Environmental Enrichment Exacerbates Amyloid Plaque Formation in a Transgenic Mouse Model of Alzheimer Disease

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Abstract. Epidemiological studies of Alzheimer patients from a wide variety of ethnic and socioeconomic backgrounds have identified education and occupation as environmental factors that can affect the risk of developing disease. A model of environmental manipulation in rodents uses enriched housing to provide cognitive and social stimulation. Previous studies have established elevations in synaptic number and function in rodents housed under enriched conditions. Recent experiments in hippocampal cultures have demonstrated that synaptic activity can influence the processing of amyloid precursor protein (APP). Here we examined whether changes in synaptic activity brought about by enriched housing might also influence the deposition of amyloid plaques in vivo using a transgenic mouse model of Alzheimer disease (AD). Mice co-expressing mutant APP and presenilin 1 (PS1) were housed in either enriched or standard cages from 2 months of age and then killed for pathological evaluation several months later. We find that, as compared to littermates housed in standard cages, the enriched APP/PS1 transgenic mice develop a higher amyloid burden with commensurate increases in aggregated and total Aβ. These results suggest that Aβ deposition can be exacerbated by the neuronal changes associated with enrichment, and demonstrate a substantial, albeit paradoxical, environmental influence on the progression of pathology in a mouse model of AD.

Key Words: Alzheimer disease; Amyloid; APP; Environmental enrichment; Neuropathology; Presenilin 1; Transgenic mouse.

INTRODUCTION

While gene mutations account for roughly 10% of Alzheimer disease (AD), the vast majority of AD cases have no identifiable cause. To date, advancing age and the inheritance of the E4 allele of apolipoprotein E are the only established risk factors for AD that affect the general population. These 2 traits cannot account for all idiomorphic AD, and much research has been devoted to identifying additional factors that may initiate or exacerbate incipient disease. More than a dozen epidemiological studies have focused on limited cognitive stimulation as a risk factor for AD (1). Education and advanced occupational attainment have been consistently found to decrease the risk of developing dementia in general and AD in particular. The protection afforded by cognitive stimulation crosses both racial and socioeconomic boundaries, and is reproduced whether cross-sectional prevalence or longitudinal incidence is observed.

One hypothesis to explain the protective effect of education is the concept of cognitive reserve (2, 3). Cognitive stimulation is thought to increase the connectivity of neurons in brain areas responsible for learning and memory, and it has been suggested that people with higher education and/or occupation may develop stronger, more elaborated neuronal networks that can withstand a greater amount of damage before showing signs of decline. Indeed, the morphological and behavioral correlates of such network strengthening have been well established in rodents exposed to complex environments. Laboratory animals housed in enriched cages generate more dendritic spines, have greater synaptic contact between cells, and show functionally stronger neuronal connections than animals housed in standard cages (for a thorough review of the effects of environmental enrichment, see [4]).

Another avenue by which environmental stimulation might alter the pathogenesis of AD is by influencing the production and turnover of neuronal proteins implicated in the disease. The notion that cognitive stimulation may directly affect the biochemistry of AD was substantially strengthened by the recent study of Kamenetz et al describing the interplay of neuronal activity and the proteolytic processing of amyloid precursor protein (APP) (5). Sequential cleavage of APP by β- and γ-secretases releases a 39–43 amino acid peptide, Aβ, which aggregates to form amyloid plaques characteristic of AD and which is thought to be central to the pathogenesis of the disease. Past work by several groups has shown that exogenously applied Aβ can substantially inhibit synaptic plasticity in the hippocampus (6–11). Complementary studies in Aβ-overproducing transgenic mice confirmed the peptide’s inhibition of synaptic enhancement (12–15). Kamenetz et al carried these studies one step further in uncovering a complete feedback loop between Aβ and synaptic activity (5). They showed that APP over-expression can modulate synaptic transmission, and that neuronal activity can in
that one was placed into an enriched cohort and the other re-
transgenic females, the sib pairs were separated at weaning so
as controls. When litters were large enough to produce multiple
female non-transgenic littermates into 2 large enrichment cages.

20 standard colony cages for the control animals were approximately
ulation; each cage housed a maximum of 16 animals (20). Stan-
were re-positioned or changed weekly to provide novel stimu-
exercise wheels, and shredded paper bedding material which
®lled with plastic tubes and hutches, small cardboard boxes,
transgenic males for backcrossing our hybrid lines.

Of course, even the best-characterized neuronal sys-
tems studied in culture do not necessarily predict what
will happen in the intact brain, and often the perturbations
used in vitro are much more severe than would be ex-
perienced in vivo. To model a functional change in syn-
aptic activity, we used environmental enrichment to pro-
mote neuronal activity in transgenic mice co-expressing
amyloidogenic variants of APP and presenilin 1 (PS1).

MATERIALS AND METHODS

Transgenic Mice

Animals used for this experiment were derived from the bi-
genic line 57 created by co-injecting separate APP and PS1
transgenes (19). The 2 constructs encoded chimeric mouse/hu-
man APP harboring the Swedish mutation K670N/M671L
(APPswe) and human PS1 encoding the exon 9 deletion mu-
tation (PS1dE9), each driven by its own mouse prion protein
promoter element. The line was maintained for several gener-
ations by backcrossing transgenic animals with non-transgenic
B6/C3 F1 hybrid mice purchased from Jackson Laboratories
(Bar Harbor, ME). Transgenic and non-transgenic female off-
spring for these experiments were derived from 5 breeding trios
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Enrichment Housing

Enrichment cages were approximately 1 meter square and
filled with plastic tubes and hutches, small cardboard boxes,
exercise wheels, and shredded paper bedding material which
were re-positioned or changed weekly to provide novel stimu-
lation; each cage housed a maximum of 16 animals (20). Standard
colony cages for the control animals were approximately
20 × 30 cm in size and contained only corn cob bedding, pellet
feed, and water; each housed a maximum of 4 mice.

Shortly after weaning at roughly 2 months of age, 16 female
APPswe/PS1dE9 transgenic mice were placed along with 16
female non-transgenic littermates into 2 large enrichment cages.
Thirty-two females of identical genotype were housed alongside
as controls. When litters were large enough to produce multiple
transgenic females, the sib pairs were separated at weaning so
that one was placed into an enriched cohort and the other re-
mained in standard caging. Both enriched and control cages
were kept in the same room and were maintained on the same
14:10 hour light:dark cycle, ad lib rodent chow and tap water,
and were exposed to the same intrusions of experimenter and
caretaker presence in the room.

All non-transgenic mice were later removed from the exper-
iment, and the transgenic animals remaining in the 2 enrichment
cages were combined. Because the combined cage contained
animals with a wide range of birthdates, young non-transgenic
females were added to keep the population constant as older
mice were removed for study. All mice were killed at 8.5
months of age by ether inhalation overdose and their brains
removed into ice-cold PBS.

Histology

One half of the brain from each transgenic animal was im-
mersed in 4% paraformaldehyde/1× PBS for histology. After
fixation for 48 hours at 4°C, brains were dehydrated in alcohols,
treated with cedarwood oil and methyl salicylate, and embed-
ded in paraf®n. Ten-µm sagittal sections were cut through the
dorsal hippocampus and processed for Hirano silver stain, thio-
®avin-S, or ubiquitin immunohistochemistry to identify amy-
loid aggregates.

Hirano Silver Stain: Silver impregnation histology was per-
formed by Hirano’s modi®cation of the Bielschowsky method
(21, 22). Briefly, sections were deparaffinized through xylene
and alcohols into tap water before being placed into fresh 20%
silver nitrate solution for 20 min. After washing thoroughly
with distilled water, slides were immersed in 20% silver nitrate
solution titrated with fresh ammonium hydroxide. After 20 min,
slides were washed with ammonia water before being individ-
ually developed with 100 µl of developer (20 ml of 37% form-
aldehyde, 100 ml of distilled water, 50 µl concentrated nitric acid,
and 0.5 g citric acid) added to 50 ml of titrated silver nitrate
solution. Slides were then rinsed in tap water, ®xed in 5% so-
dium thiosulfate, and dehydrated through alcohols and xylene.

Thio®avin-S Staining: Following deparaffinization through xylene
and alcohols, amyloid impregnation with thio-
®avin-S was performed according to the Guntern modi®cation
of the standard protocol. Slides holding the 10-µm paraf®n sec-
tions were washed twice in distilled water, then immersed for
5 min in a 0.25% potassium permanganate solution, followed
by 5 min in a 1% potassium permanganate/1% oxalic acid so-
lution. After this preparation, slides were placed into a filtered
aqueous 0.02% thio®avin-S solution (Chroma-Gesellschaft,
Schmid GmbH Co., Kongen, Germany) for 8 min. Excess stain
was removed by 2 brief rinses in 80% ethanol, then 2 in dis-
tilled water, after which slides were ®nished in aqueous mount-
ing medium for ®rescence photomicrography.

Ubiquitin Immunohistochemistry: Prior to immunostaining,
slides were deparaffinized by oven heating followed by im-
mersion in xylene. After rehydration through graded alcohols
into tap water, endogenous peroxidase activity was quenched
by incubation with 3% hydrogen peroxide in methanol. Slides
were microwaved for 5 to 7 min in water, cooled for 5 minutes,
then washed in tris-buffered saline (TBS). Nonspeci®c staining
was blocked for 1 hour with 3% normal goat serum and 0.1%
Triton-X 100 in TBS. Slides were then placed into primary
antibody (rabbit anti-ubiquitin polyclonal antibody, Dako, Car-
pinteria, CA) diluted 1:500 in TBS with 2% normal goat serum,
and incubated overnight at 4°C. After washing excess primary antibody with several changes of TBS, slides were incubated with peroxidase/anti-peroxidase reagents (Sternberger Monoclonals, Inc., Lutherville, MD) according to the manufacturer’s directions. Antibody binding was visualized with diaminobenzidine and sections were counterstained with hematoxylin.

**Stereology**

Neuritic plaque area, as defined by ubiquitin immunostaining, was quantified by unbiased stereology as described previously (23). After defining the dorsal hippocampal formation in the ubiquitin-stained sections, plaque coverage was counted using an area fractionator grid with Stereoinvestigator software (MicroBrightField, Colchester, VT). The counting frame was set to 90 × 125 μm, the scan grid 325 × 325 μm, and the Cavalieri grid spacing 15 μm, resulting in an area sampling fraction of 0.18. Percent coverage counted from 3 sections was averaged to obtain a final estimate of plaque burden for each animal.

**Filter Trap Assay**

One half of the brain from each transgenic animal was dissected to isolate the cortex and hippocampus which were snap-frozen on dry ice and later homogenized by sonication in either 5 (cortex) or 10 (hippocampus) volumes by weight of 1 × PBS with 5 mM EDTA and 1 × protease inhibitor mix (Sigma P8340, St. Louis, MO). The aliquot of each homogenate was removed and partially solubilized by the addition of SDS to a final concentration of 1%. Serial 1:1 dilutions were made with a 1 × PBS/1% SDS solution, and 100 μl of each dilution was then vacuum-filtered through a pre-wet 0.22-μm cellulose acetate membrane. Each well was washed several times with PBS, after which blots were incubated overnight with polyclonal anti-Aβ antibody (Zymed Laboratories, San Francisco, CA) diluted 1:600 in a blocking solution of 1 × TBS/5% nonfat dry milk powder. After washing the blots 3 times for 10 min each in 1 × TBS/0.1% Tween-20, the membrane was incubated for 1 hour with HRP-conjugated protein A diluted 1:5000 in blocking solution. Once again the membranes were washed three times with 1 × TBS/0.1% Tween-20, and then antibody binding was detected with enhanced chemiluminescence (New England Nuclear Life Sciences, Boston, MA). Films of the stained membranes were digitized using Photoshop software, and the intensity of Aβ staining was quantified using Quantity One image analysis software (Bio-Rad Laboratories, Hercules, CA).

**Aβ ELISA**

A 100-μl aliquot of each PBS brain homogenate prepared for filter trap assay described above was removed for ELISA analysis prior to the addition of SDS. Protein aggregates in the PBS homogenate were denatured by the addition of 164 μl of 8.2 M guanidine-HCl to a final concentration of 5 M, and mixed at room temperature for 4 hours. The denatured samples were then further diluted 250-fold in PBS containing 5% BSA, 0.03% Tween-20, and 1 × protease inhibitor cocktail (Sigma). Samples were finally prepared for application to the ELISA microplate by an additional 10-fold dilution into Standard Dilution Buffer provided by the manufacturer supplemented with 1 × protease inhibitor cocktail. Aliquots of this final dilution were assayed for both Aβ40 and Aβ42 using commercially available sandwich-ELISAs according to the manufacturer’s recommendations (BioSource International, Camarillo, CA).

**RESULTS**

We studied bigenic APPswe/PS1dE9 mice previously shown to have high expression of APP and to develop amyloid lesions typical of AD by 7 to 8 months of age (19). At roughly 2 months of age, female transgenic offspring were identified and divided into enriched and control cohorts. Control mice were maintained in standard colony cages; enriched animals were placed into large plexiglas tanks containing plastic toys, cardboard boxes, exercise wheels, bedding, and nesting material that were changed or moved to provide novel stimulation every week. The enrichment cages were approximately 1 meter square and housed up to 16 mice. The cages used in this experiment were kindly donated by Drs. van Praag and Gage, and their use for environmental enrichment of mouse colonies is well documented (20, 24). Control cages obtained from the breeding colony housed a maximum of 4 animals and were provided no supplemental items. Animals were maintained under these conditions until 8.5 months of age, at which time their brains were harvested and one half fixed by immersion in paraformaldehyde for histological analysis, and the other half dissected to isolate the cortex and hippocampus for biochemical analysis.

Two standard neuropathological stains, Hirano silver and thioflavin-S, were used to visualize plaque formation in paraffin sections from both enriched and control-housed animals. In both housing groups, amyloid deposits were found throughout the cortex and hippocampus by the age of death. The most striking comparisons in amyloid pathology between the 2 groups came from examination of the 3 sibling pairs in the experiment that had been separated at weaning into different housing conditions. In these sib sets, amyloid pathology in the cortex and hippocampus was obviously more severe in animals from enrichment cages (Fig. 1A–D).

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**Fig. 1.** Enrichment increases hippocampal plaque burden in APPswe/PS1dE9 transgenic mice. Comparison of amyloid pathology in a sib pair separated at weaning and housed for 6.5 months in either enriched (a, c, e) or control cages (b, d, f). Sagittal sections were processed for Hirano silver stain (a, b), thioflavin-S (c, d), and ubiquitin immunohistochemistry (e, f). g: The average plaque load (percentage of area covered by ubiquitin immunoreactivity) in each condition is shown, ±SEM. p = 0.043 (z-test). One outlier with plaque coverage >4 SDs from the mean of the remaining set was removed from the control group for all analyses. h: Analysis of the relationship between plaque load estimated by nonbiased stereology and total Aβ concentration measured by ELISA revealed a strong linear correlation, R² = 0.654.
Fig. 2. Hippocampal Aβ aggregates are more abundant in enriched mice than in standard-housed controls. a: Filter trap analysis was used to measure the abundance of Aβ aggregates in dilution series of PBS homogenates prepared from frozen hippocampal tissue. Three samples showing the clearest difference between conditions are shown here. b: The intensity of Aβ immunostaining at dilution 3 in the mid-linear range of each series was quantified using Quantity One image analysis software. Average intensity for each housing condition is expressed in arbitrary units ± SEM (grey: control, black: enriched).

To quantify differences in plaque burden between the enriched and control mice, we measured the area covered by deposits in each animal using nonbiased stereology. This technique provides a methodical system for estimating surface area coverage by counting a regularly spaced fraction of the total region. Because of its easily defined boundaries, we focused on measuring plaque density in the hippocampal formation. Amyloid deposits were labeled by ubiquitin immunohistochemistry, which identifies neuritic plaques by the accumulation of this protein in dystrophic neurites (Fig. 1E, F). This stain is well suited for stereology because it labels plaques with little nonspecific background as required for counting at high-power magnification. As suggested by visual inspection of plaque burden in the sib pairs, quantitation of plaque burden across the entire cohort confirmed that the enriched mice had more amyloid pathology than their control-housed counterparts. When averaged for each group, enrichment increased the percent area covered by ubiquitin-immunoreactive deposits by 68% (Fig. 1G; p < 0.05).

To determine whether the plaque burden observed in enriched mice was underpinned by a greater amount of aggregated Aβ, we used a size-exclusion filter trap assay (25) to detect and quantify detergent-resistant aggregates of Aβ. Frozen hippocampal and cortical samples were homogenized in PBS, partially solubilized with SDS, and filtered through a cellulose acetate membrane. Aggregates trapped in the filter were detected by immunostaining for Aβ (Fig. 2A). Quantitation of staining intensity within the linear range of each dilution series confirmed that overall the enriched animals contained more aggregated Aβ than control mice (hippocampus: 30%, Fig. 2B; cortex: 16%, data not shown).

Commercially available sandwich ELISAs were next used to measure the total amount of Aβ40 and Aβ42 in the PBS-homogenized tissue. To completely solubilize different forms of the peptide, aggregated or otherwise, homogenates were incubated for several hours in 5 M guanidine-HCl before being diluted in buffer for use in the ELISA. Consistent with results from the filter trap assay, ELISA measurements confirmed that the majority of enriched animals contained more Aβ than the standard-housed controls (Fig. 3A). This difference was stronger in the hippocampus than in the cortex (Fig. 3B: total Aβ, hippocampus: 52%; cortex: 31%, data not shown). Notably, in the brains of mice housed in enrichment cages, the levels of Aβ varied much more widely than in animals housed in standard cages (total Aβ and Aβ42, p < 0.05) (Fig. 3).

DISCUSSION

Using 3 separate measures of amyloid burden in the brain we demonstrate that the progression of amyloid pathology in transgenic mouse models of AD can be altered by the environment in which the animals are housed. At the outset of this work we had thought that enrichment might lessen the amyloid load because epidemiological studies had reported decreased risk for AD with advanced education or occupational attainment. Environmental enrichment in mice is thought to be a model for the kinds of changes in brain structure and function that may occur with education or a challenging vocation in humans. For example, housing rodents in enriched environments has been reported to lower the levels of spontaneous apoptosis (26), to increase neurogenesis (20, 24), and to upregulate expression of neurotrophins such as BDNF and NGF (27–30). Morbidity has been significantly lessened.
Fig. 3. Long-term enrichment increases hippocampal Aβ levels in APPswe/PS1dE9 transgenic mice. a: Hippocampal Aβ concentration (pmol/gram brain weight) measured by ELISA is plotted for each animal in both housing conditions (circles: control n = 13, diamonds: enriched n = 11). A significant increase in variance between animals occurs following enrichment. Total Aβ p = 0.034; Aβ42 p = 0.019; Aβ40 p = 0.060 (Mann-Whitney U-test). A single control outlier 7 SDs from mean has been removed from display and all analyses. b: Average hippocampal content of total Aβ, Aβ42, and Aβ40 in each condition (±SEM; grey: control, black: enriched). The difference between enriched and control groups approached significance for each measure of Aβ. Total Aβ p = 0.055; Aβ42 p = 0.057; Aβ40 p = 0.059 (z-test).

by enrichment in several other rodent disease models. Experiments using mice transgenic for huntingtin protein have shown significant improvements in motor ability and lifespan (31–33). Based on these many beneficial effects of environmental enrichment, our finding that amyloid pathology is worsened rather than attenuated in the enriched mice seems paradoxical.

A recent study by Kamenetz et al illuminated a mechanism by which enrichment might exacerbate amyloid pathology (5). Their experiments in organotypic hippocampal cultures demonstrated that neuronal stimulation indirectly modulates the activity of β-APP cleaving enzyme (BACE), which catalyzes the first proteolytic cleavage of APP in the release of Aβ. Using pharmacological manipulation of spontaneous neuronal activity in the hippocampal cultures, they found that stimulated neurons accumulate higher levels of APP β-C-terminal stubs, indicative of increased BACE activity. If this mechanism holds true in vivo, increases in synaptic activity thought to occur in response to enrichment could directly influence the processing of APP to enhance Aβ production. We note, however, that despite testing more than 32 additional transgenic animals following 8 weeks of differential housing, we did not observe any change in Aβ levels between enriched and control mice prior to the onset of plaques (data not shown). In the pre-deposit experiments, the sensitivity of the ELISA assay is an issue and a small change (on the order of 10% to 20%) would be difficult to detect. We also note that Aβ levels are typically normalized to total brain protein and the wet-weights of brains from the enriched mice were uniformly greater than that of the nonenriched mice (pre-deposit animals: hippocampus 18.95 mg (EE) vs 16.08 mg (control) p < 0.000005, Student t-test; cortex 108.8 mg (EE) vs 100.0 mg (control) p < 0.005, Student t-test). Hence, we cannot say with certainty that the increased amyloid deposition in the enriched mice is due to a change in APP processing or whether other mechanisms are responsible.

Several other factors could contribute to elevated amyloid burdens seen in the enriched animals. For example, enrichment increases neuronal number (24) and promotes synaptogenesis (4). APP is axonally transported to nerve terminals (34–37), and studies showing the absence of amyloid in the dentate gyrus following transection or lesion of the entorhinal cortex suggest that Aβ is released at the synapse-rich terminal fields of these projections (38, 39). The survival of more neurons supporting a greater number of synapses could also account for the enrichment-associated increase in Aβ deposition we observe.

Alternatively, the lesion studies of Lazarov et al (38) and Sheng et al (39) may indicate a critical role for synaptic integrity in maintaining the extracellular matrix upon which amyloid is deposited. Very little is known about how the extracellular environment may influence the aggregation of amyloid peptides into plaques. Once plaques are seeded through peptide interactions in solution, it is possible that their adherence to the local extracellular matrix may determine whether small aggregates grow into larger deposits or are instead cleared from the system (40, 41). In support of this interpretation, a recent study by Jucker et al demonstrated that wild-type neural tissues grafted into the cortex of transgenic mice expressing mutant APP develop amyloid deposits faster than surrounding transgenic tissue (42). The embryonic progenitor cells used in the graft likely produce a somewhat
different extracellular matrix than the surrounding adult tissue, which may be responsible for enhancing amyloid deposition. Perhaps the neuronal (and glial [43, 44]) changes associated with enrichment also promote the expression of matrix components that facilitate amyloid deposition.

Genetic background may also play a substantial role in the response to enrichment. The mice used for this experiment were hybrids of C3HeJ and C57BL/6J strains, and although the line was maintained at each generation by backcrossing transgenic animals to F1 hybrid mates, the genetic contribution of each parental strain can vary considerably among the offspring. Based on recent work using congenic APP/PS1 mice, we believe that the large spread in Aβ measures shown in the enriched cohort reflects differences in the response to enrichment of the parental C3HeJ and C57BL6 strains.

The present experiments provide the first experimental demonstration that AD-related pathological changes can be influenced by environmental factors. Simply altering the housing environment of transgenic APP/PS1 mice substantially accelerated the course of their disease. Given this exquisite sensitivity to environment, it may be that even the standard tasks used in cognitive behavioral testing could produce effects similar to enrichment. Thus, we caution that the housing and handling experiences of mice must be considered in interpreting pathological data in the AD mouse models.

How our findings in enriched mice relate to education in humans is difficult to gauge. We might predict that amyloid pathology is accelerated by education, but that other compensatory facets of enhanced brain function mitigate the injury caused by AD-related lesions. Certainly, genetic variation between individuals likely modulates the impact of education not only on amyloid pathology, but also on the development and progression of other aspects of the disease.

ACKNOWLEDGMENTS

We thank Henriette van Praag for kindly donating the enrichment cages, Linda Burke and Mary Brodey for advice on ELISA protocols, Gay Rudow for assistance with stereology, Alena Savonenko for help with statistics, and Marshall White for animal care. JLJ thanks Mrs. Eloise Goodhew Barnett for her generous support.

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Received June 19, 2003
Revision received August 8, 2003
Accepted August 18, 2003