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α4βδ-GABA$_A$RS IN THE HIPPOCAMPAL CA1 AS A BIOMARKER FOR RESILIENCE TO ACTIVITY-BASED ANOREXIA

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Abstract—Anorexia nervosa (AN) is a psychiatric illness characterized by restricted eating and an intense fear of gaining weight. Most individuals with AN are females, diagnosed first during adolescence, 40–80% of whom exhibit excessive exercise, and an equally high number with a history of anxiety disorder. We sought to determine the cellular basis for individual differences in AN vulnerability by using an animal model, activity-based anorexia (ABA), that is induced by combining food restriction (FR) with access to a running wheel that allows voluntary exercise. Previously, we showed that by the fourth day of FR, the ABA group of adolescent female rats exhibit >500% greater levels of non-synaptic α4βδ-GABA$_A$Rs at the plasma membrane of hippocampal CA1 pyramidal cell spines, relative to the levels found in age-matched controls that are not FR and without wheel access. Here, we show that the ABA group exhibits individual differences in body weight loss, with some losing nearly 30%, while others lose only 15%. The individual differences in weight loss are ascribable to individual differences in wheel activity that both precedes and concurs with days of FR. Moreover, the increase in activity during FR correlates strongly and negatively with α4βδ-GABA$_A$R levels ($R = -0.9$, $p < 0.01$). This negative correlation is evident within 2 days of FR, before body weight loss approaches life-threatening levels for any individual. These findings suggest that increased shunting inhibition by α4βδ-GABA$_A$Rs in spines of CA1 pyramidal neurons may participate in the protection against the ABA-inducing environmental factors of severe weight loss by suppressing excitability of the CA1 pyramidal neurons which, in turn, is related indirectly to suppression of excessive exercise. The data also indicate that, although exercise has many health benefits, it can be maladaptive to individuals with low levels of α4βδ-GABA$_A$Rs in the CA1, particularly when combined with FR.

Key words: hippocampus, alpha4-GABA-receptor, neuromodulation, adolescence, stress, anxiety.

INTRODUCTION

Anorexia nervosa (AN) is a psychiatric illness characterized by restricted eating and an intense fear of gaining weight, even when the patient is severely underweight. AN has one of the highest mortality rates among mental illnesses (10–20%) (Sullivan, 1995; Birmingham et al., 2005; Bulik et al., 2007), even surpassing depression. There are no accepted pharmacological treatments for AN (Powers and Bruty, 2009; Aigner et al., 2011; Barbarich-Marsteller et al., 2012), as its etiology remains unclear. However, the epidemiology of AN provides clues about the biological basis of the disease. No less than 40% and as many as 80% of individuals with AN exhibit excessive exercise (Davis et al., 1999; Hebebrand et al., 2003) that often precedes the formal diagnosis (Davis et al., 1997). Equally many also have a history of anxiety disorders (Kaye et al., 2004; Dellava et al., 2010; Thornton et al., 2011). The first onset of AN is most commonly at puberty, with 90–95% of the cases occurring among females (Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5)) (American Psychiatric Association, 2013), indicating that anorexic behavior during this pivotal, final stage of brain development may be associated with ovarian hormone surges of puberty that perturb anxiety regulation. Still, it is perplexing why only 0.9% of the female population is diagnosed with AN during their lifetime (Hudson et al., 2007), when nearly all females experiment with dieting during adolescence (Lucas et al., 1991). We sought to determine the cellular basis for the individual differences in AN vulnerability by using an animal model, activity-based anorexia (ABA).

The rodent ABA model captures two hallmarks of AN. One is *voluntary* excessive exercise, which is evoked by imposition of food restriction. The other is *voluntary* food restriction, as the food restricted animals paradoxically begin to choose exercise over feeding, even during the period of food access. When the ABA-inducing environment is imposed upon adolescent female rats, this combination of behaviors leads to severe body

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Abbreviations: AN, anorexia nervosa; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CON, control; EX, exercise; IgG, immunoglobulin G; PBS, phosphate-buffered saline; PSD, postsynaptic density; SIG, silver-intensified gold.

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weight loss and mortality, unless removed from the ABA-inducing environment by around the fifth day (Routtenberg and Kuznesof, 1967; Barbarich-Marsteller et al., 2013; Chowdhury et al., 2013; Gutierrez, 2013). Adolescent female rats placed in an ABA-inducing environment for 4 days exhibit a 500% greater level of non-synaptic α4βδ-$GABA_A$ receptors (α4βδ-$GABA_A$Rs) at dendritic spines of CA1 pyramidal cells, relative to controls (Aoki et al., 2012). Since the hippocampus plays an important role in anxiety regulation (Huttunen and Myers, 1986; Kataoka et al., 1991; Talaenko, 1993), this increase would be expected to reduce excitability of CA1 pyramidal cells and the animal's anxiety level. However, this rise could alternatively have exacerbated the stress-induced anxiety through desensitization of these receptors in CA1 by allopregnanolone, since allopregnanolone occurs naturally at puberty onset (Shen et al., 2007, 2010), thereby promoting excessive exercise. This study aimed to determine whether the rise of α4βδ-$GABA_A$Rs is causal to the animal's hyperactivity and to also explore the possibility that α4βδ-$GABA_A$Rs increased as a result of hyperactivity. In order to understand the relationship between hyperactivity and α4 subunit expression, we analyzed the relationship between α4βδ-$GABA_A$R expression and wheel activity and also examined brains of animals at an earlier time point of ABA induction, when only half of the animals have begun to exhibit the food restriction-evoked hyperactivity. Results indicate that heightened levels of α4βδ-$GABA_A$Rs are not evoked by hyperactivity but instead, found within brains of animals with the minimal levels of food restriction-evoked hyperactivity. This relationship is consistent with the interpretation that α4βδ-$GABA_A$R expression is a biomarker for a mechanism conferring protection against food restriction-evoked excessive exercise and weight loss.

EXPERIMENTAL PROCEDURES

Materials

The goat antibody directed against the α4 subunit of $GABA_A$ receptors ($GABA_A$Rs) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA, catalog #SC-7355). This antibody recognizes a single 67-kD band by Western blotting (Sanna et al., 2003; Griffiths and Lovick, 2005a,b; Shen et al., 2007). Preadsorption of the antibody with a synthetic peptide corresponding to the antigenic site (catalog #SC-7355p from Santa Cruz) greatly reduces immunoreactivity, as was confirmed previously by light and electron microscopy (Aoki et al., 2012; Sabaliauskas et al., 2012) and by Western blotting (Sanna et al., 2003). Application of the antibody onto the hippocampus of female mice with genetic deletion of the α4 subunit also yields much lower levels of immunolabeling, compared to the hippocampus of wildtype age-matched female mice (Sabaliauskas et al., 2012). Genetic deletion of the α4 subunits reduces the membranous expression of δ subunits at the CA1 spines, confirming that α4 and δ subunits are partners in native $GABA_A$ receptors at CA1 spines and that the immunocytochemical detections of α4 (and δ subunits) in the CA1 pyramidal cells reflect the presence of α4βδ-$GABA_A$Rs (Sabaliauskas et al., 2012). Specificity of both the membranous and cytoplasmic pools of α4-labeling was ascertained through the exhaustive controls described above (Aoki et al., 2012; Sabaliauskas et al., 2012).

The secondary antibody was rabbit anti-goat immunoglobulin G (IgG), conjugated to 0.8-nm colloidal gold (catalog #25220 from Electron Microscopic Sciences, Hatfield, PA, USA). The silver-intensification kit used to enhance 0.8-nm colloidal gold particles was purchased from KPL (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA).

Epoxy resin, grids, fixatives and most electron microscopic supplies were purchased from Electron Microscopic Sciences, while chemicals, such as bovine serum albumin, buffers and salts were purchased from Sigma Chem (St. Louis, MO, USA).

ABA induction and behavioral controls

This study describes data obtained from two sets of data: those data obtained from animals that underwent 4 days of ABA induction and euthanized on P44 and another set of data from animals that underwent 2 days of ABA induction and were euthanized on P42. The electron microscopic immunocytochemical data from the P44 set of tissue were described earlier (Aoki et al., 2012) but were re-analyzed in this study, for further analysis of the relationship to individual animal's wheel-running activity. The ABA induction and methods for electron microscopic data collection from brains of animals euthanized on P42 were never presented previously and therefore, are described in detail here.

ABA induction of the P42 group of animals was as described earlier for the P44 group of animals (Aoki et al., 2012), except that the animals were euthanized 2 days earlier. Sprague–Dawley female rats were purchased as a group of 8–12 from Taconic Farms and delivered to the New York State Psychiatric Institute's animal facility on postnatal day 21 (P21). Upon arrival, animals were individually housed in a reverse 12-h dark:light cycle in the absence of males. From P36 through P42, body weight, food intake, and wheel activity (where applicable) were measured daily within 20 min prior to the dark cycle. On P36, each group of 8–12 rats that were delivered together were divided into four treatment groups: (1) Control (CON, $N = 8$): 24 h per day food access with no wheel access; (2) Exercise (EX, $N = 7$): 24 h per day food and wheel access; (3) Food restricted (FR, $N = 8$): 1 h per day food access with no wheel access; (4) ABA ($N = 8$): 1 h per day food access and 24 h per day wheel access. Animals with access to a running wheel (EX and ABA) were housed in a standard home cage with an activity wheel attached (Med Associates, Inc., St. Albans, VT, USA). Baseline wheel activity with 24 h per day access to food was recorded for the EX and ABA groups on P37–P39; activity was quantified based on the number of wheel rotations per day, which was converted to km of running per day. On P40, restricted food access began for FR