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MEETING REPORT

Implication of Tryptophan 2,3-Dioxygenase and its Novel Variants in the Hippocampus and Cerebellum During the Developing and Adult Brain

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Abstract: Tryptophan 2,3-dioxygenase (TDO) is a first and rate-limiting enzyme for the kynurenine pathway of tryptophan metabolism. Using *Tdo*$^{-/-}$ mice, we have recently shown that TDO plays a pivotal role in systemic tryptophan metabolism and brain serotonin synthesis as well as emotional status and adult neurogenesis. However, the expression of TDO in the brain has not yet been well characterized, in contrast to its predominant expression in the liver. To further examine the possible role of local TDO in the brain, we quantified the levels of *tdo* mRNA in various nervous tissues, using Northern blot and quantitative real-time RT-PCR. Higher levels of *tdo* mRNA expression were detected in the cerebellum and hippocampus. We also identified two novel variants of the *tdo* gene, termed *tdo* variant1 and variant2, in the brain. Similar to the known TDO form (TDO full-form), tetramer formation and enzymatic activity were obtained when these variant forms were expressed *in vitro*. While quantitative real-time RT-PCR revealed that the tissue distribution of these variants was similar to that of *tdo* full-form, the expression patterns of these variants during early postnatal development in the hippocampus and cerebellum differed. Our findings indicate that in addition to hepatic TDO, TDO and its variants in the brain might function in the developing and adult nervous system. Given the previously reported associations of *tdo* gene polymorphisms in the patients with autism and Tourette syndrome, the expression of TDO in the brain suggests the possible influence of TDO on psychiatric status. Potential functions of TDOs in the cerebellum, hippocampus and cerebral cortex under physiological and pathological conditions are discussed.

Keywords: tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase (IDO), kynurenines, autism

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Introduction

Tryptophan and its metabolites, such as kynurenines and serotonin, are involved in biological functions, such as protein synthesis, energy synthesis, immune regulation, and higher-order brain functioning.[1–4] The mechanisms of mood modulation and disturbance that contribute to social functioning have become a particularly important issue in the 21st century. Identifying the mechanisms regulating tryptophan and its metabolites in the brain represents a key issue for not only understanding the mechanisms regulating emotion but also alleviating social problems. In contrast to the systemic regulation of tryptophan metabolism, molecular mechanisms and local factors regulating tryptophan (kynurenine) metabolism in the brain are not well understood. There is evidence that, in the brain, indoleamine 2,3-dioxygenase (IDO),[5–9] an initial and rate-limiting enzyme for the kynurenine pathway, is markedly induced by immune signals (including interferon-gamma) and that IDO plays an important role in kynurenine production and immune response under pathological conditions *in vivo* and *in vitro*.[6–11] In contrast, under physiological conditions, other molecule(s) are likely to play a key role in regulating kynurenine metabolism of the adult brain, as IDO levels in the adult nervous systems are extremely low.

A suitable candidate is tryptophan 2,3-dioxygenase (TDO), which is another initial and rate-limiting enzyme for the kynurenine pathway of tryptophan metabolism.[1–3,12–15] We have previously studied *tdo*-deficient (*Tdo*$^{-/-}$) mice and have demonstrated that TDO plays important roles in systemic tryptophan metabolism and brain serotonin synthesis as well as modulation of emotional status and adult neurogenesis.[16] In support of these findings, some genetic studies have demonstrated an association of human TDO2 single nucleotide polymorphisms (SNPs) with psychiatric diseases, such as Tourette syndrome and autism.[17,18] Moreover, antidepressants have been postulated to act by directly inhibiting the activity of hepatic TDO, thereby enhancing the availability of cerebral tryptophan.[19,20] These findings demonstrate that TDO play an important role not only in systemic tryptophan metabolism but also in mood regulation and psychiatric disease. However, although TDO is predominantly expressed in the liver and a few studies have suggested that *tdo* is expressed in the rodent or human brain,[11,21,22] the precise distribution and regulation of local TDO in the brain has not yet been well characterized.

Here, we describe the identification, localization and regulation of novel *tdo* variants, which we termed *tdo* variant1 and *tdo* variant2, in addition to *tdo* full-form, during brain development, and in the brain and liver of adult mice. The potential roles of TDOs in the brain under physiological and pathophysiological conditions are also discussed.

Materials and Methods

Animal

Male C57BL/6J mice were purchased from Nippon SLC (Hamamatsu, Japan) and maintained as previously described.[23] 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.

cDNA cloning of mouse *tdo* variants

cDNA cloning of mouse *tdo* variants was performed as previously decribed.[23] Briefly, cDNAs were synthesized from adult C57BL/6J 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′-TCAATCCGATTCATCGCT GC-3′. These primer sequences were designed based on the rat *tdo* minor start site.[24] Amplified cDNA fragments (about 1600 bp and 1560 bp) were subcloned into pGEM-T easy vector (Promega), which was subjected to sequence analyses.

Northern blotting

For Northern blotting, 20 μg of polyadenylated RNAs, collected from the liver of 13-week-old male mice, was electrophoresed in 1% agarose/2.2 M formaldehyde/1X MOPS gel, and blotted onto nylon filter membranes. The membranes were UV cross-linked, pre-hybridized with hybridization buffer (50% deionized formamide, 5X SSC, 5X 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 probes covering the entire

region of full-length (36 ~ 1256), exon 0 (–400 ~ –15), or exon 1 (1~70) of mouse *tdo* cDNA-labeled with [α-^{32}P] dCTP, washed twice in 2X SSC/0.1% SDS at room temperature, and then in 0.2X SSC/0.1% SDS for 30 min at 4 °C as previously described.[23] The signals were detected with a BAS 5000 autoradiography image analyzer (Fujifilm, Japan).

Quantitative real-time RT-PCR

Total RNAs were purified from tissues (liver, cerebral cortex, hippocampus, pons, striatum, midbrain, and cerebellum) of postnatal day 1 to 49. First-strand cDNA was prepared from 5 μg of DNase I-treated total RNA; quantitative real-time RT-PCR and quantitation of the *tdo* genes were performed as previously described.[23] Briefly, the expression of *tdo* genes was quantified using universal PCR master mix and TaqMan Gene Expression assays to amplify mouse *tdo* exons 4–5 (thus amplifying full-length *tdo* as well as *tdo* variant1 and variant2) and rodent GAPDH. The expression of *tdo* variants was quantified using Power SYBR Green PCR master mix and the following primer sets: *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 as described.[23] Since the expression levels of *tdo* full-form were much higher than those of *tdo* variant1 and variant2 in all indicated samples examined (for example, the levels of *tdo* variant1 and varinat2 were about 1% and 0.01% compared to those of *tdo* full-form in the liver, respectively), calculated values of *tdo* exon 4–5 (reflecting the sum of *tdo* full-form, *tdo* varinat1 and *tdo* variant2) are nearly identical to the levels of *tdo* full-form, and thus the values are expressed as the levels of *tdo* full-form. Results were expressed as the means ± S.E. of duplicate replicates.

TDO enzymatic activity

At 48 h post-Transfection with pCAGGS-*tdo*, -*tdo* variant1, or -*tdo* variant2, COS-7 cells were lysed in 0.02 M phosphate buffer (pH 7.4) containing 2 mM Trp and proteinase inhibitor cocktails (Roche Diagnostics, Germany).[23] TDO enzymatic activities were assessed by the conversion of Trp to Kyn as previously described.[23] TDO specific activities were 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 (Geneway Biotech, CA).[23] Values represent means ± S.E. (n = 5 in each group).

Results

Identification and expression of *tdo* variant1 and variant2 mRNAs, in addition to *tdo* full-form in various nervous tissues

We first performed northern blot analyses using a full-length *tdo* cDNA probe to visualize all *tdo* transcripts in various brain tissues. Liver total RNA served as a positive control for northern blot analysis. Long exposure film revealed that, in addition to the full-form size band, lower and higher bands were detected in the brainstem, hippocampus, cerebellum, and liver (see Fig. 1A in ref. 23). These results suggested that novel variants of the *tdo* gene were present.

To further examine brain *tdo* mRNA expression, we next performed quantitative real-time RT-PCR, using two primer sets, *tdo* exon 1–2 and exon 4–5, in order to detect different parts of *tdo* mRNA. Although lower than in the liver, significant levels of *tdo* mRNAs were detected in various brain regions, including the cerebral cortex, striatum, pons, and midbrain. Consistent with our northern blot findings, high expression levels of *tdo* were detected in the hippocampus and cerebellum. Interestingly, more than a 10-fold difference was detected in the expression levels of *tdo* between exon 1–2 and exon 4–5 in the cerebral cortex, striatum, cerebellum, pons, and midbrain (see Fig. 1B in ref. 23). These findings demonstrate that novel variant(s) lacking these regions of exons 1 and 2 of the *tdo* gene are present in the brain.

After additional analyses, we identified three forms of *tdo* mRNAs (Fig. 1; see details in ref. 23). One is a full-length form corresponding to a traditional *tdo* containing 12 exons. The second is a longer form that contains exon 0a + b of the *tdo* gene, which we termed *tdo* variant1.[23] The third is a shorter form that contains exon 0a but lacks exon 1, which we termed *tdo* variant2.[23] To assess the expression of *tdo* variants, we performed northern blot analyses using probes covering different parts of the *tdo* gene. One is a probe covering full-length *tdo* cDNA, which detects all three forms of *tdo* genes in Figure 1. Second is a probe

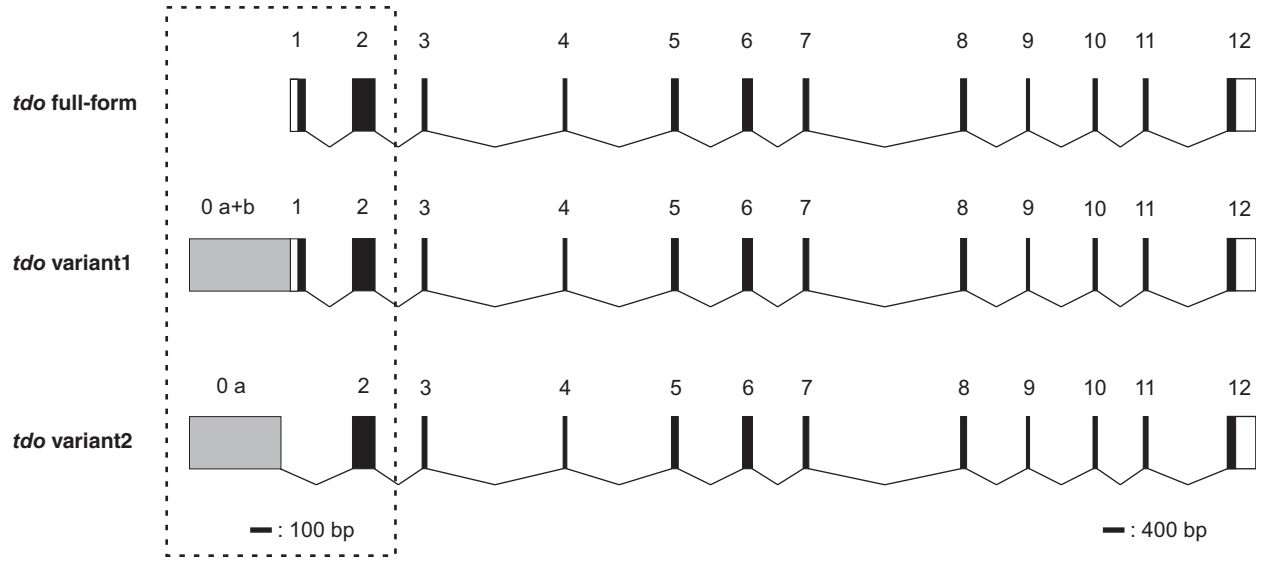

Figure 1. Schematic structure of the different forms of *tdo*. The top is a full-length form corresponding to a traditional *tdo*, which contains 12 exons. The middle is a longer form containing exon 0a + b of the *tdo* gene, which we termed *tdo* variant1. The bottom is a shorter form containing exon 0a but lacking exon 1, which we termed *tdo* variant2. Coding regions are indicated by black boxes.

covering *tdo* exon 0 (–400 ~ –15), and thus detects *tdo* variant1 and variant2 but does not detect full-length *tdo*. The last is a probe covering *tdo* exon 1 (1–70) and thus detects both full-length *tdo* and *tdo* variant1. As previously described in the brain tissues and the liver (ref. 23), northern blotting using a probe covering full-length *tdo* revealed that at least three *tdo* mRNAs are present in the liver (Fig. 2A). Using a probe covering exon 0, we found that *tdo* mRNA containing exon 0 was detected at low levels and its size was nearly identical to the full-length *tdo* mRNA in the liver. As both *tdo* variants contain a part of or whole exon 0, we cannot distinguish which variant (or a new variant) is responsible for the band in Figure 2B. But our findings clearly demonstrate that one of *tdo* variants is present and its mRNA size is close to that of full-length *tdo* (Fig. 2B, arrows). Using a probe covering exon 1, we detected three bands as indicated in Figure 2C. We speculate that additional (new) *tdo* variant(s) containing exon 1 would be present, as we identified two extra mRNA bands containing exon 1 in addition to the band correspond to the full-length *tdo* in Figure 2C.

Next, to assess the expression of *tdo* mRNAs in brain tissues, we performed quantitative RT-PCR. It revealed that both *tdo* variant1 and variant2 were expressed in various brain regions, and a relatively high expression of these variants was detected in the hippocampus and cerebellum, in addition to the *tdo* full-form, as previously described (see Fig. 2 in ref. 23). These results suggest that *tdo* and its two novel variants are expressed in various brain regions, with particularly high levels seen in the hippocampus and cerebellum.

Regulation of *tdo* mRNA expression in the hippocampus and cerebellum during development

To clarify the regulation of *tdo* mRNAs in the hippocampus during development, we examined the expression of *tdo* mRNAs in the hippocampus from postnatal day1 (P1) to P49.[23] Quantitative RT-PCR revealed that

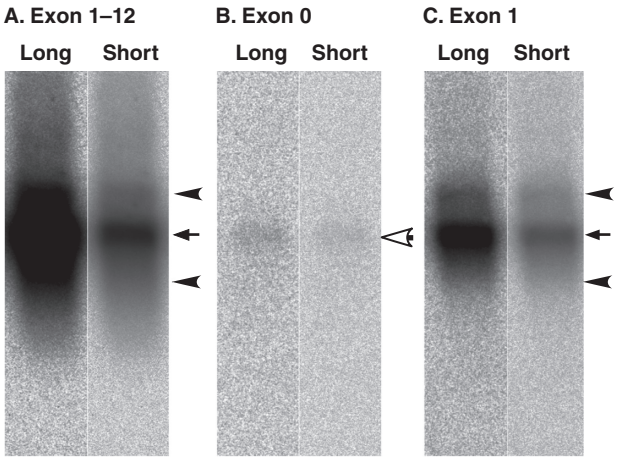

Figure 2. Expression of *tdo* mRNAs in the liver. Polyadenylated RNAs in the liver (20 μg) were hybridized with a specific probe against mouse *tdo* probes covering *tdo* full-form (exon 1 to exon 12) (**A**), exon 0 region (**B**), and exon 1 (**C**). Black arrow, full-form *tdo*. White arrow, *tdo variant* containing exon 0 region. Arrowheads, variants of *tdo*.
Abbreviations: Long, long exposure; Short, short exposure.

the levels of *tdo* full-form were high from P4, when the organization of neural networks begins, and were maintained at the same levels until P49 (levels of *tdo* full-form were calculated from the values of exon 4–5; see "Materials and Methods").[23] While expression levels of *tdo* variants were lower than the full-form, expression levels of *tdo* variants gradually increased after P14 in the hippocampus (Fig. 3A).[23] In the cerebellum, levels of *tdo* full-form were markedly increased at P14, and maintained at the same level until P49. In comparison, levels of *tdo* variant2 were low at P1, but were sustained at high levels from P4 to P49 (Fig. 3B).[23]

TDO variant1 and variant2 are functional proteins with full-TDO enzymatic activity

We synthesized each protein of TDO and its variants in COS-7 cell lines and analyzed its cell lysates by Western blotting using the antibody generated against

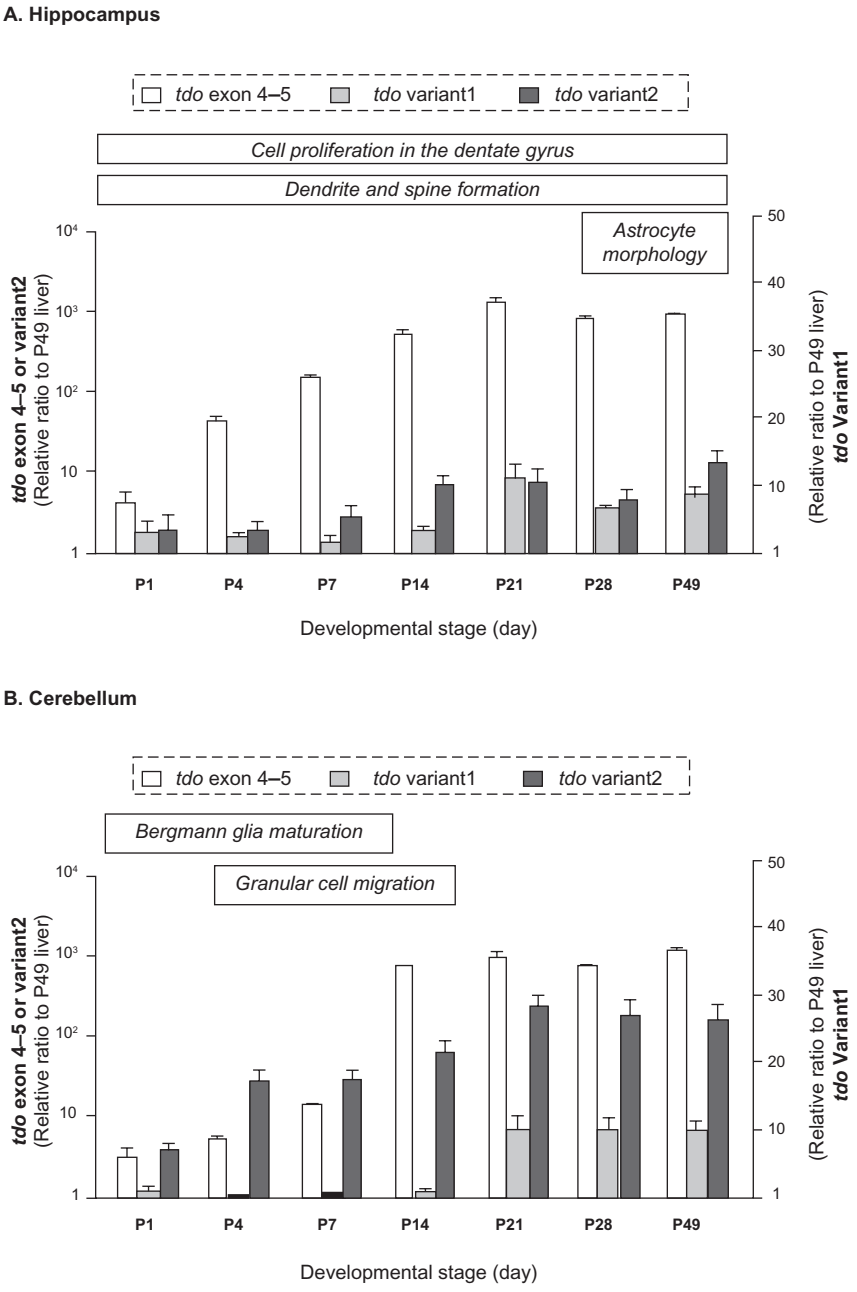

Figure 3. Regulation of the expression of *tdo* mRNAs in the hippocampus and cerebellum during development. Quantitative real-time RT-PCR was performed for *tdo* exon 4–5, *tdo* variant1, and *tdo* variant2. Total RNAs for postnatal day 1 (P1) to P49 in the hippocampus (**A**) and cerebellum (**B**) were examined. The level of *tdo* exon 4–5, *tdo* variant1, and *tdo* variant2 in 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).

rat purified TDO that can also detect mouse TDO.[23] Western blotting of cell lysates revealed that the molecular weight of mouse TDO full-form and that of TDO variant1 was identical.[23] In contrast, the molecular size of variant2 was slightly lower than that of the other forms, which fits well with the notion that translation of *tdo* variant1 would begin from exon 1, and that of variant2 would begin from exon 2, as there is no theoretical translation start site in exon 0.

We further examined whether recombinant TDO variants form tetramer, since native rat and human TDO full-form proteins form a tetramer with heme, and tetramer formation is required for TDO full enzymatic activity.[25] Western blotting showed that recombinant mouse TDO full-form migrated as a 44 kDa band at the monomer size in SDS-PAGE, while in Native-PAGE it migrated as an approximately 180 kDa band, which was 4 times higher than monomer size, indicating that it forms tetramer. TDO variant1 and variant2 were detected as a large complex in Native-PAGE, and these molecular weights were about 4 times greater than each monomer size, which demonstrates that the two novel variants form tetramer as well (see Fig. 4 in ref. 23). The enzymatic assays for TDOs revealed that all three forms had similar full-enzymatic activity (Table 1).[23] Although further work is required in order to examine the detailed kinetics of these variants, our findings demonstrate that these variants are functional proteins with full-TDO activity.

Discussion

Proper neural network development is important for cognitive development, especially in the hippocampus and cerebellum. Tryptophan is known to be involved in the development of these brain regions and their associated cognitive and behavioral functions (Fig. 4). For example, tryptophan restriction during early postnatal development reduced dendrite arborization, spine density, and cell proliferation in the dentate gyrus of the hippocampus, and caused anxiety- and depressive-like behavior.[26] Furthermore, its metabolite kynurenine, which is produced in primary neurons and glial cells, increases production of nerve growth

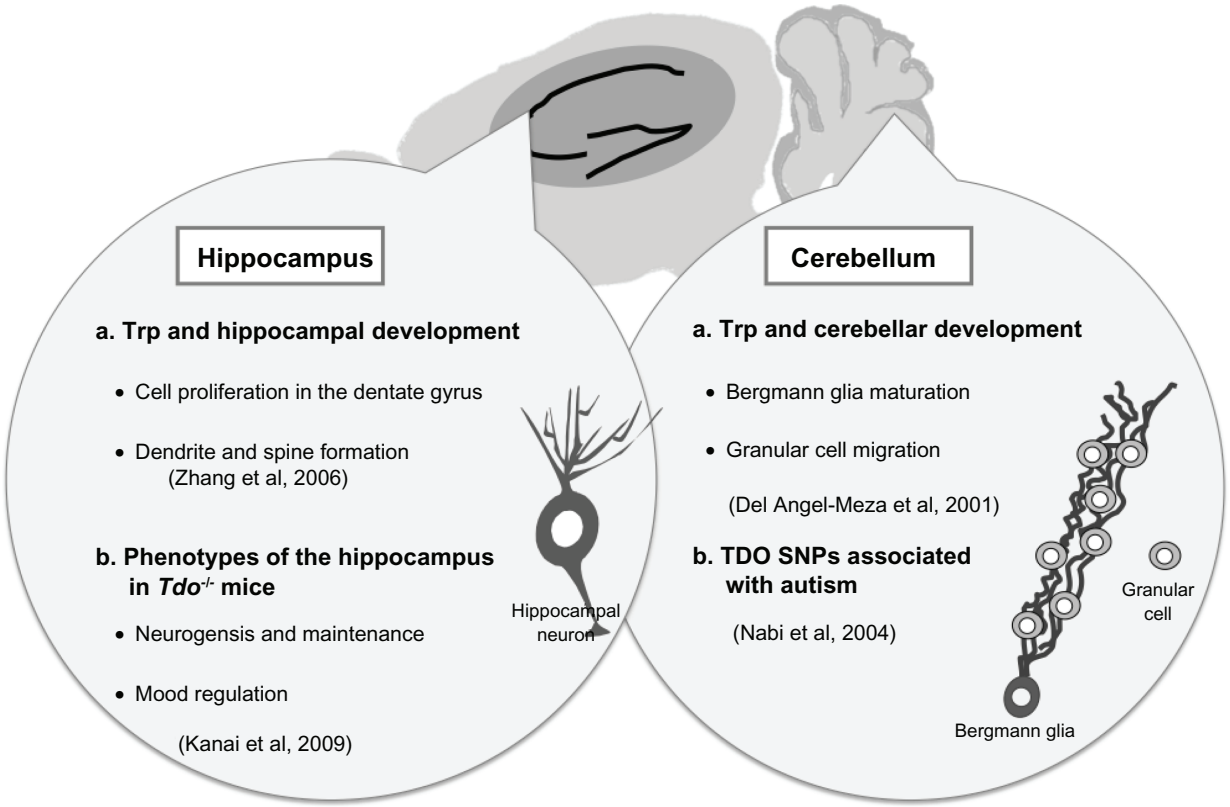

Figure 4. Tryptophan is important for hippocampal and cerebellar development and function. Restriction of tryptophan leads to abnormal development in the hippocampus and cerebellum, in turn modulating anxiety-related, depressive-like behaviors and autism.[18,26,30] TDO may also contribute to physiological mood regulation via hippocampal neurogenesis, maintenance of neural cells, and early cerebellar development.[16,23]

Table 1. Enzymatic activity of mouse TDO full-form, variant1, and variant2.

	TDO enzymatic activity (μmol Kynurenine/h/g protein)
TDO full-form	86.45 ± 4.75
TDO variant1	95.05 ± 9.01
TDO variant2	100.1 ± 11.25

Enzymatic activity for mouse TDO and its variants is examined using cell lysates of transiently transfected COS-7 cells. Values represent means ± S.E. (n = 5 in each group).

factor in astrocytes and potentially promotes survival of new neurons in the adult hippocampus.[2,10,11,27,28]

Our previous studies of *Tdo*$^{-/-}$ mice have demonstrated the critical role of TDO in hippocampal neurogenesis and the maintenance of hippocampal neurons as well as in anxiety-related behavior.[16] Moreover, the expression level of *tdo* mRNA was significantly decreased (over 90%) in the immature dentate gyrus of adult alpha-CaMKII hetero-knockout mice, which impaired working memory and mood regulation.[29] Consistent with previous studies, our quantitative study demonstrated the expression of TDOs in various brain regions, thereby suggesting that TDO and its variants, in addition to IDO, potentially produce N-formylkynurenine locally in the nervous system under physiological condition.[11,21,23] Collectively, the predominant local expression and regulation of full-form *tdo* among the various forms in the hippocampus suggests that *tdo* full-form is the dominant TDO form to modulate hippocampal functions including mood and memory, and this activity would be mediated by neurogenesis and maintenance of neural cells during development and adulthood.

In addition to its role in the hippocampus, tryptophan is thought to play an essential role in early cerebellar development. This is based on the finding that a tryptophan deficient corn-based diet begun 5 weeks before mating leads to the retardation of Bergmann glial maturation as well as a concomitant granular cell migration.[30] Furthermore, it is intriguing that human TDO SNPs have been associated with autism,[18] and that pathological changes have been reported in the postmortem cerebellum of the patients with autism (Fig. 4).[31] Since tryptophan is essential for early cerebellar development and *tdo* SNPs may be associated with autism, local predominant expression and regulation of *tdo* variant2 in the cerebellum during early postnatal development suggests that the *tdo* variant2, in addition to *tdo* full-form, plays an important role in early cerebellar development and may therefore be implicated in the development of autism.

In addition to these roles, dysregulation of the kynurenine pathway has been associated with psychiatric and neurodegenerative diseases, such as depression and Alzheimer's diseases.[2–4] There are some reports that local IDO in the brain represents one mechanism of pathology in these disorders, especially when the immune system is activated.[4,7] However, the presence of different molecular sized TDO proteins and altered immunoreactivity against TDO has been reported in the frontal cortex of individuals with schizophrenia and depression.[21] In addition, our findings of local expression of TDOs in the cerebral cortex suggests a local role of TDO and its variants in the specific regional modulation of tryptophan metabolism and subsequent behavioral and immune modulation, which would likely be regulated via a different mechanism from IDO under such pathological conditions.

In summary, in addition to the liver TDO, we present evidence that TDO, as well as newly identified TDO variants, are locally expressed and regulated in the brain, and therefore may play a critical role in the hippocampal and cerebellar development and function (Fig. 5).

Dysregulation of brain or liver TDOs may be involved in psychiatric or neurodegenerative disorders such as anxiety-related disorders, autism, or multiple sclerosis. Further analyses of brain cells expressing newly identified TDO variants in addition to the TDO full-form, the possible identification of new *tdo* variant(s), and the elucidation of the regulation and roles of these molecules may open a new avenue for understanding the role of tryptophan metabolites in higher-order brain functions in the future.

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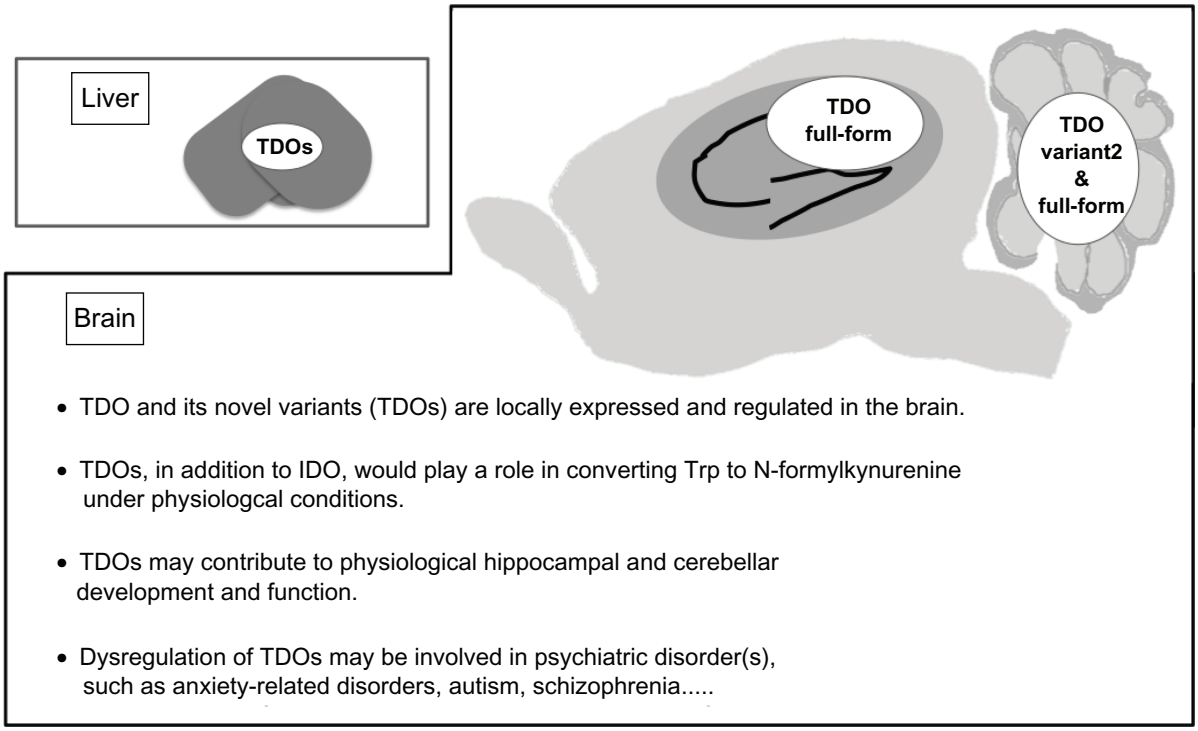

Figure 5. A working model of TDO function. In addition to the liver, *tdo* mRNAs are expressed in various brain regions, with high expression in the hippocampus and cerebellum. In the hippocampus, the level of *tdo* full-form was continuously expressed at a higher level than *tdo* variants, while *tdo* variant2 was highly expressed from early development in the cerebellum. Therefore, TDOs, in addition to IDO, potentially contribute to local tryptophan metabolism in the brain, and in turn to physiological hippocampal and cerebellar development and functions. Dysregulation of brain TDOs may, at least in part, influence the development of psychiatric disorders, such as anxiety-related disorders, autism, and schizophrenia.

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Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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