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REGULAR RESEARCH ARTICLE

LIF, a Novel Myokine, Protects Against Amyloid-Beta-Induced Neurotoxicity via Akt-Mediated Autophagy Signaling in Hippocampal Cells

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Abstract

Background: Leukemia inhibitory factor, a novel myokine, is known to be associated with neural function, but the underlying molecular mechanism remains unclear.
Methods: HT-22 mouse hippocampal cells, primary hippocampal cells, and Drosophila Alzheimer's disease model were used to determine the effect of leukemia inhibitory factor on neurons. Immunoblot analysis and immunofluorescence method were used to analyze biological mechanism.
Results: Leukemia inhibitory factor increased Akt phosphorylation in a phosphoinositide-3-kinase-dependent manner in hippocampal cells. Leukemia inhibitory factor also increased the phosphorylation of the mammalian target of rapamycin and the downstream S6K. Leukemia inhibitory factor stimulated the phosphorylation of signal transducer and activator of transcription via extracellular signal-regulated kinases. Leukemia inhibitory factor increased c-fos expression through both Akt and extracellular signal-regulated kinases. Leukemia inhibitory factor blocked amyloid β-induced neural viability suppression and inhibited amyloid β-induced glucose uptake impairment through the block of amyloid β-mediated insulin receptor downregulation. Leukemia inhibitory factor blocked amyloid β-mediated induction of the autophagy marker, microtubule-associated protein 1A/1B-light chain 3. Additionally, in primary prepared hippocampal cells, leukemia inhibitory factor stimulated Akt and extracellular signal-regulated kinase, demonstrating that leukemia inhibitory factor has physiological relevance in vivo. Suppression of the autophagy marker, light chain 3II, by leukemia inhibitory factor was observed in a *Drosophila* model of Alzheimer's disease.
Conclusions: These results demonstrate that leukemia inhibitory factor protects against amyloid β-induced neurotoxicity via Akt/extracellular signal-regulated kinase-mediated c-fos induction, and thus suggest that leukemia inhibitory factor is a potential drug for Alzheimer's disease.

Keywords: LIF, Akt, autophagy, mTOR, myokine, Alzheimer's disease

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Significance Statement

Although exercise has been reported to be beneficial in the improvement of various brain diseases, the molecular mechanism by which this occurs is unclear. The key finding of our study is that muscle-secreted myokine, LIF, may have a beneficial effect on Alzheimer's disease via Akt-mediated pathway. The implication of this study is that exercise-induced myokine influences the crosstalk between muscle and neuron. Myokine can be a promising new target for the development of drugs for Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder (Shahpasand et al., 2012). Some medications, such as AchE inhibitors and NMDA receptor antagonists, are currently used to treat the cognitive problem of AD. The benefit is small, and no medications have been clearly shown to effectively regulate the progression of AD. Epidemiologic studies have indicated that nonsteroidal antiinflammatory drugs reduce the risk of AD (Szekely et al., 2007). Although symptomatic therapies for AD are now widely available, they remain unsatisfactory for the fundamental treatment of the disease.

Physical exercise improves mental health by improving cognitive function and memory (Ratey and Loehr, 2011; Etnier et al., 2016) in neurological disorders such as depression (Russo-Neustadt et al., 1999), epilepsy (Arida et al., 2008), stroke (Zhang et al., 2012), and Parkinson's disease (Ahlskog, 2011). Physical exercise is neuroprotective in healthy people and animals and prevents the decline in cognitive function that is associated with chronic neurodegenerative processes such as AD (Weih et al., 2009). However, the underlying molecular mechanisms explaining the benefit of exercise remain unknown. Myokine is secreted from the muscle fibers during physical exercise. It has been reported that myokine may be transported to organs through the circulatory system, revealing a mechanism through which it could reach the central nervous system (Pedersen and Febbraio, 2008). Myokine, an irisin that is secreted in the form of a protein called FNDC5, induces BDNF expression in the brains of rats (Wrann et al., 2013). PEDF (Bilak et al., 1999), GDF11 (Shi and Liu, 2011), VEGF-A (Mackenzie and Ruhrberg, 2012), and FGF21 (He et al., 2016) have also been reported to be active in the nervous system. Additionally, IL-6, a major compound of myokine, has been reported to play a critical role in the nervous system (Erta et al., 2012). This suggests that there is a connection between myokines and the nervous system.

LIF was first identified as a substance that is able to induce the differentiation of macrophages (Gearing et al., 1987). LIF is secreted by cardiac muscle, neuronal tissue, and skeletal muscle and is also known as a pleiotropic cytokine (Hilton, 1992). LIF has been reported to pass through the blood brain barrier (BBB) (Pan et al., 2000) as it is small (20 kDa) and consists of 179 amino acid residues. LIF is involved in the development of astrocytes (Lee et al., 2016), the survival of oligodendrocytes (Rowe et al., 2014), and the recovery processes of injured spinal cord in mice (Li and Zang, 2014). LIF therefore has significant effects on the nervous system. However, the molecular mechanism explaining these effects remains unclear.

Autophagy is an evolutionarily conserved process that transfers cytoplasmic materials and organelles to lysosomes, leading to degradation (Eskelinen, 2008; Kundu and Thompson, 2008). The dysregulation of autophagy is involved in diseases such as cancer, Crohn's disease, and heart disease (Cadwell et al., 2008; Mathew and White, 2011; Choi et al., 2013). Abnormal autophagy activity is often observed in patients with neurodegenerative diseases such as AD, Parkinson's disease, and Huntington's disease (Martinez-Vicente, 2015). Autophagy is also associated with the removal of amyloid β (Cai et al., 2015). Several myokines, including myonectin (Seldin et al., 2013) and myostatin (Stitt et al., 2004), have been reported to block autophagy, which suggests a possible role for myokines in AD. However, due to confounding factors, the relationship between myokine and AD is not yet clear.

In this study, we investigated the effects of LIF on amyloid β-induced neurotoxicity. We found that LIF stimulated c-fos in hippocampal cells and demonstrated that LIF plays an important role in protecting against amyloid β-induced neurotoxicity and insulin signaling impairment.

Method

Reagents

Antibodies against the following proteins were used in this study: Akt, phospho-Akt (Ser473), mTOR, phosphor mTOR, p70 S6 kinase, phospho p70 S6 kinase, STAT3, phospho- STAT3 (Tyr705), and LC3II from Cell Signaling Technology, c-fos, ERK, phospho ERK and β-amyloid (immunohistochemistry) from Santa Cruz Biotechnology, Insulin receptor (Y1158) from Abcam Cambridge, β-amyloid (Immunoblot analysis) from BioLegend, Donkey anti-Mouse IgG from Thermo Fisher Scientific, β-actin from EnoGene, and horseradish peroxidase-conjugated secondary antibodies from Enzo Life Sciences. β-Amyloid (human, 1–42) was purchased from Invitrogen. LY294002 and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma. PD98059 was purchased from Calbiochem. Recombinant human LIF was purchased from Peprotech. c-fos siRNA was purchased from Santa Cruz Biotechnology.

Cell Culture

HT-22 mouse hippocampal cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum and antibiotics at 37°C in a 5% CO_2 atmosphere.

MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) was dissolved in phosphate buffered saline (PBS) at 5 mg/mL and filtered to sterilize and remove the small amount of insoluble residue that is present in some batches of MTT. The reagent solution (10 mL) was added to each well of a plate containing cells in culture medium (100 mL) and the plate was incubated for 2 hours at 37°C. Subsequently, dimethyl sulfoxide (100 mL) was added to dissolve the resulting formazan by sonication. The absorbance of each well was measured at 540 nm using a microplate reader.

siRNA Transfection

Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, siRNA for c-fos and the nontargeted control siRNA were generated. siRNA (5 μL) and 5 μL of Lipofectamine 2000 were each diluted with 95 μL of reduced serum medium (Opti-MEM; Invitrogen) and then combined. The mixtures were incubated for 25 minutes at room temperature before being added, dropwise, to a culture well containing 800 μL Opti-MEM to reach a final siRNA concentration of 100 nM.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed at 55°C for 20 minutes using the Thermoscript II one-step RT-PCR Kit (Invitrogen). cDNA amplification was performed using a GeneAmpSystem 9700 thermocycler (Applied Biosystems, Warrington, UK). The reverse transcriptase was heat-inactivated in the first step of the PCR reaction (94°C for 5 minutes). The following primers were used for amplification: β-actin, 5′-ATTTGGTCGTATTGGGCGC CTGGTCACC-3′ (sense) and 5′-GAAGATGGTGATGGGATTTC- 3′ (antisense); GLUT3, 5′-CGCAACTCTATGCTTCTAGTCAA- 3′ (sense) and 5′ -ATGCCCAGCTGGTTTAGTGT- 3′ (antisense); and an insulin receptor, 5′-gtactgggagaggcaagcag- 3′ (sense) and 5′-gtgtggtggctgtcacattc- 3′ (antisense). The amplification steps were as follows: 27 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, followed by 7 minutes at 72°C. Ten microliters of product from each RT-PCR reaction were analyzed through agarose gel electrophoresis.

Immunoblot Analysis

HT-22 cells were grown in 6-well plates and subjected to serum starvation for 12 to 24 hours before treatment. After the experimental manipulations, the medium was removed, and the cells were washed twice with ice-cold PBS and lysed with 60 μL of lysis buffer. The samples were sonicated and then centrifuged for 25 minutes. The supernatants were boiled with 5 × SDS sample buffer for 5 minutes and resolved on SDS-PAGE (10%) gels and then transferred to polyvinylidene fluoride membranes, which were incubated overnight at 4°C with primary antibodies. After 6 washes in Tris-buffered saline with 0.1% Tween 20, the membranes were incubated for 1 hour with horseradish peroxidase-conjugated secondary antibodies at room temperature. The

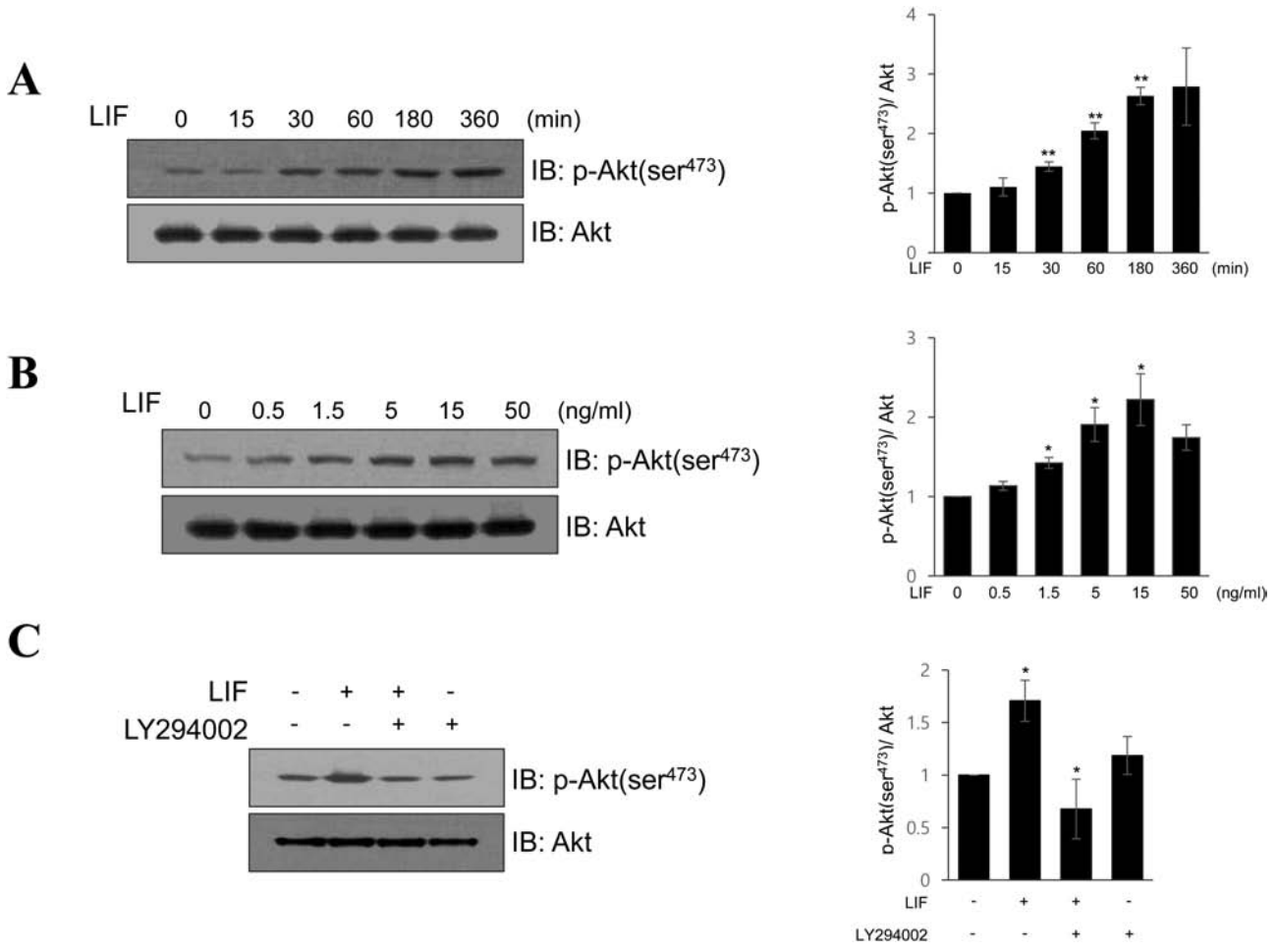

Figure 1. Leukemia inhibitory factor (LIF) stimulates Akt phosphorylation. (A) HT-22 cells were incubated with LIF (50 ng/mL) for the indicated time periods. Cell lysates were analyzed through western blot using antibodies against phospho-Akt (Ser473), with Akt serving as a control. (B) HT-22 cells were stimulated for 1 hour with several concentrations of LIF. The cell lysates were analyzed through western blot using an antibody against phospho-Akt (Ser473), with Akt serving as a control. (C) HT-22 cells were pretreated with the phosphatidylinositide 3-kinase (PI3K) inhibitor, LY294002 (10 μM, for a 30-min incubation) and then incubated with LIF for 1 hour. Cell lysates were analyzed through western blot using an antibody against phospho-Akt (Ser473), with Akt serving as a control. Experiments were performed in triplicate and repeated 3 times. Representative data are shown.

blots were washed and visualized through chemiluminescence using the Amersham Biosciences ECL western blotting detection system (Amersham International plc, Buckinghamshire, UK).

2-Deoxyglucose Uptake

HT-22 cells were seeded into 12-well plates and cultured in DMEM supplemented with 10% fetal bovine serum. After 24 hours, cells were pretreated with LIF (5 ng/mL) for 1 hour, then exposed to amyloid β1–42 (10 nM) for 48 hours. Thereafter, cells were washed with PBS and kept in prewarmed Krebs-ringer bicarbonate solution (1.2 mM $MgSO_4$, 130 mM NaCl, 130 mM KCl, 4.7 mM KH_2PO_4, 1.2 mM $CaCl_2$, 2 mM $NaHCO_3$). The uptake assay was initiated by adding 2-deoxy-D(H3)-glucose (25 mM; 10 mCi/mL) to each well and incubating for 10 minutes at 37°C and was terminated by washing the cells with ice-cold PBS. Cells were lysed in 0.5% SDS, 0.5-N NaOH to measure radioactivity.

Immunofluorescence Microscopy

Cells were seeded on 10-mm glass coverslips, fixed with 4% paraformaldehyde, and blocked/permeabilized with 0.1% Triton X-100/3% bovine serum albumin. Cells were incubated with the indicated primary antibodies. The secondary antibodies used were Alexa488–donkey anti-mouse and Alexa488–donkey anti-goat (Invitrogen). The cells were washed extensively with PBS between antibody incubations. The coverslips were mounted with immunofluorescence mounting medium. Images were recorded on a CLSM (Zeiss LSM 700) confocal microscope.

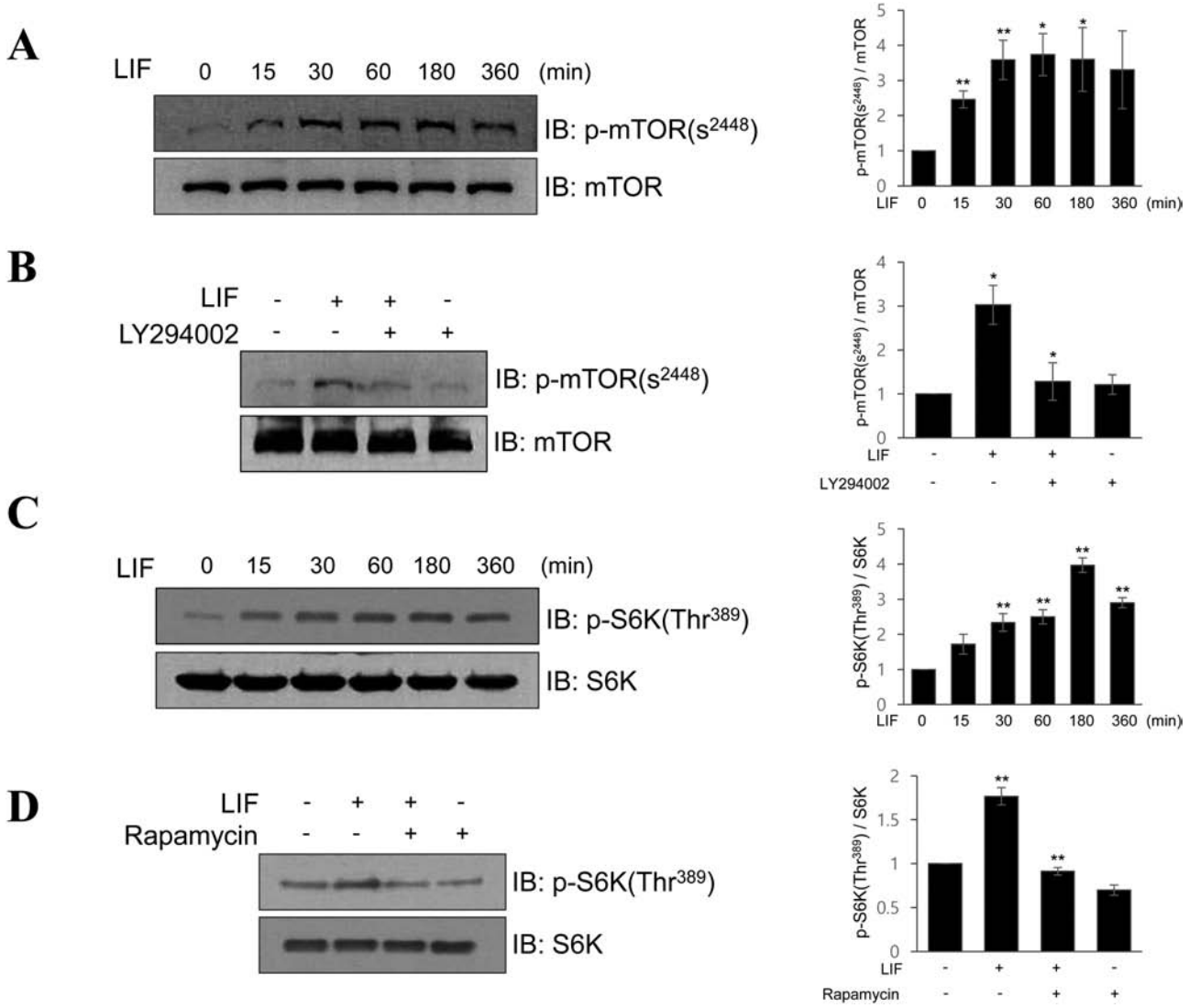

Figure 2. Leukemia inhibitory factor (LIF) stimulates the mTOR signaling pathway. (A) HT-22 cells were incubated with LIF (50 ng/mL) for the indicated times. Cell lysates were analyzed through western blot using antibodies against phospho-mTOR (Ser2448), with mTOR serving as a control. (B) HT-22 cells were pretreated with the PI3K inhibitor, LY294002 (10 μM), and then incubated with LIF for 1 hour. Cell lysates were analyzed through western blot using an antibody against phospho-mTOR (Ser2448), with mTOR serving as a control. (C) HT-22 cells were incubated with LIF (50 ng/mL) for the indicated times. Cell lysates were analyzed through western blot using antibodies against phospho-S6K (Thr389), with S6K serving as a control. (D) HT-22 cells were pretreated with the mTOR inhibitor, rapamycin (10 nM, for a 24-hour incubation) and then incubated with LIF for 1 hour. Cell lysates were analyzed through western blot using an antibody against phospho-S6K(Thr389), with S6K serving as a control. Experiments were performed in triplicate and repeated 3 times. Representative data are shown.

Primary Hippocampal Cell Preparation

Pairs of hippocampi were dissected from E17-18 mouse embryo brains. All mouse-related protocols were approved by the Institutional Animal Care and Use Committee of Korea University (KOREA-2017-0050). The meninges were thoroughly removed, and all tissue was collected in a conical tube containing Hanks' balanced salt solution at 4°C. The tissue was allowed to settle to the bottom of the tube and the supernatant was carefully removed, leaving only the tissue covered by the medium. The tissue was then enzymatically digested in trypsin-EDTA (500 μL) and Hanks' balanced salt solution (1 mL) for 10 minutes at 37°C in a water bath. The tube was gently shaken every 2 minutes and was then centrifuged for 1 minute at 1000 rpm. The supernatant was removed and the tissue was resuspended in 2 mL of 20% fetal bovine serum DMEM (1% antibiotics) by gently pipetting up and down and centrifuging for 1 minute at 1000 rpm. The supernatant was removed and the tissue was resuspended in 1 mL of neurobasal media (1% B-27, 1% L-glutamin, 1% antibiotics) by gently pipetting up and down. Cells were filtered using a cell strainer. The cells were counted using a hemocytometer, cell counter, and Trypan Blue. Approximately 2×10^5 cells were plated per well in a poly-D-lysine-coated 12-well plate. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were fed every third day by aspirating one-half of the medium from each well and replacing it with fresh medium.

Drosophila

Flies were cultured on cornmeal-based standard food on a 12-h-light/-dark (LD) cycle and maintained at 25°C with 40% to 60% relative humidity. *Elav-GAL4* (*Elav^{C155}-GAL4*) fly stocks were obtained from Bloomington *Drosophila* Stock Center (Bloomington, IN). The flies were outcrossed for 8 generations to produce a strain that was isogenic to the wild type.

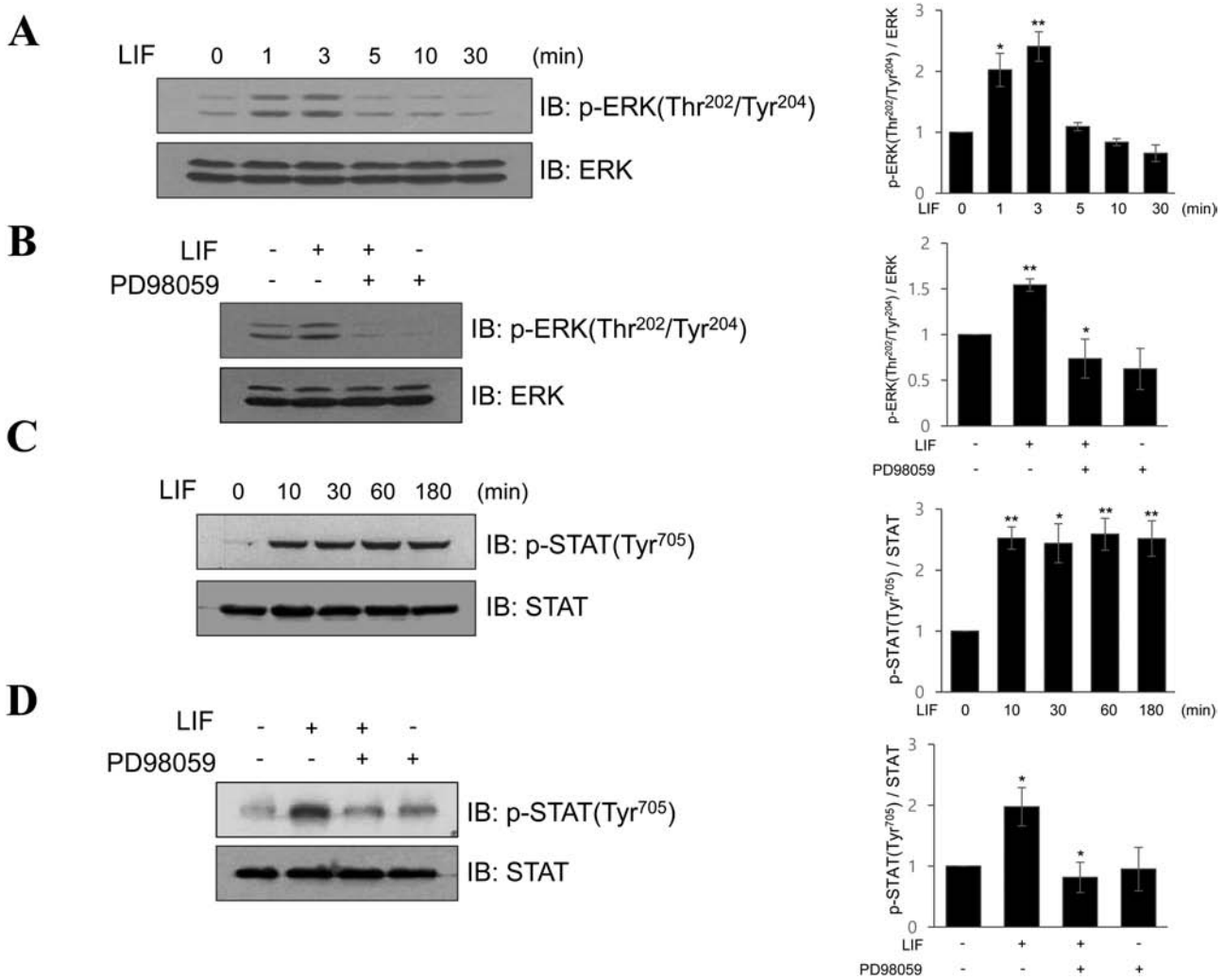

Figure 3. Leukemia inhibitory factor (LIF) is involved in neuronal activity through ERK phosphorylation. (A) HT-22 cells were incubated with LIF (50 ng/mL) for the indicated times. Lysates were analyzed through western blot using an antibody against phospho-ERK, with ERK serving as a control. (B) HT-22 cells were pretreated with the MEK inhibitor, PD98059 (50 mM, for a 30-minute incubation), and then incubated with LIF (50 ng/mL) for 3 minutes. Cell lysates were analyzed through western blot using an antibody against phospho-ERK, with ERK serving as a control. (C) HT-22 cells were incubated with LIF (50 ng/mL) for the indicated times. Lysates were analyzed through western blot using an antibody against phospho-STAT (Tyr705), with STAT serving as a control. (D) HT-22 cells were pretreated with the MEK inhibitor, PD98059 (50 mM, 30 minutes) and then incubated with LIF (50 ng/mL) for 1 hour. Cell lysates were analyzed through western blot using an antibody against phosphor-STAT(Tyr705), with STAT serving as a control. Experiments were performed in triplicate and repeated 3 times. Representative data are shown.

Statistical Analysis

In all statistical tests, *P* values <.05 are considered statistically significant. Real-time PCR and immunoblot analysis data were compared, and qualitative differences between samples were analyzed using SPSS software.

Results

LIF Stimulates Akt Phosphorylation Through PI3K

The phosphatidylinositide 3-kinase (PI3K)–AKT-mTOR signaling pathway is an important regulator of cell cycle proliferation, growth, survival, protein synthesis, and glucose metabolism (Lee et al., 2009). Akt is a key signaling molecule of the PI3K-mTOR signaling pathway. To elucidate the role of LIF in Akt activation, we examined the effects of LIF on Akt phosphorylation. Upon LIF treatment, Akt phosphorylation was increased in a time- (Figure 1A) and dose-dependent manner (Figure 1B) in HT-22 cells. The inhibition with PI3K inhibitor, LY294002, resulted in a decrease in AKT phosphorylation (Figure 1C). These results demonstrate that LIF stimulates Akt phosphorylation through PI3K in hippocampal cells.

LIF Stimulates mTOR via Akt

mTOR is downstream of Akt in the PI3K-mTOR signaling pathway and plays an important role in cell growth and protein synthesis. To confirm the role of LIF in mTOR activation, we investigated the effect of LIF on mTOR phosphorylation. Treatment with LIF significantly increased mTOR phosphorylation in a time-dependent manner (Figure 2A). We used LY294002 to confirm the involvement of Akt in this pathway. LY294002 blocked the LIF-mediated mTOR phosphorylation (Figure 2B), showing that LIF phosphorylated mTOR in a PI3K-dependent manner. LIF treatment also increased the phosphorylation of S6K downstream of mTOR (Figure 2C). We used rapamycin, an inhibitor of mTOR, to confirm the involvement of mTOR in this process. The phosphorylation of S6K was decreased by treatment with rapamycin (Figure 2D). Together, these results demonstrate

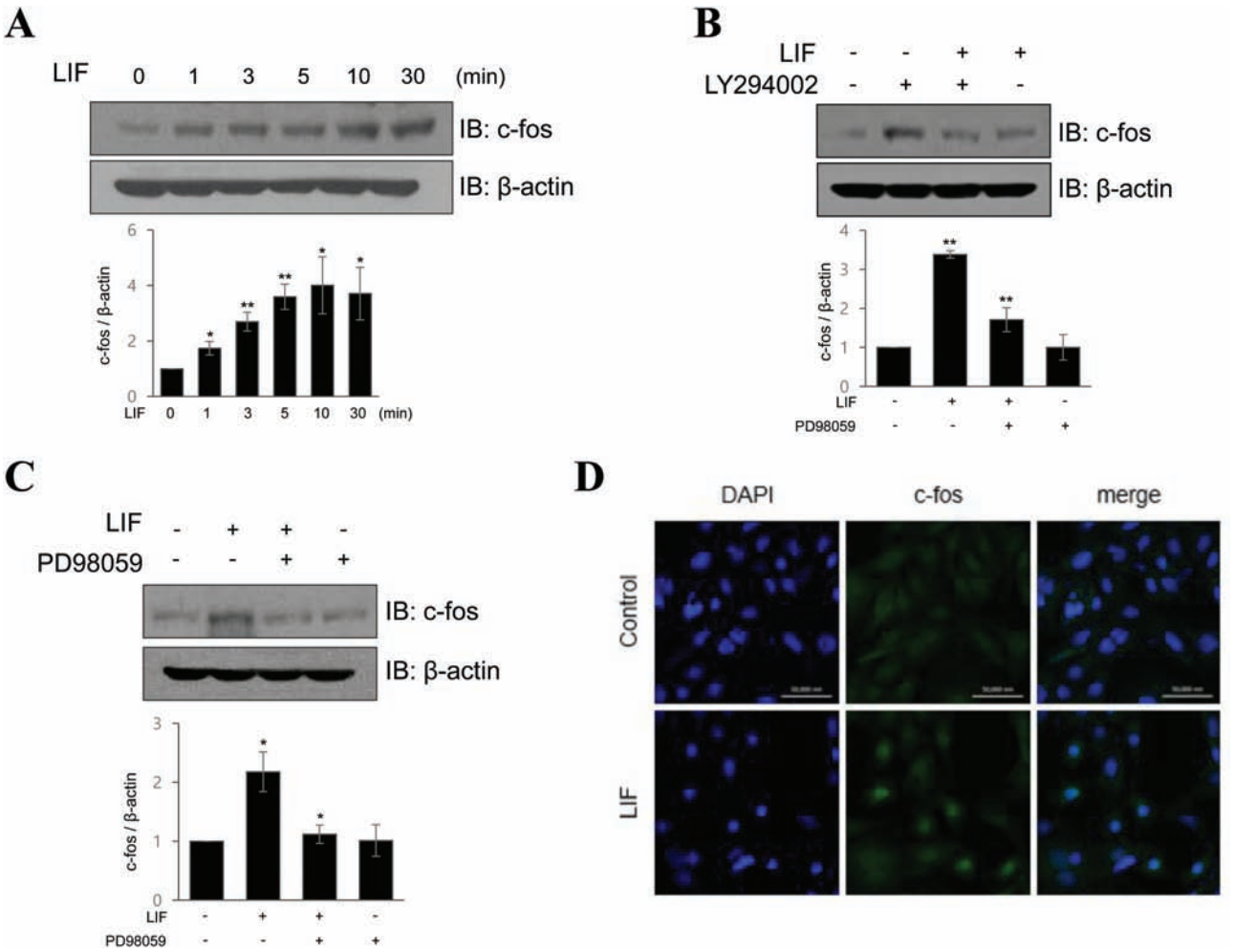

Figure 4. Leukemia inhibitory factor (LIF) stimulates neuronal activity through c-fos. (A) HT-22 cells were incubated with LIF (50 ng/mL) for the indicated times. Lysates were analyzed through western blot using an antibody against c-fos, with β-actin incubation alone serving as a control. (B) HT-22 cells were pretreated with the phosphatidylinositide 3-kinase (PI3K) inhibitor, LY294002 (10 mM, for a 30-minute incubation) and then incubated with LIF (50 ng/mL) for 15 minutes. Cell lysates were analyzed through western blot using an antibody against c-fos, with β-actin serving as a control. (C) HT-22 cells were pretreated with the MEK inhibitor, PD98059 (50 mM, for a 30-minute incubation) and then incubated with LIF (50 ng/mL) for 15 minutes. Cell lysates were analyzed through western blot using an antibody against c-fos, with β-actin serving as a control. (D) Increase in c-fos activation after LIF treatment. HT-22 cells were treated with LIF (50 ng/mL) for 15 minutes. Images were captured using a confocal microscope. Experiments were performed in triplicate and repeated 3 times. Representative data are shown.

that LIF stimulates mTOR-S6K phosphorylation through Akt phosphorylation.

LIF Increases STAT Phosphorylation via ERK

ERK regulates mitosis, proliferation, differentiation, and survival in mammalian cells during development (Widmann et al., 1999). ERK is also involved in the neural and synaptic plasticity that underlies learning and memory as well as pain hypersensitivity (Ji and Woolf, 2001; Ji et al., 2003). To gain insight into the role of ERK, we examined the effects of LIF on ERK. ERK phosphorylation was increased following LIF treatment (Figure 3A). PD98059, an MEK inhibitor, blocked the LIF-induced ERK phosphorylation (Figure 3B). This suggests that LIF phosphorylates ERK in an MEK-dependent manner. STAT3 is a transcription factor and growth factor that is activated by many cytokines and plays a key role in cell survival, proliferation, and differentiation (Wang et al., 2011). To confirm the involvement of STAT in this process, we examined the effect of LIF on STAT phosphorylation. LIF treatment increased STAT phosphorylation (Figure 3C). Phosphorylation of STAT was inhibited by an MEK inhibitor, PD98059 (Figure 3D). These results demonstrate that LIF activates STAT via ERK.

LIF Increases the Expression of c-fos Through Akt/ERK Pathways

c-fos is a known neuronal activation marker (Gao and Ji, 2009). Furthermore, ERK is known to regulate c-fos function (Monje et al., 2005). Thus, we hypothesized that LIF stimulates c-fos activation via Akt or ERK. To test this hypothesis, we examined the effects of LIF on c-fos expression. Following LIF treatment, c-fos expression increased in a time-dependent manner (Figure 4A). To confirm the involvement of Akt in c-fos activation, we performed experiments using the PI3K inhibitor, LY294002. We observed that c-fos expression was suppressed by LY294002 treatment (Figure 4B). To confirm the involvement of ERK in this process, we examined c-fos expression in the presence of PD98059. Inhibition of ERK suppressed c-fos expression (Figure 4C). Using a confocal microscope, the levels of c-fos were determined by fluorescence intensity. The fluorescence intensity was increased after LIF stimulation (Figure 4D), indicating that LIF activates c-fos through Akt/ERK pathways.

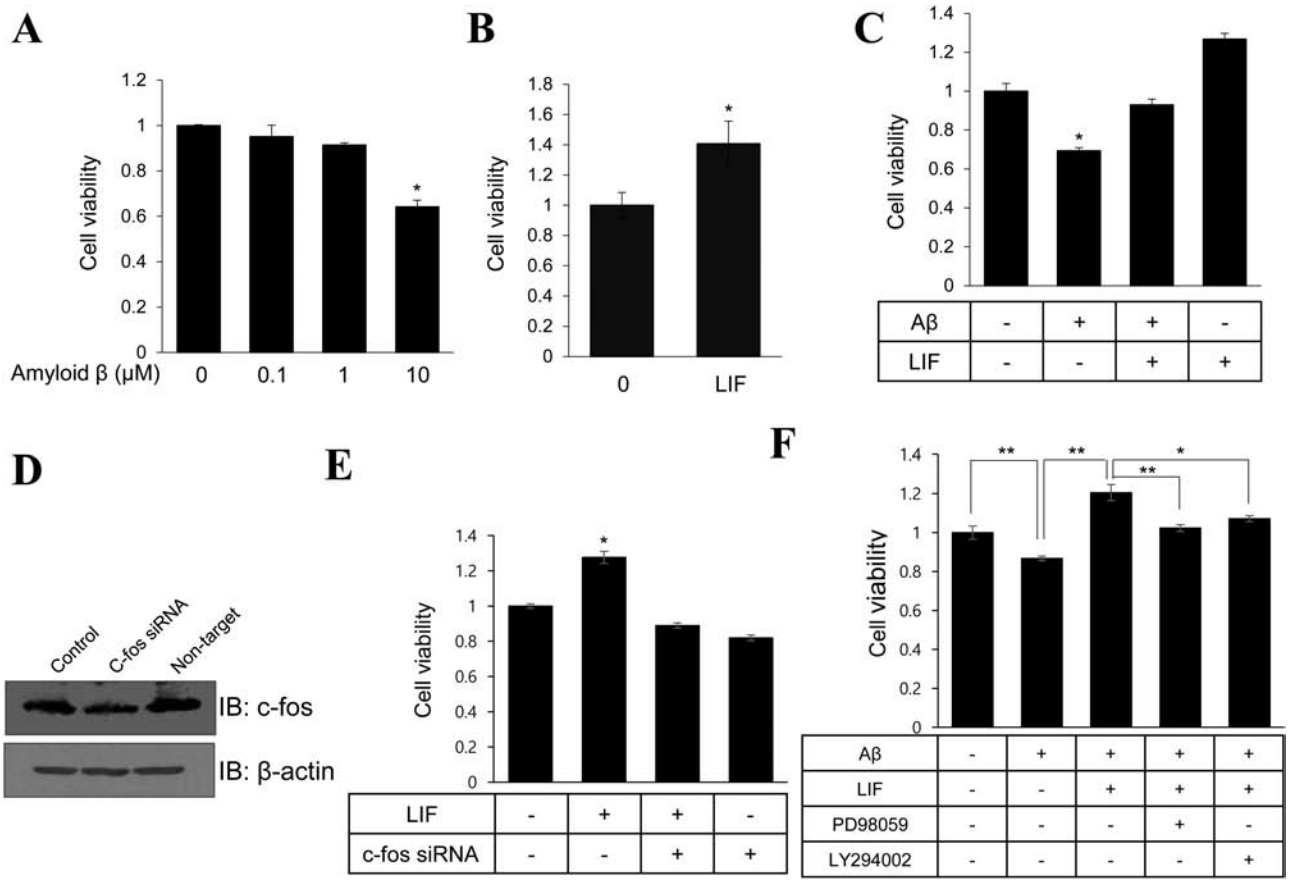

Figure 5. Leukemia inhibitory factor (LIF) increases cell viability by reducing cytotoxicity of amyloid β. (A) HT-22 cells were incubated with amyloid β at the indicated doses (for 48 hours) and then cell viability was measured. (B) HT-22 cells were incubated with LIF (50 ng/mL) for 48 hours before cell viability was measured. *P < .05 vs basal conditions. (C) HT-22 cells were pretreated with LIF for 1 hour, then incubated with amyloid β (10 μg/mL) for 48 hours, before cell viability was measured. *P < .05 vs amyloid β-treated conditions. (D) HT-22 cells were transiently transfected with c-fos siRNA (50 nM) for 48 hours. The cell lysates were analyzed through western blotting using antibody against c-fos, with β-actin serving as a control. (E) HT-22 cells were transiently transfected with c-fos siRNA (50 nM) for 48 hours and then incubated with LIF (5 ng/mL) for a further 48 hours before cell viability was measured. *P < .05 vs LIF-treated conditions. (F) Primary hippocampal cells were pretreated with LY294002 and PD98059, then incubated with indicated conditions, before cell viability was measured. **P < .01 vs amyloid β-treated conditions. Experiments were performed in triplicate and repeated 3 times. Representative data are shown.

LIF Attenuates Amyloid β-Induced Neuronal Cytotoxicity

Amyloid β is a neurotoxic substance that causes neuronal autophagy, and amyloid β accumulation in the brain causes AD. In this study, we found that c-fos was induced by LIF. Thus, we hypothesized that LIF would protect neuronal cells from amyloid β-induced neuronal toxicity. Exposure to amyloid β decreased neuronal cell viability (Figure 5A), while LIF treatment increased the viability of neuronal cells (Figure 5B). As expected, LIF treatment recovered the cell viability that was decreased by amyloid β toxicity (Figure 5C). To assay this effect further, siRNA was used to knockdown c-fos expression in HT-22 cells (Figure 5D). The LIF-induced increase in cell viability was blocked by siRNA knockdown of c-fos (Figure 5E). Inhibition with LY294002 or PD98059 suppressed LIF-induced recovery of cell viability in primary hippocampal cells (Figure 5F). These results demonstrate that LIF attenuates amyloid β-induced cell toxicity via c-fos.

LIF Recovers Amyloid β-Mediated Insulin Signal Impairment

Amyloid β accumulation leads to the dysregulation of energy metabolism, which is an early marker of AD (Querfurth and LaFerla, 2010). The brain has an abundance of GLUT3 isoforms, which are considered to be neuronal glucose transporters (Maher et al., 1994). First, we confirmed that amyloid β induces impairment of glucose uptake (Figure 6A). LIF significantly recovered the amyloid β-induced glucose uptake impairment (Figure 6B). Amyloid β also decreased insulin-mediated phosphorylation of the insulin receptor and Akt (Figure 6C). Additionally, amyloid β downregulated the mRNA expression of the insulin receptor and GLUT3. However, LIF recovered the insulin receptor and GLUT3 mRNA levels (Figure 6D). Moreover, we found that LIF restored the Akt downregulation that was caused by amyloid β (Figure 6E). These results demonstrate that LIF treatment attenuates the insulin signaling impairment that is caused by amyloid β through an increase in insulin receptor phosphorylation and GLUT3 expression.

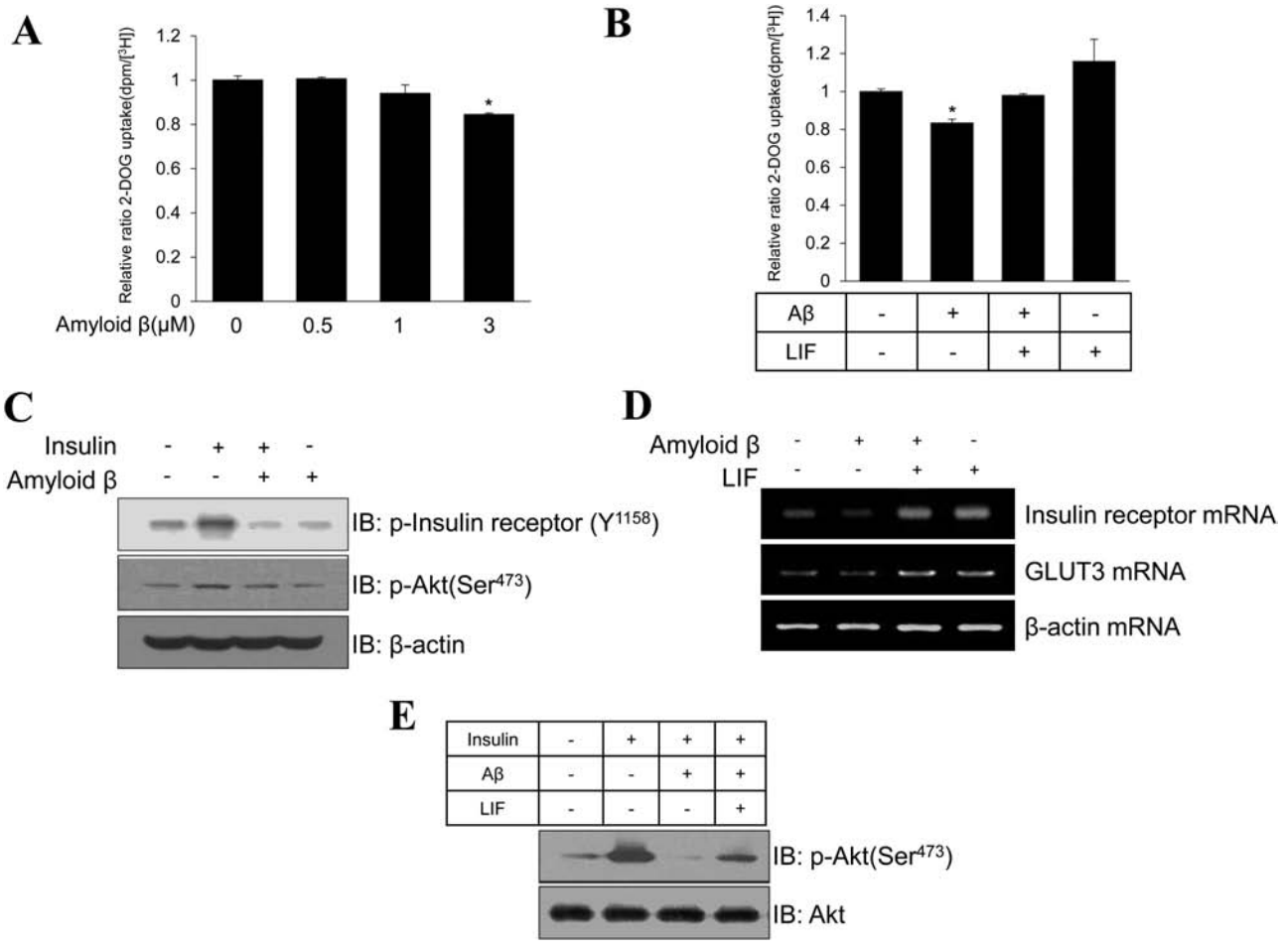

Figure 6. Leukemia inhibitory factor (LIF) enhances glucose utilization through regulation of the insulin signaling pathway (Insulin receptor, Akt, GLUT3). (A) HT-22 cells were incubated with the amyloid β at the indicated doses (for 48 hours) and then cell viability was measured. (B) HT-22 cells were pretreated with LIF for 1 hour, then incubated with amyloid β (10 μg/mL) for 48 hours, before cell viability was measured. (C) HT-22 cells were incubated with amyloid β (10 μg/mL) for 48 hours and treated with insulin (200 nM) for 1 minute. Cell lysates were analyzed through western blot using an antibody against phospho-insulin receptor, phospho-Akt (Ser473), with β-actin serving as a control. (D) To prepare mRNA, HT-22 cells were pretreated with LIF for 1 hour then incubated with amyloid β (10 μg/mL) for 48 hours, and reverse transcription-polymerase chain reaction was conducted using specific insulin receptor, GLUT3 primers. PCR products were separated on 1.5% agarose gels and visualized under ultraviolet light, with β-actin serving as a control. (E) HT-22 cells were pretreated with LIF (50 ng/mL) then incubated with amyloid β (10 μg/mL) for 48 hours and treated with insulin (200 nM) for 1 minute. Cell lysates were analyzed through western blot using an antibody against phospho-Akt (Ser473), with Akt serving as a control. *$P < .05$ vs amyloid beta-treated condition. Experiments were performed in triplicate and repeated 3 times. Representative data are shown.

LIF Prevents Amyloid β-Induced Cellular Autophagy

As amyloid β is associated with neurodegenerative diseases, we hypothesized that amyloid β causes impairment of glucose uptake and that this impairment is associated with cellular autophagy. Exposure to amyloid β increased the expression of LC3II, an autophagy marker (Figure 7A). LIF treatment prevented the LC3II induction that was caused by amyloid β (Figure 7B). The LC3II induction in response to amyloid β was also demonstrated through immunocytochemistry. The increased LC3II expression in response to amyloid β was suppressed by pretreatment with LIF (Figure 7C). These results demonstrate that LIF prevents amyloid β-induced cellular autophagy.

LIF Activates the Akt, mTOR, and ERK Signaling Pathway in Primary Cultured Hippocampal Cells

To gain insight into the role of LIF in vivo, we examined the effect of LIF on primary prepared hippocampal cells that were cultured from mouse embryos. Experiments were performed using 14-day-old cells in which neurites are fully formed (Figure 8A). The phosphorylation of Akt and S6K was higher in LIF-treated cells than in control cells (Figure 8B). The phosphorylation of ERK was also higher in LIF-treated cells than in control cells (Figure 8C). LIF recovered the reduced cell viability that was caused by amyloid β (Figure 8D). The increase in c-fos expression that was produced by LIF was blocked by LY294002 and PD98059 (Figure 8E–F). LIF prevented amyloid β-induced cellular autophagy (Figure 8G). These results suggest that LIF protects neuronal cells through the Akt, mTOR, and ERK pathway in primary prepared hippocampal cells.

LIF Reduces Autophagy Gene Expression in a *Drosophila* AD Model

We used a *Drosophila* AD model to assess the physiological relevance of our findings. We fed LIF to *Drosophila* that were exhibiting AD models over a total feeding period of 14 days from lava to adult. We then confirmed that myokine reached the brain. The expression levels of LIF in the brains of LIF-fed flies were

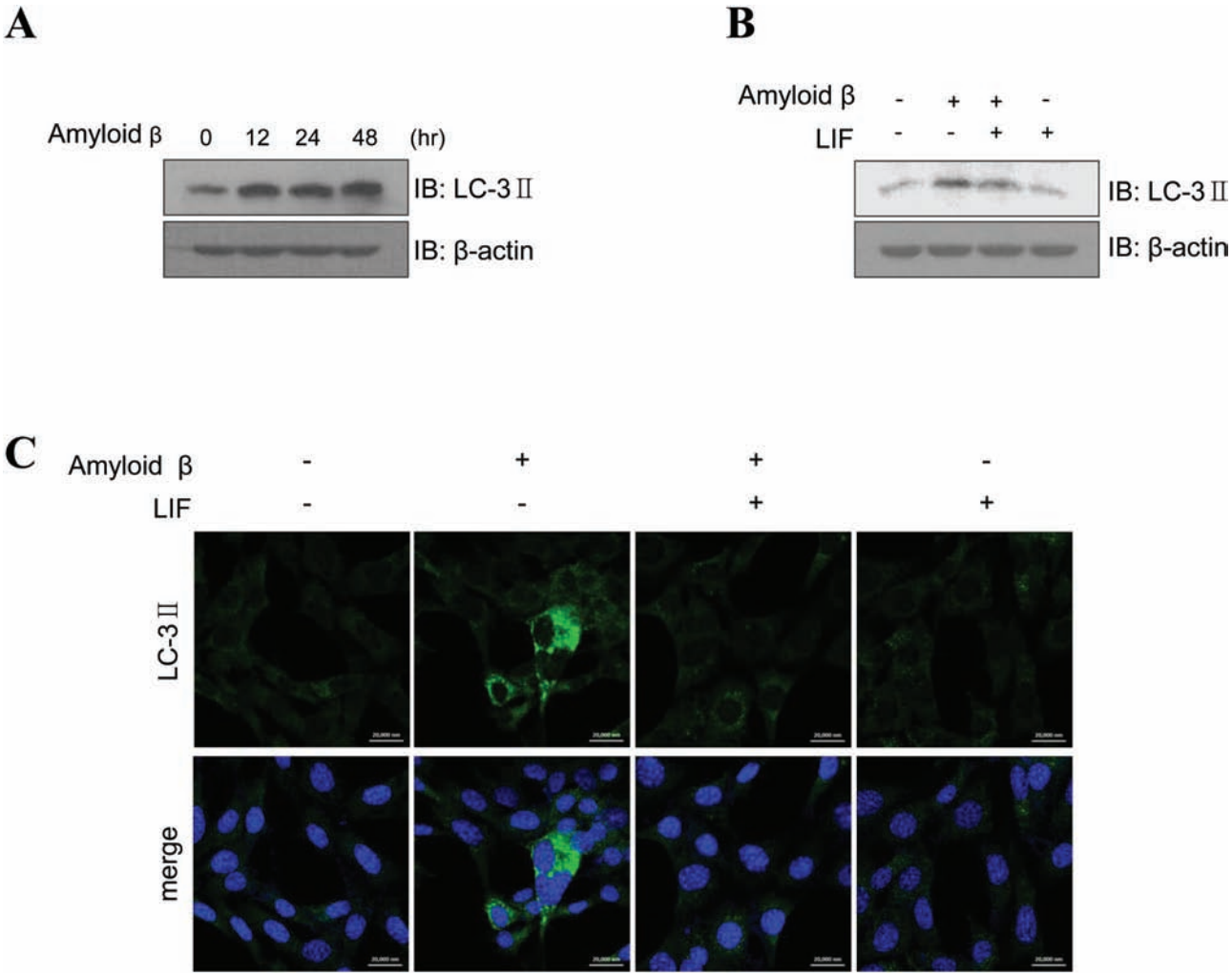

Figure 7. Leukemia inhibitory factor (LIF) prevents the cellular autophagy induced by amyloid β. (A) HT-22 cells were incubated with amyloid β (10 μg/mL) for the indicated times. Lysates were analyzed through western blot using an antibody against LC3II, with β-actin serving as a control. (B) HT-22 cells were pretreated with LIF for 1 hour, then incubated with amyloid β (10 μg/mL) for 48 hours. Lysates were analyzed through western blot using an antibody against LC3II, with β-actin serving as a control. (C) Amyloid β induces expression of intracellular autophagy genes. LIF prevents the expression of autophagy genes induced by amyloid β. HT-22 cells were pretreated with LIF for 1 hour then incubated with amyloid β (10 μg/mL) for 48 hours. Images were captured using a confocal microscope. Experiments were performed in triplicate and repeated 3 times. Representative data are shown.

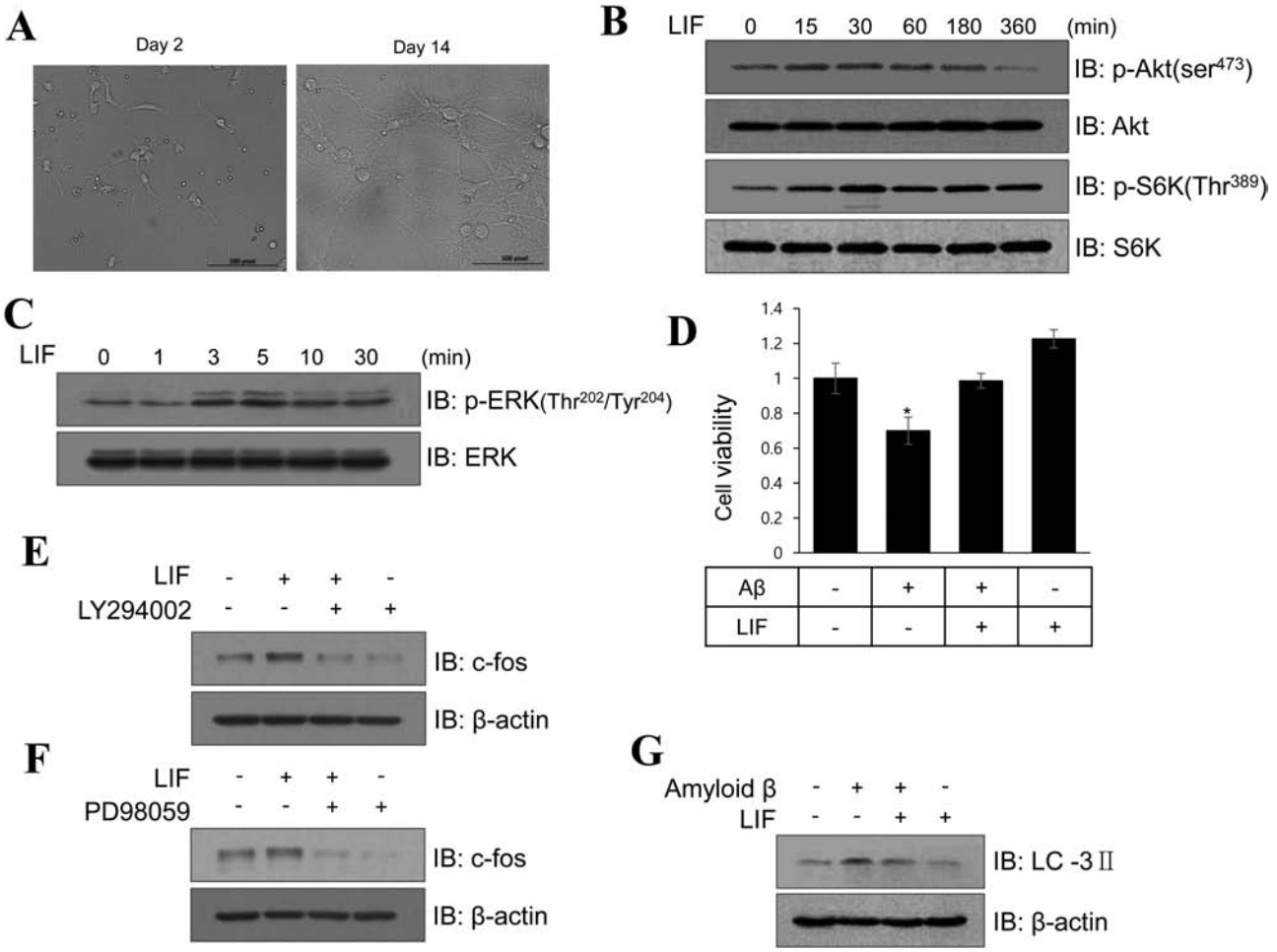

Figure 8. Leukemia inhibitory factor (LIF) activates the Akt, mTOR, and ERK signaling pathway, increases cell viability, and prevents cellular autophagy in primary hippocampal cells. (A) Culture images of primary hippocampal cells. Cells were imaged on day 1 and day 14 of culture, when cells were sufficiently differentiated. (B) HT-22 cells were incubated with LIF (50 ng/mL) for the indicated times. Lysates were analyzed through western blot using an antibody against phospho-Akt (Ser473) and phospho-S6K (Thr389), with Akt and S6K serving as controls, respectively. (C) HT-22 cells were incubated with LIF (50 ng/mL) for the indicated times. Lysates were analyzed through western blot using an antibody against phospho-ERK, with ERK serving as a control. (D) HT-22 cells were pretreated with LIF (50 ng/mL) for 1 hour, then incubated with amyloid β (10 μg/mL) for 48 hours, before cell viability was measured. (E) HT-22 cells were pretreated with the PI3K inhibitor, LY294002 (10 mM, for a 30-minute incubation), and then incubated with LIF (50 ng/mL) for 15 minutes. Cell lysates were analyzed through western blot using an antibody against c-fos, with β-actin serving as a control. (F) HT-22 cells were pretreated with the MEK inhibitor, PD98059 (50 mM, 30 minutes) and then incubated with LIF (50 ng/mL) for 15 minutes. Cell lysates were analyzed through western blot using an antibody against c-fos, with β-actin serving as a control. (G) HT-22 cells were pretreated with LIF (50 ng/mL) for 1 hour then incubated with amyloid β (10 μg/mL) for 48 hours. Lysates were analyzed through western blot using an antibody against LC3II, with β-actin serving as a control. *P < .05 vs amyloid beta-treated condition.

higher than those in non-LIF-fed flies (Figure 9A). Next, we examined the LC3II expression to confirm the effect of LIF on autophagy. In the LIF-fed flies, the level of LC3II expression in the brain was lower than in the control group according to the immunohistochemical results (Figure 9B). The expression of LC3II was reduced in LIF-fed flies (Figure 9C). These results demonstrate that LIF inhibits the expression of the autophagy gene, LC3II, in the brain in a *Drosophila* model of AD.

Discussion

Several studies have reported that LIF levels are elevated in patients with AD (Rensink et al., 2002; Soilu-Hanninen et al., 2010). It was hypothesized that amyloid β triggers LIF, which, in turn, induces inflammatory reactions in AD. Conversely, it has also been reported that LIF is not detected in patients with AD (Galimberti et al., 2008). A neuroprotective role of LIF through TNF-α-induced oligodendrocyte apoptosis has been proposed (Vanderlocht et al., 2006). These findings show that the relationship between LIF and AD is not yet clear. This controversy may be addressed by elucidating the expression patterns of the LIF receptor, which is composed of 2 components, the LIF receptor β and the gp130 (Turnley and Bartlett, 2000). LIF has a wide array of actions in multiple cell types, depending on the pattern of receptor expression. The expressions of LIF and LIFR β vary in different brain regions. The expressions of LIF and LIFR in the brain are inverted, except in the hippocampus of patients with AD, where the expression patterns of these proteins are parallel (Soilu-Hanninen et al., 2010). Further research is required to reveal the biological function of LIF and to explain the correlation between LIF expression and glucose levels. In this study, we demonstrated that LIF, a novel myokine, stimulated c-fos via the ERK and Akt pathway in hippocampal cells. Moreover, LIF treatment enhanced neuron cell viability. These results suggest that LIF plays a neuroprotective role in AD. At the same time, there are articles that showed that c-fos was implicated in

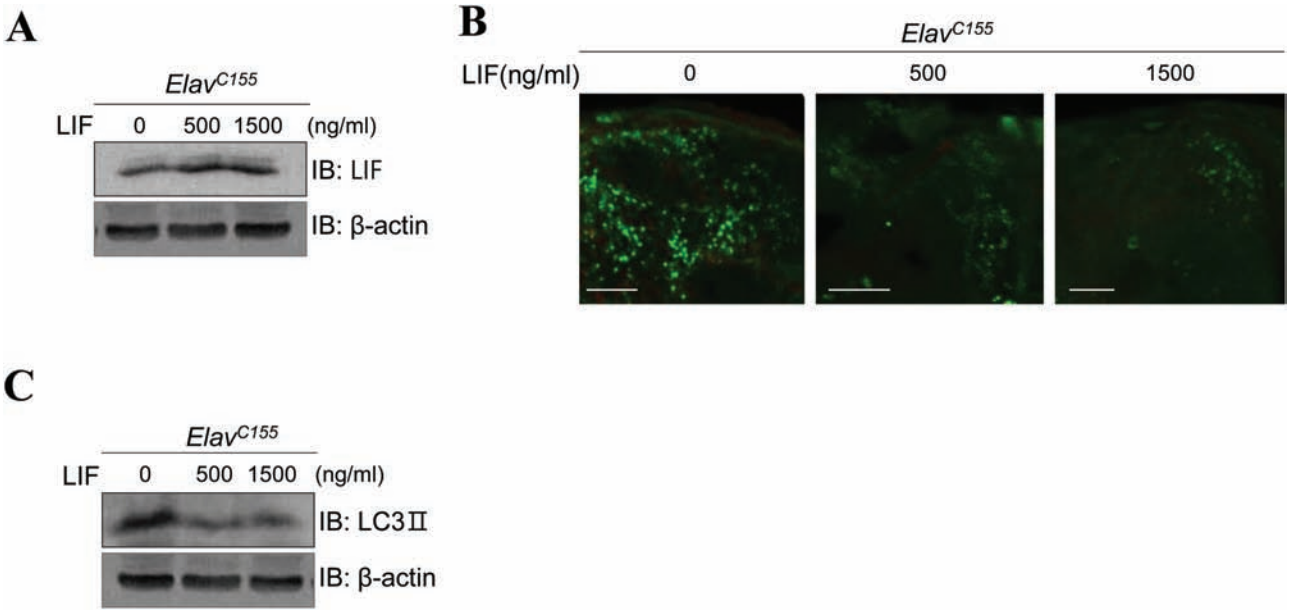

Figure 9. Leukemia inhibitory factor (LIF) reduces autophagy gene expression in a *Drosophila* model of Alzheimer's disease. (A) Amyloid β expressing *Drosophila* were fed LIF (500 or 1500 ng/mL) for 14 days. Fly head lysates were then analyzed through western blot using an antibody against LIF, with β-actin serving as a control. *P < .05 vs basal condition. (B) Decreased autophagy gene expression was observed in the LIF-fed group. Amyloid β-expressing flies were fed LIF (500 or 1500 ng/mL) for 14 days. Images were captured using a confocal microscope. *P < .05 vs basal condition. (C) Amyloid β-expressing flies were fed LIF (500 or 1500 ng/mL) for 14 days. Fly head lysates were then analyzed through western blot using an antibody against LC3II, with β-actin serving as a control. *P < .05 vs basal condition (scale bar = 50 μm).

amyloid beta-related AD or neurotoxicity (Marcus DL et al., 1998; Gillardon et al., 1996). Combined with the previous knowledge of the role of c-fos in the neuron, it can be suggested that LIF may be implicated in neuronal function via more complicated ways. Further study is necessary to explain at the molecular level.

Varieties of myokines have been subjected to drug development trials for the treatment of several diseases. For example, LIF increases neurotrophin expression and corticospinal axon growth in rats with central nervous system injuries (Blesch et al., 1999). LIF also promotes locomotor function recovery after spinal cord injury in mice (Zang and Cheema, 2003). LIF has also been shown to increase the number of nestin-positive cells in the brain in a mouse model of Parkinson's disease (Liu et al., 2013). In clinical trials, LIF may be used as a predictor of reproductive success (Mikolajczyk et al., 2007) as well as for preventing chemotherapy-induced peripheral neuropathy (Davis et al., 2005). However, it has also been shown that LIF does not increase the likelihood of implantation or pregnancy outcomes in women with recurrent implantation failure (Brinsden et al., 2009).

So far, few myokines have been successfully developed as drugs. To successfully apply myokine therapeutically to actual patients, several limitations must be overcome. First, the myokines must be purified as pure proteins or peptides. Second, the myokines must be nontoxic and overcome genetic and immune problems. Third, the effects that are induced by myokines must act specifically only on the targeted tissues. Finally, myokines must have a short time of viability in the circulation after influencing the targeted tissues. In the case of LIF, additional issues must be resolved.

The BBB is an important neurovascular interface between the central nervous system and the capillary and postcapillary venules that provide circulation. The permeability of a myokine is an important factor to consider if the myokine is to be applied in a clinical context. LIF is known to cross the BBB (Pan et al., 2000). Exogenous LIF affects the survival of oligodendrocytes in animals with experimental autoimmune encephalomyelitis (Butzkueven et al., 2002) and in patients with amyotrophic lateral sclerosis (Kurek et al., 1998). These results suggest that LIF may be a beneficial treatment regimen for AD in vivo. Moreover, the transport of LIF across the blood-spinal cord barrier is upregulated after spinal cord injury (Pan et al., 2006), indicating that the increased permeability of LIF is not explained solely by barrier disruption but that the LIFR expression status is also a factor. LIF, at 20 kDa polypeptides, is not much larger than other myokines and would be an important target for therapeutic modulation if it is stable in blood. In this study, we demonstrated that autophagy is inhibited by LIF treatment in a *Drosophila* model of AD. However, because it may not remain stable, LIF is capable of influencing neuronal tissues only for short time periods, which critically impairs its potential as a treatment for AD. If these limitations can be overcome, LIF will be a potential treatment drug for AD.

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Statement of Interest

None.

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