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Hypersensitivity to seizures in β-amyloid precursor protein deficient mice

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Abstract

Secreted forms of the β-amyloid precursor protein (β-APP) have neuroprotective properties *in vitro* and may be involved in the containment of neuronal excitation. To test whether loss of secreted forms of β-APP (sAPPs) may enhance excitotoxic responses, we injected mice homozygous for a targeted mutation of the β-APP gene (β-APP$^{\Delta/\Delta}$) intraperitoneally with kainic acid. We found that in these mice, kainic acid induced seizures initiated earlier, and acute mortality was enhanced compared to isogenic wild-type mice independently from the callosal agenesis phenotype observed to occur at increased frequency in APP mutant mice. Expression of c-*fos* in cortex and cingulate gyrus was enhanced in β-APP$^{\Delta/\Delta}$ mice, although the amount of structural damage and apoptosis in the hippocampal pyramidal cell layer and cortex was similar to that of controls. When cerebellar granule cell cultures and cortical neuronal cultures were challenged with glutamate receptor agonists, the rates of cell death and apoptosis of β-APP$^{\Delta/\Delta}$ mice were indistinguishable from those of controls. Therefore, deficiency of sAPPs causes facilitation of seizure activity in the absence of enhanced cell death. Since enhanced seizures were observed also in mice homozygous for a deletion of the entire β-APP gene, this phenotype results from a loss of APP rather than from a dominant effect of APP$^{\Delta}$.

Keywords: Alzheimer's disease; β-amyloid precursor protein; β-APP deficient mice; kainic acid; apoptosis; excitotoxicity; c-*fos*; neuronal cultures

Abbreviations: β-APP: β-amyloid precursor protein; sAPPs: secreted forms of the β-amyloid precursor protein; AD: Alzheimer's disease; APLPs: APP-like-proteins; PKC: protein kinase C; KA: kainic acid; TUNEL: In-situ nick end-labeling; GFAP: glial fibrillary acidic protein; MAP-2: microtubule associated protein-2; GCG: Cerebellar granule cell cultures

Introduction

Secreted forms of the β-amyloid precursor protein (sAPPs) are generated from the membrane-spanning holo-APP by proteolytic cleavage, and are secreted in an activity-dependant manner (Nitsch *et al*, 1993; Selkoe, 1993). sAPPs have powerful neurotrophic and neuroprotective properties *in vitro* and may be involved in the containment of neuronal excitation and in the regulation of Ca^{2+}-homeostasis (Mattson *et al*, 1993). Activation of a high conductance charybdotoxin-sensitive K^+-channel by sAPPs, leading to hyperpolarization of neurons and decrease of intracellular Ca^{2+}-concentrations, has been proposed to be a major mechanism of sAPP effect on neuronal excitation (Furukawa *et al*, 1996).

In vitro, sAPPs can protect rat hippocampal cultures against the toxic effects of glutamate and hypoglycemia by a mechanism that may involve stabilization of the intraneuronal concentration of Ca^{2+} (Mattson *et al*, 1993). *In vivo*, moderate overexpression of sAPPs in transgenic mice confers resistance to acute and chronic forms of excitotoxicity, and has a synaptotrophic effect (Mucke *et al*, 1994, 1996). However, it is presently unclear whether sAPPs are also neuroprotective at physiological levels.

Physiological processing of β-APP is thought to occur predominantly by α-secretase activity, resulting in peptide bond cleavage at position 16/17 of Aβ and secretion of the N-terminal protein as sAPP and thereby precluding production of the pathogenic full-length Aβ (Sisodia *et al*, 1990). An alternative pathway probably includes internalization of β-APP and involves the endosomal/lysosomal pathway and β-secretase cleavage at the N-terminus of Aβ, resulting in the release of potentially amyloidogenic fragments containing Aβ sequences (Haass *et al*, 1992).

In Alzheimer's disease (AD), abnormal cleavage of β-APP by β-secretase activity is thought to result in enhanced levels and altered isoforms of Aβ. One possible mechanism involves reduced amount and activity of protein kinase C (PKC) (Saitoh *et al*, 1991; Wang *et al*, 1994). Normally, activation of PKC can increase the rate of α-secretase processing (Gillespie *et al*, 1992) and reduce the secretion of the amyloidogenic Aβ-peptide (Hung *et al*, 1993). In AD, the PKC defect may lead to the diversion of a greater proportion of β-APP towards Aβ-forming pathways.

In addition, abnormal β-APP processing may also result in diminished levels of sAPPs. While Aβ-toxicity is thought to be the main pathogenetic principle underlying the neurodegeneration in AD, shortage of sAPPs has been proposed to contribute to the neurodegeneration by resulting in disturbed Ca^{2+} homeostasis and disinhibition of neuronal excitability (Furukawa *et al*, 1996). In

congruence with this hypothesis, decreased levels of soluble amyloid beta-protein precursor have been found in cerebrospinal fluid of sporadic Alzheimer disease patients (Van Nostrand *et al*, 1992). Furthermore, decreased absolute and relative amounts of α-secretase-cleaved soluble amyloid beta-protein precursor were detected in cerebrospinal fluid of Alzheimer disease patients with the Swedish mutation $APP_{670/671}$ (Lannfelt *et al*, 1995) and a PKC dependent deficiency in sAPP secretion was reported in fibroblasts from AD patients (Bergamaschi *et al*, 1995).

Another conspicuous feature of AD is the common occurrence of epileptic seizures and myoclonus. It is now recognized that AD alone is an important risk factor for new-onset epilepsy in older adults (Romanelli *et al*, 1990), with an incidence of seizures of up to ten times more than expected in a reference population (Hauser *et al*, 1986). Deficiency in sAPPs may contribute to the disinhibition of neuronal excitation underlying seizure generation by altering membrane potential and excitability (Furukawa *et al*, 1996).

To define the physiological properties of APP *in vivo*, we have taken advantage of mice with a targeted mutation in APP exon 2 (β-$APP^{\Delta/\Delta}$ mice). Due to missplicing and skipping of the targeted exon, these mice express no full-length APP and low amounts of a modified form of APP (APP^{Δ}) lacking amino acids 20–75 (Muller *et al*, 1994). Homozygous mutant mice are severely impaired in spatial learning and exploratory behavior, show reduced locomotor activity, reduced grip strength, retarded somatic growth and alterations in sensorimotor development (Muller *et al*, 1994). In addition, a high percentage of mutant mice on 129Sv(ev) genetic background show agenesis of the corpus callosum. However, no spontaneous neurodegeneration is apparent, no Alzheimer-type histopathological changes develop, and no spontaneous seizures are observed in β-$APP^{\Delta/\Delta}$ mice.

To determine whether the lack of β-APP confers enhanced vulnerability to excitotoxicity, we challenged β-$APP^{\Delta/\Delta}$ mice with injections of kainic acid (KA), and investigated the response of neuronal cultures of these mice to treatment with glutamate agonists. Kainic acid (KA) is a specific agonist of the kainate-subtype of glutamate receptors, which is expressed at high levels in the hippocampus (Lunn *et al*, 1996). Systemic administration of KA to rodents induces dose-dependent limbic and generalized seizures.

When mice with a deletion of the entire β-APP locus ($APP^{o/o}$ mice) became available (Li *et al*, 1996), the seizure experiment was repeated to exclude a confounding effect of the mutant APP^{Δ} in the previous experiments.

Results

Seizure induction

KA-induced behavioral alterations can be classified in three stages (Lothman *et al*, 1981): *Stage 1* is defined as staring, *stage 2* consists of automatisms and mild limbic convulsions. *Stage 3* is characterized by severe limbic convulsions, including forelimb clonus and loss of righting, or by the occurrence of generalized tonic-clonic seizures and status epilepticus. By electroencephalography, *stage 3* is characterized by bilateral cortical seizure activity. Barrel rotations are judged to be a particular severe form of motor seizures (Willcox *et al*, 1992), and can be induced by injections of KA or quinolinate into the striatum of rodents (Vescei and Beal, 1991), or by intraperitoneal administration of high doses of KA. Visible seizures following intraperitoneal injection of KA in mice typically occur as forelimb clonus with sudden onset

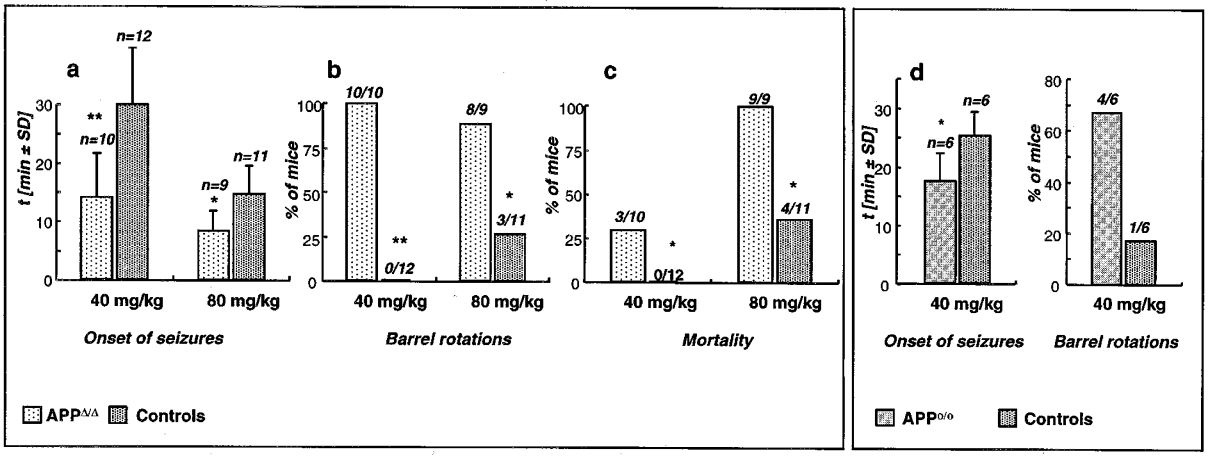

Figure 1 Response of β-$APP^{\Delta/\Delta}$ mice and controls to i.p. injection of kainic acid. (**a**) Latency from injection of kainic acid to manifestation of seizures. At 40 mg/kg KA, the mean time to the onset of seizures was 14.3 min *vs* 30.2 min in β-$APP^{\Delta/\Delta}$ mice and controls, respectively ($P<0.001$, student's *t*-test). At 80 mg/kg KA, the mean time to the onset of seizures was 8.6 min *vs* 14.9 min ($P=0.002$, student's *t*-test). (**b**) Occurrence of barrel rotations during seizures. At 40 mg/kg KA, all β-$APP^{\Delta/\Delta}$ mice displayed barrel rotations, whereas none of the controls did ($P<0.001$, chi-square test). At 80 mg/kg KA, 8/9 β-$APP^{\Delta/\Delta}$ mice and 3/11 controls displayed barrel rotations ($P=0.006$, chi-square test). (**c**) Mortality of seizures. At 40 mg/kg KA, 3/10 β-$APP^{\Delta/\Delta}$ mice died, but none of the controls ($P=0.04$, chi-square test). At 80 mg/kg KA, all β-$APP^{\Delta/\Delta}$ mice and 4/11 controls died ($P=0.003$, chi-square test). Response of β-$APP^{o/o}$ mice and controls to i.p. injection of kainic acid. (d) At 40 mg/kg KA, the mean time to the onset of seizures was 17.4 min *vs* 25.2 min in β-$APP^{o/o}$ mice and controls, respectively ($P=0.02$, student's *t*-test). 4/6 β-$APP^{o/o}$ mice displayed barrel rotations, whereas 1/6 controls did ($P=0.08$, chi-square test)

after a prodromal phase characterized by nondiscriminative behavioral alterations. In this study, the time span from the injection of KA to the manifestation of overt motor seizures in the form of forelimb clonus, loss of righting, barrel rotations or generalized tonic-clonic seizures was defined as latency.

The latency from the time point of injection to the manifestation of epileptic seizures was significantly shortened in β-APP$^{\Delta/\Delta}$ mice compared to wild-type controls (Figure 1a). The mean latency time at a dosage of 40 mg/kg KA was 14.3 min in β-APP$^{\Delta/\Delta}$ mice *versus* 30.2 min in controls ($P<0.001$), while at a dosage of 80 mg/kg KA the latency was 8.6 min in β-APP$^{\Delta/\Delta}$ mice versus 14.9 min in controls (P=0.002).

Seizures were also more severe in β-APP$^{\Delta/\Delta}$ mice (Figure 1b), with the appearance of barrel rotations in 100% of β-APP$^{\Delta/\Delta}$ mice versus none of the controls at a dosage of 40 mg/kg KA ($P<0.001$) and in 90% of β-APP$^{\Delta/\Delta}$ mice *vs* 27% of controls at a dosage of 80 mg/KA (P=0.006). In APP mutants, visible seizures often initiated directly with barrel rotations, without previous display of classical limbic seizures.

Acute mortality was also significantly enhanced in β-APP$^{\Delta/\Delta}$ mice (Figure 1c). In the group receiving 40 mg/kg KA, 3/10 mutants animals died in epileptic status in a rigid state (presumably due to ATP-depletion), compared to 0/12 animals in the control group (P=0.04). In the group receiving 80 mg/kg KA, 9/9 mutants died, compared to 4/11 animals in the control group (P=0.003).

In the β-APP$^{o/o}$ mice homozygous for the deletion of the entire β-APP locus (Figure 1d), the mean latency time at a dosage of 40 mg/kg KA was 17.4 min in β-APP$^{o/o}$ mice *versus* 25.2 min in controls (P=0.02), with the appearance of barrel rotations in 4/6 of β-APP$^{o/o}$ mice *versus* 1/6 of the controls at a dosage of 40 mg/kg KA (P=0.08). Therefore, β-APP$^{o/o}$ mice behaved similarly to β-APP$^{\Delta/\Delta}$ mice with respect to KA hypersensitivity.

To determine whether callosal agenesis was responsible for the phenotype, we asked whether absence of the corpus callosum correlates with latency time and mortality for the groups of mutant animals and wild-type controls receiving 80 mg/kg KA (Table 1). Agenesis of the corpus callosum was found in all β-APP$^{\Delta/\Delta}$ mice (n=9). However, in two groups of controls from independent experiments, agenesis of the corpus callosum was found in 3/10 and in 3/11 animals of 129 Sv(ev) genetic background.

Death occurred in a total of 8/15 controls with normal corpus callosum and in 1/6 animals with agenesis (P=0.125, chi-square-test). The mean latency time was 13.5 min in normal controls *vs* 16.4 min in animals with callosal agenesis (P=0.2, Student's *t*-test).

These data show no significant differences in the sensitivity to seizures and resulting mortality in 129Sv wild-type mice depending on the corpus callosum status, with a trend towards a less severe response to KA-induced seizures in mice with agenesis of the corpus callosum.

To exclude that the presence or absence of the corpus callosum was responsible for the differences of latency time and mortality observed between β-APP$^{\Delta/\Delta}$ mice and controls, the subgroup of 129Sv(ev) controls with callosal agenesis was compared to the group of β-APP$^{\Delta/\Delta}$ mice (with callosal agenesis in all animals) at 80 mg/kg KA (Table 1). Death occurred in 1/6 controls with callosal agenesis and in 9/9 β-APP$^{\Delta/\Delta}$ mice (P=0.005, chi-square-test). The mean latency time was 16.4 min in controls with callosal agenesis and 8.6 min in β-APP$^{\Delta/\Delta}$ mice (P=0.001, Student's *t*-test).

These data provide evidence for hypersensitivity to KA-induced seizures in β-APP$^{\Delta/\Delta}$ mice independently from callosal agenesis.

Table 1 No correlation of callosal agenesis and outcome of seizures

Genotype	Corpus callosum	*n*	Latency to seizure onset	Deaths (n)
129Sv(ev)	Agenesis	6	16.4 ± 3.7	1
129Sv(ev)	Normal	15	13.5 ± 5.8	8
			p (T) = 0.2	p (χ^2) = 0.125
129Sv(ev)	Agenesis	6	16.4 ± 3.7	1
β-APP$^{\Delta/\Delta}$	Agenesis	9	8.6 ± 3.2	9
			p (T) = 0.005	p (χ^2) = 0.001

All animals were treated with 80 mg/kg kainic acid. Callosal agenesis is a frequent anomaly in 129 mice which are wild-type for the APP gene: however, neither latency of kainate-induced seizures nor mortality correlate significantly with callosal agenesis. Instead, both parameters correlate strongly with the β-APP$^{\Delta/\Delta}$ genotype

Induction of c-*fos*

The temporo-spatial pattern of c-*fos* induction was investigated by *in situ* hybridization in order to obtain a measure of electrical activity and increased intracellular Ca^{2+}-concentration following i.p. injection of KA. In the hippocampus, the pattern of c-*fos* expression was largely similar in β-APP$^{\Delta/\Delta}$ mice and controls. The expression pattern in the different sublayers had a characteristic dynamic development (Figure 2): 30 min after injection, expression was most pronounced in the dentate gyrus. At 60 min, there was equally strong expression in the dentate gyrus and the CA1 layer, while at 120 min, expression in the dentate gyrus had faded, and expression in the CA3 layer was becoming more prominent. Relative sparing of the CA2 sublayer was evident at all time points. Low levels of expression were observed in the hippocampus at very late time points (48 h and 7 days after injection) in single scattered cells in the CA1 and CA3 layers and in the dentate gyrus in both groups (not shown).

In contrast, in β-APP$^{\Delta/\Delta}$ mice expression of c-*fos* in the cortex and cingulate gyrus was enhanced compared to controls at all time points (Figure 2). This was true both for the absolute amount of staining and for the relative staining intensity in the cortex compared to the hippocampus.

In other areas with considerable basal or KA-induced expression of c-*fos*, such as amygdala and raphe nuclei, we found no recognizable differences in the amount of expression between groups. The expression in the hippocampus and cortex was generally bilateral and symmetric in both groups. Therefore, the phenotypic characteristic of enhanced susceptibility to seizures in β-APP$^{\Delta/\Delta}$ mice is paralleled by elevated target gene transcription in the cortex and cingulate gyrus, but not in the hippocampus.

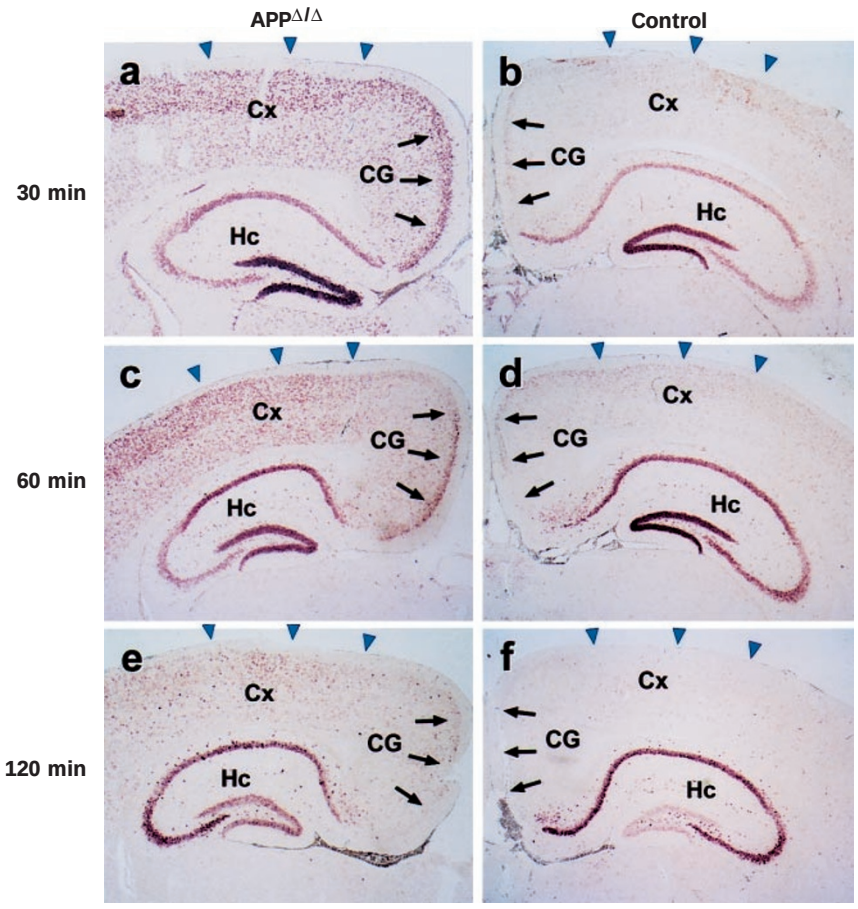

Figure 2 Temporo-spatial pattern of c-*fos* induction following i.p. injection of kainic acid. *In-situ* hybridization for c-*fos*: (**a**, **b**) 30 min after injection. (**c**, **d**) 60 min after injection. (**e**, **f**) 120 min after injection. In the hippocampus, the pattern of c-*fos* expression was largely similar in β-APP$^{\Delta/\Delta}$ mice and controls. At 30 min, expression was most pronounced in the dentate gyrus. At 60 min, there was equally strong expression in the dentate gyrus and the CA1 layer, while at 120 min, expression in the dentate gyrus had faded, and expression in the CA3 layer was becoming more prominent. Note relative sparing of the CA2 sublayer. In contrast, in β-APP$^{\Delta/\Delta}$ mice there was enhanced expression of c-*fos* in the cortex (arrowheads) and cingulate gyrus (arrows) compared to controls at all time points. CG, cingulate gyrus; Cx, cortex; Hc, hippocampus

Histopathologic changes

Morphological damage to the hippocampus and cerebral cortex was assessed by histology and immunohistochemistry. Seven days after injection of 40 mg/kg KA, the most pronounced lesions were found in the CA3 sector of the hippocampus; in some animals also the CA1 sector was affected (2/7 β-APP$^{\Delta/\Delta}$ and 5/12 wild-type mice) (Figure 3a,b) as described previously (Schwob *et al*, 1980). In addition to neuronal loss, synaptodendritic damage was apparent in immunostains for synaptophysin and MAP-2, with visibly diminished neuropil density extending into the white matter of the hippocampus (Figure 3c,e). Analysis of surviving animals from both groups which had received 40 mg/kg kainic acid evidenced some variability in the morphological degree of tissue damage after 7 days. However, differences in the severity of lesions between the two groups were not larger than individual differences within each group.

The extent of TUNEL labeling in the hippocampal formation and in the cortex was also similar 48 h and 7 days after injections (Gillardon *et al*, 1995) (Figure 3g–k).

Cytotoxicity in neuron cultures

In order to test directly whether excitotoxicity due to glutamate receptor hyperstimulation was modified by β-APP deficiency, experiments were performed in primary neuronal cultures. Incubation of cerebellar granule cells (CGC) from wild-type or β-APP$^{\Delta/\Delta}$ mice with different glutamate receptor agonists resulted in concentration-dependent, predominantly apoptotic cell death. The sensitivity of β-APP$^{\Delta/\Delta}$ neurons was not significantly different from the one of wild-type neurons (Figure 4). Similar results were obtained when cultures of cortical neurons were exposed to glutamate (10–1000 μM), kainic acid (20–200 μM) or NMDA (20–100 μM). Neither the time course nor the extent of toxicity were significantly different between wild-type or β-APP$^{\Delta/\Delta}$ cultures after NMDA-exposure for 30 min only (Figure 5a–d) or glutamate exposure over the entire period of 24 h (Figure 5e).

Discussion

We have observed that β-APP$^{\Delta/\Delta}$ mice were more vulnerable

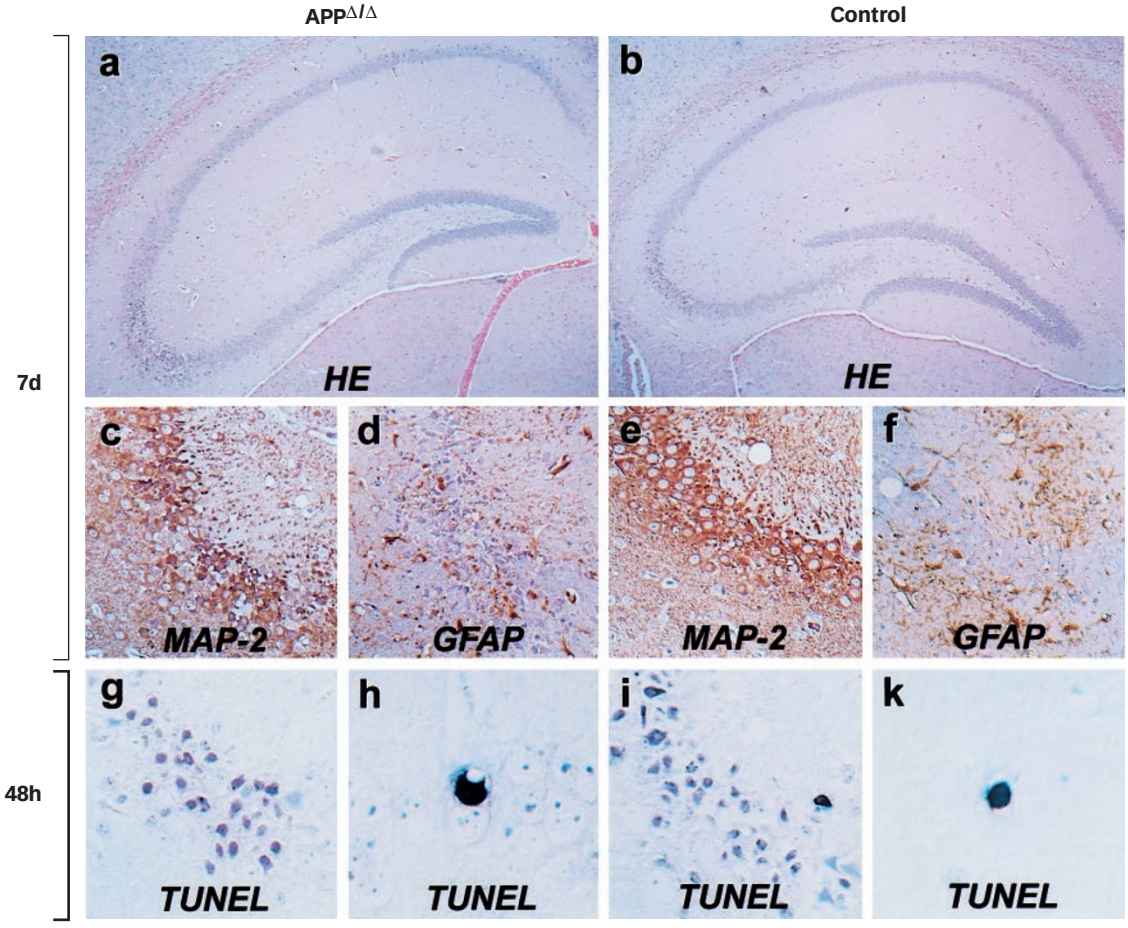

Figure 3 Tissue damage following i.p. injection of kainic acid. (**a**–**f**) Morphological changes after 7 days. (**a**, **b**) In both groups, nerve cell loss was most pronounced in the CA3 layer of the hippocampus, with diminished neuropil density in the adjacent white matter. HE staining. (**c**, **e**) Synaptodendritic damage is revealed by MAP-2 staining. (**d**, **f**) Reactive astrogliosis surrounding the pyramidal cell layer, GFAP staining. (**g**–**k**) Apoptosis after 48 h. (**g**, **i**) In the CA3 layer, there were abundant pyknotic nuclei with evidence of DNA-fragmentation, TUNEL-staining. (**h**, **k**) In addition, single apoptotic cells were found in the cortex, TUNEL-staining. No consistent differences in the amount and distribution of tissue damage and apoptosis were observed between groups

to kainic acid – induced epilepsy. Seizures started earlier, were more severe, and led to enhanced mortality compared to wild-type mice.

The visible manifestations of overt seizure activity were altered, with prominent appearance of barrel rotations, which are considered to be a more severe form of seizures than classical limbic seizures (Willcox *et al*, 1992). This is supported by the occurrence of barrel rotations also in some of the wild type controls at the high dosage of 80 mg/kg, but not at 40 mg/kg (Figure 1c). At very high dosages (100 mg/kg), barrel rotations were induced in most wild-type controls (data not shown). In addition, barrel rotations can be induced by direct stereotaxic injections of KA or quinolinate into the striatum of rodents (Vecsei and Beal, 1991). It is unclear, however, why in many β-APP$^{\Delta/\Delta}$ mice visible seizures initiated directly with barrel rotations, without previous display of classical limbic seizures, such as forelimb clonus.

A high percentage of β-APP$^{\Delta/\Delta}$ mice show agenesis of the corpus callosum (95% on pure 129Sv(ev) background), but this malformation is also observed at low frequency in the parental 129Sv strain (approximately 15% of wild-type 129Sv(ev) mice).

The corpus callosum is generally held to be a substrate for propagation, bilateralization and generalization of seizures, and section of the corpus callosum can be beneficial in most types of seizures (Spencer, 1988). However, in one study kainic acid induced seizures were aggravated by callosotomy in a rat model (Hirsch *et al*, 1992). This result was interpreted as evidence for an inhibitory influence of the non-epileptic hippocampus and neocortex on its contralateral homologue (Hirsch *et al*, 1992). In contrast, no data have been available on the effects of congenital agenesis of the corpus callosum towards the vulnerability to seizures. We have shown here, that 129Sv(ev) wild-type mice with callosal agenesis are not more sensitive to KA-induced seizures (Table 1). There is even a trend towards a slight protection from the adverse effects of KA in the animals with callosal agenesis. In addition, the differences in latency time and mortality in the subgroup of controls with callosal agenesis compared to β-APP$^{\Delta/\Delta}$ mice were undiminished and significant. Therefore,

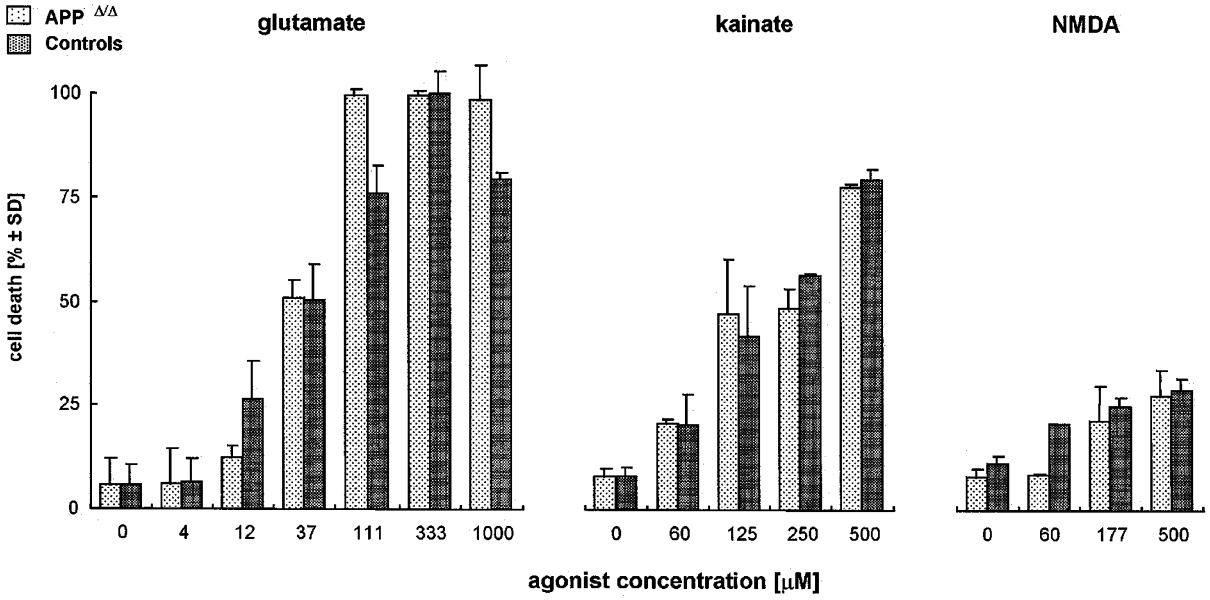

Figure 4 Glutamate receptor-mediated cytotoxicity in murine cerebellar granule cell cultures. CGC (8 DIV) from wild-type mice or β-APP$^{\Delta/\Delta}$ mice were challenged in their original medium with the glutamate receptor agonists at the indicated concentrations. The percentage of cell death was quantitated 24 h later by counting of cells with condensed nuclei and broken plasma membrane. No significant differences in the susceptibility towards either glutamate agonist were found. Data are means $\pm$ S.D. of three independent determinations

we can exclude the callosal agenesis as cause of the enhanced susceptibility to KA-induced seizures in β-APP$^{\Delta/\Delta}$ mice.

However, we could not formally exclude that the vulnerability to seizures is influenced by secretion of soluble APP$^{\Delta}$ fragments with altered biological properties. Therefore, the seizure experiment was repeated with mice bearing a deletion of the entire β-APP locus (Li *et al*, 1996), to exclude a confounding effect of the mutant APP$^{\Delta}$ in the previous experiments. Although only a small number of mice were available, which were more heterogeneous with regard to age and sex, and which were raised on a slightly different background (129Sv(ev) $\times$ 129Ola), the results confirmed enhanced vulnerability to seizures.

The bilateral and symmetric induction of c-*fos* in the cortex corresponds to the bilateral cortical seizure activity found during stage 3 seizures in electroencephalography (EEG) studies (Hirsch *et al*, 1992). The finding of enhanced induction of c-*fos* in the cortex and cingulate gyrus constitutes molecular evidence for augmented neuronal excitation in β-APP$^{\Delta/\Delta}$ mice. Such enhanced excitability in the cortex may be the substrate of enhanced vulnerability to seizures and death. This may be a consequence of diminished neuronal inhibition due to loss of sAPP function, but an influence of corpus callosum agenesis on the induction of c-*fos* cannot be ruled out with certainty. The temporospatial expression pattern of c-*fos* in controls in this study was similar to that reported previously (Smeyne *et al*, 1992).

c-*fos* can act as a mediator of cell death and apoptosis in some systems (Hafezi *et al*, 1997), and the absence of enhanced structural damage to the hippocampus maps with the failure to detect enhanced induction of c-*fos*. In the cortex, there was elevated expression of c-*fos* in β-APP$^{\Delta/\Delta}$ mice, but the amount of apoptosis was generally low, with only some scattered TUNEL-positive cells detectable in both groups. Perhaps, cortical neurons from β-APP$^{\Delta/\Delta}$ mice have a somewhat higher susceptibility to excitotoxic cell death, which does not become apparent under our experimental conditions. However, *in vitro* there were no differences in the vulnerability of β-APP$^{\Delta/\Delta}$ and wild-type cortical neuronal cultures. Thus it is likely that c-*fos* expression merely provides a marker for KA induced target gene expression and does not mediate the induction of downstream processes of cell death.

There were no differences between β-APP$^{\Delta/\Delta}$ mice and controls in the amount of morphological damage, synapto-dendritic damage and extent of TUNEL labeling. Therefore, three independent lines of evidence support the conclusion that there is no enhanced tissue vulnerability to excitotoxic insult in β-APP$^{\Delta/\Delta}$ mice.

A generally lower resistance to excitotoxic neuronal death was also not apparent in the *in vitro* studies. Both in cerebellar granule cell cultures and cortical cultures, the mode (i.e. apoptosis or necrosis) and amount of cell death following treatment with different glutamate receptor agonists was similar in both groups, and the thresholds of glutamate toxicity were the same. This appears to be in contrast with previous studies, which reported that sAPPs efficiently protect neurons against excitotoxicity *in vitro* (Mattson *et al*, 1993a,b). However, these pharmacological studies were performed in rats with exogenous sAPPs in addition to the endogenous sAPPs. The effects of loss of physiological amounts of sAPPs may be more subtle, and they can possibly be compensated by other factors, e.g. APP-like proteins (APLPs).

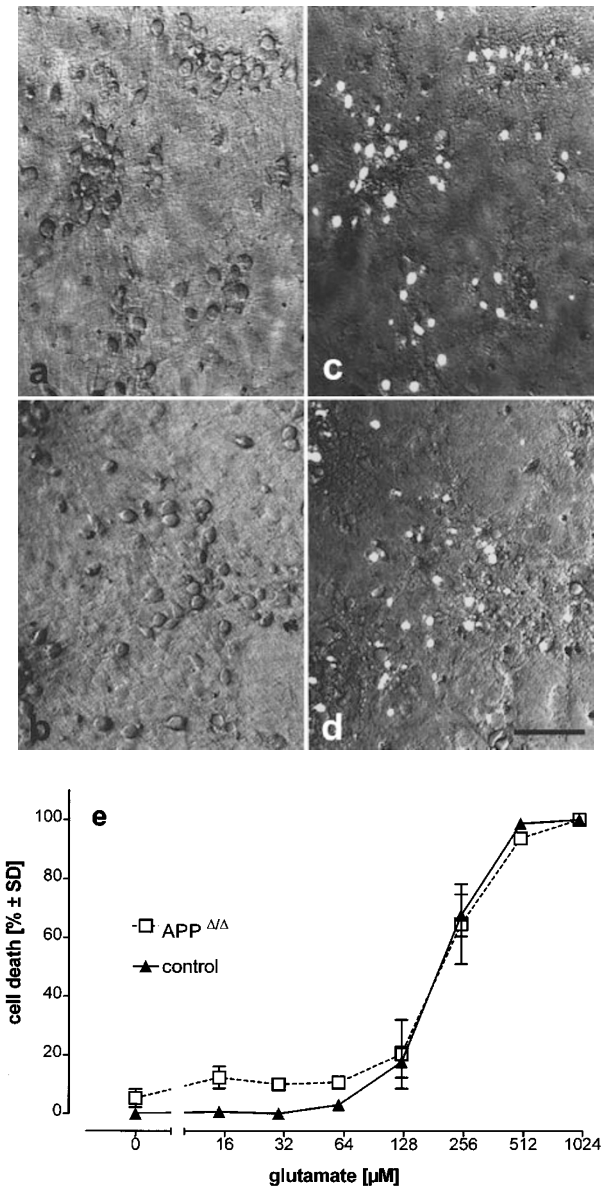

Figure 5 Glutamate receptor-mediated cytotoxicity in murine cortical neuron cultures. (**a**–**d**) Morphology of glutamate cytotoxicity on cortical neuron cultures. Cortical neuron cultures from wild-type mice (**a**, **c**) or β-APP$^{\Delta/\Delta}$ mice (**b**, **d**) were imaged under phase contrast optics (**a**, **b**) and exposed to 50 μM NMDA for 30 min in CSS-solution. After 24 h the membrane-impermeant DNA-staining dye SYTOX was added and the same fields were imaged by a combination of phase contrast and fluorescent microscopy (**c**, **d**). (**e**) Thresholds for glutamate toxicity. Cortical neurons (13 DIV) from wild-type mice or β-APP$^{\Delta/\Delta}$ mice were challenged in their original medium with glutamate. The percentage of cell death was quantitated 24 h later by counting of remaining viable cells in the same fields. Data are means $\pm$ S.D. of three independent determinations. Scale bar=40 μm

During initial stages of *sporadic* AD, seizures are usually considered relatively infrequent, but tend to become more frequent toward the end of the clinical course (Hauser *et al*, 1986; Romanelli *et al*, 1990). However, in many kindreds affected by *familial* AD caused by β-APP or PS-1 mutations, seizures and myoclonic jerks are consistent early and prominent signs (Haltia *et al*, 1994), in some pedigrees affecting more than 80% of individuals (Lampe *et al*, 1994). This corresponds to pronounced misprocessing of β-APP with consequent reduction of α-secretase cleared protective sAPPs (Lannfelt *et al*, 1995).

Our data support the hypothesis that sAPPs are involved in the containment of neuronal excitation *in vivo*, and that lack of sAPPs leads to diminished inhibition of the neuronal circuitry, and thereby lowers the threshold for seizure activity. Since K^+ channels are major determinants of membrane excitability (Tsaur *et al*, 1992), this is well compatible with *in vitro* data showing activation of a K^+-channel which modulates Ca^{2+} influx by sAPPs, thereby leading to hyperpolarization and suppression of action potentials (Furukawa *et al*, 1996).

Possibly, sAPPs serve to counteract the effect of Aβ itself, which can exert neurotoxic effects similar to glutamate *in vitro*. This is corroborated by data showing the efficiency of antiepileptic drugs against both glutamate and Aβ-induced toxicity in rat hippocampal neurons (Mark *et al*, 1995).

On the other hand, the mechanism responsible for increased vulnerability to seizures did not result in enhanced susceptibility to cell death. It is possible that compensation by other factors such as APLPs may alleviate tissue damage. In addition, excitotoxic injury seems to depend mainly on Ca^{2+} influx via NMDA channels (Rothman and Olney, 1995). There is no evidence for a direct interaction of sAPPs with NMDA channels, and enhanced Ca^{2+} influx in β-APP$^{\Delta/\Delta}$ mice may occur via pathways that are less detrimental. It has been demonstrated that Ca^{2+} entry into neurons through NMDA-channels causes substantially higher toxicity than similar elevations of cytosolic Ca^{2+} concentrations from other sources (Dugan *et al*, 1995; Tymianski *et al*, 1993).

While in β-APP$^{o/o}$ mice there is a complete absence of the β-APP gene, there may be also an additional loss of other relevant genes in the 200 kb-spanning deletion. Conversely, there is a more subtle ablation of the β-APP gene in β-APP$^{\Delta/\Delta}$ mice, but a small amount of a modified protein (APP$^{\Delta}$) is made. Since the results with both strains were congruent, we conclude that the lack of the β-APP gene is responsible for the observed hypersensitivity to KA-induced seizures rather than genetic artifacts of the recombination procedure or dominant effects of the mutant APP$^{\Delta}$. In principle, the lack of the β-APP gene will result in lack of the membrane-spanning holo-APP, lack of sAPPs and lack of Aβ. Therefore, the observed phenotype may be due to the deficit of any of these three molecules. However, it appears most likely that the lack of sAPPs is responsible for the hypersensitivity to seizures, since Aβ is a strong neurotoxic agent itself, which facilitates excitotoxicity (Koh *et al*, 1990) and induces neuronal apoptosis (Loo *et al*, 1993), and holo-APP has not been shown to be neuroprotective. The concomitant lack of both Aβ and sAPPs may even mask the effects of the loss of the latter, since it has been hypothesized that sAPPs may protect against the toxic effects of Aβ itself (Furukawa *et al*, 1996). In conclusion, our data suggest that reduced levels of sAPPs may contribute to the common occurrence of epileptic seizures in AD patients.

Materials and Methods

Animals

β-APP$^{\Delta/\Delta}$ mutants of pure 129Sv(ev) background were bred and characterized as described previously (Muller *et al*, 1994). Age-matched isogenic mice of 129Sv(ev) substrain were used as controls. β-APP$^{o/o}$ mice were bred on 129Sv(ev) $\times$ 129Ola background. All procedures were in accordance with institutional guidelines and the federal laws for animal protection and European Community Council Directive 86/609/EEC.

Seizure induction

Groups of 9–12 β-APP$^{\Delta/\Delta}$ mice and controls aged 6 weeks to 3 months were injected intraperitoneally with kainic acid (Sigma) at concentrations of 40 and 80 mg/kg. Groups of 6 β-APP$^{o/o}$ mice and controls aged 4 weeks to 3 months were injected intraperitoneally with kainic acid (Sigma) at a concentration of 40 mg/kg. Animals were placed in separate Plexiglas cages and monitored for onset of seizures.

Histology

Surviving animals were sacrificed 30, 60 and 120 min, 48 h and 7 days following the injections of kainic acid. Brains were removed, fixed overnight in 4% (wt/vol) paraformaldehyde in PBS, and embedded in paraffin. Two μm coronal and sagittal sections were mounted on silanized slides and stained with hematoxylin and eosin (H&E) and Luxol-Nissl. Immunohistochemistry was performed using polyclonal antibodies to glial fibrillary acidic protein (GFAP, DAKO, 1:300), synaptophysin (DAKO, 1:40) and microtubule associated protein-2 (MAP-2, Boehringer Mannheim, 1:1000). Biotinylated secondary antibodies (DAKO, 1:200), biotin/avidin-peroxidase (DAKO) and diaminobenzidine (Sigma) as chromogen were employed according to the instructions of the manufacturers.

TUNEL

In-situ nick end-labeling (TUNEL) was performed on sections of the hippocampus 48 h and 7days after the injections, using the *in-situ* cell death detection kit II (Boehringer Mannheim). Slides were deparaffinized, and sections were digested with proteinase K (20 μg/ml) for 15 min at 37°C, followed by incubation with terminal transferase for 1 h at 37°C in the presence of fluorescein-labelled dUTP. An alkaline phosphatase coupled anti-fluorescein Fab fragment was used for detection, and 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium (Boehringer Mannheim) were employed as chromogens.

Cell cultures and cytotoxicity

Cortical cultures of E15.5 embryos were prepared and cultured as described (Rose *et al*, 1993). Briefly, they were plated at a density of 3.2×10^5/well in 24 well plates (Costar) with poly-L-lysine (PLL)-coating or alternatively on a confluent monolayer of murine cortical glial cells. Medium was changed twice per week. Glial cell proliferation was arrested by addition of cytosine arabinoside (10 μM) between day *in vitro* (DIV) 6–8. At DIV11 cells were switched to serum-free medium and used for experiments at DIV13–17. Cerebellar granule cell cultures (CGC) were prepared from 8 day old newborn mice as described (Schousboe *et al*, 1989). Cells were seeded in 24 well plates coated with 50 μg/ml PLL at a density of 0.75×10^6 cells/ml, treated with cytosine arabinoside after 48 h (10 μM) and used for experiments without medium change at DIV 7–10.

Apoptosis of CGC was quantitated as described in detail previously (Ankarcrona *et al*, 1995; Leist *et al*, 1997a). Cells were incubated briefly with a cell-permeant (H-33342) and a non-cell-permeant (ethidiumhomodimer-1) fluorescent chromatin stain. At early time points they showed a condensation of the chromatin and maintained the integrity of the plasma membranes. When cell death was quantitated after 24 h, most nuclei of dead cells were condensed and the plasma membrane was broken. For viability assays in cortical neuron cultures, phase contrast/fluorescent images of defined fields (mask for x-y-coordinates) were recorded immediately prior to the start of the experiment and 24 h later. For visualization of broken plasma membranes, 0.5 μM of the non-toxic, membrane impermeant chromatin stain SYTOX (Molecular Probes) was added to the culture medium (Leist *et al*, 1997b). The percentage of remaining viable neurons (morphological integrity and SYTOX-exclusion) was quantitated by counting of at least 300 cells. Incubations were performed either in culture medium for 24 h or in controlled salt solution ((CSS) in mM: NaCl, 116; KCl, 5.4; 0.8; $NaHCO_3$, 25, glucose, 5.5, glycine, 0.1; HEPES, 12; pH 7.5) for 30 min and post-incubation in medium for 24 h. In control cultures the validity of the method was verified by comparison with results obtained in cultures that had been fixed and stained with antibodies against MAP-2.

In situ hybridization

Sense and antisense RNA probes were transcribed *in vitro* with T3 and T7 RNA polymerase from linearized plasmid pBFos (Hafezi *et al*, 1997) in the presence of digoxigenin-11-dUTP (Boehringer Mannheim, Germany). Fifty to 200 ng of labeled transcripts were hybridized to paraformaldehyde fixed paraffin tissue sections at 65°C as described (Weissenberger *et al*, 1997). Digoxigenin was detected with alkaline phosphatase-labeled anti-digoxigenin Fab fragments and 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim, Germany).

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