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Research article

Activity-based anorexia activates CRF immunoreactive neurons in female rats

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ABSTRACT

Activity-based anorexia (ABA) is a well-established animal model mimicking the eating disorder anorexia nervosa (AN). Since the pathophysiology of AN is yet poorly understood and specific drug treatments are lacking so far, animal models might be useful to further understand this disease. ABA consists of time-restricted access to food for 1.5 h/day and the possibility to exercise in a running wheel for 24 h/day. This combination leads to robust body weight loss as observed in AN. Here, we investigated the activation of brain corticotropin-releasing factor (CRF) neurons, a transmitter involved in the response to stress, emotional processes and also food intake. After development of ABA, rat brains were processed for c-Fos and CRF double immunohistochemistry. ABA increased the number of c-Fos/CRF double labeled neurons in the paraventricular nucleus (PVN) and the dorsomedial hypothalamic nucleus (DMH) compared to the *ad libitum* (AL, *ad libitum* fed, no running wheel) and activity (AC, *ad libitum* fed, running wheel, $p < 0.05$) but not to the restricted feeding (RF, food for 1.5 h/day, no running wheel, $p > 0.05$) group. Also the number of CRF neurons was increased in the DMH of ABA rats compared to AL and AC ($p < 0.05$). In the Edinger-Westphal nucleus (EW) the number of c-Fos positive neurons was increased in ABA and RF compared to AC ($p < 0.05$), while the number of double labeled neurons was not different ($p > 0.05$). Taken together, brain CRF activated under conditions of ABA might play a role in the development and maintenance of this animal model and possibly also in human AN.

1. Introduction

Activity-based anorexia (ABA) is the most established animal model for the eating disorder anorexia nervosa (AN) [1]. The rodent model combines time restricted access to food for 1.5 h/day and voluntary physical activity in a running wheel which leads to robust body weight loss within 14–21 days [2]. The model mimics main symptoms of AN, namely restriction of caloric intake as well as increased physical activity, reported by 80% of patients with AN [3]. Although AN is clinically well described and nutritional, educational as well as psychotherapeutic treatment options exist [4], there are still no specific pharmacological treatment options available and AN continues to be the psychiatric illness with the highest mortality rate [5]. Therefore, the ABA model might allow deeper insight into the pathophysiology contributing to the development of AN. Despite the fact that ABA is well established and has been shown to exert symptoms as observed under conditions of AN such as alterations of the endocrine system including not only food-intake regulating hormones e.g. ghrelin [6], leptin [7] and nesfatin-1 [8], but also an activation of the hypothalamus-pituitary-adrenal axis [9], intestinal barrier dysfunction [10] and the development of stress ulcers [11], the data should be interpreted with caution as animals do not voluntarily lose weight and psychological, environmental as well as genetic factors playing a role in human AN [4,12] are not mimicked by the animal model.

After establishing the ABA model in our laboratory, in a previous study we investigated neuronal changes in the brain of ABA rats using

Abbreviations: ABA, activity-based anorexia (group); AC, activity group; AL, *ad libitum* fed group; AN, anorexia nervosa; Arc, arcuate nucleus; CRF, corticotropin-releasing factor; DMH, dorsomedial hypothalamic nucleus; DR, dorsal raphe nucleus; EW, Edinger-Westphal nucleus; ir, immunoreactive; LC, locus coeruleus; NTS, nucleus of the solitary tract; PBS, phosphate buffered saline; PVN, paraventricular nucleus; RF, restricted feeding group; SO, supraoptic nucleus

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the marker c-Fos [2]. ABA activated, among others, several brain nuclei highly involved in the mediation of stress, namely the PVN, DMH, dorsal raphe nucleus (DR), locus coeruleus (LC) and the nucleus of the solitary tract (NTS) [2,8]. Brain CRF is the hallmark hormone of the stress response and modulates numerous parameters such as behavior, energy balance, endocrine, cardiovascular, immune and gastrointestinal functions [13]. Activation of CRF was demonstrated under a multitude of stressful conditions e.g. novel environment, immune challenge, immobilization and visceral stressors such as abdominal surgery [14–18].

Therefore, in the present study we investigated whether ABA activates CRF positive neurons in the brain using c-Fos/CRF double immunohistochemistry. Brain nuclei were selected based on our previous study showing neuronal (c-Fos) activation under conditions of ABA in these nuclei [2]. Moreover, these nuclei were shown to basally express CRF as shown in a previous study establishing the antibody used here [14]. The neuronal pattern of ABA was compared to three control groups established before: *ad libitum* group (AL), activity group (AC) and restricted feeding group (RF) [2]. Only female rats were used based on the higher prevalence of AN in women compared to men [4].

2. Methods

2.1. Animals

Female Sprague-Dawley rats (Harlan-Winkelmann Co., Borchen, Germany) weighing 150–180 g upon arrival were housed in groups under conditions of controlled illumination (12:12 h light/dark cycle, lights on/off: 06:00 a.m./06:00 p.m.) and temperature (21–23 °C). Rats were fed with standard rat chow (ssniff Spezialdiäten GmbH, Soest, Germany) and tap water *ad libitum* unless kept on a different protocol as described below. The study was carried out in accordance with the recommendation of the institutional guidelines; the protocol was approved by the state authority for animal research.

2.2. Activity-based anorexia protocol

After one week of acclimatization, adolescent/early adult rats were randomly assigned to one of four groups (n = 6/group): a) *ad libitum* group (AL): no extra activity and *ad libitum* access to food, b) activity group (AC): voluntary activity in a running wheel and *ad libitum* access to food, c) restricted feeding group (RF): no extra activity and restricted feeding schedule, and d) the activity-based anorexia group (ABA): voluntary activity in a running wheel and restricted feeding schedule. The experimental protocol was performed as recently described [2]. Briefly, rats were single-housed with ABA and AC having access to a running wheel inside the cage. During the first week all animals had *ad libitum* access to standard rat chow, while from day eight ABA and RF animals had restricted access to food for 1.5 h daily (09:00–10:30), whereas AC and AL animals continued their *ad libitum* feeding schedule. After 14 days of food restriction and the development of ABA [2], animals were euthanized and brains processed for immunohistochemistry as described below.

2.3. c-Fos and CRF immunohistochemistry

Animals were euthanized directly after the daytime feeding period. During this last period, food intake was restricted to 1.5 g to avoid signals due to strong distention of the stomach or nausea as described in our previous study [2]. Rats were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine (Ketanest™, Curamed, Karlsruhe, Germany) and 10 mg/kg xylazine (Rompun™ 2%, Bayer, Leverkusen, Germany) and transcardially perfused as described before [2]. Brains were removed, post-fixed overnight and then snap-frozen. Afterwards, brains were cut in 25-µm coronal sections using a cryostat (CryoStar NX70, Thermo Fisher Scientific, Waltham,MA,USA). Double labeling for c-Fos and CRF was performed as described before [14]. Briefly, every third brain section was selected and first washed in phosphate buffered saline (PBS). Next, endogenous peroxidase activity was blocked by 0.3% H_2O_2 and non-specific binding by 2% normal goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Every step of the protocol was followed by a 3 × 15 min washing step in PBS. Sections were then incubated overnight with the first primary antibody, rabbit polyclonal anti-c-Fos (1:20,000, Catalog No. ABE457, Merck Millipore, Darmstadt, Germany). The following day, incubation with biotinylated secondary goat anti-rabbit IgG Fab fragment (1:1000, Catalog No. 111-067-003, Jackson ImmunoResearch) for 2 h and then incubation with ABC (1:200, Vector Laboratories, Burlingame, CA, USA) in 0.3% Triton-PBS followed. Staining was visualized with DAB and nickel ammonium sulfate followed by a washing step in Triton-PBS. Thereafter, sections were incubated overnight in the second primary antibody, rabbit anti-rat CRF (1:10,000, CURE antibody 200101, University of California Los Angeles) established in a previous study [14]. Next, sections were incubated with biotinylated goat anti-rabbit IgG (1:1000 Catalog No. 111-065-144, Jackson ImmunoResearch) followed by ABC (1:200, Vector Laboratories) and visualization using DAB only.

Immunoreactivity of brain sections was examined using a light microscope (Axiophot, Zeiss, Jena, Germany) and images were acquired using a connected camera. For quantitative assessment the number of immunoreactive (ir) cells was counted unilaterally in at least five sections of brain nuclei selected based on two previous studies [2,14]. Coordinates of the brain nuclei were identified according to the rat brain atlas [19]: supraoptic nucleus (SO, −0.6 to −1.56), PVN (−1.56 to −1.92, corresponding to the area where the majority of CRF ir neurons is located [14]), arcuate nucleus (Arc, −1.92 to −3.06), DMH (−2.52 to −3.48), EW (−6.12 to −6.84), DR (−7.08 to −8.16), LC (−9.48 to −10.08) and the rostral part of the NTS (−13.56 to −13.2). The average number of singly or doubly labeled c-Fos and CRF ir cells per section derived from the total number of sections analyzed for each nucleus was determined for each animal and used to calculate the mean value/group. Cells with dark blue/black nuclear staining were considered c-Fos positive, cells with a brown cytoplasmatic signal as CRF ir.

2.4. Statistical analysis

Distribution of the data was determined by the Kolmogorov-Smirnov test. Data are expressed as mean ± SEM and were analyzed by one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test. Differences were considered significant when $p < 0.05$ (SigmaStat 3.1., Systat Software, San Jose, CA, USA).

3. Results

Neuronal activity was investigated when the phenotype of ABA was fully developed with ABA animals reaching an average reduction of body weight of −48.4 ± 4.9 g (−25.1 ± 2.2%), whereas RF rats showed a reduction of −31.0 ± 4.4 g (−15.3 ± 1.8%), and AL (+25.8 ± 1.9 g, +12.3 ± 0.9%) and AC (+23.0 ± 3.1 g, +10.8 ± 1.5%) rats both gained body weight. On their last day before food restriction (day 7 of training) ABA animals displayed a daily running wheel activity of 951 ± 77 m (2602 ± 211 wheel rotations) which increased during the development of ABA with a daily running wheel activity of 1381 ± 127 m (3776 ± 346 rotations, $p = 0.02$) before the end of the experimental period.

3.1. ABA activates CRF ir neurons in the PVN, DMH and EW

In the PVN ABA showed a significantly higher number of c-Fos positive cells compared to the AC group ($F_{(3,23)} = 3.6$, $\eta^2 = 0.35$, $p < 0.05$) and a higher number of c-Fos/CRF ir neurons in comparison to AL and AC ($F_{(3,23)} = 8.3$, $\eta^2 = 0.55$, $p < 0.01$; Fig. 1).