Next, for measurement of HGF protein levels, the gastrocnemius muscle of each hindlimb was removed and frozen in liquid nitrogen.

To investigate satellite cell activation for exercise intensity, 4-week-old rats were used. Rats performed walking at 16 m/min or running 24 m/min on a treadmill at -16% grade for 30 min. The exercise training protocol was referred to Smith et al.[3]. Mean walking speed in rats was 36 cm/s[14]. According to exercise walking and running, this study postulated a demarcation between walking and running of 36 cm/s (21.6 m/min). Soleus muscles were removed after 72 h. Animals were injected with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU, 3.07 mg/100 g body weight) 1 h before sampling. Right soleus muscle was used for immunofluorescence, and left soleus muscle was used to measure HGF protein levels. The right soleus muscle was placed in Tissue-Tek OCT compound (Miles, Elkhart, IN, USA), quick-frozen in liquid nitrogen-cooled isopentane, and stored at -70 °C until use.

Muscles removed from bilateral hindlimbs were homogenized in rat HGF organic extraction buffer (Institute of Immunology, Tokyo, Japan). HGF levels in solution were measured using a rat HGF enzyme immunoassay kit (Institute of Immunology).

To perform immunofluorescence for BrdU and dystrophin, 8-μm frozen sections were cut using a cryostat (Sakura Finetek, Tokyo, Japan), cooled to -20 °C, then dried for 2 h at room temperature. Frozen transverse sections were fixed in 4% paraformaldehyde for 15 min at 4 °C, then treated with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. Non-specific binding sites were blocked using 10% normal swine serum in PBS for 10 min. Sections were incubated with 1:100 monoclonal anti-dystrophin antibody (Sigma, St. Louis, MO, USA) for 30 h at 37 °C, followed by 1:600 goat anti-mouse Alexa Fluor 546 (Molecular Probes, Eugene, OR, USA) in PBS for 20 min at room temperature. After washing, BrdU was detected using a BrdU labeling and detection kit I (Roche Diagnostics, Tokyo, Japan). All nuclei were counterstained using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes). Sections were mounted in Gel/Mount (Cosmo Bio, Tokyo, Japan). Fluorescein signals in sections were observed and photographed under fluorescence microscopy (Keyence, Osaka, Japan). Levels of BrdU-positive cells inside the sarcolemma were determined.

Data are presented as mean ± standard deviation. Differences between groups were detected by one-way analysis of variance or Kruskal-Wallis analysis followed by Tukey's post hoc test, Bonferroni's post hoc test and Shirley-Williams post hoc test. For all tests, values of $P<0.05$ were considered statistically significant.

RESULTS

Volume of 2-week-old rat soleus muscle was insufficient for enzyme-linked immunosorbent assay, so gastrocnemius muscle was used. HGF levels in gastrocnemius muscle decreased significantly from 2 weeks to 4 weeks, then remained basically constant ($P<0.05$, Table 1). HGF level at 4 weeks was about one-third that at 2 weeks.

BrdU is absorbed during DNA synthesis, and dystrophin is a protein present inside the sarcolemma. Double-staining immunofluorescence showed localization of BrdU-positive nuclei and dystrophin-positive sarcolemma in soleus muscle (Fig. 1). These signals were clearly displayed. BrdU signals were seen to overlap with DAPI signals. BrdU-positive nuclei increased significantly in number with downhill running compared with controls ($P<0.05$, Table 2). HGF levels increased with downhill running, but not significantly ($P<0.06$, Table 2). Exercise training had no effect on body weight, muscle wet weight or the ratio of the two.

DISCUSSION

The number of satellite cells proliferating in soleus muscle is elevated after functional loading[15]. HGF levels were 3.2 ng/g tissue in soleus and 3.4 ng/g tissue in tibialis anterior muscle in 11-week-old rats[16]. In this study, HGF levels were 3.4-6.2 ng in gastrocnemius muscle at 4-24 weeks old. These data corresponded to data from recent studies. HGF protein levels in rat skeletal muscles were detected until 24 weeks old. HGF promotes the proliferation of adult myoblasts[17] and is present in adult tibialis anterior[13], soleus and plantaris muscles[18]. However, according to Northern blot analysis, HGF mRNA signals could be detected until 10 days after birth, but could not be detected in adult skeletal muscle[19]. Those results might support the notion that HGF level remains significantly high up to 2 weeks after birth. Increases in body and soleus

muscle during postnatal growth were not in accordance with rises in HGF levels. These findings suggest that HGF promotes satellite cell proliferation, but is unrelated to skeletal muscle fiber protein synthesis.

HGF level became basically constant by 4 weeks old. As a result, 4-week-old rats were used in this experiment with exercise training.

Satellite cells could be involved in the capacity for proliferation and differentiation, whereas muscle nuclei have no capacity to differentiate. Activated satellite cells as identified by BrdU-positive nuclei were noted inside the sarcolemma, as indicated by dystrophin signals. Recent studies have reported that mechanical stretching[13] and reloading after hindlimb suspension[18] stimulate satellite cell activation and production of HGF, and treadmill running stimulates satellite cell activation[3,20]. No recent studies have undertaken examination of satellite cells in association with differing exercise training. In this study, HGF level was not significantly increased by downhill running, tending to rise to a 1.25-fold increase, then returning to baseline. Numbers of BrdU-positive nuclei were significantly increased (2.4-fold) by downhill running. HGF is produced from inactive pro-HGF, and is transformed to active form by proteolytic processing[21]. Active-form HGF is present in the extracellular compartment of uninjured adult skeletal muscle in rats[22]. Satellite cells can be activated by binding of active HGF. These results may indicate that satellite cell activation is caused by liberation of active-form HGF from the extracellular compartment, rather than in an autocrine manner by self-production. Anew, a time lag may exist in HGF production. Further studies are thus needed to clarify the relationship between satellite cell activation and HGF production.

This study suggests that exercise intensity in excess of walking speed is required to activate satellite cells. This result may help in the development of exercise training intensities for optimal satellite cell activation to facilitate muscle strength training. After identifying such a threshold, studies will need to refer to running speed, frequency and time to apply this result to physical therapy.

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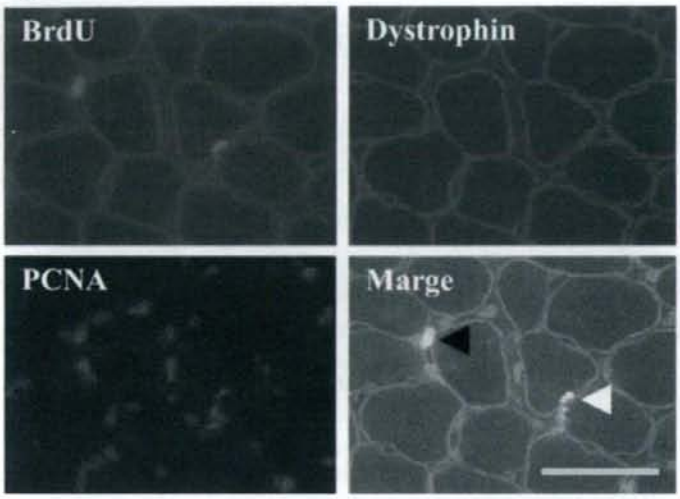
222
223 LEGENDS TO FIGURES
224 **Fig. 1** Localization of BrdU-positive nuclei in soleus muscle
225 BrdU-positive cells inside the basal membrane represent satellite cells during DNA
226 synthesis (white arrow); BrdU-positive cells outside the sarcolemma represent
227 non-muscle-derived cells (black arrow). Scale bar: 200 μm.

228

229 Figures

230 Fig. 1



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233 Tables

234 Table 1: Changes of HGF levels in gastrocnemius muscle during postnatal growth

	2 weeks (n=5)	4 weeks (n=5)	8 weeks (n=5)	16 weeks (n=5)	24 weeks (n=4)
HGF contents (ng/g tissue)	12.7±2.0	4.4±1.0 *	6.2±1.4 *	4.4±1.9 *	3.4±1.5 *

HGF protein contents are high at 2 weeks, then decrease by 4 weeks and remain unchanged from 4 to 24 weeks. *P < 0.05 vs. 2 weeks.

235

236 Table 2: Changes in number of satellite cells and HGF levels by exercise training in

237 soleus muscle.

	Control (n=5)	Downhill walking (n=4 or 6)	Downhill running (n=4 or 6)
BrdU-positive nuclei/1000fibers	3.8±1.5	4.1±1.3	9.2±0.4 **
HGF contents (ng/g tissue)	2.8±0.4	2.0±1.2	3.5±0.6 *

The number of BrdU-positive cells inside the sarcolemma is increased with downhill running. HGF level by downhill running tended to increase by around 25% compared with control, but this increase was not significant. **P < 0.05 vs. control. *P<0.06 vs. control.

238

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Identification and characterization of novel variants of the tryptophan 2,3-dioxygenase gene: differential regulation in the mouse nervous system during development

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Abbreviations used: TDO, tryptophan 2,3-dioxygenase; IDO, indoleamine 2,3-dioxygenase; Trp, tryptophan; 5-HT, 5-hydroxytryptamine or serotonin; Kyn, kynurenine; KYNA, kynurenic acid; QA, quinolinic acid; NMDA, N-methyl-D-aspartate; 3-OHKyn, 3-hydroxykynurenine

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Abstract

Tryptophan 2,3-dioxygenase (TDO), an initial and rate-limiting enzyme for the kynurenine pathway of tryptophan (Trp) metabolism, is thought to play an important role in systemic Trp metabolism as well as in emotional and psychiatric status. In contrast to its predominant expression in the liver, expression of TDO in the brain is poorly understood. Here, we show that *tdo* mRNA is expressed in various nervous tissues, including the hippocampus, cerebellum, striatum and brainstem. During development, *tdo* mRNA was differentially regulated in brain tissues. Further, we identified two novel variants of the *tdo* gene, termed *tdo* variant1 and variant2. Similar tetramer formation and enzymatic activity were obtained when these forms were expressed in wheat germ and COS-7 cells, respectively. Quantitative real-time RT-PCR revealed that *tdo* variants were expressed in various nervous tissues, with high expression in the cerebellum and hippocampus, followed by the midbrain. *Tdo* variant2 was the only variant expressed in the cerebellum from postnatal day 4 (P4) to P7, suggesting a unique role for this variant during early postnatal development. Our findings indicate that *tdo* and its novel variants may play an important role in not only the liver but also in local areas in developing and adult brain.

Keywords: tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase (IDO), cerebellum, hippocampus, serotonin (5-HT), kynurenine

1. Introduction

The metabolism of the amino acid L-tryptophan (L-Trp) is a highly regulated physiological process which leads to the generation of several neuroactive compounds within the central nervous system (CNS) (Peters, 1991). These include the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT); products of the kynurenine (Kyn) pathway of Trp metabolism, including 3-hydroxykynurenine (3-OHKyn), 3-hydroxyanthranilic acid, quinolinic acid (QA) and kynurenic acid (KYNA); and the neurohormone melatonin. Although the role of central serotonergic systems in modulating pathophysiology and behavior has long been known, the important physiological and behavioral consequences of kynurenine synthesis within the CNS have been only recently reported. Altered Kyn metabolism has been implicated in the pathophysiology of conditions such as Parkinson's disease, Huntington's disease and Alzheimer's disease (Németh et al., 2006). However, the molecular mechanisms regulating Kyn metabolism in the nervous system are not well understood, except the notion that indoleamine 2,3-dioxygenase (IDO), a first and rate-limiting enzyme for the Kyn pathway (conversion from Trp to Kyn) of Trp metabolism (Higuchi and Hayaishi, 1967; Hayaishi et al., 1993), plays a role in the immune response in the nervous system (Mellor and Munn, 2004; Takikawa, 2005). Tryptophan 2,3-dioxygenase (TDO) is thought to be the other first and rate-limiting enzyme for the Kyn pathway of Trp metabolism (Kotake and Masayama, 1937; Hayaishi et al., 1957; Maezono et al., 1990); although TDO is predominantly expressed in the liver, and is thus thought to contribute to systemic Trp metabolism, accumulating evidence suggests that TDO may play a local role in the brain, on the basis that TDO is expressed in SK-N-SH neuronal

cells and primary human brain neurons *in vitro*, and that *tdo* mRNA and TDO immunoreactivity are upregulated in postmortem anterior cingulate cortex tissue and white matter glial cells from individuals with schizophrenia, respectively (Guillemin et al., 2007; Miller et al., 2006).

To assess the role of TDO in the brain, we analyzed the expression of *tdo* mRNA in various nervous tissues in adult mice using Northern blotting. During the analyses of expression in these tissues, we identified two new mRNA transcripts, termed *tdo* variant1 and *tdo* variant2. We also assessed the expression and regulation of these genes in the adult and developing brain tissues.

2. Materials and methods

2.1. Experimental animals

Male C57BL/6 mice were purchased from Nippon SLC (Hamamatsu, Japan) and housed in groups of 3 or 4 per cage in a room with controlled light (12h light/dark cycle; lights on at 8 A.M.), humidity, and temperature, and allowed *ad libitum* access to food and water. Acquisition, care, housing, use, and disposition of the animals were in compliance with the institutional laws and regulations of Osaka University Graduate School of Medicine. All efforts were made to minimize both animal discomfort and the number of animals used.

2.2. RNA purification, Northern blot analysis, and quantitative real-time RT-PCR

The indicated brain regions (cerebral cortex, hippocampus, pons, striatum, midbrain, and cerebellum) were rapidly microdissected between 1:00 p.m and 7:00 p.m. and stored at -80 °C until analyses. Total RNAs were purified from tissues (liver and indicated brain regions) of postnatal day 1 to 13-week-old male mice using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For Northern blotting, 5 (liver) and 10 (brain) μg of total RNA were electrophoresed in 1% agarose/2.2 M formaldehyde/1 X MOPS gel, and blotted onto nylon filter membranes. The membranes were UV cross-linked (1.2 J/cm^2) and pre-hybridized with hybridization buffer (50% deionized formamide, 5 X SSC, 5 X Denhalt's solution, 0.1 mg/ml salmon sperm DNA and 1% SDS) at 42 °C for 2 h, and subsequently hybridized with hybridization buffer containing 10% dextran sulfate and full-length mouse *tdo* cDNA-labeled with [α-^{32}P]dATP (PerkinElmer) using a Megaprime DNA labeling kit (Amersham Biosciences). These filters were washed twice in 2 X SSC/0.1% SDS for 5 min at room temperature, and then in 0.2 X SSC/0.1% SDS for 30 min at 65 °C. The signals were detected with a BAS autoradiography image analyzer (Fujifilm, Japan).

First-strand cDNA was prepared from 5 μg of DNase I-treated total RNA using SuperScript III (Invitrogen) according to manufacturer's instructions. Quantitative real-time RT-PCR and quantitation of PCR product were performed using a previously described method (Nakamura K et al., 2006). Briefly, the expression of *tdo* genes was quantified using universal PCR master mix and TaqMan Gene Expression assays to amplify mouse *tdo* (FAM-labeled, exons 4 and 5, Mm00451266_m1; exons 1 and 2, Mm00451263_g1) and rodent *GAPDH* (Taq-Man rodent GAPDH control reagents, VIC probe; Applied Biosystems). The expression of *tdo variants* was quantified using Power SYBR Green PCR master mix and the following primers: *tdo* variant1, 5'-GCACTAAAGTATCTGGGAAGG-*3*' and 5'-CTCCTTTGCTGGCTCTGTTT-*3*'; *tdo* variant2,

5'-TGTAAGCTGGGTGCTGATTG-3' and 5'-GTGTATCTTTTATGTATCCTG-3'. Real-time RT-PCR was carried out with an Applied Biosystems 7900HT Fast real-time PCR system under the following conditions: 50 °C for 2 min, 95 °C for 10 min, then 50 cycles of PCR (95 °C for 15 sec; 60 °C for 1 min). Results were expressed as the mean ± S.E of duplicate replicates.

2.3. cDNA cloning of mouse tdo variants

cDNAs were synthesized from adult C57BL/6 mice midbrain and liver total RNA using oligo $d(T)_{20}$ primer and superscript III reverse transcriptase. Amplification of mouse *tdo variants* cDNA was done using TaqMan AmpliTaq Gold Master Mix and the following primer set: forward primer, 5'-ATCTCTCTCTCCCTCTACTTC-3', and reverse primer, 5'-TCAATCCGATTCATCGCTGC-3'. These primer sequences were designed based on the rat *tdo* minor start site (Schmid et al., 1982). Amplified cDNA fragments (about 1600 bp and 1560 bp) were subcloned into pGEM-T easy vector (Promega), which was subjected to sequence analyses.

2.4. RNase protection assay (RPA)

RPAs were performed using a RPAII Ribonuclease Protection Assay Kit (Ambion, TX) as described previously (Funakoshi et al., 1993, 1995). The anti-sense cRNA probe specific for *tdo* was generated in the presence of [α-^{32}P]UTP (PerkinElmer) from a linearized plasmid containing the 419 bp fragment covering nucleotides –277 – +142 of mouse *tdo* (NCBI accession number: NT_039240). The sizes of protected bands encoding full-length *tdo*, *tdo* variant1, and *tdo* variant2 were 176, 453, and 233/106 bps, respectively.

2.5. Construction of tdo, tdo variant1, tdo variant2, and tdo-HA expression vectors

To prepare expression vectors, the entire coding cDNA regions of *tdo*, *tdo* variant1, *tdo* variant2, and *hemagglutinin (HA)*-tagged *tdo (tdo-HA)* were amplified from mouse (C57BL/6) adult liver total RNA as a template using TaqMan AmpliTaq Gold Master Mix and primers (*tdo* forward, 5'-ATGAGTGGGTGCCCGTTTGC-3'; *tdo* variant forward, 5'- ATGCCATTCTCTTTAGGAT-3'; *tdo* reverse, 5'-TCAATCCGATTCATCGCTGC-3'; *tdo-HA* reverse/inside, 5'-ATCTGGAACATCGTATGGGTAATCCGATTCATCGCTGCTGAA-3'; *tdo-HA* reverse/outside, 5'- CCATACGATGTTCCAGATTACGCTTAAGCGGCCGCTAAACTAT-3') and cloned into pGEM-T easy vector. The *Eco*RI-digested cDNAs were inserted into the *Eco*RI site of the pTNT vector (Promega, WI) for a wheat germ system and the pCAGGS vector (the kind gift of Dr. J. Miyazaki, Osaka University Graduate School of Medicine, Japan) for COS-7 expression. The correct orientation and integrity of all constructs was verified by sequencing.

2.6. Cell-free transcription/translation of TDO and variants using a wheat germ system

TDO-HA, TDO variant1, and TDO variant2 were expressed *in vitro* using a wheat germ system (TNT SP6 High Yield Protein Expression, Promega) according to the manufacturer's instructions. Each reaction (50μl) contained 10 μg of pTNT/TDO-HA, pTNT/TDO variant1, and pTNT/TDO variant2 plasmid DNA. Reactions were performed at 25 °C for 120 min.

2.7. Cell culture and transfection

African green monkey kidney cells (COS-7) were maintained in low-glucose DMEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum at 37 °C in 5% CO_2 as previously described (Funakoshi and Nakamura, 2001). The cells were transiently transfected with pCAGGS-TDO, -TDO variant1, or -TDO variant2 using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

2.8. Western blotting

At 48 h post-transfection with pCAGGS-TDO, -TDO variant1, or –TDO variant2, COS-7 cells were lysed in buffer containing 2 mM Trp, 2 mM hemoglobin, and proteinase inhibitor cocktails (Complete Mini Protease Inhibitor Cocktail; Roche Diagnostics, Germany); frozen and thawed twice; sonicated; and then incubated on ice for 30 min. After centrifugation at 20,000 X *g* for 30 min at 4 °C, supernatants were separated by native or SDS polyacrylamide gel electrophoresis (Native-PAGE or SDS-PAGE) using a 4% to 20% gradient gel and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, CA). The membrane was incubated with non-fat dry milk in phosphate-buffered saline for 2 h at room temperature, immunoreacted with rabbit anti-TDO antisera (1:2,000, Niimi et al., 1983) at 4 °C overnight and subsequently labeled with HRP-conjugated antibody against rabbit immunoglobulin (1:2,000, DAKO, Denmark) for 1 h at room temperature. The resulting signals were visualized using an enhanced chemiluminescence immunoblotting detection reagent (GE Healthcare Bio-Science Corp., NJ). Densitometric quantitation of ECL immunoblots was performed using a high-resolution scanner and the Image J 1.36 program (developed by W. S. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD).

2.9. Analysis of TDO tetramer formation

TDO-HA and its variant proteins synthesized from wheat germ extracts were incubated in 2 mM Trp, 2 mM hemoglobin, and proteinase inhibitor cocktails at 37 °C for 10 min. These samples were separated by Native-PAGE (pH 7.8) using a 4% to 20% gradient Tris-acrylamide gel. Subsequent treatment was as described above.

2.10. TDO enzymatic activity

TDO enzymatic activities were assessed by the conversion of Trp to Kyn as previously described (Seglen et al., 1969) with slight modifications (Nakamura et al., 1980). We took special care to keep solutions at 4 °C at all steps except where indicated. Briefly, transfected cells (48 h after transfection) were harvested in 0.02 M phosphate buffer (pH 7.4) containing 2 mM Trp and protease inhibitor cocktails. Lysates were frozen and thawed twice, sonicated, and then incubated on ice for 30 min. After centrifugation at 20,000 X *g* for 30 min at 4 °C, the supernatants were incubated at 37 °C in 2.5 volume of 0.02 M phosphate buffer (pH 7.4) containing 2 mM Trp and 2 mM hemoglobin. Reactions were stopped with the addition of saturated trichloroacetic acid (final 10%). After centrifugation at 20,000 X *g* for 30 min at 4 °C to remove proteins, the supernatants were filtered and immediately_neutralized with NaOH. The amount of Kyn formed during incubation was

measured spectrophotometrically in the neutralized supernatant at 365 nm. A non-enzymatic blank was included as a control for all the enzymatic assays to exclude the factors modified by TCA. TDO specific activity was expressed as units (μmoles of Kyn formed per hour at 37 °C) per milligram of each protein. The amounts of each protein (TDO and its variants) were determined by Western blotting using recombinant TDO protein as a standard (Genway Biotech, CA). The supernatants revealed a Kyn spectrum comparable to a standard solution of Kyn. Statistical analysis was carried out using StatView software version 5.0.1 (SAS Institute, Cary, NC) with statistically significant differences among the three groups assessed by ANOVA followed by Fisher's PLSD test (post hoc test). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Expression of tdo mRNAs in the nervous system

Although TDO is predominantly expressed in the liver, several reports also suggest expression in the brain (Haber et al., 1993; Miller et al., 2006; Guillemin et al., 2007). To explore the expression of *tdo* mRNA in the nervous system, Northern blot analysis was carried out using a probe covering entire cDNA region of the *tdo* gene. Short-exposure film revealed the expression of *tdo* mRNAs in the hippocampus and cerebellum (Fig. 1A, upper panel, arrow) as well as liver (Fig. 1A, upper panel; Inoue et al., 1989) and long-exposure film revealed expression in the cerebral cortex. In addition, it is of note that a shorter length transcript of *tdo* mRNA was detected in the brainstem, suggesting the presence of novel *tdo* variant(s) (Fig. 1A, lower panel, arrowheads). To further assess the expression of *tdo* mRNAs, quantitative real-time RT-PCR was performed. Levels much lower than in the liver but still significant levels were detected in various regions of the brain, including the hippocampus, cerebellum, and midbrain. However, some of the tissues examined showed a more than 10-fold difference in expression when different primer sets (one primer set covered exon 1 and 2, and the second covered exon 4 and 5) were used. These findings demonstrate that *tdo* mRNA is indeed expressed in various brain tissues and raise the possibility of the presence of a novel variant form(s) lacking the part of exon 1 and 2 of the *tdo* gene in such tissues (Fig. 1B).

3.2. Identification of novel variants of the tdo gene

Based on the above findings, we predicted the possible presence of novel variant(s) in the nervous system. We used various primer sets to amplify novel variant(s) of the *tdo* gene from the brainstem as well as the liver, where *tdo* mRNA is highest. Two *tdo* mRNA fragments of different size were amplified from the brainstem as well as the liver using one of the primer sets. The forward primer used was designed based on the finding of the presence of two alternative start sites by Schmid *et al.* (Schmid et al., 1982), one of which corresponded to the upstream of the rat *tdo* transcription start site reported by Maezono *et al.* (Maezono et al., 1990). The reverse primer covered a sequence located at a stop codon of mouse *tdo* (NCBI accession number: NT_039240). Subcloning of the amplified bands into pGEM-T easy vector and subsequent repetitive sequence analyses revealed that both contained a large part of mouse *tdo* sequence, indicating that both are novel variant forms of the *tdo* gene. One is a longer form, which contains exon0a+b of the *tdo* gene

(termed mouse *tdo* variant1; Fig. 2A and B), while the second shorter form contains exon0a but lacks exon 1 of the *tdo* gene (termed mouse *tdo* variant2; Fig. 2A and B). We also identified rat *tdo* variant1 and variant2 (Kanai et al., unpublished result). To confirm the presence of all mRNAs, we performed RNase Protection Assays (RPAs) using the cRNA probe covering nucleotides –277 – +142 of mouse *tdo*. RPA identified two forms of the *tdo* gene in the liver as well as cerebellum: *tdo* full-form and *tdo* variant2 (Fig. 2C). Quantitative real-time RT-PCR using specific primer sets for *tdo* variants showed that both variants were expressed in various regions of the brain, with high expression in the hippocampus and cerebellum, followed by the cerebral cortex, striatum, brainstem and pons (Fig. 2D).

3.3. Differential regulation of tdo, tdo variant1 and tdo variant2 mRNAs during postnatal development in the nervous system

Given that *tdo* mRNA in the liver increases postnatally, TDO may play an important role in systemic Trp metabolism during postnatal development and in the adult (Franz and Knox, 1967; Killewich and Feigelson, 1977; Nagao et al., 1986). To assess the role of *tdo* and its variants in the nervous system during postnatal development, we analyzed the expression of *tdo*, *tdo* variant1 and *tdo* variant2 mRNAs in various brain tissues from postnatal day 1 (P1) to P49. Quantitative real-time RT-PCR using the primer set covering exon 4 and 5, and thus reflecting the expression of *tdo* full-form (*tdo*), revealed that *tdo* was expressed in the liver from P1 and gradually increased during postnatal development and became about 500-fold higher level at P49 compared with P1. In a similar manner, the level of *tdo* mRNA gradually increased in the hippocampus from P1 to P49, while it increased from P7 to P14 and became almost same level as P49 in the cerebellum (Fig. 3A). In contrast to *tdo* mRNA, the level of *tdo* variant1 mRNA was extremely low until P14 in all tissues examined. The level of *tdo* variant1 mRNA markedly increased from P14 to P21 in all tissues examined (Fig. 3B). The level of *tdo* variant2 mRNA in the liver increased from P1 to P49, while the mRNA level in the hippocampus was continuously low during postnatal development. The mRNA level of *tdo* variant2 was low at P1 but sustained at high levels from P4 to P49 in the cerebellum (Fig. 3C).

3.4. Tetramer formation and enzymatic activity of proteins for tdo, tdo variant1 and tdo variant2

Given that tetramer formation is essential to the enzymatic activity of TDO protein (Schutz et al., 1972), we next examined whether each protein for *tdo*, *tdo* variant1 and *tdo* variant2 could form tetramer. The respective proteins of *tdo* and its variants were synthesized from wheat germ extracts, and then incubated in the presence of Trp, hemoglobin, and proteinase inhibitor cocktails at 37 °C for 10 min. The samples were subsequently separated by Native-PAGE (pH 7.8) under non-reducing conditions and tetramer formation of TDO proteins was visualized by Western blotting using TDO-specific antiserum. Results showed that all TDO proteins have the high molecular size of the tetramer of the respective protein (Fig. 4A, upper panel). We then examined whether the protein for *tdo* and its variants retain enzymatic activity of TDO. At 48 h post-transfection of the expression vectors pCAGGS-TDO, -TDO variant1, and -TDO variant2 into COS-7 cells, the cells were lysed in lysis buffer containing protease inhibitor cocktails and parts of the resultant protein extracts were

subjected to SDS-PAGE and for the enzymatic activity assays of TDO. The TDO and TDO variant1 were of identical of molecular size, suggesting that the start site of transcription did not differ between *tdo* and *tdo* variant1. In contrast, a small difference in molecular size was detected between TDO variant1 and TDO variant2, which may indicate that the start site of *tdo* variant2 is located in exon 2 (Fig. 4B, upper panel). The proteins for *tdo*, *tdo* variant1 and *tdo* variant2 showed a similar specific enzymatic activity (Fig. 4B, lower panel).

4. Discussion

In this study, we quantitatively examined the expression of TDO in various brain tissues. Although lower than those in the liver, levels of *tdo* mRNAs in the brain were still substantial, with high expression in the hippocampus and cerebellum, followed by the cerebral cortex, striatum and midbrain. Expression was differentially regulated in various regions of the brain during development. Further, we also identified novel variants of the *tdo* gene, termed *tdo* variant1 and *tdo* variant2, which were differentially expressed and regulated in neural tissues during development and in the adult mouse. To our knowledge, this study is the first to quantitatively demonstrate the expression of TDO in various brain tissues.

Using Western blot analysis, Miller *et al.* described the presence of two as yet uncharacterized TDO proteins of different sizes in the cerebral cortex of patients with schizophrenia (Miller et al., 2004, 2006). In the present study, we identified novel variants of the *tdo* gene, one of which was smaller than TDO when expressed in COS-7 cells (Fig. 4B). In addition, two TDO proteins with slightly different sizes in Western blotting, which well correspond to our Western blot result, have been reported (Ren and Correia, 2000). The TDO variant2 protein may partly account for the size difference in these TDO proteins.

What is the role of TDO proteins in the nervous system? Metabolites/catabolites of the Trp metabolic pathway, which is modulated by TDO, include a variety of substances important to the function of the nervous system. Roles for the catabolite 5-HT have been postulated in mood, for example, such as in depression, and for melatonin in circadian rhythm. The metabolite KYNA, an endogenous N-methyl-D-aspartate (NMDA) receptor antagonist, plays a neuroprotective role, while 3-OHKyn and QA are neurotoxic, and an imbalance among them has been postulated to play a role in psychopathology and neurodegenerative diseases, such as Huntington's disease (Hilmas et al., 2001; Németh et al., 2006; Barry et al., 2008). QA is accumulated at the local site of spinal cord injury in guinea pigs, while systemic administration of a QA productive enzyme inhibitor (4-chloro-3-hydroxyanthranilate) reduces this accumulation and decreases the severity of secondary functional deficits, suggesting the importance of Trp (Kyn) metabolism at local sites of the nervous system (Yates et al., 2006). The expression pattern of *tdo* in brain tissue in our present study correlates well with that of KYNA, namely a high level of mRNA for *tdo* and its variants in the hippocampus and cerebellum, followed by the brainstem and cerebral cortex (Figs. 1 and 2). In a similar manner, the highest level of KYNA in the cerebellum, followed by the brainstem and cerebral cortex (Fukushima et al., 2007), demonstrates a significant role for locally produced TDO in various regions of the brain in these nervous system events, as well as for the protein(s) for *tdo*

variant1 and variant2. Taken together with the notion that *tdo* variants are expressed in the liver, our findings suggest that *tdo* and novel *tdo* variants in the liver play a role(s) in systemic Trp (Kyn) metabolism, but also act locally in the adult nervous system.

A role for *tdo* genes in postnatal development can be also postulated (Fig. 3). In particular, a specific role for *tdo* variant2 in the cerebellum during early postnatal development is envisaged by the finding that it was the only variant expressed in the cerebellum from P4 to P7. The finding that a Trp-deficient corn-based diet started 5 weeks before mating retards the maturation of Bergmann glial cells and migration of granule cells in the rat cerebellum (Del Angel-Meza et al., 2001) suggests that locally produced TDO variant2 in the cerebellum in early postnatal development plays a role in modulating the maturation and migration of developing cerebellar cells. Given that *tdo* is continuously expressed in the cerebellum at high levels during late postnatal development and that pathological changes have been shown in the postmortem cerebellum of patients with autism (Amara et al., 2008), the finding of an association of *tdo* gene polymorphism with autism is intriguing (Nabi et al., 2004).

Our findings demonstrate that *tdo* and novel *tdo* variants in the liver play a role(s) in systemic Trp (Kyn) metabolism, but also act locally in the adult nervous system, where they are important to the development, maturation, and maintenance of the nervous system. Elucidation of the roles of novel variants may shed further light on the roles of TDO.

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Figure Legends

Fig. 1. Expression of *tryptophan 2,3-dioxygenase (tdo)* mRNA in the brain. (A) Northern blot analysis for mouse *tdo* mRNA. Total RNA in the brain regions (10 μg) were hybridized with a specific probe against mouse *tdo* coding region. Arrow, full-form *tdo*. Arrowheads, variants of *tdo* (B) Quantitative real-time RT-PCR for *tdo* mRNA in the adult brain. Mouse *tdo/gapdh* of adult liver was assigned an arbitrary value of 10^6. Values represent means ± S.E. (n = 5 in each group). CBX (cerebral cortex); HIP (hippocampus); STR (striatum); CBL (cerebellum); MID (midbrain); and LIV (liver).

Fig. 2. Identification of novel variants for the *tdo* gene. (A) Sequence of the 5' end region of the mouse *tdo* gene. Exons (boldface type); exon0b (underline); TATA sequence (boxed); CAAT sequence (dashed box); start codon (dashed underline); and predicted DNA binding site of glucocorticoid-receptor protein (double underline; Schmid et al., 1982). (B) Schematic diagram of the gene structure of mouse *tdo* variants. Coding regions are indicated by black boxes. (C) RNase protection assay of *tdo* mRNAs in adult tissues. *tdo* full-form (arrowhead); and *tdo* variant2 (arrow). (D) Quantitative real-time RT-PCR for *tdo* variants in the adult brain. Mouse *tdo* variants of adult liver were assigned an arbitrary value of 10^6. Values represent means ± S.E. (n = 5 in each group). CBX (cerebral cortex); HIP (hippocampus); STR (striatum); CBL (cerebellum); MID (midbrain); and LIV (liver).

Fig. 3. Regulation of the expression of *tdo* mRNAs in the hippocampus, cerebellum, and liver during development. Quantitative real-time RT-PCR for *tdo* exon 4-5 mRNA (A), *tdo* variant1 (B), and *tdo* variant2 (C). Total RNA (250 ng) for postnatal day 1 (P1) to P49 was reacted. *tdo*, *tdo* variant1, and *tdo* variant2 of the liver at P49 was assigned an arbitrary value of 10^6, 60, and 10^4, respectively. Values represent means ± S.E. (n = 5 in each group).

Fig. 4. Characterization of TDO variants. (A) Tetramer formation of TDO variants using wheat germ *in vitro* translation. (B) Enzymatic activity for TDO variants using transiently transfected COS-7 cells. V1 (TDO variant 1); V2 (TDO variant 2). Values represent means ± S.E. (n = 5 in each group).

Figure 1. Kanai *et al*.