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The Neuronal Adaptor Protein X11α Reduces Aβ Levels in the Brains of Alzheimer's APPswe Tg2576 Transgenic Mice*

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Increased production and deposition of the 40–42-amino acid β-amyloid peptide (Aβ) is believed to be central to the pathogenesis of Alzheimer's disease. Aβ is derived from the amyloid precursor protein (APP), but the mechanisms that regulate APP processing to produce Aβ are not fully understood. X11α (also known as munc-18-interacting protein-1 (Mint1)) is a neuronal adaptor protein that binds APP and modulates APP processing in transfected non-neuronal cells. To investigate the *in vivo* effect of X11α on Aβ production in the brain, we created transgenic mice that overexpress X11α and crossed these with transgenics harboring a familial Alzheimer's disease mutant APP that produces increased levels of Aβ (APPswe Tg2576 mice). Analyses of Aβ levels in the offspring generated from two separate X11α founder mice revealed a significant, approximate 20% decrease in Aβ(1–40) in double transgenic mice expressing APPswe/X11α compared with APPswe mice. At a key time point in Aβ plaque deposition (8 months old), the number of Aβ plaques was also decreased in APPswe/X11α mice. Thus, we report here the first demonstration that X11α inhibits Aβ production and deposition *in vivo* in the brain.

One pathological hallmark of Alzheimer's disease is deposition of the 40–42-amino acid β-amyloid peptide (Aβ)[1] within the brains of affected individuals. Aβ is derived by proteolytic cleavage from the amyloid precursor protein (APP). APP is a type-1 membrane-spanning protein that contains a large ectodomain and a smaller, intracellular endodomain, and Aβ is derived from sequences that encompass parts of both ecto- and *trans*-membrane domains. Aberrant APP processing/metabolism leading to increased production and deposition of Aβ is believed to be central to the pathogenesis of Alzheimer's disease (for reviews see Refs. 1 and 2).

A number of proteins have now been shown to interact with the APP endodomain, including a variety of phosphotyrosine-binding domain proteins (3, 4). These phosphotyrosine-binding domain proteins comprise the Fe65 and X11 families. Disabled and JNK-interacting proteins and their interactions are mediated via the phosphotyrosine-binding domains and sequences surrounding the YENPTY motif in APP (5–18).

One such protein is the neuronal adaptor X11α, which contains a single, centrally located phosphotyrosine-binding domain through which it binds APP. However, X11α also contains a number of other protein-protein interaction domains and via these domains mediates the assembly of multi-protein complexes. Aside from APP family members, X11α-binding partners identified to date include CASK, munc-18, spinophilin/neurabin, neurexins, the N-type Ca^{2+} channel pore-forming α_{1B} subunit, the kinesin superfamily motor protein KIF17, presenilin 1, and the copper chaperone for superoxide dismutase-1 (19–30).

The binding of X11α to APP has now been shown to inhibit Aβ production in transfected non-neuronal cells, although the mechanisms that underlie this effect are not known (16, 31–33). However, whether it fulfills similar functions *in vivo* within the brain has not so far been established. This is an important omission because there is evidence that neurons can process APP differently to cell lines in culture (34–37). Here, we have utilized transgenic mice to investigate the effects of X11α on APP processing and demonstrate that X11α reduces Aβ levels in the brain.

EXPERIMENTAL PROCEDURES

Construction of Transgenic Mice—A carboxyl-terminal Myc-tagged full-length human X11α cDNA (28, 29) was inserted as a XhoI-SalI fragment into a modified mouse prion gene in which the single exon encoding the prion protein was deleted and engineered to contain a unique XhoI site (38). Vector sequences were removed, and the construct was injected into C57Bl6/SJL embryos (Xenogen Bioscience USA). Founder mice were crossed with C57Bl/6 animals and offspring backcrossed a further three times onto this background prior to analyses. APPswe Tg2576 mice (39) were obtained from Taconic (New York) and bred by mating male mice with C57Bl6/SJL F1 females as recommended by the suppliers and as described by others (40). For crossing of X11α and APPswe lines, male APPswe Tg2576 animals were mated with female X11α mice.

Northern Analyses—Mouse brains were removed and bisected along the midline. One half was utilized for analyses of mRNA/protein expression (see below), and the other half was used for Aβ assays. RNA was prepared using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were quantified by spectrophotometric analysis, and their integrity was confirmed by observing 28 and 18 S ribosomal species following electrophoresis in denaturing 1% agarose gels in MOPS buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.2) containing 2% formaldehyde. For Northern blotting, 20 μg of each sample were separated as above and transferred to GeneScreen Plus membranes (PerkinElmer Life Sciences). The probes were labeled with [γ-^{32}P]dCTP by random priming using a Prime-It II kit (Stratagene). APP mRNA was detected using a full-length human APP

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[1] The abbreviations used are: Aβ, β-amyloid peptide; APP, amyloid precursor protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay.

cDNA probe, and β-actin was detected with a commercial probe (Clontech). Hybridizations washings and autoradiography were performed as previously described (41).

SDS-PAGE and Immunoblotting—Mouse brains were weighed and prepared as 10%(w/v) homogenate in ice-cold 125 mM Tris-Cl (pH 6.8), 5 mM EDTA, 5 mM EGTA plus complete protease inhibitor mixture (Roche Applied Science). One-quarter volume of 10% SDS sample buffer was then added, and the samples were heated in a boiling water bath for 10 min. Protein concentrations were determined using Markwell assays. The samples were separated on 8% (w/v) acrylamide gels and transferred to Protran nitrocellulose membranes (Schleicher & Schuell) using a Bio-Rad TransBlot system. The blots were probed with primary antibodies, washed in phosphate-buffered saline, and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit Igs (Amersham Biosciences). The blots were developed using an enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer's instructions. X11α was detected using a rabbit polyclonal antibody (28) or mouse monoclonal antibody 9B11 (Cell Signaling) to the Myc tag on the carboxyl terminus of transgenic X11α. APP was detected using a rabbit polyclonal antibody generated using a synthetic peptide to the last 21 amino acid residues of APP (28).

To calculate the relative amounts of APP in the different mouse brain samples, increasing amounts (2.5–40 μg) of total brain proteins were probed for APP on immunoblots, and the signals were then quantified by pixel densitometry using a Bio-Rad GS710 imaging densitometer and Quantity 1 software as described (42). From these data, standard curves were generated so as to demonstrate that the protein amounts loaded on the gels gave APP signals on the immunoblots that were within the linear range.

Immunohistochemical Analyses—Transgenic X11α distribution was analyzed by immunostaining for the Myc tag using antibody 9B11. Brains from 10–12-week-old X11α transgenic and nontransgenic mice were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) and then cryoprotected at 4 °C in a solution of 30% sucrose in Tris-buffered saline (TBS; 40 mM Tris, 0.7% NaCl in 10 mM phosphate buffer, pH 7.8) containing 0.05% sodium azide prior to freezing on dry ice and storage at −80 °C. 40-μm frozen sections were cut coronally or saggitally from either hemisphere, collected in TBS-azide buffer, and stored at 4 °C prior to immunohistochemical staining. The optimal concentrations of primary and secondary antisera were determined by performing a checkerboard titration on transgenic tissue. The sections were labeled by blocking endogenous peroxidase activity with 1% H_2O_2 in TBS for 15 min and then blocking with 15% normal goat serum. The sections were then incubated with antibody 9B11 diluted 1:5000 in TBS-T (TBS containing 0.3% Triton X-100) with 10% normal goat serum for 16 h at 4 °C, washed in TBS-T, and then incubated with biotinylated goat anti-rabbit Igs (Vector Laboratories) diluted in TBS-T/10% normal goat serum. Following washing in TBS, the sections were further incubated for 2 h in avidin-biotin-peroxidase complex in TBS (Vectastain, Vector Laboratories), washed in TBS, and developed with 0.05% diaminobenzidine tetrahydrochloride and 0.001% H_2O_2 in TBS. The sections were mounted on gelatin-chrome alum-coated Superfrost slides, air-dried overnight, cleared in xylene, and coverslipped with DPX (Merck).

To detect Aβ deposits, the brains were fixed as above, embedded in paraffin wax, and stained as described previously using antibody 1E8 that detects residues 13–27 of Aβ (43). Briefly, 10-μm coronal sections through the cortex and hippocampus of APPswe and APPswe/X11α double transgenic mice were deparaffinized, rehydrated, and then treated with 85% formic acid for 8 min. Following washes and incubation with primary and secondary antibodies, reactivity was developed with Vectastain Elite ABC solution (Vector Laboratories) and diaminobenzidine tetrahydrochloride with nickel enhancement, and the sections were dehydrated and mounted in DPX.

The images were captured on a Zeiss Axioscope 2 MOT using an Axiocam and Axiovision software (Zeiss). The diameters of plaques were determined using Metamorph image analysis software.

Aβ Assays—Aβ species were assayed using commercial ELISA assay kits. Aβ(1–40) was assayed using human amyloid β(1–40)-(N) ELISA (IBL, Hamburg, Germany) and Aβ(1–42) detected using Innotest β-amyloid$_{(1-42)}$ ELISA (Innogenetics, Ghent, Belgium). Brain samples were prepared for assay by homogenization as 20% homogenates using a Dounce homogenizer as described (44). The protein concentrations were determined using Bradford assays. The data were analyzed by one-way analysis of variance tests.

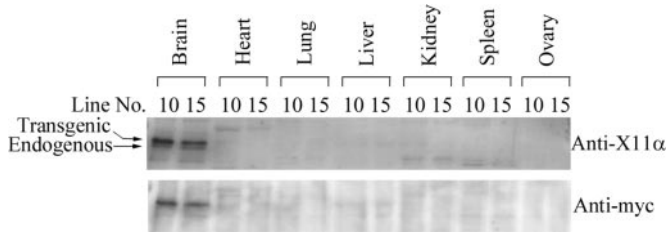

FIG. 1. **Expression of X11α in transgenic mouse tissues.** Immunoblots with antibodies to X11α (*upper panel*) and 9B11 to the Myc tag (*lower panel*) reveal that transgene-derived X11α is expressed principally in the brain. The transgenic X11α migrates slightly slower than endogenous mouse X11α because of the Myc tag. Samples from X11α transgenic lines 10 and 15 are shown. 15 μg of protein is loaded in each track.

RESULTS

We created X11α transgenic mice using the mouse prion gene promoter and regulatory elements to drive expression as previously described for other genes (38). To facilitate detection of transgene-derived X11α, we placed a Myc epitope tag on the carboxyl terminus of the protein. We obtained five transgenic founders, were able to breed from four of these, and selected the two highest level expressors as determined by immunoblot analyses (lines 10 and 15) for further study. Immunoblotting with an X11α antibody and antibody 9B11 to the Myc tag revealed that transgenic X11α protein was expressed principally in the brain, which is in agreement with previous studies that have utilized the prion promoter (38) (Fig. 1). Comparisons of X11α expression in the brains of transgenic and nontransgenic littermate mice by densitometric analyses of immunoblots revealed that X11α was overexpressed ~8-fold in line 10 and 6-fold in line 15 (Fig. 2*A*).

To analyze transgenic X11α expression in more detail, we immunostained sections of transgenic and nontransgenic mouse brains with antibody 9B11 to the Myc tag. These studies revealed that transgenic X11α protein was distributed in a similar, if not identical fashion in both transgenic lines where Myc immunoreactivity was present in neurons in a diverse number of brain regions including the cortex, hippocampus, and scattered subcortical nuclei but with no obvious expression in cells with glial morphology (Fig. 3). Myc immunoreactivity was present in apical dendrites in the cortex and hippocampus but was particularly prominent in neuronal cell bodies and proximal dendrites, and this is in agreement with studies of endogenous X11α subcellular distribution where it has been shown to be enriched in the *trans*-Golgi network; APP is also located in these regions (30, 45). Thus, transgenic X11α expression broadly mimics that of the endogenous protein.

To investigate the effect of X11α overexpression on APP and Aβ production in the brain, we crossed each of the selected X11α transgenic lines with transgenic mice expressing a familial Alzheimer's disease mutant APP harboring the double K670N/M671L Swedish mutation (transgenic APPswe mice line Tg2576). These APPswe mice secrete increased levels of human Aβ(1–40) and Aβ(1–42) (39). This approach of crossing Alzheimer's disease mutant APP mice with other transgenics to investigate the effect of a particular transgene on Aβ production has now been utilized in a number of studies. These include investigating the effects of wild-type and mutant presenilin 1 and β-secretase on Aβ production (38, 46–49) (see reviews in Refs. 50 and 51). Analyses of the offspring derived from these crosses revealed that their genotypes approximated that expected of Mendelian inheritance (one-quarter each nontransgenic, APPswe transgenic, X11α transgenic, and APPswe/X11α double transgenic). There was no evidence of any gross abnormal phenotype in any of the mice.

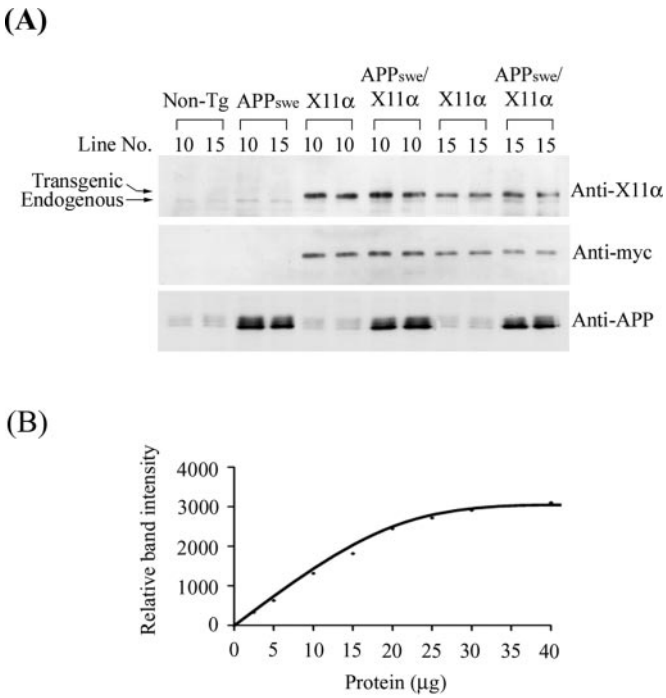

FIG. 2. **Expression of X11α and APP protein in the brains of mice derived from crosses between APPswe Tg2576 mice and X11α lines 10 and 15 mice.** *A* shows immunoblots of two animals of each genotype probed with an antibody to X11α, antibody 9B11 to the Myc tag on transgenic X11α, and an antibody to APP as indicated. 10 μg of protein is loaded in each track. Samples from other mice were also analyzed and produced identical results; all of the animals assayed for Aβ were probed for APP. *B*, quantification of APP signal on immunoblots of total protein from the brains of APPswe Tg2576 mice. *Non-Tg*, nontransgenic.

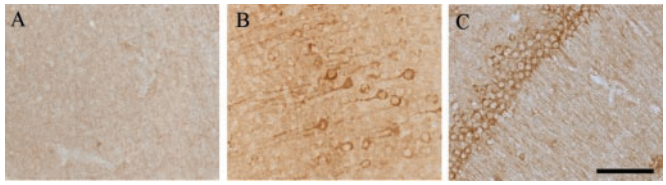

FIG. 3. **Histological detection of transgene-derived X11α in transgenic mice.** Transgenic X11α was detected using monoclonal antibody 9B11 to the Myc tag. *A* shows labeling of the cortex of a nontransgenic mouse; *B* shows labeling of the same region of an X11α transgenic mouse. Myc immunoreactive neurons were present in laminae III and V of somatosensory cortex of transgenic mice, with prominent staining of the soma and apical dendrites. Myc immunoreactive neurons (cell bodies and dendrites) were also present in the hippocampus of transgenic mice (*C*). *Scale bar*, 50 μm.

Northern blots demonstrated that overexpression of X11α had no detectable effect on the steady-state levels of either endogenous mouse or transgenic human APP mRNAs (Fig. 4). To determine whether overexpression of X11α influenced APP holoprotein levels, we performed quantitative immunoblots for APP on the different brain samples. We first generated standard curves for APP signal on the blots (Fig. 2*B*) and, by loading amounts of protein (10 μg) that fell within the linear range for this signal, analyzed the relative APP levels in the different mice generated from the various crosses. Statistical analyses of these data by one-way analysis of variance tests revealed that X11α had no effect on the steady-state levels of either endogenous mouse or transgenic human APP protein (all data not shown but see Fig. 2*A* for examples of signals obtained from nontransgenic, APPswe, X11α, and APPswe/X11α transgenic mice).

We next compared the levels of human Aβ(1–40) and Aβ(1–42) in the brains of APPswe and APPswe/X11α double transgenic mice aged between 10 and 12 weeks. For X11α line 10 transgenic crosses, we observed a significant, 24% reduction in Aβ(1–40) levels in APPswe/X11α double transgenics compared with APPswe only littermates ($p = 0.0015$). For X11α line 15 transgenic crosses, we again observed a significant 17% reduction in Aβ(1–40) levels in APPswe/X11α double transgenics compared with APPswe littermates ($p = 0.0331$) (Fig. 5). The more marked reduction of Aβ(1–40) levels in X11α line 10 is consistent with the higher levels of X11α protein detected in these animals. Analyses of Aβ(1–42) levels in the mice revealed no significant differences between any genotypes. Thus, overexpression of X11α reduces Aβ(1–40) but not Aβ(1–42) levels in the brains of the APPswe Tg2576 transgenic mice, and this effect is not mediated via a detectable effect on the steady-state levels of APP mRNA or protein.

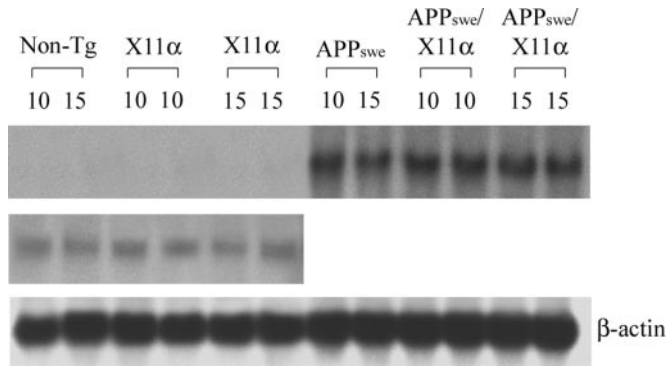

FIG. 4. **Northern analyses of APP mRNAs in the brains of mice derived from crosses between APPswe Tg2576 mice and X11α lines 10 and 15 mice.** APP mRNA is greatly overexpressed in the APPswe mice, which makes simultaneous detection of endogenous and transgenic mRNAs difficult because of their similar electrophoretic mobilities. Two different exposures of the relevant portions of the blot are thus shown. Samples from two animals of each genotype are shown, but samples from other mice were also analyzed and produced identical results. The *bottom panel* shows β-actin mRNA to demonstrate equal loading of the gel. *Non-Tg*, nontransgenic.

Finally, we analyzed Aβ deposition in the brains of APPswe and APPswe/X11α double transgenic mice. For these experiments we focused on transgenic line 10, which expressed the highest level of X11α. At 7–8 months of age, APPswe Tg2576 develop Aβ plaque deposits similar to those seen in Alzheimer's disease (40). Because this age is a key time point in the development of Alzheimer's pathology, we analyzed 8-month-old animals. Coronal sections through the cortex and hippocampus of APPswe and APPswe/X11α double transgenic mice were labeled for Aβ deposits using antibody 1E8, and the number of plaques/section was counted. Statistical analyses of these data revealed a significant reduction in the number of Aβ plaques in the APPswe/X11α double transgenic mice (Fig. 6). We also compared the size of plaques in APPswe and APPswe/X11α double transgenic mice and discovered that the mean diameter of plaques was reduced in the APPswe/X11α animals (mean diameter of plaques in APPswe mice, 28.3 μm; mean diameter of plaques in APPswe/X11α mice, 15.5 μm). Statistical analyses of these data revealed a significant difference ($p = 0.027$ by one-way analysis of variance test). Thus, the number and size of amyloid plaques in the cortex/hippocampus are reduced in APPswe/X11α mice.

DISCUSSION

The X11s are a family of adaptor proteins comprising three members, X11α, X11β, and X11γ, all of which bind to the carboxyl terminus of APP (6–8, 10, 12, 13, 16, 52). X11α and X11β are neuron-specific, whereas X11γ is ubiquitously ex-

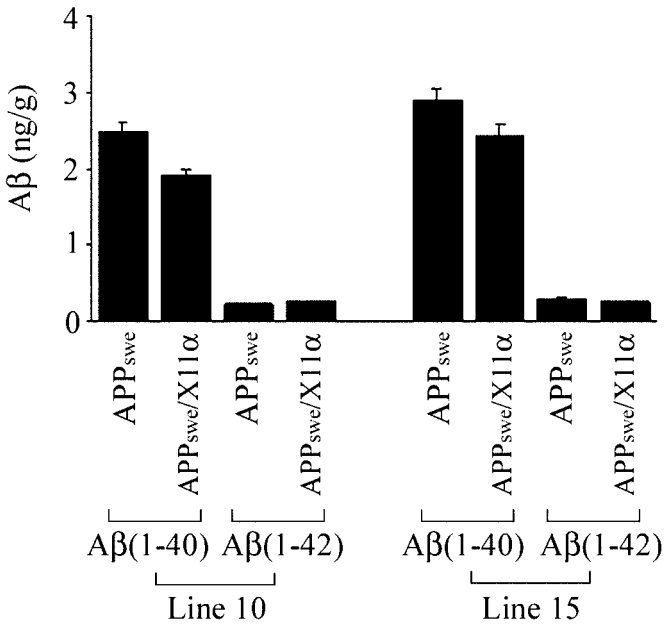

FIG. 5. **Aβ levels in the brains of mice derived from crosses between APPswe Tg2576 mice and X11α lines 10 and 15 mice.** Assays were performed on 11 APPswe and 13 APPswe/X11α (line 10 crosses) and 13 APPswe and 15 APPswe/X11α (line 15 crosses). All of the mice analyzed were aged between 10 and 12 weeks. Aβ(1–40) levels are reduced by 23.6% in X11α line 10 mice ($p = 0.0015$) and by 16.7% in X11α line 15 mice ($p = 0.0331$). Pooling data from both X11α line 10 and 15 mice also demonstrated a significant reduction in Aβ(1–40) levels ($p = 0.0008$). No significant differences in Aβ(1–42) levels were seen in X11α line 10 or 15 mice nor in data obtained from pooling of figures. The *error bars* indicate the S.E. The mouse to mouse variation in Aβ levels between mice of the same genotype was less than 17%. Normalizing Aβ levels to relative APP holoprotein levels did not significantly alter the results.

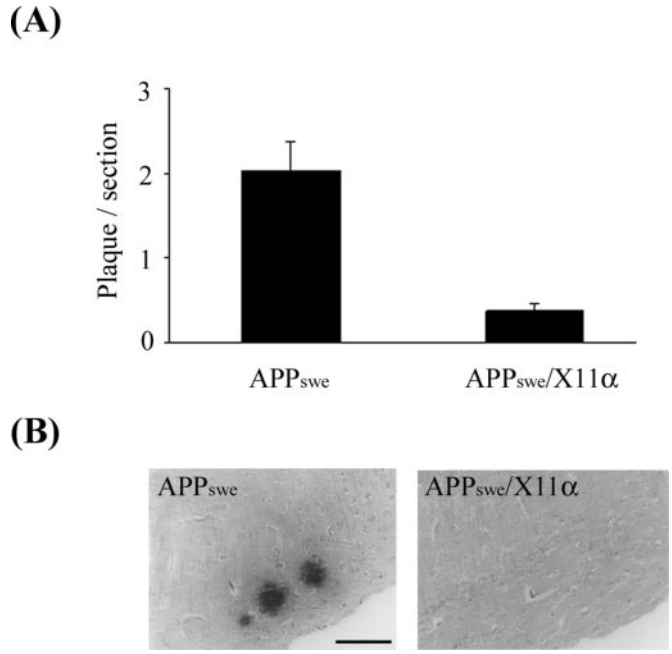

FIG. 6. **Aβ plaque deposition in the brains of mice derived from crosses between APPswe Tg2576 mice and X11α line 10 mice.** *A* shows the number of Aβ plaques in APPswe and APPswe/X11α mice ($n = 4$). The data were obtained from 27 coronal sections from each mouse cut through the cortex/hippocampal regions. Every tenth section was analyzed so each section was separated from its neighbor by ~100 μm. Aβ plaque numbers were significantly fewer in APPswe/X11α compared with APPswe mice ($p < 0.001$ analyzed by one-way analysis of variance test). The *error bars* indicate the S.E. *B* shows Aβ plaques in APPswe and APPswe/X11α mice. *Scale bar*, 100 μm.

pressed (13, 21–23, 41, 52). Both X11α and X11β have now been shown to stabilize APP and inhibit Aβ secretion in transfected cells (13, 16, 31, 32, 41), although there is currently no evidence to demonstrate that any X11 protein modulates APP processing and Aβ secretion *in vivo* in the brain. Here, we demonstrate that overexpression of X11α reduces Aβ levels and Aβ plaque numbers in the brains of APPswe transgenic mice.

Our analyses revealed that whereas Aβ(1–40) levels were significantly reduced in APPswe/X11α mice, we observed no such changes in Aβ(1–42) levels. Whether this remains the case in older mice in which Aβ(1–42) levels are 1000-fold higher (40) will require analyses of aged animals. Alternatively, X11α may have a selective effect on Aβ(1–40) production at all ages because the related X11β has been shown to inhibit secretion of Aβ(1–40) but not Aβ(1–42) in transfected cells (13). However, it is notable that even in the relatively young animals analyzed here, we see a marked reduction by ~20% in the levels of Aβ(1–40), and this is sufficient to reduce the number of Aβ plaques that are deposited in 8-month-old APPswe/X11α mice. This reduction in Aβ plaque numbers was more marked than the 24% reduction in Aβ(1–40) levels would predict, and this might suggest that overexpression of X11α acts to delay Aβ deposition. Indeed, the age at which Aβ deposition was analyzed (8 months) is a key time point in the development of Alzheimer's disease pathology in the APPswe Tg2576 model and one where Aβ plaque deposits are first observed (40). In a contrasting fashion, crossing of APPswe Tg2576 mice with Alzheimer's disease mutant presenilin 1 transgenics accelerates Aβ plaque formation at this age (46, 49). Thus, it will therefore be interesting to examine Aβ deposition in older and aged APPswe/X11α mice so as to establish the full profile of pathological changes. Additionally, because APPswe mice display spatial memory deficits (39, 53), it will be important to examine whether overexpression of X11α also reduces Aβ-associated cognitive changes by appropriate behavioral experimentation.

The mechanisms by which X11α inhibits Aβ production are not clear. In cell transfection studies, overexpression of X11α is accompanied by increased stability of full-length APP (16, 31–33), but we did not detect any changes in APP mRNA or protein levels in the presence of X11α in our transgenic mice. However, these effects of X11α on APP in the highly overexpressing transfected cells are more marked than those in our transgenic lines (*e.g.* Aβ production is decreased by ~70% in one study (31). In this context, it is notable that modulating presenilin 1 expression in transgenic mice alters Aβ production without a detectable effect on APP levels (54, 55).

Presenilin 1 is required for the proteolytic processing of APP to release Aβ (54, 56–59). Interestingly, we have demonstrated that X11α binds to presenilin 1 via its PDZ domains to facilitate the formation of APP-X11α-presenilin 1 trimeric complexes (28), and this interaction has now been confirmed by others (30). X11α may therefore modulate APP processing and Aβ production via some effect on presenilin 1 that inhibits γ-secretase activity.

Whatever the precise mechanisms by which X11α influences APP processing, our results demonstrate that altering its expression can reduce Aβ levels *in vivo* in the brain. As such, modulation of X11α function may represent a novel therapeutic strategy for Alzheimer's disease.

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