ORIGINAL INVESTIGATION

Prenatal kynurenine exposure in rats: age-dependent changes in NMDA receptor expression and conditioned fear responding

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Abstract

Rationale Levels of kynurenic acid (KYNA), an endogenous negative modulator of alpha 7 nicotinic acetylcholine receptors (α7nAChRs) and antagonist at glutamatergic *N*-methyl-D-aspartate receptors (NMDARs), are elevated in the brain of patients with schizophrenia (SZ). In rats, dietary exposure to KYNA's immediate precursor kynurenine during the last week of gestation produces neurochemical and cognitive deficits in adulthood that resemble those seen in patients with SZ.

Objectives The present experiments examined whether prenatal kynurenine exposure results in *age-dependent* changes in the kynurenine pathway (KP), expression of selected receptors, and cognitive function.

Methods Pregnant dams were fed unadulterated mash (progeny = ECON) or mash containing kynurenine (100 mg/day; progeny = EKYN) from embryonic day (ED) 15 to 22. Male offspring were assessed as juveniles, i.e., prior to puberty (postnatal day [PD] 32), or as adults (PD70) for brain KYNA levels, α7nAChR and NMDAR gene expression, and performance on a trace fear conditioning (TFC) task.

Results KYNA levels were comparable between juvenile ECON and EKYN rats, whereas EKYN adults exhibited a ~3-fold increase in brain KYNA relative to ECONs. NR2A expression was persistently reduced (30–40 %) in EKYN rats at both ages. Compared to ECON adults, there was a 50 % reduction in NR1, and a trend toward decreased α7nAChR expression, in adult EKYN rats. Surprisingly, juvenile EKYN rats performed significantly *better* in the TFC paradigm than controls, whereas adult EKYN animals showed the predicted deficits.

Conclusions Collectively, our results provide evidence that KP changes in the fetal brain alter neuronal development and cause age-dependent effects on neurochemistry and cognitive performance.

Keywords alpha7 nicotinic receptors · Glutamate · Kynurenic acid · *N*-Methyl-D-aspartate receptors · Prefrontal cortex · Schizophrenia · Trace fear conditioning

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Abbreviations

α7nACh	alpha7 nicotinic acetylcholine
CS	Conditioned stimulus
CSF	Cerebrospinal fluid
ECON	Embryonic control treatment
ED	Embryonic day
EKYN	Embryonic kynurenine treatment
KP	Kynurenine pathway
KYNA	Kynurenic acid
NMDA	*N*-Methyl-D-aspartate
PD	Postnatal day
PFC	Prefrontal cortex
SZ	Schizophrenia
TFC	Trace fear conditioning
UCS	Unconditioned stimulus

Introduction

The levels of kynurenic acid (KYNA), a major metabolite of the kynurenine pathway (KP) of tryptophan degradation, are elevated in cerebrospinal fluid (CSF) and post-mortem brain tissue of individuals with schizophrenia (SZ) and may be causally related to the pathophysiology of the disease (Erhardt et al. 2001; Linderholm et al. 2012; Schwarcz et al. 2001). Interest in this hypothesis is related to the fact that KYNA is a negative allosteric modulator of the α7 nicotinic acetylcholine receptor (α7nAChR) and, at higher concentrations, a direct antagonist of the N-methyl-D-aspartate (NMDA) receptor (Albuquerque and Schwarcz 2013; Perkins and Stone 1982), both of which play critical roles in brain development, a defining period in the etiology of SZ (Feinberg 1982; Jansson and Akerman 2014; Ohgi et al. 2015), and in several cognitive deficits seen in SZ patients (for reviews see Lewis 1997; Millan et al. 2012). Moreover, SZ is associated with the genes of two key KP enzymes, tryptophan 2,3-dioxygenase (TDO) and kynurenine 3-monooxygenase (KMO), and functional abnormalities in these enzymes may be responsible for increased KYNA neosynthesis in the brain (Miller et al. 2004; Sathyasaikumar et al. 2011; Stone and Darlington 2013; Wonodi et al. 2011).

As shown in the prefrontal cortex (PFC) of adult rats, even relatively modest KYNA increases, by negatively modulating α7nACh and/or NMDA receptor function, reduce the local extracellular levels of several major neurotransmitters known to be critically involved in cognitive processes, including glutamate (Konradsson-Geuken et al. 2010; Pershing et al. 2015; Wu et al. 2010), dopamine (Pocivavsek et al. 2016), and GABA (Beggiato et al. 2014). Alone or jointly, and possibly also affected by maturational changes in α7nACh and NMDA receptors (Lin et al. 2014a, b; Stone and Darlington 2013), these neurochemical effects may be part of developmental insults that contribute to cognitive deficits seen in people with SZ. Notably, KYNA-induced neurotransmitter changes may also contribute to the structural abnormalities that are seen in the cerebral cortex of patients, including reduced volume (Rimol et al. 2010; Wright et al. 2000), irregular cellular organization (Akbarian et al. 1993; Benes et al. 2001), and diminished dendritic spine density (Glausier and Lewis 2013).

Guided conceptually by the neurodevelopmental hypothesis of SZ, we demonstrated recently that the feeding of rat dams with KYNA's immediate bioprecursor kynurenine during the final week of gestation leads to elevated brain KYNA, diminished dendritic spine density, reduced expression of the metabotropic glutamate receptor mGluR2, reduced prefrontal glutamate levels following NMDA infusions into the nucleus accumbens, and impaired performance in the Morris water maze and the passive avoidance paradigm, in adult offspring (Pershing et al. 2015; Pocivavsek et al. 2014). Comparable results were obtained by elevating KYNA levels in the fetal brain using a KMO inhibitor, which diverts KP metabolism

toward increased KYNA formation (Forrest et al. 2013, 2015). In all these studies, animals were left undisturbed from birth until they were tested experimentally as adults. This experimental design failed to address the question about the onset of these neurobehavioral changes and whether their emergence interacted with maturational events such as adolescence.

Thus, the present study was designed to determine age dependency of these effects by measuring changes in plasma kynurenine and brain KYNA levels, as well as α7nACh and NMDA receptor gene expression, in control (normal mash during embryonic development; "ECON") and experimental rats (exposed to elevated KYNA as embryos; "EKYN") at postnatal days (PD) 32 or 70. In separate ECON and EKYN rats at both ages, we studied impairment of executive function, which is thought to be predictive of SZ progression and functional outcome (Green 1996; Green et al. 2004). To this end, we assessed trace fear conditioning (TFC), a cognitively challenging form of classical (Pavlovian) conditioning that can be readily studied in both juvenile and adult rodents (Barnet and Hunt 2005; Goodfellow et al. 2016) and that depends upon the integrity of interactions between the hippocampus and the PFC (Raybuck and Gould 2010).

Materials and methods

Animals

This study was conducted in male Wistar rats derived from in-house breeding. Animals were maintained on a 12:12 h light/dark cycle in a temperature- and humidity-controlled, AAALAC-approved animal facility at The Ohio State University, and had ad libitum access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee in accordance with the NIH Guide for Care and Use of Laboratory Animals. All necessary measures were taken to minimize pain and discomfort to the animals.

Kynurenine supplementation and treatment groups

Female breeders were habituated to wet rodent mash (Teklad Diets, Madison, WI, USA; 30 g per day) beginning on embryonic day (ED) 0. Dams received 100 mg/day of L-kynurenine sulfate ("kynurenine," 99.4 % purity; Sai Advantium, Hyderabad, India) in the wet mash each day from ED15 to ED22 (EKYN group). Control animals were fed unadulterated mash (ECON group). Standard rodent chow pellets were provided to all animals after birth. The day of birth was denoted PD0. On PD2, litters were culled to 9–11 pups to standardize growth rates across all litters and to maximize the number of males. Rats were weaned on PD21, and males were pair-housed by litter. All rats were handled approximately twice per week until selected for behavioral or biochemical experiments at one

of two different ages: as juveniles or pre-adolescents [rats ranged in age from PD31 to 34 (designated PD32 hereafter)] or as adults [rats ranged in age from PD65 to 75 (designated PD70 hereafter)]. Due to the large number of experimental outcome measures (i.e., both biochemical assessments and behavioral testing at two ages), the number of males per litter was not always sufficient to fully balance littermates across all endpoints. The typical distribution of progeny from an ECON or EKYN litter was two rats for trace fear conditioning (one at each age) and eight rats for biochemical assays (four at each age). In some cases, additional ECON or EKYN litters were added to biochemistry experiments to yield a total of $n = 8$ litters per condition at PD32 and $n = 8$–10 litters per condition at PD70. Thus, biochemical assays were conducted on litters that contributed to behavioral measures (in ~60 % of the cases) and those that did not (~40 %), but which were treated identically in all other regards (prenatal treatment, culling, housing conditions, etc.).

Biochemical analyses

Rats (PD32—ECON, $n = 9$; EKYN, $n = 10$; PD70—ECON and EKYN, $n = 10$ each) were anesthetized (CO_2), and blood was collected by cardiac puncture into tubes containing disodium EDTA. After centrifugation ($1200 \times g$, 10 min), the supernatant plasma was transferred to new Eppendorf tubes. Brains were rapidly removed from the skull, sectioned through the sagittal midline, and placed on ice. Both plasma and tissue samples were then promptly frozen on dry ice and stored at −80 °C.

Plasma kynurenine determination

On the day of the assay, plasma samples were thawed and diluted 1:2 in ultrapure water. One hundred microliters of diluted plasma was acidified with 25 μl of 6 % perchloric acid, and samples were centrifuged ($12,000 \times g$, 10 min). In the resulting supernatant, kynurenine was analyzed by high-performance liquid chromatography (HPLC) with fluorimetric detection, as previously described in Pocivavsek et al. (2014).

Kynurenic acid (KYNA) determination in tissue

Tissue was thawed, and the prefrontal cortex was dissected out and homogenized in ultrapure water (1:10 w/v). One hundred microliters of homogenate was acidified with 25 μl of 6 % perchloric acid, and samples were centrifuged ($12,000 \times g$, 10 min). In the resulting supernatant, KYNA was measured by HPLC with fluorimetric detection (Pocivavsek et al. 2014).

Quantitative polymerase chain reaction (qPCR)

Gene expression was determined for NMDA receptor subunits (NR1, NR2A, NR2B), α7nACh receptor, and GAPDH. To this end, total RNA was extracted from frontal cortices of PD32 (ECON, $n = 9$; EKYN, $n = 10$) and PD70 (ECON and EKYN, $n = 10$ each) rats using PureZol reagent (Bio-Rad, Hercules, CA, USA) and isolated using a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Genomic DNA contamination was eliminated by performing DNase digestion using RNase-free DNase (Macherey-Nagel). cDNA was obtained from the extracted RNA (up to 1 μg/reaction) using iScript reverse transcription Supermix for RT-qPCR (Bio-Rad) under the following conditions: priming at 25 °C for 5 min, reverse transcription for 30 min at 42 °C, and inactivation of transcriptase by heating at 85 °C for 5 min. The first-strand cDNA solution was diluted 5-fold with 0.5× Tris-EDTA buffer (Sigma-Aldrich, MO, USA) prior to qPCR.

qPCR was performed using the CFX96, C1000 Thermal Cycler (Bio-Rad) in a reaction mixture containing 10 μl Sso Advanced SYBR Green Supermix (Bio-Rad), 5 μl of each cDNA sample solution, 15 pmol of forward and reverse gene-specific primer pairs (Integrated DNA Technologies, Coralville, IA, USA), and DNase-free water in a total volume of 20 μl. The thermal profile for PCR was as follows: initial denaturation at 95 °C for 30 s followed by 39 temperature cycles of denaturation at 95 °C for 5 s, annealing/extension (62 °C for 30 s for α7nAChR), and melting curves. Fluorescence was acquired at the end of each extension phase. Fold changes in mRNA expression levels were calculated according to the comparative cycle threshold (CT) method and normalized using the level of GAPDH mRNA (Schmittgen and Livak 2008). The primers used for the five genes were **GAPDH** FW: 5′-CAT CAA GAA GGT GGT GAA GCA-3′, RV: 5′-CTG TTG AAG TCA CAG GAG ACA-3′ (Rao et al. 2006); **NR1** FW: 5′-GTT CTT CCG CTC AGG CTT TG-3′, RV: 5′-AGG GAA ACG TCC TGC TTC CA-3′ (Giza et al. 2006); **NR2A** FW: 5′-AGC CCC CTT CGT CAT CGT-3′, RV: 5′-GAC AGG GCA CCG TGT TCC T-3′ (Giza et al. 2006); **NR2B** FW: 5′-AGC TGG TAG CCA TGA AC-3′, RV: 5′-GAT CTT CCG GTC AGA CAT-3′ (Giza et al. 2006); and **Alpha7** FW: 5′-TGC ACG TGT CCC TGC AAG GC-3, RV: 5′-GTA CAC GGT GAG CGG CTG CG-3′ (Thomsen and Mikkelsen 2012). Primer quality was evaluated by standard curve, melting curve, and the presence of a single PCR product after gel electrophoresis.

Behavioral analyses

Conditioning apparatus

All training and testing occurred in standard operant boxes (Coulbourn Instruments, Allentown, PA), enclosed in sound-attenuating chambers. Each operant box had two stainless steel walls, two Plexiglas walls, and a grid floor composed of 0.5-cm stainless steel bars placed approximately 1.5 cm apart. A small animal shock generator (model H13-15; Coulbourn

Instruments) and neon grid scrambler connected to the grid floor provided the unconditioned stimulus (US) foot shock.

Foot shock reactivity

To ensure that TFC results were not unduly influenced by group differences in sensory perception, foot shock reactivity was assessed in juvenile (PD32) and adult (PD70) ECON and EKYN rats ($n = 3$ per condition per age). Each rat was placed into the conditioning chamber and allowed to explore for 120 s. An ascending series of 10 foot shocks (0.1 to 1.0 mA) was then administered in 0.1-mA increments with a 30 ± 5 s intertrial interval. Rat behavior was recorded using a Panasonic HC-V720 high-definition video camera, and responses were scored off-line by observers blind to the treatment condition. The following criteria, adapted from Nielsen and Crnic (2002), were used: 0 = No Response (no reaction to shock), 1 = Flinch/Rear (jerky movement, shift in body posture, attentional orienting, but with two paws remaining on floor), 2 = Hop/Run (forward or backward movement, less than half the length of the testing chamber or small vertical movement less than body height), and 3 = Jump (horizontal motion greater than half of the chamber or vertical motion greater than body height with all four paws leaving the floor in a springing motion). The shock intensity at which rat vocalizations occurred was also noted for each subject. Shock reactivity was determined by calculating the mean shock intensity at which each response occurred.

Trace fear conditioning

TFC and retention testing were conducted over three sessions, separated by ~24 h, beginning at PD32 or PD70 (ECON, $n = 10$; EKYN, $n = 12$, at each age). The conditioned stimulus (CS) was a 15-s, 2.8-kHz, 80-dB tone presented through a speaker located at the top of each chamber. The US was a 1.0-s, 1.0-mA scrambled foot shock. TFC consisted of 10 CS-US trials. For each trial, the 15-s tone CS was followed 10 s later (the trace interval) by the 1.0-s foot shock US, resulting in a 25-s interstimulus interval (ISI) from CS onset to US onset. The intertrial interval (ITI) was 240 ± 30 s. Prior to placing each subject inside, each chamber was wiped with a water/vinegar (5:1) solution. Approximately 4 min after the last trial, rats were removed from the chamber and transported to their home cages.

Approximately 24 h later, rats were returned to the conditioning chamber to measure the context-dependency of conditioned fear (i.e., freezing behavior) in the absence of the CS and the US. After a 120-s baseline, freezing was measured for 10 min (in 2-min bins). Forty-eight hours after conditioning, CS-evoked freezing was assessed independent of the conditioning context—i.e., rats were placed in a novel context located in a dark room lit with one red overhead light. The chamber was scented with Windex®, the grid bars were covered with gray opaque Plexiglas, a pink geometric shape was attached to the front door, a small magnet was placed on one inside metal wall, and a small fan was turned on, providing 60 dB white ambient noise. Unconditioned (generalized) fear to the novel context (i.e., freezing) was measured during the 1 min immediately preceding the first CS-alone trial. Rats were presented a total of 10 CS-alone trials (180 ± 30 s ITI), and freezing was measured during the 15 s tone CS and the 10 s trace interval.

Freezing analysis

Freezing was defined as cessation of all movement except that required for respiration (Fanselow 1980) and was expressed as the percentage of the recording interval that rats were engaged in freezing. Freezing behavior was recorded with a video camera (Model WDSR-2005SC; Circuit Specialists, Inc., Mesa, AZ) mounted to the top (ceiling) of the conditioning chamber. The interior was illuminated by an infrared light source to observe freezing behavior. The video signal was ported to FreezeScan (CleverSys, Inc., Reston, VA), a video-based tool that detects and quantifies freezing behavior within experimenter-defined time bins.

Statistical analyses

With the exception of body weight, which was expressed as the litter average, all data were calculated using individual subjects as the experimental unit. Body weights, plasma kynurenine, brain KYNA, gene expression, and freezing data were analyzed using single-factor, multi-factorial, or repeated-measures ANOVAs. The Huynh-Feldt correction was utilized to reduce type II errors associated with repeated-measures ANOVAs (Vasey and Thayer 1987). Significant main effects or interactions were followed by a minimum number of t tests to determine between-group effects. Data analyses were performed using the SPSS statistics program (v22; IBM Corporation, Armonk, NY), and significance was defined as $P < 0.05$.
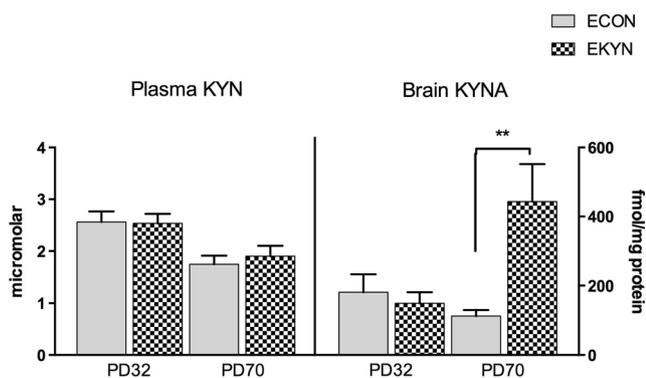
Results

Growth rate of ECON and EKYN pups

There were no apparent differences in maternal care (nest-building, nursing posture, pup retrieval) between ECONs and EKYNs (data not shown). Offspring were weighed weekly from PD7 through PD65. Average litter weights were calculated separately for ECONs ($n = 16$ litters) and EKYNs ($n = 17$ litters). Prenatal treatment with kynurenine did not affect body weight disproportionally in the offspring at any age tested, i.e., there were no significant differences in growth rate between ECON and EKYN rats during the pre-weaning (PD7–21) or post-weaning (PD28–65) periods ($P = 0.242$; repeated-measures analysis) (data not shown).

Plasma kynurenine and brain KYNA

The long-term effects of prenatal exposure to elevated kynurenine on subsequent KP metabolism was assessed by measuring plasma kynurenine and brain KYNA levels in juvenile (PD32) and adult (PD70) offspring (Fig. 1). Two-way ANOVA (age × prenatal condition) indicated that plasma kynurenine levels were similar between ECON and EKYN juvenile rats (2.6 ± 0.2 μM and 2.5 ± 0.2 μM, respectively; $P = 0.70$) and between ECON and EKYN adult rats (1.7 ± 0.2 μM and 1.9 ± 0.2 μM, respectively; $P = 0.5$). In contrast, there was a highly significant effect of age on brain KYNA levels in ECON and EKYN rats ($F_{1,35} = 8.143$, $P = 0.007$). Brain KYNA levels were similar in juvenile ECON and EKYN rats (181.4 ± 51.0 and 149.8 ± 35.3 fmol/mg protein, respectively; $t_{17} = 0.536$, $P = 0.599$). In contrast, brain KYNA levels in adult EKYN rats (443.2 ± 108.7 fmol/mg protein) were 293 % higher than in adult ECON rats (112.8 ± 17.3 fmol/mg protein), resulting in a significant group difference ($t_{18} = -3.003$, $P = 0.008$).

Gene expression: NMDA and α7nACh receptors

Several genes encoding receptors which are implicated in the etiology of SZ, are antagonized by KYNA and play crucial roles in the expression of TFC were analyzed by qPCR at PD32 and PD70 (NR1, NR2A, NR2B, and α7nAChR) (Fig. 2a). Compared to age-matched ECONs, there was a 29 % reduction in NR2A in PD32 EKYN rats ($t_{17} = 2.216$, $P = 0.041$). This decrease in EKYNs persisted through adulthood (−38 % compared to ECONs; $t_{18} = 2.314$, $P = 0.033$). At PD70, but not at PD32, there was also a significant 50 % reduction in NR1 expression in EKYN rats compared to

ECONs ($t_{18} = 2.550$, $P = 0.020$). No group differences were noted for the NR2B subunit or for α7nAChRs in either juvenile (PD32) rats or adult (PD70) rats. Of possible interest, and in line with our previous observation (Pershing et al. 2015), adult EKYN rats showed a modest yet non-significant trend toward reduced expression of α7nAChRs compared to adult ECONs (−17 %; $t_{18} = 1.153$, $P = 0.264$). Moreover, as illustrated in Fig. 2b, the ratio of NR2A/NR2B gene expression, which was comparable at PD32 and PD70 in control rats ($P = 0.76$), was slightly decreased (reflecting an increase in NR2B relative to NR2A) in EKYN rats compared to ECONs at both ages (by 29 and 39 %, respectively; $P = 0.06$ and $P = 0.07$, respectively).
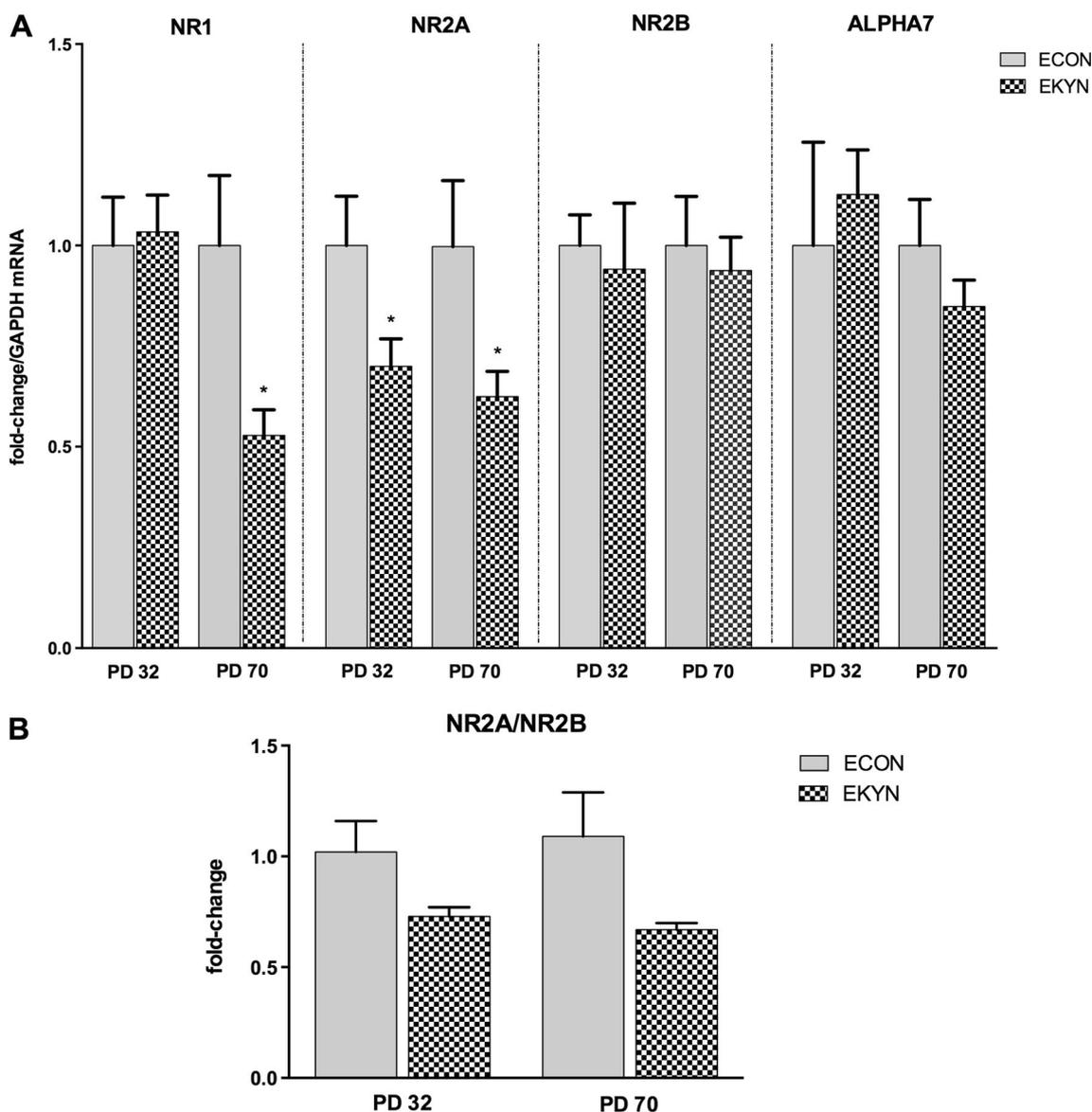
Foot shock sensitivity

Table 1 summarizes a descriptive analysis of the mean foot shock intensity (mA; range = 0.1-1.0 mA) at which various behaviors (flinch, hop/run, jump, and/or vocalizing) emerged in ECON and EKYN rats at both ages. With the exception of one ECON and one EKYN rat at PD70, rats were insensitive to the lowest shock intensity. While the sample size was relatively small ($n = 3$/group), the results were extremely similar across the two treatment groups and two ages. Thus, shock reactivity scores increased across groups in almost an identical fashion as a function of shock intensity. Vocalizations were noted at shock intensities of 0.4 mA and above. These similarities suggest that differences in freezing behavior, described below, cannot be attributed to differences in the salience of the foot shock stimulus.

Trace fear conditioning

TFC was performed to examine the effects of prenatal exposure to kynurenine on the acquisition and expression of conditioned fear in adolescence and adulthood. At either age, freezing behavior during TFC acquisition did not differ between treatment groups (data not shown). Juveniles and adults of both groups also showed similar levels of freezing throughout the 10 min context test (ranging from 34 ± 3 % to 41 ± 3 % freezing across ages and conditions) (Fig. 3a). There were no significant effects of prenatal condition ($F_{1,40} = 0.742$, $P = 0.394$), age ($F_{1,40} = 0.009$, $P = 0.926$), or age × condition interaction ($F_{1,40} = 0.941$, $P = 0.338$).

All animals were subsequently presented with 10 CS-alone test trials in a novel context. Due to equipment malfunction, CS-alone freezing data were lost for two PD32 EKYN rats. As illustrated in Fig. 3b, 2 × 2 ANOVAs showed that prenatal kynurenine treatment produced age-dependent alterations in freezing to the tone CS ($F_{1,38} = 13.664$, $P = 0.001$) and trace interval ($F_{1,38} = 9.908$, $P = 0.003$). ECON and EKYN rats were subsequently compared within each age, revealing that PD32 EKYN rats froze significantly more than ECON rats

Fig. 1 Effects of prenatal exposure to kynurenine (KYN) on levels of KYN in the plasma and kynurenic acid (KYNA) in the PFC in juvenile (PD32) and adult (PD70) rats. Data are the mean ± SEM. PD32: ECON: $n = 9$; EKYN: $n = 10$; PD70: $n = 10$ per group. Two-way ANOVA (age × prenatal condition) revealed no differences in plasma kynurenine; however, there was a significant interaction of age and prenatal condition for brain KYNA levels. EKYN versus ECON at PD70: **$P < 0.01$

Fig. 2 **a** Relative expression of mRNA levels of NMDAR subunits and α7nAChR at PD32 and PD70. Data (mean ± SEM) for EKYNs are normalized, for their respective age group, to ECONs, which were set to a value of 1.0 ($n = 11$ rats/age group). *$P < 0.05$, **$P < 0.01$. **b** Bar graphs showing a strong trend ($P < 0.06$ and $P < 0.07$ at PD32 and PD70, respectively) toward decreases in the NR2A/NR2B ratio in EKYN rats at both ages

during the CS (66 ± 4 % vs. 54 ± 3 %; $t_{18} = -2.872$, $P = 0.010$) and trace interval (69 ± 4 % vs. 56 % ± 4 %; $t_{18} = -2.349$, $P = 0.030$). In contrast, PD70 EKYN rats froze significantly less than ECONs during the CS (58 ± 3 % vs. 72 ± 5 %;

Table 1 Shock sensitivities as function of age and treatment conditions

Shock score	PD32		PD70	
	ECON	EKYN	ECON	EKYN
	Mean ± SEM (mA)	Mean ± SEM (mA)	Mean ± SEM (mA)	Mean ± SEM (mA)
1	0.32 ± 0.06	0.34 ± 0.05	0.34 ± 0.07	0.25 ± 0.06
2	0.65 ± 0.05	0.68 ± 0.05	0.63 ± 0.05	0.55 ± 0.06
3	0.92 ± 0.04	0.88 ± 0.06	0.84 ± 0.09	0.80 ± 0.06
Vocalize	0.68 ± 0.07	0.77 ± 0.05	0.77 ± 0.05	0.77 ± 0.05

Fig. 3 Percent time freezing in the training chamber during **a)** context test (absence of CS or US on day 2) and **b)** cue test (CS in a novel context on day 3). (ECON, $n = 10$; EKYN, $n = 12$ per age). Contextual fear appeared to be similar in juvenile rats and modestly, but not significantly, impaired in the adult rats. On day 3, during the critical cue test, EKYNs froze more than controls as juveniles, but less than controls as adults. *$P < 0.05$, **$P < 0.01$

Day 2: Context Test

Day 3: Cue Test
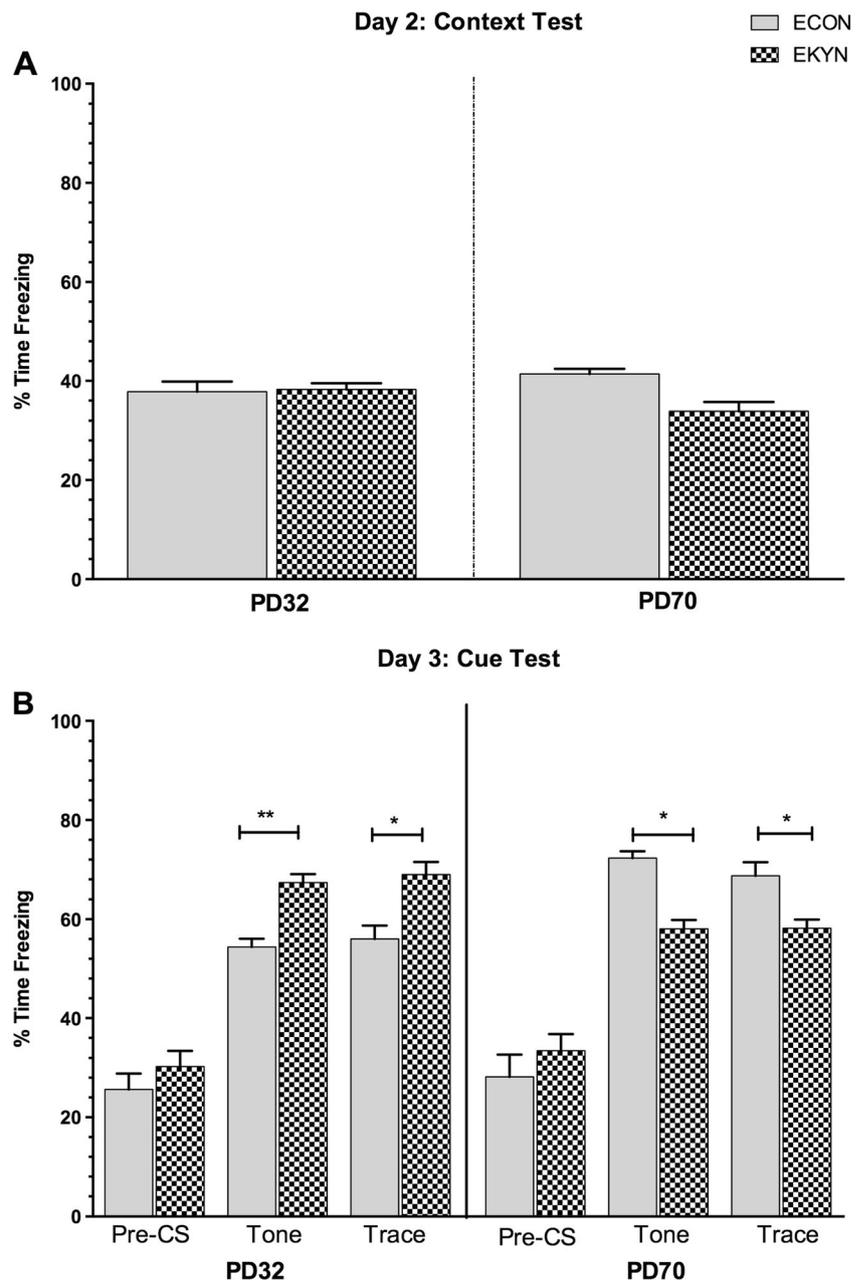
$t_{20} = 6.259$, $P = 0.021$) and trace interval (57 ± 4 % vs. 69 ± 4 %; $t_{20} = 4.414$, $P = 0.049$). This effect was not due to differences in baseline freezing in the novel context as all rats froze at roughly the same rate during the pre-tone period ($P \geq 0.228$).

Discussion

We demonstrated previously that the administration of KYNA's bioprecursor, kynurenine, to pregnant Wistar rats during the last week of gestation results in elevated brain KYNA and deficits in prefrontal- and hippocampal-mediated tasks in adulthood (Pershing et al., 2015; Pocivavsek et al. 2014). The present study was designed to expand on this work, and that of others (Forrest et al. 2015; Stone and Darlington 2013), by investigating whether prenatal exposure to kynurenine (from ED15 to 22) causes (1) *age-related* long-term changes in the gene expression of selected glutamatergic and nicotinic receptors, and/or (2) cognitive dysfunction. To that end, male ECON and EKYN rats were assessed as juveniles (PD32) or adults (PD70). We made several new observations. First, while we confirmed that prenatal kynurenine exposure causes a significant elevation in cerebral KYNA levels in adult rats (Pershing et al. 2015), no such effect was seen in juvenile animals. This indicates post-pubertal changes

in cerebral KP metabolism in EKYNs. Second, NR1 mRNA expression was substantially reduced (~50 %) in adult but not in juvenile EKYN rats. Third, NR2A mRNA expression was reduced by ~30 % in the brain of both juvenile and adult EKYN rats, leading to a lower NR2A/NR2B ratio at both ages. Finally, TFC test performance was diminished in adult EKYN rats but unexpectedly improved in juvenile EKYNs. The relevance of these findings, including the utility of prenatal kynurenine exposure as a translationally valid experimental approach for studying cognitive deficits in SZ and other major psychiatric diseases, is discussed below.

KYNA, an astrocyte-derived metabolite of the KP of tryptophan degradation, has been repeatedly postulated to contribute to the pathophysiology of SZ (for reviews see Erhardt et al. 2009; Hashimoto 2014; Schwarcz et al. 2012). This hypothesis is not only based on the observation that KYNA levels are increased in the brain and the CSF of patients with SZ (Erhardt et al. 2001; Linderholm et al. 2012; Schwarcz et al. 2001) but also on the fact that KYNA is an antagonist of NMDA and α7nACh receptors, which are both critically involved in brain development and cognitive processes (see Introduction section). Notably, the pathogenesis of SZ is related to genetics as well as environmental influences early in life. Genes encoding the KP enzymes tryptophan 2,3-dioxygenase (TDO) (Miller et al. 2004) and kynurenine 3-monooxygenase (KMO) (Aoyama et al. 2006; Wonodi et al. 2011) are abnormal in SZ patients, KMO activity is reduced in the brain of people with SZ (Sathyasaikumar et al. 2011; Wonodi et al. 2011), and known risk factors such as maternal infections and stress acutely stimulate cerebral KP metabolism in the fetus (reviewed in Notarangelo and Pocivavsek 2016). Experimentally induced prenatal changes in cerebral KP metabolism, including—but not necessarily limited to—elevations of KYNA in the fetal brain, may therefore duplicate the effects of one or more of these etiological factors.

Using an identical prenatal kynurenine treatment paradigm as in the present study, we demonstrated previously that brain KYNA levels were substantially elevated on ED22 yet returned to normal levels in pups at PD2, i.e., shortly after kynurenine was removed from the dam's diet (Pershing et al. 2015; Pocivavsek et al. 2014). However, although all experimental animals received a regular diet after birth, adult EKYN rats had significantly higher cerebral KYNA levels than age-matched ECONs. We confirmed here that brain levels of KYNA were increased in adult EKYNs but did not find differences between EKYNs and ECONs at PD32. The fact that kynurenine levels in the blood, which can readily influence cerebral KYNA formation (Gal and Sherman 1978), were unchanged in EKYN rats at PD70 suggests that the enhanced production of KYNA in adult EKYNs was caused by slowly developing alterations in KP metabolism *within the brain* (Gramsbergen et al. 1997), which do not become effective until after puberty. Underlying mechanisms may involve hormonal changes, which are known to affect KP dynamics

(McGinty and Rose 1969), perhaps leading to a secondary reduction in KMO activity and thus shunting the pathway toward enhanced KYNA formation (Abdel-Tawab et al. 1975; Danesch et al. 1983, 1987; Gibney et al. 2014). Importantly, as the present study was performed in all male offspring, ongoing experiments are designed to investigate sex differences, which may further elucidate the role of hormones in modulating cerebral KP dynamics.

Both juvenile and adult rats underwent TFC, in which a 15-s tone CS was followed by an aversive foot shock US 10 s later (the trace interval). Freezing levels during the tone and trace interval varied between ECON juvenile and adult rats, consistent with prior reports of age-dependent changes in conditioned fear (Pattwell et al. 2012). Importantly, compared to age-matched ECONs, TFC retention deficits were seen in adult, but not in juvenile, EKYN rats. Freezing behavior after re-exposure to the training context was significantly impaired in adult EKYN rats, in line with prior studies that used early-life kynurenine treatment. For example, Bucci and colleagues showed that acute KYNA elevations during early postnatal development resulted in impaired context fear conditioning in adult rats (Akagbosu et al. 2012). Notably, these results are generally consistent with the human SZ literature, which describes a variety of context-mediated impairments, including context fear conditioning (Brebion et al. 2007; Hemsley 2005; Waters et al. 2004).

Based on freezing behavior in response to the tone CS and trace interval, treatment group differences were also seen during CS-alone test trials. Relative to ECON controls, EKYN rats froze significantly less as adults and significantly more as juveniles, i.e., treatment interacted with age at the time of testing. This age-treatment interaction, which may be informative with respect to the etiology of neuropsychiatric diseases, is probably caused by abnormal maturational changes in EKYN rats. Specifically, TFC deficits in *adult* EKYNs could be causally related to the substantial, approximately 3-fold, elevation in brain KYNA levels in these animals compared to ECONs. This increase may be sufficient to reduce the release of both glutamate (Konradsson-Geuken et al. 2009, 2010; Wu et al. 2010) and acetylcholine in the PFC, especially in response to mesolimbic stimulation (Pershing et al. 2015), and thus to antagonize NMDAR and α7nAChR function (Erhardt et al. 2009). Adult EKYN rats also showed an approximately 50 % reduction in the cortical expression of the NR1 subunit, which is ubiquitously expressed in all NMDA receptors and contains the glycine B site that recognizes KYNA as a ligand (Kessler et al. 1989), and a trend (−17 %) toward a reduction in the expression of α7nAChRs, another physiological target of KYNA (Albuquerque and Schwarcz 2013). Notably, adult EKYN rats also display a significant reduction in dendritic spines on prefrontal pyramidal neurons, which mediate excitatory cortical input (Pershing et al. 2015). Collectively, these effects indicate that glutamatergic and

cholinergic neurotransmission in the PFC is markedly compromised in adult EKYN animals. TFC depends on the prefrontal cortex and working memory in order to sustain the CS neural signal across the trace interval and become associated with the US (Gilmartin et al. 2014; Gilmartin and McEchron 2005). It is plausible that CS-evoked neuronal spiking cannot be effectively maintained during the trace interval in adult EKYN rats, resulting in a weaker CS-US fear memory and significantly diminished freezing during the CS-alone test trials.

In contrast to adult EKYNs, *juvenile* EKYN rats did not show elevated brain KYNA levels or a reduction in NR1 gene expression compared to age-matched controls. This may contribute to the lack of impairment in the TFC test. In fact, the juvenile EKYN rats unexpectedly performed *better* than juvenile ECON animals, i.e., they froze more when tested during the tone CS and trace interval (cf. Fig. 3). We speculate that this improved TFC test performance may be causally related to an altered NR2A/NR2B expression ratio. This hypothesis is supported by several observations. Thus, synaptic plasticity is influenced by several factors, including the NR2A/NR2B ratio (Shouval et al. 2002; Yashiro and Philpot 2008), such that a decrease in the ratio enhances the induction and maintenance of long-term potentiation (Cui et al. 2011; Xu et al. 2009). Consequently, the overexpression of NR2B in transgenic mice leads to facilitated synaptic plasticity and superior ability in various learning and memory tasks (Tang et al. 1999). Finally, relative to NR2A, NR2B-containing NMDA receptors gate more calcium due to longer channel open times (Erreger et al. 2005), prolonging the slow reverberating neural dynamics that are required for sustained spiking (following stimulus termination) and working memory (Wang et al. 2008, 2013). In line with these findings, infusion of a NR2B antagonist (Ro25-6981) into the medial PFC of rats blocks TFC but, importantly, does not influence context fear conditioning or delay fear conditioning, in which the CS and US overlap in time (Gilmartin et al. 2013). During TFC, the decrease in NR2A expression, resulting in a proportional increase in NR2B, could therefore maintain NR2B-dependent spiking following CS offset more reliably in juvenile EKYNs than in juvenile ECONs. This would result in stronger CS-US fear memory and, at the time of testing, significantly enhance freezing throughout the tone and trace interval. Support for this idea also comes from Forrest et al. (2013), who observed enhanced long-term potentiation on PD21 in the offspring of dams that were repeatedly treated with a KMO inhibitor during the last week of gestation.

Ongoing studies in our laboratories are designed to further define the irregular maturational changes, which account for the emergence of cognitive deficits in adult EKYN animals following an adolescent period of PFC remodeling, i.e., from about PD35 to PD45 (Flores-Barrera et al. 2014; King et al. 2014). Elaboration of these abnormal developmental mechanisms will be critical for understanding—and eventually interfering with—enhanced feed-forward inhibition of PFC synapses in adult EKYN rats, which is proposed to impede CS-evoked spiking across the trace interval, yielding a weakened CS-US associative memory and impaired TFC test performance.

In summary, the current study revealed that prenatal kynurenine exposure results in distinct pre- and postpubertal changes in KYNA levels, NMDAR gene expression, and cognitive function. In light of the crucial role of developmental processes in the etiology of SZ and other major neuropsychiatric disorders (Davis et al. 2016; O'Donnell 2011; Selemon and Zecevic 2015), the present demonstration of distinct age-dependent differences in TFC following prenatal kynurenine exposure is of substantial translational interest. Together with complementary recent studies from other laboratories (Akagbosu et al. 2012; Chess et al. 2009; DeAngeli et al. 2014; Erhardt et al. 2009; Kegel et al. 2014; Stone and Darlington 2013), our findings provide further stimulus for examining KP impairments before and during adolescence, and for studying their role in the emergence of cognitive dysfunctions in adulthood. Investigations currently in progress utilize a variety of pharmacological approaches to prevent cognitive deterioration in EKYN rats by appropriately timed interventions with agents that specifically interfere with KYNA neosynthesis or function (Notarangelo and Pocivavsek 2016; Wu et al. 2014).

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