

Resilience and susceptibility to fear extinction are associated with alterations in neurogenesis and brain-wide activity

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ABSTRACT

Fear-related disorders, characterized by inappropriate learned fear and resistance to extinction, are among the most prevalent psychiatric conditions. Potential modifications in neurogenesis and brain activity were studied as possible individual factors associated with the development of these pathologies. By using Pavlovian fear conditioning and extinction paradigm, male and female mice were categorized in resilient and susceptible phenotypes based on their individual fear extinction behavior. Increased neurogenesis, as revealed by higher expression of the early neuronal marker doublecortin in the subgranular zone of the dentate gyrus, was observed in susceptible male and female mice. This result suggests the existence of a compensatory mechanism given that the DNA-alkylating agent temozolomide induced an impairment of fear extinction and a reduction of neurogenesis in male mice. The use of c-Fos immunofluorescence revealed several brain regions that were differently activated in susceptible animals, although these differences were less evident in female mice. Categorization by k-means clustering based on c-Fos labelling was significantly associated with phenotype of extinction in male, but not female, animals. Pairwise Pearson correlations between brain regions showed that resilience and susceptibility to fear extinction are related to divergent circuit-level reorganizations. These findings reveal new individual factors involved in the variability of fear extinction response which could be of interest for the development of future therapeutic strategies.

1. Introduction

Fear-related pathologies, including posttraumatic stress disorder (PTSD), panic disorder and phobias, are among the most prevalent psychiatric conditions. Patients suffering these disorders are characterized by having inappropriately learned fear and resistance to extinction as important features (Careaga et al., 2016; Maples-Keller et al., 2025). While many people who experience a traumatic experience will recover favorably, about 10% of them will develop PTSD (Kessler et al., 1995), which is a chronic and debilitating illness that can last a lifetime (Jellestad et al., 2021). Identifying the mechanisms underlying inter-individual variation in fear extinction learning, which are poorly understood at present, is crucial for setting the vulnerability to develop anxiety and fear-related disorders, and to discover new potential targets for the treatment of these diseases (Masella et al., 2024).

Pavlovian fear conditioning and cued fear extinction paradigm were used here to study this inter-individual variability in animal models.

Specifically, we assessed a between-session extinction process after an acute aversive stimulus, a model widely used to study fear extinction performance in rodents. Dissociations between within- and between-session extinction processes have been previously described by using different experimental conditions (Plendl and Wotjak, 2010; Almeida-Corrêa et al., 2015; Smith et al., 2016). The behavioral model used allows the rodent population to be stratified into three phenotypic subgroups (resilient, intermediate and susceptible) considering their individual fear extinction behavior. Based on this experimental procedure, stress-induced susceptible mice showed dysregulated corticosterone and transcriptional divergence in the basolateral amygdala (BLA) in comparison with stress-resilient control group (Daws et al., 2017) in a previous study. The amygdala neurotrophin 3-tropomyosin receptor kinase C (NT3-TrkC) pathway was also previously described to play a key role in inter-individual differences in fear extinction in mice (Masella et al., 2024). Some components of the hypothalamic-pituitary-adrenal (HPA) axis, gut microbiota diversity and

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composition, and gene expression profile of the amygdala have been also recently involved in the vulnerability to extinction of fear (Ten-Blanco et al., 2025).

Alterations in neurogenesis, brain-wide activity and neuronal network connectivity, previously associated with fear regulation, could also be potential risk factors for developing fear-related disorders. In this sense, enhanced fear memory extinction on a contextual memory learning task was observed in mice exposed to chemogenetic activation of adult hippocampal progenitors (Maheshwari et al., 2024). Increased hippocampal neurogenesis reduced PTSD-like behaviors in adult mice (Fujikawa et al., 2024). Consistently, inhibition of adult neurogenesis by deletion of ERK5 MAP kinase impaired fear extinction (Pan et al., 2012). In addition, there is growing evidence that also warns us about the importance of dysregulation in the functional interaction of various brain regions in the etiology and symptomatology of several psychopathologies, including anxiety disorders and PTSD (Becker and Milad, 2023). By using c-Fos immunohistochemistry, a functional connectivity network map was generated for remote fear memory attenuation in mice (Silva et al., 2019), shedding light on the neural mechanisms underlying this neurobiological process.

Here, we focused on potential modifications of neurogenesis, brain-wide activity and neuronal functional connectivity to better understand the inter-individual factors associated with fear extinction. For this purpose, three phenotypic subgroups (resilient, intermediate and susceptible) were identified based on their extinction behavior. We hypothesize that phenotypic subgroups will be associated with alterations in neurogenesis and brain activity. These modifications will help identify risk factors for the development of diseases characterized by pathological fear. Male and female mice were included in this study considering that most of the preclinical research in this area has exclusively used male animals, and that women have a twofold likelihood of developing PTSD compared to men (Olff et al., 2007; Velasco et al., 2019).

2. Materials and methods

2.1. Animals

Adult male and female C57BL/6 J mice (8–10 weeks old; Charles River) were used in all experiments. Animals were housed in groups of four per cage under controlled temperature ($21 \pm 1^\circ\text{C}$) and humidity ($55 \pm 10\%$) conditions, with a 12 h light/dark cycle (lights on at 08:00). All behavioral testing was performed during the light phase, and males and females were evaluated on alternate weeks. Food and water were available ad libitum. All procedures were conducted in accordance with the European Communities Directive 2010/63/EU and Spanish regulations (RD 1201/2005 and 53/2013) regulating animal research and approved by the local ethical committee (CEE-UFV).

2.2. Drugs

Temozolomide (MedChemExpress) was prepared in a vehicle solution of 90% phosphate-buffered saline (PBS) and 10% dimethyl sulfoxide (DMSO) and was intraperitoneally (i.p.) administered at the dose of 25 mg/kg (10 ml/kg of body weight). Male mice received a single cycle of treatment (3-day daily injection followed by 4 drug-free days), whereas female mice were treated with either one or three cycles (3-day daily injection separated by 4-day abstinence for three weeks). The dose regimen of temozolomide administration was based on previous reports (Yu et al., 2023; Mañas-Padilla et al., 2023). Temozolomide was administered in a group of animals that were not separated into phenotypes. Vehicle-treated animals received the corresponding injection schedule with 90% PBS and 10% DMSO.

2.3. Cued fear conditioning and extinction

Thirty male and 30 female mice were exposed to cued fear conditioning and extinction. Experiments were carried out in conditioning chambers ($25 \times 25 \times 25$ cm; LE116, Panlab, Harvard Instruments) with opaque black methacrylate walls and a transparent front door. Chambers were placed inside sound-attenuating boxes equipped with a ventilation fan to provide background noise. The floor consisted of parallel stainless-steel rods (2 mm diameter, spaced 6 mm apart) connected to a shock generator (LE100–26, Panlab, Harvard Instruments). Movement was recorded by a high-sensitivity load cell integrated in the chamber, and freezing was automatically scored using PACKWIN v2.0 (Panlab) based on the load-cell activity signal. Freezing was defined as the absence of detectable movement, except for respiration, according to the software's detection settings, which were kept constant across animals and sessions. Mice were individually conditioned after a 180 s habituation with 3 cue tones (3 kHz, 80 dB) of 20 s long (2 min interval). Each cue tone (conditioned stimulus, CS) co-terminated with a 0.7 mA foot-shock of 1 s duration (unconditioned stimulus, US). Fear extinction sessions (E1–E3) took place 24, 48 and 72 h after the conditioning day in a novel environment (white walls, transparent cylinder, and smooth floor), and after an acclimatization period (120 s in E1 and 60 s in E2 and E3), 20 cue tones (CS) were presented with an interval period of 10 s. Freezing behavior, a rodent's natural response to fear, was automatically evaluated and defined as complete lack of movement except for breathing. Between each animal trial, the chamber was cleaned with 70% ethanol to avoid olfactory cues. Data were expressed as percentage of freezing behavior during the time the sound was active and as area under curve (AUC) across extinction sessions (E1–E3), which was calculated by using a standard trapezoid method: $\text{AUC} = [0.5 \times (B1 + B2) \times h] + [0.5 \times (B2 + B3) \times h] + \dots [0.5 \times (Bn + Bn + 1) \times h]$, where Bn were the freezing percentages for each mouse and h the time between two consecutive measurements. Because extinction sessions were equally spaced, h was set to 1 (session units).

2.4. Locomotion

Locomotor activity was assessed in actimetry boxes ($27 \times 27 \times 21$ cm; Cibertec). Mice were placed individually in low-light conditions, and total activity was measured as the number of infrared beam crossings during a 15 min session. Locomotion was assessed the day before the start of the behavioral procedure in animals treated with vehicle and temozolomide. This analysis was performed in order to rule out an effect of this drug on locomotor activity.

2.5. Criteria for categorization in resilient, intermediate and susceptible mice

Two classification criteria were applied independently in males and females to categorize phenotypes, as previously reported (Ten-Blanco et al., 2025). The first criterion was based on the freezing in the last extinction session (E3), which threshold was calculated as the freezing level in $E3 \pm 40\%$ of the mean. The second criterion comprised the percentage of decrease in the freezing behavior between E1 and E3 and the threshold value was fixed in $\pm 50\%$. Therefore, resilient mice were those scoring under the low threshold in the first criterion and above 50% in the second criterion. On the contrary, susceptible mice scored above the top threshold in the first criterion and under 50% in the second criterion. These two criteria considered two important aspects: 1) the freezing levels of each mouse relative to the total mean at the end of the extinction period (percentage of freezing in E3) and 2) the intrinsic ability of each mouse to extinguish fear (percentage of decrease in freezing levels between E1 and E3 relative to the total mean). Animals that did not meet either criterion were considered intermediate.

2.6. Immunofluorescence

Tissue collection and processing. Mice were deeply anesthetized by i.p. injection of ketamine, xylazine and saline solution and transcardially perfused 90 min after the end of the last extinction session (E3). Mice were perfused with 1× phosphate buffer saline (PBS) followed by 4% paraformaldehyde. Afterward, brains were post-fixed in 4% paraformaldehyde 24 h and then dehydrated by sequential transfer to 15 and 30% of sucrose in PBS 1× (4 °C). Coronal frozen sections were cut at 30 µm thickness on a cryostat. Brain slices were stored in a cryoprotective solution (20% glycerol, 30% ethylenglycol in PBS 1×) at −20 °C until use.

Ki67 and doublecortin. Free-floating brain sections (30 µm) were first permeabilized for 30 min at room temperature in 0.5% Triton X-100 in PBS 1×. Slices were then incubated for 1.5 h at room temperature in blocking solution (2% bovine serum albumin, 0.5% Triton X-100 in PBS 1×). Sections were incubated overnight at 4 °C with rabbit anti-Ki67 (1:1000, #ab15580, Abcam) and chicken anti-doublecortin (1:100, #ab153668, Abcam), both diluted in blocking solution. The following day, after three washes in PBS 1× (10 min each), slices were incubated for 1 h at room temperature with secondary antibodies diluted in blocking solution: Alexa Fluor 488 donkey anti-rabbit IgG (1:500, #A-21206, Invitrogen) for Ki67 and Alexa Fluor 555 donkey anti-chicken IgG (1:500, #A78949, Invitrogen) for doublecortin. Sections were washed three times in PBS 1× (10 min each) and mounted with Fluoromount-G containing DAPI (Invitrogen).

c-Fos. Free-floating brain sections (30 µm) were rinsed three times in PBS 1× and incubated for 1 h at room temperature in blocking solution (4% normal donkey serum, 0.1% Triton X-100, 0.1% bovine serum albumin in PBS 1×). Sections were then incubated overnight at 4 °C with rabbit anti-c-Fos (1:2000, #226008, Synaptic Systems) diluted in blocking solution. After three washes in PBS 1× (10 min each), slices were incubated for 1 h at room temperature with Alexa Fluor 555-conjugated donkey anti-rabbit (1:500, #A-31572, Invitrogen) prepared in blocking solution. Finally, sections were rinsed three times in PBS 1× and mounted with Fluoromount-G containing DAPI (Invitrogen).

2.7. Image analysis

All images were acquired at 10× magnification using a Thunder Leica DMI8 microscope. For each animal, 6 coronal sections were analyzed, with a total of 5 animals per experimental group. Of the 20 animals with an intermediate phenotype, 5 were selected based on their extinction behavior. In this way, their extinction criteria were as close as possible to the group mean, so that these animals were clearly representative of the group.

This selection was based only on behavioral data and was performed prior to histological quantification, without reference to neurogenesis or c-Fos measures. Quantifications were manually inspected by an experimenter blind to the experimental conditions.

Ki67+ cells were quantified in the subgranular zone (SGZ) of the hippocampus and in the subventricular zone (SVZ). The SGZ area was calculated as its length (measured with the “segmented line” tool in ImageJ) multiplied by a fixed width of 25 µm. Ki67+ cells were manually counted using the “multi-point” tool, and cell density was expressed as Ki67+ cells per mm² of SGZ. Ki67+ cells in the SVZ were quantified within ROIs defined and measured using the same criteria as in the SGZ.

Doublecortin immunofluorescence was analyzed in the SGZ of the hippocampus. Images were acquired using identical microscope settings across groups to avoid pixel saturation and maintain consistent gain. Background was normalized, and images were processed to binary format in ImageJ. The “segmented line” and “analyze and measure” functions were used to quantify the integrated density of the granular cell layer, calculated as mean gray value × area. For each slice, integrated density was averaged from three independent measurements to reduce variability. Relative doublecortin integrated density was

expressed as the percentage of the intermediate phenotype group, and, in temozolomide experiments, as the percentage of the corresponding control group.

Quantification of c-Fos was performed in ten brain regions: prelimbic cortex (PL), infralimbic cortex (IL), dentate gyrus (DG), Cornu Ammonis areas 1 and 3 (Ca1, Ca3), basal amygdala (BA), central amygdala (CeA), lateral amygdala (LA), paraventricular thalamic nucleus (PVT), and habenula (HAB). For PL and IL, a 430 µm-sided square region of interest (ROI) was defined for analysis. PL and IL subregions were identified on coronal sections according to Paxinos and Franklin's mouse brain atlas, using bregma coordinates and layer structure as landmarks. For the remaining regions, DAPI staining was used to identify and delineate anatomical boundaries. Images were processed in ImageJ, and c-Fos + nuclei were quantified using the “particle counting” function with fixed size and circularity parameters. A constant threshold was applied across groups for signal-background discrimination. Data were expressed as the mean number of c-Fos + cells per mm² and normalized to the intermediate phenotype group.

2.8. Bioinformatic analysis

All analyses were performed in R (v4.3.0). Principal component analysis (PCA) was applied to the z-scored c-Fos matrices to visualize sample distribution and to explore whether resilient, intermediate, and susceptible animals clustered together. K-means clustering was applied to the first two principal components to identify group structure. Variance explained by the first ten principal components was also computed.

For each sex and phenotype, correlation matrices were generated by computing pairwise Pearson's correlation coefficients across brain regions based on c-Fos quantification values. Correlation coefficients were converted to z-scores using Fisher's r-to-z transformation. Matrices were thresholded at $p < 0.05$ to retain significant correlations.

Network construction was performed by treating brain regions as nodes and significant pairwise correlations as edges. Graph-theoretical metrics (e.g., strength, betweenness centrality, eigenvector centrality, clustering coefficient) were calculated for each node. Graph visualization and matrix plots were generated using the igraph (v1.5.1), gplots (v3.1.3), and ggcorrplot (v0.1.4) packages. Data wrangling and figure customization employed tidyverse (v2.0.0) package.

2.9. Statistical analysis

Normality of data distributions was assessed using the Kolmogorov-Smirnov test, and homogeneity of variances was verified when appropriate. Depending on the experimental design, data were analyzed using unpaired Student's *t*-test, one-way ANOVA, or two-way ANOVA of repeated measures followed by Tukey's post hoc tests when applicable. When normality assumptions were violated, non-parametric tests were employed (Mann-Whitney *U* test or Kruskal-Wallis test followed by Dunn's post hoc comparisons). A *p* value <0.05 was used to determine statistical significance. Analyses were performed using GraphPad Prism 9 (GraphPad Software). The statistical analyses performed for each experiment is described in detail in Table S1.

3. Results

3.1. Identification of resilient, intermediate and susceptible mice based on their extinction pattern

Male and female C57BL/6J mice were exposed to an auditory fear conditioning paradigm and subsequent extinction protocol for 3 days (Fig. 1A). We isolated three phenotypic subgroups (resilient, intermediate and susceptible) in both male and female mice by applying specific criteria (see Material and Methods) on their extinction profile (Fig. 1B, F), although no differences between groups were observed during the previous fear conditioning phase (Fig. S1). As shown in Fig. 1C, freezing

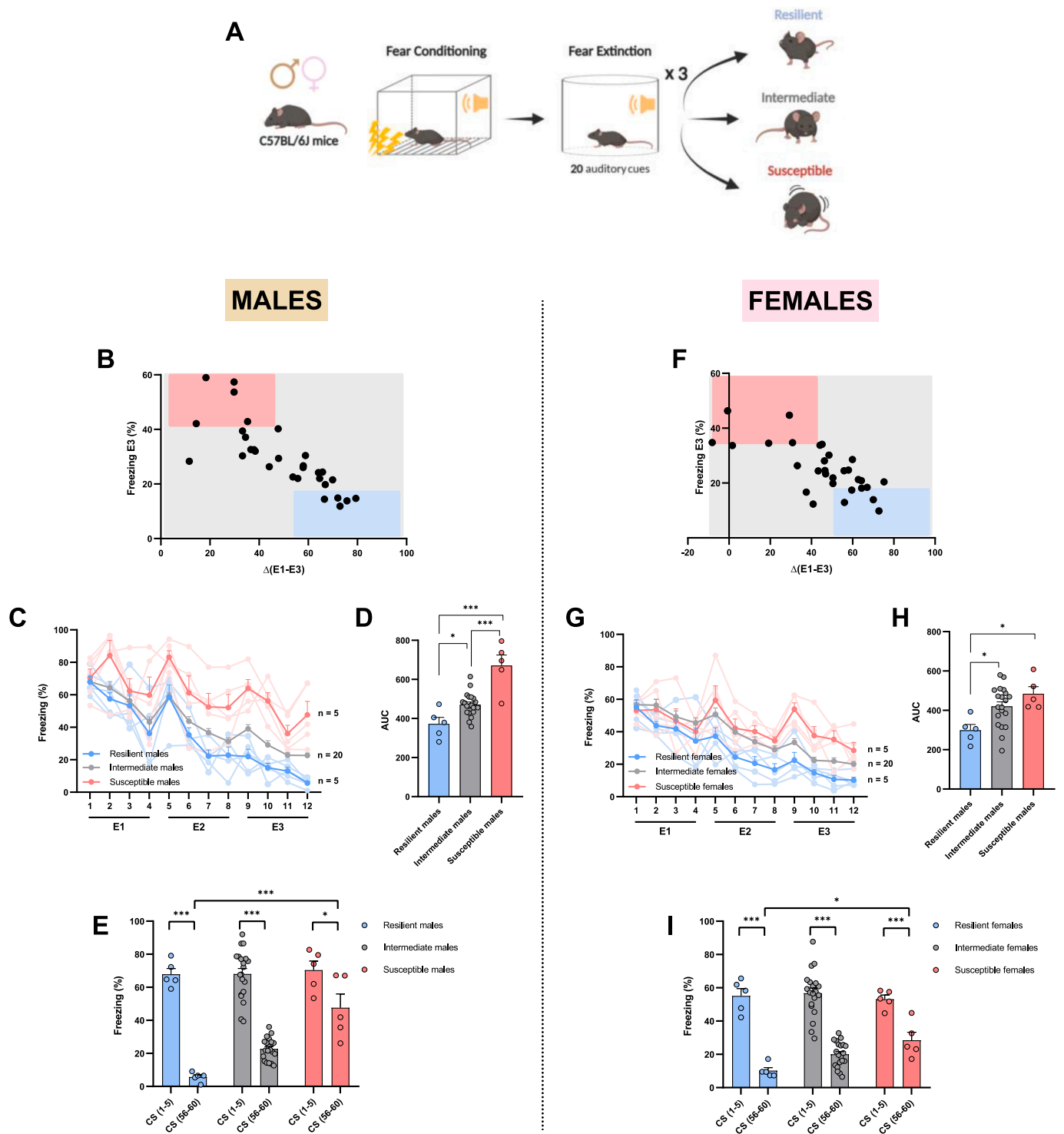


Fig. 1. Identification of resilient, intermediate and susceptible mice based on their extinction pattern. **(A)** Schematic representation of the experimental design. **(B, F)** Schematic representation of the subpopulations of resilient, intermediate and susceptible male **(B)** and female **(F)** mice considering the classification criteria used. **(C, G)** Time course of the freezing levels during cued fear extinction trials in male **(C)** and female **(G)** mice. Four bins (5 tones each) of CS presentations are shown in each extinction session. **(D, H)** AUC values of male **(D)** and female **(H)** mice for fear extinction phenotypes. **(E, I)** Measure of the extinction (freezing levels) the first bin (5 tones, extinction day 1) and the last bin (5 tones, extinction day 3) of CS presentations in resilient, intermediate and susceptible male **(E)** and female **(I)** mice considering the classification criteria. Data are expressed as mean + SEM ($n = 5-20$ mice per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (comparison between resilient, intermediate and susceptible mice). AUC, area under the curve; CS, conditioned stimulus.

levels during extinction were different between phenotypes in male mice as revealed two-way ANOVA (phenotype effect: $F_{2,27} = 22.33$, $p < 0.001$) and AUC values ($p < 0.001$, resilient versus susceptible mice) (Fig. 1D). Accordingly, freezing behavior was higher in susceptible in

comparison with resilient male mice ($p < 0.001$) when analyzing the last cluster of cues presentation the day 3 of extinction (interaction: $F_{2,27} = 6.65$, $p < 0.01$) (Fig. 1E). Likewise, fear extinction was different in resilient and susceptible female mice as revealed two-way ANOVA

(phenotype effect: $F_{2,27} = 4.96$, $p < 0.05$) (Fig. 1G) and AUC values ($p < 0.05$, resilient versus susceptible mice) (Fig. 1H). The analysis of the last bin of conditioned stimulus presentation also showed a clear difference between phenotypes (interaction: $F_{2,27} = 3.16$, $p < 0.05$) in female mice ($p < 0.05$) (Fig. 1I). A significant increase in freezing behavior of all experimental groups during the first conditioned stimulus in comparison with the previous habituation was observed, thus highlighting the discrimination between the absence and the presence of the conditioned stimulus (Fig. S2).

To explore resilience and vulnerability factors underlying inter-individual differences in fear extinction, we used the selected population of resilient/intermediate/susceptible mice to evaluate neurogenesis and brain region activity by c-Fos immunofluorescence.

3.2. Neurogenesis is altered in susceptible male and female mice to fear extinction

Adult neurogenesis has been involved in many psychiatric conditions including anxiety disorders (Chen et al., 2025a). In susceptible male mice, a non-significant increase of the cell proliferation marker Ki67 was observed in the SVZ (one-way ANOVA; $F_{2,12} = 3.11$, $p = 0.08$) (Fig. 2A) and the SGZ of the dentate gyrus (one-way ANOVA; $F_{2,12} = 2.44$, $p = 0.129$) (Fig. 2B). Interestingly, this increase was significant (one-way ANOVA; $F_{2,12} = 7.31$, $p < 0.01$) in the SGZ for the early neuron marker doublecortin ($p < 0.01$, resilient versus susceptible mice) (Fig. 2C, D). A positive correlation was also found between freezing levels in E3 and neurogenesis (Fig. S3). This unexpected result could be the consequence of a compensatory mechanism in susceptible animals (poor fear extinguishers) to counteract resistance to extinction and facilitate this process. To test this hypothesis, we studied the effects of temozolomide, a cytostatic drug capable of inhibiting neurogenesis, in the extinction of fear (Fig. 2E). Under our experimental conditions, temozolomide treated mice did not show any alteration in body weight and locomotor activity (Fig. S4). Notably, temozolomide administration induced an impairment of fear extinction (Fig. 2G), but not in the conditioning phase (Fig. 2F), in male mice as revealed two-way ANOVA (treatment: $F_{1,19} = 6.04$, $p < 0.05$) and AUC values ($p < 0.05$, vehicle versus temozolomide treatment) (Fig. 2G). Accordingly, freezing levels were higher in temozolomide treated mice as compared with controls during the extinction dynamic ($p < 0.05$ at E1 and E3) (Fig. 2H). A non-significant trend was also found in the percentage of decrease in freezing behavior (E1-E3) between groups (Fig. S5). This regimen of temozolomide administration was accompanied by a clear reduction in neurogenesis, as shown by the decrease of Ki67 in both the SVZ and the SGZ ($p < 0.01$) (Fig. 2I, J, L), and doublecortin in the SGZ ($p < 0.05$) (Fig. 2K, L). Taken together, these data reveal neurogenesis as an important inter-individual factor involved in fear extinction given that susceptible male mice could activate this process to preserve a physiological extinction course.

Congruent with the results described above, an enhancement in doublecortin expression was found in the SGZ of susceptible female mice (one-way ANOVA; $F_{2,12} = 8.30$, $p < 0.01$) ($p < 0.01$, resilient versus susceptible mice) (Fig. 3C, D). A non-significant effect was observed in the case of Ki67 in the SVZ (one-way ANOVA; $F_{2,12} = 1.43$, $p = 0.274$) and the SGZ (one-way ANOVA; $F_{2,12} = 0.809$, $p = 0.468$) (Fig. 3A, B). As in the case of male mice, a positive correlation was also observed between freezing levels in E3 and doublecortin expression (Fig. S3). Surprisingly, the same regimen dose of temozolomide administration in female mice (Fig. 3E) did not alter either fear conditioning (Fig. 3F) or extinction (Fig. 3G). Temozolomide treatment also did not modify neurogenesis in female animals (Fig. 3H, I, J), consistent with the lack of effect in the behavioral experiment.

Considering that most experimental approaches with temozolomide have been performed in male animals, we wondered whether administering a single cycle of this compound would be ineffective in female mice. However, three consecutive cycles (3-day daily injection separated by a 4-day abstinence) of temozolomide injection (Fig. S6A) also

did not modify fear extinction (Fig. S6C) or neurogenesis (Fig. S6D, E, F). Neither one nor three cycles of temozolomide changed either body weight or locomotion (Fig. S7).

3.3. Different brain region activation in susceptible male and female mice to fear extinction

We then assessed the neuroanatomical substrates that may contribute to the behavioral phenotypes described by using c-Fos immunofluorescence in brain regions associated with fear regulation. Thus, c-Fos expression in PL, IL, Ca1, Ca3, DG, LA, CeA, BA, PVT and HAB, was evaluated in resilient, intermediate and susceptible male and female mice (Fig. 4). Of the ten regions analyzed, changes specific to vulnerable versus resilient male animals were found in four regions. The expression of c-Fos increased in the IL of the susceptible group (Kruskal-Wallis; $p < 0.05$) (Fig. 4A), a critical region regulating extinction. This hyperactivation could reflect a compensatory mechanism in a vulnerable phenotype considering that earlier observations, using experimental approaches different from the one used in our study, suggest that resistance to extinction is associated with reduced c-Fos expression in the IL (Herry and Mons, 2004; Kim et al., 2010). A clear c-Fos reduction was observed in Ca1 and Ca3 regions of the dorsal hippocampus (one-way ANOVA; $F_{2,12} = 18.78$, $p < 0.001$ and $F_{2,12} = 5.63$, $p < 0.05$, respectively) ($p < 0.05$, resilient versus susceptible mice) (Fig. 4B). Finally, c-Fos expression was also reduced in the HAB of susceptible male animals ($p < 0.05$) (one-way ANOVA; $F_{2,12} = 8.89$, $p < 0.01$) (Fig. 4D). In contrast to males, we found only an increase of c-Fos in the DG of the hippocampus (Kruskal-Wallis; $p < 0.05$) in susceptible female mice in comparison with resilient animals (Fig. 4F). Taken together, these results show sex-dependent changes in the activity of different brain areas associated with phenotype of extinction.

3.4. Brain-wide activity patterns and network organization in susceptible male and female mice to fear extinction

PCA and k-means clustering algorithm, using c-Fos + counts across ten brain regions (PL, IL, Ca1, Ca3, DG, LA, CeA, BA, PVT, HAB), were used to identify brain-wide activity patterns according to fear extinction behavior. PCA analysis was sufficient to separate male mice much more clearly than females (Fig. 5A, C) into groups corresponding to their ability for fear extinction. Interestingly, this separation was less evident in the case of the intermediate group. In males, the PC1 and PC2 variances were 26.0% and 23.0%, respectively, while these variances were 30.7% and 17.2% in the case of female mice (Fig. 5A, C; scree plots in Fig. S8A, B). The corresponding PCA loading plots indicated predominant contributions from the prefrontal cortex (PL, IL) and hippocampal (Ca1, Ca3) regions to PC1 in males (Fig. 5B), whereas the hippocampal Ca1 and the HAB were the most relevant contributions in female mice (Fig. 5D). K-means cluster analysis ($k = 3$, according to our initial resilient, intermediate and susceptible phenotypes) separated animals into three clusters. Notably, this cluster assignment based on c-Fos labelling was significantly associated with phenotype in males ($\chi^2(4) = 10.4$, $p = 0.034$; ARI = 0.24) (Fig. 5A), but not in females ($\chi^2(4) = 5.6$, $p = 0.23$; ARI = -0.05) (Fig. 5C). These results indicate the existence of a sex-dependent pattern of brain-wide activity related to inter-individual differences for fear extinction.

Correlation-based networks highlighted phenotype-specific motifs (Fig. 6 and Fig. S9). Pairwise Pearson correlations between regions were calculated and significant associations ($p < 0.05$), marked by asterisks in correlation matrices and thicker edges in network graphs, are shown. These networks, characterized by both positive (green) and negative (purple) correlations, showed that extinction phenotypes are characterized by distinct topological arrangements between regions instead of uniform changes in connectivity. In males, susceptible mice were characterized by prefrontal-amygdalar (PL-LA), hippocampal-habenular (Ca1-HAB), and hippocampal-amygdalar (DG-BA) correlations

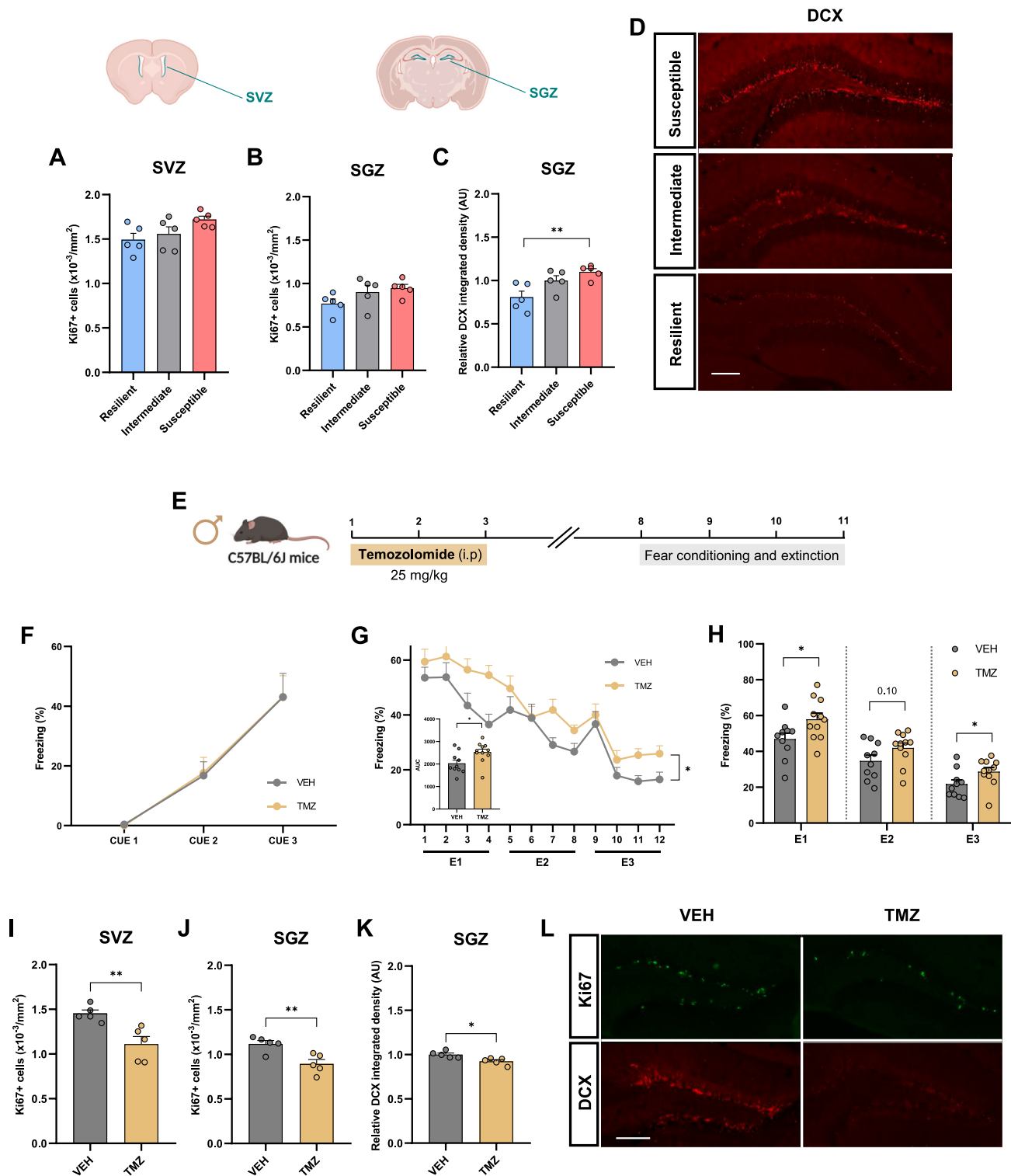


Fig. 2. Neurogenesis is altered in susceptible male mice to fear extinction. (A–C) Quantification of the cell proliferation marker Ki67 and the early neuron marker doublecortin (DCX) in the SVZ and the SGZ of resilient, intermediate, and susceptible male mice ($n = 5$ mice per group). (D) Representative immunohistochemical images of DCX+ cells in the SGZ of resilient, intermediate and susceptible mice. (E) Experimental design of the temozolomide (TMZ) treatment protocol ($n = 10$ – 11 mice per group). (F) Freezing levels during fear conditioning in VEH- and TMZ-treated animals. (G) Time course of the freezing levels and AUC values during cued fear extinction trials in VEH- and TMZ-treated groups. Four bins (5 tones each) of CS presentations are shown in each extinction session. (H) Freezing levels in VEH- and TMZ-treated animals across the extinction sessions. (I–K) Quantification of Ki67+ and DCX+ cells in SVZ and SGZ of VEH- and TMZ-treated mice ($n = 5$ mice per group). (L) Representative immunohistochemical images of Ki67+ and DCX+ cells in the SGZ of VEH- and TMZ- treated mice. Data are presented as mean + SEM. * $p < 0.05$, ** $p < 0.01$ (comparison between resilient and susceptible mice in (C); comparison between VEH- and TMZ-treated mice in (G–K)). AUC, area under the curve; CS, conditioned stimulus; DCX, doublecortin; SGZ, subgranular zone; SVZ, subventricular zone; TMZ, temozolomide; VEH, vehicle. The scale bar represents 100 μ m.

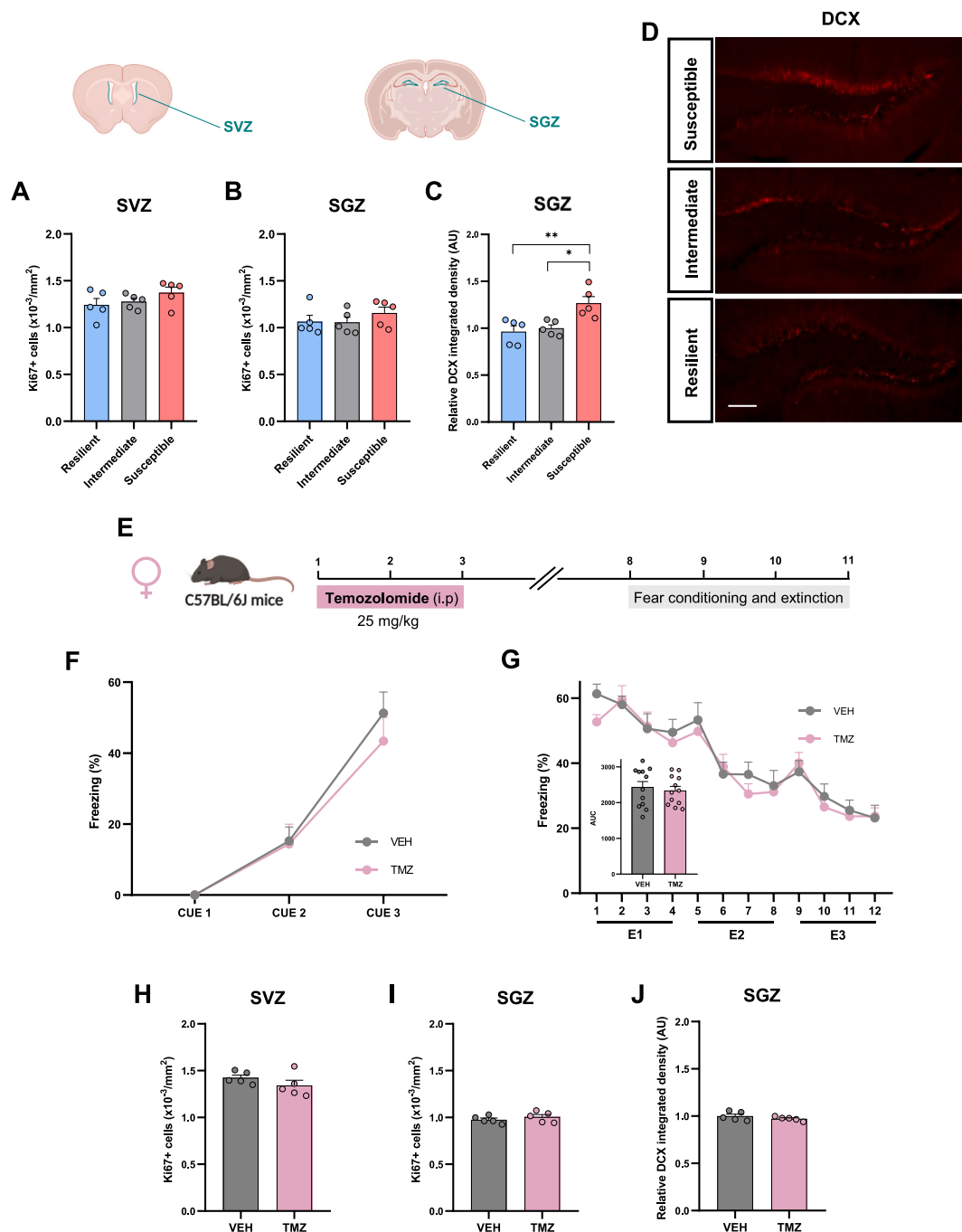


Fig. 3. Neurogenesis is altered in susceptible female mice to fear extinction. **(A–C)** Quantification of the cell proliferation marker Ki67 and the early neuron marker doublecortin (DCX) in the SVZ and the SGZ of resilient, intermediate, and susceptible female mice ($n = 5$ mice per group). **(D)** Representative immunohistochemical images of DCX+ cells in the SGZ of resilient, intermediate and susceptible mice. **(E)** Experimental design of the temozolomide (TMZ) treatment protocol ($n = 12$ mice per group). **(F)** Freezing levels during fear conditioning in VEH- and TMZ-treated animals. **(G)** Time course of the freezing levels and AUC values during cued fear extinction trials in VEH- and TMZ-treated groups. Four bins (5 tones each) of CS presentations are shown in each extinction session. **(H–J)** Quantification of Ki67+ and DCX+ cells in SVZ and SGZ of VEH- and TMZ-treated mice ($n = 5$ mice per group). Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ (comparison between resilient and susceptible mice). AUC, area under the curve; CS, conditioned stimulus; DCX, doublecortin; SGZ, subgranular zone; SVZ, subventricular zone; TMZ, temozolomide; VEH, vehicle. The scale bar represents 100 μm .

(Fig. 6C, F). In contrast, susceptible female animals showed significant hippocampal-amygdalar (Ca3-BA), hippocampal-thalamic (Ca3-PVT), and amygdalar-thalamic (BA-PVT) associations (Fig. 6I, L). However, no differences in global network connectivity between phenotypes were observed in either males or females ($p = 0.78$ and $p = 0.56$, respectively, Mantel followed by Fisher global test), maybe influenced by the inclusion of the intermediate groups, as reflected the PCA analysis. Taken together, these results suggest that resilience and susceptibility to fear

extinction are supported by divergent circuit-level reorganizations rather than global alterations in network density. Centrality metrics are reported in Fig. S9, providing quantitative support for the network configurations shown in Fig. 6.

4. Discussion

In this study, we show important intrinsic group differences in male

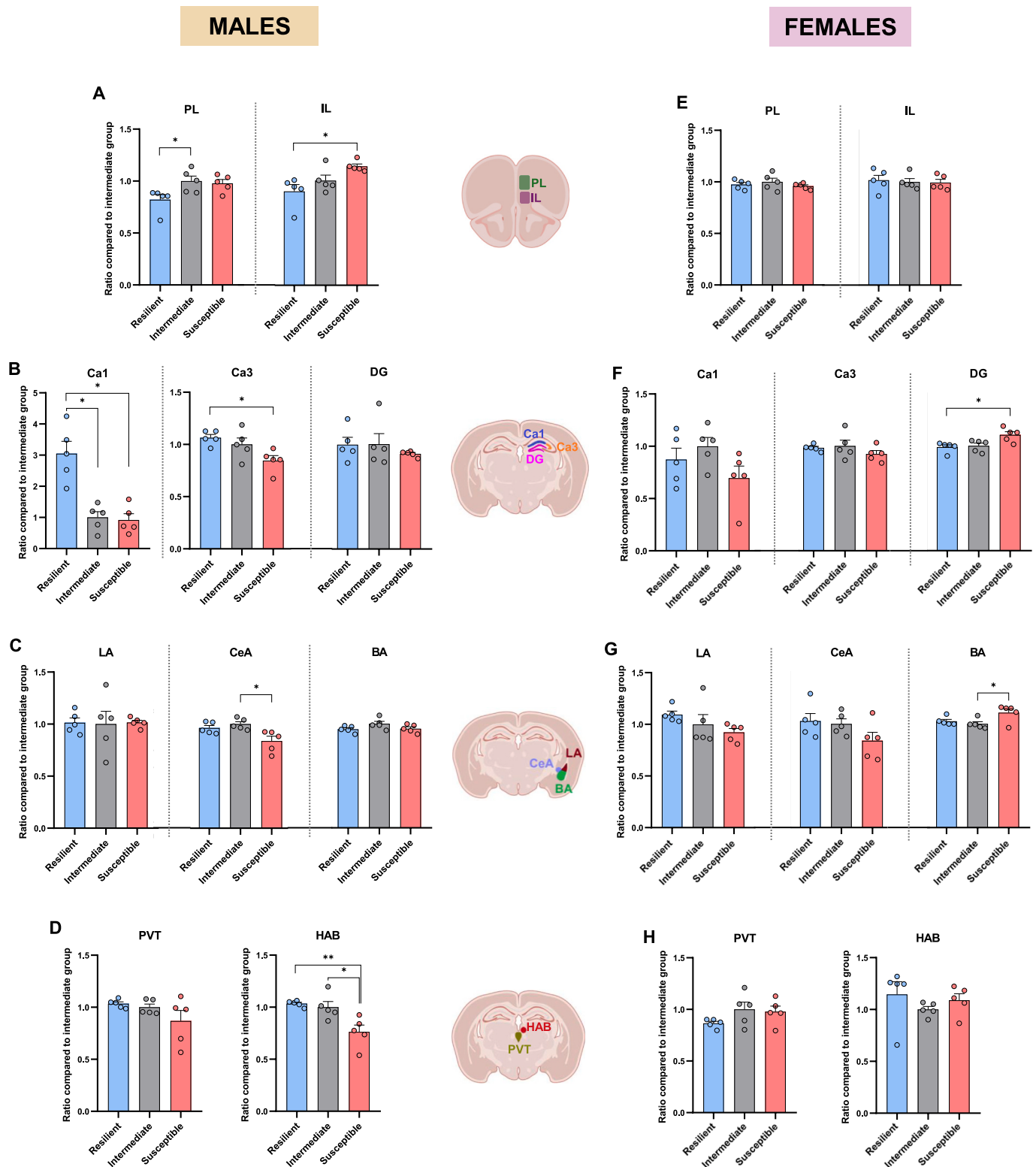
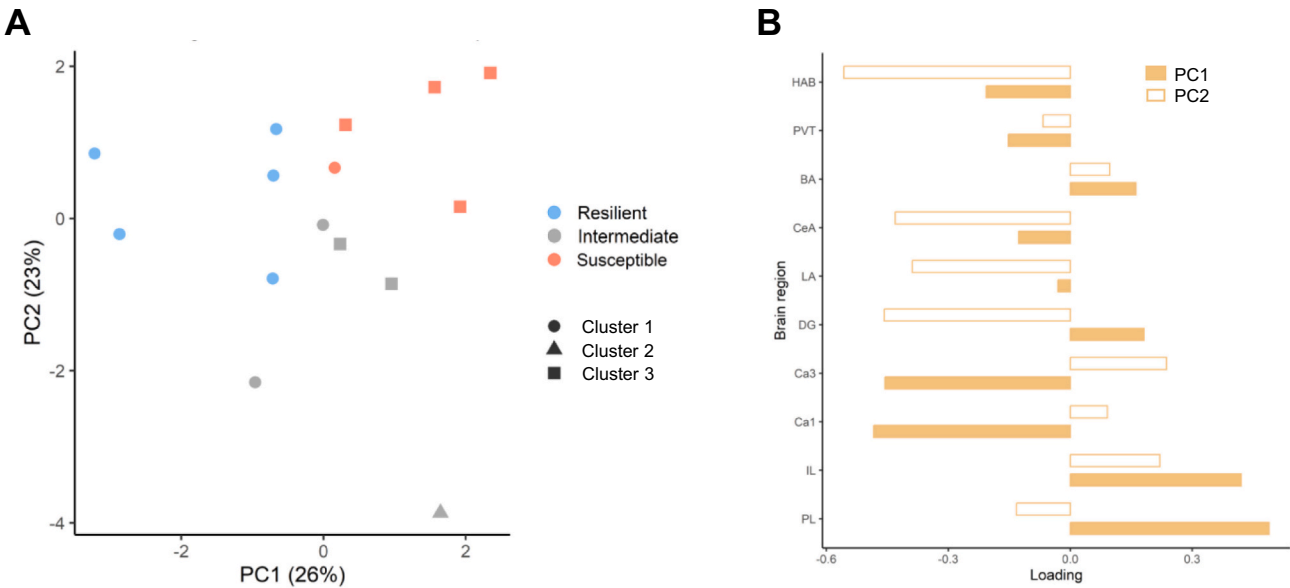


Fig. 4. Different brain region activation in susceptible male (A–D) and female (E–H) mice to fear extinction. Ratio of c-Fos + cell counts relative to the intermediate phenotype is shown. Quantification of c-Fos + cell counts in PL and IL (A, E), hippocampal regions Ca1, Ca3, and DG (B, F), LA, CeA and BA (C, G), PVT and HAB (D, H). Data are mean + SEM (n = 5 mice per group). *p < 0.05, **p < 0.01 (comparison between resilient, intermediate and susceptible mice). BA, basal amygdala; Ca1, Cornu Ammonis area 1; Ca3, Cornu Ammonis area 3; CeA, central amygdala; DG, dentate gyrus; HAB, habenula; IL, infralimbic cortex; LA, lateral amygdala; PL, prelimbic cortex; PVT, paraventricular thalamic nucleus.

MALES



FEMALES

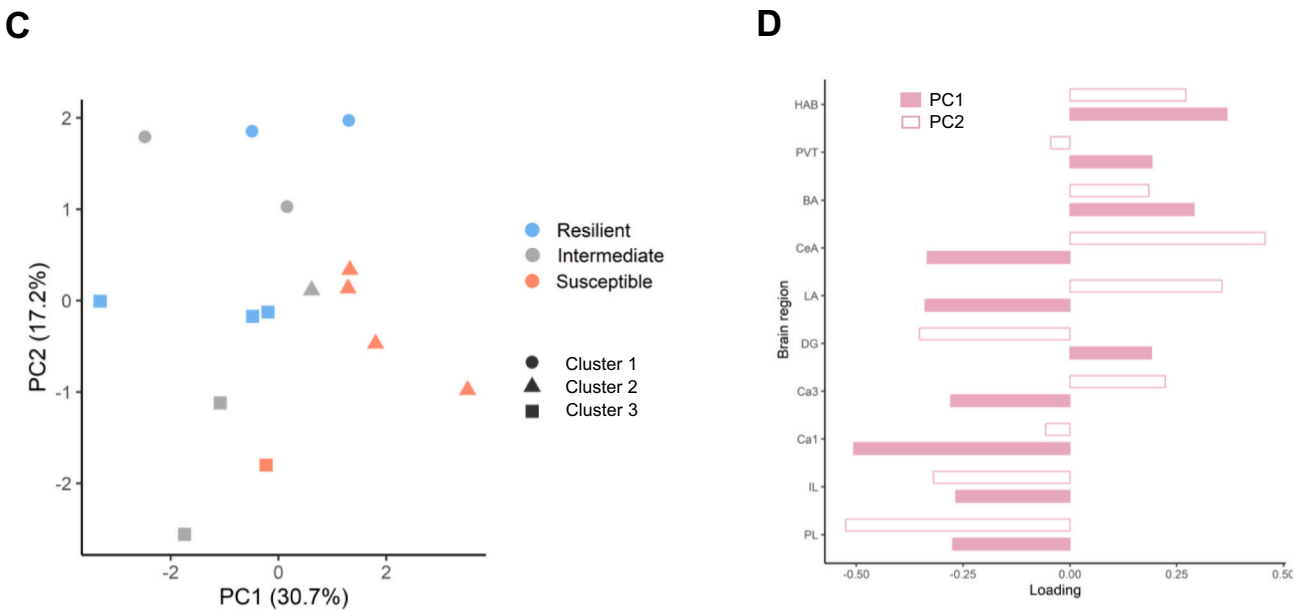


Fig. 5. Brain-wide activity is associated with the different extinction phenotypes in male mice. (A, C) PCA plots and k-means clustering based on c-Fos + cell counts across ten different brain regions in male (A) and female (C) mice. (B, D) Component loadings for PC1 and PC2 showing the relative contribution of each brain region to the principal components in male (B) and female (D) mice. BA, basal amygdala; Ca1, Cornu Ammonis area 1; Ca3, Cornu Ammonis area 3; CeA, central amygdala; DG, dentate gyrus; HAB, habenula; IL, infralimbic cortex; LA, lateral amygdala; PL, prelimbic cortex; PVT, paraventricular thalamic nucleus; PCA, principal component analysis; PC1 principal component 1; PC2, principal component 2.

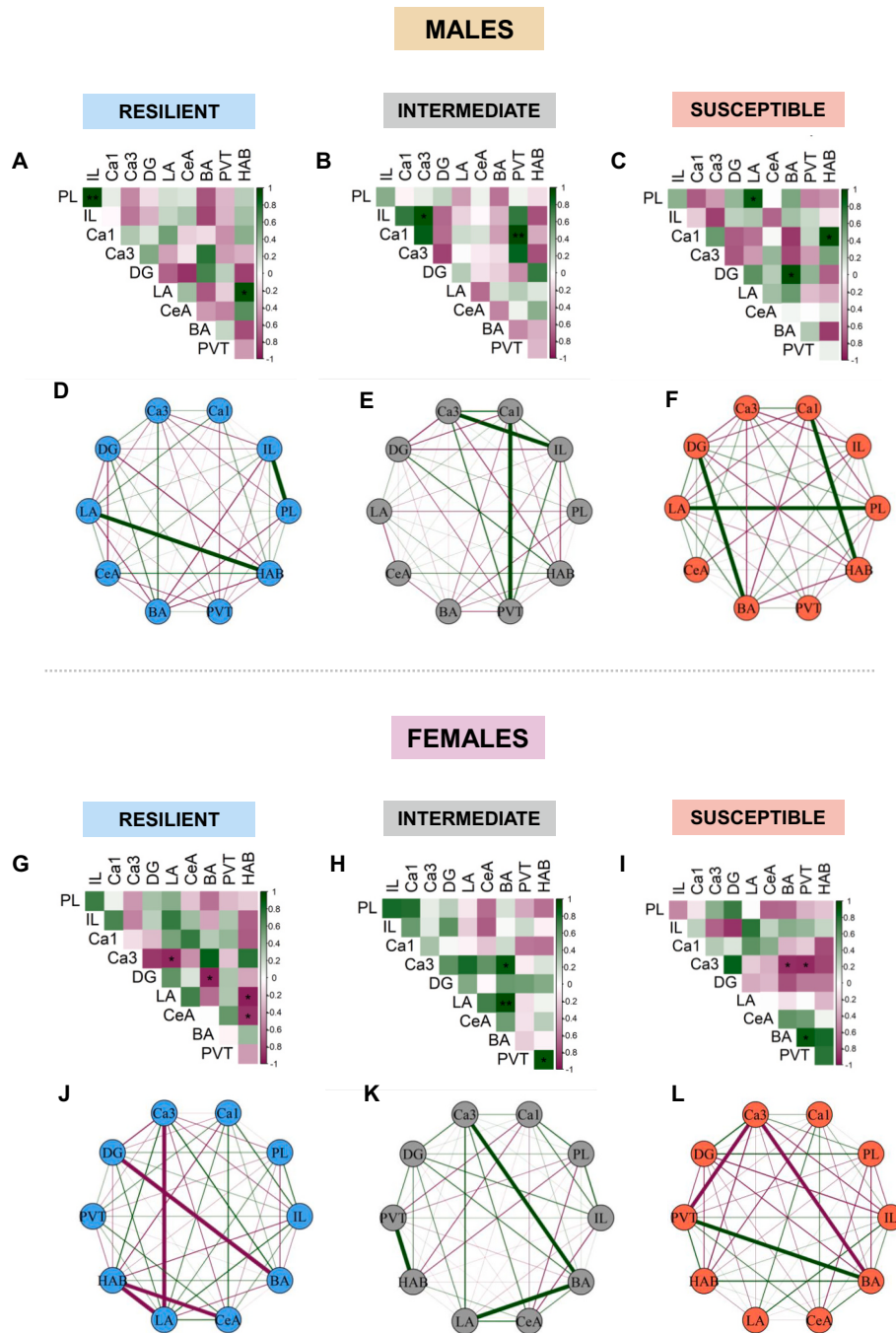


Fig. 6. Correlation matrices and network representations based on c-Fos + cell counts in the extinction phenotypes in male (A–F) and female (G–L) mice. Significant pairwise correlations are indicated by asterisks (Pearson correlation, $p < 0.05$) in males (A–C) and females (G–I). Network graphs derived from the corresponding matrices are represented in males (D–F) and females (J–L). Nodes represent brain regions, and edges represent correlations between regions. Edge thickness scales with correlation strength, and statistically significant correlations (Pearson correlation, $p < 0.05$) are displayed as thicker edges. BA, basal amygdala; Ca1, Cornu Ammonis area 1; Ca3, Cornu Ammonis area 3; CeA, central amygdala; DG, dentate gyrus; HAB, habenula; IL, infralimbic cortex; LA, lateral amygdala; PL, prelimbic cortex; PVT, paraventricular thalamic nucleus.

and female mice regarding their inter-individual variation in fear extinction performance. Thus, increased neurogenesis, as shown by the enhancement of the early neuron marker doublecortin in the SGZ, was observed in susceptible animals. In addition, a sex-dependent pattern of brain-wide activity was found according to resilient, intermediate, and susceptible phenotypes for fear extinction. Knowledge of the neurobiological mechanisms involved in the variability of fear extinction will help support the development of new therapeutic strategies for fear-related disorders.

Our behavioral model allowed us to separate mice in relation to their cued-fear extinction performance. By using specific criteria for categorization, based on freezing levels during the last session of extinction and the individual reduction in freezing behavior between the first and the last session, mice were classified in resilient (good extinguishers), intermediate and susceptible (poor extinguishers) groups. The model here described represents a powerful tool to study inter-individual differences in fear extinction since any experimental condition (e.g., pharmacological treatment, early stress or genetic alteration) was added

(Masella et al., 2024; Ten-Blanco et al., 2025). Other related experimental protocols have evaluated the role played by stress in susceptibility and resilience to PTSD (Daws et al., 2017) or how alterations in microbiota-gut-brain axis influence susceptibility to social fear extinction (Ritz et al., 2024). On the other hand, male and female animals were included in our study given that different biological contributors seem to influence the heightened risk for developing PTSD observed in women (Ponomareva and Ressler, 2021; Hiscox et al., 2023; Levesque et al., 2023). Despite these sexual differences, most preclinical research to date has used only male animals.

Neurogenesis, the process of generating new neurons from the neural stem cells or neural progenitor cells in the adult brain, is mainly restricted to specific regions of the brain including the SVZ and the SGZ of the hippocampal DG (van Praag et al., 2002).

There is much evidence in both animal and human studies that neuropsychiatric diseases are linked to alterations in adult hippocampal neurogenesis (Chen et al., 2025a). Thus, decreased neurogenesis has been implicated in depression and anxiety conditions (Tartt et al., 2022; Berger et al., 2020), although it remains unclear whether these changes are a cause or consequence of the disorder. Specifically, the regulation of emotional memory function has been also shown to be controlled by adult neurogenesis (Saxe et al., 2006; Kitamura et al., 2009; Kirby et al., 2012). Using a double trauma murine model for PTSD, different interventions that promote hippocampal neurogenesis were found to attenuate PTSD-related behavioral phenotypes (Fujikawa et al., 2024). Chemogenetic activation of adult hippocampal neuronal progenitors within the SGZ of the DG reduced anxiety-like behaviors and increased fear memory extinction in a contextual fear learning paradigm (Maheshwari et al., 2024). The psychedelic drug psilocybin facilitated fear extinction by the promotion of hippocampal neuroplasticity, including doublecortin expression in the DG (Du et al., 2023). Congruent with these results, reduced hippocampal neurogenesis and endocannabinoid levels were associated with impaired fear extinction in mice lacking diacylglycerol lipase α , the main producing enzyme of the endocannabinoid 2-arachidonoyl glycerol (Jenniches et al., 2016). Interestingly, we found an unexpected increased expression of the early neuron marker doublecortin in the SGZ of susceptible (poor fear extinguishers) male and female mice. These data reveal that neurogenesis is an important inter-individual factor involved in fear extinction and suggest the existence of a possible compensatory mechanism in susceptible mice to counteract resistance to extinction and facilitate this process.

To assess this potential compensation, we studied the effects of the DNA-alkylating agent temozolomide, which reduces adult hippocampal neurogenesis (Garthe et al., 2009; Niibori et al., 2012), in the regulation of fear extinction. This compound has been recently shown to modulate performance on a contextual fear discrimination task in male mice (Yu et al., 2023) and rats (Francis et al., 2025) through the modulation of neurogenesis. We found that a single cycle of temozolomide treatment was enough to impair fear extinction and reduce the expression of Ki67 and doublecortin in the SGZ of the DG in male mice. These results support the idea that susceptible male mice increase neurogenesis in an attempt to improve fear extinction behavior. In contrast, the same temozolomide administration regimen did not affect fear extinction or neurogenesis in female mice suggesting the existence of an intriguing sex-specific effect of this compound. Indeed, three consecutive cycles of temozolomide injection also had no effect on the females. In contrast, Balb/C female mice treated with two cycles of temozolomide showed short- and long-term decreases in Ki67+ cells and doublecortin expression in the DG (Cabrera-Muñoz et al., 2023). Different background of the two mouse strains (Balb/C versus C57BL/6 J) could explain this discrepancy. Given that most studies with temozolomide evaluating fear and anxiety have used male mice (Pereira-Caixeta et al., 2018; Mañas-Padilla et al., 2023; Yu et al., 2023; He et al., 2024), analysis of the effects of this compound in female rodents warrants further consideration in future experiments.

Adult hippocampal neurogenesis is increasingly recognized as a dynamic contributor to structural and functional hippocampal plasticity (Toda et al., 2019). Experimental modulation of neurogenesis in animal models alters structural plasticity and hippocampal-dependent behaviors (Chen et al., 2025b). The increased neurogenesis observed in susceptible mice to fear extinction could promote dendritic spine maturation and synaptic stabilization. These processes could be part of possible mechanisms for preserving extinction. Future experiments evaluating dendritic spine density and morphology, and dendritic arborization of hippocampal neurons under our experimental conditions would be of interest. Moreover, it would be also interesting to study the effects of treatment with temozolomide or neurogenic compounds such as NSI-189 (McIntyre et al., 2017; Liu et al., 2019) on these processes. On the other hand, other possible interpretations of the increased neurogenesis observed in susceptible mice are also plausible, apart from a compensatory mechanism. It is possible that extinction process could have produced increased expression of doublecortin in the SGZ at an early time point in resilient animals and at a later time point in susceptible mice. Moreover, doublecortin could not reflect strictly the formation of new neurons since it has been involved in other functions such as cytoskeleton regulation (Feng and Walsh, 2001).

Next, we assessed the neuroanatomical substrates that may contribute to the susceptible/resilient phenotypes by using c-Fos immunofluorescence. In comparison with resilient male mice, susceptible animals showed reduced c-Fos expression in the hippocampal Ca1 and Ca3 regions and the HAB while increased activity was observed in the IL, congruent with the role played by these areas in fear extinction regulation (Sierra-Mercado et al., 2011; Song et al., 2017; Lee et al., 2023). Several animal models characterized by resistance to extinction such as the 129S1/SvImJ mouse strain (Hefner et al., 2008), mice lacking dynorphin (Bilkei-Gorzo et al., 2012), or syntenin-1 knockout mice (Talukdar et al., 2018) showed IL hypoactivity, consistent with human studies showing a correlation between patients with PTSD and hypoactivation of the functionally analogous ventromedial prefrontal cortex region (Milad et al., 2009). Accordingly, extinction-facilitating effects of the orexin receptor-1 antagonist SB334867 (Flores et al., 2014) or ketamine (Glavonic et al., 2024) were related to increased c-Fos expression in this brain region. Therefore, our result could be the consequence of a compensatory mechanism to counteract the deficit in extinction and would highlight the importance of the activity of this brain area as a vulnerability factor involved in diseases related to pathological fear. On the other hand, beyond a compensatory mechanism, c-Fos expression in the IL could be lower at the end of the extinction in resilient mice because they formed an extinction memory during E1 or E2 and therefore could require less IL activity during extinction at E3. Moreover, IL stimulation has been shown to promote either more or less anxiety-like behaviors and fear-related freezing via separate projections to the lateral septum or the CeA, respectively (Chen et al., 2021).

Unlike in males, changes in brain activity were less evident in females since only one brain structure, the DG of the hippocampus, showed greater activity in susceptible compared to resilient animals. Female mice were included in our study without regard to estrus cycle phase, which could alter the expression of c-Fos (Mikosz et al., 2015), thus probably increasing the variability among individuals. Moreover, by using a different experimental approach, sex differences in c-Fos expression during the extinction of fear have been recently reported in rats (Zhang et al., 2024).

Brain function underlying behavior is the result of changes in brain-wide activity. Thus, the regulation of a fearful experience requires the coordinated activity of thousands of neurons across the brain (Bulthuis et al., 2025). Changes in brain activity and network connectivity have been recently reported in a mouse model of Alzheimer's disease showing anxiogenic-like behavior (Hunsberger et al., 2025), in social interaction associated with the presence or absence of physical contact (Zhai et al., 2025) or related to mild and strong fear memories (Haubrich and Nader,

2023). Interestingly, PCA and k-means clustering analyses, using c-Fos + counts across the different brain regions evaluated, showed the existence of a significant pattern of activity throughout the brain that coincided with resilient, intermediate, and susceptible phenotypes for fear extinction in male, but not female, mice. Moreover, network analysis using Pearson correlations between regions showed different circuit-level connectivity according to the different phenotypes in males and females. These results identify brain-wide neural activity patterns as a neurobiological factor involved in inter-individual differences in the ability to extinguish fear, which was more robust in male animals. Additionally, future research using other immediate-early genes, such as Arc and NPAS4, will be of interest to confirm and complete the results of our study as significant differences in distribution and induction patterns following learning have been recently reported with different genes of this class (Bulthuis et al., 2025; Chiaruttini et al., 2025).

As previously mentioned, most preclinical research evaluating fear extinction has used only male animals. Therefore, there is a need to change our approach to science by including female subjects at all levels of research. It is worth highlighting the challenges related to research conducted with females given the considerable variability, especially with regard to behavioral experiments, compared to male subjects, which leads to marked difficulties in achieving significant results and, in turn, clear conclusions. Here, we have found different results in male and female mice congruent with our previous study evaluating inter-individual factors in fear extinction by using a similar experimental protocol (Ten-Blanco et al., 2025). Although we are still far from understanding the basis of these differences, the estrous cycle and neuronal circuits (Bauer, 2023) appear to be important mediators of these sex-dependent modifications.

In summary, our data provide valuable insights into important inherent group differences in mice considering their individual variation in fear extinction performance. Knowledge of these inter-individual factors, which could contribute to the etiology of fear-related disorders, will allow us to advance our understanding of these pathologies for which there are currently no effective pharmacological treatments.

CRediT authorship contribution statement

María Ponce-Renilla: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Inmaculada Pereda-Pérez:** Methodology, Investigation, Formal analysis, Data curation. **Cristina Izquierdo-Luengo:** Methodology, Investigation, Formal analysis. **Rosa María Tolón:** Methodology, Investigation. **Fernando Berrendero:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Data curation, Conceptualization.

Consent for publication

All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Animal procedures were conducted with approval from the local ethical committee (CEEA-UFV) (283.2/21).

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Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nbd.2026.107300>.

Data availability

The data supporting the findings of this study are available from the corresponding author, upon reasonable request.

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