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Expression in brain of amyloid precursor protein mutated in the α-secretase site causes disturbed behavior, neuronal degeneration and premature death in transgenic mice

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A double mutation in the α-secretase site in the βA4 region of mouse amyloid precursor protein (APP) reduced its secretion from COS cells, polarized MDCK cells and rat primary neurons. Expression of this mutant in the brain of mice, using the neuron-specific elements of the mouse *Thy-1* gene promoter, resulted in transgenic mice that became progressively hyperactive, displayed seizures and died prematurely. In three different transgenic lines the severity of the phenotype was related directly to the expression levels of the transgene, estimated by both mRNA and protein levels. In addition, homozygous mice derived from each transgenic strain showed more severe symptoms which also occurred earlier in life than in heterozygotes. The observed symptoms were, however, not essentially different in the different lines. Increased aggressiveness, disturbed responses to kainic acid and *N*-methyl-D-aspartate, neophobia and deficiency in exploratory behavior were demonstrated in these mice. In the brain, the observed neuropathological changes included necrosis, apoptosis and astrogliosis in the hippocampus, cortex and other areas. The data demonstrate that incomplete or incorrect α-secretase processing of APP results in severe neurotoxicity and that this effect is expressed in a dominant manner.

Keywords: amyloid precursor protein/apoptosis/neurodegeneration/transgenic mice

Introduction

The only function of amyloid precursor protein (APP) conclusively demonstrated to date is the inhibition of serine proteinases with arginine specificity for the molecular forms of APP that contain the Kunitz inhibitor domain. The accumulated molecular evidence complicates the analysis considerably: from the single APP gene located on chromosome 21, at least seven isoforms can be generated by alternative mRNA splicing (Sandbrink *et al.*, 1994 and references therein). Moreover, the isoforms are synthesized as transmembrane proteins that can be differently proteolytically processed, *N*- and *O*-glycosylated, phosphorylated, as well as sulfated, all to varying degrees. This yields a very heterogeneous end product of a 110–130 kDa ectodomain that is secreted into the extracellular space after proteolytic cleavage (reviewed in Selkoe, 1994).

This proteolysis step, mediated by an uncharacterized proteinase named α-secretase, is an intrinsic and essential step in the mechanism of secretion of APP and has become the focal point in the analysis of processing of APP, both fundamentally and for its involvement in the pathology of Alzheimer's disease (AD). The senile plaques in the brain of AD patients that are a pathological hallmark, contain mainly the βA4 peptide (Glenner and Wong, 1984; Masters *et al.*, 1985). This 39–43 amino acid peptide represents part of the transmembrane and ectodomain of APP (Goldgaber *et al.*, 1987; Kang *et al.*, 1987; Tanzi *et al.*, 1987). Since most patients with trisomy 21 develop AD-like senile plaques in their brain (Glenner and Wong, 1984), this is to be attributed to a gene dosage effect of the APP gene (Rumble *et al.*, 1989). This indication for the narrow margin of regulation of expression of APP is corroborated further by the rare, early onset and presenile familial AD cases that are inherited in an autosomal dominant manner (Chartier-Harlin *et al.*, 1991; Goate *et al.*, 1991; Murrell *et al.*, 1991; Mullan *et al.*, 1993).

Newly synthesized membrane-bound APP is cleaved intracellularly within the βA4 peptide sequence by α-secretase, thereby generating the soluble 110–130 kDa ectodomain and leaving cell-bound 10–12 kDa C-terminal transmembrane and cytoplasmatic domains (Selkoe, 1994). In the absence of cleavage by α-secretase, production of the βA4 peptide is possible by the action of other uncharacterized cellular proteinases, named β- and γ-secretases. Cellular studies *in vitro* with the clinical APP mutants demonstrated that the α-secretase cleavage is reduced in favor of β- and γ-cleavage: considerably more βA4 peptide is produced from the Swedish APP mutant (swAPP) (Citron *et al.*, 1992) while the APP717 mutants yield relatively more of the longer 42–43 amino acid βA4 peptides (Iwatsubo *et al.*, 1994). The Swedish mutation also affects intracellular routing and processing of APP in polarized MDCK cells (Lo *et al.*, 1994; De Strooper *et al.*, 1995a; Haass *et al.*, 1995).

Whereas the known clinical mutations in APP affect the β- and γ-secretase sites, no natural mutations have been described at the α-secretase site. Located most closely are the clinical mutations Ala692→Gly and Glu693→Gln, situated downstream of the α-secretase P1 residue, Lys687. The Glu693→Gln mutation is clinically responsible for recurrent hemorrhages (HCHWA-D) in some cases combined with AD (Levy *et al.*, 1990). The Ala692→Gly mutation, resulting in a mixed clinical picture of AD and recurrent hemorrhages (Hendriks *et al.*, 1992), was shown to cause reduced secretion of APP (Haass *et al.*, 1994).

To examine the importance of the α-secretase processing step, we have previously introduced a double mutation in the α-secretase region by replacing two basic residues

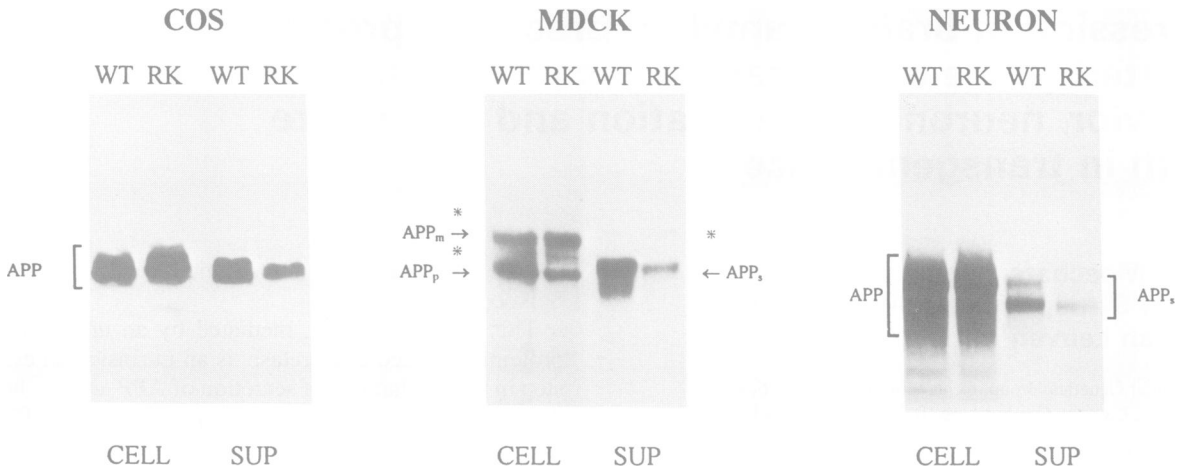

Fig. 1. Secretion of APP/WT and APP/RK in COS cells, polarized MDCK cells and primary cultures of rat hippocampal neurons. Cells were metabolically labeled and APP was immunoprecipitated from cell extracts (lanes labeled CELL) and from the conditioned medium (lanes labeled SUP) and analyzed by SDS–PAGE. In MDCK cells, precursor APP (APP_p) and the mature *N*- and *O*-glycosylated APP (APP_m) can be clearly separated, while the endogenous APP751 is indicated by asterisks.

with two acidic residues. The resulting APP/RK mutant was secreted from COS cells at a rate that was <50% relative to wild-type APP (APP/WT) (De Strooper *et al.*, 1993). We report here that, also from polarized MDCK cells, the secretion of the APP/RK mutant is markedly reduced without affecting the exclusively basolateral secretion pattern. In addition, making use of a viral infection system, we demonstrate that primary rat neurons secrete considerably less ectodomain from APP/RK than from APP/WT. The functional repercussions of the APP/RK mutant were analyzed in transgenic mice by expressing the APP/RK cDNA using the neuron-specific elements of the mouse *Thy-1* gene promoter (Vidal *et al.*, 1990). The resulting transgenic mice displayed a disturbed behavior, which deteriorated progressively with age and resulted in premature death, occurring as soon as 2 months after birth for individual homozygotes of some transgenic lines. The results of molecular, biochemical, behavioral and histochemical tests and experiments are presented and discussed.

Results

Expression of APP/RK in COS cells, polarized MDCK cells and primary neurons

COS cells were transiently transfected with constructs containing the cDNA coding for APP/RK or APP/WT under the control of the early SV40 promoter (De Strooper *et al.*, 1993). Secretion of APP/RK was ~50% relative to APP/WT, confirming our previous observations (De Strooper *et al.*, 1993).

MDCK cell lines were created that have either APP/WT or APP/RK cDNA driven by the RSV promoter stably integrated in their genome (De Strooper *et al.*, 1995a). Three independent cell lines of each were grown polarized on filters, and the apical and basolateral compartments were analyzed for APP secretion. In standard conditions, secretion of mutant APP/RK is considerably decreased relative to APP/WT and remains exclusively in the basolateral compartment (Figure 1), a situation not different from APP/WT (De Strooper *et al.*, 1995a). Primary cultures of rat hippocampal neurons were infected with recombinant Semliki forest virus carrying the APP/WT or the APP/RK mutant cDNA. The secretion of APP/RK was again <50% relative to secretion of APP/WT (Figure 1).

Table I. Overview of all founder mice with the APP/RK mutant cDNA

Founder	Copy No.	Offspring	Expression (%)
a/RK/B/1	3	+	<5
a/RK/B/2	3	+	<5
a/RK/B/3	1	–	–
a/RK/F/4	3	+	–
a/RK/F/5	2	+	<5
a/RK/F/6	1	+	–
a/RK/F/7	2	+	5
ae/RK/B/1	5	+	<5
ae/RK/B/2	3	+	<5
ae/RK/F/3	3	+	–
ae/RK/F/4	4	+	–
ae/RK/F/5	2	+	–
ae/RK/F/6	15	+	<5
ae/RK/F/7	20	+	5
ae/RK/F/8	10	+	–
ae/RK/F/9	1	+	–
ae/RK/F/10	2	+	–
t/RK/F/1	3	–	–
t/RK/B/2	1	+	50
t/RK/F/3	1	+	5
t/RK/F/4	1	+	45
t/RK/F/5	1	+	30
t/RK/F/6	2	+	10

Generation of APP/RK transgenic mice and analysis of expression

All transgenic lines derived using APP/RK cDNA in different constructs are summarized in Table I. Transgenic founders are denoted by a code identifying the promoter (t, a or ae, respectively for the mouse *Thy-1* gene promoter, the human APP gene promoter and the latter with a viral enhancer element), the APP cDNA type or mutant (in the present work only APP/RK), the mouse strain (F or B, respectively for FVB or C57Bl) and the line number. The

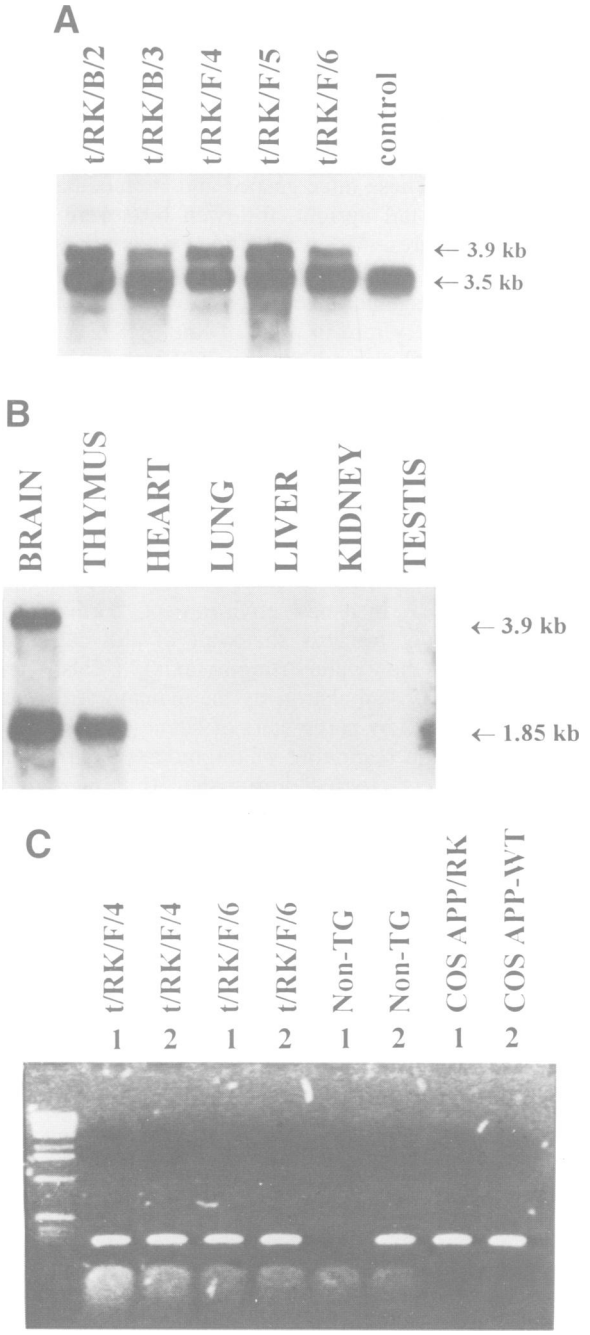

Fig. 2. Analysis of mRNA expression. (**A**) Northern blotting of total brain RNA from a non-transgenic and from five t/RK heterozygous transgenic mice. (**B**) Northern blotting of RNA from brain and from seven different tissues of a homozygous t/RK/B/2 transgenic mouse. A mouse *Thy-1* gene exon 4 probe (1.4 kb *Apa*I fragment) detected the 1.85 kb Thy-1 mRNA and the 3.9 kb APP/RK mRNA. (**C**) RT-PCR on brain mRNA using PCR primers specific for APP/RK mRNA (lanes 1, primers L921-L57) or endogenous APP mRNA (lanes 2, primers L922-L57). Positive control was RNA from COS cells transiently expressing APP/WT or APP/RK (see Figure 1).

experiments concern mainly heterozygous and homozygous offspring of founders t/RK/B/2, t/RK/F/4, t/RK/F/6 and ae/RK/F/7 (Table I).

RT-PCR established expression of the transgene in the brain in four out of 10 ae/RK transgenic strains, in four out of six a/RK lines and in five out of six t/RK lines (Table I, Figure 2). Northern blotting revealed 3.5–3.9 kb mRNA in the wild-type and t/RK strains, with the larger mRNA size due to the 3′-untranslated region of the *Thy-1* gene. Expression by the human APP gene promoter was low relative to endogenous APP. The mouse *Thy-1* gene promoter resulted in brain expression levels in heterozygotes that reached 50% of endogenous APP mRNA levels (Table I, Figure 2). In homozygotes, APP/RK mRNA levels in brain approached those of endogenous APP. Restriction of expression to brain in the t/RK lines was demonstrated by RT-PCR and Northern blotting. Endogenous *Thy-1* gene was expressed in brain and thymus (Figure 2). The thymus-specific elements of the mouse *Thy-1* gene are present in intron 3 and deleted in the construct used (Vidal *et al.*, 1990).

Western blotting of brain extracts with different polyclonal and monoclonal antibodies detected consistently more of the 110–130 kDa APP present in the brain of transgenic APP/RK mice relative to non-transgenic control mice (Figure 3A). Individual variations between mice were apparent but, overall, less important than the differences between the different transgenic strains or between mice with a different genetic status: heterozygotes expressed APP levels that are intermediate between controls and homozygotes of the same strain (Figure 3E). Semi-quantitative comparison indicated that brain APP levels in the heterozygotes were ~75% (line t/RK/F/6) and 200% (t/RK/F/4 strain) higher than in controls. The highest level of expression was noted in the brain of homozygous t/RK/F/4 mice (Figure 3C) amounting to ~3-fold the endogenous APP levels. These protein data largely paralleled the expression at the mRNA level. In all the APP/RK lines a larger APP protein band was detected that is hardly visible in control brain extracts (Figure 3). This band, representing mature, proteolytically uncleaved APP/RK is being characterized and its metabolism examined extensively, not only in the cellular paradigms used above, but also in primary cortical and hippocampal neuronal cultures derived from the transgenic mice.

Demonstration of reactive gliosis in the transgenic mice (see below) prompted us to compare the levels of glial acidic fibrillary protein (GFAP) by Western blotting. Some increase in GFAP was evident, especially in the homozygous animals, but was not nearly as prominent as the increase in APP (Figure 3). Combined immunoprecipitation and Western blotting failed to detect significant amounts of the βA4 peptide in brain extracts from normal and APP/RK transgenic mice (results not shown).

The expression of other genes in the brain was analyzed at the protein or mRNA level, including the APP-related proteins APLP1 and APLP2, the apolipoprotein E gene and its receptors VLDLR, A2MR/LRP and A2MRAP (Lorent *et al.*, 1995). The APP/RK transgenic strains did not differ appreciably in this respect from age-matched control mice.

Phenotypic characterization of transgenic APP/RK mice

Premature death. Mice from the FVB, C57Bl and CBA/J strains normally live >2 years. As progressively more APP/RK mice died prematurely, this phenomenon was monitored in a population of 64 heterozygous transgenic mice of the first generation, relative to a group of 30 genotyped, non-transgenic littermates. In the first year,

only 7% of non-transgenic mice died, while 44, 66 and 69% of transgenic mice died in lines t/RK/F/6, t/RK/F/4 and t/RK/B/2, respectively (Figure 4). The first death in lines t/RK/B/2, t/RK/F/4 and t/RK/F/6 occurred at 30, 78 and 150 days of age, respectively. This correlated with the higher APP/RK expression level in lines t/RK/B/2 and t/RK/F/4 relative to t/RK/F/6 (Table I).

All animals that died spontaneously were weighed and necropsied, but no malformations of the vital organs nor major internal bleeding were observed. Premature death was not preceded by overt signs of deteriorated health or by a marked loss of weight.

Spontaneous behavior. In lines t/RK/F4 and t/RK/F/6, heterozygotes of <3 months and homozygotes of <2 months behaved normally. Subsequently, they showed increased agitation, bouts of wild running and, among males, more frequent and fierce fighting. Many displayed mild seizures of varying severity and, upon transfer into clean cages, posture freezing and neophobia. Sex difference was not apparent. Seizures consisted of facial movements, mild fore-limb clonus and mild whole body clonus lasting from 5 to 10 s. Transgenic mice older than 6 months showed, in addition, severe tonic–clonic seizures, lasting 30–60 s. These mice gasped and stretched all four limbs with their tail upright, followed by severe whole body clonic seizures. Afterwards, mice were lethargic for variable periods of time. Seizures have been observed in >60% of all transgenic mice in these two lines. Seizures were absent or very rare in mice of line t/RK/B/2 (Table I), which were backcrossed into the C57Black background (mice analyzed were at least 87.5% of this background). This strain is known to be more resistant to seizures and to drugs inducing seizures (Ganesan *et al.*, 1995 and references therein). Spontaneous seizures were never observed in normal mice of the FVB strain in our colony, which are kept under identical conditions to the transgenic mice.

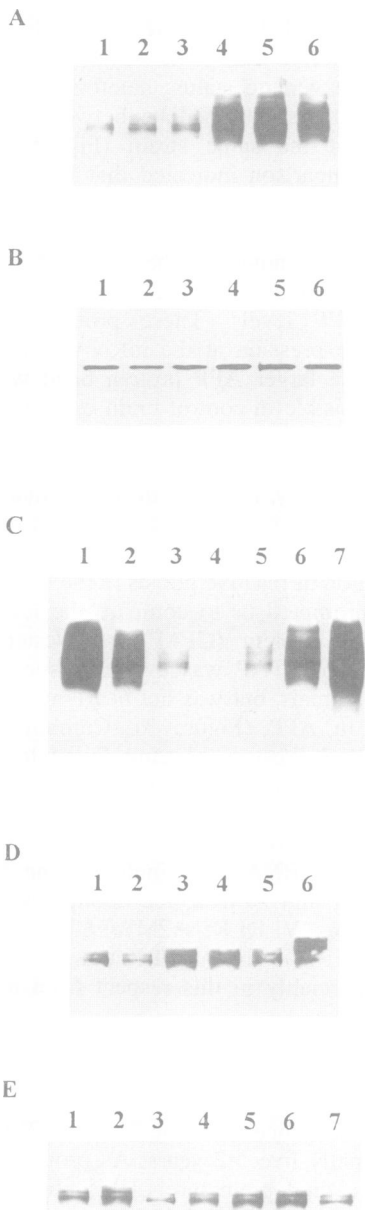

Fig. 3. Western blotting of APP and GFAP in brain of control and transgenic mice. (**A**) FVB control mice (lanes 1–3) and homozygous t/RK/F/4 transgenic mice (lanes 4–6) immunoblotted with mAb 22C11 (Hilbich *et al.*, 1993) on a 6% homogeneous SDS–PAGE gel. (**B**) The same extracts separated on a 4–20% linear SDS–PAGE gradient gel, immunoblotted with a polyclonal antibody against GFAP. (**C**) Different amounts of brain extract of FVB control (extract 1 from A) and homozygous t/RK/F/4 (sample 4 in A) on a 6% gel, stained with mAb 22C11. Lane 1: 6 µl, lane 2: 2 µl, lane 3: 0.6 µl, lane 4: 0.2 µl, lane 5: 0.2 µl, lane 6: 0.6 µl, lane 7: 2 µl. Note the equivalent staining intensity of lanes 3 and 5 (volume extract ratio of 3) and the stronger staining of the larger extra band of APP in lanes 5–7. (**D**) Brain extracts of different control and heterozygous transgenic mice, separated on a 4–20% gradient gel and stained with mAb 22C11. Lane 1: FVB control, lane 2: F1(C57Bl×CBA/J) control, lane 3: heterozygote t/RK/B/2, lane 4: heterozygote t/RK/F/4, lane 5: heterozygote t/RK/B/6, lane 6: heterozygote of a t/APP770/F/1 mouse, transgenic for mouse APP770 cDNA driven by the mouse *Thy-1* gene promoter. (**E**) Brain extracts of different heterozygous and homozygous transgenic mice separated on a 4–20% gradient gel and stained with mAb 22C11. Lanes 1 and 2: heterozygous and homozygous t/RK/F/4, lanes 3 and 4: heterozygous and homozygous t/RK/F/6, lanes 5 and 6: heterozygous and homozygous t/RK/B/2, lane 7: FVB control.

Behavioral testing. In a new environment, normal mice move immediately towards the wall of the cage and proceed along the walls (thigmotaxis) (Walsh and Cummins, 1976). Ambulation as an indicator of motor activity was scored by parameters of latency, i.e. the time elapsed before the beginning of thigmotaxis and by the number of corners crossed during the 30 s interval. In lines t/RK/F/4 and t/RK/F/6, respectively, 54 and 47% of the mice exhibited a latency of >5 s, as opposed to 5% of control FVB mice. Younger transgenic mice behaved like age-matched littermates: at 4–6 weeks, <10% of mice of both transgenic lines displayed a latency of >5 s.

Less than 2% of FVB controls showed posture freezing in a new environment, as opposed to 30% of t/RK/F/4 and 23% of t/RK/F/6 mice (3–5 months old). Transgenic mice of all ages also showed a markedly increased respiratory rate, while some mice went into seizures, indicating the severe stress.

In a group of 35 FVB control mice, the frequency of corner crossing was Gaussian distributed, centered around a mean of seven crosses (Figure 5). This distribution was

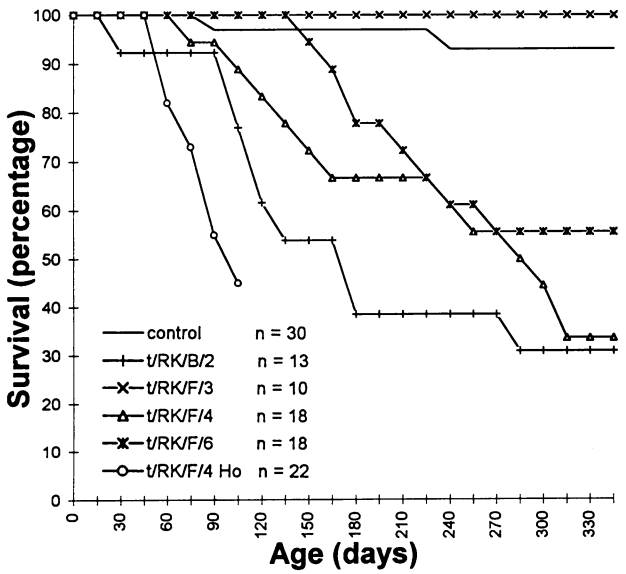

Fig. 4. Premature death of control FVB, heterozygous and homozygous APP/RK mice. The death rate in four t/RK lines that were heterozygous for the APP/RK transgene compared with non-transgenic littermates over a 1 year period. Homozygous offspring of the t/RK/F/4 line were followed for only 105 days.

similar for young or old FVB mice (2–3 or 9–12 months old). Heterozygous and homozygous mice of lines t/RK/F/4 and t/RK/F/6 (6–20 weeks old) displayed significantly reduced ambulation. Strikingly, the frequency of corner crossings was skewed towards low values, separating two subpopulations, one with low explorative behavior and another with only a slight reduction. Afterwards, the tested transgenic animals did not behave differently from untested mice. It was also evident that premature death was not confined to either subpopulation of the tested transgenic mice: two out of five t/RK/F/4 mice and two out of 11 t/RK/F/6 mice (from those tested in Figure 5) that died during the 6 months subsequent to the open field test, belonged to the low explorative groups. This proved that the reduced exploratory behavior was not a direct sign of imminent, premature death of the transgenic animals.

The augmented aggressive behavior in the t/RK/F/4 and t/RK/F/6 offspring was measured under controlled conditions. Single male FVB control and transgenic mice, homozygous for the transgene, were housed isolated for 4 weeks ('resident') before being confronted in their cage with a male FVB 'intruder' that had been reared in a group. Aggression was scored by the latency of the first attack and by the number of attacks during 3 min observation (Saudou *et al.*, 1994). APP/RK resident males attacked sooner and more often than non-transgenic FVB resident males. Transgenic males from strain t/RK/F/4 attacked sooner and more often than t/RK/F/6 residents (Figure 5), and the attacks were judged to be fiercer and more intense. When the same animals were retested, aggressiveness was increased (Figure 5).

Reactivity to glutamate analogs

The glutamatergic system is the major excitatory system involved in the generation of seizures. Its activity was tested in lines t/RK/F/4 and t/RK/F/6 with two agonists of different glutamate receptors, i.e. kainic acid (KA) and *N*-methyl-D-aspartate (NMDA).

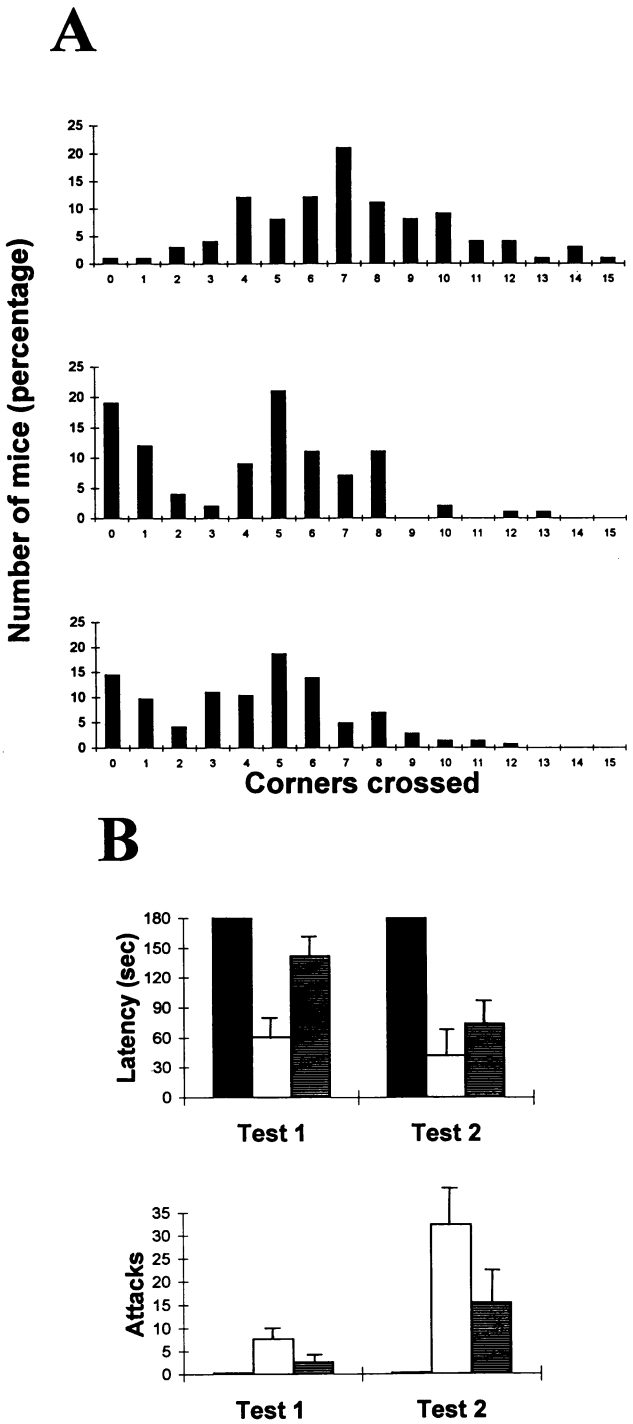

Fig. 5. Behavioral testing. (**A**) In open field testing, the number of corners crossed during a 30 s interval was measured. FVB control mice (upper panel, $n = 35$), t/RK/F/4 mice (middle panel, $n = 30$) and t/RK/F/6 mice (lower panel, $n = 50$). (**B**) In the aggression test, resident mice were FVB (black bars, $n = 4$), homozygotes of lines t/RK/F/4 (open bars, $n = 6$) and t/RK/F/6 (striped bars, $n = 5$). Latency of attack (upper panel) and the number of attacks by the resident (lower panel) in a 3 min observation period. Data are calculated mean values with standard errors. Statistical analysis showed the difference between transgenic and control mice to be significant ($P<0.02$).

Dose–response curves for KA induction of seizures and mortality in FVB, C57Bl and F1(C57Bl×CBA/J) mice demonstrated obvious strain differences (FVB being more

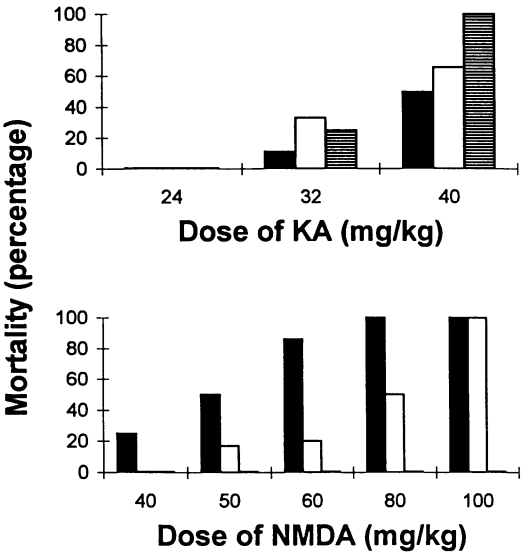

Fig. 6. Effects of glutamate analogs kainic acid and NMDA. Upper panel: mortality resulting from i.p. administration of KA to FVB mice (black bars, n = 4, 9 and 6), homozygous t/RK/F/6 mice (open bars n = 2, 8 and 4) and homozygous t/RK/F/4 mice (striped bars, n = 2, 9 and 4). Lower panel: mortality resulting from i.p. injection of NMDA in FVB mice (black bars, n = 4, 6, 7, 4 and 2), homozygous t/RK/F/6 mice (open bars, n = 2, 4, 4, 4 and 3) and homozygous t/RK/F/4 mice (n = 4, 6, 5, 6 and 2). Homozygous offspring of line t/RK/F/4 were resistant, and displayed symptoms and died only when receiving higher doses (see text).

sensitive than C57Bl mice), but KA consistently elicited, dose dependently, clonic seizures which were rated in five stages: immobilization, head nodding, forelimb clonus, rearing and rearing with falling (Racine, 1972). All APP/RK mice were more sensitive to KA than were control mice (Figure 6). KA at 40 mg/kg triggered seizures of stage 5 in all t/RK/F/4 and t/RK/F/6 mice, while only 66% of FVB control mice showed stage 4 seizures and 30% stage 5. Following the seizures, all t/RK/F/4 mice died, while 35% of t/RK/F/6 mice and 50% of control FVB mice survived (Figure 6).

The LD_{50} for NMDA in FVB control mice was ~50 mg/kg body weight (Figure 6). The APP/RK transgenic mice demonstrated a markedly diminished sensitivity for NMDA. Homozygous t/RK/F/4 mice even resisted a dose of 100 mg/kg, which was invariably lethal for control FVB as well as for all t/RK/F/6 transgenic mice (Figure 6). In t/RK/F/4 mice this high dose of NMDA failed to elicit the typical NMDA symptoms evident in control FVB mice (Leander *et al.*, 1988). These symptoms were evident at 150 mg/kg, a dose that was never tested in control or t/RK/F/6 mice and which killed all t/RK/F/4 transgenic mice in 20 min.

Histological analysis of the brain of transgenic APP/RK mice

In the brain of 24 out of 25 transgenic mice of lines t/RK/B/2 (four mice), t/RK/F/4 (15 mice), t/RK/F/6 (three mice) and ae/RK/F/7 (three mice) that died during observations, typical abnormal neurons were evident: many neurons were vacuolized or contained clear cytoplasm, surrounding compacted and densely stained nuclei (Figure 7). These aberrant neurons were most conspicuously located on the boundary of the granular layer and the hilus of the dentate gyrus and dispersed throughout the granular layer of the dentate gyrus. In the most affected animals, degenerating neurons were present in the thalamus (Figure 7) and, to a lesser extent, in the posterior cingulate cortex, in the caudate putamen, the hypothalamus and the CA1 and CA2 region of the hippocampal pyramidal layer. Typical also were the nuclei of glial cells in the corpus callosum and in the fimbria which are normally ellipsoid, but appeared rounded and more densely stained in APP/RK mice (Figure 7). The most severe signs of neurodegeneration were encountered in four t/RK/B/2 and in two t/RK/F/4 mice. The age of the animals was not the determining factor, since these mice were aged between 2.5 and 9 months.

The abnormal neurons in the dentate gyrus were reminiscent of pyknotic or apoptotic cells: condensation of the chromatin results in compact and heavily stained nuclei with blebbing of the nuclear membrane. Apoptotic cells have been demonstrated by immunochemical methods in AD brain (Su *et al.*, 1994) and in transgenic mice (LaFerla *et al.*, 1994). *In situ* detection of apoptotic cells in brain slices by terminal transferase incorporation of digoxigenin-dUTP and peroxidase-based detection, demonstrated considerable numbers of apoptotic cells in t/RK/B/2, t/RK/F/4 and ae/RK/F/7 mice (Figure 7). In mice of line t/RK/B/2 and some of line t/RK/F/4, the granular layer of the dentate gyrus contained many apoptotic neurons, while in ae/RK/F/7 mice apoptotic neurons were present in the amygdala (Figure 7).

Reactive gliosis or astrogliosis was demonstrated by immunostaining for GFAP, and proved positive even in younger transgenic mice and in mice from line t/RK/B/2 that did not exhibit seizures. An intense astrocytic reaction was observed in the cortex, the amygdala and the hippocampus; astrocytes with enlarged cell bodies and thicker processes stained intensely for GFAP (Figure 7). Brains of transgenic mice from lines t/RK/F/4 and t/RK/F/6 were stained immunochemically for the βA4 peptide with five different antibodies. Although some staining was evident in certain neurons, the reaction was weak (results not shown).

Discussion

The functional importance of correct α-secretase processing of APP was examined by creating a mutant APP that was partially defective in secretion from three different cell types: COS cells, MDCK cells and primary neurons. Expression of this α-secretase mutant in mouse brain resulted in a progressive disorganization of the central nervous system, resulting in behavioral disturbances, seizures, differential reaction to glutamate analogs, premature death and morphological neuronal changes in the brain. The histological analysis demonstrated conclusively that the brain of transgenic t/RK mice contained many abnormal neurons in the dentate gyrus and in the thalamus and, in older mice, also in the cortex and the pyramidal layer of the hippocampus. The symptoms of deranged behavior, the occurrence of seizures and the premature death are a consequence of the documented neuronal damage. This conclusion is derived from the accumulated data presented in the Results section and discussed below.

Expression of the transgene was restricted exclusively to the brain, as anticipated from the characteristics of the

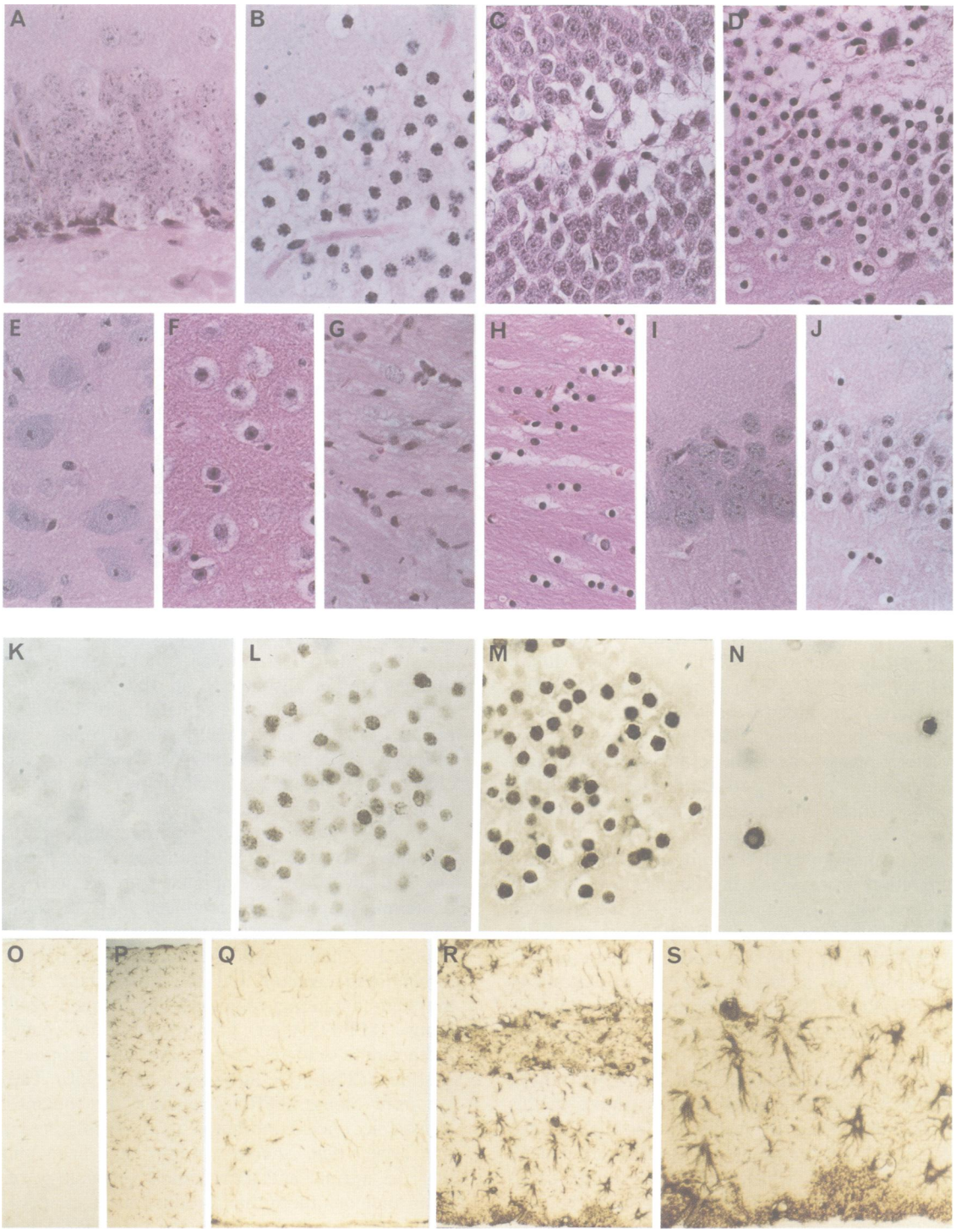

Fig. 7. Histology, apoptosis and GFAP immunochemistry of mouse brain. (A–J) Hematoxylin–eosin staining of sagittal sections of the brain of FVB and APP/RK mice. Representative photomicrographs show the granular layer of the dentate gyrus from control FVB mouse (**A**), t/RK/B/2 mouse (**B**), t/RK/F/4 mouse (**C**) and t/RK/F/6 mouse (**D**); of the thalamus from control FVB mouse (**E**) and t/RK/F/6 mouse (**F**); of the corpus callosum from control FVB mouse (**G**) and t/RK/F/6 mouse (**H**); of the hippocampal CA1 pyramidal layer from control FVB mouse (**I**) and t/RK/B/2 mouse (**J**) (×60). (K–N) *In situ* apoptosis in the brain of APP/RK transgenic mice detected on sagittal sections in the dentate gyrus from control FVB (**K**), t/RK/B/2 (**L**) and t/RK/F/4 (**M**) mice and in the amygdala of an ae/RK/F/7 mouse (**N**) (×100). (O–S) GFAP immunostaining in the cortex of control FVB (**O**) and t/RK/F/4 (**P**) mice (×10), in the dentate gyrus of FVB (**Q**) and t/RK/F/6 mice (**R**) (×20). (S) is a higher magnification (×40) of the bottom right hand corner of (R).

elements of the mouse *Thy-1* gene promoter used in the constructs injected (Vidal *et al.*, 1990). In the four independent transgenic mouse strains analyzed and described, the severity of the phenotype was related directly to the expression of the transgene in the brain both at the mRNA and protein level. Although the homozygous offspring of strain t/RK/F/4 display the most severe and spectacular phenotype, even mild overexpression, as in homozygotes of line t/RK/F/6, resulted in symptoms that are essentially the same. In addition, although not presented

here, a similar phenotype is becoming evident in transgenic mice that express the same APP/RK mutant cDNA, but under the control of the human APP gene promoter (the a/RK and ae/RK lines). The symptoms in these strains are milder and develop at a later age, which is consistent with the lower expression level attained with this promoter (unpublished results). Further, we have generated transgenic mice in which the mouse *Thy-1* gene promoter elements are used to drive expression of the mouse APP770 cDNA (Figure 3) or of the C-terminal domain of APP fused to its signal peptide (not shown). These transgenic mice show none of the phenotypic characteristics of the APP/RK mice. We conclude that the observed phenotype is specific and caused directly by the overexpression of the proven α-secretase APP/RK mutant in the brain.

Some of the phenotypic parameters of the APP/RK mice are reminiscent of AD, i.e. neurodegeneration, disturbances in behavior and premature death. Preliminary tests indicated that the short-term learning ability of the mice is not affected, but more extensive and careful testing of learning and memory capabilities is in progress. The generalized occurrence of seizures in our transgenic mice was unexpected, but literature data prove that these are frequently observed in AD patients, even as a prominent feature in familial chromosome 14-linked AD, while less prevalent in patients with the APP717 mutations (Mullan *et al.*, 1993; Lampe *et al.*, 1994). It was debated whether seizures constitute an integral characteristic of AD or are a secondary phenomenon. The loss of neurons and important alterations of the architecture of hippocampal and neocortical areas provides a suitable substrate for the generation of seizures, while seizures could enhance the progression of the disease.

Glutamate excitotoxicity has been implicated in the neurodegeneration process and in neuronal cell death in AD. In the tests with the glutamate analogs, the observed refractiveness to NMDA of the APP/RK transgenic mice could be indicative of 'NMDA receptor hypofunction' recently proposed as a novel type of excitotoxicity (Rothman and Olney, 1995). In addition, since NMDA receptor activity is thought to be linked intimately to the central mechanisms of memory building and learning (Malenka, 1994; Collingridge and Bliss, 1995), we believe that the APP/RK mice will allow further studies in this direction. The difference in reactivity in this respect, caused by the APP/RK transgene in the different genetic backgrounds (FVB or C57Black, respectively in the t/RK/F/4 or t/RK/F/6 and t/RK/B/2 transgenic lines), is most interesting: the major and so far only difference is the almost complete absence of seizures in line t/RK/B2 while, by the other parameters and criteria of molecular and histochemical analysis, this line is not notably different from the FVB transgenic lines. The C57Black mouse strain is known to be more resistant to seizures and to drugs inducing seizures (Ganesan *et al.*, 1995 and references therein). This clear genetic component in the phenotype is another strong argument that the seizures are the consequence and not the cause of the neurodegeneration.

To explain the effect of the APP/RK mutation molecularly, two possibilities need to be considered. Given the location of the double mutation, the secreted APP/RK ectodomain will contain a mutated C-terminal end when processed by α-secretase. Obviously, this is unique since the APP/RK mutant is artificial and it is impossible to predict how the double mutation at the C-terminal end of the secreted APP/RK ectodomain would disturb an (unknown) function. Alternatively, the slower processing by α-secretase should favor β-cleavage of APP/RK, resulting in secretion of β-cleaved APP/RK, a situation to be envisaged in patients carrying the Swedish APP mutation (Citron *et al.*, 1992).

These considerations should take into account the intracellular fate of unprocessed APP/RK and of cell-bound fragments containing the βA4 peptide resulting from β-cleaved APP. An interesting collateral is provided by the recent report of transgenic mice that overexpress the βA4 peptide intracellularly (LaFerla *et al.*, 1995). The many phenotypic traits that are similar to those observed in the APP/RK mice described here invite speculation about a common cause. Possibly, the cell-bound APP/RK C-terminal fragments can lead to intracellular βA4 peptide, creating a situation that is not essentially different from the one in which the βA4 peptide is produced intracellularly as instructed by the transgene (LaFerla *et al.*, 1995). This reasoning implies that intracellularly produced βA4 peptide could cause neurodegeneration and, eventually, could be more neurotoxic than extracellularly produced βA4 peptide. This would explain why transgenic mice with a massive 10-fold overexpression in brain of a clinical APP mutant protein displayed, on the one hand, massive deposits of β-amyloid in authentic extracellular amyloid plaques but, on the other, produced only a very mild phenotype (Games *et al.*, 1995). Clearly, more extensive comparative and longitudinal studies are required on the different transgenic mouse strains.

An observation that could be related directly to the mechanisms involved as discussed is the production of amyloid or βA4 peptide in mice. Little or no βA4 peptide-containing peptides are produced in COS cells, MDCK cells or neurons after transfection with mouse APP as opposed to human APP (De Strooper *et al.*, 1995b and unpublished results). On the other hand, intracellular amyloid-containing fragments are formed readily from mouse APP695 after 'humanization' by changing only three amino acid residues in the βA4 peptide region of murine APP695 (De Strooper *et al.*, 1995b). The fact that we used mouse APP to generate the mutant APP/RK transgenic mice contributed to or highlighted precisely that aspect that otherwise might have been blurred, i.e. that the normal and pathological physiology of APP in brain includes more than production of the βA4 peptide. In this respect, it is noteworthy that APP-deficient mice demonstrated several phenotypic characteristics in common with the APP/RK transgenic mice (Zheng *et al.*, 1995 and unpublished results). Eventually, not only the production of βA4 peptide but also the absence or suppression of an as yet unknown function of APP will explain this paradox.

Materials and methods

Construction of the APP695/RK minigenes

The mouse APP/RK cDNA (De Strooper *et al.*, 1993) was cloned in an adapted *Sfi*I site in the pTSCαI vector (gift from H.Van der Putten, Basel). This pUC18-based vector contained an 8.1 kb *Eco*RI fragment

comprising the mouse *Thy-1.2* gene (Vidal *et al.*, 1990). A 1.5 kb *Ban*I–*Xho*I fragment (located in exon 2 and exon 4, respectively) was replaced by the APP/RK cDNA. Alternatively, the mouse APP/RK cDNA was cloned downstream of the human APP gene promoter with a β-globin intron interspersed. A duplex polyoma virus enhancer element eventually preceded the human APP promoter.

Microinjection and analysis of founders

Linearized constructs were purified and microinjected into 1.5-day-old pre-nuclear embryos taken from superovulated FVB or F1 (C57Bl×CBA/J) females. The injected oocytes were allowed overnight to reach the two-cell stage and transferred into pseudopregnant fosters, F1 (C75Bl×CBA/J). Tail biopsies were taken 3 weeks after birth at weaning for isolation of genomic DNA and Southern blotting, as described (Umans *et al.*, 1994).

Analysis of RNA and protein

Total RNA from mouse tissues was isolated and analyzed by Northern blotting as described (Lorent *et al.*, 1995). RT-PCR was carried out with sense primers containing either the wild-type APP or the mutated APP/RK region (De Strooper *et al.*, 1993): primer L921, 5′ttgaagtcCGCcatcaaAaA3′ or primer L922, 5′ttgaagtcGATcatcaaGaG3′ (the five nucleotide differences are capitalized). The antisense primer was L57: 5′ ccgatgggtagtgaagcaatggtt 3′. RT-PCR was done with touch-down temperature programming: four series of three cycles each (1 min denaturation at 92°C, 2 min annealing at 68, 67, 66 and 65°C and 1 min extension at 72°C) followed by 35 cycles (1 min 92°C, 2 min 64°C, 1 min 72°C). For Western blotting, brain tissue was homogenized in 10 vol of buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS) with proteinase inhibitors (25 mM EDTA, 1 μg/ml pepstatin, 1000 U/ml aprotinin) in a motor-driven mechanical homogenizer. After centrifugation (12 000 *g*, 20 min), the clear supernatant was made 1% in SDS and 2-mercaptoethanol, boiled for 5 min and analyzed on Tris–glycine-buffered polyacrylamide gels (6% or 4–20%).

Brain histology and immunostaining

Brain tissue was fixed overnight in 4% paraformaldehyde, dehydrated and paraffin embedded for sectioning (5 μm). For *in situ* apoptosis detection, sections were mounted on silanized slides and treated for terminal transferase incorporation of digoxigenin-dUTP and immunoperoxidase detection and staining (Apoptag kit, Oncor). For GFAP immunostaining, 5 μm paraffin sections were dewaxed, rehydrated and treated with hydrogen peroxide to eliminate endogenous peroxidase activity. After blocking with normal goat serum (5% in 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100), sections were incubated overnight at 4°C with rabbit anti-cow GFAP antibody (Dakopatts, Denmark) diluted 1:1000, followed by peroxidase-conjugated goat anti-rabbit antibody (dilution 1:100). The reaction was developed with diaminobenzidine and hydrogen peroxide.

Open field test

All mice used were kept under a light cycle of 12 h, with food and water *ad libitum*. The corner index test is a variant open field test to measure activity and ambulation. The mice were placed in the middle of a clean, unused opaque cage (20 cm square) and allowed to move under dim, indirect light for 30 s. Between tests, cages were cleaned. Controls were age matched and subjected at random to the tests. Results were scored independently by two observers, one of which was unaware of the genetic status of the mice. The mice were tested twice with a 3 day interval.

Aggression test

FVB or transgenic male 'residents' (tested at 10–12 weeks old) were housed alone for 4 weeks in normal cages in the same room under the same conditions as other mice. Bedding was changed once a week without handling the animal, with the last change 6 days before the first test. 'Intruders' were male FVB mice (8 weeks old) kept in groups of eight in larger cages. After the intruder was placed in the resident cage, attack latency and number of attacks were recorded during 3 min. An attack was scored only when the resident bit the intruder. One week later the same resident mice were tested a second time with different intruders.

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