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Positive modulation of α5 $GABA_A$ receptors in preadolescence prevents reduced locomotor response to amphetamine in adult female but not male rats prenatally exposed to lipopolysaccharide

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ABSTRACT

We previously demonstrated that lipopolysaccharide (LPS) administered intraperitoneally (i.p.) to pregnant Wistar rat dams, at embryonic days 15 and 16 (E15/16), induced a decrease of baseline locomotor activity and diminished reactivity to amphetamine in adult female offspring. In the present study we aimed to assess the duration of LPS-induced maternal immune activation (MIA) and investigate possible changes in levels of main neurotransmitters in fetal brain during MIA. We hypothesized that the observed behavioral changes may be linked with MIA-induced disturbance of prenatal GABAergic system development, especially with α5 $GABA_A$ receptors (α5$GABA_A$Rs), expression of which takes place between E14 and E17. Thereafter, we set to investigate if later potentiation of α5$GABA_A$Rs in offspring's preadolescence (from postnatal day 22–28) could prevent the deficit in locomotor reactivity to amphetamine observed in adulthood, at postnatal day P60. The elevation of IL-6 in amniotic fluid 6 h after LPS treatment (100 μg/kg, i.p.) at E15 was concurrent with a significant increase of GABA and decrease of glutamate concentration in fetal brain. Moreover, repeated administration of MP-III-022, a selective positive allosteric modulator of α5$GABA_A$Rs, at a dose (2 mg/kg daily, i.p.) derived from a separate pharmacokinetic study, prevented the LPS-induced decrease in locomotor reactivity to amphetamine (0.5 mg/kg, i.p.) in adult females. These results were not mirrored in the parallel set of experiments with male offspring from LPS-treated rats. The results suggest that pharmacological potentiation of α5$GABA_A$Rs activity in preadolescence may ameliorate at least some of adverse consequences of exposure to MIA *in utero*.

1. Introduction

Maternal immune activation (MIA) is one of the most investigated prenatal influences with potentially harmful effects on brain development. Epidemiological studies have linked the emergence of schizophrenia and several other neurodevelopmental disorders with an increased incidence of MIA caused by bacterial or viral infections during pregnancy (Brown and Derkits, 2010). MIA is suggested to lead to a disbalance between pro- and anti-inflammatory cytokines in the fetal brain, which may ultimately impact *in utero* neurodevelopment and result in sustained long-lasting alterations in postnatal brain (Meyer et al., 2009, 2011). Several animal models of prenatal exposure to MIA, designed to mimic behavioral symptoms and elucidate pathophysiological mechanisms underlying the disorders related to abnormal fetal brain development, differ with respect to the challenging agents and the time of exposure (Harvey and Boksa, 2012).

Lipopolysaccharide (LPS), a cell wall component of gram negative bacteria, induces febrile response and cytokine release when administered intraperitoneally to pregnant rodents. Due to a significant variability in LPS exposure protocols, a diversity of findings was reported, describing acute changes in fetal compartment, as well as behavioral symptoms and morphological alterations in offspring. Nevertheless, the changes observed in a number of studies on LPS-treated rodents are consistent with those seen in schizophrenia patients, including

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behavioral changes or postmortem findings in brain tissue (reviewed in Boksa, 2010). A growing body of evidence supports the concept that development of GABAergic system may be a major convergence point for both genetic and environmental susceptibility factors for schizophrenia, as reviewed by Schmidt and Mirnics (2015). Oskvig et al. (2012) showed that a substantial number of GABA-related genes are acutely dysregulated by LPS treatment at E15, such as genes for GAD (glutamate decarboxylase) 67 and GAD65, as well as those which expression enables the hippocampal and cortical migration (Dlx1, 2, 5, and 6) (Cobos et al., 2005). In line with reports on reduction in mRNA and protein for GAD67 (e.g. Benes et al., 2007) and reelin (e.g. Eastwood and Harrison, 2006) in various brain regions, including the hippocampus, of schizophrenia subjects, Nouel et al. (2012) found that LPS (100 μg/kg) treatment of pregnant rat dams at embryonic day (E) 15 and 16 led to a reduction of GAD67-immunoreactive and reelin-immunoreactive hippocampal neurons in preadolescent rat offspring. Moreover, an identical protocol of exposure to LPS resulted in a decreased prepulse inhibition of acoustic startle in adult offspring, indicating the deficits in sensorimotor gating, which is a finding consistent with schizophrenia (Fortier et al., 2007). Nouel et al. (2012) suggested that the changes in prepulse inhibition, as a form of behavior partially modulated by the hippocampus, may be influenced by alterations in levels of GABA or reelin, as key neurochemical components of hippocampal function. The postnatal persistence of developmental MIA-induced changes in GABAergic system is still to be unequivocally assessed. For example, in the hippocampus, LPS treatment on E15/16 has not only immediate effects on dentate cells, but also results in a decreased proliferation and survival of dentate cells generated at postnatal day (P) 14, whereas adult hippocampal neurogenesis at P60 was not affected (Cui et al., 2009).

We have previously shown that LPS (100 μg/kg) administered to pregnant Wistar dams at E15/16 resulted in a significantly diminished baseline locomotor activity and reduced locomotor response to amphetamine in adult female offspring (P60), while the changes in adult males were less pronounced (Batinić et al., 2016); the reduction in spontaneous and amphetamine-induced locomotor activity was also observed in studies using the similar protocol of LPS exposure in rats (Harvey and Boksa, 2014a, 2014b). Importantly, the time of LPS treatment in our protocol (E15/16) correlates with the emergence of α5 $GABA_A$ receptors (α5$GABA_A$Rs) and migration of interneurons (Lauri et al., 1992). In the period between E14 and E17, the α5 subunit of $GABA_A$ receptor is the only one among all six α subunits that is in the process of expression in important brain structures: fetal cortex, fetal thalamus and fetal hippocampus (Lauri et al., 1992). Thus, we hypothesized that behavioral alterations observed in our model may at least partly derive from LPS-mediated disturbance of α5$GABA_A$Rs function, possibly occurring in the developing brain at E15/16. We aimed to investigate whether the onset of behavioral symptoms in adulthood may be affected by preadolescent modulation of α5$GABA_A$Rs, accomplished after GABA excitatory/inhibitory shift (Ben-Ari, 2002) and relocalization from the cell bodies to the dendritic layers have taken place (Ramos et al., 2004). To potentiate the activity of α5$GABA_A$Rs in the settings of decreased GABA-ergic neurotransmission demonstrated in the hippocampus of LPS-exposed offspring at P14, and especially P28 (Nouel et al., 2012), we repeatedly administered MP-III-022, a thoroughly profiled selective positive allosteric modulator (PAM) (Stamenić et al., 2016), during the fourth week of postnatal development.

2. Materials and methods

2.1. Animals

Male and female Wistar rats were obtained from Military Farm (Belgrade, Serbia) at the age of two months, weighting 180–220 g, and were housed separately in male and female animal rooms. The rats were housed in transparent plastic cages and had free access to food pellets and tap water. The temperature of the animal room was 22 ± 1°C, the relative humidity 40–70%, the illumination 120 Lx, and the 12/12 h light/dark period (light on at 6:00 h). All handling and testing took place during the light phase of the diurnal cycle. All procedures in the study conformed to EEC Directive 86/609 and were approved by the Ethical Committee on Animal Experimentation of the Faculty of Pharmacy in Belgrade.

2.2. LPS treatment

The procedure of handling and treatment of animals was replicated from our previous study (Batinic et al., 2016). After joining a male and female rat in a single cage, vaginal smears were taken daily at 9 am and if found positive for spermatozoa females were separated from the males. To avoid a social isolation context, pregnant dams were housed three per cage until gestation day 19 and then individually until delivery. On gestation (embryonic) days 15 and 16 (E15/16) pregnant dams were treated intraperitoneally either with LPS (from Escherichia coli, serotype 0111:B4, Sigma L2630) at a dose of 100 g/kg per day, or with 0.9% saline (2 mL/kg per day). After delivery, offspring were left with their mothers undisturbed until postnatal day 21, and then were weaned, separated by gender and housed up to five and not less than three per cage. The pups from different litters were not mixed. In the further text, offspring rats born to dams treated with LPS will be referred to as LPS offspring or LPS males or LPS females.

2.3. Cytokine determination

For the determination of cytokines at both E15 and E16, pregnant dams of separate cohorts were sacrificed 2 h and 6 h after LPS or SAL treatment, as suggested by Bell et al. (2004). The following samples were harvested: dam's blood, placenta, amniotic fluid, and fetal brain. To determine possible cytokine induction in adult offspring of both genders, their blood and brains were taken at P60. Blood was collected in heparinized tubes, centrifuged at 1000*g* for 10 min and plasma was collected. The placenta and brain tissue samples were placed in tubes with buffer (50 mM Tris-HCl, 0,6 M NaCl, 0,2% Triton X-100, 0,5% BSA and protease inhibitors), then homogenized and centrifuged at 14000*g* at 4 °C, and supernatants were collected. Plasma, amniotic fluid and supernatants were stored at − 80 °C until analysis. Cytokine assays using enzyme-linked immunosorbent assay (ELISA) kits (Rat TNF-alpha ELISA Ready-SET-Go!®, eBioscence, USA and IL-6 ELISA Kit, Rat, Life Technologies, USA) were processed according to the manufacturer's instructions. The detection limits were 16 pg/mL for TNF-α and 23.5 pg/mL for IL-6.

2.4. Neurotransmiters quantification

Five neurotransmitters: glutamate (GLU), gamma-Aminobutyric acid (GABA), dopamine (DA), noradrenaline (NA) and serotonin (5-HT) were determined using the protocol described in Huang et al. (2014) with additional determination of acetylcholine (Ach), in fetal brain in total and selected parts of adult brain. Calibration solutions for acetylcholine were the same concentration range as for dopamine, noradrenaline and serotonin.

Whole fetal brains were collected 2 h and 6 h after LPS or SAL treatment at E15 and E16, while adult brain tissues (prefrontal cortex, dorsal striatum, nucleus accumbens and ventral hippocampus) were sampled from experimentally naive rats decapitated at P60. Brains of adult animals were instantly removed from the skull, washed with ice-cold saline and put into the cold brain matrices (Zivic Instruments). Brain sections were cut according to Paxinos-Watson atlas, and removed from the matrix onto ice-cold pad to isolate the analyzed structures. The whole hippocampus was separated from the rest of its brain section and ventral two-fifths of the structure were cut. Samples

were immediately weighted, frozen in liquid nitrogen and afterwards collected and kept at −80 °C until analysis. Ice cold methanol (0.1% formic acid) was added in a ratio 1:12.5 (tissue (mg): methanol (μL)) and the sample was homogenized. A 100 μL of homogenate was diluted 3.2 × (in total, 40 × dilution) with 175.2 μL methanol (0.1% formic acid) and 44.8 μL of internal standard (30 μg/mL benzylamine). The samples were then vortexed and centrifuged at 18,000*g* for 10 min at 4 °C. The supernatant was evaporated to dryness under nitrogen stream. The residue was reconstituted with 350 μL of mobile phase (0.1% formic acid in water/acetonitrile, 98:2, v/v), and put into the LC–MS/MS system for analysis.

LC–MS/MS analyses were performed using an Accela LC system coupled to a triple-quadrupole mass spectrometer (Thermo Scientific TSQ Quantum Access Max, San Jose, CA, USA) with heated electrospray ionization (HESI). The optimized parameters of the mass analyzer's interface were as follows: positive-ion mode; spray voltage, 4000 V; vaporizer temperature, 325 °C; sheath gas (nitrogen) pressure, 30; aux gas (nitrogen) pressure, 10; ion sweep gas (nitrogen) pressure, 1.0; capillary temperature, 300 °C; scan time, 0.2 s; Q1 (FWHM), 0.70; collision gas (argon) pressure, 1.5 mTorr. Analytes were monitored by selected reaction monitoring (SRM) mode as follows: GABA *m/z* 104 → 87 (CE, 10 V), Ach *m/z* 146 → 87 (CE, 14 V), GLU *m/z* 148 → 84 (CE, 16 V), DA *m/z* 154 → 137 (CE, 8 V), NE *m/z* 170 → 152 (CE, 5 V), 5-HT 177 → 160 (CE, 8 V) and benzylamine *m/z* 108 → 91 (CE, 10 V).

2.5. Kinetics of repeated MP-III-022 administration

In our previous paper (Stamenić et al., 2016) we established that the 2.5 mg/kg (i.p.) dose of MP-III-022, given acutely, exerts selective potentiation of α5GABA$_A$Rs and produces no muscle relaxation, ataxia, sedation, or an influence on the anxiety level. To obtain kinetics of MP-III-022 (2.5 mg/kg) during 7 days of repeated administration, we used a separate cohort of pups, delivered from untreated Wistar dams. MP-III-022 (synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee) was suspended in a solvent (SOL) containing 85% distilled water, 14% propylene glycol and 1% Tween-80. We administrated MP-III-022 at a dose of 2.5 mg/kg (i.p.) once daily from P22 to P28, to both female and male pups. At five different time points (20 min after the 1st dose, 24 h after the 1st dose, 20 min after the 3rd dose, 20 min after the 7th dose, and 24 h after the 7th dose) we sacrificed 3 rats per gender, per time point. Rats were decapitated and brains were weighed, homogenized in 2 mL of methanol and centrifuged at 3400*g* for 20 min. To determine the concentration of MP-III-022 in supernatants of brain tissue homogenates, MP-III-022 was extracted from these samples by solid phase extraction, using Oasis HLB cartridges (Waters Corporation, Milford, Massachusetts). The procedure of sample preparation and determination of MP-III-022 by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) with Thermo Scientific Accela 600 UPLC system connected to a Thermo Scientific TSQ Quantum Access MAX triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, California), equipped with an electrospray ionization (ESI) source, has been already described in detail (Stamenić et al., 2016)

2.6. MP-III-022 administration

For postnatal treatment in the main experiment, pups were divided by gender (male or female), prenatal treatment (LPS or SAL) and postnatal treatment (MP-III-022 or SOL) into eight testing groups in total. After analyzing kinetics of MP-III-022 dosed at 2.5 mg/kg during 7 days of repeated intraperitoneal administration, we decided to use a 20% lower dose (2 mg/kg) due to observations commented in the results Section 3.3. MP-III-022 or SOL were repeatedly administered (5 mL/kg, i.p.) using 26 gauge needles, once daily for 7 days (P22 to P28), at the same time around 14:00 h. Each day before the treatment pups were weighted, and the average weight recorded was from around 25 g (P22) to 50 g (P28).

2.7. Baseline and amphetamine induced locomotor activity

As detailed in Batinić et al. (2016), locomotor activity testing was conducted in four blurred plexiglas chambers (40 × 25 × 35 cm) under dim red light (20 Lx) and tested animals weighted 180–220 g. Testing was performed in three consecutive trials. In the first trial, a single rat was placed in the apparatus for 30 min of habituation and baseline locomotor activity was recorded. This was followed by saline application (i.p.) and rat's activity was recorded in the second 30-min trial to examine whether injection stressor per se induced a locomotor response. Finally, the third trial started with an intraperitoneal administration of 0.5 mg/kg of amphetamine (Sigma-Aldrich) and locomotor activity was recorded for 60 min. Before testing the next group of animals olfactory traces were removed using diluted ethanol. Besides the total distance travelled, behavior was analyzed by dividing the locomotor activity data into 10-min bins. Behavior was recorded using camera mounted on the ceiling of the testing room and connected to a computer equipped with AnyMaze software (Stoelting Co.).

2.8. Experimental design

The design of the present study is graphically represented on an experimental timeline in Fig. 1.

2.9. Statistical analysis

Concentrations of each neurotransmitter in fetal brains were statistically analyzed using Student's *t*-test to compare samples from LPS- and SAL-treated dams, and separate analyses were done for each of four time points: 2 h and 6 h after treatment on both, E15 and E16. Brain concentrations of each neurotransmitter in a specific brain tissue at P60 were analyzed using two-way ANOVA with LPS and MP-III-022 as between-subject factors, separately for male and female rats.

Data from AILA testing obtained from male and female rats were analyzed separately, and values from 2 or 3 animals within a litter were averaged and a single value per litter was used in statistical analysis as suggested by Lazic and Essioux (2013). We used a three-way ANOVA with repeated measures (LPS and MP-III-022 treatments as two between-subject factors and time as within-subject factor). If significant ($p < 0.05$), the main effect was further analyzed using pairwise

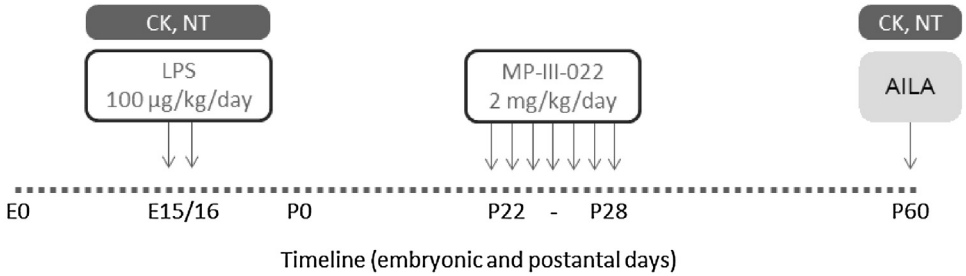

Fig. 1. Experimental design. Pregnant dams were treated with LPS or saline on embryonic days 15 and 16 (E15/16), while offspring were treated with MP-III-022 or solvent repeatedly from postnatal day 22–28 (P22-P28). Cytokine (CK) and neurotransmitter (NT) analysis were conducted on E15/16 and P60. Baseline and amphetamine induced locomotor activity (AILA) was recorded on P60.

Table 1
Concentrations of TNF-α and IL6 in dam's blood, placenta, amniotic fluid and fetal brain 2 h and 6 h after of LPS treatment on E15 and E16; after SAL treatment no detectable levels of cytokines were found in any sample analyzed. Samples were taken from 3 dams per treatment at each time point. Data are presented as mean value ± SD (pg/mL), except the values below the limit of detection (ND).

Cytokine	Tissue	Time point			
		E15 (2 h)	E15 (6 h)	E16 (2 h)	E16 (6 h)
TNF-α	Dam's blood	137.05 ± 71.12	17.22 ± 4.91	ND	ND
	Placenta	145.71 ± 63.08	33.36 ± 12.67	18.33 ± 6.73	ND
	Amniotic fluid	ND	ND	ND	ND
	Fetal brain	ND	ND	ND	ND
IL-6	Dam's blood	> 3000	2585.75 ± 243.71	723.33 ± 297.14	ND
	Amniotic fluid	ND	48.50 ± 65.48	ND	ND

multiple comparison procedures (Student–Newman–Keuls method). A trend denotes p values between 0.05 and 0.1. Data were processed using SPSS Statistics 20 (IBM).

3. Results

3.1. Cytokine determination in dams' and fetal tissues

Cytokine concentrations in samples analyzed 2 h or 6 h after LPS treatment on E15 and E16 are presented in Table 1. In the samples taken from dams treated with SAL, no detectable levels of cytokines were found. Unfortunately, in solid tissues (placenta and fetal brain) we were not unable to obtain interpretable results of IL-6 concentrations.

3.2. Neurotransmitter quantification in fetal rat brain

Fetal brain concentrations of all neurotransmitters are presented in Table 2. Twenty-four Student's *t*-test calculations in total were conducted to analyze concentrations of all six neurotransmitters at four time points; only reports bearing significant differences are presented in this section. The LPS treatment on E15 induced a significant increase of GABA concentration ($t(16) = 2.655$, $p = 0.017$) and a significant decrease in glutamate concentrations ($t(16) = -2.346$, $p = 0.032$) in fetal brain 6 h after treatment, in comparison to control tissue samples. Of note, a trend of decrease in glutamate concentration ($t(13) = -1.801$, $p = 0.095$) was found at the previous time point, 2 h after treatment on E15.

3.3. MP-III-022 kinetics

The total and estimated free brain concentrations of MP-III-022, dosed at 2.5 mg/kg and administered to pups during 1, 3 or 7 days from P22, are presented in Table 3. As the maximum estimated free brain concentrations obtained in the present kinetic study using a dose of 2.5 mg/kg were at the level of 100 nM, which is connected with a slight positive modulation of α2 and α3 $GABA_A$ receptors (Stamenić et al., 2016), we opted to use in the treatment protocol a 20% lower dose of MP-III-022 (2 mg/kg). Based on the elaborate analysis presented in Stamenić et al., 2016, and the current pharmacokinetic study in pups, this dose is expected to enable a protracted slight to moderate positive modulation of $GABA_A$ receptors containing the α5 subunit without a concomitant modulation of other receptor subtypes.

3.4. Baseline and amphetamine induced locomotor activity

The results of locomotor performance of female and male adult animals are presented, respectively, in Figs. 2 and 3. Six 3-way RM ANOVA calculations were done in total to analyze effects of LPS and MP-III-022 across time in each of three testing trials, separately for male and female rats. General description of significance of between-subject factors (LPS and MP-III-022) in respective analyses is presented in panel A, while the results of pairwise multiple comparison procedures are shown in graphs B–F of Figs. 2 and 3. The exact values of statistical analysis are presented in Supplementary Table S1.

Table 2
Concentrations of dopamine (DA), γ-aminobutyric acid (GABA), glutamate (Glu), acetylcholine (ACh), noradrenaline (NA) and serotonin (5-HT) in fetal brains. Tissues were sampled 2 h and 6 h after treatment of dams with LPS or SAL on E15 and E16. Data are presented as mean value ± SE (μg/g) and analyzed using Student's *t*-test. *($p < 0.05$) and ^{t}($p < 0.10$) relative to control at the given time point. Samples were taken from 3 dams per treatment at each time point.

Treatment/time point	Neurotransmitter					
	DA	GABA	GLU	ACH	NA	5-HT
LPS E15 (2 h)	0.14 ± 0.01	15.82 ± 0.71	543.88 ± 9.99 ^{t}	0.25 ± 0.10	10.12 ± 0.88	0.07 ± 0.00
SAL E15 (2 h)	0.14 ± 0.01	16.64 ± 0.66	575.75 ± 15.77	0.28 ± 0.08	9.90 ± 0.87	0.08 ± 0.01
LPS E15 (6 h)	0.11 ± 0.01	27.17 ± 3.49 *	567.04 ± 12.42*	0.12 ± 0.04	9.48 ± 0.50	0.06 ± 0.00
SAL E15 (6 h)	0.10 ± 0.01	13.66 ± 1.33	624.90 ± 24.87	0.15 ± 0.06	8.70 ± 0.71	0.06 ± 0.00
LPS E16 (2 h)	0.12 ± 0.01	15.36 ± 1.17	508.48 ± 27.77	0.24 ± 0.09	9.86 ± 0.79	0.06 ± 0.00
SAL E16 (2 h)	0.11 ± 0.01	14.64 ± 1.78	540.62 ± 12.33	0.21 ± 0.09	9.74 ± 0.86	0.05 ± 0.00
LPS E16 (6 h)	0.11 ± 0.00	16.74 ± 1.28	498.60 ± 13.69	0.19 ± 0.05	9.15 ± 0.49	0.06 ± 0.00
SAL E16 (6 h)	0.11 ± 0.01	14.44 ± 5.31	515.28 ± 30.79	0.28 ± 0.11	8.98 ± 0.87	0.06 ± 0.01

Table 3
Total and estimated free brain concentrations of MP-III-022, dosed at 2.5 mg/kg, administered to pups. Data are presented as mean ± SEM (nmol/kg), n = 3.

Time point	Total brain concentration		Estimated free brain concentration	
	Males	Females	Males	Females
20 min after 1 st dose	1408.64 ± 330.42	981.65 ± 285.25	93.11 ± 0.02	64.89 ± 0.02
24 h after 1 st dose	139.76 ± 70.27	38.95 ± 8.71	9.24 ± 0.01	2.57 ± 0.01
20 min after 3rd dose	1653.90 ± 696.19	517.77 ± 118.06	109.32 ± 0.05	34.22 ± 0.01
20 min after 7th dose	1318.81 ± 1119.20	345.53 ± 177.92	87.17 ± 0.07	22.84 ± 0.01
24 h after 7th dose	148.41 ± 8.55	216.03 ± 71.92	9.81 ± 0.01	14.28 ± 0.01

3.5. Cytokine determination in adult rat tissues

In blood and brain tissue samples of adult rats of both genders, prenatally treated with LPS or SAL, no detectable levels of TNF-α and IL-6 were found at P60.

3.6. Neurotransmitters quantification in adult rat brain

Forty-eight two-way ANOVA calculations were conducted in total to analyze effects of LPS and MP-III-022 on each of six neurotransmitters in each of four brain regions, separately for male and female rats at P60. Only reports bearing significant differences are presented in this section. In male rats the LPS treatment induced a significant increase in

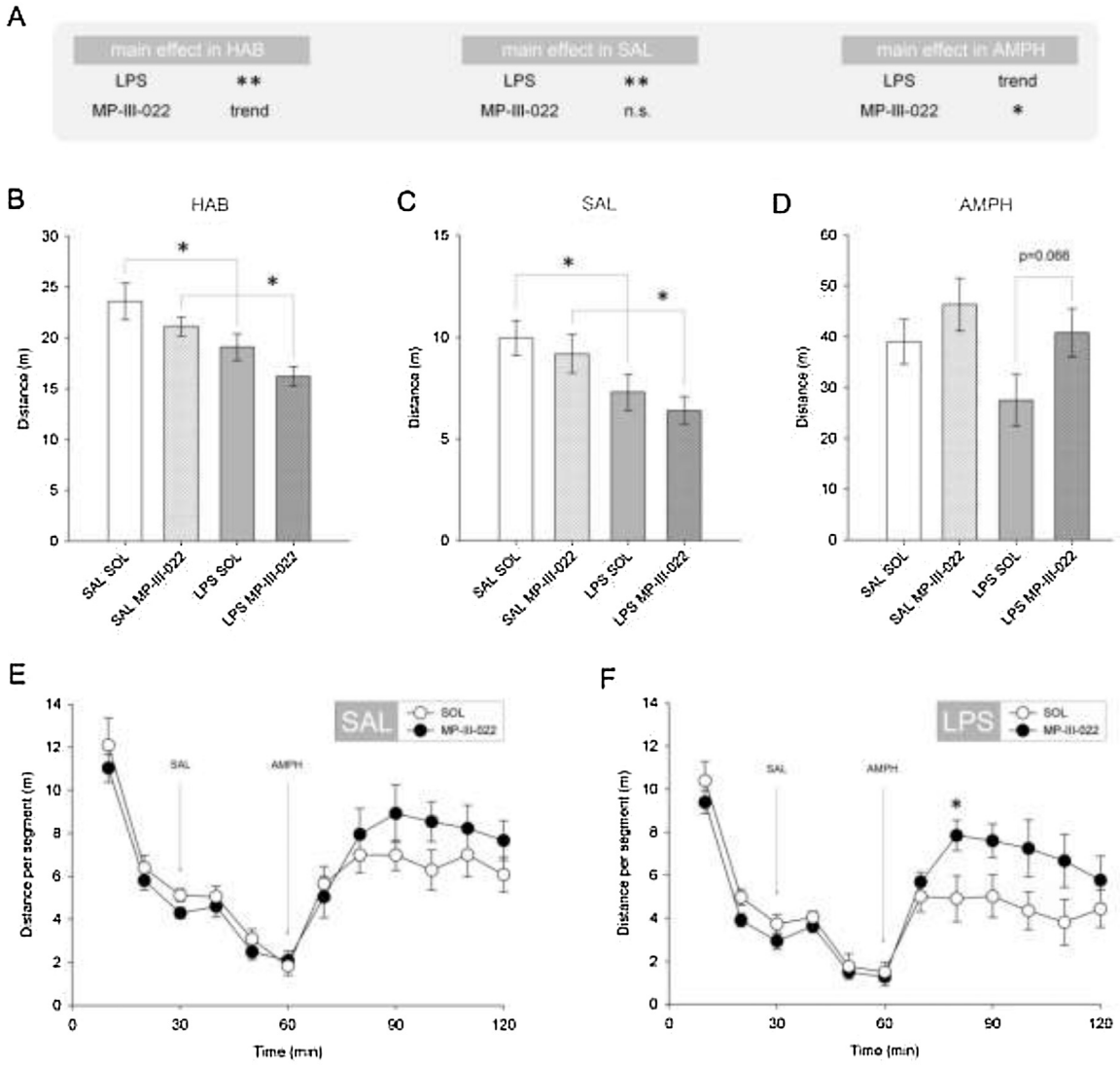

Fig. 2. Effects of prenatal LPS (E15/16) and postnatal MP-III-022 (P21-28) treatments on distance traveled by female Wistar offspring at P60. Activity was recorded during 30 min of habituation and baseline activity (HAB trial), then 30 min after saline challenge (SAL trial) and finally 60 min after amphetamine (0.5 mg/kg, i.p.) administration (AMPH trial). Panel A: the main effects of between-subject factors (LPS and MP-III-022) in each trial, as analyzed by three-way RM ANOVA. Graphs B, C and D: pairwise multiple comparison (Student–Newman–Keuls method) of LPS and MP-III-022 effects on total distance traveled in each of three trials (HAB, SAL and AMPH, respectively). Graphs E and F: effects of MP-III-022 on distance per time bin analyzed by pairwise multiple comparison procedures (Student–Newman–Keuls method) within SAL or LPS cohorts, respectively. Data (mean value ± SEM) are shown as total distance per trial (B, C and D) or distance traveled in 10 min bins (E and F). *($p < 0.05$) and **($p < 0.01$). A trend denotes p values between 0.05 and 0.1.

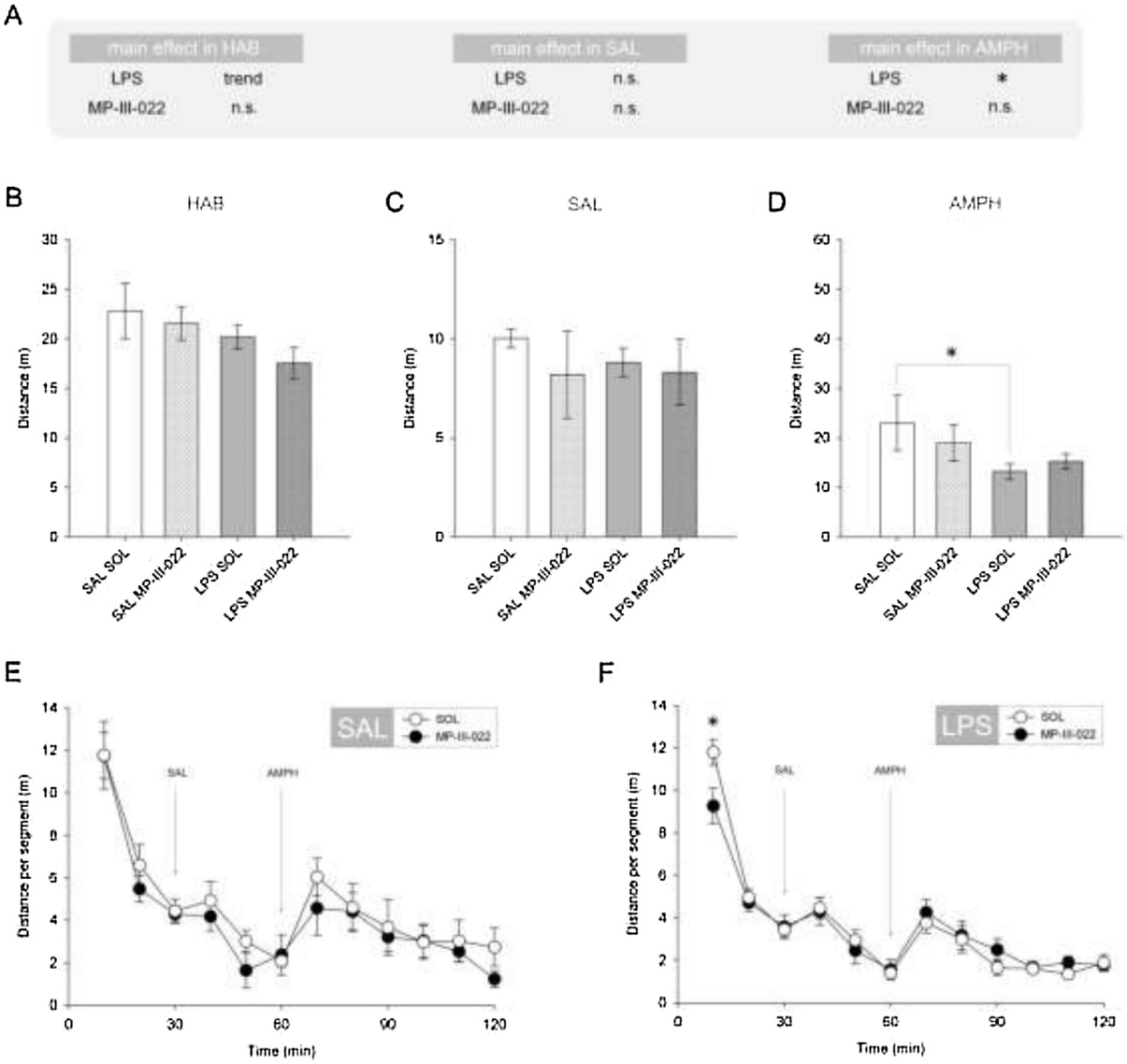

Fig. 3. Effects of prenatal LPS (E15/16) and postnatal MP-III-022 (P21-28) treatments on distance traveled by male Wistar offspring at P60. Activity was recorded during 30 min of habituation and baseline activity (HAB trial), then 30 min after saline challenge (SAL trial) and finally 60 min after amphetamine (0.5 mg/kg, i.p.) administration (AMPH trial). Panel A: the main effects of between-subject factors (LPS and MP-III-022) in each trial, as analyzed by three-way RM ANOVA. Graphs B, C and D: pairwise multiple comparison (Student–Newman–Keuls method) of LPS and MP-III-022 effects on total distance traveled in each of three trials (HAB, SAL and AMPH, respectively). Graphs E and F: effects of MP-III-022 on distance per time bin analyzed by pairwise multiple comparison procedures (Student–Newman–Keuls method) within SAL or LPS cohorts, respectively. Data (mean value ± SEM) are shown as total distance per trial (B, C and D) or distance traveled in 10 min bins (E and F). *($p < 0.05$). A trend denotes p values between 0.05 and 0.1.

ACh concentration in striatum ($F(18, 1) = 5.357$, $p = 0.033$), and an increase in glutamate concentration in ventral hippocampus ($F(18, 1) = 6.513$, $p = 0.020$). In female rats the MP-III-022 treatment induced a significant decrease in ACh concentration in ventral hippocampus ($F(20, 1) = 6.533$, $p = 0.019$). Concentrations of all neurotransmitters in specific brain regions and significant differences obtained in pairwise multiple comparison procedure (SNK method) are presented in Supplementary Table 2.

4. Discussion

This is the first study that reports the changes of neurotransmitter concentrations in rat fetal brain during LPS-induced MIA. We have shown that LPS treatment at E15 induced an acute GABAergic/glutamatergic imbalance in fetal brain, followed further by both, a decreased baseline locomotor activity and reactivity to amphetamine in adult offspring. Moreover, we report that repeated potentiation of $\alpha5GABA_ARs$ in preadolescence prevented the deficit in locomotor response to amphetamine in adult LPS females.

4.1. Cytokine induction and GABA/glutamate imbalance

In the present study, LPS-induced MIA was confirmed via detection of TNF-α and IL-6 release in dams' blood at the earliest time point after immune challenge (E15, 2 h). To our knowledge, none of the published studies on prenatal LPS exposure has assessed cytokine induction after the second LPS dose, which is routinely administered used in similar protocols. Here, the second LPS challenge, at E16, did not additionally induce these cytokines in dams' blood, and also, TNF-α elevation in the placental tissue detected at E15 diminished gradually during the next 24 h. While there was no TNF-α elevation in amniotic fluid or fetal brains at any time point, IL-6 concentration in amniotic fluid was increased 6 h after LPS challenge at E15; unfortunately, the data on IL-6 concentration in fetal brain was uninterpreTable Such differing findings on these two pro-inflammatory cytokines in amniotic fluid are not surprising given a study in the human placental perfusion model

showing that IL-6, but not TNF-α, exhibits substantial transfer to the fetal circulation (Zaretsky et al., 2004).

It should be underlined that increased levels of cytokines in amniotic fluid may as well derive from LPS-induced immune activation of the membrane that surrounds the fetus during gestation, as discussed by Gayle et al. (2004). Namely, the same dose of LPS (100 μg/kg, 0111:B4 serotype) administered to pregnant Sprague–Dawley dams, but at E18, induced an increase in TNF-α and IL-6 concentrations in dams' blood and amniotic fluid, and upregulated cytokine mRNA in both placenta and chorioamnion, but not in the fetal brain (Gayle et al., 2004). In line with the latter finding, TNF-α and IL-6 concentrations in fetal brain were not significantly changed 4 h after treatment with 50 μg/kg of LPS (0111:B4 serotype) to Sprague–Dawley dams at E18 (Ashdown et al., 2006). However, in a study with another LPS serotype (05:B55, from *Escherichia coli*), administered to Sprague–Dawley dams at E15 at a significantly higher dose (250 μg/kg), both TNF-α and IL-6 were detected in fetal brains and amniotic fluid after 4 h, but their corresponding concentrations in dams' blood were more than 100-fold (for TNF-α) or 5-fold (for IL-6) higher compared to our present report (Oskvig et al., 2012). Thus, in our settings of less vigorous immune activation, an increase of IL-6 in fetal brain around the second time point at E15 cannot be excluded, especially having in mind that the permeability of rat placental barrier to IL-6 is expected to be higher at E15 than at E18 (Dahlgren et al., 2006).

Although we found no direct support for acute fetal neuroinflammation, the surge of IL-6 in amniotic fluid occurred concurrently with a significant GABAergic/glutamatergic imbalance in fetal brains. If we put aside the probable involvement of other cytokines, and assume that fetal brain was exposed to an increased concentration of IL-6 for a short time, we may hypothesize that IL-6 elevation upregulated the expression of neurotrophin-3 (März et al., 1999), which in turn was reported to increase activity of GAD67, an enzyme that converts glutamate to GABA (Pérez-Navarro et al., 1999). In the current experiment, GABA concentration in fetal brain reached values twice higher in comparison to control tissue, while glutamate concentration at the same time point (E15, 6 h) was significantly decreased, following a statistical trend in concentration decrease observed as early as 2 h after the LPS challenge. No additional imbalances of other main neurotransmitters in fetal brain were discovered, and concentrations of GABA and glutamate were not affected after the second LPS challenge at E16.

Bearing in mind that rat fetal brain develops at a very high rate, due to the short gestation period, even a few hours of GABA/glutamate imbalance at E15 might have been sufficient to protractedly affect numerous regulatory processes in the developing GABAergic system. Here, attention should be given to α5GABA$_A$R emergence in the developing hippocampus, which occurs at the very time of MIA onset and GABA imbalance in the present study; namely, at E14 α5GABA$_A$Rs are absent, while by E17 they become abundant in hippocampus and fetal cortex (Laurie et al., 1992). Interestingly, in a different LPS challenge protocol, among 3285 genes dysregulated just 4 h after LPS treatment, Oskvig et al. (2012) observed a significant down-regulation of genes for both GAD67 and GAD65. While this finding may be seen as at odds with a significant embryonic GABA increase and glutamate decrease revealed in our present study, one must notice major divergences between these two MIA models. The differences in the rat strain, LPS serotype and mostly LPS dosage regimen used may have resulted in a full-blown immune response in the study by Oskvig et al. (2012).

Additionally, in the current study we looked for possible changes in levels of analyzed cytokines and neurotransmitters in adult offspring at P60. An absence of detectable concentrations of TNF-α and IL-6 in adult brain or blood suggests that our LPS exposed offspring did not develop a persistent inflammation, possibility of which was discussed in Meyer et al. (2011). For comparison, increased cytokines in blood were detected in adult LPS exposed animals, but in protocols substantially different from ours, which involved subcutaneous treatment with a 2 mg/kg dose of LPS (serotype 026:B6) daily (Romero et al., 2010), or 1 mg/kg dose of LPS (serotype 026:B6) on alternate days (Borrell et al., 2002) during the whole period of rat pregnancy.

In the present experiment, a significant increase of glutamate in ventral hippocampus and acetylcholine in dorsal striatum was detected in LPS males; it would by highly speculative to try and answer how these findings may have affected the behavioral output in the used behavioral paradigm. Nonetheless, a significant decrease of ACh detected in the ventral hippocampus of MP-III-022-treated females supports at least partly the revealed tendency of decreased locomotor activity in the habituation trial, considering that ACh release in the ventral hippocampus correlates with an increased exploratory activity (Bianchi et al., 2003).

4.2. Behavioral effects of LPS

Our previous study showed that LPS treatment (100 μg/kg) at E15/16 resulted in a significant decrease of baseline locomotor activity as well as reactivity to amphetamine (0.5 mg/kg) in Wistar female offspring at P60 (Batinić et al., 2016). Hereby, in an elaborate design, involving a repeated administration during the fourth postnatal week of the selected PAM at α5GABA$_A$Rs or the corresponding solvent, we replicated a significant decrease of distance traveled in the habituation and saline trials in the LPS-females, while the hyporeactivity to amphetamine reached a statistical trend in comparison to SAL females. While concordant to each other, these two sets of our data are apparently at odds with reports from two other studies which were also conducted on Wistar rats and used LPS exposure protocol identical to ours (Wischhof et al., 2015a, 2015b). In one study authors reported an increased baseline locomotion in LPS females at P60, relative to controls (Wischhof et al., 2015a), while in the other study no difference in distance travelled between LPS and control females (P100-120) was observed (Wischhof et al., 2015b). However, it needs to be emphasized that these authors have reported a substantially higher overall activity, at the levels of up to 35 m during the first 5 min, and up to 200 m during 60 min testing (Wischhof et al., 2015a), or around 350 m in just 30 min (Wischhof et al., 2015b); such oversized and inconsistent values impede any comparison with the current results. On the other hand, in a study conducted in 60 day old Sprague Dawley rats born to dams treated with a 50 μg/kg dose of LPS at E15/16, a non-significant decrease of baseline locomotion in LPS females during 60 min of recording was observed, with absolute levels of activity corresponding well to our data (Harvey and Boksa, 2014a).

Our previous findings on locomotor activity of adult males (without any treatment in preadolescence), indicated a consistently lower locomotor activity of LPS vs. SAL rats in every time bin of each trial, with a significant decrease obtained in the second trial, after saline injection stressor (Batinić et al., 2015). In the current study, locomotor activity of LPS vs. SAL rats was significantly decreased in the third (amphetamine) trial, while a trend of hypolocomotion was observed in the first (habituation) trial. The present set of results corresponds well with a significant reduction of baseline locomotion in adult, 60 days old Sprague-Dawley males born to dams treated with 50 μg/kg of LPS on E15/16 (Harvey and Boksa, 2014a). Moreover, the same authors reported a decreased locomotor reactivity of 70 day old LPS males during the first 30 min after amphetamine (2 mg/kg) administration (Harvey and Boksa, 2014b). Apart from these corresponding results, Wischhof et al. (2015a, 2015b) reported no difference in baseline locomotion in their studies in male rats; however, the above-discussed inconsistency of absolute distance values applies to data obtained in males as well.

The detailed observations on behavioral effects of prenatal LPS admnistration in our MIA model may be summarized in a conclusion that both female and male offspring prenatally treated with 100 μg/kg of LPS at E15/16 manifest analogous deficits in baseline locomotor activity and reactivity to amphetamine at their young adult age, and that a matching deficit can be observed after challenge with 50 μg/kg of LPS at E15/16, at least in male Sprague Dawley rats (Harvey and Boksa,

2014a, 2014b).

4.3. Behavioral effects of positive modulation at α5GABA$_A$ receptors

According to the analysis of pharmacokinetic and electrophysiological data presented in Stamenić et al. (2016), the ligand MP-III-022 at doses 1–10 mg/kg, which correspond to mild via moderate to strong potentiation at α5GABAARs not accompanied by any activity at other subtypes of benzodiazepine-sensitive GABAARs, was devoid of ataxic or sedative action, as assessed in the rotarod and spontaneous locomotor activity test in rats, respectively. The results of our current pharmacokinetic study in preadolescent rats have helped to choose the dose of 2 mg/kg daily as appropriately selective for one-week administration in young rats. The major novelty we report here is that a repeated potentiation of α5GABA$_A$Rs in early rat adolescence, from postnatal day 22–28, prevented the later emergence of a deficit in locomotor response to amphetamine in LPS female offspring at P60. Otherwise, the hint of such hyporeactivity to amphetamine can be traced back to the locomotor assay conducted at P40 in our previous study (Batinić et al., 2015). Additionally, after the emergence of GABA$_A$α5Rs in fetal brain between E14 and E17 (Laurie et al., 1992), the second critical period in their development can be broadly connected with postnatal day 10 and 30 (Yu et al., 2006). Following that observation we assumed that selective modulation of α5GABA$_A$Rs activity should take place before the period of adolescence.

The observed gender difference in the relative role of selective modulation of α5GABA$_A$Rs is a novel, but not an unexpected finding; importantly, a number of gender differences in behavioral and electrophysiological parameters related to the hippocampus have been described (Monfort et al., 2015). Moreover, it has been demonstrated that gender affects persistent neuroinflammation, with GABA$_A$ receptors described as less active in females in these settings (Tonsfeldt et al., 2016), which may be an indirect explanation for more useful consequences of positive modulation of α5GABA$_A$Rs in females than males seen in the present study. Finally, it was recently found that both, single and protracted treatment with another ligand selective for α5GABA$_A$Rs, structurally similar to, but less receptor subtype-selective than MP-III-022, can produce a pattern of decreased stress-induced behaviors across a battery of tests in female, but not in male mice exposed to unpredictable chronic mild stress (Piantadosi et al., 2016). However, for a more detailed conclusion in the current model, one would need to perform studies of gene expression of α5GABA$_A$Rs and the electrophysiological activity of these receptors ex vivo.

4.4. Conclusion

Prenatal exposure to LPS is a common model of MIA with relevance to schizophrenia and/or autism onset (Harvey and Boksa, 2012; Kirsten et al., 2013). In contrast to the protocols of repeated exposure to the effects of high LPS doses during the whole pregnancy, which resulted in a prolonged postnatal inflammation in offspring (Borrell et al., 2002; Romero et al., 2010), the present exposure to a low-to-moderate dose of LPS resulted in one-day MIA with a delayed occurrence of behavioral abnormalities, proving it as a translationally-realistic and replicable neurodevelopmental model. Clinical trials of various psychotropic drugs in adolescent subjects with schizophrenia prodrome (e.g. Cornblatt et al., 2007) have prioritized the need for animal models which enable investigation of early interventions and preventive treatments (Meyer et al., 2010). Preclinical studies that introduced the concept of peri-adolescent preventive treatment in prenatal PolyI:C model of immune activation have reported that treatment with antipsychotic and/or antidepressant drugs successfully blocked some of schizophrenia-related abnormalities (Meyer et al., 2010; Piontkewitz et al., 2009). Within the research focusing on the role of GABA-ergic system in the schizophrenia development (Schmidt and Mirnics, 2015), the present finding on amelioration of at least some of adverse consequences of MIA by pharmacological modulation of α5GABA$_A$Rs activity in preadolescence may indicate a new target in the strategy of preventive treatments.

Conflict of interest

We declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijdevneu.2017.06.001.

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