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STAT1 Negatively Regulates Spatial Memory Formation and Mediates the Memory-Impairing Effect of Aβ

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Signal transducer and activator of transcription-1 (STAT1) has an important role in inflammation and the innate immune response, but its role in the central nervous system is less well understood. Here, we examined the role of STAT1 in spatial learning and memory, and assessed the involvement of STAT1 in mediating the memory-impairing effect of amyloid-beta (Aβ). We found that water maze training downregulated STAT1 expression in the rat hippocampal CA1 area, and spatial learning and memory function was enhanced in *Stat1*-knockout mice. Conversely, overexpression of STAT1 impaired water maze performance. STAT1 strongly upregulated the expression of the extracellular matrix protein laminin β1 (LB1), which also impaired water maze performance in rats. Furthermore, Aβ impaired spatial learning and memory in association with a dose-dependent increase in STAT1 and LB1 expression, but knockdown of STAT1 and LB1 both reversed this effect of Aβ. This Aβ-induced increase in STAT1 and LB1 expression was also associated with a decrease in the expression of the N-methyl-D-aspartate receptor (NMDAR) subunits, NR1, and NR2B. Overexpression of NR1 or NR2B or exogenous application of NMDA reversed Aβ-induced learning and memory deficits as well as Aβ-induced STAT1 and LB1 expression. Our results demonstrate that STAT1 negatively regulates spatial learning and memory through transcriptional regulation of LB1 expression. We also identified a novel mechanism for Aβ pathogenesis through STAT1 induction. Notably, impairment of spatial learning and memory by this STAT1-mediated mechanism is independent of cAMP responsive element-binding protein signaling.
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INTRODUCTION

We have previously demonstrated that protein inhibitor of activated signal transducer and activator of transcription-1 (STAT1) (PIAS1) has an important role in spatial learning and memory formation in rats. Fast learners from the water maze learning task showed higher levels of PIAS1 expression in the hippocampal CA1 area, and overexpression of PIAS1 facilitated water maze performance in rats. Consistent with this, knockdown of PIAS1 expression by PIAS1 siRNA transfection impaired water maze performance (Tai *et al*, 2011). PIAS1 has also been shown to facilitate spatial learning and memory through enhanced sumoylation and decreased phosphorylation of STAT1 (Tai *et al*, 2011). PIAS1 was originally identified as an inhibitor of STAT1 that blocks STAT1 DNA binding and inhibits STAT1 transcriptional activity (Liu *et al*, 1998). PIAS1 also has an important role in the innate immune response by downregulating the expression of inflammatory genes through negative regulation of STAT1 and other transcription factors (Liu *et al*, 2007). Because PIAS1 inhibits STAT1 activity, these results imply that STAT1 may negatively regulate learning and memory function. Interestingly, *laminin β1* (LB1) is a target of STAT1, has been implicated in the impairment of spatial learning and memory (Yang *et al*, 2011).

The possible involvement of STAT1 in the negative regulation of learning and memory formation suggests that STAT1 may have a role in cognitive impairment in patients with certain neurodegenerative diseases, such as Alzheimer's disease (AD). The brain of AD patients is characterized by the accumulation of senile plaques, the major component of which is amyloid-beta ($A\beta_{1\text{-}42}$). Aβ is generated by sequential and proteolytic cleavage of the amyloid precursor protein (APP) by β-secretase and γ-secretase (De Strooper and Annaert, 2000). Aβ is known to cause lipid peroxidation, free radical production, caspase activation, protein cleavage, and DNA damage, which eventually lead to neuronal death (Butterfield *et al*, 2001; Hardy and Selkoe, 2002). Aβ has also been shown to promote the expression of some pro-inflammatory genes, such as cyclooxygenase-2 and interleukin (IL)-1β; notably, the activation of these signaling pathways also results in apoptosis (Lukiw and Bazan, 2006). In addition, the Aβ protein or overexpression of Aβ causes cognitive impairment in animals (Chen *et al*, 2000; Cleary *et al*, 2005). This

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cognitive impairment correlates with amyloid plaque formation (Chen *et al*, 2000; Hsiao *et al*, 1996) or precedes it (Hsia *et al*, 1999; Kamenetz *et al*, 2003). Aβ has also been shown to suppress N-methyl-D-aspartate (NMDA)-evoked current by decreasing the surface expression of the NMDA receptor (NMDAR) subunits, NR1 and NR2B, and reducing the phosphorylation level of NR2B (Snyder *et al*, 2005). Decreased surface expression of NMDAR, in turn, has been shown to contribute to synaptic defects caused by Aβ and in APP transgenic mice (Dewachter *et al*, 2009).

Because Aβ and STAT1 are both involved in inflammation, we speculated that Aβ and STAT1 might also interact in the nervous system. On the basis of the observation that PIAS1 facilitates learning and memory, we hypothesized that STAT1 impairs learning and memory performance. We also examined whether STAT1 mediates the memory-impairing effect of Aβ and examined the role of LB1 and NMDAR subunits in this process.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (250–350 g) bred at the Animal Facility of the Institute of Biomedical Sciences, Academia Sinica in Taiwan were used. Adult male 129S6/SvEv mice and homozygous *Stat1*$^{-/-}$ male mice (24–29 g) were purchased from Taconic Farms (Germantown, NY). Animals were housed in a room (23 ± 2 °C) maintained on a 12/12 h light/dark cycle (light on at 0630 hours) with food and water continuously available.

Drugs

Aβ and rAβ were purchased from AnaSpec (San Jose, CA). NMDA was purchased from Tocris Bioscience (St Louis, MO). All drugs were injected to the CA1 area bilaterally at 0.7 μl each side.

Water Maze Learning

The water maze used for rats was a plastic, circular pool with 2.0 m in diameter and 0.6 m in height that was filled with water (25 ± 2 °C) to a depth of 20 cm. A circular platform (13 cm in diameter) was placed at a specific location away from the edge of the pool. The top of the platform was submerged 1.5 cm below the water surface. Procedures for spatial learning are described previously (Tai *et al*, 2011) and detailed in Supplementary Information.

DNA/Polyethyleneimine Complex Preparation and siRNA Injection

Before injection, plasmid DNA was diluted in 5% glucose to a stock concentration of 2.77 μg/μl. Branched polyethyleneimine (PEI) of 25 kDa (Sigma, St Louis, MO) was diluted to 0.1 M concentration in 5% glucose and added to the DNA solution. 0.1 M PEI was added to reach a ratio of PEI nitrogen per DNA phosphate equals to 10 (Chao *et al*, 2011). For plasmid DNA transfection, 0.7 μl plasmid DNA complex (1.5 μg/μl) was injected to the CA1 area bilaterally. For siRNA injection, 0.7 μl of various siRNAs (8 pmol/μl) or control siRNA was transfected to each CA1 area using the transfection agent PEI.

Western Blot

The CA1 tissue was lysed in RIPA buffer. The lysate was resolved by 8% SDS-PAGE. Proteins resolved by SDS-PAGE were transferred to the PVDF membrane (Millipore, Bedford, MA) and incubated with primary and peroxidase-conjugated secondary antibodies (see Supplementary Information). Membrane was developed by reacting with chemiluminescence HRP substrate (Millipore) and exposed to the LAS-4000 mini image system (Fujifilm, Tokyo, Japan). Protein bands were visualized and quantified using the NIH Image J Software.

Immunohistochemistry and DAB Staining

Sixty hours after plasmid/PEI or Aβ injection, rats were anesthetized with pentobarbital (40 mg/kg, i.p.) and perfused with ice-cold PBS followed by 4% paraformaldehyde. Brains were frozen, cut into 30-μm sections on a cryostat, and mounted on gelatin-coated slides. Immunofluorescence detection of injected Aβ, Aβ aggregation, and amyloid plaque is described in Supplementary Information. In addition, 3,3′-diaminobenzidine (DAB) staining was performed for the visualization of Aβ deposit 14 days after Aβ injection. The procedures are described in detail in Supplementary Information.

Statistics

Water maze data were analyzed by analysis of variance (ANOVA) with repeated measure followed by *post hoc* Newman–Keuls multiple comparisons (represented by *q*-value). Probe trial data and biochemical data were analyzed with one-way ANOVA followed by the Newman–Keuls multiple comparisons (for more than two groups) or with Student's *t*-test (for two groups).

RESULTS

Spatial Training Decreases STAT1 Expression, and Overexpression of STAT1 Impairs Spatial Learning

Rats were divided into a trained group and a swimming control group ($n = 8$ each group). Animals were killed at the end of training and their CA1 tissues were biopsied for various biochemical determinations. Results revealed that spatial training markedly decreased STAT1 protein expression and STAT1 DNA binding ($t_{1,14} = 30.02$ and 15.44, both $P < 0.001$) (Figure 1a). These results demonstrate a negative relationship between spatial learning and STAT1 expression in the rat hippocampus. To further examine this relationship, we divided rats into two groups ($n = 8$ each group). One group of rats received Flag-vector and the other group of rats received Flag-STAT1 wild-type (WT) plasmid transfection and they were subject to water maze learning. Results showed that overexpression of STAT1 markedly impaired acquisition performance ($F_{1,14} = 8.68$, $P = 0.01$) (Figure 1b) and decreased the amount of time animals spent in the target quadrant in the probe trial test ($t_{1,14} = 3.61$,

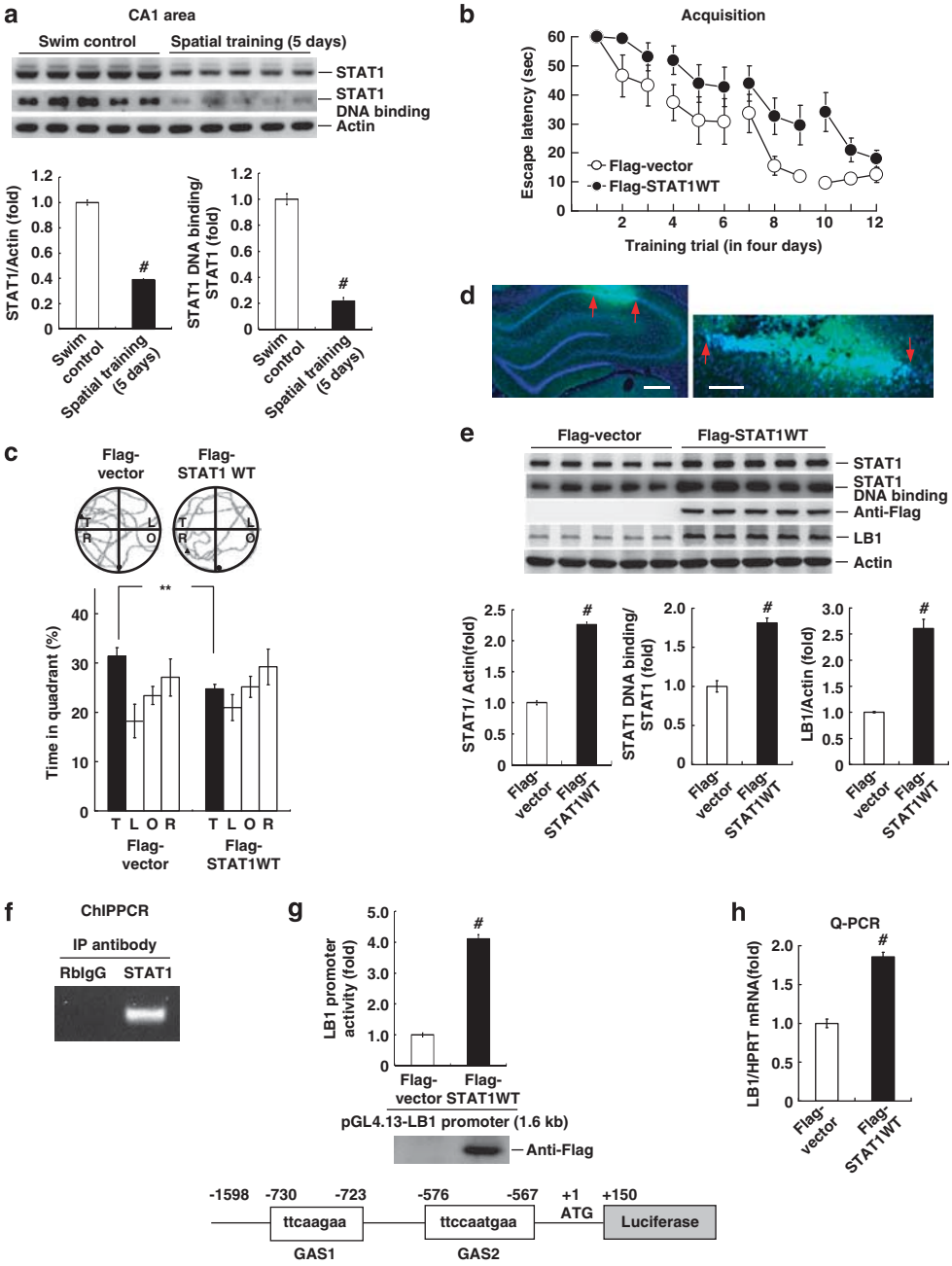

Figure 1 Spatial training decreases signal transducer and activator of transcription-1 (STAT1) expression and overexpression of STAT1 impairs spatial learning and memory, increases laminin β1 (LB1) promoter activity and LB1 expression. (a) Gel pattern of western blotting (WB) and analysis of STAT1 protein level and STAT1 DNA binding from trained and swim control rats in the CA1 area. Flag-STAT1WT plasmid or Flag-vector was transfected to rat CA1 area and animals were subject to (b) water maze learning and (c) probe trial test. T: target quadrant, L: left quadrant, O: opposite quadrant, R: right quadrant. ●: start point, ▲: end point (d) Immunohistochemistry (IHC) showing the area of EGFP-STAT1WT transfection and expression in the CA1 area at different magnifications. Cells that show both green fluorescence (EGFP) and blue fluorescence (DAPI) are cells successfully transfected with the plasmid. Red arrows indicate the range of expression. Scale bar equal 400 μm for the left panel and 100 μm for the right panel. (e) Gel pattern and analysis of STAT1 DNA binding, STAT1, and LB1 protein expression after the probe trial test. WB for STAT1 and anti-Flag verifies Flag-STAT1WT transfection and expression. (f) Results from chromatin immunoprecipitation (ChIP) assay showing STAT1 binding to the LB1 promoter in N2A cells using the STAT1 antibody (or rabbit IgG) and the primer for the LB1 promoter. Experiments are in duplicates. (g) Flag-STAT1 plasmid (or Flag-vector) was co-transfected with the LB1 promoter to N2A cells and LB1 promoter activity was determined 48 h later. The lower panel shows the position of two GAS elements on the LB1 promoter. Experiments are in triplicates. (h) Analysis of LB1 mRNA level in Flag-STAT1WT-and Flag-vector-transfected animals. Data are mean ± SEM. $^{**}P<0.01$ and $^{\#}P<0.0001$.

$P<0.01$) (Figure 1c). Immunohistochemistry (IHC) clearly revealed EGFP fluorescence (green) and its colocalization with the nuclear dye DAPI (blue), confirming effective transfection and expression of STAT1WT in the CA1 area (Figure 1d). The transfected area was about 655 μm in length, or ~28% of the total CA1 area viewed from a single plane (Figure 1d). STAT1 overexpression following STAT1WT transfection was also confirmed by an increase

in STAT1 protein in the transfected area ($t_{1,8} = 21.94$, $P < 0.001$) (Figure 1e).

Overexpression of STAT1 Enhances STAT1 DNA Binding and LB1 Expression

We next examined the molecular events linking STAT1 overexpression with spatial memory impairment. Result revealed that STAT1WT transfection increased STAT1 DNA binding ($t_{1,14} = 8.54$, $P < 0.001$) (Figure 1e), suggesting that STAT1 acted through a downstream target gene involved in memory impairment. We tested this possibility focusing on LB1 because the promoter of the rat *LB1* gene contains two GAS elements that are specific for STAT1 binding (Figure 1g, bottom), and because LB1 has been found to impair spatial learning and memory (Yang *et al*, 2011). Chromatin immunoprecipitation (ChIP) assays revealed that STAT1 directly bound to the LB1 promoter (Figure 1f). These results were confirmed by luciferase reporter assay, which showed that STAT1WT transfection increased LB1 promoter activity ($t_{1,4} = 19.16$, $P < 0.001$) (Figure 1g). Flag-STAT1WT transfection and expression were confirmed by western blotting (WB) using an anti-Flag antibody (Figure 1g). In a separate experiment ($n = 7$ each group), we examined whether transfection of STAT1WT increased the transcription and expression of LB1. Results revealed that the transfection of STAT1WT increased LB1 at both the mRNA ($t_{1,12} = 11.96$, $P < 0.001$) (Figure 1h) and protein ($t_{1,14} = 9.3$, $P < 0.001$) levels (Figure 1e).

STAT1-Knockout (*Stat1*$^{-/-}$) Mice Show Enhanced Spatial Learning and Memory

We further examined the role of STAT1 in spatial learning and memory using *Stat1*$^{-/-}$ mice and *Stat1*WT mice ($n = 8$ each group). Results revealed that *Stat1*$^{-/-}$ mice exhibited enhanced acquisition performance compared with *Stat1*WT mice ($F_{1,14} = 45.21$, $P < 0.001$) (Figure 2a) and spent more time in the target quadrant in the probe trial test ($t_{1,14} = 2.13$, $P < 0.05$) (Figure 2b). WB and RT-PCR analyses confirmed the absence of STAT1 expression in *Stat1*$^{-/-}$ mice (Figure 2c and d) and demonstrated that STAT2 and STAT3 expressions were unaltered (Figure 2c). In addition, the expression level of LB1 was significantly lower in *Stat1*$^{-/-}$ mice ($t_{1,14} = 12.82$, $P < 0.001$) (Figure 2c), confirming that LB1 is regulated by STAT1 in association with the STAT1 expression-dependent impairment in spatial memory. Similar results were obtained in rats transfected with STAT1 siRNA (Supplementary Figure S1).

Because cAMP responsive element-binding protein (CREB) has an important role in long-term memory formation (Silva *et al*, 1998), it is conceivable that STAT1 could impair spatial learning and memory through a mechanism involving inactivation of CREB. We therefore examined the phosphorylation level of CREB in the same animals used in the experiments described above. Results showed no alteration in either the phosphorylation or expression level of CREB in *Stat1*$^{-/-}$ mice ($t_{1,14} = 0.02$ and 0.42, both $P > 0.05$) (Figure 2c). We further examined the

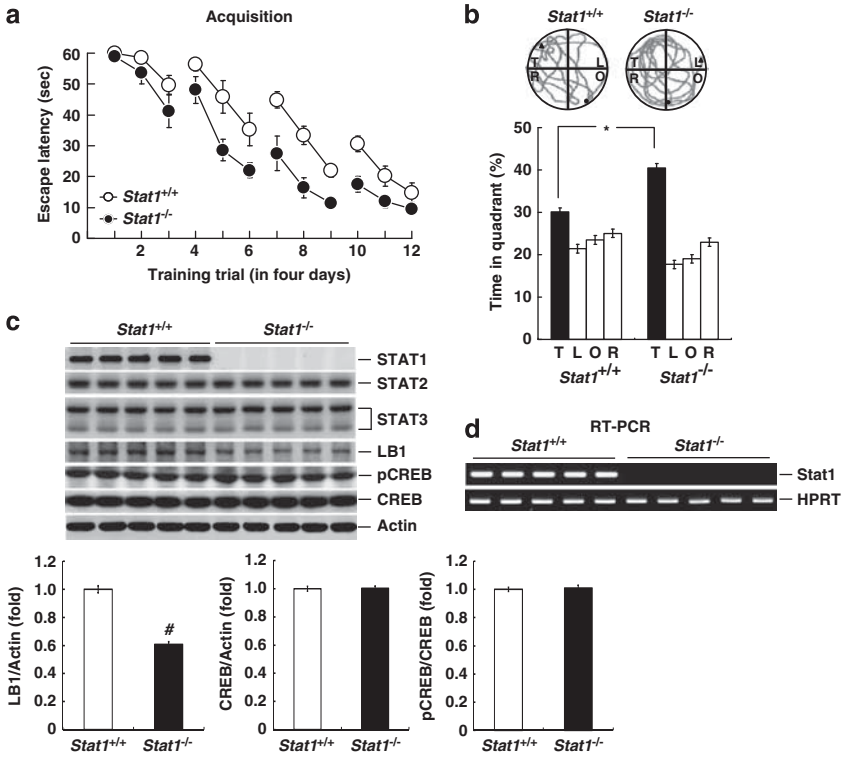

Figure 2 *Stat1*$^{-/-}$ mice show enhanced spatial learning and memory. *Stat1*$^{-/-}$ mice and *Stat1*WT mice were subject to (a) water maze learning and (b) probe trial test. (c) Gel pattern of signal transducer and activator of transcription-1 (STAT1), STAT2, STAT3, laminin β1 (LB1), cAMP responsive element-binding protein (CREB), and pCREB as well as analysis of protein expression of LB1, CREB, and pCREB in *Stat1*$^{-/-}$ and *Stat1*WT mice after the probe trial test. (d) Gel pattern from RT-PCR showing the *Stat1* and *HPRT* bands from *Stat1*$^{-/-}$ and *Stat1*WT mice. Data are mean ± SEM. $^{*}P < 0.05$ and $^{\#}P < 0.001$.

relationship between STAT1 and CREB by transfecting STAT1 siRNA into the rat CA1 area. Results revealed that STAT1 siRNA transfection did not affect the expression and phosphorylation levels of CREB. Similarly, transfection of CREB siRNA into the rat CA1 area did not affect the expression level of STAT1 either (Supplementary Figure S2).

Aβ Increases the Expression of STAT1 and LB1 and Decreases the Expression of NMDA Receptor Subunits in Association with Impairment of Spatial Memory

The above results implicate that both STAT1 and LB1 negatively regulate spatial learning and memory. Because Aβ also impairs spatial memory (Chen *et al*, 2000; Cleary *et al*, 2005), we speculated that Aβ-induced memory impairment might be associated with an increase in the expression of STAT1 and LB1. This issue was examined here. A dose-response study with Aβ was first carried out. Rats were divided into four groups to receive 1% NH_4OH or different doses (0.2 μg, 2.1 μg, and 21 μg) of Aβ injection to the CA1 area and were subject to water maze learning 10 days later. Results revealed that Aβ impaired acquisition performance in a dose-dependent manner ($F_{3,28}=70.99$, $P<0.001$). Further analyses showed that both 2.1 μg Aβ and 21 μg Aβ significantly impaired spatial acquisition ($q=10.63$ and $q=18.59$, respectively, both $P<0.001$) (Figure 3a). There was also a dose-dependent significant effect of Aβ on retention performance in the probe trial test ($F_{3,28}=11.36$, $P<0.001$). Additional analyses indicated that both 2.1 μg Aβ and 21 μg Aβ markedly impaired memory retention ($q=5.51$, $P<0.01$ and $q=7.55$, $P<0.001$, respectively) (Figure 3b).

We also examined whether Aβ-induced memory impairment was associated with a decrease in the expression of NMDAR subunits using WB. These analyses revealed that Aβ induced a dose-dependent decrease in the expression of NR1 ($F_{3,28}=39.67$, $P<0.001$), NR2A ($F_{3,28}=41.16$, $P<0.001$), and NR2B ($F_{3,28}=78.17$, $P<0.001$). Further analyses indicated that Aβ at doses of 0.2, 2.1, and 21 μg all significantly decreased NR1 expression ($q=3.87$, $P<0.05$; $q=8.93$, $P<0.01$, and $q=13.7$, $P<0.001$, respectively) and NR2A expression ($q=3.76$, $P<0.05$; $q=9.86$, $P<0.001$, and $q=14.47$, $P<0.001$, respectively). Aβ at doses of 2.1 and 21 μg both significantly decreased NR2B expression ($q=12.54$ and $q=19.15$, both $P<0.001$) (Figures 3c and d). Moreover, Aβ induced a dose-dependent increase in the expression of STAT1 and LB1 in these animals ($F_{3,28}=94.71$ for STAT1 and $F_{3,28}=216.91$ for LB1, both $P<0.001$). Further analyses revealed that Aβ at doses of 2.1 and 21 μg both significantly increased the expression of STAT1 ($q=9.66$ and $q=20.61$, respectively, both $P<0.001$)

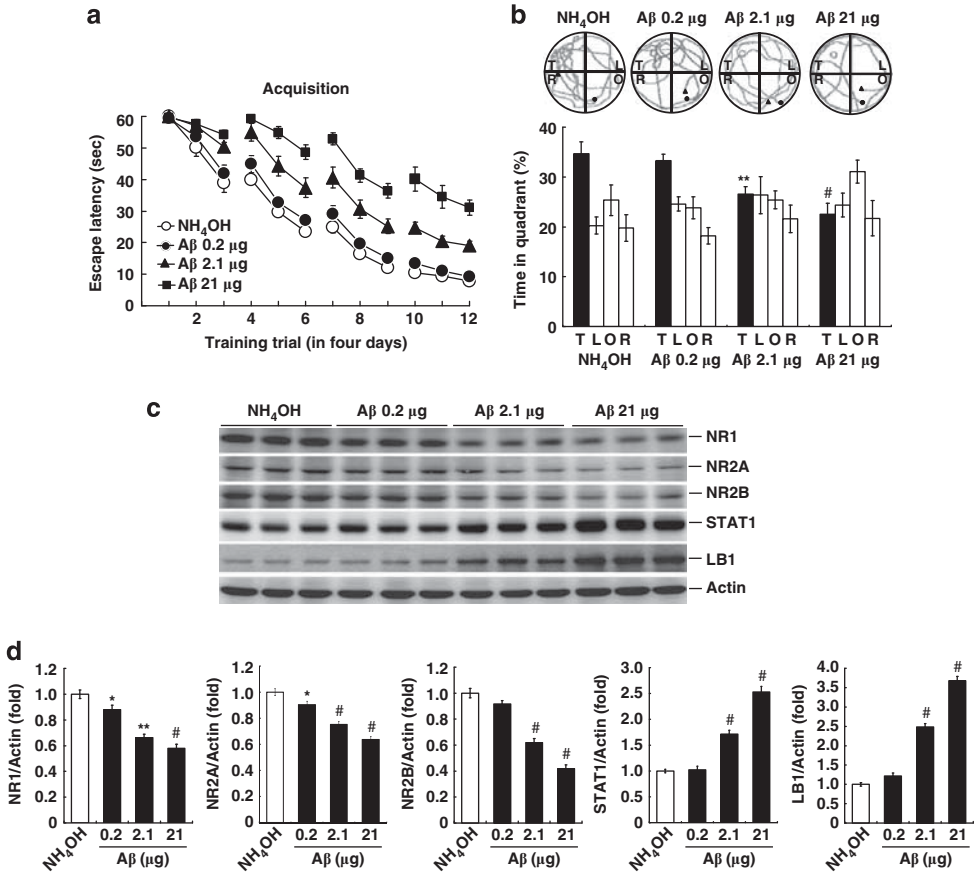

Figure 3 Aβ impairs spatial learning and memory in a dose-dependent manner. Rats received NH_4OH or different concentrations of Aβ injection to the CA1 area and were subject to (a) water maze learning and (b) probe trial test. (c) Gel pattern and (d) analysis of protein expression of NR1, NR2A, NR2B, signal transducer and activator of transcription-1 (STAT1), and laminin β1 (LB1) after the probe trial test. Data are mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ and $^{\#}P<0.001$.

and LB1 ($q = 17.57$ and $q = 31.76$, respectively, both $P < 0.001$) (Figure 3c and d).

Because a 21-μg dose of Aβ produced the most significant effects in the above experiments, we adopted this dose of Aβ for subsequent experiments to further characterize the behavioral, biochemical, and immunohistochemical changes produced by Aβ. Animals were divided into three groups to receive 1% NH_4OH, rAβ, or Aβ (both at 21 μg) injections to the CA1 area and were subject to water maze learning 10 days later. Both NH_4OH and rAβ-injected animals served as control groups because rAβ was previously shown not to produce toxicity to the hippocampal neurons (Hsu *et al*, 2009). Results revealed that Aβ, but not rAβ, markedly impaired acquisition performance in rats ($F_{2,20} = 20.41$; $q = 8.28$, $P < 0.001$) (Figure 4a). Aβ-injected animals also spent less time in the target quadrant in the probe trial test compared with those in the NH_4OH and rAβ groups ($F_{2,20} = 3.66$; $q = 3.5$, $P = 0.05$ and $q = 3.08$, $P < 0.05$, respectively) (Figure 4b).

Further analyses from WB revealed that Aβ increased the expression of STAT1 and LB1 ($F_{2,18} = 184.3$; $q = 23.37$, $P < 0.001$ for STAT1 and $F_{2,18} = 20.87$; $q = 7.97$, $P < 0.001$ for LB1), whereas rAβ did not ($q = 0.28$ and 0.11, $P > 0.05$) (Figure 4c). Moreover, Aβ, but not rAβ, decreased the expression of NR1 ($F_{2,18} = 42.09$; $q = 12.24$, $P < 0.001$), NR2A ($F_{2,18} = 8.56$; $q = 4.13$, $P < 0.01$), and NR2B ($F_{2,18} = 19.17$; $q = 7.12$, $P < 0.001$) (Figure 4c). Because it has been found that CREB signaling is altered in AD patients (Saura and Valero, 2011), we also examined the phosphorylation and expression levels of CREB in the same animals. Results revealed that neither Aβ nor rAβ affected the levels of pCREB and CREB at the time point measured ($F_{2,18} = 0.66$ for pCREB and $F_{2,18} = 0.08$ for CREB, both $P > 0.05$) (Figure 4c).

Aβ, Aβ aggregates, and amyloid plaque in the CA1 area were visualized by IHC staining of tissue slices from a separate group of animals injected with 21 μg Aβ ($n = 3$ and three slices per rat). Consecutive photomicrographs were collected and merged for subpanels A–D in Figure 4d. IHC analyses with anti-Aβ antibody at different magnifications clearly showed Aβ immunofluorescence in the CA1 area over a range of ~535 μm in length (Figure 4d (A and E); extra-hippocampal deposits of Aβ were not seen (Figure 4d (A)). Thioflavin S staining (Figure 4d (B and F)) and ProteoStat dye staining (Figure 4d (C and G)) indicated that the areas of Aβ aggregation and amyloid plaque overlapped with that of Aβ deposition. (Figure 4d (D)) is an adjacent tissue section showing the needle track and merged image of Aβ, thioflavin S, and ProteoStat dye staining. (Figure 4d (H))is the merged image of (Figure 4d (E–G)) at a higher magnification. Photomicrographs taken at higher magnifications are shown to allow clearer visualization of Aβ aggregation and amyloid plaques. (Figure 4e (A and B)) shows distinct Aβ aggregates and amyloid plaques in the CA1 area, respectively. Further costaining of thioflavin S and DAPI as well as ProteoStat dye and DAPI at a higher magnification revealed that both Aβ aggregation and amyloid plaques are mostly found surrounding nuclear areas (Figure 4e (C and D)), respectively. To further illustrate Aβ deposition and aggregation, we performed DAB staining. (Figure 4f (A)) shows Aβ deposits in the CA1 area. Aβ deposits at a higher magnification are shown in (Figure 4f (B)). An adjacent tissue section was stained with thioflavin S (Figure 4f (C and D)). The results indicate that Aβ aggregation takes place where Aβ deposits are located.

STAT1 Mediates the Memory-Impairing Effect of Aβ

This experiment was designed to examine whether the increased level of STAT1 following Aβ injection mediates the memory-impairing effect of Aβ. Rats were divided into three groups ($n = 7$ each group) to receive NH_4OH (0.5 μl) + control siRNA (0.25 μl, a sub-threshold volume), Aβ (0.5 μl) + control siRNA (0.25 μl), or Aβ (0.5 μl) + STAT1 siRNA (0.25 μl) injections and were subject to water maze learning. Aβ was given 10 days before spatial learning and STAT1 siRNA was given 60 h before spatial learning. The second STAT1 siRNA was given before the beginning of spatial training on day 2. Results revealed that Aβ consistently impaired acquisition performance in rats ($F_{2,18} = 16.49$; $q = 6.29$, $P < 0.001$), but transfection of a sub-threshold volume of STAT1 siRNA reversed this effect of Aβ ($q = 7.59$, $P < 0.001$) (Figure 5a). Probe trial analyses indicated that Aβ-injected animals spent less time in the target quadrant ($F_{2,18} = 3.49$; $q = 3.29$, $P < 0.05$), and this effect was similarly prevented by transfection with a sub-threshold volume of STAT1 siRNA ($q = 3.51$, $P < 0.05$) (Figure 5b). Furthermore, WB analyses indicated that Aβ consistently increased the expression of STAT1 and LB1 ($F_{2,18} = 120.68$; $q = 14.69$, $P < 0.001$ for STAT1 and $F_{2,18} = 34.58$; $q = 9.81$, $P < 0.001$ for LB1), but these effects were also prevented by transfection with a sub-threshold volume of STAT1 siRNA ($q = 21.49$ for STAT1 and $q = 10.52$ for LB1, both $P < 0.001$) (Figure 5c).

LB1 Mediates the Memory-Impairing Effect of Aβ

Similar experiment was designed to test whether Aβ-induced LB1 expression mediates the memory-impairing effect of Aβ. Rats were divided into three groups ($n = 7$ each group) to receive NH_4OH (0.5 μl) + control siRNA (0.25 μl, a sub-threshold volume), Aβ (0.5 μl) + control siRNA (0.25 μl), or Aβ (0.5 μl) + LB1 siRNA (0.25 μl) injections and were subject to water maze learning. The protocol used is the same as that described in the above experiment. Results revealed that Aβ consistently impaired acquisition performance in rats ($F_{2,18} = 18.66$; $q = 4.82$, $P < 0.01$), but transfection with a sub-threshold volume (0.25 μl) of LB1 siRNA reversed this effect of Aβ ($q = 8.57$, $P < 0.001$) (Figure 5d). Probe trial analyses indicated that Aβ-injected animals spent less time in the target quadrant ($F_{2,18} = 9.33$; $q = 3.89$, $P < 0.05$), and this effect was similarly prevented by transfection with a sub-threshold volume of LB1 siRNA ($q = 6.18$, $P < 0.001$) (Figure 5e). In addition, WB analyses indicated that Aβ consistently increased the expression of STAT1 in both Aβ-treated groups ($F_{2,18} = 15.83$; $q = 6.76$ for Aβ + control siRNA group and $q = 7.02$, for Aβ + LB1 siRNA group, both $P < 0.001$) (Figure 5f). Aβ also increased the expression of LB1 ($F_{2,18} = 43.11$; $q = 10.27$, $P < 0.001$), but this effect was prevented by transfection with a sub-threshold volume of LB1 siRNA ($q = 12.22$, $P < 0.001$) (Figure 5f).

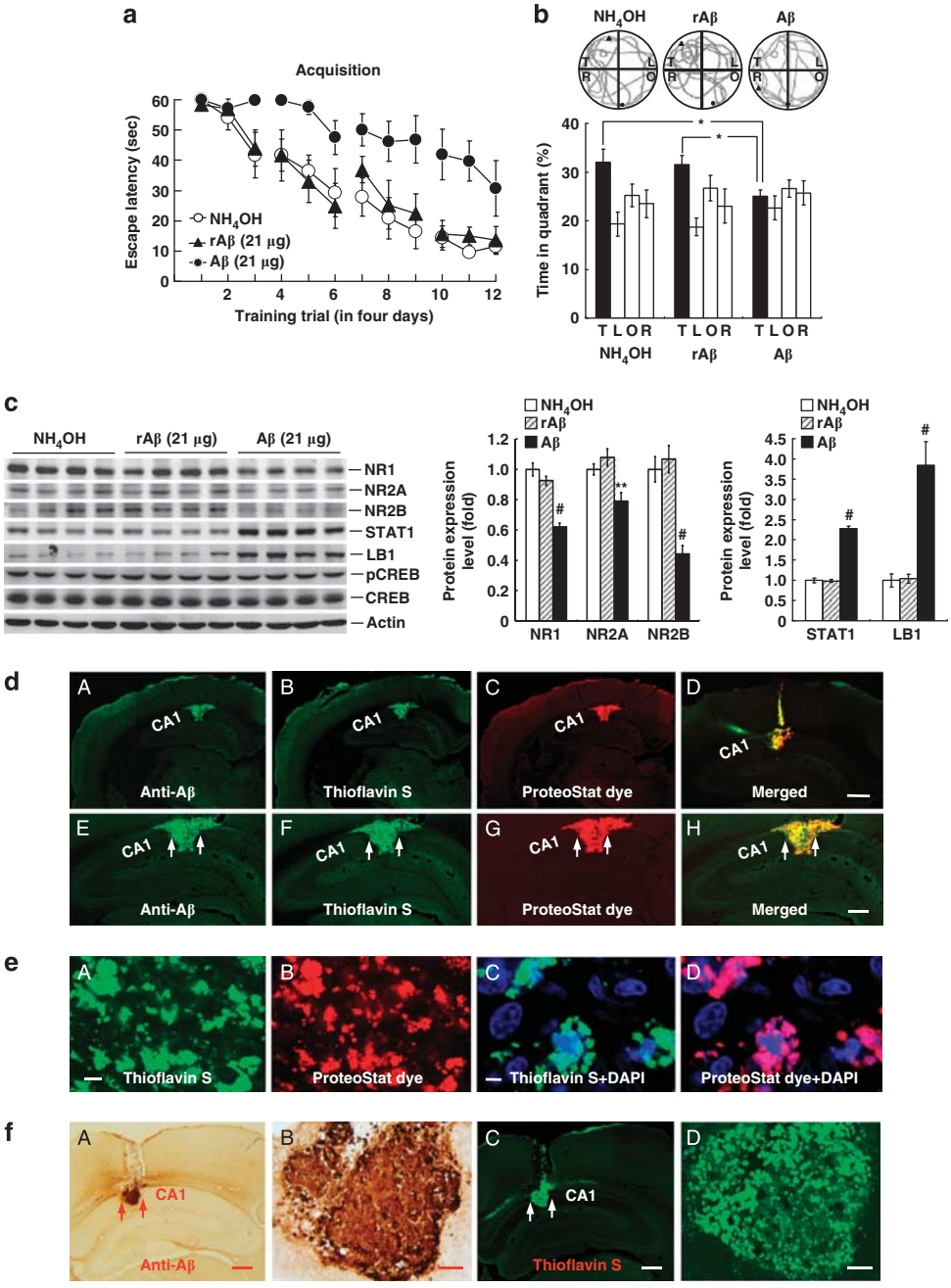

Figure 4 Aβ impairs spatial learning and memory, decreases the expression of NR1, NR2A and NR2B, but increases the expression of signal transducer and activator of transcription-1 (STAT1) and laminin β1 (LB1). Rats received 21 μg Aβ, rAβ, or NH_4OH injection and were subject to (a) water maze learning and (b) probe trial test. (c) Gel pattern and the analysis of protein expression of NR1, NR2A, NR2B, STAT1, and LB1 after the probe trial test. Data are mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, and $^{\#}P<0.001$. (d) Immunohistochemistry (IHC) showing 'A' Aβ staining using anti-Aβ antibody, 'B' Aβ aggregation with thioflavin S staining, and 'C' amyloid plaque with ProteoStat dye staining in the CA1 area 14 days after Aβ injection. Consecutive photomicrographs were taken and merged for (d (A–D)). Photomicrographs for A–C taken at a higher magnification are shown in (d (E–G)) in order and the merged image is shown in (d (H)). (d (D)) shows the merged image of an adjacent tissue section showing need track at a lower magnification. Scale bar equals 800 μm for the upper panels and scale bar equals 400 μm for the lower panels. (e) Photomicrographs taken at a higher magnification show distinct populations of 'A' Aβ aggregates and 'B' amyloid plaques. Scale bar equals 10 μm. Further illustrations show the relationship between 'C' Aβ aggregation and DAPI as well as 'D' amyloid plaque and DAPI. Scale bar equals 2.5 μm. (f) 3,3′-diaminobenzidine (DAB) staining shows 'A' Aβ staining using anti-Aβ antibody. Photomicrograph at a higher magnification is shown in 'B'. IHC shows 'C' thioflavin S staining of an adjacent tissue section. Photomicrograph taken at a higher magnification is shown in 'D'. Scale bars equal 400 μm for (f (A and C)) and scale bars equal 50 μm for (f (B and D)). Anti-Aβ staining overlaps with thioflavin S staining in photomicrographs taken at both magnifications.

LB1 mediates the Memory-Impairing Effect of STAT1

The above results demonstrated that both STAT1 and LB1 mediate the memory-impairing effect of Aβ. Here, we examined whether STAT1 acts through LB1 to mediate this effect. Rats were divided into three groups ($n=7$ each group) to receive Flag-vector (0.5 μl) + control siRNA (0.25 μl, a sub-threshold volume), Flag-STAT1WT (0.5 μl) +

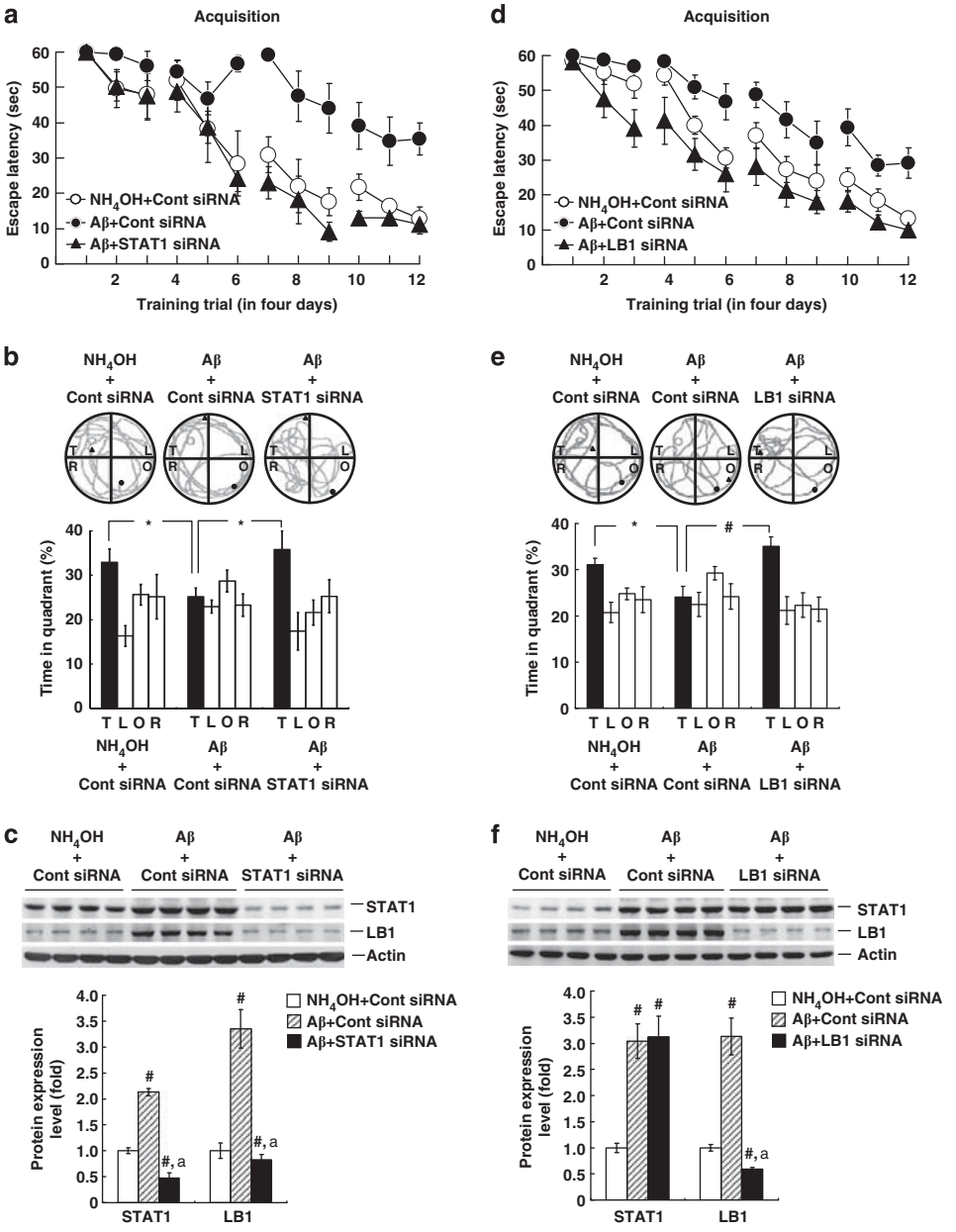

Figure 5 Both signal transducer and activator of transcription-1 (STAT1) and laminin β1 (LB1) mediate the memory-impairing effect of Aβ. Rats received Aβ + STAT1 siRNA transfection, Aβ + control siRNA transfection, or NH_4OH + control siRNA transfection and were subject to (a) water maze learning and (b) probe trial test. (c) Gel pattern and analysis of STAT1 and LB1 protein expression after the probe trial test. A separate group of rats received Aβ + LB1 siRNA transfection, Aβ + control siRNA transfection, or NH_4OH + control siRNA transfection and were subject to (d) water maze learning and (e) probe trial test. (f) Gel pattern and analysis of STAT1 and LB1 protein expression after the probe trial test. Data are mean ± SEM. $^{*}P<0.05$ and $^{\#}P<0.001$. a: compared with the corresponding Aβ + control siRNA group.

control siRNA (0.25 μl), or Flag-STAT1WT (0.5 μl) + LB1 siRNA (0.25 μl) injections and were subject to water maze learning. The protocol used is the same as that described in the above experiments. Results revealed that STAT1WT transfection consistently impaired water maze performance in rats ($F_{2,18}=6.74$, $P<0.01$; $q=4.9$, $P<0.01$), but transfection with a sub-threshold volume of LB1 siRNA reversed this effect of STAT1 ($q=3.94$, $P=0.01$) (Figure 6a). Probe trial analyses indicated that animals received STAT1WT transfection spent less time in the target quadrant ($F_{2,18}=6.55$; $q=3.72$, $P<0.05$), and this effect was similarly prevented by transfection with a sub-threshold volume of LB1 siRNA ($q=4.91$, $P<0.01$) (Figure 6b). WB analyses indicated that STAT1WT transfection increased the level of STAT1 expression in both STAT1-transfected groups ($F_{2,18}=185.81$, $q=23.55$ and $q=23.67$ for Flag-STAT1WT + control siRNA and Flag-STAT1WT + LB siRNA groups, respectively, both $P<0.001$). STAT1WT transfection also increased the expression of LB1 ($F_{2,18}=80.49$, $q=12.78$, $P<0.001$), but this effect was prevented by transfection with a sub-threshold volume of LB1 siRNA ($q=17.3$, $P<0.001$) (Figure 6c). Immunofluorescence of

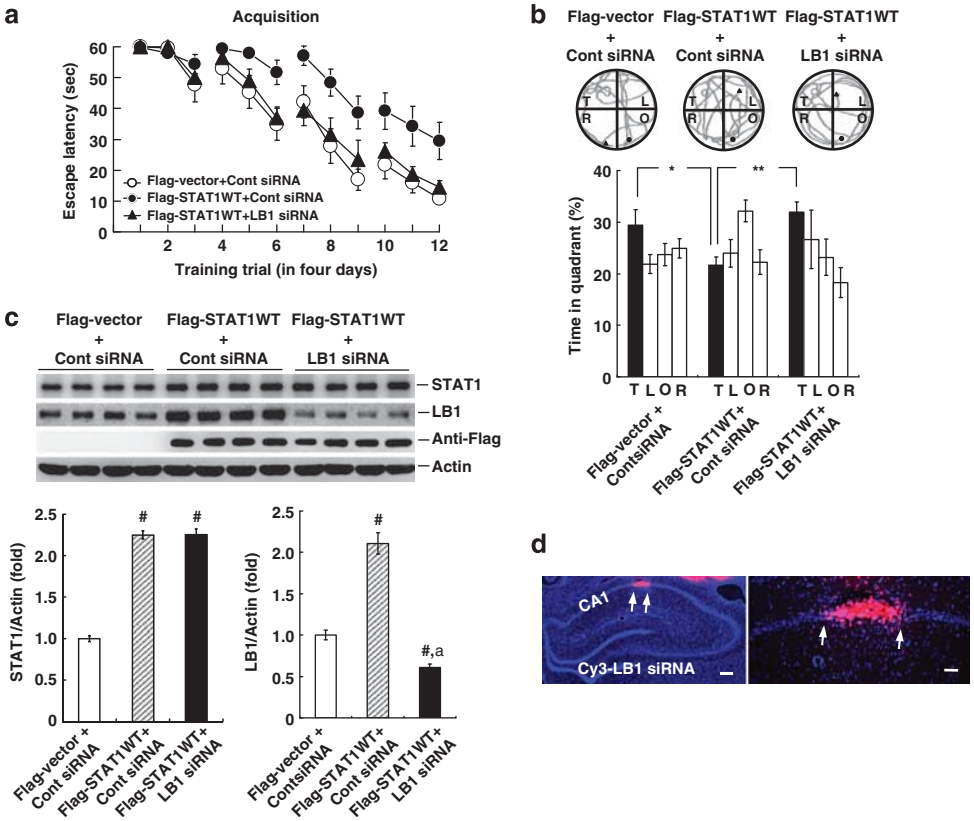

Figure 6 Laminin β1 (LB1) mediates the memory-impairing effect of signal transducer and activator of transcription-1 (STAT1). Animals received Flag-vector + control siRNA, Flag-STAT1WT + control siRNA, or Flag-STAT1WT + LB1 siRNA co-transfections and were subject to (a) water maze learning and (b) probe trial test. (c) Gel pattern and analysis of STAT1 and LB1 protein expression after the probe trial test. Western blotting (WB) using anti-Flag antibody confirms the transfection and expression of Flag-STAT1WT. (d) Immunofluorescence showing the area of Cy3-LB1 siRNA transfection in the CA1 area. Cells that show both red fluorescence (Cy3) and blue fluorescence (DAPI) are the cells successfully transfected with the LB1 siRNA. Scale bar equals 200 μm for the left panel and 50 μm for the right panel. $N = 7$ each group. Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{\#}P < 0.001$. $^{\#}$: compared with the control group and a: compared with Flag-STAT1WT + control siRNA group.

Cy3 and DAPI shows the area of sub-threshold volume of LB1 siRNA transfection in the CA1 area (Figure 6d).

Overexpression of NR1 and NR2B Prevents the Memory-Impairing Effect of Aβ and Aβ-induced STAT1 and LB1 expression

The above results show that Aβ decreased the expression of NR1, NR2A, and NR2B, and increased the expression of STAT1 and LB1 in association with impairment of spatial memory. Because Aβ produced a greater effect on NR1 and NR2B than NR2A, we examined whether the decreased expression of NR1 and NR2B mediates the memory-impairing effect of Aβ and the effect of Aβ on STAT1 and LB1 induction. Rats were divided into four groups ($n = 8$ each group) to receive NH_4OH (0.5 μl) + pCI vector (0.25 μl, a sub-threshold volume), Aβ (0.5 μl) + pCI vector (0.25 μl), Aβ (0.5 μl) + NR1 plasmid (0.25 μl), or Aβ (0.5 μl) + NR2B plasmid (0.25 μl) transfection and were subject to water maze learning. Aβ was given 10 days before spatial learning and NR1-pCI plasmid or NR2B-pCI plasmid was transfected 60 h before spatial learning. The same plasmid was transfected again on day 2 during water maze learning. Results revealed that Aβ consistently impaired acquisition performance in rats ($F_{3,28} = 13.03$, $q = 5.82$; $P < 0.001$), but overexpression of a sub-threshold volume of NR1 and NR2B plasmids both effectively reversed the memory-impairing effect of Aβ ($q = 5.37$ and $q = 8.65$, both $P < 0.001$ comparing the Aβ + NR1 group with Aβ group and comparing the Aβ + NR2B group with Aβ group) (Figure 7a). Probe trial analyses indicated that Aβ-injected animals spent less time in the target quadrant ($F_{3,28} = 23.66$, $q = 7.58$; $P < 0.001$), but animals received a sub-threshold volume of NR1 plasmid or NR2B plasmid transfection both reversed the impairing effect of Aβ on memory retention ($q = 7.95$ and $q = 11.58$, both $P < 0.001$ comparing the Aβ + NR1 group with Aβ group and comparing the Aβ + NR2B group with Aβ group) (Figure 7b). Moreover, WB analyses indicated that Aβ consistently increased the expression of STAT1 and LB1 ($F_{3,28} = 26.84$, $q = 10.13$; $P < 0.001$ for STAT1 and $F_{3,28} = 146.42$, $q = 21.1$; $P < 0.001$ for LB1), but overexpression of a sub-threshold volume of NR1 plasmid reversed these effects of Aβ ($q = 8.09$ and $q = 21.94$ for STAT1 and LB1, both $P < 0.001$ comparing the Aβ + NR1 group with Aβ group). Similarly, overexpression of a sub-threshold volume of NR2B plasmid also reversed these effects of Aβ ($q = 10.89$ and $q = 21.94$ for STAT1 and LB1, both $P < 0.001$ comparing the Aβ + NR2B group with Aβ group) (Figure 7c). Plasmid

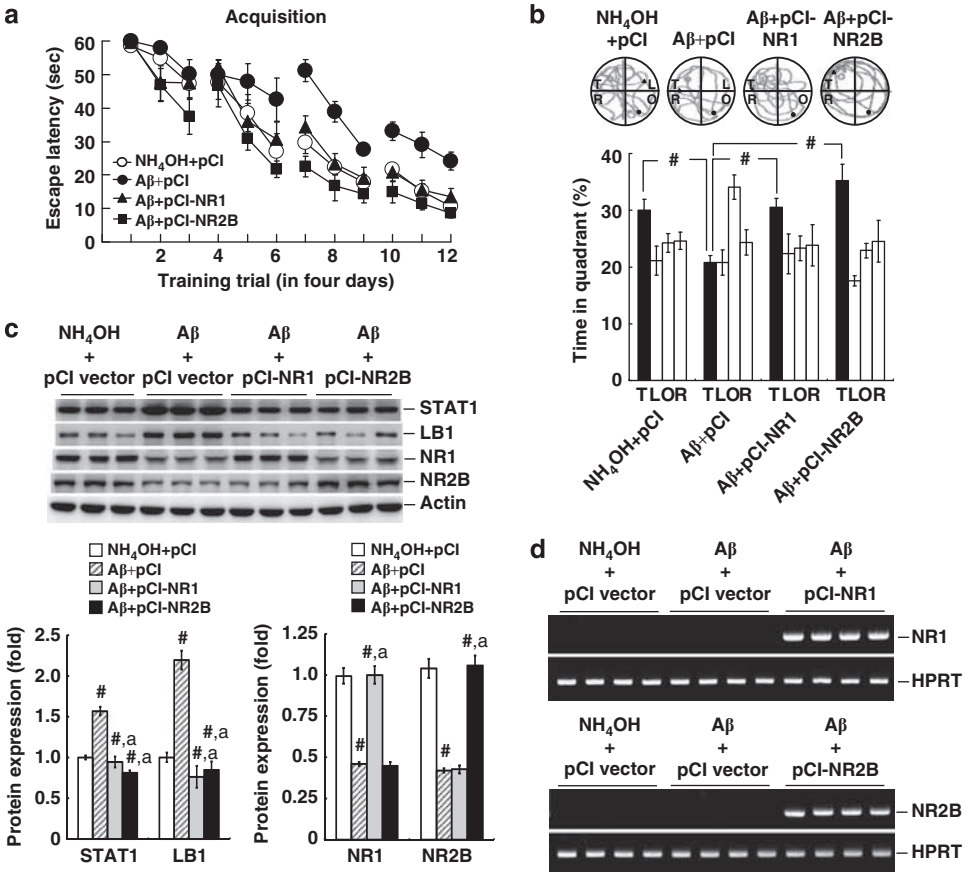

Figure 7 Overexpression of NR1 and NR2B blocks the memory-impairing effect of Aβ and Aβ-induced signal transducer and activator of transcription-1 (STAT1) and laminin β1 (LB1) expression. Animals received Aβ + NR1 plasmid transfection, Aβ + NR2B plasmid transfection, Aβ + pCI vector transfection, or NH_4OH + pCI vector transfection (served as the control group) and were subject to (a) water maze learning and (b) probe trial test. (c) Representative gel pattern and quantitative analysis of STAT1, LB1, NR1, and NR2B protein expression after the probe trial test. $N = 8$ each group. (d) Representative gel pattern from RT-PCR showing the NR1 and NR2B bands after NR1 plasmid and NR2B plasmid transfection, respectively. The designed primers contain sequences in the pCI vector and in NR1 or in the pCI vector and NR2B plasmid. Data are mean ± SEM. $^{\#}P < 0.001$. a: compared with the corresponding Aβ + pCI group.

transfection and expression of NR1 and NR2B was confirmed by RT-PCR with primers specific to the pCI-NR1 fragment and the pCI-NR2B fragment. Thus, the NR1 and NR2B bands were observed in the overexpression groups only (Figure 7d). In addition, NMDA injection produced a similar effect to that of NR1 and NR2B overexpression (Supplementary Figure S3) and transfection of either NR1 siRNA or NR2B siRNA upregulated STAT1 and LB1 expression (Supplementary Figure S4a–c).

DISCUSSION

In this study, we demonstrated a novel role for STAT1 in the negative regulation of spatial learning and memory formation, showing that spatial training decreases the transcription and expression of STAT1, and spatial learning and memory performance are enhanced in *Stat1*$^{-/-}$ mice. These results are consistent with our previous report that PIAS1, an inhibitor of STAT1, enhances spatial learning and memory formation (Tai *et al*, 2011). We further showed that STAT1 impairs spatial learning and memory through the mediation of the extracellular matrix protein LB1. These results are congruent with reports showing that LB1 negatively regulates spatial learning and memory in rats (Yang *et al*, 2011) and that vascular dementia patients have higher levels of anti-LB1 immunoreactivity (Matsuda *et al*, 2002). Importantly, we also identified a novel mechanism of Aβ pathogenesis, demonstrating that Aβ induces upregulation of STAT1 and LB1 via a mechanism that depends on the downregulation of the NMDAR subunits, NR1 and NR2B.

Because STAT1 must form dimers (homo- or heteromeric) before it can be translocated to the nucleus to regulate gene expression, we have tested the effects of transfecting the *Stat1/Stat2* or *Stat1/Stat3* plasmids to HEK293T cells and examined their effects on LB1 promoter activity. Results revealed that *Stat1/Stat3*, but not *Stat1/Stat2*, also increased LB1 promoter activity (Supplementary Figure S5a). These results are consistent with our observation that spatial training decreased the expression of STAT3 in the CA1 area without affecting the expression of STAT2 (Supplementary Figure S5b). These results further suggest that, in addition to STAT1 homodimers, STAT1/STAT3 heterodimers may also have a role in impairing learning and memory through binding to the same GAS element on gene

promoters. Identification of other candidate target genes in addition to LB1 would help to clarify this issue. It is possible, however, that the role of STAT1/STAT3 heterodimers is different from that of STAT3/STAT3 homodimers because STAT3 signaling was found to have a role in mediating spatial working memory (Chiba *et al*, 2009) and inducing NMDAR-dependent long-term depression (Nicolas *et al*, 2012). The possible involvement of STAT3 in spatial learning and memory requires further investigation. In addition, the effect of STAT1WT transfection on impairment of acquisition performance seems reduced during the last few learning trials. To address this issue, we have conducted an additional experiment with more learning trials and results revealed that additional training overcomes the acquisition deficit caused by the overexpression of STAT1 (Supplementary Figure S6). This probably reflects the operation of compensatory mechanisms that increase the neuronal plasticity for spatial learning. Because STAT1 is involved in the immune response (Shuai and Liu, 2003), we also examined whether spatial training affected the expression of STAT1 through alteration of the immune response. However, we found no evidence that spatial training affected the expression of tumor necrosis factor (TNFα) or IL-6 in the same animals (Supplementary Figure S5b).

A previous study showed that Aβ decreases the phosphorylation level of STAT3, and that colivelin, an agent that activates the JAK2/STAT3 signaling pathway, rescues memory impairment in a transgenic mouse model of AD (Chiba *et al*, 2009). The authors of this study suggested that Aβ-dependent inactivation of the JAK2/STAT3 axis mediates memory impairment in AD. On the other hand, Aβ was shown to increase the phosphorylation of STAT3, whereas decreased STAT3 activation or expression attenuated Aβ-induced neurotoxicity, an effect that is likely mediated through the Tyk2/STAT3 pathway (Wan *et al*, 2010). Although these studies examined the involvement of STAT1 and STAT3 signaling in Aβ-induced cognitive dysfunction and neurotoxicity, they did not address whether Aβ regulates the expression of STAT1. Here, we provide the first evidence that Aβ increases the expression of STAT1 and further shows that STAT1 mediates the memory-impairing effect of Aβ. Whether the JAK2/STAT1 pathway mediates the neurotoxicity of Aβ is a question that requires further investigation.

In study of the upstream molecule that mediates Aβ-induced STAT1 and LB1 expression, we found that the NMDAR has an important role. These results are consistent with the observations that Aβ suppresses NMDA-evoked current, decreases the phosphorylation level of NR2B, and reduces surface expression of NR1 and NR2B (Snyder *et al*, 2005). In this same context, decreased surface expression of NMDAR has been shown to contribute to synaptic defects caused by Aβ and observed in APP transgenic mice (Dewachter *et al*, 2009). Our findings are also consistent with reports that Aβ accumulation impairs long-term potentiation in the CA1 area and spatial memory in mice (Chapman *et al*, 1999; Freir *et al*, 2001). Further support for the involvement of NMDARs in mediating Aβ effects comes from the observation that an NMDAR antagonist blocks Aβ-induced suppression of synaptic transmission (Kamenetz *et al*, 2003). However, our results are inconsistent with reports that Aβ induces early neuronal dysfunction through activation of NR2B-containing NMDARs (Ronicke *et al*, 2011) and that NMDAR activation is required for Aβ-mediated reduction in the spine number and plasticity (Wei *et al*, 2010). These apparent discrepancies may reflect differences in experimental design and the location (synaptic *vs* extrasynaptic) of the NMDARs under investigation (Li *et al*, 2011). In this study, we also found that knockdown of NR1 and NR2B both upregulated the expression of STAT1 and LB1. Exogenous application of NMDA also prevented Aβ-induced STAT1 and LB1 expression. One possible explanation for the negative regulation of STAT1 and LB1 by NMDAR activation could be mediated through the JAK/STAT pathway. Our preliminary results revealed that NMDA injection to the CA1 area rapidly increased the phosphorylation level of JAK2 (Supplementary Figure S4d and e). This result is consistent with the report that activation of NMDAR produces a transient activation of JAK2 and STAT3, and the JAK/STAT pathway is involved in synaptic plasticity (Nicolas *et al*, 2012). Activation of JAK/STAT signaling pathway may increase the expression of PIAS1 because the promoter region of the *pias1* gene contains three GAS elements that are specific for STAT1/STAT1 and STAT1/STAT3 binding (Supplementary Figure S4g). Increased PIAS1 expression would further downregulate the expression of STAT1 and, consequently, LB1. Thus, a negative auto-regulation loop may exist between STAT1 activation and STAT1 expression. But the exact mechanism and signaling pathway between NMDAR activation and decrease in STAT1 expression requires further investigation.

In the present study, we found that Aβ impaired spatial learning and memory 10–14 days after administration. At this stage, about a 4% neuronal loss was observed in the CA1 area (Supplementary Figure S7). These results are consistent with reports showing correlations between Aβ levels, learning/memory deficits, and amyloid plaques in the transgenic mouse model of AD (Chen *et al*, 2000; Hsiao *et al*, 1996). They are also congruent with the notion that synaptic deficits and memory impairment occur before neuronal degeneration takes place in AD patients (Selkoe 2002). On the other hand, an increased level of Aβ has been reported to cause synaptic deficits and learning impairment before, or independent of, the accumulation of amyloid plaques (Hsia *et al*, 1999; Kamenetz *et al*, 2003; Mucke *et al*, 2000). Our results are not inconsistent with these reports but measures of amyloid plaque and learning/memory at earlier time points after Aβ treatment should help to clarify this issue.

It should also be noted that we used a high dose of Aβ (21 μg) in the present study. This was because Aβ at this dose produced the most marked biochemical changes and learning and memory impairment (Figure 3). These observations are consistent with other studies that have used a similar or higher dose of Aβ (Tsai *et al*, 2007; Chacon *et al*, 2004). Using a high dose of Aβ that causes a significant impairment in cognitive function is also beneficial from the standpoint of observing the preventive effects of transfecting STAT1 siRNA and LB1 siRNA. We further examined the effects of different Aβ doses and injection times on Aβ aggregation and amyloid plaque formation, and found that a single injection of Aβ at 21 μg produced an effect similar

to that of three injections of Aβ at a lower dose (2.1 μg) over a 14-day interval (Supplementary Figure S8), whereas 2.1 μg Aβ is a dose that is commonly used in the literature. In addition, we found that three injections of 2.1 μg of Aβ over a longer 28-day interval showed the most significant effect on Aβ aggregation and amyloid plaque formation (Supplementary Figure S8). Accordingly, we used a high dose of Aβ (21 μg) to mimic the effect of three injections of a lower dose (2.1 μg) of Aβ, without risking the greater tissue damage caused by multiple injections. Although a dose of 2.1 μg Aβ produces a concentration that is still about 1 000 000 times higher than endogenous levels of Aβ in the rat brain, a lower dose of Aβ (0.2 μg) did not produce apparent alterations in behavioral, biochemical (Figure 3), and immunohistochemical measures (Supplementary Figure S8).

In conclusion, we have identified STAT1 as a novel negative regulator of spatial memory formation and further showed that STAT1 acts through LB1 to mediate the memory-impairing effect of Aβ via a mechanism that is dependent on downregulation of the NMDAR subunits, NR1 and NR2B. The impairment of memory formation by STAT1, whether considered in isolation or in response to Aβ, is independent of CREB signaling. Visible learning performance was not affected in animals in all experiments (Supplementary Figure S9). Identification of other candidate genes downstream of STAT1 signaling may reveal novel mechanisms underlying the synaptic toxicity produced by Aβ and suggests therapeutic strategies for managing AD.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

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