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Stressful environmental and social stimulation in adolescence causes antidepressant-like effects associated with epigenetic induction of the hippocampal BDNF and mossy fibre sprouting in the novelty-seeking phenotype

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Abstract

An outbred rat model of novelty-seeking phenotype can differentiate between rats that show high rates (high responders; HRs) versus low rates (low responders; LR) of locomotor reactivity to a novel environment. In the present study, LR and HR rats were exposed to a regimen of environmental and social stimuli (ESS) consisting of 14 random exposures of isolation, crowding or novel environment, once per day during the peripubertal-juvenile period (postnatal days 28–41) or handled as controls. Twenty-four hours after the last ESS exposure or control handling, all animals were tested on the forced swim and social interaction tests for depressive-like and social anxiety-like behaviors respectively. The ESS exposure during the peripubertal-juvenile period led to antidepressant-like effects on the forced swim test associated with increase in acetylation of histones 3 and 4 at the promoter regions P2 and P4 of the brain-derived neurotrophic factor (BDNF) gene in the dorsal hippocampus of HRs. Moreover, epigenetic activation of the hippocampal BDNF in the HRs following ESS exposure was accompanied by increase in the supra-pyramidal mossy fibre (SP-MF) and total mossy fibre terminal field volumes compared to handled controls. These findings suggest that the ESS exposure in the peripubertal-juvenile period may constitute an example of environmental induction of the hippocampal BDNF, and may mimic behavioral effects of exogenous antidepressants in the HR phenotype.

Keywords

forced swim test; neuroplasticity; histone acetylation; design-based stereology; Timm staining; chromatin immunoprecipitation assay

Neurobiological differences between individuals contribute a significant variance in reactivity to stressful stimuli [1, 9, 28]. To study such individual differences in responsiveness to stressful stimulation, an outbred rat model of novelty-seeking phenotype was introduced [20], where experimentally naïve rats were screened based on degrees of locomotor reactivity in a novel environment. In such settings, some rats display high rates of locomotor reactivity to novelty (high responders; HR), and others display low rates (low

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responders; LR). HR rats repeatedly choose novel environments over familiar ones [20], appear less anxious in the light–dark box and the elevated plus maze [12], and demonstrate prolonged stress-induced secretion of corticosterone when compared to LR rats [21]; validating this phenotype in the study of stress and anxiety-like behaviors. We have previously shown that a chronic non-habituating exposure to ESS composed of a regimen of stressors commonly used in the literature (e.g., isolation, crowding, novel environment) during the peripubertal-juvenile period (postnatal day, PN 28-56) led to downregulation in the hippocampal glucocorticoid receptor (GR) and elevated corticosterone response, however these effects were reversed with maturation into young adulthood [10]. The same ESS exposure during adolescence prevented the development of behavioral sensitization to amphetamine in adulthood [14] associated with inhibition in dopaminergic markers in the striatum [13]. In the present study, we will behaviorally characterize the LRHR phenotype following the chronic, peripubertal-juvenile ESS exposure or control handling in depressive-like behavior measured on the forced swim test and in social anxiety-like behavior measured on the social interaction test. Moreover, differential acetylation of histones at the promoter regions of the hippocampal BDNF gene is assessed following the ESS exposure or control handling in the LRHR rats, and such effects are followed by morphological assessments of the mossy fibre terminal fields.

Male Sprague-Dawley rats (N=108, Charles River, Wilmington, MA) arrived at weaning (PN 22), were housed in $43 \times 21.5 \times 25$ cm clear acrylic cages, and were allowed ad libitum access to food and water. Animals were kept on a 12 hr light/dark cycle (lights on at 7:00 A.M.). On PN 25, animals underwent locomotor screening for 60 min in commercially-available locomotion chambers (San Diego Instruments, San Diego, CA). Locomotor reactivity to novelty was tested in $43 \times 43 \times 24.5$ cm clear Plexiglas cages with stainless steel grid flooring. Activity was monitored by means of two banks of photocells (total of photocells X=16; Y=16) connected to a microprocessor. Two frames of photocells were used; the lower frame was located 2.5 cm above the grid floor and the upper frame was located 12.5 cm above the floor of the box. Horizontal locomotion was monitored by the lower bank of photocells. Each horizontal activity count recorded a minimum 14.3 cm traversing of the cage. Rearing was monitored by activity on the upper bank of photocells. At the end of a 60 min screening session, total locomotor activity (i.e., X, Y and Z locomotion) were pooled and the rats were ranked as HRs (i.e., rats that exhibited locomotor scores in the highest third of the sample, $n = 36$) or LRs (i.e., rats that exhibited locomotor scores in the lowest third of the sample, $n = 36$).

For environmental and social stimulation (ESS), three stressors were selected from the literature [10, 13]. Stressors were applied in a systematic random order and at varying times of day (one stressor per day) for a total of 14 stress exposures to avoid habituation. ESS or control handling occurred between PN 28-41 ($n=18$ per group). 1) Isolation: Animals were transferred into cages similar to home cages and placed in a room other than the home colony for 2-hr with free access to food and water. 2) Novel environment: Animals were individually placed in novel environments that consist of boxes with different geometric shapes (circular, rectangular, triangular), color and luminosity for 2-hr. 3) Crowding: A group of animals ($n=20$) were placed in one cage similar to the home cage for 2-hr in the home colony. As animals matured, total number of animals placed in a cage during crowding session was decreased to $n=8$.

Twenty-four hours after the last stress exposure or control handling (PN 42), a subgroup of animals ($n=10$ per group) was tested on the social interaction and forced swim tests. Order of tests was counterbalanced between subjects. For social interaction test, an experimentally naive experimental rat was placed in a $23 \times 15 \times 13$ cm rubber rectangular box and allowed to habituate to its surroundings for 8 min. After the initial 8 min, an age- and weight-

matched rat was placed in the box and both rats were allowed to interact for 5 min. Social behaviors elicited by the experimental rat towards the resident rat (i.e., sniffing, following, grooming and crawling over or under) were measured. In forced swim test, rats were placed in a 30 cm diameter cylinder filled with water (25°C) and were tested for 5 min. Behavior constituting as immobile (lack of movement except necessary movements to keep the head above water) was scored. The remaining animals (n=8 per group) were tested under an acute stressor (15 min exposure to elevated plus maze). Blood samples were collected at the basal condition (0') and 15', 30', 60' and 90' following the onset of stress via tail nick according to our published protocol [10]. Plasma corticosterone (CORT) levels were assessed using the Diagnostic Systems Laboratories (DSL) radioimmunoassay kit.

At the completion of forced swim and social interaction tests, animals were sacrificed by rapid decapitation. A set of animals (n=4 per experimental group) was used for the chromatin immunoprecipitation (ChIP) assay. Remaining animals (n=6 per experimental group) were used for harvesting hippocampi for Timm's method for silver sulfide staining and subsequent morphological analyses using design-based stereology. For the ChIP assay, tissue punches from dorsal hippocampi were pooled and fixed to cross-link DNA with associated proteins. Fixed hippocampal tissue was sonicated twice for 10 sec at low setting and then sonicated three more times for 15 sec at maximum power to shear the chromatin to 400–500 bp using a cell lysis buffer (1% SDS (10%), 1 mM Tris-HCl (pH 8.0) and 100 mM 0.5 M EDTA). After determination of optical densities, equal amounts of chromatin lysate (60 µg) were diluted with ChIP dilution buffer to a final volume of 1 ml. Samples were then immunoprecipitated overnight at 4°C with the following antibodies: 5 µg of anti-acetyl H3 antibody (acetylated on Lys9 and Lys14 Millipore, CA), 5 µg of anti-acetyl H4 antibody (acetylated at Lys5, Lys8, Lys12, and Lys16 Millipore, CA) and 5 µg nonimmune rabbit IgG antibody (Millipore, CA) used as control. The immunoprecipitant was collected using Protein A beads from Thermo Scientific (Rockford, IL). The beads were washed once with low salt, high salt, and LiCl and washed twice with TE buffers. After washing, the DNA–histone complexes were eluted from the beads and then reverse cross-linked using proteinase K. DNA was extracted, purified and quantified using quantitative real time-PCR (qPCR).

qPCR was performed to determine the levels of acetylated H3 (acH3) and H4 (acH4) at the BDNF gene promoters P2 and P4. Specifically, experimental primers were custom designed as the following: BDNF P2: 5'-TGAGGATAGGGGTGGAGTTG-3', 5'-GCAGCAGGAGGAAAAGGTTA-3'; BDNF P4: 5'-TGCAGGGGAATTAGGGATAC-3', 5'-TCTTCGGTTGAGCTTCGATT-3'. Endogenous control (GAPDH, Applied Biosystems, CA) was used for normalization. qPCR was performed on MxPro-Mx3005P QPCR System (Agilent Technologies, CA) and the reaction mixture consisted of 12.5 µl of Stratagene Brilliant II SYBR® Green Low ROX QPCR Master Mix (Agilent Technologies, CA), 2.5 µl of forward and backward primers and 4 µl of DNA in a total volume of 25 µl. Cycling conditions comprised of 10 min polymerase activation at 95°C, an amplification step consisting of 40 cycles at 95°C for 30 sec each and 60°C for 1 min and followed by 1 cycle each of 95°C for 1 min, 55°C for 30 sec and 95°C for 30 sec to obtain the dissociation curve. Immunoprecipitated DNA were PCR amplified in duplicate, and Ct values from each sample were calculated by using the MxPro software. Ct values obtained from the intermediary responders (animals ranking in the middle 1/3rd of population in total locomotor reactivity screening) were used as controls.

For Timm staining, coronal brain sections were collected at 160µm intervals throughout the hippocampus, and were kept at –80°C until staining. Sections were air-dried and immersed in a phosphate-buffered (pH 7.4) 0.5% sodium sulfide solution for 2 min, y rinsed in two changes of phosphate buffer, fixed in 96% ethanol and rehydrated. Sections were stained as

described previously [11]. The volumes of the two major components (SP-MF and intra/intra-pyramidal terminal field, IIP-MF) of the mossy fiber system were estimated using the Cavalieri estimator as described in previously published work [11, Stereoinvestigator, Micro-BrightField; Colchester, VT].

Two-way ANOVAs were conducted for (1) percent time spent in social interaction (2) percent time spent in immobility on the forced swim test (3) amounts of acH3 and acH4 in the P2 and P4 promoters of the BDNF gene, (4) the SP-MF, IIP-MF, and total MF terminal field volumes between phenotypes (LR, HR) and stress conditions (ESS, CONT). A repeated-measures ANOVA was used to analyze plasma CORT levels between phenotypes (LR, HR), stress conditions (ESS, CONT) and Time (0, 15, 30, 60, 90 min). Significant interactions and main effects of ANOVAs were followed by posthoc comparisons.

Figure 1A shows the percent time engaged in social interaction in the LRHR rats. A two-way ANOVA revealed no significant interaction or main effects of Phenotype (LR, HR) or Stress (ESS, CONT) in social interaction between groups. Figure 1B shows the percent time spent in immobility in LRHR rats in forced swim test. A two-way ANOVA showed a significant interaction between Phenotype (LR, HR) and Stress (ESS, CONT) in immobility behavior [$F = 5.760$, $p = 0.023$]. Subsequent posthoc comparisons showed a significant decrease in percent time spent in immobility in HRs exposed to ESS compared to control animals [$p = 0.047$]. Figure 1C shows plasma CORT levels to elevated plus maze exposure across time points for LRHR rats under ESS or control handling conditions. Repeated-measures ANOVA showed a significant interaction between Phenotype and Stress, and main effect of Stress in CORT levels [$F \geq 6.31$, $ps \leq 0.221$]. Posthoc comparisons showed a significantly lower peak in CORT response (at 30 min) in HR rats exposed to ESS compared to handled controls [$p = 0.245$].

Figures 2A and 2B show levels of acH3 at the P2 promoter of the BDNF gene and levels of acH4 at the P4 promoter of the BDNF gene respectively in the dorsal hippocampus of the LRHR rats exposed to ESS or control handling. A two-way ANOVA revealed a significant interaction between Phenotype (LR, HR) and Stress (ESS, CONT) in the acH3 levels at the BDNF P2 promoter [$F = 8.007$, $p = 0.015$]. Posthoc comparisons showed that control HRs had lower levels of acH3 at the BDNF P2 promoter compared to control LRs [$p = 0.031$]. Furthermore, acH3 levels at the P2 promoter were increased with ESS exposure in HRs compared to control levels [$p = 0.028$]. No significant effects were detected in the acH3 levels at the P2 promoter of the BDNF gene in the dorsal hippocampus of the LR rats, as well as acH3 levels at the P4 promoter of the BDNF gene between phenotypes and stress conditions (data not shown). Moreover, a two-way ANOVA showed a significant interaction between Phenotype (LR, HR) and Stress (ESS, CONT), and significant main effects of Phenotype and Stress in the acH4 levels at the BDNF P4 promoter [$Fs \geq 21.058$, $ps \leq 0.001$]. Posthoc comparisons showed that control HRs had lower levels of acH4 at the BDNF P4 promoter compared to control LRs [$p = 0.001$]. Furthermore, ESS caused an increase in acH4 at the P4 promoter in HRs compared to handled controls [$p < 0.001$]. No significant effects were detected in acH4 levels at the P2 promoter of the BDNF gene between phenotypes and stress conditions (data not shown).

Figure 2C depicts representative sections of the Timm-stained septal hippocampi from LRHR rats exposed to ESS or control handled. Figure 2D and 2E show estimates of terminal field volumes for SP-MF and total mossy fibres (SP-MF + IIP-MF) respectively. Two-way ANOVAs showed significant interactions between Phenotype (LR, HR) and Stress (ESS, CONT), and main effects of Phenotype in SP-MF and total mossy fibre terminal field volumes [$Fs \geq 3.191$, $ps \leq 0.039$], whereas no significant effects were detected in IIP-MF terminal field volume (data not shown). Subsequent posthoc comparisons showed a

significant increase in the SP-MF and total mossy fibre terminal field volumes in HRs exposed to ESS compared to handled controls [$ps \leq 0.041$].

Our findings showed that ESS exposure during adolescence results in antidepressive-like behavior (i.e., decreased amount of immobility) in HRs on the forced swim test along with suppressed peak in CORT response to acute stress, leaving the performance on the social interaction test unaffected. Moreover, although basal levels of histone acetylation at the BDNF gene is lower in HR compared to LR hippocampus between respective handled controls, levels of acetylated H3 and H4 at the BDNF promoters P2 and P4 respectively are increased with the ESS exposure in the HR hippocampus compared to levels observed in handled controls. These findings suggest increased epigenetic activation of the hippocampal BDNF gene in HRs with chronic stressful stimulation during the peripubertal-juvenile period. Concurrent with the findings in the hippocampal BDNF levels, our results showed a significant increase in total mossy fibre volume, particularly in SP-MF terminal field size in HRs following the ESS exposure compared to handled controls. These findings suggest that fluctuations in the hippocampal BDNF levels induced by stressful stimulation during the peripubertal-juvenile period, particularly those that are mediated by histone modifications at the BDNF gene, may sustain observed structural plasticity at the mossy fibre terminals, which may mediate antidepressive-like effects in the HR phenotype.

We report emergence of an antidepressive-like effect in the HR phenotype following the peripubertal-juvenile (PN 28-41) ESS exposure. This is in striking contrast to numerous reports showing increased depressive-like behavior following exposure to chronic stress. In fact, paradigms of chronic stress are widely accepted as animal models for studying enhanced depressive-like state [see review; 7]. Although individual components of our chronic ESS regimen are commonly employed as stressors in the literature such as novelty [17], isolation and crowding [8, 22], and generally considered as “negative” experiences, a regimen of combined stressors applied intermittently at varying times of day and at varying durations during the developmental period of adolescence may indeed mimic an enrichment-like effect, especially in the stress responsive HR phenotype. It is not uncommon to induce a robust physiological stress response in conjunction with rewarding environmental stimuli during adolescence. For example, rough- and tumble-play in juveniles induces positive experiences and is rewarding [4], although marked with increased levels of glucocorticoids [24]. Therefore, we can argue that environmental stimulation induced by crowding, isolation and exposure to novel environment in the critical period of adolescence, although highly stressful in the physiological sense, may result in enrichment-like outcome. Classic postweaning enrichment paradigm produces an antidepressive-like effect on the forced swim test [3], such as the one we report in the HR rats following the ESS exposure applied postweaning into adolescence (PN 28-41).

In parallel with the antidepressant-like effects, we have also shown increased chromatin plasticity at the hippocampal BDNF gene with the ESS exposure during the peripubertal-juvenile period in the HR rats. Namely decreased levels of helplessness measured as decreased immobility on the forced swim test were associated with increased levels of acH3 at the BDNF P2 promoter and increased levels of acH4 at the BDNF P4 promoter, suggesting epigenetic induction of the BDNF gene in the HR rats. Chromatin plasticity in terms of increased histone methylation at the BDNF promoter is previously linked to stress-induced suppression of the hippocampal BDNF gene, and antidepressant treatments led to hyperacetylation of H3 at the BDNF promoter [25]. Our adolescent ESS exposure appear to mimic previously reported effects of antidepressants on histone acetylation at the BDNF promoter in the HR phenotype, depicting a novel mechanism for environmental-induction of the BDNF gene. In addition, recent reports showed that the classic environmental enrichment procedure in adolescence and early environmental enrichment such as the

communal nest can stimulate expression of the hippocampal BDNF gene through chromatin-specific events [16, 2], providing further support for enriching-like quality of our ESS paradigm in the HR rats.

We showed increased mossy fibre terminal field size, particularly the SP-MF volume, in the HR animals following the ESS regimen that is associated with increased H3 and H4 acetylation at the P2 and P4 promoters respectively. These findings strongly suggest BDNF-mediated synaptic plasticity at the mossy fibre terminals of the HR rats following stressful environmental stimulation during adolescence. Since hippocampal BDNF is primarily stored in the axon terminals of the mossy fibres, ESS-induced changes in the hippocampal BDNF levels could mediate structural changes in the mossy fibre-CA3 synapse in the HRs. Intrahippocampal microinfusion of the BDNF is shown to increase mossy fibre content at the stratum oriens of the CA3 [23]. Although mossy fibre plasticity is linked to cognitive function [19], implications in depressive-like behavior are, for the most part, unexplored. Functional maturation of the DG and its output mossy fibres are implicated in depressive-like behavior [18, 27], suggesting granule neurons and their axonal projections onto CA3 may be critically involved in pathogenesis of depression and antidepressant effects [15]. In support of this, repeated electroconvulsive stimulation (ECS) is shown to induce mossy fibre sprouting [26]. Similar to ECS, we report increased mossy fibre terminal fields in the HR phenotype following the ESS exposure and accompanying antidepressant-like effects on forced swim test; which implicate the mossy fibre system as a target for the neuroadaptations induced by environmental stimulation of stressful nature during adolescence.

Optimally, the LRHR phenotype is manifested in locomotor reactivity to the mild stress of a novel environment in the experimentally naïve animals. Phenotypic locomotor differences are eliminated in the light-dark box when HR rats are socially isolated for a week [12]. Hence locomotor effects of the LRHR phenotype can be neutralized by a factor of stressor severity or duration. In our forced swim paradigm, control LRHR rats do not exhibit locomotor effects observed in phenotype screening in response to novelty. Novelty has been shown to induce lower glucocorticoid response than forced swim [5, 6]. Hence, forced swim, by virtue of being a more severe stressor, may not be an optimal condition for the novelty-seeking phenotype to manifest its locomotor effects.

In conclusion, exposure to a chronic nonhabituating regimen of environmental and social stimuli during the critical period of adolescence may induce antidepressant-like effects in the stress responsive HR phenotype that is associated with epigenetic induction of the hippocampal BDNF gene and subsequent mossy fibre sprouting. Hence, the ESS regimen during adolescence may be useful as an environmental therapy that mimics effects of exogenous antidepressants in a subpopulation of individuals that are responsive to stress.

Highlights

- ESS exposure during adolescence results in antidepressant-like effect in HRs.
- ESS exposure during adolescence results in epigenetic induction of BDNF in HRs.
- ESS exposure during adolescence results in mossy fibre sprouting in HRs.
- ESS exposure during adolescence may constitute environmental therapy in HRs.

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Figure 1.

Percent time spent in social interaction (**A**), percent time spent in immobility on the forced swim test (**B**) and CORT response to an acute stressor (**C**, 15 min exposure to an elevated plus maze) in LRHR rats exposed to ESS or control handling. Means are plotted in bar graphs $\pm$ SEMs in **A**, **B**. Means are plotted in a line graph $\pm$ SEMs in **C**. *: $p \leq 0.05$.

Figure 2.

Levels of acH3 at the BDNF P2 promoter (**A**), and acH4 at the BDNF P4 promoter (**B**) in the LRHR rats exposed to ESS or handled as controls. Fold changes in Ct values of immunoprecipitated samples were calculated with the Ct values obtained from intermediary responders (rats that rank in the middle 1/3rd of the population in total locomotor reactivity scores at the time of phenotype screening) that serve as phenotype control, and quantified using real-time PCR. Data are expressed as means $\pm$ SEMs; *: $p \leq 0.05$. Coronal hemisections of the dorsal hippocampus showing Timm-stained mossy fibre terminal fields that are counterstained by cresyl violet are depicted in (**C**). Black arrows are pointing at the SP-MF compartment of the mossy fibre projections. Volume estimates pertaining to SP-MF terminal fields (**D**) and total mossy fibre terminal fields (**E**, SP-MF + IIP-MF) are expressed as means $\pm$ SEMs in bar graphs. *: $p \leq 0.05$. Scale bar = 250 μ m.