Mice with combined gene knock-outs reveal essential and partially redundant functions of amyloid precursor protein family members

Heber, S; Herms, J; Gajic, V; Hainfellner, J; Aguzzi, A; Rülicke, T; von Kretzschmar, H; von Koch, C; Sisodia, S; Tremml, P; Lipp, H P; Wolfer, D P; Müller, U

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Abstract

The amyloid precursor protein (APP) involved in Alzheimer's disease is a member of a larger gene family including amyloid precursor-like proteins APLP1 and APLP2. We generated and examined the phenotypes of mice lacking individual or all possible combinations of APP family members to assess potential functional redundancies within the gene family. Mice deficient for the nervous system-specific APLP1 protein showed a postnatal growth deficit as the only obvious abnormality. In contrast to this minor phenotype, APLP2(-/-)/APLP1(-/-) and APLP2(-/-)/APP(-/-) mice proved lethal early postnatally. Surprisingly, APLP1(-/-)/APP(-/-) mice were viable, apparently normal, and showed no compensatory upregulation of APLP2 expression. These data indicate redundancy between APLP2 and both other family members and corroborate a key physiological role for APLP2. This view gains further support by the observation that APLP1(-/-)/APP(-/-)/APLP2(+/-) mice display postnatal lethality. In addition, they provide genetic evidence for at least some distinct physiological roles of APP and APLP2 by demonstrating that combinations of single knock-outs with the APLP1 mutation resulted in double mutants of clearly different phenotypes, being either lethal, or viable. None of the lethal double mutants displayed, however, obvious histopathological abnormalities in the brain or any other organ examined. Moreover, cortical neurons from single or combined mutant mice showed unaltered survival rates under basal culture conditions and unaltered susceptibility to glutamate excitotoxicity in vitro.
Mice with Combined Gene Knock-Outs Reveal Essential and Partially Redundant Functions of Amyloid Precursor Protein Family Members

Sabine Heber,1 Jochen Herrms,2 Vladan Gajic,7 Johannes Hainfellner,3 Adrian Aguzzi,3 Thomas Rülicke,4 Hans Kretzschmar,2 Cornelia von Koch,5 Sangram Sisodia,4 Phillippe Trembl,6 Hans-Peter Lipp,8 David P. Wolfe,6 and Ulrike Müller1,2

1Department of Neurochemistry, Max-Planck-Institute for Brain Research, D-60528 Frankfurt, Germany, 2Department of Neuropathology, University of Göttingen, Göttingen, Germany, 3Institute of Neuropathology, and 4Biologisches Zentrallabor, University Hospital, 8091 Zürich. 5Department of Neurobiology, Pharmacology and Physiology, University of Chicago, Chicago, Illinois 60637, and Institutes for 6Anatomy, and 7Molecular Biology, University of Zürich, 8057 Zürich, Switzerland

The amyloid precursor protein (APP) involved in Alzheimer’s disease is a member of a larger gene family including amyloid precursor-like proteins APLP1 and APLP2. We generated and examined the phenotypes of mice lacking individual or all possible combinations of APP family members to assess potential functional redundancies within the gene family. Mice deficient for the nervous system-specific APLP1 protein showed a postnatal growth deficit as the only obvious abnormality. In contrast to this minor phenotype, APLP2−/−/APP−/− and APLP2−/−/APP−/− mice showed no compensatory upregulation of APLP2 expression. APLP1−/−/APP−/−/APLP2−/− mice display postnatal lethality. In addition, they provide genetic evidence for at least some distinct physiological roles of APP and APLP2 by demonstrating that combinations of single knock-outs with the APLP1 mutation resulted in double mutants of clearly different phenotypes, being either lethal, or viable. None of the lethal double mutants displayed, however, obvious histopathological abnormalities in the brain or any other organ examined. Moreover, cortical neurons from single or combined mutant mice showed unaltered survival rates under basal culture conditions and unaltered susceptibility to glutamate excitotoxicity in vitro.

Key words: amyloid precursor protein; amyloid precursor-like protein; knock-out mice; functional redundancy; excitotoxicity; cortical neurons; Alzheimer’s disease

Neurofibrillary tangles and senile neuritic plaques are the major pathological features of Alzheimer’s disease. The predominant constituent of neuritic plaques is the β-amyloid peptide (βA4), proteolytically derived from the larger β-amyloid precursor protein (APP). APP is a member of a larger gene family including the two amyloid precursor-like proteins APLP1 and APLP2 from mammals (Wasco et al., 1992, 1993; Sprecher et al., 1993; Sandbrink et al., 1994; Slunt et al., 1994). Both APLPs are highly homologous to APP and are proteolytically processed in a similar way, leading to the secretion of the large ectodomains (sAPP and sAPLP) (Slunt et al., 1994; Paliga et al., 1997). Using in situ hybridization and RT-PCR analysis, we and others have demonstrated that APP and APLP2 are expressed ubiquitously in largely overlapping patterns during embryonic development and in adult tissue (Slunt et al., 1994; Lorent et al., 1995). In summary, APP/APLP proteins are highly related, are similarly processed, share overlapping domains of expression, and may therefore also be functionally conserved.

Multiple functions have been proposed for APP, mainly based on in vitro experiments (for review, see Mattson, 1997). To address the physiological functions of APP directly, we and others have generated mice carrying a hypomorphic mutation of APP (APPΔ; Müller et al., 1994) and APP-deficient null mutants (Zheng et al., 1995; Li et al., 1996). The phenotypes of these mutants suggested that APP may play a role in neurite outgrowth and the formation of forebrain commissures, postnatal somatic growth and neurobehavioral development, locomotor activity and grip strength, copper homeostasis, and the susceptibility to epileptic seizures and excitotox agents (Zheng et al., 1995; Li et al., 1996; Perez et al., 1997; Steinbach et al., 1998; Trembl et al., 1998; Magara et al., 1999; White et al., 1999a,b). APLP2−/− mice showed no apparent abnormalities, but double mutants obtained by crossing APP−/− mice (obtained from H. Zheng, H. Chen, M. Trumbauer, and L. H. T. van der Ploeg) to APLP2−/− mice were perinatally lethal, suggesting functional redundancy (van Koch et al., 1997).

This study investigates the phenotype of APLP1−/− mice and addresses the question of functional complementation within the gene family by analyzing all possible combinations of double mutants. We show that APP family members serve essential, at least partially redundant functions by demonstrating early postnatal lethality for both APLP2−/−/APP−/− and APLP2−/−/APP−/−/double mutants. In addition, we provide genetic evidence for distinct physiological roles of APP and APLP2, by showing that crosses of the respective single knock-outs with APLP1 knock-out mice result in double mutants of clearly different phenotypes, being either lethal (APLP2−/−/APLP1−/−), or viable (APP−/−/...
APLP1−/−). Moreover, we investigated the presumed neuroprotective role of endogenous APP family members.

MATERIALS AND METHODS

Generation of APLP1−/− embryonic stem cells

Using as a probe a 0.4 kb PsI fragment derived from the 5’-end of the APLP1 cDNA (Wasco et al., 1992) two overlapping Al clones encompassing 19 kb of genomic sequence were isolated from an isogenic AEC1/11 genomic library constructed from AB-1 [129/Sv(ey)] embryonic stem (ES) cells. As judged from hybridization experiments with various oligonucleotides derived from different regions of the APLP1 cDNA and partial sequencing the genomic clones contained the whole coding region including the putative first exon (cDNA positions 9–22) harboring the ATG start codon. Apart from exon1 and exon2 the precise location of the other putative exons was not mapped. The targeting vector pAPL1tag was constructed by inserting a blunted 0.9 kb SacI/BglII fragment lying 2.5 kb upstream of putative exon1 into the NotI site of PTKNEOMSPSA, a slightly modified vector (containing additional restriction sites) derived from the previously described plasmid PTKNEOMS (Ruffner et al., 1993). Subsequently a blunt-ended 4.7 kb XhoI/PvuII fragment was inserted as the “long arm” of homology into the unique ClaI site of the vector. In this construct the neo gene under the control of the phosphoglycerate kinase (PGK) promoter was in antisense orientation to the transcriptional direction of the APLP1 gene and the thymidine kinase (HSV-TK) cassette was inserted at the 3’ end to allow for counterselection. Homologous recombination leads to a ~8 kb genomic deletion containing ~2.5 kb of the promoter region. To determine how much of the coding region would be deleted by gene targeting, hybridization experiments were performed showing that the deleted region contained coding sequences up to approximately position 1000 of the 2.3 kb cDNA, as judged by hybridization to a genomic DNA pool. The targeting vector was subsequently linearized with SacII at the 5’ border of the APLP1 genomic region, electroporated into GS1 ES cells (established by Gerlindie Stark from 129 Sv(ey) mice) grown on irradiated mouse embryos. Electroporated ES cells were cultured in medium containing 20% and calf serum (D20). Colonies resistant to neomycin and 1-(2-deoxy-2-flouro-β-D-arabinofuranosyl) -5-iodoauracil (FIAU) were selected in D20 containing 400 μg of G418 and 0.2 μm FIAU and screened as described (Müller et al., 1994). The primer pair flanking the 3’ end of the Aplp1 cDNA (P3: 5’-ATCCCAGCGCATGCCCTTATCGCC-3’) and a primer corresponding to a genomic sequence 5’ from the targeting vector (UM20: 5’-GGATTCCGCTTGTTCCATGTTACC-3’) were the templates for PCR amplification of DNA colonies. A typical PCR product was ~1.4 kb in size indicating ~20% homology of the PCR products for APLP1−/− mice (65 animals analyzed).

Generation of APLP1−/− mice

ES cells homologous for the mutant APLP1 gene were injected into 3.5-d-old C57BL/6 blastocysts that were transferred into the uteri of pseudopregnant NMRI foster mothers. One of four clones investigated (clone GS-34.4) gave rise to a chimeric male that was mated to C57BL/6 females [and 129 Sv(ey) females for pure genetic background, respectively] and transmitted the mutant allele in the germline as revealed by PCR and Southern blot analysis. Homozygous offspring were intercrossed and animals heterozygous for both loci were backcrossed to 129Sv(ev) mice and obtained as APLP1−/−/− animals (65 animals analyzed).

Animals and PCR genotyping

APLP1 knockout mouse [of either pure 129Sv(ey) or mixed 129Sv(ey)/C57BL/6 genetic background] were initially screened with primers UM20 and P3-neo (5’-AGCGGCTGGAAGATGACCATC-3 and UM31 (5’-ATCCCAGCGCATGCCCTTATCGCC-3’) derived from the targeting vector or from the region replaced by neo, respectively. For the mutant allele a 450 bp product was obtained with primers UM30 and P4-neo (5’-ATGCGGCTGGAAGATGACCATC-3’) derived from the PGKNeo cassette. A typical PCR product was ~1.4 kb in size indicating ~20% homology of the PCR products for APLP1−/− animals (65 animals analyzed).

Generation of combined mutants

Double mutants were generated by three consecutive crosses. Single mutants of APP family members (e.g., APLP1−/− × APLP2−/−) were intercrossed, and animals heterozygous for both loci were backcrossed to single mutants (e.g., APLP1−/− × APLP2−/− × APLP1−/−) to obtain 25% offspring. Homozygous knockout mice of either kind and heterozygous for the other (e.g., APLP1+/− × APLP1−/−) were intercrossed, to obtain double knock-outs in the next generation (e.g., APLP1−/− × APLP2−/− × APLP1−/−) to obtain double APP/APP and APP/APLP mutants the respective analogous crosses were set up, and double knock-outs were generated in the third round of crossing from the following matings: (APP × APP) × (APLP1 × APLP2) or (APP × APLP1) × (APLP2 × APP). Alternatively, to obtain APP/APLP2 and APP/APLP mutants the respective analogous crosses were set up, and double knock-outs were generated in the third round of crossing from the following matings: (APP × APP) × (APLP1 × APLP2) or (APP × APLP1) × (APLP2 × APP). APLP1−/− × APLP2−/− mice were generated by crossing APP−/− mice with APP−/− × APLP2−/− mice.

Southern and Northern blot analysis of APLP1−/− mice

Southern blots were prepared from APLP1 ES cell or tail DNA digested with HindIII or EcoRI, cutting outside of the targeting vector and separated by conventional or pulsed-field gel electrophoresis (1% agarose in 45 mM Tris-borate, 1 mM EDTA run for 15 hr at 63°C, 1200 V, switch time being from 0.5–2 sec in a Bio-Rad (Hercules, CA) DRIII apparatus). Hybridization was performed with a random primer 32P-labeled (Premix; Stratagene, La Jolla, CA) genomic 0.5 kb PsI fragment (Fig. 1, probe B) and a 0.6 kb PsI/XbaI fragment of pGKneo.

For Northern blots, total RNA (prepared as described in Muller et al., 1994) was hybridized with 32P-labeled 490 bp RNA comprising exon 2-17 of the APP gene and were digested with 200 units of HindIII and BamH I and were re-labeled with 32P. Hybridizations were performed at 65°C for 17 hr with wet transfer equipment (Bio-Rad). Filters were blocked for 1 hr in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk. The blot was incubated overnight with the primary antibody (e.g., rabbit antiserum C7-11 at a dilution of 1:2000 in TBST and 1% nonfat dry milk) followed by an 1 hr incubation with HRP-coupled secondary antibody (swine anti-rabbit IgG from Dako (Carpinteria, CA) at a dilution of 1:2500 in TBST and 1% nonfat dry milk and developed by the ECL reaction (Pierce).

Western blot analysis

Mouse organs were homogenized in ~10 vol of homogenization buffer (0.5% SDS and 50 mM Tris-HCl, pH 6.8, 5 mM EDTA, 50 μg/ml peptatin, and 0.25 mM PMSF) per gram of tissue, using a Ultra-Turrax T25 (IKA) mechanical blender. The tissues were homogenized with 200 units of collagenase from Clostridium histolyticum (Boehringer, Mannheim, Germany), sodium-streptomycin (1500, Dako), glibal biliaric acid protein (GFAP, 1:50, Dako), and β-III tubulin (1:2000, Promega) diluted in PBS for 2 hr at room temperature. This was followed by incubation with the secondary antibody (rabbit anti-mouse IgG) diluted 1:500 in PBS for 45 min at room temperature. Bound secondary antibody was detected by using the alkaline phosphatase-anti-alkaline phosphatase complex (APAAP; mouse monoclonal, Dako diluted 1:40 in PBS and incubated for 45 min at room temperature. The alkaline phosphatase activity was visu-
and 25 mM Tris-HCl, pH 7.5] for 60 min at 37°C. After rinsing in exam-
ined under the electron microscope (Zeiss EM 10C). Images were

Ultracut E). Sections were stained with uranyl acetate/lead citrate and

ny). Ultrathin 75 nm sections were cut with an ultramicrotome (Reichert

embedding in Epon 812 substitute (Fluka, Neu-Ulm, Ger-

were dissected and fixed by immersion in 2% glutaraldehyde in 0.1 M

cacodylate buffer, pH 7.4, containing 2% polyvinyl-pyrrolidone (PVP) for

post-fixed in 1% OsO4 in cacodylate buffer followed by dehydration in

95% as determined by immunohistochemical

alized by using Astrapenfuchins (Aldrich, Milwaukee, WI). The sections

counterstained with hemalaun. The immunohistochemical methods

represents restriction sites that were destroyed during cloning.

indicate the direction of transcription. The

Figure 1. Disruption of the APLP1 gene by gene targeting in ES cells. Our targeting strategy was aimed at abolishing transcription and translation by generating an ~8 kb genomic deletion comprising 2.5 kb of the putative promoter, the first exon containing the ATG translational start codon and genomic sequences containing ~50% of the APLP1 coding region. Top, Genomic segment containing the APLP1 locus. Apart from the first and second exon the precise location of the other exons (Exn) was not mapped. The stippled box represents an arbitrary positioned exon corresponding to coding sequences around cDNA position 1000. Parentheses indicate a restriction site derived from one of two overlapping phages. Middle, Targeting vector pAPLP1-targ. Horizontal arrows indicate the direction of transcription. The dashed box represents a HSV-TK gene. Bottom. The disrupted allele after homologous recombination. E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SacI; X, Xhol; neo-probe, 0.6 kb PstI/Xhol fragment of pGKneo; probe B, genomic 0.5 kb PstI fragment. 

Brains from newborn mice obtained from wt matings or by intercrosses of APLP1+/−/APLP2−/− mice, or APP+/−/APLP2−/− mice, respectively, were dissected and fixed by immersion in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 2% polyvinyl-pyrrolidone (PVP) for 2 hr. After several rinses in cacodylate buffer with 2% PVP, the brainstems were post-fixed in 1% OsO4 in cacodylate buffer followed by dehydration in ethanol and embedding in Epon 812 substitute (Fluka, Neu-Ulm, Germany). Ultrathin 75 nm sections were cut with an ultramicrotome (Reichert Ultracut E). Sections were stained with uranyl acetate/lead citrate and examined under the electron microscope (Zeiss EM 10C). Images were recorded with a BioScan Camera (Gatan).

Primary cultures from mouse cortex

Cultures of cortical neurons were prepared with some minor modifications in analogy to the procedure described by Banker and Goslin (1998) for hippocampal neurons. Embryos for cortical cultures were obtained from intercrosses of APLP1+/−/APLP2−/− mice or APP+/−/APLP2−/− mice, respectively. Genotyping was done by PCR on tail tissue. Wild-type control mice were obtained from 129 Svej × C57Bl6 matings and processed in parallel. Cortices from single E14.5 mouse embryos were collected in Ca2+− and Mg2+-free HBSS/10 mM HEPES, pH 7.2 (Life Technologies, Gaithersburg, MD). After addition of trypsin (final concentration 0.05%) neuronal tissue was incubated for 3 min at 37°C. Trypsin was removed, the suspension was washed with 15 ml of HBSS/HEPES, and cortices were triturated with 5 ml glass pipettes in plating medium (serum-free Neurobasal medium supplemented with B27, 0.5 mM glutamine, and 50 U of penicillin–streptomycin; Life Technologies). Cells were seeded in 500 μl of plating medium in 24-well plastic dishes (coated overnight with 10 μg/ml poly-I-lysine hydrobromide in 0.1 mM borate buffer, pH 8.5) at a density of 130,000 cells/well (~73,000 cells/well) and maintained at 37°C in 5% CO2. Neuronal cultures were treated with cytosineβ-D-arabinofuranoside (ara-C; final concentration 3 μM, Sigma) dissolved in 250 μl of plating medium on day 3 in vitro (DIV 3) to prevent glial proliferation. Neuronal purity was >95% as determined by immunohistochemical staining against GFAP, performed on DIV 10. Cultures were kept without further medium change until DIV 15 when survival experiments were done.

Survival assays and glutamate treatment

To assess spontaneous survival rates, neurons from individual embryos were cultured on gridded celloate coverslips (Eppendorf, Hamburg). Live neurons, as judged by their morphological integrity (with an extended neurite network, smooth membrane appearance, and noncondensed soma) were counted within the same area (six nonoverlapping gridded fields, each covering a total area of 350 × 350 μm, containing ~40–60 neurons per field, on average 240–360 neurons per sample) once on DIV 1 and again on DIV 7. Neuron counts were performed for two or three independent embryos for each genotype. Values represent averages ± SEM. The rate of spontaneous survival on DIV 7 was calculated relative to neuron counts obtained on DIV 1.

Cell survival after glutamate treatment was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT) assays: yellow MTT (Sigma) dissolved in PBS was added to the neuronal cultures to a concentration of 0.5 mg/ml and incubated for 1 hr at 37°C. A violet formazan product is produced by viable cells because of the metabolic activity of mitochondrial enzymes. The formazan product is dissolved in 250 μl of DMSO and detected by its optical density (OD) at 550–690 nm. The OD550–690 takes as a measure of the number of living cells (>95% neurons) in the culture (Mosmann, 1983).

To investigate cell survival in response to glutamate, neurons were prepared from individual embryos (8–12 processed in parallel), and cell suspensions of two cultures were counted before seeding. To correct for
small differences in initial plating densities between individual cultures, cell survival was always expressed as relative values of glutamate-treated cultures normalized to values obtained from untreated cultures derived from the same embryo. For dose–response experiments different concentrations of glutamate (10, 25, 50, 100 µM) dissolved in plating medium were added to cortical neurons for 24 hr followed by MTT assays. Average values from triplicate cultures were calculated relative to values from untreated cultures for which the mean was set as 100% cell survival. For each experiment two or three independent embryos were used, and the experiment was replicated at least three times. In addition, cortical neurons were treated with 50 µM glutamate for 1 or 3 hr. For each time point, a replicate of 24-well cultures were processed in parallel, and mean values were determined. In addition, triplicate sets of nontreated cultures were analyzed for each time point. The mean MTT value obtained from these nontreated cultures (after 0, 1, and 3 hr of incubation) was set as 100% cell survival for each embryo. Percentage of survival was calculated relative to these mean values.

RESULTS

Generation and characterization of APLP1 knock-out mice

Inactivation of the murine APLP1 gene was achieved by gene targeting with a strategy depicted in Figure 1. By homologous recombination in ES cells we generated an ~8 kb genomic deletion comprising 2.5 kb of the putative promoter, the first exon containing the ATG translational start codon and genomic sequences containing ~50% of the APLP1 coding region. Deletion of the transcription and translation start sites should completely abolish APLP1 expression. Mutant ES cells were injected into blastocysts and gave rise to one chimera that transmitted the mutant allele in the germ line. Correct homologous recombination was confirmed by Southern blot analysis of tail DNA that showed the expected pattern for an interrupted APLP1 locus (Fig. 2A,B). Heterozygous animals were intercrossed and yielded homozygous APLP1 knock-out animals at a normal Mendelian frequency. To determine whether the APLP1 gene was also functionally inactive, Northern and Southern blot analysis was performed on brain tissue. An antisense RNA probe (cDNA position 1788 –2360) lying downstream of the targeted deletion revealed a band of ~2.6 kb on brain of wt animals, whereas no transcript was detected in APLP1+/− mutants (Fig. 2D). Western blot analysis with the antibody CT-11, which is directed against the 11 C-terminal amino acids of APLP1 and discriminates against the related APP and APLP2 proteins, gave rise to a set of APLP1-specific bands of 85–100 kDa for wt brain, but not for mutants, indicating that the gene had been completely inactivated. Expression of the other family members APP and APLP2 was unaltered, as judged from Northern (data not shown) and Western blot analysis (Fig. 3A, lane 1, lane 2).

APLP1+/− animals were viable, fertile, and showed, apart from a significant body weight deficit of ~10% (mutant males, 25.37 ± 0.70 gm; wt males, 27.72 ± 0.76 gm; mutant females, 20.01 ± 0.47 gm; wt females, 22.12 ± 0.55 gm; average age, 9 weeks; n = 59; p < 0.001 by two-way ANOVA), emerging in the second week postnatally (data not shown), no overt abnormalities until at least 18 months of age. In contrast to APP-deficient mice that exhibit reduced locomotor activity and reduced forelimb grip strength (Müller et al., 1994; Zheng et al., 1995; Tremml et al., 1997), APLP1+/− mice showed, compared to wt controls, normal locomotor activity, and in adult animals grip strength was unchanged (data not shown). A subtle retardation of neurobehavioral development [as assessed by tests of the Fox (1965) battery] was observed early postnatally (Tremml et al., 1997; data not shown). Histopathological analysis of brain sections (see Materials and Methods) showed no morphological alterations; in particular no agenesis of the corpus callosum was observed [even on a pure 129Sv(ev) genetic background], as opposed to APP-deficient mice exhibiting commissure defects and a high incidence of callosal agenesis on a 129-specific background (Magara et al., 1999). Spatial learning was analyzed by the Morris water maze test, which revealed similar cognitive abilities of APLP1+/− animals as wt controls (as assessed by their normal performance in reversal learning). Surprisingly, however, APLP1-deficient mice showed improved acquisition learning, which could be attributed to altered noncognitive components of the behavior, in particular reduced thigmotaxis (Tremml et al., 1997; P. Tremml, U. Müller, H.-P. Lipp, and D. Wolfer, manuscript in preparation). In summary, ablation of the neuron-specific APLP1 gene results in a rather minor phenotype that is clearly distinct from that of APP-deficient mice, suggesting at least some different functions of both proteins.

Generation of mutants with combined gene deficiencies

To address the question of whether the minor phenotype of single mutants is caused by functional compensation by other APP family members, we set out to generate all three possible combinations of double mutants (APP+/−/APLP2+/−, APLP1−/−/APLP2−/−, APLP1+/−/APLP2−/−).
and APP<sup>−/−</sup>/APLP1<sup>−/−</sup> mice by crossing the respective single mutants (see Materials and Methods for details). To obtain APP<sup>−/−</sup>/APLP2<sup>−/−</sup> mice, animals deficient for APLP2 and heterozygous for APP (APLP2<sup>−/−</sup>/APP<sup>+/−</sup>) were intercrossed and expected to yield 25% offspring deficient for both proteins. When we genotyped the offspring at weaning (−4 weeks of age) initially none and, after analysis of a larger sample of 355 mice, only a single surviving double mutant was found (Table 1A), suggesting that a combined deficiency of APP/APLP2 is lethal either during development or within the first weeks after birth. Analysis of the genotype distribution of offspring at E19 and shortly after birth (P0) showed that almost the theoretically expected number of double mutants (21% of 131 animals screened; Table 1A) survived until this time point. A smaller sample of 26 mice was analyzed at postnatal day 1 (P1), however, only two APP<sup>−/−</sup>/APLP1<sup>−/−</sup> mice were found (one of which died at P2 and the other after 4 weeks) indicating postnatal lethality, predominantly within the first day after birth.

Analogously, APLP1<sup>+/−</sup>/APLP2<sup>−/−</sup> mice were intercrossed, and the offspring were genotyped at weaning. As seen for APP<sup>−/−</sup>/APLP2<sup>−/−</sup> mice, a combined APLP1/APLP2 deficiency proved lethal, in this case with 100% penetrance. Among 326 animals analyzed at weaning, not a single surviving double mutant was found (Table 1B). A smaller sample of pups was screened shortly after birth (P0), and another set on P1 (Table 1B). Whereas APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> mice were born at normal Mendelian frequency (29% of 171 animals), no surviving double mutants were found at P1, indicating that a combined APLP1/APLP2 deficiency results in lethality within the first day after birth. These results demonstrate that APP family members serve essential but at least partially redundant functions in vivo and corroborate a key physiological role for APLP2.

To our surprise, APP<sup>−/−</sup>/APLP1<sup>−/−</sup> mice generated in a similar manner were viable, fertile, and showed no apparent abnormalities, apart from a body weight deficit comparable to that of single mutants, until at least 18 months of age. This unexpected finding that not all three possible combinations of the single mutants result in a lethal phenotype has crucial implications for the specific and redundant functions exerted by APP family members (see Discussion).

The key physiological function or functions of APLP2 are further supported by recent preliminary data demonstrating postnatal lethality for APP<sup>−/−</sup>/APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> mice, which suggests haploinsufficiency for the remaining single APLP2 allele. In an attempt to ultimately generate triple knock-outs, which should theoretically be feasible by intercrossing APP<sup>−/−</sup>/APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> mice, we set up appropriate matings (see Material and Methods) that were expected to yield 25% offspring of the respective (APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> genotype. Among 350 animals screened at weaning, we found only four APP<sup>−/−</sup>/APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> mice (1% instead of the expected 25%). As observed for double mutants (see below) APP<sup>−/−</sup>/APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> mice showed (when analyzed on P0) no apparent histopathological abnormalities (data not shown). The surviving animals were severely impaired in breeding, and we have so far been unable to obtain any litters from these mice. Again, lethality occurred postnatally, because an APP<sup>−/−</sup>/APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> allele frequency of 27% was found in a smaller sample of mice, analyzed shortly after birth (data not shown). We conclude from these data that the presence of a single APLP2 allele in the absence of other APP family members is not sufficient for survival.

**Analysis of APP/APLP expression in double mutants**

We then asked whether the viable phenotype of APP<sup>−/−</sup>/APLP1<sup>−/−</sup> mice may be attributed to a compensatory upregulation of APP and APLP2 expression. As expected, APP expression was abolished in APP<sup>−/−</sup>/APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> mice (lanes 1, 2) compared to the wt control. Note that in heterozygous APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> animals (lanes 3, 4) expression is reduced to ~50% and abolished in APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> double mutants (lanes 5, 6). Genotypes of animals analyzed are as indicated above blot panels.

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**Figure 3.** Western blot analysis of APP/APLP expression in single and double mutants. A, Total brains of newborn APP<sup>+/+</sup> (lane 9), APLP2<sup>+/+</sup> (lane 7), and wt (lane 8) mice and in addition brains of newborn mice generated by intercrossing heterozygous APP<sup>−/−</sup>/APLP1<sup>−/−</sup> mice (lanes 1–6) were homogenized, and equal amounts of protein (20 μg/lane) were resolved on 8% PAA gels. From each blot the bottom half was cut off and probed with an Actin-specific antibody to monitor loading (second row of each panel). Top, Note that probing with an antisemur specific for APLP2 (D2II) showed no major alterations of APLP2 expression in animals of different APP/APLP1 genotype compared to wt levels (lane 8). Bottom, Probing with an APP-specific antibody (22C11) showed comparable amounts of total APP expression in APLP1<sup>−/−</sup> (lanes 1, 2) and wt (lane 8) mice. The seemingly higher expression of APP in APLP2<sup>−/−</sup> mice (lane 7) is attributable to unequal loading as evidenced by more intense actin staining. As expected, APP expression was abolished in APP<sup>−/−</sup>/APLP1<sup>−/−</sup> single mutants (lane 9) and APP<sup>−/−</sup>/APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> double mutants (lanes 5, 6). Note that in heterozygous APP<sup>−/−</sup>/APLP1<sup>−/−</sup> mice APP expression is reduced to ~50%, arguing against a compensatory upregulation of APP expression. B. Total brains of newborn pups obtained from heterozygous APLP2<sup>+/−</sup>/APLP2<sup>−/−</sup> intercrosses were homogenized, and equal amounts of protein (20 μg/lane) were separated on a 8% PAA gel. Top, Probing with an APP-specific antibody (22C11) showed similar amounts of total APP expression in both viable APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> heterozygotes (lanes 3, 4) or in lethal APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup> double knock-outs (lanes 5, 6), compared to the amount of APP expression in APLP2<sup>−/−</sup> single mutants (lanes 1, 2) or in a wt control (lane 7). Note that no significant compensatory upregulation of APP expression was found. Bottom, Probing with an antisemur specific for APLP1 (CT11) showed similar APLP1 expression levels in APLP2<sup>−/−</sup> mice (lanes 1, 2) compared to the wt control.
tion of APLP2 (Fig. 3). Western blot analysis of brain homogenates prepared from newborn mice showed APLP2 levels comparable to those found in single mutants or wt animals (Fig. 3A, top) suggesting that basal wt-APLP2 levels are not limiting for survival. More subtle or region-specific alterations in APLP2 expression cannot be ruled out, however. Likewise, APP protein levels were not upregulated in heterozygous, viable APP1+/-APLP1+/- littermates, showing an ~50% reduction of APP as compared to APP1+/+/APLP1+/- single knock-outs (Fig. 3A, bottom). We then determined whether in the lethal APLP2+/-/APLP1+/- double knock-out, expression of the remaining third family member, APP, is altered. Both Northern (data not shown) and Western blot analysis failed to reveal changes in the expression level of APP in the brains of both newborn viable APLP2+/-/APLP1+/- pups and of APLP2+/-/APLP1+/- littermates that would have died within the next hour (Fig. 3B, bottom). However, APP1 expression was found to be reduced to ~50% of wt level in viable APLP2+/-/APLP1+/- heterozygous newborns (Fig. 3B, bottom). Thus, a loss of APP/APLP family members does not cause compensatory up-regulation of related family members in double knock-outs.

Gross and histopathological analysis of double mutants

Newborn double mutants were initially (for several hours) indistinguishable from their littermates and were able to suckle. Be-
Viability of primary neuronal cultures derived from single and combined mutants

Viability of cortical neurons derived either from APLP2−/− single mutants or from both types of lethal double mutants (APP−/−APLP2−/− and APLP1−/−APLP2−/−) was investigated by counting viable neurons on gridded coverslips after plating (DIV 1) and again after 7 d in culture (DIV 7; Fig. 6). No significant differences relative to wt neurons were detectable for any of the mutants analyzed, indicating that under the culture conditions used, loss of endogenous APLP2 alone, or in combination with APLP1 (see Fig. 6A for APLP1−/−APLP2−/−) or with APP (see Fig. 6B for APP−/−APLP2−/−), respectively, does not affect neuronal survival. Moreover, we saw no significant differences in plating efficiency of viable neurons between wt and mutant cultures on DIV 1 (data not shown), indicating that lack of APP family members did not affect the viability of major neuronal subpopulations in these cultures.

Survival of neuronal cultures derived from single or combined mutants against excitotoxic stress

Studies by Mattson et al. (1993) and Mattson (1994) have demonstrated that primary neuronal cultures can be protected from glutamate-induced excitotoxicity by addition of exogenous secreted forms of human APP (sAPP) to the culture medium. We have previously shown that APP-deficient mice are hypersensitive to seizures induced by the glutamate receptor agonist kainate, although the amount of tissue damage was similar to that observed for wt animals (Steinbach et al., 1998). However, using primary neuronal cultures derived from APP mutant mice, we and others (Steinbach et al., 1998; White et al., 1998) have been unable to show differences in the glutamate sensitivity of APP-deficient neurons. To unravel whether endogenous APP plays any role in the protection of neurons against glutamate excitotoxicity and whether the related APLPs may compensate for such a protection by APP, we compared the survival of neurons derived from single APP family mutants and from lethal mutants deficient for a combination of APLP2/APP, or APLP2/APLP1, respectively. Two sets of experiments were performed. We first investigated the response of neurons prepared from single embryos obtained by intercrossing heterozygous APP+/−APLP2−/− mice and as controls, neurons prepared from wt embryos of 129Sv(ev)xC57BL/6 matings (Fig. 7,A,C). Second, we analyzed neurons of embryos derived from intercrosses of APLP1−/−APLP2−/− mice in comparison to wt neurons of 129Sv(ev)xC57BL/6 matings (Fig. 7B,D). Cortical neurons were used because sufficient numbers of cells can be obtained from individual cortices and moreover, this cell type has previously been shown to be protected from glutamate excitotoxicity by (hu) sAPP (Mattson et al., 1993). Cortical neurons cultured under serum-free conditions for 15 d in vitro were treated with increasing amounts of glutamate (10−100 μM) for 24 hr, and their survival was assessed by MTT assays. This resulted in a glutamate concentration-dependent reduction in cell survival for all cultures examined. Neither APLP2-deficient neurons nor APP−/−APLP2−/− double knock-out cultures differed, however, significantly in their survival rates compared to wt control neurons at any glutamate concentration used (Fig. 7B). Likewise, APLP1−/−APLP2−/− double knock-out cultures showed similar survival rates as APLP2−/− single knock-outs or wt control cultures (Fig. 7A). To test for possible differences in the response kinetics of the various mutants we investigated cell survival at 1, 3, and 24 hr after addition of a constant amount of 50 μM glutamate (Fig. 7C,D for 1 and 3 hr, Fig. 7A,B for 24 hr time point). Again, no significant differences for both sets of mutants compared to wt neurons were found at any time point investigated. As expected from these results, we were also unable to detect significant differences when we tested the whole panel of single mutants APP−/−, APLP2−/−, and APLP1−/− relative to wt controls (data not shown).
We have conducted MTT assays to monitor cell death in response to glutamate challenge. Because no difference was found between mutants and wt neurons either at different concentrations or after different length of exposure to glutamate, we did not extensively investigate the actual mode of cell death. Apoptotic cell death has been reported in cultures of cortical neurons when assayed late (18–24 hr) after exposure to excitotoxins (Tenneti and Lipton, 2000). After incubation with 50 μM glutamate for 24 hr we...
DISCUSSION

This paper investigates the phenotype of APLP1 knock-out mice and a complete set of double mutants comprising all combinations of known APP family members. A summary of our findings is given in Table 2. To analyze the physiological role of the nervous system-specific APLP1 protein, we have generated APLP1−/− mice that were viable and fertile, but revealed a body weight deficit, similar to that observed for APP knock-out mice (Zheng et al., 1995; Tremml et al., 1997; Magara et al., 1999). Thus, both APP and APLP1 appear to play a role in postnatal somatic growth. At least for APLP1-deficient mice, this phenotype might reflect a neural deficit, because APLP1 expression is restricted to the nervous system. Other abnormalities, including those observed for APP−/− mice, were absent in APLP1-deficient mice, suggesting either functional compensation by other family members or distinct functions for APLP1 and APP.

Postnatal lethality of double mutants

In contrast to the minor phenotype of mice singly deficient for individual APP family members, two of three double mutants generated in this study (APP−/−/APLP2−/− and APLP1−/−/APLP2−/−) proved perinatally lethal, indicating essential but partially redundant functions within the gene family (Table 2). Our findings that APP−/−/APLP2−/− mice die shortly after birth are in agreement with previous results from von Koch et al. (1997), who had crossed the same line of APLP2−/− mice used here to the APP−/− line generated by Zheng et al. (1995). Interestingly, we observed an almost complete penetrance of early postnatal lethality for our strain of APP−/−/APLP2−/− mice, whereas 26% of double mutants generated by von Koch et al. (1997) survived well into adulthood. This difference in penetrance is likely attributable to the respective APP knock-out strains, which differ in the targeted deletion introduced into the APP locus and in the composition of genetic background alleles. Because the phenotypes of both APP single knock-out lines are largely congruent, it appears more likely that differences in the genetic backgrounds affected penetrance (Müller, 1999).

Given the similarities of the phenotypes of the two lethal double mutants (APP−/−/APLP2−/− and APLP1−/−/APLP2−/−), i.e., death within the first day or days in the absence of gross morphological anomalies, a common underlying mechanism seems likely, although the actual cause is presently unknown. Because APLP1 is, unlike the ubiquitously expressed APP and APLP2 proteins, restricted to the nervous system, it is conceivable that the cause of lethality resides, at least for APLP1−/−/APLP2−/− mice, in the nervous system. This hypothesis gains further support from recent studies on the Caenorhabditis elegans homolog APL-1 by Li et al.
Most interestingly, a knock-out of the ubiquitously expressed apl-1 gene proved lethal in *C. elegans*, but could be rescued by transgenic expression of APL-1 in neurons, indicating lethal neuronal deficits in the absence of APL-1 (Hornsten et al., 1999).

APP family members could be compensating for each other by binding to and stimulating a common, as yet unidentified receptor. Alternatively, APP/APLPs might act themselves as receptors for a common ligand. Several proteins containing phosphotyrosine-binding (PTB) domains (e.g., Fe65- and X11-family proteins) interact with the intracellular C-terminal domain of APP/APLPs, including the cytoplasmic adapter protein Dab1 (Homayouni et al., 1999). Dab1 is involved in the reelin (Reln) signal transduction pathway, which directs cell positioning in the developing CNS (Rice and Curran, 1999). Our histopathological analysis of single or combined APP/APLP mutants failed to reveal alterations in the formation of laminated brain structures, typically seen in *Dab1* −/− and *Reln* −/− mice (Ohshima et al., 1996). Recently, APP has been shown to have antiapoptotic effects in a neuroblastoma (B103) cell line challenged with apoptosis-inducing insults (Xu et al., 1999). It will therefore be interesting to see whether neurons derived from APP/APLP knock-out mice are, accordingly, more sensitive to apoptotic challenge. In this study, however, no increase in the amount of spontaneous apoptosis was detectable, as evidenced by normal, low levels of TUNEL-positive cells in the brains of newborn mutant mice. It is evident from the lack of obvious morphological abnormalities that a more detailed analysis is necessary, addressing for example possible deficits in synaptogenesis and/or electrophysiological alterations.

**Neuronal survival and response to glutamate-induced excitotoxicity**

One aim of this work was to assess the role of endogenous APP family members for neuronal survival and protection against excitotoxicity.
Double mutants

**APP**/**APLP2**

- Viable and fertile
- Reduced body weight
- Reduced grip strength
- Reduced locomotor activity
- Alterations in sensorimotor development
- Commissure defects
- Hypersensitivity to epileptic seizures

**APLP1**/**APLP2**

- Viable and fertile
- Reduced body weight

**APLP2**/**APLP1**

- Viable and fertile
- Reduced body weight
- No apparent abnormalities

Table 2. Phenotypes of APP family knock-outs

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<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Reference</th>
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<tr>
<td><strong>Single mutants</strong></td>
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<tr>
<td><strong>APP</strong>/−/−</td>
<td>Viable and fertile</td>
<td>Zheng et al.</td>
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<td></td>
<td>Reduced body weight</td>
<td>Müller et al.</td>
</tr>
<tr>
<td></td>
<td>Reduced grip strength</td>
<td>Li et al.</td>
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<td></td>
<td>Reduced locomotor activity</td>
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<td></td>
<td>Alterations in sensorimotor</td>
<td>Tremml et al.</td>
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<td></td>
<td>development</td>
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<td></td>
<td>Commissure defects</td>
<td>Magara et al.</td>
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<tr>
<td><strong>APLP1</strong>/−/−</td>
<td>Viable and fertile</td>
<td>Steinbach et al.</td>
</tr>
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<td></td>
<td>Reduced body weight</td>
<td>This paper</td>
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<tr>
<td><strong>APLP2</strong>/−/−</td>
<td>Viable and fertile</td>
<td>von Koch et al.</td>
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<td>No apparent abnormalities</td>
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<td><strong>Double mutants</strong></td>
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<td><strong>APLP2</strong>/−/−, <strong>APP</strong>/−/−</td>
<td>Perinatally lethal</td>
<td>This paper</td>
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<tr>
<td></td>
<td>No histopathological anomalies</td>
<td>von Koch et al.</td>
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<tr>
<td><strong>APLP2</strong>/−/−, <strong>APLP1</strong>/−/−</td>
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<td>No histopathological anomalies</td>
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Redundancy within the gene family

Interestingly, lack of **APLP2** in combination with a deficiency of either **APP** or **APLP1** was perinatally lethal, whereas the third double mutant, **APP**/−/−/**APLP1**/−/− was viable and apparently normal (Table 2). These data corroborate a key physiological role for **APLP2** and indicate redundancy between **APLP2** and both other family members. The viability of **APP**/−/−/**APLP1**/−/− mice could not be explained by an upregulation of **APLP2**, because **APLP2** expression was unaltered (Fig. 3). Likewise, viable heterozygous litters of lethal double mutants showed no upregulation of **APP**/**APLP** expression. Even in the absence of **APLP2** upregulation, viability of **APP**/−/−/**APLP1**/−/− mice might still be attributable to compensation by **APLP2**, a view that is further supported by the postnatal lethality of **APP**/−/−/**APLP1**/−/−/**APLP2**/−/− mice. It will be interesting to see whether lack of the entire **APP** family will result in an even more pronounced phenotype.

The unexpected finding that **APP**/−/−/**APLP1**/−/− mice are viable has also crucial implications for the specific physiological functions exerted by **APP** and **APLP2**. So far, it has been widely assumed (von Koch et al., 1997; Steinbach et al., 1998; White et al., 1998) that both proteins may serve primarily overlapping functions because (1) **APP** and **APLP2** are highly homologous, (2) they are similarly processed, (3) they are expressed in a virtually overlapping pattern, and (4) deficiency of both proteins results in a synthetic lethal phenotype suggesting functional redundancy. This
view, however, is challenged by our data showing that double mutants obtained by crosses of APP-/- or APLP2-/- single mutants with APLP1-/- mice have clearly different phenotypes, being either viable (APP-/-/APLP1-/) or lethal (APLP2-/-/APLP1-/-). We thus provide genetic evidence that APP and APLP2 must serve, besides overlapping functions, also at least some distinct, nonredundant functions in vivo.

REFERENCES


