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Functional expression of carnitine/organic cation transporter OCTN1 in mouse brain neurons: Possible involvement in neuronal differentiation

Noritaka Nakamichi^a, Takayuki Taguchi^a, Hiroshi Hosotani^a, Tomohiko Wakayama^b,

Takuya Shimizu^a, Tomoko Sugiura^a, Shoichi Iseki^b, Yukio Kato^{a,*}

^a *Faculty of Pharmacy and* ^b *Department of Histology and Embryology, Institute of Medical,
Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Ishikawa 920-1192,
Japan*

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OCTN1 regulates neuronal differentiation

*Corresponding author:

Prof. Yukio Kato, Ph.D

Faculty of Pharmacy,

Kanazawa University

Kakuma-machi, Kanazawa 920-1192, Japan

Tel/Fax:(81)-76-234-4465 / Email: ykato@p.kanazawa-u.ac.jp

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Abbreviations: ATRA, all-*trans* retinoic acid; CL, cerebellum; CX, cerebral cortex; DIV, days in vitro; DMEM, Dulbecco's modified Eagle's medium; ERGO, ergothioneine; FBS, fetal bovine serum; GAP43, growth-associated protein 43; HC, hippocampus; HO-1, heme oxygenase-1; HPLC, high-performance liquid chromatography; HT, hypothalamus; *i.c.v.*, intracerebroventricular; MAP2, microtubule-associated protein 2; MB, midbrain; MP, medulla and pons; Nrf2, nuclear factor-erythroid 2 p45-related factor-2; OCTN1, carnitine/organic cation transporter 1; PA, paraformaldehyde; PBS, phosphate-buffered saline; ROS, reactive oxygen species; siOCTN1; siRNA targeting the mouse OCTN1 gene; siRNA, small interfering RNA; SLC, solute carrier; Sox2, sex determining region Y-box 2; ST, striatum; Syn1, synapsin I; xCT, cystine glutamate exchanger subunit; Ucp2, mitochondrial uncoupling protein 2; 36B4, acidic ribosomal phosphoprotein P0

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ABSTRACT

The aim of the present study is to clarify the functional expression and physiological role in brain neurons of carnitine/organic cation transporter OCTN1/SLC22A4, which accepts the naturally occurring antioxidant ergothioneine (ERGO) as a substrate *in vivo*. After intracerebroventricular administration, the distribution of [³H]ERGO in several brain regions of *octn1*^{-/-} mice was much lower than that in wild-type mice, whereas extracellular marker [¹⁴C]mannitol exhibited similar distribution in the two strains. The [³H]ERGO distribution in wild-type mice was well correlated with the amount of ERGO derived from food intake and the OCTN1 mRNA level in each brain region. Immunohistochemical analysis revealed colocalization of OCTN1 with neuronal cell markers microtubule-associated protein 2 (MAP2) and β III-tubulin in mouse brain and primary cultured cortical neurons, respectively. Moreover, cultured cortical neurons exhibited time-dependent and saturable uptake of [³H]ERGO. These results demonstrate that OCTN1 is functionally expressed in brain neurons. The addition of ERGO simultaneously with serum to culture medium of cortical neurons attenuated mRNA and protein expressions of MAP2, β III-tubulin and synapse formation marker synapsin I, and induced those of sex determining region Y-box 2 (Sox2), which is required to maintain the properties of undifferentiated neural stem cells. In neuronal model Neuro2a cells, knockdown of OCTN1 by siRNA reduced the uptake of [³H]ERGO with

concomitant up-regulation of oxidative stress marker HO-1 and Sox2, and down-regulation of neurite outgrowth marker GAP43. Interestingly, the siRNA knockdown decreased the number of differentiated Neuro2a cells showing long neurites, but increased the total number of cells. Thus, OCTN1 is involved in cellular differentiation, but inhibits their proliferation, possibly via the regulation of cellular oxidative stress. This is the first evidence that OCTN1 plays a role in neuronal differentiation and proliferation, which are required for brain development.

Keywords: Brain neuron, Differentiation, Proliferation, Transporter, Neurite outgrowth,

Ergothioneine

1. Introduction

Solute carrier (SLC) superfamily includes more than 50 transporter families, which consist of approximately 400 genes encoding membranous proteins. SLC transporters have been divided into so-called physiological transporters that are involved in various biological events by recognizing neurotransmitters, including dopamine, serotonin and glutamic acid, as substrates, and xenobiotic transporters that have relatively broad substrate specificity. The roles of the physiological transporters in the brain have generally been well characterized, but the roles of the xenobiotic transporters are less well understood.

Carnitine/organic cation transporter OCTN1/SLC22A4, classified as a xenobiotic transporter, is ubiquitously expressed in the body, and transports various therapeutic agents including organic cations and zwitterions (Tamai et al., 1997; Meier et al., 2007; Taubert et al., 2009). However, OCTN1 has mainly been characterized *in vitro*, and little is known about its function *in vivo*. We recently succeeded in producing *octn1* gene knockout (*octn1*^{-/-}) mice, and found by means of metabolome analysis that the naturally occurring antioxidant ergothioneine (ERGO) is a good *in vivo* substrate of OCTN1 (Kato et al., 2010). OCTN1 is functionally expressed in mouse small intestine, liver and kidney (Kato et al., 2010; Sugiura et al., 2010). In peripheral organs, OCTN1 has been proposed to be involved in proliferation and differentiation of erythroid cells (Nakamura et al., 2007), and to be associated with

development of rheumatoid arthritis (Tokuhiro et al., 2003) and Crohn's disease (Kato et al., 2010).

OCTN1 is also expressed in the brain (Alnouti et al., 2006; Lamhonwah et al., 2008; Okura et al., 2008), although its function has not been clarified. The expression level of OCTN1 was minimal in immortalized rat brain endothelial cells (Okura et al., 2008). In fact, the brain/plasma concentration ratio of [^3H]ERGO after intravenous administration was similar in wild-type and *octn1*^{-/-} mice (Sugiura et al., 2010), suggesting that OCTN1 may not be functionally expressed in the blood-brain barrier. Inazu et al. (2006) could not detect OCTN1 mRNA in rat cultured cortical astrocytes, whereas Lamhonwah et al. (2008) suggested that OCTN1 may be expressed in neuronal cell bodies and dendrites on the basis of morphological observation, although the functional relevance of such expression remains unclear.

The aim of the present study is to clarify the physiological role of OCTN1 in the brain, especially in neurons. First, we investigated the functional expression of OCTN1 in the brain *in vivo* by means of intracerebroventricular (*i.c.v.*) administration of [^3H]ERGO to wild-type and *octn1*^{-/-} mice, followed by measurement of radioactivity in various brain regions. To confirm functional expression of OCTN1 in brain neurons, we examined uptake of [^3H]ERGO in mouse primary cultured cortical neurons. Furthermore, to examine the physiological role of OCTN1 expressed in neurons, we performed an OCTN1 knockdown

experiment using undifferentiated neuronal model Neuro2a cells. Our results indicate for the first time that OCTN1 plays a role in neuronal differentiation and proliferation, which are required for brain development.

2. Materials and methods

2.1. Materials

[³H]ERGO (1 Ci/mmol) and [¹⁴C]mannitol (55 mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA, USA). Clearsol I was obtained from Nacalai Tesque (Kyoto, Japan). Monoclonal antibodies against microtubule-associated protein 2 (MAP2), β III-tubulin and sex determining region Y-box 2 (Sox2), polyclonal antibody against synapsin I (Syn1), Dulbecco's modified Eagle's medium (DMEM), poly-L-lysine, cytosine arabinoside, all-*trans* retinoic acid (ATRA), and RNA*later* were from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was supplied by Biowest (Nuaille, France). Antiserum against the carboxyl terminus of mouse OCTN1 was produced in our laboratory (Sugiura *et al.* 2010). Secondary antibodies conjugated with Alexa Fluor series, Lipofectamine RNAiMAX, Opti-MEM, small interfering RNA (siRNA) targeting the mouse OCTN1 gene (siOCTN1),

and non-targeting (negative control) siRNA were provided by Invitrogen (San Diego, CA, USA). ISOGEN was purchased from Nippon Gene (Tokyo, Japan). MultiScribe™ Reverse Transcriptase was obtained from Applied Biosystems (Foster City, CA, USA). THUNDERBIRD SYBR qPCR Mix was from TOYOBO (Osaka, Japan). Mouse neuroblastoma Neuro2a cells were supplied by ATCC (Manassas, VA, USA). Block Ace was provided by DS Pharma Biomedical (Suita, Japan). All other chemicals and reagents were of the highest purity available and were purchased from commercial sources.

2.2. Animals

The *octn1*^{-/-} mice were generated according to the previous report (Kato et al., 2010). Seven- to nine-week-old male *octn1*^{+/+} (wild-type) and *octn1*^{-/-} mice were used in this study. Pregnant ICR mice for neuronal culture were purchased from Japan SLC (Hamamatsu, Japan). The mice were kept in a temperature- and light-controlled environment with standard food and tap water provided *ad libitum*. Animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals in Kanazawa University, with efforts to minimize the number of animals used and their suffering.

2.3. Distribution of [³H]ERGO in the brain after intracerebroventricular administration

Wild-type and *octn1*^{-/-} mice were fasted overnight with access to water and anesthetized by inhalation of diethylether. [³H]ERGO and [¹⁴C]mannitol were dissolved in artificial cerebrospinal fluid buffer (145 mM NaCl, 0.6 mM KCl, 1.0 mM MgCl₂, 1.2 mM CaCl₂ and 0.2 mM ascorbic acid, dissolved in 2 mM potassium phosphate buffer, pH 7.4) at a concentration of 23 ng/μl and 110 ng/μl, respectively. This radiolabeled mixture was injected into the right lateral cerebral ventricle (1.0 mm lateral from bregma and 2.5 mm below the surface of the skull) in a volume of 1 μl at a constant rate of 2 μl/min using a 5 μl Hamilton microsyringe. The injection site was verified in pilot experiments by administration and localization of trypan blue dye. At 4 h after the *i.c.v.* administration, the mice were decapitated, and the brain was excised and divided into seven parts [cerebellum (CL), medulla and pons (MP), hypothalamus (HT), striatum (ST), midbrain (MB), hippocampus (HC) and cerebral cortex (CX)]. Each tissue was weighed and solubilized with Solene-350 at 50 °C overnight. The solution was treated with hydrogen peroxide and neutralized with 5 M HCl. After the addition of scintillation cocktail (Clearsol I), the radioactivity was measured with a liquid scintillation counter (LSC-5100; Aloka, Tokyo, Japan).

2.4. Distribution of feed-derived ERGO in the brain

Wild-type mice were decapitated, and each brain was excised and divided into seven parts as described above. Measurement of ERGO by high-performance liquid chromatography (HPLC) was performed as described previously (Kato et al., 2010). All the tissue samples were weighed and homogenized with 14 volumes of distilled water. The homogenized tissues were deproteinized with acetonitrile and subjected to HPLC after centrifugation. The HPLC system incorporated a constant flow pump (LC-10AVP; Shimadzu, Kyoto, Japan) and a UV detector (SPD-10AV; Shimadzu) with a normal-phase column (Cosmosil HILIC, 4.6×250 mm; Nacalai Tesque). The mobile phase was 10 mM ammonium acetate and acetonitrile in a ratio of 20:80. The flow rate was 1 ml/min. The wavelength for UV detection was 260 nm. Cefoperazone was used as an internal standard.

2.5. Cell Cultures

Primary cortical neuronal cultures were carried out according to the method of di Porzio et al. (1980), with minor modifications (Nakamichi et al., 2005). In brief, cerebral cortices from 15-day-old embryonic ICR mice were dissected and incubated with 0.25% trypsin in phosphate-buffered saline (PBS) containing 28 mM glucose at 37 °C for 20 min. Cells were mechanically dissociated by using a 1,000 μ l pipette tip in culture medium and plated at a density of 1.5×10^5 cells/cm² on plastic dishes coated with 7.5 μ g/ml poly-L-lysine.

Cortical neurons were cultured in DMEM 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 in either the presence or absence of 200 µM ERGO for the initial 4 h at 37 °C in a humidified 5% CO₂ incubator. Further culture was performed in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 28 mM glucose, 2 mM glutamine, 5 mM HEPES, 50 µg/ml apo-transferrin, 500 ng/ml insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 8 ng/ml sodium selenite and 100 µM putrescine in either the presence or absence of 200 µM ERGO. During 2 to 3 days *in vitro* (DIV), cells were treated with 5 µM cytosine arabinoside for 12 h to reduce proliferating cells. Under these culture conditions, almost all cells were immunoreactive for the neuronal marker MAP2 in double immunocytochemical analysis using antibodies against MAP2 and glial fibrillary acidic protein (Nakamichi et al., 2005).

Neuro2a cells derived from mouse neuroblastoma exhibited the characteristic ability of neuronal progenitor cells to differentiate into neuron-like cells in the presence of ATRA, as described previously (Nakamura et al., 2012). Undifferentiated Neuro2a cells were cultured in DMEM supplemented with 10% FBS and passaged at least three times prior to the treatment with ATRA. Neuro2a cells were then plated at 3×10^4 cells/ml in DMEM supplemented with 10% FBS for 24 h, followed by medium change to DMEM supplemented with 2% FBS and

20 μ M ATRA for neuronal differentiation. Cultures were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C.

2.6. Knockdown of OCTN1 by siRNA

Undifferentiated Neuro2a cells were transiently transfected with siOCTN1 or negative control siRNA by using lipofectamine RNAiMAX in Opti-MEM according to the manufacturer's instructions. Culture medium was replaced with DMEM supplemented with 2% FBS and 20 μ M ATRA at 24 h after the transfection, and culture was continued for an additional 2 DIV. The knockdown efficiency of siRNA was checked by determining the expression level of OCTN1 mRNA.

2.7. Quantitative RT-PCR

After dissection of brains of wild-type mice into seven parts as described above, the samples were immediately transferred to RNAlater, incubated at 4 °C overnight, and stored at -30 °C until RNA extraction. Total RNA was extracted from the brain samples and cultured cells according to the standard ISOGEN procedure. cDNA was synthesized with oligo (dT)₁₂₋₁₈ primer, deoxy nucleotide triphosphate mix, RT buffer and MultiScribe™ Reverse

Transcriptase and amplified on a Mx3005P (Agilent Technologies, Santa Clara, CA, USA) in a reaction mixture containing a cDNA aliquot, 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 to an internal standard acidic ribosomal phosphoprotein P0 (36B4), which is more stable reference gene for the normalization than very often used housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase, β -actin and ribosomal RNA 18S, in some neurodegenerative models (Pernot et al., 2010).

2.8. Immunohistochemical analysis

Wild-type mice were deeply anesthetized for intracardial perfusion with PBS containing 4% paraformaldehyde (PA). Brains were removed, postfixed overnight, and cryoprotected in 30% sucrose. Free-floating sections were cut at 50 μ m using a cryostat (Leica, Wetzlar, Germany) and stored at -30 °C in cryoprotective solution (30% sucrose, 30% ethyleneglycol and 1% polyvinylpyrrolidone in PBS) until processing as described previously (Tamaki et al., 2008). Sections were washed in PBS, incubated for 2 h in blocking solution (10% normal goat serum), and then incubated at 4 °C for 48 h in PBS containing 0.15%

Triton X-100 and supplemented with antiserum against OCTN1 (1 : 100) and antibody against MAP2 (1 : 500). The sections were further rinsed in PBS, incubated again for 2 h in blocking solution, and then reacted with Alexa Fluor series-conjugated secondary antibodies (1 : 800; Invitrogen) at 4 °C overnight. The sections were washed again with PBS and mounted onto slides for observation with a confocal laser scanning microscope (LSM710; Carl Zeiss, Jena, Germany).

Cortical neurons or Neuro2a cells were washed with PBS, then fixed with 4% PA for 20 min at 25 °C and incubated for 30 min in blocking solution (3% bovine serum albumin and 0.2% Triton X-100 in PBS) at 25 °C. They were then incubated overnight in 10-times-diluted blocking solution containing antiserum against OCTN1 (1 : 500) and antibody against β III-tubulin (1 : 4000) at 4 °C, washed with PBS, and reacted with Alexa Fluor series-conjugated secondary antibodies (1 : 2000) for 1 h at 25 °C. The cells were rinsed again with PBS, treated with mounting medium including DAPI, and observed under a confocal laser scanning microscope (LSM710).

2.9. Uptake of [³H]ERGO in cultured cells

Cortical neurons or Neuro2a cells were washed twice with transport buffer (125 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KaH₂PO₄, 5.6 mM glucose and

25 mM HEPES, pH 7.4) and incubated with 0.9 μM [^3H]ERGO in transport buffer at 37°C for various periods in either the presence or absence of 1-200 μM unlabeled ERGO. Incubation was terminated by rapid aspiration of the buffer, followed by rinsing with ice-cold transport buffer three times and solubilization with 0.2 M NaOH at 25 °C overnight. The solubilized solution was neutralized with 5 M HCl, followed by addition of Clearsol I for liquid scintillation spectrometry. Protein concentration was determined with a Bio-Rad Protein Assay Kit. K_m values were calculated by fitting the data of dose-dependent inhibition by unlabeled ERGO to the Michaelis-Menten equation.

2.10. Western blotting

Western blot analysis was carried out according to the method of Nakamichi et al. (2005), with minor modifications. Cortical neurons 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, 10 mM sodium fluoride, 10 mM sodium β -glycerophosphate, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate and 1 $\mu\text{g/mL}$ of various protease inhibitors [(*p*-amidinophenyl)methanesulfonyl fluoride, leupeptin, antipain and benzamidine]. Suspensions were added at a volume ratio of 4:1 to 10 mM Tris-HCl buffer (pH 6.8)

containing 10% glycerol, 2% sodium dodecylsulfate, 0.01% bromophenol blue and 5% mercaptoethanol, followed by mixing and heating at 100 °C for 10 min. Protein concentration was determined with a Bio-Rad Protein Assay Kit. Each aliquot of 5 µg proteins 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 by Block Ace solution, the membrane was reacted with an antibody against β III-tubulin (1 : 5000), MAP-2 (1 : 5000), Syn1 (1 : 2000) or Sox2 (1 : 2000), diluted with the buffer containing 10% Block Ace solution, followed by a reaction with an anti-mouse IgG (1 : 10000) or anti-rabbit IgG (1 : 100000) antibody conjugated with peroxidase. Proteins reactive with those antibodies were detected with the aid of ECLTM detection reagents using a lumino image analyzer (LAS-4000; FUJIFILM, Tokyo, Japan). Densitometric determination was carried out using the ImageJ software.

2.11. Statistical analysis

All experiments were performed at least three times. Data are expressed as the mean $\pm$ 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 test and $p < 0.05$ was regarded as

denoting a significant difference.

3. Results

3.1. OCTN1 is functionally expressed in brain parenchymal cells in vivo

We first confirmed functional expression of OCTN1 in the brain, using [^3H]ERGO as a tracer. Namely, in order to assess the involvement of OCTN1 in the distribution of ERGO to brain parenchymal cells, the concentrations of [^3H]ERGO and [^{14}C]mannitol, an extracellular space marker, in different brain regions after *i.c.v.* administration were compared between wild-type and *octn1*^{-/-} mice. After injection into the right lateral cerebral ventricle, [^3H]ERGO was distributed to all seven brain regions examined in wild-type mice, while the concentration of [^3H]ERGO in *octn1*^{-/-} mice was significantly lower than that in wild-type mice in the CL, MP, HT and MB (Fig. 1A). The differences of [^3H]ERGO distribution between the two strains were observed in both the right (Fig. 1A, upper panel) and left (Fig. 1A, lower panel) hemispheres. In the ST, HC and CX, [^3H]ERGO concentration in *octn1*^{-/-} mice tended to be lower than that in wild-type mice, but without statistical significance. On the other hand, the distribution of [^{14}C]mannitol exhibited no difference between wild-type and *octn1*^{-/-} mice in

any brain region examined (Fig. 1B).

To further confirm that OCTN1 is involved in ERGO distribution to brain parenchymal cells, the distribution of feed-derived ERGO in the brain was also examined. Mammals are considered to derive ERGO from food, because they cannot synthesize ERGO (Mayumi et al., 1982). We found that the ERGO concentration was below the detection limit in all organs, including brain, in *octn1*^{-/-} mice, probably because of the lack of OCTN1-mediated absorption (Kato et al., 2010). Concentrations of ERGO in the seven brain regions were measured by HPLC in wild-type mice given standard feed. Concentrations of ERGO in the CL, MP and HT were clearly higher than those in the ST, HC and CX (Fig. 2A). The distribution of feed-derived ERGO (Fig. 2A) coincided with that of exogenous [³H]ERGO (Fig. 1A, wild-type).

Moreover, we examined the relationship between expression level of OCTN1 and distribution of ERGO. Quantitative PCR analysis revealed that OCTN1 mRNA was highest in the CL (Fig. 2B). The expression levels of OCTN1 in the MP and MB were higher than those in the ST, HC and CX (Fig. 2B). In these brain regions with higher expression levels of OCTN1, the distribution of [³H]ERGO was significantly higher in wild-type mice compared to *octn1*^{-/-} mice (Fig. 1A). The relationship between OCTN1 mRNA expression level and food-derived ERGO concentration is shown in Figure 2C. The expression pattern of OCTN1 in the brain was well correlated with the distribution of ERGO to brain parenchyma (Fig. 2C).

The expression level of MAP2 mRNA was the highest in the HC, and tended to be higher in the CL, MB and CX than in the MP, HT and ST (Fig. 2D). There was no correlation between the expression of OCTN1 and MAP2.

3.2. OCTN1 is localized and functionally expressed in brain neurons

We then examined whether OCTN1 is expressed in brain neurons. Immunoreactivity to OCTN1 antiserum was observed in brain tissue slices of wild-type mice and was at least partially colocalized with that to MAP2, a mature neuronal marker protein, in both MB (Fig. 3A) and CX (Fig. 3B). Similar immunoreactivity was also seen in the other five brain regions.

To further confirm expression of OCTN1 in brain neurons, we performed quantitative PCR and immunocytochemical analysis of OCTN1 in primary cultured cortical neurons. The expression level of OCTN1 mRNA was not significantly changed between CX dissected from wild-type mice and mouse cultured cortical neurons (Fig. 4A). Immunoreactivity to OCTN1 antiserum was seen in both neuronal cell bodies and dendrites, and showed good colocalization with β III-tubulin, an immature neuronal marker protein (Fig. 4B). Such localization of OCTN1 in cultured neurons is also consistent with the result of immunohistochemical analysis of mouse CX (Fig. 3B). To establish whether OCTN1 expressed in brain neurons is functional, we examined uptake of the OCTN1 substrate

ERGO into primary cultured cortical neurons. Uptake of [^3H]ERGO into cultured cortical neurons increased time-dependently (Fig. 4C) and was inhibited by simultaneous addition of unlabeled ERGO with K_m value of 3.08 μM in a dose-dependent manner over the concentration range of 1 to 200 μM (Fig. 4D). This K_m value is close to the value of 4.68 μM obtained in human embryonic kidney 293 cells overexpressing mouse OCTN1 (Kato et al., 2010).

3.3. Typical substrate of OCTN1 suppresses early neuronal maturation

To examine the physiological function of OCTN1 expressed in neurons, 200 μM ERGO was added to the culture medium of cortical neurons at the various time points after the start of primary culture. When ERGO and FBS were simultaneously present in the culture medium for the initial 4 h, expressions of MAP2, β III-tubulin and Syn1, a marker of synapse formation, were significantly reduced, whereas that of Sox2, which is required to maintain the properties of undifferentiated neural stem cells (Cavallaro et al., 2008; Pevny and Nicolis, 2010), was clearly increased at 24 h (Fig. 5A, gray columns). The reduction in expressions of MAP2, β III-tubulin and Syn1, and the induction in expression of Sox2 were observed regardless of the presence or absence of ERGO for the last 20 h (Fig. 5A, gray and black columns). On the other hand, when ERGO was added to the culture medium for the latter 20 h

only, MAP2 was still decreased, β III-tubulin and Syn1 were not markedly changed, and Sox2 tended to be increase at 24 h (Fig. 5A, diagonal columns). When ERGO was present in the culture medium throughout the 72 h culture period, the decrease in expressions of MAP2, β III-tubulin and Syn1, and the increase in that of Sox2 were no longer significant, but the expressions of MAP2 and β III-tubulin were still tended to be reduced (Fig. 5B). Expression of growth-associated protein 43 (GAP43), a marker of neurite outgrowth, or OCTN1 was not significantly affected by ERGO under any culture conditions (Fig. 5A, B). To examine whether the addition of ERGO for 24 h significantly changes expressions of MAP2, β III-tubulin, Syn1 and Sox2, at the protein levels as well as the mRNA levels, western blot analysis was performed. Protein levels of β III-tubulin and Syn1 were markedly reduced, and that of Sox2 was significantly increased (Fig. 5C). Protein level of MAP2 was not significantly changed, but tended to be decreased. We have confirmed that expressions of these neuronal maturation-related factors were changed by the addition of ERGO not only at the mRNA levels but also at the protein levels.

3.4. OCTN1 is functionally expressed in Neuro2a cells

To further clarify the physiological role of OCTN1 in neurons, we performed knockdown of OCTN1 using undifferentiated neuronal model Neuro2a cells. We first

confirmed that OCTN1 is functionally expressed in Neuro2a cells, as in primary cultured neurons. Neuro2a cells expressed OCTN1 at a similar level to that seen in CX from wild-type mice and in primary cultured cortical neurons (Fig. 4A). Immunocytochemical analysis showed that OCTN1 is colocalized with a neuronal marker β III-tubulin, and is localized in neurites as well as the cell body (Fig. 6A). Uptake of [3 H]ERGO increased linearly up to 4 h in Neuro2a cells (Fig. 6B). This uptake of [3 H]ERGO was dose-dependently inhibited by unlabeled ERGO with K_m value of 3.78 μ M (Fig. 6C), as in the case of primary cultured neurons (Fig. 4D). Thus, functional expression of OCTN1 was also confirmed in Neuro2a cells.

3.5. Knockdown of OCTN1 affects intracellular oxidative state

We next transfected siRNA for OCTN1 into Neuro2a cells to suppress the expression of OCTN1. The mRNA expression of OCTN1 was remarkably and continuously reduced for up to 72 h in siOCTN1-transfected cells compared to cells transfected with negative control siRNA (Fig. 7A). To confirm functional suppression of OCTN1, we investigated the transport activity of [3 H]ERGO in siOCTN1-transfected Neuro2a cells. Uptake of [3 H]ERGO was markedly decreased in siOCTN1-transfected cells compared to cells transfected with negative control siRNA (Fig. 7B). Thus, the reduction in transport activity of [3 H]ERGO was

consistent with the knockdown of OCTN1 gene expression.

Because OCTN1 recognizes the stable antioxidant ERGO as a good *in vivo* substrate, we examined the effect of knockdown of OCTN1 on expression of genes relevant to oxidative stress in Neuro2a cells, focusing on heme oxygenase-1 (HO-1), nuclear factor-erythroid 2 p45-related factor-2 (Nrf2), cystine glutamate exchanger subunit (xCT) and mitochondrial uncoupling protein 2 (Ucp2). It was reported that these four genes are induced as cellular antioxidant defense mechanisms in cells exposed to oxidative stress (Degasperi et al., 2008; Seng et al., 2010; Lewerenz et al., 2012). Expression of HO-1 was significantly increased in siOCTN1-transfected cells compared to negative control siRNA-transfected cells, whereas knockdown of OCTN1 did not markedly affect expression of Nrf2, xCT or Ucp2 in Neuro2a cells (Fig. 7C).

3.6. OCTN1 promotes neuronal differentiation and suppresses neuronal proliferation

Finally, we investigated the influence of knockdown of OCTN1 on neuronal proliferation and differentiation. Knockdown of OCTN1 tended to increase expression of Sox2, and significantly decreased expression of GAP43, but not Syn1 in Neuro2a cells (Fig. 7D). Further, the morphology of the cells was examined at 72 h after the transfection of siRNA for either negative control (Fig. 8A, upper panel) or OCTN1 (Fig. 8A, lower panel). We

considered cells with longer neurites than the cell diameter as differentiated cells. Arrowheads in Fig. 8A indicate typical differentiated cells having long neurites. Interestingly, differentiated cells were decreased in the siOCTN1-treated group as compared to the negative control treated group (Fig. 8A). Moreover, the number of round undifferentiated cells with a smaller cell body than differentiated cells seemed to be increased in the siOCTN1-treated group (Fig. 8A). Quantitative analysis showed that the percentage of differentiated cells was significantly decreased in the siOCTN1-treated group (Fig. 8B), though the total number of cells was increased in the same group (Fig. 8C). These results suggest that OCTN1 may promote neuronal differentiation and suppress neuronal proliferation, possibly via its role in the regulation of cellular oxidative stress.

4. Discussion

The present study is the first to demonstrate functional expression of OCTN1 in the brain. Furthermore, we established that OCTN1 is functionally expressed in brain neurons, and we suggest that it may promote neuronal differentiation and suppress neuronal proliferation, possibly via regulation of cellular oxidative stress. We found that not only exogenous [^3H]ERGO, which is a typical substrate of OCTN1, but also feed-derived

unlabeled ERGO was distributed widely to seven different brain regions (Fig. 1A, 2A). The concentration of ERGO was higher in the brain stem and epencephalon than in the telencephalon, and this is compatible with mRNA expression levels of OCTN1 (Fig. 2A-C). These results indicate that OCTN1 is functional in the brain and is involved in the differential distribution of its substrate(s) to various brain regions. There was no correlation between the mRNA expression levels of OCTN1 and MAP2 (Fig. 2B, D), indicating that the expression pattern of OCTN1 in the brain is not due to differences in neuronal populations. Brain neurons, however, exhibit characteristic distribution patterns according to the primary neurotransmitter involved in neurotransmission. Further studies will be needed to clarify the relationship between OCTN1 expression pattern and neuronal subtypes. The physiological significance of the higher functional expression of OCTN1 in brain stem and epencephalon also remains unknown, but considering the fact that the OCTN1 gene is universally conserved in vertebrata, including amphibian, birds, fishes and mammals, OCTN1 could play a pivotal role in early brain development.

In the brain, OCTN1 is not functionally expressed in brain capillary endothelial cells (Okura et al., 2008) and is not expressed at the mRNA level in astrocytes (Inazu et al., 2006). Therefore, the distribution of ERGO to brain parenchyma (Fig. 1A, 2A) seems to principally reflect the uptake by neurons, although we also need to clarify whether OCTN1 is functionally expressed in other brain constituent cells, such as oligodendrocytes and microglia.

The above conclusion is supported by immunohistochemical analysis revealing that OCTN1 is colocalized with a neuronal marker protein *in vivo* in the brain (Fig. 3) and *in vitro* in primary cultured cortical neurons (Fig. 4B). Time-dependent and saturable uptake of [³H]ERGO in cultured cortical neurons (Fig. 4C) further supports the functional expression of OCTN1 in brain neurons. Considering the multi-substrate specificity of OCTN1, it seems noteworthy that OCTN1 is present throughout neuronal cell bodies and dendrites: functional expression of this transporter in neurons may imply that OCTN1 is involved in neuronal uptake of various substrates, and therefore the subsequent biological activities. For example, ERGO is an antioxidant and may protect neurons from oxidative stress after being taken up by OCTN1. Although there is little information on possible transport of neurotransmitters by OCTN1, Pochini et al. (2011) have recently clarified that OCTN1 transports acetylcholine in both uptake and efflux directions. Thus, further studies are needed to clarify the possible contribution of OCTN1 to signal transduction in neurons. Kim et al. (2010) recently reported that donepezil, which is used to treat Alzheimer's disease, is a substrate of OCTN1. Donepezil inhibits acetylcholine esterase, which is thought to operate in the extracellular space, and so cellular uptake of donepezil via OCTN1 may reduce its pharmacological activity. Jong et al. (2011) recently reported OCTN1-mediated uptake of oxaliplatin in cultured dorsal root ganglion neurons, and this may be relevant to the neurotoxicity caused by this anticancer drug.

Because the antioxidant ERGO is a good *in vivo* substrate of OCTN1 (Kato et al., 2010), and intracellular reactive oxygen species (ROS) promote proliferation of neural stem cells (Yoneyama et al., 2010), OCTN1 expression in undifferentiated neurons, *i.e.* neuronal progenitor cells, may suppress neuronal proliferation. In the present study, to clarify the role of OCTN1 in neuronal proliferation, we used mouse neuroblastoma Neuro2a cells, which are undifferentiated and proliferative cells that are induced to differentiate into neuron-like cells with neurites by retinoic acid. Actually, knockdown of OCTN1 in Neuro2a cells increased the number of undifferentiated cells (Fig. 8), suggesting that OCTN1 may suppress neuronal proliferation. Knockdown of OCTN1 increased expression of HO-1 (Fig. 7C), which is known to be induced in response to intracellular oxidative stress (Seng et al., 2010), indicating that OCTN1 may have a role in regulating the intracellular oxidative state in neurons. It should be noted that ERGO is present in serum, which is included in the culture medium. ERGO is known to be present in mammal brain *in vivo* at the level of 0.2 to 1.0 mg per 100 g tissue (Fig. 2A; Kato et al., 2010; Cheah et al., 2011) and may protect neurons from cytotoxicity induced by a variety of neurotoxins, including *N*-methyl-D-aspartate, β -amyloid and cisplatin (Moncaster et al., 2002; Jang et al., 2004; Song et al., 2010). Thus, OCTN1 may transport antioxidant into neurons and consequently reduce the intracellular ROS level, resulting in suppression of neuronal proliferation.

Interestingly, knockdown of OCTN1 decreased expression of GAP43 (Fig. 7D),

which is induced during neurite extension. The knockdown of OCTN1 also reduced the number of differentiated Neuro2a cells, which possess long neurites and a big soma (Fig. 8), suggesting that OCTN1 may promote differentiation from neuronal progenitor cells into neurons. Knockdown of OCTN1 tended to increase the expression of Sox2, which is required to maintain the properties of neural stem cells (Fig. 7D). Thus, OCTN1 may decrease the number of undifferentiated cells via reduction of Sox2 expression, resulting in suppression of neuronal proliferation and promotion of neuronal differentiation. However, it is unclear whether intracellular oxidative state is involved in the regulation mechanism of Sox2 expression by OCTN1. Neuronal differentiation, moreover, might be regulated by some unidentified substrate of OCTN1: in primary cultured cortical neurons, which would differentiate into neurons without proliferation immediately after preparation under the culture conditions used in the present study, addition of ERGO to the culture medium (in the presence of serum) for the initial 4 h inhibited expressions of MAP2, β III-tubulin and Syn1, and induced that of Sox2, whereas addition of ERGO to the medium for the latter 20 h (in the absence of serum) did not (Fig. 5A), suggesting that ERGO may act as an inhibitor of OCTN1 in this case and inhibit uptake of an unidentified substrate of OCTN1 from serum, resulting in the suppression of neuronal differentiation or maturation. Even when ERGO was added to culture medium in the presence of serum for the initial 4 h, the significant difference in expression levels of MAP2, β III-tubulin, Syn1 and Sox2 were no longer observed after 3 days

of culture (Fig. 5B), indicating that the inhibitory effect of ERGO on OCTN1 in the initial 4 h may have disappeared during the latter culture period because ERGO alone does not affect neuronal differentiation or maturation in the absence of serum. These results suggest that an unidentified substrate of OCTN1 that promotes neuronal differentiation or maturation may exist in serum.

It is considered that neurodegeneration owing to oxidative stress is related to the development of various brain diseases, including Alzheimer's, Parkinson's and Huntington's diseases (Sayre et al., 2008; Cassano et al., 2012). OCTN1 may therefore influence the development of neurodegenerative diseases via its role in transporting endogenous antioxidants. On the other hand, OCTN1 may be involved in the pathogenesis of neurodegenerative diseases via its cation transport activity: for example, it is well known that the cation 1-methyl-4-phenylpyridinium ion (MPP⁺) induces symptoms like parkinsonian syndromes (Sonsalla et al., 2008), although the endogenous causative compound has not yet been identified. Taubert et al., (2007) reported that organic cation transporter OCT2 as well as dopamine transporter may also be involved in the etiology of Parkinson's disease. So far, there is no evidence that OCTN1 transports endogenous organic cations other than ERGO, but further work is needed to search for other endogenous substrates in the brain.

In conclusion, our findings indicate for the first time that the xenobiotics transporter OCTN1 is functionally expressed in brain neurons and plays a role in neuronal differentiation

and proliferation, possibly via the regulation of cellular oxidative stress. Dysfunction of OCTN1 may contribute to the development of neurodegenerative diseases. Thus, therapeutic drugs and other compounds that target OCTN1 may influence neuronal differentiation or proliferation.

5. Conflict of interest

All authors have no conflict of interest.

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Figure legends

Fig. 1. Distribution of [^3H]ERGO and [^{14}C]mannitol to different brain regions after *i.c.v.* administration to wild-type and *octn1*^{-/-} mice. [^3H]ERGO (A) and [^{14}C]mannitol (B) were injected into the right lateral cerebral ventricle of wild-type (black columns) and *octn1*^{-/-} (white columns) mice. At 4 h after the injection, brains were separated into hemispheres (right hemisphere, upper panel; left hemisphere, lower panel) and each hemisphere was divided into seven parts (1, cerebellum; 2, medulla and pons; 3, hypothalamus; 4, striatum; 5, midbrain; 6, hippocampus; 7, cerebral cortex). The radioactivity of each tissue was measured after solubilization. Each value represents the mean $\pm$ S.E.M. (n = 4). * $p < 0.05$, significant difference from the corresponding value in wild-type mice.

Fig. 2. Relationship between ERGO distribution and OCTN1 expression level in mouse brain. Brains of wild-type mice were divided into seven parts (1, cerebellum; 2, medulla and pons; 3, hypothalamus; 4, striatum; 5, midbrain; 6, hippocampus; 7, cerebral cortex). (A) Concentration of feed-derived ERGO was measured by HPLC after homogenization and deproteinization. The mRNA levels of OCTN1 (B) and MAP2 (D) were determined by quantitative RT-PCR after extraction of total RNA. Each value was normalized by the mRNA level of 36B4 and expressed as a relative value to the cortex. (C) Correlation between

expression level of OCTN1 mRNA and concentration of feed-derived ERGO in each brain region. Each value represents the mean $\pm$ S.E.M. (n = 3-4).

Fig. 3. Colocalization of OCTN1 with MAP2 in mouse midbrain and cerebral cortex.

Wild-type mice were perfused with 4% PA, and frozen sagittal sections were prepared.

OCTN1 (green) and neuronal marker MAP2 (red) were visualized immunohistochemically in the midbrain (A) and cerebral cortex (B). The merged images are also shown.

Fig. 4. Functional expression of OCTN1 in mouse primary cultured cortical neurons. (A) The

expression level of OCTN1 mRNA was determined by quantitative RT-PCR after extraction of total RNA from mouse cerebral cortex (CX; black columns), primary cortical neurons

cultured for 3 DIV (PN; gray columns) and differentiated Neuro2a cells (N2A; white

columns). Each value is normalized by the expression level of 36B4 mRNA and expressed as

a relative value to the cortex. (B) Primary cortical neurons cultured for 3 DIV were fixed with

4% PA, followed by immunocytochemical detection of OCTN1 (green), neuronal marker

β III-tubulin (red) and nuclear marker DAPI (blue). The merged image is also shown. (C)

Cultured cortical neurons were incubated with [3 H]ERGO in transport buffer at 37°C for up to

30 min. (D) Incubation was done with [3 H]ERGO for 30 min in either the presence or absence

of unlabeled ERGO in the concentration range of 1 to 200 μ M. Each value represents the

mean $\pm$ S.E.M. ($n = 3-9$). $*p < 0.05$, significant difference from the control value obtained in neurons incubated with [^3H]ERGO alone.

Fig. 5. Effect of ERGO on expression of neuronal maturation-related genes in mouse primary cultured cortical neurons. Mouse cortical neurons were cultured in DMEM supplemented with 5% FBS in either the presence (gray columns) or absence (white columns) of ERGO for the initial 4 h, followed by further culture in DMEM in the absence of ERGO up to 24 h (A), in DMEM supplemented with 5% FBS in either the presence (black columns) or absence (diagonal columns) of ERGO for the initial 4 h, followed by further culture in the presence of ERGO up to 24 h (A), and in DMEM supplemented with 5% FBS for the initial 4 h and subsequent culture in either the presence (black columns) or absence (white columns) of ERGO up to 72 h (B). After culture for each period, total RNA was extracted for quantitative RT-PCR analysis. Each value is normalized by the expression level of 36B4 mRNA and expressed as a relative value to the corresponding control value obtained in the absence of ERGO, and represents the mean $\pm$ S.E.M. ($n = 8-12$). (C) Cortical neurons were cultured in DMEM supplemented with 5% FBS for the initial 4 h, followed by further culture in DMEM up to 24 h, in either the presence (black columns) or absence (white columns) of ERGO throughout the culture period. After the end of the culture period, cells were homogenized, followed by SDS-PAGE for immunoblotting using an antibody against MAP2, β III-tubulin,

Sox2 or Syn1. Typical immunoblots are shown in the left panel, while in the middle panel each value is expressed as a relative value to the corresponding control value obtained in the absence of ERGO, and represents the mean $\pm$ S.E.M. (n = 6-9). * $p < 0.05$, significant difference from the corresponding control value obtained in the absence of ERGO.

Fig. 6. Functional expression of OCTN1 in Neuro2a cells. (A) Neuro2a cells were fixed with 4% PA, followed by immunocytochemical detection of OCTN1 (green), neuronal marker β III-tubulin (red) and nuclear marker DAPI (blue). The merged image is also shown. (B) Neuro2a cells were incubated with [3 H]ERGO in transport buffer at 37°C for up to 240 min. (C) Incubation was done with [3 H]ERGO for 240 min in either the presence or absence of unlabeled ERGO in the concentration range of 1 to 200 μ M. Each value represents the mean $\pm$ S.E.M. (n = 3-9). * $p < 0.05$, significant difference from the corresponding control value obtained in cells incubated with [3 H]ERGO alone.

Fig. 7. Effect of OCTN1 knockdown on expression of oxidative stress- or neuronal differentiation-related genes in Neuro2a cells. (A) Neuro2a cells were transiently transfected with siOCTN1 (white columns) or negative control siRNA (black columns) for 24 h, and further cultured for an additional period up to 48 h. Total RNA was extracted, followed by measurement of OCTN1 expression level by means of quantitative RT-PCR. Each value is

normalized by the expression level of 36B4 mRNA and expressed as a relative value to the corresponding control value obtained in cells transfected with negative control siRNA. (B) Neuro2a cells were transiently transfected with siOCTN1 (white symbols) or negative control siRNA (black symbols) for 24 h, and further cultured for 48 h. Neuro2a cells were incubated with [³H]ERGO in transport buffer at 37°C for up to 240 min. (C, D) At 72 h after the transfection of siRNA, total RNA was extracted from Neuro2a cells for quantitative RT-PCR analysis. The expression levels of oxidative stress- (C) or neuronal differentiation- (D) related genes were determined. Each value is normalized by the expression level of 36B4 mRNA and expressed as a relative value to the control value obtained in cells transfected with negative control siRNA. Each value represents the mean $\pm$ S.E.M. (n = 3-8). **p* < 0.05, significant difference from the corresponding control value obtained in cells transfected with negative control siRNA.

Fig. 8. Effect of OCTN1 knockdown on neuronal differentiation and proliferation of Neuro2a cells. (A) Neuro2a cells were transiently transfected with siOCTN1 (lower panel) or negative control siRNA (upper panel) for 24 h, and further cultured for 48 h. Typical phase-contrast micrographs are shown. White arrowheads indicate typical differentiated cells with longer neurites than the cell diameter. The number of differentiated cells (B) and the total number of cells (C) per field were counted for quantitative analysis in Neuro2a cells transfected with

siOCTN1 (white column) or negative control siRNA (black column). Each value represents the mean $\pm$ S.E.M. (n = 6). * p < 0.05, significant difference from each control value obtained in cells transfected with negative control siRNA.

Table 1

Primers used for real-time PCR analysis in the present study.

Gene	Sense primer (5'-3')	Antisense primer (3'-5')
MAP2	TCAGGAGACAGGGAGGAGAA	GTGTGGAGGTGCCACTTTTT
βIII-tubulin	CATGGACAGTGTTCTGGTCTG	CGCACGACATCTAGGACTGA
Sox2	AACGCCTTCATGGTATGGTC	TCTCGGTCTCGGACAAAAGT
GAP43	CTGCTGTCACTGATGCTGCT	GGTTTGGCTTCGTCTACAGC
Synapsin 1	CACCGACTGGGCAAAATACT	TCCGAAGAACTTCCATGTCC
Nrf2	AACGAGAGGACACCTGGTCA	CGGGGGACTCACTCTCACTA
HO-1	TGCTCGAATGAACACTCTGG	TCCTCTGTCAGCATCACCTG
xCT	AAACCCAAGTGGTTCAGACG	ATCTCAATCCTGGGCAGATG
Ucp2	AGCCTGAGACCTCAAAGCAG	ACATCTGTGGCCTTGAAACC
36B4	ACTGGTCTAGGACCCGAGAAG	TCCACCTTGTCTCCAGTCT

Figure 1

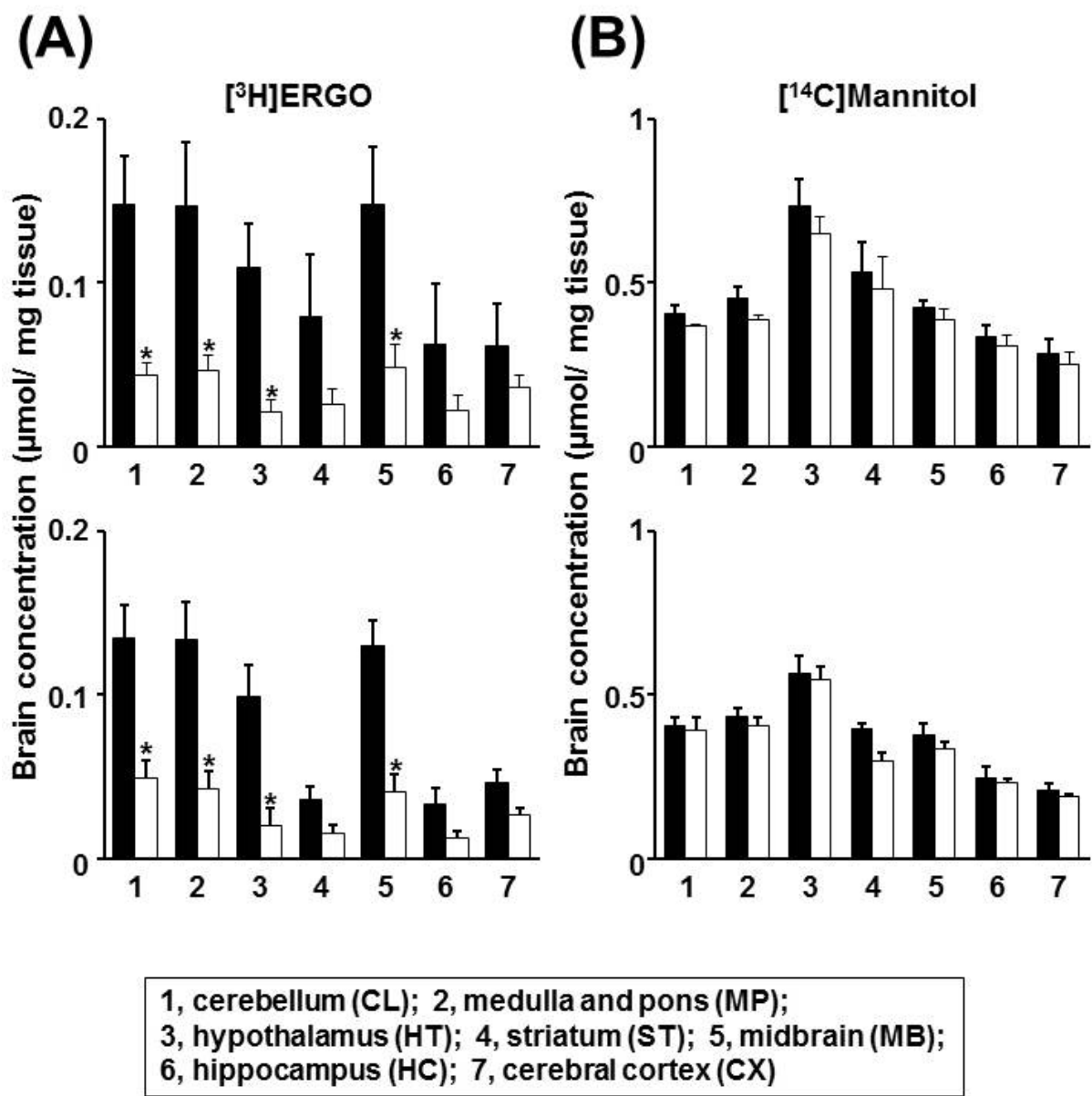


Figure 2

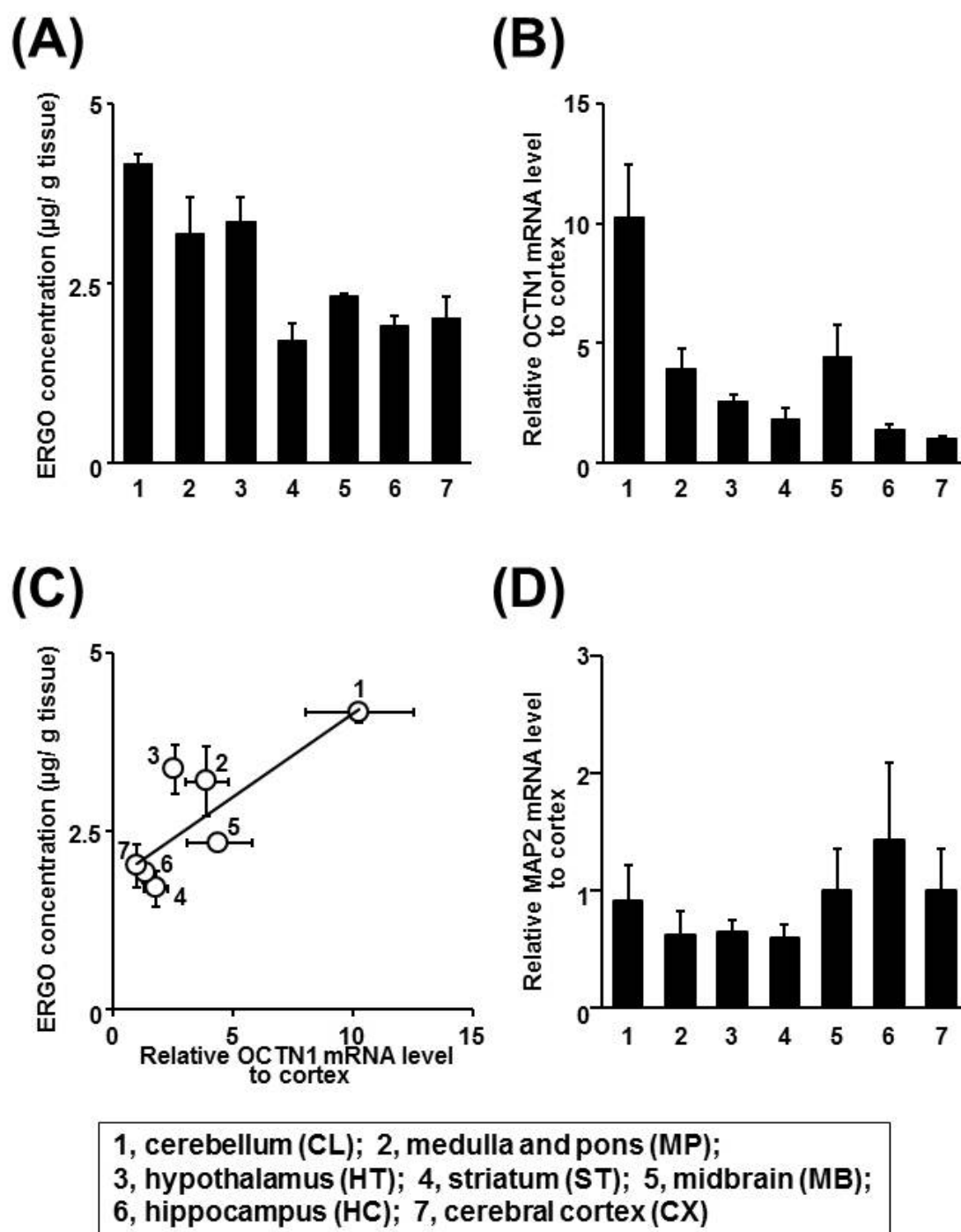


Figure 3

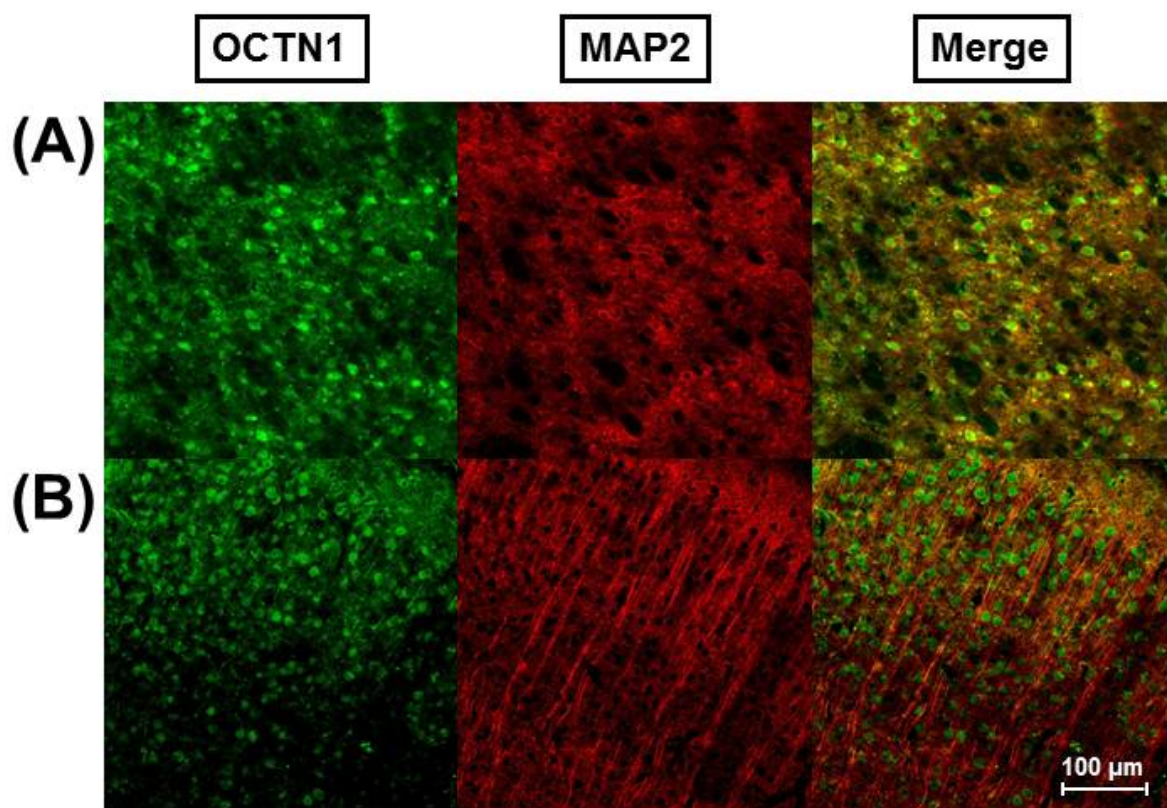


Figure 4

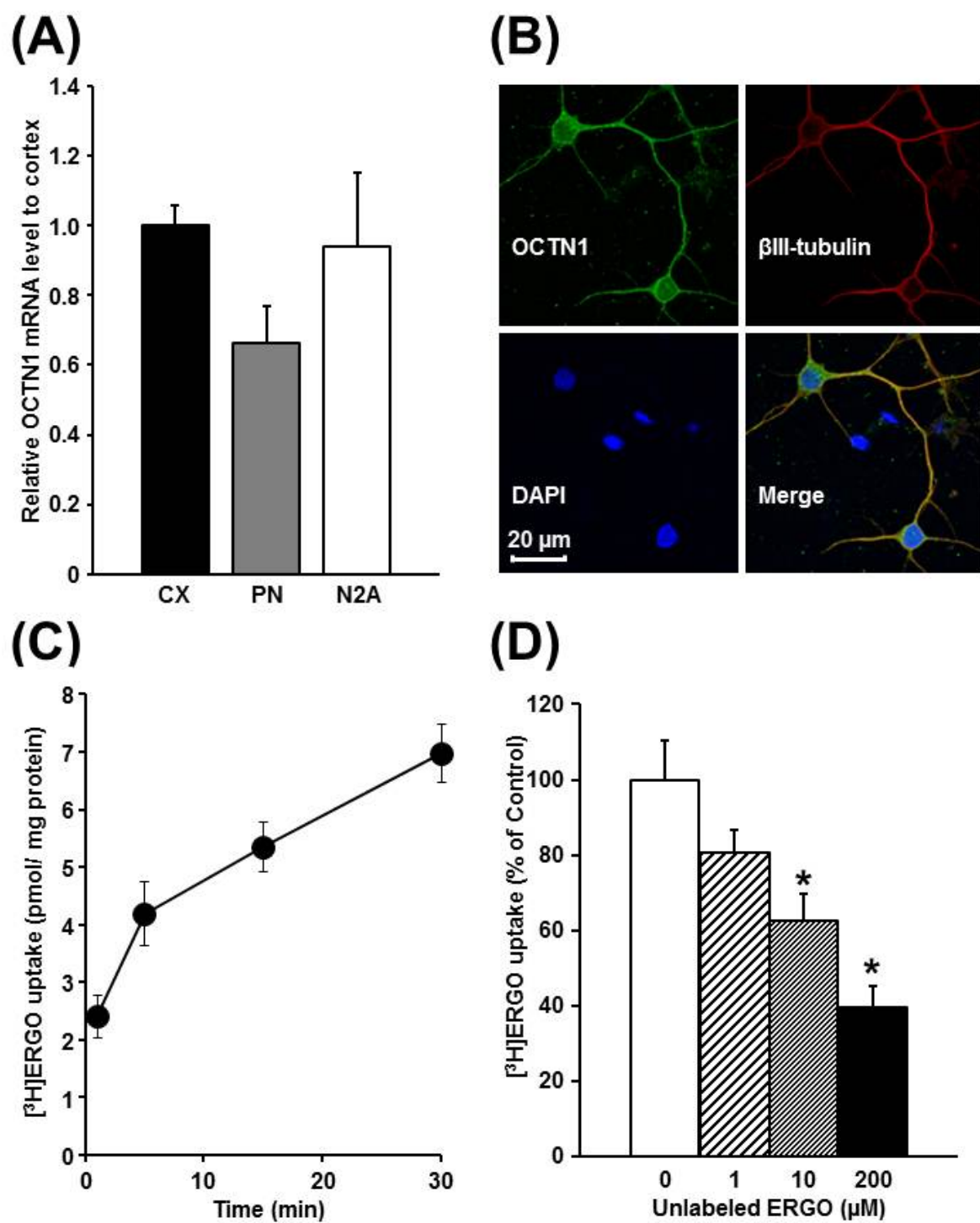


Figure 5

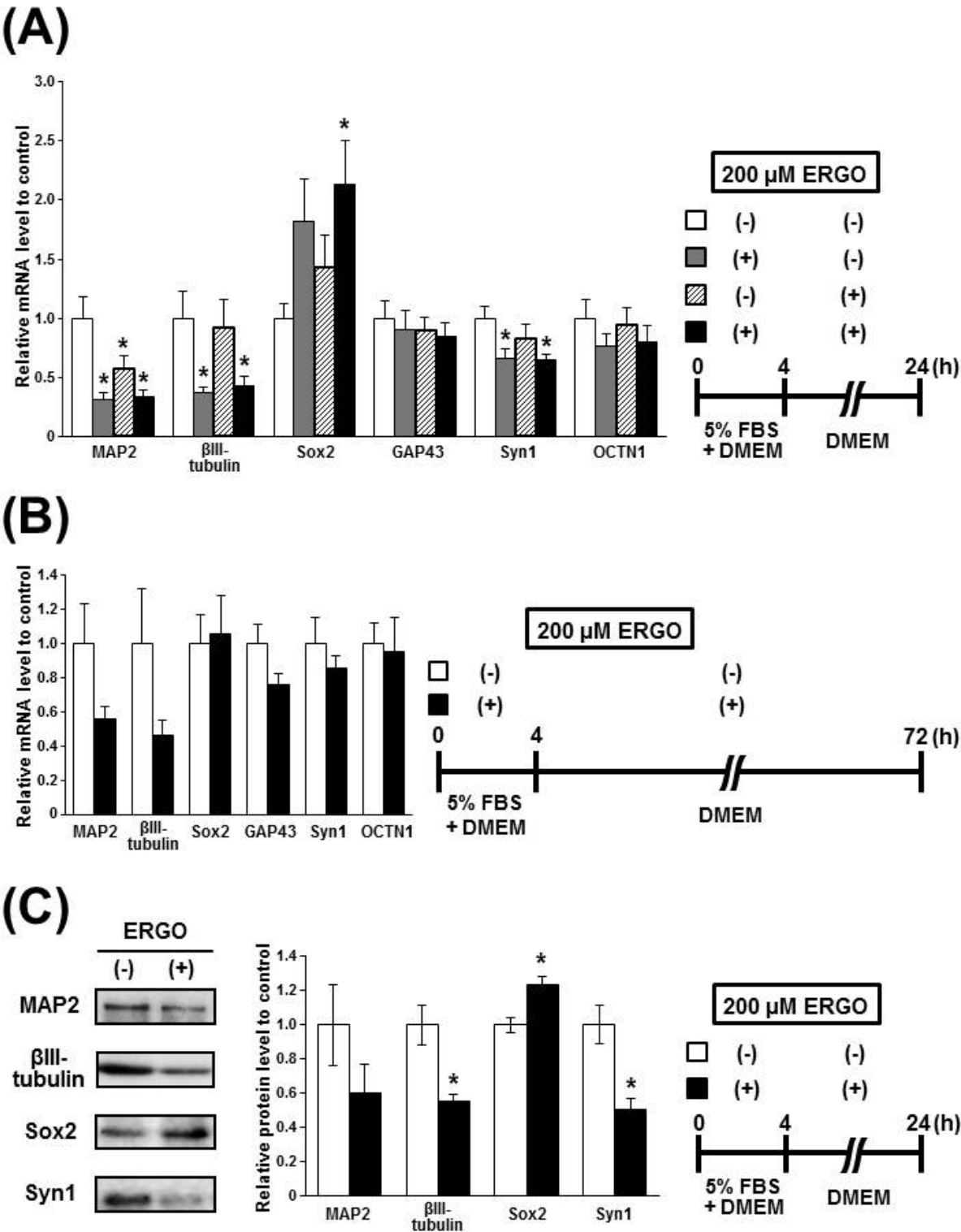


Figure 6

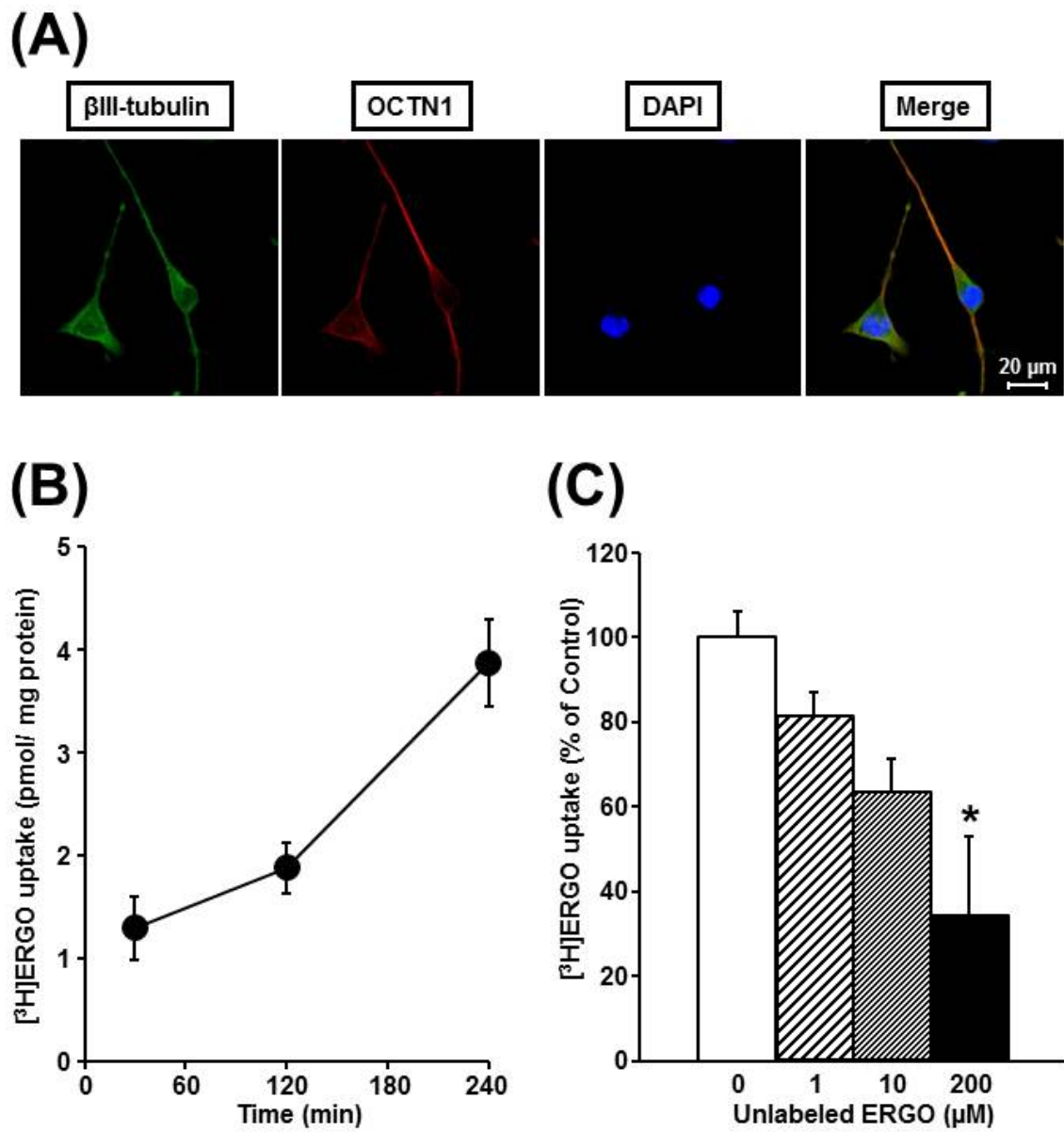


Figure 7

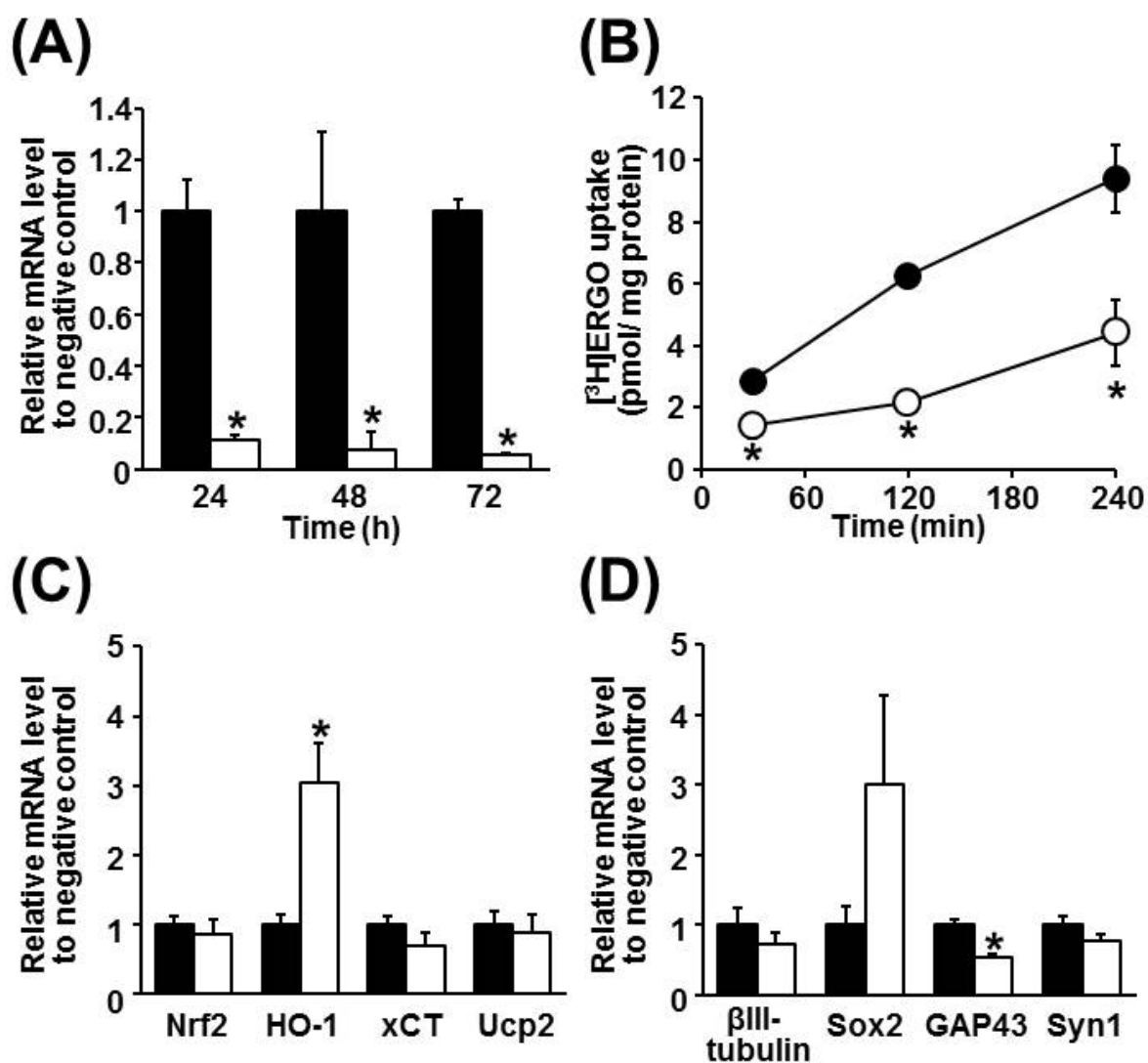


Figure 8

