Pharmacology, Biochemistry and Behavior 100 (2011) 25–32

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Post-weaning environmental enrichment alters affective responses and interacts with behavioral testing to alter nNOS immunoreactivity

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ARTICLE INFO

Article history:
Received 20 December 2010
Received in revised form 29 June 2011
Accepted 7 July 2011
Available online 18 July 2011

Keywords:
Wheel running
Enrichment
Social housing
Isolation
Anxiety
Depression
Aggression
NOS-1

ABSTRACT

Challenging early life events can dramatically affect mental health and wellbeing. Childhood trauma and neglect can increase the risk for developing depressive, anxiety, and substance abuse disorders. Early maternal separation in rodents has been extensively studied and induces long-lasting alterations in affective and stress responses. However, other developmental periods (e.g., the pubertal period) comprise a critical window whereby social and environmental complexity can exert lasting changes on the brain and behavior. In this study, we tested whether early life environmental complexity impacts affective responses, aggressive behaviors, and expression of neuronal nitric oxide synthase (nNOS), the synthetic enzyme for nitric oxide, in adulthood. Mice were weaned into social + nonsocial enrichment, social only enrichment, or standard (isolated) laboratory environments and were tested in open field, elevated plus maze, forced swim, and resident–intruder aggression tests 60 days later. Social + nonsocial enrichment reduced locomotor behavior and anxiety-like responses in the open field and reduced depressive-like responses in the forced swim test. Social housing increased open arm exploration in the elevated plus maze. Both social + nonsocial enrichment and social housing only reduced aggressive behaviors compared with isolation. Social + nonsocial enrichment also increased body mass gain throughout the study. Finally, socially-housed mice had reduced corticosterone concentrations compared with social + nonsocial-enriched mice. Behavioral testing reduced nNOS-positive neurons in the basolateral amygdala and the ventral lateral septum, but not in the social + nonsocial-enriched mice, suggesting that environmental complexity may buffer the brain against some environmental perturbations.

1. Introduction

Aversive early life experiences have enduring maladaptive consequences. Childhood abuse and neglect have been linked to the development of health problems and result in early onset of depression and lack of coping mechanisms (Shonkoff et al., 2009). Individuals who experience stress early in life are hypothesized to develop pathophysiological changes in the central nervous system, increasing their vulnerability to stressors later in life, and predisposing them to psychological and physical disorders (Neigh et al., 2009). In rodents, early adverse experiences such as long-term maternal separation or pre- or neonatal stress can increase anxiety- and depressive-like responses and alter the HPA-axis in adulthood (Chung et al., 2005; Fumagalli et al., 2007; Marais et al., 2008; Meaney, 2001). Further, neonatal communal nesting, which may be a form of social enrichment has enduring effects, as well as transgenerational effects on behavior (Branchi, 2009; Curley et al., 2009. Manipulations of environmental complexity (opportunities for wheel running and social, tactile, and visual stimulation) during the pubertal period may provide insights into how environmental factors interact with gene products during adolescence to influence affective and stress-related disorders in adulthood.

'Environmental enrichment,' a housing manipulation that commonly includes opportunities for social interactions, wheel running, and 'toys' such as tubes and nest boxes, has been extensively used to study the impact of environmental factors on learning and memory (Huang et al., 2006; Leggio et al., 2005; Olson et al., 2006; van Praag et al., 2000; Volkmar and Greenough, 1972). Because environmental complexity induces hippocampal growth (i.e., neurogenesis and dendritic complexity), it may provide a means for studying the role of environmental factors in reducing or preventing anxiety- and depressive-like responses (Pittenger and Duman, 2008), both of which have been associated with hippocampal function and morphology. Major depressive-disorder is often accompanied by hippocampal atrophy (Sheline et al., 1996) and treatment with selective serotonin reuptake inhibitors can reinstate hippocampal neurogenesis and

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doi:10.1016/j.pbb.2011.07.008

integration of newly born neurons (Wang et al., 2008). Because environmental complexity enhances hippocampal plasticity, we hypothesized that a more complex environment with both social and nonsocial factors would also reduce depressive-like and anxiety-like responses.

In some cases, early social complexity in the form of communal nesting increased anxiety- and depressive-like responses such as in male CD-1 mice (Branchi et al., 2006). Voluntary wheel running also increased anxiety-like responses in female C57Bl6/J mice (Pietropaolo et al., 2006). In other cases, complexity reduced anxiety- and depressive-like responses induced by maternal behavior of BALB/C mice (Iwata et al., 2007) and basal anxiety- and depressive-like responses in rats (Brenes Saenz et al., 2006). Furthermore, environmental complexity during the peripubertal period reverses the effects of early life maternal separation on both HPA axis reactivity and behavior in a novelty suppressed feeding test (Francis et al., 2002). Thus, the peripubertal period may be a critical period for social and physical environmental factors to influence adult phenotype (Brenes et al., 2009; Pietropaolo et al., 2004). Moreover, continuous enrichment, rather than discrete periods of enrichment, may be necessary to elicit behavioral changes (Bennett et al., 2006; Pietropaolo et al., 2004). We tested the hypothesis that environmental enrichment beginning directly after weaning and lasting throughout testing (early adulthood) reduces anxiety- and depressive-like responses. We assessed aggressive and social behaviors as well because men may externalize depression in the form of irritability or aggression (Radloff, 1975). Furthermore, because early social impoverishment reduces NADPH-diaphorase activity (an indirect marker for NO production) in the orbitofrontal cortex of degus (*Octodon degus*) (Poeggel et al., 2005) we hypothesized that the expression of neuronal nitric oxide synthase (nNOS), the synthetic enzyme for the atypical neurotransmitter nitric oxide (NO), would vary in response to the differential environmental conditions.

NO may also play a regulatory role in depressive- and anxiety-like responses (Volke et al., 2003), but studies investigating the role of NO in anxiety have yielded mixed results. For example, although systemic administration of nonspecific NOS inhibitors increases anxiety-like responses (Czech et al., 2003; DeOliveira et al., 1997), systemic treatment with relatively specific nNOS inhibitors decreases anxiety-like responses (Dunn et al., 1998; Volke et al., 1997). However, nonspecific NOS inhibition also affects endothelial (e) NOS inhibition, which may contribute to changes in behavior via changes in vascular tone. In contrast, NO inhibition has yielded consistent behavioral effects in the forced swim test (FST), a pharmacologically-validated model for depression (Porsolt et al., 1977). NO inhibition, either through genetic or pharmacological manipulation, decreases immobility time, which suggests that NO inhibition ameliorates behavioral despair (Spiacci et al., 2008; Volke et al., 2003). At moderate doses, two NOS inhibitors achieved similar immobility responses as the tricyclic antidepressant, imipramine (Harkin et al., 2004). Genetic or pharmacological suppression of nNOS results in dramatic increases in aggressive and sexual behavior (Nelson et al., 1995; Nelson et al., 2006), and altered stress responses (Bilbo et al., 2003).

The goal of this study was to determine the effects of peripubertal environmental and social complexity on affective responses and aggressive behaviors in adult mice and nNOS expression in limbic and prelimbic areas. We predicted that early environmental enrichment would reduce anxiety- and depressive like-responses and aggressive behavior. We also predicted that environmental enrichment would increase nNOS expression in brain regions important for affective responses. Early, as well as contemporaneous, social environment may alter behavior and nNOS expression within limbic and prelimbic areas. The role of enrichment on NO in the context of spatial learning has been previously reported (Arnaiz et al., 2004), but not in the context of emotional behaviors. Additionally, because repeated handling and behavioral testing may influence histological outcomes, we also included a group of undisturbed mice to control for this possibility.

2. Methods

2.1. Animals

Fifteen dams with nursing litters were obtained from Jackson Laboratory (Bar Harbor, ME) and arrived at our laboratory on postnatal day (PND) 17. A total of 60 male C57Bl6/J mice were used in the study. Litter sizes ranged from 2 to 7 male pups and averaged 4 mice per litter. Mice were weaned on PND 21 and immediately randomly assigned to one of 6 conditions such that no siblings were placed into the same treatment group (thereby mitigating any potential litter or stress effects from shipping): socially housed, no behavior, $n=10$; socially isolated, no behavior, $n=10$; environmentally-enriched, no behavior, $n=10$; socially housed, behavior, $n=10$; socially isolated, behavior, $n=10$; environmentally-enriched, behavior, $n=10$. Mice in the no-behavior group were left unhandled with the exception of body mass assessment and cage changes every two weeks. Mice in the behavior group also underwent body mass measurements and cage changes every two weeks, as well as a behavioral test battery designed to assess affective responses and social behaviors. Tissue collection took place 2–5 days after cessation of behavioral testing. Mice in the isolated and social groups were housed in standard cages ($27.8\times17.5\times13$ cm), whereas mice in the environmentally-enriched group were housed in large cages ($45\text{ cm}\times24\text{ cm}\times20$ cm), each equipped with a running wheel, a nest box, and a Habitrail® tube and 2–3 socially-novel cage mates (Workman et al., 2009; Workman et al., 2010). Mice in the socially housed group were also housed with 2–3 socially novel cage mates. All mice had ad libitum access to food (Harlan Teklad 8640 rodent diet, Indianapolis, IN, USA) and filtered tap water. All mice were housed in the same room with a 16:8 light–dark cycle; lights-off at 1500 h Eastern Standard Time (EST). All procedures were approved by The Ohio State Institutional Animal Care and Use Committee prior to the study and met guidelines published in the National Institutes of Health (1986) Guide for the Care and Use of Laboratory Animals.

2.2. Behavioral testing

At 60 days of age, mice were tested in a battery of behavioral tests designed to assess affective responses and aggressive behaviors. Mice were tested in the following order: open field, elevated plus maze, forced swim test, and resident–intruder aggression test on consecutive days. (See below for detailed methods for each test.) All behavioral testing began at 1500 h EST coincident with lights-off and mice were allowed 30 min to habituate to the testing rooms. Behavioral testing was conducted under dim red light illumination.

2.2.1. Locomotor behavior

To assess total locomotor behavior and anxiety-like responses, mice were placed in a 40×40 cm clear acrylic chamber (open field) lined with ~1 cm corncob bedding, inside a ventilated cabinet. The center of the open field was defined as the central 30×30 cm. A frame at the base of the chamber consisting of 32 photobeams in a 16×16 arrangement, in addition to a row of beams above, detected the location of horizontal movements and rearing, respectively (Open Field Photobeam Activity System, San Diego Instruments, Inc., San Diego, CA, USA). Total movement was tracked for 30 min and analyzed for the percentage of time spent in the center of the open field, number of rears, and total locomotor behavior.

2.2.2. Elevated plus maze

To assess anxiety-like responses, mice were tested in an elevated plus maze. The maze was ~1 m above the floor and consisted of two open

arms bisected by two arms enclosed with opaque black acrylic. The room was illuminated with a dim red light. Mice were placed into the center of the maze, facing a closed arm and recorded on video for 5 min. The maze was thoroughly cleaned with mild soapy water between tests. An open arm entry was scored when all 4 limbs had entered an open arm. Measures analyzed include total time spent in the open arms, number of open arm entries, and percent of transitions into the open arms.

2.2.3. Forced swim test

To assess depressive-like responses, mice were placed in room-temperature water ~17 cm deep within an opaque, cylindrical tank (24 cm diameter, 53 cm height). Swimming behavior was recorded on video for 7 min and scored by a blind observer with Observer software to quantify total number of floating bouts and total time spent floating. A float was scored when a mouse was immobile, and only engaging in movement to keep the head above water. One float bout is any continuous amount of time during which a mouse is immobile. There was no minimum for float time. A float was scored as soon as the animal attained immobility.

2.2.4. Resident–intruder aggression test

For each aggression test, a sexually inexperienced age-matched male was introduced into each resident's home cage for 10 min and recorded on video. For residents in the socially-housed and enriched groups, cage mates where removed while each animal was tested. Intruders were kept in the same animal room as the residents. Biting, boxing, and aggressive grooming/sniffing were quantified as aggressive behaviors. Aggressive grooming and sniffing was operationally defined as when the resident mouse sniffs or grooms the intruder with forepaws on the dorsum of the intruder, the intruder is immobilized, and the resident repeatedly grooms and sniffs the intruder (Clipperton-Allen et al., 2011; Kudryavtseva et al., 1999). We also quantified nonaggressive ano-genital sniffing. All behaviors were scored from videotape by an experimenter uninformed of the treatment groups using Observer software (Version 8.0, Noldus Information Technology, Leesburg, VA, USA).

2.3. Tissue collection and processing

After behavioral testing, a terminal blood sample was collected and kept on ice until centrifugation. Mice were administered an overdose of sodium pentobarbital and perfused through the heart with ice-cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Brains were post-fixed overnight, cryoprotected in 30% sucrose until they sank, rapidly frozen on dry ice, and stored at −80 °C until slicing. Brains were sectioned at 40 μm on a cryostat, and free-floating sections were processed for nNOS immunocytochemistry. Sections were washed three times (5 min each) in phosphate-buffered saline (PBS) and then incubated in 1% sodium borohydride (Fisher, Pittsburgh, PA, USA) in PBS for 10 min. Sections were then blocked in 20% normal goat serum (NGS; Sigma, St. Louis, MO, USA) and 0.3% hydrogen peroxide (Sigma) in PBS for 20 min. Sections were incubated in primary nNOS antibody (No. 23287; ImmunoStar, Hudson, WI, USA; 1:20,000) in PBS with 0.5% Triton-X (TX; Sigma; PBS + TX) and 1% NGS for 48 h. Next, the sections were rinsed three times (5 min each) in PBS and incubated for 90 min with biotinylated goat-antirabbit antibody (Vector Laboratories, Burlingame, CA, USA) in PBS + TX. The sections were then rinsed three times (5 min each) in PBS and then incubated for 30 min in avidin–biotin complex (ABC Elite kit; Vector Laboratories). After three rinses (5 min each) in PBS, the sections were developed in diaminobenzidine in the presence of hydrogen peroxide and nickel (DAB kit; Vector Laboratories) for 90 s. After two rinses (5 min each) in PBS, sections were dipped in distilled water, mounted on gel-coated slides, dehydrated, and coverslipped with Permount (Fisher).

Sections containing the prelimbic cortex (PLC, 2.34 mm), infralimbic cortex (2.34 mm) ventral lateral septum (vLS; 0.26 mm), bed nucleus of the stria terminalis (BNST; 0.02 mm), the paraventricular nucleus (PVN, 0.94 mm), the ventromedial hypothalamus (VMH, −1.70 mm), the medial amygdala (MeA, −1.70 mm), and the CA1 field of the dorsal hippocampus (−2.06 mm) were identified with the mouse brain atlas (Franklin and Paxinos, 2008). Two representative regions from both hemispheres for each brain area were visualized using a Nikon E800 microscope. Immunopositive cells in each area were counted with the aid of Neurolucida software (Microbrightfield, Burlington, VT, USA) and averaged. Positively stained cells were counted if the border was visible against the background and if stained processes could be seen. Overlapping cells were counted as two cells only if there was a distinct border between them. In order to maintain the same area for cell counting in particular brain regions across animals, gridlines in Neurolucida were used to maintain the same region of interest for each brain area. Control sections in which primary antibodies were not added showed no specific staining.

2.4. Corticosterone assay

Blood samples were centrifuged at 4 °C for 30 min at 3300 × *g*. Plasma was aspirated and stored in polypropylene microcentrifuge tubes at −80 °C until radioimmunoassay. Total serum corticosterone concentrations for mice were determined in duplicate in an assay using an ^{125}I double-antibody kit (ICN Diagnostics, Costa Mesa, CA). The high and low limits of detectability of the assay were 1200 and 3 ng/mL, respectively.

2.5. Statistical analyses

All behavioral data were analyzed with housing environment as the independent factor using one-factor ANOVAs and Fisher's PLSD tests for post-hoc comparisons following significant omnibus ANOVAs.

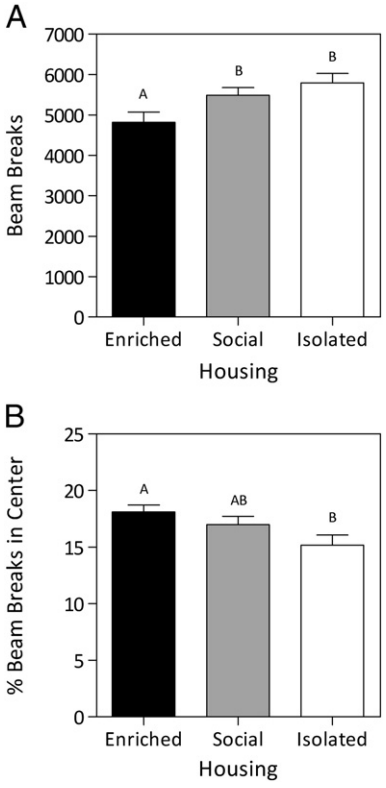

Fig. 1. Open field. A) Mean ± standard error of the mean (SEM) total beam breaks in 30 min of testing. Enrichment significantly reduced total locomotor counts compared with both social housing and isolation. B) Mean ± SEM percent of beam breaks in the center of the locomotor chamber. Enrichment increased central tendency compared with isolation. Nonshared letters denote significant differences ($p \leq 0.05$).

Change in body mass (grams at final body mass − grams at initial body mass), corticosterone concentrations, and histological data were analyzed by a 3×2 ANOVA with housing environment and behavioral testing as independent factors. Because body mass may be a predictor of dominance in some species (Grant, 1970), mice in social + nonsocial enrichment were given a rank based on body mass to determine if body mass predicted corticosterone concentrations or performance in the elevated plus maze. Mice within cages were assigned numbers in order from heaviest to lightest (1 through 4). One-way ANOVAs were then conducted using within-cage body mass rank as the independent variable and corticosterone concentrations and elevated plus maze measures as the dependent variables (Workman et al., 2009). A repeated measures ANOVA was conducted to assess locomotor activity over time. Pearson's product–moment correlations were conducted on nNOS cell counts and behavioral measures. Tests were conducted in StatView software (v. 5.0.1, Cary, NC, USA). All mean differences and correlation coefficients were considered statistically significant if $p \leq 0.05$.

3. Results

3.1. Behavioral measures

In the open field test, housing environment altered total locomotor activity ($p<0.05$; $F_{2,27}=4.972$; Fig. 1A). Specifically, social + nonsocial enrichment reduced total locomotor behavior compared with both social and isolated environments ($p<0.05$ in both cases). Housing environment altered locomotor activity over time ($p<0.05$), but did not significantly interact with time ($p>0.05$). Rather, all mice regardless of housing condition significantly reduced locomotor activity over time ($p<0.0001$; data not shown). Housing also altered the percent of beam breaks in the center of the open field ($p<0.05$; $F_{2,27}=3.797$; Fig. 1B). Specifically, social + nonsocial enrichment increased central tendency compared with isolation ($p<0.05$). Housing environment did not affect rearing behavior ($p>0.05$; $F_{2,27}=1.044$; data not shown).

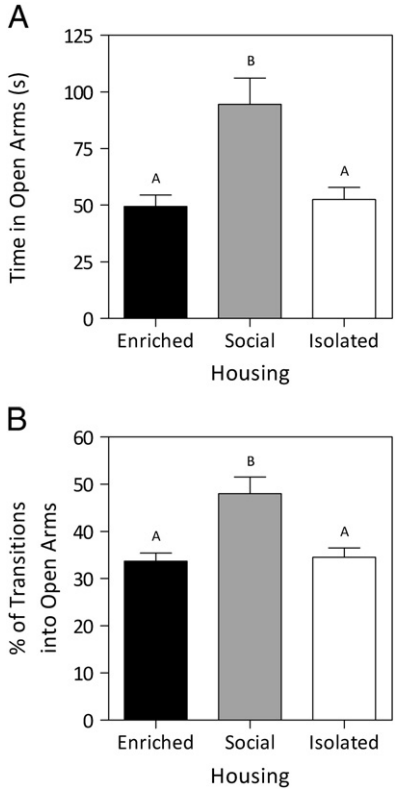

Fig. 2. Elevated plus maze. A) Mean ± SEM time (s) spent in the open arms of the maze. B) Mean ± SEM percent of transitions that were into the open arms. Social housing increased time in open arms and percent of transitions into the open arms compared with both enriched and isolated mice. Nonshared letters denote significant differences ($p \leq 0.05$).

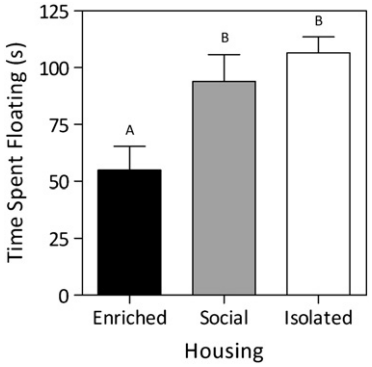

Fig. 3. Forced swim test. Mean ± SEM of time (s) spent floating. Enrichment significantly reduced time spent floating compared with both social and isolated housing. Nonshared letters denote significant differences ($p \leq 0.05$).

In the elevated plus maze, housing environment significantly altered time spent in the open arms ($p<0.01$; $F_{2,27}=9.854$; Fig. 2) such that social housing significantly increased the amount of time spent in the open arms compared with social + nonsocial enrichment and isolation ($p \leq 0.01$ in both cases). Housing environment did not alter the number of open arm entries ($p>0.05$; $F_{2,27}=2.323$), but significantly altered percent of transitions into the open arms ($p<0.01$; $F_{2,27}=10.328$) such that social housing increased percent of open arm entries compared with both social + nonsocial enrichment and isolation ($p<0.01$ in both cases). In the social + nonsocial enrichment condition, assumed dominance (as indicated by body mass) did not alter number of open arm entries ($p>0.05$; $F_{1,8}=0.051$), percent of entries into the open arms ($p>0.05$; $F_{1,8}=0.404$), or time spent in the open arms ($p>0.05$; $F_{1,8}=0.372$).

Housing environment significantly altered time spent floating in the forced swim test ($p<0.01$; $F_{2,27}=7.255$) such that social + nonsocial enrichment significantly reduced the time spent floating compared with both isolation and social housing ($p \leq 0.01$ in both cases; Fig. 3). Housing environment did not significantly alter number of float bouts ($p>0.05$; $F_{2,27}=2.892$).

In the resident–intruder aggression test, housing environment had no effect on bouts of boxing ($p>0.05$; $F_{2,27}=2.559$), time spent boxing ($p>0.05$; $F_{2,27}=2.716$), bouts of biting ($p>0.05$; $F_{2,27}=0.9$), or time spent biting ($p>0.05$; $F_{2,27}=0.6902$). Housing significantly altered bouts of aggressive grooming and sniffing ($p<0.01$; $F_{2,27}=13.714$; Fig. 4A) and time spent aggressively grooming and sniffing ($p<0.01$; $F_{2,27}=8.968$; Fig. 4B). Specifically isolation increased time ($p<0.05$ in both cases) and bouts of ($p<0.01$ in both cases) aggressive grooming and sniffing compared with both social + nonsocial enrichment and isolation. Housing did not alter bouts of ano-genital sniffing ($p>0.05$; $F_{2,27}=0.858$; Fig. 4C) but altered time engaged in ano-genital sniffing ($p<0.01$; $F_{2,27}=20.08$; Fig. 4D) such that social + nonsocial enrichment increased ano-genital sniffing compared with isolation and social housing ($p<0.01$ in both cases).

3.2. Body mass and corticosterone concentrations

Housing significantly affected incremental change in body mass ($p<0.05$; $F_{2,52}=3.581$) such that social + nonsocial enrichment increased body mass gain compared with both social housing and isolation ($p<0.05$ in both cases; Fig. 5). Behavioral testing did not alter body mass ($p>0.05$; $F_{1,52}=0.134$), thus, data were collapsed for graphs. All mice gained weight over time, but social + nonsocial

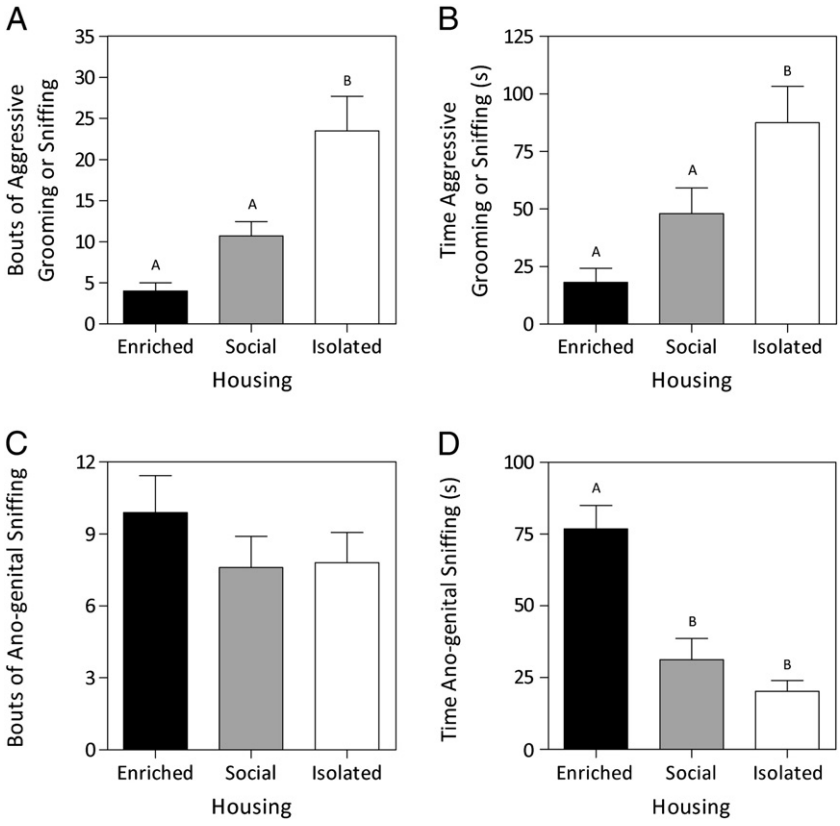

Fig. 4. Resident–intruder aggression test. A) Mean ± SEM bouts of aggressive grooming and sniffing and B) mean ± SEM time spent aggressive grooming and sniffing. Isolation significantly increased aggressive grooming and sniffing of the intruder mouse. C) Mean ± SEM bouts of ano-genital sniffing and D) mean ± SEM time spent ano-genital sniffing. Enrichment significantly increased time but not bouts of ano-genital sniffing. Nonshared letters denote significant differences ($p \leq 0.05$).

enrichment induced a greater weight gain compared with social housing and isolation.

Housing environment significantly altered corticosterone concentrations ($p = 0.05$; $F_{2,49} = 3.176$) such that social + nonsocial enrichment significantly elevated corticosterone concentrations compared with social housing ($p < 0.05$; Fig. 6). Behavioral testing did not affect corticosterone concentrations ($p > 0.05$; $F_{1,49} = 0.862$) and data were again collapsed across behavioral testing groups for depiction in the figures. In the social + nonsocial enrichment condition, within-cage body mass rank had a significant effect on corticosterone concentrations ($p < 0.05$; $F_{3,15} = 6.854$). Third-ranking males had significantly higher corticosterone concentrations compared with dominant and second-ranking males ($p < 0.05$ in both cases). Fourth-ranking males had significantly lower corticosterone concentrations than second- and third-ranking males ($p < 0.05$ in both cases). This pattern was not evident in socially housed mice as body mass rank had no effect on corticosterone concentrations ($p > 0.05$; $F_{3,15} = 0.748$).

3.3. Histology

Behavioral testing significantly reduced nNOS positive neurons in the BLA ($p < 0.05$; $F_{1,44} = 5.013$; Fig. 7A), but this effect was driven by the isolated and socially housed mice. Among the behaviorally-tested mice, isolation reduced nNOS-positive cells in the BLA compared with enriched mice ($p < 0.05$). Behavioral testing also decreased nNOS-positive cells in the vLS ($p = 0.05$; $F_{1,43} = 3.973$; Fig. 7B), but this effect was primarily driven by the isolated group. Post-hoc comparisons revealed that among isolated mice only, behavioral testing reduced

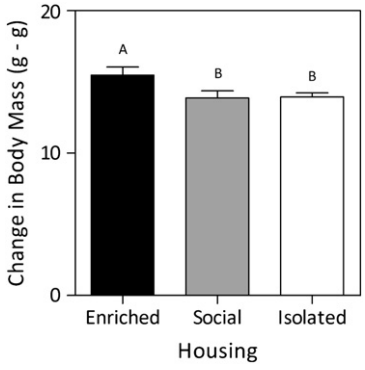

Fig. 5. Mean ± SEM change in body mass ($g_{final} - g_{intial}$). Enrichment significantly increased body mass gain compared with both social and isolated conditions. Nonshared letters denote significant differences ($p \leq 0.05$).

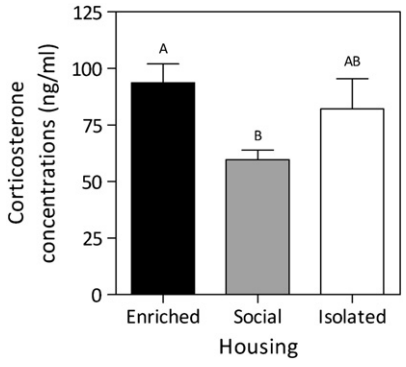

Fig. 6. Mean ± SEM corticosterone concentrations. Enriched and socially-housed mice were significantly different from one another, but not from isolated mice. Nonshared letters denote significant differences ($p \leq 0.05$).

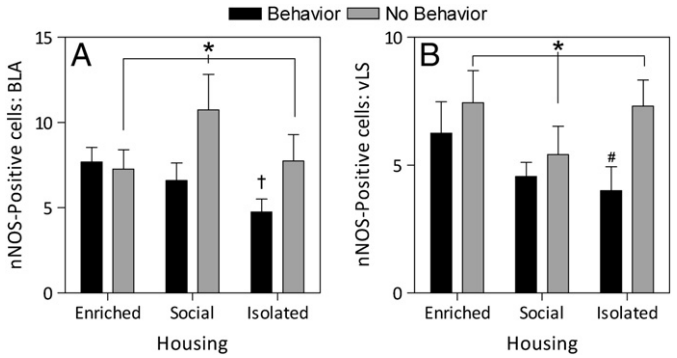

Fig. 7. nNOS cells counts in the A) BLA and B) vLS. Behavioral testing significantly reduced nNOS cell counts in the BLA, but this effect was driven by the social and isolated groups. Behavioral testing also significantly reduced nNOS positive cells in the vLS, but this effect was limited to the isolated mice. *Main effect of behavioral testing; †significantly different from enriched, behaviorally-tested group; #significantly different from isolated, unhandled group ($p \leq 0.05$).

nNOS-positive cells in the vLS ($p<0.05$). Neither housing nor testing affected nNOS cell counts in the VMH, PVN, MeA, IL, PRL, or BNST ($p>0.05$ for all interaction terms and main effects; see Table 1 for cell counts).

4. Discussion

In this study, we report that social + nonsocial enrichment that begins at weaning and lasts into adulthood altered adult affective responses and social behaviors as assessed in the open field, elevated plus, forced swim, and resident-intruder aggression tests. Collectively, these data suggest that opportunities for a physically, visually, and socially complex environment reduced some depressive- and anxiety-like responses and behavioral indices of aggressive motivation.

In the forced swim test – a task commonly used to assess 'behavioral despair' (Porsolt et al., 1977) – social + nonsocial enrichment significantly reduced the time spent floating compared with both isolation and social housing. This suggests that early social + nonsocial enrichment reduces depressive-like responses in adulthood. Although, floating in the forced swim test is not typically interpreted as the consequence of physical exhaustion, it remains possible that enriched mice are more physically fit, which may enable them to swim longer. It is more likely, however, that floating represents a motivational difference in search of escape. Social housing itself was not sufficient to significantly alter floating compared with isolated housing suggesting that social factors likely do not account for the reduction in immobility in the social + nonsocial enrichment.

Housing environment did not alter overt measures of aggressive behaviors such as biting or boxing, however, housing did alter aggressive grooming and sniffing. This behavior is qualitatively different from other social investigation. We operationally defined aggressive grooming and sniffing as residents pinning and immobilizing the intruders while sniffing and grooming the intruders' backs (Clipperton-Allen et al., 2011; Kudryavtseva et al., 1999). Isolation significantly increased both time spent aggressive grooming and sniffing as well as bouts engaging in aggressive sniffing and grooming, which may indicate aggressive motivation. Interestingly, social + nonsocial enrichment increased nonaggressive social investigation during the resident–intruder test, i.e., ano-genital sniffing, compared with both social housing and isolation.

Housing exerted subtle but significant effects on total locomotor behavior and central tendency in the open field test. Specifically, social + nonsocial enrichment significantly reduced total locomotor activity in the open field. This may indicate that enrichment reduces novelty-induced hyperlocomotion; enriched mice may habituate to a novel environment more quickly than isolated or socially-housed mice (Duman et al., 2008). Social + nonsocial enrichment also increased the percentage of beams broken in the center of the open field compared with isolated mice, indicating that enriched mice display less anxiety-like behavior than isolated mice. These data also suggest that the effects of nonsocial factors and social interactions on some affective responses may be additive because socially-housed mice displayed an intermediate number of beam breaks within the center of the open field. Social housing and nonsocial factors may alter the brain via different pathways, which may account for the additive behavioral effect in this particular test.

In the elevated plus maze (another test designed to assess anxiety-like responses) social + nonsocial enrichment did not alter time in the open arms compared with isolated mice, indicating that a complex environment does not reduce anxiety-like responses in the elevated plus maze. However, socially-housed mice spent significantly more time in the open arms than either enriched or isolated mice, suggesting that social housing alone reduced anxiety-like responses. Social housing also significantly increased the percent of transitions into the open arms. The results in the anxiety-like tests may reflect that these two tests assess different forms of anxiety that arise from different neurochemical mechanisms (Green, 1991).

Wheel running reduces anxiety-like responses in mice (Fox et al., 2008) and in Sprague Dawley rats (Fulk et al., 2004). Thus, it is curious that the social + nonsocial enrichment did not increase time spent in the open arms. It is possible that a more complex cage allows formation of dominance hierarchies (Mackintosh, 1970), which can lead to altered stress responses (DeVries et al., 2007; DeVries et al., 2003) and perhaps running wheel competition. Indeed, resting corticosterone concentrations were higher in social + nonsocial enrichment compared with social housing. Prior research has shown that communal nesting prior to weaning reduces corticosterone responses to a social interaction test but yields similar basal corticosterone concentrations (Branchi et al., 2010). Thus, housing conditions at different developmental time points may differentially organize the HPA axis. In the social + nonsocial enrichment condition, corticosterone varied as a function of body mass—which may predict dominance (Grant, 1970). Corticosterone concentrations were higher in third-ranking males compared with both dominant and lowest-ranking males. We found no effect of body mass rank on corticosterone concentrations in the socially-housed mice. These data support the hypothesis that complexity or partitions facilitate territoriality—a phenomenon that has been suggested in previous work (Mackintosh, 1970). Further, it is possible that social factors (establishment of dominance hierarchies) preclude the effects of wheel running on

Table 1
Mean ± SEM of nNOS positive cells. Neither housing environment nor behavioral testing had an effect on cells in the VMH, PVN, MeA, IL, PRL, or BNST.

		VMH	PVN	MeA	IL	PRL	BNST
Enriched	Behavior	20.333 ± 3.327	15.4 ± 2.009	25.5 ± 2.99	3.5 ± 0.453	4.938 ± 0.684	8.65 ± 1.453
	No behavior	14.571 ± 2.532	14 ± 1.21	22.286 ± 2.246	2.813 ± 0.559	3.938 ± 0.53	8.11 ± 1.314
Social	Behavior	20.167 ± 1.951	13.778 ± 1.746	26.15 ± 1.436	3.8330 ± 759	4.111 ± 0.644	6.889 ± 0.794
	No behavior	13.833 ± 3.589	14.167 ± 1.682	24.583 ± 3.097	3.786 ± 0.474	3.857 ± 0.459	9.75 ± 1.352
Isolated	Behavior	17.571 ± 3.279	16.625 ± 2.373	24.5 ± 4.461	3.417 ± .0271	4.833 ± 1.202	6.75 ± 0.901
	No behavior	14.8 ± 3.632	15.313 ± 2.283	25 ± 3.983	3.938 ± 0.406	3.438 ± 0.57	9.063 ± 1.037

anxiety-like responses and this may explain why social housing reduced anxiety-like responses in the EPM. These data however, should be interpreted with caution. In laboratory mice, body mass is not always associated with social dominance as reliably as in *Peromyscus* sp. (Hilakivi et al., 1989; Kaliste-Korhonen and Eskola, 2000). Additionally, we did not tag or ear-punch mice. Thus, stability of body mass within the enriched cages is unknown.

Because NO plays an important role in social behaviors, affective regulation, and brain plasticity, we investigated whether environment altered nNOS immunoreactivity in brain regions important for affective responses and social behaviors (Table 1). Environmental factors can indeed alter the immunoreactivity of nNOS. For example, exposure to short day lengths reduces the number of nNOS positive cells within the PVN (Wen et al., 2004). Exposure to behavioral testing significantly decreased nNOS immunoreactivity in the BLA and the vLS. However, this effect was not consistent across all housing groups. For instance, socially-housed and isolated, undisturbed mice had higher nNOS immunoreactivity in the BLA compared with those mice in the tested group; an effect that did not extend to the enriched groups of mice. Previous research has implicated the basolateral amygdala in anxiety-like responses (Mitra et al., 2005; Ono et al., 2008). Similarly, in the vLS, housing condition exerted a significant main effect on nNOS-positive neurons; again, behavioral testing reduced nNOS immunoreactivity. However, this effect appears to be driven by the isolated group as *post hoc* tests revealed that within the enriched and social housing conditions, vLS nNOS-positive cells did not differ between behavioral testing groups, but did differ in the isolated group. The lateral septum has also been associated with anxiety (Henry et al., 2006) and aggression (Beiderbeck et al., 2007). Thus, these results may indicate that enriched or social housing conditions buffer against behavioral testing effects on nNOS immunoreactivity. A final note of caution is warranted: shipping during such a sensitive developmental time point is not optimal. However, we can rule out any impact of litter because no siblings were assigned to the same group. This manipulation, however does not rule out the possibility that all mice enter the experiment from a different baseline compared with mice bred in-facility. Thus we may be investigating an interaction between early life stress and environment.

5. Conclusions

This research establishes that enrichment beginning directly after weaning and lasting into adulthood is associated with reduced negative affective responses in adulthood and may buffer the brain from stressful environmental factors. These results may help elucidate contradictory findings regarding enrichment and affective responses. Further, these results suggest that environmental factors (i.e., housing and behavioral testing) interact to influence the immunoreactivity of nNOS, the synthetic enzyme for NO, and may have implications for the role of environmental factors and NO in mood and anxiety disorders.

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