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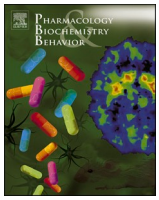

Intermittent environmental enrichment induces behavioral despair, while intermittent social isolation impairs spatial learning in rats

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ABSTRACT

Environmental enrichment and social isolation constitute two well-studied experimental manipulations that result in several behavioral, neural, and molecular changes in rodents. Enrichment is linked to enhanced cognitive performance, and mitigation of different nervous system injuries and disorders. In contrast, social isolation or impoverished environment often induce negative effects on cognitive and affective systems. Both manipulations are typically examined with a short-term or chronic exposure, which cannot capture the actual human experiences. In this study, we explored the behavioral and neural alterations led by intermittent environmental enrichment or social isolation in adult Wistar rats. Animals were assigned to an enriched condition (EC), isolation/impoverished condition (IC), or standard condition (SC). The differential housing protocol involved transferring the animals to their respective cages for two days at the end of each five-day standard housing period for 8 weeks. Enriched animals exhibited behavioral despair in the forced swim test without differential overall locomotor activity. In the Morris water maze, impoverished animals displayed a slower learning rate compared to the SC and EC groups. In line with this, the IC group had fewer parvalbumin (PV) immunopositive (+) cells in the CA1 and dentate gyrus. No differences were observed in PV+ cell levels in the amygdala, while the IC group had more c-Fos+ cells in the same region following acute restraint stress. These findings implicate that intermittent isolation or enrichment are sufficient to trigger distinct behavioral changes at the cognitive and affective domains, and pinpoint PV as a biomarker for environmentally induced alterations in hippocampal memory performance.

1. Introduction

The non-pharmacological treatments of mood and anxiety disorders, such as psychotherapy or light therapy, are widely used to complement or replace pharmacotherapy. Similarly, experimental animal models (Gencturk and Unal, 2024) do not only serve as a platform for assessing the impact of different drug treatments (Ecevitoglu et al., 2019; Kingir et al., 2023), but also as a valuable tool for revealing the behavioral and neural effects of different environmental manipulations (Brydges et al., 2011; Guven et al., 2022). In this regard, environmental enrichment in rodents has been extensively studied as a therapeutic model (Fox et al., 2006; Simpson and Kelly, 2011), while the opposite manipulation, social isolation, constitutes a widely employed stress paradigm (Mumtaz et al., 2018; Unal, 2021). Previous studies employing these manipulations have shown considerable variability in their experimental designs, utilizing either short-term, acute applications or long-term protocols. However, these time scales may not align with actual human experiences, as individuals frequently undergo intermittent forms of environmental stress or treatment, such as weekly psychotherapy sessions. To address this issue, we implemented a realistic experimental design, examining the behavioral and neural effects of intermittent environmental enrichment and social isolation in adult Wistar rats. Animals that underwent 2 days of differential housing at the end of each week for a total of eight weeks were tested in the forced swim test (FST), open field test (OFT), elevated plus maze (EPM), and Morris water maze (MWM). We revealed c-Fos-immunopositive (+) cells following acute restraint stress, and compared parvalbumin (PV)+ neurons in the hippocampus and basolateral amygdala complex.

Donald Hebb's (1947) original observation on the procognitive effects of environmental enrichment in rats has subsequently evolved to be a standard model in rodent research (Diamond et al., 1964; Rosenzweig, 1966; Rosenzweig et al., 1962). This model, widely applied in both

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juvenile and adult animals, significantly contributed to our understanding of the power of neural plasticity (Diamond, 2001; Kentner et al., 2019). Environmental enrichment in rats (Ismail et al., 2021) and mice (Sztainberg and Chen, 2010) typically involve providing the animals with a variety of novel sensory stimuli in a large cage, access to a running wheel, and a greater number of conspecifics to engage in social interaction. Hence, rodent enrichment often combines sensory-motor and social enhancements that trigger several experience-dependent neurobiological alterations (Kempermann, 2019; Nithianantharajah and Hannan, 2006). At the molecular level, it changes the expression of immediate early genes (Grimm et al., 2016), brain-derived neurotrophic factors (Ickes et al., 2000), and nuclear receptors (Soares et al., 2015). At the neuronal level, enrichment facilitates adult neurogenesis and synaptogenesis (Birch et al., 2013; van Praag et al., 2000, 2002). It may also increase cortical thickness (Bennett et al., 1962) and dendritic branching (Mora et al., 2007). These neurobiological alterations are accompanied by improved cognitive performance in different types of memory tasks (Bechara and Kelly, 2013; Cortese et al., 2018; Simpson and Kelly, 2011), and amelioration of the cognitive impairment led by neurological diseases (Nithianantharajah and Hannan, 2006), brain lesions (Urakawa et al., 2007) or addiction (Grimm and Sauter, 2020). Environmental enrichment can also prevent or treat behavioral despair (Brenes et al., 2008; Normann et al., 2021) and anxiety-like states (Ashokan et al., 2016; Sampedro-Piquero et al., 2013; Soares et al., 2015) in a time dependent manner (Amaral et al., 2008).

Social isolation in a relatively small cage constitutes the opposite of environmental enrichment in rodents. Social isolation cages restrict the exposure of animals to sensory stimuli and block opportunities for social interaction. This model mirrors certain human experiences such as the self-isolation widely practiced during the COVID-19 pandemic (Unal, 2021). Exposing rodents to an impoverished environment can induce several detrimental alterations in both cognitive and affective processes (see Mumtaz et al., 2018 for a review). Limiting environmental stimuli around an animal leads to a decrease in synaptic plasticity (Cui et al., 2006), a decreased volume in the prefrontal cortex (Day-Wilson et al., 2006), and other types of impaired brain development (Narducci et al., 2018). In the affective domain, social isolation induces depressive- and anxiety-like states (Brenes et al., 2008; Carnevali et al., 2012; Zlatković et al., 2014), and increases reactivity to novelty (Fone and Porkess, 2008). It also leads to deteriorating effects on different types of learning and memory (Hellemans et al., 2004; Li et al., 2007), pre-pulse inhibition (Wilkinson et al., 1994), and sensory gating (Witten et al., 2014). Due to the similarities between the behavioral and neural alterations observed in social isolation and the symptoms of schizophrenia, this paradigm is also employed to model schizophrenia in rodents (Fone and Porkess, 2008).

These findings collectively demonstrate the influence of environmental changes on the brain and behavior across various levels, eliciting both positive and negative alterations in cognitive and affective domains. In laboratory settings, environmental manipulations are applied for different durations. Typically, environmental enrichment lasts 30–40 days (Harland and Dalrymple-Alford, 2020), while social isolation protocols range from a few days to several weeks. Although there are protocols involving a 6-hour daily application of environmental enrichment (Bindu et al., 2005), no study has explored the effects of differential intermittent housing on a weekly basis. A weekly interval, as applied in this study, would more closely resemble the varied stress levels intermittently experienced by humans or the weekly interventions such as psychotherapy sessions. Here, we adopted a translational approach to mirror the oscillating life patterns of humans, investigating the affective and cognitive behavioral outcomes resulting from intermittent exposure to a standard, impoverished, or enriched environment. We evaluated the neural effects of these differential housing conditions by examining PV+ cell levels in the amygdala and hippocampus, as well as c-Fos+ cells in the amygdala.

2. Materials and methods

2.1. Subjects

Twenty-four experimentally naïve adult (5–8 months old) male Wistar rats ($M = 369.88$ g, $SD = 22.92$ g) were used for the experiment. The animals were housed in a vivarium (21 ± 1 °C; 40–60 % humidity; 12:12 day/night cycle, lights on at 08:00) with *ad libitum* access to food and water. They were assigned to a standard condition (SC), isolation/impoverished condition (IC) or enriched condition (EC) by systematic sampling based on their initial weights ($n = 8$ per condition). The experimental procedures were approved by the Boğaziçi University Ethics Committee for the Use of Animals in Experiments (Approval No: 2021-11).

2.2. Experimental design

All animals were housed in standard cages containing 4 animals for 5 consecutive days, which was followed by a 2-day differential housing. The animals in the IC group were transferred to small isolation cages, while the EC animals were together placed in an enrichment cage. Animals in the SC group were transferred to clean standard cages for these 2 days. This procedure consisting of a 5-day standard housing and 2 days of differential housing was repeated for 8 weeks. The animals were weighted at the beginning of each weak. All animals were placed to standard cages and remained there during behavioral testing. Behavioral despair, general locomotor activity, anxiety-like behavior and spatial memory were respectively assessed in the FST, OFT, EPM, and MWM. A 50 min-long restraint stress was applied to each animal following behavioral testing in order to recruit stress-related neural circuits for *ex vivo* c-Fos analysis. Perfusion-fixations were done 90 min after the restraint stress application (Fig. 1).

2.3. Environmental enrichment and social isolation

A custom-made transparent Plexiglas cage (66 × 66 cm) was used for environmental enrichment (Aykan et al., 2024). It contained an elevated platform (25 × 25 cm), a running wheel, two small shelter boxes (10 × 10 × 10 cm), a tunnel, a food pellet, and several objects with different shapes, colors, and texture. The objects and the location of the food pellet were changed each week to induce novelty. Social isolation was done in relatively small metal cages (36.5 × 16.5 × 12.5 cm) with bedding.

2.4. Forced swim test

The forced swim test consisted of a 15-minute pretest session (FST-1) and a 5-minute test session (FST-2) conducted 24 h after. Each animal was placed into a transparent cylindrical Plexiglas apparatus (height = 45 cm, diameter = 30 cm) filled with 30 cm-high water (23 ± 1 °C) in order to prevent the hindlimbs of the animal from touching the ground. The sessions were recorded with a video camera for offline analyses with EthoVision XT (Noldus Information Technology, Leesburg, VA). Periods of immobility, swimming, and struggling were quantified and compared between the groups (Porsolt et al., 1977; Slattery and Cryan, 2012).

2.5. Open field test

The 5-minute open field test was conducted in an enclosed square arena (45 × 45 × 35 cm) illuminated from above (center = 90 ± 5 lx, periphery = 55 ± 5 lx). The sessions were recorded for offline analyses with EthoVision XT. Duration of locomotor activity, total distance traveled, and periods of thigmotaxis were detected in addition to the frequency of grooming and rearing. The number of fecal boli were also counted. The testing arena was cleaned with 70 % ethanol after each session to prevent olfactory cues for the next animal.

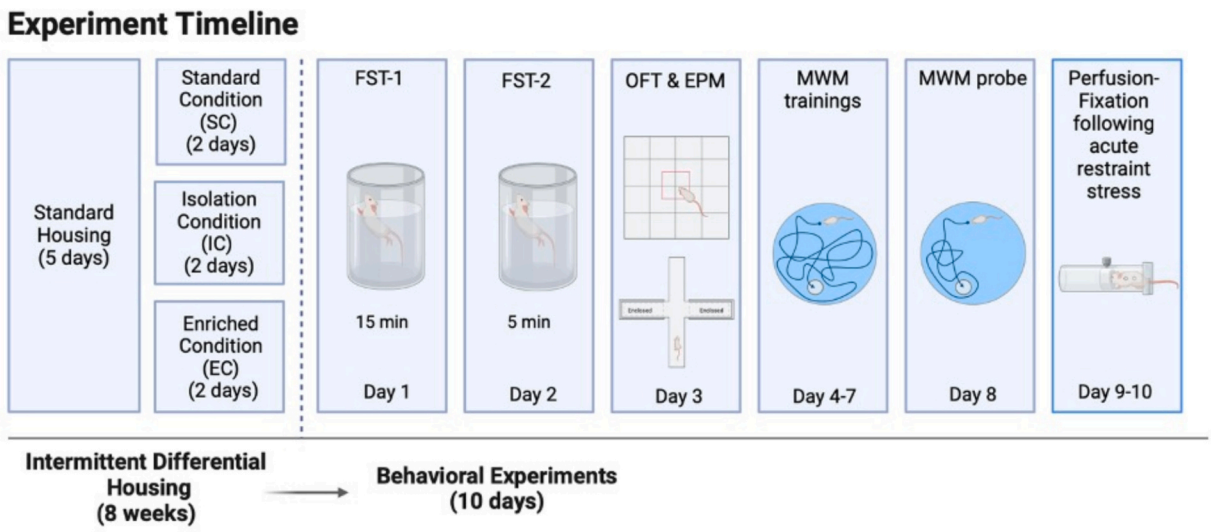

Fig. 1. Experimental timeline. The differential intermittent housing for 8 weeks was followed by the forced swim test (FST), open field test (OFT), elevated plus maze (EPM), and Morris water maze (MWM).

2.6. Elevated plus maze

A 50 cm-high plus-shaped maze had two open arms with transparent Plexiglas walls and two closed arms with opaque walls (arm length: 50 cm, width: 10 cm). The open arms were substantially brighter (230 ± 5 lx) than the closed arms (60 ± 5 lx). Each animal was gently placed in the center of the maze and allowed to move around freely for 5 min. The sessions were video-recorded for offline EthoVision XT analyses. Total locomotor activity in the maze and the time spent in open *vs.* closed arms were quantified. The maze was cleaned with 70 % ethanol after each session.

2.7. Morris water maze

The Morris water maze experiments consisted of four days of training followed by a single day of probe trial (Vorhees and Williams, 2006). The pool (diameter = 120 cm) was filled with water up to 30 cm (24 ± 1 °C), and a small transparent escape platform was placed 2 cm below the water level. Opacifying milk powder was added to the water in order to further reduce the visibility of the escape platform. The pool was separated into four virtual quadrants, and different geometrical shapes in different colors were placed at the center of the walls of each quadrant to provide visual cues. The quadrant with the escape platform is designated as the "target quadrant".

The animals underwent four one-minute trials in each training day with an inter trial interval of 3 min. The starting quadrant of the animals were changed each day in order to prevent any possible order effect. In each trial, the animals were allowed to locate the hidden platform within 1 min. Animals that fail to locate the platform within a minute were gently pulled to it at the end of the session. Each animal was allowed to stay on the platform for 15 s once they locate it, or are placed on it at the end of an unsuccessful trial. On the last day of MWM experiments, the escape platform was removed for a single probe trial session. The experimenter placed the animals from the opposite quadrant of the target quadrant and allowed them to swim for 1 min. The time spent in each quadrant and the duration to find the hidden platform (*i.e.*, escape latency) were analyzed using EthoVision XT.

2.8. Tissue preparation and immunohistochemistry

The animals were perfused with 0.9 % saline and 4 % depolymerized paraformaldehyde (PFA) following deep anesthesia with a mixture of ketamine (100 mg/kg, IP) and xylazine (10 mg/kg, IP). The brains were post-fixated in PFA overnight at 4 °C, thoroughly rinsed in phosphate buffer saline (PBS; 3 × 10 min), and stored in 0.01 % PBS-azide. Serial 50 μm-thick coronal sections were obtained with a vibratome (VT1000 S, Leica Biosystems, Nussloch, Germany), and collected in a 0.01 % PBS-azide solution.

We performed free-floating fluorescence immunohistochemistry for PV and c-Fos as described previously (Akmese et al., 2023; Unal et al., 2015). The sections were washed with 0.3 % Triton X-100 in PBS (PBS-Tx; 3 × 10 min), incubated in a blocking solution of 20 % normal horse serum (NHS, Vector Laboratories) in PBS-Tx for 1 h at room temperature, and transferred to the primary antibody (rabbit anti-parvalbumin, ab11427, Abcam or guinea pig anti-c-Fos, 226 308, Synaptic Systems) solution (1:2000 for PV and 1:4000 for c-Fos in 1 % NHS in PBS-Tx) for 72 h at 4 °C. The sections were washed in PBS-Tx (3 × 10 min) and incubated with the secondary antibody in 1 % NHS in PBS-Tx for 4 h at room temperature. For PV immunohistochemistry, donkey anti-rabbit secondary antibodies (Alexa Fluor 405, ab175649, Abcam; 1:250 or Alexa Fluor 488, ab150071, Abcam; 1:500) were used, while donkey anti-guinea pig secondary antibody (Alexa Fluor 647, AB_2340476, Jackson ImmunoResearch; 1:250) was used for c-Fos detection. The sections were then rinsed with PBS-Tx (3 × 10 min) and stored in PBS until permanent mounting with methyl salicylate or Fluoromount-G. Some sections were double-labeled with a mouse monoclonal anti-GAD-65 antibody (Iowa DSHB deposited by Gottlieb, D.I.; 1:500 in 1 % NHS in PBS-Tx), and a donkey anti-mouse secondary antibody (Alexa Fluor 555, ab150110, Abcam; 1:500 in 1 % NHS in PBS-Tx) following the same procedure.

2.9. Microscopy and cell counting

For PV and c-Fos+ cell quantification, 12 randomly selected sections from 4 to 5 animals were analyzed per experimental group. Each hemisphere was treated as a separate data point ($n = 24$ data points per group). The sections were selected at rostro-caudal levels between −2.92 and −3.60 mm to the Bregma point (Paxinos and Watson, 2006). Target region boundaries were identified with reference to the rat brain atlas (Paxinos and Watson, 2006) and the GAD-65 labeled sections. We counted PV immunopositive cells in the CA1, CA3, and dentate gyrus (DG) of the hippocampus, and the lateral (LA) and basolateral (BL) nuclei of the amygdala. C-Fos immunopositive cells were detected and quantified in the LA and BL.

An epifluorescence microscope (Olympus BX53) equipped with a monochrome CCD camera (Olympus XM10) and a confocal laser

scanning microscope (Leica SP8) were used for image acquisition. PV immunopositive cells were counted manually by observers who were blind to the experimental conditions. C-Fos immunopositive cells were counted with the Cellpose algorithm in an automatic manner (Stringer et al., 2021). For this purpose, acquired images were cropped to isolate the region of interest and were auto-adjusted for contrast. In Cellpose, *cell diameter* was set to 3.5, *flow_threshold* was set to 0.7, and *cellprob_threshold* was set to −4. The Cyto2 model of Cellpose was used for automated cell counting.

2.10. Statistical analysis

The statistical analyses were performed using GraphPad Prism (v. 9.5) and R Statistical Software (v. 4.3.1; R Core Team, 2023). All data was checked for the assumptions of the parametric tests for normality with Shapiro-Wilk and sphericity. In the case of violation of normality, non-parametric tests (*e.g.*, Kruskal Wallis) and their corresponding post-hoc tests (*e.g.*, Dunn's test) were used. Depending on the dataset, one-way, two-way, or mixed-design ANOVA was used to compare the experimental groups. When significant main effects were found, post-hoc analyses were performed using Tukey's test or Šidák-corrected paired-samples *t*-tests. Results were considered as statistically significant if $p < .05$. The graphical data were prepared using R Statistical Software (v. 4.3.1; R Core Team, 2023) and Adobe Illustrator (v. 27.3). All data were presented as mean (*M*) and standard deviation (*SD*) in the text. Error bars in the figures indicate standard error of the mean (*SEM*).

3. Results

3.1. Behavioral despair

The intermittent changes in environmental conditions altered the immobility levels in the test phase of the FST ($F(2,21) = 4.15$, $p = .030$, $\eta^2 = 0.28$, one-way ANOVA; Fig. 2A). Further analysis with Tukey's multiple comparison test revealed that enriched animals ($M = 48.59$, $SD = 16.22$) displayed significantly longer immobility compared to the animals continuously housed in standard conditions ($M = 26.00$, $SD = 16.95$, $p = .029$, Cohen's $d = 1.36$). Intermittently impoverished animals ($M = 31.85$, $SD = 15.68$), however, did not differ from the standard housing group.

Differential housing also had a significant effect on swimming behavior in FST-2 ($F(2, 21) = 6.52$, $p = .006$, $\eta^2 = 0.38$, one-way ANOVA; Fig. 2B). Tukey's post-hoc test revealed that the IC group ($M = 221.0$, $SD = 19.51$) displayed more swimming compared to the EC ($M = 192.30$, $SD = 6.60$, $p = .006$, Cohen's $d = 1.97$), but not the SC group ($M = 212.80$, $SD = 19.48$). In contrast, struggling behavior in FST-2 did not change between the SC ($M = 61.23$, $SD = 13.90$), IC ($M = 47.17$, $SD = 10.70$), or EC animals ($M = 59.11$, $SD = 13.67$) ($F(2, 21) = 2.79$, $p = .084$, $\eta^2 = 0.21$, one-way ANOVA; Fig. 2C).

3.2. Weight change and locomotor activity

The animals showed a consistent increase in weight across the experimental timeline, as indicated by the percentage of weight change compared to their baseline weights prior to differential intermittent housing ($F(9, 189) = 19.90$, $p < .001$; 2-way mixed ANOVA). Furthermore, there were no significant differences in the percentage of weight gain between the groups throughout the experimental timeline ($F(2, 21) = 0.99$, $p = .388$, one-way ANOVA).

The experimental conditions did not produce an effect in total distance traveled ($F(2, 21) = 0.05$, $p = .957$, $\eta^2 = 0.004$, one-way ANOVA) or the overall duration of locomotor activity ($F(2, 21) = 0.16$, $p = .857$, $\eta^2 = 0.01$, one-way ANOVA; Fig. 3A) in the OFT, indicating that the observed immobility differences in FST-2 did not emerge as a result of a general alteration in locomotor activity (Unal and Canbeyli, 2019).

3.3. Anxiety-like behavior

The anxiety-like behavior was assessed in the EPM by comparing the overall time spent in the open arms of the maze between the groups. There was a significant difference between animals that underwent different intermittent housing conditions ($H(2) = 6.84$, $p = .03$, $\eta^2[H] = 0.23$, Kruskal Wallis nonparametric test, Fig. 3B). Dunn's multiple comparison test showed that EC animals ($M = 94.01$, $SD = 123.17$) spent significantly more time in the open arms compared to the standard condition group ($M = 5.70$, $SD = 9.67$), indicating an anxiolytic effect of intermittent environmental enrichment ($p = .03$, Cliff's *delta* = −0.78). Notably, two animals from the enriched environment group that almost exclusively remained in the open arms of the EPM substantially contributed to this difference (Fig. 3B).

3.4. Spatial learning and memory

The animals displayed a significant decrease in escape latency across training days of the MWM ($F(3, 63) = 45.57$, $p < .001$, $\eta^2p = 0.68$, 4 × 3 mixed ANOVA; Fig. 4A), indicating successful spatial learning for all groups. The housing conditions, however, did not have a main effect on escape latency in training sessions ($F(2, 21) = 2.87$, $p = .07$, $\eta^2p = 0.21$, 4 × 3 mixed ANOVA; Fig. 4A). We then compared the performance on the first day of MWM with that of subsequent training days to identify

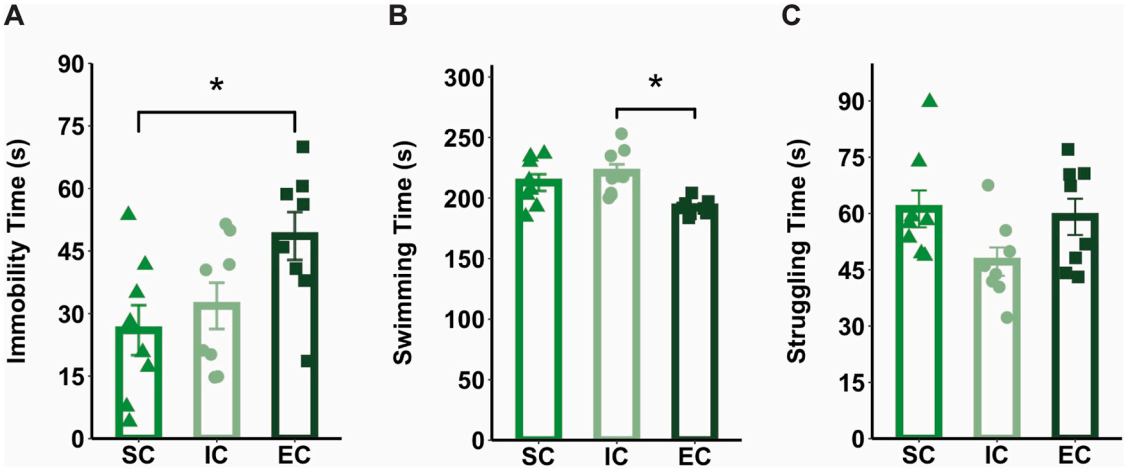

Fig. 2. Assessment of behavioral despair in the forced swim test. A) Overall duration of immobility. B) Total swimming time. C) Total struggling time. Error bars depict SEM. *$p < .05$.

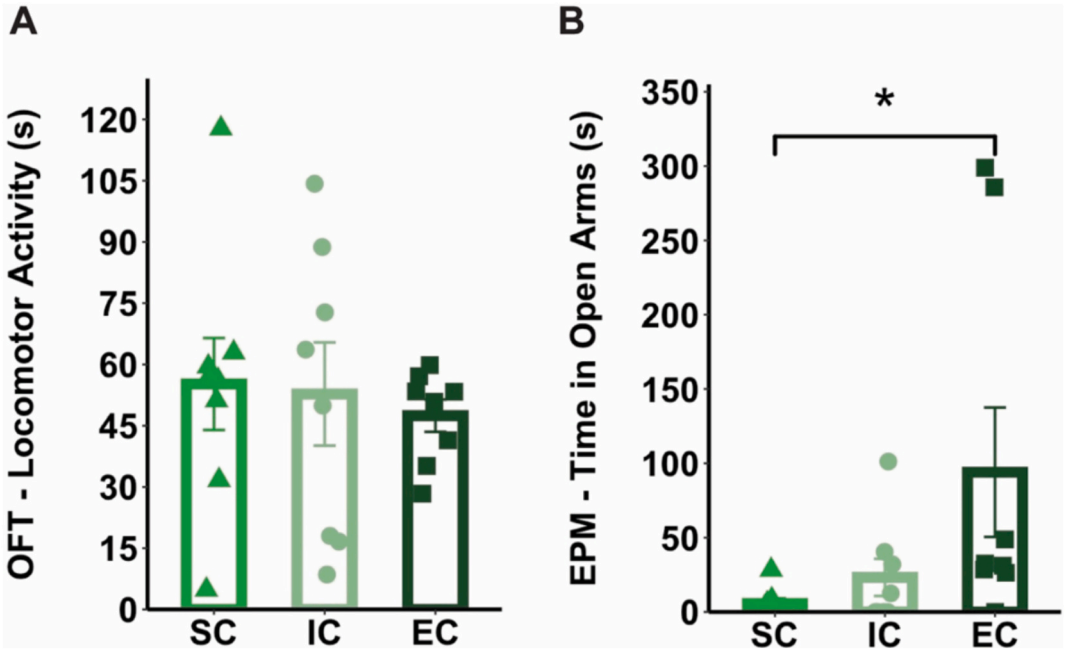

Fig. 3. Locomotor activity and anxiety-like behavior assessed in the open field test (OFT) and elevated plus maze (EPM). A) Overall duration of locomotor activity in the OFT. B) Time spent in the open arms of EPM. Error bars depict SEM. *$p < .05$.

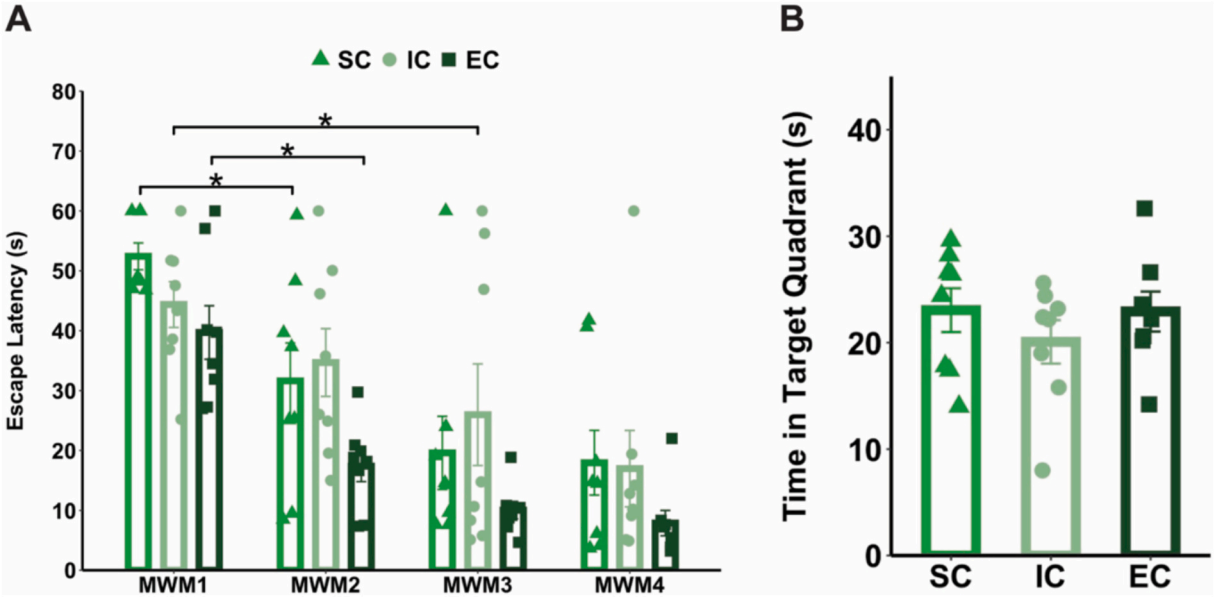

Fig. 4. Spatial memory performance in the Morris water maze (MWM). A) Escape latency duration (time spent to locate the hidden platform) across four training days. B) Time spent in the target quadrant during the probe trial. Error bars depict SEM. *$p < .05$.

the stage at which significant learning was achieved. The standard condition animals exhibited a significantly lower escape latency on the second day ($M = 31.62$, $SD = 17.92$) compared to the first day ($M = 52.41$, $SD = 6.32$) ($t(63) = 4.14$, $p < .001$, Cohen's $d = 1.55$, Šidák corrected paired samples t-test; Fig. 4A). Intermittently enriched animals also had a lower escape latency score on the second day ($M = 17.38$, $SD = 7.30$) compared to their initial performance on day one ($M = 39.70$, $SD = 12.64$) ($t(63) = 4.44$, $p < .001$, Cohen's $d = 2.16$, Šidák corrected paired samples t-test; Fig. 4A). In contrast, the IC animals showed a significant decrease in escape latency not on the second ($M = 34.68$, $SD = 16.02$), but third training day ($M = 25.97$, $SD = 23.99$) compared to their first day performance ($M = 44.37$, $SD = 10.82$) ($t(63) = 3.66$, $p = .003$, Cohen's $d = 0.98$, Šidák corrected paired samples t-test; Fig. 4A).

In the probe trial, all animals spent more time in the target quadrant ($M = 22.02$, $SD = 5.57$) compared to the overall duration they spent in non-target quadrants ($M = 12.33$, $SD = 1.89$) ($F(1,21) = 40.09$, $p < .001$, $\eta^2p = 0.67$, 3 × 2 two-way mixed ANOVA). The SC ($t(21) = 4.10$, $p = .002$, Cohen's $d = 2.35$, Šidák corrected paired samples t-test), IC ($t(21) = 2.67$, $p = .043$, Cohen's $d = 3.17$, Šidák corrected paired samples t-test), and EC ($t(21) = 4.197$, $p = .001$, Cohen's $d = 2.36$, Šidák corrected paired samples t-test) groups did not differ in their time spent in the target quadrant ($F(2, 21) = 0.71$, $p = .502$, $\eta^2 = 0.06$, one-way ANOVA; Fig. 4B).

3.5. Alterations in PV+ neurons and cellular activity

The eight-week-long differential intermittent housing altered the number of PV+ cells in the CA1 ($F(2, 69) = 6.58$, $p = .002$, $\eta^2 = 0.16$, one-way ANOVA; Fig. 5), and DG ($F(2, 69) = 3.68$, $p = .03$, $\eta^2 = 0.10$, one-way ANOVA; Fig. 5), but not of CA3 ($F(2, 69) = 1.00$, $p = .373$, $\eta^2 = 0.03$, one-way ANOVA; Fig. 5) of the hippocampus. Tukey's multiple comparisons tests showed that the IC group ($M = 30.54$, $SD = 9.86$) had significantly fewer number of PV+ cells in the CA1 compared to both the SC ($M = 48.75$, $SD = 19.1$, $p = .005$, Cohen's $d = 1.20$) and EC groups ($M = 47.38$, $SD = 25.80$, $p = .01$, Cohen's $d = 0.86$). There was no difference between the SC and EC animals ($p = .967$, Cohen's $d = 0.06$).

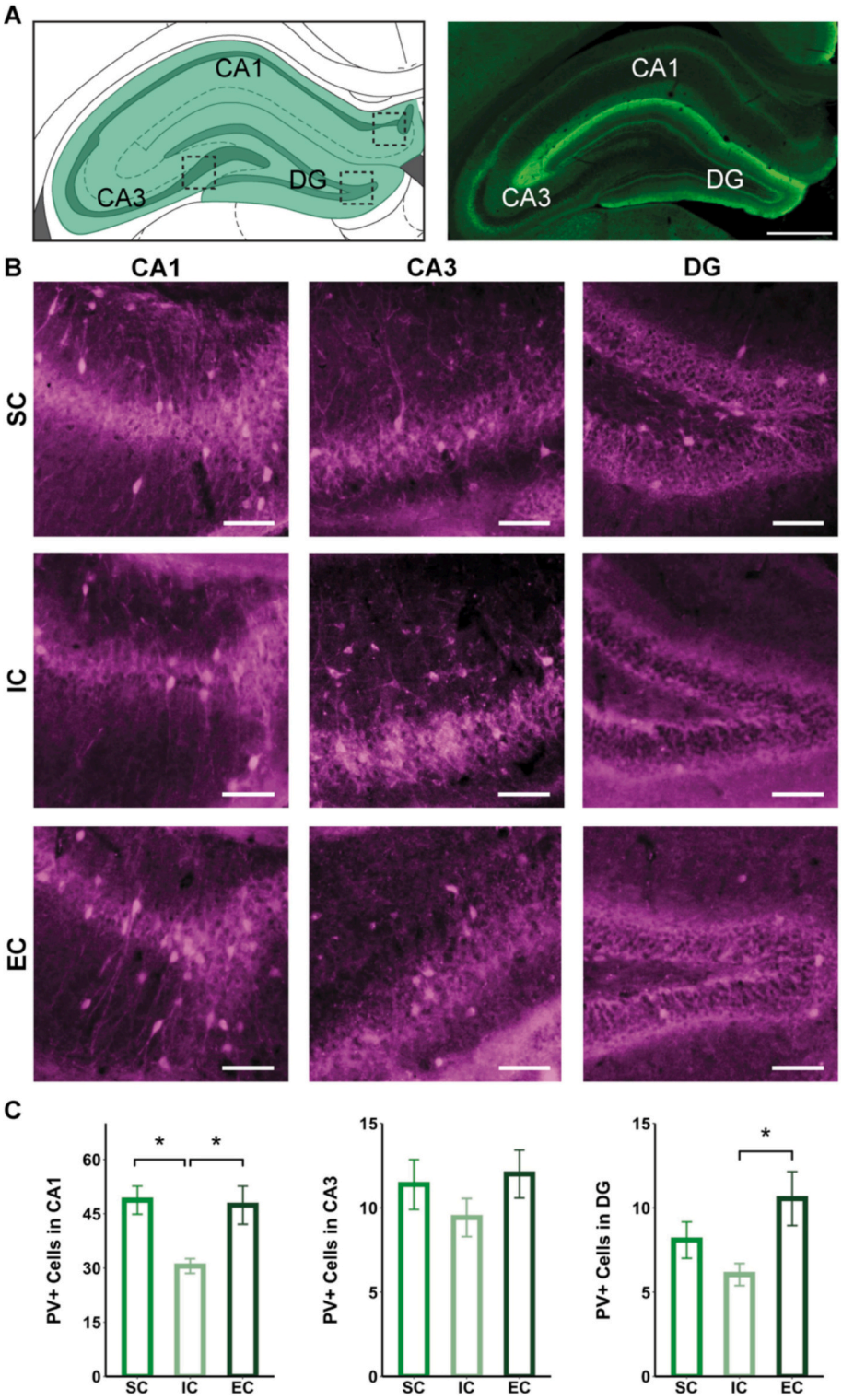

Fig. 5. Immunohistochemistry for GAD-65 and parvalbumin (PV) in the hippocampus. A) A GAD-65-immunolabeled coronal section with the corresponding atlas image (−3.24 mm to the Bregma point), depicting the approximate locations (squares) of the shown CA1, CA3, and dentate gyrus (DG) photographs. Scale bar: 1 mm. B) Representative fluorescence images of PV+ cells. Scale bars: 100 μm. C) The total number of PV+ cells. Error bars depict SEM. *$p < .05$.

Intermittently isolated animals also had significantly fewer PV+ cells in the dentate gyrus ($M = 6.04$, $SD = 3.20$), as compared to the EC animals ($M = 10.54$, $SD = 7.84$, $p = .023$, Cohen's $d = 0.75$, Tukey's multiple comparisons), with no difference between the IC and SC groups ($M = 8.08$, $SD = 5.27$, $p = .441$, Cohen's $d = 0.47$), or between the SC and EC groups ($p = .307$, Cohen's $d = 0.37$). The number of PV+ cells in the CA3 showed no significant difference between the groups ($p = .373$, Cohen's $d = 0.03$).

Differential housing did not alter the number of PV+ cells in the LA ($F(2, 66) = 0.75$, $p = .478$, $\eta^2 = 0.02$, one-way ANOVA; Fig. 6) or BL nuclei ($F(2, 65) = 2.30$, $p = .109$, $\eta^2 = 0.07$, one-way ANOVA; Fig. 6) of the amygdala. Furthermore, *ex vivo* immunohistochemistry for c-Fos following a 50 min-long restraint stress revealed that chronic intermittent housing changes altered the cellular activation in both the LA ($F(2, 68) = 5.43$, $p = .006$, $\eta^2 = 0.14$, one-way ANOVA; Fig. 6) and BL ($F(2, 67) = 17.32$, $p = .0001$, $\eta^2 = 0.34$, one-way ANOVA; Fig. 6). Intermittently isolated animals had significantly more c-Fos+ cells in their LA ($M = 82.35$, $SD = 38.1$), as compared to the EC animals ($M = 55.83$, $SD = 21.6$, $p = .005$, Cohen's $d = 0.86$, Tukey's multiple comparisons), with no difference between the IC and SC groups ($M = 65.75$, $SD = 20.9$, $p = .109$, Cohen's $d = 0.54$), or between the SC and EC groups ($p = .437$, Cohen's $d = 0.47$). The isolated group also had more c-Fos+ cells in the BL ($M = 121.0$, $SD = 49.7$) than both the enriched ($M = 67.67$, $SD = 23.9$, $p < .0001$, Cohen's $d = 1.37$) and standard ($M = 73.5$, $SD = 20.8$, $p < .0001$, Cohen's $d = 1.25$) groups. No significant difference was observed between the EC and SC groups in terms of BL cellular activation ($p < .818$, Cohen's $d = 0.26$).

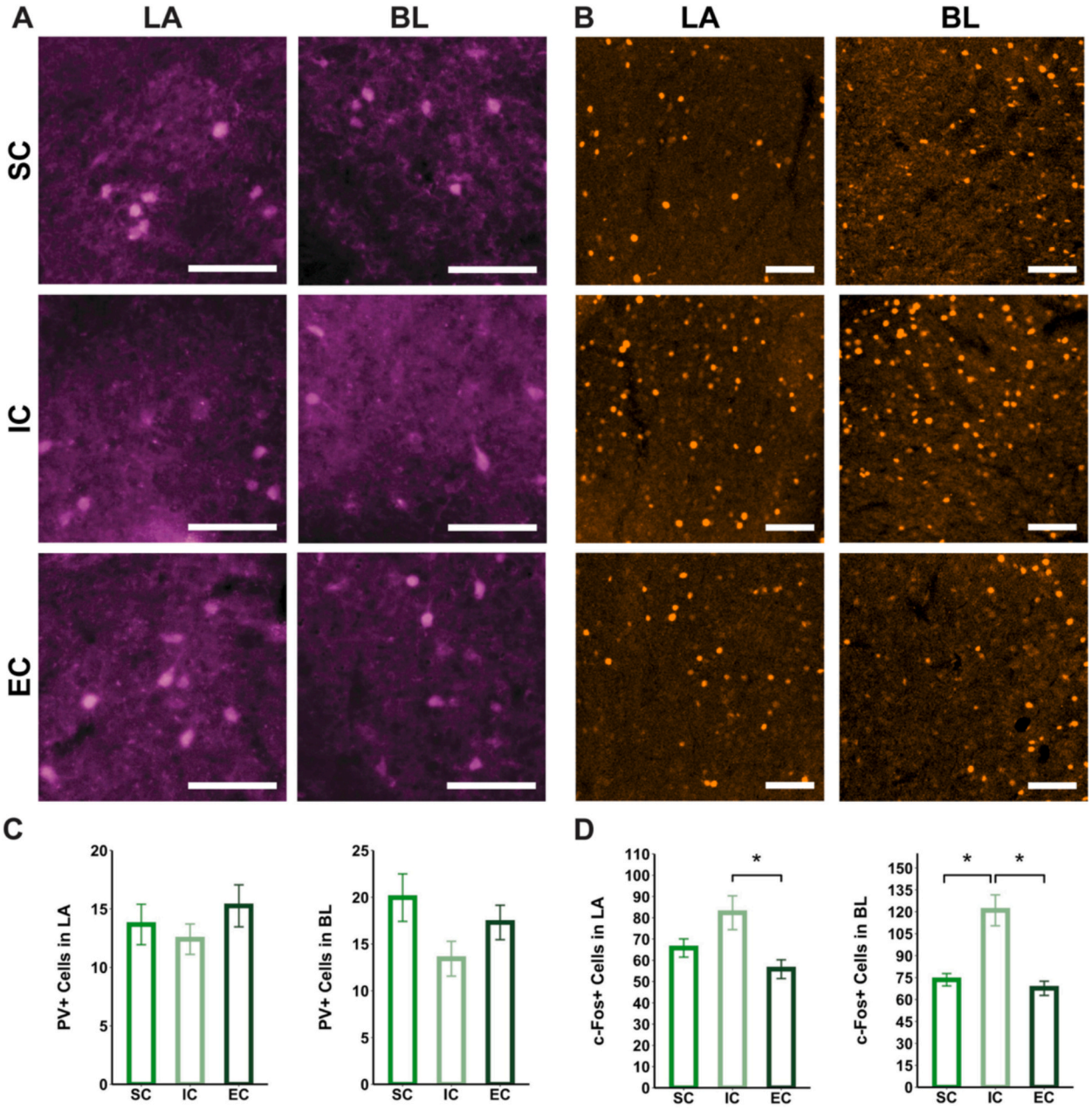

Fig. 6. Immunohistochemistry for parvalbumin (PV) and c-Fos in the lateral amygdala (LA) and basolateral amygdala (BL). A) Representative fluorescence images of PV+ cells. Scale bars: 100 μm. B) Representative fluorescence images of c-Fos+ cells. Scale bars: 100 μm. C) The total number of PV+ cells. D) The number of c-Fos+ cells following acute restraint stress. Error bars depict SEM.

4. Discussion

This study adopted a translational approach, utilizing environmental enrichment and social isolation paradigms intermittently to mirror the dynamic conditions of daily human life. The findings revealed distinct effects at both the behavioral and neural levels. Intermittent enrichment induced depressive-like behavior in the FST, but had an anxiolytic effect in the EPM. Weekly social isolation, in contrast, did not affect depressive- or anxiety-like behavior, but slowed down spatial learning in the MWM. This partial memory impairment was accompanied by significantly reduced number of PV-immunopositive cells in the CA1, and dentate gyrus of the hippocampus, suggesting the use of PV as a biomarker for environmentally induced changes in spatial memory performance.

In our study, we found that brief and intermittent exposure to environmental enrichment induced behavioral despair. Importantly, we ruled out the possibility that the difference in immobility scores in the FST-2 arose from a differential metabolic effect of different housing conditions, as all three groups exhibited similar levels of locomotor activity in the OFT (Unal and Canbeyli, 2019). Previous research has suggested that environmental enrichment typically induces an antidepressant-like effect rather than despair (Brenes et al., 2008; Normann et al., 2021; Singhal et al., 2019). However, earlier studies have also shown that the removal of environmental enrichment, known as enrichment-loss, can lead to depression-like behavior in both male (Kent et al., 2022; Smith et al., 2017) and female rats (Morano et al., 2019). Therefore, the intermittent exposure to enrichment followed by a return to standard cages in our study may have functioned as a repeated enrichment-loss paradigm, ultimately leading to behavioral despair in the environmentally enriched group. In this context, returning to a standard cage after a two-day period of enrichment could be seen as a negative punishment for the animals.

The enrichment loss hypothesis, while relevant, may not fully explain the behavioral despair observed in the intermittently enriched group. This is supported by a previous study from our laboratory, where we found that brief enrichment following social isolation also resulted in behavioral despair compared to a group that was continuously isolated (Guven et al., 2022). This suggests that factors beyond the removal of enrichment, such as the stress of environmental switching or specific stressors within an enriched environment like novelty-induced stress or crowding, may contribute to inducing behavioral despair.

The inoculation stress hypothesis suggests that environmental enrichment shares similarities with the chronic mild stress paradigm (see Kingir et al., 2023), where exposure to mild stress in an enriched environment can lead to adaptive responses and resilience against future stress (Crofton et al., 2015). However, in our experiment involving intermittent housing changes, the animals might not have had adequate time to acclimate to the mild stress of enrichment and develop resilience. Moreover, alternating toys weekly could have introduced unpredictability stress, potentially shifting the stress response of the animals from adaptive to maladaptive. Interestingly, the impoverished group did not exhibit elevated levels of immobility compared to the standard housing group, which appears contradictory to prior findings (Brenes et al., 2008; Djordjevic et al., 2012; Jahng et al., 2012). However, this result can also be explained by the cage-switching procedure. Returning to a standard cage from an impoverished environment could constitute a positive reward, without the novelty stressors of the enriched environment.

Intermittent enrichment, despite its antidepressant effect in the FST, elicited an anxiolytic response, consistent with findings from continuous enrichment studies (Ashokan et al., 2016; Sampedro-Piquero et al., 2013; Soares et al., 2015) and our previous research (Guven et al., 2022). We assessed anxiety-like behavior using the elevated plus maze (Walf and Frye, 2007), a well-established unconditioned anxiety measure that relies on the approach-avoidance conflict of rodents and their natural tendency to seek refuge in enclosed spaces (Gencturk and Unal, 2024). The weekly introduction of new toys may have led to habituation to novelty in the enriched group (Lambert et al., 2016; Zimmermann et al., 2001), potentially contributing to a shift in their approach-avoidance conflict towards more exploratory behavior. However, it is important to note that the anxiolytic impact of enrichment observed in this study was primarily influenced by the behaviors of two animals within the group (Fig. 3B).

The cognitive effects of differential intermittent housing were evaluated in the Morris water maze, a hippocampus dependent test on spatial memory. Each group learned to locate the escape platform at the end of the four-day training period, and performed equally well on the subsequent probe trial. However, intermittently isolated animals displayed slower learning compared to the standard and enriched groups, and required an extra day to reach their performance level. This observation is in line with previous research indicating the deleterious cognitive effects of physical and social impoverishment (Gardner et al., 1975; Unal, 2021).

The decline in learning speed observed in the impoverished group was reflected at the neural level by a relatively reduced number of PV+ cells in the hippocampus. PV is a calcium-binding protein that is co-expressed in certain GABAergic interneurons, including PV-expressing basket cells (Freund and Buzsáki, 1996). These PV+ GABAergic interneurons are involved in several intra- and extra-hippocampal (Hu et al., 2014; Unal et al., 2015, 2018) feedback and feedforward inhibitory connections, significantly contributing to the temporal dynamics of hippocampal circuit operations (Klausberger et al., 2003; Klausberger and Somogyi, 2008). The disturbance in PV+ hippocampal interneurons is associated with schizophrenia (del Pino et al., 2013), clinical depression (Zhou et al., 2015), Alzheimer's disease (Verret et al., 2012), and other memory impairments (Ji et al., 2017). In addition, environmental stress and developmental disturbances lead to a decrease in PV+ neurons in the hippocampus (Zaletel et al., 2016) and prefrontal cortex (Brenhouse and Andersen, 2011). In our study, the decreased number of PV+ hippocampal cells in the IC group extend these findings, indicating that even brief and intermittent exposure to an impoverished environment during adulthood could disrupt hippocampal circuits and impair hippocampus-dependent memory functions. Yet, the *ex vivo* immunohistochemical detection of PV+ cells does not definitively clarify whether the observed reduction in these cells resulted from suppressed PV expression, loss of PV+ cells, or reduced levels of neurogenesis (Stranahan et al., 2006).

Differential housing conditions did not alter the level of PV+ cells in the examined amygdaloid nuclei. Consequently, the intermittent application of enrichment was insufficient to increase the number of PV+ interneurons in the basolateral amygdala (see Wolff et al., 2014), in contrast to an earlier observation in young rats raised in an enriched environment (Urakawa et al., 2013). At the end of behavioral testing, we induced restraint stress to activate stress-related neural circuits for subsequent analysis of c-Fos, an immediate-early gene protein used as a cellular activation marker (Cullinan et al., 1995). Impoverished animals had more c-Fos-labeled cells in both the LA and BL of the basolateral amygdala, a key region for emotional processing, indicating increased cellular activation in response to acute stress following chronic intermittent isolation. This finding supports previous studies (Stanisavljević et al., 2019; Wang et al., 2022) demonstrating that chronic social isolation elevates c-Fos expression in the BLA. Our results further suggest that even intermittent isolation, rather than continuous isolation, is sufficient to alter the basolateral amygdala's response to stress.

5. Conclusion

The results of this study reveal that intermittent environmental enrichment over an eight-week period induced depressive-like behavior in the forced swim test while also producing an anxiolytic response in the elevated plus maze. Enrichment had no observable positive impact on hippocampal memory performance, as assessed in the Morris water

maze. In contrast, intermittent social isolation slowed down spatial learning, which was accompanied by a significant decrease in PV+ cells in the CA1 and dentate gyrus of the hippocampus. Chronic intermittent isolation also increased c-Fos+ cells in the LA and BL of the basolateral amygdala in response to acute stress. These findings suggest that intermittent environmental changes, akin to real-life human experiences, are sufficient to recruit distinct limbic circuits, resulting in significant cognitive and affective alterations.

CRediT authorship contribution statement

Aybuke Akkaya: Writing – original draft, Methodology, Investigation. **Deren Aykan:** Writing – original draft, Methodology, Investigation. **Sinem Gencturk:** Writing – original draft, Methodology, Investigation. **Gunes Unal:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

None.

Data availability

Data will be made available on request.

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