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Laminin-β1 Impairs Spatial Learning through Inhibition of ERK/MAPK and SGK1 Signaling

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Laminin is a major structural element of the basal lamina consisting of an α-chain, a β-chain, and a γ-chain arranged in a cross-like structure, with their C-terminal inter-coiled. Laminin is abundantly expressed in the hippocampus of mature brain and is implicated in several psychiatric disorders, but its possible role involved in learning and memory function is not known. This issue was examined here. Our results revealed that water maze training significantly decreased laminin-β1 (LB1) expression in the rat hippocampal CA1 area. Transfection of LB1 WT plasmid to hippocampal CA1 neurons impaired water maze performance in rats. Meanwhile, it decreased the phosphorylation level of ERK/MAPK and protein kinase serum- and glucocorticoid-inducible kinase-1 (SGK1). By contrast, knockdown of endogenous LB1 expression using RNA interference (LB1 siRNA) enhanced water maze performance. Meanwhile, it increased the phosphorylation level of ERK/MAPK and SGK1. The enhancing effect of LB1 siRNA on spatial learning and on the phosphorylation of ERK/MAPK and SGK1 was blocked by co-treatment with the MEK inhibitor U0126 at a concentration that did not apparently affect spatial learning and ERK/MAPK phosphorylation alone. Further, the enhancing effect of LB1 siRNA on spatial learning and SGK1 phosphorylation was similarly blocked by co-transfection with SGK1 siRNA at a concentration that did not markedly affect spatial learning and SGK1 expression alone. These results together indicate that LB1 negatively regulates spatial learning in rats. In addition, LB1 impairs spatial learning through decreased activation of the ERK/MAPK–SGK1 signaling pathway in the rat hippocampus.
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INTRODUCTION

Extracellular matrix (ECM) is a complex network of macromolecules, including glycoproteins, polysaccharides, and proteoglycans (Bukalo *et al*, 2001). ECM is involved in neuronal migration, neurite outgrowth, and synaptic function (Dityatev and Schachner, 2003; Sanes, 1989). In the adult brain, many studies have demonstrated the role of ECM in some neuropathological conditions, such as Alzheimer's disease (Bruckner *et al*, 1999) and multiple sclerosis (Sobel and Ahmed, 2001). In addition to collagen, laminin is another major structural element of the basal lamina (Timpl, 1996). Laminin is a heterotrimeric molecule consisting of an α-chain, a β-chain, and a γ-chain (Colognato and Yurchenco, 2000). Mammals express at least 5 α-chains, 3 β-chains, and 3 γ-chains (Colognato and Yurchenco, 2000; Tunggal *et al*, 2000). These laminin subunits show different spatial and temporal distribution patterns, which suggests that they may participate in different physiological functions (Ekblom *et al*, 2003).

Laminins are widely distributed in the central nervous system and the basement membrane of the blood vessels. In the adult brain, laminins are predominantly expressed in the hippocampus and neocortex (Hagg *et al*, 1989). Further study indicates that the laminin-β1 (LB1) subunit is highly expressed in the brain, with laminin-10 ($\alpha5\beta1\gamma1$) as the major isoform (Indyk *et al*, 2003). Transgenic mice overexpressing the *laminin-β1* promoter driving β-galactosidase show that LB1 is widely expressed in various brain regions, including the hippocampus, the entorhinal cortex, and the striatum. Similar expression pattern was found with the endogenous LB1 protein (Sharif *et al*, 2004). Further, laminin also has a role in synaptic plasticity because laminin degradation by plasmin impairs the maintenance, but not the induction, of long-term potentiation (LTP) in hippocampal neurons (Nakagami *et al*, 2000). But whether laminin also has a role in learning and memory function is not known.

The expression of laminin is also related to several psychiatric disorders. For example, prolonged stress decreases laminin expression in the frontal cortex and hippocampus in rats, whereas chronic antidepressant treatment increases the expression of laminin in the hippocampus (Laifenfeld *et al*, 2005a). Further, laminin expression was decreased in the parieto-occipital cortex of the postmortem brain from

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depression patients, but antidepressant treatment reversed this effect (Laifenfeld *et al*, 2005b). A significant decrease in laminin expression was also observed in the same area from schizophrenia and bipolar disorder patients (Laifenfeld *et al*, 2005b). On the other hand, laminin expression was increased in the prefrontal cortex of schizophrenia patients (Laifenfeld *et al*, 2005b). More related to the present study, there is a higher level of laminin-α1 and laminin-γ1 in the frontal cortex of Alzheimer's disease patients (Palu and Liesi, 2002). Moreover, vascular dementia patients show a significantly higher anti-LB1 immunoreactivity in their cerebrospinal fluid (Matsuda *et al*, 2002). These latter results suggest that there is a negative relationship between laminin expression and memory function in humans.

Other than the roles described above, laminin also has a role in signal transduction. In neurons, laminin-1 consistently activates calcium/calmodulin-dependent kinase-II (CaMKII), and laminin-1 activation of CaMKII stabilizes newborn embryonic axons through its influence on the actin cytoskeleton (Easley *et al*, 2006). Laminin also activates the transcription factor, signal transducer and activation of transcription (STAT1) during macrophage maturation (Coccia *et al*, 1999), and we have demonstrated recently that STAT1 phosphorylation impairs spatial learning in rats (Tai *et al*, 2011). Furthermore, STAT1/STAT2 could be activated by serum- and glucocorticoid-inducible kinase-1 (SGK1) to mediate amyloid-β-induced defense mechanism (Hsu *et al*, 2009), and we have shown previously that the protein kinase SGK1 facilitates spatial learning and hippocampal LTP in rats (Tsai *et al*, 2002; Ma *et al*, 2006). Much evidence supports the notion that the mechanism associated with neuronal plasticity is also implicated in the therapeutic action of antidepressants. For example, chronic treatment with different classes of antidepressants, including fluoxetine, tranylcypromine, and reboxetine, increases neurogenesis in the adult rat hippocampus (Malberg *et al*, 2000). Together with the observation that laminin expression is negatively associated with memory performance in Alzheimer's disease patients, these results suggest that laminin may also negatively regulate learning and memory function in animals. The present study was aimed to examine this hypothesis focusing on the role of LB1. We also examined whether LB1 alters learning and memory performance through alteration of SGK1 signaling.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats (250–350 g) bred at the Institute of Biomedical Sciences, Academia Sinica, Taiwan, were used. They were housed in a room (23 ± 2 °C) maintained on a 12-h light/dark cycle (lights on at 0600 hours), with food and water continuously available. The experimental procedures followed the Guidelines of Animal Use and Care of the National Institute of Health.

Drug

A selective inhibitor of MEK1 and MEK2, U0126, was purchased from Calbiochem (Darmstadt, Germany). U0126 was dissolved in 100% DMSO to 13.3 mg/ml and stored at −20 °C. Before use, U0126 was further diluted to a final concentration of 2 mg/ml in 12.5% DMSO with 0.9% NaCl.

Water Maze Learning

The water maze used was a plastic, circular pool, 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 (8 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. Water was made cloudy by adding milk powder. Distinctive, visual cues were set on the wall.

For spatial learning, animals were subjected to three trials a day, with one administered early in the morning, one administered in the early afternoon, and another administered in the late afternoon. The training procedure lasted for 4 days and a total of 12 trials were given. This procedure was adopted because spaced training is a better paradigm to facilitate memory consolidation. In addition, because the transfection method we used for plasmid DNA and siRNA transfection is a transient transfection method. It would require repeated injection (transfection), if the training procedure lasts for more than 4 days, it causes certain extent of tissue inflammation. Therefore, we have adopted the training procedure for 4 days, with two transfections being made to each animal to last through the probe trial test. For these trials, the animals were placed at different starting positions spaced equally around the perimeter of the pool in a random order. The animals were given 60 s to find the platform. If an animal could not find the platform, it was guided to the platform and was allowed to stay there for 20 s. The time that each animal took to reach the platform was recorded as the escape latency. A probe trial of 60 s was administered on day 5 to test their memory retention. The animals were placed in the pool with the platform removed and the time they spent in each quadrant (target quadrant, left quadrant, opposite quadrant, and right quadrant) was recorded. For the trained and swimming control experiments, animals in the trained group were subjected to the regular water maze learning procedure. The animals in the swimming control group swam for the same period of time for each trial as the trained group (use the mean latency value for each trial) except that the visual cues and the platform were removed. Thus, the spatial relationship between these two cannot be established. In order to know whether a different training paradigm also yields similar result on LB1 expression, in a separate experiment,animals were subjected to four trials a day space apart by 2 h between trials for 7 days in all. The escape latency is 120 s. The animals in the swimming control group swam for the same period of time for each trial as the trained group but with the visual cues and the platform removed.

For visible platform learning, a flag was mounted on the platform and the platform was raised 2.5 cm above the surface of water. In addition, milk powder was not added so the animals could see the location of the platform from the water. Separate animals were used for visible platform learning in each experiment.

Plasmid DNA Construction and DNA/PEI Complex Preparation

For construction of the Flag-tagged *laminin-β1* plasmid, full-length *laminin-β1* was cloned by amplifying the rat

hippocampal *laminin-β1* cDNA using the primers 5′-CC GCTCGAGCATGGAAAGGCCCCTCTCCTCTCTCC-3′ and 5′-GGGGGCCCTTATAAGCAGGTGCTGTAAACGGCAAC-3′. The PCR product was sub-cloned between the *Xho*I and *Apa*I sites of the mammalian expression vector pCMV-Tag2A (Stratagene, La Jolla, CA). For construction of the GFP-tagged *laminin-β1* plasmid, full-length *laminin-β1* was sub-cloned into the pEGFP-C1 expression vector with *Rsr*II sites. The method used for plasmid DNA transfection to brain tissues was adopted from that of a previous study (Abdallah *et al*, 1996) with modifications. The non-viral vector transfection reagent, polyethylenimine (PEI), was used because we have demonstrated previously that PEI does not produce toxicity to hippocampal neurons (Chao *et al*, 2011). Before injection, plasmid DNA was diluted in 5% glucose to a stock concentration of 2.77 μg/μl. Branched PEI of 25-kDa (Sigma, St Louis, MO) was diluted to 0.1 M concentration in 5% glucose and added to the DNA solution. Immediately before injection, 0.1 M PEI was added to reach a ratio of PEI nitrogen per DNA phosphate of 9. The mixture was subjected to vortexing for 30 s and allowed to equilibrate for 15 min.

RNA Interference

Rat LB1 siRNA was used to knock down LB1 expression in CA1 area. The sense and antisense sequences used were adopted from that published on NCBI (XM_001075963). The sequence for the sense strand is 5′-GCAUUUCUGC CUUGAUCCATT-3′ and that for the antisense strand is 5′-UGGAUCAAGGCAGAAAUGCTG-3′. For the purpose of immunohistochemistry, the LB1 siRNA was conjugated to the Cy3 dye. Rat SGK1 siRNA was adopted from that of a previous study (Yang *et al*, 2006). The sequence for the sense strand is 5′-GUCCCUCUCAACAAAUCAAtt-3′ and that for the antisense strand is 5′-UUGAUUUGUUGAG AGGGACtt-3′. The Silencer Negative Control number-1 siRNA (control siRNA) was used as control. These are siRNAs with sequences that do not target any gene product (Ambion, Austin, TX). All the siRNAs used were synthesized from Ambion.

Intra-Hippocampal Gene Transfection and siRNA Injection

Rats were anesthetized with pentobarbital (40 mg/kg, i.p.) and subjected to stereotaxic surgery. Two 23-gauge, stainless-steel, thin-wall cannulae were implanted bilaterally to the CA1 area of the dorsal hippocampus at the following coordinates: 3.5 mm posterior to the bregma, 2.5 mm lateral to the midline, and 3.4 mm ventral to the skull surface. One week was allowed for the animals to recover from the surgery. After recovery from the surgery, the animals were handled gently and 0.7 μl of plasmid DNA complex (1.5 μg/μl) was injected to the CA1 area at a rate of 0.1 μl/min. For siRNA injection, 0.7 μl of LB1 siRNA (8 pmol/μl) or control siRNA was transfected to the CA1 area by using the cationic polymer transfection reagent jetSI™ at 10 mM (Polyplus-Transfection, New York, NY). For the LB1 siRNA and SGK1 siRNA co-transfection experiment, 0.7 μl of each siRNA was injected 2 h apart. For the LB1 siRNA and U0126 co-injection experiment, 0.7 μg of U0126 (0.7 μl) was injected 30 min after the first LB1 siRNA (0.7 μl) injection, and 0.7 μg of U0126 (0.7 μl) was injected again on each day 30 min before water maze learning. This injection paradigm was adopted because U0126 has a short half-life of approximately 30 min (Zhang *et al*, 2004). In these studies, because the PEI reagent and control siRNA were used as the proper control group for plasmid DNA transfection and LB1/SGK1 siRNA transfection, respectively, we have performed additional control experiments to examine whether PEI alone and control siRNA alone may have an effect on spatial acquisition and LB1 expression. Results revealed that animals receiving PEI injection to the CA1 area showed a similar acquisition curve compared with animals receiving phosphate-buffered saline (PBS) injection ($t_{1,6} = 1.36$, $p > 0.05$). Their LB1 expression level is also similar (Supplementary Figure S1a). Meanwhile, animals receiving control siRNA transfection to the CA1 area also had a similar acquisition curve compared with animals receiving jetSI transfection (jetSI is the solution used to mix with siRNA for transfection) ($t_{1,6} = 0.63$, $p > 0.05$). Their LB1 expression level is also similar (Supplementary Figure S1b). Therefore, PEI and jetSI were used for all plasmid DNA and siRNA injection experiments, respectively. The inner diameter of the injection needle is 0.31 mm and the wall thickness of the injection needle is 0.12 mm each side. The injection needle was left in place for 5 min to limit the backflow of injected DNA, siRNA, and drug. Spatial training started 48 h after DNA injection or 72 h after siRNA injection. Plasmid DNA or siRNA was injected again at the beginning of the second training day. One hour was allowed between the second injection and spatial training.

The animals were killed after the probe trial test or after spatial training. Their brains were removed and hippocampal tissue slices (2-mm thickness of each slice, two slices in all) were dissected out by using a brain slicer. The CA1 tissue was further punched out by using a punch with 1.8 mm diameter.

RNA Preparation and Q-PCR

Total RNA from CA1 tissue was isolated by using the RNAspin mini kit (GE Healthcare). The cDNA was generated from total RNA with Superscript-III reverse transcriptase (Invitrogen). Real-time PCR analysis was performed with the ABI PRISM-7500 real-time PCR system by using the Power SYBR Green PCR Master Mix according to the instruction manual (Applied Biosystems (ABI), Foster City, CA). The PCR parameters used were as follows: 50 °C for 2 min for 1 cycle, 95 °C for 10 min for 1 cycle, 95 °C for 15 s followed by 60 °C for 1 min for 40 cycles. The primer sequences for *laminin-β1* are as follows: Forward: 5′-CCGG GCTCAAGATACGTTGT-3′ and reverse: 5′-AACCGCACG GTGTAGTTCATC-3′. The primer sequences for *laminin-β2* are as follows: Forward: 5′-GGCGAGGTCATCTATCGTGT-3′ and reverse: 5′-GCATGTCCATAGCAGAAGCA-3′. These sequences were designed based on the Primer Design Program 'Primer 3' Software (http://frodo.wi.mit.edu/primer3). The primer sequences for *HPRT*, forward: 5′-GCAGACTTTGC TTTCCTTGG-3′ and reverse: 5′-TCCACTTTCGCTGATGA CAC-3′, are also designed based on the 'Primer 3' Software. The relative amount of *laminin-β1* and *laminin-β2* mRNA expression was estimated by using a standard curve that was

generated by serial dilution of the reverse transcription product from control samples.

Western Blot

The CA1 tissue lysate was lysed in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% IGEPAL CA-630, 1 mM phenymethylsulfonyl fluoride, 20 μg/ml pepstatin-A, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 50 mM NaF, and 1 mM Na_3VO_4). The lysate was resolved by 8% SDS-PAGE. The proteins resolved by SDS-PAGE were transferred to a PVDF membrane (Millipore, Bedford, MA) and western blot was performed by using the following antibodies: rabbit anti-LB1, anti-pS422 SGK1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-laminin-β2 (LB2), anti-Akt, anti-pS473 Akt (Cell Signaling, MA), anti-SGK1, anti-ERK1/2, anti-pERK1/2 (Millipore), and anti-actin (Chemicon, Temecula, CA). The secondary antibody used was an HRP-conjugated goat-anti rabbit IgG antibody (Chemicon). The membrane was developed by reacting with chemiluminescence HRP substrate and exposed to the LAS-3000 image system (Fujifilm, Tokyo, Japan) for visualization of protein bands. The protein bands were quantified by using the NIH ImageJ Software.

Immunohistochemistry

Rats were anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused with ice-cold PBS followed by 4% paraformaldehyde. Brains were removed and post-fixed in a 20% sucrose/4% paraformaldehyde solution for 20–48 h. The brains were then frozen, cut into 30-μm sections on a cryostat, and mounted on gelatin-coated slides. The brain sections were rinsed with 1 × PBS for 10 min and permeabilized with pre-cold EtOH/CH3COOH (95% : 5%) for 10 min, followed by 1 × PBS for 10 min for three times. The sections were pre-incubated in a blocking solution containing 3% normal goat serum, 3% BSA, and 0.2% Triton X-100 in 1 × PBS for 2 h followed by 1 × PBS for 10 min for three times. For immunofluorescence detection of nucleus, the tissue sections were treated with 20 μl of the VECTASHIELD mounting medium with DAPI (1.5 μg/ml) (Vector Laboratories, Burlingame, CA). To confirm siRNA transfection, the Cy3-labeled LB1 siRNA was transfected to the hippocampal CA1 area and brain sections were prepared 72 h after siRNA injection for visualization of Cy3 fluorescence under a confocal microscope. For immunofluorescence detection of LB1 transfection and expression, the GFP-tagged LB1 plasmid was transfected to the CA1 area and brain sections were prepared 48 h after GFP-LB1 transfection for visualization of GFP fluorescence under a confocal microscope. Digital photomicrographs were taken with an Olympus digital C-3030 camera mounted on a Zeiss microscope.

Statistics

Behavioral data were analyzed by analysis of variance (ANOVA) with repeated measure followed by *post-hoc* Newman–Keuls multiple comparisons (represented by *q*-value). Biochemical data were analyzed by Student's *t*-test or one-way ANOVA followed by Newman–Keuls comparisons.

RESULTS

Spatial Training Decreases LB1 Expression in the Rat Hippocampus

Animals were randomly divided into the trained and the swimming control groups ($n = 8$ each group) as described under Materials and methods. They were killed immediately after training (and swimming) and their hippocampal CA1 tissues were dissected out and subjected to quantitative real-time PCR (Q-PCR) and western blot analyses for *laminin-β1* and *laminin-β2* mRNA and protein level determination. Results revealed that spatial training markedly decreased the mRNA level of *laminin-β1* ($t_{1,14} = 3.45$, $p < 0.01$) but not that of *laminin-β2* ($t_{1,14} = 0.92$, $p > 0.05$) in the hippocampal CA1 area when compared with the swimming controls (Figure 1a). Spatial training similarly decreased LB1 protein expression in the CA1 area ($t_{1,14} = 5.98$, $p < 0.001$) without affecting that of LB2 in the same area ($t_{1,14} = 0.19$, $p > 0.05$) (Figure 1b). There is no apparent alteration of LB1 expression in the amygdala and striatum ($t_{1,14} = 0.52$ and 0.72, respectively; both $p > 0.05$), areas that are not implicated in spatial learning (Figure 1c and d). In a separate experiment, animals were also randomly divided into the trained and the swimming control groups ($n = 5$ each group), and subjected to a longer training period for 7 days (as described under Materials and methods). They were killed immediately after training (and swimming) and their hippocampal CA1 tissues and frontal cortex were dissected out and subjected to western blot analyses for LB1 expression. Their acquisition curve is shown in Supplementary Figure S2a. The result revealed that such a training paradigm also yielded a significant reduction in LB1 expression in the CA1 area ($t_{1,8} = 11.29$, $p < 0.001$) (Supplementary Figure S2b). But it did not affect the expression of LB1 in the frontal cortex of these animals ($t_{1,8} = 0.08$, $p > 0.05$) (Supplementary Figure S2c). Because both training paradigms markedly decreased LB1 expression in the CA1 area, we have adopted the former paradigm for the rest of the experiments.

Overexpression of LB1 Impairs Spatial Learning

Results from the above experiments demonstrate a negative relationship between spatial learning and LB1 expression in the hippocampal CA1 area. But these results do not reveal whether there is a casual relationship between these two. In this experiment, we examined the effect of LB1 overexpression on spatial learning. Animals were randomly divided into two groups to receive Flag-vector transfection or Flag-LB1 WT transfection in the CA1 area ($n = 10$–11 each group) and subjected to water maze learning. Results revealed that LB1 overexpression markedly impaired the acquisition performance ($F_{1,19} = 9.46$, $p < 0.01$) (Figure 2a). For the probe trial performance conducted one day later, animals receiving LB1 transfection spent less time in the target quadrant than did the control animals ($t_{1,19} = 2.06$, $p < 0.05$) (Figure 2b). Separate groups of animals received the same transfection as described above ($n = 7$ each) and were subjected to visible platform learning. Results indicated that their performance under visible platform learning is similar ($n = 7$ each group; $F_{1,12} = 0.33$, $p > 0.05$)

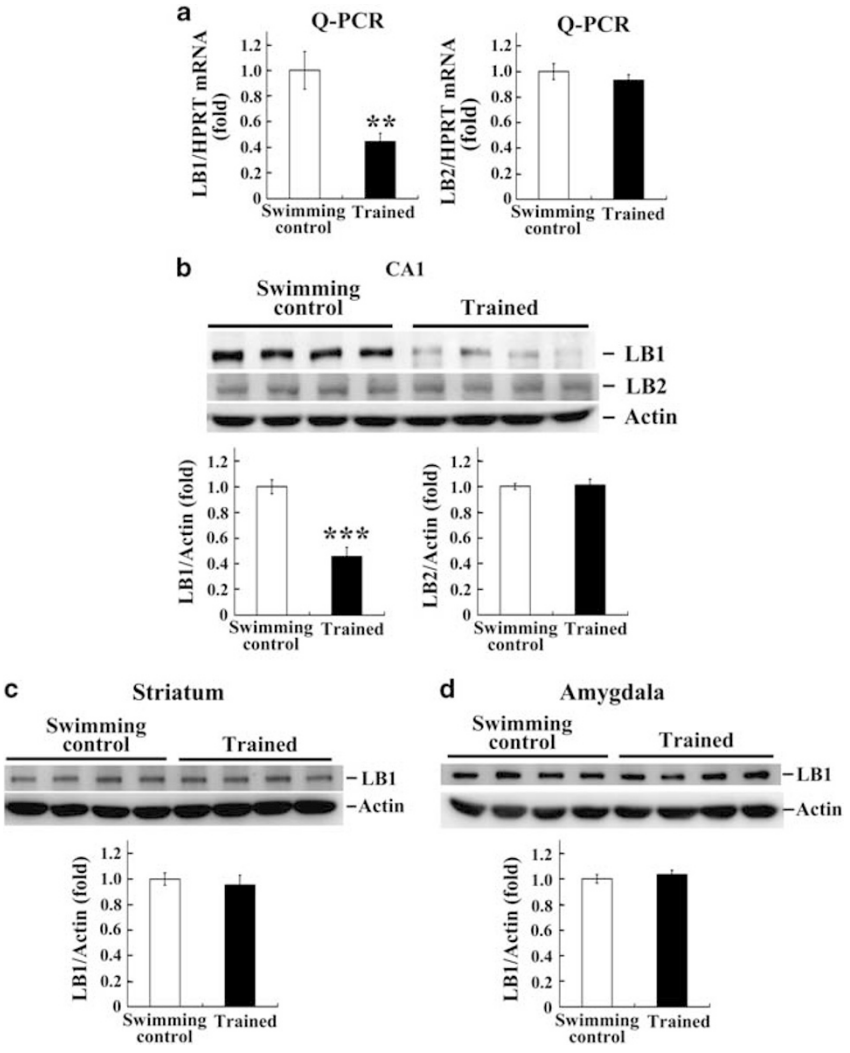

Figure 1 Spatial training decreases the expression of LB1, but not LB2, in the rat hippocampus. (a) Analysis of *laminin-β1* and *laminin-β2* mRNA levels in the swimming control animals and trained animals by Q-PCR. (b) A representative gel pattern and analysis of LB1 and LB2 protein levels in the CA1 area of the swimming control rats and trained rats by western blotting. (c) A representative gel pattern and analysis of LB1 protein level in the striatum from the swimming control rats and trained rats by western blotting. (d) A representative gel pattern and analysis of LB1 protein level in the amygdala from the swimming control rats and trained rats by western blotting ($n = 8$ in each group). The data are the mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$.

(Figure 2c). This result indicated that the animals' visual and motor functions were not altered by LB1 overexpression. To confirm the transfection and the expression of LB1WT plasmid in the CA1 area, an EGFP-tagged LB1WT plasmid was transfected to the CA1 area and DAPI was added to tissue sessions for identification of the nuclei. Immunohistochemistry was performed for visualization of fluorescence for EGFP (green) and DAPI (blue) (Figure 2d and e). The transfected area is approximately 21% of the total CA1 area viewed from a single plane (Figure 2d, upper left panel). Images at a higher magnification show the entire area of EGFP-LB1WT transfection from the most left to the most right tissue sessions (Figure 2e). The two white arrows indicate the range of LB1 plasmid transfection, which approximates 535 μm in length (Figure 2e). To estimate the transfection efficiency, we have counted the number of DAPI-positive cells that are surrounded by EGFP over that of total DAPI-positive cells in the CA1 area from Slide-2 to Slide-6 in Figure 2e. They are 146/166, 202/203, 164/164, 225/225, and 186/186 in order, which together yielded the averaged transfection efficiency of about 97.5%. This is adopted because LB1 is expressed in the extracellular space surrounding the cells (Figure 2f). The reason why we observed overlapping images of EGFP and DAPI in Figure 2d is because of the intense EGFP signal shown in these slides. On the other hand, animal receiving EGFP vector transfection (control group) only showed background EGFP fluorescence in the CA1 area (Figure 2g). Moreover, transfection and expression of LB1 in the CA1 area was further confirmed by immunoprecipitation of the Flag tag and immunoblotting of LB1 and Flag (Figure 2h).

LB1 Impairs Spatial Learning Associated with Decreased Phosphorylation Level of ERK/MAPK and SGK1

The above result demonstrated that overexpression of LB1 impairs spatial learning in rats. In this experiment, we further examined the downstream signaling that mediates

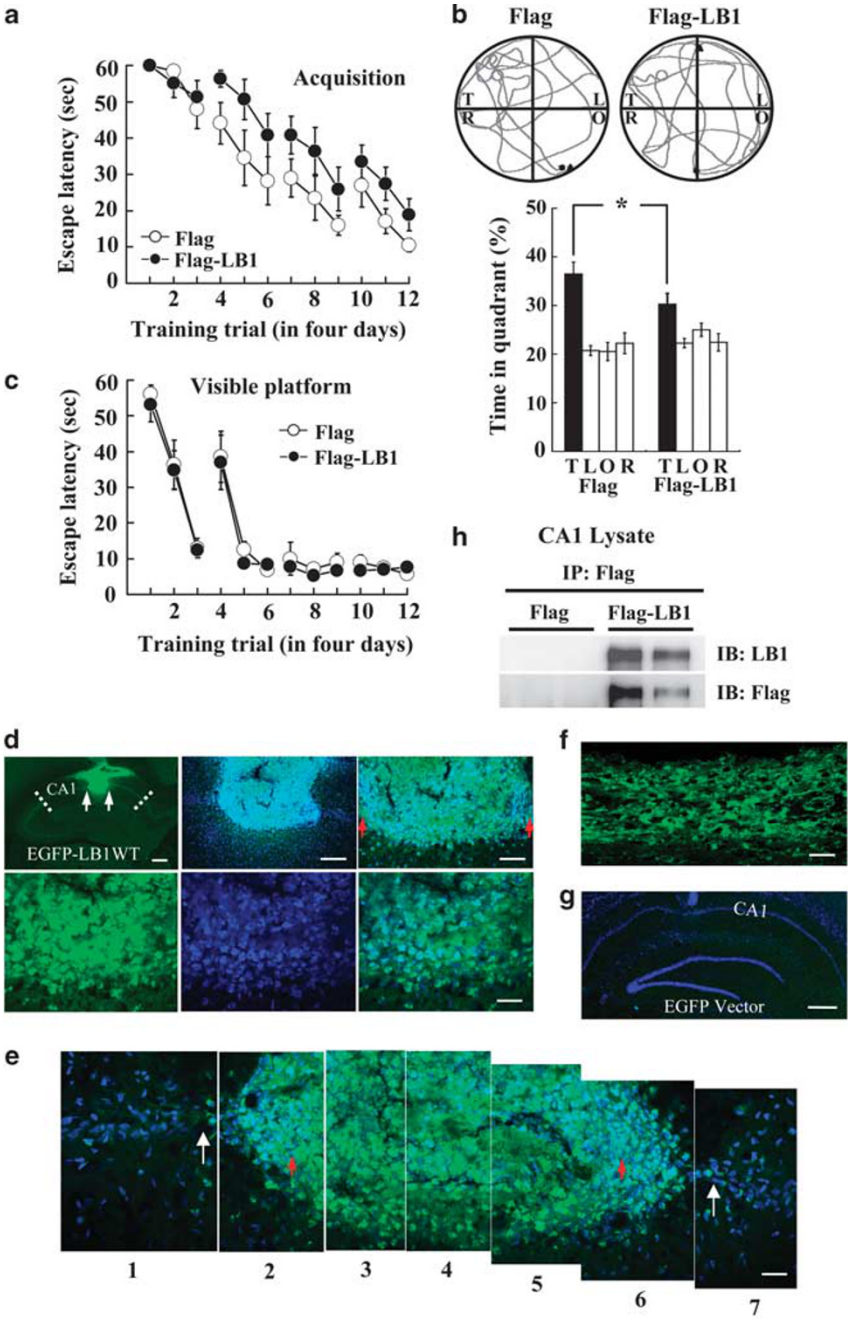

Figure 2 Overexpression of LB1 impairs spatial learning in rats. LB1 WT plasmid or Flag-vector was transfected to the rat CA1 area and the animals were subjected to (a) water-maze learning and (b) probe trial test. T, target quadrant; L, left quadrant; O, opposite quadrant; R, right quadrant; ●, start point, ▲, end point ($n = 10$–11 in each group). (c) The same transfection was made to different groups of rats and they were subjected to visible platform learning ($n = 7$ in each group). (d) Immunohistochemistry showing the area of EGFP-LB1WT transfection and the expression of LB1 in the CA1 cell layer at different magnifications. Cells that show both green fluorescence (EGFP) and blue fluorescence (DAPI) are cells successfully transfected with the plasmid. The dotted line indicates the CA1 area. The white arrows indicate the area of transfection and the red arrows are markers for visualization of enlarged images in panel e. The scale bars equal 400 μm for the upper-left image in panel d; 100 μm for the upper-middle image in panel d; 50 μm for the upper-right image in panel d; and 25 μm for the lower image in panel d. The white arrows in Slide-1 and Slide-7 in panel e correspond to the two white arrows seen in the upper-left image in panel d. (f) Additional immunohistochemistry of EGFP at a later stage after transfection shows that LB1 is present in the extracellular space only. The scale bar equals 25 μm. (g) Immunohistochemistry of control animals receiving EGFP vector transfection only was shown. The scale bar equals 400 μm. (h) Immunoprecipitation and western blot showing that Flag-LB1WT plasmid is indeed transfected and expressed in the CA1 area. The data are the mean ± SEM. *$p < 0.05$.

the effect of LB1 overexpression. ERK/MAPK was shown to have an essential role in learning and memory formation (Atkins *et al*, 1998; Adams and Sweatt, 2002), and we have demonstrated previously that protein kinase SGK1 also has an important role in spatial learning (Tsai *et al*, 2002). Further, ERK/MAPK was found to activate SGK1 in the rat hippocampus (Lee *et al*, 2006). Thus, we examined whether overexpression of LB1 alters the activation of ERK/MAPK

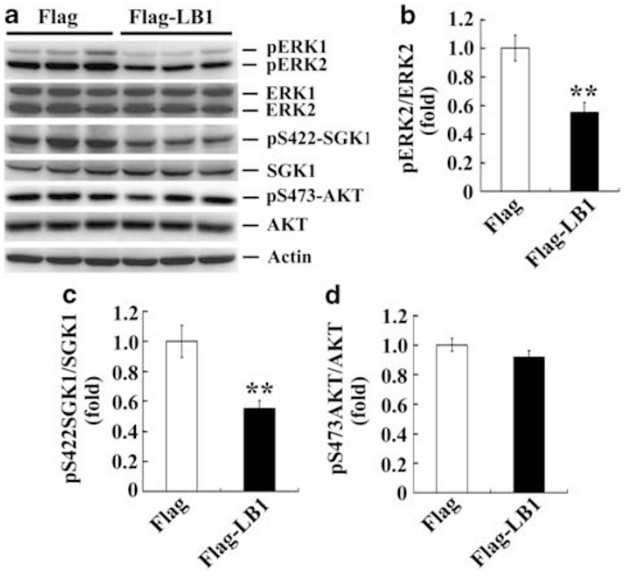

Figure 3 Overexpression of LB1 decreases the phosphorylation level of ERK and SGK1. (a) A representative gel pattern showing the protein level of ERK1/2, phospho-ERK1/2, SGK1, phospho-SGK1, AKT, and phospho-AKT in CA1 neurons after LB1WT transfection and after the probe trial test. Quantitative analyses showing the phosphorylation level of (b) ERK2 and (c) SGK1 at Ser-422, and (d) AKT at Ser-473 ($n=7$ in each group). The data are the mean ± SEM. $^{**}p<0.01$.

and SGK1. Animals were killed immediately after the probe trial test and their CA1 tissues were dissected out for western blot analysis of the phosphorylation level of ERK/MAPK and SGK1. The phosphorylation level of Akt, another protein kinase that shares 50% sequence homology to that of SGK1 (Kobayashi and Cohen, 1999), was also examined. A representative profile of the western blot is shown in Figure 3a. Further analyses indicated that overexpression of LB1 markedly decreased the phosphorylation level of ERK2 ($t_{1,12}=3.85$, $p<0.01$) (Figure 3b) and SGK1 ($t_{1,12}=3.74$, $p<0.01$) (Figure 3c), without altering the phosphorylation level of Akt ($t_{1,12}=1.8$, $p>0.05$) (Figure 3d). The protein level of ERK1/2, SGK1, and Akt was not altered (all $p>0.05$).

Knockdown of LB1 Expression Enhances Spatial Learning

Previous results demonstrate that overexpression of LB1 impairs spatial learning. In this experiment, we further examined the role of LB1 in spatial learning by knocking down endogenous LB1 expression. Animals were randomly divided into two groups to receive control siRNA (without Cy3) or Cy3-LB1 siRNA transfection to the CA1 area ($n=11$ each group) and were subjected to water maze learning. Results revealed that LB1 siRNA transfection markedly facilitated acquisition performance in rats ($F_{1,20}=19.12$, $p<0.001$) (Figure 4a). Analysis of probe trial performance indicated that the LB1 siRNA-transfected animals spent more time in the target quadrant than the control animals ($t_{1,20}=2.19$, $p<0.05$) (Figure 4b). Separate groups of animals received the same manipulations as described above ($n=8$ each group) and were subjected to visible platform learning. Results revealed that their acquisition performance under visible platform learning is not different ($F_{1,14}=0.02$, $p>0.05$) (Figure 4c). For immunohistochemistry, DAPI was added to tissue sections for identification of nuclei. Immunohistochemistry was performed for visualization of the fluorescence of Cy3 (red) and DAPI (blue). Results revealed that control siRNA transfection (without Cy3) only yielded background fluorescence in the CA1 area (Figure 4d, upper-left panel). But LB1 siRNA transfection showed specific fluorescence image in the CA1 neurons (other panels in Figure 4d). Serial tissue sections containing CA1 neurons revealed a total transfection area measuring approximately 628 μm in length, as indicated by the two arrows in Figure 4e. To estimate the transfection efficiency, we have counted the number of Cy3-positive neurons over that of DAPI-positive neurons from Slide-2 to Slide-6. They are 46/47, 44/44, 42/43, 45/46, and 46/55 in order, which yielded an averaged transfection efficiency of approximately 95.4% (Figure 4e). The effectiveness of LB1 siRNA transfection was further confirmed by a significant reduction of LB1 protein expression in the CA1 area ($t_{1,14}=4.85$, $p<0.001$) (Figure 4f).

Knockdown of LB1 Enhances Spatial Learning Associated with Increased Phosphorylation Level of ERK/MAPK and SGK1

The above results demonstrate that knockdown of LB1 expression facilitates spatial learning. Here, we examined the effect of LB1 siRNA transfection on the phosphorylation of ERK/MAPK and SGK1. A representative profile of the western blot is shown in Figure 5a. Further analyses indicated that LB1 siRNA transfection markedly increased the phosphorylation level of ERK2 ($t_{1,14}=5.26$, $p<0.001$) (Figure 5b) and SGK1 ($t_{1,14}=5.64$, $p<0.001$) (Figure 5c) without apparently altering their protein levels (both $p>0.05$). On the other hand, LB1 siRNA transfection did not alter the phosphorylation level of Akt ($t_{1,14}=0.24$, $p>0.05$) (Figure 5d) and its protein level ($p>0.05$). Because both overexpression of LB1 and knockdown of LB1 expression do not affect the expression and phosphorylation level of Akt, we no longer measure Akt and phospho-Akt levels in future experiments.

ERK/MAPK Activation Mediates the Enhancing Effect of LB1 siRNA on Spatial Learning

The above results revealed that LB1 negatively regulates spatial learning associated with decreased phosphorylation of ERK/MAPK. But it is not known whether decreased phosphorylation of ERK/MAPK actually mediates the impairing effect of LB1 on spatial learning. This hypothesis was tested here using the specific MEK inhibitor U0126. We first performed a dose–response study using U0126 on spatial learning. Animals were randomly divided into three groups ($n=5$ each group) to receive DMSO (12.5%), U0126 (1 μg/μl), and U0126 (2 μg/μl) infusions to the CA1 area. The animals were subjected to water maze learning and their CA1 tissues were dissected out for western blot analysis of the phosphorylation level of ERK1 and ERK2. Results revealed that there is an overall significant effect of U0126 treatment on spatial acquisition ($F_{2,12}=6.5$, $p<0.05$). Further analyses indicated that U0126 at 2 μg/μl

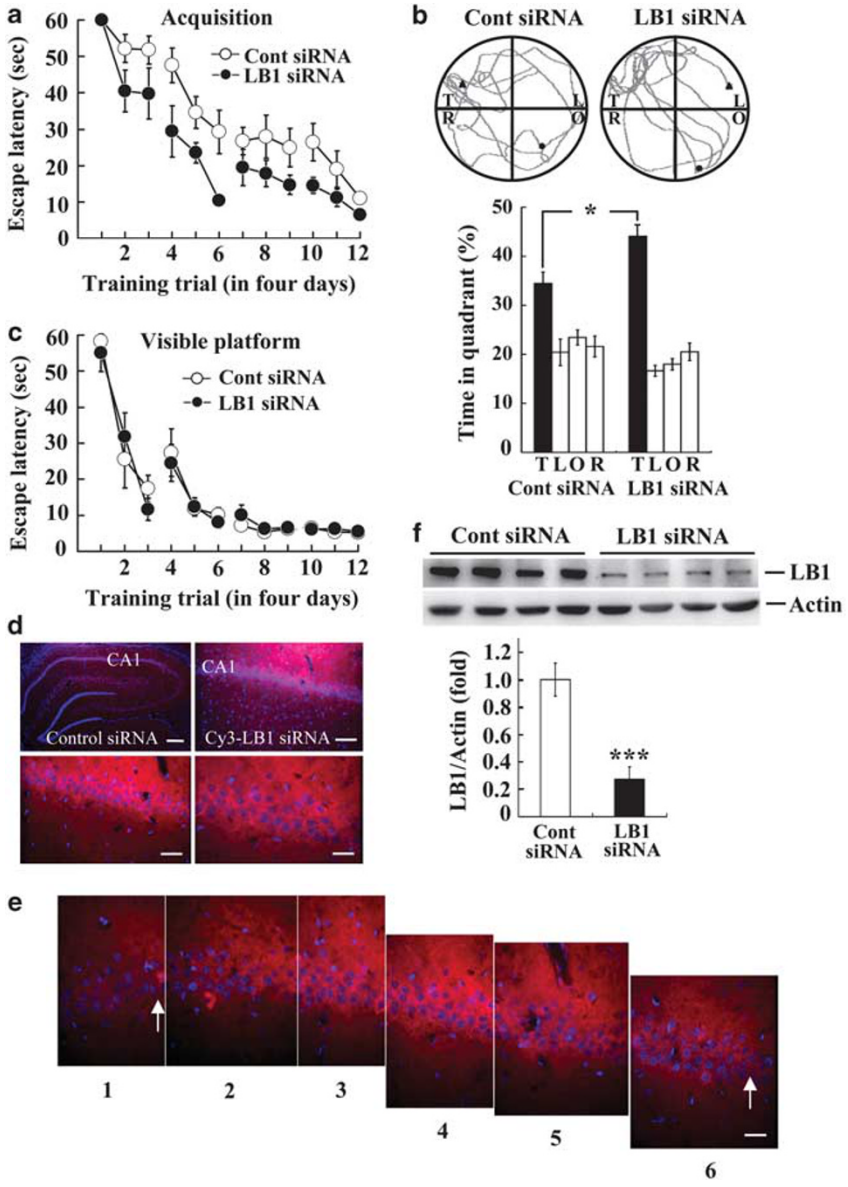

Figure 4 Transfection of LB1 siRNA facilitates spatial learning in rats. LB siRNA or a control siRNA was transfected to the CA1 area and the rats were subjected to (a) water-maze learning and (b) probe trial test. The quadrants are the same as in Figure 2 ($n = 11$ in each group). (c) The same transfection was made to different groups of rats and they were subjected to visible platform learning ($n = 8$ in each group). (d) Immunohistochemical staining against Cy3 and DAPI showing LB1 siRNA transfection to the CA1 area at different magnifications. Cells showing both red fluorescence (Cy3) and blue fluorescence (DAPI) are cells successfully transfected with the LB1 siRNA. Immunohistochemistry of control siRNA transfection was also shown. The scale bar equals 400 μm for the upper-left image; 100 μm for the upper-right image; 50 μm for the lower-left image; and 25 μm for the lower-right image. (e) Continuous tissue sessions showing the range of LB1 siRNA transfection in the CA1 area as indicated by the two arrows. The scale bars equal 25 μm. (f) A representative gel pattern and quantitative analysis showing LB1 protein level in the CA1 area after LB1 siRNA transfection and after the probe trial test ($n = 8$ in each group). The data are the mean ± SEM. $^{*}p < 0.05$ and $^{***}p < 0.001$. Cont, control.

significantly impaired spatial acquisition ($q = 4.95$, $p = 0.01$), but U0126 at 1 μg/μl did not have such an effect ($q = 1.17$, $p > 0.05$) (Figure 6a). Analysis of probe trial performance revealed a similar result. Animals receiving U0126 infusion at 2 μg/μl spent significantly less time in the target quadrant than the control animals ($F_{2,12} = 5.08$, $p < 0.05$; $q = 4.5$, $p < 0.05$), but U0126 at 1 μg/μl did not yield a significant effect ($q = 2.05$, $p > 0.05$) (Figure 6b). Because U0126 at 1 μg/μl did not significantly affect spatial acquisition and probe trial performance, visible platform learning was not performed. Further western blot analyses revealed that U0126 at 2 μg/μl markedly decreased the phosphorylation level of ERK1 ($F_{2,12} = 9.41$, $p < 0.01$; $q = 6.05$, $p < 0.01$) and ERK2 ($F_{2,12} = 8.07$, $p < 0.01$; $q = 5.67$, $p < 0.01$), but U0126 at 1 μg/μl did not have a significant effect on these measures ($q = 2.18$ and 2.54, both $p > 0.05$) (Figure 6c). These results are consistent with that of Shi and McGinty (2006) and Zhao *et al* (2010) that U0126 at 1 μg/μl does not affect the basal phosphorylation level of ERK1/2 and novel object recognition. We have therefore used 1 μg/μl U0126 for the following interaction study. Animals were randomly divided into three groups ($n = 8$

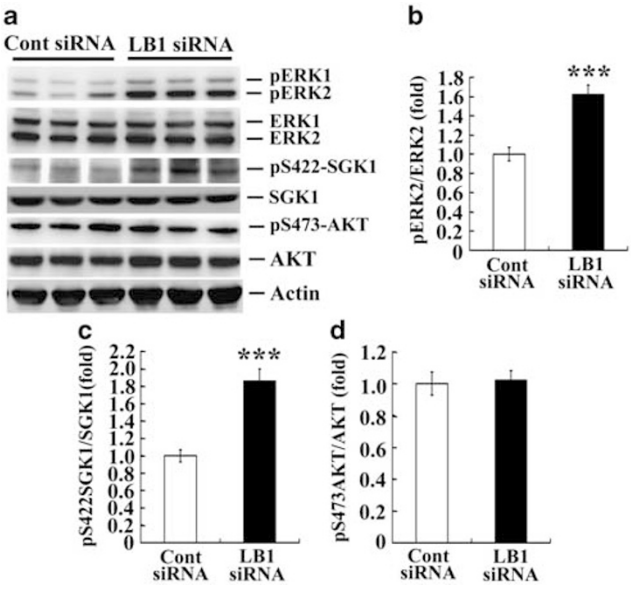

Figure 5 Transfection of LB1 siRNA increases the phosphorylation level of ERK and SGK1. (a) A representative gel pattern showing the protein level of ERK1/2, phospho-ERK1/2, SGK1, phospho-SGK1, AKT, and phospho-AKT in CA1 neurons after LB1 siRNA transfection and after the probe trial test. Quantitative analyses showing (b) the phosphorylation level of ERK2, (c) the phosphorylation level of SGK1 at Ser-422, and (d) AKT at Ser-473 ($n=8$ in each group). The data are the mean ± SEM. ***$p<0.001$.

each group) to receive control siRNA + DMSO, LB1 siRNA + DMSO, and LB1 siRNA + U0126 co-treatment to CA1 neurons. The animals were subjected to water maze learning and their CA1 tissues were dissected out for further western blot analysis. Results revealed that transfection of LB1 siRNA consistently enhanced acquisition performance in rats ($F_{2,21}=6.81$, $p<0.01$; $q=3.26$, $p<0.05$ comparing the LB1 siRNA + DMSO group with the control group), but co-administration of the MEK inhibitor U0126 antagonized the enhancing effect of LB1 siRNA on spatial learning ($q=5.11$, $p<0.01$ comparing the LB1 siRNA + DMSO group with the LB1 siRNA + U0126 group) (Figure 7a). Further probe trial analysis indicated that the LB1 siRNA-transfected animals spent more time in the target quadrant than the control animals ($F_{2,21}=11.59$, $p<0.01$; $q=3.72$, $p<0.05$ comparing the LB1 siRNA + DMSO group with the control group), but U0126 co-treatment blocked this effect of LB1 siRNA ($q=6.81$, $p<0.01$ comparing the LB1 siRNA + DMSO group with the LB1 siRNA + U0126 group) (Figure 7b). Their acquisition performance under visible platform learning was similar ($n=6$ each group; $F_{2,15}=0.44$, $p>0.05$) (Figure 7c). A representative profile of the western blot is shown in Figure 7d. Further analyses indicated that LB1 siRNA transfection markedly decreased LB1 protein level in both LB1 siRNA-transfected groups of rats ($F_{2,21}=9.83$, $p=0.001$; $q=4.61$ and 4.77, $p=0.01$ and $p<0.01$ for the LB1 siRNA + DMSO group and the LB1 siRNA + U0126 group, respectively) (Figure 7e). By contrast, LB1 siRNA transfection markedly increased the phosphorylation level of ERK2 ($F_{2,18}=8.23$, $p<0.01$; $q=5.54$, $p<0.01$ comparing the LB1 siRNA + DMSO group with the control group), but this effect was blocked by U0126 co-treatment ($q=4.41$, $p<0.01$ comparing the LB1 siRNA + DMSO group with the LB1 siRNA + U0126 group) (Figure 7e). Meanwhile, there is a parallel alteration of SGK1 phosphorylation at Ser-422 by LB1 siRNA ($F_{2,18}=27.72$, $p<0.001$; $q=9.9$, $p<0.001$ comparing the LB1 siRNA + DMSO group with the control group), but this effect was similarly blocked by U0126 co-treatment ($q=8.96$, $p<0.001$ comparing the LB1 siRNA + DMSO group with the LB1 siRNA + U0126 group) (Figure 7e).

SGK1 Activation Mediates the Enhancing Effect of LB1 siRNA on Spatial Learning

The above results revealed that LB1 negatively regulates spatial learning associated with decreased phosphorylation of SGK1 at Ser-422, and SGK1 activation seems to be a downstream event of ERK/MAPK activation. But these results do not reveal whether SGK1 inactivation indeed mediates the impairing effect of LB1 on spatial learning. This issue was examined here. We first performed a dose–response study using SGK1 siRNA on spatial learning. Animals were randomly divided into three groups ($n=6$ each group) to receive transfections of control siRNA, SGK1 siRNA at 4 pmol, and SGK1 siRNA at 8 pmol, and subjected to water maze learning. They were killed after the probe trial test and their CA1 tissues were dissected out for western blot analysis of SGK1 expression. Results revealed that there is an overall significant effect of SGK1 siRNA transfection on spatial acquisition ($F_{2,15}=7.24$, $p<0.01$). Further analyses revealed that SGK1 siRNA at 8 pmol significantly impaired spatial learning ($q=5.26$, $p<0.01$), but SGK1 siRNA at 4 pmol did not have a significant effect on this measure ($q=1.5$, $p>0.05$) (Figure 8a). The same results were observed for their probe trial performance. Animals receiving SGK1 siRNA transfection at 8 pmol spent significantly less time in the target quadrant than the control animals ($F_{2,15}=3.38$, $p>0.05$; $q=3.19$, $p<0.05$), but animals receiving SGK1 siRNA transfection at 4 pmol did not show such an effect ($q=0.07$, $p>0.05$) (Figure 8b). Because SGK1 siRNA at 4 pmol did not have a significant effect on spatial acquisition and probe trial performance, visible platform learning was not performed. Biochemical analyses revealed that there is an overall significant effect of SGK1 siRNA transfection on SGK1 expression ($F_{2,15}=12.36$, $p<0.001$). Further analyses indicated that SGK1 siRNA at 8 pmol markedly decreased the expression level of SGK1 ($q=6.86$, $p<0.001$), but SGK1 siRNA at 4 pmol did not significantly alter SGK1 expression ($q=2.09$, $p>0.05$) (Figure 8c). Because SGK1 siRNA at 4 pmol did not significantly affect spatial learning and SGK1 expression, we have used 4 pmol SGK1 siRNA for the following interaction study. Animals were randomly divided into three groups ($n=8$ each group) to receive control siRNA + control siRNA, LB1 siRNA + control siRNA, and LB1 siRNA + SGK1 siRNA co-transfection to CA1 neurons. The animals were subjected to water maze learning and their CA1 tissues were dissected out for further western blot analysis. Results revealed that transfection of LB1 siRNA consistently enhanced acquisition performance in rats ($F_{2,21}=12.18$, $p<0.001$; $q=2.97$, $p<0.05$ comparing the LB1 siRNA + control siRNA group with the control group), but co-transfection of SGK1 siRNA antagonized the enhancing effect of LB1 siRNA on spatial learning ($q=3.89$,

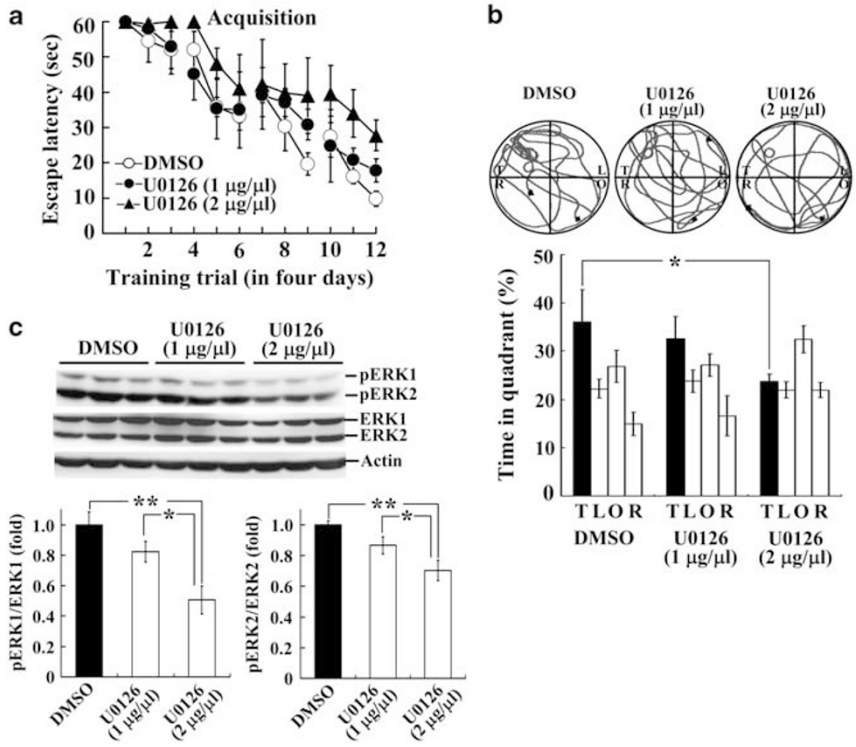

Figure 6 The dose–response effect of U0126 treatment on spatial learning in rats. DMSO (12.5%) or different concentrations of U0126 were injected to the rat CA1 area and the animals were subjected to (a) water-maze learning and (b) probe trial test. The quadrants are the same as in Figure 2 ($n = 5$ in each group). (c) A representative gel pattern and quantitative analysis of ERK1 and ERK2 phosphorylation level in the CA1 area after the probe trial test. The data are the mean ± SEM. *$p < 0.05$ and **$p < 0.01$.

$p = 0.001$ comparing the LB1 siRNA + control siRNA group with the LB1 siRNA + SGK1 siRNA group) (Figure 9a). Further probe trial analysis indicated that the LB1 siRNA-transfected animals spent more time in the target quadrant than the control animals ($F_{2,21} = 4.25$, $p < 0.05$; $q = 3.01$, $p < 0.05$ comparing the LB1 siRNA + control siRNA group with the control group), but SGK1 siRNA co-transfection blocked this effect of LB1 siRNA ($q = 3.95$, $p < 0.05$ when comparing the LB1 siRNA + control siRNA group with the LB1 siRNA + SGK1 siRNA group) (Figure 9b). Their acquisition performance under visible platform learning was similar ($n = 6$ each group; $F_{2,15} = 0.02$, $p > 0.05$) (Figure 9c). A representative gel pattern of the western blot is shown in Figure 9d. Further analyses indicated that LB1 siRNA transfection markedly decreased LB1 protein level in both LB1 siRNA-transfected groups of rats ($F_{2,21} = 21.69$, $p < 0.001$; $q = 8.25$, $p < 0.001$ and $q = 7.87$, $p < 0.001$ for the LB1 siRNA + control siRNA group and the LB1 siRNA + SGK1 siRNA group, respectively) (Figure 9e). In addition, SGK1 siRNA transfection markedly decreased SGK1 protein level ($F_{2,21} = 5.23$, $p < 0.05$; $q = 3.69$, $p < 0.05$ comparing the LB1 siRNA + SGK1 siRNA group with the LB1 siRNA + control siRNA group), but LB1 siRNA did not affect this measure ($q = 0.42$, $p > 0.05$ comparing the LB1 siRNA + control siRNA group with the control group) (Figure 9f). Meanwhile, LB1 siRNA transfection increased the phosphorylation level of SGK1 at Ser-422 ($F_{2,21} = 9.76$, $p < 0.001$; $q = 6.2$, $p < 0.001$ comparing the LB1 siRNA + control siRNA group with the control group), whereas this effect was partially, but significantly, blocked by SGK1 siRNA co-transfection ($q = 3.88$, $p = 0.01$ comparing the LB1 siRNA + SGK1 siRNA group with the LB1 siRNA + control siRNA group) (Figure 9f). On the other hand, LB1 siRNA transfection markedly increased the phosphorylation level of ERK2 ($F_{2,21} = 90.5$, $p < 0.001$; $q = 16.96$, $p < 0.001$ comparing the LB1 siRNA + control siRNA group with the control group), but this effect was not affected by SGK1 siRNA co-transfection ($q = 1.69$, $p > 0.05$ comparing the LB1 siRNA + SGK1 siRNA group with the LB1 siRNA group) (Figure 9f). This is probably because SGK1 is a downstream molecule of ERK/MAPK signaling. The protein levels of ERK1 and ERK2 were not altered (Figure 9d) (both $p > 0.05$).

DISCUSSION

In the present study, we have demonstrated that spatial training significantly decreased LB1 transcription and protein expression in hippocampal CA1 neurons. This is not due to differences of endogenous LB1 expression in different animals because we have shown that the LB1 expression level is similar in several randomly chosen naïve rats (Supplementary Figure S3a). One would also suspect that the stress level may be different in the trained and the swimming control animals, and stress may affect LB1 expression. But our results showed that there is no apparent difference in LB1 expression between control animals with handling and animals introduced into the water maze ($t_{1,8} = 1.39$, $p > 0.05$) (Supplementary Figure S3b). The swim speed of the swimming control animals and the trained animals were not different either ($t_{1,10} = 0.3$, $p > 0.05$) (Supplementary Figure S3c). To further test the possible

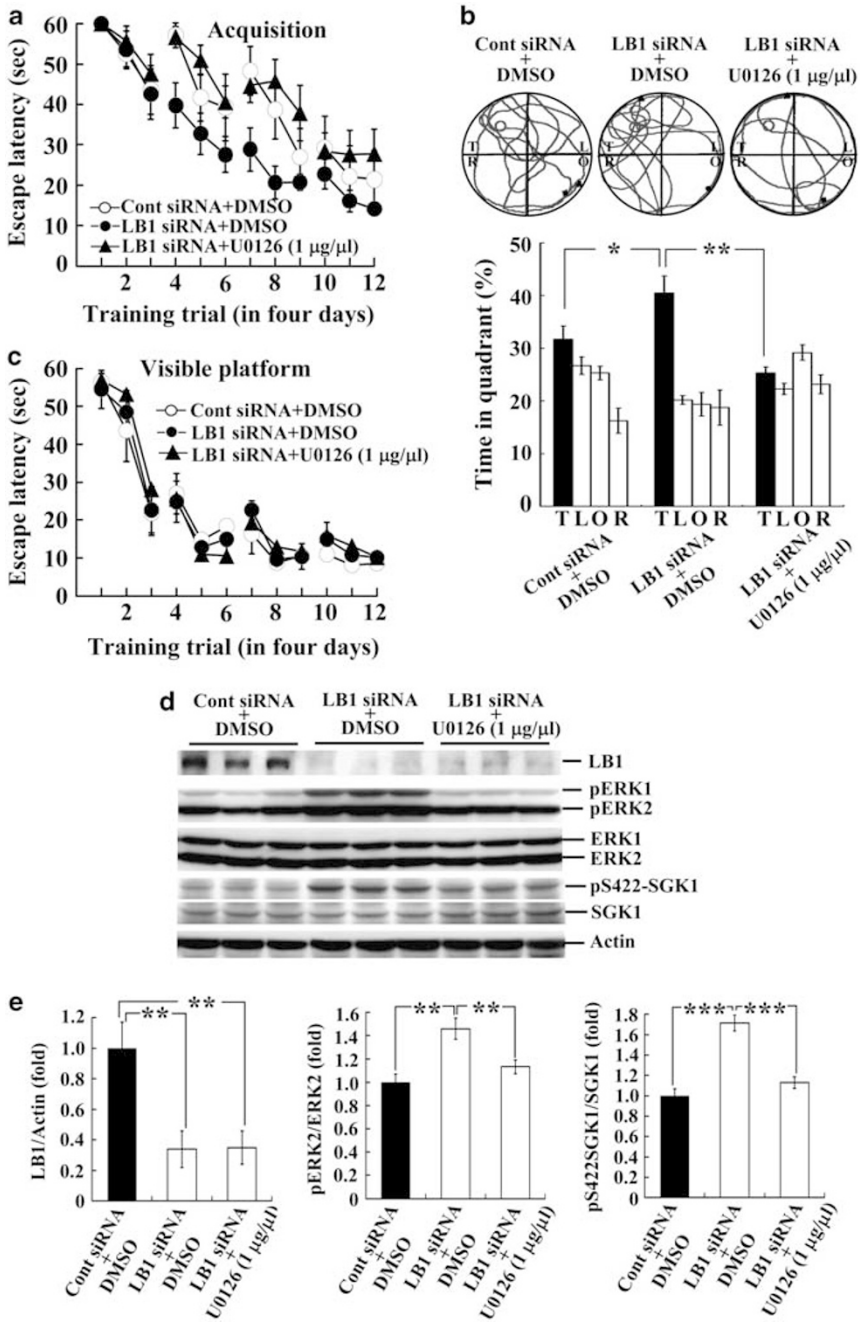

Figure 7 Infusion of U0126 (1 μg/μl) blocks the enhancing effect of LB1 siRNA on spatial learning. Control siRNA, LB1 siRNA, or LB1 siRNA together with U0126 infusion (1 μg/μl) was made to the rat CA1 area and the animals were subjected to (a) water-maze learning and (b) probe trial test. The quadrants are the same as that in Figure 2 ($n = 8$ in each group). (c) The same transfection/infusion was made to different groups of rats and they were subjected to visible platform learning. (d) A representative gel pattern showing the protein level of LB1, ERK1/2, phospho-ERK1/2, SGK1, and phospho-SGK1 in CA1 neurons after these transfections/infusions and after the probe trial test. (e) Quantitative analyses of LB1 protein level, the phosphorylation level of ERK2, and the phosphorylation level of SGK1 at Ser-422 ($n = 6$ in each group). The data are the mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

effect of stress on LB1 expression, some animals were subjected to foot shock stress whereas others stayed in the home cage. Results showed that foot shock stress did not alter LB1 expression in the CA1 neurons either ($t_{1,8} = 0.39$, $p > 0.05$) (Supplementary Figure S3d). These results together indicate that acute stress does not affect LB1 expression in the hippocampus. Further, overexpression of LB1 impairs spatial learning, whereas knockdown of LB1 expression facilitates spatial learning. These results demonstrate that LB1 negatively regulates spatial learning. These results are consistent with reports that the expression level of laminin-α1 and laminin-γ1 is higher in the frontal cortex of Alzheimer's disease patients (Palu and Liesi, 2002), and that the *laminin-β1* and *laminin-γ1* mRNA level is elevated in the brain of Alzheimer's disease patients compared with age-matched controls (Murtomaki *et al*, 1992). They are also congruent with the report that vascular dementia patients have a higher anti-LB1 immunoreactivity (Matsuda

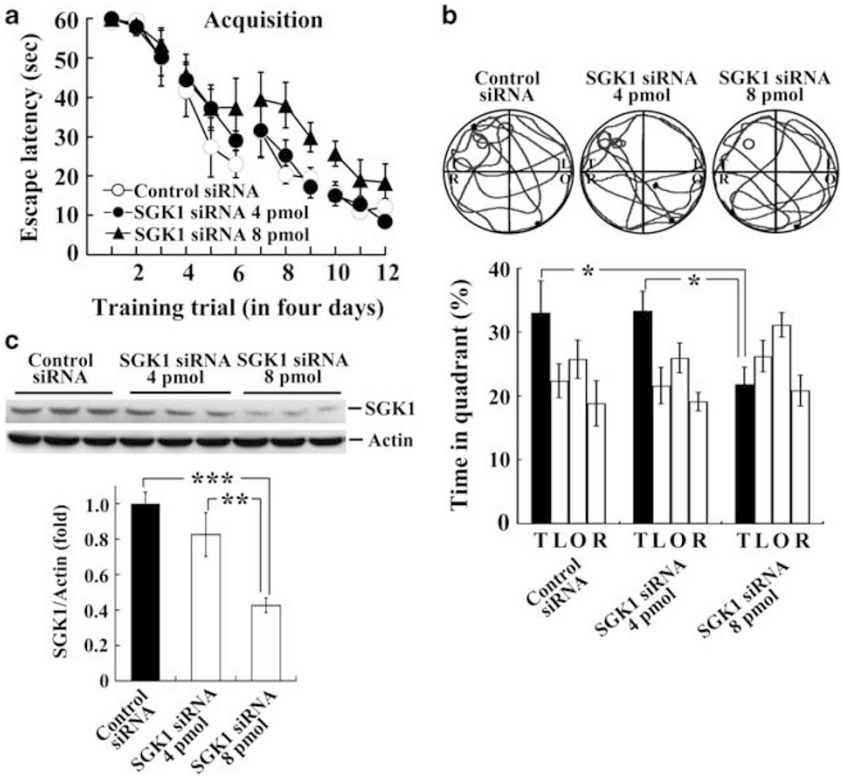

Figure 8 The dose–response effect of SGK1 siRNA transfection on spatial learning in rats. A control siRNA and different concentrations of SGK1 siRNA were transfected to the rat CA1 area and the rats were subjected to (a) water-maze learning and (b) probe trial test. The quadrants are the same as in Figure 2 ($n = 6$ in each group). (c) A representative gel pattern and quantitative analysis of SGK1 expression level in the CA1 area after the probe trial test. The data are the mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

et al, 2002). Because laminin expression is increased in the frontal cortex of Alzheimer's disease patients, we have also measured LB1 level in the frontal cortex of rats subjected to water maze training. Results revealed that, unlike that observed in the CA1 area (Supplementary Figure S2b), spatial training for 7 days did not alter the expression level of LB1 in the frontal cortex (Supplementary Figure S2c). This result does not necessarily mean that LB1 is not involved in memory storage because these animals were subjected to training for 7 days only, whereas it takes a longer time for memory storage to take place in the frontal cortex (Liang *et al*, 1996). Future study with longer time intervals, such as 21 days, is helpful to answer this question. In addition, the LB1WT-transfected animals showed a comparable within-session acquisition but poorer day-to-day retention compared with the control animals (Figure 2a). This suggests that LB1 primarily impairs the consolidation process. This speculation is supported by the observation that hippocampal LB1 expression is decreased after spatial training for 7 days (Supplementary Figure S2b), a time period long enough for memory consolidation to take place. Owing to the consideration that repeated injection (transfection) may cause tissue inflammation (it was only injected twice in the present study), the effects of LB1WT and LB1 siRNA transfection on memory consolidation were not examined in this study. But it would be important to investigate the role of LB1 and other laminin subunits on memory consolidation and memory storage in future studies. On the other hand, the present results are not consistent with the observation that laminin-1 degradation by plasmin impairs the maintenance of hippocampal LTP (Nakagami *et al*, 2000). The reason for this discrepancy is not known. It could be that different laminin proteins or different subunits of the laminin protein were examined in these two studies. Alternatively, plasmin also degrades other ECM proteins and proteoglycans in addition to laminin (Alexander and Werb, 1989), so the observed effect in this study could be a mixed result. Matrix metalloproteinase (MMP) is a family of enzymes that are able to cleave ECM and reconfigure ECM proteins (Woessner and Nagase, 2000). The activity of both MMP-3 and MMP-9 was shown to be associated with synaptic plasticity or learning and memory function. For example, inhibition of MMP-3 and MMP-9 in the hippocampus was shown to disrupt spatial learning and memory in rats (Meighan *et al*, 2006; Wright *et al*, 2007). MMP-9 is activated by inhibitory avoidance learning and is required for long-term memory (Nagy *et al*, 2007). Further, MMP-9 is activated upon LTP induction and LTP maintenance, and MMP-9-induced synaptic potentiation is mediated, in part, through integrin-β1 receptors (Wang *et al*, 2008). In another study, MMP-9 was found to control *N*-methyl-D-aspartate receptor surface diffusion also through mediation of integrin-β1 (Michaluk *et al*, 2009). Because laminin, along with other ECM proteins, was identified as a substrate of MMP-9 *in vivo* (Zamilpa *et al*, 2010), it is possible that MMP-9 may enhance synaptic potentiation and memory function through cleavage of laminin and, consequently, activation of integrin receptor signaling. This suggestion is supported by our finding that LB1 is associated with both integrin-α3 and integrin-β1 in

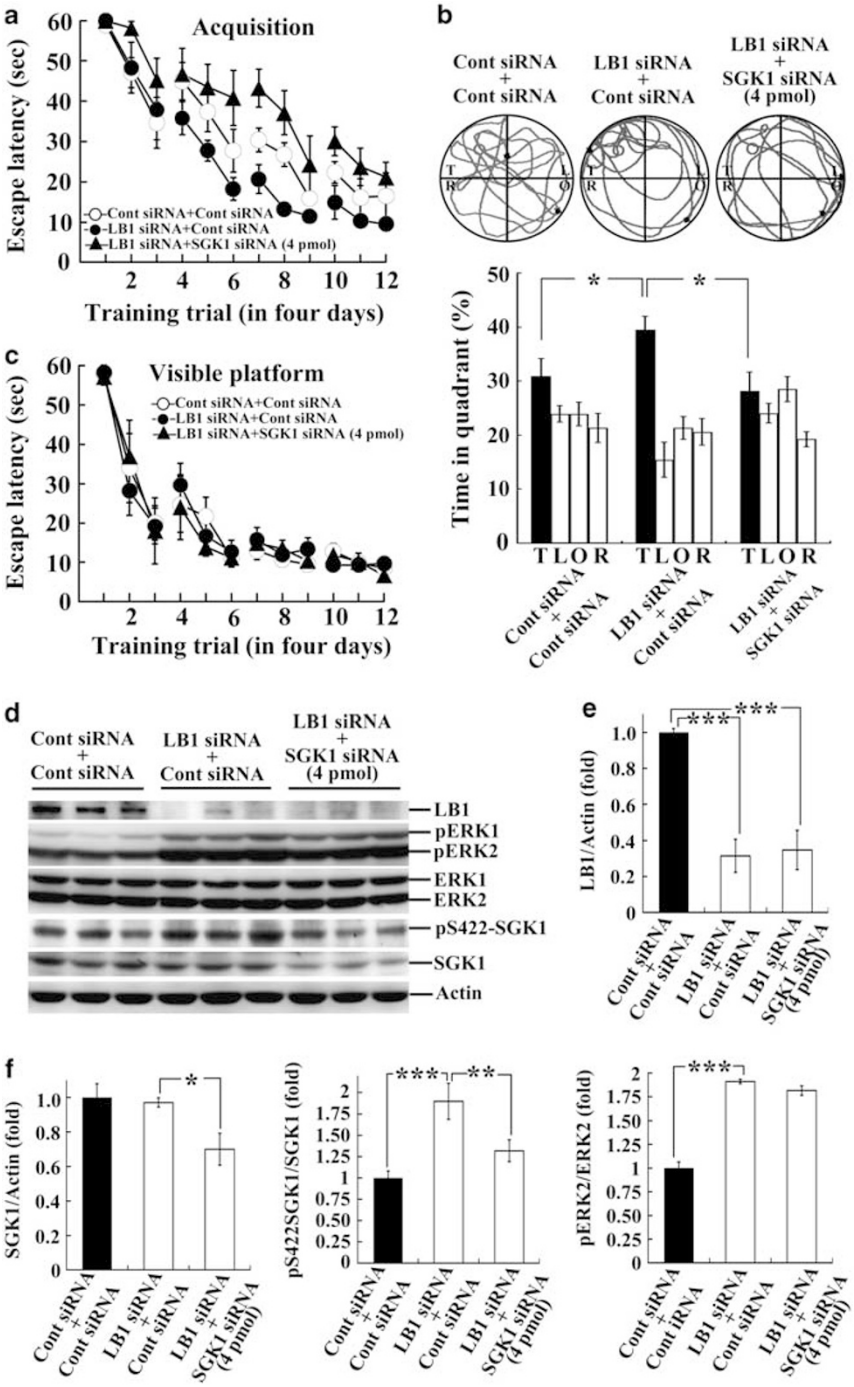

Figure 9 Transfection of SGK1 siRNA (4 pmol) blocks the enhancing effect of LB1 siRNA on spatial learning. Control siRNA, LB1 siRNA, or LB1 siRNA together with SGK1 siRNA (4 pmol) was transfected to the rat CA1 area and the animals were subjected to (a) water-maze learning and (b) probe trial test. The quadrants are the same as in Figure 2 ($n = 8$ in each group). (c) The same transfections were made to different groups of rats and they were subjected to visible platform learning ($n = 6$ in each group). (d) A representative gel pattern showing the protein level of LB1, SGK1, phospho-SGK1, and phospho-ERK2 in CA1 neurons after these transfections and after the probe trial test. (e) Quantitative analyses showing the protein level for LB1, (f) SGK1, the phosphorylation level of SGK1 at Ser-422, and ERK2 ($n = 8$ in each group). The data are expressed as in Figure 7. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

the rat hippocampus, and the association between LB1 and integrin-α3 is decreased after spatial training (Supplementary Figure S4). It will be interesting to examine whether spatial training may result in the cleavage of LB1 in future studies. The present results are also inconsistent with the finding that laminin is a plasticity gene involved in norepinephrine-mediated neuritogenesis in neuroblastoma cells (Laifenfeld *et al*, 2002). Whether this is also due to the different laminin subunits examined in these two studies is not known. On the other hand, although LB2 was also found to be expressed in the hippocampus (Egles *et al*, 2007), other than its well-documented role at the neuromuscular junction (Noakes *et al*, 1995), spatial training did not alter the expression of LB2. These results indicate the specific

relationship between downregulation of LB1 expression and spatial learning.

In examination of the signaling pathway that mediates the impairing effect of LB1 on spatial learning, we have found that LB1 overexpression decreased the phosphorylation level of ERK1/2 and SGK1, whereas knockdown of LB1 expression enhanced the phosphorylation level of ERK1/2 and SGK1. These results are consistent with reports that ERK1/2 activation and SGK1 activation both have an important role in facilitating spatial learning and memory formation (Atkins *et al*, 1998; Adams and Sweatt, 2002; Tsai *et al*, 2002). They are also congruent with the finding that ERK1/2 directly phosphorylates SGK1 *in vitro* and ERK1/2 increases the phosphorylation of SGK1 at Ser-422 in the hippocampus (Lee *et al*, 2006). In looking for the downstream molecule of SGK1 that may mediate the impairing effect of LB1 on spatial learning, we think STAT1 could be a possible candidate. This is based on the report that laminin could activate STAT1 during macrophage maturation (Coccia *et al*, 1999) and STAT1 inhibition by the protein inhibitor of activated STAT1 facilitates spatial learning (Tai *et al*, 2011). Besides, laminin-111, together with prolactin, was found to sustain STAT5 activation and STAT5-mediated β-casein expression in epithelial cells (Xu *et al*, 2010). Whether laminin also regulates STAT5 activity in the hippocampus and whether laminin may also regulate other transcription factors to negatively regulate spatial learning requires further investigation.

Integrin was shown to be a receptor type for LB1. Laminins could mediate different physiological functions through interaction with different subunits of the integrin receptor. For example, laminin-5 regulates the adhesion of epithelial cell through interaction with integrin-α6β4, whereas laminin-5 regulates the proliferation of human epithelial cell through interaction with integrin-α3β1 (Gonzales *et al*, 1999). We wonder whether the interaction between LB1 and integrin may also have a role in mediating the impairing effect of LB1 on spatial learning. Our preliminary results revealed that spatial training apparently increased the expression of integrin-α3 in the hippocampal neurons at the same time that it decreased the expression of LB1. But expression of integrin-β1 was not altered (Supplementary Figure S4a). Further, there is an obvious decrease in the association between LB1 and integrin-α3 (but not between LB1 and integrin-β1) after spatial training (Supplementary Figure S4b). This probably resulted from the decreased expression of LB1 following training. These results are consistent with reports that integrin facilitates synaptic plasticity and spatial memory in mice (Chan *et al*, 2003), and that the integrin-associated protein enhances the memory retention of inhibitory avoidance learning in rats (Huang *et al*, 1998). They are also consistent with the findings that integrin-α3 is involved in the consolidation of LTP (Kramar *et al*, 2002). In addition to integrin, there are also non-integrin type of laminin receptors in the brain, such as the cellular prion protein (PrPc) (Graner *et al*, 2000) and α-dystroglycan (McDearmon *et al*, 2006). Further, PrPc–laminin interaction was shown to have a role in neuritogenesis in PC12 cells and memory consolidation in rats (Graner *et al*, 2000; Coitinho *et al*, 2006). Whether there is also a decreased association between LB1 and PrPc after spatial training requires further investigation.

In the present study, transfection was made only to a limited area in CA1 neurons, but significant behavioral and biochemical changes were observed. Similar results were found in other studies that transfection of plasmid DNA/siRNA to approximately the same percentage of amygdala neurons and CA1 neurons affects fear conditioning and spatial learning in mice and rats, respectively (Han *et al*, 2007; Tai *et al*, 2011). Further, selective depletion of those amygdala neurons with overexpression blocks that fear memory (Han *et al*, 2009). Although the exact mechanism of communication between the transfected neurons and untransfected neurons is not known, this result suggests that activation of a subpopulation of neurons and their neuronal activity is sufficient to mediate the behavioral changes. In addition, the protein extraction method we used is suitable for extraction of proteins in the cell, but there are more fibers than cells in the punched area. When biochemical assays were performed, total amount of proteins instead of total tissue volume was used as a criterion. This reasonably explains why significant biochemical changes were observed from the punched tissue. To know whether LB1 plasmid and LB1 siRNA transfection to the CA1 area may also affect LB1 expression and its downstream biochemical changes in other regions of the hippocampus, we have measured LB1 expression and the phosphorylation level of ERK1/2, SGK1, and Akt in the rest of the hippocampus excluding CA1 and have found that both treatments did not significantly alter these measures (Supplementary Figure S5). These results indicate that LB1 plasmid and LB1 siRNA transfections to the CA1 area preferentially affect LB1 expression, ERK1/2, and SGK1 phosphorylation in this area. But given the interconnectivity of the hippocampus and the difficulty of dissecting the hippocampal tissue into small sub-regions for biochemical determination, indirect changes in LB1 expression, ERK1/2, and SGK1 phosphorylation in other regions of the hippocampus cannot be excluded. In this study, we have shown that spatial training decreased the expression of LB1 in the hippocampal CA1 area. But how LB1 is being regulated upon spatial training is not known. According to the promoter analysis, the *laminin-β1* gene contains several binding elements, such as GAS, AP1, and RARE (Okano *et al*, 1992; Vasios *et al*, 1989). Whether *laminin-β1* transcription is regulated by the corresponding transcription factors of these elements waits to be studied. In addition, the signaling pathway that mediates the downregulation of LB1 expression upon spatial training also requires further examination. Moreover, based on reports that laminin is implicated in antidepressant action, it would be as important to investigate the role of LB1 involved in fear memory formation.

Previous reports have shown that chronic stress decreased laminin-γ1 expression in the rat brain and that laminin-γ1 expression is decreased in depression patients, whereas antidepressant treatment reverses these effects (Laifenfeld *et al*, 2005a, b). These results seem to indicate that the cognitive function of these patients is enhanced, but antidepressant treatment would have a deteriorating effect. This is a complicated issue and a simple relationship between these two cannot be established. First, different laminin subunits were examined in these studies (laminin-γ1) and in our study (LB1). Different laminin subunits may have a different role in cognitive function. For example, we

have demonstrated that spatial training does not alter LB2 expression. The role of laminin-γ1, and perhaps laminin-α1, involved in learning and memory function should also be examined in the future. Second, laminin-γ1 expression was decreased in the parietal–occipital cortex of schizophrenia patients, whereas laminin-γ1 expression was increased in the prefrontal cortex of schizophrenia patients (Laifenfeld *et al*, 2005b). These findings indicate that a compensation mechanism may exist for the expression of laminin under a disorder state that makes the relationship between antidepressant treatment and cognitive performance more complicated. Third, although chronic stress decreases laminin-γ1 expression whereas antidepressant increases laminin-γ1 expression in rat hippocampus (Laifenfeld *et al*, 2005a), chronic stress was found to impair various learning and memory performance in rats, including water maze learning (Sandi, 2004). Whether chronic stress increased LB1 expression and whether antidepressant reverses this effect should be examined in the future. Examination of the expression level of various laminin subunits in depression patients following learning as compared with that in depression patients not subject to learning is as important. In addition, study of the effect of antidepressant on cognitive function in depression patients associated with alternations of their laminin subunit expression should also be helpful in answering this question.

In summary, laminin is an ECM protein that has an important role in neuronal differentiation and migration. In some studies, laminin is implicated in depression, schizophrenia, and Alzheimer's disease. The expression of laminin is abundant in the adult hippocampus and cortex, but its role involved in learning and memory function was not known. In the present study, we have demonstrated that overexpression of LB1 impairs spatial learning, whereas knockdown of endogenous LB1 expression enhanced spatial learning. Further, decreased phosphorylation of ERK/MAPK and protein kinase SGK1 mediates the impairing effect of LB1 on spatial learning. The present study reveals a novel role and mechanism of LB1 in the central nervous system.

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DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

Abdallah B, Hassan A, Benoist C, Goula D, Behr JP, Demeneix BA (1996). A powerful nonviral vector for *in vivo* gene transfer into the adult mammalian brain: polyethylenimine. *Hum Gene Ther* **7**: 1947–1954.

Adams JP, Sweatt JD (2002). Molecular psychology: roles for the ERK MAP kinase cascade in memory. *Annu Rev Pharmacol Toxicol* **42**: 135–163.

Alexander CM, Werb Z (1989). Proteinases and extracellular matrix remodeling. *Curr Opin Cell Biol* **1**: 974–982.

Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, Sweatt JD (1998). The MAPK cascade is required for mammalian associative learning. *Nat Neurosci* **1**: 602–609.

Bruckner G, Hausen D, Hartig W, Drlicek M, Arendt T, Brauer K (1999). Cortical areas abundant in extracellular matrix chondroitin sulphate proteoglycans are less affected by cytoskeletal changes in Alzheimer's disease. *Neuroscience* **92**: 791–805.

Bukalo O, Schachner M, Dityatev A (2001). Modification of extracellular matrix by enzymatic removal of chondroitin sulfate and by lack of tenascin-R differentially affects several forms of synaptic plasticity in the hippocampus. *Neuroscience* **104**: 359–369.

Chan CS, Weeber EJ, Kurup S, Sweatt JD, Davis RL (2003). Integrin requirement for hippocampal synaptic plasticity and spatial memory. *J Neurosci* **23**: 7107–7116.

Chao CC, Ma YL, Lee EHY (2011). BDNF enhances Bcl-xL expression through protein kinase CK2-activated and NF-κB-mediated pathway in rat hippocampus. *Brain Pathol* **21**: 150–162.

Coccia EM, Del Russo N, Stellacci E, Testa U, Marziali G, Battistini A (1999). STAT1 activation during monocyte to macrophage maturation: role of adhesion molecules. *Int Immunol* **11**: 1075–1083.

Coitinho AS, Freitas ARO, Lopes MH, Hajj GNM, Roesler R, Walz R *et al* (2006). The interaction between prion protein and laminin modulates memory consolidation. *Eur J Neurosci* **24**: 3255–3264.

Colognato H, Yurchenco PD (2000). Form and function: the laminin family of heterotrimers. *Dev Dyn* **218**: 213–234.

Dityatev A, Schachner M (2003). Extracellular matrix molecules and synaptic plasticity. *Nat Rev* **4**: 456–468.

Easley CA, Faison MO, Kirsch TL, Lee JA, Seward ME, Tombes RM (2006). Laminin activates CaMK-II to stabilize nascent embryonic axons. *Brain Res* **1092**: 59–68.

Egles C, Claudepierre T, Manglapus MK, Champliaud MF, Brunken WJ, Hunter DD (2007). Laminins containing the beta2 chain modulate the precise organization of CNS synapses. *Mol Cell Neurosci* **34**: 288–298.

Ekblom P, Lonai P, Talts JF (2003). Expression and biological role of laminin-1. *Matrix Biol* **22**: 35–47.

Gonzales M, Haan K, Baker SE, Fitchmun M, Todorov I, Weitzman S *et al* (1999). A cell signal pathway involving laminin-5, alpha3beta1 integrin, and mitogen-activated protein kinase can regulate epithelial cell proliferation. *Mol Biol Cell* **10**: 259–270.

Graner E, Mercadante AF, Zanata SM, Forlenza OV, Cabral ALB, Veiga SS *et al* (2000). Cellular prion protein binds laminin and mediates neuritogenesis. *Mol Brain Res* **76**: 85–92.

Hagg T, Muir D, Engvall E, Varon S, Manthorpe M (1989). Laminin-like antigen in rat CNS neurons: distribution and changes upon brain injury and nerve growth factor treatment. *Neuron* **3**: 721–732.

Han JH, Kushner SA, Yiu AP, Cole CJ, Matynia A, Brown RA *et al* (2007). Neuronal competition and selection during memory formation. *Science* **316**: 457–460.

Han JH, Kushner SA, Yiu AP, Hsiang HL, Buch T, Waisman A *et al* (2009). Selective erasure of a fear memory. *Science* **323**: 1492–1495.

Hsu WL, Chiu TH, Tai DJ, Ma YL, Lee EH (2009). A novel defense mechanism that is activated on amyloid-beta insult to mediate cell survival: role of SGK1–STAT1/STAT2 signaling. *Cell Death Differ* **16**: 1515–1529.

Huang AM, Wang HL, Tang YP, Lee EH (1998). Expression of integrin-associated protein gene associated with memory formation in rats. *J Neurosci* **18**: 4305–4313.

Indyk JA, Chen ZL, Tsirka SE, Strickland S (2003). Laminin chain expression suggests that laminin-10 is a major isoform in the mouse hippocampus and is degraded by the tissue plasminogen activator/plasmin protease cascade during excitotoxic injury. *Neuroscience* **116**: 359–371.

Kobayashi T, Cohen P (1999). Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphotidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. *Biochem J* **339**: 319–328.

Kramar EA, Bernard JA, Gall CM, Lynch G (2002). Alpha3 integrin receptors contribute to the consolidation of long-term potentiation. *Neuroscience* **110**: 29–39.

Laifenfeld D, Karry R, Grauer E, Klein E, Ben-Shachar D (2005a). Antidepressants and prolonged stress in rats modulate CAM-L1, laminin, and pCREB, implicated in neuronal plasticity. *Neurobiol Dis* **20**: 432–441.

Laifenfeld D, Karry R, Klein E, Ben-Shachar D (2005b). Alterations in cell adhesion molecule L1 and functionally related genes in major depression: a postmortem study. *Biol Psychiatry* **57**: 716–725.

Laifenfeld D, Klein E, Ben-Shachar D (2002). Norepinephrine alters the expression of genes involved in neuronal sprouting and differentiation: relevance for major depression and antidepressant mechanisms. *J Neurochem* **83**: 1054–1064.

Lee CT, Tyan SW, Ma YL, Tsai MC, Yang YC, Lee EHY (2006). Serum- and glucocorticoid-inducible kinase (SGK) is a target of the MAPK/ERK signaling pathway that mediates memory formation in rats. *Eur J Neurosci* **23**: 1311–1320.

Liang KC, Hu SJ, Chang SC (1996). Formation and retrieval of inhibitory avoidance memory: differential roles of glutamate receptors in the amygdala and medial prefrontal cortex. *Chin J Physiol* **39**: 155–166.

Ma YL, Tsai MC, Hsu WL, Lee EHY (2006). SGK protein kinase facilitates the expression of long-term potentiation in hippocampal neurons. *Learn Mem* **13**: 114–118.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* **20**: 9104–9110.

Matsuda K, Tashiro K, Hayashi Y, Monji A, Yoshida I, Mitsuyama Y (2002). Measurement of laminins in the cerebrospinal fluid obtained from patients with Alzheimer's disease and vascular dementia using a modified enzyme-linked immunosorbent assay. *Dement Geriatr Cogn Disord* **14**: 113–122.

McDearmon EL, Combs AC, Sekiguchi K, Fujiwara H, Ervasti JM (2006). Brain a-dystroglycan displays unique glycoepitopes and preferential binding to laminin-10/11. *FEBS Lett* **580**: 3381–3385.

Meighan SE, Meighan PC, Choudhury P, Davis CJ, Olson ML, Zornes PA *et al* (2006). Effects of extracellular matrix-degrading proteases matrix metalloproteinases 3 and 9 on spatial learning and synaptic plasticity. *J Neurochem* **96**: 1227–1241.

Michaluk P, Mikasova L, Groc L, Frischknecht R, Choquet D, Kaczmarek L (2009). Matrix metalloproteinase-9 controls NMDA receptor surface diffusion through integrin b1 signaling. *J Neurosci* **29**: 6007–6012.

Murtomaki S, Risteli J, Risteli L, Koivisto UM, Johansson S, Liesi P (1992). Laminin and its neurite outgrowth-promoting domain in the brain in Alzheimer's disease and Down's syndrome patients. *J Neurosci Res* **32**: 261–273.

Nagy V, Bozdagi O, Huntley GW (2007). The extracellular protease matrix metalloproteinase-9 is activated by inhibitory avoidance learning and required for long-term memory. *Learn Mem* **14**: 655–664.

Nakagami Y, Abe K, Nishiyama N, Matsuki N (2000). Laminin degradation by plasmin regulates long-term potentiation. *J Neurosci* **20**: 2003–2010.

Noakes PG, Gautam M, Mudd J, Sanes JR, Merlie JP (1995). Aberrant differentiation of neuromuscular junctions in mice lacking s-laminin/laminin beta 2. *Nature* **374**: 258–262.

Okano R, Mita T, Matsui T (1992). Characterization of a novel promoter structure and its transcriptional regulation of the murine laminin β1 gene. *Biochim Biophys Acta* **1132**: 49–57.

Palu E, Liesi P (2002). Differential distribution of laminins in Alzheimer disease and normal human brain tissue. *J Neurosci Res* **69**: 243–256.

Sandi C (2004). Stress, cognitive impairment and cell adhesion molecules. *Nat Rev* **5**: 917–930.

Sanes JR (1989). Extracellular matrix molecules that influence neural development. *Ann Rev Neurosci* **12**: 491–516.

Sharif KA, Baker H, Gudas LJ (2004). Differential regulation of laminin β1 transgene expression in the neonatal and adult mouse brain. *Neuroscience* **126**: 967–978.

Shi X, McGinty JF (2006). Extracellular signal-regulated mitogen-activated protein kinase inhibitors decrease amphetamine-induced behavior and neuropeptide gene expression. *Neuroscience* **138**: 1289–1298.

Sobel RA, Ahmed AS (2001). White matter extracellular matrix chondroitin sulfate/dermatan sulfate proteoglycans in multiple sclerosis. *J Neuropathol Exp Neurol* **60**: 1198–1207.

Tai DJC, Hsu WL, Liu YC, Ma YL, Lee EHY (2011). Novel role and mechanism of protein inhibitor of activated STAT1 in spatial learning. *EMBO J* **30**: 205–220.

Timpl R (1996). Macromolecular organization of basement membranes. *Curr Opin Cell Biol* **8**: 618–624.

Tsai KJ, Chen SK, Ma YL, Hsu WL, Lee EH (2002). *Sgk*, a primary glucocorticoid-induced gene, facilitates memory consolidation of spatial learning in rats. *Proc Natl Acad Sci USA* **99**: 3990–3995.

Tunggal P, Smyth N, Paulsson M, Ott MC (2000). Laminins: structure and genetic regulation. *Micros Res Tech* **51**: 214–227.

Vasios GW, Gold JD, Petkovich M, Chambon P, Gudas LJ (1989). A retinoic acid-responsive element is present in the 5′ flanking region of the laminin β1 gene. *Proc Natl Acad Sci USA* **86**: 9099–9103.

Wang XB, Bozdagi O, Nikitczuk JS, Zhai ZW, Zhou Q, Huntley GW (2008). Extracellular proteolysis by matrix metalloproteinase-9 drives dendritic spine enlargement and long-term potentiation coordinately. *Proc Natl Acad Sci USA* **105**: 19520–19525.

Woessner JF, Nagase H (2000). *Matrix Metalloproteinases and TIMPs*. Oxford UP: Oxford, New York.

Wright JW, Brown TE, Harding JW (2007). Inhibition of hippocampal matrix metalloproteinase-3 and -9 disrupts spatial memory. *Neural Plast* **2007**: 73813–73820.

Xu R, Spencer VA, Groesser DL, Bissell MJ (2010). Laminin regulates PI3K basal localization and activation to sustain STAT5 activation. *Cell Cycle* **9**: 4315–4322.

Yang YC, Lin CH, Lee EH (2006). Serum- and glucocorticoid-inducible kinase 1 (SGK1) increases neurite formation through microtubule depolymerization by SGK1 and by SGK1 phosphorylation of tau. *Mol Cell Biol* **26**: 8357–8370.

Zamilpa R, Lopez EF, Chiao YA, Dai Q, Escobar GP, Hakala K *et al* (2010). Proteomic analysis identifies *in vivo* candidate matrix metalloproteinase-9 substrates in the left ventricle post-myocardial infarction. *Proteomics* **10**: 2214–2223.

Zhang HT, Zhao Y, Huang Y, Dorairaj NR, Chandler LJ, O′Donnell JM (2004). Inhibition of the phosphodiesterase 4 (PDE4) enzyme reverses memory deficits produced by infusion of the MEK inhibitor U0126 into the CA1 subregion of the rat hippocampus. *Neuropsychopharmacology* **29**: 1432–1439.

Zhao Z, Fan L, Frick KM (2010). Epigenetic alterations regulate estradiol-induced enhancement of memory consolidation. *Proc Natl Acad Sci USA* **107**: 5605–5610.

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