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# Tryptophan 2,3-Dioxygenfase and Indoleamine 2,3-Dioxygenase 1 Make Separate, Tissue-Specific Contributions to Basal and Inflammation-Induced Kynurenine Pathway Metabolism in Mice

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## Abstract

In mammals, the majority of the essential amino acid tryptophan is degraded via the kynurenine pathway (KP). Several KP metabolites play distinct physiological roles, often linked to immune system functions, and may also be causally involved in human diseases including neurodegenerative disorders, schizophrenia and cancer. Pharmacological manipulation of the KP has therefore become an active area of drug development. To target the pathway effectively, it is important to understand how specific KP enzymes control levels of the bioactive metabolites *in vivo*. Here, we conducted a comprehensive biochemical characterization of mice with a targeted deletion of either tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO), the two initial rate-limiting enzymes of the KP. These enzymes catalyze the same reaction, but differ in biochemical characteristics and expression patterns. We measured KP metabolite levels and enzyme activities and expression in several tissues in basal and immune-stimulated conditions. Although our study revealed several unexpected downstream effects on KP metabolism in both

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest with the contents of this article. 1

**AUTHOR CONTRIBUTIONS**

PBL designed and coordinated the study, made the figures and wrote the initial draft of the paper. PBL, KVS and FMN conducted experiments. PBL, KVS, FMN, RS and PJM analyzed and interpreted data and revised the paper. All authors reviewed the results and approved the final version of the manuscript.

knockout mice, the results were essentially consistent with TDO-mediated control of basal KP metabolism and a role of IDO in phenomena involving stimulation of the immune system.

**Keywords**

Tryptophan; Inflammation; Neurodegeneration; Cancer; Schizophrenia

## INTRODUCTION

The essential amino acid tryptophan is incorporated into protein or degraded to a variety of bioactive molecules, including 5-hydroxytryptamine (serotonin), melatonin, tryptamine and metabolites of the kynurenine pathway (KP) (1). Generation of KP metabolites is initiated by the enzymes tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO), which catalyze the conversion of tryptophan to N-formylkynurenine, which is then degraded further to the pivotal KP metabolite kynurenine. From kynurenine, one branch of the KP leads to the formation of kynurenic acid (KYNA), and another results in the production of $NAD^+$ via 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN) (Fig. 1).

TDO and IDO are crucial to understanding KP metabolism, which accounts for the majority of tryptophan degradation in mammals. The two enzymes catalyze the oxidative opening of tryptophan's indole ring at a comparable rate and have only subtle differences in substrate binding (2), but differ greatly in tissue distribution and regulation of expression. Under physiological conditions, TDO activity is highest in the liver (3), but the enzyme and its mRNA are also detectable in a number of other organs, including brain (4–7) and endometrium (8). Enzyme activity is induced by tryptophan and glucocorticoids (9–10), and allosterically modulated by the downstream KP metabolite 3-hydroxyanthranilic acid (11). In contrast, the less substrate-specific IDO, which was discovered much later than TDO (12–13), is expressed in a wide variety of tissues including epididymis, gut, lung, spleen, kidney, vascular endothelium and brain (14–16). Notably, IDO is only expressed at low levels under basal conditions, but is strongly induced by interferon-γ, lipopolysaccharide (LPS), or several other pro-inflammatory stimuli (17–18).

A second isoform of IDO (IDO2) also catalyzes the conversion of tryptophan to kynurenine (19–20). This enzyme, which likely arose via gene duplication of IDO, has distinctive kinetic characteristics and substrate specificity (21–22), is expressed in different tissues, and responds to immune stimulation differently than IDO (19,23). The physiological relevance of IDO2 is still being elaborated, but the enzyme, like IDO (now re-named IDO1) and TDO, may have a role in cancer(20).

The diverse physiological functions of the various KP metabolites, as well as the realization that impaired KP metabolism is *causally* involved in a number of grave disorders in humans (24–34), have prompted efforts to unravel the intricacies of KP regulation in health and disease. As entry points to the enzymatic cascade, TDO and IDO naturally attract special attention in this regard. In addition to ongoing attempts to specifically target these enzymes pharmacologically (35–36), genetic tools have been generated for experimental use. For example, mice genetically deficient in TDO (*Tdo*$^{-/-}$ mice) (37) or IDO1 (*Ido*$^{-/-}$ mice) (38)

have been successfully used for specific hypothesis testing in the neurosciences (6,37,39–41). However, these mutant animals have so far not been used for a comprehensive investigation of KP metabolism. The present study was designed to fill this void by directly comparing adult *Tdo*$^{-/-}$ and *Ido*$^{-/-}$ mice, as well as respective wild-type animals, under basal conditions and in response to a stimulation of the immune system (by LPS). Our results, which revealed several tissue- and KP metabolite-specific phenomena in these mutants, jointly emphasize the separate biological roles of TDO and IDO1, with the latter being of special relevance in events involving activation of the immune system.

## EXPERIMENTAL PROCEDURES

### Mice

All animals (C57BL/6 background) were bred, housed and handled in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals”, and all experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Animals were housed in a pathogen-free facility on a 12 h/12 h light/dark cycle. *Tdo*$^{-/-}$ mice were originally generated in our laboratory in Japan (37), and *Ido*$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). For each strain, heterozygote-to-heterozygote breedings provided gene deficient ($^{-/-}$) mice and wild-type ($^{+/+}$) littermates. All mice received an intraperitoneal (i.p.) injection 24 h before tissues were collected (see below), and all studies were performed using 6–10 mice per group.

### LPS injection and tissue collection

LPS from *Escherichia coli* K-235 was purchased from Sigma-Aldrich (St. Louis, MO; lot #020M4060). Mice received an i.p. injection of 1 mg/kg LPS, prepared freshly using sterile saline, or an equal volume of saline and were euthanized with an overdose of avertin 24 h later. After blood was collected into EDTA-containing tubes via cardiac puncture, animals were transcardially perfused with saline. Forebrain (i.e. brain minus cerebellum) and liver were rapidly removed and frozen on dry ice. Blood was promptly centrifuged to obtain plasma, which was subsequently also frozen on dry ice. All samples were then stored at −80°C.

### Genotyping

Mouse tail DNA was analyzed by PCR to determine genotype. The *Tdo*$^{+/+}$ and *Tdo*$^{-/-}$ alleles were amplified in separate reactions. The forward [AGC AAA CCT GTG TGG TCC TG] and reverse [GCC ATA GAT AAG TCC TCC T] primers were used to amplify the *Tdo* $^{+/+}$ allele, while the forward [CTT GGG TGG AGA GGC TAT TC] and reverse [AGG TGA GAT GAC AGG AGA TC] primers were used to amplify the Neo cassette in the *Tdo*$^{-/-}$ allele.

The *Ido*$^{+/+}$ and *Ido*$^{-/-}$ alleles were amplified in the same reaction with the forward [TGG AGC TGC CCG ACG C] and reverse [TAC CTT CCG AGC CCA GAC AC] primers for the *Ido*$^{+/+}$ allele and the forward [CTT GGG TGG AGA GGC TAT TC] and reverse [AGG TGA GAT GAC AGG AGA TC] primers for the *Ido*$^{-/-}$ allele.

## Chemicals

[$^{2}H_{6}$]-L-kynurenine, pentafluoropropionic anhydride (PFPA) and 2,2,3,3,3-pentafluoro-1-propanol (PFP) were obtained from Sigma-Aldrich. Ro 61-8048 was a generous gift from Dr. W. Fröstl (Novartis, Basel, Switzerland). 4-Chloro-3-hydroxyanthranilic acid (4-Cl-3-HANA) was kindly provided by Drs. W.P. Todd and B.K. Carpenter (Department of Chemistry, Cornell University, Ithaca, NY). [$^{2}H_{3}$]-QUIN was purchased from Synfine Research (Richmond Hill, Ontario, Canada), and [$^{2}H_{5}$]-L-tryptophan was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). [1-$^{14}$C]-3-Hydroxyanthranilic acid (6 mCi/mmol) was obtained from Dupont/New England Nuclear (Boston, MA). All other fine biochemicals and chemicals were purchased from various commercial suppliers and were of the highest available purity

## Enzyme assays

On the day of the assays, tissues were thawed and homogenized 1:5 (w/v) in ultrapure water. Enzyme activities were then determined as described and referenced below. Only essential information, including methodological modifications when indicated, is spelled out here.

**Kynurenine 3-monooxygenase (KMO; E.C. 1.14.13.9)—**The original tissue homogenate was diluted 1:5 (forebrain) or 1:6,000 (liver) (v/v) in 100 mM Tris–HCl buffer (pH 8.1) containing 10 mM KCl and 1 mM EDTA. The preparation was incubated for 40 min in the presence of 100 μM L-kynurenine, and the reaction product, 3-HK, was analyzed by HPLC with electrochemical detection. Blanks were obtained by including the KMO inhibitor Ro 61-8048 (100 μM) in the incubation solution(42).

**Kynureninase (KYNU; E.C. 3.7.1.3)—**The original tissue homogenate was diluted 1:40 (forebrain) or 1:4,000 (liver) (v/v) in 5 mM Tris-HCl buffer (pH 8.4) containing 10 mM of 2-mercaptoethanol and 50 μM pyridoxal-5′-phosphate. After incubation for 2 h at 37°C in the presence of 4 μM DL-3-HK, the reaction product, 3-hydroxyanthranilic acid, was measured by HPLC with fluorimetric detection. To obtain blank values, tissue homogenate was added at the end of the incubation period (43).

**3-Hydroxyanthranilic acid dioxygenase (3-HAO; E.C. 1.13.11.6)—**The original tissue homogenate was diluted 1:5 (forebrain) in 60 mM MES [2-(N-morpholino)ethanesulfonic acid] buffer (pH 6.0), and then incubated for 1 h at 37°C with 3 μM (3.4 nCi) [1-$^{14}$C]-3-hydroxyanthranilic acid. The reaction product ($^{14}$C-QUIN) was quantitated by liquid scintillation spectrometry. Blanks were obtained by including the 3-HAO inhibitor 4-Cl-3-HANA (100 μM) in the incubation solution (44).

**Kynurenine aminotransferases I and II (KAT I; E.C. 2.6.1.64; KAT II; E.C. 2.6.1.7)—**The original brain tissue homogenate was further diluted (1:2, v/v) in 5 mM Tris-acetate buffer (pH 8.0) containing 10 mM 2-mercaptoethanol and 50 μM pyridoxal-5′-phosphate, and then dialyzed overnight at 4°Cagainst 4 L of the same buffer.

For the determination of KAT I activity, the dialyzed homogenate was incubated for 2 h at 37°C in 150 mM 2-amino-2-methyl-1-propanol (AMP) buffer (pH 9.5) containing 100 μM

L-kynurenine and 1 mM pyruvate. For the measurement of KAT II, we used the same incubation conditions except that 150 mM Tris-acetate buffer (pH 7.4) was substituted for the AMP buffer. Blanks were prepared by adding the non-specific KAT inhibitor aminooxyacetic acid (1 mM) to the incubation solution. The reaction product, KYNA, was determined by HPLC with fluorimetric detection (45).

## Determination of KP metabolites

Thawed tissues or plasma samples (see above) were processed as detailed below. Assays were then performed according to established methodologies, which are briefly described below.

**Tryptophan, kynurenine, 3-HK (liver) and QUIN—**The tissue levels of these metabolites were quantified by gas chromatography/mass spectrometry (GC/MS). To this end, tissues were homogenized (1:20, w/v) in an aqueous solution containing 0.1% ascorbic acid and internal standards ([$^2H_5$]-L-tryptophan, [$^2H_6$]-L-kynurenine and [$^2H_3$]-QUIN). Proteins were denatured using acetone and then removed by centrifugation. Fifty μL of methanol:chloroform (20:50, v/v) were added to the supernatant, and the samples were centrifuged. GC/MS analysis in samples of the supernatant was carried out as described (46).

For kynurenine measurement in plasma, 50 μL of 6% perchloric acid were added to 100 μL of the sample, and the precipitated proteins were removed by centrifugation. Twenty μL of the supernatant were applied to a 3 μm $C_{18}$ reverse phase column (HR-80; 80 mm × 4.6 mm; ESA Inc., Chelmsford, MA), and kynurenine was isocratically eluted using a mobile phase containing 250 mM zinc acetate, 50 mM sodium acetate and 3% acetonitrile (pH 6.2) at a flow rate of 1 mL/min. In the eluate, kynurenine was detected fluorimetrically (excitation wavelength: 365 nm; emission wavelength: 480 nm; S200 fluorescence detector; Perkin-Elmer, Waltham, MA).

**3-HK (brain, serum)—**Forebrain was homogenized (1:5, w/v), and plasma was diluted (1:2, v/v), in ultrapure water. Twenty-five μL of 6% perchloric acid were added to 100 μL of the samples. After thorough mixing, the precipitated proteins were removed by centrifugation (16,000 × *g*, 15 min). Twenty μL of the resulting supernatant were applied to a 3 μm HPLC column (HR-80; 80 mm × 4.6 mm; ESA, Chelmsford, MA, USA), using a mobile phase consisting of 1.5 % acetonitrile, 0.9 % triethylamine, 0.59 % phosphoric acid, 0.27 mM EDTA and 8.9 mM sodium heptane sulfonic acid, and a flow rate of 0.5 mL/min. In the eluate, 3-HK was detected electrochemically using a HTEC 500 detector (Eicom Corp., San Diego, CA, USA; oxidation potential: +0.5 V). The retention time of 3-HK was ~11 min.

**KYNA—**Tissues were homogenized (forebrain: 1:5, liver: 1:50, w/v), and plasma was diluted (1:10, v/v), in ultrapure water. After acidification, precipitated proteins were removed by centrifugation, and 20 μL of the resulting supernatant were subjected to HPLC analysis with fluorometric detection(48).

### Quantitative real-time PCR

RNA from mouse brain or liver was harvested with an RNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA was immediately reverse transcribed to cDNA with Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and stored at −20°C.

Quantitative real-time PCR (qPCR) was performed using FAST SYBR Green Mastermix (Applied Biosystems), and thermal cycling was performed using an ABI Prism 7900 HT Sequence Detector (Applied Biosystems) with the following program: UNG activation (50°C for 2 min), initial denaturation (95° for 10 min), 40 cycles of denaturation (95°C for 15 sec), annealing and extension (60°C for 1 min), followed by a disassociation stage for melting curve analysis. Analysis of fluorescence data from genes of interest and from the control gene β-actin was performed using the online analysis tool QPCR (49).

Primers were designed using the online tool Primer-BLAST (NCBI), and primer specificity was evaluated using Primer-BLAST and melting curve analysis. All data are expressed as a fold change compared to wild-type mice treated with saline, and all primers are listed in Table 1. Statistics: In all analyses, measurements that deviated more than 4 standard deviations from the mean were considered outliers and were excluded from analysis. All statistical tests were conducted using Prism (Graphpad, La Jolla, CA, USA), except where noted. Data sets were tested for normality using a D'Agostino & Pearson omnibus normality test and for homoscedasticity using Bartlett's test (http://home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/BartletTest.htm). Then, group means were compared with either a t-test for normal, homoscedastic data; a t-test with Welch's correction for normal, heteroscedastic data; or a Mann Whitney U-test for non-normal, homoscedastic data. To control for multiple comparisons, the family-wise error rate was corrected for the four planned comparisons.

## RESULTS

### Tdo$^{-/-}$ mice

**Basal status of tryptophan, KP metabolites and KP enzymes—**Compared to *Tdo*$^{+/+}$ mice, brain tryptophan levels were significantly elevated (>9–fold) in *Tdo*$^{-/-}$ mice (Fig. 2A). Similar significant increases were observed in brain kynurenine content in the mutant animals (Fig. 2B). Hepatic tryptophan and kynurenine levels, too, were much higher (~6-fold) in *Tdo*$^{-/-}$ than in *Tdo*$^{+/+}$ mice (Table 2). In contrast, plasma kynurenine levels in the mutants were only slightly (~2-fold) increased compared to wild-type controls (Table 2).

Metabolites downstream from kynurenine were also abnormal in *Tdo*$^{-/-}$ mice. 3-HK levels were elevated in brain and liver of *Tdo*$^{-/-}$ mice, but statistical significance was reached only in the liver (Fig. 2D, Table 2). This increase was accompanied by higher activity of its biosynthetic enzyme, KMO, in both tissues, though statistical significance was only attained in the brain (Figs. 3A,B). Plasma 3-HK levels (Table 2), too, tended to be higher in the *Tdo*$^{-/-}$ genotype but were not significantly different from wild-type controls. Interestingly, the TDO genotype did not influence brain QUIN (Fig. 2E), whereas liver QUIN levels were substantially (>70%) lower in *Tdo*$^{-/-}$ than in *Tdo*$^{+/+}$ mice (Table 2). In the other arm of the

KP, there was a non-significant trend towards higher basal KYNA levels in brain, liver and plasma of *Tdo*$^{-/-}$ compared to *Tdo*$^{+/+}$mice (Fig. 2C, Table 2).

The activities of the KP enzymes KAT I, KAT II, KYNU and 3-HAO in the brain were not altered in the mutant animals(Table 3).

**Expression of KP enzymes—**As expected, TDO mRNA was not detectable in *Tdo*$^{-/-}$ mice (Table 4). Notably, IDO mRNA in brain did not differ between *Tdo*$^{+/+}$ and *Tdo*$^{-/-}$ mice, i.e. TDO deletion was not accompanied by an increased expression of IDO1. Similarly, the mRNA of several other KP enzymes was not changed in brain or liver of *Tdo* $^{-/-}$ mice (Table 4).

**Effects of LPS treatment—**Compared to their respective control values, the brain levels of tryptophan, kynurenine and 3-HK increased in *Tdo*$^{+/+}$ mice after an immune challenge. However, LPS had no significant effect on the levels of these metabolites in *Tdo*$^{-/-}$ mice (Figs. 2A,B,D). Brain QUIN levels were elevated in both *Tdo*$^{+/+}$ and *Tdo*$^{-/-}$ mice, but the increase reached significance only in *Tdo*$^{-/-}$ mice (Fig. 2E). Brain KYNA levels in *Tdo*$^{+/+}$ and *Tdo*$^{-/-}$ mice were not changed 24 h after the systemic administration of LPS (1 mg/kg) (Figs. 2C).

In the periphery, LPS significantly increased plasma but not liver kynurenine concentrations in both genotypes (Table 2). Similarly, LPS-induced changes in the downstream metabolites 3-HK and KYNA were more pronounced in plasma than in liver, and there was no effect of LPS on liver QUIN and tryptophan levels (Table 2).

LPS treatment reduced the expression of liver KYNU and 3-HAO in *Tdo*$^{+/+}$ mice (Table 4), but did not alter the activity or the expression of other KP enzymes measured in brain or liver in either of the two genotypes (Tables 3 and 4).

## Ido$^{-/-}$ mice

**Basal status of tryptophan, KP metabolites and KP enzymes—**Brain and liver levels of tryptophan and kynurenine were not affected by the deletion of IDO1 (Figs. 4A,B, Table 5), though plasma kynurenine levels tended to be lower in *Ido*$^{-/-}$ than in *Ido*$^{+/+}$ mice (Table 5). Moreover, deletion of the *IDO1* gene did not influence basal KYNA or QUIN levels in brain, liver and plasma (Figs. 4C,E, Table 5). 3-HK levels in brain were lower in *Ido*$^{-/-}$ mice than in *Ido*$^{+/+}$ mice, whereas 3-HK levels in liver and plasma were unaffected by the elimination of IDO1 (Fig. 4D, Table 5).

Deletion of IDO1 did not influence the activity of KMO, KYNU, 3-HAO, KAT I and KAT II in the brain, or KMO activity in the liver (Figs. 5A,B, Table 6).

**Expression of KP enzymes—**IDO mRNA was not detectable in *Ido*$^{-/-}$ mice. Notably, *Ido*$^{-/-}$ mice did not compensate for the loss of IDO1 by up-regulating TDO mRNA expression in either brain or liver (Table 7). The IDO genotype also did not affect the mRNA expression of KMO and 3-HAO in brain, or of KMO, KYNU and 3-HAO in the liver (Table 7).

**Effects of LPS treatment—**LPS caused an increase in tryptophan levels in the brain of both *Ido*$^{+/+}$ mice and *Ido*$^{-/-}$ mice, but statistical significance was attained only in the mutant animals (Fig. 4A). Kynurenine levels, too, were increased in the brain of both *Ido*$^{+/+}$ and *Ido*$^{-/-}$ mice, but statistical significance was only attained in the wild-type animals (Fig. 4B). In contrast, LPS treatment affected neither tryptophan nor kynurenine levels in either genotype in the liver (Table 5). LPS induced significant increases in 3-HK levels in brain, liver and plasma of *Ido*$^{+/+}$ mice, but this effect was not seen in any of these tissues in *Ido*$^{-/-}$ mice (Fig. 4D, Table 5). Similarly, LPS raised brain QUIN levels significantly in wild-type but not in mutant animals (Fig. 4E). However, no effect of LPS was seen in hepatic QUIN levels in either genotype (Table 5). Notably, LPS treatment did not affect KYNA levels in brain, liver and plasma in either *Ido*$^{+/+}$ or *Ido*$^{-/-}$ mice (Fig. 4C, Table 5).

LPS treatment did not affect the activity of the KP enzymes measured (KMO, KYNU, 3-HAO, KAT I and KAT II in the brain, and KMO in the liver) in either *Ido*$^{+/+}$ or *Ido*$^{-/-}$ mice (Figs. 5A,B, Table 6).

LPS treatment increased gene expression of IDO and KMO in the brain of *Ido*$^{+/+}$ mice and decreased expression of KYNU in the liver of both *Ido*$^{+/+}$ and *Ido*$^{-/-}$ mice. In addition, LPS treatment decreased liver 3-HAO expression in both *Ido*$^{+/+}$ and *Ido*$^{-/-}$ mice, though statistical significance was only attained in the mutant mice. LPS did not alter expression of the other KP enzymes tested (Table 7).

## DISCUSSION

Our comparative biochemical analysis of *Tdo*$^{-/-}$ and *Ido*$^{-/-}$ mice provides evidence for the view that TDO is the major KP enzyme that is responsible for the oxidative degradation of tryptophan under normal physiological conditions, whereas the role of IDO1 is largely confined to situations in which the immune system is activated. In addition, the present study revealed several unexpected, tissue-specific metabolic features and other previously unrecognized downstream effects of TDO and IDO elimination. These effects shed new light on the regulation of the KP, which metabolizes the vast majority of dietary tryptophan in mammals (50), and have added relevance due to the fact that a number of KP metabolites have distinct, biologically significant properties (14,51–53). Notably, we found no evidence that IDO can substitute for TDO in the absence of the latter or, conversely, that TDO performs IDO-like functions in *Ido*$^{-/-}$ mice.

As we reported previously, *Tdo*$^{-/-}$ mice showed a substantial increase in tryptophan levels in plasma, brain and liver, indicating that TDO plays an important role in controlling the basal levels of this essential amino acid (6,37). In the present study, this increase was accompanied by significantly higher kynurenine levels in the plasma, and especially in liver and brain, of the mutant animals. These increases in kynurenine were not caused by a compensatory up-regulation of IDO mRNA levels in the brain of *Tdo*$^{-/-}$ mice, and liver IDO mRNA remained undetectable in these animals (data not shown). In principle, reduced degradation by any of its catabolic enzymes could also have caused the accumulation of kynurenine in *Tdo*$^{-/-}$ mice (cf. Fig. 1), but no such changes were seen in the activities of KAT I, KAT II, KMO or KYNU in these animals. On the contrary, KMO activity was unexpectedly *enhanced* when

TDO was eliminated, and this increase was especially pronounced in the brain. Increased IDO activity caused by posttranslational mechanisms, and/or alternative biosynthetic routes, possibly involving microbes (54), may therefore be responsible for the enhanced kynurenine levels seen in plasma, liver and brain of *Tdo*$^{-/-}$ animals.

Surprisingly, in spite of the large increase in cerebral kynurenine levels and enhanced KMO activity, we observed only modest increases in basal 3-HK levels, and no change in QUIN levels, in the brain of *Tdo*$^{-/-}$ mice. The effect of TDO elimination on downstream KP metabolism was qualitatively very different in the periphery, however. Thus, in spite of only a modest trend towards enhanced KMO activity, liver 3-HK levels in the knockout animals increased 4-fold, and this elevation was accompanied by a >70% *reduction* in hepatic QUIN levels. The most parsimonious explanation for these results is that elimination of TDO, for reasons which remain to be elaborated, stimulates the production of anthranilic acid, which can serve as an efficient bioprecursor of 3-hydroxyanthranilic acid in the brain but not in the liver (55) (Figure 1). This phenomenon, which may also underlie qualitatively similar differences between central and peripheral QUIN levels in *Kmo*$^{-/-}$ mice or after the administration of a selective KMO inhibitor (43,56), would also be in line with the observation that urinary QUIN levels are greatly reduced in *Tdo*$^{-/-}$ animals (57). Unfortunately, limited tissue availability prevented the determination of anthranilic acid and 3-hydroxyanthranilic acid levels in the present study.

In contrast to *Tdo*$^{-/-}$ mice, *Ido*$^{-/-}$ mice had normal basal levels of most KP metabolites in brain, liver and plasma. The only exception was a small decrease in 3-HK levels in the brain, which is in agreement with a reported reduction in 3-HK levels in the striatum of these animals (41). Taken together, these findings indicate that elimination of IDO during normal pre- and postnatal development does not have a major permanent impact on tryptophan metabolism. Specifically, no compensatory up-regulation of TDO mRNA was observed in IDO knockout animals.

Pro-inflammatory conditions, modeled by systemic administration of LPS, had qualitatively similar effects in *Tdo*$^{+/+}$ and *Ido*$^{+/+}$ mice. However, although both of these wild-type cohorts were generated on the same background (C57BL/6), some quantitative differences were observed, possibly due to the fact that the animals were obtained from different providers. Interestingly, whereas LPS did not affect brain or liver tryptophan levels in *Tdo*$^{-/-}$ mice and, unexpectedly, did not raise cerebral and hepatic kynurenine levels further in these tissues, the effect of LPS treatment on brain QUIN levels was more pronounced in *Tdo*$^{-/-}$ than in *Tdo*$^{+/+}$ mice, suggesting a higher sensitivity of IDO to cerebral immune stimulation in the absence of TDO.

Finally, in line with the well-established major role of IDO as a regulator of the QUIN branch of the KP under pro-inflammatory conditions (58–60), almost all LPS-induced increases in metabolite levels in both brain and periphery were abolished in *Ido*$^{-/-}$ mice in this arm of the KP. Notably, other KP enzymes, including KMO, did not compensate for the absence of IDO.

In contrast to KMO knockout animals, where comparatively smaller increases in kynurenine levels are accompanied by large elevations in KYNA levels in brain, liver and plasma (43), the relationship between these two KP metabolites was inverted in *Tdo*$^{-/-}$ mice, i.e. only moderately elevated KYNA concentrations were seen in the mutant animals in spite of substantial increases in kynurenine levels (see above). In agreement with recent studies in flies (61), these observations further support the critical importance of KMO in KP metabolism (62–64) and confirm that fluctuations in the levels of the pivotal KP metabolite kynurenine are not the sole determinant of KYNA formation in mammals. Of note, both central and peripheral KYNA levels tended to be reduced by LPS in *Ido*$^{-/-}$ mice, again without obvious correlations with respective kynurenine levels.

Taken together, the present set of experiments and analyses highlight the complexity of mammalian KP metabolism and provide rationale and directions for follow-up studies. Thus, it will be necessary to clarify the mechanisms underlying several of the unexpected findings reported here, including the dissociation between 3-HK and QUIN fluctuations in response to discrete metabolic manipulations upstream, the distinct roles of TDO and IDO in affecting KMO activity and KYNA formation, and, more generally, the frequently observed qualitative differences between central and peripheral KP metabolism. These studies will require the use of novel pharmacological as well as genetic tools that can target individual KP enzymes *in vivo* specifically in individual brain regions or in various peripheral tissues and distinct cell populations (65–66), attention to age-specific phenomena (cf. (67), and careful experimental control of nutritional and other environmental influences. Notably, future investigations of KP metabolism may uncover physiologically relevant species or strain differences.

Because of the surge of interest in the role of the KP in a variety of pathological events and the realization that several KP metabolites are actively involved in oxidative processes (68–71) and can affect an array of receptor targets (52,72–78), *Tdo*$^{-/-}$ and especially *Ido*$^{-/-}$ mice have been increasingly used as experimental tools in recent years. So far, studies in *Tdo*$^{-/-}$ mice have focused mainly on brain and behavioral phenomena, including effects on cerebral serotonin metabolism, hippocampal morphology and anxiety (37,79), but these animals are clearly also valuable for investigating catabolic events following tryptophan ingestion (80) as well as cellular and molecular phenomena involved in inflammation, infection and other states of immunopathology (81). In contrast, as IDO is increasingly recognized as a key regulator of the immune system, and IDO1 has emerged as an attractive pharmacological target for the prevention or treatment of illnesses ranging from viral diseases and cancer (82–84) to neurological and psychiatric disorders (58–60), *Ido*$^{-/-}$ mice are already being widely used to investigate both the beneficial and the possible detrimental effects of IDO manipulation (40,85–87). Using peripheral LPS administration, the present study confirmed that the enzyme is indeed a major determinant of downstream events in the 3-HK branch of the KP when the immune system is stimulated, though the choice of a single time point of analysis, i.e. 24 h after LPS, may have obscured additional effects on KP metabolism both in the periphery and in the brain (88–90). Moreover, and of special translational relevance, future studies with *Tdo*$^{-/-}$ and *Ido*$^{-/-}$ animals should examine the distinct effects of localized cerebral inflammation, which is seen in several major brain diseases (91–92).

In conclusion, the experimental results described here demonstrated that mice lacking TDO or IDO have discrete impairments in KP metabolism. Our study revealed that the effects of these genomic enzyme deletions are complex and possibly adaptive in nature, and not always predictable. Further careful analysis of the organ- and cell-specific changes that occur in *Tdo*$^{-/-}$ and *Ido*$^{-/-}$ mice over time is therefore necessary in order to optimize the use of these mutant animals. To address the many challenges of KP research more comprehensively, conditional or cell type-specific mutants and pharmacological agents that target TDO, IDO and other KP enzymes specifically, will constitute valuable additional investigative tools for the examination of KP metabolism in physiology and pathology.

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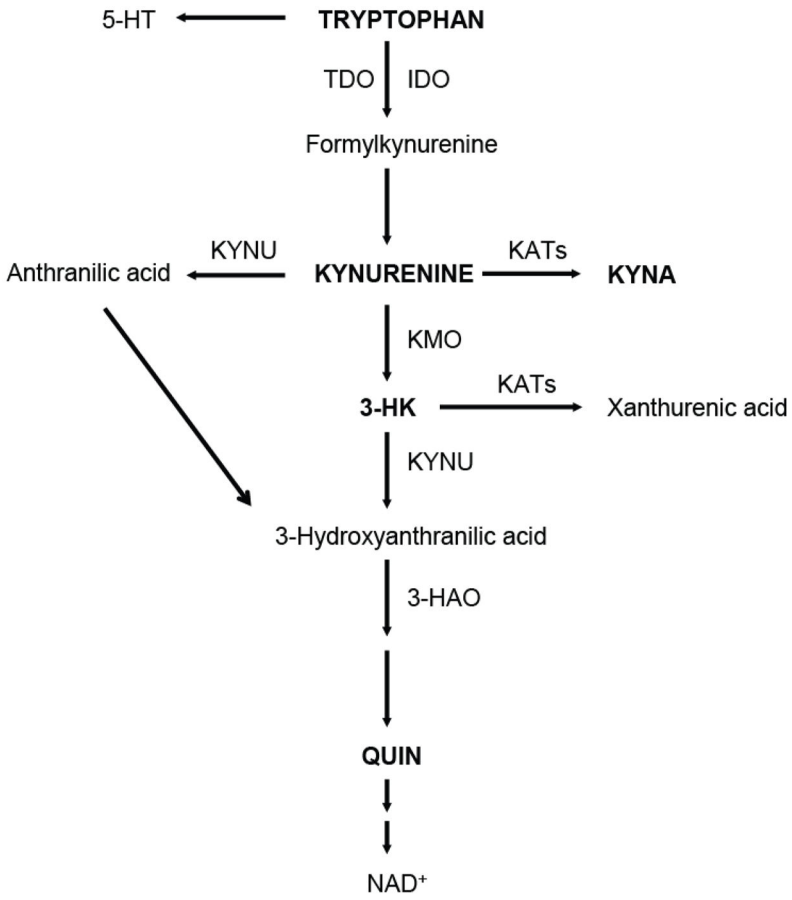


**Figure 1.**

Kynurenine pathway of tryptophan degradation in mammals. Indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) catalyze the first step of the kynurenine pathway. 5-HT: serotonin; KATs: kynurenine aminotransferases; KYNU: kynureninase; KMO: kynurenine 3-monooxygenase; KYNA: kynurenic acid; 3-HK: 3-hydroxykynurenine; 3-HAO: 3-hydroxyanthranilic acid dioxygenase; QUIN: quinolinic acid.

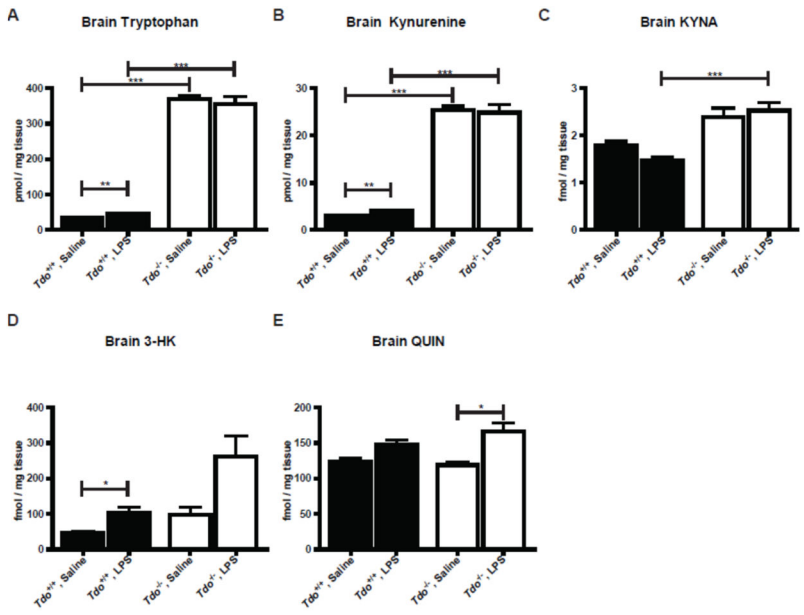


**Figure 2.**

Brain KP metabolites in *Tdo*$^{-/-}$ mice. Data are the mean ± SEM (n=6–10 per group). Statistical significance is indicated by asterisks. One symbol: $p<0.05$, two symbols: $p<0.01$, three symbols: $p<0.001$.

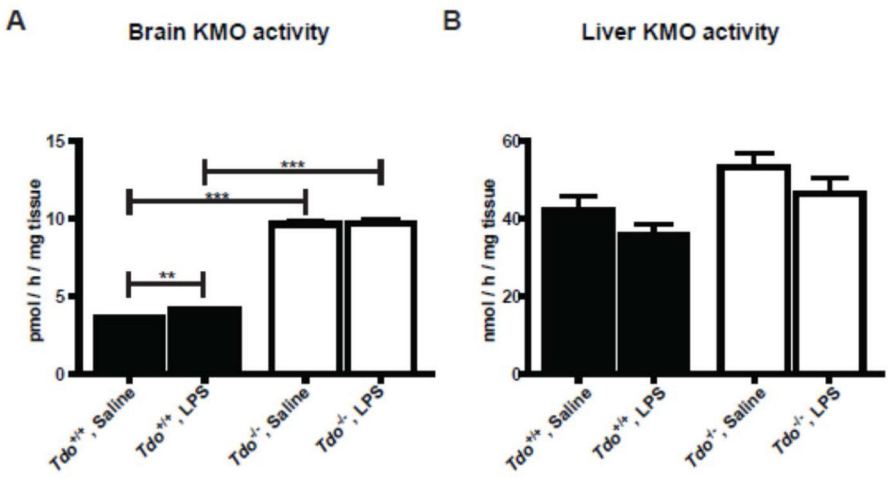


**Figure 3.**

KMO activity in *Tdo*$^{-/-}$ mice. Data are the mean ± SEM (n=8–10 per group). Statistical significance is indicated by asterisks. Two symbols: p<0.01, three symbols: p<0.001.

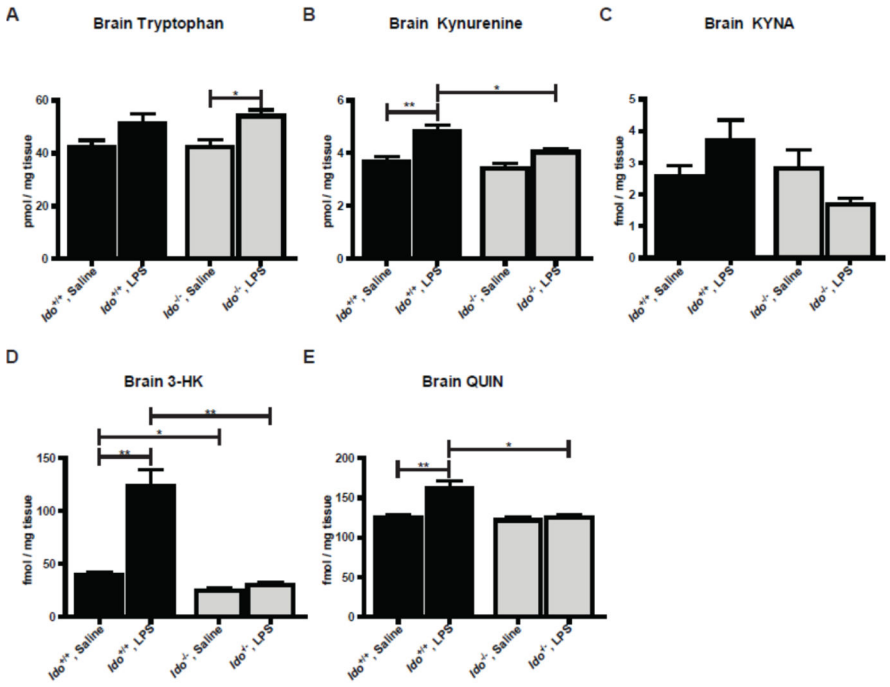


**Figure 4.**

Brain KP metabolites in *Ido*$^{-/-}$ mice. Data are the mean ± SEM (n=8–10 per group). Statistical significance is indicated by asterisks. One symbol: $p<0.05$, two symbols: $p<0.01$.

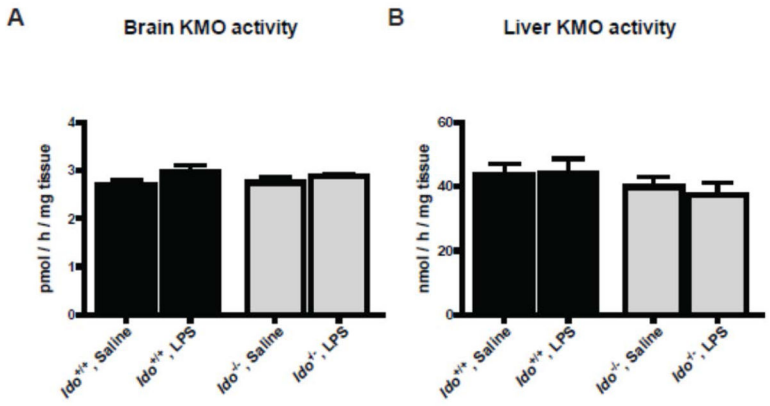


**Figure 5.**
KMO activity in *Ido*$^{-/-}$ mice. Data a re the mean ± SEM (n=8–10 per group).

**Table 1**

Primers used for qPCR experiments

| Gene symbol | Gene name | Accession # | Forward Primer | Reverse primer |
|---|---|---|---|---|
| 3-HAO | 3-Hydroxyanthranilate 3,4-dioxygenase | NM_025325 | ctt tca gcc tcc ggt ttg | gcc tcc aac gaa cat gat tt |
| IDO | Indoleamine-2,3-dioxygenase | NM_008324 | ggg ctt ctt cct cgt ctc tc | tgg ata cag tgg gga ttg ct |
| KMO | Kynurenine 3-monooxygenase | NM_133809 | tcc ttt caa taa gca gag aaa act ta | ctt cgc att ggc ata gga ct |
| KYNU | Kynureninase | NM_027552 | caa acc ctc cca ttt tgt tg | cgc agt cat agt tgc ttg ct |
| TDO | Tryptophan-2,3-dioxygenase | NM_019911 | tcc agg gag cac tga tga ta | ctg gaa agg gac ctg gaa tc |

## Table 2

Metabolite levels in liver and plasma (TDO cohort)

| | ***Tdo*$^{+/+}$, Saline** | ***Tdo*$^{+/+}$, LPS** | ***Tdo*$^{-/-}$, Saline** | ***Tdo*$^{-/-}$, LPS** |
|---|---|---|---|---|
| **Liver Tryptophan (pmol/mg tissue)** | 51 +/− 3 | 56 +/− 3 | 370 +/− 16 $^{aaa}$ | 300 +/− 25 $^{ccc}$ |
| **Liver Kynurenine (pmol/mg tissue)** | 2 +/− 0 | 3 +/− 0 | 12 +/− 0 $^{aaa}$ | 11 +/− 0 $^{ccc}$ |
| **Liver KYNA (fmol/mg tissue)** | 32 +/− 4 | 45 +/− 5 | 54 +/− 8 | 55 +/− 8 |
| **Liver 3-HK (fmol/mg tissue)** | 58 +/− 5 | 100 +/− 13 $^{a}$ | 230 +/− 33 $^{aa}$ | 200 +/− 25 $^{c}$ |
| **Liver QUIN (pmol/mg tissue)** | 4 +/− 0 | 4 +/− 0 | 1 +/− 0 $^{aaa}$ | 1 +/− 0 $^{ccc}$ |
| **Plasma Kynurenine (pmol/μL)** | 1 +/− 0 | 2 +/− 0 $^{aaa}$ | 2 +/− 0 $^{a}$ | 3 +/− 0 $^{bb}$ |
| **Plasma KYNA (fmol/μL)** | 96 +/− 11 | 140 +/− 22 | 123 +/− 7 | 170 +/− 14 $^{b}$ |
| **Plasma 3-HK (fmol/μL)** | 59 +/− 7 | 250 +/− 54 $^{a}$ | 270 +/− 81 | 760 +/− 240 |

Values are the mean +/− SEM (n=7–10 per group). Statistical significance is indicated by letters:

$^{a}$(vs *Tdo*$^{+/+}$, saline);

$^{b}$(vs *Tdo*$^{-/-}$, saline);

$^{c}$(vs *Tdo*$^{+/+}$, LPS).

One symbol: p<0.05, two symbols: p<0.01, three symbols: p<0.001.

**Table 3**

Enzyme activity in brain (TDO cohort)

| | ***Tdo*$^{+/+}$, Saline** | ***Tdo*$^{+/+}$, LPS** | ***Tdo*$^{-/-}$, Saline** | ***Tdo*$^{-/-}$, LPS** |
|---|---|---|---|---|
| **KAT I (pmol/h/mg tissue)** | 13 +/− 1 | 14 +/− 1 | 14 +/− 1 | 14 +/− 1 |
| **KAT II (pmol/h/mg tissue)** | 8 +/− 0 | 8 +/− 0 | 7 +/− 0 | 7 +/− 0 |
| **KYNU (pmol/h/mg tissue)** | 4 +/− 0 | 4 +/− 0 | 4 +/− 0 | 4 +/− 0 |
| **3-HAO (pmol/h/mg tissue)** | 9 +/− 1 | 10 +/− 1 | 9 +/− 1 | 10 +/− 1 |

Values are the mean +/− SEM (n=8 – 10 per group).

**Table 4**

Gene expression in brain and liver (TDO cohort)

| | ***Tdo*$^{+/+}$, Saline** | ***Tdo*$^{+/+}$, LPS** | ***Tdo*$^{-/-}$, Saline** | ***Tdo*$^{-/-}$, LPS** |
|---|---|---|---|---|
| **Brain IDO** | 1.0 +/− 0.1 | 1.0 +/− 0.0 | 0.9 +/− 0.1 | 1.1 +/− 0.1 |
| **Brain TDO** | 1.0 +/− 0.0 | 0.9 +/− 0.0 | -- | -- |
| **Brain KMO** | 1.0 +/− 0.1 | 1.1 +/− 0.1 | 0.9 +/− 0.1 | 1.0 +/− 0.1 |
| **Brain 3-HAO** | 1.0 +/− 0.1 | 1.0 +/− 0.1 | 1.1 +/− 0.1 | 1.1 +/− 0.1 |
| **Liver TDO** | 1.0 +/− 0.2 | 0.6 +/− 0.0 | -- | -- |
| **Liver KMO** | 1.0 +/− 0.1 | 1.1 +/− 0.1 | 0.9 +/− 0.1 | 1 +/− 0.2 |
| **Liver KYNU** | 1.0 +/− 0.1 | 0.7 +/− 0.1 [a] | 0.9 +/− 0.1 | 0.8 +/− 0.1 |
| **Liver 3-HAO** | 1.0 +/− 0.2 | 0.4 +/− 0.0 [a] | 0.8 +/− 0.1 | 0.9 +/− 0.2 |

Gene expression is presented relative to *Tdo*$^{+/+}$, Saline. Values are the mean +/− SEM (n=8–10 per group).

[a] $p<0.05$ vs *Tdo*$^{+/+}$, saline.

**Table 5**

Metabolite levels in liver and plasma (IDO cohort)

| | *Ido*$^{+/+}$, Saline | *Ido*$^{+/+}$, LPS | *Ido*$^{-/-}$, Saline | *Ido*$^{-/-}$, LPS |
|---|---|---|---|---|
| **Liver Tryptophan (pmol/mg tissue)** | 58 +/− 3 | 65 +/− 5 | 54 +/− 3 | 61 +/− 4 |
| **Liver Kynurenine (pmol/mg tissue)** | 3 +/− 0 | 3 +/− 0 | 3 +/− 0 | 3 +/− 0 |
| **Liver KYNA (fmol/mg tissue)** | 70 +/− 8 | 86 +/− 10 | 98 +/− 29 | 51 +/− 6 $^{b}$ |
| **Liver 3-HK (fmol/mg tissue)** | 76 +/− 6 | 130 +/− 14 $^{a}$ | 80 +/− 7 | 87 +/− 9 |
| **Liver QUIN (pmol/mg tissue)** | 3 +/− 0 | 3 +/− 0 | 3 +/− 0 | 3 +/− 0 |
| **Plasma Kynurenine (pmol/μL)** | 2 +/− 0 | 4 +/− 1 $^{a}$ | 1 +/− 0 | 1 +/− 0 $^{bb}$ |
| **Plasma KYNA (fmol/μL)** | 240 +/− 48 | 240 +/− 25 | 220 +/− 30 | 160 +/− 16 |
| **Plasma 3-HK (fmol/μL)** | 90 +/− 14 | 290 +/− 48 $^{aa}$ | 68 +/− 8 | 81 +/− 4 $^{bb}$ |

Values are the mean +/− SEM (n=8–10 per group). Statistical significance is indicated by letters:

$^{a}$(vs *Ido*$^{+/+}$, saline);

$^{b}$(vs *Ido*$^{+/+}$, LPS).

One symbol: $p<0.05$, two symbols: $p<0.01$.

**Table 6**

Enzyme activity in brain (IDO cohort)

| | ***Ido*$^{+/+}$, Saline** | ***Ido*$^{+/+}$, LPS** | ***Ido*$^{-/-}$, Saline** | ***Ido*$^{-/-}$, LPS** |
|---|---|---|---|---|
| **KAT I (pmol/h/mg tissue)** | 13 +/− 1 | 13 +/− 0 | 13 +/− 0 | 13 +/− 0 |
| **KAT II (pmol/h/mg tissue)** | 7 +/− 0 | 7 +/− 0 | 7 +/− 0 | 7 +/− 0 |
| **KYNU (pmol/h/mg tissue)** | 1 +/− 0 | 1 +/− 0 | 1 +/− 0 | 1 +/− 0 |
| **3-HAO (pmol/h/mg tissue)** | 10 +/− 1 | 10 +/− 0 | 9 +/− 2 | 9 +/− 1 |

Values are the mean +/− SEM (n=9–10 per group).

**Table 7**

Gene expression in brain and liver (IDO cohort)

| | ***Ido*$^{+/+}$, Saline** | ***Ido*$^{+/+}$, LPS** | ***Ido*$^{-/-}$, Saline** | ***Ido*$^{-/-}$, LPS** |
|---|---|---|---|---|
| **Brain IDO** | 1.0 +/− 0.0 | 1.2 +/− 0.1 *a* | -- | -- |
| **Brain TDO** | 1.0 +/− 0.1 | 0.9 +/− 0.0 | 1.0 +/− 0.1 | 1.0 +/− 0.1 |
| **Brain KMO** | 1.0 +/− 0.0 | 1.2 +/− 0.1 *a* | 1.1 +/− 0.0 | 1.1 +/− 0.1 |
| **Brain 3-HAO** | 1.0 +/− 0.1 | 1.1 +/− 0.1 | 1.0 +/− 0.1 | 0.9 +/− 0.1 |
| **Liver TDO** | 1.0 +/− 0.2 | 0.7 +/− 0.2 | 1.2 +/− 0.3 | 0.8 +/− 0.1 |
| **Liver KMO** | 1.0 +/− 0.1 | 1.2 +/− 0.1 | 1.2 +/− 0.1 | 1.2 +/− 0.1 |
| **Liver KYNU** | 1.0 +/− 0.1 | 0.6 +/− 0.1 *aa* | 1.0 +/− 0.1 | 0.6 +/− 0.1 *b* |
| **Liver 3-HAO** | 1.0 +/− 0.1 | 0.7 +/− 0.2 | 1.0 +/− 0.1 | 0.6 +/− 0.0 *b* |

Gene expression is presented relative to *Ido*$^{-/-}$, Saline. Values are the mean +/− SEM (n=6 –10 per group). Statistical significance is indicated by letters:

*a* (vs *Ido*$^{+/+}$, saline);

*b* (vs *Ido*$^{-/-}$, saline).

One symbol: $p<0.05$, two symbols: $p<0.01$.