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Enhanced novelty-seeking after early adolescent exposure to vicarious social defeat predicts the vulnerability of female mice to cocaine reward

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ABSTRACT

Stressful experiences can have a serious impact on adolescents, as the process of brain maturation, particularly of the prefrontal cortex, takes place during this developmental period. In animal models, male mice exposed to social defeat during early or late adolescence show increased vulnerability to cocaine reward, but this effect has only been studied in late adolescent female mice exposed to Vicarious Intermittent Social Defeat (VISD). The aim of the present study was to investigate the biochemical and behavioural effects of exposure to VISD during early adolescence in female mice. VISD only induced anxiety-like symptoms in the elevated plus maze (EPM) and increased novelty-seeking behaviour in the hole-board test. Furthermore, the behavioural profile of VISD-exposed mice in these tests was associated with their vulnerability or resilience to cocaine reward in adulthood. Female mice that exhibited a higher frequency of entries in the closed arms of the EPM and a lower latency of dips in the hole-board subsequently acquired cocaine-induced conditioned place preference. Thus, exposure of female mice to VISD during early adolescence also induced short-term changes that increased sensitivity to cocaine reward in susceptible individuals.

1. Introduction

Adolescence is a period during which the process of brain maturation is completed. Adversity during this phase can have a significant impact on development (Spear, 2000a), particularly in the case of females, which are more sensitive to the consequences of stress (Bangasser and Wiersielis, 2018; Swaab and Bao, 2020). During adolescence, peer relationships develop thus contributing to an optimal socioemotional development (Smetana et al., 2015). In this sense, studies with animal models have demonstrated that female mice exposed to a social isolation protocol during early adolescence display an increase in cocaine and heroin-seeking and anxiety- and depression-like symptoms (Caruso et al., 2018; Challa et al., 2023; Fosnocht et al., 2019; Li and Yan, 2023; Singh et al., 2022).

Another model shown to be effective for evaluating the effects of social stress in female mice is that of vicarious social defeat, in which females witness the defeat of a conspecific male by another male of a more aggressive strain (Decker-Ramirez et al., 2024; Franco et al., 2022; Iñiguez et al., 2018; Ródenas-González et al., 2023; Warren et al., 2020). We have previously studied the effects of social stress on different parameters, including vulnerability to cocaine, by exposing late adolescent (LA) female mice to a protocol of intermittent vicarious defeats. In this protocol, that we denominate Vicarious Intermittent Social Defeat (VISD), female mice experience one episode of vicarious defeat every 72 h (PND 47–50–53–56), as an alternative the protocol employed by other research teams in which animals are exposed to an episode of vicarious defeat on 10 consecutive days (Decker-Ramirez et al., 2024; Franco et al., 2022; Iñiguez et al., 2018). VISD decreased corticosterone levels, increased interleukin-6 (IL-6) levels, enhanced novelty-seeking in the hole-board test, improved memory in the object recognition test and induced anxiety- and depression-like symptoms in the elevated plus maze and splash test, respectively (Martínez-Caballero et al., 2024).

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Although LA females exposed to VISD did not acquire cocaine-induced conditioned place preference (CPP), we observed an increase in the rewarding effects of cocaine in adulthood in some of them (susceptible mice). This indicated that not all mice are vulnerable to the effects of stress, as occurs in humans. We also demonstrated that vulnerability and resilience to the effects of VISD on cocaine reward were associated with different behavioural profiles. Mice resilient to developing cocaine CPP were characterized by less novelty-seeking in the hole-board and an absence of social avoidance in the social interaction test and depression-like behaviour in the splash test (Martínez-Caballero et al., 2024).

As explained previously, early adolescence is a period of development during which individuals are particularly vulnerable; however, the effects of social defeat at this age have been poorly studied. We have identified only one study of the effects of vicarious defeat in female rats exposed at this stage of their development (Liu et al., 2018). In the study in question, EA female rats witnessed for 7 consecutive days (PND 21–27) how their dams or another adult female rat were attacked by a male of the same species. On PND 64, female rats that saw how their dams (but not a new female rat) were attacked showed depression-like behaviour in the forced swimming test (Liu et al., 2018). In relation to resilience/susceptibility to stress, a more recent study analysed the differences between the two profiles of EA females using a different paradigm of social defeat. An EA female mouse (PND 25) was attacked by a male mouse twice a day for 4 consecutive days. Twenty-four hours after the last social defeat session, the female mice were assessed in a social interaction test and divided into resilient or vulnerable types. Differences in anxiety-like behaviours in the elevated plus maze were not found between the two groups. However, differences in inhibitory control were observed in adulthood, since only the resilient female mice showed deficits in impulse control in a Go/No-Go task (Pantoja-Urbán et al., 2024). It is important to note that there are no studies about the consequences of vicarious defeat in EA female rodents on the rewarding effects of cocaine.

Research in male mice has revealed the existence of differences in the effects of intermittent social defeat (ISD) stress between EA and LA males. In our laboratory, we observed that LA male mice exposed to four episodes of defeat (PND 47–50–53–56) showed anxiety- and depressive-like symptoms (in the elevated plus maze and splash test, respectively), social avoidance in the social interaction test, stress hyperreactivity in the tail suspension test, and enhanced sensitivity to the rewarding effects of cocaine (Calpe-López et al., 2020). In contrast, anxiety-like symptoms and hyperreactivity to stress were not seen when EA male mice were exposed to defeat (PND 27–30–33–36); moreover, their preference for the cocaine reward was lower in comparison to LA mice exposed to ISD, despite receiving a higher dose of cocaine (Calpe-López et al., 2023). The resilience profile of the two age groups was also different; all the mice resilient to developing cocaine CPP after ISD exposure showed an active coping response and an avoidance of potential dangers in unknown environments, while LA (but not EA) male mice exposed to stress showed lower novelty-seeking in the hole-board test and reduced acute stress reactivity in the tail suspension test (Calpe-López et al., 2020, 2023).

All of the experimental evidence described above highlights the importance of investigating the influence of age on the effects of defeat stress in female mice. Therefore, we set out to evaluate the biochemical and behavioural effects of exposure to the VISD protocol in female EA mice (one episode of vicarious defeat every 72 h, PND 27–30–33–36). In particular, we assessed IL-6 levels as a marker of stress-induced inflammation after the first episode of social defeat, as well as the short-term behavioural effects of VISD on anxiety-like behaviour, novelty-seeking behaviour, social behaviour, depression-like behaviour, stress reactivity and recognition memory; in order to evaluate the effects of VISD on these behaviours, we used the elevated plus maze, the hole-board test, the social interaction test, the splash test, the tail suspension test and the object recognition test, respectively. In addition, we assessed the long-term effects of VISD on cocaine-induced CPP and explored whether the behavioural profile of mice exposed to VISD was associated with their subsequent vulnerability to cocaine reward. Based on our previous results with LA and EA male and LA female mice, we hypothesised that EA female mice would be resilient to most of the effects of VISD, since EA males were more resilient than LA males to the effects of ISD in the elevated plus maze, hole-board and tail suspension tests and LA female mice were also resilient to the effects of VISD in the tail suspension test, social interaction and cocaine CPP.

2. Material and methods

2.1. Subjects

46 Female and 32 male mice of the C57BL/6 strain and 32 male mice of the OF1 strain were acquired from Charles River (France). The C57BL/6 animals arrived at the laboratory on PND 21 and were housed in groups of four in plastic cages (25 × 25 × 14.5 cm). The OF1 mice arrived on PND 42 and were housed individually (23 × 32 × 20 cm) to induce aggression (Rodríguez-Arias et al., 1998). All mice were maintained under the same conditions: constant temperature, reversed light schedule (white light on 19:30–07:30) and food and water available ad libitum, except during behavioural tests. All experimental protocols were conducted according to the Directive 210/63/EU and were approved by the Ethics Committee for Experimental Research of the University of Valencia (A20210217012657, 2021 – VSC – PEA – 0083).

2.2. Drugs

Physiological saline (NaCI 0.9 %) or 1.5 mg/kg of cocaine (Alcaliber Laboratory, Madrid, Spain) was injected intraperitoneally in a volume of 0.01 ml/g of weight. Physiological saline was used to dissolve the cocaine. The dose of cocaine was selected on the basis of a pilot study and similar studies in the literature (Flores-Ramirez et al., 2018; Martínez-Caballero et al., 2024; Ródenas-González et al., 2023).

2.3. Experimental design

Two groups of female mice were used to evaluate the effects of stress on IL-6 levels, a control group not exposed to stress ($n = 6$), and a stressed group ($n = 8$). For the behavioural experiments, two groups of female mice were used: a control group (no stress, n = 8) and another exposed to stress by Vicarious Intermittent Social Defeat (VISD) paradigm ($n = 24$). The VISD sample was larger in order to allow us to segregate the group into two subgroups according to the response of mice in the behavioural tests and thus be able to study resilience or vulnerability to stress.

2.3.1. Vicarious intermittent social defeat (VISD)

The VISD protocol took place on PND 27, 30, 33 and 36 to induce social stress in early adolescent female mice. The age at which episodes of VISD were initiated was selected based on the literature. A developmental timeline in rodents is accepted, and distinguished early (PND 25–45) and late (PND 46–65) adolescence, corresponding to 10–18 and 18–25 years of age in humans, respectively (Laviola et al., 2003; Spear, 2015, 2000b; Vetter-O'Hagen and Spear, 2011, 2012). In each episode, separated by intervals of 72 h, the experimental female mice witnessed male mice of the same strain being defeated in an agonistic encounter with another more aggressive male mouse. At the start of each episode, a C57BL/6J female mouse was placed in the home cage of an aggressor male mouse of the OF1 strain (resident), with both mice separated by a perforated methacrylate wall. Then, a C57BL/6J male mouse (intruder) was introduced into the compartment of the resident male, and the two males were allowed to confront each other for a period of 5 min. After each episode the females and intruder males were returned to their home cages. The perforated methacrylate wall ensured the perception of olfactory and chemosensory stimuli of the defeat episode, which induced emotional/psychological stress in the female mice (Iñiguez et al., 2018). In each episode, the intruder mouse was exposed to a different resident mouse. The first (1st) and fourth (4th) episode were recorded to evaluate the locomotor activity of the females, as well as the time and frequency that female paid attention to the agonistic encounter. The non-stressed female mice (control group) underwent the same protocol without the presence of an aggressor male mouse.

2.3.2. IL-6 level determination

Blood was obtained from the saphenous vein between 10 a.m. and 1 p.m. and was collected in BD Vacutainer Plus serum blood collection tubes. Immediately afterwards, plasma was separated from whole blood by centrifugation (5 min, 5000*g*), transferred to sterile 2 ml micro-centrifuge tubes and stored at −80 °C until determination of IL-6. The concentration of IL-6 in plasma was analysed with the Mouse IL-6 ELISA Kit (Catalog no.: ab100712, Abcam, Cambridge, UK), following the manufacturer's instructions.

2.3.3. Behavioural testing

After VISD, female mice performed the Elevated plus maze (EPM), Hole-board (HB) and Splash (SP) tests (PND 37) and the Object Recognition (OR), Social Interaction (SI) and Tail Suspension (TS) tests (PND 38). All experiments took place during the dark period (8:30–16:30 h) and mice were transported to a dimly lit experimental room during the dark period 1 h before the behavioural tests initiated, in order to facilitate adaptation. Female mice were subjected to these tests, with an interval of at least 1 h between each test, and in a specific sequence determined by the degree of stress induced in the mice. This methodology has been previously employed, with the objective of preventing the impact of prior test experiences on subsequent test results (Calpe-López et al., 2020). Finally, after 3 weeks during which animals were undisturbed in the vivarium, they underwent the CPP procedure (PND 57–64) after conditioning with 1.5 mg/kg of cocaine (Fig. 1).

2.3.3.1. Elevated plus maze (EPM). The EPM was used to evaluate the presence of anxiety-like symptoms based on the aversion of rodents to open spaces (Campos et al., 2013; Rodgers and Johnson, 1995). The apparatus consisted of a central platform (5 × 5 cm) with two open arms and two closed arms (30 × 5 cm each) that was elevated 45 cm above floor level. Each mouse was placed on the central platform, facing an open arm, and was allowed to explore the maze for 5 min. The behaviour of the mice was videotaped and analysed using a computerised method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The time, entries and latency of entry into the open arms (OA) and closed arms (CA), and time and entries into the central platform were measured. The percentage of time spent in the OA [time spent in OA / (time spent in OA + time spent in CA) * 100] and the percentage of entries into the OA [entries into OA / (entries into OA + entries into CA) * 100] were calculated. Anxiety levels are considered to be lower when measurements in the open arms (time spent, entries or their percentages) are higher and measurements in the closed arms are lower, and vice versa (Rodgers and Johnson, 1995). Moreover, the total number of entries into the arms is regarded as a score of locomotor activity (Campos et al., 2013).

2.3.3.2. Hole-board (HB)test. The HB test was used to evaluate the novelty-seeking behaviour of the mouse (Arenas et al., 2014). The test apparatus was a square box (28 × 28 × 20.5 cm) with 16 equidistant holes of 3 cm in diameter on the floor and transparent plexiglas walls (CIBERTEC SA, Madrid, Spain). The mouse was introduced in a corner of the box and was allowed to explore the cage for 10 min. The total number of dips (frequency) and latency to perform the first dip were recorded by the apparatus via photocells located beneath the surface of the holes. These measures are used to define animals as low or high novelty seekers, the latter of the two performing more dips and presenting lower latencies to perform this behaviour (Arenas et al., 2014).

2.3.3.3. Splash (SP) test. The SP test was carried out in order to evaluate the presence of depressive-like symptoms (Smolinsky et al., 2009). Each mouse was placed in a transparent cage (15 × 30 × 20 cm) and sprayed on the dorsal coat with a 10 % sucrose solution to stimulate grooming, which is a motivational and self-care behaviour. Each trial was videotaped and the behaviour of the mice was analysed using a computerised method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The time spent in grooming, the latency to perform the first grooming and the frequency of grooming was recorded. A decrease in the time or frequency of grooming and an increase in the latency to perform this behaviour have been interpreted as depressive-like behaviour (Smolinsky et al., 2009).

2.3.3.4. Object recognition (OR) test. The OR test was used to evaluate explicit memory (Montesinos et al., 2015). It consisted of three phases: habituation, training session (T1) and test session (T2). During

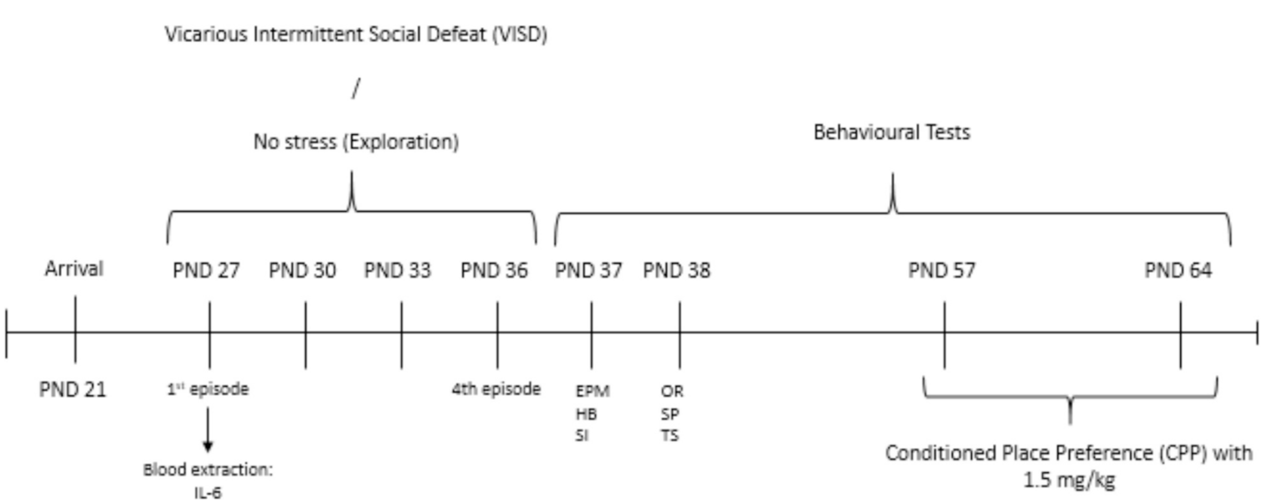

Fig. 1. Experimental design. Two groups of female mice were used. One group was exposed to four episodes of Vicarious Intermittent Social Defeat (VISD) on PND 27–36, and the other group explored an empty cage (no stress). Thirty minutes after the 1st episode of VISD/exploration, blood samples were collected from a set of stressed female (VISD, $n = 8$) or control ($n = 6$) to determinate IL-6 levels. On PND 37–38, the remaining female mice (VISD, $n = 24$; Control, $n = 8$) performed the elevated plus maze (EPM), hole-board (HB), social interaction (SI), object recognition (OR), splash (SP) and tail suspension (TS) tests. After an interval of 3 weeks, the same female mice underwent the conditioned place preference (CPP) paradigm with 1.5 mg/kg of cocaine (PND 57–64).

habituation (on PND 37), the mouse was given 2 min to explore a plastic cage (25 × 25 × 14.5 cm). On PND 38, T1 and T2 were performed. During T1, the mouse was placed in the same plastic cage for 5 min, allowing exploration of two stones placed in two different corners. It was then returned to its home cage for 1 min (the memory retention interval). In the T2 phase, the mouse was reintroduced to the cage for 5 min, allowing exploration of a familiar stone and a new small plastic toy. The total time spent exploring each object was recorded and analysed using Raton Time 1.0 software (Fixma SL, Valencia, Spain) to calculate a discrimination index (DI): [DI = (Tnovel − Tfamiliar) / (Tnovel + Tfamiliar) × 100 %]. If the DI is positive, it is considered that the animal recalls the familiar object and has spent more time exploring the new one.

2.3.3.5. Social interaction (SI) test. The SI test was used to evaluate social avoidance or social approach (Krishnan et al., 2007). The female mouse was placed in the centre of an open field (37 × 37 × 30 cm) that contained a perforated plexiglass cage (10 × 6.5 × 30 cm) in the middle of one wall. During the first phase (object phase), the perforated plexiglass cage was empty and the female was allowed to explore the open field for 10 min. Subsequently, she was returned to her home cage for 2 min. A C57BL/6 male mouse was confined to the perforated cage and the female was reintroduced in the open field for 10 min (social phase). The time spent in the 8 cm area surrounding the perforated cage (interaction zone) during both phases was automatically registered and sent to a computer using the Ethovision 2.0 software package (Noldus, Wageningen, The Netherlands). These measures were employed to calculate an index of social interaction (ISI) [time spent in the interaction zone during the social phase / (time spent in the interaction zone during the social phase + time spent in the interaction zone during the object phase)] (Henriques-Alves and Queiroz, 2016). An ISI of 0.5 indicates that the mouse did not exhibit social approach or social avoidance, while values of ISI below 0.5 are interpreted as a reduction of social behaviour.

2.3.3.6. Tail suspension (TS) test. During the TS test, usually employed to evaluate depression-like behaviour, the female mouse was suspended by the tail using adhesive tape for a period of 6 min (Vaugeois et al., 1997). The behaviour of the mouse was videotaped and analysed using a computerised method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). Specifically, the time spent immobile and the latency to show immobility were recorded (Pollak et al., 2010). Enhanced immobility in the TS test is interpreted as despair behaviour, an indicator of a state resembling depression.

2.3.3.7. Conditioned place preference (CPP) protocol. The CPP procedure was used to evaluate the conditioned rewarding properties of cocaine (Maldonado et al., 2007). The apparatus used for the CPP comprised eight Plexiglas boxes. Each box had two compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) with different coloured walls (white vs. black) and floor textures (wide grid vs. fine grid). The compartments are separated by a central grey area (13.8 cm long × 31.5 cm wide × 34.5 cm high), and the position of the mouse is recorded by four infrared light beams in each compartment and six in the central area. The equipment was controlled by three IBM PC computers using MONPRE 2Z software (Cibertec SA, Madrid, Spain). The CPP procedure was carried out in three phases: Pre-Conditioning (Pre—C, PND 57–59), Conditioning (PND 60–63) and Post-Conditioning (Post-C, PND 64). During the Pre-C phase, the mouse was given 15 min to explore the box, after which the time spent in each compartment was recorded. Mice that showed a strong unconditioned aversion or a preference for a specific compartment were excluded from the study. In the conditioning phase, mice were injected with saline before being confined to the vehicle-paired compartment for 30 min. Four hours later, mice were injected with 1.5 mg/kg of cocaine before being confined to the drug-paired compartment for 30 min. During the four days of this phase, the order of injections (saline or cocaine) was alternated. In the final phase (Post-C), the mouse was allowed to explore the box for 15 min, and the time spent in each compartment was recorded. A group was considered to have acquired CPP if the time spent in the drug-compartment in Post-Conditioning (Post-C) was significantly higher than the time spent in this compartment in Pre-Conditioning (Pre-C).

2.4. Statistical analysis

The short-term effects of VISD on the different biochemical and behavioural measurements were evaluated by unpaired Student's *t*-tests. A repeated measures two-way ANOVA with a within-subjects variable – Days, with two levels (Pre-C and Post-C) – and a between-subjects variable – Stress, with two levels (Control and VISD) – was used to evaluate the effects of VISD on cocaine CPP. Pearson correlation tests were employed in order to detect relationships among the behaviour of female mice during episodes of defeat and in the different behavioural tests. In the case of CPP, the conditioning score (time spent in Post-C minus time spent in Pre—C) was used.

For each behavioural measurement that correlated with cocaine CPP, the whole group of female mice exposed to VISD was separated into two subgroups (High Score and Low Score). To evaluate differences between these two subgroups and the control group, a one-way ANOVA with a between-subjects variable –Groups, with three levels (Control, High Score and Low Score) – was performed. Additionally, repeated measures two-way ANOVAs with a within-subjects variable – Days, with two levels (Pre-C and Post-C) – and a between-subjects variable – Group, with three levels (Control, High Score and Low Score) were performed to determine possible behavioural markers of subsequent vulnerability or resilience to cocaine CPP. Paired Student's *t*-tests were used to evaluate differences in the behaviours showed by female mice exposed to VISD in the first and fourth episode of vicarious defeat. To evaluate the potential influence of behavioural profile during these episodes on the acquisition of cocaine CPP, the whole group of female mice exposed to VISD was separated into two subgroups (High Score and Low Score) and a repeated measures two-way ANOVA (Days, two levels; Group, two levels) was performed. After ANOVAs, post hoc comparisons were performed with Bonferroni's correction if the Levene's test for homogeneity of variances did not indicate significant differences. Otherwise, post hoc comparisons were performed with the Games-Howell test. All statistical analyses were performed with the SPSS program.

3. Results

3.1. Biochemical and behavioural effects of VISD

There was a trend towards higher levels of IL-6 in VISD-exposed mice, though the difference with respect to control mice did not reach statistical significance [$t(9.006) = -1.964$; $p = 0.08$] (Fig. 2).

VISD decreased the Time [$t(30) = 2.617$; $p < 0.05$] (Fig. 3a), Percentage of Time [$t(30) = 2.584$; $p < 0.05$] (Fig. 3b), and Percentage of Entries [$t(30) = 2.028$; $p = 0.05$] (Fig. 3c) into the open arms of the EPM; increased the time spent [$t(30) = -3.048$; $p < 0.01$] (Fig. 3d) and the number of entries into the closed arms [$t(30) = -2.316$; $p < 0.05$] (Fig. 3e); and decreased the latency to enter the closed arms of the EPM [$t(30) = 2.873$; $p < 0.01$] (Fig. 3f). VISD did not affect other indices in the EPM, including the number of entries and latency to enter the open arms, time spent and number of entries into the centre of the EPM, and total arm entries (data not shown).

In the HB test, VISD decreased the latency to perform the first dip [$t(24) = 2.271$; $p < 0.05$] (Fig. 4) but did not affect the frequency of this behaviour. No significant differences were observed in the SI, OR, SP, TS and CPP tests (see Supplementary materials, Figs. S1–S4).

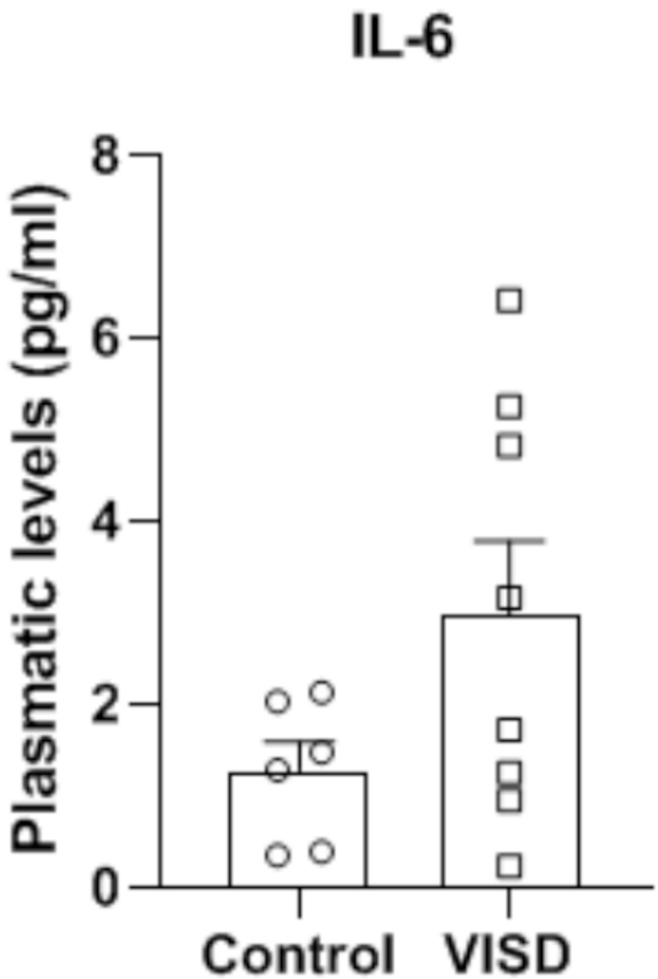

Fig. 2. Biochemical effects of VISD. One group of EA female mice was exposed to VISD ($n = 8$) on PND 27 while the other group was not exposed to stress (Control, $n = 6$). Bars represent mean (±SEM) plasmatic levels of IL-6.

3.2. Correlations between behavioural measurements

Significant Pearson correlations were observed between some behavioural measures in stressed female mice (Table 1). As expected, correlations were observed among several measures of EPM (see also Supplementary material, Table S1) as they also were between latency and frequency of grooming in the SP test. However, no significant correlation was observed between measures in the TS test, or between measures in the HB test. In addition, latency and frequency of grooming correlated with measures of the EPM, while the number of dips correlated negatively with ISI and frequency of grooming. These correlations suggested that female mice with less depression-like symptoms displayed less anxiety-like symptoms and more novelty-seeking behaviour. Interestingly, there was also a correlation between CPP score and both entries into the closed arms (ECA) in the EPM and latency of dips in the HB, suggesting that the performance of mice in these tests indicated vulnerability to cocaine CPP. No other measure obtained during episodes of VISD or in the behavioural tests performed shortly after the last episode correlated with cocaine CPP. Regression analyses involving the CPP score and the behavioural measurements revealed that the frequency of ECA [$R^2 = 0.174$; $F(1,22) = 5.619$, $p < 0.05$], along with the latency of dips [$R^2 = 0.242$; $F(1,18) = 5.735$, $p < 0.05$], were effective predictors of the CPP score (see Supplementary material, Figs. S5–S6). With the exception of the correlations between the EPM measurements, the correlations reported in female mice exposed to VISD were not observed in control mice (see Supplementary material, Table S1). In addition, Pearson correlations revealed significant correlations between the measures taken during the episodes of VISD and during behavioural tests (see Supplementary material, Table S2).

3.3. Behavioural profile of stressed female mice during behavioural tests and vulnerability to cocaine CPP

The correlation between CPP score and both ECA and latency of dips suggested that these measurements might allow us to discriminate between mice that are vulnerable or resilient to the effects of cocaine. To test this, female mice exposed to VISD were divided into two subgroups according to the frequency of ECA (Low or High ECA) (below or above the median of the defeat group, 10 times). One-way ANOVA revealed a significant effect of the variable Group [$F(2,29) = 19.697$; $p < 0.001$], the High ECA group was significantly different from the control and Low EOA groups (ps < 0.001) (Fig. 5a). ANOVA of the CPP data of the control group and the two groups of stressed mice (Low and High ECA) revealed the variable Days [$F(1,29) = 4.441$; $p < 0.05$] and the Interaction Days × Group [$F(2,29) = 3.525$; $p < 0.05$] to be significant. Bonferroni post hoc comparisons revealed that only the High ECA group developed CPP ($p < 0.01$) (Fig. 5b).

Similarly, stressed female mice were separated into Low or High LDips (below or above the median of the defeated group, 25.5 s). One-way ANOVA revealed a significant effect of the variable Group [$F(2,29) = 11.122$; $p < 0.001$], the Low LDips group was significantly different from the control and the High LDips groups ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 6a). ANOVA of the CPP data of the control group and the two groups of stressed mice (Low and High LDips) revealed the variable Days [$F(1,25) = 5.507$; $p < 0.05$] to be significant. Simple effects of the Interaction showed that the effect of Days was only significant in the Low LDips group [$F(1,25) = 6.990$; $p < 0.05$] (Fig. 6b).

3.4. Behavioural profile of stressed female mice during episodes of vicarious defeat and vulnerability to cocaine CPP

All behaviours in female mice analysed during the episodes of vicarious defeat (motor activity, frequency of attention and time in attention) showed lower values in the fourth than in the first episode (see Supplementary material, Table S3). When stressed female mice were segregated into two subgroups (High or Low) according to these behaviours, none of these behavioural measures allowed us to discriminate between mice that acquired CPP and those that did not (see Supplementary material, Table S4).

4. Discussion

The results of the present study indicate that exposure of female mice to VISD during EA induces inflammation, anxiety-like symptoms in the EPM, and more pronounced novelty-seeking behaviour in the HB test shortly after the last episode of defeat. Furthermore, the behaviour displayed during the EPM and HB test was associated with vulnerability or resilience to cocaine reward in adulthood. Defeated female mice that performed more entries in closed arms of the EPM and showed a shorter latency to perform the first dip in the HB test acquired cocaine-induced CPP.

Our results indicated that exposure to VISD was an effective protocol to induce stress in early adolescent female mice. Firstly, a trend of increased IL-6 levels, a marker of stress-induced neuroinflammation, was observed after the 1st episode of VISD. A similar result has been observed in brain samples (paraventricular nucleus of the hypothalamus and ventral hippocampus) from female rats exposed to an inescapable footshock for 1 or 2 h (PND 30–31) (Marsland et al., 2022). Secondly, exposure to VISD during EA also induced the development of short-term anxiety-like symptoms, however, this result contrasted with that reported by other studies. For example, EA female mice exposed to restraint (PND 33–39) did not show anxiety-like symptoms in the EPM

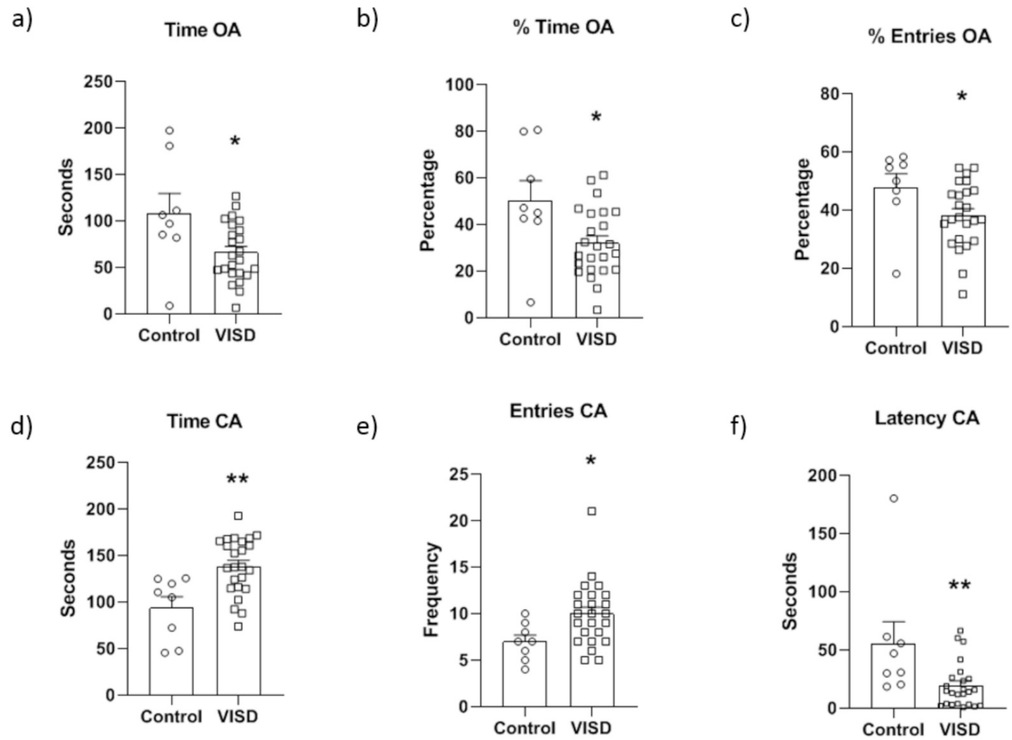

Fig. 3. Short-term behavioural effects of VISD on the EPM. One group of EA female mice was exposed to VISD ($n = 24$) on PND 27, 30, 33 and 36, while the other group was not exposed to stress (Control, $n = 8$). Bars represent mean (±SEM) values in the open arms (OA) and closed arms (CA). VISD reduced the time spent in OA (a), the percentage of time spent in OA (b), and the percentage of entries into OA (c). VISD increased the time spent in CA (d) and the entries into CA (e). VISD reduced the latency to enter CA (f). $^{*}p < 0.05$, $^{**}p < 0.01$, significant difference with respect to the control group.

(PND 42) (He et al., 2020). Similarly, exposure to the repeated multiple concurrent stressors (RMS) paradigm (PND 30–40) did not induce anxiety in the Light/dark box test (PND 41) (Fariborzi et al., 2021). These divergent results are probably related with the different paradigms used to induce stress. Thirdly, VISD reduced the latency to perform the first dip in the HB test, that may be interpreted as an increase of novelty-seeking or stress-induced exploration, in concordance with that observed in adolescent female rodents exposed to other paradigms of stress (Sexton et al., 2022; Toledo-Rodriguez and Sandi, 2011). Alternatively, the reduction in the latency to perform the first dip in the hole-board test could also be interpreted as low avoidance behaviour, high arousal or more risky behaviour in VISD-exposed female mice.

In contrast, we observed no differences between controls and stressed female mice in terms of depression-like symptoms in the SP and TS tests. Although the effects of VISD during EA on depression have not previously been evaluated, similar results have been obtained in the forced swim (FS) test and sucrose preference test among female mice exposed to restraint, RMS paradigm or predatory stress (Eidson et al., 2019; Fariborzi et al., 2021; He et al., 2020). However, one study reported that chronic unpredictable stress (CUS) during EA (PND 28–40) induced depression-like symptoms in female mice performing the sucrose preference test (PND 40) (Yohn and Blendy, 2017). Such discrepancies between studies are likely to be due to the different levels of stress induced by each paradigm of stress; for example, CUS is a more stressful protocol, since mice are chronically exposed to three different stressors, three times a day, every day throughout the protocol.

In the present study, the VISD procedure did not induce effects in the SI and OR tests. Indeed, the effects of stress experienced during EA on female mice in the SI test have been studied only after isolation. In line with our results, socially-housed and isolated female mice (PND 21–60) exhibited a similar social preference in a three-chamber social interaction task (PND 45) (Myers et al., 2024). In the case of discrimination memory, a previous study with female rats exposed to chronic unpredictable stress (PND 26–44) did not find differences between control and stressed groups in the OR test (PND 49–51) (Sexton et al., 2022). The age of the animals may have had an impact on the results observed, since female mice exposed to social isolation on PND 29–52 were found to spend less time interacting with a novel female in the SI test (PND 54–55) and scored lower on the discrimination index in the OR test (PND 56–58) (Lodha et al., 2023).

In relation to the long-term effects of VISD on cocaine CPP, we observed that both stressed and control female mice did not acquire CPP after conditioning with a low dose of cocaine. Although the long-term effects of VISD on EA females have not been evaluated to date, it is relevant that research exploring the impact of social isolation (PND 21 – adulthood) on cocaine self-administration found that isolated female mice displayed higher motivation for cocaine and cue-induced reinstatement of cocaine seeking (Fosnocht et al., 2019). These divergent results are probably linked to the protocol used to evaluate cocaine reward (primary reinforcing vs conditioned rewarding effects) and the protocol used to induce stress (social isolation until adulthood is presumably more stressful than VISD). In the present study we have observed that the behavioural profile of stressed female mice in the EPM and HB test is associated with their subsequent vulnerability to cocaine CPP. Only VISD-exposed female mice that displayed more entries in the closed arms of the EPM subsequently acquired CPP. Although the increase in closed arms entries could be interpreted as anxiety-like behaviour it seems more adequate to think that it reflected enhanced exploration for several reasons. The frequency of entries in closed arms did not correlate with other measures of anxiety (for example, time and percentage of time in open arms or time in closed arms) but positively correlated with entries in the centre of the EPM and with total entries, considered a measure of locomotor activity or exploration (Campos et al., 2013). In addition, we previously observed in late adolescent female mice exposed to VISD, that their behavioural profile in the EPM did not predict resilience or vulnerability to cocaine CPP (Martínez-

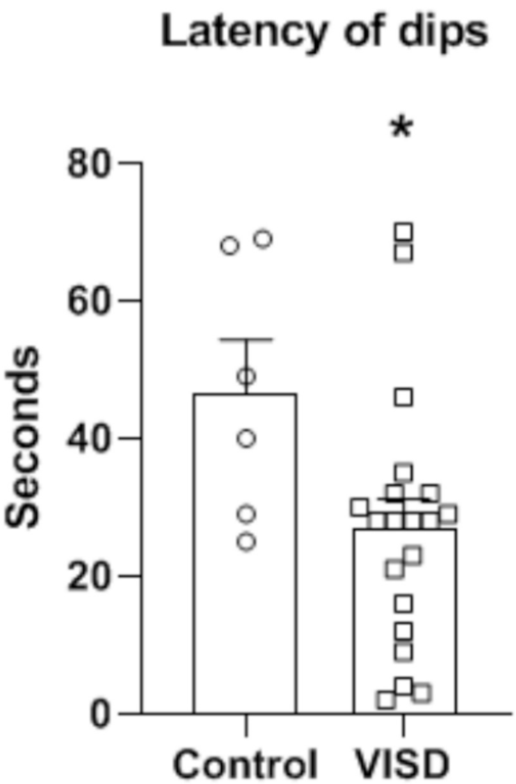

Fig. 4. Short-term behavioural effects of VISD in the HB test. One group of EA female mice was exposed to VISD (n = 24) on PND 27, 30, 33 and 36, while the other group was not exposed to stress (Control, n = 8). Bars represent mean (±SEM) latency to perform the first dip. VISD decreased the latency of dips. *p < 0.05 significant difference with respect to the control group.

Caballero et al., 2024). Furthermore, only VISD-exposed early adolescent female mice with lower latency to perform the first dip acquired CPP. This result is in concordance with previous research carried out in our laboratory showing that novelty-seeking behaviour predicts sensitivity to the conditioned rewarding effects of cocaine in naïve mice. Specifically, we found that adolescent female mice which were high-novelty seeking in the HB test acquired cocaine-induced CPP (Arenas et al., 2014). Finally, in agreement with the present results, we observed that only female mice displaying more novelty-seeking after exposure to VISD during late adolescence subsequently develop cocaine CPP (Martínez-Caballero et al., 2024). It is important to note that the resilience or susceptibility of female mice to the rewarding effects of cocaine was not a function of the degree of the attention they paid to the social interactions between male mice during the VISD episodes. The frequency of attention and time in attention during the VISD episodes did not predict resilience or vulnerability to cocaine CPP (see Supplementary material, Table S4).

In conclusion, this work indicates that the VISD protocol is effective in inducing social stress in early adolescent females, which develop anxiety-like symptoms and more pronounced novelty-seeking behaviour. Furthermore, some behavioural characteristics of stressed mice, such as enhanced exploration or novelty-seeking, predict subsequent vulnerability to cocaine reward. Thus, from a translational point of view, our results highlight the importance of identifying the phenotype of adolescent females that are more vulnerable to the negative consequences of stress in order to implement preventive strategies to reduce the development of stress-related disorders in such individuals.

Welfare of animals

All experimental protocols were conducted according to the Directive 210/63/EU and were approved by the Ethics Committee for Experimental Research of the University of Valencia (A20210217012657, 2021 – VSC – PEA – 0083).

CRediT authorship contribution statement

María Ángeles Martínez-Caballero: Visualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Claudia Calpe-López:** Visualization, Formal analysis, Writing – original draft. **María Pilar García-Pardo:** Formal analysis, Writing – review & editing. **María Carmen Arenas:** Methodology, Writing – review & editing. **Jose Enrique de la Rubia Ortí:** Formal analysis, Writing – review & editing. **María Benlloch:** Investigation. **Carmen Manzanedo:** Methodology, Writing – review & editing. **María Asunción Aguilar:** Supervision, Project administration, Funding acquisition, Conceptualization, Writing – review & editing.

Role of the funding source

This work is part of the R + D + i project PID2020-118945RB-I00,

Table 1
Coefficients of Pearson's correlation between the measurements obtained in the different behavioural tests in the VISD-exposed female mice. The upper part of the table shows the values of the Pearson coefficients (r) and the lower part shows the observed significance level for each one (p <). Only behavioural measures that presented a significant correlation are included and specific correlations between behavioural indices of the EPM are not featured (see Table S1). CPP score, conditioned place preference score, Dips and Ldips, number of dips and latency to perform the first dip in the hole-board test, respectively; ISI, index of social interaction; TOA and EOA, time spent and number of entries in the open arms of the elevated plus maze (EPM), respectively; TCA and ECA, time spent and number of entries in the closed arms of the EPM, respectively; %TOA and %EOA, percentage of time spent and percentage of entries in the open arms of the EPM, respectively; LGr and FGr, latency and frequency of grooming behaviour in the splash test, respectively.

	CPP score	Dips	LDips	ISI	TOA	EOA	TCA	ECA	%TOA	%EOA	LGr	FGr
CPP score		ns	r = -0.492	ns	ns	ns	ns	r = 0.417	ns	ns	ns	ns
Dips	ns		ns	r = -0.523	ns	ns	ns	ns	ns	ns	ns	r = -0.558
Ldips	0.05	ns		ns	ns	ns	ns	ns	ns	ns	r = 0.495	ns
ISI	ns	0.05	ns		ns	ns	ns	ns	ns	ns	ns	ns
TOA	ns	ns	ns	ns		r = 0.724	r = -0.793	ns	r = 0.980	r = 0.825	r = -0.530	ns
EOA	ns	ns	ns	ns	0.001		r = -0.511	ns	r = 0.673	r = 0.679	r = -0.536	r = 0.425
TCA	ns	ns	ns	ns	0.001	0.05		ns	r = -0.893	r = -0.581	r = 0.421	r = -0.402
ECA	0.05	ns	ns	ns	ns	ns	ns		ns	r = -0.417	ns	ns
%TOA	ns	ns	ns	ns	0.001	0.001	0.001	ns		r = 0.795	r = -0.525	ns
%EOA	ns	ns	ns	ns	0.001	0.001	0.01	0.05	0.001		r = -0.656	ns
LGr	ns	ns	0.05	ns	0.01	0.01	0.05	ns	0.01	0.001		r = -0.530
FGr	ns	0.01	ns	ns	ns	0.05	0.05	ns	ns	ns	0.01	

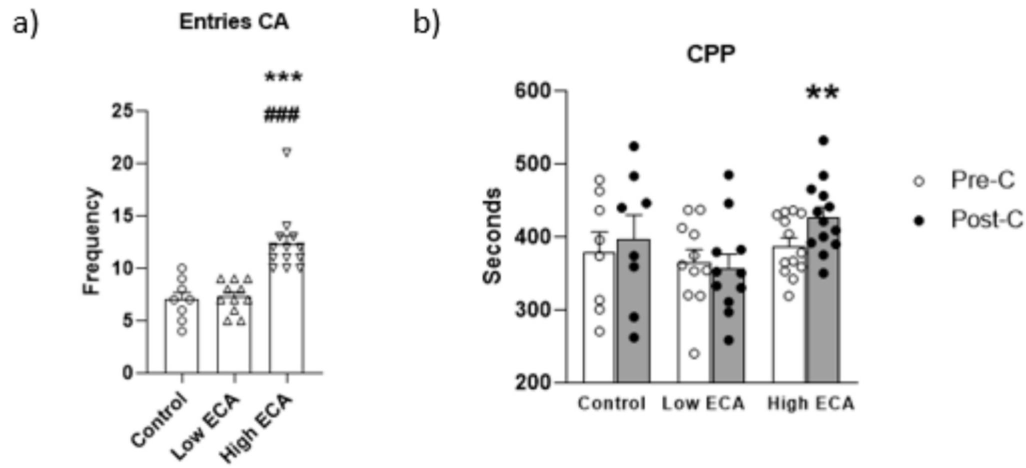

Fig. 5. Behavioural profile during the EPM and cocaine reward (Entries CA). One group of EA female mice was exposed to VISD ($n = 24$) on PND 27, 30, 33 and 36, while the other group was not exposed to stress (Control, $n = 8$). A) Stressed female mice were divided into two groups according to frequency of entries into the closed arms (ECA). Bars represent mean (±SEM) number of entries into the closed arms. ***$p < 0.001$, significant difference with respect to the control group. ###$p < 0.001$ significant difference between Low ECA and High ECA groups. B) Effects of VISD on the CPP induced by cocaine (1.5 mg/kg) according to the ECA. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning test (Pre—C) and in the post-conditioning (Post-C). Only the High ECA group acquired CPP. **$p < 0.01$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

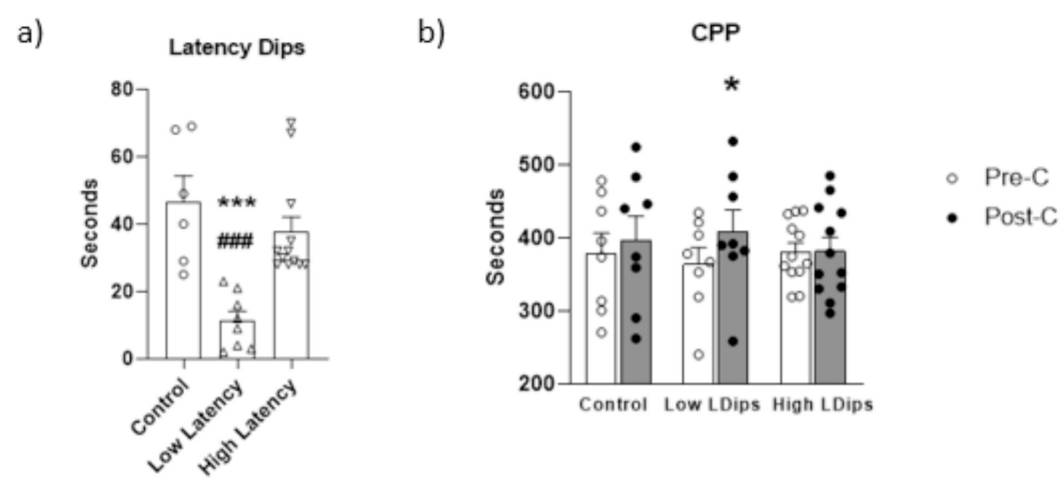

Fig. 6. Behavioural profile during the HB test and cocaine reward. One group of EA female mice was exposed to VISD ($n = 24$) on PND 27, 30, 33 and 36, while the other group was not exposed to stress (Control, $n = 8$). a) Stressed female mice were divided into two subgroups according to the latency to perform the first dip in the hole-board. Bars represent mean (±SEM) latency of dips. **$p < 0.01$, significant difference with respect to the control group. ###$p < 0.001$ significant difference between Low and High Latency of Dips groups. B) Effects of VISD on the CPP induced by cocaine (1.5 mg/kg) according to the latency to perform the first dips. Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning (Pre—C) and post-conditioning (Post-C) tests. Only the Low LDips group acquired CPP. *$p < 0.05$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

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Declaration of competing interest

All the authors declare no financial/personal interest that could affect the objectivity of their work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pbb.2025.174039.

Data availability

Data will be made available on request.

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