

DMD # 80838

Short Communications

Sulfotransferase 4A1 (SULT4A1) increases its expression in mouse neurons as they mature

Takuyu Hashiguchi, Sawako Shindo, Shih-Heng Chen, Jau-Shyong Hong, Masahiko Negishi

Pharmacogenetics section, Reproductive and Developmental Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA (T.H., S.S., and M.N.); Neurobiology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA (S.H.C., and J.S.H.)

DMD # 80838

Running title: SULT4A1 expression during neuron maturation

Address correspondence to: Dr. Masahiko Negishi, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina, 27709, USA. Telephone, 919-541-2404; Fax, 919-541-0696; E-mail address, negishi@niehs.nih.gov

Number of text pages: 15

Number of tables: 0

Number of figures: 4

Number of supplemental figures: 1

Number of references: 20

Number of words in Abstract: 169

Number of words in Introduction: 294

Number of words in Discussion: 565

Abbreviations: SULT, cytosolic sulfotransferase; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; E14, embryonic day 14; NeuN, neural nuclei; GFAP, glial fibrillary acidic protein; IBA-1, ionized calcium binding adaptor molecule 1; TH, tyrosine hydroxylase; CAR, constitutive androstane receptor; PXR, pregnane X receptor; FXR, farnesoid X receptor; ER α , estrogen receptor alpha; PB, phenobarbital; E2, 17 β -estradiol; T3, 3,3',5-triiodo-L-thyronine; DHEA, dehydroepiandrosterone; PREG, pregnenolone; RIF, rifampicin; LPS, lipopolysaccharide; EGF, epidermal growth factor; HGF, hepatocyte growth factor; PKA, protein kinase A; PCR, polymerase chain reaction; DAPI, 4',6-diamidino-2-phenylindole

DMD # 80838

Abstract

Cytosolic sulfotransferases (SULTs) catalyze sulfation and play essential roles in detoxification of xenobiotics as well as inactivation of endo-biotics. Sulfotransferase 4A1 (SULT4A1) which was originally isolated as a brain-specific sulfotransferase, is the most highly conserved isoform among SULTs in vertebrates. Here, expression of SULT4A1 was examined in neuron-enriched and neuron-glia mixed cells derived from mouse embryo brains at day 14 gestation and mixed glia from 2 days-old neonate brains. Western blots showed an increase of SULT4A1 expression as neurons matured. RT-PCR and agarose gel analysis found two different forms (variant and wild type) of SULT4A1 mRNA in neurons; the level of wild type correlates with the protein level of SULT4A1. SULT1E1 was expressed neither in mouse brains, neuron-enriched cells nor mixed glia cells. SULT1A1 protein was only detected in adult brains. Immunofluorescence staining of neuron-glia mixed cells confirmed selective expression of SULT4A1 in neurons including dopaminergic neurons, but not in either astrocytes or microglia. Thus, SULT4A1 is a neuron-specific sulfotransferase and may play a role in neuronal development.

Introduction

Cytosolic sulfotransferases (SULTs) are enzymes which catalyze sulfation by transferring the sulfonate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to substrate (Gamage et al., 2006). The total 15 human and 22 mouse SULTs are divided into four and six families in humans and mice, respectively, ubiquitously expressed in various tissues and organs and collectively sulfate numerous xenobiotics and endo-biotics. Characteristically, SULT1 enzymes sulfate a simple phenol group such as *p*-nitrophenol, while the SULT2 enzymes are known to sulfate steroid hormones such as DHEA, estrogen and bile acids (Gamage et al., 2006; Wang and James, 2005). Exception to these characteristics, SULT1E1, the only member within the SULT1E subfamily and called estrogen sulfotransferase, sulfates E2 with high affinity at nM of *K_m* values (Falany et al., 1995).

Contrary to the other ubiquitous expression, SULT4A1 (the only member within the SULT4 family) is only expressed in brains. Although cDNAs were cloned from human and rodents brains more than 15 years ago (Falany et al., 2000; Sakakibara et al., 2002), neither endogenous nor exogenous substrates have yet been identified. Amino acid sequence of SULT4A1 is highly conserved from mice to humans. These cross-species conservation and brain-specific expression suggest yet unknown physiological functions. In fact, SULT4A1 was associated with susceptibility of schizophrenia in humans (Lewis and Minchin, 2009). Recently-generated SULT4A1 KO mice developed severe and progressive neurological symptoms such as tremor, rigidity and seizure and died postnatally at days 21-25 (Garcia et al., 2018). Here, we have utilized mouse brains, mouse primary neurons and glia cells for Western blot, real-time PCR and immunofluorescence staining analyses to examine expression of SULT4A1, and have reported that SULT4A1 is expressed in neurons including dopaminergic neurons but not in glia

DMD # 80838

cells. Based on our finding, we will discuss possible roles of SULT4A1 in the brain.

Materials and Methods

Materials: Anti-NeuN antibody (ABN78) was obtained from Millipore (Billerica, MA). SULT1A1 (10911-2-AP), SULT1E1 (12522-1-AP), and SULT2B1 polyclonal antibody (12879-1-AP) were purchased from Proteintech (Rosemont, IL); SULT4A1 mouse monoclonal antibody (sc-374545) from Santa Cruz Biotechnology (Santa Cruz, CA); IBA-1 antibody (GTX100042) from GeneTex (Irvine, CA); Alexa Fluor 488 goat anti-mouse IgG antibody and Alexa Fluor 594 goat anti-rabbit IgG antibody from Life Technologies (Carlsbad, CA); Rabbit anti-tyrosine hydroxylase (TH) polyclonal antibody from Chemicon (Temecula, CA); Mounting Medium with DAPI and Mouse on Mouse (M.O.M.TM) Blocking Reagent from Vector Laboratories (Burlingame, CA); Polyclonal rabbit anti-Glial Fibrillary Acidic Protein (Z0334) from Dako Cytomation (Carpinteria, CA); TaqMan Gene Expression Assays (probe and primer sets) for SULT4A1 (AssayID: Mm00489166_m1) (FAM), SULT2B1 (AssayID: Mm00450550_m1) (FAM) and mouse GAPDH (FAM) from Applied Biosystems (Foster, CA). All other chemicals were of the highest grade commercially available.

Animals and cell cultures: C57BL/6 mice were maintained under the standard condition at the National Institute of Environmental Health Sciences, and animal experiments were conducted according to protocols approved by the animal ethics committee at NIEHS/National Institutes of Health. The primary neuron-glia mixtures, neuron-enriched cells and mixed glia cells were prepared according to the previous study (Chen et al., 2013). Brains were isolated from 8 weeks-old male mice and 2 day-pup for subsequent Western blot analysis.

Western blotting: The brain derived cells and tissues were homogenized in the lysis buffer [8M

DMD # 80838

Urea/1% SDS in TBS-T]. The lysate was centrifuged and the supernatant was collected. Five micrograms of proteins were electrophoresed on a 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes at 10 V for 1 h. The membranes were probed with a given antibody overnight at 4°C, reacted with horseradish peroxidase-conjugated secondary antibody using WesternBright Sirius kit (Advansta, Menlo Park, CA), visualized by a C-DiGit Blot Scanner (LI-COR, Inc). As a loading control, β -actin levels were detected using anti- β -actin antibody (sc-47778) from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunocytochemistry: Neuron-glia mixed cells were prepared on glass bottom dishes as mentioned above. The cells were fixed with 4% formaldehyde in PBS, rinsed, and blocked with MOM mouse IgG blocking reagent. The cells were subsequently blocked with the reagent containing 0.4% Triton-X100/PBS, 1% bovine serum albumin, and 4% normal goat serum and washed. The primary antibody against SULT4A1, NeuN, IBA-1, GFAP, or TH was added onto the cells. After overnight reaction at 4°C, another primary antibody was added after PBS wash, followed by incubation at 4°C overnight. Alexa Fluor 488 goat anti-mouse IgG antibody and Alexa Fluor 594 goat anti-rabbit IgG antibody were reacted for 2 h at room temperature. The mounting reagent containing DAPI for nuclei staining were added and observed with Zeiss LSM 710 inverted confocal microscope.

Real-time PCR (qPCR) and RT-PCR: Total RNA was extracted from mouse brain tissues and cells using TRIZOL reagent, with which cDNAs were synthesized using a High Capacity cDNA Archive kit (Applied Biosystems). Real-time PCR was conducted using a TaqMan Universal PCR Master mix and TaqMan probes with a 7900HT Fast Real-Time PCR System (Applied Biosystems). Primers for SULT4A1 wild type, SULT4A1 variant, and β -actin were designed and

DMD # 80838

amplified according to the previous report (Sidharthan et al., 2014). The products were then separated by electrophoresis on 2.0% agarose gels, stained with ethidium bromide, and visualized by UV illumination.

Statistical Analysis: Multiple groups were analyzed by one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test. The statistical analyses was conducted using a software GraphPad Prism 7.04 (San Diego, CA).

Results

SULT4A1 expression in mouse brains: Extracts were prepared from brains of 8 weeks-old adult mice and 2 days-old neonates for subsequent Western blot analysis. Primary brain cell cultures were prepared from the mesencephalic tissues of E14 embryos, from which neurons were enriched to about 96% purity and cultured by employing an earlier described protocol (Chen et al., 2013). Mixed glia cell cultures mainly containing 70% astroglia and 30% microglia were prepared from cerebral hemisphere and midbrains of 2-day pups (Chen et al., 2013). In Fig. 1A, Western blot analysis showed higher expression of SULT4A1 in adults than in neonate brains. SULT4A1 was also expressed in neuron enriched cells but not mixed glia cells.

SULT1A1 was detected in only adult brains. No SULT1E1 protein was detected with any of these brains and cells. Real-time PCR was performed with RNAs to examine levels of SULT4A1 mRNA, which correlated with the protein levels in each brain and cells, except that a slight disconnect was noted between protein and mRNA levels in brains (Fig. 1B). SULT4A1 mRNA was also present in neonate brains and neuron enriched cells in addition to adult brains. As to SULT1E1, no mRNA was detected (data not shown). These observations indicate that primary neurons express only SULT4A1 at protein levels. It is also noted that none of these SULTs are expressed in glia cells.

SULT4A1 expression during neuron maturation: Primary midbrain neuron-glia cells were prepared from E14 embryos. During 7 day culturing period, neurons mature by extending axons and dendrites to establish cell-cell interactions (Supplemental fig. 1A) (Chen et al., 2013). To examine SULT4A1 expression, protein and RNA extracts were prepared from 1, 3, 5 and 7 days of culture after initial seeding for subsequent Western blot and real-time PCR analyses,

DMD # 80838

respectively (Fig. 2). SULT4A1 protein was barely detected at day 1 and increased as culture continued, which correlated with neuron maturation (Fig. 2A). Like SULT4A1, SULT2B1 protein increased as neuron matured. SULT1A1 and SULT1E1 were not detected at any time points of culture (Fig. 2A). Real-time PCR analysis revealed that SULT4A1 mRNA levels did not correlate with its protein levels; mRNA was already expressed before its protein was detected and peaked at 5 days while protein levels peaked at 7 days (Fig. 2B). To examine this discrepancy, SULT4A1 transcripts were analyzed on an agarose gel (Fig. 2C). One splice variant and wild type mRNAs were detected. Variant was expressed during the initial period of culture. Reciprocal to variant decrease, wild type increased in time-dependent manner. This increase of wild type appeared to correlate with protein levels. The variant mRNA contains a pseudo exon 6 and translates a truncated/inactive enzyme (Sidharthan et al., 2014). These observations were consistent with previous findings with rat brains and human neuroblastoma cell lines (Falany et al., 2000; Sidharthan et al., 2014). SULT2B1 mRNA increased as its protein increased (Fig. 2D). No mRNAs were detected for SULT1A1 and SULT1E1 in neuron glia mixed cells at any time points (data not shown). While SULT4A1 was found not to be expressed in glia cells in our present work, it was previously reported in glia-derived cell lines (Sidharthan et al., 2014). To examine this difference, Western blot analysis was employed to examine the expression in glioblastoma-derived U373-MG cells and dopaminergic neuron-derived N27 cells. SULT4A1 could not be detected in these immortalized cells (data not shown).

SULT4A1 expression upon chemical treatments: Neuron enriched cultures were treated with various exogenous chemicals and endogenous hormones for subsequent Western blot analysis of SULT4A1. Since Toll-like receptor (TLR) signaling is known to regulate axonal growth and

DMD # 80838

neuronal plasticity (Okun et al., 2011), neuron cultures were treated with increased concentration of LPS, but no changes were found in SULT4A1 levels at any concentrations (Fig. 3A). Nuclear receptors such as CAR, PXR and FXR are known to regulate expressions of SULTs (Kodama and Negishi, 2013). Neuron enriched cultures were treated with their activators and ligands such as phenobarbital, CITCO, RIF and GW4064. None of these tested affected levels of SULT4A1 (Fig. 3B). Neither growth factors EGF, HGF, endocrine hormones T3, pregnenolone, DHEA nor DHEA-sulfate (DHEAS) altered SULT4A1 levels. Moreover, a PKA activator forskolin also showed no effect (Fig. 3B).

Immunofluorescence staining of neurons and glia for SULT4A1: Mixed neuron-glia cells were cultured for 7 days for subsequent double immunofluorescence staining by an anti-SULT4A1 antibody with an anti-neuronal marker NeuN antibody, an anti-dopaminergic neuron marker tyrosine hydroxylase (TH) antibody, an anti-astrocyte marker glial fibrillary acidic protein (GFAP) antibody or a microglia marker ionized calcium binding adaptor molecule 1 (IBA-1) antibody. All of neurons stained by an anti-NeuN antibody were also stained by an anti-SULT4A1 antibody (Fig. 4A). About 2% of cultured cells are dopaminergic neurons (Gao et al., 2002). There were two dopaminergic neurons in this section, both of which were co-stained by an anti-SULT4A1 antibody (Fig. 4B). Neither astrocytes nor microglia were co-stained (Figs. 4C and 4D), confirming that SULT4A1 was not expressed in glia cells.

Discussion

As to glia cells, astrocytes produce and provide energy sources to other brain cells while microglia are resident macrophages and are the only immune cells in the brain. The finding that SULT4A1 was not expressed in either glia cells indicates that SULT4A1 is not involved in their respective cell functions. On the other hand, SULT4A1 may play roles in the neuronal maturation and/or in protecting neurons from degeneration. Since SULT4A1 KO mice survive through embryonic stages (Garcia et al., 2018), mixed neuron-glia cells can be prepared from E14 embryos from this mutant mouse, providing us with an excellent experimental system to determine physiological roles of SULT4A1 in neurons.

Since ablation of the *Sult4a1* gene caused severe neuronal abnormalities (Garcia et al., 2018), SULT4A1 must critically regulate various neuronal functions. With this respect, our finding of SULT4A1 expression in dopaminergic neurons is informative, since abnormalities of dopaminergic neurons have been associated with the development of various neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases (Block and Hong, 2005; Cepeda et al., 2014). Neuroinflammation mediated through dysregulated microglia have been linked to the pathogenesis of Parkinson's disease (Gao et al., 2002). However, since SULT4A1 is not expressed in microglia, if SULT4A1 has a direct role in these neuronal degenerations, it is not initiated from microglia. While neuron-specific expression of SULT4A1 is an informative finding, SULT1E1 not being expressed in brains and neurons is a surprising but equally informative finding. Brain is a major estrogen-targeted organ (Gillies and McArthur, 2010). Besides exciting numerous physiological functions, estrogens are also toxic chemicals that adversely affect organs. SULT1E1 is the enzyme that inactivates estrogen. Sulfated estrogen can be reactivated through desulfation by steroid sulfatase (Purohit et al., 1994). Thus, SULT1E1 is

DMD # 80838

ubiquitously expressed as a key enzyme that controls the metabolic balance of estrogen activity, which is intriguing it is not expressed in the brain. Although SULT1A1 and SULT2A1 were reported to sulfate estrogen, but poorly compared with SULT1E1 (Harris et al., 2000; Wang and James, 2005), they were not expressed in mouse primary neurons (Fig. 1A) and brains (Salman et al., 2011), respectively. As to estrogen sulfation, it was reported that SULT4A1 bound 2-hydroxyestradiol in thermostability screening assays (Allali-Hassani et al., 2007). Moreover, SULT4A1 and ER α concomitantly increased their expression levels in neurons during maturation (Supplemental fig. 1B) and our preliminary work with recombinant SULT4A1 enzyme suggested that it may sulfate estrogen (unpublished). However, SULT4A1 being enzyme that sulfates estrogen is uncertain now and remains to be explored in further investigations.

Expression of SULT4A1 increased in primary neurons as they matured and were higher in adult than neonate brains. Two forms of SULT4A1 mRNA, wild type and a splice variant, were expressed in primary neurons. Of which the increase of the wild type correlated with that of SULT4A1 protein. However, the cell signal mechanism that developmentally activates the *Sult4a1* gene remains uninvestigated. Nearly 10 endo-biotics and exogenous chemicals were examined, none of which affected SULT4A1 expression in neurons. Finding the regulatory mechanism of this expression should help us to understand the neuronal function of SULT4A1.

In conclusion, SULT4A1 appears to play a role in maturation of neurons. Expression of SULT4A1 in dopaminergic neurons is an indicative of its involvement in neuronal functions such as movements. Although SULT4A1 may regulate both beneficial and adverse effects of estrogens, the involvement of SULT4A1 in estrogen metabolism remains urgent subject of future investigations.

DMD # 80838

Acknowledgements

We thank the fluorescence microscopy and imaging center and DNA sequence and histology cores at NIEHS for their excellent assistance. Hashiguchi, T. was supported by the Strategic Young Researcher Overseas Visits Program for Accelerating Brain Circulation, Japan Society for the Promotion of Science (JSPS).

Authorship Contributions

Participated in research design: Negishi and Hashiguchi.

Conducted experiments: Hashiguchi, Shindo and Chen.

Performed data analysis: Hashiguchi.

Wrote or contributed to the writing of the manuscript: Negishi, Hashiguchi, and Hong.

References

- Allali-Hassani A, Pan P, Dombrowski L, Najmanovich R, Tempel W, Dong A, Loppnau P, Martin F, Thornton J, Edwards AM, Bochkarev A, Plotnikov AN, Vedadi M, and Arrowsmith CH (2007) Structural and Chemical Profiling of the Human Cytosolic Sulfotransferases. *PLoS Biol* 5: e97.
- Block ML, and Hong JS (2005) Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Prog Neurobiol* 76: 77-98.
- Cepeda C, Murphy KP, Parent M, and Levine MS (2014) The role of dopamine in Huntington's disease. *Prog Brain Res* 211: 235-254.
- Chen SH, Oyarzabal EA, and Hong JS (2013) Preparation of rodent primary cultures for neuron-glia, mixed glia, enriched microglia, and reconstituted cultures with microglia. *Methods Mol Biol* 1041: 231-240.
- Falany CN, Krasnykh V, and Falany JL (1995) Bacterial expression and characterization of a cDNA for human liver estrogen sulfotransferase. *J Steroid Biochem Mol Biol* 52: 529-539.
- Falany CN, Xie X, Wang J, Ferrer J, and Falany JL (2000) Molecular cloning and expression of novel sulphotransferase-like cDNAs from human and rat brain. *Biochem J* 346: 857-864.
- Gamage N, Barnett A, Hempel N, Duggleby RG, Windmill KF, Martin JL, and McManus ME (2006) Human sulfotransferases and their role in chemical metabolism. *Toxicol Sci* 90: 5-22.
- Gao HM, Jiang J, Wilson B, Zhang W, Hong JS, and Liu B (2002) Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. *J Neurochem* 81: 1285-1297.
- Garcia PL, Hossain MI, Andrabi SA, and Falany CN (2018) Generation and Characterization of

DMD # 80838

- SULT4A1 Mutant Mouse Models. *Drug Metab Dispos* 46: 41-45.
- Gillies GE and McArthur S (2010) Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. *Pharmacol Rev* 62: 155-198.
- Harris RM, Waring RH, Kirk CJ, and Hughes PJ (2000) Sulfation of "estrogenic" alkylphenols and 17beta-estradiol by human platelet phenol sulfotransferases. *J Biol Chem* 275: 159-166.
- Kodama S, and Negishi M (2013) Sulfotransferase genes: regulation by nuclear receptors in response to xeno/endo-biotics. *Drug Metab Rev* 45: 441-449.
- Lewis A, and Minchin R (2009) Lack of exonic sulfotransferase 4A1 mutations in controls and schizophrenia cases. *Psychiat Genet* 19: 53-55.
- Liu MC, and Lipmann F (1984) Decrease of tyrosine-O-sulfate-containing proteins found in rat fibroblasts infected with Rous sarcoma virus or Fujinami sarcoma virus. *Proc Natl Acad Sci U S A* 81: 3695-3698.
- Okun E, Griffioen KJ, and Mattson MP (2011) Toll-like receptor signaling in neural plasticity and disease. *Trends Neurosci* 34: 269-281.
- Purohit A, Dauvois S, Parker MG, Potter BV, Williams GJ, and Reed MJ (1994) The hydrolysis of oestrone sulphate and dehydroepiandrosterone sulphate by human steroid sulphatase expressed in transfected COS-1 cells. *J. Steroid Biochem Mol Biol* 50: 101-104.
- Sakakibara Y, Suiko M, Pai TG, Nakayama T, Takami Y, Katafuchi J, and Liu MC (2002) Highly conserved mouse and human brain sulfotransferases: molecular cloning, expression, and functional characterization. *Gene* 285: 39-47.
- Salman E, Faye-Petersen O, and Falany CN (2011) Hydroxysteroid sulfotransferase 2B1b expression and localization in normal human brain. *Horm Mol Biol Clin Investig* 8: 445-

DMD # 80838

454.

- Sidharthan N, Butcher N, Mitchell D, and Minchin R (2014) Expression of the Orphan Cytosolic Sulfotransferase SULT4A1 and Its Major Splice Variant in Human Tissues and Cells: Dimerization, Degradation and Polyubiquitination. *PLoS One* 9: e101520.
- Wang LQ, and James MO (2005) Sulfotransferase 2A1 forms estradiol-17-sulfate and celecoxib switches the dominant product from estradiol-3-sulfate to estradiol-17-sulfate. *J Steroid Biochem Mol Biol* 96: 367-374

DMD # 80838

Footnotes

This work was supported by the Intramural Research Program of the National Institutes of Health and National Institute of Environmental Health Sciences [Grant Z01ES71005-01].

Figure Legends

Fig. 1. Neuron specific expression of SULT4A1. A. Western blots. SULT expressions were examined in 8 weeks-old adult mouse brains, 2 days-old neonate brains, mixed glia cells and neuron enriched cells using an anti-SULT4A1, SULT1A1 or SULT1E1 antibody. Sample preparations were independently conducted three times and the representative Western blot image was indicated. B, Real-time PCR. SULT4A1 mRNA levels using the tissues or cells as described in Fig. 1A legend. Each value is shown as the mean \pm S.D (n=3). Asterisks indicate significant differences between experimental groups (*p<0.05)

Fig. 2. SULT4A1 expression during neuron maturation. A. Mesencephalic neuron-glia cells were cultured as described in the Materials and Methods section. Western blots were performed as described in the Fig. 1 legend. A representative image of three independent experiments was indicated. B. Real-time PCR. Total RNAs were extracted from each stage of neuron glia cultures. C. SULT4A1 mRNAs were amplified by RT-PCR. D. Real-time PCR. SULT2B1 mRNAs were amplified as indicated in Fig. 2B. Each value is shown as the mean \pm S.D (n=3). Asterisks indicate significant differences between experimental groups (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

Fig. 3. Effects of various compounds on SULT4A1 expression in mouse primary neurons. A. Mouse neuron enriched cells were treated with LPS (1-15 μ g/ml) for 24 h and harvested for Western blot analysis. B. The cells were treated with EGF (10 ng/ml), HGF (50 ng/ml), PB (1 mM), CITCO (1 μ M), RIF (10 μ M), GW4064 (5 μ M), forskolin (10 μ M), T3 (10 nM), PREG (500 nM), DHEA (100 nM) or DHEAS (100 nM) for 24 h, followed by Western blot analysis. A representative Western blot image of three independent experiments was indicated.

DMD # 80838

Fig. 4. Double fluorescence staining of mouse primary neuron/glia cells. Mesencephalic primary neuron-glia cells were prepared from E14 mouse embryos and cultured for 7 days. Cells were double stained by an anti-SULT4A1 antibody with NeuN (A), TH (B), GFAP (C) or IBA-1 (D). Nuclei was stained by DAPI.

Figure 1

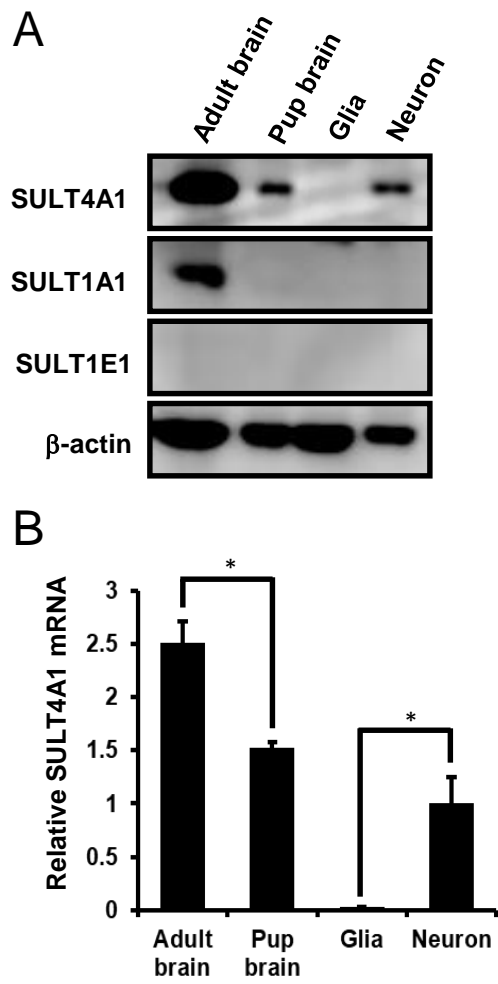


Figure 2

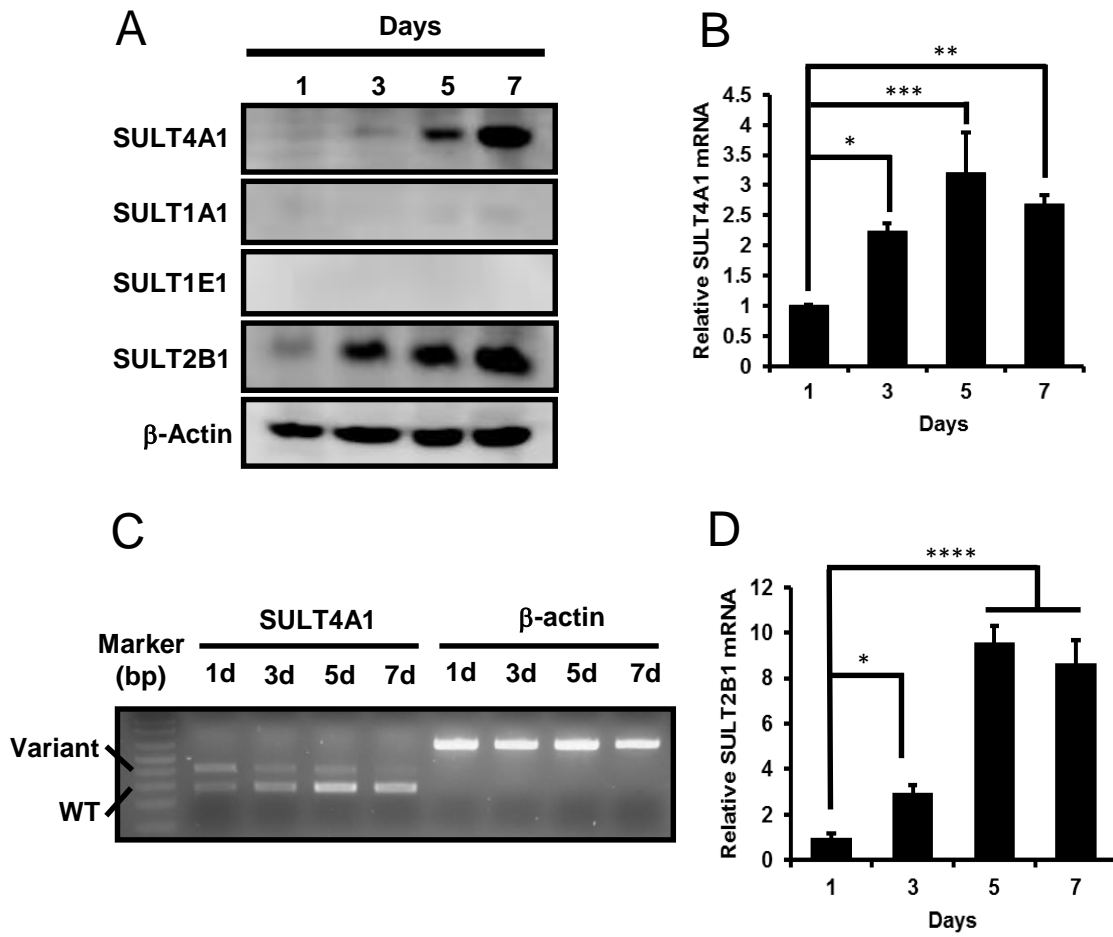
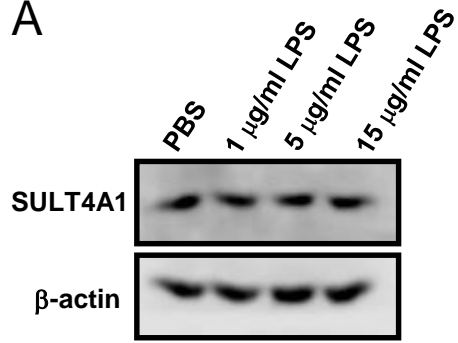


Figure 3

A



B

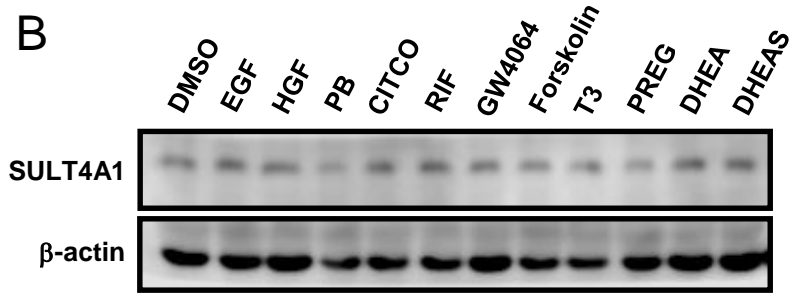
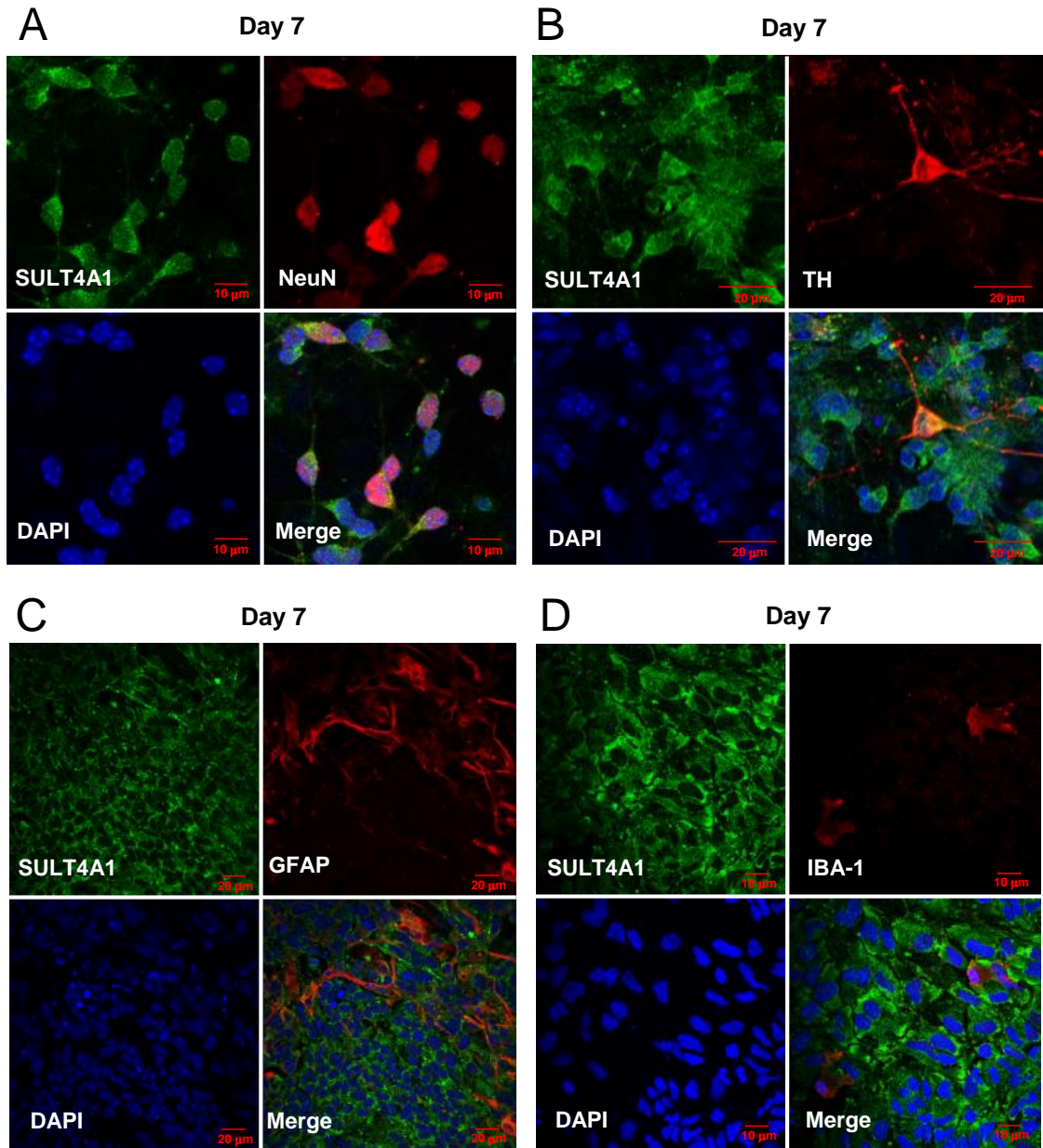


Figure 4



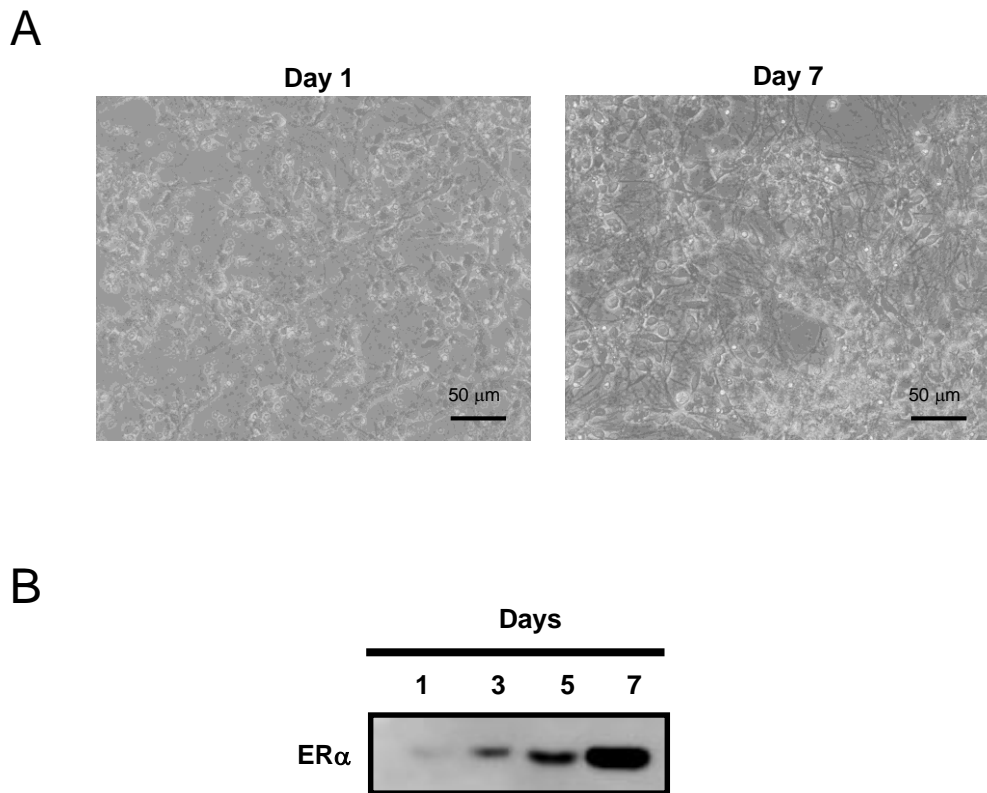
Sulfotransferase 4A1 (SULT4A1) increases its expression in mouse neurons as they mature

Takuyu Hashiguchi, Sawako Shindo, Shih-Heng Chen, Jau-Shyong Hong, Masahiko Negishi

Pharmacogenetics section, Reproductive and Developmental Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA (T.H., S.S., and M.N.); Neurobiology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA (S.H.C., and J.S.H.)

Address correspondence to: Dr. Masahiko Negishi, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina, 27709, USA. Telephone, 919-541-2404; Fax, 919-541-0696; E-mail address, negishi@niehs.nih.gov

Supplemental figure 1



Supplemental figure 1. Microscopic images of neuron glia mixed cultures at Day 1 and 7. Mesencephalic primary neuron-glia cells prepared from E14 mouse embryos were cultured for 7 days and observed in bright-field. **B.** Expression of ER α . Protein extracts prepared from neuron glia mixture cells at Days 1, 3, 5 and 7 were subjected to Western blot analysis using an anti-ER α antibody as shown in Fig. 2A