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Ergothioneine-induced neuronal differentiation is mediated through activation of S6K1 and neurotrophin 4/5-TrkB signaling in murine neural stem cells

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ABSTRACT

The promotion of neurogenesis is considered to be an effective therapeutic strategy for neuropsychiatric disorders because impairment of neurogenesis is associated with the onset and progression of these disorders. We have previously demonstrated that orally ingested ergothioneine (ERGO), a naturally occurring antioxidant and hydrophilic amino acid, promotes neurogenesis in the hippocampal dentate gyrus (DG) with its abundant neural stem cells (NSCs) and exerts antidepressant-like effects in mice. Independent of its antioxidant activities, ERGO induces in cultured NSCs this differentiation through induction of the basic helix-loop-helix transcription factor Math1. However, the upstream signaling of Math1 in the mechanisms underlying ERGO-induced neuronal differentiation remains unclear. The purpose of the present study was to elucidate the upstream signaling with the aim of discovering novel targets for the treatment of neuropsychiatric disorders. We focused on neurotrophic factor signaling, as it is important for the promotion of neurogenesis and the induction of antidepressant effects. We also focused on the signaling of the mammalian target of rapamycin (mTOR) complex 1 (mTORC1), a known amino acid sensor, and the members of this signaling pathway, mTOR and p70 ribosomal protein S6 kinase 1 (S6K1). Exposure of cultured NSCs to ERGO significantly increased the expression of phosphorylated S6K1 (p-S6K1) at Thr389 in only 1 h, of phosphorylated mTOR (p-mTOR) in 6 h, and of the gene product of neurotrophin 4/5 (NT5) which activates tropomyosin receptor kinase B (TrkB) in 24 h. ERGO increased the population of βIII-tubulin-positive neurons, and this effect was suppressed by the inhibitors of S6K1 (PF4708671), mTORC1 (rapamycin), and TrkB (GNF5837). Oral administration of ERGO to mice significantly increased in the DG the expression of p-S6K1 at Thr389, the gene product of NT5, and phosphorylated TrkB but not that of p-mTOR. Thus, neuronal differentiation of NSCs induced by ERGO is mediated, at least in part, through phosphorylation of S6K1 at Thr389 and subsequent activation of TrkB signaling through the induction of NT5. Thus, S6K1 and NT5 might be promising target molecules for the treatment of neuropsychiatric disorders.

Abbreviations: BBB, blood-brain barrier; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; bHLH, basic helix-loop-helix; CNTF, ciliary neurotrophic factor; DAPI, 2-(4-amidinophenyl)-1H-indole-6-carboxamidine; DG, dentate gyrus; DMEM, Dulbecco's modified Eagle's medium; DMEM/F12, DMEM/Nutrient Mixture F-12 Ham; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; ERGO, ergothioneine; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; ICR-NSCs, NSCs derived from ICR mice; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; NGF, nerve growth factor; NSC, neural stem cell; NT3, neurotrophin 3; NT5, neurotrophin 4/5; OCTN1, carnitine/organic cation transporter 1; *octn1*$^{-/-}$, *octn1*-deficient; *octn1*$^{-/-}$-NSC, NSC derived from *octn1*$^{-/-}$-mouse; PA, paraformaldehyde; PBS, phosphate-buffered saline; PDK1, 3-phosphoinositide-dependent protein kinase 1; p-mTOR, phosphorylated mTOR; p-S6K1, phosphorylated S6K1; p-TrkB, phosphorylated TrkB; p-4EBP1, phosphorylated 4EBP1; RT-PCR, reverse transcription polymerase chain reaction; SSRIs, selective serotonin reuptake inhibitors; S6K1, p70 ribosomal protein S6 kinase 1; SCF, stem cell factor; TrkB, tropomyosin receptor kinase B; vEGF, vascular endothelial growth factor; WT, wild-type; WT-NSC, NSC derived from wild-type mouse; 36B4, acidic ribosomal phosphoprotein P0; 4EBP1, eukaryotic initiation factor 4E-binding protein 1

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1. Introduction

Neural stem cells (NSCs) have self-renewal ability as well as pluripotentiality to differentiate into neurons, astrocytes, and oligodendrocytes [1,2]. New neurons are generated from NSCs in the hippocampus, subventricular zone, and olfactory bulb throughout the lifetime of the mammalian brain [3–6]. Because severely damaged neurons cannot regenerate in mammals [7,8], including humans, neurogenesis is essential for sustaining normal brain functions. Suppression of neurogenesis leads to the onset and progression of neuropsychiatric disorders, and this suppression, in conjunction with environmental stressors, causes major depression and dementia [6,9,10]. Amelioration of suppressed neurogenesis improves depression symptoms [11,12] and age-dependent decline of learning and memory [13]. Promotion of neurogenesis is important for the efficacy of clinically used antidepressants [14–16]. Hence, the promotion of neurogenesis would be an effective therapeutic strategy for neuropsychiatric disorders [17,18], and the discovery of compounds promoting neurogenesis without adverse effects and the elucidation of their mechanisms of action is desired.

Ergothioneine (ERGO) is a naturally occurring, food-derived, hydrophilic amino acid and antioxidant. It is found in many foods, including edible mushrooms. ERGO is highly distributed to the brain via the carnitine/organic cation transporter 1 (OCTN1) after oral ingestion, and it has been shown to promote neurogenesis in the hippocampal dentate gyrus (DG) of mice [19]. In mice, oral intake of ERGO exerts antidepressant-like effects [19] and improves the impairment of learning and memory caused by administration of D-galactose [20]. Little is known about hydrophilic compounds, like ERGO, that promote neurogenesis via oral administration, because the compounds that are reported to promote neurogenesis are mostly hydrophobic in nature [21,22]. For example, resveratrol and curcumin are known to promote neurogenesis and are both hydrophobic compounds. However, their brain distribution after oral administration is limited, owing to their low oral bioavailability and permeability across the blood-brain barrier (BBB) [23]. An overdose of these compounds is necessary to induce an improvement of brain functions; however, long-term overdoses of resveratrol and curcumin can cause adverse effects, such as liver injury [24,25]. In contrast, ERGO is hydrophilic and its disposition is highly controlled by the ERGO-specific transporter OCTN1/SLC22A4. Therefore, ERGO can be expected to safely improve brain functions, even with long-term use [26]. In fact, ERGO is highly distributed to the brain across the BBB after oral ingestion and exhibits antidepressant-like effects at relatively low doses [19]. We have previously demonstrated that ERGO promotes neuronal differentiation through induction of the basic helix-loop-helix (bHLH) transcription factor Math1 via unidentified mechanisms that are distinct from its antioxidant activities in NSCs [27]. The mechanisms underlying ERGO-induced neuronal differentiation, however, remain unclear. The identification of the mechanisms that drive ERGO-induced neuronal differentiation could lead to the discovery of novel therapeutic targets for neuropsychiatric disorders, so we tried to elucidate those mechanisms in the present study.

Differentiation of NSCs is regulated by multiple extracellular and intracellular factors, including neurotransmitters, neurotrophic factors, kinases, and transcription factors [28,29]. Activation of neurotrophic factor signaling is associated with the amelioration of depression [14,30,31], and ERGO is known to have antidepressant-like properties [19]. Brain-derived neurotrophic factor (BDNF) promotes neuronal differentiation of NSCs via induction of Math1 in a manner similar to ERGO [27,32]. Therefore, we examined the involvement of BDNF and other neurotrophic factors, nerve growth factor (NGF), neurotrophin 3 (NT3), and neurotrophin 4/5 (NT5), in the mechanisms that underlie ERGO-induced neuronal differentiation. In addition, we also investigated the involvement of mammalian target of rapamycin (mTOR) complex 1 (mTORC1) signaling in this process. mTORC1 is a known sensor for intracellular amino acids [33,34] and is a master growth controller [34]. It regulates differentiation of NSCs [35–38] and promotes secretion of BDNF [39]. Since ERGO is an amino acid and is transported into the intracellular space by OCTN1, ERGO-induced activation of mTORC1 may be involved in neuronal differentiation. Thus, in the present study, we examined whether ERGO activates mTORC1 and neurotrophic factor signaling to clarify the mechanisms underlying ERGO-induced neuronal differentiation.

2. Materials and methods

2.1. Materials

Anti-mTOR, phosphorylated mTOR (p-mTOR) at Ser2448, p70 ribosomal protein S6 kinase 1 (S6K1), phosphorylated S6K1 (p-S6K1) at Thr371, p-S6K1 at Thr389, eukaryotic initiation factor 4E-binding protein 1 (4EBP1), phosphorylated 4EBP1 (p-4EBP1) at Thr37/46, and tropomyosin receptor kinase B (TrkB) antibodies, and anti-rabbit IgG antibody conjugated with peroxidase were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-phosphorylated TrkB (p-TrkB) at Tyr816 antibody was purchased from Merck Millipore (Darmstadt, Germany). Block Ace™ was provided by DS Pharma Biomedical (Suita, Japan). Dulbecco's modified Eagle's medium (DMEM), DMEM/Nutrient Mixture F-12 Ham (DMEM/F12), poly-L-lysine, and monoclonal antibodies against βIII-tubulin and glial fibrillary acidic protein (GFAP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was supplied by Biowest (Nuaillé, France). GNF5837 was purchased from SYNkinase Pty (Melbourne, Australia). ISOGEN was purchased from Nippon Gene (Tokyo, Japan). MultiScribe™ reverse transcriptase was obtained from Applied Biosystems (Foster City, CA, USA). NeuroCult Chemical Dissociation Kit was purchased from Stem Cell Technologies Inc. (Cambridge, UK). Neurotrophin 4/5 and BDNF Rapid™ enzyme-linked immunosorbent assay (ELISA) Kits were purchased from Biosensis (Thebarton, Australia). Rapamycin was purchased from AdipoGen Life Sciences (San Diego, CA, USA). Recombinant human basic fibroblast growth factor (bFGF) and recombinant human epidermal growth factor (EGF) were provided by PeproTech Inc. (Rocky Hill, NJ, USA). Recombinant mouse neurotrophin 4/5 was purchased from GenScript (Piscataway, NJ, USA). Secondary antibodies conjugated with the Alexa Fluor series were provided by Invitrogen (San Diego, CA, USA). THUNDERBIRD™ SYBR qPCR Mix and Can Get Signal™ were purchased from TOYOBO (Osaka, Japan). All other chemicals and reagents were of the highest purity available and were purchased from commercial sources.

2.2. Animals

ICR mice were purchased from Japan SLC (Hamamatsu, Japan). *octn1*-deficient mice (*octn1*$^{-/-}$-mice) were established in a previous paper [26] and backcrossed to a C57BL/6 J strain [27]. The mice were kept in a temperature- and light-controlled environment with standard food and tap water provided ad libitum. This study was carried out in strict accordance with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Kanazawa (Permit Number: AP-132875) with an effort to minimize the number of animals used and their suffering.

2.3. Preparation of NSCs

Cortical NSC culture was carried out according to the method of Ishimoto et al. [27] with minor modifications. In brief, cerebral cortices from 15-day-old embryonic mice were dissected and incubated at 37 °C for 20 min with 0.25% trypsin in phosphate-buffered saline (PBS) containing 28 mM glucose. Cells were mechanically dissociated, using a 1000 μL pipette tip, in culture medium and plated at a density of 1×10^6 cells/2 mL/well on 0.2% agarose-coated 6-well dishes for

culture, under floating conditions. First, cortical NSCs were cultured in the growth medium DMEM/F12 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL apo-transferrin, 20 nM progesterone, 5.2 ng/mL sodium selenite, 60 μM putrescine, 10 ng/mL EGF, and 10 ng/mL bFGF at 37 °C in a humidified incubator with 5% CO_2. These primary NSC cultures were kept for 6 days in this growth medium with half its volume changed on day 3. Neurospheres were formed from clusters of proliferating cells under floating culture conditions in a culture period-dependent manner and were transferred to uncoated 6-well dishes at day 3. On the sixth day in culture, primary neurosphere cultures were dispersed by using a NeuroCult Chemical Dissociation Kit, and then replated at a density of 2×10^5 cells/2 mL/well and 5×10^4 cells/0.5 mL/well into 6- and 24-well dishes, respectively, as secondary cultures. The cells were kept in the growth medium for various times, for 3, 6, or 9 days, under the same conditions as the primary cultures. Half of the secondary culture medium was changed every 3 days. Experiments in the present study were performed using NSCs obtained from these secondary cultures. The cells in the secondary cultures were cultured in either the absence or presence of ERGO for 1, 3, or 9 days. The secondary ERGO-treated cultures were also treated with either several inhibitors or a vehicle. Cultured NSCs were exposed to ERGO at 500 μM. We have clarified that 500 μM is an appropriate concentration to evaluate the effect of ERGO on cellular differentiation of cultured NSCs in our previous study [27].

In order to evaluate differentiation of NSCs, neurospheres cultured for 3, 6, or 9 days in secondary cultures were dispersed by using a NeuroCult Chemical Dissociation Kit, followed by seeding them at a density of 7.9×10^4 cells/cm^2 into 4-well dishes coated with poly-L-lysine for culture under adhesion conditions. The subsequent culture was conducted in the absence of growth factors for an additional 3 days in DMEM media supplemented with 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 28 mM glucose, 2 mM glutamine, 5 mM HEPES, 25 μg/mL apo-transferrin, 250 ng/mL insulin, 0.5 pM β-estradiol, 1.5 nM triiodothyronine, 10 nM progesterone, 4 ng/mL sodium selenite, and 50 μM putrescine. Removal of growth factors led to the complete abolition of nestin immunoreactivity along with a drastic increase in the number of cells immunoreactive for either βIII-tubulin or GFAP on day 3 [27].

2.4. Administration of ERGO to mice

Five-week-old mice were purchased, and ERGO, dissolved in autoclaved pure water, was orally administered at 0 or 50 mg/kg on experimental day 0, 2, 4, 7, 9, and 11 by gavage. We have clarified that 50 mg/kg is an appropriate dose to evaluate the effect of ERGO on activation of mTOR signaling in mice in our preliminary study. The mice were then decapitated 4 h after the administration on experimental day 2 or on experimental day 19. The hippocampal DG and cortex were collected in accordance with the method of Hagihara et al. [40]. The tissue samples were weighed, homogenized, and used for western blot analysis and enzyme immunoassays.

2.5. Western blot analysis

Western blot analysis was carried out according to the method of Ishimoto et al. with minor modifications [27]. In brief, samples, including cultured NSCs, DG, and cortex, were washed twice with ice-cold PBS, followed by centrifugation at 4 °C for 5 min at 15,000 ×*g*. Pellets thus obtained were suspended and sonicated in 20 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, protease inhibitors (0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 μg/mL leupeptin, 1 μg/mL antipain, and 0.5 mM benzamidine hydrochloride hydrate), and phosphatase inhibitors (10 mM sodium fluoride, 10 mM β-glycerophosphate disodium salt hydrate, 10 mM sodium pyrophosphate decahydrate, and 1 mM sodium orthovanadate), followed by protein concentration measurements with a Bio-Rad Protein Assay Kit. Solubilized lysates were added at a volume ratio of 4:1 to 10 mM Tris–HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue, and 5% 2-mercaptoethanol. Each aliquot of five micrograms of protein was loaded on a 10% polyacrylamide gel for electrophoresis at a constant current of 21 mA/plate for 30 min at room temperature using a compact-slab size PAGE system (ATTO, Tokyo, Japan), followed by blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After blocking with 4% (*w/v*) Block Ace solution, the membrane was probed with antibodies against mTOR, p-mTOR at Ser2448, S6K1, p-S6K1 at Thr371, p-S6K1 at Thr389, 4EBP1, p-4EBP1 at Thr37/46, TrkB, β-actin (all 1: 1000), and p-TrkB at Tyr816 (1: 00) diluted with Can Get Signal, followed by probing with a secondary anti-rabbit IgG antibody conjugated with peroxidase (1: 2000) or anti-mouse IgG antibody conjugated with peroxidase (1: 10,000). Proteins that reacted with those antibodies were detected with the aid of ECL™ detection reagents, using a lumino image analyzer (LAS-4000; FUJIFILM, Tokyo, Japan). Densitometric analysis of the western blots was performed using ImageJ software.

2.6. Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from cultured cells according to the standard ISOGEN procedure. cDNA was synthesized with oligo $(dT)_{12–18}$ primers, deoxynucleotide triphosphate mix, RT buffer, and MultiScribe™ Reverse Transcriptase and was amplified using Mx3005P (Agilent Technologies, Santa Clara, CA, USA) in a reaction mixture that contained cDNA with relevant sense and antisense primers (Table 1) and THUNDERBIRD SYBR qPCR Mix. PCR reactions were initiated by template denaturation at 95 °C for 15 min, followed by 40 cycles of amplification (denaturation at 95 °C for 10 s and primer annealing and extension at 60 °C for 30 s. The expression levels of mRNA were normalized by an internal standard, acidic ribosomal phosphoprotein P0 (36B4).

Table 1
Primers used for real-time polymerase chain reaction analysis in the present study.

Genes	Sense primers (5′-3′)	Antisense primers (3′-5′)
NGF	TCTATACTGGCCGCAGTGAG	GGACATTGCTATCTGTGTACGG
BDNF	GCGGCAGATAAAAAGACTGC	TCAGTTGGCCTTTGGATACC
NT3	GGAGGAAACGCTATGCAGAA	GTCACCCACAGGCTCTCACT
NT5	CCCAAGTTGAGGGAAAACAA	TCCTCCGGGAGAACTCCTAT
bFGF	CGGCTCTACTGCAAGAACG	TGCTTGGAGTTGTAGTTTGACG
EGF	CCATCCATTGGCAAAACC	AACCTTCACGACACAAACACC
vEGF	AAAAACGAAAGCGCAAGAAA	TTTCTCCGCTCTGAACAAGG
CNTF	CTGGAACAGAAGGTCCCTGA	AAGCCCCACAGCTTCTTCTC
Haptoglobin	GAGAGGTCCACGATGAGAGC	ACTCCACAGCAAAAAGCTGAC
SCF	CAGAACAGCTAAACGGAGTCG	TTTGTGTCTTCTTCATAAGGAAAGG
Math1	ACATCTCCCAGATCCCACAG	GGGCATTTGGTTGTCTCAGT
36B4	ACTGGTCTAGGACCCGAGAAG	TCCCACCTTGTCTCCAGTCT

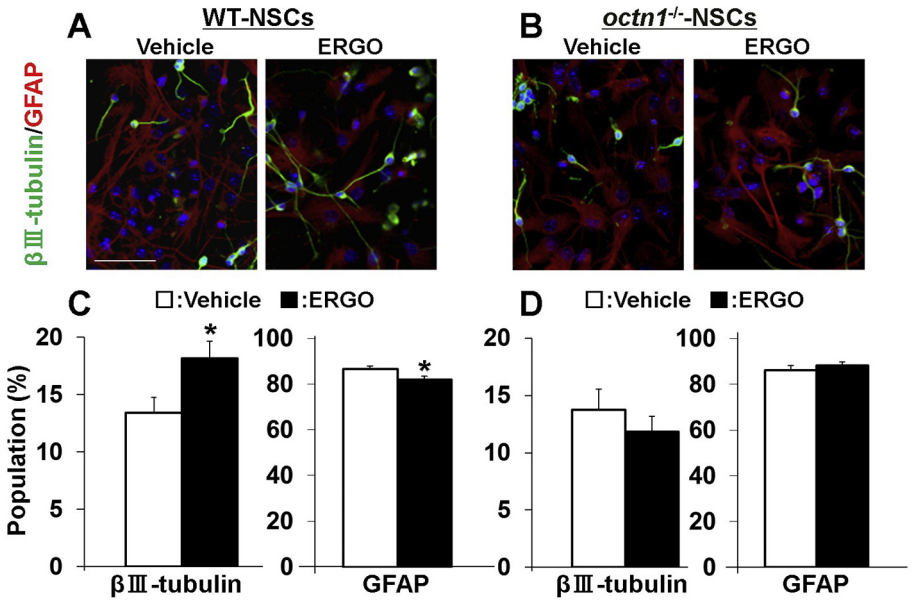

Fig. 1. Effects of ERGO on the cellular differentiation in cultured WT- and *octn1*$^{-/-}$-NSCs. WT- and *octn1*$^{-/-}$-NSCs derived from C57BL/6 J mouse embryonic cortex were exposed to vehicle or ERGO (500 μM) for 9 days, and then induced to differentiate by adhesion culture for an additional 3 days. (A, B) The cells were fixed with 4% PA, followed by immunocytochemical detection of the neuronal marker βIII-tubulin (green), the astroglial marker GFAP (red), and nuclei (blue). *Scale bar*: 50 μm. (C, D) The number of cells positive for each marker was counted by using ImageJ and normalized by the number of the nuclear marker DAPI-positive cells to calculate the population of cells positive for each marker. White columns show NSCs treated with vehicle, and black columns show NSCs treated with ERGO. Each value represents the mean ± S.E.M. (n = 6). *p < .05, significant difference from the control value obtained in NSCs treated with vehicle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.7. Enzyme immunoassays

Enzyme immunoassays were performed according to the recommended protocol of the Neurotrophin 4/5 and BDNF Rapid™ ELISA Kits. In brief, samples, including cultured NSCs, DG, and cortex, were washed twice with ice-cold PBS, followed by centrifugation at 4 °C for 5 min at 15,000 ×*g*. The cellular pellets and tissues were then homogenized by a tip sonicator (TOMY SEIKO, Tokyo, Japan) in nine volumes of distilled water containing the same protease inhibitors and phosphatase inhibitors as was used for western blotting, followed by centrifugation at 4 °C for 5 min at 15,000 ×*g*. The protein concentration of the supernatant was measured with the Bio-Rad Protein Assay Kit, and the protein expression of NT5 and BDNF was examined by their respective ELISA kit. The absorbance of each sample was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a test wavelength of 450 nm. Each obtained value was normalized by the protein concentration of the respective sample.

2.8. Immunocytochemical analysis

Neural cells derived from cultured NSCs were washed with PBS, then fixed with 4% paraformaldehyde (PA) for 20 min at 25 °C, and incubated for 30 min in a blocking solution (PBS containing 3% bovine serum albumin and 0.2% Triton X-100) at 25 °C. They were then incubated overnight at 4 °C in a 10-times-diluted blocking solution containing antibody against βIII-tubulin (1: 1000) or GFAP (1: 1000), followed by washing with PBS and then probing with secondary antibodies conjugated with Alexa Fluor dyes (1:1000) for 1 h at 25 °C. The cells were rinsed again with PBS, treated with mounting medium that contained 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI), and were observed under an LSM710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). The neuronal marker βIII-tubulin and the astrocyte marker GFAP were visualized in green and red, respectively. Quantification was performed by counting the number of the cells immunoreactive for either βIII-tubulin or GFAP in a double immunocytochemical analysis, followed by calculation of the individual percentages versus the total number of cells stained with DAPI.

2.9. Statistical analysis

All experiments were performed at least three times. Data are expressed as the mean ± S.E.M. The statistical significance of differences was determined by means of Student's *t*-test or one-way ANOVA with the Bonferroni/Dunn or Dunnett test, and $p < .05$ denoted a significant difference.

3. Results

3.1. OCTN1 is essential for ERGO-induced neuronal differentiation in NSCs

It has already been demonstrated that exposure to ERGO promotes neuronal differentiation in a primary NSC culture [27], although an involvement of the ERGO uptake transporter OCTN1 in this differentiation process remains unclear. We first compared the effects of ERGO on neuronal differentiation in NSCs derived from wild-type mice (WT-NSCs) or *octn1*$^{-/-}$-mice (*octn1*$^{-/-}$-NSCs), which minimally incorporate ERGO [27]. Exposure to ERGO appeared to increase the number of βIII-tubulin-positive cells in WT-NSCs (Fig. 1A) but not in *octn1*$^{-/-}$-NSCs (Fig. 1B). Quantitative analysis showed that the population of βIII-tubulin-positive cells was significantly increased by ERGO exposure, whereas ERGO decreased that of GFAP-positive cells in WT-NSCs (Fig. 1C). On the other hand, the population of βIII-tubulin- and GFAP-positive cells were minimally affected by ERGO exposure in *octn1*$^{-/-}$-NSCs (Fig. 1D). We also examined the effects of ERGO on cellular proliferation in WT-NSCs and *octn1*$^{-/-}$-NSCs. Exposure to ERGO significantly decreased the area of neurospheres in WT-NSCs (Fig. S1A) but not in *octn1*$^{-/-}$-NSCs (Fig. S1B). These results indicate that OCTN1 is essential for both the promotion of neuronal differentiation and the suppression of cellular proliferation by ERGO in NSCs. These data also suggest that ERGO acts in the intracellular space after incorporation by OCTN1 to promote neuronal differentiation.

3.2. Exposure of NSCs to ERGO for 1 day promotes neuronal differentiation

Next, we determined the NSC culture time and the ERGO exposure time that are required for the promotion of neuronal differentiation by ERGO in cultured NSCs derived from ICR mice (ICR-NSCs). NSCs cultured for 3, 6, or 9 days were exposed to ERGO during the last 1 or 3 days and were evaluated for cellular differentiation (Fig. 2A). The population of βIII-tubulin-positive cells gradually decreased (Fig. 2B), whereas that of GFAP-positive cells gradually increased (Fig. 2C), in a culture period-dependent manner. These results are comparable with a previous study, in that NSCs gradually lose their ability to differentiate into neurons the longer they are in culture [41]. Exposure to ERGO for

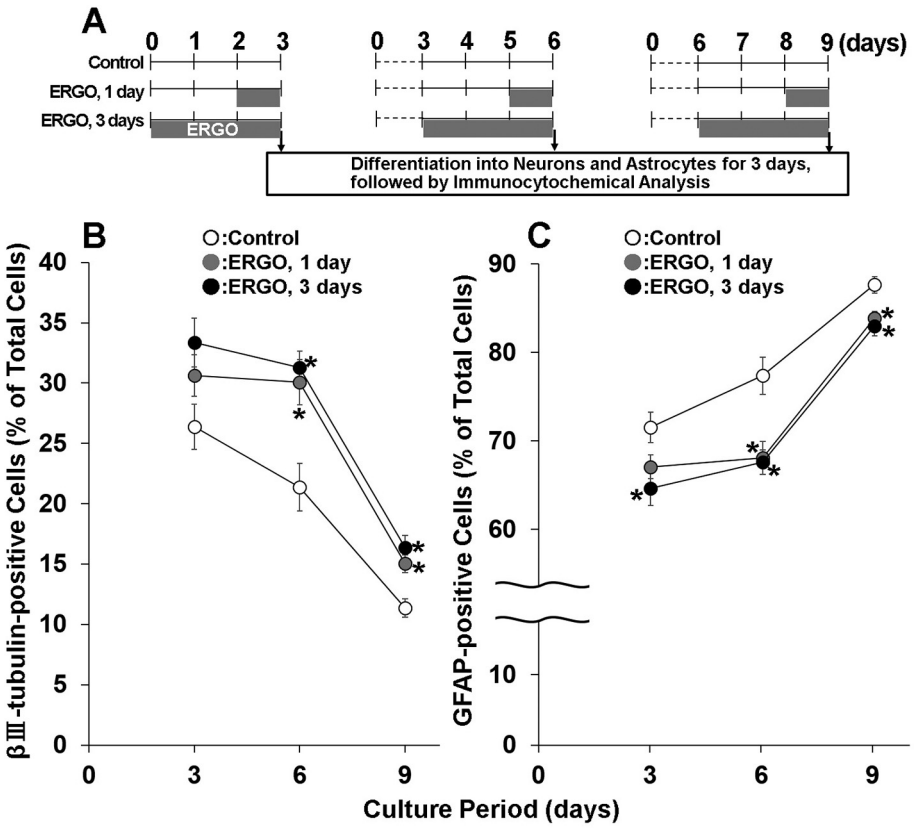

Fig. 2. Effects of culture period and exposure time to ERGO on the cellular differentiation of NSCs. (A) Schematic representation of experimental schedule. (B, C) ICR-NSCs were cultured for 3, 6, or 9 days in either the absence (white symbols) or presence of 500 μM ERGO during the last 1 day (gray symbols) or 3 days (black symbols) in culture and then induced to differentiate by adhesion culture for a further 3 days. The cells were fixed with 4% PA, followed by immunocytochemical detection of the neuronal marker βIII-tubulin (green), the astroglial marker GFAP (red), and nuclei (blue). The number of cells positive for each marker was counted by using ImageJ and normalized by the number of the nuclear marker DAPI-positive cells. Each value represents the mean ± S.E.M. (n = 8). *p < .05, significant difference from the corresponding control value obtained in NSCs not treated with ERGO. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the last 1 or 3 days of culture increased the population of βIII-tubulin-positive cells and decreased GFAP-positive cells to similar extents in NSCs cultured for either 6 or 9 days (Fig. 2B, C), whereas the effects of ERGO were marginal in NSCs cultured for 3 days (Fig. 2B, C). This may be attributed to a change in the expression of OCTN1 in cultured NSCs. The expression of the OCTN1 gene product in NSCs cultured for 3 days is lower than that of NSCs cultured for 6 and 9 days [27]. These results indicate that ERGO promotes neuronal differentiation in cultured NSCs in as little as 1 day of exposure.

3.3. Exposure of NSCs to ERGO selectively induces NT5 among neurotrophins

The effects of ERGO on the induction of neurotrophins were examined as a possible mechanism underlying the ERGO-induced neuronal differentiation in NSCs. With reference to the studies by Ito et al. [32] and Mosher et al. [42], we examined expression of the following neurotrophins: NGF, BDNF, NT3, NT5, bFGF, EGF, vascular endothelial growth factor (vEGF), ciliary neurotrophic factor (CNTF), Haptoglobin, and stem cell factor (SCF). Exposure of WT-NSCs to ERGO for 9 days significantly increased the mRNA expression of the bHLH transcription factor Math1, a neuronal differentiation activator, and the neurotrophic factor NT5 (Fig. 3A), whereas exposure of *octn1*$^{-/-}$-NSCs to ERGO only minimally affected the mRNA expression of any neurotrophin (data not shown). Exposure of ICR-NSCs to ERGO for 9 days also remarkably increased the mRNA expression of NT5 and Math1 (Fig. S2), indicating that there is no difference in the ERGO-induced expression of the neurotrophic factor or the bHLH transcription factor between the two mouse strains C57BL/6 J and ICR. We next investigated in cultured ICR-NSCs the time profile of the mRNA expression of the neurotrophic factors after the addition of ERGO. ERGO increased the gene expression of NT5 at 6 h and significantly increased the NT5 expression after treatment for 12 or 24 h (Fig. 3B). ERGO increased the gene expression of Math1 at 12 h and significantly increased the Math1 expression at 24 h (Fig. 3B). Furthermore, exposure to ERGO for 24 h significantly increased the expression of the gene product of NT5 but not BDNF in cultured NSCs (Fig. 3C). These results suggest that, in cultured NSCs, exposure to ERGO induced the neurotrophic factor NT5 within several hours, and that the ERGO-induced elevation of Math1 expression could be mediated through the induction of NT5.

3.4. ERGO-induced neuronal differentiation is mediated through activation of TrkB by NT5

We next examined whether an inhibitor of TrkB which is a receptor for NT5 prevents the promotion of neuronal differentiation and induction of Math1 by ERGO. ICR-NSCs were exposed to ERGO in either the absence or presence of the TrkB inhibitor GNF5837, followed by an evaluation of the cellular differentiation and the expression of p-TrkB and Math1 mRNA. Exposure to ERGO or NT5 significantly increased the population of βIII-tubulin-positive cells and decreased that of GFAP-positive cells in cultured ICR-NSCs (Fig. 4A-C). Simultaneous treatment with GNF5837 prevented these changes by ERGO or NT5 exposure in the cellular population (Fig. 4A-C). On the other hand, GNF5837 alone did not significantly affect the population of βIII-tubulin- and GFAP-positive cells (Fig. 4A-C). In addition, exposure of cultured NSCs to ERGO elevated the expression of p-TrkB, and this elevation by ERGO was suppressed to the control level by GNF5837 treatment (Fig. 4D). GNF5837 treatment also markedly inhibited the induction of Math1 mRNA by ERGO exposure in cultured NSCs (Fig. 4E). These results suggest that ERGO may promote neuronal differentiation, at least in part, through activation of TrkB by NT5 and subsequent induction of Math1 in NSCs.

3.5. Exposure of NSCs to ERGO activates mTORC1 signaling

To clarify the upstream signaling of ERGO-induced NT5 upregulation, a possible involvement of mTORC1 known as an amino acid sensor

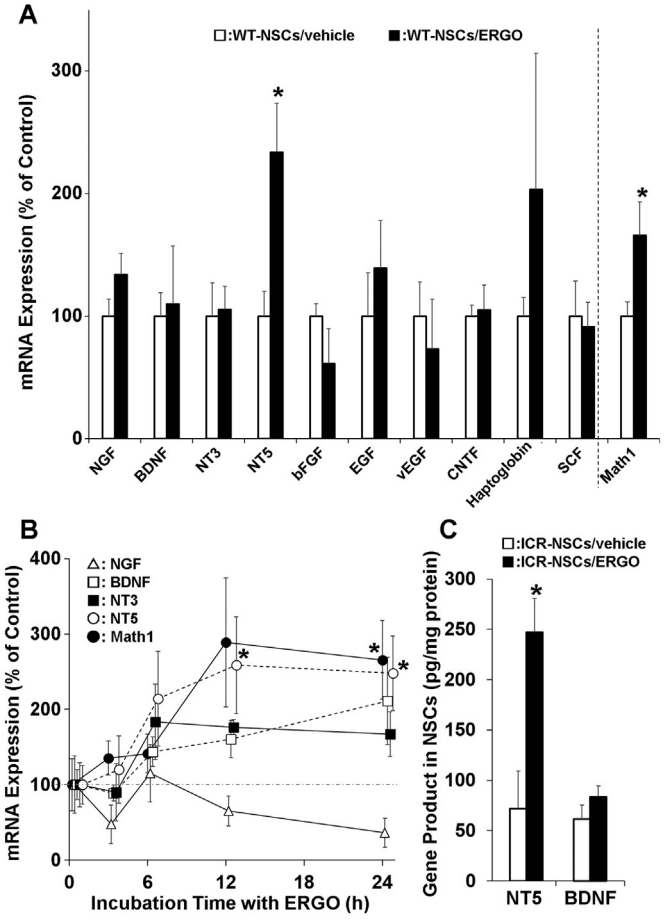

Fig. 3. Effects of ERGO on the expression of neurotrophins in cultured NSCs. (A) WT-NSCs were exposed to vehicle or ERGO (500 μM) for 9 days, and total RNA was extracted for quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. mRNA expression of neurotrophins and Math1 were determined and normalized by that of 36B4. Each value represents the mean ± S.E.M. (n = 3–6). *p < .05, significant difference from the control value obtained in NSCs treated with vehicle. (B) ICR-NSCs were cultured for 6 days and exposed to ERGO (500 μM) during the last 0, 3, 6, 12, or 24 h in culture. Total RNA was then extracted for quantitative RT-PCR analysis. mRNA expression of NGF (△), BDNF (□), NT3 (■), NT5 (○), and Math1 (●) was determined and normalized by that of 36B4. Each value represents the mean ± S.E.M. (n = 4–6). *p < .05, significant difference from the corresponding control value obtained in NSCs not treated with ERGO. (C) ICR-NSCs were cultured for 6 days and exposed to vehicle or ERGO (500 μM) during the last 24 h in culture. Expression of NT5 and BDNF at the protein level was determined by enzyme immunoassay. Each value represents the mean ± S.E.M. (n = 3–6). *p < .05, significant difference from the control value obtained in NSCs treated with vehicle.

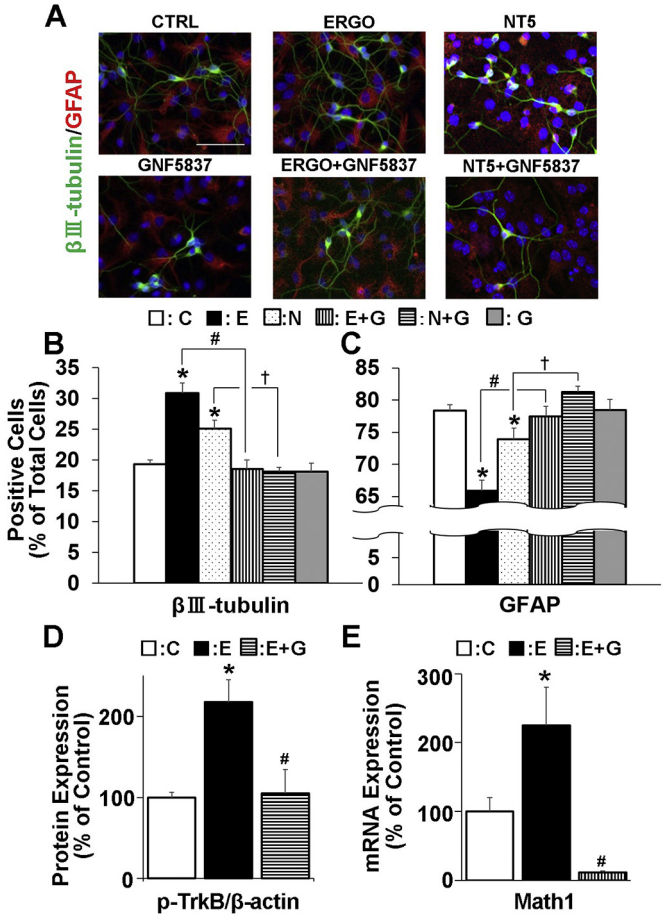

Fig. 4. Effects of a TrkB inhibitor on the neuronal differentiation provoked by ERGO or NT5 in cultured NSCs. (A-C) ICR-NSCs were cultured for 6 days and exposed to vehicle (C; control), ERGO (E; 500 μM), or NT5 (N; 100 ng/mL) in either the absence or presence of the TrkB inhibitor GNF5837 (G; 100 nM) during the last 3 days in culture. The NSCs were then induced to differentiate by adhesion culture for an additional 3 days. (A) The cells were fixed with 4% PA, followed by immunocytochemical detection of the neuronal marker βIII-tubulin (green), the astroglial marker GFAP (red), and nuclei (blue). *Scale bar*: 50 μm. (B, C) The number of cells positive for each marker was counted using ImageJ and normalized by the number of the nuclear marker DAPI-positive cells. Each value represents the mean ± S.E.M. (n = 10–20). (D) Phosphorylation of TrkB was examined by western blot after incubation with vehicle or ERGO in either the absence or presence of GNF5837 during the last 3 days in culture. Each value is normalized by the protein level of β-actin and represents the mean ± S.E.M. (n = 3–4). (E) Expression of Math1 mRNA was also determined by quantitative RT-PCR after incubation with vehicle or ERGO in either the absence or presence of GNF5837 during the last 3 days in culture. Each value is normalized by the expression level of 36B4 mRNA and represents the mean ± S.E.M. (n = 3–6). *p < .05, significant difference from the control value obtained in NSCs treated with vehicle. #p < .05, significant difference from the value obtained in NSCs treated with ERGO alone. †p < .05, significant difference from the value obtained in NSCs treated with NT5 alone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was examined. mTOR is a catalytic component of mTORC1, downstream effectors of mTORC1 are S6K1 and 4EBP1, and rapamycin is an inhibitor of mTORC1 signaling. Exposure of cultured NSCs to ERGO for 3 days significantly increased the expression of p-mTOR, p-S6K1 at Thr389, and p-S6K1 at Thr371, whereas simultaneous addition of rapamycin remarkably suppressed this increase in p-mTOR, p-S6K1 at Thr389, and p-S6K1 at Thr371 provoked by ERGO (Fig. 5A, B). On the other hand, ERGO minimally affected the expression of total mTOR and S6K1 (Fig. 5A, C) as well as p-4EBP1 and total 4EBP1 (Fig. 5A-C). In rodents, three isoforms of 4EBP1 are detected: α is the least phosphorylated form, β is an intermediate form, and γ is a hyperphosphorylated isoform [43]. Treatment with rapamycin alone significantly decreased β and γ forms of p-4EBP1 and tended to increase the α form of p-4EBP1 (Fig. 5A, B), which is in agreement with previous studies [43,44]. Time profiles of the expression of mTORC1 signaling-related proteins during ERGO exposure were also examined in cultured NSCs. ERGO significantly increased the expression of p-S6K1 at Thr389 at just 1 h after the start of the exposure, and the expression of p-mTOR tended to be increased at 3 h and significantly increased at 6 h (Fig. 5D, E). Thus, the increase in expression of p-S6K1 and p-mTOR occurred in a shorter time than the induction of NT5 observed at 12 h (Fig. 3B). The expression of β and γ forms of p-4EBP1 was also increased within 6 h, but these increases were not statistically significant (Fig. 5D, E). These

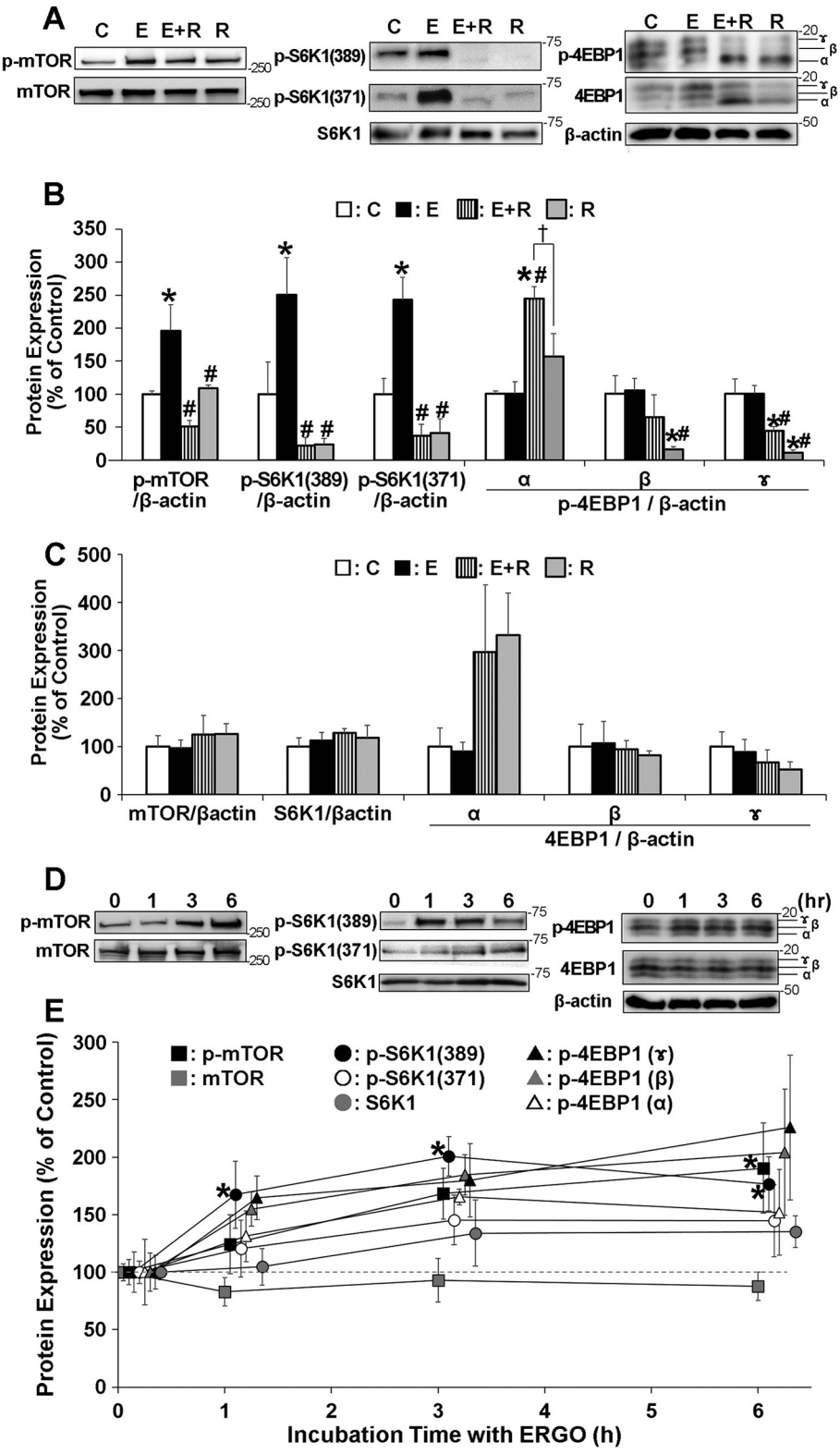

Fig. 5. Effects of ERGO on the activation of mTORC1 signaling in cultured NSCs. (A-C) ICR-NSCs were cultured for 6 days and exposed to vehicle (C; control) or ERGO (E; 500 μM) in either the absence or presence of the mTORC1 inhibitor rapamycin (R; 100 nM) during the last 3 days in culture. Phosphorylation of mTORC1 signaling-related proteins (mTOR, S6K1, and 4EBP1) was examined by western blot. Typical examples of western blotting of phosphorylated and total proteins are shown in panel (A). Intensity of each band is normalized by that of β-actin. Each value represents the mean ± S.E.M. (n = 3–4). *p < .05, significant difference from the control value obtained in NSCs treated with vehicle. #p < .05, significant difference from the value obtained in NSCs treated with ERGO alone. (D, E) ICR-NSCs were cultured for 6 days and then exposed to ERGO (500 μM) during the last 0, 1, 3, or 6 h in culture. Phosphorylation of the mTORC1 signaling-related proteins was examined by western blot. Typical examples of western blotting are shown in panel (D). Intensity of each band is normalized by that of β-actin (■: p-mTOR, ■: mTOR, ●: p-S6K1 at Thr389, ○: p-S6K1 at Thr371, ●: S6K1, △: p-4EBP1(α), ▲: p-4EBP1(β), ▲: p-4EBP1(γ)). Each value represents the mean ± S.E.M. (n = 4–7). *p < .05, significant difference from the corresponding control value obtained in NSCs not treated with ERGO.

results suggest that in NSCs ERGO may activate mTORC1 signaling in a relatively short time, followed by induction of NT5.

3.6. ERGO-induced neuronal differentiation is mediated through activation of mTORC1 signaling and subsequent activation of TrkB

A possible involvement of mTORC1 signaling in the ERGO-induced neuronal differentiation of NSCs was examined using several inhibitors. GSK2334470 is an inhibitor of 3-phosphoinositide-dependent protein kinase 1 (PDK1), which is one of the upstream kinases that can phosphorylate S6K1, while PF4708671 directly inhibits S6K1. All of the inhibitors rapamycin, GSK2334470, and PF4708671 suppressed the increase in the population of βIII-tubulin-positive cells (Fig. 6A, B) and the decrease in the number of GFAP-positive cells (Fig. 6A, C) provoked by ERGO in cultured NSCs. Rapamycin or PF4708671 alone minimally affected the population of βIII-tubulin- or GFAP-positive cells, but

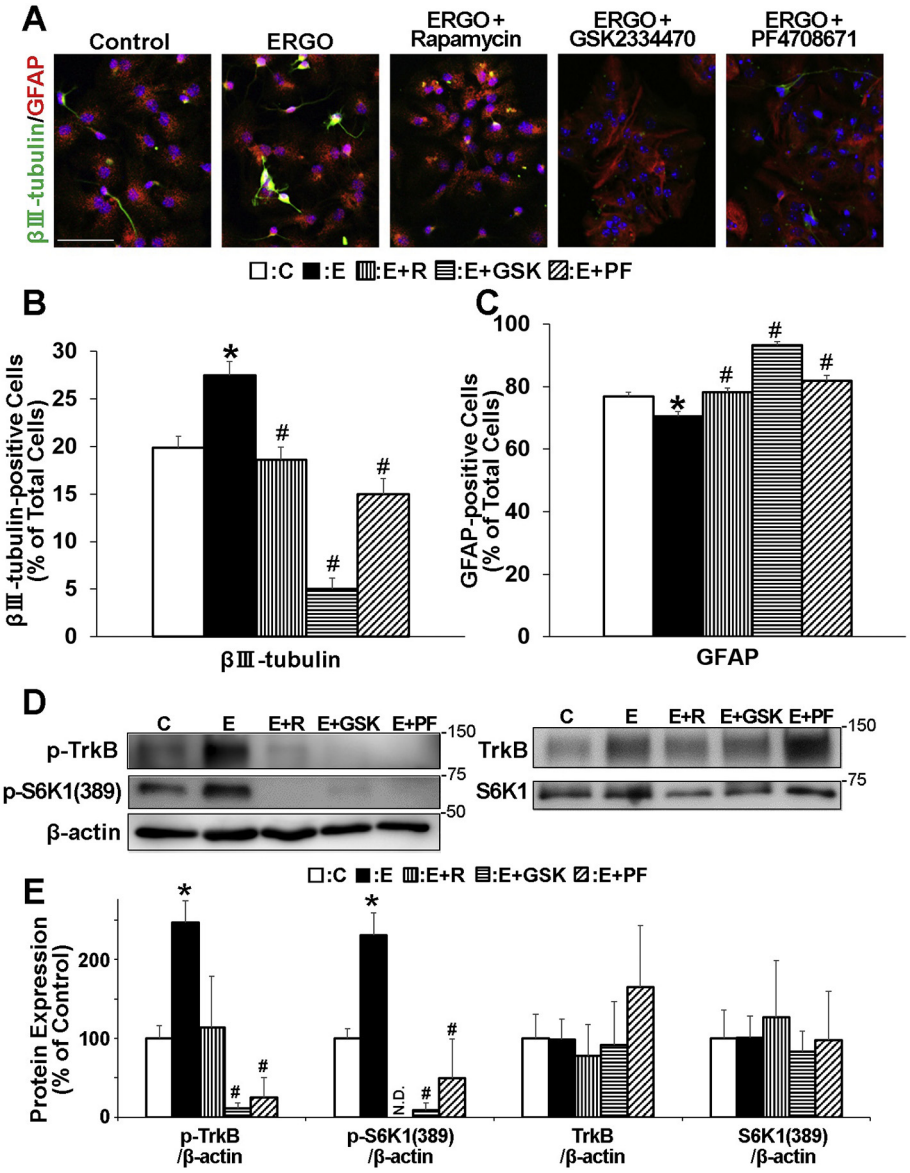

Fig. 6. Effects of mTORC1 signaling inhibitors on ERGO-induced neuronal differentiation and activation of TrkB in cultured NSCs. ICR-NSCs were cultured for 6 days and exposed to vehicle (C; control) or ERGO (E; 500 μM) in either the absence or presence of rapamycin (R; 100 nM), the PDK1 inhibitor GSK2334470 (GSK; 5 μM), or the S6K1 inhibitor PF4708671 (PF; 1 μM) during the last 3 days in culture. The NSCs were then induced to differentiate by adhesion culture for an additional 3 days. (A) The cells were fixed with 4% PA, followed by immunocytochemical detection of the neuronal marker βIII-tubulin (green), the astroglial marker GFAP (red), and nuclei (blue). *Scale bar*: 50 μm. (B, C) The number of cells positive for each marker was counted by using ImageJ and normalized by that of the nuclear marker DAPI-positive cells. Each value represents the mean ± S.E.M. (n = 10–20). (D, E) Phosphorylation of TrkB and S6K1 was examined by western blot. Typical examples of western blotting are shown in panel (D). Intensity of each band is normalized by that of β-actin. Each value represents the mean ± S.E.M. (n = 3–4). *N.D.*: not detectable. *p < .05, significant difference from the control value obtained in NSCs treated with vehicle. #p < .05, significant difference from the value obtained in NSCs treated with ERGO alone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GSK2334470 alone decreased the population of βIII-tubulin-positive cells and increased that of GFAP-positive cells (data not shown). These results suggest that ERGO may promote neuronal differentiation through activation of mTORC1 and S6K1 in cultured NSCs. GSK2334470 seemed to suppress spontaneous neuronal differentiation in NSCs (Fig. 6B), and the inhibitory effects may be caused by the inhibition of Akt signaling because PDK1 activates not only S6K1 but also Akt signaling which is important for neuronal differentiation [45].

Effects of mTORC1 signaling inhibitors on phosphorylation of TrkB were examined to clarify that in NSCs exposed to ERGO mTORC1 signaling is upstream of TrkB phosphorylation. In cultured NSCs, exposure to ERGO significantly increased the expression of p-TrkB and p-S6K1 at Thr389, whereas ERGO minimally affected the expression of total protein of TrkB and S6K1 (Fig. 6D, E). All of the inhibitors rapamycin, GSK2334470, and PF4708671 suppressed the ERGO-induced phosphorylation of TrkB and S6K1 at Thr389 (Fig. 6D, E). These results suggest that ERGO-induced neuronal differentiation may be mediated, at least in part, through activation of mTORC1 signaling and subsequent activation of TrkB in NSCs.

3.7. Oral administration of ERGO activates S6K1 and NT5-TrkB signaling in the hippocampal DG

Finally, it was examined whether oral administration of ERGO activates mTORC1 and NT5-TrkB signaling in mice. Oral administration of ERGO three times per week for 2 weeks to ICR mice significantly increased the expression of p-TrkB and p-S6 K1 at Thr389 in the hippocampal DG (Fig. 7A, C) but not in the cortex (Fig. 7B, D). In contrast to the present in vitro results, the oral administration of ERGO did not increase the expression of p-mTOR in the DG and the cortex (Fig. 7A–D). Minimal effects on total protein of TrkB and S6K1 after oral ERGO administration were observed in the DG and cortex (Fig. 7A, B). The oral ERGO administration also increased the gene product of NT5 in the DG but not in the cortex (Fig. 7E). These changes in TrkB and S6K1 phosphorylation and NT5 expression may be attributed to NSCs. NSCs are abundantly present in the DG but not in the cortex, and OCTN1 is highly expressed in NSCs [27]. On the other hand, oral administration of ERGO twice for 2 days to ICR mice significantly increased the protein expression of p-S6K1 at Thr389 but not p-TrkB in the DG (Fig. S3), supporting the hypothesis that phosphorylation of TrkB occurs downstream of that of S6K1 with regard to activation of cellular signaling by ERGO. These in vivo results are comparable with

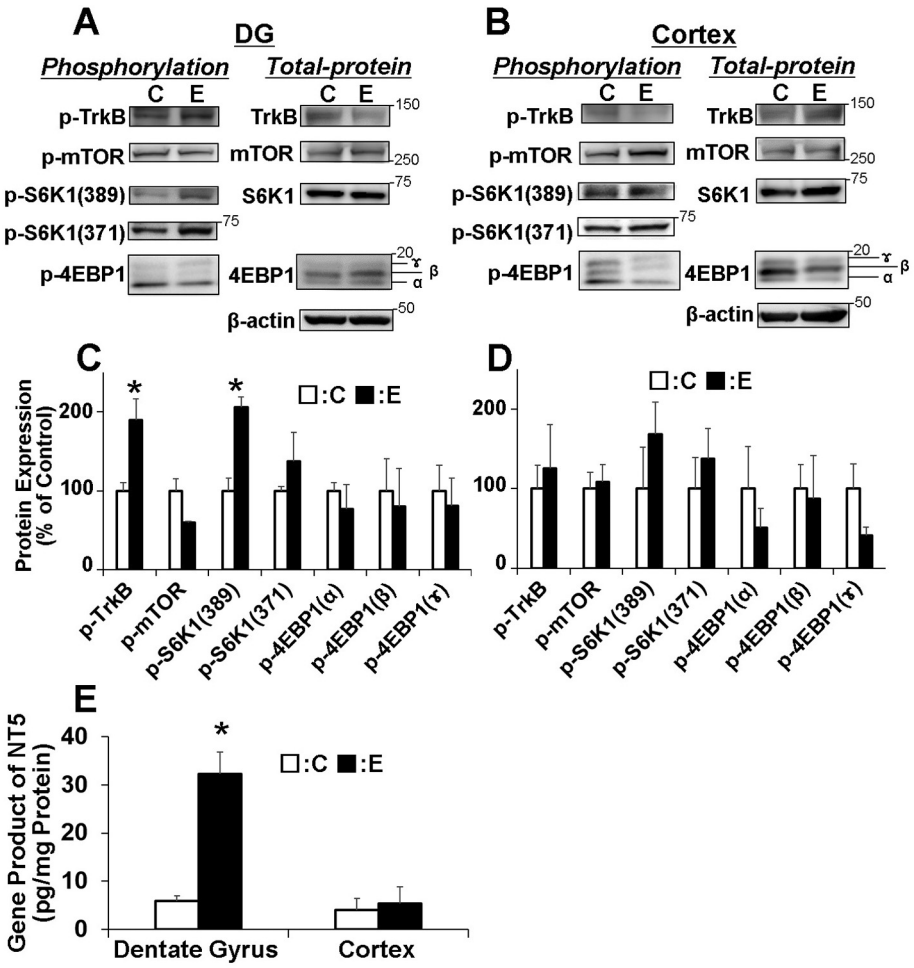

Fig. 7. Effects of 2-week oral administration of ERGO on the activation of mTORC1 and NT5-TrkB signaling in the hippocampal DG and the cortex of mice. Mice were orally administered with 0 or 50 mg/kg ERGO on day 0, 2, 4, 7, 9, and 11. Hippocampal DG and cortex were collected on day 19. (A-D) Phosphorylation of TrkB and mTORC1 signaling-related proteins (mTOR, S6K1, and 4EBP1) was examined by western blot. Typical examples of western blotting of DG and cortex samples are shown in panel (A) and (B), respectively. Intensity of each band is normalized by that of β-actin in DG (C) and cortex (D). Each value represents the mean ± S.E.M. (n = 3). (E) The amount of the gene product of NT5 in DG and cortex samples was determined by enzyme immunoassay. Each value is normalized by the protein concentration and represents the mean ± S.E.M. (n = 3). *p < .05, significant difference from the control value obtained in mice administered with vehicle.

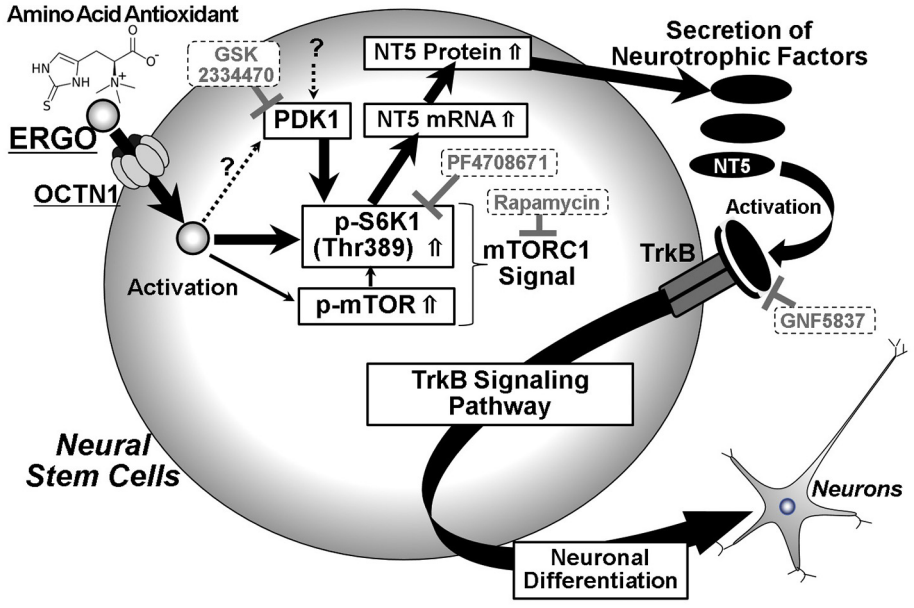

Fig. 8. Mechanisms underlying the neuronal differentiation provoked by ERGO in NSCs. Intracellular ERGO incorporated by OCTN1 phosphorylates S6K1 at Thr389 and/or mTORC1, followed by induction of NT5, activation of the TrkB signaling pathway, and subsequent promotion of neuronal differentiation of NSCs. Solid and dot arrows represent demonstrated results and hypothesis, respectively. Gray characters stand for inhibitors.

the present in vitro findings using cultured NSCs.

4. Discussion

The present study is the first to demonstrate that in cultured NSCs, ERGO-induced neuronal differentiation is mediated through phosphorylation of S6K1 at Thr389 and subsequent activation of NT5-TrkB signaling (Figs. 3–6, 8). This study is also the first to show that oral administration of ERGO to mice phosphorylates S6K1 at Thr389, which then activates NT5-TrkB signaling in the hippocampal DG, a brain region with NSC abundancy (Fig. 7, Fig. S3). Our previous study showed that orally ingested ERGO promotes neurogenesis in the DG of

mice [19]. Therefore, ingestion of ERGO may promote neurogenesis through phosphorylation of S6K1 at Thr389 and activation of NT5-TrkB signaling in the DG. Promotion of neurogenesis alleviates the symptoms of neuropsychiatric disorders, such as depression [11–13], and several drugs used for the treatment of these psychiatric disorders can induce neurogenesis [14–16]. The mechanisms underlying the induction of neuronal differentiation by ERGO are different from those by the clinically used drugs: ERGO promotes neuronal differentiation through activation of S6K1 by phosphorylation at Thr389 and induction of NT5, whereas the conventional drugs induce differentiation through the activation of mTORC1 and BDNF [30,31,46]. Thus, p-S6K1 at Thr389, NT5, and their signaling pathways could be novel targets for the treatment of neuropsychiatric disorders. Drugs currently used to treat neuropsychiatric disorders cause several problems, e.g., severe adverse effects, such as serotonin syndrome, delayed onset of therapeutic effects, among others. These problems could be solved by using compounds such as ERGO, which could induce neuronal differentiation via mechanisms of action that are different from conventional drugs.

In cultured NSCs, exposure to ERGO selectively induced NT5 expression and phosphorylated TrkB, which is a receptor for NT5 (Figs. 3, 5D, E). Oral administration of ERGO also increased the expression of the gene product of NT5 and p-TrkB in the DG of mice (Fig. 7). Both ERGO and NT5 increased the population of βIII-tubulin-positive cells, and this increase was inhibited by the TrkB inhibitor GNF5837 (Fig. 4). Nakamichi et al. reported that dietary ingestion of ERGO causes antidepressant-like effects via the promotion of hippocampal neurogenesis in mice [19]. Like BDNF, NT5 promotes neuronal differentiation through activation of TrkB [16,32,47]. NT5 has been suggested as a clinical marker of depression [30], because its plasma level decreases with the worsening of depressive symptoms [48,49]. TrkB signaling is involved in the alleviation of depressive symptoms via the promotion of neurogenesis, spine formation, and synaptic transmission [14,31] and can affect the patient's sensitivity to antidepressants via regulation of hippocampal neurogenesis [14–16]. Taken together, ERGO may promote neuronal differentiation and exert antidepressant effects, at least in part through induction of NT5 and subsequent activation of TrkB in NSCs.

The therapeutic effects of increased cerebral concentrations of TrkB ligands, such as BDNF, on depression are well studied [50–52]. Because it is difficult for peripherally administered neurotrophins to cross the BBB, this route of administration is unlikely to cause sufficient antidepressant effects [53]. In contrast, oral administration of ERGO could be useful for the treatment of neuropsychiatric disorders because orally ingested ERGO is absorbed from the gastrointestinal tract and is highly distributed to the brain across the BBB [19]. In addition, orally administered ERGO increased the expression of NT5 and activated TrkB in the DG (Fig. 7). Compounds that can induce NT5 in the brain have not been reported yet. Therefore, ERGO, with the characteristics of a cerebral NT5 inducer, can be considered as an interesting seed compound for the development of new therapeutic drugs for neuropsychiatric disorders. In particular, ERGO would be useful for the treatment of depression in BDNF Met allele carriers, because ERGO induced NT5 but not BDNF in NSCs (Fig. 3) and the DG (Fig. 7E). BDNF Met allele carriers show resistance to treatments with selective serotonin reuptake inhibitors (SSRIs) [54], due to the decrease in secretion of BDNF [55]. Additionally, plasma concentrations of ERGO in patients with Parkinson's disease or mild cognitive impairment are significantly lower than those in healthy people [56,57]. Reduction of the promotive effects on neuronal differentiation and/or the protective effects against neurotoxicity by ERGO may, in part, be involved in the onset and development of some neuropsychiatric disorders [58].

ERGO-induced neuronal differentiation was in NSCs suppressed by the inhibitors of mTORC1, S6K1, and TrkB (Figs. 4, 6). Exposure of NSCs to ERGO (Figs. 3B, 5D, E) and oral administration of ERGO to mice (Fig. 7, Fig. S3) first increased the expression of p-S6K1 at Thr389 and then via induction of NT5 that of p-TrkB. These results suggest that the mechanisms underlying ERGO-induced neuronal differentiation involve the upstream activation of S6K1 and that S6K1 activation is the signal for the induction of NT5 and subsequent activation of TrkB (Fig. 8). Unexpectedly, phosphorylation of S6K1 at Thr389, which is a downstream signal of mTOR phosphorylation in an mTORC1 signaling pathway [59], was induced by ERGO in a shorter time, compared to the phosphorylation of mTOR (Fig. 5D, E). ERGO may induce phosphorylation of S6K1 at Thr389 in an mTOR-independent manner, in addition to an mTOR-dependent manner (Fig. 8). GSK2334470, an inhibitor of PDK1 that phosphorylates S6K1 suppressed the ERGO-induced phosphorylation of S6K1 at Thr389 and TrkB and the promotion of neuronal differentiation (Fig. 6). Taken together, intracellular ERGO may activate S6K1 signaling via the activation of PDK1 as well as mTOR and/or via unidentified molecules, and spontaneous neuronal differentiation of NSCs may be regulated through activation of S6K1 by PDK1 (Fig. 8). Further studies are required to elucidate more details of this process.

Intriguingly, ERGO activated mTORC1 and NT5-TrkB signaling and promoted neuronal differentiation in a relatively short time. Exposure to ERGO increased in NSCs the expression of p-S6K1 at Thr389 in only 1 h and that of p-mTOR in 6 h (Fig. 5D, E), and exposure to ERGO for 24 h increased the population of βIII-tubulin-positive cells (comparison between white and gray symbols in Fig. 2B). Fast incorporation of ERGO into NSCs via OCTN1 [27] may rapidly activate intracellular signaling, such as mTORC1 signaling that is able to promote neuronal differentiation. Like ERGO, SSRIs also phosphorylate mTOR and S6K1 and promote neuronal differentiation [60–64]. While the SSRI paroxetine is able to phosphorylate mTOR and S6K1 and to promote neuronal differentiation, it requires much longer time compared to that of ERGO. Exposure to paroxetine requires about 7 days to promote neuronal differentiation in cultured NSCs [64] and about 4 days to increase the expression of p-mTOR and p-S6K1 in cultured neurons [60]. The slow induction of neuronal differentiation by SSRIs is considered to be one of the reasons for their delayed onset of antidepressant effects. In fact, it has been recently reported that rapid activation of mTORC1 and promotion of neurogenesis are essential for a rapid onset of antidepressant effects [16,39,61,65–67]. Ketamine shows rapid antidepressant effects within hours of the administration through activation of mTORC1, whereas the clinically used antidepressants like paroxetine take several weeks to exert antidepressant effects [39,65–67]. In cultured NSCs, ketamine activates mTORC1 signaling 1 h after the treatment [68], and ERGO also activated S6K1, the downstream molecule of p-mTOR in mTORC1 signaling, in only 1 h (Fig. 5D, E). Therefore, ERGO is expected to show rapid antidepressant effects through the activation of mTORC1 signaling, including mTOR and S6K1. Oral administration of ERGO phosphorylated in the DG S6K1 at Thr389 after 2 days (Fig. S3) as well as TrkB and S6K1 at Thr389 after 19 days (Fig. 7A, C). Oral ingestion of ERGO for 2 weeks promotes hippocampal neurogenesis and exhibits antidepressant-like effects in mice [19]. Further studies are required to elucidate the administration periods and doses of ERGO necessary to promote hippocampal neurogenesis and to exert antidepressant effects.

5. Conclusions

Intracellular ERGO incorporated by OCTN1 promotes in NSCs neuronal differentiation, at least in part, through phosphorylation of S6K1 at Thr389 and subsequent activation of TrkB signaling by induction of NT5 (Fig. 8). S6K1 and NT5 are possible targets for the treatment of neuropsychiatric disorders, and ERGO is a promising candidate as a seed compound in the discovery of drugs for these disorders.

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Author contributions

Conceived and designed the experiments: TI YK NN. Performed the experiments: TI NN. Analyzed the data: TI YM YK NN. Contributed reagents/materials/analysis tools: TI YM YK NN. Wrote the paper: TI YK NN.

Conflict of interest

The authors declare that they have no conflicts of interest.

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References

[1] C.B. Johansson, S. Momma, D.L. Clarke, M. Risling, U. Lendahl, J. Frisén, Identification of a neural stem cell in the adult mammalian central nervous system, Cell 96 (1999) 25–34.
[2] B.A. Reynolds, S. Weiss, Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell, Dev. Biol. 175 (1996) 1–13.
[3] R. Knoth, I. Singec, M. Ditter, G. Pantazis, P. Capetian, R.P. Meyer, V. Horvat, B. Volk, G. Kempermann, Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years, PLoS One 5 (2010) e8809.
[4] G.L. Ming, H. Song, Adult neurogenesis in the mammalian brain: significant answers and significant questions, Neuron 70 (2011) 687–702.
[5] C.L. Zhang, Y. Zou, W. He, F.H. Gage, R.M. Evans, A role for adult TLX-positive neural stem cells in learning and behaviour, Nature 451 (2008) 1004–1007.
[6] A.J. Eisch, D. Petrik, Depression and hippocampal neurogenesis: a road to remission? Science 338 (2012) 72–75.
[7] M.A. Hilliard, Axonal degeneration and regeneration: a mechanistic tug-of-war, J. Neurochem. 108 (2009) 23–32.
[8] P.J. Horner, F.H. Gage, Regenerating the damaged central nervous system, Nature 407 (2000) 963–970.
[9] L.D. Ladage, Environmental change, the stress response, and neurogenesis, Integr. Comp. Biol. 55 (2015) 372–383.
[10] J.N. Le Grand, L. Gonzalez-Cano, M.A. Pavlou, J.C. Schwamborn, Neural stem cells in Parkinson's disease: a role for neurogenesis defects in onset and progression, Cell. Mol. Life Sci. 72 (2015) 773–797.
[11] A. Sahay, R. Hen, Adult hippocampal neurogenesis in depression, Nat. Neurosci. 10 (2007) 1110–1115.
[12] J.S. Snyder, A. Soumier, M. Brewer, J. Pickel, H.A. Cameron, Adult hippocampal neurogenesis buffers stress responses and depressive behaviour, Nature 476 (2011) 458–461.
[13] S. Bolognin, M. Buffelli, J. Puoliväli, K. Iqbal, Rescue of cognitive-aging by administration of a neurogenic and/or neurotrophic compound, Neurobiol. Aging 35 (2014) 2134–2146.
[14] Y. Li, B.W. Luikart, S. Birnbaum, J. Chen, C.H. Kwon, S.G. Kernie, R. Bassel-Duby, L.F. Parada, TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment, Neuron 59 (2008) 399–412.
[15] T.D. Perera, S. Park, Y. Nemirovskaya, Cognitive role of neurogenesis in depression and antidepressant treatment, Neuroscientist 14 (2008) 326–338.
[16] J.L. Warner-Schmidt, R.S. Duman, Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment, Hippocampus 16 (2006) 239–249.
[17] E. Horgusluoglu, K. Nudelman, K. Nho, A.J. Saykin, Adult neurogenesis and neurodegenerative diseases: a systems biology perspective, Am. J. Med. Genet. B Neuropsychiatr. Genet. 174 (2017) 93–112.
[18] A. Thompson, K. Boekhoorn, A.M. Van Dam, P.J. Lucassen, Changes in adult neurogenesis in neurodegenerative diseases: cause or consequence? Genes Brain Behav. 1 (2008) 28–42.
[19] N. Nakamichi, K. Nakayama, T. Ishimoto, Y. Masuo, T. Wakayama, H. Sekiguchi, K. Sutoh, K. Usumi, S. Iseki, Y. Kato, Food-derived hydrophilic antioxidant ergothioneine is distributed to the brain and exerts antidepressant effect in mice, Brain Behav. 6 (2016) e00477.
[20] T.Y. Song, H.C. Lin, C.L. Chen, J.H. Wu, J.W. Liao, M.L. Hu, Ergothioneine and melatonin attenuate oxidative stress and protect against learning and memory deficits in C57BL/6J mice treated with D-galactose, Free Radic. Res. (2014) 1049–1060.
[21] C. Herrera-Arozamena, O. Martí-Marí, M. Estrada, M. de la Fuente Revenga, M.I. Rodríguez-Franco, Recent advances in neurogenic small molecules as innovative treatments for neurodegenerative diseases, Molecules 21 (2016) (pii: E1165).
[22] D. Halder, G.H. Kim, I. Shin, Synthetic small molecules that induce neuronal differentiation in neuroblastoma and fibroblast cells, Mol. BioSyst. 11 (2015) 2727–2737.
[23] P. Ganesan, H.M. Ko, I.S. Kim, D.K. Choi, Recent trends in the development of nanophytobioactive compounds and delivery systems for their possible role in reducing oxidative stress in Parkinson's disease models, Int. J. Nanomedicine 10 (2015) 6757–6772.
[24] M.S. Kumaran, G. Dabas, A.B. Kapadia, D. Parsad, Resveratrol-induced thrombocytopenia: inadvertent side-effect of a commonly used antioxidant, Dermatol. Ther. 31 (2018) e12578.
[25] P. Qiu, S. Man, J. Li, J. Liu, L. Zhang, P. Yu, W. Gao, Overdose intake of curcumin initiates the unbalanced state of bodies, J. Agric. Food Chem. 64 (2016) 2765–2771.
[26] Y. Kato, Y. Kubo, D. Iwata, S. Kato, T. Sudo, T. Sugiura, T. Kagaya, T. Wakayama, A. Hirayama, M. Sugimoto, K. Sugihara, S. Kaneko, T. Soga, M. Asano, M. Tomita, T. Matsui, M. Wada, A. Tsuji, Gene knockout and metabolome analysis of carnitine/organic cation transporter OCTN1, Pharm. Res. 27 (2010) 832–840.
[27] T. Ishimoto, N. Nakamichi, H. Hosotani, Y. Masuo, T. Sugiura, Y. Kato, Organic cation transporter-mediated ergothioneine uptake in mouse neural progenitor cells suppresses proliferation and promotes differentiation into neurons, PLoS One 9 (2014) e89434.
[28] A.M. Bond, G.L. Ming, H. Song, Adult mammalian neural stem cells and neurogenesis: five decades later, Cell Stem Cell 17 (2015) 385–395.
[29] C.J. Hong, H. Park, S.W. Yu, Autophagy for the quality control of adult hippocampal neural stem cells, Brain Res. 1649 ((2016) 166–172.
[30] E. Shimizu, K. Hashimoto, N. Okamura, K. Koike, N. Komatsu, C. Kumakiri, M. Nakazato, H. Watanabe, N. Shinoda, S. Okada, M. Iyo, Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants, Biol. Psychiatry 54 (2003) 70–75.
[31] E. Castrén, T. Rantamäki, The role of BDNF and its receptors in depression and antidepressant drug action: reactivation of developmental plasticity, Dev. Neurobiol. 70 (2010) 289–297.
[32] H. Ito, A. Nakajima, H. Nomoto, S. Furukawa, Neurotrophins facilitate neuronal differentiation of cultured neural stem cells via induction of mRNA expression of basic helix-loop-helix transcription factors Mash1 and Math1, J. Neurosci. Res. 71 (2003) 648–658.
[33] L. Bar-Peled, D.M. Sabatini, Regulation of mTORC1 by amino acids, Trends Cell Biol. 24 (2014) 400–406.
[34] R.L. Wolfson, L. Chantranupong, R.A. Saxton, K. Shen, S.M. Scaria, J.R. Cantor, D.M. Sabatini, Sestrin2 is a leucine sensor for the mTORC1 pathway, Science 351 (2015) 43–48.
[35] N.W. Hartman, T.V. Lin, L. Zhang, G.E. Paquelet, D.M. Feliciano, A. Bordey, mTORC1 targets the translational repressor 4E-BP2, but not S6 kinase 1/2, to regulate neural stem cell self-renewal in vivo, Cell Rep. 5 (2013) 433–444.
[36] J.E. Lee, M.S. Lim, J.H. Park, C.H. Park, H.C. Koh, S6K promotes dopaminergic neuronal differentiation through PI3K/Akt/mTOR-dependent signaling pathways in human neural stem cells, Mol. Neurobiol. 53 (2016) 3771–3782.
[37] L. Magri, R. Galli, mTOR signaling in neural stem cells: from basic biology to disease, Cell. Mol. Life Sci. 70 (2013) 2887–2898.
[38] T. Takarada, N. Nakamichi, R. Nakazato, T. Kakuda, H. Kokubo, S. Ikeno, S. Nakamura, N. Kuramoto, E. Hinoi, Y. Yoneda, Possible activation by the green tea amino acid theanine of mammalian target of rapamycin signaling in undifferentiated neural progenitor cells in vitro, Biochem. Biophys. Rep. 5 (2016) 89–95.
[39] Z.M. Ignácio, G.Z. Réus, C.O. Arent, H.M. Abelaira, M.R. Pitcher, J. Quevedo, New perspectives on the involvement of mTOR in depression as well as in the action of antidepressant drugs, Br. J. Clin. Pharmacol. 82 (2016) 1280–1290.
[40] H. Hagihara, K. Toyama, N. Yamasaki, T. Miyakawa, Dissection of hippocampal dentate gyrus from adult mouse, J. Vis. Exp. 17 (2009) (pii: 1543).
[41] H. Kitajima, S. Yoshimura, J. Kokuzawa, M. Kato, T. Iwama, T. Motohashi, T. Kunisada, N. Sakai, Culture method for the induction of neurospheres from mouse embryonic stem cells by coculture with PA6 stromal cells, J. Neurosci. Res. 80 (2005) 467–474.
[42] K.I. Mosher, R.H. Andres, T. Fukuhara, G. Bieri, M. Hasegawa-Moriyama, Y. He, R. Guzman, T. Wyss-Coray, Neural progenitor cells regulate microglia functions and activity, Nat. Neurosci. 15 (2012) 1485–1487.
[43] T.A. Lin, X. Kong, A.R. Saltiel, P.J. Blackshear, J.C. Lawrence Jr., Control of PHAS-I by insulin in 3T3-L1 adipocytes. Synthesis, degradation, and phosphorylation by a rapamycin-sensitive and mitogen-activated protein kinase-independent pathway, J. Biol. Chem. 270 (1995) 18531–18538.
[44] L. Beretta, Y.V. Svitkin, N. Sonenberg, Rapamycin stimulates viral protein synthesis and augments the shutoff of host protein synthesis upon picornavirus infection, J. Virol. 70 (1996) 8993–8996.
[45] K. Oishi, K. Watatani, Y. Itoh, H. Okano, F. Guillemot, K. Nakajima, Y. Gotoh, Selective induction of neocortical GABAergic neurons by the PDK1-Akt pathway through activation of Mash1, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 13064–13069.
[46] M.K. Seo, C.M. Choi, R.S. McIntyre, H.Y. Cho, C.H. Lee, R. Mansur, Y. Lee, J.H. Lee, Y.H. Kim, S.W. Park, J.G. Lee, Effects of escitalopram and paroxetine on mTORC1 signaling in the rat hippocampus under chronic restraint stress, BMC Neurosci. 18 (2017) 39.
[47] Y. Shen, N. Inoue, K. Heese, Neurotrophin-4 (ntf4) mediates neurogenesis in mouse embryonic neural stem cells through the inhibition of the signal transducer and activator of transcription-3 (stat3) and the modulation of the activity of protein kinase B, Cell. Mol. Neurobiol. 30 (2010) 909–916.
[48] E.A. Ogłodek, M.J. Just, A.R. Szromek, A. Araszkiewicz, Assessing the serum concentration levels of NT-4/5, GPX-1, TNF-α, and l-arginine as biomediators of depression severity in first depressive episode patients with and without posttraumatic

stress disorder, Pharmacol. Rep. 69 (2017) 1049–1058.
[49] J.C. Walz, P.V. Magalhães, L.M. Giglio, A.B. Cunha, L. Stertz, G.R. Fries, A.C. Andreazza, F. Kapczinski, Increased serum neurotrophin-4/5 levels in bipolar disorder, J. Psychiatr. Res. 43 (2009) 721–723.
[50] S.R. Vaka, S.N. Murthy, A. Balaji, M.A. Repka, Delivery of brain-derived neurotrophic factor via nose-to-brain pathway, Pharm. Res. 29 (2012) 441–447.
[51] A. Nitta, M. Ito, H. Fukumitsu, M. Ohmiya, H. Ito, A. Sometani, H. Nomoto, Y. Furukawa, S. Furukawa, 4-methylcatechol increases brain-derived neurotrophic factor content and mRNA expression in cultured brain cells and in rat brain in vivo, J. Pharmacol. Exp. Ther. 291 (1999) 1276–1283.
[52] M. Tikhonova, A.V. Kulikov, Antidepressant-like effects of central BDNF administration in mice of antidepressant sensitive catalepsy (ASC) strain, Chin. J. Physiol. 55 (2012) 284–293.
[53] S.R. Alcalá-Barraza, M.S. Lee, L.R. Hanson, A.A. McDonald, W.H. Frey, L.K. McLoon, Intranasal delivery of neurotrophic factors BDNF, CNTF, EPO, and NT-4 to the CNS, J. Drug Target. 18 (2010) 179–190.
[54] R. Colle, F. Gressier, C. Verstuyft, E. Deflesselle, J.P. Lépine, F. Ferreri, P. Hardy, J.P. Guilloux, A.C. Petit, B. Fève, B. Falissard, L. Becquemont, E. Corruble, Brain-derived neurotrophic factor Val66Met polymorphism and 6-month antidepressant remission in depressed Caucasian patients, J. Affect. Disord. 175 (2016) 233–240.
[55] M.F. Egan, M. Kojima, J.H. Callicott, T.E. Goldberg, B.S. Kolachana, A. Bertolino, E. Zaitsev, B. Gold, D. Goldman, M. Dean, B. Lu, D.R. Weinberger, The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function, Cell 112 (2003) 257–269.
[56] T. Hatano, S. Saiki, A. Okuzumi, R.P. Mohney, N. Hattori, Identification of novel biomarkers for Parkinson's disease by metabolomic technologies, J. Neurol. Neurosurg. Psychiatry 87 (2016) 295–301.
[57] I.K. Cheah, L. Feng, R.M. Tang, K.H. Lim, B. Halliwell, Ergothioneine levels in an elderly population decrease with age and incidence of cognitive decline; a risk factor for neurodegeneration? Biochem. Biophys. Res. Commun. 478 (2016) 162–167.
[58] N. Nakamichi, Y. Kato, Physiological roles of carnitine/organic cation transporter OCTN1/SLC22A4 in neural cells, Biol. Pharm. Bull. 40 (2017) 1146–1152.
[59] M. Saitoh, N. Pullen, P. Brennan, D. Cantrell, P.B. Dennis, G. Thomas, Regulation of an activated S6 kinase 1 variant reveals a novel mammalian target of rapamycin phosphorylation site, J. Biol. Chem. 277 (2002) 20104–20112.
[60] S.W. Park, J.G. Lee, M.K. Seo, C.H. Lee, H.Y. Cho, B.J. Lee, W. Seol, Y.H. Kim, Differential effects of antidepressant drugs on mTOR signalling in rat hippocampal neurons, Int. J. Neuropsychopharmacol. 17 (2014) 1831–1846.
[61] D. Xu, Y. Sun, C. Wang, H. Wang, Y. Wang, W. Zhao, G. Bao, F. Wang, Z. Cui, B. Jiang, Hippocampal mTOR signaling is required for the antidepressant effects of paroxetine, Neuropharmacology 128 (2017) 181–195.
[62] M.E. Glover, P.C. Pugh, N.L. Jackson, J.L. Cohen, A.D. Fant, H. Akil, S.M. Clinton, Early-life exposure to the SSRI paroxetine exacerbates depression-like behavior in anxiety/depression-prone rats, Neuroscience 284 (2015) 775–797.
[63] T. Deltheil, B.P. Guiard, J. Cerdan, D.J. David, K.F. Tanaka, C. Repérant, J.P. Guilloux, F. Coudoré, R. Hen, A.M. Gardier, Behavioral and serotonergic consequences of decreasing or increasing hippocampus brain-derived neurotrophic factor protein levels in mice, Neuropharmacology 55 (2008) 1006–1014.
[64] Z.W. Peng, F. Xue, H.N. Wang, R.G. Zhang, Y.C. Chen, Y. Wang, L.Y. Zhang, J. Fan, Q.R. Tan, Paroxetine up-regulates neurogenesis in hippocampus-derived neural stem cell from fetal rats, Mol. Cell. Biochem. 375 (2013) 105–113.
[65] W. Zhou, N. Wang, C. Yang, X.M. Li, Z.Q. Zhou, J.J. Yang, Ketamine-induced antidepressant effects are associated with AMPA receptors-mediated upregulation of mTOR and BDNF in rat hippocampus and prefrontal cortex, Eur. Psychiatry 29 (2014) 419–423.
[66] N. Li, B. Lee, R.J. Liu, M. Banasr, J.M. Dwyer, M. Iwata, X.Y. Li, G. Aghajanian, R.S. Duman, mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists, Science 329 (2010) 959–964.
[67] J.F. Cryan, O.F. O'Leary, Neuroscience. A glutamate pathway to faster-acting antidepressants? Science 329 (2010) 913–914.
[68] S. Mansouri, I. Agartz, S.O. Ögren, C. Patrone, M. Lundberg, PACAP protects adult neural stem cells from the neurotoxic effect of ketamine associated with decreased apoptosis, ER stress and mTOR pathway activation, PLoS One 12 (2017) e0170496.