

Original Research

Oxidative and Excitatory Neurotoxic Stresses in CRISPR/Cas9-Induced Kynurenine Aminotransferase Knockout Mice: A Novel Model for Despair-Based Depression and Post-Traumatic Stress Disorder

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Abstract

Backgrounds: Memory and emotion are especially vulnerable to psychiatric disorders such as post-traumatic stress disorder (PTSD), which is linked to disruptions in serotonin (5-HT) metabolism. Over 90% of the 5-HT precursor tryptophan (Trp) is metabolized via the Trp-kynurenine (KYN) metabolic pathway, which generates a variety of bioactive molecules. Dysregulation of KYN metabolism, particularly low levels of kynurenic acid (KYNA), appears to be linked to neuropsychiatric disorders. The majority of KYNA is produced by the *aadat* (*kat2*) gene-encoded mitochondrial kynurenine aminotransferase (KAT) isotype 2. Little is known about the consequences of deleting the KYN enzyme gene. **Methods:** In CRISPR/Cas9-induced *aadat* knockout (*kat2*^{−/−}) mice, we examined the effects on emotion, memory, motor function, Trp and its metabolite levels, enzyme activities in the plasma and urine of 8-week-old males compared to wild-type mice. **Results:** Transgenic mice showed more depressive-like behaviors in the forced swim test, but not in the tail suspension, anxiety, or memory tests. They also had fewer center field and corner entries, shorter walking distances, and fewer jumping counts in the open field test. Plasma metabolite levels are generally consistent with those of urine: antioxidant KYNs, 5-hydroxyindoleacetic acid, and indole-3-acetic acid levels were lower; enzyme activities in KATs, kynureninase, and monoamine oxidase/aldehyde dehydrogenase were lower, but kynurenine 3-monooxygenase was higher; and oxidative stress and excitotoxicity indices were higher. Transgenic mice displayed depression-like behavior in a learned helplessness model, emotional indifference, and motor deficits, coupled with a decrease in KYNA, a shift of Trp metabolism toward the KYN-3-hydroxykynurenine pathway, and a partial decrease in the gut microbial Trp-indole pathway metabolite. **Conclusions:** This is the first evidence that deleting the *aadat* gene induces depression-like behaviors uniquely linked to experiences of despair, which appear to be associated with excitatory neurotoxic and oxidative stresses. This may lead to the development of a double-hit preclinical model in despair-based depression, a better understanding of these complex conditions, and more effective therapeutic strategies by elucidating the relationship between Trp metabolism and PTSD pathogenesis.

Keywords: post-traumatic stress disorder (PTSD); depression; anxiety; tryptophan; kynurenine; microbiota; oxidative stress; transgenic mice; translational medical research; CRISPR/Cas9

1. Introduction

The interaction between memory and emotion involves a complex interplay of neural, cognitive, and physiological processes involving the amygdala, hippocampus, and prefrontal cortex [1–6]. Orderly function at multi-layered levels is essential to maintaining sound mental well-being [7–10]. The reciprocal interaction between cogni-

tive function and affective states can significantly impact each other. Cognitive impairment can lead to affective disturbances, triggering emotional responses such as frustration, anxiety, and stress, particularly when individuals feel a loss of control over their cognitive abilities [11]. Similarly, emotional disturbances such as depression and anxiety can influence memory function, increasing vulnerability



to cognitive challenges [12–15]. This intricate bidirectional link between cognition and emotions can lead to changes in brain structure, function, behavior, lifestyle, and neurotransmitter systems [15–17]. Memory impairment and emotional disturbance are associated with a wide range of systematic diseases and neuropsychiatric disorders such as Alzheimer's disease (AD), Parkinson's disease, traumatic brain injury, major depressive disorder (MDD), and post-traumatic stress disorder (PTSD) [18–26].

The serotonergic nervous system plays an important role in regulating mood, anxiety, and cognition [27–30]. Serotonin (5-hydroxytryptamine, 5-HT) is involved in cognitive processes such as attention, learning, and memory [31–34]. Studies indicate that 5-HT enhances long-term memory consolidation and improves cognitive flexibility, which is the ability to switch between different cognitive tasks or mental sets [35–44]. 5-HT is implicated in regulating mood and anxiety, influencing cognitive function [45,46]. Mental illnesses like MDD, eating disorders, obsessive-compulsive disorder, schizophrenia (SCZ), and PTSD are associated with dysregulation of 5-HT [47–52]. Selective serotonin reuptake inhibitors (SSRIs) are commonly used for these conditions, targeting the serotonergic nervous system [53–55]. Furthermore, abnormalities in the serotonergic system also affect norepinephrine and dopamine [56–58].

The complex interplay of tryptophan (Trp)-kynurenine (KYN) and 5-HT metabolism is crucial for comprehending the pathogenesis of mental illnesses [59,60]. The Trp-KYN metabolic system, closely associated with 5-HT metabolism, plays a pivotal role in the production of prooxidants and antioxidants, regulation of the immune system, and the balance between neurotoxicity and neuroprotection [61,62]. Approximately 2% of L-Trp undergoes metabolism through the 5-HT metabolic pathway; however, over 90% of Trp is catabolized through the KYN route, which safely to say that it governs Trp metabolism (Fig. 1a,b, Ref. [63–83]) [84]. Various factors, including stress, inflammation, and the gut microbiome, influence this system [85–88]. Dysregulation of the KYN route has been linked to mental health conditions such as MDD, SCZ, and AD [89]. About 5% of dietary Trp is converted by gut bacteria, like *E. coli* and *Clostridium sporogenes*, into indole and its derivatives (e.g., indole-3-acetic acid, indoxyl sulfate) (Fig. 1c) [63,90–98]. Disruptions in this pathway are linked to gastrointestinal and liver conditions (e.g., colorectal cancer, irritable bowel syndrome, non-alcoholic fatty liver disease, hepatic encephalopathy) and affect brain neurotransmitters and communication via the vagus nerve [64,95,99–109]. The gut microbial indole pathway is increasingly recognized for its role in mental health disorders like depression, anxiety, autism, SCZ, and AD [103,110–116].

However, the understanding of the interplay between Trp-KYN, 5-HT, and indole metabolism in the patho-

genesis of mental illnesses remains limited. Kynurenine aminotransferases (KATs) are members of the pyridoxal-5'-phosphate-dependent enzyme family involved in the KYN metabolic pathway. The KYN metabolism is responsible for the conversion of L-KYN to kynurenic acid (KYNA), an antioxidant and neuroprotective metabolite with implications for various central nervous system (CNS) diseases [73,117,118]. Among the KAT enzymes, kynurenine/alpha-aminoadipate aminotransferase (KAT/AadAT, aka KAT II) is a mitochondrial enzyme encoded in the gene *aadat* (*kat2*) [119]. KAT II is considered to play the most important role among the four isozymes in the cellular environment due to its highest enzymatic activity close to the physiological pH. Thus, KAT II plays a prominent role in KYNA production in the human brain and is considered a crucial target for managing CNS disorders [120].

Preclinical research has significantly contributed to our understanding of mental illnesses by elucidating the underlying pathomechanisms and identifying potential therapeutic targets [121–129]. Researchers have employed preclinical animal models to examine the causes and effects of mental disorders, thereby attaining a comprehensive understanding of their underlying pathology [130–137]. *In vitro* models, such as cell cultures and organoids, have facilitated the investigation of complex molecular pathways linked to mental disorders [138–141]. Animal models, along with other *in vivo* models, have been instrumental in studying the behavioral, cognitive, and physiological dimensions of mental disorders [142–148]. These models allow researchers to simulate disease conditions, assess symptomatology, and evaluate the efficacy of potential interventions [148,149]. Transgenic animals are vital in biomedical research, enabling the replication of human conditions through gene deletion or the introduction of altered genes into their genome [150]. These animals offer indispensable insights into human diseases, facilitating the exploration of disease mechanisms, experimentation with potential treatments, and assessment of therapeutic effectiveness [151–155]. Moreover, they offer crucial insights into changes in structure and imaging techniques in clinical cases [156–175]. Preclinical and clinical research collaboratively contribute to innovative therapeutics and personalized medicine [176–182].

This study involved manipulating the gene *kat2* in mice to create a knockout (*kat2*^{-/-}) model, allowing us to observe the behavioral consequences of KAT II deficiency. By focusing on negative valence in emotional domain, memory acquisition, and motor function, we aimed to gain insights into the role of KAT II in these specific behavioral domains in young adult *kat2*^{-/-} mice. Furthermore, we assess the levels of Trp and its metabolites in three distinct metabolic pathways in both plasma and urine samples, the enzyme activities of Trp metabolism, and the oxidative stress and excitotoxicity indices of KYN metabolites, with

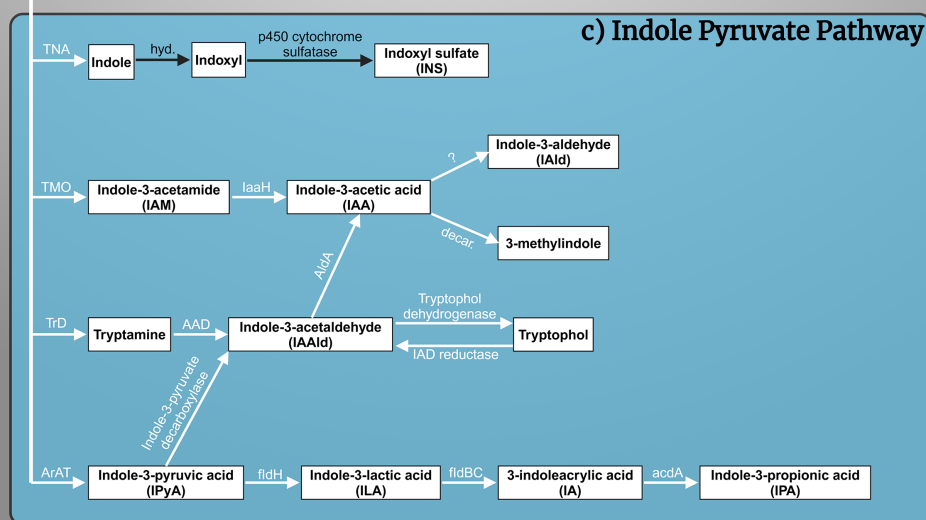
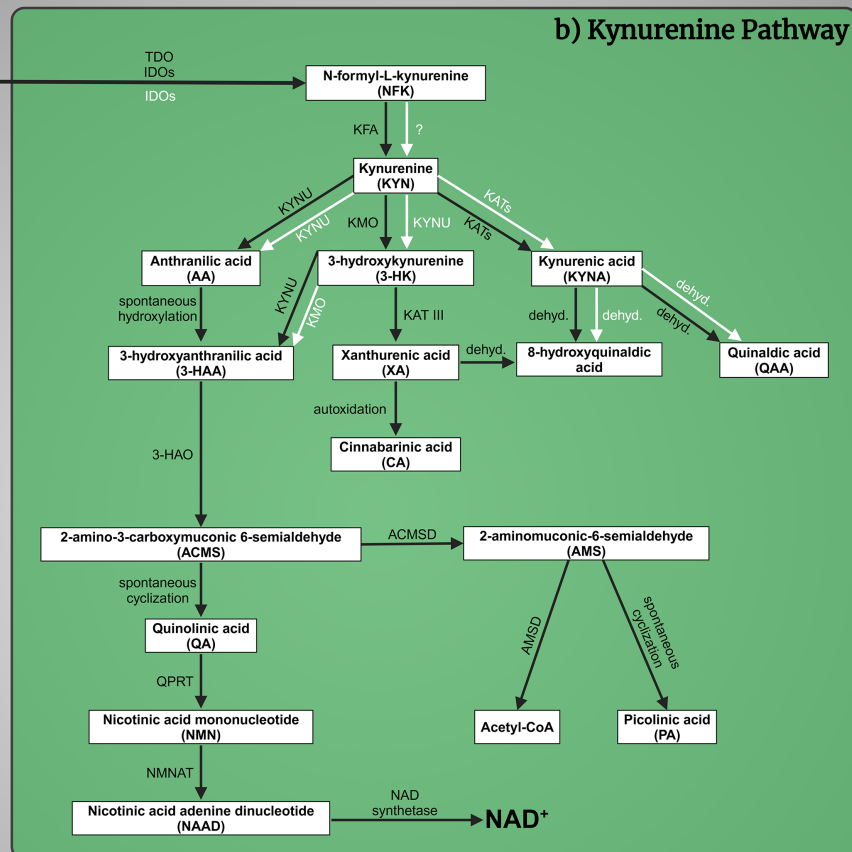
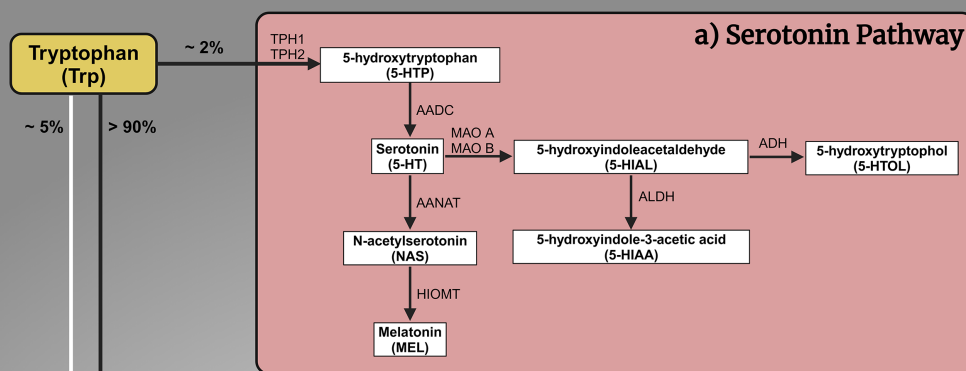


Fig. 1. Tryptophan (Trp) metabolism. (a) The serotonin (5-HT) pathway: A fraction exceeding 2% of L-Trp is metabolized within the 5-HT pathway. The rate-limiting enzyme tryptophan hydroxylase 1 and 2 (TPH1, TPH2) converts Trp to 5-hydroxytryptophan (5-HTP), which is then decarboxylated by aromatic L-amino acid decarboxylase (AADC) to 5-HT. 5-HT is oxidized by monoamine oxidase A and B (MAO A, MAO B) in different tissues to 5-hydroxyindoleacetaldehyde (5-HIAL), which is subsequently further oxidized to 5-hydroxyindoleacetic acid (5-HIAA) by aldehyde dehydrogenase (ALDH) or reduced to 5-hydroxytryptophol (5-HTOL) by alcohol dehydrogenase (ADH). 5-HIAA is the main metabolite and a marker of serotonergic activity, whereas 5-HTOL is a minor pathway of 5-HT degradation [65]. On the other hand, 5-HT synthesizes melatonin (MEL, N-acetyl-5-methoxytryptamine). First, 5-HT is converted into N-acetylserotonin (NAS) by arylalkylamine N-acetyltransferase (AANAT), then hydroxyindole-O-methyltransferase (HIOMT) transform MEL [66–69]. (b) The kynurenine (KYN) pathway: More than 90% of Trp enters the KYN pathway, which produces a variety of biomolecules. The primary metabolites include N-formyl-L-kynurenine (NFK), KYN, kynurenic acid (KYNA), anthranilic acid (AA), 3-hydroxykynurenine (3-HK), xanthurenic acid (XA), 3-hydroxyanthranilic acid (3-HAA), quinolinic acid (QA), picolinic acid (PA), and nicotinamide adenine dinucleotide (NAD^+). These metabolites are produced through the catalytic actions of various enzymes, namely tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenases (IDOs), kynurenine formamidase (KFA), kynurenine 3-monooxygenase (KMO), kynurenine aminotransferases (KATs), kynureninase (KYNU), 3-hydroxyanthranilate oxidase (3-HAO), quinolinate phosphoribosyl transferase (QPRT) [70], nicotinamide mononucleotide adenylyltransferase (NMNAT) [71], NAD synthetase [72], amino- β -carboxymuconate-semialdehyde-decarboxylase (ACMSD) and 2-aminomuconic-6-semialdehyde dehydrogenase (AMSD) [73–75]. KYNA is subsequently metabolized by the gut microbiome to quinaldic acid (QAA) and 8-hydroxyquinaldic acid [76]. 8-hydroxyquinaldic acid can be dehydroxylated from XA [77,78]. (c) The gut microbial indole pyruvate pathway: The metabolism of Trp is accomplished through four distinct pathways, which include the indoxyl sulfate pathway, the indole-3-acetamide (IAM) pathway, the tryptamine pathway, and the indole-3-propionic acid (IPA) pathway. The pyridoxal phosphate-dependent tryptophanase (TNA) enzyme serves as the rate-limiting component of the indoxyl sulfate pathway. Its primary function is to facilitate the transformation of Trp into indole, which is passing through the gut epithelium, then hydroxylated into 3-hydroxyindole (indoxyl) and ultimately transformed into indoxyl sulfate (INS) by p450 cytochrome and sulfanate in the liver [79]. In the IAM pathway, tryptophan-2-monooxygenase (TMO) catalyzes the conversion of Trp to IAM. This is followed by the conversion of IAM to indole-3-acetic acid (IAA) by indole-3-acetamide hydrolase (IaaH), which can then be further metabolized into indole-3-aldehyde (IAld) or decarboxylized into 3-methylindole (skatole) [63,80]. Tryptophan decarboxylase (TrD) catalyzes the conversion of Trp to tryptamine by amino acid decarboxylase (AAD), which subsequently undergoes conversion into indole-3-acetaldehyde (IAAld) [81]. IAAld can be further converted into IAA by indole-3-acetaldehyde dehydrogenase (AldA). It is also worth noting that IAAld can be reversibly converted into indole-3-ethanol (tryptophol) by IAD reductase and tryptophol dehydrogenase [82]. The transformation of Trp into indole-3-pyruvic acid (IPyA) is catalyzed by aromatic amino acid aminotransferase (ArAT), resulting in the formation of either tryptamine, or indole-3-lactic acid (ILA) by phenyllactate dehydrogenase (fldH), then 3-indoleacrylic acid (IA) by phenyllactate dehydratase (fldBC), and ultimately IPA by acyl-coenzim A dehydrogenase (acdA) [64,82,83]. Black arrows: the host pathways, yellow arrows: the gut microbiome pathways. AA, anthranilic acid; acdA, acyl-coenzim A dehydrogenase; AAD, amino acid decarboxylase; AADC, aromatic L-amino acid decarboxylase; AANAT, arylalkylamine N-acetyltransferase; ACMSD, amino- β -carboxymuconate-semialdehyde-decarboxylase; ADH, alcohol dehydrogenase; AldA, indole-3-acetaldehyde dehydrogenase; ALDH, aldehyde dehydrogenase; AMSD, 2-aminomuconic-6-semialdehyde dehydrogenase; ArAT, aromatic amino acid aminotransferase; decar., decarboxylation; dehyd., dehydroxylation; fldBC, phenyllactate dehydratase; fldH, phenyllactate dehydrogenase; 3-HAA, 3-hydroxyanthranilic acid; 3-HAO, 3-hydroxyanthranilate oxidase; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HIAL, 5-hydroxyindoleacetaldehyde; HIOMT, hydroxyindole-O-methyltransferase; 3-HK, 3-hydroxykynurenine; 5-HT, serotonin/5-hydroxytryptamine; 5-HTOL, 5-hydroxytryptophol; 5-HTP, 5-hydroxytryptophan; hyd., hydroxylation; IA, 3-indoleacrylic acid; IAA, indole-3-acetic acid; IaaH, indole-3-acetamide hydrolase; IAAld, indole-3-acetaldehyde; IAld, indole-3-aldehyde; IAA, indole-3-acetic acid; IAM, indole-3-acetamide; IDOs, indoleamine 2,3-dioxygenases 1 and 2; ILA, indole-3-lactic acid; INS, indoxyl sulfate; IPA, indole-3-propionate; IPyA, indole-3-pyruvic acid; KAT III, kynurenine aminotransferase III/cysteine conjugate beta-lyase 2; KATs, kynurenine aminotransferases; KFA, kynurenine formamidase; KMO, kynurenine 3-monooxygenase; KYN, kynurenine; KYNA, kynurenic acid; KYNU, kynureninase; MAO A, monoamine oxidase A; MAO B, monoamine oxidase B; MEL, melatonin/N-acetyl-5-methoxytryptamine; NAAD, nicotinic acid adenine dinucleotide; NAD^+ , nicotinamide adenine dinucleotide; NAS, N-acetylserotonin; NFK, N-formyl-L-kynurenine; NMN, nicotinic acid mononucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase; PA, picolinic acid; QA, quinolinic acid; QAA, quinaldic acid; QPRT, quinolinate phosphoribosyl transferase; TDO, tryptophan-2,3-dioxygenase; TMO, tryptophan-2-monooxygenase; TNA, tryptophanase; TrD, tryptophan decarboxylase; Trp, tryptophan; TPH1/2, tryptophan hydroxylase 1 and 2; XA, xanthurenic acid; ?, unknown. The figure was created with Scientific Image and Illustration Software [Biorender](#).

the aim of elucidating the Trp metabolic profiles that underlie the behavioral phenotype. This research contributes to our understanding of the genetic factors influencing behaviors related to emotional valence, memory, and motor function and Trp catabolism.

2. Materials and Methods

CRISPR/Cas9 was applied on C57BL/6N and CD1 (ICR; Institute for Cancer Research) mice to generate knockout *kat2*^{-/-} mice, and Taqman allelic discrimination was used to prove that the gene had been deleted. The emotional domain, including depression-like and anxiety-like behaviors, was evaluated with the modified forced swim test (FST), tail suspension test (TST), elevated plus maze (EPM) test, open field (OF) test, and light dark box (LDB) test; the cognitive domain was evaluated with the passive avoidance test (PAT); and the motor domain was evaluated with the OF test. Furthermore, the levels of Trp and its major metabolites, as well as enzyme activities in plasma and urine samples, were determined, and oxidative stress and excitotoxicity indices were calculated.

2.1 Ethical Approval

Animal experiments were conducted humanely in accordance with the Regulations for Animal Experiments of Kyushu University and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions governed by the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and with the approval of the Institutional Animal Experiment Committees of Kyushu University (A29-338-1 (2018), A19-090-1 (2019)). The Department of Nature Conservation of the Ministry of Agriculture has authorized us to use genetically modified organisms in a closed system of the second security isolation level (TMF/43-20/2015). The import of genetically modified animals has been approved by the Department of Biodiversity and Gene Conservation of the Ministry of Agriculture (BGMF/37-5/2020). In accordance with the guidelines of the 8th Edition of the Guide for the Care and Use of Laboratory Animals, the Use of Animals in Research of the International Association for the Study of Pain, and the directive of the European Economic Community (2010/63/EU), the experiments conducted in this study received ethical approval from two committees. The Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XI/95/2020, CS/I01/170-4/2022) and the Committee of Animal Research at the University of Szeged (I-74-10/2019, I-74-1/2022) both approved the experiments. Furthermore, Directive 2010/63/EU on the protection of animals used for scientific purposes provides guidance for the ethical evaluation of animal use proposals. The directive allows individual institutions to make determinations based on the recommendations of their ethical review committees. These ethical guidelines and regulations

ensure that the experiments conducted on animals adhere to the highest standards of animal welfare and scientific integrity. The approval from the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board and the Committee of Animal Research at the University of Szeged demonstrates that the study was conducted in compliance with these ethical principles and regulations.

2.2 Animals

C57BL/6N and CD1 (ICR) mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and Charles River Laboratories International, Inc. (Yokohama, Japan), respectively, in order to generate *kat2*^{-/-} mice utilizing the CRISPR/Cas9 technique. After genetic modifications, breeding, and transport from Japan to Hungary, the animals were housed in groups of 4–5 in polycarbonate cages (530 cm² floor space) under pathogen-free conditions in the Animal House of the Department of Neurology, University of Szeged, maintained at 24 ± 1 °C and 45–55% relative humidity under a 12:12-h light:dark cycle. Throughout the duration of the investigation, mice had unrestricted access to standard rodent food and water.

The deletion was introduced into the KATs gene using the CRISPR/Cas9 method. The single guide RNAs (sgRNA) were selected using the CRISPRdirect software [183]. Artificially synthesized sgRNA were purchased from FASMAC (Atsugi, Japan). The 8–12 weeks old female C57BL/6N mice were injected with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) with a 48-h interval, and mated with 8–20 weeks old male C57BL/6N mice. The fertilized one-cell embryos were collected from the oviducts. Then, 25 ng/μL of the sgRNA and 75 ng/μL Guide-it™ Recombinant Cas9 protein (TaKaRa, Kusatsu, Japan) were injected into the cytoplasm of these one-cell-stage embryos. The injected two-cell embryos were then transferred into pseudo-pregnant ICR mice (Fig. 2) anesthetized with a combination anesthetic (M/M/B: 0.3/4/5) [184] prepared with 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol by intraperitoneal injection.

The *kat2*^{-/-} mouse line expresses a carboxy-terminal truncated polypeptide consisting of the first 47 amino acids of the intact KAT II with a 2-nucleotide deletion (CCDS nucleotide sequence 32–33) in the mRNA.

2.3 DNA Extraction and Sequencing

Genomic DNA of tails collected from mice was extracted using NucleoSpin Tissue (MACHEREY-NAGEL GmbH&Co, KG, Düren, Germany). Each targeted fragment around the sgRNA targeting site from the extracted genomic DNA as a part of the KATs genes was amplified with TAKARA Ex Taq (Takara Bio, Kusatsu, Japan) and the 1st primers pair and subsequently with 2nd primers pair (Table 1). The polymerase chain reaction (PCR) product was

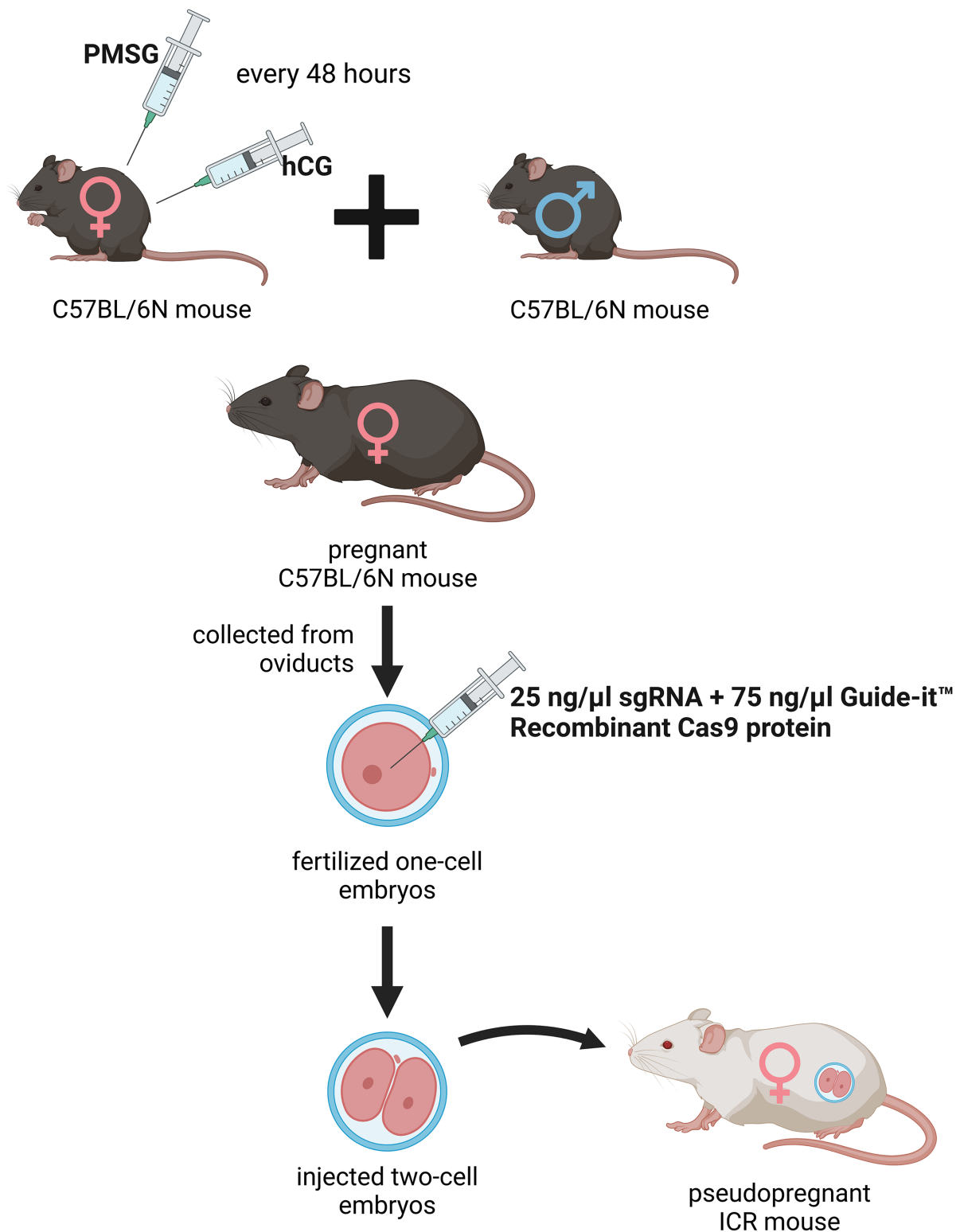


Fig. 2. Generation of the knockout *kat2*^{-/-} mice. Female C57BL/6N mice were treated with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) with a 48-hour interval between administrations, then mated with male C57BL/6N mice. From the oviducts, fertilized one-cell embryos were collected and injected with single guide RNA (sgRNA) and Guide-it™ Recombinant Cas9 protein. At the two-cell stage, the embryos were transferred into pseudopregnant Institute for Cancer Research (ICR) mice. PMSG, pregnant mare serum gonadotropin; hCG, human chorionic gonadotropin; sgRNA, single guide RNA. The figure was created with Scientific Image and Illustration Software [Biorender](#).

Table 1. Properties of sgRNA, primers and KAT gene.

Name of sgRNA		Sequence	
M-KAT II-2		GTTCTCACTGCAACGAGCCguuuuagagcuagaaauagcaaguu- aaaaaaggcuaguccguuaucaacuugaaaaaguggcacggacucggugcuuuu	
Name of primer		Sequence	
M-KAT II_1st_F		CCCTCTGTGGATGGACTTTG	
M-KAT II_1st_R		TTGAAAGATGTGCCTCATGC	
M-KAT II_2nd_F		GGATGGACTTTGTCCCTTCT	
M-KAT II_2nd_R		ATGTGCCTCATGCTTGGCCC	
Name of KAT gene	Transcript ID	CCDS	CCDS Nucleotide Sequence
<i>Aadat-201</i>	ENSMUST00000079472.4	CCDS22320	32–33 (2 nucleotide deletion)

sgRNA, single guide RNA; KAT, kynurenine aminotransferase; KAT II, aminoadipate aminotransferase; CCDS, Consensus Coding Sequence.

purified with a Fast Gene Gel/PCR Extraction Kit (Nippon Genetics Co., Ltd., Tokyo, Japan), and the PCR products were purified by agarose gel electrophoresis and Monarch Gel Extraction Kit (NEW ENGLAND BioLabs Inc., Ipswich, MA, USA). Then, the PCR products were sequenced with M-KAT II_2nd_R (Table 1).

2.4 Western Blotting

For Western blotting, tissue extracts from the liver (20 mg) of the knockout and wild-type (WT) mice were prepared by the Total Protein Extraction Kit for Animal Cultured Cells and Tissues (Invent Biotechnologies, Plymouth, MN, USA) according to the manufacturer's instructions. Subsequently, the tissue extracts were passed through Protein G HP SpinTrap™ (Cytiva, Buckinghamshire, UK) to remove immunoglobulin G. 14 µL of each sample were mixed with 7 µL of SDS Blue Loading Buffer (New England BioLabs Inc.) and separated on a 12% SDS-polyacrylamide gel. Subsequently, the protein was transferred to the membranes. The membranes were blocked and incubated with anti-human KAT II rabbit polyclonal antibody (1:500, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 2 h, followed by combination with alkaline phosphatase-labeled secondary goat anti-rabbit immunoglobulin G (IgG) FC antibody (1:10,000, Sigma-Aldrich Co. LLC, St. Louis, MO, USA) at room temperature for 2 h, followed by visualization of dystrophin and utrophin using Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega, Madison, WI, USA). The Multicolor Protein Ladder (10–315 kDa) from Nippon Gene Co., Ltd. (Tokyo, Japan) was used as a molecular weight marker for western blotting, allowing visualization and size estimation of target proteins.

2.5 Phenotype Analysis with Modified SHIRPA Test

The 8–48 weeks old male and female mice mated in August 2023 and became pregnant about three to four weeks later. The RIKEN (The Institute of Physical and Chemical Research) modified SHIRPA (SmithKline

Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment) test was conducted to ascertain the comprehensive phenotypic traits of the mutant rodents. The assessment included the evaluation of diverse behaviors and physical attributes such as motion, bowel movements, urination, locomotor activity, startle response, tactile escape, pinna reflex, trunk curling, limb grasping, contact-righting reflex, grip strength, wire maneuver test, corneal reflex, toe pinching, and overall appearance. The animals were also monitored for vocalization, aggression, head bobbing, jumping, circling, retropulsion, grooming, and tail-wagging [185,186]. The experiment was captured on video using a camera (Basler ace Classic acA1300 - 60gm, Basler AG, Ahrensburg, Germany) and software (EthoVision XT14, Noldus Information Technology BV, Wageningen, the Netherlands).

2.6 Behavioral Tests

The 8–48 weeks old male and female mice mated between April 2021 and April 2022 and became pregnant approximately three to four weeks later. 8-week-old male C57BL/6N and *kat2^{-/-}* mice (n = 10–13) were tested. In order to make the results comparable, all behavioral experiments were performed between 8 a.m. and 12 p.m. The animals were transferred to the laboratory, where the measurements were made, one hour before the start of the experiment, thus they had time to acclimatize to the environmental conditions.

2.6.1 Modified Forced Swim Test (FST)

The modified FST was performed as reported previously. The mice were placed individually in a glass cylinder of 12 cm in diameter and 30 cm in height. Water (25 ± 1 °C) was filled to a height of 20 cm. Fresh water was used for each mouse. A 15-min pretest was carried out 24 hours before the 3-min test session. A time-sampling technique was conducted to count the duration of time spent with climbing, swimming, and immobility [187,188].

2.6.2 Tail Suspension Test (TST)

The mice were placed in a $28 \times 28 \times 23.5$ cm wooden box with three side walls and a clip hanging from the top of the box. The animals were suspended by their tails from the base to the middle two-thirds using a clip and allowed to hang for 6 minutes. We measure the duration of immobility. A cotton swab was pre-attached to the clip's interior to prevent the mice's tails from injuring or severely restricting blood circulation. If the animal is able to climb or falls off the clip, it is removed from the experiment and its results are discarded [189,190]. The experiment was captured on video using a camera (Basler ace Classic acA1300 -60gm, Basler AG, Ahrensburg, Germany) and software (EthoVision XT14, Noldus Information Technology BV, Wageningen, the Netherlands).

2.6.3 Elevated Plus Maze (EPM) Test

The animals were positioned in a plus-shaped apparatus with four arms measuring 35×10 cm. Two of the opposite arms are open, while the other two are closed, forming an angle of 90 degrees. The open arms have no side walls, while the closed arms have walls that are 20 cm tall. The entire apparatus is situated 50 cm off the ground. The device is surrounded by a screen that does not display any visual signals. The mouse was placed in the device's center with its nose facing an open arm and allowed it to explore for 5 minutes. We measure the time spent in each part (open arms, closed arms, and central part). The experiment was captured on video using a camera (Basler ace Classic acA1300 -60gm, Basler AG, Ahrensburg, Germany) and software (EthoVision XT14, Noldus Information Technology BV, Wageningen, the Netherlands). Between each animal, the apparatus was disinfected with 70% ethanol and left exposed to the air for 5 minutes [191,192].

2.6.4 Light Dark Box (LDB) Test

The LDB apparatus is comprised of larger illuminated (2/3 of the box) and smaller dark (1/3 of the box) compartments that are connected by a 5×5 cm door. The length of time a mouse spent in the lighted compartment during the 5-minute session was determined 5 seconds after a mouse was placed in the bright area. After each session, the box was cleaned with 70% ethanol and allowed to air for 5 minutes [192–194].

2.6.5 Passive Avoidance Test (PAT)

Each mouse was individually placed in a box containing two apparatuses with distinct lighting. The animals began in the bright compartment and had 5 minutes to pass through the 5×5 cm door into the dark, smaller portion of the box. As soon as the animals entered the dark compartment, they received a 0.3 mA electroshock through their paws, and the door shut. After 10 seconds, the animals were removed, and the experiment was repeated 24 hours later. Those animals that did not enter the dark area within

5 minutes during the pre-testing phase were omitted from the measurement. The box was cleaned with 70% ethanol and left to air for 5 minutes between mice [195].

2.6.6 Open Field (OF) Test

A standard table lamp illuminated the center of the 48×40 cm OF box, while the Conducta 1.0 system (Experimetria Ltd., Budapest, Hungary) monitored the mouse's movements. Each mouse was placed individually in the center of the box. Ambulation distance, time spent in the center zone, and number of entries to the center zone were measured for 10 minutes. After each session, the box was wiped down with 70% ethanol and allowed to for 5 minutes [196,197].

Throughout the experiment, the animals' general physical condition was constantly assessed using a scoring scale, which included body weight, appearance and overall condition, respiration, mobility, and the presence of basic reflexes. Humane endpoints were determined using the scales. If any animal reached the required score for withdrawal from the behavioral assessments, it was euthanized via transcardial perfusion under isoflurane anesthesia, effectively terminating its participation in the evaluation.

2.7 Ultra-High-Performance Liquid Chromatography with Tandem Mass Spectrometry

The 8–48 weeks old male and female mice mated in August 2023 and became pregnant about three to four weeks later. The urine samples were collected before anesthesia, and were immediately stored at -80°C after the sample collection. For plasma collection, the mice were anesthetized with 2% isoflurane, and after exposing their chest, blood samples were taken from the left heart ventricle using a syringe into Eppendorf tubes containing disodium ethylenediaminetetraacetate dihydrate. Plasma was separated by centrifugation (10,300 rpm for 10 minutes at 4°C). The supernatant plasma samples were pipetted into new Eppendorf tubes. The samples were stored at -80°C until use. The animals were perfused with artificial cerebrospinal fluid for 5 minutes to remove additional organs for later use. Trp and its metabolites were measured in plasma and urine using previously published protocols [198,199] using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Picolinic acid multiple reaction monitoring (MRM) showed a change from 124.0 to 106.0 over 1.21 minutes, with 75 V acting as the declustering potential and 13 V acting as the collision energy. All reagents and chemicals were of analytical or liquid chromatography–mass spectrometry grade. Trp and its metabolites, and their deuterated forms: d4-serotonin, d5-tryptophan, d4-kynurenine, d5-kynurenine acid, d4-xanthurenic acid, d5-5-hydroxyindole-acetic acid, d3-3-hydroxyanthranilic acid, d4-picolinic acid, and d3-quinolinic acid were purchased from Toronto Research Chemicals (Toronto, ON, Canada).

d3-3-hydroxykynurenine was obtained from Buchem B. V. (Apeldoorn, The Netherlands). Acetonitrile (ACN) was provided by Molar Chemicals (Halásztelek, Hungary). Methanol (MeOH) was purchased from LGC Standards (Wesel, Germany). Formic acid (FA) and water were obtained from VWR Chemicals (Monroeville, PA, USA). The UHPLC-MS/MS system consisted of a PerkinElmer Flexar UHPLC system (two FX-10 binary pumps, solvent manager, autosampler and thermostatic oven; all PerkinElmer Inc. (Waltham, MA, USA)), coupled to an AB SCIEX QTRAP 5500 MS/MS triple quadrupole mass spectrometer and controlled by Analyst 1.7.1 software (both AB Sciex, Framingham, MA, USA).

2.8 The Enzyme Activities of Tryptophan (Trp) Metabolism

The enzyme activities of each Trp metabolism were determined by dividing the concentration of the product by the concentration of the substrate.

2.9 Oxidative Stress and Excitotoxicity Indices

The oxidative stress index was calculated as the ratios of putative prooxidant metabolite 3-hydroxykynurenine (3-HK) concentrations to the sums of putative antioxidant metabolite concentrations (KYNA, anthranilic acid (AA), and xanthurenic acid (XA)) (Eqn. 1) [200–202].

$$\text{Oxidative stress index} = \frac{[3 - \text{Hydroxykynurenine}]}{\{[\text{Kynurenic acid}] + [\text{Anthranilic acid}] + [\text{Xanthurenic acid}]\}} \quad (1)$$

The excitotoxicity index is calculated by dividing the concentration of N-methyl-D-aspartate (NMDA) receptor agonist quinolinic acid (QA) by that of NMDA receptor antagonist KYNA (Eqn. 2) [203–205].

$$\text{Excitotoxicity index} = \frac{[\text{Quinolinic acid}]}{[\text{Kynurenic acid}]} \quad (2)$$

2.10 Statistical Analysis

We used IBM SPSS Statistics 28.0.0.0 (IBM SPSS statistics, Chicago, IL, USA) for the statistical analysis. The Shapiro–Wilk test was used to determine the distribution of data. In addition, we used a Q-Q plot to find out if two sets of data come from the same distribution. Our data followed a normal distribution. One-way ANOVA test was used to evaluate the results of the behavioral tests and neurochemical measurements followed by the Tamhane post hoc test. Values $p < 0.05$ were considered statistically significant. Our data are reported as means $\pm$ SD for all parameters and groups.

3. Results

3.1 DNA Sequence Analysis and Western Blot

To generate knockout mice of *kat2* gene, 25 ng/ μ L of sgRNA and 75 ng/ μ L Cas9 protein were injected into

the cytoplasm of the one-cell-stage embryos. Sequencing analyses with their founder mice showed that various deletions and/or insertions were introduced in the target sequence. One of the founders was selected and established the homozygous mouse line for further analyses. KAT II knockout mouse line expresses a carboxy-terminal truncated polypeptide consisting of the first 47 amino acids of the intact KAT II with 2 nucleotides deletion (CCDS nucleotide sequence 32–33) in the mRNA. Western blotting with antibodies against KAT II revealed that the band with approximately 50-kDa supposed to be KAT II was not detected in the knockout mice, while it was detected in the wild-type (WT) counterparts (Fig. 3).

3.2 Phenotype Analysis with SHIRPA Protocol

We did not detect any significant differences between the knockout mice and their wild-type counterparts.

3.3 Behavioral Tests

3.3.1 Forced Swim Test (FST)

The immobility time was significantly longer and the swimming time was significantly shorter in *kat2*^{−/−} mice than in WT mice (Fig. 4a,b; Table 2). There were no significant differences in climbing time (Table 2).

3.3.2 Open Field (OF) Test

The ambulation distance of the *kat2*^{−/−} mice was significantly shorter in the first 10-minute timeframe than that of their WT counterparts (Fig. 4c; Table 2). The number of jumps was significantly fewer in the *kat2*^{−/−} mice than that of their WT counterparts (Fig. 4d; Table 2). There were significantly fewer entries into the center and corner zones compared to their WT counterparts (Fig. 4e; Table 2).

3.3.3 Other Behavioral Tests

There were no statistically significant distinctions observed between the transgenic mice and their WT counterparts in TST, PAT, EPM test, and LDB test (Table 2).

3.4 Ultra-High-Performance Liquid Chromatography with Tandem Mass Spectrometry

Transgenic mice had significantly lower levels of KYN, KYNA, XA, AA, 5-hydroxyindoleacetic acid (5-HIAA), indole-3-acetic acid (IAA), and higher levels of 3-HK in plasma samples than wild-type mice. In urine samples, KYNA, XA, and IAA were significantly lower, whereas KYN, 3-HK, and 5-HT were significantly higher than those of the wild-type counterparts (Fig. 5; Table 3).

3.5 Enzyme Activities in Tryptophan (Trp) Metabolism

The transgenic mice showed significantly lower KATs, kynureninase (KYNU), KAT III, monoamine oxidase (MAO), aldehyde dehydrogenase (ALDH), and tryptophan-2-monooxygenase (TMO) activities and significantly higher kynurenine 3-monooxygenase (KMO) activ-

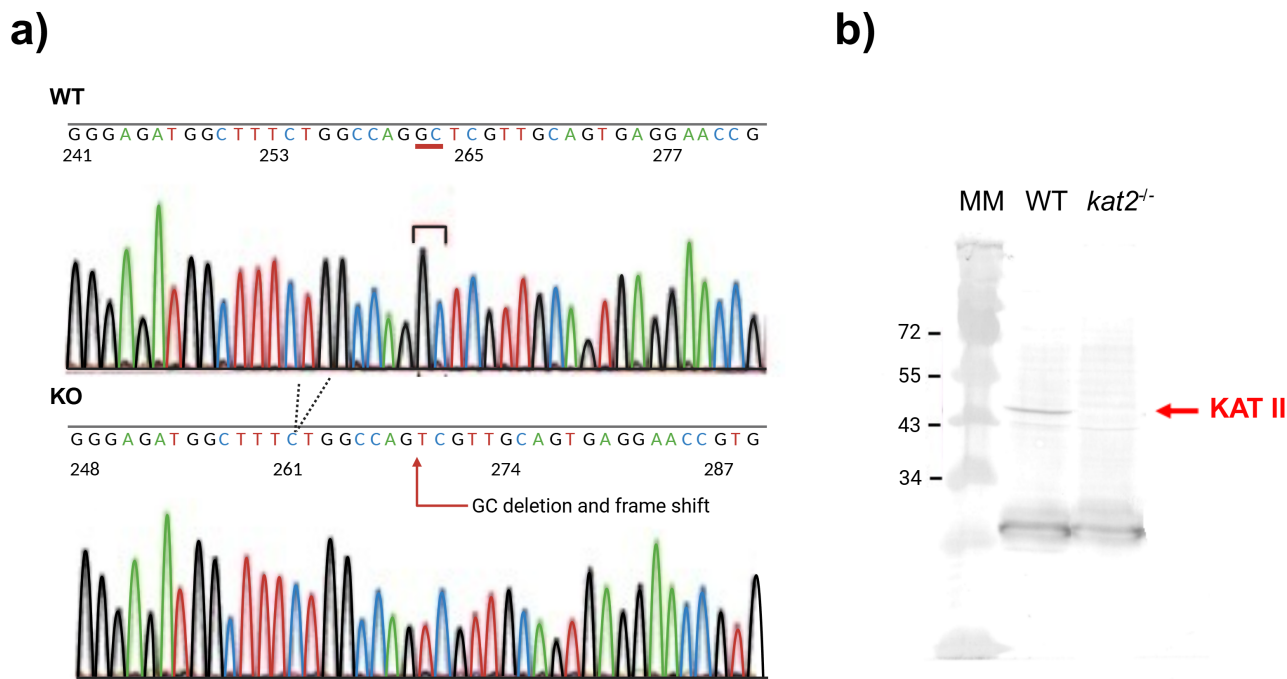


Fig. 3. DNA sequence and western blot analysis of knockout *kat2*^{-/-} mouse line. (a) Genomic sequences around the mutation site of knockout *kat2*^{-/-} mouse strain. (b) Western blot analysis of knockout *kat2*^{-/-} mouse line. MM: molecular weight marker, WT: wild-type mouse. The figure was created with Scientific Image and Illustration Software [Biorender](#).

Table 2. Behaviors of *kat2*^{-/-} mice and the wild-type counterparts.

Test type	Number of animals	Perspectives	Mean ± SD of wild-type	Mean ± SD of <i>kat2</i> ^{-/-}	<i>p</i> -value
Modified forced swim test (FST)	WT: n = 12	Immobility time (s)	157.73 ± 17.23	174.09 ± 6.64	0.022 *
	<i>kat2</i> ^{-/-} : n = 11	Swimming time (s)	18.18 ± 15.37	3.18 ± 4.62	0.014 *
		Climbing time (s)	4.09 ± 4.37	1.82 ± 6.03	0.681
Tail suspension test (TST)	WT: n = 10 <i>kat2</i> ^{-/-} : n = 13	Immobility time (s)	194.50 ± 66.76	209.58 ± 67.23	0.625
Passive avoidance test (PAT)	WT: n = 12	Time spent in the lit box on the training day (s)	48.33 ± 29.24	64.67 ± 55.78	0.979
	<i>kat2</i> ^{-/-} : n = 12	Time spent in the lit box on the test day (s)	256.00 ± 76.94	283.75 ± 39.60	0.822
Elevated plus maze (EPM) test	WT: n = 10 <i>kat2</i> ^{-/-} : n = 11	Time spent in the open arms (s)	42.90 ± 61.60	30.64 ± 43.70	0.500
Light dark box (LDB) test	WT: n = 12 <i>kat2</i> ^{-/-} : n = 11	Time spent in the lit box (s)	119.00 ± 31.38	113.91 ± 24.41	0.957
Open field (OF) test	WT: n = 12 <i>kat2</i> ^{-/-} : n = 11	Number of entries to the center zones (times)	281.67 ± 69.13	210.73 ± 65.20	0.011 *
		Number of entries to the corner zones (times)	83.08 ± 26.95	51.27 ± 17.88	0.001 ***
		Ambulation distance (cm)	2191.75 ± 364.45	1609.27 ± 381.96	0.002 **
		Number of jumps (times)	7.33 ± 4.94	2.45 ± 3.08	0.034 *

*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001.

ity in plasma samples than wild-type mice. In the urine samples, the transgenic mice showed significantly lower KATs, KYNU, KAT III, MAO, ALDH, and TMO activities, and significantly higher tryptophan-2,3-dioxygenase (TDO)/

indoleamine 2,3-dioxygenases (IDOs) (KFA), KMO, and aromatic L-amino acid decarboxylase (AADC) activities compared to the wild-type counterparts (Fig. 6, Table 4).

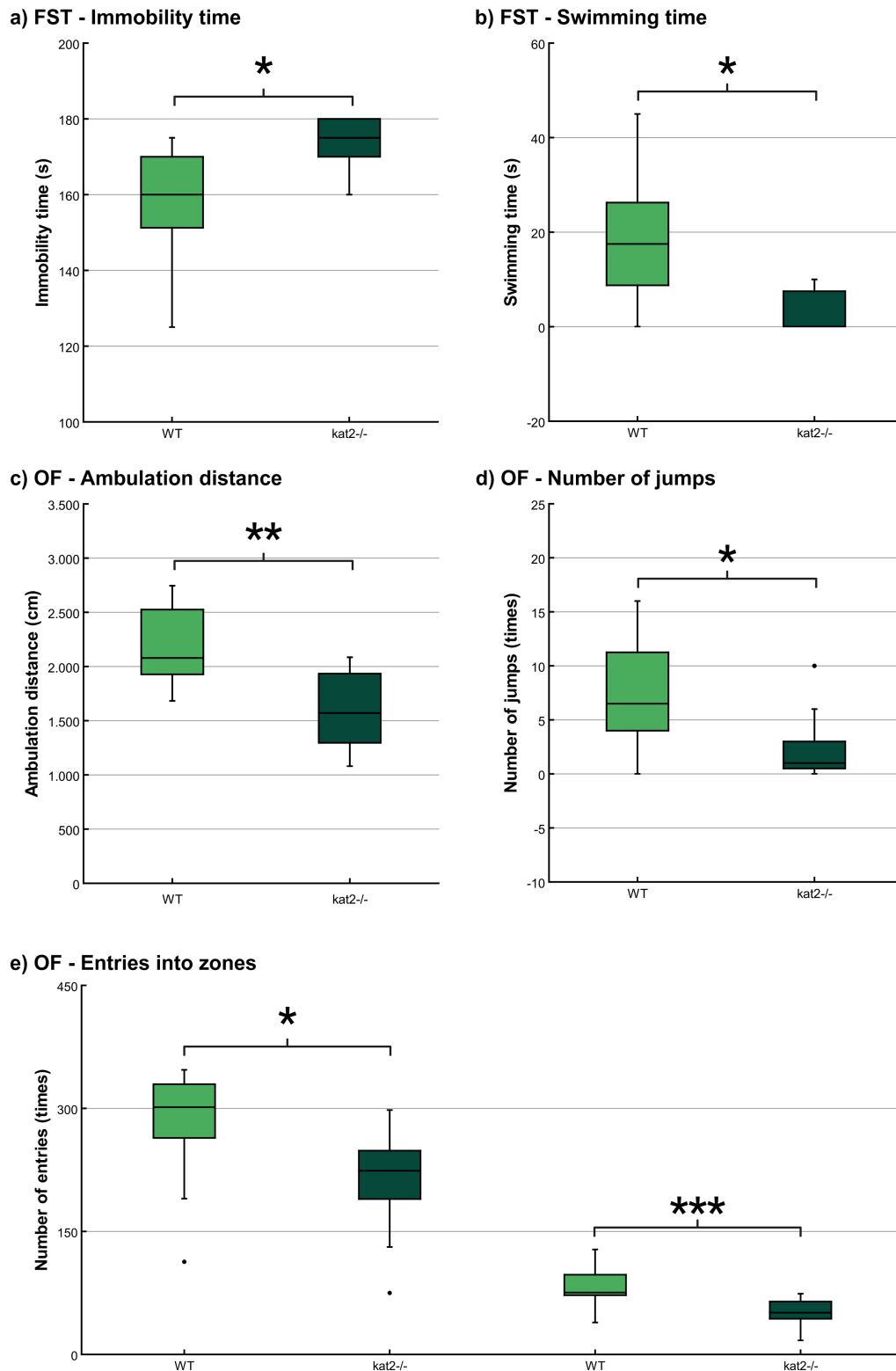
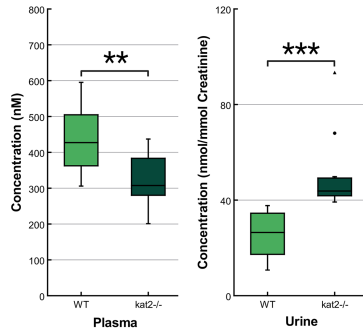
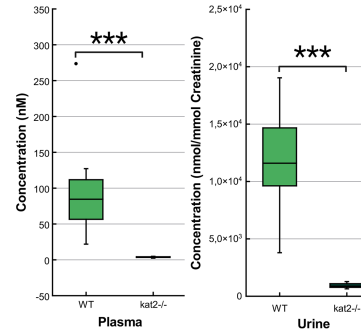


Fig. 4. Behavioral tests. (a) Time spent immobile in the modified forced swim test (FST). (b) Time spent swimming in the modified FST. (c) Ambulation distance in the open field (OF) test. (d) Number of jumps in the OF test; and (e) Number of entries into the center and corner zones in the OF test. Wild-type mice (light green); *kat2*^{-/-} mice (dark green). WT, wild-type; *kat2*^{-/-}, kynurenine aminotransferase II knockout mice; FST, forced swim test; OF, open field test; •, outlier. Mean $\pm$ SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The figure was created with Labplot 2.9.0 (KDE, Berlin, Germany) and Scientific Image and Illustration Software [Biorender](#).

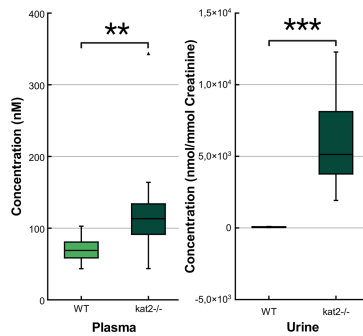
a) KYN



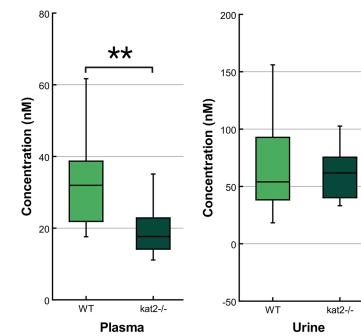
b) KYNA



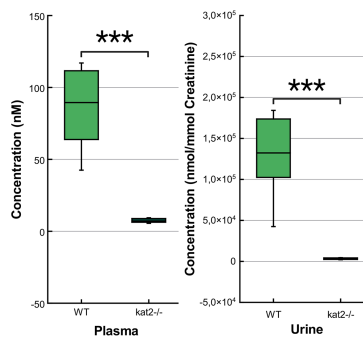
c) 3-HK



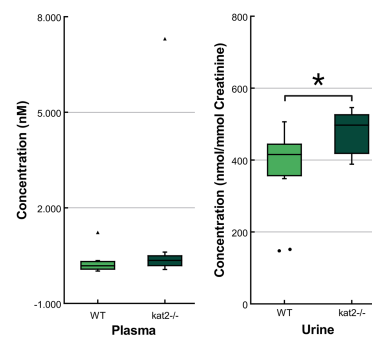
d) AA



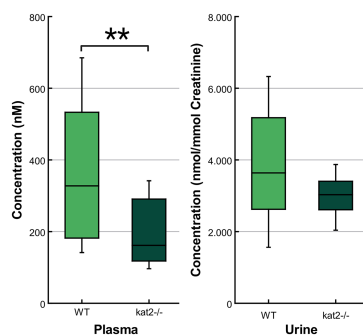
e) XA



f) 5-HT



g) 5-HIAA



h) IAA

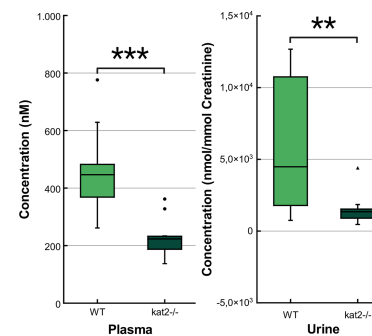


Fig. 5. Concentration level of tryptophan metabolites in plasma and urine. (a) Kynurenine. (b) Kynurenic acid. (c) 3-hydroxykynurenine. (d) Anthranilic acid. (e) Xanthurenic acid. (f) Serotonin/5-hydroxytryptamine. (g) 5-hydroxyanthranilic acid. (h) Indole-3-acetic acid. We marked wild-type mice with light, and *kat2*^{-/-} mice results with dark green boxes. WT, wild-type; *kat2*^{-/-}, kynurenine aminotransferase II knockout; 3-HK, 3-hydroxykynurenine; 5-HIAA, 5-hydroxyanthranilic acid; 5-HT, serotonin/5-hydroxytryptamine; AA, anthranilic acid; IAA, indole-3-acetic acid; XA, xanthurenic acid; •, outlier; ▲, far out. Mean ± SD; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The figure was created with Labplot 2.9.0 (KDE, Berlin, Germany) and Scientific Image and Illustration Software [Biorender](#).

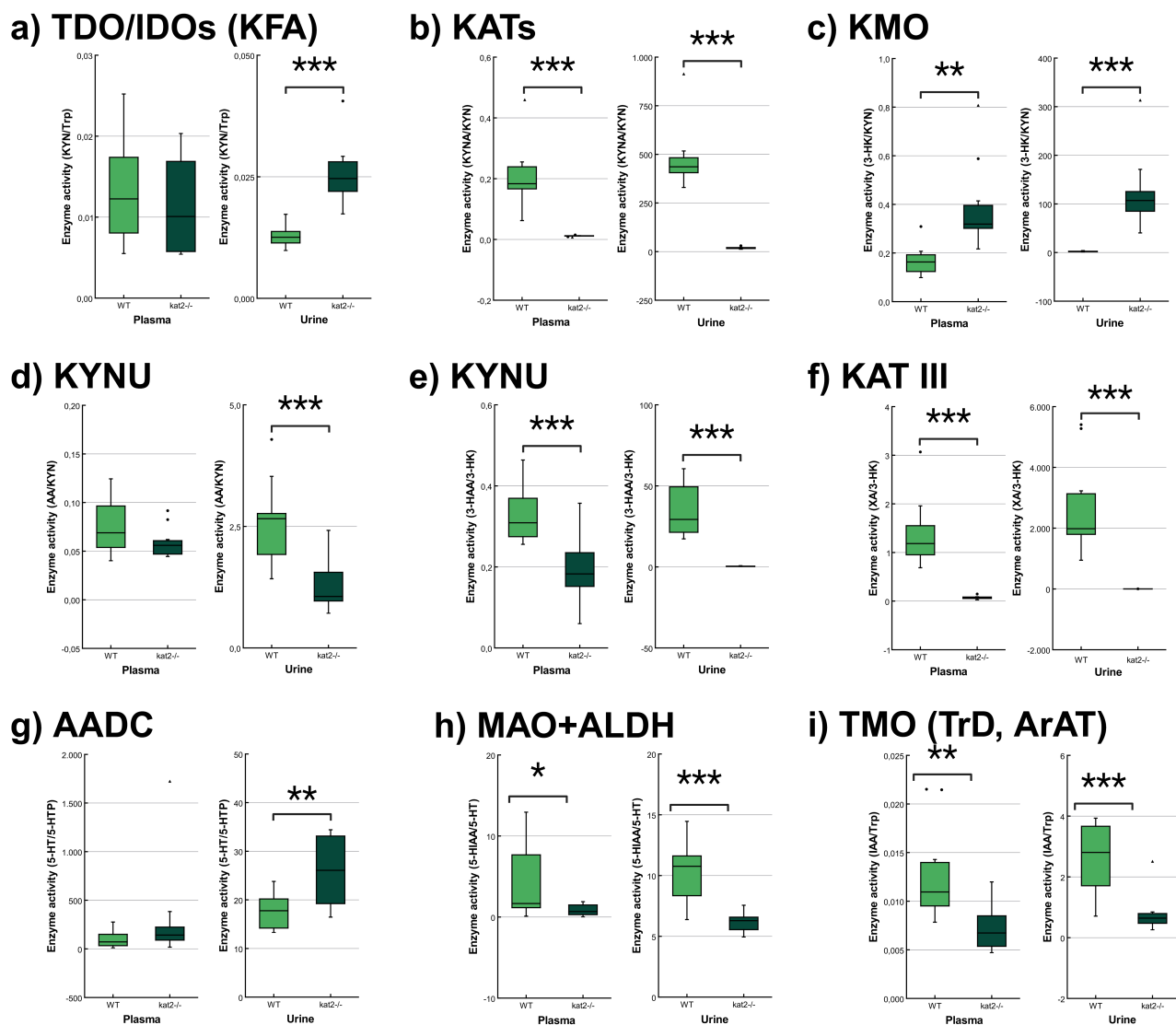


Fig. 6. Tryptophan metabolism's enzyme activity in plasma and urine. (a) Tryptophan 2,3-dioxygenase/indoleamine 2,3-dioxygenases (kynurenine formamidase). (b) Kynurenine aminotransferases. (c) Kynurenine 3-monooxygenase. (d,e) Kynureninase. (f) Kynurenine aminotransferase III/cysteine conjugate beta-lyase 2. (g) Aromatic L-amino acid decarboxylase. (h) Monoamine oxidases + aldehyde dehydrogenase. (i) Tryptophan-2-monooxygenase (tryptophan decarboxylase, aromatic amino acid aminotransferase). We marked wild-type mice with light, and *kat2*^{-/-} mice results with dark green boxes. 3-HAA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin/5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; AADC, aromatic L-amino acid decarboxylase; ALDH, aldehyde dehydrogenase; ArAT, aromatic amino acid aminotransferase; IAA, indole-3-acetic acid; IDOs, indoleamine 2,3-dioxygenases; KAT III, kynurenine aminotransferase III/cysteine conjugate beta-lyase 2; *kat2*^{-/-}, kynurenine aminotransferase II knockout; KATs, kynurenine aminotransferases; KFA, kynurenine formamidase; KMO, kynurenine 3-monooxygenase; KYNU, kynureninase; MAO, monoamine oxidase; TDO, tryptophan 2,3-dioxygenase; TMO, tryptophan-2-monooxygenase; TrD, tryptophan decarboxylase; Trp, tryptophan; WT, wild-type; XA, xanthurenic acid; KYN, kynurenine; KYNA, kynurenic acid; AA, anthranilic acid; •, outlier; ▲, far out. Mean ± SD; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The figure was created with Labplot 2.9.0 (KDE, Berlin, Germany) and Scientific Image and Illustration Software Biorender.

Table 3. Quality control samples of mouse plasma and urine (runtime 25 h, (mean concentrations, 14-14 replicates of each, the given n is the samples size of the pooled individual samples)).

	Plasma (nM)			Urine (nmol/mmol Creatinine)		
	Mean $\pm$ SD		<i>p</i> value	Mean $\pm$ SD		<i>p</i> value
	WT	<i>kat2</i> ^{-/-}		WT	<i>kat2</i> ^{-/-}	
Tryptophan (Trp)	40,901.678 $\pm$ 21,056.888	35,543.573 $\pm$ 16,203.237	0.532	2022.196 $\pm$ 908.643	1972.014 $\pm$ 286.954	0.870
Kynurenine (KYN)	440.674 $\pm$ 102.886	327.348 $\pm$ 76.385	0.012 **	25.238 $\pm$ 10.185	50.883 $\pm$ 17.134	<0.001 ***
Kynurenic acid (KYNA)	96.960 $\pm$ 70.837	3.654 $\pm$ 0.860	<0.001 ***	11,783.938 $\pm$ 5040.178	920.990 $\pm$ 215.223	<0.001 ***
Quinaldic acid (QAA)	6.884 $\pm$ 5.397	5.608 $\pm$ 1.234	0.476	14.248 $\pm$ 9.716	12.014 $\pm$ 7.490	0.572
3-hydroxykynurenine (3-HK)	70.714 $\pm$ 18.994	130.851 $\pm$ 82.199	0.037 **	55.472 $\pm$ 31.438	5986.833 $\pm$ 3157.255	<0.001 ***
Xanthurenic acid (XA)	93.624 $\pm$ 45.637	7.406 $\pm$ 1.452	<0.001 ***	127,228.662 $\pm$ 52,582.223	3273.334 $\pm$ 1021.511	<0.001 ***
Anthranilic acid (AA)	32.655 $\pm$ 13.114	19.335 $\pm$ 7.280	0.012 **	69.112 $\pm$ 45.347	60.862 $\pm$ 22.368	0.612
3-Hydroxyanthranilic acid (3-HAA)	22.992 $\pm$ 6.140	20.920 $\pm$ 5.921	0.452	1741.538 $\pm$ 824.887	1789.475 $\pm$ 454.422	0.874
Quinolinic acid (QA)	132.185 $\pm$ 75.409	112.000 $\pm$ 41.600	0.468	10,059.485 $\pm$ 4601.597	11,718.491 $\pm$ 2401.051	0.326
Picolinic acid (PA)	193.797 $\pm$ 93.230	154.895 $\pm$ 88.753	0.352	190.435 $\pm$ 91.394	193.898 $\pm$ 113.072	0.941
5-Hydroxytryptophan (5-HTP)	2.790 $\pm$ 1.577	2.708 $\pm$ 1.297	0.901	21.742 $\pm$ 8.520	19.297 $\pm$ 3.833	0.419
Serotonin (5-HT)	277.309 $\pm$ 353.179	1010.379 $\pm$ 2219.355	0.316	371.974 $\pm$ 125.489	479.383 $\pm$ 63.304	0.027 *
5-hydroxyindoleacetic acid (5-HIAA)	362.241 $\pm$ 199.450	201.217 $\pm$ 99.184	0.035 **	3774.968 $\pm$ 1666.005	2969.725 $\pm$ 598.373	0.167
Indole-3-acetic acid (IAA)	457.329 $\pm$ 153.046	229.142 $\pm$ 68.266	<0.001 ***	6030.306 $\pm$ 4737.901	1513.400 $\pm$ 1097.122	0.009 **
Indoxyl-sulphate (INS)	6738.111 $\pm$ 3559.896	5404.257 $\pm$ 2292.535	0.332	400,636.750 $\pm$ 185,880.105	497,063.585 $\pm$ 190,235.646	0.267

SD, standard deviation; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 4. Enzymes activities in plasma and urine.

Enzyme	Product/Substrate	Plasma			Urine		
		Mean $\pm$ SD		<i>p</i> value	Mean $\pm$ SD		<i>p</i> value
		WT	<i>kat2</i> ^{-/-}		WT	<i>kat2</i> ^{-/-}	
TDO/IDOs (KFA)	KYN/Trp	0.013 $\pm$ 0.007	0.011 $\pm$ 0.006	0.532	0.013 $\pm$ 0.002	0.026 $\pm$ 0.006	<0.001 ***
KATs	KYNA/KYN	0.205 $\pm$ 0.107	0.011 $\pm$ 0.002	<0.001 ***	476.464 $\pm$ 164.156	18.937 $\pm$ 5.057	<0.001 ***
KMO	3-HK/KYN	0.168 $\pm$ 0.062	0.386 $\pm$ 0.180	0.002 **	2.219 $\pm$ 0.827	122.983 $\pm$ 75.543	<0.001 ***
KYNU	AA/KYN	0.075 $\pm$ 0.028	0.059 $\pm$ 0.016	0.120	2.593 $\pm$ 0.862	1.253 $\pm$ 0.529	<0.001 ***
KYNU	3-HAA/3-HK	0.330 $\pm$ 0.070	0.194 $\pm$ 0.080	<0.001 ***	35.177 $\pm$ 16.776	0.372 $\pm$ 0.182	<0.001 ***
KAT III	XA/3-HK	1.374 $\pm$ 0.714	0.070 $\pm$ 0.033	<0.001 ***	2702.990 $\pm$ 1524.430	0.629 $\pm$ 0.229	<0.001 ***
3-HAO	QA/3-HAA	5.1771 $\pm$ 2.978	5.486 $\pm$ 1.994	0.804	6.240 $\pm$ 2.487	6.856 $\pm$ 1.779	0.532
3-HAO + ACMSD	PA/3-HAA	8.797 $\pm$ 4.263	7.681 $\pm$ 4.872	0.592	0.123 $\pm$ 0.071	0.119 $\pm$ 0.085	0.906
TPHs	5-HTP/Trp	<0.001 $\pm$ <0.001	<0.001 $\pm$ <0.001	0.128	0.011 $\pm$ 0.002	0.010 $\pm$ 0.003	0.410
AADC	5-HT/5-HTP	97.585 $\pm$ 87.384	307.233 $\pm$ 509.276	0.216	17.608 $\pm$ 3.583	25.997 $\pm$ 7.185	0.004 **
MAOs + ALDH	5-HIAA/5-HT	4.217 $\pm$ 4.818	0.905 $\pm$ 0.712	0.045 *	10.209 $\pm$ 2.530	6.181 $\pm$ 0.859	<0.001 ***
TMO (TrD, ArAT)	IAA/Trp	0.013 $\pm$ 0.005	0.007 $\pm$ 0.002	0.005 **	2.570 $\pm$ 1.243	0.786 $\pm$ 0.636	<0.001 ***
TNA	INS/Trp	0.208 $\pm$ 0.178	0.170 $\pm$ 0.089	0.555	215.671 $\pm$ 100.757	248.916 $\pm$ 81.413	0.428

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 5. The oxidative stress and excitotoxicity indices in the plasma and urine.

Oxidant/antioxidant metabolites	Oxidative stress index					
	Plasma (nM)			Urine (nmol/mmol Creatinine)		
	Mean $\pm$ SD		<i>p</i> value	Mean $\pm$ SD		<i>p</i> value
	WT	<i>kat2</i> ^{-/-}		WT	<i>kat2</i> ^{-/-}	
3-HK/KYNA+AA+XA	0.378 $\pm$ 0.163	4.090 $\pm$ 1.478	<0.001 ***	0.085 $\pm$ 0.011	1.352 $\pm$ 0.473	<0.001 ***

agonist/antagonist metabolites	Excitotoxicity index					
	Plasma (nM)			Urine (nmol/mmol Creatinine)		
	Mean $\pm$ SD		<i>p</i> value	Mean $\pm$ SD		<i>p</i> value
	WT	<i>kat2</i> ^{-/-}		WT	<i>kat2</i> ^{-/-}	
QA/KYNA	1.648 $\pm$ 0.810	30.514 $\pm$ 8.618	<0.001 ***	0.884 $\pm$ 0.320	13.092 $\pm$ 2.833	<0.001 ***

***, *p* < 0.001.

3.6 Oxidative stress and Excitotoxicity indices

Transgenic mice had higher levels of oxidative stress and excitotoxicity in both plasma and urine than wild-type mice (Fig. 7, Table 5).

4. Discussion

Dysregulation of 5-HT metabolism is a key factor in mental symptom development, with attention focused on its imbalance with neurotransmitters like dopamine, norepinephrine, and biosystems such as substance P [206–210]. Alterations in 5-HT precursor Trp metabolism are noted in mental illnesses, but their connection with the Trp-KYN metabolic system remains poorly understood [211–213]. Growing evidence suggests that the gut microbial indole pyruvate pathway can influence the microbiome-gut-brain axis, implying that intestinal Trp metabolism may play a significant role in psychological health. The microbiome-gut-brain axis is responsible for regulating mood, cognition, stress response, and behavior [101]. As a result, the gut-microbial indole pyruvate pathway can influence the microbiome-gut-brain axis by controlling the production and availability of neurotransmitters, hormones, cytokines, and bioactive metabolites involved in neuropsychiatric conditions.

KATs are cytosolic and mitochondrial aminotransferases that convert KYN to KYNA [74,214–216]. The mitochondrial isoform KAT II exclusively influences cellular bioenergetics due to its exclusive location in the mitochondria [117,205]. CRISPR/Cas9 was employed to knock out the *kat2* gene, creating *kat2*^{-/-} mice. This study aimed to examine the negative emotional aspects and evaluate any behavioral alterations caused by the knockout of the *kat2* gene in young adults aged 8 weeks. *kat2*^{-/-} mice, studied in 8-week-old adults, induce a unique depression-like phenotype marked by increased immobility in FST, likely linked to serotonergic pathways. TST did not show significant differences, possibly due to FST conditioning. The results that the PAT did not show a significant difference may suggest

that depression-like behavior is more likely to be related to depression-like behavior caused by despair experiences than to aversive-conditioned memory. Anxiety-like behaviors (EPM and LDB) showed no difference, but the OF test revealed shorter ambulation distance, fewer jumping counts, and fewer entries into both center field and corners, suggesting a la belle indifference-like trait. *kat2*^{-/-} mice exhibited despair-based depression-like behavior without anxiety-like traits, demonstrating motor deficits. The study suggests the *kat2* gene deletion potentially leads to a PTSD-like phenotype, including a la belle indifference trait, indicative of complex PTSD with emotional dysregulation [217–219].

The gene knockout significantly alters Trp metabolism in both 5-HT, KYN, and indole pathways in plasma and urine. A major 5-HT metabolite, 5-HIAA, is markedly reduced, possibly explained by scarce mitochondrial enzyme activity. Lower levels of KYNA and antioxidant KYNs indicate decreased production in peripheral tissues of *kat2*^{-/-} mice. Conversely, 3-HK is significantly elevated. The levels of the gut microbial metabolite IAA, an antioxidant and anti-inflammatory molecule, were reduced. The disruption of the KAT II gene may lead to a reduction in the levels of IAA in the indole pathway of the gut microbiota, as the enzyme plays a role in controlling Trp metabolism. KAT II has an impact on the availability of Trp and its subsequent metabolic pathways, including the production of IAA. In the absence of KAT II, the Trp metabolite balance may shift, resulting in less IAA synthesis by gut bacteria. This change may disrupt the gut-brain axis and have an impact on intestinal health, as IAA is required for immune response regulation, intestinal barrier integrity, and modulating the production of other indole derivatives. Furthermore, gene knockout affects enzyme activity, puts organisms under oxidative stress, imposes high excitotoxicity and neurotoxicity, and alters immune responses. The study demonstrates that the deletion of the *kat2* gene leads to a specific set of characteristics, including behavior similar to depression, impaired

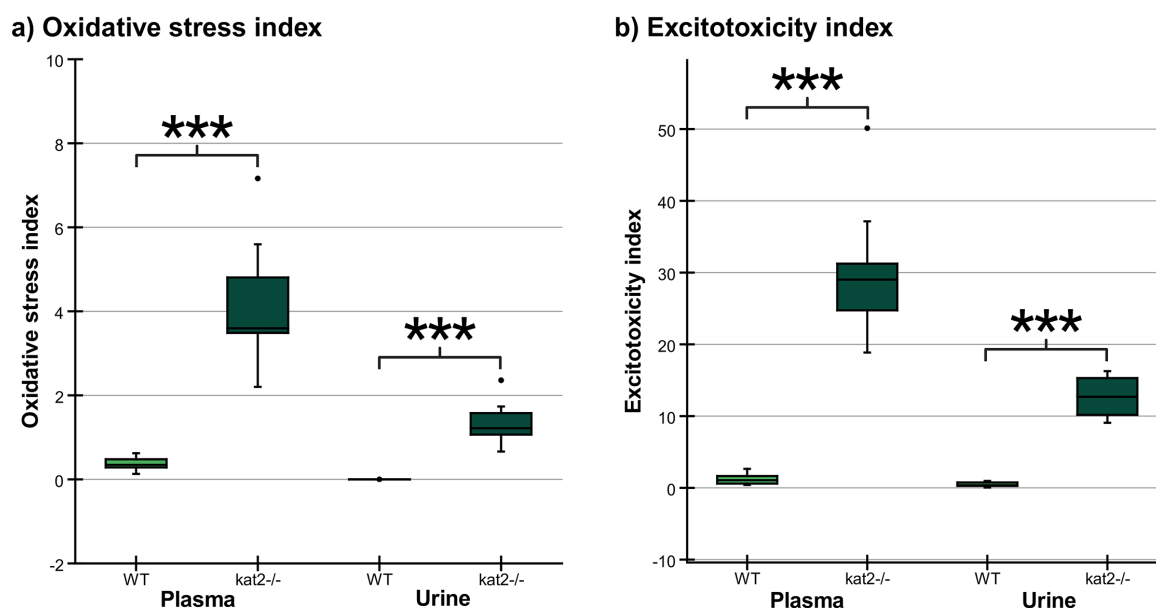


Fig. 7. Oxidative stress and excitotoxicity indices in plasma and urine. (a) The oxidative stress indices in *kat2*^{-/-} mice's plasma and urine samples are significantly higher than those in the wild-type. (b) The excitotoxicity indices in *kat2*^{-/-} mice's plasma and urine samples are significantly higher than those in the wild-type. We marked wild-type with light, and *kat2*^{-/-} mice results with dark green boxes. WT, wild-type; *kat2*^{-/-}, kynurenine aminotransferase II knockout; *, outlier. Mean ± SD; ***, $p < 0.001$. The figure was created with Labplot 2.9.0 (KDE, Berlin, Germany) and Scientific Image and Illustration Software Biorender.

motor function, decreased levels of KYNA, and a change in the way Trp is metabolized towards the KYN pathway. This phenotype exhibits similarities to PTSD in humans, potentially indicating the presence of complex PTSD due to the observed belle indifference-like trait.

The amygdala encodes and stores fear memory after receiving sensory input from the thalamus, which also consolidates and retrieves memories from the initial stimuli that induce fear [220–222]. Fear memory is associated with the release of stress hormones such as adrenaline and cortisol, which stimulate the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis [46,223–227]. This study does not show evidence of fear memory acquisition. In contrast, the encoding and storage of memories associated with despair occur in the prefrontal cortex, which plays a crucial role in the cognitive and emotional processing of negative experiences [228]. Recalling distressing memories, triggered by cues linked to the initial negative encounter, results in the disruption of 5-HT, norepinephrine, and dopamine regulation. Although fear and despair memories have similarities in terms of encoding and retrieval processes, they are associated with different brain regions, neurotransmitters, and neural circuits [229,230].

Furthermore, despair memory and despair experience differ. The latter pertains to an instantaneous, personal feeling of despair or hopelessness, prompted by present circumstances, as opposed to a remembrance of past experiences [231]. Despair memory involves the consolidation and retrieval of long-term memories, influenced by stress and

emotion [232]. In contrast, a despair experience entails immediate emotional responses influenced by factors like cognitive assessments, environmental cues, and physiological states [233]. Additionally, la belle indifference arises from a discrepancy between cognitive and emotional symptom processing, including altered emotional processing in the amygdala and insula, changed self-awareness in the medial prefrontal cortex, and adjusted activity in the somatosensory cortex influenced by dopamine and 5-HT [234]. Thus, *kat2*^{-/-} mice show more despair-based depression-like behavior involving a change in 5-HT metabolism.

Approximately 60% of individuals on antidepressants, including SSRIs, for two months experience a 50% reduction in depression symptoms [235]. The observation aligns with the monoamine hypothesis, suggesting depression's pathogenesis is linked to low 5-HT levels. Transgenic models are used to study 5-HT dysmetabolism behaviors, with a focus on the *Tph* gene, which encodes tryptophan hydroxylase, a key enzyme in 5-HT synthesis [236]. Pre-clinical studies found normal 5-HT levels with no behavioral changes in *Tph1*^{-/-} mice, while *Tph2*^{-/-} mice's behaviors are inconclusive [237,238]. The knock-in mice of the TPH2 variant (R439H) showed depression-like behavior in TST [239]. Intriguingly, *Tph1/Tph2*^{-/-} mice exhibited contrasting behaviors: antidepressant-like in FST, depressive in TST, and anxious in the MB test, accompanied by low 5-HT levels in the brain and periphery [240]. 5-HT1A receptor knockout (*5-HT1AR*^{-/-}) mice display heightened fear memory to contextual cues, suggesting a role for 5-HT re-

ceptors in PTSD-like phenotype [241]. 5-HT 2C receptor knockout *5-HT2CR^{-/-}* mice attenuates fear responses in contextual or cued but not compound context-cue fear conditioning [242]. Knockout of the 5-HTT gene in mice (*5-HTT^{-/-}*) leads to impaired stress response, fear extinction, and abnormal corticolimbic structure [243].

Over 90% of 5-HT precursor Trp undergoes catabolism in the Trp-KYN metabolic system, generating a variety of bioactive molecules including prooxidants, antioxidants, inflammation suppressants, neurotoxins, neuroprotectants, and/or immunomodulators [244]. Growing evidence indicates disrupted KYN metabolism in MDD, bipolar disorder, and SCZ [245–247]. Earlier, KYN metabolites were suggested to be either neuroprotective or neurotoxic [248]. However, increasing evidence suggests that KYN metabolites exhibit versatile actions, potentially influenced by concentrations and the microenvironment [249]. Previously, cognitive and motor functions of 129/SvEv *kat2^{-/-}* mice were reported. These transgenic mice exhibited transient hyperlocomotive activity and motor coordination issues at postnatal day 21. However, from postnatal day 17 to 26, they demonstrated notable improvements in cognitive functions, particularly in object exploration and recognition tasks in PAT and T-maze tests [250,251].

Other biosystems play an important role in the pathogenesis of PTSD, including dopaminergic and gamma-aminobutyric acid (GABA)ergic, and cannabinoid systems. Catechol-*O*-methyltransferase (COMT) degrades dopamine. *COMT^{-/-}* mice exhibited an increased response to repeated stress exposures [252]. Glutamic acid decarboxylase (GAD) synthesizes GABA [253]. *GAD6^{-/-}* mice shows increased generalized fear and impaired extinction of cued fear [254]. GABA receptor subunit B1a knockout *GABAB1a^{-/-}* mice showed a generalization of conditioned fear to nonconditioned stimuli [255]. Cannabinoid 1 receptor (CB1R) knockout *CB1R^{-/-}* mice showed an increased response to repeated stress exposures [256].

The potential of this study is to characterize the negative valence of emotional domain in context with aversive-conditioned memory and despair experience in the young adult (8 week) of *kat2^{-/-}* mice. The findings complement the previous studies of *kat2^{-/-}* mice in the early adolescence (2 and 1/2 to 4 weeks) to reveal that, toward adulthood, there is a dynamic change in emotional susceptibility and motor function derived from despair experience in adjunct to Trp metabolism. Furthermore, urinary Trp metabolite levels were generally consistent with plasma levels, suggesting that urinary samples may serve as non-invasive biomarkers for Trp metabolism status. This study may shed new light on the deletion of the *kat2* gene as a new avenue toward understanding a KYN metabolite as an oxidative stressor, a potential barrier between aversive-conditioned memory and despair experience, a distinction between memory and experience, their mechanism for the formation of intrusive

memories, and the pathogenesis of PTSD. The ultimate goal is to probe a potential interventionable stage in age where the progression of PTSD is preventable and to identify targets which drugs or psychotherapy can relieve symptoms of PTSD. The greatest challenge lies in preclinical animal models that are difficult to simulate and interpolate to mental illnesses to achieve high model validity.

This research on transgenic mice offers great potential for future studies. By examining the link between behavioral changes and variations in Trp and its metabolites in plasma and urine, scientists can gain insights into Trp metabolism's role in emotional and cognitive functions. Additionally, assessing enzyme activities related to Trp metabolism and their effects on oxidative stress and neurochemical imbalances may uncover mechanisms behind observed behavioral differences. This thorough approach could reveal causal or parallel relationships, shedding light on how altered Trp metabolism impacts neurochemical imbalances and oxidative stress, contributing to conditions like depression and PTSD. The results may help identify specific biomarkers and therapeutic targets, opening new pathways for precision medicine and more effective treatments for neuropsychiatric disorders. The study's findings could lead to the development of customized therapies, enhancing mental health by focusing on unique metabolic pathways and genetic factors. This research highlights the importance of combining metabolic, behavioral, and genetic data to deepen our understanding of complex psychiatric disorders [257].

This study suggests that behavioral sampling in rodents can distinguish between fear-, memory-, and despair-based depression-like behavior associated with Trp metabolism gene deletions. Further research incorporating neurochemical, neurogenetic, and electrophysiological biomarkers may reinforce this finding. Additionally, using inhibitory RNA or antisense RNA on neurotransmitters in specific brain regions could elucidate the precise mechanisms underlying emotional behaviors. Preclinical research drives advances in clinical applications like precision medicine and drug discovery [258–260]. The study acknowledges weaknesses, noting distinctions in interpreting animal behaviors and drug responses compared to humans. Recent perspectives consider depression-like behavior in FST as related to different stages of stress-coping behaviors [261]. Consequently, Translational research has limitations that necessitate careful interpretation [262–264]. This study employed animal models with standard protocols, focusing on the negative valence of the emotional domain and motor function in *kat2^{-/-}* mice. Further exploration with diverse models such as sucrose preference tests, fear condition tests, and those using non-standard protocols is crucial for a more accurate characterization of *kat2^{-/-}* mouse behavior. Notably, the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, emphasizes four symptom clusters in PTSD diagnosis [265–267]. The transgenic mice

in this study did not exhibit signs related to negative cognitions and mood, and arousal state and reactivity were not investigated.

5. Conclusions

Psychiatric disorders, including PTSD, have a significant impact on memory and emotion, and disruptions in 5-HT metabolism have been associated with these disorders. The Trp-KYN metabolic pathway plays a crucial role in metabolizing over 95% of the 5-HT precursor Trp. To investigate the effects of gene deletion on negative valence in emotion, memory, and motor function, transgenic *kat2*^{-/-} mice were created and compared to WT mice. The *kat2*^{-/-} mice exhibited depression-like behavior characterized by despair experiences, diminished motor functions, and la belle indifference-like characteristics without anxiety-like behavior. This study provides insights into the negative valence of the emotional domain in the context of aversive-conditioned memory and despair experiences in 8-week-old *kat2*^{-/-} mice. Understanding the complex interplay between memory, emotion, and genetic factors is crucial for advancing our knowledge of psychiatric disorders [268,269]. By elucidating the specific effects of gene deletion on negative valence and related behaviors, this research contributes to our understanding of the underlying mechanisms and potential interventions.

Declaration of AI and AI-assisted Technologies in the Writing

During preparation for this work, the authors used QuillBot for grammar and style checks in the main text. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

Abbreviations

3-HAA, 3-hydroxyanthranilic acid; 3-HAO, 3-hydroxyanthranilate oxidase; 3-HK, 3-hydroxykynurenine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin/5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; AA, anthranilic acid; AADC, aromatic L-amino acid decarboxylase; ACMSD, amino- β -carboxymuconate-semialdehyde-decarboxylase; AD, Alzheimer's disease; ALDH, aldehyde dehydrogenase; ArAT, aromatic amino acid aminotransferase; CB1R, cannabinoid 1 receptor; CNS, central nervous system; COMT, catechol-O-methyltransferase; EPM, elevated plus maze; FST, forced swim test; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; hCG, human chorionic gonadotropin; IAA, indole-3-acetic acid; ICR: Institute of Cancer Research; IDOs, indoleamine 2,3-dioxygenases; IgG, immunoglobulin G; INS, indoxyl sulfate; KAT II, α -amino adipate aminotransferase/kynurenine aminotransferase II; KAT III, kynurenine aminotransferase

III/cysteine conjugate beta-lyase 2; KATs, kynurenine aminotransferases; KFA, kynurenine formamidase; KMO, kynurenine 3-monooxygenase; KYN, kynurenine; KYNA, kynurenic acid; KYNU, kynureninase; LDB, light dark box; MAO, monoamine oxidase; MDD, major depressive disorder; MRM, multiple reaction monitoring; NMDA, N-methyl-D-aspartate; OF, open field; PA, picolinic acid; PAT, passive avoidance test; PCR, polymerase chain reaction; PMSG, pregnant mare serum gonadotropin; PTSD, post-traumatic stress disorder; QA, quinolinic acid; SCZ, schizophrenia; sgRNA, single guide RNA; SSRI, selective serotonin reuptake inhibitor; TDO, tryptophan 2,3-dioxygenase; TMO, tryptophan-2-monooxygenase; TNA, tryptophanase; TPHs, tryptophan hydroxylases; TrD, tryptophan decarboxylase; Trp, tryptophan; TST, tail suspension test; UHPLC-MS, ultra-high performance liquid chromatography-tandem mass spectrometry; WT, wild-type; XA, xanthurenic acid.

Availability of Data and Materials

The data presented in this study are available on request from the corresponding author.

Author Contributions

Conceptualization, ÁS, JT, EO, LV, and MT; methodology, ÁS, ZG, ES, MS, DM, KT, KO, HI, SY, and MT; software, ÁS, ES, MS, KO; validation, ÁS, ZG, ES, MS, KT, KO, and MT; formal analysis, ÁS, ZG, ES, MS, KO, and MT; investigation, ÁS, ZG, ES, MS, DM, KT, KO, and MT; resources, ÁS and EO; data curation, ÁS, ZG, ES, MS, KO; writing—original draft preparation, ÁS, ZG, MT; writing—review and editing, ÁS, ZG, ES, MS, DM, JT, EO, LV, and MT; visualization, ÁS, KO; supervision, JT, EO, LV, and MT; project administration, JT, EO, and LV; funding acquisition, JT, EO, LV, and MT. All authors have read and agreed to the published version of the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Regulations for Animal Experiments of Kyushu University, the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions governed by the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and with the approval of the Institutional Animal Experiment Committees of Kyushu University (A29-338-1 (2018), A19-090-1 (2019)), and approved by the National Food Chain Safety Office (XI/95/2020, CS/I01/170-4/2022) and the Committee of Animal Research at the University of Szeged (I-74-10/2019, I-74-1/2022).

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Conflict of Interest

Given his role as a Guest Editor, Masaru Tanaka had no involvement in the peer-review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Thomas Heinbockel. The authors declare no conflict of interest.

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