Neonatal exposure to the experimental environment or ketamine can induce long-term learning dysfunction or overmyelination in female but not male rats

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Ketamine can induce neurotoxicity after exposures to the developing brain. To investigate whether ketamine at subanesthetic dosage or its environmental condition can cause long-term cognitive dysfunction after multiple exposures in male or female neonatal rats, postnatal day 5 (P5)-day-old Sprague-Dawley rats were randomized into three groups: ketamine group, vehicle group, and control group (no disturbance). Learning and memory abilities from P60 to P65 and immunofluorescence tests for myelin basic protein (MBP) in gray matter on P65 were conducted. The results showed that in female rats, the path length on day 1 in ketamine group and on days 1 and 2 in vehicle group was longer than that in control ($P < 0.05$), but there was no difference between ketamine and vehicle groups ($P > 0.05$). The mean density of MBP in the medial prefrontal cortex (mPFC) was significantly increased in vehicle and ketamine groups compared with that in control ($P < 0.05$), and there was a significant difference between vehicle and ketamine groups ($P < 0.05$), but MBP density was not changed in CA1 or CA3 region ($P > 0.05$). In male rats, there were no significant differences in path length among the groups, and the density of MBP in the mPFC and hippocampus in vehicle or ketamine group was not different from that in control ($P > 0.05$). Pearson's correlation analysis showed that there was a positive correlation between MBP density in the mPFC and path length in adult female rats ($r = 0.753$, $P < 0.01$). Overall, the results suggested that neonatal female rats exposed to multiple episodes of the experimental environment can develop learning dysfunction in adulthood, which may result from overmyelination in the mPFC, but male rats were not affected. Ketamine could increase myelination in the mPFC in female rats, but it did not induce learning dysfunction in adulthood; therefore, ketamine may be a safe drug for pediatric anesthesia. *NeuroReport* 30:491–497 Copyright © 2019 The Author(s). Published by Wolters Kluwer Health, Inc.

Introduction

Inhalational or intravenous general anesthetic agents can induce widespread neuronal apoptosis or long-term cognitive dysfunction after multiple exposures in neonatal animals [1–3]. Clinical studies have shown that infants less than 4 years old exposed to general anesthesia three or more times are likely to develop impaired learning and memory later in life [4,5].

Ketamine, a noncompetitive *N*-methyl-D-aspartic acid (NMDA) receptor antagonist, is widely used in pediatric anesthesia at subanesthetic dosage, and it can provide sedation for diagnostic or invasive procedures. Multiple exposures to ketamine at subanesthetic dosage can induce widespread neuronal apoptosis in the developing brain [6], but it is unclear whether it can induce cognitive dysfunction in adulthood, which is the biggest concern in pediatric anesthesia in the clinic.

Myelination can be inhibited in white matter in newborn animal brains by propofol, an intravenous general anesthetic agent [7]. Myelination reaches the peak level ∼2 weeks after birth in rodents [8]. Myelin, either in white matter or in gray matter, is necessary for myelinated nerve fibers and plays a key role in brain development and cognitive function [9]. NMDA receptor is involved in myelination in the central nervous system [10], but whether ketamine can inhibit myelination when exposed in the developing brain and consequently impair cognitive function in adulthood has not been thoroughly elucidated to date, and it should be studied to clarify concerns with ketamine usage in pediatric anesthesia.

In the previous studies [11–13], when the neurotoxicity of ketamine in the developing brain was studied, vehicle control was necessary in which the solvent of ketamine was administered, but it is clear now, whether in ketamine anesthesia or vehicle control condition, that the

experimental environment was considerably different from normal living conditions but very similar to those in clinical pediatric anesthesia, such as maternal separation, oxygen delivery, pain on injection, and even fear. However, whether multiple short-episode exposures to the experimental environment in neonatal animals can cause long-term cognitive impairment needs to be verified. In addition, several anesthetic agents including sevoflurane and isoflurane can exhibit sex differences in inducing cognitive dysfunction [14], and whether ketamine exhibits sex differences is unclear to date.

Thus, in this study, neonatal rats were exposed to ketamine at a subanesthetic dosage, and the effects of ketamine and the experimental environment on myelination and long-term cognitive function were studied. The relationship between myelination and long-term cognitive dysfunction was investigated. Furthermore, we tried to figure out whether sex differences exist in ketamine-induced neurotoxicity.

Materials and methods
Animals
Postnatal day 5 (P5) Sprague-Dawley rat pups of either sex with their lactating dams were purchased from the Animal Center of Chongqing Medical University, China (permission number: SCXK 2012-0001). All rat pups were housed in a reversed 12/12-h light–dark cycle (light at daytime) at 22°C and allowed access to food and water *ad libitum*. Rat pups were weighed throughout the experiment, weaned on P21, and then male and female pups from the same litter were housed separately. All animal experiments were approved by the Ethics Committee of Chongqing University Cancer Hospital and followed the Guidance Suggestions for the Care and Use of Laboratory Animals formulated by Ministry of Science and Technology of China. Every effort was made to minimize animal suffering.

Experimental protocol
The flowchart is shown in Fig. 1. P5 pups of either sex from different litters were randomly divided into three groups ($n = 36$, 18 of each sex). For ketamine group, the pups received intraperitoneal injections of 50 mg/kg

ketamine (1 mg/ml), once a day with a 24 h interval from P5 to P9. For vehicle group, the pups received intraperitoneal injections of saline with equivalent volume and were exposed to the same environmental conditions as in ketamine group. For control group, the pups were housed in normal living conditions (in feeding cages with their mother) and were not disturbed. During anesthesia, the pups were placed on a heating pad, and their rectal temperature was maintained at 37 ± 1°C. Oxygen saturation was monitored using pulse oxymetry and maintained at more than 95%. In our preliminary experiment, the duration of loss of righting reflex after each intraperitoneal injection of 50 mg/kg ketamine was ~180 min, and a tail-pinch was used intermittently to measure the depth of ketamine anesthesia and whether body movement could be induced from every pinch. Therefore, 50 mg/kg of intraperitoneal ketamine was considered a subanesthetic dosage, as it only caused deep sedation (loss of the righting reflex) and did not inhibit body movement from pain stimulation. In the three groups, the tail-pinch was not used to minimize the suffering of the animals as 50 mg/kg was proved to be subanesthetic in the preliminary experiment. Some pups were used for transcardial arterial blood gas analysis before full recovery from ketamine anesthesia (six in each group), and the other pups were returned to their dams after anesthesia and used for behavioral tests from P60 to P65 and the immunofluorescence test on P65.

Morris water maze
Spatial learning and memory tests were performed as described in a previous study [2]. In brief, a round platform (9 cm in diameter) was placed 1.0 cm below the water surface in the northeast quadrant of a circular pool. In the acquisition trials, rats were placed into the water facing the wall at a random starting position and were allowed to search for the platform for 60 s. There were four quadrants of the pool: northeastern, southeastern, northwestern, and southwestern quadrants. The starting position was set at the middle point of the wall of each quadrant. Each rat received four trials per day from days 1 to 5, and the starting position and sequence were determined using the random number table. If the rat could

Fig. 1

Flowchart of the experiment. Rat pups were injected intraperitoneally with ketamine (50 mg/kg) from P5 to P9. The pups were weaned from their mothers on P21. Rats were subjected to behavioral tests from P60 to P65 and the immunofluorescent test on P65. MWM, Morris water maze; P, postnatal day.

not locate the platform within 60 s, it was guided to the platform and allowed to stay on it for 15 s. On day 6, the platform was taken out of the pool. Rats were placed into the farthest starting position from the previous platform location and allowed to swim for 60 s. A continuous video tracking system recorded the swimming motions of each rat, and the data were analyzed using Morris water maze (MWM) motion-detection software (Zhenghua Biotech Co. Ltd, Huaibei, China). Learning ability was evaluated by the path length from the starting position to locating the platform. Memory was evaluated by counting the number of times the rat crossed the previous platform position.

Tissue preparation
As described in our previous study [2], rats were anesthetized with 4% sevoflurane for 3 min and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde solution. The brains were removed and postfixed in 4% paraformaldehyde solution for 24 h and cryoprotected with 25% sucrose solution until they sunk. The brains were cut on the coronal plane into 10 μm sections.

Immunofluorescence
Immunofluorescence was performed as previously described [2]. Frozen sections were blocked with 3% normal goat serum and 0.1% Triton-X 100 in PBS for 1 h at 37°C after antigen retrieval. The sections were incubated with the primary polyclonal mouse myelin basic protein (MBP) antibody (1 : 500; BioLegend, San Diego, California, USA) for 16 h at 4°C and incubated with the second Alexa Fluor 488 goat anti-mouse (1 : 200; Invitrogen, St. Louis, Missouri, USA) for 2 h at 37°C, and then counterstained with 0.5% 4′,6-diamidino-2-phenylindole (Sigma, St. Louis, Missouri, USA) for 5 min at 37°C. All images were taken under a confocal microscope (Leica TCS SP8; Leica Microsystems, Wetzlar, Germany), images were collected using a ×40 objective (NA0.85), and the immunofluorescent density of MBP was analyzed using Image-Pro Plus software (Media Cybernetics, Rockville, Maryland, USA) and obtained using the immunofluorescent integrated optical density divided by the defined staining area (mm^2). Five microscopic areas were selected in each slice from the stratum radiatum and lacunosum-moleculare of the hippocampus (CA1 and CA3), and layers IV and V of the medial prefrontal cortex (mPFC). Three slices were used for each brain region (hippocampus or mPFC), and six animals were included in each group.

Statistical analysis
Data were expressed as mean ± SEM and analyzed using SPSS 20 software (IBM Corp., New York, New York, USA). One-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test was used to analyze the data of the body weight gain, blood gas analysis, and mean MBP density. Three-way ANOVA with repeated measures, including the within factor, days of learning and independent factors, sex (levels: male and female), and treatment (levels: control, vehicle, and ketamine), was used to analyze the data in the acquisition trial of the MWM. The data from the probe trial of the MWM were non-normally distributed and expressed as a median and analyzed using a Kruskal–Wallis test. Pearson's correlation analysis was used to determine the correlation between

Table 1 Arterial blood gas analysis in P9 pups of female and male rats

	Control ($n = 6$)	Vehicle ($n = 6$)	Ketamine ($n = 6$)
Female			
pH	7.34 ± 0.02	7.32 ± 0.03	7.28 ± 0.02
PaCO$_2$ (mmHg)	48.9 ± 4.1	48.6 ± 4.8	51.6 ± 4.7
PaO$_2$ (mmHg)	90.0 ± 4.5	89.2 ± 5.1	88.2 ± 3.9
Glucose (mM)	8.1 ± 0.2	8.2 ± 0.4	8.4 ± 0.5
Lactate (mM)	3.2 ± 0.5	3.5 ± 0.3	4.0 ± 0.4
Male			
pH	7.32 ± 0.03	7.31 ± 0.04	7.27 ± 0.04
PaCO$_2$ (mmHg)	49.8 ± 3.6	48.7 ± 5.1	52.5 ± 4.9
PaO$_2$ (mmHg)	91.2 ± 3.5	90.3 ± 5.2	87.5 ± 4.6
Glucose (mM)	8.3 ± 0.3	8.4 ± 0.4	8.5 ± 0.2
Lactate (mM)	3.1 ± 0.2	3.4 ± 0.4	4.1 ± 0.3

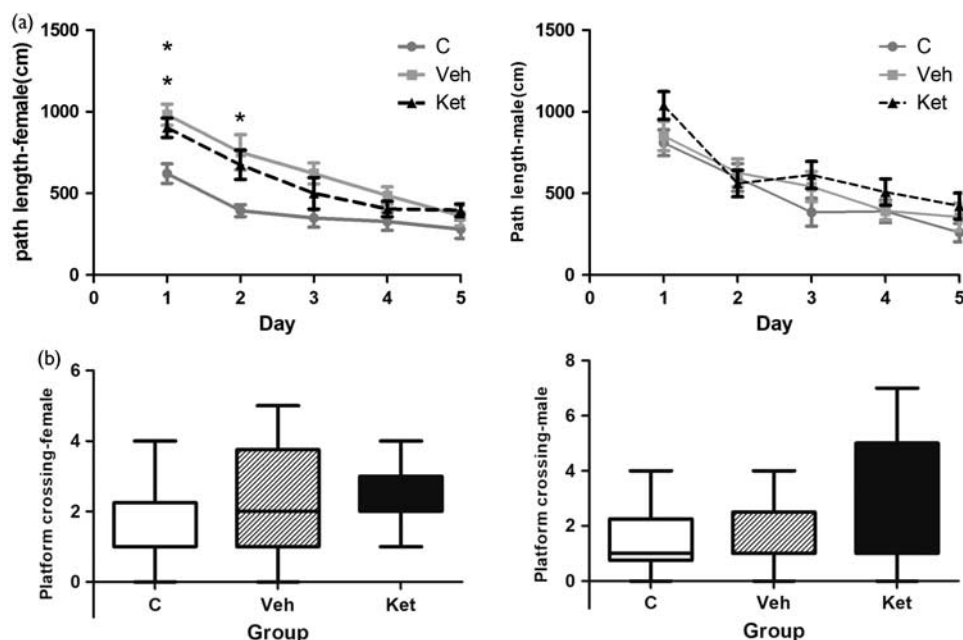Data were expressed as the mean ± SEM.
PaCO$_2$, partial pressure of carbon dioxide; PaO$_2$, partial pressure of oxygen.

Fig. 2
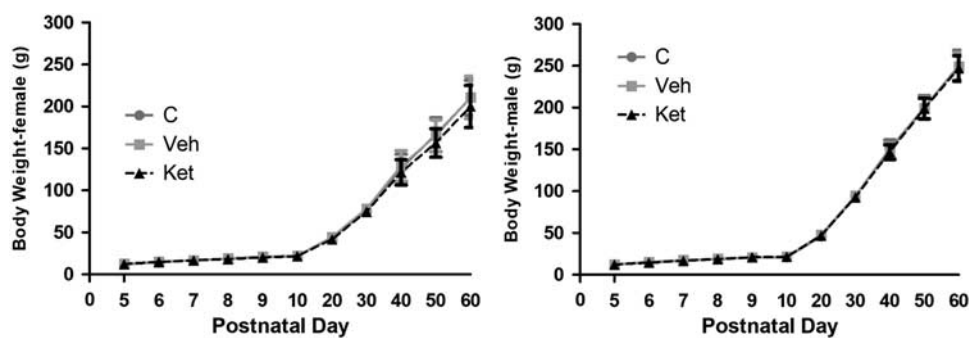
Body weight gain of both sexes during development. Data are expressed as mean ± SEM ($n = 12$). C, control; Ket, ketamine; Veh, vehicle.

Fig. 3

Effects of ketamine on spatial learning and memory. (a) The path length in the acquisition trial (mean ± SEM, $n = 12$), *$P < 0.05$ versus control. (b) Crossing times in the probe trial. Data are expressed as median ($n = 12$). C, control; Ket, ketamine; Veh, vehicle.

MBP density and the path length on the MWM. A P value less than 0.05 was considered to be a statistically significant difference.

Results

Body weight gain during development and respiratory and metabolic function during anesthesia
Body weight was recorded every day from P5 to P9 and every 10 days from P10 to P60. There were no differences in weight gain among the groups for either sex (Fig. 2). The results of the blood gas analysis on P9 showed that exposures to the experimental environment or ketamine did not cause significant respiratory or metabolic distress (Table 1). No rat pups died during ketamine anesthesia.

Effect of the experimental environment or ketamine on spatial learning and memory
In the acquisition trial of the MWM test, the average velocity increased in ketamine group and vehicle group compared with control on days 4 and 5 in females ($P < 0.05$) and on days 1 and 5 in males ($P < 0.05$). The path length on the MWM test was used to represent learning ability.

In the acquisition trial of the MWM test, three-way ANOVA analysis revealed a significant interaction effect of the days of learning, animal sex, and treatment ($P < 0.05$). The path length on day 1 in ketamine group and on days 1 and 2 in vehicle group was longer than that in control female rats ($P < 0.05$), and there was no difference between ketamine and vehicle groups ($P > 0.05$). In male rats, there were no significant differences among the three groups (Fig. 3a).

In the probe trial of the MWM test, there were no significant differences between groups in crossing times in either female or male rats ($P > 0.05$, Fig. 3b).

Effect of the experimental environment or ketamine on myelin basic protein expression
In female rats, the mean density of MBP was significantly increased in the mPFC in vehicle and ketamine groups compared with controls ($P < 0.05$), and there was a significant difference between vehicle and ketamine groups ($P < 0.05$). There were no differences in hippocampal CA1 and CA3 regions ($P > 0.05$, Fig. 4a and b). In male rats, the density of MBP in the mPFC, CA1, and CA3 areas in vehicle and ketamine groups was not different from controls ($P > 0.05$, Fig. 4c).
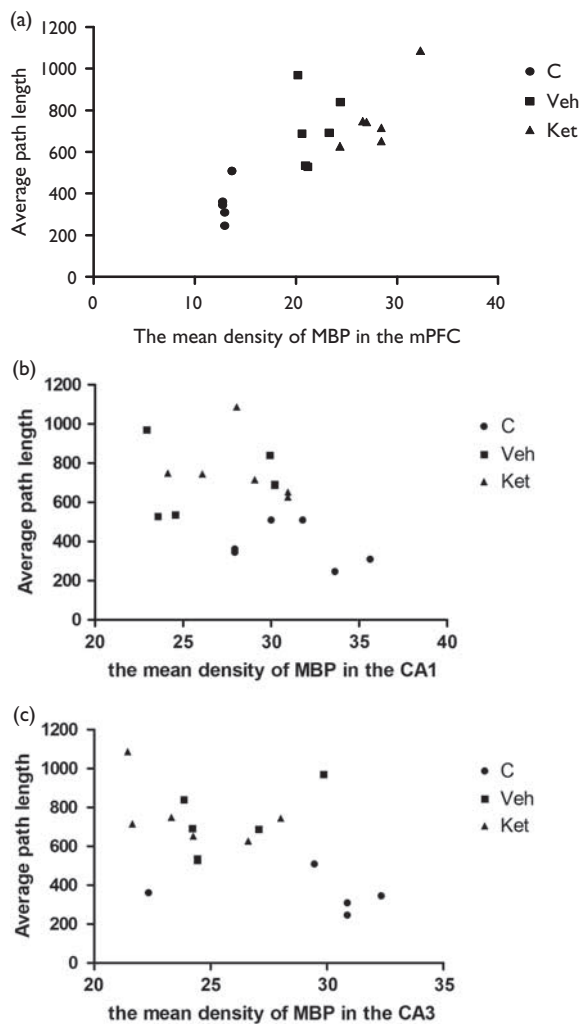
Linear regression analysis
Pearson's correlation analysis was conducted to determine the correlation between MBP expression in the mPFC and hippocampus and the average path length on the MWM test in female rats. The results showed that there was a positive correlation between MBP density in the mPFC and the path length in adult female rats ($r = 0.753$, $P < 0.01$, Fig. 5a). There was no correlation between MBP density in CA1 ($P > 0.05$, Fig. 5b) or CA3

Fig. 4
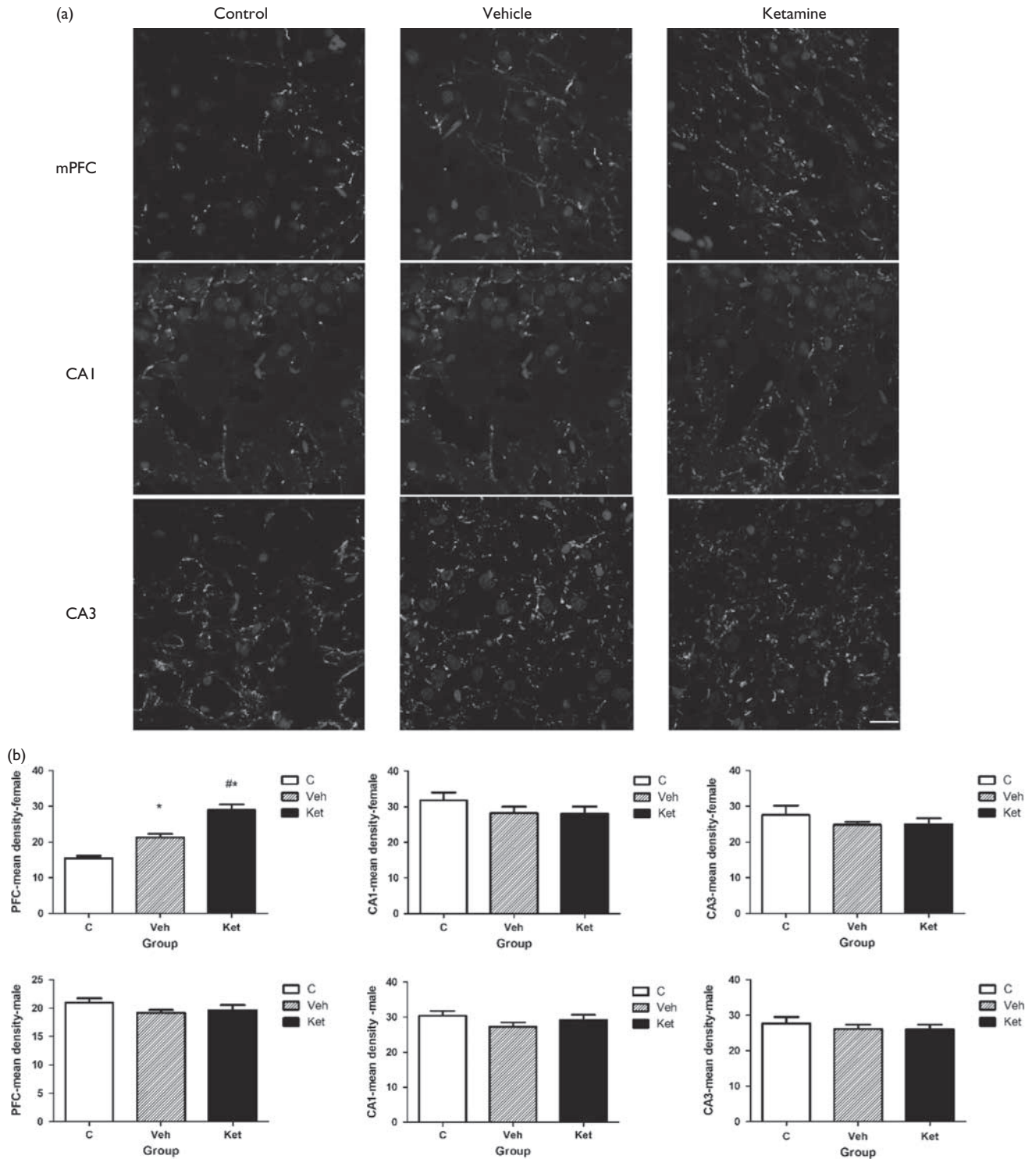
Effect of ketamine on MBP expression. (a) The representative images of immunofluorescent staining of MBP in the mPFC and hippocampus in female rats. scale bar = 20 μm. (b) The density of MBP in female rats and male rats in the mPFC and hippocampus. Data are expressed as mean ± SEM (*n* = 6); *P < 0.05 versus control, #P < 0.05 versus vehicle. C, control; Ket, ketamine; MBP, myelin basic protein; mPFC, medial prefrontal cortex; PFC, prefrontal cortex; Veh, vehicle.

Fig. 5

Scatter plot from linear regression analysis between the average path length and MBP density in female rats. (a) There was a close correlation between path length and MBP density in the mPFC ($r = 0.753$, $P < 0.01$). (b) In CA1, there was no correlation ($P > 0.05$). (c) In CA3, there was no correlation ($P > 0.05$). C, control; Ket, ketamine; MBP, myelin basic protein; mPFC, medial prefrontal cortex; Veh, vehicle.

($P > 0.05$, Fig. 5c) and the average path length on the MWM test. As increased path length meant impaired learning ability, it can be stated that learning dysfunction in adult female rats was closely correlated with over-myelination in the mPFC.

Discussion

The results of this study showed that the path length on the MWM test was increased after multiple exposures to the experimental environment or ketamine at a sub-anesthetic dose in female rats. The increase in the average path length on the MWM test indicates impaired learning, as the path length did differ between ketamine and vehicle groups. Therefore, it can be said that the impaired learning ability was induced by the experimental environment but not by ketamine. The crossing times through the platform were not changed, which indicates that memory was not affected.

It has been reported that myelin development is fundamental for the formation of myelinated nerve fibers and for neural information transmission in the central nervous system. Thus, myelination is extremely important for maintaining synaptic plasticity [15]. The results of this study showed that neonatal exposure to the experimental environment or ketamine increased MBP expression in the mPFC in adulthood female rats, and greater MBP expression was induced in ketamine group, but the experimental environment contributed to the majority of the increasing of MBP expression after ketamine exposures. Ketamine itself increased MBP expression to a limited extent. It is unclear how the experimental environment enhanced myelination in the mPFC. Ketamine can cause NR1 subunit upregulation in the frontal cortex after multiple exposures in neonatal rodents [16,17], and NMDA receptor plays an important role in myelin development [10], which may be the mechanism by which ketamine promotes myelination in the mPFC in female rats. From the results, the path length on the MWM test was closely correlated with MBP level in the mPFC, which indicates that learning dysfunction in adult female rats may result from over-myelination in the mPFC.

In animal studies on ketamine neurotoxicity, environmental exposure in neonatal animals to either ketamine anesthesia or vehicle control is similar to the clinical environment in pediatric anesthesia, such as maternal separation, fear, pain stimulation, and oxygen delivery. From the results of this study, multiple short episodes of the environmental exposures can induce a decline in learning ability and an increase in MBP expression in the mPFC in adulthood. It can be stated that environmental exposures can cause neurotoxicity directly, and although ketamine could increase MBP expression in the mPFC to some extent, it may not impair learning ability. Therefore, neurotoxicity after ketamine exposures can be attribute to the effect of the experimental environment. How to minimize the effect of these environmental factors on pediatric anesthesia is a very important issue in the clinic. In our previous study, propofol, another commonly used intravenous anesthetic agent, caused long-term learning and memory impairment after multiple exposures in rat developing brain but environmental exposures did not. In that study, the cognitive function was tested from P35 to P41, and the escape latency was used for learning ability on the MWM test [2]. However, whether propofol can affect the cognitive function in adulthood after neonatal exposures needs to be verified.

Neonatal male mice exposed to a single episode of sevoflurane anesthesia have facilitated escape latency on

the MWM, but female mice do not [18]. After neonatal mice are exposed to isoflurane anesthesia, long-term cognitive function is less impaired in female mice than in male [19]. In this study, neonatal female rats exposed to multiple episodes of the experimental environment or ketamine had increased path length on the MWM test and MBP expression in the mPFC in adulthood, but male rats did not. During brain development, the expression of NMDA receptor or NMDA to AMPA receptor ratio exhibits a sex difference, which can lead to different responses to physiological stimulus and alterations in neural ultrastructure between male and female rodents [20]. It has been reported that different sexes can exhibit different or even opposite development of dendritic spines under external stimuli [21]. Therefore, sex differences in behaviors in this study may result from the sex differences in NMDA receptor expression or the development of dendrite spines.

Conclusion

Multiple episodes of exposures to the experimental environment in neonatal female rats induced learning dysfunction in adulthood, which may result from overmyelination in the mPFC. The experimental environment contributes to neurotoxicity from multiple exposures of ketamine at subanesthetic dosage, and although ketamine itself can induce overmyelination in the mPFC to a certain extent, it may not impair learning ability in adulthood, which suggests that ketamine may be a safe drug for pediatric anesthesia. Male rats were not affected through myelination or learning ability in adulthood after neonatal exposures.

Acknowledgements

Conflicts of interest
There are no conflicts of interest.

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