β-Amyloid infusion results in delayed and age-dependent learning deficits without role of inflammation or β-amyloid deposits

Tarja Malm*, Michael Ort[†‡], Leena Tähtivaara[§], Niko Jukarainen[¶], Gundars Goldsteins*, Jukka Puoliväli[§], Antti Nurmi[§], Raimo Pussinen[§], Toni Ahtoniemi*, Taina-Kaisa Miettinen[§], Katja Kanninen*, Suvi Leskinen*, Nina Vartiainen[§], Juha Yrjänheikki[§], Reino Laatikainen[¶], Marni E. Harris-White[‖], Milla Koistinaho*[§], Sally A. Frautschy[‖**], Jan Bures[†,††], and Jari Koistinaho*[†††‡‡]

*A. I. Virtanen Institute and [¶]Department of Chemistry, University of Kuopio, and [‡‡]Department of Oncology, Kuopio University Hospital, P.O. Box 1627, FI-70211, Kuopio, Finland; [†]Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4-Krc, Czech Republic; [‡]Department of Psychiatry, Charles University, Ke Karlovu 11, 121 28 Prague 2, Czech Republic; [§]Cerebricon, Ltd., Microkatu 1, FI-70210, Kuopio, Finland; [‖]Department of Medicine, University of California, Los Angeles, and the Veterans Administration Greater Los Angeles Healthcare System, Sepulveda, CA 91343; and **Department of Neurology, University of California, Los Angeles, and the Geriatric Research Education and Clinical Core, North Hills, CA 91343

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β-Amyloid (Aβ) polypeptide plays a critical role in the pathogenesis of Alzheimer's disease (AD), which is characterized by progressive decline of cognitive functions, formation of Aβ deposits and neurofibrillary tangles, and loss of neurons. Increased genetic production or direct intracerebral administration of Aβ in animal models results in Aβ deposition, gliosis, and impaired cognitive functions. Whether aging renders the brain prone to Aβ and whether inflammation is required for Aβ-induced learning deficits is unclear. We show that intraventricular infusion of $Aβ_{1–42}$ results in learning deficits in 9-month-old but not 2.5-month-old mice. Deficits that become detectable 12 weeks after the infusion are associated with a slight reduction in Cu,Zn superoxide dismutase activity but do not correlate with Aβ deposition and are not associated with gliosis. In rats, Aβ infusion induced learning deficits that were detectable 6 months after the infusion. Approximately 20% of the Aβ immunoreactivity in rats was associated with astrocytes. NMR spectrum analysis of the animals cerebrospinal fluid revealed a strong reduction trend in several metabolites in Aβ-infused rats, including lactate and myo-inositol, supporting the idea of dysfunctional astrocytes. Even a subtle increase in brain $Aβ_{1–42}$ concentration may disrupt normal metabolism of astrocytes, resulting in altered neuronal functions and age-related development of learning deficits independent of Aβ deposition and inflammation.

aging | cerebrospinal fluid | learning | oxidative stress | Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of progressive impairment of cognitive functions in humans during aging (1). Diagnosis is confirmed by the presence of neurofibrillary tangles (formed via hyperphosphorylation of tau) and β-amyloid (Aβ)-containing plaques (2). Aβ, a 39- to 43-aa-long polypeptide derived from the Aβ precursor protein (APP), is neurotoxic and plays a critical role in the pathogenesis of AD (1, 2). Mutations in APP or presenilin genes that lead to the elevation of fibrillogenic form of Aβ are responsible for only a small portion of AD cases (1, 3, 4). In addition, triplication of APP gene-containing chromosome 21 in Down's syndrome results in AD, and the transgenic (TG) mouse models (5) overexpressing human mutant APP or presenilin genes several-fold, show progressive accumulation of Aβ deposits, gliosis, and cognitive decline (2). The relationship between Aβ deposition and cognitive decline is unclear because the severity of dementia does not correlate with Aβ plaques (6). In TG models, cognitive deficits can occur in the absence of Aβ deposits (7–10). Recent findings emphasize the role of soluble Aβ oligomers. Aβ oligomers are highly neurotoxic (11), and their amount in the brain correlates with the severity of AD and with spatial learning deficits in APP TG mice (12). However, it is not completely clear whether the neuropathology in these mice is solely a consequence of Aβ accumulation or whether increased concentrations of other cleavage products of APP, including the carboxyl-terminal fragments, also contribute to the dysfunction of brain cells and cognitive disturbances (13). This concern also is raised by the fact that overexpression of mutant presenilin gene, which increases productions of $Aβ_{1–42}$, is not sufficient to cause impairment in cognitive functions without APP mutation (13).

Neuroinflammation, manifested as activated microglia and astrocytes and increased expression of proinflammatory proteins in association with Aβ deposits, is an invariant feature of AD brain (14). Similarly, activated glia and production of proinflammatory mediators are observed in almost all TG and Aβ infusion models of AD at the time amyloid deposits become detectable (15, 16). In addition, fibrillogenic Aβ triggers activation of microglia *in vitro*, resulting in the release of neurotoxic molecules (17). Epidemiological studies suggest that patients treated with high doses of nonsteroidal antiinflammatory drugs (NSAIDs) have a lower risk of developing AD (18). Moreover, numerous compounds with antiinflammatory activity reduce progression of cognitive deficits or Aβ burden in AD models (18–22). Therefore, inflammation is considered as a promising target for developing AD therapy. Recent studies have challenged the role of inflammation in the development of cognitive deficits, suggesting that the role of glial activation is more complicated than previously thought. Activated glia may participate in Aβ clearance and plastic restorative changes (23–26). Activation of glia may be triggered by Aβ deposition and neuronal injury, and some studies have shown that both fibrillar and soluble Aβ have the potential to activate glia (27–29). However, NSAIDs may lower the secretion of the amyloidogenic $Aβ_{1–42}$ (30, 31), offering an inflammation-independent alternative for the potentially beneficial effects of antiinflammatories in AD.

Another characteristic feature in humans and animal models is the strong influence of aging on the development of AD pathology and the long maturation time before gene mutations in APP and/or presenilin result in behavioral deficits. It has been hypothesized that with time, the increased Aβ production begins

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Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; CSF, cerebrospinal fluid; HDL, high-density lipoprotein; MWM, Morris Water Maze; TG, transgenic.

[††]To whom correspondence may be addressed. E-mail: bures@biomed.cas.cz or jari.koistinaho@uku.fi.

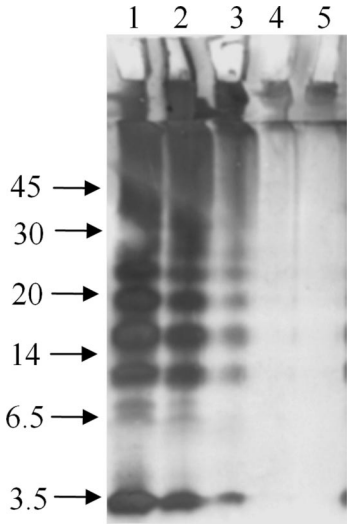

Fig. 1. Western blot analysis of the $A\beta_{1-42}$-HDL preparation released from the osmotic pumps. Samples were taken twice a week for 2 weeks, and 0.5 μg samples were run on gel and blotted against antibody recognizing human $A\beta_{1-42}$. Lane 1 represents the form of $A\beta_{1-42}$ filled in the pumps, and lanes 2–5 (2, 3 days; 3, 7 days; 4, 10 days, 5, 14 days) represent the $A\beta_{1-42}$ released over the 2-week period.

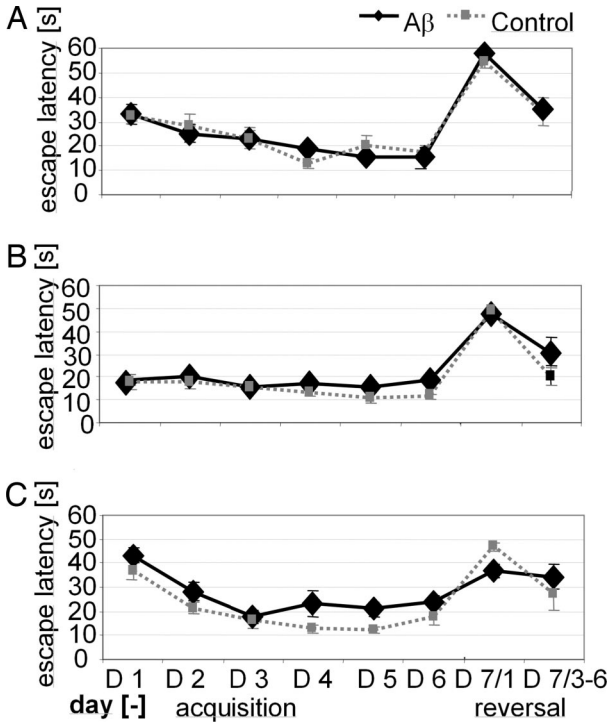

Fig. 2. $A\beta_{1-42}$ infusion results in delayed learning deficits in 9-month-old mice. (*A*) Learning curves 6 weeks after infusion, the first test in MWM. $A\beta_{1-42}$-HDL (Aβ)-infused and HDL (control) animals learned equally well, both groups improved their performance, and no differences in their learning abilities were found. Reversal trials were significant in comparison with previously reached asymptotes in both groups but did not differ from each other. (*B*) Learning curves 12 weeks after infusion, the second test of the mice (shown in *A*) in MWM. HDL- and $A\beta_{1-42}$-HDL-infused mice learned differentially, their learning curves differed significantly, and they reached asymptotic levels at a significantly different pace. Reversal trials were significant in comparison with previously reached asymptotes in both groups but did not differ from each other. (*C*) Learning curves 12 weeks after infusion, the first test in MWM. $A\beta_{1-42}$-HDL-infused mice performed significantly worse than the controls, asymptotic values of $A\beta_{1-42}$-HDL-infused mice were significantly worse than asymptotic values in controls. Escape latencies in reversal trials were significantly longer than previously reached asymptotes in both groups, but in the later trials, control animals improved significantly in the search of the new target position, whereas $A\beta_{1-42}$-HDL infused animals did not.

to result in formation of Aβ deposits, and after reaching a critical threshold, the latter will disturb neuronal functions and trigger inflammation (13, 14). On the other hand, behavioral defects can be observed without detectable Aβ deposits in aged TG APP mice in which total Aβ concentrations are only 2- to 3-fold compared with control mice or do not even reach detectable Aβ levels (9, 10). These observations challenge the hypothesis that the cognitive deficits are a result of increased concentration and long exposure of Aβ. It is possible that the brain becomes more prone to the detrimental effects of Aβ when the nervous system ages. Because in APP TG mice APP itself and many different APP cleavage products are produced in excess in neurons, sensitivity to long-term overproduction of APP and carboxyl-terminal fragments also may play a role. Thus, it is difficult to address the specific role of Aβ peptides *per se* in the development of learning deficits in AD.

Here we demonstrate that aged wild-type mice are selectively prone to monomeric, oligomeric, and more aggregated forms of $A\beta_{1-42}$, which results in delayed, Aβ deposit-independent learning deficits without inflammation and neuronal death in 9-month-old but not in 2.5-month-old mice. Brain Cu,Zn-superoxide dismutase (SOD1) levels are significantly reduced after $A\beta_{1-42}$ infusion, suggesting compromised antioxidant defenses; $A\beta_{1-42}$ infusion leads to delayed learning deficits also in rats, indicating species independence of the Aβ effects. Importantly, $A\beta_{1-42}$ infusion also alters the metabolite profile in the rat cerebrospinal fluid (CSF).

Results

Analysis of $A\beta_{1-42}$ Preparation. The biochemical analysis of the $A\beta_{1-42}$ released from the pumps contained monomers, dimers, various forms on oligomers, and larger aggregates that moved very little or not at all in the gel. (Fig. 1). The immunoreactivity decreased over the course of time so that at the end of the infusion period, only faint amounts of oligomeric Aβ forms were released, and the majority of the Aβ moved very little on the gel, reflecting high molecular weight aggregates.

When cortical neuronal cultures were exposed to $A\beta_{1-42}$-high-density lipoprotein (HDL) preparation, a concentration-dependent death of neurons was observed so that the exposure to 10 μM $A\beta_{1-42}$-HDL preparation resulted in a loss of 80–90% of the neurons (data not shown). Exposure of primary mouse microglia to the same $A\beta_{1-42}$ preparation at 5 or 10 μM Aβ did not significantly increase NO or TNF-α production, whereas lipopolysaccharide, a strong activator of inflammation, induced a statistically significant 2-fold increase in NO release [normalized values (mean $\pm$ SD, $n = 6$ per group): 1.0 $\pm$ 0.1, 1.0 $\pm$ 0.1, and 2.6 $\pm$ 0.1 for vehicle, 10 μM soluble $A\beta_{1-42}$, and lipopolysaccharide, respectively) and 9-fold increase in TNF-α release [normalized values (mean $\pm$ SD, $n = 6$ per group): 1.0 $\pm$ 0.3, 1.5 $\pm$ 0.3, and 9.1 $\pm$ 0.1 for vehicle, 10 μM soluble $A\beta_{1-42}$, and lipopolysaccharide, respectively).

Behavioral Testing. ***Mouse Morris Water Maze (MWM).*** Six weeks after infusion of 9-month-old mice, the performance was significantly improved over the six training days in both $A\beta_{1-42}$-HDL and control HDL group [F (1, 14) = 0.03; $P < 0.1$; Fig. 2*A*], and no significant difference was observed in escape latencies between the groups. The reversal trials also did not reveal any differences between the groups. When these mice were retested 12 weeks after the infusion, the control HDL mice but not the $A\beta_{1-42}$-HDL mice improved their performance significantly [F (1, 14) =

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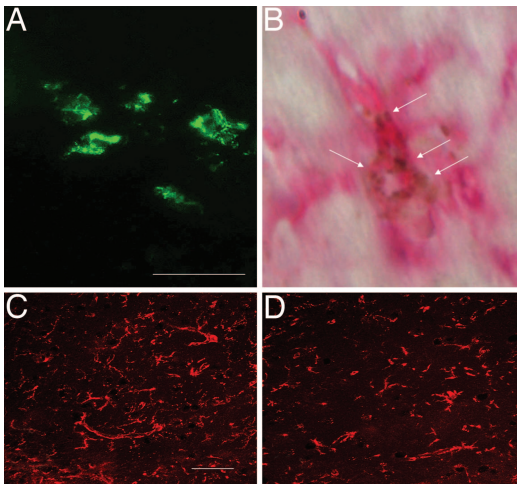

Fig. 3. Representative images showing histopathological changes observed in Aβ_{1-42}-HDL infused mice and rats. (*A*) Aβ immunoreactive material in the mouse brain at 12 months of age (12 weeks after the infusion). (*B*) Aβ immunoreactive material in association with an astrocyte (arrows point to brown Aβ granules and pink staining represents glial fibrillar acidic protein) in the rat brain 6 months after Aβ_{1-42}-HDL infusion. (*C* and *D*) CD11b staining for microglia in the mouse brain 12 weeks (*C*) after Aβ_{1-42}-HDL and HDL infusion at the age of 12 months (*D*). (Scale bars: *A* and *B*, 25 mm; *C* and *D*, 100 mm.)

8.49; $P < 0.05$; Fig. 2*B*]. Reversal trials did not show differences between the groups. In the separate cohort of Aβ_{1-42}-HDL and HDL mice tested for the first time 12 weeks after the infusion (Fig. 2*C*), the Aβ_{1-42}-HDL mice performed significantly worse than the HDL mice regarding both of the learning curves during the 6 training days [F (1, 15) = 5.98; $P < 0.05$), and a significant difference was observed in reversal trials [t (14) = 15.38; $P < 0.05$]. In fact, the Aβ_{1-42}-HDL mice did not improve significantly at all in the search of the new target position.

For the mouse groups infused with Aβ_{1-42}-HDL and HDL at the age of 2.5 months, no statistically significant differences were detected when the tests were performed 12 weeks after the infusion (data not shown).

Rat Y maze. In rat studies, compared with HDL infusion, the Aβ_{1-42}-HDL infusion impaired the spontaneous alternation behavior in Y maze (Aβ_{1-42}-HDL infusion: 70.9 ± 2.3; HDL infusion 81.7 ± 4.4, mean ± SEM, t test; $P = 0.04$). Aβ_{1-42}-HDL infusion had no effect on the number of arm entries (Aβ_{1-42}-HDL infusion: 16.4 ± 1.3; HDL infusion 13.4 ± 0.7, mean ± SEM; $P = 0.06$).

Histology. No obvious neuronal loss was seen in either the mice or rats infused with Aβ_{1-42}-HDL when compared with HDL-infused control groups. Because of the different brain size of the mouse and rat, the number of the brain sections and the brain area included in Aβ analyses also were different. Aβ-immunoreactive deposits were detected in 50% of the Aβ_{1-42}-HDL infused mice and in all Aβ_{1-42}-HDL infused rats. In mice, the number of the deposits varied from 1 to 41 deposits in 6 sections (Fig. 3*A*), and in the rats, from 16 to 2,159 deposits in 15 sections. No Aβ-immunoreactive deposits were seen in HDL-infused control animals. Approximately 18% of the Aβ-immunoreactivity in Aβ_{1-42}-HDL infused rats was closely associated with astrocytes (Fig. 3*B*) but not with microglia, as evidenced by Aβ-ED1 double labeling (data not shown). The areas covered by immunoreactive microglia (mean and SD values in mouse model: Aβ_{1-42}-HDL infused, 4.1 ± 3.5; HDL-infused, 4.9 ± 3.2; in rat model: Aβ_{1-42}-HDL infused, 3.8 ± 1.5; HDL-infused, 4.0 ± 2.3) (Fig. 3 *C* and *D*) or astrocytes (mean and SD values in mouse model: Aβ_{1-42}-HDL infused, 8.1 ± 1.1; HDL-infused, 7.5 ± 0.3; in rat model: Aβ_{1-42}-HDL infused, 13.7 ± 5.4; HDL-infused, 14.6 ± 1.1) were not different between the Aβ_{1-42}-HDL and HDL-infused mice or rats. Importantly, we did not observe any correlation between the performance in behavioral tests and the density of Aβ-immunoreactive deposits, the performance in behavioral tests and gliosis, or between the density of Aβ-immunoreactive deposits and gliosis.

Brain Enzyme Studies. Oxidative stress is implicated in AD pathology, and in several models of AD, alterations in markers of oxidative stress, including SOD1 and glutathione, have been observed (32–37). Moreover, oxidative stress and Aβ-induced neuronal damage are mediated partially by GSK-3β in cell culture and some animal models of AD (38, 39). We observed a mild but statistically significant reduction in hippocampal SOD1 activity in the 9-month-old Aβ_{1-42}-HDL-infused mice compared with controls (35.0 ± 3.5 versus 30.6 ± 3.9 relative units, respectively; $P < 0.05$, t test). No corresponding alteration in the glutathione activity or phosphorylation of Ser-9 residue of GSK-3β, a marker of the GSK-3β deactivation, was detected (data not shown).

NMR. The NMR analysis of the metabolite concentrations of rat CSF revealed prominent alterations in all of the 14 metabolites. The average concentrations were decreased by up to 60% in Aβ_{1-42}-HDL-infused rats when compared with HDL-infused rats for all metabolites except glutamate. For valine, myo-inositol, creatine, acetate, glutamine, lactate, glucose, and hippurate, the decrease was >20%, and for α-hydroxy isovalerate and citrate, the decrease still was >10%. Unfortunately, because of the small number (3 + 3) of samples, the difference of a single component between the two groups was statistically significant only for acetate (with $P = 0.01$).

Discussion

We found that the Aβ preparation, containing monomers, dimers, oligomers, and larger molecular weight forms (aggregates) of Aβ_{1-42} peptide, results in delayed learning deficits, which do not correlate with Aβ deposition. Moreover, these learning deficits were not associated with gliosis but caused a mild reduction in the brain SOD1 activity and a mild alteration in the metabolite pattern in the CSF. Importantly, the learning deficits were observed in 9-month-old but not 2.5-month-old mice, indicating that aging renders the brain sensitive to Aβ_{1-42}. Previous studies have demonstrated that antiinflammatory compounds (18–22) and antioxidants (16, 20, 22, 36, 37) limit Aβ deposition, glial activation, and even behavioral deficits in animal models of AD. Our Aβ_{1-42} preparation, which contained similar forms of the peptide as found in AD brains, did not activate inflammatory response of microglia *in vitro* or *in vivo*. However, the reduced SOD1 activity in Aβ-infused mice reflects a slightly compromised antioxidant defense system, suggesting that Aβ-induced oxidative stress may contribute to the learning deficits before clear neuroinflammation is triggered. Many studies suggest that oxidative stress plays a role in early AD and Aβ_{1-42}-induced learning deficits (22, 32–37) and proteomic analyses have demonstrated oxidative damage of brain proteins in advanced stages of AD (40, 41). Also, the susceptibility of the brain to lipid peroxidation increases with aging (42). Our results agree with previous data because we found learning deficits in aged but not young mice after Aβ_{1-42} infusion. Whether development of brain sensitivity to Aβ_{1-42} continues at a very old age remains to be investigated. Our preliminary results with 18- to 22-month-old C57BL mice suggest that the Aβ_{1-42} sensitivity in mice may peak at ≈12 months (unpublished data). However, because our aged mouse cohorts were accompanied with a variety of physical changes and overall deterioration that may mask the specific, Aβ_{1-42} in-

duced cognitive impairment, further careful behavioral analysis is needed to verify these pilot findings.

In TG AD models, APP is overexpressed severalfold, resulting in increased concentrations of not only Aβ peptides but also APP itself and $CT_{100-105}$ fragments. Several studies have indicated that APP decreases antioxidant capacity and is a potent activator of microglia to produce neurotoxins (43, 44). $CT_{100-105}$ fragments, on the other hand, can disrupt intracellular calcium homeostasis, render neuronal cells vulnerable to excitotoxicity, induce gliosis *in vivo*, and cause changes in behavior (45–47). Because abnormally high levels of APP and $CT_{100-105}$ fragments may result in changes that are not relevant for AD, we chose to test the role of $A\beta_{1-42}$ alone by infusing intraventricularly the preparation that contains several different forms of the peptide present in the AD brain. $A\beta_{1-42}$ induced learning deficits without glial activation. These results differ from most previous studies on AD models (2, 15, 16) and underline the specific role of Aβ peptides in slow development of cognitive defects. The difference in brain pathology between our study and previous studies with Aβ infusion models may not be due to different animal species or strains, because we observed learning deficits without gliosis in both mouse and rat models. The more likely explanation for the different results may be the form of Aβ in the infusion solution. Aβ can be very unstable, and the stability and solubility depend on the preparation protocol, time, and presence of carriers, such as HDL in our study (32). Also, the infusion rate and the final concentration of the peptide reaching different brain structures may easily vary from one laboratory to another. In fact, the Aβ released from the osmotic pump in the course of time rarely has been analyzed. Electrophoretic analysis of the infused Aβ preparation in our study confirmed that Aβ peptide existed not only in oligomeric form but also as monomers and larger molecular weight forms (aggregates), thus resembling the endogenous Aβ naturally present in the brain.

The fibrillar $A\beta_{1-42}$ and APP are known to activate microglia to release proinflammatory molecules and neurotoxins (1–4). Whether soluble or oligomeric Aβ can stimulate microglia, is not well known. One recent study showed that primary rat microglia released TNFα upon stimulation with certain preparations of soluble Aβ (48). Also, cultured rat astrocytes respond to oligomeric $A\beta_{1-42}$ by increased production of various cytokines (28). We tested the effect of our $A\beta_{1-42}$ preparation on cultured mouse microglia and did not observe consistent or significant induction of TNF-α or NO release. Even though we cannot exclude the possibility that soluble or oligomeric Aβ in certain preparation can stimulate microglia to release neurotoxins, the infused Aβ preparation did not activate microglia in the brain, although it resulted in learning deficits and changes in CSF metabolites.

The NMR analysis of CSF metabolites showed that acetate, α-hydroxyisovalerate, creatine, lactate, and myo-inositol all were decreased in $A\beta_{1-42}$-HDL-infused rats. Because of the small sample number, clearly significant differences could not be observed, indicating that the metabolite data and conclusion need to be taken with caution. Although little is know regarding the role of α-hydroxyisovalerate, an intermediate of valine catabolism, myo-inositol is known to reflect astrocytic metabolic activity (49) and extracellular lactate is a widely used marker of brain activity in humans (50). Lactate is almost exclusively synthesized by astrocytes (49). Creatine, on the other hand, is synthesized in all brain cells but taken up only by oligodendrocytes and neurons (51). Previous studies have shown that oxidative stress reduces lactate, myo-inositol, and creatine synthesis in astrocytes (49). Acetate, which also showed a reduction in $A\beta_{1-42}$-HDL-infused rats, is mainly metabolized to glutamine in astrocytes and, to some extent, to glutamate in neurons (52). Overall, our findings together with the previous studies suggest that oxidative stress in $A\beta_{1-42}$-HDL-infused animals might impair metabolic activity and functions of astrocytes, leading to reduced lactate, myo-inositol, and even creatine levels in the CSF. This hypothesis is supported further by the finding that in the rat model, the CSF glutamine levels were decreased, possibly reflecting reduced astrocytic metabolism of acetate. Because lactate is known to contribute to the synaptic function in adult neurons (53), we further hypothesize that Aβ-induced oxidative stress and astrocytic dysfunction could result in learning deficits by impairing synaptic functions in the hippocampus and cortex.

Materials and Methods

Animals. Altogether 34 9-month-old and 38 2.5-month-old male C57BL mice (University of Kuopio), and 20 2.5-month-old male SHR rats (Taconic Farms) were used. To ensure the development of Aβ histopathology and development of behavioral deficits in the young rats, animals were fed with a diet containing 2% cholesterol and 1% sodium cholate (TestDiet catalog no. 57UJ; LabDiet, Richmond, IN) starting 1 month before the Aβ infusion and continuing until the start of behavioral testing. All animal work was approved by the Animal Care and Use Committee of the University of Kuopio and performed according to the guidelines of National Institutes of Health for animal care.

Aβ Infusion. The infusion model was adopted from previous work on the rat infusion model (16). $A\beta_{1-42}$ (American Peptide, Sunnyvale, CA) was oligomerized (54). The anesthetized animals were placed in a stereotaxis (model 940, David Kopf Instruments, Tujunga, CA), and stainless steel catheters were attached to miniosmotic pumps (model 1002; Alzet, Palo Alto, CA) were implanted into the following coordinates: mouse (unilaterally): +1.0 mm medial/lateral (m/l), −0.46 mm anterior/posterior (a/p), −1.75 mm dorsal/ventral (d/v); rat (bilaterally): ±1.9 mm m/l, −0.9 mm a/p, −4.5 mm d/v. The pump contents were released over a period of 2 weeks consisting either the total of 40 μg Aβ with 1.0 mg HDL ($n = 18$ for mice, $n = 10$ for rats) or 1.0 mg HDL only ($n = 16$ for mice, $n = 10$ for rats) per each pump.

For the analysis of Aβ oligomerization and the time course of Aβ release, extra pumps were prepared in each experiment. Sample solution was collected to Eppendorf tubes at 37°C, crosslinked with glutaraldehyde (55), and run as 0.5-μg samples on the 18% multiphasic buffer system SDS/PAGE (56). The separated proteins were transferred onto Hypond-P membrane (Amersham Pharmacia-Amersham Pharmacia Biotech) and detected by using human Aβ antibody (clone 6E10, 1:1,000 dilution; Signet Laboratories, Dedham, MA), secondary HRP-labeled anti-mouse antibody (Amersham Pharmacia Life Science) and an ECL Plus -kit (Amersham Pharmacia-Amersham Pharmacia Biotech). Finally, the membranes were scanned on STORM 860 (Molecular Dynamics) fluoroimager.

The toxicity and inflammatory potential of $A\beta_{1-42}$ preparation was tested in mixed cortical cultures prepared from embryonic day 15 rat fetal brains (57) and mouse microglia cultures (58), respectively. At 11 days, cultured neurons were treated with $A\beta_{1-42}$-HDL preparations for 48 h (5 μM and 10 μM). Cell death was assessed by cell counting and by measuring kinetically the release of lactate dehydrogenase by using pyruvate and NADPH as substrates. In microglia cultures, the released TNF-α was measured by ELISA kit (Endogen, Woburn, MA) and NO metabolites in a Multiskan ELISA reader (Labsystems, Vantaa, Finland) after adding Griess reagent (58).

Behavioral Testing. ***Mouse MWM.*** The learning and memory deficits of the infused mice were analyzed as described in ref. 10. The mice infused at the age of 9 months were randomly divided into two groups. One half of the mice in these two groups were tested at 6 and 12 weeks after infusion, and the other half was tested only at 12 weeks. Because no deficiencies were detected at 6

weeks, the mice that were infused with Aβ at the age of 2.5 months were analyzed 12 weeks after infusion only.

Because there was a high positive correlation between the swim path lengths and escape latencies, and no sensorimotor impairment was observed, only the latter values were used for evaluation of the animals' performance. Asymptotic latency was taken as the average latency on days 4–6 when no significant difference between days occurred. A reversal experiment was made on day 7 when the platform was placed into the opposite side of the pool, and the mice were given six trials to find it. The statistical analysis was performed with STATISTICA (StatSoft, Tulsa, OK). The data were evaluated by two-way ANOVA with repeated measures on one factor followed by Newman–Keuls post hoc tests and individual contrasts where appropriate. The differences between means were accepted as significant at the $P < 0.05$ level.

Rat Y maze test. The Y maze apparatus was made of black plastic with the following dimensions: arm length 35 cm, height 25 cm, and width 10 cm. The animal was placed at the end of one arm and allowed to move freely through the maze for 8 min. The sequence of arm entries was recorded manually. Total number of arm entries during the observation period was recorded. Spontaneous alternation behavior that is considered to reflect a primitive form of spatial working memory behavior was defined as the entry into all three arms on consecutive choices in overlapping triplet sets. The percent spontaneous alternation score was calculated as the ratio of actual to possible alternations. The behavioral data were analyzed by using the Student *t* test.

Immunohistochemistry. ***Mouse brains.*** After perfusion with heparinized saline, one brain hemisphere of each mouse was processed for immunohistochemistry and the other one for biochemical analyses. For immunohistochemistry, the hemispheres were immersion-fixed with 4% PFA for 24 h, followed by cryoprotection in 30% sucrose for 3 days. Frozen brains were cut into 20-μm-thick cryosections. The hemispheres used for biochemical analyses were snap-frozen on liquid nitrogen. The hippocampal microgliosis and astrogliosis were analyzed by CD11b (1:500 dilution; Serotec) and glial fibrillar acidic protein, 1:500, DAKO) antibodies, respectively. Treatments with biotinylated secondary antibodies (Vector Laboratories) were followed by the use of a TSA-amplification system (PerkinElmer) for CD11b and an avidin–biotin complex (Vectastain Elite Kit, Vector Laboratories) system for astrogliosis. The glial fibrillar acidic protein immunoreactivity was visualized with H_2O_2 and nickel-enhanced diaminobenzidine. Four to six sections at 200-μm intervals through the hippocampi were evaluated per animal. For quantification, the immunoreactive areas were imaged by using an AX70 microscope (Olympus) equipped with a digital camera (Color View 12 or F-View, Soft Imaging System, Munster, Germany) and running ANALYSIS (Soft Imaging System). The immunoreactive area was quantified by using IMAGEPRO PLUS (Media Cybernetics, Silver Spring, MD). Data are expressed by the area of hippocampi occupied by immunoreactivity and represented as the mean ± SEM. The brain Aβ immunoreactivity was detected by using pan-Aβ antibody (1:250 dilution; BioSource, Nivelles, Belgium) after incubation with Alexa Fluor 488-conjugated secondary anti-rabbit antibody (1:200 dilution; Molecular Probes).

Rat brains. All rat brains were processed for immunocytochemistry similar to the mouse brains, and 20-μm-thick coronal sections were cut with a 200-μm interval from anterioposterior levels +2.0 to −2.0, and with a 500-μm interval, from levels + 2.0 to +0.5 and −2.0 to −3.5. Analyses of the Aβ immunoreactivity and gliosis in the rat brain sections were similar to those in mouse sections, except that Aβ immunoreactivity was visualized by using nickel-enhanced diaminobenzidine, and for microgliosis, an ED1 antibody (Serotec; 1:200) was used as a marker. To analyze the association of Aβ immunoreactivity and gliosis, Aβ-glial fibrillar acidic protein and Aβ-ED1 double-labeling was performed.

Brain Enzyme Studies. For SOD1 activity measurements, tissues were homogenized in 10 vol of 20 mM Tris buffer, pH 7.4. Samples were centrifuged for 5 min at 20,000 × *g*. SOD1 activity was assayed by a method described in ref. 59. Reduced glutathione concentration in the tissues extracts was measured with 2,3-naphthalenedicarboxaldehyde derivatization as described in ref. 60.

For phospho-GSK-3β proteins, the hippocampal samples were homogenized in 20 mM Tris buffer (pH 7.4), containing Complete inhibitory mixture (Roche Diagnostics, Mannheim, Germany) and boiled at 90°C for 5 min. Ten milligrams of proteins per lane were run in 10% SDS/PAGE gel by using a MiniProtean (Bio-Rad) apparatus according to manufacturer's instructions. Separated proteins were transferred onto Hybond-P membrane (Amersham Pharmacia Biosciences), and the membranes were blocked in 5% skimmed milk solution in PBS Tween-20 before incubation with primary phospho-GSK-3β (Ser-9; Cell Signaling Technology, Beverly, MA; 1:1,000) in blocking buffer. The blots were stripped and reprobed with anti-actin antibody (Santa Cruz Biotechnology) as a loading control. Secondary anti-rabbit IgG HRP conjugate (Amersham Pharmacia) was diluted 1:2,000 in blocking buffer and the horseradish peroxidase label was detected by using the ECL Plus kit (Amersham Pharmacia Biosciences). Membranes were directly scanned on STORM 860 (Amersham Pharmacia Biosciences) fluoroimager, and detected bands quantified by using IMAGEQUANT (Molecular Dynamics).

Metabolite NMR Analysis. ***CSF sampling.*** A rat was anaesthetized and placed prone on the stereotaxic instrument. A sagittal incision of the skin was made inferior to the occiput. After blunt dissection of the s.c. tissue and neck muscles through the midline, the dura was penetrated between the third and fourth vertebra by a 26G needle, and ≈100 μl of CSF was collected. CSF from one to three similarly treated rats was pooled for each sample.

NMR analysis. The freeze-dried samples were stored at −20°C in sealed vials, reconstituted in 75 μl of D_2O (99.98%-D, Merck) before NMR measurements, and 5 μl of 21.5 mM TSP-d4 (3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid) in D_2O was added and used as an internal standard of known concentration. Spectra were measured by using an AVANCE DRX 500 instrument operating at 500.13 MHz (Bruker-Biospin, Karlsruhe, Germany) equipped with a broadband inverse probe (2.5 mm BBI BB, ^{1}H, Z-Grad) for all 1D ^{1}H quantitative measurements. Bruker XWIN-NMR version 3.5 pl 6 was used for acquisition of all spectra. All spectral processing before the model creation was done by using PERCH NMR (61). The assignation of the spectral signals was done according to the available chemical shift and coupling constant information in the literature. Some compounds were verified from 2D spectra. The quantification of the spectra was performed with the constrained total-line-shape fitting method by using PERCH NMR (39). The constraints defining relative line-positions and intensities were written on basis of the theoretical spectral structures of the metabolites. The 14 metabolites, corresponding to nearly 90% of the total area of the spectrum, were quantified as follows: acetate, α-hydroxy isovalerate, β-hydroxy butyrate, α- and β-glucose, citrate, creatine, glutamate, glutamine, hippurate, myo-inositol, lactate, lysine, and valine.

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