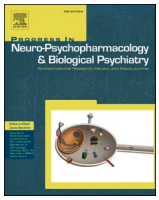
Progress in Neuropsychopharmacology & Biological Psychiatry 141 (2025) 111484

Contents lists available at ScienceDirect

Progress in Neuropsychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp



The impact of prior exposure to severe stressors on fear learning: The critical contribution of the type of stressor and the rat strain

Sara Serrano [a,b], Xavier Belda [a,b], Antonio Armario [a,c,d,*]

[a] Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain
[b] Animal Physiology Unit, Departament de Biologia Cellular, Fisiologia I Immunologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Barcelona, Spain
[c] Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Madrid, Spain
[d] Unitat de Neurociència Traslacional, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc Taulí (I3PT), Sabadell, Spain

ARTICLE INFO

Keywords:
PSTD animal models
Inescapable foot-shocks
Immobilization
Context fear conditioning
Fear generalization
Strain differences
Long-Evans rats
Sprague-Dawley rats

ABSTRACT

The appearance of long-lasting behavioral alterations is considered critical for the characterization of acute stressors as putative animal models of PTSD. However, the traumatic nature of the different stressors used is objectively difficult to demonstrate and literature is plagued by inconsistent results. In the present study we wanted to demonstrate the relevance of qualitative aspects of stressors not linked to their severity (as evaluated by classical biological markers) and how the use of different mouse or rat strains can contribute to the inconsistencies. We then exposed Sprague-Dawley (SD) and Long-Evans (LE) rats to two different severe stressors of roughly similar intensity, immobilization on boards (IMO) and inescapable foot-shocks (IS), and studied their impact on contextual fear conditioning, generalization, extinction and extinction recall. The results confirmed that the two stressors are of similar severity (IMO a little bit more severe) in terms of biological markers of stress, but their impact of fear conditioning was strongly dependent on the stressor and the strain, with a strong impact of IS in LE rats, a modest impact of IMO in the latter strain and almost null impact of the two stressors in SD rats. We thus confirm the relevance of both qualitative aspects of stressors and the strain used in order to characterize appropriate models of PTSD. Deciphering the processes underlying the contribution of the two factors is fundamental and requires comparison of stressors and strains at different neurobiological levels.

1. Introduction

In the last decades, there has been a great interest for the long-term behavioral and neuroendocrine effects of a single exposure to severe stressors (Armario et al., 2008; Schöner et al., 2017; Deslauriers et al., 2018; Richter-Levin et al., 2019). The protracted consequences of stress, typically observed several days or weeks after initial exposure, are considered to be an animal reflection of the alterations observed in posttraumatic stress disorder (PTSD). Studies have demonstrated that a single exposure to some severe stressors, including immobilization on boards (IMO), inescapable foot-shocks (IS) and the single prolonged stress paradigm (SPS) can result in potentiated shock-induced fear conditioning and/or impaired extinction (Rau et al., 2005; Yamamoto et al., 2009; Lisieski et al., 2018). However, results are very often inconsistent, and we lack a clear picture about the origin of the inconsistencies.

Most of the data about the influence of prior stress exposure on FC have been obtained using the SPS paradigm that is likely to be currently the most popular animal model of PTSD. The original version consisted of the sequential exposure of rats (or mice) to 2 h of restraint, 20 min forced swim and ether anesthesia (Liberzon et al., 1999). Other variants have introduced immobilization on boards instead of restraint (Laukova et al., 2014) or other anesthetics (Ganon-Elazar and Akirav, 2012). Prior exposure to SPS has been found to potentiate cue and context FC in some cases (e.g., Imanaka et al., 2006; Iwamoto et al., 2007; Harada et al., 2008; Xiao et al., 2020), but in other studies, fear learning was not affected, whereas it was fear extinction and/or fear extinction recall (Yamamoto et al., 2008; Ganon-Elazar and Akirav, 2012; Knox et al., 2012; Keller et al., 2015; Vanderheyden et al., 2015; Lin et al., 2016a, 2016b).

In our hands, either a single exposure to IMO or a SPS procedure that included IMO did not affect, 7 days later, context fear acquisition and only modestly impaired fear extinction (Sanchís-Ollé et al., 2023). Similarly, a single exposure of SD rats to IMO in plastic bags did not alter

* Corresponding author at: Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain.
E-mail address: Antonio.armario@uab.cat (A. Armario).

https://doi.org/10.1016/j.pnpbp.2025.111484
Received 3 April 2025; Received in revised form 1 September 2025; Accepted 1 September 2025
Available online 2 September 2025

either context FC acquisition or extinction and, in fact, modestly improved fear extinction recall (Kirby et al., 2013). Interestingly, studies in C57 mice have observed consistent impairment of tone fear extinction in mice exposed to restraint or IMO at least 6–7 days before conditioning (Andero et al., 2011; Chauveau et al., 2012; Sawamura et al., 2016; Velasco et al., 2022). We are not aware of similar studies in other mouse strains, and it is plausible that C57 mice are particularly susceptible to the impact of severe stressors.

Whereas the picture obtained after restraint, IMO or SPS is far from being consistent, Fanselow's laboratory have repeatedly found in Long-Evans rats that a single exposure to IS resulted in long-lasting (up to 3 months) potentiation of FC in both males and females (Rau et al., 2005; Rau and Fanselow, 2009; Perusini et al., 2016). They termed this effect as stress-enhanced fear learning (SEFL), implicitly assuming that this might reflect a sensitization phenomenon that might be induced by severe stressors rather than a specific effect of IS.

We reasoned that the high consistency of the results obtained by Fanselow and collaborators might have been due to two major factors. First, the use of the same aversive stimulus (foot-shocks) to cause stress and to induce FC. Secondly, the choice of the LE strain, which is characterized by a marked hyperresponsiveness of the hypothalamic-pituitary-adrenal (HPA) axis to stress as compared with Sprague-Dawley (SD) and other rat strains (Sanchís-Ollé et al., 2021). Importantly, in our hands, LE rats are particularly sensitive to CFC compared with SD rats (unpublished data). We then hypothesized that the impact of prior exposure to stress on CFC would be more evident after IS than IMO and stronger in LE than SD rats. IS and IMO were chosen because, based on biological markers of stressor intensity, they are both severe (e.g., Márquez et al., 2002; Belda et al., 2016). Defining the characteristics of stressors and strains which determine the impact of prior severe stressors on fear learning is a critical step to study underlying neurobiological processes.

2. Materials and methods

2.1. Animals and general procedure

Seven-week-old male Sprague-Dawley and Long-Evans rats were purchased from Janvier Labs (France). The animals were housed in pairs and maintained under standard conditions of temperature (22 ± 1 °C) and in a 12 h light-dark cycle (lights on at 8:00 am). Food and water were available ad libitum, and no specific environmental enrichment was used. The experimental protocol was approved by the Ethics Committee at the Universitat Autònoma de Barcelona and the Generalitat de Catalunya, and it was carried out in accordance with the European Council Directive (2010/63/UE) and Spanish legislation (RD 53/2013).

All experimental procedures were conducted during the light period. Nine days after their arrival, animals were habituated to handling (2 min/day for three days) before starting the experiment. Animals were blood sampled by the tail-nick procedure, which allows obtaining true resting levels of hormones, as described previously (Belda et al., 2004).

2.2. Experimental design and procedures

The average age of the animals on experimental day 1 was 70 days. Rats of each strain were randomly assigned to three experimental group, thus resulting in: SD – control ($n = 10$); SD – IMO (n = 10); SD – IS (n = 10); LE – control (n = 10); LE – IMO (n = 10) and LE – IS (n = 10). Due to the high number of animals, stress exposure was done in two consecutive days, with the same number of animals per group each day. Exposure to IMO and IS took place in two different rooms having similar characteristics and animals were transported from the vivarium to the experimental rooms in their homecages. Then, rats were exposed to 1 h immobilization on boards, following our routine protocol (Gagliano et al., 2008), or were exposed to IS in a shock chamber (context A). In this case, rats were left 2 min of habituation to context A and then received 30 foot-shocks (1 mA, 2 s, 2 min inter-trial interval). After that, they were removed from the IS chamber. Context A (27 × 25,5 × 24,5 cm) had three black walls, a transparent wall, and a grid floor of 20 stainless steel rods (Panlab S.L.U., Barcelona, Spain) through which the shock was administered. The apparatus was cleaned before the first animal and between animals with ethanol (70 %, *v*/v). Control animals remained undisturbed on their home cages during the exposure. The detailed experimental design is illustrated in Fig. 1.

Blood samples were taken under resting conditions 3 days before stress exposure. On the stress day, samples were taken immediately after stress exposure and 1 h after stress termination. Cage-mates were always sampled simultaneously. Blood (300 μl) was collected within 2 min into ice-cold EDTA capillary tubes (Microvette®, SARSTEDT) and centrifuged at 4 °C and 4500 rpm, the plasma being frozen and stored at −20 °C until analysis. Plasma ACTH and corticosterone levels were determined by well-stablished double-antibody radioimmunoassay (RIA) as described previously (Muñoz-Abellán et al., 2011).

One week later (day 8), all animals were trained for contextual fear conditioning in a different context B. Context B was a clear Plexiglas box (57 × 41 × 70 cm) with a removable grid floor with 44 stainless steel rods (Panlab S.L.U., Barcelona, Spain). The apparatus was placed in another room with black walls and fluorescent light and was cleaned before the first animal and between animals with water solution containing soap. Rats were transported to the experimental room in a small white plastic box (29 × 27 × 14 cm) without bedding and a transparent ceiling. This context is larger than those typically used for fear conditioning in rats to allow displaying of exploratory activity. After 3 min of habituation to the new chamber, rats received only one shock (1 mA, 2 s), remaining for 1 additional min in the chamber.

On day 10, all rats were exposed to a novel environment (open-field, Context C) for 8 min without receiving shocks in order to study contextual fear generalization. Animals were exposed to Context C before being tested in Context B to prevent possible extinction. Context C consisted of a rectangular grey plastic cage (56 × 36,5 × 31 cm) without ceiling, located in a room with black walls and dimmed light. The apparatus was cleaned with diluted acetic acid before the first animal and between animals. Rats were transported to the experimental room in a white circular container with a red lid without bedding.

On day 12, the animals were exposed again to context B for 20 min to test for fear conditioning and extinction. In this case, behavior was recorded during the first 8 min and again in the last 4 min to evaluate extinction. Finally, on day 18, all groups were exposed again to context B for 8 min to evaluate recall of extinction. All sessions were recorded with a video camera for further analysis.

2.3. Food intake and body weight gain

To have additional information about the impact of the stressors, food intake per cage and body weight gain was measured. Food intake per cage was measured for 3 days before exposure to the stressors (baseline) and in the 24 h and 48 h following exposure. Similarly, rats were weighed regularly and during the 48 h following exposure.

2.4. Behavioral measures

Behavior was recorded manually (the experimenter blind with each particular experimental group) and included freezing time, distance travelled and frequency of rearing. Freezing was considered as the absence of all movements except for respiration. Distance travelled was assessed by video tracking analysis using the centre of gravity of the animal (Smart, Panlab S.L.U., Barcelona, Spain). Rearing was registered when the animals had the two front legs off the floor. Regarding fear acquisition, freezing time was measured before and after shock administration. Generalization, test/extinction, and the extinction recall were measured in blocks of 4 min.

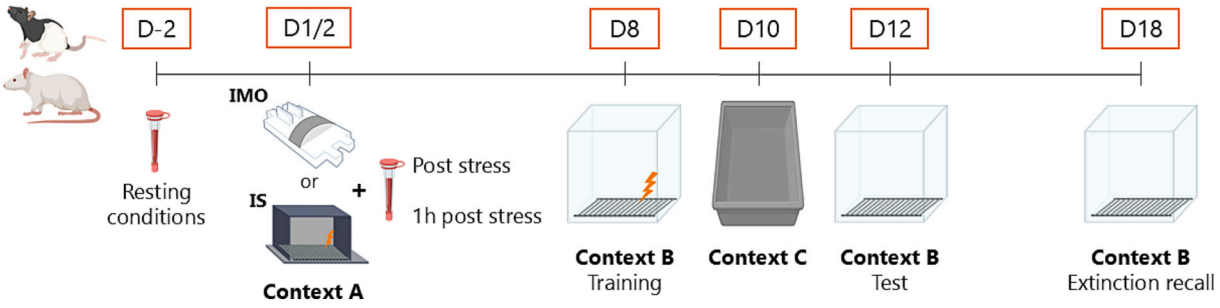


Fig. 1. Time-line of the experimental procedures. Animals were randomly assigned to six experimental groups ($n = 10$/group). Three days before stress exposure, blood samples were taken under basal conditions from all animals. Either on days 1 or 2, rats were exposed to 30 inescapable foot-shocks (IS) in context A, or to 1 h immobilization (IMO), or left undisturbed (controls). Then, blood samples were taken immediately after the exposure and 1 h later. On day 8, all animals underwent fear conditioning training in context B. On day 10, all animals were exposed to a novel environment (context C). On day 12, all groups were tested for fear conditioning and extinction in context B. Finally, on day 18, all rats were exposed again to context B for extinction recall.

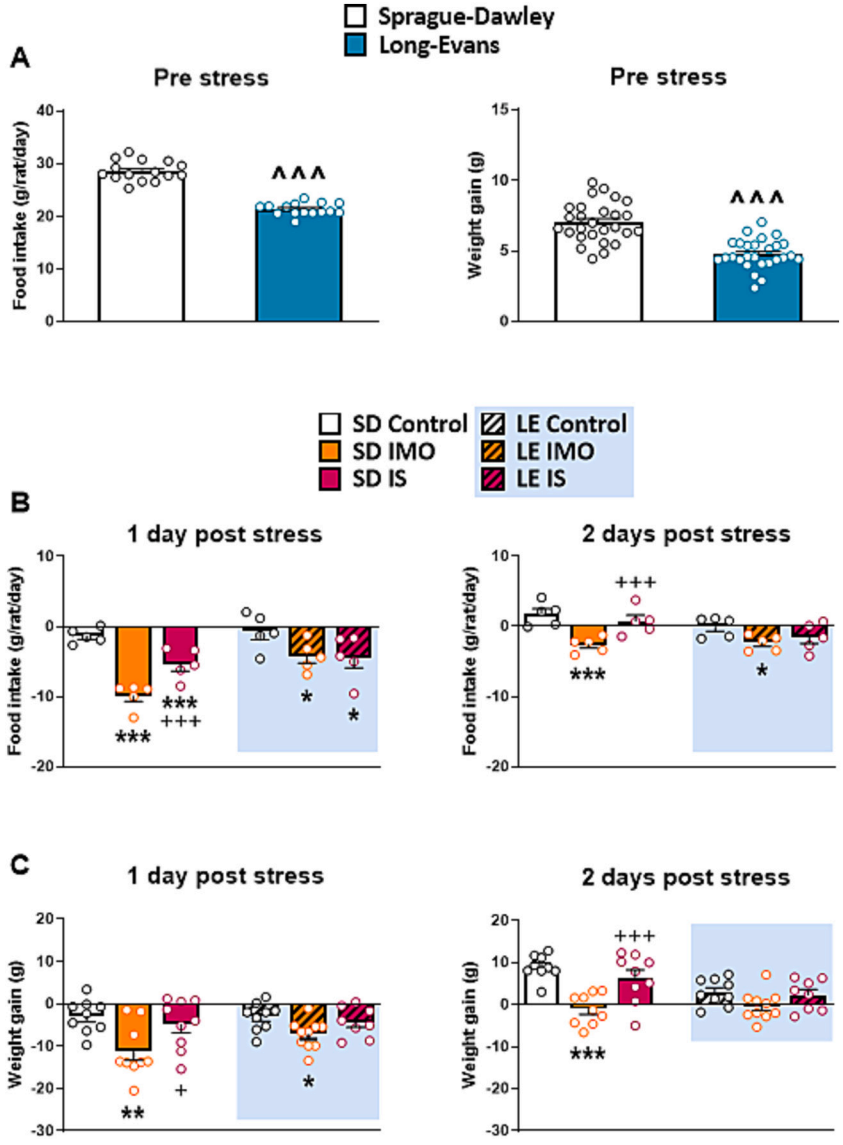


Fig. 2. Physiological response to IMO and IS. Data shown as mean + S.E.M. Food intake and body weight changes are represented in Sprague-Dawley (SD) Long-Evans (LE) rats. Food intake and body weight gain on the 3 days preceding stress exposure (A). Food intake (B) and body weight changes (C) with respect to the pre-stress days on day 1 and 2 after stress exposure (B), and body weight gain 1 and 2 days after stress exposure (C). Food intake and body weight gain prior stress were lower in LE than SD rats ($n = 30$/group, ^^^ $p < 0.001$). After exposure to the stressors, overall differences between strains were still observed (see text), but differences in the Figure refers to the impact of stressors within each strain. * indicates significant differences with respect to the respective control group. + indicates significant differences between IS and IMO. In all cases, one symbol indicates $p < 0.05$, two symbols $p < 0.01$, and three symbols $p < 0.001$.

2.5. Statistical analysis

Data were analyzed by the Statistical Program for Social Sciences (SPSS) version 24 for Windows using generalized linear model (GzLM; McCulloch and Searle, 2001) that does not require normal distribution and equality of variances. When the variables were measured over time (food intake, body weight gain, hormones) the data were segregated for each particular time to avoid complex interactions. The initial analysis included the factor strain (two levels) and the factor stress (three levels: control, IMO and IS) to show main effects of strain. However, when an interaction stress × strain was found, we further analyzed the impact of stress separately for each strain, further comparison between the three stress groups being corrected by sequential Sidak. In some cases, data were log-transformed to improve homogeneity of variances, although data are represented without transformation to better visualize group differences. The criterion for significance was set at $p < 0.05$.

3. Results

3.1. Physiological impact of the stressors

Food intake and body weight gain were measured for 3 days before stress exposure to detect baseline strain differences and possible pre-stress differences among the three experimental groups. No differences were found among the rats assigned to each experimental group, but lower food intake ($t = 32.20$, df = 28, $p < 0.001$) and body weight gain ($t = 6.68$, df = 52, $p < 0.001$) were found in LE compared with SD rats (Fig. 2A). After that, the impact of stress on food intake and body weight was separately analyzed on the first and second day post-stress.

Changes in food intake on the first post-stress day (Fig. 2B) were affected by strain ($\chi_{(1)} = 10.2$, $p = 0.001$), stress ($\chi_{(2)} = 45.3$, $p < 0.001$) and the interaction strain x stress ($\chi_{(2)} = 9.7$, $p = 0.008$), whereas on the second day it was affected by strain ($\chi_{(1)} = 6.0$, $p = 0.015$) and stress ($\chi_{(2)} = 28.3$, $p < 0.001$), but the interaction did not reach significance ($p = 0.11$). The analysis reflected an overall greater impact in SD than LE

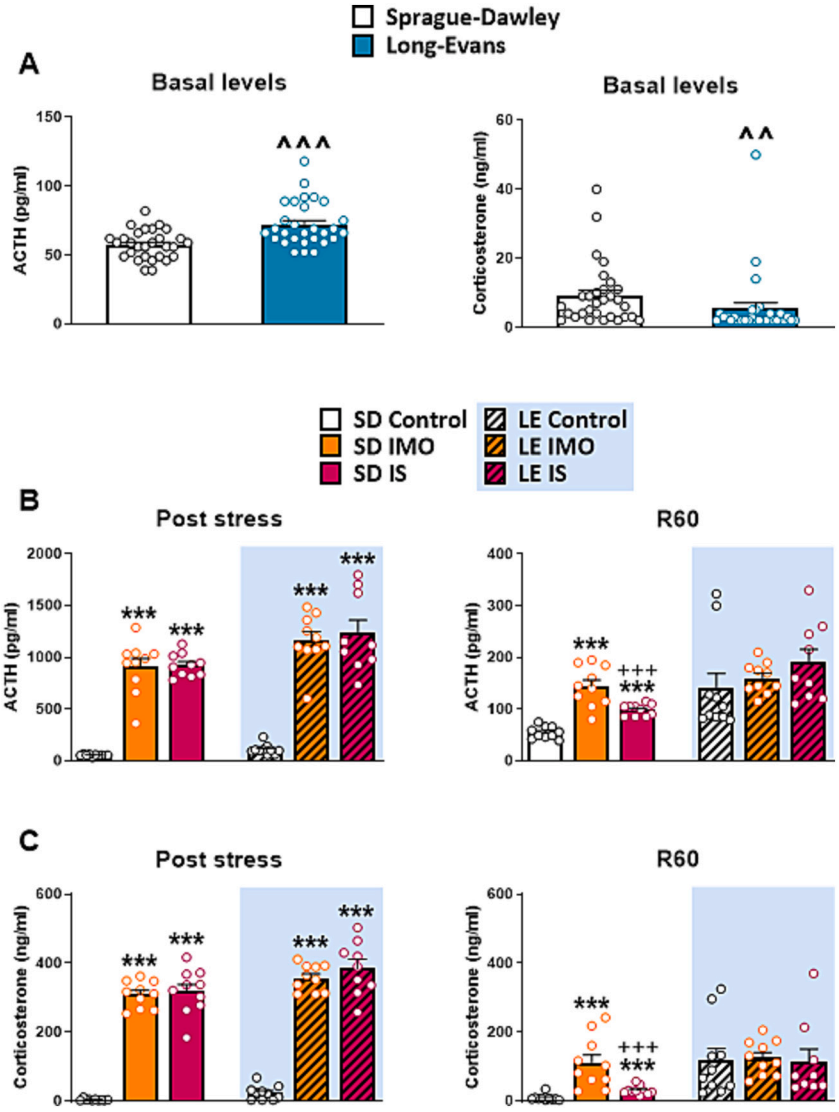


Fig. 3. Basal and stress plasma levels of ACTH and corticosterone. Data shown as mean + S.E.M. Basal levels of ACTH and corticosterone were measured 3 days prior to stress exposure (A): higher ACTH levels (^^^ $p < 0.001$) and lower corticosterone levels (^^ $p < 0.01$) were found in LE compared with SD rats (n = 30/strain). The ACTH (B) and corticosterone (C) responses to IMO or IS were evaluated immediately after the stressor (post-stress) or after a 60 min recovery period (R60). Note that the graphs presented on post stress and R60 ACTH levels use different scales, which should be considered when making comparisons. After exposure to the stressors some differences between strains were still observed (see text), but differences in the Figure refers to the impact of stressors within each strain. *** indicates significant differences with respect to the respective control group ($p < 0.001$). +++ indicates significant differences between IS and IMO ($p < 0.001$).

rats. When segregating by strain, in SD rats, the effect of stress on food intake was significant in both the first and the second post-stress day ($\chi_{(2)} = 73.4$, $p < 0.001$, $\chi_{(2)} = 25.0$, $p < 0.001$, respectively). Post-hoc comparisons showed reduced intake in IMO and IS rats in the first day compared with controls ($p < 0.001$), with a greater reduction after IMO than after IS ($p < 0.001$). In the second day only IMO resulted in reduced intake compared with controls ($p < 0.001$) and IS ($p = 0.001$) (Fig. 2B). In LE rats, the effect of stress on food intake was significant in both the first and the second post-stress day ($\chi_{(2)} = 7.6$, $p = 0.022$, $\chi_{(2)} = 7.1$, $p = 0.029$, respectively). Post-hoc comparisons showed that both IMO and IS groups showed lower food intake than controls on the first post-stress day ($p = 0.042$ in the two cases), whereas only IMO differed from controls on the second day ($p = 0.026$).

Changes in body weight on the first post-stress day were only affected by stress ($\chi_{(2)} = 20.7$, $p < 0.001$), whereas on the second day it was affected by strain ($\chi_{(1)} = 9.8$, $p = 0.015$), stress ($\chi_{(2)} = 34,1$, $p < 0.001$) and the interaction strain x stress ($\chi_{(2)} = 8.5$, $p = 0.014$) (Fig. 2C). Overall, the impact was greater in SD than LE rats. In SD rats, the effect of stress to reduce body weight gain was significant at days 1 and 2 post-stress ($\chi_{(2)} = 12.4$, $p = 0.002$; $\chi_{(2)} = 30.4$, $p < 0.001$). Post-hoc comparisons showed that the effect was observed only after IMO ($p = 0.002$ day 1, $p < 0.001$, day 2 vs controls), with reduced weight gain in IMO than IS rats on the two days ($p = 0.019$ day 1, $p < 0.001$, day 2). In LE rats, the effect of stress on body weight was significant on the first ($\chi_{(2)} = 8.5$, $p = 0.014$), but not on the second, post-stress day. Post-hoc comparisons showed that only IMO resulted in lower body weight than controls on the first day after stress ($p = 0.014$).

Basal levels of ACTH were slightly higher in LE than SD rats ($t = -4.30$, df = 56, $p < 0.001$), whereas basal corticosterone levels were slightly lower ($t = 3.08$, df = 57, $p = 0.003$) (Fig. 3A). The ACTH and corticosterone responses to the stressors were analyzed including the factor stress and strain, but separately for each time (immediately after stress and at 60 min post-stress, R60), given the markedly different magnitude of hormone values at the two times.

The analysis of ACTH immediately after stress revealed significant effects of strain ($\chi_{(1)} = 31.0$, $p < 0.001$), stress ($\chi_{(2)} = 1261$, $p < 0.001$) and the interaction strain x stress ($\chi_{(2)} = 6.5$, $p = 0.039$), with overall higher levels of ACTH in LE than SD rats. Then, when segregating by strain, a significant effect of stress was found in both SD and LE rats ($\chi_{(2)} = 997$, $p < 0.001$; $\chi_{(2)} = 422$, $p < 0.001$, respectively). Post-hoc comparisons showed no differences between IMO and IS either in SD or LE rats, with both stressors showing higher ACTH levels than controls (always $p < 0.001$) (Fig. 3B). The analysis of ACTH at R60 revealed significant effects of strain ($\chi_{(1)} = 43.0$, $p < 0.001$), stress ($\chi_{(2)} = 46.5$, $p < 0.001$) and the interaction strain x stress ($\chi_{(2)} = 13.8$, $p = 0.001$), with overall higher levels in LE than SD rats. When segregated by strain, a significant effect of stress was found in SD ($\chi_{(2)} = 109$, $p < 0.001$), but not LE rats. In SD rats, both IMO and IS increased ACTH levels, with a lower response after IS than IMO (always $p < 0.001$) (Fig. 3B).

The analysis of corticosterone immediately after stress revealed significant effects of strain ($\chi_{(1)} = 15.0$, $p < 0.001$) and stress ($\chi_{(2)} = 716$, $p < 0.001$), with no interaction. Overall, LE rats showed higher levels than SD rats, regardless of stress. When segregating by strain, significant effects of stress were found in both SD ($\chi_{(2)} = 368$, $p < 0.001$) and LE ($\chi_{(2)} = 350$, $p < 0.001$). Post-hoc comparisons showed no differences between IMO and IS, which showed higher levels than controls in SD and LE (in all cases $p < 0.001$) (Fig. 3C). The analysis of corticosterone at R60 revealed significant effects of strain ($\chi_{(1)} = 56.8$, $p < 0.001$), stress ($\chi_{(2)} = 49.9$, $p < 0.001$) and the interaction strain x stress ($\chi_{(2)} = 30$, $p < 0.001$). When segregating by strain, significant effect of stress was found in SD ($\chi_{(2)} = 91.7$, $p < 0.001$), but not LE rats. In SD rats, both IMO and IS increased corticosterone levels, with a lower response after IS than IMO (always $p < 0.001$) (Fig. 3C).

3.2. Impact of stressor on behavioral response to the conditioning context B before training

GzLM analysis of freezing in context B prior to shock exposure showed significant effects of strain ($\chi_{(1)} = 60.1$, $p < 0.001$), stress ($\chi_{(2)} = 84.4$, $p < 0.001$) and the interaction strain x stress ($\chi_{(2)} = 80.6$, $p < 0.001$). Overall higher levels of freezing were observed in LE than SD rats. When segregating by strain we did not observe significant stressor effect in SD rats but did in LE rats ($\chi_{(2)} = 64.1$, $p < 0.001$), which showed higher levels in IS as compared with both control and IMO rats ($p < 0.001$ in the two cases) (Fig. 4A).

Analysis of distance prior to shock exposure showed no effect of strain, but significant effects of stress ($\chi_{(2)} = 162.3$, $p < 0.001$) and the interaction strain x stress ($\chi_{(2)} = 29.1$, $p < 0.001$). When segregating by strain, we observed significant stress effect in SD and LE rats ($\chi_{(2)} = 32.3$, $\chi_{(2)} = 148.1$, respectively, $p < 0.001$ in the two cases) (Fig. 4B). Post-hoc comparisons showed that in SD rats, only IS significantly decreased activity as compared with controls and IMO ($p < 0.001$ in the two cases) and similar results were found in LE rats. The interaction reflects the fact that the reduction of activity after IS was stronger in LE than SD rats.

Analysis of rearing prior to shock exposure showed significant effects of strain ($\chi_{(1)} = 4.8$, $p = 0.028$), stressor ($\chi_{(2)} = 107$, $p < 0.001$) and their interaction ($\chi_{(2)} = 18.7$, $p < 0.001$). When segregating by strain, we observed significant stress effect in SD and LE rats ($\chi_{(2)} = 24.6$, $\chi_{(2)} = 146.4$, respectively $p = 0.000$ in the two cases) (Fig. 4C). Post-hoc comparisons showed that in SD rats, only IS significantly decreased activity as compared with controls ($p < 0.001$) and IMO ($p = 0.001$). Similar results were found in LE rats, the interaction reflecting the fact that the reduction of rearing after IS was stronger in LE than SD rats.

3.3. Impact of prior IS exposure and contextual fear conditioning on generalization of fear to an open-field (context C)

Freezing in the novel environment was significantly affected by strain ($\chi_{(1)} = 34.3$, $p < 0.001$), stress ($\chi_{(2)} = 84.3$,$p < 0.001$) and the interaction strain x stress ($\chi_{(2)} = 18.4$, $p < 0.001$). When segregated by strain, no effect of stress was found in SD rats, but it was in LE rats ($\chi_{(1)} = 80.7$, $p < 0.001$). Post-hoc comparisons in LE rats showed higher levels in IS than control and IMO rats ($p < 0.001$ in the two cases) (Fig. 5A).

Distance travelled in the novel environment was significantly affected by strain ($\chi_{(1)} = 15.2$, $p < 0.001$) and stress ($\chi_{(2)} = 32.0$, $p < 0.001$), with no interaction. However, when segregating by strain, no effect of stress was found in SD rats, but it was in LE rats ($\chi_{(2)} = 25.4$, $p < 0.001$). Post-hoc comparisons in LE rats showed lower levels in IS than control and IMO rats ($p < 0.001$, $p = 0.002$ respectively) (Fig. 5B).

Rearing in the novel environment was marginally affected by strain ($\chi_{(1)} = 3.8$, $p = 0.053$), but significantly by stress ($\chi_{(2)} = 31.8$, $p < 0.001$) and the interaction strain x stress ($\chi_{(2)} = 6.6$, $p = 0.036$). When segregating by strain, no effect of stress was found in SD rats, but it was in LE rats ($\chi_{(2)} = 30.8$, $p < 0.001$). Post-hoc comparisons in LE rats showed lower levels in IS than control and IMO rats ($p < 0.001$ in the two cases) (Fig. 5C).

3.4. Fear conditioning memory, extinction and extinction recall (context B)

Rats were exposed for 20 min to the shock context to induce extinction and their behavior measured in the first 8 min to evaluate context fear conditioning and the last 4 min to demonstrate extinction. As high correlations were found between distance travelled and rearing frequency in the generalization test, we focused only on freezing and rearing.

The analysis of freezing during the first 8 min of exposure to context B showed significant effects of strain ($\chi_{(1)} = 43.2$, $p < 0.001$), stress ($\chi_{(2)} = 27.9$, $p < 0.001$) and the interaction strain x stress ($\chi_{(2)} = 13.3$, $p = 0.001$). When segregating by strain, no effect of stress was found in SD

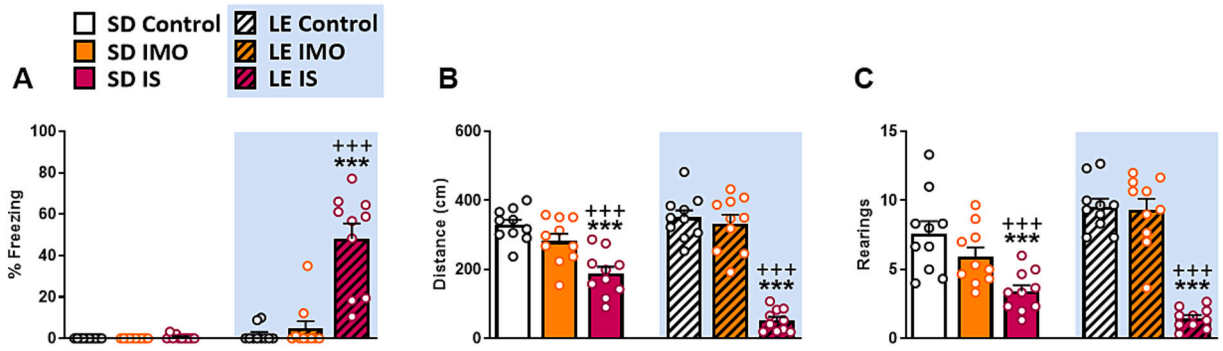


Fig. 4. Behavioral response to the conditioning context B prior to shock exposure for training in rats previously exposed to IMO or IS 7 days before. Data shown as mean + S.E.M of time spent freezing (A), distance travelled (B), and rearing episodes (C) in Sprague-Dawley (SD) and Long-Evans (LE) rats. Overall differences between strains were observed (see text), but differences in the Figure refers to the impact of stressors within each strain. *** indicates significant differences with respect to the control group ($p < 0.001$). +++ indicates significant differences compared to the IMO group ($p < 0.001$).

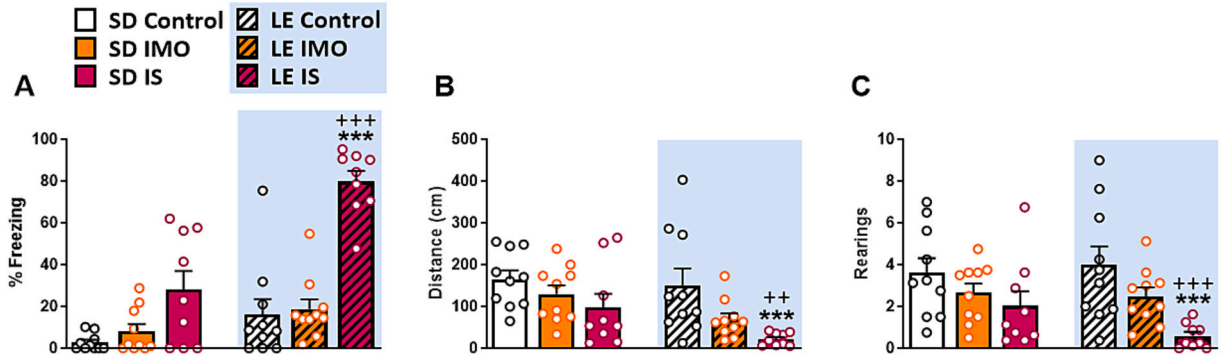


Fig. 5. Behavioral response to a completely novel environment (context C) after contextual fear conditioning in context B. Data shown as mean + S.E.M of time spent freezing (A), distance travelled (B), and rearing episodes (C) in Sprague-Dawley (SD) and Long-Evans (LE) rats. Overall differences between strains were observed (see text), but differences in the Figure refers to the impact of stressors within each strain. * indicates significant differences with respect to the control group. + indicates significant differences compared to the IMO group. In all cases, two symbols indicate $p < 0.01$, and three symbols $p < 0.001$.

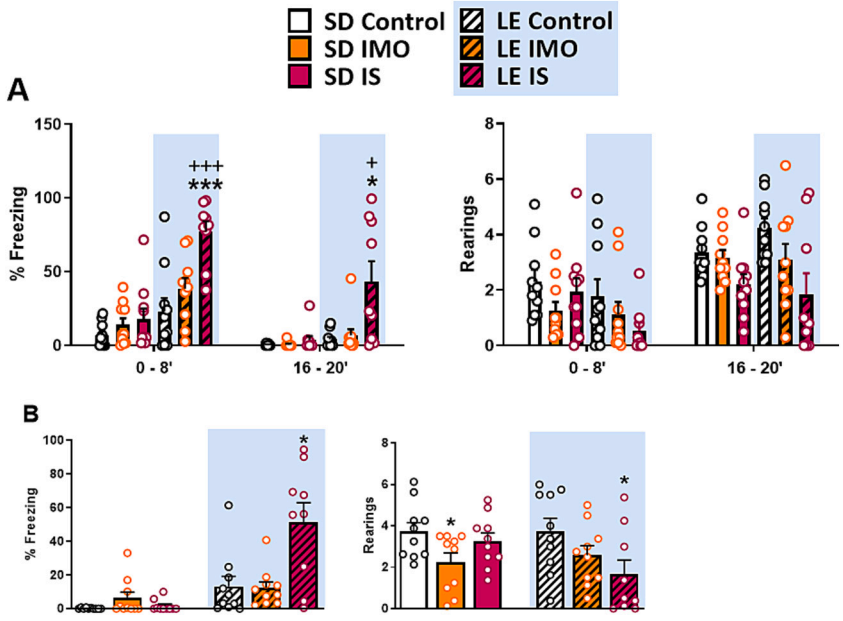


Fig. 6. Behavioral response during test/extinction and extinction recall sessions in context B. Data shown as mean + S.E.M of time spent freezing and rearing episodes during the test/extinction session (A) and during extinction recall session (B) in Sprague-Dawley (SD) and Long-Evans (LE) rats. Overall differences between strains were observed (see text), but differences in the Figure refers to the impact of stressors within each strain. * indicates significant differences with respect to the control group. + indicates significant differences compared to the IMO group. In all cases, one symbol indicates $p < 0.05$, and three symbols $p < 0.001$.

rats, but it was significant in LE rats ($\chi_{(2)} = 27.3$, $p < 0.001$), with higher levels of freezing in IS compared with control and IMO groups ($p < 0.001$, $p = 0.001$, respectively) (Fig. 6A). In the last 4 min, significant effects of strain ($\chi_{(1)} = 27.1$, $p < 0.001$) and stress ($\chi_{(2)} = 14.6$, $p = 0.001$) were found, indicating overall high levels of freezing in LE than SD rats. However, when segregating by strain, no effect of stress was found in SD rats, but it was in LE ($\chi_{(2)} = 8.5$, $p = 0.014$). Post-hoc comparisons in LE rats showed higher levels of freezing in IS than in control or IMO rats ($p = 0.019$, $p = 0.047$, respectively) (Fig. 6A).

Regarding rearing, during the first 8 min in context B, only marginal effects of strain ($\chi_{(1)} = 3.7$, $p = 0.055$) and stress ($\chi_{(2)} = 5.0$, $p = 0.08$) were found, with lower levels of rearing in LE than SD rats (Fig. 6A). In the last 4 min, only a significant effect of stress was found ($\chi_{(2)} = 16.5$, $p < 0.001$), with lower levels after IS versus controls ($p < 0.001$) and IMO ($p = 0.012$), regardless of the strain (Fig. 6A).

The analysis of freezing during re-exposure to the shock context 6 days later (extinction recall) showed significant effects of strain ($\chi_{(1)} = 61.6$, $p < 0.001$), stress ($\chi_{(2)} = 6.7$, $p = 0.035$) and a marginal interaction strain x stress ($\chi_{(2)} = 4.8$, $p = 0.091$). When segregating by strain, the effect of stress was not significant in SD rats, but it was in LE rats ($\chi_{(2)} = 7.5$, $p = 0.024$). Post-hoc comparisons in LE rats showed higher levels of freezing in IS than controls ($p = 0.020$) (Fig. 6B).

Regarding rearing, only a significant effect of stress was found ($\chi_{(2)} = 9.7$, $p = 0.008$). When segregated by strain, the effect of stress was significant in both SD ($\chi_{(2)} = 7.1$, $p = 0.029$) and LE ($\chi_{(2)} = 6.9$, $p = 0.032$). Post-hoc comparisons showed lower levels of rearing in IMO than control SD rats ($p = 0.027$), and lower levels in IS than control LE rats ($p = 0.027$) (Fig. 6B).

4. Discussion

The present study compared the relevance of the strain of rats (SD vs LE rats) and the type of stressor (IMO vs IS) regarding the long-term impact of a single stress exposure on contextual FC, fear generalization to a novel context and the pattern of fear extinction and fear extinction recall. The physiological impact of the stressors was evaluated by changes in food intake, body weight and HPA hormones. The results demonstrate that the long-lasting impact of these two severe stressors is markedly dependent on the qualitative aspects of stressors and the particular rodent strain used, essentially confirming our initial hypotheses. More precisely, we demonstrated a strong impact of prior exposure to IS on the acquisition of fear learning in LE rats, and a lesser, although significant, impact of prior exposure to IMO. In striking contrast, basically no effect of prior IS or IMO exposure was found in SD rats. These effects were not explained by a different severity of the two stressors or major strain differences in their physiological response to them.

IMO and the particular IS procedure used in the present experiment have been repeatedly reported to be severe stressors on the basis of classical biological markers of stress such as ACTH, corticosterone, prolactin and changes in food intake and body weight (e.g., García et al., 2000; Belda et al., 2016; Úbeda-Contreras et al., 2018; Molina et al., 2023). Direct comparison of the two stressors indicated that they were of high intensity, but the overall impact of IMO was greater in terms of the physiological response they elicited (Márquez et al., 2002; Rabasa et al., 2011). In the present study we observed similar initial ACTH and corticosterone responses to both stressors either in SD or LE rats, with an overall higher response in LE rats. However, during the post-stress period, slower recovery of HPA hormones was observed after IMO than after IS in SD rats, in accordance with our previous results. In LE rats, no clear conclusion about post-stress recovery can be achieved as control rats markedly responded to the blood sampling procedure despite a prior experience with the procedure and no difference with respect to IMO or IS was found. Accordingly with post-stress recovery of the HPA axis, a higher impact of IMO compared with IS was observed on body weight and food intake in SD rats, confirming previous reports (Márquez et al., 2002), whereas in LE rats differences between the two stressors were subtle.

We have demonstrated a greater responsiveness of the HPA axis to stress in LE compared to other rat strains using stressors of lower intensity than those used in the present study (Sanchís-Ollé et al., 2021). The present results support our previous findings and confirm that LE rats showed a generalized greater ACTH responsiveness to predominantly emotional stressors, that is paralleled by corticosterone, suggesting that main differences are likely to be at the upper levels of the HPA axis rather than at the adrenal gland. In fact, relative adrenal weight was similar in the two strains in the present experiment (not shown).

Prior exposure of LE rats to IS in context A resulted in markedly lower activity and higher levels of freezing in context B before the FC acquisition procedure, whereas no evidence for altered behavior was found in prior IMO-exposed LE rats. In SD rats, activity and freezing were insensitive to prior IMO exposure, but prior IS did reduce activity, suggesting subtle effects. These data suggest some generalization of fear to context B after IS exposure in context A despite changes in walls and background odor, perhaps because of the presence of the grid in the two contexts. The extent of generalization to novel contexts is a complex issue with important differences between the available studies, although the presence of grids can be important (see Armario et al., 2024 for further discussion). In any case, it is clear that fear generalization was restricted to IS and that LE rats were more sensitive than SD rats.

Exposure to IS strongly potentiated contextual fear memory in LE rats assessed the day after training. The potentiation of contextual FC in LE rats after prior exposure to IS was strong and entirely consistent with the previous reports from Fanselow's laboratory that also used LE rats. These authors have nicely described, using high intensity IS, some of the main characteristics of this phenomenon that they call stress-induced enhanced fear learning (SEFL): maintenance for a long period of time (months), dependence on the number of shocks and persistence despite fear extinction, thus strongly suggesting that it reflects sensitization (e.g., Rau et al., 2005; Rau and Fanselow, 2009; Ponomarev et al., 2010; Poulos et al., 2015; Perusini et al., 2016). It is unlikely that generalization of fear observed in IS-exposed LE rats in the context B before contextual fear conditioning training was responsible for the potentiation of fear learning as the Fanselow's lab has demonstrated that this potentiation is observed using the immediate shock deficit procedure (Landeira-Fernandez et al., 2006), which impedes contextual fear learning (Fanselow, 1980; Landeira-Fernandez et al., 2006) and it is maintained intact after the extinction of fear to the IS context (Rau et al., 2005; Long and Fanselow, 2012). Moreover, we did not find a correlation between freezing in context B prior to FC and freezing in context B when tested the day after, suggesting that they are two independent phenomena.

In contrast to prior IS, prior exposure of LE rats to IMO only caused a non-significant trend to enhance contextual FC. The greater impact of IS versus IMO on fear learning in LE rats cannot be attributed to differences in intensity as evaluated by classical markers of stress. The finding that prior IMO caused a much lower potentiation of FC than IS might suggest that this potentiation is likely to be greater when the aversive stimulus is the same during prior stress and conditioning. Alternatively, the long-term consequences of severe stressors might be dependent on certain characteristics of stressors other than intensity. Our results remark another source of complexity by demonstrating that SD rats were unsensitive to the impact of the stressors, either IMO or IS, in terms of further potentiation of context FC. The lack of effect of the two stressors on context FC in SD rats is supported by previous unpublished results from our laboratory using both IS and IMO, and by the lack of effect of prior IMO or a modified SPS procedure that included IMO, where only a marginal effect on extinction was found (Sanchís-Ollé et al., 2023).

The relevance of the characteristics of stressors in their long-term consequences are poorly characterized given the scarcity of studies comparing stressors. In this regard, Iwasaki et al. (2015) observed in C57

mice that a single prior shock further potentiated contextual fear learning, whereas this was not found by prior swim or tail-pinch. Prior IS exposure has been found to potentiate context FC in SD rats (Szczytkowski-Thomson et al., 2013; Jones et al., 2015; Jones et al., 2018a, 2018b). In the latter studies, the animals were individually housed and stressed and tested in the dark phase of the circadian cycle and this might contribute to the discrepancies with our data. In addition, the rats were provided by Charles-River instead of Janvier and differences have been described between rats of the same strain provided by different vendors (Turnbull and Rivier, 1999; Pecoraro et al., 2006; Tsuda et al., 2020). Since our two strains were obtained from the same commercial breeder, factors other genetics are unlikely to contribute to the observed differences. However, differences in the protocols used for breeding and maintenance of animals can be considered when comparing studies.

On the basis of the results from studies using prior IS exposure, it appears that this stressor is prone to induce SEFL, particularly if combined with a sensitive animal strain. However, the results regarding other stressors are more controversial in the literature. For instance, in rats, no major alterations have been reported after prior exposure of SD rats to IMO in plastic bags (Kirby et al., 2013). Results regarding the effects of the single prolonged stress paradigm on subsequent fear conditioning (e.g., 1 week later) in rats are controversial, with some studies reporting enhanced fear conditioning, whereas other studies found normal fear memory but impaired fear extinction or fear extinction recall (see refences in the Introduction). In this regard, prior IMO or restraint of C57 for 2 h result in normal fear acquisition but impaired fear extinction (Andero et al., 2011; Chauveau et al., 2012; Daws et al., 2017; Velasco et al., 2022), supporting the idea that C57 mice might be quite sensitive to the impact of severe stressors.

In addition to the evaluation of CFC and extinction, we evaluated, before testing for CFC per se, the influence of prior stress followed by CFC on activity of animals in a different context C (open-field). It has been repeatedly reported that exposure to IS or the brief exposure to shocks for fear conditioning induced hypoactivity in novel environments completely different from the shock context (van Dijken et al., 1992a; Van Dijken et al., 1992b; Radulovic et al., 1998; Daviu et al., 2010, 2014, 2024; Armario et al., 2024). We have termed this phenomenon cognitive fear generalization as it is completely independent of similarities with the shock context (Daviu et al., 2014; Fuentes et al., 2018; Armario et al., 2024). It is of note that in contrast to SEFL, hypoactivity in novel environments is in great part an associative phenomenon dependent on the acquisition of CFC (Radulovic et al., 1998; Daviu et al., 2014). Nevertheless, a non-associative component of hypoactivity has been demonstrated after exposure to severe IS (Daviu et al., 2024). Our results indicate that prior IS but not IMO reduced horizontal and vertical (rearing) activity and enhanced freezing in SD rats, but the effect of IS was greater in LE rats, which also showed a moderated impact of IMO. These results paralleled the effects of prior IS or IMO on CFC conditioning, thus supporting the close relationship between CFC and cognitive generalization.

An important limitation of the present study was that we only used male rats due to the complexity of the design that simultaneously assessed the contribution of both stressor nature and strain. Although sex differences have been observed in both behavioral and physiological consequences of stress and learning, including fear learning, the picture is still complex (Cohen and Yehuda, 2011; Kokras and Dalla, 2014; Day and Stevenson, 2020; Mancini et al., 2025). We focused on male rats that appear to be more vulnerable than females to the effects of inescapable shocks and fear conditioning (e.g., Daviu et al., 2014; Armario et al., 2024). Nevertheless, we are currently doing a new experiment comparing the response to IS in male and female LE rats.

Establishing animal models of psychiatric diseases is a critical step for the understanding of their neurobiological underpinnings. The validity and reliability of animal models in terms of behavioral outcomes constitute the bases for further characterization of neurobiological mechanisms involved and the efficacy of drugs. Unfortunately, literature is riddled with controversial results. Our results indicate that some rat or mice strains are more prone to develop altered FC after prior exposure to severe stressors, but IS exposure appears to be particularly potent in potentiating FC. Thus, when combining IS exposure and a sensitive strain, results are highly consistent, but the opposite can occur when using stressors other than IS and a particularly resistant strain. Controversial results in literature can be in great part explained by the contribution of these two factors.

CRediT authorship contribution statement

Sara Serrano: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Xavier Belda:** Writing – review & editing, Methodology, Investigation. **Antonio Armario:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Ethical statement

The experimental protocol was approved by the Ethics Committee at the Universitat Autònoma de Barcelona and the Generalitat de Catalunya, and it was carried out in accordance with the European Council Directive (2010/63/UE) and Spanish legislation (RD 53/2013).

Declaration of competing interest

The authors have nothing to declare.

Acknowledgements

This research was funded by Spanish grants (PID2020-118844RB-I00) and the Generalitat de Catalunya (SGR21-00158) to AA and RN. Our group belongs to REIS (Red Temática Española de Investigación en Estrés), Ministerio de Ciencia e Innovación, REF. RED2022-134191-T.

Data availability

Data will be made available on request.

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