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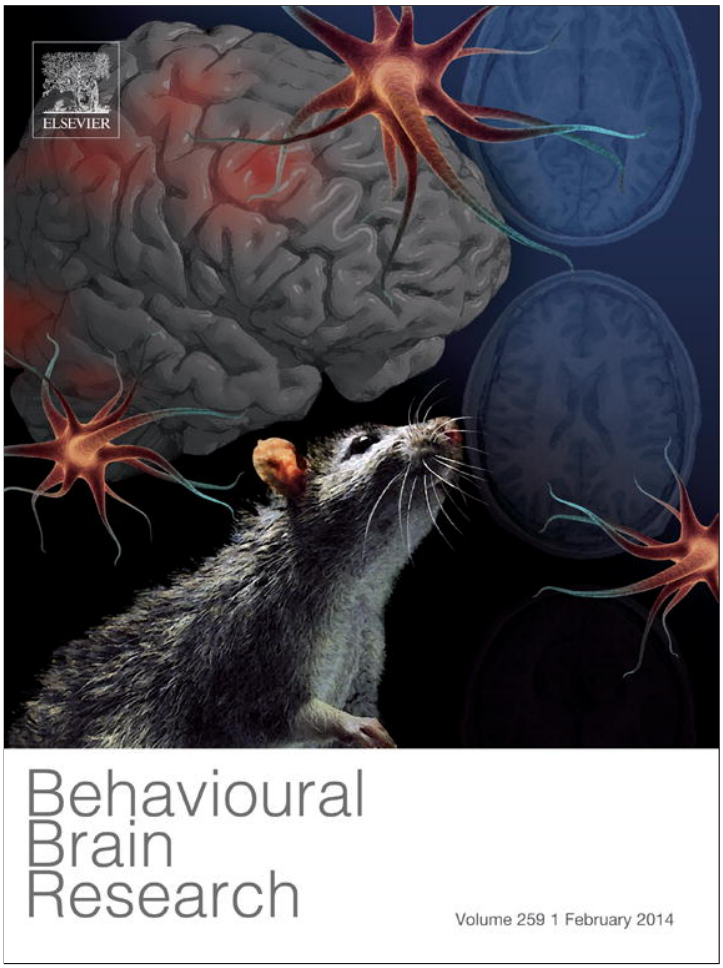


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Research report

# Effects of chronic stress in adolescence on learned fear, anxiety, and synaptic transmission in the rat prelimbic cortex

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## HIGHLIGHTS

- Chronic stress in adolescence decreases excitatory synaptic transmission in the prelimbic cortex.
- Chronic stress in adolescence slows the extinction of learned fear and enhances anxiety-like behavior.
- Stress-induced alterations of the prelimbic cortex and learned fear were reversed in adulthood.
- The anxiogenic effect of chronic stress in adolescence was still present in adulthood.

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## ABSTRACT

The prelimbic cortex and amygdala regulate the extinction of conditioned fear and anxiety, respectively. In adult rats, chronic stress affects the dendritic morphology of these brain areas, slowing extinction of learned fear and enhancing anxiety. The aim of this study was to determine whether rats subjected to chronic stress in adolescence show changes in learned fear, anxiety, and synaptic transmission in the prelimbic cortex during adulthood. Male *Sprague Dawley* rats were subjected to seven days of restraint stress on postnatal day forty-two (PND 42, adolescence). Afterward, the fear-conditioning paradigm was used to study conditioned fear extinction. Anxiety-like behavior was measured one day (PND 50) and twenty-one days (PND 70, adulthood) after stress using the elevated-plus maze and dark-light box tests, respectively. With another set of rats, excitatory synaptic transmission was analyzed with slices of the prelimbic cortex. Rats that had been stressed during adolescence and adulthood had higher anxiety-like behavior levels than did controls, while stress-induced slowing of learned fear extinction in adolescence was reversed during adulthood. As well, the field excitatory postsynaptic potentials of stressed adolescent rats had significantly lower amplitudes than those of controls, although the amplitudes were higher in adulthood. Our results demonstrate that short-term stress in adolescence induces strong effects on excitatory synaptic transmission in the prelimbic cortex and extinction of learned fear, where the effect of stress on anxiety is more persistent than on the extinction of learned fear. These data contribute to the understanding of stress neurobiology.

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## 1. Introduction

The Austro-Hungarian researcher Hans Selye introduced the concept of stress, which he defined as a complex biological response common to all living organisms induced by environmental threats (i.e., stressors) [55]. Stress is oriented to restoring homeostasis and adaption to environment pressures [7,32]. When the threat is too intense and persistent, stress responses may become maladaptive and affect the brain [32,52]. In adulthood, chronic stress impairs limbic structures such as the hippocampus and amygdaloid complex, and the medial prefrontal cortex (mPFC) in both animal models and humans [27,42,62–64]. These brain areas regulate anxiety and fear [14,22,40].

Anxiety is a long-lasting state of apprehension elicited by threats that are not immediately present [12], while fear is an adaptive state activated by real threats, which begins rapidly and dissipates once the threat disappears [12]. The amygdala and *Bed Nucleus of Stria Terminalis* (BNST) are involved in anxiety-like behaviors, while the central nucleus of the amygdala (CeA) and the periaqueductal gray (PAG) nucleus neuronal pathway are associated with fear

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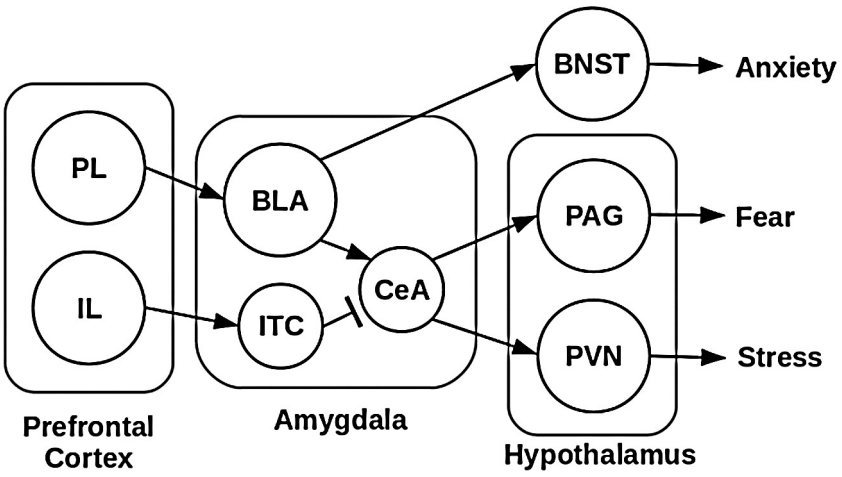


**Scheme 1.** Simplified schematic diagram of the neural circuitry involved in fear and anxiety-like behavior responses. The amygdala complex, mPFC and hypothalamus are the main structures involved in fear and anxiety. During fear conditioning, sensory information about the US and CS converges on the BLA, leading to the storage of the CS-US association within this nucleus. The BLA is also activated by integrated sensory information from PL. In consequence, the presentation of the CS alone, which after conditioning is considered a predictor of explicit threat, activates a set of BLA neurons, which in turn activates the CeA, but not the BNST. The CeA projects to PAG and PVN, triggering the physiological and behavioral responses of freezing and stress, respectively. During extinction of conditioned fear, CS sensory information enters the mPFC and BLA. It activates IL in the mPFC, which along with the BLA, projects and activates ITC cells. The ITC are GABAergic neurons that project to the CeA, inhibiting this nucleus, leading to the reduction of freezing and stress responses. Sensory information, which is a less explicit predictor of threat, enters a set of neurons in the BLA, which now instead of activating the CeA, activate the BNST. This nucleus activates the PAG and PVN, triggering the physiological and behavioral responses of freezing and stress, respectively.

regulation [12,13,22] (Scheme 1). Anxiety levels are higher in rats subjected to chronic stress during adulthood [9,62], which is associated with dendritic hypertrophy in both the basolateral amygdala (BLA) and BNST [61,63,64]. In addition, chronic stress induces dendritic atrophy in the pyramidal neurons of layers II/III and V of the mPFC in adult rats [5,31,42,49], a brain area that regulates the recall of learned fear [22,38,47,56].

### 1.1. Effects of stress in adolescence and adulthood

Adolescence is the only transitional period between childhood and adulthood that is characterized in all mammals by several behavioral, hormonal, and neural changes [57,58]. Adolescence is an extended period that includes puberty and culminates in reproductive maturation. Adolescence in male rats is considered to last from post-natal day (PND) 35 to 55 [39], with physical markers of sexual maturation observed from PND 45 to 48 in males [26].

The brain is particularly sensitive to stress in the adolescence [11]. It has been shown that adolescent rats display higher levels of stress responses than do adult animals [31,45]. For example, adolescent rats subjected to chronic stress have higher plasma corticosterone levels than do adults under same condition. As well, the corticosterone levels of adolescent can take twice as long as those adults to return to the baseline after acute stress [16,19]. The rat mPFC and amygdala, and the neuronal connectivity between them begin developing during adolescence and continue into adulthood [8,24]. Chronic stress affects the dendritic morphology of these brain areas in adolescence and adulthood [10,15,63]. As well, it has been shown that after 21 days of stress-free recovery, adult rats continue showing enhanced anxiety-like behavior and dendritic hypertrophy in the amygdala, while mPFC neurons completely restore their dendritic structure and functions [18,48,64]. A comparable study with adolescent mice treated with corticosterone, shows that this hormone decreases spinal density in the infralimbic and orbitofrontal cortices of the mPFC, while in the amygdala this treatment increases spinal density [21]. After a corticosterone-free recovery period, dendritic changes in the prelimbic cortex and amygdala were reversed, while the dendritic changes in the orbitofrontal cortex remained unchanged after the recovery period [21]. It is not known whether the effects of stress on the mPFC and amygdala during adolescence are reversed in adulthood.

### 1.2. Fear conditioning

Fear conditioning is a three-phase behavioral paradigm used to study the neural circuit of fear [53]. The first phase is conditioning in which the rats are trained in a shuttle box to associate a conditioned stimulus (CS) (a tone) with an unconditioned stimulus (US) (e.g. a foot shock). Once learned, the CS will by itself elicit a conditioned response. For instance, in the fear conditioning paradigm freezing is a behavioral and physiologically conditioned response to fear [25]. It is supposed that fear memories acquired during conditioning are consolidated in the BLA area of the brain [25,43,54] (Scheme 1). The recall of learned fear is associated with increased neuronal activity in the BLA, which in turn activates the CeA. Direct projections are sent from this brain structure to the PAG and paraventricular nuclei (PVN) to elicit defensive fear-behaviors (Scheme 1) [25]. During the fear extinction phase, the CS is presented several times to the rats, which allows them to acquire a new memory that suppresses the retrieval of the previously learned fear. The mPFC regulates the expression of extinction memory via the amygdala [46]. In the recall phase, the CS is presented to the rats and they recall the conditioned fear extinction learned during the extinction phase, which in turn decreases the freezing behavior elicited in the animals [47].

The prelimbic cortex (PL) integrates auditory, contextual, and stress-related signals from several brain areas during fear conditioning to regulate fear expression by the basal amygdala (Scheme 1) [6]. PL activity is key for the expression of fear and memory extinction. For example, in vivo stimulation of the PL increases freezing during the conditioning phase [60]. Chronic stress-induced dendritic atrophy in the PL and dendritic hypertrophy in the lateral amygdala, both in adulthood, have been correlated to failure to express fear extinction [36,48,63,66].

In general, the acquisition of the conditioned fear is regulated by BLA [25,43,54], while the mPFC controls the recall of the extinction of conditioned fear [47,56]. In adulthood, hypertrophy of the rat BLA neurons induced by chronic stress persists after 3 weeks of stress-free recovery, while the morphological alterations induced

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by stress on the PL were reversed [48,64]. However, the effects of stress on the PL during adolescence remain unclear.

We hypothesize that seven days of restraint stress applied in adolescence decreases excitatory synaptic transmission in the PL and slows extinction of learned fear, while enhancing anxiety-like behavior levels. These electrophysiological and behavioral alterations display different recovery patterns during adolescence and adulthood. The objective of this study was to test whether adolescent chronic stress affects synaptic transmission in the PL and recall of conditioned fear extinction as well as anxiety-like behavior. The main results of this study were that rats subjected to restraint stress in adolescence had significantly higher anxiety levels during adulthood, whereas stress-induced decreases in excitatory transmission in the PL and slowing of extinction of learned fear were reversed in adulthood.

## 2. Materials and methods

### 2.1. Animals

Male *Sprague Dawley* rats (80–100 g, 21 days old at the start of the experiment) were housed in groups of three animals per cage, under a 12/12 light/dark cycle (lights on at 8:00 am). They were maintained in a temperature and humidity controlled room ($20 \pm 1\,^{\circ}C$, 55%) and weighed every day on a digital scale (Model WLC2/A1, Radwag, Poland). All procedures relating to animal experimentation were in strict accordance with animal care standards outlined in the National Institute of Health (USA) guidelines and approved by the Institutional Animal Ethics Committee of the Universidad de Valparaíso. Efforts were made to minimize the number of animals used and their suffering.

### 2.2. Experimental design

Fig. 1A is a schematic drawing of the experimental design used in this study. Rats were maintained with ad libitum access to food (rat chow, Champion®, Santiago, Chile) and water during all experiments. Rats were randomly assigned to two experimental groups: control ($n = 20$) and stress ($n = 20$) for all experiments. Control and stress group rats were littermates and after weaning were housed in separate rooms. Rats of the control group were never exposed to any type of experimental stress or to the stressed rats. The restraint stress protocol was applied in a separate room. Experiment 1 analyzed whether the repeated restraint stress paradigm affects locomotor activity, anxiety-like behavior, and fear conditioning during adolescence. Locomotor activity and anxiety-like behavior were evaluated in the open field test twenty-four hours after the stress period ended. Fifteen seconds after the open field was applied, anxiety-like behavior was assessed in each rat by the elevated-plus maze test. The next day rats were subjected to fear conditioning and extinction, and twenty-four hours later recall of the fear learned during the conditioning phase was assessed (recall-1). In Experiment 2 we studied the effect of restraint stress on anxiety-like behavior and the recall of learned fear in adulthood. Twenty-one days after the stress period, anxiety was evaluated by the dark/light box test. One day after this, the rats were returned to the conditioning chamber and the recall of learned fear was evaluated (recall-2). None of the rats of either experimental group were subjected to any type of the experimental stress during adolescence or adulthood. Experiment 3 analyzed whether the restraint stress paradigm affects excitatory synaptic transmission in the PL during adolescence and adulthood. A different set of rats was used for experiments 1 and 2 (control, $n = 12$, stress, $n = 12$), and for the experiment 3 (control, $n = 8$, stress, $n = 8$).

### 2.3. Handling procedure and restraint stress

Rats were removed every day by hand and transferred to another cage on the pan of a balance to be weighed. Different investigators did this procedure from those applying the restraint stress. All rats in every group were handled with the same procedures. Animals were placed in acrylic restrainers (6 cm wide × 12 cm long and then 6 cm wide × 20 cm long as the rats grew) in their home cages. They were subject to restriction for 3 h every day, beginning at 10 am, from PND 42 to 49 (7 days of restraint stress). Restrainers were perforated at each end to allow ventilation and avoid overheating the animals. During the stress protocol, animals could breathe without difficulty and urinate and defecate without being in constant contact with their waste. It is difficult to apply this stress protocol before PND 42 because some rats die during restraint stress.

### 2.4. Stress markers

To monitor the overall effects of the stress protocol the percentage gains in body weight of all the animals were determined (net change in weight after experiment × 100/weight at the beginning of experiment). In addition, we measured the adrenal weight [(wet weight of adrenal glands in mg) × 100/body weight in g)] in another set of rats [control ($n = 6$) and stress ($n = 6$)] one day after the stress period ended.

### 2.5. Behavioral experiments

#### 2.5.1. Behavioral testing

The open field and elevated plus-maze tests were conducted twenty-four hours after completion of the stress protocol (Fig. 1A). All animals were naive to the test situations. Behavioral tests were carried out from 10 am to 2 pm in the test room. The activity of each rat was recorded by IP cameras fixed above the behavioral apparatus and connected to a computer in another room. Videos were acquired by Nuuo software (Nuuo, Taipei, Taiwan) and analyzed using the ANY-maze video tracking system (Stoelting Co., Illinois, USA). All mazes were thoroughly cleaned with a 5% ethanol solution after each trial. In all experiments, animals from control and stress experimental groups were evaluated at the same time.

#### 2.5.2. Open field test

Behavioral tests were conducted in a soundproof and temperature-controlled ($21 \pm 1\,^{\circ}C$) room. Each rat was placed in the center of a black Plexiglass cage (70 cm × 70 cm × 40 cm) for 5 min. The background noise level in the open field was 40 dB (Precision sound level meter, Model # 1100, Quest Technologies, Oconomowoc, WI) and the arena was illuminated to 300 lx (measured by digital lux meter, Model # LX-1010B, Weafo Instrument Co., Shanghai, China). The total distance traveled and average speed were analyzed from video recordings using the ANY-maze video tracking system (Stoelting Co., Illinois, USA). Entry to a zone was defined as occurring when an animal placed all four limbs onto the center or perimeter.

#### 2.5.3. Elevated-plus maze

Immediately after the analysis of the open field (approximately 10 s) we measured anxiety-like behavior levels using an elevated plus-maze test. Each rat was placed individually in an elevated plus-maze, consisting of two open arms (60 cm × 15 cm each), two closed arms (60 cm × 15 cm × 20 cm each) and a central platform (15 cm × 15 cm), arranged so that the two arms of each type were opposite to each other. The maze was elevated 100 cm above the floor. The illumination was 300 lx in the open arms and 210 lx in the closed arms. At the beginning of each trial animals were placed at the center of the maze, facing an open arm. During a 5-min test

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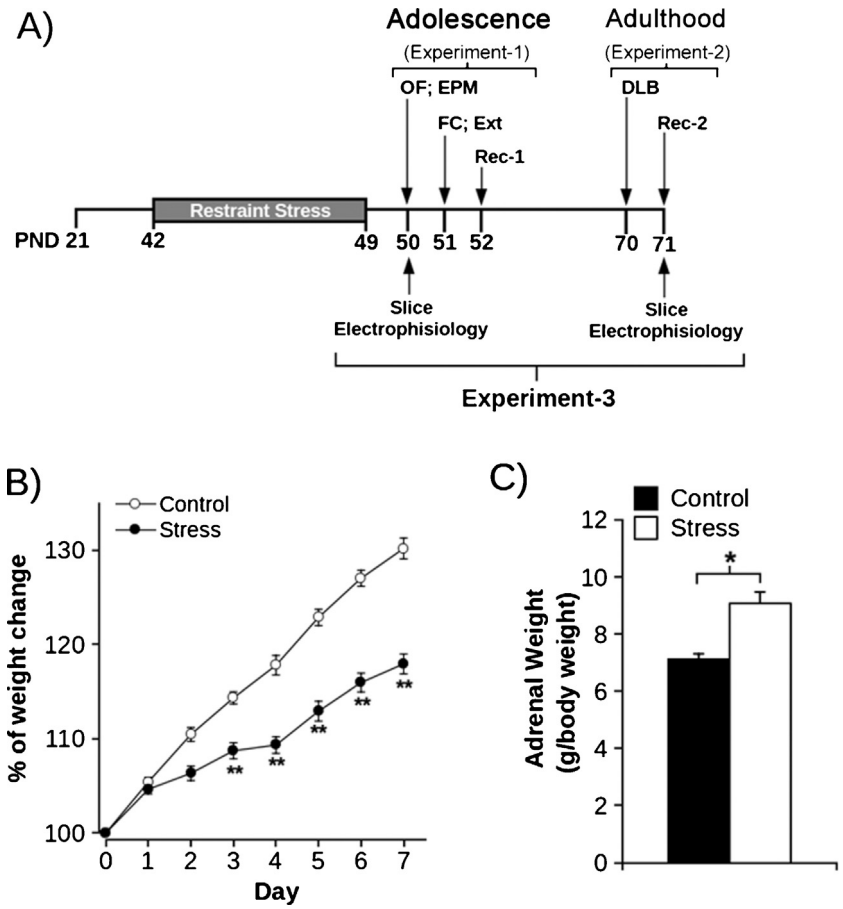


**Fig. 1.** Experimental design and the effect of restraint stress on physiological stress markers. (A) Rats ($n = 40$) were handled and weighed daily from weaning (post-natal day 21) until the end of stress. During adolescence (post-natal day, PND, 42), rats of the stressed group ($n = 20$) were subjected to three hours of restraint stress for seven consecutive days until PND 49 (adolescence). Control animals were not subjected to any type of stress. One day after stress (Experiment-1, Adolescence), locomotor activities and anxiety-like behavior were evaluated in both groups with the open field (OF) and elevated-plus maze (EPM) test. At PND 51, rats were subjected to fear conditioning and extinction, and one day later (PND 52), we evaluated the recall of the extinction of conditioned fear (Rec-1) in adolescence. Rats were returned to the vivarium, and during adulthood (Experiment-2, PND 70, Adulthood), we evaluated anxiety-like behavior with the dark-light box test. One day later (pnd 71), we evaluated the recall of the extinction of conditioned fear (Rec-2). Synaptic transmission was analyzed in layer I of the prelimbic cortex during adolescence and adulthood (Experiment-3, Adolescence and Adulthood). (B) Body weight change expressed as a percentage of the weight at the beginning of the stress protocol (**: significant difference between control and stressed rats with $p < 0.01$; $n = 12$ per group). (C) Seven days of chronic juvenile stress increased adrenal weight (*$p < 0.05$; $n = 4$ per group). Data are presented as mean ± SEM.

period we recorded the frequency of entries to the open and closed arms, the total number of arm entries, and the amount of time spent in each section of the maze. The number of entries and time spent in the open arms, and the ratio of open to total arm entries (open/total × 100) were used as measures of the anxiety level. Total arm entries were taken as an indicator of general locomotor activity. Entry into an arm was defined as having occurred when the animal placed all four limbs onto the arm floor.

*2.5.4. Fear conditioning and extinction of learned fear*

*2.5.4.1. Apparatus and stimuli delivery.* Rats were trained in two shuttle boxes (50 cm × 25 cm × 25 cm) (Model LE 916, Panlab Instruments, Barcelona, Spain). The grid floor of each shuttle box had 38 stainless steel bars arranged parallel to the dividers. The conditioning chamber was placed in a 110 cm (length) × 50 cm (height) × 84 cm (width) sound-attenuating cubicle lined with 7.5 cm acoustic foam (Vroka S.A., Santiago, Chile). The inside of the cubicle was dimly illuminated to 250 lx with a 0.5-W light Led (measured by a digital lux meter, Model # LX-1010B, Weafo Instrument Co., Shanghai, China) and the background noise level was 30 dB (Precision sound level meter, model # 1100, Quest Technologies, Oconomowoc, WI). A 3-kHz tone amplified to 80 dB, with the speaker mounted in front of the pellet receptacles, was presented to the subjects as a CS signaling the upcoming US in the form of a foot shock (0.5 mA, 500 ms) delivered by a shocker (LE 100-26, Panlab S.L., Barcelona, Spain). The CS was delivered simultaneously by speakers located on opposite walls of the chamber (20 cm high). The delivery of both the CS and US delivery was regulated by Shutavoid computer-based operant software (Panlab S.L., Barcelona, Spain). Behavior was videotaped for analysis using IP cameras mounted in front of the shuttle box. Videos were acquired by Nuuo software (Nuuo, Taipei, Taiwan). The fear conditioning chambers were cleaned with 5%-ethanol each time a rat was removed from the chamber.

*2.5.4.2. Behavioral training.* Fear conditioning was conducted over two days, beginning one day after the end of stress period. The rats were placed in the shuttle box and trained individually. During the training sessions the rats were subjected to a 10-min stimulus-free acclimation period, without CS presentation (day 0). Rats were then returned to their home cages. On day 1, all rats were first exposed to a 3-min acclimation period, followed by five habituation trials in which rats received a CS tone for 30 s, with an average inter-trial interval (ITI) of 30 s without presenting the US. Rats were not returned to their home cages. After a 3-min acclimation period, seven conditioning trials were applied. Each trial consisted of the presentation of 30 s of CS overlapping at the end with a 0.5 s foot shock. Rats were returned to their home cage and to the colony

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room. Freezing was continuously recorded during the conditioning session and later scored to determine the degree to which rats acquired the conditioned association.

*2.5.4.3. Extinction procedure.* Rats were returned to the conditioning chamber one hour later. After a 3-min acclimation period, the rats received extinction trials consisting of CS alone. To ensure comparable levels of extinction learning between control and stress groups, the extinction trials on day 1 continued until the rats exhibited less than 10% (3 s) freezing in four consecutive trials. The number of trials to criterion was similar across experimental groups. After the extinctions trials, the rats were returned to their home cages in the housing room.

*2.5.4.4. Recall of extinction of conditioned fear.* On day 2, rats were placed in the conditioning chamber for a 3-min acclimation period, followed by extinction trials consisting of fifteen CS alone to analyze the recall of fear learned during the conditioning trials (recall-1). Twenty-one days after the stress protocol ended, animals were returned to the conditioning chamber and twelve CS were delivered (recall-2). Freezing was continuously recorded during the trials and later scored to determine the degree to which rats acquired the conditioned association (see Section 2.5.4.5). The ITI used throughout the experiment was applied randomly with an average of $240 \pm 30$ s.

*2.5.4.5. Measurement of freezing behavior.* Freezing was used to measure the conditioned emotional fear response and was defined as the absence of any visible movements with the exception of respiration-related movement and non-awake or rest body posture [37,40]. For all trials, the duration of freezing during the 30-s CS was measured with a digital stopwatch by an observer blind to experimental conditions. The percentage of freezing (seconds spent freezing/30-s CS) during habituation, fear conditioning, extinction, and recall was calculated and compared across groups.

We evaluated freezing responses in the intervals without tone presentation to determine the effect of context on fear responses [4]. We then measured freezing 30 s before and after the first extinction trial (points A and B, respectively), 30 s after the last trial of extinction (point C), and 30 s before of the first trial of recall-1 (point D) and recall-2 (point E).

*2.5.4.6. Sensitivity to foot shock.* One day after the completion of recall-2, animals were tested for sensitivity to foot shock. Rats were placed in the conditioning chamber and given unsignaled foot shocks of increasing amplitudes, beginning with 0.05 mA. The foot shock was increased by 0.05 mA increments until a jumping response was reached in the animal. An observer blind to the assignment of experimental groups measured the thresholds.

*2.5.5. Dark-light box test*

The test lasted 5 min and was performed in a two-compartment Plexiglas box consisting of a lighted chamber (50 cm × 50 cm × 40 cm) illuminated from above with a white light producing an intensity of 500 lx at floor level, and a dark chamber (50 cm × 50 cm × 40 cm) with a light intensity of 5 lx at floor level. The chambers were separated by a black partition with a small opening (8 cm × 8 cm) at the bottom. At the start of the experiment a rat was placed in the center of the lighted box with its head facing the wall opposite to the door. The time spent in the lighted and dark compartments was measured ANY-maze software (Stoelting, Wood Dale, IL, USA). Time in a zone was considered to have begun when the animal placed all four paws in that zone.

*2.6. Electrophysiology*

*2.6.1. Slices preparation*

A new set of rats (control, $n = 8$, stress, $n = 8$) was used to study the effects of chronic stress on synaptic transmission in the PL. One or twenty-one days after completion of the stress protocol rats were decapitated under deep anesthesia with halothane. The brain was removed quickly and submerged for 30 s in cold (~4 °C) dissection buffer (in mM: 300 Sucrose; 6 $MgSO_4$; 4 KCl, 1 $Na_2HPO_4$; 0.5 $CaCl_2$, 26 $NaHCO_3$, 10 D-glucose). The pH of the dissection buffer was stabilized at 7.4 by bubbling carbogen (95% $O_2$, 5% $CO_2$). Coronal brain slices (400 μm) containing PL (3.70–2.20 mm from bregma) were cut with a Vibratome (The Vibratome Company, 1000 Plus Sectioning System, MO, USA) in cold dissection buffer. Slices were then transferred to a holding chamber immersed in artificial cerebrospinal fluid (in mM: 124 NaCl; 4 KCl; 1 $Na_2HPO_4$; 26 $NaHCO_3$; 1 $MgCl_2$; 2 $CaCl_2$, 10 D-glucose). Slices were superfused with carbogen-bubbled artificial cerebrospinal fluid (2 ml/min) and maintained at room temperature ($28 \pm 1$ °C) for at least 2 h.

*2.6.2. Recording*

A bipolar concentric tungsten stimulating electrode was positioned in layer I of the PL. Field excitatory post-synaptic potentials (fEPSP) were evoked by the application of monophasic constant current square pulses at an intensity range of 10–50 μA, a duration of 200 μs and a frequency of 0.033 Hz with an stimulus isolator (model SIU91A, Cygnus Technology Delaware Water Gap, PA). The evoked fEPSP was recorded with a borosilicate pipette filled with 2 M NaCl placed in layer I at approximately 100 μm from the stimulating electrode. Recordings were amplified 1000×, bypass filtered at 1 Hz-5 kHz (amplifier model 1700, A-M system amplifier; Sequim, WA, U.S.A.), and acquired using an analog/digital converter interface (Model BNC-2090, National Instruments). Recordings were acquired and analyzed offline with IgorPro software.

To evaluate the basal synaptic transmission an input-output curve was generated for each slice. The average of three pulses delivered at 0.33 Hz, with each intensity in the range of 10–50 μA in steps of 10 μA, was considered for the analysis. Synaptic strength was determined measuring the peak amplitude of the negative component of fEPSPs.

*2.7. Statistical analysis*

Data from the open field (time, total distance traveled, and average speeds) and the adrenal weight were analyzed with the Student's *t*-test.

The percentage of body weight gain, fear conditioning, and results from electrophysiological experiments were analyzed using two-way repeated-measures ANOVA [Body weight [groups (control, stress) × restraint stress days (1–7)]; Fear Conditioning, Extinction, Recall-1 and 2 [groups (control, stress) × trials]; Electrophysiology [groups (control, stress) × Stimulus Intensity (10–50)]] followed by Bonferroni post hoc comparison tests.

Data from entries and time in the arms measured in the elevated-plus maze, trials to extinction criterion, context trials, threshold, dark/light box test were compared across groups using a one-way ANOVA. Results are presented as the mean ± SEM. A probability level of 0.05 or less was accepted as significant.

## 3. Results

*3.1. The effects of restraint stress on the stress markers and locomotor activity*

The two-ways repeated-measures ANOVA (group × days) showed that chronic restraint stress significantly reduced

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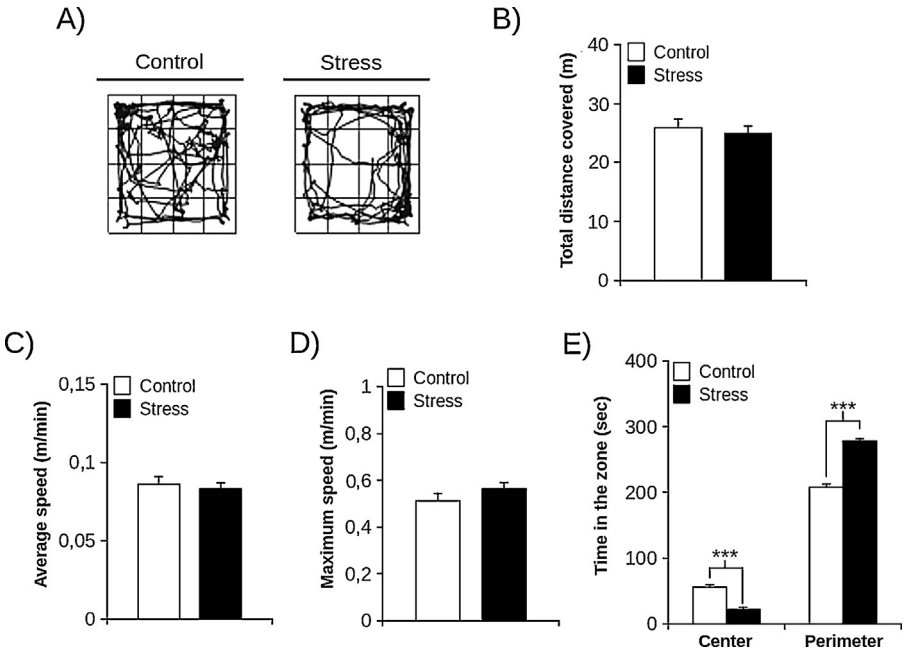


**Fig. 2.** Effects of restraint stress on locomotor activity and anxiety-like behavior in adolescence. (A) Representative tracking plots from control and stressed rats in the open field. Chronic restraint stress did not affect total distance traveled (B), average speed (C) and maximum speed (D). (E) Stressed rats spent less time in the center and more time in the perimeter of the open field compared to control rats (***: significant difference between control and stressed rats with $p < 0.001$; $n = 12$ per group). Data are mean ± SEM.

body weight gain [$F(1,220) = 401.6$; $p < 0.0001$] (Fig. 1B). There was a interaction between days and experimental groups [$F(9,220) = 31.90$; $p = 0.0001$].

Rats subjected to restraint stress had significantly higher adrenal weights than controls (Stress = 9.08 ± 0.69; Control = 7.14 ± 0.35; $p < 0.05$) (Fig. 1C).

Restraint stress did not affect the total distance ($p = 0.6496$), average speed ($p = 0.6709$), and maximum speed ($p = 0.1762$) in the open field (Fig. 2).

### 3.2. Experiment 1

#### 3.2.1. The effects of chronic stress on anxiety-like behavior during adolescence

Stress group rats spent less time in the center of the open field and more time in the perimeter than did controls (Center: stress = 21.6 ± 3.3; control = 55.9 ± 3.6, $p < 0.001$; Perimeter: stress = 278.4 ± 3.6; control = 207.9 ± 5.1, $p < 0.001$) (Fig. 2A and E). A two-way ANOVA (group × zone) showed a significant effect of group on the time spent in the center and perimeter ($F(1,40) = 25.0$, $p < 0.0001$), as well as a significant interaction between group and zone ($F(1,40) = 200.3$, $p < 0.0001$) (Fig. 2E).

To confirm this finding, we measured the anxiety-like behavior with the elevated-plus maze paradigm. A two-way ANOVA (group × arms) showed that there was no significant effect of group on the percentage of entries into the open and closed arms ($F(1,44) = 0.88$; $p = 0.352$), or the percentage of time spent in the open and closed arms ($F(1,44) = 0.11$; $p = 0.7384$) (Fig. 3B and C). However, there was a significant effect of the interaction between groups and arms on the percentage of time spent in the open and closed arms ($F(1,44) = 250.0$; $p < 0.0001$) (Fig. 3C). Rats subjected to restraint stress had significantly lower ratio of open to total arm entries than controls (Stress = 22.01 ± 3.01; Control = 34.22 ± 3.40; $p < 0.05$) (Fig. 3D), without affecting the number of total entries ($p = 0.143$) (Fig. 3E).

#### 3.2.2. The effects of chronic stress on the recall of learned fear

Fig. 4A shows the percentage of conditioned responses (% of freezing) to the tone in control and stress groups during the fear conditioning. The two-way repeated-measures ANOVA shows that during the habituation phase, restraint stress did not affect the percentage of freezing ($F(1,70) = 0.5263$; $p = 0.4706$) and no interaction was found between group and trial ($F(4,70) = 1.741$; $p = 0.1506$).

During the conditioning phase, control and stress groups rats showed similar increases of freezing (Fig. 4A). A two-way ANOVA for group and trial revealed that there was a significant effect of the number of trials ($F(6,98) = 18.94$; $p < 0.001$), stressed and control group rats reached similar levels of freezing at the end of the conditioning phase. Restraint stress did not affect either the percentage of freezing in the conditioning trials [$F(1,98) = 0.0044$; $p > 0.05$] or the interaction between groups and trials [$F(6,98) = 0.441$; $p > 0.05$].

In the extinction phase, there were a significant effect of block of trials on the percentage of freezing [$F(7,91) = 44.42$; $p < 0.001$], rats of both experimental groups significantly reduced of freezing (Fig. 4A). Rats from the stress group showed similar percentage to freezing [$F(1,91) = 0.069$; $p = 0.796$] and the interaction between block of trials and group ($F(7,91) = 0.573$; $p = 0.776$) during the extinction phase (Fig. 4A).

Rats subjected to restraint stress had a significantly higher percentage of freezing than controls in the adolescence (recall-1: effect of group $F(1,56) = 7.80$; $p < 0.0001$) (Fig. 4A and B). There were similar levels of freezing for rats from both experimental groups in the first block of trials in recall-1, and then freezing decreased in the subsequent trials (significant effect of trials, $F(4,56) = 42.2$; $p < 0.05$). There was an interaction between groups and blocks of trials [$F(4,56) = 5.02$; $p < 0.01$].

Fig. 4B shows the effects of restraint stress on trials to extinction criterion. A two-way ANOVA (group × phase) showed a significant effect of the phase ($F(2,39) = 14.17$; $p < 0.0001$), but not of

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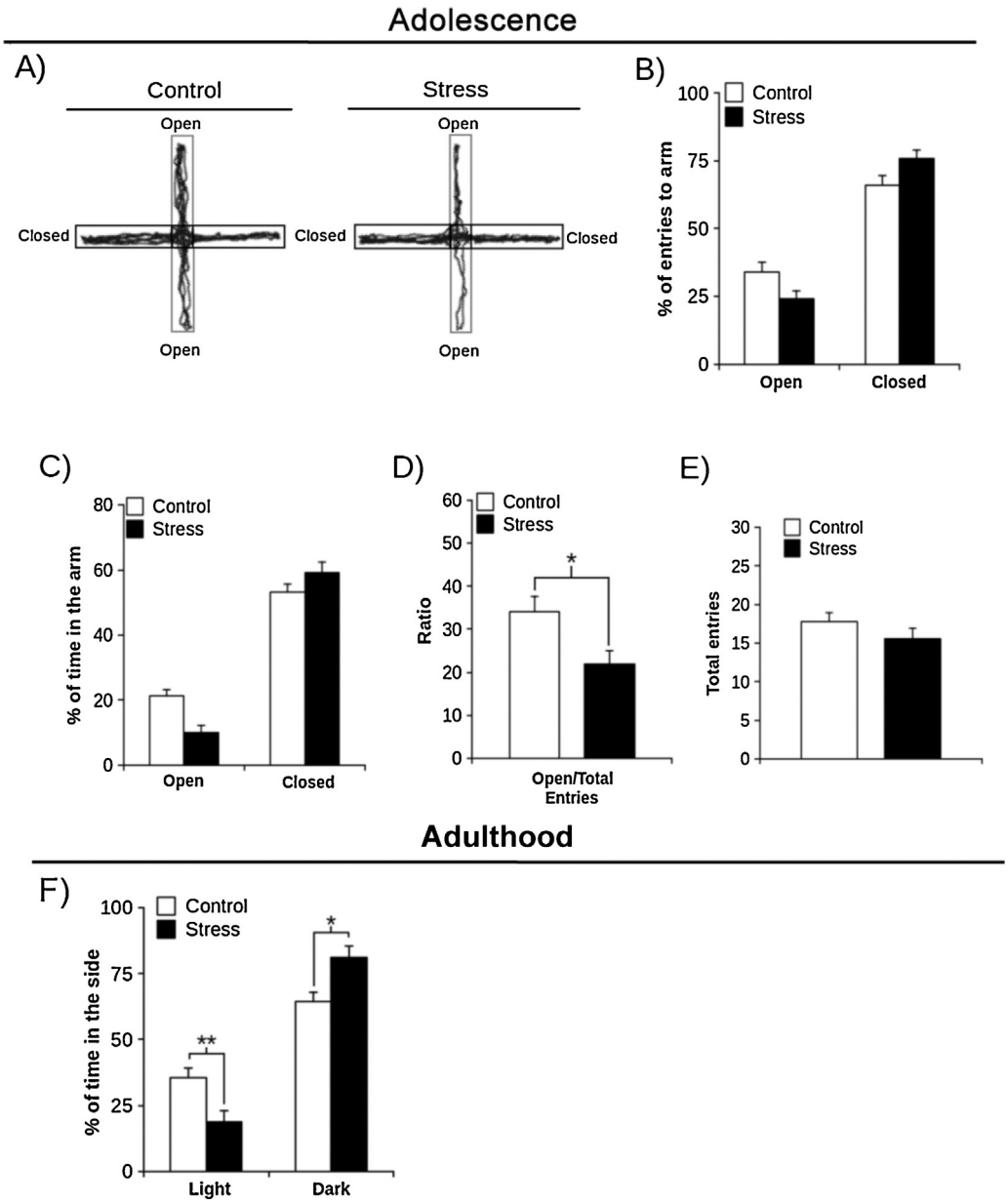


**Fig. 3.** Effects of restraint stress on anxiety-like behavior in adolescence and adulthood. (A) Representative tracking plots from control and stressed rats in the elevated plus maze. (B) During adolescence, there was no difference between control and stressed rats in the percentage of entries to the open and closed arms, and (C) the percentage of time spent in the open and closed arms in the elevated-plus maze. (D) Restraint stress decreased the ratio of entries (open arm entries/total entries to the open and closed arms)(*: significant difference between control and stressed rats with $p < 0.05$; $n = 12$ per group). (E) The stress protocol did not affect the total number of entries to the arm. (F) In adulthood, stressed animals spent less time in the light side (**$p < 0.01$) and more in the dark side of the box than control animals (*$p < 0.05$; $n = 8$ per group). All data are represented as mean ± SEM.

the group [$F(1,39) = 3987$; $p = 0.052$]. Conversely, an interaction between group and phase was found in the recall-1 [$F(2,39) = 3.429$; $p < 0.05$]. There was a no effect between groups in the extinction phase ($p > 0.05$). Rats that had been subjected to restraint stress in adolescence required a significantly higher number of trials to reach the extinction criterion (Stress = 13.25 ± 1.02; Control = 8.25 ± 0.53; $p < 0.01$).

Fig. 4C shows the effect of context on freezing responses during the extinction and recall-1 trials. There was a significant effect of the context on trials [$F(4,66) = 19.89$; $p < 0.001$]. However, there was no effect of group [$F(1,66) = 1.09$; $p = 0.3003$] and the interaction between groups and trials [$F(4,66) = 0.6463$; $p = 0.6315$] (Fig. 4C).

### 3.3. Experiment 2

#### 3.3.1. The effects of restraint on anxiety-like behavior and the recall of learned fear in the adulthood

The rats that were subjected to restraint stress spent significantly less time in the light side (Stress = 18.76 ± 4.26; Control = 35.64 ± 3.56; $p < 0.01$), and more time in the dark side of the box than did the controls (Stress = 81.23 ± 4.26; Control = 64.36 ± 3.56; $p < 0.05$) (Fig. 3F). There was a interaction between group and side ($F(1,28) = 18.56$; $p < 0.001$).

Fig. 4A shows the effect of restraint stress on freezing in adulthood (recall-2). There was no effect of the group ($F(1,39) = 1.47$; $p = 0.245$) and interaction between groups rats and

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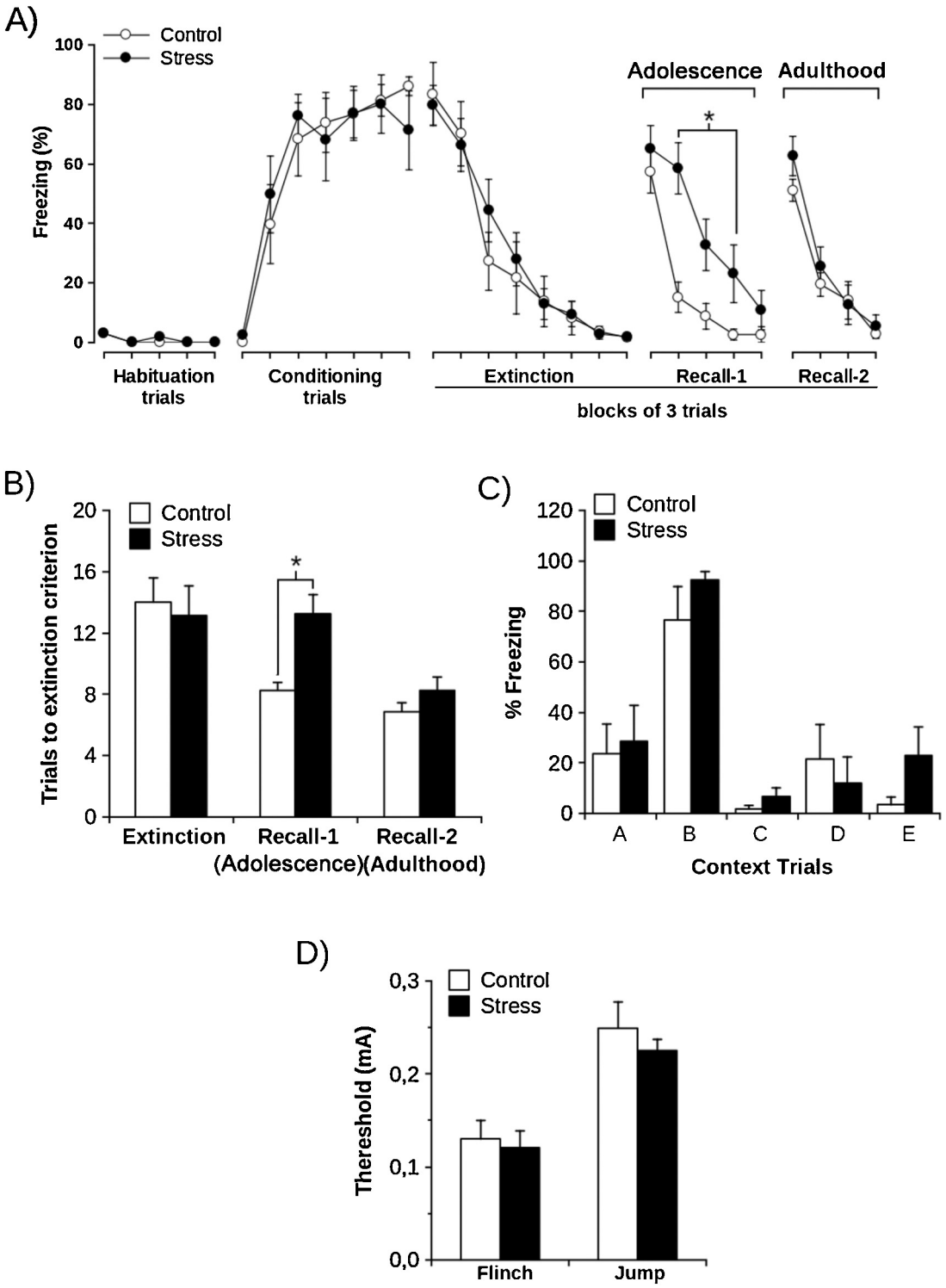


**Fig. 4.** Effects of restraint stress on fear conditioning and extinction of learned fear in the adolescence and adulthood. (A) Chronic restraint stress increased the percentage of freezing time to conditioned stimulus during adolescence (recall-1; $^{*}p < 0.05$; $n = 8$ per group). The stress protocol did not affect the percentage of freezing time during conditioning, extinction, and recall-2 (adulthood). (B) Stressed rats required more trials to reach the extinction criterion (less than 20% of freezing time during four consecutive tone trials) in adolescence (recall-1) compared to controls ($^{**}p < 0.01$; $n = 8$ per group). There was no difference in extinction and recall-2 during adulthood. (C) Chronic stress did not produce significant differences in the percentage of freezing to context measured in the contextual trials (A and B: 30 s immediately before and after the first extinction trial, respectively; C, 30 s before the last extinction trial; D and E, 30 s before the first trial of recall-1 and 2, respectively) ($n = 7$–8 per group). (D) Chronic stress did not affect the level of current needed to evoke the flinching or jumping responses. The values are presented as mean ± SEM.

trial blocks on the percentage of freezing time ($F(1,39) = 1.04$; $p = 0.382$). Likewise, the analysis of the number of trials to the extinction criterion (Fig. 4B) revealed that there was no significant difference between control and stress group rats in the adulthood (Stress = 8.25 ± 2.49; Control = 6.68 ± 1.68; $p > 0.05$).

We also evaluated whether there is a long-term effect of chronic stress on contextual freezing in the adulthood. Fig. 4C shows that

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there was no effect of trials ($F(1,27) = 1.034$; $p = 0.3182$), group ($F(1,27) = 2.948$; $p = 0.0974$) or interaction between context trials and groups ($F(1,27) = 1.561$; $p = 0.2223$).

### 3.4. Sensitivity to the foot-shock

Fig. 4D shows that rats of both groups required significantly more current for the jump response ($F(1,19) = 34.09$; $p < 0.0001$). However, restraint stress did not affect the sensitivity to the foot shock ($F(1,19) = 0.746$; $p = 0.398$).

### 3.5. Experiment 3

#### 3.5.1. The effects of chronic stress on excitatory synaptic transmission in the PL

To determine whether the chronic stress affects the basal excitatory synaptic transmission of PL, we evoked fEPSP by applying a square pulse current protocol. We observed that during adolescence the stressed rats had significantly lower fEPSP amplitude in the PL compared to controls ($F(1,95) = 22.80$, $p < 0.0001$; effect of stimulus intensity: $F(4,95) = 16.81$, $p < 0.0001$) (Fig. 5A). Moreover, we observed that restraint stress had no effect on the fiber volley amplitude ($F(1,95) = 0.2032$, $p = 0.6532$; effect of stimulus intensity: $F(4,95) = 11.01$, $p < 0.0001$) (Fig. 5A).

We evaluated the long-term effect of chronic stress on synaptic transmission in the PL. Fig. 5B shows that there was no effect on the fEPSP ($F(1100) = 0.027$, $p = 0.869$) and the fiber volley amplitude ($F(1100) = 0.302$, $p = 0.5839$) in the adulthood.

## 4. Discussion

The present study shows that chronic stress applied in the course of adolescence decreases excitatory synaptic transmission in the rat PL and extinction of learned fear. These alterations were reversed in adulthood. Moreover, stressed rats that showed increased anxiety-like behavior levels in adolescence were still anxious in adulthood.

The first step of our investigation was to analyze whether our stress protocol was effective in triggering stress responses in adolescence. The rats subjected to restraint stress had less body weight gain than controls (Fig. 1B). Comparable results have been reported using similar stress paradigms [36]. Glutamate release in limbic brain areas is higher in rats subjected to chronic stress [3,51], which in turn may increase the energetic demand supplied by the lipid catabolism. As a result, in the case of our experiments the stressed rats had less weight gain than controls (Fig. 1B). In addition, we found adrenal gland hypertrophy in the stressed rats (Fig. 1C), which we argue was induced by hyperactivation of the HPA axis. In support of this idea, chronic stress increases plasma corticosterone levels and induces adrenal gland hypertrophy and hyperplasia in an ACTH-dependent manner [41,59]. The results shown in Fig. 2 demonstrate that the stress protocol used in this study was effective with adolescent rats.

### 4.1. Effects of chronic stress in adolescence

Restraint stress did not affect locomotor activity (Fig. 2A–D), although stressed rats spent significantly less time in the center of the open field (Fig. 2E) and the ratio of their open arm entries in the elevated-plus maze was lower than that of the controls (Fig. 3D). These results indicate that rats subjected to seven days of restraint stress in adolescence had higher levels of anxiety-like behavior. Comparable results were obtained in the adult rats using another chronic restraint stress paradigm [9,62,64]. Anxiety is mainly regulated by the BLA and the BNST [12,13]. In fact, some chronic stress paradigms that enhance anxiety-like behavior have been correlated to dendritic hypertrophy in the BLA and BNST [61,63,64]. It is possible that in our study the chronic stress applied in adolescence induced hyperactivation of the BLA and/or BNST by plastic neuronal changes that significantly increased anxiety-like behavior (Figs. 2E and 3D and F).

Restraint stress did not affect freezing during the conditioning trials (Fig. 4A and B). The neuronal pathway formed by the CeA-PAG-brainstem regulates fear responses [12,13]. Thus, we suggest that the neuronal morphology of the CeA is not affected by restraint stress in adolescence. It has been shown that in a comparable chronic stress paradigm, stress did not affect the CeA in adulthood [61].

The IL and PL cortices regulate retrieval of learned fear and extinction memories in control rats [35]. However, expression of fear and extinction memories in stressed rats can be affected by the stress-induced dendritic alterations in the PL and amygdala. The PL converts transient lateral amygdala input into a sustained PL output, which could drive fear responses via projections to the basal amygdala [35]. Therefore, we speculate that chronic stress in our study induced dendritic hypertrophy in BLA of adolescent rats, which in turn increased neuronal activity in this brain area and enhanced anxiety-like behaviors (Fig. 3A). If the stressed rats had higher BLA activity, then poor retrieval extinction could be induced in them given that BLA activity regulates fear expression. It has been reported that chronic stress induces dendritic atrophy in the PL of adolescent rats [5,28]. We suggest that this morphological alteration can decrease excitatory synaptic transmission in the PL, as shown in Fig. 5A. The excitatory neurons in layers II-III of the PL partly regulate neuronal activity in the IL, which in turn modulates GABAergic transmission in the BLA by ITC (Scheme 1) [35]. Therefore, a stress-induced decrease of excitatory transmission in the PL could indirectly reduce inhibitory activity in the BLA and as a result retrieval of extinction memories is slowed in stressed adolescent rats (Fig. 4A).

We found that chronic stress decreased the amplitude of evoked fEPSPs in the PL during adolescence (Fig. 5A), which appears to contradict previous findings that show chronic stress does not affect the amplitude of evoked fEPSPs in the mPFC [18]. A possible explanation for our finding is the age of the animals. We used adolescent rats while Goldwater et al. used adult rats [18]. Neuronal maturation in the PFC occurs largely during adolescence [30] so it is possible that the effects of stress on the PFC are more pronounced in adolescence than in adulthood. This is supported by the fact that prenatal stress disrupts PFC development in adolescents, while it does not affect spinal density in the PFC of adult rats [30,34].

Reduction of AMPA currents has been associated with morphologic alterations in the mPFC of stressed rats [67]. It is possible that changes in AMPA receptor functions underlie the reduction of evoked fEPSPs amplitude in the PL of stressed rats. We think that restraint stress decreased proplastic protein levels in the mPFC, which in turn induced dendritic atrophy. Proplastic proteins are implicated in neurite extension, cell survival and synaptic plasticity [23].

It have been reported that seven days of restraint increases plasma corticosterone levels [5], while the AMPA receptors in the mPFC have fewer GluR2/3 subunits in rats treated with corticosterone [20]. Therefore, it is possible that higher plasma corticosterone levels in rats subjected to seven days of restraint stress affect excitatory synapse transmission in the PL, which in turn decreases the pace of learned fear extinction in our experiments.

### 4.2. Effects in adulthood of chronic stress during adolescence

In our experiments, the animals were still anxious in adulthood (Fig. 3F). It is possible that dendritic hypertrophy in the

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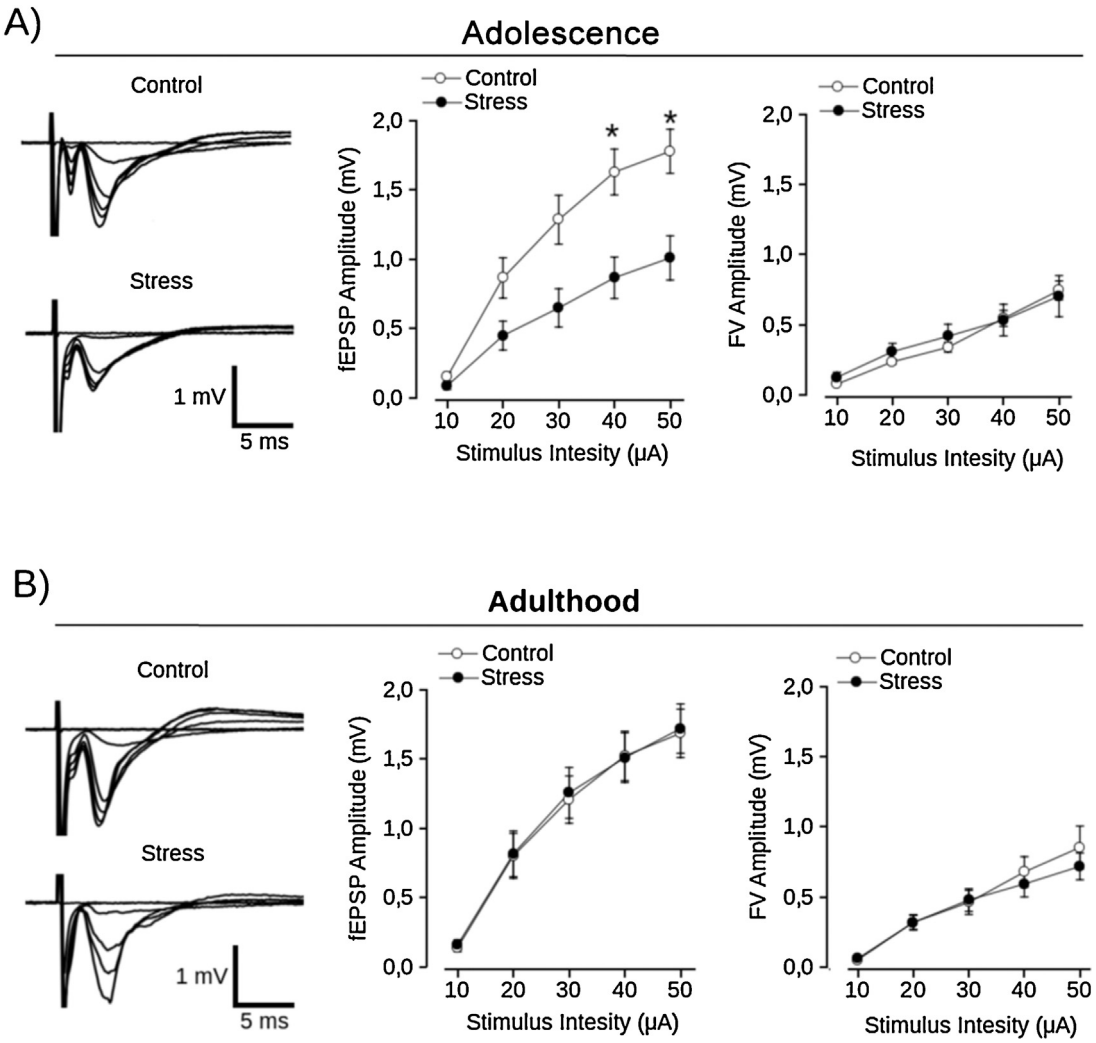


**Fig. 5.** Synaptic transmission in the prelimbic cortex. (A) Left panel, representative evoked fEPSP traces under different intensities of stimuli during adolescence. Middle and right panels, input–output relationship of the fEPSP average and the presynaptic fiber volley amplitude, respectively, as a function of the intensity of stimuli (control, $n = 9$; stressed, $n = 12$). (B) Left panel, representative evoked fEPSP traces under different intensities of stimuli during adulthood. Middle and right panels, input–output relationship of the fEPSP average and the presynaptic fiber volley amplitude, respectively, as a function of the intensity of stimuli. (control, $n = 9$; stress, $n = 13$). Data are expressed as mean ± SEM. An asterisk (*) indicates significant differences.

BLA induced during adolescence by stress persists into adulthood. Indeed, a comparable but more aversive stress protocol, the chronic immobilization stress paradigm, enhanced anxiety-like behavior twenty-one days after stress, which correlated with dendritic hypertrophy in the BLA [61].

Figs. 4 and 5 shows that stress-induced slowing of extinction of learned fear and decreases in excitatory synaptic transmission in the PL were reversed in adulthood. At the cellular level, it has been shown that after 3 weeks of stress-free recovery pyramidal neurons of the mPFC restore their dendritic morphology [48]. Likewise, we suggest that in our experiments the PL pyramidal neurons of the stressed rats were recovered in adulthood and that excitatory synaptic transmission increased in these neurons (Fig. 5B). Conversely, anxiety-like behavior produced by stress in adolescence continued into adulthood, probably due to dendritic hypertrophy in the BLA of the adult rats (Fig. 3F).

Recovery of the effects of stress on learned fear might be due to direct action via the glucocorticoid receptors (GR) in the PL neurons. The rat frontal cortex expresses higher levels of GR and distress decreases GR protein levels in this area of the brain [1,2]. After stress-free recovery, GR expression could be higher in the PL of adult rats. Corticosterone binds to cytosolic GR, inducing GR dimerization and translocation to the nucleus, thereby increasing the gene expression of neurotrophins such as BDNF in the PL [50,65,68]. These molecules are implicated in synaptic plasticity [29]. As a result, synaptic plasticity induced by GR in the PL restores the dendritic morphology of pyramidal neurons, which in turn improves retrieval of memory extinction in adult rats.

Fig. 4C shows that restraint stress did not affect the freezing to context in every fear-conditioning session. The hippocampus regulates the context memory [44] and it has been reported that seven days of restraint stress does not affect the neuronal morphology of the hippocampus [33]. We suggest that in our experiments the hippocampus of the stressed rats was not affected and they responded to context (Fig. 4C). However, it is necessary to evaluate fear conditioning and extinction in a different context [17].

## 5. Conclusions

The data presented here demonstrate that chronic stress in adolescence decreased the amplitude of evoked fEPSPs in the PL and slows the extinction of learned fear, while anxiety levels were higher in stressed rats than controls. The anxiogenic effect of restraint stress was still present in adulthood, while alterations in

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excitatory synaptic transmission in the PL were reversed and the recall of extinction of learned fear was recovered. Interestingly, it has been shown that psychosocial stress in humans impairs the functions of the frontal cortex and this alteration is reversed after stress [27]. Finally, this study gives a new approach to understanding the neurobiology of stress and stress-related disorders.

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