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# Intermittent voluntary wheel running promotes resilience to the negative consequences of repeated social defeat in mice

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ABSTRACT

A novel approach to reduce the incidence of substance use disorders is to promote resilience to stress using environmental resources such as physical exercise. In the present study we test the hypothesis that Voluntary Wheel Running (VWR) during adolescence blocks the negative consequences of stress induced by intermittent repeated social defeat (IRSD). Four groups of adolescent male C57BL/6 mice were employed in the experiment; two groups were exposed to VWR (1 h, 3 days/week) from postnatal day (PND) 21 until the first social defeat (PND 47), while the remaining two groups did not have access to activity wheels (controls). On PND 47, 50, 53 and 56 mice, who had performed VWR, were exposed to an episode of social defeat by a resident aggressive mouse (VWR+IRSD group) or allowed to explore an empty cage (VWR+EXPL group). The same procedure was performed with control mice that had not undergone VWR (CONTROL+IRSD and CONTROL+EXPL groups). On PND 57, all the mice performed the Elevated Plus Maze (EPM), Hole-Board, Social Interaction, Tail Suspension and Splash tests. After an interval of 3 weeks, all mice underwent a conditioned place preference (CPP) procedure with 1 mg/kg of cocaine. Exposure to VWR prevented the negative consequences of social stress in the EPM, splash test and CPP, since the VWR+IRSD group did not display anxiety- or depression-like effects or the potentiation of cocaine reward observed in the Control+IRSD group. Our results support the idea that physical exercise promotes resilience to stress and represents an excellent target in drug abuse prevention.

## 1. Introduction

Stress is part of our lives and necessary for survival, but chronic stress can induce negative consequences. In this context, it is important to note that each person copes with stress differently and that responses to adversity vary among individuals. While some people develop psychiatric conditions after stressful experiences, such as major depressive disorder or post-traumatic stress disorder, others recover from stress without presenting significant symptoms [1]. The phenomenon of resilience, understood as the ability of subjects to overcome the negative effects of stress, is the focus of the present work.

It is well-known that social stress plays an important role in the incidence of several mental diseases, including the development of addictive behaviors. Numerous reports have demonstrated that exposure to intermittent repeated social defeat (IRSD), an animal model of social stress, results in a significant increase in the rewarding effects of different drugs of abuse, such as MDMA [2], alcohol [3] or cocaine [4–6]. Cocaine use disorder is a pressing problem with limited therapeutic options, and it is of enormous importance to understand the impact of social stress on the development of this disorder and find ways to promote resilience to the negative effects of stress. In a recent study in our laboratory, we observed that mice exposed to IRSD performed differently in a series of behavioral tests compared to mice that had not suffered stress. In particular, exposure to IRSD increased anxiety-like behavior in the elevated plus maze (EPM) and reactivity in the tail suspension test (TST), as well as reducing social interaction and grooming behavior in the splash test - indicative of depressive-like behavior - and increasing the rewarding effects of cocaine in the conditioned place preference paradigm (CPP) [7]. However, in the same study, we also demonstrated that some defeated mice did not go on to develop cocaine CPP. Several behavioral traits were associated with resilience to the effects of IRSD on cocaine reward. First, resilient defeated mice exhibited a behavioral profile characterized by an active coping response (less submission) during episodes of defeat. Additionally, in the short-term period following IRSD, resilient mice showed greater aversion to potential dangers (lower novelty-seeking in the

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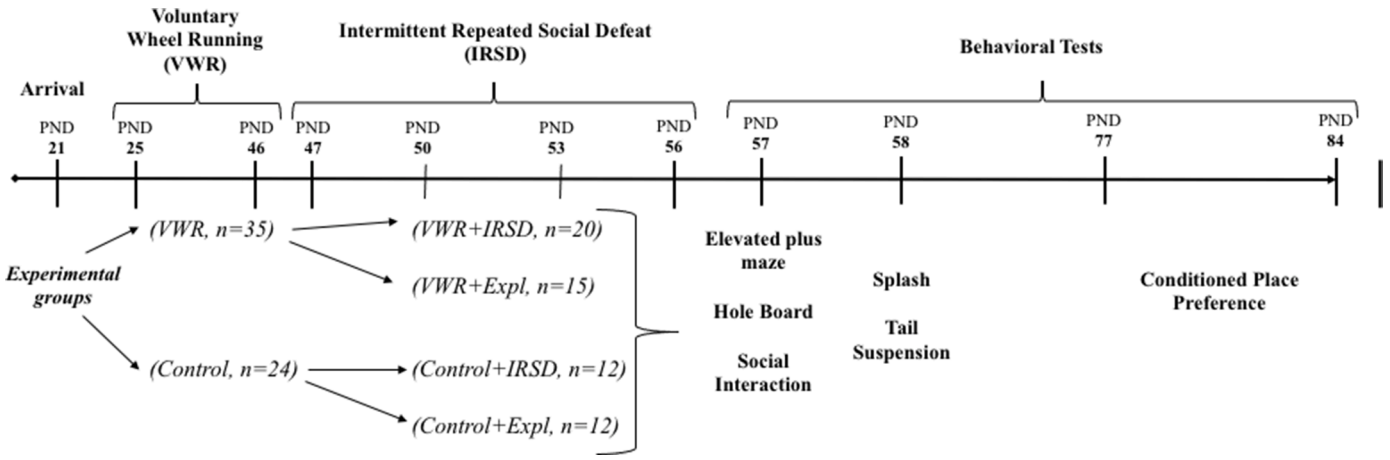


**Fig. 1.** Timeline of experimental protocols and experimental design.

hole-board test), as well as displaying less reactivity in a situation of inevitable moderate stress (e.g. the TST) and fewer depressive-like symptoms after stress (less social avoidance in the social interaction test and a lack of anhedonia in the splash test) [7]. Surprisingly, this subgroup of defeated mice which did not develop CPP was also characterized by a lower percentage of time spent in the open arms of the EPM than both the non-stressed mice and another subgroup of defeated mice that were resilient to the IRSD-induced anxiety [7].

A growing amount of research is currently focused on the development of pharmacological and environmental strategies that promote resilience in order to prevent the development of stress-related mental disorders, including cocaine addiction. Among possible environmental manipulations, physical exercise has been demonstrated to prevent and improve the pathophysiology of illnesses and to promote healthy aging [8]. Voluntary Wheel Running (VWR) is a rodent model that mimics aspects of human physical exercise and can be effective in reducing cocaine CPP [9] and promoting resilience to the effects of chronic SD stress in mice [10,11]. In particular, sedentary mice exposed to stress were shown to develop a depressive-like state, characterized by anhedonia and social avoidance, whereas stressed mice that had engaged in wheel running showed resilience to these effects of stress [10]. Similarly, VWR was found to reverse the impairment of social preference and the deficiency of social interaction induced by exposure to chronic social defeat [11]. In this line, Reguilon and colleagues showed that VWR reverses the increase in ethanol intake induced by IRSD [12].

Thus, the aim of the present study was to test the hypothesis that physical activity promotes the development of resilience to the negative effects of IRSD; namely, the development of anxiety and depression-like behavior and a potentiation of the rewarding effects of cocaine. We endorse VWR as an effective tool to prevent the short- and long- term detrimental effects of social stress.

## 2. Materials and methods

### 2.1. Animals

A total of 59 male of the C57BL/6 strain and 15 male mice of the OF1 strain (Charles River, France) were delivered to our laboratory on postnatal day (PND) 21. All mice (except those used as aggressive opponents) were housed in groups (4–5 mice per cage) in plastic cages (25 × 25 × 14.5 cm). All the mice housed in the same cage underwent the same experimental conditions and the composition of each cage remained stable throughout the whole study. Mice employed as aggressive opponents were individually housed in plastic cages (23 × 13.5 × 13 cm) for a month before the experiments to induce heightened aggression [13]. To reduce the animals' stress levels in response to experimental manipulations, grouped mice were handled for 5 min per day on each of the 3 days prior to initiation of the experimental procedures.

All mice were housed under the following conditions: constant temperature; a reversed light schedule (white lights on 19:30–07:30); and food and water available ad libitum, except during behavioral tests. Mice were fed a standard chow diet (Teklad Global Diet 2014, ENVIGO rms, Spain) containing 14% protein and 4% fat. All mice were provided by shredded paper strips and wooden gnawing sticks in their home cage in order to increase their welfare. Procedures involving mice and their care were conducted according to national, regional and local laws and regulations, which are in compliance with the Directive 2010/63/EU. The protocol was approved by the Ethics Committee of Experimental Research (Experimentation and Animal Welfare) of the University of Valencia (A1549371980205, 2019-VSC-PEA-056).

### 2.2. Drugs

Animals were injected intraperitoneally with 1 mg/kg of cocaine (Alcaliber Laboratory, Madrid, Spain) or physiological saline (NaCl 0.9%) in a volume of 0.01 ml/g of weight. The same physiological saline was also used to dissolve the cocaine. The dose of cocaine administered was selected based on previous studies [5,7,14], which have shown that it induces CPP in mice exposed to IRSD but not in naive mice.

### 2.3. Experimental design

Four groups of mice were used for this study. On PND 25–46, a set of mice ($n = 35$) performed a physical activity protocol (VWR) over a total of 11 h (1 h, 3 days per week: Monday, Wednesday and Friday) while another set of mice (Control) did not engage in physical activity ($n = 24$). On PND 47, 24 h after the last session of VWR, the mice that had engaged in physical activity were assigned to one of two groups: one group was subsequently exposed to four episodes of social defeat (IRSD on PND 47, 50, 53 and 56) in the cage of a resident mice (VWR+IRSD, $n = 20$), while the other group did not undergo stress and explored an empty cage on the same days (VWR+EXPL, $n = 15$). Control mice, which did not undergo the VWR protocol, were also assigned to two groups: one group was exposed to IRSD on PND 47, 50, 53 and 56 (Control+IRSD, $n = 12$), and the other group explored an empty cage on the same days (Control+EXPL, $n = 12$). A lower number of mice were used in these groups because we have previously evaluated and confirmed the effects of IRSD and EXPL (7).

On PND 57–58, all mice underwent a series of behavioral tests: elevated plus maze (EPM), hole-board (HB), social interaction (SI), splash (SH) and tail suspension (TS) tests. On PND 57, the mice

performed first the EPM, followed by the HB and then the SI test, with an interval of 1 h between each test. On PND 58, mice performed the SH test and, after an interval of 1 h, the TS test. The order of tests was based on a previous study carried out in our laboratory (7), according to the degree of stress that the tests had induced in the mice; in this way, we hoped to prevent previous experience in a test from affecting the performance in subsequent tests. As the open arms measurements are very sensitive to environmental conditions and prior manipulation of the animal, we decided to perform the EPM first. And, as the TS is the most stressful test, it was performed last. Afterwards, all mice were housed in the vivarium for 3 weeks, after which they underwent the CPP procedure (PND 77–84) in order to evaluate the long-term effects of the experimental manipulations undergone during adolescence on cocaine reward in the adult mice (see Fig. 1). All experiments took place during the dark period (8.30 h–16.30 h) and in a different environment to that of the confrontation sessions. In order to facilitate adaptation, mice were transported to the dimly illuminated experimental room 1 h prior to testing. During the behavioral tests the experimental room was illuminated with a dim red light (40 lux at 1 m above floor level).

### 2.4. Experimental protocols

#### 2.4.1. Voluntary wheel running (VWR)

Eight low-profile running wheels (Med Associates Inc.) were employed in the experiments. Each wheel, made entirely of plastic (10.25 × 15.5 × 13.7 cm), rotates on a central axis in a horizontal plane, allowing physical activity through spontaneous locomotion. All animals in the running condition (VWR+IRSD and VWR+EXPL groups) were distributed in batches of eight and allowed to run individually on the wheel (which was located in a plastic cage different to the home cage) for 1 h, three times per week (Monday, Wednesday and Friday). Control animals (Control+IRSD and Control+EXPL groups) were placed in the same plastic cages (different from their own) without any running wheel. A total of 11 VWR sessions took place (see Fig. 1). The procedure and duration of VWR was based on the work of Reguilon and colleagues, who also studied the effects of IRSD on vulnerability to drugs of abuse, in particular to alcohol [12]. The cohort of 35 VWR mice were exposed to the wheels during 5 rounds between 9.30 h and 15.00 h. The mice included in each round varied every day according to a standardized schedule (for example, mice in the first round on the first day were exposed to the wheels in the second round on the second day, and so on, successively).

#### 2.4.2. Intermittent repeated social defeat (IRSD)

The IRSD procedure consisted of four encounters, separated by intervals of 72 h (PND 47, 50, 53 and 56), between an experimental C57BL/6 mouse (intruder) and an isolated OF1 mouse (resident), which culminated in the defeat of the experimental animal (IRSD groups). Each encounter lasted for 25 min and consisted of three phases, which began by introducing the experimental animal into the home cage of the aggressive opponent and leaving it there for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall, which allowed social interaction and threats from the aggressive resident male. The wire mesh was then removed from the cage and confrontation between the two mice initiated and was allowed to last for 5 min. In the third phase, the wire mesh was returned to the cage to separate the two animals once again for another 10 min to allow for social threats by the resident. Intruder mice were exposed to a different aggressor mouse during each episode of social defeat. 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 [15–17]. All the experimental mice displayed defeat and submission postures, given that they all faced resident mice with high levels of aggression. Previous studies in our laboratory have demonstrated that this protocol of IRSD is effective in increasing corticosterone levels and inducing behavioral alterations related with stress [2,7,18]. The non-stressed mice underwent the same protocol but without the presence of a ‘‘resident’’ mouse in the cage; in other words, they simply explored the cage (EXPL groups) (see Fig. 1).

#### 2.4.3. Elevated plus maze (EPM)

The effects of IRSD on anxiety were evaluated on PND 57 using the EPM paradigm. This test is based on the natural aversion of mice to open elevated areas, as well as on the natural spontaneous exploratory behavior they exhibit in novel environments; in this way, it measures the extent to which rodents avoid high open spaces. The apparatus consisted of two open arms (30 × 5 cm) and two enclosed arms (30 × 5 cm), and the junction of the four arms formed a central platform (5 × 5 cm). The floor of the maze was made of white Plexiglas and the walls of the enclosed arms were made of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide the animals with additional grip. The entire apparatus was elevated 45 cm above floor level. The total time spent in the open and closed arms, the number of entries into the open and closed arms, and the percentage of time and entries into the open arms are commonly considered indicators of open space-induced anxiety in mice. Thus, anxiety levels are considered to be lower when the measurements in the open arms are higher and those in the closed arms are lower, and vice versa [19,20]. The total number of entries into the arms is a score of locomotor activity scores [21,22].

At the beginning of each trial, subjects were placed on the central platform facing an open arm and allowed to explore for 5 min. The maze was cleaned with a 7% alcohol swab after each test and left to dry completely. The behavior of the mice was video recorded and automatically sent to a computer using the Ethovision 2.0 software package (Noldus, Wageningen, The Netherlands). An arm was considered to have been visited when the animal placed all four paws on it. The following measures were taken into account for the statistical analyses: the latency to first enter the open arms; the time and percentage of time [(open / open + closed) × 100] spent in the open arms; and the number and percentage of open arm entries and total number of entries into the arms.

#### 2.4.4. Hole board test

The mice’s novelty-seeking was evaluated in the hole board test 24 h after the last defeat or exploration (PND 57). This test was carried out in a square box (28 × 28 × 20.5 cm) with transparent Plexiglas walls and 16 equidistant holes with a diameter of 3 cm in the floor (CIBERTECSA, Madrid, Spain). Photocells below the surface of the holes detected the number of times a mouse performed a head-dip. At the beginning of the test, mice were placed in the same corner of the box and were allowed to freely explore the apparatus for 10 min. The latency to the first dip and the frequency of dips were automatically recorded by the apparatus.

#### 2.4.5. Social interaction test

Twenty-four hours after the last defeat or exploration (PND 57), the social behavior of the mice was evaluated in an open field (37 × 37 × 30 cm). A perforated plexiglass cage (10 × 6.5 × 30 cm) was placed in the middle of one wall of the open field. After habituation to the room, each animal was placed in the center of the open field and was allowed to explore it twice, under two different experimental conditions.

The first time (object phase), the perforated plexiglass cage was empty. After 10 min of exploration, the experimental mouse was returned to its home cage for 2 min. Next, a mouse of the OF1 strain was placed inside the perforated cage (which safeguarded the experimental mouse from attack) and the experimental mouse was reintroduced into the open field for 10 min (social phase). The OF1 mouse was unfamiliar to the experimental mouse (i.e., it was different from the one used in the IRSD episodes). In both phases, the time spent in an 8 cm wide corridor surrounding the perforated cage—the interaction zone—was automatically registered using the Ethovision 2.0 software package (Noldus, Wageningen, The Netherlands). As in Henriques-Alves and Queiroz

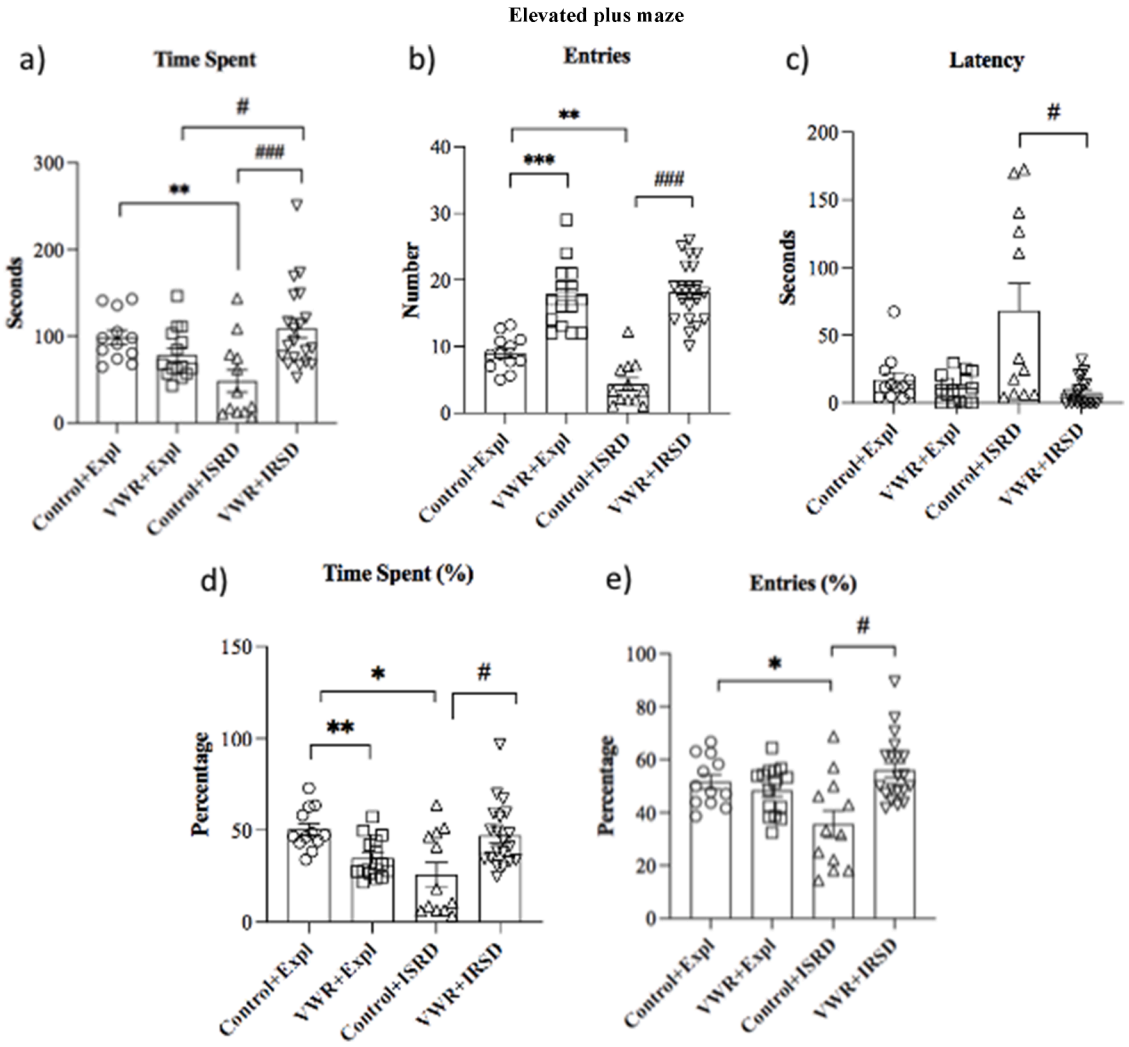


**Fig. 2.** Effects of voluntary wheel running (VWR) and intermittent repeated social defeat (IRSD) in the elevated plus maze. Naive control mice explored an empty cage (Control+EXPL, $n = 12$) or were exposed to IRSD (Control+IRSD, $n = 12$) during late adolescence (PND 47, 50, 53 and 56). Similarly, mice with access to the Voluntary Wheel Running (from PND 25 to 46) explored an empty cage (VWR+EXPL, $n = 15$) or were subjected to IRSD (VWR+IRSD, $n = 20$) in late adolescence (PND 47, 50, 53 and 56). The animals' behavior in the maze was evaluated on PND 57. (a) Time spent in the Open Arms (OA). Bars represent the mean (±SD) time spent by each group in the OA of the maze. $^{**}p < 0.01$, significant difference with respect to the Control+Expl group; $\#p < 0.05$, $\#\#\#p < 0.001$, significant difference with respect to the VWR+IRSD group; (b) Number of entries into the OA. Bars represent the mean (±SD) number of entries into the OA for each group. $^{**}p < 0.01$, $^{***}p < 0.001$, significant difference with respect to the Control+Expl group; $\#\#\#p < 0.001$, significant difference with respect to the VWR+IRSD group; (c) Latency to enter the OA. Bars represent the mean (±SD) latency to enter the OA for each group. $\#p < 0.05$, significant difference with respect to the VWR+ISRD group; (d) Percentage of time spent in the OA. Bars represent the mean (±SD) percentage of time spent by each group in the OA.* $p < 0.05$, $^{**}p < 0.01$, significant difference with respect to the Control+Expl group; $\#p < 0.05$, significant difference with respect to the VWR+IRSD group; (d) Percentage of entries into the OA. Bars represent the mean (±SD) percentage of entries into the OA for each group. $^{*}p < 0.05$, significant difference with respect to the Control+Expl group; $\#p < 0.05$, significant difference with respect to the VWR+ISRD group.

[23], an index of social interaction (ISI) was obtained [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). The ISI is commonly used as the social preference-avoidance index [24].

### 2.4.6. Splash test

The splash test consisted of spraying a 10% sucrose solution (to stimulate grooming behavior) on the dorsal coat of a mouse that had been placed in a transparent cage (15 × 30 × 20 cm) containing bedding. The behavior of the mice was videotaped for 5 min and later analyzed by an observer who was blind to the treatment received by the animal using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The time spent engaged in grooming and the frequency of this behavior were recorded forty-eight hours after the last defeat or exploration (PND 58). A decrease in the time and/or frequency of grooming is interpreted as depressive-like behavior [25].

### 2.4.7. Tail Suspension test (TST)

The tail suspension test (TST) measures the behavioral variable of immobility, which is considered to represent despair [26]. It is based on the observation that rodents, after initial escape-oriented movements, adopt an immobile posture when placed in an inescapable, stressful situation. In the case of the TST, the stressful situation involves the hemodynamic stress of being hung by their tail so that they are immobile [27]. This is used as a measure of behavioral depression because, when antidepressant treatments are administered prior to the test, subjects engage in escape-directed behaviors for longer periods of time than after treatment with a vehicle [26].

Forty-eight hours after the last defeat or exploration (PND 58), we

investigated whether our procedure of social defeat modified the length of time spent in immobile positions in the TST. Following the protocol described by Vaugeois and colleagues, mice were suspended by the tail (using adhesive tape) from a hook during a 6 min test period [28]. The behavior displayed by the mice was video recorded and later analyzed by an observer who was blind to the treatment received by the animal using a computerized method (Raton Time 1.0 software; Fixma SL, Valencia, Spain). The parameter considered for the statistical analyses was the total time spent immobile.

#### 2.4.8. Conditioned Place preference (CPP) paradigm

Three weeks after the last episode of social defeat, the animals underwent the CPP procedure. For place conditioning, we employed eight identical Plexiglas boxes with two equal-sized compartments (30.7 cm long × 31.5 cm wide × 34.5 cm high) separated by a gray central area (13.8 cm long × 31.5 cm wide × 34.5 cm high). The compartments had different colored walls (black vs. white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animals and their crossings from one compartment to the other. The equipment was controlled by three IBM PC computers using MONPRE 2Z software (Cibertec SA, Madrid, Spain).

CPP consisted of three phases and took place during the dark cycle following an unbiased procedure in terms of initial spontaneous preference (for detailed explanations of the procedure, see [29]). In brief, during pre-conditioning (Pre-C), the time spent by the animal in each compartment during a 15 min period was recorded on 3 consecutive days. Animals showing a strong unconditioned aversion (less than 250 s) or a preference (more than 650 s) for a particular compartment in the last pre-conditioning session were excluded from the study ($n = 3$). In the second phase (conditioning), which lasted 4 days, experimental animals received saline before being confined to the vehicle-paired compartment for 30 min. Subsequently, after an interval of 4 h, they were injected with 1 mg/kg of cocaine immediately before being confined to the drug-paired compartment for 30 min. During the third phase, or post-conditioning (Post-C), the time spent by the untreated mice in each compartment was recorded during a 15 min period.

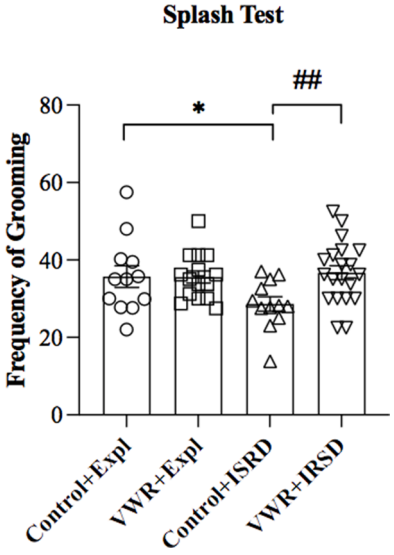


**Fig. 3.** Effects of voluntary wheel running (VWR) and intermittent repeated social defeat (IRSD) on the splash test. Naive control mice explored an empty cage (Control+EXPL, $n = 12$) or were exposed to IRSD (Control+IRSD, $n = 12$) in late adolescence (PND 47, 50, 53 and 56). Similarly, mice with access to the Voluntary Wheel Running (from PND 25 to 46) explored an empty cage (VWR+EXPL, $n = 15$) or were exposed to IRSD (VWR+IRSD, $n = 20$) in late adolescence (PND 47, 50, 53 and 56). The animals' behavior in the maze was evaluated on PND 58. Bars represent the mean (±SD) frequency of grooming in each group. $^{*}p < 0.05$, significant difference with respect to the Control+Expl group; $\#\#p < 0.01$, significant difference with respect to the VWR+IRSD group.

### 2.5. Statistical analysis

The data of the behavioral tests were first analyzed by means of a Levene test to check the variance of data. Levene's tests confirmed homogeneous variance among the data for: time spent in [$F(3,55) = 1.913$; $p > 0.05$] and entries into [$F(3,55) = 1.730$; $p > 0.05$] the open arms of the EPM; number of dips in the hole board test [$F(3,55) = 1.533$; $p > 0.05$]; frequency of grooming in the splash test [$F(3,55) = 0.895$; $p > 0.05$]; and length of time spent immobile in the TST [$F(3,55) = 0.294$; $p > 0.05$]. Conversely, they revealed unequal variance among the data for: percentage of time spent in [$F(3,55) = 5.260$; $p < 0.05$] and percentage of entries into [$F(3,55) = 2.915$; $p < 0.05$] the open arms of EPM; total entries into the arms [$F(3,55) = 5.506$; $p < 0.05$] of the EPM; and ISI in the social interaction test [$F(3,55) = 3.293$; $p < 0.05$]. The behavioral effects of VWR and IRSD on time spent in/entries into the open arms of the EPM, number of dips in the hole board test, frequency of grooming in the splash test and length of time immobile in the TST (data sets with homogeneous variance) were evaluated using a two-way ANOVA with two between-subjects variables; Voluntary Wheel Running, with two levels (Control and VWR), and Defeat, with two levels (Expl and IRSD). Post hoc comparisons were performed with Bonferroni tests, which allow multiple hypotheses to be tested simultaneously, thus limiting the type I error rate without increasing the probability of a type II error occurring. The behavioral effects of VWR and IRSD on percentage of time spent in and percentage of entries into the open arms of EPM, total entries into the arms of the EPM, and ISI in the social interaction test (data sets with unequal variance) were analyzed by means of an ANOVA with one variable - Group (with four levels: Control+Expl, VWR+Expl, Control+IRSD and VWR+IRSD) - and post-hoc comparisons with the Games-Howell Test. The effects of VWR and IRSD on the CPP paradigm (data set with homogeneous variance) were evaluated using a three-way ANOVA with the between-subjects variables Voluntary Wheel Running and Defeat (described above) and a within-subjects variable; Days, with two levels (Pre-C and Post-C). Post hoc comparisons were performed with Bonferroni tests. All statistical analyses were carried out with the SPSS program.

## 3. Results

### 3.1. Physical activity prevented the anxiogenic effects of IRSD in the EPM

The ANOVA of the time spent in the open arms of the EPM revealed that the Interaction of the variables Stress X Running was significant [$F(1,55) = 14.405$; $p < 0.001$] (Fig. 2a). Post-hoc comparisons showed that control mice exposed to defeat (Control+IRSD) spent less time in the open arms of the EPM than mice in the Control+Expl ($p < 0.01$) and VWR+IRSD ($p < 0.001$) groups. In addition, mice in the VWR+IRSD group spent more time in the open arms than those in the VWR+Expl group ($p < 0.05$).

The ANOVA of the number of entries into the open arms of the EPM revealed that the variable Running [$F(1,55) = 104.223$; $p < 0.001$] and the Interaction of the variables Stress X Running [$F(1,55) = 6.182$; $p < 0.05$] were significant (Fig. 2b). Mice engaging in physical activity performed more entries into the open arms of the EPM than control mice ($p < 0.001$). Post-hoc comparisons of the Interaction showed that control mice exposed to defeat (Control+IRSD) performed less entries into the open arms than those in the Control+Expl ($p < 0.01$) and VWR+IRSD ($p < 0.001$) groups. In addition, mice in the VWR+Expl group performed more entries into the open arms than mice in the Control+Expl group ($p < 0.001$).

The ANOVA of the latency to enter the open arms of the EPM was

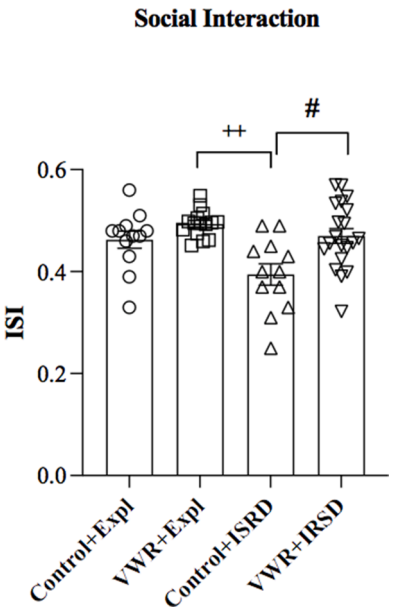


**Fig. 4.** Effects of Voluntary Wheel Running (VWR) and Intermittent Repeated Social Defeat (IRSD) on the Social Interaction Test. Naive control mice explored an empty cage (Control+EXPL, $n = 12$) or were exposed to IRSD (Control+IRSD, $n = 12$) in late adolescence (PND 47, 50, 53 and 56). Similarly, mice engaging in Voluntary Wheel Running (from PND 25 to 46) explored an empty cage (VWR+EXPL, $n = 15$) or were exposed to IRSD (VWR+IRSD, $n = 20$) in late adolescence (PND 47, 50, 53 and 56). The animals' behavior in the maze was evaluated on PND 57. Bars represent the mean (±SD) index of social interaction (ISI) in each group. ++ $p < 0.01$, significant difference with respect to the VWR+EXPL group; #$p < 0.05$, significant difference with respect to the VWR+IRSD group.

significant [F(3,55) = 9.796; $p < 0.001$] (Fig. 2c). Post-hoc comparison showed that the VWR+IRSD group displayed a lower latency to enter the open arms than the Control+IRSD group ($p < 0.05$).

The ANOVA of data of the percentage of time spent in the open arms of the EPM was significant [F(3,55) = 6.509; $p < 0.001$] (Fig. 2d). Post-hoc comparison showed that the groups VWR+Expl and Control+IRSD spent a lower percentage of time in the open arms of the EPM than mice in the Control+Expl ($p < 0.01$ and $p < 0.05$, respectively); in addition, the Control+IRSD group spent a lower percentage of time in the open arms than mice in the VWR+IRSD group ($p < 0.05$).

The ANOVA of the percentage of entries into the open arms of the EPM was significant [F(3,55) = 7.013; $p < 0.001$] (Fig. 2e). Post-hoc comparison highlighted a lower percentage of entries into the open arms by mice in the Control+IRSD group than by mice in the Control+Expl ($p < 0.05$) or VWR+IRSD ($p < 0.05$) groups.

The ANOVA of the total entries into the arms of the EPM was significant [F(3,55) = 64.988; $p < 0.001$] (data not shown). Post-hoc comparison showed that the Control+IRSD group performed a lower total number of entries into the arms than the rest of the groups (ps < 0.001); in addition, the VWR+IRSD and VWR+Expl groups performed a higher number of total entries into the arms than the Control+Expl group (ps < 0.001).

### *3.2. Physical activity prevented the depression-like effects of IRSD in the splash test*

The ANOVA of the frequency of grooming revealed that the Interaction of the variables Stress X Running was significant [F(1,55) = 4; $p < 0.05$] (Fig. 3). Post-hoc comparison showed a lower frequency of grooming in the Control+IRSD group than in the Control+Expl ($p < 0.05$) and VWR+IRSD ($p < 0.01$) groups.

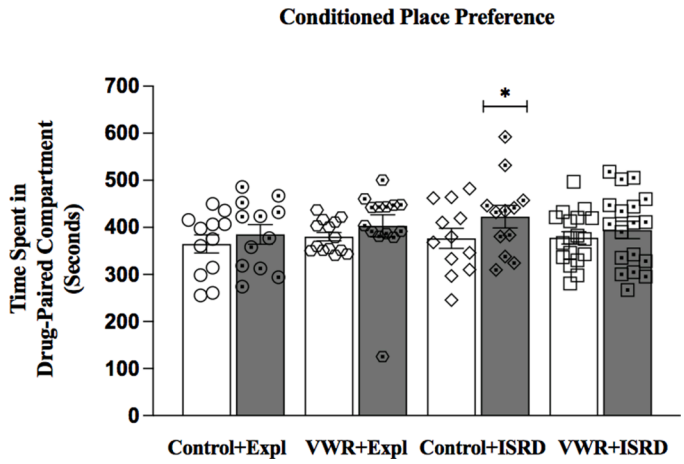


**Fig. 5.** Effects of voluntary wheel running (VWR) and intermittent repeated social defeat (IRSD) on the conditioned place preference (CPP) paradigm. Naïve control mice explored an empty cage (Control+EXPL, $n = 12$) or were exposed to IRSD (Control+IRSD, $n = 12$) in late adolescence (PND 47, 50, 53 and 56). Similarly, mice with access to the Voluntary Wheel Running (from PND 25 to 46) explored an empty cage (VWR+EXPL, $n = 15$) or were exposed to IRSD (VWR+IRSD, $n = 20$) in late adolescence (PND 47, 50, 53 and 56). After behavioral tests on PND 57–58 and an interval of 3 weeks, mice were conditioned with cocaine (1 mg/kg). Bars represent the mean (±SEM) time (in seconds) spent in the drug-paired compartment in the pre-conditioning (Pre-C, black bars) and post-conditioning (Post-C, gray bars) tests. *$p < 0.05$, significant difference in the time spent in the drug-paired compartment in Post-C vs. Pre-C test.

### *3.3. Physical activity prevented the social interaction deficit induced by IRSD*

The ANOVA of the ISI data was significant [F(3,55) = 7.057; $p < 0.001$] (Fig. 4). Post-hoc comparison showed that the group Control+IRSD had a lower ISI than the VWR+Expl ($p < 0.01$) and VWR+IRSD ($p < 0.05$) groups.

### *3.4. Physical activity did not modify the effects of IRSD in the hole-board and tail suspension test*

The ANOVA of the number of dips revealed Stress to be the only significant variable [F(1,55) = 5.211; $p < 0.05$] (data not shown). Mice exposed to defeat showed a lower number of head dips than mice that were not exposed to stress ($p < 0.05$).

The ANOVA of the time spent in immobile in the tail suspension test revealed that only the variable Stress was significant [F(1,55) = 10.045; $p < 0.01$] (data not shown). Mice exposed to defeat spent less time immobile than those not exposed to stress ($p < 0.01$).

### *3.5. Physical activity prevented the potentiation of cocaine CPP induced by IRSD*

The ANOVA of the time spent in the drug-paired compartment revealed that only the variable Days was significant [F(1,52) = 6.519; $p < 0.05$] (Fig. 5). Although the Interaction Days X Stress X Running was not significant, post-hoc comparisons showed that mice in the Control+IRSD group spent more time in the drug-paired compartment in Post-C than in Pre-C ($p < 0.05$). Additional Student t tests comparing the time spent by each group in the drug-paired compartment in Pre-C vs Post-C confirmed a significant difference only in the Control+IRSD group ($p < 0.05$).

## 4. Discussion

The present study reveals that physical activity during adolescence prevents some of the short-term effects of subsequent exposure to social stress in late adolescent mice, such as the induction of anxiogenic- and depressive-like effects in the EPM and the splash test, respectively, and the development of social avoidance in the social interaction test. In addition, we have seen how VWR during adolescence also prevents the long-term effects of IRSD on drug reward; in particular, the potentiation of cocaine-induced CPP in adult mice. Thus, our results indicate that physical activity during adolescence can enhance resilience to the short- and long-term negative consequences of subsequent social stress.

Influence of VWR on the short-term behavioral effects of IRSD

Exposure to IRSD increased anxiety in the EPM, which is in line with previous studies carried out in our laboratory [7,30]. Similarly, other research using the chronic SD stress (CSDS) paradigm has demonstrated that male mice exposed to defeat on 5–10 consecutive days display anxiety-like symptoms [31–39]. Regarding the influence of VWR on the anxiogenic effects of IRSD, we observed that physical activity during adolescence prevented the reduction in open arm measurements induced by social stress. Interestingly, this effect of VWR on defeated mice was observed despite the fact that mice exposed only to VWR (VWR+EXPL group) spent a higher percentage of time in the open arms and more time in the closed arms with respect to the Control+Expl group. These results suggest that physical activity, while inoculating against the anxiogenic effects of a subsequent stressor, can itself slightly increase anxiety. Although we have not found any study in the literature concerning changes in corticosterone after limited VWR, there are reports that continuous exposure to VWR for 4–5 weeks increased corticosterone levels in comparison to control mice that did not run and induced changes in the hypothalamic-pituitary-adrenal system [40,41]. The fact that VWR also increased the number of entries into the open arms appears to be related with an increase of general activity, since both the groups engaging in VWR performed a higher number of total entries. Only one previous study has evaluated the influence of physical activity on the anxiety-like effects of social defeat [42]. As in the present work, the authors reported that Syrian hamsters engaging in physical activity 21 days before defeat (three 5 min defeat sessions) exhibited less risk assessment behavior in the EPM than defeated hamsters (controls, without running), which was indicative of lower anxiety. However, no significant differences were observed between the two groups in the time or percentage of time spent in the open arms of the EPM. In addition, socially defeated hamsters exposed to physical activity showed less defensive/submissive behaviors than control/defeated hamsters when confronted by a resident aggressor [42]. In the same line, another study demonstrated that engaging in treadmill activity for two weeks after defeat (30 min daily for 7 days) reversed anxiety-like behavior (in the EPM, light-dark and open field tests) induced by social defeat in rats [43]. All these results, together with those of the present study, suggest that VWR promotes resilience to the anxiogenic effects of social defeat stress.

The social interaction deficit is probably the most studied behavioral consequence of CSDS exposure and has been used to model the social avoidance that characterizes depression in human beings [38,44,45]. In accordance with this, we observed that exposure to IRSD induced such a deficit, similarly to previous studies carried out in our laboratory [7,46]. We also found that exposure to VWR was effective in preventing social avoidance induced by IRSD. Although no previous studies have evaluated the effects of physical activity on the social interaction deficit induced by IRSD, our results are in line with those observed in mice exposed to other procedures of defeat, in which VWR prevented the social avoidance induced by 5 [47] or 10 consecutive days of defeat (CSDS) [10,11,35,48].

In the present research, exposure to IRSD also induced depression-like symptoms in the splash test; in particular, an increase in the latency to perform grooming behavior, in line with previous results obtained by our group showing that defeated mice exhibit a decrease in the frequency of grooming [7] or an increase in the latency to perform this behavior [46]. We also observed that exposure to physical activity prevented the effects of IRSD in the splash test. Similar results were reported by Mul and colleagues, who found that mice engaging in VWR for 21 days before CSDS did not develop the reduction in sucrose preference observed in defeated mice [10]. Similarly, using a 7-day protocol of vicarious SD, Kochi and colleagues reported that rats with trauma witness spent more time immobile in the forced swim test than non-defeated rats, an effect indicative of depression that was absent in rats given access to the VWR for 14 days before vicarious SD [49]. Conversely, we have not observed a preventive effect of VWR on the changes induced by defeat in the tail suspension test. However, it must be taken into account that, in our model, exposure to IRSD did not increase immobility in said test, as would have been expected if such an effect were indicative of depression-like behavior. In contrast, as in a previous study carried out in our laboratory, IRSD decreased the time spent immobile in the tail suspension test, an effect that we interpret as representing a higher level of reactivity of defeated mice to a subsequent stressful situation [7]. In the same way, VWR did not prevent the effects of IRSD on the hole-board test, since a reduction of novelty-seeking was observed in both groups of defeated mice, irrespective of whether or not they had engaged in physical activity. These results are in accordance with those of a recent study in our laboratory, which demonstrated that exposure to a brief period of maternal separation – which has been shown to prevent other effects of IRSD - did not modify the reduction in novelty-seeking induced by exposure to defeat [46].

Influence of VWR on the long-term effects of IRSD on cocaine reward

As expected, adult mice exposed to IRSD during late adolescence displayed enhanced sensitivity to the rewarding effects of cocaine in the CPP paradigm, since they spent more time in the compartment paired with a low dose of cocaine (1 mg/kg) - which was ineffective in inducing CPP in non-stressed mice - in the Post-C vs. Pre-C test. This result confirms our previous observations that exposure to IRSD potentiates the rewarding effects of different drugs of abuse, including cocaine [5,7,14,18,46,50], MDMA [2] and alcohol [51]. Similar results have been observed with animals exposed to the IRSD or CSDS paradigms, in which defeated rats and mice have been shown to develop enhanced sensitivity to different drugs of abuse [52,53].

The VWR procedure employed in our study induced resilience to the potentiating effects of IRSD on cocaine reward, since mice that performed voluntary physical activity before exposure to IRSD did not develop CPP in adulthood. No previous studies have evaluated the influence of VWR during adolescence on the long-term effects of social stress. However, in line with the present results, it has been reported that mice exposed to VWR during and after IRSD are protected against the increase in ethanol consumption induced by IRSD [12]. In addition, in the absence of stress, 1 or 4 weeks of VWR after cocaine conditioning was shown to abolish CPP in mice [9,54], while 6 weeks of VWR lowered breaking points in the cocaine self-administration paradigm [55], and post-extinction VWR attenuated priming-induced reinstatement of cocaine self-administration [56]. However, in other studies, 3 weeks of VWR before cocaine conditioning did not modify CPP in mice [57], while VWR did not prevent stress-induced reinstatement of cocaine seeking [56]. Considered as a whole, these results suggest that running acts as an alternative reinforcer to cocaine; in fact, it has been well demonstrated that physical activity is a motivating and rewarding behavior for rodents [58,59]. A potential enriching component of VWR has also been hypothesized in order to explain the inhibition of cocaine CPP in mice exposed to physical activity [9]. As in the study of Lespine and Tirelli [57], mice in the present study performed VWR during adolescence and underwent cocaine CPP in adulthood, but there is an important difference between the two studies; namely, the exposure to social stress to which our animals were submitted. Our results indicate that, though it did not modify the rewarding effects of cocaine, physical activity exerted a preventive effect on the negative long-term

consequences of social stress for cocaine reward.

One limitation of our study is the use of a single dose of cocaine. However, it must be taken into account that one of the aims was to test whether exposure to VWR can prevent the potentiation of cocaine reward induced by IRSD. In order to observe this potentiation, we needed to use a subthreshold dose of cocaine that was ineffective in inducing CPP in non-stressed mice. If we had used a moderate-to-high dose, it is probable that all the groups would have acquired cocaine CPP due to the strong rewarding effects of this drug. In such a case, we would probably have observed differences between groups regarding the maintenance or reinstatement of CPP. In this regard, we have previously seen that exposure to IRSD prolonged drug-induced CPP and enhanced the vulnerability of mice to priming-induced reinstatement [2,60,61]. We would also expect VWR to prevent these effects of IRSD. This hypothesis should be tested in future studies.

Potential mechanisms underlying the protective effects of VWR on social stress

In the present study we have shown that VWR during adolescence prevents anxiety- and depression-like behaviors (including social avoidance), as well as undermining the potentiation of cocaine reward induced by exposure to IRSD. Regarding the mechanisms underlying the protective influence of VWR on the negative consequences of defeat stress, we hypothesize the involvement of different neurotransmitter systems, neuroplasticity, and neuroinflammatory and epigenetic processes. Physical activity induces changes in different markers of neurotransmitter systems in reward-related brain areas that are also affected by stress and drugs of abuse, such as the dopaminergic and glutamatergic systems [62–64]. For example, VWR reverses the decrease in levels of tyrosine hydroxylase in the ventral tegmental area and of DA D2 receptors in the nucleus accumbens shell induced by CSDS [11]. VWR increases the production of new neurons [54,65], activates BDNF/TRKB signaling in the hippocampus [36,66,67], and induces ΔFosB and altered dendritic morphology in the nucleus accumbens [10], and reverses the neuroinflammatory response induced by IRSD [12]. In addition, exposure to physical activity after CSDS counteracts the increase in oxidative stress through epigenetic mechanisms, including acetylation of histone H3 and modulation of methyl-CpG-binding in the hippocampus [43]. In the same line, when lactate (a metabolite produced during physical activity) is administered before each episode of CSDS, it has been shown to promote resilience to stress by restoring the activity of hippocampal H1 deacetylase (HDAC2/3) [68]. It is important to take into account that most of these studies have employed continuous access to a running wheel, while the limited and intermittent VWR applied in the present study can induce different neurobiological adaptations that underly its protective influence on the negative consequences of defeat stress.

## 5. Conclusions

CSDS (defeat on several consecutive days) is the most frequently used animal model to study the negative consequences of social stress, such as anxiety- and depression-like symptoms [38,44] and enhanced sensitivity to drugs of abuse [53]. In the same way that most humans exposed to stress do not develop mental disorders, chronically defeated rodents respond to CSDS differently; some develop anxiety-, depression- and addiction-like symptoms, whereas others remain resilient to stress [38,50]. In our laboratory, we have used a different protocol of defeat in mice consisting of intermittent exposure to an episode of defeat that is repeated four times, every 72 h. With this protocol, we have seen how mice exposed to IRSD exhibit an increase in anxiety- and depression-like behavior, an alteration of social interaction, and an impairment of learning [7,30], as well as enhanced sensitivity to drugs of abuse [2,7,14,51]. In addition, we have observed that some mice are vulnerable, while others are resilient to the effects of IRSD stress [7], and that a brief stressful event during childhood (i.e., an acute 6 h episode of maternal separation) can induce resilience to some effects of subsequent IRSD exposure, including depression-like behavior in late adolescence and potentiation of cocaine reward in adulthood [46]. In the present study we have demonstrated that exposure to VWR, a preclinical model of human voluntary exercise [58], also enhances the resilience of mice and protects them against the anxiogenic effects, social interaction deficit, depression-like behavior and potentiation of the rewarding effects of cocaine observed in mice under social stress. A comparison of the behavioral profile of the VWR+IRSD mice in the present study and that of the resilient mice in our previous study is provided in Table 1. Our results endorse the idea that physical activity can prevent the development of stress-related disorders, both in animals and in humans. Nevertheless, it is necessary to carry out more research to determine other protective factors that promote the development of resilience to stress during childhood or early adolescence.

**Table 1**
Behavioral profile of VWR+IRSD and resilient mice. = mice showed similar values to mice without exposure to social defeat. ↓ mice showed lower values than mice without exposure to social defeat.

| | VWR+IRSD mice | Resilient defeated mice |
|---|---|---|
| Time in open arms (EPM) | = | ↓ |
| Dips (Hole-board) | ↓ | ↓ |
| ISI (Social interaction test) | = | = |
| Grooming (Splash test) | = | = |
| Immobility (TST) | ↓ | = |
| Cocaine reward (CPP) | = | = |

In summary, our findings suggest that physical activity during adolescence is an excellent tool to improve resilience to the negative effects of subsequent social stress on an individual's vulnerability to mental and addictive disorders later in life. Future studies should be conducted to disentangle the mechanisms underlying VWR-promoted stress prevention. In addition, it is important to include females in studies of resilience to social stress, as their response to VWR or other potentially preventive environmental manipulations may differ from that observed in males. Such research could contribute to developing new approaches aimed at avoiding mental stress-related disorders and new therapeutic strategies for treating people at risk of developing a drug use disorder following stressful experiences.

## Author contributions

MAA and MPG-P contributed conception and design of the study; CC-L and MAM-C performed the experiments, organized the databases and performed the statistical analyses; CC-L and MAM-C wrote some sections of the manuscript, CC-L and MPG-P wrote the complete first draft of the manuscript and MAA wrote the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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## Declaration of Competing Interest

The authors declare no conflict of interest.

## Data availability

Data will be made available on request.

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