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

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Abstract: Memory and emotion are highly vulnerable to psychiatric disorders like post-traumatic stress disorder (PTSD), which has been linked to serotonin (5-HT) metabolism disruptions. In fact, over 90% of the 5-HT precursor tryptophan (Trp) is metabolized via the Trp-kynurenine (KYN) metabolic pathway, producing a variety of bioactive molecules. The *aadat* (*kat2*) gene encodes mitochondrial kynurenine aminotransferase (KAT) isotype 2, responsible for kynurenic acid (KYNA) production. Little is known about its role in behavior. 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 the urine of 8-week-old males compared to wild-type mice. 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: KYN, antioxidant KYNs, 5-hydroxyindolacetic acid, and indole-3-acetic acid levels are lower; enzyme activities in KATs, kynureninase, and monoamine oxidase/aldehyde dehydrogenase are lower, but kynurenine 3-monooxygenase is higher; and oxidative stress and excitotoxicity indices are higher. Transgenic mice show 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-HK pathway, and a partial decrease in the gut microbial Trp-indole pathway metabolite. This is the first evidence that deleting the *aadat* gene causes depression-like behaviors that are unique to despair experience, which appears to be linked to excitatory neurotoxic and oxidative stresses. This may lead to the development

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of a double-hit preclinical model in experience-based depression, 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; tryptophan; kynurenine; microbiota; transgenic mice

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 (1-5). Orderly function at multi-layered levels is essential to maintaining sound mental well-being (6-9). The reciprocal interaction between cognitive 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 (10). Similarly, emotional disturbances such as depression and anxiety can influence memory function, increasing vulnerability to cognitive challenges (11, 12). This intricate bidirectional link between cognition and emotions can lead to changes in brain structure, function, behavior, lifestyle, and neurotransmitter systems (13, 14). 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) (15-20).

The serotonergic nervous system plays an important role in regulating mood, anxiety, and cognition (21-24). Serotonin (5-hydroxytryptamine, 5-HT) is involved in cognitive processes such as attention, learning, and memory (25-27). 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 (28-37). 5-HT is implicated in regulating mood and anxiety, influencing cognitive function (38, 39). Mental illnesses like MDD, eating disorders, obsessive-compulsive disorder, schizophrenia (SCZ), and PTSD are associated with dysregulation of 5-HT (40-45). Selective serotonin reuptake inhibitors (SSRIs) are commonly used for these conditions, targeting the serotonergic nervous system (46-48). Furthermore, abnormalities in the serotonergic system also affect norepinephrine and dopamine (49-51).

The complex interplay of tryptophan (Trp)-kynurenine (KYN) and 5-HT metabolism is crucial for comprehending the pathogenesis of mental illnesses (52, 53). 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 (54, 55). Approximately 2% of L-Trp undergoes metabolism through the 5HT metabolic pathway; however, over 90% of Trp is catabolized through the KYN route, which safely to say that it governs Trp metabolism (Figure 1, a,b) (56). Various factors, including stress, inflammation, and the gut microbiome, influence this system (57-59). Dysregulation of the KYN route has been linked to mental health conditions such as MDD, SCZ, and AD (60).

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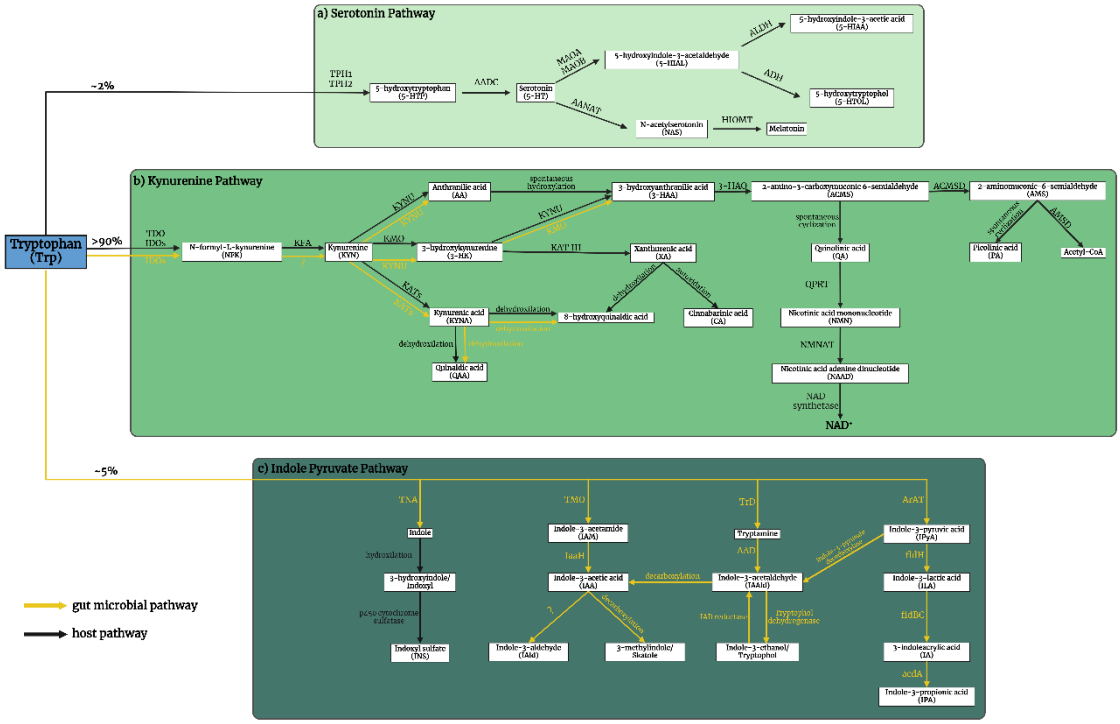

Figure 1. Tryptophan (Trp) metabolism. (a) The serotonin (5-HT) pathway: A fraction exceeding 2% of L-Trp is metabolized within the 5-HT pathway. The rate-limiting enzyme tryptophan hydroxylase 1 and 2 (TPH1, TPH2) converts Trp to 5-hydroxytryptophan (5-HTP), which is then decarboxylated by aromatic L-amino acid decarboxylase (AADC) to 5-HT. 5-HT is oxidized by monoamine oxidase A and B (MAOA, MAOB) in different tissues to 5-hydroxyindole acetaldehyde (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 (61). On the other hand, 5-HT synthetize melatonin (N-acetyl-5-methoxytryptamine). First, 5-HT is converted into N-acetylserotonin (NAS) by arylalkylamine N-acetyltransferase (AANAT), then hydroxyindole-O-methyltransferase (HIOMT) transform melatonin (62-64). (**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) (65), nicotinamide mononucleotide adenylyltransferase (NMNAT)(66)**,** NAD synthetase (67), amino-β-carboxymuconate-semialdehyde-decarboxylase (ACMSD) and 2-aminomuconic-6-semialdehyde dehydrogenase (AMSD) (68-70). KYNA is subsequently metabolized by the gut microbiome to quinaldic acid (QAA) and 8-hydroxyquinaldic acid (71). 8-hydroxyquinaldic acid can be dehydroxylated from xanthurenic acid (72, 73)**.** (**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 (74). 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-

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acetamide hydrolase, which can then be further metabolized into indole-3-aldehyde (IAld) or decarboxylized into 3-methylindole (skatole) (75, 76). Tryptophan decarboxylase (TrD) catalyzes the conversion of Trp to tryptamine by amino acid decarboxylase (AAD), which subsequently undergoes conversion into indole-3-acetaldehyde (IAAld) (77). IAAld can be further converted into IAA by indole-3-acetaldehyde dehydrogenase. It is also worth noting that IAAld can be reversibly converted into indole-3-ethanol (tryptophol) by IAD reductase and tryptophol dehydrogenase (78). 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), indole acrylic acid (IA), and ultimately IPA (78-80). 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, ArAT: aromatic amino acid aminotransferase, flBC: phenyllactate dehydratase, fldH: phenyllactate dehydrogenase, 3-HAA: 3-hydroxyanthranilic acid, 3-HAO: 3-hydroxyanthranilate oxidase, 5-HIAA: 5-hydroxyindoleacetic acid, 5-HIAL: 5-hydroxyindole-3-acetaldehyde, HIOMT: hydroxyindole-O-methyltransferase , 3-HK: 3-hydroxykynurenine, 5-HT: serotonin/5-hydroxytryptamine, 5-HTOL: 5-hydroxytryptophol, 5-HTP: 5-hydroxytryptophan, IA: indole acrylic 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, IDO1/2: 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, MAOA/B: monoamine oxidase A and B, 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.

Approximately 5% of dietary Trp undergoes conversion to indole and its derivatives via the gut microbial indole pyruvate pathway, facilitated by gut microbiota like Escherichia coli and Clostridium sporogenes (81-83). These bacteria produce indole and its derivatives, including indole-3-acetic acid (IAA), indole-3-aldehyde, and indoxyl sulfate (ISN), among others (Figure 1, c) (76, 84, 85). These signaling molecules influence a variety of physiological processes in the host, including the intestinal barrier, inflammation, oxidative stress, hormone secretion, and nuclear receptor activation (86-88). Many gastrointestinal and hepatic conditions, such as colorectal cancer, irritable bowel syndrome, non-alcoholic fatty liver disease, and hepatic encephalopathy, are linked to the gut microbial indole pyruvate pathway (80, 86, 89). Disruptions in the gut microbial indole pyruvate pathway can impact the neurotransmitters, hormones, and immune factors in the brain (90-92). This can also impair the communication between the gut and the brain through the vagus nerve (93-95). The increasing attention towards the role of the gut microbial indole pathway in mental health disorders such as depression, anxiety, autism, SCZ, and AD highlights its potential significance in the field of neuropsychiatry (96-100).

However, the understanding of the interplay between Trp-KYN, 5-HT, and indole metabolism in the pathogenesis 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 (68, 101, 102). Among the KAT enzymes, kynurenine/alpha-aminoadipate aminotransferase (KAT/AadAT, aka KAT II) is a mitochondrial enzyme encoded in the gene *aadat* (*kat2*) (103). KAT II is considered to play the most important role among the four isozymes

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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 (104).

Preclinical research has significantly contributed to our understanding of mental illnesses by elucidating the underlying pathomechanisms and identifying potential therapeutic targets (105-113). Researchers have employed preclinical animal models to examine the causes and effects of mental disorders, thereby attaining a comprehensive understanding of their underlying pathology (114-121). In vitro models, such as cell cultures and organoids, have facilitated the investigation of complex molecular pathways linked to mental disorders (122-125). Animal models, along with other in vivo models, have been instrumental in studying the behavioral, cognitive, and physiological dimensions of mental disorders (126-132). These models allow researchers to simulate disease conditions, assess symptomatology, and evaluate the efficacy of potential interventions (132, 133). 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 (134). These animals offer indispensable insights into human diseases, facilitating the exploration of disease mechanisms, experimentation with potential treatments, and assessment of therapeutic effectiveness (135-139). Moreover, they offer crucial insights into changes in structure and imaging techniques in clinical cases (140-158). Preclinical and clinical research collaboratively contribute to innovative therapeutics and personalized medicine (159-164).

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 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) 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), 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

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 (86/609/ECC), 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) 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

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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 cm^2 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. 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. After the transport of the animals, the studies complied with the Ethical Codex of Animal Experiments, were approved by the Committee of the Animal Research of the University of Szeged (I-74-10/2019, I-74-1/2022), were authorized by the National Food Chain Safety Office with permission number XI./95/2020, and complied with the guidelines for the Use of Animals in Research of the International Association for the Study of Pain and the directive of the European Parliament (2010/63/EU).

The deletion was introduced into the KATs gene using the CRISPR/Cas9 method. The single guide RNAs (sgRNA) were selected using the CRISPRdirect software. Artificially synthesized the sgRNA were purchased from FASMAC (Atsugi, Japan). The 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 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, Japan) were injected into the cytoplasm of these one-cell-stage embryos. The injected two-cell embryos were then transferred into pseudopregnant ICR mice (Figure 2).

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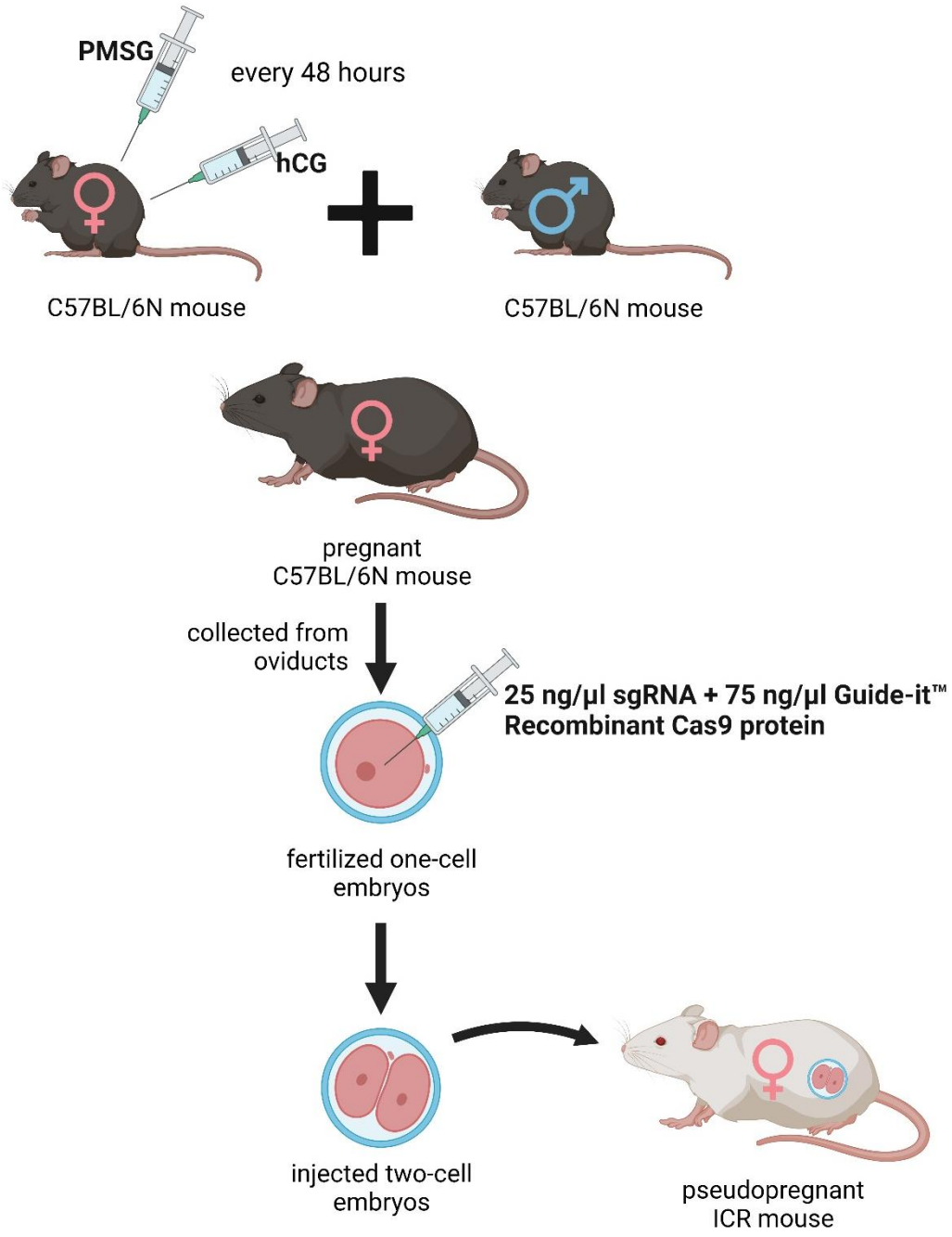

Figure 2. Generation of the knockout *kat2*$^{-/-}$ mice. Female C57BL/6N mice were treated with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) with a 48-hour interval between administrations, then mated with male C57BL/6N mice. From the oviducts, fertilized one-cell embryos 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 ICR mice. PMSG: pregnant mare serum gonadotropin; hCG: human chorionic gonadotropin; sgRNA: single guide RNA.

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.

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2.3. DNA Extraction and Sequencing

Genomic DNA of tails collected from mice was extracted using NucleoSpin Tissue (MACHEREY-NAGEL GmbH&Co, KG, 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, Japan) and the 1st primers pair and subsequently with 2nd primers pair (Table 1). The polymerase chain reaction (PCR) product was 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.). Then, the PCR products were sequenced with M-KAT II_2nd_R (Table 1).

Table 1. Properties of sgRNA, primers and KAT gene. sgRNA: single guide RNA; KAT: kynurenine aminotransferase; KAT II: aminoadipate aminotransferase; CCDS: Consensus Coding Sequence.

Name of sgRNA	Sequence
M-KAT II-2	GTTCCTCACTGCAACGAGCCguuuuagagcuagaaauagcaaguuaaaaaaggcuaguccguuaucaacuugaaaaaguggcacggacucggugcuuuu

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
Aadat-201	ENSMUST00000079472.4	CCDS22320	32-33 (2 nucleotide deletion)

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, Minnesota, 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 3X SDS Blue Loading Buffer (New England BioLabs) 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, Massachusetts, USA) at room temperature for 2 h, followed by combination with alkaline phosphatase-labeled secondary goat anti-rabbit IgG FC antibody (1:10000, Sigma-Aldrich) 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).

2.5. Phenotype Analysis with Modified SHIRPA Test

The RIKEN modified SHIRPA 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 (165, 166).

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2.6. Behavioral Tests

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 (167, 168).

2.6.2. Tail Suspension Test (TST)

The mice were placed in a 28x28x23.5 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 (169, 170).

2.6.3. Elevated Plus Maze (EPM) Test

The animals were positioned in a plus-shaped apparatus with four arms measuring 35x10 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 allow 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 and software (EthoVision XT14). Between each animal, the apparatus was disinfected with 70% ethanol and left exposed to the air for 5 minutes (171, 172).

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 5x5 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 (172-174).

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 5x5 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 (175).

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2.6.6. Open Field (OF) Test

A standard table lamp illuminated the center of the 48x40 cm OF box, while the Conducta 1.0 system (Experimetria Ltd.) 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 (176, 177).

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

Trp and its metabolites were measured in plasma and urine using previously published protocols (178, 179) 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.

2.8. The Enzyme Activities of Trptophan (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-HK concentrations to the sums of putative antioxidant metabolite concentrations (KYNA, AA, and XA) (1) (180-182).

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

The excitotoxicity index is calculated by dividing the concentration of NMDA receptor agonist QA by that of NMDA receptor antagonist KYNA (2)(183-185).

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

3.0. Statistical Analysis

We used IBM SPSS Statistics 28.0.0.0 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 TST, OF, FST, and EPM followed by the Tamhane post hoc test. Values $p < 0.05$ were considered statistically significant. Our data are reported as means ± SEM for all parameters and groups.

3. Results

3.1. DNA Sequence Analysis and Western Blot

To generate knockout mice of *kat2* gene, 25 ng/µl of sgRNA and 75ng/µl 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 WT counterparts (Figure 3).

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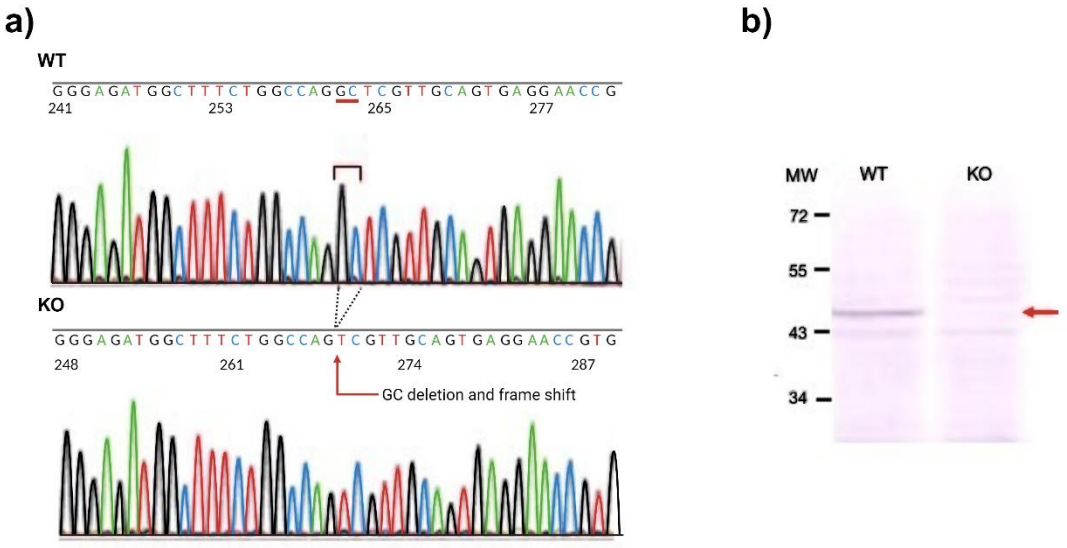

Figure 3. DNA sequence analysis of knockout *kat2*$^{-/-}$ mouse line. a) Genomic sequences around the mutation site of knockout *kat2*$^{-/-}$ mouse strain. b) Western blot analysis of knockout *kat2*$^{-/-}$ mouse line.

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 (Figure 4, a,b; Table 2). There were no significant differences in climbing time (Table 2).

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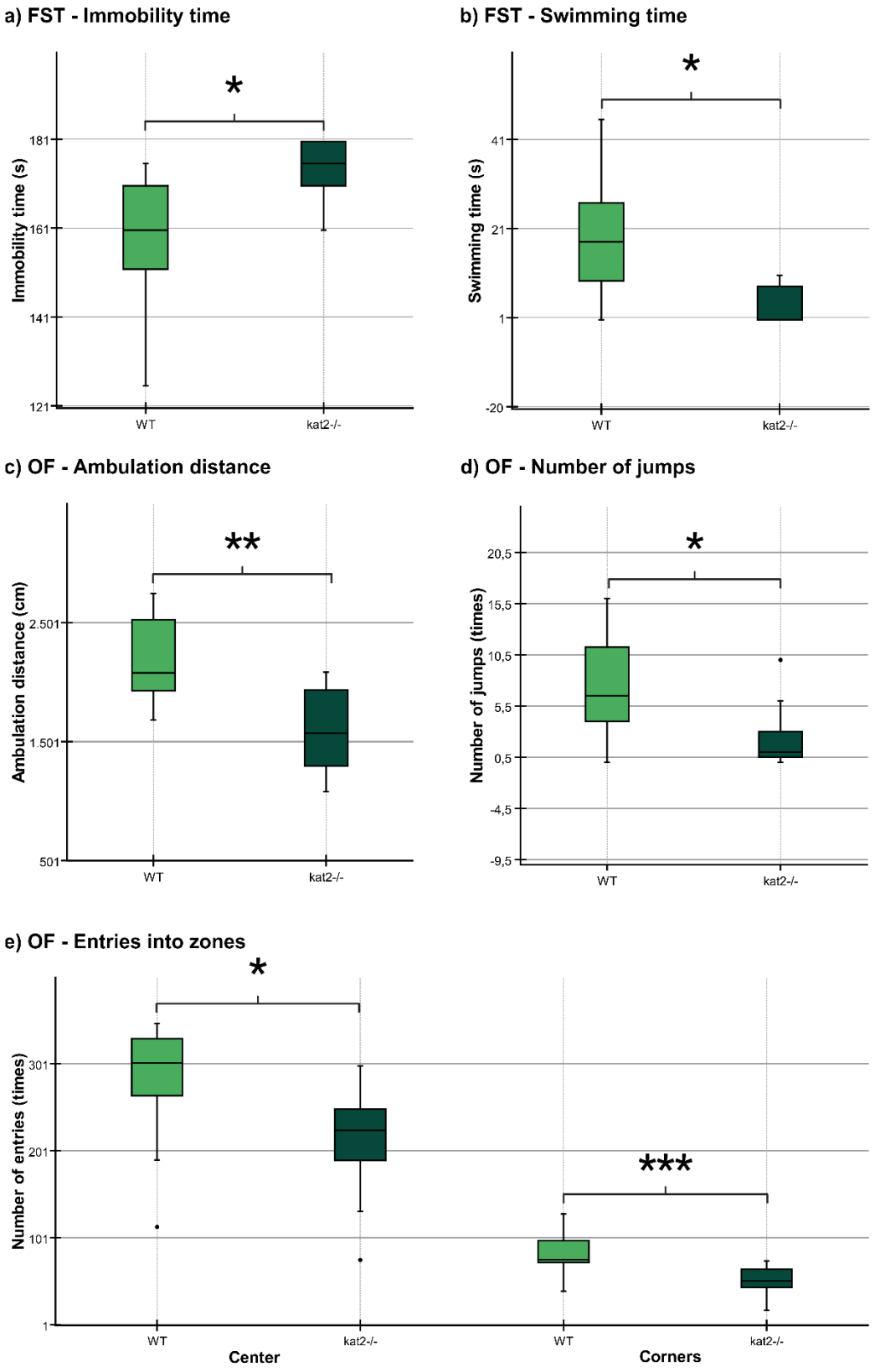

Figure 4. Behavioral tests. a) Time spent immobile in the modified forced swim test (FST); b) Time spent swimming in the modified FST; c) Ambulation distance in the open field (OF) test; d) Number of jumps in the OF test; and e) Number of entries into the center and corner zones in the OF test. Wild-type mice (light green); kat2$^{-/-}$ mice (dark green). WT: wild-type; kat2$^{-/-}$: kynurenine aminotransferase

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II knockout mice; FST: forced swim test; OF: open field test; •: outliner. Mean ± SEM. *: p ≤ 0.05; **: p ≤ 0.01; ***: p ≤ 0.001.

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

Test type	Number of animals	Perspectives	Mean ± SEM of wild-type	Mean ± SEM of *kat2*$^{-/-}$	p-value
Modified forced swim test (FST)	WT: n = 12 *kat2*$^{-/-}$: n = 11	Immobility time (s)	157,73 ± 4,97	174,09 ± 2,00	0,022 *
		Swimming time (s)	18,18 ± 4,44	3,18 ± 1,39	0,014 *
		Climbing time (s)	4,09 ± 1,26	1,82 ± 1,82	0,681
Tail suspension test (TST)	WT: n = 10 *kat2*$^{-/-}$: n = 13	Immobility time (s)	194,50 ± 21,11	209,58 ± 18,65	0,625
Passive avoidance (PA) test	WT: n = 12 *kat2*$^{-/-}$: n = 12	Time spent in the lit box on the training day (s)	48,33 ± 8,44	64,67 ± 16,10	0,979
		Time spent in the lit box on the test day (s)	256,0 ± 22,2	283,8 ± 11,4	0,822
Elevated plus maze (EPM) test	WT: n = 10 *kat2*$^{-/-}$: n = 11	Time spent in the open arms (s)	42,90 ± 19,48	30,64 ± 13,17	0,500
Light-dark box (LDB) test	WT: n = 12 *kat2*$^{-/-}$: n = 11	Time spent in the lit box (s)	119,00 ± 9,06	113,91 ± 7,36	0,957
Open-field test (OFT)	WT: n = 12 *kat2*$^{-/-}$: n = 11	Number of entries to the center zones (times)	281,67 ± 19,96	210,73 ± 19,66	0,011 *
		Number of entries to the corner zones (times)	83,08 ± 7,78	51,27 ± 5,39	0,001 ***
		Ambulation distance (cm)	2191,75 ± 105,21	1609,27 ± 115,17	0,002 **
		Number of jumps (times)	7,33 ± 1,43	2,45 ± 0,93	0,034 *

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 (Figure 4, c; Table 2). The number of jumps was significantly fewer in the *kat2*$^{-/-}$ mice than that of their WT counterparts (Figure 4, d; Table 2). There were significantly fewer entries into the center and corner zones compared to their WT counterparts (Figure 4, e; 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-HIAA, and IAA in plasma samples than wild-type mice (Figure 5, Table 3).

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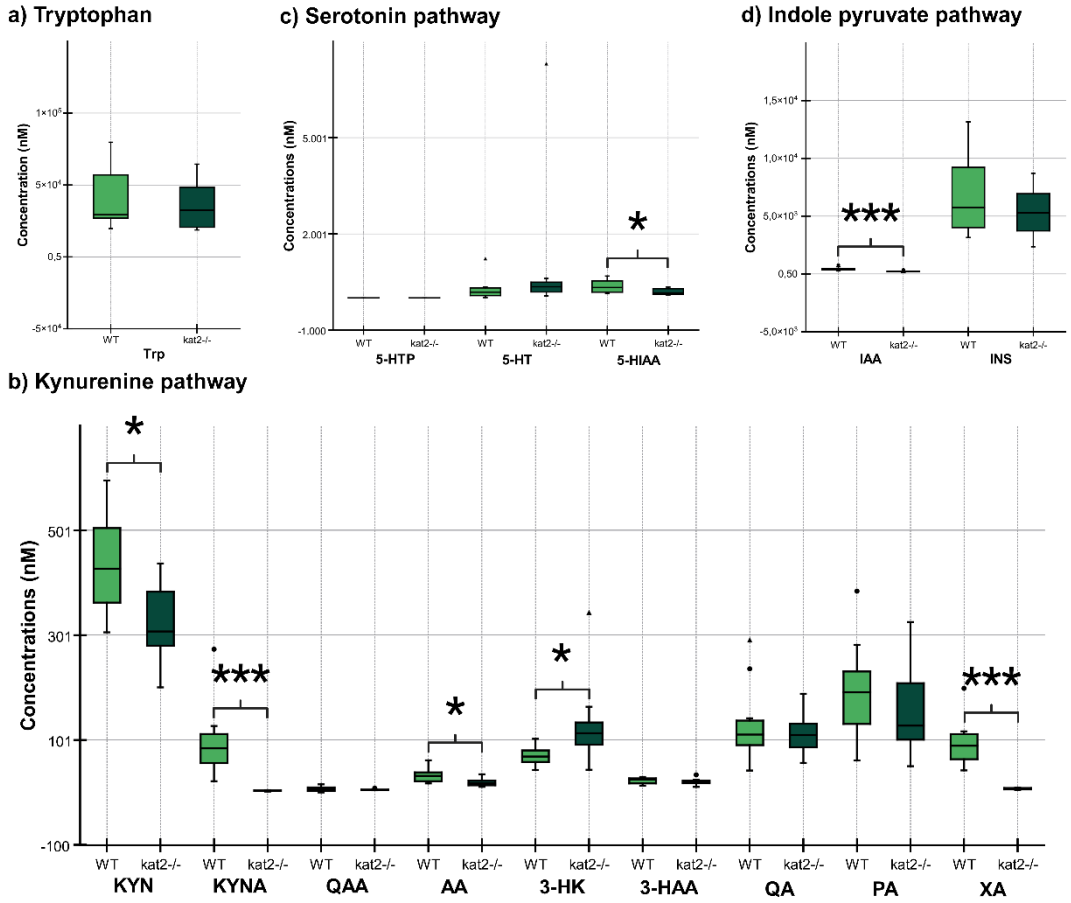

Figure 5. Concentration of tryptophan and its metabolites in plasma. We marked wild-type with light, and *kat2*$^{-/-}$ mice results with dark green boxes. WT: wild-type; *kat2*$^{-/-}$: kynurenine aminotransferase II knockout; Trp: tryptophan; 5-HTP: 5-hydroxytryptophan; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid; IAA: indole-3-acetic acid; INS: indoxyl-sulphate; KYN: kynurenine; KYNA: kynurenic acid; QAA: quinaldic acid; AA: anthranilic acid; 3-HK: 3-hydroxykynurenine; 3-HAA: 3-hydroxyanthranilic acid; QA: quinolinic acid; PA: picolinic acid; XA: xanthurenic acid; •: outliner; ▲: far out. Mean+SEM; * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001.

Table 3. QC 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)). SD: standard deviation; CV%: coefficient of variation (should be <20%).

	Plasma (nM)			Urine (nmol/mmol Creatinine)		
	Mean ± SD		p value	Mean+SD		p value
	WT	kat2$^{-/-}$		WT	kat2$^{-/-}$	
Tryptophan (Trp)	40901.678 ± 21056.888	35543.573 ± 16203.237	0.532	2022.196 ± 908.643	1972.014 ± 286.954	0.870
Kynurenine (KYN)	440.674 ± 102.886	327.348 ± 76.385	0.012 **	25.238 ± 10.185	50.883 ± 17.134	< 0.001 ***
Kynurenic acid (KYNA)	96.960 ± 70.837	3.654 ± 0.860	< 0.001 ***	11783.938 ± 5040.178	920.990 ± 215.223	< 0.001 ***
Quinaldic acid (QAA)	6.884 ± 5.397	5.608 ± 1.234	0.476	14.248 ± 9.716	12.014 ± 7.490	0.572
3-hydroxykynurenine (3-HK)	70.714 ± 18.994	130.851 ± 82.199	0.037 **	55.472 ± 31.438	5986.833 ± 3157.255	< 0.001 ***

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Xanthurenic acid (XA)	93.624 ± 45.637	7.406 ± 1.452	< 0.001 ***	127228.662 ± 52582.223	3273.334 ±1021.511	< 0.001 ***
Anthranilic acid (AA)	32.655 ± 13.114	19.335 ± 7.280	0.012 **	69.112 ± 45.347	60.862 ± 22.368	0.612
3-Hydroxyanthranilic acid (3-HAA)	22.992 ± 6.140	20.920 ± 5.921	0.452	1741.538 ± 824.887	1789.475 ± 454.422	0.874
Quinolinic acid (QA)	132.185 ± 75.409	112.000 ± 41.600	0.468	10059.485 ± 4601.597	11718.491 ± 2401.051	0.326
Picolinic acid (PA)	193.797 ± 93.230	154.895 ± 88.753	0.352	190.435 ± 91.394	193.898 ± 113.072	0.941
5-Hydroxytryptophan (5-HTP)	2.790 ± 1.577	2.708 ± 1.297	0.901	21.742 ± 8.520	19.297 ± 3.833	0.419
Serotonin (5-HT)	277.309 ± 353.179	1010.379 ± 2219.355	0.316	371.974 ± 125.489	479.383 ± 63.304	0.027 *
5-hydroxyindoleacetic acid (5-HIAA)	362.241 ± 199.450	201.217 ± 99.184	0.035 **	3774.968 ± 1666.005	2969.725 ± 598.373	0.167
Indole-3-acetic acid (IAA)	457.329 ± 153.046	229.142 ± 68.266	< 0.001 ***	6030.306 ± 4737.901	1513.400 ± 1097.122	0.009 **
Indoxyl-sulphate (INS)	6738.111 ± 3559.896	5404.257 ± 2292.535	0.332	400636.750 ± 185880.105	497063.585 ± 190235.646	0.267

In urine samples, KYN, KYNA, XA and IAA were significantly lower, whereas 3-HK, and 5-HT were significantly higher than those of the wild type (Figure 6, Table 3).

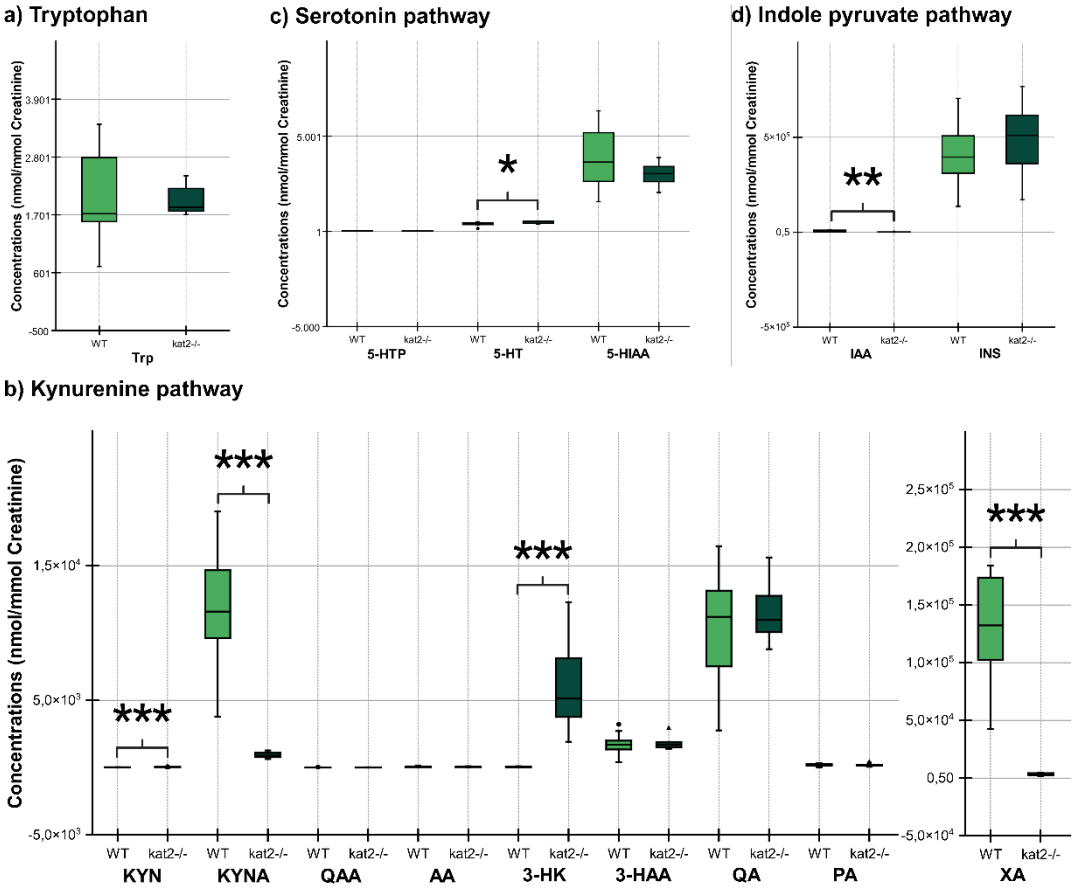

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Figure 6. Concentration of tryptophan and its metabolites in urine. We marked wild-type with light, and *kat2*$^{-/-}$ mice results with dark green boxes. WT: wild-type; *kat2*$^{-/-}$: kynurenine aminotransferase II knockout; Trp: tryptophan; 5-HTP: 5-hydroxytryptophan; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid; IAA: indole-3-acetic acid; INS: indoxyl-sulphate; KYN: kynurenine; KYNA: kynurenic acid; QAA: quinaldic acid; AA: anthranilic acid; 3-HK: 3-hydroxykynurenine; 3-HAA: 3-hydroxyanthranilic acid; QA: quinolinic acid; PA: picolinic acid; XA: xanthurenic acid; •: outliner; ▲: far out. Mean+SEM; * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001.

3.5. Enzyme Activities in Trptophan (Trp) Metabolism

The transgenic mice showed significantly lower KATs, kynureninase, MAO, ALDH, and TMO activities and significantly higher kynurenine 3-monooxygenase activity in plasma samples than wild-type mice. In the urine samples, the transgenic mice showed significantly lower KATs, 3-HA, TPH, and TMO activities, and significantly higher TDO/IDOs (KFA), KMO, KYNU, AADC, MAO, and ALDH activities compared to the wild type (Table 4).

Table 4. Enzymes activities in plasma and urine.

Enzyme	Product/Substrate	Plasma			Urine		
		Mean ± SD		p value	Mean ± SD		p value
		WT	kat2$^{-/-}$		WT	kat2$^{-/-}$	
TDO/IDOs (KFA)	**KYN/Trp**	0.013 ± 0.007	0.011 ± 0.006	0.532	0.013 ± 0.002	0.026 ± 0.006	< 0.001 ***
KATs	**KYNA/KYN**	0.205 ± 0.107	0.011 ± 0.002	< 0.001 ***	476.464 ± 164.156	18.937 ± 5.057	< 0.001 ***
KMO	**3-HK/KYN**	0.168 ± 0.062	0.386 ± 0.180	0.002 **	2.219 ± 0.827	122.983 ± 75.543	< 0.001 ***
KYNU	**AA/KYN**	0.075 ± 0.028	0.059 ± 0.016	0.120	2.593 ± 0.862	1.253 ± 0.529	< 0.001 ***
KYNU	**3-HAA/3-HK**	0.330 ± 0.070	0.194 ± 0.080	< 0.001 ***	35.177 ± 16.776	0.372 ± 0.182	< 0.001 ***
KAT III	**XA/3-HK**	1.374 ± 0.714	0.070 ± 0.033	< 0.001 ***	2702.990 ± 1524.430	0.629 ± 0.229	< 0.001 ***
3-HAO	**QA/3-HAA**	5.l771 ± 2.978	5.486 ± 1.994	0.804	6.240 ± 2.487	6.856 ± 1.779	0.532
3-HA	**PA/3-HAA**	8.797 ± 4.263	7.681 ± 4.872	0.592	1.286 ± 0.594	0.119 ± 0.085	< 0.001 ***
TPH	**5-HTP/Trp**	< 0.001 ± <0.001	< 0.001 ± < 0.001	0.128	15.653 ± 6.024	0.010 ± 0.003	< 0.001 ***
AADC	**5-HT/5-HTP**	97.585 ± 87.384	307.233 ± 509.276	0.216	10.209 ± 2.530	25.997 ± 7.185	< 0.001 ***
MAO & ALDH	**5-HIAA/5-HT**	4.217 ± 4.818	0.905 ± 0.712	0.045 *	< 0.001 ± < 0.001	6.181 ± 0.859	< 0.001 ***
TMO (TrD, ArAT)	**IAA/Trp**	0.013 ± 0.005	0.007 ± 0.002	0.005 **	5.243 ± 1.794	0.786 ± 0.636	< 0.001 ***
TNA	**INS/Trp**	0.208 ± 0.178	0.170 ± 0.089	0.555	203.069 ± 108.000	248.916 ± 81.413	0.298

2.9. Oxidative Stress and Excitotoxicity Indices

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

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

Oxidative stress index						
Oxidant/antioxidant metabolites	**Plasma (nM)**			**Urine (nmol/mmol Creatinine)**		
	Mean ± SD		**p value**	**Mean ± SD**		**p value**
	WT	***kat2*$^{-/-}$**		**WT**	***kat2*$^{-/-}$**	
3-HK/KYNA+AA+XA	0.378 ± 0.163	4.090 ± 1.478	< 0.001 ***	0.085 ± 0.011	1.352 ± 0.473	< 0.001 ***
Excitotoxicity index						
NMDA agonist/antagonist metabolites	**Plasma (nM)**			**Urine (nmol/mmol Creatinine)**		
QA/KYNA	1.648 ± 0.810	30.514 ± 8.618	< 0.001 ***	0.884 ± 0.320	13.092 ± 2.833	< 0.001 ***

3. 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 (186-189). Alterations in 5-HT precursor Trp metabolism are noted in mental illnesses, but their connection with the Trp-KYN metabolic system remains poorly understood (190-192). Growing evidence suggests that the gut microbial indole pyruvate pathway can influence the microbiome-gut-brain axis, implying that intestinal tryptophan metabolism may play a significant role in psychological health. The microbiome-gut-brain axis is responsible for regulating mood, cognition, stress response, and behavior. 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 (69, 193-195). The mitochondrial isoform KAT II exclusively influences cellular bioenergetics due to its exclusive location in the mitochondria (101, 187). 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, induces a unique depression-like phenotype marked by increased immobility in FST, likely linked to serotonergic pathways. TST didn't show significant differences, possibly due to FST conditioning. PAT indicated despair-induced depression rather than average-conditioned memory. Anxiety-like behaviors (EPM, LDB, and MB tests) 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 leads to a PTSD-like phenotype, including a la belle indifference trait, indicative of complex PTSD with emotional dysregulation (196-198).

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. Furthermore, gene knockout affects enzyme activity, puts organisms under oxidative stress, and imposes high excitoneurotoxicity. The study demonstrates that the deletion of the *kat2* gene leads to a specific set of characteristics, including behavior similar to depression, impaired motor function, decreased levels of KYNA, and a change in the way tryptophan 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.

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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 (199-201). 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 (39, 202-205). 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 (206). Recalling distressing memories, triggered by cues linked to the initial negative encounter, results in the disruption of serotonin, 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 (207, 208).

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 (209). Despair memory involves the consolidation and retrieval of long-term memories, influenced by stress and emotion (210). In contrast, a despair experience entails immediate emotional responses influenced by factors like cognitive assessments, environmental cues, and physiological states (211). 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 serotonin (212). 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 (213). 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 (214). Preclinical studies found normal 5-HT levels with no behavioral changes in *Tph1*$^{-/-}$ mice, while *Tph2*$^{-/-}$ mice's behaviors are inconclusive (215, 216). The knock-in mice of the TPH2 variant (R439H) showed depression-like behavior in TST (217). 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 (218). 5-HT1A receptor knockout (*5-HT1AR*$^{-/-}$) mice display heightened fear memory to contextual cues, suggesting a role for 5-HT receptors in PTSD-like phenotype (219). 5-HT 2C receptor knockout *5-HT2CR*$^{-/-}$ mice attenuates fear responses in contextual or cued but not compound context-cue fear conditioning (220). Knockout of the 5-HTT gene in mice (*5-HTT*$^{-/-}$) leads to impaired stress response, fear extinction, and abnormal corticolimbic structure (221).

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 (222). Growing evidence indicates disrupted KYN metabolism in MDD, bipolar disorder, and SCZ (223-225). Earlier, KYN metabolites were suggested to be either neuroprotective or neurotoxic (226). However, increasing evidence suggests that KYN metabolites exhibit versatile actions, potentially influenced by concentrations and the microenvironment (227). 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 (228, 229).

Other biosystems play an important role in the pathogenesis of PTSD include dopaminergic and gamma-aminobutyric acid (GABA)ergic system and cannabinoids. Catechol-*O*-methyltransferase (COMT) degrades dopamine. *COMT*$^{-/-}$ mice exhibited an increased response to repeated stress exposures (230). Glutamic acid decarboxylase (GAD) synthesizes GABA (231). *GAD6*$^{-/-}$ mice shows

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increased generalized fear and impaired extinction of cued fear (232). GABA receptor subunit B1a knockout *GABAB1a*$^{-/-}$ mice showed a generalization of conditioned fear to nonconditioned stimuli (233). Cannabinoid 1 receptor (CBIR) knockout *CB1R*$^{-/-}$ mice showed an increased response to repeated stress exposures (234).

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 week) to reveal that, toward the 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 are 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 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. 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 (235). Consequently, Translational research has limitations that necessitate careful interpretation. 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 (236-238). 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 serotonin 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 (239, 240). 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.

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Abbreviations

5-HT: serotonin
AD: Alzheimer's disease
CBIR: 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
ISN: indoxyl sulfate
KAT II: α-aminoadipate aminotransferase/kynurenine aminotransferase II
KATs: kynurenine aminotransferases
KYN: kynurenine
KYNA: kynurenic acid
LDB: light dark box
MDD: major depressive disorder
MRM: multiple reaction monitoring
OF: open field
PAT: passive avoidance test
PCR: polymerase chain reaction
PMSG: pregnant mare serum gonadotropin
PTSD: posttraumatic stress disorder
SCZ: schizophrenia
sgRNA: single guide RNA
SSRI: selective serotonin reuptake inhibitors
TNA tryptophanase
Trp: tryptophan
TST: tail suspension test

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UHPLC-MS: ultra-high performance liquid chromatography-tandem mass spectrometry
WT: wild-type

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