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RESEARCH ARTICLE

Mouse model of panic disorder: Vulnerability to early environmental instability is strain-dependent

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Abstract

Early life experiences and genetic background shape phenotypic variation. Several mouse models based on early treatments have evaluated short- and long-term phenotypic alterations and explored their molecular mechanisms. The instability of maternal cues was used to model human separation anxiety in outbred mice, one of the etiopathogenetic factors that predict panic disorder (PD). Application of the repeated cross-fostering (RCF) protocol to inbred strains (C57 and DBA) allowed us to measure differential responses to the same experimental manipulation. Ultrasounds emitted during isolation indicated that after RCF, pups from both strains lose their ability to be comforted by nest cues, but the frequency modulation of separation calls increased in RCF-C57 and decreased in RCF-DBA mice. No strain-specific difference in olfactory ability explained these responses in RCF-exposed mice. Rather, disruption of the infant-mother bond may differentially affect separation calls in the two strains. Moreover, the RCF-associated increased respiratory response to hypercapnia–an endophenotype of human PD documented among mice outbred strains–was replicated in the C57 strain only. We suggest that RCF-induced instability of the early environment affects emotionality and respiratory physiology differentially, depending on pups' genetic background. These strain-specific responses provide a lead to understand differential vulnerability to emotional disorders.

KEYWORDS
6%CO_2, C57BL/6J and DBA/2, instability of early environment, interference with maternal care, mouse model of panic disorder, repeated cross-fostering, respiratory endophenotype, tidal volume, ultrasonic vocalizations, unrestrained plethysmograph

1 | INTRODUCTION

Clinical and epidemiological studies have shown how early adverse experiences shape the liability to psychiatric disorders; one key epidemiological finding is that adversities affect the occurrence and development of psychopathology depending on the individual genetic, environmental, and epigenetic background (Battaglia, 2012). Adversities at an early age can have far-reaching effects due to the immaturity of physiological, behavioral, and hormonal coping systems (Vazquez, 1998). On the other hand, the plasticity of the neural system allows for adaptive responses to the environment that are a function of the organism's complexity. Finally, the effects of early adversities are often long-lasting and detectable later in life, when physiological and psychological mechanisms have already primed dysfunctions in the organism.

Early-life adversities are critical etiopathogenetic factors that underlie the onset of several psychiatric disorders, including panic disorder (PD; Heim & Nemeroff, 2001; Horesh et al., 1997; McEwen, 2000). In contrast to other anxiety disorders, the onset of PD is usually

not associated with altered hypothalamic-pituitary-adrenal axis function (HPA: Klein, 1993), respiratory symptoms (hyperventilation and smothering sensation), and hypersensitivity to CO_2 constituting validated markers of the disorder (Battaglia et al., 2007, 2008, 2014; Leibold et al., 2016). Acute anxiety and hyperventilation can be reproduced in humans at risk for PD through CO_2 inhalation or lactate infusion (both sharing the mechanism of blood acidification by hydrogen ions' (H^+) elevation; Vollmer et al., 2015; Wiese et al., 2019), with the elicitation of both physical and cognitive symptoms that are typical of PD (Battaglia et al., 2007). A further validated risk factor that is linked to both PD and CO_2 hypersensitivity is childhood parental loss, encompassing childhood loss/separation from parents due to a number of events such as separation/divorce, military assignment, or death (Battaglia et al., 2009; Spatola et al., 2011).

To model the connections between early-life adversities, CO_2 hypersensitivity, and PD in a laboratory context, we have developed the repeated cross-fostering (RCF) protocol of interference with the early maternal environment. The RCF is based on the early removal of newborn mice from the biological mother, followed by multiple fostering to other lactating females (D'Amato et al., 2011; Luchetti et al., 2015). During 6% CO_2-enriched air breathing, RCF mice show the key response of exaggerated hyperventilation that marks human PD; this is stable from childhood into adulthood and is compounded by enhanced avoidance of CO_2-enriched environments (D'Amato et al., 2011). Such respiratory and avoidance responses are non-inferential by nature and constitute key assets of the RCF model. Moreover, RCF is associated with enhanced separation anxiety behavior (pups' ultrasonic calls to mother); it does not evoke maternal neglect/maltreatment, offspring abnormal behavior (e.g., stereotypies, aggression, isolation), and does not affect body weight development (Luchetti et al., 2015). Also, akin to humans at the onset of PD (Klein, 1993), the HPA axis is not affected by RCF (D'Amato et al., 2011; Luchetti et al., 2015; Di Segni et al., 2016). The interplays among sensitivity to heightened [CO_2], blood acidification, and early maternal separation have become an increasingly popular preclinical model of human PD and have been adopted by several independent laboratories with comparable results, supporting the validity of these approaches (Battaglia et al., 2019; D'Amato et al., 2011; Dumont et al, 2011; Leibold et al, 2016; Winter et al., 2017).

The function of genetic variation in driving CO_2 responses has also been examined in both man and animals. A twin study showed that a common genetic background explains the association between childhood separation anxiety, adult PD, and anxious responses to 35%CO_2–65%O_2 stimulation, with childhood parental loss contributing to this longitudinal covariation (Battaglia et al., 2007). A recent preclinical study (Schlenker et al., 2006) showed marked variability for the CO_2-evoked behavioral responses among outbred versus inbred rat strains, echoing previous results of differential sensitivity to CO_2 and maternal behavior (D'Amato et al., 2005; Millstein & Holmes, 2007) among genetically different mouse strains. By applying severely hypercapnic and normoxic conditions, Tankersley et al. (1994) classified the C57BL/6J strain as being the most highly respondent to hypercapnic stimulation among eight tested strains.

Here, to further characterize the genetic and environmental components of the respiratory responses to CO_2 inhalation after the RCF procedure, we studied two mouse strains that are known to differ for CO_2 sensitivity: the high-responder C57BL/6J and the low-responder DBA/2J strain (Tankersley et al., 1994). We hypothesized that the RCF protocol would affect the behavioral and neurobiological responses of mice in both strains, but the direction and intensity of responses would depend on the animal's genetic background.

We first determined whether the RCF procedure interferes with infant-mother communication among C57BL/6J and DBA/2J inbred mice, compared to outbred mice. Then, maternal behavior was evaluated as a possible rescue versus precipitating factor for the development of the main RCF-associated phenotypes. Finally, the respiratory response to CO_2 was measured in young and adult RCF and control mice to examine phenotypic stability and strain-dependent gene-environment interplay.

2 | MATERIALS AND METHODS

2.1 | Experimental subjects

C57BL/6J (C57) and DBA/2J (DBA) mice (Charles River) were used in this study. All procedures were conducted in parallel in the two strains. Mice were mated when 12 weeks old: mating consisted of housing two females with one male in transparent polysufone cages (26.7 × 20.7 × 14.0 cm) with water and food available *ad libitum*. Paper was provided as nesting material. Room temperature (21 ± 1°C) and a 12:12 h light-dark cycle (lights on at 7.00 p.m.) were kept constant; testing occurred during the light phase of the day. After 15 days, males were removed and pregnant females were isolated, left in clean cages, and inspected twice a day for live pups. Litters were left with the biological mother the day of birth (PND0).

Litter sizes were not modified after birth, but only litters ranging from 4 to 10 pups were included in the experiment. Animals were weaned at PND28, separated by sex, and housed in groups of four to five same-sex/strain/treatment subjects from different litters.

All experiments were conducted under license from the Italian Department of Health and in accordance with the Italian regulations on the use of animals for research (legislation DL 116/92 and 26/2014) and European guidelines on animal care.

The procedure for RCF has been previously described (D'Amato et al., 2011; Luchetti et al., 2016) and is briefly summarized here. Pups of the same litter spent the first postnatal day (PND0) with their biological mother. On PND1, litters were randomly selected and assigned to experimental (RCF) or control treatment (CONT). Each experimental litter was fostered by replacing the mother with a novel lactating female of the same strain, caring for pups of the same age, but mated with a different male; this procedure was repeated daily (4 times, from PND1 up to PND4) until the fourth adoptive mother was reached (Luchetti et al., 2015; 2016). Pups were left with the last adoptive mother until weaning. Control mothers and pups were left together until weaning; from PND1 to PND4, pups were briefly picked up and

put back into the nest area. Pups were partially covered with home cage bedding, before reintroducing the mothers. This procedure lasted no more than 30 s.

2.2 | Maternal behavior

Maternal behavior was observed daily from PND1 to PND7 by an observer unaware of the litter's status (RCF/control) in two daily sessions (12.00–12.30 p.m. and 4.00–4.30 p.m.), the morning session taking place 1 h after the cross-fostering procedure on PND1–PND4. Maternal behavior was monitored with an instantaneous sampling method (one sampling every 2 min), for a total of 16 sampling points/session. The following behaviors were considered: (a) nursing–including the arched-back and blanket postures, and (b) GP/L: grooming and licking pups (D'Amato et al., 2011). The analyses of maternal behavior were based on 10 litters of RCF and 7 litters of control in the C57 strain and 10 litters of RCF and 7 litters of control in the DBA strain. In the analysis, the first week of life of pups was split up into two different periods: the RCF period (PND1–4: 4 days of data collection) and the stable period (PND5–7: 3 days of data collection). Because of different time duration of the two blocks, the mean daily value for nursing and GP/L behaviors was used in the statistical analyses.

2.3 | Offspring behavior

Pups' behavior was evaluated after the completion of the RCF manipulation. The impact of RCF was assessed in pups at PND8 by measuring the ultrasonic vocalizations (USVs) emitted during isolation and at PND10 by evaluating pups' ability to orient toward and approach maternal/home cage bedding (homing; D'Amato et al., 2011).

2.3.1 | USVs

No more than four pups x litter (two males and two females) were tested: Each pup was individually placed into a beaker containing (i) home cage bedding (USVs-home cage) or (ii) clean bedding (USVs-clean), and vocalizations were recorded during a 5 min session. USVs were recorded and analyzed via a dedicated software (Avisoft Bioacoustics). The sample size for each experimental group was 9–11 and pups from each group belonged to five to six different litters. Further details concerning methods and vocalization parameters analyzed can be found in the supporting information (Methods-SuppInfo.pdf).

2.3.2 | Homing

The ability of pups to orient toward familiar odorous cues was evaluated in a small apparatus (5 × 33 × 10 h cm) with a central Plexiglas part (5 × 5 cm, starting point) that separated (with sliding doors) two differently scented arms. One arm was covered with the pup's home cage bedding, while the other one was covered with clean bedding. The pup was left 45–60 s in the central part of the apparatus before opening doors and allowing it to move freely for 5 min. The behavior of the pup was video-recorded, and the time spent in the different arms of the apparatus was then evaluated by video-tracking software (SMART 1.1, Panlab). The apparatus was carefully cleaned with 10% ethanol solution at the end of each test session. No more than four pups (two males and two females) per litter were tested. Further details are given as supporting information (Methods-SuppInfo.pdf).

2.4 | Respiratory responses

We used an unrestrained plethysmograph (PLY4211, Buxco Electronics) carrying two separate Plexiglass chambers of 450 ml, allowing for the parallel assessment of two animals/session to measure tidal volume (TV). The apparatus was located in a dedicated room adjacent to the animal house facility, with controlled temperature (21 ± 1°C; Methods_SuppInfo.pdf).

On PNDs 16–20, pups were tested for their respiratory response to air mixture enriched with 6% CO_2, according to a shortened version of a validated protocol (D'Amato et al., 2011). Body weights were measured at the end of the respiratory test described below and are shown in the Supporting information (TABLE_1_SuppInfo.pdf). Each subject was positioned in one of the chambers of the plethysmograph for a 40-min acclimatization period. Subsequently, the recording of respiratory parameters started under air condition (baseline-pre) for 20 min. Next, the challenge began: this lasted 20 min and consisted of administration of a 6% CO_2 air mixture. A 20 min recovery period (baseline-post) took place ending the trial and the recording time. A complete respiratory recording session thus lasted 60 min per animal (plus 40 min for habituation to the chamber), and all breaths in each 20 min session were analyzed to overcome the variability of respiratory parameters due to awake/sleeping state, movements' artifacts, and behaviors. Quindry et al (2016) demonstrated reproducibility of respiratory function data in mice, using the above procedures with the whole body plethysmography. Sample sizes of the four experimental groups were: C57 CONT: $n = 15$; C57 RCF: $n = 14$; DBA CONT: $n = 12$; DBA RCF: $n = 16$. No more than two animals (one male and one female) per litter were tested for CO_2 responsiveness, each experimental group belonging from six to eight different litters. At the end of the test, animals were marked to avoid retesting them later. TV and respiratory rate were evaluated as dependent variables.

In adulthood, only male subjects were tested (METHODS_SuppInfo). On PND 90–120, the respiratory response to 6% CO_2 of 52 adult male mice was evaluated with the same device and procedures as described above. Sample sizes of the four experimental groups were: C57 CONT: $n = 12$; C57 RCF: $n = 12$; DBA CONT: $n = 14$; DBA RCF: $n = 14$. No one of the adult mice that underwent the respiratory challenge had previously been exposed to the test as a pup.

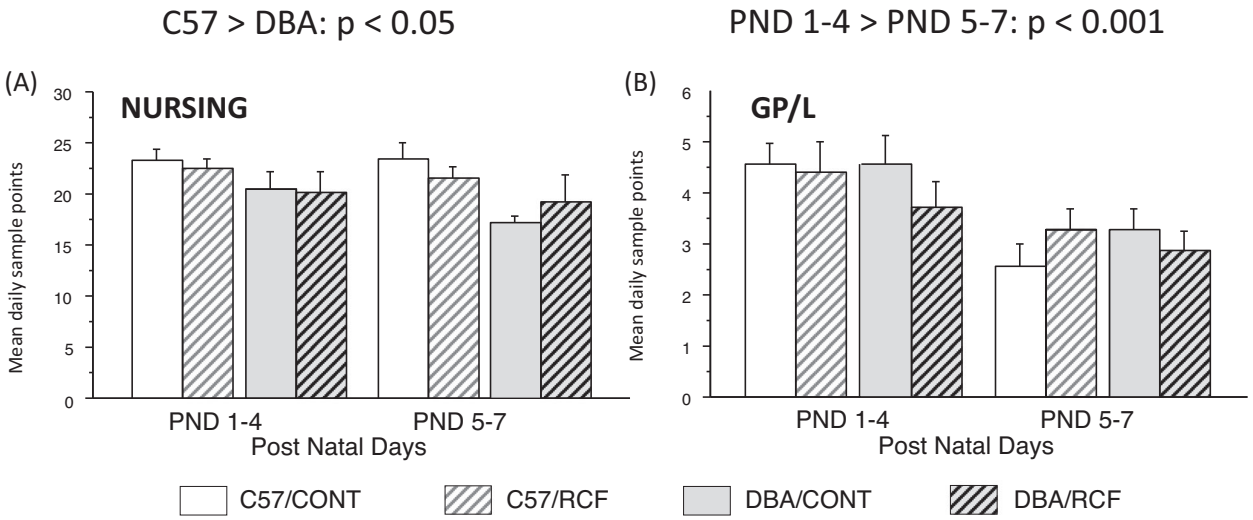

Figure 1 The amount of maternal behaviors ((A) nursing , (B) grooming/licking) received by pups was not affected by the repeated cross-fostering (RCF) protocol, in both strains. Sample size (litters): C57/CONT = 7, C57/RCF = 10, DBA/CONT = 7, DBA/RCF = 10

2.5 | Statistical analysis

Data were analyzed by analyses of variance (ANOVAs) using the Statview 5.0PowerPC (SAS Institute Inc.) and the Statistica software packages. Maternal behaviors were analyzed by three-way ANOVAs for repeated measures with strain, RCF manipulation, and age as factors. As for pups' vocalizations, 3-way ANOVAs (with strain, early manipulation, and bedding as factors) were used to evaluate differences in total time spent calling, and number, duration, mean peak amplitude, frequency, and frequency modulation of calls.

A three-way repeated measure ANOVA (with strain, postnatal manipulation, and bedding as factors) was used to evaluate pups' homing behavior (clean vs. home cage bedding).

Finally, because of the relation between body weight and TV (METHODS_SuppInfo), a composite measure was used to evaluate respiratory performance under CO_2 exposure, according to early manipulation and strain. Changes in respiratory parameters (%ΔTV and %ΔRR) induced by hypercapnia were evaluated by two-way ANOVAs followed by Tukey's honest significance test, with strain and early manipulation as factors.

3 | RESULTS

3.1 | Maternal behavior

The RCF protocol was not associated with different amounts of maternal behavior. C57 mothers showed nursing behavior (Figure 1A) more frequently than DBA mothers ($F_{1/30} = 5.90$, $p < 0.05$) and independently of postnatal manipulation ($F_{1/30} = 0.05$, ns) and developmental time period: PND 1–4 versus PND 5–7: ($F_{1/30} = 1.47$, ns). In addition, no between-factor interactions reached a significant level. For all experimental groups, the time spent in daily grooming of the pups showed a significant decrease ($F_{1/30} = 16.83$, $p < 0.001$), independently of strain ($F_{1/30} = 0.61$, ns) and postnatal manipulation ($F_{1/30} = 0.26$, ns); no significant between-factor interaction emerged from the ANOVA (Figure 1B).

3.2 | Offspring behavior

In spite of no differences in maternal care, both C57 and DBA pups exposed to RCF differed from their respective controls for the number of ultrasonic calls measured from PND8, that is, 4 days after the last cross-fostering manipulation. As shown in Table 1 and Figures 2A,B, ultrasounds' parameters were strain-dependent. Considering the time spent in vocalizing, RCF 8-day old C57 vocalized less (total duration, Figure 2A) than DBA pups (strain effect: $p < 0.0001$), pups of both strains vocalized less when in home cage bedding (bedding effect: $p < 0.01$), and postnatal manipulation had no effect *per se* (RCF effect: ns). Home cage bedding confirmed its soothing effect on pups' calls (mean duration, total duration, and mean peak amplitude), with home cage bedding reducing USVs in comparison with exposure to clean, odorless bedding. However, as clearly shown in Figure 2A, this effect disappeared in RCF mice (both C57 and DBA) that seemed unable to detect/benefit from the familiar home cage cues (Table 1, RCF x bedding effect: total duration: $p < 0.005$; the number of calls: $p < 0.005$).

Frequency modulation of pups' ultrasounds was differently affected by the RCF protocol in the two strains (Figure 2B, Table 1: strain x RCF effect: $p < 0.01$) suggesting increased "arousability" in the C57 strain; by contrast, DBA RCF mice showed no changes in calls' modulation in comparison with their DBA controls. Mean values for all considered USV parameters are reported in TABLE_1_SuppInfo.pdf.

Results from the homing test, where pups were evaluated for their capability to discriminate odors, and for their preference for their home cage bedding, confirmed that all pups spent more time in the familiar-scented section of the apparatus (Table 2, Figures 2C,D), which in turn indicates intact olfactory discrimination.

TABLE 1 Effects of strain (S), repeated cross-fostering (RCF), and bedding exposure (B) on ultrasonic vocalizations (USVs) emitted by postnatal day 8 (PND8) pups during 5 min of isolation

Strain	RCF	Bedding	S x RCF	S x B	RCF x B	S x RCF x B	Analysis of variance (ANOVA) effects: USVs parameters
54.72***	0.55	6.66*	1.16	1.03	9.45**	0.13	Total duration (s)
20.17***	0.01	2.13	0.37	0.03	9.56**	0.03	Mean number
90.72***	5.95*	10.35**	2.23	0.81	1.76	0.26	Mean duration (s)
67.14***	1.27	2.35	0.56	0.12	0.07	0.11	Mean peak frequency (kHz)
41.65***	0.03	7.52**	0.01	0.02	0.05	0.01	Mean peak amplitude (dB)
18.51***	1.43	0.12	8.41**	0.04	0.88	1.18	Mean frequency modulation (kHz)

Note: Data reported are *F* values (df = 1/69).
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.0001$.

TABLE 2 Effects of S, RCF. and B on homing behavior shown by PND10 pups, during 5 min of exposure to differently scented arms of the apparatus. Two-way ANOVA for repeated measures: *F* values are reported in the table

Strain	RCF	Bedding	S x RCF	S x B	RCF x B	S x RCF x B	Homing
0.15	0.12	174.26***	1.95	0.99	2.14	0.09	Time spent

Legend: df = 1/76 for strain, RCF, and strain x RCF interaction; df = 2/152 for bedding preference, and all other interactions.
***$p < 0.0001$.

3.3 | Respiratory responses

RCF manipulation was associated with the percentage of increase in TV under 6% CO_2 in C57, but not in DBA, in pups (Figure 3A: %ΔTV: strain x RCF effect: $F = 5.91$, df = 1/53, $p < 0.05$) as well as in adult mice (Figure 3C: %ΔTV: strain x RCF effect: $F = 5.02$, df = 1/48, $p < 0.05$). As for respiratory rate, DBA adult mice showed a larger increase in this respiratory parameter during hypercapnia, in comparison with C57 mice. No other significant effect was detected on respiratory parameters.

4 | DISCUSSION

This study shows that some behavioral (USVs) and respiratory physiological (TV) responses associated with an unstable maternal environment (modeled via the RCF) are strain-specific among C57 versus DBA mice. The exaggerated responses to CO_2 stimulation also appear as a trait that is stably acquired among C57 mice after early exposure to RCF, similar to NMR1 (D'Amato et al., 2011) and Swiss (Battaglia et al., 2019) outbred mice.

These findings support the role of genetic elements in shaping short- and long-term responses to early perturbations of maternal care and fall under the broader category of genetic sensitivity to the environment. The highly standardized RCF protocol has been used as an "environmental instability condition" and entails exposing mouse litters to sequential adoptions by different dams. Previous studies conducted in outbred mice have demonstrated that lactating RCF females do not reject alien litters and take adequate care of pups (D'Amato et al., 2011; Luchetti et al., 2015). Similarly, other cross-fostering procedures do not alter strain-specific differences in maternal behavior (Brown et al., 1999; Ward, 1980), and our analyses conducted separately during the RCF (Day 1–4) and stable period (Day 5–7) confirmed the lack of an effect of RCF on maternal care during and after the adoption period.

Nonetheless, the RCF procedure impacts the behavioral and neurobiological profiles of developing outbred mice (D'Amato et al., 2011; Luchetti et al., 2015).

In this study, USVs indicated both strain-specific and RCF-related effects on pups' emotionality. C57 and DBA control pups differed significantly in the number and duration of ultrasounds during isolation and in the sonographic characteristics of calls, such as peak amplitude, peak frequency, and frequency modulation. The sonographic parameters are strain-characteristics, less affected by early environment manipulation and experimental condition (for a review, Caruso et al., 2018). In contrast, the number, mean duration, and total time spent by pups emitting USVs were strongly affected by odor cues and early manipulation (Wöhr et al., 2008). As reported, pups vocalized more consistently in the absence of familiar cues (clean condition), indicating that the home cage bedding reduced the emotional impact of social isolation (Cinque et al., 2012; Moles et al., 2004). This effect was evident in both strains, despite the differences in absolute values, with C57 vocalizing less than DBA pups.

However, for RCF-exposed pups, the "soothing/anxiolytic" effect of their home cage bedding was lost in both strains. Home cage bedding, for a still blind, isolated pup searching for safety and led by olfaction, represents the mother. This result may offer different leads. One may hypothesize that RCF pups lost some olfactory abilities, or did not recognize their "current" home cage bedding as being familiar, or that RCF-related impairments in the formation of mother-infant bond affected

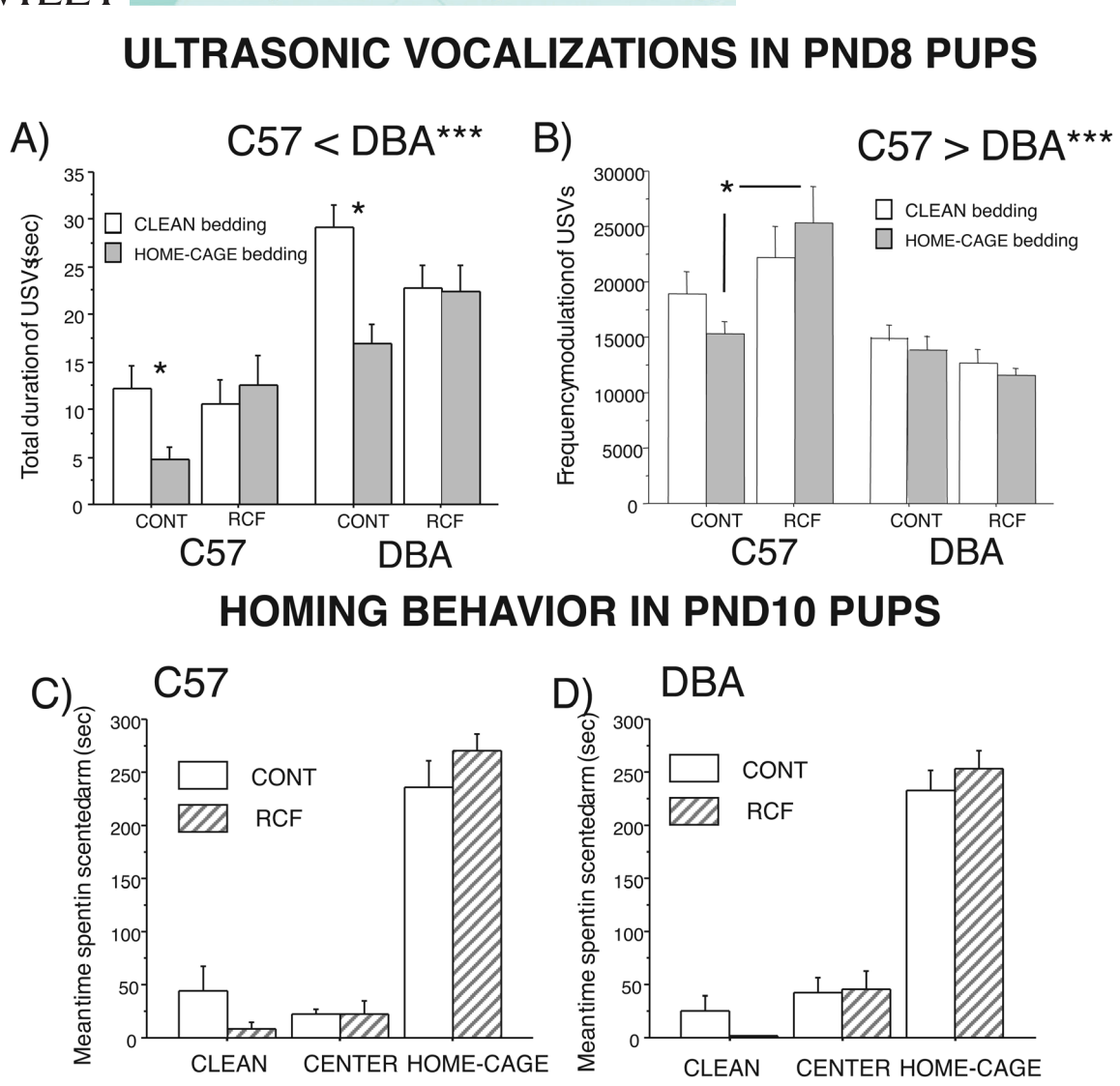

Figure 2 RCF pups of both C57 and DBA strain showed defective mother-infant bond but no deficits in olfaction. (A) RCF 8-day-old C57 spent less time vocalizing than DBA pups, and control pups of both strains vocalized less when in the presence of home cage bedding cues. Significant early manipulation x bedding interaction suggested no calming effect of the home cage bedding in RCF pups. (B) Strain differences in ultrasonic vocalizations' bandwidth (frequency modulation) were emphasized by the RCF manipulation. Group sample sizes ranged from 9 to 11 pups. RCF and CONT 10-day-old pups of the C57 (C) and DBA strain (D) spent more time in the home cage scented part of the apparatus (homing test), suggesting intact olfactory capability and preference for familiar cues. Sample size (pups): C57/CONT = 20, C57/RCF = 20, DBA/CONT = 24, DBA/RCF = 20. * $p < 0.05$ post hoc Tukey HSD

the development of the reward circuit, thus lowering the salience of positive (and negative) signals in modulating behavior. Results of the homing experiment excluded the first two hypotheses, leaving the possibility that RCF mice experienced dysfunction of the reward system. We have already tested this latter hypothesis in outbred mice (Ventura et al., 2013) and inbred strains (Di Segni et al., 2016; 2017) and confirmed altered sensitivity to natural and pharmacological rewards associated with RCF.

C57 and DBA mouse pups reacted differently to RCF in terms of frequency modulation of ultrasounds (Table 1 and Figure 2): Independent of bedding exposure, RCF increased the frequency modulation in C57 but had no effect in DBA pups. Frequency modulation can be considered a measure of the complexity of ultrasonic calls; thus, RCF accentuated the differences between strains, with C57 and DBA pups showing a larger and narrower frequency bandwidth, respectively. A different explanation could be based on the earlier hearing loss that characterizes the DBA strain, in comparison with the C57. DBA mothers could be less responsive to pups' calls because of their precocious hearing handicap; the absence of DBA mothers' response to pups' calls could prevent frequency modulation modifications. An increase in frequency bandwidth has been reported after prenatal high ethanol exposure in rats (Shahrier & Wada, 2018), a condition that leads to insecure attachment in humans (Kelly et al., 2000). Notably, these changes in distress vocalizations were observed in prenatally ethanol exposed pups, together with other altered pup-dam behaviors (e.g., latency and duration of nursing, maternal retrieving: Barron et al., 1992; Ness &Franchina,

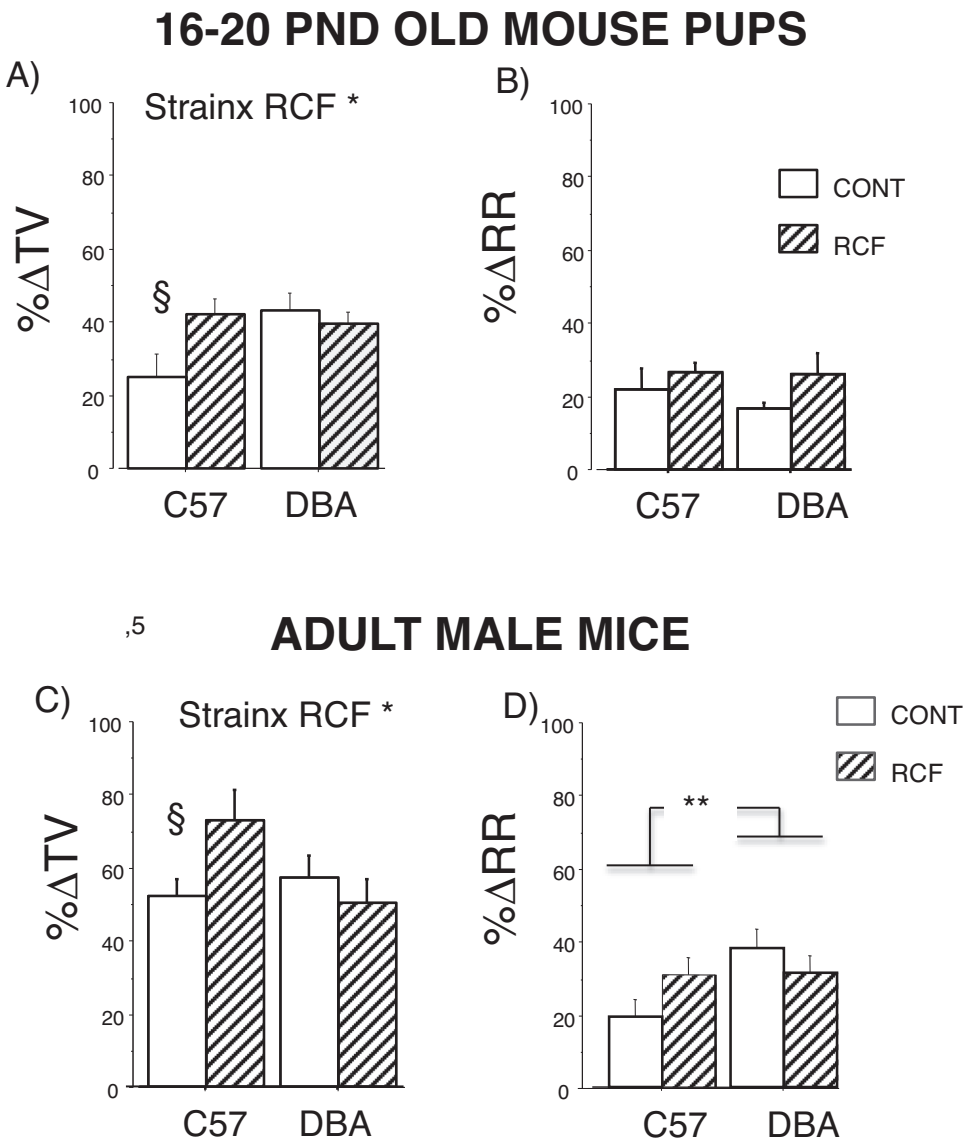

Figure 3 RCF treatment stably affected C57 but not DBA respiratory response to 6% CO_2 exposure in breathing air. Changes in tidal volume (%ΔTV) and respiratory rate (%ΔRR) in juvenile (A-B) and adult mice (C-D) during hypercapnia are reported for early manipulated (RCF) and control mice (CONT). Analysis of variance: ** $p < 0.001$. Tuckey post hoc test: §$p < 0.05$ in comparison with same-strain RCF group

1990; Rockwood & Riley,1990). These modifications synergistically affect the quality of mother-infant interactions and can thereby influence the infant's behavioral development (Ness & Franchina, 1990).

USVs at PND8 appeared to anticipate adult strain-dependent phenotypic differences in respiratory response to CO_2, suggesting that stable modifications developed shortly after the pups' exposure to RCF. Isolated RCF C57 pups vocalized more intensively (increased total time and greater frequency modulation) and overreacted (increase in TV of respiratory acts) to hypercapnia; RCF DBA pups merely showed no reduction in USVs in home cage bedding and did not differ from their controls in respiratory profile during CO_2 exposure. Further studies could clarify whether the RCF protocol exacerbated inter-strain differences in mother-infant bond.

The increased hyperventilation that occurs during panic attacks is associated in humans with a series of changes in autonomic nervous system function, such as chest pain, sweating, tingling, and dizziness (Anderson et al., 1984). Hypercapnic hyperventilation, in contrast to the other symptoms above, can be easily measured in the mouse, thus representing a useful endophenotype in animal models of PD. However, although mice from both strains responded to instability in the early environment, the gene-environment interplay suggested different strain-dependent responses to early-life events. These two strains have been compared extensively and already show differences in corticolimbic processing of aversive and rewarding stimuli (Andolina et al., 2015; Cabib et al., 2002; Grice et al., 2007).

The lower sensitivity to CO_2 of DBA, compared with C57 mice (Tankersley et al., 1994) could be responsible in part for the absence

of the respiratory PD endophenotype in the RCF-treated DBA mice. However, the USV data suggested that the early manipulation differentially affected pups of the two strains, and permanent effects could be seen in various neurobiological systems. Early aversive events affected the development of the subject—precocious manipulations can be more intrusive than later ones. The RCF protocol, applied during the *stress*-hyporesponsive-period (which begins immediately after birth in mice, Schmidt et al., 2003) did not appear to affect the activity of the HPA axis as evidenced by the lack of HPA hormonal responses to stress in outbred RCF mice (D'Amato et al., 2011; Luchetti et al., 2015).

The functionality of the reward system appeared affected in these RCF animals. Coherently, a series of studies identified changes in behavioral profiles in C57 and DBA in their responses to drugs of abuse and stress in adulthood (Di Segni et al., 2016; 2017; Ventura et al., 2013). When exposed to acute stress (forced-swimming test), RCF-C57 adult mice showed increased sensitivity for a natural reinforcing stimulus, RCF-DBA mice responded with an "anhedonic-like" phenotype in comparison with their controls (Di Segni et al., 2016). Early adversities promote addiction in C57 and depression in DBA mice.

The results of this study confirm that the early environment in immature species modulates several behavioral and homeostatic systems and that the resulting adaptation follows disparate strategies, according to the genetic profile of the individual. DNA methylation has been proposed as one of the molecular mechanisms for how early environment could influence transcription processes modulating biological processes (Szyf & Bick, 2013). Czamara et al. (2021) reported as five cohorts of human patients revealed combined effects of genotype and childhood adversity in shaping variability of DNA methylation. Coherently, we found histonic protein and DNA-related enrichments of genes (such as the acid-sensing ion channel family) that are simultaneously subserving anxiety, learning, and respiration (Cittaro et al., 2016; Giannese et al., 2018) associated with the RCF protocol. Coherent with these findings, this study stresses that CO_2 exposure in adulthood results from different susceptibilities to acidosis, likely deriving from the G x E interaction that programs the individual phenotype during early life. The RCF procedure affected the infant-mother relationship, disrupting the pups' attachment behavior with their caretaker. This early manipulation did not result in overt maladaptive behaviors under basal conditions. However, exposure to challenges in adulthood revealed an underlying fragility of various systems that, according to the genetic makeup of the individual, led to differential behavioral and physiological profiles.

DATA AVAILABILITY STATEMENT

All data in the manuscript are available on reasonable request.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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