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Prenatal Exposure to Ketamine Leads to Anxiety-Like Behaviors and Dysfunction in Bed Nucleus of Stria Terminalis

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ABSTRACT

Background: Both the clinical and preclinical studies have suggested embryonic or infant exposure to ketamine, a general anesthetic, pose a great threat to the developing brain. However, it remains unclear how ketamine may contribute to the brain dysfunctions.

Methods: A mouse model of prenatal exposure to ketamine was generated by i.m. injection and continuous i.p. infusion of pregnant mice. Open field test and elevated plus maze test were used to analyze the behavioral alterations induced by ketamine. Immunostaining by c-Fos was used to map the neuron activity. Chemogenetic modulation of the neurons was used to rescue the abnormal neuron activity and behaviors.

Results: Here we show that mice prenatally exposed to ketamine displayed anxiety-like behaviors during adulthood, but not during puberty. C-Fos immunostaining identified abnormal neuronal activity in Bed Nucleus of the Stria Terminalis, the silencing of which by chemogenetics restores the anxiety-like behaviors.

Conclusions: Taken together, these results demonstrate a circuitry mechanism of ketamine-induced anxiety-like behaviors.

Keywords: anxiety, Bed Nucleus of the Stria Terminalis (BNST), chemogenetics, ketamine

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Significance Statement

A growing body of evidence suggests ketamine, a very common anesthetic, could affect the brain development of fetuses and babies. However, it is still unclear about how ketamine may contribute to brain dysfunctions. Here, using a mouse model, our study demonstrates that prenatal exposure to ketamine results in anxiety-like behaviors in adulthood. Furthermore, a circuitry mechanism was proposed to underlie such behavioral abnormalities. These results not only shed new light on how ketamine might induce neurodevelopmental defects but also have implications on how to restore such defects in human.

Introduction

Up to 2% percent of women had to receive surgery during pregnancy because of fetal problems, laparoscopy, and other medical needs (Goodman, 2002). Although the general anesthetics have been proven to be safe to human health, there is still a great concern about their risk of affecting the fetus. The process of neural development is highly orchestrated and may be rather vulnerable to anesthetics. This concept was supported by several retrospective clinical investigations showing a clear association between anesthesia and/or surgery during early life and late-onset learning disabilities (Hansen et al., 2011; Ing et al., 2012; Stratmann et al., 2014). Moreover, the severity of cognitive and behavioral impairment correlated to the species, dose of anesthesia, and the exposure duration as shown by prospective General Anesthesia compared with Spinal Anesthesia study (Davidson et al., 2016; McCann et al., 2019) and 3 large-scale, 2-way observational cohort studies: Pediatric Anesthesia Neurodevelopmental Assessment (Sun et al., 2016), Mayo Anesthesia Safety in Kids (Wilder et al., 2009; Sprung et al., 2012), and University of California, San Francisco (Stratmann et al., 2014). (Andropoulos, 2018; Davidson and Sun, 2018; Warner et al., 2018). In 2017, the US Food and Drug Administration warned that prolonged or repeated exposure to general anesthetics may affect neurodevelopment in children (http://www.fda.gov/Drugs/DrugSafety/ucm532356.htm; accessed November 10, 2017). This warning is also underpinned by several preclinical studies (Palanisamy et al., 2011; Davidson and Sun, 2018).

Ketamine is a very common anesthetic in clinical practice (Iacobucci et al., 2017). Since its first usage as an anesthetic in the 1970s, ketamine has been widely adopted in pediatric anesthesia, sedation, analgesia, and auxiliary anesthesia as well as anti-depression because of its low cost, high efficiency at relatively lower dose, and quick and long-term action (Duman et al., 2012; Zanos et al., 2016; Aleksandrova et al., 2017; Iacobucci et al., 2017). In addition, the clinical dose of ketamine has little impact on human respiration and circulation. However, with the extensive application of ketamine, increasing side effects have also been reported in recent decades, such as addiction, hallucination, and cognitive impairment (Fitzgerald, 2012; Iacobucci et al., 2017). Much ketamine is consumed by the public including pregnant women as a result of drug abuse in Asia-Pacific in recent years (Rofael et al., 2003; Cheung and Yew, 2019). Even though large doses of ketamine are rarely used for anaesthetizing pregnant women, a small dose of ketamine is still required in some cases like hypovolemia and asthma (Akamatsu et al., 1974; Mercier and Benhamou, 1998). Therefore, it is very necessary to evaluate the effect of ketamine on the fetus.

Ketamine can serve as a noncompetitive N-Methyl-D-aspartic acid receptor antagonist whereby it exerts broad function in the nervous system (Iacobucci et al., 2017). It can also interfere with opioids, monoamines, choline, purine, and adenosine receptor systems (Lois and De Kock, 2008). A preclinical study has shown that a 2-hour exposure to ketamine during the late second trimester of pregnant rats could lead to increased apoptotic neuronal death, impaired neurogenesis, and induced anxiety-like and depression-like behaviors in offspring (Zhao et al., 2014). Additionally, oxidative stress, defects in neurogenesis, and synaptogenesis have also been proposed to underlie the neurotoxicity of ketamine to developing brains (Cheung and Yew, 2019). However, it is largely unknown about the circuitry mechanism of ketamine-induced brain dysfunctions.

Here, our study demonstrates a causal role of prenatal exposure to ketamine in the development of anxiety-like behaviors and abnormal activities in Bed Nucleus of the Stria Terminalis (BNST). Silencing of the neuron activity in BNST could rescue the anxiety-like behaviors. Our study provides a circuitry mechanism underlying the neuropathological effect of ketamine.

MATERIALS AND METHODS

Subjects

All husbandry and experimental procedures in this study were approved by Animal Care and Use Committees at the Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, and Southern Medical University, China. All efforts were made to minimize the number of animals used. Adult (8 weeks) C57BL/6J mice (Guangdong Medical Laboratory Animal Center, Guangzhou, China) were group-housed, accessible to food pellets and water ad libitum, and maintained on a 12-hour-light/-dark cycle (lights on at 7:00 AM). The timed-pregnant C57BL/6J mice were housed in the same specific pathogen free environment as the adult mice. Dams were used for experiments on gestation day 12.

Anesthesia and Experimental Design

On gestation day 12, 16 dams were randomly divided into control (n = 8) and ketamine (n = 8) groups. Controls were left undisturbed in their home cages. The ketamine group received a bolus dose (60 mg/kg) (Zhao et al., 2014) of ketamine (Gutian Pharmaceutical Co., Ltd. Fujian province, China) via i.m. injection at the right lower extremity of femoral muscle, followed by continuous i.p. infusion through a soft plastic tube at a rate of 60–90 mg/kg/h (10 mg/mL diluted with saline) for 2 hours. This duration of ketamine infusion is clinically relevant while the infusion rate was distinct with an aim 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 control group only received an equal volume of saline via i.m. injection at the right lower extremity of femoral muscle.

Blood was drawn from the left ventricle of mice and subjected to arterial blood gas analysis on a blood gas analyzer

(EDANi15, Shenzhen, China; see the Results section in Table 1). The core body temperature was measured with a rectal probe and maintained between 36.5°C 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. All dams were allowed to give birth naturally. All pups were allowed to grow up with their mothers until postnatal day (P) 21 (the day of birth was designated as P0; 3–4 cages/dam), and 3 to 4 male pups from each dam were selected for later behavioral tests on P35 and P56. Immediately after behavioral tests, brain samples were harvested for immunohistochemistry. Whole brain-wide slices were screened to identify the key brain regions. Furthermore, remodeling and chemogenetic for specific nuclei, that is, drug-specific receptors exclusively activated by designer drugs, for intervention experiments until P56. Four weeks later (P86), the virus was expressed and behavioral experiments were conducted. A schematic representation of experimental protocols is shown in Figure 1.

Behavioral Test

Male offspring of control and ketamine-treated mice with 3 to 4 pups from each dam were then subject to the open field test (OFT) and elevated plus maze test (EPM) at P35. At P56, their behaviors were analyzed again by OFT and EPM, followed by fear conditioning test (FCS). To minimize the influence of related factors on animal behavior tests, each behavioral test of the 2 groups of offspring was performed at the same time interval on the day of testing. Prior to behavioral testing, all mice were handled for elimination of tension for 3 consecutive days.

Table 1. Blood Gas and Sugar Tests of Pregnant Rats After Anesthesia

Blood gas test	Control (n = 6)	Ketamine (n = 6)
pH	7.38 ± 0.01	7.39 ± 0.01
$PaCO_2$ (mmHg)	41.33 ± 0.30	41.54 ± 0.51
PaO_2 (mmHg)	106.14 ± 0.51	105.65 ± 0.20
$HCO3^-$ (mmol/L)	24.34 ± 0.33	24.45 ± 0.31
Glucose (mg/dL)	105.83 ± 0.40	105.87 ± 0.42
Temperature (°C)	36.43 ± 0.35	36.54 ± 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 2 groups. Data are presented as mean ±SEM (n = 6).

OFT

The OFT is used for assessing general locomotor activity and anxiety-like behaviors (Prut and Belzung, 2003). An OF arena (50 cm × 50 cm × 50 cm) made of white PVC placed in a quiet environment was used to assess anxiety-related behavior and locomotor activity (Prut and Belzung, 2003). The center of the arena was defined as a smaller concentric square inside the arena covering 25% of the area of the arena floor (i.e., 25 cm × 25 cm). Mice were allowed to freely explore the OF for 5 minutes, following 5 minutes of EPM as described below. The mice were placed in the center of the activity chamber equipped with an overhead camera to record activity. Images were captured for 5 minutes per animal and were analyzed using Image Anymaze software. The number of entries to the center, time spent in the center, and the total distance traveled in OF (total activity) were recorded and then analyzed by Anymaze software (Stoelting, Co., IL). The OF was cleaned after each test with 25% ethanol solution to avoid the disturbance by smell from other mice.

EPM

The EPM is widely used for assessing depressive behavior (Sartor and Aston-Jones, 2012; Li et al., 2018). Mice were placed on a 4-arm plus maze with 2 open and 2 closed arms (white PVC, 30 cm long per arm, 35 cm wide) raised 50 cm above the ground for 5-minute sessions. The EPM was cleaned after each test with 25% ethanol solution. The number of entries to the open arms and time spent in the open arms were recorded and analyzed by Anymaze software.

FCS

The contextual and cued FCS is one of the behavioral tests that assesses the ability of mice to learn and remember an association between environmental cues and aversive experiences. To reduce possible influences of cage transportation on behavior and to adapt the mice to the experimental environment, the cages containing the mice were transferred from the animal holding room into a soundproof waiting room adjacent to a soundproof testing room at least 30 minutes before each test began.

Following the EPM at P56, mice were exposed to the fear conditioning chamber (24 × 24 × 30 cm, L × W × H, with Plexiglas walls and a metal fence floor) by the Shanghai XinRuan Conditional Fear Test System and submitted to the FCS (11 minutes, animals were given 5 presentations of the 0.8-mA foot shock lasting 1 second [unconditioning test; US]). The first US stimuli was presented 139

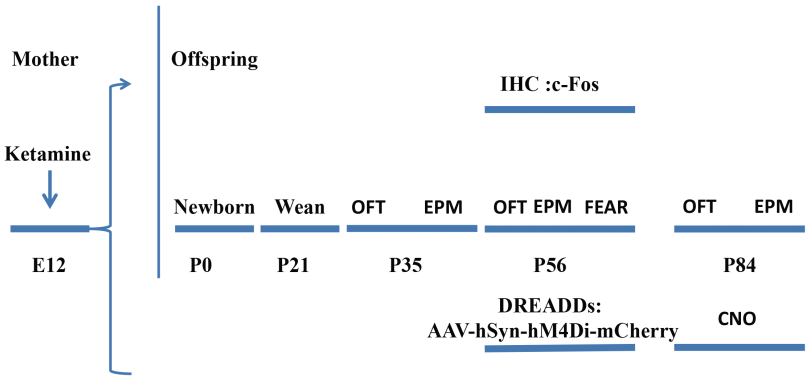

Figure 1. The flow chart of the experimental procedure. CNO, clozapine N-oxide; DREADDs, designer receptors exclusively activated by designer drugs; E, gestation day; EPM, elevated plus maze; FCS, fear conditioning test; IHC, immunohistochemistry; OFT, open field test; P, postnatal day.

seconds into the session, and the intertrial interval between US presentations was 105 seconds on average (range 90–120). Five conditioning trials were applied to test fear acquisition. Individual mice were returned to their home cages 100 seconds after the last trial of conditioning. Light (660 seconds, 25 lux) was as the background. After the conditioning session was completed, the mice were returned to the same conditioning chamber and scored for freezing behavior to measure contextually conditioned fear (context test). A delay interval between the conditioning and the context test has been generally set at 24 hours. To test mouse freezing response to contextual stimuli that were no longer coupled to footshocks (fear extinction), the mice were placed in the conditioning chamber and allowed to freely explore the chamber for 300 seconds without US presentations. Freezing was defined as the absence of movements, except for those related to respiration and slight head tremble. The freezing time was recorded and analyzed by Anymaze software and Shanghai XinRuan Conditional Fear Analyze System (Shanghai XinRuan Information Technology Co., LTD).

Histology

Tissue Section Preparation—Six animals were randomly selected from each group (n=2/dam) at P56. After 90 minutes of the EPM test, mice were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). Fixed brains were cryoprotected in 30% sucrose in PBS pH 7.35 and cut on a cryostat in 40-mm slices. Immunohistochemistry (IHC) was performed to map (1) the c-Fos activation in the global brain after OFT and EPM and (2) the expression of AAV-hSyn-hM4Di-mCherry virus in BNST. Antibody staining was performed on single-well floating tissue sections. Sections were incubated for 48 hours in primary antibodies at 4°C followed by overnight incubation with secondary antibodies at 4°C. Primary antibodies used in this study were rabbit anti-c-Fos (2250, Cell Signaling Technology; 1:500) and mouse anti-c-Fos (ab208942, Abcam; 1:500). For counterstaining, sections were incubated for 10 minutes with 40, 6-diamidin-2-phenylindol (0.4 mg/mL, Sigma). All of the images were captured with a Zesis LSM 880 confocal microscope or an Olympus VS120 virtual microscopy slide scanning system.

Count of c-Fos–Positive Cells—For each group, 5 animals were analyzed. Six brain regions were scanned by c-Fos immunostaining after behavioral assays. Five consecutive slices were examined for each brain structure. The cell density was defined as the number of double-positive dots of c-Fos and 40, 6-diamidin-2-phenylindol staining in a given area. The dots were counted in a double-blinded manner (Lammel et al., 2012; Zhou et al., 2019).

Stereotaxic Surgery and Virus Injection—Stereotaxic surgeries and virus injections were performed as described previously (Zhang et al., 2007; Wei et al., 2015) at P56. Briefly, animals were anesthetized with sodium pentobarbital (80 mg/kg body weight [BW]) and placed in a stereotaxic apparatus (RWD, China) where anesthesia was maintained with 1% isoflurane. Injections were made using a microsyringe pump (UMP3/Micro4) using a 10-mL syringe connected to a 33-Ga needle (Neuros, Hamilton, Reno, NV). The syringe was not removed until 10 minutes after the end of infusion to allow diffusion of the virus. AAV9 viruses encoding hSyn-hM4Di-mCherry (for chemogenetic experiment) were packaged by BrainVTA, China. Viral vector titers were in the range of 3 to 6×10^{12} genome copies/mL. The BNST coordinates were AP: 0.14 mm, ML: ±0.9 mm, and DV: – 4.0 approximately – 4.3 mm. For chemogenetic experiment, the BNST received bilateral injections.

In Vivo Chemogenetic Manipulation—The offspring mice whose dams received ketamine anesthesia were used for chemogenetic experiments. All mice were injected bilaterally by AAV9-hSyn-hM4Di-mCherry at P56. The mice received CNO (0.3 mg/kg BW, clozapine N-oxide, C0832 Sigma) via i.p. injection was used as the BNST inhibition group, while 0.9% saline i.p. injection were used for control group. After 4-week injection of virus, mice underwent in vivo chemogenetic inhibition of BNST prior to 30 minutes of the OFT and EPM test. Possible side effects of CNO injections on locomotor activity were evaluated by comparing performance in an OFT 30 minutes after CNO injection (n=5–7/group).

Quantification and Statistical Analysis

All the statistical data are represented as mean±SEM. All n values represent the number of mice used in each experiment. Data distribution was assumed to be normal, but this was not formally tested. Unpaired Student t test was used to detect effects of treatment. All statistics were performed with Graph Pad Prism (GraphPad Software, Inc.) unless otherwise indicated. Statistical significance was set at $P<.05$. Asterisks are defined as follows: $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$, and $^{****}P<.0001$. All statistical tests are specified in each figure legend.

RESULTS

Early Exposure to Ketamine-Induced Anxiety-Like Behaviors

To generate the mouse model of prenatal exposure to ketamine, pregnant mice on gestation 12 (n=8) received ketamine via i.m. injection at right lower extremity of femoral muscle (72 mg/kg) followed by continuous i.p. injection by pump at a rate of 90 mg/kg/h for 2 hours. The control group was composed of age-matched pregnant mice without any treatment except for the initial via lower back i.m. injection of the same volume of saline (n=8).

Since ketamine has broad functions in the nervous system such as providing sedation, the mood-related behaviors were analyzed in mice exposed to ketamine prenatally by OFT and EPM. We speculate deficit might be manifested by the developing brain. Unexpectedly, no significant difference was found between the 2 groups in their anxiety-like behaviors at P35, as shown by the time spent in the center of open field and in the open arm zone of EPM of around 11% and 35%, respectively (Figure 2). In accordance with the behavioral data, similar neuron activity was found between 2 groups, as shown by the c-Fos staining data (Figure 2). It is also concordant with the observation from Coronel-Oliveros and Pacheco-Calderon (Coronel-Oliveros and Pacheco-Calderon, 2018).

In contrast to the juvenile mice, the mice at P56 that received ketamine displayed obvious avoidance of the central region of OFT and the open arm of EPM with unaltered total activity (Figure 3). It indicates increased anxiety-like behaviors caused by prenatal exposure to ketamine. Counting of the defecate particles also favors this conclusion (Figure 3F–G).

The increased anxiety-like behaviors and abnormal activity in BNST persist to P84, as shown by OFT and EPM assays and c-Fos staining (supplementary Figure 1). Taken together, it suggests that prenatal exposure to ketamine could induce a long-lasting adult-onset dysfunction in BNST.

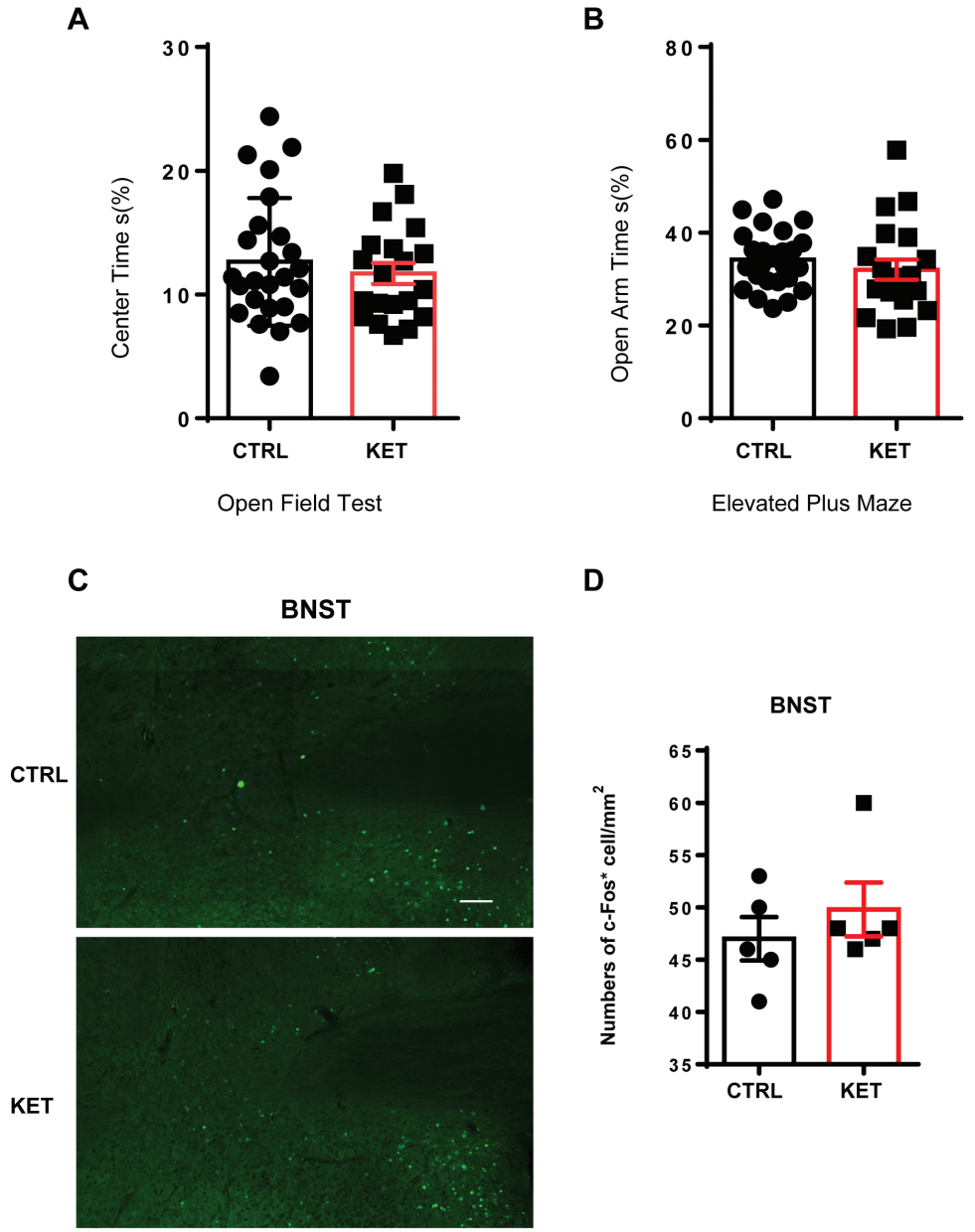

Figure 2. Mice prenatally exposed to ketamine displayed no significant anxiety-like behaviors at postnatal day (P) 35. Mice that received ketamine anesthesia at gestation day (E) 12 had no significant change in anxiety-like behaviors, as measured by the time spent in the center of the open field test (OFT) (A: n = 25 mice for control group, n = 20 mice for ketamine group, t = 0.6817, P > .05; unpaired Student's *t* test and in the open arms of elevated plus maze (EPM); B: n = 25 mice for control group, n = 20 mice for ketamine group, t = 0.8889, P > .05; unpaired Student's *t* test) at P35. No significant difference was found in c-Fos signaling and D, P > .05; unpaired Student's *t* test). CTRL , control group; KET, experiment group that received ketamine anesthesia at E12.

In addition, the associated learning ability was also evaluated by FCS. There is no significant difference between ketamine and control groups (Figure 3E). It consists with the notion that ketamine mainly affect mood- and addiction-related behaviors.

Anxiety-Like Behaviors Are Associated Abnormal Activities in BNST

To explore the mechanism underlying ketamine-induced behavioral alterations, the neuron activity was evaluated by c-Fos staining directly after behavioral assays in locus coeruleus, ectorhinal cortex, hippocampus, basolateral amygdaloid nucleus, central amygdala, lateral amygdaloid nucleus, bed nucleus of the stria terminalis, ventromedial thalamic nucleus, and dorsomedial hypothalamic nucleus. All these brain regions have a close relationship with anxiety-like behaviors. Then we found neurons in BNST have obviously higher c-Fos expression in ketamine-exposed mice. It suggests the altered activity of BNST might contribute to ketamine-induced anxiety-like behaviors (Figure 4).

Chemogenetic Inhibition of BNST Restores Anxiety-Like Behaviors

To test whether dysfunction of BNST is responsible for the above-mentioned anxiety-like behaviors, we silenced BNST neurons by chemogenetics. AAV virus carrying hM4Di was

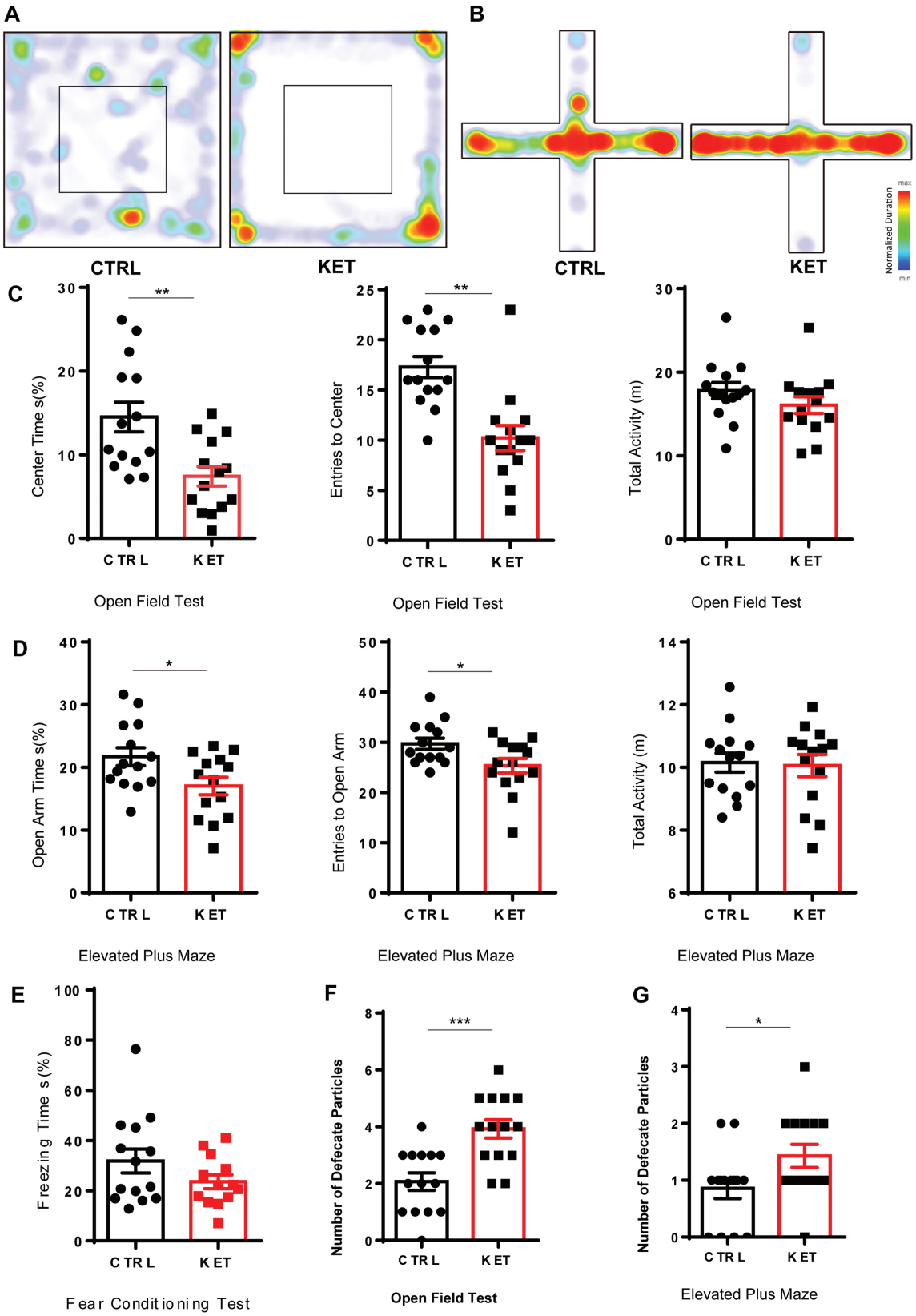

Figure 3. Mice prenatally exposed to ketamine exhibit more anxiety-like behaviors at postnatal day (P) 56. Mice prenatally exposed to ketamine (gestation day [E] 12) explored the center of the open field test (OFT) (A and C) and the open arm area of the environment (B and D) for a shorter duration and less frequently than controls. (C: n = 14 mice for each group, t = 3.340 and t = 3.331, $^{**}P < .01$; unpaired Student's *t* test; D: n = 14 mice for each group, t = 2.325 and t = 2.404, $^{*}P < .05$; unpaired Student's *t* test. Horizontal arms indicate closed arms of elevated plus maze [EPM]). The group treated with ketamine had no significant differences in locomotor activity. (E) There was no statistical difference in freezing time between the 2 groups at P56 for contextual fear condition (FCS) test. (E: n = 14 mice for each group, t = 2.195, $P > 0.05$; unpaired Student's *t* test). (F and G) Increased defecate particles were observed during OFT and EPM assay in the ketamine-treated group. (F: n = 14 mice for each group, t = 4.184, $^{***}P < .001$; G: n = 14 mice for each group, t = 2.126, $^{*}P < .05$; unpaired Student's *t* test).

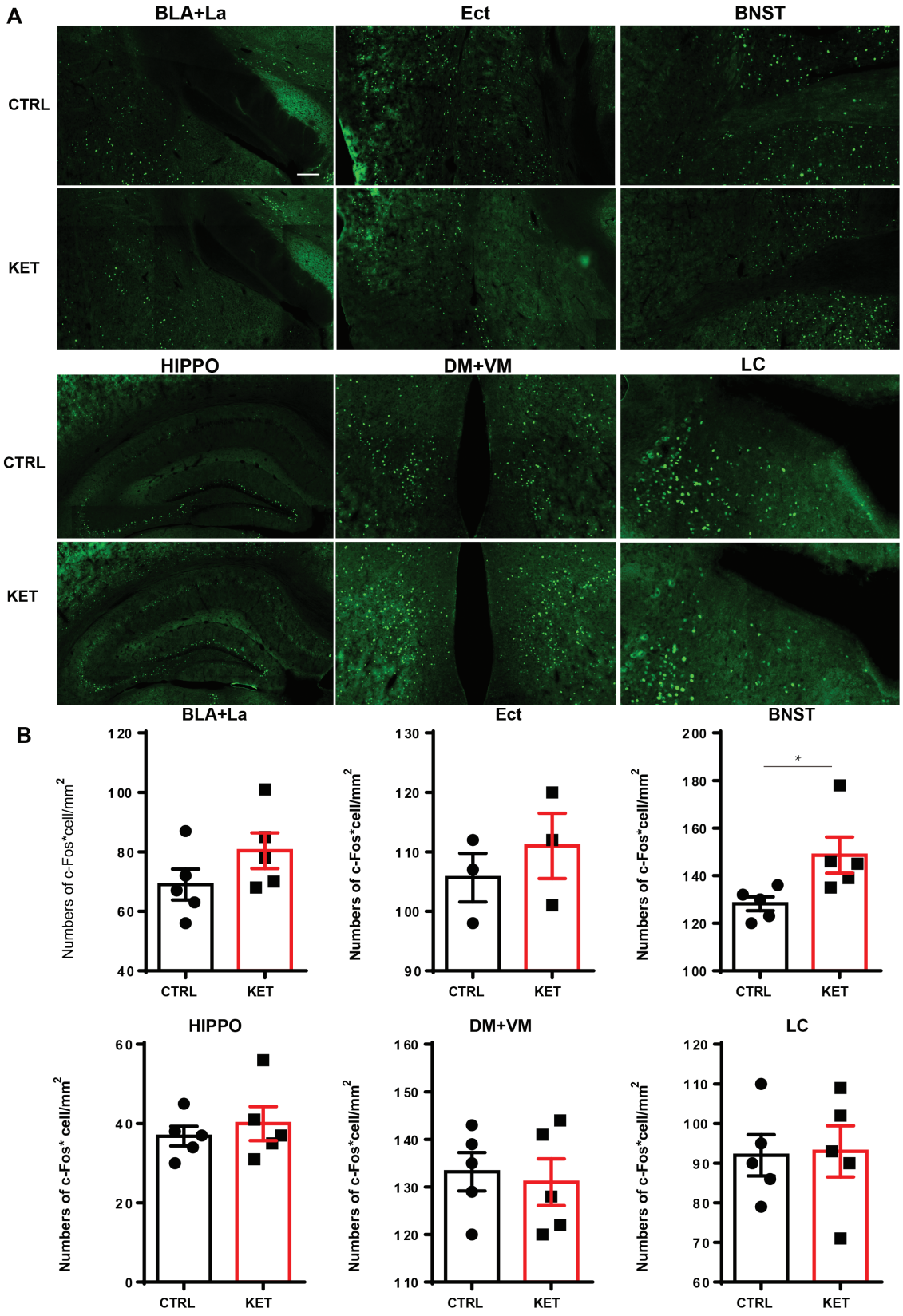

Figure 4. Screening for the abnormal nuclei related to the increased anxiety-like behaviors. (A) Representative images of c-Fos immunostaining in locus coeruleus (LC), ectorhinal cortex (Ect), hippocampus (HIPPO), basolateral amygdaloid nucleus (BLA), central amygdala (CEA), lateral amygdaloid nucleus (La), bed nucleus of the stria terminalis (BNST), ventromedial thalamic nucleus (VM), and dorsomedial hypothalamic nucleus (DM) (scale bars = 100 μm). (B) The c-Fos expression in BNST of the ketamine group was higher than that of controls. (Five consecutive sections for each brain region, animal number n = 5, t = 2.498, *P < .05; unpaired Student's t test. The calculation of the dots is double-blind.)

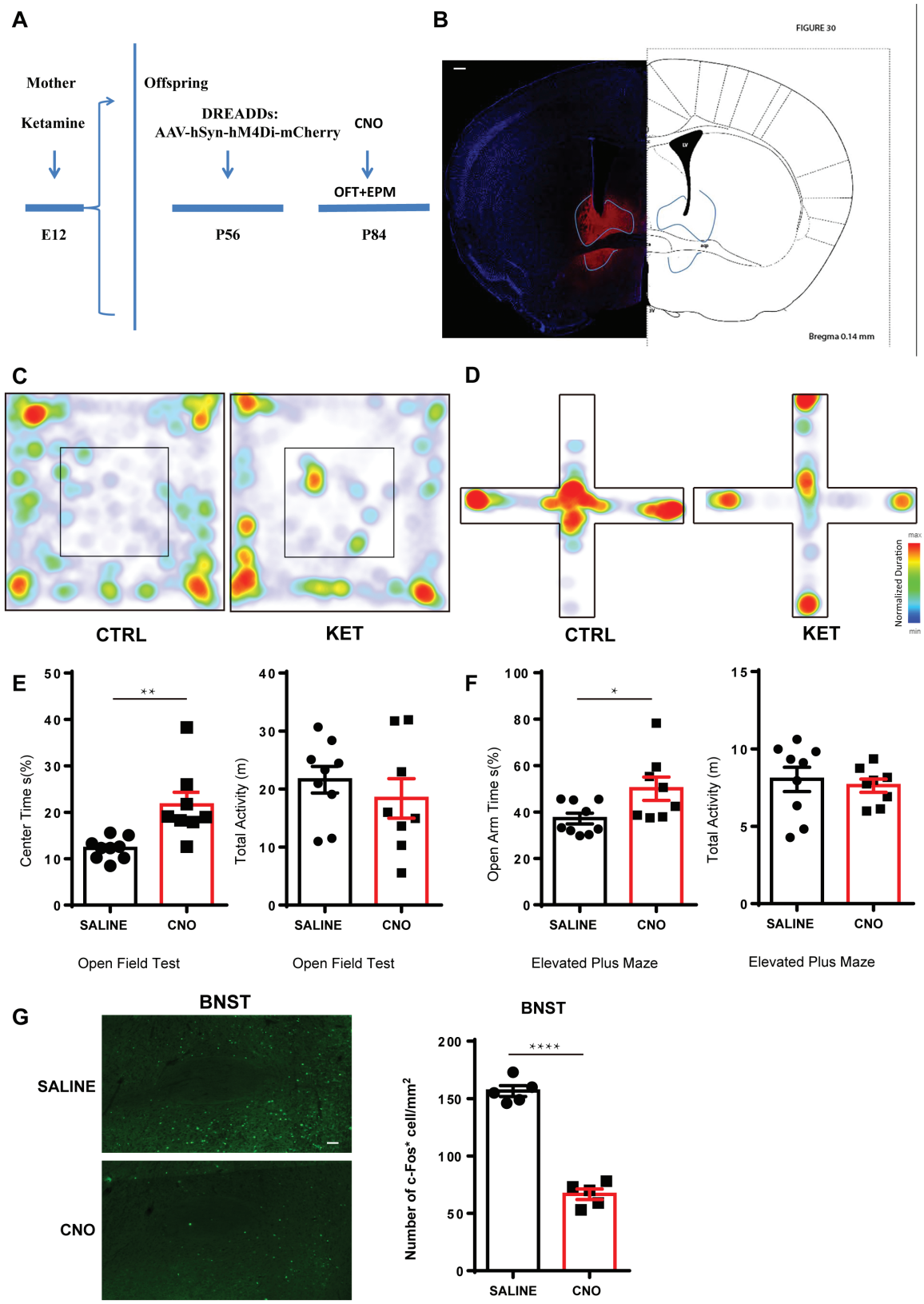

Figure 5. Chemogenetic inhibition of Bed Nucleus of the Stria Terminalis (BNST) neurons rescued the increased anxiety-like behaviors. (A) Chemogenetic strategy and experimental timeline, bilateral LC inhibition. (B) Injection of hM4Di-mCherry to BNST neurons (left: blue, DAPI; red, hM4Di-mCherry; scale bars = 100 μm; bregma: 0.14 mm. Right: sketch map). (C–F) Inhibition of BNST by CNO restores the increased anxiety-like behaviors as measured by the time spent in the center of OFT and in the open arms of EPM (E, n = 9 mice for saline control, n = 8 mice for CNO group, t = 3.489, **P < .01; F, n = 9 mice for saline control, n = 8 mice for CNO group, t = 2.428, *P < .05; each dot represents the result of 1 trial of test of the animal; unpaired Student's *t* test). Injection of hM4Di-mCherry had no effect on the locomotor of the mice (data not shown). (G) CNO injection significantly inhibited the neuron activity in BNST. (Five consecutive sections for each brain region, animal number n = 5, t = 13.58, ****P < .001; unpaired Student's *t* test).

bilaterally injected into BNST. Then CNO (0.3 mg/kg BW) was given by i.p. injection. At 30 minutes post-CNO administration, mice were subjected to OFT and EPM tests. The results show that anxiety-like behaviors of the CNO group were markedly lower than those of the saline control group (Figure 5), and the neuron activity was apparently inhibited by CNO injection (Figure 5). This indicates silencing of BNST is sufficient to rescue the anxiety-like behaviors induced by ketamine.

Discussion

Despite increasing awareness of the risk of ketamine to perturb early brain development, it remains unclear which brain functions might be affected. Inspired by the clinical retrospective studies, much emphasis has been laid on the impairment of cognitive abilities. Here our results demonstrate causality between prenatal exposure to ketamine and anxiety-like behaviors. Of note, these abnormal behaviors were not obvious at P35. We suppose that it might be due to the immaturity of anxiety-related neural circuits (Walker et al., 2003; Tovote et al., 2015) such as BNST. This nucleus is vulnerable during sexual differentiation (Chung et al., 2002). Moreover, the sex-related hormones are highly dynamic during this stage (Altemus, 2017, 2019), which might affect the manifestation of anxiety-like behaviors. Actually, similar effects were also observed in other studies. For example, it has been shown that phase spatial memory impairment induced by anesthetics occurs during the developmental stage from 6 weeks (Olney, 2002; Constantinidis and Wang, 2004; Viberg et al., 2008; Satomoto et al., 2009; Stratmann et al., 2009; Fang et al., 2012). In Fang's (Fang et al., 2012) research, 7-day-old rats treated with sevoflurane showed cognitive impairment via the Morris Water Maze test at 42 days, but not at 21 days. In previous studies on sevoflurane (Olney, 2002), the results showed that cognitive function via FCS in mice was significantly worse than that of the control group from 8 weeks to 14–17 weeks.

Furthermore, a circuitry mechanism was proposed underlying such brain dysfunction. An abnormally higher c-Fos expression in BNST was found to be correlated with the anxiety-like behaviors. Silencing BNST by chemogenetics rescues the increased anxiety level. It indicates a pivotal role of BNST in ketamine-induced anxiety-like behaviors. The BNST was first described nearly a century ago and has since emerged as a region central to motivated behavior and affective states, including anxiety, fear, aversion, stress, and reward (Lebow and Chen, 2016). Multiple studies have indicated that the BNST send projections into the parabrachial nucleus, the lateral hypothalamus, the periaqueductal gray, the central nucleus of the amygdala, and the ventral tegmental area (Jennings et al., 2013). BNST is a component of the extended amygdala that modulates affective behavior (Alheid, 2003; Ostrander et al., 2003). Stimulating BNST has been shown to increase anxiety-like behaviors, which is consistent with our findings. Further studies will be needed to elucidate how BNST and related circuits were affected by ketamine.

Despite these findings, we also have to note some limitations in this study. First, this study only focused on 1 aspect of affective disorder, that is, anxiety-like behavior. In the future, the behavioral paradigm of depressive emotion, Forced Swimming Test, and Sucrose Preference Test should be included. In addition to analyzing the anxiety-like behaviors, fear-conditioned assay was also conducted. Unexpectedly, no significant learning disabilities were found in mice exposed to ketamine, which contradicts previous reports showing that cognitive ability could be impaired by ketamine (Zhao et al., 2014). However, it should be noted that the behavioral paradigm is the MWM. More behavioral paradigms of learning and memory may be needed to give a more comprehensive depiction of the mice's learning abilities. Lastly, more detailed information should be given about the cellular and molecular alterations that underlie BNST dysfunction.

Nonetheless, this study demonstrates a causal role of prenatal exposure to ketamine in the development of anxiety-like behaviors. Moreover, BNST was found to be responsible for these behavioral alterations. It provides a targetable neural circuit to intervene such brain dysfunction. These results not only shed new light on the neurological mechanism of ketamine-induced neurodevelopmental defects but also have implications on how to restore such defects in humans.

Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

Acknowledgments

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Statement of Interest

None.

References

Akamatsu TJ, Bonica JJ, Rehmet R, Eng M, Ueland K (1974) Experiences with the use of ketamine for parturition. I. Primary anesthetic for vaginal delivery. Anesth Analg 53:284–287.

Aleksandrova LR, Phillips AG, Wang YT (2017) Antidepressant effects of ketamine and the roles of AMPA glutamate receptors and other mechanisms beyond NMDA receptor antagonism. J Psychiatry Neurosci 42:222–229.

Alheid GF (2003) Extended amygdala and basal forebrain. Ann N Y Acad Sci 985:185–205.

Altemus M (2017) Neuroendocrine Networks and Functionality. Psychiatr Clin North Am 40:189–200.

Altemus M (2019) Neuroendocrine Networks and Functionality. Med Clin North Am 103:601–612.

Andropoulos DB (2018) Effect of anesthesia on the developing brain: infant and fetus. Fetal Diagn Ther 43:1–11.

Cheung HM, Yew DTW (2019) Effects of perinatal exposure to ketamine on the developing brain. Front Neurosci 13:138.

Chung WC, De Vries GJ, Swaab DF (2002) Sexual differentiation of the bed nucleus of the stria terminalis in humans may extend into adulthood. J Neurosci 22:1027–1033.

Constantinidis C, Wang XJ (2004) A neural circuit basis for spatial working memory. Neuroscientist 10:553–565.

Coronel-Oliveros CM, Pacheco-Calderón R (2018) Prenatal exposure to ketamine in rats: implications on animal models of schizophrenia. Dev Psychobiol 60:30–42.

Davidson AJ, Sun LS (2018) Clinical evidence for any effect of anesthesia on the developing brain. Anesthesiology 128:840–853.

Davidson AJ, et al.; GAS consortium (2016) Neurodevelopmental outcome at 2 years of age after general anaesthesia and awake-regional anaesthesia in infancy (GAS): an international multicentre, randomised controlled trial. Lancet 387:239–250.

Duman RS, Li N, Liu RJ, Duric V, Aghajanian G (2012) Signaling pathways underlying the rapid antidepressant actions of ketamine. Neuropharmacology 62:35–41.

Fang F, Xue Z, Cang J (2012) Sevoflurane exposure in 7-day-old rats affects neurogenesis, neurodegeneration and neurocognitive function. Neurosci Bull 28:499–508.

Fitzgerald PJ (2012) The NMDA receptor may participate in widespread suppression of circuit level neural activity, in addition to a similarly prominent role in circuit level activation. Behav Brain Res 230:291–298.

Goodman S (2002) Anesthesia for nonobstetric surgery in the pregnant patient. Semin Perinatol 26:136–145.

Green CJ, Knight J, Precious S, Simpkin S (1981) Ketamine alone and combined with diazepam or xylazine in laboratory animals: a 10 year experience. Lab Anim 15:163–170.

Hansen TG, Pedersen JK, Henneberg SW, Pedersen DA, Murray JC, Morton NS, Christensen K (2011) Academic performance in adolescence after inguinal hernia repair in infancy: a nationwide cohort study. Anesthesiology 114:1076–1085.

Iacobucci GJ, Visnjevac O, Pourafkari L, Nader ND (2017) Ketamine: an update on cellular and subcellular mechanisms with implications for clinical practice. Pain Physician 20:E285–E301.

Ing C, DiMaggio C, Whitehouse A, Hegarty MK, Brady J, von Ungern-Sternberg BS, Davidson A, Wood AJ, Li G, Sun LS (2012) Long-term differences in language and cognitive function after childhood exposure to anesthesia. Pediatrics 130:e476–e485.

Jennings JH, Sparta DR, Stamatakis AM, Ung RL, Pleil KE, Kash TL, Stuber GD (2013) Distinct extended amygdala circuits for divergent motivational states. Nature 496:224–228.

Lammel S, Lim BK, Ran C, Huang KW, Betley MJ, Tye KM, Deisseroth K, Malenka RC (2012) Input-specific control of reward and aversion in the ventral tegmental area. Nature 491:212–217.

Lebow MA, Chen A (2016) Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders. Mol Psychiatry 21:450–463.

Li L, Feng X, Zhou Z, Zhang H, Shi Q, Lei Z, Shen P, Yang Q, Zhao B, Chen S, Li L, Zhang Y, Wen P, Lu Z, Li X, Xu F, Wang L (2018) Stress accelerates defensive responses to looming in mice and involves a locus coeruleus-superior colliculus projection. Curr Biol 28:859–871.e5.

Lois F, De Kock M (2008) Something new about ketamine for pediatric anesthesia? Curr Opin Anaesthesiol 21:340–344.

McCann ME, et al.; GAS Consortium (2019) Neurodevelopmental outcome at 5 years of age after general anaesthesia or awake-regional anaesthesia in infancy (GAS): an international, multicentre, randomised, controlled equivalence trial. Lancet 393:664–677.

Mercier FJ, Benhamou D (1998) Promising non-narcotic analgesic techniques for labour. Baillieres Clin Obstet Gynaecol 12:397–407.

Olney JW (2002) New insights and new issues in developmental neurotoxicology. Neurotoxicology 23:659–668.

Ostrander MM, Badiani A, Day HE, Norton CS, Watson SJ, Akil H, Robinson TE (2003) Environmental context and drug history modulate amphetamine-induced c-fos mRNA expression in the basal ganglia, central extended amygdala, and associated limbic forebrain. Neuroscience 120:551–571.

Palanisamy A, Baxter MG, Keel PK, Xie Z, Crosby G, Culley DJ (2011) Rats exposed to isoflurane in utero during early gestation are behaviorally abnormal as adults. Anesthesiology 114:521–528.

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.

Rofael HZ, Turkall RM, Abdel-Rahman MS (2003) Immunomodulation by cocaine and ketamine in postnatal rats. Toxicology 188:101–114.

Sartor GC, Aston-Jones G (2012) Regulation of the ventral tegmental area by the bed nucleus of the stria terminalis is required for expression of cocaine preference. Eur J Neurosci 36:3549–3558.

Satomoto M, Satoh Y, Terui K, Miyao H, Takishima K, Ito M, Imaki J (2009) Neonatal exposure to sevoflurane induces abnormal social behaviors and deficits in fear conditioning in mice. Anesthesiology 110:628–637.

Sprung J, Flick RP, Katusic SK, Colligan RC, Barbaresi WJ, Bojanić K, Welch TL, Olson MD, Hanson AC, Schroeder DR, Wilder RT, Warner DO (2012) Attention-deficit/hyperactivity disorder after early exposure to procedures requiring general anesthesia. Mayo Clin Proc 87:120–129.

Stratmann G, Sall JW, May LD, Bell JS, Magnusson KR, Rau V, Visrodia KH, Alvi RS, Ku B, Lee MT, Dai R (2009) Isoflurane differentially affects neurogenesis and long-term neurocognitive function in 60-day-old and 7-day-old rats. Anesthesiology 110:834–848.

Stratmann G, et al. (2014) Effect of general anesthesia in infancy on long-term recognition memory in humans and rats. Neuropsychopharmacology 39:2275–2287.

Sun LS, Li G, Miller TL, Salorio C, Byrne MW, Bellinger DC, Ing C, Park R, Radcliffe J, Hays SR, DiMaggio CJ, Cooper TJ, Rauh V, Maxwell LG, Youn A, McGowan FX (2016) Association between a single general anesthesia exposure before age 36 months and neurocognitive outcomes in later childhood. Jama 315:2312–2320.

Tovote P, Fadok JP, Luthi A (2015) Neuronal circuits for fear and anxiety. Nat Rev Neurosci 16:317–331.

Viberg H, Pontén E, Eriksson P, Gordh T, Fredriksson A (2008) Neonatal ketamine exposure results in changes in biochemical substrates of neuronal growth and synaptogenesis, and alters adult behavior irreversibly. Toxicology 249:153–159.

Walker DL, Toufexis DJ, Davis M (2003) Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. Eur J Pharmacol 463:199–216.

Warner DO, Shi Y, Flick RP (2018) Anesthesia and neurodevelopment in children: perhaps the end of the beginning. Anesthesiology 128:700–703.

Wei P, Liu N, Zhang Z, Liu X, Tang Y, He X, Wu B, Zhou Z, Liu Y, Li J, Zhang Y, Zhou X, Xu L, Chen L, Bi G, Hu X, Xu F, Wang L (2015) Processing of visually evoked innate fear by a non-canonical thalamic pathway. Nat Commun 6:6756.

Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, Mickelson C, Gleich SJ, Schroeder DR, Weaver AL, Warner DO (2009) Early exposure to anesthesia and learning disabilities in a population-based birth cohort. Anesthesiology 110:796–804.

Zanos P, Moaddel R, Morris PJ, Georgiou P, Fischell J, Elmer GI, Alkondon M, Yuan P, Pribut HJ, Singh NS, Dossou KS, Fang Y, Huang XP, Mayo CL, Wainer IW, Albuquerque EX,

Thompson SM, Thomas CJ, Zarate CA Jr, Gould TD (2016) NMDAR inhibition-independent antidepressant actions of ketamine metabolites. Nature 533:481–486.

Zhang F, Wang LP, Brauner M, Liewald JF, Kay K, Watzke N, Wood PG, Bamberg E, Nagel G, Gottschalk A, Deisseroth K (2007) Multimodal fast optical interrogation of neural circuitry. Nature 446:633–639.

Zhao T, Li Y, Wei W, Savage S, Zhou L, Ma D (2014) Ketamine administered to pregnant rats in the second trimester causes long-lasting behavioral disorders in offspring. Neurobiol Dis 68:145–155.

Zhou Z, Liu X, Chen S, Zhang Z, Liu Y, Montardy Q, Tang Y, Wei P, Liu N, Li L, Song R, Lai J, He X, Chen C, Bi G, Feng G, Xu F, Wang L (2019) A VTA GABAergic neural circuit mediates visually evoked innate defensive responses. Neuron 103:473–488.e6.