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Research paper

Pre-adolescence repeat exposure to sub-anesthetic doses of ketamine induces long-lasting behaviors and cognition impairment in male and female rat adults

Amir Barzegar Behrooz [a], Mahdieh Nasiri [a], Soheila Adeli [a], Maryam Jafarian [b], Seyed Khalil Pestehei [c,d,*,1], Javad Fahanik Babaei [a,**,2]

[a] Electrophysiology Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran
[b] Brain and Spinal Cord Injury Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran
[c] Department of Anesthesiology, Tehran University of Medical Sciences, Tehran, Iran
[d] Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

In pre-adolescence, repeated anesthesia may be required for therapeutic interventions. Adult cognitive and neurobehavioral problems may result from preadolescent exposure to anesthetics. This study examined the long-term morphological and functional effects of repeated sub-anesthetic doses of ketamine exposure on male and female rat adults during pre-adolescence. Weaned 48 pre-adolescent rats from eight mothers and were randomly divided into four equal groups: control group and the ketamine group of males and females (20 mg/kg daily for 14 days); then animals received care for 20–30 days. Repeated exposure to sub-anesthetic doses of ketamine on cognitive functions was assayed using Social discrimination and novel object tests. Besides, an elevated plus maze and fear conditioning apparatus were utilized to determine exploratory and anxiety-like behavior in adults. Toluidine blue stain was used to evaluate the number of dead neurons in the hippocampus, and the effects of ketamine on synaptic plasticity were compared in the perforant pathway of the CA1 of the hippocampus. Our study indicates that repeated exposure to sub-anesthetic doses of ketamine during pre-adolescence can result in neurobehavioral impairment in male and female rat adulthood but does not affect anxiety-like behavior. We found a significant quantifiable increase in dark neurons. Recorded electrophysiologically, repeat sub-anesthetic doses of ketamine resulted in hampering long-term potentiation and pair pulse in male adult animals. Our results showed that repeated exposure to sub-anesthetic doses of ketamine during pre-adolescence can induce hippocampus and neuroplasticity changes later in adulthood. This study opens up a new line of inquiry into potential adverse outcomes of repeated anesthesia exposure in pre-adolescent rats.

1. Introduction

Many people undergo surgery and procedures requiring general anesthesia each year. Using anesthesia during pre-adolescence may be necessary to perform therapeutic interventions such as laser therapy, radiotherapy, and ambulatory surgery (Lee et al., 2014; Lunardi et al., 2010). Repeated dressing changes may necessitate sedation or anesthesia for severe burn patients. Thus, the anesthetic may be given during or after essential brain development. Several clinical studies have shown that general anesthesia exposure during childhood or infancy can cause a variety of clinical disturbances, including impaired memory, executive function, neuroapoptosis, and long-term neurocognitive difficulties (Creeley et al., 2014; Lee et al., 2014; Steinmetz et al., 2009). Several laboratory observations in rodents and nonhuman primates suggest that exposure to general anesthetics during the neonatal or early post-natal period may result in cell death, impaired synaptic growth, neurogenesis, and subsequent cognitive and behavioral disorders, which could lead to neurobehavioral problems in later childhood and adulthood

* Correspondence to: Neuroscience Research Center Institute, Tehran University of Medical Sciences, Tehran, Iran.
** Correspondence to: Electrophysiology Research Center, Neuroscience Research Center Institute, Tehran University of Medical Sciences, Tehran, Iran.
E-mail addresses: skhpestehei@sina.tums.ac.ir (S.K. Pestehei), J-fbabaei@farabi.tums.ac.ir (J.F. Babaei).
[1] ORCID ID: 0000–0002-0538–8898
[2] ORCID ID: 0000–0001-6707–132X

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(DiMaggio et al., 2012; Fan et al., 2021; Hasegawa et al., 2019; Jevtovic-Todorovic, 2013; Lin et al., 2014; Wilder et al., 2009).

The dentate gyrus and the hippocampal formation, also known as the Cornus ammonia (CA), comprise the hippocampal formations, which are important structures linked to memory functions and affective responses. Ketamine has dissociative and antidepressant effects on the senses, but in healthy people, it interferes with the hippocampus's contribution to memory retrieval and decoding (Honey et al., 2005). It has been shown that there is an increase in dark neurons in the hippocampus after long-term ketamine exposure. These neurons feature pyknotic nuclei and acidophilic cytoplasm, often associated with damaged neurons' morphological changes (Ahmadpour et al., 2016). Dark neurons may be easily identified from normal neurons since they exhibit aberrant basophilia and severe shrinkage (Jafarian et al., 2010).

Ketamine can also affect neuroplasticity, behavioral changes, and learning and memory effects. Synaptic dysfunction can contribute to neurodegenerative diseases such as Alzheimer's disease by impairing well-tuned synaptic transmission and controlling excitatory synaptic transmission (Vazquez-Juarez et al., 2023a; Zhang et al., 2023). A great deal of research has been conducted on the long-term potentiation (LTP) of the hippocampus, which has been implicated in mechanisms that influence memory formation (Feifel, 2016; Vazquez-Juarez et al., 2023b). Synaptic plasticity in the hippocampal region plays a significant role in several learning and memory processes (Drewniany et al., 2015; Granry et al., 2000; Hirota and Lambert, 2011a; Kurdi et al., 2014). Hence, it is postulated that an impairment in synaptic plasticity within the hippocampus is the underlying mechanism for the observed learning impairments in individuals who have received anesthesia.

In the past 65 years, ketamine has been used as an anesthetic drug as a non-selective antagonist of the N-methyl-D-aspartate (NMDA) receptor (Feifel, 2016). Due to its affordability and wide availability in developing countries, ketamine is commonly used for anesthesia and to treat depression, seizures, chronic pain, and headaches (Drewniany et al., 2015; Hirota and Lambert, 2011b; Kurdi et al., 2014; Strasburger et al., 2017). There is, however, evidence that ketamine exposure during early post-natal development and beyond may cause learning and behavioral problems later in life among rodents and nonhuman primates (Brambrink et al., 2012; Huang and Yang, 2015). According to studies conducted on rodents, repeated exposure to anesthetics during neonatal development may lead to long-term impairments of the central nervous system (CNS) (Amrock et al., 2015; Brambrink et al., 2012; Makaryus et al., 2015; Shen et al., 2013). Even though several studies have been conducted on the long-term effects of anesthetic drugs in neonates, little research has been conducted on their long-term impact on adolescents. Additionally, there is a lack of studies examining possible sex-related changes in the effects of sub-anesthetic doses of ketamine.

This study provides a comprehensive morphological and functional analysis of the effects of repeated sub-anesthetic doses of ketamine exposure over the long term. In addition, we investigated the effects of repeated exposure to male and female rats model of pre-adolescence on induced ketamine-related neurotoxicity in the hippocampus and the effects of long-term use on behavior, histology, and electrophysiology. Lastly, we examined the induction of LTP in the perforant pathway of the adult hippocampus following ketamine exposure in adolescents.

2. Material and method

2.1. Design of experiment

This study used pre-adolescent male and female rats weighing 35–40 gr. This age was chosen based on prior literature describing the adolescent period in rodents. Post-natal day 25–30 marks the beginning of early adolescence, while post-natal day 60–70 marks the beginning of early adulthood (L. Spear, 2000; L. P. Spear, 2004; L. P. Spear and Brake, 1983). Mother rats and their offspring were obtained from the experimental study center at the Tehran University of Medical Sciences throughout the experimentation. We kept the rats in cages of four rats each, at a temperature of $22 \pm 2\,^{\circ}C$ under 12-hour light/dark cycles. The animals were provided with a standard diet and water throughout the study. All experiments were carried out between 9 a.m. and 4 p.m. NIH guidelines for the care and use of laboratory animals (NIH No: 8023, revised 1978) were followed for all experimental protocols and procedures. This experiment was approved by the Ethics and Research Committee of the Tehran University of Medical Sciences (IR TUMS.NI.REC.1400,05).

Rats were separated and adapted for three days after weaning, and then ketamine was administered on day 24. Weaned 48 pre-adolescent Wistar rats from eight mothers were randomly divided into four equal groups: a control group of males and females and a ketamine group of males and females (20 mg/kg daily for 14 days, intraperitoneally). The same dosage of ketamine was used throughout the experiment. A loss of eyelid and righting reflexes was used to confirm deep anesthesia. The respiratory frequency of the rats and the color of their skin were monitored during anesthesia. All groups were cared for 30 to 35 days following the last administration of ketamine in preparation for the planned experiments. During days 0, 5, 10, and 15, each experimental group's animals were weighed, and their weight gain was monitored. The experimental design of the study is illustrated in Fig. 1.

2.2. Behavioral tests

2.2.1. Social discrimination test

The main principle of the Social discrimination test is that a subject rat may choose to spend time in any of three compartments in the box during two experimental sessions. Rats were placed in a social interaction apparatus of three communicating chambers ($90 \times 45 \times 45$ cm, elevated 50 cm). Three 10-minute sessions were conducted as part of the Social discrimination test to assess habituation, sociability, and novelty preference. A test rat was initially placed in the middle chamber and allowed to explore it freely for 10 min. In the left chamber of the test apparatus, a stimulus rat is placed under a wire cage following the habituation period. In the right chamber of the test apparatus, a similar wire cage is located without a stimulus rat. One enclosure was introduced with an unfamiliar conspecific (Stranger 1), and the test rat was allowed to sniff either the stranger or an empty enclosure. Despite having similar backgrounds, genders, and ages, the stranger rat was unfamiliar with the test rat. An unfamiliar conspecific (Stranger 2) was introduced into the other enclosure after 24 h, and the test rat was allowed to sniff both conspecifics. Using a video camera-based system (EthoVision, Noldus, Version 14), the total exploration time for each object was automatically recorded. After each trial, the stranger was placed on either the social apparatus's left or right side and cleaned with 20% alcohol to minimize olfactory distraction. In sessions for sociability and preference, the amount of time spent exploring the target chamber (stranger 2) was measured (Blázquez et al., 2018).

2.2.2. Novel object recognition (NOR) task

Rat cognition can be assessed using the novel object recognition (NOR) test, based on the animal's natural tendency to examine a novel object more than a familiar item. Our test (habituation, familiarization, recognition) was completed in three days. For habituation, the day before starting the test, the animals were allowed to explore the empty arena ($60 \times 60 \times 50$ cm) for 5 min. After 24 h, each animal was placed in an arena with two similar objects attached to the floor at an equal distance from the walls and each other. During the first (familiarization) session, rats investigated two comparable items. The animal was taken and returned to its cage after 10 min. The animal was taken and returned to its cage after 10 min. On the third day, the rat was placed in the arena for 10 min with a novel object replaced by one of the familiar objects. The new thing was the same height, material, and volume as the known object but was different in form and color. Item exploration is defined as the animal's nostrils being in the zone at least 2 cm from the item. The

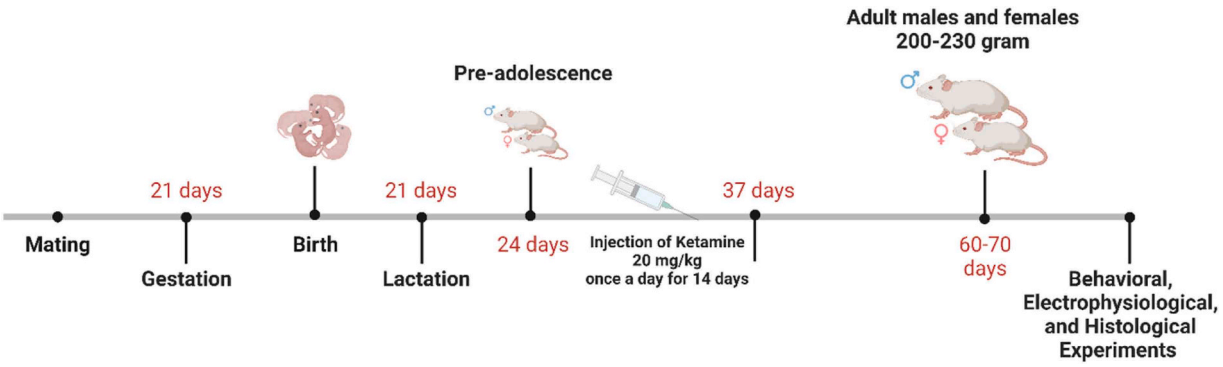

Fig. 1. Schematic diagram of the study's experimental design.

animals had never seen our goods before they were examined. The total time spent investigating each object was automatically recorded using a video camera-based system (EthoVision, Noldus, Version 14). "The recognition Index (RI) was computed using the following formula: [RI = (TN) / (TN + TF) × 100]. Where TN is the novel object spent time, TF is the familiar object spent time.

2.2.3. Elevated plus maze (EPM)

The elevated plus maze (EPM) often evaluates a laboratory animal's anxiety-like behavior. There were two open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm) with an open roof, so the two open arms were opposite. All four arms terminated in a central area of 10 cm. The maze is located 50 centimeters above the ground. For behavioral testing, the animals were placed individually in the elevated plus maze in the center, facing an open arm, and were given five minutes to explore. Each arm's entrance had to be crossed by the hind paws of the rats before they were permitted entry. Anxiety-like behavior in control and experimental rats was evaluated by measuring the time spent on the open, close arm and time spent in the center of total time. Before assessing a new animal, the equipment was cleaned with 20% ethanol to remove any potential bias caused by the odor of the preceding animal. All measurements were conducted in a quiet atmosphere with consistent light (Pellow et al., 1985) and automatically recorded using a video camera-based system (EthoVision, Noldus, Version 14).

2.2.4. Fear conditioning

The experimental cage for fear conditioning is set up inside a soundproof enclosure with a shocking floor and sound and light generators. The cage was 30 cm high and featured a 30 cm by 30 cm floor. An electric shock generator (Tajhiz Gostar Iranian, Iran) was attached to a stainless-steel grid (inter-bar spacing: 1.0 cm) installed on the chambers' floor. Rats were handled for three successive days for 5 min daily. During day one, the animal was placed in a chamber for 5 min. The initial habituation period on the first day allowed acclimation to the experimental environment and reduced orienting responses. The freezing behavior of the rat was monitored 24 h after training. During the second day of a conditioning session, the animal was exposed to tones and unpaired foot shocks (first tones, then foot shocks). A tone was played for 30 s, and then two extended 3-mA foot shocks were delivered at 2.5 and 4.0 min after placement in the chamber. On the third day of the testing session, rats were exposed to the same conditioning context without being shocked for 5 min. Freezing time in 60 s and 80 s after sound was assayed and analyzed.

2.3. Histological evaluation

The hippocampal samples of randomly selected rats were studied histologically (n = 6/experimental group). A high dose of ketamine (150 mg/kg) was administered to rats before they were transcardially perfused with 50 ml of normal heparinized saline and 50–75 ml of a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). The brains were removed after 24 h and stored in closed plastic containers with 10% formaldehyde. To prepare the samples for embedding, they were dehydrated in ascending ethanol series, cleared with xylene, and embedded in paraffin. Tolonidine blue was used to stain a hippocampal block cut into 7 mm coronal sections. CA1 and CA2 areas of the hippocampus and the dentate gyrus of the hippocampus were counted for neurons. This study counted dark neurons (DNs) (neuronal shrinkage, nuclear pyknosis, cytoplasmic eosinophilia, and surrounding spongiosis) and cells with visible cytoplasmic boundaries and an evident nucleus. Each section was counted twice.

2.4. Electrophysiological study

2.4.1. Long-term potential

An extracellular bipolar stainless-steel stimulating electrode with a diameter of 0.125 mm was inserted in the medial perforant pathway (4.2 mm lateral to the lambda, −3.2 mm ventrally) to record fEPSP. A recording electrode made of stainless steel was positioned in the DG with the maximum response (−3.8 mm posterior and 2.2 mm lateral to the bregma). Extracellular field potentials were amplified 1000 times, digitized at 10 kHz, and filtered with a differential amplifier at frequencies ranging from 0.1 Hz to 10 kHz. Biphasic square waves (with a width of 200 ms) were used as stimuli. Signals were sent to a computer using an A/D interface (Science Beam Co., Iran), and data were analyzed with Eprobe software. The intensity of the stimulation was set to evoke 40% of the maximum response (Population Spike (PS) and field Excitatory Post-Synaptic Potential (fEPSP)). We performed stimulus-response curves with a range of stimulus intensities (100–1200 μA) before LTP induction. After at least 30 min of steady baseline recording, LTP occurred through delivering high-frequency stimulation (HFS) (10 trains of 10 pulses at 200 Hz separated by 10 s). The baseline stimulation was restarted after the tetanic stimuli, and the recording continued for at least 60 min. Five consecutive evoked responses were averaged at 10-second intervals (Nabavi Zadeh et al., 2023).

2.4.2. Paired-pulse response

The facilitation of paired pulses was determined after recording a baseline for 30 min. Following the paired-pulse stimulation, evoked responses were recorded and delivered at 40% of the maximal stimulus intensity at 20, 30, 50, 70, 100, 150, and 300 ms intervals. Ten consecutive evoked responses were averaged for each of them. The fEPSP slope ratio [percentage of the second fEPSP slope to the first fEPSP slope; fEPSP2/fEPSP1%] and the population spike amplitude ratio [percentage of the second population spike amplitude to the first population spike amplitude; PS2/PS1%] were determined using various interstimulus intervals (Nabavi Zadeh et al., 2023).

2.5. Statistical analysis

The statistical analysis used Graph-Pad Prism Version 8.0 for Windows (Graph-Pad Software, USA). Bonferny post hoc tests were used to determine whether there were statistical differences between the two-way and one-way ANOVA analyses. P values less than 0.05 were considered statistically significant. All values were expressed as the Mean ± SEM.

3. Results

3.1. Effect of repeated ketamine exposure on weight gain

Fig. 2 shows the effects of repeated ketamine exposure on weight gain. According to the results of our three-way ANOVA with Bonferroni's post hoc test, there were significant differences between male and female animals in the day [$F_{(3,24)}$= 269.9, P = 0.0001] and treatment [$F_{(1,8)}$= 0.596.9, P = 0.0001] and sex [$F_{(1,8)}$= 23.00, P = 0.001]. Our results revealed an immediate increase in body weight gain patterns in male and female ketamine groups during days 0 to 15 after the first sub-anesthesia treatment compared to the control groups. This increase in body weight was statistically significant on days 5 to 15 (P < 0.05, P < 0.001, and P < 0.0001 in male and P < 0.05, P < 0.001, and P < 0.001 in female group in 5,10 and 15 days respectively). In addition, there was a gender difference within the groups (Fig. 2).

3.2. Effect of repeated ketamine exposure on social interaction

To examine sociability and social memory, we conducted the three-chamber test. As seen in Fig. 3**A**, animals with normal sociability preferred a wire cage with a strange rat over an empty cage. Compared with their control animal littermates, the male animals of the ketamine group demonstrated significantly close social interaction. Both male and female animals of the ketamine group, however, failed to distinguish the novel stranger rat from the familiar rat when the task required normal hippocampal function. According to the results of our three-way ANOVA with Bonferroni's post hoc test, there were significant differences between male and female animals in sociability in target [$F_{(1,7)}$= 6.803, P = 0.035] but not in sex [$F_{(1,7)}$= 0.53, P = 0.48] and treatment [$F_{(1,7)}$= 0.33, P = 0.58]. Our results also showed there were significant differences between male and female animals in social memory in target [$F_{(1,6)}$ = 22.81, P = 0.003] and treatment [$F_{(1,6)}$ = 7.51, P = 0.033] but not in sex [$F_{(1,6)}$ = 0.66, P = 0.44] (n = 7). As a result, the control rat spent significantly more time exploring the novel stranger rat (Fig. 3**B**), indicating a deficit in social memory in ketamine-treated animals.

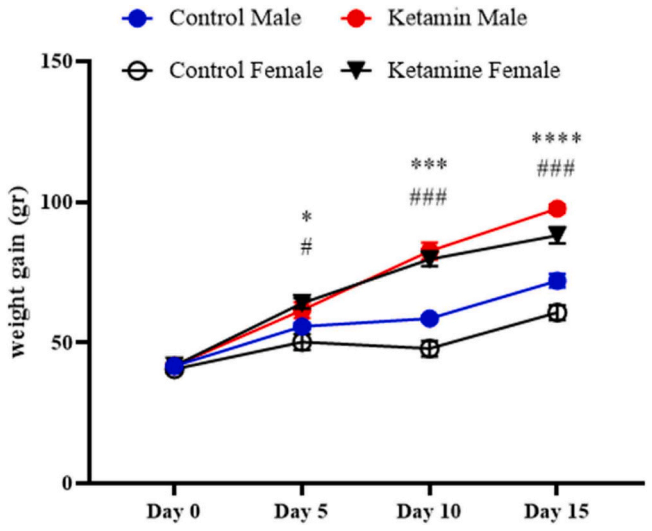

Fig. 2. Effects of repeated ketamine exposure on weight gain. Compared to the control groups, bodyweight gain patterns in the ketamin groups (male and female) during day 0 to 15 after the first sub-anesthesia treatment. (*P < 0.05, ***P < 0.001 and ****P < 0.0001 in male and #P < 0.05, ###P < 0.001 in female group in 5,10 and 15 days respectively). There was gender difference within the groups. All graphs were plotted as mean± SEM.

3.3. Effect of repeated exposure to ketamine on novel object recognition

Performance on the NOR task was measured by the total exploration time and recognition index. According to our three-way ANOVA with Bonferroni's post hoc test results, male and female control group animals compared to animals of sub-anesthesia exposure groups (both male and female ketamine group) indicated a tendency to spend more time exploring a novel object than a familiar object. Our results show there were significant differences between male and female animals in sex [$F_{(1,7)}$= 6.803, P = 0.035] but not in object [$F_{(1,7)}$= 0.58, P = 0.46] and treatment [$F_{(1,7)}$= 5.45, P = 0.052]. However, in total exploration time for each object, results revealed a significant influence on the interaction between the objects and groups [$F_{(1,7)}$ = 13.92, P = 0.007]. Our results indicated as compared to controls (61.9% ± 6.9% for males and 59.7 ± 4.6%), the recognition index is significantly lower in the repeated exposure anesthesia groups (25.6 ± 2.281%; P < 0.001 for male ketamine vs. 38.3% ± 6.8% for female ketamine), the recognition index is significantly lower in the repeated exposure groups (n = 8, Fig. 3**C and D**). According to the results of our three-way ANOVA with Bonferroni's post hoc test, our results indicate that repeated exposure to Ketamine at pre-adolescence leads to persistent impairment in recognition memory in adulthood [$F_{(1,7)}$= 39.34, P = 0.0004]. Although, there were significant differences between male and female animals [$F_{(1,7)}$= 1.27, P = 0.29].

3.4. Effect of repeated exposure to ketamine on anxiety-like behavior

Fear conditioning test and EPM assessed anxiety-like behavior in control and experimental groups. During the conditioning or acquisition phase of fear conditioning, an emotionally intense, naturally unpleasant, unconditioned stimulus, such as an electric shock, is matched with an emotionally neutral, conditioned stimulus, such as a tone. According to this paradigm, freezing is a rodent's usual natural defense strategy. The apparent lack of movement serves as feedback on memory formation and illustrates the health of the hippocampus. Fig. 4**A** shows contextual fear conditioning, experimental design, and fear training scheme in the fear conditioning test. As shown in Fig. 4**B**, the effect of repeat exposure to ketamine on freezing time during the 5-minute test has no significant difference between groups. One way-ANOVA revealed that compared to control groups (male and female), there was no significant effect of repeat exposure to ketamine on freezing time [freezing time 1, F (3, 25) = 2.07, P = 0.12 and freezing time 2 F (3, 25) = 1.69, P = 0.19] (n = 7). For EPM, as shown in Fig. 4, there was no significant difference in time spent in open arms (F (3, 25) = 0.57, P = 0.63; Fig. 4**B**) and time spent in closed arms [F (3, 31) = 0.44, P = 0.72] (Fig. 4**C**) among groups both male and female rats. Our results indicated that there was no significant difference in time spent in the center of EPM between animals of control and ketamine groups [F (3, 30) = 0.86, P = 0.47] (Fig. 5**D**). According to the results of our two-way ANOVA with Bonferroni's post hoc test there were no significant differences between male and female animals in anxiety-like behavior tests **(**Fear conditioning and EPM**).** Data are expressed as mean± SEM (n = 8). Besides, the heat map shows time spent in zones of EPM (Fig. 5**A**).

3.5. Histological result

In the histological assessment, the appearance of neurons with condensed darkly stained nuclei and bright eosinophilic cytoplasm was used to identify neuronal injury. Dark neurons (DNs) were diagnosed

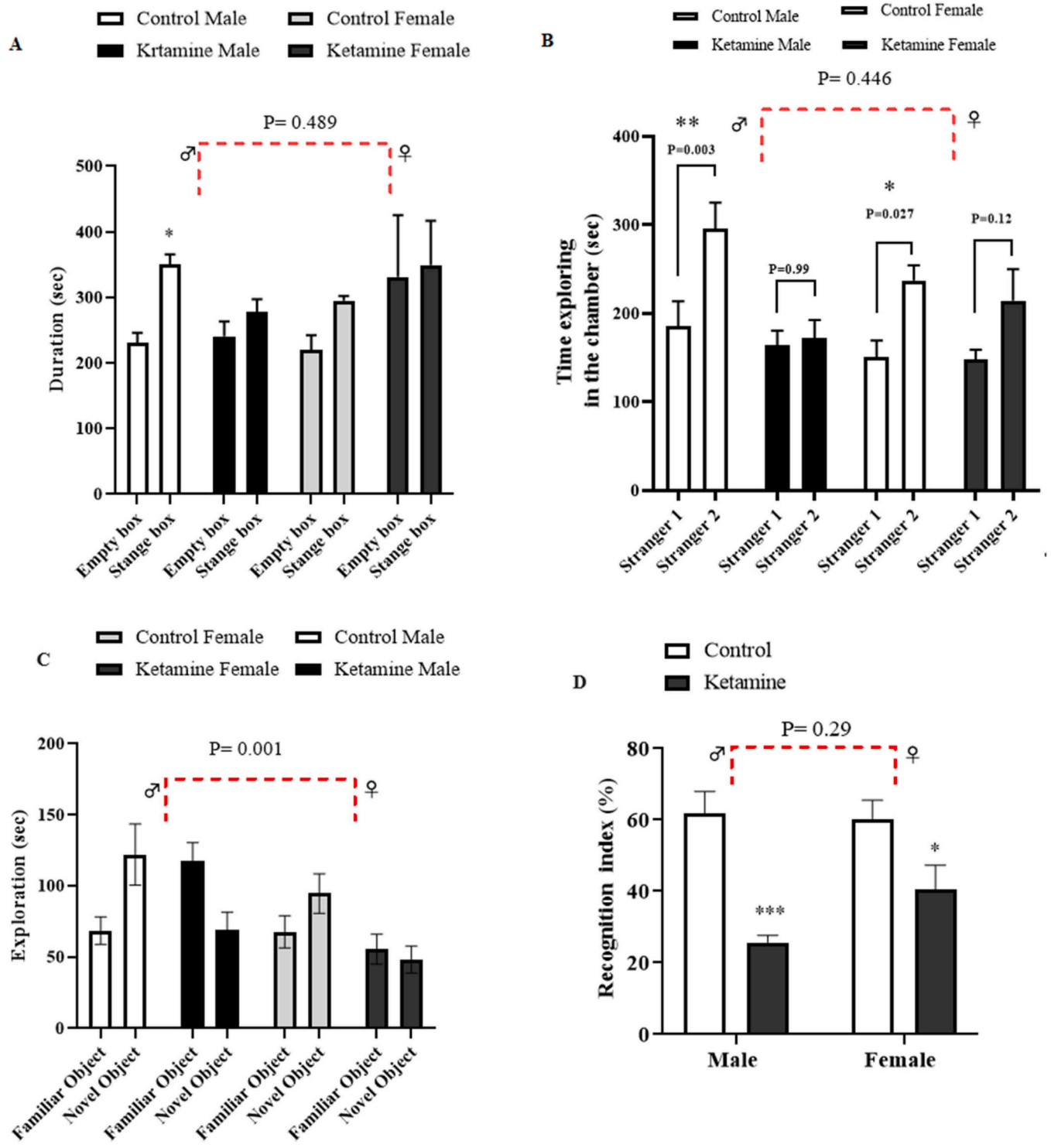

Fig. 3. The three-chamber test measures social recognition memory and performance on the NOR task for total exploration time, and the recognition index, and the three-chamber test measures social recognition memory. (A) Exploration time for the empty (E) cage and the cage with a stranger (S) rat in the control and ketamine groups during the socialization in the three-chamber sociability test (n = 7; S vs. E * $P < 0.05$ in the male control group). (B) Exploration time for the cage with the familiar mouse (S1) and the cage with the stranger mouse (S2) for the male and female control and ketamin groups during the sociability in the three-chamber test (n = 7; S1 vs. S2 **$P < 0.01$ in the male control group and *$P < 0.05$ in the female control group). (C) Comparison of total exploration time for familiar and novel objections. There was gender difference within the groups. (D) Comparison of the recognition index assay between the male and female ketamine groups and the control groups (***$P < 0.001$ in male *$P < 0.05$ for Female (n = 7)). All graphs were plotted as mean ± SEM.

with neuronal shrinkage, nuclear pyknosis, cytoplasmic eosinophilia, and surrounding spongiosis. The numbers of DNs per unit area in the hippocampus's CA1, CA2, and DG subdivisions were counted. Then, the mean number of DNs in the hippocampus of the experimental groups (Ketamine) was compared with the control groups. As shown in Fig. 6**A**, only a few DNs were found in different hippocampus regions in the control groups (male and female); in comparison to them, in ketamine male and female groups, DNs per unit area in the CA1, CA2, and DG of

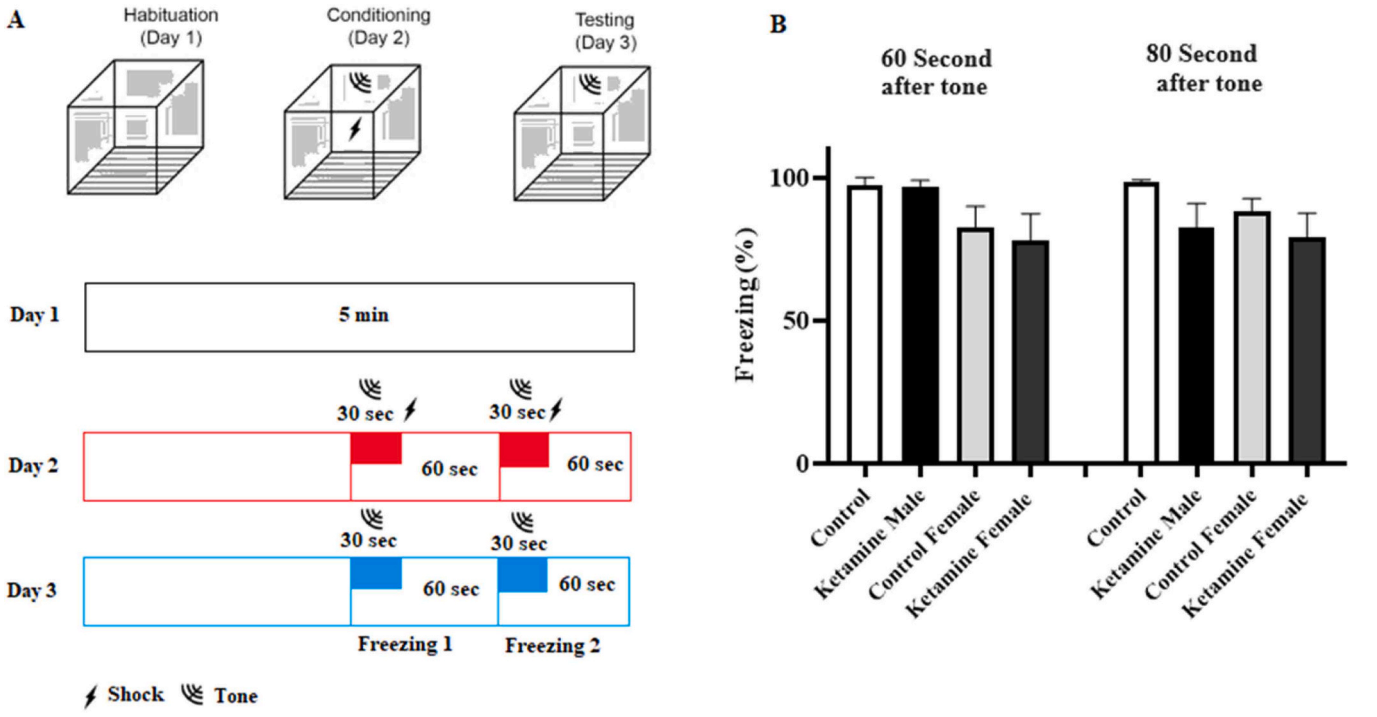

Fig. 4. Effect of Ketamine on conditioning behavior in fear conditioning maze. Experimental design (A) and fear training scheme (B). Result of repeat exposure to ketamine on freezing time during the 5-minute test in mean freezing levels (± SEM) of male and female controls and male and female rats of the ketamine group (n = 7). All graphs were plotted as mean± SEM.

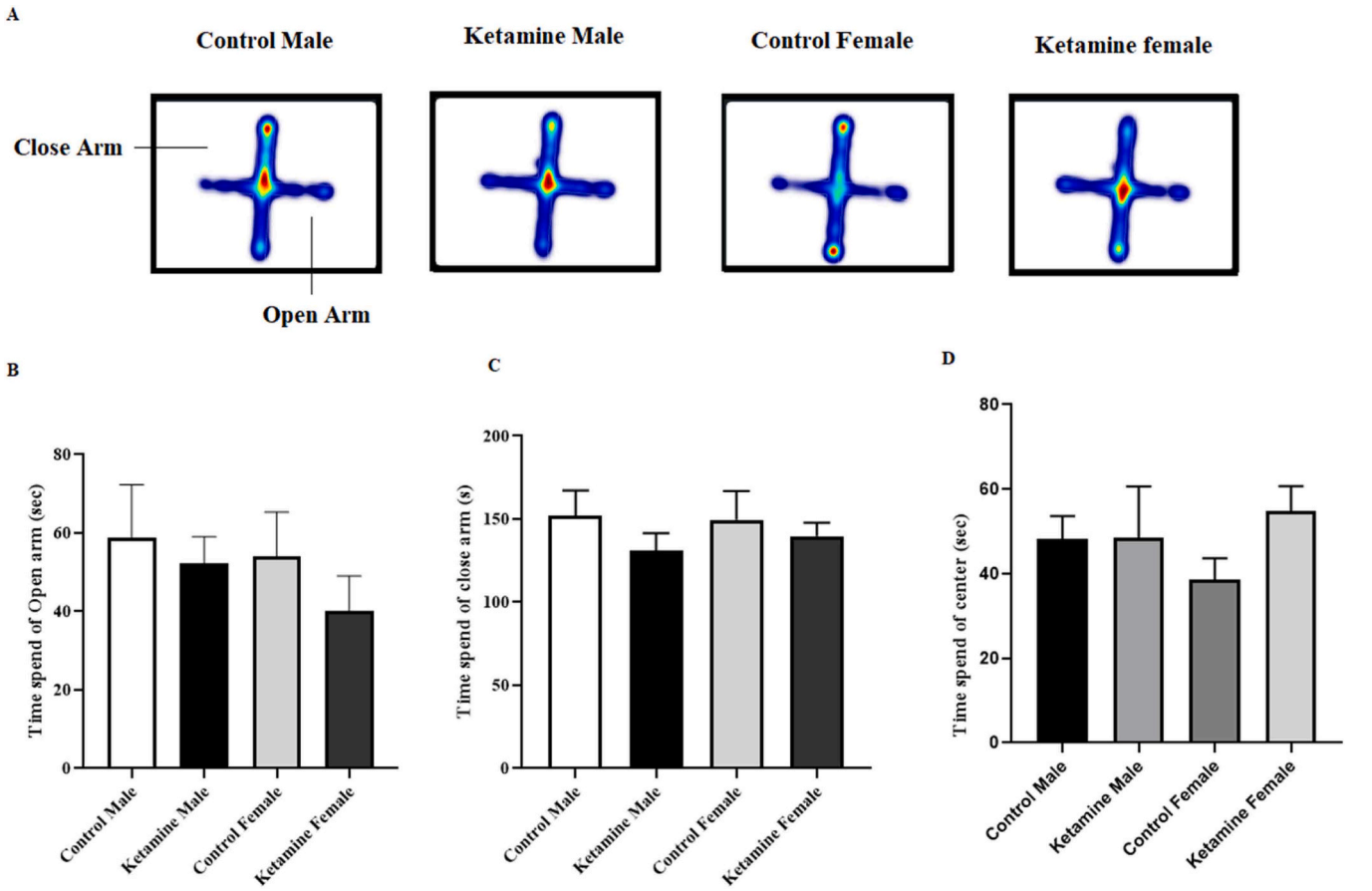

Fig. 5. Effect of Ketamine on anxiety behavior in Elevated Plus Maze. The time spent in the open, closed arms and center of the elevated plus maze (sec) in the adult rats subjected to repeated doses of pre-adolescence ketamine exposure. All graphs were plotted as mean ± SEM (n = 9 ± 1).

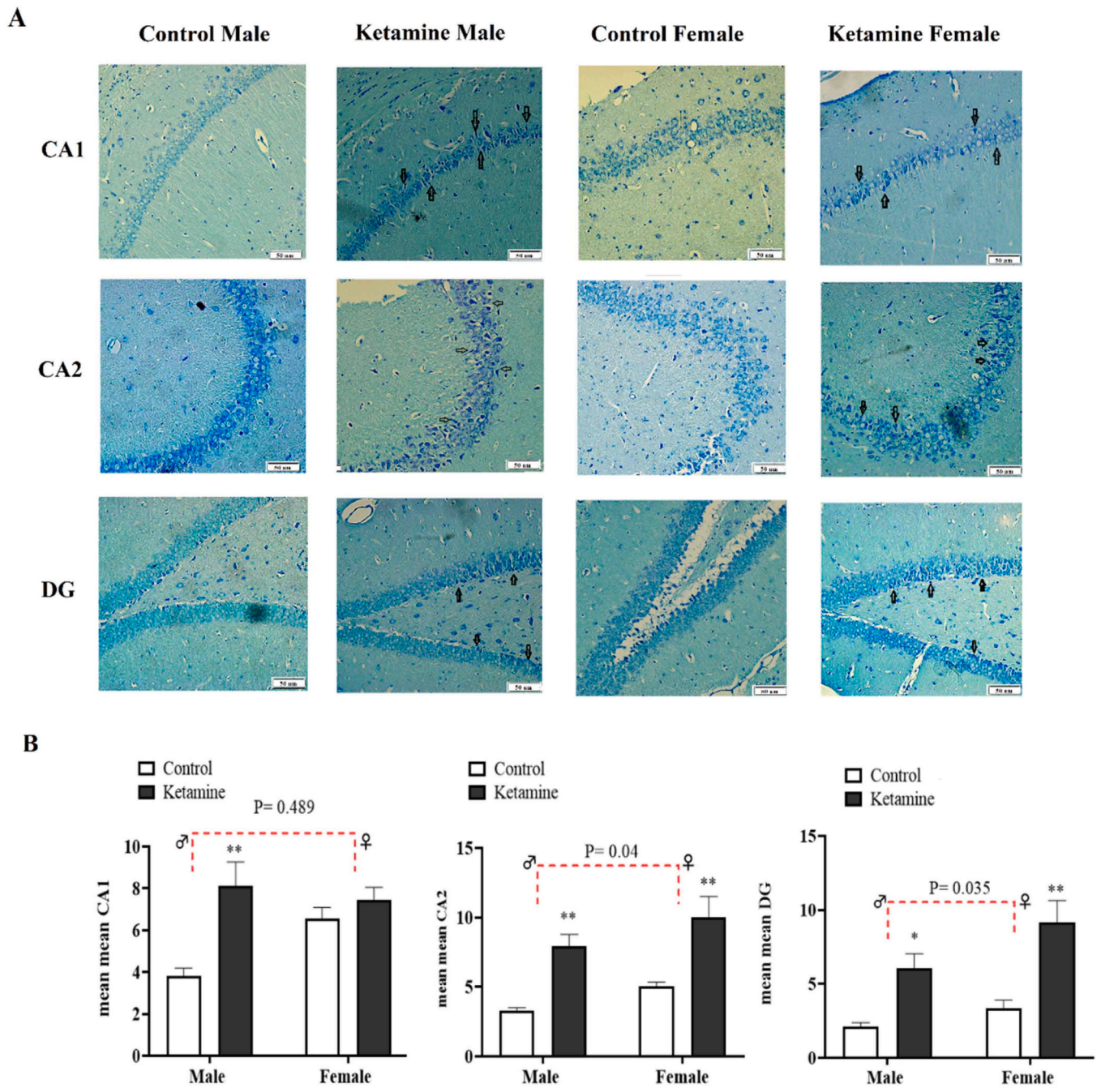

Fig. 6. Production of dark neurons in the hippocampal CA1, CA2, and dentate gyrus areas in adult rats after repeated exposure to ketamine in pre-adolescence. (A) The light-microscopic appearance of toluidine blue-stained dark neurons in 10-μm sections of the hippocampus's CA1, CA2, and DG areas. (B) Dark neuron numbers in the CA1 region of the ketamine male (no female) group were significantly more than the control group (**p < 0.01). In the CA2 region, dark neuron numbers of the ketamine either male or female group were significantly more than the control group (**P < 0.01in male and female). Dark neuron numbers in the region of DG in the ketamine group (both sexes) were significantly more than the control group (*P < 0.05 in male groups and **P < 0.01 in female groups). There was gender difference within the groups. All graphs were plotted as mean± SEM.

the hippocampus increased. Two-way ANOVA with Bonferroni's Post hoc analyses indicated that there was a significant increase in the mean number of dark neurons in the CA1 area in the male samples of the ketamine group but not in the female samples of the ketamine group compared to the control groups ($P < 0.01$); furthermore, there was no significant difference in sex factor for CA1 area [$F_{(1,16)} = 2.11$, $P = 0.16$]. In CA2 and DG area, our results indicated there was a significant increase of DNs in the CA2 and DG area in both male and female ketamine groups compared to control groups (treatment factor) [$F_{(1,16)} = 12.79$, $P = 0.002$ for CA2 and $F_{(1,16)} = 26.43$, $P = 0.0001$]. However, there were significant differences in sex factor for the DG area [[$F_{(1,16)} = 5.27$, $P = 0.035$] but not for the CA2 area [[$F_{(1,16)} = 2.11$, $P = 0.16$]. Based on our findings in adult rats indicate that repeated exposure to ketamine in pre-adolescence causes cell death in the hippocampus (Fig. 6**B**).

3.6. *Electrophysiological result*

To further investigate the long-term effects of ketamine sub-anesthesia on neuronal activity, we assessed basal synaptic

transmission and plasticity in the perforant pathway of the hippocampal at one month after exposing rats to the anesthesia. Basal synaptic transmission was studied by analyzing stimulus-response (I/O) curves and short- and long-term potentiation by assessing paired-pulse facilitation (PPF) and long-term potentiation (LTP). As shown in Fig. 7**A,** the acquired input/output (I/O) curve from recorded 60 s periods revealed that there is a substantial difference in the amplitude of PS between the control and the ketamine groups (Fig. 7**A**). According to the results of our three-way ANOVA with Bonferroni's post hoc test, there were significant differences between male and female animals in sex [$F_{(11, 192)} = 10.26$, $P = 0.001$] and treatment [$F_{(1192)} = 197.7$, $P = 0.0001$]. In addition, the fEPSP slope of the exposure groups was also reduced significantly compared to the control group. Fig. 7**B** illustrates the effect of repeated exposure to ketamine on LTP induction and maintenance in the dentate gyrus. A repeated measure two-way ANOVA followed by Bonferroni's post hoc test revealed that slop-LTP after tetanization (HFS) was significantly lower in the ketamine group relative to the control group [$F_{(3.151,18.91)} = 4.143$, $P < 0.05$]. High-frequency stimulation (400 Hz) of the medial perforant path produced a long-lasting synaptic potentiation in the control group compared to anesthesia groups ($P < 0.001$) up to 60 min after HFS. The LTP level, measured as the mean slope (0 to 90%) at 0 to 60 min after HFS stimulation, differed in all groups (Fig. 7**C and D**). According to our results, there were significant differences between male and female animals.

As shown in Fig. 7**E**, in short intervals between two strictly paired stimuli, the amplitude of PS prompted by paired-pulse did not increase in the ketamine groups. Two-way ANOVA with Bonferroni's post hoc analyses indicated a significant difference in the paired-pulse ratio of PS amplitude [$F_{(2.5,9.9)} = 4.4$ $P < 0.05$] in the male ketamine (no female) group compared to the control group, especially in intervals of 20 to 70 msec. In addition, in the paired-pulse ratio of PS amplitude, more analysis with three-way ANOVA with Bonferroni's post hoc analyses indicated that there were no significant differences between male and female animals (sex factor) [$F_{(1,4)} = 0.84$, $P = 0.41$]. Similar results were observed for the fEPSP slope by assessing pair pulse facilitation (PPF) and long-term potentiation (LTP) (data not shown).

4. Discussion

To the best of our knowledge, this is the first study to determine the gender differences in the pre-adolescence repeat exposure to sub-anesthetic doses of ketamine (20 mg/kg daily for 14 days, i.p.) in female vs. male rat adults. According to the results of the present study, ketamine administered frequently during pre-adolescence can have long-term effects that can manifest in adults. The main findings of this study showed: (1) Social interaction behavior (sociability and social memory) can be affected by ketamine in both male and female rats; however, no gender difference was observed. (2) The novel object test showed a significant difference between male and female rats in the exploratory time. Also, there was a significant difference between the control and ketamine-receiving groups in the recognition index in both males and females. However, no significant difference was observed. (3) In an investigation of anxiety behaviors using fear conditioning and elevated plus mazes, there was no significant difference between the

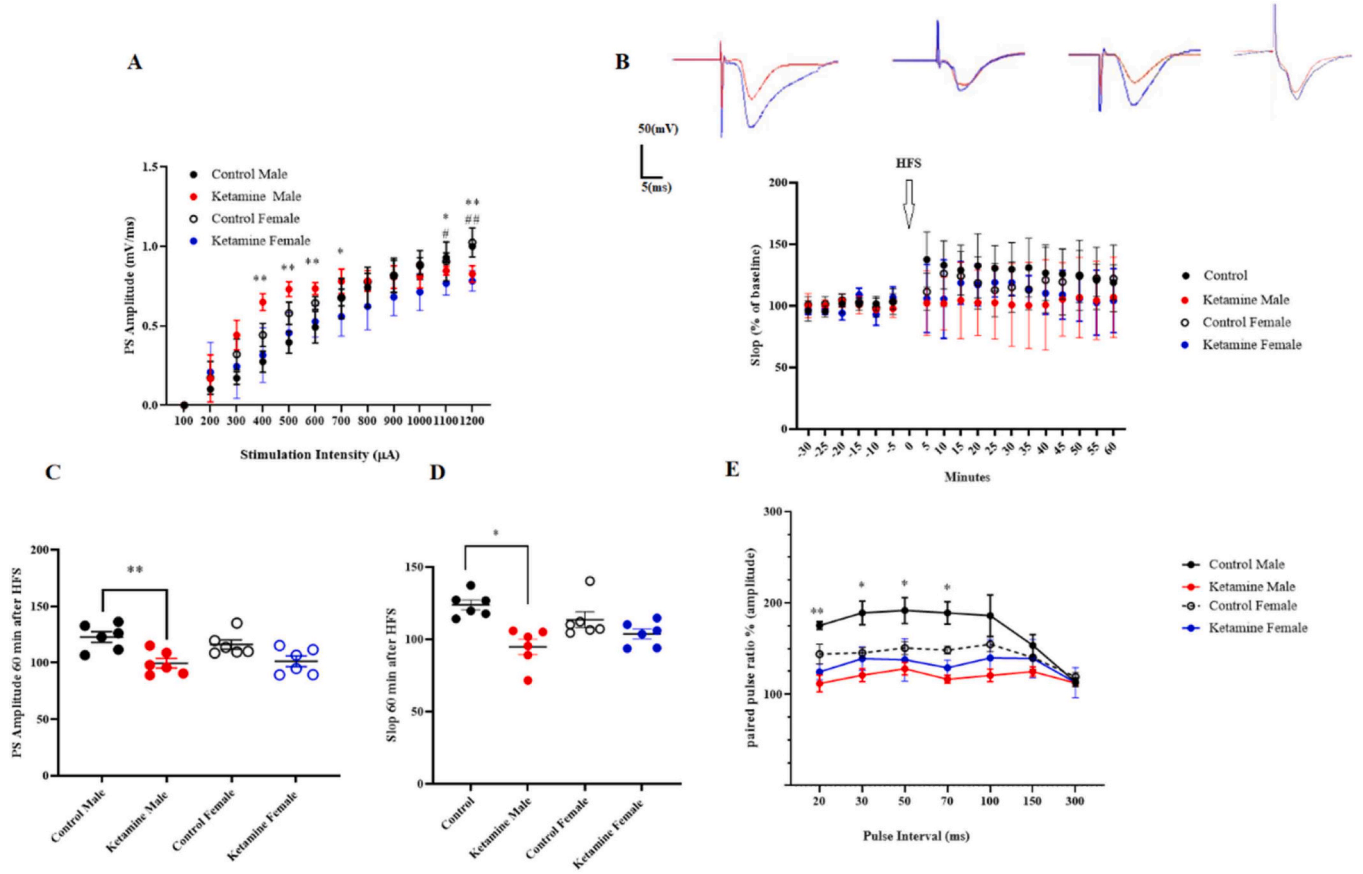

Fig. 7. Effect of ketamine exposure on neuronal synaptic plasticity. (A) The input/output curve was acquired from a 60 s recording and single traces of the DG neurons' reaction to numerous stimulus intensities (100–1200 mA). (B) The effect of repeated exposure to ketamine on LTP induction and maintenance in the dentate gyrus up to 60 min after tetanization (HFS). Data were normalized against the baseline period (−30 to +60 min), and the single traces were recorded before/after HFS. (C & D) The PS amplitude and slop mean were recorded at 0 to 60 min after applying HFS (n = 6 animals/group). (E) In short intervals between two strictly paired stimuli, the amplitude of PS was prompted by a paired-pulse in all male and female groups. (*$P < 0.05$, **$P < 0.01$ in male and #$P < 0.05$, ##$P < 0.01$ in female groups). All graphs were plotted as mean± SEM.

control and ketamine groups, as well as between the gender groups. (4) Electrophysiological studies on long-term and short-term memory showed significant differences between control rats and male (not female) ketamine rats. As well as gender differences in long-term and short-term memory in this context, we observed differences between males and females. (5) A significant difference was found in the average number of dark neurons in the hippocampus region of rats treated with ketamine compared to those in the control group. In addition, male rats were more affected by ketamine during pre-adolescence than female rats. Fig. 8.

As anesthesia is widely used in children and adolescents, its safety is of great concern. Ketamine is widely used as an anesthetic, analgesic, and sedative in pediatric patients and acts primarily through the NMDA receptors. However, more and more studies have shown that repeated use of ketamine could trigger hydroxyl radical generations, oxidative stresses, and neuronal apoptotic deaths by directly blockading NMDA receptors in neonates and adults (Aligny et al., 2014; Bates and Trujillo, 2019; Melo et al., 2016; Onaolapo et al., 2019). During neonatal brain development, using anesthesia drugs has been linked to memory impairment, neuroapoptosis, long-term neurocognitive deficits, and behavioral defects (Amrock et al., 2015; Huang and Yang, 2015; Lee et al., 2014; Steinmetz et al., 2009). Several studies have examined the long-term effects of anesthesia agents on pre-adolescents (single or multiple doses, high-dose versus low-dose). It remains unclear whether anesthesia in pre-adolescence has similar effects as neonatal development, how anesthesia affects the brain beyond development, and whether these adverse effects persist into adulthood. Given that repeated ketamine administration produced long-lasting behavioral effects, we were interested in determining if such ketamine would have deficits in cognition behaviors and even anxiety.

It was shown that anesthesia drugs such as ketamine significantly affect neuroapoptosis, long-term neurocognitive deficits, behavioral abnormalities, and memory impairments in pediatric and outpatient surgeries (Hollmann et al., 2001; Nishikawa and Harrison, 2003). NMDA receptors facilitate neuronal and behavioral plasticity processes and the development of new brain cells (Bliss and Lomo, 1973; McCabe and Horn, 1988). According to conducted studies, NMDA-Rs concentrations peak during adolescence, particularly in the hippocampus, and decrease as animals approach adulthood (Insel et al., 1990; Sava et al., 2012). This means that blocking these receptors, especially before adolescence, may have long-term effects on memory and learning. Animal models revealed that repeated- or high-dose administration of ketamine suppressed neural excitability and caused apoptosis in the hippocampus, a significant component of the brain associated with memory and learning (Brown et al., 2015; Cao et al., 2015; Huang et al., 2013; Jiang et al., 2014). Also, our findings indicated that ketamine administration to pre-adolescent rats (24 days to 37 days post-birth) was associated with deficits in social behavior and learning as well as

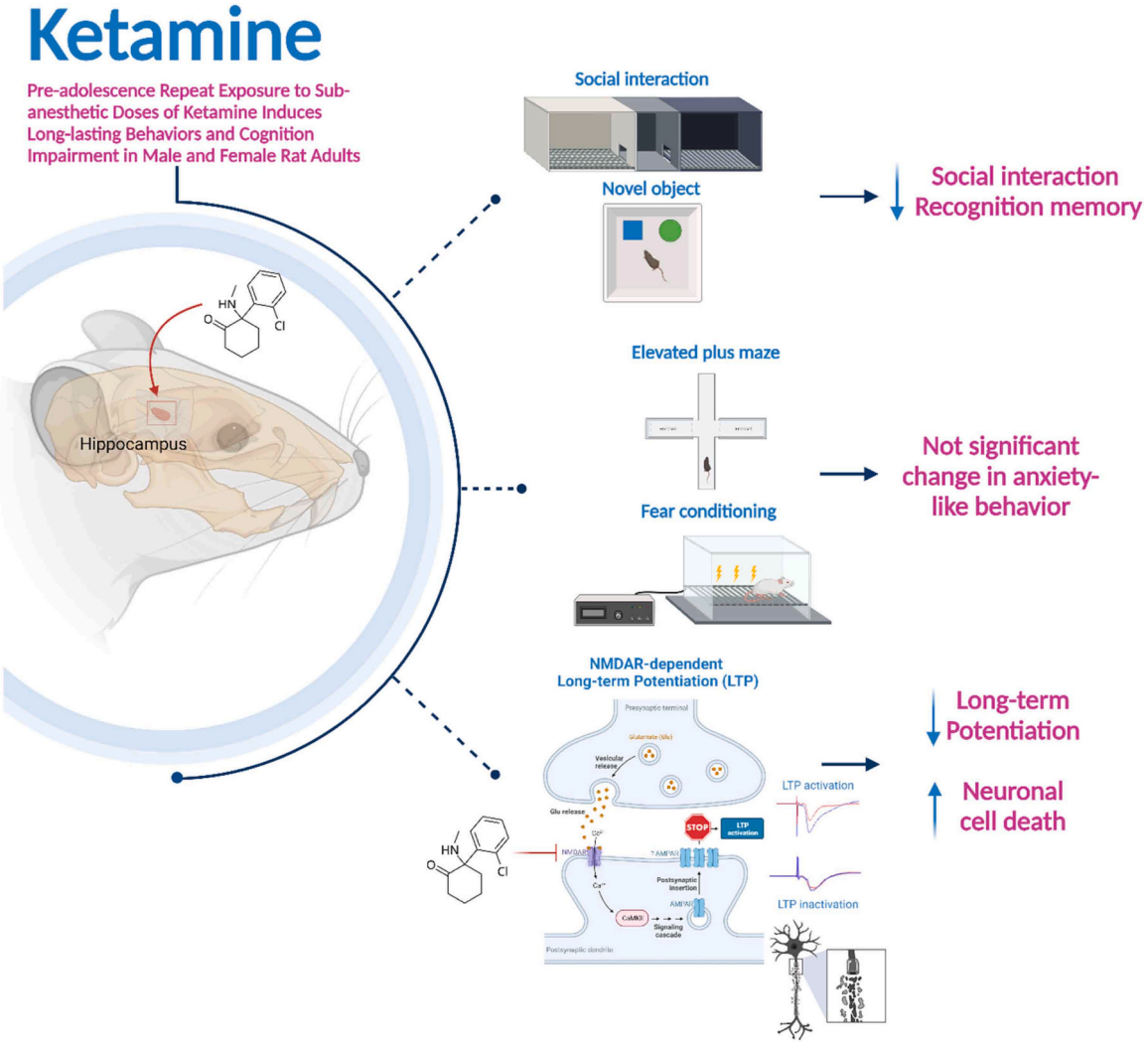

Fig. 8. Summary of main findings of pre-adolescence repeat exposure to Sub-anesthetic Doses of Ketamine induces behaviors and cognition impairment in male and female rat adults. (Created with BioRender.com).

ketamine-mediated neurotoxicity in the hippocampus, which resulted in several behavioral changes that can be related to each other.

Novel object recognition and social interaction deficits have been associated with impaired recognition memory and social cognition and have been utilized extensively to evaluate the effects of NMDA receptor antagonists (Neill et al., 2010; Pitsikas, 2018). In the social discrimination test, our results indicated that in comparison with control rats, male and female ketamine-exposed rats failed both to show decreased contact time with the empty enclosure significantly and did not significantly decrease contact time with the unfamiliar rat, which reduced contact with a novel conspecific may be associated with depressive- and/or anxiety-like behaviors, which would be following our other behavioral results. Our results are consistent with previous studies involving chronic 25 mg/kg ketamine administration to adolescent rats, which reported decreased social contact (Gama et al., 2012). Wedzony and colleagues 2008 used post-natal blockade of NMDA receptors with increasing doses (administered on day 21) in neonatal male rats to model schizophrenia (Wedzony et al., 2008). It appeared that NMDA receptor blockers such as ketamine or CGP 40116 have a long-lasting effect on social interaction behavior in adults by decreasing social non-aggressive behavior and increasing the duration of aggressive interactions. According to Beeker and colleagues (Becker and Grecksch, 2004), administering ketamine (30 mg/kg, 5 consecutive days) did not adversely affect social interactions in adult rats. In another study, Huang and colleagues administered ketamine anesthesia at P7, P9, and P11, corresponding to neonatal development, and found no noticeable behavioral changes or learning deficits in mice with just one injection of an anesthetic (Huang *et al.,* 2017). A novel object test demonstrated that ketamine had adverse effects on neurodevelopmental models. The present study used a 24-hour delay timepoint between the familiarization and test session for assay long-term memory. Subchronic ketamine (30 mg/kg, i.p.) administration from the post-natal day (PND) 41 to 45 consistently reduced recognition memory abilities in rats in the object recognition test (ORT) when assessed from the post-natal day (PND) 108 to PND 109, according to Ram and colleges (Ram et al., 2013). As well, subcutaneous administration of ketamine (30 mg/kg) on PND 7, 9, and 11 in different species (mouse) led to a loss of recognition memory ability in mice assessed with the ORT (Jeevakumar et al., 2015). However, several investigations found that ketamine had no effect (Huang et al., 2013; Schumacher et al., 2016) or improved ORT and the object location test (OLT) (Schumacher et al., 2016). In light of the evidence, ketamine administration in varying dosages at later stages of development can substantially influence animal social behavior or recognition memory.

We evaluated the reliability and validity of EPM and fear conditioning tests for detecting anxiety-related behaviors and their relationship to cognition. Anxiogenic features include decreased entry into open arms, decreased time spent on an open or central platform, decreased head dipping, augmented scanning of the environment, etc. NMDAR blockade during the developmental period has previously been associated with persistent anxiety in adulthood (Wedzony et al., 2008). In the EPM maze study, Akillioglu and Karadepe demonstrated that single and repetitive doses of 10 and 20 mg/kg on the 7th post-natal day increased anxiety-like behavior in adulthood (Akillioglu and Karadepe, 2021). Onaolapo's study (Onaolapo et al., 2019) showed that subchronic ketamine administration increased time spent in the open arm of the EPM at all doses studied (7, 15, or 30 mg/kg per day for 8 weeks), indicating that ketamine may have an anxiolytic effect. In addition, Amorim and colleagues, in a 2018 study (Manuela Amorim et al., 2018), reported that repeated ketamine administration (30 mg/kg, intraperitoneally for seven days) in adolescent male Wistar rats at 35 post-natal days (PND) resulted in increased anxiety in adults' rats as indicated by the results of the EPM, open field, and social contact tests. The study stated that repeat exposure to ketamine in pre-adolescence rats does not induce anxiety-like behaviors in adult animals. In contrast, former research using a similar exposure to ketamine found decreased anxiety in the EPM (Parise et al., 2013; Silvestre et al., 2002). Ketamine's effect on anxiety-related behavior may depend on some factors, including the duration and dosage of administration, the strain tested, and the sex of the individual.

Fear conditioning is an evolutionary-conserved form of associative learning, making it a suitable model organism for examining the regulation and dysregulation of aversive associative memories(Milad and Quirk, 2012; Pattwell et al., 2012). Results of the present study indicated that neither males nor females had a significant difference in rats' freezing time after hearing tone. Previous studies on fear conditioning in rodents have inconsistent findings, with fear memory either increasing, decreasing, or remaining unchanged following ketamine administration. Studies on ketamine's effects on aversive associative learning have focused primarily on cued tone-shock learning, mediated by the amygdala, rather than single-trial contextual fear conditioning controlled by the hippocampus. The effects of pre- and post-training ketamine on the consolidation and extinction of contextual fear memory in rats were studied by Clifton and colleagues. It was shown that pre-training ketamine (25 mg/kg) impaired the extinction of the conditioned fear response, as did a lower dose (8 mg/kg). Consolidation or extinction of conditioned fear was not affected by post-training ketamine (25 mg/kg) (Clifton et al., 2018). When state dependency effects were controlled, administering ketamine (25 mg/kg) before fear conditioning did not affect fear conditioning consolidation. Ketamine pre-training (25 mg/kg) inhibited the extinction of the conditioned fear response, mirrored by a lower dose (8 mg/kg). Post-training ketamine administration (25 mg/kg) did not affect the consolidation or extinction of conditioned fears. In an experiment conducted by Pietersen et al.(Pietersen et al., 2006), ketamine (16 mg/kg) was administered before training to prevent fear conditioning in rats. Contrary to this, some researchers found that high doses of ketamine administered after training (four injections of 100/50/50/50 mg/kg spaced at 60-minute intervals) or pre-training (8 mg/kg) had no impact on cued fear conditioning (Bolton et al., 2012; Groeber Travis et al., 2015). Moreover, ketamine (8 mg/kg) had no discernible impact on contextual fear conditioning. However, these conflicting results may be explained by factors such as route, dose, and stress exposure during ketamine administration.

As a result of our histological studies on male and female rats, interesting results were discovered regarding the effects of ketamine on neuronal death. As a result of our study, we found that male rats showed cell death throughout the entire hippocampus. In contrast, female rats demonstrated cell death only in the dentate gyrus of the hippocampus. The discrepancy may be due to interactions between circulating gonadal hormones and ketamine since gonadal hormones can influence ketamine sensitivity (Saland et al., 2016). Consequently, it has been reported that 17β-estradiol provides neuroprotection in numerous brain injury models in both sexes and alleviates ketamine-induced neurodegeneration (J. Li et al., 2014; J. Li et al., 2013; W. Li et al., 2019). It seems ketamine in different regions of the brain in both sexes could have had various effects, although it is still necessary to conduct further research to confirm this claim. In the present study, repeated ketamine injection was administered before hormone release during adulthood, which could have affected the results. It is possible, however, that the destructive effects of ketamine and the elimination of NMDA receptors continue even after puberty, in which our electrophysiology data could justify this issue. Conversely, our study focused on hippocampus-dependent memory formation and apoptosis in the hippocampus. So, for further research, we suggest that other areas of the brain might be considered; this point of view was a limitation of our study.

Despite the negative effects of the administration of ketamine during brain development, some reports have demonstrated neuroprotection effects from ketamine in adults (Huang and Yang, 2015; Parise et al., 2013; Ye et al., 2018). Based on behavioral findings and their correlation with histology and electrophysiological findings, it appears that ketamine has various effects throughout the brain. Thus, the results of ketamine on sensitivity and anxiety are the same in male and female rats;

however, when it comes to memory and learning, ketamine has more negative effects on males. Many factors must be considered, including age, gender, species, and race of the animals. In contrast, histological results indicated more cell death in the ketamine male group; different types of anesthesia affect cell function differently. Therefore, this field needs more studies at the molecular level and cellular signaling pathways. According to behavioral tests and histological investigations related to cognition and memory, the concentration and duration of anesthesia exposure may differ between species/strains of animals. Cell and death processes may be affected differently depending on the type of anesthetic used, which may influence animal behavior.

NMDA receptors are ligand-gated cation channels (calcium channels) that allow the passage of nerve messages between neurons in the brain and spinal cord. These receptors provide a slow synaptic response and play essential roles in brain development, learning, or memory. It has also been reported that abnormal or excess NMDA receptor signals may negatively influence synaptic maturation (De Roo et al., 2009; Koyama et al., 2012). Anesthesia with ketamine passively modulates synaptic formations or synaptic development. According to Huang & Yang, in male rats, repeated exposure to ketamine-xylazine during early development results in behavioral and motor impairment and learning-dependent dendritic spine plasticity later on in life (Huang and Yang, 2015). Inducing abnormal synaptic formation or ectopic neuronal cell distribution results in impairments of synaptic plasticity, such as facilitating paired pulse or inducing hippocampal LTP in the CA1 region. Our results revealed suppressed LTP and larger PPF in the hippocampus of 60-day-old rats after exposure to ketamine in male rats. These findings suggest that pre-adolescent anesthesia exposure induced lasting effects on neural transmission, at least at the pre-synaptic level. The changes observed in LTP and PPF may be due to increased pre-synaptic calcium concentrations so that ketamine depresses neuronal activity and reduces Ca^{2+} influx into neurons primarily through the blockade of NMDA receptors (Franks and Lieb, 1994; Yamakura et al., 2001). NMDA receptor–mediated Ca^{2+} influx is critical for neuronal differentiation, migration, and synaptogenesis (Lau et al., 2009). Consequently, ketamine-induced inactivation of the NMDA receptor may significantly reduce activity-mediated calcium influx into neurons, affecting synapse formation and plasticity. A study by Luhman (2014) demonstrated that synapse maturation accelerated after 2 weeks after birth due to increased levels of NMDA receptors as well as 3-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors and glutamatergic synaptic transmission, as indicated by the increase in miniature and spontaneous excitatory post-synaptic currents (Lohmann and Kessels, 2014). The observed behavioral changes in the present study correlate with synaptic structural and functional plasticity alterations, as detected in previous studies, which occurred with repeated exposures (Huang et al., 2016; Huang and Yang, 2015). According to histology and electrophysiological data and their correlation, it is possible that ketamine's long-term effects progress after puberty and that sex hormones protect against future brain damage produced by ketamine injection.

Sex hormones, specifically progesterone and estrogen, impact the response across all levels investigated; thus, sex becomes a pivotal variable to assess when investigating the ketamine response. Thus, according to the data, females exhibit greater ketamine sensitivity than males. It has been consistently observed that females exhibit greater sensitivity to ketamine in dosage and extent of the behavioral response, whereas males display a prolonged response (Ponton et al., 2022). Notably, female rodents with ovariectomies, similar to their male counterparts, exhibit no response to low-dose treatment (2.5 mg/kg). Nevertheless, ketamine sensitivity can be restored through hormone replacement therapy, indicating that hormonal fluctuations associated with female sex hormones may influence behavioral responses (Carrier and Kabbaj, 2013; Saland et al., 2016). Mainly, corticosterone, the hormonal stress response, is known to be modulated by ketamine. The potential variation in the acute impact of ketamine on corticosterone between the sexes and the subsequent behavioral consequences remain uncertain. A single i.p. injection of (R, S)-ketamine (30 mg/kg) with or without unpredictable chronic stress (UCS) was found to have distinct long-lasting effects on the behavior of female C57BL/6 J mice in the forced swim test (FST), elevated zero maze (EZM), and open field test (OFT) (Fitzgerald et al., 2021). In addition to increasing body weight systemically, acute and chronic ketamine administration can reverse the effects of chronic moderate stress-induced adrenal hypertrophy. Obesity seems to be a predictor of depression's onset, and depression appears to be a predictor of obesity's subsequent episodes of depression. Transient antidepressant response to ketamine was better observed in patients with higher body mass index and obesity (Freeman et al., 2020).

Several limitations are worth considering. First, whether repeated administration of ketamine before or after pre-adolescence can produce results similar to ours, which is a limitation of our study and should be explored. Second, female rats may have been examined on different estrus days; however, since our aim was not to investigate this issue, this was one of our limitations.

5. Conclusion

It has been shown that repeated pre-adolescent ketamine exposure can rapidly alter the hippocampus and that these transient changes persist into adulthood, leading to cell death, impaired synapse development, cognitive and behavioral problems, and neurobehavioral problems. Electrophysiological recordings reveal impaired PPF and LTP in male adult animals administered anesthesia during adulthood. According to the findings of cell death and behavioral studies, repeated exposure to this chemical may result in persistent central nervous system impairment. Through this investigation, it may be possible to understand the mechanism by which repeated ketamine exposure induces toxic effects. Further exhaustive research is required to clarify the safe doses, developmental stages, and durations of ketamine administration in the neonatal period for pediatric patients.

Declarations

Ethics Approval All experiments were conducted according to the Guide for Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80–23, revised 1996). Additionally, the Research and Ethics Committee of Tehran University of Medical Sciences ((IR TUMS.NI) reviewed and confirmed all procedures.REC.1400.05).

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CRediT authorship contribution statement

Amir Barzegar Behrooz: Data curation; Visualization; Roles/Writing original draft; and Writing - review & editing. **Mahdeh Nasiri:** Data curation; Investigation Methodology. **Soheila Adeli:** Data curation; Validation; Roles/Writing an original draft**. Maryam Jafarian:** Resources; Software. **Seyed Khalil Pestehei:** Resources; Software; Writing - review & editing. **Javad Fahanik-babaei:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing original draft; and Writing - review & editing.

Competing Interests

The authors declare no competing interests.

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