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ORIGINAL PAPER

Environmental Enrichment Differentially Activates Neural Circuits in FVB/N Mice, Inducing Social Interaction in Females but Agonistic Behavior in Males

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Abstract

Environmental enrichment induces behavioral and structural modifications in rodents and influences the capability of mice to cope with stress. However, little is understood about hippocampal neurogenesis and the appearance of social/agonistic (aggressive) behavior upon activation of different neuronal circuits in FVB/N mice. Thus, in this study we hypothesized that environmental enrichment differentially regulates neurogenesis, neural circuit activation and social/agonistic behavior in male and female FVB/N mice. We explored the (1) neurogenic process as an indicative of neuroplasticity, (2) neuronal activation in the limbic system, and (3) social behavior using the resident-intruder test. On postnatal day 23 (PD23), mice were assigned to one of two groups: Standard Housing or Environmental Enrichment. At PD53, rodents underwent the resident-intruder test to evaluate social behaviors. Results revealed that environmental enrichment increased neurogenesis and social interaction in females. In males, environmental enrichment increased neurogenesis and agonistic behavior. Enriched male mice expressed higher levels of agonistic-related behavior than female mice housed under the same conditions. Neural circuit analysis showed lower activation in the amygdala of enriched males and higher activation in enriched females than their respective controls. Enriched females also showed higher activation in the frontal cortex without differences in male groups. Moreover, the insular cortex was less activated in females than in males. Thus, our results indicate that environmental enrichment has different effects on neuroplasticity and social/agonistic behavior in FVB/N mice, suggesting the relevance of sexual dimorphism in response to environmental stimuli.

Keywords Environmental enrichment · Neurogenesis · Hippocampus · Social behavior · Agonistic behavior · Neuronal activation · FVB/N mice · Adolescence

Introduction

Environmental enrichment is a paradigm providing social, cognitive, and physical stimuli to rodents [1]. It induces structural alterations in the brain and behavioral modifications [1]. Regardless of the exposure time, environmental enrichment increases cortical thickness, dendrite maturation, the establishment and maturation of dendritic spines, and neurogenesis in the dentate gyrus (DG) [2–7]. This paradigm also induces neurochemical changes such as increased levels of brain-derived neurotrophic factor and vascular endothelial growth factor, and modifications in neurotransmitters [1, 4, 5]. Studies have proven that environmental enrichment favors social interaction and has a positive effect on learning and memory processes and mood-related behaviors [8–11] [6–13]. Despite the effects on social behavior, controversial

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evidence indicates that the benefits of environmental enrichment are influenced by mouse strain, age, or sex [14–24]. For instance, adult male 129S6/SvEv mice exhibited agonistic social behavior (i.e., increased number of attacks to their conspecifics), which decreased after housing in environmental enrichment [18]. Moreover, environmental enrichment increased aggressive behavior in adult male CD-1 mice but not in male Balb/C mice [14]. Further, adolescent male NMRI mice housed in environmental enrichment spent more time exhibiting agonistic behavior than mice housed in standard conditions [13]. These studies suggest that environmental enrichment favors social behavior depending on the mouse strain [25, 26]. It is important to note, however, that most of these studies have been performed in male mice. Thus, further research is needed to understand the influence of enriched environments on social behavior in both male and female mice [13, 27], as well as the neuroplastic changes occurring in brain regions related to social behavior, such as the frontal cortex (FCx), the insular cortex (ICx), the amygdala (Am), and the DG [28, 29].

In the present study, we analyzed the impact of environmental enrichment on social behavior, the neurogenic process, and neuronal activation in the limbic system. We hypothesized that housing female and male FVB/N mice in an enriched environment increases neurogenesis in the DG and elicits different social behaviors concomitantly with the activation of the neuronal circuit of the limbic system. Neuronal circuit activation was mapped with the expression of immediate early genes (IEGs), such as the activity-regulated cytoskeletal-associated protein (Arc) [16, 30], after the behavioral test [31]. IEGs have a temporal expression induced in response to several stimuli [31, 32]. Here, we used male and female FVB/N mice. The basal neurogenesis of this inbred strain is similar to that of Balb/C and 129/SvJ mice but lower to that of C57/BL/6 mice [33].

Materials and Methods

Animals

Female and male GFAP-GFP transgenic FVB/N mice were used for this study and kindly donated by Professor Helmut Kettenmann (Max Delbrück Center for Molecular Medicine, Berlin, Germany). They express the green fluorescent protein (GFP) under the control of human glial fibrillary acidic protein promoter (GFAP) [34]. Mice were housed in standard laboratory cages under a 12-h light/12-h dark cycle (light phase: ZT0 = 1900, dark phase: ZT12 = 0700) at a temperature of 23 ± 1 °C with free access to food and water. Animal use and handling procedures complied with the Mexican Official Standard for animal care (NOM-062-ZOO-1999) and were approved by the local Institutional Ethics Committee of the National Institute of Psychiatry "Ramón de la Fuente Muñiz" (IACUC: CEI/C/009/2013). On postnatal day 23 (PD23), rodents were divided into two groups: (1) Standard Housing (SH) or (2) Environmental Enrichment. The animals were given a single dose of BrdU injected intraperitoneally (50 mg/Kg; Sigma) on PD37, and the behavioral test was performed on PD53.

Experimental Design

At PD23, an age that corresponds to the preadolescence period [35, 36], mice were randomly assigned to one of the following groups: (a) Standard Housing (For males: MSH, $n = 7$. For females: FSH, $n = 7$) or (b) Environmental Enrichment (For males: MEE, $n = 6$. For females: FEE, $n = 8$) (Fig. 1A, B). The enriched environment contained large boxes (34 × 44 × 20 cm) with running wheels and tunnels of different colors and shapes. Mice were placed in

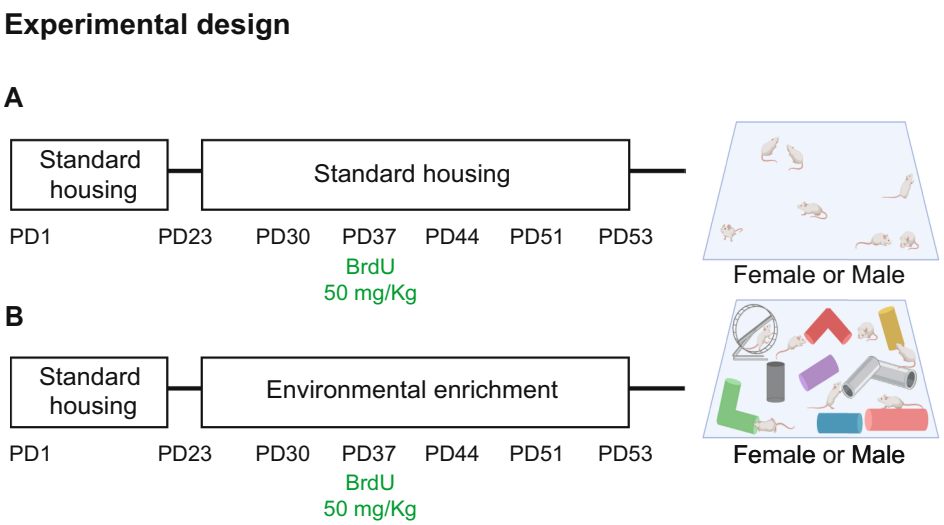


Fig. 1 Experimental design. FVB/N mice were exposed to *enriched* or standard housing to evaluate the effects of environmental enrichment on aggression. Female and male FVB/N mice ($N = 28$) were housed in standard conditions (**A**) or environmental enrichment (**B**) from preadolescence until late adolescence (PD23-53). On PD37, mice received a single BrdU injection (50 mg/kg). Then, on PD53, rodents were exposed to the resident-intruder test

the enriched environment or normal housing conditions for 30 days, from PD23 to PD53. The complexity of the tunnels was modified every third day. Mice in standard conditions were housed in cages of similar size to environmental enrichment cages (Fig. 1B).

Resident-Intruder Paradigm

At the end of the environmental enrichment (PD53), an open field test was performed, followed by the resident-intruder test to evaluate social behaviors (Fig. 2A). Between 0700 and 0800 h, mice were tested in a transparent Plexiglass box (L: 50 cm × l: 25 cm × H: 31 cm) in an experimental room and under red lighting (44 lx). Observations of enriched and control mice were alternated to minimize any possible influence of the time of exposure to the test. The floor of the cage was covered with clean sawdust. The camera was outside the plexiglass box, and all the recordings were done in a visual horizontal field for 5 min. Positive social behaviors (following, oral-anal sniffing and allogrooming) and agonistic (aggressive) behaviors (mounting and biting/fighting) were analyzed. Further, the number of behavioral events, the duration of the events, and the latency to the first occurrence of an event were quantified (Figs. 2, 3).

Histology

Two hours after the behavioral test, the animals were euthanized and the brain was collected to analyze neurogenesis by evaluating markers that indicate cell proliferation and survival (Ki67, BrdU, respectively). Neuronal activation was analyzed by identifying the Arc protein. Brains were removed and fixed with 4% p-formaldehyde (PFA) in 0.1 M phosphate buffer (pH = 7.4) for five days before being stabilized in phosphate buffer containing 30% sucrose. Serial coronal sections were cut at a thickness of 40 µm using a sliding microtome (Leica) and stored at 4 °C in a cryoprotective solution until required. Brain coronal sections were incubated with primary antibodies to detect Ki67 and BrdU, and the peroxidase DAB method was performed [37]. Positive cells were quantified in every 6th section from all animals. Ki67- or BrdU-positive cells were visualized using a 40× objective throughout the rostrocaudal axis. Counting was done as previously described using the modified optical dissector method under bright field light microscopy (Leica). The cells appearing in the uppermost focal plane were excluded to avoid over-sampling. The resulting numbers were multiplied by six to obtain the estimated total number of Ki67, BrdU-, and Arc-labeled cells per granule cell layer. Also, cellular activation was quantified with Arc detection in the DG, FCx, ICx, and Am. Arc-positive cells were quantified in the FCx (interaural 2.10 mm to 0.16 mm), DG (Bregma Bregma −1.34 to −3.20), ICx (Bregma 2.34 to −0.94), and Am (Bregma −0.10 to −0.3.16) [38]. The antibodies used were rabbit anti-Ki67 (1:1000; Abcam), rat anti-BrdU (1:500; Accurate Chemical), or rabbit anti-Arc (1:1000; Santa Cruz Biotech). GFP expression in the DG was analyzed with a confocal microscope (LSM510 META, Zeiss) to identify GFAP + cells corresponding to radial glial cells (RGCs) or cells with horizontal projections to the DG. In this case, nuclei were stained with propidium iodide (Santa Cruz Biotech). In addition, newborn neurons were analyzed by identifying the co-labeling of BrdU (1:250; Accurate Chemical) with calbindin (1:500; Abcam) under a confocal microscope (LSM 510 META, Zeiss).

Statistical Analysis

Analyses were performed using Prism 5.0 (GraphPad). The results are presented as mean ± standard error of the mean (SEM). For comparisons between both groups, we used an unpaired Student t-test. For additional parameters, we performed a one-way ANOVA followed by the Bonferroni post-hoc test. When the normality test failed, we applied a non-parametric Kruskal–Wallis one-way ANOVA on ranks followed by the appropriate multiple comparison methods. In other cases, we performed a two-way ANOVA with the factors housing condition (factor A) and sex (factor B) followed by the Bonferroni post-hoc test. To verify the effect size (ES), η2 was calculated. Values above 0.6 represent a high ES, values between 0.3 and 0.6 represent a moderate effect, and values < 0.3 represent a low effect. Additionally, a Pearson coefficient was performed to find a correlation between brain activation and behaviors. Differences were considered statistically significant at $p \leq 0.05$.

Results

Effects of Environmental Enrichment on Social Behaviors in Female and Male Mice

We analyzed the behaviors that female and male FVB/N mice exhibited in the resident-intruder paradigm after housing them in standard conditions or enriched environments for 30 days (Fig. 2A, B). Behaviors were positive (following, oral-anal sniffing and allogrooming; Fig. 2) or agonistic (mounting and biting/fighting; Fig. 3).

The number of events for following behavior showed the main effect caused by sex ($F1,27 = 116.32$, $p < 0.001$). The number of following events was higher in females than in males, regardless of the housing condition ($p < 0.001$; Fig. 2C1). However, the analysis of time spent in this behavior showed an interaction between housing (factor A) and sex (factor B; $F1,27 = 12.98$, $p = 0.002$; Fig. 2C2). Female mice spent more time exhibiting following behavior

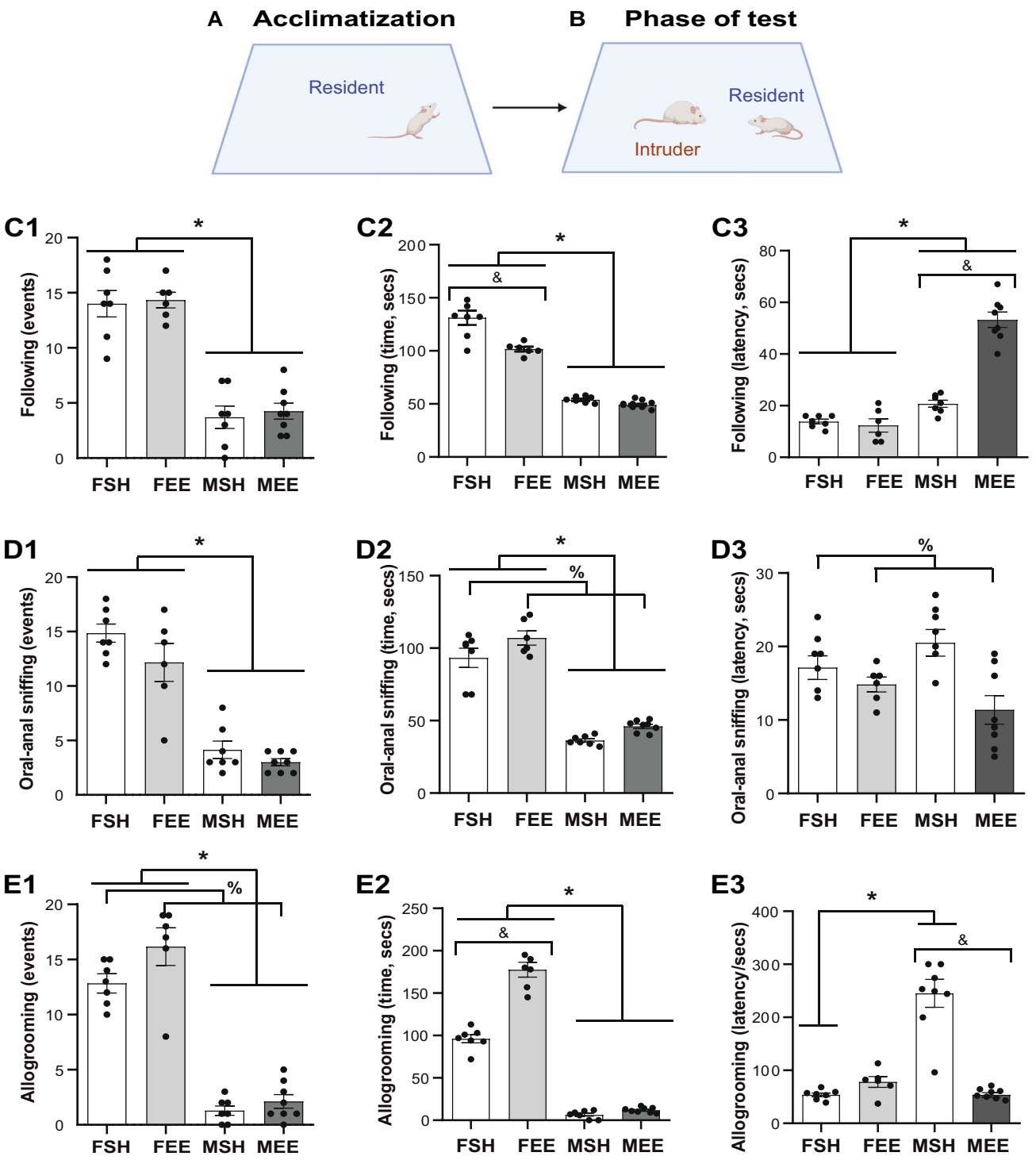


Fig. 2 Social behaviors evaluated after environmental enrichment in FVB/N mice. Environmental enrichment (EE) or standard housing (SH) applied to female and male FVB/N mice. **A**, **B** Resident-intruder paradigm representation. Individual rodents were placed in a plexiglass box with clean sawdust on the floor for ten minutes. Five minutes later, social behavior was manually filmed and analyzed. The groups of animals were females or males housed in standard conditions (FSH or MSH) or environmental enrichment (FEE or MEE). (**C1-E1**) Events of positive behaviors, (**C2-E2**) time in every behavior, (**C3-E3**) latency to the first event. $n = 6–8$; Significant differences are indicated with * for males versus females; % for housing; & for males or females housed in environmental enrichment versus males or females housed in standard conditions. Differences were considered statistically significant at $p \leq 0.05$

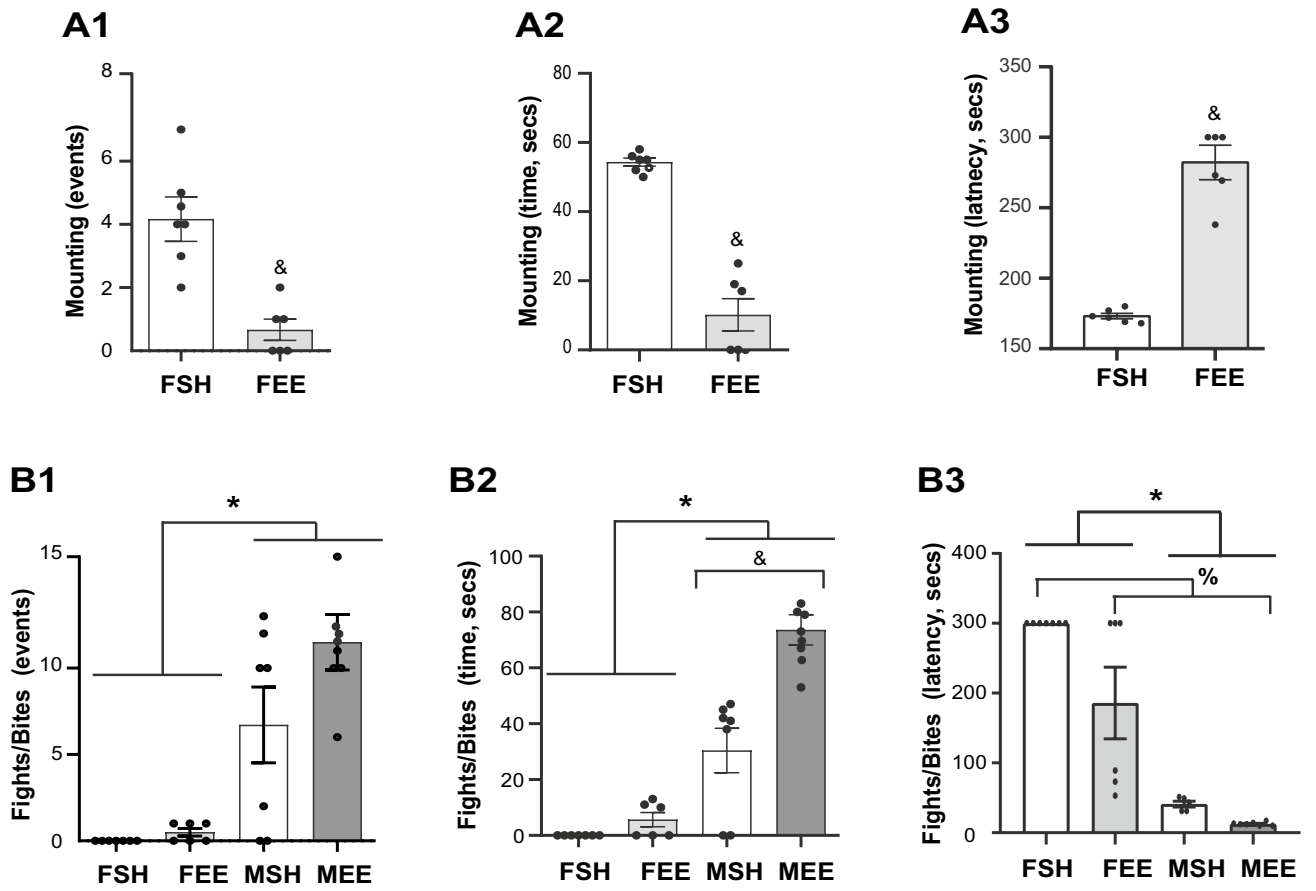


Fig. 3 Agonist behaviors evaluated after environmental enrichment in FVB/N mice. Environmental enrichment (EE) or standard housing (SH) applied to female and male FVB/N mice. **A** Mounting or **B** fights/bites were analyzed in the resident-intruder paradigm representation. Individual rodents were placed in a plexiglass box with clean sawdust on the floor for ten minutes. Five minutes later, agonist behavior was manually filmed and analyzed. The groups of animals were females or males housed in standard conditions (FSH or MSH) or environmental enrichment (FEE or MEE). (**A1-B1**) Events of agonistic behaviors, (**A2-B2**) time in every behavior, or (**A3-B3**) latency to the first event. $n = 6–8$; significant differences are indicated with * for males versus females; & for males or females housed in environmental enrichment versus males or females housed in standard conditions. Differences were considered statistically significant at $p \leq 0.05$

than males, regardless of the housing condition ($p < 0.001$, $p < 0.001$). When comparing between both groups of females, we found that females housed in standard conditions spent more time expressing following behavior than enriched females ($p < 0.001$). Similar effects were seen in the latency to exhibit the first following event (Fig. 2C3). Post hoc analysis comparison after a two-way ANOVA (housing, factor A; sex, factor B; F1,27 = 59.74, $p < 0.001$) revealed a significant increase in latency to the first following event in enriched male mice compared with standard housing male mice ($p < 0.001$). Also, male mice showed increased latency to the first following event compared with female mice independently of the pre-housing condition (SH, p = 0.038; EE, $p < 0.001$). The ES for following behavior was high for the number of following events, time spent exhibiting these events, and latency to the first event (η2 = 0.83, 0.67, and 0.91, respectively).

The analysis of the number of oral-anal sniffing events showed the main effect caused by sex (F1,27 = 107.70, $p < 0.001$). Again, female mice displayed more oral-anal sniffing events than male mice ($p < 0.001$; Fig. 2D1). Regarding the time spent in this behavior (Fig. 2D2), the analysis showed main effects caused by housing (F1,27 = 7.72, $p = 0.011$) and sex (F1,27 = 195.86, $p < 0.001$). Mice housed in environmental enrichment spent more time oral-anal sniffing than mice pre-housed in standard conditions ($p = 0.011$), and female mice spent more time in this behavior ($p < 0.001$). However, the latency to the first oral-anal sniffing event (Fig. 2D3) showed the main effect caused by housing (F1,27 = 10.94, $p = 0.003$). Mice pre-housed in standard conditions showed higher latency to the first oral-anal sniffing event than enriched mice ($p < 0.003$) (Fig. 2D3). The ES for oral-anal sniffing behavior was high for the number of events,

time spent in this behavior, and latency to the first event (η2 = 0.86, 0.79, and 0.36, respectively).

Analysis of the number of allogrooming events showed main effects caused by sex (F1,27 = 181.57, $p < 0.001$) and housing (F1,27 = 4.82, $p = 0.038$). Female mice exhibited more allogrooming events than male mice ($p < 0.001$). Also, enriched mice showed more allogrooming events than mice housed in standard conditions ($p = 0.038$; Fig. 2E1). However, post hoc analysis following a two-way ANOVA (housing, factor A; sex, factor B; F1,27 = 75.79, $p < 0.001$) revealed that enriched female mice spent significantly increased time in allogrooming than mice pre-housed in standard conditions ($p < 0.001$). Female mice also invested more time in this behavior than male mice independently of the pre-housing conditions ($p < 0.001$; Fig. 2E2). Besides, a similar two-way ANOVA interaction (housing, factor A; sex, factor B; F1,27 = 49.76, $p < 0.001$) showed a significant increase in the latency to exhibit the first allogrooming event in standard housing male mice compared with enriched male mice ($p < 0.001$, Fig. 2E3). Male mice pre-housed in standard conditions also showed increased latency to the first allogrooming event compared with female mice $p < 0.001$). The ES for allogrooming was high for number of allogrooming events and time spent in this behavior (η2 = 0.89 and 0.92, respectively), and moderate for the latency to the first event (η2 = 0.77).

Regarding the number of aggressive behavior *events* (mounting; Fig. 3A1, A2), we found that, independently of housing conditions, males did not present mounting behavior. Female mice pre-housed in standard conditions presented more mounting events than females housed in environmental enrichment (Fig. 3A1; t = 4.49, d.f. = 11, $p = 0.001$). Regarding the total time spent in the mounting behavior (Fig. 3A2; t = 9.16, d.f. = 11, $p < 0.0001$), female mice pre-housed in standard conditions spent more time in this behavior than enriched female mice. Similar effects were seen in the latency to the first mounting event (Fig. 3A3; t = 9.70, d.f. = 11, $p < 0.0001$). Also, the ES for number of mounting events and time spent in these events was high (η2 = 0.82 and 0.95, respectively), while latency to the first mounting was moderate (η2 = 0.50).

Analysis of the number of bites/fights revealed a main effect of sex (Fig. 3B1; F1,27 = 39.76, $p < 0.001$). Male mice showed more biting/fighting events than female mice ($p < 0.001$). Post-hoc comparison following a two-way ANOVA (housing, factor A; sex, factor B; F1,27 = 13.23, $p = 0.002$; Fig. 3B2) showed that enriched mice spent more time biting/fighting ($p < 0.001$) than mice pre-housed in standard conditions. Independently of the housing condition, male mice spent more time in this aggressive behavior than females ($p < 0.001$, $p < 0.001$). Regarding the latency to the first biting/fighting event (Fig. 3B3), the main effects were caused by housing (F1,27 = 7.54, $p = 0.012$) and sex (F1,27 = 69.59, $p < 0.001$). In these cases, female mice pre-housed in standard and enrichment conditions exhibited a higher latency to the first event than males ($p < 0.001$ and $p = 0.003$, respectively, Fig. 3B3). The ES for biting/fighting behavior was high for number of events, time spent in this behavior, and latency to the first event (η2 = 0.67, 0.93, and 0.82, respectively).

These results suggest that the exposure to environmental enrichment increases aggressive behavior in males, while in females it decreases aggressive behavior, promoting social interaction.

Effect of Environmental Enrichment on Some Parameters Involved in the Neurogenic Process in the Dentate Gyrus

Previous studies indicated that housing in environmental enrichment promotes the generation of new neurons [4, 5, 7]. The neurogenic process initiates with neural stem cells proliferating to rapidly amplifying cells that reach intermediate stages until newborn granule cells maturate [4, 5, 7]. First, we analyzed RGCs in female and male GFAP-GFP FVB/N mice housed in standard conditions or environmental enrichment (Fig. 4A). Female mice had more RGCs (FSH: 469 ± 10 and FEE: 480 ± 44.8) than male mice (MSH: 263 ± 22.017 and MEE: 265 ± 11.393). The main effect was caused by sex (F1,13 = 80.76, $p < 0.001$; Fig. 4A). However, the quantification of GFAP cells with horizontal processes, also known as type 2 cells, showed a significant increase in enriched females compared with standard housing females (FSH: 484 ± 32.76 and FEE: 715 ± 24.48; $p < 0.001$). Similar effects were seen between enriched male mice and males housed in standard conditions (MSH: 273 ± 17.64 and MEE: 524 ± 38.52; $p < 0.01$), although the main effect was caused by housing (F1,13 = 27.36; $p < 0.001$). It should be noted that the enriched females presented a higher number of GFAP type 2 cells than enriched males ($p < 0.001$). The ES was moderate for RGCs (η2 = 0.48) and high for type 2-positive cells (η2 = 0.81), respectively.

Given the increasing number of type 2 cells, we quantified the number of Ki67 cells (Fig. 4B). A two-way ANOVA yielded the following values: housing condition (factor A) F1,13 = 107.51, $p < 0.001$; sex (factor B) F1,12 = 42.76, $p < 0.001$; and the interaction AxB F1,13 = 42.16, $p < 0.001$. Cellular quantification of proliferative cells showed an increasing number in female (27 ± 1.92) and male (56 ± 0.74) mice previously exposed to environmental enrichment compared with females (19 ± 2.52; $p = 0.025$) and males (19 ± 2.76; $p < 0.001$) in standard housing, respectively. It should be noted that enriched males presented more Ki67 cells compared to females pre-housed in environmental enrichment ($p < 0.001$). The ES for Ki67 cells was high (η2 = 0.96).

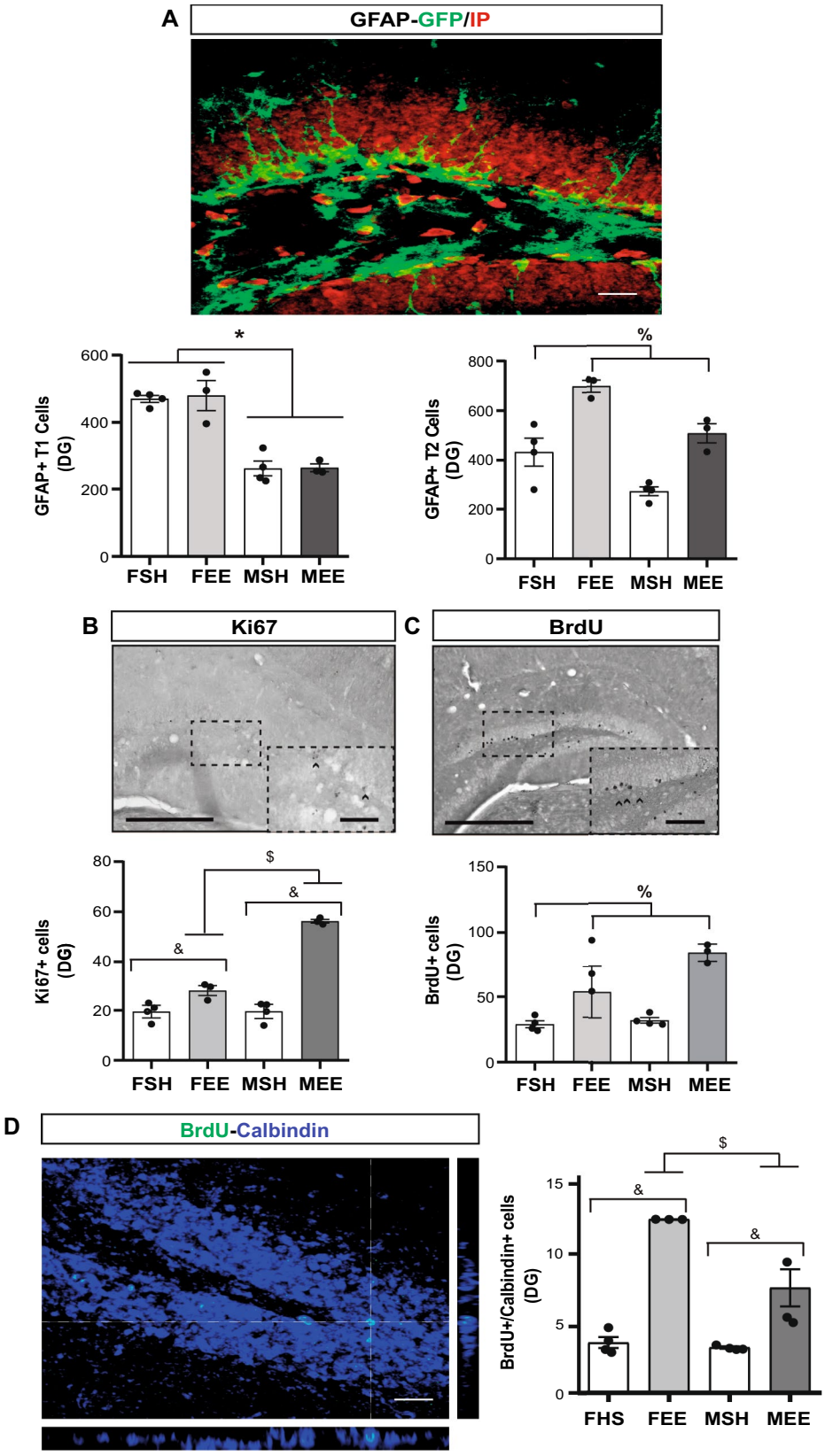
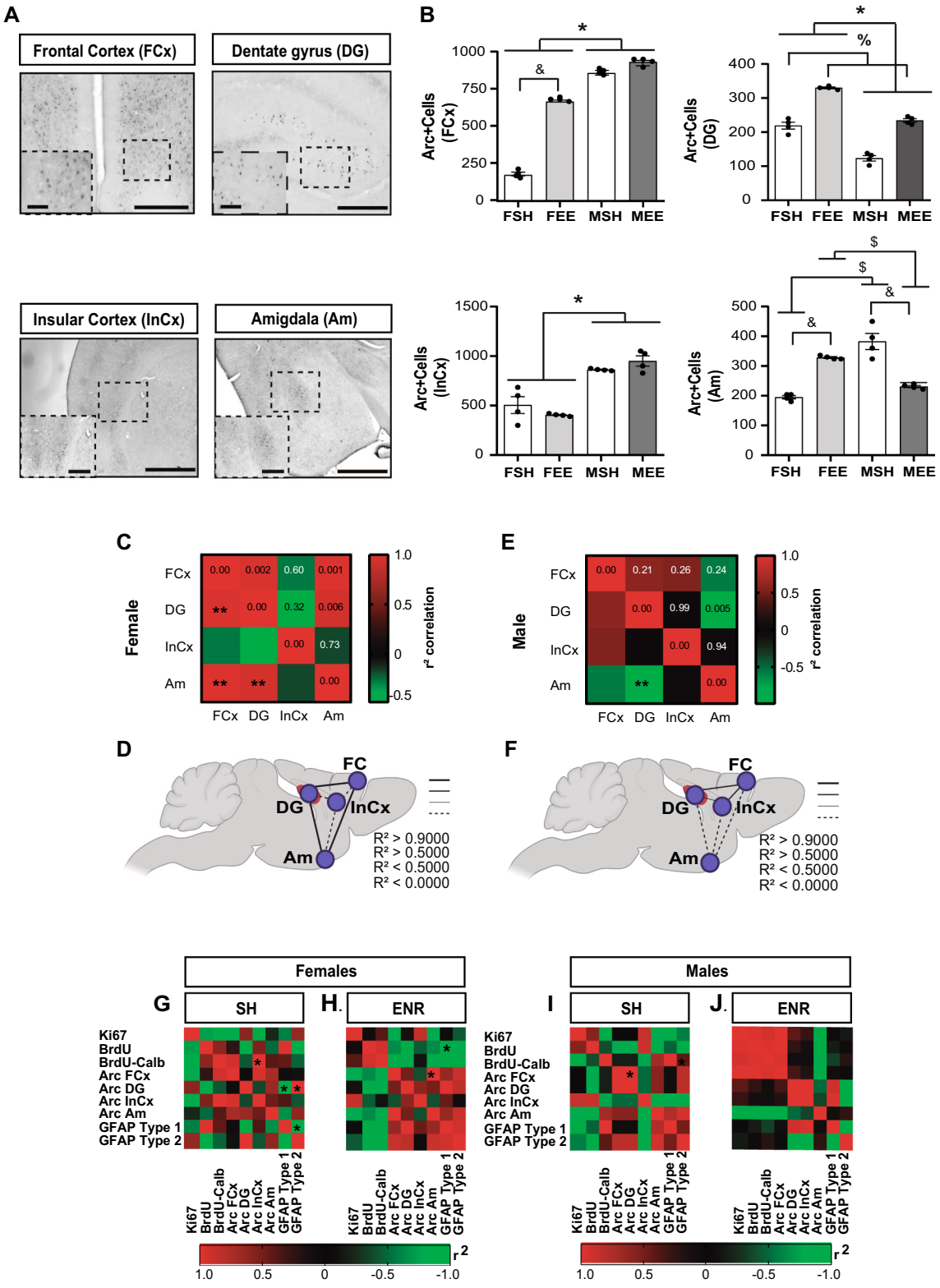


Fig. 4 Neurogenic process in the dentate gyrus. **A** Representative image of *a* coronal section within the dentate gyrus showing GFAP-GFP radial glial cells (type 1) or type 2 cells with horizontal processes. Scale bars 50 µm. The groups consisted of females or males housed in standard conditions (FSH or MSH) or environmental enrichment (FEE or MEE). Quantification of type-1 or type-2 GFAP-GFP cells is shown in the left and right histograms, respectively. $n = 3–4$. Significant differences are indicated with * for males versus females; % for housing; & for males or females housed in environmental enrichment versus males or females housed in standard conditions. Differences were considered statistically significant at $p \leq 0.05$. Representative micrographs of Ki67 (**B**) or BrdU-labeled cells (**C**) or BrdU/Calbindin co-labeled cells (**D**) are shown. Scale bars 400 µm, 100 µm, 50 µm; respectively. Cellular quantification of Ki67 (**B**) BrdU (**C**) labeled cells or BrdU/Calbindin co-labeled cells (D) are shown. Positive cells are indicated with arrowheads (**B**, **C**). $n = 3–4$. Significant differences are indicated with * for males versus females; % for housing; & for males or females housed in environmental enrichment versus males or females housed in standard conditions. Differences were considered statistically significant at $p \leq 0.05$



For BrdU-positive cells (Fig. 4C), we found a main effect produced by the housing factor ($F1,13 = 58.2$, $p < 0.001$). Both female and male mice of the environmental enrichment groups (72 ± 11.41 and 84 ± 6.55, respectively) showed a higher number of positive cells compared with female and male mice of the standard housing groups (28 ± 2.75 and 32 ± 2.11; $p < 0.001$; Fig. 4C). The ES for BrdU-positive cells was high ($\eta 2 = 0.81$). Also, we quantified BrdU-positive

◂**Fig. 5** Neural circuit after the resident-intruder test. **A** Representative micrographs depict Arc-positive cells in coronal sections with the frontal cortex (FCx), dentate gyrus (DG) of the hippocampus, insular cortex (ICx), and amygdala (Am) of female and male FVB/V mice after standard housing (SH) or environmental enrichment (EE). Scale bars 100 µm and 400 µm. **B** The groups of animals were females or males housed in standard conditions (FSH or MSH) or environmental enrichment (FEE or MEE). Quantification of Arc-positive cells in each of these regions. $n=4$. Significant differences are indicated with * for males versus females; % for housing; & for males or females housed in environmental enrichment versus males or females housed in standard conditions. Differences were considered statistically significant at $p \leq 0.05$. **C**, **E** Correlation matrices indicating limbic regions in which the quantification of Arc-labeled cells was performed in female and male mice. Pearson correlation $*p<0.05$, $n=4$. **D**, **F** Schematic representation of the interaction of the limbic system regions and correlation by sex. The positive and negative correlations are shown (continuous and discontinuous lines, respectively). **G**–**J** Individual correlations among the parameters analyzed. Correlation matrices elaborated per group. Female mice housed in standard conditions (SH, **G**) or environmental enrichment (EE, **H**). Male mice housed in SH (**I**) or EE (**J**). Pearson correlation $*p=0.05$; $**p<0.005$, $n=4$ per group

cells co-expressing calbindin (Fig. 4D). Post hoc comparison following a significant two-way ANOVA (housing, factor A; sex, factor B; F1,13 = 7.69, $p=0.021$) showed that enriched female ($p<0.001$) and male ($p=0.004$) mice have more BrdU/Calbindin cells than mice housed in standard conditions (FEE = 13 ± 0.004, MEE = 8 ± 1.42 as compared with FSH = 3 ± 0.45 and MSH = 3 ± 0.02, respectively). Enriched female mice also have more newborn neurons than males ($p=0.002$). The ES for BrdU/Calbindin was high ($\eta 2=0.84$).

These results suggest that the exposure to environmental enrichment differentially affects the ratio of cellular populations involved in the neurogenic process in male and female FVB/N mice.

Activation of the Aggression Circuit after the Resident-Intruder Paradigm

Once we found the differential impact of environmental enrichment on social/agonistic behavior and the neurogenic process in male and female FVB/N mice, we conducted the resident-intruder test to explore the activation of the aggression circuit (Fig. 5). We analyzed neuronal activation *by* identifying the immediate early gene Arc in the FCx, ICx, Am, and DG (Fig. 5A, B). In the FCx (Fig. 5B) post hoc comparison following a two-way ANOVA (housing, factor A; sex, factor B; F1,15 = 62.08, $p<0.001$) showed that enriched female mice have more Arc-positive cells than female mice housed in standard conditions (694 ± 37.05 and 198 ± 14.162, $p<0.001$). Independently of the housing conditions, male mice have more Arc-positive cells (835 ± 3.88 and 894 ± 38.76) in the FCx than females (198 ± 14.16 and 694 ± 37.05; SH $p<0.001$; EE $p<0.001$; respectively). The *ES* was high ($\eta 2=0.98$).

For the DG, a two-way ANOVA revealed the main effects of housing (Factor A; F1,15 = 203.38 $p<0.001$) and sex (Factor B; F1,15 = 173.64, $p<0.001$). Thus, female mice showed more Arc-positive cells (226 ± 5.12 and 336 ± 2.44; $p<0.001$) than male mice (111 ± 3.87 and 227 ± 2.56; $p<0.001$), and mice of the environmental enrichment groups showed a higher number of Arc-positive cells than mice housed in standard conditions ($p<0.001$). The ES was high ($\eta 2=0.97$).

Regarding neuronal activation in the ICx, the main effect caused by sex (F1,15 = 115.48, $p<0.001$) indicated that male mice have more Arc-positive cells than females ($p<0.001$) but no significant differences were observed between SH compared to EE condition (MSH = 862 ± 55.12; MEE = 951 ± 52.56, FSH = 505 ± 85.43, and FEE = 360 ± 40.17). The ES was high ($\eta 2=0.94$). For Am, post hoc comparison following a two-way ANOVA (housing, factor A; sex, factor B; F1,15 = 237.19, $p<0.001$) showed that enriched female mice have more Arc-positive cells than female mice housed in standard conditions (325 ± 2.27 and 195 ± 5.74, respectively; $p<0.001$). However, males housed in standard conditions exhibit more Arc-positive cells than enriched male mice (382 ± 26.87 and 231 ± 3.87, respectively; $p<0.001$). Also, males housed in standard conditions have more Arc-positive cells than females ($p<0.001$). In the environmental enrichment groups, females exhibited more Arc cells than males. The ES was high ($\eta 2=0.62$).

Furthermore, we made correlation matrices (Fig. 5C, E) and plotted the value of r2 obtained from Pearson's correlations. For the correlation matrices, we considered the sex factor to elucidate the contribution of every analyzed region of the limbic system. In female FVB/N mice (Fig. 5C), the FCx was strongly correlated with the Am (r2 = 0.977, $p=0.001$) and DG (r2 = 0.960, $p=0.002$), and the DG was strongly correlated with the Am (r2 = 0.937, $p=0.006$). However, in male FVB/N mice (Fig. 5E), we found a negative correlation between the Am and DG (r2 = −0.994, $p=5.22 \times 10–5$). Thus, the representation of interactions (Fig. 5D) among the regions analyzed in females, considering the correlation coefficients for each interaction, suggests a strong interaction between the FCx, DG, and Am in female social behavior. However, in males (Fig. 5F), a strong interaction exists between the FCx, DG, and ICx but not Am.

Finally, we explored the impact of housing conditions on relationships between neuronal activation and neurogenesis (Fig. 5G–J). Females housed in standard conditions (Fig. 5G) showed positive correlations between BrdU/Calbindin cells and Arc cells in the ICx (r2 = 0.98, $p=0.043$) or between Arc cells in the DG and GFAP type 2 cells (r2 = 0.00, $p=0.035$). Also, negative correlations were found between Arc cells and the DG and GFAP type 1 cells

(r2 = −0.99, $p = 1.89 \times 10–8$) or between GFAP type 1 cells and GFAP type 2 cells (r2 = −0.99, $p = 0.035$). Conversely, enriched females (Fig. 5H) showed a positive correlation in Arc cells in the FCx and Arc cells in the Am (r2 = 0.99 $p = 0.0001$), and a negative correlation in BrdU cells and GFAP type 1 cells (r2 = −0.99, $p = 0.004$). Regarding male mice in standard conditions (Fig. 5I), a negative correlation was found between BrdU/Calbindin cells and GFAP type 2 cells (r2 = −0.99, $p = 0.009$), and a positive correlation was found between Arc cells in the DG and Arc cells in the FCx (r2 = 0.99, $p = 0.0001$). Interestingly, enriched male mice (Fig. 5J) did not show significant correlations between neuronal activation and neurogenic-associated parameters.

Discussion

This study analyzed the effect of environmental enrichment on social behavior, hippocampal neurogenesis, and neuronal activation in some regions of the limbic system after social behavior evaluation through the resident-intruder paradigm in female and male FVB/N mice. Female mice showed increased positive social behavior, but males exhibited increased agonistic behavior. However, enriched female mice showed decreased behavior related to aggression compared to standard housing female mice. Moreover, we confirmed that housing rodents in environmental enrichment is beneficial for increasing neuroplasticity; in this case, by favoring the neurogenic process but at different rates between female and male mice. We also found differential neuronal activation in regions of the limbic system in female and male FVB/N mice after the resident-intruder test.

Environmental Enrichment Modulates Social Behavior

Social interaction is important for reproduction, the care of the young, and the defense of a territory [28, 29]. One of the models widely used to evaluate social behavior in rodents is the resident-intruder paradigm [18]. This test allows researchers to evaluate positive (following, oral-anal sniffing and allogrooming) or agonistic (mounting and biting/fighting) behaviors [28, 29] to determine the influence of environmental stimuli [13, 18]. In fact, environmental enrichment has been proven to enhance learning and memory processes and mood-related behaviors [8–11]. However, several studies reported differential behavior in male mice of different strains [13, 14, 18, 39, 40]. For example, male 129S6/SvEv mice showed more agonistic behavior than male C57BL/6 mice, but there were no differences between these strains after housing in environmental enrichment [18]. However, male CD-1 mice housed in environmental enrichment became aggressive compared to male Balb/C mice [14]. And male NMRI mice showed agonistic behavior after environmental enrichment [13]. Interestingly, many studies analyzing the impact of environmental enrichment on behavior have been performed in male rodents [41, 42], but only a few have examined the effects of this paradigm on both sexes [22, 24, 43]. In this regard, the sex factor is critical due to the differential behavioral response displayed between male and female C57BL/6 mice exposed to environmental enrichment, as was shown in emotionality-related behavior [43]. Here we found that, independently of the housing condition, females exhibited more positive social behavior events (following, oral-anal sniffing and allogrooming) than males. However, diminished aggressive behavior (mounting) in females and increased aggressive behavior (biting/fighting) in males exposed to environmental enrichment were observed in FVB/N mice. Besides, enriched male mice showed more biting/fighting behavior than standard housing male mice. Interestingly, environmental enrichment decreased mounting events in females. We also found that enriched female mice spent more time in allogrooming and less time in mounting behavior, whereas male mice spent more time biting/fighting with decreased latency to the first biting/fighting event. Further, enriched male FVB/N mice showed a high latency to the first event of following, while male mice under standard conditions showed a high latency to the first allogrooming event. Our results support the previous report indicating differences in social behavior in female and male CD-1 mice [43]. However, CD-1 mice, independent of their sex, spent more time in agonistic than in social behavior. Regardless, females were less aggressive than males in establishing dominance over a same-sex conspecific [44]. And the effects of environmental enrichment on agonistic behavior are similar to those found in male CD-1 mice and male NMRI mice [13, 18]. Moreover, the effects identified in female mice are supported by a previous study in which female C57BL/6 mice housed in environmental enrichment showed more social behavior than females exposed to standard conditions analyzed in their housing cage [12]. The differences in social behavior among strains may be due to the susceptibility of certain factors to the environment [23, 45, 46]. As for the mechanisms underlying the differential effects of environmental enrichment it is known that single nucleotide polymorphisms and copy number variations might be responsible for the differences in aggressive behavior in male mice of the BALB/cJ and BALB/cByJ substrains [45]. In addition, C57BL/6 and 129S6/SvEv mice housed in environmental enrichment showed differential expression of limbic system-associated membrane proteins A and B (Lsamp-1a and Lsamp-1b). These proteins are neural cell adhesion molecules expressed in neuronal dendrites and somata, and they enhance synaptic integrity and stability [23]. Also, the overexpression of the regulator of G-protein signaling 2 (Rgs2) in serotoninergic neurons of Rgs2-/-C57Bl6 mice

is sufficient to induce male aggression [46]. These studies support the notion that intrinsic differences among mouse strains could explain inconsistences in social behavior.

Effect of Environmental Enrichment on Brain Plasticity

Several studies have shown that environmental enrichment positively affects neuroplasticity in the brain, including new neurons [1, 14, 35, 47–50]. Our results are similar to those of previous reports in which environmental enrichment increased neuronal proliferation, survival, and maturation [6, 7]. The strain of mice used in our study showed basal neurogenesis like that of Balb/C and 129/SvJ mice but lower than that of C57/BL/6 mice [33]. Despite the differences observed between male and female FVB/N mice, environmental enrichment increased the neurogenic process in both cases. We analyzed the initial cellular populations of the neurogenic process identified by the expression of GFAP [37]. In our study FVB/N mice expressed the GFP protein under the GFAP promoter [34]. Analysis showed that, independent of the housing condition, male mice have a lower number of RGCs and type 2 cells than female mice [37]. Enriched mice had more type 2 cells than those housed in standard conditions. Our results are similar to those in adult male GFAP-GFP FVB/N mice exposed to voluntary physical exercise for two weeks [51]. In that study, male mice exhibited an increased number of proliferative (Ki67) and surviving (BrdU) cells than control mice [51]. The authors also found an increased number of RGCs after two weeks of physical activity [51], an effect that we did not see in our males or females housed in an enriched environment. It is possible that the housing conditions in both studies differentially impact on aspects of the neurogenic process, especially because environmental enrichment with tunnels favors the survival of newborn cells, but physical activity with the running wheel affects cell proliferation [5, 52]. Interestingly, adult female C57BL/6 or male Sprague–Dawley rats exposed to ENR showed an increased number of astrocytes in the DG, but not in the CA1, CA3, or cortex [51, 53, 54]. However, these effects depend on the species and strain used to analyze the benefits of environmental enrichment or physical activity [i.e. 55]. Nevertheless, we confirmed that environmental enrichment favors the neurogenic process in the DG of the hippocampus in mice [4].

Effect of Environmental Enrichment on Cellular Activation

The hippocampus is a region of the limbic system where new neurons are generated [37]. Although the hippocampus is related to learning and memory processes and mood-related behavior, its participation in social behavior may be relevant [43]. The neural circuit for social behavior could be elucidated using immediate-early gene expression [55]. We analyzed cellular activation after the resident-intruder test because the FCx, ICx, and Am areas of the limbic system, as well as the hippocampus, are related to social behaviors or aggression [56, 57]. Thus, the Arc immunoreactivity indicated differential activation of the neural circuit in enriched females with increasing numbers of Arc-positive cells in the FCx, DG, and Am compared to females in standard housing. Also, the number of Arc-positive cells in enriched males increased in the FCx and DG but decreased in the Am compared to males in standard housing. Interestingly, the number of Arc-positive cells in the FCx, DG, and Am are lower in males than in females pre-housed in an enriched environment. Our results suggest that differential neural circuit activation occurs after the social behavioral test in male and female mice previously housed in environmental enrichment. The FCx is implicated in decision-making, goal-directed behavior, and social behaviors [58–63]. It is also related to the nucleus accumbens, Am, DG, and hypothalamus, which are implicated in the neural circuit of aggression or emotional regulation [31]. Interestingly, male FVB/N mice showed increased neuronal activation in the ICx. This brain region is associated with aggressive behavior and empathy [31]. However, the hypoactivation seen in male mice previously housed in environmental enrichment is consistent with the findings reported in mice with repeated experience of victory [65]. In that study, male C57Bl6 mice showed a decreased number of c-fos positive cells in the Am and an increased number of activated cells in the hippocampus [64].

Female mice displayed a strong positive correlation in the activation of the FCx, DG, and Am and in the DG with Am. But in male mice, a strong negative correlation was seen between the DG and Am. These results and the representation of the interactions suggest a strong interaction among FCx, DG and Am in female mice, but in males the strong interactions were among FCx, DG and ICx, indicating the differential neuronal activation that could be associated with the circuits of social or agonistic behaviors in female and male FVB/N mice.

These differences provide partial evidence about the neural circuit involved in social behavior exhibited by female and male FVB/N mice previously housed in an enriched environment or standard conditions.

Limitations and Conclusion

Unfortunately, there are aspects that deserve exploration to explain the differences in social behavior such as hormone involvement. For instance, testosterone is related to aggressive behaviors in males and females [65], and estradiol increases aggressive behavior specifically in females [65,

66]. In addition to these hormones, their receptors are associated with territorial, hierarchical, advertent, and aggressive behavior [65, 66]. We did not preserve serum samples to perform hormone quantifications, but our results confirm the importance of conducting studies with female and male mice to evaluate the impact of environmental stimuli on behavior. Future studies should also consider analyzing newborn neurons expressing IEGs to understand their participation in the regulation of social behavior.

Our study shows that FVB/N mice display sexual dimorphism in social behavior influenced by environmental enrichment. It also confirms that environmental enrichment increases neurogenesis and activates different regions of the limbic system when male and female FVB/N mice display social or agonistic behavior. This study furthers our understanding about the regulation of social behavior and the involvement of increased neurogenesis induced by environmental stimuli in FVB/N mice.

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Data Availability The relevant data are within the manuscript. However, the datasets analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest The authors declare no conflict of interest.

Ethical Approval All institutional and legal regulations regarding animal ethics, care, and handling were performed following the Mexican Official Standard for animal care (NOM-062-ZOO-1999) and approved by the local Institutional Ethics Committee of the National Institute of Psychiatry "Ramón de la Fuente Muñiz" (IACUC: CEI/C/009/2013).

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