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Ketamine treatment upon memory retrieval reduces fear memory in marmoset monkeys

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Abstract

Emotionally arousing experiences are retained very well as seen in posttraumatic stress disorder (PTSD). Various lines of evidence indicate that reactivation of these memories renders them labile which offers a potential time-window for intervention. We tested in non-human primates whether ketamine, administered during fear memory reactivation, affected passive (inhibitory) avoidance learning. For the consolidation of contextual emotional memory, the unescapable foot-shock paradigm in a passive avoidance task with two compartments (dark vs illuminated) was used. After entering the dark compartment, marmoset monkeys received four random foot-shocks (1 mA, 4 s) within 15-min. This stressful exposure increased the saliva cortisol and heart rate and impaired REM-sleep ($p<0.05$). One week later the monkeys were re-exposed to the stressful situation for the reconsolidation of the fearful experience. During the re-exposure the monkeys were treated with ketamine (0.5 mg/kg) or saline. In week 3, the monkeys were placed in the experimental setting to test their memory for the fearful experience. In contrast to the vehicle-treated monkeys, who avoided the dark compartment, the ketamine-treated monkeys entered the dark compartment that was previously associated with the fearful experience ($p<0.05$). Post-mortem analysis of the hippocampus showed that ketamine-treated animals exhibited less doublecortin positive neurons and BrdU-labeled cells in the dentate gyrus. This study reveals that a single low dose of ketamine, administered upon fear retrieval in monkeys, reduce contextual fear memory and attenuate neurogenesis in the

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hippocampus. These are important findings for considering ketamine as a potential candidate to target traumatic memories in PTSD.

1. Introduction

Ketamine is increasingly being popularized for the treatment of a variety of psychiatric disorders, including post-traumatic stress disorder (PTSD) (Feder et al., 2021; Rosenblat et al., 2019). The mechanisms of action of ketamine, in particular in emotional memory processing in PTSD, are not fully understood.

PTSD is a debilitating disorder, which can develop after a traumatic experience like combat, assault, abuse or a natural disaster (Thakur et al., 2015; Yehuda et al., 2015). The DSM-5 characterizes PTSD by vivid memories, flashbacks and nightmares of the traumatic event, avoidance of reminder cues, and hyper-arousal like anxiety and sleep disturbances (American Psychiatric Association, 2013). Sleep disturbances such as insomnia, disturbed REM-sleep and vivid nightmares are one of the most pronounced symptoms (Germain, 2013; Spoormaker and Montgomery, 2008). Related to hyper-arousal, PTSD is characterized by enhanced adrenergic tone, and reduced heart rate variability (Cohen et al., 2000; Sammito et al., 2015; van Liempt et al., 2013) keeping the body in an aroused state (Gorman and Sloan, 2000). However, the most important hallmark of PTSD is the strong memory of a traumatic experience and the inability of the brain to control acquired fear (Joels et al., 2006; LeDoux, 1994; Milad et al., 2009).

Formation of fearful and emotionally arousing memories are believed to depend on changes in synaptic function (Mitsushima et al., 2011; Rumpel et al., 2005), and mechanisms that underlie long-term potentiation (LTP) (Nabavi et al., 2014). Activation of N-methyl-D-aspartate receptors (NMDARs) is critical for induction of LTP (Morris et al., 1986; Tsien et al., 1996) and, accordingly, targeting NMDARs affects acquisition of fear memories (Bauer et al., 2002). The notion that NMDARs play a crucial role in synaptic plasticity and memory formation warrants the question whether targeting these receptors is effective in reducing fear memories such as seen in PTSD. This may be particularly relevant at the time window when fear memories are labile, i.e. after fear memory reactivation (Kindt et al., 2009; Kroes et al., 2014; Nader et al., 2000). Therefore, the non-competitive NMDAR-antagonist ketamine might be an interesting candidate to reduce the expression of fear. First, ketamine decreases depression-like symptoms in rodents (Moda-Sava et al., 2019; Zhang et al., 2015), and prevents structural alterations induced by prolonged stress (Krzystyniak et al., 2019). In addition, ketamine at the reconsolidation window reduces alcohol intake and when used as an anesthetic during surgery ketamine reduced the prevalence of PTSD (McGhee et al., 2008).

For the translation of the findings in rodents to the clinic, the marmoset monkey provides an appropriate animal model for human validity. The mechanisms involved in stress processing are similar to humans. Neuro-anatomical studies show many similarities with regard to regional distribution of neurotransmitter receptor types in the hippocampus, in which the marmoset monkey, more strongly than the rat, resembles humans (Kraemer et al., 1995). Furthermore, the evolutionary preservation of the structural plasticity in the amygdala in the marmoset appears to correspond to other primates and humans (Marlatt et al., 2011), and the prefrontal cortex (PFC) (Etkin and Wager, 2007; Shin et al., 2006) has the most connections to the rest of the brain in both man and monkey (Ongur and Price, 2000; Petrides et al., 2012) that strongly influences perceptual, motor, attention, and emotional systems of the frontal cortex. The evolutionary anatomical and functional organization of the PFC is relatively well preserved in primate species (Burman et al., 2006; Ongur and Price, 2000; Petrides et al., 2012). This makes monkeys, and the marmoset in particular (Oikonomidis et al., 2016), valuable for translational studies towards emotional memory in PTSD. In addition, monkeys, like humans, are diurnal and the sleep stages are similar to those in humans as opposed to rodents that have fragmented daytime sleep (Philippens et al., 2004; Verhave et al., 2011).

In this study, the effect of a single low subanesthetic dose of ketamine was tested during reconsolidation in the marmoset on fear memory (assessed in the passive avoidance task), heart rate, cortisol levels and sleep, which are affected in humans after traumatic fear. Additionally, we examined neurogenesis in the dentate gyrus, which has been implicated in discrimination of aversive from safe environments and generalization of fear, a hallmark of PTSD (Besnard and Sahay, 2016), and structural changes in the hippocampal formation. As recent studies are progressing in treatment of PTSD with ketamine (Feder et al., 2021, 2014), this study is needed to contribute to the potential of ketamine as a treatment for the emotional memory in PTSD (Durand et al., 2019).

2. Experimental procedures

2.1. Subjects

Women are more likely than men to develop persistent PTSD symptoms following trauma exposure (Breslau and Davis, 1992; Roth et al., 1997), but fewer than 45% of animal studies used females to investigate these disorders (Zucker and Beery, 2010). We therefore used adult female common marmoset monkeys (*Callithrix jacchus*) (3-6 years; 340-380 g; $N = 12$; Biomedical Primate Research Centre (BPRC), Rijswijk, NL). Prior to the study, the monkeys underwent a health check and were experimentally naïve. They were paired-housed in cages (76 × 71 × 190 cm) under the standard controlled conditions (23-25 °C; humidity >60%; lights off 7 pm-7 am) and had access to pellet monkey-chow (Special Duit Services, Essex, UK), daily enrichments like fruit, peanuts or raisins and free access to water. Cages were enriched with branches and varying toys. All aspects of animal care are described in Standard Oper-

ating Procedures in accordance with the European Community directives. According to the Dutch law on animal experimentation, the Institute's Ethics Committee reviewed and approved the study protocol and experimental procedure (DEC#716). All monkeys were under veterinary care throughout the study.

2.2. Study design

For the inescapable foot-shock paradigm a passive avoidance task with two compartments (dark vs illuminated) was used to measure emotional/fear memory. The effect on neurogenesis was examined with two markers: 5-bromo-2′-deoxyuridine (BrdU) for the survival of newborn cells (Wojtowicz and Kee, 2006), and doublecortin (DCX) for the maturation of newborn cells (Couillard-Despres et al., 2005).

Before the start of the study, the animals were trained to enter a transport tube voluntarily to be transported to the passive avoidance apparatus which was located in the room next door. This handling was done by an animal caretaker. Before the start of the experiments, all monkeys were habituated to the passive avoidance apparatus for 10 min during four days and trained to perform actions needed for the saliva sampling for three weeks. The animals were randomly divided in a treatment and a control group ($N = 6$/group). The study was divided into three experimental periods in which two monkeys of each group were tested. Each experimental period lasted three weeks (Fig. 1).

Week 1: Consolidation of emotional memory by an unescapable foot-shock procedure in the passive avoidance test. During the first day behavioral observations, saliva sampling and EEG/ECG measurements were performed in the home cage. On the second day, these procedures were repeated after the acquisition in the passive avoidance test. Before the acquisition trial, BrdU (200 mg/kg s.c.) was given to all animals. The procedures on the third day were the same as the first day. All measurements were done between 9:00 and 13:00. Clinical observations took place at 8:30 am every test day and two days after the test days.

Week 2: Reconsolidation of emotional memory in the passive avoidance test. The procedure was similar as in Week 1, only the foot-shock was escapable. Ketamine (0.5 mg/kg) or saline was injected intramuscularly (i.m.) immediately before the reconsolidation trial on day two.

Week 3: During the follow-up phase, the same procedure was followed as in Week 1, but no foot-shock was given and the sliding door between the two compartments stayed open. Latency times between opening the door and entering the other compartment were measured, with a maximum of 10 min. After the last measurements on day 3 the monkeys were euthanized under anesthesia to isolate the brains for histology. The clinical observations were performed by an animal caretaker. The sleep EEG was analyzed by an external sleep expert. Cortisol was analyzed in a blinded fashion in another laboratory. All personnel performing the assays were blinded for treatment.

2.3. Drugs

One single dose of racemic ketamine (Ketamine 10%, Alfasan Nederland BV, Woerden, NL) of 0.5 mg/kg i.m. was administered to the animals of the treatment group. This subanesthetic low dose of 0.5 mg/kg was based on the effective dose in monkeys used for sedation (25 mg/kg) (Bakker et al., 2013). The T_{max} of ketamine after an i.m. injection is 10–15 min (Aroni et al., 2009). The control group received saline. Due to a technical failure one animal received a higher dose of ketamine (5 mg/kg), but still below the anesthetic dose of 25 mg/kg (Bakker et al., 2013). This monkey did not show any differences in performance and behavior compared to the other monkeys from the same group.

2.4. Passive (inhibitory) avoidance test

Effects on emotional memory were measured with the use of the passive avoidance test (Model 256000, TSE-Systems, Bad Homburg, Germany) (Baarendse et al., 2008). The apparatus consisted of two same-sized compartments (30 × 25 × 30 cm), one illuminated and one dark compartment. A computer-controlled sliding door (9 × 11.5 cm) divided the compartments. At the start of each trial the animal is placed in the illuminated compartment where it could habituate for one minute. Thereafter, the sliding door between the compartments opened. The latency time for the animal to enter the dark compartment was recorded after all four limbs had entered the compartment.

During the 'acquisition phase' the door closed after entering the dark compartment and an inescapable foot-shock (1 mA) for 4 s on four random moments within a 15-min time-frame was given, creating a negative emotional memory for the dark compartment. During the re-exposure (reconsolidation), the 4-second foot-shock was escapable as the door remained opened. The foot-shock (1 mA) was given after the monkey completely went in the dark compartment, which was detected by infra-red detectors in the connecting door. As the door stays open, the monkeys could escape already immediately. During the 'follow-up phase' to test whether the animals were less inclined to enter the dark compartment, no electrical foot-shocks were given and the door stays open. If the animal did not enter the dark compartment a latency time of 10 min was noted.

2.5. Biopotential transmitter implantation

To measure the sleep electroencephalogram (EEG) and the heart rate by electrocardiogram (ECG), in all animals of both groups a bio-potential transmitter (TL10M3-F50-EET, Data Sciences International, St. Paul, Minnesota, USA) was placed internally under anesthesia with 10 mg/kg alphaxalone i.m. (Alfaxan, Vétoquinol, 's Hertogenbosch, NL, 10 mg/ml) and 0.1 mg/kg medetomidine i.m. (Sedastart; AST Farma, Oudewater, NL, 1 mg/ml). During sedation, the animal was placed on a heating pad of 35 °C to prevent a drop of body temperature. To prevent dehydration of the eyes, eyes drops were administered (ceva santé Animale B.V. Naaldwijk, NL). During surgery, a sterile bio-electric transmitter was placed intraperitoneally. Two ECG-electrodes were tunneled subcutaneously to the chest and fixed to the Pectoralis major. Two EEG-electrodes were tunneled subcutaneously to the skull. The EEG-electrodes were placed on the right hemisphere, one above and one 7 mm anterior to the intra-aural line, 3 mm from the sutura sagittalis, leaving the dura mater intact. The EEG-electrodes were fixed by a surgical screw and dental cement. In preparation of the surgery premedication was given. One day prior to the surgery until two days after the surgery the animals orally received twice a day 12.5 mg/kg amoxicillin (Synulox®, Pfizer Animal Health, Capelle a/d IJssel, NL). One hour prior to surgery 20 μg/kg buprenorphine (Buprecare®, AST farma, Oudewater, NL) has been provided (i.m.) as an analgesic. After surgery, animals orally received one daily meloxicam 0.10-mg/kg PO (Novacam 0.5 mg/ml, AST Beheer, Oudewater, NL) and three times a day buprenorphine (20 μg/kg i.m.) for two days. Because technical problems with the transmitters developed over the weeks in both groups due to the loosening of the dental cement and electrodes, the group sizes became too small for the analysis of ketamine treatment effects on ECG and EEG in week 3.

2.6. Clinical observations

The general clinical assessment was done every morning (8:30 am). The observations were done with a scoring list based on the Unified Parkinson's Disease Rating Scale (Philippens et al., 2014; van Vliet et al., 2006), that has been adapted for stress research. Item scored

Week 1: Consolidation
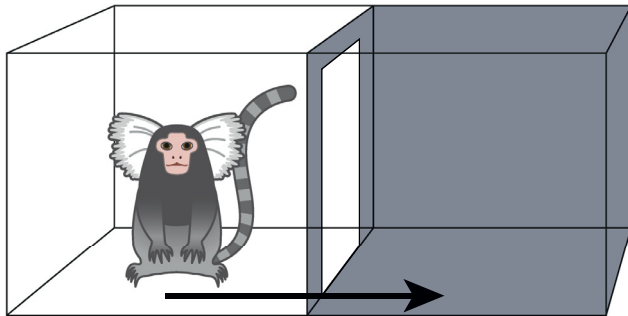
door closes
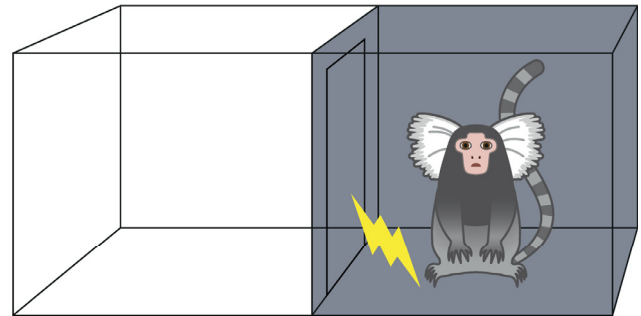
4 foot-shocks (0.5mA) of 4s in 15'

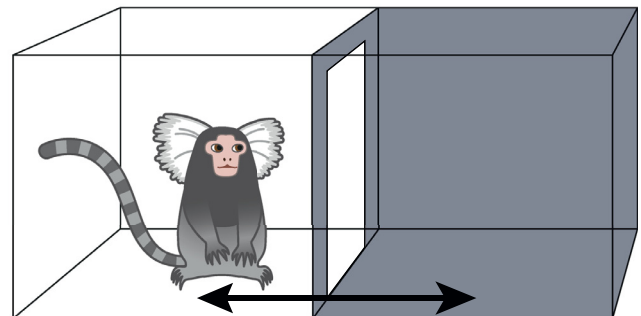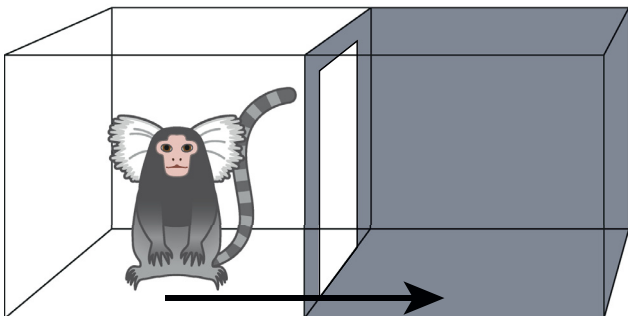**Week 2: Reconsolidation**
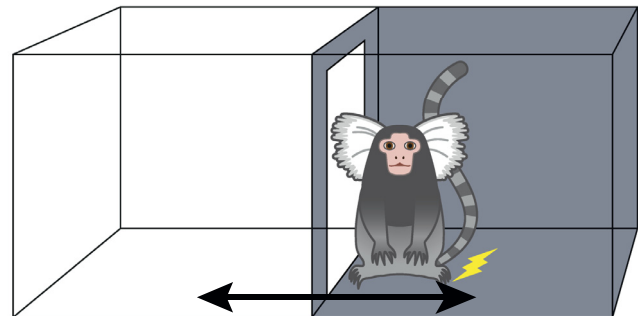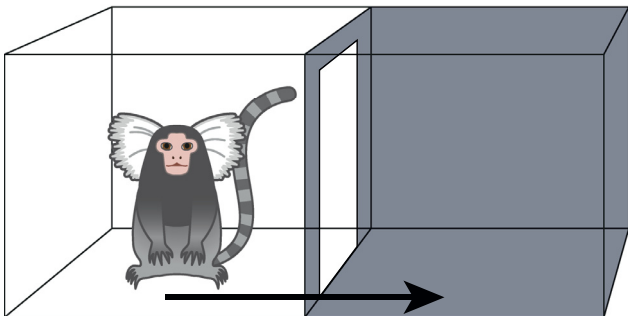
foot-shock (0.5mA)

Week 3: Follow-up phase

Fig. 1 Unescapable foot shock paradigm in a passive avoidance task to model PTSD. The apparatus consists of a light and a dark compartment (30 × 25 × 30 cm) connected by a sliding door (9 × 11.5 cm) and an electric grid floor. The monkeys enter the passive avoidance test in the illuminated compartment. The door opens after one minute. Only during week 1 (consolidation) the door closes after entering the dark compartment for delivering four random unescapable foot-shock (0.5 mA, 4 s) within a 15-minute time frame. In week 2 (reconsolidation) the monkeys are re-exposed to the stressful situation. Prior to re-exposure the monkeys received a treatment. In week 3 (follow-up phase) the monkeys re-experience the stressful situation without foot-shocks to measure the memory for the stressful situation by recording the latency time to enter the dark compartment. The arrows indicates the direction the monkey can choose. In case of two arrow points the monkey is able to switch in both direction as the door stays open.

are: apathy, immobility, inadequate grooming, huddle (fetus-like posture), stereotypic behavior (repeated movement of a limb or the head or motion without a goal). The scoring ranged from 0 (normal) to 4 (severe). The sum of these scores were used for the general clinical assessment of each individual monkey.

2.7. Sleep analysis

Sleep behavior was studied with the use of EEG. Sleep was monitored during the three test days for three weeks. The EEG/ECG data were collected between 16.30–7 h by a receiver (RMC-1, Data Sciences International, St. Paul, Minnesota, USA) outside the cage. The raw data were collected with acquisition software (Ponehma, Data Sciences International, St. Paul, Minnesota, USA). EEG was recorded in 30-s epochs at a frequency of 1000 Hz. The raw data were sorted and converted to European Data Format (EDF) with data analyses software Neuroscore (Data Sciences International, St. Paul, Minnesota, USA). An experienced sleep technologist manually analyzed the EEG-data of every animal into four different sleep stages based on the method of Rechtschaffen (1968) and Verhave et al. (2011). Total REM sleep was used as a measure for stress.

Table 1 Clinical score of stress-related signs (average ± SEM).

Treatment		Control	Ketamine
Week 0	Baseline clinical score	6.5 ± 2.6	5.8 ± 1.8
Week 1	After acquisition trial	11 ± 3.1	8 ± 2.4
Week 2	After reconsolidation trial and treatment	9.2 ± 3.7	6.5 ± 2.3
Week 3	After the follow-up trial	10.5 ± 2.9	6.3 ± 1.3

2.8. Heart rate

Heart rate was measured using ECG. The ECG data were collected simultaneously with the EEG data (4:30 pm-7:00 am). The number of heartbeats was analyzed with Neuroscore software (Data Sciences International, St. Paul, Minnesota, USA) and the data were further analyzed in Microsoft Excel. The mean heart rate per 30-s ECG epoch during wake were analyzed for every experimental day.

2.9. Cortisol levels

Saliva samples were collected between 9:00 and 11:00 am to measure cortisol levels. The timing of the saliva collection was kept standard to prevent the circadian effects on the cortisol level. The swabs were put in separate Eppendorf tubes and centrifuged at a speed of 3000 rpm for 10 min. The liquid residue of the saliva was stored at $-20\,°C$ until further analysis. Cortisol was measured with a commercially available enzyme-linked immunosorbent assay (Salimetrics®, Pennsylvania, USA.). Cortisol collected over the last week of testing (week 3) was analyzed elsewhere, where the samples were too much diluted resulting in cortisol levels below the detection limit.

2.10. Brain tissue preparation

The brains were fixated in formalin for 24 h and thereafter stored in buffered PBS. Preserved brains were cryoprotected in 30% w/v sucrose in PBS. After hemisection, the middle part of the right hemisphere, containing the hippocampus and amygdala, was sliced at 40 μm into a 10-series. The sections were preserved in anti-freeze (50% glycerol, 25% aquadest, 25% ethylene glycol).

2.11. Immunohistochemistry

To investigate the effects of ketamine on neurogenesis, we stained for BrdU and DCX. Between each step, sections were washed with TBS (pH 7.6, 0.05M) unless specified otherwise. All incubations and washing steps were at room temperature (RT), unless specified otherwise. All sections were counterstained using haematoxylin, dehydrated and cover slipped using entallan.

For the BrdU staining, sections were microwaved in citrate buffer (0.01 M, pH 6.0) for 5 min at 800 W, 400 W and 200 W for antigen retrieval. Thereafter, sections were cooled and incubated for 30 min in 0.3% H_2O_2 in tris-buffered saline (TBS) to block endogenous peroxidase activity. After the peroxidase block, sections were incubated for 1 hour in incubation mix (1% BSA and 0.3% Triton-X in TBS) to block for non-specific antibody binding. Immediately after blocking, sections were incubated for 1 h with 1:500 monoclonal rat α-BrdU antibodies in incubation mix, and overnight at 4 °C (Accurate Chemical Westbury NY USA Clone BU1/75 (ICR1). The next day, sections were incubated for 2 h with 1:500 goat-α-Rat antibodies in incubation mix. The sections were incubated for 90 min with 1:800 avidin-biotin-peroxidase (ABC) complex, and then washed with tris buffer (TB; pH 7.6, 0.05 M). Staining was performed using the DAB-reaction, using 3,3-diaminobenzidine (0.5 mg/mL DAB, 0.01% H_2O_2 in TB, pH 7.58) for 45 min.

For the DCX staining, sections were stained in vials. Endogenous peroxidase activity was blocked by incubating sections for 15 min in 0.5% H_2O_2 in TBS. After the peroxidase block, non-specific antibody binding block was performed by incubating sections for 30 min in 2% w/v milk powder in TBS. Immediately after blocking, sections were incubated in 1:800 polyclonal goat-α-DCX (Santa Cruz Biotechnology, Santa Cruz CA, USA sc-8066) in a buffer containing 0.25% gelatin/0.1% Triton-X in TBS 1 h, then overnight at 4 °C. The next day, sections were incubated for 2 h in 1:800 donkey-α-goat antibody in a buffer containing 0.25% gelatin/0.1% Triton-X in TBS. Afterwards, sections were incubated in 1:800 ABC for 90 min. After incubation for 30 min in 1:500 tyramide in 0.01% H_2O_2 in TBS, sections were again incubated for 90 min in 1:800 ABC. Sections were washed with TB. Staining was performed using the DAB-reaction, with 3,3-diaminobenzidine (0.5 mg/mL DAB, 0.01% H_2O_2 in TB, pH 7.59) for 40 min.

2.12. Cell counting

Sections were counted for BrdU or DCX positive cells in the dentate gyrus (DG) as described before (Marlatt et al., 2011, 2012). The DG was counted from ($+2.9$ to Bregma; to -3.2 mm Bregma) (Palazzi and Bordier, 2008). For the DCX+ cells, the sub granular zone (SGZ) and granular cell layer (GCL) were also counted separately. The number of cells per slice were calculated per animal. All cells-counts were performed while blinded to experimental conditions of the animals.

2.13. Statistical analysis

The behavioral data have been tested for normality using the Shapiro-Wilk test. A repeated measures two-way ANOVA was performed to measure interaction effects between time and condition. To measure differences between condition and time t-tests were performed per session. To measure the within group differences on specific time points a paired student t-test with Welch's correction was used. For the histology data, analysis was performed on cells per section to correct for variability in total sections between animals (Bunk et al., 2011). For BrdU student's t-test was used and for DCX (rostral, mid and caudal) a Mann-Whitney U test was used performed, as the assumption of normality was not met. A level of $p < 0.05$ was considered significant (Prism 6.0e for Mac OS X; GraphPad Software, San Diego California USA; Histology: counts were analyzed in SPSS Statistics 22). Justification of the number of animals: The formula used is: $N = 2(Z\alpha/2 + Z\beta)^2 * (SD/ES)^2$. The statistical exact power calculation is based on simple between group t-tests with an α of 0.05 and a power of 80%. With α set at 0.05, $Z\alpha/2 = 1.96$; β set at 0.2 (80% power), $Z\beta = 0.84$; $2(Z\alpha/2 + Z\beta)^2 = 15.7$. The latency time in the passive avoidance test was selected as primary outcome measure. We expect a short latency during the first exposure of 100 to 200 s with a variation
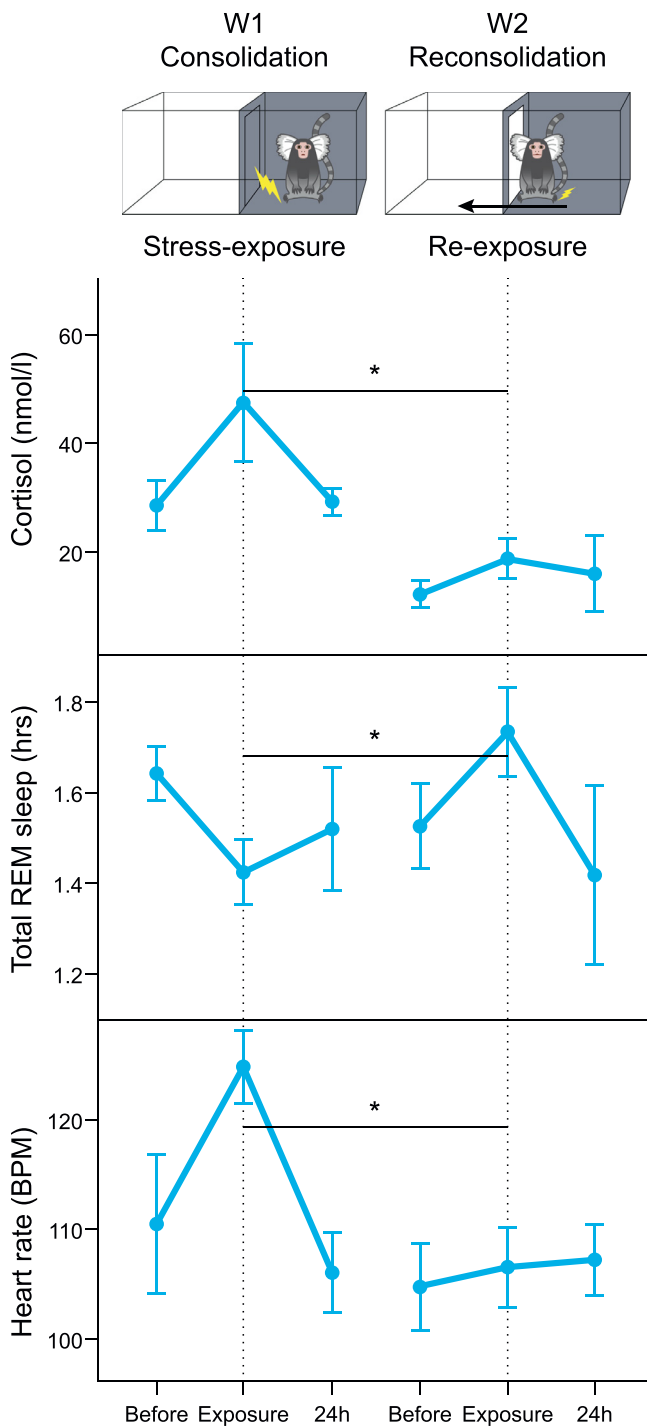
Fig. 2 Stress-exposure effects on saliva cortisol level, total REM-sleep time and heart rate. The effect of stress exposure of the control group ($n = 6$) during the memory reconsolidation (W1: week 1) and of the re-exposure during the reconsolidation (W2: week 2) are shown. During the stress exposure the saliva cortisol level (top) and heart rate (bottom) are significantly increased after the stress exposure compared to the re-exposure one week later (resp. $P = 0.0456$ and $P = 0.0133$). After the stress exposure the control monkeys spent less time in REM-sleep (middle) compared to the re-exposure ($P = 0.0380$). * Significant effect (t-test with Welch's correction, two-tailed $P<0.05$).
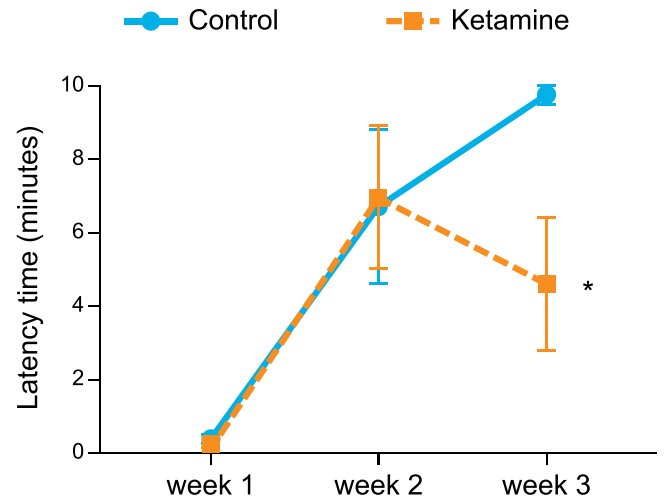

Fig. 3 Effect on emotional memory in the passive avoidance task. During the re-exposure in week 2, all monkeys showed a delay in entering the dark compartment compared to week 1. One week after treatment in week 3, the vehicle-treated (saline control) monkeys avoided entering the dark compartment, whereas the ketamine-treated monkeys entered the dark compartment significantly faster and more frequently. * Two-way repeated measures ANOVA, Time: $F(2,20) = 19.18$, $P < 0.0001$. A t-test with a correction for multiple testing week 3 control vs ketamine: $t(30)$ 2.64, $P = 0.038$.

of 55 s (SD) that can increase by several minutes to 400-500 s during the second exposure. With a SD of 55, and effect size of 100: $N = 15.7 * (55/100)^2 = 5$ (assuming a normal distribution). To adjust to student t distribution the N is increased to 6 ($5 + 1 = 6$) for each group.

3. Results

3.1. Effect on stress-related parameters

The clinical score was not affected in both groups compared to the baseline score (Table 1). No significant effect was found between the treatment groups by a non-parametric Wilcoxon signed rank test ($P > 0.9$).

Saliva cortisol levels, in the non-treated control group, after the inescapable foot-shock exposure (consolidation phase, week 1) were significantly higher compared to the re-exposure in week 2, in which the foot-shock was escapable (Fig. 2; t-test with Welch's correction, $t = 2.507$, df $= 6.166$, two-tailed $P = 0.0456$). The same effect was found on the heart rate (BPM) (Fig. 2; t-test with Welch's correction, $t = 3.765$, df $= 4.963$, two-tailed $P = 0.0133$). As expected, the total REM sleep was lower during the first night after the stress exposure compared to the night after the re-exposure (Fig. 2; t-test with Welch's correction, $t = 2.563$, df $= 6.859$, two-tailed $P = 0.0380$).

3.2. Effect on memory

In Fig. 3 the effect of ketamine on fear memory in the passive avoidance task is shown. In the first week all mon-
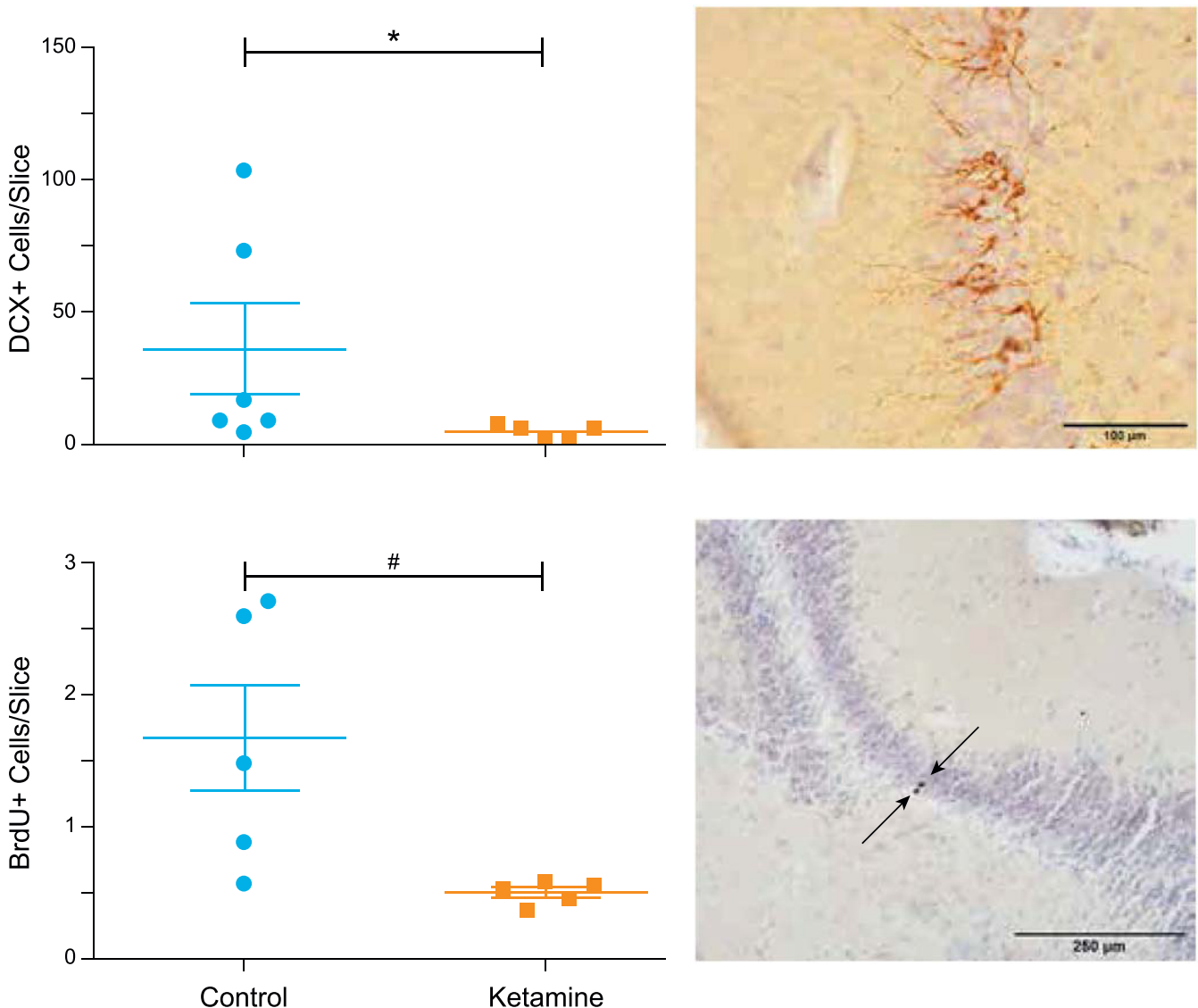
Fig. 4 Brain histology of the hippocampus one week after a low dose of ketamine. Histological results are shown of the dentate gyrus (DG) of the hippocampus with a DCX-staining (top) and BrdU-staining (bottom) with a haematoxylin counter staining. The pictures at the right side represent examples of a slice from a stress-exposed control monkey. BrdU+ cells are marked with downward arrows in the granular cell layer (GCL) and with upward arrow in the sub granular zone (SGZ). Only DCX+ cells with the whole cell body in the plane of sight and in the DG were counted. Ketamine treated monkeys expressed significantly less neurogenesis in the DG. A single injection of ketamine (0.5 mg/kg) during reconsolidation (week 2) reduced the number of DCX+ cells compared to vehicle treated controls. * $P = 0.030$, # $P < 0.1$ and > 0.05 (Mann–Whitney U test).

keys entered the dark compartment immediately. In the second week all monkeys remembered the aversive effect of entering the dark compartment: it took them on average 6.7 ± 2.1 min to enter the dark compartment (two-way repeated measures ANOVA, Time: $F(1,20) = 20.96$, $P = 0.0002$). In the third week, the effect on emotional memory was tested one week after treatment with vehicle or ketamine. The vehicle-treated monkeys largely avoided entering the dark compartment in week 3 (9.8 min $\pm$ 0.3). The ketamine-treated monkeys entered the dark compartment significantly faster (4.6 min $\pm$ 1.8) (vehicle versus ketamine: two-way repeated measures ANOVA, Time: $F(2,20) = 19.18$, $P < 0.0001$; t-test with a correction for

multiple testing Week 3 control vs ketamine: $t(30)$ 2.64, $P = 0.038$).

3.3. Effect on neurogenesis

Fig. 4 shows the effect of a single ketamine injection, during the reconsolidation, one week after treatment, on DCX+ and BrdU labeled neurons in the DG of the hippocampus, which reflect young immature neurons and newly born cells respectively. Data points smaller than Q1 – 1.5*IQR or greater than Q3 + 1.5*IQR were considered as outliers. One animal in the ketamine group was an outlier, and was ex-

cluded from the analysis. BrdU positive (BrdU+) cells are found in the SGZ and GCL. In contrast to ketamine-treated monkeys, vehicle-control monkeys displayed considerable variation in BrdU+ cells. The same was found with the DCX-staining. For the BrdU+ cells, no significant difference was found between the experimental groups, although a trend towards a decrease in the number of BrdU+ cells in the DG was observed in ketamine-treated monkeys ($t = 2.077$, $df = 5.063$, $P = 0.092$). As the number of BrdU+ cells was quite low in most sections, no distinction between SGZ and GCL was made in the analysis. On the other hand, a significant decrease in DCX+ cells is observed in ketamine-treated monkeys ($P = 0.030$). In the rostral region, no significant difference was found ($P = 0.126$), whereas in the mid region a significant difference was observed between control and ketamine-treated monkeys ($P = 0.017$), and in the caudal region a trend could be seen ($P = 0.052$).

4. Discussion

In this study we report that a single low dose of the NMDAR-antagonist ketamine immediately before retrieval/reconsolidation of the traumatic exposure reduces acquired fear in the marmoset monkey. The present study shows that ketamine treatment during retrieval/reconsolidation reduced fear for the aversive compartment since ketamine-treated monkeys entered the aversive compartment. Retrieval/reconsolidation itself was most likely free of trauma as the cortisol levels stayed at a normal baseline value and the heart rate did not increase when compared to the effect of the trauma-exposure one week before. Additionally, the REM-sleep was only declined after the trauma exposure compared to the re-exposure period and the following week after re-exposure.

These findings are in accordance with reports that REM-sleep is inhibited after exposure to a fearful experience (Mellman et al., 2007). The absence of this decline during the re-exposure indicated that the re-exposure itself was not stressful. Although the re-exposure did not evoke a stress response, the earlier exposure was remembered well during the re-exposure since all monkeys showed a significant delay in entering the trauma-related compartment compared to the acquisition trial. Interestingly, one week after the ketamine treatment during the re-exposure these monkeys did enter the fear-related compartment. In contrast, the vehicle-treated control monkeys avoided this compartment. This effect is not the result of attenuation of learned fear during the re-exposure, as ketamine in mice only show prophylactic effects when given one week before the stress exposure and not one hour before the exposure (McGowan et al., 2017). These findings suggest that ketamine reduces reconsolidation of fear memory, presumably independently of modification of sleep, HPA-axis activation or modifying the autonomic nervous system.

Several studies have reported that in rodents, ketamine at subanesthetic doses, exerts rapid antidepressant-like effects within hours after administration (Duman et al., 2019; Li et al., 2010; Moda-Sava et al., 2019). Since NMDARs are critical for synaptic plasticity and memory formation, targeting these receptors might be effective to reduce fear memory when applied upon memory reactivation as seen in

rodent models (Nader et al., 2000) and humans (Kindt et al., 2009; Kroes et al., 2014). This offers a potential therapeutic intervention (Monfils et al., 2007; Schiller et al., 2000). In a rat model of PTSD, the NMDAR-antagonist Xenon impaired fear memory reconsolidation (Meloni et al., 2014), and the NMDAR-antagonist memantine, promoted the forgetting of hippocampus-dependent fear memories after reactivation (Ishikawa et al., 2016). Additionally, the NMDAR-antagonist MK-801 disrupts fear memory reconsolidation, whereas the NMDAR-agonist D-cycloserine facilitates reconsolidation (Lee et al., 2006; Merlo et al., 2014).

Histological data, obtained after the last measurement (one week after ketamine treatment) shows that the ketamine treatment in combination with trauma-exposure reduced neurogenesis. Neurogenesis in the DG is linked to pattern separation which is a process that allows similar events displaying overlap to be encoded as different memories, preventing mixing up of memories (Kheirbek et al., 2012). Aberrant neurogenesis has been implicated in PTSD (Colgin et al., 2008; Kheirbek et al., 2012; Sodic et al., 2007). This is possibly related to impaired pattern separation which may lead to generalization of fear-related cues and impaired discrimination between safe environments and trauma-related environments (Besnard and Sahay, 2016). An earlier study on neurogenesis in the marmoset showed an immediate reduction of neurogenesis after stress (Gould et al., 1998). This effect was normalized after a two-week period in a non-stress environment, as no difference in neurogenesis in the DG could be found after this two-week period (Marlatt et al., 2011). Remarkably, in the present study, treatment with a single low dose of ketamine further reduced neurogenesis. This is in line with the findings that ketamine treatment in seven-days old rats reduces neurogenesis, which was indicated by reduced number of BrdU+ neurons in the hippocampus (Huang et al., 2016). Other studies also reported an increase of the β-tubulin III (TuJ-1) marker for immature cells (Dong et al., 2012). A similar result was found in another study, in which an increase of DCX+ cells was observed immediately after ketamine treatment (Clarke et al., 2017). In these studies there was a short latency of only one day between ketamine administration and the histological examination of the brain. In our studies, we report a reduced number of DCX+ cells in the dentate gyrus. This reduction in DCX+ cells could potentially be the result from cells maturing faster after ketamine administration, which needs further investigation. One interpretation of our findings on DCX and BrdU could be that increased maturation of earlier-born cells, in the days following ketamine treatment during the re-exposure trial, might reflect increased functional integration of these cells and promote the forgetting of memories due to integration of the recently-matured neurons into the existing hippocampal circuit, as was found in earlier studies towards effects of neurogenesis on memory (Akers et al., 2014; Ishikawa et al., 2016).

4.1. Conclusion

Although present medications help ease associated symptoms of depression and anxiety and seize sleep disturbances, there is an urgent need for early effective in-

terventions targeting more elementary processes in the pathophysiology of PTSD (Wright et al., 2019). Despite research efforts to develop more effective therapies, PTSD remains difficult to treat (Jonas et al., 2018; Lee et al., 2016). Patients are treated by several treatment modalities, like a variety of forms of psychotherapy and drug therapy (Charney et al., 2018; Veen et al., 2018; Watkins et al., 2018). In the absence of novel compounds, ketamine has been favored as a novel therapeutic for PTSD focusing on memory consolidation and synaptic connectivity (Krystal et al., 2017a, 2017b). A single low dose of ketamine, given during reconsolidation, reduces the expression of fear memory in a passive avoidance paradigm measured one week after ketamine treatment.

This ketamine treatment also reduced neurogenesis in the hippocampus, which might affect the formation of new neuronal connections and can be an explanation how ketamine influences the reconsolidation process. This study provides important findings for ketamine as a potential candidate to target traumatic memories in PTSD.

Role of the funding source

Contributors

IP (project leader) designed the study, did the interpretation of the data, wrote the manuscript and the request for the involvement of animals and is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

LD did the data acquisition, data analysis and helped with writing the manuscript.

GB planned the study and did the data acquisition.

HK designed the study, performed the histology, helped with the interpretation of the data and with writing the manuscript.

EV designed the study, helped with the interpretation of the data.

Declaration of Competing Interest

None.

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