Sustained hippocampal IL-1β overexpression mediates chronic neuroinflammation and ameliorates Alzheimer plaque pathology

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Introduction

The proinflammatory cytokine IL-1β is a key regulator of acute inflammatory processes in the CNS (1). IL-1β upregulation is part of a patterned response that unfolds after a wide range of CNS insults including infection, trauma, and stroke (2). This response, termed neuroinflammation, is characterized by activation of neuroglia and liberation of inflammatory mediators into the local milieu. Recent work has established increased IL-1β activity as a central driving force in acute neuroinflammation and has provided evidence for a harmful role of IL-1β in this setting (2, 3).

IL-1β–driven neuroinflammation is also thought to contribute to the pathophysiology of chronic neurodegenerative diseases (4–7), but attempts to define the functional significance of sustained increases in IL-1β expression have been hindered by a deficiency in models of long-term expression. Coexistence of robust neuroinflammation and IL-1β expression were first documented in Alzheimer disease (AD) (4, 8). Until now, ascribing a role to IL-1β in AD pathogenesis has relied on indirect evidence from exposure paradigms in normal animals.

In hopes of better defining the role of IL-1β in chronic neuroinflammation and AD, we engineered a transgenic model that allows for spatial and temporal control of the initiation of IL-1β overexpression within the mouse brain. We demonstrated that sole expression of IL-1β was sufficient to drive a robust, sustained neuroinflammatory response within the mouse hippocampus. Based on inferences drawn from acute neuroinflammatory models, we hypothesized that IL-1β overexpression would exacerbate AD pathology when we crossed these animals with APPswe/PS1dE9 (APP/PS1) mice. Surprisingly, we discovered an adaptive role for IL-1β in this setting as evidenced by significant reductions in measures of amyloid pathology. This effect was likely mediated through enhancement of microglia-dependent plaque degradation.

Results

Engineering of the IL-1βXAT mouse model. In order to study the role of sustained cytokine expression within the brain, we employed an excisional activation transgene (XAT) (9) cassette to drive eventual transcription of IL-1β in the CNS (10). We subcloned the hybrid cDNA sstIL-1β—which incorporates the signal sequence (ss) from human IL-1 receptor antagonist (hIL-1ra) fused in frame to the coding sequence for mature hIL-1β, thus directing extracellular release and eliminating the need for caspase-1 cleavage of IL-1β (11)—into a universal XAT vector along with a LacZ reporter gene (9). We chose a human transgene because of its ability to signal through IL-1 receptor 1 (IL-1R1) in the mouse and because of the capability of distinguishing the transgene from its murine counterpart. Functionality of the transgene construct was established in the 293<sup>GLVp/CrePr</sup> stable cell line, which is capable of producing Cre when treated with RU486 (Figure 1A) (12). Cre expression led to DNA excisional recombination and dropout of the approximately 2.5-kb loxp-flanked transcriptional stop sequence as well as induction of hLIL-1β relative to GAPDH. Additionally, increased LacZ activity was observed using X-gal histochemistry (Figure 1A).

We substituted a GFAP promoter to direct CNS-specific expression of the transgene and used the resulting IL-1βXAT construct to create parallel lines of transgenic mice (Figure 1B). Following microinjections, we identified 2 heterozygous transgenic founders designated A/a and B/b (Figure 1C). We used an infection-compe tent, replication-incompetent feline immunodeficiency virus (FIV) to deliver Cre to the brain, which allowed for both temporal and spatial control of the initiation of IL-1β production. Production

Nonstandard abbreviations used: Aβ, amyloid β; AD, Alzheimer disease; APP, amyloid precursor protein; APP/PS1 mouse, APPswe/PS1dE9 mouse; APP/PS1-IL-1β mouse, mouse expressing the APP/PS1 and IL-1β<sup>XAT</sup> transgenes; FIV, feline immunodeficiency virus; h-, human; Iba-1, ionized calcium-binding adaptor molecule 1; IL-1R1, IL-1 receptor 1; m-, murine; MCP, monocyte chemoattractant protein; PS1, presenilin 1; qRT-PCR, quantitative real-time PCR; ra, receptor antagonist; ss, signal sequence; XAT, excisional activation transgene.

Conflict of interest: The authors have declared that no conflict of interest exists.

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of hIL-1β protein in FIV-Cre–infected IL-1βXAT primary astrocyte cultures confirmed the function of the transgene construct (Figure 1D). Stereotactic injection of FIV-Cre into the dentate gyrus of IL-1βXAT mice led to viral transduction of both neuronal and non-neuronal cells (Figure 1E). FIV-Cre–mediated hIL-1β protein expression was detected in astrocytes (Figure 1F) but not in microglia or neurons (data not shown). LacZ activity was not detected in transgenic animals (data not shown). Finally, following stereotactic injections of FIV-Cre into the dentate gyrus of the mouse brain, we demonstrated spatially restricted MHC class II expression in the injected ipsilateral hippocampus (Figure 1G).

Neurominflammatory phenotype of IL-1βXAT mice. After successful creation of the IL-1βXAT model, our initial efforts were directed at defining the downstream effects of sustained IL-1β expression in the adult murine CNS (Figure 2). Two weeks following intrahippocampal FIV-Cre injections, we sought to characterize phenotypic and transcriptional changes in glia residing in the hippocampus of IL-1βXAT A/a and B/b animals compared with WT animals. FIV-Cre injections in WT mice controlled for inflammation resulting from the stereotactic surgeries and host response to the viral vector. Most of the phenotypic changes were seen in the region of the dentate gyrus, where the stereotaxic injections were directed (Figure 2, A...
and B). Microglial activation was most prominent in B/b animals, as demonstrated by dramatic increases in ionized calcium-binding adaptor molecule 1 (Iba-1) staining through much of the hippocampus (Figure 2A). Many microglia assumed a highly activated amoeboid state, with fewer cellular processes than in WT animals (Figure 2A, insets). The B/b animals also exhibited robust MHC class II expression — in both perivascular and parenchymal sites — that colabeled with Iba-1. In the A/a animals there was very mild activation of microglia and scant MHC class II expression limited to perivascular Iba-1–positive cells (Figure 2A). Astrocyte activation, as evidenced by increased GFAP staining intensity of Iba-1, was demonstrated in the dentate gyrus of B/b mice only. Scale bars: 50 μm (A and B); 10 μm (insets in A). (C) GFAP mRNA expression was significantly increased only in B/b mice (2.9-fold; Figure 2D). Intrahippocampal injections using GFP-expressing FIV in A/a and B/b animals failed to precipitate a neuroinflammatory response, establishing its dependence upon exposure to Cre (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI31450DS1). Based on this demonstration of a more potent neuroinflammatory phenotype in B/b versus A/a IL-1βXAT mice, we used only B/b animals in our subsequent experiments.

In order to prove that IL-1β was in fact mediating the inflammatory response seen, we crossed B/b mice with il1r1−/− animals (Figure 3). These animals lack IL-1R1, the sole biologically active receptor for IL-1β (13). As expected, il1r1−/− animals failed to demonstrate increases in neuroinflammatory indices 2 weeks following transgene activation. Relative MHC class II expression was unchanged in Il1r1−/− B/b compared with WT animals (0.84-fold that of WT), whereas Il1r1−/− B/b animals demonstrated an intermediate phenotype compared with Il1r1−/− B/b mice (20.4- and 46.3-fold increases, respectively, relative to WT; Figure 3, A and B). GFAP mRNA expression in Il1r1−/− B/b mice was 1.03-fold that of WT controls, but this was significantly upregulated in both Il1r1−/− B/b and Il1r1−/− B/b mice (2.7-fold and 2.5-fold, respectively, that of WT; Figure 3C).

IL-1βXAT model of chronic neuroinflammation. To establish the IL-1βXAT mouse as a useful model of chronic CNS inflammation, we followed cohorts of adult B/b and WT mice for an extended time course after transgene activation (Figure 4). Expression of MHC class II gene transcripts, representative of sustained micro-
glial activation, remained significantly elevated compared with WT controls between 1 week and 10 months after FIV-Cre injection (Figure 4A). An analogous pattern of expression was determined for GFAP expression, attributable to chronic astrocyte activation (Figure 4C). Elevated MHC class II and GFAP expression were detected histochemically 1 year after FIV-Cre injection, the latest time point at which the mice were examined (Figure 4, B and D). The chronology of glial activation was mirrored by expression of the activated hIL-1β transgene, which peaked early in the time course and was last detected 4 months after transgene activation (Figure 4E). In addition to glial activation markers, we sought to determine whether transgene activation could drive induction of inflammatory cytokines classically associated with acute hIL-1β activity. Indeed, transgene activation resulted in chronic, increased expression of all members of the murine IL-1 (mIL-1) family: mIL-1α, mIL-1β, and mIL-1ra (Figure 4F). In addition, hIL-1β activity drove chronic expression of the proinflammatory cytokines TNF-α and IL-6 (Figure 4G).

**IL-1β overexpression reduces amyloid pathology.** Our primary impetus for creating the IL-1βXAT mouse model was a desire to understand the functional role of sustained IL-1β upregulation in AD. Having confirmed their ability to mount an IL-1β-driven chronic neuroinflammatory response, we next crossed the IL-1βXAT B/b animals with the APP/PS1 mouse model of AD (14). This model coexpresses a chimeric mouse/human amyloid precursor protein (APP) along with human mutant presenilin 1 (PS1) and features accelerated plaque deposition beginning at 4 months of age (15, 16). We hypothesized that sustained IL-1β overexpression would exacerbate the plaque pathology seen in these animals based on studies linking IL-1β expression to disease pathogenesis (17). Pathologic lesions were characterized in 7-month-old animals 4 weeks after FIV-Cre injections (Figure 5). We were surprised to discover substantially reduced pathologic indices in animals exposed to sustained levels of IL-1β expression. Using Aβ antibody and Congo red staining, we observed dramatically reduced plaque pathology in the injected ipsilateral hippocampi of mice expressing the APP/PS1 and IL-1βXAT transgenes (APP/PS1+IL-1β mice; Figure 5, A and B).

To account for wide variability in pathologic indices in this mouse model at the time point assayed, we determined the ratio of pathologic indices between the FIV-Cre–injected ipsilateral and un.injected contralateral hemispheres within individual animals. Detailed histological analysis of fibrillar plaques stained with Congo red revealed 59% and 46% reductions in plaque area fraction and frequency, respectively, in APP/PS1+IL-1β compared with APP/PS1 mice (Figure 5C). Analogous observations were made using Thioflavine-S staining (data not shown). Amelioration of plaque pathology was further substantiated by determining the concentration of hippocampal-insoluble amyloid β (Aβ) peptide, the major component of plaques. Aβ40 and Aβ42 peptides were reduced by 46% and 36%, respectively, in APP/PS1+IL-1β compared with APP/PS1 mice (Figure 5D). No significant differences were observed for their soluble counterparts (Figure 5E and Supplemental Table 1). This finding, together with the lack of evidence that IL-1β modulates APP expression or BACE-1 expression and activity (Supplemental Figure 2), suggests that the observed reduction in plaque pathology in this model is likely not mediated through regulation of Aβ synthesis.

**Enhanced microglial activation may underlie reductions in plaque pathology.** Based in part on their physical association with plaques in AD (4), ability to efficiently phagocytose amyloid (18, 19), and heightened activation states in the IL-1βXAT mouse model as demonstrated in the present study, microglia represent an attractive candidate cell type for mediating the observed reductions in plaque pathology in APP/PS1+IL-1β mice. Using confocal microscopy and To-Pro-3 nuclear stain, we demonstrated that direct interaction readily occurred between microglial processes and amyloid plaques in APP/PS1+IL-1β mice (Figure 6A). IL-1β overexpression caused both a dramatic spatial shift in the relationship between microglial cell nuclei and amyloid plaque and an increased Iba-1 staining intensity within these cells (Figure 6A). Quantitative analysis revealed a 4.4-fold increase in the number of microglial nuclei overlapping plaques in APP/PS1+IL-1β compared with APP/PS1 mice (4.27 and 0.96 nuclei per plaque, respectively; Figure 6B). MHC class II, a classic marker of activated scavenger cells (20), was highly expressed among amoeboid Iba-1–expressing microglia directly contacting amyloid plaques (Figure 6, C and D). Moreover, monocyte chemotactic protein–1 (MCP-1) expression was significantly increased within the hippocampi of APP/PS1+IL-1β mice (Figure 6C).
Discussion
Two decades of intense research have established neuroinflammation as an integral feature of AD, sparked by initial discoveries of colocalization of MHC class II–immunoreactive microglia with neuritic plaques (8, 21). Shortly thereafter, the importance of IL-1 signaling to AD pathology was recognized, based on observations in reactive glia surrounding plaques (4). In the ensuing years neuroinflammation became implicated as a primary contributor to AD pathogenesis based on epidemiologic studies linking chronic nonsteroidal antiinflammatory drug (NSAID) use to reduced AD incidence (22, 23), and IL-1β was identified as a key instigating factor (24).

Previously, assigning a functional role to IL-1β expression in AD relied largely on indirect evidence. This evidence included reported associations between AD pathology and IL-1 expression patterns (25), disease risk and genetic polymorphisms (26, 27), and the downstream effects of IL-1β treatment in vitro (28) and in normal animals (17). Based on these reports, along with demonstrations of detrimental roles in acute neuroinflammatory settings (2), we expected that our study would reveal IL-1β–driven exacerbation of AD neuropathology.

Instead, this work provides the first evidence to our knowledge that IL-1β expression may underlie a beneficial neuroinflammatory response in AD. Enhanced IL-1β expression is a part of a stereotypical host-defense reaction to a wide range of injuries to the CNS (1), and its expression is augmented in mouse models of AD (29, 30) and in response to Aβ peptide exposure (31). In AD, IL-1β expression may serve as an effective homeostatic mechanism to...
the complex role that IL-1β plays in AD pathogenesis. Our study focused on a single time period during the disease course, shortly after plaque pathology is first observed (16), to facilitate detection of the anticipated IL-1β–mediated exacerbation in plaque pathology. Triggering early, localized IL-1β expression mediated a substantial reduction in plaque pathology (Figure 5). As described above, our results suggest that IL-1 expression in AD, which is thought to parallel worsening of disease pathology (25), represents a response to counteract plaque accumulation within the brain parenchyma. Ultimately, this balance may be overwhelmed by ongoing plaque deposition, which would explain the prominence of plaques in late-stage AD. We are currently investigating whether IL-1β overexpression can mediate analogous reductions in plaque pathology at later time points in APP/PS1 mice. Additionally, it has been suggested that IL-1β is capable of triggering a vicious cycle of increased APP expression, Aβ synthesis, and further IL-1β elevation in AD (34). However, in contrast to previously published studies in human cells (35), we found no evidence for upregulation of the murine APP gene in our model (Supplemental Figure 2E).

Our findings suggest that microglia are the principal mediators of the beneficial effects of IL-1β overexpression in APP/PS1 mice. Through studies of microglial morphology and MHC class II expression, we showed that IL-1β overexpression was capable of driving phenotypic and immunologic activation of microglia (Figure 2, A and C). In addition to our present results, previous studies have shown microglial activation to be beneficial in other mouse models of AD (36, 37). Microglial phagocytosis of plaque has been established in vivo and is thought to be a key mechanism underlying effective Aβ immunotherapy (38, 39). IL-1β overexpression in the APP/PS1 model resulted in increased overlap of activated microglial nuclei with plaques, which could potentially increase the efficiency of plaque phagocytosis (Figure 6, A, B, and D). This may be coupled with degradation of plaques by microglia-produced proteases (40). IL-1β–driven enhancement of plaque degradation may be explained by increased seeding of bone marrow–derived microglia in the brain, as evidenced by the amoeboid-shaped perivascular cells coexpressing Iba-1 and MHC class II seen in IL-1βSTAT mice (Figure 2A). This microglial subpopulation is recruited to sites of Aβ deposition and is thought to be the most effective at restricting plaque
formation (31, 41). Robust MCP-1 upregulation in the hippocampi of APP/PS1+IL-1β mice may serve as a potent chemotactic force for such recruitment (Figure 6C). Enhanced recruitment of bone marrow–derived microglia may be an important mechanism of the IL-1β–driven reductions in plaque pathology seen in the APP/PS1 mice. However, our results do not eliminate the possibility that other cell populations participate in reductions in amyloid pathology. Future studies using GFP expressing bone marrow chimeras will help establish a functional role for these microglial cells.

This study provides the first description to our knowledge of a mouse model capable of chronic IL-1β overexpression and emphasizes the centrality of IL-1β signaling to inflammatory processes of the brain. We have shown that solitary IL-1β overexpression was capable of driving a robust neuroinflammatory response in the mouse hippocampus. We believe that the IL-1βXAT mouse presents an invaluable model system that enables exploration of the functional significance of IL-1β overexpression in a wide range of chronic neuroinflammatory disorders. Moreover, this work provides the first functional characterization to our knowledge of the role of IL-1β–driven neuroinflammation in a mouse model of AD. We have provided evidence that IL-1β–driven neuroinflammation may perform a beneficial, adaptive role in mediating a reduction in AD pathology, perhaps through enhancement of microglial phagocytosis of amyloid plaque.

**Methods**

Cloning of the IL1β-XAT vector. The ssIL-1β construct (539 bp) consists of the signal sequence from hIL-1ra (75 bp) fused to the cDNA sequence of mature hIL-1β (464 bp) (42). The cloning of the CMV-IL1βXAT vector has been described previously (43). The murine GFAP promoter was kindly provided by I. Campbell (University of Sydney, Sydney, New South Wales, Australia; ref. 44) and excised by NotI/EcoRI digestion of the plasmid pGFGH. It was then blunt ended and inserted into the XbaI site of a modified pCRII-TOPO vector (Invitrogen) containing a custom-made PacI cloning site (43). The resultant vector was then PacI digested, and the GFAP promoter was ligated into the PacI site of CMV-IL1βXAT in place of the CMV promoter, creating the final IL1βXAT vector.

**In vitro studies.** Testing of CMV-IL1βXAT was performed in the 293GLV/CrePr cell line as previously described (42). Briefly, Cre expression was initiated following successful transfection by addition of RU486 at a final concentration of 1 × 10⁻⁷ M. RNA and DNA extraction (TRIZol and DNAzol, Invitrogen) as well as X-gal (Invitrogen) histochemistry were performed 60 hours later. Primers specific for ssIL-1β (HIL-1β-FIXUP, 5’-ATGGAAATCTGCAGAGGCCTCC-3’; IL1β-17kD-LP, 5’-CTTTAGGAAGACACAAATTGCATGG-3’)}
and GAPDH (5′-AGGTGAAGGTCGGAGTCAAC-3′; 5′-TGGGGGAGATCATATTGGGAC-3′) were used. To demonstrate successful Cre-mediated loxP recombination, PCR was performed with primers HEX-XAT-UP (5′-AATGTCTGAAACTCCG-3′) and IL1β-17kD-UP, binding to the 3′ ends of the CMV promoter and ssIL-1β transgene, respectively. Primary mouse astrocyte cultures were derived from homogenized cortices of P0–P1 animals plated in complete media with 10% fetal bovine serum for 5–7 days until confluent. After 2 days of constant agitation at 300 rpm on a rotary shaking platform, adherent astrocytes were segregated according to transgene status, pooled, and plated overnight in complete media, resulting in astrocyte cultures that were greater than 98% pure (45). Viral infections were performed for 15 hours in the presence of 6 μg/ml polybrene (Sigma-Aldrich) followed by a media change. Supernatants were harvested 60 hours later and analyzed using an hIL-1β–specific ELISA (R&D Systems).

**Transgenic mice.** All animal procedures were reviewed and approved by the University Committee on Animal Resources of the University of Rochester Medical Center for compliance with federal regulations prior to the initiation of the study. The IL1βloxP transgene construct was linearized from its vector by NotI digestion and purified using QIAEX II columns (QIAGEN). The approximately 10-kb construct was microinjected into fertilized C57BL/6 mouse eggs and implanted into pseudopregnant females by the University of Rochester Transgenic Facility, yielding 11 live-born progeny. Genomic DNA was isolated from tail snips of potential founders using the Wizard SV Genomic kit (Promega) and screened using the ssIL-1β–specific primers described above. Founders were maintained on a pure C57BL/6 background (The Jackson Laboratory). Maintenance genotyping of lines A/a and B/b was performed by qRT-PCR using the following ssIL-1β–specific primers: forward, 5′-TTCTCCCAACTGTACATCAGC-3′; reverse, 5′-CCGGCTTCTTGGCTCCTCC-3′; probe, 5′-CCCTCTCAAGGAGAAACAAAA-GCCGTCCTTT-3′. Progeny of IL1βloxP founders appeared healthy and without anatomic or behavioral abnormalities. Il1r1−/− (stock no. 3245) and AP5we/PS1dE9 (stock no. 4462) mice were obtained from The Jackson Laboratory and genotyped according to the manufacturer’s recommended protocols. Experimental APP2PS1 and APP2PS1/IL1β−/− mice on a mixed B6C3F1/J × C57BL/6J genetic background were produced from an F1 cross of APP2PS1 with IL1β−/− transgenic animals. Intrahippocampal injections were performed at 8–12 weeks of age, and equal numbers of male and female mice were used.

**FIV.** The construction and packaging of FIV-Cre has been described previously (43). Briefly, the FIV-Cre virus encodes the nuclear localization sequence, Cre recombinase protein, and V5 epitope tag under the control of a CMV promoter. FIV-Cre and FIV-GFP (System Biosciences) were packaged for a final titer of approximately 1 × 10^10 infectious viral particles per milliliter. For in vitro experiments, an MOI of approximately 0.25 was achieved. In vivo stereotactic injections contained 1.5 μl of FIV-Cre or FIV-GFP in order to deliver approximately 1.5 × 10^6 infectious viral particles to the mouse hippocampus. Viral titration was performed in the 293T cell line using an anti-V5 antibody or GFP fluorescence (Invitrogen).

**Stereotaxic injections.** Mice were anesthetized with 1.75% isoflurane in 30% N2O and 67% O2 in a Kopf stereotaxic apparatus. To prevent sacca, lubricant was applied to the cornea. The scalp was thoroughly scrubbed with betadine before making a midline incision. A burr hole (0.5 mm) was then drilled in the skull at ~1.8 mm caudal and 1.8 mm horizontal from bregma. A 33-gauge needle mounted to a 10-μl syringe (Hamilton) preloaded with virus was lowered 1.75 mm from the brain surface over a 2-minute period. A Micro-1 microsyringe pump controller (World Precision Instruments) injected 1.5 μl of virus at a constant rate over a 10-minute period. After allowing an additional 5 minutes for diffusion of the virus, the needle was raised slowly over a 2-minute period. The burr hole was sealed with bone wax, and the scalp incision was closed with 6-0 nylon suture (Ethicon).

**qRT-PCR.** Mice were deeply anesthetized i.p. with ketamine and xylazine (60–90 and 4–8 mg/kg, respectively) and sacrificed by decapitation. The hippocampus ipsilateral and contralateral to the injection site was immediately dissected and snap-frozen in isopentane chilled with dry ice and stored in sterile tubes at –80°C. RNA was isolated using TRIzol (Invitrogen) and an Omni 2000 tissue homogenizer according to the manufacturer’s suggested protocol. cDNA was generated using 1 μg DNase-treated total RNA, oligo (dt) and random hexamer primers, and SuperScript III reverse transcriptase (Invitrogen). Quantification of relative mRNA abundance was determined by using custom-designed primer (Invitrogen) and FAM 490 probes (Biosearch Technologies) or proprietary predesigned primer/probe sets (Applied Biosystems) and the iCycler (Bio-Rad). For in vitro experiments, an MOI of approximately 0.25 was used. The construction and packaging of FIV-Cre has been described previously (43). Briefly, the FIV-Cre virus encodes the nuclear localization sequence, Cre recombinase protein, and V5 epitope tag under the control of a CMV promoter. FIV-Cre and FIV-GFP (System Biosciences) were packaged for a final titer of approximately 1 × 10^10 infectious viral particles per milliliter. For in vitro experiments, an MOI of approximately 0.25 was achieved. In vivo stereotactic injections contained 1.5 μl of FIV-Cre or FIV-GFP in order to deliver approximately 1.5 × 10^6 infectious viral particles to the mouse hippocampus. Viral titration was performed in the 293T cell line using an anti-V5 antibody or GFP fluorescence (Invitrogen).
stored at -80°C until use. Samples containing formic acid were diluted 1:20 in neutralization buffer (1 M Tris, 0.5 M Na2HPO4) prior to addition to the assay plate. For Western blot, hippocampal lysates were diluted in 2x sample buffer (125 mM Tris-HCl, 4% SDS, and 20% glycerol), and protein concentration was determined by a bicinchoninic assay (Pierce). Protein (15 μg) was electrophoresed on a Tris-HCl polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad) to detect APP, while analysis of the β-carboxyterminal fragment of APP (β-CTF) was performed using a Tris-Tricine gel and PVDF membrane (Bio-Rad). After 1 hour in Western blocking reagent (Roche Diagnostics), membranes were incubated overnight with primary antibodies to APP (diluted 1:2,000; clone 6E10; Chemicon), GAPDH (diluted 1:5,000; Ambion), or β-CTF (diluted 1:1,000; Sigma-Aldrich). After rinsing, blots were incubated with peroxidase-linked secondary antibodies (provided in Supersignal West Dura Kit; Pierce), and bands were visualized using either X-AR film or the Image Station 440 CF (Kodak).

Immunohistochemistry. Mice were deeply anesthetized with ketamine/xylazine as described above and then intracardially flushed, first briefly with –25°C with more than 50 ml of ice-cold 4% paraformaldehyde (PFA) in 0.15 M PB (pH 7.2).Brains were carefully removed and postfixed in 2 hours for 4% PFA before overnight equilibration in 30% sucrose in 0.15 M PB. Whole brains were then snap-frozen in isopentane and stored at –80°C until cryosectioning into 30-μm sections on a sliding microtome. The resulting free-floating sections were stored in cryoprotectant until processing. Visualization of antibody-bound sections on a slidding microtome with a –25°C freezing stage. The resulting free-floating sections were stored at –80°C until use. Samples containing formic acid were diluted 1:20 in neutralization buffer (1 M Tris, 0.5 M Na2HPO4) prior to addition to the assay plate. For Western blot, hippocampal lysates were diluted in 2x sample buffer (125 mM Tris-HCl, 4% SDS, and 20% glycerol), and protein concentration was determined by a bicinchoninic assay (Pierce). Protein (15 μg) was electrophoresed on a Tris-HCl polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad) to detect APP, while analysis of the β-carboxyterminal fragment of APP (β-CTF) was performed using a Tris-Tricine gel and PVDF membrane (Bio-Rad). After 1 hour in Western blocking reagent (Roche Diagnostics), membranes were incubated overnight with primary antibodies to APP (diluted 1:2,000; clone 6E10; Chemicon), GAPDH (diluted 1:5,000; Ambion), or β-CTF (diluted 1:1,000; Sigma-Aldrich). After rinsing, blots were incubated with peroxidase-linked secondary antibodies (provided in Supersignal West Dura Kit; Pierce), and bands were visualized using either X-AR film or the Image Station 440 CF (Kodak).

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