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Research report

Activity-based anorexia is associated with reduced hippocampal cell proliferation in adolescent female rats

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HIGHLIGHTS

- Activity-based anorexia is an animal model of anorexia nervosa.
- This model is being used to study starvation-induced brain changes.
- We report reduced hippocampal cell proliferation following induction of the model.
- The results suggest a primary effect on gliogenesis rather than neurogenesis.

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ABSTRACT

Activity-based anorexia (ABA) is an animal model of anorexia nervosa that mimics core features of the clinical psychiatric disorder, including severe food restriction, weight loss, and hyperactivity. The ABA model is currently being used to study starvation-induced changes in the brain. Here, we examined hippocampal cell proliferation in animals with ABA (or the appropriate control conditions). Adolescent female Sprague-Dawley rats were assigned to 4 groups: control (24 h/day food access), food-restricted (1 h/day food access), exercise (24 h/day food and wheel access), and ABA (1 h/day food access, 24 h/day wheel access). After 3 days of ABA, 5-bromo-2′-deoxyuridine (BrdU; 200 mg/kg, i.p.) was injected and the rats were perfused 2 h later. Brains were removed and subsequently processed for BrdU and Ki67 immunohistochemistry. The acute induction of ABA reduced cell proliferation in the dentate gyrus. This effect was significant in the hilus region of the dentate gyrus, but not in the subgranular zone, where adult neurogenesis occurs. Marked decreases in cell proliferation were also observed in the surrounding dorsal hippocampus and in the corpus callosum. These results indicate a primary effect on gliogenesis rather than neurogenesis following 3 days of ABA. For each brain region studied (except SGZ), there was a strong positive correlation between the level of cell proliferation and body weight/food intake. Future studies should examine whether these changes are maintained following long-term weight restoration and whether alterations in neurogenesis occur following longer exposures to ABA.

1. Introduction

Anorexia nervosa is a life-threatening psychiatric disorder with an initial onset that occurs primarily during adolescence in young women [1]. The disorder is characterized, in part, by unrelenting food restriction, severe weight loss, and in many cases hyperactivity [1–3]. The lack of effective treatments [4,5] and high mortality rate [6,7] provides strong justification for utilizing animal models to identify neurobiological mechanisms that may play a role in perpetuating self-starvation and hyperactivity.

Activity-based anorexia is a translational model of anorexia nervosa that combines unlimited access to a running wheel with limited access to food (1 h/day), resulting in significant weight loss, hyperactivity, and a failure to adapt food intake to increasing energy demands [8–13]. Remarkably, animals continue to run throughout the period of food access, thereby promoting *self-starvation* and, if not stopped, death. The severity of weight loss and hyperactivity in ABA rats escalates rapidly and can be used to study starvation-induced changes in the brain.

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The neurobiology driving the maladaptive cycle of self-starvation and hyperactivity in ABA rats is not well understood, yet our recent work indicates increased expression of α4 and δ subunits of α4βδ $GABA_A$ receptors in the hippocampus following the initial onset of ABA [12]. In the current study, we extended our analysis of the hippocampus to cell proliferation (the initial step towards neurogenesis) in adolescent female rats following 3 days of ABA. Proliferation levels were compared to three control groups (cage control, food-restricted control, and exercise control), thus allowing each component of the model to be studied separately.

2. Materials and methods

2.1. Subjects

All animal procedures followed the principles of laboratory animal care (NIH publication No. 86-23, revised 1985) and were approved by the Institutional Animal Care and Use Committee at Princeton University. Thirty-two adolescent female Sprague-Dawley rats were obtained from Taconic Farms, Inc. (Germantown, NY) and individually housed in standard home cages. Adolescent females (37–39 days old) were chosen in order to closely mimic the stereotypical onset of anorexia nervosa in adolescent girls [1]. Animals were maintained on a reversed 12 h light/12 h dark cycle (lights off at 0600 h or 1000 h) and were fed Purina Rodent Chow 5001. For animals with running wheel access, 8 rat activity wheel cages that consisted of a home cage with a running wheel attached were used (Med Associates, Inc., St. Albans, VT; ENV-046). Wheel turns were automatically monitored by computer (Med-PC IV, wheel counter program) and a back-up manual counter.

2.2. Study design

At the start of the study, rats were assigned to four experimental groups (n = 8/group) that were matched for baseline body weight: (1) cage control (24 h/day food access), (2) exercise control (24 h/day food and wheel access), (3) food-restricted control (1 h/day food access) and (4) activity-based anorexia (ABA; 1 h/day food access, 24 h/day wheel access). Body weight and food intake were recorded daily ~15 min prior to the start of the dark cycle. On days −1 and 0, the exercise and ABA groups were given 24 h/day access to both food and the running wheel in order to record baseline wheel running activity. On days 1–3, the food-restricted and ABA groups were given unlimited access to food for 1 h/day at the onset of the dark cycle in order to mimic the typical time *ad libitum* fed animals begin food intake. At the start of food access, food pellets were weighed and placed on top of the cage for 1 h/day; pellets were then removed for the remaining 23 h/day and weighed again to determine food intake. The cage was checked for any pieces of food that may have been hidden by the animal. Individual housing was also used to increase the accuracy of individual food intake measurements. Wheel access was not restricted during the period of food access.

At the end of the light cycle on day 3, rats received a single i.p. injection of 200 mg/kg of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) to label proliferating cells in the hippocampus. This dose represents a near-maximal dose for labeling cells and does not induce any discernible signs of toxicity in the animal [14]. The BrdU (Sigma–Aldrich, St. Louis, MO) was dissolved in sterile 0.9% (w/v) saline (containing 0.007 N NaOH) and given in a volume of 10 ml/kg of body weight. Immediately after the injection, food was removed from the cages (or withheld in the case of the food-restricted animals), and all running wheels were locked. This was done in order to prevent any direct effects of eating and/or exercise on the systemic absorption of BrdU and its uptake into the brain. Two hours after the BrdU injection, animals were deeply anesthetized with chloral hydrate (~1.75 g/kg, i.p.) and were perfused transcardially with cold physiological saline (containing 10 IU heparin/ml), followed by paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4). Brains were removed, postfixed in paraformaldehyde for 24 h at 4 °C, transferred to sucrose (30% in 0.1 M phosphate-buffered saline, PBS) for 2–3 days until equilibrated and then sectioned with a microtome.

2.3. BrdU and Ki67 immunohistochemistry

Frozen coronal sections (40-μm thick) were cut throughout the entire rostral-caudal extent of the hippocampus (bregma, approximately −1.80 mm to −6.80 mm [15]) and a 1-in-12 series of tissue was then processed for BrdU or the intrinsic mitotic marker Ki67 using a slide-mounted immunoperoxidase technique, as described previously [16]. The latter marker was used to rule out the possibility that any observed changes in BrdU labeling were due to alterations in BrdU bioavailability, as a result of rapid changes in body weight and/or composition. Furthermore, unlike BrdU which labels cells only during DNA synthesis (S-phase), Ki67 labels proliferating cells during all phases of mitosis [17], and therefore can be used to confirm the general findings obtained with BrdU.

For BrdU staining, sections were heated in citric acid (0.01 M, pH 6.0), digested with trypsin (0.1% in 0.1 M Tris buffer, pH 7.5, containing 0.1% $CaCl_2$), denatured with 2.4 M hydrochloric acid, and then incubated with a mouse monoclonal antibody raised against BrdU (1:200 in PBS containing 0.5% Tween 20; NCL-BrdU; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) for 48 h at 4 °C. For Ki67 staining, sections were heated in citric acid (0.01 M, pH 6.0), and then incubated with a mouse monoclonal Ki67 antibody (1:200 in PBS containing 0.5% Tween 20; NCL-Ki-67-MM1; Novocastra Laboratories Ltd.) for 48 h at 4 °C. Following the primary antibody incubation, sections were incubated with a biotinylated horse anti-mouse IgG (1:200 in PBS; Vector Laboratories, Burlingame, CA) and with avidin-biotin complex (1:100 in PBS; Vectastain® Elite ABC kit, Vector Laboratories) for 60 min, and then reacted with 3,3′-diaminobenzidine (DAB), to visualize labeled cells. Sections were then counterstained with cresyl violet, dehydrated and coverslipped with DPX.

2.4. Tissue analysis

All slides were analyzed blind with respect to treatment using an Olympus BX-60 light microscope (Olympus America Inc., Melville, NY, USA). Immunopositive cells were identified by their distinct dark-brown nuclear staining. In every 12th section, BrdU/Ki67 labeled cells were counted bilaterally in the dentate gyrus (DG), the dorsal hippocampus (minus the DG), and in the medial corpus callosum at high magnification (400× or 600×). Cell counts per region were summed across all sections for each animal and then multiplied by 12 (the inverse of the sampling interval) to obtain an estimate of the total number of labeled cells in each region. In addition, the DG was divided into anterior (dorsal) and posterior (ventral) portions, as in Guzmán-Marin et al. [18]. The boundary separating the anterior and posterior hippocampus corresponded to the region where the CA2 and CA3 pyramidal cell layers coalesce into a continuous cell layer in the coronal plane (approximately −4.5 mm from bregma, according to the atlas of Paxinos and Watson [15]). Typically, there were 6 anterior and 4 posterior sections for each animal. Labeled cells in the dentate gyrus were also counted separately in the subgranular zone (SGZ) and in the hilus. Cells located within two cell-body widths of the granular cell layer were considered to be in the SGZ; cells located more distally were considered to be in the hilus.

2.5. Statistical analysis

The behavioral data were analyzed using a two-way repeated measures analysis of variance (ANOVA) with time as the within subjects factor and treatment as the between subjects factor. For statistical analysis of the cell proliferation data (BrdU and Ki67) in each brain region, a one-way ANOVA was used. When a significant F value was calculated, *post hoc* comparisons between groups were made using Bonferroni multiple comparison test. Correlations between BrdU and Ki67, or BrdU and the various behavioral/physiological measures were analyzed using Pearson correlation coefficient. The statistical analyses were carried out using Prism version 5.0c for Mac OS (GraphPad Software, San Diego, CA). All data are expressed as means ± standard error of the mean (SEM). In all cases, a probability value $p < 0.05$ was taken as statistically significant.

3. Results

3.1. Behavior

Fig. 1 displays changes in wheel running activity, food intake, and body weight over the course of the study for the different treatment groups. For wheel running activity (top panel), ANOVA revealed significant main effects of both treatment ($F(1,42) = 11.82$, $p < 0.005$) and time ($F(3,42) = 16.98$, $p < 0.0001$). The ABA group ran significantly more than the exercise group on days 2 (+129.6%, $p < 0.05$) and 3 (+227.7%, $p < 0.0001$).

For food intake (middle panel), an ANOVA beginning on day 0 indicated significant main effects of both treatment ($F(3,84) = 243.5$, $p < 0.0001$) and time ($F(3,84) = 34.80$, $p < 0.0001$). Compared to the control group, the food-restricted group consumed significantly less food on days 1 (−93.1%, $p < 0.0001$), 2 (−75.7%, $p < 0.0001$), and 3 (−67.1%, $p < 0.0001$), and the ABA group also consumed significantly less food on days 1 (−91.7%, $p < 0.0001$), 2 (−76.4%, $p < 0.0001$), and 3 (−75.2%, $p < 0.0001$). Compared to the exercise group, the food-restricted and ABA groups consumed significantly less food on days 1 (−91.8% and −90.2%, $p < 0.0001$), 2 (−72.4% and −73.2%, $p < 0.0001$), and 3 (−62.9% and −72.1%, $p < 0.0001$).