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A ROLE FOR PROGESTERONE AND α4-CONTAINING $GABA_A$ RECEPTORS OF HIPPOCAMPAL PYRAMIDAL CELLS IN THE EXACERBATED RUNNING RESPONSE OF ADOLESCENT FEMALE MICE TO REPEATED FOOD RESTRICTION STRESS

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Abstract—Adolescent females are particularly vulnerable to mental illnesses with co-morbidity of anxiety, such as anorexia nervosa (AN). We used an animal model of AN, called activity-based anorexia (ABA), to investigate the neurobiological basis of vulnerability to repeated, food restriction (FR) stress-evoked anxiety. Twenty-one of 23 adolescent female mice responded to the 1st FR with increased wheel-running activity (WRA), even during the limited period of food access, thereby capturing AN's symptoms of voluntary FR and over-exercise. Baseline WRA was an excellent predictor of FR-elicited WRA (severity of ABA, SOA), with high baseline runners responding to FR with minimal SOA (i.e., negative correlation). Nine gained resistance to ABA following the 1st FR. Even though allopregnanolone (3α-OH-5α-pregnan-20-one, THP), the metabolite of progesterone (P4), is a well-recognized anxiolytic agent, subcutaneous P4 to these ABA-resistant animals during the 2nd FR was exacerbative, evoking greater WRA than the counterpart resistant group that received oil vehicle, only. Moreover, P4 had no WRA-reducing effect on animals that remained ABA-vulnerable. To explain the sensitizing effect of P4 upon the resistant mice, we examined the relationship between P4 treatment and levels of the α4 subunit of $GABA_A$Rs at spines of pyramidal cells of the hippocampal CA1, a parameter previously shown to correlate with resistance to ABA. α4 levels at spine membrane correlated strongly and negatively with SOA during the 1st ABA (prior to P4 injection), confirming previous findings. α4 expression levels were greater among P4-treated animals that had gained resistance than of vehicle-treated resistant animals or of the vulnerable animals with or without P4. We propose that α4-$GABA_A$Rs play a protective role by counterbalancing the ABA-induced increase in excitability of CA1 pyramidal neurons, and although exogenous P4's metabolite, THP, enhances α4 expression, especially among those that can gain resistance, it also interferes with α4-$GABA_A$Rs' protective role by desensitizing α4-$GABA_A$Rs.

Key words: activity-based anorexia, anxiety, anorexia nervosa, resilience, exercise, allopregnanolone.

INTRODUCTION

Adolescence is a period of great vulnerability to mental illness (Spear, 2000). The stress response system in the brain and periphery exhibit strong and long-lasting (Andersen, 2003; Romeo, 2010) plasticity during adolescence, perhaps because it is still continuing to develop (Romeo, 2005; Romeo et al., 2006; McCormick and Mathews, 2007). As a consequence, a stressful event during adolescence affects the mental health outcome into adulthood (Andersen, 2003; Romeo, 2010). In puberty and adolescence, there is an interaction of the following factors: an increase of environmental stressors, gonadal hormone fluctuations, and a growth spurt in the hippocampus (Mannan and O'Shaughnessy, 1991; Palumbo et al., 1995; Spear, 2000; Romeo et al., 2006; Chowdhury et al., 2014). Estrogen, progesterone and testosterone are the major peri-pubertal hormones influencing plasticity and signaling in the hippocampus, along with reproductive function (Smith and Woolley, 2004). In adulthood, estrogen has excitatory effects in several brain regions, including the hippocampus, hypothalamus, and amygdala, with the impact of increasing or decreasing anxiety, increasing stress responses and decreasing depression (Nabekura et al., 1986; Isgor et al., 2003; Walf and Frye, 2006; Harte-Hargrove et al., 2013). Among the questions that remain to be answered is the role of progesterone (P4) in altering the stress response of adolescents. We sought to address this gap in knowledge by imposing an animal model of stress called activity-based anorexia (ABA) upon adolescent mice and testing P4's cellular and behavioral effects upon ABA response.

The stressor in the ABA model is food restriction (FR) and the response to this stress is a marked increase in voluntary wheel-running activity (WRA), causing exacerbated weight loss. WRA is a reliable measure of stress response, because it correlates positively with levels of corticosterone (cort) released after FR (Duclos

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Abbreviations: ANOVA, analysis of variance; ABA, activity-based anorexia; AN, anorexia nervosa; FR, food restriction; PND, postnatal day; PSD, postsynaptic density; SIG, silver-intensified colloidal gold; SR, stratum radiatum; WRA, wheel-running activity.

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et al., 2005, 2009). The WRA is also robustly correlated to anxiety levels measured during ABA (Wable et al., 2015). Stress usually afflicts an individual more than once. The change in physiology evoked by repeated stress can also be analyzed in this animal model by imposing a second episode of ABA after recovery from the first. Such repeated stress exposures enable investigation of individual variability in habituation, adaptation or sensitization to the repeated stressor. Indeed, one of the strengths of the ABA model is that there is considerable individual variability in the WRA response to the first as well as the repeated episode of the FR stressor. The variability in behavior is not noise, because it is correlated in meaningful ways to variability in biological markers, such as the levels of cort (Duclos et al., 2005, 2009). The extent of GABAergic innervation as well as expression levels of the α4 subunit of $GABA_A$Rs by pyramidal neurons in the CA1 of the dorsal hippocampus is also correlated strongly and negatively with the FR-evoked increase of WRA, suggesting that up-regulation of the hippocampal GABAergic system may contribute toward suppression of WRA and thereby resilience to ABA (Chowdhury et al., 2013; Aoki et al., 2014).

P4 plays an important role in regulating anxiety. P4's metabolite, THP (3αOH-5α[β]-pregnan-20-one), reduces anxiety (Frye and Paris, 2011) (Bitran et al., 1995) in late adolescence, adulthood (Canonaco et al., 1993; Frye and Walf, 2004a; Reddy et al., 2005; Engin and Treit, 2007; Koonce and Frye, 2013) and pre-pubertally (Shen et al., 2007) through positive modulation of $GABA_A$ receptors ($GABA_A$Rs) (Belelli and Lambert, 2005). These findings, together with our recent observation of the strong correlation between WRA response and anxiety (Wable et al., 2015), led us to test the following prediction: P4 treatment could decrease an animal's excessive WRA response to FR when imposed at late adolescence, which in turn would minimize weight loss and serve to protect animals from ABA. We chose a dosage of subcutaneous P4 that has been demonstrated to be anxiolytic in female mice of similar age (Frye et al., 2004).

To identify a possible molecular mechanism underlying the behavioral effect of P4, we measured plasmalemmal and cytoplasmic levels of the α4 subunit of $GABA_A$Rs in pyramidal neurons of the dorsal hippocampal CA1 of these mice. Our reason for measuring the α4 subunit level in the CA1 was twofold: (1), the administration as well as withdrawal of P4 and THP increase the expression of α4βδ-$GABA_A$R in the hippocampus over 2–3 days (Shen et al., 2007); and (2) ABA was previously shown to increase the expression of α4βδ-$GABA_A$Rs in the hippocampal CA1 (Aoki et al., 2012, 2014). We surmised that, if heightened levels of α4βδ-$GABA_A$R in the hippocampal CA1 contributed to the suppression of FR-evoked increase in WRA, thereby reducing ABA vulnerability (Aoki et al., 2014), then P4 could induce the expression of α4βδ-$GABA_A$R, which would also be protective against ABA. However, while P4's metabolite, THP, is, in general, anxiolytic, THP can also be anxiogenic. This is because THP has an additional property of desensitizing α4βδ-$GABA_A$R, when expressed in pyramidal cells of the CA1 (Shen et al., 2007). This additional desensitizing property of THP upon α4βδ-$GABA_A$Rs expressed by CA1 pyramidal cells (as opposed to the consistently positive modulating effect of THP upon α4βδ-$GABA_A$Rs expressed by granule cells of the dentate gyrus, involving the difference in the direction of Cl^- flux across the two regions) has the potential consequence of increasing excitability of the CA1 pyramidal neurons and thus of anxiety (Shen et al., 2007). Because of this dual potential action of THP, knowledge about the changing level of α4 subunits in the hippocampal CA1 was needed for assessing the action of P4 that would include the interaction of α4βδ-$GABA_A$R in the animal's response to ABA. Here, we report that, indeed, P4 led to exacerbated WRA during the second ABA, specifically among individuals that had attained resilience during recovery from the 1st ABA and a rise of α4βδ-$GABA_A$Rs. These observations are consistent with the idea that up-regulation of α4βδ-$GABA_A$R in the dorsal hippocampal CA1 during recovery from the first ABA contributed toward attainment of resilience to the second ABA, while desensitization of α4βδ-$GABA_A$Rs by exogenous P4 contributed to anxiogenesis and exacerbated animals' responses to the second ABA.

EXPERIMENTAL PROCEDURES

General aspects about the animals

All procedures relating to the use of animals were according to the NIH Guide for the Care and Use of Laboratory Animals and also approved by the Institutional Animal Care and Use Committee of New York University.

Altogether, 23 animals were used for this study. All animals were female C57BL6 mice, bred at New York University's animal facility in a 12-h:12-h light:dark cycle (lights on at 7 am). At postnatal day (PND) 25, they were weaned and group-housed with same sex littermates. If any litter contained only a single female mouse, she was not included in the experiment. Food was provided *ad libitum* when the animals were not undergoing the FR portion of the paradigm [dry chow (PMI Mouse Diet 5001; 336 kcal per 100 g, 28.507% protein, 57.996% carbohydrates, 13.496% fat) and soft food (Clear H_2O DietGel® 76A; 99.8 kcal per 100 g, 4.7% protein, 17.9% carbohydrates, 1.5% fat, 73.4% moisture)] and water was provided *ad libitum* throughout the experiment.

Non-ovariectomized mice were used for the study because ovariectomy has been shown to alter CNS neurosteroid metabolism following injection of exogenous P4 (Corpechot et al., 1993). Also, P4 is produced by the adrenal gland in stress so ovariectomy does not guarantee depletion of circulating P4 (Romeo et al., 2005).

ABA induction and P4 injection

ABA induction consisted of combining FR with access to a running wheel, as described in the ABA protocol established in our laboratory (Chowdhury et al., 2013).

Running wheels were purchased from Med Associates, called Low-Profile Wireless Running Wheel

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