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REGULAR RESEARCH ARTICLE

Electroconvulsive Seizures Induce Autophagy by Activating the AMPK Signaling Pathway in the Rat Frontal Cortex

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Abstract

Background: It is uncertain how electroconvulsive therapy-induced generalized seizures exert their potent therapeutic effects on various neuropsychiatric disorders. Adenosine monophosphate-activated protein kinase (AMPK) plays a major role in maintaining metabolic homeostasis and activates autophagic processes via unc-51-like kinase (ULK1). Evidence supports the involvement of autophagy system in the action mechanisms of antidepressants and antipsychotics. The effect of electroconvulsive therapy on autophagy-related signaling requires further clarification.
Methods: The effect of electroconvulsive seizure on autophagy and its association with the AMPK signaling pathway were investigated in the rat frontal cortex. Electroconvulsive seizure was provided once per day for 10 days (E10X), and compound C or 3-methyadenine was administered through an intracerebroventricular cannula. Molecular changes were analyzed with immunoblot, immunohistochemistry, and transmission electron microscopy analyses.
Results: E10X increased p-Thr172-AMPKα immunoreactivity in rat frontal cortex neurons. E10X increased phosphorylation of upstream effectors of AMPK, such as LKB1, CaMKK, and TAK1, and of its substrates, ACC, HMGR, and $GABA_BR2$. E10X also increased p-Ser317-ULK1 immunoreactivity. At the same time, LC3-II and ATG5–ATG12 conjugate immunoreactivity increased, indicating activation of autophagy. An intracerebroventricular injection of the AMPK inhibitor compound C attenuated the electroconvulsive seizure-induced increase in ULK1 phosphorylation as well as the protein levels of LC3-II and Atg5–Atg12 conjugate. Transmission electron microscopy clearly showed an increased number of autophagosomes in the rat frontal cortex after E10X, which was reduced by intracerebroventricular treatment with the autophagy inhibitor 3-methyadenine and compound C.
Conclusions: Repeated electroconvulsive seizure treatments activated in vivo autophagy in the rat frontal cortex through the AMPK signaling pathway.

Keywords: adenosine monophosphate-activated protein kinase, autophagy, electroconvulsive therapy

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Significance Statement

Electroconvulsive therapy (ECT) has been widely used to treat depression, schizophrenia, bipolar disorder and the neuropsychiatric symptoms of various neurological disorders. However, it is uncertain how ECT-induced generalized seizures exert their potent therapeutic effects on various neuropsychiatric disorders. Autophagy is a basic cellular process that preserves the balance among the synthesis, degradation, and recycling of cellular components; therefore, it is essential for neuronal survival and function. Autophagy is thought to predispose individuals to psychotic symptoms, mood disorders, and various behavioral changes. Adenosine monophosphate-activated protein kinase (AMPK) is a serine–threonine kinase that plays a major role in maintaining metabolic homeostasis, and it plays a critical role in the autophagy process. Our results demonstrated that repeated ECT treatments activated autophagy in the rat frontal cortex through the AMPK signaling pathway in vivo. The findings suggest that AMPK-related autophagy induction may play a role in mediating the superior efficacy of ECT.

Introduction

Electroconvulsive therapy (ECT) has been widely used to treat depression, schizophrenia, bipolar disorder, and the neuropsychiatric symptoms of various neurological disorders. The therapeutic effect of ECT is dependent on the induction of generalized seizures, and repeated treatments are required to induce the therapeutic actions of ECT (Lisanby, 2007; Weiner and Reti, 2017). Repeated electroconvulsive seizure (ECS) treatment, an animal model of ECT, induces cellular proliferation, synaptic modifications, increased expression of neurotrophic factors, and changes in the activity of intracellular signaling molecules (Segi-Nishida, 2011), resulting in the long-term plastic changes induced by ECS. However, the mechanism of action whereby ECT-induced generalized seizures alter brain physiology and relieve the symptoms of psychiatric and neurological disorders needs further clarification.

Autophagy is an intracellular process that delivers cytoplasmic components to the lysosomes for degradation (Mizushima and Komatsu, 2011; Menzies et al., 2017). Autophagy is a basic cellular process that maintains homeostasis; preserves the balance among the synthesis, degradation, and recycling of cellular components; and selectively targets dysfunctional organelles, intracellular microbes, and pathogenic proteins (Levine and Kroemer, 2019). Therefore, impairments in the autophagic process in neurons lead to intracellular accumulation of toxic materials and disrupt homeostasis, which induces dysfunction and death of neurons (Nixon, 2013; Polajnar and Zerovnik, 2014). Impaired autophagy is associated with neurodegenerative diseases, such as Parkinson's disease and Huntington's disease (Nixon, 2013; Menzies et al., 2017), for which ECT has demonstrated beneficial therapeutic effects (Beale et al., 1997; Mughal et al., 2011; Narang et al., 2015). A dysfunctional autophagic process can disrupt neuronal cell biology, which predisposes individuals to psychotic symptoms, mood disorders, and various behavioral changes (Polajnar and Zerovnik, 2014).

Repeated ECS treatments have been reported to enhance autophagy signaling in the rat hippocampus, which may be related to ECS-induced expression of brain-derived neurotrophic factor (BDNF) (Otabe et al., 2014). However, it has also been reported that BDNF signaling suppresses autophagy in the forebrain of adult mice (Nikoletopoulou et al., 2017). Otabe et al. (2014) suggested the possible involvement of Akt/mammalian target of rapamycin (mTOR) signaling in the activation of autophagy signaling by ECS, but the mechanisms were not investigated in detail. In our previous studies, ECS was found to affect several signaling pathways, including Akt-glycogen synthase kinase 3β (Roh et al., 2003; Kang et al., 2004) and extracellular signal-regulated kinase1/2 (ERK1/2) (Kang et al., 1994, 2006). ERK1/2 and Akt are important regulators of mTOR and interact with the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway to regulate major cellular functions, including autophagy (Corcelle et al., 2007; Heras-Sandoval et al., 2014). Evidence indicates that AMPK is a crucial component of the autophagy process (Poels et al., 2009). The antipsychotic agent clozapine activates the AMPK signaling pathway (Kim et al., 2012) and induces autophagy via the AMPK–unc-51-like kinase (ULK1)–Beclin1 signal pathway in the rat frontal cortex (Kim et al., 2018).

AMPK is activated primarily through phosphorylation of the Thr172 residue in its catalytic α subunit by upstream regulators, including liver kinase B1 (LKB1), Ca^{2+}/calmodulin-dependent protein kinase kinase-β (CaMKK-β), and transforming growth factor-beta-activated kinase-1 (TAK-1) (Woods et al., 2003, 2005; Xie et al., 2006; Hardie et al., 2016). Activation of AMPK promotes autophagy through ULK1, which is the mammalian homologous protein of the Atg1 gene. ULK1 is part of a protein complex consisting of Atg13, Atg101, and FIP200 (Lin and Hurley, 2016). AMPK directly activates ULK1 through phosphorylation, which initiates autophagy (Egan et al., 2011b; Kim et al., 2011).

This study examined whether repeated ECS treatments induced autophagy in vivo and investigated the AMPK signaling pathway, including both upstream kinase and substrates as the underlying molecular mechanisms of ECS-induced autophagy in the rat frontal cortex. The frontal cortex is one of the major brain regions implicated in the pathogenesis and treatment of depressive disorder and schizophrenia (Drevets et al., 1997; MacDonald et al., 2005), and ECS-induced autophagy signaling has been reported only in the hippocampus (Otabe et al., 2014). Autophagy is differentially regulated in different brain regions (Nikoletopoulou et al., 2017). Repeated ECS treatments induced autophagy in the rat frontal cortex via the AMPK signaling pathway in vivo.

Methods

Animals and ECS Treatment

The animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and formal approval to conduct this experiment was obtained from the Animal Subjects Review Board of Dongguk University Hospital. Male Sprague-Dawley rats (150–200 g, 6 weeks old) were housed for 1 week before the experiments and maintained under a 12-hour-light/-dark cycle with food and water available ad libitum. Rats were divided into 2 groups: sham (sham treatment for 10 days) and E10X (daily ECS for 10 days). The ECS and sham groups were treated once per day for 10 days at the same time (12:00 PM to 1:00 PM) for a total of

10 treatments. The rats were decapitated 24 hours after the last treatment, and their frontal cortices were dissected.

As previously reported (Park et al., 2014), ECS was administered to the rats via ear-clip electrodes and a pulse generator (UgoBasile ECT Unit-57800-001; UgoBasile, VA, Italy) at a frequency 100 pulses/s, with a pulse width of 0.5 milliseconds, shock duration of 0.5 seconds, and current of 55 mA. Sham-treated control animals were handled in the same fashion as the ECS treatment group, but no electric current was delivered. An ECS-induced seizure was validated by observing general convulsions consisting of tonic and clonic phases and measuring the duration of the convulsions. ECS-treated animals that underwent a generalized convulsion for more than 30 seconds were included for further analysis.

Intracerebroventricular Injection of Compound C and 3-MA

Intracerebroventricular (i.c.v.) cannulae were placed to perform intrabrain injections of compound C (also called dorsomorphin), an AMPK inhibitor (Zhou et al., 2001), and 3-methyladenine (3-MA), an autophagy inhibitor (Seglen and Gordon, 1982; Petiot et al., 2000). The i.c.v. cannula placement surgery was performed as described previously (Kim et al., 2008). The rats were anesthetized with an i.p. injection of tribromoethanol (Avertin; Sigma-Aldrich, St. Louis, MO) at a dose of 250 mg/kg and mounted on a stereotaxic apparatus (Narishige, Tokyo, Japan). The skull was exposed, and a hole was drilled to place a guide cannula (21 gauge; Plastics One, Roanoke, VA) into the lateral ventricle (Bregma coordinates: AP –1 mm, ML 2.5 mm, DV 3.5 mm) (Paxinos and Watson, 1998). Guide cannulae were anchored with dental cement and capped with dummy cannulae (Plastics One) until compound C or 3-MA was injected i.c.v. Verification of cannula placements was made by visual observation when dissecting the rat brain, and the verified samples were included in the analysis.

Compound C or 3-MA was administered through the i.c.v. cannula for 1 minute using a syringe pump (KD Scientific, Holliston, MA). A 5-μL aliquot of compound C (Millipore, Bedford, MA; 25 nM) or vehicle (20% dimethyl sulfoxide) was administered 1 hour before decapitating the rats. A 5-μL aliquot of 3-MA (0.6 μM; Sigma-Aldrich) or its vehicle (0.9% saline) was injected i.c.v. 24 hours before decapitation. The experimental method used for compound C was based our previous study (Kim et al., 2018), and that for 3-MA was based on a preliminary experiment referenced in the previous study (Gao et al., 2012). The rats were decapitated, and the frontal cortices were dissected and used for further analyses.

Immunoblot Analysis

The dissected frontal cortical tissues were homogenized as described previously (Kim et al., 2008; Park et al., 2014). After centrifugation at 20000 × *g* for 20 minutes at 4°C, the supernatants were boiled in Laemmli sample buffer. The immunoblot analysis was performed as described previously (Kim et al., 2008; Park et al., 2014). The membranes were incubated with primary antibodies overnight at 4°C, followed by a second incubation with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories Inc., West Grove, PA). We used the following primary antibodies specific for the following molecules, which are presented with their molecular weight, Research Resource Identifier, and dilution ratio, respectively. Primary antibodies specific for *β-actin* (42 kDa, AB_330331, 1:10000) (Sigma-Aldrich), *AMPK* (62 kDa, AB_330331, 1:1000), *ACC* (280 kDa, AB_2219397, 1:1000), *microtubule-associated protein 1 light chain 3* (14~16 kDa, LC3, AB_2137703, 1:1000), *autophagy protein 5* (ATG5, 55 kDa, AB_10829153, 1:1000), *CaMK4* (60 kDa, AB_2068389, 1:1000), *TAK1* (82 kDa, AB_490858, 1:1000), *p-Thr172-AMPKα* (62 kDa, AB_331250, 1:1000), *p-Ser317-ULK1* (160 kDa, AB_2687883, 1:1000), *p-Ser79-ACC* (280 kDa, AB_330337, 1:1000), *p-Thr184/187-TAK1* (82 kDa, AB_561317, 1:1000) (Cell Signaling Technology, Beverly, MA), *ULK1* (160 kDa, AB_2214706, 1:500), *HMG-CoA reductase* (HMGR, 97 kDa, AB_2118193, 1:1000), *metabotropic $GABA_B$ receptor subtype 2* ($GABA_BR2$, 102 kDa, AB_640747, 1:000), *LKB1* (52kDa, AB_2198344, 1:500), *p-Ser431-LKB1* (52kDa, AB_2198347, 1:500), *p-Thr196-CaMK4* (60 kDa, AB_2068399, 1:500) (Santa Cruz Biotechnology, Santa Cruz, CA), *p-Ser783-$GABA_BR2$* (102 kDa, AB_2492117, 1:1000) (PhosphoSolutions, Aurora, CO), and *p-Ser872-HMGR* (97 kDa, AB_10925160, 1:1000) (Biorbyt, Cambridge, UK) were used. The membranes were developed using the enhanced chemiluminescence system (Pierce Biotechnology, Rockford, IL) and were then exposed to X-ray film (AGFA CurixRPI, Mortsel, Belgium). Immunoblot signals on developed X-ray film were quantified with the TINA program, version 2.10G (Raytest, Straubenhardt, Germany).

Immunohistochemistry

Immunohistochemistry was performed using a free-floating method. Rats were anesthetized with urethane (1.5 g/kg, i.p.) 24 hours after the last ECS treatment and perfused intracardially with 0.1 M phosphate-buffered saline (PBS) (pH 7.4) followed by 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M PBS (pH 7.4). The brains were sectioned at 25 μm on a cryostat (Leitz, Wetzlar, Germany) and immediately immersed in a cryoprotectant of 50% glycerol in 0.1 M PBS. Immunohistochemistry was performed with the ABC system (Invitrogen, Carlsbad, CA). The sections were washed with and incubated in 0.3% H_2O_2 for 30 minutes to quench endogenous peroxidase activity. After extensive washing with 0.1 M PBS, the sections were blocked with 5% normal goat serum at room temperature for 30 minutes then incubated overnight with primary antibodies against p-AMPK (Thr172) or p-ULK1 (Ser317) (Cell Signaling Technology) at a dilution of 1:500 at 4°C. The sections were incubated with biotinylated secondary antibodies and then incubated with horseradish peroxidase-conjugated streptavidin. The signals were detected using a 3, 3'-diaminobenzidine substrate. Subsequently, the sections were mounted with DPX Mountant (Fluka, Basel, Switzerland). Images of the prefrontal, orbital, cingulate, and insular cortices were obtained according to the rat brain atlas (Paxinos and Watson, 1998) using an Olympus microscope connected to a Leica DFC280 digital camera with software (Leica Application Suite V3, Wetzlar, Germany) under a 40× microscope objective, and all the p-AMPK- or p-ULK1-positive cells in the 300- × 400-μm areas of each brain region were counted for the quantitative analysis.

Double-Labeled Immunofluorescence

The sections were blocked for 1 hour with 3% bovine serum albumin in tris-buffered saline/Tween 20 and incubated with primary antibodies (1:100 anti-p-AMPK [Thr172] and 1:100 anti-neuronal nuclear protein [Neu-N; Chemicon, Temecula, CA]) at 4°C overnight. After washing in TBST, the sections were incubated with secondary antibodies (1:100 Alexa Fluor488 and 555; Molecular Probes, Eugene, OR) for 1 hour at room temperature.

Next, the sections were fluorescently labeled, and the nuclei were stained with 4',6'-diamidino-2-phenylindole (Sigma-Aldrich). Then the sections were mounted with DPX Mountant and analyzed on a Meta confocal microscope (Model LSM 510; Carl Zeiss MicroImaging, Inc., Jena, Germany) equipped with 4 lasers (Diode 405, Argon 488, HeNe 543, and HeNe633). Each channel was separately scanned using a multitrack photomultiplier tube configuration to avoid crosstalk between fluorescent labels. Images were acquired and 3-dimensionally reconstructed using Zeiss LSM software.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was conducted with the rat frontal cortical sections. The brains were fixed overnight in a mixture of cold 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and 2% paraformaldehyde in 0.1 M phosphate or cacodylate buffer (pH 7.2), and tissue blocks (1 mm^3) were obtained from the prefrontal cortical regions. The tissues were post-fixed for 1.5 hours in 2% osmium tetroxide in 0.1 M phosphate or cacodylate buffer for 1.5 hours at room temperature. The samples were washed briefly with deuterated H_2O_2; dehydrated throughout a graded 50, 60, 70, 80, 90, 95, and 100% ethanol (×2) series; infiltrated with propylene oxide and EPON epoxy resin mix (Embed 812, Nadic methyl anhydride, poly Bed 812, dodecenyl succinic anhydride, dimethylaminomethyl phenol; Polysciences, Warrington, PA), and embedded with epoxy resin only. The epoxy resin-mixed samples were loaded into capsules and polymerized at 38°C for 12 hours and 60°C for 48 hours. Sections for light microscopy were cut at 500 nm and stained with 1% toluidine blue for 45 seconds on a hot plate at 80°C. Thin sections were made using an ultramicrotome (RMC MT-XL) and collected on a copper grid. Areas appropriate for thin sectioning were cut at 65 nm and stained with saturated 6% uranyl acetate and 4% lead citrate before examination by TEM (JEM-1400; Tokyo, Japan) at 80 Kv.

Statistical Analyses

The immunoblot results are expressed as relative optical densities (ODs) (i.e., the OD percentages relative to sham control mean values). Results are presented as mean ± SEM. The mean relative immunoreactivity ODs on the immunoblot and the number of immunoreactive-positive cells identified by immunohistochemistry were compared between the sham and E10X groups using an independent *t* test. $P < .05$ was considered significant. The immunoreactivity ODs from compound C- and/or E10X-treated samples were analyzed with respect to compound C and the E10X treatments using 2-way ANOVA, and the comparison between groups was performed with a Tukey's post hoc test; a Bonferroni-adjusted *P* value < .008 for multiple comparisons was considered significant. All tests were performed using SPSS 19.0 for Windows (IBM SPSS, Inc., Chicago, IL).

Results

Repeated ECS Treatments Increase AMPK (Thr172) Phosphorylation Levels in Rat Frontal Cortex

Phosphorylation of the AMPK α subunit at Thr172 reflects the catalytic activity of AMPK (Carling, 2004). The immunoreactivity of p-Thr172-AMPKα increased significantly at 24 hours after E10X compared with the sham-treated control group ($t = 10.241$, $P < .001$). The total protein level of AMPK was unchanged. β-Actin immunoreactivity was demonstrated as a loading control (Figure 1A).

An immunohistochemical analysis was performed to investigate the detailed anatomical localization of p-Thr172-AMPKα-positive cells within the frontal cortical regions after E10X. The density of p-Thr172-AMPKα-positive neurons increased significantly after E10X. The number of p-Thr172-AMPKα-positive cells increased significantly in the E10X-treated animals in the prefrontal (Fr; $t = 7.526$, $P = .001$), orbital (lateral orbital [LO] and ventrolateral orbital [VLO]; $t = 2.9674$, $P = .024$), insular (anterior insular [AI]; $t = 2.912$, $P = .020$), and cingulate (Cg; $t = 3.646$, $P = .014$) cortices (Paxinos and Watson, 1998) (Figure 1B). The immunoreactivity of p-Thr172-AMPKα was co-localized with that of Neu-N, a neuronal cell marker, but was not co-localized with 4',6'-diamidino-2-phenylindole immunoreactivity, a fluorescence stain that labels cell nuclei by binding to DNA (Figure 1C). These findings demonstrate that increased phosphorylation of AMPKα after the E10X treatment occurred in the cytoplasm of neurons in the rat cortical regions, including the prefrontal, cingulate, orbital, and insular cortices.

Repeated ECS Treatments Increase Phosphorylation Levels of LKB (Ser431), CaMK4 (Thr196), and TAK1 (Thr184/187) in Rat Frontal Cortex

LKB1 acts as a master upstream kinase activating AMPK and can be identified by the phosphorylation level of LKB1 at Ser431 (Woods et al., 2003). The E10X treatments increased p-Ser431-LKB1 immunoreactivity in the rat frontal cortex compared with the sham group ($t = 6.000$, $P < .001$). CaMKKβ is also well characterized as an upstream kinase of AMPK (Woods et al., 2005), and CaMKKβ activity can be determined by the phosphorylation level of CaMK4 at Thr196 (Swulius and Waxham, 2008). The E10X treatment also increased p-Thr196-CaMK4 immunoreactivity ($t = 22.296$, $P < .001$). TAK1 kinase is another direct upstream kinase of AMPK (Xie et al., 2006). The immunoreactivity of p-Thr184/187-TAK1 was also increased in the E10X group compared with the sham group ($t = 4.839$, $P = .003$). The immunoreactivity of total LKB1, CaMK4, and TAK1 remained unchanged (Figure 2A).

Repeated ECS Treatments Increase Phosphorylation Levels of ACC (Ser79), HMGR (Ser871), and $GABA_BR2$ (Ser783) in Rat Frontal Cortex

We examined the phosphorylation levels of the AMPK substrates, including ACC, HMGR, and $GABA_BR2$. The phosphorylation level of ACC at Ser79 accompanied by phosphorylation of AMPK at Thr172 is widely used as an index of true in vivo AMPK activity (Park et al., 2002). p-Ser79-ACC immunoreactivity increased significantly in the E10X group compared with the sham group ($t = 8.849$, $P < .001$). Phosphorylation of HMGR increased in response to E10X ($t = 4.013$, $P = .007$). p-Ser783-$GABA_BR2$ immunoreactivity also increased significantly ($t = 5.092$, $P = .002$) after E10X. The increased phosphorylation of various AMPK substrates, including ACC, HMGR, and $GABA_BR2$, indicates that the E10X treatment induced activation of AMPK in the rat frontal cortex. The immunoreactivity of total ACC, HMGR, and $GABA_BR2$ did not change after the E10X treatment (Figure 2B).

ECS Increases ULK1 (Ser317) Phosphorylation in Rat Frontal Cortex

p-Ser317-ULK1 immunoreactivity increased significantly in the E10X group compared with the sham group ($t = 11.085$,

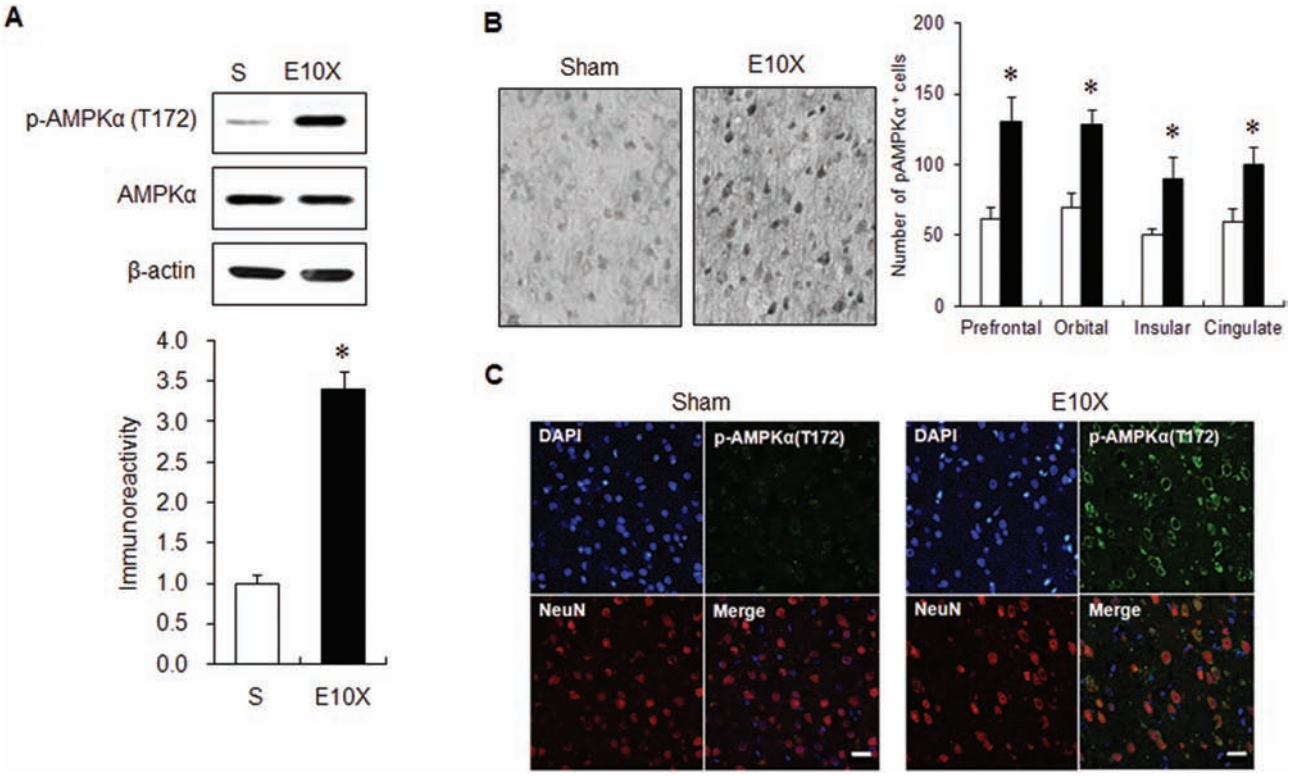

Figure 1. Repeated electroconvulsive seizure (ECS) treatment increases the AMPKα phosphorylation level in neurons of the rat frontal cortex (A) p-AMPKα (T172) immunoreactivity increased 24 hours after the E10X treatment. S and E10X indicate the sham-treated control and daily ECS for 10 days-treated sample, respectively. Representative immunoblots are presented. Immunoreactivity was quantified by densitometric analysis of band intensity and is presented as the optical density value relative to that of the control. Data are mean ± standard error of the mean (*P < .05; n = 6–8/group). (B) Increased immunostaining of p-AMPKα (T172) in the rat frontal cortex after the E10X treatment. The number of p-AMPKα-positive cells (T172) was counted, and they increased in the prefrontal, orbital, insular, and cingulate cortices after the E10X treatment. Data are mean ± standard error of the mean (*P < .05; n = 4/group). (C) p-AMPKα (T172) (green) immunofluorescence was co-localized with that of NeuN (red) but not with that of DAPI (blue) in the rat frontal cortex. Magnification bar = 20 μm.

$P < .001$). The total ULK1 protein level tended to increase in response to the E10X treatment ($t = 1.964$, $P = .062$) (Figure 2C). p-Ser317-ULK1 immunoreactivity was examined in the frontal cortical regions. Based on cell morphology, p-Ser317-ULK1 immunoreactivity was localized to the cytoplasmic part of the neurons, and the density of p-Ser317-ULK1-positive neurons increased in the frontal cortex after E10X compared with that of the sham group (Figure 2D). The p-Ser317-ULK1-positive cells increased significantly in the E10X group compared with the sham group in the prefrontal (Fr; $t = 4.215$, $P = .021$), orbital (LO and VLO; $t = 2.970$, $P = .024$), insular (AI; $t = 5.023$, $P = .006$), and cingulate (Cg; $t = 4.204$, $P = .014$) cortices (Paxinos and Watson, 1998) (Figure 2D).

Repeated ECS Treatments Activate Autophagy Signaling in Rat Frontal Cortex

The protein levels of LC3-II and the ATG–ATG12 conjugate have been used as biochemical markers of autophagy (Sharifi et al., 2015). LC3 immunoreactivity was examined in the rat frontal cortex 24 hours after the E10X treatment. LC3-II immunoreactivity was significantly higher in the E10X group compared with the sham group ($t = 9.924$, $P = .001$). The molecular weight of ATG5 is 35 kDa, and the immunoreactivity signal against the anti-ATG5 antibody is detected at around 55 kDa when ATG5 is conjugated with ATG12 (Jounai et al., 2007; Sharifi et al., 2015). The immunoreactivity of the ATG5–ATG12 conjugate also increased significantly after the E10X treatment ($t = 6.554$, $P = .003$) (Figure 3). These findings indicate that the repeated ECS treatments activated autophagy signaling in the rat frontal cortex.

Effects of i.c.v. Injection of Compound C on E10X-Induced Autophagy in Rat Frontal Cortex

To examine whether the AMPK pathway mediates the E10X-induced autophagy signal, the effects of an i.c.v. injection of the AMPK inhibitor compound C (Zhou et al., 2001) on the E10X-induced changes were examined. Two-way ANOVA indicated that p-Ser79-ACC, p-Ser872-HMGR, p-Ser783-$GABA_BR2$, and p-Ser317-ULK1 immunoreactivity was significantly affected by the compound C ($F(1, 20) = 14.965$, $P = .002$; $F(1, 20) = 9.302$, $P = .016$; $F(1, 20) = 11.212$, $P = .004$; $F(1, 20) = 18.686$, $P = .001$, respectively) and E10X ($F(1, 20) = 24.327$, $P < .001$; $F(1, 20) = 9.352$, $P = .014$; $F(1, 20) = 16.124$, $P = .008$; $F(1, 20) = 18.242$, $P = .004$, respectively). Group comparisons were performed with post hoc Tukey's tests, and a $P < .008$ for multiple comparisons was considered significant. The immunoreactivity of p-Ser79-ACC, p-Ser872-HMGR, and p-Ser317-ULK1 reflected a more pronounced trend toward a reduction in the compound-C group (ComC) than in the sham group (S) ($P = .014$, $P = .010$, and $P = .012$, respectively). The immunoreactivity of p-Ser79-ACC, p-Ser872-HMGR, p-Ser783-$GABA_BR2$, and p-Ser317-ULK1 was significantly lower in the compound C/E10X group (ComC/E10X) than in the E10X group (E10X) ($P = .007$, $P = .004$, $P < .001$, and $P < .001$, respectively). The total protein levels of ACC, $GABA_BR2$, HMGR, and ULK1 did not

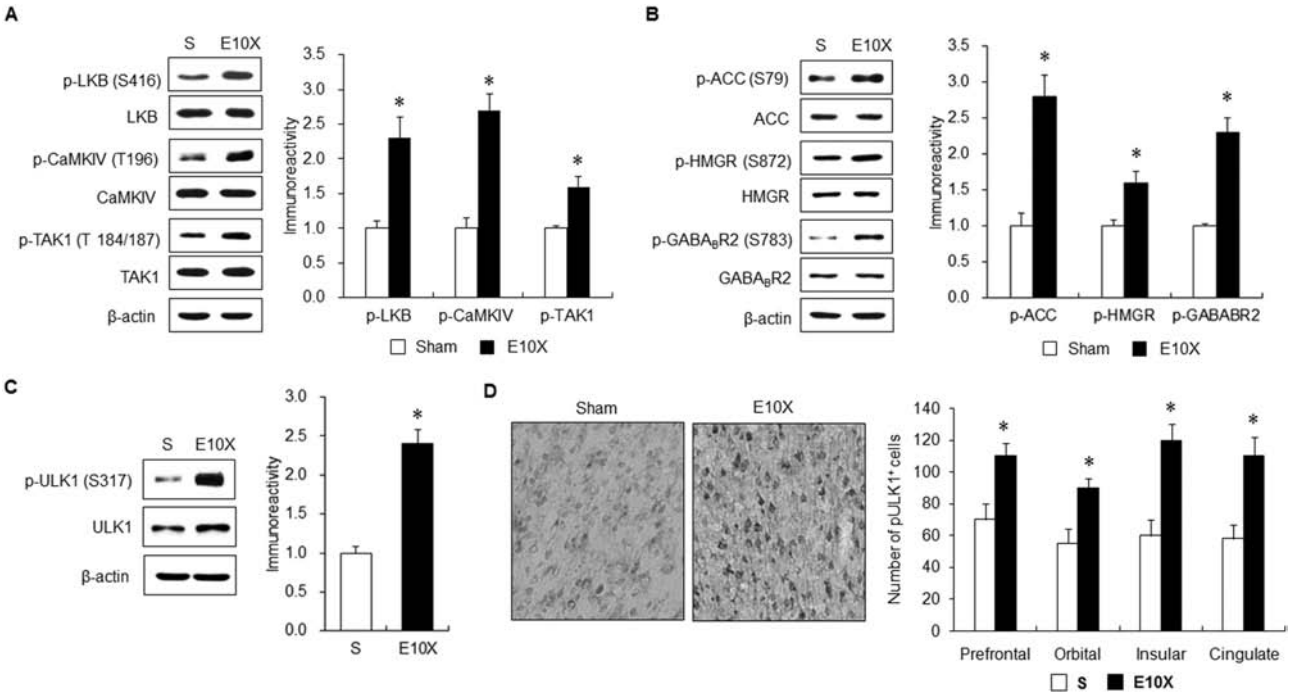

Figure 2. Effects of repeated electroconvulsive seizure (ECS) treatment on AMPK up and downstream molecules in the rat frontal cortex S and E10X indicate the sham-treated control and daily ECS for 10 days-treated sample, respectively. (A) Immunoreactivity of p-LKB1 (S431), p-CaMK4 (T196), and p-TAK1 (T184/187), upstream kinases of AMPK, increased in the rat frontal cortex 24 hours after the E10X treatment. (B) Immunoreactivity of p-ACC (S79), p-HMGR (S872), and p-GABABR2 (S783), substrates of AMPK, increased in the rat frontal cortex 24 hours after the E10X treatment. (C) p-ULK1 immunoreactivity (S317) increased 24 h after the E10X treatment. Representative immunoblots are presented, and the immunoblot data were quantified by densitometric analysis of band intensity (A–C; *$P < .05$; n = 6–8/group). (D) Increased immunostaining of p-ULK1 (S317) in the rat frontal cortex after the E10X treatment. The number of p-ULK1-positive cells (S317) was counted and increased in prefrontal, orbital, insular, and cingulate cortices after the E10X treatment. Data are mean ± standard error of the mean (*$P < .05$; n = 4/group).

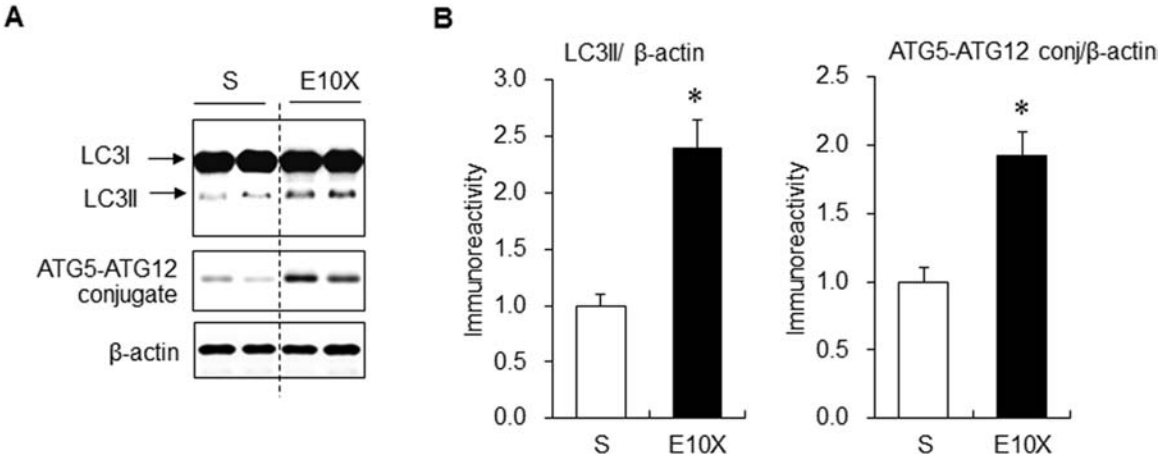

Figure 3. Repeated electroconvulsive seizure (ECS) treatment increases LC3 and ATG–ATG12 conjugate protein levels in the rat frontal cortex S and E10X indicate the sham-treated control and daily ECS for 10 days-treated sample, respectively. The immunoreactivity of LC3-II and the ATG5–ATG12 conjugate was elevated 24 h after E10X treatment. (A) Representative immunoblots of the rat frontal cortex 24 hours after the E10X treatment. (B) The immunoblot data were quantified by densitometric analysis of band intensity (*$P < .05$; n = 6–8/group).

change. Two-way ANOVA indicated that the immunoreactivity of LC3-II and the Atg5–Atg12 conjugate was also significantly affected by the compound C ($F [1, 20] = 18.384$, $P = .001$ and $F [1, 20] = 11.452$, $P = .003$, respectively) and E10X treatments ($F [1, 20] = 12.606$, $P = .003$ and $F [1, 20] = 8.278$, $P = .018$, respectively). The trend toward a reduction in the immunoreactivity of LC3-II and the Atg5–Atg12 conjugate was more pronounced in the ComC group than in the S group ($P = .011$ and $P = .021$). The immunoreactivity of LC3-II and the Atg5–Atg12 conjugate was significantly lower in ComC/E10X than in E10X ($P < .001$ and $P = .003$) (Figure 4). These findings indicate that AMPK mediates the E10X-induced autophagy in the rat frontal cortex.

Repeated ECS Treatments Increase Number of Autophagosomes via AMPK Signaling Pathway in Rat Frontal Cortex

Ultrastructural changes in the rat frontal cortical sections were examined by TEM after the sham or E10X treatments. Bubble-like vacuoles containing recognizable cytoplasmic

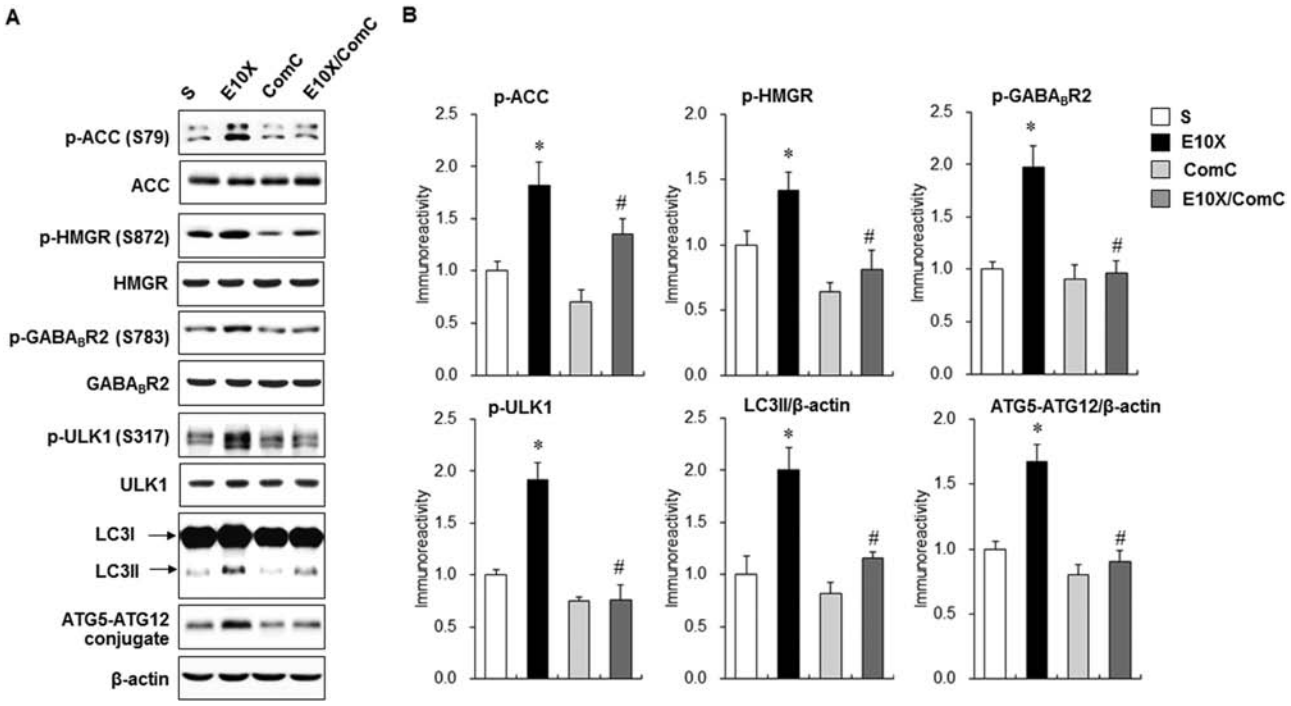

Figure 4. Intracerebroventricular administration of compound C inhibits the repeated electroconvulsive seizure (ECS) treatment-induced activation of the AMPK signaling pathway and autophagy in the rat frontal cortex S, E10X, ComC, and E10X/ComC indicate sham-treated control, daily ECS for 10 days-treated sample, compound C-treated sample, and E10X/compound C-treated sample, respectively. (A) Representative immunoblots of the rat frontal cortex after treatment with sham, E10X, compound C, or E10X/compound C. (B) Quantification of immunoblot data by densitometric analysis of band intensity. Immunoreactivity of p-ACC (S79), p-HMGR (S872), p-GABABR2 (S783), p-ULK1 (S317), LC3-II, and the ATG5–ATG12 conjugate all increased in the E10X group, and immunoreactivity in the E10X/ComC group was significantly lower than that in the E10X group. *$P < .008$, compared to the S group; #$P < .008$, compared to the E10X group (n = 6/group).

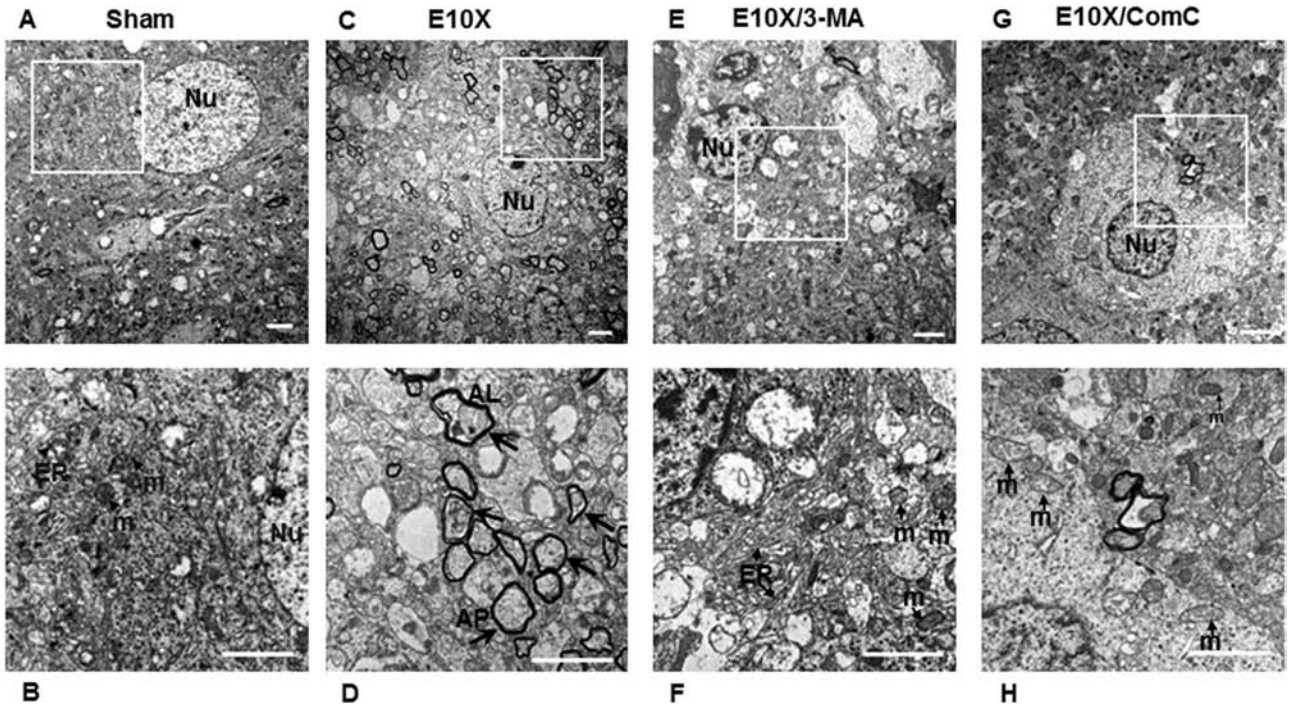

Figure 5. Transmission electron microscopy (TEM) shows an increased number of autophagosomes in the rat frontal cortex in response to the repeated electroconvulsive seizure (ECS) treatment S and E10X indicate the sham-treated control and daily ECS for 10 days-treated sample, respectively. Brain sections were obtained from the prefrontal cortical area 24 hours after the sham or E10X treatment. TEM images from sham-treated (A, B), E10X-treated (C, D), E10X/3-MA-treated (E, F), and E10X/compound C-treated (G, H) rats are shown. B, D, F, and H are higher-magnification images of the white-lined rectangular parts of A, C, E, and G, respectively. Selected representative autophagosomes and autophagolysosomes containing inclusion materials are indicated by arrows. Representative autophagosome and autophagolysosome are indicated by AP and AL, respectively (D). Nu, nucleus; m, mitochondria; ER, endoplasmic reticulum; Scale bars = 2 μm.

material represent the gold standard EM manifestation of autophagosomes (Brunk and Terman, 2002; Wang et al., 2008; Sharifi et al., 2015). A few small autophagosomes were observed in the sham-treated frontal cortical sections (Figure 5A-B). An increasing number of autophagosomes showing double-membraned cistern structures containing inclusion materials was clearly demonstrated in the E10X-treated frontal cortex (Figure 5C-D). To confirm that the increased number of autophagosomes actually reflected the increased autophagy process in response to E10X, the autophagy inhibitor 3-MA was injected i.c.v. after the E10X treatment. The E10X-induced increase in autophagosomes was clearly inhibited by the i.c.v. injection of 3-MA (Figure 5E–F). Additionally, the i.c.v. injection of compound C also inhibited the E10X-induced increase in autophagosomes, which is consistent with the immunoblot findings showing E10X-induced autophagy via AMPK signaling (Figure 5G–H).

Discussion

Repeated ECS treatments activated the AMPK signaling pathway in the rat frontal cortex. The repeated ECS treatments increased the phosphorylation levels of both AMPKα at the Thr172 residue and its downstream molecules, including ACC, HMGR, and $GABA_BR2$, which could reflect a true in vivo increase in AMPK activity (Park et al., 2002; Hardie et al., 2016). AMPK phosphorylation was evident in the neurons of the frontal cortical regions, including the prefrontal, orbital, insular, and cingulate cortices. The findings show that the repeated ECS treatments induced activation of the AMPK signaling pathway in the neuronal cells of the rat frontal cortex.

Among the examined AMPK substrates, ULK1 is one of the major regulators of autophagy (Lin and Hurley, 2016). E10X-induced phosphorylation of ULK1 at Ser317, an AMPK-mediated type of phosphorylation (Egan et al., 2011b; Kim et al., 2011; Lin and Hurley, 2016), was accompanied by increased LC3-II and ATG5–ATG12 conjugate protein levels, indicating activation of autophagy processes (Jounai et al., 2007; Tanida et al., 2008; Sharifi et al., 2015); this was inhibited by i.c.v. injection of compound C, an AMPK inhibitor. An increase in the number of autophagosomes by E10X was also demonstrated by TEM. Intrabrain inhibition of autophagy through an i.c.v. injection of 3-MA, a PI3K autophagy inhibitor (Seglen and Gordon, 1982; Petiot et al., 2000), reduced the E10X-induced increase in autophagosomes, indicating activation of autophagy by E10X. The increase of autophagosomes following E10X was inhibited by intrabrain inhibition of AMPK. The results demonstrate that repeated ECS treatments activate autophagy via the AMPK/ULK1 signaling pathway in the rat frontal cortex.

The beneficial effects of ECT may be related to the role of autophagy in the cellular quality control process, that is, detecting and eliminating misfolded or aggregated proteins and damaged organelles (Mizushima and Komatsu, 2011; Nikoletopoulou et al., 2015). Autophagy activation by ECT may serve a therapeutic function for diseases in which impairments of the autophagy process have been reported, such as major depressive disorder, schizophrenia, Parkinson's disease, and Huntington's disease (Tharyan and Adams, 2005; Lisanby, 2007; Mughal et al., 2011; Narang et al., 2015; Weiner and Reti, 2017). Impairments of the autophagic process in neurodegenerative disorders, such as Parkinson's disease and Huntington's disease, are well recognized, and stimulating autophagy enhances the treatment response (Lynch-Day et al., 2012; Nixon, 2013; Radad et al., 2015; Boland et al., 2018). Possible dysregulation of autophagy in major depressive disorder has been suggested (Jia and Le, 2015). Various antidepressants, such as fluoxetine, imipramine, sertraline, and amitriptyline, have been reported to induce autophagy in cell lines (Bowie et al., 2015; Cho et al., 2016; Xia et al., 2017; Gulbins et al., 2018). Molecular findings suggesting impaired autophagy in schizophrenia have also been reported (Merenlender-Wagner et al., 2014, 2015; Schneider et al., 2016). Several antipsychotics induce autophagy (Zhang et al., 2007; Shin et al., 2012, 2013; Yin et al., 2015).

The antipsychotic agent clozapine induces autophagy in the rat frontal cortex (Kim et al., 2018). Clozapine, an atypical antipsychotic agent with superior efficacy compared with other antipsychotic agents, is used for the treatment of severe and refractory psychotic disorders (Kane et al., 1988; Kane, 1992; Lewis et al., 2006; McEvoy et al., 2006). Clozapine activates AMPK via increased phosphorylation of its substrates, including ACC, HMGR, and $GABA_BR2$, which mediate clozapine-induced autophagy in the rat frontal cortex (Kim et al., 2018). The results of this study regarding ECS are very similar to those of clozapine. The superior efficacy of clozapine and ECT for treating a broad range of psychiatric disorders may be related to AMPK signaling-mediated autophagy activation in the brain. Clozapine-resistant patients with schizophrenia who received repeated ECT exhibited rapid and substantial reduction of psychotic symptoms (Petrides et al., 2015; Kim et al., 2017). Combined clozapine and ECT treatment may synergistically augment AMPK and autophagy activation in the brain and could induce a beneficial effect by normalizing neuronal function; however, this requires further study.

AMPK is involved in many biochemical pathways related to physiology and pathology, including energy homeostasis, lipid metabolism, mitochondrial biogenesis, cell cycle regulation, immune responses, and autophagy (Carling et al., 2012; Hardie et al., 2016). In this study, phosphorylation of AMPK substrates, including ACC, HMGR, and $GABA_BR2$, increased after the E10X treatment. ACC catalyzes the production of malonyl CoA, which is both a major building block for de novo fatty acid synthesis and an allosteric inhibitor of CPT1 (Hardie and Carling, 1997). HMGR is a membrane-bound enzyme that catalyzes the rate-limiting step in sterol and isoprenoid biosynthesis, and AMPK is a primary HMGR phosphoregulatory kinase (Burg and Espenshade, 2011). AMPK phosphorylates $GABA_BR2$ at Ser783, which reduces excitotoxicity and promotes neuronal survival (Kuramoto et al., 2007). Investigations of the consequences of AMPK-mediated phosphorylation of ACC, $GABA_BR2$, and HMGR could enhance understanding of the action mechanism of ECT. Additionally, multiple upstream kinases of AMPK, including LKB1, CaMKK, and TAK1, were activated by the repeated ECS treatments. AMPK is widely recognized as an antagonist of mTOR (Egan et al., 2011a; Kim et al., 2011). Activation of AMPK and inhibition of mTOR results in activation of ULK1, followed by autophagy (Kim et al., 2011). To reveal the regulating mechanisms of ECS-induced AMPK activation, further research is required, including studies on the effect of ECS on the mTOR pathway.

Additional research regarding the neurobehavioral consequences of the ECS-induced activation of the AMPK-mediated autophagy system in the frontal cortex is needed. As noted above, ECS treatment has beneficial therapeutic effects on neurodegenerative disorders such as Parkinson's disease and Huntington's disease (Beale et al., 1997; Mughal et al., 2011; Narang et al., 2015), which reportedly involve impairments in the autophagy process (Nixon, 2013; Menzies et al., 2017). For example, we can use animal models of neurodegenerative disorders to study the effects of ECS on behavioral and/or neurochemical changes to clarify the implications of ECS-induced autophagy activation. We previously reported that repeated ECS treatments induce ERK1/2 activation, increase BDNF (Kang et al.,

2006), and induce histone deacetylase 2 expression (Park et al., 2014) in the rat frontal cortex. As diverse molecules are interconnected and cross-talk between signaling pathways is important, we must investigate the relationships among these factors.

In summary, repeated ECS treatment-induced autophagy in the rat frontal cortex in vivo as evidenced by the increased levels of LC3-II and the ATG5–ATG12 conjugate as well as the ultrastructural TEM findings showing an increase in the number of autophagosomes. The AMPK/ULK1 pathway, a major signaling pathway of autophagy induction, was activated and an i.c.v. injection of the AMPK inhibitor compound C inhibited ECS-induced autophagy. Taken together, our results demonstrated that repeated ECS treatments activated autophagy in the rat frontal cortex through the AMPK signaling pathway in vivo.

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Statement of Interest

There is no actual or potential conflict of interest in relation to this article. SH Kim declares that he has received research grants from and/or served as a lecturer for Janssen and Eli Lilly. YM Ahn declares that he has received grants, research support, and/or honoraria from Janssen, Otsuka, and Lundbeck. UG Kang declares that he has received research grants from and/or served as a lecturer for Janssen and Otsuka. YS Kim declares that he has received grants, research support, and/or honoraria from Janssen and Otsuka. The present study was not related to any of these professional relationships.

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