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Chapter 15

Activity-Based Anorexia, an Animal Model of Anorexia Nervosa for Investigating Brain Plasticity Underlying the Gain of Resilience

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Abstract

Anorexia nervosa (AN) is a mental illness characterized by continuously severe, self-imposed starvation and intense anxiety, manifested as fear of gaining weight. An increasing number of individuals are diagnosed with AN, especially among men. AN is now recognized to include those serving the military as well. With no accepted pharmacological treatments available, coupled with its high mortality and relapse rates, better understanding of the neurobiological basis of this mental illness is needed. This chapter describes the animal model of AN, called activity-based anorexia (ABA), that captures multiple core features of AN successfully, including voluntary food restriction, heightened anxiety, and excessive exercise, culminating in severe body weight loss. Also described in this chapter is how individual differences in vulnerability to ABA can be quantified. This chapter will include examples of synaptic plasticity measurements that may underlie the gain of resilience, quantified as the suppression of two maladaptive behaviors – excessive exercise and voluntary food restriction. Finally, the chapter will describe potential uses of the ABA model for exploring pharmacological treatments to reduce the maladaptive behaviors elicited in the ABA model.

Key words Food restriction, Food-anticipatory activity, Hippocampus, Prefrontal cortex, Dopamine, Serotonin, GABA, Glutamate, Synaptic plasticity, Rodents

1 Introduction

1.1 The Human Condition of Anorexia Nervosa

Anorexia nervosa (AN) is a mental illness characterized by continuously severe, self-imposed starvation and intense anxiety [1], manifested as fear of gaining weight [2]. Approximately 0.3–0.4% of young women are diagnosed with this grave mental illness [3, 4]. The mortality rate for AN is the second highest of all mental illnesses (10–15%) [5], surpassed only by opioid addiction [6]. The mortality rate is more than 200 times greater than the suicide rate in the general population [7] and has a high relapse rate as well (25%) [3, 8]. In spite of these indications of significant clinical burden, there are at present no accepted pharmacotherapy for AN [9]. For example, comorbidity of anxiety and AN is high [1, 10],

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but anxiolytics, such as benzodiazepines, are of limited efficacy for reducing the maladaptive behavior of food restriction. Body dysmorphia is another comorbidity of AN. Antipsychotics that are used to treat hallucinations are of limited efficacy for reducing obsessions that perpetuate food restriction by patients with AN, although it is modestly helpful in weight restoration [11, 12]. Ketamine, an FDA-approved antidepressant, has been reported to ameliorate the obsessive-compulsive thoughts that propagate restricted eating behaviors [13], even though the more traditional antidepressants, such as the serotonin reuptake inhibitors, are of limited efficacy. These observations reflect the paucity of knowledge linking the etiology of the illness to pharmacological treatments.

Although not listed in the Diagnostic and Statistical Manual of Mental Disorders fifth Edition (DSM-5), excessive exercise is one of the core symptoms of AN. Incessant, excessive exercise exacerbates the severe weight loss associated with food restriction [14–16]. 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 [17]. Many of those individuals who successfully restore their body weight continue to stay on their feet, and this activity pattern contributes toward recurrent body weight loss [18]. Amenorrhea is one other commonly observed symptom among individuals diagnosed with AN, although this condition is no longer considered an essential diagnostic criterion [2].

Analysis of the demographics of individuals with AN provides some clues about the etiology of AN. The onset is almost always during early adolescence and much more prevalent among females (previously reported ratio was 9 females to 1 male, but more recent estimates indicate a ratio of 3 to 1) [3, 19, 20]. AN is higher among those individuals with a pre-existing history of anxiety [21, 22]. These demographics suggest that gonadal hormonal surges at puberty are risk factors, perhaps due to changes in brain regions and synapses that are modulated dually by gonadal hormones and stress hormones [23–27]. As noted above, individuals engaged in and able to tolerate strenuous physical training are also at risk. This may be one reason eating disorders, including AN, are prevalent among individuals in military services [28].

1.2 Activity-Based Anorexia (ABA), an Animal Model of AN

First reported in 1954 [29], activity-based anorexia (ABA) is a widely used rodent model of AN. ABA captures multiple clinical features of AN:

Voluntary food restriction.

Severe weight loss.

Excessive exercise that is elicited by hunger [30].

Heightened anxiety [31, 32].

Cessation of the estrous cycle for females.

Heightened vulnerability during adolescence, compared to early adulthood [15, 30] (also reviewed in [27] and unpublished observations).

These parallelisms between humans and rodents indicate that identification of biological mechanisms underlying these behavioral phenotypes in rodents can provide clues for understanding the condition of AN as well as the gain of resilience that prevents relapse. Indeed, this laboratory has used the ABA model to reveal several forms of synaptic plasticity that are induced by the experience of ABA during adolescence. For example, within brains of animals that exhibited signatures of resilience to ABA, such as the suppression of food restriction-induced hyperactivity, we observed increased expression of $GABA_A$ receptor at excitatory synapses on pyramidal neurons in the hippocampus [33, 27, 34, 35], and at excitatory synapses on inhibitory interneurons in the dorsolateral amygdala [36]. Increased GABAergic innervation of pyramidal neurons in the hippocampus [37, 38] and prefrontal cortex [39] was also observed among animals that exhibited abilities to suppress hyperactivity. Conversely, within hippocampus of animals that exhibited increased signatures of vulnerability to ABA – hyperactivity, the expression of NR2B-containing NMDA receptors at excitatory synapses in the hippocampus was significantly increased [40]. In a study that examined the risk factor of sex, we learned that both sexes of laboratory rodents exhibit similar levels of vulnerability to ABA, but the cellular mechanisms underlying the gain of resilience differ across the sexes [41].

Another chapter in a previous edition of this book [42] provides comprehensive coverage of the methodological details of the rat model of ABA. Multiple review articles also exist, pertaining to the revelations of the underlying neurobiology of AN based on data from the rat model of ABA [15, 27, 43–46] (see more references listed under Subheading 1.6). Our lab has invested in developing the mouse model of ABA. This effort was driven by the hopes that a mouse model could provide additional advantages due to the much wider availability of strains, mutants, and transgenic animals as tools for unraveling the genetic, cellular, and molecular mechanisms underlying individual differences in ABA vulnerability and resilience [37]. Thus, this chapter will describe the mouse model of ABA, with added notations of slight differences between the rat and mouse models of ABA. Moreover, this chapter will also describe methods we use to highlight individual differences in vulnerability to ABA. Finally, we believe that the ABA model is particularly useful for revealing individual differences in the gain of resilience when animals undergo repeated exposures to ABA induction. Thus, we describe the protocols that we have used for repeated ABA

inductions and methods we have developed to quantify the gain of resilience.

1.3 Key Steps for Inducing Activity-Based Anorexia That Capture the Symptoms of AN

Activity-based anorexia (ABA) protocol begins by acclimating animals to a wheel within its home cage, for providing ad libitum opportunity for voluntary exercise. Within this environment, voluntary wheel running distance and duration are measured 24/7, with temporal resolution of 1 min. Following this phase of acclimation, starvation is induced by limiting the hours of food access without limiting the amount of food available. The extent of starvation is aimed to be sufficiently severe so as to induce up to 20% body weight loss within 3–5 days, but not so severe as to cause death by food restriction alone. For rats, limiting the food access to 1 h. per day but of unlimited amount meets this condition. For adolescent mice, access to unlimited amount of food for 2 h per day meets this condition. Interestingly, most adult mice that undergo three episodes of ABA gain the ability to eat sufficiently during the 2 h. to retain its body weight, even in the presence of a wheel.

Curiously and most importantly, after losing body weight due to food restriction, adolescent animals increase the extent of wheel running severalfold. Wheel running becomes so excessive as to continue even during the limited hours of food access. Thus, one is able to quantify the emergent behavioral phenotype of voluntary food restriction. This seemingly maladaptive behavior exacerbates body weight loss far beyond the extent of body weight loss due to food restriction, alone. Unless the wheel is removed and the hours of food access are lengthened, these animals can die, even though food restriction, alone, is not lethal. To be sure, the behavioral trait of voluntary food restriction emerges only after the imposition of food restriction. The heightened wheel activity, especially during the hours leading up to the time of feeding, termed food-anticipatory activity (FAA) becomes evident within 24 h. of the imposed food restriction.

1.4 Theories Regarding Individual Differences in ABA Inducibility

As noted above, the greatest change in food restriction-evoked behavior is hyperactivity, especially during the hours leading up to the feeding hour, FAA. There is evidence to indicate that FAA reflects the animals' learning of the hours of food availability [47]. This learning requires the expression of dopamine D1 receptors by GABAergic neurons in dorsal striatum [48]. However, even if FAA indicates that the hours of food availability has been learned, animals continue running during the hours of food availability, albeit less than during the hours preceding food availability. The extent of wheel running (distance and duration) during the hours of food access as well as the hours leading up to feeding (FAA) can be used to quantify the maladaptive anorexic behavior (hence, the name "activity-based anorexia").

While approximately 80% of female mice in mid-adolescence become hyperactive, when food restricted, the proportion drops to 50% when the same ABA-inducing environment is imposed during late adolescence. The greater vulnerability for ABA during mid-adolescence may be due to asynchrony in the maturation of brain regions, causing an imbalance in the interregional connectivity [49, 50]. In addition, individual differences in the gain of resilience during the mid to late adolescent may reflect individual differences in the progression of the last phase of brain maturation. In what ways might brain maturation differ across individuals? Possibilities include the cellular events underlying microcircuit-level fine-tuning, such as synapse pruning, increased myelination of the prefrontal cortical pyramidal cells, and changes in the balance of excitatory-to-inhibitory synapses, among others [27, 51, 52].

The causal-effect relationship between food restriction, anxiety, and exercise remains unclear, but there are at least two prevailing views (reviewed in [27]). One view is that food restriction and excessive exercise are evoked due to a preexisting condition of anxiety and that patients deliberately choose these as anxiolytic behaviors to abate the intense fear of weight gain. In support of this view, preexisting conditions of anxiety and overexercise are common among individuals with AN [21]. Similarly, mice carrying genes that elevate trait anxiety exhibit stronger hyperactivity when stressed [53, 54]. Moreover, results from animal models indicate that exercise can be anxiolytic [55] and stress-relieving [56] through the production of BDNF [57, 58]. Food restriction can also be anxiolytic, through the production of ghrelin [59, 60], for which there are ubiquitous binding sites throughout the brain, including the hippocampus [61].

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 [62]. Although food restriction-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 [62]. 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 [63].

Additional explanation about food restriction-evoked hyperactivity is that it is an animal's attempt to counter the hypothermia caused by food restriction. In support of this idea, elevating ambient temperature from 21 °C to 31 °C effectively reduces body

weight loss caused by hyperactivity of food-restricted adult rats [64]. For the explanation of the incessant wheel running, in spite of the progressive weight loss, it has also been hypothesized that running is an addictive behavior associated with the release of endorphins. In support of this explanation, antagonists of endogenous opioid peptides (naloxone) can elicit withdrawal-like symptoms, such as teeth-chattering much more strongly among animals that had undergone ABA compared to animals that were acclimated to a wheel without food restriction [65].

1.5 ABA as an Experimental Tool for Preclinical Studies

As described above, AN is a grave mental illness without accepted pharmacological treatment. Studies to investigate pharmacological manipulations that reduce ABA vulnerability could provide insight into the efficacy of drugs for treating AN. The ABA model can be of use for quantifying the effect of drugs or environmental factors, hormonal status, sex, earlier sensory and emotional experiences, etc., upon ABA vulnerability (*see* Subheading 3.4 for methods to quantify ABA vulnerability). For such studies, one can compare ABA-induced changes in cognitive and anxiety-like behavior, brain anatomy, synaptic physiology, neurochemistry, gene expression, etc., with and without the treatments that are applied at different time points within the ABA schedule. One of our preclinical pharmacological study was to test whether ketamine could ameliorate the maladaptive behavior of excessive exercise and voluntary food restriction. We learned that a single dose of ketamine administered during mid-adolescent ABA induction could ameliorate both of these maladaptive behaviors during the second ABA induction that followed recovery after the first ABA induction [66]. This gives us hope that ketamine may somehow rewire brain in ways that boost the suppression of maladaptive behavior later in life, when stress-inducing environmental factors recur.

1.6 Video and Review Articles on ABA

This chapter describes details of the procedures, rationale of the procedures, and notes to enhance success. As an additional source for familiarizing with the ABA procedure, a visual description of the mouse ABA procedure is available [67]. Many excellent review articles on the theory of ABA exist [15, 30, 27, 44, 68–70] as well as an up-to-date comprehensive summary of ABA studies [46]. Since the focus of this chapter is not theory or review of the literature but presentation of procedural details, reader is urged to examine these other resources for additional information.

2 Materials

2.1 Animals

A key factor for ABA induction is stress-induced anxiety associated with the imposed food restriction. It is therefore important to

minimize all other sources of stress that may potentially interact with the experimental manipulation of food restriction stress.

For studying the effects of ABA during adolescence, it is best to obtain mice of specified ages, strains, and genotypes through breeding in the home institution's animal facility, so as to minimize the uncontrolled levels of stress associated with shipment [71, 72]. Depending on the extent of stress-associated shipment, acclimation to the new facility could require as long as 4 weeks, precluding the ability to begin ABA induction at a desired developmental stage. One recommendable source of mouse breeders is Jackson Laboratories. If in-house breeding is not possible, then one should consider the possibility that shipment of young rodents can incur more or less stress, depending on the age at the time of shipment. We have aimed for the transportation to occur during the fourth postnatal week for female rats. It is desirable to schedule at least 7 days for acclimation to the animal facility in the absence of wheels or food restrictions. Group-housing during this period is desirable, for minimizing stress associated with isolation. For analysis of ABA vulnerability during mid-adolescence, mice and rats begin to be housed individually in cages with free access to a wheel starting around postnatal day 36, an age that is significantly separated from puberty onset, when gonadal hormone fluctuations are known to influence stress-induced anxiety [23, 34].

2.2 Equipment

The desirable features of equipment for measuring wheel activity are that they be able to record wheel activity of multiple animals synchronously, automatically, and over multiple days. Med Associates sells such a model for rats (ENV-046, *see* Fig. 1) and mice (ENV-044, *see* Fig. 2), but multiple other competing companies also sell equipment with these features. The model sold by Med Associates for mice is the Low-Profile Wireless Activity Wheels (ENV-044), that fits within most mouse cages, including the Optimouse "pizza pie" cages (*see* Fig. 2) and Allentown cages with flat tops. The shown rat wheel is made freely accessible through a guillotine door connected to a plastic shoebox-style home cage.

2.3 Water and Food

Food restriction is imposed through limitations in the hours of food access but not in amount. Since mice and rats are nocturnal, the food access should be scheduled during the dark phase. Setting the hour(s) of food access to be at the start of the dark phase has the advantage of maximizing capture of FAA, which can be contrasted to the circadian behavior of non-food-deprived individuals that are usually asleep during the hours leading up to the first hours of the dark period.

It is recommended that the animals be pre-acclimated to the specific foods planned for feeding during the limited hours of food access, so as to avoid hyponeophagia. Hyponephagia is shyness that healthy animals exhibit toward a newly encountered food [73]. A

Fig. 1 Rat wheel access connected to a home cage. A model from Med Associates

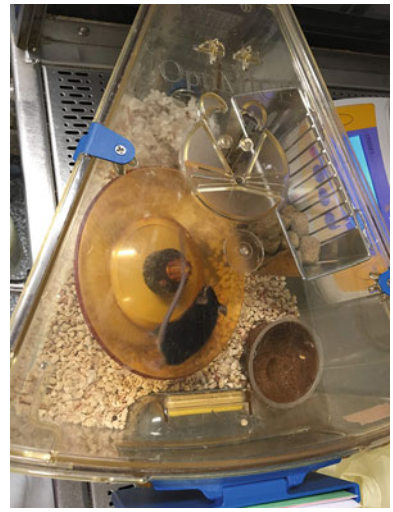

Fig. 2 Mouse wheel (Med Associates) arranged within the cage (Optimouse brand), together with a food hopper that allows easy weighing of dry food. Wet food, kept in the manufacturer's plastic cup (Clear H_2O brand DietGel 76A), is also weighed daily. Nesting material can be found, away from the wheel, to ensure free rotation of the wheel

good time to begin food acclimation is the time animals begin to be acclimated to single-housing and wheel access.

It is recommended that dry food pellet (LabDiet PMI Nutrition Int'l, Brentwood, MO's #5001,10% fat, 20% protein, 70%

Fig. 3 The same mouse wheel arranged within the cage, with an empty dry food hopper and Hydrogel that is 0 cal (Clear H_2O brand)

carbohydrate, 4.07 gross energy kcal/g, 3.02 metabolizable kcal/g; alternatively LabDiet Rodent Diet 20 EXT (5053), 20% protein, 4.5% crude fat, 6% crude fiber, 7% ash, 12% maximum moisture with 4.07 gross energy kcal/g, equal to 3.07 metabolizable energy kcal/g) be dispensed in readily removable food hopper, synchronously with soft wet food (Clear H_2O brand DietGel 76A in plastic cups, 0.998 kcal/g, 4.7% protein, 17.9% carbohydrates, 1.5% fat, 73.4% moisture) which is easier for animals to ingest, but of lower caloric content. During the hours of no food, water gels (Clear H_2O brand Hydrogels Produce #70-01-5022) and empty food hoppers are placed in positions within the cage that are identical to those during the hours of food availability, so as to minimize changes to the context (*see* Fig. 3).

3 Methods

3.1 General Comments about Animals

This study was preceded by works from other labs examining comparisons of ABA vulnerability in other strains of mice that were adult males [53, 54, 74, 75] and mutants that were much younger (anx/anx because they do not survive to adolescence) [76, 77].

If the subjects of the research are to be adolescents, it is recommended that adolescent animals be of similar body weight (<10% variation of mean body weights across groups) and postnatal ages (±5 days) at the start of the acclimation period (however, *see* Subheading 4.1 about animal weights and ages). They need to be individually housed, so as to be able to measure their daily intake of dry and wet food and wheel activity.

Animals should be housed in a rodent vivarium with a 12:12 light/dark cycle maintained at 21 °C and controlled humidity. All experimental procedures (including handling, housing, husbandry,

food restriction) must be conducted in accordance with National and Institutional Guidelines for the Care and Use of Laboratory Animals and University Institutional Animal Care and Use Committee protocols. Since food restriction is a USDA Category E procedure, involving imposition of "more than slight or momentary distress that cannot be treated with anesthetics and analgesics" (USDA Policy #111997 on the use of animals), it is important to provide the strongest scientific justification and to have procedures in place to minimize distress. The procedures for food restriction should include daily monitoring of body weight twice – before and after the hour(s) of food access – and a plan to provide additional food when an animal's body weight decreases to be less than 80% of the baseline body weight that is assessed just before the food restriction period has begun.

It is recommended that animals of a single cohort be divided across ABA and control groups. ABA animals receive the combined treatment of food restriction and wheel access. For the initial establishment of the ABA paradigm, there are three desirable control groups. They are FR (food restricted only for equivalent days and ages), EX (exercise only, with access to the identical wheel model for equivalent days and ages), and CON (no food restriction and no wheel access).

3.2 General Comments About Caging, Acclimation, and Ambient Temperature

Body weight loss associated with restricted food access causes body temperature to decrease. Conversely, raising the ambient temperature reduces wheel running and body weight loss [64, 78]. Based on these observations, it is likely to be important that nesting material be provided, so as to enable individuals to create thermally insulating nests (*see* Fig. 4). If so, then the nesting material should be equalized across cages, so as to minimize differences in body temperature and stress arising from differences in the thermally insulating nesting material.

Fig. 4 A typical nest that an adolescent female mouse creates using three kinds of products: facial tissue, paper strips, and compressed cotton squares

A substantial amount of food remains at the bottom of cages. In order to ensure that food availability is restricted to the hours of the experimental design, the cage bedding must be refreshed completely at the end of the feeding hour, with the bottom of cages wiped clean to remove all dry food crumbs and dust. Water should be freely accessible at all times, with additional hydration available through water gel cups during the food-restricted hours, in the place of wet food gel cups. The water gel assures that animals can remain hydrated while acclimating to the new water delivery system, such as the automatic water delivery systems.

If animals are to be shipped, they should be allowed a minimum of 7 days of acclimation to the new facility [72] while group-housed. Group-housing should strive to maintain same cagemates as found during the shipment, so as to avoid stress due to aggressive social interactions.

3.3 The Daily Schedule of ABA and Control Groups

The environmental treatment of ABA is comprised of a combination of food restriction and exercise. In order to be able to identify behavioral and brain changes elicited mainly by food restriction, mainly exercise or through their interaction, it is useful to set up four groups: ABA (food restricted in the presence of wheel), FR (food restricted, with schedule equalized to that of ABA animals), EX (given wheel access, to which it has acclimated as are the ABA animals), and CON (neither food-restricted nor given wheel access) [34, 37]. Listed below are the procedures scheduled for each experimental phase for animals undergoing one to three bouts of ABA (ABA1, ABA2, ABA3), with ABA2 and ABA3 designed to measure adaptive changes evoked by the previous bout of ABA (*see* Fig. 5).

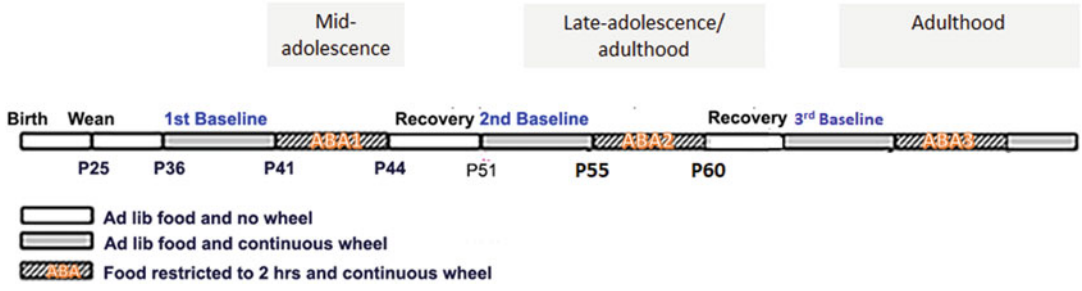

Fig. 5 Timeline for three ABA inductions spanning adolescence to adulthood, each preceded by an acclimation phase and followed by a recovery phase

3.3.1 Acclimation – 4 Days

This is the phase for acclimating animals to single housing, wheel in the cage, and food type. For studies of adolescents, the recommended start time is 6 h prior to the beginning of the dark phase (e.g., 1 pm, for rooms that become darkened at 7 pm) of postnatal day (P) 36, ending at 1 pm on P40. [For the sake of simplicity, postnatal ages will be considered to increase by 1 day at 1 pm. Thus, 1 pm of P40 is equal to the beginning of P41]. Adolescent animals exhibit daily increases in body weight. Wheel acclimation is indicated by daily increments in wheel counts per day, as animals gain speed of running.

Introduction

Wheels

Animals designated for wheel access (EX and ABA) are acclimated to the wheel in their cages starting the first day of acclimation. Be sure that the wheel can rotate freely, not obstructed by other items in the cage. In tightly fitting cages, such as the Optimouse cages, it is important to be vigilant that wheel turning is not obstructed by the food hopper, wet food cup, or nesting material *(see* Figs. 2 and 3). If the wheel counting system relies on battery, be sure that the battery voltage is safely above the cutoff (e.g., >4 volts for the ENV-044, manufactured by Med Associates).

Food

Rodents exhibit hyponeophagia – shyness to new source of food. Hyponeophagia is minimized by introducing the dry and wet foods for a minimum of 4 days, starting on the first day of acclimation.

Bedding and Nesting Material

All animals should receive equal amount of bedding and nesting material. Nesting material can comprise of tissue, shredded paper, and compressed cotton pads, which mice shred to mix with shredded paper and tissue to create thermally insulating nests (*see* Fig. 4). Since animals' body temperature can decrease during food restriction, nesting material can play an important factor of thermal regulation. The nesting material should be equalized across the cages.

Record Keeping

Wet and dry food consumptions, body weight, and wheel activities are recorded throughout the entire experiment. The frequency of recording can be kept to once per day during the acclimation phase, although it should be at least twice per day during the food-restricted, ABA-inducing periods, timed to be just before and at the end of the hours of food availability. It is important to record wheel activities at least once per day, even if the wheel activity system is recording animals' activity every minute automatically. This is because more often than not, the data acquisition system is interrupted unexpectedly. The daylight savings day is one predictable cause for failures, as are the days of power failures.

3.3.2 ABA1–3 Days

General Comments

This is the first phase in which food restriction is combined with wheel access for the ABA group of animals. The FR group of animals begin to be food-restricted synchronously with the ABA animals, in the absence of wheel access. ABA and EX groups continue to receive wheel access, as is done during the acclimation phase. The type of food remains unchanged for all animals, but the food access becomes limited to 2 h per day for mice and 1 h. per day for rats in the ABA and FR groups. For adolescent mice undergoing their first experience of food restriction, 3 consecutive days of food restriction is the maximum duration before body weight loss exceeds 20%. For animals that have begun acclimation on P36, ABA1 begins on P40 and ends at the end of P43 (which equals the beginning of P44).

Details of the Schedule

During the food-restricted days, food access should be scheduled for the first hour of the dark period for rats and the first 2 hours of the dark period for mice. The first food-restricted day of ABA1 (FR1) should begin 6 h prior to the beginning of the dark phase. Thus, all food should be removed 6 h prior to the beginning of the dark phase. For example, for animals housed in rooms with light hours set to be from 7 am to 7 pm, all food is removed from the cage at 1 pm, returned to the cage at 7 pm, and taken away again at 8 pm for rats or at 9 pm for mice.

Details of the Procedure for Food Restriction

Food removal is achieved by removing the wet gel food cup and the hopper containing dry pellets. Bedding material can contain crumbs from the dry pellet. Therefore, the bedding needs to be exchanged with new bedding material at the end of the feeding period and the interior surface of the cage wiped with a dry paper cloth. However, as much as is possible of the nesting material is returned to the cage, in order to minimize changes to the cage interior. As further effort to minimize changes to the cage interior, an empty food hopper and zero-calorie water gel are placed in the same positions as the food hopper with food and wet food cups. Nesting material should be placed in the original position while ensuring that none of the cage objects interfere with free movement of the running wheel. This is the procedure that should be followed at 1 pm of ABA1-FR1 and at the end of the feeding period of FR2 and FR3.

Daily Data Collection

For calculating food consumption, dry pellets and wet gel food are weighed in their containers every time food is removed from the cage and re-weighted every time that food is returned to the cage. For monitoring animal's body weight fluctuation associated with food restriction and feeding, it is recommended that animals' body weight be recorded at the time of food removal and at the time food is returned to the cage. The body weight at the time of food removal of the first day of food restriction (ABA1-FR1) can be considered the animal's baseline body weight (*see* Fig. 6a).

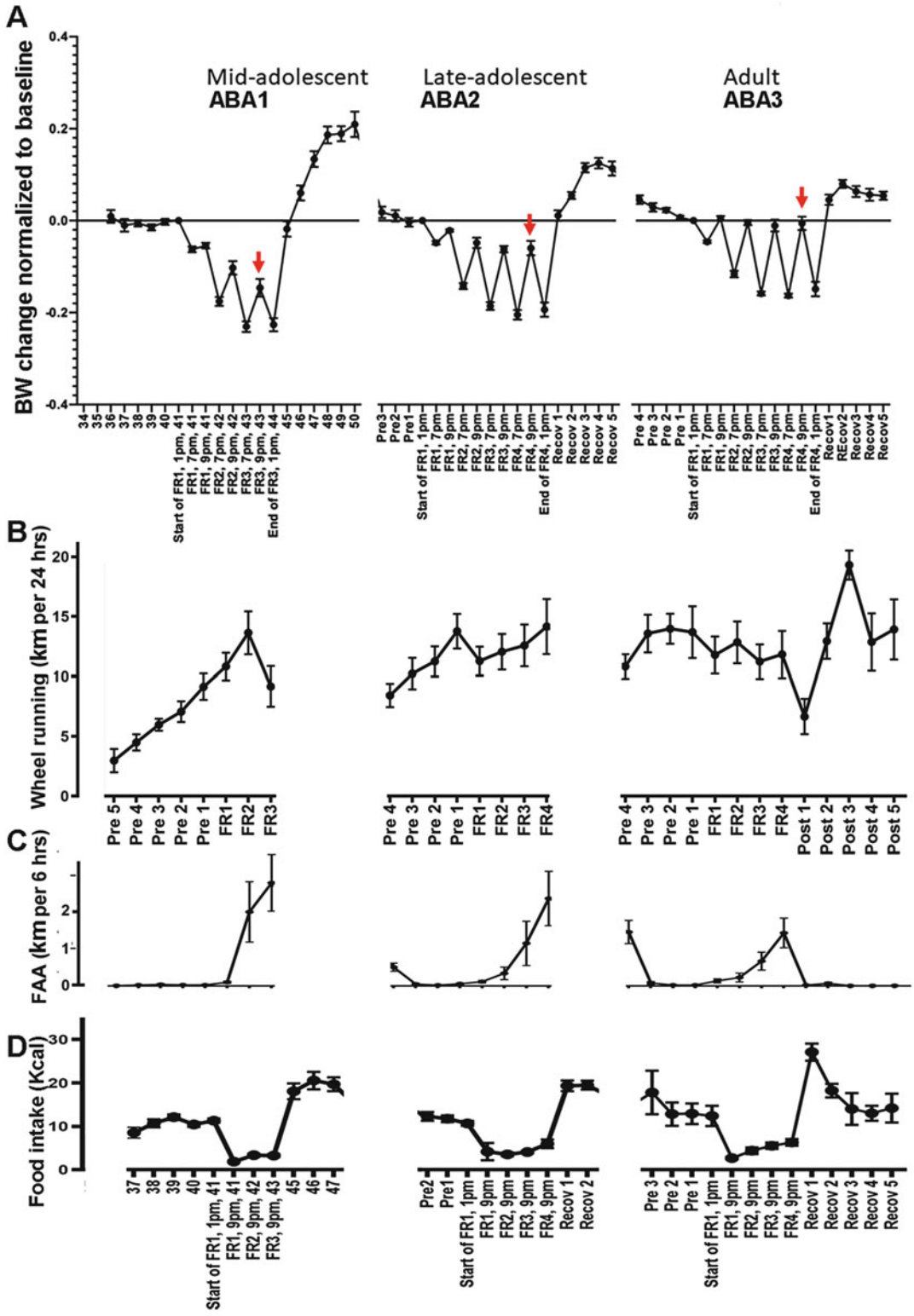

Fig. 6 Group average of body weight change (panel A), wheel running distance per day (panel B), and FAA (food-anticipatory activity), spanning 1 pm to 7 pm on food-restricted days (panel C) and food intake (panel D) of 9 female mice that underwent three ABA inductions. The red arrow in panel A highlights the progressive decrease in body weight, as animals undergo the successive ABAs

Summary and Conditions Requiring Supplemental Feeding

In short, during the first day of the first ABA (ABA1-FR1, P40), body weight, dry food weight, wet food weight, and wheel counts are recorded three times: at 1 pm, 7 pm, and 9 pm. On subsequent days (FR2 and FR3), these measurements are made twice per 24 h: at 7 pm and 9 pm. If, at the scheduled time of food removal (9 pm), measurement indicates that the animal has failed to retain at least 80% of the baseline body weight, and/or if the animal is exhibiting stillness, these behaviors are signs that the animal may not survive to the next feeding time. For them, additional pre-weighed amount of food, roughly equal to half of prior days' food consumption, should be placed at the bottom of the cage, for animals to have the easiest access to them, so as to avoid lethality. Approximately one out of eight mice exhibit gravely reduced locomotion by FR3 and need to receive supplemental food at 9 pm (unpublished observations). This is rarely observed for the FR-only group of animals and is more often observed for the ABA animals with high levels of food restriction-evoked wheel running. Food restriction-evoked wheel running increases most dramatically during the hours preceding the feeding hours and is thus called "food-anticipatory activity" (FAA) (*see* Fig. 6c).

3.3.3 Recovery from ABA1–5 Days

At the end of ABA1, which for the example above is 1 pm of FR3 (beginning of P44 or end of P43), animal's body weight is measured, as are dry and wet food, just before returning them to the cage, and the wheel is removed from the cage. During the recovery days, body weight and food consumption are measured once per day. Almost all animals restore their body weight within 1 day. Adolescent animals resume the daily body weight gain, as shown during acclimation. For animals that enter the experiment on P36, the ages of recovery is the beginning of P44 to the beginning of P49.

3.3.4 Reacclimation – 4 Days

For animals scheduled to return to a second ABA (ABA2) or EX, wheels are returned to the cage at the end of recovery so that animals can reacclimate to the wheel. Feeding condition remains ad libitum during this period for all groups. Most animals exhibit wheel activity that is similar to the extent observed on ABA1-FR1, indicating that they retain the state of acclimation to the wheel (*see* Fig. 6b). Body weight, food consumption, and wheel counts are recorded once per day during reacclimation. For animals that enter the experiment on P36, the age of reacclimation is the beginning of P49 to the beginning of P54. The average of wheel running during the last 2 days of reacclimation is considered the baseline wheel activity, to be used for calculating food restriction-evoked increase in wheel running during the succeeding ABA2.

3.3.5 ABA2–4 Days

ABA2 is similar to ABA1, except that the duration of food restriction can be extended to 4 days or longer. This may be due to behavioral adaptation, such as more efficient eating during the hours of food access, decreased wheel running during the hours of food access, and/or increased baseline body weight, all of which are questions that can be addressed experimentally for pursuing cell biological and neurochemical understanding.

ABA2 begins by removing food at 1 pm, returning the food to the cage for 1 or 2 h at the beginning of the dark phase, and removing the food after 1 h (for rats) or 2 h (for mice). The body weight at 1 pm at the beginning of ABA2 is considered the new baseline body weight. The average of wheel running during the last 2 days of reacclimation is considered the baseline wheel activity, to be used for calculating food restriction-evoked increase in wheel running during ABA2.

As was cautioned for ABA1, bedding material should be exchanged completely, and the interior surface of the cage should be wiped to avoid residual food availability during the hours of food deprivation. During the first day of the second ABA (ABA2-FR1), body weight, dry food weight, wet food weight, and wheel counts are recorded three times: at 1 pm, 7 pm, and 9 pm for mice and at 8 pm for rats. On subsequent days (FR2, FR3, FR4), these measurements are made twice per 24 h: at 7 pm and 9 pm for mice and at 7 pm and 8 pm for rats. If at the scheduled time of food removal body weight measurement indicates that the animal has failed to retain at least 80% of the baseline body weight, and/or if the animal is exhibiting inability to locomote, additional pre-weighed amount of food, roughly equal to half of prior days' food consumption, should be placed in the cage, so as to avoid lethality. However, this is rarely observed during ABA2. For animals that enter the experiment on P36, the age of reacclimation is the beginning of P54 to the beginning of P59, which is considered a stage for transitioning into adulthood.

The wheel activity observed during ABA1 typically increases multiple folds relative to the level observed during acclimation (~80% of the population of C57BL6-J strain). In contrast, *group average* of total wheel activity observed during ABA2 is only minimally increased relative to the level recorded during reacclimation (*see* Fig. 6b). Interestingly, closer examination of individuals reveals that some of the animals increase wheel running in response to food restriction during ABA2, while others reduce wheel running in response to food restriction, yielding a group average value that suggests no overall change (*see* Figs. 7 and 8). This observation fits with previous reports that studied ABA behavior of adult mice, indicating that C57BL6 mice are not vulnerable to ABA while other strains with known traits of heightened anxiety are [53]. However, individual differences revealed during ABA2 is an opportunity to identify brain and physiological changes that

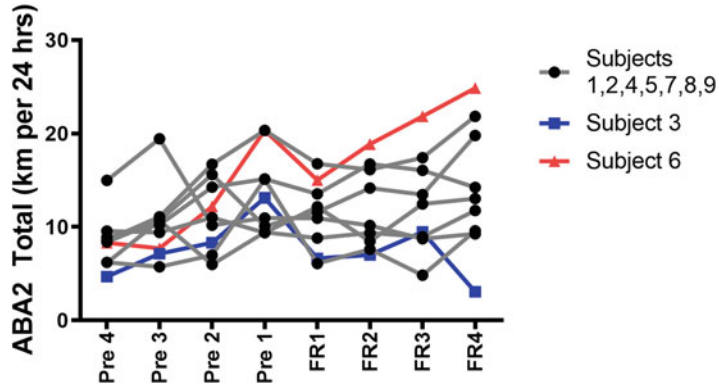

Fig. 7 Individual records of wheel activity per day, during ABA2 of the same nine mice used to calculate the group average shown in Fig. 6a. Subject 6 exhibited progressive increase in wheel running during the days of food restriction (FR1 through FR4), while subject 3 exhibited no significant increase in food restriction-evoked wheel running. These individual differences are examples of ABA vulnerability and resilience, respectively

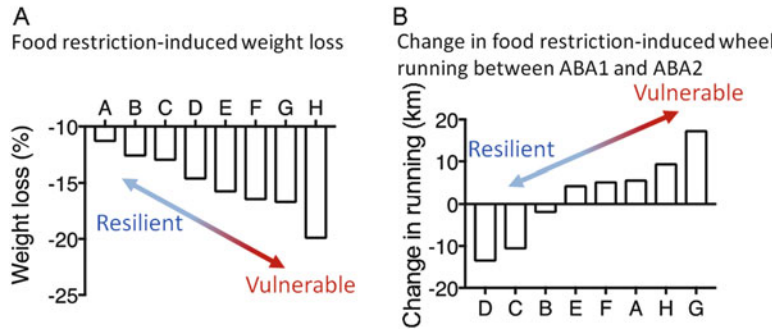

Fig. 8 The relationship between body weight loss and wheel activity was examined using another cohort of adolescent female mice that underwent ABA1 and ABA2. Each alphabet corresponds to datum from an individual mouse. The severity of food restriction-induced body weight loss (panel A) aligns somewhat, but not exactly with hyperactivity (panel B), presumably because differences in food intake during the limited hours of food availability and individual differences in energy metabolism contribute to body weight losses as well

correlate with persistence versus suppression of food restriction-evoked hyperactivity which, in turn, contributes toward ABA vulnerability versus resilience, respectively. Indeed, comparison of brain circuitry across vulnerable versus resilient mice and rats has revealed individual differences in the GABAergic synapses and $GABA_A$ receptor subunit expressions, with the resilient mice showing enhancement of these measures, compared to CON. Strong correlations ($r > 0.8$, $p < 0.05$ by Pearson correlation test) between wheel activity and these measures in dorsal

hippocampus [35, 38, 41, 79] and prelimbic area of prefontal cortex [39] have also been revealed. Conversely, this approach has revealed strong positive correlations between body weight loss and NMDAR levels at postsynaptic density of excitatory synapses in dorsal hippocampus (higher levels of NMDARs, the more they had lost body weight), while the levels of NMDARs sequestered in the postsynaptic spine cytoplasm correlated with suppression of hyperactivity [40]. These correlation analyses provide the data necessary for forming working hypotheses about the cellular and molecular basis of ABA vulnerability/resilience, which can be tested for causality using pharmacological and molecular approaches, such as knockin/knockdown of genes of interest within specific brain regions.

3.3.6 Recovery from ABA2–5 Days

For animals that are scheduled to continue onto ABA3, 5 days of recovery is recommended. The details of the procedure are as described for recovery from ABA 1 (*see* Subheading 3.3.3).

3.3.7 Reacclimation – 4 Days

For animals that are scheduled to continue onto ABA3, 4 days of reacclimation is recommended. The details of the procedure are as described above (*see* Subheading 3.3.4).

3.3.8 ABA3–4 Days or longer

For animals that are scheduled to continue onto ABA3, 4 days of food restriction is recommended. The details of the procedure are as described for ABA2 (*see* Subheading 3.3.5).

3.3.9 Euthanasia

The end point of the experiment should be decided based on the experimental question. For studies intending to correlate antemortem behavior with neurochemistry or brain anatomy, euthanasia may be scheduled on the last day of food restriction of the ABA period [34, 36, 40, 80]. On the other hand, for capturing brain mechanisms underlying the stable gain of resilience versus the persistent vulnerability to ABA, a better time point for euthanasia may be the end of the recovery phase that follows ABA [41]. We have also attempted to capture brain changes evoked during ABA, for which animals were euthanized on FR2 of ABA1, when body weight had begun to decrease and food restriction-evoked hyperactivity was on the rise but not yet maximal [35].

For optimal brain preservation for light and electron microscopic immunocytochemistry, see previously published methodological papers on transcardial perfusions to preserve synaptic structure [81, 82].

3.4 Quantification of ABA Vulnerability

As was remarked in Introduction, ABA is useful for capturing major characteristics of AN, consisting of body weight loss, voluntary reduction of food consumption, hyperactivity, and increased anxiety-like behavior. Individual differences in ABA vulnerability within and across cohorts can be quantified by making

measurements of these variables. Examples of procedures used to measure these changes are described below.

3.4.1 Body Weight Loss

Adolescent rodents on ad libitum food access gain weight continuously, but this body weight gain is severely interrupted when food access is reduced to 1 or 2 h per day (*see* Fig. 6 for an example of mouse data; *see* Fig. 8 for an example of rat data). Interestingly, body weight loss due to food restriction (in grams or percent of baseline) is less during ABA2 than observed during ABA1 (*see* Fig. 6), with body weight gain (a turning point) exhibited by the fourth day, in spite of the continuation of restricted food access. Analysis of individual animals' daily body weight changes can reveal differences, with some animals showing less weight loss and earlier turning points. The degree of weight loss is not paralleled exactly by individual differences in wheel activity (*see* Fig. 8), suggesting that individual differences in food consumption during the restricted hours of food availability and intrinsic differences in metabolism may contribute additionally to weight loss.

3.4.2 Reduction of Food Consumption and Suppression of Running during the Hours of Food Availability

Although the imposition of food restriction causes inevitable reductions of food intake, the extent to which animals reduce food intake can still vary. This is due, at least in part, to some animals "choosing" to run more than others during the hours of food availability. During ABA1 and ABA2, almost all animals run maximally during the first night of food restriction. Their running decreases progressively during FR2 to FR3, presumably reflecting their having learned the restricted feeding schedule. Some animals "learn" better than others, evidenced by their greater suppression of wheel running during the feeding hours (*see* Fig. 9). This is one other variable that can be used to quantify individual differences in ABA vulnerability (i.e., less suppression of running equals greater vulnerability).

3.4.3 Food Restriction-Evoked Increase in Wheel Running

In our experience, food restriction-evoked increase in wheel running is the variable that provides the greatest precision for quantifying individual and group (e.g., with versus without drug) differences in ABA vulnerability. By comparing the extent of voluntary wheel running during the days before versus the days after food restriction (i.e., acclimation versus ABA), one is able to quantify wheel activity associated with the introduction of the environmental stressor – food restriction. The comparisons may be of the total activity over a 24-hr period, or focused upon specific time bins of interest, such as the hours of FAA (food-anticipatory activity, when an animal must make a choice between foraging and conserving energy), the hours of food availability (when an animal must make a choice between running and eating), and the postprandial hours. Similar measurements can be compared just prior to versus just following drug administration (see an example of the analysis of the

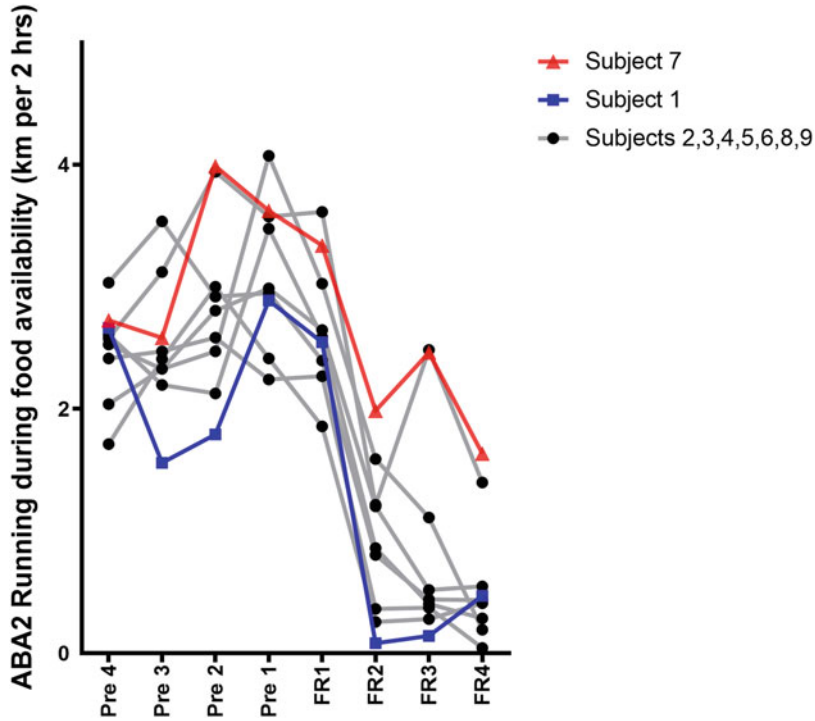

Fig. 9 Individual records of wheel activity during the 2 h of food availability during ABA2 of the same nine mice whose data are shown in Figs. 6 and 7. The red and blue lines depict the extreme examples of hyper- and hypoactivity, respectively

influence of ketamine upon ABA vulnerability, *see* Figs. 10 and 11). Additionally, combinations of time segments, such as the light versus dark hours, may be useful for examining the impact of ABA and/or of drugs upon circadian rhythm. Individual differences or treatment effects may be more evident when comparing wheel running in terms of distance run, duration run, or velocity of running. Finally, treatment effects (e.g., drug versus vehicle) may require analytic approaches that eliminate preexisting individual or group differences, such as of wheel running or of body weight. This comparison can be achieved by normalizing the degree of change evoked by the treatment, relative to pretreatment levels (e.g., body weight loss following food restriction during ABA minus baseline body weight measured just prior to the start of ABA divided by baseline body weight; *see* Figs. 10a and 11).

One powerful use of the ABA model is to identify potential cellular substrates for individual differences in ABA vulnerability. Figure 12 shows an example of a strong correlation revealed between wheel running during the food-restricted period (ABA2) and sizes of GABAergic axon terminals forming axosomatic inhibitory synapses onto layer 5 pyramidal neurons in the Cg1 area of prefrontal cortex. In contrast, the wheel activities of the same

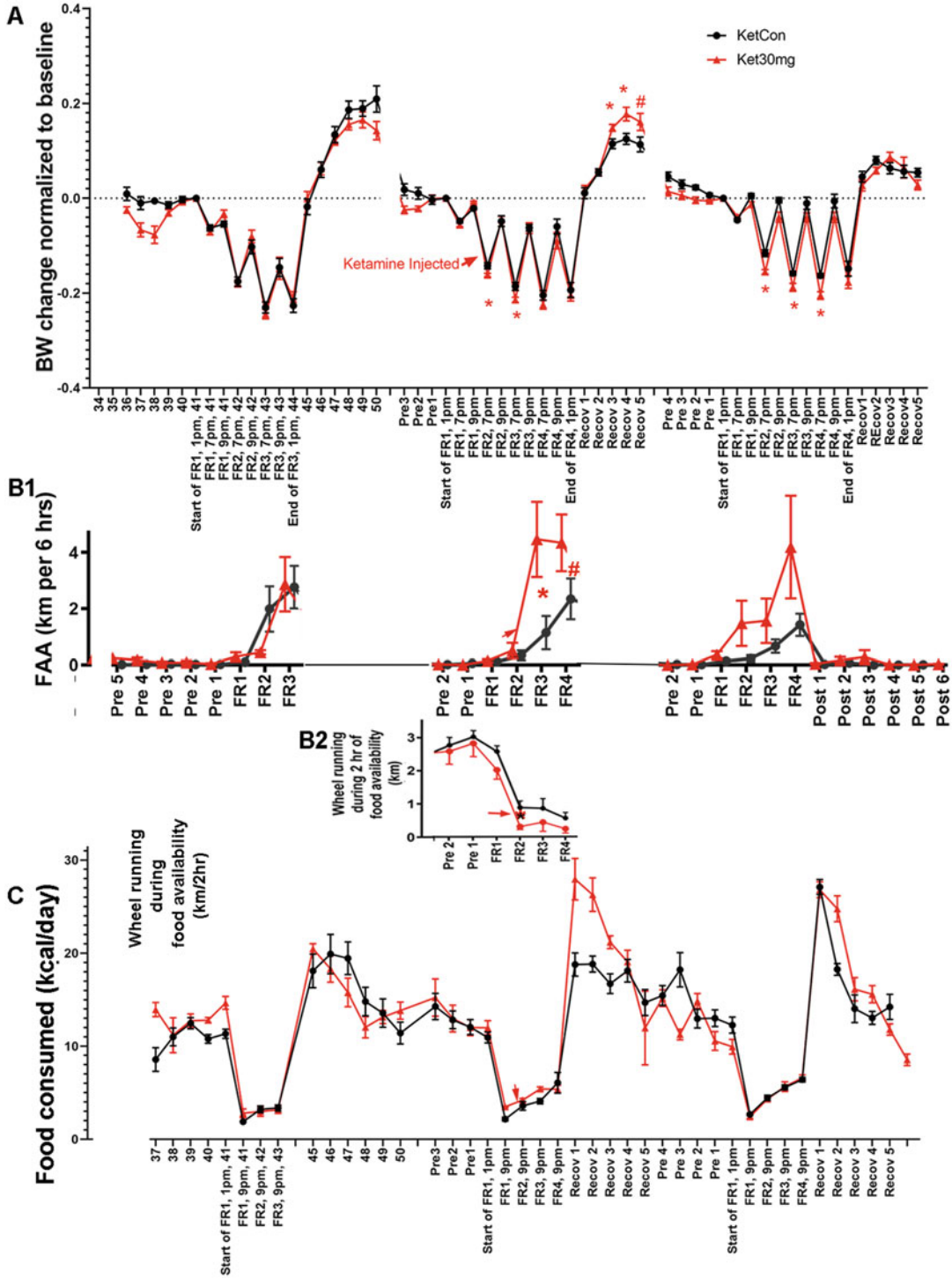

Fig. 10 An example of the use of the ABA paradigm to explore drug effects on ABA vulnerability/resilience. Data shown here suggest that ketamine exacerbates body weight loss, when administered during ABA2 in late adolescence. We had previously shown that a single injection of ketamine (30 mg/kg body weight) during

animals during acclimation/baseline showed no correlation with GABAergic axon terminal sizes. Also, note that the GABAergic axon terminal sizes within the corresponding brain region of control animals that experienced neither wheel activity nor food restriction were significantly smaller, indicating that the ABA experience stimulated enlargement of GABAergic axon terminal sizes. This finding has prompted us to prepare for the next phase of study, which will be to modulate GABAergic interneurons in the region using DREADD technology, for determining whether boosting the activity of these neurons can suppress the maladaptive wheel running during FAA and during the 2 h of food availability, thereby minimizing body weight losses.

3.4.4 Increase in Food Restriction-Evoked Anxiety-like Activity

A major feature of AN is anxiety. All animals that undergo food restriction express food restriction-evoked stress, as is indicated by the rise of circulating cortisol, but the extent to which animals increase anxiety-like behavior differs across individuals [31]. Specifically, individual differences in anxiety (before versus during food restriction) correlate significantly with the extent of increase in wheel running ($p = 0.004$, $R^2 = 0.56$) [31] (*see* Fig. 13), indicating that wheel running can be used to assess changes in anxiety. Unlike anxiety tests, which cannot be repeated and must be limited in number (we used open field before and elevated plus maze after food restriction), wheel running can be used to monitor changes in food restriction-evoked anxiety continuously.

3.4.5 Altered Cognition

Individuals diagnosed with AN are often characterized as high achievers but with rigidity in their decision-making [83]. Do

Fig. 10 (continued) mid-adolescence (P42) could increase food intake and decrease food restriction-evoked hyperactivity, leading to reduced body weight loss [66]. Encouraged by this result, the follow-up study shown here was to assess the influence of 30 mg/kg ketamine injection that is delayed by 10 days, corresponding to an age during late adolescence. Ketamine was injected on FR2 (second day of food restriction) of *ABA2* at 6 pm, corresponding to 1 h. preceding the time of feeding. Preliminary results indicate that ketamine treatment at this age does not yield protection against body weight loss.
The red line depicts group average of body weight changes of the cohort that received 30 mg/kg of ketamine on the second day of food restriction of ABA2. The black line depicts group average of the control group that received vehicle injection (saline), instead of ketamine, on the injection day. Asterisks indicate significant group difference ($p < 0.05$, unpaired t-test), while # depicts a trend. Red arrows point to the time of ketamine/vehicle injections. The exacerbated body weight loss is evident during FR2 and FR3 of ABA2 (panel A) and is persistently exacerbated even during ABA3 (panel A). FAA, calculated as the extent of wheel running from 1 pm to 7 pm, is increased on days following the ketamine treatment (panel B1, red asterisk). This increase in FAA is likely to be causal to the exacerbated weight loss. In contrast, ketamine's effect of reducing wheel running during the hours of food availability (7 pm to 9 pm) (panel B2) and the slight increase in food intake during those hours did not protect animals from body weight loss. Ketamine-treated animals consumed significantly more food, and especially so during the days of recovery from ABA2 (panel C), but this did not protect them from the exacerbated weight loss during the subsequent ABA3

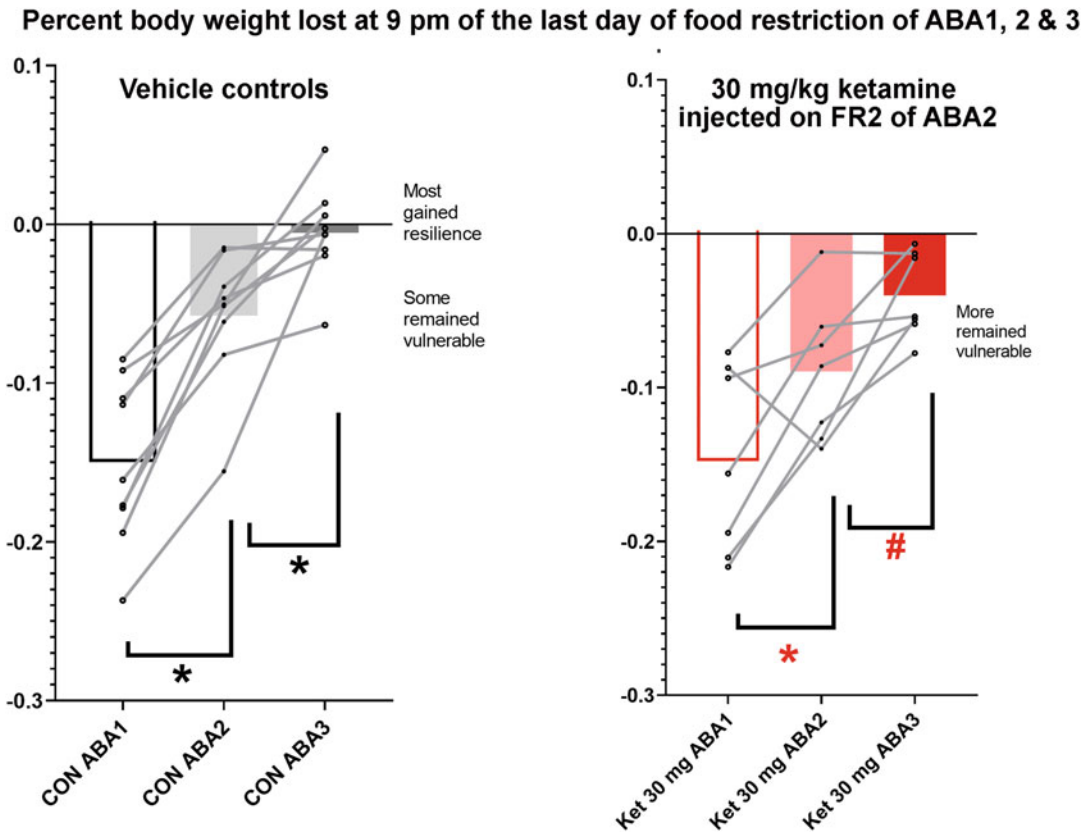

Fig. 11 Individualized analysis of food restriction-evoked body weight losses following ketamine treatment on FR2 of ABA2. Each data point reflects body weight measurement of one animal at one time point, with each time point taken at 9 pm of the last day of the food restriction period, corresponding to FR3 of *ABA1,* FR4 of ABA2, and FR4 of ABA3. These data were taken from the same cohort of animals whose group averages are shown in Fig. 10. Ketamine (30 mg/kg, right graph with red bars) or vehicle only (saline, left graph with gray bars) was injected intraperitoneally at 6 pm on FR2 of ABA2. All but one animal exhibited progressively less body weight loss, as the ABA induction was repeated. For both ABA2 and ABA1, ABA vulnerability, measured as the extent of body weight loss, tended to be more for the individuals of the ketamine group, compared to the group average of the values obtained from the CON group

animals that have undergone ABA also exhibit inflexibility or impairments in updating memory? Does the experience of ABA interfere with spatial memory formation? Our unpublished observations indicate that the answer is "no." We observed that ABA animals exhibit superior cognitive abilities in the active place avoidance (APA), a hippocampus-dependent spatial memory task [84], relative to CON with no experience of food restriction or wheels [85, 86]. Moreover, ABA animals with the greatest FAA were also the best performers in APA but only if the APA was delayed by 9–10 days after the end of food restriction [85, 86]. These observations suggest that the experience of ABA does not impair but rather enhances hippocampus-dependent cognition.

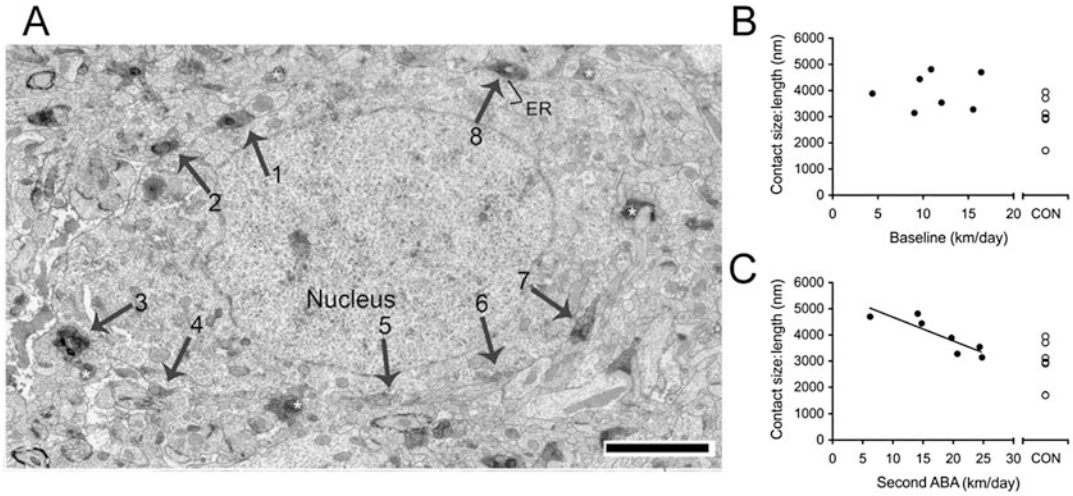

Fig. 12 Individualized analysis of food restriction-evoked change in wheel running revealed a strong correlation with the extent of GABAergic inhibitory synaptic input to pyramidal neurons in Cg1 area of medial prefrontal cortex. Panel A shows an example of electron micrographs used to assess GABAergic synapse lengths formed onto cell bodies of layer 5 pyramidal neurons in Cg1 of medial prefrontal cortex. Calibration bar = 2 μm. Panels B and C show correlation analyses of these synaptic lengths with the individual animal's extent of wheel running prior to (panel B) and during (panel C) ABA2 induction. (From Chen et al. [39], reproduced with permission)

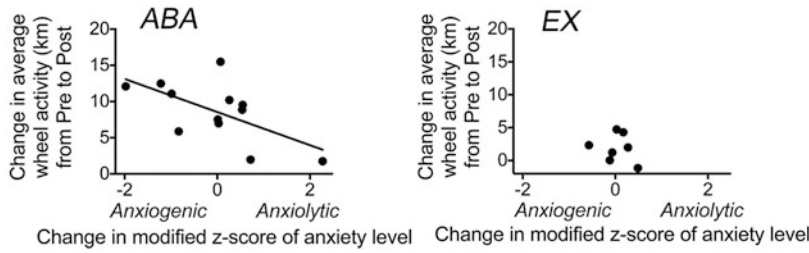

Fig. 13 Food restriction-evoked increase in wheel activity correlates with food restriction-evoked increase in anxiety. Anxiety of adolescent female mice that had acclimated to the wheel was measured before and after food restriction (ABA, left panel) and compared to those that acclimated to the wheel without the experience of food restriction (EX, right panel). In the absence of food restriction, the animals' anxiety-like measurements were similarly ranked during the first open field test and the second elevated plus maze test conducted 21 h later. For those that underwent food restriction, the two anxiety-like tests revealed wide individual differences. Those that exhibited more anxiety-like behavior (anxiogenic) were the same ones that had increased their wheel running the most. (Adapted from Ref. [31], *see* Fig. 4b)

4 Notes

4.1 Notes on Animals

In Subheading 3.1, it was advised that the baseline body weight and age be kept to within 10% variance. However, both of these parameters remain to be explored as potential risk factors. Do animals

with lower body weight but of equal postnatal age exhibit greater vulnerability? Are animals undergoing puberty onset more at risk than those that are mid-adolescent? For addressing these questions, experiments can be conducted in which the schedule is kept identical, for running correlation analyses between the baseline body weight or the age of entry into the experimental schedule and the extent of food restriction-evoked wheel running, increase of anxiety-like behavior, or change in cognition.

Sex of the animal is also an important parameter that should continue to be explored. Most published studies have used adult male rats. We have examined ABA inducibility (vulnerability) of adolescent males versus females and shown that both sexes are vulnerable, but resilience is attained using molecularly distinct mechanisms across the sexes [41]. In experiments examining the efficacy of certain drugs for reducing ABA vulnerability, differences in the outcome across the sexes could also shed lights upon the differences in the mechanisms underlying the gain of resilience.

4.2 Notes about Caging, Acclimation, and Ambient Temperature

We have run the mouse ABA paradigm using a variety of cage models. Obviously, it is desirable for the caging to remain unchanged throughout the ABA paradigm and for comparisons across cohorts. One parameter that the experimenter should strive to keep constant is the amount of nesting material, since this could affect the thermal insulation provided to the animal. It has been shown that elevated ambient temperature reduces the food restriction-evoked hyperactivity [64, 78], suggesting that animals (in this study, the subjects were adult rats) may increase wheel running so as to raise body temperature under the starvation mode. Based on this observation, one must consider the possibility that the degree of thermal insulation provided by the nesting material may also interact with animals' food restriction-evoked wheel running.

4.3 Notes about the ABA Schedule

Variations can be introduced easily into the ABA schedule, for addressing many different questions. The schedule described under Subheading 3.3 was for a preset duration of food restriction. An alternative might be to let the number of days of food restriction to be a set according to an individual animal's body weight change, such as 80% of baseline. The number of consecutive days of food restriction could be prolonged, as animals undergo repeated ABA sessions and the baseline body weight increases with maturity. Alternatively, although our earlier study indicated that many of the mid-adolescent mice do not survive when food access is limited to 1 h. per day, this shorter duration may be possible for late adolescence and adulthood. Conversely, increasing the number of hours of food access to be greater than 2 h for mice or greater than 1 h. for rat will definitely reduce hyperactivity, as animals will be able to maintain their baseline body weight sufficiently.

The duration of wheel acclimation is recommended based on the baseline wheel activity that is usually attained for adolescent mice and rats. It is helpful to wait until an animal's pre-food restriction wheel running attains a steady level, for enabling measurements of food restriction-evoked increases. For adults, a much longer acclimation period may be required, compared to animals described here that undergo two sessions of ABA during adolescence.

5 Conclusion

Anorexia nervosa is increasing in prevalence, especially among the male population. Although recognized to be more prevalent among athletes, dancers, and models, the Department of Defense recognizes that those serving the military may also be at risk. This author agrees with the viewpoint of the DoD since individuals serving the military are selected based on their ability to tolerate the combination of extreme stress and strenuous exercise, the two environmental risk factors for AN and reliable factors that induce ABA in rodents. The animal model of ABA promises to serve as a useful preclinical trial tool for exploring pharmacological treatments that ameliorate the addictive, maladaptive aspects of excessive exercise and food restriction and for understanding the neurobiological basis for brain plasticity that enables suppression of these maladaptive behaviors.

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