

Activation of Brain Indoleamine-2,3-dioxygenase Contributes to Depressive-Like Behavior Induced by an Intracerebroventricular Injection of Streptozotocin in Mice

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Abstract There is a lack of information concerning the molecular events underlying the depressive-like effect of an intracerebroventricular injection of streptozotocin (ICV-STZ) in mice. The elevated activity of the tryptophan-degrading enzyme indoleamine-2,3-dioxygenase (IDO) has been proposed to mediate depression in inflammatory disorders. In the present study, we reported that ICV-STZ activates IDO in the hippocampus of mice and culminates in depressive-like behaviors, as measured by the increased duration of immobility in the tail suspension test and decreased sucrose intake in the sucrose preference test. The blockade of IDO activation by the IDO inhibitor 1-methyltryptophan (1-MT) prevents the development of depressive-like behaviors and attenuates STZ-induced up-regulation of proinflammatory cytokines in the hippocampus. 1-MT abrogates kynurenine production and normalizes brain-derived neurotrophic factor (BDNF) and the kynurenine/tryptophan ratio, but does not protect the biomarkers of the serotonin (5-HT) system in the hippocampus of STZ-injected mice. These results implicate IDO as a critical molecular mediator of STZ-induced depressive-like behavior, likely through activation of the kynurenine pathway and subsequent reduction of BDNF levels. Impairment of the 5-HT system may reflect the inflammatory response induced by STZ and also contributes to observed depression symptoms. The present study not only provides evidence that IDO plays a critical role in mediating inflammation-induced depression but also supports the notion that

neuroinflammation and the kynurenine pathway are important targets for novel therapeutic drugs for depression. In addition, this study provides new insights on the neurobiological mechanisms underlying ICV-STZ and indicates that this model could be employed in preclinical research of depression.

Keywords Streptozotocin · Depressive-like behavior · Indoleamine-2,3-dioxygenase · Inflammation · Brain-derived neurotrophic factor

Abbreviations

BDNF	Brain-derived neurotrophic factor
i.c.v.	Intracerebroventricular
IDO	Indoleamine, 2-3-dioxygenase
IL-1 β	Interleukin-1beta
IL-6	Interleukin-6
KYN	Kynurerine
OFT	Open-field test
s.c.	Subcutaneously
STZ	Streptozotocin
SPT	Sucrose preference test
TRP	Tryptophan
TST	Tail suspension test
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	Serotonin
1-MT	1-Methyltryptophan

Introduction

Depression is a common psychological disorder affecting up to 15% of the population over the life course. Depression was the third leading cause of burden among all diseases in the year 2004 and is expected to be the greatest

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cause in high-income countries by 2030 [1]. This condition is associated with marked impairments in daily functioning and work and creates significant demands on service providers in terms of workload. Reflecting the high prevalence and negative impact on the quality of life of the world population, a better understanding of the neurobiological mechanisms of depression is of paramount importance to develop new treatment drugs.

While the pathophysiology of depression has not been fully elucidated, increasing evidence has shown that neuroinflammation may play a role in the development of this disorder [2–4]. Specifically, it has been reported that patients with depression frequently show immune system alterations, such as elevated proinflammatory cytokines levels in plasma and cerebrospinal fluid [5]. Additionally, cytokine immunotherapy in the treatment of hepatitis C or cancer induces depression symptoms in otherwise psychiatrically normal individuals [6]. Further evidence supporting an inflammatory basis for depression comes from studies reporting the induction of depressive-like behaviors in animals upon the administration of inflammatory cytokines [3, 7]. In contrast, inhibiting proinflammatory cytokines may improve depressed mood and increase the treatment response to conventional antidepressant medication [5]. Thus, it has been proposed that depression is an inflammatory-associated disorder.

An impaired central serotonin (5-hydroxytryptamine, 5-HT) system has been implicated in the pathogenesis of depression [8, 9]. In the past 20 years, increasing interest has focused on the correlation between inflammation and low 5-HT. Proinflammatory cytokines, including interleukin-6 (IL-6) and interleukin-1beta (IL-1 β), secreted by glial cells within the brain in response to injury and infection, impair the 5-HT system through activation of the tryptophan (TRP)-metabolizing enzyme indoleamine-2,3-dioxygenase (IDO) [10]. This enzyme, primarily observed in glial cells, is the first and rate-limiting enzyme in the kynurenine (KYN) pathway, the major metabolic pathway for TRP in the body [11]. IDO activation results in elevated KYN production, and several downstream metabolites have been correlated with inflammation-associated depression [12, 13]. Consequently, IDO induction has been proposed as a mechanism by which inflammation can precipitate depression, either reducing the availability of TRP for 5-HT synthesis, or by increasing KYN formation [14]. It has also been proposed that the neurotoxic metabolites of the KYN pathway can lead to neurodegeneration and reduce neurogenesis in the brain [15]. Indeed, a previous study demonstrated that the systemic inflammatory challenge with bacterial lipopolysaccharide (LPS) reduced the expression of the neurotrophin brain-derived neurotrophic factor (BDNF) in rat brain [16]. Considering the role of BDNF in driving neurogenesis, a process implicated in the

pathogenesis of depression and in the therapeutic response to antidepressants [17, 18], this event may contribute to the depressive behavior that occurs secondary to inflammation, thereby implicating IDO as a critical molecular mediator of inflammation-induced depressive-like behavior.

Streptozotocin (STZ), a glucosamine compound with betacytotoxic action, is commonly used to induce diabetes in laboratory animals. Recently, numerous studies have suggested that the intracerebroventricular (i.c.v.) administration of a subdiabetogenic dose of STZ in rodents is a valid experimental model to study pathophysiological alterations in sporadic Alzheimer's disease (AD) [19–22]. The i.c.v. injection of STZ (ICV-STZ) induces cognitive dysfunction, oxidative stress, neuroinflammation and other neuropathological and biochemical changes similar to those observed in AD [19]. However, there is a lack of information concerning the noncognitive behavioral effects of ICV-STZ in rodents, such as depressive-like states. In a previous study, we provide the first evidence that ICV-STZ induced depressive-like behavior in the tail suspension test and anhedonia-like behavior, a core symptom of depression [23]. The behavioral deficits were followed by an increase in tumor necrosis factor-alpha (TNF- α) in the hippocampus, and treatment with fluoxetine and anti-TNF- α therapies prevented these alterations. Recently, we also reported that ICV-STZ activated IDO in the hippocampus and culminated in depressive-like behavior, measured in forced swimming and splash tests. The indirect blockade of IDO activation with the cytokine inhibitor minocycline prevented the development of depressive symptomatology [24]. Despite this information, the molecular events underlying the depressive-like effect of ICV-STZ are not well understood.

Therefore, the purpose of the present study was to determine the extent to which ICV-STZ-induced depressive-like behavior in mice was dependent on IDO activation. In addition, the ability of ICV-STZ to induce the tryptophan degrading enzyme IDO and the impact of ICV-STZ on 5-HT, KYN and BDNF levels were investigated as potential mechanisms linking STZ-induced neuroinflammation and depressive-like behaviors. The competitive inhibitor of IDO, 1-methyl-tryptophan (1-MT), was used to investigate the involvement of IDO in the mechanisms of STZ neurotoxicity.

Methods

Animals

The experiments were performed using male C57/BL6 mice (25–35 g, 60 days old). The animals were maintained at 22–25 °C with free access to water and food, under a

12:12 h light/dark cycle, with lights on at 7:00 a.m. All manipulations were conducted during the light phase of the day. All experiments were performed in separate groups of animals, and each animal was used only once in each test. The procedures of the present study were conducted according to the guidelines of the Committee on the Care and Use of Experimental Animal Resources.

Drugs

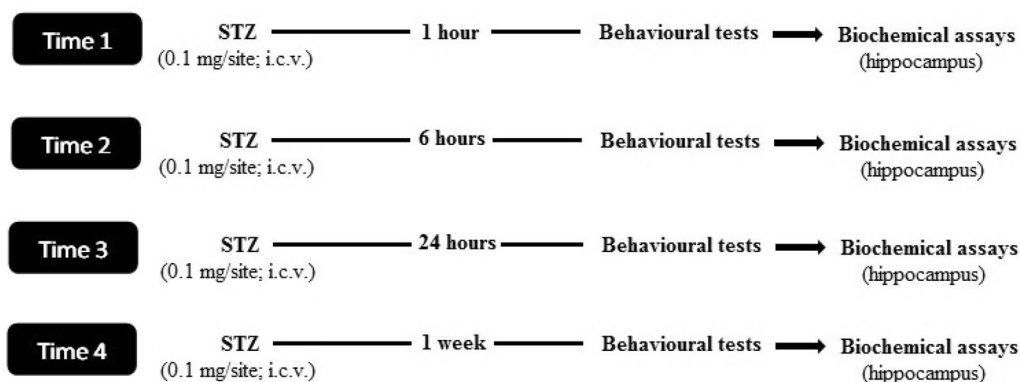
The biochemicals STZ and 1-methyltryptophan (1-MT) were purchased from Sigma-Aldrich (USA) and dissolved in saline solution prior to administration.

Experimental Design

Two experiments were conducted. In the first experiment, the mice ($n=6-8$ animals per group) received an i.c.v. injection of STZ (ICV-STZ) to investigate depressive-like behavior and IDO activity in a time-course curve (times: 1, 6, and 24 h and 1 week), as previously described [23].

In the second experiment, the mice were divided into four groups ($n=6-8$ animals per group): saline + saline (sham), saline + 1MT, STZ + saline and STZ + 1MT. Six hours after the ICV-STZ (peak effect), the mice were subjected to behavioral tests. Subsequently, the animals were euthanized, and the hippocampus was removed for neurochemical determinations (Fig. 1).

Experiment 1 (time-course curve)



Experiment 2 (IDO antagonist 1-MT)

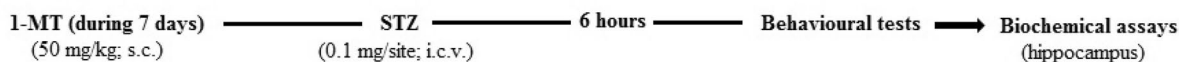


Fig. 1 Overview of study design. *STZ* streptozotocin, *i.c.v.* intracerebroventricular, *IDO* indoleamine-2,3-dioxygenase, *1-MT* 1-methyltryptophan, *s.c.* subcutaneously

Intracerebroventricular Injection of Streptozotocin (STZ) and 1-Methyltryptophan Administration (1-MT)

STZ groups were administered an ICV-STZ (0.1 mg/site, total volume of 4 μ l), whereas the sham groups received an i.c.v. injection of saline solution (total volume of 4 μ l) as previously described [23, 25]. The mice were anesthetized with an i.p. injection of sodium pentobarbital (0.067 mg/g). A single ICV-STZ or saline was injected into the left ventricle of the brain using a stereotaxic apparatus. The bregma coordinates used for injection were -1.0 mm lateral, -0.3 mm posterior, and -2.5 mm below.

1-MT was administered subcutaneously at the dose of 50 mg/kg (administration volume of 5 ml/kg) as previously described [4, 26]. The injections were administered twice daily at 12-h intervals for 7 days prior to STZ injection. According to Xie et al. [4], the dose used in the present study produces an effect equal to that observed in previous studies using 5 mg/day pellets [27]. We prepared 1-MT using 0.1 M NaOH and adjusted the pH to 9.0 using 1 M HCl.

Behavioral Assessment

Open-Field Test (OFT)

The OFT was performed to evaluate whether the drugs produced effects on locomotor activity. The animals were

submitted individually to an OFT apparatus (Insight model EP 154C) for 5 min. The total distance (unit: mm) was computed [28, 29].

Tail Suspension Test (TST)

The mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail, and the immobility time was recorded for 6 min. The immobility behavioral was determined according to the method of Steru et al. [30].

Sucrose Preference Test (SPT)

An independent group of mice was evaluated in the SPT test to investigate hedonic alterations. Anhedonic state (decreased libido and lack of pleasure from enjoyable experiences) is a classical feature of depression [31] and considered a core symptom of depressive-like behavior in rats [32]. At the beginning of the test, all the groups were singly housed in individual cages for 48 h. After 24 h of food and water deprivation, the mice were treated with STZ (0.1 mg/site, i.c.v.) or saline. 1-MT was administered for 7 days prior to exposure to the test. Two bottles were available in each cage, one bottle contained 100 ml of 1% sucrose and the other bottle contained 100 ml of tap water. The total intake of sucrose was calculated from the amount of sucrose solution consumed and expressed as a percentage of the total amount of liquid drunk [23, 31].

Biochemical Assays

After behavioral tests, the mice were euthanized, and blood samples were collected and the hippocampus was removed. The hippocampus was homogenized in 50 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at $2400\times g$ for 15 min at 4 °C, and a low-speed supernatant fraction (S_1) was used for assays. The blood samples were collected directly from the ventricle of the heart in anesthetized animals, using heparin as an anticoagulant, and plasma was separated by centrifugation ($2400\times g$) for 15 min.

Blood Glucose Determination

To confirm that ICV-STZ (0.1 mg/site) is a subdiabetogenic dose, the plasma glucose level was determined by enzymatic colorimetric methods using a commercial kit (Labtest Diagnostica, MG, Brazil). The glucose level was expressed as mg/dl.

Pro-inflammatory Cytokines Levels

The levels of interleukin-6 (IL-6) and interleukin 1-beta (IL-1 β) in the hippocampus were determined using commercially available ELISA assays, according to the manufacturer's instructions (DuoSet Kits, R&D Systems; Minneapolis). The results are shown as pg/mg tissue.

Brain-Derived Neurotrophic Factor (BDNF) Levels

The levels of BDNF in the hippocampus were measured using a BDNF E_{max} ImmunoAssay System kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The supernatants were collected to determine the BDNF levels. The BDNF content was recorded as pg/mg tissue.

Tryptophan (TRP) and Kynurenine (KYN) Levels

The levels of TRP and its metabolite KYN in the hippocampus were determined using a Shimadzu LC-10A liquid chromatograph, according to Silva et al. [33]. The chromatographic separation was achieved using a 250- by 4.6-mm (inner diameter) C_{18} reverse-phase column (particle size, 4 μ m; Aquapore RP-300C-18). For TRP measurement, the column was isocratically eluted at flow rate of 1.0 ml/min with 0.015 M sodium acetate (pH 4.5) containing 15% methanol. For KYN determination, the column was eluted with acetonitrile at a 1:47 dilution in 0.1 M acetic acid–0.1 M ammonium acetate (pH 4.65). The absorbance of the column effluent was monitored at 280 and 365 nm for TRP and KYN, respectively. The TRP or KYN peaks were identified by comparison with the retention times of standard compounds (Sigma), and quantification was based on the ratios of the peak areas of compound to the internal standard. The tissue levels were expressed as pg/mg tissue.

Serotonin (5-HT) and 5-Hydroxyindoleacetic acid (5-HIAA) Levels

The levels of 5-HT and its metabolite 5-HIAA in the hippocampus were analyzed using high performance liquid chromatography (HPLC) with electrochemical detection, according to Ferraz et al. [34]. The mobile phase, used at a flow rate of 0.8 ml/min, comprised 0.02 M phosphate/citrate buffer and 90/10 methanol (v/v), 0.12 mM Na_2 EDTA, and 0.0556% heptane sulfonic acid as an ion pair. The pH was adjusted to 2.64 with H_3PO_4 at 22 °C. A 5- μ m (220×4.6) Spheri-5 RP-18 column from Brownlee Laboratory was used. Electrochemical detection was performed using a Shimadzu L-ECD-6A electrochemical

detector with a potential of 0.75 V. The peak area of the internal standard (DHBA) was used to quantify the sample peaks. The tissue levels were expressed in pg/mg tissue.

Indoleamine-2,3-dioxygenase (IDO) Activity

IDO activity in the hippocampus was determined as previously described [35]. The supernatants (0.2 ml) were added to 0.8 ml of the reaction mixture containing 400 μ M L-tryptophan, 20 mM ascorbate, 10- μ M methylene blue, and 100 μ g of catalase in 50 mM potassium phosphate buffer, pH 6.5. The reaction was performed at 37 °C under agitation for 60 min. Subsequently, the reaction was blocked by adding 0.2 ml of 30% trichloroacetic acid and further incubated at 50 °C for 30 min to convert the *N*-formylkynurenine to L-kynurenine. The samples were centrifuged at 13,000 \times *g* for 10 min at 4 °C. The supernatants were filtered via microspin ultrafiltration with a cut-off of 10,000 M_r prior to measuring IDO activity.

The amount of L-kynurenine formed from tryptophan was determined by reversed phase high-pressure liquid chromatography (HPLC). 100 μ l of the reaction product was injected onto a Merck LiChrospher column (150 mm long, 4.6 mm diameter, packed with 5 μ m silica beads holding 18C long carbon chains). A cartridge guard column containing the same material as the analytical column was used. The mobile phase comprised 0.1 M ammonium acetate buffer (pH 4.65) with 5% acetonitrile. The flow rate was 1 ml/min. KYN was detected using a spectrometer measuring absorbency at a wavelength of 365 nm and was quantified using known amounts of L-kynurenine. The retention time of KYN was approximately 5.35 min. All determinations were performed in duplicate. One unit of the activity was defined as 1 pmol KYN/h/mg protein at 37 °C.

Protein Determination

The protein concentration was measured according to the Bradford method [36], using bovine serum albumin as the standard.

Statistical Analysis

The results are presented as the means \pm standard error of the mean (SEM). Comparisons between the experimental and control groups were performed using one-way (experiment 1) or two-way (experiment 2) analysis of variance (ANOVA), followed by Newman–Keuls and Bonferroni post hoc tests when appropriate. A value of $p < 0.05$ was considered significant. All tests were performed using GraphPad Prism software 5.0 (San Diego, CA, USA).

Results

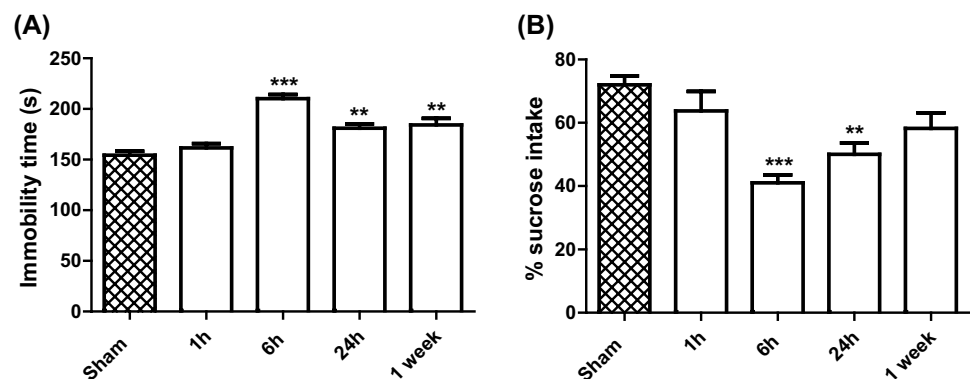
ICV-STZ Induced Depressive-Like Behavior and Anhedonic-Like Behavior

Injection of STZ at 6, 24 h and 1 week prior to the TST increased the immobility time compared to saline-treated controls (sham group) [$F(4,35)=21.56$; $p < 0.001$]. Post hoc comparisons demonstrated that the peak effect occurred at the 6-h time point (Fig. 2a).

The injection of STZ prior to SPT significantly decreased sucrose intake compared to saline-treated controls, producing an anhedonic effect [$F(4,35)=7.91$; $p < 0.001$]. Post hoc comparisons demonstrated that the injection of STZ produced an anhedonic effect at 6 and 24 h (Fig. 2b). The observed reduced preference for sucrose was not related to a change in total fluid intake, as no change in this parameter was observed when saline and STZ-treated mice were compared (data not shown).

The STZ injection did not cause significant alterations in locomotor activity in the OFT [$F(4,35)=0.03$;

Fig. 2 Effect of ICV-STZ (0.1 mg/site) on immobility time in the TST (a), sucrose intake in the SPT (b) in groups tested 1, 6, 24 h and 1 week after an STZ injection. Values are mean \pm SEM (n=6–8). ** $p < 0.01$, *** $p < 0.001$ compared with the sham group (one-way ANOVA, Newman–Keuls post hoc test)



$p < 0.99$] and glucose plasma levels [$F(4,35) = 0.05$; $p < 0.99$] (data not shown).

STZ Induced Neuroinflammation and Down-Regulation of BDNF in the Hippocampus

The neuroinflammatory response induced by STZ was characterized by an increase in the levels of pro-inflammatory cytokines IL-6 and IL-1 β .

In response to STZ injection, a significant increase in IL-6 levels was observed in the hippocampus compared to saline-treated controls [$F(4,35) = 25.85$; $p < 0.001$]. Post hoc comparisons showed that the injection of STZ increased IL-6 levels at 1, 6, and 24 h and 1 week. The peak effect occurred at the 6-h time point (Fig. 3a).

Compared to saline-treated controls, STZ-treated mice showed a significant increase of IL-1 β in the hippocampus [$F(4,35) = 42.51$; $p < 0.001$]. Post hoc comparisons showed that the injection of STZ increased IL-1 β levels at all time points analyzed. The peak effect occurred at 6 h (Fig. 3b).

In response to STZ injection, BDNF levels were significantly reduced in the hippocampus compared to saline-treated controls [$F(4,35) = 5.70$; $p < 0.001$]. Post hoc comparisons showed that the injection of STZ reduced BDNF levels at 6 and 24 h, returning to control levels at the 1-week time point (Fig. 3c).

STZ Caused IDO Activation Coupled with an Increase of TRP Levels and KYN Production in the Hippocampus

Compared to saline-treated controls, STZ-treated mice showed a significant increase in IDO activity in the hippocampus [$F(4,35) = 10.86$; $p < 0.001$]. Post hoc comparisons showed that the injection of STZ increased IDO activity at 6 and 24 h, returning to baseline levels at 1 week (Fig. 4a).

Increased levels of TRP were observed in the hippocampus after STZ injection [$F(4,35) = 12.67$; $p < 0.001$]. Post hoc comparisons showed that increased TRP was observed at 6 and 24 h post STZ injection, returning to control levels at 1 week (Fig. 4b).

The injection of STZ also significantly increased KYN levels in the hippocampus [$F(4,35) = 24.12$; $p < 0.001$]. Post hoc comparisons showed a peak effect of STZ at 6 h. Moreover, increased KYN levels were maintained significantly elevated until the 1-week time point (Fig. 4c). This finding translated to an increased KYN/TRP ratio post-STZ injection [$F(4,35) = 5.94$; $p < 0.001$] at 6 h (peak effect), maintaining significantly elevated levels at 24-h and 1-week time points (Fig. 4d).

STZ Increased 5-HIAA Levels in the Hippocampus

In response to STZ, no change in 5-HT levels was detected in the hippocampus at any time point analyzed [$F(4,35) = 0.73$; $p < 0.58$] (data not shown). However,

Fig. 3 Effect of ICV-STZ (0.1 mg/site) on the levels of IL-6 (a), IL-1 β (b) and BDNF (c) in hippocampus of mice in groups tested 1, 6, 24 h and 1 week after an STZ injection. Values are mean \pm SEM (n=6–8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the sham group (one-way ANOVA, Newman–Keuls post hoc test)

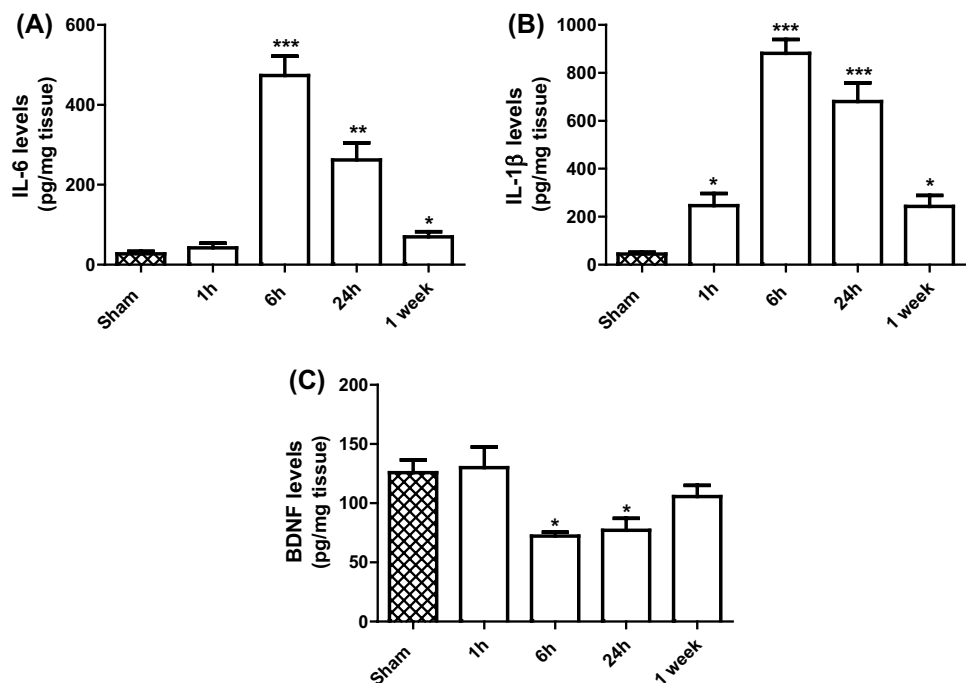


Fig. 4 Effect of ICV-STZ (0.1 mg/site) on the IDO activity (a), TRP levels (b), KYN levels (c) and KYN/TRP ratio (d) in hippocampus of mice in groups tested 1, 6, 24 h and 1 week after an STZ injection. Values are mean \pm SEM (n=6–8). * p <0.05, ** p <0.01, *** p <0.001 compared with the sham group (one-way ANOVA, Newman–Keuls post hoc test)

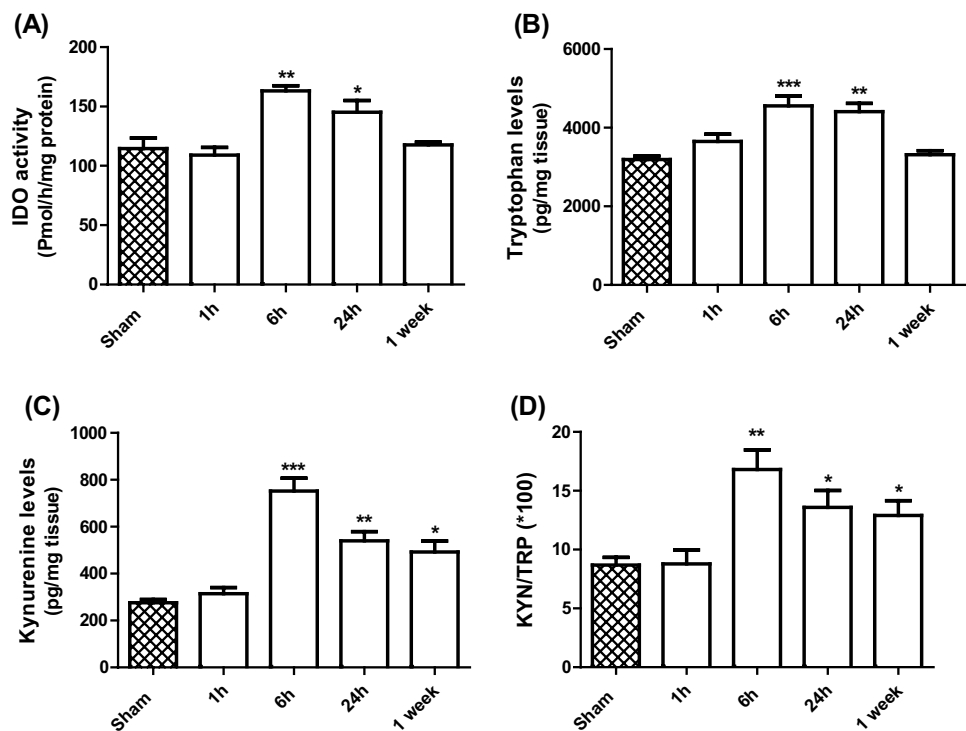
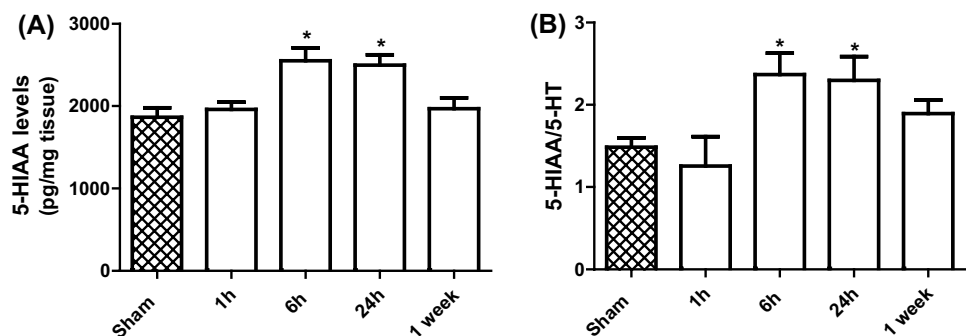


Fig. 5 Effect of ICV-STZ (0.1 mg/site) on the 5-HIAA levels (a) and 5-HIAA/5-HT ratio (b) in hippocampus of mice in groups tested 1, 6, 24 h and 1 week after an STZ injection. Values are mean \pm SEM (n=6–8). * p <0.05 compared with the sham group (one-way ANOVA, Newman–Keuls post hoc test)



an increased level of the 5-HT metabolite 5-HIAA was observed in response to STZ injection [$F(4,35)=6.80$; p <0.01], indicative of increased 5-HT metabolism. Post hoc comparisons showed that the STZ injection increased 5-HIAA levels at 6 and 24 h, returning to control levels at 1 week (Fig. 5a). An increased 5-HT/5-HIAA ratio was observed after STZ injection [$F(4,35)=3.70$; p <0.05]. Post hoc comparisons showed that this reduction significantly occurred at the 6- and 24-h time points (Fig. 5b).

STZ-Induced Depressive-Like Behavior and Anhedonia is Blocked by 1-MT Pretreatment

In experiment 2, the mice were pretreated with 1-MT for 7 days prior to STZ injection. Depressive-like behavior and locomotor activity were assessed at 6 h (peak effect) post-STZ administration.

Two-way ANOVA of the immobility time in TST revealed a significant STZ \times 1-MT interaction [$F(1,24)=10.29$; p <0.01]. Post hoc comparisons showed that the 1-MT pretreatment significantly blocked the increase in immobility time induced by STZ (Fig. 6a).

The statistical analysis of sucrose intake in SPT revealed a significant STZ \times 1-MT interaction [$F(1,24)=32.55$; p <0.001]. Post hoc comparisons demonstrated that the 1-MT pretreatment significantly protected against the decrease of sucrose intake caused by STZ. The observed reduced preference for sucrose was not related to a change in total fluid intake, as no change in this parameter was observed in all treatment groups (data not shown) (Fig. 6b).

Two-way ANOVA showed that the locomotor activity in OFT was not significantly altered by STZ injection [$F(1,24)=0.17$; p <0.68], 1-MT

Fig. 6 Effects of 1-MT (50 mg/kg; s.c.) on the immobility time in the TST (a), sucrose intake in the SPT (b) 6 h after ICV-STZ (0.1 mg/site). Values are mean ± SEM (n=6–8). @*p*<0.05 when compared STZ+saline with saline+saline. #*p*<0.05 when compared STZ/1-MT with STZ+saline (two-way ANOVA, Bonferroni post hoc test)

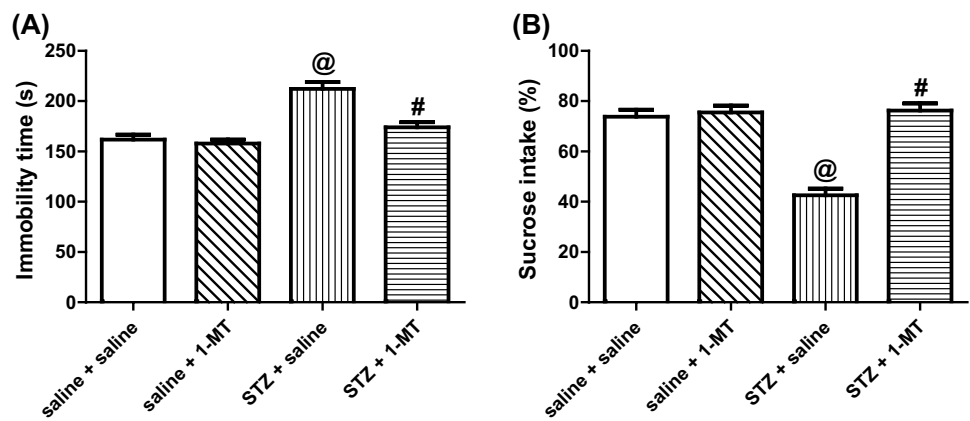
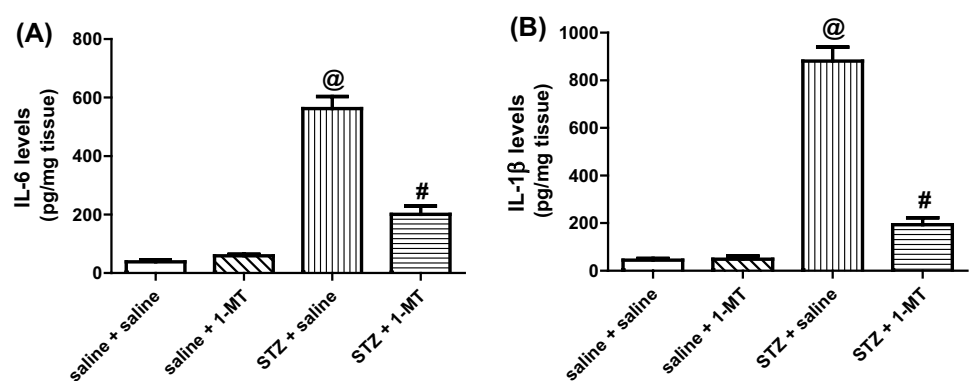


Fig. 7 Effects of 1-MT (50 mg/kg; s.c.) on the levels of IL-6 (a) and IL-1β (b) in the hippocampus of mice 6 h after ICV-STZ (0.1 mg/site). Values are mean ± SEM (n=6–8). @*p*<0.05 when compared STZ+saline with saline+saline. #*p*<0.05 when compared STZ/1-MT with STZ+saline (two-way ANOVA, Bonferroni post hoc test)



pretreatment [*F*(1,24)=0.01; *p*<0.99] or their interaction [*F*(1,24)=0.03; *p*<0.99] (data not shown).

Plasma glucose levels were also not significantly altered by STZ injection [*F*(1,24)=0.89; *p*<0.36], 1-MT pretreatment [*F*(1,24)=1.44; *p*<0.25] or their interaction [*F*(1,24)=0.03; *p*<0.86] (data not shown).

STZ-Induced Neuroinflammation in the Hippocampus is Attenuated by 1-MT

Two-way ANOVA of IL-6 in the hippocampus demonstrated a significant STZ×1-MT interaction [*F*(1,24)=21.46; *p*<0.001]. Post hoc comparisons revealed that STZ significantly increased IL-6 levels in the hippocampus of mice compared to the sham group. 1-MT pretreatment attenuated the increase of IL-6 caused by STZ (Fig. 7a).

Statistical analysis of IL-1β in the hippocampus revealed a significant STZ×1-MT interaction [*F*(1,24)=107.62; *p*<0.001]. Post hoc comparisons demonstrated that the increase of IL-1β induced by STZ was significantly attenuated by 1-MT pretreatment (Fig. 7b).

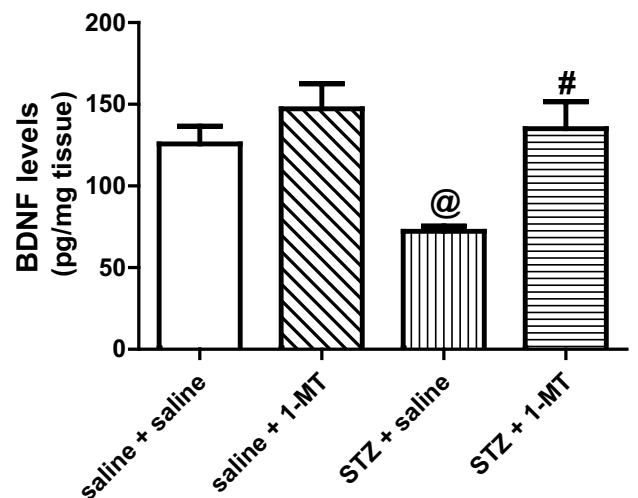


Fig. 8 Effects of 1-MT (50 mg/kg; s.c.) on the BDNF levels in hippocampus of mice 6 h after ICV-STZ (0.1 mg/site). Values are mean ± SEM (n=6–8). @*p*<0.05 when compared STZ+saline with saline+saline. #*p*<0.05 when compared STZ/1-MT with STZ+saline (two-way ANOVA, Bonferroni post hoc test)

STZ-Induced Down-Regulation of BDNF in the Hippocampus is Normalized by 1-MT

Two-way ANOVA of BDNF levels in the hippocampus revealed a significant STZ×1-MT interaction [$F(1,24)=5.67$; $p<0.01$]. Post hoc comparisons showed that the decreased BDNF levels induced by STZ were significantly normalized by 1-MT pretreatment (Fig. 8).

1-MT Blocks STZ-Induced IDO Activation in the Hippocampus

Statistical analysis of IDO activity in the hippocampus demonstrated a significant STZ×1-MT interaction [$F(1,24)=14.26$; $p<0.01$] and a main effect of 1-MT

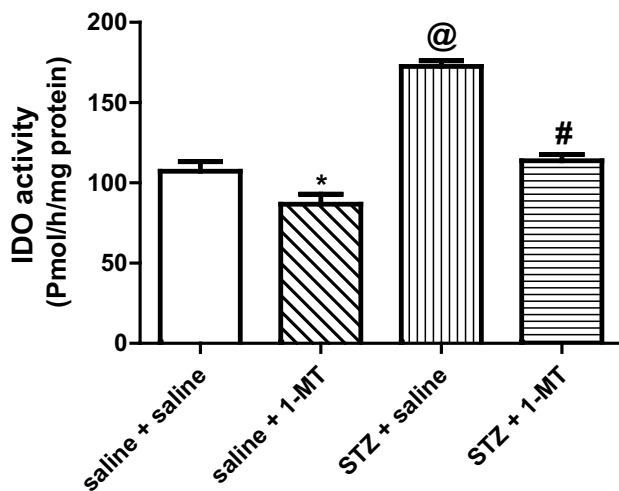


Fig. 9 Effects of 1-MT (50 mg/kg; s.c.) on the IDO activity in hippocampus of mice 6 h after ICV-STZ (0.1 mg/site). Values are mean \pm SEM (n=6–8). @ $p<0.05$ when compared 1-MT + saline with saline + saline. * $p<0.05$ when compared STZ + saline with saline + saline. # $p<0.001$ when compared STZ/1-MT with STZ + saline (two-way ANOVA, Bonferroni post hoc test)

[$F(1,24)=61.21$; $p<0.001$]. Post hoc comparisons revealed that STZ significantly increased IDO activity in the hippocampus of mice compared to the sham group. 1-MT pretreatment blocked the increase of IDO activity caused by STZ. A per se effect of 1-MT on IDO activity was observed (Fig. 9).

1-MT Normalized KYN Levels and KYN/TRP Ratio, But Not TRP Levels in the Hippocampus

Two-way ANOVA of KYN levels in the hippocampus demonstrated a significant STZ×1-MT interaction [$F(1,24)=38.49$; $p<0.001$]. Post hoc comparisons revealed that STZ significantly increased KYN levels in the hippocampus of mice compared to the sham group. 1-MT pretreatment significantly normalized the KYN levels (Table 1).

Statistical analysis of KYN/TRP ratio in the hippocampus revealed a significant STZ×1-MT interaction [$F(1,24)=12.93$; $p<0.01$]. Post hoc comparisons demonstrated that the increased KYN/TRP ratio induced by STZ was significantly normalized by 1-MT pretreatment (Table 1).

Two-way ANOVA of TRP levels in the hippocampus demonstrated a significant main effect of STZ [$F(1,24)=56.00$; $p<0.001$]. Post hoc comparisons revealed that STZ significantly increased TRP levels in the hippocampus of mice compared to the sham group. 1-MT pretreatment failed to protect against the increase in TRP levels induced by STZ (Table 1).

1-MT Fails to Prevent the Serotonergic Changes in the Hippocampus

Statistical analysis of 5-HIAA levels in hippocampus revealed a significant main effect of STZ [$F(1,24)=25.25$; $p<0.001$]. Post hoc comparisons revealed that STZ significantly increased 5-HIAA levels in hippocampus of mice

Table 1 Effect of STZ and 1-MT on TRP and 5-HT levels and their metabolites in hippocampus of mice

ng/mg tissue	Saline		STZ 6 h	
	Saline	1-MT	Saline	1-MT
TRP	3195 \pm 80.42	3226 \pm 116.7	4560 \pm 244.2 ^a	4534 \pm 218.7
KYN	275.8 \pm 13.58	141.0 \pm 12.56	752.0 \pm 54.72 ^a	284.5 \pm 28.35 ^b
KYN/TRP	8.680 \pm 0.66	6.529 \pm 0.34	16.05 \pm 1.40 ^a	6.351 \pm 0.76 ^b
5-HT	1261 \pm 38.56	1247 \pm 98.27	1072 \pm 135.9	1045 \pm 113.2
5-HIAA	1867 \pm 115.5	1814 \pm 82.9	2552 \pm 156.0 ^a	2482 \pm 167.8
5-HIAA/5HT	0.6861 \pm 0.060	0.6929 \pm 0.066	0.4176 \pm 0.037 ^a	0.4325 \pm 0.065

The values were analyzed by two-way ANOVA and Bonferroni post hoc test, each value is expressed as the mean \pm S.E.M. (n=10)

^a $p<0.05$ when compared saline + saline with STZ + saline

^b $p<0.05$ when compared STZ + 1-MT with STZ + saline

compared to sham group. 1-MT pretreatment failed to protect against the increase in 5-HIAA levels induced by STZ (Table 1).

Two-way ANOVA of 5-HIAA/5-HT ratio in hippocampus demonstrated a significant main effect of STZ [$F(1,24)=22.89$; $p<0.001$]. Post hoc comparisons revealed that STZ significantly increased 5-HIAA/5-HT ratio in hippocampus of mice compared to sham group. 1-MT pretreatment failed to protect against the decrease in 5-HIAA/5-HT ratio induced by STZ (Table 1).

5-HT levels in hippocampus of mice were not altered significantly by STZ injection [$F(1,24)=0.89$; $p<0.36$], 1-MT pretreatment [$F(1,24)=1.44$; $p<0.25$] or their interaction [$F(1,24)=0.03$; $p<0.86$] (Table 1).

Discussion

In the present study, we used an intracerebroventricular injection of streptozotocin (ICV-STZ) to investigate the link between neuroinflammation and depression in mice, and we determined the impact of STZ-induced inflammatory response on the BDNF levels and on the KYN/5-HT axis, two systems implicated in the pathogenesis of depression.

ICV-STZ Induced Depressive-Like Behavior and Anhedonic-Like Behavior

We previously demonstrated that ICV-STZ induced a depressive-like effect [23, 24]. We showed that STZ-injected mice displayed increased immobility time in the tail suspension test (TST) and an anhedonia-like response characterized by reduced sucrose intake in the sucrose preference test (SPT) [23].

In the present study, we observed similar results. In response to STZ, the depressive-like behaviors in the TST and SPT occurred at 6-, 24-h and 1-week time-points, with a peak effect at 6 h. As expected, ICV-STZ did not alter locomotor activity in the OFT or plasma glucose levels. The depressive like-behavior induced by STZ has previously been reported [37, 38]. However, these studies used STZ-induced diabetes model in rodents, in which STZ was administered through an intraperitoneal route. In contrast, the present study is the first to show depression-like effects when STZ is directly administered into the brain. Based on these findings, we also confirmed that ICV-STZ (0.1 mg/site) has the capacity to elicit depression-like states in mice.

STZ Induced Neuroinflammatory Response

In a previous study, we demonstrated that the depressive-like behavior induced by ICV-STZ was accompanied by an

increase in levels of the pro-inflammatory cytokine TNF- α in the hippocampus of mice [23]. To confirm the interrelationship between neuroinflammation and STZ-induced depression states, we examined the levels of proinflammatory cytokines IL-6 and IL-1 β in the hippocampus of mice, a brain region that constitutes part of the cortical-limbic neural circuits implicated with depression [39].

Increased levels of IL-6 and IL-1 β were detected following ICV-STZ, with maximal levels observed at 6 h, a time at which depressive-like behaviors were also most evident. Recently, several studies have confirmed the proinflammatory action of ICV-STZ, showing that STZ produced a significant increase in IL-6 and IL-1 β levels in the hippocampus of rats [40–43]. Previous studies have demonstrated a role for these cytokines in the development of depressive-like behaviors in rodents. For example, the administration of the endotoxin lipopolysaccharide (LPS) either intraperitoneally or intracerebroventricularly induced neuroinflammation and depressive-like effects similar to those observed in the present study [13, 44, 45]. A role for IL-6 and IL-1 β in the development of depressive-like symptoms has also been proposed by studies using stress protocols [46, 47]. Therefore, we suggest that the up-regulation of inflammatory cytokines observed in the present study is likely one of the mechanisms mediating the behavioral effects of ICV-STZ.

STZ Caused a Down-Regulation of BDNF in the Hippocampus

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and key regulator of neural circuit development and function. This molecule mediates many processes in the mammalian brain, including synaptic plasticity, neuronal survival and neurotransmission release [48]. An increasing body of evidence has demonstrated that an impairment of synaptic plasticity (neurogenesis, axon branching, dendritogenesis and synaptogenesis) in the hippocampus, particularly due to BDNF deficits, may be a core factor in the pathophysiology of depression [47, 49–51]. In fact, alterations in the BDNF pathway are central to the ‘neurotrophin hypothesis of depression’. This theory is largely based on observations that decreases in hippocampal BDNF levels are correlated with depressive symptoms and antidepressant treatment enhances the expression of this neurotrophin [49].

Here, we observed that ICV-STZ reduced BDNF levels in the hippocampus of mice. This finding is consistent with previous studies indicating that inflammation clearly affects the expression of BDNF within the brain [16, 51]. It has been reported that the administration of bacterial LPS, an agent that induces a strong immune response, causes a significant reduction of BDNF gene expression in

the hippocampus of rats [16]. Recently, Gibney et al. [51] observed the down-regulation of brain BDNF in response to polyinosinic:polycytidylic acid (POLY I:C), a toll-like receptor-3 agonist. In fact, IL-1 β is considered one of the main cytokines to compromise the BDNF pathway. For example, the mRNA levels of BDNF were significantly decreased in the rat hippocampus at 4 h after the intraperitoneal injection of IL-1 β [50]. In recent years, a number of studies have shown a mechanistic link between neuroinflammation and neuroplasticity [51, 52, 53]. In this sense, pathological conditions may lead to the overproduction of proinflammatory cytokines that may damage neuronal structure and function, leading to detrimental effects on the BDNF pathway. Consequently, neuronal protection and neurogenesis are affected [53].

Thus, we suggest that the decreased hippocampal BDNF levels in response to ICV-STZ are related to the depressive-like behavior observed in the present study. In addition, we indicated that one of the mechanisms by which the central administration of STZ may modulate BDNF levels in the hippocampus of mice could involve the up-regulation of proinflammatory cytokines.

STZ Induced IDO Activation, Kynurenine Production and Serotonin Metabolism Alterations

It has been proposed that IDO plays a pivotal role in mediating the depression-like behaviors in response to immune activation [4, 27]. The increase of proinflammatory cytokines, such as IL-1 β and IL-6 may trigger IDO activation in the brain [45]. Consequently, the proinflammatory cytokine-induced activation of IDO leads to the depletion of TRP and reduces the synthesis of 5-HT in the brain, which eventually may induce depressive symptoms [14, 54]. In addition, the induction of IDO causes KYN pathway activation, thereby increasing the generation of KYN and its neuroactive metabolites, including the free-radical generator, 3-hydroxyanthranilic acid (3-HAA) and the excitotoxin and *N*-methyl-D-aspartate (NMDA) receptor agonist, quinolinic acid (QUIN) [11]. Several studies have shown that these neurotoxic metabolites are related to the development of depressive symptomatology in both laboratory animals and depressed patients [3, 44, 55, 56].

In the present study, we demonstrated that STZ induced an increase in IDO activity in the hippocampus of mice at 6–24 h post-administration. This activation of IDO coincides with the appearance of the depression-like behaviors in the same time course, confirming the key role of this enzyme in initiating depression symptoms. In fact, the induction of brain IDO has been extensively demonstrated by studies using the intraperitoneal administration of LPS [13, 44, 57]. Consistent with the results of the present study, Gibney et al. [51] recently showed

the induction of IDO in the hippocampus of mice following the administration of the cytokine-inducer POLY I:C, with a peak effect occurring at 6 h, the same time point observed in the present study. In a recent study, we provided the first evidence that hippocampal IDO activation plays a key role in ICV-STZ-induced depressive-like behavior [24]. Therefore, here we confirmed that ICV-STZ might cause IDO activation in the hippocampus of mice, consistent with studies reporting depressive-like behavior induced by immune system-activating drugs [13, 44, 51].

In the present study, the increase of IDO activity induced by STZ was followed by increased levels of both TRP and its metabolite KYN. These findings are somewhat counterintuitive, and certainly argue against the hypothesis that IDO activation depletes TRP bioavailability for 5-HT synthesis. Thereby, we suggest that the behavioral changes induced by STZ are not involved with an impairment of 5-HT synthesis in the hippocampus of mice. The finding that the hippocampal levels of TRP are increased by STZ is supported by previous studies in which the administration of inflammatory cytokines, LPS or POLY I:C also induced an increase in brain TRP [7, 51, 58]. Importantly, we demonstrated that central STZ injection precipitated depression-like behaviors coupled with increased KYN levels and KYN:TRP ratio, supporting a role for brain KYN metabolism in driving depression. Increased IDO enzymatic activity and elevated KYN levels have been correlated with inflammation-associated depression. Accordingly, Lawson et al. [45] showed that mice treated with i.c.v. LPS injection exhibited elevated brain KYN levels and increased KYN:TRP ratio. In addition, the administration of KYN has been shown to directly induce a depressive phenotype in mice [27]. Thus, these findings indicate that the activation of the KYN pathway may be necessary for ICV-STZ to induce depressive-like behaviors.

Depression is associated with impaired central 5-HT metabolism [8]. Here, while the levels of 5-HT were not significantly altered by STZ, a decreasing trend of 5-HT levels at all time points tested was observed in the hippocampus of mice. Consistently, an increase in the 5-HT metabolite 5-HIAA was observed in the hippocampus of mice following STZ injection. This finding is consistent with previous studies where inflammatory cytokines, LPS or POLY I:C provoke an increase in brain 5-HIAA levels [51, 59]. Here, we also observed that the 5-HIAA/5-HT ratio, an indicator of 5-HT turnover, is increased in the hippocampus of mice following STZ injection. Indeed, altered 5-HT turnover in the brain is often observed in depressed patients [60]. Thus, these data suggest that, although ICV-STZ did not affect 5-HT synthesis, it may promote 5-HT function abnormalities, primarily reflecting increasing 5-HT breakdown. These effects may explain the tendency

of the lower 5-HT levels observed in the hippocampus, contributing to depressive symptoms.

Taken together, these data demonstrated that ICV-STZ induced depressive-like behavior in mice that is related with a neuroinflammatory response, the activation of the KYN pathway and serotonergic alterations in the hippocampus. Thus, the present study provides evidence that the central administration of STZ mimics some characteristics of depressive-like phenotype, supporting the hypothesis that this model may be a useful experimental model for studying depression in mice.

1-MT Blocked Depressive-Like Behaviors via Attenuation of Neuroinflammatory Response and Inhibition of IDO Activation in the Hippocampus of STZ-Injected Mice

To examine the role of the enzyme IDO in mediating STZ-induced depressive-like behavior, we administered the IDO inhibitor 1-MT twice daily for 1 week prior to i.c.v. STZ injection (experiment 2).

Here, we demonstrated that the pharmacological blockade of hippocampal IDO activation with 1-MT prevented the development of depressive-like behaviors in STZ-injected mice. The data obtained in the present study suggest a novel mechanistic link between STZ and depression via IDO activation in the hippocampus. This finding is consistent with several studies using 1-MT to prevent depressive-like behaviors associated with peripheral and central inflammatory changes. For example, 1-MT blocked depressive complications associated with the i.p. or i.c.v. injection of LPS [44, 45]. 1-MT was also effective in limiting depressive-like behavior in a model where microglia regulation is impaired [13]. It has been demonstrated that pharmacological blockade of IDO can reverse the depressive-like phenotype in response to Bacille Calmette–Guéri (BCG) inoculation [61], and IDO deficient mice were resistant to BCG-induced depressive-like behavior [62].

Here, we also demonstrated that 1-MT pretreatment attenuated the up-regulation of IL-6 and IL-1 β levels and normalized BDNF levels in the hippocampus of STZ-treated mice. This result is relevant because it shows that most of the neuroinflammation caused by STZ is mediated by IDO and also suggests the involvement of this enzyme in neurotrophin deficit observed in depression.

In the present study, we also demonstrated that 1-MT pretreatment abrogated KYN production and normalized the KYN/TRP ratio in the hippocampus of STZ-injected mice, consistent with the results of a previous study [51, 63]. These findings showed that increased levels of KYN and the KYN/TRP ratio in the hippocampus were regulated by IDO activity. Therefore, the results of the present study indicate increased KYN levels as a potential precipitant of neuropsychiatric-like

behaviors induced by ICV-STZ. An interesting finding from the present study was that 1-MT pretreatment did not prevent STZ-associated 5-HT alterations in the hippocampus. This result is consistent with a previous report of O'Connor et al. [27], showing that 1-MT intervention blocked depressive-like behavior, but had no effect on 5-HT turnover in CD-1 mice at 24 h after LPS injection. Plausible explanations for this finding include different mouse strains and time points analyzed. This finding may also indicate that the increased production of KYN and its subsequent downstream neurotoxic metabolites plays a more direct role in the development of STZ-induced depressive-like behavior than decreases in TRP levels or 5-HT synthesis impairment. Thus, 5-HT alterations in the hippocampus of STZ-injected mice may be indicative of increased cytokine exposure. In support of this premise, it has been reported that altered cytokine secretion may play an important role in the termination of serotonergic neurotransmission by 5-HT uptake into presynaptic neurons [64]. Proinflammatory cytokines might trigger the increased synthesis of the serotonin transporter, leading to 5-HT uptake into presynaptic neurons, the down-regulation of 5-HT levels, and eventually the onset of depression.

In summary, the results of the present study support the idea that ICV-STZ may cause depressive-like behaviors in mice through IDO activation and IDO-independent mechanisms in the hippocampus, and the up-regulation of cytokine levels is the initial step. First, increased levels of IL-6 and IL-1 β following STZ injection activate IDO and subsequently increase the levels of KYN, which may directly mediate depressive-like behaviors. Moreover, increased KYN levels may activate neurodegenerative pathways that ultimately provoke a decrease in BDNF levels; this neurotrophic deficit could contribute to the depression symptoms observed. Second, the increased levels of proinflammatory cytokines may cause a direct impairment on 5-HT function, via increasing the breakage or turnover rate of this compound. Future studies are required to determine the precise causal relationship between the depressive complications observed and the STZ-induced changes in KYN, 5-HT and BDNF pathways.

Conclusions

The results of the present study clearly confirmed that ICV-STZ has the capability to reproduce the core components of inflammation-associated depression in mice, with similar findings to other depression modeling paradigms. The present study provides new insights on the neurobiological mechanisms underlying ICV-STZ. Thus, these results indicate that this model could be employed in preclinical research of depression.

Notably, these data implicate hippocampal IDO activation in STZ-associated depressive-like behavior. Therefore,

the present study not only presents evidence that IDO plays a critical role in mediating inflammation-induced depression but also supports the notion that neuroinflammation and the kynurenine pathway are important targets of novel therapeutic drugs for depression.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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