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β-Amyloid Precursor Protein-Deficient Mice Show Reactive Gliosis and Decreased Locomotor Activity

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Summary

In several pedigrees of early onset familial Alzheimer's disease (FAD), point mutations in the β-amyloid precursor protein (APP) gene are genetically linked to the disease. This finding implicates APP in the pathogenesis of Alzheimer's disease in these individuals. To understand the in vivo function of APP and its processing, we have generated an APP-null mutation in mice. Homozygous APP-deficient mice were viable and fertile. However, the mutant animals weighed 15%–20% less than age-matched wild-type controls. Neurological evaluation showed that the APP-deficient mice exhibited a decreased locomotor activity and forelimb grip strength, indicating a compromised neuronal or muscular function. In addition, four out of six homozygous mice showed reactive gliosis at 14 weeks of age, suggesting an impaired neuronal function as a result of the APP-null mutation.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that disproportionately affects the population over 65 years of age. It is characterized pathologically by the presence of senile plaques, neurofibrillary tangles, and extensive neuronal cell loss. Senile plaques are extracellular deposits of heterogeneous substances of which the major component is a 39–43 amino acid peptide referred to as β-amyloid peptide or Aβ (Glenner and Wong, 1984). The 4 kDa Aβ peptide is derived by proteolytic cleavage of a larger β-amyloid precursor protein (APP; Kang et al., 1987). APP is an integral membrane glycoprotein consisting of an extracellular domain, a single transmembrane domain, and a short cytoplasmic tail. About two-thirds of the Aβ peptide is extracellular, and the remaining sequence is embedded in the membrane (Selkoe, 1993). The APP gene, located on the long arm of chromosome 21 (Goldgaber et al., 1987), spans approximately 400 kb (at least 18 exons; Yoshikai et al., 1990; Lamb et al., 1993). Alternative splicing generates APP mRNAs encoding several isoforms that range from 365 to 770 amino acid residues (Kosik, 1993). The major Aβ peptides encoding proteins measure 695, 751, and 770 amino acids (referred to as APP695, APP751, and APP770). APP751 and APP770 contain a domain homologous to the Kunitz-type serine protease inhibitors (KPIs; Kitaguchi et al., 1988; Ponte et al., 1988) that is absent in APP695. APP695 is expressed predominantly in neurons. APP751 and APP770 can be detected in all tissues examined (Neve et al., 1988). APP is also expressed during early mouse embryonic development (Fisher et al., 1991).

APP undergoes at least three different processing pathways. In the constitutive secretory pathway, the molecule is cleaved within the Aβ region, thus preventing β-amyloid formation (Esch et al., 1990; Sisodia et al., 1990). Alternatively, APP can be internalized from the cell surface and targeted to the endosomal–lysosomal compartment, where APP fragments containing the intact Aβ are generated (Golde et al., 1992; Haass et al., 1992). Finally, the Aβ peptide is normally secreted from cultured cells and is found in cerebrospinal fluid (CSF) of patients with AD as well as in that of healthy individuals (Selkoe, 1993). The functions of APP and Aβ in vivo are still uncertain. APP has been implicated as a growth factor in fibroblast cultures (Saitoh et al., 1989), as a mediator of cell adhesion and neurite outgrowth (Schubert et al., 1989; Milward et al., 1992), as a regulator of intraneuronal calcium (Mattson et al., 1993), and as a neuronal receptor regulating the GTP-binding protein G_o (Nishimoto et al., 1993). The Aβ peptide has been shown to have neuroprotective and neurotoxic actions, dependent on the cell line and protein preparations tested (Kosik and Coleman, 1992). The fact that intraneuronal expression of Aβ peptide in transgenic mice leads to neurodegeneration and apoptosis strongly suggests that Aβ is neurotoxic in vivo (LaFerla et al., 1995). The KPI domain may serve a role in regulating protein half-life (Van Nostrand et al., 1990; Miyazaki et al., 1993).

Abnormal regulation and misprocessing of APP may contribute to AD. In Down's syndrome (DS) patients, the APP gene is overexpressed, owing to the presence of an extra copy of a portion of chromosome 21 encoding the APP gene. Individuals with DS who live into their thirties invariably develop early onset AD pathology (Mann, 1989), suggesting that an increase in APP gene dosage may contribute to AD in these patients. In addition, five different mutations have been identified in the human APP (hAPP) gene, linking the hAPP gene to early onset familial AD (FAD) and cerebral hemorrhage in several unrelated fami-

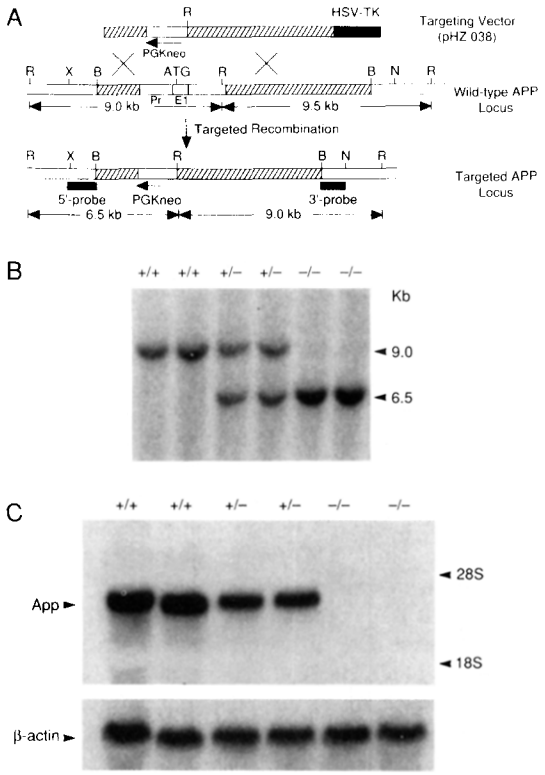

Figure 1. Generation of APP-Deficient Mice

(A) Targeted disruption of the APP gene in ES cells. The targeting vector (pHZ 038) contains the following, from left to right: a 1.4 kb segment preceding the APP promoter (hatched 5′ rectangle); a PGK–*neo* expression cassette inserted in an orientation opposite to that of the APP gene; a fragment of 7.1 kb homologous to the first intron of the APP gene (hatched 3′ rectangle); and an *MC1-TK* gene for negative selection (Mansour et al., 1988; labeled HSV-TK, shown as a closed box). Probes used (shown as black boxes below the physical map) for detecting a targeted event were the following: a 1.0 kb XbaI–BglII (5′ probe); a 0.8 kb BglII–NcoI (3′-probe); and the *neo* sequence. Digestion with the restriction endonuclease EcoRI was used to separate the wild-type and the targeted APP alleles. R, EcoRI; X, XbaI; B, BglII; N, NcoI. Pr, mouse APP promoter; E1, exon 1 of the mouse APP gene; PGK, phosphoglycerate kinase promoter.

(B) Southern blot analysis of representative offspring from heterozygous matings. Genomic DNA isolated from the tails of 2-week-old pups generated from crosses of heterozygous mice was digested with EcoRI, transferred to membranes, and hybridized with the 5′ probe. (+/+), wild-type; (+/−), heterozygotes; (−/−), homozygous APP-deficient mice.

(C) APP gene expression. Total RNA (20 μg) from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) APP mice was isolated (two mice each) from brain and kidney (data not shown) by using the RNAzol B method (Biotecx Laboratories, Incorporated) and hybridized with the full-length *APP695* cDNA sequence. APP mRNA is absent in (−/−) mice in both tissues. Lower panel, mouse β-actin cDNA hybridization for loading control.

lies (Mullan and Crawford, 1993). These affected families provide the strongest evidence yet for the notion that APP processing and Aβ peptide accumulation serve a central role in AD progression. Indeed, in vitro studies have shown that these mutations alter APP processing to generate increased levels of Aβ (Citron et al., 1992; Cai et al., 1993) or to produce longer forms of Aβ peptides, which are potentially more amyloidogenic (Suzuki et al., 1994).

We have generated a complete deficiency of APP in mice by homologous recombination in embryonic stem (ES) cells. Neither APP mRNA nor protein can be detected in the mutant animals, and the mice show reactive gliosis and behavioral abnormalities.

Results

Generation of APP-Deficient Mice

We inactivated the mouse APP gene by deleting a 3.8 kb sequence encoding its promoter (Izumi et al., 1992) and first exon, which encodes the ATG translation initiation codon and the signal peptide of the APP (Figure 1A). The linearized targeting vector (pHZ 038) was electroporated into AB2.1 ES cells, and transfected cells were selected in the presence of G418 and 1-(2′-deoxy, 2′-fluoro-β-δ-arabinofuranosyl)-5-iodouracil (FIAU). A 4.4-fold enrichment was achieved by FIAU counter selection as compared with G418 selection alone. Double-resistant colonies were expanded and screened by a mini-Southern blot protocol (Ramírez-Solis et al., 1992). A total of six targeted clones were identified from 193 double-resistant colonies (a targeted recombination versus random integration event of 1 in 140). The 5′ and 3′ probes used for Southern blot analysis of the targeted locus immediately flanked the targeting sequences (Figure 1A). The expected polymorphic EcoRI restriction enzyme fragments were detected for the wild-type (9.0 kb) and the targeted (6.5 kb) APP alleles with the 5′ probe (Figure 1B) and 3′ probe (data not shown).

A total of seven male chimeras were produced from two of the ES clones (numbers 76 and 174), with ES cell contributions ranging from 60% to 100%, as judged by the percentage of agouti coat color in the chimeric mice. Successful germline transmission of ES cells was achieved with both clones, and approximately 50% of the agouti pups contained the disrupted APP allele. Heterozygous mice were indistinguishable from their normal littermates. To produce mice homozygous for the disrupted APP gene, cross-matings between heterozygous mice were set up. Normal litter sizes were observed, and the genotypes of the resulting pups were analyzed (Figure 1B). Of 87 mice analyzed at 2 weeks of age, 30 (34%) were wild type (+/+), 36 (41%) were heterozygous (+/−), and 21 (24%) were homozygous (−/−) for the mutated APP allele, representing a normal 1:2:1 Mendelian inheritance of the mutated APP allele. This result ruled out an essential function of APP in mouse embryogenesis. The homozygous APP knockout mice appeared normal and healthy up to 6 months of age.

Molecular Characterization of APP-Deficient Mice

To determine whether the APP gene is completely inactivated following the deletion of its promoter and first exon, Northern blot analysis was performed to determine APP mRNA levels with brain and kidney RNA, using *APP695* cDNA as a probe. APP mRNA could not be detected in mice homozygous for the targeted allele. The APP message was reduced by approximately 50% in the heterozygous mice compared with amounts in wild-type controls (Figure 1C). Western blot analysis using an APP-specific

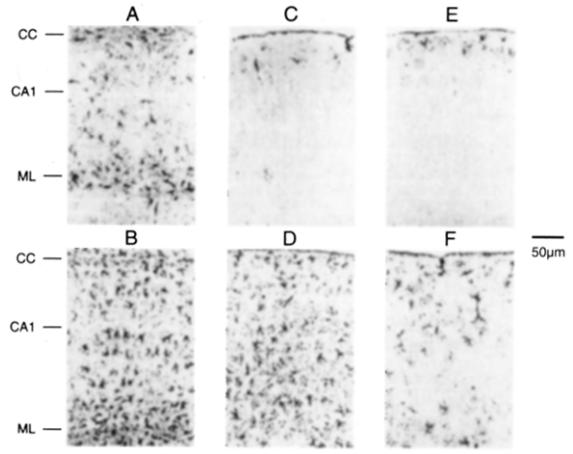

Figure 2. Astrogliosis in the Brains of APP-Null Mice

Representative photomicrograph of GFAP-immunostained coronal brain sections through the hippocampus ([A], +/+; [B], −/−), entorhinal cortex ([C], +/+; [D], −/−) and parietal cortex ([E], +/+; [F], −/−) of a wild-type (+/+) and an APP-deficient (−/−) mouse. CC, cerebral cortex; CA1, hippocampal field 1; ML, molecular layer.

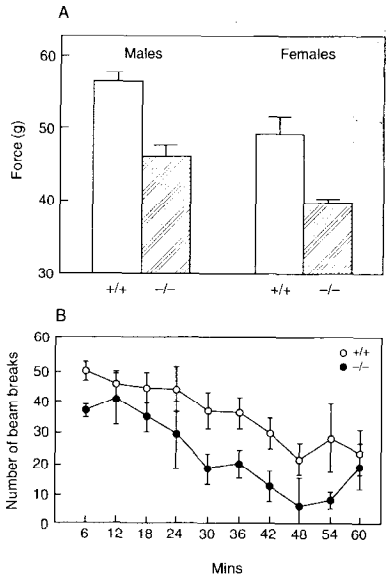

Figure 3. Neurological Evaluation of APP-Deficient Mice

(A) Reduced forelimb grip strength in homozygous APP knockout mice ($p < 0.05$) (males, n = 8; females, n = 7). (+/+), wild-type controls; (−/−), homozygous APP-null mice. See Experimental Procedures for details.

(B) Reduced locomotor activity in APP homozygous knockout mice ($p < 0.001$). The spontaneous motor activity of APP-deficient mice ([−/−], n = 8 males and 7 females) and their age- and sex-matched controls ([+/+], n = 8 males and 7 females) are shown at successive 6 min time intervals during the 60 min session.

antibody (CT15) could not detect any APP protein in brain and other tissues of the homozygous APP knockout mouse (data not shown).

In situ hybridization was carried out with different ^{35}S-labeled oligonucleotide probes, each specific for the mouse *APP695*, *APP751*, and *APP770*. Although APP mRNA was expressed throughout the brain in the wild-type mouse, it could not be detected in any brain region analyzed in the homozygous (−/−) APP knockout mouse (data not shown). We can thus exclude that local regions of gene expression, below the sensitivity of Northern blot analyses, were present in the brain.

Neuroanatomical and Histopathological Analysis of APP-Deficient Mice

Since APP is expressed at the highest level in the brain, immunohistochemical studies were performed on two 6-week-old and two 14-week-old APP-null mice to check for developmental or other neuroanatomical changes. Immunostaining of brain sections from wild-type animals with the N-terminal APP antibody 22C11 showed normal homogeneous APP immunoreactivity in neurons in all areas of the cortical mantle and in the hippocampus and cerebellum, which have the highest densities of APP staining (data not shown). Immunoreactive APP neurons could not be detected in a total of four homozygous APP-null mice in any of the above outlined brain regions (data not shown). Hematoxylin- and eosin-stained brain sections from these APP-null mice did not reveal evidence for cellular abnormalities in cortical, hippocampal, or cerebellar neurons in terms of swelling, atrophy, increase in basophilic material, or granulovacuolations (data not shown).

When the above mice and four additional mice at 14 weeks of age were stained with antibodies for the glial fibrillary acidic protein (GFAP), four out of six homozygous mutants showed reactive gliosis (Figure 2). In the brains of wild-type animals, a low level of GFAP immunoreactivity was principally localized in the hippocampus and layer 1 of the cerebral cortex (Figures 2A, 2C, and 2E). In contrast, brain sections from APP-null mice showed greatly enhanced GFAP immunoreactivity and typical reactive astrocytes with thicker and more numerous processes in the hippocampus and various parts of the neocortex (Figures 2B, 2D, and 2F). In the hippocampus, extensive astrogliosis was seen in the CA1 region and in the molecular layer (ML), while in the neocortex, reactive gliosis was observed throughout the cortical layers. The onset of reactive gliosis may be age dependent, since we had not observed this in younger animals. However, a detailed time course study has to be performed to determine the age of onset.

A total of six knockout mice and four controls (10 weeks old) were also processed for a detailed histopathological evaluation of brain and other tissues. A summary of the tissues examined appears in the Experimental Procedures in the section describing gross and microscopic analysis. Abnormal structures could not be found in any of the tissues examined in the APP-null mice.

Neurological Evaluation of APP-Deficient Mice

Although homozygous APP mutant mice were apparently healthy and fertile, the body weight of both males and females was 15%–20% less at all ages compared with that of their age-matched controls. For example, at 9 weeks of age, the average body weights of male and female wild-type mice were 28.7 ± 1.1 g and 23.6 ± 0.9 g, respectively, while the corresponding APP-deficient mice mea-

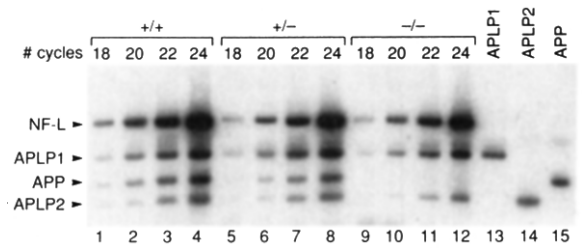

Figure 4. Quantitative RT–PCR Analysis of APP Homologs

Hemibrain RNA from wild-type ([+/+], lanes 1–4), heterozygous ([+/−], lanes 5–8), and homozygous ([−/−], lanes 9–12) APP knockout mice were reverse transcribed and PCR amplified at 18 (lanes 1, 5, and 9), 20 (lanes 2, 6, and 10), 22 (lanes 3, 7, and 11), and 24 (lanes 4, 8, and 12) cycles, respectively. The NF-L was used as amplification control. Control plasmids for *APLP1* (lane 13), *APLP2* (lane 14), and APP (lane 15) were also PCR amplified as molecular weight standards, which show sizes of 500 bp, 150 bp, and 250 bp, respectively.

sured 23.0 ± 0.3 g and 18.3 ± 0.3 g, respectively (n = 10 per sex group). A group of eight male and seven female knockout mice and the same number of age- and sex-matched controls were subjected for preliminary neurological examinations.

Determination of the forelimb grip strength of both the male and female knockout mice showed a significantly reduced grip strength compared with that of age- and sex-matched controls (Figure 3A; $p < 0.05$). The locomotor activity was measured as the total number of beam breaks in successive 6 min intervals for a total of 60 min. Since sex had no effect on these data, ($p = 0.203$), the data were collapsed across this variable. The APP-deficient mice showed a decreased locomotor activity compared with that of wild-type controls (Figure 3B; $p < 0.001$). The total activity in the 60 min measurement for the control and APP knockout mice measured 660 ± 54 beam breaks and 410 ± 52 beam breaks, respectively.

Expression of APP Homologs in APP Knockout Mice

Two APP homologs, amyloid precursor-like protein 1 (APLP1) and APLP2, have been identified in mouse and human (Wasco et al., 1992; Wasco et al., 1993; Slunt et al., 1994). The homologs share significant similarity in their amino acid sequence, and several potentially functional domains are conserved. Like APP, both members of the APP family are expressed at high levels in the brain. The expression pattern of APLP2 in the brain is similar to that of APP (Slunt et al., 1994). Therefore, the expression of APP homologs could theoretically compensate for the loss of the APP in the mutant mice. Direct evidence for this could not be obtained, since quantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis of RNA levels of both homologs did not reveal significant differences in APLP1 or APLP2 expression when compared among the wild-type, heterozygous, and homozygous mice (Figure 4). This result was further confirmed by Northern blot and in situ hybridization analysis (data not shown).

Discussion

Inactivation of the 400 kb APP gene, which undergoes alternative splicing, requires a careful design of the targeting vector to assure that an APP-null mutation can be generated. We chose to delete the APP promoter and first exon, with the assumption that the mutation would prevent APP gene transcription. If an alternative promoter and ATG translation initiation codon were used, transport of the protein to the membrane should be impaired, since the signal peptide was deleted. As a result, we generated a complete deficiency for APP at the mRNA and protein levels. Targeting the promoter and ATG translation initiation codon may serve as an approach to inactivate relatively large genes for which a deletion of the entire gene is impractical.

In this report, we show that the APP-deficient mice were 15%–20% underweight compared with age-matched controls, exhibited behavioral abnormalities, and exhibited significant reactive astrocytosis. Reactive gliosis occurs in many disease states and in response to neuronal injury (Eddleston and Mucke, 1993). The fact that four of six homozygous APP-deficient mice showed reactive gliosis at 14 weeks of age suggests that an impairment of neuronal function may occur as a result of the loss of APP. Indeed, APP and its derivatives have been shown to be involved in the regulation of neurite outgrowth through interactions with neuronal cell adhesion molecules and extracellular matrix proteins (Koo et al., 1993; Small et al., 1993, 1994) and through the regulation of intracellular Ca^{2+} levels (Mattson et al., 1993). However, since APP is also expressed in nonneuronal cells and is involved in numerous extraneuronal activities in vitro (Mattson et al., 1993), and since we did not observe any neuronal cell damage or loss in the brains of APP-deficient mice, the mechanisms responsible for the reactive gliosis in these mice are not clear at present.

Decreased body weight in the mutant animals correlated with a decreased food and water intake. Wild-type animals of varying ages and weights show similar grip strength and locomoter activity, thus indicating that body weight is not a major determinant of these behavioral parameters (data not shown). These results indicate that neuronal or muscular function may be compromised in the mutant animals. These findings appear consistent with published evidence that APP may play a role in the process of nerve and muscle degeneration and regeneration: increased expression of APP mRNA has been shown in motor neurons taken from rats following peripheral nerve axotomy (Sola et al., 1993) and in regenerating human muscle fibers from patients with neuromuscular diseases (Sarkozi et al., 1994).

APP is conserved among mammalian species (Shivers et al., 1988), and APP homologs have been identified in human (*APLP1*, *APLP2*), mouse (*APLP1*, *APLP2*), Drosophila melanogaster (*Appl*), and Caenorhabditis elegans (*apl-1*) (Rosen et al., 1989; Daigle and Li, 1993). These APP homologs lack the Aβ region. The conservation among members of the APP gene family outlines that they

may be functionally related. Indeed, the behavioral deficit in Drosophila containing a deletion of its APP homolog (*Appl*) can be partially rescued by the *hAPP695* (Luo et al., 1992). Mutant mice with a modified APP (a deletion of exon 2) showed behavioral deficits, the result of which was published during the course of preparation of this manuscript (Müller et al., 1994).

A comparison of the APP-null mice described here with those described by Müller et al. (1994) highlights unique aspects of the role of APP. First, the ability to maintain a normal APP-null litter size shows that the murine APP is either dispensable for development, or that the APP homologs compensate for the loss of APP. Second, in contrast with the APP exon 2 deletion mice generated by Müller et al. (1994), approximately 65% of the APP-null mice over three months of age described here developed reactive gliosis. This could argue for a partial retention of function in the APP exon 2 deletion, preventing reactive gliosis. Third, the modified APP described by Müller et al. (1994) in some instances resembles the null mutation described here, showing significant though less severe reductions in body weight and locomotor activity, again indicating a partial rescue of the APP-null phenotype in the exon 2 deletion mutant. Fourth, agenesis of the corpus callosum could not be detected in the APP-null mice, although mice of the same 129/Sv and C57BL/6 hybrid genetic background were used in both studies. Given that the APP exon 2 deletion mouse exhibits a separate phenotypic change with respect to corpus callosum development, this mutant protein has a distinct dominant effect, which may also have affected other aspects of neuronal development and function.

In attempts to generate a murine model of AD, transgenic mice have been produced that express various forms of the hAPP gene (Price and Sisodia, 1994). Recently, successful AD-like neuropathology was reported in mice overexpressing a mutant form of the hAPP (V717F) gene (Games et al., 1995). A very high level of expression of hAPP was required to produce a mouse with AD-like neuropathology. This does not reflect the APP level in AD patients, and it is unclear whether overexpression of APP itself or β-amyloid plaque deposition is responsible for some of the pathology seen in these mice. Indeed, synaptic loss can occur by overexpressing hAPP alone without plaque formation (Mucke et al., 1994). The requirement for a high level of expression of hAPP in transgenic mice may be explained by, among other factors, interference from the endogenous mouse APP, which has three amino acid differences in the Aβ region compared with human Aβ. APP-deficient mice may therefore serve as a valuable strain for insertion of the hAPP gene in our efforts to generate mouse models of AD, mimicking human physiological conditions.

Experimental Procedures

Construction of the APP-Targeting Vector

The APP promoter region was isolated from a 129 mouse library by using a 1.0 kb HindIII–PvuII fragment from the APP promoter as a probe (Izumi et al., 1992). The targeting vector was constructed by ligating a 1.4 kb BglII–XhoI fragment from the 5′ portion of the APP promoter and a 7.1 kb XhoI–BglII fragment from the 3′ portion of exon 1. A 1.5 kb XhoI–SalI PGK-*neo* cassette (Ramírez-Solis et al., 1993) was inserted at the site of the deletion. The final vector was made by ligating the 2 kb XhoI fragment of pMC-TK (Mansour et al., 1988) to the 3′ end of the 7.1 kb targeting arm.

Gene Targeting in Murine ES Cells

The targeting vector was linearized by digestion with NotI prior to electroporation, leaving the pKS backbone attached to the end of the TK gene. AB2.1 cells (1×10^7) (pg14) and 25 μg of linear DNA were resuspended in phosphate-buffered saline and used for one electroporation, which was carried out by use of a Bio-Rad Gene Pulser apparatus at 230 V and 500 μF. The cells were plated onto five 90 mm dishes with γ-irradiated SNL76/7 feeder cells. G418 (200 μg/ml of active ingredient) and FIAU (0.4 μM) were applied 24 hr and 48 hr later, respectively. The double-resistant colonies were picked 10 days after G418 selection and subjected to mini-Southern blot analysis as previously described (Ramírez-Solis et al., 1992).

Blastocyst Injection and Mouse Breeding

Targeted APP ES clones were injected into C57BL/6J (Jackson Laboratories) recipient blastocysts by using techniques described previously (Bradley, 1987). The injected embryos were reimplanted into the uteri of day 3 pseudopregnant Tac:SW (fBR, Taconic) mice and allowed to develop to term. The male chimeras were bred with C57BL/6J females to assess their potential for germline colonization, which was determined by the presence of agouti pups in the offspring. The (+/+) and (−/−) APP-null breeding colonies were housed in a virus antibody–free (VAF) facility. The care for animals was conducted as approved by the Institutional Animal Care and Use Committee of Merck and Company, Incorporated.

Initial Neurological Evaluation

A blind study was carried out to determine forelimb grip strength and locomotor activity. The grip strength apparatus consisted of a 30 mm metal equilateral triangle (3 mm thickness) secured to a strain gauge transducer (Pioden Controls, Limited). The transducer was connected to an amplifier (Micro Movement, Limited), which in turn was connected to a chart recorder (Pharmacia LKB, model Brommo 2210). To measure forelimb grip strength, mice were held by the tail until they grasped the metal triangle with both forepaws. The animal was then gently pulled away from the transducer, and the maximum force generated was recorded. Three successive readings were taken for each mouse.

Locomotor activity measurements were performed with mice placed in individual activity cages (230 mm × 280 mm × 210 mm). The walls, floor, and ceiling were lined with wire mesh to allow climbing all around the cage. Each cage was equipped with four parallel infrared beams that traversed the front and the back of the cage at floor and ceiling level. Total activity was measured by adding together the number of times each of the four beams was broken.

The data were analyzed by using analysis of variance (ANOVA) followed by Newman–Keuls' multiple comparison test. Analysis of grip strength was performed by using one-way ANOVA; locomotor activity was analyzed by three-way ANOVA using group, sex, and time as factors; and group differences were determined by one-way ANOVA.

Quantitative RT-PCR Analysis of *APLP1* and *APLP2* Expression

RNA was prepared from mouse brain by homogenizing the tissue in guanidinium isothiocyanate and centrifugation through a CsCl cushion. From each RNA preparation, 2 μg was reverse transcribed in the presence of 50 pmol of random hexamer primers (Pharmacia, and Reverse Transcriptase from Bethesda Research Laboratories). For each sample, four tubes each containing one tenth of the RT mixture (equivalent to 200 ng of RNA) were subjected to PCR with 5′-end ^{32}P-labeled APP/APLP sense primer (0.5 pmol), APP/APLP antisense primer at 50 μM, and neurofilament L (NF-L) oligos at 20 μM. The annealing temperature was 58°C. The tubes were removed at 18, 20, 22, and 24 cycles. Previous results had shown that amplification is constant over this cycling range (Slunt et al., 1994). For each control,

0.5 ng of plasmid template was used, and the PCR was performed for20 cycles. Each reaction was subjected to phenol–chloroform extraction, and the PCR products were precipitated with ammonium acetate and ethanol. Resulting PCR products were subsequently digested with BamHI–HindIII–XhoI and fractionated on 2% agarose gels. Gels were dried and exposed to X-ray film. The sequences of the oligo primers for PCR can be obtained on request.

Gross and Microscopic Analyses

Mice were anesthetized with Isoflurane via an open drop method in an enclosed container. While the animals were anesthetized, blood was removed via cardiocentesis, and mice were returned to the container with Isoflurane until euthanized. Mice were subjected to diagnostic necropsies, and all tissues were fixed in 10% buffered formalin. Bones were decalcified by use of formic acid. Sections of the following tissues were prepared by routine methods and stained with hematoxylin and eosin: salivary gland, tongue, esophagus, stomach, small intestine, large intestine, liver, gall bladder, pancreas, adrenal gland, thyroid gland, parathyroid gland, pituitary gland, kidney, urinary bladder, ovary, uterus, vagina, testis, epididymis, seminal vesicle, prostate gland, bulbourethral gland, penis, preputial gland, skin, mammary gland, lung, nasal turbinates, heart, aorta, spleen, lymph node, thymus, skeletal muscle, bone, bone marrow, brain (including cerebral cortex, subcortical white matter, cerebellum, pons, and corpus callosum), cervical spinal cord, sciatic nerve, eye (including optic nerve), harderian gland, and brown adipose tissue.

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