Published in final edited form as:

Horm Behav. 2014 August ; 66(3): 517–524. doi:10.1016/j.yhbeh.2014.07.010.

Temperament moderates the influence of periadolescent social experience on behavior and adrenocortical activity in adult male rats

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Abstract

Adolescence is a period of significant behavioral and physiological maturation, particularly related to stress responses. Animal studies that have tested the influence of adolescent social experiences on stress-related behavioral and physiological development have led to complex results. We used a rodent model of neophobia to test the hypothesis that the influence of adolescent social experience on adult behavior and adrenocortical function is modulated by preadolescent temperament. Exploratory activity was assessed in 53 male Sprague-Dawley rats to classify temperament and then they were housed in one of three conditions during postnatal days (PND) 28-46: (1) with familiar kin, (2) with novel social partners, or (3) individually with no social partners. Effects on adult adrenocortical function were evaluated from fecal samples collected while rats were individually-housed and exposed to a 1-hour novel social challenge during PND 110-114. Adolescent-housing with novel or no social partners led to reduced adult glucocorticoid production compared to adolescent-housing with familiar littermates. Additionally, highly-exploratory pre-weanling rats that were housed with novel social partners during adolescence exhibited increased exploratory behavior and a more rapid return to basal glucocorticoid production in adulthood compared to those housed with familiar or no social partners during adolescence and compared to low-exploratory rats exposed to novel social partners. In sum, relatively short-term adolescent social experiences can cause transient changes in temperament and potentially longer-term changes in recovery of glucocorticoid production in response to adult

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social challenges. Furthermore, early temperament may modulate the influence of adolescent experiences on adult behavioral and adrenocortical function.

Keywords

Behavioral inhibition; Neophobia; Adolescence; Fecal corticoid; Social experience

Adolescence is a unique period for social and adrenocortical development; it is a period when non-familial social experiences become more frequent while significant neuronal and adrenocortical maturation is occurring (Spear 2000; Douglas, Varlinskaya, & Spear, 2004; Romeo 2010). In particular, the acute glucocorticoid response in human and rodent adolescents requires a longer period to return to baseline levels compared to adults and prepubertal youth, and this may be an important developmental period for programming of adult adrenocortical responses (Walker, Walder, & Reynolds, 2001; Stroud et al., 2009; Romeo, 2010; Folib, Lui, & Romeo, 2012). Adaptations to current and future environments are also driven by adolescent social experiences and interact with glucocorticoid hormones to shape adult behavioral profiles (Sachser, Kaiser, & Hennessy, 2013).

The rodent adolescent period is typically defined as postnatal day (PND) 28-46, with a characteristic increase in non-familial social interactions first evident at approximately PND 28 (Spear, 2000), and maturation of hypothalamic-pituitary-adrenal (HPA) axis negative feedback mechanisms throughout (Romeo, 2010). Adolescent social experiences may shape adult behavior and HPA function via interactions between these two developmental phenomena.

Long-term effects of peripubertal experiences on behavioral and HPA axis profiles have been reported. For example, in rodents, adolescent chronic social stress (isolation, social reorganization, subordination during PND 28-70+) causes protracted corticosterone (CORT) responses and elevated basal CORT levels in adulthood and these effects can be prevented by anti-depressant (paroxetine) or corticotropin-releasing hormone 1 receptor antagonist (DMP696) administration during the stress procedure (Schmidt et al., 2007; Sterlemann et al., 2008; Ros-Simó & Valverde, 2012; Toth, Mikics, Tulogdi, Aliczki, & Haller, 2011). However, models of adolescent stress are highly variable (e.g. social vs. physical stressor, novel social partners vs. social isolation, etc.), and outcomes of these models are inconsistent with numerous reports of unaffected adult adrenocortical activity after peripubertal stress (Isgor et al., 2004; McCormick, Smith, & Mathews, 2008; McCormick, Robarts, Gleason, & Kelsey, 2004; Lukkes, Mokin, Scholl, & Forster, 2009). In the current study, we tested the hypothesis that long-term influences of adolescent experiences may be temperament-specific.

Behavioral inhibition (BI) or neophobia is a relatively stable behavioral trait in humans and rodents that emerges early in development (infancy) and is characterized by fear and avoidance of novel or unfamiliar situations and objects, and heightened cortisol reactivity after exposure to psychosocial stress, novel social situations, and novel objects (Kagan, Reznick, & Snidman, 1987; Schmidt et al., 1997; Cavigelli & McClintock, 2003; Cavigelli et al., 2007; Walker & Mason 2012). In humans, this trait has been associated with increased

risk of adolescent and adult mood disorders, which are often associated with altered adrenocortical regulation (e.g. Rosenbaum et al., 1993; Schwartz, Snidman, & Kagan, 1999). Incidentally, some studies (human and rodent) have also shown greater glucocorticoid responses to novelty in non-inhibited (i.e. approach-oriented) individuals compared to inhibited ones (e.g. Dellu et al., 1996; de Haan et al., 1998; Stansbury & Harris, 2000), and that behavioral responses to psychological stress that are incongruent with an individual's preferred coping strategies may account for some of these seemingly contradictory findings (Stansbury & Harris, 2000; Tarullo, Mliner, & Gunnar, 2011). For example, in a novel peer interaction test children who exhibited behavior that was incongruent with their self-reported peer competence had larger HPA responses than children exhibiting congruent behavior, regardless of temperament (Stansbury & Harris, 2000). Furthermore, increased HPA activity was observed for highly inhibited children who were more social than those that were less social, and for uninhibited children who were less social than their uninhibited counterparts (Tarullo et al., 2011). Given increased fear-related responses to novel experiences, BI or neophobia may represent a specific trait that modulates how particular adolescent social experiences (novel social partners vs. lack of social partners) shape adult behavior and adrenocortical regulation. In the present study, we used a rodent model of neophobia to experimentally test the influence of several different adolescent social experiences (familiar kin partners, novel social partners, and no social partners) on the development of adult behavioral responses and glucocorticoid responses to novelty. We predicted that individuals that do not readily engage novelty (neophobic) will exhibit greater adrenocortical upregulation after complex, novel adolescent experiences (e.g. exposure to novel social partners) compared to after simple adolescent experiences (e.g. social isolation or familiar social partners). Additionally, we predicted that individuals that readily engage novelty (neophilic) will exhibit adrenocortical upregulation in response to simple adolescent environments such as social isolation compared to more complex social experiences like exposure to novel partners.

Previously we found that rat (Sprague-Dawley) neophobia/philialia, characterized by locomotion in an unfamiliar and protected arena, was related to latency to approach novelty, and was moderately stable from pre-weaning age throughout adulthood, and was reproducible across studies (Cavigelli & McClintock, 2003; Cavigelli et al., 2007; Cavigelli, Ragan, Michael, Kovacsics, & Bruscke, 2009). Neophobic or inhibited males also had greater plasma CORT responses to novelty and stress compared with neophilic or non-inhibited males (Takahashi, 1992; Cavigelli & McClintock, 2003; Veenema, Sijtsma, Koolhaas, & de Kloet, 2005; Cavigelli et al., 2007; Qi et al., 2010; Díaz-Morán et al. 2013; c.f. Dellu et al. 1996). Furthermore, neophobia is associated with decreased voluntary interactions with enriched environments and early environmental conditions can influence the development of this trait and its associated glucocorticoid profile (Tang, Reeb-Sutherland, Romeo, & McEwen, 2012). To our knowledge no one has assessed whether adolescent social experiences modify the development of this trait, and/or if this temperament dimension modifies the influence of adolescent social experiences on behavioral and glucocorticoid development into adulthood.

We tested an interactional 'temperament x adolescent social experience' hypothesis – i.e. that temperament modulates the influence of adolescent social challenges on adult

behavioral and glucocorticoid response development. Specifically, we tested a ‘congruent-incongruent’ hypothesis that neophilic rats exposed to novel, complex social experiences would be less challenged by this ‘congruent’ adolescent experience than neophobic rats exposed to the same novel complexity, and/or neophilic rats exposed to no social partners (i.e. ‘incongruent’ with their temperament), and that temperament-adolescent ‘incongruent’ experiences will lead to relatively long-term upregulation of glucocorticoid production. In addition, because previous work showed that temperamental traits observed in rodents and humans can be stable throughout life, we expected that locomotion in a novel environment would be relatively stable within individuals during the peripubertal and young adult periods, but that this behavioral trait may be modulated by adolescent social experiences.

Methods

Animals

Fifty-three male Sprague-Dawley rats from 15 litters were housed in solid-bottom plastic cages (43.5 × 23.5 × 20.5 cm). Rats were maintained on a 14L:10D lighting schedule with lights on at 2000 h (central standard time, CST) and *ad libitum* access to food and water. Cages were cleaned twice a week by trained animal facility personnel. The colony room was maintained at 22°C with ~50% humidity. All methods detailed below were approved by the University of Chicago Institute for Animal Care and Use Committee and adhered to the methods specified in the *Guide for the Care and Use of Laboratory Animals* (1996).

Overall Design

Rats were housed with the dam and littermates from birth (i.e. PND 0) until PND 22. At PND 18, each pup was given an individually-unique ear notch, and at PND 20 pups were tested on the Exploration Arena to estimate neophobia. To our knowledge, there are no studies evaluating the duration or magnitude of the effects of the ear notch procedure on subsequent locomotion, however, our personal observations of pups ear-notched at this age is that they return to original behavioral profiles within hours of the procedure. Rats were weaned at PND 22 and housed in same-sex sibling trios with similar temperament distribution in each cage (one neophobic rat, one neophilic rat, and one non-responsive rat – see ‘Exploration arena’ below). During PND 28-46, rats were placed in one of three experimental adolescent social conditions: (1) a control group (KIN) in which rats remained in groups of three same-sex littermates, (2) a social reorganization group (SRO) for which three unrelated same-sex novel social partners were housed together, or (3) an individual group (IND) in which rats were housed alone. In the KIN and SRO conditions, each group included one neophobic rat, one neophilic rat, and one non-responsive rat to ensure that social experiences were similar across all cages. These housing manipulations were developed to mimic social experiences that may be considered common during adolescent development in social species (e.g. moving to a new environment, social isolation, etc.) and were considered to be relatively short-lived and benign manipulations. On PND 46 all rats were rehoused in the original same-sex littermate trios.

To determine if adult exploratory behavior and/or glucocorticoid production were altered by these adolescent experiences and/or by a congruent-incongruent interaction between

temperament and adolescent social experience, rats were again tested on the Exploration Arena at PND 60 and 85, and from PND 110-114 fecal samples were collected and analyzed for fecal corticosteroid levels (see Glucocorticoid Measure below). To sample feces from individuals and to provide a complex novel challenge to stimulate glucocorticoid production, rats were placed in individual hanging cages on PND 110 ('Day 1') and then exposed to three novel social partners for one hour on PND 111 ('Day 2'; see Social Challenge below). Fecal boli were regularly collected across all days.

Behavioral Response to Novelty

Behavioral testing was conducted in a non-colony room during the rat active period (4-6 hours after lights off). The room was illuminated with a red light providing approximately 6 lux of light at the center of the testing arena.

Exploration arena—This arena was used to assess rat exploratory behavior on PND 20, 40, 60, and 85. It was square with tall opaque walls ($92 \times 92 \times 23$ cm area for rats at PND 20; $122 \times 122 \times 46$ cm for older rats) and a Plexiglas cover with a 3×3 grid that divided it into 9 equal divisions for quantification of locomotion. Inside the arena were three rat-sized objects that were placed 13 cm from each corner; objects were different for each test age to ensure object novelty. To provide familiar odors from cagemates and from colony room members, the floor was covered with clean wood chips that were then sprinkled with a small amount of bedding from all colony cages. If a rat defecated in the arena during testing, feces were removed and no further cleaning occurred. To begin the test the rat was placed in a ceramic bowl with 5 cm-high walls and lowered into the empty corner of the arena. The test lasted 5 minutes and each rat was video-recorded for the duration of the test. At the conclusion each rat was returned to the home cage, transported back to the colony room, and then the test bowl rinsed with water and dried for the next rat. Locomotion in the arena was used to estimate neophobia/neophilia; locomotion was quantified as the number of times a rat crossed all 4 limbs over a line of the 3×3 grid on the arena cover. In prior studies, we have found that this measure of locomotion in the arena was closely related to latency to approach the novel objects in the arena at several different ages (Cavigelli et al. 2007). The locomotion score is a measure of behavioral activity in a novel environment, but it does not quantify which grid squares were visited or how much time was spent within each square.

Rat temperament (neophobic or neophilic) was assigned based on locomotion in the Exploration Arena at PND 20. Locomotion scores varied both between and within litters, thus to control for litter effects, we assigned temperament within litters, identifying 1-2 neophobic and 1-2 neophilic males from each litter. Within each litter, males with the highest locomotion scores were classified as neophilic and males with the lowest, non-zero locomotion scores as neophobic. The range of litter locomotion scores at PND 20 for neophobic vs. neophilic rats was 6-34 (for neophobic) and 33-54 (for neophilic). Rats that did not move in the arena were considered non-responsive and were not included in analyses.

Glucocorticoid Measure

Fecal corticoids—To assess basal and glucocorticoid responses to a complex, novel social experience we evaluated glucocorticoid diurnal rhythms in feces in response to individual housing and a social challenge (see below). Fecal corticosteroid metabolite measures provide a minimally-invasive method to evaluate basal and long-term corticosterone production in response to complex and lengthy experimental manipulations while minimizing the influence of frequent blood sampling (Cavigelli et al., 2005). During the fecal collection period, rats were placed in cages with hanging wire bottoms through which feces dropped into a pan of standard wood-shaving bedding (Sani-Chips, Laboratory grade). Fecal samples were removed from the pan with forceps and placed in Whirl-pak bags (Nasco, Fort Atkinson, WI, USA), labeled, and stored at -30 °C until extraction. Forceps were cleaned with ethanol after each collection to avoid cross-contamination. Samples were collected at 3-hour intervals (600, 900, 1200, 1500, 1800, 2100, and 2400 hr) for 4 consecutive days. Given the ~6-12-hr lag time required for circulating steroids to be excreted in rat feces (Cavigelli et al., 2005; Harper & Austad, 2000), we were able to assess basal corticoid levels (from Day 1 samples), corticoid responses to novel individual-housing and novel social partners (from Days 2-3 samples), and recovery after novelty (Day 4 samples).

Fecal corticoid extraction—Fecal steroids were extracted using methods previously described (Cavigelli et al., 2005). Frozen samples were allowed to thaw and desiccated in a centrifugal evaporator and dry weights recorded. Dry samples were crushed into a fine powder, 0.2 g placed in a 15 ml polypropylene centrifuge tube and 10 ml of 100% ethanol added to each sample. Samples were boiled in a water bath for 20 min at 78°C, centrifuged at 2000g for 15 min, and the supernatant poured off into a borosilicate glass culture tube. Another 5 ml 100% ethanol was added to the fecal pellet and samples were vortexed for 1 min, centrifuged for 15 min at 2000g, and the supernatant added to the previous 10 ml of extract. Samples were evaporated with air and reconstituted with 1 ml of methanol. After reconstitution all samples were stored at -80°C until assay. The outcome measure was total fecal corticoids excreted during each collection day. We used this total production measure to estimate total corticosterone production across a whole day as opposed to short-term responses to acute challenges (Cavigelli et al., 2005; Lepschy et al. 2010). We also analyzed total dried fecal mass to determine if fecal mass was driving any of the total fecal corticoid results reported.

Radioimmunoassay—Fecal corticoid metabolites were measured using commercially-available [¹²⁵I] Radioimmunoassay for rat and mouse serum/plasma (MP Biomedicals, Solon, OH). The antibody in this assay binds to 3 of 6 corticosterone metabolites in rats and thus provides a broad estimate of corticosterone production (Cavigelli et al., 2005), and the fecal corticoid metabolite measure is closely related to circulating corticosterone levels (Thanos et al., 2009). Samples were diluted 1:50 with the steroid diluent provided with the assay kit which ensured antibody binding on the linear portion of the standard curve (20-80% binding). The range of the standard curve was 12.5 to 1000 ng/ml. Duplicates were run for all samples and any sample with a coefficient of variation above 10% was re-

analyzed. Inter-assay and intra-assay coefficients of variance were 10.7 and 8.6, respectively.

Social challenge—At 4 months of age rats were tested in a novel social situation. During a 1-hour social challenge, 3 rats that had no prior interactions with each other were placed in an arena with similar dimensions as the one used in the adult exploration arena tests. The rats were of similar age and size. Each social challenge group included of one neophobic rat, one neophilic rat, and one non-responsive rat. After the social challenge rats were placed in their wire-bottom home cages and returned to the colony room.

Statistical Analyses

Prior to statistical analyses we tested whether data were normally-distributed (according to Kolmogorov-Smirnov tests). Because fecal corticoid output for days 2 through 4 were not normally-distributed, we used log-transformed values to satisfy distribution requirements for parametric statistical analyses. For clarity, data are presented as untransformed values in figures and the table. All analyses were conducted with SPSS version 22.

To test the hypothesis that individual temperament status was stable over time, repeatability of locomotor behavior in the test arena was estimated from an intraclass correlation coefficient (ICC) using locomotion scores across the 4 test ages (PND 20,40,60,85). The ICC is based on variance components derived from a one-way analysis of variance (ANOVA) and is calculated as $r = s^2_a / (s^2 + s^2_a)$, where s^2 is within-individual variance overtime and s^2_a is the among-litter variance (Lessels & Boag, 1987). ICC is often used to determine individual consistency in repeatedly measured behaviors both in the field and laboratory (Bell, Hankinson, & Laskowski, 2009; Lessels & Boag, 1987). To determine if adult exploratory behavior was affected by weanling exploratory behavior, adolescent social conditions, and/or an interaction between weanling temperament and adolescent experiences, a 2×3 (temperament $\times$ adolescent condition) ANOVA was used with adult (PND 60 and 85) locomotion values as dependent variables ($n=53$). To verify that weanling locomotor behavior did not differ among the adolescent experimental groups, a similar ANOVA was conducted PND 20 locomotion values as the dependent variable ($n=53$). To estimate effect sizes for all ANOVA main and interaction effects, eta squared (η^2) was calculated to indicate the proportion of variance in the dependent variable that was accounted for by the independent variable. Cohen's d was used to estimate effect size for pairwise comparisons. Because locomotion varied both within and between litters (e.g., the range of locomotion scores for two extreme litters were 22-79 vs. 69-117), litter mean locomotion scores were included as a covariates in the above models to control for between-litter variance.

To compare daily fecal corticoid production across sampling days, a repeated measures ANOVA was used with time as the within subjects factor. To assess the effects of temperament and adolescent social conditions on adult baseline fecal corticoid output, a 2×3 (temperament $\times$ adolescent condition) ANOVA was conducted using Day 1 fecal measures ($n=45$; not all males defecated during this baseline period). To determine if adult glucocorticoid responses to novelty were affected by temperament, adolescent social

conditions or an interaction between these factors, two 2×3 (temperament $\times$ adolescent condition) ANOVAs were conducted with total corticoid metabolite production and total daily fecal mass production on the second and third days of fecal collection, when fecal corticoid levels were highest ($n=53$). Last, to determine if weanling temperament and/or adolescent social conditions affected adult rates of glucocorticoid recovery (i.e. the ability to return to basal levels), two similar ANOVAs were conducted with total fecal corticoid levels and fecal mass on the last day of sampling – the fourth day after adult individual housing began ($n=53$). Total daily fecal mass production was assessed to ensure that differences in fecal corticoid levels were not due to differences in the amount of fecal mass produced between groups. Total dry fecal mass was not included in the final model because it did not significantly affect fecal corticoid levels. All post-hoc analyses were conducted with a Bonferroni correction for multiple comparisons, $p < 0.05$.

Daily total fecal corticoid production on days 2, 3, and 4 were calculated as the sum total (ng) of fecal corticoids produced from 900-900 hr the following day. Mean total daily fecal corticoid production varied considerably between and within litters, thus mean litter production was used as a covariate in the above models. Eight rats did not defecate during day 1 of individual housing: 2 neophilic males (1 KIN, 1 SRO), and 6 neophobic males (1 KIN, 4 IND, 1 SRO). Independent-samples t-tests were conducted to compare behavior in the exploration arena and daily fecal corticoid output between the males that were removed from analyses and those that were not removed. Males that did not defecate on day 1 had significantly lower locomotion in the exploration arena on PND 20 than males that did defecate on day 1 ($\bar{X} \pm \text{SD}$: 21 ± 19 vs. 36 ± 17 , $t_{51} = 2.28$, $p < 0.05$, Cohen's $d = 0.63$). There were no other differences between males that did and did not defecate on day 1 in behavior or in total fecal corticoid output over days 2-4.

Results

Weanling and Adult Behavioral Response to Novelty

An intraclass correlational analysis revealed that locomotion in a novel, complex environment was relatively stable (i.e. repeatable) within individuals across PND 20, 40, 60, and 85 ($r_{49,147} = 0.75$, $p > 0.05$). There were no main effects of weanling-based temperament or adolescent social condition on young adult (PND 60) locomotion in the exploration arena ($F_{1,51} = 0.16$, $p = 0.70$, $\eta^2 = 0.00$; $F_{2,51} = 0.51$, $p = 0.60$, $\eta^2 = 0.00$), but there was an interaction effect of temperament and adolescent condition ($F_{2,51} = 4.33$, $p < 0.05$, $\eta^2 = 0.05$; Figure 1A). Interestingly, this interaction effect was no longer present at PND 85 ($F_{2,52} = 0.67$, $p = 0.52$, $\eta^2 = 0.01$), and there was only a main effect of weanling-based temperament on adult (PND 85) locomotion in the exploration arena: males identified as neophilic at weaning crossed significantly more lines as adults in the exploration arena than did males identified as neophobic at weaning ($F_{1,52} = 4.64$, $p < 0.05$, $\eta^2 = 0.05$; Figure 1B). At this later age, there were no significant differences in locomotion among the three adolescent social conditions ($F_{2,52} = 1.67$, $p = 0.20$, $\eta^2 = 0.03$). Weanling locomotion on PND 20 did not differ among the KIN, SRO, and IND males ($\bar{X} \pm \text{S.E.M.}$: 34 ± 2 vs. 35 ± 2 vs. 33 ± 2 ; $F_{2,52} = 0.19$, $p = 0.83$, $\eta^2 = 0.00$) nor was there an interaction effect of

temperament and adolescent condition on locomotion at this age ($F_{2,52} = 1.51, p = 0.23, \eta^2 = 0.02$).

Adult Fecal Corticoids

Adult males displayed a circadian rhythm in fecal corticoid production across the four consecutive sampling days, with a significant effect of day on total daily fecal corticoid production ($F_{1,52} = 4.24, p < 0.05, \eta^2 = 0.08$): males produced significantly more fecal corticoids on Day 3 than on Day 4 (Figure 2).

Baseline—Adolescent social conditions had a significant effect on adult basal fecal corticoid production (on ‘Day 1’ or PND 110; $F_{2,44} = 4.83, p < 0.05, \eta^2 = 0.07$). KIN males had significantly higher baseline fecal corticoid output than SRO males, and IND males did not differ from KIN or SRO males (Figure 3). Weanling temperament tended to predict mean baseline fecal corticoid production, but this effect did not reach statistical significance ($F_{1,44} = 3.83, p < 0.10, \eta^2 = 0.03$), and there was no interaction between temperament and adolescent condition ($F_{2,44} = 0.68, p = 0.51, \eta^2 = 0.00$). Total dry fecal mass produced during day 1 did not differ between neophobic and neophilic males ($X \pm \text{S.E.M.}$: 1.75 ± 0.44 vs. 1.58 ± 0.14 ; $F_{2,44} = 0.62, p = 0.44, \eta^2 = 0.01$), nor among KIN, SRO, and IND males ($X \pm \text{S.E.M.}$: 1.89 ± 0.17 vs. 1.71 ± 0.18 vs. 1.40 ± 0.19 ; $F_{2,44} = 1.83, p = 0.17, \eta^2 = 0.05$), and there was no interaction of temperament and adolescent condition ($F_{2,44} = 1.70, p = 0.20, \eta^2 = 0.05$).

Social Challenge—Neither weanling temperament ($F_{1,52} = 1.50, p = 0.23, \eta^2 = 0.03$) nor adolescent social condition ($F_{2,52} = 1.03, p = 0.37, \eta^2 = 0.03$) affected day 2 fecal corticoid production, nor was there an interaction ($F_{2,52} = 0.15, p = 0.87, \eta^2 = 0.00$). Adolescent social condition affected day 3 fecal corticoid production with KIN males producing more fecal corticoids compared to SRO and IND males ($F_{2,52} = 3.26, p < 0.05, \eta^2 = 0.08$; Table 1), however day 3 fecal corticoid production did not differ between neophobic and neophilic males ($F_{1,52} = 0.15, p = 0.70, \eta^2 = 0.00$), and there was no interaction between weanling temperament and adolescent social condition ($F_{2,52} = 0.13, p = 0.88, \eta^2 = 0.00$). Fecal corticoid recovery levels (day 4) were significantly affected by adolescent social condition with KIN males producing significantly more fecal corticoids than SRO males ($F_{2,52} = 4.15, p < 0.05, \eta^2 = 0.06$). In relation to the congruent-incongruent hypothesis, there was a significant interaction between weanling temperament and adolescent social condition ($F_{2,52} = 4.67, p < 0.05, \eta^2 = 0.07$; Figure 4); neophilic males that had experienced novel social partners during adolescence (SRO) produced less fecal corticoids than neophobic males that had this same adolescent experience (SRO) and neophilic males that experienced no new social partners during adolescence (IND, KIN). Further, individual-housing negated the difference in fecal corticoid production between neophobic and neophilic males. There was no main effect of weanling-based temperament on day 4 fecal corticoid recovery ($F_{1,52} = 0.97, p = 0.33, \eta^2 = 0.00$). Total dry fecal mass produced on each day did not differ between weanling-based temperament categories ($X \pm \text{S.E.M.}$ for days 2, 3, 4: neophobic = $4.70 \pm 0.20, 5.13 \pm 0.30, 5.70 \pm 0.22$, neophilic = $4.86 \pm 0.20, 5.14 \pm 0.32, 5.38 \pm 0.22$; $F_{1,52} = 0.40, p = 0.53, \eta^2 = 0.00, F_{1,52} = 0.00, p = 0.99, \eta^2 = 0.00, F_{1,52} = 1.03, p = 0.32, \eta^2 = 0.02$) or among adolescent social conditions ($X \pm \text{S.E.M.}$ Days 2, 3, 4: KIN = $4.74 \pm 0.24,$

5.31 $\pm$ 0.38, 5.81 $\pm$ 0.27, SRO = 4.82 $\pm$ 0.25, 4.41 $\pm$ 0.40, 5.03 $\pm$ 0.28, IND = 4.76 $\pm$ 0.24, 5.69 $\pm$ 0.38, 5.79 $\pm$ 0.27; $F_{2,52} = 0.03$, $p = 0.97$, $\eta^2 = 0.00$, $F_{2,52} = 2.78$, $p = 0.07$, $\eta^2 = 0.09$, $F_{2,52} = 2.56$, $p = 0.09$, $\eta^2 = 0.08$). There were no interactions of temperament and adolescent social condition for days 2, 3, or 4 ($F_{2,52} = 0.08$, $p = 0.92$, $\eta^2 = 0.00$; $F_{2,52} = 0.48$, $p = 0.62$, $\eta^2 = 0.02$; $F_{2,52} = 0.64$, $p = 0.53$, $\eta^2 = 0.02$).

Discussion

Temperament/Exploratory Behavior

The results of the present study replicate previous findings that male Sprague-Dawley rats, like human children, display relatively stable individual differences in exploratory behavioral responses to novelty, with weanling exploratory responses predicting adult exploratory behavior (Cavigelli & McClintock, 2003; Cavigelli et al., 2007; Cavigelli et al., 2009). Furthermore, the results indicate that these traits were relatively stable from weaning to young adulthood, even after experiencing divergent adolescent social experiences (i.e. familiar social partners vs. novel social partners vs. social isolation). However, there was evidence of a short-term alteration in exploratory behavior at PND 60 that reflected an interaction of temperament with adolescent social experiences. Specifically, neophobic weanling rats that were housed with novel social partners (SRO) during adolescence (i.e. a hypothesized incongruent social experience relative to temperament) showed decreased exploratory behavior at PND 60 compared to neophobic rats that had been housed with familiar littermates (KIN) or alone (IND) during adolescence (i.e. temperament-congruent social experiences; Figure 1A). On the other hand, neophilic weanling rats exposed to novel social partners during adolescence (i.e. temperament-congruent social experience) showed increased exploratory behavior at PND 60 compared to neophilic rats that were housed with familiar social partners and/or alone during adolescence (i.e. hypothesized temperament-incongruent conditions). Although this interaction of temperament and adolescent social experience was evident at PND 60, this effect dissipated by PND 85 (Figure 1B).

The tendency to approach or avoid novel objects and people, and the amount of interaction with novel stimuli, are frequently-used method for classifying a child as non-inhibited or inhibited (Kagan et al. 1987); similar methods can be used to classify animals as non-inhibited or inhibited. As in humans, animal behavioral inhibition tends to be associated with elevated glucocorticoid production, and is believed to be highly conserved across species (Takahashi, 1994; Schulkin, Gold, & McEwen, 1998; Gosling & John, 1999; Cavigelli, 2005; Cavigelli et al. 2007; Roseboom et al. 2007). These results indicate that naturally-occurring individual differences in exploratory behavior in outbred rodents provide a viable model of human behavioral inhibition.

Recent evidence suggests that the developmental stability of temperament is not as stable as once thought. Environmental factors, in particular maternal factors, may influence the plasticity of temperament (Tang et al. 2012). However, non-maternal social influences may also affect temperament development. For example, in rats the ratio of males to females in a litter predicts 4-month-old, although not 3-month-old, locomotion in an open field (Gracceva, Koolhaas, & Groothuis, 2011). Furthermore, the stability of personality increases significantly during the transition from adolescence to young adulthood in humans (Roberts,

Caspi, & Moffit, 2001). The results from the current study indicate that ‘congruent’ vs. ‘incongruent’ social experiences during adolescence may lead to short-term alterations in exploratory behavior, but that original levels of exploration re-emerge over time. Similar results have been shown in orange-winged Amazon parrots: birds that were hand-reared by humans as neonates showed transient reductions in neophobic behavior, compared to birds that were reared by parents, and that this change disappeared in adulthood (Fox & Millam, 2004). Prior studies have suggested greater behavioral plasticity during infancy and adolescence as compared to adulthood, although this area requires further experimental work (Moretz, Martins, & Robison, 2007; Roberts et al., 2001). Thus, infancy and adolescence may represent a period of plasticity in the development of temperament, with short-term alterations in social conditions leading to transient changes in behavior. Future studies will need to determine if longer-term (congruent or incongruent) social experiences during these early developmental periods lead to long-term alterations in temperament.

Adult Glucocorticoid Production

A characteristic circadian rhythm is evident in adult male fecal corticoid production (Figure 2). Similar results have been observed in prior studies and these results indicate that fecal corticoid production can accurately portray daily fluctuations in adrenal activity as well as adrenal response to an environmental challenge (Cavigelli et al., 2005). At baseline, there was a trend for neophobic males to produce more fecal corticoids, although this difference was not statistically significant. These results differ from prior studies in rats that used the same behavioral protocol and circulating corticosterone measures and in humans in which BI children, teens, and adults showed elevated afternoon cortisol and enhanced cortisol reactivity (Kagan et al., 1987; Schmidt et al., 1997; Cavigelli & McClintock 2003; Cavigelli et al. 2007; Tyrka et al., 2007; Essex et al., 2010). In the current study, there was a significant bias in the number of males that produced feces during this period, with the neophobic males producing feces less often than neophilic males, which may have led to both decreased statistical power and a sampling bias. Interestingly, stable BI is associated with chronic constipation in human children (Reznick, et al., 1986). Additionally, daily integrated measures of fecal corticoid production may not be sensitive enough to detect small, albeit likely functionally-significant, differences in glucocorticoid production between inhibited and non-inhibited individuals (Thanos et al., 2009). Temperament differences in circulating glucocorticoids must be substantial and persist for several hours in order to be detected in fecal samples (Siswanto, Hau, Carlsson, Goldkuhl, & Abelson, 2008).

Social experiences in adolescence shape adult behavioral and neuroendocrine responses (Sachser et al., 2013). In the current study, two-weeks of an adolescent social experience led to significant alterations in adult corticoid excretion at baseline and during exposure to social novelty, and these effects persisted longer than observed alterations in behavior. Specifically, rats that lived with novel social partners during adolescence (SRO) produced less fecal corticoids at baseline and in response to individual-housing and brief exposure to novel social partners in adulthood compared to rats that continuously lived with familiar siblings during adolescence (KIN; Table 1, Figures 3 & 4). This alteration in adult glucocorticoid production was documented more than 2-months following the adolescent

social experience suggesting that there was a long-term recalibration of adrenocortical activity. Similar findings of adult hypothalamic-pituitary-adrenal (HPA) hyporeactivity have been documented in humans and rodents that experienced more severe physical and social stressors during adolescence or adulthood (e.g., four to six weeks of exposure to immobilization, change of cage mate, cage tilt, exposure to white noise, exposure to predator odor; Bazak et al., 2009; Goliszek et al., 1996; Toth et al., 2008; Schmidt et al., 2007; Engert et al., 2010; Rós-Simo & Valverde, 2012; Ostrander et al., 2006). Fries, Hesse, Hellhammer, and Hellhammer (2005) proposed that chronic stress exposure may lead to an initial period of glucocorticoid hypersecretion followed by subsequent HPA hypoactivity. With regard to this theory, adolescent experiences may prepare an organism for its future social environment and regulate HPA axis activity accordingly (Sachser et al., 2013). Hypoactive HPA activity may be an adaptation to prevent negative effects of prolonged stress hormone exposure. However, an alternative interpretation of these results is possible. SRO males may not have produced as much corticosterone as KIN males in response to the adult novel social challenge because it was not as novel an experience; SRO males had already experienced similar social novelty during adolescence which may have led to habituation of the HPA response. Future research would be necessary to determine whether adolescent social experience causes short-term HPA hyperreactivity followed by hypoactivity postulated in Fries and colleagues' theory of the development of hypocortisolism.

The last result from the current study indicates that stable inter-individual differences in behavioral responses to novelty may moderate the influence of adolescent social experiences on adult adrenocortical activity. Specifically, neophilic males that were exposed to novel social partners during adolescence showed significantly shorter increase in adult glucocorticoid production after a complex social challenge compared to neophilic males that received no novel social stimulation during adolescence (SRO vs KIN/IND; Figure 4). This is comparable to a handful of studies which indicate that children that behave in a manner that is congruent with their preferred behavioral coping strategies (e.g., approach or avoid novel or challenging situations) exhibited lower cortisol responses than those that behaved in an incongruent fashion (Stansbury & Harris, 2000; Tarullo et al., 2011). Of note, these children chose to approach or avoid social interaction in these studies, whereas in the current experiment, rats were assigned to a particular adolescent social condition. These prior findings in children, in conjunction with the current longer-term study with rodents, suggest that temperament may modulate the degree to which adolescent social experiences have long-term impacts on adrenocortical function.

Genetics and early life experiences are additional factors that may influence the development of temperament over and above the effects of adolescent social experiences. BI is moderately heritable (Smoller et al., 2005; Oler et al., 2010). In humans, genetic markers associated with BI include single nucleotide polymorphisms (SNPs) in the gene encoding corticotropin releasing hormone (CRH) which regulates fear behavior and HPA axis activation (Smoller et al., 2005). In a non-human primate model of BI, SNPs in the CRH receptor 1 gene and metabolic activity in the hippocampus, which mediates inhibition of the HPA axis stress response via negative feedback, in response to social novelty were heritable and predicted BI (Oler et al., 2010; Rogers et al., 2013). Maternal caretaking also influences behavior and HPA axis activity via epigenetic modifications of HPA axis regulatory genes

(Weaver et al., 2004). In the current study, we controlled for covariation in exploratory behavior and HPA axis activity between litters. Future research is necessary to determine the effect of these early life experiences and genetic factors on the development of temperament and HPA axis activity.

Conclusion

Stable individual differences in rodent exploratory temperament were established early in life based on methods that were comparable to those used to classify childhood behavioral inhibition in humans. Rats that exhibited low-exploration at weaning also exhibited low-exploration in adulthood, although this behavioral disposition was temporarily shifted depending on adolescent social experiences. Furthermore, a short-term novel social experience in adolescence, as compared to stable social housing with littermates/kin, was associated with a significant decrease in adult basal glucocorticoid secretion and a tendency towards low total daily glucocorticoid production after social novelty. Finally, return to basal glucocorticoid production was more rapid in rats that experienced adolescent social conditions that were congruent with their weanling-established temperament. Specifically, neophilic rats that experienced novel social partners during adolescence (i.e., a temperament-congruent social experience) had lower adult glucocorticoid production four days into a novel social experience than neophilic rats that had no novel social partners during adolescence and/or neophobic rats that were exposed to social partners during adolescence (i.e., temperament-incongruent social experiences). In sum, relatively short-term adolescent social experiences can lead to transient changes in temperament and longer-lasting changes in HPA axis regulation, with the specific influence of adolescent social experiences on HPA axis function potentially moderated by early temperament and its relative congruency/incongruency with the adolescent social experience. Interestingly, some long-term physiological consequences of early life experiences may persist despite a lack of persistently altered behavior.

Acknowledgments

Notes Support: This research was supported by National Institute of Child Health and Human Development Grant F32 HD08693 (to S.A.C.), National Institute on Aging Grant PO1 AG018911 (to M.K.M), and National Institute of Mental Health Grant R37 MH41788 (to M.K.M).

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Highlights

- Adult temperament is transiently altered by adolescent social experiences.
- Novel adolescent social experiences reduce adult basal glucocorticoid production.
- Temperament and adolescent social experience interact to alter adult glucocorticoid production.

Figure 1.

Estimated marginal means for locomotion for 53 male rats during 5-minute trials in the exploration arena. (A) * On postnatal day (PND) 60, males identified as neophilic at weaning and that experienced adolescent social reorganization (SRO) crossed significantly more lines than neophobic males that experienced SRO. † Conversely, neophobic males that remained with siblings during adolescence (KIN) crossed significantly more lines than neophilic KIN males. (B) ‡ On PND 85, males identified as neophobic at weaning crossed significantly fewer lines than did neophilic males (all p 's < 0.05). Error bars indicate S.E.M.

Figure 2.

Mean fecal corticoid production at 6 hr intervals for 53 adult male rats over a 4 day period. *

Males produced significantly more fecal corticoids on Day 3 compared to Day 4, $p < 0.05$.

Error bars indicate S.E.M.

Figure 3.

Estimated marginal means for basal fecal corticoid production prior to adult individual housing and social challenge (day 1). * Males that remained with siblings in adolescence (KIN) males had significantly higher basal fecal corticoid output than social reorganization (SRO) males, $p < 0.05$. Error bars indicate S.E.M.

Figure 4.

Estimated marginal means for total fecal corticoid production during recovery from adult individual housing and social challenge (day 4). * Males that experienced adolescent social reorganization (SRO) had significantly lower total fecal corticoid production than males that remained with siblings (KIN). † Males that were identified as neophilic at weaning and exposed to SRO during adolescence had lower total fecal corticoid production than neophobic males that experienced SRO. This temperament-specific corticoid production was not evident in the males that had experienced KIN or individual housing (IND) during adolescence. (All p 's < 0.05). Error bars indicate S.E.M.

Table 1

Mean (S.E.M) total daily fecal corticoid production (ng) in response to individual housing and adult social challenge.

Time (day):	<u>Pubertal Condition</u>		
	KIN (<i>n</i> =18)	SRO (<i>n</i> = 17)	IND (<i>n</i> = 18)
Day 2	2562 (217)	2263 (224)	2045 (217)
Day 3	2930 (229) ^a	2254 (232)	2277 (227)

Note. KIN = Control; SRO = social reorganization; IND = individual housing;

^aDay 3: KIN < SRO and IND, $p < 0.05$.