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Intracellular mechanisms and behavioral changes in mouse model of attention deficit hyperactivity disorder: Importance of age-specific NMDA receptor blockade

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ABSTRACT

Exposure of NMDA receptor antagonists during developmental stages leads to behavioral consequences like attention deficit hyperactivity disorder (ADHD). However, the underlying molecular mechanisms have remained poorly understood. Herein, we studied the phosphorylated Akt (pAkt) and caspase-3, the key regulators of neuronal cell survival/death, as the probable downstream targets of MK-801 often used to engender ADHD-like condition. Swiss albino mice at postnatal days (PND) 7, 14 or 21 were injected with a single dose of MK-801 and evaluated for hyperactivity (open field test) and memory deficit at adolescence (PND 30) and adult stages (PND 60). PND 7 or 14 treatment groups (but not PND 21) consistently showed hyperactivity at the adolescence stage. A significant increase in working and reference memory errors in radial arm maze was noted at the adolescence age. PND 7 group continued to display the symptoms even in adulthood. All the treatment groups showed a significant decrease in the percent alterations (Y-maze) and discrimination index (novel object recognition test) at adolescence age. A significant increase in caspase-3 expression was noted in the prefrontal cortex (PFC) and hippocampus, whereas increased pAkt was noticed only in the hippocampus, following a single injection of MK-801 at PND 7. Concurrently, PND 7 treatment group showed significantly decreased neuronal nuclei (NeuN) expression (a marker for mature neurons) in the dentate gyrus, cornu ammonis-3 and PFC, but not in cornu ammonis-1, at adolescence age. We suggest that the observed symptoms of ADHD at adolescence and adulthood stages may be linked to alteration in pAkt and caspase-3 followed MK-801 treatment at PND 7.

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by hyperactivity, impaired attention and impulsiveness (Sroubek et al., 2013). Individuals with ADHD also show poor working memory and executive function (Holmes et al., 2014). ADHD is highly prevalent during childhood and frequently persists into adulthood (Kooij et al., 2010). It affects approximately 5–10% of children worldwide (Willcutt, 2012). Several studies correlate dysregulation in the neurotransmission of dopamine, norepinephrine, glutamate and serotonin systems with the appearance of ADHD symptoms (Russell, 2002; Sagvolden et al., 2005; Feldman and Reiff, 2014). Imaging studies showed the involvement of frontal-motor cortex circuitry in ADHD patients (Giedd et al., 2001; Clark et al., 2007). Previous reports indicate an involvement of multiple genetic factors as the etiological basis of ADHD, however, exposure to various environmental agents during neuronal development have been suggested as causal factors (Brondum, 2007; Faraone and Larsson, 2019). It is also reveal that ADHD is not a single pathophysiological entity, but involves multiple interacting factors (Faraone et al., 2015).

Exposure to various pharmacological agents (ethanol, phencyclidine, ketamine, nitrous oxide, barbiturates, benzodiazepines, halothane, isoflurane and propofol) during synaptogenesis triggers apoptosis throughout the brain and leads to psychiatric disorders later in life (Ikonomidou et al., 1999; Olney et al., 2000). Largely synaptogenesis and other constructive anatomical developments occur during the first three weeks in the life of rodents. During this period, age-specific synaptic density changes take place in the developing brain which

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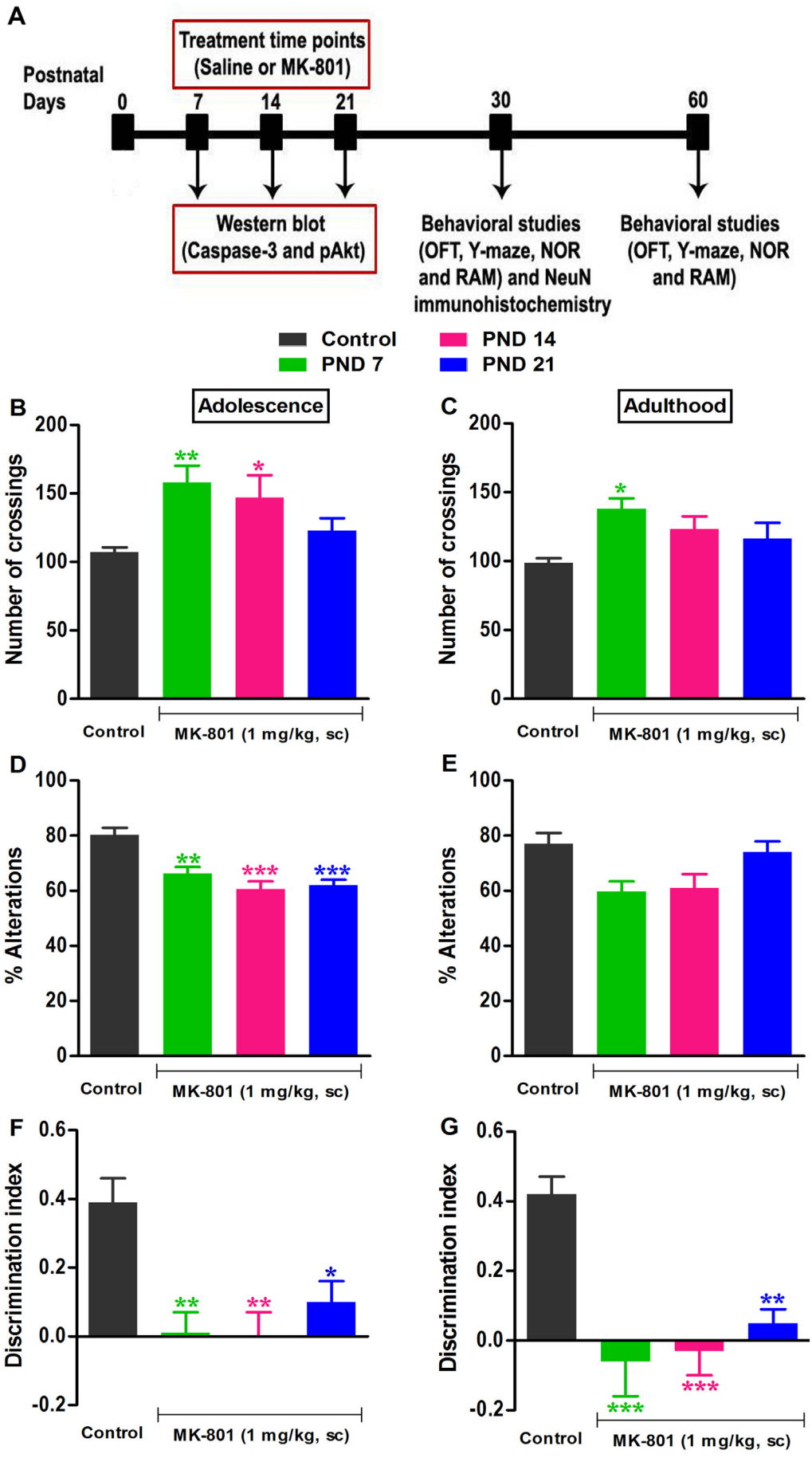

Fig. 1. Diagrammatic representation illustrating the time points of drug administration, behavioral and molecular analysis (A). Effect of MK-801 (1 mg/kg, sc), administered at postnatal day (PND) 7, 14 or 21 and tested at adolescence and adulthood stage, on the number of crossings (open field test, OFT; B–C), percent (%) alterations (Y-maze; D–E) and discrimination index (novel object recognition; F–G) in mice. Note that MK-801 treatment at PND 7 and 14 showed a significant higher number of crossings in OFT as compared to saline treated (control) animals when tested at adolescence, which was sustained up to adulthood only in the PND 7 group. The percent (%) alterations in Y-maze have also been significantly reduced in all the treatment groups only at adolescence stage. However, discrimination index was significantly blocked by MK-801 treatment throughout the study groups on both stages. The data are expressed as means ± SEM and analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. *P < 0.05, **P < 0.01 and ***P < 0.001 vs control (saline) (n = 5–9/group).

involves elimination of excessive synapses and formation of mature neural circuitries. For instance, synaptic density in rodents is low at the first week, peaks at postnatal day (PND) 10 and equals to that in the adults by PND 30 (Semple et al., 2013). Similarly, dramatic changes were also observed in *N*-methyl-D-aspartate (NMDA) receptor expressions which peak at the age of three weeks in rat hippocampus and cortex (Zhong et al., 1995). A wealth of data showed that MK-801, an NMDA receptor antagonist induced ADHD-like symptoms (Fredriksson and Archer, 2003, 2004; Oliveira-Pinto et al., 2015).

Neuronal proliferation and apoptosis are key processes regulated by various protein kinases (Akt, PI-3K, Src, etc.) and caspases (McIlwain et al., 2013; Yu and Cui, 2016). Alteration in these processes during synaptogenesis might lead to the development of behavioral deficits like ADHD (Jevtovic-Todorovic et al., 2003; Galvez-Contreras et al., 2017). Akt is a key protein in PI3K-Akt pathway that is shown to regulate cell survival and cell growth (Song et al., 2005). While administration of MK-801 increased the phosphorylated ser473-Akt (pAkt) in adult rat prefrontal cortex (PFC), a decrease was observed in developing brain (PND 7) (Ahn et al., 2005; Kim et al., 2010). pAkt acts upstream of glycogen synthase kinase-3β (GSK-3β) and impairment of these signaling has been demonstrated in various psychiatric disorders (Ahn et al., 2005; Jope and Roh, 2006). Amphetamine treatment reduced behavioral hyperactivity via GSK-3β in a hyperactive multigenetic animal model of ADHD (Yen et al., 2015). On the other hand, caspase-3 is a cysteine-aspartic acid protease, whose activation leads to the initiation of apoptosis (Porter and Jänicke, 1999). Administration of MK-801 to rat at PND 7 resulted in an increase in caspase-3 immunoreactivity in various brain regions including PFC and hippocampus (Coleman et al., 2009; Lyall et al., 2009). Moreover, Turner et al. (2009) demonstrated increased casapse-3 expression following MK-801 injection at PND 7, 14 or 21 in the cingulate cortex, somatosensory cortex and caudate putamen. However, the effect of MK-801 administration at different age time points (PND 14 or 21) on pAkt and caspase-3 in PFC and hippocampus for ADHD has not been studied. In this background, we propose to investigate the factors like development and environment causal to ADHD. Therefore, in the present study, we administered MK-801 at different time points in the life of the animal and assessed the pAkt and caspase-3 in the PFC and hippocampus. We also monitored the behavior of the animals for ADHD-like symptoms at adolescence and adulthood stages.

Adverse changes in PFC and hippocampus may manifest as ADHD (Sweatt, 2004; Zang et al., 2005; Plessen et al., 2006). While PFC regulates attention, executive function, working memory and impulsivity, lesions in these areas are known to produce symptoms similar to ADHD (Itami and Uno, 2002). Hypoactivation of PFC was found in ADHD patients (Cortese et al., 2012). Apoptotic cell death and long-term behavioral deficit were noted in the various regions of the brain after postnatal MK-801 treatment (Ikonomidou et al., 1999; Fredriksson and Archer, 2004). Alteration in pAkt and caspase-3 were evaluated at 8 h followed MK-801 treatment as a probable mechanism of ADHD-like phenotype in the mouse. Neonate mice were treated with MK-801 (1 mg/kg, sc) and screened for behavioral symptoms of ADHD-like hyperactivity and memory deficits at their adolescence (PND 30) and adulthood stage (PND 60). Further neuronal nuclei (NeuN) immunoreactivity was assessed at the adolescence stage in PFC, dentate gyrus (DG), cornu ammonis-1 (CA1) and cornu ammonis-3 (CA3) to understand the long-term effect of MK-801 treatment on neuronal population.

2. Experimental procedures

2.1. Animals

Swiss albino mice were maintained under controlled light (lights on 07:00–19:00 h), temperature 25 ± 1 °C, relative humidity (50–70%), and food and tap water were provided *ad libitum*. The pregnant mice were identified, isolated and offspring weaned on PND 21 were group housed with 5 same-sexed cage-mates. All the experimental protocols were approved and performed in compliance with the Institutional Animal Ethics Committee (IAEC), Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, India. The behavioral protocols were undertaken in room illuminated with ~250 lx.

2.2. Drug preparation and administration

(+)-MK-801 hydrogen maleate (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. D054) was dissolved in 0.9% normal saline. Animals were randomly assigned to different treatment groups and were administered with MK-801 (1 mg/kg, sc) or vehicle (Saline, 0.05–0.1 ml/mouse, sc) on PND 7, PND 14 or PND 21 (Fig. 1A). Animals were subjected to the assessment of hyperactivity or memory at adolescence and adult stage. Saline administration on PND 7, 14 or 21 (control groups) did not show any difference in the hyperactivity or memory deficit, when noted at adolescence and adulthood stages. Thus, to simplify the comparison, we combined and averaged the data from these three groups, represented as control.

2.3. Behavioral assessments

2.3.1. Hyperactivity using open-field test (OFT)

The hyperactivity (reactivity to the novel environment a key feature of ADHD) in mice was measured at adolescence and adulthood in control and treatment groups by placing each mouse in the center of the circular open field (Grailhe et al., 1999). The apparatus was made up of acrylic (nontransparent surrounding walls that prevent escape with white floor, 80 cm diameter) and divided into square areas of 10 cm^2 each marked on the floor. The animal was released in the arena and the number of crossings was counted during 5 min of the test period. Before each test, the arena was cleaned with 70% alcohol to eliminate the possible bias due to the odor that could be left by a previous animal (Borkar et al., 2018).

2.3.2. Novel object recognition task

In the control or MK-801 treated mice, recognition memory was assessed by using the NOR test. This non-rewarded paradigm is used to assess animals' spontaneous exploratory behavior and reactivity to discrete novel stimuli (Ennaceur and Delacour, 1988; Zhuang et al., 2001). The experiments were carried out in an open field chamber (40 × 40 × 30 cm) made of non-transparent plexiglass. The NOR procedure consisted of habituation, acquisition and test phases. In the habituation phase, the mice were placed in an open field for 10 min without any object and subjected to an acquisition phase following an interval of 24 h. During acquisition, the mouse was allowed to freely explore two identical objects [square white plastic bottle (length 6 cm, height 12 cm)] for 5 min. The objects were located at the center of the chamber and positioned 10 cm apart, equidistant from the walls. Thus, mice had an equal chance to explore both the objects in order to avoid the possibility of side bias. Thirty minutes following the acquisition trial, the mice were re-subjected to the testing phase. One of the identical objects was replaced with a novel object (N) [circular black glass bottle (diameter 6 cm, height 12 cm)] while keeping another familiar object (F) undisturbed. These objects were placed in the same locations as in the acquisition phase. Object exploration was measured using two stopwatches during the experimental session by the observer blind to the treatment. With a view to eliminating olfactory cues of the previous animal, the chamber and objects were cleaned by using 70% ethanol, following each animal. Behavior like sniffing or touching the object with the nose at a distance of not > 2 cm, was considered as the exploration. Exploration time for each object was recorded along with the discrimination index (DI) calculated as, DI = (N − F)/(N + F), representing difference in exploration time expressed as a proportion of

the total time spent exploring the two objects in testing phase (Ennaceur and Delacour, 1988; Borkar et al., 2019).

2.3.3. Y-maze test

The spontaneous alteration was analyzed using the Y-maze apparatus as described previously (Alkam et al., 2007). Y-maze consisted of three radial arms (50 cm long, 12 cm high and 4 cm wide) positioned at an equal angle (120°). Each mouse was placed at the cross points of arms and allowed to move freely throughout the maze for an 8 min session. The sequence of arm entries was recorded manually. Spontaneous alteration is defined as the entry into all three arms on consecutive choices in overlapping triplet sets of a non-repeated pattern. The percent (%) spontaneous alteration behavior was calculated as the ratio of actual to possible alterations (defined as the total number of arm entries − 2) × 100. Herein, the spontaneous alterations (correct preference) were assessed in control and MK-801 treatment mice.

2.3.4. Radial arm maze task

For the assessment of working and reference memory, 8-arm radial maze elevated 25 cm from the floor and each arm (9 × 40 cm) radiated from an octagonal central starting platform (perimeter 12 × 8 cm) was used (Olton and Samuelson, 1976). Animals were subjected to an 11 day session divided into three phases; acclimation, training and testing. During the entire 11 days period, animals were provided with a restricted food to maintain their body weights at 90–95% of pretest weight. During acclimation (days 1–3) animals were allowed to explore the radial arm maze with randomly placed food pellets (45 mg pellets) throughout the maze. In the training phase (days 4–6), the mice were placed in the center of the maze, facing towards arm 1, arms 1, 2, 4 and 7 were baited with a food pellet at the end of each arm, whereas arms 3, 5, 6 and 8 were closed. Internal maze visual cues (white tape) were provided at the entry and end of each baited arm. External visual cues such as wall-paper, location of door and experimenter provided the additional spatial reference cues. Four training sessions were performed for each animal on days 4–6, each lasting until all food pellets were retrieve or 5 min duration whatever is earlier. The testing phase was conducted over days 7–11. Throughout the trials, all the arms of the radial maze were kept open. At the beginning of each testing session, the animals were placed in the maze facing arm 1. Four testing sessions were conducted each day for every animal and lasted until all food pellets were retrieved or 5 min duration whatever is earlier. Entries into non-baited arms were counted as reference memory errors and re-entry into previously baited arms were counted as working memory errors (Hillman et al., 2011).

2.4. Immunohistochemical analysis of NeuN

Immunohistochemical analysis was carried out to assess the effect of MK-801 at PND 7, 14 or 21 on the number of mature neuronal cells in PFC and hippocampal DG, CA1 and CA3 regions using NeuN expression at adolescence stage (PND 30) in mice. Animals were deeply anesthetized with an overdose of thiopentone sodium (60 mg/kg, intraperitoneally, ip), perfused transcardially with heparinized phosphate-buffered saline (PBS; pH 7.45) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were post-fixed in the same fixative overnight, cryoprotected in 30% sucrose solution and serially sectioned on a cryostat (CM1850, Leica Microsystems, Germany) at 30-µm thickness in coronal plane. Sections were processed for NeuN immunolabeling using the immunofluorescence method described earlier (Borkar et al., 2018). Thereafter, sections were treated with 0.5% Triton X-100 for 20 min and incubated in 5% bovine serum albumin (BSA) for 60 min, followed by incubation in the mouse NeuN antibody (Millipore, USA, Cat No. MAB377; 1:500 dilution) overnight at 4 °C. After rinsing in PBS, sections were incubated separately in anti-mouse Alexa Fluor® 568 IgG (Jackson Immunoresearch) at 1:500 dilution for 3 h at room temperature. Finally, the sections were mounted and observed under Leica DM-2500 fluorescence microscope using the filters for Alexa Fluor® 568.

2.5. Morphometric analysis

Transverse sections of PFC and hippocampal DG, CA1 and CA3 of control (saline), PND 7, 14 and 21 group mice were scanned. The images were captured, adjusted for brightness and contrast and merged using Adobe Photoshop CS4 software (Adobe Systems Inc., San Jose, CA, USA). The percentage (%) immunoreactive area occupied by NeuN was evaluated. The images were digitized and analyzed by using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The background was considered as threshold and area occupied by immunostained cells was measured based on individual pixel intensity in all the treatment groups. The data were collected from the predetermined areas (as demarcated by squares in the Fig. 3E, J, O and T) from both the sides of five sections of each brain, drawn from five animals in each group. The morphometric data obtained from five sections of each animal were averaged, thus representing an individual animal. The percent (%) immunoreactive area has been automatically generated by using ImageJ software. The data for each group were collated, and mean ± standard error of the mean (SEM) was calculated (Borkar et al., 2018, 2019).

2.6. Western blot analysis

Immunoblot assay was performed to evaluate the levels of activated caspase-3 and pAkt in PFC and hippocampus of the mice pups treated with MK-801 on PND 7, 14 or 21. Mice pups were decapitated 8 h after MK-801 or saline treatment, the brains were rapidly removed, and various brain regions (PFC and hippocampus) were dissected on ice. Tissue samples were homogenized in RIPA buffer (150 mM NaCl, 1% v/v TritonX-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris-HCl, 1 mM EDTA and 500 nM PMSF, adjust pH 8.0) incubated on ice for 20 min, and centrifuged at 10,000 rpm for 20 min at 4 °C. Supernatants were removed and frozen at −20 °C for analysis of activated caspase-3 and pAkt.

Bradford assay was used to analyze the total protein concentrations in the cell lysates. Lysates were mixed with an equal quantity of 0.05% w/v bromophenol blue and heated at 95 °C for 5 min. The supernatant was loaded (20 µg/lane, protein) along with protein marker (Puregene, Cat. PG500-0500PI) in the adjacent lane and resolved on 10% SDS–PAGE. Proteins on the gel were then transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane (Sigma) using semi-dry transfer method (BioRad). The blots were blocked in 3% BSA and incubated with either rabbit activated caspase-3 antibody (Sigma, Cat No. C8487; 1:5000 dilution) or rabbit $pAkt^{Ser\text{-}473}$ antibody (Cell signaling Technologies, USA, Cat. No. 4058; 1:2000 dilution) for 16 h at 4 °C. This was followed by incubation in goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies (Cell Signaling, dilution 1:10,000), individually. The signal was detected in ChemiDoc (BioRad) using chemiluminescent HRP substrate (Millipore). The intensity of activated caspase-3 or $pAkt^{Ser\text{-}473}$ immunoreactive band in the PFC or hippocampus of the mice from different age group was measured using Quantity One software. The percentage intensity of activated caspase-3 or $pAkt^{Ser\text{-}473}$ protein relative to GAPDH (loading control) was calculated and the values were represented as mean ± SEM (Kumar et al., 2017).

2.7. Statistical analysis

Data obtained from behavioral, immunohistochemistry and western blot were expressed in mean ± SEM. Statistical significance of a number of crossings, percent (%) alterations, DI, body weight change and morphometric data based on immunohistochemistry were assessed by the one-way analysis of variance (ANOVA) followed by the

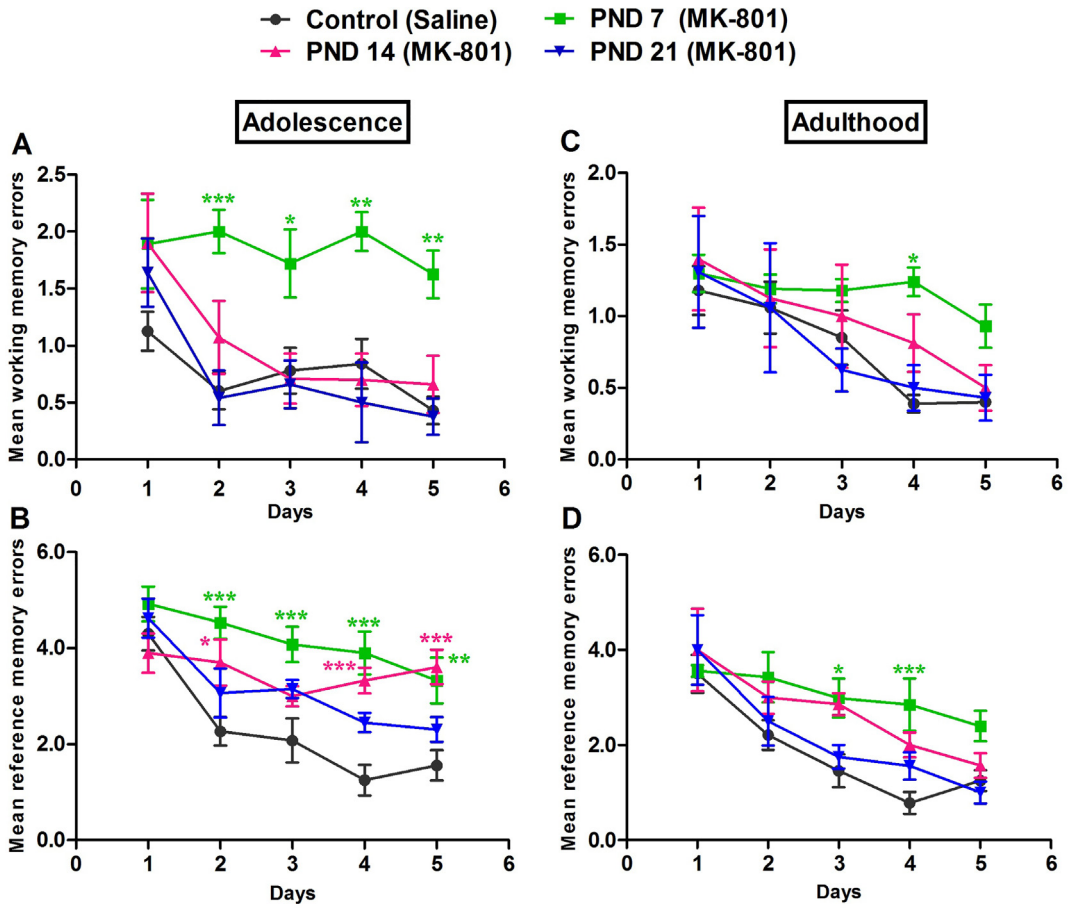

Fig. 2. Effect of MK-801 (1 mg/kg, sc), administered at postnatal day (PND) 7, 14 or 21 and tested at adolescence (A–B) and adulthood stage (C–D), on working memory (A and C) and reference memory (B and D) assessed using radial arm maze test in mice. While PND 7 treatment group showed a significant higher working and reference memory error, PND 14 group showed only significant higher memory errors for reference memory at adolescence. At adulthood stage, the mice treated with MK-801 at PND 7 were continued to show higher working as well as reference memory errors as compared to saline treated (control) group. The data are expressed as means ± SEM and analyzed by two-way ANOVA followed by Bonferroni's multiple comparison tests. *P < 0.05, **P < 0.01 and ***P < 0.001 vs control (saline) (n = 5–9/group).

Bonferroni's multiple comparison test, using Prism 5.0 (GraphPad Software Inc., CA, USA). Working and reference memory errors between control (saline) and treated (MK-801) mice were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. Densitometry data obtained was analyzed using Student's *t*-test. Differences were considered significant at P < 0.05.

3. Results

3.1. Postnatal MK-801 treatment induces locomotor hyperactivity

Mice were treated with MK-801 (1 mg/kg, sc) at PND 7, 14 or 21 and assessed at the adolescence (PND 30) and adulthood (PND 60) for the number of crossings in OFT (n = 5–9/group). One-way ANOVA showed that, MK-801 treatment significantly influenced the number of crossings in mice at adolescence [F(3,25) = 5.506, P < 0.01] and adulthood [F(3,25) = 4.572, P < 0.05]. The post-hoc Bonferroni's multiple comparison test revealed that, at adolescence, MK-801 treatment significantly increased the number of crossings in PND 7 (P < 0.01) and 14 (P < 0.05) groups, as compared to control (saline administered) animals. However, it remained unaffected when treatment given at PND 21 (P > 0.05), and McIlwain et al., 2013thus single dose treatment of MK-801 failed to induce hyperactivity in the animals in the late phase (Fig. 1B). On the other hand, Bonferroni's multiple comparison test indicates that only PND 7 group showed significantly increase in a number of crossings (P < 0.05) as compared to control when tested at adulthood stage (Fig. 1C).

3.2. Postnatal MK-801 treatment reduces spontaneous alterations in Y-maze

To estimate the effect of neonatal blockade of NMDA receptor on spontaneous alteration, mouse pups were treated with MK-801 (1 mg/kg, sc) at PND 7, 14 or 21 and subjected to Y-maze (n = 5–9/group). Application of one-way ANOVA revealed that MK-801 treatment significantly affect percent (%) spontaneous alternations in mice when assessed at adolescent [PND 30, F(3,25) = 14.26, P < 0.0001] and adult stage [PND 60, F(3,25) = 4.240, P < 0.05]. The Bonferroni's multiple comparison test showed that, MK-801 treatment significantly reduced the spontaneous alterations, when assessed at day 30 [PND 7 (P < 0.01), 14 and 21 (P < 0.001), Fig. 1D], but not at day 60 [PND 7, 14 and 21; P > 0.05 (Fig. 1E)] as compared to control group.

3.3. Postnatal MK-801 treatment impaired object recognition memory in NOR test

Another set of animals was administered with MK-801 (1 mg/kg, sc) and object recognition memory was assessed in PND 7, 14 and 21 treatment groups using NOR test (ITI-30 min) at adolescence and adulthood (n = 5–9/group). Application of one-way ANOVA showed a significant effect of MK-801 treatment on the DI at both adolescence [F(3,24) = 8.180, P < 0.001] and adulthood [F(3,24) = 14.41,

P < 0.0001]. Bonferroni's multiple comparison test applied to the data on animals at adolescence stage revealed that MK-801 significantly decreased the DI as compared to saline injected group of PND 7 (P < 0.01), 14 (P < 0.01) and 21 (P < 0.05) (Fig. 1F). Moreover, when assessed at adulthood stage all treatment groups showed significant decrease in the DI as compared to saline injected group [PND 7, 14 (P < 0.001) and 21 (P < 0.01); Fig. 1G].

3.4. Postnatal MK-801 treatment impaired working and reference memory in radial arm maze

To estimate the effect of NMDA receptor blockade on working and reference memory, mouse pups were treated with MK-801 (1 mg/kg, sc) at PND 7, 14 or 21 and were subjected to radial arm maze at adolescent and adulthood (n = 5–9/group). Application of two-way ANOVA showed no significant effect of interaction between variables viz., treatment and testing days for working memory errors [F (12,110) = 1.164, P > 0.05] and reference memory errors [F (12,110) = 1.685, P > 0.05] when tested at adolescence. Furthermore, two-way ANOVA revealed a main effect of MK-801 treatment [working memory errors, F(3,110) = 22.48, P < 0.0001; reference memory errors, F(3,110) = 20.73, P < 0.0001] and testing days [working memory errors, F(4,110) = 7.052, P < 0.0001; reference memory errors, F(4,110) = 13.88, P < 0.0001]. Post-hoc Bonferroni's multiple comparison test revealed the increase in working memory errors in the PND 7 group [working memory errors, at day 2 (P < 0.001), 3 (P < 0.05), 4 and 5 (P < 0.01)], however other groups (PND 14 and PND 21) did not show significant difference in working memory errors as compared to control group (Fig. 2A). PND 7 [reference memory errors, at day 2, 3, 4 (P < 0.001) and 5 (P < 0.01)], PND 14 [at day 2 (P < 0.05), 4 and 5 (P < 0.001)], but not PND 21 group showed significant difference in reference memory errors as compared to control group (Fig. 2B).

Later on, the effect of postnatal MK-801 treatment on working and reference memory was also assessed at the adulthood stage. Application of two-way ANOVA showed no significant effect of interaction between variables viz.; treatment and testing days for working memory errors [F (12,110) = 0.5631, P > 0.05] and reference memory errors [F (12,110) = 0.9795, P > 0.05]. However, two-way ANOVA also revealed a main effect of treatment [working memory errors, F (3,110) = 3.768, P < 0.05; reference memory errors, F (3,110) = 8.820, P < 0.0001] and testing days [working memory errors, F(4,110) = 7.449, P < 0.0001; reference memory errors, F (4,110) = 18.97, P < 0.0001]. As revealed in post-hoc Bonferroni's multiple comparison test, working and reference memory errors vary in the groups; PND 7 (working memory errors, day 4, P < 0.05), but other groups (PND 14 and PND 21) did not show significant difference in working memory errors as compared to control group (Fig. 2C). While reference memory errors were higher in PND 7 group [at day 3 (P < 0.05) and 4 (P < 0.001)], treatment at PND 14 or 21 did not produce a significant difference in reference memory errors as compared to control group (Fig. 2D).

3.5. Postnatal MK-801 treatment reduced NeuN immunoreactivity in PFC and hippocampal DG and CA3 regions

Fig. 3 summarizes the effect of postnatal administration of MK-801 in PND 7, 14 or 21 groups assessed at adolescent stage (n = 5–6/group). MK-801 treatment significantly reduced the percent (%) NeuN immunoreactive area in the PFC regions when administered at PND 7 or 14, but not at PND 21, as compared to the control animals (Fig. 3E, P < 0.01). On the other hand, the percent (%) area covered by NeuN immunoreactive cells was significantly reduced in the hippocampal DG and CA3 but not in CA1 region when MK-801 treatment was given at PND 7 (Fig. 3J, T and O, P < 0.001, P < 0.05 and P > 0.05, respectively). However, no significant changes were noted in percent (%) NeuN immunoreactivity area in PND 14 or 21 groups in DG, CA1 and CA3 (P > 0.05).

3.6. Effect of postnatal MK-801 treatment on caspase-3 and pAkt expression in PFC and hippocampus

To estimate the effect of NMDA receptor blockage on pAkt and caspase-3 expression in PFC and hippocampus, mouse pups were treated with MK-801 (1 mg/kg, sc) or saline at PND 7, 14 or 21 (n = 4/group). Eight hours after the treatments, the brains were isolated and samples were prepared for Western blot analysis. Fig. 4 represents the relative expression of $pAkt^{Ser\text{-}473}$ (C–D) and caspase-3 (E–F) in PFC (A, C and E) and hippocampus (B, D and F) in MK-801 and age matched saline control group. Application of Student's *t*-test showed a significant increase in pAkt in the hippocampus (P < 0.05) as compared to the age matched control. Similarly, MK-801 treatment at PND 7 also showed increase in expression of caspase-3 in PFC and hippocampus (P < 0.001). However, no significant effect was observed when MK-801 treatment given at PND 14 or 21 in the caspase-3 and pAkt expression (P > 0.05).

4. Discussion

4.1. Methodological consideration

The pattern of neurodegeneration, triggered by NMDA receptor antagonist, seems to follow a precise time line during developmental age. MK-801 induced apoptosis was high on both PND 0 and PND 3, increased further between PND 3 and PND 7, and then decreased sharply between PND 7 and PND 14. A low abundance of apoptotic neurons was detected at PND 21 in MK-801 treated animals (Ikonomidou et al., 1999). Moreover, region specific apoptotic neurodegeneration was observed in rodents on postnatal MK-801 treatment (Lema Tomé et al., 2006; Turner et al., 2007). However, mechanisms underlying variation in these apoptotic pattern induced by NMDA receptor blockade are still unknown. In this background, we investigate the intracellular mechanisms following MK-801 treatment at three different postnatal developmental phases (PND 7, 14 or 21). We also try to correlate the changes with the ADHD-like condition with the profile of mature neuronal cell density in PFC and hippocampus of mice. Compromised attention/alertness, sensory processing, working memory deficits, and hyperactivity are well recognized symptoms of ADHD. We employed OFT for the assessment of hyperactivity, while the Y-maze, NOR test and radial arm maze were used to analyze deficit in attention and working/reference memory (Zhuang et al., 2001; Fredriksson and Archer, 2004; Bouchatta et al., 2018).

4.2. ADHD induction with MK-801

In rodents, synaptogenesis mostly occurs during the first three weeks after birth with a peak at two weeks, which corresponds to the third trimester of pregnancy and several years after birth in humans (Semple et al., 2013). During this period, the constructive anatomical processes like rapid increase in brain weight, the proliferation of astroglial and oligodendroglial cells, neuronal axonal elongation and dendritic arborization have been noted (Ikonomidou et al., 1999). Follow-up studies revealed that glutamate mediates its neurotrophic role via modulation of intracellular calcium concentration through NMDA receptor (Bhave and Hoffman, 1997; Dammerman and Kriegstein, 2000). Moreover, NMDA receptor antagonism triggers apoptotic neurodegeneration in the developing brain (Olney et al., 2000). These neuropathological changes might be causal to the induction of hyperactivity and attention/memory deficits in animals, resembling symptoms of ADHD. Importantly, we observed core symptoms of ADHD (hyperactivity and attention/memory deficit) in mice at the adolescent stage following MK-801 treatment during brain

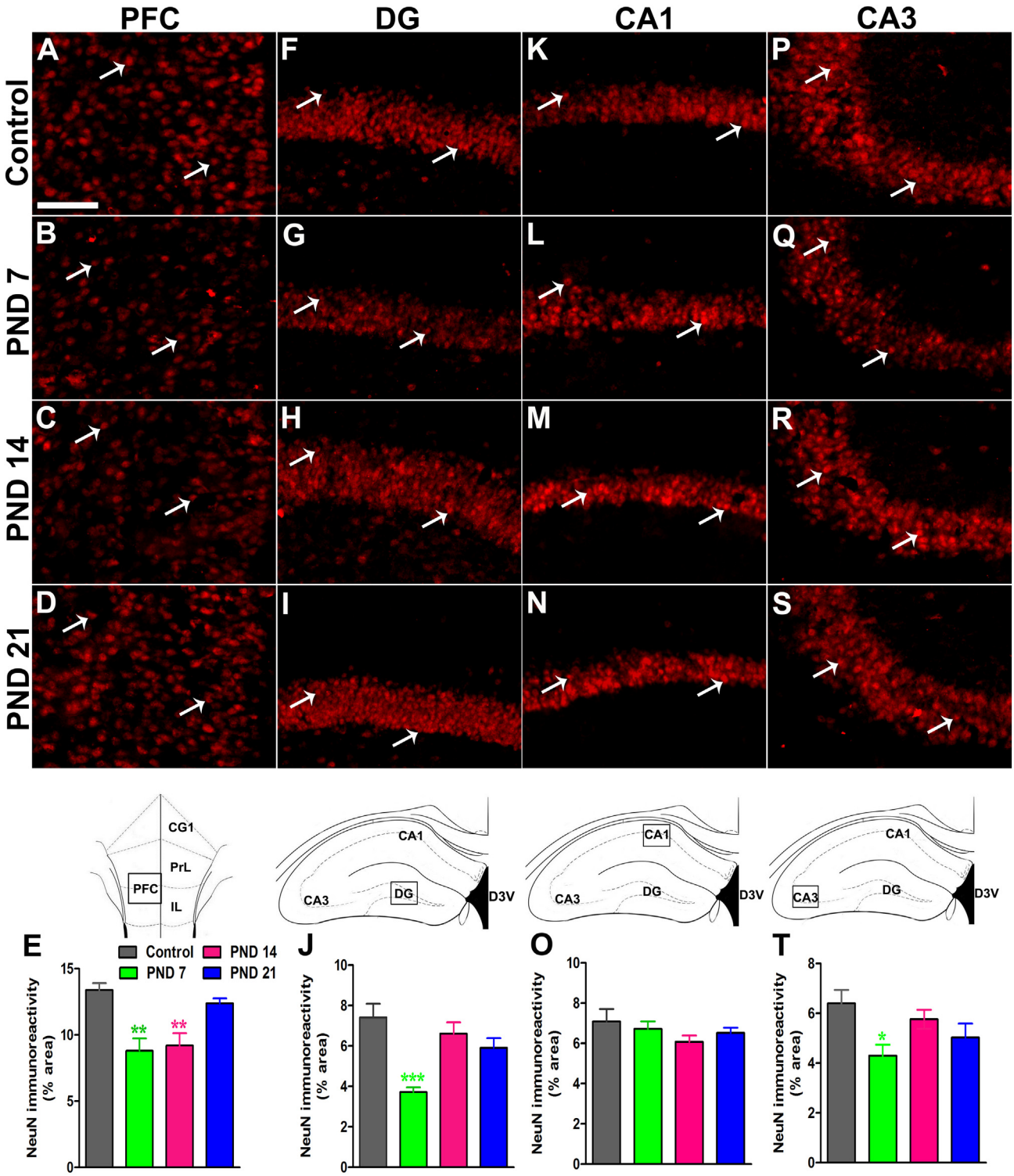

Fig. 3. Effect of saline (control; A, F, K and P) and MK-801 (1 mg/kg, sc) administered at postnatal day (PND) 7 (B, G, L and Q), 14 (C, H, M and R) or 21 (D, I, N and S) on neuronal nuclei (NeuN) immunoreactivity (arrows) within prefrontal cortex (PFC) (A–D) and hippocampal dentate gyrus (DG) (F–I), cornu ammonis 1 (CA1) (K–N) and cornu ammonis 3 (CA3) (P–S) regions in adolescence (PND 30) mice. Each bar represents the mean ± SEM of ten measurements taken from predetermined fields of PFC, DG, CA1 and CA3, from both the sides of each brain (n = 5–6/group). Note a significant decrease in the NeuN immunoreactivity percent (%) area in the PFC, DG and CA3 regions but not within CA1 in the PND 7 group. PND 14 treatment group has also shown the reduced NeuN immunoreactivity only in the PFC region. The outline of the coronal sections showing different regions of the PFC, DG, CA1 and CA3 (coordinates from bregma: PFC, +1.54 mm; DG, −1.94 mm; CA1, −1.94 mm; CA3, −1.94 mm) and indicated by square (not to scale) from which the measurements were collated (Paxinos and Franklin, 2001). The morphometric data on percent (%) area occupied by NeuN immunoreactive elements in PFC (E), DG (J), CA1 (O) and CA3 (T) were individually analyzed using one-way ANOVA followed by Bonferroni's multiple comparison tests. *P < 0.05, **P < 0.01, ***P < 0.001 vs control group. CG1, cingulate gyrus 1; PrL, prelimbic cortex; IL, infralimbic cortex; D3V, dorsal 3rd ventricle. Scale bar: 100 μm.

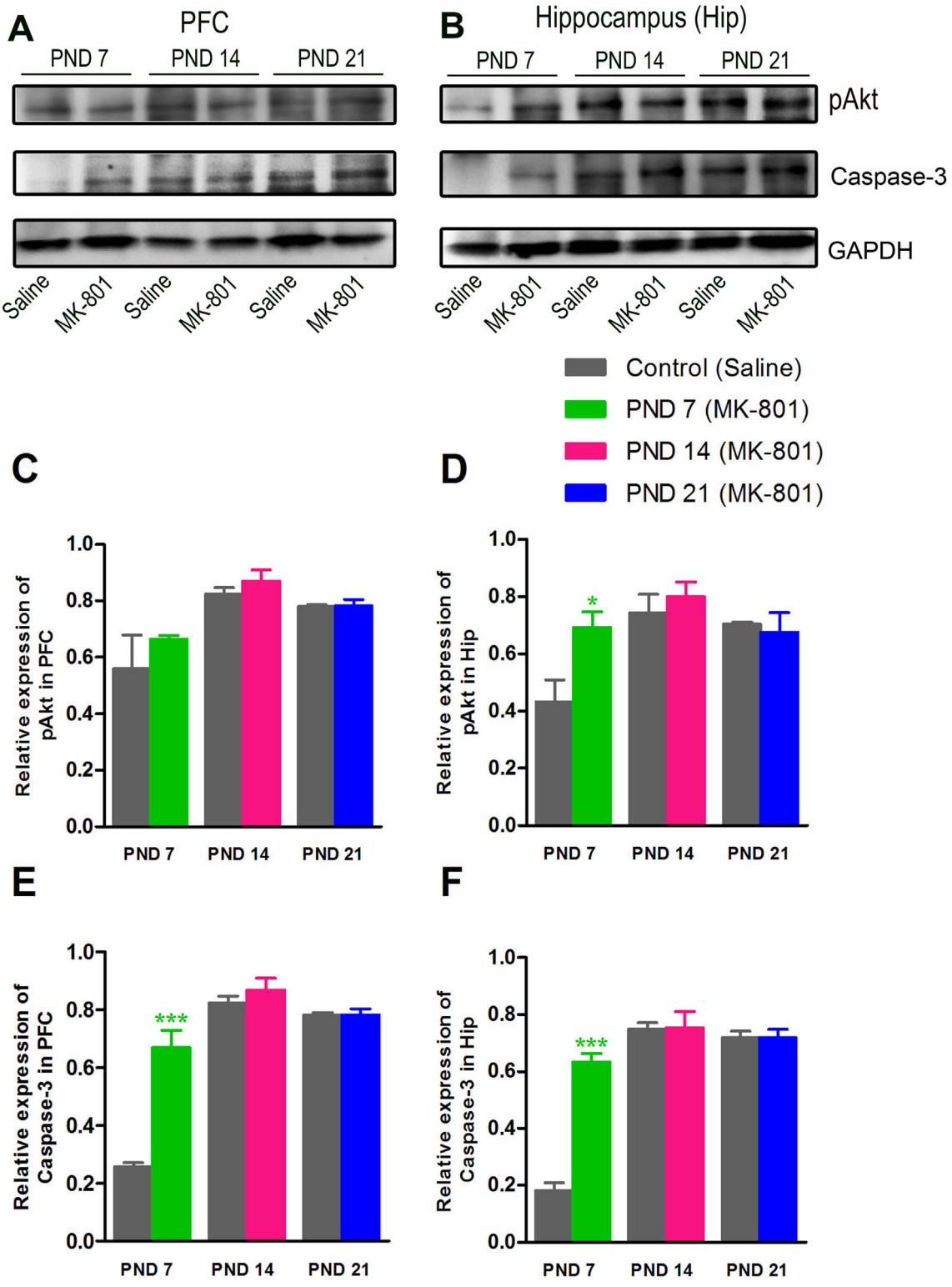

Fig. 4. Western blot data represents effect of saline (control) and MK-801 (1 mg/kg, sc) administered at postnatal day (PND) 7, 14 or 21 on pAkt (C–D) and caspase-3 (E–F) within prefrontal cortex (PFC) (A, C and E) and hippocampus (B, D and F) in mice. Each bar represents the mean ± SEM of four measurements (n = 4/group). Note a significant increase in the caspase-3 expression in the PFC and hippocampus regions following MK-801 treatments at PND 7. However, pAkt expression was only increased in the hippocampus of PND 7 treatment group. The data on relative expression of activated caspase-3 and pAkt were individually analyzed using Student's *t*-test. *P < 0.05, ***P < 0.001 vs control (saline). Hip, hippocampus; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

development.

4.3. Age-specific changes followed MK-801 treatment

Fredriksson and Archer (2004) reported that postnatal administration of MK-801 at PND 11 leads to hyperactivity and attention/memory deficit, a characteristic feature of ADHD in mice. However, data on the effect of age-specific MK-801 (1 mg/kg, sc) treatment on behavior (hyperactivity and attention/memory deficit) at the adolescence stage is not available. Mice administered with MK-801 particularly on PND 7 and 14, produced marked hyperactivity and attention/memory deficit later in life. However, MK-801 treatment at PND 21 did not produce similar results.

While MK-801 treatment at PND 7 or 14 decreased the population of mature neurons in PFC, no change was observed in PND 21 group, when assessed at the adolescent stage. Imaging studies indicate that ADHD patients often have smaller PFC volume (Castellanos et al., 1996; Mahone et al., 2011). Lesions of the PFC produce symptoms like ADHD (Itami and Uno, 2002). Akin to ADHD patients, PFC lesioned patients have poor concentration and organization, are vulnerable to disruption, impulsivity, and may have difficulty in controlling overt behaviors (Brennan and Arnsten, 2008). NMDA receptor antagonism at PND 7 decreased the parvalbumin containing neurons in primary somatosensory, motor and retrosplenial cortices (Wang et al., 2008). In this background, we suggest that observed symptoms in PND 7 or 14 groups following MK-801 treatment might be attributed to the neuronal loss in PFC.

Hippocampus regulates attention, executive function, working and visuospatial memory (Baddeley et al., 2011). Damage to the hippocampus in a neonate disrupts PFC development and results in cognitive and working memory dysfunctions in rats (Marquis et al., 2008). Plessen et al. (2006) reported morphological changes in the hippocampus of ADHD children and adolescents and associated these changes with ADHD symptoms. We found a decrease in the number of mature neurons of DG and CA3 in PND 7, but not in PND 14 or 21 groups. Previous studies support these findings since MK-801 exposure at PND 7 resulted in a decrease in the neuronal population in the hippocampus (Harris et al., 2003; Li et al., 2015). MK-801 treatment has been shown to produces an age-specific pattern of neurodegeneration in different brain regions (Ikonomidou et al., 1999). We suggest that NMDA receptor blockade at PND 7 significantly affect the brain morphology (PFC, DG and CA3) and results in ADHD-like symptoms in mice.

4.4. Corresponding intracellular changes

Akt is a serine/threonine protein kinase which regulates cell growth, proliferation and survival (Franke et al., 1997; Song et al., 2005). For robust activation of Akt pathway, it is essential to phosphorylate at both Ser-473 and Thr-308 sites (Alessi et al., 1996). Phosphorylated Akt activates many downstream substrates including GSK-3β (Carracedo and Pandolfi, 2008). GSK-3β is a protein kinase shown to be regulating neurogenesis, neurodevelopment and cell fate (Hur and Zhou, 2010). Several studies suggested dysfunction of GSK-3β signaling in various psychiatric and neurological disorders (Jope and Roh, 2006; Beurel et al., 2015). Signaling molecules like Akt and wnt, inhibit the activity of GSK-3β (Peineau et al., 2008; Valvezan and Klein, 2012). MK-801 or phencyclidine treatment in rat induces psychotomimetic effect through the phosphorylation of GSK-3β (Svenningsson et al., 2003; Ahn et al., 2005). While some studies showed NMDA receptor antagonism leads to increase in $pAkt^{Ser\text{-}473}$ (Ahn et al., 2005; Seo et al., 2007), others demonstrated a decrease in $pAkt^{Ser\text{-}473}$ (Lei et al., 2008; Kim et al., 2010). We observed that a single dose MK-801 administration (1 mg/kg, sc) at PND 7 (but not at PND 14 or 21) induced region specific (hippocampus) increase in $pAkt^{Ser\text{-}473}$. Our results seem to vary from the previous studies which showed that administration of phencyclidine or MK-801 at PND 7 decreased the $pAkt^{Ser\text{-}473}$ (Lei et al., 2008; Kim et al., 2010). This difference might be explained partially by biphasic dose response to MK-801 (Lei et al., 2008). For instance, Ahn et al. (2005) showed that doses up to 1 mg/kg of MK-801 increased $pAkt^{Ser\text{-}473}$, whereas higher dose led to a decrease in $pAkt^{Ser\text{-}473}$. Further, Kim et al. (2010) demonstrated decreased $pAkt^{Ser\text{-}473}$ in PFC after two injections (8 h apart) of MK-801, but no change has observed followed single injection. Similarly, we found no change in $pAkt^{Ser\text{-}473}$ expression in PFC after a single dose of MK-801 administration. Although studies correlate phosphorylation of $Akt^{Ser\text{-}473}$ with Akt kinase activity (Fu et al., 2016; Kuttikrishnan et al., 2019), basically phosphorylation of Akt at either Ser-473 or Thr-308 has very little effect on Akt activity (Alessi et al., 1996; Song et al., 2005). Moreover, increase in $pAkt^{Ser\text{-}473}$ alone did not prevent cell death occurred due to exposure of glutamate in hippocampal neurons (Kitagawa et al., 2002). Similarly we observed an increase in $pAkt^{Ser\text{-}473}$ followed MK-801 (1 mg/kg, sc) treatment at PND 7 in hippocampus, but phosphorylation of the $Akt^{Ser\text{-}473}$ did not prevent cell death as reflected in the increase in caspase-3 (cell death marker). Further, it was shown that MK-801 treatment phosphorylates $Akt^{Ser\text{-}473}$, but not $Akt^{Thr\text{-}308}$ (Ahn et al., 2005). We suggest that the activation of $pAkt^{Ser\text{-}473}$ might not be sufficient to prevent cell death in hippocampus followed MK-801 treatment at PND 7. We speculate that simultaneous activation of both $Akt^{Ser\text{-}473}$ and $Akt^{Thr\text{-}308}$ might fully activate Akt kinase and prevent neuronal cell death in hippocampus followed NMDA receptor blockade.

Apoptosis or programmed cell death is the process of death and removal of unwanted cells during brain development to form normal neuronal circuitries. Caspase-3 is death protease which activates and cleaves many key cellular proteins during the natural cell death process (Porter and Jänicke, 1999). MK-801 exposure at brain development (PND 7) activates caspase-3 and abnormally potentiates the programmed cell death in various brain regions including PFC and hippocampus (Coleman et al., 2009; Lyall et al., 2009; Turner et al., 2009). But the comparative age-specific effect of MK-801 treatment at different time points (PND 14 or 21) on caspase-3 in PFC and hippocampus has not been studied yet. We found an increase in caspase-3 at PND 7 followed MK-801 injection in PFC and hippocampus, but no change has been observed in PND 14 and 21 groups. PND 7 and PND 14 treatment groups showed ADHD phenotype when assessed at adolescence, but only PND 7 group continue these symptoms in adulthood. We suggest that exposure of MK-801 at PND 7 (but not PND 14 or 21) triggered the natural cell death process in PFC and hippocampus via activation of caspase-3. The observed ADHD-like symptoms at adolescent and adulthood stages may be a consequence of the drastic changes in synaptogenesis at PND 7.

5. Conclusion

The present study reveals that, blockade of NMDA receptors, preferentially at PND 7, elicit changes in the intracellular signaling pathways (increase in caspase-3) in the mouse PFC and hippocampus. This might be responsible for observed decrease in the mature neuronal population as a result of apoptosis and behavioral deficit resembling to ADHD, at adolescence stage of life. The early life (PND 7) exposure of NMDA receptor antagonist produced long lasting behavioral changes and endured in the adulthood stage of the life.

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Declaration of competing interest

None.

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