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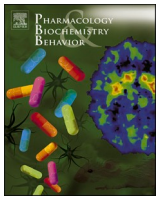

Environmental enrichment enhances the antidepressant effect of ketamine and ameliorates spatial memory deficits in adult rats

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ABSTRACT

Ketamine is a rapid-acting antidepressant associated with various cognitive side effects. To mitigate these side effects while enhancing efficacy, it can be co-administered with other antidepressants. In our study, we adopted a similar strategy by combining ketamine with environmental enrichment, a potent sensory-motor paradigm, in adult male Wistar rats. We divided the animals into four groups based on a combination of housing conditions and ketamine versus vehicle injections. The groups included those housed in standard cages or an enriched environment for 50 days, which encompassed a 13-day-long behavioral testing period. Each group received either two doses of ketamine (20 mg/kg, IP) or saline as a vehicle. We tested the animals in the novel object recognition test (NORT), forced swim test (FST), open field test (OFT), elevated plus maze (EPM), and Morris water maze (MWM), which was followed by ex vivo c-Fos immunohistochemistry. We observed that combining environmental enrichment with ketamine led to a synergistic antidepressant effect. Environmental enrichment also ameliorated the spatial memory deficits caused by ketamine in the MWM. There was enhanced neuronal activity in the habenula of the enrichment only group following the probe trial of the MWM. In contrast, no differential activity was observed in enriched animals that received ketamine injections. The present study showed how environmental enrichment can enhance the antidepressant properties of ketamine while reducing some of its side effects, highlighting the potential of combining pharmacological and sensory-motor manipulations in the treatment of mood disorders.

1. Introduction

Clinical depression is a devastating mood disorder that affects several different neural circuits, producing a broad range of symptoms (Unal, 2021). Conventional antidepressants that target the monoaminergic system raised concerns due to their slow onset of action and inefficacy in treatment-resistant depression (TRD) (Schwartz et al., 2016). This gave rise to the search for novel antidepressants that produce a rapid therapeutic response with a wide margin of safety (aan het Rot et al., 2010; Sanacora and Schatzberg, 2015). Ketamine, a non-competitive *N*-methyl-*D*-aspartate receptor (NMDAR) antagonist binding to the phencyclidine (PCP) site in the transmembrane domain of the receptor, provided the desired fast-onset and efficacy in clinical trials (Li and Vlisides, 2016), although it produces several side effects in a dose-dependent manner (Hashimoto, 2020; Short et al., 2018). While esketamine, the *S*(+) enantiomer of ketamine, is utilized as the last treatment choice for patients who cannot benefit from typical antidepressants (Bozymski et al., 2020), it has the same side effects. For this reason, administering ketamine in combination with other drugs to achieve a synergistic effect while minimizing its side effects has been a major research pursuit (Pitsikas and Carli, 2020; Pitsikas and Markou, 2014; Verma and Moghaddam, 1996). In our study, we adopted a non-pharmacological approach for the same purpose by utilizing environmental enrichment (EE) (Hebb, 1947; Rosenzweig and Bennett, 1996) in adult male Wistar rats. We assessed the impact of this potent sensory-motor paradigm on various cognitive and affective properties of ketamine, revealing their synergistic therapeutic effect.

In recent years, intranasal or intravenous (IV) administration of ketamine has emerged as a leading choice for treating patients with TRD (Berman et al., 2000; Zarate Jr et al., 2006) and reducing suicidal ideation in severe cases (Ballard et al., 2014; Serafini et al., 2014). Behavioral studies in rats (Ecevitoglu et al., 2019; Engin et al., 2009; Garcia et al., 2008; Gigliucci et al., 2013; Kingir et al., 2023; Yilmaz et al., 2002) and mice (Autry et al., 2011; da Silva et al., 2010; Maeng

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et al., 2008; Salat et al., 2015) have replicated the potent antidepressant effects of ketamine in the forced swim test (Porsolt et al., 1977; Porsolt et al., 1978a, 1978b; Unal and Canbeyli, 2019). However, systemic administration of ketamine in rodents have also been associated with various cognitive side effects, including impairments in object recognition memory (Goulart et al., 2010; Pitsikas et al., 2008; Pitsikas and Boultadakis, 2009), spatial memory (Moosavi et al., 2012; Pitsikas et al., 2008; Pitsikas and Boultadakis, 2009) and working memory (Enomoto and Floresco, 2009; Imre et al., 2006; Verma and Moghaddam, 1996). The therapeutic effects of ketamine may be accompanied by these impairments and other behavioral side effects depending on the dose and length of treatment (Hashimoto, 2020; Short et al., 2018).

Environmental factors can alter or modify the multifaceted cognitive and affective properties of ketamine. Both the desired therapeutic outcomes and the side effects may be modulated by sensory stimulation and locomotor activity via bottom-up processes. Environmental enrichment, originally observed by Donald O. Hebb (1947), became the standard rodent behavioral paradigm to test the effects of enhanced sensorymotor experience under different conditions (Rosenzweig and Bennett, 1996). This procedure leads to experience-dependent changes in several limbic and cortical circuits and improves performance in different cognitive tasks (Simpson and Kelly, 2011). Environmental enrichment promotes adult neurogenesis and synaptogenesis (Birch et al., 2013; Kempermann et al., 1996; van Praag et al., 2000), cell proliferation (Tanti et al., 2013), cortical thickness (Rosenzweig et al., 1962), and dendritic branching (Mora et al., 2007). It alters the expression of immediate early genes (Leger et al., 2012), growth factors (Rossi et al., 2006), and nuclear receptors (Soares et al., 2015). These neurobiological changes underlie the procognitive effects of EE in neurodegenerative diseases like Alzheimer's, Parkinson's, and Huntington's disease (Nithianantharajah and Hannan, 2006). The neuroprotective and neuroregenerative properties of EE were also observed in central nervous system injuries (Alwis and Rajan, 2014).

In addition to its versatile cognitive effects leading to enhanced performance in different behavioral paradigms (Bechara and Kelly, 2013; Cortese et al., 2018; Leggio et al., 2005; Sampedro-Piquero et al., 2013), EE may also suppress anxiety-like behavior and ameliorate depressive-like states in rodents (Ashokan et al., 2016; Brenes et al., 2020; Sampedro-Piquero et al., 2016; Urakawa et al., 2007). These findings, altogether, led to the consideration of EE as a non-pharmacological treatment option for perinatal (Forbes et al., 2020), traumatic (Alwis and Rajan, 2014), and post-stroke brain injury (McDonald et al., 2018), as well as other types of neurogenic and psychogenic stress (Fox et al., 2006). Here, we investigated whether these protective and ameliorative effects can be utilized to suppress the side effects of ketamine (20 mg/kg, IP) or produce a synergistic therapeutic action with it. We formed four groups by combining standard or enriched housing with ketamine or vehicle injections, and tested the contribution of EE to different cognitive and affective properties of ketamine in the novel object recognition test (NORT), forced swim test (FST), open field test (OFT), elevated plus maze (EPM), and Morris water maze (MWM). We combined these results with ex vivo c-Fos immunohistochemistry to delineate brain regions that display altered neuronal activity in response to ketamine administration, environmental enrichment, or their combination.

2. Materials and methods

2.1. Subjects

Thirty-two experimentally naïve adult male Wistar rats (8 months old; $M = 359.8$ g, $SD = 35.04$ g) were used for the experiment. The animals were housed in a controlled environment (21 ± 1 °C; 40–60 % humidity; 12:12 day/night cycle, lights on at 09:00) with ad libitum access to food and water. They were assigned to control and experimental groups by systematic sampling based on their weight at the beginning of the experiment. One group was housed in standard cages and received vehicle injections (Standard Environment, SE; $n = 8$). Another group was housed together in an enrichment cage and received vehicle injections (Enriched Environment, EE; n = 8). The third group was housed in standard cages and received IP ketamine injections (Ketamine + Standard Environment, Ket SE; n = 8). The other ketamine group was housed in an enriched environment (Ketamine + Enriched Environment, Ket EE; n = 8). The enrichment cages housed all eight belonging to the same group (EE or Ket EE), whereas each standard cage housed four animals from the same group (SE or Ket SE). All experimental procedures were approved by the Boğaziçi University Ethics Committee for the Use of Animals in Experiments.

2.2. Experimental design

Differential housing started at the beginning of the experiment and lasted for 50 days until perfusion-fixation (Fig. 1). To minimize handling stress during testing, all animals underwent 2-minute handling sessions per day for one week prior to behavioral testing. Experiments started with the habituation phase of the NORT on the 38th day of differential housing. This was respectively followed by the FST, OFT, EPM, and MWM (Fig. 1). All behavioral procedures were carried out between 10:00 and 19:00 in the same test room. Behavioral analyses were done by experimenters who were blind to the experimental conditions.

We administered two doses of IP ketamine injections to Ket SE and Ket EE groups to achieve an effective antidepressant dose in each behavioral test. The SE and EE groups received IP saline injections as a vehicle. The first doses of ketamine (20 mg/kg) and vehicle (1 ml/kg) were applied immediately after the training phase of NORT, while the second injections were done following EPM (Fig. 1). Ketamine (Brema-Ketamine, 100 mg/ml; Warburg, Germany) was dissolved in saline (0.9 %) at a 1 ml/kg volume. The effective dosage (20 mg/kg) was chosen based on previous studies that reported antidepressant effects following a single IP injection (Wang et al., 2011) or repeated administration (Parise et al., 2013). This dose was also observed to induce impairment in the consolidation of object recognition memory (Goulart et al., 2010).

2.3. Environmental enrichment

We followed the environmental enrichment procedure of Slater and Cao (2015) with minor adjustments. Eight animals were housed in a custom-made transparent Plexiglas cage (66 × 66 × 66 cm) with bedding. The cage contained a running wheel, two shelter huts, a 20 cm-high platform (25 × 25 cm), a tunnel, and several objects/toys with different colors, textures and shapes (see Guven et al., 2022). The objects were replaced each week with new ones until the beginning of behavioral testing to induce novelty (Nithianantharajah and Hannan, 2006). In addition, the location of each object was changed in the middle (during the fourth day) of the week.

2.4. Novel object recognition test

We used a square arena enclosed with opaque walls (45 × 45 × 35 cm). The task consisted of two habituation, one familiarization, and one test session run on separate days (Fig. 1). During habituation sessions, the animals explored the empty maze for 5 min, and their overall locomotor activity was recorded. For familiarization, each rat was placed in the apparatus with two identical objects (A1 and A2) for 10 min. The amount of time spent exploring A1 and A2, and overall locomotor activity were recorded. The test session was conducted 24 h after familiarization to assess object recognition memory. In the test phase, one of the identical objects was kept in the test apparatus (A), while the other was replaced with a novel object (B). Time spent exploring the novel versus the familiar object, and overall locomotor activity in the maze were recorded. The animal was considered to exhibit exploratory behavior when the tip of its nose was within 2 cm of an object. Turning

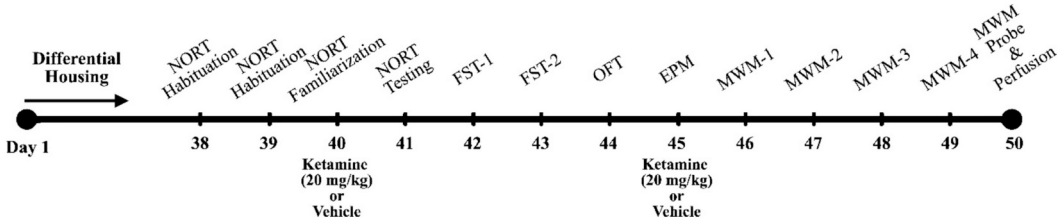

Fig. 1. Experimental timeline showing the order of behavioral experiments and IP ketamine injections. NORT, novel object recognition test; FST, forced swim test; OFT, open field test; EPM, elevated plus maze; MWM, Morris water maze.

around or sitting on the object was not considered as exploratory behavior (Antunes and Biala, 2012). Object locations were counterbalanced during familiarization and test sessions. Both the maze and the objects were cleaned with 70 % ethanol between each session to eliminate olfactory cues for the next animal. A discrimination index was calculated as T_N / (T_N + T_F); where T_N denotes the time (s) spent with the novel object, and T_F stands for the time (s) spent with the familiar object (Akkerman et al., 2012; de Lima et al., 2008). A discrimination index exceeding 0.5, the chance level, suggests that the animal has allocated more time to exploring the novel object. This behavior aligns with the typical inclination of rodents to preferentially explore novel objects compared to familiar ones, indicating the presence of recognition memory for the familiar object.

2.5. Forced swim test

We measured immobility, struggling, and swimming displayed in the pretest (FST-1) and test (FST-2) sessions as outlined by Slattery and Cryan (2012). The FST apparatus was a transparent cylindrical Plexiglas chamber (height = 45 cm, r = 15 cm). The chamber was filled with water (23 ± 1 °C), ensuring that it reached a depth of 30 cm above the ground to prevent the hindlimbs from touching the chamber's bottom. Each rat was placed in the apparatus for 15 min during FST-1 and for 5 min during FST-2, which was conducted 24 h later. Each session was recorded with a video camera for offline analyses. The first 5 min of FST-1 and the FST-2 were analyzed for predominant behaviors (i.e. immobility, struggling, and swimming) in each 5-s interval as previously reported (Slattery and Cryan, 2012). Two types of active behavior, headshaking and diving were also quantified (Akmese et al., 2023). Each session was independently analyzed by three observers, and the mean value of their scores was calculated.

2.6. Open field test

The test apparatus was a square arena (70 × 70 × 35 cm) enclosed with opaque walls. The field was illuminated from above, making the center brighter (90 ± 5 lx) compared to the periphery of the arena (55 ± 5 lx). Each session, which lasted 5 min, was recorded with a video camera for subsequent analyses of overall mobility, total travel distance, and the time spent in the center zone (35 × 35 cm) versus periphery of the maze. Instances of rearing, a type of exploratory behavior (Guven et al., 2022), grooming, and the number of fecal boli were counted manually. Duration analyses were made with a modified version of DeepLabCut (Mathis et al., 2018), which was trained to generate consistent results with other major open-source analysis software (Isik and Unal, 2023). The arena was cleaned with 70 % ethanol between each session to eliminate olfactory cues for the next animal.

2.7. Elevated plus maze

An elevated (50 cm above the ground) plus-shaped apparatus with two open and two closed arms (length: 50 cm, width: 10 cm) was used to assess anxiety-like behavior. The open arms had transparent Plexiglas walls, while the closed arms were enclosed by opaque wooden walls. The open arms were significantly more illuminated (230 ± 5 lx) than the closed arms (60 ± 5 lx). Each trial started by gently placing a rat in the center of the maze facing an open arm. Overall time spent in open vs. closed arms and the total duration of locomotor activity was recorded for 5 min. The apparatus was cleaned with 70 % ethanol after each session.

2.8. Morris water maze

We used a circular pool (diameter: 120 cm) filled with tap water up to 30 cm (24 ± 1 °C). The swimming area was separated into four virtual quadrants, and each quadrant had a unique cue attached to the maze wall. The cues were two-dimensional geometrical shapes with different colors. A transparent circular escape platform was placed 2 cm below the water surface in one of the virtual quadrants, which is designated as the "target quadrant". Milk powder was added to the water to ensure that the escape platform was not visible to the animals.

The procedure consisted of four days of training and a final day of probe trial as described in Vorhees and Williams (2006). Each animal was subjected to four trials per training day with 2-minute intertrial intervals. Rats started each trial from a different, randomly chosen, quadrant, and were given 1 min to locate the hidden platform, on which they were allowed to remain for 15 s. Animals that failed to find the platform were gently pulled towards it at the end of the 1-minute session. A 1-min single-session probe trial was run on the final day of the MWM. On the probe trial, the hidden platform was removed from the maze, and each animal started the maze from the opposite quadrant of the target quadrant. All sessions were recorded with a video camera for subsequent analyses with a custom-made version of DeepLabCut. Time spent to locate the hidden platform (i.e. escape latency), time spent in each virtual quadrant, swimming speed, trajectory, and thigmotaxis were analyzed.

2.9. Tissue preparation and immunohistochemistry

We performed transcardial perfusion-fixation 90 min after the probe trial of MWM. Animals received terminal ketamine-xylazine anesthesia (100 mg/kg-10 mg/kg, IP), and perfused with 0.9 % saline and 4 % depolymerized paraformaldehyde (PFA). The brains underwent post-fixation in the same PFA solution and were subsequently rinsed in phosphate-buffered saline (PBS). Coronal brain sections (50–70 μm) were collected with a vibrating microtome (VT1000 S, Leica Biosystems, Nussloch, Germany) and stored in 0.01 % PBS-azide.

Fluorescent immunohistochemistry and microscopic observations were done as previously described (Akmese et al., 2023; Kingir et al., 2023; Unal et al., 2015). Briefly, coronal sections were washed 3 times for 10 min with Triton X-100 in PBS (PBS-Tx) to achieve tissue penetration. They were blocked in 20 % normal horse serum (NHS, Vector Laboratories, France) in PBS-Tx for 1 h at room temperature. We investigated the effects of differential housing on neuronal activity by c-Fos immunohistochemistry. Sections were incubated in rabbit polyclonal anti-c-Fos antibody (sc-52, Santa Cruz Biotechnology, 1:250 in 1 % NHS PBS-Tx) for 72-hour at 4 °C. After primary incubation, the sections were washed with PBS-Tx 3 times for 10 min at room temperature,

and treated with donkey anti-rabbit secondary antibody (Alexa Fluor 488, ab150073, Abcam, 1:250 in 1 % NHS PBS-Tx) for 4 h at room temperature. The sections were then washed with PBS-Tx 3 times for 10 min and transferred into a PBS solution for subsequent mounting. For DAPI staining, sections were washed 3 times for 5 min with PBS-Tx, and incubated with DAPI (D3571, ThermoFisher, 1:1500 in PBS) for 15 min.

2.10. Microscopy and cell counting

We obtained 36 sections from 12 animals (3 animals per group) and analyzed each hemisphere as separate data points. We counted c-Fos immunopositive (c-Fos+) cells in the medial habenula (MHb), lateral habenula (LHb), perirhinal cortex (PRh), and dorsolateral entorhinal cortex (DLEnt) at rostrocaudal levels between −3.12 mm to −3.36 mm to the Bregma point (Paxinos and Watson, 2006). Analyzed brain sections were chosen before the immunohistochemistry, and no further sections were processed after data acquisition. Quantification was done under an epifluorescence microscope (Olympus BX53) equipped with a monochrome CCD camera (Olympus XM10). We utilized the MIA module of cellSens (Olympus LS) to capture the complete regions of interest on DAPI-stained coronal sections. Acquired images were only subjected to uniform brightness and contrast processing.

2.11. Statistical analysis

The sample size was determined by using GPower (v. 3.1; Faul et al., 2007) to achieve a large effect size ($d = 2$). Paired samples *t*-tests, two-way repeated measures ANOVA, and two-way mixed ANOVA were used to compare groups and test sessions in JASP (v. 0.18.3). Statistical outliers were detected by the iterative Grubbs' method. Two animals that displayed freezing in the center of the maze (7.51 and 13.70 SD away from the group mean) were removed from the OFT analyses. Another animal (9.35 SD away from the group mean) was removed from the OFT locomotor activity analysis. All statistical tests were two-tailed with an alpha level of 0.05. Statistically significant effects were followed by post-hoc analyses with Tukey-corrected multiple comparisons. Graphical data were produced by using GraphPad Prism (v. 10.2) and Inkscape (v. 1.3.2).

3. Results

3.1. Object recognition memory

All animals spent sufficient time with the two identical objects during the familiarization session of NORT (total exploration time: $M = 17.60$, $SD = 12.16$). Overall time spent with the identical objects A1 and A2 did not differ ($t(31) = 0.38$, $p = .71$, Cohen's $d = 0.07$, paired samples *t*-test; Fig. 2A). In the test phase of NORT, the SE ($t(6) = -1.04$, $p = .34$, Cohen's $d = -0.39$, paired samples *t*-test), EE ($t(7) = -0.12$, $p = .91$, Cohen's $d = -0.04$, paired samples *t*-test), and Ket EE animals ($t(7) = 0.98$, $p = .36$, Cohen's $d = 0.35$, paired samples *t*-test) displayed similar exploration time with both objects; while the Ket SE animals spent significantly more time with the novel object ($t(7) = -7.01$, $p < .001$, Cohen's $d = -2.48$, paired samples *t*-test; Fig. 2B). One animal from this group did not exhibit any interaction on the test day, and therefore not included in the comparisons.

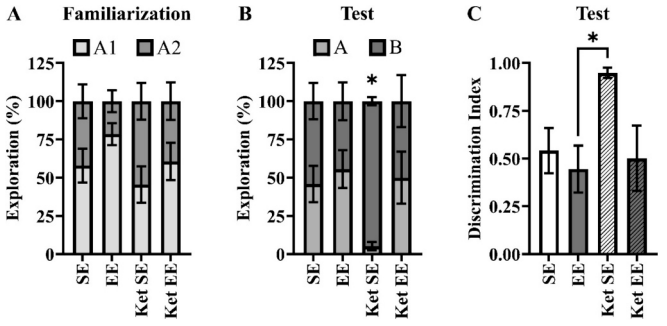

Fig. 2. Object recognition memory performance in the NORT. (A) The percentage of time spent exploring each identical object (A1 and A2) during the familiarization session. (B) The percentage of time spent exploring the familiar (A) and the novel object (B) during the test session. (C) Test session performance as assessed with an object discrimination index [T_n / (T_n + T_f)]. Error bars depict SEM. *$p < .05$.

The effect of housing conditions on the consolidation of object recognition memory was also revealed with a discrimination index ($F(1, 27) = 4.98$, $p = .03$, $\eta^2 = 0.13$, 2 × 2 two-way mixed ANOVA; Fig. 2C). The Ket SE group showed significantly higher discrimination as compared to the EE group ($t(27) = 2.97$, $p = .03$, Cohen's $d = 1.48$, 2 × 2 two-way mixed ANOVA; Fig. 2C). There was no main effect of housing conditions ($F(1, 27) = 0.25$, $p = .62$, $\eta^2 = 0.008$, 2 × 2 two-way mixed ANOVA) or IP injections ($F(1, 27) = 3.36$, $p = .08$, $\eta^2 = 0.11$ 2 × 2 two-way mixed ANOVA; Fig. 2C) in total exploration time.

3.2. Behavioral despair

Behavioral despair analysis revealed a main effect of housing conditions on the immobility scores of FST-1 ($F(1, 28) = 5.13$, $p = .03$, $\eta^2 = 0.15$, 2 × 2 two-way mixed ANOVA), with no group-level difference in Tukey-corrected post-hoc analysis. In FST-2, SE, EE, Ket SE, and Ket EE groups displayed similar levels of immobility, showing no effect of ketamine administration ($F(1, 28) = 3.28$, $p = .08$, $\eta^2 = 0.10$, 2 × 2 two-way mixed ANOVA) or environmental enrichment ($F(1, 28) = 0.26$, $p = .62$, $\eta^2 = 0.008$, 2 × 2 two-way mixed ANOVA; Fig. 3A). The Ket EE group showed a significant decrease in immobility durations between the two FST sessions ($t(7) = 3.03$, $p = .02$, Cohen's $d = 1.07$, paired samples *t*-test; Fig. 3A). Diminished immobility durations were also observed in the other ketamine-receiving group, Ket SE as a non-significant trend; while the inverse pattern, relatively higher freezing in FST-2, was observed in vehicle-receiving groups (Fig. 3A).

Struggling scores in FST-1 revealed a main effect of ketamine ($F(1, 28) = 14.83$, $p < .001$, $\eta^2 = 0.31$, 2 × 2 two-way mixed ANOVA; Fig. 3B), resulting from a significant difference between EE and Ket EE groups ($t(28) = -3.27$, $p = .01$, Cohen's $d = -1.63$, Tukey corrected). Struggling behavior in FST-2 also differed between the groups, as ketamine-receiving animals (Ket SE and Ket EE) displayed higher levels of struggling compared to the SE and EE animals ($F(1, 28) = 31.73$, $p < .001$, $\eta^2 = 0.51$, 2 × 2 two-way mixed ANOVA; Fig. 3B). Ket SE animals struggled more than SE animals ($t(28) = 3.07$, $p = .02$, Cohen's $d = 1.54$, Tukey corrected). Similarly, the Ket EE group showed more struggling behavior than the EE group ($t(28) = 4.89$, $p < .001$, Cohen's $d = 2.45$, Tukey corrected). Furthermore, ketamine administration led to more struggling behavior in FST-2, irrespective of housing conditions (Ket SE: $t(7) = -3.18$, $p = .02$, Cohen's $d = -1.13$; Ket EE: $t(7) = -3.65$, $p = .008$, Cohen's $d = -1.29$, paired samples *t*-tests).

Ketamine-receiving groups displayed significantly less swimming compared to the vehicle groups in FST-1 ($F(1, 28) = 54.67$, $p < .001$, $\eta^2 = 0.66$, 2 × 2 two-way mixed ANOVA; Fig. 3C), as SE animals swam more than Ket SE animals ($t(28) = 4.92$, $p < .001$, Cohen's $d = 2.46$, Tukey corrected), and EE animals swam more than Ket EE animals ($t(28) = 5.53$, $p < .001$, Cohen's $d = 2.77$, Tukey corrected). FST-2 swimming durations were also higher in vehicle groups ($F(1, 28) = 60.32$, $p < .001$, $\eta^2 = 0.66$, 2 × 2 two-way mixed ANOVA; Fig. 3C), as SE animals swam more than Ket SE animals ($t(28) = 4.71$, $p < .001$, Cohen's $d = 2.35$, Tukey corrected), and EE animals swam more than Ket EE animals ($t(28) = 6.28$, $p < .001$, Cohen's $d = 3.14$, Tukey corrected). In line with struggling results, ketamine-receiving groups displayed more swimming in FST-1 compared to FST-2 (Ket SE: $t(7) = 2.36$, $p = .05$, Cohen's $d = 0.83$; Ket EE: $t(7) = 2.49$, $p = .04$, Cohen's $d = 0.88$, paired samples *t*-tests; Fig. 3C).

The total number of headshakes varied across housing conditions in

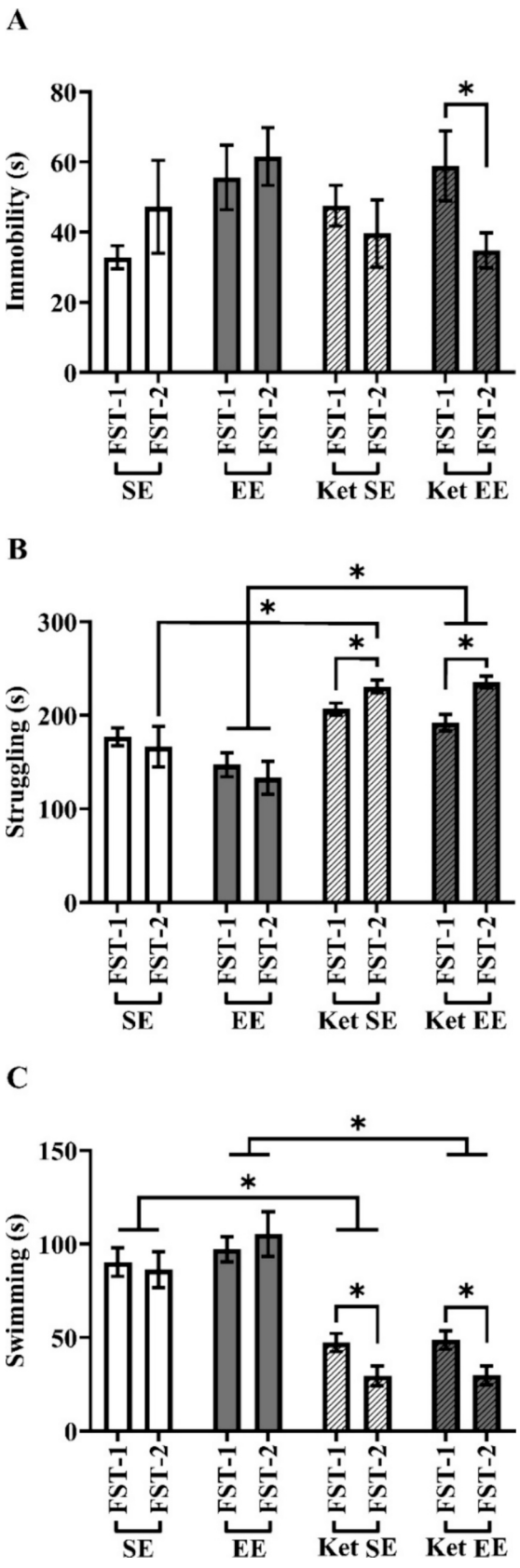

Fig. 3. Forced swim test analyses show periods of (A) immobility, (B) struggling, and (C) swimming exhibited during the first 5 min of FST-1 and FST-2. Error bars depict SEM. *$p < .05$.

FST-2 ($F(1, 28) = 7.09$, $p = .01$, $\eta^2 = 0.19$, 2 × 2 two-way mixed ANOVA). Ket EE animals exhibited more headshakes ($M = 54.75$, $SD = 8.78$) compared to Ket SE animals ($M = 43.63$, $SD = 9.59$) ($t(28) = 2.84$, $p = .04$, Cohen's $d = 1.42$, Tukey corrected). There were no effects of ketamine or enrichment on diving behavior on either day of the FST.

3.3. Locomotor activity and anxiety

The overall duration of locomotion in the OFT did not differ between the groups due to ketamine administration ($F(1, 25) = 0.50$, $p = .49$, $\eta^2 = 0.02$, 2 × 2 two-way mixed ANOVA) or environmental enrichment ($F(1, 25) = 0.64$, $p = .43$, $\eta^2 = 0.02$, 2 × 2 two-way mixed ANOVA; Fig. 4A), indicating that the FST results did not arise due to differences in locomotor activity. Anxiety-like behavior in the OFT was assessed based on the time spent in the center of the maze, which yielded a significant main effect of both ketamine ($F(1, 26) = 74.41$, $p < .001$, $\eta^2 = 0.65$, 2 × 2 two-way mixed ANOVA) and enrichment ($F(1, 26) = 7.20$, $p = .01$, $\eta^2 = 0.06$, 2 × 2 two-way mixed ANOVA; Fig. 4B). Animals from SE spent more time in the center of the maze compared to EE ($t(26) = 3.74$, $p = .005$, Cohen's d = 1.87, Tukey corrected) as well as Ket SE animals ($t(26) = 8.12$, $p < .001$, Cohen's $d = 4.06$, Tukey corrected). Furthermore, the EE group spent more time in the center compared to the Ket EE group ($t(26) = 4.24$, $p = .001$, Cohen's $d = 2.29$, Tukey corrected).

Rearing frequency in the OFT did not change with ketamine treatment ($F(1, 26) = 0.77$, $p = .39$, $\eta^2 = 0.03$, 2 × 2 two-way mixed ANOVA) or housing conditions ($F(1, 26) = 0.04$, $p = .85$, $\eta^2 = 0.001$, 2 × 2 two-way mixed ANOVA). Likewise, there was no group-level difference in boli counts due to ketamine treatment ($F(1, 26) = 3.08$, $p = .09$, $\eta^2 = 0.1$, 2 × 2 two-way mixed ANOVA) or enrichment ($F(1, 26) = 0.04$, $p = .52$, $\eta^2 = 0.01$, 2 × 2 two-way mixed ANOVA).

EPM analysis, a more robust measure of anxiety-like behavior (Gencturk and Unal, 2024), showed no effect of ketamine ($F(1, 28) = 0.38$, $p = .54$, $\eta^2 = 0.01$, 2 × 2 two-way mixed ANOVA) or enrichment on the time spent in open arms ($F(1, 28) = 0.79$, $p = .38$, $\eta^2 = 0.27$, 2 × 2 two-way mixed ANOVA; Fig. 4C). Ketamine administration ($F(1, 28) = 3.61$, $p = .07$, $\eta^2 = 0.11$, 2 × 2 two-way mixed ANOVA) or enrichment ($F(1, 28) = 0$, $p = 1$, $\eta^2 = 0$, 2 × 2 two-way mixed ANOVA) did not alter the number of open arm entries.

3.4. Spatial memory

MWM analyses revealed a significant main effect of the training day on escape latency ($F(3, 84) = 43.23$, $p < .001$, $\eta^2 = 0.36$, 2 × 2 × 4 repeated measures ANOVA; Fig. 5A), indicating successful spatial learning. The SE group displayed significantly lower escape latency on day three ($t(84) = 5.53$, $p < .001$, Cohen's $d = 2.04$, Tukey corrected) and day four ($t(84) = 6.05$, $p < .001$, Cohen's $d = 2.23$, Tukey corrected) compared to the first day. The final day performance of the SE animals

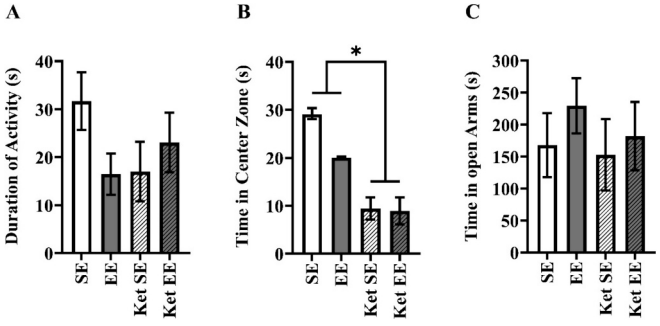

Fig. 4. Locomotor activity and anxiety-like behavior assessed in the OFT and EPM. (A) Locomotor activity in terms of total duration of activity in the OFT. (B) Average time spent in the center of the OFT. (C) Average time spent in the open arms of the EPM. Error bars depict SEM. *$p < .05$.

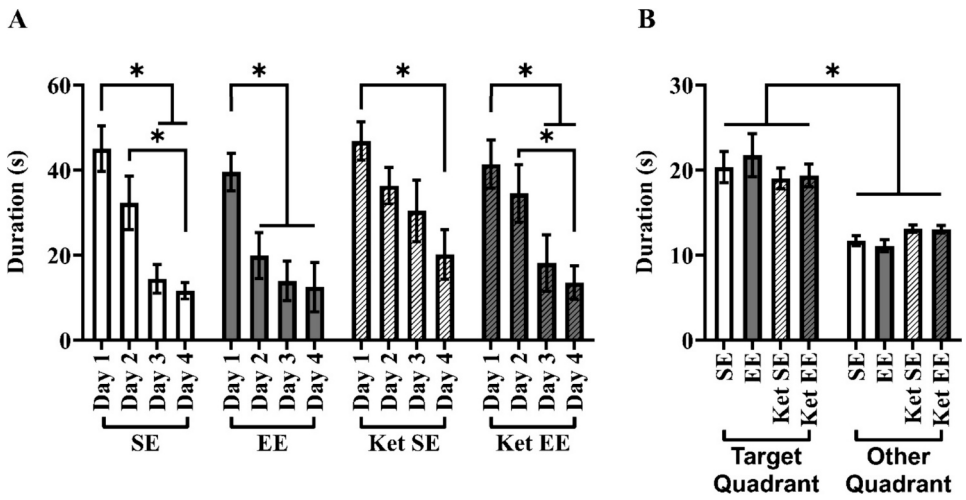

Fig. 5. Spatial memory performance in the MWM. (A) Escape latency scores across the four training days. (B) Overall time spent in the target quadrant vs. the average of other quadrants during the probe trial. Error bars depict SEM. *$p < .05$.

was also better than their second day ($t(84) = 3.73$, $p = .03$, Cohen's $d = 1.38$, Tukey corrected; Fig. 5A). EE animals performed significantly better at locating the platform on the second day in comparison to the first day ($t(84) = 3.57$, $p = .05$, Cohen's $d = 1.31$, Tukey corrected; Fig. 5A). This difference was sustained on the third ($t(84) = 4.65$, $p = .001$, Cohen's $d = 1.71$, Tukey corrected) and fourth day of training ($t(84) = 4.90$, $p < .001$, Cohen's $d = 1.80$, Tukey corrected; Fig. 5A).

Escape latency of the Ket SE animals significantly decreased on the fourth day compared to the first day of training ($t(84) = 4.83$, $p < .001$, Cohen's $d = 1.78$, Tukey corrected; Fig. 5A); while the Ket EE group displayed better memory performance on the third day ($t(84) = 4.21$ $p = .006$, Cohen's $d = 1.55$, Tukey corrected) and the fourth day ($t(84) = 5.04$, $p < .001$, Cohen's $d = 1.86$, Tukey corrected; Fig. 5A) compared to the first day. In addition, similar to the SE group, the final day performance of the Ket EE animals was also better than the second day of training ($t(84) = 3.80$, $p = .02$, Cohen's $d = 1.40$, Tukey corrected; Fig. 5A).

The probe trial of MWM revealed no group-level differences in the time spent in the target quadrant. There was no main effect of ketamine ($F(1, 28) = 1.08$, $p = .31$, $\eta^2 = 0.04$, 2 × 2 two-way mixed ANOVA) or housing conditions ($F(1, 28) = 0.24$, $p = .63$, $\eta^2 = 0.01$, 2 × 2 two-way mixed ANOVA; Fig. 5B). However, as indicative of successful learning, all animals spent significantly more time in the target quadrant ($M = 20.10$, $SD = 4.95$) compared to other quadrants ($M = 12.26$, $SD = 1.73$) ($t(31) = 6.8$, $p < .001$, Cohen's $d = 1.20$, paired samples t-test; Fig. 5B). This finding held true for the SE ($t(7) = 3.61$, $p = .01$, Cohen's $d = 1.27$, paired samples t-test), EE ($t(7) = 3.34$, $p = .01$, Cohen's $d = 1.18$, paired samples t-test), Ket SE ($t(7) = 3.61$, $p = .009$, Cohen's $d = 1.28$, paired samples t-test;), and Ket EE groups ($t(7) = 3.57$, $p = .009$, Cohen's $d = 1.26$, paired samples t-test; Fig. 5B).

3.5. Neuronal activity

Quantification and comparison of c-Fos+ cells were done in the MHb, LHb, PRh, and DLEnt. We observed that ketamine had a significant effect on the number of cFos+ cells in MHb ($F(1, 62) = 9.88$, $p = .003$, $\eta^2 = 0.12$, 2 × 2 two-way mixed ANOVA; Fig. 6), which was also affected by differential housing ($F(1, 62) = 5.59$, $p = .02$, $\eta^2 = 0.07$, 2 × 2 two-way mixed ANOVA; Fig. 6). Higher neuronal activity was observed in the MHb of EE animals compared to SE ($t(62) = 3.16$, $p = .01$, Cohen's $d = 1.07$, Tukey corrected), Ket SE ($t(62) = 3.82$, $p = .002$, Cohen's $d = 1.36$, Tukey corrected), and Ket EE animals ($t(62) = 3.73$, $p = .002$, Cohen's $d = 1.26$, Tukey corrected; Fig. 6). Similarly, antidepressant ketamine administration ($F(1, 64) = 7.98$, $p = .006$, $\eta^2 = 0.1$, 2 × 2 two-way mixed ANOVA) and housing conditions ($F(1, 64) = 6.01$, $p = .02$, $\eta^2 = 0.08$, 2 × 2 two-way mixed ANOVA; Fig. 6) led to neuronal alterations in the number of cFos+ cells in LHb following the probe trial of the MWM. A higher number of cFos+ cells was counted in the LHb of EE animals than Ket SE animals ($t(64) = 3.61$, $p = .003$, Cohen's $d = 1.29$, Tukey corrected; Fig. 6). There was no effect of ketamine ($F(1, 49) = 2.12$, $p = .15$, $\eta^2 = 0.04$, 2 × 2 two-way mixed ANOVA) or enrichment ($F(1, 49) = 1.27$, $p = .27$, $\eta^2 = 0.02$, 2 × 2 two-way mixed ANOVA; Fig. 6) in the number of cFos+ cells in PRh. Similarly, cFos immunoreactivity levels did not differ between the four groups in the DLEnt ($F(1, 41) = 0.4$, $p = .53$, $\eta^2 = 0.01$, 2 × 2 two-way mixed ANOVA for ketamine; $F(1, 41) = 1.79$, $p = .19$, $\eta^2 = 0.04$, 2 × 2 two-way mixed ANOVA for housing; Fig. 6).

4. Discussion

This study examined the effects of environmental enrichment on the cognitive and affective outcomes of an antidepressant dose of ketamine in adult male Wistar rats. Behavioral findings showed that environmental enrichment and ketamine (20 mg/kg, IP) administration led to a synergistic antidepressant effect. Furthermore, enrichment ameliorated the spatial memory deficits of ketamine in the MWM. These effects were accompanied by enhanced neuronal activity in the MHb and LHb regions of the EE group following the probe trial of the MWM. The combination of enrichment with ketamine had no effect on simple object recognition memory or anxiety-like behavior. Ketamine administration enhanced object recognition memory in the standard environment group, while it increased anxiety-like behavior in the OFT irrespective of housing conditions.

We assessed behavioral despair (Unal and Canbeyli, 2019) by comparing the immobility scores of FST-1 and FST-2, following the common procedure for experiments that utilize chronic manipulations (Brenes et al., 2009; Ecevitoglu et al., 2019; Kingir et al., 2023). Albeit previous studies have highlighted the positive impact of environmental enrichment on antidepressant efficacy (Castrén and Hen, 2013; Ramírez-Rodríguez et al., 2021), the synergistic potential of enrichment and ketamine has not been investigated. The combinatorial treatment utilized in this study improved the fast-onset antidepressant properties of ketamine, producing a significant synergistic effect. The analysis of mobility periods in the FST revealed that both of the ketamine-receiving groups exhibited higher levels of struggling and lower periods of swimming compared to groups that did not receive ketamine. Thus, injections of an antidepressant dose of ketamine led to enhanced active coping in the FST (Armario, 2021), switching the major behavioral

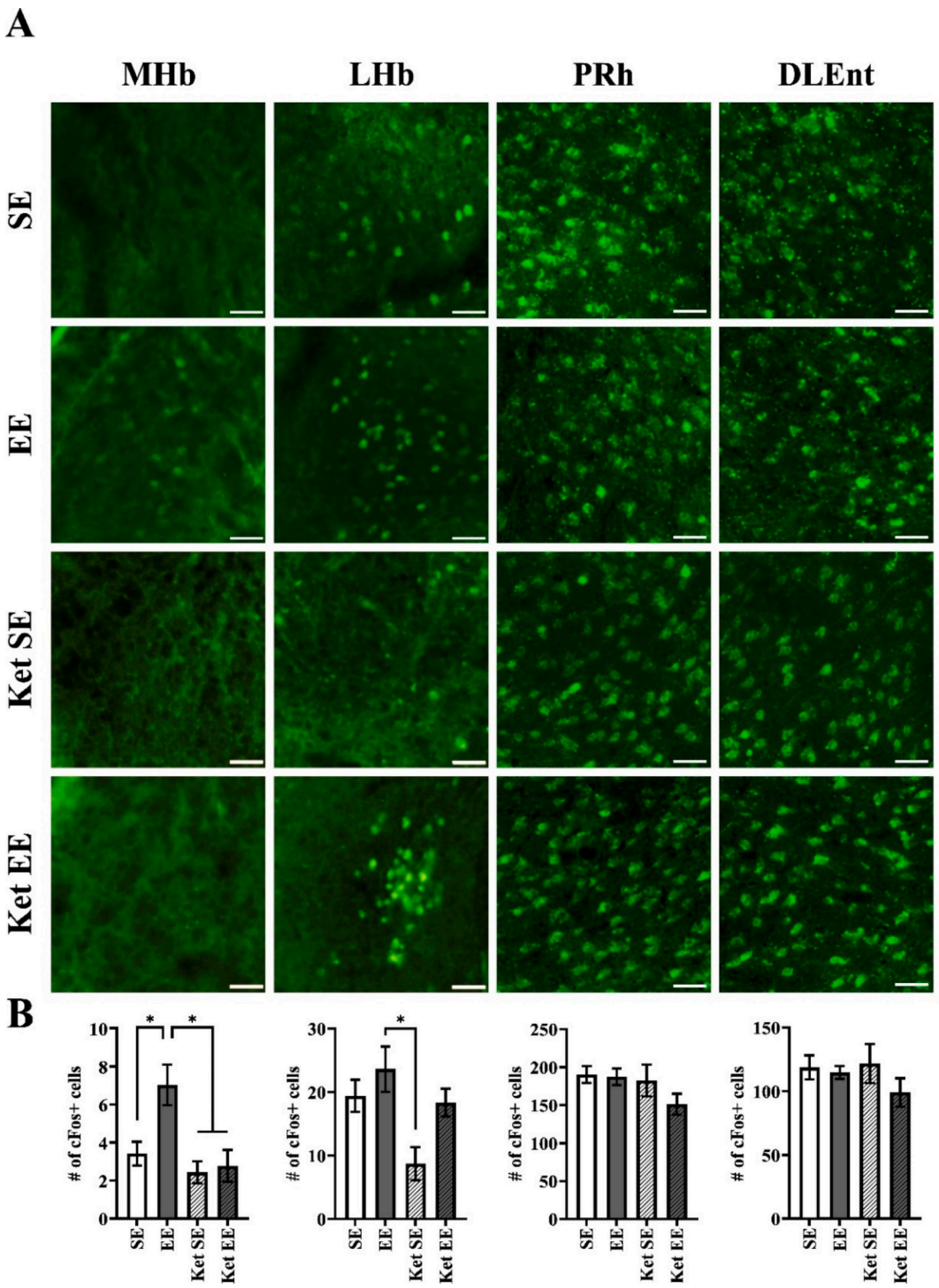

Fig. 6. Immunohistochemistry for c-Fos. (A) Fluorescence images of c-Fos+ cells in the medial habenula (MHb), lateral habenula (LHb), perirhinal cortex (PRh), and dorsolateral entorhinal cortex (DLEnt). Scale bars: 50 μm. (B) The average number of c-Fos+ cells in the corresponding regions of the SE, EE, Ket SE, and Ket EE groups. Error bars depict SEM. *$p < .05$.

strategy from swimming to struggling, irrespective of housing conditions.

The FST is differentially sensitive to different antidepressant drugs and applications (Cryan et al., 2005a; Unal and Canbeyli, 2019), and may trigger distinct coping mechanisms in response to different groups of antidepressants. Selective reuptake inhibitors (SSRIs), which are the most widely prescribed antidepressants, tend to increase swimming (Cryan et al., 2002; Cryan et al., 2005b; Detke et al., 1995). In contrast, antidepressants that enhance catecholaminergic neurotransmission, like the norepinephrine reuptake inhibitor (NRI) reboxetine, increase climbing or struggling behavior in the FST (Page et al., 2003). The atypical antidepressant we used in this study may also follow this pattern. Further evidence comes from in vivo microdialysis studies on the bed nucleus of the stria terminalis (BNST), a region critical for sustained defensive responses to environmental threats (Gungor and Paré, 2016; Ressler et al., 2011). Both reboxetine (Cadeddu et al., 2014) and ketamine (Cadeddu et al., 2016) substantially enhanced dopaminergic and noradrenergic tone in the BNST, which may contribute to the

increased struggling response led by both antidepressants in the FST.

The timing of ketamine administration may have also influenced the outcomes observed in the FST. In drug testing experiments, the antidepressant is typically given after the acclimation phase of the two-day rat FST protocol (FST-1), with immobility scores compared during the test phase (FST-2) (Connor et al., 2000; Page et al., 1999). In long-term behavioral designs, where within-group comparisons can be made between the acclimation and test sessions (Atesyakar et al., 2020), pharmacological interventions are typically administered before FST-1 (Chen et al., 2017; Cryan et al., 2005a; Rygula et al., 2006).

The enrichment procedure in our study did not significantly decrease FST immobility scores on its own. Previous research has shown conflicting results regarding the antidepressant effects of enrichment. Some studies have shown a therapeutic result (Brenes et al., 2008; Porsolt et al., 1978a), while others have failed to replicate this effect (Cui et al., 2006; Simpson et al., 2012). These inconsistencies could be attributed to variations in the duration of exposure to EE, as well as differences in the strain, age, and sex of the animals (Bogdanova et al., 2013). However, our study focused exclusively on male rats, limiting our ability to explore potential sex differences in behavior or neuronal responses. Earlier studies have reported various sex-specific variations in behavioral tests, neural plasticity, and hormonal levels in response to EE (Simpson and Kelly, 2011). For instance, while EE thickens the occipital cortex in male rats, it increases the somatosensory cortex thickness only in females (Diamond, 2001). Additionally, EE affects stress hormones in a sex-specific manner, resulting in different adrenocorticotrophic hormone levels (Bakos et al., 2009), which could influence stress response in the FST and MWM.

In spatial memory, no group-level effect was observed in the probe trial of MWM, indicating that the second dose of ketamine administered 24 h before the first MWM trial did not block spatial learning. However, IP ketamine injections led to significant differences in the pace of learning across the training days. Animals housed in a standard environment and received ketamine located the escape platform significantly faster on the last day, exhibiting the slowest rate of learning, likely resulting from the disruptive effects of ketamine on spatial memory (Moosavi et al., 2012; Pitsikas et al., 2008; Pitsikas and Boultadakis, 2009). The EE group, in contrast, was able the locate the platform on the second day, showing that enrichment had a positive effect on the acquisition rate of spatial memory. We found that environmental enrichment ameliorated the ketamine-led impairment in spatial memory, as the Ket EE animals significantly decreased their escape latency on the third day of MWM, similar to the SE animals. In short, the EE group displayed the best performance, which was followed by the SE and Ket EE groups, while the Ket SE group exhibited the slowest rate of learning.

The ex vivo immunohistochemistry for c-Fos was conducted following the probe trial of MWM. The four groups did not differ in their probe trial performance as well as the number of c-Fos+ cells in the dorsolateral entorhinal cortex, a key region for spatial memory (Steffenach et al., 2005). The habenula was also implicated in spatial memory as habenular lesions impair performance in the MWM (Lecourtier et al., 2004). In the present study, c-Fos+ cell quantification revealed substantially higher neuronal activity in the medial and lateral habenula of EE animals. This difference may have emerged due to the stress-inducing nature of the MWM, which activates not only spatial memory-related regions (Vorhees and Williams, 2006), but also stress-related limbic circuits (Mondoloni et al., 2022; Schulz et al., 2004, 2007). The medial habenula is often associated with aversive memories (Soria-Gómez et al., 2015) and functions as a center for regulating aversion (Boulos et al., 2020; McLaughlin et al., 2017). The lateral habenula is a phylogenetically conserved structure implicated in reward omission, and learning under aversive conditions (Hikosaka, 2010; Sosa et al., 2021). Lateral habenula neurons are activated by environmental stressors (Kingir et al., 2023; Lei et al., 2020; Park et al., 2017) and correlate with depressive-like behavior (Li et al., 2011; Nair et al., 2013). In line with these findings, the MWM used in this study constitutes an aversive condition due to its water-based nature, while removal of the escape platform during the probe trial constitutes reward omission. The increased habenular activation observed in the EE animals may have resulted from enhanced reward (i.e. escape platform) anticipation of this group, which exhibited the fastest rate of spatial learning. Having acquired the location of the escape platform earlier during training may have led to higher reward expectancy in the probe trial.

The combination of enrichment and ketamine administration did not affect simple object recognition memory. However, ketamine administration alone (Ket SE) enhanced the performance in the test session of the NORT, a paradigm that relies on innate exploratory behavior to assess object recognition (Ennaceur et al., 2006). A similar finding was reported with lower doses of ketamine (10 mg/kg) in mice (Fan et al., 2021). Interestingly, however, an earlier study reported impaired object recognition memory following a single IP injection at 20 mg/kg in 3-month-old Wistar rats housed under standard conditions (Goulart et al., 2010). The disparity between our observations and this study may have resulted from differences in the age of animals or inherited dissimilarities between the rat colonies. The positive effect of ketamine on object recognition memory was lost when combined with environmental enrichment. It is possible that stimulus salience in the NORT may not have been sufficient to capture the attention of the enriched animals, which had been consistently exposed to novelty for 40 days. Likewise, environmental enrichment may attenuate novelty exploration by altering habituation to novelty as suggested previously (Zimmermann et al., 2001). Both social enrichment (Elliott and Grunberg, 2005) and physical enrichment (Schrijver et al., 2002) have been associated with faster habituation to novelty, which may have obstructed the enhancing effect of ketamine in the test phase. Furthermore, the quantification of c-Fos expression levels in the PRh, a cortical region crucial for object recognition memory (Aggleton et al., 2010; Mumby et al., 2002; Unal et al., 2012) showed no significant differences between the groups. Although the ex vivo immunohistochemistry was conducted several days after the NORT, the probe trial of the MWM may have differentially recruited perirhinal circuits, as observed previously (Shires and Aggleton, 2008).

Anxiety-like behaviors were assessed with the OFT and EPM. Ketamine administration caused an anxiogenic effect in the OFT, in which both the Ket SE and Ket EE animals spent less time in the center of the maze compared to the vehicle-receiving groups. This anxiogenic effect was not observed in the EPM conducted 24 h after the OFT. It must be noted that the anxiety-like measure of the OFT and EPM, a more common rodent test of anxiety (Gencturk and Unal, 2024), do not necessarily correlate (Belzung and Le Pape, 1994; Gokalp and Unal, 2024). Enhanced thigmotaxis displayed by ketamine-receiving groups in the OFT was not affected by environmental enrichment.

5. Conclusion

The combination of environmental enrichment and ketamine led to a synergistic antidepressant effect and ameliorated the spatial memory deficits of ketamine. The positive impact of heightened sensory stimulation and increased motor activity on affective states extends beyond environmental enrichment (Brenes Sáenz et al., 2006). The therapeutic potential of sensory-motor stimulation has been observed in light therapy (Ashkenazy et al., 2009) and voluntary wheel running in animal models (Greenwood and Fleshner, 2008). Similarly, exercise (Blumenthal et al., 2007), bright-light therapy (Pail et al., 2011), and their combination (Leppämäki et al., 2004) produced antidepressant effects in humans. On the other hand, alterations between different environmental conditions, such as switching from social isolation to environmental enrichment, have been associated with depressive effects (Guven et al., 2022). These findings support a sensory-motor understanding of clinical depression and antidepressant activity, emphasizing a bottom-up approach, as opposed to a cognition-driven top-down approach (Canbeyli, 2010, 2013, 2022; Curlik 2nd and Shors, 2013; Eyre and

Baune, 2012). The present study demonstrated how environmental enrichment, a potent sensory-motor manipulation, can synergize with ketamine to produce antidepressant and procognitive effects, highlighting the potential for combining environmental and pharmacological interventions in treating mood disorders.

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CRediT authorship contribution statement

Deren Aykan: Writing – original draft, Investigation, Conceptualization. **Mert Genc:** Writing – original draft, Investigation, Conceptualization. **Gunes Unal:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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