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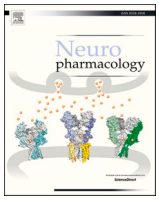

Stress inoculation during adolescence attenuates social stress-induced increase in ethanol intake in adult male mice

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ABSTRACT

Social stress exposure heightens the risk of substance abuse disorder development, especially when endured during adolescence, influencing long-term mental health. This study investigates early-life stress's potential to confer resilience against later-life stressors. To investigate this hypothesis, we examined the impact of a single social defeat (SD) incident during adolescent mice's lives on subsequent voluntary ethanol consumption following repeated adult social stress exposure. Half of the adolescent mice experienced SD at postnatal day 28. Three weeks later (postnatal day 49), defeated groups encountered four confrontations with aggressive residents every 72 h, while control groups were exposed to non-resident exploration. A day after the last SD, defeated mice were classified as resilient or susceptible based on their response to a social interaction test (SIT), a model for depressive behavior. To assess ethanol consumption during young adulthood, researchers used the 'drinking in the dark' and oral ethanol self-administration paradigms. Stress inoculation (IS) slightly increased resilient animals in the SIT. In mice without IS exposure during adolescence, susceptible defeated mice displayed higher ethanol consumption and motivation than control and resilient mice. IS in adolescence effectively counteracted this effect, as IS-SD groups, whether resilient or susceptible, showed no increase in ethanol intake. These groups also exhibited similar motivation to control, measured by the progressive ratio. Notably, elevated IL-6 levels seen in SD-S mice were absent in IS-exposed mice. Additionally, IS-exposed groups had lower prefrontal cortex IL-6 and CX3CL1 levels. These findings support the hypothesis that IS, induced by moderate-intensity stress during adolescence, can enhance resilience to more severe stressors in adulthood.

1. Introduction

It is extensively documented that exposure to intense stressors in adult life is associated with the development of addictive behaviors, such as alcoholism. These circumstances can be further exacerbated if unfavorable situations have been experienced during early stages of life (Loman and Gunnar, 2010; Lucassen et al., 2013). Adolescence is a critical period of biological, behavioral, and social changes, which, together with family and social experiences, can act as risk factors for adverse behaviors and situations in adulthood (Crews et al., 2007; Forbes and Dahl, 2010). Adverse situations during adolescence have been strongly associated with the onset of mental illness in adulthood, such as anxiety and depression, as well as being related to substance use disorders (SUD), and an increase in violent behaviors (Ho and King, 2021; Lee et al., 2012).

In preclinical studies, the ethologically validated model of social defeat (SD; Miczek, 1979) induces long-term physiological and behavioral changes consistent with the characteristic symptoms of anxiety and depression (Iñiguez et al., 2014; Macedo et al., 2018) and produces modifications in important neurotransmitter systems (Burke and Miczek, 2015), vulnerability to develop drug addiction (Burke and Miczek, 2015; Macedo et al., 2018; Montagud-Romero et al., 2018), and leads to an increase in the neuroimmune response (Ballestín et al., 2021; Reguilón et al., 2021a, 2021b). Despite the adverse effects that social stress can induce on individuals, not everyone develops these negative effects uniformly. In both humans and animal models, the phenomenon of resilience has been observed, involving complex neurobiological mechanisms (Cathomas et al., 2019; Feder et al., 2019; Murrough and Russo, 2019; Russo et al., 2012; Wood and Bhatnagar, 2015). Rodents resistant to the adverse effects of SD during encounters showed proactive behaviors, resisted showing defeat/submission behaviors, and developed adaptive changes in corticotropin-releasing factor (CRF) signaling, such as habituation of the hypothalamic-pituitary-adrenal (HPA) axis with decreased CRF efficacy and optimal activity of the dopaminergic system (Ballestín et al., 2021; Friedman et al., 2014;

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Abbreviations

BP	breaking point
CRF	cortocitropin-releasing factor
CTRL	control
CX3CL1	C-X3-C motif ligand 1 (fractalkine)
DID	drinking in the dark
ELISA	enzyme-linked immunosorbent assay
FR1	fixed ratio 1
HPA	hypothalamic-pituitary- adrenal
IL-6	interleukin 6
IS	inoculated stress
PFC	prefrontal cortex
PND	postnatal day
PR	progressive ratio
SA	self-administration
SD	social defeat
SIT	social interaction test
SUD	substance used disorders

Giménez-Gómez et al., 2021; Reguilón et al., 2021a; Rodenas-Gonzalez et al., 2021; Wood et al., 2010). The innate immune system has also been associated with the resilient response to social stress. Subjects resilient to the effects of SD exhibit a normalized immune response compared to susceptible subjects, who experience an increase in inflammatory cells and pro-inflammatory mediators (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Hodes et al., 2014; Pfau et al., 2019; Reguilón et al., 2021a, 2022; Rodenas-Gonzalez et al., 2021).

In recent decades, several models and strategies involving stress have been developed to promote resilience in animal models (for a review, see Ashokan et al., 2016). In our laboratory, we have enhanced resilience through environmental enrichment, physical exercise, and oxytocin administration prior to SD in adulthood (Giménez-Gómez et al., 2021; Reguilón et al., 2020, 2021a, 2021b). In the scientific literature, positive results have been observed following mild exposure to predator odors during adulthood (Wang et al., 2020) and sensory contact during agonistic social encounters in adulthood (Ayash et al., 2020; Brockhurst et al., 2015).

Early life stages represent critical periods for an individual's development, and adverse experiences during this period can alter sensitivity to challenges later in life, potentially contributing to an individual's vulnerability or resilience to psychopathologies such as anxiety, depression, or addiction (Daskalakis et al., 2013; Russo et al., 2012). When a stressful experience overwhelms an individual's coping skills, it is perceived as a threat, leading to a loss of control and negative impacts on emotions and behavior. However, if the individual can effectively cope with the adverse situation, it is perceived as a challenge (Southwick and Charney, 2012). It is important to note that resilience is a dynamic response that evolves throughout life. In our laboratory, we observed that only 20% of defeated adolescent mice exhibited a phenotype resilient to depressive-like behaviors, reinforcing and motivational effects on drug intake after SD (Reguilón et al., 2022). In this line, we successfully increased resilience by providing environmental enrichment to adolescent mice before subjecting them to a SD protocol in adulthood (Reguilón et al., 2021a).

Another challenging approach involves the use of mild to moderate stressors during early life to enhance the resilient response. One of the most widely used paradigms for inducing early stress experiences is maternal separation (for a review, see Alves et al., 2020). Moderate maternal separation has been shown to prevent an increase in anxious behaviors and reduce neural excitability in the basolateral amygdala in mice subjected to chronic SD during adulthood (Qin et al., 2019). Similarly, brief maternal separation has been reported to enhance resistance to cocaine-conditioned reward effects induced by repeated SD in late adolescence (Calpe-López et al., 2022). Another study conducted during the pre-weaning period, employing the limited nesting and bedding material paradigm (Hsiao et al., 2016), demonstrated a milder deficit in social interaction following chronic SD exposure during adolescence. It's worth noting that the studies mentioned above have primarily focused on adult animals or the pre-weaning period, and there is a lack of research evaluating the long-term effects of stress inoculation during adolescence.

Protective interventions during adolescence affect brain development and shape neural circuits regulating future responses to stress. In the present study we aimed to enhance resilience to the negative effects of adult exposure to SD by a moderate-intensity exposure to social stress during adolescence. We hypothesized that this intervention would induce a protective effect against exposure to more intense stressful situations during adulthood. To this end, we inoculated stress (IS) by exposing mice to a single SD during adolescence. Subsequently, the animals were subjected to four SD sessions during young adulthood, and 24h later, all animals were classified according to their depressive-like behavior using the social interaction test (SIT). Three weeks later, ethanol sensitivity was assessed using the 'drinking in the dark' (DID) and oral self-administration (SA) paradigms. Finally, since previous reports have shown that SD induces an increase in the pro-inflammatory cytokine interleukin 6 (IL-6) and the chemokine C-X3-C motif ligand 1 (CX3CL1) (Reguilón et al., 2021; Ferrer-Pérez et al., 2022), the neuroinflammatory response was determined by analyzing striatal and prefrontal levels of these two markers.

2. Methodology

2.1. Animals

A total number of 77 adult male C57BL/6 mice (Charles River, France) were delivered to our laboratory at postnatal day (PND) 21. Experimental mice were housed in groups of five in plastic cages (27 × 27 × 14 cm) during the entire experimental procedure. OF1 adult mice (Charles River, France) were used as aggressive opponents (N = 20) and were individually housed in plastic cages (21 × 32 × 20 cm) for at least one month prior to initiation of the experiments in order to heighten aggression. All mice were housed in controlled laboratory conditions: constant temperature and humidity and a reversed light schedule (red light from 8:00 to 20:00). Food and water were available *ad libitum* to all the mice used in this study, except during behavioral tests. All procedures were conducted in compliance with the guidelines of the European Council Directive 2010/63/UE regulating animal research and were approved by the local ethics committees of the University of Valencia (number 2017-VSC-PEA-00224, on December 11th, 2017).

2.2. Drugs

For the DID and oral SA procedures, absolute ethanol (Merck, Madrid, Spain) was diluted in water using a 20% (v/v) ethanol solution.

2.3. Experimental design

All the animals were delivered to our laboratory on PND 21 and were housed in regular condition throughout the study. Four experimental groups were randomly assigned. On the one hand, a control group and a defeated group that remained undisturbed until PND 47, and on the other hand, a control group and a defeated group that were exposed to a single SD encounter on PND 28. Subsequently, all mice were exposed to the SD procedure or exploration from PND 47 to 56. Twenty-four hours after the last SD episode, animals performed the social interaction test (SIT) to evaluate depressive-like behaviors and were characterized as resilient or susceptible depending on their ratio obtained on the SIT. From this point on, the SD groups were divided into susceptible and

resilient groups, forming six experimental groups: CTRL; SD-R; SD-S; IS-CTRL; IS-SD-R; IS-SD-S. Three weeks after the last defeat, the animals initiated the DID test for four days and, in the following week, animals initiated the ethanol SA protocol for approximately 22 days. At the end of this test, all the animals were sacrificed to obtain the prefrontal cortex (PFC) and striatum for further analysis of the cytokine (IL-6) and chemokine (CX3CL1) levels.

The experimental design is depicted in Fig. 1.

2.4. Procedure and apparatus

2.4.1. Brief stress inoculation (IS)

Animals randomly distributed to the IS-CTRL and IS-SD groups were subjected to exposure to a single SD at PND 28. This agonistic encounter was conducted following the same protocol as the SD detailed below in section 2.4.2. The only difference is that only one encounter was conducted during the adolescence stage.

2.4.2. Procedure of social defeat (SD)

Mice in the stress/defeat groups were exposed to four episodes of SD during adulthood on PND 47, 50, 53 and 56. At each of these PNDs, mice were exposed to SD for 25 min. The SD consisted of three phases: the initial phase began by introducing the "intruder" (the experimental animal) into the home cage of the "resident" (the aggressive opponent) for 10 min (Tornatzky and Miczek, 1993). During this initial phase, the intruder was protected from attack, but the wire mesh walls of the cage allowed for social interactions and species-typical threats from the male aggressive resident, thus facilitating instigation and provocation (Covington and Miczek, 2001). In the second phase, the wire mesh was removed from the cage to allow confrontation between the two mice over a 5-min period. The second phase of each SD protocol was video-recorded and ethologically analyzed. Finally, the wire mesh was returned to the cage to separate the two mice once again for another 10 min to allow for social threats by the resident. The non-stressed exploration groups underwent the same protocol, but without the presence of a "resident" mouse in a clean cage. The intruder mice were exposed to a different aggressor mouse during each SD episode. The criterion used to define an animal as defeated was the adoption of a specific posture signifying defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears (the time spent in each behavior was subsequently measured in the ethological analyses) (Miczek et al., 1982; Rodri guez-Arias et al., 1998). A detailed description of these behaviors can be found in Rodri;guez-Arias et al. (1998).

2.4.3. Social interaction test (SIT)

The SIT is based on the social approach-avoidance test previously described by Berton et al. (2006) following the temporal modifications performed by Henriques-Alves and Queiroz (2016). The test took place 24 h after the last SD during the dark light cycle and in a different environment of the confrontation sessions. First, mice were transferred to a quiet, dimly lit room 1 h before the test was initiated. After habituation, each animal was placed in the center of a square arena (white Plexiglas open field, 30 cm on each side and 35 cm high) and its behavior was monitored by video (EthoVision XT 15, 50 fps; camera placed above the arena). Mice were allowed to explore the arena twice, for 600 s in each session, during two different experimental sessions. In the first (object session), an empty perforated Plexiglas cage (10 × 6.5 × 35 cm) was placed in the middle of one wall of the arena. In the second session (social session), an unfamiliar C57BL/6 male mouse was introduced into the cage as a social stimulus. Before each session, the arena was cleaned with 5% alcohol solution to minimize odor cues. Between sessions, the experimental mouse was removed from the arena and returned to its home cage for 2 min.

Arena occupancy during object and social sessions were determined using the animals' horizontal positions, determined by commercial video tracking software (EthoVision XT 15, Noldus). The time spent in the interaction zone was quantified. This measure is commonly used as social preference-avoidance score being calculated by measuring the time spent in a 6.5 cm wide corridor surrounding the restraining cage.

2.4.4. Drinking in the dark (DID)

Following the basic paradigm of Rhodes et al. (2005), the test consists of two phases. The first is habituation, in which the mice are removed from their cages to be housed individually 2 h per day for one week to habituate them to the cages and to the sipper tubes (containing a ball bearing at the end to prevent leakage and to be used throughout the test) with water. In the second phase of the protocol, the test begins 3 h after lights out. The mice are then housed in the individual cages and exposed to the 10 ml graduated tubes containing a 20% (v/v) ethanol solution. This procedure lasts 2 h. After this 2-h period, the mice are returned back to their grouped cages, with food and water bottles available *ad libitum*. This procedure is repeated on days 2 and 3, and the procedure lasts for 4 h on day 4. In addition, immediately after each day, the volume consumed was recorded. A fresh ethanol solution is prepared each day. In our case, we will maintain the protocol for two consecutive weeks, one for habituation with water and one for ethanol testing.

2.4.5. Oral ethanol self-administration (SA)

This procedure is based on that employed by Navarrete et al. (2014). Oral ethanol SA was carried out in 8 modular operant chambers (MED Associated Inc., Georgia, VT, USA). A software package (Cibertec, SA, Spain) controlled stimuli and fluid delivery and recorded operant responses. The chambers were placed inside noise isolation boxes equipped with a chamber light, two nose-poke holes, one receptacle to drop a liquid solution, one syringe pump, one stimulus light and one buzzer. Active nose-pokes delivered 20 μl of fluid combined with a 0.5s stimulus

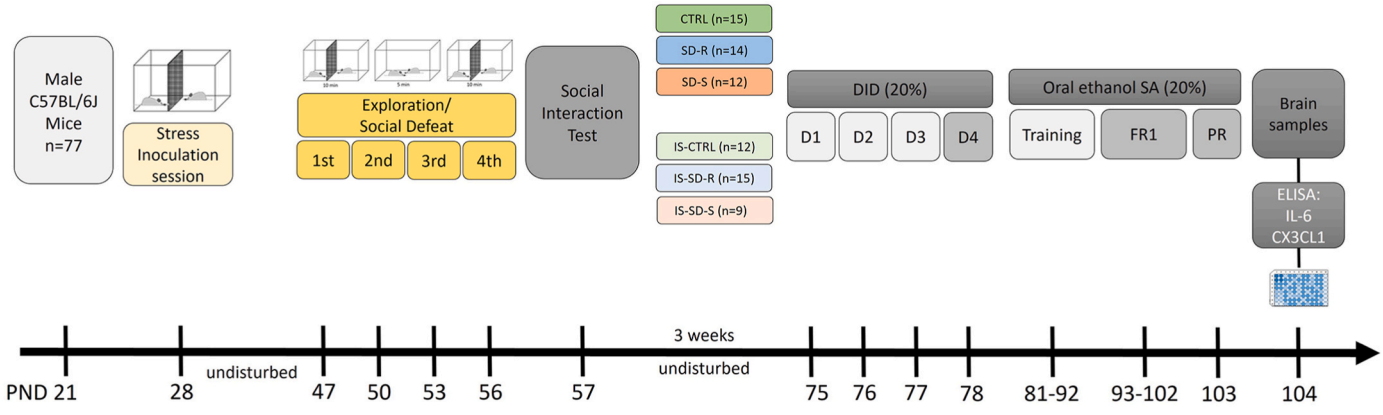

Fig. 1. Experimental design.

light and a 0.5s buzzer beep, which was followed by a 6-s time-out period. Inactive nose-pokes did not produce any consequence.

This protocol did not involve daily water and food deprivation. Food and water were only restricted during the sessions inside the operant chambers (1 h or 2 h depending on the experimental phase). In all sessions, mice had access to both active and inactive nose-pokes.

To evaluate the consequences of SD on the acquisition of oral ethanol SA, mice underwent an experiment carried out in three phases: training, fixed ratio 1 (FR1) and progressive ratio (PR) with a 20% ethanol concentration.

Training phase (12 days): Mice were trained to discriminate between both nose-pokes and learn to select the active nose-poke to receive a 20 μl of 20% (v/v) ethanol reinforcement. This phase lasted approximately 12 consecutive days, during which the mice were exposed to the operant chamber for 1 h per day, with each session repeated every 24 h. To evaluate the mice's learning and consider that they had acquired the operant behavior, several requirements had to be achieved: showing a preference for the active nose-poke of at least 60% over the inactive nose-poke for at least 3 consecutive days and exhibiting a deviation of less than 30 points during the last 3 days of this phase. The liquid consumed is not taken into account during this session.

FR1 (10 days): This phase lasted for 10 consecutive days and aimed to evaluate the number of responses in the active nose-poke and the intake of 20% (v/v) ethanol. The number of effective responses and ethanol consumption (μl) were measured for each session. The remaining ethanol in the receptacle was collected and measured using a micropipette to account for each mouse's voluntary liquid consumption in each session.

PR (1 day): In the third and final phase, a PR session was conducted to establish the breaking point (BP) for each animal (the maximum number of nose-pokes each animal is able to perform to earn one reinforcement). This phase was conducted on a single day, at the end of the procedure, and lasted for 2 h. The response requirement to achieve reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation toward ethanol consumption, the breaking point was calculated for each animal as the maximum number of consecutive responses it performed to achieve one reinforcement according to the previous scale (Navarrete et al., 2014). For example, if an animal activated the nose-poke a total of 108 times, this meant that it was able to respond a maximum of 40 times consecutively for one reinforcement. Therefore, the BP value for this animal would be 40.

2.5. Immunoassay analysis (ELISA)

Samples from the striatum and the PFC were obtained 24 h after SA. To obtain tissue samples, mice were sacrificed by cervical dislocation and then decapitated. Brains were rapidly removed, and the striatum and PFC dissected with a brain slicer matrix with 1 mm coronal section slice intervals using mouse brain atlas coordinates (Franklin and Paxinos, 2008), which were then kept in dry ice until storage at −80 °C. Before IL-6 and CX3CL1 determination, brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al. (2010). Frozen brain cortices were homogenized in 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 40 mM DTT, 1 mM Na3VO4, and 10 mM PMSF). Brain homogenates were kept on ice for 30 min and centrifuged at the maximum speed for 15 min; the supernatant was collected, and protein levels were determined by the Bradford assay from ThermoFisher (Ref: 23227).

The concentrations of CX3CL1 and IL-6 in homogenized extracts were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits in 96-well strip plates (Abcam, ab100683, ab100712). We determined CX3CL1 and IL-6 concentration in the striatum and PFC. All reagents and standard dilutions were prepared following the manufacturer's instructions. To determine absorbance, we employed an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. Optical density of plates was read at 450 nm and the results were calculated using a standard curve following the manufacturer's instructions. Total protein concentrations were determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) to determine the number of nanograms of CX3CL1 and picograms of IL-6. Data are expressed as ng/mg or pg/mg of protein for tissue samples.

2.6. Statistical analysis

Mice were previously classified into resilient and susceptible groups based on the SIT. A ratio was calculated by considering the time spent by an experimental mouse in the interaction zone when a social target is present divided by the time it spends in the interaction zone when the target is absent. A ratio equal to 1 means that equal time has been spent in the presence versus absence of a social target. Based on the regular behavior of control C57BL/6 mice, animals with a ratio under 1 are classified as susceptible, while those with a ratio equal to or higher than 1 are classified as resilient (Golden et al., 2011). To analyze social interaction proportions, a UNIANOVA with two between-subjects variables –SD, with three levels (CTRL, SD-S and SD-R) and IS with two levels (not exposed to IS and exposed to IS). In addition, we used a three-way ANOVA with two between-subjects variables –SD with three levels and IS with two levels– and a within-subjects variable –Session, with two levels of SIT session– for analysis of total time in the interaction zone when the target is absent or present for each session.

The data of the ethological analyses of resident and intruder mice were analyzed by a three-way ANOVA with two between-subjects variables –SD with two levels and IS with two levels– and a within-subjects variable –Days with two levels: 1st and 4th agonistic encounter.

To analyze the acquisition of ethanol SA, a three-way ANOVA was performed with two between-subjects variables –SD with three levels and IS with two levels– and a within-subjects variable –Days, with ten levels of FR1. The effects of SD and IS on BP values and ethanol consumption during PR was analyzed by a one-way ANOVA, with two between-subjects variables –SD and IS.

The data of the CX3CL1 and IL-6 levels were analyzed using a one-way ANOVA with two between-subjects variables –SD, with three levels and IS, with two levels.

In all the studies, following the ANOVA, Bonferroni post-hoc tests were calculated whenever required. All statistical analyses were performed using SPSS Statistics (v.28; IBM, NY, USA) for behavioral data and GraphPad Prism (v8; GraphPad Software Inc., CA, USA) for graph design. Data were expressed as mean ± SEM and a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Inoculation of stress slightly increases the percentage of resilient defeated mice

The ANOVA of the social interaction ratio performed 24 h after the last defeat showed an effect of the variables SD [$F(2,71) = 22.136$; $p < 0.001$] and IS [$F(1,71) = 8.635$; $p < 0.001$] (Fig. 2a). The post-hoc comparison revealed greater social interaction scores among control (CTRL and IS-CTRL groups) and resilient mice (SD-R and IS-SD-R groups) in comparison with susceptible mice (SD-S and IS-SD-S groups; p's < 0.001). Additionally, resilient mice (SD-R and IS-SD-R groups) showed a greater SIT score than control groups (CTRL and IS-CTRL; $p < 0.05$).

Moreover, all stress-inoculated mice (IS-CTRL, IS-SD-R and IS-SD-S groups) showed smaller SIT scores in comparison with all non-stress-inoculated mice (CTRL, SD-R and SD-S groups; $p < 0.01$). Distribution of the mice according to the SIT can be seen in Fig. 2b.

Following the SIT calculation criteria (Fig. 2c), the CTRL group ($n = 15$) presented a mean ratio >1. In the SD group of mice ($n = 26$), 46.15%

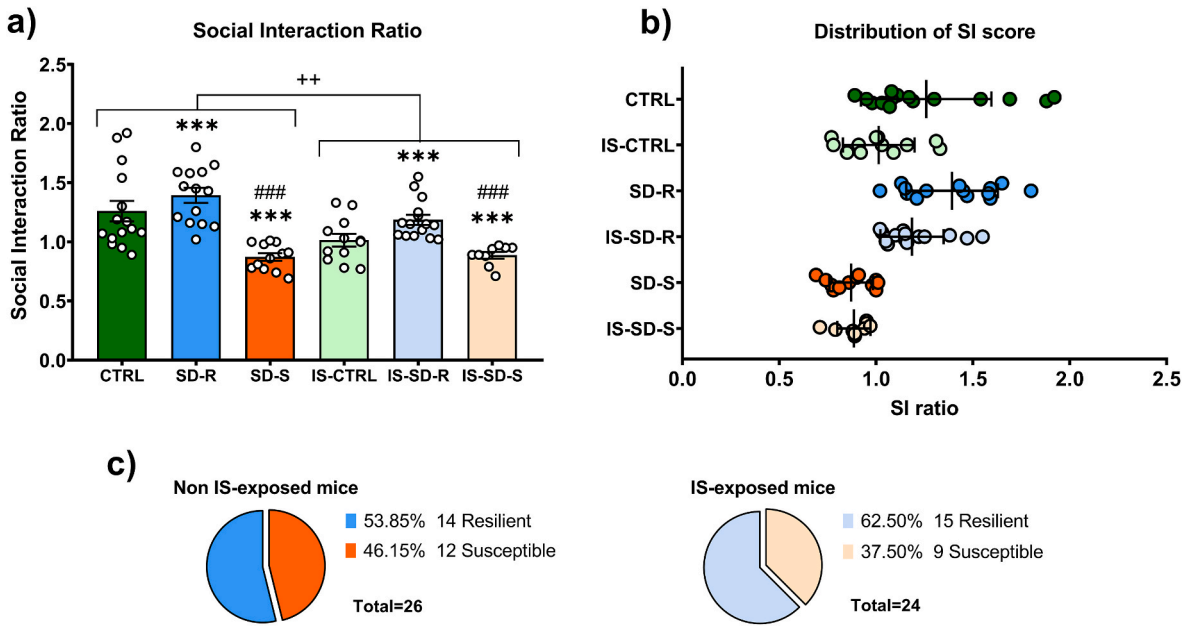

Fig. 2. Social defeat stress induces avoidance behavior in susceptible mice. (a) Repeated social defeat stress results in a spectrum of avoidance behavior, divided between susceptible and resilient phenotypes as a function of their social interaction ratio score. (b) Distribution of social interaction scores. (c) Percentages of resilient and susceptible mice in both conditions (non-IS and IS). Error bars represent means ± SEM. ***$p < 0.001$ vs. control mice. $^{###}p < 0.001$ vs. resilient mice. $^{++}p < 0.01$ vs. non-stress-inoculated mice.

of the mice showed a ratio <1, which classifies them as susceptible (SD-S) animals (n = 12). The remaining 53.85% of the mice showed a ratio equal to or higher than 1, which classifies them as resilient (SD-R) mice (n = 14). Furthermore, mice of the IS-CTRL group (n = 12) showed a mean ratio >1. In the IS-SD group of mice (n = 24), 37.5% of the animals showed a ratio <1, which classifies them as susceptible (IS-SD-S) (n = 9), and the remaining 62.5% of the mice showed a ratio equal to or higher than 1, which classifies them as resilient (IS-SD-R) mice (n = 15).

3.2. Exposure to inoculation of stress does not affect behavior during SD

The ANOVA for the time employed in Avoidance/Flee and Defensive/Submissive behaviors showed an effect of the variable Days [$F(1,44) = 16.303$; $p < 0.001$] and [$F(1,44) = 42.505$; $p < 0.001$]. All mice increased the time spent in Avoidance/Flee behavior, but spent less time in Defensive/Submissive behavior during the 4th SD ($p < 0.001$ in both cases).

The ANOVA for the time employed in Attack and Threat behaviors by resident mice showed an effect of the variable Days [$F(1,44) = 69.641$; $p < 0.001$] and [$F(1,44) = 10.167$; ($p < 0.01$)]. Resident mice increased the time spent in attack and threat behavior during the 4th SD ($p < 0.001$ and $p < 0.01$, respectively).

All these analyses are shown in Table 1.

Finally, we assessed the relationship between the time spent in Avoidance/Flee and Defensive/Submissive behaviors during the 1st and the 4th social defeat encounters and the SIT ratio shown 24h after the fourth SD. This analysis evaluates whether the strategies to cope with stress could be an indicator of the depressive-like behavior phenotype (Fig. 3a and b). A significant Pearson correlation coefficient was only obtained between the SIT ratio and these behaviors for susceptible mice not exposed to stress inoculation ($r = 0.623$, $p < 0.041$ for time in avoidance/flee during the 1st SD and $r = 0.705$, $p < 0.015$ for time in

Table 1
Ethological analyses of social defeat. Behavior of intruder and resident mice during 5-min agonistic encounters. ***$p < 0.001$, **$p < 0.01$ significant differences between the 1st and 4th SDs.

			Without inoculated stress				With inoculated stress			
		Encounters	1st		4th		1st		4th	
			Resilient	Susceptible	Resilient	Susceptible	Resilient	Susceptible	Resilient	Susceptible
Intruder mice	Avoidance/ Flee	Total Time (s)	28.7±1.5	28.8±1.9	36.2±3	35.7±2.1 ***	28.5±2.2	26.5±3.7	36.6±2.4	33.3±3.9 ***
	Defense/ Submission	Total Time (s)	9.8±1.5	8.8±1.1	19.4±1.9	17.6±2.2 ***	9.7±1.9	6.7±2.2	18.4±2.8	17.7±1.5 ***
Resident mice	Attack	Total Time (s)	16±1.2	15.4±1.3	25±2.5	27.4±1.1 ***	14.2±1.4	16.3±2.9	24.5±1.5	27.1±1.7 ***
	Threat	Total Time (s)	9.2±1.2	11.1±0.7	12.6±1.4	12.7±1.1 **	10.2±1.7	9.6±1.4	13.2±1.4	13.7±1.6 **

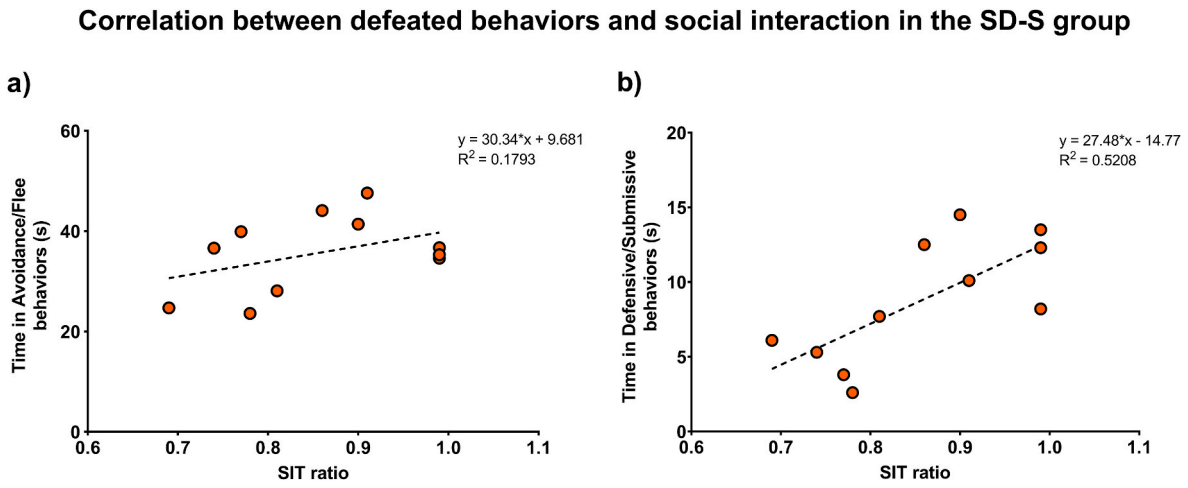

Fig. 3. Regression plot for the Pearson correlation between Avoidance/Flee and Defensive/Submissive behaviors during the 1st SD and the SIT ratio in SD-S group. The trend line represents the linear regression of data.

defensive/submissive during the 1st SD). The higher the time spent in these behaviors, the higher the social interaction during SIT. The lack of correlation during the 4th social defeat indicates that experience overruled the initial strategy and equalized the response. However, this correlation was absent in mice exposed to stress inoculation, meaning that previous exposure to a small stress during adolescence abolishes the influence of coping strategies and depressive-like behaviors phenotype.

3.3. Stress inoculation during adolescence leads to decreased ethanol consumption in the DID procedure

The ANOVA for ethanol consumption revealed a significant effect of the interactions Days × IS [$F(3,204) = 3.007$; $p < 0.05$], and SD × IS [$F(2,68) = 4.230$; $p < 0.05$] (Fig. 4). The post-hoc comparison showed that all animals consumed a higher ethanol on day 4 compared to days 1, 2 and 3 (p's < 0.001 in all cases). In addition, less ethanol was consumed by the inoculated mice during the 2nd day of DID, compared to those not stressed during adolescence ($p < 0.001$). In addition, the SD-S group consumed higher amounts of ethanol than the SD-R ($p < 0.01$) and IS-SD-S ($p < 0.001$) groups during the DID test.

3.4. Stress inoculation during adolescence leads to decreased ethanol consumption in susceptible mice

No differences were found between the animals during the training phase, showing that SD did not induce any learning deficit (data not shown).

The ANOVA for the number of active responses during the FR1 schedule of ethanol SA revealed a significant effect of the variable Days [$F(9,639) = 3.694$; $p < 0.001$], SD [$F(2,71) = 4.333$; $p < 0.05$], and the interaction Days × SD × IS [$F(18,639) = 2.317$; $p < 0.01$] (Fig. 5a). The post-hoc comparison revealed that mice performed fewer active

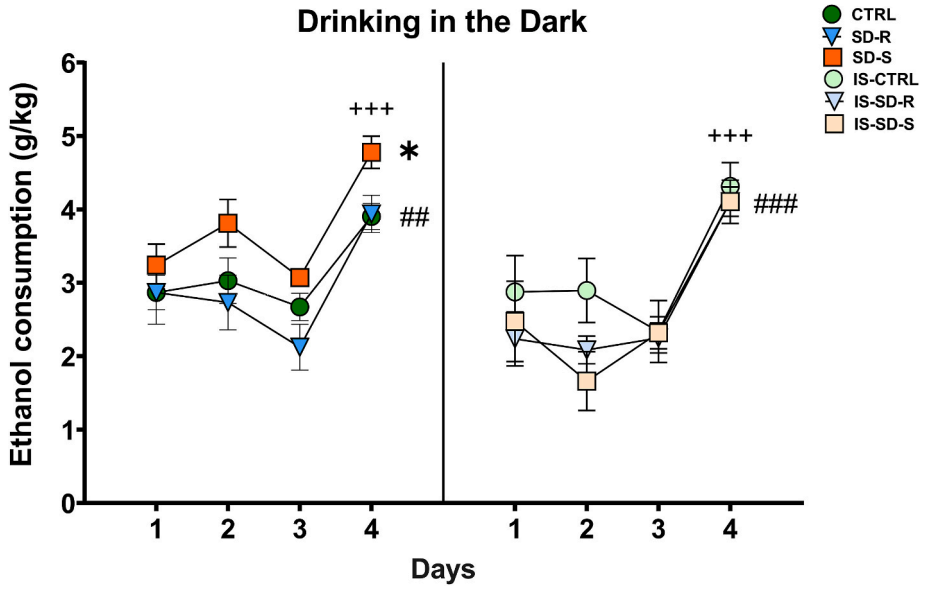

Fig. 4. Effect of adolescent stress inoculation on ethanol intake during DID. Animals were divided into the following six treatment groups: CTRL group not exposed to SD in adulthood (CTRL, n = 15) or CTRL group exposed to IS in adolescence, but not exposed to SD in adulthood (SI-CTRL, n = 12); Resilient SD group exposed to SD in adulthood (SD-R, n = 14) or Resilient SD exposed to IS in adolescence and exposed to SD in adulthood (IS-SD-R, n = 15); and Susceptible SD group exposed to SD in adulthood (SD-S, n = 12) or Susceptible SD group exposed to IS in adolescence and exposed to SD in adulthood (IS-SD-S, n = 9). Defeated mice were characterized as resilient or susceptible depending on their SIT. The dots represent means and the vertical lines ± SEM of the g/kg of ethanol at 20% consumed. *p < 0.05 vs. CTRL group; ###p < 0.001, ##p < 0.01 vs SD-S group; $^{+++}$p<0.001 vs. days 1, 2 and 3.

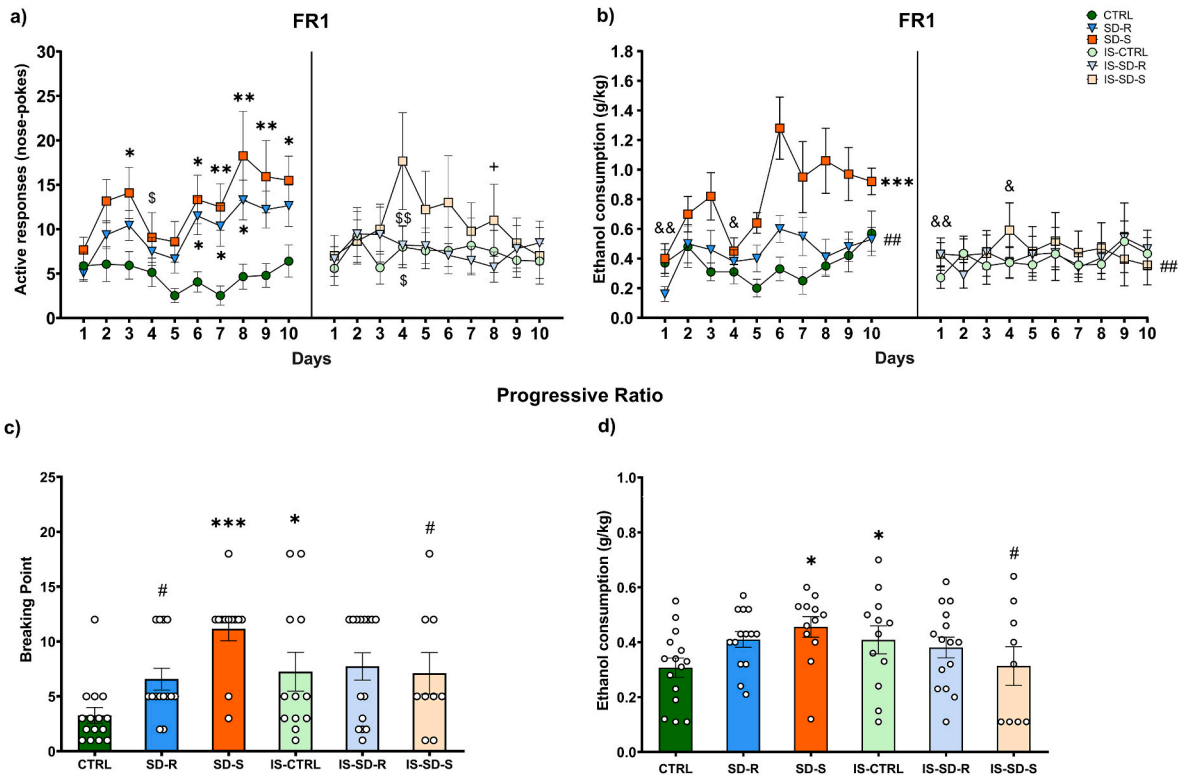

Fig. 5. Effects of stress inoculation during adolescence on the increase in oral ethanol self-administration induced by social stress in C57BL/6J mice. Animals were divided into the following six treatment groups: CTRL group not exposed to SD in adulthood (CTRL, n = 15) or CTRL group exposed to IS in adolescence, but not exposed to SD in adulthood (IS-CTRL, n = 12); Resilient SD group exposed to SD in adulthood (SD-R, n = 14) or Resilient SD exposed to IS in adolescence and exposed to SD in adulthood (IS-SD-R, n = 15); and Susceptible SD group exposed to SD in adulthood (SD-S, n = 12) or Susceptible SD group exposed to IS in adolescence and exposed to SD in adulthood (IS-SD-S, n = 9). Defeated mice were characterized as resilient or susceptible depending on their SIT. The dots represent means and the vertical lines ± SEM of (a) the number of active responses and (b) the g/kg of ethanol at 20% consumed during FR1 schedule. The columns represent the mean and the vertical lines ± SEM of (c) the breaking point values, and (d) the g/kg of ethanol at 20% consumed during PR. ***p < 0.001, **p < 0.01, *p < 0.05 vs. CTRL group; ## p < 0.01, # p < 0.05 vs. SD-S group; + p < 0.05 vs. SD-R group; $$ p < 0.01, $ p < 0.05 vs. IS-SD-S; && p < 0.01, & p < 0.05 vs. day 10.

responses on day 1 compared to days 2 ($p < 0.001$), 3 ($p < 0.01$), 4 ($p < 0.05$), 8, and 10 (p's < 0.01). Moreover, the SD-S group performed more active responses than the CTRL group on days 3, 6 (p's < 0.05), 7, 8, 9 and 10 (p's < 0.01). The SD-S group also performed more active responses than the SD-R group on day 7 ($p < 0.05$). Likewise, the IS-SD-S group performed more active responses on day 4 compared to the IS-CTRL ($p < 0.05$) and IS-SD-R ($p < 0.05$) groups. Finally, the post-hoc comparison showed a significant decrease in active responses in the IS-SD-R group on day 8 compared to the SD-R group ($p < 0.05$).

With respect to ethanol consumption, the ANOVA revealed a significant effect of the variable Days [$F(9,630) = 2.976$; $p < 0.01$] and a significant effect of the interaction SD × IS [$F(2,70) = 3.558$; $p < 0.05$] (Fig. 5b). The post-hoc comparison showed that during day 10 all animals consumed a significantly higher amount of ethanol than during days 1 ($p < 0.01$) and 4 ($p < 0.05$). Also, the SD-S group showed significantly higher consumption than the CTRL ($p < 0.001$), SD-R ($p < 0.01$) and IS-SD-S ($p < 0.01$) groups.

During the PR, the ANOVA for the breaking point values of ethanol SA revealed a significant effect of the interaction SD × IS [$F(2,71) = 4.710$; $p < 0.05$] (Fig. 5c). The post-hoc comparisons showed that the SD-S group achieved a significantly higher BP than the CTRL ($p < 0.001$) and SD-R ($p < 0.05$) groups, while no significant differences were observed between the groups exposed to a brief social defeat. Moreover, a higher BP was observed in the IS-CTRL group compared to the CTRL ($p < 0.05$) group, and a higher BP was observed in the SD-S group compared to the IS-SD-S ($p < 0.05$) group.

The ANOVA for ethanol consumption during PR revealed a significant effect of the interaction SD × IS [$F(2,71) = 4.298$; $p < 0.05$] (Fig. 5d). The post-hoc comparisons showed that the SD-S group had a higher ethanol consumption than the CTRL ($p < 0.05$) and IS-SD-S ($p < 0.05$) groups, although IS-CTRL group consumed significantly more ethanol than the CTRL group ($p < 0.05$).

3.5. *Stress inoculation during adolescence leads to decreased neuroinflammatory response in susceptible mice*

The ANOVA for IL-6 levels in PFC showed an effect of the variable IS [$F(1,68) = 15.821$; $p < 0.001$] (see Fig. 6a). Post-hoc comparisons showed lower IL-6 levels in the IS-exposed mice (IS-CTRL, IS-SD-R and IS-SD-S groups) compared to non-IS-exposed mice (CTRL, SD-R and SD-S groups; $p < 0.001$). Moreover, the ANOVA for the striatal IL-6 levels showed an effect of the interaction SD × IS [$F(2,70) = 3.173$; $p < 0.05$] (see Fig. 6c). A significant increase in IL-6 levels was observed in the SD-S group compared to the CTRL ($p < 0.01$) and SD-R groups ($p < 0.05$). Additionally, both SD-S and SD-R groups showed higher IL-6 striatal levels than the corresponding groups exposed to IS ($p < 0.001$ for IS-SD-S, and $p < 0.01$ for IS-SD-R). A positive correlation was found between the IL-6 striatal levels and the total amount of ethanol consumption during the oral ethanol SA procedure ($r = 0.311$, $p < 0.006$) (Fig. 7). No correlation was found with the amount of ethanol during the DID test.

The ANOVA of CX3CL1 levels in PFC also revealed a significant effect of the interaction SD × IS [$F(2,70) = 5.760$; $p < 0.01$] (see Fig. 6b). The post-hoc comparisons revealed a significant increase in CX3CL1 levels in the SD-S group compared to the CTRL and IS-SD-S groups ($p < 0.01$ in all cases). Moreover, the results showed an increase in CX3CL1 levels in the IS-CTRL group compared to the CTRL group ($p < 0.05$). The ANOVA of

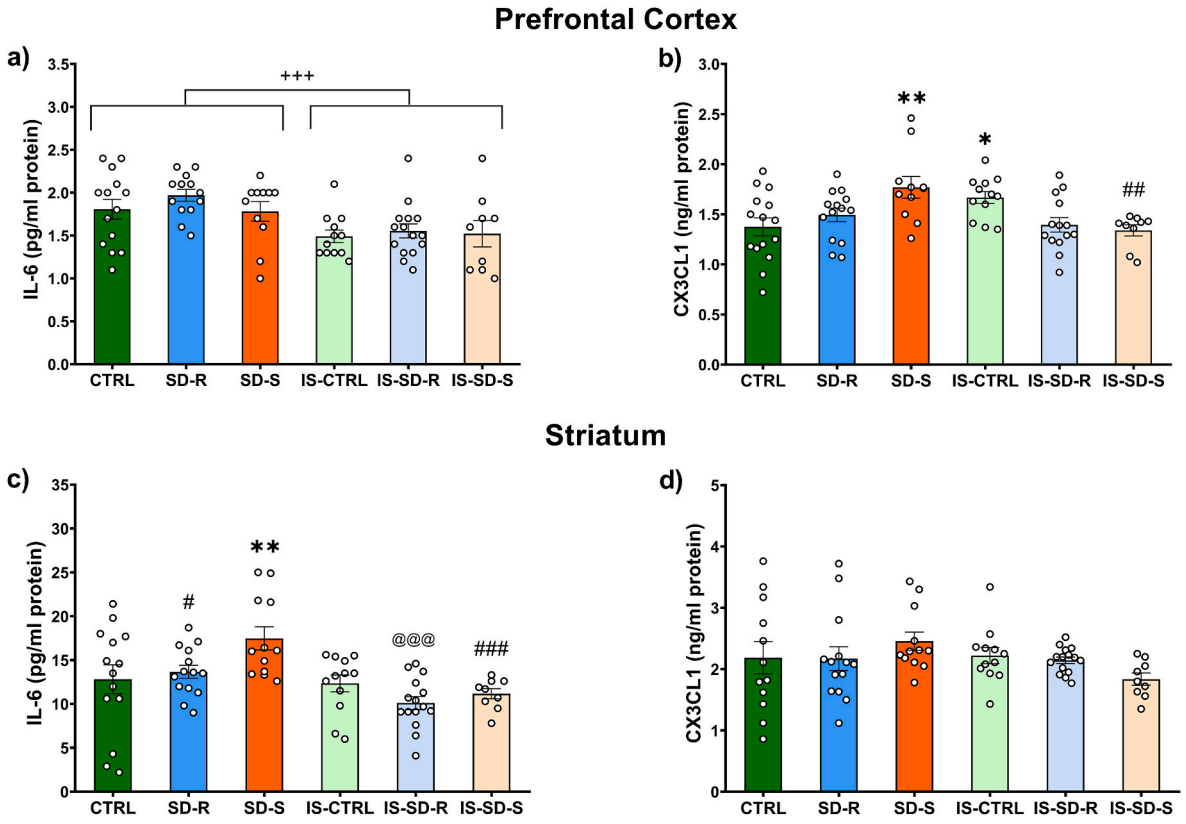

Fig. 6. Effects of stress inoculation during adolescence in the neuroinflammatory response induced by social stress in C57BL/6J mice. Animals were divided into the following six treatment groups: CTRL group not exposed to SD in adulthood (CTRL, n = 12–15) or CTRL group exposed to IS in adolescence, but not exposed to SD in adulthood (IS-CTRL, n = 12); Resilient SD group exposed to SD in adulthood (SD-R, n = 13–14) or Resilient SD exposed to IS in adolescence and exposed to SD in adulthood (IS-SD-R, n = 15); and Susceptible SD group exposed to SD in adulthood (SD-S, n = 11–12) or SD group exposed to IS in adolescence and exposed to SD in adulthood (IS-SD-S, n = 9). Defeated mice were characterized as resilient or susceptible depending on their SIT. The columns represent the mean and the vertical lines ± SEM of the striatal (a) and cortical (c) levels (in pg/mg) of the pro-inflammatory cytokine IL-6, and of the striatal (b) and cortical (d) levels (in ng/mg) of the chemokine CX3CL1. **p < 0.01, *p < 0.05 vs. CTRL group; ### p < 0.001, ## p < 0.01, # p < 0.05 vs. SD-S group; +++ p < 0.001 vs. IS-exposed mice; @@@ p < 0.001 vs. IS-SD groups.

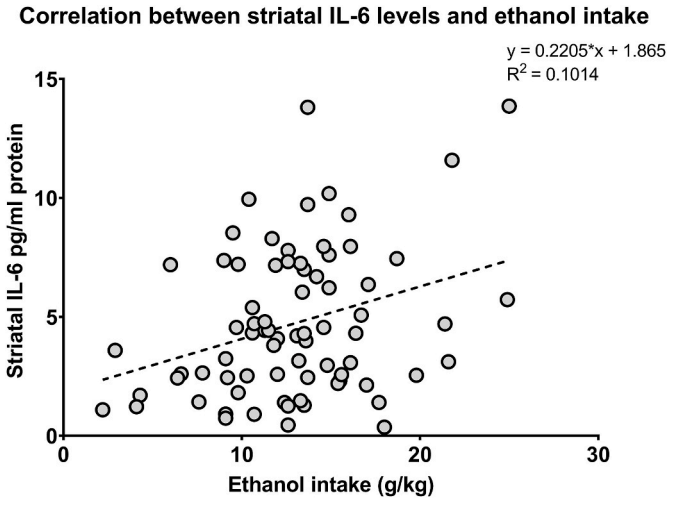

Fig. 7. Regression plot for the Pearson correlation between striatal IL-6 levels and ethanol intake during SA procedure. The trend line represents the linear regression of data.

striatal CX3CL1 levels revealed no significant differences (Fig. 6d).

4. Discussion

The study of the mechanisms underlying more effective coping strategies to social stress, which will increase the likelihood of resilience, can unexpectedly include adverse events (Seery et al., 2010). The experience of mild-to-moderate stress prior to a more intense stressful encounter has been shown to enhance resilience. Most of this research has been conducted in pre-weaning or adult age (Alves et al., 2020; Calpe-López et al., 2022; Hsiao et al., 2016; Qin et al., 2019). Studies focusing on adolescence remain unexplored to date.

Previous studies showed that resilient mice exhibit a decreased response to SD-induced cocaine and ethanol reward enhancement, coupled with a normalized neuroinflammatory response (Ballestín et al., 2021; Reguilón et al., 2021a). In the present study, as we expected, SD induced an increase in ethanol consumption in mice susceptible to SD, which also showed an increase in protein levels of IL-6 in the striatum and CX3CL1 in the PFC. Furthermore, our results showed that exposure to a unique episode of SD (IS) during adolescence buffers the negative effects of intermittent SD experience during adulthood. Although there were no behavioral differences in flight and submissive behaviors exhibited by the intruder mice during social encounters, the correlation between the SIT and the intruder susceptible mice behavior was not observed in animals exposed to stress inoculation. In addition, exposure to IS during adolescence led to a 9% increase in the resilient phenotype assessed with the SIT. IS also blocked the increased ethanol consumption

observed in susceptible mice during the DID and FR1 schedule of oral ethanol SA. We also observed a decreased neuroinflammatory response in all animals exposed to IS with reduced IL-6 levels in PFC and in the striatum of susceptible mice subjected to IS, as well as decreased levels of CX3CL1 in PFC. Interestingly, we observed increased consumption and motivation for ethanol of control mice exposed to IS during adolescence compared to the non-inoculated control group, as well as increased levels of IL-6 in the PFC.

4.1. Defeated mice exposed to stress inoculation during adolescence induce a slight increase in social interaction in adulthood

Across various studies, a consistent proportion of around 50% of resilient mice among the defeated group is observed using the SIT, despite differences in the social defeat process (Alves-dos-Santos et al., 2020; Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021a; Rodenas-Gonzalez et al., 2021). Similar results have been seen in mice subjected to environmental enrichment during adolescence (Reguilón et al., 2021a). However, exposure to IS during adolescence increases this percentage. As depicted in Fig. 2, the percentage of IS-exposed animals resilient to SD effects (62.5%) surpassed that of resilient defeated animals without intervention (53.8%). Limited studies have assessed IS impact on adult social interaction. Consistent with our findings, Ayash et al. (2020) observed a significant increase in social interaction in SIT in mice exposed during adolescence to an IS based on 11 sensory stress sessions compared to controls. Similarly, Hsiao et al. (2016) observed an increase in resilience to chronic SD-induced depressive-like behaviors in adolescence with SI exposure, recording a 60% resilience rate versus 33% in controls. In our study, IS-exposed mice exhibited a modest 9% increase in the resilient subpopulation compared to non-exposed resilient mice. Based on these few studies, we speculate that IS might facilitate better adaptation to social stress, possibly mitigating the development of depressive-like behaviors in adulthood. Although the experimental design is not comparable, brief maternal separation (6h during PND 9) failed to boost social interaction after repeated SD in late adolescence (Calpe-López et al., 2022). These authors suggest that the short separation duration may hinder SI's protective impact at this developmental stage.

Interestingly, we also observed a positive correlation between time spent in avoidance and defensive behaviors in non-inoculate susceptible mice and the social interaction ratio in the SIT. The longer the time spent in these behaviors, the higher the ratio score in the SIT. Therefore, susceptible mice showing more active coping strategies would be less affected by stress-induced depressive-like behaviors. However, this correlation is absent in susceptible mice with stress inoculation, highlighting the effect of this previous stress experience on coping strategies.

4.2. Stress inoculation enhances resilience to the increased ethanol consumption induced by social defeat

The schedule of SD employ in this study consistently leads to increased voluntary ethanol intake, consumption escalation, and heightened substance motivation (Montagud-Romero et al., 2021; Newman et al., 2018; Reguilón et al., 2020, 2021a, 2021b, 2022; Rodriguez-Arias et al., 2016). In previous studies, we reported that SD-susceptible mice exhibited an increase in ethanol consumption and an increase in motivation to obtain the substance, whereas SD-resilient mice showed resistance to escalated drug effects (Ballestín et al., 2021; Giménez-Gómez et al., 2021; Reguilón et al., 2021a; Rodenas-Gonzalez et al., 2021). The present results confirm that mice susceptible to depressive-like behaviors increase ethanol consumption and motivation, while resilient mice do not display this ethanol intake surge. We also showed that IS, despite only increasing resilience to SD-induced depressive-like behaviors by 9%, buffered the increased ethanol consumption in all defeated mice populations, both resilient and susceptible. Therefore, IS slightly increased resilience to depressive-like behaviors and strongly reduced SD-induced ethanol intake. Therefore, we only observed an increase in alcohol consumption in susceptible animals (SD-S) during the DID paradigm, used as a pre-exposure to ethanol. Similar results were obtained during the FR1 schedule of the oral ethanol SA, since IS-SD-S susceptible mice consumed similar amounts as resilient counterparts (SD-R and IS-SD-R), and substantially less than non-exposed susceptible mice (SD-S). Enhanced drug motivation and consumption were only evident in the SD-S groups during progressive ratio. Interestingly, the IS-exposed control group (IS-CTRL) displayed higher ethanol consumption and breakpoints than the non-exposed control (CTRL) during PR schedule, suggesting that a single SD exposure in adolescence may predispose vulnerability to adult ethanol consumption (for review, see Camarini et al., 2018).

Although no comparable studies exist, our results point to IS promoting resilience to future stress, and suggest that IS could mitigate substance use escalation following stress. For example, the preventive effect of brief maternal separation on the increased cocaine-induced CPP by repeated SD during early adulthood has been described (Calpe-López et al., 2022). Although the study is not comparable, Ordoñes Sanchez et al. (2021) used the limited bedding and nesting model of adversity to assess IS on phenotypes related to opiate addiction in adulthood in male mice. These authors found that IS reduced impulsive choice, decreased morphine SA, and dampened morphine-induced plasticity in the nucleus accumbens. Collectively, limited research suggests early-life IS may induce resilience against addiction-related traits. Notably, these conclusions are largely based on male animals; female-specific IS studies remain absent. Further investigation is warranted to comprehend IS's role in subsequent stressful experiences and adult addictive behaviors across sexes.

4.3. Stress inoculation reduces the neuroinflammatory response induced by social defeat

Previous studies have highlighted an increased neuroinflammatory responses in animals displaying susceptibility to depressive-like symptoms after SD exposure (Ballestín et al., 2021; Nasca et al., 2019; Pfau et al., 2019; Reguilón et al., 2021a). Increased levels of pro-inflammatory cytokines such as MCP-1, IL-17, and IL-1β, accompanied by the activation of signaling complexes (NFkBp-p65, COX-2, NLRP3), have been observed following exposure to social stress (Hodes et al., 2014; Stewart et al., 2015; Wood and Bhatnagar, 2015; Yang et al., 2021). Conversely, anti-inflammatory cytokines like IL-4 and IL-10 have been identified in resilient animals (Hodes et al., 2014; Stewart et al., 2015). Using a similar protocol, we have identified a sustained elevation of the pro-inflammatory cytokine IL-6 in the PFC, striatum, and hippocampus of susceptible mice subjected to SD (Ballestín et al., 2021; Reguilón et al., 2021a). However, responses of the chemokine CX3CL1 exhibited variability. SD led to reduced CX3CL1 levels in specific brain regions such as the striatum or the hippocampus (Ballestín et al., 2021; Montagud-Romero et al., 2020; Reguilón et al., 2021a), while increases were observed in others (hypothalamus, rostral cortex) within the C57BL/6 strain (Wohleb et al., 2013). Remarkably, CX3CL1 protein levels showed an increase in the striatum of a different mouse strain (OF1; Reguilón et al., 2020, 2021b).

Our findings showed that SD exposure induces increased levels of proinflammatory cytokines such as IL-6 in the striatum of susceptible mice (SD-S), whereas no increase was observed in defeated mice (either resilient or susceptible) exposed to IS during adolescence. This protecting effect was also observed in the PFC as all mice exposed to IS presented lower levels of IL-6 when compared to those without this intervention. We also assessed the relationship between the levels of these two neuroinflammatory markers and the amount of ethanol intake during SA (Fig. 7). For all mice, a significant Pearson correlation coefficient was obtained between the striatal levels of IL-6 and the total amount of ethanol intake ($r = 0.311$, $p < 0.006$). The higher the ethanol intake, the higher the concentration of IL-6 in the striatum. This analysis

confirms that regardless of the stress experience, ethanol intake modulates IL-6 production. This correlation is clear in the SD-S mice, those with higher alcohol consumption and therefore higher IL-6 concentration.

The chemokine CX3CL1 showed significantly elevated levels in the PFC in the SD-S group only. No increases were observed in defeated mice exposed to IS, confirming the protective effect of this intervention. No changes were observed in the striatum for this chemokine. As we have previously pointed out, in response to SD we have reported no changes in CX3CL1 after cocaine-induced CPP (Ballestín et al., 2021) or lower levels in the PFC and striatum after 6% ethanol SA in susceptible mice (Reguilón et al., 2021a). Based on studies in humans and animal models in which increases in the levels of this chemokine have been observed in the striatum during alcohol exposure, dependence and withdrawal (Chen et al., 2011; Pascual et al., 2015), we hypothesized that the increase observed in the present study could be due to the exposure to a 20% ethanol during the DID and SA procedures, which could have altered CX3CL1-CX3CR1 signaling.

Our results confirm that IS during adolescence mitigate the increase in neuroinflammation caused by social stress. Aligning with these findings, a lower neuroinflammatory response was noted in environmentally enriched susceptible mice during adolescence (Reguilón et al., 2021a). Interestingly, a significant difference between control groups was obtained, with heightened CX3CL1 levels in the PFC of the IS-exposed control group (IS-CTRL) compared to the control non-stressed group. Previous studies suggest that adolescent stress affects the immune system, affecting the reward pathway and increasing vulnerability to substance abuse (Lo Iacono and Carola, 2018; Rodriguez-Arias et al., 2016). Although no changes in the striatum have been observed in our study, these data highlight the importance of social stress on the CX3CL1-CX3CR1 signaling pathway and should be further investigated.

The beneficial effects of IS on the reduction of SD-induced ethanol intake could be due to its dampening effect on neuroinflammation. The present results have confirmed the relation between the resilient phenotype and increased ethanol intake with a contained neuroinflammatory response. However, other mechanisms must be also involved. The decrease in the social stress-induced detrimental effects produced by IS may be due to a lower reactivity of the HPA axis to a subsequent stress experience and/or the development of a higher neuroplasticity or behavioral flexibility that characterizes the resilient phenotype (Hawley et al., 2010; Lambert et al., 2014; Lee et al., 2014). Taking into consideration the U-shaped function, mild and intense stressors would show an extreme HPA axis response, although moderate stress would show a moderate axis response (Macrì et al., 2011). There are hardly any studies evaluating the HPA axis response in rodents subjected to IS during adolescence and the results are controversial. Some studies demonstrated reduced corticosterone response following IS training in adult animals (Brockhurst et al., 2015), although opposite results were obtained in adolescents (Mancini et al., 2021). Further studies need to be done to clarify the HPA response after IS experience.

5. Conclusion

The study of the mechanisms that explain the development of depression and vulnerability to drug addiction after exposure to social stress highlights the importance of developing preventive strategies that promote resilience to stress. The present study highlights the role of moderate IS in enhancing resilience to the effects of social stress. In summary, our results corroborate that SD produces depressive-like behaviors, enhances the reinforcing and motivational effects of ethanol, and induces a greater neuroinflammatory response in susceptible mice, in contrast to resistant animals. Our results indicated that by training individuals exposed to stressful situations, they can develop adaptive responses to social stress. To date, the data suggest that a mild social stressor could confer resilience. However, other non-social stressors (such as restraint stress) must be evaluated. Adolescence is a critical developmental period for later mental illnesses, and an important period on which to focus intervention strategies. Further studies assessing the role of interventions during this period are needed to develop effective and individualized preventive strategies.

CRediT authorship contribution statement

Marina D. Reguilón: Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft. **Carmen Manzanedo:** Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft. **José Miñarro:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Marta Rodríguez-Arias:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

This work has not been published previously and is not under consideration for publication elsewhere. The authors have no possible conflict of interest in the carrying out and reporting of this research.

Data availability

Data will be made available on request.

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