

Review

Synaptic changes in the hippocampus of adolescent female rodents associated with resilience to anxiety and suppression of food restriction-evoked hyperactivity in an animal model for anorexia nervosa



Chiye Aoki, Tara G. Chowdhury, Gauri S. Wable, Yi-Wen Chen

Center for Neural Science, 4 Washington Place, Room 809, New York, NY 10003, United States

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ABSTRACT

Anorexia nervosa is a mental illness that emerges primarily during early adolescence, with mortality rate that is 200 times higher than that of suicide. The illness is characterized by intense fear of gaining weight, heightened anxiety, obstinate food restriction, often accompanied by excessive exercise, in spite of mounting hunger. The illness affects females nine times more often than males, suggesting an endocrine role in its etiology. Its relapse rate exceeds 25%, yet there are no accepted pharmacological treatments to prevent this. Here, we summarize studies from this laboratory that have used adolescent female rodents in activity-based anorexia (ABA), an animal model of anorexia nervosa, with the goal of identifying neurobiological underpinnings of this disease. We put forth a hypothesis that a GABAergic mechanism within the hippocampus is central to regulating an individual's anxiety which, in turn, strongly influences the individual's resilience/vulnerability to ABA. In particular, we propose that ionotropic GABA_A receptors containing the subunits alpha4 and delta, are at play for exerting shunting inhibition upon hippocampal pyramidal neurons that become more excitable during ABA. Since these receptors confer insensitivity to benzodiazepines, this pharmacological profile of ABA fits with lack of report indicating efficacy of benzodiazepines in reducing the anxiety experienced by individuals with anorexia nervosa. The idea that the GABAergic system of the hippocampus regulates resilience/vulnerability to anorexia nervosa complements current opinions about the important roles of the prefrontal cortex, amygdala, striatum, gustatory pathways and feeding centers of the hypothalamus and of the neuromodulators, serotonin and dopamine, in the etiology of the disease.

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Abbreviations: ABA, activity-based anorexia; AN, anorexia nervosa; CON, control; EX, exercise; FR, food restriction; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; LTP, long-term potentiation; MWM, Morris water maze; Rm, membrane resistance; SR, stratum radiatum; SLM, stratum lacunosum-moleculare; THP, 3 α ,5 α [β]-tetrahydroprogesterone; Rs, receptors; Vm, membrane potential

E-mail address: ca3@nyu.edu (C. Aoki).

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1. Introduction

By now, it is firmly established that neurons of juvenile sensory neocortex are exquisitely sensitive to an individual's environment, culminating in receptive field properties that closely mirror an individual's sensory experience (Aoki and Erisir, 2012; Bear and Singer, 1986; Hubel and Wiesel, 1970; Rauschecker and Singer, 1979; Wiesel, 1981). However, the final stage of brain development lies beyond the juvenile stage, and for some brain regions, even beyond the end of adolescence. With this final stage of development comes bursts of creativity and energy that lead many to outperform their younger siblings and parents (see the Time magazine Nov 9 2015 issue for examples of teen exuberance (TIME staff, 2015), but also the emergence of many mental disorders. The residual growth that continues through adolescence can be the window of opportunity for the environment to exert life-long influences that are more cognitive or emotional than sensory. The advantage of such a window is that it allows for mental capacity to become finely tuned to life's challenges and opportunities, well timed to the stage when an individual prepares to leave the nest and face more unpredictable and complex sensory and social settings. However, the mechanisms enabling experience to sculpt brain function may also be the substrates that contribute to an individual's vulnerability to mental illnesses.

This chapter will report on recent findings pertaining to the neurobiological basis of anorexia nervosa, a mental disorder that emerges most often at early adolescence and especially in females. A number of excellent review articles on the subject of anorexia nervosa (Casper et al., 2008; Compan et al., 2012, 2015; Kaye et al., 2009, 2011) and animal models for anorexia nervosa (Casper et al., 2008; Gutierrez, 2013) already exist, but these do not consider the possible involvement of the hippocampal formation. This chapter is intended to complement the pre-existing reviews by presenting data indicative of additional contributions made by the hippocampus in anxiety regulation and resilience/vulnerability to anorexia nervosa. These ideas are based mostly on data derived from an animal model of anorexia nervosa, called activity-based anorexia (ABA), applied to adolescent female rodents.

2. Anorexia nervosa

2.1. General description

Anorexia nervosa is a mental illness characterized by continuously severe, self-imposed starvation and intense anxiety (Kaye et al., 2004), manifested as fear of gaining weight (APA, 2013). The estimate of lifetime prevalence is 1–2% world-wide (Hudson et al., 2007; Smink et al., 2014). About 25% of the individuals with anorexia nervosa suffer from a chronic and relapsing course (Hudson et al., 2007; Steinhausen, 2002). The

mortality rate for anorexia nervosa is the highest of all mental disorders (Arcelus et al., 2011). Among females diagnosed with anorexia nervosa (1–2% of the general population, world-wide), the mortality rate is estimated to be 0.56% per year. This rate is more than twice that of the female psychiatric inpatients 10–39 years old, 12 times higher than the annual death rate for females 15–24 years old in the general population, and more than 200 times greater than the suicide rate in the general population (Sullivan, 1995). One longitudinal study indicated that 15.6% of the patients in the study that were diagnosed with anorexia nervosa died from causes related to anorexia nervosa within 21 years (Zipfel et al., 2000). In spite of these indications of significant clinical burden, there are, to date, no accepted pharmacological treatments for anorexia nervosa, reflecting the paucity of knowledge regarding its etiology and ineffectiveness of a number of pharmacological treatments that have been tried. In particular, anxiolytics, such as benzodiazepines, are not efficacious for treating anorexia nervosa or in preventing its relapse, even though anxiety is highly comorbid with anorexia nervosa (Kaye et al., 2004). We will discuss below how these features of anorexia nervosa provide clues about the etiology of AN.

2.2. The role of exercise in the etiology of the disease

Excessive exercise is one of the core symptoms of anorexia nervosa, and this behavior exacerbates the severe weight loss associated with food restriction (FR) (Beumont et al., 1994; Casper et al., 2008; Davis et al., 1997). One study noted hyperactivity in 25 out of 33 patients and that all were hyperactive at some time during the course of the illness and 21 were hyperactive prior to dieting and weight loss (Kron et al., 1978). MRI analysis shows that excessive exercise routine leads to enlargement of the hippocampus, which is restored to sizes equivalent to healthy controls' (5% decrease) within 10 weeks of weight restoration and cessation of exercise (Beadle et al., 2015).

The causal-effect relationship between anxiety, FR, and exercise remains unclear but there are at least two prevailing views (Fig. 1). One view is that FR and excessive exercise are evoked due to a pre-existing condition of anxiety, and that patients deliberately choose these behaviors as anxiolytic agents to abate the intense fear of weight gain. In support of this view, pre-existing conditions of anxiety and over-exercise are common among individuals with anorexia nervosa (Dellava et al., 2010). Similarly, mice carrying genes that elevate trait anxiety exhibit stronger hyperactivity when stressed (Gelegen et al., 2007, 2010). Moreover, results from animal models indicate that exercise can be anxiolytic (Sciolino and Holmes, 2012) and stress-relieving (Schoenfeld et al., 2013) through the production of BDNF (brain-derived neurotrophic factor) (Hill and Martinowich, 2015; Rasmussen et al., 2009). FR can also be anxiolytic, through the production of ghrelin (Chuang and Zigman, 2010; Lutter et al., 2008), for which there are ubiquitous

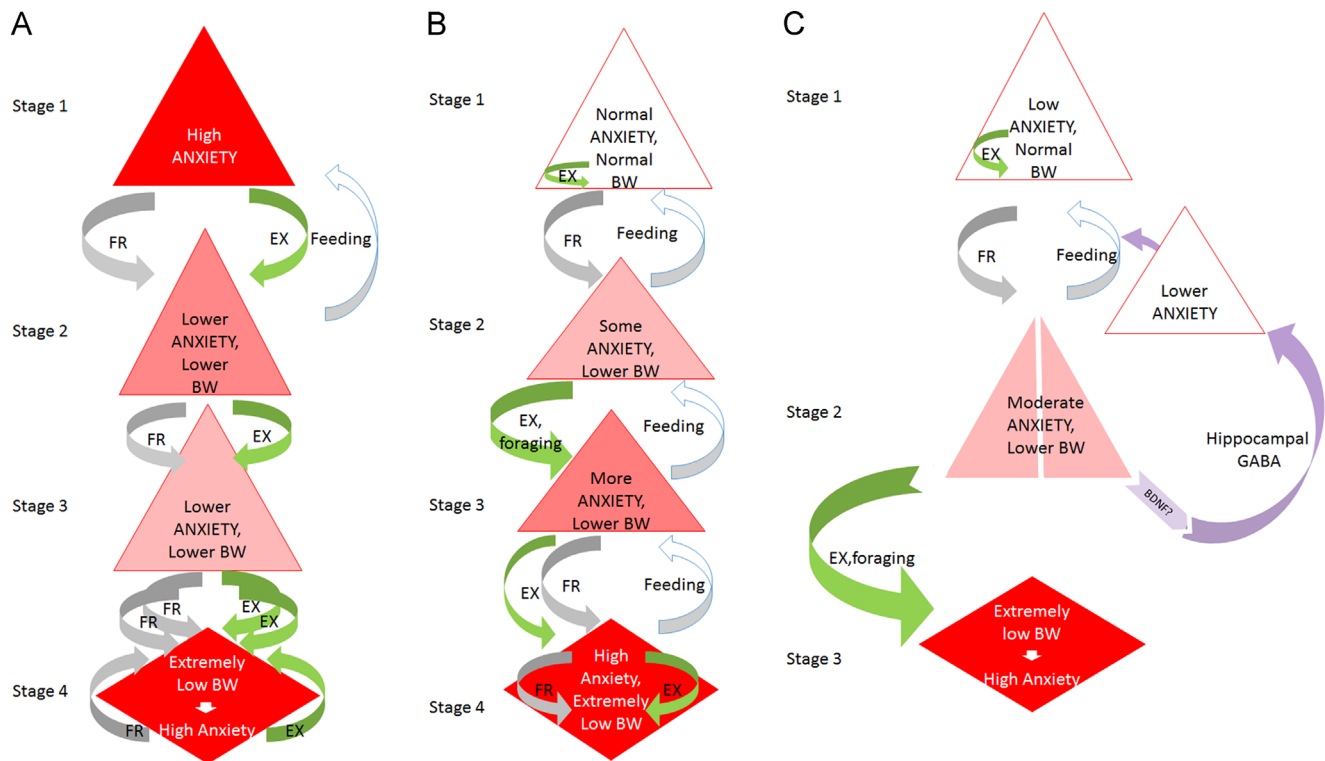


Fig. 1. Working hypotheses. There are at least two prevalent ideas regarding the etiology of anorexia nervosa, depicted here in panels A and B, both of which revolve relentless FR and excessive exercise (EX) as the key features of anorexia nervosa (Stage 4). One view (panel A) is that excessive FR and excessive EX emerge due to a pre-existing condition of elevated anxiety (Stage 1). FR and EX are regarded as voluntary activities chosen by patients to ameliorate high anxiety. Since FR and EX are effectively anxiolytic (Stage 2), patients tend to perpetuate FR and EX, rather than feed themselves. However, this leads inevitably to unhealthy BMI (body mass index) (stage 3). Eventually, the extremely low BW becomes a new source for elevated anxiety. However, patients have taken on a habit of perpetuating FR and EX (Stage 4), and this habit is difficult to break. An alternative view (panel B) is that some degree of EX is appetitive, as has been shown even for animals in the wild (Stage 1) but excessive EX emerges as a result of hunger (Stage 2 to Stage 3), as is observed by healthy individuals facing famine or animals in the wild that forage. Refeeding can easily restore normal body weight and return subjects' anxiety to the low level seen before they became hungry. For animals in captivity, wheel running is not effective for generating a new source of food, thereby exacerbating the weight loss and hunger, causing further elevation of anxiety (Stage 4). At any of these stages, re-feeding is the most effective treatment to reduce anxiety and with it, the excessive EX. However, the habit that has formed may interfere with the restorative behavior of re-feeding (Stage 4). Panel C depicts the goal of this review article, which is to consider the neurochemical and anatomical basis for individual differences in activity-based anorexia, an animal model of anorexia nervosa. We adopt the idea that hunger (Stage 2) is anxiogenic and evokes hyperactivity, even among healthy individuals (Stage 1), leading individuals to transition from Stage 2 to Stage 3. However, some individuals (symbolized by the right half of the triangle of Stage 2) are able to respond to the rise of anxiety associated with the stress of FR by evoking up-regulation of the GABAergic system in the hippocampus, while others cannot (left half of the triangle of Stage 2). Up-regulation of the GABA system, in turn, contributes towards lowered anxiety, even if still hungry. The suppression of EX enables animals to maximize feeding during the limited hours of FR and minimize energy loss, thereby maximizing weight restoration, whenever food is available. As for the neurochemical basis for the GABAergic responsiveness of individuals to the FR-evoked stress and anxiety, we propose BDNF as one possibility.

binding sites throughout the brain, including the hippocampus (Ferrini and De Koninck, 2013).

The other prevailing view is that anxiety and hyperactivity are inevitable behaviors stemming from starvation. There is a wealth of evidence indicating that many species, including healthy humans, rodents and even pigs become hyperactive following starvation (Guisinger, 2003). For rodents housed in cages, hyperactivity is measured by their wheel running (Gutierrez, 2013). Although FR-evoked hyperactivity seems paradoxical, it may have an evolutionary advantage of propelling foraging behavior, an innate behavior that is adaptive for organisms encountering insufficient food supply in the wild (Guisinger, 2003). However, for animals in captivity, the incessant voluntary wheel running that is evoked by FR is clearly maladaptive, because it exacerbates the negative energy balance without bringing the animal closer to a new source of food. Although the incessant voluntary wheel running appears stereotypical, it is not an artifact of captivity: voluntary wheel running is a behavior that can be elicited repeatedly, even by feral mice (Meijer and Robbers, 2014). When faced with FR, caged rodents become so hyperactive that they run, even during the limited hours of food access. In this way, the imposed FR becomes voluntary FR. The voluntary FR among animals exposed to the wheel is called activity-based anorexia (ABA) and has

been used as an animal model to investigate the neurobiological (i.e., non-social, non-cultural) risk factors and neural circuit changes associated with anorexia nervosa. In our opinion, although ABA is not able to investigate the causes that evoke humans to initiate voluntary FR, it is a useful model for analyzing voluntary FR that is perpetuated by individuals with anorexia nervosa, after starvation. The work that we summarize below used this ABA model to probe for the etiology of individual differences in ABA vulnerability, by quantifying FR-evoked hyperactivity, anxiety, and the neuroanatomical correlates of these measures.

2.3. Why study the hippocampus?

2.3.1. Pharmacological profile

The most common age of onset of AN is 15–19 (Lucas et al., 1991), although the incidence is increasing for ages less than 13, based on the British Pediatric Surveillance System (Nicholls et al., 2011), as is the onset of puberty (Biro et al., 2010). Moreover, anorexia nervosa affects females nine times more often than males. This pattern strongly suggests that the gonadal hormone surge at puberty is a contributing factor to anorexia nervosa. Increased stress that comes with transition from childhood to adolescence is also likely to contribute to an individual's vulnerability to anxiety

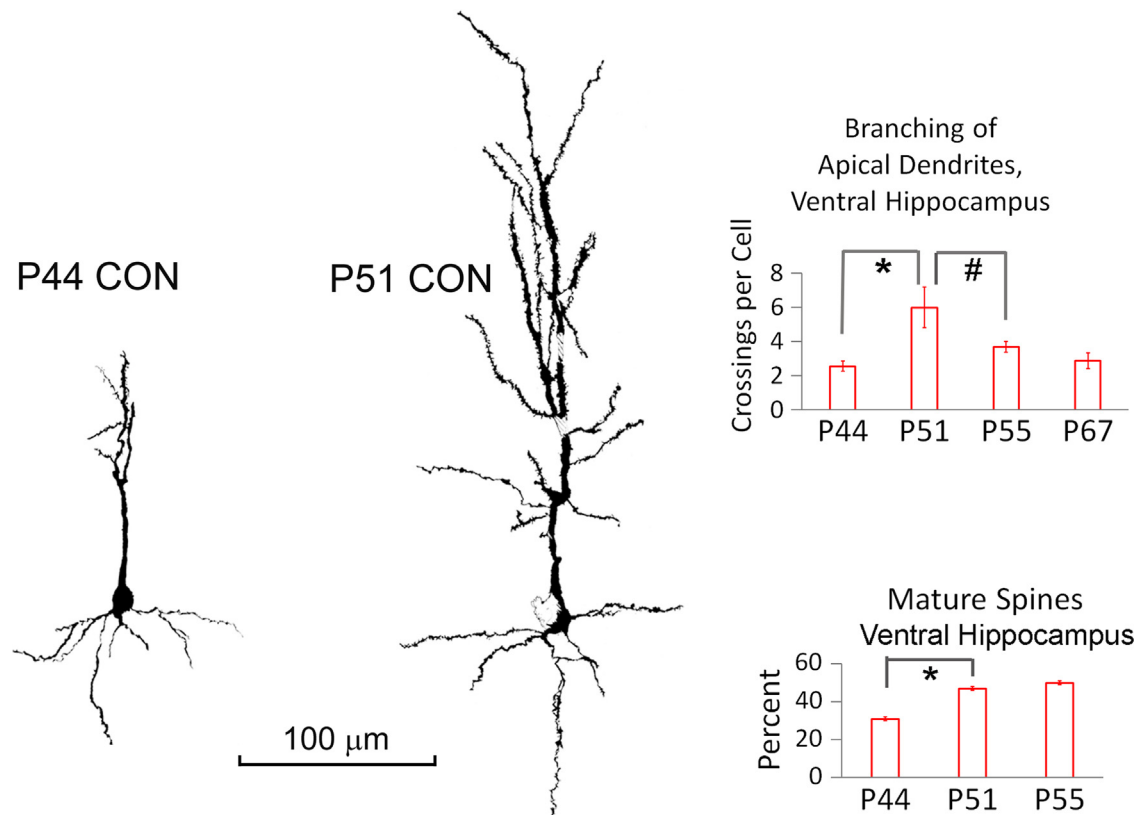


Fig. 2. Adolescent hippocampus is still growing. Unlike most neocortical brain regions, where morphological, biophysical and neurochemical changes have reached near maturity by the end of the juvenile stage (ca P21 for rodents), neurons of the hippocampus are still undergoing robust growth and maturation during adolescence. This growth during adolescence was discovered by sampling the hippocampal tissue at multiple ages during adolescence. The two drawings were made using the *camera lucida*, sampled from the ventral hippocampus of adolescent female rats at two ages – P44 and P51, within a control environment (i.e., no FR, no access to a wheel). Based on Sholl analysis of proximal portions of the apical dendrites of ventral hippocampal CA1, in SR (80 to 200 μm from the soma), there is a dramatic increase in dendritic branching (upper bar graph). This increase is transient, indicating that dendritic branches also retract during adolescence, thereby showing no statistically significant differences in dendritic complexity between the earlier and later phases of adolescence. Analysis of spine morphology (lower graph) indicates that much of synapse maturation also occurs during adolescence, since there is a substantial increase in the proportion that is mature (mushroom and stubby), relative to the immature (filopodia) during mid-adolescence, and nearly half of the spines are still immature even towards end of adolescence (P55). These data are excerpts from a previous publication (Chowdhury et al., 2014a). *indicates significance at $p < 0.05$; # indicates significance at $p = 0.05$.

disorders and anorexia nervosa. The hippocampus is a brain structure endowed with particularly high levels of receptors for both the gonadal and adrenal steroidal hormones (Hojo et al., 2011; McEwen et al., 1993, 1994; McEwen and Woolley, 1994), making this structure particularly sensitive to gonadal and stress hormone fluctuations. In addition, ghrelin, which rises systemically and centrally during FR, enhances hippocampal function, increases spine density within the hippocampus, generates LTP, and stimulates neurogenesis in the adult hippocampus (Chuang and Zigman, 2010).

2.3.2. Growth

For the sensory cortex, synaptic plasticity is most robust during the juvenile developmental stages of synaptogenesis and pruning (Aoki and Erisir, 2014; Ramaswamy and Markram, 2015). As for the hippocampus, there is now evidence to indicate that their neurons continue to undergo robust synaptogenesis during adolescence, accompanied by synapse pruning. Dendritic arbors of pyramidal neurons in the ventral CA1 hippocampus more than double in branching over a period of a week during adolescence, then return to the level of branching complexity seen prior to the growth spurt (Fig. 2). This is paralleled by a 50% increase in the proportion of dendritic spines in stratum radiatum (SR) that are mature (mushroom and stubby spines) (Chowdhury et al., 2014c) (Fig. 2). Conversely, dendritic branches of pyramidal neurons in the dorsal CA1 hippocampus exhibit a 37% retraction during adolescence (Chen et al., 2015a). The changes in both the dorsal and

ventral hippocampus are transient, returning to pre-pubertal levels by the time animals reach late adolescence. These dramatic increases and retractions may have been missed in previous anatomical studies, due to sparsity of time points sampled during adolescence (Cintra et al., 1997; Harris et al., 1992; Pokorny and Yamamoto, 1981a, 1981b). Furthermore, because the changes are in the opposite directions for the dorsal versus ventral hippocampus, the developmental changes may have been missed, if the entire hippocampus was analyzed with the assumption that it is a homogeneous functional unit. We will discuss below (Section 3.4) what functional significance may be attributed to the changes in opposite directions across the rostral-dorsal versus caudal-ventral sectors of the rodent hippocampus.

The hippocampus is required for the generation of the cognitive map, and this, in turn, is created through an ensemble of hippocampal place cells. These hippocampal place cells fire in relation to a specific place that the animal occupies within space. The only developmental study on these place cells indicates that well-defined CA1 place cells in rats do not develop until postnatal day (P) 50, near the end of adolescence (Martin and Berthoz, 2002). In comparison, spatial abilities, such as searching behavior, emerge around P35–40 (Schenk, 1985). These developmental changes in the hippocampus are in sharp contrast to the sensory cortex, where neurons attain adult-like tuning, although still malleable, by around P21. The delayed maturation of the hippocampus is also in contrast to the cingulum, where head-direction cells are detectable as early as P30 (Martin and Berthoz, 2002).

Presumably, since sensory afferents define a place field, those afferents must first mature in order for the postsynaptic place cells to attain maturity.

2.3.3. Anxiety regulation

The hippocampus has received relatively less attention in studies of anorexia nervosa, in part, because anorexia nervosa is an eating disorder, and the hippocampus is not part of the brain's feeding center. Many more investigators are studying the relationship between anorexic behavior and chemical transmissions occurring within the hypothalamus, the brain's feeding center (Kaye et al., 2009, 2011) or of the involvement of neurotransmitter systems in behavior related to feeding and reward (Compan et al., 2012; Hammer et al., 2009; Verhagen et al., 2009). Prefrontal cortical activity is strongly implicated in individuals' food choices and decisions to eat or exercise (Ehrlich et al., 2015; Kaye et al., 2009). Involvement of the frontal-striatal pathway for the persistence of maladaptive (low-fat) food choices by individuals with anorexia nervosa has also been discovered recently (Foerde et al., 2015). However, anorexia nervosa is not only an eating disorder. It is also an anxiety disorder. Although the amygdala and prefrontal cortex are the brain structures most often considered in relation to anxiety, there is a growing body of evidence for the involvement of the hippocampus (and its ventral sector, especially) in anxiety regulation (Adhikari et al., 2010, 2011; Bannerman et al., 2004; Fanselow and Dong, 2010), such as to control the amygdala's drive in the prefrontal cortex (Thomases et al., 2014). We propose that the role of the hippocampus in anxiety regulation, decision-making (to eat or to run?) and behavioral flexibility, together with its pharmacological attributes (expression of receptors for sex hormones, stress hormones and the growth hormone secretagogue, ghrelin), makes this structure a good candidate as a node that influences an individual's vulnerability to anorexia nervosa.

2.4. Estrogen and progesterone metabolites...risk factors for anorexia nervosa?

Estrogens generally inhibit food intake (Young, 1991), and estrogen receptor beta (ESR2) polymorphism (1082G > A) has been shown to be associated with anorexia nervosa (Eastwood et al., 2002; Rosenkranz et al., 1998). Most reviews consider the role of estrogen in anorexia nervosa, as it interacts with the dopamine, serotonin and neuropeptide systems within brains of individuals with anorexia nervosa (Casper et al., 2008; Kaye et al., 2009). In addition, the metabolite of progesterone, THP (3 α ,5 α [β]-tetrahydroprogesterone, or 3 α OH-5 α [β]-pregnan-20-one), may increase an individual's vulnerability to anorexia nervosa, through a Cl⁻ flux-dependent desensitization of the ionotropic GABA (γ -aminobutyric acid) receptors expressed by CA1 pyramidal cells (Shen et al., 2007; Smith et al., 2009; Wable et al., 2015a).

2.5. Heritability of anorexia nervosa

Twin studies indicate that genetic heritability accounts for 50–70% of the condition of anorexia nervosa (Bulik et al., 2007, 2010; Klump et al., 2001; Wade et al., 2000). The largest reported genome-wide association study (GWAS) of anorexia nervosa was based on 5551 anorexia nervosa cases from 14 countries and 21,080 controls. However, this study led to no genome-wide significance. A suggestive association was indicated for two intronic variants in SOX2OT (SOX2 overlapping transcript) and PPP3CA (protein phosphatase 3, catalytic subunit, alpha isozyme), previously associated with Alzheimer's disease and known to be highly expressed in the brain. Two additional signals were identified only for Europeans descents, located between CUL3 (cullin 3) and FAM124B (family with sequence similarity 124B and near

SPATA13 (spermatogenesis associated 13). Four neurodevelopmental genes regulating synapse and neuronal network formation (SYN2, NCAM2, CNTNAP2 and CTNNA2) and ATP2B1 (ATPase, calcium transporting, plasma membrane 1) were suggestive of involvement for early-onset severe AN cases (Boraska et al., 2014). Without diminishing the socio-cultural risk factors, these genetic heritability studies point to the strong risk for anorexia nervosa that is associated with genes encoding synaptogenesis within the brain.

3. Activity-based anorexia (ABA), an animal model of anorexia nervosa

3.1. General description

The majority of ABA studies have used rats as the animal model (Gutierrez, 2013). Our lab has opted to use mice as well as rats as animal models, in preparation for future studies that will assess gene-based differences in ABA vulnerability (Aoki et al., 2012; Chowdhury et al., 2015a, 2013). For both species, ABA is induced by first acclimating animals to the wheel for 4 days. This is followed by 3–4 days of restricted food access (food restriction, FR). For rats, food access is limited to 1 hour per day. For mice, food access is limited to 2 hours per day (Fig. 3) (Chowdhury et al., 2015a). Within 24 h of wheel access, rats and mice learn to ambulate on the wheel and exhibit voluntary wheel running. Analysis of animals in the wild indicates that wheel running is intrinsically rewarding and not a stereotypy due to captivity: when running wheels are placed in the forest, wild mice repeatedly visit and use the wheel, even without any extrinsic reward, and the bout lengths match those of captive mice (Meijer and Robbers, 2014). Within 24 h of the imposition of FR, captive mice begin to exhibit significant increases (frequently more than two-fold) in voluntary running. The wheel running becomes obsessive, in that animals will choose to run on the wheel, even during the limited hours of food access, hence exhibiting voluntary FR. The animals' extent of wheel running correlates positively with anxiety-like measures on the elevated plus-maze (Wable et al., 2015b). While ABA cannot capture the socio-cultural triggers leading to the first bout of anorexia nervosa, it can serve to shed light on the neurobiological changes that ensue due to starvation, especially among individuals with anorexia nervosa that become hospitalized, due to their: (1) relentless dieting; (2) restlessness and inability to curb excessive exercise; (3) severe weight loss; (4) heightened anxiety.

3.2. Applicability of the ABA model to mice

Previous reports had indicated that the C57BL6 strain, used most commonly as the background to genetically modified mice, show no FR-evoked hyperactivity (Gelegen et al., 2006). However, we have observed FR-evoked hyperactivity in C57BL6 mice, possibly because we are using adolescent females, rather than adult males. Importantly, we observe large individual differences in the extent of FR-evoked hyperactivity, even within single litters of C57BL6 mice. Because of this large variance, a statistically significant change in wheel running following FR may have been difficult to detect. In our opinion, the individual differences in vulnerability to ABA can provide valuable clues about the molecular, subcellular, cellular, network and pre-adolescent rearing conditions that contribute to ABA vulnerability and resilience.

3.3. Developmental changes to the dendritic arbors of hippocampal pyramidal neurons by ABA, exercise and FR

Are the growths and retractions of dendritic branches that

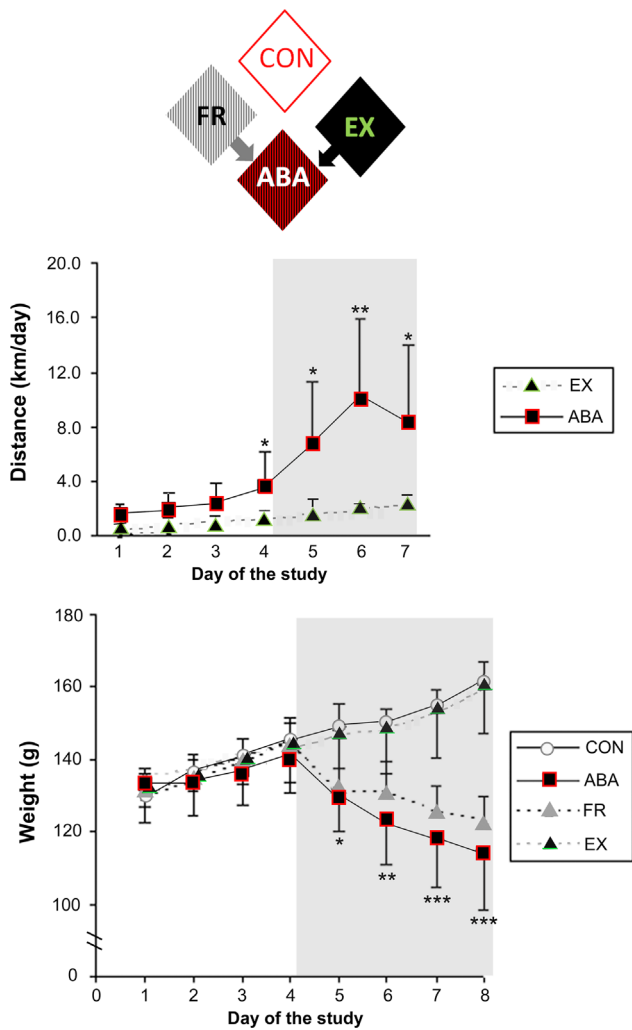


Fig. 3. The ABA paradigm. Top panel. Food restriction (FR), when combined with wheel access to allow voluntary exercise (EX), triggers hyperactivity. This phenomenon is called activity-based anorexia (ABA), because animals exposed to the combination of FR and EX run excessively, even during the limited hours of food availability. Middle Panel. The sudden change in wheel running is shown for the ABA group of adolescent female rats, quantified as an average daily running of nine ABA animals that were exposed to FR (shaded region), after 4 days of acclimation to the wheel without FR. In comparison, animals exposed to the wheel for 7 days without any food restriction show only a modest increase in daily running. Bottom Panel. Body weight of these animals are strongly affected by the environment. Without wheel access or food restriction (CON), adolescent females continue to show daily increases in body weight across 7 days of the study (P37 to P44). Those with wheel access (EX) show slightly lowered body weight, but the difference from CON is not statistically significant. The animals that are subjected to FR+ wheel access (=ABA) show highly significant weight loss, compared to CON's. However, their body weights are not significantly different from those of the FR group that were without wheel access. In addition to measuring the daily running activity, the ABA model can be used to assess the animal's entrainment to the feeding schedule, by measuring the food anticipatory activity (FAA) that spans the hours preceding the restricted food access hours or of the animals' extent of running during the hours of food access, to measure how well animals suppress the wheel running activity, so as to maximize feeding. See Fig. 5, bottom, for examples of how these additional measurements were employed to uncover the neurochemical bases for entrainment to the restricted feeding schedule. These data were taken from a publication (Aoki et al., 2012) and modified from a figure appearing in that publication. Reproduced with permission from the publisher.

occur during adolescence subject to experience-dependent perturbations? Our analysis of Golgi-stained neurons indicates that they are (Chowdhury et al., 2014c) (Fig. 4). The adolescent period for rats spans from around P32 to P35 (marked by vaginal opening) to P55 (characterized by the establishment of a more regular estrous cycle) (Frisch et al., 1975, 1977, also reviewed by Piekarski

et al., in this issue). Following an ABA induction, consisting of four days of FR, from P40 to P44, overlapping with 8 days of wheel access (from P36 to P44), apical dendrites of pyramidal neurons in the CA1 of the dorsal hippocampus exhibit a 35% decrease in total length. This is due mainly to a 39% decrease of dendritic branching in stratum radiatum (SR), corresponding to the portion where Schaffer collaterals from the CA3 form excitatory synapses. Since this change is not accompanied by a compensatory increase in spine density, the loss of dendritic branches in the dorsal hippocampus equates to the loss of CA3-to-CA1 synapses. This change is not likely to be attributable simply to malnutrition, since the ventral hippocampus exhibits a change in the opposite direction. In the ventral hippocampus, dendritic lengths in SR are increased by 70%, contributing to the increased branching in SR by 44%, again with no change in spine density, indicating a substantial increase of excitatory synapses formed between CA3 afferents to CA1 pyramidal cells. The effect of ABA appears to be to induce precocious maturation of the apical dendrites in the ventral hippocampus, since the dendritic exuberance observed at P44 by the ABA animals is similar to the pattern observed a week later in the ventral hippocampus of control animals that were never exposed to FR or a wheel (Chowdhury et al., 2014c). This interpretation is supported by data on spine morphology: more of the spines of the P44-ABA animals appear mature (mushroom and stubby spines, rather than filopodia), compared to those of age-matched controls (P44-CON) but are similar to the proportion observed a week later in the hippocampus of controls (P51-CON) (Chowdhury et al., 2014c).

In order to determine whether the changes in dendritic arbors were evoked by FR, exercise or by their combination (i.e., ABA), we also analyzed the effect of FR and exercise, alone, upon age-matched cohorts of rats. A two-way ANOVA revealed that the contrasting effect of ABA across the dorsal versus ventral hippocampus in SR is attributable to the main effect of exercise. Exercise, alone, can evoke a reduction of dendritic arbors in the dorsal hippocampal CA1 and an increase of dendritic arbors in the ventral hippocampal CA1. These observations suggest that the balance between the ventral and dorsal hippocampus becomes altered by exercise, in favor of the ventral hippocampus for through-put of the hippocampal trisynaptic circuitry via excitatory synapses in SR (Andersen et al., 2007).

FR is not without its own effects. Rather, CA1 of animals that have undergone FR, alone, exhibit retraction of dendritic arbors consistently across the dorsal and ventral hippocampal CA1 in stratum lacunosum-moleculare (SLM). This stratum contains afferents from the entorhinal cortex that form excitatory synapses onto distal dendrites of CA1 pyramidal cells. These afferents strongly potentiate excitatory synapses in the more proximal SR, depending on the relative timing of synaptic inputs across the two strata (Spruston, 2008). Pushing the interpretation further, it is possible that FR renders CA1 cells' excitability to become less receptive to entorhinal cortex's direct modulation of CA1 cells, as the CA1 cells respond to the excitatory input via the tri-synaptic circuitry within the hippocampus.

Previous studies of adult hippocampus have noted alterations in dendritic spine density for the CA1 cells in response to fluctuating levels of gonadal hormones, but without changes to dendritic branching patterns (Woolley et al., 1990; Woolley and McEwen, 1993). Running has also been shown to induce structural changes to adult pyramidal neurons, including the branches of CA1 pyramidal neurons, but only after a much longer period of running (2 months) (Stranahan et al., 2007). Short-term glucocorticoid manipulations (3 or 7 days) influence the survival and morphology of neurons in the adult dentate gyrus, without altering the morphology or survival of pyramidal cells in the CA1 (Gould et al., 1990). In comparison with these reports revolving the adult

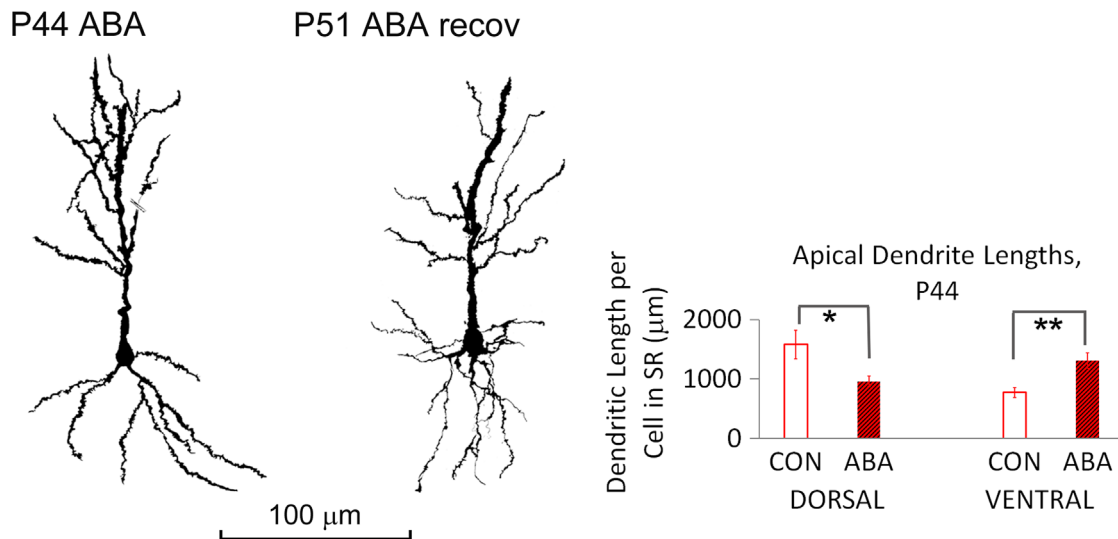


Fig. 4. Pyramidal neurons of adolescent hippocampus are highly susceptible to environmental perturbations. Adolescent female rats were subjected to 4 days of FR in an ABA paradigm (FR + wheel access). At the end of the 4th day of FR, on postnatal day 44, brains were collected and stained by the Golgi method. An example of the CA1 pyramidal neuron from the ventral hippocampus of this cohort is shown (P44 ABA, left cell). Other animals underwent a 7-day recovery phase, whereby FR was converted to *ad libitum* food access, in the absence of a wheel. Their body weights restored within 2 days. Brains from recovered animals were collected at P51 (P51 ABA recov, right cell). Analysis of pyramidal neurons of P51 ABA recov showed no difference in dendritic branching in the ventral hippocampus, relative to those of age-matched P51 animals that were never exposed to ABA (not shown). Apical dendrites of hippocampal CA1 at P44 spanning SR and SLM underwent Sholl analysis, using NeuroLucida, to quantify dendritic branching and dendritic lengths. This quantification revealed significant (* indicates $p=0.03$), 39% retraction of dendritic branches within SR of the rostral-dorsal CA1 ("DORSAL" in the graph), after 4 days of ABA. In contrast, dendritic branches in SR of the caudal-ventral CA1 ("VENTRAL" in the graph) exhibited a highly significant (** indicates $p=0.002$), 70% increase, following 4 days of ABA. These expanded ventral pyramidal neurons of P44-ABA animals were indistinguishable from those of the P51-CON, indicating that 4 days of FR + wheel (from P40–44) greatly accelerated development. These observations indicate that pyramidal neurons of adolescent hippocampus are strongly affected by environmental factors, even after a short period of 4 days, and that these changes are distinct for the dorsal and ventral hippocampus. Moreover, severe weight loss that is associated with FR, whether with or without wheel access, do not cause irreversible atrophy of hippocampal neurons. These are part of the data that appear in publications (Chowdhury et al., 2014a, 2014c). This figure was modified from a figure of a book chapter (Aoki and Erisir, 2014).

hippocampus, the morphological changes we report for the adolescent CA1 are more pronounced (changes in dendritic branches, in addition to spine morphology) and more rapid (4 days of FR or within 8 days of wheel access). We think these differences reflect the greater structural plasticity of adolescent hippocampal neurons that accompany the growth spurt that recurs during this last phase of neural development.

3.4. Perturbation of hippocampus-dependent behavior by ABA, in light of the known functional segregation of the dorsal versus ventral hippocampus and the morphological changes in the hippocampus

Previous work involving lesions of the dorsal versus ventral hippocampus indicate that the dorsal hippocampus is functionally linked to spatial cognition, while the ventral hippocampus is linked to anxiety regulation and less so to spatial cognition (Fanselow and Dong, 2010). This notion is most strongly influenced by the earlier works of Moser et al. (1993) and Bannerman et al. (2004). They observed that lesion of the dorsal hippocampus impairs spatial cognition tasks, such as a rat's performance on the Morris water maze (MWM), without altering basal (trait) anxiety. Conversely, lesions of the ventral hippocampus spared spatial cognition tasks, such as the MWM, but reduced unconditioned anxiety traits. This notion is supported by more recent works, showing that the ventral hippocampus fires more synchronously with the amygdala (Likhtik et al., 2014) and the prefrontal cortex (Adhikari et al., 2010) than does the dorsal hippocampus during animals' exposure to anxiogenic environments. Anatomically, the ventral hippocampus is bi-directionally connected with the prefrontal cortex and the amygdala, while the dorsal hippocampus has no direct connection with either of these structures (Fanselow and Dong, 2010). On the other hand, the entrainment of hippocampal theta to the prefrontal cortical theta may require

preservation of the pathways connecting the dorsal and ventral hippocampus (Spellman et al. (2015) and personal communication with Josh Gordon). Therefore, the dorsal hippocampus may have 'access' to and modulate learning that involves avoidance of anxiety-inducing spatial cues.

If lesion of the ventral hippocampus reduces trait anxiety, then expansion of the dendritic arbors of the ventral hippocampus, as was observed among the ABA animals, might be expected to generate an opposite effect - increased anxiety. Indeed, mice subjected to ABA increase wheel running much more than do mice exposed to a wheel without FR, and the extent to which ABA mice increase wheel running correlates tightly with anxiety measures on the elevated plus maze that are conducted during the FR phase (Wable et al., 2015b). As for this cohort of rats, we only have measurements of anxiety-like behavior during recovery - i.e., 2–3 days after the FR phase and 9–10 days after the FR phase. These animals do not exhibit increased anxiety. Instead, a two-way ANOVA indicates that the main effect of exercise (a treatment shared by the EX-only group and ABA) is anxiolysis (i.e., greater exploration in the open field) that emerges 9 to 10 days after the FR phase, as the animals are approaching the end of the adolescent period (P53–54) (Chowdhury et al., 2014b, 2015b).

Retraction of dendritic branches in the dorsal hippocampus might be expected to reduce rats' ability to perform in spatial cognition tasks. We tested this hypothesis by challenging animals to learn a spatial cognition task, called active place avoidance, whereby animals must learn to avoid an electrified sector by using distal room cues and ignoring proximal cues associated with a rotating arena (Cimadevilla et al., 2001; Pastalkova et al., 2006). This was followed by a test for retention on the next day and cognitive flexibility, whereby animals had to learn to associate the same distal cues and a new electrified sector. This behavioral task was subjected to four groups of animals: ABA, FR only, exercise only and naïve controls 3–4 days after the respective treatment

ended. To our surprise, the ABA animals were not significantly impaired, compared to age-matched naïve control animals. Moreover, when allowed to recover for 9–10 days, rather than just 3–4 days, ABA animals, together with the FR-only animals, outperformed the CON and the exercise-only cohorts, indicating the main effect of FR. No difference in their retention, cognitive flexibility or anxiety was detected between any of the groups of rats (Chowdhury et al., 2014b, 2015b).

The good news is that ABA during adolescence does not impair hippocampal function. We offer three explanations for this outcome, which are contrary to expectations, based on morphological data. Clearly, more research is required to test these ideas:

- 1) The reduced branching of neurons in the dorsal hippocampus would be expected to increase membrane resistance, if no change occurs to the channels dictating resting membrane potential or excitability. This increase in membrane resistance would have an effect of increasing the amplitude of the excitatory post-synaptic potential evoked for the same amount of incoming currents (Ohm's law, $V_m = I * R_m$). This could have compensated for the reduction of afferent excitatory inputs, allowing each of the remaining afferent inputs to exert more excitatory influence upon excitability of dorsal hippocampal neurons. This compensatory mechanism explains the lack of spatial cognitive impairment 3–4 days after ABA, in spite of the reduced dendritic branching in the dorsal hippocampus.
- 2) The improved cognitive performance that emerged with a delay among the ABA and FR-only groups, relative to that of the CON group, suggests the involvement of a slower, growth-like maturational process. The expanded dendritic arbors of the ventral hippocampus could have contributed to enhanced excitatory drive to the prefrontal cortex and amygdala, thereby increasing the animal's sensitivity to the unconditioned stimulus (electric shock) and fear evoked by the unconditioned stimulus. Both of these are parameters known to strengthen learning (Shettleworth, 2010).
- 3) Although the impact of FR upon cognitive performance during adolescence has not been studied before, its impact upon adult brains has been reported to enhance adult hippocampal neurogenesis, possibly through the cooperative effect of increasing BDNF and corticosterone levels in the brain (Mattson, 2000; Stangl and Thuret, 2009). Although neurogenesis does not result in net gain of neuron number, the newly born neurons are relatively more important for the maintenance of synaptic plasticity in the adult hippocampus (Gould and Gross, 2002). It is possible that the delayed cognitive improvement observed among the FR-only and ABA animals reflects increased plasticity, beyond what is observed for the adolescent hippocampus, due to boosting of neurogenesis in the dentate gyrus.
- 4) Although previous Golgi analysis of neurons indicated differential changes to the dendritic arbors of the dorsal and ventral hippocampus immediately following ABA induction, these anatomical changes may not have persisted after 9–10 days of recovery. This is based on our observation that 7 days of recovery from ABA (P51 ABA recov in Fig. 4) is sufficiently long to eliminate the anatomical differences between the ABA and CON groups' ventral hippocampus (unfortunately, we have not conducted Golgi analysis of the dorsal hippocampus of the ABA group after recovery) (Chowdhury et al., 2014c). It is likely that adolescent hippocampal neurons exhibit greater plasticity than adults', and this allows for normalization of ABA-induced changes. Such normalization does not preclude the possibility that molecular changes at synapses persist beyond restoration of body weight, dendritic branches and synapse number.
- 5) The improvements in cognitive function among the ABA and FR-only animals that emerge 9–10 days after ABA may be a

response to the cognitive challenge during the critical period of adolescence, prompting animals to create a model of the external world that is based on experience (Rescorla, 1988), such as the "idea" that food is not going to be available all the time anymore, that food will become available during the first hour of the dark phase only, but of unlimited amounts. In accordance with this view, we observed a tight correlation between the animals' performance in spatial learning and their food anticipatory activity 10 days earlier, measured by the increase in wheel running during the 6 h preceding the hour of food access (Chowdhury et al., 2014b, 2015b).

Individuals with anorexia nervosa are characterized as perfectionistic, high-achieving and goal-oriented (Beals, 2004), even after recovery. Association of a single nucleotide polymorphism (SNP) (C-521T) in the dopamine D4 receptor with perfectionism has been identified for individuals with AN and control healthy subjects (Bachner-Melman et al., 2007). Although the rats used in our study are not expected to carry the C-521T SNP, individual differences in the expression of dopamine receptors or of dopaminergic afferents may have enabled some to become more entrained to the feeding schedule than others. Dopaminergic modulation of the striatum has been shown to be required for entrainment of animals to a feeding schedule (Gallardo et al., 2014) and dopaminergic signaling at the cellular and behavioral levels are potentiated by FR (Carr, 2011). Since the dopaminergic potentiation of behavior includes conditioned place preference, which is a spatial cognition task requiring hippocampal connectivity to the prefrontal cortex and nucleus accumbens (Ferbințeanu and McDonald, 2001), it is possible that dopaminergic modulation of the hippocampus or of its connectivity to the prefrontal cortex contributes to better spatial cognitive performance of the FR-only and ABA rats.

3.5. ABA resilience relates to differences in the responsiveness of the hippocampal GABAergic system

Relapse rate among individuals with anorexia nervosa is unacceptably high – 25% (Hudson et al., 2007; Steinhilber, 2002). This rate is especially grave, since mortality rate of 0.56% deaths per year is evaluated among those that have been diagnosed at least once with the condition of AN (Sullivan, 1995).

We have modeled the anorexia nervosa relapse by exposing rodents to two bouts of ABA induction during adolescence, separated by 1 week of recovery. One might argue that this fails to capture the nature of anorexia nervosa relapse, because FR is imposed, rather than voluntary. We agree with this criticism but also recognize that the double exposure to ABA induction captures the neurobiological changes that ensue during the conversion of voluntary FR (in humans) to inevitable continuation of FR, as is observed for starving rodents that continue to run on the wheel, even during the hours of food access.

We observe greater variance in the animals' response to the second ABA induction, compared to the first (Fig. 5) (Chowdhury et al., 2013). During the first ABA induction, the majority of C57BL/6 adolescent females become hyperactive and exhibit running even during the hours of food access. In comparison, FR-evoked hyperactivity is seen among half of the animals following the second ABA, while the remaining animals exhibit reductions in wheel running. This suppression of wheel running is adaptive, because it conserves energy and spares them from death. Conversely, those animals that resume their FR-evoked hyperactivity must be removed from the FR+wheel environment within four days, in order to prevent their death (Chowdhury et al., 2013). These differences in FR-evoked wheel activity correlate with differences in the GABAergic innervation of pyramidal neurons in the dorsal

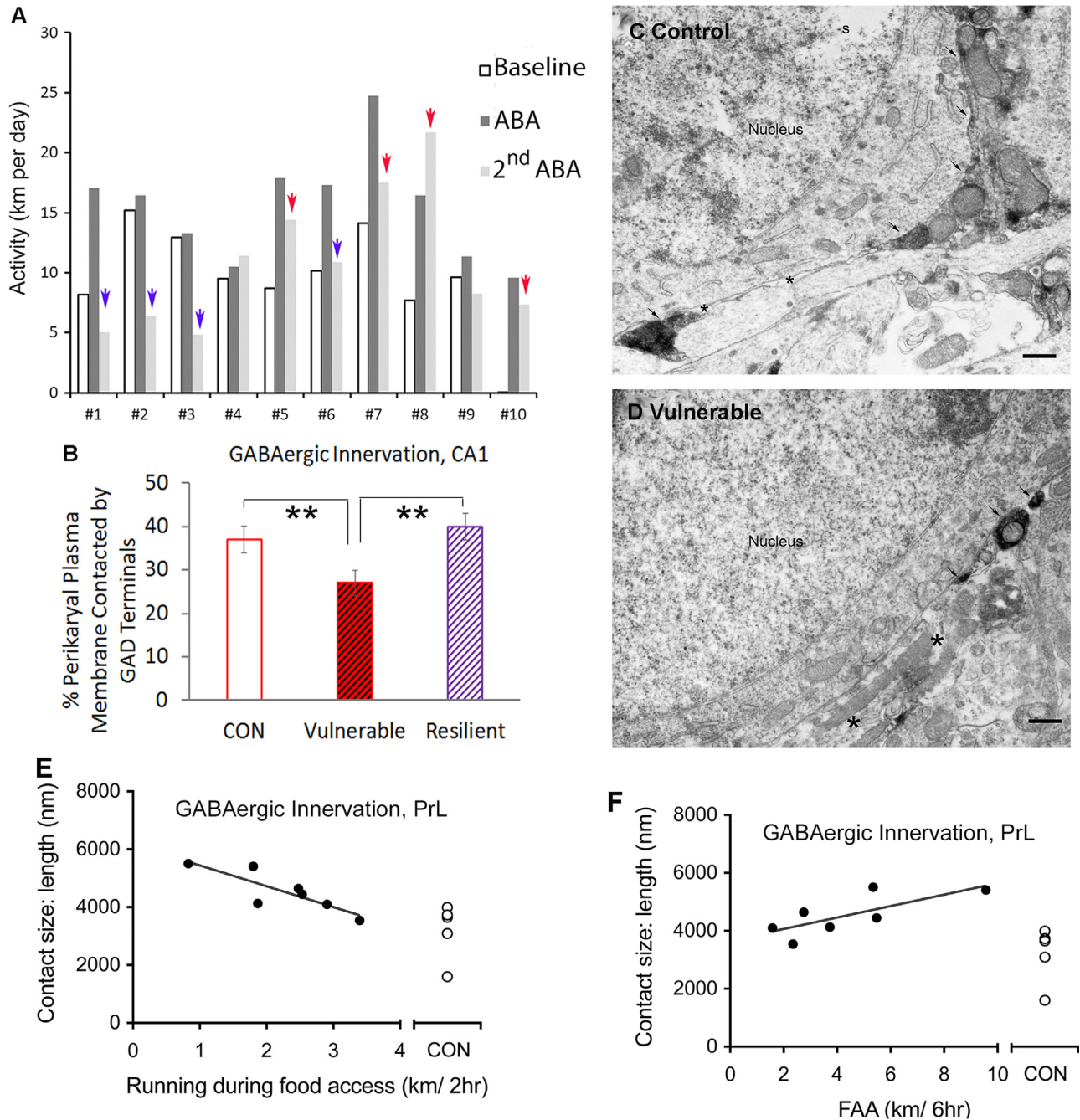


Fig. 5. GABAergic innervation of pyramidal neurons are highly variable and correlate with individual differences in resilience to ABA. Adolescent female mice were the subjects of this study. These animals underwent two episodes of ABA induction during adolescence, separated by a week. These animals showed great variability in the FR-evoked running, which can be quantified in many ways. Panel A. FR-evoked running was compared by measuring each animal's average running per day during the last two of the three FR-days of the first ABA and during the first three of the four FR-days of the second ABA. We categorized animals as vulnerable and hyperactive, if they exhibited FR-evoked increase in running during the second ABA, relative to the baseline running measured before the first ABA (red arrows). Animals were categorized as resilient, if the FR-evoked running during the second ABA was equal to or less than the baseline running (blue arrows). All animals were euthanized after four FR-days of the second ABA. Panel B. The bar graph in panel B shows the mean \pm SEM values of the percent of cell body plasma membrane lengths in the hippocampal CA1 that are directly contacted by GAD-immunoreactive axon terminals. This axo-somatic GABAergic innervation was reduced significantly for those neurons from animals categorized as vulnerable, relative to the neurons from the hippocampus of resilient and CON animals (** depicts $p < 0.005$). Panels C and D. Examples of electron micrographs depicting GABAergic innervation of pyramidal neurons in the dorsal hippocampal CA1. Panel C shows an example from a CON animal that experienced no ABA but was singly housed for equivalent periods as the ABA animals. Panel D shows an example from an ABA animal (#7 in panel A) that exhibited the highest FR-evoked activity during the first ABA and persistent hyperactivity during the second ABA. HRP-DAB labeling depicts immunoreactivity to glutamic acid decarboxylase (GAD), the rate-limiting synthetic enzyme for GABA. Axo-somatic synaptic junctions formed by the GAD-positive axon terminals are indicated by black arrows. Asterisks indicate the cytoplasm of astrocytic processes that envelope perikarya of pyramidal neurons where axo-somatic synapses are absent. Calibration bars = 500 nm. Panels E and F. FR-evoked hyperactivity can be quantified in additional ways: the extent to which they continue to run, even during the 2 h of food access during the FR days (panel E) and the food anticipatory activity (FAA) that they exhibit during the 6 hours preceding the scheduled feeding time (panel F). These running activities show reversal of correlation with axon terminal sizes impinging upon layer 5 pyramidal neurons in the prelimbic area of the medial prefrontal cortex (PrL). The greater the contact size of the GABAergic axon terminals, the more that they are able to suppress running during the hours of feeding ($p < 0.05$ by Pearson correlation). The more that they anticipate the scheduled feeding period, indicating entrainment to the feeding schedule, the larger are the GABAergic contact sizes (panel F). These data were taken from (Chen et al., 2015b; Chowdhury et al., 2013). Figures were modified from published sources (Chen et al., 2015b; Chowdhury et al., 2013). Reproduced with permission from the publisher.

hippocampal CA1 (Chowdhury et al., 2013) and in the medial prefrontal cortex (Chen et al., 2015b) (Fig. 5).

Besides this synaptic mechanism, a second non-synaptic GABAergic mechanism is also recruited to modify excitability of CA1 pyramidal neurons. This involves the up-regulation of $\alpha 4\beta\delta$ -GABA_A receptors (GABA_AR) that occur adjacent to excitatory synapses formed onto dendritic spines in SR of the CA1. Quantitative electron microscopic immunocytochemistry reveals that the more that animals were able to suppress wheel running, the higher the frequency of $\alpha 4\beta\delta$ -GABA_AR occurring adjacent to excitatory axo-spinous synapses of the dorsal hippocampal CA1 pyramidal cells (Wable et al., 2015a) (Fig. 6). Since wheel running correlates with anxiety levels (Wable et al., 2015b), the suppression of wheel running by individuals exhibiting up-regulation of $\alpha 4\beta\delta$ -GABA_AR suggests that these receptors are mediating anxiolysis through increased inhibition of the hippocampal pyramidal cells. This may not seem surprising, given the widely recognized role of GABA agonists as anxiolytic agents. However, this relationship between $\alpha 4\beta\delta$ -GABA_AR levels and anxiety is also contrary to expectation, because the rise of these receptors specifically at puberty and in the CA1 has been shown to underlie the stress-evoked anxiety upon female mice (Shen et al., 2007). The significant difference between those pubertal mice and the ABA-induced mice could be

the level of circulating THP. For pubertal mice, it is the circulating THP that desensitizes $\alpha 4\beta\delta$ -GABA_AR expressed by the CA1 pyramidal cells, specifically at puberty (Shen et al., 2007). This desensitization leads to increased excitability of pyramidal neurons and increased expression of anxiety-like behavior (Shen et al., 2007). In contrast, the $\alpha 4\beta\delta$ -GABA_AR expressed by the CA1 pyramidal neurons of ABA animals would not be expected to be desensitized, due to the greatly lowered levels of gonadal hormones associated with starvation (Bruni et al., 2000; Dellava et al., 2011; Kaye et al., 2009; Lucas et al., 1991) and with it, the depletion of progesterone's metabolite, THP (Frye et al., 2000). This idea was further supported by our most recent observation, namely that administration of progesterone converted the resilient ABA-mice to become vulnerable. This fits with the idea that resilience, prior to progesterone treatment, was conferred through the up-regulation of $\alpha 4\beta\delta$ -GABA_AR expression by the CA1 pyramidal cells, while their conversion to becoming vulnerable was associated with the desensitization of the $\alpha 4\beta\delta$ -GABA_AR by exogenous progesterone (Wable et al., 2015a).

How quickly do these GABAergic adaptive changes occur? This question was addressed by analyzing brains of ABA-induced animals at earlier time points after FR have begun. Analysis of the hippocampus immediately at the end of the first ABA (after 4 days of FR) (Aoki et al., 2014b) revealed that some animals expressed levels of $\alpha 4\beta\delta$ -GABA_AR that were nearly five-fold those of controls, while others exhibited levels no different from controls. Moreover, there was, again, a strong correlation between the animals' FR-evoked running and the level of $\alpha 4\beta\delta$ -GABA_AR at spine membranes immediately adjacent to excitatory axo-spinous synapses (Fig. 2B in Aoki et al. (2014b)), with the most resilient animals having the most increased levels of $\alpha 4\beta\delta$ -GABA_AR. When examined after only 2 days of FR, we, again, observed a strong correlation between $\alpha 4\beta\delta$ -GABA_AR levels and the extent of the animals' suppression of FR-evoked running. However, approximately half of the $\alpha 4\beta\delta$ -GABA_AR resided in the spine cytoplasm, rather than at the plasma membrane (Fig. E in Aoki et al. (2014b)). We interpret the cytoplasmic $\alpha 4\beta\delta$ -GABA_AR to be representative of the reserve pool, available to be mobilized to the plasma membrane, as environmental stressors (such as FR) are prolonged from 2 days to 4 days. Those animals with minimal weight loss following 4 days of FR – i.e., the most resilient to ABA induction – were the same ones that form the most extensive GABAergic axo-somatic inhibitory synapses upon pyramidal neurons of the dorsal CA1 (Aoki et al., 2014a).

To our surprise, we observed no detectable change in GABAergic innervation of dendritic spines, where we observed an increase of $\alpha 4\beta\delta$ -GABA_AR. This indicates that $\alpha 4\beta\delta$ -GABA_AR are activated primarily by ambient GABA, which is estimated to be 1 μ M, rather than the phasically released GABA that are precisely targeted to GABAergic synaptic junctions (see Shen et al. in this issue for further discussion about this point). Together, these findings indicate that the ABA-resilient animals can begin to utilize two GABAergic mechanisms to dampen excitability of CA1 pyramidal neurons – increase $\alpha 4\beta\delta$ -GABA_AR for shunting inhibition within 2 days and/or increase GABAergic innervation of pyramidal cells – within 4 days.

3.6. Perturbations to excitatory synapses in the hippocampus by ABA

Neuronal excitability is determined by the strengths of both excitatory and inhibitory inputs. Since excitatory axon terminals target dendritic spines of pyramidal neurons, one can estimate the extent of excitatory input upon pyramidal neurons by assessing their spine density and lengths of dendritic branches. We looked for correlations between dendritic branching patterns, spine density and resilience/vulnerability to ABA and did not find any.

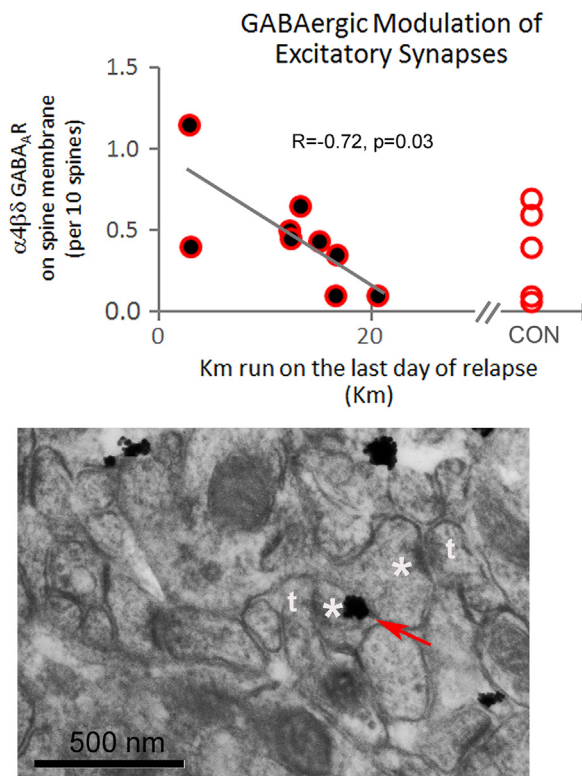


Fig. 6. $\alpha 4\beta\delta$ -GABA_AR expression at excitatory synapses are highly variable and correlate with individual differences in resilience to ABA. The hippocampus of the same animals shown in Fig. 5 were subjected to electron microscopic analysis of $\alpha 4\beta\delta$ -GABA_AR immunoreactivity at dendritic spines in SR of the hippocampal CA1. As shown in the electron micrograph, $\alpha 4$ subunits of $\alpha 4\beta\delta$ -GABA_AR (red arrow) occur at the plasma membrane of dendritic spines forming asymmetric axo-spinous synaptic junctions, evident by the thick postsynaptic density (asterisks) and vesicles within the adjacent terminal (t). In this example, the $\alpha 4$ -immunoreactivity is associated with the spinous plasma membrane of one or both spines forming axo-spinous excitatory junctions. Graph: FR-evoked running was compared across animals by measuring the distance run on the fourth food-restriction day, corresponding to the last day before euthanasia. This value is strongly correlated with $\alpha 4\beta\delta$ -GABA_AR immunoreactivity at the plasma membrane of dendritic spines. Data from another cohort of adolescent female mice with two ABA exposures appear in (Wable et al., 2015a). The electron micrograph also appeared in the article by Wable et al., 2015a and is reproduced here, with permission from the publisher.

Neuronal excitability could still be altered by changes in the local concentration of glutamate receptors at spines. Therefore, we have also analyzed the level of expression of NMDA receptors (NMDARs) at dendritic spines in SR, which is where $\alpha 4\beta\delta$ -GABA_AR expression increases in the resilient individuals after ABA induction. Our rationale for beginning with the analysis of NMDARs, rather than AMPA receptors, was that NMDARs contribute more significantly to the firing property of pyramidal neurons (Palmer et al., 2014) and to long-term synaptic plasticity. NR2A and NR2B subunit levels occurring precisely at post-synaptic membranes were assessed (Klingensmith et al., 2015, Chen et al., under review), using the post-embed immunogold approach (Aoki et al., 2009). These analyses indicated that ABA induces a 34% increase in NR2B levels and a 21% increase in the proportion of spines with detectable levels of NR2B at the postsynaptic plasma membrane of spines within 4 days of FR under the ABA paradigm. Moreover, these increases were associated with the extent to which the animals lost weight by the third day of FR, with greater NR2B levels detected among individuals with more weight loss. NR2A levels also augmented significantly, by 38%. The association between NR2B levels and weight loss was specific to the ABA group, and not for the FR group, even though the weight loss was not significantly different between the two groups. This suggests that the greatly exaggerated wheel running that is provoked by FR may have triggered the up-regulation of NR2B-containing NMDARs which, in turn, contributed to the elevated anxiety. Since the individuals with increases in NR2B levels overlap with the individuals that fail to up-regulate $\alpha 4\beta\delta$ -GABA_AR expression, both the GABA and glutamate neurotransmitter systems are likely to contribute additively or synergistically to ABA vulnerability (Fig. 7).

We have also noted that the non-synaptic cytoplasmic NR2A levels are elevated the most within spines of individuals with the greatest resilience to ABA, characterized by the minimal increase

in FR-evoked, exaggerated wheel running. This suggests that a mechanism exists to suppress the insertion of NR2A-NMDARs from the cytoplasm to the post-synaptic plasma membrane, thereby minimizing excitability of hippocampal neurons, ultimately contributing towards ABA resilience. Since levels of cytoplasmic NR2A and post-synaptic NR2B are both correlated to the level of an F-actin binding protein, drebrin, within spines, we propose that this cytoskeletal protein may be participating in the homeostatic control of NMDAR-mediated excitability of hippocampal neurons (Chen et al., under review).

Besides modulating excitability, shunting inhibition that is mediated by the $\alpha 4\beta\delta$ -GABA_ARs prevents the membrane depolarization-dependent unblocking of Mg²⁺ from NMDAR channels, thereby reducing the NMDA/AMPA receptor current ratio, synaptic plasticity in the form of LTP and spatial cognition (Shen et al., 2010) (also Shen et al., in this Special Issue). As was discussed above, individuals that have undergone ABA induction exhibit superior performance in active place avoidance tests than the age-matched controls after a 10 day period of recovery. At least some of this difference in performance may be ascribable to the relatively modest $\alpha 4\beta\delta$ -GABA_AR and the relatively greater NR2B-containing NMDARs in SR of the CA1, if these synaptic changes can persist for as long as 10 days.

3.7. Summary

Three synaptic molecules in the hippocampus are closely associated with ABA resilience/vulnerability: NR2B-containing NMDARs at spines of CA1 pyramidal cells in SR; $\alpha 4\beta\delta$ -GABA_ARs at spines of CA1 pyramidal cells in SR; and GABAergic innervation of pyramidal cells. Of these three parameters, $\alpha 4\beta\delta$ -GABA_ARs emerge as the molecule most tightly correlated with the FR-evoked elevation in wheel running ($R = -0.7, p < 0.05$) (Aoki et al., 2014b); On the other hand, NR2B-NMDAR levels are correlated with the ABA animals' extent of weight loss ($R = 0.7, p = 0.05$) (Klingensmith et al., 2015; Chen et al., under review). Therefore, at least for some animals, the ABA-induced increase in excitability of the pyramidal neurons in the CA1, due to the rise of NR2B-NMDAR elevation in the SR may be effectively dampened by the up-regulation of $\alpha 4\beta\delta$ -GABA_ARs, in time to quell the anxiety-evoked hyperactivity and the weight loss that follows from it (Fig. 1C).

The ABA animal model can be used to assess different aspects of the altered state: hyperactivity throughout the day and night that relates to the animal's ABA-induced rise in anxiety (Wable et al., 2015b) and neurochemical changes in the hippocampus; hyperactivity that reflects the animal's entrainment to the feeding schedule, as measured by their food anticipatory activity during the 6 hours preceding feeding and the suppression of running during the hours of food availability, both of which may relate to the animal's ability to create and act upon a working model of the world, via GABAergic inhibition of the prefrontal cortex (Chen et al., 2015b); activity levels that precedes the days of FR, which may relate to the basal (trait) anxiety levels that are unrelated to FR but are nevertheless related to $\alpha 4\beta\delta$ -GABA_AR levels in the hippocampus (Aoki et al., 2014b).

4. Closing remarks

Starting from the foundational works of Torsten Wiesel and David Hubel, much has been learned about the molecular mechanisms underlying developmental plasticity of the brain that is exquisitely dependent on childhood experience. In contrast, relatively less is known about the developmental events during the succeeding developmental stage – adolescence. Our data add to a growing body of evidence indicating that the hippocampus grows

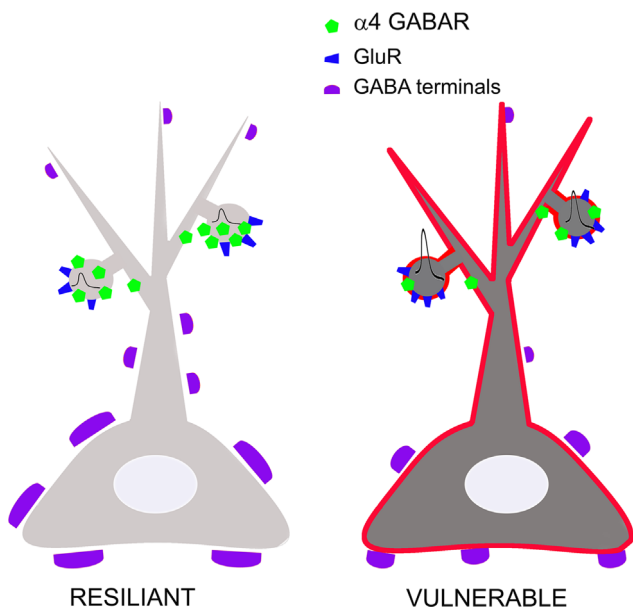


Fig. 7. $\alpha 4\beta\delta$ -GABA_AR expression at excitatory synapses, together with increased GABAergic innervation, counter the elevation of NR2B-NMDAR and the FR-evoked hyperactivity in ABA animals. The convergent effects of three synaptic molecules – GAD, NR2B-NMDARs and $\alpha 4\beta\delta$ -GABA_AR – are hypothesized to alter excitability of pyramidal neurons in the hippocampal CA1 which, in turn, manifest as individual differences in the extent of FR-evoked hyperactivity of female rodents that undergo ABA induction during adolescence. Note that $\alpha 4\beta\delta$ -GABA_ARs expressed by neurons of the resilient animal also occur in the spine cytoplasm. These depict the reserve pool of receptors that can be recruited quickly to the plasma membrane, in response to heightened excitatory input. This figure was modified from a published figure (Wable et al., 2015a).

extensively during adolescence and that the growth there is strongly influenced by environmental factors.

At the beginning of this chapter, we presented two views regarding the causal-effect relationship between anxiety, FR and hyperactivity. There is strong evidence supporting the view that hyperactivity is an innate behavior evoked even in healthy individuals following the imposition of FR. This view is supported by our observation that easily more than half of healthy laboratory wildtype rodents become hyperactive, following imposition of severe FR. How might this be of relevance to the clinical setting, where individuals with anorexia nervosa succumb to the illness without ever having been forced to FR? In our opinion, at the time of admission to the hospital, individuals with anorexia nervosa that compulsively over-exercise are exhibiting the inevitable, perhaps innate, starvation-evoked hyperactivity and anxiety. The ABA animal model can provide a means to explore behavioral therapy and pharmacological approaches to break the inevitable, compulsive behavior. Our findings indicate that treatments to boost the GABAergic system and more specifically, the $\alpha 4\beta\delta$ -GABARs, together with treatments to dampen the NMDARs might be helpful for putting a break upon the strong, innate behavior, while managing to restore patients' body weight. It has been shown that fluctuating levels of progesterone and its metabolite, THP, up-regulate $\alpha 4\beta\delta$ -GABAR expression in the CA1 (Shen et al., 2007). However, our attempt to up-regulate $\alpha 4\beta\delta$ -GABARs through an exogenous supply of progesterone (Shen et al., 2005) failed to confer protection against ABA vulnerability (Wable et al., 2015a), probably due to THP's second action – desensitization of already existing $\alpha 4\beta\delta$ -GABARs (Shen et al., 2007; Smith et al., 2007, 2009). Another agent known to up-regulate $\alpha 4\beta\delta$ -GABARs and to promote the growth and maturation of GABAergic synapses is BDNF (Jiao et al., 2011; Marty et al., 1996; Mizuno et al., 1994). However, BDNF can exert multiple actions, including the one of dampening GABAergic synaptic transmission and enhancing glutamatergic synaptic transmission (Henneberger et al., 2002; Wardle and Poo, 2003). These latter actions could increase excitability of hippocampal pyramidal neurons, thereby again exacerbating the FR-evoked anxiety. Yet another route might be to develop drugs with specificity for the $\alpha 4\beta\delta$ -GABARs. While there are drugs that can exclude activation of $\alpha 4\beta\delta$ -GABARs, such as the benzodiazepines (Brown et al., 2002), gaboxadol (THIP, 4,5,6,7-tetrahydroisoxazolo [5,4-c]pyridine-3-ol) can be applied at a low concentration *in vitro* (Brown et al., 2002) and *in vivo* (Gulinello et al., 2003) to preferentially activate $\alpha 4\beta\delta$ -GABARs. This treatment has been suggested for treating premenstrual anxiety (Gulinello et al., 2003) and may also be helpful for treating relapse of anorexia nervosa.

We also support the second view, namely that hyperactivity and FR are manifestations of an anxiety disorder. This is based on our observation that there are large individual differences in animals' vulnerability/suppression of wheel running, even within co-housed wildtype litters, and that these differences map onto their pre-FR differences in running (Fig. 2F from the Aoki et al. (2014b)). These behavioral differences suggest that some environmental factor(s) pre-dating the experimental manipulation of ABA may have altered the brain circuit underlying trait anxiety and hyperactivity, both of which contributed to ABA vulnerability. This idea will be tested in future studies that examine the impact of experimentally induced neonatal stress upon trait anxiety and ABA vulnerability during adolescence.

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