

Differential long-term neuroendocrine and behavioral impact of controllable and uncontrollable foot-shock exposure during adolescence in male rats

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ABSTRACT

There is substantial evidence that control over stress affects both its short- and long-term consequences. However, much remains unknown about the impact of controllability, particularly during adolescence. In the present study, we examined in adolescent male rats the long-term effects of repeated exposure to controllable footshocks in a two-way active avoidance (TWAA) task (master group) versus exposure to identical but uncontrollable footshocks (yoked group). Neither the master nor the yoked procedure affected adult basal levels of ACTH and corticosterone. No evidence of altered anxiety-like behavior in the elevated plus-maze was found in either the master or the yoked groups compared with stress-naïve animals, whereas active social interaction was reduced in yoked but not master rats. As expected, prior adolescent experience of control over the TWAA task improved performance in an adult TWAA session. However, a significant, although less marked, improvement in performance was also found in rats previously assigned to the adolescent yoked condition. Improved performance in master and yoked versus stress-naïve rats were observed despite behavioral and endocrine (ACTH and corticosterone) evidence of shuttle-box context conditioning. Improved TWAA performance was paralleled by reduced ACTH response to the task. Finally, adolescent yoked rats showed a greater freezing response to the shuttle-box context of the shuttle after the adult sessions, suggesting long-lasting fear potentiation. These results confirm that stress during adolescence has long-term behavioral consequences, controllability over stress mitigating some of its effects.

1. Introduction

Early life stress (ELS), which typically covers from the prenatal period to childhood, has been repeatedly found to exert long-lasting programming effects that can be detected in adulthood in both animals and humans (Gunnar and Quevedo, 2007; Weinstock, 2008; Heim et al., 2010). Not surprisingly, the impact of ELS is greatly dependent on the age at which stress exposure occurs, as well as on the nature and duration of such exposure. In this regard, the consequences of exposure to stress during youth and adolescence have been poorly studied in humans and largely neglected in animal models, with a major

resurgence of interest in the last two decades (Andersen and Teicher, 2008; Teicher et al., 2016).

Unfortunately, results from animal models regarding the impact of adolescent stress are controversial. This can be partly due to the diversity of experimental models used, which includes social instability and conflict, repeated exposure to a particular stressor (typically restraint or electric foot-shocks), or exposure to various stressors either for a relatively short period of time (e.g., 3 days) or for several weeks, the latter models being included within the concept of chronic unpredictable stress (CUS) (McCormick and Mathews, 2010; Horovitz et al., 2012; Neigh et al., 2013; Eiland and Romeo, 2013; Hollis et al., 2013; Burke

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et al., 2017). The overall picture is that juvenile-adolescent stress can often exert detrimental physiological and behavioral effects, but in some cases protective or presumably positive effects have been reported (e.g., Parihar et al., 2009; Suo et al., 2013), with the impact being opposite in juvenile (stress exposure PN22–33) as compared with mid-adolescence (stress exposure PN35–46) rats (Wilkin et al., 2012). Moreover, little attention has been devoted to the differential impact of controllability over stress during this period.

It has been proposed that repeated experience with controllable versus uncontrollable aversive (and rewarding) stimuli can shape future behavior toward proactive versus reactive (Moscarello and Hartley, 2017). Interestingly, in the laboratory setting, this influence appears to be stronger in adolescents than in children or adults (Raab et al., 2024), in accordance with a field study demonstrating a negative impact of lack of control and perceived severity of stressors on depression and anxiety symptoms in youth (Fassett-Carman et al., 2019).

Controllability over stress has typically been studied in adult animals using the triadic design developed by Weiss and collaborators, and later extensively used in Maier's laboratory (Weiss et al., 1970; Maier and Watkins, 2005). The design includes three groups: stress-naïve, master, and yoked. The stress-naïve rats are minimally stressed and do not receive shocks. The master rats are exposed to tail-shocks while maintained in a small chamber where they can stop an ongoing shock by turning a wheel with their front paws (escapable, master group, ES). Finally, each yoked rat is paired with a master rat, and it will receive the same amount of shock as the master rat, regardless of its own behavior (inescapable, yoked group, IS). Using this paradigm, it has been repeatedly found that the possibility to escape protects from some of the negative consequences of inescapable stress, including performance deficits in a two-way active avoidance (TWAA) task, enhanced anxiety, predisposition to subordination, and potentiation of foot-shock induced fear conditioning (Maier, 1984; Maier and Watkins, 2005).

Using the tail-shock procedure, it has been reported that, in adolescent rats, a single IS exposure decreased social interaction when evaluated 24 h later, whereas ES had no effect compared with stress-naïve animals. Importantly, prior juvenile ES protects against the negative consequences of adult IS on social exploration (Kubala et al., 2012). It was also found that these beneficial effects of ES were not observed after lesions of the medial prefrontal cortex, in accordance with previous results in adult rats. We then reasoned that repeated experience of control or lack of control over stress during adolescence could exert a longer lasting and stronger impact than during adulthood due to the reorganization of brain circuits involving the frontal cortex during this period (McEwen and Morrison, 2013). We then used a TWAA master-yoked design in which some adolescent rats could not only escape but also avoid the occurrence of foot-shocks (master), whereas other rats could not (yoked). We found a lower activation of the hypothalamic-pituitary-adrenal (HPA) axis, the classical biological marker of stress (Armario et al., 2020), in master as compared with yoked rats after repeated experience with the stressful situation (Sanchís-Ollé et al., 2019). Moreover, in adulthood, yoked rats showed increased motor, but not cognitive, impulsivity as well as greater perseverative behavior.

In the present work, we wanted to study, using the same experimental design, whether adolescent experience with controllable versus uncontrollable stress can exert long-lasting effects on the activity of the HPA axis, anxiety-like behavior, social interaction, and the performance of the animals in the TWAA task during adulthood. We hypothesized that early experience of control over stress could reduce the long-term negative consequences of uncontrollable stress and improve performance in the adult TWAA task. We included only males in the study because of the high number of animals required and the evidence that females are rather insensitive to stress controllability (Dalla et al., 2008; Baratta et al., 2018).

2. Materials and methods

2.1. Subjects and general procedure

Male Sprague-Dawley rats from the breeding center of the Universitat Autònoma de Barcelona were used. Animals were always maintained under standard conditions of light (lights on from 8 am to 8 pm), temperature (21 ± 1 °C) and humidity (40–60%). All protocols were in accordance with the European Communities Council Directive 2010/63/EU and the Spanish legislation (RD53/2013). They were approved by the Ethics Committee at the Universitat Autònoma de Barcelona and the Generalitat de Catalunya.

2.2. Experimental design

Ninety male Sprague-Dawley rats were used. Rats were 25–27 days old when arriving at the laboratory and were left undisturbed for one week. The experimental phase started when they were on average 34 days old. They were maintained 2 per cage throughout the experimental period. All the experimental procedures were carried out in rooms distinct from the housing room. Housing in pair avoids the social isolation but allows us to accommodate housing to all the procedures in which the two rats of a cage were handled, stressed and blood sampled together by two independent researchers, thus minimizing disturbances. They were semi-randomly assigned to three experimental adolescent groups on the basis of their body weight ($n = 30$ per group): (i) stress-naïve; (ii) master, exposed to the TWAA task; and (iii) yoked, rats exposed to the TWAA task but having no control over the foot-shocks. As our aim was to cover all the adolescent period, stress exposure consisted of 8 sessions given in an unpredictable way (changing the time of day and the interval of days between sessions) throughout a 25-day period. Each adolescent stress session was performed over three consecutive days because of the high number of animals. Exposure to the TWAA task during adolescence took place between 8 a.m. and 4 p.m., and both the behavioral tests and the adult TWAA task took place between 8 a.m. and 1 p.m. The timeline of the experiment can be seen in Fig. 1.

The TWAA task was performed in 4 rectangular shuttle-boxes, each consisting of 3 dark Plexiglas walls and one transparent frontal wall. Each shuttle has two identical chambers (25x25x25 cm) connected by a door (8 × 10 cm), and a grid floor (19 bars 0.3 cm diameter separated by 1 cm center to center). Shocks were delivered by a LEI100–26 shocker (Panlab S.L.U, Harvard, Barcelona, Spain). The shuttle-boxes were insulated inside metal boxes. Master rats were exposed to a TWAA task consisting of a 5 min habituation period with no stimuli, followed by 50 trials of a light-tone conditioned stimulus (CS, 10 s) immediately followed by a 0.7 mA foot-shock lasting a maximum 10 s (unconditioned stimulus, US). Crossing from one side to the other terminated the CS (avoidance) or US (escape) presentation and was followed by a variable inter-trial-interval (ITI, 5–55 s, average 30 s). Each yoked rat was paired with a master rat and received the same number and duration of shocks as the corresponding master, with their own behavior having no consequences over the shocks. Stress-naïve rats were exposed to the shuttle-box for the same period of time as the other groups without shocks. Avoidances of master rats were recorded along the 8 sessions and time spent freezing was evaluated in master and yoked rats on sessions 1, 2 and 8.

When the rats were about 3 months old, they were evaluated either in the elevated plus-maze (EPM) test ($n = 42$) or in the social interaction (SI) test ($n = 48$). The EPM test was conducted simultaneously for the two rats of a cage in two apparatuses placed in adjacent rooms. In the SI test, two cages were also tested simultaneously, placing together two animals from the same experimental group but from different home-cages. Animals were recorded on video using a camera placed in a zenith position.

The EPM, adapted from Pellow and File (1986), consisted of 4 black arms forming the shape of a plus sign and elevated 50 cm above the

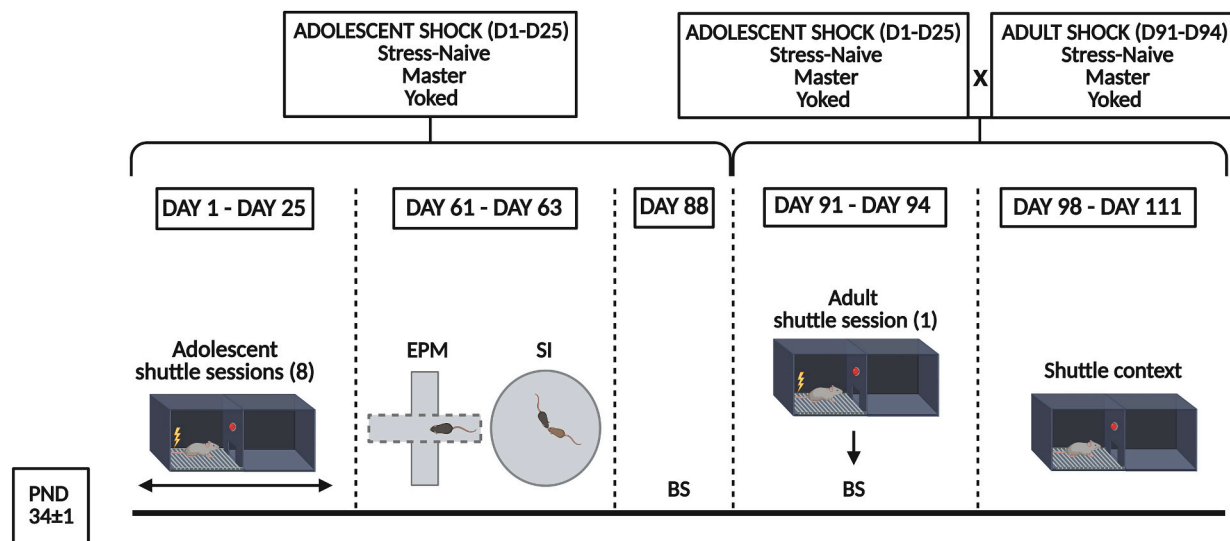


Fig. 1. Timeline of the experimental design. Adolescent rats were assigned to three experimental adolescent shock exposure (D1–15) groups when they were about 34 days old: stress-naïve, only exposed to the shuttle-box without shocks, master, exposed to two-way active avoidance (TWAA) task in the shuttle-box, and yoked, which received the same amounts of shocks as the corresponding master rats. Starting on D1, eight shuttle sessions were semi-randomly distributed over 25 days. On D61–63 some rats were exposed to the elevated-plus-maze (EPM) or to a social interaction test (SI). On D88 some rats from each group were randomly chosen to be blood sampled both in the morning and the evening. On D91–94 rats from each adolescent group were exposed to one of three adult shock conditions in the shuttle-box: non-shocked, master or yoked. A week later the rats were exposed to the shuttle-box again to evaluate fear conditioning to the apparatus.

floor. Each arm was 46 cm long and 10 cm wide. Two of the opposite arms had high walls (enclosed arms, 43 cm high), whereas the other two were the open arms, which had a 0.7 cm ridge to provide an additional grip. The illumination was provided by a white bulb (25 W). The rat was placed for 5 min in the apparatus, starting in the center facing a closed arm. A video tracking system (Smart version 2.5.10, Panlab SLU, Harvard, Barcelona, Spain) was used to measure distance travelled into open and closed arms. Time spent in open and closed arms and number of entries in each type of arm were manually measured. A subject was considered to have entered a given arm when all paws were inside.

The SI test was conducted in a white circular wooden open field (80 cm in diameter and 34 cm in height) with a smooth black floor. The illumination was provided by a white bulb (25 W). No habituation to the apparatus was performed. The two animals were from the same stress adolescent group, but were neither housed together nor siblings, and they differed less than 20 g in weight (except in 3 cases in which differences were of about 30 g). Individual animals were identified by black marks in the head and the shoulder. Two pairs were evaluated simultaneously in two separate rooms. The apparatuses were cleaned with water and soap. The test duration was 5 min. The variables measured using the Observer software (Noldus, Wageningen) were as follows (File, 1980): (i) active social behavior, which includes social grooming, jumping over or passing underneath (pass and jump), or sniffing the other subject; (ii) following, when one animal moved in coordination with the other, and (iii) passive social behavior, when subjects maintain body contact without engaging in active behaviors. In addition, the distance travelled by each individual subject was measured using the video tracking system. The rat pair was considered the statistical unit, except for activity that was independently assessed for each individual rat.

When the rats were about 4 months old, blood sampling was done under resting conditions between 9 and 10 h and between 19 and 20 h in ten randomly chosen rats from each group. Three days later, re-exposure to the shuttle-box started. Rats from each adolescent experimental group were assigned to three experimental adult conditions (non-shocked, master and yoked) and exposed exactly to the same apparatuses and procedures as previously described for adolescent rats. This results in 9 experimental groups. Finally, a week after the adult shuttle exposure, rats were again exposed to the shuttle for 10 min in the absence of shocks

to evaluate contextual fear conditioning.

2.3. Blood sampling and biochemical analyses

To study HPA axis activity, blood samples were obtained from the caudal vein by the tail-nick procedure without anesthesia (Belda et al., 2004; Vahl et al., 2005), using capillary tubes containing EDTA (Microvette CB 300, Sarstedt). Samples were obtained under resting conditions in the morning (9–10 h) and the evening (19–20 h) or after exposure to the adult TWAA task (in the morning). In the latter case, immediately after the task or after a recovery period of 45 min.

Plasma ACTH and corticosterone were determined by double-antibody radioimmunoassays that have been extensively validated and used in our laboratory (Muñoz-Abellán et al., 2011). All samples to be statistically compared were processed in the same assay to avoid inter-assay variability.

2.4. Statistical analysis

Data were analyzed with the Statistical Program for Social Sciences, SPSS-IBM (version 30). We used a *t*-test when only two groups were compared and generalized linear models (GzLM) otherwise (Hardin and Hilbe, 2002; McCulloch and Searle, 2001). GzLM includes one between-subjects factor (adolescent condition, with three levels: stress-naïve, master and yoked) or two between-subjects factors (adolescent condition and adult condition, the latter also with 3 levels: non-shocked, master, and yoked). Log-transformations were made when this procedure improved the homogeneity of variances. Where appropriate, post-hoc comparisons were made using the Sidak sequential correction. Alpha level for significance was $p < 0.05$, but trends for significance were also mentioned.

3. Results

A significant effect of adolescent stress on body weight gain during the period of exposure to the shuttle (Table 1) was found ($\chi^2(2) = 7.1$, $p = 0.029$), with a statistically significant decrease in the master group and a marginally significant decrease in the yoked group compared with stress-naïve rats ($p = 0.049$ and $p = 0.056$, respectively).

Table 1
Impact of adolescent stress on body weight.

Group	Initial weight (g)	Weight gain (g)	Adult weight (g)
Stress-naïve	69.3 ± 2.2	177.5 ± 3.9	449 ± 10.9
Master	69.8 ± 1.8	163.7 ± 3.5 *	437 ± 8.6
Yoked	70.2 ± 2.1	164.9 ± 4.9 (*)	439 ± 10.9

Means ± SEM are represented ($n = 29$ – 30 /group). Weight gain refers to body weight gain during the period of adolescent exposure to stress. (*) marginally significant ($p = 0.056$), * $p = 0.049$ versus stress-naïve rats.

The performance of adolescent master rats in the eight TWAA sessions can be seen on Fig. 2A. Avoidance increased during the first sessions but then stabilized with no improvement in the last four sessions. No differences between master and yoked rats were observed regarding freezing levels, rearings, and intercrossings in the habituation period corresponding to the second and the last session (Fig. 2B).

A significant time of day effect, but no effect of adolescent group, was observed regarding plasma ACTH and corticosterone levels ($\chi^2(1) = 53.8$, $\chi^2(1) = 808.1$, $p < 0.001$ in both cases), evening levels being higher than morning levels, as expected (Table 2).

In the EPM, three stress-naïve rats were discarded because they jumped from the apparatus. EPM behavior can be seen in Fig. 3. The statistical analysis showed a significant effect of adolescent condition on the number of closed arm entries ($\chi^2(2) = 23.3$, $p < 0.001$), but not on the number of open arm or total arm entries. Post-hoc comparisons of closed arm entries showed lower number of entries in master than in the stress-naïve ($p < 0.001$) and yoked ($p = 0.004$) groups, with a marginal decrease in yoked compared with stress-naïve ($p = 0.069$). The analysis

Table 2
Adolescent stress exposure and adult circadian pattern of ACTH and corticosterone.

Group	ACTH (pg/ml)		Corticosterone (ng/ml)	
	morning	evening	morning	evening
Stress-naïve	75 ± 2.2	94 ± 4.7	6 ± 1.7	164 ± 16.4
Master	70 ± 6.6	107 ± 10.8	5 ± 0.7	172 ± 17.4
Yoked	74 ± 5.0	95 ± 5.3	8 ± 2.0	160 ± 10.3

Means ± SEM are represented ($n = 10$ /group). The statistical analyses revealed significant effects for time of day but not groups.

of time spent in each arm revealed no group effects. The distance travelled in the open and closed arms was also evaluated. No group differences were found in distance in the open arms, whereas a group effect was found in distance in the closed arms ($\chi^2(2) = 26.1$, $p < 0.001$), with lower distance in master and yoked versus stress-naïve ($p < 0.001$ and $p = 0.013$, respectively) and in master versus yoked ($p = 0.01$).

In the social interaction test (Fig. 4) a pair was discarded because of camera recording problems. The analysis revealed an adolescent condition effect regarding active social interaction ($\chi^2(2) = 6.4$, $p = 0.042$), with reduced time in yoked versus stress-naïve groups ($p = 0.050$). Also, a significant group effect was found in time spent following ($\chi^2(2) = 8.3$, $p = 0.016$), yoked showing marginally lower levels than stress-naïve ($p = 0.078$) and lower levels than master ($p = 0.017$). Only a trend for differences was observed in passive social behavior ($\chi^2(2) = 5.3$, $p = 0.072$), with a trend for the yoked group to increase time spent in passive interaction compared with stress-naïve group ($p = 0.089$). When

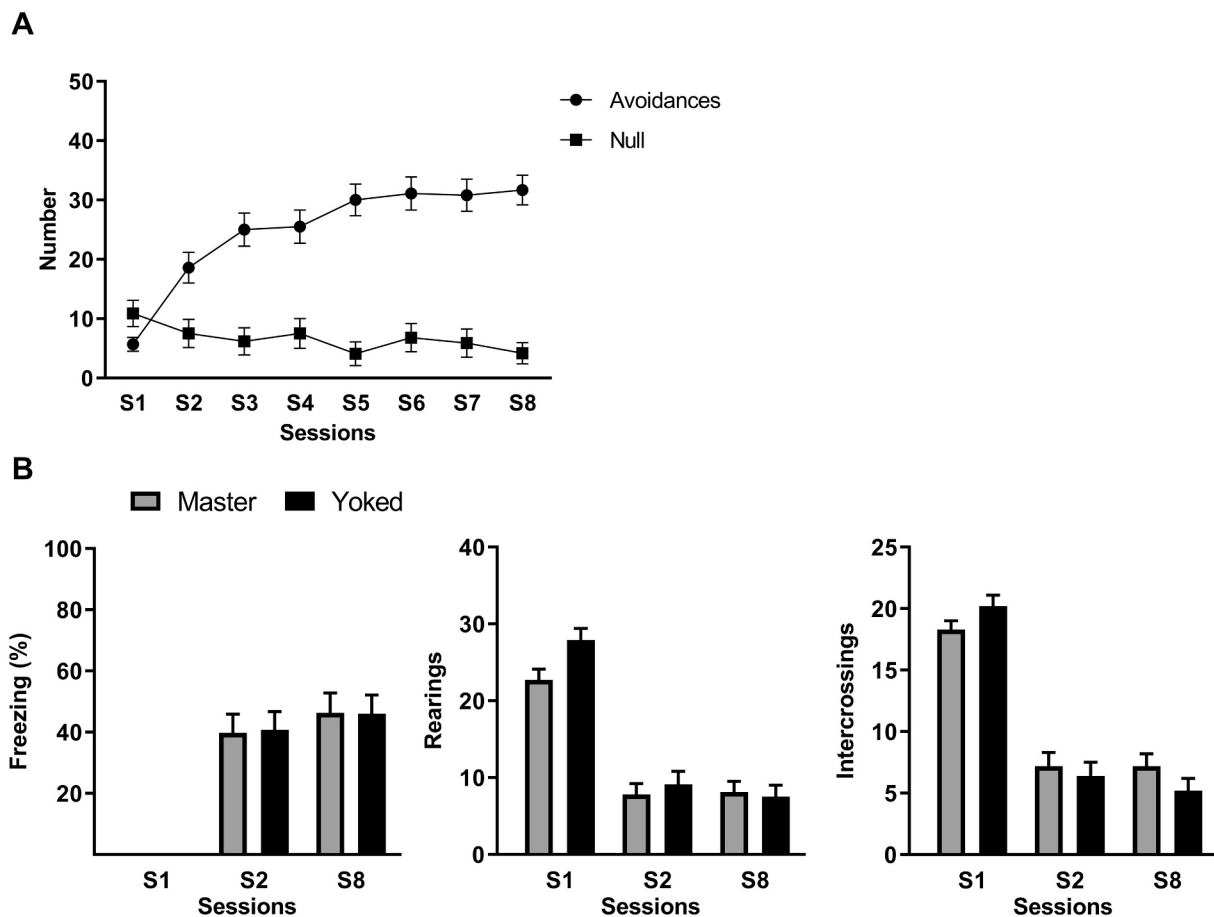


Fig. 2. Behavior of adolescent rats over the foot-shock sessions. Means ± SEM ($n = 30$ /group) are represented. Panel A illustrates the performance (avoidances and null responses) of adolescent master rats in the eight sessions of the TWAA task. Panel B indicates the behavior of master and yoked rats in the 5 min habituation period of the first, second and eighth shuttle sessions. No behavioral differences were observed between master and yoked rats.

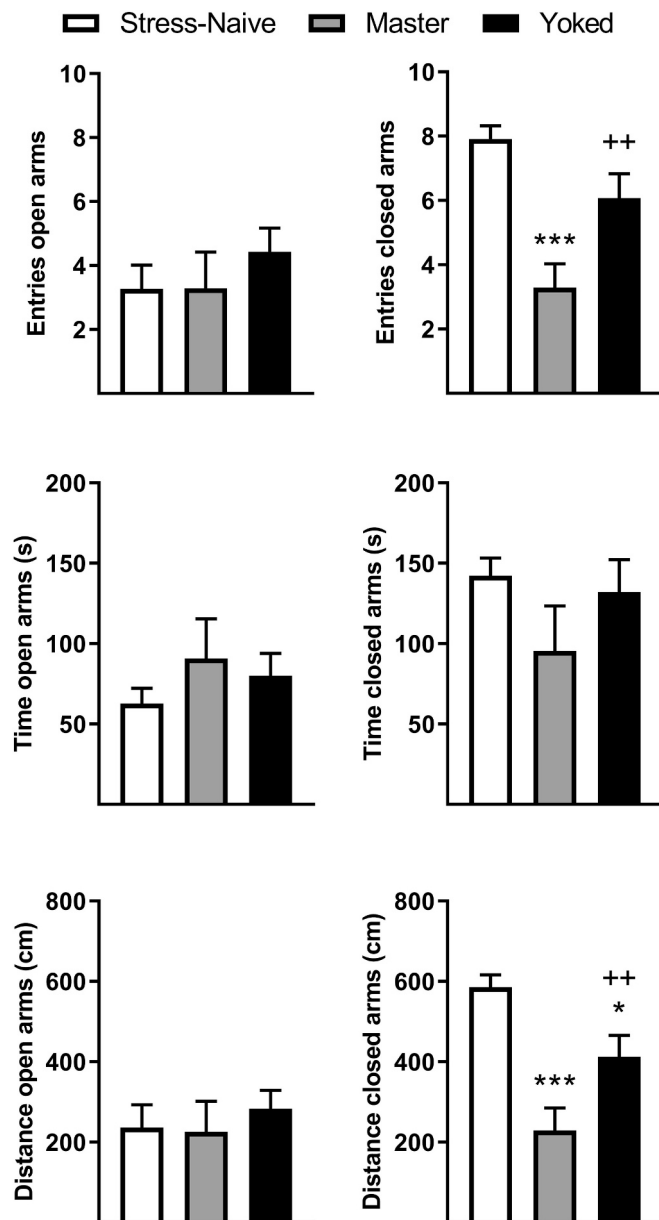


Fig. 3. Impact of adolescent stress on adult EPM behavior. Means $\pm$ SEM are represented ($n = 14$ per group, except for the stress-naïve group in which 3 rats were discarded because they jumped from the EPM). * Indicates significant differences with respect to stress-naïve, + indicates significant difference versus masters; one symbol p at least <0.05 , two symbols p at least <0.01 , three symbols $p < 0.001$.

individual distance travelled for each individual rat was analyzed, a significant group effect was found ($\chi^2(2) = 24.7, p < 0.001$), yoked rats displaying less activity than both stress-naïve and master ($p < 0.001$ in both cases).

The long-term fear memory was evaluated during the 5 min habituation (pre-shock) period when rats were exposed to the shuttle-box in adulthood (Fig. 5A). The analysis revealed significant adolescent group effects on time spent freezing ($\chi^2(2) = 370.1, p < 0.001$), number of rearings ($\chi^2(2) = 95.8, p < 0.001$) and number of crossings ($\chi^2(2) = 73.9, p < 0.001$). Further comparisons showed higher levels of freezing in yoked and master compared with stress-naïve rats ($p < 0.001$ in both cases), with yoked rats showing higher levels than master ($p = 0.016$). The opposite pattern was found regarding rearings and crossings. Both master and yoked rats showed lower number of rearings and crossings

than stress-naïve (always $p < 0.001$), with yoked rats showing also lower number of rearings and crossings than master ($p = 0.011$ and $p < 0.001$, respectively). The data support a greater fear of the shuttle context in yoked than master rats.

During the adult TWAA task (Fig. 5B), significant adolescent group effects were found for avoidances ($\chi^2(2) = 47.9, p < 0.001$) and escapes ($\chi^2(2) = 50.1, p < 0.001$), but not for failures (null responses). Further comparisons showed higher number of avoidances in adolescent master than both adolescent stress-naïve and yoked ($p < 0.001$ in both cases), with yoked rats performing better than stress-naïve ($p = 0.048$). The mirror pattern was found regarding escapes, with adolescent master rats showing much lower levels than stress-naïve and yoked ($p < 0.001$ in both cases) and yoked rats lower levels than stress-naïve ($p = 0.003$).

Plasma ACTH and corticosterone levels were measured in all nine groups just after completion of the TWAA task and after a 45 min recovery period (Fig. 6). Given the marked reduction of hormone levels during the post-stress recovery and the complexity of factors, we decided to segregate hormonal data by time.

The analysis of ACTH levels just after the adult task showed no effect of adolescent condition, but significant effects of adult group ($\chi^2(2) = 356.8, p < 0.001$) and the interaction between adolescent condition and adult group ($\chi^2(4) = 15.8, p = 0.003$). Similar results were obtained with corticosterone: a significant effect of adult group ($\chi^2(2) = 72.3, p < 0.001$), and a significant interaction between adolescent condition and adult group ($\chi^2(4) = 19.1, p < 0.001$). Considering these interactions, we analyzed the impact of the three adolescent conditions for each adult condition. In non-shocked adults, a significant effect of adolescent conditions was found on ACTH ($\chi^2(2) = 9.5, p = 0.009$) and corticosterone ($\chi^2(2) = 14.4, p = 0.001$). Post-hoc comparisons revealed marginally significant higher levels of ACTH ($p = 0.069$) and significantly higher levels of corticosterone ($p = 0.021$) in adolescent master than in adolescent stress-naïve rats, whereas adolescent yoked rats did show clearly higher levels of both ACTH and corticosterone than stress-naïve animals ($p = 0.008, p = 0.001$, respectively). In response to the adult TWAA task (master condition), a significant effect of adolescent condition was found on both ACTH ($\chi^2(4) = 7.2, p = 0.028$) and corticosterone ($\chi^2(2) = 13.0, p = 0.002$), with adolescent master rats showing lower levels of ACTH and corticosterone than adolescent stress-naïve ($p = 0.023, p = 0.002$, respectively). In addition, adolescent masters showed lower corticosterone levels than adolescent yoked ($p = 0.011$). No effect of adolescent condition on the ACTH or corticosterone response to the adult yoked condition was found.

The analysis of hormone levels at R45 revealed only significant adult group for both ACTH ($\chi^2(2) = 42.8, p < 0.001$) and corticosterone ($\chi^2(2) = 28.4, p < 0.001$), with higher levels in adult master and yoked rats than non-shocked rats ($p < 0.001$ in both cases).

When rats were again exposed to the shuttle-box after the adult task to assess fear to the context (Fig. 7), significant effects of adolescent condition ($\chi^2(2) = 11.5, p = 0.003$) and adult condition ($\chi^2(2) = 120.3, p < 0.001$) were found. Appropriate post-hoc comparisons revealed that both master and yoked adult condition, as expected, markedly enhanced freezing compared to adult non-shocked rats ($p < 0.001$ in both cases), this effect being greater in adult yoked versus adult master rats ($p = 0.044$). When the adolescent condition was considered (regardless of the adult condition), adolescent yoked condition markedly enhanced freezing compared with stress-naïve rats ($p = 0.002$), whereas adolescent master condition only marginally enhanced freezing versus stress-naïve rats ($p = 0.08$).

4. Discussion

The present results support previous findings that rats having prior experience of control over the same task showed much better performance than both stress-naïve and yoked groups. In addition, our results indicate for the first time that prior repeated experience of male rats with uncontrollable foot-shock stress during adolescence improves

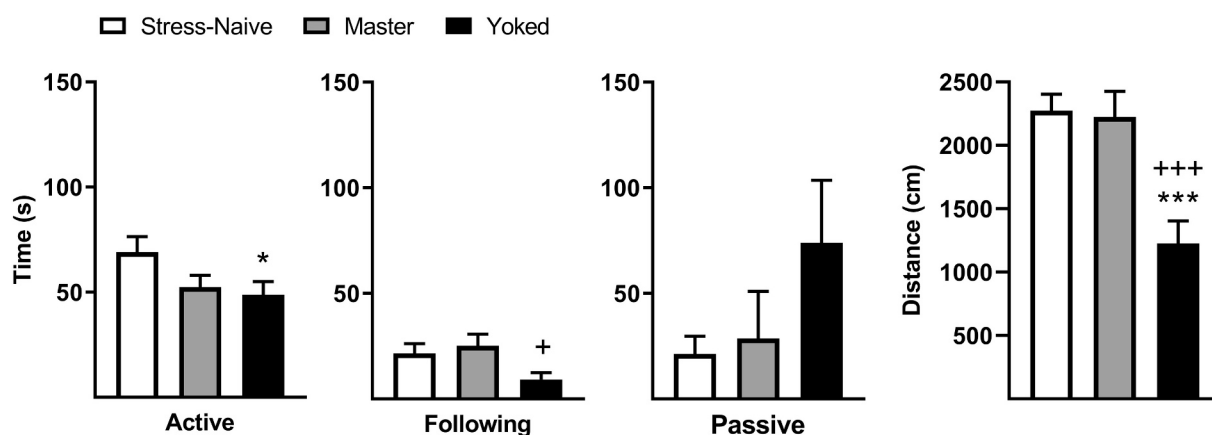


Fig. 4. Impact of adolescent stress on adult social interaction and activity. Means $\pm$ SEM are represented. Eight pairs of rats from each adolescent group were tested, except in the stress-naïve groups in which recording failed in a pair. Note that activity was individually measured and therefore sample size was double. * Indicates significant differences with respect to stress-naïve, + indicates significant difference versus masters; one symbol p at least <0.05 , three symbols $p < 0.001$.

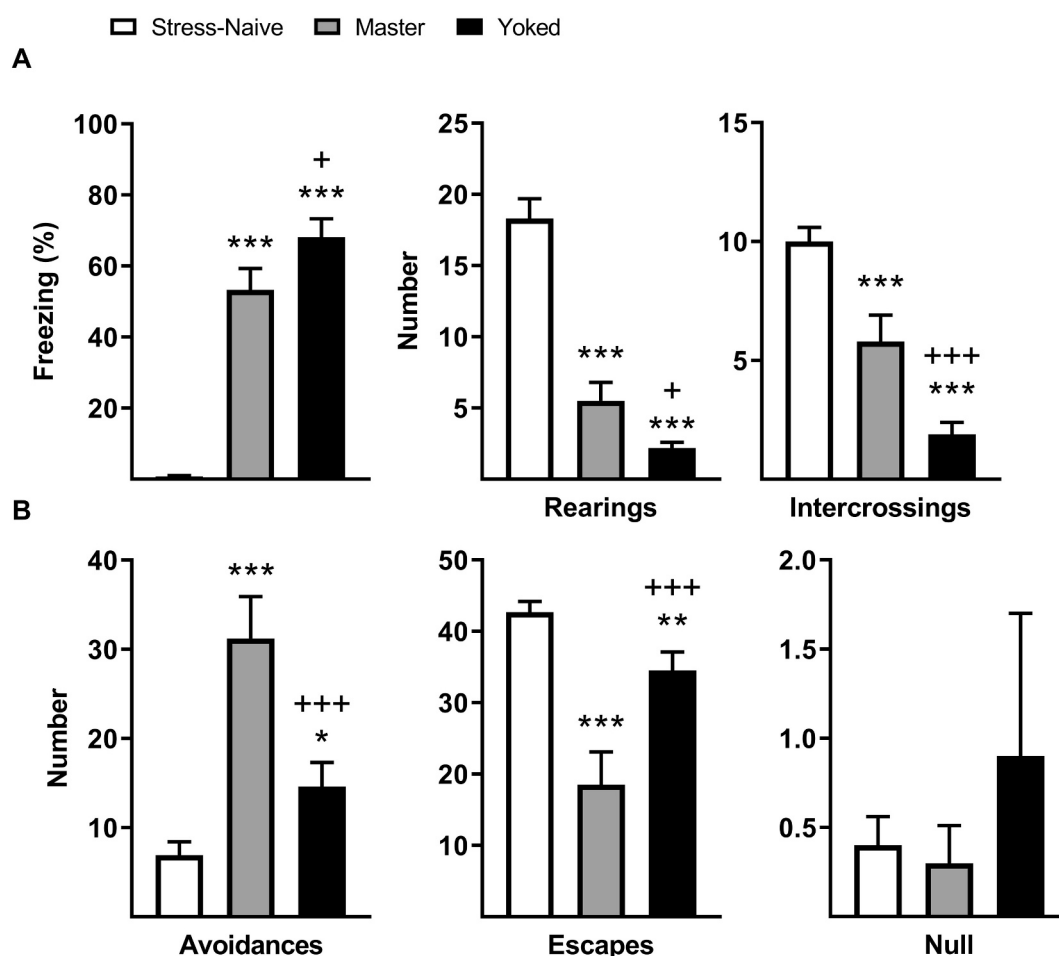


Fig. 5. Adolescent stress conditions affect behavior of animals during adult exposure to the shuttle-box. Means $\pm$ SEM are represented. Rats from each adolescent condition ($n = 30$ per group) were exposed when adults to the same apparatus (shuttle-box) without receiving shocks to evaluate long-term fear conditioning (Panel A). Panel B indicates the performance of the rats exposed to the adult master condition ($n = 10$ per group). * Indicates significant differences with respect to stress-naïve, + indicates significant difference versus masters. One symbol indicates p at least <0.05 , two symbols p at least <0.01 , three symbols $p < 0.001$.

avoidance performance in a TWAA task during adulthood as compared with stress-naïve rats. This improved performance in the two stressed groups was observed despite behavioral and endocrine evidence of contextual fear conditioning to the shuttle-box. Some differences were also observed between master and yoked rats regarding adult anxiety-

like and social behaviors, with a greater negative impact in yoked than in master rats.

It has been repeatedly demonstrated in adult rats, using the classical learned-helplessness procedure developed by Weiss and extensively studied by Maier's lab, that prior experience with inescapable tail-shocks

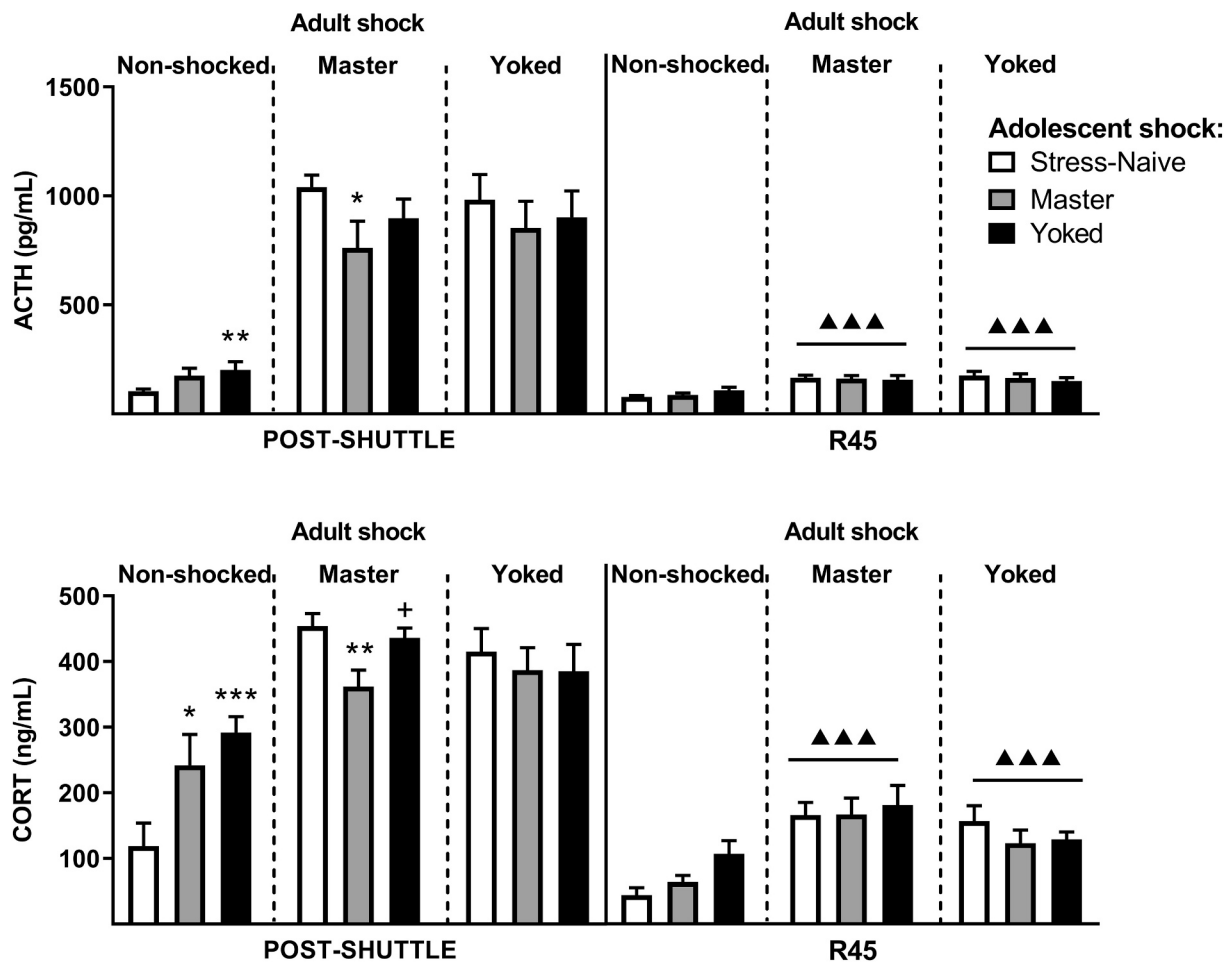


Fig. 6. Impact of adolescent stress groups on plasma ACTH and corticosterone responses to the various adult shock conditions. Means $\pm$ SEM are represented ($n = 10$ per group). Rats from each adolescent group were exposed when adults to each of the three adult conditions (letters above the bars): the apparatus (shuttle) without receiving shocks (non-shocked), the master condition or the yoked condition. Blood samples were taken immediately after the adult session (post-shuttle) and again 45 min later (R45). * Indicates significant differences with respect to adolescent stress-naïve within the same adult condition; indicates significant difference versus adult non-shocked condition; one symbol p at least <0.05 , two symbols p at least <0.01 , three symbols $p < 0.001$.

(IS) resulted in impaired performance in a two-way active escape task (Maier and Watkins, 2005). The deficit typically lasted for a few days, and it is not observed in those animals that could terminate the tail-shocks by turning a wheel with their front paws and, therefore, had control over the termination of shocks (ES group). Importantly, seminal studies demonstrated that a single prior ES experience protected rats from the negative consequences of further exposure to IS, a phenomenon called immunization (Seligman et al., 1975; Williams and Maier, 1977; Williams and Lierle, 1986). Later, similar results were obtained in juvenile rats (Kubala et al., 2012).

In the present experiment, we studied the long-term endocrine and behavioral impact of repeated experience of controllability or uncontrollability over shocks during adolescence using a TWAA task that involves foot-shock rather than tail-shock exposure. At adulthood (PND 93–95), they were tested for anxiety-like behavior in the EPM or for social interaction. None of the two stressed groups showed evidence of enhanced anxiety-like behavior in the EPM. Nevertheless, master rats showed fewer entries in the closed arms and moved less in them than stress-naïve rats, effects that were less evident in yoked animals. There is no clear explanation for these results. In fact, there are very controversial data about the impact of inescapable shocks on the EPM, including some reluctance to enter and remain in the closed arms (e.g., Steenbergen et al., 1990, 1991; Radulovic et al., 1998; Belda et al., 2004; Daviu et al., 2010; Girardi et al., 2013; Korem and Akirav, 2014; Viviani et al., 2012, 2015). It has been suggested that this reluctance to enter the

closed arms might be related to the association of the enclosed arms with the dark chamber where they received the shocks (Viviani et al., 2015). The reasons for this association being stronger in ES rats are at present unclear, although there is a possibility that the ES rats learned to be still and attentive to the CS during the TWAA task, transferring this learned strategy to other spaces that resemble the shock chamber.

In the social interaction test, master rats behaved normally, but yoked rats showed a reduced distance travelled and time spent following (a type of active social interaction), as well as a trend toward increased passive social interaction. Whether these effects on social interaction are related to reduced activity in yoked rats remains unclear. Although master rats also tended to spend less time engaging in active social interaction, they did not differ from stress-naïve in other measures and, in fact, showed higher levels of following and overall activity than yoked rats. Therefore, the results suggest that control over stress during adolescence might mitigate some of the negative consequences of uncontrollable ELS. To our knowledge, there are no previous studies on the impact of controllable foot-shocks during adolescence on adult anxiety-like behavior. Other studies in male rats have found that exposure to inescapable foot-shocks during adolescence reduced activity in an open-field (OF) (Lytle et al., 2015) or both OF activity and social interaction (Aga-Mizrachi et al., 2014; Zhao et al., 2020). Hypoactivity in an OF and enhanced anxiety in the EPM have also been reported in other studies using the same stressor (Li et al., 2015; Gomes and Grace, 2017; Liu et al., 2017; Zhao et al., 2020). In mice, no effect of adolescent foot-

Adolescent shock: □ Stress-Naïve ■ Master ■ Yoked **

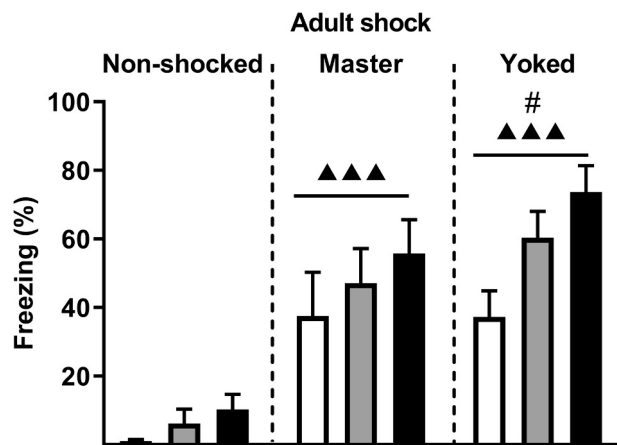


Fig. 7. Impact of both adolescent and adult conditions on contextual freezing in the shuttle-box. Means $\pm$ SEM are represented ($n = 10$ per group). Rats from each adolescent group were exposed when adults to each of the three adult conditions (letters above the bars): the apparatus (shuttle) without receiving shocks (non-shocked), the master condition or the yoked condition. The symbols above the bars indicate differences between the three adult conditions. The * besides the identification of the adolescent groups indicate significant effects of the adolescent group, regardless of the adult condition; $\blacktriangle$ indicates significant difference versus adult non-shocked condition, regardless of adolescent group; # Indicates significant differences versus adult master condition, regardless of the adolescent group; one symbol p at least <0.05 , three symbols $p < 0.001$.

shock exposure on the EPM was found despite clear OF hypoactivity (Spinelli et al., 2013). Using the same procedure, another group did report reduced social interaction, although changes in anxiety-like behavior were inconsistent (Cavicholi et al., 2023). The reasons for the inconsistent results in the EPM remain unclear. Age might be a contributing factor, as Zhu and Grace (2023), combining repeated exposure to foot-shocks and restraint in adolescent rats of both sexes, observed enhanced anxiety only in females exposed to stress between PN21–30, whereas exposure between PN41–50 had no effect in either sex. Another putative factor is the extent to which the closed arms of the EPM might be reminiscent of the small boxes where the rats are typically exposed to foot-shocks (Daviu et al., 2010).

When the rats were exposed to a TWAA task approximately two months after the adolescent stress period (PND 120), both master and yoked rats showed greater freezing than stress-naïve rats during the habituation period preceding the task, demonstrating that fear conditioning to the context (shuttle box) persisted over this extended time period. Importantly, this contextual freezing was lower in master than in yoked rats, suggesting that control over the shocks caused less persistent fear, in accordance with data in adult rats (Ilin and Richter-Levin, 2009; Hadad-Ophir et al., 2017). This differential contextual fear between master and yoked animals was not observed during the adolescent task (present data and Sanchís-Ollé et al., 2019), suggesting either a ceiling effect during adolescent exposure or a stronger long-term fear memory in yoked rats.

Despite marked contextual freezing before the adult task, both master and yoked rats showed improved performance in the TWAA task with respect to stress-naïve rats, although this effect was much stronger in the master rats. That prior experience with a similar task during the peri-adolescent period enhances performance of the same task during adulthood is in accordance with prior experiments in rats (Gruss et al., 2010; Bock et al., 2014), although contextual fear conditioning was not assessed by these authors. It thus appears that prior repeated experience of control in the TWAA task can clearly overcome the possible drawback of freezing before the task. However, the finding that yoked

rats showed better performance in the TWAA task than stress-naïve rats was unexpected. A possible interpretation is that learning was improved because prior experience with shocks reduced their aversive properties. In fact, this was also the case when the ACTH response to the first and last adolescent session was compared in both master and yoked rats (Sanchís-Ollé et al., 2019), and similar results have been observed in adult rats repeatedly exposed to inescapable foot-shocks when their intensity was of a magnitude similar to the one used in the present study (Rabasa et al., 2011).

In other studies, rats exposed during adolescence for 3 days to three different stressors (swim, elevated platform and foot-shocks, one per day), showed impaired TWAA performance in adulthood (Tsoory and Richter-Levin, 2006), although it remains unclear whether this effect was specifically driven by foot-shock exposure, as a similar outcome was observed following exposure to a combination of swim, elevated platform, and restraint (Tsoory et al., 2008). The present results are in partial agreement with prior studies in adult rats comparing exposure to a TWAA task (master) with the corresponding yoked condition (Ilin and Richter-Levin, 2009; Lucas et al., 2014; Hadad-Ophir et al., 2017). Thus, prior exposure to 6 sessions of a TWAA task resulted in less hypoactivity in a novel setting than the corresponding yoked rats and less freezing to both the shuttle context and the CS 2–4 weeks later. Also, master rats showed better performance in a shuttle-box active avoidance task than stress-naïve and yoked rats, although the authors changed the CS (lights instead of noise) and some contextual aspects of the shuttle during the final TWAA task (Lucas et al., 2014). Interestingly, in the latter study, yoked rats showed similar levels of avoidances than stress-naïve rats, but less escape and more null responses in the second study, suggesting some performance deficit. This discrepancy can be due to the different age of the rats or to procedural variations. These authors did not use paired master-yoked animals, but instead calculated the averaged latency to avoid/escape per trial in the master rats and applied the same shock schedule to all yoked rats. It is unclear whether these are relevant factors. It is noteworthy that several studies in adult rats support a positive effect of prior foot-shock exposure on a subsequent two-way active escape/avoidance task (Koba et al., 2001; Sawamura et al., 2004; Wakizono et al., 2007; Lee et al., 2008; Kikuchi et al., 2008). Interestingly, it has been demonstrated in adult mice that repeated experience of control over shocks favors active strategies in response to a novel aversive stimulus (Daviu et al., 2020), although no stress-naïve group was included. It would be relevant in future experiments to study whether the improved performance observed in yoked rats could extend to situations involving aversive stimuli other than footshock.

To obtain additional information about how prior adolescent experience altered the stressful properties of uncontrollable and controllable foot-shocks, the HPA response to adult exposure was also studied. We firstly demonstrated that prior adolescent treatment did not alter basal levels of ACTH or corticosterone, as these levels were similar in all groups both at the nadir and the peak of the circadian rhythm. The circadian pattern observed is fully in accordance with previous findings that ACTH levels hardly double during the circadian peak whereas a 10-fold increase is observed in corticosterone levels (e.g., Dallman et al., 1978; Atkinson and Waddell, 1997; Fuentes et al., 2014). In those rats solely exposed to the shuttle box in adulthood but not shocked, both plasma ACTH and corticosterone levels were higher in master and yoked compared with stress-naïve rats, reflecting the conditioned response to the context, although no differences were found between master and yoked despite their differential levels of contextual freezing. Thus, the present data confirm that HPA hormones are sensitive to fear conditioning (e.g., Van De Kar et al., 1991; Merino et al., 2000; Daviu et al., 2010, 2014), but they also suggest that behavioral parameters are better than the endocrine response at discriminating between different levels of fear (Daviu et al., 2024).

The ACTH and corticosterone levels in response to shocks followed the expected temporal pattern, with a marked decline during the 45 min recovery period. Therefore, it is more relevant to focus on their levels

just after the termination of shock exposure. It is of note that the information we can obtain from these plasma ACTH and corticosterone levels is complicated by the fact that the actual response to the task must consider the levels observed in the absence of shocks (fear conditioned levels). Plasma ACTH and corticosterone are good markers of stress intensity, although corticosterone is more appropriate for low intensity stressors (see Armario et al., 2020), as is the case of the non-shocked adult rats. Both ACTH and corticosterone levels after the adult TWAA task (master condition) were lower in adolescent master than in adolescent stress-naïve rats. These lower levels were observed despite the already high levels in the rats merely exposed to the shuttle-boxes (no adult shocks) that reflected conditioning. The lower actual ACTH response to the TWAA task in the adolescent master rats was not unexpected considering the much-improved avoidance during the task, which reduced the time receiving shocks and the stressful properties of the situation. Did prior adolescent yoked experience change the response to the adult TWAA task? A reduced response was not found in absolute terms, but it is plausible if we consider the already high levels of hormones observed in the absence of shocks. This apparently reduced response of adolescent yoked rats could be explained by the better performance (and less shock time) in the adult TWAA task compared with adolescent naïve rats.

To determine whether the adolescent condition could have affected the development of contextual fear conditioning related to adult foot-shock exposure, all rats were exposed a week after the adult sessions to the shuttle box, without shocks. As expected, high levels of freezing were observed in all rats having master or yoked adult experience as compared to those not receiving shocks, but overall levels of freezing were higher in rats that experienced the yoked condition during adolescence, whereas adolescent master rats were similar to adolescent stress-naïve animals. It thus appears that a history of uncontrollable foot-shock experience during adolescence exerts a long-lasting potentiation of fear conditioning. These results are, at first glance, similar to those showing that previous exposure to severe inescapable footshocks can further enhance fear conditioning (Rau et al., 2005; Perusini et al., 2016; Serrano et al., 2025). However, in the latter studies conditioning was done in a context B different from that of the inescapable footshocks, and the phenomenon appears to involve sensitization rather than potentiated conditioning.

In conclusion, the present results indicate that repeated experience of adolescent rats with controllable foot-shocks (the TWAA task) did not exert major long-term negative consequences on the HPA axis or behavior, whereas exposure to the same conditions of uncontrollable foot-shocks exerted mixed effects, with some evidence for a negative impact on social interaction and activity in novel environments, and also regarding adult foot-shock induced fear conditioning. The present data encourage further studies about the differential impact of control over stress in adult animals, but particularly during youth and adolescence.

CRedit authorship contribution statement

María Sanchís-Ollé: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Roser Nadal:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Silvia Fuentes:** Writing – review & editing, Validation, Methodology, Data curation. **Gisela Rusiñol:** Writing – review & editing, Visualization, Validation, Data curation. **Antonio Armario:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Ethical statement

The experimental protocol was approved by the Committee of Ethics of the Universitat Autònoma de Barcelona and by the Generalitat de

Catalunya and was carried out in accordance to the European Communities Council Directive (2010/63/EU) and Spanish Legislation (RD53-2013).

Declaration of competing interest

None.

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Data availability

Data will be made available on request.

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