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RESEARCH ARTICLE

The differential effects of brief environmental enrichment following social isolation in rats

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Abstract

Environmental enrichment (EE) in rodents is associated with a wide range of physiological, affective, and cognitive benefits. A seemingly opposite housing condition, social isolation (SI), is used as a rodent model of stress, negatively affecting several neurobiological mechanisms and hampering cognitive performance. Experimental designs that involve switching between these housing conditions produced mixed results. We evaluated different behavioral and cognitive effects of brief EE following long-term, SI-induced stress. We revealed the influence of enrichment after 30 days of isolation on behavioral despair, anxiety-like behavior, and spatial working memory in adult male Wistar rats and found a substantial anxiolytic effect in the experimental (SI to EE) group. Interestingly, rats exposed to EE also showed increased behavioral despair compared with the control (continuous SI) group. There was no difference in spatial working memory performance at the end of a 5-day water Y-maze (WYM) test. However, the SI to EE animals displayed better memory performance in the first 2 days of the WYM, indicating faster learning. In line with this difference, we recorded significantly more c-Fos-immunopositive (c-Fos+) cells in the retrosplenial and perirhinal cortices of the SI to EE animals. The lateral and basolateral nuclei of the amygdala showed no such difference. These results suggest that brief enrichment following isolation stress leads to differential results in affective and cognitive systems.

Keywords Environmental enrichment · Social isolation · Forced swim test · Elevated plus maze · c-Fos · Spatial memory

Introduction

Chronic stress may precipitate the development of several types of psychopathology, including depression, anxiety disorders, and posttraumatic stress disorder (Coyne, 1991; McEwen, 2004). Different types of stressors act on particular neurobiological pathways (Alleva & Santucci, 2001) and have differential effects at the neuronal, hormonal, and behavioral level (Oishi et al., 2003; Pijlman et al., 2003). Evidence from human (Cacioppo & Hawkley, 2003) and other animal studies (Cacioppo et al., 2015; Filipović et al., 2017) demonstrate that psychosocial stressors lead to unique physiological and emotional outcomes, not observed with other types of stressors. Social isolation (SI), a phenomenon widely experienced during the COVID-19 pandemic (Unal, 2021), is a major source of psychosocial stress associated with health problems, including depression (House et al., 1988). To mimic human SI in animal models, rodents are exposed to individual housing with regular auditory and olfactory stimulation but limited visual and tactile input (Garzón & Del Río, 1981). Individually housed rodents demonstrate prolonged impairment in reward-seeking behavior and various cognitive tasks, often accompanied with increased levels of anxiety and depressive-like behavior (Carnevali et al., 2012; Nakayasu & Ishii, 2008; Von Frijtag et al., 2000). Long-term SI hinders hippocampal neurogenesis (Stranahan et al., 2006), causes autonomic and cardiac dysregulation (Grippo et al., 2007), and alters the neuroinflammatory response to stroke (Karelina et al., 2009).

Elif Beyza Guven and Nicole Melisa Pranic contributed equally to this work.

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Several forms of nonpharmacological treatment have been tested to block or reverse the consequences of long-term stress in rodents. Environmental enrichment (EE), put forward by Donald Hebb (1947), proved very successful in blocking the adverse effects of different forms of chronic stress, including SI. Pioneering studies in the 1960s transformed EE to a standard rodent behavioral paradigm (Rosenzweig, 1966; Rosenzweig et al., 1962), by demonstrating the effects of environmental stimuli on a variety of large-scale neurobiological parameters, such as the "total brain weight" or "total DNA or RNA content" in the brain (Bennett et al., 1969; Rosenzweig et al., 1967; Rosenzweig & Bennett, 1969). Subsequent work showed its ameliorative effects on neurodegenerative diseases, traumatic brain injury, neurodevelopmental disorders, and psychopathologies, such as schizophrenia, depression, and anxiety (Laviola et al., 2008; Nithianantharajah & Hannan, 2006; Renoir et al., 2013). EE applications produced consistent findings in reversing the neuronal (Biggio et al., 2019; Cao et al., 2018; Monteiro et al., 2014), physiological (Vitalo et al., 2012; Watanasriyakul et al., 2019), and cognitive (Lambert & Guillette, 2021) deficits of SI but led to mixed results in terms of its affective consequences. Switching from SI to EE produced an antidepressant effect in rats (Brenes et al., 2020) and socially monogamous prairie voles (Grippo et al., 2014; Normann et al., 2021). For anxiety-like behavior, no anxiolytic effect was observed in rats following a switch from SI to EE (Mora-Gallegos & Fornaguera, 2019), unlike EE experiments that involve no change in living conditions (Peña et al., 2006). A more recent study found a significant anxiolytic effect in prairie voles when EE provided opportunity for voluntary exercise (Normann et al., 2021). Another study applying an SI to EE switch, in contrast, observed that isolated mice had lower levels of anxiety compared with grouped-housed mice (Lopez & Laber, 2015). In order to obtain a comprehensive understanding of the effects of EE on long-term SI, we assessed behavioral despair, anxiety-like behavior, and spatial working memory in the same experimental design. We combine these behavioral results with immunohistochemistry for the c-Fos protein and reveal neuronal correlates of the EE procedure in different cortical and amygdaloid structures.

Isolation and enrichment studies often utilize behavioral despair and anhedonia—two psychiatric endophenotypes that recapitulate the pathology of depressive disorders (Carrier & Kabbaj, 2012; Djordjevic et al., 2012; Gould & Gottesman, 2006; Zlatković et al., 2014). Studies using group-housed animals as a control demonstrate that a 21-day SI stress leads to behavioral despair, defined as increased immobility in the forced swim test (FST) (Unal & Canbeyli, 2019), as well as anhedonia, reflected as a decrease in sucrose preference. In contrast to SI, enrichment procedures offer therapeutic effects for both phenomena (Ashokan et al., 2018; Mitra & Sapolsky, 2009; Veena et al., 2009). Aforementioned work utilizing an isolation to enrichment switch replicated these results, showing an antidepressant effect in the EE group (Brenes et al., 2020; Grippo et al., 2014; Normann et al., 2021).

Unlike rodent models of depressive behavior, measures of anxiety produced contradictory findings in research that involve either SI or EE. Several long-term EE studies reported anxiolytic effects (Brenes Sáenz et al., 2006; Harati et al., 2013; Leal-Galicia et al., 2007; Leal-Galicia et al., 2008; Peña et al., 2006), while some others found no performance change on the elevated plus maze (EPM) (Goes et al., 2015), or report an opposite, anxiogenic effect (Mann & Gervais, 2011). The effects of SI on anxiety-like behavior also produced mixed results (Butler, Carter, & Weiner, 2014b); some reported anxiolytic effects (Chappell et al., 2013; McCool & Chappell, 2009; Zhang et al., 2012), whereas others showed no influence of SI on arm preference in the EPM (Butler, Ariwodola, & Weiner, 2014a; Butler, Carter, et al., 2014; Simpson et al., 2012). This inconsistency persists for the few experiments involving an SI to EE switch. An anxiolytic effect was found in prairie voles (Normann et al., 2021), while no significant change was observed in rats (Mora-Gallegos & Fornaguera, 2019), and an anxiogenic effect was found in enriched mice (Lopez & Laber, 2015).

The cognitive effects of switching from SI to EE are straightforward compared with the affective consequences. Different types of SI impair spatial working memory in humans (Volkers & Scherder, 2011) and other animals (Fischer et al., 2012; Gregory & Szumlinski, 2008; Zorzo et al., 2019), especially when it is introduced after weaning (Kosten et al., 2012). Long-term EE, in contrast, improves several types of memory (Harati et al., 2011), as observed in the radial arm maze (Bell et al., 2009), the Hebb-Williams maze (Kobayashi et al., 2002), and the Morris water maze (Nilsson et al., 1999; Schrijver et al., 2002). As in isolation protocols, the emergence of cognitive effects following EE is time-dependent. Birch et al. (2013) showed that a 6-week continuous EE period, but not 3 weeks, was able to improve working memory. EE-led increase in memory performance was associated with long-term synaptic plasticity (Stein et al., 2016) and hippocampal neurogenesis (Nilsson et al., 1999).

The positive cognitive effects of EE may or may not emerge following a stress paradigm. Some studies demonstrated that short-term EE following chronic stress overcame the stress-induced deficits in spatial memory (Hutchinson et al., 2012; Veena et al., 2009), while others indicated that spatial memory performance following acute stress was not influenced by housing conditions (Del Arco et al., 2007; Garrido et al., 2013; Segovia et al., 2008). Resocialization in standard cages following SI was sufficient to overcome

the cognitive deficits triggered by this stress model (Chen et al., 2016). Switching aged rats from SI to EE for 3 months led to a better performance in complex, blind-alley mazes, whereas switching from standard or enriched cages to isolation impaired this performance (Winocur, 1998).

These studies altogether show that the affective and cognitive alterations observed by enrichment often require relatively long periods of differential housing. Brief or acute enrichment models were mostly utilized in the aforementioned sucrose consumption studies (Grimm et al., 2013; Grimm et al., 2018; Grimm et al., 2019; Slaker et al., 2016). No study has investigated the effects of brief EE following long-term isolation stress. Therefore, we tested whether a very brief, 3-day, EE manipulation could reverse the affective and cognitive alterations led by long-term, SI-induced stress in adult Wistar rats. We assessed behavioral despair in the FST, anxiety-like behavior in the EPM, locomotor activity in the open field test (OFT), and spatial working memory performance in a water Y-maze (WYM) task. We recorded and correlated c-Fos immunoreactivity in different memory-related cortical and amygdaloid structures.

Materials and methods

Subjects

Adult male Wistar rats (290-340 g, $n = 16$) were housed individually (21 ± 1 °C; ~50% humidity; 12:12 day/night cycle, lights on at 7:00 a.m.) in small SI cages (36.5 × 16.5 × 12.5 cm) for 30 days, until being divided into experimental and control conditions based on their body weight and home cage (Fig. 1). Animals in the experimental group ($n = 8$) were placed together in a single, large EE cage (SI to EE), whereas control animals ($n = 8$) remained in their SI cages (continuous SI). Food and water were provided *ad libitum* for both groups throughout the experiment. All procedures were performed as approved by the Boğaziçi University Ethics Committee for the Use of Animals in Experiments.

Before the experiment, animals were housed in standard cages that contain four animals. The 16 animals in the study and the 8 animals in each group come from 4 different home cages.

Experimental design and environmental enrichment

After 30 days of SI, half of the rats were moved to the EE cage and remained there for a total of 10 days until perfusion-fixation (Fig. 1). The experimental testing started with the FST after 3 days of enrichment (of the SI to EE group) on Day 34 (Fig. 1). This was followed by the OFT, EPM, and WYM. Accordingly, behavioral despair analysis of the 2-day FST depends on 3-4 days of enrichment, whereas the results of the final test, the 5-day WYM reflect 5-10 days of differential housing (Fig. 1). Animals were perfused 24 h after they were returned to their cages following the last WYM trial (Fig. 1).

The EE procedure (Hebb, 1947; Krech et al., 1960) was implemented in a square (66 × 66 cm) transparent Plexiglas cage. It contained a running wheel, a small mirror, a nest box (10 × 10 × 10 cm), a ramp connecting to a platform (25 × 25 cm) 20 cm above the ground level, and six different plastic toys that were rearranged daily. As an additional enrichment procedure, animals were handled daily for approximately 2 min.

Forced swim test

The FST is a stress-inducing behavioral test developed to assess the efficacy of antidepressant agents and manipulations in rodents (Porsolt et al., 1977; Unal & Canbeyli, 2019). The stress response of the test, known as behavioral despair, was assessed over two consecutive days in an acrylic glass cylinder (diameter: 30 cm, height: 45 cm) filled with 30 cm water at 25 ± 0.5 °C. Following the standard procedure in rats, each animal was placed in the FST chamber for 15 min on the acclimatization/pretest day (FST-1) and for 5 min on the test day (FST-2) conducted after 24 h (Porsolt et al., 1977). Following FST-2, each animal was placed in a standard cage for drying for 30 min and then returned to its home cage. Each session was recorded with a video camera and coded by two observers blind to the experimental conditions. Periods of immobility (immobility scores) were

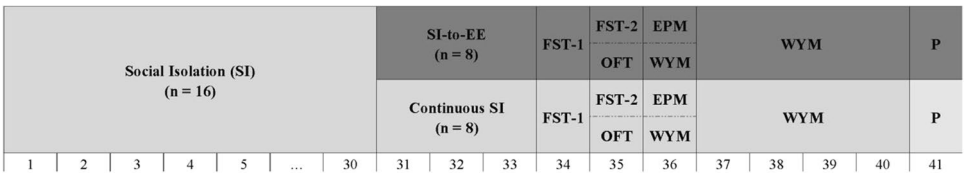

Fig. 1 Experimental timeline shows environmental manipulations and behavioral tests (above) along 41 days (below). Colors indicate social isolation (light grey) and environmental enrichment (dark grey). FST, forced swim test; OFT, open field test; EPM, elevated plus maze; WYM, water Y-maze; P, perfusion-fixation

averaged (interrater reliability: $r = 0.99$) and compared using an independent samples *t*-test.

Open field test

An open field (72 × 72 × 45 cm) was utilized to measure general locomotor activity levels. Each animal was taken to the test room 5 before the test and placed in the OFT chamber for another 5 min. Sessions were recorded on video by using the animal tracking software EthoVision (Noldus, Wageningen, NL) to assess total duration of mobility and specific behavioral patterns, including rearing and thigmotaxis.

Elevated plus maze

An EPM (Handley & Mithani, 1984; Pellow et al., 1985; Pellow & File, 1986) set up at 51 cm above the ground level was used to assess anxiety-like behavior. It consists of a wooden base with four narrow arms (50 × 10 cm). Two opposite arms are covered with transparent acrylic glass sides and are known as open arms, whereas the other two are closed with wooden sides, leading to substantially darker corridors. Each animal was placed in the central compartment and left in the maze for 5 min. Total time spent in the center of the maze, in open arms and in closed arms, was recorded and calculated with EthoVision. Time spent in open arms indicates an anxiolytic effect while closed arms are associated with anxiety-like behavior (Walf & Frye, 2007).

Water Y-maze

We utilized a modified version of the water T-maze developed by Del Arco et al. (2007) and validated by Locchi et al. (2007). An acrylic glass Y-maze consisting of three 120° apart arms (45 × 15.5 × 45 cm each) was filled with 30 cm water at 24 ± 0.5 °C to assess spatial working memory performance via spontaneous alternation. Each rat was tested for 5 days with six consecutive trials per day, separated by an intertrial interval of 30 s. Animals were brought to the test room 5 min before the first trial for familiarization with the environment. In each trial, animals were placed on the far side of the starting arm with their head pointing to the center of the maze, towards which they swim to choose one of the two target arms (Arm A or Arm B). A small transparent platform was placed 1 cm below the water level in one of the target arms (Arm A or Arm B) in a counterbalanced fashion at the beginning of the first trial and alternated between the two target arms for each trial. Animals that were not able to locate the platform in 60 s were gently guided toward it by their tail. Following the last trial, they were placed in a small cage to dry for 40 min before being returned to their home cages. Each session was recorded on video using EthoVision. Latency to locate the hidden platform in each trial, and the number of wrong arm entries (errors) were coded.

Perfusion-fixation, tissue processing, and immunohistochemistry

Perfusion-fixation of the animals was performed on Day 41, 24 h after the last behavioral test (WYM Day 5). Animals were removed from their (SI or EE) cages, deeply anesthetized with a ketamine-xylazine solution (100-15 mg/kg, IP), and perfused with 4% depolymerized paraformaldehyde (pH 7.4). Following overnight post-fixation in the same solution, coronal sections (40-50 µm) were cut with a vibratome (VT1000 S, Leica Biosystems, Nussloch, Germany) and rinsed in phosphate-buffered saline (PBS; pH 7.4).

Expression of the c-Fos protein was revealed by immunohistochemistry to assess the effect of housing conditions on the number of spontaneously active neurons in various brain regions. Tissue penetration was achieved by adding 0.05% Triton X-100 (pH 7.4) to PBS (PBS-Tx). Sections were treated with hydrogen peroxide (0.6%) for 30 min at room temperature (RT) before being transferred to the blocking solution. They were blocked with 20% normal goat serum (NGS; Vector Laboratories) in PBS-Tx for 30 min at RT and incubated with an anti-c-Fos antibody (mouse monoclonal, Santa Cruz 8047, 1:500 concentration) in PBS-Tx with 1% NGS for 3 nights at 4 °C. Sections were then rinsed in PBS (3 × 10 min), transferred to a secondary antibody solution (biotinylated anti-mouse IgG, 1:250 in PBS-Tx with 1% NGS), and incubated for 2 h at RT. Following another series of rinsing, they were incubated with avidin-biotinylated peroxidase (ABC kit, Vector Laboratories) in PBS for 20 min at RT. The peroxidase product emerged with a nickel-enhanced 3,30-Diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma-Aldrich D5637).

Microscopy and cell counting

We processed a total of 48 sections from 12 animals (6 Continuous SI and 6 SI to EE; 4 sections per animal) with good perfusion quality. The number of c-Fos-immunopositive (c-Fos+) nuclei was quantified for the retrosplenial cortex (RSC), agranular perirhinal cortex (Area 35; PRC), lateral nucleus of the amygdala (LA), and basolateral nucleus of the amygdala (BL). Regions of interest were located by cytological comparison with a rat brain atlas (Paxinos & Watson, 2006). Images were obtained with an epifluorescent microscope (Olympus BX53) equipped with a monochrome CCD camera (Olympus XM10). Analyses were restricted to the tissue selected before immunohistochemistry and no additional sections were processed after data acquisition.

Labelled cells were counted in a semi-automatic fashion. First, the number of c-Fos+ cells in the aforementioned regions were detected and quantified with a cell-counting plugin (ITCN) in ImageJ (Schneider et al., 2012). The ITCN produced output images showing the detected cells within each region. Two independent scorers blind to the experimental conditions evaluated these images to identify and count false-negative cases (i.e., faintly labelled cells missed by the software). The arithmetic average of these two false-negative cell counts was added to the total number of cells detected by the ITCN for each section.

Results

Weight change

Body weight of the animals were recorded at four time points, both before (Days 1, 15, and 31) and after the EE manipulation (Day 41). We found an effect of time on body weight (F (3, 42) = 7.69, $p < 0.001$) and a weighing time-group interaction (F (3, 42) = 3.30, $p = 0.029$, 2 × 4 two-way mixed ANOVA), pointing to a significant weight loss in EE animals following enrichment: weights on Day 1 (M = 324.6, SD = 15.7), Day 15 (M = 334.6, SD = 13.3), and Day 31 (M = 325.5, SD = 21.6) were higher than that of Day 41 (M = 311.9, SD = 14; all ps < 0.05). There was no such change in the continuous SI group (Day 1, M = 321, SD = 12.5; Day 15, M = 325, SD = 15.5; Day 31, M = 322.3, SD = 15.5; Day 41, M = 320.1, SD = 12.9).

Behavioral despair

We assessed the effects of brief EE manipulation on behavioral despair following SI-induced stress by recording and comparing the overall FST-2 immobility scores (Slattery & Cryan, 2012; Yankelevitch-Yahav et al., 2015). The immobility level of the SI to EE group (M = 49.04, SD = 21.77) was significantly higher than that observed in the continuous SI group (M = 29.47, SD = 13.04; t (14) = 2.18, $p = 0.047$, d = 1.09, independent samples t-test; Fig. 2), pointing to behavioral despair in animals exposed to brief EE, and a relative antidepressant effect of continuous SI. It should be noted that the baseline, i.e., FST-1, immobility scores of the continuous SI (M = 41.32, SD = 17.02) and SI to EE animals (M = 38.18, SD = 11.42) were very similar. Hence, the difference observed in the test phase of the FST was not due to a baseline difference in locomotor activity. Likewise, no alternation in general locomotor activity levels was observed following FST-2, as assessed in the OFT (below).

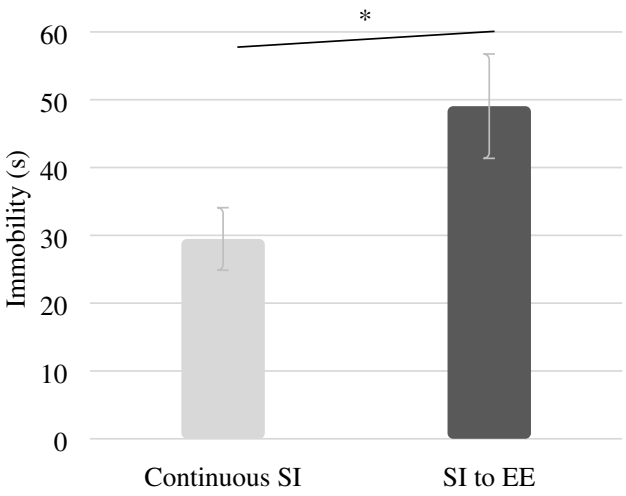

Fig. 2 Overall FST-2 immobility durations of the continuous SI (light grey) and SI to EE (dark grey) animals show a significant difference between the groups at α level 0.05 (asterisk), indicating behavioral despair in the SI to EE group. Error bars denote SEM

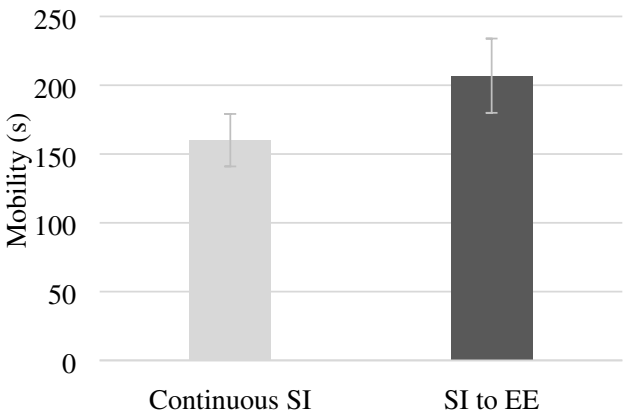

Fig. 3 Total duration of mobility in the OFT for continuous SI (light grey) and SI to EE (dark grey) animals depict no difference. Error bars denote SEM

Locomotor activity and exploratory behavior

We utilized the OFT to assess potential differences in locomotor activity between the continuous SI and SI to EE animals. There was no difference between the control (M = 160.05, SD = 53.95) and experimental animals (M = 206.93, SD = 76.36; t (14) = 1.42, $p = 0.178$, independent samples t-test; Fig. 3), showing that the metabolic (i.e., weight change) effect of brief EE did not alter overall locomotor activity and interfere with the FST. Rearing behavior, an indicator of exploratory behavior, did not show a significant difference between the groups either, although SI to EE animals often displayed more rearing (M = 38.75, SD = 31.91) compared with continuous SI animals (M = 18.75,

SD = 11.83; *t* (14) = 1.66, *p* = 0.119, independent samples *t*-test). The result did not change when the observations were restricted to the center (SI to EE *M* = 2.25, *SD* = 1.75 vs. continuous SI *M* = 2.38, *SD* = 2.77) or the periphery (SI to EE *M* = 34.25, *SD* = 34.44 vs. continuous SI *M* = 16.38, *SD* = 9.93) of the OFT (all *p*s > 0.05). Irrespective of the groups, the majority of the rearing behavior was displayed at the periphery, often against the walls (*M* = 25.31, *SD* = 25.34) as opposed to the center (i.e., free-standing rearing; *M* = 2.31, *SD* = 2.17; *t* (15) = 3.54, *p* = 0.003, *d* = 1.28, paired samples *t*-test).

Anxiety

Differential rearing and total duration spent at the center versus periphery of the OFT indicates an alteration of general anxiety levels. Following this observation, we employed a standard anxiety measure—the EPM—and compared the anxiety-like behavior of the continuous SI and SI to EE animals. We found that the SI to EE group spent significantly more time in the open arms (*M* = 169.25, *SD* = 106.59) compared with control animals (*M* = 60.87, *SD* = 77.51; *t* (14) = −2.33, *p* = 0.036, *d* = 1.16, independent samples *t*-test; Fig. 4), indicating an anxiolytic effect of brief enrichment following SI-induced stress.

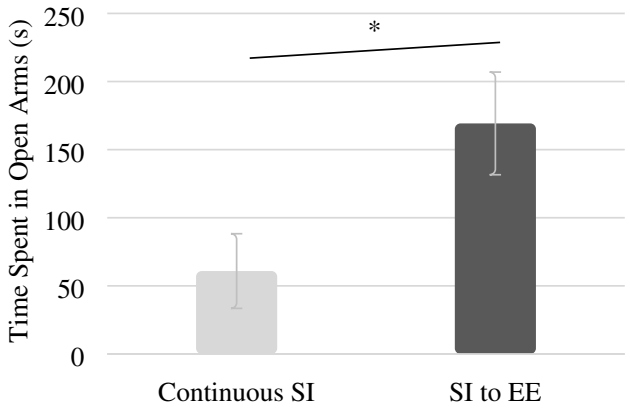

Fig. 4 Time spent in the open arms of the EPM by continuous SI (light grey) and SI to EE (dark grey) animals, point to an anxiolytic effect of the brief EE. Asterisk stands for statistical significance at α level 0.05. Error bars denote SEM

Spatial working memory

Spatial working memory performance was assessed across five consecutive days in the WYM. We found a significant effect of the test day, as measured by mean latency to locate the hidden escape platform (*F* (1.47, 20.64) = 12.95, *p* = 0.001, 2 × 5 two-way mixed ANOVA), revealing that learning was achieved. Bonferroni corrected post-hoc tests showed that latency to locate the platform was significantly higher on the first day (*M* = 15.26, *SD* = 10.13) compared with the third (*M* = 6.79, *SD*= 2.25), fourth (*M* = 4.73, *SD* = 1.44), and fifth (*M* = 6.29, *SD* = 2.03) days of the WYM (all *p*s < 0.05; Fig. 5). Neither a test day-condition interaction (*F* (1.47, 20.64) = 2.54, *p* = 0.115) nor a main effect of the experimental condition (*F* (1, 14) = 3.87, *p* = 0.069, 2 × 5 two-way mixed ANOVA) was found.

We analyzed the total number of wrong arm entries (i.e., errors) across trials as an additional measure of working memory performance and found a significant effect of the test day (*F* (4, 56) = 4.71, *p* = 0.002, 2 × 5 two-way mixed ANOVA). Bonferroni corrected post-hoc tests revealed that the total number of errors decreased significantly on the fourth day (*M* = 8.69, *SD* = 1.82) compared with the first day of the WYM (*M* = 12.69, *SD* = 4.27; *p* < 0.05), indicating learning was achieved. There was no main effect of the

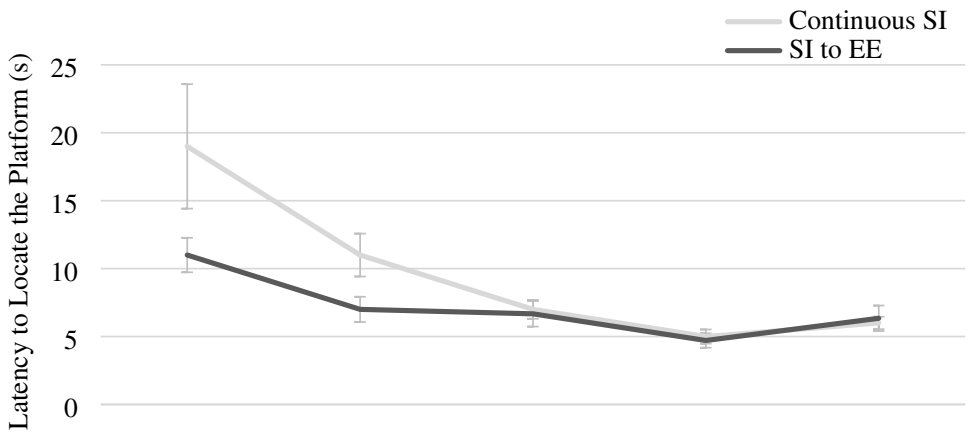

Fig. 5 Mean latency to locate the escape platform for continuous SI (light grey) and SI to EE (dark grey) animals across 5 days of the WYM. Error bars denote SEM

experimental condition (F (1, 14) = 2.46, p = 0.139, 2 × 5 two-way mixed ANOVA) or a test day-condition interaction (F (4, 56) = 1.34, p = 0.269). The error rate analyses indicate that learning was achieved by both groups on the fourth day. However, the SI to EE animals displayed a seemingly better performance in the first 2 days of the WYM (Fig. 5). This difference in latency to locate the platform became significant on the second day of WYM (SI to EE M = 7.13, SD = 2.63 vs. continuous SI M = 11.09, SD = 4.47; t (14) = 2.17, p = 0.048, d = 1.08, independent samples t-test), pointing to faster learning in the early phase of the memory task.

Neuronal activity

We quantified the number of c-Fos+ cells in each region of interest, took the arithmetic average for each animal, and made group-level comparisons (Fig. 6). Examined sections were acquired at similar rostra-caudal levels (−3.0 to −3.6 from the Bregma point; Paxinos & Watson, 2006) to minimize the effect of topographical variability on neuronal activity. As shown in representative c-Fos immunolabeling depictions (Fig. 6), we found that the number of c-Fos+ cells in the RSC were significantly different for continuous SI (M = 2356.5, SD = 438.4) and SI to EE animals (M = 3443.7, SD = 856.3; t (10) = 2.77, p = 0.02, d = 1.6, independent samples t-test; Fig. 7). Similarly, in the PRC, continuous SI animals (M = 739.7, SD = 201.4) had significantly lower number of c-Fos+ cells compared with the experimental group (M = 2398.3, SD = 1070.6; t (10) = 3.73, p = 0.004, d = 2.15, independent samples t-test; Fig. 7).

No such difference was observed in the LA (continuous SI: M = 823.6, SD = 225.6 vs. SI to EE: M = 957, SD = 584.4; t (8) = 0.48, p = 0.647, independent samples t-test; Fig. 7) or BL (continuous SI: M = 588.2, SD = 414.5 vs. SI to EE: M = 1176.4, SD = 677.7; t (8) = 1.66, p = 0.136, independent samples t-test; Fig. 7).

Discussion

We show that a brief EE following 30 days of social isolation produced opposing results in depression- and anxiety-like behavior. Compared with the control group, animals switched from SI to EE showed increased behavioral despair in the FST. In contrast, we found a substantial anxiolytic effect in the same group after 5 days of enrichment. Briefly, enriched animals following SI displayed a better spatial working memory performance during the first 2 days of the WYM compared with the continuous SI group, indicating faster learning. Brief enrichment was associated with

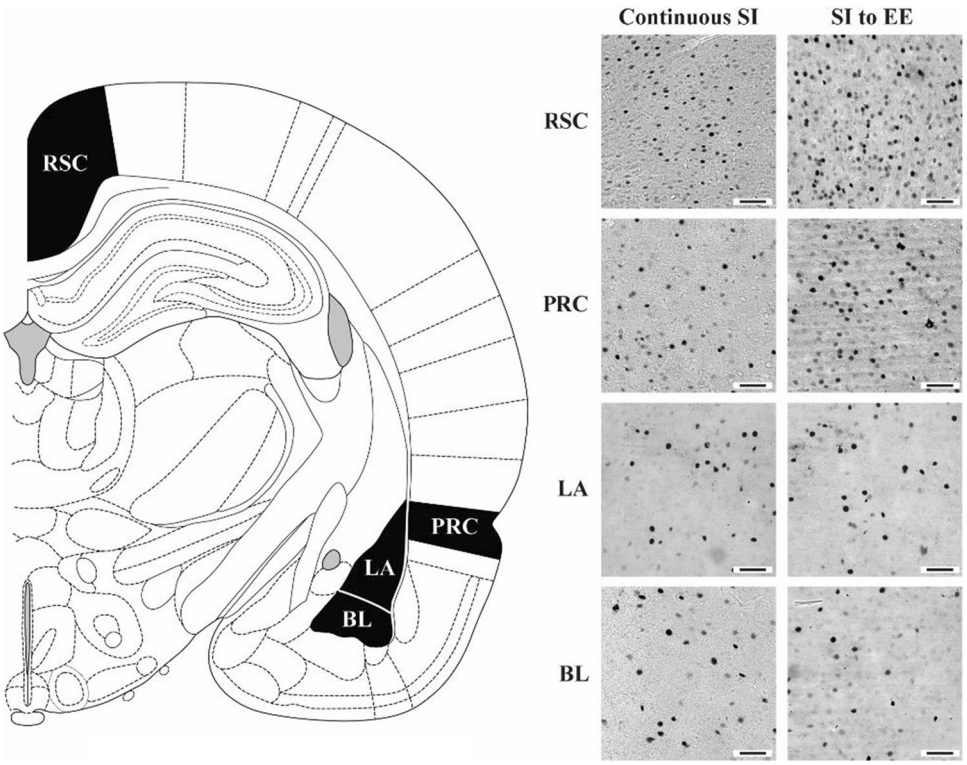

Fig. 6 c-Fos-immunopositive cells were counted in the retrosplenial (RSC) and perirhinal cortices (PRC), and the lateral (LA) and basolateral nuclei of the amygdala (BL; black regions on the coronal image). The panel on the right shows representative light microscopic images from each group (columns) for each region of interest (rows). Scale bars denote 50 µm

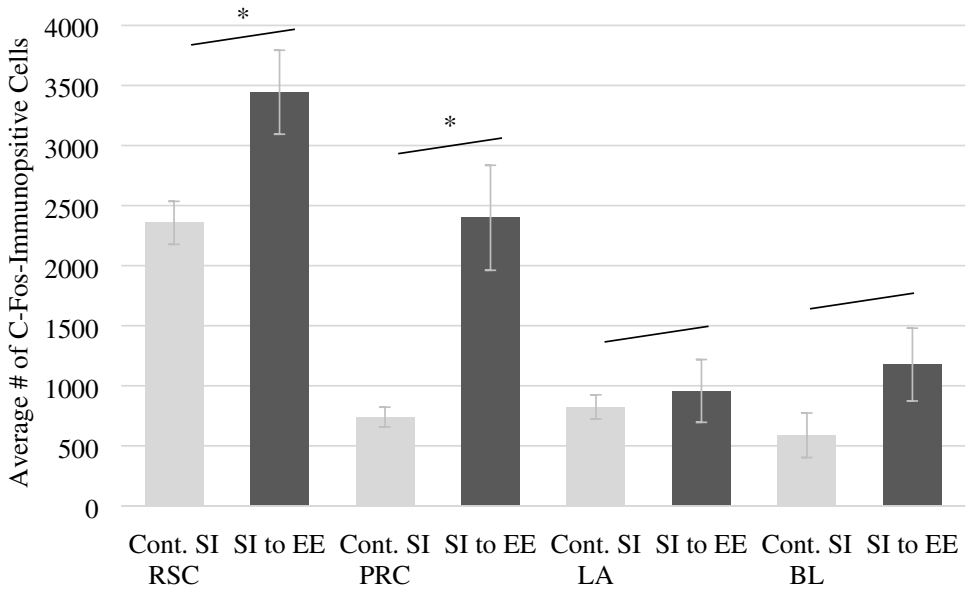

Fig. 7 Comparison of the number of c-Fos-immunopositive cells between continuous SI and SI to EE animals in each region of interest. RSC, retrosplenial cortex; PRC, perirhinal cortex; LA, lateral nucleus of the amygdala; BL, basolateral nucleus of the amygdala. Depicted with the same y-axis for simplicity, please note that different regions of interest (horizontal square brackets) cannot be compared with each other due to differences in surface area (see the coronal image in Fig. 6) and cytoarchitecture. Asterisks point to a significant difference between the groups at α level 0.05. Error bars denote SEM

changes in neuronal activity levels in the retrosplenial and perirhinal cortices. SI to EE animals had significantly higher number of c-Fos+ cells in these cortical regions as compared to the control animals.

Replicating previous research stating a weight drop in EE (Harati et al., 2011; Moncek et al., 2004; Zaias et al., 2008), body weight of the SI to EE animals decreased significantly following enrichment. This was in contrast to other work reporting no effect of chronic (Glueck et al., 2017; Grimm et al., 2016, 2018) or brief enrichment (Beale et al., 2011) on weight. The running wheel provided in the enrichment cage (Augustsson et al., 2002; Harati et al., 2011; Stein et al., 2016) and the initial short-term feeding suppression observed in pair-housed animals following isolation (Lopak & Eikelboom, 2000; O'Connor & Eikelboom, 2000; Weisinger et al., 1989) may have accounted for this difference. As long-term isolation does not alter food consumption (Hellemans et al., 2004) or body weight (Fone & Porkess, 2008), no such weight change was observed in continuous SI animals.

It should be noted that behavioral despair observed in the SI to EE group was not a reflection of alterations in metabolic difference, as the overall locomotor activity in the OFT did not differ between the groups. This finding is noteworthy given that chronic or subchronic SI can itself be used as a rodent model of depression (Djordjevic et al., 2015; Stanisavljević et al., 2019). In contrast, there are contradicting results for the effects of EE on behavioral despair. Some studies report no significant effect (Cui et al., 2006; Simpson et al., 2012), whereas others found increased mobility in the test phase of the FST, indicating an antidepressant effect (Brenes et al., 2008; Cui et al., 2006; Porsolt et al., 1977). One reason for the depressant effects of brief EE observed in our study could be the novelty stress induced by the enrichment cage following relatively long-term isolation with minimal environmental stimulation (Hennessy & Foy, 1987; Miura et al., 2002). A phenomenon also observed in humans, novelty stress was associated with alterations in synaptic monoamine levels and heightened HPA axis activity (Miura et al., 2002). However, the depressant effect of brief EE in our study was accompanied by a substantial anxiolytic effect observed in the EPM (Fig. 4).

Environmental enrichment can also function as a social stressor by producing crowding stress, especially in male rats that are not familiar with each other (Brown & Grunberg, 1995). It should be noted that the eight animals in the SI to EE group originally came from four different home cages, making most of the other animals in the EE cage unfamiliar. Crowding, like the isolation stress, is correlated with elevated plasma levels of adrenocorticotropic hormone (ACTH) and corticosterone (CORT) (Dronjak et al., 2004). Albeit these potential alterations, brief enrichment in this study produced a profound anxiolytic effect, as observed in the majority of earlier research (Galani et al., 2007; Harati et al., 2013; Leal-Galicia et al., 2008; Leal-Galicia et al., 2007; Peña et al., 2006; Sampedro-Piquero et al., 2014; but

see Goes et al., 2015; Mann & Gervais, 2011). Long-term isolation, in contrast, often leads to an anxiogenic effect in the EPM (Djordjevic et al., 2015; Hall, 1998), as well as other measures of anxiety-like behavior (Spasojevic et al., 2007; Zlatković et al., 2014). Similar to the design of the present study, Ravenelle et al. (2013) showed that rats bred to display high-anxiety and exposed to post-weaning EE spent more time in the open arms of the EPM as compared to impoverished high-anxiety rats.

These finding show that, unlike behavioral despair, the relationship between living (environmental) conditions and anxiety is straightforward: increase in enrichment decreases anxiety (Benaroya-Milshtein et al., 2004; Ravenelle et al., 2014; Sampedro-Piquero et al., 2013). In line with the present findings, other brief EE experiments reported anxiolytic effects for as short as 2 weeks of enrichment (Briones-Aranda et al., 2020). The length and intensity of enrichment may not have been sufficient in the few studies that did not report an anxiolytic effect of enrichment (see Goes et al., 2015; Simpson & Kelly, 2012). EE cage in the current study included a running wheel for voluntary exercise, which further decreases anxiety-like behavior in the EPM (Binder et al., 2004; but see Burghardt et al., 2004).

To assess cognitive effects of brief enrichment, we chose a simple but versatile memory task, the WYM spontaneous alternation test. This spatial working memory measure depends on several structures including the prefrontal cortex, hippocampus and basal forebrain. Impoverished housing conditions and social isolation lead to several plasticity-related alterations in the cortex (Gregory & Szumlinski, 2008; Ieraci et al., 2016; Popa et al., 2020), accompanied by an impairment in spatial working memory tasks (Gregory & Szumlinski, 2008; Melendez et al., 2004). Enrichment, in contrast, often produces the opposite effect at the neuronal and behavioral level (Sampedro-Piquero et al., 2013). Our findings show that 5 days of enrichment was strong enough to increase spatial working memory performance following 30 days of SI stress. This difference did not persist in the third day of the test when both groups displayed a significant decrease in their latency to locate the correct platform. The stressful nature of the WYM may have contributed to these results. The WYM task, like other water mazes, relies on the animal's inherent motivation to escape an aversive condition (i.e., water). Differences in anxiety levels may effect memory performance in the initial sessions before animals learn that there is an escape platform. Such a differential effect would be transient as there were six consecutive trials per day and animals that were not able to locate the platform in 60 s were gently guided toward it at the end of each trial.

We correlated these differential behavioral findings with c-Fos immunohistochemistry. As the peak level of c-Fos protein expression occurs approximately 90 min before perfusion, the group-level differences observed in neuronal activity mainly reflect the different environmental conditions. However, some of these differences may be due to sustained long-term alterations in neuronal circuits caused by brief EE and the differential effects of subsequent behavioral testing. In theory, brief EE may have formed new circuits and modified old ones in specific regions, which would result in differences in the number of active neurons under the same conditions (Nikolaev et al., 2002; Zorzo et al., 2019). In our study, it was not possible to isolate these sustained alterations in neuronal activity from the transient differences reflecting activity approximately 90 min before perfusion.

In SI to EE animals, we recorded significantly more c-Fos+ cells in the retrosplenial cortex, an association cortex involved in allocentric spatial navigation and memory among several other functions (Hindley et al., 2014; Vann & Aggleton, 2002). In addition, these animals had more c-Fos+ cells in the perirhinal cortex, the cortical region underlying object recognition and associative memory (Samarth et al., 2017; Unal et al., 2012). This structure, providing the densest afferent of the entorhinal cortex, is also required for spatial working memory (Liu & Bilkey, 2001). Both results complement the faster learning observed among SI to EE animals in the early phase of the WYM.

There was no group-level difference in c-Fos-immunolabeling in the lateral and basolateral nuclei of the amygdala. An earlier study on FosB/DFosB-immunoreactivity in the basolateral amygdala complex (BLA) and medial prefrontal cortex (mPFC) following an SI to EE (4 weeks each) switch found significantly more immunolabeling in the BLA of isolated animals and in the mPFC of SI to EE animals (Watanasriyakul et al., 2019). The higher number of c-Fos+ cells found in the retrosplenial and perirhinal cortices of SI to EE animals in our findings are in line with the aforementioned observation made in the mPFC. In contrast, we did not observe a meaningful difference in the LA or BL. It is likely that the c-Fos immunoreactivity observed in the amygdala mostly reflects transient changes in neuronal activity: neurons with a high level of firing approximately 90 min before perfusion. Hence, the differential behavioral results observed in the FST and EPM do not correlate with a sustained difference in neuronal activity.

Albeit its popularity in behavioral neuroscience, there is a major discussion on the validity of the FST as a rodent model of clinical depression. One line of criticism considers the immobility in the FST as an adaptive behavior rather than an indication of behavioral despair (Anyan & Amir, 2018; Borsini et al., 1986; Molendijk & de Kloet, 2015, 2019). According to this point of view, increased FST-2 immobility of the SI to EE group may not be indicative of behavioral despair; but reflect better behavioral adaptation (or learning) of the briefly enriched group. This possibility cannot be ruled out completely (see Unal

& Canbeyli, 2019 for a detailed survey of the behavioral adaptation theory of the FST). However, if the increased immobility of the experimental group were solely arising from their enhanced capacity for adaptation, it would likely be reflected in their overall WYM performance. The FST-2 was conducted with four days of enrichment, whereas the last WYM session was done after spending 10 days in the EE cage. It is therefore likely that most, if not all, of the immobility difference in the FST reflects group-level differences in affective processing.

One limitation of the present study was the exclusive use of male rats. Laboratory rats of different strains, like humans, show sex differences in susceptibility to anxiety and different depressive symptoms like anhedonia (Unal & Moustafa, 2021). As for the majority of neurobiological research using rodent models, this technical constraint restricts the generalization of our findings. Another limitation was the lack of stress hormone measurements. Our design was confined to behavioral measures of stress and *ex vivo* assessment of recent neuronal activity by means of c-Fos immunohistochemistry.

Overall, we showed that a brief EE procedure was sufficient to produce a substantial anxiolytic effect following SI stress. Strikingly, it produced the opposite result in behavioral despair. SI to EE animals displayed significantly higher immobility in the test phase of the FST, while continuous SI had a relative antidepressant effect. The brief enrichment period was sufficient to accelerate spatial working memory performance in the early phase of the WYM. Significantly higher expression of the c-Fos protein in retrosplenial and perirhinal cortices of the SI to EE animals complemented this observation. As social isolation went beyond its use as a laboratory model and became a usual part of daily life with the COVID-19 pandemic (Unal, 2021), environmental enrichment attracted attention as a potential translatable paradigm against human isolation (Davim et al., 2020; Rojas-Carvajal et al., 2021). Our results revealed differential effects in depressive- and anxiety-like behavior of enrichment following relatively long-term isolation. This indicates that unlike the cognitive effects, the affective consequences of switching from an impoverished to an enriched condition are not straightforward. An important question is whether these findings apply to humans who are exposed to relatively enriched conditions following long-term social isolation.

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Declarations

Conflicts of interest None

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Open practices statement Numerical data, video recordings, and photomicrographs for all experiments reported are available per request.