Brain Trauma Induces Massive Hippocampal Neuron Death Linked to a Surge in β -Amyloid Levels in Mice Overexpressing Mutant Amyloid Precursor Protein

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Although brain trauma is a risk factor for Alzheimer's disease, no experimental model has been generated to explore this relationship. We developed a model of brain trauma in transgenic mice that overexpress mutant human amyloid precursor protein (PDAPP) leading to the appearance of Alzheimer's disease-like β -amyloid ($A\beta$) plaques beginning at 6 months of age. We induced cortical impact brain injury in the PDAPP animals and their wild-type littermates at 4 months of age, ie, before $A\beta$ plaque formation, and evaluated changes in posttraumatic memory function, histopathology, and regional tissue levels of the $A\beta$ peptides $A\beta_{1-40}$ and $A\beta_{1-42}$. We found that noninjured PDAPP mice had impaired memory function compared to noninjured wild-type littermates ($P < 0.01$) and that brain-injured PDAPP mice had more profound memory dysfunction than brain-injured wild-type littermates ($P < 0.001$). Although no augmentation of $A\beta$ plaque formation was observed in brain-injured PDAPP mice, a substantial exacerbation of neuron death was found in the hippocampus ($P < 0.001$) in association with an acute threefold increase in $A\beta_{1-40}$ and sevenfold increase in $A\beta_{1-42}$ levels selectively in the hippocampus ($P < 0.01$). These data suggest a mechanistic link between brain trauma and $A\beta$ levels and the death of neurons. (*Am J Pathol* 1998, 153:1005-1010)

Although circumstantial evidence suggests that traumatic brain injury is a risk factor for Alzheimer's disease (AD), the mechanisms underlying this relationship remain unknown. Previously, postmortem histopathological analysis of brains from boxers with dementia pugilistica (punch-drunk syndrome) revealed neurofibrillary tangles

and diffuse plaques composed of β -amyloid peptides ($A\beta$ s) similar to AD lesions.^{1,2} Indeed, a single incident of brain trauma may result in a widespread deposition of $A\beta$,^{3,4} and a history of brain trauma increases an individual's risk for AD.⁵ Moreover, marked accumulations of β -amyloid precursor proteins have been seen after brain trauma in humans and experimental animals.^{6,7,8,9} Although this suggests that ample substrates are available for pathological $A\beta$ production after trauma, the investigation of mechanisms whereby brain trauma induces deposition of $A\beta$ has been hampered by the inability of experimental models of brain trauma in rodents to produce $A\beta$ -containing plaques.^{6,7} Although there are several potential explanations for this, amino acid sequence differences in human *versus* rodent $A\beta$ are known to limit the ability of rodent $A\beta$ to form amyloid.⁶

To overcome this technical obstacle, we developed a model of brain trauma in transgenic mice that develop AD-like $A\beta$ plaques in a specific brain region beginning at 6 months of age. These mice were generated using a construct with the platelet-derived growth factor promoter driving a human β -amyloid precursor protein minigene containing the familial AD mutation V→F at APP position 717 (PDAPP).^{10,11} In the present study, we evaluated the effects of brain trauma in the PDAPP mice at 4 months of age, ie, 2 months before the appearance of AD-like pathology, on memory function, histopathology, and regional tissue levels of $A\beta$ peptides.

Materials and Methods

Brain Injury

To induce brain trauma, we used a recently described mouse model of rigid cortical indentation that dynamically deforms the left parietal cortex.¹² Briefly, brain trauma was induced by impacting a 3-mm diameter impounder onto the cortex (5 m/s, 1 mm depth) through a 5-mm craniectomy. We induced brain trauma or sham treatment (surgery without brain impact) in PDAPP neu-

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tered female mice at 4 months of age. As controls, we also induced brain injury in or sham treated 4-month-old neutered female wild-type littermates.

Memory Evaluation

The water maze paradigm has been described in detail.^{12,13} Briefly, the water maze is a circular pool 1 m in diameter. PDAPP and wild-type mice were trained to swim to a plexiglas platform submerged 1 cm, which they found by navigating using external visual cues. The mice were given 20 to 25 trials over 2 to 3 days to reach criterion, the last trial being 1 hour before brain damage or sham treatment. Only those reaching criterion were used. The animals that could perform the task were subjected to brain trauma ($n = 14$ wild-type, $n = 13$ PDAPP) or sham treatment ($n = 14$ wild-type, $n = 15$ PDAPP), and evaluated 1 week later for their ability to recall the platform location. The platform was removed and the mice were given 1-minute probe trials in the water maze while a video computer recording unit tracked their swim path. A memory score was derived by determining the relative amount of time spent in or near the former platform site. Statistical analyses were performed using two-way analysis of variance for all groups followed by a posthoc *t*-test with Bonferroni correction for individual comparisons.

Histopathological Analysis

The mice were humanely euthanized with an overdose of sodium pentobarbital (200 mg/kg) and transcardially perfused with saline after brain injury or sham treatment at 1 week ($n = 5$ injured, $n = 3$ sham wild-type mice; $n = 5$ injured, $n = 3$ sham PDAPP mice), 2 weeks ($n = 5$ injured, $n = 3$ sham wild-type mice; $n = 5$ injured, $n = 3$ sham PDAPP mice) and 2 months ($n = 8$ injured, $n = 3$ sham wild-type mice; $n = 8$ injured, $n = 3$ sham PDAPP mice). The brains were removed and fixed in 70% ethanol, embedded in paraffin, and cut into 6- μ m sections. Brain sections were immunostained with a variety of previously characterized monoclonal and polyclonal antibodies that are highly specific for distinctive epitopes within the A β peptides, including 2332, BA-27, and BC-05.^{7,14–16} Alternate sections were stained with hematoxylin, eosin, and cresyl violet. Light microscopic examination was then performed on the sections. Because we found an extreme loss of hippocampal CA₂ and CA₃ neurons in the brain-injured PDAPP mice, we selected one region of the dorsal hippocampus to perform neuron cell counting. Three adjacent sections from bregma–1.7¹⁷ were selected for each sham or injured animal sacrificed 1 to 2 weeks posttrauma ($n = 10$ /injured group, $n = 6$ /sham group). Stained neurons with clearly identifiable nuclei were manually counted via light microscopy in the CA₂ and CA₃ regions of the hippocampus ipsilateral to injury. These regions, selected according to atlas determination,¹⁷ encompassed the entire CA₂ and the CA₃ from the margin of the CA₂ to the most lateral extension of the dentate gyrus. The average number of neurons from each region of the three sections for each animal were determined and used to determine group

means. Statistical analysis of the neuron loss was performed using a two-way analysis of variance for all groups followed by a posthoc *t*-test with Bonferroni correction for individual comparisons.

A β Tissue Levels

PDAPP mice and wild-type littermates were euthanized (see above) at 0 hours (sham), 2 hours, 6 hours, 24 hours, 3 days, and 7 days posttrauma. Following sacrifice, the brains were rapidly removed and dissected on a chilled plate isolating cortex, hippocampus, thalamus, brainstem, and cerebellum. These samples were Dounce-homogenized in 6.7 volumes (w/v) of 70% formic acid at 4°C. Homogenates were centrifuged at 45,000 rpm, 4°C for 1 hour. The supernatant was diluted 1:20 in 1 mol/L Tris base. Samples ($n = 4$ –12/group) were evaluated with sandwich enzyme-linked immunosorbent assays for A β peptides as previously described.^{10,18} Absorbencies falling within the standard curve for each assay were converted to fmole. Sandwich enzyme-linked immunosorbent assays for A β were prepared with the capture antibody BAN-50, a monoclonal antibody specific for amino acids 1–16 of A β , and reporting antibodies BA-27, specific for A β ending at A β 40, and BC-05, specific for A β ending at A β 42.⁴³ The assay has a detection limit of <6 fmole/well for A β _{1–40} and A β _{1–42}. The monoclonal antibodies BAN-50, BA-27, and BC-05 were prepared as described previously.¹⁹ Statistical analyses were performed using a two-way analysis of variance for all groups followed by a posthoc *t*-test with Bonferroni correction for individual comparisons.

Results

Memory Evaluation

We found that although all wild-type mice easily learned the spatial memory task before injury, approximately 20% of the transgenic animals did not reach criterion and were excluded from further analysis. Of the animals that did reach criterion, several significant differences were found

Figure 1. Histogram demonstrating relative memory retention of a water maze task of wild-type and PDAPP mice one week following brain trauma or sham treatment. **P* < 0.01, ***P* < 0.001.

Figure 2. Representative Nissl-stained coronal brain sections of the dorsal hippocampi of PDAPP and wild-type mice one week following sham treatment or brain trauma. **A:** Normal neuron density and orientation of the hippocampus in a PDAPP mouse. **B:** Typical loss of neurons in the CA₂ and CA₃ subfield of brain injured wild-type mice (small arrows). **C-F:** Hippocampi from four brain-injured PDAPP mice demonstrating an almost complete loss of CA₃ neurons (large arrow, c).

between groups evaluated one week later for memory retention of the task. Consistent with previous reports, wild-type brain-injured animals demonstrated significant memory dysfunction compared to their respective sham group ($P < 0.001$).¹² In addition, a significant impairment of memory retention was found in the sham PDAPP mice compared to sham wild-type animals ($P < 0.01$). Moreover, brain-injured PDAPP mice had a more profound impairment of memory function than did injured wild-

type animals ($P < 0.001$) (Figure 1). Importantly, the swimming speed of the mice in all groups was identical, demonstrating that potential motor impairment did not influence performance.

Histopathological Analysis

Brain sections immunostained with a variety of antibodies that are highly specific for distinctive epitopes within the

Figure 3. Histogram demonstrating neuron cell loss in the CA₂ and CA₃ region of the hippocampus of brain injured wild-type and PDAPP mice. **P* < 0.001 compared to respective sham group. †*P* < 0.001 compared to brain injured wild-type mice.

A β peptides revealed the presence of only a few A β -containing plaques, primarily in the hippocampus, that were indistinguishable between the 4-month-old brain-injured and sham-treated PDAPP animals. Although 6-month-old PDAPP animals demonstrated several more A β -containing plaques, consistent with previous reports,^{10,11} there was no discernable difference in the number or distribution of plaques between brain-injured (2 months postinjury) and sham-treated animals. Thus, brain trauma did not accelerate or exacerbate A β deposition in the PDAPP mice.

Nonetheless, we did observe a dramatic difference in hippocampal pathology in the brain-injured PDAPP versus the wild-type mice. At both 1 and 2 weeks after injury, the wild-type mice demonstrated a modest loss of hippocampal neurons in a patchy distribution throughout the ipsilateral CA₂ and CA₃ subfield exactly as described earlier.²⁰ In striking contrast, the entire length of the CA₃ pyramidal cell layer of the brain-injured PDAPP mice degenerated completely with virtually no or very few neurons remaining (Figure 2). Quantitative analysis of the CA₂ and CA₃ regions showed that the number of neurons in the hippocampi of wild-type and PDAPP sham animals were nearly the same. Brain injury in both the wild-type and PDAPP mice induced a highly significant loss of neurons in the CA₂ and CA₃ regions of the hippocampus

Figure 4. Histogram demonstrating the temporal changes in the concentration of A β ₁₋₄₀ and A β ₁₋₄₂ following brain trauma in PDAPP mice in the hippocampus and cortex. **P* < 0.01, ***P* < 0.001 compared to respective sham A β levels. Data are not shown for wild-type mice because their A β levels were at the minimal detection limit (6 fmol/well) or below for all time points.

compared to respective sham groups (*P* < 0.001). The wild-type animals had a 52% loss of CA₂ neurons compared to a 58% loss for the PDAPP animals, an insignificant difference. However, in the CA₃ region the wild-type brain-injured mice demonstrated a 36% loss of neurons whereas the PDAPP brain-injured mice showed a remarkable 84% loss, compared to respective sham groups. This difference between injured groups was found to be highly significant (*P* < 0.001). These results are shown in Figure 3.

A β Tissue Levels

A β ₁₋₄₀ and A β ₁₋₄₂ levels in samples from both sham and brain-injured wild-type mice were either very low or below the level of detection at all timepoints. Thus, no injury effect on A β levels could be discerned in the wild-type mice. In the sham PDAPP mice, the highest levels of A β ₁₋₄₀ and A β ₁₋₄₂ were expressed in the hippocampus at twice the concentration found in the cortex, with the A β ₁₋₄₂ present at levels similar to those previously reported in PDAPP mice at 4 months (Figure 4).^{10,11} In addition, very low amounts of A β ₁₋₄₀ and A β ₁₋₄₂ were found in the thalamus, cerebellum, and brainstem in the sham PDAPP mice. Two hours after trauma in PDAPP mice, a dramatic sevenfold increase in the concentration

of A β_{1-42} and threefold increase in A β_{1-40} were observed in hippocampal tissue ($P < 0.001$), whereas smaller but significant increases were seen in cortical tissue ($P < 0.01$). Thus, the ratio of the levels of A β_{1-40} compared to A β_{1-42} also changed in the hippocampus after injury. In sham animals A β_{1-42} was found at three times the concentration of A β_{1-40} , but at 2 hours post-trauma, the concentration of A β_{1-42} became 10-fold greater than A β_{1-40} . However, by 6 hours posttrauma, A β_{1-40} and A β_{1-42} concentrations in the hippocampus had returned to baseline values and by 24 hours, A β_{1-42} levels fell below baseline in the cortex (Figure 4). No other regions demonstrated detectable changes in A β concentrations after brain injury in PDAPP mice.

Discussion

These results demonstrate that brain trauma in PDAPP mice induces massive neuron death selectively in the hippocampus, the extent of which has never been observed following the same or greater levels of brain trauma in wild-type mice.¹² Additionally, acute and marked increases in A β peptide levels were also found selectively in the hippocampi of PDAPP animals after brain trauma. However, despite these posttraumatic increases in A β brain trauma did not accelerate or augment A β plaque formation 2 months after injury in PDAPP mice. In this study we also found that PDAPP mice demonstrate memory dysfunction before the appearance of overt AD-like pathology, consistent with previous reports using other transgenic mouse strains.^{20,21} Although there appeared to be an exacerbation of posttraumatic memory dysfunction in the PDAPP mice compared to wild-type littermates, the nature of this difference could not be determined due to the baseline deficit in the noninjured PDAPP animals.

The acute posttraumatic increase of A β_{1-42} in the 4-month-old PDAPP mice reached levels similar to those found in 1-year-old noninjured PDAPP mice when substantial A β deposition is observed throughout the hippocampus.^{10,11} Notably, the high levels of A β in the brain-injured PDAPP mice were associated not with plaque formation but with neuron death. However, hippocampal neuron death has never been reported in the absence of brain injury in this or another line of transgenic mice that overexpress mutant β -amyloid precursor proteins and develop increasing numbers of AD-like amyloid plaques with advancing age.^{22,23} Moreover, no induction of neuron death has been seen after A β injections in rat brains.^{24,25} In association, these findings suggest that *in vivo* A β may not be neurotoxic unless a second stress or insult triggers A β to induce neuron death. In support of this hypothesis, a very recent report demonstrated increased susceptibility to ischemic brain damage in another line of transgenic mice overexpressing mutant APP.²⁶ Although there was no difference in A β levels between ischemic and nonischemic hemispheres at 24 hours postinjury, high baseline levels of A β were present in the brains of these transgenic animals. Thus, we propose a "two-hit" hypothesis for AD wherein A β is neces-

sary but not sufficient to cause neuron death until a second pathological process potentiates A β neurotoxicity.

Because an acute and marked posttraumatic increase in the concentrations of both A β_{1-40} and A β_{1-42} implicates A β peptides as potential deleterious agents in the posttraumatic sequelae, these findings suggest a mechanistic link in the pathogenesis of brain trauma and AD which may have important clinical implications. Accordingly, recent reports have shown that brain trauma patients with apolipoprotein E-4 genotypes, a genetic risk factor for AD, have much worse outcomes after brain trauma, even in the very acute setting.^{27,28,29} Taken together, these findings suggest that the role of brain trauma in AD can be explored in greater detail using the model presented here.

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