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Age-dependent effects of (+)-MK801 treatment on glutamate release and metabolism in the rat medial prefrontal cortex

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A B S T R A C T

NMDAR antagonist treatments in adolescent/young adult rodents are associated with augmented glutamate (Glu) release and perturbed Glu/glutamine (Gln) metabolism in the medial prefrontal cortex (mPFC) resembling those found in first-episode schizophrenia. Few studies, however, investigated NMDAR antagonist-induced changes in the adult mPFC and whether there is an age-dependence to this end. In this study, the effects of acute/repeated (+)-MK801 treatment on Glu release/metabolism were measured in the mPFC of male adolescent (postnatal day 30) and adult (14 weeks) rats. Acute (+)-MK801 treatment at 0.5 mg/kg body weight induced an approximately 4-fold increase of extracellular Glu concentration in the adolescent rats, and repeated treatment for 6 consecutive days significantly increased the levels of Glu + Gln (Glx) and glial metabolites 7 days after the last dose. Histologically (+)-MK801 treatments induced reactive astrocytosis and elevated oxidative stress in the mPFC of adolescent rats, without causing evident neuronal degeneration in the region. All (+)-MK801-induced changes observed in the mPFC of adolescent rats were not present or evident in the adult rats, suggesting that the treatments might have caused less disinhibition in the adult mPFC than in the adolescent mPFC. In conclusion, the effects of (+)-MK801 treatments on the Glu release/metabolism in the mPFC were found to be age-dependent; and the adult mPFC is likely equipped with more robust neurobiological mechanisms to preserve excitatory-inhibitory balance in response to NMDAR hypofunction.

1. Introduction

Acute/repeated treatment of N-methyl-D-aspartate receptor (NMDAR) antagonists, including dizocilpine (MK801), ketamine and phencyclidine (PCP), are frequently used animal models to study the roles of NMDAR hypofunction in the emergence of schizophrenia (SZ)-related symptoms/behaviors (Cadinu et al., 2018; Frohlich and Van Horn, 2014). Most of these studies used animals with ages around postnatal day (PND) 60 (i.e., body weights of 250–300 g for rats and 25–30 g for mice), when the animals have just attained sexual maturation (i.e., gonadarche). PND 60 in rodents is equivalent roughly to 16 years of age in human (Dutta and Sengupta, 2016; Markham et al., 2013). The majority of these studies focused on the medial prefrontal cortex (mPFC), mainly due to its protracted maturation and involvement in the early emergence of psychiatric disorders (Cass et al., 2013; Gilmartin et al., 2013; Hoftman and Lewis, 2011; Konstantoudaki et al., 2018). It is well-established that NMDARs play important roles in the plasticity and maturation of mPFC (Flores-Barrera et al., 2014; Thomases et al., 2013, 2014).

The human prefrontal cortex (PFC) is known to reach full maturation at an age around 22–25 years old (Cohen et al., 2016; Tunbridge et al., 2007). In rodents, many developmental processes in the mPFC are still in progress after PND 60, including remodeling of the local neural circuit and changes in the connectivity with subcortical structures (Caballero et al., 2016; Caballero and Tseng, 2016; Insel, 2010). Surprisingly, few previous studies have investigated the effects of NMDAR antagonists on the brain of fully matured (i.e., beyond PND 90) animals (Zhang et al., 2008). It is less clear how the fully matured mPFC responds to acute/chronic NMDAR hypofunction, and whether the responses are significantly different from those observed in the mPFC that is still in development.

One of the most documented responses of the adolescent/young adult mPFC to acute NMDAR antagonist treatment is enhanced release of excitatory neurotransmitter glutamate (Glu) (Amitai et al., 2012; Chan et al., 2008; Lefevre et al., 2016; Lorrain et al., 2003; Pietraszek et al., 2009; Zuo et al., 2006), likely mediated by a disinhibition-related mechanism (Jackson et al., 2004; Liu et al., 2017; Rujescu et al., 2006). Acute NMDAR antagonist treatments at doses that induce SZ-like phenotypes also caused significant increases of cortical glutamine (Gln) level observable to *in vivo/ex vivo* ^{1}H-magnetic resonance spectroscopy (MRS) (Brenner et al., 2005; Iltis et al., 2009; Napolitano et al., 2014). Repeated NMDAR antagonist treatment decreased baseline

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extracellular Glu level in the mPFC (Murai et al., 2007; Zuo et al., 2006), and resulted in dose-dependent (i.e., 0.1–0.7 mg/kg body weight (+)-MK801) changes of Glu level in the frontal and parietal lobes (Eyjolfsson et al., 2006; Kondziella et al., 2006; Sun et al., 2013a). It is generally believed that NMDAR antagonist-augmented Glu release underpins the metabolic perturbations observed in repeat treatment models (Kim et al., 2011; Kondziella et al., 2006).

In this study, we aimed to compare the effects of acute/repeated MK801 treatment on Glu release/metabolism in the mPFC of adolescent (PND 30) and adult (14 weeks) male rats. These ages of the rats were selected, as they corresponded roughly to 13 and 22 years old in humans, respectively (Dutta and Sengupta, 2016; Markham et al., 2013). MK801 was chosen because it is a strictly selective NMDA receptor antagonist, while ketamine and PCP have affinity not only for NMDA receptors but also for other receptors (e.g., D_2 receptors and 5-HT_2 receptors) (Kapur and Seeman, 2002). MK801 has two stereoisomers (+)-MK801 and (−)-MK801, with the latter being only one-seventh as potent as the former at the NMDA receptors (Wong et al., 1986).

In vivo ^{1}H spectra were acquired from bilateral mPFC of the rats treated with (+)-MK801, (−)-MK801 or saline for 6 consecutive days at a daily dose of 0.5 mg/kg body weight/day. This treatment regimen in young adult rats had been shown to result in cortical metabolic abnormalities resembling those seen in drug-naive first-episode SZ patients (Kondziella et al., 2006). Histological/immunohistochemical assessments, including hematoxylin and eosin (HE), glial fibrillary acidic protein (GFAP) and dihydroethidium (DHE) staining, were performed. Western blotting was used to assess the expression of key proteins involved in glutamatergic neurotransimission and Glu transport/metabolism, including glutamate-aspartate transporter (GLAST), glutamate transporter-1 (GLT-1), glutamine synthetase (GS), metabotropic glutamate receptor 5 (mGLUR5) and postsynaptic density protein 95 (PSD95). Extracellular Glu concentration was measured in the mPFC with a time-resolved *in vivo* microdialysis technique before and after an acute (+)-MK801 injection at 0.5 mg/kg body weight.

2. Experiment procedures

2.1. Animals, treatments and experimental design

All animal protocols followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the institutional internal review board (approval #: WIPMA-20170801). Repeated treatment experiments and acute treatment experiments were performed at Wuhan and Beijing, respectively. All rats were housed in group of 2-3 per cage, and acclimated to the laboratory environment for 1 week before any experiments. Standard pellet food and tap water were available *ad libitum*. The animals were randomly assigned into the treatment groups and control groups. Saline solutions of (+)-MK801 or (−)-MK801 (Cat# M107/M108, Sigma, St. Louis, MO) were prepared at a concentration of 0.25 mg/ml.

In the repeated treatment experiments (Fig. 1A), pregnant Sprague-Dawley (SD) rats at 15–17 days of gestation and adult male SD rats (91–98 days old) were purchased from Hunan SJA Laboratory Animal Company (Changsha, China). Male offsprings born to the pregnant rats were weaned at PND 21, and subjected to drug treatment at PND 30 (i.e., adolescence groups). The rats in the treatment groups received daily intraperitoneal injections of (+)-MK801 or (−)-MK801 solution at 0.5 mg/kg body weight for 6 consecutive days, while those in the control groups were injected with the same amount of saline. Two separated cohorts of rats were subjected to *in vivo* ^{1}H-MRS measurements and decapitated for Western blotting measurements, respectively, 7 days after the last MK801/saline injection (Table 1). For the *in vivo* ^{1}H-MRS cohort, randomly selected rats were decapitated after the spectroscopy session, among which three from each group were used for HE staining, and the other three for GFAP and DHE staining. For the Western blotting cohort, the expression levels of GLAST, GLT-1, GS, PSD95 and mGLUR5 in the mPFC were measured semi-quantitatively.

In the acute treatment experiments (Fig. 1B), adolescent (n = 11, 21–28 days old, 158 ± 12 g) and adult SD rats (n = 11, 91–98 days old, 397 ± 14 g) were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China). The extracellular Glu levels in the mPFC were measured before and after a single dose of (+)-MK801 at 0.5 mg/kg body weight with the *in vivo* microdialysis technique. Given that the half-time of (+)-MK801 in rat brain was about 2 h (Vezzani et al., 1989), all rats were decapitated 3.5 h after the (+)-MK801/saline injection and used for GFAP and DHE staining.

2.2. In vivo ^{1}H-MRS

In vivo ^{1}H-MRS experiments were performed on a 7.0 T/20 cm Bruker Biospec scanner (Ettlingen, Germany), with a 72 mm-diameter volume coil for radiofrequency (RF) pulse transmission and a 40 mm-diameter quadrature surface coil for signal detection. Anesthesia during the experiments was maintained with 1.8–2.5% isoflurane in pure oxygen delivered via a nose cone, with the respiratory rate and body temperature of the animals monitored continuously. The core body temperature was maintained at 37 ± 1 °C with a warm water circulation system. Localizer T_2-weighted anatomical images were acquired from 20 contiguous coronal slices covering the whole brain with a two-dimensional rapid acquisition with relaxation enhancement (RARE) sequence, field of view (FOV) 30 mm × 30 mm, matrix size 256 × 128, slice thickness 0.8 mm, repetition time (TR) 2764 ms, effective echo time (TE_{eff}) 40 ms, RARE factor 4 and number of averages 8.

In vivo ^{1}H-spectra were acquired from a voxel (2.5 mm × 2.4 mm × 2.0 mm) containing mainly bilateral mPFC with a point resolved spectroscopy (PRESS) sequence, TR/TE 4000/15 ms, spectral bandwidth 4 kHz, 2048 data points and 512 averages. Hyperbolic secant RF pulses with a bandwidth of 13,500 Hz were used for outer volume suppression with 5.0 mm-thick saturation slices 0.5 mm apart from the voxel. First- and second-order shim currents were adjusted using fast automatic shimming technique by mapping along projections (FASTMAP) (Gruetter, 1993). All unsuppressed water signals had a full width at half maximum less than 9 Hz. Variable power radiofrequency pulses with optimized relaxation delays (VAPOR) was used for water suppression (Tkáč et al., 1999).

All proton spectra were processed and analyzed with the LCModel software (version 6.3-1 A; Stephen Provencher Inc., Oakville, Canada). Eddy current, frequency and phase corrections were done automatically by the LCModel pipelines. The signals within the chemical shift range of 0.2-4.2 ppm were fitted as a superposition of a set of basis spectra coming with the LCModel package using a constrained regularization algorithm. Only the metabolites with Cramér-Rao lower bounds (CRLB) less than 20% in all spectra were selected for quantitative analysis, and included Glu, Gln, Glu + Gln (i.e., Glx), myo-inositol (Ins), taurine (Tau), glutathione (GSH), N-acetyl aspartate (NAA), total creatine (tCr) and total phosphocholine (tPC). Metabolite quantification was done using unsuppressed water signal as the internal reference, with an assumed water concentration of 43,300 mM/kg wet tissue weight.

2.3. Histological/immunohistological staining

Each rat was anesthetized by an intraperitoneal injection of chloral hydrate (350 mg/kg body weight), and perfused transcardially with 500 ml cold saline solution, followed by 250 ml 4% paraformaldehyde (PFA) dissolved in 0.01 M phosphate buffered saline (PBS). The brains were removed, postfixed in the same fixative overnight, and dehydrated in 20% and 30% sucrose solutions, respectively, at 4 °C for three days.

For HE staining, the brains were embedded in paraffin wax, and sectioned into 2.5 μm-thick coronal slices on a rotary microtome (RM 2016, Leica, Germany). For staining, the sections were dewaxed in xylene, rehydrated with ethanol solutions with decreasing concentrations, and washed in tap water. After staining with hematoxylin for

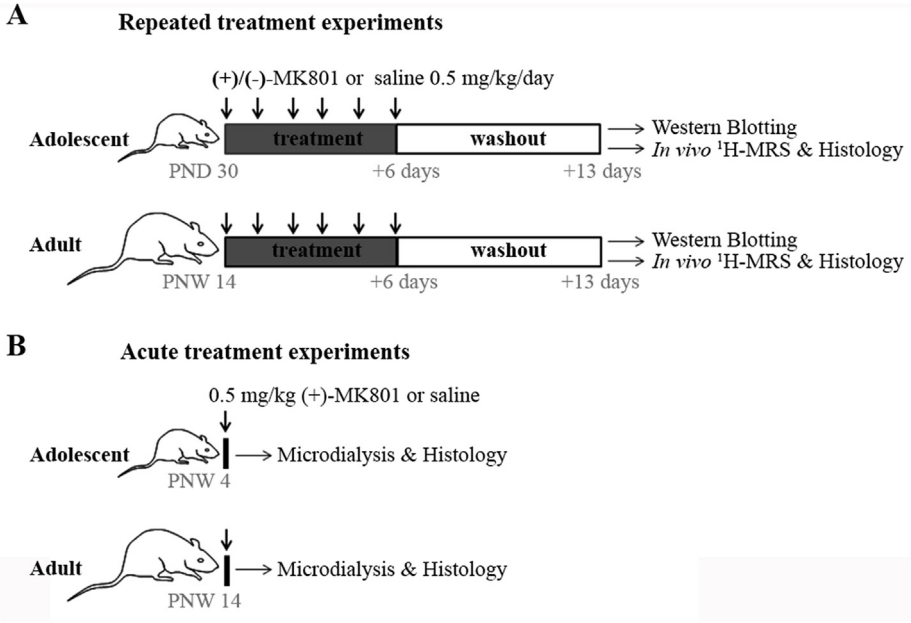

Fig. 1. Timelines of the repeated treatment experiments (A) and acute treatment experiments (B). MRS: magnetic resonance spectroscopy; PND: postnatal day; PNW: postnatal week.

Table 1
Animals used in the repeated treatment experiments.

Cohorts	Age group	Treatment	N	Body weight (g)	
				pre-treatment	7 days after treatment
In vivo ^{1}H-MRS cohort	Adolescent	(+)-MK801	13	117 ± 24	225 ± 27
		Saline	12	117 ± 23	243 ± 27
		(−)-MK801	17	110 ± 18	214 ± 23
		Saline	17	114 ± 15	224 ± 18
	Adult	(+)-MK801	13	519 ± 24	545 ± 33
		Saline	12	509 ± 25	561 ± 28
Western blotting cohort	Adolescent	(+)-MK801	5	108 ± 20	187 ± 26
		Saline	5	101 ± 18	188 ± 20
	Adult	(+)-MK801	5	510 ± 39	522 ± 41
		Saline	5	508 ± 18	538 ± 29

5 min, the sections were washed with tap water, dehydrated through ethanol solutions of increasing concentrations, and stained with eosin for 5 min. Finally, the sections were dehydrated, hyalinized in xylene and mounted.

For GFAP and DHE staining, the brains were sectioned into 30-μm-thick contiguous coronal slices on a freezing microtome (Leica Biosystems Inc., Wetzlar, Germany) and stored in a −20 °C refrigerator until use. For GFAP immunofluorescence staining, the brain sections were first rinsed with 0.01 M PBS for 5 min followed by incubation in a mixed solution of 0.5% Triton X-100 and 10% normal goat serum at room temperature for 60 min. The brain sections were then incubated with a mouse monoclonal anti-GFAP (1:400 dilution, Cat# G3893, Sigma) primary antibody at 4 °C overnight. After rinses with PBS, the brain sections were incubated with an FITC goat anti-mouse IgG secondary antibody (1:200 dilution, Lot MG141818, Thermo Fisher Scientific) at 37 °C for 90 min. For DHE staining, the brain slices were first rinsed with 0.01 M PBS for 5 min, followed by incubation in a DHE solution (0.05 mM, Cat# 37291, Sigma) in a dark chamber at 37 °C for 30 min. DHE is a superoxide-sensitive fluorescence dye frequently used as a marker for oxidative stress (Aoyama et al., 2008). After staining, the brain sections were washed with PBS, mounted, air-dried and coverslipped.

All stained brain sections were photographed on an Olympus BX-UCB microscope (Tokyo, Japan) under × 10 total magnification. The numbers of necrotic neurons (i.e., contracted soma, pyramidal in shape, bright red cytoplasm and pyknotic nuclei) and GFAP-positive cells with an astrocyte-like morphology, as well as the optical density of DHE staining, were quantified in the mPFC. Four observation fields were selected (size: 0.36 mm^2, two in each hemisphere) on each brain section, and the results from five non-consecutive brain sections (Bregma 4.68 to 2.52 mm) were averaged. The counts were reported as number of cells/mm^2. The Image-Pro Plus 7 (IPP7, http://www.mediacy.com/imageproplus) was used to measure the integrated optical density of DHE staining. Positioning of the observations fields and cell counting/optical density measurements were performed by an observer blinded to the treatment condition.

2.4. Western blotting

Brain tissues in the mPFC were dissected on ice and lysed in a cold lysis buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0) containing 1 mM phenylmethanesulfonyl fluoride (Beyotime, Cat# ST506) and a protease inhibitor cocktail (Roche, Cat# 4693132001). After centrifugation at 12,000 rpm/4 °C for 15 min, 200 μl supernatant was collected, mixed with 2 × Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl, pH 6.8), boiled for 10 min and irradiated with 60 Hz ultrasonic wave for 15 min. The proteins in the samples were first separated by Tris-HCl 10% polyacrylamide gels, and then transferred onto an Immobilon-P membrane (Merck Millipore, Darmstadt, Germany, Cat# IPVH00010) using a Mini-PROTEIN® Tetra System (Bio-Rad Laboratories, Berkeley, CA). After blocking the membranes with 5% non-fat milk in a Tris-buffered saline added with 0.1% Tween 20 (TBST) at room temperature for 1 h, the membranes were incubated with rabbit anti-glyceraldehyde phosphate dehydrogenase (GAPDH) (1:20000 dilution, Cat# ABS16, Milipore), rabbit anti-GLAST (1:2000 dilution, Cat# PA5-80012, Invitrogen), rabbit anti-GLT-1 (1:250 dilution, Cat# PB0293, Boster), rabbit anti-GS (1:5000 dilution, Cat# G2781, Sigma-Aldrich), rabbit anti-PSD95 (1:1000 dilution, Cat# AF1096, Byeotime)

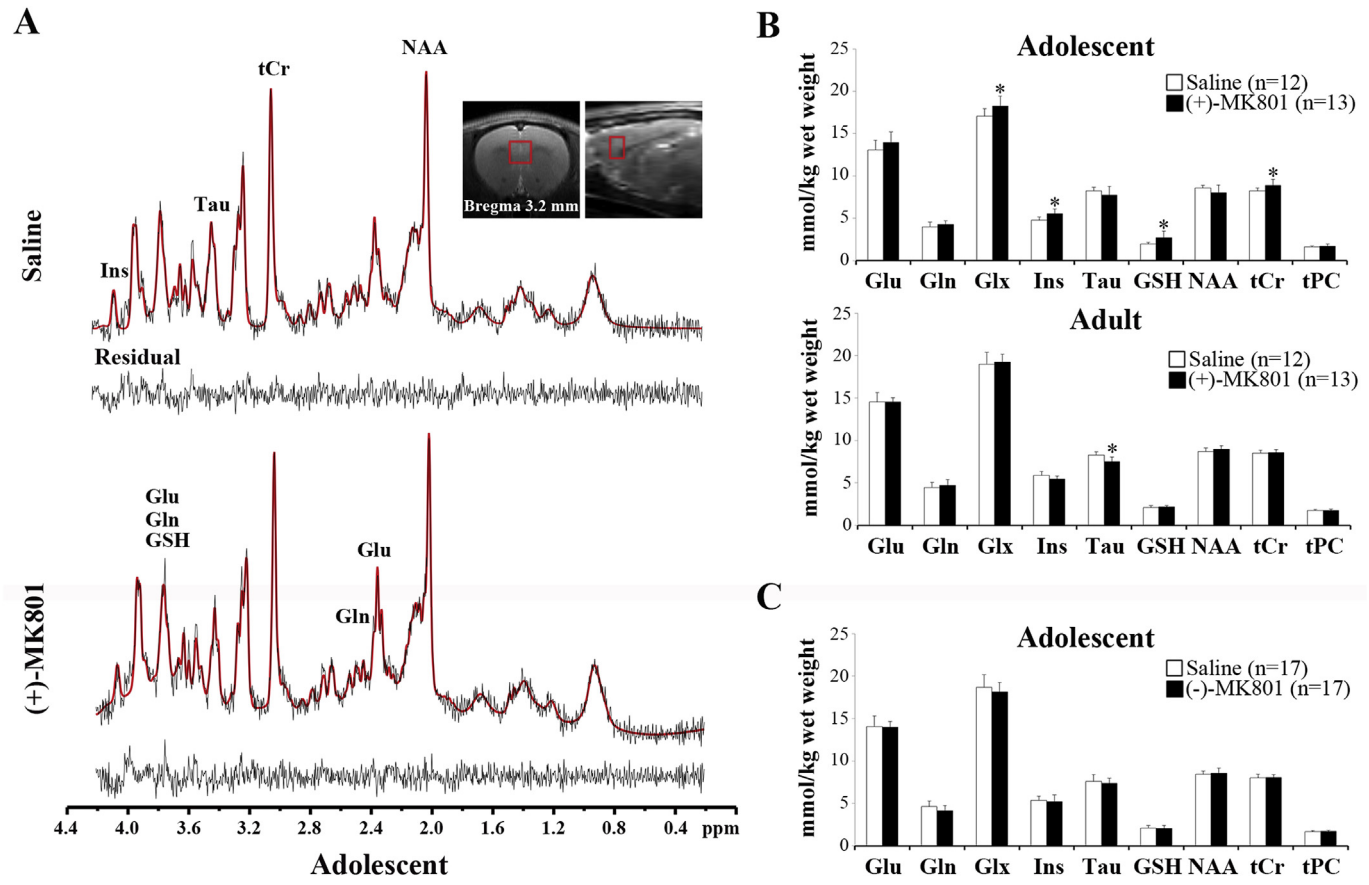

Fig. 2. Representative *in vivo* ^{1}H spectra acquired from bilateral medial prefrontal cortex (mPFC) of adolescentrats treated with (+)-MK801 and saline, respectively (A). Location and size (2.5 mm × 2.4 mm × 2 mm, red rectangle) of the voxel are depicted on T_2-weighted anatomical images (insert, A). Representative raw spectra (black solid lines), LCModel fits (red solid lines) and fitting residuals from the adolescent rats are presented in (A). Absolute metabolite concentrations using water signal as the internal references are plotted in (B and C). *: $p < 0.05$, compared to the corresponding saline-treated group, false discovery rate corrected for multiple comparisons among nine metabolites. Glu: glutamate; Gln: glutamine; Glx: Glu + Gln; Ins: *myo*-inositol; Tau: taurine; GSH: glutathione; NAA: N-acetyl aspartate; Cr: creatine; PCr: phosphocreatine; tCr: Cr + tCr; tPC: total phosphocholine. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and rabbit anti-mGLUR5 (1:1000 dilution, Cat# AF1744, Byeotime) primary antibodies, respectively, at 4 °C overnight. The membranes were then incubated with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:20000 dilution, Cat# A9169, Sigma-Aldrich) at room temperature for 1 h, and exposed to a chemiluminescent HRP substrate (Millipore, Cat# WBKLS0500). Finally, the membranes were analyzed on a ChemiDocTM MP Imaging System (Bio-Rad Laboratories, Berkeley, CA). The integrated immunoreactivities of GLAST, GLT-1, GS, PSD95 and mGLUR5 were normalized using the immunoreactivity of GAPDH from the same sample on the same membrane as the internal reference.

2.5. Extracellular Glu level measurement by microdialysis

The animals were anesthetized with an intraperitoneal dose of chloral hydrate (350 mg/kg body weight) and immobilized on a stereotaxic frame with the incisor bar set at 3.3 mm below the interaural line. A microdialysis guide cannula (CMA/12 Guide Cannula, CMA Microdialysis AB, Kista, Sweden) was implanted into the left mPFC. The stereotaxic coordinates for the adults rats were taken from the rat brain atlas (Paxinos and Watson, 2007), from which the coordinates for the adolescent rats were calculated by multiplying a coefficient to account for brain size differences. Dental acrylic cement was used to attach the cannula to three screws fixed onto the skull. Each rat was placed into a warm incubator for recovery after the surgery, followed by microdialysis sampling 24 h later. The rats implanted with the guide cannula were anesthetized with intraperitoneal injections of chloral hydrate (induction dose: 350 mg/kg body weight, maintaining dose: 35 mg/kg body weight every 60 min). A microdialysis probe (2 mm in active length; 0.5 mm outside diameter; CMA 12 Elite Microdialysis Probe, CMA Microdialysis AB, Kista, Sweden) was inserted through the implanted guide cannula and perfused with an artificial cerebrospinal fluid (aCSF, in mM: 126 NaCl, 2.4 KCl, 1.1 $CaCl_2$, 0.85 $MgCl_2$, 27.5 $NaHCO_3$, 0.5 Na_2SO_4, 0.5 KH_2PO_4 and pH 7.0) at a flow rate of 2 µl/min driven by a microinjection pump (CMA/100; CMA Microdialysis AB, Stockholm, Sweden). For each animal, three baseline samples were collected (30 min each) after a 90-min equilibrium period, followed by an intraperitoneal injection of either (+)-MK801 at 0.5 mg/kg body weight or the same amount of saline. Microdialysis samples were collected every 30 min until 150 min after the drug injection. The samples were stored in −20 °C refrigerator until use.

The Glu concentrations in the microdialysis samples were measured on a Shimadzu HPLC system (LC-20AD, Tokyo, Japan) equipped with a 250 mm-long Shimadzu C18 reversed-phase column (diameter: 4.6 mm, particle size: 5 µm). A filtered and degassed methanol-phosphate buffer mixture (0.02 mol/l, pH = 6.8; 65:35, v/v) was used as the mobile phase. Elution was performed at a flow rate of 1.0 ml/min at 25 °C (i.e., column temperature). After derivatization with orthophosphoric acid in a 4 °C refrigerator for 1 min, the Glu concentration in each sample was measured with fluorescence detection at 472 nm wavelength (Groton Technologies, Boxborough, MA) (Piepponen and Skujins, 2001).

2.6. Statistical analyses

All data were expressed as mean ± standard deviations, except for the microdialysis data, which were expressed as mean percentage of the baseline levels ± standard errors. Two-sample student's t-tests were used to analyze the inter-group differences in body weight, metabolite concentrations and relative protein levels obtained from the Western blotting experiments. False discovery rate (FDR) corrections were applied for multiple comparisons among nine metabolites for the *in vivo* ^{1}H-MRS data. For the histology/immunohistology data, the effects of (+)-MK801 treatment and age were assessed with two-way analysis of variance (ANOVA), followed by post-hoc Sidak's tests to assess the differences between the group pairs. Two-way repeated measures ANOVA was used for statistical analysis of the microdialysis data, with treatment and age as the independent variables and time as the repeated measure. Post-hoc Sidak's tests were used to assess inter-group differences in the time series curves. Within each individual group, post-hoc Dunnett's tests were used to assess the differences between the post-treatment data and the baseline. A $p < 0.05$ was considered to be statistically significant.

3. Results

Compared to the saline-treated rats, the rats subjected to repeated (+)-MK801 treatment did not show any statistically significant difference in body weight gain, regardless of the age (Table 1). Neither were the (−)-MK801-treated adolescent rats. By visual observation, treatment-induced hyperlocomotion was more evident in the adolescent rats subjected to repeated (+)-MK801 treatment, relative to the (+)-MK801-treated adult rats or (−)-MK801-treated adolescent rats.

Fig. 2 shows representative raw spectra acquired from the mPFC of adolescent rats subjected to repeated (+)-MK801/saline treatments (Fig. 2A), LCModel fits (red solid lines) to the raw spectra and fitting residuals. Compared to the saline-treated controls, the adolescent rats subjected to repeated (+)-MK801 treatment had significantly increased Glx (18.3 ± 1.2 vs. 17.1 ± 0.9, $p = 0.03$), Ins (5.6 ± 0.6 vs. 4.8 ± 0.4, $p = 0.009$), GSH (2.7 ± 0.8 vs. 2.0 ± 0.2, $p = 0.02$) and tCr (8.9 ± 0.7 vs. 8.2 ± 0.4, $p = 0.02$) concentrations in the mPFC. The only statistically significant metabolic change observed in the mPFC of (+)-MK801-treated adult rats was decreased Tau concentration (7.5 ± 0.5 vs. 8.3 ± 0.4, $p = 0.03$) (Fig. 2B). Repeated (−)-MK801 treatment induced no statistically significant metabolic perturbations in the mPFC of adolescent rats (Fig. 2C).

Repeated (+)-MK801 treatment induced significantly increased GFAP expression ($p < 0.001$) and oxidative stress ($p < 0.001$) in the mPFC of adolescent rats, in the absence of evident neuronal degeneration (Fig. 3A). Two-way ANOVA revealed statistically significant main effects of treatment (GFAP: [$F(1, 8) = 26.069$, $p = 0.001$; DHE: $F(1, 8) = 45.924$, $p < 0.001$]), age (GFAP: [$F(1, 8) = 7.533$, $p = 0.025$]; DHE: $F(1, 8) = 76.733$, $p < 0.001$) and treatment × age interaction (GFAP: [$F(1, 8) = 19.1$, $p = 0.002$]; DHE: $F(1, 8) = 35.437$, $p < 0.001$). Post-hoc Sidak's tests demonstrated that the increases of GFAP expression and DHE staining in the (+)-MK801-treated adolescent group were statistically significant, compared to the saline-treated adolescent ($p \leq 0.001$) and (+)-MK801-treated adult ($p < 0.01$) groups. Relative to the saline treatment, the repeated (−)-MK801 treatment in adolescent rats resulted in no statistically significant histologic changes in the mPFC (two-sample t-tests, Fig. 3B).

Fig. 4 shows the results of Western blotting in the repeated treatment groups. Relative to the age-matched controls, the (+)-MK801-treated adolescent rats had significantly increased levels of GLAST ($p = 0.049$) and GS ($p < 0.001$) in the mPFC. The (+)-MK801-treated adult rats had a trend of increased GLT-1 level ($p = 0.078$) in the mPFC, compared to the age-matched controls.

Fig. 5 shows the results of microdialysis experiment. Comparing the time series from the four groups together, two-way repeated measures ANOVA revealed statistically significant main effects of time [$F(7, 126) = 10.108$, $p < 0.001$], treatment [$F(1, 18) = 7.053$, $p = 0.016$], time × treatment interaction [$F(7, 126) = 5.506$, $p = 0.002$], time × age interaction [$F(7, 126) = 3.114$, $p = 0.034$], treatment × age interaction [$F(1, 18) = 7.140$, $p = 0.016$] and time × treatment × age interaction [$F(7, 126) = 4.453$, $p = 0.008$]. The main effect of age, however, was not statistically significant [$F(1, 18) = 3.033$, $p = 0.099$]. Post-hoc Sidak's tests revealed that the time series in the (+)-MK801-adolescent group was significantly different from those in the saline-adolescent ($p = 0.008$) and (+)-MK801-adult ($p = 0.025$) groups. The time series in the (+)-MK801-adult and saline-adult groups were not significantly different from each other ($p = 1.000$).

Post-hoc Dunnett's tests within the groups indicated that the (+)-MK801-treated adolescent rats had a significantly increased extracellular Glu level in the mPFC at 105 min post-treatment (394 ± 87% of the baseline, $p < 0.001$). The extracellular Glu level in the mPFC of (+)-MK801- and saline-treated adult rats appeared to have increased at 135 min post-treatment, but only the increase in the (+)-MK801-adult group reached statistical significance (170 ± 62% of the baseline; $p = 0.018$, post-hoc Dunnett's test). No statistically significant inter-group difference was found for the (+)-MK801- and saline-treated adult groups at this time point ($p = 0.856$, two-sample t-tests).

For the adolescent rats, acute (+)-MK801 treatment increased GFAP expression and oxidative stress in the mPFC contralateral to the microdialysis probe implantation (right panel, Fig. 5C), as well as the death of astrocytes in the vicinity of probe implantation (left panel, Fig. 5C). Acute (+)-MK801 treatment induced no evident histological changes in the contralateral (Fig. 5D) or ipsilateral mPFC of the adult rats (data not shown). Comparing the semi-quantitative GFAP and DHE measurements in the contralateral mPFC with two-way ANOVA revealed significant main effects of age × treatment interactions (GFAP: [$F(1, 18) = 9.030$, $p = 0.008$]; DHE: [$F(1, 18) = 4.366$, $p = 0.051$]). Post-hoc Sidak's tests indicated that the (+)-MK801-treated adolescent rats had significantly increased GFAP and DHE levels in the contralateral mPFC ($p < 0.05$) compared to the saline-treated adolescent rats and (+)-MK801-treated adult rats (Fig. 5D).

4. Discussion

It was observed in this study that acute (+)-MK801 treatment at a moderate dose of 0.5 mg/kg body weight augmented Glu release, induced astrocytosis and elevated oxidative stress in the mPFC of adolescent rats. In parallel, a 6-day repeated (+)-MK801 treatment with a daily dose of 0.5 mg/kg body weight in the adolescent rats resulted in significant metabolic perturbations in the mPFC, manifesting as increased Glx, Ins, tCr and GSH levels. Histologically the mPFC of adolescent rats treated with (+)-MK801 repeatedly showed evident signs of reactive astrocytosis and increased oxidative stress, but without apparent neuronal degeneration. Interestingly none of the changes observed in the (+)-MK801-treated adolescent rats was found in the (+)-MK801-treated adult animals, nor in the (−)-MK801-treated adolescent animals. Taken together, these results suggest that the effects of acute/repeated (+)-MK801 treatment on Glu release/metabolism in the mPFC are age-dependent.

4.1. Effects of acute (+)-MK801 treatment on Glu release

Extracellular Glu level monitored by *in vivo* microdialysis reflects the balance between neuronal Glu release and reuptake into surrounding nerve terminals and glial elements (Herrera-Marschitz et al., 1996). Previous studies have demonstrated that acute (+)-MK801 treatments at doses of 0.3-1 mg/kg body weight augmented Glu release in the mPFC of adolescent/young adult animals (Lopez-Gil et al., 2007; Pietraszek et al., 2009; Roenker et al., 2011). Agreeing with these

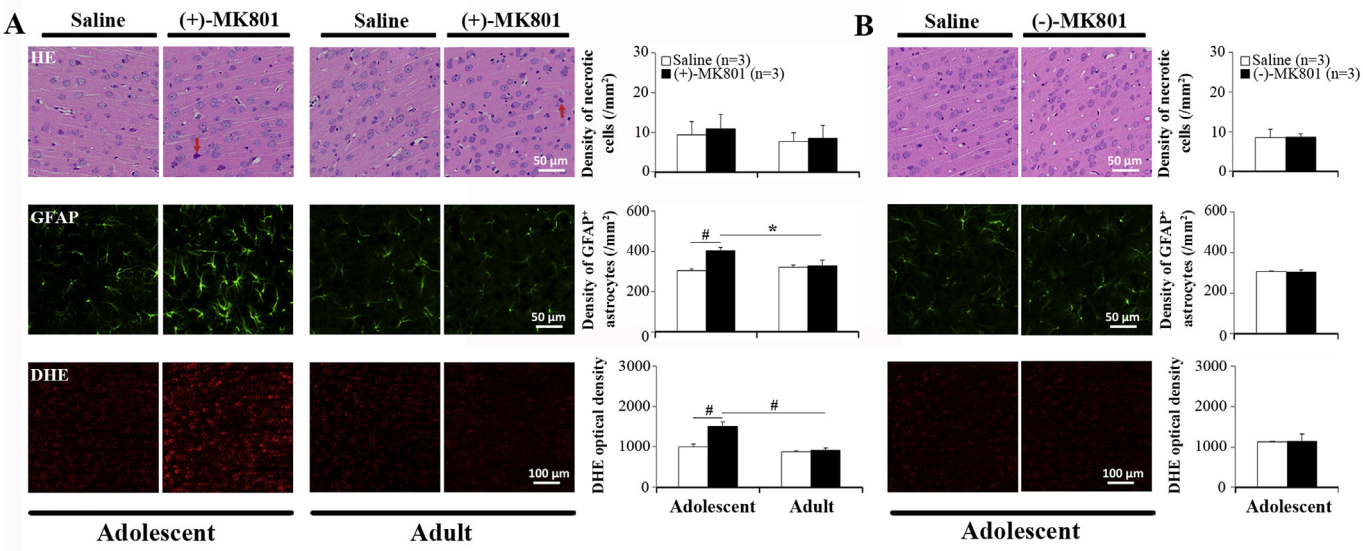

Fig. 3. Representative histological/immunohistological results from the medial prefrontal cortex (mPFC) of rats subjected to repeated MK801 and saline treatments, respectively. Significant increases in GFAP expression and DHE staining were observed only in the (+)-MK801-treated adolescent rats (A), but not in the (+)-MK801-treated adult rats (A) or (−)-MK801-treated adolescent rats (B). HE staining revealed that MK801 treatments resulted in no evident neuronal degeneration (A and B). Red arrows: necrotic neurons. *: $p < 0.05$ and #: $p < 0.005$, post-hoc Sidak's tests. HE: hematoxylin and eosin; GFAP: glial fibrillary acidic protein; DHE: dihydroethidium. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

results, an approximately 4-fold increase of extracellular Glu level was observed in the mPFC of (+)-MK801-treated adolescent rats. It was further demonstrated in this study that the same dose of (+)-MK801 induced little changes of Glu release in the mPFC of adult rats, indicative of an age-dependence. NMDAR antagonist-augmented Glu release may trigger increases in oxidative stress via activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Behrens et al., 2008; Brennan et al., 2009). Astrocytes play important roles in clearing Glu from the extracellular space via Glu transporters GLAST/GLT-1 and preventing excitotoxicity; and they also play a role in modulating neuronal antioxidant status via ascorbate and GSH (Swanson et al., 2004). Corroborating with these views, the increases of Glu release in the mPFC of (+)-MK801-treated adolescent rats was found to be accompanied with increased oxidative stress and reactive astrocytosis in a homologous region contralateral to the microdialysis probe implantation. It is interesting to note that (+)-MK801 treatment in the adolescent rats, and presumably the massive Glu release induced, appeared to be lethal to the reactive astrocytes in the vicinity of microdialysis probe, which had already become activated before the treatment probably due to the injuries caused by probe insertion.

Under normal physiological conditions, cortical pyramidal neurons regulate their own firing and Glu release by sending recurrent inhibitory collaterals to the NMDARs on the parvalbumin (PV) fast-spiking interneurons that feed back onto the primary neurons, forming the so-called feedback inhibitory loop (Olney et al., 1999). At low-to-moderate doses, NMDAR antagonists are thought to preferentially block the NMDARs on the PV-interneurons due to their tonic firing (Huettner and Bean, 1988; Wang and Gao, 2009), resulting in disinhibition and hyperactivity of the pyramidal neurons (Jackson et al., 2004; Liu et al., 2017; Rujescu et al., 2006). (+)-MK801-induced increases of Glu release in the adolescent mPFC had been previously attributed to this mechanism (Amitai et al., 2012; Moghaddam et al., 1997; Wang and Gao, 2012). The presynaptic NMDARs, which are abundant in the developing brain and known to facilitate Glu release, might have also played a role (Bouvier et al., 2015).

In contrast to systemic administration, local application of NMDAR antagonists into the mPFC of young adult animals did not seem to have any significant effects on Glu release and/or spontaneous firing (Jodo et al., 2005; Lopez-Gil et al., 2007; Lorrain et al., 2003). On the other hand, local application of these agents to the hippocampus enhanced excitatory activities in the mPFC (Jodo et al., 2005). It is shown recently that multiple long-range inputs, such as those from the ventral hippocampus and mediodorsal thalamus, can evoke NMDAR responses in the mPFC PV-interneurons and pyramidal neurons (Bogart and O'Donnell, 2018). These results suggest that NMDAR antagonist-induced disinhibition in the mPFC of adolescent/young adult rats may, at least partially, be driven by long-range glutamatergic afferents (Celada et al., 2013; Sharp et al., 2001; Thomases et al., 2013; Tseng et al., 2009). Furthermore, NMDAR antagonist-induced Glu release in the mPFC (Lopez-Gil et al., 2007) and neurodegeneration in the cingulate and retrosplenial cortex (Sharp et al., 1995) can be prevented by local application of AMPA/kainate receptor antagonists, suggestive of the involvement of these receptors.

4.2. Effects of repeated (+)-MK801 treatment on Glu metabolism

A number of previous *ex vivo* and *in vivo* ^{1}H-MRS studies have demonstrated that adolescent/young adult rats subjected to repeated NMDAR antagonist treatment had significant metabolic perturbations in the frontal and parietal cortices, involving mainly Glu and glial metabolites (i.e., Ins, GSH, and Gln). For example, increased Glu, GSH and Tau levels were observed in the cingulate + retrosplenial + middle frontal cortices (CRFC) after a 6-day repeated (+)-MK801 treatment at a daily dose of 0.5 mg/kg body weight (Kondziella et al., 2006), but not at a daily dose of 0.1 mg/kg body weight (Eyjolfsson et al., 2006). Repeated ketamine treatment at a moderate daily dose of 30 mg/kg body weight for 6 days significantly increased the Glu level in the PFC (Kim et al., 2011). Human subjects were found to have increased Glu/Gln levels in the anterior cingulate after hours of ketamine infusion (Rowland et al., 2005; Stone et al., 2012).

Most of these studies acquired spectra from a relatively large region containing heterogeneous cortical areas, such that the metabolic perturbations could not be attributed unequivocally to the mPFC, which showed significantly different metabolic profile even from the neighboring cingulate cortex (Zhang et al., 2019). In these studies, metabolic changes were often measured 20–30 min after the last dose of repeated treatment, when the acute pharmacological effects of the drug might still be present (Vezzani et al., 1989). In addition, few previous studies

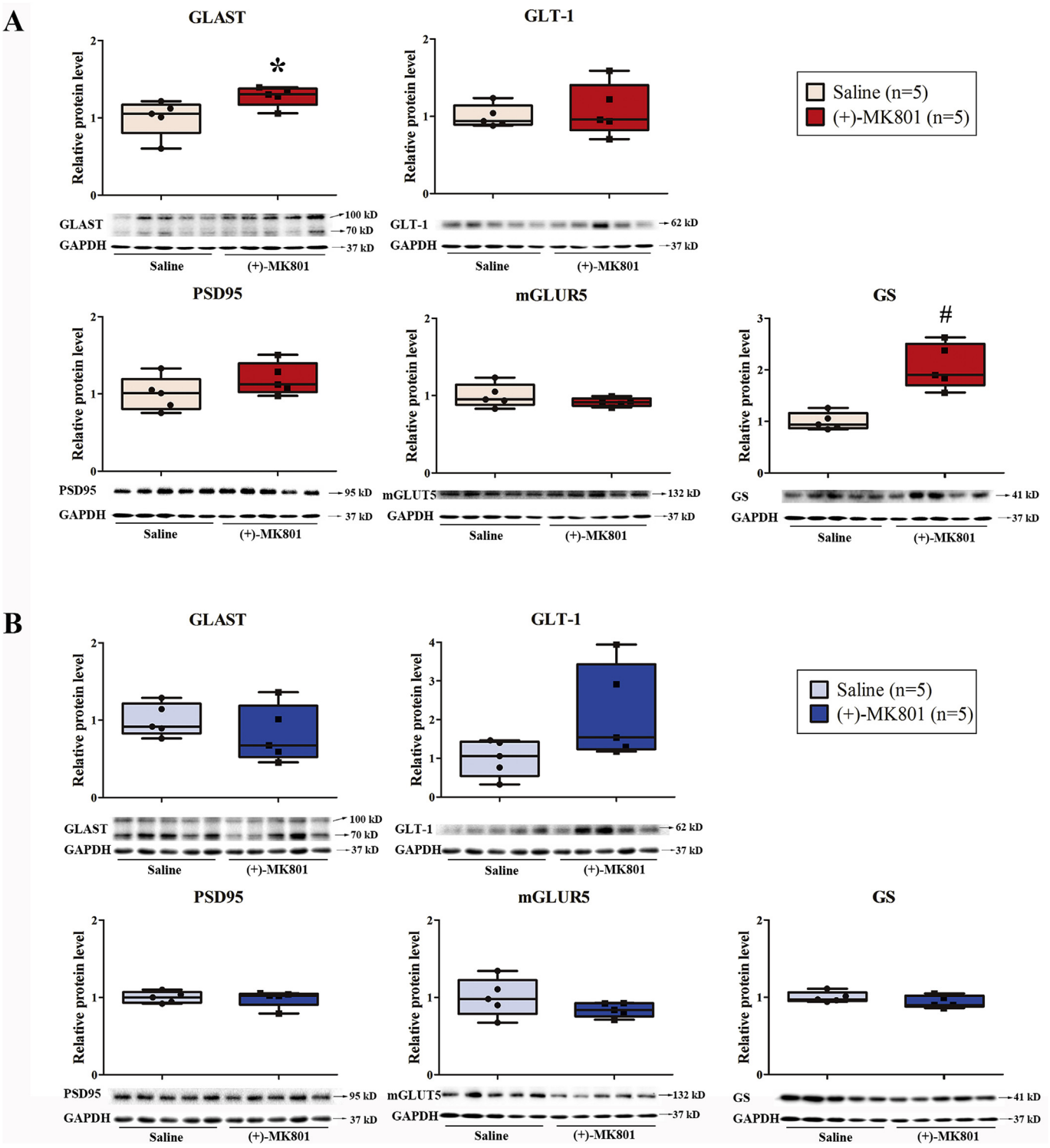

Fig. 4. The results of Western blotting experiments. Repeated (+)-MK801 treatment induced significant increases of GLAST and GS protein levels in the adolescent mPFC (A), and a trend of increased GLT-1 protein level in the adult mPFC (B). *: $p < 0.05$, #: $p < 0.005$, two-sample *t*-tests. Data are expressed as boxplots showing the median (line) with 25/75 percentile (box) and the minimal/maximal values (whiskers). GLAST: astrocytic glutamate transporters glutamate-aspartate transporter; GLT-1: glutamate transporter-1; GS: glutamine synthetase; mGLUR5: metabotropic glutamate receptor 5; PSD95: postsynaptic density protein 95.

have linked the ^{1}H-MRS-observable neurochemical changes induced by NMDAR antagonist treatments to histological alterations.

In this study, a small voxel was used for better spatial localization in bilateral mPFC, and the *in vivo* ^{1}H-MRS measurements were performed after a 7-day wash-out period to rule out the possible direct pharmacological effects. The metabolic perturbations and histological changes in the mPFC of (+)-MK801-treated adolescent rats were found not only to be consistent, in general, with the previous findings (Hajszan et al., 2006; Kondziella et al., 2006; Li et al., 2016; Zhou et al., 2015), but also corroborating with each other. For instance, the (+)-MK801-treated adolescent rats showed significantly increased Ins and tCr levels in the mPFC, in line with the observation of astrocytosis (Arif et al., 2007; Brenner et al., 2005; Hajszan et al., 2006; Murai et al., 2007); both increased GSH level and elevated oxidative stress pointed to regional

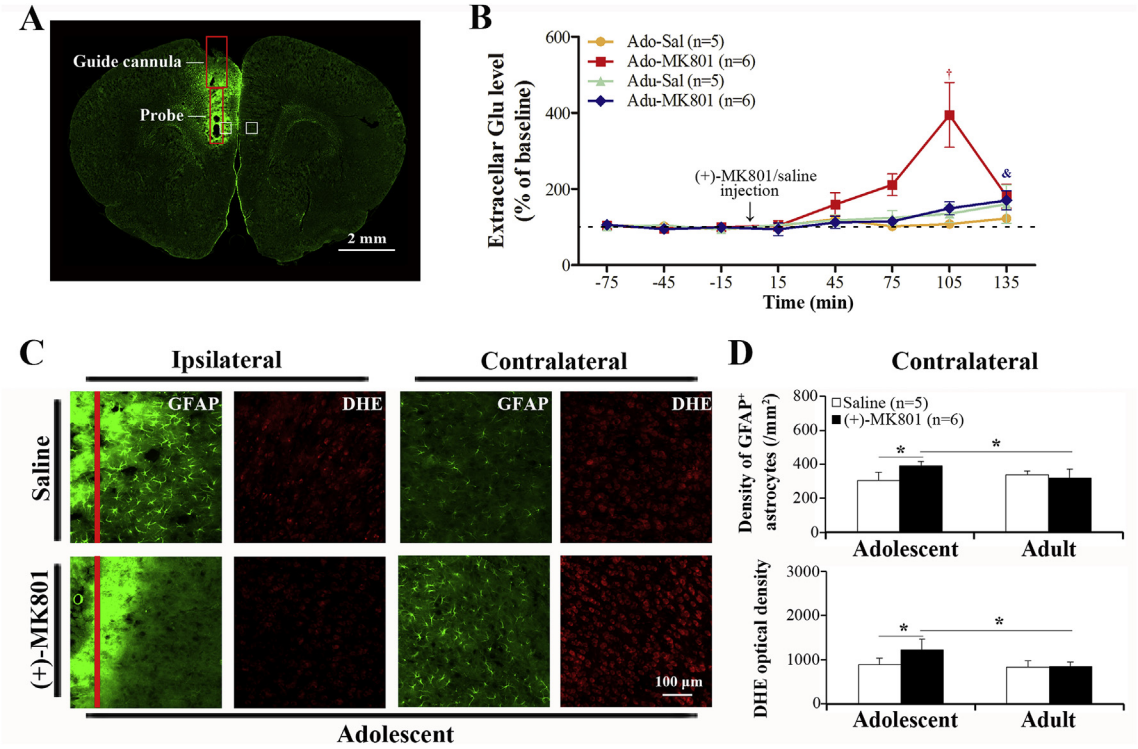

Fig. 5. Extracellular glutamate (Glu) concentrations (B) in the medial prefrontal cortex (mPFC, A) of rats subjected to acute (+)-MK801/saline treatments (single dose, 0.5 mg/kg, i. p.), measured by time-resolved *in vivo* microdialysis. The sizes and locations of the guide cannula and active microdialysis probe are depicted on a GFAP-stained brain slice from a saline-treated adolescent rat (A). Panel (C) presents GFAP and DHE staining of the bilateral mPFC (white squares, A) of representative (+)-MK801- and saline-treated adolescent rats. Panel (D) plots the semi-quantitative results of GFAP and DHE straining in the adolescent/adult mPFC contralateral to the microdialysis probe implantation. †: $p < 0.001$, &: $p < 0.05$: the post-treatment measurement significantly different from the baseline of the corresponding group, post-hoc Dunnett's test. *: $p < 0.05$, post-hoc Sidak's test. Ado: adolescent; Adu: adult; Sal: saline; GFAP: glial fibrillary acidic protein; DHE: dihydroethidium.

redox dysregulation (Behrens et al., 2007; Rae and Williams, 2017; Zhou et al., 2015); unchanged NAA level was consistent with the fact that no evident neuronal degeneration was observed in the region (Barnes et al., 2015; Li et al., 2016).

Increased Glu and/or Gln levels in response to acute NMDAR antagonist treatment have been reported in human anterior cingulate (Rowland et al., 2005; Stone et al., 2012) and the PFC of young adult rats (Brenner et al., 2005; Iltis et al., 2009; Napolitano et al., 2014). Such metabolic alterations have been attributed to metabolic feedbacks or adaptations to augmented Glu release and/or glutamatergic hyperactivity induced by NMDAR antagonism (Brenner et al., 2005; Napolitano et al., 2014). Supporting these arguments, it has been shown that the mPFC of young adult animals subjected to repeated NMDAR antagonist treatment has decreased resting state extracellular Glu concentration (Murai et al., 2007; Zuo et al., 2006), up-regulation of GLAST and S100 calcium-binding protein B (S100B) (Murai et al., 2007) and decreased GABA concentration (Amitai et al., 2012; Boczek et al., 2015; Zhou et al., 2015), indicative of, respectively, decreased spontaneous Glu release, enhanced neuronal-glial interactions (i.e., Glu-Gln shuttle) and reduced GABA synthesis with Glx as the precursor (Boczek et al., 2015; Walls et al., 2015). All these mechanisms might have contributed to the increased Glx level observed in this study. The increased Glx level might also have played a role in the upregulation of GSH by providing more precursors for GSH synthesis (Dringen and Hirrlinger, 2003; Rae and Williams, 2017).

On the other hand, there are also studies in the literature that reported decreased Glu concentration in the cortex of animals subjected to repeated NMDAR antagonist treatment (Bustillo et al., 2012; Sun et al., 2013a). These studies often used higher doses of NMDAR antagonist or longer period of drug administration, as such the decreased Glu levels were likely a metabolic manifestation of neuronal degeneration (Bustillo et al., 2012; Sun et al., 2013a). Clinically, the high-risk/first-episode SZ patients often show increased Glu (or Glx) concentration in the mPFC (Fuente-Sandoval et al., 2015; Kegeles et al., 2012; Purdon et al., 2008; Tibbo et al., 2004). In contrast, the chronic SZ patients are consistently reported to have significantly reduced cortical Glu (or Glx) levels (Lutkenhoff et al., 2010; Natsubori et al., 2014; Tsai et al., 1995) and reduced GLAST expression (Bauer et al., 2008). Decreased NAA concentration and/or grey matter atrophy, indicators of neuronal degeneration, were often observed in these patients (Brugger et al., 2011; Natsubori et al., 2014; Premkumar et al., 2008; Velakoulis et al., 2002).

The levels of GS and GLAST, in the adolescent mPFC increased significantly after repeated (+)-MK801, indicative of upregulated astrocyte uptake of Glu and conversion into Gln. Meanwhile, the levels of PSD95 and mGLUR5 did not exhibit any significant changes, suggestive of unaffected postsynaptic glutamatergic function, consistent with a previous report showing that the mGLUR5 level in the PFC of young adult rats had no significant changes after PCP treatments at a dose of 7.5 mg/kg body weight (Abe et al., 2001).

The only statistically significant metabolic perturbation observed in the (+)-MK801-treated adult rats was the decrease of Tau, which probably had reflected Tau efflux in responding to astrocyte swelling (Schober and Mongin, 2015). However, the contributions from other mechanisms could not be excluded, as Tau is also known to have antioxidant, anti-inflammatory and neuromodulatory functions (Albrecht and Schousboe, 2005; Chung et al., 2012).

4.3. Mechanisms underlying age-dependent effects of (+)-MK801 treatment

In clear contrast to the adolescent rats, the adult rats showed little changes of Glu release/metabolism in the mPFC in response to acute/repeated (+)-MK801 treatment at the doses used. Neither did the

immunohistological and Western blotting assessments reveal any evident abnormalities in this age group. It has been observed in the mPFC of adult rats that while low dose(s) of (+)-MK801 (i.e., ≤0.1 mg/kg) preferentially block the NMDARs in PV-interneurons, higher doses can act on the NMDARs in both PV-interneurons and pyramidal neurons (Moghaddam et al., 1997; Xi et al., 2009). Interestingly, the effects of (+)-MK801 on these two types of neurons could cancel each other completely at an optimal dose of 0.33 mg/kg (Xi et al., 2009). Acute ketamine treatment appeared to have a similar dose-dependence in this regard (Moghaddam et al., 1997). The daily dose of (+)-MK801 used in this study was close to the optimal dose reported by Xi et al., such that the lack of marked disinhibition/excitotoxicity in the adult mPFC might be attributed to the effects of (+)-MK801 on the pyramidal neurons and interneurons cancelling each other.

It has also been shown that the acute neurotoxic effects of single-dose (+)-MK801 treatment on adult brain are reversible at the dose range of 0.3–1.0 mg/kg body weight, and tend to resolve 18–24 h after the treatment (Horvath et al., 1997; Olney et al., 1989, 1991; Sharp et al., 1991). Repeated (+)-MK801 treatment at 0.5 mg/kg body weight induced no cumulative neurotoxic effects when the injections were given 24 h apart (Olney et al., 1989). In comparison, repeated injections given at shorter intervals (i.e., 8 h) or higher doses were shown to result in irreversible neuronal damages (Horvath et al., 1997). The absence of significant (+)-MK801-induced changes in the adult brain therefore can also be due to the fact that the treatment regime used might have not exceeded the threshold to induce irreversible neuronal damages.

The developing mPFC differs from the fully matured mPFC not only in the quantity and cell type-specific distribution of NMDAR, but also in the circuit property with respect to excitatory-inhibitory balance/integration (Chung et al., 2017; Insel et al., 1990; Wang and Gao, 2009). The density of total NMDAR was found to about 60% higher in the adolescent mPFC than in the adult mPFC (Insel et al., 1990). The PV-interneurons in the mPFC showed significantly different electrophysiological properties between PND 30-60 and PND 90-110, with more PV-interneurons in the adolescent PFC exhibiting NMDA currents and Ca^{2+}-permeable AMPA receptors-mediated currents (Wang and Gao, 2009, 2010). There is a developmental pruning of glutamatergic synaptic inputs to the PV-interneurons from adolescence to adulthood, accompanied by increases in the densities of glutamatergic synaptic proteins (i.e., vGLUT1 and PSD95) on the remaining excitatory synapses (Chung et al., 2017) and upregulation of glutamatergic synaptic transmission onto the PV-interneurons (Caballero et al., 2014). In addition, the subunits of NMDAR on the PV-interneurons undergo a switch from NR2B to NR2A after PV appearance (Zhang and Sun, 2011), and the functional emergence of NR2B in the prefrontal pyramidal neurons occurs only after PND 40 (Flores-Barrera et al., 2014). Such switch of sub-unit composition might have affected Glu sensitivity of the NMDARs (Paoletti, 2011). Furthermore, the age-related changes of astrocytes might have also affected the Glu reuptake/release, metabolism and signaling at the tripartite synapse (Sun et al., 2013b; Yang et al., 2019; Zhou et al., 2006; Ziemens et al., 2019). All these developmental changes may influence the sensitivity and susceptibility of the feedback inhibitory loop in the mPFC to NMDAR antagonism, and how the excitatory-inhibitory balance is maintained or re-established in response to (+)-MK801 treatment.

4.4. Comparison between (+)-MK801 and (−)-MK801

(−)-MK801 is only one-seventh as potent as (+)-MK801 at the NMDA receptors (Wong et al., 1986). Previous studies have demonstrated that acute/repeated (−)-MK801 treatment in rodents resulted in less prominent behavioral phenotypes (Geter-Douglass and Witkin, 1997; Yang et al., 2016) and apoptotic responses (Ikonomidou et al., 1999), relative to (+)-MK801 treatment at the same dose(s). Agreeing with these previous results, it was observed in this study that repeated (−)-MK801 treatment was associated with no significant changes in all the measurements performed, confirming that (+)-MK801-induced neurobiological changes in the adolescent brain are mediated by NMDAR hypofunction, rather than the other pharmacological properties of the agent.

4.5. Limitations and perspectives

There are some limitations to this study. Firstly, the age-dependent effects of acute/repeated (+)-MK801 treatments were demonstrated only in the mPFC. Future studies are needed to investigate the age-dependence in brain regions with different developmental trajectories, for instance the cingulate cortex and visual cortex. Secondly, the current study investigated the age-dependent effects of (+)-MK801 treatment only in the male rats. It is known that the effects of (+)-MK801 on adult brain are sexually dimorphic, with females showing higher sensitivity and susceptibility than males (Feinstein and Kritzer, 2013; Olmos et al., 2008). Thus the age-dependence of NMDAR treatments may also be sexually dimorphic. Thirdly, only one dose was used in this study and the dose-dependent effects were not evaluated. Last but not least, whether the age-dependence of (+)-MK801 treatment observed in this study can be generalized to other NMDAR antagonists, such as ketamine and PCP, remains to be investigated. This is an important issue since off-label clinical usage of ketamine (Newport et al., 2015) and PCP/ketamine abuse are on the rising worldwide, especially among the adolescence/young adult age groups (Morgan et al., 2010).

5. Conclusion

It was observed in this study that acute/repeated (+)-MK801 treatments induced age-dependent changes of Glu release/metabolism in the mPFC of male adolescent and adult rats. At the dose used, disinhibition and reactive astrocytosis in the mPFC were observed only in the adolescent rats (PND 30), but not in the adult rats with an age around PND100. These results suggest that the adult rats must have developed more robust mechanisms, as-yet-unknown, to maintain excitatory-inhibitory balance in response to NMDAR hypofunction. This study highlights the need to consider the modulating effects of age when using NMDAR antagonists under experimental or clinical settings.

Statement of interest

Declarations of interest: none.

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