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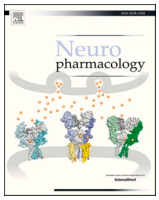

Antidepressant ketamine via oral gavage impairs fear memory, suppresses 22 kHz ultrasonic vocalizations, lowers GluN2A/B expression, and reduces medial habenula activity in rats

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ABSTRACT

Ketamine, an N-methyl-D-aspartate receptor (NMDAR) antagonist known for its rapid-acting antidepressant properties, has been extensively studied through intravenous and intraperitoneal routes. However, despite its growing use in pharmacotherapy, research on oral administration of ketamine remains limited. This study aims to investigate the behavioral and neural effects of fixed-dose oral ketamine delivered via gavage. Adult male Wistar rats were assigned to four groups receiving either saline (vehicle) or ketamine at 15 mg/kg, 30 mg/kg, or 45 mg/kg doses. Animals were tested in the forced swim test (FST), open field test, cued fear conditioning, and elevated plus maze (EPM). Three doses of ketamine were administered via oral gavage every two days, each given 30 min before the FST, fear extinction, and EPM. ELISA was used to measure expression levels of the NMDAR subunit GRIN1 (GluN1), while immunohistochemistry was used for the GluN2A and GluN2B subunits as well as c-Fos. 45 mg/kg ketamine reduced immobility in the FST, transiently impaired fear memory retrieval and reduced ultrasonic vocalizations during Extinction 1. GRIN1 levels were reduced in the hypothalamus for all doses, but increased in the thalamus for higher doses. The antidepressant-like dose decreased the number of GluN2A and GluN2B expressing neurons in the paraventricular nucleus of the thalamus, basolateral amygdala, and habenula. High dose groups also showed diminished c-Fos + cells in the medial habenula following acute stress. These results suggest that 45 mg/kg ketamine via oral gavage produces antidepressant-like effects, through regulation of NMDAR subunits in several depression-related structures like the medial habenula.

1. Introduction

Ketamine, initially synthesized from phencyclidine as an anesthetic (Maddox et al., 1965), has since proven useful as an analgesic (Grathwohl, 2011; Persson, 2013), sedative (Krystal, 1994), and antidepressant (Sofia and Harakal, 1975). When administered intravenously in sub-anesthetic doses (0.1–0.5 mg/kg), it provides rapid relief from depressive symptoms, often within minutes, offering a valuable option for treatment-resistant patients (Ballard and Zarate, 2020; Zarate et al., 2006). The route of administration plays a pivotal role in ketamine's bioavailability and pharmacological effects, with clinical and preclinical studies utilizing diverse methods, including intravenous (i.v.) (Clements et al., 1982; Malinovsky et al., 1996; Chong et al., 2009; Fanta et al., 2015; Fava et al., 2020; Spencer et al., 2022), intraperitoneal (i.p.) (Aykan et al., 2024; Gokalp and Unal, 2024), intramuscular (Grant et al., 1981; Clements et al., 1982; Beaglehole et al., 2025), transdermal (Akan et al., 2023), oral (Grant et al., 1981; Clements et al., 1982; Bitter, 2011; Andrade, 2019; Domany et al., 2019; Ecevitoglu et al., 2019; Kingir et al., 2023), and intranasal delivery (Malinovsky et al., 1996; Yanagihara et al., 2003; Bitter, 2011; Fu et al., 2020; Halpape et al., 2025). Among these, oral ketamine has gained traction in clinical trials, promising broader accessibility (Glue et al., 2024). Yet fixed-dose oral administration remains underexplored in animal models, largely due to methodological challenges such as variable absorption and metabolism (Lou et al., 2023). Previous work from our group has indicated that low-dose oral ketamine, when administered ad libitum, may produce antidepressant effects (Ecevitoglu et al., 2019), prevent anhedonia under chronic stress, and modulate reward-circuit activation (Kingir et al., 2023). However, voluntary oral consumption of ketamine does not allow for precise control over dosage, making it difficult to reliably assess receptor-level alterations induced by ketamine. In the present study, we addressed this limitation by using oral gavage, enabling a

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comprehensive assessment of ketamine's effects on behavioral despair, locomotor activity, fear memory, anxiety-like behavior, and fear-induced ultrasonic vocalizations (USVs), as well as its impact on NMDAR subunit levels and stress-induced neuronal activity in depression-related regions.

Oral ketamine displays distinct pharmacokinetic characteristics with notably low bioavailability (17–29 %) (Grant et al., 1981; Clements et al., 1982; Ganguly et al., 2018) due to extensive hepatic first-pass metabolism. This metabolic process generates active metabolites, most notably norketamine and hydroxynorketamine (Ganguly et al., 2018; Kharasch and Labroo, 1992; Peltoniemi et al., 2012), that reach plasma levels comparable to the parent compound and exert antidepressant-like effects, albeit with reduced potency (Sałat et al., 2015; Yokoyama et al., 2020). Ketamine's therapeutic mechanism involves not only NMDARs but also multiple other receptors and downstream signaling pathways (Zanos et al., 2016). We have previously shown that the antidepressant-like effects of ketamine depend on metabotropic glutamate receptor 5 (mGluR5) activity (Gokalp and Unal, 2024). Additional studies have implicated downstream signaling cascades, including the BDNF/VGF/GluA1 axis (Teng et al., 2024; Wang et al., 2025) and the PI3K/AKT pathway (Liu et al., 2024). Ketamine also binds to and activates μ-opioid receptors (MORs) (Bonaventura et al., 2021), and blockade of these receptors with naltrexone has been shown to attenuate ketamine's antidepressant and antisuicidal effects in clinical studies (Williams et al., 2018, 2019). In mice, naltrexone similarly inhibits several behavioral effects of ketamine, including locomotor activation, analgesia, and antidepressant-like responses in the FST (Pomrenze et al., 2025). The interaction between NMDARs and MORs appears to be influenced by the enantiomeric form of ketamine (Levinstein et al., 2023, 2025), and involves independent signaling pathways (Rodríguez-Muñoz et al., 2012). Together, these findings underscore the complex, multifaceted nature of ketamine's mechanism of action, which integrates both glutamatergic and non-glutamatergic systems.

While ketamine's therapeutic efficacy involves multiple receptors and downstream pathways, its non-competitive antagonism of NMDAR (Anis et al., 1983; MacDonald et al., 1987) is considered a basic mechanisms in many models. Its rapid antidepressant action has been linked to the blockade of NMDAR-mediated glutamatergic transmission in GABAergic interneurons (Gerhard et al., 2020; Stone et al., 2012), particularly within reward-related brain regions (Kingir et al., 2023). The receptor's subunit composition contributes to these therapeutic effects: GluN1/GRIN1, the obligatory subunit of the tetrameric NMDAR, is essential for receptor function and contains the binding site for glycine and serine (Paoletti et al., 2013; Beaurain et al., 2024). Among the glutamate-binding subunits, GluN2B has been most consistently implicated in mediating ketamine's antidepressant actions (Li et al., 2010; Miller et al., 2014; Poleszak et al., 2013).

To examine the relationship between ketamine's pharmacological profile and NMDAR-related plasticity, we administered three oral doses (15, 30, and 45 mg/kg) via gavage. These doses were chosen based on ketamine's reported oral bioavailability in rodents (17–29 %, see Grant et al., 1981; Clements et al., 1982; Ganguly et al., 2018), aiming to approximate the effective intraperitoneal dose range typically used in behavioral studies (10–30 mg/kg, i.p.). This pharmacokinetic rationale allowed us to examine dose-dependent effects through a clinically relevant and translationally appropriate route of administration. The animals were tested in the forced swim test (FST), open field test (OFT), cued fear conditioning, and elevated plus maze (EPM). In a subset of animals, GRIN1 subunit levels were quantified in the hypothalamus, thalamus, and basolateral amygdala using ELISA. Following behavioral testing, we examined GluN2A, GluN2B, and stress-induced c-Fos expression in the paraventricular nucleus of the hypothalamus (PVN), paraventricular nucleus of the thalamus (PVT), basolateral amygdala (BLA), medial habenula (MHb), and lateral habenula (LHb) via immunohistochemistry.

2. Experimental procedures

2.1. Subjects

Forty-eight experimentally naïve adult male Wistar rats (3–5 months old; M = 324.4 g, SD = 25.98 g) were used. Animals were housed in groups of four under controlled conditions (21 ± 1 °C; 40–60 % humidity; 12:12 h light/dark cycle, lights on at 08:00) (Aykan et al., 2024), with ad libitum access to food and water. Rats were randomly assigned to one of four experimental groups based on the dose of ketamine administered (Keta-control, Doğa İlaç, 100 mg/ml, Istanbul, Turkey; diluted in 0.9 % saline): low dose (Ket15: 15 mg/kg, n = 12), medium dose (Ket30: 30 mg/kg, n = 12), high dose (Ket45: 45 mg/kg, n = 12), or vehicle (saline, n = 12). All procedures were approved by the Boğaziçi University Ethics Committee for the Use of Animals in Experiments (Protocol # 2024-02). As this is the first study to examine oral gavage administration of ketamine in rats for behavioral despair, only male animals were used to facilitate comparison with prior rodent studies using other administration routes, the vast majority of which employed males (19 of 20 studies; Choi et al., 2020).

2.2. Experimental design

All underwent a three-day acclimatization period with daily handling to minimize stress from oral gavage. The acute experiment spanned six days, with ketamine or saline administered via oral gavage on Days 2, 4, and 6, each 30 min before behavioral testing: the test session of the FST, the first fear extinction session, and the EPM (Fig. 1). The FST was followed by the OFT, conducted 50 min after the first ketamine administration. At this point, 4 animals per group were sacrificed by decapitation for ELISA analysis, while the remaining 8 animals completed the behavioral protocol. Cued fear conditioning was conducted on Days 3–5, with acquisition and extinction sessions performed in distinct contexts. Behavioral testing concluded with the EPM, followed by 30 min of restraint stress in plastic tubes. Ninety minutes after stress exposure, animals were perfused for c-Fos immunohistochemistry. All tests were conducted between 9:00 and 19:00 in the same behavioral room, and data were analyzed using EthoVision XT 17 (Noldus, Wageningen, Netherlands) by experimenters blind to group assignments.

2.3. Forced swim test

A two-day rat FST protocol was used to assess behavioral despair, consisting of a 15-min initial session (FST1) followed by a test session on the next day (FST2), as previously described (Guven et al., 2022; Gokalp and Unal, 2024). The test was conducted in a transparent acrylic cylinder (height = 45 cm, radius = 15 cm) filled with 30 cm of tap water maintained at 23 ± 1 °C. The first 5 min of both FST1 and FST2 were analyzed for immobility, struggling, and swimming behaviors.

2.4. Open field test

We used an opaque, square-shaped wooden apparatus (70 × 70 × 45 cm) with sidewalls to assess locomotor activity. The box was divided into two defined zones: a brightly lit central area (45 × 45 cm, 133 ± 5 lx) and a dimly lit peripheral area (55 ± 5 lx). The apparatus remained in the same position for all animals. Following the FST sessions, animals were placed in the center of the arena and allowed to explore freely for 5 min. Total distance traveled and time spent in the center were recorded. To eliminate residual olfactory cues, the surface and walls of the apparatus were cleaned with 70 % ethanol between each test.

2.5. Auditory cued fear conditioning

We employed a modified version of auditory cued fear conditioning adapted from Totty et al. (2023), pairing an auditory cue (CS) with a

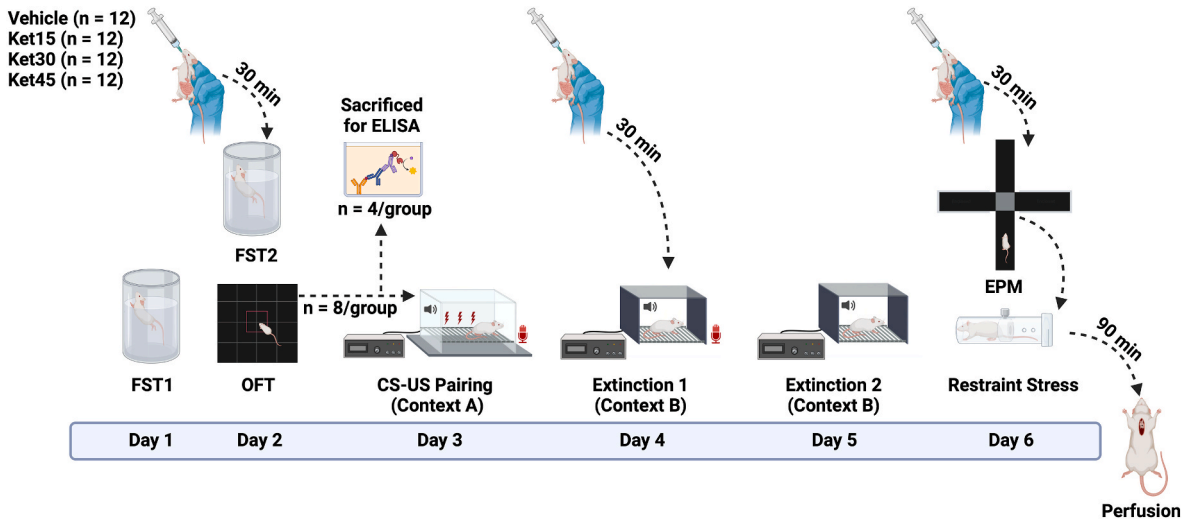

Fig. 1. Schematic overview of the experimental timeline, depicting the sequence of behavioral tests and ketamine administration via oral gavage. FST, forced swim test; OFT, open field test; CS, conditioned stimulus; US, unconditioned stimulus; EPM, elevated plus maze. USV recordings are depicted by a microphone symbol.

mild foot shock (US) in a specific context, followed by two days of extinction training in a different context. Ketamine was administered prior to the initial conditioning session. On the conditioning day (Acquisition), animals were placed in Context A (21 × 45 × 27 cm; floor: 32 parallel metal bars spaced 1.4 cm apart; walls and lid: transparent dark grey acrylic) and, after a 3-min baseline, were presented with five CS–US pairings (CS: 75 kHz tone, 10 s; US: foot shock, 1.02 mA, 2 s, co-terminating with CS). Shocks were delivered with randomized intertrial intervals (ITIs) of 50–70 s, and a 1-min post-shock recording followed the final trial. On Extinction Day 1, the context was changed to a customized top-open chamber (Context B, 41 × 43 × 31 cm; transparent acrylic walls with black opaque floor), and animals received their assigned ketamine or saline injections prior to placement in the chamber. After a 3-min baseline, animals were exposed to forty CS presentations with randomized ITIs of 20–40 s, followed by a 30-s post-session recording. Extinction Day 2 followed the same procedure as Extinction Day 1, without any drug administration.

2.6. Ultrasonic vocalization recordings

USVs were recorded during fear acquisition and the first extinction session to detect stress-induced alarm calls. Vocalizations were captured using a CM16/CMPA condenser ultrasound microphone (20 Hz–460 kHz). The microphone was mounted on a tripod approximately 15 cm above the recording chamber (Context A or B) and angled downward at approximately 35°, directed toward the center of the chamber. Data acquisition was performed with a digital recorder (Model #116H, Avisoft Bioacoustics) at a sampling rate of 50–1000 kHz. Analysis focused on 22 kHz distress calls (Brudzynski, 2015) using DeepSqueak (Coffey et al., 2019) to assess call duration, frequency, and total count, including only calls with a minimum detection score of 0.525 and a maximum frequency shift of 1 kHz.

We used the Long Rat Detector YOLO R1 pretrained neural network for USV analyses. The score parameters provided by DeepSqueak served as a quantitative measure for call-to-noise discrimination. An empirically determined threshold of 0.525 was applied, which consistently ensured accurate call detection across different recordings and settings (Context A or B). Each detection file generated by the network was manually reviewed on a per-call basis, with calls either accepted or rejected accordingly. Missed calls were manually selected and assigned a score of 1 automatically by the program. Adjusted detection files were saved separately and exported as CSV files. During the manual review, bounding boxes were adjusted to match the exact length of each observed call. Thus, both under- and overestimations in automated detections were corrected individually for each call. This review was performed by an observer blinded to the experimental group of the recorded animal.

2.7. Elevated plus maze

The apparatus consisted of a black, plus-shaped wooden maze elevated 61.5 cm above the floor, with two open and two closed arms (each 50 cm long and 10 cm wide). Open arms were illuminated at 90 ± 10 lx, while closed arms had lower light intensity (33.7 ± 5 lx). Following OFT principles, open arms indicated anxiolytic behavior, whereas preference for closed arms reflected anxiety-like avoidance (Gencturk and Unal, 2024). Each animal was placed at the maze center and allowed to explore freely for 5 min. Behavior was assessed by the number of entries into and total time spent in open and closed arms. An open arm entry was counted when more than half the animal's body, including the center, extended beyond the boundary of a closed arm. The maze was cleaned with 70 % ethanol between animals to minimize olfactory cues.

2.8. ELISA

To quantify expression levels of the NMDA receptor subunit 1 (GluN1), an ELISA assay was performed using the Rat Glutamate [NMDA] Receptor Subunit Zeta-1 (GRIN1) kit (E1005Ra, Bioassay Technology Laboratory). Fresh brain tissues were collected immediately following decapitation, and specific regions, including the entire thalamus, hypothalamus, and basolateral amygdala, were isolated as previously described (Jia et al., 2012; Salehi, 2013). The tissues were homogenized in ice-cold phosphate-buffered saline (PBS) at a ratio of 9 ml per gram of tissue. To ensure complete cellular disruption, samples underwent multiple freeze-thaw cycles followed by vortexing. The homogenates were then centrifuged at 4 °C for 5 min at 5000×*g*, and the resulting supernatants were collected and stored at −80 °C until analysis. Following the manufacturer's protocol, tissue samples and standards were sequentially processed through biotinylated antibody binding, Streptavidin-HRP incubation, wash steps, and enzymatic reactions. Absorbance was measured at 450 nm using a microplate reader, with the standard curve detection range set between 3.75 and 240 ng/ml. Sample concentrations were determined by plotting absorbance values against the standard curve.

2.9. Restraint stress

Rats were physically restrained in ventilated polypropylene conical tubes (100 cm length) for 30 min. Each animal's tail extended outside the tube, and the lid was adjusted to securely immobilize without causing harm, based on the animal's size and weight. Tubes were thoroughly cleaned with 70 % ethanol after each session to eliminate residual olfactory cues.

2.10. Tissue preparation and immunohistochemistry

Rats were anesthetized with urethane (2 mL/kg, i.p.; U-2500, Sigma) and transcardially perfused with 0.9 % saline followed by 4 % paraformaldehyde (PFA), 90 min after exposure to restraint stress. Brains were extracted and post-fixed in 4 % PFA at 4 °C for 24 h, then rinsed in 1 × PBS and stored in PBS containing 0.01 % sodium azide (PBS-azide) at 4 °C until further processing. Coronal sections (50 μm) were cut using a vibratome (VT1000 S, Leica Biosystems, Nussloch, Germany) and preserved in PBS-azide solution.

We carried out immunohistochemistry for GluN2A (N327/95, DSHB; 1:50), GluN2B (N59/36, DSHB; 1:50) and c-Fos (sc-52, Santa Cruz; 1:500). Briefly, brain sections were washed three times in PBS containing 0.1 % Triton X-100 (PBS-Tx), then incubated for 1 h at room temperature (RT) in blocking solution: 10 % normal goat serum (NGS; Vector Laboratories) for GluN2A and GluN2B, or 10 % normal horse serum (NHS; Vector Laboratories) for c-Fos, prepared in PBS-Tx. Sections were subsequently incubated with their respective primary antibodies for 48 h at 4 °C. After washing, sections were incubated with secondary antibodies, either goat anti-mouse Alexa Fluor 488 (AB150117; 1:250 in 1 % NGS PBS-Tx) or donkey anti-rabbit Alexa Fluor 488 (AB150073; 1:250 in 1 % NHS PBS-Tx), for 3 h at RT. Finally, sections were mounted using a DAPI-containing mounting medium.

2.11. Microscopy and cell counting

Two rostrocaudal levels were selected for microscopic observation and cell quantification. One set of sections included the PVN (Bregma −2.04 to −2.16), while the second set encompassed the PVT, anterior BLA, MHb, and LHb (Bregma −2.04 to −2.28) (Paxinos and Watson, 2006). Slides were imaged using an epifluorescence microscope (Olympus BX43) equipped with a monochrome CCD camera (Olympus XM10). All images were acquired at 10 × or 20 × magnification using consistent exposure settings and contrast adjustments. For GluN2A and GluN2B immunolabelling, 12 data points were collected from two groups per target region. For c-Fos immunolabelling, 24 data points were obtained from all groups, using three animals per group. Regions of interest were visually identified based on neuroanatomical landmarks, and images were cropped accordingly (mean surface areas: PVN, 0.2599 mm^2; PVT, 0.2737 mm^2; BLA, 0.6676 mm^2; MHb, 0.1182 mm^2; LHb, 0.1324 mm^2). Automated quantification of c-Fos-immunopositive cells was performed using Cellpose 3.0 with the Cyto3 model, optimized for each region (Stringer et al., 2021). All automated counts were manually verified for accuracy.

2.12. Statistical analysis

All data were analyzed using GraphPad Prism version 10.4.1. Normality was assessed separately for each group using the Shapiro–Wilk test, and homogeneity of variances was evaluated by visual inspection of residual plots. One-way ANOVA was used to assess the effects of drug treatment on the FST, OFT, EPM, USV recordings, ELISA results, and neuronal activity measures. Cued fear conditioning data were analyzed using two-way repeated measures ANOVA to evaluate the main effects of drug treatment, cue exposure, and their interaction. Differences in GluN2A and GluN2B expression levels between vehicle and Ket45 groups were analyzed using independent t-tests. Statistical outliers, defined as values exceeding 2.5 standard deviations from the group mean in behavioral tests, were excluded from analysis. Group-wise post hoc comparisons were performed using Tukey's test, and Bonferroni correction was applied for freezing rate comparisons between Extinction 1 and Extinction 2. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Behavioral despair

FST1, which was conducted before drug administration, showed no differences were among the four groups in immobility, struggling, or swimming (all p values > 0.05, one-way ANOVAs). On the test day, however, ketamine treatment significantly reduced immobility time ($F(3, 42) = 4.311$, $p = 0.010$, $R^2 = 0.235$, one-way ANOVA) in Ket45 group ($q = 5.082$, $p = 0.005$, Cohen's $d = 1.550$, Tukey's corrected; $M = 9.577$, $SD = 11.280$), compared to the vehicle group ($M = 28.21$, $SD = 12.71$), indicating an antidepressant-like effect. No significant differences were observed between vehicle and Ket15 ($q = 2.697$, $p = 0.241$, Cohen's $d = 0.681$, Tukey's corrected; $M = 18.32$, $SD = 16.07$) or Ket30 groups ($q = 2.402$, $p = 0.337$, Cohen's $d = 0.834$, Tukey's corrected; $M = 18.97$, $SD = 9.172$; Fig. 2A).

There was no effect of ketamine on struggling in FST2 ($F(3, 43) = 0.236$, $p = 0.871$, $R^2 = 0.016$, one-way ANOVA) for Ket15 ($q = 1.140$, $p = 0.851$, Cohen's $d = 0.418$, Tukey's corrected; $M = 126.3$, $SD = 25.30$), Ket30 ($q = 0.855$, $p = 0.930$, Cohen's $d = 0.226$, Tukey's corrected; $M = 123.0$, $SD = 47.06$), and Ket45 ($q = 0.643$, $p = 0.968$, Cohen's $d = 0.189$, Tukey's corrected; $M = 120.7$, $SD = 39.43$) groups compared to the vehicle ($M = 113.7$, $SD = 34.32$; Fig. 2B). Likewise, no effect of ketamine treatment was observed on FST2 swimming time ($F(3, 44) = 1.038$, $p = 0.385$, $R^2 = 0.066$, one-way ANOVA; Fig. 2C).

3.2. Locomotor activity

Ketamine treatment did not influence distance traveled in the OFT ($F(3, 42) = 1.210$, $p = 0.318$, $R^2 = 0.080$, one-way ANOVA): Ket15 ($q = 0.923$, $p = 0.914$, Cohen's $d = 0.373$, Tukey's corrected; $M = 61.39$, $SD = 31.25$), Ket30 ($q = 1.035$, $p = 0.884$, Cohen's $d = 0.340$, Tukey's corrected; $M = 97.50$, $SD = 57.58$), and Ket45 ($q = 1.486$, $p = 0.721$, Cohen's $d = 0.367$, Tukey's corrected; $M = 105.3$, $SD = 88.17$) groups showed comparable activity compared to the vehicle ($M = 78.23$, $SD = 55.59$; Fig. 2D), ensuring that the results of the FST are not due to varied locomotor activity levels between the groups. Additionally, no significant effect of ketamine treatment was observed on time spent in the center of the maze ($F(3, 44) = 0.389$, $p = 0.762$, $R^2 = 0.026$, one-way ANOVA).

3.3. Fear memory

Repeated CS presentation increased freezing during fear acquisition for all groups ($F(4, 112) = 32.19$, $p < 0.0001$, $\eta 2 = 0.367$, two-way repeated measures ANOVA). Freezing rates significantly increased following the first CS-US pairing, indicating successful fear acquisition, yet there was no significant effect of treatment ($F(3, 28) = 0.444$, $p = 0.724$, $\eta 2 = 0.013$, two-way repeated measures ANOVA) or group-cue interaction ($F(12, 112) = 1.032$, $p = 0.425$, $\eta 2 = 0.035$; Fig. 3A).

The first extinction session revealed a significant main effect of the CS ($F(7, 196) = 8.349$, $p < 0.0001$, $\eta 2 = 0.100$, two-way repeated measures ANOVA) as well as a group-cue interaction ($F(21,196) = 1.906$, $p = 0.012$, $\eta 2 = 0.069$, two-way repeated measures ANOVA). While ketamine treatment did not have a main effect on its own ($F(3, 28) = 1.877$, $p = 0.156$, $\eta 2 = 0.083$, two-way repeated measures ANOVA), Tukey's post hoc comparisons demonstrated that Ket45 group froze significantly less than both the Ket15 ($q = 3.715$, $p = 0.045$, $M = 69.161$, $SD = 29.097$) and vehicle groups ($q = 5.005$, $p = 0.003$, $M =$

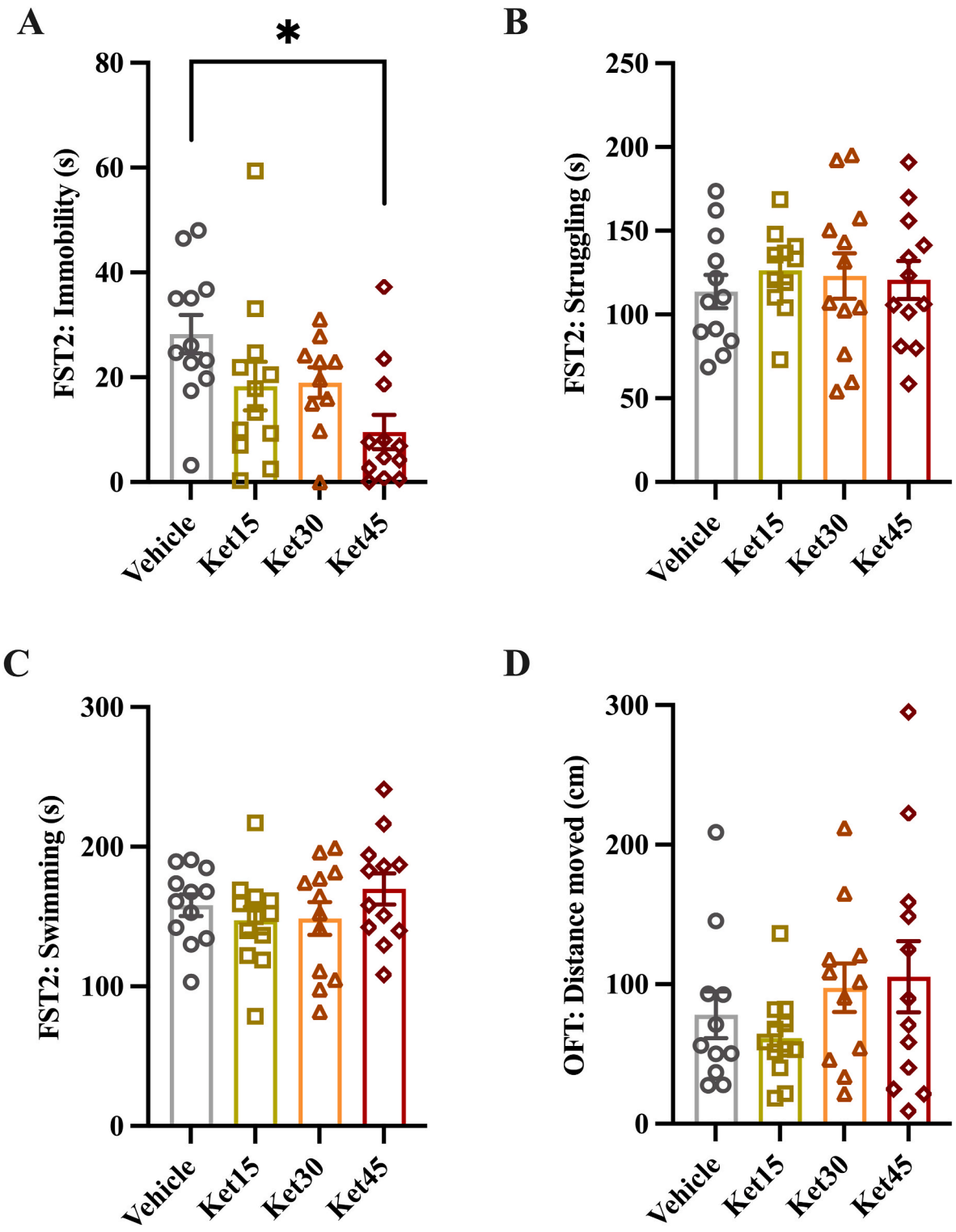

Fig. 2. Behavioral outcomes in the forced swim test (FST) and open field test (OFT). (**A**) Time spent immobile, (**B**) struggling, and (**C**) swimming during the FST2. (**D**) Total locomotor activity measured in the OFT. Error bars represent SEM. Asterisks denote statistical significance ($p < 0.05$) based on Tukey's post hoc comparisons.

77.726, SD = 26.703; Fig. 3B), suggesting that antidepressant ketamine transiently impaired retrieval of conditional fear memory.

Extinction 2 analyses also revealed a main effect of CS presentation on overall freezing rates ($F(7, 196) = 15.55$, $p < 0.0001$, $\eta 2 = 0.211$, two-way repeated measures ANOVA). There was no group effect ($F(3, 28) = 1.692$, $p = 0.191$, $\eta 2 = 0.058$, two-way repeated measures ANOVA) or a group-cue interaction ($F(21, 196) = 0.728$, $p = 0.801$, $\eta 2 = 0.030$, two-way repeated measures ANOVA; Fig. 3C). When the average freezing levels of Extinction 1 and Extinction 2 were compared within all groups, only the vehicle group showed significantly reduced freezing from the first to the second session ($t(28) = 2.830$, $p = 0.034$, two-way repeated measures ANOVA, Bonferroni corrected; M = 69.565, SD = 16.444; Fig. 3D), showing that control animals exhibited better extinction learning across sessions.

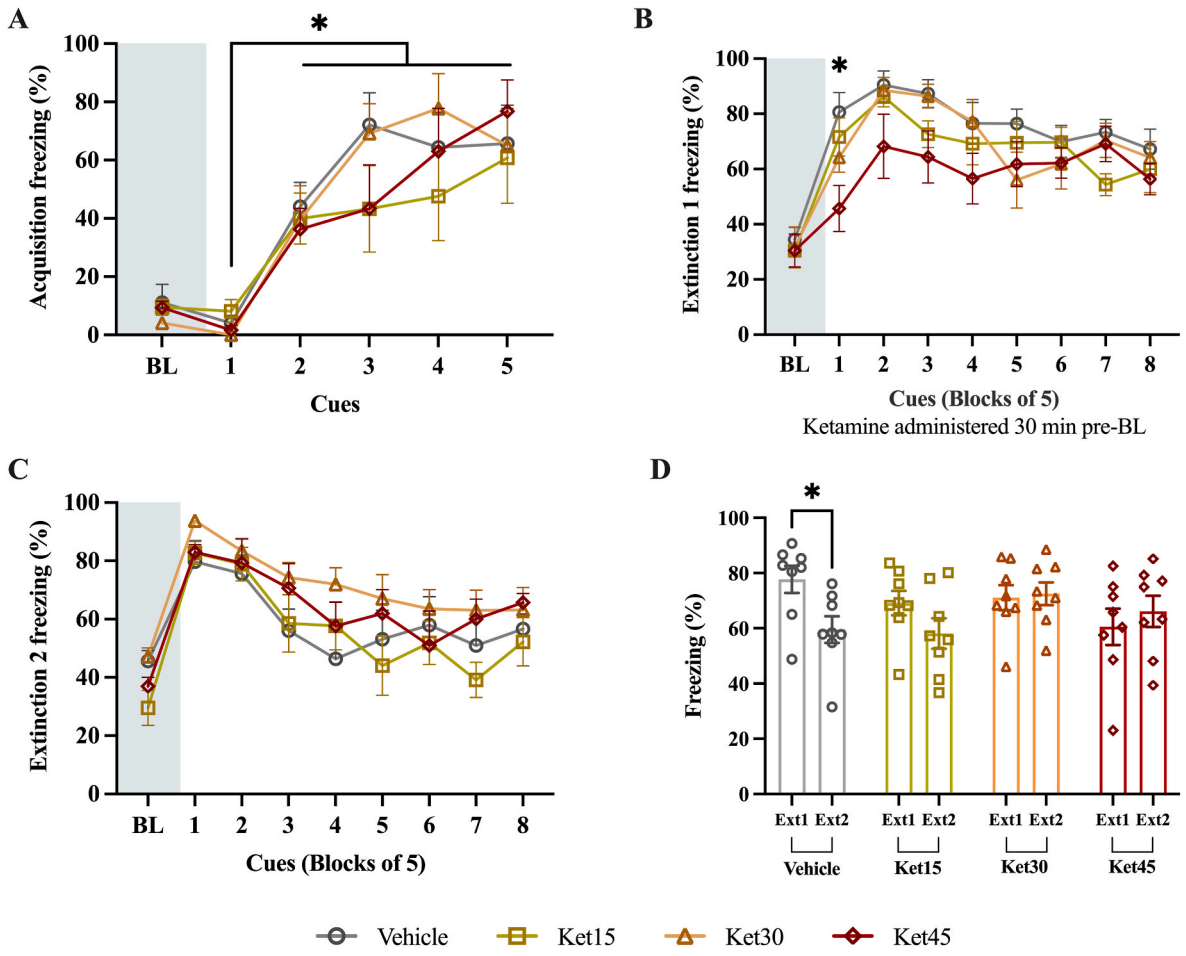

Fig. 3. Cued fear conditioning and extinction sessions. (**A**) Percentage of freezing during the baseline period (grey shading) and in response to the conditioned stimulus (CS) during fear acquisition in Context A. (**B, C**) Freezing responses to blocks of five CS presentations during Extinction 1 and Extinction 2 sessions in Context B. (**D**) Average freezing percentage across all 40 CS presentations in Extinction 1 and Extinction 2. Error bars represent SEM. Asterisks denote statistical significance ($p < 0.05$) based on post hoc tests: Tukey's comparisons for cue effects across all groups (A) and for freezing differences between Ket45 and control in Ext 1 (B); and Bonferroni correction for freezing differences between Ext 1 and Ext 2 (D).

3.4. Alarm calls

Animals produced detectable USV signals mostly during fear acquisition and Extinction 1. Ketamine treatment had no effect on the number of calls ($F(3, 28) = 1.022$, $p = 0.398$, $R^2 = 0.099$, one-way ANOVA; Fig. 4A) or overall call duration ($F(3, 28) = 0.973$, $p = 0.419$, $R^2 = 0.094$, one-way ANOVA; Fig. 4B) during acquisition. In contrast, the extinction session led to a greater number of alarm calls, on which ketamine treatment had a significant main effect ($F(3, 28) = 3.380$, $p = 0.032$, $R^2 = 0.266$, one-way ANOVA). In comparison to the vehicle group ($M = 118.1$, $SD = 104.8$), the number of calls was significantly reduced in Ket45 ($q = 4.317$, $p = 0.024$, Cohen's $d = 1.513$ Tukey's corrected; $M = 5.00$, $SD = 14.14$; Fig. 4C). An identical pattern was observed for call durations, which was also affected by ketamine treatment ($F(3, 28) = 3.390$, $p = 0.032$, $R^2 = 0.266$, one-way ANOVA). The Ket45 group ($q = 4.145$, $p = 0.032$, Cohen's $d = 1.616$, Tukey's corrected; $M = 7.75$, $SD = 21.92$) exhibited a significant reduction in total call duration compared to the vehicle group ($M = 160.2$, $SD = 131.6$; Fig. 4D). The other ketamine groups did not differ from the vehicle group in either call frequency or duration (all p values > 0.05, Tukey's corrected), as also illustrated by representative 5-s USV recordings during Extinction 1 (Fig. 4E).

3.5. Anxiety-like behavior

Ketamine treatment did not affect the overall duration spent in the closed arms of the EPM ($F(3, 28) = 0.266$, $p = 0.849$, $R^2 = 0.028$, one-way ANOVA; Fig. 5A). Likewise, no group effect was found on time spent in the open arms ($F(3, 28) = 0.211$, $p = 0.888$, $R^2 = 0.022$, one-way ANOVA; Fig. 5B). The Ket15 ($q = 1.019$, $p = 0.888$, Cohen's $d = 0.374$, Tukey's corrected; $M = 79.55$, $SD = 80.38$), Ket30 ($q = 0.723$, $p = 0.9156$, Cohen's $d = 0.248$, Tukey's corrected; $M = 90.23$, $SD = 96.11$), and Ket45 ($q = 0.916$, $p = 0.916$, Cohen's $d = 0.290$, Tukey's corrected; $M = 83.28$, $SD = 114.5$) groups displayed comparable open arm duration with the vehicle group ($M = 116.4$, $SD = 113.9$). Locomotor activity levels were also similar among the groups ($F(3, 28) = 2.125$, $p = 0.120$, $R^2 = 0.185$, one-way ANOVA; Fig. 5C).

3.6. GRIN1 expression levels

ELISA analysis showed that ketamine treatment altered GRIN1 expression in the hypothalamus ($F(3, 12) = 4.928$, $p = 0.019$, $R^2 = 0.552$, one-way ANOVA) as well as the thalamus ($F(3, 12) = 9.414$, $p = 0.002$, $R^2 = 0.702$, one-way ANOVA). Interestingly, no difference was found among the groups in the isolated BLA tissue ($F(3, 12) = 0.501$, $p = 0.688$, $R^2 = 0.111$, one-way ANOVA; Fig. 6).

Post-hoc comparisons revealed suppressed GRIN1 expression in extracted hypothalamic tissue for the Ket15 ($q = 4.707$, $p = 0.027$,

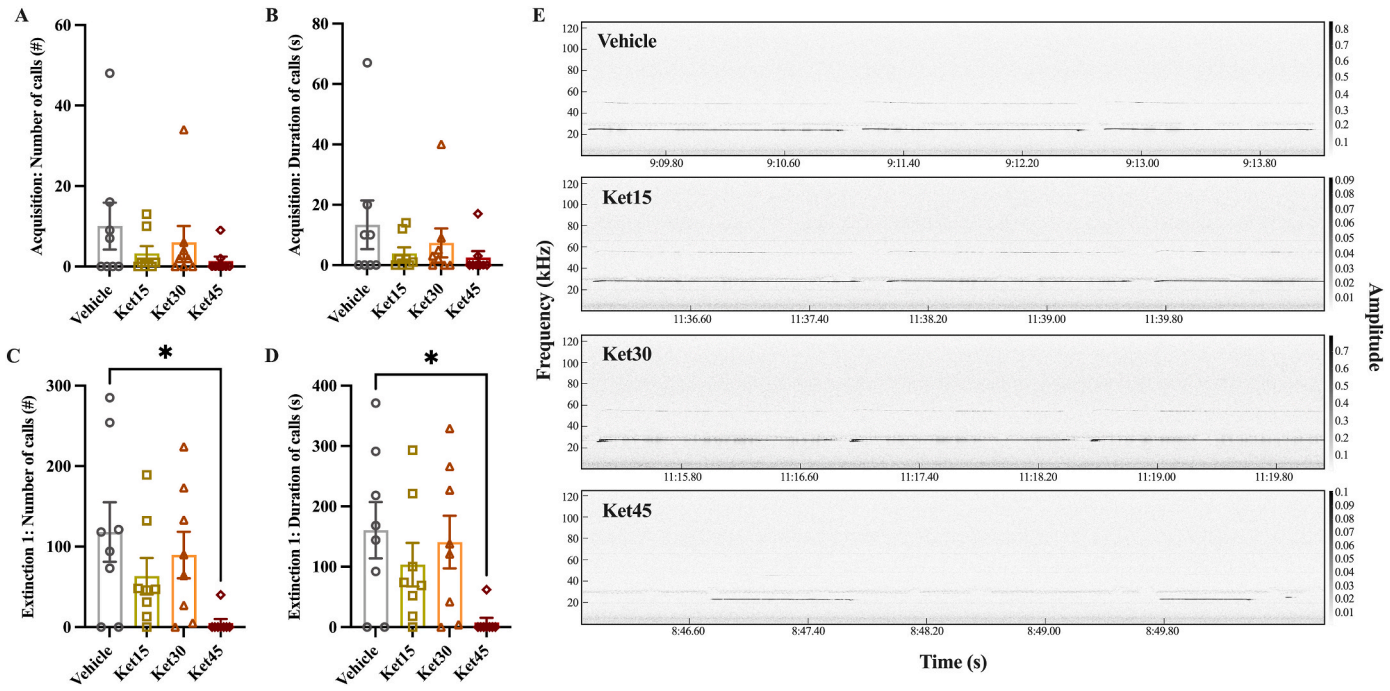

Fig. 4. Ultrasonic vocalization (USV) recordings during cued fear conditioning and Extinction 1. (**A**) Number and (**B**) duration of USV calls during fear acquisition. (**C**) Number and (**D**) duration of USV calls during Extinction 1. (**E**) Representative 5-s USV recording fragments for each group during Extinction 1. Error bars represent SEM. Asterisks denote statistical significance ($p < 0.05$) based on Tukey's post hoc comparisons.

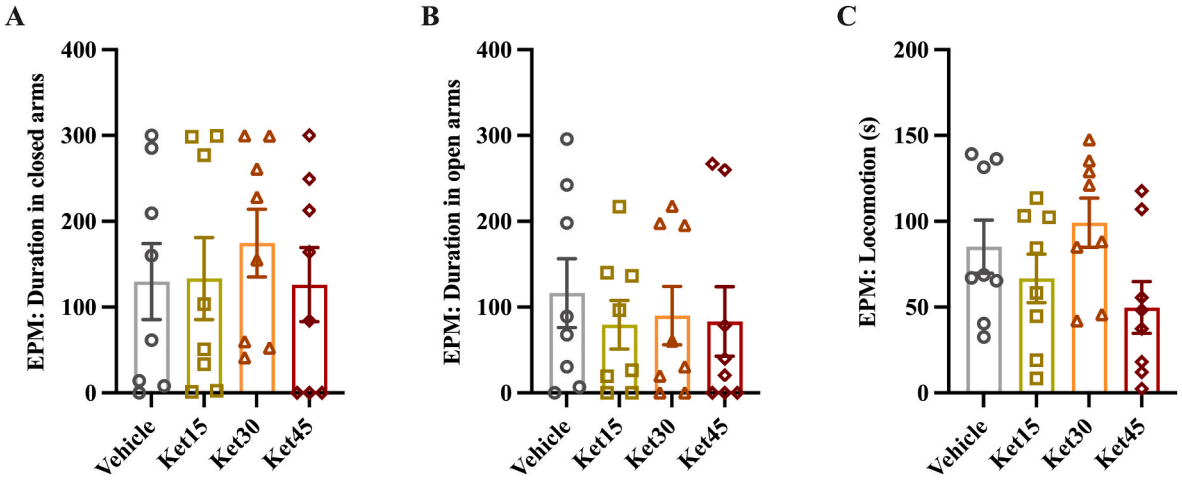

Fig. 5. Elevated plus maze (EPM) performance. (**A**) Time spent in the closed arms, (**B**) time spent in the open arms, and (**C**) total locomotor activity during the test session. Error bars represent SEM. Asterisks denote statistical significance ($p < 0.05$) based on Tukey's post hoc comparisons.

Cohen's d = 1.679, Tukey's corrected; M = 60.35, SD = 6.346) and Ket45 (q = 4.447, p = 0.037, Cohen's d = 1.583, Tukey's corrected; M = 65.04, SD = 7.845) groups as compared to the vehicle group (M = 145.3, SD = 71.23); while the Ket30 group remained slightly above the significance threshold (q = 4.074, p = 0.058, Cohen's d = 1.455, Tukey's corrected; M = 71.77, SD = 5.753; Fig. 6A).

In the thalamus, GRIN1 expression was significantly increased in the Ket30 (q = 5.064, p = 0.017, Cohen's d = 2.404, Tukey's corrected; M = 69.74, SD = 11.27) and Ket45 (q = 4.663, p = 0.028, Cohen's d = 1.972, Tukey's corrected; M = 67.16, SD = 14.89) groups, but not the Ket15 (q = 0.823, p = 0.936, Cohen's d = 0.422, Tukey's corrected; M = 31.91, SD = 8.495), compared to the vehicle group (M = 37.19, SD = 15.49). GRIN1 levels of the Ket30 (q = 5.887, p = 0.006) and Ket45 (q = 5.486, p = 0.010) groups were also significantly higher than that of the Ket15 group (Fig. 6B).

GRIN1 subunit levels did not change in the BLA for the Ket15 (q = 0.705, p = 0.958, Cohen's d = 0.356, Tukey's corrected; M = 61.85, SD = 25.61), Ket30 (q = 1.660, p = 0.654, Cohen's d = 1.447, Tukey's corrected; M = 70.71, SD = 14.49) or Ket45 groups (q = 1.180, p = 0.837, Cohen's d = 0.686, Tukey's corrected; M = 66.26, SD = 22.19) in comparison to the vehicle group (M = 55.32, SD = 4.011; Fig. 6C).

3.7. *GluN2A and GluN2B expression levels*

After assessing GRIN1 (GluN1) levels via ELISA, we performed immunohistochemistry for GluN2A (Fig. 7A) and GluN2B (Fig. 7C) to quantify neurons expressing these receptor subunits and compare their distribution between the vehicle and antidepressant ketamine groups. Ketamine (45 mg/kg) significantly reduced GluN2A expression in the PVT (t(10) = 2.252, p = 0.0481), BLA (t(10) = 2.949, p = 0.0145), and

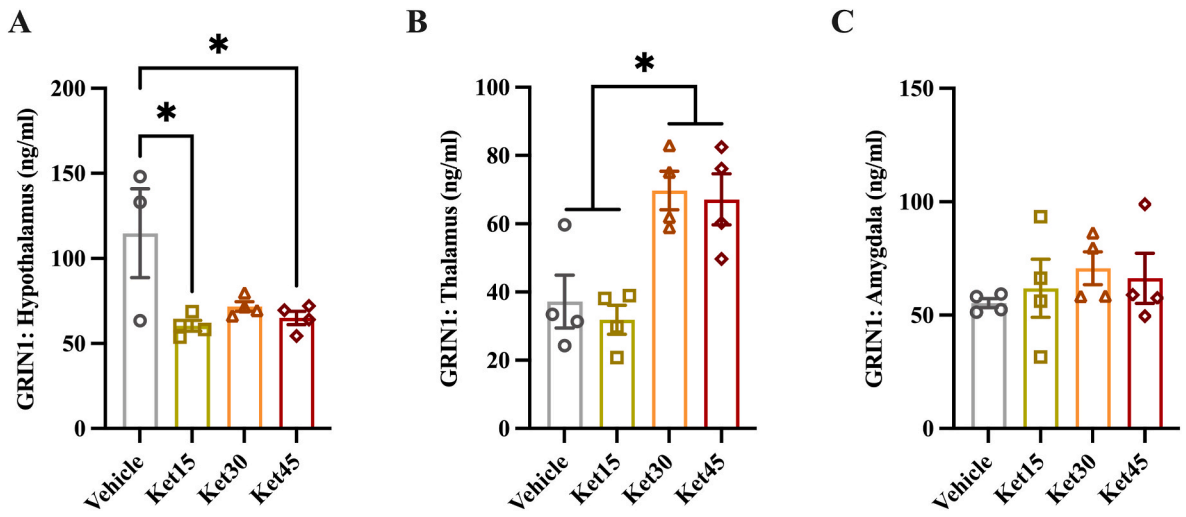

Fig. 6. GRIN1 protein concentrations measured by ELISA in the (**A**) the hypothalamus, (**B**) thalamus, and (**C**) basolateral amygdala. Error bars represent SEM. Asterisks denote statistical significance ($p < 0.05$) based on Tukey's post hoc comparisons.

MHb ($t(10) = 9.968$, $p < 0.0001$), with no significant differences in the PVN ($t(10) = 1.470$, $p = 0.1722$) or LHb ($t(10) = 1.828$, $p = 0.0975$; independent samples t-tests; Fig. 7B).

Similarly, the number of GluN2B-decorated neurons significantly decreased in the PVT ($t(10) = 3.261$, $p = 0.0082$), BLA ($t(10) = 2.336$, $p = 0.0416$), MHb ($t(10) = 5.486$, $p = 0.0003$), and LHb ($t(10) = 3.717$, $p = 0.0040$), with no difference observed in the PVN ($t(10) = 0.9627$, $p = 0.3584$; Fig. 7D).

3.8. Stress-induced neuronal activity

Immunohistochemistry for the c-Fos protein revealed sufficient number of activated neurons in response to restraint stress in all regions of interest (Fig. 8A). However, the overall number of c-Fos immunopositive cells in the PVN ($F(3, 20) = 1.958$, $p = 0.153$, $R^2 = 0.227$, one-way ANOVA), PVT ($F(3, 20) = 0.537$, $p = 0.663$, $R^2 = 0.075$, one-way ANOVA), and BLA ($F(3, 20) = 0.834$, $p = 0.491$, $R^2 = 0.111$, one-way ANOVA) were similar among the vehicle and ketamine groups (Fig. 8B).

In contrast, oral ketamine treatment had a significant effect on the number of habenular c-Fos + cells in both the MHb ($F(3, 20) = 5.370$, $p = 0.007$, $R^2 = 0.446$, one-way ANOVA) and LHb ($F(3, 20) = 4.525$, $p = 0.014$, $R^2 = 0.404$, one-way ANOVA). Yet, post hoc comparisons revealed a significant group difference only in the MHb. Higher doses of ketamine decreased c-Fos + cells in this nucleus, as observed for the Ket30 ($q = 4.506$, $p = 0.022$, Tukey's corrected; $M = 9.167$, $SD = 5.231$) and Ket45 groups ($q = 4.135$, $p = 0.039$, Tukey's corrected; $M = 10.33$, $SD = 4.633$) in comparison to the vehicle group ($M = 23.33$, $SD = 12.32$; Fig. 8B).

4. Discussion

Using controlled oral administration via gavage, this study demonstrated that 45 mg/kg oral ketamine alleviates behavioral despair without affecting locomotor activity or anxiety-like behavior, as assessed in the OFT or EPM. At this antidepressant-like dose, ketamine also impaired the retrieval of conditioned fear memory and significantly reduced aversive 22 kHz USV calls. While all ketamine doses lowered GRIN1 expression in the hypothalamus, higher doses enhanced its expression in the thalamus. The antidepressant-like dose also decreased the number of GluN2A and GluN2B expressing neurons in the paraventricular nucleus of the thalamus, basolateral amygdala, and habenula. Moreover, higher ketamine doses attenuated stress-induced neuronal activation in the medial habenula.

Antidepressants often promote active coping behaviors in rodents, typically reflected by reduced immobility in the FST (Cryan et al., 2005; Unal and Canbeyli, 2019). In the present study, oral administration of 45 mg/kg ketamine via gavage achieved sufficient bioavailability to alleviate behavioral despair. Importantly, no group differences were observed in FST1, prior to ketamine treatment. Similarly, locomotor activity and anxiety-like behavior remained unaffected in the EPM and in thigmotaxis behavior in the OFT, a species-typical indicator of stress-induced locomotion (Gencturk and Unal, 2024; Gould et al., 2009). These findings align with previous work employing i.p. administration of 5–10 mg/kg ketamine, where behavioral testing occurred 30 min post-injection (Botanas et al., 2017). Also note that in this study, the OFT was conducted 20–30 min after FST2. The Ket45 group showed a non-significant trend toward increased distance traveled and displayed normal exploratory behavior, with no signs of sedation or motor suppression, consistent with the reduced immobility observed in FST2.

Regarding the observed effects on behavioral despair, it is important to note that the current study used racemic ketamine, which contains both [R]- and [S]-enantiomers. These enantiomers have distinct pharmacological profiles: [S]-ketamine has greater affinity for NMDA receptors and is currently approved for clinical use in treatment-resistant depression (Arendt-Nielsen et al., 1996; Bonaventura et al., 2021; Oye et al., 1992; Ulrich Zeilhofer et al., 1992), while [R]-ketamine has shown promising antidepressant-like effects in preclinical models, with fewer side effects (Arendt-Nielsen et al., 1996; Chen et al., 2020; Chong et al., 2009; Clements et al., 1982; Grant et al., 1981; Yang et al., 2015; Zhang et al., 2014). Our study, like many others using racemic ketamine, did not differentiate between enantiomer-specific effects. As such, both the FST results, and the molecular analyses reflect the combined action of both enantiomers.

A major finding of this study is the impairment of conditional fear memory retrieval by the antidepressant-like dose of ketamine. Since ketamine was administered 30 min before the first extinction session, and the significantly reduced freezing in the Ket45 group emerged only during the first set of CS presentations, the effect likely reflects a disruption in memory retrieval rather than extinction learning. Although freezing levels increased in subsequent blocks, they remained lower than those of the control and lower-dose groups. The rapid onset of this effect is more consistent with acute disruption of memory retrieval than with new learning processes. We have previously shown that ketamine (30 mg/kg, i.p.) has stage-dependent effects on fear memory reconsolidation, as it disrupts extinction when administered before conditioning or retrieval but shows no effect when given prior to extinction trials (Yuksel et al., 2024). Other studies report that ketamine can facilitate fear extinction in PTSD models (Liu et al., 2024; Teng et al.,

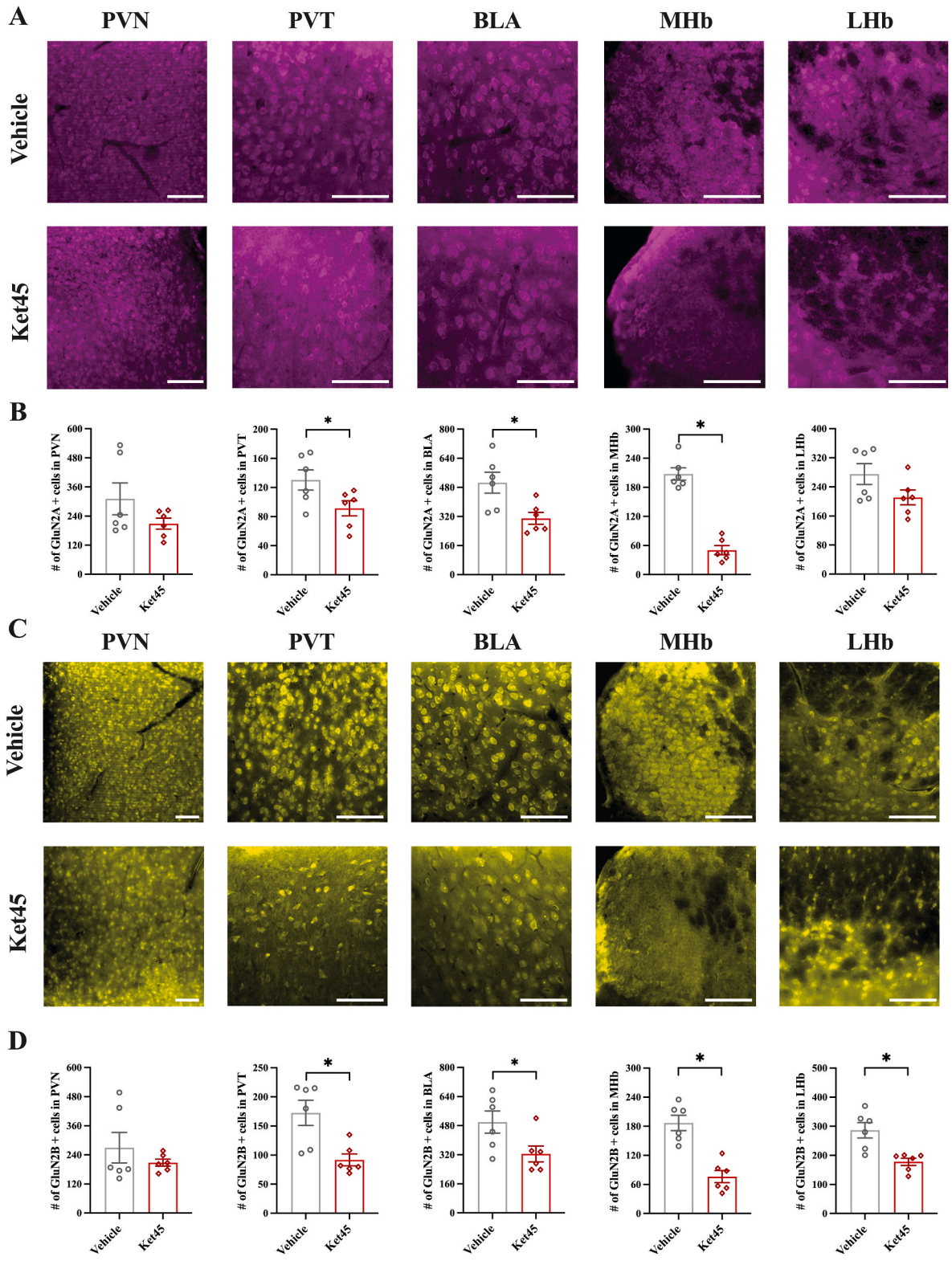

Fig. 7. Immunohistochemical analysis of GluN2A and GluN2B expression. (**A**) Representative fluorescence images of GluN2A+ and (**C**) GluN2B + cells in the paraventricular nucleus of the hypothalamus (PVN), paraventricular nucleus of the thalamus (PVT), basolateral amygdala (BLA), medial habenula (MHb), and lateral habenula (LHb). Scale bars: 100 μm. (**B**) Quantification of GluN2A+ and (**D**) GluN2B + cell counts in vehicle and Ket45 groups. Error bars represent SEM. Asterisks denote statistical significance ($p < 0.05$).

2024) and reverse extinction deficits associated with aging, accompanied by restored long-term potentiation in hippocampal neurons (Zhao et al., 2023). These findings underscore how subtle differences in dose or conditioning protocols can yield divergent effects on extinction learning, highlighting the complexity of ketamine's actions across different stages of fear processing (Choi et al., 2020; Yuksel et al., 2024).

Pharmacokinetic differences between intraperitoneal and intravenous ketamine administration may contribute to the divergent

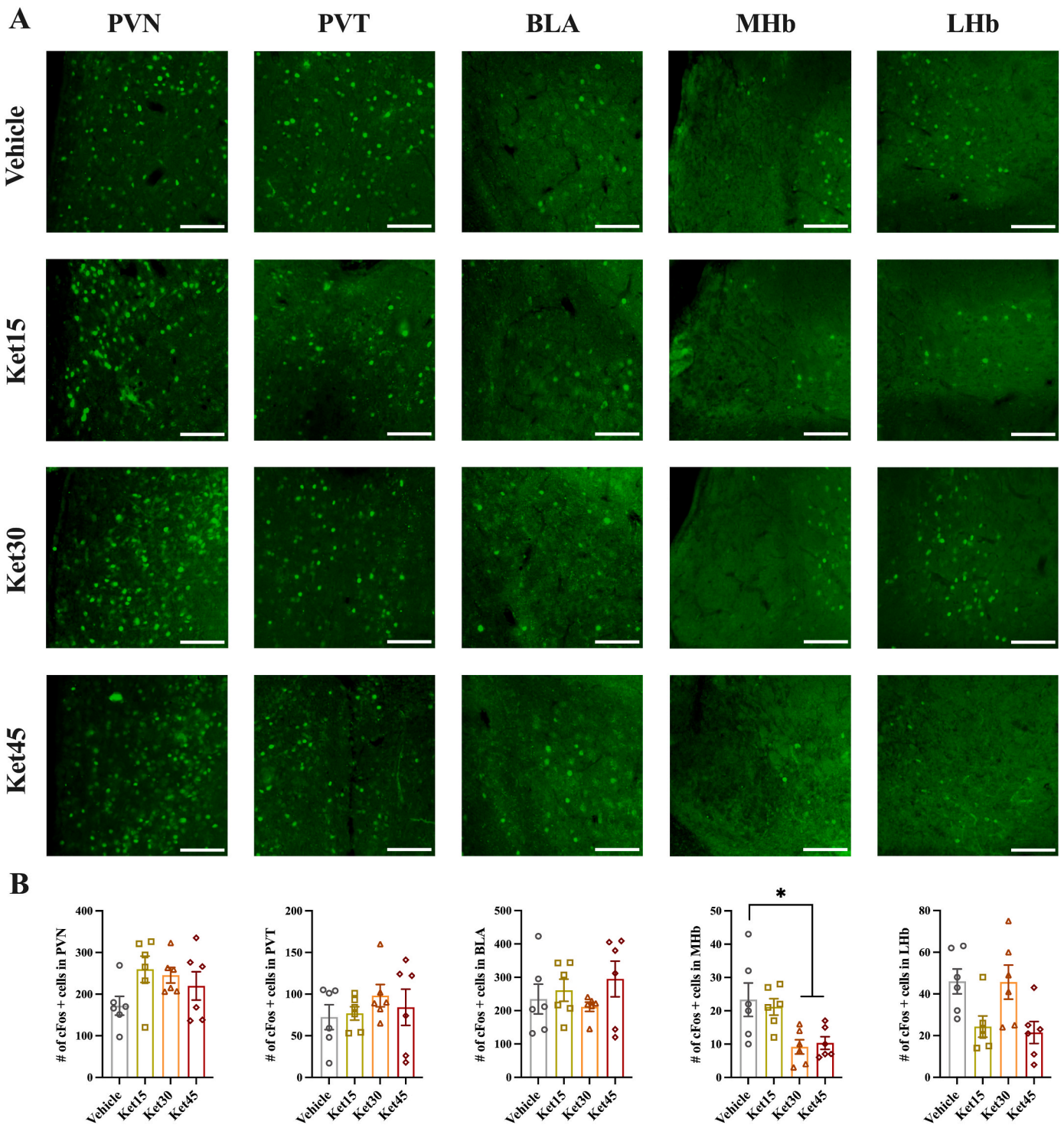

Fig. 8. Immunohistochemical analysis of c-Fos expression following acute restraint stress. (**A**) Representative fluorescence images showing c-Fos + cells in each group. Scale bars: 100 μm. (**B**) Quantification of c-Fos + cells presented as group means. Error bars represent SEM. Asterisks denote statistical significance ($p < 0.05$) based on Tukey's post hoc comparisons.

behavioral outcomes observed. Intraperitoneal injection of ketamine at 10 mg/kg has been shown to facilitate fear extinction (Duclot et al., 2016; Ito et al., 2015), whereas the same dose delivered via i.v. infusion, offering 100 % bioavailability and limited initial metabolism, does not affect extinction learning (Radford et al., 2020). In contrast, the ketamine metabolite hydroxynorketamine (2R,6R-HNK) suppresses fear retrieval when administered systemically or directly into the BLA (Xu et al., 2023) or lateral ventricle (Wang et al., 2025). Since oral gavage introduces additional metabolic variability (Fanta et al., 2015; Highland et al., 2019), the observed behavioral effects may reflect a combined influence of ketamine and its active metabolites.

Consistent with their lower freezing rates during extinction, the Ket45 group remained almost completely silent during Extinction 1, whereas the other groups consistently produced 22 kHz USVs throughout the extinction sessions. Although the Ket45 group also showed a trend toward reduced vocalizations during fear acquisition,

this effect was not statistically significant and occurred during a relatively drug-free interval, as ketamine had been administered 24 h earlier. Thus, it is unlikely to reflect acute sedative effects, but rather attenuated emotional arousal. While the precise function of these calls remains debated in the literature, and it is unclear whether they serve to communicate danger to conspecifics (Blanchard et al., 1991; Kim et al., 2010), the reduction observed in the antidepressant ketamine group could reflect impaired retrieval of conditioned fear memory. Alternatively, this effect may result from mechanical alterations in the laryngeal muscles, which are essential for generating such aerodynamic whistles (Riede et al., 2017). A previous study reported a reduction in 50 kHz USVs, typically associated with appetitive states, during social interaction following antidepressant ketamine administration (Popik et al., 2017). Conversely, GLYX-13, an NMDAR glycine site agonist, has been shown to restore USV production deficits (Burgdorf et al., 2013). Regarding aversive USVs, total vocalization time has been shown to decrease in rat pups treated with compounds such as fluoxetine (1–30 mg/kg), benzodiazepines (1–10 mg/kg), and the mGluR antagonist MTEP (1–30 mg/kg) (Hodgson et al., 2008). Additionally, analgesics such as morphine and paracetamol reduce aversive USV calls (Jourdan et al., 1998). Taken together with our findings, these results suggest that ketamine and other antidepressants induce a unidirectional suppression of vocal output, irrespective of USV frequency range.

The aforementioned behavioral results may be influenced by potential sedative or dissociative-like effects of the high dose ketamine (45 mg/kg, gavage) used here. However, oral ketamine undergoes extensive first-pass metabolism and exhibits low bioavailability (17–29 %), meaning that this dose results in systemic exposure roughly equivalent to the antidepressant-range i.p. doses commonly used in rodents, rather than anesthetic doses (Botanas et al., 2017; Gokalp and Unal, 2024). In contrast, sedative and dissociative effects are more reliably observed following intravenous administration, which bypasses gastrointestinal metabolism, achieves higher peak plasma concentrations, and maintains steadier systemic levels (Radford et al., 2017, 2018, 2020). With i.p. and oral routes, ketamine is rapidly metabolized into active metabolites such as hydroxynorketamine, which contribute significantly to its antidepressant effects, rather than its dissociative properties (Marietta et al., 1976; Radford et al., 2017). Supporting this pharmacokinetic profile, our behavioral data indicate no evidence of sedation or dissociation in the Ket45 group: animals displayed normal locomotor activity in the OFT and exhibited intact fear learning and behavioral responsiveness.

To investigate potential NMDAR-dependent plastic changes in response to ketamine, we first assessed GRIN1 expression levels in extracted and homogenized tissue using ELISA. For GluN2A and GluN2B, immunohistochemistry was sufficiently effective to allow detection of neurons expressing these subunits in histological sections. While ELISA requires complete homogenization of the hypothalamus, thalamus, and basolateral amygdala (BLA), thus limiting the ability to draw nucleus-specific conclusions, our findings suggest that antidepressant-dose ketamine induces significant plastic changes in both the hypothalamus and thalamus. This aligns with proposed mechanisms of ketamine's action involving modulation of the HPA axis (Anderzhanova et al., 2020; Fitzgerald et al., 2019).

Immunohistochemistry for GluN2A and GluN2B also revealed marked differences in the PVT, a thalamic center involved in stress mediation, as well as in the BLA and habenula. These observations are consistent with ketamine's established effects on fear memory (Girgenti et al., 2017; Glavonic et al., 2024), which are frequently attributed to its modulation of ionotropic glutamate receptors and resulting synaptic changes in glutamatergic transmission (Vieira et al., 2015; Zhang et al., 2017). Notably, ketamine at an antidepressant-like dose reduced the number of neurons expressing both GluN2A and GluN2B subunits across these structures, indicating a downregulation of both receptor types. This finding aligns with previous studies showing that antagonism of either GluN2A- or GluN2B-containing NMDARs is sufficient to produce antidepressant-like effects (Jiménez-Sánchez et al., 2014). In particular, blockade of GluN2B-containing NMDARs has been closely linked to ketamine's antidepressant action (Li et al., 2010; Miller et al., 2014; Poleszak et al., 2013).

To determine whether the observed differences in NMDAR subunit expression corresponded with changes in stress-induced neuronal activity, we assessed c-Fos expression across key brain regions. In the PVN, we found no change in c-Fos levels, consistent with previous findings using 16 mg/kg subcutaneous ketamine (Pietersen et al., 2006). Similarly, no differences were observed in the PVT, in line with our earlier work using low-dose oral ketamine (Kingir et al., 2023). Interestingly, a higher dose of ketamine (30 mg/kg, i.p.), but not its metabolite (2S, 6S)-HNK, increased c-Fos expression in the PVT (Mastrodonato et al., 2024). In the BLA, as in the PVT, ketamine (16 mg/kg, s.c.) did not alter c-Fos expression alone but normalized the elevated c-Fos + cell counts observed in fear-conditioned animals (Pietersen et al., 2006). In contrast, (2R,6R)-HNK at 10 and 30 mg/kg (i.p.) significantly increased c-Fos + cell numbers in the BLA and reduced fear memory expression (Xu et al., 2023). Lower doses of (2S,6S)-HNK and ketamine itself did not produce this effect, though they still suppressed fear-related behaviors (Mastrodonato et al., 2024). The lack of change in c-Fos expression in our study is consistent with these findings and suggests that the oral gavage doses used here did not generate sufficient bioavailability or metabolic activation to modulate neuronal activity in the BLA.

Interestingly, higher doses of ketamine reduced the number of c-Fos + cells in the medial habenula, a region implicated in aversion and reward regulation (Hsu et al., 2014; McLaughlin et al., 2017; Soria-Gómez et al., 2015). In a recent whole-brain light-sheet microscopy study, ketamine (10 mg/kg, i.p.) induced relatively higher c-Fos + cell densities in several brain regions compared to other psychedelic and psychoactive compounds (Aboharb et al., 2025). In contrast, our findings show that ketamine attenuated stress-induced neuronal activity in the medial habenula, alongside a marked downregulation of GluN2A and GluN2B subunit expression. Notably, previous work has implicated habenular GluN1/GluN3A-containing NMDA receptors in mediating conditioned aversion (Otsu et al., 2019), suggesting that ketamine's effects on different types of NMDAR in this region may underlie its distinct properties.

5. Limitations of the study

This study included only male rats to align with existing ketamine literature (see Choi et al., 2020) and to reduce variability associated with hormonal fluctuations of the estrous cycle (Carrier and Kabbaj, 2013; Chen et al., 2020; Franceschelli et al., 2015), which limits the generalizability of the findings. Additionally, repeated ketamine administration over a short time span may have resulted in cumulative effects that were not independently controlled or evaluated. However, the oral route of administration and known pharmacokinetics suggest minimal systemic accumulation. It should also be noted that our analysis focused only on NMDAR subunits and c-Fos expression, without assessing other molecular mediators such as MOR activity or BDNF levels, which are critical contributors to the antidepressant effects of ketamine. Future studies should aim to clarify these mechanisms using both sexes and a design that distinguishes between acute and cumulative effects of ketamine.

6. Conclusion

Oral ketamine administration, utilized here through gavage to ensure fixed dosing, emerges as a promising alternative route for antidepressant treatment (Dutton et al., 2023). In the present study, this approach ameliorated behavioral despair, attenuated fear memory retrieval, and significantly abolished ultrasonic alarm calls, without inducing locomotor side effects. These behavioral outcomes were accompanied by altered glutamate receptor expression in the hypothalamus and thalamus, downregulation of GluN2A and GluN2B

subunits in key limbic regions, as well as a stress-driven decrease in neuronal activity within the habenula. By enabling sustained delivery of low yet effective doses, oral ketamine offers a potentially viable therapeutic strategy. Nonetheless, its pharmacokinetics require further investigation before this route can be confidently advanced toward preclinical validation.

CRediT authorship contribution statement

Beenish Asrar: Writing – original draft, Investigation, Conceptualization. **Sude Metin:** Writing – original draft, Investigation, Conceptualization. **Zeynep Sen:** Writing – original draft, Investigation, Conceptualization. **Gunes Unal:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors have no conflict of interest, financial or otherwise.

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Data availability

Data will be made available on request.

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