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Brief communication

Social stress-related behavior affects hippocampal cell proliferation in mice

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Abstract

Although social stress inhibits neurogenesis in the adult hippocampus, the extent to which individual differences in stress-related behavior affect hippocampal cell proliferation is not well understood. Based on results from resident–intruder stress tests administered to adult male mice, here we report that individual differences in hippocampal cell proliferation are related to the frequency of defensive behavior, and not the amount of aggression received or the frequency of fleeing. In contrast, access to voluntary wheel-running exercise did not affect hippocampal cell proliferation in either stressed or non-stressed mice. Social stress-induced inhibition of cell proliferation was restricted to the hippocampus, as neither stress nor access to wheel-running exercise altered cell proliferation in the amygdala. These findings indicate that individual differences in stress-related behavior influence cell proliferation in the mouse hippocampus, and may have important implications for understanding structural and functional hippocampal impairments in human psychiatric patients.

Keywords: Amygdala; BrdU; Cell proliferation; Hippocampus; Resident–intruder; Stress

1. Introduction

Exposure to social stress inhibits hippocampal neurogenesis in a variety of animals, including rats, tree shrews and primates [1–4]. The most common stressor employed in this research is the resident–intruder paradigm in which a male intruder is placed in the home cage of an aggressive male resident. Individual differences in the behavior of intruders in response to resident aggression have been well documented and in rodents range from interactive upright defense to avoidant fleeing [5–8]. In this study, we examine whether individual differences in these behavioral coping strategies are related to differences in cell proliferation in the adult hippocampus and also in amygdala, another brain area where experience-induced plasticity has been reported [9].

2. Materials and methods

Young adult male C57BL mice, obtained from Taconic (Germantown, NY), were pair-housed in 27.9×17.7×12.7-cm polypropylene cages on wood chip bedding at the Stanford Research Animal Facility. Rodent chow (PMI Nutrition International, Brentwood, MO) and water were available ad libitum. Animals were housed and tested in climate-controlled rooms on a 12:12-h light–dark cycle at an ambient temperature of 22 °C. Subjects were randomized to either a social stress ($n=14$) or a no stress handled control condition ($n=13$) at 5–7 weeks of age. In each condition, a functional running wheel (Penn-Plax Inc., Garden City, NY) or a non-functional wheel was also provided in the 2×2 experimental design ($n=5$–7 mice per group). Identical running wheels were continuously available throughout the study in each home cage, but the wheels were locked stationary for half of the subjects to prevent running-related exercise. All procedures were conducted in accordance with the Animal Welfare Act and were approved by Stanford University's Administrative Panel on Laboratory Animal Care.

For the social stress condition, mice were exposed to a repeated resident–intruder protocol similar to that described elsewhere in detail [10,11]. Briefly, each C57BL intruder mouse was placed in the cage of a singly housed Swiss–Webster male mouse resident obtained from Taconic (Germantown, NY). Residents were larger and older than intruders and were pre-screened to ensure that they consistently responded to intruders

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with aggression ($n=15$ residents). The resident and intruder were allowed to interact for 2 min and then a wire-mesh barrier was placed between the mice to prevent direct social interaction for an additional 8 min. This 10-min manipulation was subsequently repeated six times. All occurrences of three predefined categories of behavior were recorded during each 2-min session of direct social interaction. (1) *Aggression*—resident bites, holds or attacks the intruder. (2) *Flee*—intruder runs away in response to aggression. (3) *Upright defense*—intruder exhibits a defensive upright posture [12] in response to aggression from the resident. Thereafter, the intruder remained behind the wire-mesh barrier in the resident's cage for an additional 60 min. After completion of the 2-h test, the intruder was returned to his home cage. In keeping with previously published research [10], resident–intruder social stress tests were repeated each morning between 08:00 and 10:00 for 7 consecutive days. Intruders interacted with a different resident male during each daily test session. Immediately before each daily social stress test, intruders were administered a single intraperitonial injection of bromodeoxyuridine (BrdU, Sigma, Inc.; 50 mg/kg body weight in 0.9% saline) to label newborn cells. Non-stressed mice were also administered BrdU at the same time each day, but otherwise remained undisturbed.

Immediately after completion of the 7th daily 2-h stress test and at matched time points for the non-stressed controls, mice were euthanized and transcardially perfused with chilled saline and 4% paraformaldehyde. Brains were extracted, immersion fixed overnight in 4% paraformaldehyde at 4 °C, equilibrated in 30% sucrose and frozen for sectioning in the coronal plane. All serial 40 μm sections that encompassed the hippocampus and amygdala were stored frozen in a cryoprotectant solution at −25 °C. The amygdala was examined to assess whether stress and wheel-running exercise effects were specific to the hippocampus.

A randomly selected 1-in-6 series of tissue sections that encompassed the hippocampus and amygdala was washed in PBS (phosphate buffer saline), incubated in 2 N hydrochloric acid (HCl) at 37 °C for 1 h and neutralized with boric acid buffer (pH 8.5). Sections were then incubated in 0.3% H_2O_2 in methanol, washed in PBS, blocked in 3% serum and incubated overnight at 4 °C with primary mouse anti-BrdU antibody (Chemicon International, 1:1000). Subsequently, sections were stained using the peroxidase method (ABC system with biotinylated donkey antimouse antibodies and diaminobenzadine as chromogen; Vector Laboratories, Burlingame, CA) and counterstained with Gill's Formulation #1 Hematoxylin (Fisher Scientific).

Immunolabeled tissue sections were coded for counting without knowledge of the treatment conditions. Cells labeled with BrdU in the hippocampal dentate gyrus (granule cell layer and hilus) and basolateral amygdala were each counted separately on each brain side with a Nikon microphot-SA microscope, 60× oil objective and StereoInvestigator software (MicroBrightField, Williston, VT). On average, 10 to 12 sections were analyzed for hippocampus and 5 to 6 sections were analyzed for amygdala. All BrdU+cells in each region of interest were counted using unbiased stereology using a modified optical dissector protocol that excluded cells cut in the outermost focal plane to avoid double counting cell caps. The volume of each region was also determined using StereoInvestigator software (MicroBrightField Inc., Williston, VT).

To confirm that the resident–intruder test induced in the intruder an adrenocortical stress response, blood samples for corticosterone determinations were collected from an additional group of non-stressed handled control ($n=6$) and socially stressed ($n=8$) mice. Immediately after completion of the 2-h resident–intruder stress test and at matched time points for the non-stressed controls, subjects were rapidly decapitated for sample collection (median latency from capture to sample collection = 18 s, range = 3 to 38 s). Blood samples were centrifuged, and the serum fraction was transferred to a polypropylene tube and frozen on dry ice. Samples were stored at −80 °C prior to hormone quantification. Corticosterone was measured in duplicate using a commercially prepared radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). All samples were quantified in a single assay run and the intra-assay coefficient of variation was less than 10%. The sensitivity of the corticosterone assay was 5.7 ng/ml.

Group differences in the numbers of BrdU+cells and measures of social behavior were examined with ANOVAs using Systat v10.2 (Richmond, CA). The social stress and wheel-running conditions were analyzed as between-subjects factors, and brain side and test days were considered repeated within-subject factors. Relationships between the behavioral

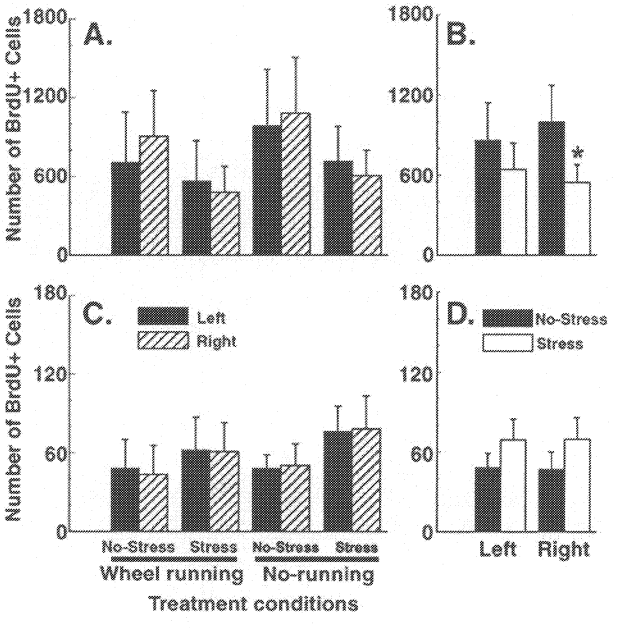

Fig. 1. Social stress and wheel-running exercise effects on cell proliferation in hippocampus and amygdala. Total number of BrdU+cells (mean±S.E.M.) in hippocampal dentate gyrus (A) and amygdala (C) are presented for male mice exposed to social stress, a functional running wheel, both treatment conditions or an undisturbed control ($n=5$–7 mice per condition). The social stress main effect on BrdU+cell numbers in hippocampus (B) and amygdala (D) is also presented with the wheel-running and non-running exercise conditions combined (*$p<0.05$).

measures and numbers of BrdU+cells were assessed with Pearson correlation coefficients and multiple regression analysis. The level of aggression received by the intruder was included as covariate for the correlation analysis. Group differences in corticosterone levels were examined with *t*-tests. Descriptive statistics are presented as mean±S.E.M. and all test statistics are evaluated with two-tailed probabilities ($p<0.05$).

3. Results

The resident–intruder paradigm induced a robust adrenocortical response as intruder mice demonstrated a seven-fold increase in plasma corticosterone concentrations compared to handled control mice (429±14 ng/ml vs. 55±21 ng/ml, $t=15.15$, $df=12$, $p<0.0001$). Hippocampal and amygdala volumes did not differ, however, in mice exposed to social stress, wheel-running exercise, both treatment conditions or undisturbed controls. Significantly, more BrdU+cells were evident in mice with larger hippocampal ($r=0.59$, dfs=23, $p=0.002$) and amygdala ($r=0.58$, $df=23$, $p=0.002$) volumes. To control for section series variation related to differences in volume, BrdU+cell numbers were analyzed for social stress, running exercise and brain side effects with volume included as a statistical covariate.

Hippocampal cell proliferation did not differ significantly in the running versus non-running conditions (Fig. 1A), but a stress-by-brain side interaction was discerned for the number of BrdU+cells in hippocampus ($F(1,19)=4.65$, $p=0.044$). Simple main effects analysis confirmed that stress inhibited cell proliferation in the right ($F(1,21)=4.38$, $p=0.049$) but not the left ($p=0.335$) hippocampus. Data presented in Fig. 1A are replotted in Fig. 1B to show the main effect of stress on hippocampal cell proliferation with the running and non-running conditions combined. While stress caused a significant decrease in the number of BrdU+cells in the right hippocampus (46% reduction compared to the no-stress control group), the 25% reduction observed in BrdU+cell numbers in the left hippocampus failed to reach statistical significance. Relative to the hippocampus, 12-fold fewer BrdU+cells were evident in the amygdala, and the effects of social stress, running exercise and brain side for the amygdala were not significant (Fig. 1C,D).

The frequency of aggression received by intruders from residents (77±7 events/day) and upright defense exhibited by intruders (33±3 events/day) did not differ significantly over repeated test days. The frequency of fleeing by intruders (32±3 events/day) decreased significantly over repeated test days ($F(6,72)=3.29$, $p=0.006$), but none of the measures of social behavior differed significantly in mice from the running and non-running conditions. Behavior scores summed across repeated days revealed that aggression received from the resident correlated with the frequency of fleeing by intruders ($r=0.85$, $df=12$, $p<0.001$) and tended to correlate inversely with the frequency of upright defense ($r=-0.46$, $df=12$, $p=0.094$).

Relationships between social behavior and BrdU+cell numbers were assessed with multiple regression analysis to control for inter-correlations between the three different measures of behavior. Of the behavioral measures examined, only interactive upright defense by intruders showed a unique direct correlation with the number of BrdU+cells in hippocampus ($F(1,10)=6.11$, $p=0.033$; Fig. 2). Mice that more often responded to aggression with upright defense showed significantly fewer BrdU+cells in the left ($r=-0.60$, $df=10$, $p=0.038$) and right ($r=-0.62$, $df=10$, $p=0.032$) hippocampal dentate gyrus. None of the behavioral measures predicted differences in BrdU+cell numbers in the left or right amygdala.

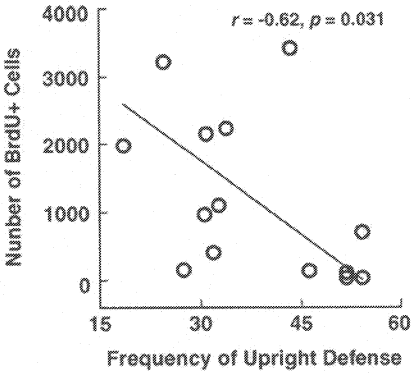

Fig. 2. Regression of upright defensive behavior on BrdU+cell numbers in the hippocampus (left and right hippocampi combined).

4. Discussion

These results demonstrate that intruders who respond to resident male aggression with more interactive defensive behavior show diminished hippocampal cell proliferation, whereas neither aggression received nor the frequency of fleeing correlate with BrdU+cell numbers in the hippocampus. Interactive defensive behavior evoked in resident–intruder social stress tests has been linked to increased secretion of glucocorticoids in male rats [13]. Our study likewise shows that intruder male mice exposed to an aggressive resident have significantly higher post-stress plasma corticosterone levels compared to non-stressed control mice. Because glucocorticoids are known to diminish hippocampal cell proliferation [14–17], glucocorticoid-mediated mechanisms might account for the correlation between hippocampal cell proliferation and defensive behavior in mice.

Similar to previous reports, our study demonstrated stress-induced reductions in hippocampal cell proliferation. The extent of this reduction was significant for the right hippocampus, but failed to reach statistical significance for the left hippocampus. Lateral asymmetries have been previously reported in rodent models of stress disorders [18–20], and magnetic resonance imaging studies of hippocampal volume in humans with depression or posttraumatic stress disorder (PTSD) [21,22]. Although we did not predict lateral differences a priori, greater stress-induced suppression of right hippocampal cell proliferation parallels studies of humans with PTSD in which the right hippocampus is preferentially affected by stress exposure [23–25]. Lateral differences in stress-induced suppression of

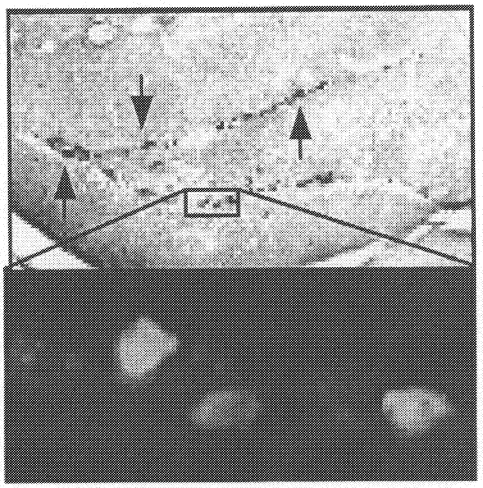

Fig. 3. Typical profile of BrdU+cells (top panel, arrows) in the hippocampal dentate gyrus and a magnified image of BrdU+cells (bottom panel, bright green) co-labelled with the early neuronal marker doublecortin (blue).

hippocampal cell proliferation warrant further attention in neurobiological research.

Social stress-related behavioral regulation of cell proliferation is evidently restricted to the hippocampus as no differences were observed in the amygdala. Although dendritic arborization in the amygdala is enhanced by exposure to repeated immobilization stress [9], cell proliferation in the amygdala is not affected by repeated exposure to social stress. Very few BrdU+cells were observed in the amygdala and, consequently, the proportions of specific BrdU+cell phenotypes could not be reliably determined. In the hippocampus, we found that BrdU+cells were co-labeled with doublecortin, an early neuronal marker (Fig. 3). A detailed quantitative analysis was not performed, however, because numerous studies have established that most of these cells mature into neurons in rats and mice [3,26,27].

In contrast to reports that wheel-running exercise increases hippocampal neurogenesis in rodents [27,28], access to wheel-running did not enhance hippocampal cell proliferation in stressed or non-stressed mice in our study. Several factors may explain these results, including the subjects' age (i.e., mice in our study were 5–7 weeks of age as opposed to more mature animals used in other studies), wheel-running access [29,30], and the effects of dominant–subordinate relationships formed between males housed together before and after each social stress test [26]. The large individual variability observed in our measures of BrdU+cell numbers is probably due to dominant–subordinate relationships from housing male mice in pairs. Most previous work in this area has examined female mice housed in small groups where dominant–subordinate relationships are generally less problematic. Studies of mature, singly housed, adult male intruder mice are needed to further investigate the potential protective effects of exercise on stress-induced inhibition of cell proliferation. It should be noted, however, that a recent study of adult male rats indicates that individual housing precludes wheel-running enhancement of hippocampal neurogenesis, and combined with stress exposure, individual housing and wheel-running exercise may actually suppress hippocampal neurogenesis [31].

In summary, our finding that specific aspects of behavior evoked by stress predict individual differences in hippocampal cell proliferation suggest that coping strategies and their presumed physiological correlates (e.g., glucocorticoid secretion) are important determinants of plasticity in the hippocampus. Stress-related inhibition of cell proliferation in the hippocampus has been implicated in the pathogenesis of human psychiatric disorders, including depression and PTSD [32]. Behavioral correlates of cell proliferation may thus provide important new insights on individual differences in vulnerability to these stress-related neuropsychiatric conditions.

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