

The Amyloid Precursor Protein Represses Expression of Acetylcholinesterase in Neuronal Cell Lines*

Received for publication, February 18, 2013, and in revised form, July 13, 2013. Published, JBC Papers in Press, July 29, 2013, DOI 10.1074/jbc.M113.461269

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Background: Acetylcholinesterase (AChE) and amyloid precursor protein (APP) are implicated in Alzheimer's disease, but their specific biological roles remain unclear.

Results: Overexpression or knockdown of neuronal APP modulates AChE mRNA, protein levels, and enzyme activity.

Conclusion: APP can act as a transcriptional regulator through a novel mechanism independently of γ -secretase.

Significance: Understanding the physiological functions of APP will lead to a greater understanding of Alzheimer's disease etiology.

The toxic role of amyloid β peptides in Alzheimer's disease is well documented. Their generation is via sequential β - and γ -secretase cleavage of the membrane-bound amyloid precursor protein (APP). Other APP metabolites include the soluble ectodomains sAPP α and sAPP β and also the amyloid precursor protein intracellular domain (AICD). In this study, we examined whether APP is involved in the regulation of acetylcholinesterase (AChE), which is a key protein of the cholinergic system and has been shown to accelerate amyloid fibril formation and increase their toxicity. Overexpression of the neuronal specific isoform, APP₆₉₅, in the neuronal cell lines SN56 and SH-SY5Y substantially decreased levels of AChE mRNA, protein, and catalytic activity. Although similar decreases in mRNA levels were observed of the proline-rich anchor of AChE, PRiMA, no changes were seen in mRNA levels of the related enzyme, butyrylcholinesterase, nor of the high-affinity choline transporter. A γ -secretase inhibitor did not affect AChE transcript levels or enzyme activity in SN56 (APP₆₉₅) or SH-SY5Y (APP₆₉₅) cells, showing that regulation of AChE by APP does not require the generation of AICD or amyloid β peptide. Treatment of wild-type SN56 cells with siRNA targeting APP resulted in a significant up-regulation in AChE mRNA levels. Mutagenesis studies suggest that the observed transcriptional repression of AChE is mediated by the E1 region of APP, specifically its copper-binding domain, but not the C-terminal YENTPY motif. In conclusion, AChE is regulated in two neuronal cell lines by APP in a manner independent of the generation of sAPP α , sAPP β , and AICD.

The amyloid precursor protein (APP)² and acetylcholinesterase (AChE) are both multifaceted proteins with a wide range of functions. Although they are both linked with growth and developmental processes, especially in the brain (1–3), they are also crucially linked with the pathogenesis of Alzheimer's disease (AD). APP is the precursor of the A β peptide, which is suggested to be the pathological agent in AD (4), with its oligomers considered the most toxic (5, 6). AChE has also been linked to the disease pathogenesis by exacerbating amyloid fibril formation and toxicity (7–10) and is the main target of clinically available AD drugs (11).

AChE is a key protein in cholinergic signaling, of which there are several main systems in the brain. Among these, the basal forebrain cholinergic system (12) is strongly linked to AD (13). There are reports specifically and consistently linking cholinergic hypofunction and cognitive decline. For reasons unknown, cholinergic neurons of the basal forebrain are specifically affected in AD (14). The predominant neurotransmitter in this system is acetylcholine (ACh), which, like AChE, has been suggested to have trophic functions (15).

The individual subunits of AChE can associate with each other, forming both dimers and tetramers. The proline-rich membrane anchor (PRiMA) is a 20-kDa protein responsible for both AChE tetramerization and its anchorage to the membrane in neuronal cells. This is a crucial role because tetramers of AChE form the functional units at cholinergic synapses (16–19). PRiMA has a proline-rich attachment domain like its counterpart, ColQ, which serves as a membrane anchor for AChE at neuromuscular junctions. The AChE-PRiMA association occurs between the C-terminal t peptides of AChE and the pro-

* This work was supported by the UK Biotechnology and Biological Sciences Research Council (studentship support to D. A. H.), the UK Medical Research Council, Alzheimer's Research UK, Russian Foundation for Basic Research Grant FBR 13-04-00388, and the Fundamental Sciences to Medicine Programme of the Russian Academy of Sciences.

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² The abbreviations used are: APP, amyloid precursor protein; AChE, acetylcholinesterase; ACh, acetylcholine; AD, Alzheimer's disease; A β , amyloid β peptide; PRiMA, proline rich membrane anchor; AICD, amyloid precursor protein intracellular domain; CuBD, copper-binding domain; BChE, butyrylcholinesterase; qPCR, quantitative PCR; CHT, choline transporter; DAPT, *N*-(*N*-(3,5-difluorophenacetyl)-*L*-alanyl)-*S*-phenylglycine *t*-butyl ester.

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line-rich attachment domain of PRiMA. It has also been suggested that disulfide bonds form between four Cys residues at the N terminus of PRiMA and the C-terminal Cys in the AChE t peptide (17). Immunofluorescence studies have shown strong colocalization between AChE and PRiMA in cholinergic neurons but no localization of PRiMA in either dopaminergic or GABAergic neurons (18).

APP is a type I integral membrane protein (see Fig. 1A) (20). It exists in three isoforms (APP₆₉₅, APP₇₅₁, and APP₇₇₀) generated by differential splicing of exons 7 and 8 (21, 22). In terms of distribution, APP mRNA is expressed in almost every tissue, where only the isoform ratio differs (23). It is APP₆₉₅ that predominates in neurons (2), and APP mRNA represents 0.2% of the total mRNA in these cells (24). There are two proteolytic pathways of APP processing. Amyloidogenic processing is the minor pathway (~5%) and involves sequential cleavage of APP by β -secretase and the γ -secretase complex, both aspartic proteases. These proteolytic events release A β and sAPP β , with the former, although linked to AD, having physiological roles, such as ion channel regulation (25). The second, non-amyloidogenic pathway involves α -secretase cleavage of APP, which occurs within the A β region (26, 27), precluding formation of A β , while generating a large, soluble ectodomain, the neuroprotective sAPP α (27–29). Both pathways result in the generation of the amyloid precursor protein intracellular domain (AICD), which can act as a transcriptional regulator (30–34), although there remains some controversy about the precise cohort of genes involved in AICD-mediated transcriptional regulation (35). Important residues in APP₆₉₅ for transcriptional regulation are Tyr-682 and Tyr-687, which represent the N- and C-terminal residues of the YENPTY motif in the cytoplasmic domain of APP (Fig. 1A) (36). Functional AICD is mostly formed in the amyloidogenic pathway, predominantly from the APP₆₉₅ isoform (37–39). Much of the transcriptional regulation by AICD is dependent on binding of the Fe65 protein, which may facilitate its nuclear translocation (40, 41). Although the APP C terminus is the predominant region for protein-protein interactions, other regions are also involved, e.g. via the extracellular E1 region with reelin (42), fibulin-1 (43), and integrin β 1 (44, 45) and also in dimerization of APP (46). Within the E1 domain, there are subdomains, including the His-rich copper-binding domain (CuBD) (36, 47), which has an important role in mediating APP dimerization (48).

The main goal of this study was to investigate whether APP₆₉₅ regulates AChE expression in neuronal cells. The concept of the cholinergic system regulating APP has been well documented (49, 50), but data about APP regulation of the cholinergic system are sparse. Here we show that overexpression or, conversely, knockdown of APP₆₉₅ can modulate mRNA, protein, and activity levels of AChE. In addition, PRiMA appears to be regulated via the same mechanism, whereas BChE and CHT remain unaffected. This novel mechanism is γ -secretase-independent and is likely mediated via the E1 domain of APP, specifically the CuBD therein.

EXPERIMENTAL PROCEDURES

Cell Culture—Cholinergic SN56 cells (provided by Prof. A. Szutowicz, Medical University of Gdańsk, Poland) and GD25/

GD25 β 1 cells (a gift from Prof. S. Johansson, Uppsala University, Sweden) were cultured in DMEM (Lonza, Basel, Switzerland), and SH-SY5Y cells were cultured in DMEM F-12 (Lonza). All media were supplemented with 10% fetal bovine serum and 1% penicillin, streptomycin, and L-glutamine. For SH-SY5Y cells, 1% non-essential amino acids were added. For transfected cells, either 150 μ g/ml hygromycin B (wild-type APP₆₉₅ or APP₆₉₅ with tyrosine/histidine mutations) (Invitrogen) or 10 μ g/ml blasticidin S (APP Δ E1) (Invitrogen) was added. All cells were incubated at 37 °C and 5% CO₂ in a humidified atmosphere.

Constructs—The APP₆₉₅ (pIRESHyg) construct was provided by Dr. A. R. Whyte (University of Leeds). APP₆₉₅ constructs expressing tyrosine mutations (all pIRESHyg, Y682G, Y687G and Y682G + Y687G double mutant) were prepared as follows. These APP Y682G and Y687G constructs were generated using site-directed mutagenesis to convert tyrosine codons (TAC) to glycine (GGC) according to the instructions of the manufacturer (Agilent Technologies UK Ltd, Stockport, UK) using pIRESHyg-wt-APP₆₉₅ as a template. The APP Y682G + Y687G double mutant was then generated in the same manner but using previously mutated APPY682G as a template. The APP Δ CuBD (H147A, H149A, H151A triple mutant) was a gift from Dr. Edward Neale (Lancaster University, UK). The APP Δ E1 (pLBCX, c-myc, 6 \times His tag) was provided by Prof. C. U. Pietrzik (Department of Pathobiochemistry, University Medical Center of the Johannes Gutenberg University, Mainz, Germany).

Transformation—Where appropriate, JM109-competent cells (Agilent Technologies) were transformed and cultured on ampicillin agar plates overnight. Plasmid DNA was extracted according to the instructions of the manufacturer using the Plasmid Maxi kit (Qiagen, Crawley, UK), checked for APP expression by polymerase chain reaction, and then further verified by sequencing (GATC Biotech, Konstanz, Germany).

Transfection—SH-SY5Y-APP₆₉₅ cells were provided by Dr. I. J. Whitehouse (University of Leeds). SN56 cells were grown to 50–80% confluence and incubated for 3–4 h in 5 ml of OptiMEM (Invitrogen) containing 2 μ l of Lipofectamine (Invitrogen) and 5 μ g of DNA (absent in mock-transfected cells) encoding APP₆₉₅ (or mutants thereof). Standard culture medium was added after 3–4 h, and the medium was changed after 24 h. At 48 h, cells were either assayed (for transient transfection) or split 1:50 and grown in 300 μ g/ml hygromycin B or 20 μ g/ml blasticidin S to select transfected cells (for stable transfection).

Cell Treatment—SN56-APP₆₉₅ or SH-SY5Y-APP₆₉₅ cells at \geq 80% confluence were incubated for 24 h in OptiMEM containing either vehicle (dimethyl sulfoxide, control), 25 μ M GM6001 (InSolutionTM, Calbiochem, Merck Chemicals, Nottingham, UK), 1 μ M β -IV (Calbiochem), or 10 μ M N-(3,5-difluorophenyl)-L-alanyl)-S-phenylglycine *t*-butyl ester (DAPT; InSolutionTM γ -secretase inhibitor IX, Calbiochem). The latter treatment was also performed in wild-type cells. After incubation, media samples were taken and concentrated, and cell lysates were prepared as described below. Alternatively, RNA was extracted from the cells for PCR analysis.

Preparation of Cell Lysates for Analysis of Cellular Proteins—Cells at \geq 80% confluence or after pharmacological treatment,

as indicated above, were washed twice in ice-cold PBS and harvested in 10 ml PBS. Cells were pelleted at $2700 \times g$ for 5 min (4°C) and resuspended in $6 \times$ volume of lysis buffer (50 mM Tris-HCl (pH 7.4) with 1% Triton X-100 and 0.5% sodium deoxycholate) with a 21-gauge needle and syringe. Lysis was performed for 30 min on ice, followed by centrifugation at $2700 \times g$ for 5 min to clarify the lysates. Supernatants were collected for assays.

Preparation of Cell Media for the Analysis of Secreted Proteins—Cells were washed with OptiMEM and incubated for 24 h in OptiMEM. The cell medium was then collected, and 5 ml was centrifuged ($2400 \times g$, 5 min, 4°C) to remove cell debris. Media samples were then added to a 6-ml, 10-kDa molecular weight cutoff Spin X-UF 20 concentrator (Corning Life Sciences, Amsterdam, The Netherlands). This was followed by centrifugation ($2400 \times g$, 4°C) until the volume reached 0.75–1.0 ml. At this point, the medium samples were centrifuged ($10,000 \times g$, 10 min) in a new Eppendorf tube to clear cell debris.

Cholinesterase Assay—This followed the classical method (51), modified as described before (52). Absorbance changes were measured using a plate reader (412 nm) (Fluostar Omega, BMG LabTech, Aylesbury, Buckinghamshire, UK). Type IV AChE from *Electrophorus electricus* (diluted 1:12500) (Sigma-Aldrich) was used as a positive control in the assays.

Determination of Protein Concentration—The BCA assay method was used for determining protein concentration. Both the bicinchoninic acid and 4% copper (II) pentahydrate solutions were supplied by Sigma-Aldrich.

SDS-PAGE and Western blotting—An 8% gel was used unless stated otherwise. Protein samples (20–50 μg) were run for 90 min (30 mA and 300 V) using a Bio-Rad gel rig and Invitrogen PowerEase 500 power supply. Western blotting was performed as described previously (37). Primary antibodies used were for AChE (AChE (C-16) catalog no. sc-6430, goat, 1:250, Santa Cruz Biotechnology), APP (22C11, mouse, 1:2000, Millipore, Billerica, MA or anti-C-terminal fragment, rabbit, 1:1000, Sigma-Aldrich), sAPP β (rabbit, 1:250, Signet Laboratories), and β -actin (1:10000, mouse, Sigma-Aldrich).

RT-PCR—RNA was isolated using the RNeasy kit (Qiagen) according to the instructions of the manufacturer. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad) and amplified using conventional PCR with TaqDNA polymerase (New England Biolabs, Hitchin, UK). Conditions were as follows: 94°C (5 min), 60°C (30 s), and 68°C (50 s) for 35 cycles and then 68°C (10 min) using a PTC-200 Peltier thermal cycler (MJ Research). Amplified DNA was resolved on 1% agarose gels with 50 μg of ethidium bromide and visualized on the Molecular Imager Gel Doc XR system with the Quantity One 4.6.1 program (Bio-Rad).

Primers (Sigma-Aldrich) were as follows: APP, AAGAAGC-CGATGATGACGAG (forward) and TTCTCATCCCCAG-GTGTCTC (reverse) and GAPDH, AACTTTGGCATTGTG-GAAGG (forward) and CACATTGGGGGTAGGAACAC (reverse).

Quantitative PCR (qPCR)—RNA was isolated and cDNA synthesized as above. Transcript levels were assessed using SensiMix SYBR Green (Bioline Reagents, London, UK) on a Rotor-Gene 6000 (Corbett Life Sciences, Cambridge, UK).

Primers used were for human genes as follows: AChE, TTC-CTCCCCAAATTGCTCAG (forward) and TCCAGTGCACC-ATGTAGGAG (reverse); PRIMA, TGATCATCATTGCCGT-ATGC (forward) and GGTGCCATTTTCGTCTTTTC (reverse); neprilysin, CCTGGAGATTATAATGGATCTTG (forward) and AAAGGGCCTTGCGCAAAG (reverse); and GAPDH, CAATGACCCCTTCATTGACC (forward) and GA-CAAGCTTCCCGTTCTCAG (reverse).

Primers used were for mouse genes as follows: PRIMA, ATCATTGTCGCTGTGGTCTG (forward) and GGTGCCA-TTTCATCCTTTC (reverse); BChE, TTACAACCAAGAC-CGGAAGG (forward) and GTTGTGCATAGGGGATACCG (reverse); CHT-F, ATATGGGCTGCATGGAAAAC (forward) and CACCAACCAACAAACCAATG (reverse); and U6-F, CTCGCTTCGGCAGCAC (forward) and AACGCTTCAC-GAATTTGCGT (reverse). The AChE primers were used in both cell lines because they bind in a conserved area of the transcript.

siRNA Knockdown—Wild-type SN56 cells were transfected with either 25 nM siRNA targeting APP (SMARTpool, Dharmacon, Thermo Scientific) or a scrambled sequence (siRNA negative control, Ambion, Invitrogen) at an equivalent concentration using Lipofectamine (Invitrogen). The transfection medium was replaced with OptiMEM after 6–8 h, and cells were harvested after 24 h.

Statistical Analysis—All statistical analyses were performed using an unpaired, two-tailed Student's *t* test (Microsoft Excel 2007). All error bars displayed are \pm S.E. Statistical significance is defined as follows: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

RESULTS

Overexpression of APP₆₉₅ in Neuronal Cell Lines—To investigate the possible regulation of AChE by APP, two neuronal cell lines, the human SH-SY5Y neuroblastoma cell line and the mouse cholinergic SN56 cell line, were used. The SN56 cell line is a neuronal cell line derived from murine septal neurons of the basal forebrain (53). A stable SH-SY5Y (APP₆₉₅) cell line has been generated and characterized previously (37). We also generated a stable SN56 (APP₆₉₅) cell line that showed substantial increases in APP₆₉₅ protein (mature and immature) and also in mRNA transcripts as compared with mock-transfected cells (Fig. 1, B and C).

Analysis of AChE Activity, Protein, and mRNA in Transfected Cell Lines—AChE activity was assessed in cell lysates from both the SN56 and SH-SY5Y cell lines (Fig. 2). The activity was significantly reduced in cell lines stably overexpressing APP₆₉₅ compared with mock-transfected cells, by 75% in SN56 and by 40% in SH-SY5Y cells (Fig. 2, A and B). This correlated with protein level changes caused by APP₆₉₅ overexpression, as shown by Western blot analysis (Fig. 2, A and B, top panels). To assess the effects of APP₆₉₅ overexpression on AChE transcript levels, quantitative RT-PCR was used (Fig. 2, C and D). The results of mRNA analyses correlate with AChE activity and protein levels, with a 95% reduction in AChE mRNA in SN56 (APP₆₉₅) cells and a 50% mRNA reduction in SH-SY5Y (APP₆₉₅) cells. Furthermore, AChE activity was decreased in the medium of SN56 (APP₆₉₅) cells in proportion to the decrease seen in the lysates (data not shown).

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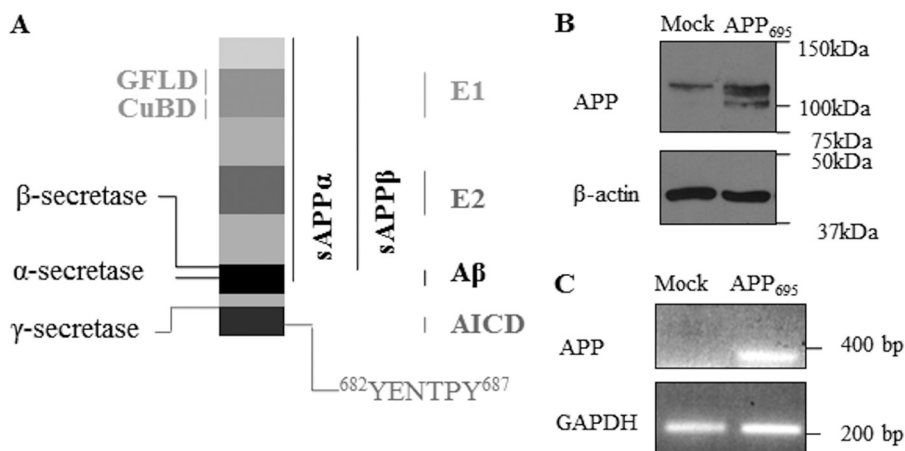


FIGURE 1. Schematic representation of APP₆₉₅ and data of overexpression of APP₆₉₅ in cholinergic SN56 cells. *A*, the amyloid precursor protein is a multidomain protein. The key domains growth factor-like domain (GFLD), CuBD, E1, E2, Aβ, and AICD are indicated. The YENTPY motif in the C-terminal region encompasses residues 682–687 in APP₆₉₅, which are crucial in the interaction of APP with intracellular binding partners. The full-length protein can be cleaved sequentially by α- and γ-secretases at the indicated sites, yielding the sAPPα fragment and AICD. Alternatively, APP can be cleaved sequentially by β- and γ-secretases, yielding sAPPβ, Aβ, and functional AICD. *B*, Western blot analysis for APP (top panel) and β-actin (bottom panel) in lysates of mock-transfected SN56 cells and SN56 cells overexpressing APP₆₉₅. *C*, RT-PCR of DNA extracted from the same cell lines, assessing levels of APP (top panel) and GAPDH (bottom panel).

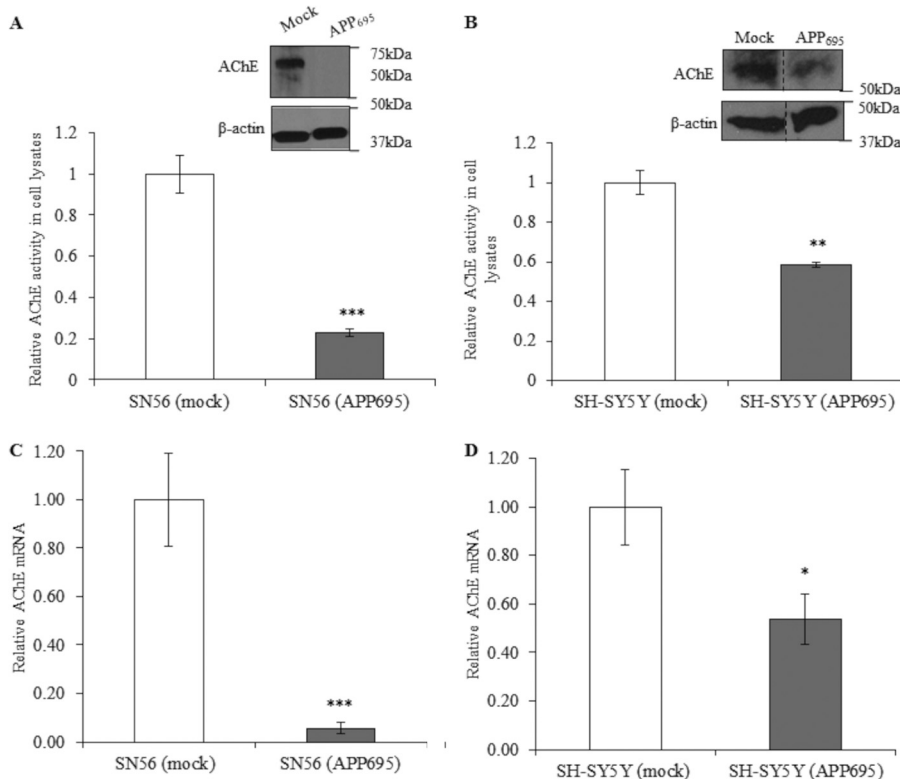


FIGURE 2. Effect of APP₆₉₅ overexpression on AChE activity, protein, and mRNA levels and mRNA levels of other cholinergic genes. *A*, AChE activity in SN56 (Mock) and SN56 (APP₆₉₅) cell lysates ($n = 6$, $p < 0.001$) and Western blot analysis for AChE (top panel) and β-actin (bottom panel) in lysates of SN56 cells (using 10% gel). *B*, AChE activity assays and Western blot analysis for AChE in SH-SY5Y cells ($n = 9$, $p = 0.01$). The vertical dashed line on the blot indicates alignment of samples from distal lanes run on the same immunoblot analysis. *C*, qPCR analysis of AChE mRNA transcripts ($n = 6$, $p = 0.001$) in SN56 cells and in SH-SY5Y cells ($n = 6$, $p = 0.02$) (*D*). *, $p \leq 0.05$; **, $p \leq 0.01$; or ***, $p \leq 0.001$.

Regulation of Other Cholinergic Genes by APP₆₉₅—Because catalytically active AChE is PRiMA-bound (17), we investigated whether PRiMA expression also changed with APP₆₉₅ overexpression, possibly either through coregulation or in response to the significant decrease in AChE expression (Fig. 3A). Quantitative PCR showed a significant 50% decrease in PRiMA transcripts in SN56 (APP₆₉₅) cells ($p < 0.001$). We further investi-

gated the transcript levels of another cholinergic protein, namely BChE, which, however, showed no change with overexpression of APP₆₉₅ (Fig. 3B, $p = 0.51$). To further expand our assessment of a more global regulatory effect by APP on the cholinergic system, we again utilized qPCR to assay mRNA levels of the high-affinity choline transporter (CHT). We confirmed expression of this protein in SH-SY5Y and SN56 cells by

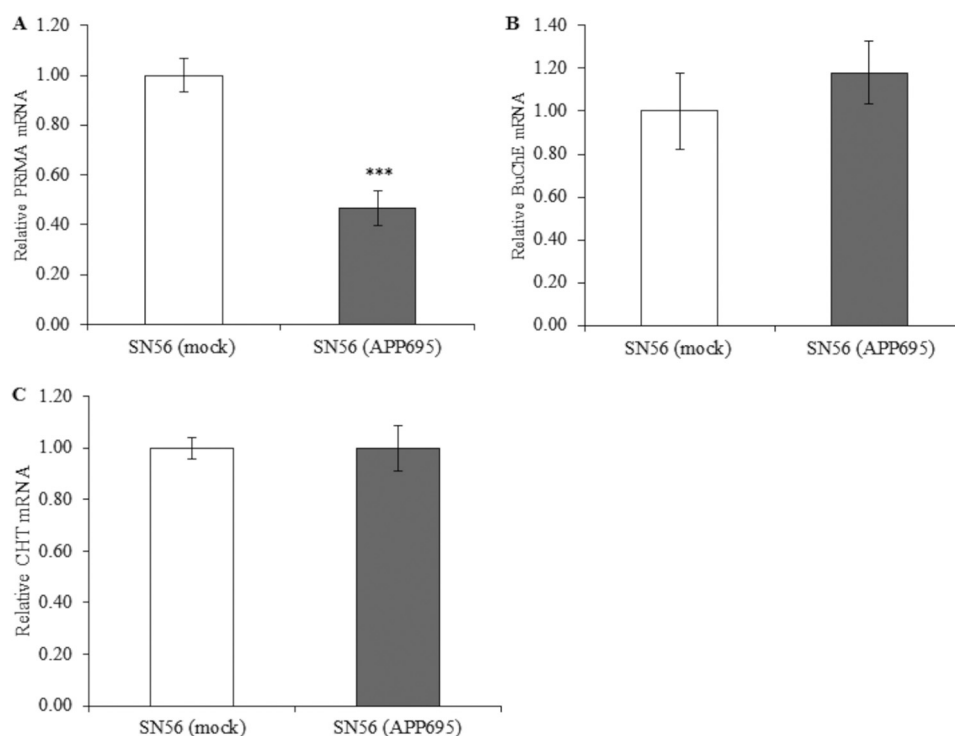


FIGURE 3. **qPCR analysis of mRNA levels of cholinergic genes upon stable overexpression of APP₆₉₅ in SN56 cells.** A, qPCR analysis of PRiMA mRNA transcripts ($n = 7, p < 0.001$) (A), BChE mRNA transcripts ($n = 6, p = 0.51$) (B), and CHT transcripts ($n = 9, p = 0.99$) (C). ***, $p \leq 0.001$.

Western blot analysis (data not shown). In SN56 cells overexpressing APP₆₉₅, there were no changes in mRNA levels of CHT (Fig. 3C, $p = 0.99$).

Assessment of AChE mRNA Levels after siRNA-mediated Knockdown of Endogenous APP—After demonstrating the effects of APP overexpression on AChE mRNA levels and activity, we next examined the effects of knockdown of endogenous APP using siRNA. Transfection of wild-type SN56 cells with a scrambled sequence oligonucleotide showed no effect on APP expression, but the APP-specific siRNA caused large reductions in APP protein levels (Fig. 4, inset). We then assessed AChE mRNA levels after siRNA knockdown of APP. Again, the scrambled sequence had no effect, but siRNA knockdown of APP resulted in a significant increase in transcription of AChE (Fig. 4).

Investigation of the Role of APP Metabolites in AChE Regulation—We next examined the potential role of AICD in the APP-mediated regulation of AChE expression by using the γ -secretase inhibitor DAPT to block AICD production (Fig. 5). Inhibition of the γ -secretase complex precludes generation of AICD and up-regulation of neprilysin (33, 38). Inhibition of AICD generation had no effect on total AChE activity in SH-SY5Y (APP₆₉₅) or SN56 (APP₆₉₅) cells (Fig. 5, A and B). Furthermore, analysis of AChE mRNA levels by quantitative RT-PCR revealed no changes in these cell lines (Fig. 5, C and D). Similarly, DAPT had no effect on AChE activity in wild-type SN56 cells (data not shown). As with AChE, PRiMA mRNA transcripts showed no change on treatment with DAPT (data not shown). Successful inhibition of γ -secretase was confirmed by a significant reduction in transcript levels of the AICD-regulated gene neprilysin (Fig. 5E).

To assess the potential roles of the soluble ectodomains sAPP α and sAPP β in the transcriptional repression of AChE,

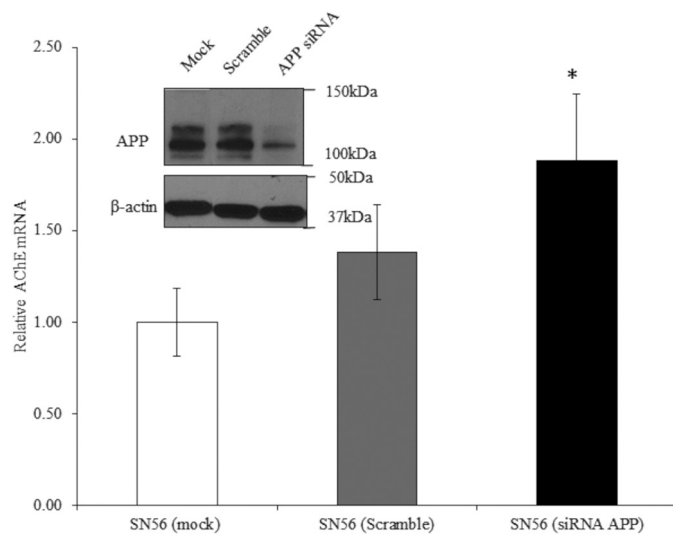


FIGURE 4. **Effect of APP knockdown on AChE mRNA.** qPCR analysis of AChE mRNA transcripts (scramble, $n = 7, p = 0.43$; siRNA APP, $n = 9, p = 0.03$) and (inset) Western blot analysis for APP (top panel) and β -actin (bottom panel) in lysates of SN56 cells treated with buffer (mock), scrambled oligonucleotide, or siRNA targeting APP. *, $p \leq 0.05$.

we blocked their formation in SH-SY5Y (APP₆₉₅) and SN56 (APP₆₉₅) cells using the α -secretase inhibitor GM6001 and the β -secretase inhibitor β -IV. Neither of these inhibitors was able to alter cellular AChE activity in SH-SY5Y (APP₆₉₅) or SN56 (APP₆₉₅) cells (Fig. 6, A and B). Confirmation that the inhibitors were functional at the concentrations used was demonstrated by Western blot analysis for the APP ectodomains sAPP α and sAPP β , the production of which is dependent on α -secretase and β -secretase, respectively (Fig. 6C).

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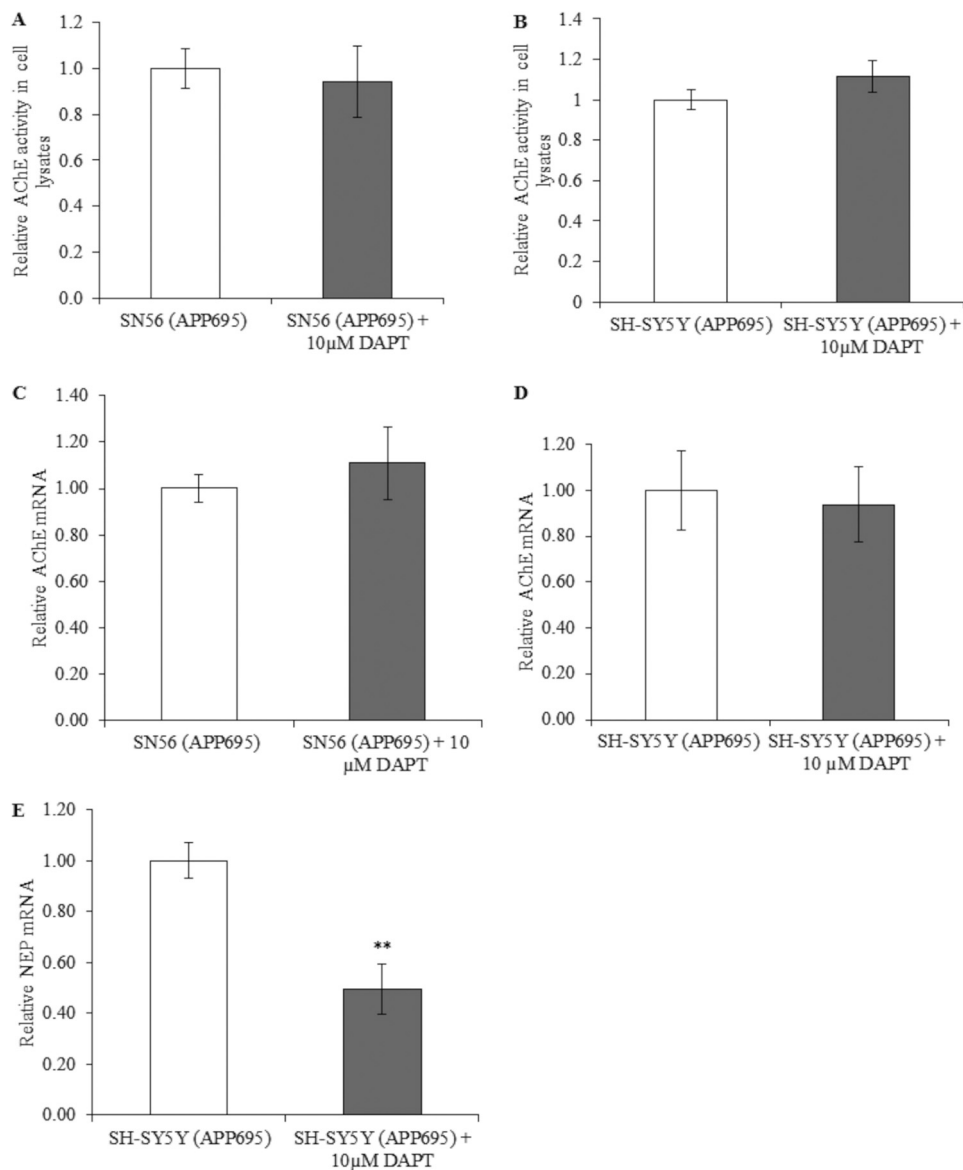


FIGURE 5. Effects of γ -secretase inhibition on AChE activity and mRNA levels. A, AChE activity in cell lysates from SN56 (APP₆₉₅) and SN56 (APP₆₉₅) cells treated with 10 μ M DAPT for 24 h ($n = 6$, $p = 0.41$) and in SH-SY5Y cells ($n = 6$, $p = 0.26$) (B). C, qPCR analysis of AChE mRNA transcripts with same treatment in SN56 cells ($n = 6$, $p = 0.54$) and SH-SY5Y cells ($n = 6$, $p = 0.55$) (D). E, qPCR for neprilysin in SH-SY5Y (APP₆₉₅) cells, either control or 24 h of 10 μ M DAPT ($n = 5$, $p = 0.001$). **, $p \leq 0.01$

Effects of Mutagenesis of Key Residues and Domains in APP₆₉₅ on AChE Regulation.—To elucidate the regions of APP that may be responsible for mediating transcriptional repression of AChE, we overexpressed three APP₆₉₅ mutant constructs in SN56 cells (Fig. 7, A and B). Numerous interactions of proteins with APP are mediated through its C-terminal region (36), the majority of which involve the YENTPY motif. The key residues therein, Tyr-682 and Tyr-687, were mutated both individually and together. These mutations did not change the ability of APP to exert its repressive effects on AChE. When assessing both AChE activity and mRNA in these cell lines, we have found significant reductions after overexpression of the APP mutants (Fig. 7, C and D). Although these APP constructs can undergo proteolysis in a slightly different manner compared with the wild-type protein, we have already shown that proteolytically derived APP metabolites have no effect on AChE expression

(Figs. 5 and 6), which would suggest that differential proteolysis would not have any effect on AChE.

Another region of APP that has several interacting partners is the extracellular E1 domain. To assess a possible role of this APP domain in transcriptional repression of APP, we expressed a deletion construct (APP Δ E1) in SN56 cells (Fig. 7E). Assessment of AChE activity and mRNA levels in these cells showed, in contrast to wild-type APP₆₉₅, no AChE-repressing effect of the APP Δ E1 construct (Fig. 7, F and G). Similarly, expression of this construct did not result in any significant change in PRiMA mRNA levels (Fig. 7H).

A significant reduction in AChE activity and mRNA levels was seen after transient transfection with the APP₆₉₅ construct (Fig. 8, B and C). However, transient transfection of wild-type SN56 cells with the APP Δ E1 construct showed no change in AChE activity or mRNA levels compared with mock-trans-

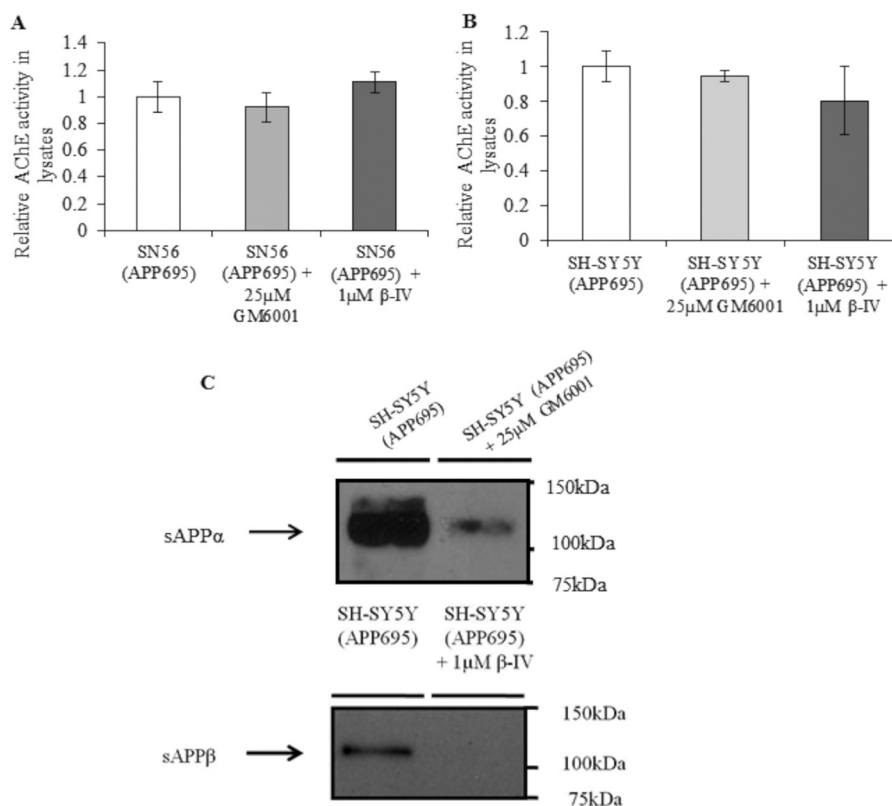


FIGURE 6. **Effects of α - and β -secretase inhibition on AChE activity.** A, AChE activity in cell lysates from SN56 (APP₆₉₅) treated with 25 μ M GM6001 for 24 h ($n = 6, p = 0.78$) or 1 μ M β -IV for 24 h ($n = 6, p = 0.52$). B, AChE activity in cell lysates from SH-SY5Y (APP₆₉₅) cells ($n = 7; p = 0.83$ (GM6001); $p = 0.51$ (β -IV)). C, Western blot analyses for sAPP α in cell culture medium from SH-SY5Y (APP₆₉₅) cells, either control or after 24 h of 25 μ M GM6001 treatment or for sAPP β in medium from SH-SY5Y (APP₆₉₅) cells after 24 h of 1 μ M β -IV treatment.

affected cells (Fig. 8, E and F), further supporting a role for the E1 domain in mediating AChE repression.

Effects of Overexpression of APP₆₉₅ (Δ CuBD)—The E1 domain has been linked to a number of APP-interacting proteins and also as containing critical residues in mediating APP dimerization (36, 45, 46). Indeed, studies of the APP interactome frequently list APP/APLP (APP-like protein) as an interacting partner for APP itself (54). Therefore, we mutated a key motif in APP dimerization and protein-protein interactions, the CuBD, mutating three critical His residues to Ala, generating APP₆₉₅ Δ CuBD. We overexpressed this APP construct in SN56 cells (Fig. 9A) to assess whether mutation of the CuBD would compromise the ability of APP to repress the transcription of AChE. These data showed that the SN56 (APP Δ CuBD) cells were unable to repress AChE activity in cell lysates (Fig. 9B). These findings were recapitulated at the mRNA level (Fig. 9C).

Integrin β 1 Is Not Involved in Mediating the Transcriptional Repression of AChE by APP₆₉₅—Integrins are transmembrane proteins with an intracellular NPXY motif, downstream of which intracellular signaling is better defined than any other candidate APP N-terminal ligands (44, 45). Therefore, we investigated whether ITGB1 may be the transmembrane interacting partner of APP through the C-terminal region of which intracellular signals are transduced. We used GD25 cells that are null for ITGB1 (55) and their ITGB1-expressing counterparts, GD25 β 1. Transient transfection of both cell lines with wild-type APP₆₉₅ resulted in non-significant decreases in AChE mRNA levels, and there was no significant difference in the

magnitude of this effect between GD25 and GD25 β 1 cells (data not shown).

DISCUSSION

Although full-length APP has a number of putative functions, a detailed investigation of these has been diverted by substantial focus on the amyloid hypothesis, dominated by $A\beta$ (56). Similarly, with AChE, attention has been diverted from the investigation of its non-catalytic physiological roles. Although a protein with significant links with AD, AChE research has mostly focused on inhibition of its catalytic activity for therapeutic benefits rather than understanding its physiology and metabolism in relation to the AD pathology.

To elucidate any possible direct relationship between APP and AChE, the possible regulation of AChE expression by APP was investigated. Our data show that, in two different neuronal cell lines, SN56 and SH-SY5Y, APP₆₉₅ overexpression repressed expression of AChE. This negative regulation is occurring on a transcriptional level with consequent reductions in AChE protein levels and enzyme activity. Although the data from our overexpression studies showed a clear relationship between the levels of APP and AChE expression and activity, we deemed it important to also investigate the effect of endogenous APP. Using siRNA, we were able to show that endogenous APP exerts a repressive effect on AChE transcription, showing that APP is a physiologically relevant modulator of AChE transcription. Furthermore, it clearly demonstrates that our data are not artifacts of an overexpression system, with the relationship

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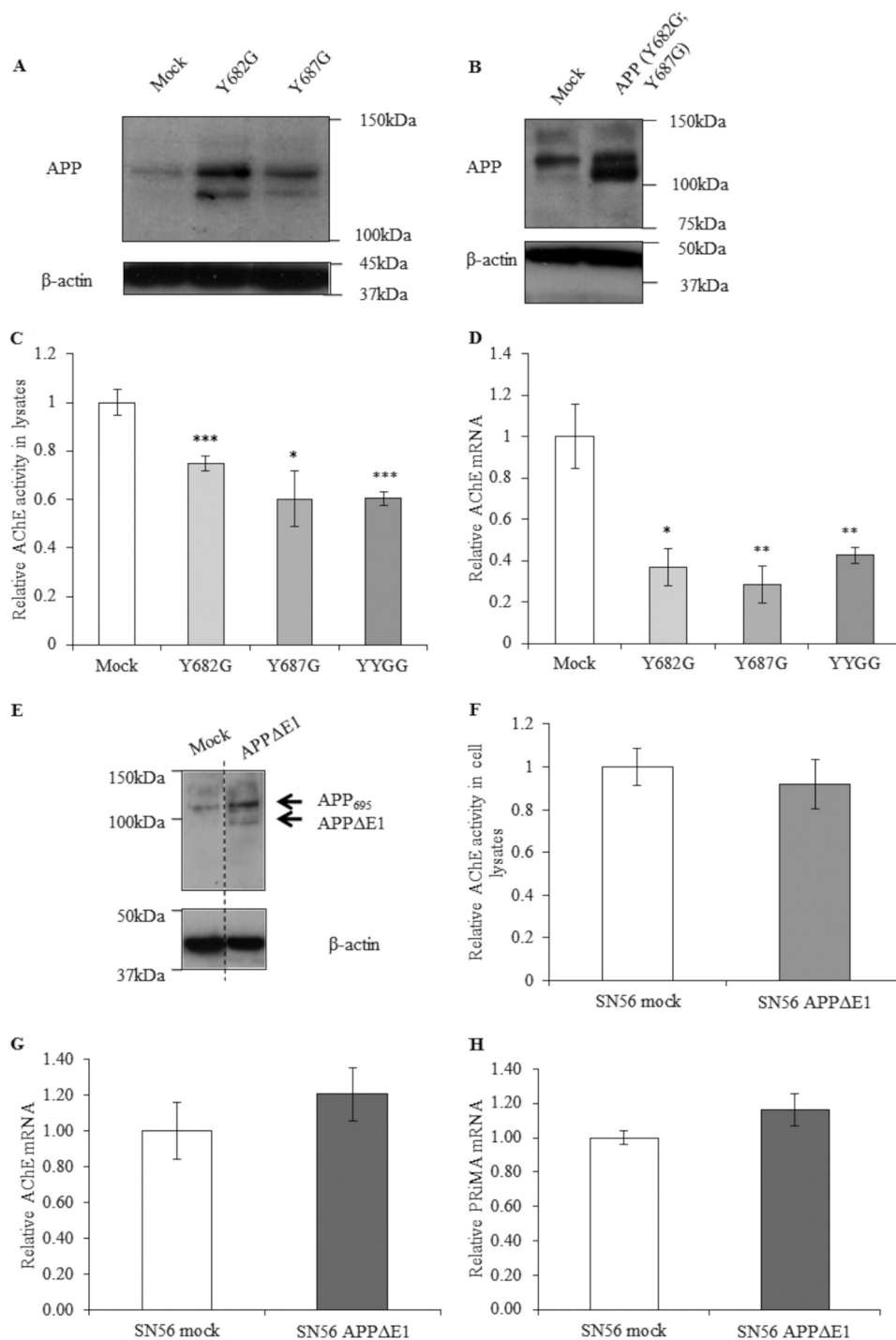


FIGURE 7. Effect of APP₆₉₅ mutagenesis and overexpression on AChE activity and mRNA. Western blot analysis for APP (top panel) and β -actin (bottom panel) in lysates of mock-transfected SN56 cells SN56 cells overexpressing mutant APP₆₉₅, specifically Y682G and Y687G (A) and the Y682G, Y687G double mutant (YYGG) (B). C, AChE activity in SN56 (mock) and SN56 (mutant APP₆₉₅) cell lysates (Y682G, $n = 6$, $p < 0.001$; Y687G, $n = 6$, $p = 0.02$; YYGG, $n = 6$, $p < 0.001$). D, qPCR analysis of AChE mRNA transcripts (Y682G, $n = 6$, $p = 0.01$; Y687G, $n = 6$, $p = 0.005$; YYGG, $n = 6$, $p = 0.009$). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. E, Western blot analysis for APP (1:1000 anti-C-terminal fragment) (top panel) and β -actin (bottom panel) in lysates of mock-transfected SN56 cells or SN56 cells overexpressing APP Δ E1. The vertical dashed line on the blot indicates alignment of samples from distal lanes run on the same immunoblot analysis. F, AChE activity in SN56 (mock) and SN56 (APP Δ E1) cell lysates ($n = 27$, $p = 0.51$). G, qPCR analysis of AChE mRNA transcripts ($n = 9$, $p = 0.33$). H, qPCR analysis of PRIMA mRNA transcripts ($n = 6$, $p = 0.4$).

induced by large increases in protein translation. Some studies *in vivo* have reported changes in AChE activity when APP is overexpressed. A study by Van Dam *et al.* (57) using the APP23 mouse model showed a significant reduction in AChE activity in the basal forebrain but not other regions examined. A further

study in APP23 mice observed moderate decreases in AChE activity but also reported reductions in cholinergic fiber density (58), and Boncristiano *et al.* (59) reported reduced neuronal volume in the basal forebrain of APP23 mice. These data show that the degenerative phenotype in these AD model mice may

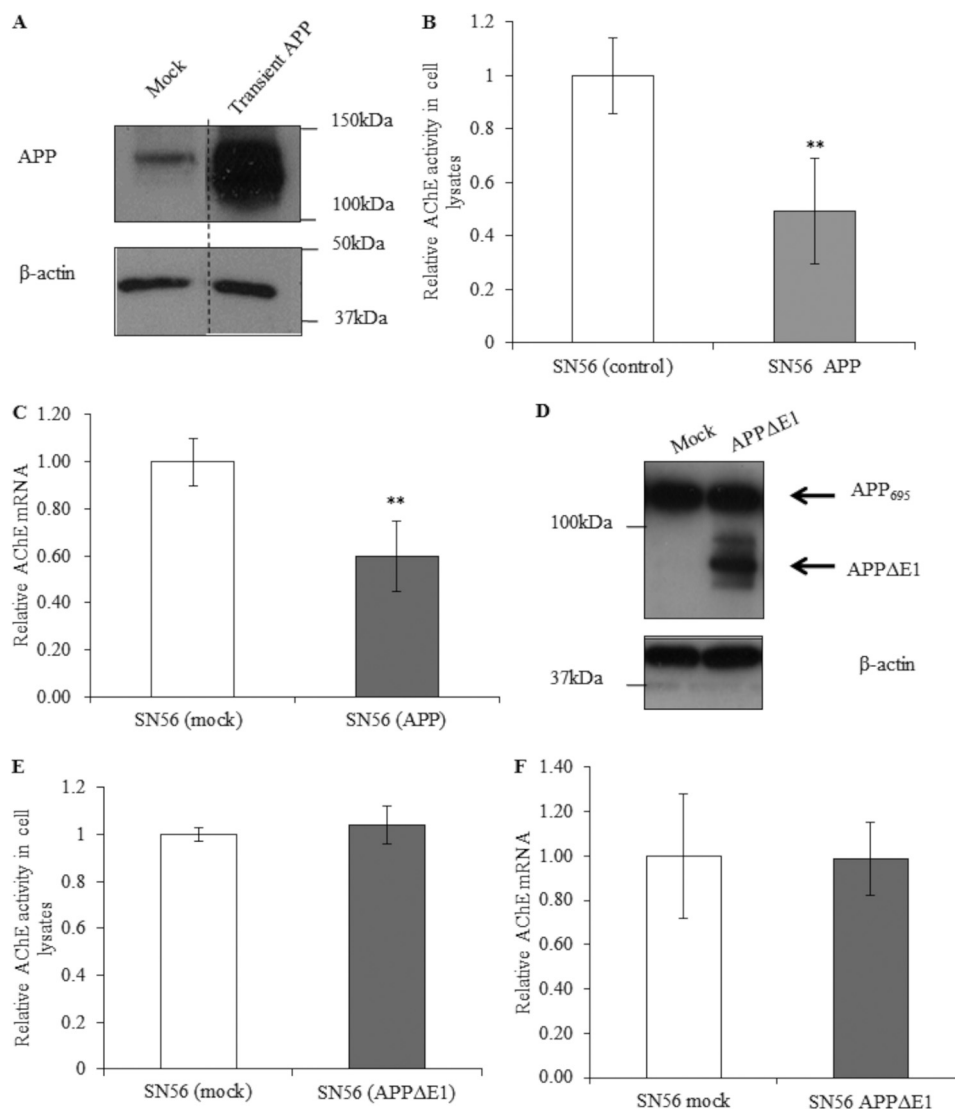


FIGURE 8. Effects of transient APP₆₉₅ overexpression on AChE activity and mRNA levels. *A*, Western blot analysis for APP (1:2000, 22C11) (*top panel*) and β -actin (*bottom panel*) in lysates of mock-transfected SN56 cells and SN56 cells transiently transfected with APP₆₉₅. The vertical dashed line on the blot indicates alignment of samples from distal lanes run on the same immunoblot analysis. *B*, AChE activity in SN56 (*mock*) and SN56 (transient APP₆₉₅) cell lysates ($n = 3$, $p = 0.005$). *C*, qPCR analysis of AChE mRNA transcripts in mock SN56 cells and cells transiently transfected with APP₆₉₅ ($n = 5$, $p = 0.01$). *D*, Western blot analysis for APP (1:1000, anti- C-terminal fragment) (*top panel*) and β -actin (*bottom panel*) in lysates of mock-transfected SN56 cells and cells transiently transfected with APP Δ E1. *E*, AChE activity in SN56 (*mock*) and SN56 (transient APP Δ E1) cell lysates ($n = 9$, $p = 1.0$). *F*, qPCR analysis of AChE mRNA transcripts in SN56 (*mock*) and SN56 (transient APP Δ E1) cells ($n = 6$, $p = 0.96$). **, $p \leq 0.01$.

be a confounding factor in the interpretation of AChE activity differences between wild-type and transgenic mice. We explored the possibility that APP had some pan-regulatory role in the cholinergic system, perhaps regulating a cohort of cholinergic genes rather than one in particular.

Apart from AChE, we also found a regulatory relationship between APP and another cholinergic system protein, namely PRiMA, which, like AChE, was down-regulated after APP₆₉₅ overexpression. This shows that, at least in this pathway, AChE and PRiMA levels are closely coupled. However, it is still unclear whether PRiMA is directly regulated by APP or whether its down-regulation is a consequence of modifications in AChE levels.

On the contrary, APP had no effect on another mammalian cholinesterase, BChE, because its levels were unchanged after APP₆₉₅ overexpression. Similarly, we were unable to find any

effect on mRNA levels of the high-affinity choline transporter CHT. This suggests that the regulatory effects downstream of APP₆₉₅ overexpression exhibit some selectivity and specificity. Our data, then, argue against regulation of a subset of cholinergic genes by APP, with only AChE and its membrane anchor directly modulated.

In addition to APP itself, its metabolites have also been shown to exert biological effects (60, 61). These metabolites include AICD, which regulates expression of numerous genes (61, 62), as well as sAPP α and sAPP β , whose mechanisms of action are still poorly understood (60). Of the APP fragments, AICD is by far the best characterized as a transcriptional regulator (31, 61–63). A large number of genes have been linked to AICD (64), with some consistently shown to be up-regulated, such as neprilysin (33, 34), and a much smaller number to be down-regulated, such as the EGF receptor (65).

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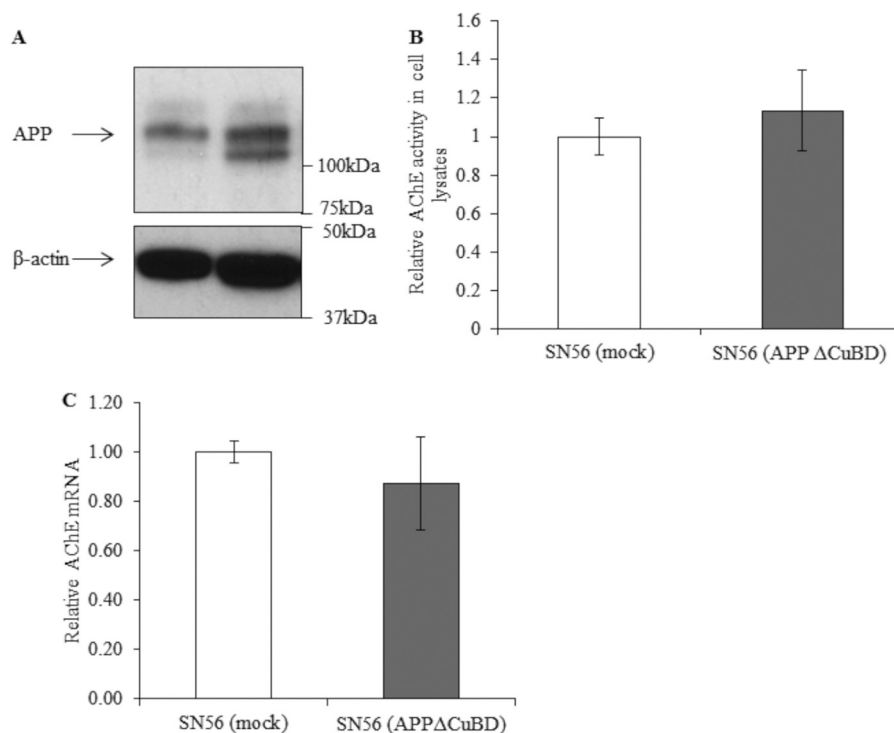


FIGURE 9. Analysis of AChE activity and mRNA levels after overexpression of APP₆₉₅ (ΔCuBD). A, Western blot analysis for APP (1:1000, 22C11) (top panel) and β-actin (bottom panel) in lysates of mock-transfected SN56 cells and SN56 cells stably overexpressing APP₆₉₅ (ΔCuBD). B, AChE activity in lysates of mock-transfected SN56 cells and those overexpressing either of the aforementioned APP construct ($n = 12$, $p = 0.53$) followed by qPCR analysis for AChE in these cell lines ($n = 6$, $p = 0.32$) (C).

However, the failure of a γ -secretase inhibitor to affect AChE mRNA or activity levels demonstrates that AICD does not regulate AChE in the mammalian neuronal cell lines used, with the repression of AChE expression unchanged by γ -secretase inhibition and, hence, ablation of AICD production. Because the effects of APP on AChE expression did not involve AICD, the possible involvement of the soluble APP ectodomains was also examined because they may have some roles in gene expression (60). sAPP α has well reported neuroprotective and neurotrophic effects (23, 66), and there are data reporting an increase in neuronal survival and neurite outgrowth promoted by sAPP α in rat cortical neurons (23). Further works have implicated enhancement of mitogenesis and synapse formation and stability as features of sAPP α action (67, 68). Neuroprotective roles of sAPP α against glucose deprivation, glutamate toxicity, and ischemia have also been reported (66, 69, 70). Although there is scant evidence linking sAPP α to any specific genes, (60), sAPP β has been linked to transcriptional regulation of transthyretin and Klotho (71). However, despite such a range of physiological properties, we failed to observe any effect of inhibition of sAPP α or sAPP β production on AChE activity.

The elimination of the major secretase-derived APP fragments (AICD, sAPP α , and sAPP β) as having a contributory role in repression of AChE transcription leaves full-length APP as the most likely responsible species. It is not surprising that full-length APP is involved in gene regulation because it is linked to numerous signaling pathways and other processes (1, 2). In terms of interactions of APP, the intracellular C-terminal region has the most documented interacting proteins, with key residues demonstrated to be Tyr-682 and Tyr-687 within the

YENPTY motif (36). However, our data demonstrate that mutation of these residues did not reduce the repressive effect exerted by APP and suggest that the observed transcriptional repression is unlikely to be mediated by the C-terminal region.

For further investigation of the role of APP in AChE repression, we analyzed the effect of deletion of the E1 region in APP. One possibility is that deletion of this domain prevents binding of an APP-interacting protein. Several N-terminal APP binding partners have been reported, namely fibulin-1, reelin, F-spondin, Lingo-1, contactin 2, pancortins 1 and 3, and integrin β 1 (ITGB1) (36, 44, 45). Of these, ITGB1 is capable of binding copper-binding domains (72), although this has not been directly shown for APP. A necessary requirement of a binding partner is that the interacting protein must be able to transduce intracellular signals, which ultimately results in transcriptional repression of AChE. Most of the APP binding partners listed are adhesion proteins, and many do not have any defined intracellular signaling activity. However, integrins do have defined intracellular signaling activity, modulating such proteins as focal adhesion kinase and Akt (73). However, our studies have shown that, although ITGB1 is an interacting partner of APP, it is unlikely to be the N-terminal binding partner through which repression of AChE is transduced.

The data obtained in our work have clearly indicated that deletion of the E1 domain ablates the repressive activity of APP₆₉₅, which suggests that this domain can mediate transcriptional repression of AChE. Previous studies have shown that the APP Δ E1 construct traffics and localizes in the same manner as wild-type APP₆₉₅ (74). We were able to characterize the CuBD within E1 as the region responsible for this transcrip-

tional repression of AChE. This APP Δ CuBD construct has been shown to traffic and undergo proteolysis in a manner very similar wild-type APP₆₉₅, although sAPP β is reduced relative to wild-type.³ Copper binding has been linked to promoting APP dimerization (48), but binding of interacting proteins to this region has also been suggested (75), and previous work has implicated integrins as being capable of binding CuBDs (72). Although ITGB1 does not appear to be the relevant interacting partner in the transduction mechanism, other interacting partners or even perturbation of dimerization may be factors.

The data provide an insight into the relationship between APP and AChE and highlight a novel function for APP in the regulation of gene expression independent of its intracellular domain. Although AD-linked genes have been shown to be regulated by APP, none of these regulatory pathways have been shown, so far, to involve the E1 domain of full-length APP, although the holoprotein has been recently linked to regulation of cholesterol metabolism at a transcriptional level (76). It is possible that repression of AChE serves to maintain cholinergic signaling by reducing ACh hydrolysis in a neurodegenerative environment. However, it is also possible that this relationship is independent of the catalytic function of AChE. AChE and APP both have roles in cell adhesion and synaptic integrity (3, 77), so perhaps this novel relationship serves to regulate these non-catalytic functions. Finally, AChE has been shown to have a role in apoptosis, being up-regulated by certain apoptotic stimuli and then participating in the process of apoptosis (78, 79), including in AD (80). As such, this down-regulation of AChE might be an example of a novel neuroprotective function of the APP holoprotein.

To elucidate any direct links between APP and the key cholinergic system proteins in mammalian cells, we have shown that APP₆₉₅ represses transcription of AChE in SH-SY5Y and SN56 cells. The mechanism is not dependent on APP metabolites produced by α -, β -, or γ -secretases because their inhibition in cell culture does not ablate the effect of APP₆₉₅ overexpression. Furthermore, mutagenesis studies show that the C-terminal region of APP is unlikely to have any involvement in this process. However, the E1 region of APP appears to play a role in the transcriptional repression of APP. Because current therapy focuses on inhibition of AChE, an understanding of the APP-AChE regulatory axis and the manipulation thereof may lead to novel therapeutic strategies.

Acknowledgments—We thank Dr. S. Isbert (University Medical Center of the Johannes Gutenberg University, Mainz, Germany) for assistance with the APP Δ E1 construct and Dr. C. Kerridge (University of Leeds) for transformation of tyrosine mutant APP plasmids into competent cells and subsequent extraction. We also thank Prof. N. M. Hooper (University of Leeds) for helpful discussion of the manuscript.

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