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ORIGINAL ARTICLE

Brain-derived neurotrophic factor (BDNF) induces antagonistic action to Nogo signaling by the upregulation of lateral olfactory tract usher substance (LOTUS) expression

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Abstract

Neurons in the central nervous system (CNS) have limited capacity for axonal regeneration after trauma and neurological disorders due to an endogenous nonpermissive environment for axon regrowth in the CNS. Lateral olfactory tract usher substance (LOTUS) contributes to axonal tract formation in the developing brain and axonal regeneration in the adult brain as an endogenous Nogo receptor-1 (NgR1) antagonist. However, how LOTUS expression is regulated remains unclarified. This study examined molecular mechanism of regulation in LOTUS expression and found that brain-derived neurotrophic factor (BDNF) increased LOTUS expression in cultured hippocampal neurons. Exogenous application of BDNF increased LOTUS expression at both mRNA and protein levels in a dose-dependent manner. We also found that pharmacological inhibition with K252a and gene knockdown by siRNA of tropomyosin-related kinase B (TrkB), BDNF receptor suppressed BDNF-induced increase in LOTUS expression. Further pharmacological analysis of the TrkB signaling pathway revealed that BDNF increased LOTUS expression through mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) cascades, but not phospholipase C-γ (PLCγ) cascade. Additionally, treatment with c-AMP response element binding protein (CREB) inhibitor partially suppressed BDNF-induced LOTUS expression. Finally, neurite outgrowth assay in cultured hippocampal neurons revealed that BDNF treatment-induced antagonism for NgR1 by up-regulating LOTUS expression. These findings suggest that BDNF may acts as a positive regulator of LOTUS expression through the TrkB signaling, thereby inducing an antagonistic action for NgR1 function by up-regulating LOTUS expression. Also, BDNF may synergistically affect axon regrowth through the upregulation of LOTUS expression.

KEYWORDS
axon regrowth, brain-derived neurotrophic factor (BDNF), lateral olfactory tract usher substance (LOTUS), nogo receptor-1 (NgR1), tropomyosin-related kinase B (TrkB)

Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BLyS, B lymphocyte stimulator; BSA, bovine serum albumin; CNS, central nervous system; CREB, c-AMP response element binding protein; CSPGs, chondroitin sulfate proteoglycans; DIV, days in vitro; hiPSC-NS/PC, human induced pluripotent stem cell-derived neural stem/progenitor cell; HRP, horseradish peroxidase; LOTUS, lateral olfactory tract usher substance; MAG, myelin-associated glycoprotein; MAP2, microtubule-associated protein 2; MAPK, mitogen-activated protein kinase; mRNA, messenger ribonucleic acid; NgR1, nogo receptor-1; OMgp, oligodendrocyte myelin glycoprotein; p75NTR, p75 neurotrophin receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PFA, paraformaldehyde; PI3K, phosphatidylinositol 3-kinase; PIR-B, paired immunoglobulin receptor-B; PLCγ, phospholipase C-γ; PLL, poly-l-lysine; RhoA, ras homolog family membrane A; RRID, research resource identifier; RT, room temperature; SCI, spinal cord injury; SEM, standard error of the mean; siRNA, small interfering ribonucleic acid; TBS, tris-buffered saline; TrkB, tropomyosin-related kinase B.

1 | INTRODUCTION

Neuronal regeneration capacity after traumatic injury and neurodegenerative diseases in an adult mammalian central nervous system (CNS) is remarkably limited. In order to regain functional restoration, it is necessary to protect the surviving neural circuit and form a new functional neural circuit (He & Jin, 2016; Varadarajan et al., 2022). However, several inhibitory molecules derived from myelin and glial cells after injury prevent the restoration of the CNS environment (Varadarajan et al., 2022; Yiu & He, 2006). Nogo receptor-1 (NgR1) has been identified as the major obstacle to neuronal regeneration (Fournier et al., 2001; Fujita & Yamashita, 2014; Schwab & Strittmatter, 2014). The interaction of Nogo receptor-1 (NgR1) with its ligands, such as Nogo (Fournier et al., 2001), myelin-associated glycoprotein (MAG) (Domeniconi et al., 2002), oligodendrocyte myelin glycoprotein (OMgp) (Wang et al., 2002), B lymphocyte stimulator (BLyS) (Zhang et al., 2009) and chondroitin sulfate proteoglycans (CSPGs) (Dickendesher et al., 2012) leads to growth cone collapse and neurite outgrowth inhibition through the activation of Ras homolog family membrane A (RhoA) (Fournier et al., 2003; Fujita & Yamashita, 2014; Yiu & He, 2006), thereby failing CNS regeneration. Therefore, inhibiting NgR1 function is a promising therapeutic target for neuronal regrowth and functional restoration after CNS injury.

Lateral olfactory tract usher substance (LOTUS) has been identified as a neural circuit formation factor of the lateral olfactory tract in the developing brain and as an endogenous antagonist of Nogo receptor-1 (Sato et al., 2011). Previous studies show that LOTUS suppresses NgR1-mediated growth cone collapse and neurite outgrowth inhibition induced by all of the five NgR1-ligands (Nogo-A, MAG, OMgp, BLyS, and CSPGs) (Kawakami, Kurihara, et al., 2018; Kawakami, Saito, et al., 2018; Kurihara et al., 2014, 2017; Sato et al., 2011). LOTUS was also reported to suppress axonal growth inhibition by blocking the binding of Nogo to paired immunoglobulin receptor-B (PIR-B) (Kurihara et al., 2020), which plays an essential role during developmental and adult CNS in one of the neuronal inhibitory signals (Atwal et al., 2008). We have also shown that genetic over-expression of LOTUS in the CNS enhanced significant functional recovery after spinal cord injury (SCI) (Hirokawa et al., 2017; Ito et al., 2018; Ito et al., 2021), brain ischemia (Takase et al., 2017) and unilateral pyramidotomy (Ueno et al., 2020). Furthermore, we have recently reported that LOTUS enhances synapse formation and memory function (Nishida et al., 2021). Thus, LOTUS functions as a potent neuronal regeneration agent, and LOTUS is, therefore, expected to be useful for future therapy of CNS damage through inhibition of NgR1 and PIR-B functions. However, the level of LOTUS expression drastically decreases after CNS injury. For example, LOTUS expression in the injured spinal cord drastically decreases, and down-regulation of LOTUS delays the level of motor recovery (Hirokawa et al., 2017). Additionally, the cerebrospinal fluid concentration of LOTUS negatively correlates with the morbidity of neuroinflammatory diseases, such as multiple sclerosis and meningitis (Takahashi et al., 2015, 2018). Moreover, the expression level of LOTUS in rat hippocampus was reported to decline along with aging and correlate with age-related cognitive impairment (Starkey et al., 2013). Altogether, the decrease in LOTUS expression can be considered as one of the main causes of the delay in CNS regeneration and plasticity. However, how LOTUS expression is regulated and which substance regulates LOTUS expression remains to be elucidated. Therefore, we first employed a molecular screen for changes in LOTUS expression in cultured hippocampal neurons. Among the many candidate substances, we found brain-derived neurotrophic factor (BDNF) as a strong candidate for an enhancer of LOTUS expression (data not shown).

BDNF is a member of major and potent neurotrophins in the CNS. BDNF is constructed by 247 amino acids polypeptide precursor and synthesized as the mature BDNF, secreted extracellularly (Brigadski & Leβmann, 2014; Mowla et al., 2001). Mature BDNF binds mainly to tropomyosin-related kinase receptor type B (TrkB) with high affinity and p75 neurotrophin receptor (p75NTR) with low affinity (Chao, 2003). The binding of BDNF to TrkB is essential for maintaining cell survival, neural development, and synaptic plasticity (Park & Poo, 2013). Previous studies have demonstrated neuroprotective functions of BDNF–TrkB interaction: BDNF–TrkB contributes to enhancing axonal regeneration (Nagahara & Tuszynski, 2011); delivering BDNF mRNA nanomicelles to the injury site restores motor function after SCI (Crowley et al., 2019); lentivirus-assisted transduction of TrkB enhances corticospinal regeneration (HollisII et al., 2008); BDNF rescues the dysfunction of memory formation in the model of Alzheimer's disease (Nagahara et al., 2009; Pins et al., 2019). In contrast, genetic knockdown of BDNF or TrkB diminished spontaneous recovery of the corticospinal tract function after brain injury (Ueno et al., 2012). Therefore, BDNF functions mainly with TrkB and plays an essential role in CNS regeneration and neuroprotection, and also offers therapeutic potential for restoring CNS functions. Interestingly, the expression level of BDNF decreases after SCI and with aging (Erickson et al., 2010; Miranda et al., 2019; Sada et al., 2020; Ueno et al., 2012). Additionally, the expression levels of LOTUS and BDNF increase after stimulation of kainic acid in mice (Karlsson et al., 2017). Moreover, a recent study reported that BDNF is upregulated in LOTUS-expressing human-induced pluripotent stem cell-derived neural stem/progenitor cells (hiPSC-NS/PC) (Ito et al., 2021). However, the precise molecular mechanism of how LOTUS expression is regulated by BDNF has not been investigated.

Here, we focused on BDNF and examined the effect of BDNF on LOTUS expression level. This study showed that BDNF increases the expression level of LOTUS in cultured hippocampal neurons through the TrkB signaling pathway. Moreover, BDNF-induced increase in LOTUS expression suppresses neurite outgrowth inhibition induced by Nogo. Our data indicate that BDNF acts as a positive regulator of LOTUS expression, suggesting BDNF may synergistically affect axon regrowth through the upregulation of LOTUS expression.

2 | MATERIALS AND METHODS

2.1 | Animals and ethical standards

C57BL/6J Wild-type mice (RRID: IMSR_JAX:000664) were purchased from Japan SLC. The *lotus* mutant mice were generated as previously described (Sato et al., 2011; Hirokawa *et al*., 2017). In brief, the first exon of *lotus* was replaced with a Neomycin selection cassette. LOTUS mutants were assessed in C57BL/6 background. These mice were housed (one to five mice in each rectangular plastic cages) in a specific-pathogen-free facility under 12 h/12 h light/dark conditions with ad libitum access to autoclaved water and food. Genotype of the mutant mice and their offspring were confirmed with polymerase chain reaction (PCR) using the following primer sequences to target the intronic region of the mouse *lotus* locus (forward: 5'-TAGCTCTTCTCCCGGGAAGC-3′; reverse: 5'-CTTGCACCCATCCCAGAAGG-3′) or the genomic region containing the *neomycin* gene (forward, 5'-GGATTCATCGACTGTGGCCG-3′). A total of 52 pregnant mice were used to obtain the E17.5 embryos (wild-type, 47; *lotus* mutant mice, 5). Six-11 embryos were obtained from each pregnant mice and used to implement primary-dissociated cultures of hippocampal neurons.

Throughout the experimental procedures, all efforts were made to minimize the number of animals used and their suffering. Isoflurane (cat. No. 099-06571, Wako Pure Chemical Industries) anesthesia was used in sacrificing all pregnant mice. The mice were placed in isoflurane vapor-filled container until confirming the death. Surgically extracted embryos were immediately placed on ice and sacrificed by decapitation to obtain the brains for primary-dissociated cultures of hippocampal neurons. The experimental procedures were approved by the institutional animal care and use ethical committee of Yokohama City University (approval number #T-A-21-002).

2.2 | Antibodies and reagents

Hamster monoclonal antibodies against mouse LOTUS (named H24G11-mAb, RRID: AB_2819119) were generated as described previously (Sato et al., 2011). Additionally, mouse monoclonal antibodies against rat LOTUS (ITM, RRID: AB_2819118), rabbit monoclonal antibodies against TrkB (cat. no. ab187041, Abcam, AB_2892613), mouse monoclonal antibodies against β-actin (cat. no. A5316, Sigma-Aldrich, RRID: AB_476743), rabbit polyclonal antibodies against rat tubulin β-3 (cat. no. 802001, Biolegend, RRID: AB_2564645), rabbit polyclonal antibodies against human MAP2 (cat. no. 4542, Cell Signaling Technology, RRID: 10693728), Alexa Fluor 488-labeled Goat antibodies against rabbit IgG (cat. no. 111-545-003, Jackson ImmunoResearch, RRID: AB_2338046), Alexa Fluor 594-labeled goat antibodies against hamster IgG (cat. no. 127-585-099, Jackson ImmunoResearch, RRID: AB_2338998), Horseradish peroxidase (HRP)-labeled goat antibodies against mouse IgG (cat. no. 115-035-003, Jackson ImmunoResearch, RRID: AB_10015289), HRP-labeled goat antibodies against rabbit IgG (cat. no. 111-035-003, Jackson ImmunoResearch, RRID: AB_2313567), Recombinant BDNF (cat. no. 450-02, Peprotech), K252a (cat. no. K1639, Sigma-Aldrich), Recombinant Mouse IgG2A Fc (cat. no. 4460-MG, R&D Systems), Recombinant Mouse TrkB Fc Chimera (cat. no. 10266-TB, R&D Systems), PD98059 (cat. no. 10006726, Cayman Chemical), LY-294002 Hydrochloride (cat. no. L96002, LKT Laboratories), U-73122 (cat. No. 662035, Merck Millipore), CREB inhibitor 666-15 (cat. no. 538341, Merck Millipore), and Rat Nogo66-Fc (cat. no. 3728-NG, R&D Systems) were obtained commercially.

2.3 | Cell lysate preparation

The cultured primary hippocampal neurons from E17.5 mouse embryos were placed on ice for 10 min and washed twice with cold phosphate-buffered saline (PBS). The cells were lysed with lysis buffer containing 20 mM Tris–HCl pH 8.0, 150 mM NaCl, 10 mM NaF, 1 mM EDTA-Na pH 8.0, 1% Nonidet P-40, 1 mM Na_3VO_4 pH 10.0 (Sigma Aldrich), 0.05 mM (*p*-amidinophenyl) methanesulfonyl fluoride (Wako), and 0.1 U/ml aprotinin (Sigma-Aldrich). The lysate was centrifuged at 20400 g for 10 min at 4°C, and the supernatant was mixed with 4 × Laemmli buffer (40% glycerol, 8% SDS, 250 mM Tris–HCl pH 6.8, 0.03% bromophenol blue) containing 10% β-mercaptoethanol, boiled for 7 min, and stored at −80°C until use.

2.4 | Western blotting

The samples were loaded onto 8% acrylamide gels and separated with SDS-PAGE. The protein was transferred onto polyvinylidene difluoride membrane (Immobilon-P Transfer Membrane, cat. no. IPVH00010, Merck Millipore) by semidry blotting for 1 h. The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween20 (TBS-T) for 1 h at 22°C and incubated with primary antibody against LOTUS (0.425 μg/μl, custom-made, ITM, RRID: AB_2819118), TrkB (0.167 μg/μl, cat. no. ab187041, Abcam, AB_2892613), or β-actin (1/10000 dilution, cat. no. A5316, Sigma-Aldrich, RRID: AB_476743) in TBS-T containing 5% bovine serum albumin (BSA) or 1% skim milk for overnight at 4°C. After the membrane was washed with TBS-T three times, the membrane was incubated with HRP-labeled goat antibodies against mouse IgG (0.08 μg/μl or 0.16 μg/μl, cat. no. 115-035-003, Jackson ImmunoResearch, RRID: AB_10015289), or HRP-labeled goat antibodies against rabbit IgG (0.16 μg/μl, cat. no. 111-035-003, Jackson ImmunoResearch, RRID: AB_2313567) for 1 h at 22°C. The membrane was incubated with ECL Western Blotting Detection Reagents (cat. no. RPN2109, GE Healthcare Life Sciences), Immobilon Western Chemiluminescent HRP Substrate (cat. no. WBKLS0100, Merck Millipore), or Western blot Ultra-Sensitive HRP Substrate (cat. no. T7104A, Takara) at 22°C and the chemiluminescent signals were detected with ImageQuant LAS 4000 mini (GE Healthcare Life Sciences) using ImageQuant TL software (GE Healthcare Life Sciences, RRID: SCR_014246).

2.5 | RNA isolation and Real-time PCR

Total RNA was isolated from the primary hippocampal neurons using NucleoSpin RNA Plus (cat. no. 740984.50, MACHREY-NAGAL). The isolated total RNAs were reverse-transcribed to complementary DNA with PrimeScript RT Master Mix (Perfect Real Time) (cat. no. RR036A, Takara Bio). Expression levels of mRNA encoding *Lotus* and *β-actin* were determined by real-time PCR using TB Green Premix Ex TaqII (Tli RNaseH Plus) (cat. no. RR820S, Takara) on a Thermal Cycler Dice Real Time System (Takara Bio). Relative mRNA expression level was calculated using the ΔΔCt method. The expression level of LOTUS mRNA was normalized against the expression of β-actin mRNA. The synthesized cDNAs were amplified using the following specific primer sequences: Mouse *Lotus* forward primer: 5′-ATGGCTCCGAGCGCTGACCCGGGC, reverse primer: 5′-CTCCATAGTTGAGCTGGGTGG-3′; Mouse *β-actin* forward primer: 5′-CATCCGTAAAGACCTCTATGCCAAC-3′, reverse primer: 5′-ATGGAGCCACCGATCCACA-3′.

2.6 | Primary culture

The primary-dissociated cultures of hippocampal neurons from E17.5 mouse embryos were performed as previously reported (Nishida et al., 2021). In brief, a mean value of 8 brains were pooled per cell culture from pregnant mice and embryonal hippocampus were dissected in ice-cold Hank's balanced salt solution (HBSS). Cells were dissociated with 0.25% trypsin (15090-046, Gibco) at 37°C for 12 min. After gentle pipetting, cells were further incubated using 100 μg/ml DNaseI and equivalent amount of fetal bovine serum at 37°C for 5 min. Dispersed cells were seeded at the density of 2.5×10^4 cells/well on the glass coverslips (φ12 mm, cat. no. C012001, Matsunami glass), which were placed in a cell culture multi-well plate (cat. no. 170740, Thermo Fisher Scientific) and coated with poly-L-lysine (PLL) (100 μg/ml, cat. no. 163-19 091, Wako) or 5×10^5 cells/well on six-well plate (cat. no. 657160, Greiner Bio-One) which were coated with Polyethyleneimine (0.001%, cat. no. P3143, Sigma Aldrich), and cultured in Neurobasal medium (cat. no. 21103-049, Gibco) containing 10% fetal bovine serum (cat. no. S1400, Biowest) and 10 mM Hepes-NaOH pH 7.3 for 3 h to confirm that almost all the cells were attached on the bottom of the culture plate. Then, the medium was exchanged for neurobasal medium 2% B-27 supplement (cat. no. 17504044, Gibco), 10 mM Hepes-NaOH pH 7.3, and 2 mM Glutamax (cat. no. 35050061, Gibco).

Purchased recombinant BDNF was added into the culture medium at 100 ng/ml on days *in vitro* (DIV) 1 and incubated for 48 h or DIV 6 and incubated for 24 h at 37°C with 5% CO_2. K252a, recombinant TrkB-Fc, PD98059, LY-294002, U-73122, or CREB inhibitor 666-15 were added at 200 nM, 2 μg/ml, 100, 50, 10 or 500 nM, respectively, into the culture medium 30 min before the BDNF treatment.

2.7 | Transfection of small interfering RNA (siRNA)

The 27mer dicer-substrate small interfering RNAs (DsiRNAs) to target TrkB or negative control were obtained from Integrated DNA Technologies and chemically synthesized the following sequences: Non-targeting negative control sense: 5′-CGUUAAUCGCGUAUAA UACGCGUAT-3′, anti-sense: 5′-AUACGCGUAUUAUACGCGAUUAA CGAC-3′; TrkB sense: 5′-GGCUUACAAAGCGUUUCUGAAAAAC-3′, anti-sense: 5′-GUUUUUCAGAAACGCUUUGUAAGCCAC-3′.

At DIV 4, Lipofectamine RNAiMAX transfection reagent (Invitrogen) was used in transfecting the cultured primary hippocampal neurons on 6-well plates with 40 nM of negative control or TrkB siRNA based on the manufacturer's instructions. The transfected cells were treated with BDNF at 100 ng/ml on DIV 6 and further incubated for 24 h at 37°C with 5% CO_2.

2.8 | Immunocytochemistry

The primary-cultured hippocampal neurons were treated with primary antibodies for LOTUS (1 μg/ml, H24G11-mAb, RRID: AB_2819119) in the culture medium for 1 h at 37°C with 5% CO_2 and fixed with warmed 4% paraformaldehyde (PFA) in the culture medium for 10 min at 37°C followed by 10 min at 22°C. After blocking with 5% Normal goat serum in TBS-T for 30 min at 22°C, an anti-tubulin β3 antibody (1/2000, cat. no. 802001, Biolegend, RRID: AB_2564645) was applied, and cells were incubated for 1 h at 22°C. After washing with TBS-T three times, Alexa Fluor 488-labeled Goat antibodies against rabbit IgG (cat. no. 111-545-003, Jackson ImmunoResearch, RRID: AB_2338046) and Alexa Fluor 594-labeled goat antibodies against hamster IgG (cat. no. 127-585-099, Jackson ImmunoResearch, RRID: AB_2338998) were applied, and cells were incubated for 1 h at 22°C in the dark. After washing with TBS-T three times, the cells were loaded with Prolong Diamond Antifade Mountant (cat. no. P36965, Thermo Fisher Scientific), and was mounted on a slide glass (cat. no. FRC-04, Matsunami). Fluorescent images were obtained using a TCS SP8 microscope (Leica Microsystems) equipped with a 63× oil-immersion objective lens and LAS X software (Leica Microsystems, RRID: SCR_013673).

2.9 | Neurite outgrowth assay

The primary-cultured hippocampal neurons were obtained from E17.5 wild-type, or *lotus*-deficient mice were seeded (2.5×10^4 cells/well) on the PLL-coated glass coverslips and cultured in neurobasal medium 2% B-27 supplement (cat. no. 17504044, Gibco), 10 mM Hepes-NaOH pH 7.3 and 2 mM Glutamax (cat. no. 35050061, Gibco) for 24 h. After that, cells were treated with BDNF (100 ng/ml, cat. no. 450-02, Peprotech) and Nogo66-Fc (400 nM, cat. no. 3728-NG, R&D Systems). K252a (200 nM) was added into the culture medium 30 min before the BDNF treatment. After treatment, cells were

further cultured for 48 h and fixed with a warmed 4% PFA in the culture medium for 10 min at 37°C followed by 10 min at 22°C. After blocking 1% BSA in TBS-T for 30 min at 22°C, an anti-MAP2 antibody (cat. no. 4542, Cell Signaling Technology, RRID: 10693728) was applied and cells were incubated for 1 h at 22°C. After washing with TBS-T three times, Alexa Fluor 488-labeled Goat antibodies against rabbit IgG (cat. no. 111-545-003, Jackson ImmunoResearch, RRID: AB_2338046) were applied, and cells were incubated for 1 h at 22°C in the dark. After washing with TBS-T three times, the cells were loaded with Prolong Diamond Antifade Mountant, and mounted on a sliding glass. Fluorescent images were obtained using a BZ-8100 microscope (Keyence). The distance from an initial neurite segment to the tip of the longest neurite in each neuron was measured as the neurite length of the hippocampal neuron using ImageJ software (National Institutes of Health, RRID: SCR_003070).

2.10 | Study design and statistical analysis

The study was not pre-registered. No sample calculation was performed, and the sample sizes in each study were estimated based on previous studies in the field (Caldeira et al., 2007; Kawakami, Kurihara, et al., 2018; Kurihara et al., 2020; Melo et al., 2013). Post hoc power analysis was performed by using G*Power software version 3.1 (Faul et al., 2007). The α error was set at 0.05. Based on each experiment, the effect size d or f was calculated from means and standard deviation or variance explained by a special effect and variance within groups (the effect size d: 15.51 ~ 16.25, the effect size f: 0.99 ~ 4.76). The power was analyzed for >89% of all experiments. No randomization and blinding were performed in this study. The experiments were conducted between 9 a.m. and 10 p.m.

All tests were performed in more than three independent experiments, and the data were presented as mean ± standard error of the mean (SEM). The data were analyzed for normal distribution by Shapiro–Wilk test. Statistically significant differences were analyzed by Student's *t*-test, one-way ANOVA followed by post hoc Tukey–Kramer or Holm multiple comparison test, and two-way ANOVA followed by post hoc Tukey–Kramer multiple comparison test. All statistical tests were two-tailed. The analyses were performed using BellCurve for Excel (Social Survey Research Information Co., Ltd). Statistical significance was defined as $p < 0.05$. No test for outliers was conducted and no exclusion criteria was predetermined on the data.

3 | RESULTS

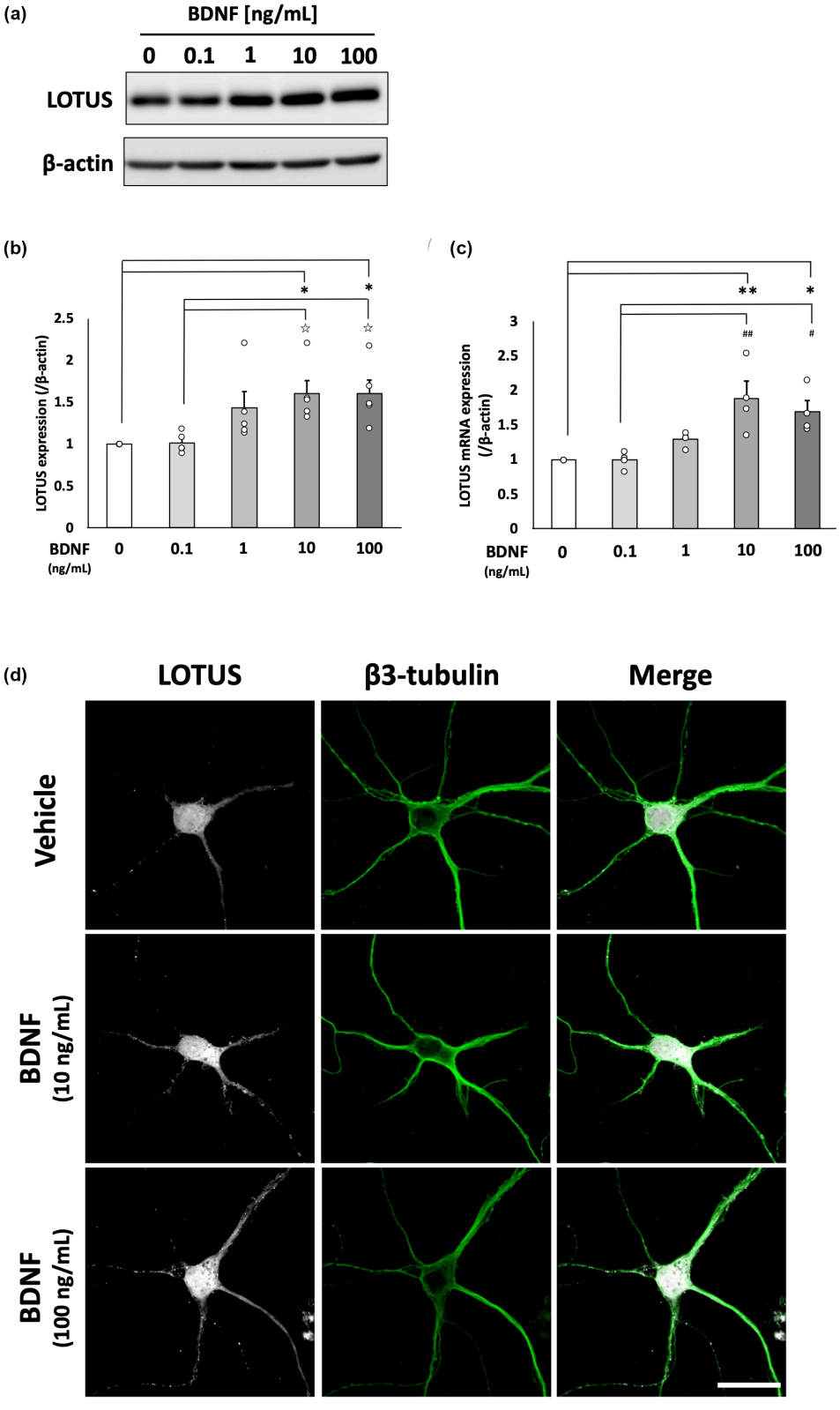
3.1 | BDNF increases LOTUS expression level in cultured hippocampal neurons

To assess the effect of BDNF on endogenous LOTUS expression, we used cultured hippocampal neurons in which LOTUS expresses (Nishida et al., 2021). The primary-cultured hippocampal neurons were treated with recombinant BDNF on days in vitro (DIV) 6 and cultured for 24 h at various concentrations. The Western blotting analysis revealed that LOTUS protein expression in cultured hippocampal neurons on DIV 7 significantly increased at 10 ng/ml and 100 ng/ml of BDNF concentration (Figure 1a,b). To further investigate whether BDNF increases LOTUS mRNA, we performed real-time PCR with the lysate samples of cultured hippocampal neurons on DIV 7, and found that the BDNF treatment increased the expression of LOTUS at the mRNA level (Figure 1c). LOTUS is a membrane protein that contains a signal peptide, four phenylalanyl-glycyl and glycyl-alanyl-prolyl (FG-GAP) domains, a UnbV/ASPIC (UA) domain, and an EGF-like calcium binding (EC) domain (Sato et al., 2011). To confirm whether BDNF increases LOTUS expression on the cell surface, we performed immunocytochemistry in cultured hippocampal neurons by treating with the antibody before fixation, which allows the antibody to specifically bind to LOTUS on the cell surface and found that BDNF enhanced the expression of membranous LOTUS (Figure 1d). Accordingly, these findings suggest that BDNF may increase the expression of LOTUS in cultured hippocampal neurons.

3.2 | BDNF induces an increase in LOTUS expression through TrkB

Next, we examined the molecular mechanism of BDNF-induced increase in LOTUS expression. TrkB is the high-affinity receptor for BDNF, and the intracellular tyrosine kinase domains of TrkB are phosphorylated, followed by the binding of BDNF. The phosphorylation activates the downstream signal transduction leading to gene transcription (Chao, 2003; Cocco et al., 2018). To determine whether BDNF increases LOTUS expression through TrkB, we examined the pharmacological effect of TrkB inhibition using K252a, a TrkB inhibitor, on LOTUS expression in cultured hippocampal neurons. The cultured hippocampal neurons were pretreated with K252a (200 nM) before the BDNF treatment (100 ng/ml) on DIV 6, and further cultured for 24 h. Western blotting, real-time PCR, and fluorescent immunostaining revealed that K252a suppressed the BDNF-induced increase in LOTUS expression (Figure 2a–d). Treatment with K252a alone did not decrease mRNA and protein levels of LOTUS expression (Figure 2b,c). Gene knockdown was performed using siRNA of TrkB to further investigate whether BDNF increases LOTUS expression by TrkB. The cultured hippocampal neurons were transfected with siRNA of TrkB (40 nM) at DIV 4, treated with BDNF (100 ng/ml) at DIV 6, and cultured for an additional 24 h. TrkB expression was reduced by ~50% (Figure 2e,f). The Western blotting results presented that TrkB gene knockdown significantly suppressed the BDNF-induced increase in LOTUS expression (Figure 2g,h). Moreover, treatment with soluble recombinant TrkB-Fc protein (2 μg/ml) presented a blocking effect, since the added BDNF was trapped by TrkB-Fc, which result in reduced LOTUS expression at both mRNA and protein levels (Figure S1).

These results suggest that exogenous application of BDNF may increase LOTUS expression through TrkB activation.



3.3 | MAPK and PI3K signal cascades involve in BDNF-induced LOTUS expression

To further determine the intracellular signaling pathway of BDNF-induced increase in LOTUS expression, we focused on mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase C-γ (PLCγ) cascades, which are known to be the downstream signaling of TrkB phosphorylation followed by binding of BDNF (Chao, 2003). First, we applied several inhibitors, PD98059 (100 μM), a specific MEK inhibitor, or LY-294002 (50 μM), a specific PI3K inhibitor, or U-73122 (10 μM), a specific phospholipase C inhibitor before the BDNF treatment in cultured hippocampal

FIGURE 1 BDNF increases the expression level of LOTUS in cultured hippocampal neurons. (a) Immunoblot analysis of LOTUS expression in BDNF-treated cultured hippocampal neurons on DIV 7. The primary-cultured hippocampal neurons on DIV 6 were treated with BDNF (0, 0.1, 1, 10, 100 ng/ml) for 24 h. β-actin was used as an internal control. (b) Quantitative analysis of LOTUS expression in (a). The relative intensity of LOTUS to β-actin with BDNF treatment was normalized to the intensity of control (BDNF 0 ng/ml). Data are means ± SEM from the five independent cell culture preparations. $^{*}p < 0.05$ versus BDNF 0 ng/ml, $\star p < 0.05$ versus BDNF 0.1 ng/ml, one-way ANOVA post hoc Tukey–Kramer (df = 4, $F = 4.9063$, $p = 0.0064$). (c) Real-time PCR analysis of LOTUS mRNA expression in BDNF-treated DIV 7-cultured hippocampal neurons. mRNA levels were assessed by ΔΔCt, and β-actin was used as an internal control. The relative mRNA level of LOTUS to β-actin with BDNF treatment was normalized to the mRNA levels of control (BDNF 0 ng/ml). Data are means ± SEM from the four independent cell culture preparations. $^{*}p < 0.05$, $^{**}p < 0.01$ versus BDNF 0 ng/ml, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus BDNF 0.1 ng/ml, one-way ANOVA post hoc Tukey–Kramer (df = 4, $F = 8.7656$, $p < 0.001$). (d) Confocal microscopic images of βIII-tubulin (green) and LOTUS (white) in cultured hippocampal neurons treated with BDNF (10 and 100 ng/ml) on DIV 7. Scale bars, 25 μm. Abbreviations: BDNF, brain-derived neurotrophic factor; DIV, days in vitro; LOTUS, lateral olfactory tract usher substance; mRNA, messenger RNA; SEM, standard error of the mean.

neurons on DIV 6. The Western blotting analysis revealed that blocking MEK with PD98059 prevented the BDNF-induced increase in LOTUS expression (Figure 3a), while treatment with PD98059 alone did not affect the expression level of LOTUS compared with controls. Next, we found that blocking PI3K with LY294002 also prevented the BDNF-induced increase in LOTUS expression, although LOTUS was decreased by LY294002 alone (Figure 3b). Herein, treatment with U-73122 showed no suppressive effect on the BDNF-induced increase in LOTUS expression (Figure 3c).

CREB is activated by BDNF and promotes the transcription of target genes (Du, k., & Montminy, M., 1998; Gokce et al., 2009). To investigate whether CREB was involved in the BDNF-increased LOTUS expression, we used CREB inhibitor 666-15 and applied it before the BDNF treatment on DIV 6. Treatment with 666-15 suppressed the BDNF-induced increase in LOTUS expression, although a complete blocking effect was not observed (Figure 3d). These results suggest that BDNF increases LOTUS expression through MAPK-CREB pathway and/or PI3K/AKT-CREB pathway.

3.4 | Exogenous application of BDNF suppresses Nogo66-mediated neurite outgrowth inhibition by up-regulation of LOTUS expression

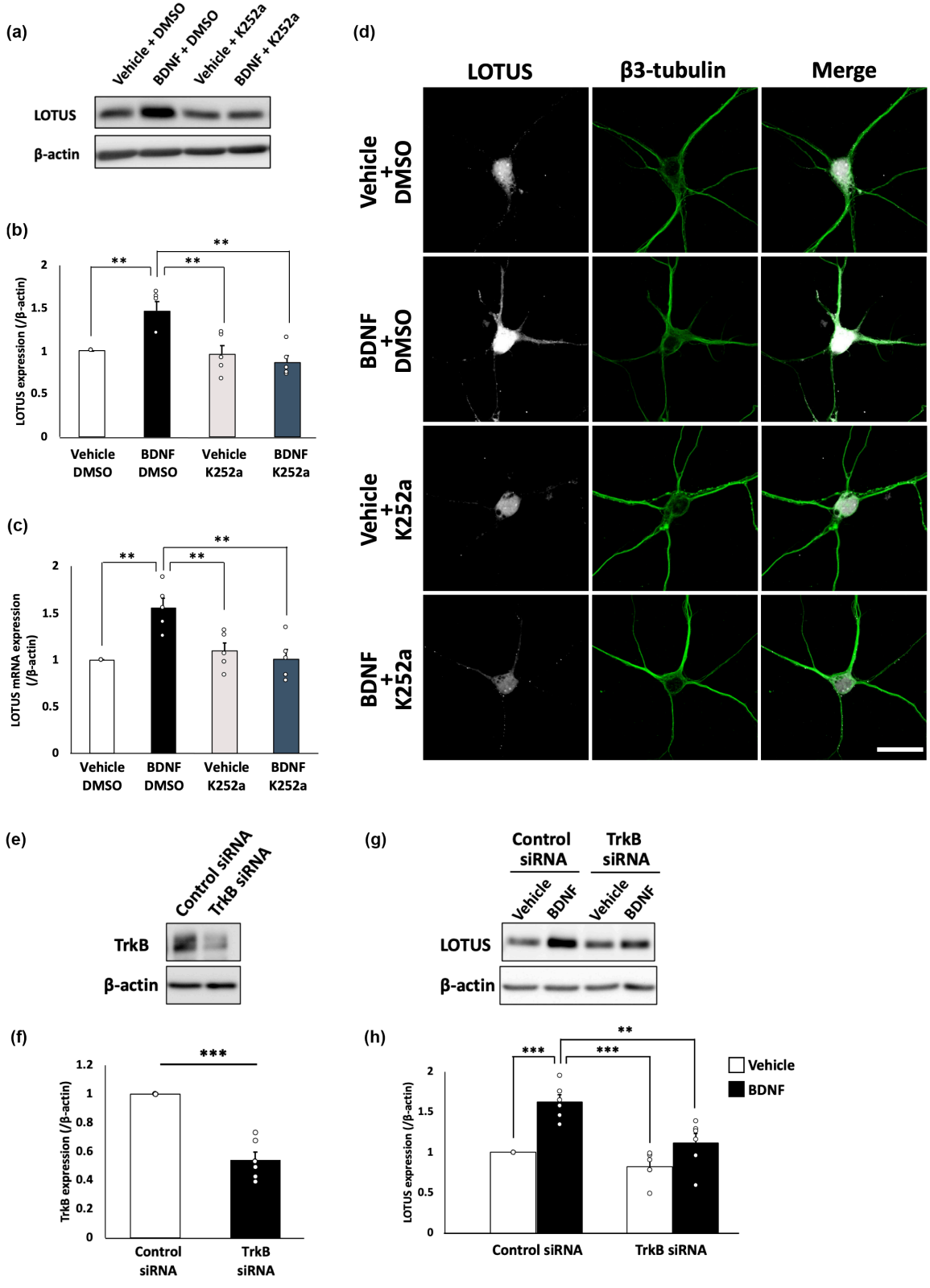
LOTUS is a potent antagonist for NgR1 and suppresses neurite outgrowth inhibition induced by the interaction of Nogo66 with NgR1 (Kawakami, Kurihara, et al., 2018; Kurihara et al., 2014, 2017; Sato et al., 2011), suggesting that upregulation of LOTUS induced by exogenous application of BDNF may enhance the suppressive effect of LOTUS on NgR1 function. To verify this hypothesis, we conducted a neurite outgrowth assay in cultured hippocampal neurons at DIV 3 from wild-type and *lotus*-deficient mice. We confirmed that the expression level of LOTUS in cultured hippocampal neurons at DIV 3 is also increased by BDNF (Figure 4a). The maximum neurite length in each neuron was measured at DIV 3 following treatment with BDNF (100 ng/ml) and Nogo66-Fc (400 nM) on DIV 1. Nogo66 inhibited neurite outgrowth in both wild-type and *lotus*-deficient neurons at almost equivalent levels (Figure 4b,c left). In cultured hippocampal neurons from wild-type mice, treatment with BDNF significantly suppressed neurite outgrowth inhibition induced by Nogo66 to its control level, while no suppressive effect on Nogo66-induced neurite outgrowth inhibition was observed in *lotus*-deficient mice. In our experimental conditions of cultured hippocampal neurons, treatment with BDNF itself did not affect neurite outgrowth activity compared with controls (vehicle) as previously reported (Shih et al., 2013; Yang et al., 2016). The data show that exogenous application of BDNF suppresses Nogo66-mediated neurite outgrowth inhibition, whereas loss of LOTUS has no inhibitory effect, suggesting that the inhibitory effect of BDNF is caused by LOTUS expression (Figure 4c right).

To explore whether the BDNF-induced increase in LOTUS suppresses Nogo66-mediated neurite outgrowth inhibition, we further performed a neurite outgrowth assay in wild-type hippocampal neurons pretreated with K252a. Treatment with K252a also suppressed the increase of LOTUS by BDNF-like cultured neurons at DIV 7 (Figure 2b,d). We also confirmed no effect on LOTUS expression with K252a (Figure 2b,d). The neurite length of the neurons treated with BDNF was unchanged in the presence of Nogo66 (Figure 4e,f). However, K252a canceled BDNF-induced suppressive action to Nogo66-mediated neurite outgrowth inhibition, although K252a decreased neurite length in the absence of Nogo66 (Figure 4e,f). These findings suggest that Nogo66-mediated neurite outgrowth inhibition in cultured hippocampal neurons is blocked by the increase in LOTUS expression through BDNF–TrkB signaling.

4 | DISCUSSION

In this study, we found that BDNF increases LOTUS expression in cultured hippocampal neurons by activating the TrkB receptor and its downstream MAPK and/or PI3K signaling pathways. Moreover, we found that a BDNF-induced increase in LOTUS expression blocks the inhibition of neurite outgrowth by Nogo66 (Figure 5).

Polarity changes in cultured hippocampal neurons formed and established axons and dendrites at week 1 (Dotti et al., 1988). Synaptogenesis in cultured hippocampal neurons is confirmed at DIV 7 (Grabrucker et al., 2009). Previous studies have shown that BDNF increases the synaptic plasticity-related proteins, such as AMPA receptor subunits (Caldeira et al., 2007) and VGLUT (Melo et al., 2013) in developing cultured hippocampal neurons. In this



study, exogenous administration of BDNF increased LOTUS expression in cultured hippocampal neurons of DIV 7 in a dose-dependent manner (Figure 1b). We have reported that LOTUS is expressed in the synaptic region and influences the synapse formation in cultured hippocampal neurons (Nishida et al., 2021). Thus, upregulation of LOTUS expression induced by BDNF in developing hippocampal neurons may contribute to synapse formation.

TrkB is one of the Trk family receptors consisting of two cysteine-rich clusters C1 and C2, the three leucine-rich clusters, and two immunoglobulin-like motifs Ig1 and Ig2 in the extracellular

FIGURE 2 Inhibition of TrkB suppresses BDNF-induced increase in LOTUS expression. (a) Immunoblot analysis of LOTUS expression in DIV 7-cultured hippocampal neurons. The primary-cultured hippocampal neurons on DIV 6 were treated with BDNF (100 ng/ml) in the presence of K252a (200 nM). K252a was preincubated for 30 min before the BDNF treatment. (b) Quantitative analysis of LOTUS expression in (a). The relative intensity of LOTUS to β-actin with BDNF or K252a treatment was normalized to the intensity level of control (vehicle and DMSO treatment). Data are means ± SEM from the five independent cell culture preparations. $^{**}p < 0.01$ versus BDNF treatment alone, one-way ANOVA post hoc Tukey–Kramer (df = 3, $F = 9.8375$, $p < 0.001$). (c) Real-time PCR analysis of LOTUS mRNA expression in DIV 7-cultured hippocampal neurons treated with BDNF or K252a. mRNA levels were assessed by ΔΔCt, and β-actin was used as an internal control. The relative mRNA level of LOTUS to β-actin with BDNF or K252a treatment was normalized to the mRNA level of control (vehicle and DMSO treatment). Data are means ± SEM from the five independent cell culture preparations. $^{**}p < 0.01$ versus BDNF treatment alone, one-way ANOVA post hoc Tukey–Kramer (df = 3, $F = 9.3169$, $p < 0.001$). (d) Confocal microscopic images of βIII-tubulin (green) and LOTUS (white) in cultured hippocampal neurons treated with BDNF (100 ng/ml) or K252a (200 nM) on DIV 7. Scale bars, 25 μm. (e) Immunoblot analysis of TrkB expression in DIV 7-cultured hippocampal neurons. The primary-cultured hippocampal neurons on DIV 4 were transfected with TrkB siRNA (40 nM) and further cultured for 72 h. (f) Quantitative analysis of TrkB expression in (e). The relative intensity of TrkB to β-actin with TrkB siRNA treatment was normalized to the intensity level of control siRNA. Data are means ± SEM from the six independent cell culture preparations. $^{***}p < 0.001$ versus control siRNA, Student's *t*-test (df = 10, $t = 8.2270$, $p < 0.001$). (g) Immunoblot analysis of LOTUS expression in DIV 7-cultured hippocampal neurons. The primary-cultured hippocampal neurons transfected with TrkB siRNA were treated with BDNF (100 ng/ml) on DIV6. (h) Quantitative analysis of TrkB expression in (g). The relative intensity of LOTUS to β-actin with TrkB siRNA or BDNF treatment was normalized to the intensity level of control (control siRNA and vehicle treatment). Data are means ± SEM from the six independent cell culture preparations. $^{**}p < 0.01$, $^{***}p < 0.001$ versus BDNF treatment alone, one-way ANOVA post hoc Tukey–Kramer (df = 3, $F = 17.4166$, $p < 0.001$). Abbreviations: BDNF, brain-derived neurotrophic factor; DIV, days in vitro; LOTUS, lateral olfactory tract usher substance; mRNA, messenger RNA; SEM, standard error of the mean; siRNA, small interfering RNA; TrkB, tropomyosin-related kinase B.

region, a transmembrane region, and the intracellular kinase domain. BDNF binds to the Ig2 domain with high affinity, resulting in homodimerization followed by the autophosphorylation of tyrosine residues (Cocco et al., 2018). TrkB phosphorylation transduces intracellular signaling pathways, such as the MAPK/Ras, PI3K/Akt/mTOR, and PLCγ signaling cascades (Park & Poo, 2013). In this study, the treatment with K252a, a TrkB inhibitor, suppressed the BDNF-induced increase in LOTUS expression at both the mRNA and protein levels (Figure 2b,c). K252a treatment did not affect LOTUS expression. Aside from the inhibitor-based approach, TrkB knockdown by siRNA (Figure 2g,h) and treatment with soluble recombinant TrkB-Fc protein as a decoy protein suppressed the BDNF-induced increase in LOTUS expression (Figure S1). These results suggest that exogenous administration of BDNF may increase LOTUS expression by activating TrkB and exert a boosting effect on endogenous LOTUS expression. Transient and sustained activation of TrkB by BDNF play distinct roles in cultured hippocampal neurons (Ji et al., 2010). Therefore, it would be interesting to investigate the effects of sustained BDNF administration on LOTUS expression and the effect of BDNF-induced LOTUS on neural development and regeneration.

The signaling cascades for BDNF-induced upregulation of LOTUS expression involved MAPK and PI3K (Figure 3a,b) and BDNF-induced LOTUS expression was suppressed by LY294002, while treatment with LY294002 alone resulted in a level of slight reduction. These data imply that the PI3K signaling cascade may regulate LOTUS expression levels independently of BDNF–TrkB signaling. For example, PAC1, a class B-G-protein coupled receptor expressed in the CNS and a pituitary adenylate cyclase-activating polypeptide (PACAP)-selective high-affinity receptor, transduces PI3K (Hirabayashi et al., 2018; May et al., 2010). Alternatively, previous studies have reported that deletion of phosphatase and tensin homolog (PTEN), a negative regulator of PI3K/mTOR pathway, promotes axonal regeneration after CNS injury (Liu et al., 2010; Park et al., 2008). It is interesting to note that the activation of PACAP-PAC1 and PTEN is possibly involved in LOTUS expression. The treatment with U-73122 presented no inhibitory effect of BDNF-induced LOTUS expression (Figure 3c). The binding of BDNF leads to tyrosine 532 (Y532) and 833 (Y833) phosphorylation in the intracellular kinase domain of human TrkB. The phosphorylation of Y532 leads to the binding of TrkB to SHC-transforming protein (SHC) and fibroblast growth factor receptor substrate 2 (FRS2), which results in the activation of MAPK and PI3K signaling cascades. However, Y833 phosphorylation leads to the binding of TrkB to PLCγ and the binding transduces downstream signaling (Cocco et al., 2018). Therefore, there is a possibility that the BDNF-induced increase in LOTUS expression may be induced through specific TrkB kinase domains, for example, tyrosine 532.

Additionally, we showed that the CREB inhibitor 666-15 partially suppressed the BDNF-induced increase in LOTUS expression (Figure 3d). Previous reports have shown that TrkB activation by BDNF causes not only CREB phosphorylation but also EIK-1 phosphorylation, both of which induce gene transfer (Besnard et al., 2011; Gokce et al., 2009). Therefore, Elk-1, in addition to CREB, may be involved as a transcription factor in the induction of LOTUS expression by BDNF. Further studies are required to elucidate the detailed gene expression mechanism of LOTUS.

Our previous studies have shown that LOTUS interacts with both NgR1 and PIR-B, thereby suppressing Nogo66-related functions of both receptors (Kawakami, Kurihara, et al., 2018; Kurihara et al., 2020; Sato et al., 2011). In this study, we tested whether the upregulation of endogenous LOTUS expression suppresses Nogo66-induced inhibition of neurite outgrowth. The BDNF-induced increase in LOTUS expression was also observed at DIV 3 (Figure 4a). The effect of BDNF on the LOTUS expression levels was much more

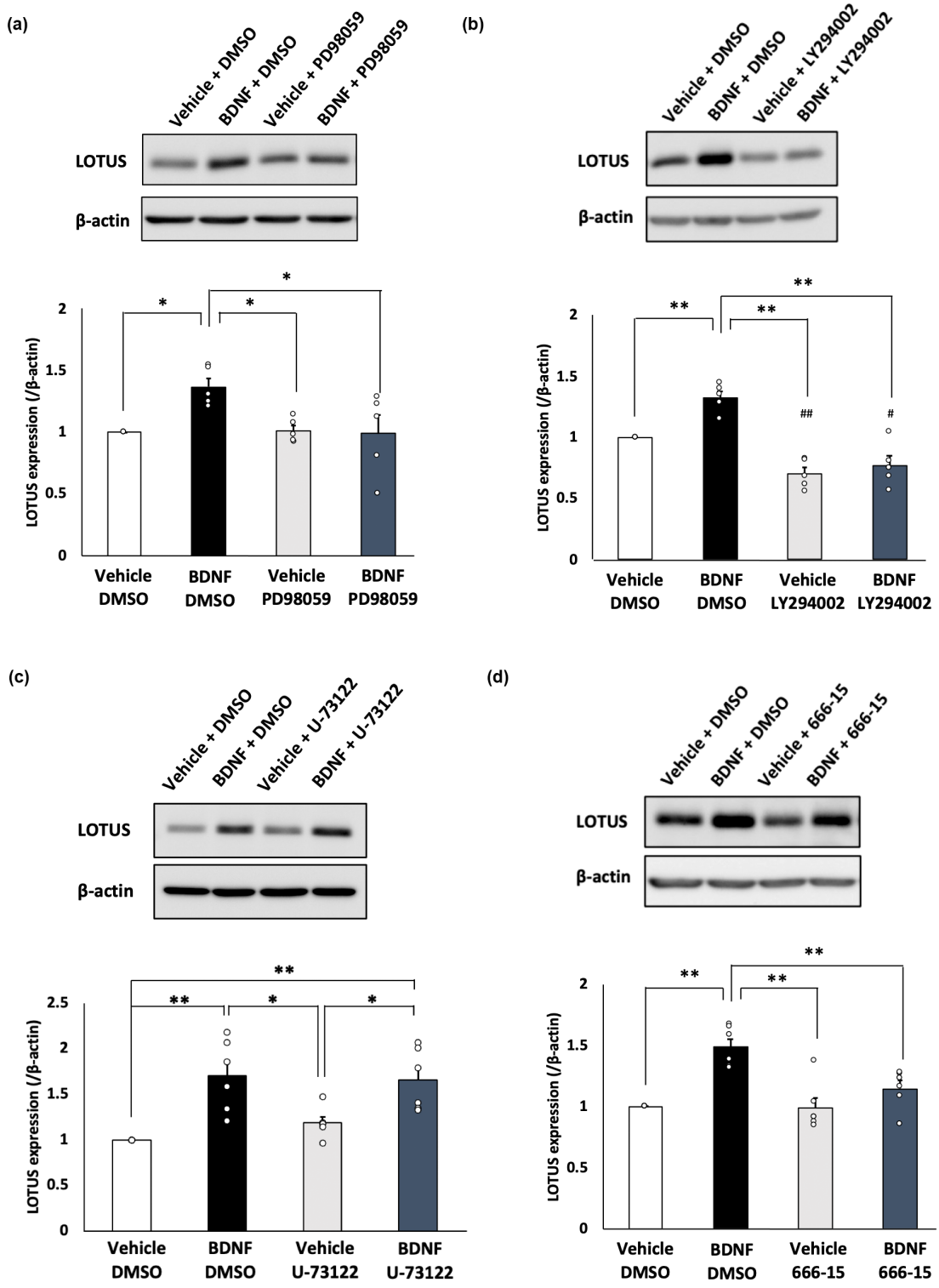


FIGURE 3 Inhibition of MAPK and PI3K signaling pathways suppresses BDNF-induced increase in LOTUS expression. (a–d) Immunoblot analysis of LOTUS expression in cultured hippocampal neurons on DIV 7. The primary-cultured hippocampal neurons on DIV 6 were treated with BDNF (100 ng/ml) in the presence of MAPK inhibitor, PD98059 (100 μM) (a) or PI3K inhibitor, LY294002 (50 μM) (b) or PLCγ inhibitor, U-73122 (10 μM) (c) or CREB inhibitor, 666–15 (500 nM) (d). Each inhibitor was preincubated for 30 min before the BDNF treatment. The relative intensity of LOTUS to β-actin with BDNF was normalized to the intensity of control (vehicle and DMSO treatment). Data are means ± SEM from the five (a and b) or the six (c and d) independent cell culture preparations. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus control, $^{*}p < 0.05$, $^{**}p < 0.01$, one-way ANOVA post hoc Tukey–Kramer (PD98059; df = 3, $F = 4.7265$, $p = 0.0151$: LY294002; df = 3, $F = 26.4821$, $p < 0.001$: U-73122; df = 3, $F = 9.7787$, $p < 0.001$: 666–15; df = 3, $F = 13.9201$, $p < 0.001$). Abbreviations: BDNF, brain-derived neurotrophic factor; CREB, c-AMP response element binding protein; DIV, days in vitro; LOTUS, lateral olfactory tract usher substance; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PLCγ, phospholipase c-γ; SEM, standard error of the mean.

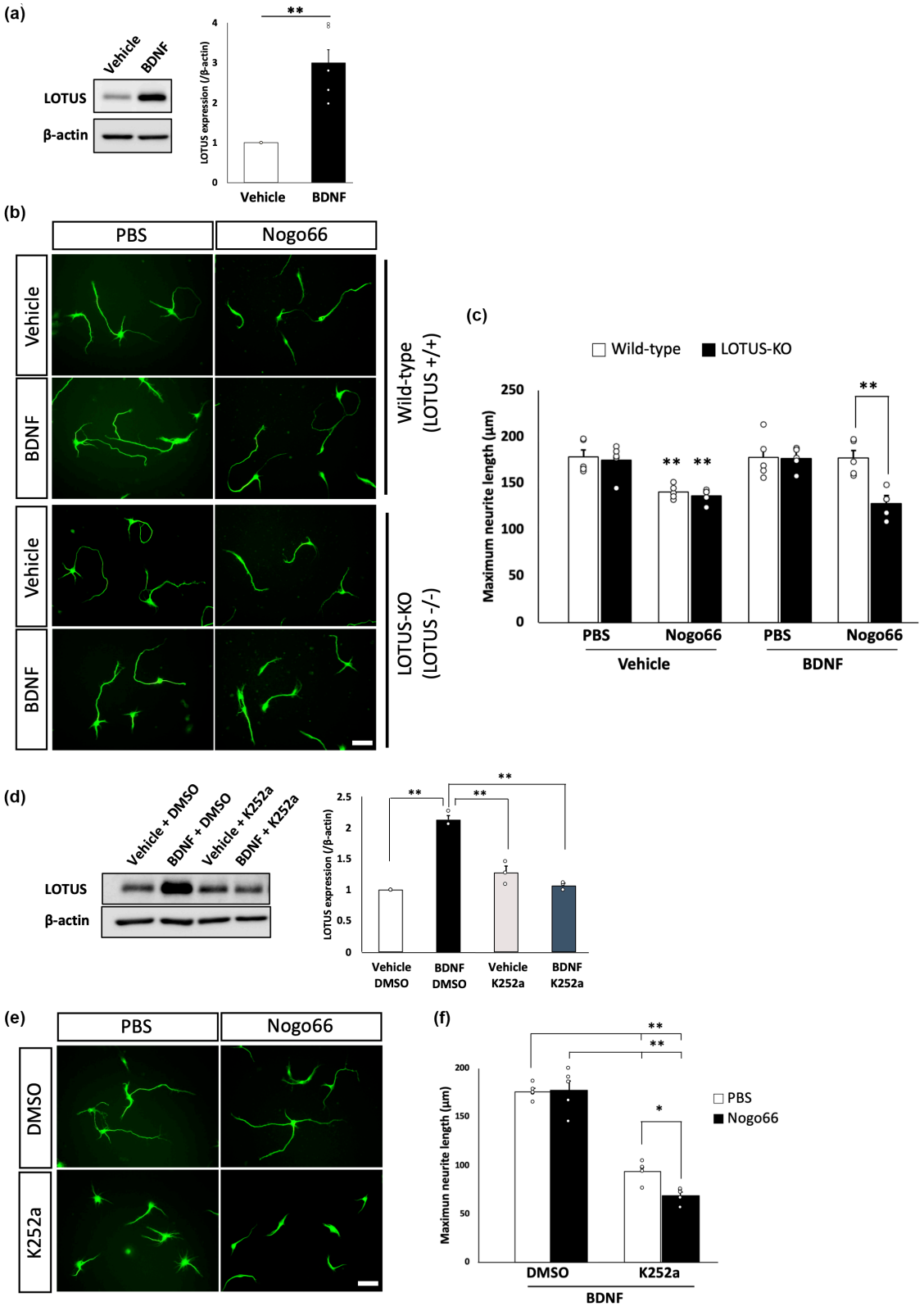


FIGURE 4 Legend on next page

significant in cultured hippocampal neurons at DIV 3 than at DIV 7. This might be caused by the difference in BDNF exposure time in the experiments; in DIV 7, the exposure time to BDNF is 24 h, whereas, in DIV 3, the exposure time is 48 h. Another reason might be the different developmental stages of cultured hippocampal neurons (Dotti et al., *Journal of Neuroscience*, 8, 1454–1468, 1988), which leads to the idea that BDNF may exert a more significant effect on immature DIV 3 neurons.

Addition of BDNF into cultured hippocampal neurons of *lotus*-deficient mice showed no inhibitory effect on Nogo66-induced

FIGURE 4 BDNF-induced LOTUS upregulation suppresses neurite outgrowth inhibition by Nogo66. (a) Immunoblot analysis of LOTUS expression in DIV 3-cultured hippocampal neurons from wild-type mice treated with BDNF on DIV 1. Data are means ± SEM from the five independent cell culture preparations. $^{**}p < 0.01$ versus vehicle control, Student's *t*-test (df = 8, $t = 4.9104$, $p = 0.0012$). (b) Representative images of DIV 3 cultured hippocampal neurons from wild-type and *lotus*-deficient mice. The neurons were immunostained by anti-MAP2 antibody to visualize neurites. The cultured neurons were treated with BDNF (100 ng/ml) or vehicle, and Nogo66 (400 nM) or PBS. Scale bars, 50 μm. (c) Quantification of neurite length in (b). White bars indicate the longest neurite length of neurons from wild-type mice and black bars indicate the longest neurite length of neurons from *lotus*-deficient mice. The neurite length was shown as the longest neurite from an initial neurite segment to the neurite tip in each neuron. Data are means ± SEM (total neurite analyzed = 616–719 per each group) from the five independent cell culture preparations. $^{**}p < 0.01$, two-way ANOVA post hoc Tukey–Kramer (genotype; df = 1, $F = 8.0090$, $p = 0.0080$: treatment group; df = 3, $F = 14.4606$, $p < 0.001$: genotype * treatment group; df = 3, $F = 5.3798$, $p = 0.0041$). (d) Immunoblot analysis of LOTUS expression in DIV 3 cultured hippocampal neurons treated with BDNF (100 ng/ml) in the presence of K252a (200 nM) in DIV 1. K252a was applied 30 min before the BDNF treatment. Data are means ± SEM from the three independent cell culture preparations. $^{**}p < 0.01$ versus BDNF treatment alone, one-way ANOVA post hoc Tukey–Kramer (df = 3, $F = 60.4640$, $p < 0.001$). (e) Representative images of DIV 3–cultured hippocampal neurons immunostained by anti-MAP2 antibody. The cultured neurons were preincubated with K252a (200 nM) or DMSO before treatment of BDNF and Nogo66. Scale bars, 50 μm. (f) Quantification of neurite length in (e). White bars indicate the longest neurite length of neurons without Nogo66 and black bars indicate the longest neurite length of neurons with Nogo66. The neurite length was shown as the longest neurite from an initial neurite segment to the neurite tip in each neuron. Data are means ± SEM (total neurite analyzed = 657–692 per each group) from the five independent cell culture preparations. $^{*}p < 0.05$, $^{**}p < 0.01$, one-way ANOVA post hoc Holm (df = 3, $F = 89.5547$, $p < 0.001$). Abbreviations: BDNF, brain-derived neurotrophic factor; DIV, days in vitro; LOTUS, lateral olfactory tract usher substance; MAP2, microtubule-associated protein 2; SEM, standard error of the mean.

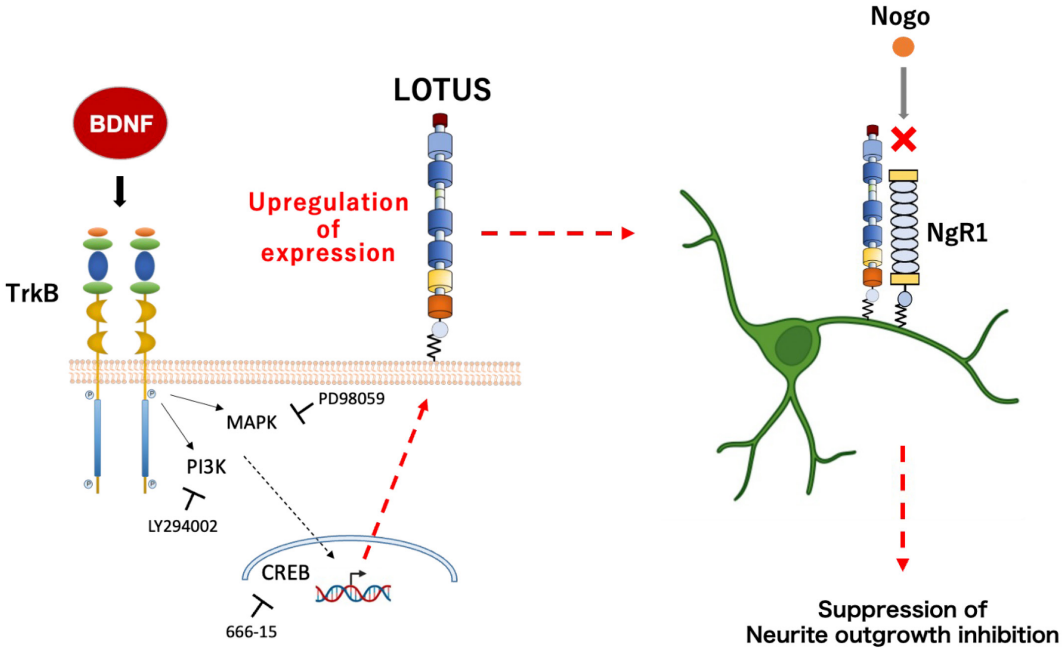


FIGURE 5 Schematic showing the upregulation of LOTUS expression induced by BDNF. The binding of BDNF to TrkB leads to increase of LOTUS expression via MAPK-CREB and/or PI3K-CREB pathways, resulting in the suppression of Nogo-mediated neurite outgrowth inhibition in cultured hippocampal neurons. Abbreviations: BDNF, brain-derived neurotrophic factor; CREB, c-AMP response element binding protein; LOTUS, lateral olfactory tract usher substance; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; TrkB, tropomyosin-related kinase B.

inhibition of neurite outgrowth compared with WT mice (Figure 4c right), and treatment with K252a prevented BDNF-induced suppression in Nogo66-induced inhibition of neurite outgrowth in WT neurons (Figure 4f). These findings suggest that BDNF suppresses Nogo66-induced inhibition of neurite outgrowth by up-regulating LOTUS expression, as LOTUS exerts inhibitory effects on NgR1-ligand binding-induced inhibition of neurite outgrowth (Kawakami, Kurihara, et al., 2018; Kurihara et al., 2014, 2017). We speculate that the BDNF-induced increase in LOTUS expression may also exert inhibitory effects on NgR1-ligands other than Nogo66.

The binding of MAG to PIR-B, which interacts with p75NTR, leads to dephosphorylation of TrkB and inhibition of axon growth (Fujita, Endo, et al., 2011; Fujita, Takashima, et al., 2011). There is a possibility that the interaction of MAG with PIR-B may attenuate the BDNF-induced

increase in LOTUS expression through dephosphorylation of TrkB. In contrast, our previous studies demonstrated that LOTUS interacts with both p75NTR and PIR-B, resulting in the suppression of growth cone collapse and neurite outgrowth inhibition (Kawakami, Kurihara, et al., 2018; Kurihara et al., 2020). It is interesting to consider the possibility that LOTUS, upregulated by BDNF, controls TrkB activation by inhibiting the interaction of TrkB with the p75NTR/PIR-B complex. Furthermore, recent studies have reported that increased expression of BDNF and other neurotrophic factors, such as neurotrophin-3 (NT-3) and nerve growth factor (NGF), are increased in human iPS cell-derived neuronal cell or precursor cell (hiPSC-NS/PC) that express LOTUS (Ito et al., 2021). Moreover, there is a positive feedback system in BDNF regulation (Cheng et al., 2011; Tuvikene et al., 2016). Thus, we hypothesize that the BDNF-induced LOTUS expression accelerates the BDNF-positive feedback loop via TrkB activation, but it is unclear whether LOTUS interacts with TrkB. It will be of great interest for future research to examine whether the BDNF-induced increase in LOTUS expression is involved in these mechanisms.

Previous studies have demonstrated the efficacy of several therapeutic strategies using LOTUS, such as soluble form of LOTUS protein (Kawakami, Kurihara, et al., 2018), adeno-associated virus encoding *lotus* gene (Hirokawa et al., 2017), and transplantation of hiPSC-NS/PC expressing LOTUS (Ito et al., 2021) in various CNS injury. In addition to these applications, enhancing LOTUS expression by BDNF may be a beneficial therapeutic strategy for neurological disorders as it is expected to be synergistic with the axon growth activity of BDNF (Nagahara & Tuszynski, 2011).

Conclusively, we found that BDNF increases LOTUS expression in cultured hippocampal neurons via TrkB and its downstream signaling, thereby promoting the antagonistic action of NgR1 function. Furthermore, these findings provide new insight into the notion that axon growth-promoting effects of BDNF include an indirect action of LOTUS and that BDNF acts as a positive regulator of LOTUS expression. Finally, the increased LOTUS expression induced by BDNF may represent a novel therapeutic strategy for neuronal regeneration after CNS injury. To address this possible idea, it is required in future studies to confirm the positive effects of BDNF-induced upregulation in LOTUS expression on neural plasticity and neuronal regeneration *in vivo* systems.

AUTHOR CONTRIBUTIONS

J.M. and K.T. originally designed the study. J.M. and Y. Kawaguchi conducted the experiments and analyzed the data. J.M. wrote the manuscript. K.T. and Y. Kawakami edited the manuscript. K.T. had full access to all data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis.

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All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

OPEN RESEARCH BADGES

This article has been awarded Open Materials Badge for making the components of the research methodology needed to reproduce the reported procedure and analysis publicly available at: https://cos.io/our-services/open-science-badges/

DATA AVAILABILITY STATEMENT

The data sets analyzed in the study and noncommercial materials used are available from the corresponding authors upon reasonable scientific requests.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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