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Prenatal THC exposure raises kynurenic acid levels in the prefrontal cortex of adult rats

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Abstract

Cannabis remains one of the most widely used illicit drugs during pregnancy. The main psychoactive component of marijuana (^{9}-tetrahydrocannabinol, THC) is correlated with untoward physiological effects in the offspring. Neurobehavioral and cognitive impairments have been reported in longitudinal studies on children and adolescents prenatally exposed to marijuana, and a link to psychiatric disorders has been proposed. Interestingly, the deleterious effects of prenatal cannabis use are similar to those observed in adult rats prenatally exposed to (L)-kynurenine, the direct bioprecursor of the neuroactive metabolite kynurenic acid (KYNA). We therefore investigated whether alterations in KYNA levels in the rat brain might play a role in the long-term consequences of prenatal cannabinoid exposure. Pregnant Wistar rats were treated daily with THC [5 mg/kg, p.o.] from gestational day (GD)5 through GD20. Using in vivo microdialysis in the medial prefrontal cortex, adult animals were then used to determine the extracellular levels of KYNA and glutamate. Compared to controls, extracellular basal KYNA levels were higher, and basal glutamate levels were lower, in prenatally THC-exposed rats. These rats also showed abnormal short-term memory. Following an additional acute challenge with a low dose of

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Author contributions

S.B. and L.F. designed the study. S.B. and A.I. performed the experiments. S.B. analyzed the data and wrote the manuscript. M.C.T. compiled the figures. S.B., L.F. and R.S. acquired funding. All authors read, critically revised, and approved the final manuscript.

Ethical statement

The study was carried out in accordance with current Italian legislation (D.L. 26/2014), which allows experimentation on laboratory animals only after approval by the Ministry of Health (Rome, Italy), and in strict accordance with the European Council Directives on animal use in research (n. 2010/63/EU). According to the ARRIVE guidelines, all possible efforts were made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

Declaration of Competing Interest

The authors declare no conflict of interest.

kynurenine (5 mg/kg i.p.) in adulthood, the increase in extracellular KYNA levels in the mPFC was more pronounced in in prenatally THC-exposed rats. These effects could be causally related to the cognitive dysfunction seen in prenatally THC-exposed rats. In the translational realm, these experiments raise the prospect of prevention of KYNA neosynthesis as a promising novel approach to combat some of the detrimental long-term effects of prenatal cannabis use.

Keywords

Cognition; Kynurenic acid; Kynurenine; Prefrontal cortex; Prenatal; THC

1. Introduction

Marijuana (*Cannabis sativa*) is the most extensively used illicit drug worldwide. As the content of ^{9}-tetrahydrocannabinol (THC), its main psychotomimetic component, has steadily increased in recent years (Calvigioni et al., 2014; Ryan et al., 2018), there is growing apprehension about adverse effects of the drug. Special concerns have been raised with regard to marijuana use during pregnancy since 2–5% of pregnant women use the drug, with the number reaching 15–28% in urban, low-income populations (Metz and Stickrathm, 2015; Chasnoff, 2017). Concerns have been further substantiated by the increasing legalization of marijuana (State Medical Marijuana Laws, 2016; UNODC, 2019) and the notable fact that, in contrast to tobacco and alcohol usage, cannabis use during pregnancy has not decreased in recent years (Agrawal et al., 2019).

Studies in both humans and experimental animals provide evidence that attention to this phenomenon is warranted. THC is highly lipophilic and therefore crosses the placenta freely, accumulating rapidly in fetal tissues, particularly in the brain (Grant et al., 2018). After marijuana use during pregnancy, THC concentrations in fetal blood reach one-third to one-tenth of maternal concentrations (Hutchings et al., 1989; Grant et al., 2018). Notably, longitudinal studies of children and adolescents who were prenatally exposed to marijuana have revealed significant impairments of higher cognitive functions (El Marroun et al., 2011; Huizink, 2014; Calvigioni et al., 2014). In rats, prenatal exposure to cannabinoids (i.e. THC or the synthetic compound WIN 55,212-2) disrupts normal brain development, leading to cognitive deficits in the offspring (Ferraro et al., 2009; Calvigioni et al., 2014). These impairments are associated with long-lasting alterations of aminoacidergic neurotransmission in the hippocampus and the prefrontal cortex (PFC) (Mereu et al., 2003; Antonelli et al., 2004; Beggiato et al., 2017). Although the mechanisms mediating these deleterious drug effects have not been clarified so far, it is now increasingly realized that the effects of prenatal marijuana use predispose the offspring to neurodevelopmental abnormalities and may therefore also be causally related to the pathophysiology major neurodevelopmental brain diseases such as schizophrenia (SZ) (Jutras-Aswad et al., 2009; Mathews et al., 2014; Alpár et al., 2016).

Some of the negative effects of prenatal THC treatment on cognitive functions in the offspring resemble those observed in adult rats that had been prenatally exposed to L-kynurenine (“kynurenine”), a pivotal metabolite of the kynurenine pathway (KP) of

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tryptophan degradation (Pocivavsek et al., 2014, 2019) during the final week of gestation. Circulating kynurenine readily crosses the blood-brain barrier, as well as the placental barrier, and is then readily converted to kynurenic acid (KYNA), which has remarkable neuroactive properties (Fig. 1; Notarangelo and Pocivavsek, 2017). In the adult brain, KYNA can act as a negative allosteric modulator of the α7 nicotinic acetylcholine (α7nACh) receptor and as a competitive antagonist of the obligatory glycine co-agonist site of the NMDA receptor (Parsons et al., 1997; Flores-Barrera et al., 2017), i.e. two receptors which play central roles in cognitive function (Newcomer and Krystal, 2001; Levin, 2012). Notably, even relatively modest fluctuations in brain KYNA bi-directionally affect the extracellular levels of major neurotransmitters which are critically involved in cognitive processes, such as dopamine (Rassoulpour et al., 2005), glutamate (Wu et al., 2010), and GABA (Beggiato et al., 2014). Dysfunction in KP metabolism, and specifically abnormal KYNA function, has therefore been plausibly linked to cognitive impairments (Wonodi and Schwarcz, 2010). In humans, particular attention has been paid to the cognitive deficits of persons with SZ in this regard since KYNA levels are elevated in cerebrospinal fluid and in the PFC (assessed post mortem) in the disease (Erhardt et al., 2001; Schwarcz et al., 2001; Wonodi and Schwarcz, 2010; Sathyasaikumar et al., 2011; Linderholm et al., 2012). Of considerable interest in this context, the cognitive abnormalities seen in adult offspring of rats which were administered kynurenine prenatally (see above) are associated with increased cerebral KYNA levels (Pocivavsek et al., 2014, 2019).

Because of the apparent similarities of the long-term cognitive impairments caused by treatment with THC and kynurenine during pregnancy, we speculated that a causal connection may exist between the effects of prenatal THC exposure and KYNA-related biochemical and cognitive abnormalities in the adult offspring. The present study was designed as a first attempt to test this hypothesis experimentally. To this end, we exposed pregnant Wistar rats daily to THC during a sensitive period of prenatal brain development and examined extracellular KYNA and glutamate levels and the expression of KYNA's biosynthetic enzymes kynurenine aminotransferases I and II (KAT I and II) in the brain, and also assessed performance in a Y-maze, in adult offspring. Additionally, to assess the "two-hit" hypothesis of SZ (Feigenson et al., 2014; Debnath et al., 2015; Owen et al., 2016) in the context of our model, we investigated the effects of an acute systemic challenge with kynurenine in adult rats that had been prenatally exposed to THC. Our results provided novel and translationally relevant insights into the molecular mechanisms that underlie the detrimental long-term consequences and hazards of prenatal THC exposure.

2. Methods

2.1. Animals and chemicals

2.1.1. Animal care—All animals were bred and kept in a temperature-controlled animal housing facility at the University of Ferrara. The study was carried out in accordance with current Italian legislation (D.L. 26/2014), which allows experimentation on laboratory animals only after approval by the Ministry of Health (Rome, Italy), and in strict accordance with the European Council Directives on animal use in research (n. 2010/63/EU). According

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to the ARRIVE guidelines, all possible efforts were made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

2.1.2. Animals and treatment conditions—Adult, primaparous female Wistar rats were obtained from Charles River Laboratories (Milan, Italy) and were then housed for one week. The animals were kept on a 12 h:12 h light:dark cycle, with free access to food and water. Pairs of females were then placed with single male rats in the late afternoon. Vaginal smears were taken the following morning at 09.00. The day on which sperm was present was designated as gestational day 0 (GD 0). Prenatal treatments were performed as previously described (Castaldo et al., 2010). Pregnant rats were administered THC (5 mg/kg), dissolved in sesame oil and prepared as described (Molina-Holgado et al., 1993), daily by oral gavage from GD 5 through GD 20. This dose is considered to be equivalent to moderate human gestational exposure to THC after correction for route of administration and body surface area (Molina-Holgado et al., 1996). Control dams were treated with vehicle (sesame oil).

A group of dams was euthanized on GD 20 for biochemical analyses of maternal and fetal plasma as well as fetal brain. The other dams gave birth, and litters were reduced to a standard size of six male pups (when possible) within 24 h after birth. Only male offspring were retained. One offspring from each litter was used in each experimental group, and each animal was used only on a single test. After weaning on postnatal day (PD) 21, animals were fed a normal diet and received no other drug treatment until PD 65-90.

The number of dams giving birth as well as the length of pregnancy were monitored. Litter size at birth, postnatal mortality (the number of male pups that died before weaning) and weight gain of the pups were recorded.

Biochemical, neurochemical and behavioral studies were performed in offspring of THC- and vehicle-treated dams in adulthood (PD 65-90).

2.1.3. Chemicals—THC and L-kynurenine sulfate were purchased from Sigma (Milan, Italy). Other chemicals were obtained from various commercial suppliers and were of the highest available purity.

2.2. Serum and tissue analyses

Dams were euthanized on GD 20 using an anesthetic overdose, and maternal and fetal blood was collected in tubes containing 25 μl of 0.5 M EDTA as an anticoagulant. After centrifugation, the supernatant plasma was frozen on dry ice and stored at −80 °C. On the day of the assays, maternal and fetal plasma samples were diluted (1:2, *v*/v and 1:10, v/v, respectively) in ultrapure water. Next, 100 μl of each preparation were acidified with 25 μl of 6% perchloric acid. After centrifugation (16,000 × *g*, 15 min), kynurenine and KYNA were measured by high performance liquid chromatography (HPLC). To this end, 20 μl of the supernatant were injected onto a 3 μm C18 reverse phase HPLC column (100 mm × 4 mm; Dr. Maisch GmbH, Ammerbuch, Germany), using a mobile phase containing 50 mM sodium acetate and 4% acetonitrile (pH adjusted to 6.2 with glacial acetic acid) at a flow rate of 0.5 ml/min. Zinc acetate (0.5 M, not pH adjusted) was delivered postcolumn by a peristaltic pump (Dionex AXP, Thermo Fisher, Waltham, MA, USA) at a flow rate of 0.1 ml/min. In

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the eluate, kynurenine (excitation: 365 nm, emission: 480) and KYNA (excitation: 344 nm, emission: 398 nm) were detected fluorimetrically (Jasco fluorescence spectrophotometer FP2020 Plus, Jasco, Tokyo, Japan). The retention times of kynurenine and KYNA were ~6 min and ~14 min, respectively.

Using the same dams, these procedures were also used to determine kynurenine and KYNA levels in the fetal brain. To this end, the brain (minus cerebellum) was rapidly dissected out, frozen on dry ice and stored at − 80 °C. On the day of the assays, the tissue was thawed and sonicated in ultrapure water (1:5, *w/v*). Tissue homogenates were further diluted (1:2, *v*/v) in ultrapure water, and 100 μl of the homogenate were acidified with 25 μl of 25% perchloric acid. After centrifugation (16,000 × *g*, 15 min), 20 μl of the resulting supernatant were subjected to HPLC analysis as described above.

2.3. mRNA expression of KAT I and KAT II

For total RNA extraction, fetal brain (minus cerebellum) and prefrontal cortex from adult male rats were processed using Directzol RNA MiniPrep (Zymo Research, purchased by Euroclone, Milan, Italy) according to manufacturer's instructions and quantified by spectrophotometer analysis. One μg of RNA was used to synthesize the cDNA by the iScript kit (Biorad, Milan, Italy) according to manufacturer's instructions. Samples were then processed for quantitative real time PCR by a CFX Connected Real-Time System (Biorad) using iTaq Universal SYBR Green Supermix (Bio-Rad) as previously described (Mallei et al., 2018). PCR cycling conditions were: 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 15 s at 60 °C. Relative expression was determined using the 2^{-Ct} method using S18 as the reference gene. The following primers were used: KAT I: Fw: 5′ CAATGATGGCTGGAGGTTGS-3′, Rev.: S18 5′-GTTGTTGGGTGTGTTGAGGA-3′ (Liu et al., 2015). KAT II: Fw: 5′-CCCTGTACTTTATCACAGCTC-3′, Rev.: 5′ AAACCACGTAACCACTTGTC-3′ (Clark et al., 2019). S18: Fw: 5′-CATGCAGAACCCACGACAAT-3′ Rev.: 5′-CTTCCCATCCTTCACGTCCT-3′ (Tornese et al., 2019).

2.4. In vivo microdialysis

2.4.1. Surgery—For implantation of the microdialysis probe, animals were anesthetized (1.5% mixture of isoflurane and air) and mounted in a David Kopf stereotaxic frame (Tujunga, CA, USA) with the upper incisor bar set at 2.5 mm below the interaural line. A small hole was drilled on one side of the exposed skull. A microdialysis probe of concentric design (CMA12; molecular weight cutoff: 20 kD; outer diameter: 0.5 mm; length of the dialysis membrane: 2 mm; Alfatech S.p.A., Genova, Italy) was implanted vertically in the medial PFC (mPFC) and then fixed to the skull with dental cement. The coordinates used were: AP: 3.5 mm from bregma, L: ± 0.8 mm from the midline and V: 4.0 mm below the dura (Paxinos and Watson, 2007). The probe was secured to the skull with anchor screws and acrylic dental cement. After surgery, the animals were housed individually in microdialysis chambers, with food and water available ad libitum.

2.4.2. Microdialysis—Experiments were performed in freely moving rats 24 h after probe implantation. To this end, the probe was connected to a microperfusion pump (CMA

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100; Carnegie Medicin, Stockholm, Sweden) set to a speed of 1.5 μl/min and perfused with Ringer solution containing (in mM) NaCl, 144; KCl, 4.8; $MgSO_4$, 1.2 and $CaCl_2$, 1.7 (pH 6.7). Collection of dialysate samples commenced 5 h after the onset of perfusion to achieve stable glutamate levels (Beggiato et al., 2014), and perfusates were collected every 30 min thereafter. Following the collection of three stable basal values, kynurenine (5 mg/kg) or saline was administered i.p., and microdialysate samples were collected for another 4.5 h. The rats were then euthanized using an anesthetic overdose, the brain was removed from the skull, and the position of the dialysis probe was verified using 30 μm-thick coronal cryostat sections. Only animals with a correctly located probe were included in the analysis.

2.4.3. Glutamate and KYNA determination—In the dialysate, glutamate was measured by HPLC with fluorimetric detection. Briefly, 10 μl were transferred into glass microvials and placed in a temperature-controlled (4 °C) Triathlon autosampler (Spark Holland, Emmen, The Netherlands). Fifteen microlitres of o-phthaldialdehyde/mercaptoethanol reagent were added to each sample, and 15 μl of the mixture were injected onto a Chromsep analytical column (3 mm inner diameter, 10 cm length; Chrompack, Middelburg, The Netherlands). The column was perfused at a flow rate of 0.5 ml/min (Beckman 125 pump; Beckman Instruments, Fullerton, CA, USA) using a mobile phase containing 0.1 M sodium acetate, 10% methanol and 2.2% tetrahydrofuran (pH 6.5). In the eluate, glutamate was detected by means of a Jasco fluorescence spectrophotometer FP2020 Plus (Jasco, Tokyo, Japan). The retention time of glutamate was ~3.5 min, and the limit of detection was ~20 fmol.

To determine the extracellular concentration of KYNA, 20 μl of the microdialysate samples were directly injected onto a 3 μm C18 reverse phase HPLC column and detected fluorimetrically in the eluate, as described in Section 2.2. above. The limit of detection for KYNA was ~10 fmol.

2.5. Behavior (Y-maze)

The effect of prenatal THC exposure on short-term memory was evaluated using a Y-maze consisting of three interconnected closed arms, each 120° from the other. For the habituation phase, the rat was placed in the middle of the maze and was allowed to freely explore for 5 min. The movement of the animal was recorded by an overhead camera. The number of arm entries was used as a marker of locomotor activity, and the number of spontaneous alternations, i.e. the entry into three different arms in sequence (triad), was used as a measure of short-term working memory. The percentage of spontaneous alternations was calculated from the number of triads and arm entries using the following equation: Y = number of triads/(total number of arm entries-2) × 100 (Nookala et al., 2018).

2.6. Data management and statistical analysis

Serum and tissue levels of kynurenine and KYNA, as well as gene expression of KAT I and KAT II, were analyzed by Student's *t*-test.

Microdialysis data were not adjusted for recovery from the dialysis probe. Basal values were calculated as the mean of three consecutive samples (differing by no more than 15%)

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collected immediately preceding drug or vehicle treatment. The area under the curve (AUC), reflecting the duration of the effect, was determined for each animal. Area values (overall effects) were calculated as percentages of changes in baseline value over time by using the trapezoidal rule. Statistical analysis was carried out by analysis of variance (ANOVA), followed by Newman-Keuls or Tukey's test for multiple comparisons when appropriate.

For the Y-maze test, the differences across three days were assessed in each group by using the Mann Whitney *U* test.

All data are expressed as the mean ± SEM. A *P* value of < 0.05 was considered significant in all cases.

All statistical analyses were performed using GraphPad Prism 6.0.

3. Results

3.1. Reproduction data

Prenatal THC treatment did not affect pregnancy duration, the dam's weight during gestation or lactation, litter size at birth, pup weight gain and postnatal survival (*data not shown*).

3.2. Kynurenine and KYNA in maternal plasma, fetal plasma and fetal brain on GD 20

Following gestational THC exposure, no differences in kynurenine and KYNA levels were found in maternal (Fig. 2A,B) and fetal (Fig. 2C,D) plasma or in the fetal brain (Fig. 2E,F) on GD 20.

3.3. mRNA brain levels of KAT I and KAT II on GD 20 and in adulthood

Prenatal THC exposure did not affect mRNA expression levels of either KAT I or KAT II in the fetal brain (Fig. 5A,B). In contrast, in adult rats prenatally exposed to THC, mRNA expression levels of KAT I were significantly elevated compared to vehicle-treated animals ($p < .05$, Fig. 5C), whereas no differences were observed in the mRNA expression of KAT II (Fig. 5D).

3.4. Extracellular KYNA and glutamate levels in adulthood

3.4.1. Effect of prenatal THC exposure on basal KYNA and glutamate levels

—Prenatal THC exposure differently affected KYNA and glutamate levels in the mPFC of adult rats. Thus, compared to vehicle-treated animals, extracellular KYNA levels were significantly elevated (Fig. 3A), whereas extracellular glutamate levels were significantly reduced (Fig. 3B), in the offspring of THC-treated dams.

3.4.2. Effect of an acute challenge with kynurenine on extracellular KYNA and glutamate levels

—To further investigate the impact of prenatal THC treatment on the KP in adult rats, we next examined the consequences of an acute challenge with kynurenine (5 mg/kg, i.p.) on extracellular KYNA and glutamate levels in the mPFC of rats which had been exposed to THC during the prenatal period. These animals showed a more pronounced kynurenine-induced increase in KYNA than vehicle-exposed animals ($p < .001$)

(Fig. 4A,B). Kynurenine administration did not affect extracellular glutamate levels in either of the two groups, however (Fig. 4C,D).

3.5. Short-term memory in adulthood

Short-term memory was examined in adult offspring of dams which had received prenatal treatment with THC or vehicle. As illustrated in Fig. 6, prenatal THC exposure negatively affected short-term memory (reduced spontaneous alternation), but not locomotor activity (arm entries), in adult animals ($p < .01$).

4. Discussion

The present study revealed notable biochemical and behavioral effects in adult animals following prenatal THC exposure, for the first time linking this gestational treatment to abnormally high increases in the brain levels of KYNA later in life. Specifically, we demonstrated that basal extracellular KYNA levels were higher, and basal extracellular glutamate levels were correspondingly lower, in the PFC of adult offspring of dams that had been treated with THC during the last week of gestation. In addition and in line with our previous observations (Ferraro et al., 2009), we found that prenatal exposure to THC led to impaired short-term memory in adulthood, tested here using the Y maze. These neurochemical and behavioral alterations were associated with a significant increase in KAT I mRNA levels. In a second set of experiments, rats which had been treated with THC in utero were challenged with an acute systemic injection of a small dose of KYNA's immediate bioprecursor, the pivotal KP metabolite kynurenine, in adulthood. Compared to the effect of kynurenine in normal adult rats, these animals showed a significantly greater increase in extracellular KYNA levels in the PFC. Jointly, these results not only substantiated previously established functional links between THC and KYNA (cf. Introduction) but also generated new concepts of translational significance.

In spite of the fact that both THC and KYNA affect major neurotransmitters including glutamate, acetylcholine and dopamine, and can influence learning, memory and other cognitive functions in adult rodents (Zou and Kumar, 2018; Schwarcz et al., 2012), only very few studies have so far shown mutual interactions between the drug – and by extension endocannabinoids – and KYNA. In the adult brain, such interactions clearly exist, though many questions remain (Justinova et al., 2013; Colín-González et al., 2016; Nagy-Grócz et al., 2017; Secci et al., 2019). In the present study, prenatal treatment with THC did not influence the levels of either kynurenine or KYNA in maternal and fetal plasma or, importantly, in the fetal brain. Accordingly, no alterations in the mRNA expression levels of KAT I and KAT II (i.e. two enzymes converting kynurenine to KYNA) were observed in the fetal brain after prenatal exposure to THC. Therefore, unlike prenatal kynurenine treatment, which readily raises KYNA in the fetal brain and causes KYNA elevations in the adult brain (Notarangelo and Pocivavsek, 2017; Pocivavsek et al., 2014), the KYNA elevations in the adult brain described here do not appear to be caused by THC-induced abnormal KP metabolism in the prenatal period.

The long-term impact of prenatal THC on cerebral KYNA levels in adulthood suggests a substantive role of epigenetic processes, as also observed in other studies following prenatal

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THC exposure (Hurd et al., 2019). While details of the cellular and molecular mechanisms which underlie the delayed KYNA increase following prenatal THC treatment on brain chemistry and behavior in adulthood will need to be clarified in future studies, mechanism(s) may involve an unexpected role of KAT I, which does not normally make significant contributions to KYNA neosynthesis in the mammalian brain (Guidetti et al., 1997; see also Kocki et al., 2018). Regardless of the underlying mechanism(s), the neurochemical and behavioral consequences seen in adult animals after prenatal exposure to THC should be considered to be functionally linked to elevated brain KYNA levels. Thus, as exogenous application of KYNA causes a prompt reduction in extracellular glutamate levels in the rat PFC (Wu et al., 2010; Beggiato et al., 2014), the decrease in basal extracellular glutamate seen in the PFC in the present study (Fig. 3; cf. also Campolongo et al., 2007) may be secondary to the observed KYNA increase. A similar argument can be made in the behavioral realm since high concentrations of KYNA disrupt sensory gating (Erhardt et al., 2004; Nilsson et al., 2006; Shepard et al., 2003) and impair spatial working memory (Chess et al., 2007), contextual fear conditioning (Chess et al., 2009), and attentional set shifting (Alexander et al., 2013) – all presumably related to the KYNA-induced reduction in glutamatergic tone. Notably, these and other cognitive impairments are seen at various postnatal stages in the offspring of prenatally THC-treated rats (Ferraro et al., 2009), as also confirmed here by assessing short-term memory function in adulthood.

Exposure to THC during pregnancy may have implications for the pathophysiology of psychiatric diseases, several of which are increasingly understood to originate early in life (Silbereis et al., 2016; Hurd et al., 2019; Scheyer et al., 2019a). This hypothetical link is especially intriguing in persons with SZ, who have elevated KYNA levels in brain and cerebrospinal fluid (Erhardt et al., 2001; Schwarcz et al., 2001; Wonodi and Schwarcz, 2010; Linderholm et al., 2012; Sathyasaikumar et al., 2011). Data from longitudinal studies have demonstrated that people exposed perinatally to cannabis display cognitive impairments and deficits in executive function as adults (Hurd et al., 2019; Scheyer et al., 2019a). Accordingly, studies in experimental animals showed that cannabinoids disrupt and re-wire the fetal endocannabinoid system, in turn affecting brain development both pre- and postnatally (Hurd et al., 2019; Frau et al., 2019). The emerging consensus holds that exposure to THC in utero might constitute a “first hit”, which has only subtle deleterious consequences in the offspring unless it is paired with genetic vulnerabilities or environmental insults. The latter, termed the “second hit”, may be experienced at various stages in life and include adverse phenomena such as stressful events, physical trauma, infections and drug use (Debnath et al., 2015; Owen et al., 2016; Feigenson et al., 2014).

Interestingly, several of these detrimental “second hits” are known to be associated with, and may in fact be causally related to, increased KYNA neosynthesis (Liu et al., 2014; Nold et al., 2019). Additivity with the elevated brain KYNA levels which we observed here several weeks after prenatal THC administration would for the first time suggest a distinct molecular mechanism – namely increased KYNA function – by which a “second hit” may exert detrimental effects. In preparation of experiments using less specific challenges such as stressors, infectious agents, etc., we therefore decided to examine if an acute stimulation of KYNA production with a low dose (5 mg/kg, i.p.; Swartz et al., 1990) of its direct bioprecursor kynurenine would further increase KYNA levels in the brain of adult animals

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which had received THC prenatally. This was indeed the case though, as expected the relatively mild acute challenge was unable to reduce glutamate levels further (Konradsson-Geuken et al., 2010). This finding provides the basis for a large number of follow-up experiments using informative biochemical and functional outcome measures. Because of its obvious translational relevance, questions will clearly also have to be addressed by using *adolescent* animals that were exposed to THC in utero. In fact, adolescent rats that had been exposed to THC prenatally indeed show reduced extracellular glutamate levels (Castaldo et al., 2010) and, in a recent preliminary study, we observed elevated extracellular KYNA levels in the mPFC of these animals on postnatal days 35–15 (Beggiato et al., 2018). Thus, it seems likely that the neurochemical alterations seen here in adulthood are also present in adolescence, i.e. during a critical neurodevelopmental period (Meyer et al., 2018) which is fine-tuned by the endocannabinoid system (Parsons and Hurd, 2015). One plausible molecular mediator is brain-derived neurotrophic factor (BDNF), which is induced by chronic THC treatment (Segal-Gavish et al., 2017) and shows increased expression in adult rats that had received THC prenatally (Beggiato et al. unpublished data).

In general, the results of the present study provide additional support for the "double-hit" model, which posits that adverse external events can precipitate and/or exacerbate brain dysfunction in vulnerable individuals later in life. While our new data focused specifically on the consequences of prenatal THC exposure, other "first hits" may have similar effects on cerebral KYNA in adolescence and adulthood. These include, among others, various recreational or illicit drugs, bacterial and viral infections, and abnormally stressful maternal experiences. Genetic predispositions, including variations in KP genes (Beggiato et al., 2018), too, should be conceptualized as risk factors in this context. As these "first hits", alone or in combination, are believed to account for enhanced postnatal vulnerabilities (Richardson et al., 2016), it seems worthwhile to examine their long-term effects on KYNA levels and function.

The present findings indicate that abnormal KP metabolism, and specifically a chronic increase in brain KYNA function, by reducing cortical glutamate transmission, could play a role in the cognitive impairments and in the attenuated adaptation to untoward challenges (stress, drug abuse, etc.) that is observed in children affected by cannabinoid exposure in utero (Alpár et al., 2016). As reviewed in detail elsewhere (Erhardt et al., 2009; Szalardy et al., 2012; Notarangelo and Pocivavsek, 2017), cognitive impairments should receive special attention in this regard. From a translational point of view, it is worth noting that among the cognitive domains observed in schizophrenic patients, memory, working memory and executive function appear to be most influenced by the glutamatergic pathway (Thomas et al., 2017). Neuroimaging studies demonstrate that the glutamatergic pathways in the PFC are potential molecular mechanisms for executive function and working memory deficits in schizophrenia (Thomas et al., 2017). Furthermore, low glutamate and high KYNA levels have been reported in various brain regions, particularly in the PFC of chronic schizophrenia patients (Wonodi and Schwarcz, 2010; Rigucci et al., 2018). These findings assume particular relevance since hypofunctioning glutamate neurotransmission has been postulated to be the primary deficit inducing the alterations in dopamine transmission consistently reported in schizophrenia (Snyder and Murphy, 2008; Howes et al., 2015). Interestingly, either alterations in the above cognitive domains or changes in PFC glutamate and KYNA

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levels have been reported after human or rat prenatal exposure to cannabinoids (Fried and Smith, 2001; Ferraro et al., 2009; Hurd et al., 2019; present study). Overall, the above findings lead to the suggesting hypothesis that the long-term impairment of PFC glutamate transmission together with the impairment of LTP observed in rat offspring induced by prenatal cannabinoid exposure (Mereu et al., 2003) could explain, at least in part, the behavioral alterations and the executive function deficiency (Fried and Smith, 2001) observed in school-aged children born to women who used marijuana during pregnancy. This hypothesis is also supported by a recent study demonstrating that cannabis use is significantly associated with decreased PFC glutamate levels in early psychosis, with some relevance for the progression of the disease (Rigucci et al., 2018). Finally, chronic cannabis use decreases the levels of metabolites derived from glutamate, particularly in cortical and subcortical area, possibly through an excessive down-regulation of the glutamatergic signalling (Colizzi et al., 2016; Colizzi and Bhattacharyya, 2018). Finally, it is worth noting that the present study focused on prenatal THC-induced alterations in the PFC, a brain region that seems particularly sensitive to prenatal cannabis insult (Hurd et al., 2019). However, the appropriate connectivity between the PFC and other brain structures, as the hippocampus, the amygdala and mesolimbic regions is basilar for the adaptive organization of goal-directed behavioral responses and cognition (Negrón-Oyarzo et al., 2016), and it is also argued that neuropsychiatric disorders involve alterations to the behavioral and cognitive control system supported by the PFC and its connectivity with other brain systems (Negrón-Oyarzo et al., 2016). In this context, it becomes relevant that several clinical and preclinical studies suggested that chronic neurodevelopmental exposure to cannabinoids can alter various neural regions that functionally interact with the PFC (Mereu et al., 2003; Ferraro et al., 2009; de Salas-Quiroga et al., 2015; Renard et al., 2016; Grant et al., 2018; Hurd et al., 2019; Scheyer et al., 2019b). Thus, it seems likely that prenatal THC-induced alterations of development and fine-tuning of PFC circuits, involving other neurotransmitters as GABA and dopamine, could also be involved in the observed changes in PFC KYNA and/or glutamate levels. Research conducted in humans is, however, still too limited to reach final conclusions and further studies are necessary to address the relevance of the present findings for the clinical phenotype of prenatal cannabis-associated schizophrenia.

5. Conclusions

In a clinical context, the results of the present study raise the possibility that moderate prenatal cannabinoid exposure may act as a “first hit”, triggering comparatively subtle changes in the developing brain but leading to harmful effects by causing abnormal increases in the brain levels of KYNA in adulthood. In addition, demonstrated here by an exaggerated response to an acute challenge with KYNA’s bioprecursor kynurenine, exposure to THC during pregnancy increases vulnerability to an adverse “second hit” later in life. Jointly, these findings suggest that KP dysfunction, and specifically elevated brain KYNA levels, may play a significant role in the negative long-term consequences of prenatal THC use.

From a translational point of view, interventions aimed at preventing the increase in KYNA levels in the brain may therefore provide a novel approach to combat the detrimental long-term effects and vulnerabilities caused by prenatal cannabis use. Of note in this regard,

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selective genetic or pharmacological interference with KYNA formation has been repeatedly shown to have pro-cognitive effects in rodents.(Potter et al., 2010; Rossi et al., 2010; Kozak et al., 2014; Pocivavsek et al., 2019)

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Abbreviations:

GD	gestational day
KAT	kynurenine aminotransferase
KP	kynurenine pathway
KYNA	kynurenic acid
PD	postnatal day
PFC	prefrontal cortex
SZ	schizophrenia
THC	^{9}-tetrahydrocannabinol

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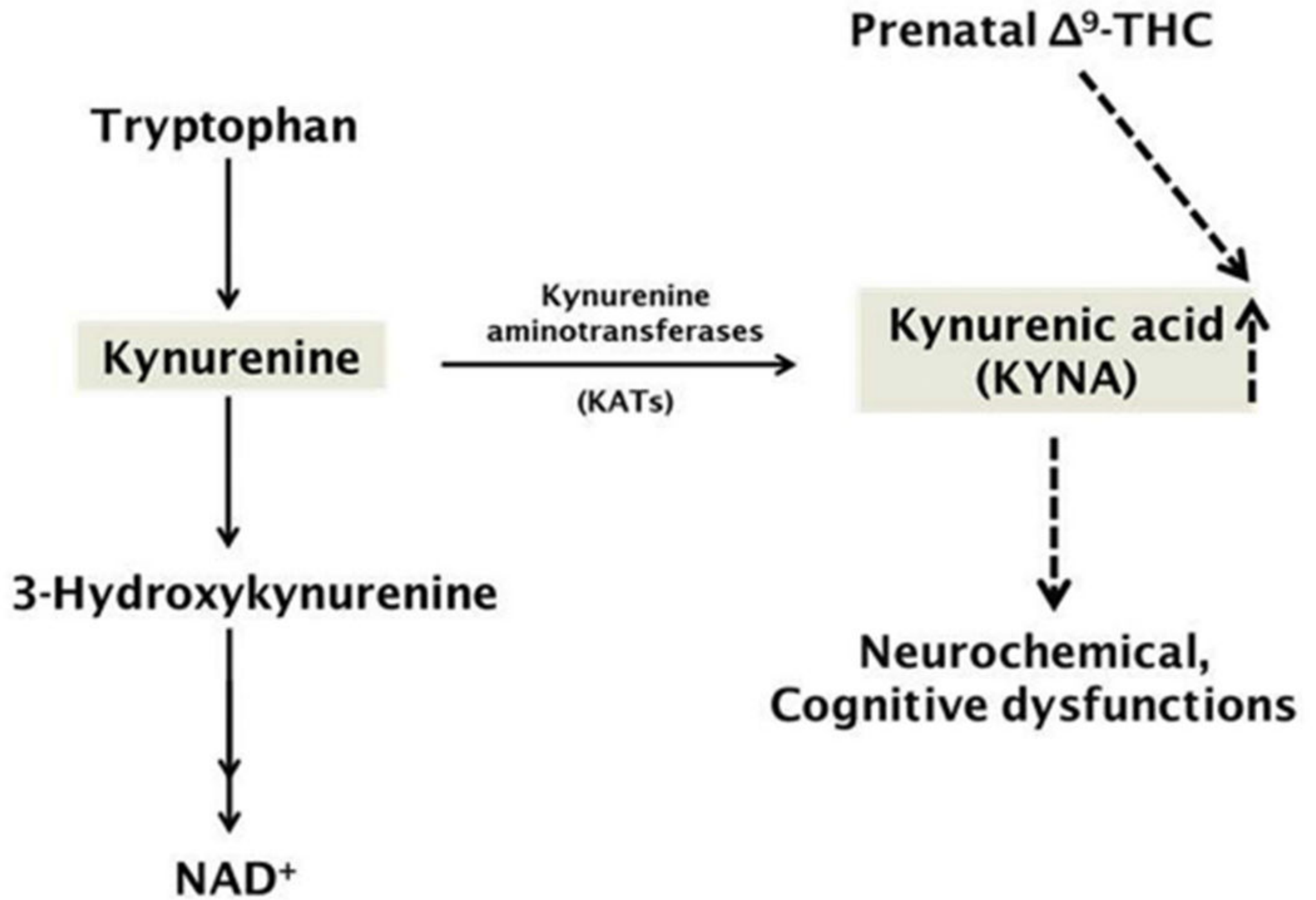

Fig. 1. Prenatal 9-THC treatment by increasing KYNA levels, induces neurochemical and cognitive dysfunctions in the adult offspring.

Schematic representation of the kynurenine pathway (continuous arrows) and hypothesized (dashed arrows) mechanisms involved in the long-term effects of prenatal THC treatment.

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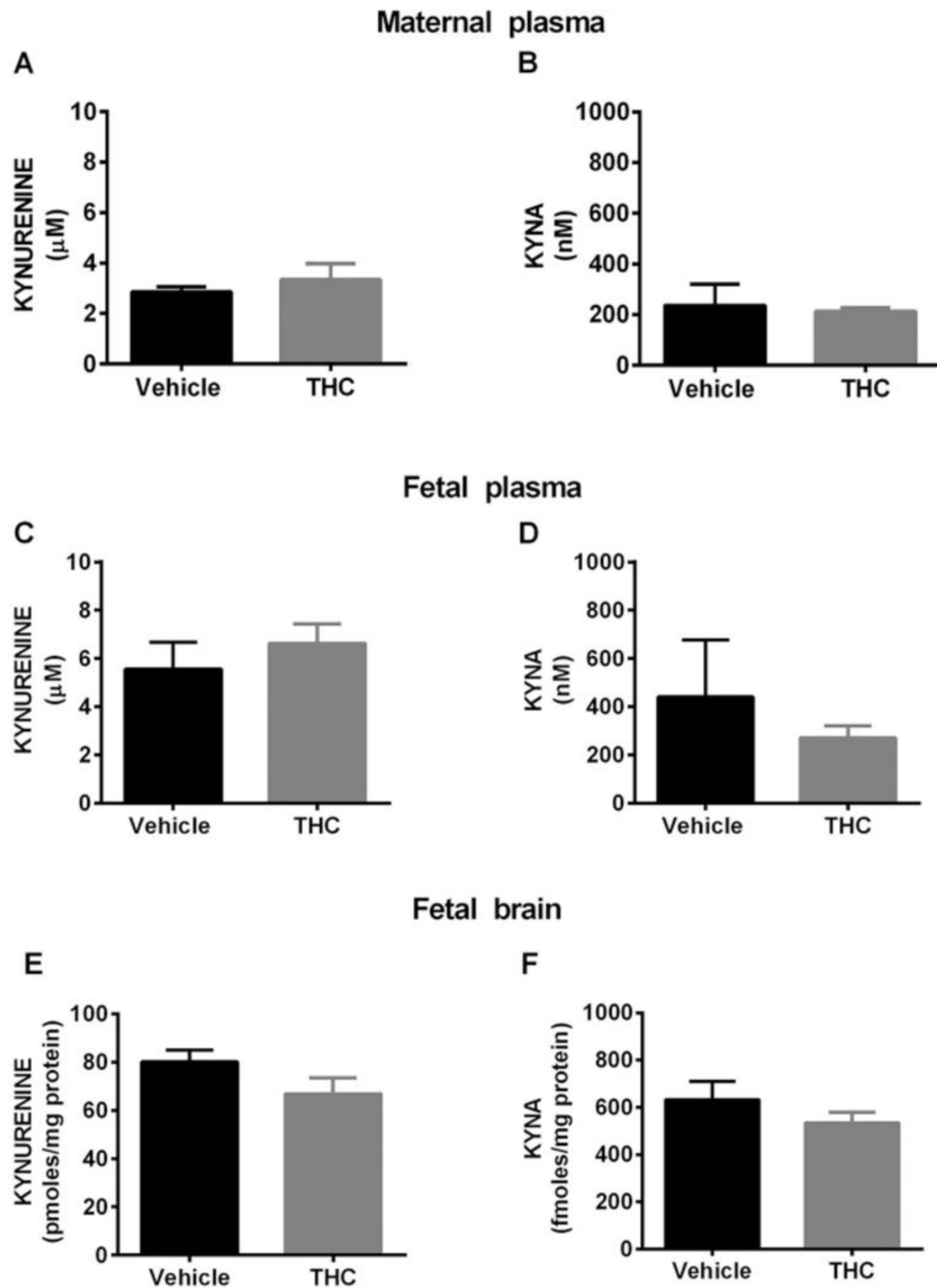

Fig. 2. Gestational THC exposure does not induce any changes of kynurenine and KYNA levels in the maternal and fetal plasma, and fetal brain.

Kynurenine (**A, C, E**) and KYNA (**B, D, F**) content of maternal and fetal plasma, and fetal brain, on gestational day (GD) 20. Pregnant rats were treated daily with THC (5 mg/kg) or vehicle, administered daily by oral gavage from gestational day (GD) 5 through GD 20. Data are the mean ± standard error of the mean (SEM). Vehicle: $n = 3$ dams per group, n = 3–4 embryos per dam; THC: $n = 5$ dams per group, n = 3–4 embryos per dam. No statistical differences were observed between groups.

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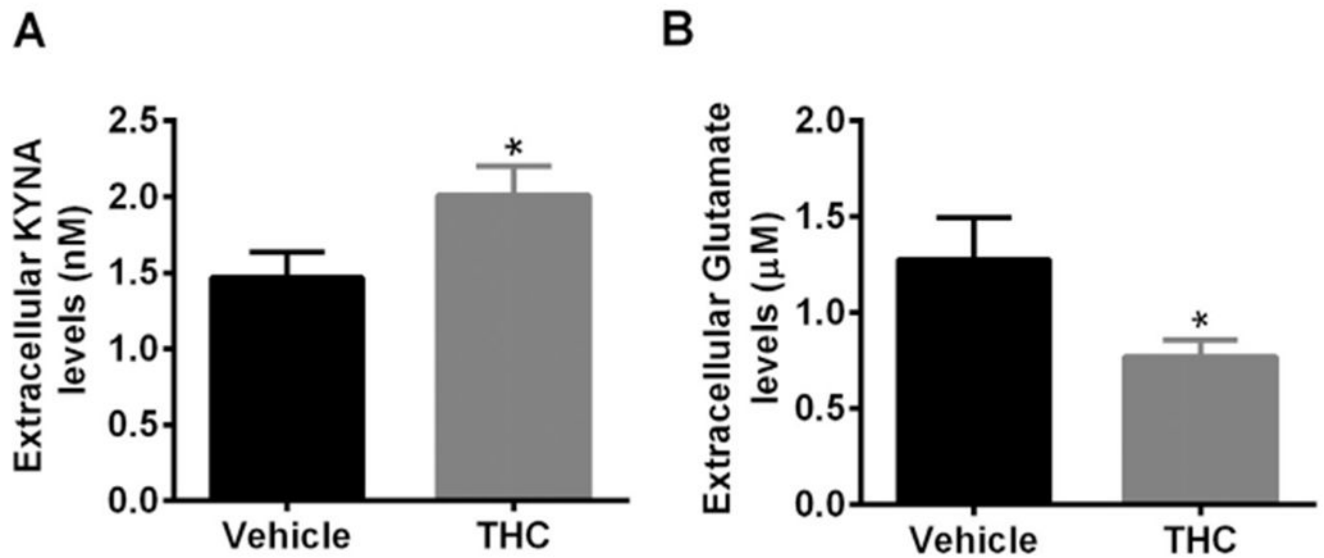

Fig. 3. Prenatal THC leads to higher basal extracellular KYNA and lower basal extracellular glutamate in the mPFC in adulthood.

Basal extracellular KYNA (**A**) and glutamate (**B**) levels in the mPFC of adult rats prenatally exposed to THC (5 mg/kg) or vehicle, administered daily by oral gavage from gestational day (GD) 5 through GD 20. Data are the mean ± SEM; $n = 8–10$/group, 1 male per litter. * $p < .05$ vs. vehicle (Student's t-test).

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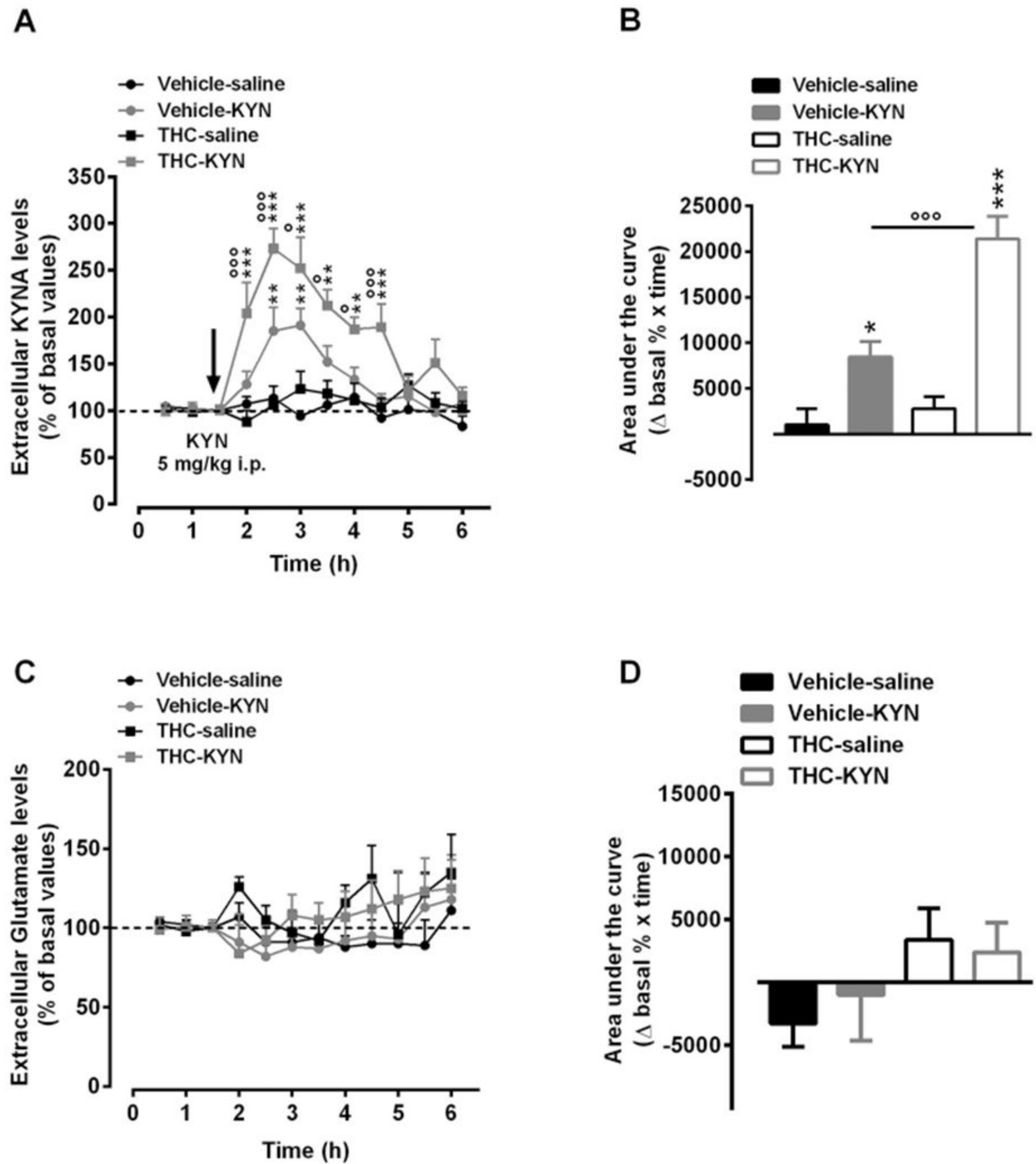

Fig. 4. An acute challenge with kynurenine induces a greater increase in extracellular KYNA levels in the mPFC of prenatally THC-exposed than in prenatally Vehicle-exposed offspring.
Extracellular KYNA (**A, B**) and glutamate (**C, D**) levels in the mPFC of adult male rats prenatally exposed to THC (5 mg/kg) or vehicle, administered daily by oral gavage from gestational day (GD) 5 through GD 20. Kynurenine (KYN) or saline was injected i.p. (arrows). **A, C:** data (means ± SEM) are expressed as a percentage of the averaged three baseline values prior to treatment. In vehicle-exposed offspring basal extracellular KYNA and glutamate levels were 1.40 ± 0.19 nM and 1.32 ± 0.21 μM respectively. In THC-exposed

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offspring basal extracellular KYNA and glutamate levels were 2.10 ± 0.18 nM and 0.67 ± 0.12 μM respectively.

C, D: areas under the curves (means ± SEM) calculated as the percentage of changes in basal values over time. Data were analyzed by two-way (**A, C**) or one-way (**B, D**) ANOVA and Tukey's post-hoc test for multiple comparisons. **A:** *** $p < .001$, ** $p < .01$ vs. saline; °°° p < .001, ° p < .05 vs. vehicle-KYN. **B:** * p < .05 vs. saline; *** p < .001 vs. all, °°° p < .001 vs. vehicle-KYN, $n = 6–9$/group (1 male per litter).

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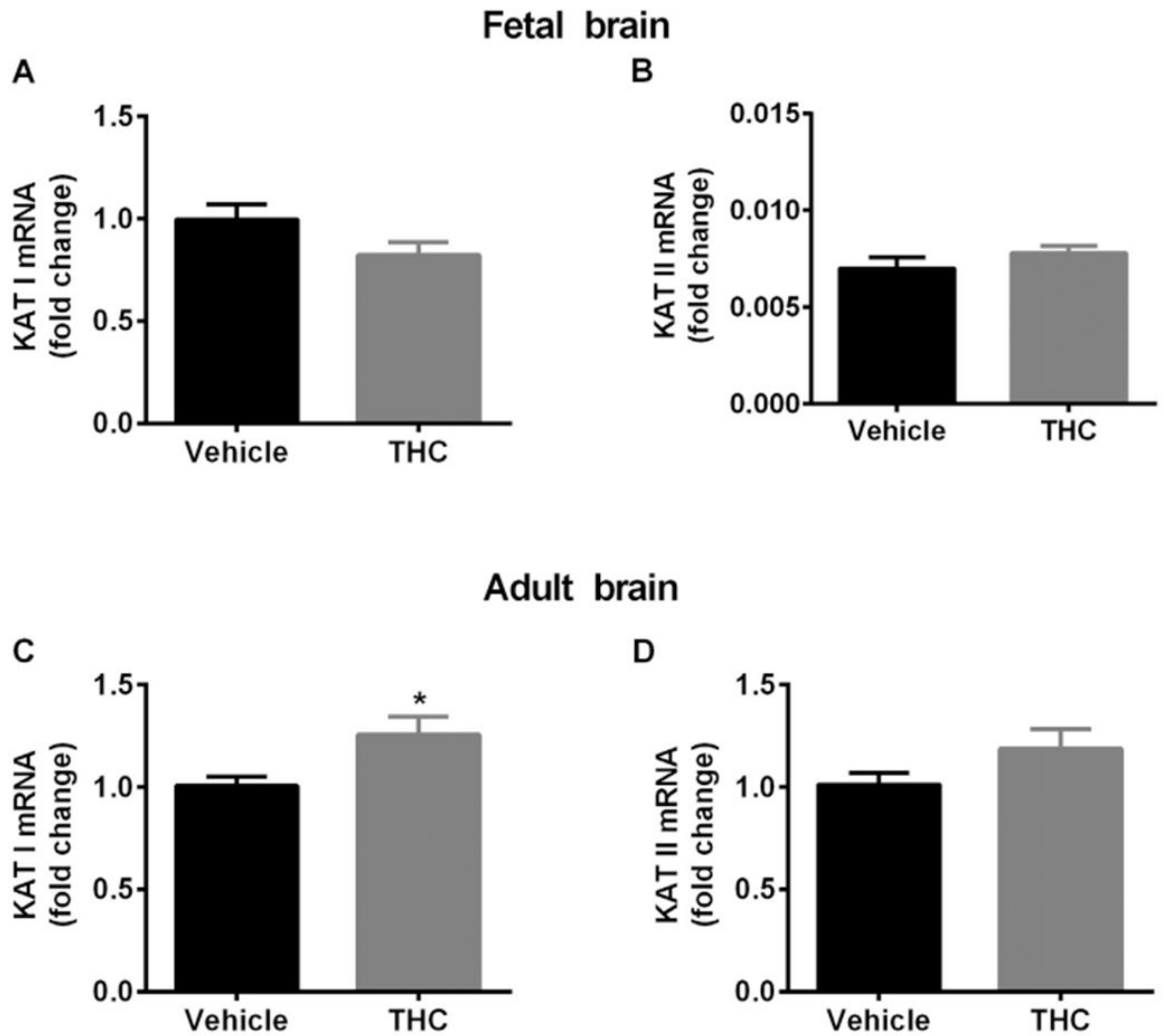

Fig. 5. Prenatal THC exposure differentially affects KAT I and KAT II mRNA in the offspring.
Brain KAT I and KAT II mRNA levels in the fetal brain (GD 20, **A, B**) and in the PFC of the adult brain (PD 60–90; **C, D**). Pregnant rats were treated daily with THC (5 mg/kg) or vehicle, administered daily by oral gavage from gestational day (GD) 5 through GD 20. Bars represent the mean ± SEM (*n* = 7–9/group. *p < .05 vs. vehicle (Student's t-test).

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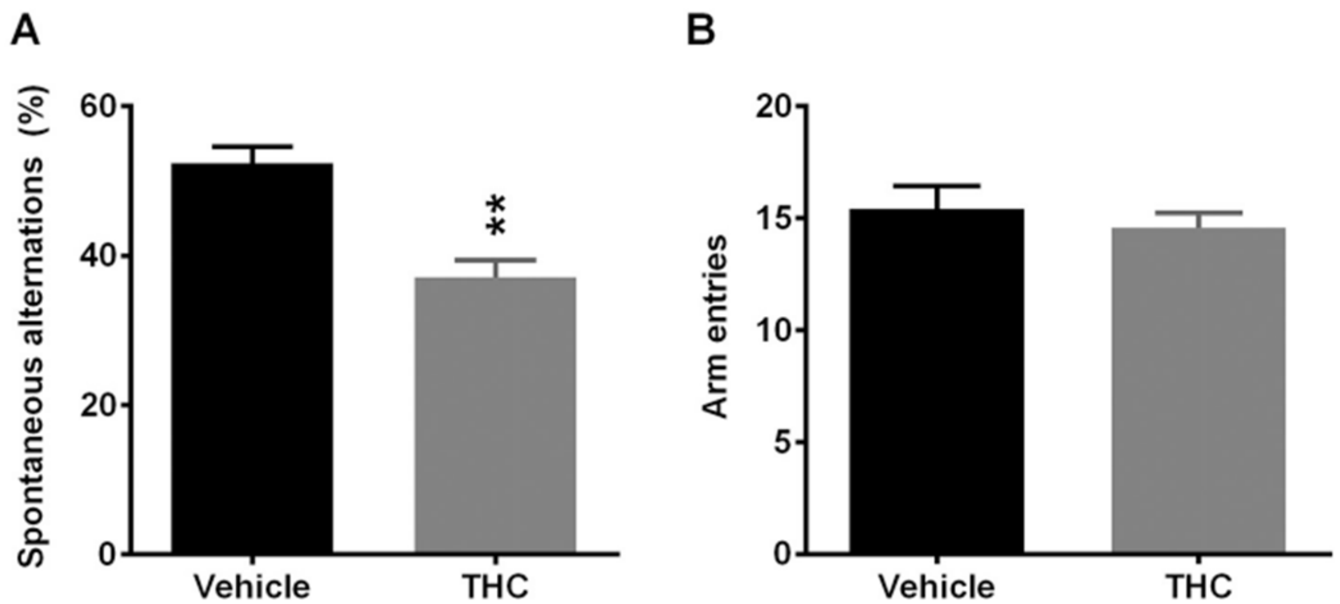

Fig. 6. Prenatal THC exposure induces impaired short-term memory in adulthood, as evaluated by the Y maze.

Percentage of spontaneous alternations (**A**) and the number of arm entries (**B**) in adult rats prenatally exposed to THC (5 mg/kg) or vehicle from gestational day (GD) 5 through GD 20 by oral gavage. Bars represent the mean ± SEM (n = 7/group. *p < .01 vs. vehicle (Mann Whitney *U* test).