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Corticosterone may interact with peripubertal development to shape adult resistance to social defeat

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ABSTRACT

Studies of social stress in adult mice have revealed two distinct defeat-responsive behavioral phenotypes; "susceptible" and "resistant," characterized by social avoidance and social interaction, respectively. Typically, these phenotypes are observed at least 1 day after the last defeat in adults, but may extend up to 30 days later. The current study examined the impact of *peripubertal* social defeat on immediate (1 day) and adult (30 day) social stress phenotypes and neuroendocrine function in male C57BL/6 mice. Initially, peripubertal (P32) mice were resistant to social defeat. When the same mice were tested for social interaction again as adults (P62), two phenotypes emerged; a group of mice were characterized as susceptible evidenced by significantly lower social interaction, whereas the remaining mice exhibited normal social interaction, characteristic of resistance. A repeated analysis of corticosterone revealed that the adult (P62) resistant mice had elevated corticosterone following the social interaction test as juveniles. This was when all mice, regardless of adult phenotype, displayed equivalent levels of social interaction. Peripubertal corticosterone was positively correlated with adult social interaction levels in defeated mice, suggesting early life stress responsiveness impacts adult social behavior. In addition, adult corticotropin-releasing factor (CRF) mRNA in the paraventricular nucleus of the hypothalamus (PVN) was elevated in all defeated mice, but there were no differences in CRF mRNA expression between the phenotypes. Thus, there is a delayed appearance of social stress-responsive phenotypes suggesting that early life stress exposure, combined with the resultant physiological responses, may interact with pubertal development to influence adult social behavior.

1. Introduction

Adolescence is a critical developmental period during which social stress can have lasting emotional and behavioral consequences (Buwalda et al., 2011; Paus et al., 2008; Spear, 2000). Several animal studies demonstrate a significant impact of early life stress exposure on neuroendocrine function and behavioral outcomes. For example, a single exposure to elevated platform stress as a juvenile combined with adult swim stress increases anxiety-like behavior as measured by open-field and acoustic startle (Avital and Richter-Levin, 2005). Thus, juvenile stress exacerbates the consequences of adult stress. Similarly, a 3 day stress exposure in adolescent rats (PND 34, 45 or 55) reduces locomotion and increases acoustic startle when animals are tested as adults (Cymerblit-Sabba et al., 2015). Other studies show that juvenile and adult neuroendocrine responses to stress are different. Juvenile exposure to several acute stress procedures increases adrenocorticotropic hormone (ACTH) and corticosterone secretion that persists for twice as long compared with adult rats (Goldman et al., 1973; Romeo et al., 2006b; Romeo et al., 2006c; Romeo et al., 2004; Vázquez and Akil, 1993). Exposure to chronic restraint stress, however, produces an elevated corticosterone response in juveniles and a faster return to baseline compared to adults (Romeo et al., 2006b). The latter effect is due to increased activation of corticotropin-releasing factor (CRF) neurons in the paraventricular nucleus (PVN) of the hypothalamus in juveniles compared with adults, and suggests pubertal maturation reorganizes neuroendocrine stress responses (Romeo et al., 2006b).

Although these studies have uncovered important differences in physiological and behavioral responses to non-social stress between juveniles and adults, they provide little information on specific effects of juvenile *social* stress on these outcomes. Studies in Syrian hamsters show that males exposed to repeated social stress as juveniles display an accelerated transition of agonistic behavior from play fighting to adult aggression (Wommack and Delville, 2003; Wommack et al., 2003). This effect is due, in part, to elevated secretion of glucocorticoids (Wommack and Delville, 2007; Wommack et al., 2005), suggesting that early hypothalamic-pituitary-adrenal (HPA) axis function can shape adult behavior. It is not completely clear if a more social species, such as mice, show similar alterations in aggression in response to juvenile or pubertal social stress. The limited studies available find that defeated juvenile or pubertal mice display reduced social interaction, increased

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anxiety-like behavior, and spatial learning deficits as adults (Jacobson-Pick et al., 2011; Novick et al., 2013; Vidal et al., 2007).

Most social stress studies in mice focus on adults and have revealed two distinct defeat-responsive behavioral phenotypes; "susceptible" and "resistant" (aka unsusceptible or resilient) characterized by social avoidance and social interaction, respectively. These phenotypes are observed at least 1 day after the last defeat session, but may extend up to 30 days later (Dulka et al., 2015; Gilman et al., 2015; Krishnan et al., 2007; Meduri et al., 2013). In addition, although a large literature has identified several neurobiological mechanisms underlying susceptibility and resistance to social defeat in adult animals (Berton et al., 2006; Cao et al., 2010; Gilman et al., 2015; Jasnow et al., 2004a; Jasnow et al., 1999; Jasnow et al., 2004b; Jasnow and Huhman, 2001; Jasnow et al., 2005; Krishnan et al., 2007; Romeo et al., 2007; Vialou et al., 2010) few studies examine the ontogeny of these behavioral phenotypes by documenting the longitudinal effects of juvenile social stress.

In the current set of experiments we examine the immediate and long term effects of peripubertal social stress in mice and focus on identifying predictive markers and mechanisms underlying the divergent phenotypic behavioral responses to peripubertal social defeat that are present in adult mice. Specifically, we exposed peripubertal male mice to mild repeated social defeat followed by social interaction tests 1 day and 30 days later. We measured serum corticosterone and testosterone as well as brain CRF mRNA levels to identify behavioral, physiological, and molecular indicators associated with adult social defeat-responsive behavioral phenotypes.

2. Methods

2.1. Animals

All mice were male C57BL/6 mice bred in our animal facility and weaned 21 days after birth (P21). Experimental C57BL/6 mice were group housed (2–5 per cage) with male littermates until the first day of defeats, after which they were singly housed for the duration of the experiment (32 days). Animals were left undisturbed throughout the duration of behavioral testing except for routine animal care. Male CD — 1 mice used as aggressors were individually housed and prescreened for aggression. Group housed, age-matched male CD — 1 mice were used as social targets during social interaction. All mice were housed on a 12:12 light:dark cycle with lights on at 7 a.m. and allowed access to food and water *ad libitum*. Experiments and procedures were approved by Kent State University Institute of Animal Care and Use Committee (IACUC) and conducted in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals, 8th Ed.

2.2. Experiment 1: longitudinal behavioral responses to peripubertal social defeat

Thirty-three C57BL/6 mice were used in Experiment 1. Experimental mice were housed and weaned as described above and were exposed to 2 days of social defeat or control procedures and tested for social avoidance behavior 1 day (P32) and again 30 days (P62) after the last day of defeat.

2.2.1. Social defeat procedure

P28–31 male C57BL/6 was randomly assigned to treatment groups (control or defeat). Social defeat involved placing the experimental mouse into the home cage of an aggressive, territorial CD — 1 mouse for 5 min or 3 attacks, whichever came first (modified to "3 attacks" from Meduri et al., 2013). The number of attacks was recorded to ensure that each experimental mouse was attacked equally. Additionally, we have previously shown that animal-to-animal variability in attacks does not influence the development of susceptible and resistant phenotypes (Meduri et al., 2013). Following exposure to attacks, mice were separated across a Plexiglas divider for the remainder of an hour for sensory exposure to the CD — 1 mouse and their cage (Fig. 1B). After the 55-minute separation, mice were transferred into the cage of a different CD — 1 mice and the defeat was repeated until each mouse encountered 4 different aggressors per day for 2 consecutive days. Control mice were placed across a Plexiglas divider for an entire hour with no physical contact. Social defeat occurred between 11 a.m. and 5 p.m.

2.2.2. Social interaction procedure

Experimental mice were tested in social interaction approximately 24 h after the last social defeat procedure and within 4 h of the onset of the dark cycle (P30–33) (referred to as P32). These same experimental mice were tested again 30 days after the initial interaction test (P60–63) (referred to as P62; Fig. 1A). During each social interaction test, animals were placed in an open field arena (46 cm × 46 cm × 39 cm; Coulbourn Instruments) where social behavior was measured under dim red light. A Galaxy cup with metal bars (Spectrum, Streetsboro, OH; Moy et al., 2004; Nadler et al., 2004) was placed upside-down against one of the four corners. The metal bars are spaced to allow sensory (olfactory, visual and auditory), but not physical, contact. During the first trial, experimental mice were placed into the center of the arena and allowed to explore for 300 s. During the second trial, social

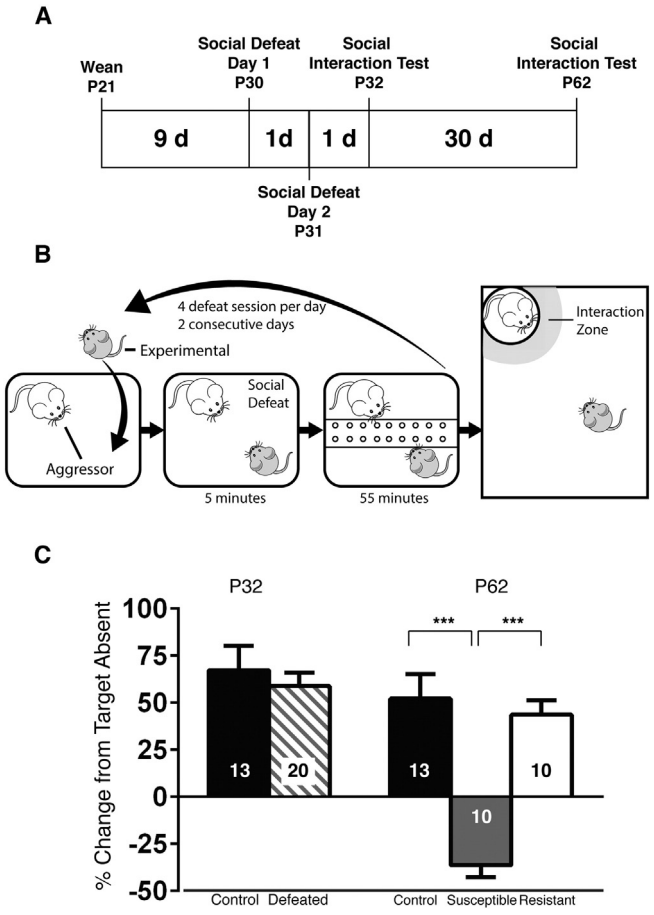

Fig. 1. Peripubertal social defeat results in a delayed emergence of susceptible and resistant phenotypes. (A) Timeline for peripubertal social defeat (P30) and social interaction (P32), as well as adult social interaction tests (P62). (B) Schematic representation of the social defeat procedure and social interaction test. (C) At P32, peripubertal defeated mice interact with a target mouse at similar levels compared with controls ($p > 0.05$). The same mice tested again as adults at P62 display two behavioral phenotypes. A subset of mice exhibit a susceptible phenotype characterized by significantly less interaction compared to controls ($p < 0.001$) whereas the remainder exhibit a resistant phenotype characterized by social interaction similar to non-defeated controls. Control and resistant mice interact at a similar level ($p > 0.05$). Data are expressed as mean ± SEM. $^{***}p < 0.001$.

targets were placed in the Galaxy cup and experimental mice were again allowed to explore for 300 s. Time spent interacting with social targets was measured *via* AnyMaze 4.99 software (Stoelting, Wood Dale, IL). We calculated the percent change in interaction from when the social target was absent (% change from target absent) = (time spent interacting with target present) / (time spent interaction with target absent) × 100 − 100. This way, defeated mice with scores >0 were classified as resistant, whereas defeated mice with scores <0 were classified as susceptible (Dulka et al., 2015; Gilman et al., 2015; Krishnan et al., 2007; Meduri et al., 2013).

2.3. Experiment 2: do neuroendocrine and molecular responses predict adult phenotypic responses to peripubertal social defeat?

Fifty-one C57BL/6 mice were used in Experiment 2. In this experiment we looked for predictive markers of the behavioral phenotypes that emerge in adulthood following peripubertal social defeat. In order to determine individual differences in baseline corticosterone and testosterone following social defeat, we used repeated blood collection. Blood was collected at baseline (P23–24) before any behavioral manipulations or testing, and again 30 min following each social interaction test (P32 and P62). Cheek blood from the submandibular vein was collected for baseline, after the first social interaction test, and after the first defeat by using a 4 mm lancet (Golde et al., 2005). Thirty minutes after the adult (P62) interaction test, animals were sacrificed and trunk blood was collected. All blood samples were collected within a 2-hour time window and within 4 h of the onset of the dark cycle to control for circadian variation in corticosterone. Samples were allowed to clot for 1 h at room temperature, then centrifuged at 3500 rpm for 1 h at 4 °C. Blood serum was stored at −80 °C until analysis (Jasnow et al., 2000). Social defeat and social interaction procedures were identical to those describes in Experiment 1. After the last social interaction, animals were sacrificed, trunk blood was taken and brains were extracted and immediately frozen on powdered dry ice and stored at −80 °C for later sectioning and analysis using *in situ* hybridization.

2.3.1. Serum hormone immunoassays

Serum corticosterone and testosterone were measured using Enzo Life Sciences corticosterone and testosterone enzyme-linked immunosorbent assay (ELISA) kits (Farmingdale, NY) according to the manufacturer's instructions. The plate was read at 405 nm with correction at 595 nm. The cross reactivity for the corticosterone assay was 28.6% for deoxycorticosterone, 1.7% for progesterone, and <0.3% for all other hormones. The inter-assay variability was <15% for all plates and the intra-assay variability was <10%. The cross reactivity for the testosterone assay was 14.64% for 19-hydroxytestosterone, 7.20% for androstendione, and <0.01% for all other hormones. The inter-assay variability was <10% for all plates and the intra-assay variability was <5%. The sensitivity of the assay was 27 pg/ml.

2.3.2. In situ hybridization

For production of the CRF riboprobe, a pBluescript SK vector containing a 1.2-kb fragment of the rat CRF cDNA was linearized with *Xho*I to make antisense cRNA probes. Labeled RNA probes were synthesized by *in vitro* transcription of linearized DNA templates using the appropriate T3 polymerase with S35-labeled UTP (Romeo et al., 2007). Full-length probes were separated from labeling reactions *via* size-exclusion columns before being mixed with hybridization buffer and measured for specific activity. *In situ* hybridization was performed on 16 μm sections sliced on a cryostat and mounted on Superfrost Plus slides (Fisher Scientific, Waltham, MA). A standard hybridization procedure was used with slight modifications (Jasnow et al., 2013). Briefly, slides were fixed in 4% paraformaldehyde for 30 min, rinsed through a series of phosphate-buffered saline (PBS) washes, followed by rinses in triethanolamine with acetic anhydride. Slides were then washed in PBS and dH_2O and dehydrated through a series of graded alcohols. For prehybridization, slides were exposed to hybridization solution in the absence of labeled probe for 2 h at 55 °C in a humidified chamber. Following our prehybridization procedure, the sections were hybridized with S^{35}-labeled CRF probes at 55 °C for 16 h in a humidified chamber and then underwent a series of rigorous washes, exposure to RNase and dehydration through a series of ethanol washes containing NaOAc. Slides were air dried and then exposed to Kodak BioMax MR film for 14 days to generate autoradiograms.

Relative densities were measured from the autoradiograms using standard computerized image analysis software (NIH Image; ImageJ). The paraventricular nucleus (PVN) was traced and densities were collected for the left and right hemisphere for each animal and averaged together. The average densities were calculated and grouped according to adult phenotypic behavior.

2.4. Experiment 3: do juvenile corticosterone responses after social stress differ between phenotypes?

Twenty-three C57BL/6 mice were used to determine whether peripubertal social stress-induced corticosterone responses would differ between adult behavioral phenotypes. In Experiment 3, the social defeat procedure was modified slightly in order to measure corticosterone in response to a single social defeat, but also measure behavioral phenotypes in response to repeated social defeat. Thus, mice were exposed to a single 5-minute defeat or 3 attacks and then separated for sensory contact. Thirty minutes after the end of the defeat, blood was drawn as described above and then animals were placed back in the cage to finish the remainder of the 55-minute sensory contact. All blood was taken within a 2-hour period and within 4 h of the onset of the dark cycle to control for circadian variation in corticosterone. To control for altered defeat responses as a result of the blood draw, the remaining 3 rounds of defeats were carried out the following day (rounds 2, 3, 4). Twenty-four hours later, defeats resumed normally as described in Experiment 1.

2.5. Statistical analysis

Results of behavioral tests are expressed as the individual interaction ratio scores, and with indicators of each respective mean ± standard error of the mean (SEM). Behavioral tests were analyzed using a *t*-test or one-way analysis of variance (ANOVA), and Tukey's post hoc analyses were run where appropriate. Testosterone data was analyzed using one-way ANOVA and Tukey's post hoc analysis. Corticosterone results were analyzed using a two-way ANOVA with repeated measures, followed by a Tukey's post hoc analysis where appropriate. Area means for PVN measurements were averaged together and analyzed using a one-way ANOVA test and Tukey's post hoc analysis. Significant level was set *a priori* to $p < 0.05$. A Pearson's correlation coefficient was run between interaction ratios and corticosterone levels. Effect size estimates were assessed using G*Power 3 (Faul et al., 2007) and determined according to Cohen (1988) for *t*-tests and reported as eta^2 for ANOVA analyses.

3. Results

3.1. Experiment 1: delayed emergence of social stress-responsive behavioral phenotypes

In Experiment 1, peripubertal social defeat had no immediate effect on social interaction when animals were tested 24 h later. Nearly all defeated animals displayed high levels of social interaction with a novel conspecific target mouse, similar to non-defeat controls. A *t*-test on P32 defeated (n = 20) and control (n = 13) mice revealed no significant difference between levels of social interaction ($t_{(31)} = 0.61$, $p > 0.05$, d = 0.17) (Fig. 1C).

After 30 days at P62, these same animals were tested again for social interaction. Half of the peripubertal defeated mice displayed social avoidance behavior indicative of a susceptible phenotype (n = 10). The remaining mice displayed a resistant phenotype (n = 10) as evidenced by continued social interaction. One-way ANOVA analysis revealed a significant difference between groups ($F_{(2, 30)} = 21.26$, $p < 0.001$, $\eta^2 = 0.59$). Post hoc comparisons revealed that control and resistant mice spent significantly more time interacting than susceptible mice ($p < 0.001$). No differences in interaction time between control and resistant mice were observed ($p > 0.05$) (Fig. 1C).

3.2. Experiment 2: peripubertal corticosterone after social interaction predicts an adult-emergent resistant phenotype

In Experiment 2, we investigated predictive markers of the behavioral phenotypes that emerge in adulthood following peripubertal social defeat. We replicated the initial behavioral effects observed in Experiment 1. A *t*-test on social interaction behavior on P32 between control (n = 16) and defeated (n = 35) mice revealing no significant difference between levels of interaction ($t_{(49)} = 0.19$; $p > 0.05$, d = 0.16) (Fig. 2B). When these same mice were tested for social interaction as adults at P62, divergent stress-responsive behavioral phenotypes were again

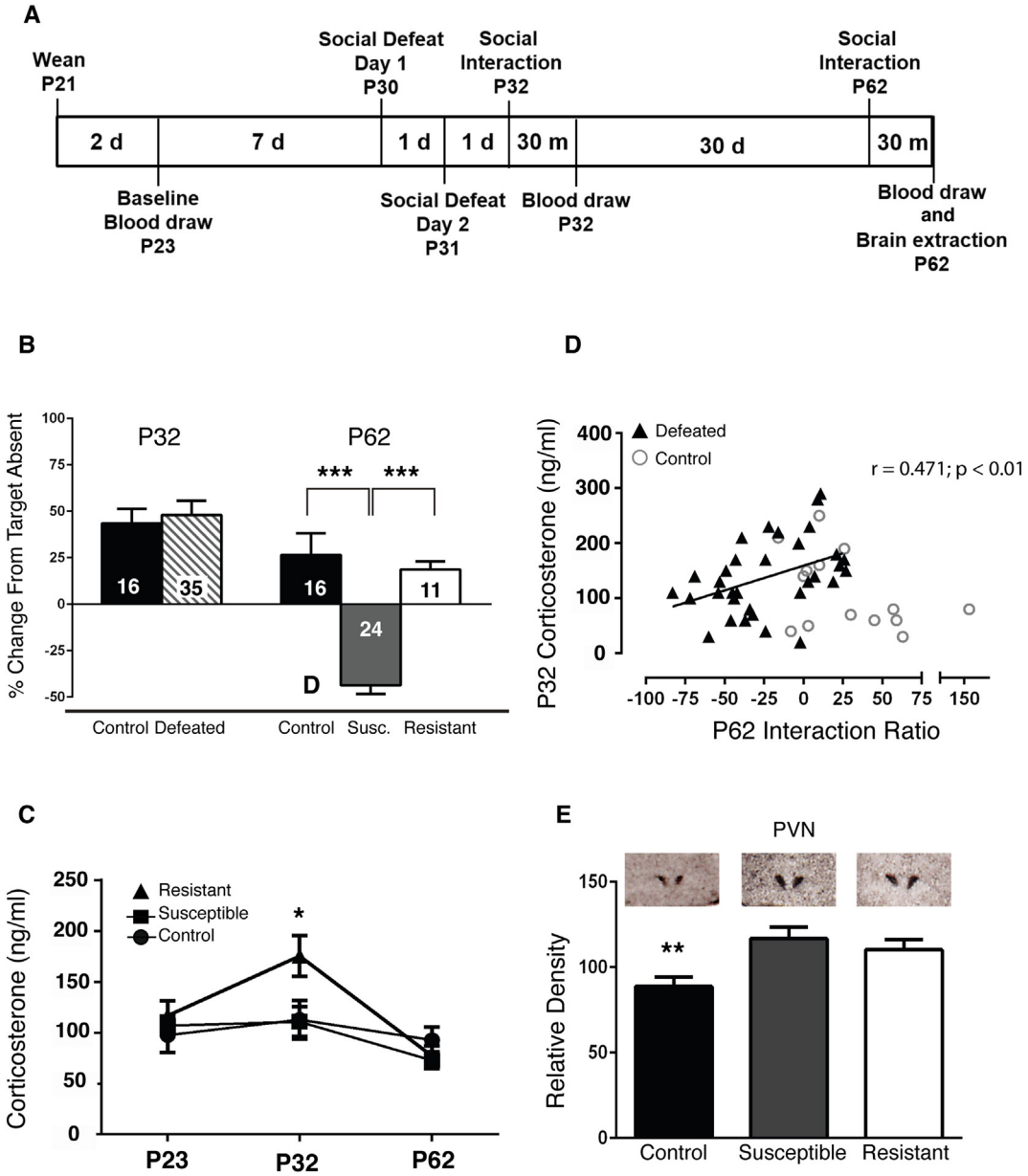

Fig. 2. Peripubertal corticosterone response predicts adult behavioral phenotype. (A) Timeline for peripubertal social defeat (P30) and social interaction (P32), as well as adult social interaction tests (P62) and blood draws. (B) At P32, peripubertal defeated mice interact with a target mouse at similar levels compared with controls ($p > 0.05$). The same mice tested again as adults at P62 display two different phenotypes. A subset of mice exhibit a susceptible phenotype characterized by social avoidance whereas the remainder exhibit a resistant phenotype characterized by social interaction similar to non-defeated controls. Control and resistant mice interact at a similar level ($p > 0.05$). (C) Mice that maintain the resistance in adulthood (P62) displayed significantly elevated corticosterone during social interaction testing as juveniles (P32). Resistant mice had significantly elevated corticosterone at P32 compared to their baseline (P23) levels ($p < 0.05$) and after adult interaction (P62) ($p < 0.001$). Resistant mice also displayed significantly greater corticosterone at P32 compared to susceptible and control (*$p < 0.05$) mice. Susceptible mice displayed significantly elevated corticosterone at P32 compared to P62 ($p < 0.05$). (D) Peripubertal corticosterone during social interaction testing at P32 is significantly positively correlated with social interaction levels at P62 for defeated mice ($r = 0.47$; $p < 0.01$), but not for control mice. (E) As adults (P62) susceptible and resistant mice have significantly elevated CRF mRNA in the PVN compared to controls, but are not different from each other (**$p < 0.01$). Data are expressed as mean ± SEM. ***$p < 0.001$.

observed. A one-way ANOVA revealed a significant difference between interaction ratios ($F_{(2, 48)} = 29.27$; $p < 0.001$, $\eta^2 = 0.55$). Post hoc comparisons revealed that control (n = 16) and resistant mice (n = 11) spent significantly more time interacting compared to susceptible mice (n = 24) ($p < 0.001$). No differences in interaction time between control and resistant mice were observed ($p > 0.05$) (Fig. 2B).

Serum was collected at P23 for baseline corticosterone levels and again 30 min following social interaction testing on P32 and P62 for post interaction corticosterone responses. After social interaction on P62, peripubertal defeated mice were classified into susceptible and resistant phenotypes based on their social interaction scores. Analysis of serum collected at baseline (P23), P32, and P62 were based on the phenotypic groups determined *after P62 social interaction*. Neuroendocrine exclusion criteria were applied to animals with corticosterone levels that were 2 or more standard deviations away from the mean (n = 4). For the corticosterone analysis, a two-way repeated measure ANOVA revealed a significant main effect for time, ($F_{(2, 88)} = 15.21$, $p < 0.001$; $\eta^2 = 0.13$) and phenotype ($F_{(2,44)} = 4.17$, $p < 0.05$; $\eta^2 = 0.03$) and a significant time × phenotype interaction ($F_{(4,88)} = 2.57$, $p < 0.05$; $\eta^2 = 0.06$). Post hoc analysis revealed that resistant mice had significantly elevated corticosterone during the P32 social interaction test compared to baseline ($p < 0.05$) and P62 ($p < 0.001$) and significantly elevated corticosterone during the P32 social interaction test compared to susceptible ($p < 0.01$) and control ($p < 0.05$) mice. This analysis also revealed that susceptible mice had elevated corticosterone at P32 compared to P62 ($p < 0.05$) (Fig. 2C). We hypothesized that the P32 corticosterone response would be associated with interaction levels at P62 such that increased peripubertal corticosterone predicts higher levels of interaction in adulthood. A Pearson's correlation coefficient analysis indicated a significant positive correlation between P32 corticosterone in defeated mice, regardless of phenotype ($r = 0.47$; $p < 0.01$) (Fig. 2D), but no correlation for control mice ($p > 0.05$). Therefore, higher peripubertal corticosterone after P32 social interaction appears to predict higher social interaction at P62 in defeated mice, but not in control mice.

To further identify differences in HPA axis responsivity to juvenile social defeat stress, CRF mRNA in the PVN was measured 30 min following P62 social interaction. A one-way ANOVA revealed a significant difference in the expression of CRF mRNA in the PVN among the groups ($F_{(2,53)} = 9.28$; $p < 0.001$; $\eta^2 = 0.28$). Post-hoc comparisons revealed that susceptible and resistant mice had significantly elevated CRF mRNA in the PVN compared with control mice ($p < 0.01$, $p < 0.001$ respectively). No differences were observed between susceptible and resistant mice ($p > 0.05$) (Fig. 2E).

In addition to measuring corticosterone, testosterone was measured at P32 after social interaction to verify peripubertal endocrine status (control = 11; susceptible = 22; resistant = 11) (Fig. 3B). We also evaluated whether testosterone differed between phenotypes in adulthood (P62). A one-way ANOVA revealed no difference in testosterone levels between control, susceptible, and resistant mice ($F_{(2, 41)} = 1.77$, $p > 0.05$; $\eta^2 = 0.79$) (Fig. 3C). There was also no correlation between social interaction at P62 and testosterone in defeated mice in general ($r = -0.20$; $p > 0.05$) or control mice ($r = 0.52$; $p > 0.05$) (data not shown).

3.3. Experiment 3: peripubertal corticosterone after social defeat is not different between adult-emergent behavioral phenotypes

We identified that elevated corticosterone 30 min after the peripubertal social *interaction* test was associated with the resistant phenotype and increased social interaction in adulthood, but we were interested in identifying if juvenile corticosterone responses to social *defeat* also differ between the adult-emergent phenotypes. In a separate group of mice, blood serum was collected 30 min after the first social defeat encounter. A *t*-test on interaction ratios at P32 between control (n = 6) and defeated (n = 17) mice revealed no significant differences between levels of social interaction ($t_{(21)} = 0.33$; $p > 0.05$; d = 0.13)

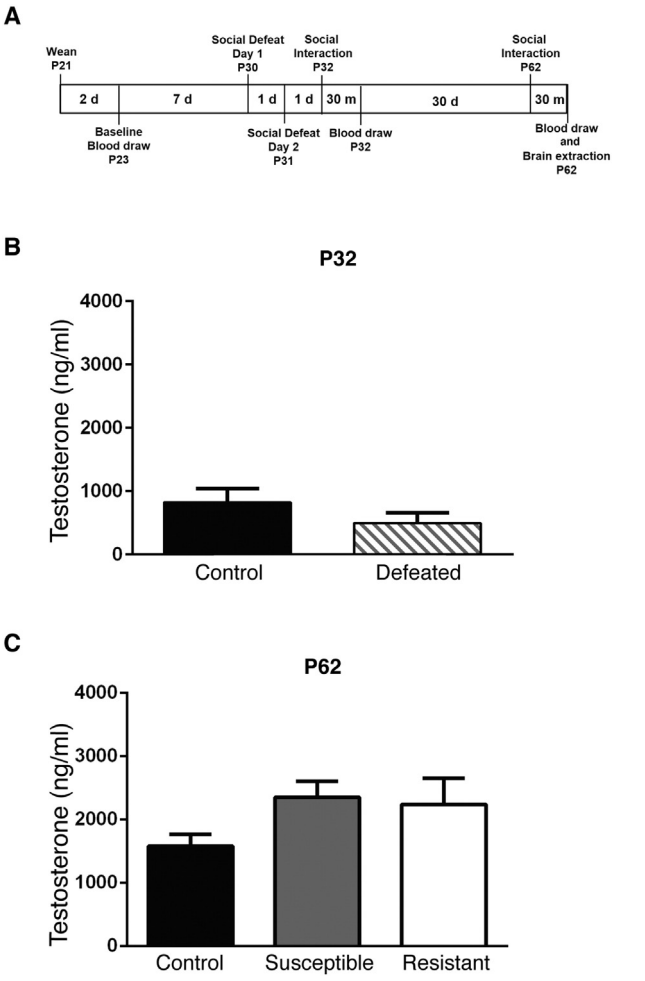

Fig. 3. Testosterone is not associated with behavioral phenotype. (A) Timeline for experiments (same as Fig. 2). (B) Peripubertal testosterone levels at P32 after social interaction confirming peripubertal state and are no different between control and defeated mice ($p > 0.05$). (C) Testosterone levels measured after social interaction at P62 were not different among the behavioral groups.

(Fig. 4B). When these same animals were tested at P62, susceptible and resistant phenotypes were observed. A one-way ANOVA revealed a significant difference between social interaction levels ($p < 0.001$). Post hoc comparisons revealed that control (n = 6) and resistant (n = 13) mice spent significantly more time interacting than susceptible mice (n = 4) ($F_{(2, 20)} = 10.09$; $p < 0.001$; $\eta^2 = 0.50$). No differences in interaction time between control and resistant mice were observed ($p > 0.05$) (Fig. 4B). The corticosterone analysis *via* one-way ANOVA revealed a significant difference in corticosterone levels among the groups ($F_{(2, 20)} = 8.93$; $p < 0.01$; $\eta^2 = 0.47$). Post hoc comparison revealed that resistant and susceptible mice had significantly elevated corticosterone immediately following social defeat stress compared with control mice ($p < 0.01$). No differences in corticosterone between susceptible and resistant mice were observed ($p > 0.05$) (Fig. 4C).

4. Discussion

In the current set of experiments, we demonstrate for the first time that peripubertal social defeat results in a delayed emergence of social stress-responsive behavioral phenotypes. In particular, when peripubertal mice were tested for social behavior 1 day after experiencing social defeat, nearly all mice displayed resistance characterized by increased social interaction with a novel conspecific. When the same mice were tested again as adults (P62), however, a susceptible phenotype emerged in a significant percentage of the mice. The remaining

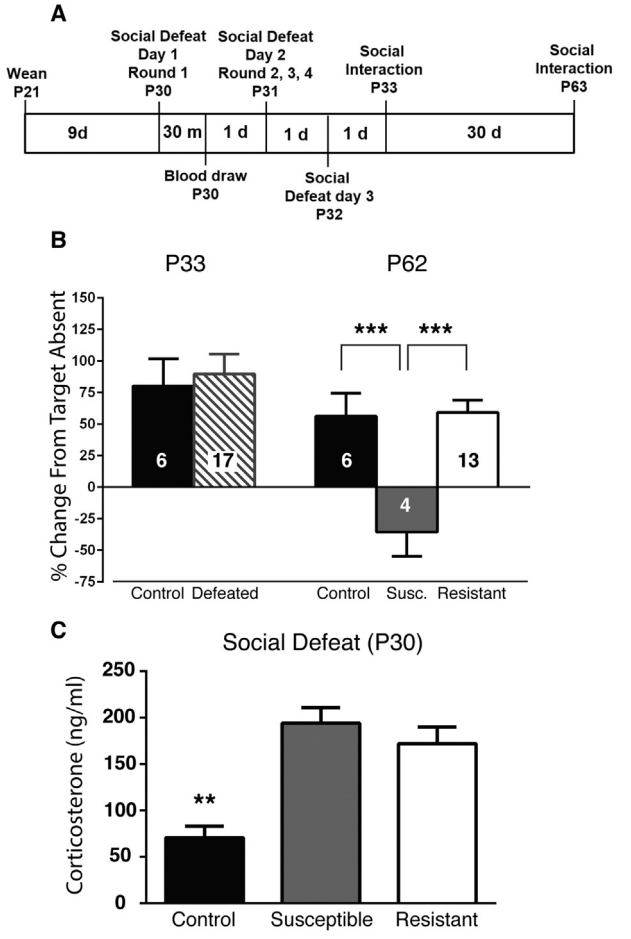

Fig. 4. Susceptible and resistant mice show equivalent corticosterone responses after social defeat. (A) Timeline for peripubertal social defeat (P30) and social interaction (P32), as well as adult social interaction tests (P62) and blood draws. (B) At P32, peripubertal defeated mice interact with a target mouse at similar levels compared with controls ($p > 0.05$). The same mice tested again as adults at P62 display two different phenotypes. A subset of mice exhibit a susceptible phenotype characterized by social avoidance whereas the remainder exhibit a resistant phenotype characterized by social interaction similar to non-defeated controls. Control and resistant mice interact at a similar level ($p > 0.05$). (C) Serum corticosterone response 30 min after the initial social defeat encounter. Susceptible and resistant mice both display significantly elevated corticosterone compared to non-defeated controls, but are not different from each other. Data are expressed as mean ± SEM.$^{**}p < 0.01$; $^{***}p < 0.001$.

mice continued to display resistance. The emergence of susceptibility in adulthood suggests that the effects of peripubertal social stress either require an incubation period before becoming apparent, or that the appearance of different phenotypes in adulthood is potentially related to peripubertal HPA-axis activity interacting with development to affect adult social behavior. For instance, in the present study corticosterone levels measured after peripubertal social interaction significantly correlated with adult social interaction in defeated mice (Fig. 2D). These data suggest increased peripubertal corticosterone could influence adult social behavior phenotypes; higher juvenile corticosterone was associated with adult resistance and increased social interaction. Similarly, exposure to repeated juvenile social stress alters the development and trajectory of agonistic behavior in Syrian hamsters (Wommack and Delville, 2003; Wommack et al., 2003), that is dependent upon glucocorticoid release (Wommack and Delville, 2007; Wommack et al., 2005). In the present study, however, corticosterone measured 30 min after a single peripubertal social stress exposure did not differ between adult behavioral phenotypes. The only time when corticosterone differed between the behavioral phenotypes was *after* social interaction testing at P32. There are a few possible explanations for this finding. First, we only sampled corticosterone 30 min after the first defeat rather than after the full two days of defeats. It is possible that the phenotypes differ in their corticosterone response to repeated social defeat over the 2 days and our sampling method would have missed this difference. It is also possible that prolonged glucocorticoid release over the two days of defeat may be necessary to establish adult behavioral phenotypes and is likely regulated by glucocorticoid and/or mineralocorticoid receptors. Future studies manipulating corticosterone, blocking corticosterone synthesis or secretion, and examining the role of glucocorticoid and mineralocorticoid receptors will be required to examine this hypothesis in more detail.

Finally, we hypothesized that differences in CRF mRNA expression in the PVN might underlie elevated corticosterone in resistant mice during the initial social interaction test. Both susceptible and resistant mice exhibited increased CRF mRNA expression in the PVN compared with control mice. These data suggest that peripubertal social defeat has prolonged consequences within the PVN, resulting in increased CRF mRNA levels into adulthood regardless of phenotype. This is unlike what was found for peripubertal chronic non-social stress where increased activity of PVN CRF neurons was broadly related to an increased peripubertal neuroendocrine stress response (Romeo et al., 2006b). In our set of experiments, however, we were unable to *a priori* identify the resistant and susceptible phenotypes at P32 when a significant difference in corticosterone response to social interaction occurred, and consequently we could not examine peripubertal CRF mRNA. Thus, it remains possible that at P32 there exist differences in PVN CRF mRNA among mice who eventually develop resistant and susceptible phenotypes. Alternatively, the driving mechanism for increased juvenile corticosterone in resistant mice might be downstream of PVN CRF or in its negative feedback pathway.

In all experiments, we demonstrated that peripubertal social defeat produced delayed effects on subsequent social interaction. Peripubertal mice displayed social-approach behavior resembling resistance during a social interaction test 1 day after social defeat at P32. However, a delayed stress-responsive phenotype emerged when mice were tested as adults at P62. Although a population of mice remained resistant, a substantial number of mice displayed a susceptible behavioral phenotype characterized by reduced social interaction. These two phenotypic responses to social defeat observed in adults here are consistent with previous observations in our lab and by others (Berton et al., 2006; Cao et al., 2010; Dulka et al., 2015; Gilman et al., 2015; Krishnan et al., 2007; Meduri et al., 2013). One possible caveat to using the interaction ratio (Target present/Target absent), however, is that the two behavioral phenotypes may represent the habituation to the chamber irrespective of whether a conspecific target is present, rather than a measure of social interaction phenotypes.

Previous studies have observed increased corticosterone levels, reduced locomotor behavior and increased startle in adult rats that had been stressed during adolescence but not necessarily at other times during development (Avital and Richter-Levin, 2005; Cymerblit-Sabba et al., 2015). These studies, however, did not examine social behavior, nor were they able to examine differences in stress phenotypes. A study by Iñiguez et al. (2014) used chronic (10 days) social defeat stress beginning at P35 and examined its effects across a number of behavior measures. This study found that social stress during adolescence produced decreased social interaction and increased depressive- and anxiety-like responses; indexed by reduced sucrose preference, decreased latency to immobility in a force swim test, and reduced time in open arms on the elevated plus maze. Taken together, these studies demonstrate that adolescence is a sensitive period during which social or environmental stressors can have both immediate and lasting effects on behavior. These studies, however, were not able to determine individual differences in social stress responsiveness and how they may relate to adult social behavior. In the present study, we show peripubertal (P32) social stress had delayed effects on social behavior, but we did not examine startle and other measures of depression- and anxiety-like behavior.

Peripubertal animals display increased and prolonged corticosterone responses to restraint stress (Romeo, 2010), but these data have not been previously associated with behavioral phenotypes. Krishnan et al. (2007) found that resistant (unsusceptible) and susceptible mice displayed increased corticosterone in response to a forced swim test 1 day after the end of a 10-day social stress procedure. Thirty days after the end of social defeat, however, resistant (unsusceptible) mice showed increased AM corticosterone levels, whereas susceptible mice exhibited decreased corticosterone compared to non-defeated controls. In the current study, both resistant and susceptible mice display increases in corticosterone immediately after social defeat. Yet, only mice that remain resistant in adulthood display elevated corticosterone during social interaction 24 h after peripubertal social defeat. In addition, the P32 corticosterone response to social interaction was significantly correlated with adult (P62) social interaction for all defeated mice, suggesting that the peripubertal corticosterone could have a direct impact on adult social behavior. Although our study shows no difference in corticosterone between resistant and susceptible mice in adulthood, resistance may be characterized by a more sensitized peripubertal physiological response to stress. For example, increases in corticosterone can alter stress response trajectory, in which injections of corticosterone can protect against PTSD-like symptoms in rodents (Rao et al., 2012; Zohar et al., 2011). The current data are consistent with these findings that increased corticosterone plays a protective role against stress-responsive physiology and behavior. As noted above, future studies manipulating corticosterone or its receptors will be required to test this hypothesis directly.

The current experiments contribute novel and important findings to the ontogeny of social stress responsiveness. Peripubertal social defeat produced a latent emergence of stress-responsive behavioral phenotypes. Adult resistance was associated with increased peripubertal corticosterone during a social interaction test, and peripubertal corticosterone significantly correlated with adult social behavior, suggesting early life corticosterone can help shape adult social behaviors. It is not currently clear if the increased peripubertal corticosterone secretion in resistant mice is only in response to a social challenge or represents a prolonged response to social defeat. It seems unlikely, though, that corticosterone would be elevated 1 day after social defeat given the relatively rapid recovery observed in both juvenile and adult rats in response to restraint stress (Romeo, 2010; Romeo et al., 2006a). Future experiments will systematically assess the peripubertal neuroendocrine response to social stress to determine if individual response differences can help shape adult behavior. The current set of experiments has begun to define the mechanisms underlying the delayed emergence of social stress-responsive phenotypes in adulthood. These findings and future studies will help determine the role of stress hormones on the ontogeny of individual responses to adolescent social stress.

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