Neurobiology of Disease 68 (2014) 145–155

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Ketamine administered to pregnant rats in the second trimester causes long-lasting behavioral disorders in offspring

Tianyun Zhao [a], Yuantao Li [a,*], Wei Wei [b], Sinead Savage [c], Libing Zhou [d,**], Daqing Ma [c]

[a] Department of Anesthesiology, Shenzhen Maternity and Child healthcare Hospital, Southern Medical University, Shenzhen 518028, China
[b] Department of Anesthesiology, Meizhou people's hospital, Meizhou, Guangdong 514031, China
[c] Anaesthetics, Pain Medicine & Intensive Care, Department of Surgery & Cancer, Faculty of Medicine, Imperial College London, Chelsea & Westminster Hospital, London, UK
[d] Guangdong-Hongkong-Macau Institute of CNS Regeneration, Jinan University, Guangzhou, Guangdong 510632, China

ARTICLE INFO

Article history:
Received 16 November 2013
Revised 21 February 2014
Accepted 25 February 2014
Available online 26 April 2014

Keywords:
Ketamine
Brain development
Pregnancy
Cognition
Depression
NMDA receptor

ABSTRACT

Commonly used anesthetic agents, e.g. ketamine, may be neurotoxic to the developing brain but there has been little attention to the neurobehavioral consequences for offspring when used for maternal anesthesia. We hypothesize that treatment of pregnant rats with ketamine during the second trimester would affect brain development of the offspring. Pregnant rats on gestational day 14, about equal to midtrimester pregnancy in humans, received a sedative dose of ketamine intravenously for 2 h. Brain hippocampal morphology of their pups at postnatal days 0 (P0) and P30 was examined by Nissl-staining and the characteristics of dendrites were determined using the Golgi-Cox staining, while cell proliferation in subventricular zone (SVZ) and dentate gyrus (DG) was labeled with bromodeoxyuridine (BrdU). Their neurobehavioral functions were tested at P25–30 after which the NR1 and NR2 subunits of N-methyl-D-aspartate (NMDA) receptor, brain-derived neurotrophic factor (BDNF) and postsynaptic density protein 95 (PSD-95) in the hippocampus were analyzed by western blot. When pregnant rats were exposed to ketamine, there was neuronal loss, pyramidal neuronal abnormality and reduced cell proliferation in the hippocampus of offspring. These morphological abnormalities were associated with depression- and anxiety-like behaviors, and impaired memory up to young adult age. The treatment further caused NR2A receptor subunit up-regulation and NR2B receptor subunit, BDNF and PSD-95 down-regulation. These data suggest that maternal anesthesia with ketamine during the fetal brain development period can cause fetal brain damage and subsequent neurobehavioral abnormality, which is likely associated with the imbalanced expression of NMDA receptor subunits.

Introduction

There is great concern about the safety of currently used anesthetics in the young. Indeed, preclinical data suggests that commonly used anesthetics including ketamine are neurotoxic to the developing brain in rodents and even primates (Brambrink et al., 2012; Jevtovic-Todorovic et al., 2003; Satomoto et al., 2009; Slikker et al., 2007). This concept is also supported by retrospective clinical data in which there was an association between anesthesia and/or surgery early in life leading to late-onset learning disabilities (Hansen et al., 2011; Ing et al., 2012; Kalkman et al., 2009). Unfortunately, only a few previous reports looked into the effects of general anesthesia on neurodevelopmental consequences for the fetus before birth (Kong et al., 2012; Palanisamy et al., 2011; Slikker et al., 2007; Zheng et al., 2013). For example, rats exposed to isoflurane for 4 h in utero caused memory and learning deficits, as well as abnormal behaviors (Palanisamy et al., 2011). A 24-h exposure of ketamine anesthesia during late second trimester in pregnant macaques caused apoptotic neuronal death in the fetal brain (Slikker et al., 2007). However, most studies of this kind mainly concentrated on acute effect of anesthetics on neuroapoptosis without further investigation of neuronal development and neurogenesis. In particular, long-term learning and spatial memory impairments and other neurological disorders including depression, anxiety, emotional blunting and apathy were not well studied.

Ketamine is often consumed as a drug of abuse by the public, including pregnant women (Rofael et al., 2003). In addition, between 0.75% and 2% of pregnant women require surgery that is related to either the pregnancy or other medical problems (Goodman, 2002). This number is increasing, partly because of laparoscopic procedures and fetal surgery becoming more widely performed (Cheek and Baird, 2009). It is therefore important to ensure that anesthetics, such as ketamine, used in surgery or as a drug of abuse during pregnancy do not pose a risk to the unborn baby. In this study, pregnant rats on gestational day

* Correspondence to: Y Li, Department of Anesthesiology, Shenzhen Maternity and Child healthcare Hospital, Southern Medical University, Shenzhen 518028, China. Fax: +86 755 83228832.
** Correspondence to: L. Zhou. Fax: +86 20 85223563.
E-mail addresses: sylyt6788@sina.com (Y. Li), tlibingzh@jnu.edu.cn (L. Zhou).

http://dx.doi.org/10.1016/j.nbd.2014.02.009
0969-9961/

14, equivalent to the second trimester of human pregnancy (Clancy et al., 2001), were administered ketamine intravenously, and its impact on neuronal development, neurogenesis and behavior, together with underlying molecular mechanisms, were investigated in offspring.

Materials and methods

Subjects

All experimental procedures were performed according to the guidelines that have been approved by the Ethics Committees of Jinan University, Guangdong, China. All efforts were made to minimize the number of animals used. The timed-pregnant Sprague–Dawley rats were housed in polypropylene cages in a temperature and humidity regulated room with a 12 h light/dark cycle and had access to water and food ad libitum. Dams were used for experiments on gestational day 14.

Anesthesia

On the gestational day 14, 12 dams were randomly divided into control (n = 6) and ketamine (n = 6) groups. Controls were left undisturbed in their home cages while the ketamine group received a bolus dose (40 mg/kg) of ketamine (Gutian Pharmaceutical Co., Ltd. Fujian province, China) via lower back intramuscular injection, followed by continuous intravenous infusion with a pump (Sinomdt Co., Ltd, Shenzhen, China) via a tail vein at a rate of 40–60 mg/kg/h (10 mg/ml diluted with saline) for 2 h. This duration of ketamine infusion is clinically relevant while the infusion rate was varied to induce a sedative state between light anesthesia and deep sedation, evidenced by a lack of voluntary movement, decreased muscle tone, and minimal reaction to painful stimulation with the maintenance of an intact palpebral reflex but without any cardiorespiratory function being compromised (Green et al., 1981). The latter was confirmed with the blood gas analysis in a separate cohort (Nova Biomedical blood gas analyser, Massachusetts, USA) (see the Results section). The core body temperature was measured with a rectal probe and maintained between 36.5 and 37.5 °C by a servo-controlled infrared lamp and heating pad throughout experiments (RWD life science Co. Ltd., Shenzhen, China). At the end of infusion, dams were returned to their home cages after the righting reflex was recovered. Three dams per group were used for the neurogenesis study on gestational day 22 (see below) while another three in each group were allowed to give birth naturally. Two pups from each dam (total 6 pups in each group) were killed about 6 h after birth for brain histology examination. The remaining pups were allowed to grow up with their mothers until postnatal day (P) 21 (the day of birth was designated as P0) at which time pups were weaned (3–4 cages/dam) for behavioral tests. Subsequently, their ex vivo brain samples were harvested at P30 for histology, immunostaining, Golgi stain and western blot (n = 1–2/dam for each examination) (see below). A schematic representation of experimental protocols is shown in Fig. 1.

Behavioral test

Two groups (n = 10 or 11) of rats were randomly selected from each group (3–4 from each dam) at P25 to 30 for open field activity test (OFT) and forced swimming test (FST) or Morris water maze test (MWM) and sucrose preference test (SPT) for 3 consecutive days respectively. In order to avoid possible behavioral biases, each behavioral test of the two groups of animals was performed at the same time interval on the day of testing.

Open field activity test (OFT)

In order to assess general locomotor activity and anxiety-like behaviors (Prut and Belzung, 2003), the open field test was adopted. Rats (n = 10) were placed gently in a dimly lit open-field apparatus (50 cm × 50 cm × 37 cm) constructed of Plexiglas wall and black floor, and allowed to move freely for 15 min. The arena was cleaned with 70% ethanol between each trial. Data were recorded with a video camera and analyzed using EthoVision XT 7.0 (Noldus, Wageningen, Netherlands). Locomotor activity was measured with the traveling distance and speed while anxiety-like behaviors were evaluated with the duration and the frequency of the center zone visits.

Forced swimming test (FST)

The FST is widely used for assessing depressant behavior (Cryan et al., 2005). To measure this the same cohort of rats, after OFT testing, were individually placed into a transparent Plexiglas cylinder (50 cm high and 15 cm diameter) filled with 25 cm deep water at 24 ± 1 °C. Each animal was left to swim for 5 min. Data were recorded with a video camera and analyzed using EthoVision XT 7.0 (Noldus) to calculate the duration of their immobility. A rat was judged to be immobile when the velocity of center point was less than 1.7 cm/s.

Morris water maze (MWM)

MWM test was performed to assess spatial learning and memory in the animals. A water tank 120 cm in diameter and 50 cm in height was filled with water to a depth of 35 cm and maintained at a temperature of 24 ± 1 °C. It was equally divided into four quadrants. An escape platform (10 × 10 cm^2) was submerged 1.5 cm below the water surface in one quadrant. Rats were placed into the pool and allowed to search for the platform for 180 s for four trials (once from each quadrant; 15 min gap between each trial). Animals were pre-trained for 3 days before the formal test was started and guided to the platform if they could not find the platform at the end of the test, in which case the latency was defined as 180 s. Data were recorded with a video camera and analyzed using EthoVision XT 7.0 (Noldus) to determine the total distance traveled and the total latency to platform.

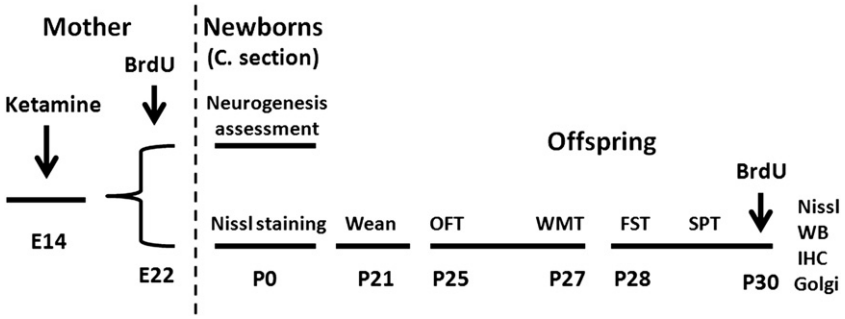

Fig. 1. The flow chart of the experimental protocols. C. section = cesarean section; E = gestation day; BrdU = Bromodeoxyuridine; Nissl staining = cresyl violet staining; P = postnatal day; OFT = open field activity test; FST = forced swimming test MWM = Morris water maze; SPT = sucrose preference test. WB = western blot; IHC = immunohistochemistry; Golgi = Golgi staining.

Sucrose preference test (SPT)

Anhedonia is a core feature of depression symptomatology, which can be defined by a blunting of sensitivity to rewarding stimuli (Rygula et al., 2005). As described previously (Bambico et al., 2009) with modifications, all the animals were initially exposed to a bottle containing 1% (wt./vol.) sucrose in tap water 1 h daily for 3 days. They were then completely deprived of water for 23 h. Next, at the beginning of the 4th day, they were subjected to a 1-h trial where they were allowed to drink from 2 water bottles, one of which contained the sucrose solution while the other was filled with tap water. The two identical size plain water bottles were placed in the designed cage and their position was swapped over after the first half of the test to minimize egocentric orientation bias. Sucrose preference was quantified as the ratio of sucrose intake to total fluid intake.

Histology and immunohistochemistry

Tissue section preparation

Six animals were randomly selected from each group (n = 2/dam) at P0 or P30. All the animals were decapitated and perfused via the left ventricle with chilled saline followed with 4% paraformaldehyde in 0.01 M phosphate buffered saline pH 7.35 (PBS). Brains were then removed and post-fixed for 24 h for either paraffin or frozen sections.

Histology and immunohistochemistry

Ten paraffin coronal sections (5 μm) of dorsal hippocampus (referring to Atlas of the Neonatal Rat brain edited by Renuka Ramachandra) in which the anatomical structure is similar to adult rat atlas around −3.8 mm from bregma and +5.2 mm from interaural landmarker of each pup (n = 6) were stained with 0.5% cresyl violet to assess the cell density in the CA1 and CA3 region. Microphotographs were taken using a microscope digital camera system (Leica, DM6000b, Germany) at 40× magnification. Nissl-positive healthy neuronal cell numbers were counted in a blinded manner using ImageJ (National institute of health, USA) and expressed as cell number/mm^2.

Proliferation was assessed through bromodeoxyuridine (BrdU) incorporation into nuclei during the S phase of the cell cycle (Taupin, 2007). In order to determine the long term effects of the drug treatment on brain cell proliferation, BrdU was administered directly to dams at the latest gestation day or to offspring. On gestational day 22, pregnant mothers received i.p. injection of 50 mg/kg BrdU dissolved in saline. Two hours after injection, the pups were obtained via cesarean section and decapitated to prepare for frozen section. Similarly, offspring of natural birth (1–2/dams) received once daily i.p. injection of 50 mg/kg BrdU for 3 consecutive days (from P28 to P30). They were killed by decapitation 12 h after the last injection for frozen sections (Taupin, 2007). The coronal sections (10 μm) were rinsed with 0.01 M PBS three times for 5 min. Then the sections were incubated in 2 M HCl for DNA denaturation, neutralized with 0.1 M borate buffer, and further incubated with 10 g/L bovine serum albumin at room temperature for 1 h. Then the sections were incubated with a rat monoclonal antibody to BrdU (1:500, Sigma) in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 for 24 h at 4 °C. After several rinses in PBS, slices were incubated with a donkey anti-rat-IgG Alexa Fluor594 (1:1000, Invitrogen, USA) for 2 h at room temperature, then mounted and examined using a fluorescence microscope (Leica DM6000B, Germany). Some sections from P30 offspring were co-probed with a rat monoclonal antibody to BrdU (1:500, Sigma) and rabbit polyclonal to doublecortin (DCX, a neuronal marker) (1:1000, Abcam, HK, China) in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 for 24 h at 4 °C. After several rinses in PBS, slices were incubated with a donkey anti-rat-IgG Alexa Fluor594 (1:1000, Invitrogen, USA) and a donkey anti-rabbit-IgG Alexa Fluor488 (1:1000, Invitrogen, USA) for 2 h at room temperature, then mounted and examined using a Zeiss LSM 700 laser scanning confocal microscope using 10× objective for DG area and 20× objective for SVZ area respectively.

Golgi stain

The Golgi-Cox stain was performed on 150 μm-thick frozen brain sections (around −3.8 mm from bregma and +5.2 mm from interaural relative to rat atlas) obtained from three P30 rats in each group after behavioral tests, using the FD Rapid Golgi Stain kit (FD NeuroTechnologies, Inc. Columbia, USA) according to the manufacturer's protocol. Twenty well individualized CA1 or CA3 neurons in the CA1 or CA3 region of hippocampus were randomly selected from each rat, and sequential optical sections of 1392 × 1040 pixels were taken at 1.5 μm intervals along the z-axis (Leica, DM6000B, Germany). The Imaris software (BitPlane AG, Zurich, Switzerland) was used for tridimensional reconstruction and total branch number and dendritic length measurements. The complexity of total dendritic trees was estimated using Sholl analysis (Sholl, 1953). To measure spine density, 20 neurons from each animal were selected and matching regions of distal branch dendrites were photographed using a 100× objective for counting of spine numbers in 40 μm segments, with results expressed as the number of spines/10 μm.

Western blot

Hippocampal fragments (n = 3/group) were isolated after behavioral test and stored in a −80 °C freezer until use. At the time of processing, tissue lysis buffer (containing protease inhibitors, 50 mM Tris–HCl pH 7.6) was added to the samples on ice and then the tissue was homogenised via ultrasonification (Ningbo scientz biotechnology Co. Ltd., Ningbo, China) and centrifuged at 12,000 *g* for 10 min at 4 °C. The protein concentration in the supernatant was measured with a BCA assay kit (Beyotime Institute of Biotechnology, China). Then the supernatants were mixed with gel loading buffer (50 mM Tris–HCl, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml bromophenol blue) in a ratio of 1:1 and boiled for 5 min and then subjected to SDS page (12% acrylamide gels for BDNF and 10% acrylamide gels for PSD-95 and NR1, 120 V, 1.5 h, and 8% acryl-amide gels for NR2A and NR2B, 30 V overnight transfer at 4 °C and 70 V for 1 h the next morning). The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes and incubated with rabbit anti-BDNF, NR1, NR2A, NR2B, PSD-95 (1:250, Biosynthesis Biotechnology Co. Ltd., Beijing, China) or mouse anti-GAPDH (1:1000, Biosynthesis Biotechnology Co. Ltd., Beijing, China) overnight at 4 °C. The membrane was exposed to either rabbit or mouse secondary antibody (1:2000, Biosynthesis Biotechnology Co. Ltd., Beijing, China) for 1.5 h at room temperature. The blots were visualized by a Scanmaker 3836 (Microtek Technology co., LTD, Shanghai, China) and quantified with the Quantity One software (Bio-Rad Laboratories, Inc., USA). The expressions of BDNF, PSD-95 and the NMDA receptor (NMDAR) subunits NR2A, NR2B, and NR1 were determined by calculating their density ratio to the GAPDH band, and then normalized to the control group.

Table 1
Blood gases, glucose and temperature measurements in age-matched pregnant and unpregnant rats during ketamine administration.

Arterial blood gas	Unpregnancy	Pregnancy
PH	7.38 ± 0.02	7.36 ± 0.01
$PaCO_2$ (mm Hg)	39.73 ± 1.47	43.73 ± 3.12
PaO_2 (mm Hg)	104.33 ± 3.28	103.67 ± 3.38
HCO_3^-	24.30 ± 1.03	25.60 ± 1.61
Glucose (mg/dl)	104.33 ± 1.86	108.67 ± 2.91
Temperature (°C)	36.97 ± 0.35	37.03 ± 0.50

Arterial blood samples were sampled from the left cardiac ventricle immediately at the end of the 2-hour ketamine administration. All measurements were within the normal physiological ranges and no statistical significance was found between the two groups. Data are presented as mean ± S.E.M. (n = 3).

Statistical analysis

All data were presented as mean ± SEM. Homogeneity of variance was verified using Levene's test and then single comparison between both groups was made using unpaired two-tailed Student *t* test. The parametric Bonferroni and nonparametric Kruskal–Wallis H tests were used to analyze dendrite branching with the Sholl method (Sholl, 1953). A p value < 0.05 was considered to be statistically significant. All analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

Physiologic response to ketamine infusion

The total dose of ketamine used in each dam was 141.2 ± 4.7 mg/kg, and all the anesthetized dams recovered fully without any complications. The recovery time from anesthesia was 36 ± 3 (n = 6) min, and the total anesthetic time (from onset time of initial intramuscular injection to recovery time) was 174 ± 4 min. Maternal blood gas, blood glucose, and body temperature measurements were within normal physiological range as obtained from unpregnant rats (Table 1). At P28, the body weight of rats whose mother received ketamine was significantly lower than that of controls (66 ± 3 g vs. 90 ± 4 g of controls; n = 22 in the controls and n = 24 in the ketamine treated group; t = 4.963, p < 0.001). There were two offspring with extreme low body weight (36 g and 44 g) excluded from further studies.

Ketamine induced multiple behavioral deficits in offspring

Ketamine caused offspring to display anxiety-like behavior

For locomotor activity measured by total distance traveled and velocity, there was no difference between the two groups. Decreased time spent and the number of entries into the central squares are utilized

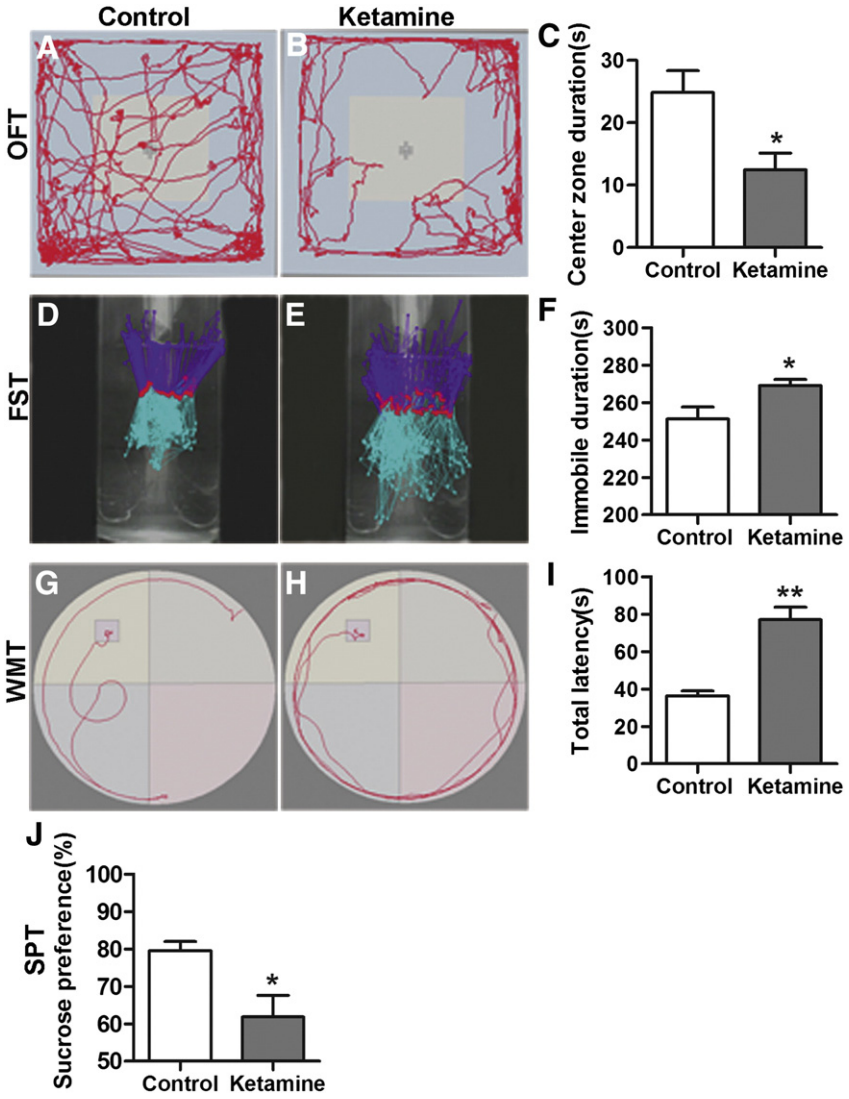

Fig. 2. Ketamine induced multiple behavioral deficits in offspring. Female Sprague–Dawley rats on gestation 14 (n = 3) received ketamine with an initial intramuscular injection (40 mg/kg) followed by continuous intravenous infusion by pump via tail vein at a rate of 40–60 mg/kg/h for 2 h while no treatment was made to the pregnant age matched controls (n = 3). Two group (n = 10/11) cohort rats were randomly selected from each group (3–4 from each dam) at the postnatal days (PND) 25 to 30 for open field activity test (OFT) and forced swimming test (FST) or Morris water maze (MWM) and sucrose preference test (SPT) for 3 consecutive days respectively. Example recording trails from controls (A), ketamine treated offspring (B) and mean time spent in the center zone of OFT (C); example recording trails from controls (D), ketamine treated offspring (E) and mean immobile duration of FST (F); example recording of MWM from controls (G), ketamine treated offspring (H) and mean latency of MWM (I); (J) mean consumption of sucrose containing water vs. normal water (J). Data are mean ± SEM (n = 10/11); *p < 0.05, **p < 0.01 vs. control.

as indices of anxiogenic-like behavior (Prut and Belzung, 2003). Rats from the ketamine treated dams explored the center area of the environment for a shorter duration and less frequently than controls (Figs. 2A–B). The cumulative time spent in the center zone of the treated offspring was significantly decreased when compared with that of controls (Fig. 2C, 12.5 ± 2.7 s vs. 24.8 ± 3.5 s of controls, n = 10, t =2.816, p = 0.0114). These data indicated that ketamine treated offspring showed anxiety-like behavior without locomotor functional impairment.

Ketamine induced depression-like behavior in offspring

To further clarify the effect of the maternal administration of ketamine on offspring's mood behaviors, we did forced swimming test at P28–30, which is usually used to test the capability of rodents to handle stress (Cryan et al., 2005). Rodents usually struggle to escape from these situations instinctively. When rats remained floating with all limbs and tail motionless, they were considered to be immobile and this was noted as depressive-like behavior. This paradigm can be reversed with antidepressant treatments which has been documented previously (Cryan et al., 2005). Maternal administration of ketamine significantly prolonged the immobility time of their offspring (Figs. 2D–F, 269.1 ± 3.1 s vs. 251.2 ± 6.5 s of controls, n = 10, t = 2.499, p = 0.0223).

Ketamine induced impaired learning and spatial memory in offspring

The Morris water maze (MWM) is a test for learning and spatial memory in rodents that relies on distal cues to navigate from the start locations around the perimeter of an open swimming arena to locate a submerged escape platform (D'Hooge and De Deyn, 2001). Extensive evidence supports its validity as a measure of hippocampal dependent spatial learning and memory. Control offspring performed well in water maze tests. In contrast, ketamine treated offspring took more time to escape onto the platform (Figs. 2G–I, 77.0 ± 6.6 s vs. 36.3 ± 2.6 s of controls; n = 11, t = 5.709, p < 0.001).

Ketamine caused depression-like behaviors in offspring

The sucrose preference test (SPT) was designed to measure anhedonia (Rygula et al., 2005). To evaluate whether ketamine treatment affects emotional state of offspring, we have performed a modified sucrose preference test after MWM at P28–30. No significant differences

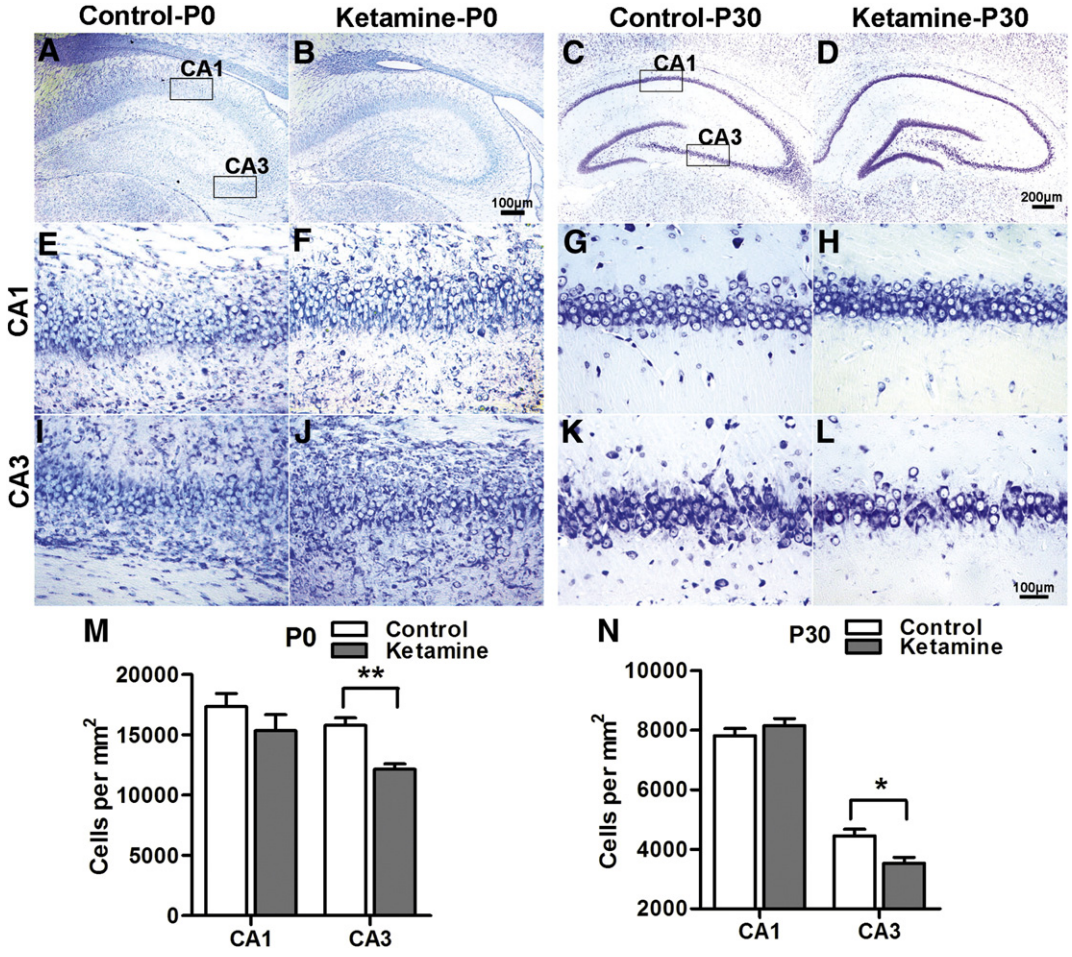

Fig. 3. Ketamine administrated in second trimester induced neuronal cell loss in the hippocampal CA3 region of offspring. Female Sprague–Dawley rats on gestation 14 (n = 3) received ketamine with an initial intramuscular injection (40 mg/kg) followed by continuous intravenous infusion by pump via tail vein at a rate of 40–60 mg/kg/h for 2 h while no treatment was made to the pregnant age matched controls (n = 3). After treatment, dams were allowed to give birth naturally. Coronal sections of dorsal hippocampal from six animals were randomly selected from each group (n = 2/dam) at the postnatal day (PND) 0 or PND 30 (after behavioral tests) for Nissl-staining with cresyl violet. Gross architecture of the hippocampus in the control and ketamine treated offspring at PND 0 (A and B, respectively) and PND 30 (C and D, respectively); the cellular layer in the CA1 region of hippocampus of control and ketamine treated offspring at PND 0 (E and F, respectively) and PND 30 (G and H, respectively), from which the cell density was calculated. There is no significant difference in cell density in CA1 between the two groups (M, p = 0.272 at the PND 0 and N, p = 0.973 at the PND 30). In contrast, the cell density of the CA3 region was significantly lower in the ketamine-treated offspring than in the control offspring at PND 0 (M) and PND 30 (N), as can be seen in the cellular layer of the CA3 region of control and ketamine treated offspring at PND 0 (I and J, respectively) and PND 30 (K and L, respectively). Data are mean ± SEM (n = 6); *p < 0.05, **p < 0.01 vs. control.

were observed between the two groups of animals in terms of total fluid consumption. However, the offspring of ketamine treated mothers had a significant reduction in sucrose intake compared with that of controls (Fig. 2J, 61.8 ± 5.8% vs. 79.5 ± 2.4%; n = 11, $t = 2.812$, $p = 0.0108$). These results indicate that exposure to ketamine in the second trimester induces a state of anhedonia in offspring.

Ketamine administrated in second trimester induced neuronal cell loss in the hippocampal CA3 region of offspring

The hippocampus is highly related to learning and memory and mood behaviors (Femenia et al., 2012; Smith and Mizumori, 2006). Based on this, we studied the hippocampal architecture at P0 and P30 using Nissl staining. There was no significant difference in cell density in the CA1 region at P0 and P30 between the control and treated groups (Figs. 3E–H, $p = 0.3537$). In contrast, cell density of ketamine treated offspring was decreased by 23% at P0 and 21% at P30 in the CA3 region (Figs. 3I–L and M, N, n = 6, $t = 4.720$, $p < 0.01$ and $t = 3.058$, $p < 0.05$, respectively). Thus, neurons in the CA3 are more susceptible to the ketamine treatment.

Golgi stain demonstrated less maturation of pyramidal neurons in CA3 in ketamine treated offspring

To study the morphology of CA1 and CA3 pyramidal neurons, we used Golgi-Cox impregnation and reconstructed tridimensional basal and apical dendritic trees in 120 pyramidal neurons selected from three brains in each group (Figs. 4A, B). Pyramidal dendrites in the ketamine treated pups were less branched (Fig. 4C, n = 3, $t = 4.930$, $p = 0.0079$), and total branch length was shorter (Fig. 4D, n = 3, $t = 7.344$, $p = 0.0018$) than in controls in the CA3, but not in the CA1 region. A phenotype was also confirmed by the Sholl analysis (Fig. 4E, n = 3, $p < 0.01$). Furthermore, spine density was significantly decreased in the ketamine treated offspring compared with that of the controls in the CA3 region (Figs. 4A′, B′ and F, n = 3, $t = 3.674$, $p = 0.0213$).

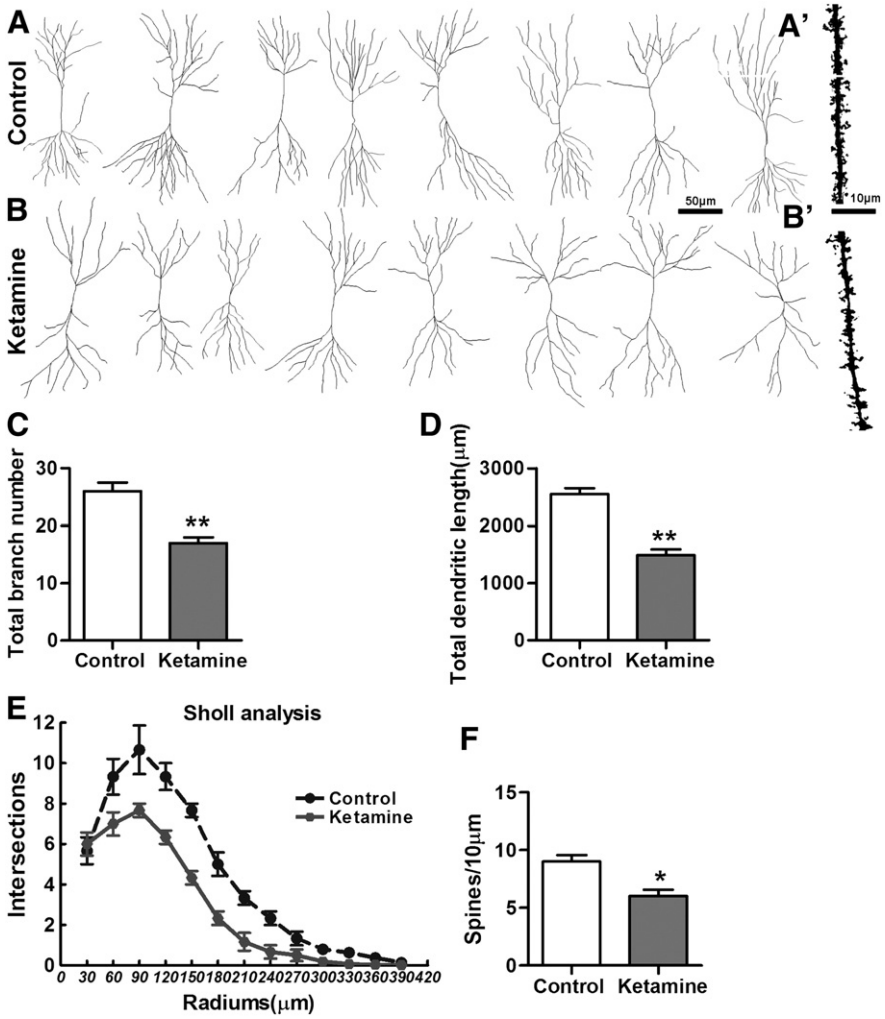

Fig. 4. Golgi stain demonstrated less maturation of pyramidal neurons in the CA3 region of hippocampus in the ketamine-treated offspring. Female Sprague–Dawley rats on gestational day 14 (n = 3) received ketamine with an initial intramuscular injection (40 mg/kg) followed by continuous intravenous infusion by pump via tail vein at a rate of 40–60 mg/kg/h for 2 h, while no treatment was made to the pregnant age matched controls (n = 3). After treatment, dams were allowed to give birth naturally. On postnatal day PND 30, after behavioral tests, three brains (one/dam) were harvested for Golgi-Cox impregnation. The Imaris software (BitPlane AG) was used for tridimensional reconstruction and measurement of total branch number and dendritic length. Examples of reconstructed Golgi-impregnated pyramidal neurons in the CA3 region of the control (A) and ketamine-treated offspring (B) are shown. Compared with the controls, the pyramidal neurons in the CA3 subfield show less complexity in the ketamine-treated offspring and also the total branch number (C) and dendritic length were significantly decreased compared with controls (D) (**$p < 0.01$, n = 60 neurons). Dendritic atrophy was found in the ketamine-treated offspring assessed with Sholl analysis (E, **$p < 0.01$, Kolmogorov–Smirnov test); as can be seen in examples of the reconstructed spines in the CA3 region of the control (A′) and ketamine-treated offspring (B′). Spine density was decreased in the ketamine-treated offspring vs. controls (F). Data are mean ± SEM (n = 3); *$p < 0.05$, **$p < 0.01$ vs. control.

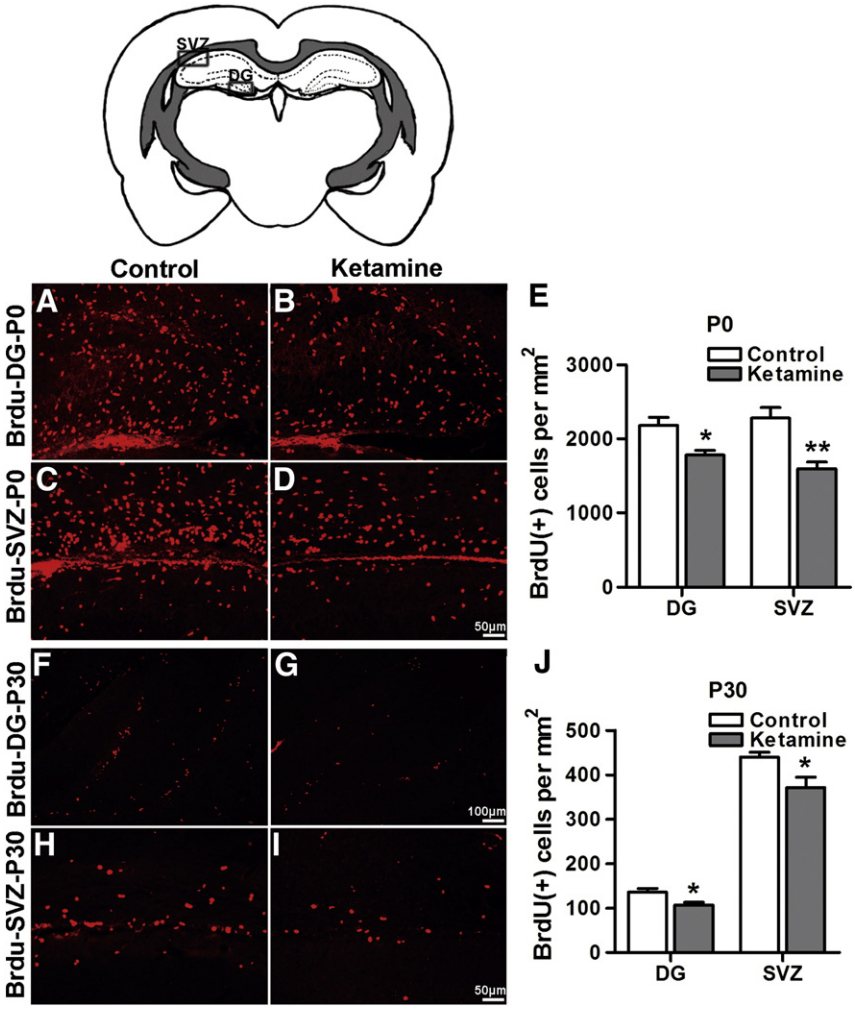

Fig. 5. Ketamine administrated in the second trimester impaired postnatal brain cell genesis in offspring. Female Sprague–Dawley rats on gestational day 14 (n = 6) received ketamine with an initial intramuscular injection (40 mg/kg) followed by continuous intravenous infusion by pump via tail vein at a rate of 40–60 mg/kg/h for 2 h while no treatment was made to the pregnant age matched controls (n = 6). The pups from three dams from each group were used for the neurogenesis study at postnatal day (PND) 0, while another three in each group were allowed to give birth naturally and pups were used for the neurogenesis study for PND 30. On the gestational day 22, 2 h after injection 50 mg/kg i.p. Bromodeoxyuridine (BrdU), the pups were obtained via cesarean section (the pups were designated as PND 0) and decapitated to prepare for frozen section. Offspring of natural birth (1–2/dams) received once daily i.p. injection of 50 mg/kg BrdU for 3 consecutive days (from PND28 to PND 30) and then were killed by decapitation 12 h after the last injection for frozen sections. $BrdU^+$ cells were subjected to quantitative analysis of ongoing cell proliferation in the 2-boxed area. Representative images of $BrdU^+$ cells are shown for PND 0 of controls and ketamine treated offspring in the DG (A and B, respectively) and the subventricular zone (SVZ) (C and D, respectively) and mean data is shown in cells/mm^2 (E); The same data for PND 30 of the control and ketamine-treated offspring are shown in the DG (F and G, respectively) and SVZ regions (H and I, respectively) and mean data are again expressed as cells/mm^2 (J). Data are mean ± SEM (n = 5), $^*p < 0.05$ and $^{**}p < 0.01$ vs. control.

Ketamine administrated during midtrimester impaired postnatal neurogenesis

The dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles have been established as the primary sites of neurogenesis in adults (Taupin, 2007). When compared to the controls at P0, ketamine-treated pups via mother resulted in a significant decrease in BrdU+ cells in both the DG and SVZ regions (Figs. 5A–E, decreased by 18% and 30%, respectively; n = 5, t = 3.154, $p = 0.0135$ at DG and t = 3.985, $p = 0.004$ at SVZ). This phenomenon persisted up to P30, as the number of BrdU+ cells was reduced by 23% and 15% respectively in the DG and SVZ regions (Figs. 5F–J, n = 5, t = 2.763, $p = 0.0246$; and t = 2.661, $p = 0.0288$, respectively). When compared to the controls, ketamine exposure caused a decrease of co-labeled BrdU/DCX cells relative to total BrdU + cells in both the DG and SVZ regions but this only reached to a statistical significance in the DG area (Fig. 6). All these indicated that the proliferating function of brain cells including neurons was impaired.

Ketamine administrated in the second trimester perturbed NMDA receptor subunits and down-regulated BDNF and PSD-95 in the hippocampus

NMDA receptor activation modulates the mammalian target of rapamycin (mTOR) signaling pathway (Duman et al., 2012), which leads to activity-dependent release of BDNF and stimulation of signaling cascades including Akt. All these activate the mTOR translational system in neuronal dendrites to cause an increased level of PSD-95 and other synaptic proteins triggering synaptogenesis and spine formation (Duman et al., 2012). Subunits (NR1, NR2A, NR2B) of the NMDA receptor, as well as its downstream effectors BDNF and PSD-95 proteins in the hippocampus were analyzed with western blot analysis in our study (Fig. 5A). There were no significant differences of NR1 protein levels between the two groups (n = 3, $t = 0.0280$, $p = 0.9790$). The expression level of NR2A (1.31 ± 0.08) was significantly higher in the ketamine treated rats than in the controls (1.00 ± 0.09, $t = 2.816$, $p = 0.048$). In contrast, NR2B was decreased by over a quarter of control levels (NR2B 0.21 ± 0.06 vs. 1.00 ± 0.15 of control, $t = 5.002$, $p = 0.0075$).

The expression levels of BDNF and PSD-95 levels were significantly lower in the ketamine treated offspring than in the control offspring (Figs. 7A and B, BDNF 0.65 ± 0.09 vs. 1.00 ± 0.09 of control, $t = 3.369$, $p = 0.0281$ and PSD-95 0.30 ± 0.06 vs. 1.00 ± 0.11 of control, $t = 5.488$, $p = 0.0054$).

Discussion

The present study provides preclinical evidence that pregnant rats receiving ketamine during the second trimester causes memory impairment, depression-like and anxiety-like behavioral disorders together with lower body weight in their litters. These neurological abnormalities found in the offspring were associated with neuronal loss, pyramidal cell abnormality and reduced cell proliferation in the hippocampus, together with mismatched expression of NMDA receptor subunits, which, in turn, caused a reduction of BDNF and PSD-95 in the hippocampus.

It is well known that alcohol consumption during pregnancy is associated with a constellation of developmental disabilities characterized by physiological, cognitive, and behavioral impairments (Brocardo et al., 2012; Famy et al., 1998; O'Connor and Paley, 2006; Olney et al., 2002). It was reported that 72% of fetal alcohol syndrome (FAS) patients experienced learning disorders in childhood, as well as requiring

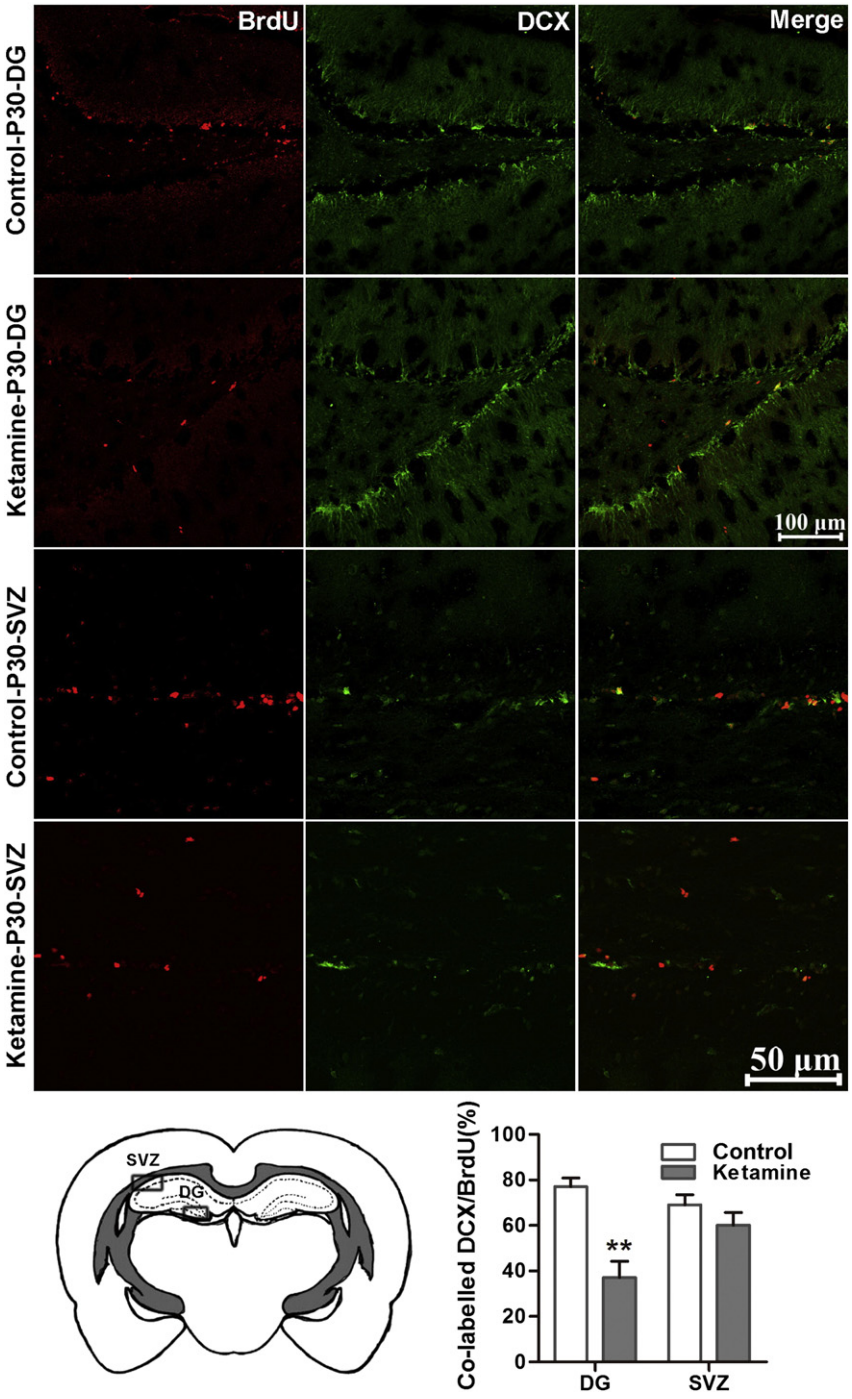

Fig. 6. Ketamine administrated in the second trimester impaired postnatal neurogenesis in offspring. Offspring received i.p. injection of 50 mg/kg BrdU once daily for 3 consecutive days from the postnatal day 28 to day 30. They were killed by decapitation 12 h after the last injection for frozen sections. $BrdU^+$ cells and co-labeled of BrdU and doublecortin (DCX) cells were subjected to confocal microscopic quantitative analysis in the 2-boxed areas indicated in the schematic draw. Fluorescent immunohistochemistry for BrdU (red) and DCX (green), merged image showing co-localization of BrdU and DCX. Co-localized cells with DCX/BrdU relative to BrdU+ cells are expressed as mean ± SEM (n = 5); $^{**}p < 0.01$ vs. control.

psychiatric care for adult-onset disturbances, including a 44% incidence of major depression and 40% incidence of psychosis (Famy et al., 1998). Given that ethanol has both NMDA antagonist and GABA mimetic properties (Ikonomidou et al., 2000), any drugs with NMDA antagonistic and/or GABA mimetic properties, e.g. anticonvulsants, sedatives or anesthetics, could have detrimental effect on babies if such drugs are abused by pregnant women or used for surgery during pregnancy. Our paradigm of behavioral disorders, albeit obtained from an animal model, is similar to those seen as a result of maternal alcohol and drug consumption during pregnancy (Brocardo et al., 2012; Famy et al., 1998). In fact, all general anesthetics can cross the placenta and there is convincing evidence showing that anesthetics, including ketamine, can cause neuronal apoptosis during synaptogenesis and behavioral deficits in later life (Brambrink et al., 2012; Palanisamy et al., 2011; Slikker et al., 2007; Zheng et al., 2013). Depression is characterized by lowered mood, a loss in the ability to experience pleasure (anhedonia), suicidal thoughts and changes in sleep, appetite, sexual desire and often gastrointestinal motility (Alladin, 2013). In rodents, these depression- and anxiety-like behaviors can be measured by SPT, FST and OFT (Nestler and Hyman, 2010). Our behavioral data clearly demonstrates that midtrimester exposure to ketamine induces depression-like consequences in adolescent rats. In addition, our data also shows that ketamine treated offspring display deficits in spatial memory capabilities as manifested by increases in the latency in the MWM, consistent with previous studies (Kong et al., 2012; Zheng et al., 2013). Taken together, our data suggests that maternal exposure to ketamine causes mixed neurobehavioral disorders that may include both affective disorders and cognitive deficiency up to adolescence. The affective disorders, especially depression and anxiety, found in our study are novel and have been not reported before, although both pre-clinical experimental data and human epidemiologic evidence suggest an association between anesthesia and surgery early in life and late-onset learning disabilities (Hansen et al., 2011; Jevtovic-Todorovic et al., 2003; Kalkman et al., 2009; Satomoto et al., 2009; Viberg et al., 2008). It should be pointed out that, as demonstrated through a number of behavioral measures aimed at demonstrating behavioral disorders in the present study, we found that our model is analogous to the previous studies of maternal alcohol and other abused drug consumption during pregnancy, most of which are concerned with long-term developmental consequences of their impact on attention, memory and mood disorders (Brocardo et al., 2012; Day et al., 2011; Richardson et al., 2011). Thus, the work reported here may have enormous social implications as memory impairment and mood disorders have a significant negative impact to people's daily lives (Crocker et al., 2013; Iosifescu, 2012; Papazacharias and Nardini, 2012).

NMDA receptors in the hippocampus play an important role in both learning and anxiety (Femenia et al., 2012; Shapiro, 2001). In addition, although a predominant view that the hippocampus is primarily involved in memory formation, recent studies have also highlighted that it plays a role in emotion, particularly in anxiety and depression (David et al., 2009; Femenia et al., 2012; Masi and Brovedani, 2011). Our present study demonstrated a significant reduction of the NMDA receptor NR2B subunit expression in the ketamine treated offspring, while levels of the NR2A subunit were significantly increased, with the NR1 subunit unaffected. This is in line with previous studies showing that no changes in expression of the NR1 subunit were observed in the amygdala, locus coeruleus or cerebellum in depression (Karolewicz et al., 2005), although changes in NR2 subunit expression were not investigated in those studies. In addition, the abnormalities of the NMDA receptor system have been previously observed in postmortem brain tissue from major depressives and suicide victims; indeed, NR2A subunit up-regulation in the amygdala was reported in depression (Karolewicz et al., 2009). All these indicate that anxiety- and depression-like behaviors found in the ketamine treated offspring may be due to NR2A over expression.

Activation of synaptic NMDA receptors results in BDNF release and also evokes Akt and ERK signaling activation, which in turn stimulate mTOR and synaptic protein (including GluR1, PSD95, and synapsin I) synthesis, leading to insertion of GluR1 and increased synaptogenesis (Duman et al., 2012). Our study showed that an NMDAR antagonist, in this case ketamine, administrated in the second trimester may weaken the mTOR signaling pathway and disturb NMDA receptors, as well as induce lower expression of downstream effectors BDNF and PSD-95. This is in accordance with the studies of depression and/or anxiety both in rodents (Castren and Rantamaki, 2010; Duman and Monteggia, 2006; Ladurelle et al., 2012; Toro and Deakin, 2005) and humans (Feyissa et al., 2009). In those studies, subjects exposed to different types of physical or social stress had decreased BDNF level in the hippocampus and prefrontal cortex (PFC). Conversely, anti-depressant treatment increases the expression of BDNF in the hippocampus, amygdala and/or PFC (Castren and Rantamaki, 2010; Duman and Monteggia, 2006; Ladurelle et al., 2012). In models of depression and stress, such

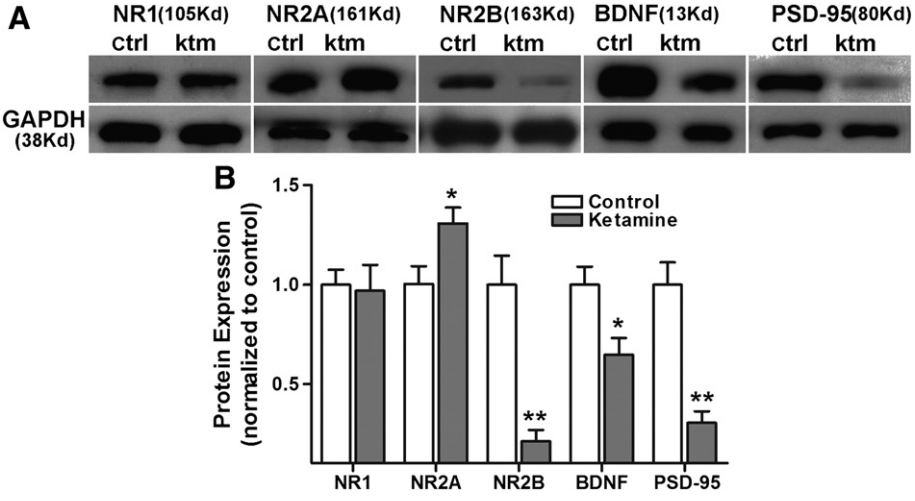

Fig. 7. Ketamine administrated in the second trimester perturbed NMDA receptor subunits and down-regulated BDNF and PSD-95 in the hippocampus in offspring. Female Sprague–Dawley rats on gestational day 14 (n = 3) received ketamine with an initial intramuscular injection (40 mg/kg) followed by continuous intravenous infusion by pump via tail vein at a rate of 40–60 mg/kg/h for 2 h, while no treatment was given to pregnant age matched controls (n = 3). After treatment, dams were allowed to give birth naturally. On postnatal day (PND) 30, after behavioral test, brains were harvested for western blot analysis to determine the expression of subunits NR1, NR2A and NR2B of the N-methyl-D-aspartate (NMDA) receptor, and the downstream effectors brain-derived neurotrophic factor (BDNF) and postsynaptic density protein 95 (PSD-95). Shown are example bands (A) and mean densitometric data normalized to controls (B). Data are mean ± SEM (n = 3); *$p < 0.05$, **$p < 0.01$ vs. control.

as chronic unpredictable stress (CUS), atrophy and reduced dendritic arborization of the pyramidal neurons in the CA3 region of hippocampus were clearly demonstrated (McEwen et al., 2012; Stewart et al., 2005). Interestingly, these effects can be reversed by antidepressants (Hajszan et al., 2005; McEwen et al., 1997; Watanabe et al., 1992). These stress paradigms all reduce neural progenitor proliferation and neurogenesis in the hippocampus of adult animals, similar to the results we have shown here (Malberg and Duman, 2003; Pham et al., 2003). It has been suggested that PSD-95 plays a crucial role in the trafficking, membrane targeting and internalization of NMDA receptor complexes and BDNF. It is also particularly abundant in the hippocampus and in the cerebral cortex, where it regulates neurogenesis, neuronal survival, maturation of connections and plasticity (axonal branching, dendritic arborization) (Chao, 2003; Ladurelle et al., 2012). As found in our study, both were down-regulated after ketamine administration to pregnant rats, subsequently causing remarkable neurological disorders in offspring. Collectively, although the dose of ketamine is relatively low and the administration duration is just 2 h in this study, the long term cellular and neurobehavioral effects are very profound, likely due to the biological effects of these molecular cascades.

In summary, our data shows that maternal exposure to ketamine during the second trimester induces anxiety- and depression-like behaviors, together with cognitive impairment in offspring rats which is likely associated with its adverse effects on NMDA receptor subunits and mTOR signaling pathway. The good news is that ketamine is rarely used in pregnant women in the developed countries but it is still often being used as a “rescuing” anesthesia, for example when regional anesthesia cannot meet surgery requirement, in the developing countries (Craven, 2007). In addition, the risk of increasing use of ketamine as a recreational drug, particularly by female abusers of child-bearing age in Southeast Asia cannot be ignored (Dargan and Wood, 2012). If our data can be extrapolated to clinical settings, then more rigorous epidemiologic studies and clinical trials to assess the long-term effects of maternal exposure to anesthesia on their babies are urgently needed. The most important finding is the clear need to prohibit mothers abusing drugs such as ketamine to prevent long term damage to the child.

Disclosure

Authors claim no conflict of interest.

Acknowledgments

This work was supported by grants from the Program of Shenzhen Science and Technology Innovation Committee, China (knowledge innovation program, JCYJ20130402090541514).

References

Alladin, A., 2013. The power of belief and expectancy in understanding and management of depression. Am. J. Clin. Hypn. 55, 249–271.
Bambico, F.R., et al., 2009. Decline in serotonergic firing activity and desensitization of 5-HT1A autoreceptors after chronic unpredictable stress. Eur. Neuropsychopharmacol. 19, 215–228.
Brambrink, A.M., et al., 2012. Ketamine-induced neuroapoptosis in the fetal and neonatal rhesus macaque brain. Anesthesiology 116, 372–384.
Brocardo, P.S., et al., 2012. Anxiety- and depression-like behaviors are accompanied by an increase in oxidative stress in a rat model of fetal alcohol spectrum disorders: protective effects of voluntary physical exercise. Neuropharmacology 62, 1607–1618.
Castren, E., Rantamaki, T., 2010. The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of developmental plasticity. Dev. Neurobiol. 70, 289–297.
Chao, M.V., 2003. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat. Rev. Neurosci. 4, 299–309.
Cheek, T.G., Baird, E., 2009. Anesthesia for nonobstetric surgery: maternal and fetal considerations. Clin. Obstet. Gynecol. 52, 535–545.
Clancy, B., et al., 2001. Translating developmental time across mammalian species. Neuroscience 105, 7–17.
Craven, R., 2007. Ketamine. Anaesthesia 62 (Suppl. 1), 48–53.
Crocker, L.D., et al., 2013. Relationships among cognition, emotion, and motivation: implications for intervention and neuroplasticity in psychopathology. Front. Hum. Neurosci. 7, 261.
Cryan, J.F., et al., 2005. Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. Neurosci. Biobehav. Rev. 29, 547–569.
Dargan, P.I., Wood, D.M., 2012. Recreational drug use in the Asia Pacific region: improvement in our understanding of the problem through the UNODC programmes. J. Med. Toxicol. 8, 295–299.
David, D.J., et al., 2009. Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression. Neuron 62, 479–493.
Day, N.L., et al., 2011. The effects of prenatal marijuana exposure on delinquent behaviors are mediated by measures of neurocognitive functioning. Neurotoxicol. Teratol. 33, 129–136.
D'Hooge, R., De Deyn, P.P., 2001. Applications of the Morris water maze in the study of learning and memory. Brain Res. Brain Res. Rev. 36, 60–90.
Duman, R.S., Monteggia, L.M., 2006. A neurotrophic model for stress-related mood disorders. Biol. Psychiatry 59, 1116–1127.
Duman, R.S., et al., 2012. Signaling pathways underlying the rapid antidepressant actions of ketamine. Neuropharmacology 62, 35–41.
Famy, C., et al., 1998. Mental illness in adults with fetal alcohol syndrome or fetal alcohol effects. Am. J. Psychiatry 155, 552–554.
Femenia, T., et al., 2012. Dysfunctional hippocampal activity affects emotion and cognition in mood disorders. Brain Res. 1476, 58–70.
Feyissa, A.M., et al., 2009. Reduced levels of NR2A and NR2B subunits of NMDA receptor and PSD-95 in the prefrontal cortex in major depression. Prog. Neuropsychopharmacol. Biol. Psychiatry 33, 70–75.
Goodman, S., 2002. Anesthesia for nonobstetric surgery in the pregnant patient. Semin. Perinatol. 26, 136–145.
Green, C.J., et al., 1981. Ketamine alone and combined with diazepam or xylazine in laboratory animals: a 10 year experience. Lab. Anim. 15, 163–170.
Hajszan, T., et al., 2005. Short-term treatment with the antidepressant fluoxetine triggers pyramidal dendritic spine synapse formation in rat hippocampus. Eur. J. Neurosci. 21, 1299–1303.
Hansen, T.G., et al., 2011. Academic performance in adolescence after inguinal hernia repair in infancy: a nationwide cohort study. Anesthesiology 114, 1076–1085.
Ikonomidou, C., et al., 2000. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287, 1056–1060.
Ing, C., et al., 2012. Long-term differences in language and cognitive function after childhood exposure to anesthesia. Pediatrics 130, e476–e485.
Iosifescu, D.V., 2012. The relation between mood, cognition and psychosocial functioning in psychiatric disorders. Eur. Neuropsychopharmacol. 22 (Suppl. 3), S499–S504.
Jevtovic-Todorovic, V., et al., 2003. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J. Neurosci. 23, 876–882.
Kalkman, C.J., et al., 2009. Behavior and development in children and age at the time of first anesthetic exposure. Anesthesiology 110, 805–812.
Karolewicz, B., et al., 2005. Elevated levels of the NR2C subunit of the NMDA receptor in the locus coeruleus in depression. Neuropsychopharmacology 30, 1557–1567.
Karolewicz, B., et al., 2009. Elevated levels of NR2A and PSD-95 in the lateral amygdala in depression. Int. J. Neuropsychopharmacol. 12, 143–153.
Kong, F.J., et al., 2012. Fetal exposure to high isoflurane concentration induces postnatal memory and learning deficits in rats. Biochem. Pharmacol. 84, 558–563.
Ladurelle, N., et al., 2012. Agomelatine (S20098) modulates the expression of cytoskeletal microtubular proteins, synaptic markers and BDNF in the rat hippocampus, amygdala and PFC. Psychopharmacology (Berl.) 221, 493–509.
Malberg, J.E., Duman, R.S., 2003. Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. Neuropsychopharmacology 28, 1562–1571.
Masi, G., Brovedani, P., 2011. The hippocampus, neurotrophic factors and depression: possible implications for the pharmacotherapy of depression. CNS Drugs 25, 913–931.
McEwen, B.S., et al., 1997. Prevention of stress-induced morphological and cognitive consequences. Eur. Neuropsychopharmacol. 7 (Suppl. 3), S323–S328.
McEwen, B.S., et al., 2012. Stress and anxiety: structural plasticity and epigenetic regulation as a consequence of stress. Neuropharmacology 62, 3–12.
Nestler, E.J., Hyman, S.E., 2010. Animal models of neuropsychiatric disorders. Nat. Neurosci. 13, 1161–1169.
O'Connor, M.J., Paley, B., 2006. The relationship of prenatal alcohol exposure and the postnatal environment to child depressive symptoms. J. Pediatr. Psychol. 31, 50–64.
Olney, J.W., et al., 2002. The enigma of fetal alcohol neurotoxicity. Ann. Med. 34, 109–119.
Palanisamy, A., et al., 2011. Rats exposed to isoflurane in utero during early gestation are behaviorally abnormal as adults. Anesthesiology 114, 521–528.
Papazacharias, A., Nardini, M., 2012. The relationship between depression and cognitive deficits. Psychiatr. Danub. 24 (Suppl. 1), S179–S182.
Pham, K., et al., 2003. Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. Eur. J. Neurosci. 17, 879–886.
Prut, L., Belzung, C., 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur. J. Pharmacol. 463, 3–33.
Richardson, G.A., et al., 2011. Prenatal cocaine exposure: effects on mother- and teacher-rated behavior problems and growth in school-age children. Neurotoxicol. Teratol. 33, 69–77.

Rofael, H.Z., et al., 2003. Immunomodulation by cocaine and ketamine in postnatal rats. Toxicology 188, 101–114.
Rygula, R., et al., 2005. Anhedonia and motivational deficits in rats: impact of chronic social stress. Behav. Brain Res. 162, 127–134.
Satomoto, M., et al., 2009. Neonatal exposure to sevoflurane induces abnormal social behaviors and deficits in fear conditioning in mice. Anesthesiology 110, 628–637.
Shapiro, M., 2001. Plasticity, hippocampal place cells, and cognitive maps. Arch. Neurol. 58, 874–881.
Sholl, D.A., 1953. Dendritic organization in the neurons of the visual and motor cortices of the cat. J. Anat. 87, 387–406.
Slikker, W.J., et al., 2007. Ketamine-induced neuronal cell death in the perinatal rhesus monkey. Toxicol. Sci. 98, 145–158.
Smith, D.M., Mizumori, S.J., 2006. Hippocampal place cells, context, and episodic memory. Hippocampus 16, 716–729.
Stewart, M.G., et al., 2005. Stress suppresses and learning induces plasticity in CA3 of rat hippocampus: a three-dimensional ultrastructural study of thorny excrescences and their postsynaptic densities. Neuroscience 131, 43–54.
Taupin, P., 2007. BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res. Rev. 53, 198–214.
Toro, C., Deakin, J.F., 2005. NMDA receptor subunit NRI and postsynaptic protein PSD-95 in hippocampus and orbitofrontal cortex in schizophrenia and mood disorder. Schizophr. Res. 80, 323–330.
Viberg, H., et al., 2008. Neonatal ketamine exposure results in changes in biochemical substrates of neuronal growth and synaptogenesis, and alters adult behavior irreversibly. Toxicology 249, 153–159.
Watanabe, Y., et al., 1992. Tianeptine attenuates stress-induced morphological changes in the hippocampus. Eur. J. Pharmacol. 222, 157–162.
Zheng, H., et al., 2013. Sevoflurane anesthesia in pregnant mice induces neurotoxicity in fetal and offspring mice. Anesthesiology 118, 516–526.