Passive Immunization against β-Amyloid Peptide Protects Central Nervous System (CNS) Neurons from Increased Vulnerability Associated with an Alzheimer’s Disease-causing Mutation*

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To characterize the effects of the familial Alzheimer’s disease-causing Swedish mutations of amyloid precursor protein (SwAPP) on the vulnerability of central nervous system neurons, we induced epileptic seizures in transgenic mice expressing SwAPP. The transgene expression did not change the seizure threshold, but consistently more neurons degenerated in brains of SwAPP mice as compared with wild-type littermates. The degenerating neurons were stained both by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling and by Gallyas silver impregnation. A susceptible population of neurons accumulated intracellular Aβ and immunoreacted with antibodies against activated caspase-3. To demonstrate that increased Aβ levels mediated the increased vulnerability, we infused antibodies against Aβ and found a significant reduction in neuronal loss that was paralleled by decreased brain levels of Aβ. Because the SwAPP mice exhibited no amyloid plaques at the age of these experiments, transgenic overproduction of Aβ in brain rendered neurons susceptible to damage much earlier than the onset of amyloid plaque formation. Our data underscore the possibility that Aβ is toxic, that it increases the vulnerability of neurons to excitotoxic events produced by seizures, and that lowering Aβ by passive immunization can protect neurons from Aβ-related toxicity.

Alzheimer’s disease (AD) is the most common cause of dementia in the elderly. Both familial and sporadic cases share the deposition in brain of extracellular β-amyloid plaques as a common pathological hallmark. β-Amyloid is associated with the formation of neurofibrillary tangles, impaired synaptic functions, and the loss of neurons (1–4). β-Amyloid deposits are composed of amyloid-β peptides (Aβ) that aggregate to form amyloid fibrils (5). Increased generation of the highly amyloidogenic 42-residue form (Aβ42) is associated with most, if not all, mutations that cause familial AD (6–12). In AD patients there is an inverse correlation between brain levels of Aβ, synaptic density, and cognitive functions (13–15). Oligomers of Aβ, proteofibrils, and fibrillar forms of Aβ are neurotoxic (16–22). This toxicity may be mediated by oxidative stress (23–26). Injections of Aβ fibrils into mammalian brains causes neuronal loss accompanied by microglial proliferation, abnormal tau phosphorylation, loss of microtubule binding, and the formation of neurofibrillary tangles (1, 27, 28). Transgenic expression of the familial AD-causing mutant APP in brains of mice increases Aβ generation causing both amyloid plaque formation and abnormal phosphorylation of tau. However, neither neurofibrillary tangles nor neuronal loss were consistently observed in these mice (11, 29–38). The reasons for this discrepancy to AD pathology are unknown but may involve the young age of the mouse neurons along with the absence of pathogenic factors that may occur while aging.

To examine whether transgenic expression of SwAPP in mouse brain increases the vulnerability of neurons to cellular stress, we induced seizures in mice expressing SwAPP and analyzed the neurodegeneration and apoptotic cell death in brains at different times following the seizures (39–41). We determined whether the increased levels of Aβ mediated the increased vulnerability of the SwAPP neurons. We report that brain neurons of SwAPP mice were more vulnerable than non-transgenic littermates, even before the onset of amyloid plaque formation, and that passive immunization with antibodies against Aβ protected the neurons from degeneration.

EXPERIMENTAL PROCEDURES

SwAPP Mice—SwAPP mice were obtained from Dr. K. Hsiao (University of Minnesota, Minneapolis, MN) (30). The colony was maintained by backcrossing transgenic males to non-transgenic (non-tg) females from the same colony. Mice were housed under a light cycle of 12 h with dry food pellets and water available ad libitum, and experiments were done according to the guidelines for animal care of the University of Zurich, Zurich, Switzerland. Mice were weaned at 4 weeks of age, and the presence of the SwAPP transgene was determined by PCR using genomic DNA isolated from tail biopsies and specific primers 5'-GTG GAT ACC CCC TCC CCC AGC CTA GAC CA-3' and 5'-CTG ACC ACT CGA CCA GGT TCT GGG T-3' (30).

Seizures—The induction of epileptic seizures was done as described (40). In brief, 8-week-old SwAPP and non-transgenic littermates received an intraperitoneal injection of pilocarpine (360–400 mg/kg, Sigma) 30 min after receiving an injection of scopolamine methylbromide (1 mg/kg, Sigma) to reduce peripheral cholinergic activation (40). Seizure severity was staged according to the clinical phenotype by two independent observers as follows: stage 1 (mild seizures): wet dog shakes, arrest, rigid posture, facial twitches; stage 2: head nodding, chewing; stage 3 (moderate seizures): forelimb clonus, continuous shaking, rearing, but not falling; stage 4: falling on forelimbs, rearing and falling; stage 5 (severe seizures): falling on side or back, hindlimb clonus, seizure of all limbs, repetitive seizures of body (42–44). Only mice that were rated identically by both observers were used in the...
study. We analyzed mice with stages 1, 3, and 5. The time of onset and the severity of the seizures were monitored throughout the experiment (42–44). The duration of the seizures was kept constant in all groups by terminating the seizures with 10 mg/kg diazepam (Roche Molecular Biochemicals) 30 min after the onset of seizures (40, 45). To ensure exact duration of seizures, mice with continued seizures, with the last 40 min after diazepam injections were eliminated from the study. Control mice were treated identically but received PBS instead of pilocarpine.

**Tissue Preparation and Histochecmy**—To determine the effects of transgene expression on numbers of degenerating neurons, 5–10 mice per group following various seizures were anesthetized 1 and 7 days after the seizures and perfused transcardially with ice-cold PBS (pH 7.4) and 4% paraformaldehyde. Brains were postfixed overnight in 4% paraformaldehyde at 4 °C and washed, and 35-μm thick serial coronal sections were cut through the hippocampus. To quantify the levels of Aβ in brains of SwAPP mice after the seizures, mice (n = 5 per group) were perfused with ice-cold PBS (pH 7.4), and brains, excluding cerebellum, were homogenized in lysis buffer.

Passively immunized and control mice were analyzed by both ELISA and histochemistry. These mice were perfused transcardially with ice-cold PBS (pH 7.4), and the frontal parts of the brains containing inter-aural regions 6–4 (46) of both hemispheres were homogenized in lysis buffer. The remaining brain tissue was fixed for 24 h in 4% paraformaldehyde at 4 °C, washed several times in PBS, and processed for histology. Sections were stained by a terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) according to the manufacturer’s instructions (Roche Molecular Biochemicals) and Gallyas silver impregnation protocols (47) to assess the degree of cell damage. Immunostaining was performed with an antibody against activated caspase-3 (Cell Signaling Technology) combined with anti-Aβ antibodies. Anti-Aβ antibodies used were 4G8 (Senetec), pan-Aβ, and anti-β-amyloid 40 (both from BIOSOURCE International) all giving similar results. Neither pan-Aβ nor anti-β-amyloid 40 antibodies cross-react with full-length APP (48). Adjacent sections were stained with antibodies against glial fibrillary acidic protein (Sigma and Inno-Genex) and MAP-2 (Chemicon). Combined MAP-2 and Gallyas staining of same sections was done sequentially. 22C11 (Roche Molecular Biochemicals) and 6E10 (Senetec) antibodies were used to determine the amounts of full-length APP in Western blots. The spatial patterns of same sections was done sequentially. 22C11 (Roche Molecular Biochemicals) and 6E10 (Senetec) antibodies were used to determine the amounts of full-length APP following the seizures (not shown). Cells in areas of the hippocampus were determined at 400× magnification in three standardized frontal adjacent sections, each 105 μm apart from the middle section at 1.70 mm posterior to bregma according to a stereological mouse brain atlas (46). Images were digitized by a CCD camera (Sony), and positive cells were counted by using the KAPPA Imagebase software (KAPPA opto-electronics, Gießen, Germany). The average number of positive cells in each hippocampus was compared for each group and group means were compared statistically by the non-parametric Mann-Whitney U test.

**Aβ ELISA**—For quantification of concentrations of SDS-soluble Aβ, we homogenized brain tissue in lysis buffer containing 2% SDS, 1% Triton X-100 (pH 7.6) for 2 h, the supernatant fluids by adding 20 volumes 1 M Tris base. Microtiter plates (Maxi sorb, Nunc) were coated overnight at 4 °C and then incubated 1 h at room temperature with 1% bovine serum albumin, 1% gelatin in 100 mM Tris, 5 mM EDTA, and 0.1% Tween 20 (pH 7.6) for 4 h at 37 °C and then washed three times with PBS containing 0.02% Tween 20. 150 μl of diluted samples or serial dilution of standard (Aβ1–40, Bachem) in the above buffer was incubated overnight and washed, and bound Aβ was detected with biotinylated monoclonal mouse antibody 6H1 (Evotec) directed against amino acids 1–17 of human Aβ incubated at a concentration of 1 μg/ml in blocking buffer for 4 h at 37 °C and visualized by a peroxidase reaction using tetramethylbenzidine as substrate at 450 nm. The linear range (r² ≥ 0.99) of the used ELISA system was between 2.5–100 ng Aβ/ml. Serial dilutions of samples were used for measurements in the linear range. In parallel, the concentration of Aβ42 in each sample was determined using a commercially available kit (Innogenics) according to the provider’s instructions.

**Passive Immunization against Aβ**—Passive immunization was done by intravenous injections of an Aβ-specific monoclonal antibody into the tail vein. SwAPP mice were immunized by injections of 22C4 antibody, 9 mg/kg body weight, diluted in sterile PBS (pH 7.4) (n = 5). Injections were carried out every 5 days beginning at 6 weeks of age. Each mouse received a total of four injections. Severe seizures were induced 1 day after the last injection with pentyleneetetrazole (60 mg/kg, i.p.) and by electrical brain stimulation, 7 days later. Control groups comprised of non-tg (n = 5) and SwAPP mice (n = 5) that had received intravenous PBS injections instead of antibodies and that had undergone identical seizures.

**Statistical Analysis**—Data were collected by investigators blinded to the treatment and genotype of the mice and were analyzed by non-parametric Mann-Whitney U tests. All in tests, means ± S.E. are shown, and asterisks indicate statistically significant differences.

**RESULTS**

**Transgenic Expression of SwAPP in Neurons Increased Their Vulnerability to Seizures**—Seven days after seizures, many hippocampal pyramidal cells were stained by both Gallyas silver impregnation and TUNEL, whereas no stained neurons were present in the not-seizured control mice (Fig. 1A). In general, the spatial distribution of Gallyas- and TUNEL-positive neurons were similar. Quantitative analysis of Gallyas-positive cells in hippocampal CA1 and CA3 regions revealed that their numbers increased with seizure severity and with post-seizure time and were consistently higher in the SwAPP mice with the maximum numbers of positive cells in both groups at 7 days after the seizures (Fig. 1B). For both genotypes, cell death started in the medial part of the CA1 region of the hippocampus. The lateral portion of the CA1 and the CA3 region of non-tg mice were unaffected 1 day after severe seizures, whereas in SwAPP mice severe seizures caused significant numbers of degenerating cells in these regions (Fig. 1C). In addition, neurons in the amygdala and in the striatum were heavily affected by the seizures in SwAPP but not in non-tg mice (Fig. 1D). The time of onset of seizures following the pilocarpine injections did not differ in SwAPP and non-transgenic littermates. No Gallyas- or TUNEL-positive cells were found in corresponding areas of control mice that had been injected with PBS instead of pilocarpine and had not undergone seizures (Fig. 1, A and D).

Reactive astrogliosis was higher in both SwAPP and non-tg mice after the seizures when compared with not-seizured controls. Dense glial fibrillary acidic protein (GFAP)-positive astrocytes populated areas of degenerating neurons, and the extent of astrogliosis correlated with the degree of cell loss (Fig. 1E). In contrast to SwAPP mice, little astrogliosis was found in amygdala of non-tg mice 7 days after severe seizures (Fig. 1E), paralleling the very little degeneration in this region in non-tg mice (Fig. 1D).

**Increased Tissue Concentrations of Aβ after Seizures and Activated Caspase-3**—Total Aβ levels in brains of SwAPP mice were measured 1 and 7 days after severe seizures. One day after the seizures, the levels of transgenic total Aβ in brains of SwAPP mice were not significantly different from control not-seizured SwAPP littermates that had undergone the same treatment but received PBS instead of pilocarpine. However, this level was 2-fold higher 7 days after the seizures (Fig. 2A), possibly due to the accumulation of Aβ-positive cells in hippocampus, amygdala, and striatum of these mice (Fig. 1, A, B, and D). Western blotting with anti-Aβ antibodies 6E10 and 22C11 revealed no consistent changes in the amount of full-length Aβ among control groups following various seizures in non-tg mice and that had undergone identical seizures.

**Double Immunostaining revealed that Aβ-positive cells were also stained with an antibody that detects the activated pro-apoptotic form of caspase-3** (Fig. 2B).
Neuronal damage in hippocampus following seizures. A, Gallyas and TUNEL staining revealed a similar pattern of degenerating cells. Representative Gallyas and TUNEL staining of coronal sections of not-seizured SwAPP mice (SwAPP/CNTR), non-tg, and SwAPP mice 7 days after severe seizures are shown. Not-seizured control mice did not exhibit any degeneration in hippocampus. In contrast, both seized groups...
Passive Immunization against Aβ Ameliorated the Neuronal Damage—To determine whether the higher vulnerability of SwAPP neurons to seizures was mediated by increased tissue levels of Aβ, we passively immunized SwAPP mice by intravenous injections of Aβ-specific antibodies. The 22C4 monoclonal antibody, a monoclonal antibody that specifically recognizes the C termini of Aβ40 and Aβ42, was used for passive immunization. Again, the time of onset of seizures did not vary between antibody-treated SwAPP mice (SwAPP/22C4) and control mice that had received intravenous injections of PBS (SwAPP/PBS). SwAPP/PBS and SwAPP/22C4 mice seized within 27 ± 10 and 26 ± 13 min after pilocarpine injections, respectively (means ± S.D.). The passive immunization protocol reduced significantly the brain levels of total Aβ and amyloidogenic Aβ42 (Fig. 3A). After SDS extraction, no formic acid-extractable Aβ could be detected in the SDS-insoluble pellets, indicating that brain Aβ in our mice was SDS-soluble at the age of 10 weeks, i.e. at the end of these experiments. Importantly, passive immunization significantly reduced the numbers of seizure-induced degenerating cells in the hippocampus (Fig. 3B). The number of degenerating cells in passively immunized SwAPP mice did not differ from the corresponding number of the not-immunized non-transgenic littermates (Fig. 3B). To evaluate the role of intracellular Aβ accumulation in degenerating cells, the number of Aβ-positive cells was quantified in adjacent sections. The number of Aβ-positive neurons was significantly higher in SwAPP showed numerous Gallyas- and TUNEL-positive neurons. Scale is 1 mm. B, the extent of neuronal damage in hippocampus following pilocarpine lesioning was consistently larger in SwAPP mice than in their non-tg littermates. The number of degenerating cells increased with seizure severity and post-seizure time in both groups. Whereas the difference of numbers of damaged neurons between non-tg and SwAPP mice did not reach the statistical significance for mild lesions, the number of degenerating cells depended positively on the presence of the SwAPP transgene after moderate and severe seizures. *, p ≤ 0.05; **, p ≤ 0.01. C, one day after severe seizures, no degenerating neurons were observed in lateral portion of the CA1 or CA3 region in non-tg mice. These neurons in CA1 (arrow) and CA3 areas (arrowhead), however, exhibited many degenerating cells in SwAPP mice indicating higher vulnerability of SwAPP hippocampal pyramidal neurons to pilocarpine-induced damage as early as 1 day after the seizures. Scale is 1 mm. D, Gallyas-stained degenerating cells were detected in striatum and amygdala of both groups 7 days after severe seizures, whereas control mice injected with PBS instead of pilocarpine (SwAPP/CNTR) did not exhibit any degenerating cells in these areas. Note that the SwAPP neurons were highly affected in these areas, whereas only a few degenerating cells were detected in amygdala and striatum of non-tg mice. Scale is 100 μm. E, astroglia paralleled the degeneration of cells after pilocarpine lesioning. Seven days after seizures, higher numbers of reactive astrocytes were found in hippocampus of severely lesioned mice of both groups when compared with control mice injected with PBS instead of pilocarpine (SwAPP/CNTR). Whereas high levels of GFAP positivity were found in hippocampus of both seizure groups, strong astroglia was only found in amygdala of SwAPP mice, paralleling the high level of degeneration in this region. Scale is 200 μm.
mice when compared with non-tg littermates. Passive immunization with the monoclonal anti-\(\text{A}\beta\) antibody 22C4 significantly reduced this number to the number observed in non-transgenic littermates (Fig. 3C).

DISCUSSION

The results of this study show that brain neurons of mice expressing SwAPP are more vulnerable to seizure-induced damage than their non-tg littermates and that passive immunization against \(\text{A}\beta\) reduced this vulnerability. Importantly, the transgenic expression of SwAPP did not affect the seizure threshold indicated by identical time intervals between the pilocarpine injection and initial signs of seizures, by identical doses of pilocarpine used, and by identical presentations of seizure severity in both genotype groups. Because both genetic backgrounds and seizure phenotypes of mice were identical and because the duration of the seizure was kept constant by terminating the seizures with diazepam, the observed differences in the susceptibility of brain neurons to damage were related to the expression of the SwAPP transgene. The Gallyas protocol was shown to selectively detect degenerating neurons in many pathogenic conditions including kainate-induced apoptotic cell death in hippocampal neurons after ischemic lesions or progressive motor neuron disease (50–53). It also detects damaged and degenerating neurons in human neurodegenerative diseases including AD. In general, the SwAPP neurons were more vulnerable to pilocarpine-induced seizures as compared with their non-transgenic littermates. One day after the seizures, the medial part of the CA1 region was labeled by the Gallyas stain in all groups, whereas neurons within the lateral CA1 region were stained only in SwAPP but not in non-tg control mice. In non-tg mice these neurons became Gallyas-positive 7 days after severe seizures. Substantial numbers of Gallyas-positive cells occurred also in the amygdala and the striatum of the SwAPP but not non-tg mice, indicating that transgenic expression of the SwAPP mutation increased the vulnerability of neurons within hippocampus, the amygdala, and the striatum.

In addition, seizures caused intracellular accumulations of \(\text{A}\beta\) in affected neurons throughout the brain. This increase was associated with activated caspase-3. Together with increased TUNEL stains, the activation of caspase-3 clearly suggest that intracellular \(\text{A}\beta\) was associated with apoptotic cell degeneration that led to the staining of these cells by the Gallyas silver impregnation protocol.

Passive immunization of mice for 3 weeks with a monoclonal anti-\(\text{A}\beta\) antibody reduced both total \(\text{A}\beta\) and \(\text{A}\beta_{42}\) in brain tissue and protected neurons against seizure-induced cell damage. All existing forms of \(\text{A}\beta\) in the 10-week-old mice examined here were SDS-soluble; there was no \(\text{A}\beta\) in formic acid extracts of SDS-insoluble pellets. The pool of SDS-insoluble \(\text{A}\beta\) may well contain monomers as well as higher molecular mass oligomers (14, 54). Therefore, these data suggest a crucial role of SDS-soluble \(\text{A}\beta\) in the vulnerability to toxic events that occur during and after seizures and demonstrates that antibodies against \(\text{A}\beta\) can have neuroprotective activities. Because the
numbers of damaged cells in the 22C4-immunized SwAPP mice were reduced to the corresponding levels in non-tg mice, passive immunization essentially blocked the increased vulnerability caused by SwAPP mutation. Our data suggest that intracellular Aβ was involved in this neurodegeneration because the number of Aβ-positive cells correlated to the number of Gallyas-positive cells in adjacent sections and passive immunization with 22C4 also reduced the number of Aβ-positive cells, again in correlation with the number of Gallyas-positive cells in adjacent sections. Both ELISAs used in this study selectively detected human but not mouse Aβ; therefore, we were unable to measure whether the mouse Aβ was decreased in response to passive immunization. Because the 22C4 antibody recognizes specifically the C termini of Aβ and Aβ42, our data strongly suggest that the reduction of Aβ levels in brain by passive immunization was associated with the reduced vulnerability of neurons to external noxious stress. Another theoretical possibility is that Aβ antibodies bind to Aβ-containing derivatives, thereby blocking potential toxic functions of these proteins. Importantly, no amyloid plaques and no formic acid-extractable amyloid fibrils were present in these mice, strongly suggesting an Aβ-related toxic event that is unrelated to amyloid plaque deposits. If SDS-soluble Aβ is toxic \textit{in vivo}, it may link the expression of SwAPP transgene to increased neuronal vulnerability. This data is consistent with behavioral deficits in transgenic mice with high brain levels of Aβ before the onset of amyloid plaque formation (30, 55).

Our data demonstrate that the combination of two pathogenic factors, the neuronal expression of an AD-causing mutant APP along with additional excitotoxicity caused by seizures, resulted in additive toxic effects and accelerated neurodegeneration. Because lowering Aβ by passive immunization blocked the increased vulnerability and protected neurons from seizure-induced degeneration, it may have therapeutic potential for AD.

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REFERENCES

1. Gotz, J., Chen, F., van Dorpe, J., and Nitsch, R. M. (2001) \textit{Science} 293, 1491–1495.
2. Lewin, J. D., Dickson, D. W., Lin, W. L., Chisholm, L., Corral, A., Jones, G., Yen, S. H., Sahara, N., Skipper, L., Yager, D., Eckman, C., Hardy, J., Hutton, M., and McGowan, E. (2001) \textit{Science} 293, 1487–1491.
3. Hardy, J. (1999) \textit{Neurobiol. Aging} 20, 85.
4. Selko, D. J. (1999) \textit{Nature} 399, A29–A31.
5. Jarrett, J. T., and Lansbury, P. T., Jr. (1993) \textit{Cell} 73, 1055–1058.
6. Borchelt, D. R., Matsumoto, M., Cai, Z., De Strooper, B., and Ihara, Y. (1991) \textit{J. Biol. Chem.} 266, 19065–19071.
7. Younkin, S. G. (1995) \textit{Ann. Neurol.} 37, 287–288.
8. Borchelt, D. R., Thun, G., Eckman, C. B., Lee, M. K., Davenport, R., Ferrante, A. W., Kim, G., Seigerman, S. M., Gajewski, B., Sturk, H. H., Wang, R., Seeger, M., Levey, A. I., Gandy, S. E., Copeland, N. G., Jenkins, N. A., Copeland, N. G., Price, D. L., and Sisodia, S. S. (1997) \textit{Neuron} 19, 939–945.
9. Vousden, K. H. (1993) \textit{Ann. Rev. Cell Dev. Biol.} 9, 171–197.
10. Citron, M., Levey, A. I., Gandy, S. E., Copeland, N. G., Jenkins, N. A., Copeland, N. G., Price, D. L., and Sisodia, S. S. (1999) \textit{Neuron} 23, 1179–1191.
11. Rudge, J., and Seed, P. (1996) \textit{J. Biol. Chem.} 271, 13004–13010.
12. Racine, R. J., Gartner, J. G., and Burnham, W. M. (1972) \textit{Brain Res.} 47, 270–285.
13. Racine, R., Oktawia, J., and Chasiotis, S. (1972) \textit{Electroencephalogr. Clin. Neurophysiol.} 32, 295–299.
14. Franklin, R. J. B., and Paxinos, G. (1996) \textit{The Mouse Brain in Stereotaxic Coordinates}, Academic Press, San Diego, CA.
15. Gallay, P. (1971) \textit{J. Neurosci.} 10, 1–8.
16. Franklin, R. J. B., Paxinos, G., and Chater, F. (2001) \textit{J. Neurosci.} 21, 2161–2173.
17. Teichert-Noldt, G., Breuer, K. H., and Dawirs, R. R. (1991) \textit{Dev. Neurosci.} 13, 151–163.
18. Pollard, H., Charriaut-Marlangue, C., Sagarel, S., Regassa, A., Robain, O., Moreau, J., and Ben-Ary, Y. (1994) \textit{Neuroscience} 63, 7–18.
19. Renouleau, S., Aggoun-Zaroulia, D., Ben-Ary, Y., and Charriaut-Marlangue, C. (1998) \textit{Stroke} 29, 1454–1460.
20. Kawarabayashi, T., Younkin, L., Saido, T., Shoji, M., Ashe, K., and Younkin, S. (1999) \textit{J. Biol. Chem.} 274, 4583–4586.