65 or TiP60 (15, 16), could regulate the transcription of target genes, including CD82/KAI, neprilysin, GSK3β, APP, Tip60, and other genes (17–20). However, transcriptome analysis of human neuronal cell culture inducible for expression of AICD, Fe65, or both did not confirm differential regulation of previously described AICD target genes such as KAI1, SERCA2B, or GSK3β (40). In addition, inhibition of AICD production using pharmacological agents or by presenilin knockout did not affect the expression of proteins encoded by these target genes, and Fe65 was able to transactivate a wide variety of different promoters independently of AICD (22, 41, 42). Therefore, the role of AICD in the transcriptional regulation of these genes remains questionable.

To study whether APP is able to regulate gene transcription, we compared the transcripts of MEFs expressing endogenous APP to those of APP-knockout MEFs from the same genetic background. This allowed us to avoid overexpression artifacts that could occur in cells overexpressing exogenous APP. None of the previously reported APP target genes were differently expressed in $APP^{+/+}$ and $APP^{-/-}$ MEFs, confirming that APP (and consequently AICD) is a poor transcription stimulator of these genes. However, we identified 14 genes highly down-regulated and 8 genes highly up-regulated by APP, including the AQP1 gene. In several $APP^{+/+}$ and $APP^{-/-}$ cell lines that we tested (n=5), a systematic 6- to 10-fold down-regulation of AQP1 mRNA and protein was observed in $APP^{-/-}$ MEFs.

The APP gene belongs to a gene family including APLP-1 (43) and APLP-2 (44), and the corresponding proteins, sharing structural features with APP, have overlapping functions *in vivo* (45). APLP2 did not affect AQP1 expression in MEFs. Since APLP1 is not expressed in MEFs (31), we conclude that the regulation of AQP1 expression in these cells is APP specific and does not involve other members of the APP/APLP family.

Expression of full-length human APP was able to rescue AQP1 expression in $APP^{-/-}$ MEFs. In contrast, expression of APP lacking AICD was unable to rescue them, indicating that AICD is needed for triggering AQP1 expression. In addition, the decrease in AQP1 expression observed in $PS^{-/-}$ MEFs strongly suggests that AICD must be released from APP by a PS-dependent γ-secretase activity to be able to regulate AQP1 expression. The γ-secretase cleavage of several substrates is performed by multiprotein complexes containing either Aph-1a or Aph-1b (6, 46, 47), as well as PS1 or PS2 as an enzymatic activity (48). Emerging evidence suggests that these 6 possible multiprotein complexes may contribute to diverse biological functions. PS1- and PS2-knockout mice reveal dramatically different phenotypes. PS1-deficient mice show serious developmental abnormalities with prenatal lethality (49), whereas PS2-deficient mice are fertile and viable, presenting only a temporary lung fibrosis and hemorrhages (25, 50). In fibroblasts, deletion of PS1 strongly inhibits Aβ production, contrary to deletion of PS2 (51). We demonstrate here that the C-terminal domain of APP has to be released by a PS2-mediated γ-secretase activity to control AQP1 expression, suggesting that PS2

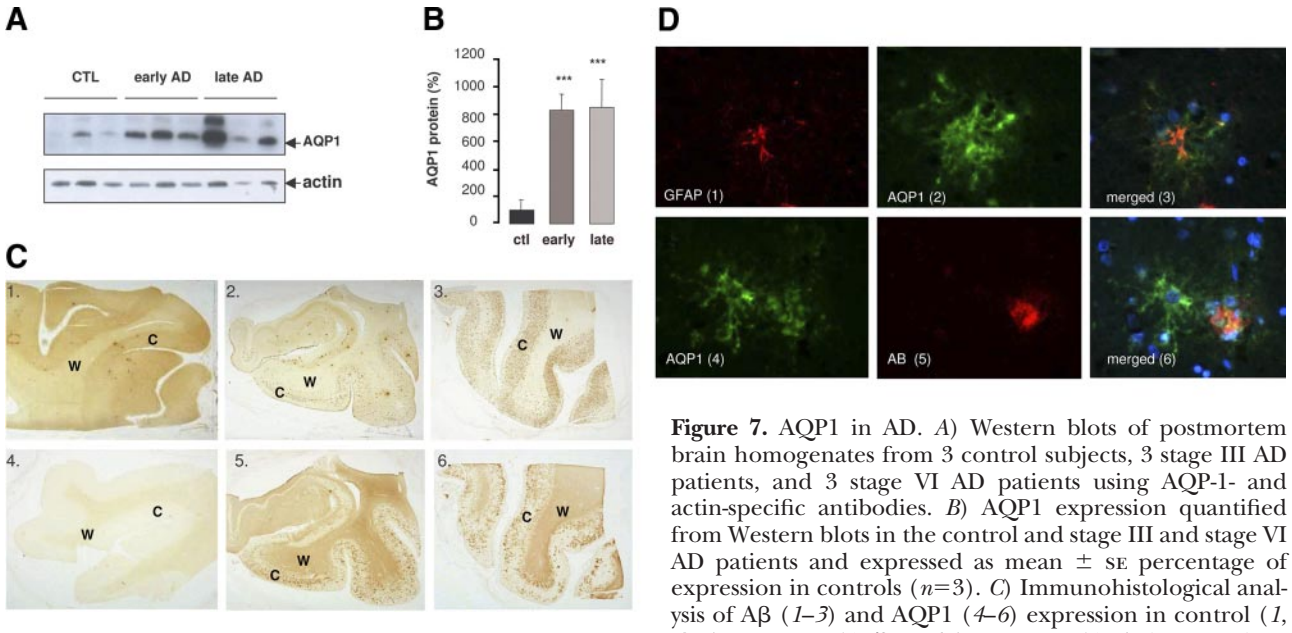

Figure 7. AQP1 in AD. *A*) Western blots of postmortem brain homogenates from 3 control subjects, 3 stage III AD patients, and 3 stage VI AD patients using AQP-1- and actin-specific antibodies. *B*) AQP1 expression quantified from Western blots in the control and stage III and stage VI AD patients and expressed as mean ± SE percentage of expression in controls (n=3). *C*) Immunohistological analysis of Aβ (*1–3*) and AQP1 (*4–6*) expression in control (*1, 4*), AD stage III (*2, 5*), and AD stage VI (*3, 6*) tissue sections of the frontal cortex (*1, 4*) and of the hippocampus (*2, 5*) (macroscopic view). Patchy Aβ-immunoreactive deposits are present in the gray matter of the cortical ribbon in the AD patients (stages III and VI; *2, 3*). In an adjacent tissue section, numerous AQP1-immunoreactive patches, corresponding to clusters of AQP1-positive astrocytes associated to senile plaques are present in the gray matter of the cortical ribbon in the AD patents (stages III and VI; *5, 6*) but not in the control subject (*4*). C, gray matter in the cortical ribbon; W, white matter. *D*) Double-labeling immunofluorescence enabled visualization of AQP1 (green; *2*) and glial fibrillary acidic protein (red; *1*). Merged image (*3*) indicated that astrocytes expressed AQP1. Double-labeling immunofluorescence of AQP1 (green; *4*) and Aβ (red; *5*) indicated that astrocytes located near the senile plaques expressed AQP1 (merged, *6*). ***$P < 0.001$.

could be more involved in AICD production than in Aβ production.

AQP1 mRNA was not stabilized by APP, and APP was not able to transactivate the AQP1 gene promoter in luciferase reporter assays. Therefore, the differences in AQP1 mRNA levels between $APP^{+/+}$ and $APP^{-/-}$ MEFs were likely to result from mechanisms that cannot be measured using reporter genes. Epigenetic control of expression related to chromatin organization is difficult to study using reporter genes transiently expressed as extrachromosomic constructs (32). In addition, in $APP^{+/+}$, but not in $APP^{-/-}$ MEFs, transcription of the AQP1 gene was inhibited by TSA, a specific inhibitor of HDAC type I and II activities (52). This highly suggests that APP could regulate transcription of the AQP1 gene by modification of histone acetylation and chromatin remodeling. In coimmunoprecipitation experiments, full-length APP but not APP lacking AICD was associated with HDAC activities inhibited by TSA. Identification of these HDAC activities will need further investigation, in particular using specific antibodies in Western blotting experiments. This association between AICD and HDACs is in agreement with recent data indicating that, in yeast 2-hybrid studies, Fe65 can simultaneously recruit SET, one of the main components of inhibitor of acetyl transferase (INHAT), and Teashirt, which in turn recruits HDACs (23). Epigenetic control of neprilysin expression has also been recently reported in which HDAC1 was demonstrated to be associated with the neprilysin gene promoter (53). In transgenic models for neurodegeneration, p25 age-related and AD-related stress factors inhibit HDAC1 in adult neurons (54), causing DNA breaks and cell cycle reactivation before the neuronal death. While HDACs were extensively studied in cancer therapy (33, 52), much attention has recently been focused on the potential function of HDAC inhibitors for treatment of neurodegenerative diseases, since chromatin remodeling through increased histone acetylation by the use of HDAC inhibitors is associated with the recovery of learning and memory in transgenic mice (55, 56).

Since epigenetic regulations could be cell specific, we analyzed the role of APP in AQP1 expression *in vivo* in tissues where AQP1 and APP are highly expressed. AQP1 expression was significantly decreased in the kidneys of APP-deficient mice, indicating that the APP-mediated control of AQP1 expression is not restricted to a particular cell type (MEFs) and to *in vitro* observations. In the brain, AQP1 is constitutively and exclusively expressed at the apical pole of the choroid plexus (57, 58) and participates in the production of cerebrospinal fluid (59). In the AD brain, we observed a very important up-regulation of AQP1 expression in the early and late stages of AD. In these brains, AQP1 was expressed by astrocytes located closely to amyloid plaques. Similar data were reported by Misawa *et al.* (37). Absence of AQP1 up-regulation in stages V-VI of AD (30) suggests that up-regulation of AQP-1 is an early event in AD. AQP1 expression by astrocytes in the AD brain could be important in increasing cell motility (60). Interestingly, AICD was previously demonstrated to up-regulate genes involved in actin dynamics and

cytoskeletal organization (40). In astrocytes, activation of these genes could be important in promoting Aβ clearance at the amyloid deposition site (38, 39).

CONCLUSIONS

We demonstrate that a PS2-mediated cleavage of APP allows the release of AICD, which interacts with HDACs to control AQP1 expression both in cultured cells and *in vivo* in transgenic mice. Modifications of HDAC activities play a key role in the etiology of several diseases, including neurodegenerative diseases. Direct interaction between AICD and HDACs confers to APP a key role in the control of histone acetylation and chromatin remodeling. Epigenetic control of AQP1 expression could be involved in its up-regulation in astrocytes located near the amyloid deposits of AD. FJ

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