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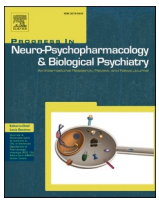

# A mouse model of the 3-hit effects of stress: Genotype controls the effects of life adversities in females

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ABSTRACT

Helplessness is a dysfunctional coping response to stressors associated with different psychiatric conditions. The present study tested the hypothesis that early and adult adversities cumulate to produce helplessness depending on the genotype (3-hit hypothesis of psychopathology). To this aim, we evaluated whether Chronic Unpredictable Stress (CUS) differently affected coping and mesoaccumbens dopamine (DA) responses to stress challenge by adult mice of the C57BL/6J (B6) and DBA/2J (D2) inbred strains depending on early life experience (Repeated Cross Fostering, RCF). Three weeks of CUS increased the helplessness expressed in the Forced Swimming Test (FST) and the Tail Suspension Test by RCF-exposed female mice of the D2 strain. Moreover, female D2 mice with both RCF and CUS experiences showed inhibition of the stress-induced extracellular DA outflow in the Nucleus Accumbens, as measured by *in vivo* microdialysis, during and after FST. RCF-exposed B6 mice, instead, showed reduced helplessness and increased mesoaccumbens DA release. The present results support genotype-dependent additive effects of early experiences and adult adversities on behavioral and neural responses to stress by female mice. To our knowledge, this is the first report of a 3-hit effect in an animal model. Finally, the comparative analyses of behavioral and neural phenotypes expressed by B6 and D2 mice suggest some translationally relevant hypotheses of genetic risk factors for psychiatric disorders.

## 1. Introduction

Approaches to mental diseases based on 'precision,' 'individualized,' or 'stratified' strategies might help to increase the now-limited effects of therapeutic strategies. These approaches require the identification of markers of individual variability for risk, severity, and sensitivity to treatments (Schumann et al., 2014; Dean, 2019; Northoff and Tumati, 2019; Gratton et al., 2020). Coping strategies represent valuable markers because coping is the main moderator of the impact of adversities, and dysfunctional stress coping is associated with different psychiatric conditions (Folkman et al., 1986; Mikulincer and Florian, 2003; Taylor and Stanton, 2007; Brousse et al., 2011; Cabib and Puglisi-Allegra, 2012; Moritz et al., 2016; de Kloet et al., 2019; Roelofs and Dayan, 2022). Life adversities are pathogenic determinants of several mental diseases, with genetic influences moderating their impact at individual levels. Thus, genetic determinants of individual variability have been investigated with increasingly potent tools such as Genome-Wide Association Studies (GWAS). However, several technical obstacles prevent a significant translation of the acquired information at clinical levels. Moreover, since mental diseases are polygenic multifactorial phenotypes, biological sex, and early and proximal experiences must be considered as moderators (Hall et al., 2016; Mathieson, 2021). A classic hypothesis views the action of these factors as cumulative. Thus, genes set susceptibility to environmental challenges (stress-diathesis), leading to a more severe impact of early life experience (ELE) that, in turn, promotes dysfunctional adaptation to adult life experience (Daskalakis et al., 2012; Malave et al., 2022). Clinical and preclinical studies have demonstrated a genetically driven preference toward specific coping

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strategies (coping styles) (Kendler et al., 1991; Cabib et al., 1997; Koolhaas et al., 1999; Cline et al., 2015; Santarnecchi et al., 2018). Moreover, there is compelling evidence that coping strategies are controlled by previous experiences depending on the genotype (Alcaro et al., 2002; Mineur et al., 2006; Mozhui et al., 2010; Cabib et al., 2012; Campus et al., 2016; Ventura et al., 2021).

We have recently reported that exposure to an early unstable environment (Repeated Cross-Fostering: RCF) determines opposite changes in the stress-coping strategies and mesolimbic DA response to stress challenge by adult female mice of the inbred strains C57BL/6 (B6) and DBA/2 (D2). Indeed, adult female mice of the B6 strain exposed to the RCF protocol during the first four days of life showed increased levels of active coping with acute stress challenges compared to same strain controls, whereas RCF-exposed D2 females showed increased levels of passive coping. Moreover, during an acute stressful experience, the stress-induced increase of DA release in the limbic striatum (Nucleus Accumbens: NAc), measured by *in vivo* intracerebral microdialysis, was greater in RCF-exposed B6 females than in same strain Controls, while this response was reduced in RCF-exposed D2 females. Finally, the molecular analysis of the primary source of NAc DA: the Ventral Tegmental Area (VTA), performed by next-generation RNA-Seq, revealed RCF-induced strain-specific alterations almost exclusively in females (Lo Iacono et al., 2021; Di Segni et al., 2016, 2019). The sex-specific effects of RCF align with the preponderance of mood disorders in women (Seney et al., 2022; Pitzer et al., 2022; Abdoli et al., 2022; Hyde and Mezulis, 2020).

Moreover, the increase in passive coping exhibited by RCF D2 female mice indicates a genotype-specific liability to develop helplessness behavior. The new Research Domain Criteria initiative of National Institute of Mental Health (NIMH RDoC, https://www.nimh.nih.gov/research/research-funded-by-nimh/rdoc/index.shtml) describes Helplessness behavior within the Negative Valence Systems Domain as "An aversive emotional state caused by prolonged (i.e., weeks to months) exposure to internal and/or external condition(s), state(s), or stimuli that are adaptive to escape or avoid…". The RDoC approach, that is now guiding the granting activity of NIMH, considers psychopathology in the context of major domains of basic human neurobehavioral functioning along a continuum from normal to abnormal. A human 'state' cannot be translated in animals unless it is associated with a measurable behavioral outcome. A state of helplessness severely interferes with active stress coping. Thus, this state is modeled in animals by the severe reduction of attempts to actively cope with an observable stressor following chronic/repeated adverse experience ('sustained threat': NIMH RDoC). Indeed, passive and active coping are both adaptive responses to stress, but dysfunctional adaptation can push these responses to extreme, pathological, levels (Douma and de Kloet, 2020; de Kloet and Molendijk, 2016; Cabib et al., 2012; Campus et al., 2016; Forgeard et al., 2011; Maier and Seligman, 2016).

Finally, the strain-specific effects of RCF on mesoaccumbens DA response to stress point to this phenotype as a genotype-specific neural target of life adversities. Mice from the D2 and B6 inbred strains are good models to test how the genotype moderates the effects of life experiences. Indeed, comparative studies of phenotypes expressed by inbred mouse strains can best reveal the relative influence of genetic and non-genetic risk factors (Dickson and Mittleman, 2021). Moreover, approaches that can target genotype-specific polygenic composites are more useful than genetic manipulations targeting a single or a few genes to unveil the genetic of complex polygenic multifactorial phenotypes (Vrantsidis et al., 2021). D2 and B6 genotypes differ for~5 million known sequence variants (Wang et al., 2016), and the two strains are the founders of the Recombinant Inbred (RI) lines, proposed amongst the best systems approaches to discover genetic mechanisms underlying phenotypic variation (Ashbrook et al., 2021; Knoll et al., 2018). Indeed, these lines possess recombinant chromosomes containing B6 or D2 haplotype segments. Thus, they are used for quantitative trait loci (QTLs) mapping, the typical approach for identifying the naturally occurring polymorphisms.

For example, B6 mice are less impulsive than D2, as measured by a cognitive test derived from human reaction-time tasks (Loos et al., 2014). A significant QTL for this phenotype was identified in BxD RI strains (derived from a cross between B6 and D2), around marker rs6197032 on chromosome 14: the Impu1 locus, located in a region syntenic with the human 10q22-q23 region (Loos et al., 2014). Targeted genetic manipulations in B6 mice unveiled the involvement of Neuregulin-3 (NrG3) polymorphisms in the variability of the impulsive phenotype in mice (Loos et al., 2014). This phenotype is also greatly influenced by variants of the tryptophan hydroxylase 2 (mTph2) gene known to modulate neurodevelopment, adaptation to stress, and different behavioral disturbances (Siesser et al., 2010; Herrmann et al., 2007; Markett et al., 2017; Waider et al., 2011; Nakamura and Hasegawa, 2007; Chen et al., 2023). B6 mice are homozygous for the 1473C variant of Tph2, whereas D2 mice are homozygous for the 1473G variant (Zhang et al., 2004). Finally, the development of striatal DA synapses is controlled by NrG3 in DA neurons (Cui et al., 2020), and in RCF *versus* control female B6 mice, we have found that the majority of differentially expressed genes in the VTA, the main source of DA afferents to the limbic striatum, are related to synapses, including DA synapses (Lo Iacono et al., 2021).

Lastly, the Chronic Unpredictable Stress (CUS) protocol is a good model of 'sustained threat' in adulthood (NIMH RDoC) which, as discussed above, is considered the main pathogenic pathway to helplessness behavior. Indeed, it models a sequence of unexpected adversities by exposing rodents to various unpredictable stressors. This protocol does not include social stressors, such as social defeat, with some potential translational limitations. However, CUS consistently promotes the significant increase of the immobility observable in the Forced Swimming Test (FST) or the Tail Suspension Test (TST), modeling helplessness behavior induced by Sustained Threat (Tye et al., 2013; Yu et al., 2016; Garcia-Gutierrez et al., 2010; Mineur et al., 2006; Bittar et al., 2021; Karisetty et al., 2017; Woodward et al., 2023; Bai et al., 2017). Finally, CUS and CUS-like protocols induce plasticity within the limbic DA circuit leading to low DA tone in target brain areas (Rincon-Cortes and Grace, 2017; Douma and de Kloet, 2020; Moreines et al., 2017; Tye et al., 2013). Therefore, in the reported experiments, we tested the additive effects of genotype, ELE (RCF), and CUS on 1) measures of anxiety, motor functioning, and sociality, 2) measures of passive coping, and 3) the mesoaccumbens stress-induced DA outflow as evaluated by *in vivo* intracerebral microdialysis in mice exposed to FST.

## 2. Methods

### 2.1. Animals

C57BL/6J (B6) and DBA/2J (D2) female and male mice (Charles River Laboratories, Italy) were housed in transparent polysulfone cages (26.7 × 20.7 × 14.0 cm) with water and food available *ad libitum*, 12:12 h light-dark cycle (lights on 7 AM, lights off 7 PM) and room temperature stable at 21 ± 1 °C. Virgin naive females and males were mated when 12 weeks old. The mating protocol (Di Segni et al., 2016) housed two females and one male in transparent polysulfone cages with water and food *ad libitum*. After 15 days, males were removed, and pregnant females were isolated in clean cages with nesting material and inspected twice daily for delivery. On delivery day (P0) litters were assigned by simple randomization to ELE or Control (RCF/Cont, see below) manipulation, and on P28 animals were weaned, separated by sex, and housed in groups of 4 littermates and left undisturbed until adulthood (P90). To avoid litter effects, experimental subjects were sorted by collecting not > two individuals per cage/litter (D'Amato et al., 2011; Ventura et al., 2013; Di Segni et al., 2016). To minimize pain in mice, adequate measures were taken, and all experiments were carried out according to Italian law regarding the protection and welfare of animals used in scientific procedures (DL 116/92 and DL 26/2014) and European Communities Council Directives (86/609/EEC and 2010/63/UE). The

experimental protocol (no. 769/2017) was approved by the Italian Ministry of Health. 10–12 weeks old mice (for RCF and Control groups: 8–9 female B6, 6–8 male B6, 6–7 female D2, 5–6 male D2) were used for behavioral and *in vivo* microdialysis experiments.

### 2.2. Repeated Cross-Fostering (RCF)

Repeated Cross Fostering procedure, as ELE, was performed as previously described between 10.00 and 10.30 AM (D'Amato et al., 2011; Ventura et al., 2013; Di Segni et al., 2016; Lo Iacono et al., 2021). Briefly, pups from the same litter spent the first postnatal day (P0) with their biological mother. On P1, RCF pups were fostered by moving the entire litter from the home cage to a different adoptive mother's cage, whose pups had just been removed. This procedure was repeated four times, from P1 up to P4, until the fourth adoptive mother was reached (Fig. 1a). Pups were left with the last adoptive mother until weaning (P28). Control (Cont) litters (from P1 to P4) were only picked up daily, reintroduced in their home cage, covered with home-cage bedding, and had their mothers returned within 30 s.

### 2.3. Chronic Unpredictable Stress (CUS)

For three weeks (from P90 to P111), RCF and Cont groups of B6 and D2 female and male mice were exposed to a chronic unpredictable stress procedure (modified version of van Boxelaere et al., 2017; Fig. 1a). The CUS procedure was characterized by stressors presented in the dark (7 PM-7 AM) or light phases (11 AM-5 PM) of the day (Supplementary Table 1), like: exposure to wet mash (200 ml of water was added to home cage bedding material, and after 4 h, mice were moved to cleaned cages), overnight illumination (cages were moved to a different room of animal facility with same conditions except for lights on all night long), food restriction (for 4 h, from 2 to 6 PM, food was removed to the home cage but the water was maintained *ad libitum*) and brief social isolation (mice were housed individually for 3 days). Interspersed with unpredictable stressors (see Fig. 1a and Supplementary Table 1), behavioral tests that belong to the negative valence system (Söderlund and Lindskog, 2018) were performed to assess the effects of the CUS procedure on anxiety (elevated plus maze), social preference (social preference test), and coping strategy (tail suspension test and forced swimming test). Before any testing procedure, animals were habituated to the experimental room for 1 h to avoid confounding effects and once a week (after the elevated plus maze test), the weight of mice was recorded.

### 2.4. Social Preference Test (SPT)

At P 90–100-105 (between 9 and 12 AM), Social Preference Test was conducted in a gray rectangular plexiglass apparatus (60 × 40 × 24 cm) consisting of a central starting chamber connected with two chambers containing two identical clear cylinders (plexiglass, 8 cm diameters) with multiple small holes (Fiori et al., 2015). In the habituation session (10 min), mice were placed in the starting chamber and left free to explore the whole apparatus. During the test session (10 min), a novel social stimulus (age and sex-matched mouse) was introduced into one cylinder and a neutral object was introduced into the other. The position of stimuli within cylinders was alternated to avoid potential confounding effects (Lo Iacono et al., 2021) and a different social stimulus for each week was used to reduce the habituation effect. Sessions were recorded and the time spent exploring each cylinder (*i.e.* contact with social stimulus and object) was manually scored with "Boris" software (Friard and Gamba, 2016) by a trained observer blinded to the animals' treatment, sex, and genotype. Percentages of time spent in contact with the social stimulus (social stimulus interaction (s)*100/social stimulus contact (s) + object contact (s)) were evaluated. The apparatus was carefully cleaned with a 5% ethanol solution at the end of each test session.

### 2.5. Tail Suspension Test (TST)

At P91–99-106 (between 1 and 6 PM), Tail Suspension Test was conducted (Yan et al., 2015). Mice were individually suspended by the tail 60 cm above the floor in a neutral plastic chamber using adhesive tape placed 1 cm from the top of the tail (Yan et al., 2015). Behavior was video recorded for 10 min with a digital camera placed in front of the apparatus. The duration of immobility (period when the animals stopped struggling for ≥1 s) and active behavior was manually scored by "Boris" software (Friard and Gamba, 2016) by a trained observer blinded to the animals' treatment, sex, and genotype. Time spent in immobility and active behavior (s) was taken as dependent variables. The apparatus was carefully cleaned with 5% ethanol solution at the end of each test session.

### 2.6. Forced Swimming Test (FST)

At P96–103-108 (between 1 and 6 PM), the Forced Swimming Test was performed as previously described (Lo Iacono et al., 2021). Mouse was individually placed in a glass cylinder (height 40 cm, diameter 18 cm) filled with 20 cm of water (28 ± 2C°). The behavioral response was video recorded for 10 min using a digital camera placed in the front of the cylinder. The duration (s) of immobility (absence of movement) and swimming (active swimming) were taken as the dependent variables and manually scored with "Boris" software (Friard and Gamba, 2016) by a trained observer blinded to the animals' treatment, sex, and genotype. In addition, an extended version of the Forced Swimming Test (20 instead of 10 min, as reported in Babicola et al., 2020) was performed during the microdialysis experiment (see Section 2.9).

### 2.7. Calculation of Unified Passive Score

Time spent in immobility (as outcome of passive coping strategy) in FST and TST was normalized to obtain a Unified Passive Score (UPS) as previously reported (Harrison et al., 2020; Thornton et al., 2021). For each test, the passive score was calculated using the following formula:

$$MS = X(i)/X(m)$$

where MS: passive score for each test, X(i): time spent in immobility, X(m): maximum measure datum in the cohort (all mice for each sex). The passive score normalized was a value between 0 (low passive coping) and 1 (high passive coping) for each mouse in each test. The UPS for each mouse was subsequently calculated as the mean value of passive scores obtained in FST and TST.

### 2.8. Elevated Plus Maze (EPM)

At P93–98-108 (between 9 and 12 AM) the Elevated Plus Maze test was performed (Cabib et al., 2003; Di Segni et al., 2016). Mouse was individually tested in a single 5-min session in a gray plexiglass apparatus elevated 38.5 cm above the floor, consisting of two open (27× 5 cm) and two closed (27 × 5 × 15 cm) arms connected by a central area (5 × 5 cm) representing the starting point of the session. The percentage of time spent in the open arms (time in open (s) *100 /time in open (s) + time in closed (s)) and moved distance (cm) were recorded and automatically scored by "EthoVision" (Noldus, the Netherlands) video tracking system. The apparatus was carefully cleaned with a 5% ethanol solution at the end of each test session. After the test session, animals' weight was recorded and was taken as a dependent variable.

### 2.9. In vivo microdialysis

At the end of the CUS procedure, mice were implanted with a microdialysis probe in the Nucleus Accumbens (NAc) and dopamine (DA) release during the extended version of the forced swimming test

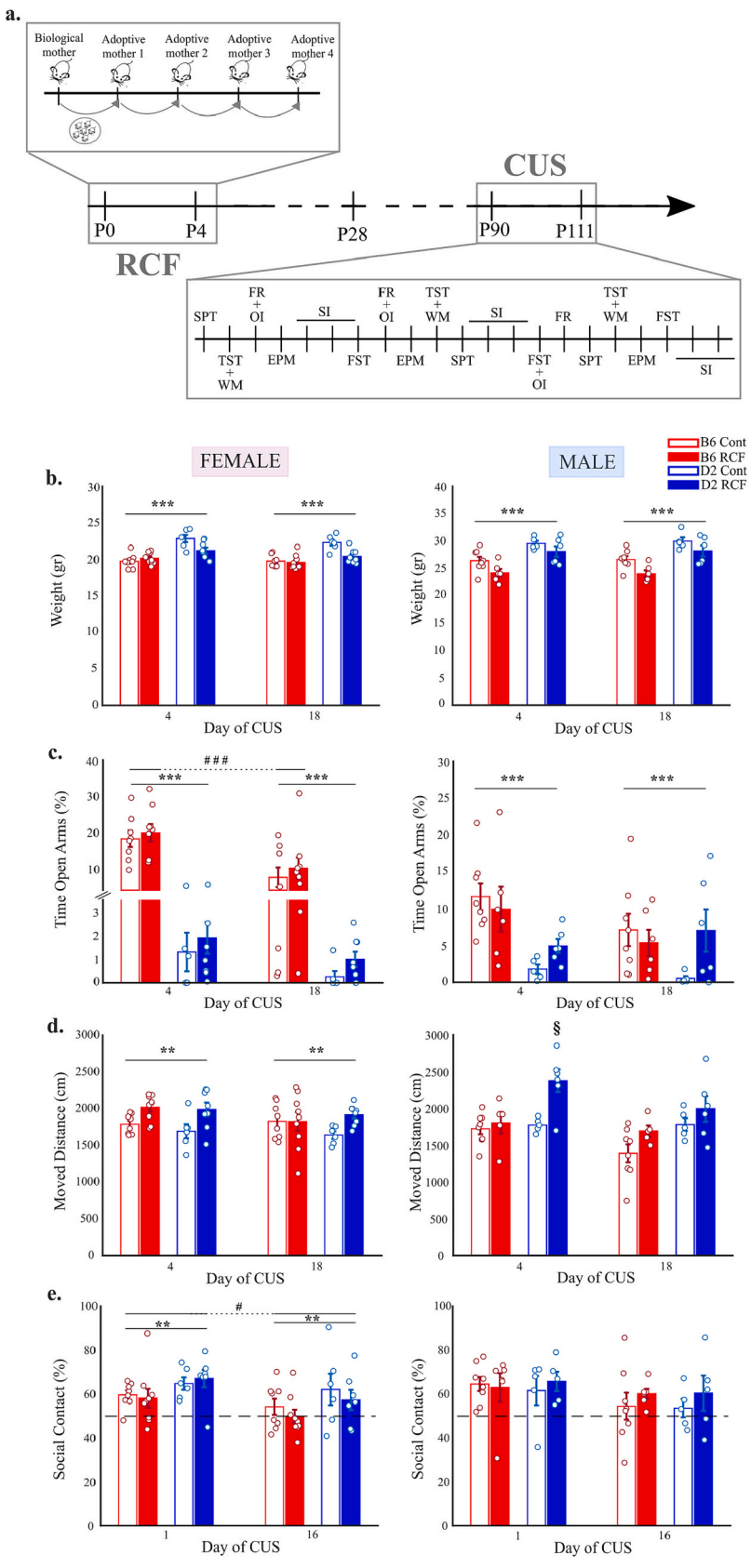


**Fig. 1.** Impact of CUS on weight, anxiety, and preference for social stimuli. (a) Graphic representation of Repeated Cross Fostering (RCF, from Postnatal day 1 (P1) to P4) experience and experimental timeline of Chronic Unpredictable Stress protocol (CUS, from P90 to P111; SPT: Social Preference Test, TST: Tail Suspension Test, WM: Wet Mash, FR: Food Restriction, OI: overnight illumination, EPM: elevated plus maze, SI: Social Isolation, FST: Forced Swimming Test). (b) Body weight in the first and last day of the CUS protocol of RCF (filled bar) and Cont (empty bar) groups of B6 (red) and D2 (blue) female (left) and male (right) mice (c) Percentage of time spent in open arms and moved distance (d.) during EPM, measured during the first and last time point of CUS in all groups. (e). Percentage of time spent interacting with the social stimulus at the first and last time point of CUS in all groups. All Data are represented as mean ± SEM. Number of animals for RCF and Control groups: 8–9 female B6, 6–8 female D2, 6–8 male B6, 5–6 male D2; ** $p < 0.01$, *** $p < 0.001$: Main effect of Strain, comparison between B6 and D2; # $p < 0.05$, ### $p < 0.001$: Comparison between first and last time point (pairwise comparisons); § $p < 0.05$: Difference with all other groups on the same day. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was analyzed. Animals were anesthetized with Zoletil 100 (Virbac, Milano, Italy (tiletamine HCl 50 mg/ml + zolazepam HCl 50 mg/ml) and Rompun 20 (Bayer S.p.A Milano, Italy (xylazine 20 mg/ml), dissolved in a volume of 4.1 and 1.6 mg/ml, respectively, in saline and injected in a volume of 7.3 ml/kg and mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) equipped with a mouse adapter. NAc probe implantation was counterbalanced in right and left hemispheres.

The coordinates from bregma for probe implantation were + 1.6 AP, ±0.6 L (B6) and + 1.1 AP; ±0.6 L (D2) (Franklin and Paxinos, 2001) Mouse Brain Atlases, The Mouse Brain Library, www.nervenet.org) with different depths ventral to the skull surface for each strain (B6 5.5 mm; D2 5.0 mm). Vertical concentric dialysis probes implanted were prepared with AN69 fibers (Hospal Dasco, Bologna, Italy), according to the previously reported method (Di Chiara et al., 1993; Ventura et al., 2013; Di Segni et al., 2016) with different shaft lengths for B6 (4.5 mm) and D2 (4.0 mm) with the same dialysis membrane length (1 mm o. d. 0.24 mm), and were tested to verify *in vitro* dopamine recovery. The microdialysis probe was connected to a CMA/100 pump (Carnegie Medicine Stockholm, Sweden) through PE-20 tubing and an ultra-low torque liquid swivel (Model 375/D/22QM, Instech Laboratories, Inc., Plymouth Meeting, PA, USA) to allow free movement. Artificial cerebrospinal fluid was pumped through the dialysis probe at a constant 2 μl/min flow rate.

*In vivo* microdialysis sessions were conducted 48 h after probe placement, and dialysate samples were collected every 10 min (20 μl each). The mean concentration of three samples collected immediately before the forced swimming test (<10% variation) was taken as basal concentration. Samples were collected during exposure to the extended version of forced swimming test (20 min duration; 2 dialysate samples) and 10 min after its end (post-FST) (Babicola et al., 2020). Dialysate samples were analyzed as reported (Di Segni et al., 2016, 2019) by ultra-high performance liquid chromatography (UHPLC) consisting of an UltiMate 3000 (Thermo Fisher Scientific S.p.A. Italy) system and a coulometric detector (UltiMate 3000 ECD-3000RS) provided with an analytical cell (6011RS ultra Coulometric Cell). Electrode 1 was set at 100 mV, and electrode two at 250 mV. A C18 column (ACCLRSLC PA2 2.2 U2.1 × 100, Thermo Fisher Scientific S.p.A. Italy) maintained at 35 °C was used coupled with a Sentry Guard pre-column (ACCLAIM, V-2 GUARD). The mobile phase consisted of 3% methanol in 0.1 M Na phosphate buffer, 1.3 mM Na2 EDTA, 0.34 mM 1-octane sulfonic acid Na salt (Sigma Aldrich, USA), pH 3.5. The concentrations (pg/15 μl) were corrected for probe recovery.

### 2.10. Probe placement

At the end of the experiments, mice were sacrificed, and brains were postfixed in 4% paraformaldehyde overnight and then cryoprotected in PBS-30% sucrose solution. Correct probe placement was evaluated by visually examining the probe tracks on Nissl-stained coronal sections (40 μm). Only mice with correct probe placement were considered in the analyses.

### 2.11. Statistics

Behavioral data of Control groups were investigated by 2-way ANOVAs (2 independent factors: Strain, 2 levels: B6 and D2; and Sex, 2 levels: Female and Male). Moreover, Behavioral data and UPS of RCF and Control (Cont) groups were statistically evaluated by 3-way ANOVAs (2 independent factors: Strain, 2 levels: B6 and D2, and ELE, 2 levels: Cont and RCF; 1 repeated measure CUS, 2 levels: first and last week of experience). Pairwise comparisons between groups were used to test group differences when significant interaction was attained. For microdialysis data, analysis of DA release in the NAc was conducted by 3-way ANOVA, for each sex, with time-point of sample collection (pg/μl) (Time, 4 levels: baseline, 10- and 20-min time FST, post FST) as repeated measure, and Strain (2 levels: B6 and D2) and ELE (2 levels: Cont and RCF) as independent factors. Moreover, to evaluate the effects of FST exposure on DA outflow, 2-way ANOVA with time-point of sample collection (pg/μl) (Time, 4 levels: baseline, 10- and 20-min time FST, post FST) as the repeated measure, and ELE (2 levels: Cont and RCF) as independent factor was performed within each genotype. Basal DA levels in NAc (pg/μl) of D2 and B6 (females and males, RCF and Cont groups) were compared by Student *t*-test. In a subsequent analysis, to compare the difference in % DA modifications induced by FST between RCF and Control within each strain, we performed separate 2-way ANOVAs (ELE x Time) on data collected as percentage modification over basal value (1 independent factor: ELE, 2 levels: Cont and RCF; 1 repeated measure: Time, 3 levels: 10 and 20 Time FST, post FST). Post-hoc analysis was performed when appropriate.

## 3. Results

### 3.1. Weight and behavioral differences between control groups

Weight and behavioral parameters investigated in Control groups are reported in Supplementary Fig. 1 and Supplementary Table 2. Strain and sex differences are evident only in weight analysis (main effect of Strain: $p < 0.0001$; main effect of Sex: $p < 0.0001$), with male mice being heavier than females and D2 mice heavier than B6. Analysis of behavioral parameters shows that only the factor strain drives differences in EPM (main effect of Strain: $p < 0.0001$), FST (main effect of Strain: $p < 0.0001$), TST (main effect of Strain: $p < 0.0001$) and UPS (main effect of Strain: $p < 0.0001$). Accordingly, D2 mice show higher open arms avoidance compared to B6, and less passive coping behavior as observed by FST, TST and UPS.

### 3.2. Effect of 3-hit interaction on weight of female and male mice

Weight (Mean g ± SEM) measured on the first (I, P93) and final day (Week III, P107) of the CUS protocol in the six groups of mice used in these experiments are reported in Fig. 1 (top; Females on the right, Males on the left), and statistical results are reported in Supplementary Table 3. Strain differences are evident both in female and male mice (Main effect of Strain: Female = $p < 0.0001$, Males = $p < 0.0001$) with D2 mice heavier than B6. Moreover, interaction between ELE and Strain ($p < 0.01$) shows that RCF female D2 mice are lighter than their Controls, whereas in males this difference is present regardless of the genotype (main effect of ELE: $p < 0.05$). In conclusion, CUS protocol induces weight loss only in female groups (main effect of the factor CUS: $p < 0.001$).

### 3.3. Effect of 3-hit interaction in Elevated Plus Maze of female and male mice

Data collected in the EPM are presented in Fig. 1(c,d) and all statistical results are reported in Supplementary Table 4. Both female (left) and male (right) mice of the D2 strain show extreme levels of open arms avoidance (Main effect of Strain: Females = $p < 0.0001$, Males = $p < 0.01$). The CUS experience in adulthood reduces the time spent in the open arms by female and male mice, regardless of ELE (Female: Fig. 1b. left: interaction Strain x CUS: $p < 0.001$; Male: Fig. 1b. right: interaction Strain x CUS: $p < 0.05$). Specifically, the open arms avoidance evident in female mice is driven by B6 mice with a significant reduction between the first and the last week. None of these effects could be accounted for by changes in motor performance (distance traveled) in female mice. Indeed, the only finding is a main effect of the ELE factor ($p < 0.01$) driven by longer distance traveled by RCF mice (Fig. 1d. left). However, differences are observed in moved distance with a significant Strain x CUS x ELE interaction (Fig. 1d. right; $p < 0.05$) in male mice. Post-hoc analysis reveals that D2 RCF male mice traveled a longer distance compared to the Control group ($p < 0.05$) in the first week.

### 3.4. Effect of 3-hit interaction in Social Preference of female and male mice

Statistical analyses of data collected from SPT (Fig. 1e left) is reported in Supplementary Table 5. A significant main effect of Strain on social preference in females ($p < 0.01$) is due to higher preference expressed by D2 mice. Moreover, CUS affects the social preference overall in female mice regardless of Strain and ELE (main effect CUS: $p < 0.05$). The preference for social stimulus in male mice is not influenced by CUS, ELE or Strain (Fig. 1e right).

### 3.5. Effect of 3-hit interaction on coping strategy of female and male mice and Unified Passive Score

Statistical analyses of data collected in female and male mice in FST and TST are reported in Supplementary Table (Supplementary Table 6 and 7, respectively). Analysis of immobility and active behavior (swimming for FST and active behavior for TST) in female mice reveals a significant main effect of CUS (FST: immobility and swimming $p < 0.0001$; TST: immobility and active $p < 0.0001$) and Strain (FST: immobility and swimming $p < 0.0001$; TST: immobility and active $p < 0.0001$) and a significant Strain x ELE interaction (FST: immobility and swimming $p < 0.0001$; TST: immobility and active behavior $p < 0.001$). Moreover, a significant CUS x Strain interaction for immobility and active behavior expressed in TST ($p < 0.01$) is evident. Data and pairwise comparisons reported in Fig. 2 (left) indicate that female D2 mice of the Control groups are less immobile, and more active, than B6 mice in both tests. Moreover, Control and RCF B6 mice exposed to CUS show a significant increase of immobility and a reduction of swimming, expressed in the FST test (I time point *vs* III time point), whereas only RCF D2 mice show this response. Finally, RCF eliminates strain differences in coping strategies expressed in the two tests. In conclusion, ELE experience induces differences in immobility and swimming in a strain-dependent manner in the FST. Statistical analyses of data collected in male mice reveals a significant main effect of CUS for both test in immobility ($p < 0.0001$) and swimming/active behavior ($p < 0.0001$), while of Strain ($p < 0.0001$) and ELE (immobility $p < 0.05$, swimming $p < 0.01$) for immobility and swimming in the FST. Moreover, a significant interaction between Strain x CUS for immobility and swimming/active behavior expressed in FST ($p < 0.01$) and TST ($p < 0.0001$), and a significant interaction Strain x ELE only for the immobility in FST ($p < 0.05$) are observed. Data and pairwise comparisons reported in Fig. 2 (right) indicate that CUS only increases Immobility and reduces active behavior expressed by mice of the D2 strain in both tests. Moreover, Control and RCF mice of the D2 strain show less immobility and more swimming than B6 mice in the FST. In conclusion, statistical analysis conducted on the UPS (Supplementary Table 8) shows a significant main effect of CUS and Strain both in female ($p < 0.0001$) and in male (CUS: $p < 0.0001$, Strain: $p < 0.001$) mice. Moreover, a significant Strain x ELE interaction is evident in female mice ($p < 0.0001$), and between CUS x Strain in males ($p < 0.0001$).

### 3.6. Effect of 3-hit interaction on stress-induced dopaminergic release in the Nucleus Accumbens of female and male mice

Data collected in the microdialysis experiments are presented in Fig. 3 as percent changes from baseline. 3-way ANOVA analysis performed on raw data (pg/μl; Supplementary Table 9) reveals a significant main effect of "Time" both in female ($p < 0.01$) and male ($p < 0.05$) mice and a significant Strain x ELE x Time interaction ($p < 0.01$) in females. These data indicate that the mesoaccumbens DA response to FST by female mice differed depending on the strain and ELE experience. Therefore, we performed separate 2-way ANOVAs (ELE x Time) on data collected from female mice of each strain. A significant interaction between the two factors is observable in females of the B6 strain ($p < 0.001$). RCF B6 females show a significantly increased DA outflow in the first 10 min of FST, followed by a return to baseline levels. Control mice, instead, show FST-induced decreased mesoaccumbens DA outflow at 20 min that was still significant 10 min after the end of the experience (post FST) (Fig. 3 top, left). Statistical analyses of data collected in D2 females (Fig. 3 bottom left) reveal the main effect of the Time ($p < 0.05$) and ELE ($p < 0.0001$). Indeed, only RCF D2 females show reduced DA outflow at 10 and 20 min that was still evident after the end of the stressful experience (post FST). The same analysis conducted on data collected from male mice of each strain, highlights that only B6 mice show the main effect of the Time ($p < 0.05$) and ELE ($p < 0.05$). Accordingly, only RCF B6 males show reduced DA outflow after 10 min of FST. Moreover, Student *t*-test performed on basal DA outflow in NAc between RCF and Control groups showed a significant difference in DA release between RCF and Control B6 mice regardless of sex (females: $t = 3.07$, $p < 0.05$; males:$t = 2.42$, $p < 0.05$) and in D2 female mice ($t = 3.744$, $p < 0.01$). In a subsequent analysis, to compare the difference in % DA modifications induced by FST, between RCF and Control within each strain, we performed separate 2-way ANOVAs (ELE x Time) (Supplementary Table 10). Statistical analysis shows a significant ELE effect in female B6 ($p < 0.001$) and in D2 regardless of sex (females: $p < 0.0001$, males: $p < 0.05$). Post-hoc analysis shows differences between RCF and Control groups at 10 min of FST in B6 female mice, at 20 min of FST in B6 mice of both sexes, while 10 min after the end of the FST (post FST) in D2 female mice.

## 4. Discussion

The present study was planned to test the additive effects of different risk factors on developing a dysfunctional stress coping. We found evidence of a female-specific 3-hit effect of genotype, ELE, and CUS. Moreover, the findings of the present study point to mesoaccumbens DA response to stress as the target of the different risk factors.

### 4.1. Additive effects of genotype, ELE, and CUS on the development of helplessness behavior by female mice

This study evaluated the genotype, ELE, and CUS coaction on different measures. However, previous findings suggested a modulatory effect of sex on genotype-dependent phenotypes (Lo Iacono et al., 2021; Di Segni et al., 2019). Thus, we first tested whether genotype and sex influenced the chosen measures in baseline conditions. Only weight varied depending on genotype and sex. Moreover, FST and TST immobility levels and the Unified Passive Scores (UPS) were higher in B6 mice than in D2, regardless of sex. The observation that D2 male mice are active copers whereas B6 mice are passive ones is in line with previous findings on immobility levels exhibited by mice of the two inbred strains either in FST or in TST (Pitzer et al., 2022; Mozhui et al., 2010; Van der Veen et al., 2008; Mineur et al., 2006; Ventura et al., 2002).

A strain difference was also evident for open arms avoidance in EPM, a measure of anxiety in animal models, because D2 mice showed extreme levels of avoidance in comparison with B6 mice, regardless of the sex, as previously observed (Ventura et al., 2021; van Boxelaere et al., 2017; Van der Veen et al., 2008; Mathiasen et al., 2008; Mineur et al., 2006; Voikar et al., 2005). The observation that active coping mice are more anxious than passive copers is in line with conflicting findings on the relationship between coping responses and open-arm avoidance in EPM (Blanchard, 2022; Van der Veen et al., 2008; Voikar et al., 2005). Moreover, in mice of the D2 strain, either acute or chronic treatment with the tricyclic antidepressant imipramine is ineffective in reducing arm avoidance in EPM but effective in reducing immobility in TST (Ripoll et al., 2003; Cole and Rodgers, 1995). This finding indicates that, in these mice, open arms avoidance does not model depression-associated anxiety. Finally, neither strain nor sex difference was observed for SPT, in line with previous reports (van Boxelaere et al., 2017; Moy et al., 2004). Based on these findings, we tested the

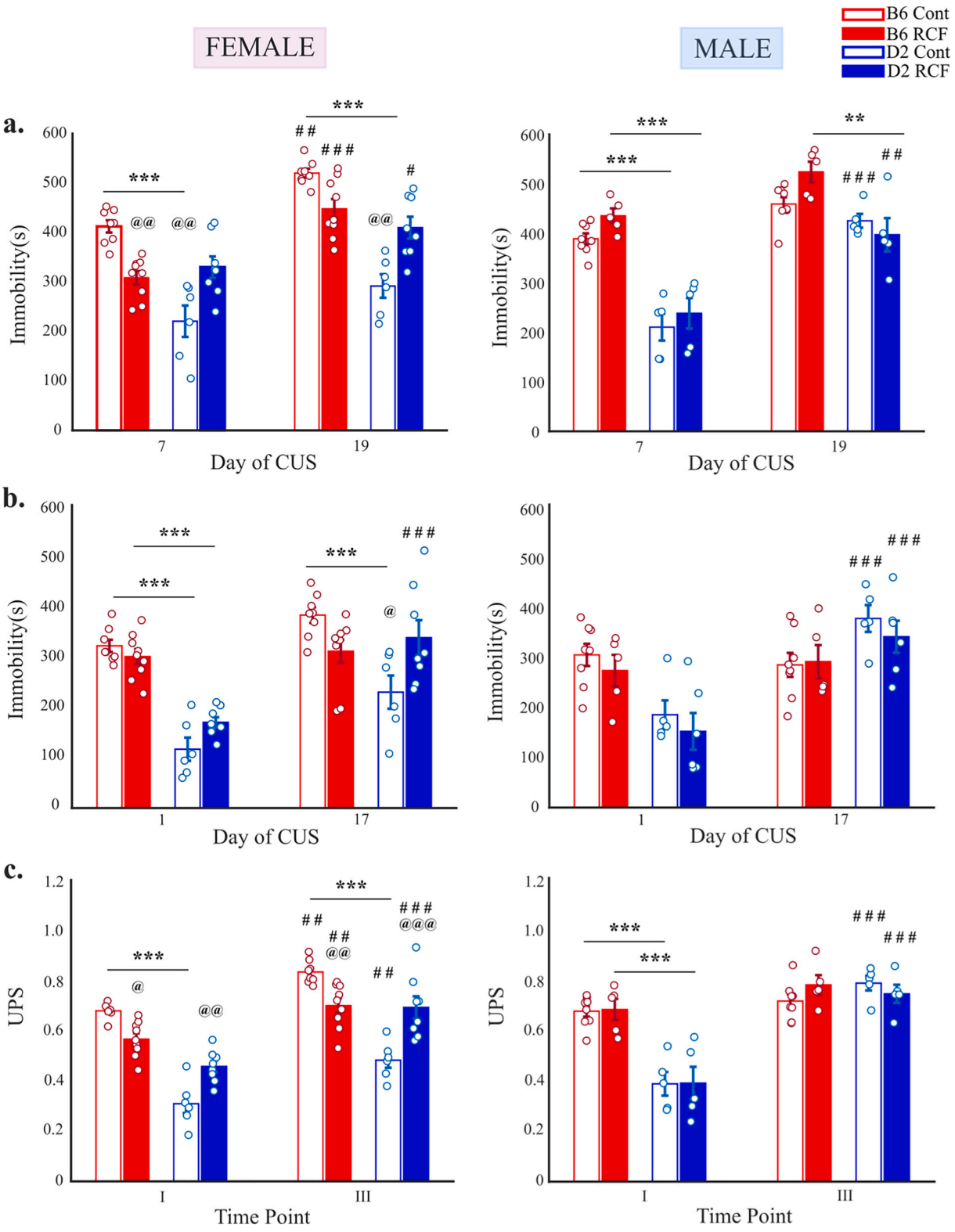


**Fig. 2.** Coping strategies adaptation results from strain, sex, RCF and CUS interplay. Time spent in passive coping behavior (Immobility, s) during FST (a), TST (b) and Unified Passive Score (UPS) (c) in the RCF (filled bar) and Control (empty bar) groups of B6 (red) and D2 (blue) female (left) and male (right) mice. All data are represented as mean ± SEM. Number of animals for RCF and Control groups: 8–9 female B6, 6–8 female D2, 6–8 male B6, 5–6 male D2; ** $p < 0.01$, *** $p < 0.001$: Comparison between B6 and D2; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$: Comparison between first and last time point; @@ $p < 0.01$, @ $p = 0.05$: comparison between RCF and Control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

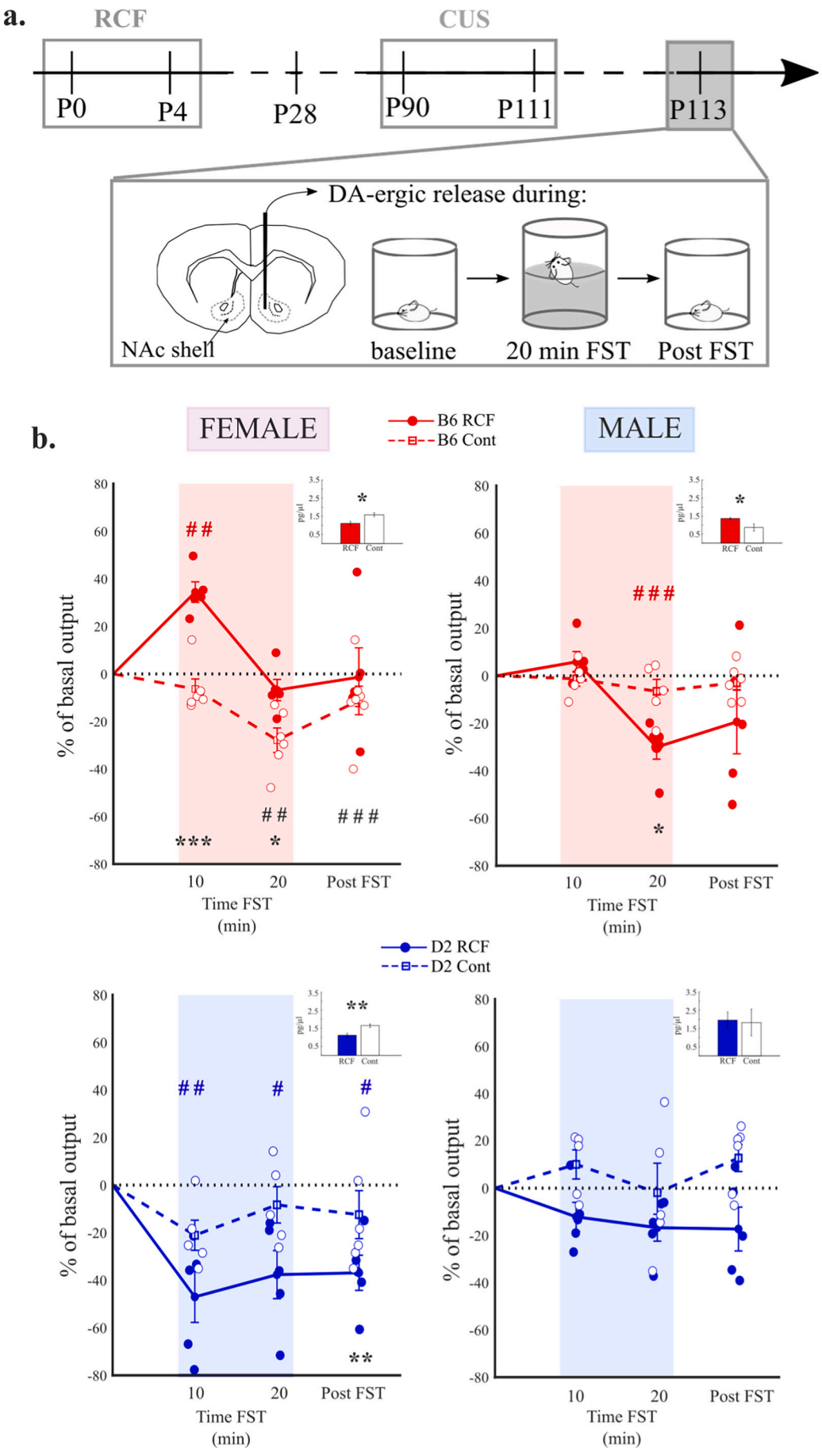


**Fig. 3.** FST-induced dopaminergic outflow in NAc after early and adult adversities. (a) Experimental timeline and graphic representation of *in vivo* intracerebral microdialysis experiment in the NAc following CUS. (b) Extracellular DA-ergic release during (20 min) and after a prolonged FST experience. The results are expressed as percent values from baseline levels of each group with the start of the FST as time 0. Upper side of graphs, analysis of basal DA-ergic outflow (pg/µl) in female (left) and male (right), RCF (filled) and Cont (empty black), B6 (red) and D2 (blue) mice. All data are expressed as mean ± SEM. Number of animals for RCF and Control groups: 6–5 female B6, 5 female D2, 5 male B6, 5 male D2; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$: Comparison with baseline (dotted line for Control groups, solid red line for RCF (red for B6; blu for D2), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: Comparison between RCF and Control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interaction between the effects of genotype and early (ELE) and late (CUS) life adversities separately in male and female mice. The main finding of the present study is that a 3-hit process selectively influences the coping style of female mice. Indeed, CUS increased helplessness expressed by female mice of both strains, but the effect was largest in RCF D2 mice. Moreover, CUS significantly increased the difference between RCF and Control D2 mice, supporting an additive effect. This is the first report of a 3-hit effect involving genotype and early and adult adversities in an animal model (Daskalakis et al., 2013; Peña et al., 2019). Several aspects of the study add to the translational value of its findings. Thus, the RCF protocol involves disrupting the mother-infant relationship (Ventura et al., 2021), a major source of mood disorders (Tamman et al., 2021; Adams et al., 2018; Ponizovsky and Drannikov, 2013). Moreover, the sex-specificity of the additive effects of genotype, early experience, and CUS on helplessness behavior aligns with the preponderance of mood disorders in women (Seney et al., 2022; Pitzer et al., 2022; Abdoli et al., 2022; Hyde and Mezulis, 2020). Finally, our CUS protocol did promote helplessness, increased anxiety, and reduced social preference in mice, although the different effects of CUS involved the interplay of different factors. Thus, the D2 male mice were especially susceptible to CUS-induced helplessness, females of this same strain required the RCF experience to express this phenotype and, in B6 females, CUS increased helplessness while RCF decreased it. Moreover, neither CUS nor RCF altered open arms avoidance expressed by D2 mice, whereas CUS significantly increased this phenotype in B6 females regardless of the early experience. Finally, a modest but significant reduction of social preference was observable in CUS-exposed female mice, regardless of the strain or early experience. These data indicate that a complex interaction between the different risk factors moderates the impact of potentially pathogenic experiences on different behavioral phenotypes.

Variable effects of CUS on the different behavioral measures could suggest limitations of the protocol used in the present experiments. However, it is worth pointing out that a variable relationship between behavioral effects also characterizes the outcome of chronic/repeated defeat experiences (Lyons et al., 2023), and defeated mice of the B6 strain do not show helplessness behaviors regardless of their individual susceptibility to this stressor (Krishnan et al., 2007). On the other hand, variable susceptibility of different behavioral phenotypes could relate to the variability of symptom profiles associated with mental disturbances. Indeed, the same symptom can be associated with different pathological conditions, while no single symptom is sufficient to identify a specific disease.

### 4.2. An interaction between genotype, early experience, and sex controls measoaccumbens DA responses to stress challenge following CUS

In the present study, we evaluated DA outflow by *in vivo* intracerebral microdialysis, during and following a prolonged experience with FST, in the NAc of mice from the same four experimental groups subjected to the CUS protocol (Fig. 3). The microdialysis data collected during and following a prolonged exposure to FST in CUS-experienced male mice only revealed a temporary significant deep in DA release after 20 min in the NAc of RCF-exposed B6 mice. As for females, we observed major strain differences for the response to 10 min of FST experience because RCF-exposed mice of the B6 strain responded with a significant increase of DA release in the NAc whereas D2 RCF mice responded with a significant decrease below baseline levels that was also evident at 20 min and at the end of FTS. We measured mesoaccumbens DA outflow during and following a prolonged FST experience because of previous data obtained in mice and rats. Indeed, mesoaccumbens DA response to an inescapable stressful experience measured by intracerebral microdialysis in rodents is polyphasic: a temporary increase in DA release is observable in the first minutes of the experience, it is followed by a decrease below baseline levels that lasts as long as the stressful experience. Finally, a significant increase of DA outflow follows the end of the stress experience (Cabib and Puglisi-Allegra, 2012; Di Segni et al., 2016). The prolonged exposure to FST was designed to capture this dynamic. However, none of the groups tested in the present study showed this response, supporting the influence of CUS. Although twenty minutes of FST exposure could have induced physical exhaustion in mice (Yi et al., 2021; Anand et al., 2012), the deep in NAc DA could be detected before, as in the case of D2 females or not observed as in the case of B6 RCF-exposed females, B6 Control males, D2 Control females and Control and RCF D2 males.

CUS has been reported to reduce the activation of VTA DA neurons (Tye et al., 2013; Holly and Miczek, 2016; Rincon-Cortes and Grace, 2017; Douma and de Kloet, 2020), and compelling evidence points to the mesoaccumbes DA controlling coping with stress challenges (Cabib and Puglisi-Allegra, 2012; Tye et al., 2013; de Kloet and Molendijk, 2016; Bai et al., 2017; Cui et al., 2020). CUS was also reported to increase NAc DA outflow in rats challenged with 10 min of tail pinch (Di Chiara et al., 1999). This finding, however, is difficult to evaluate because the authors reported a tail pinch-induced decrease of DA outflow in control rats, while other studies report that a tail pinch experience of the same or longer duration increases mesoaccumbens DA outflow (Rougé-Pont et al., 1993; Amato et al., 2011). On the other hand, repeated experience of restraint stress progressively reduces the initial increase of DA release in the NAc of rats, whereas the late inhibition of NAc DA did not change (Cabib and Puglisi-Allegra, 2012).

In the present study, only RCF female B6 mice showed increased mesoaccumbens DA outflow after 10 min of FST experience, followed by a return to baseline levels. Control female mice of this same strain only showed a DA decrease below baseline after 20 min of FST that was still evident 10 min after the end of the stressful experience (Fig. 3 top, left). These findings align with the previous report that a different inescapable stressor (restraint) elicited a larger increase of DA release in the NAc of RCF B6 females compared to their Controls (Di Segni et al., 2016) Therefore, they support the conclusion that RCF experience protects the NAc DA response to stress by female mice with the B6 genotype. The different effect evident in B6 RCF males supports the hypothesis of a sex-dependent effect of RCF experience in mice of this inbred strain. CUS-exposed female D2 mice of the Control group responded to 10 min of FST with a temporary non-significant decrease in DA levels, followed by a return to baseline (Fig. 3 bottom left). D2 female mice with no experience of RCF or CUS were previously reported to respond with an early large increase of DA to a different inescapable stressor (restraint), followed by a decrease below basal level (Di Segni et al., 2016). This initial stress-elicited increase of mesoaccumbens DA outflow was significantly reduced in RCF-exposed female mice of this inbred strain (Di Segni et al., 2016). Thus, the observation that CUS-exposed RCF D2 females respond with a large and significant decrease of DA outflow to FST experience is coherent with the hypothesis of a genotype-specific additive effect of CUS and RCF on stress-induced inhibition of NAc DA.

Due to technical problems, we were unable to collect enough data to evaluate the behavior expressed by mice during microdialysis experiments. This missing data could have certainly added to the interpretation of these findings. However, it is worth pointing out that the fluctuations of tonic DA levels in the NAc during stressful experiences cannot be directly related to specific behavioral responses, due to the large time-window (minutes) required for its accumulation. On the other hand, studies on the relationship between expression of active responses (struggling) in TST and phasic DA transients, which are measurable in seconds, have obtained contrasting results (Tye et al., 2013; Cui et al., 2020). Nonetheless, optogenetic activation of the VTA DA neurons, the major source of mesoaccumbens DA (Douma and de Kloet, 2020), increases behavioral resilience to social defeat stress (Chaudhury et al., 2013) and promotes active coping in TST (Tye et al., 2013). Moreover, converging evidence indicates that CUS-reduced DAergic population activity in the VTA is responsible for the increased passive coping expressed in FST and TST (Douma and de Kloet, 2020; Rincon-Cortes and Grace, 2017; Tye et al., 2013). Low population activity of VTA DA

neurons reduces tonic DA levels in target areas measured by intracerebral microdialysis (Floresco et al., 2001) a condition that could reduce motivation to sustain effortful active coping response mediated by the NAc (Cabib et al., 2012; Mourra et al., 2020; Treadway and Salamone, 2022; Iodice et al., 2017). However, fluctuations of tonic DA levels in the NAc during stressful experiences and the expression of specific coping responses cannot be directly related, while studies on the relationship between expression of active responses (struggling) in TST and phasic DA transients in the NAc have obtained opposite results (Tye et al., 2013; Cui et al., 2020).

Over the past years, human functional data have identified several brain areas involved in stress and anxiety responses. Interestingly, alterations of the connectivity between mesolimbic areas have been proposed to contribute to the etiology of many stress-induced disorders in animal models (Daviu et al., 2019). In particular, circuits involving bidirectional signaling between medium spiny neurons of the NAc and DA neurons of the VTA, as well as between VTA and amygdala have shown to be involved in mediating the disruptive effects of adult adversities, as chronic stress (Madur et al., 2023). Further studies will be necessary to deeply investigate possible mesolimbic circuit alterations induced by the 3-hit effect of genotype, early and adult adversities.

### 4.3. Limitations of the study

The most relevant limitation of the present study is the absence of data collected in mice naïve to all the tests. Unfortunately, the CUS protocol chosen did not allow for pre-tests. We considered the first measure collected in the different tests as baseline because it mostly replicated previous findings from our or different laboratories. The only discrepancy found was the absence of the sex difference in B6 mice tested in FST (females more immobile than males, Di Segni et al., 2019) and the absence of the RCF effects on immobility expressed by D2 females exposed to TST (Lo Iacono et al., 2021). Both might be due to the experience of other stressors. Nonetheless, the effects of CUS were statistically demonstrated by the comparisons between the two time-points chosen for the analysis in both tests.

Although we investigated the sex effect in control animals before the exposure to the CUS, changes across sexes after environmental stressful exposure had been not analyzed. This represents a potential limitation of this study.

Stressful factors are presented in an unpredictable order during CUS protocol, these factors are multimodal, and each of them was repeated 3 times. Existing data indicate that repeated exposure to the same stressor is less stressful for the animal compared to its first presentation (Markov and Novosadova, 2022). This observation represents a potential limitation of the results reported in any study involving repeated/chronic stress experiences. However, there is also evidence that repeated/chronic stressful events sensitize rather that habituate to adversities (Kaye et al., 2023), and it is well-known that coping responses are learnt through previous experiences with the same or different stressors (Cabib et al., 2012; Campus et al., 2016; Maier and Seligman, 2016). Therefore, the effects of each stressful factor on the organism and their cumulative effect during CUS are probably determined by the complex interaction of habituation, desensitization, facilitation, learning, and memory formation. Moreover, performing more stressful behavioral tests, such as FST, before less stressful ones, such as EPM or social interaction test, can add a possible heightened bias of the effect of the previous behavioral exposure on the latter ones. Finally, the effect of repeated exposure to the same behavioral apparatus for some tests has not been specifically addressed in this study.

## 5. Conclusion

Our results show for the first time the 3-hit effect of genotype, early experiences, and adult adversities in a rodent model. Moreover, they indicate that females are most affected by this pathogenic pathway and support the influence of the 3 factors on the mesoaccumbens DA response to stress. Epigenetic modifications (Alyamani and Murgatroyd, 2018) are increasingly being recognized as critical to understanding sex differences in brain development and response to early life experience (Keller and Roth, 2016). The sex-related impact of early and adult adversities observed in present study could be explained by several complementary factors: first, the existence of different developmental paths between males and females (Chocyk et al., 2015; Gillies et al., 2014; Hodes and Epperson, 2019) that may be temporally shifted in their critical plasticity windows for the dopaminergic system development (Bath, 2020); second, the differential impact of stress hormones on dopaminergic developmental trajectories in male and female brains (Gillies et al., 2014); third, gonadal steroid hormones represent one of the major drives for sexually dimorphic brain and one mechanism by which stress can affect DA-related motivated behavior is *via* regulating gonadal hormones in a different manner in males and females (Eck and Bangasser, 2020; Zachry et al., 2021). Further studies will be necessary to clarify these points.

Finally, our findings align with the complexity of genetic influences on neural phenotypes associated with liability to mental diseases. This complexity escapes classic monogenic or oligogenic approaches but can be accessed through comparative studies in the B6 and D2 mice, QTLs analyses in BxD recombinant inbred strains, and hypothesis-driven genetic targeting based on these findings.

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## Ethical statement

All experiments were carried out according to Italian law regarding the protection and welfare of animals used in scientific procedures (DL 116/92 and DL 26/2014) and European Communities Council Directives (86/609/EEC and 2010/63/UE). The experimental protocol (no. 769/2017) was approved by the Italian Ministry of Health.

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## CRediT authorship contribution statement

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## Declaration of Competing Interest

None.

## Data availability

Data will be made available on request.

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