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α4-GABA$_A$ receptors of hippocampal pyramidal neurons are associated with resilience against activity-based anorexia for adolescent female mice but not for males

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ABSTRACT

Activity-based anorexia (ABA) is an animal model of anorexia nervosa, a mental illness with highest mortality and with onset that is most frequently during adolescence. We questioned whether vulnerability of adolescent mice to ABA differs between sexes and whether individual differences in resilience are causally linked to α4βδ-GABA$_A$R expression. C57BL6/J WT and α4-KO adolescent male and female mice underwent ABA induction by combining wheel access with food restriction. ABA vulnerability was measured as the extent of food restriction-evoked hyperactivity on a running wheel and body weight losses. α4βδ-GABA$_A$R levels at plasma membranes of pyramidal cells in dorsal hippocampus were assessed by electron microscopic immunocytochemistry. Temporal patterns and extent of weight loss during ABA induction were similar between sexes. Both sexes also exhibited individual differences in ABA vulnerability. Correlation analyses revealed that, for both sexes, body weight changes precede and thus are likely to drive suppression of wheel running. However, the suppression was during the food-anticipatory hours for males, while for females, suppression was delayed by a day and during food-access hours. Correspondingly, only females adaptively increased food intake. ABA induced up-regulation of α4βδ-GABA$_A$Rs at plasma membranes of dorsal hippocampal pyramidal cells of females, and especially those females exhibiting resilience. Conversely, α4-KO females exhibited greater food restriction-evoked hyperactivity than WT females. In contrast, ABA males did not up-regulate α4βδ-GABA$_A$Rs, did not exhibit genotype differences in vulnerability, and exhibited no correlation between plasmalemmal α4βδ-GABA$_A$Rs and ABA resilience. Thus, food restriction-evoked hyperactivity is driven by anxiety but can be suppressed through upregulation of hippocampal α4βδ-GABA$_A$Rs for females but not for males. This knowledge of sex-related differences in the underlying mechanisms of resilience to ABA indicates that drugs targeting α4βδ-GABA$_A$Rs may be helpful for treating stress-induced anxiety and anorexia nervosa of females but not males.

1. Introduction

Anorexia nervosa (AN) is a mental illness with a high mortality rate (Hoek, 2006; Smink et al., 2012). AN is reported to be nine times more prevalent among females than males (Hoek, 2006; Smink et al., 2012), but the incidence of AN among males is on the rise, changing this ratio to be as low as 3-to-1, by some estimates (Hudson et al., 2007; Weltzin et al., 2012; Sabel et al., 2014), as the disorder is becoming recognized to be relatively more gender-neutral (NEDA, 2017). It has been suggested that the greater prevalence of AN among females may be due to females' greater vulnerability to anxiety disorders (McLean et al., 2011) but this explanation leads to another question, What is the neurobiological basis of females' greater vulnerability to anxiety disorders? One of multiple possible origins of anxiogenesis in females is the rapidly fluctuating levels of progesterone and its metabolite, allopregnanolone, especially at puberty onset (Shen et al., 2007).

Although commonly believed to arise from societal propagation of thinness as a desirable body image, animal models can provide clues regarding the neurobiological basis of AN. Activity-based anorexia (ABA) is an animal model, whereby severe voluntary hyperactivity and weight loss are induced by restricting food access while providing ad libitum access to a running wheel (Gutierrez, 2013). Among female rodents, ABA captures four hallmarks of AN (American Psychiatric Association, 2013): heightened anxiety (Wable et al., 2015b; Chen

Abbreviations: α4βδ-GABA$_A$R, GABA$_A$ receptor containing α4 and δ subunits; ABA, activity-based anorexia; BDNF, brain-derived neurotrophic factor; BDNF$^{Val/Met}$, Val-to-Met substitution at amino acid 66 of BDNF; EX, exercise; FAA, food anticipatory activity; FR, food restriction; KO, knockout; SIG, silver-intensified gold immunolabeling

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et al., 2017a), severe weight loss, over-exercise (Wable et al., 2015b) and voluntary food restriction (FR) (Chen et al., 2016). Regarding the hallmark of voluntary FR, this point is based on the observation that once female rodents begin losing body weight, voluntary wheel activity becomes so excessive that animals choose wheel running over eating, even during the limited hours of food access.

Previous studies investigating sex differences in ABA mainly involved adult rodents and found conflicting results, with some reporting greater vulnerability among males than of females (Doerries et al., 1991; Achamrah et al., 2017), while others show no sex difference (Lambert and Kinsley, 1993; Boakes et al., 1999) or greater vulnerability among females (Pare et al., 1978; Hancock and Grant, 2009). Importantly, none of these studies examined males versus females undergoing ABA vulnerability during adolescence (P28–60). This study aimed to address this gap in knowledge by comparing ABA vulnerability between the two sexes during adolescence, given that the onset of AN is most common during adolescence (Hoek, 2006; Smink et al., 2012). Weight loss is exacerbated by hyperactivity. Thus, besides weight loss, we compared adolescent males' and females' resilience to FR stress by measuring FR-evoked hyperactivity during discrete sectors of the circadian rhythm, including the food-anticipatory hours, feeding hours, and post-prandial hours.

We also aimed to determine the role of dorsal hippocampal CA1 in sex differences to ABA vulnerability. The hippocampus shows plasticity in response to stress (McEwen, 1999; Conrad et al., 2017) and gonadal hormones (Woolley and McEwen, 1992) independently and jointly (McLaughlin et al., 2010). The dorsal hippocampus is required for spatial learning and memory performance (Moser et al., 1993; Fanselow and Dong, 2010) but also participates in anxiety regulation, since infusion of anxiolytics into the dorsal hippocampus decreases, while infusion of inverse agonists of GABA-benzodiazepine receptors exacerbates anxiety (Huttunen and Myers, 1986; Kataoka et al., 1991). Enriched environment induces greater brain-derived neurotrophic factor (BDNF) expression in the dorsal hippocampus of females than males (Zhu et al., 2006; Bakos et al., 2009), while stress, such as social isolation during adolescence, decreases BDNF transcription in the hippocampus of female, only (Weintraub et al., 2010). Furthermore, ABA during adolescence alters dendritic branching in the hippocampus of female rats in ways that are distinct from food restriction, only and exercise, only (Chowdhury et al., 2014). Compared to the ventral tegmental area, striatum, hypothalamus, parabrachial nucleus and thalamus that have been studied extensively for food intake control (Wu et al., 2009; Ferrario et al., 2016), relatively less is known about the influence of hippocampus in food intake, especially during adolescence. Thus, we analyzed sex-dependent effects of ABA and its two components separately-food restriction, only, and exercise, only, as was tested earlier for the rat ABA model (Aoki et al., 2012; Chowdhury et al., 2014) and for the mouse model (Wable et al., 2015b).

As cited earlier, dorsal hippocampus regulates anxiety via $GABA_A$ receptors ($GABA_A$Rs). $GABA_A$Rs containing α4 and δ subunits (α4βδ-$GABA_A$Rs) exhibit properties that are unique among $GABA_A$Rs (reviewed by (Smith and Woolley, 2004; Smith et al., 2009)). At extrasynaptic sites that are adjacent to excitatory synapses in the dorsal hippocampus, α4βδ-$GABA_A$Rs mediate shunting inhibition, which can reduce long-term potentiation (LTP) of excitatory synapses and contribute towards spatial memory impairment (Shen et al., 2010; Shen et al., 2017). α4βδ-$GABA_A$Rs also contribute to regulation of anxiety—both anxiolysis and anxiogenesis (Shen et al., 2007)—through the dual modulatory actions of allopregnanolone (3αOH-5αβ-OH-pregnan-20-one), a stress steroid and a metabolite of progesterone. During adulthood and pre-pubertally, α4βδ-$GABA_A$Rs occur in low abundance in dorsal hippocampal CA1 (Shen et al., 2007; Shen et al., 2010). At puberty onset, its expression in dorsal CA1 of female rodents increases transiently in response to the rapid rise, then the decline in the level of allopregnanolone (Shen et al., 2007; Aoki et al., 2012). ABA increases α4βδ-$GABA_A$R expression at excitatory synapses of stratum radiatum of dorsal hippocampal CA1 of adolescent female rodents (Aoki et al., 2012; Wable et al., 2015a; Aoki et al., 2017). However, whether ABA increases α4βδ-$GABA_A$R expression in adolescent males remained unknown. We provide the first evidence for sex differences in multiple parameters of ABA during adolescence, shown to arise from differences in α4βδ-$GABA_A$R expression in the dorsal hippocampal CA1 and magnified in females lacking α4βδ-$GABA_A$Rs.

2. Materials and methods

2.1. Animals

All animals used in the study were bred at New York University's animal facility. Two different strains of animals were used in this study. All male and female C57Bl/6J wild-type (WT) littermates were the $BDNF^{Val/Val}$ animals that derived from heterozygous $BDNF^{Val/Met}$ (Val-to-Met substitution at amino acid 66 of BDNF) breeding pairs (Chen et al., 2006; Chen et al., 2017a). An additional experiment used animals in which the α4-subunit of $GABA_A$ receptors (α4) was genetically deleted (knockout (KO)), together and their WT littermates (Chandra et al., 2006). Homozygous α4-KO animals and WT littermates of the F3–4 generations used in the study were derived from mixed background (C57Bl/6J and Strain 129S1/X1) heterozygous parents as described (Chandra et al., 2006), generously provided to us from the laboratory of Dr. Gregg Homanics, U of Pittsburgh. All procedures relating to the use of animals were according to the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees of New York University (A3317-01). All animals were kept on a 12/12 light-dark cycle (lights on at 0700 h) with food and water available ad libitum. After weaning at postnatal day (P) 25 (P25), the animals were group-housed 2–4 per cage with same-sex littermates.

2.2. Activity-based anorexia (ABA) induction and behavioral controls

On P36, thirty-five male and thirty-eight female littermates were randomly assigned to one of four experimental groups – activity-based anorexia (ABA), exercise control (EX), food restriction control (FR) or control (CON). The ABA group was given access to a running wheel but were also food-restricted (N = 10 for male, N = 10 for female). The EX group of animals were given access to a running wheel but without food restriction, for assessing the effect of wheel running, alone (N = 9 for male, N = 8 for female). The FR group of animals underwent food restriction without wheel access for assessing the effect of food restriction, alone (N = 8 for male, N = 9 for female). The CON group of animals were singly housed, without food restriction and without wheel access (N = 8 for male, N = 11 for female). For animals, food consisted of both dry chow, PMI Mouse Diet 5001 pellets, not grounded (336 kcal per 100 g, 28.507% protein, 57.996% carbohydrates, 13.496% fat) and soft food (Clear H_2O DietGel 76A, 99.8 kcal per 100 g, 4.7% protein, 17.9% carbohydrates, 1.5% fat, 73.4% moisture). These foods were given from P36–60, both ad libitum, allowing the animal to choose between the two food sources. Caloric intake was calculated based on the daily change in weight (g) of dry chow and soft food, multiplied by calorie/g of each food. Body weight and caloric intake of animals from P40–43 can be found in Supplementary Table 1.

Starting at noon on P36, each animal in the ABA and EX group was placed in a standard home cage with a running wheel attached (Low-Profile Mouse Wheel, Med Associates, Inc., St. Albans, VT) and with ad libitum access to food, to record baseline running activity. Baseline (set to 100%) food intake and weight were calculated as the food intake and weight on P40. Starting at noon on P41 and until noon on P44, ABA and FR animals' food access became limited to only the first two hours of the dark cycle. To ensure that food was not available for the remaining 22 h per day, animals were moved to a fresh standard cage on P41, with transfer of some of the bedding from the previous cage, but with no

residual food crumbs. EX animals continued to have unrestricted access to food. Body weight, food intake, and wheel running activity of each mice were measured daily within 20 min prior to the start of the dark cycle and at 2100 h. After 3 days of FR, starting at 1 PM on P44, ad libitum food access was restored and the running wheel was removed from the cage. The animals were allowed to recover until the day of euthanasia (P60). Further details of the ABA induction procedure are as reported previously (Wable et al., 2015b; Chen et al., 2017a).

For the last experiment, on P36, nine α4-KO males and ten α4-KO females and their WT littermates ($N = 8$ for both male and female) were assigned to ABA rearing condition. The ABA procedure was the same as described above for the other experiments in this study.

2.3. Quantitative ultrastructural analysis of α4-immunoreactivity in pyramidal neurons of dorsal CA1 of the hippocampus

2.3.1. Animals used for electron microscopic immunocytochemistry

C57Bl/6 J WT male mice ($N = 8$ for CON and $N = 7$ for ABA) and female mice (N = 7 for CON and N = 8 for ABA) were euthanized on the morning of P60, between 8:00 and 10:30 am. The mice were deeply anesthetized with urethane (i.p. 0.34 g/g body weight), then transcardially perfused with 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde (PFA, EM Sciences, Hatfield, PA, USA). Glutaraldehyde-fixation was withheld until after immunocytochemistry, so as to optimize antigenicity. The brain of each mouse was removed from the skull, stored in 4% paraformaldehyde in 0.1 M phosphate buffer, and later blocked for slicing on a vibratome (Leica VT1000M) into 50-μm sections (Leica Microsystems GmbH, Wetzlar, Germany). Coronal sections containing optimal cross sections of the dorsal hippocampus (Bregma −1.58 ~ −1.94 mm) were collected.

2.3.2. α4-immunoreactivity/silver-intensified gold tissue processing

The primary antibody directed against the α4-subunit of $GABA_A$ receptor ($GABA_AR$) was obtained from Santa Cruz Biotechnology (catalog #SC7355, lot J1912, Antibody Registry #AB_640770, RRID:AB_640770). Previous studies demonstrated specificity of this antibody for the α4-subunit of $GABA_AR$ using three EM-immunocytochemical procedures: reduction of immunoreactivity at the plasma membrane and in the cytoplasm of pyramidal neurons in the CA1 of hippocampus of α4-KO animals at the transition between prepuberty and puberty onset (Sabaliauskas et al., 2012), which is when immunoreactivity for the α4 and δ subunits of $GABA_AR$ emerge at the spine plasma membrane of the hippocampal CA1 (Shen et al., 2007). Reduction of immunoreactivity was also verified in the CA1 when applying the antibody solution after preadsorption with the antigen corresponding to amino acids 1–14 of the α4-subunit and when the primary antibody was omitted from the incubation procedure (Sabaliauskas et al., 2012). This antibody has also been shown to recognize a single band at 67 kDa by Western blotting (Sanna et al., 2003; Griffiths and Lovick, 2005) and to recognize no band following preadsorption of the antibody with a peptide corresponding to the target sequence (Sanna et al., 2003). The secondary antibody was obtained from Electron Microscopic Sciences [Rabbit anti-goat IgG conjugated with ultra-small (0.8 nm) gold particles (catalog #25220, lot 20126/1)]. The silver intensification kit used was purchased from KPL (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA).

The immunocytochemical procedure commenced by incubating the free-floating sections in PBS azide containing 1% bovine serum albumin (Sigma Chem) and 1:100 dilution of the goat primary antibody directed against the α4 subunit of $GABA_ARs$. The free-floating sections were agitated continuously at room temperature for 3 days, after which time, excess unbound primary antibodies were removed by rinsing in PBS. Sections were then incubated overnight at room temperature in the rabbit secondary antibody, consisting of an anti-goat IgG conjugated to 0.8 nm colloidal gold particles. On the following day, excess unbound secondary antibodies were removed by rinsing in PBS, then post-fixed by immersing the sections in PBS containing 2% glutaraldehyde (EMSciences EM grade) for 10 min. After rinsing in PBS, sections were stored overnight at 4 °C, then processed for silver-intensification of the colloidal gold particles, so as to enlarge the gold particles to sizes detectable by EM. The silver-intensified colloidal gold particles (SIG) ranged in sizes from 10 to 100 nm, even though all procedures were run strictly in parallel. The silver-intensified sections were processed osmium-free (Phend et al., 1995), so as to avoid oxidation of the SIG particles. The heavy metals used to generate contrast were uranyl acetate, which also served to improve ultrastructural preservation (Terzakis, 1968; Lozsa, 1974) and Reynold's lead citrate. Vibratome sections were then infiltrated with EMBED-812, flat-embedded between two sheets of Aclar plastic, then ultrathin sectioned at a thickness setting of 70 nm at a cutting plane tangential to the vibratome-cut surface, so as to maximize capture of the EPON-vibratome section surface, where penetration by immunoreagents was maximal.

2.3.3. Electron microscopic image collection and analysis for α4-subunits of $GABA_A$ receptors

From the brain of each animal, two-hundred spines receiving asymmetric (presumably excitatory (Peters et al., 1991)) synapses in stratum radiatum (SR) of area CA1 in the dorsal hippocampus were analyzed at a direct magnification of 40,000×. Digitized images were captured using AMT's XR80 CCD camera system (Boston, MA) connected to the JEOL 1200XL electron microscope. Spines were identified using criteria described before (Aoki et al., 2014), which consisted of a prominence of thick postsynaptic density (PSD), lack of mitochondria or microtubules, parallel alignment of the plasma membrane associated with the PSD with that of the axon terminal containing clusters of vesicles. All spines encountered at the surface-most regions of vibratome-cut surfaces of sections were analyzed for the position and level of immunoreactivity to the α4-subunits, strictly in the order of encounter, so as to ensure randomness of sampling, until a minimum of 200 spines were encountered from tissue of each animal. The electron microscopist remained blind to the rearing condition and sex of the animal throughout the sessions for EM image capture and during quantitative analyses.

Dendritic shafts of pyramidal neurons were also sampled from SR of CA1 of the dorsal hippocampus of each animal. These were observed at a direct magnification of 20,000×, using the same camera and microscope as stated above, and also analyzed in the order that they were encountered along the vibratome-cut surfaces of sections. Dendritic shafts were identified by the presence of mitochondria and microtubules. Pyramidal neurons receive asymmetric synapses exclusively at dendritic spines, while aspiny interneurons receive excitatory inputs directly along dendritic shafts (Peters and Jones, 1984). Only those dendritic shafts that were coursing parallel to one another and lacking asymmetric synapses were sampled, to ensure that they were apical dendrites originating from pyramidal cells.

For both the spines and dendritic shafts, the location of SIG was noted as being at the plasma membrane or intracellular. The number of each type of SIG particles as well as the total number of SIG particles (plasmalemmal plus intracellular) was recorded. The length of the plasma membrane and area of each dendritic shaft were measured using the segmented line tool and polygon tool, respectively, of NIH's software, Image J (version 1.46r). The entire plasma membrane of dendritic shafts was not always visible. Only the visible plasma membrane was counted in the length measure used for analysis. The number of plasmalemmal SIGs for each animal was divided by the total length of the plasma membrane to obtain a density value (# SIG/unit plasma membrane length) for that animal. The total plasma membrane length sampled was equalized across all animals (~85 μm). The density of intracellular SIG was determined by calculating the ratio of intracellular SIG per area of dendritic shaft cytoplasm within which the SIG particles were found. The density of total dendritic shaft SIG was

determined by first obtaining the sum of intracellular and plasmalemmal SIGs, then dividing this sum value by the area of the shaft within which the SIG particles were found.

2.4. Statistical analysis

Experimenters remained blind to the sex and rearing condition of animals until the time when statistical analyses began. Normality of the distribution of measures was tested using the D'Agostino & Pearson omnibus normality test, Shapiro-Wilk normality test, and Kruskal-Wallis test. Two-way analysis of variance (ANOVA) was used to evaluate significance of the differences between the four groups within each sex, using food restriction and exercise as the two factors, followed by Fisher's Least Significant Difference (LSD) post hoc analysis. When three-way or two-way ANOVA was performed to evaluate the effect of experimental days on weight changes, food intake, and wheel running, within each sex, the day of experiment was used as repeated measure, followed by Fisher's LSD post hoc analysis. When three-way ANOVA was performed to evaluate the effect of experimental days, sex, and food restriction on wheel running, the day of experiment was used as repeated measure, sex and food restriction were used as two between groups measurements, followed by Fisher's LSD post hoc analysis. Pearson's correlation was computed to assess the significance of correlations for normally distributed variables. Spearman correlation was used if the measures were not distributed normally. Comparisons of the sex-differences in baseline body weight and food consumed were made using Student's *t*-test. All the results are expressed as mean ± SEM. *p*-values of < 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism Version 7.01, and IBM SPSS 24.0.

3. Results

The goal of this study was to investigate whether there are sex differences in behavioral responses and body weight of animals following the imposition of food-restriction stress during adolescence. We report on two behavioral parameters - food consumption and voluntary wheel running – together with body weight changes.

3.1. Food consumption during the days with limited food access are similar between the females and males

From P41 to P43, activity-based anorexia (ABA) and food restriction control (FR) animals' food access became limited to only the first 2 h of the dark cycle. Accordingly, the ABA and FR animals' food intake on the first day of food restriction (P41) were reduced dramatically, to about 20% of the baseline food intake on P40. Two-way ANOVA, using food restriction (ad libitum fed vs food restricted) and wheel access (no wheel access vs wheel access) as factors, showed main effects of food restriction on food intakes for both females (Fig. 1A) and males (Fig. 1B) on P41 (females: $F(1,34) = 189.517$, $p < 0.001$; males: $F(1,32) = 502.763$, $p < 0.001$), P42 (females: $F(1,34) = 127.993$, $p < 0.001$; males: $F(1,34) = 73.139$, $p < 0.001$), and P43 (females: $F(1,34) = 33.938$, $p < 0.001$; males: $F(1,34) = 109.888$, $p < 0.001$). ABA females increased food intake after three days of food restriction (P43) compared to the first day of food restriction (P41). Although slight, this increase was significant (Fig. 1A, $t(9) = 2.978$, $p = 0.016$), indicating an adaptation in females' feeding behavior. In contrast, no change of daily food intake was detected among ABA males (Fig. 1B). Female and male exercise controls (EX) animals reduced their food intake after 8 days of wheel access, on P43, compared to their food intake on P41 (female, $t(7) = 2.65$, $p = 0.033$; male, $t(8) = 4.564$, $p = 0.002$). No difference in baseline caloric intake was found between sexes (Supplementary Table 1).

3.2. Body weight changes following food restriction are similar between females and males

Large differences in baseline body weight were found between sexes among all treatment groups (Supplementary Table 1). To compare the treatment effects between sexes, each animal's average daily body weight change was normalized to the individual's weight on P40, corresponding to the day preceding food restriction. As expected, females (Fig. 1C) and males (Fig. 1D) of both the FR and ABA groups lost weight following food restriction. Repeated-measures three-way ANOVA, using food restriction (ad libitum fed vs food restricted) and wheel access (no wheel access vs wheel access) as between group measurements and experimental days (6 days from P38–43) as repeated measures, showed main effects of food restriction for both females (Fig. 1C $F(1,34) = 204.483$, $p < 0.001$) and males (Fig. 1D, $F(1,31) = 184.197$, $p < 0.001$). Among females, there was no main effect of wheel access. Nor were there interactions of wheel access with food restriction or succeeding days of food restriction (wheel access: $F(1,34) = 0.979$, $p = 0.329$; days x food restriction x wheel access 3 ways interaction: $F(5,170) = 0.777$, $p = 0.567$, Food restriction × wheel access: $F(5,170) = 1.626$, $p = 0.156$). Among males, there was no main effect of wheel access ($F(1,31) = 1.907$, $p = 0.177$) nor interaction of days x food restriction × wheel access (3 ways interaction: $F(5,155) = 1.547$, $p = 0.179$), but marginally significant interaction between the food restriction days and exercise ($F(5,155) = 2.133$, $p = 0.064$). On P42, two-way ANOVA showed significant main effect of wheel access for males (Fig. 1D, $F(1,31) = 5.226$, $p = 0.03$), and marginal effect of wheel access for females (Fig. 1C, $F(1,34) = 3.607$, $p = 0.066$). FR males reduced significantly less weight on the second (P42, $t(16) = 3.003$, $p = 0.008$) and the third (P43, $t(16) = 1.785$, $p = 0.093$) days of food restriction compared to the ABA males. In contrast, no difference was found between FR females and ABA females on corresponding days. No difference was found in weight changes between EX and control (CON) animals of either sex.

3.3. Wheel running activities following food restriction are similar between sexes although males reduce wheel running earlier

Previously, we had shown that food restriction robustly increases voluntary wheel running of adolescent female mice and that this behavior contributes to weight loss during the food-restricted period (Chowdhury et al., 2013; Wable et al., 2015b). However, it remained untested whether adolescent males would also respond to food restriction with increased wheel running. This question was addressed by analyzing the daily wheel running pattern of both female and male mice that had undergone ABA induction and compared to age-matched EX animals of both sexes that had access to the running wheel without food restriction. The baseline wheel running activity was determined by taking an average of the daily activity of the two days preceding the onset of food restriction for the ABA group (P39–40). The wheel running activity following food restriction for the ABA animals was quantified by taking an average of the daily activity during the first two days when food restriction was imposed (P41–42). The running activities on the corresponding days for the EX animals were also quantified.

As was shown previously, ABA females increased their wheel running after the onset of food restriction (mean increase = 10.3665 km, Fig. 2A). No increase was found in the running activity of EX females on the corresponding days. In males, similar results were found. ABA males increased their running after the onset of food restriction (mean increase = 14.2475 km, Fig. 2B). No increase was found in the running activity of EX males on the corresponding days. Repeated measures three-way ANOVA, using sex (male vs female) and food restriction (ABA vs EX) as between group measurements and experimental days (P39–40 vs P41–42) as repeated measures, revealed a significant food restriction X experimental days interaction ($F(1,33) = 30.619$, $p < 0.001$) and significant main effects of food restriction (F

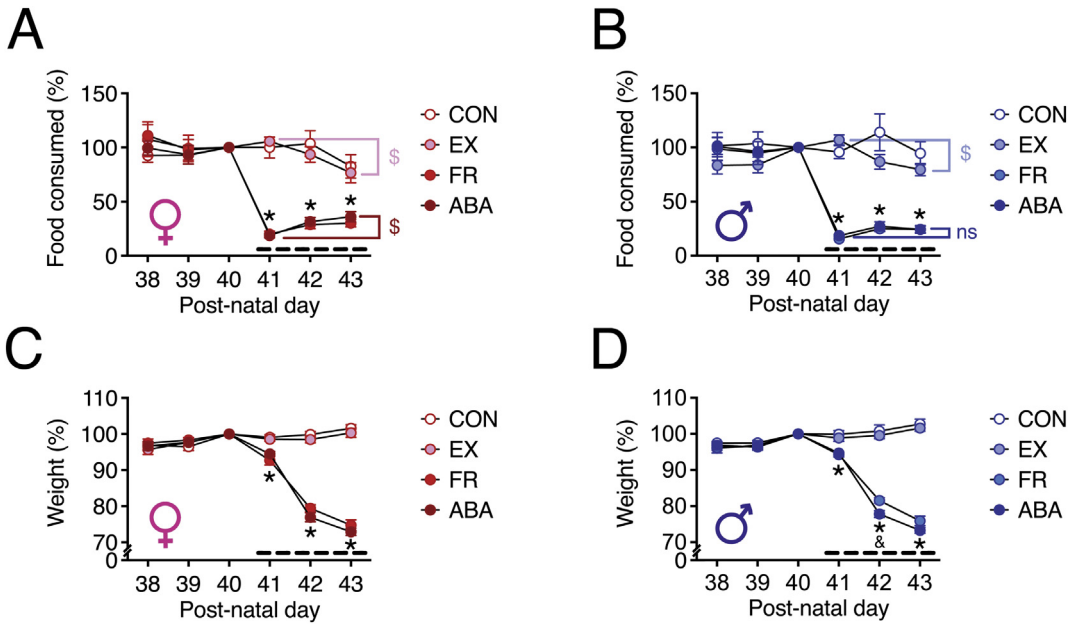

Fig. 1. Daily weight changes and food consumed.

Panels A and B: Food consumed daily in 4 groups of female (panel A) and male (panel B) animals from P38–43. The average daily food intake (kcal) relative to P40 was calculated as the food intake during each of the day divided by the total food intake of the animal on P40. Exercise alone reduces animal's food intake in both females and males from P41 to P43. ABA females increased food intake on day 3 of food restriction (P43) compared to the first 24 h of food restriction (P41), while no change of daily food intake was detected among ABA males during the three days of food restriction.

Panels C and D: Daily weight changes in 4 groups of female (panel C) and male (panel D) animals from P38–43 are shown. The average daily body weight relative to P40 was calculated as the weight on each day, divided by the animal's weight on P40. Food restriction reduces animal's weight in both male and female mice. No difference was found between food restriction controls (FR) and activity-based anorexia (ABA) groups of females. However, FR males reduce significantly less weight on the second (P42) and the third (P43) day of food restriction compared to the ABA males.

* indicates significant main effects ($p < 0.05$) of food restriction. & indicates significant main effect of wheel access. $ indicates $p < 0.05$. "ns" indicates no difference. The dotted line parallel to the x-axis indicates the days of food restriction for the FR and ABA groups.

(1,33) = 10.917, $p = 0.002$) and experimental days ($F(1,33) = 77.911$, $p < 0.001$). The main effect of sex did not reach significant level ($F(1,33) = 2.095$, $p = 0.157$), which indicated that there was overall no difference in wheel running between sexes.

Disruption of circadian rhythm following food restriction was previously shown for adolescent female mice that had undergone ABA induction (Chowdhury et al., 2013). We sought to investigate whether changes in circadian rhythm would be different between female and male animals. ABA females showed food restriction-evoked increases of wheel running activities during the light cycle (Fig. 2C; 7 am–1 pm, $t(9) = 2.143$, $p = 0.039$; 1 pm–7 pm, FAA, $t(9) = 4.494$, $p < 0.001$) and during the second half of the dark cycle (1 am–7 am; $t(9) = 4.927$, $p < 0.0001$). ABA males showed a similar pattern of changes in circadian rhythm following food restriction (Fig. 2D; 7 am–1 pm, $t(9) = 3.431$, $p = 0.008$; 1 pm–7 pm, FAA, $t(9) = 7.892$, $p < 0.0001$; 1 am–7 am: $t(9) = 10.82$, $p < 0.0001$).

Although subtle, sex differences emerged through analysis of animals' daily wheel running activities (Fig. 2E and F). Repeated measures three-way ANOVA, using sex (male vs female) and food restriction (ABA vs EX) as between-group measurements and experimental days as repeated measures, revealed a significant food restriction X experimental days interaction ($F(5,165) = 10.755$, $p < 0.001$), a significant main effect of postnatal days ($F(5,165) = 20.42$, $p < 0.001$), and a marginal significant main effect of sex ($F(1,33) = 3.223$, $p = 0.082$).

On the first day of food restriction, ABA animals of both sexes increased their running much more than EX animals which were fed ad libitum (female: $t(16) = 2.885$, $p = 0.011$; male: $t(17) = 3.90$, $p = 0.001$) (Fig. 2E and F). ABA females remained hyperactive on the second day of food restriction, as no difference was found between the wheel running activity on the first two days of food restriction (P41 and P42; $p = 0.23$). However, the wheel running activity of ABA females on the third day of the food restriction (P43) was significantly lower than the first two days of food restriction (P41: $p < 0.0001$; P42: $p < 0.0001$). The wheel running activity of the ABA females on P43 trended to be lower than that of the EX females on the corresponding day (P43; $t(16) = 1.812$, $p = 0.08$).

ABA males exhibited reduction in wheel running earlier - on the second day of food restriction, as the wheel running activity of ABA males on the second day of the food restriction (P42) was lower than on the first day of food restriction (P41, $p = 0.0002$) (Fig. 2F). By P43, the wheel running activity of ABA males returned to the level preceding food restriction (compared to P40: $p = 0.89$). By P43, no difference was found in wheel running activities between ABA and EX males ($t(17) = 1.398$, $p = 0.18$) (Fig. 2F).

3.4. Correlation analyses reveal striking sex differences in the relationship between food restriction-induced weight changes and wheel running during the hours of food-access: Females exhibit correlation but males do not

Food restriction of the ABA and FR groups began at 1 pm on P41 (FR1), 6 h before the light turns off and resumed after 9 pm of that day (Fig. 3A). By the end of the initial 6 h of food restriction, weight loss was already detectable for the ABA females (mean decrease = 5.5538%, Fig. 3B). This weight change was significantly larger than the weight changes shown by EX females over the same period of time but in the absence of food restriction (mean change = −1.464%; Fig. 3B; $t(16) = 4.592$, $p < 0.001$). ABA males lost weight similarly after the initial 6 h of food restriction (mean decrease = 5.3035%, Fig. 3C). This weight change was significantly larger than the weight changes shown by EX males (mean change = −1.13%; Fig. 3C; $t(17) = 4.383$, $p < 0.001$).

To determine whether the excessive running of animals during the

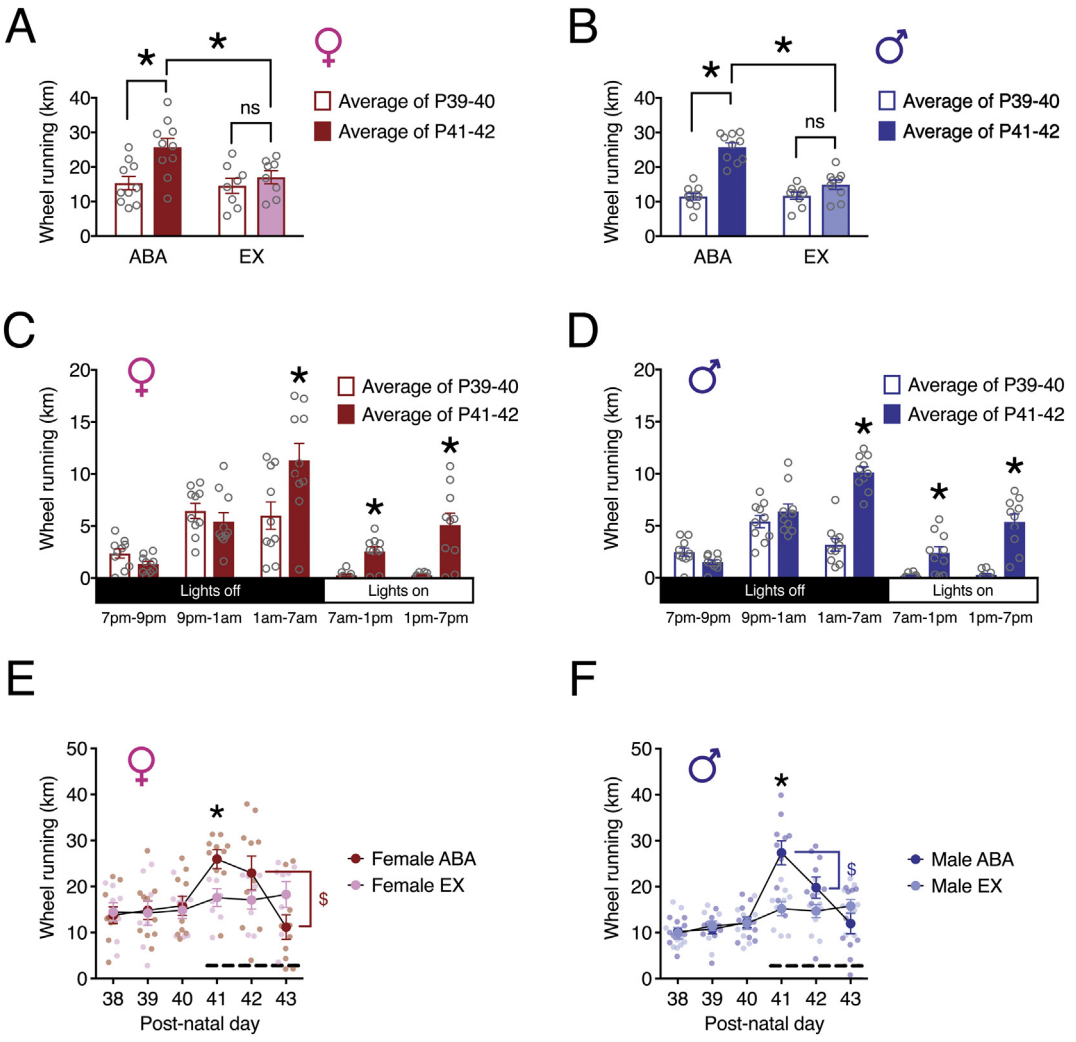

Fig. 2. Running wheel activities of ABA and EX animals.
Panels A and B: Averaged daily wheel activity, measured as the distance run on the wheel, of ABA and EX females (panel A) and males (panel B) preceding and following food restriction. Food restriction significantly increased wheel running activity in both ABA females and males. The white bars represent average baseline wheel activity of ABA and EX animals during the hours indicated under the x-axis at ages P39–40. The color bars represent average wheel activity measured during the first two days of the food restriction phase for ABA animals and corresponding days for EX animals that were fed ad lib during the phase, P41–42. Here and in all other panels, each small circle represents value of an individual. * indicates $p < 0.05$, "ns" indicates no difference.
Panels C and D: Food restriction increased wheel running activities during the second half of the dark cycle (1–7 am), as well as the first half (7 am–1 pm) and the second half (1–7 pm, food anticipatory activity, FAA) of the light cycle for both females (panel C) and males (panel D). * indicates $p < 0.05$, comparing color bars with respective white bars of the same group.
Panels E and F: Daily wheel activity of ABA and EX females (panel E) and males (panel F). The dotted line parallel to the x-axis indicates the days of food restriction for the ABA group. During food restriction, ABA animals increased their running much more than the EX animals which were fed ad libitum for both females and males during the first two days of food restriction. Wheel running activity of ABA females on the third day of the food restriction (P43) was significantly lower than that during the first two days of food restriction (P41 and P42). The wheel running activity of the ABA females on P43 was also lower than that of the EX females on the corresponding day (P43). The wheel running activity of ABA males on the second day of the food restriction (P42) was lower than that on the first day of food restriction (P41). No difference is found in wheel running activities between ABA and EX males on P43. Larger data points and bars represent means ± SEM for each group. $ indicates $p < 0.05$.

food-restricted periods was induced by weight loss, correlation analyses of factors along a series of time points was performed. For ABA females, the initial weight change, measured at 7 pm on FR1, correlated negatively and strongly with wheel running activity during the 2 h of food access from 7 to 9 pm ($R = -0.8461$, $p = 0.001$, Fig. 3B). This correlation indicates that, paradoxically, the ABA animals that lost more weight initially ran more during the time with food access. This is probably due to greater extent of anxiety experienced by animals that lost the greatest amount of weight (Wable et al., 2015b). In contrast, such a correlation was absent for the ABA males, EX females, and EX males ($R = 0.119$, $p = 0.745$, Fig. 3C; $R = -0.143$, $p = 0.752$, Fig. 3B; $R = -0.106$, $p = 0.785$, Fig. 3C, respectively).

To determine whether the negative relationship persisted to the next day, the 24-h weight change from 7 pm on P41 (FR1) to 7 pm on P42 (FR2) was calculated. As expected, ABA females lost weight due to food restriction (mean decrease = 17.7906%, Fig. 3D). In sharp contrast to the negative correlation observed for the initial 6 h of FR1 (Fig. 3B), the weight change over the subsequent 24-h period correlated positively with wheel running activity during the hours of food access that followed immediately after ($R = 0.86$, $p = 0.001$, Fig. 3D). This positive correlation indicates that the more that ABA females lost weight during the day before, the less that they ran during the hours of food access on the day after. Such a correlation was absent for ABA males, EX females, or EX males ($R = 0.242$, $p = 0.501$, Fig. 3E; $R = -0.412$, $p = 0.311$,

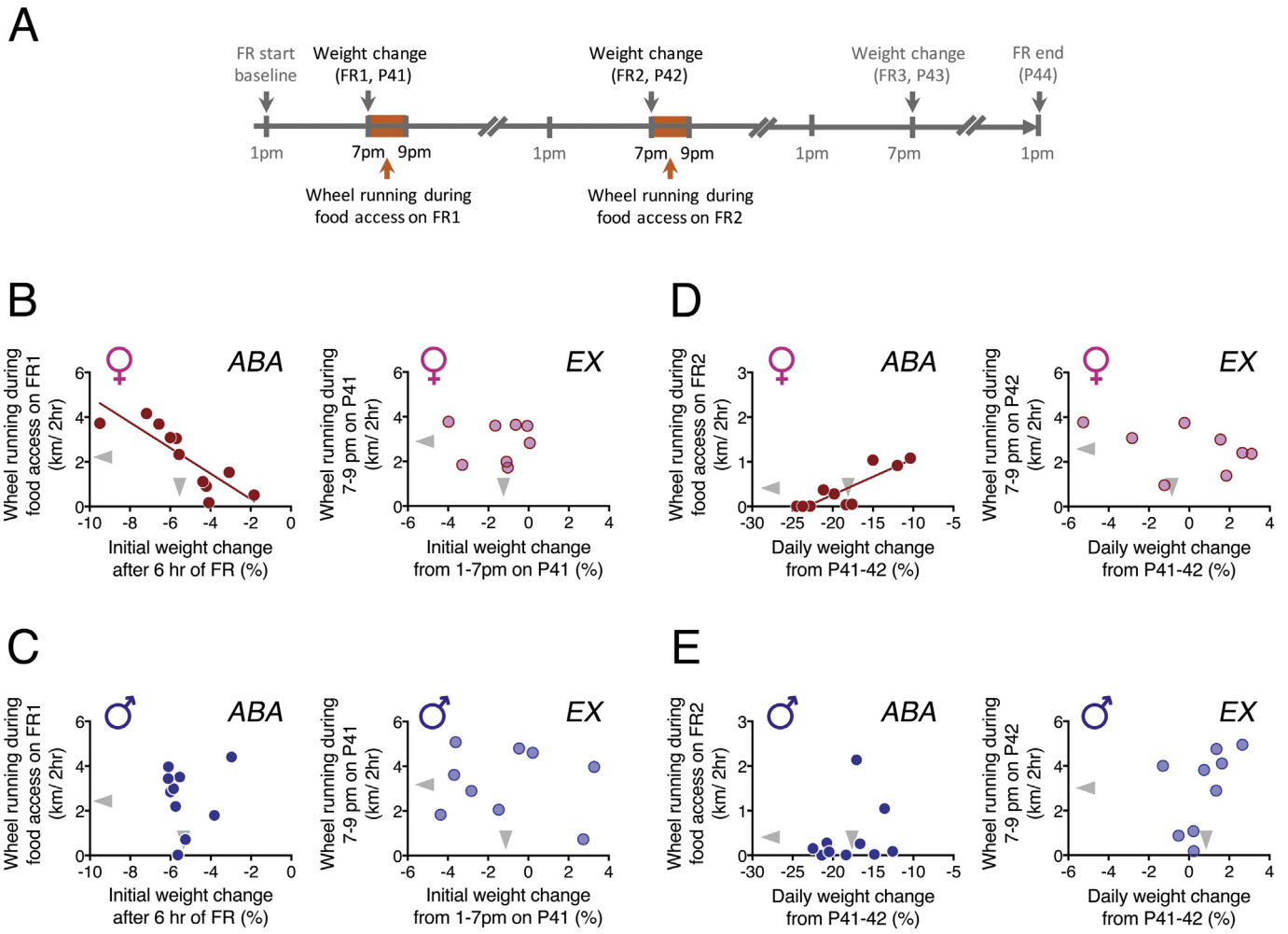

Fig. 3. Weight changes evoked by food restriction correlate with wheel activity during food access for the ABA females, but not for the ABA males, nor with wheel activity in the EX animals during the corresponding hours.
Panel A: A schematic of the measurements of weight changes in relation to wheel running activities during food access period. Panel B: The weight changes within the initial 6 h of food restriction (1–7 pm) correlates negatively with wheel activity during the hours of food access (7–9 pm) of ABA females on the first day of food restriction (FR1), but not for the EX females on the corresponding day. Panel C: For both the ABA males and the EX males, the weight change from 1 to 7 pm on P41 are not correlated with wheel activities during the hours of 7–9 pm on P41. Panel D: The weight changes from P41–42 (7–7 pm) correlate positively with the wheel activity during the hours of food access (7–9 pm) of ABA females on the second day of food restriction (FR2), but not for EX females on the corresponding day. Panel E: For both ABA males and EX males, the weight changes from P41–42 (7–7 pm) are not correlated with the wheel activities during 7–9 pm on P42. Regression lines indicate a significant correlation with $p < 0.05$. Grey arrows indicate the mean value of each group.

Fig. 3D; $R = 0.509$, $p = 0.162$, Fig. 3E, respectively). All but one ABA male ran < 0.3 km during the 2-h of food access, which is much less than the running activity shown by EX males (mean activity = 2.962 km, Fig. 3E).

3.5. Correlation analysis reveals a strong sex-difference in the relationship between food restriction-induced weight changes and food anticipatory activity (FAA): males exhibit correlation but females do not

FAA is an important feature of the ABA model. In the ABA model, a rodent placed in a cage with full access to a running wheel and on a restricted feeding schedule shows increases in its wheel running activity level during the hours prior to feeding (Wu et al., 2014); (Fig. 4A). As the name implies, FAA is interpreted to reflect the animal's learning of the circadian feeding schedule (Luby et al., 2012; Michalik et al., 2015). Decrease of FAA is partially or potentially contributing to reduction of body weight loss or improved survival rate (Lambert and Porter, 1992; Hillebrand et al., 2005; Atchley and Eckel, 2006; Verhagen et al., 2009; Klenotich et al., 2012; Wu et al., 2014; Chen et al., 2016). As shown previously (Chowdhury et al., 2013; Chen et al., 2016), ABA females expressed FAA during the 6-h preceding the feeding hours during FR2, corresponding to 30-h of food restriction (mean running = 6.439 km, Fig. 4B). The wheel running activity of ABA females was significantly higher than the running shown by EX females over the same period of time but in the absence of food restriction (mean activity = 0.1163 km, Fig. 4B, $t(16) = 3.418$, $p = 0.004$).

FAA was also found in ABA males. ABA males expressed FAA after 30-h of food restriction (mean running = 6.001 km, Fig. 4C). This running activity was also significantly higher than the running by EX males (mean running = 0.0932 km, Fig. 4C, $t(17) = 2.775$, $p = 0.013$).

Both the ABA females and males showed FAA on P43, after 54-h of food restriction (mean running = 3.738 km, 4.716 km, respectively). These running activities remained higher than the running shown by EX females or males (mean activity for EX females = 0.041 km, Fig. 4D; mean activity for EX males = 0.1268 km, Fig. 4E). No sex-related difference was found on the FAA on P42 or P43 between ABA females and males (P42: $t(18) = 0.1686$, $p = 0.868$; P43: $t(18) = 0.4355$, $p = 0.668$).

To determine whether the excessive FAA of the animals during food restriction was induced by the weight loss during the day before, we

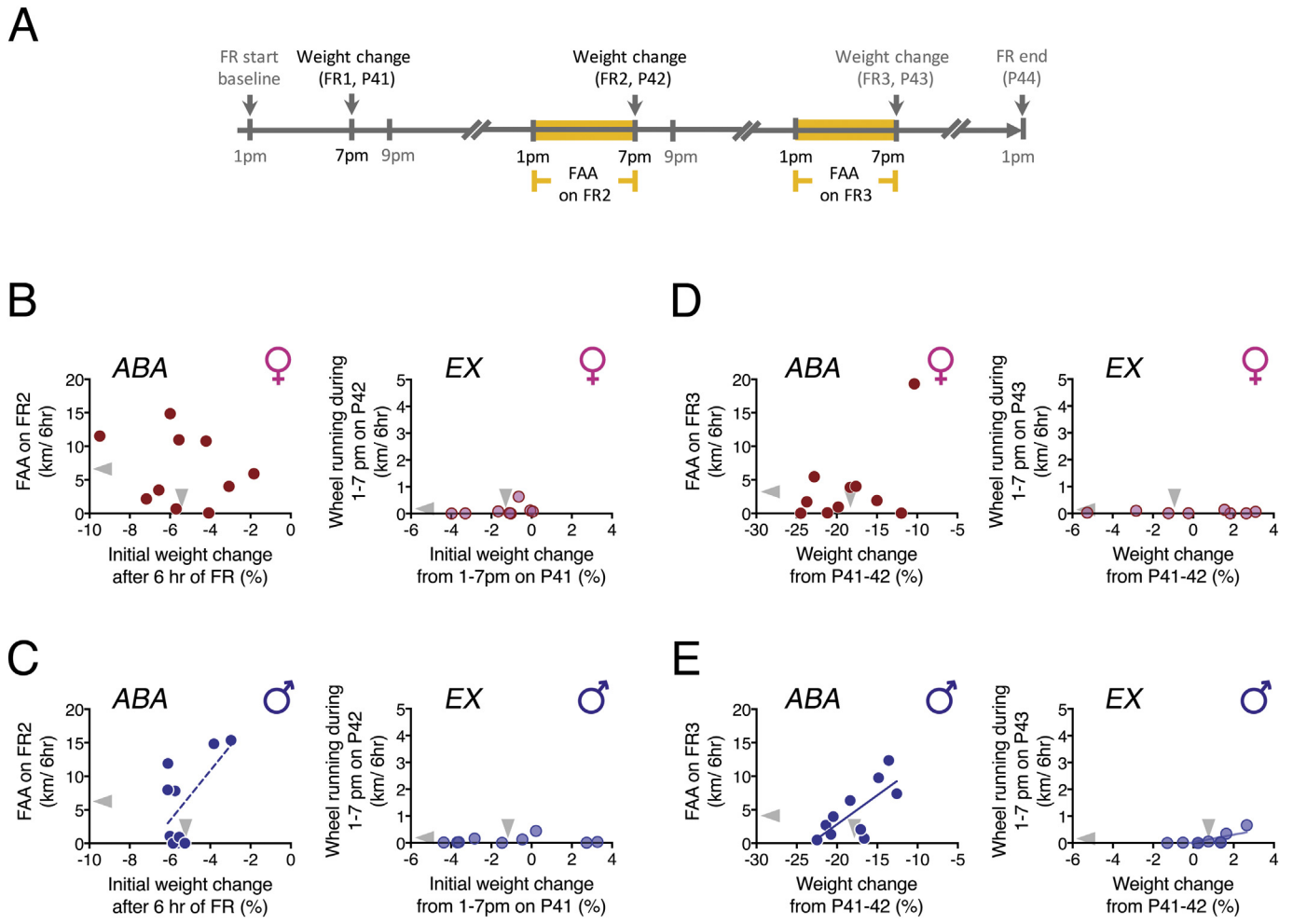

Fig. 4. Weight changes evoked by food restriction are correlated with FAA of ABA males, but not for ABA females, nor with the wheel activity of EX animals of either sexes

Panel A: A schematic of weight changes measured in relation to FAA. Panel B: For both female ABA and EX groups, the initial weight change from 1 to 7 pm on P41 are not correlated with the wheel activities during 1–7 pm on P42. Panel C: The weight changes within the initial 6 h of food restriction (1–7 pm) correlates positively with the food anticipatory activity (1–7 pm) of ABA males on the second day of food restriction (FR2), but not for EX males on the corresponding day. Panel D: For both female ABA and EX groups, weight changes from P41–42 (7–7 pm) are not correlated the wheel activities during 1–7 pm on P43 (FAA on FR3). Panel E: Weight changes from P41–42 (7–7 pm) correlate positively with the food anticipatory activity (1–7 pm) of ABA males on the third day of food restriction (FAA on FR3), but not for EX males on the corresponding day. Regression lines indicate a significant correlation with $p < 0.05$. Dash regression lines indicate a marginal significant correlation with $p = 0.057$. Grey arrows indicate the mean value of each group.

assessed the extent of correlation of weight changes of the animals to their FAA on the next day. For ABA males, the initial weight change following 6-h of food restriction on P41 correlated positively with FAA 1-7 pm on P42 ($R = 0.618$, $p = 0.057$, Fig. 4C). This correlation indicates that ABA males which lost more weight initially showed less FAA next day. ABA females, EX females, and EX males showed no such correlation ($R = -0.24$, $p = 0.504$, Fig. 4B; $R = 0.524$, $p = 0.197$, Fig. 4B; $R = 0.217$, $p = 0.581$, Fig. 4C, respectively).

We determined whether the positive relationship remained on the next day. For ABA males, the 24-h weight change from P41 to P42 correlated positively and more strongly with FAA on the next day ($R = 0.73$, $p = 0.017$, Fig. 4E). EX males also showed similar positive correlation between the 24-h weight change and running during the period of 1 pm to 7 pm on the next day ($R = 0.8$, $p = 0.014$, Fig. 4E). However, the ABA females and EX females showed no such correlation ($R = 0.382$, $p = 0.279$, Fig. 4D; $R = -0.009$, $p = 0.984$, Fig. 4D, respectively).

3.6. Food restriction-induced up-regulation of α4-subunit immunoreactivity at asymmetric (presumably excitatory) synapses of stratum radiatum of the dorsal hippocampus in ABA females but not in ABA males

Up-regulation of α4-subunits of $GABA_ARs$ was previously shown to occur within the hippocampus of adolescent female ABA rats (Aoki et al., 2012; Aoki et al., 2014; Aoki et al., 2017) and mice (Wable et al., 2015a). However, none of those studies compared α4-immunoreactivity between the sexes. We sought to investigate whether the changes in α4-subunit immunoreactivity would be different between female and male animals, and if so, whether such a difference could underlie the sex-differences in modulation of wheel running during different circadian compartments by weight changes.

While animals of all four groups showed α4-subunit immunoreactivity in dendritic shafts (Fig. 5A and B), two-way ANOVA revealed no significant main effect of sex or ABA treatment on α4-immunoreactivity at the plasma membrane (Fig. 5C, top panel), at intracellular compartments (Fig. 5C, middle panel), or total labeling (total = plasma membrane plus intracellular) in dendritic shafts (Fig. 5C, bottom panel).

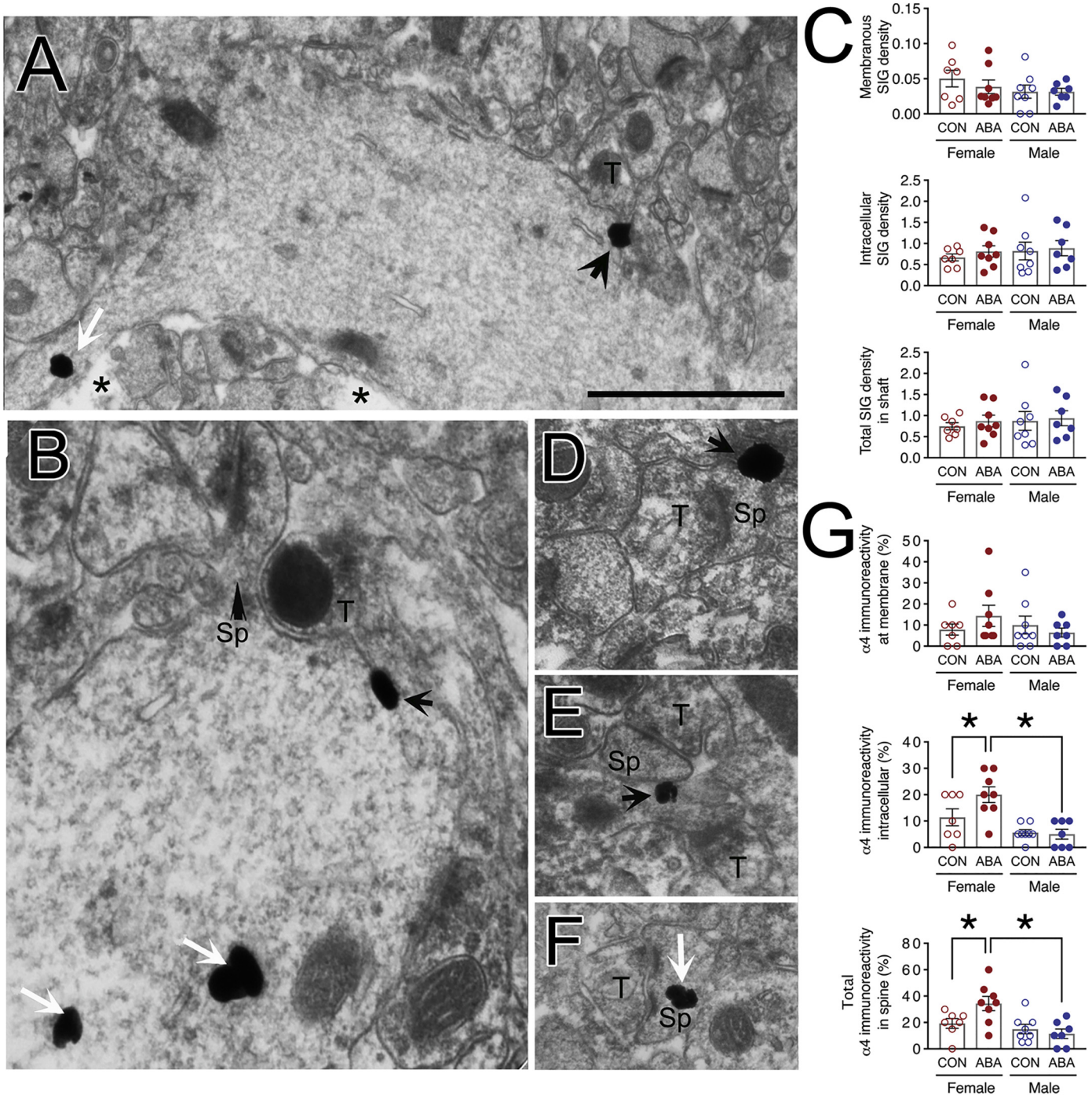

Fig. 5. Food restriction induces upregulation of α4-subunit immunoreactivity near asymmetric (presumably excitatory) synapses in stratum radiatum of the dorsal hippocampus of ABA female but not of ABA males. Panels A–B, D–F: The electron micrographs show the range of subcellular locations where a4-immunoreactivity can be found. α4-subunit immunoreactivity was detected by the presence of SIG (silver-intensified gold) labels, which occurred on the plasma membrane (black short arrows) and intracellularly (white arrows) within dendritic shafts (Panels A, B) and spines (Panels D, E, F). T = terminal; Sp = dendritic spine. Plasmalemmal SIGs on dendritic shafts are near terminals forming symmetric, presumably inhibitory, synapses. Synapses associated with dendritic spines are asymmetric, based on the presence of thick postsynaptic densities, and presumably excitatory. These micrographs were taken from tissue of a CON female (Panel A), a CON male (Panel B), and females that underwent ABA (Panels D, E, F). Asterisks in panel A indicate the surface-most regions of vibratome sections, where transitions to no-tissue zones are apparent. Calibration bar equals 1 μm for panels A, D, E and F and equals 640 nm for panel B. Panel C: Group comparison of the density of α4-subunit immunoreactivity at dendritic shafts. ABA did not evoke a statistically significant difference from the CON in the plasmalemmal (upper panel), intracellular (middle panel), or total (plasmalemmal plus intracellular) labeling (lower panel) for females or males. Panel G: Group comparison of α4-subunit immunoreactivity at the plasma membrane of dendritic spines, at intracellular sites of dendritic spines, and generally within dendritic spines (plasmalemmal plus intracellular). ABA did not evoke a statistically significant difference from CONs in membranous labeling (upper panel) of either sex. For females, α4-subunit immunoreactivity was significantly increased at intracellular sites of dendritic spines (middle panel) and generally in association with dendritic spines (plasma membrane plus intracellular components) (lower panel), relative to CON tissue. Such a difference was not observed for the ABA males. * indicates $p < 0.05$.

Within dendritic spines (Fig. 5D–F), which are the sites for excitatory synaptic inputs (Yuste, 2010), two-way ANOVA revealed a significant main effect of sex ($F(1,26) = 18.15$, $p < 0.001$) and marginally significant sex × ABA interaction ($F(1,26) = 3.546$, $p = 0.071$) for intracellular labeling of α4-subunit immunoreactivity (Fig. 5G, middle panel). Post hoc analysis revealed that female ABA hippocampi exhibited significantly greater intracellular α4-subunit immunoreactivity compared with ABA males ($p < 0.001$) and CON females ($p = 0.02$). Two-way ANOVA also revealed a significant main effect of sex ($F(1,26) = 10.5$, $p = 0.003$) and sex × ABA interaction ($F(1,26) = 4.929$, $p = 0.035$) for overall α4-subunit immunoreactivity at spines (plasmalemmal + intracellular compartments) (Fig. 5G, bottom panel). Post hoc analysis revealed that ABA females exhibited significantly greater α4-subunit immunoreactivity compared with ABA males ($p < 0.001$) and CON females ($p = 0.018$) at spines. While animals of all four groups showed α4-subunit immunoreactivity at the plasma membrane of asymmetric (presumably excitatory, (Peters et al., 1991)) synapses, two-way ANOVA revealed no significant main effect of sex ($F(1,26) = 0.564$, $p = 0.46$) or ABA ($F(1,26) = 0.145$, $p = 0.706$). Although female ABA tissue exhibited a greater occurrence of α4-subunit immunoreactivity at plasma membrane, relative to female CON tissue, this difference did not reach statistical significance because of the large variance (Fig. 5G, top panel). This observation led us to wonder whether the variance, especially within the ABA groups, could be related to individual differences in reactivity to environmental factors. To test this idea, we determined the Pearson correlation between α4-subunit immunoreactivity and each animal's response to food restriction and wheel access.

3.7. α4-subunit immunoreactivity at dendritic shaft of stratum radiatum of the dorsal hippocampus is correlated with wheel running activity induced by food restriction in ABA females but not in ABA males

For the tissue obtained from ABA females, Pearson correlation analysis revealed a negative relationship between the density of α4-subunit labeling at plasma membrane of dendritic shafts and food restriction-induced increase in wheel running, calculated as the extent to which each animal's wheel running activity changed during the three days of food restriction, relative to its running activity during the three days preceding food restriction (Fig. 6A, $R = -0.719$, $p = 0.044$). This negative correlation indicates that the greater that an animal expressed α4-subunit immunoreactivity at plasma membrane of dendritic shafts, the less excessive was the running induced by food restriction. Membranous α4-subunit immunoreactivity at dendritic shaft in ABA males did not correlate with their food restriction-induced increase in wheel running (Fig. 6E, $R = -0.337$, $p = 0.46$).

For the tissue obtained from ABA females, Pearson correlation analysis revealed negative relationships between membranous α4-subunit immunoreactivity at dendritic shaft and the average wheel running during the following three periods: the three food-restricted days (Fig. 6B, $R = -0.651$, $p = 0.081$); the last two food-restricted days (FR2 + FR3, Fig. 6C, $R = -0.612$, $p = 0.1$); and the last day (FR3, Fig. 6D, $R = -0.694$, $p = 0.056$). The correlations were slightly weaker than that obtained after relating individual differences in food restriction-induced increases, presumably because individual differences in baseline activity, which is subtracted to calculate food restriction-induced increases, is another source for variance in individual differences (Fig. 6A). Nevertheless, these negative correlations (Fig. 6B) also indicate that the greater the expression of α4-subunit immunoreactivity at dendritic shafts, the less excessive was that animal's running during food restriction, especially during the later phase of food restriction. On the other hand, the same measurements of wheel running activity of ABA males did not correlate with membranous α4-subunit immunoreactivity at dendritic shafts (Fig. 6F, $R = 0.436$, $p = 0.328$). Males with the highest levels of α4-subunit immunoreactivity showed the highest amount of wheel running activity during the last two days of food restriction (FR2 + FR3, Fig. 6G, $R = 0.741$, $p = 0.057$; FR3, Fig. 6H, $R = 0.697$, $p = 0.082$).

3.8. α4-subunit immunoreactivity near excitatory synapses is correlated with weight loss during food restriction of ABA females but not of ABA males

For the tissue obtained from ABA females, Pearson correlation analysis revealed a strong positive relationship between the frequency of α4-subunit immunoreactivity on spinous plasma membrane and weight change after 3 days of food restriction, calculated as the extent to which each animal's body weight changed during food restriction, relative to its body weight just before food restriction began (Fig. 7A, $R = 0.856$, $p = 0.007$). This positive correlation indicates that the greater that an animal expressed α4-subunit immunoreactivity at spinous plasma membrane, the more body weight the animal retained during the food-restricted period. No such correlation was observed for ABA males (Fig. 7E, $R = -0.075$, $p = 0.873$).

3.9. α4-subunit immunoreactivity near excitatory synapses is correlated with wheel running activity during the first day of food restriction in the female but not in the male ABA animals

For the tissue obtained from ABA females, Pearson correlation analysis revealed a strong negative relationship between the frequency of α4-subunit immunoreactivity on spinous plasma membrane and wheel running activity, summed over 24 h of the first day of food restriction (Fig. 7B, $R = -0.725$, $p = 0.04$). This negative correlation indicates that greater the α4-subunit immunoreactivity at spinous plasma membrane, less excessive was the wheel running activity during the first day of food restriction. No such correlation was observed for ABA males (Fig. 7F, $R = 0.509$, $p = 0.244$). Pearson correlation analysis revealed no relationship between the frequency of α4-subunit immunoreactivity on spinous plasma membrane and wheel running activity on the last two days of food restriction (FR2: $R = 0.183$, $p = 0.664$; FR3: $R = 0.584$, $p = 0.128$) for ABA females. Similarly, no correlation was found in ABA males (FR2: $R = 0.105$, $p = 0.823$; FR3: $R = -0.633$, $p = 0.127$).

3.10. ABA males differ from ABA females in the circadian compartments of wheel running activity that are correlated with α4-subunit immunoreactivity near excitatory synapses

For the tissue obtained from ABA females, Pearson correlation analysis revealed a strong negative relationship between the frequency of α4-subunit immunoreactivity on spinous plasma membranes and food restriction-induced wheel running during food access of the first day of food restriction, calculated as the extent to which each animal ran during food access period (7-9 pm) of the first day of food-restricted, relative to its running activity during 7–9 pm on days preceding food restriction (Fig. 7C, $R = -0.77$, $p = 0.025$). This negative correlation indicates that greater the α4-subunit immunoreactivity at spinous plasma membrane, less excessive was the running of the animal during the food-access period of the food-restricted day. Plasmalemmal α4-subunit immunoreactivity at dendritic spines of ABA males showed no such correlation (Fig. 7G, $R = -0.179$, $p = 0.7$).

One the other hand, for the tissue obtained from ABA males, Pearson correlation analysis revealed a strong positive relationship between the intracellular α4-subunit immunoreactivity within spines and food restriction-induced FAA, calculated as the extent to which each animal exhibited increased FAA (from 1 to 7 pm) while food-restricted, relative to its running activity during 1-7 pm on days preceding food restriction (Fig. 7H, $R = 0.893$, $p = 0.007$). This positive correlation indicates that greater the intracellular α4-subunit immunoreactivity in spine, the more FAA that the animal showed during the food-restricted days. Intracellular α4 subunit immunoreactivity at dendritic spine in ABA females did not correlate with their food

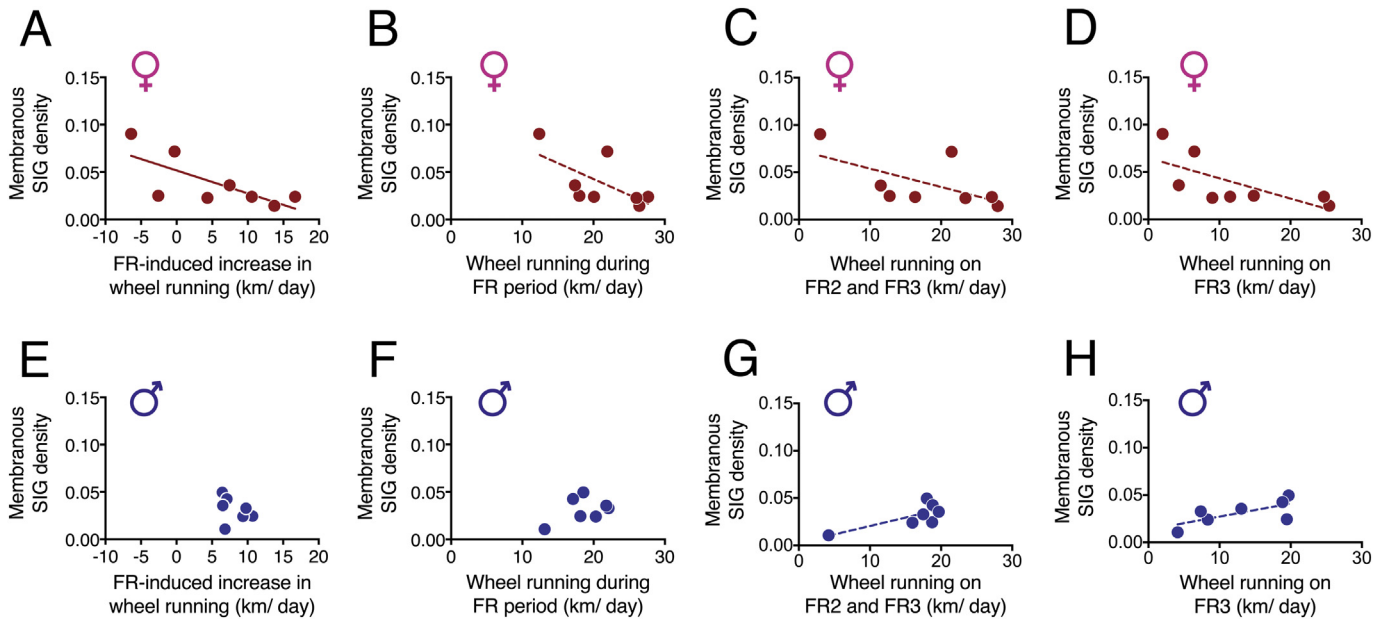

Fig. 6. Density of α4-subunit immunoreactivity at dendritic shaft plasma membranes correlates with wheel activity during food restriction for ABA females, but not for ABA males.

Panels A and B: Food restriction (FR)-induced increase in wheel running activity was calculated as the average running activity during the three food-restricted days minus the average wheel running activity during the three days preceding food restriction. This value is negatively correlated with the density of α4-subunits at the plasma membrane of ABA females (panel A), but not of ABA males (panel E). Females with the highest levels of α4-subunit immunoreactivity showed the least amount of food restriction-induced increase in wheel running. Panels B – D: For ABA females, the average running activity during the three food-restricted days (panel B), the average running activity during the last two day of food restriction (FR2 + FR3, panel C), and the wheel running activity on the last day (FR3, panel D), are negatively correlated with the density of α4-subunits at the plasma membrane. Females with the highest levels of α4-subunit immunoreactivity showed the least amount of wheel running activity during these food-restricted periods (panels B, C and D). Panels F–H: On the other hand, α4-subunit immunoreactivity of ABA males did not correlate with their wheel running during the three food-restricted days (panels F). Males with the highest levels of α4-subunit immunoreactivity showed the highest amount of wheel running activity during the last two days of food restriction (panel G-H). Regression lines indicate a significant correlation with $p < 0.05$. Dashed regression lines indicate a marginal significant correlation with $p \leqq 0.1$.

restriction-induced FAA (Fig. 7D, $R = -0.627$, $p = 0.097$).

3.11. Knockout of α4-subunit of $GABA_A$Rs exacerbates food restriction-evoked wheel running hyperactivity, especially in female mice

The relationship between body weight loss and α4βδ-$GABA_A$Rs suggested that α4βδ-$GABA_A$Rs are playing a protective role against ABA vulnerability for females (Fig. 7A) but not for males (Fig. 7E). The strong negative correlation between spinous α4-subunits and wheel running by females during the first day of food restriction (P41, Fig. 7B) further suggested α4βδ-$GABA_A$Rs' protective role for females (Fig. 7B), but not males (Fig. 7F). To test for a causal link between the α4-subunit of $GABA_A$Rs and the food restriction-evoked increase in wheel running, and to determine whether this relationship is specific for females, we took advantage of the α4-KO mice. We analyzed the wheel running pattern of α4-KO mice and their WT littermates during the first day (P41) and one-day preceding (P40) the food-restricted days. Repeated measure two-way ANOVA revealed a significant main effect of genotype ($F(1,16) = 10.08$, $p = 0.005$) and food restriction ($F(1,16) = 17.69$, $p = 0.001$) on wheel running (Fig. 8A). Post hoc analysis revealed that α4-KO females increased their wheel running after the onset of food restriction (Fig. 8A, $t(16) = 4.58$, $p < 0.001$). Their wheel running after the onset of food restriction was significantly higher than the wheel running shown by their female WT littermates (Fig. 8A, $t(32) = 3.796$, $p < 0.001$).

For males, repeated measure two-way ANOVA revealed a significant main effect of food restriction ($F(1,15) = 50.68$, $p < 0.0001$) but no significant effect of genotype ($F(1,15) = 1.63$, $p = 0.221$) on wheel running during the first day of food restriction (Fig. 8B). Post hoc analysis revealed that α4-KO males increased their wheel running after the onset of food restriction (Fig. 8B, $t(15) = 5.534$, $p < 0.0001$), and so as WT males ($t(15) = 4.567$, $p = 0.0004$). Their wheel running after the onset of food restriction was more than that of male WT littermates, but this difference was marginal (Fig. 8B, $t(30) = 1.355$, $p = 0.186$).

In order to analyze this genotype-related difference in wheel running between sexes further, we quantified animals' wheel activity during each of the four 6-h bins of the first day of food restriction, i.e., their circadian rhythm. For females, α4-KO exacerbated wheel running activities in all three compartments of the dark cycle of the first day of food restriction (food access period, 7-9 pm, $t(16) = 2.171$, $p = 0.045$; 9 pm-1 am, $t(16) = 2.495$, $p = 0.024$; 1-7 am, $t(16) = 2.319$, $p = 0.034$), as well as during the hours of 1–7 pm that develop to becoming FAA after entrainment to the feeding hours (Fig. 8C; 1–7 pm; $t(16) = 2.266$, $p = 0.038$). In contrast, no genotype difference was found among males for any of the five compartments of circadian within one-day following food restriction in males (Fig. 8D; food access period, 7–9 pm, $t(15) = 1.208$, $p = 0.246$; 9 pm–1 am, $t(15) = 1.221$, $p = 0.241$; 1–7 am, $t(15) = 1.244$, $p = 0.233$; 7 am–1 pm, $t(15) = 0.333$, $p = 0.744$; 1–7 pm, $t(15) = 0.793$, $p = 0.44$). The mean FAA (1–7 pm) of α4-KO males was even lower than the FAA shown by their WT male littermates (Fig. 8D).

Notably, there was no genotype effect on wheel running during the second or the third day of food restriction in females (FR2: WT running = 7.074 ± 2.15 km, α4-KO running = 10.122 ± 1.389 km, $t(16) = 1.236$, $p = 0.234$; FR3: WT running = 7.926 ± 2.526 km, α4-KO running = 7.781 ± 1.052 km, $t(16) = 1.236$, $p = 0.955$), or in males (FR2: WT running = 12.484 ± 2.075 km, α4-KO running = 9.433 ± 2.378 km, $t(15) = 0.9552$, $p = 0.355$; FR3: WT running = 4.676 ± 1.063 km, α4-KO running = 6.681 ± 1.393 km, $t(15) = 1.122$, $p = 0.28$).

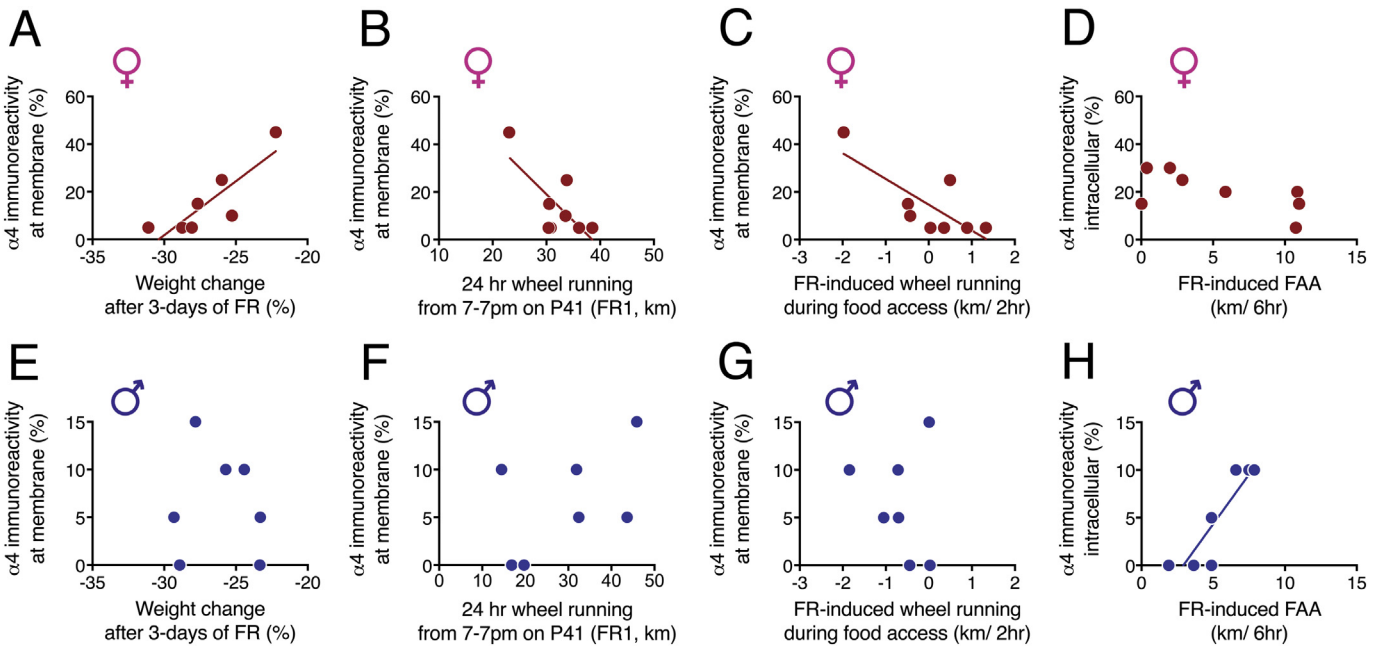

Fig. 7. Correlation between α4-subunit immunoreactivity at dendritic spines and weight loss or wheel running.
Panels A and E: Body weight loss after three days of food restriction (FR) is negatively correlated with α4-subunit labeling at the plasma membrane of ABA females (panel A) but not of ABA males (panel E). Females with the highest levels of α4-subunit immunoreactivity show the least amount of weight loss during the food-restricted period. Panels B and F: Wheel running activity during the first day of food restriction is negatively correlated with α4-subunit labeling at the plasma membrane of ABA females (panel B) but not of ABA males (panel F). Females with the highest levels of α4-subunit immunoreactivity show the least amount of wheel running activity during the first day of food restriction. Panels C-D, G-H: Female and male hippocampi differ in the subcellular compartment exhibiting correlation between α4-subunit immunoreactivity and wheel running activity. Food restriction-induced increase in wheel running activity during the food access period was calculated by the running activity during the food-access period on P41 minus the wheel running activity during 7–9 pm of the evening preceding food restriction. This value is negatively correlated with α4-subunits' plasma membrane labeling of ABA females (panel C), but not of ABA males (panel G). Females with the highest levels of α4-subunit immunoreactivity show the least amount of food restriction-induced increase in wheel running during the food-restricted days. Food restriction-induced FAA during food-restricted days was calculated as the difference between FAA during P42 and the wheel running activity during the hours of 1–7 pm on the day preceding food restriction. This value is positively correlated with the level of α4-subunits at intracellular locations of ABA males (panel H), but not of ABA females (panel D). Males with the highest levels of α4-subunit immunoreactivity show the highest amount of food restriction-induced FAA during food-restricted days. Regression lines indicate a significant correlation with $p < 0.05$.

4. Discussion

Our focus on sex differences (Shansky and Woolley, 2016; Bale and Epperson, 2017) revealed that neither male nor female adolescent mice are resistant to ABA, in that both sexes exhibit hyperactivity and weight loss within the first day of encounter with FR. However, we identified differences between the sexes in their underlying mechanisms for resilience, which emerge at different time points, as quantified based on suppression of wheel running (Fig. 2E vs F), decrease in daily weight loss (Fig. 1C vs 1D) and up-regulation of hippocampal α4βδ-GABA$_A$R levels (Fig. 5G).

4.1. Females exhibit more adaptive feeding behavior but the two sexes lose weight similarly

In contrast to adults (Li et al., 2015; Achamrah et al., 2017), both female and male mice exhibit similar patterns and levels of weight losses in response to ABA during adolescence. For both sexes, the presence of the wheel, alone (i.e., the EX group), suppresses feeding on the days corresponding to food restriction of the ABA group, suggesting that exercise suppresses appetite (Bi et al., 2005). We also identified a number of differences between sexes. For males but not females, ABA causes larger body weight loss than does food restriction alone, reflecting the additional weight loss due to hyperactivity. ABA females but not ABA males progressively increased food intake during the three days of food restriction, indicating that the feeding behavior of females are more adaptable, culminating in weight loss that was no greater than by food restriction, alone.

4.2. Weight loss may drive suppression of food restriction-evoked hyperactivity of both sexes but sooner and during food-anticipatory hours for males and during the feeding hours for females

As the name implies, food "anticipatory" activity (FAA) is widely regarded to be cognitively driven, since heightened activity emerges during the hours preceding feeding only after animals experience and entrain to restricted food access with circadian periodicity (Luby et al., 2012) and not simply in response to starvation (Mistlberger, 2011). The expression of dopamine D1 receptors by medium spiny neurons in the striatum is obligatory for the development of FAA (Gallardo et al., 2014). This supports the idea that activity of the cortico-striatal pathway may underlie cognitive modulation of FAA. Previous studies showed that adult males express significantly more FAA than adult females (Li et al., 2015; Michalik et al., 2015). In contrast, our study indicates that adolescents of both sexes exhibit FAA after having experienced a single day of food restriction (Fig. 2C and D), indicating that both sexes learn the circadian periodicity of food access within 24 h.

Differences between sexes in wheel running pertains to the directionality of its change relative to body weight change: body weight losses during the first day of food restriction (weight change from P41 to P42) correlated with suppression of wheel running on subsequent days for males, while body weight losses correlated with augmented wheel running on subsequent days for females. Secondarily, the sexes differed in the temporal window of food restriction-evoked hyperactivity. Specifically, males, but not females, that lost the most weight due to food restriction were also the ones that were the most efficient in

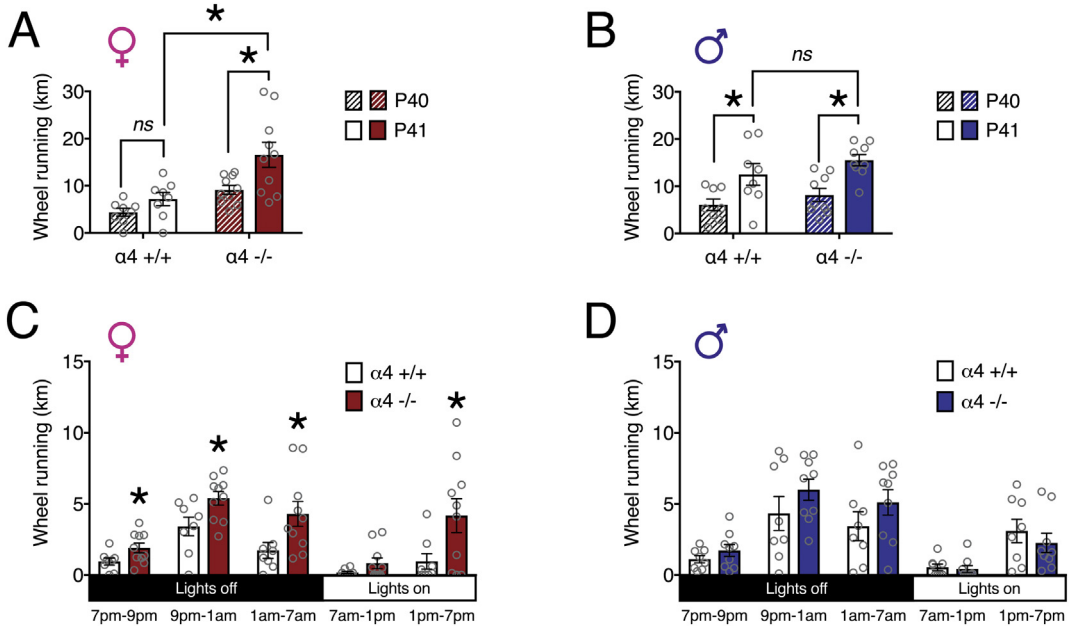

Fig. 8. α4-subunit of $GABA_A$ receptors knockout (KO) mice show increased food restriction-evoked wheel running activity following ABA, especially for females. Panels A and B: Daily wheel activity, measured as the distance run on the wheel, of α4-$GABA_A$R KO (α4 −/−) and their wild-type (WT) littermate (α4 +/+) female (panel A) and male (panel B) animals one-day preceding and following food restriction. Food restriction significantly increased wheel running activity of both female and male KO animals. The extent of increase of running induced by food restriction is greater for KO animals, compared to their WT littermates, especially among the female cohorts. The dashed bars represent baseline wheel activity of WT and KO animals (P40). The bars with no pattern represent wheel activity measured on the first day of food restriction phase for WT and KO animals (P41). * indicates $p < 0.05$. "*ns*" indicates no significant difference.
Panels C and D: Food restriction-evoked wheel running activities are significantly higher during the first half (7 pm–1 am, including the period of food access from 7 to 9 pm) and the second half of the dark cycle (1–7 am), also during the second half (1–7 pm, FAA) of the light cycle for female KO animals compared to the WT females (panel C). No difference between genotypes was found in the food restriction-evoked wheel running activity in any of the five circadian compartments for males (panel D). * indicates $p < 0.05$, comparing color bars with respective white bars of the same circadian compartment.

suppressing FAA on the next day (Fig. 4E). This suppression contributed towards decreased total wheel running activity by males within the first 30-h of food restriction (Fig. 2F). In contrast, weight loss for females does not appear to have been a factor suppressing FAA, as there was no correlation between weight change during the first day of food restriction and FAA on the subsequent day (Fig. 4B and D). In sharp contrast to males, females that lost more weight during the first 6 h of food restriction paradoxically ran more during the food-access period that followed immediately after (FR1, Fig. 3B). Furthermore, 8 out of 10 ABA females developed FAA on the next day, regardless of their extent of weight change (Fig. 4B). As a consequence, adolescent female animals remained hyperactivity for longer – through the second day of food restriction (Fig. 2E).

Females did exhibit individual differences in resilience to ABA - via suppression of wheel running during the hours of food access, but only after a delay by a full day (FR2). Individual differences in this suppression for females correlated with weight loss during the 24 h preceding food access (Fig. 3D). Thus, both males and females exhibited individual differences in resilience to ABA. Since weight changes preceded suppression of wheel running, we surmise that weight change 'drives' or serves as a physiological signal to suppress wheel running. Importantly, the wheel running suppression occurred during different compartments of circadian rhythm (FAA for males, feeding hours for females) and with a delay for females. This result concurs with an earlier study showing that adult females' body weight loss does not correlate with FAA (Wu et al., 2014). The difference between the sexes that we revealed suggests that there are subtle sex-related differences in brain circuits linking energy homeostasis to wheel activity and feeding behavior.

Past studies have shown that exercise can be anxiolytic to adult rats (Duman et al., 2008), mice (Binder et al., 2004; Schoenfeld et al., 2013) and humans (DeBoer et al., 2012). On the other hand, there are studies to support the view that exercise can be anxiogenic, especially for animals that have undergone the stress of being reared in isolation (Fuss et al., 2010; Hare et al., 2012; Onksen et al., 2012). We have shown previously that adolescent females that experience ABA exhibit heightened anxiety-like behavior even after 7 days of weight restoration from ABA (Chen et al., 2017a). This could be because the ABA animals were also reared in isolation.

4.3. *α4βδ-$GABA_A$Rs in the dorsal hippocampal CA1 may contribute to ABA resilience of females*

Deletion of the gene encoding α4 subunits result in the loss of expression of δ-subunits of $GABA_A$R and gaboxadol-sensitive δ-containing $GABA_A$R currents (Sabaliauskas et al., 2012; Peng et al., 2014), while up-regulation of α4 subunits are accompanied by up-regulation of δ-subunits within brains of animals that have undergone ABA induction (Aoki et al., 2012). Based on these findings, the increased expression of α4 subunits in the current study is likely to reflect increased expression of α4βδ-$GABA_A$Rs which reside at sites that are extrasynaptic to GABAergic synapses but, instead, nearer to glutamatergic synapses (Shen et al., 2007). Due to their location near glutamatergic synapses within dendritic spines and lower rate of desensitization, these extrasynaptic α4βδ-$GABA_A$Rs are activated by ambient GABA to tonically mediate shunting inhibition and prevent LTP of excitatory synapses in the dorsal CA1 (Shen et al., 2010; Sabaliauskas et al., 2015; Shen et al., 2017).

α4-subunits of $GABA_A$Rs in dendritic spines of stratum radiatum of the dorsal hippocampal CA1 increased for ABA females but not for the male littermates (Fig. 5). Given that food restriction was imposed from P41 to 43, the persistent up-regulation of α4 subunits at dendritic spines and shafts of female hippocampi at P60, well beyond the age of weight restoration (ca. P46, data not shown), is another finding that extends our knowledge regarding α4 subunit up-regulation, which was

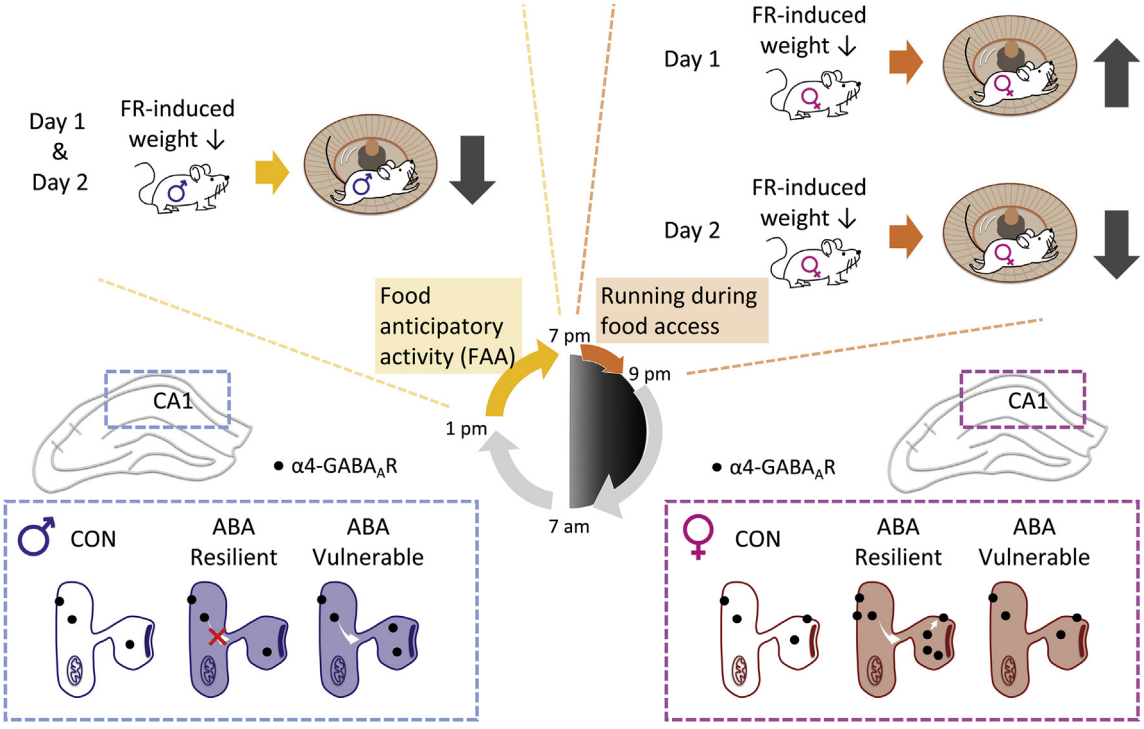

Fig. 9. Summary of results.
The schematic summarizes sex differences in response to ABA, revolving wheel running activity and α4-subunit immunoreactivity in the dorsal hippocampal CA1. Top left: ABA males which lost the most weight due to food restriction (FR) were also the most efficient in suppressing food-anticipatory activity on the next day. This suppression of wheel running during the food-anticipatory hours contributed towards decreased wheel running activity within 30-h of food restriction.
Top right: In contrast, adolescent ABA females which lost more weight during the first 6 h of food restriction paradoxically ran more during food access period on the first day. Adolescent females remained hyperactivity for longer – through the second day of food restriction. On the second day of food restriction, those that lost the most body weight due to food restriction were the ones able to suppress their running during the food-access hours.
α4βδ-GABA$_A$R in the dorsal hippocampal CA1 contribute to ABA resilience of females but not males. The increased plasmalemmal α4 immunoreactivity of CA1 pyramidal neurons by food restriction indicates that shunting inhibition by ambient GABA contributes to the suppression of food restriction-evoked hyperactivity, thereby protecting females from excessive weight loss.

previously shown immediately at the end of food restriction at P44 (Aoki et al., 2012; Aoki et al., 2017) and at the end of second ABA at PN60, (Wable et al., 2015a) for females.

The functional significance of the up-regulated α4βδ-GABA$_A$Rs was elucidated through correlation analyses of their levels with wheel activity and body weight. The strongly negative correlation between plasmalemmal levels of α4-subunit immunoreactivity in CA1 pyramidal neurons and food restriction-induced increase in wheel running (Fig. 6A) suggests that shunting inhibition of excitatory inputs at dendritic spines may contribute to the suppression of food restriction-evoked hyperactivity, thereby protecting females from excessive weight loss, which we also observed (Fig. 7A). Since anxiety-like level is positively correlated with food restriction-induced wheel running in female mice (Wable et al., 2015b), suppression of wheel running activity may conversely reflect decrease in anxiety-like levels, especially since this relationship has been shown previously to be associated with up-regulation of α4βδ-GABA$_A$R currents and protein levels in homogenates of CA1 pyramidal neurons of females at puberty (Shen et al., 2007). α4βδ-GABA$_A$Rs can also mediate anxiogenesis (Shen et al., 2007; Wable et al., 2015a)—through desensitization of these receptors by allopregnanolone (3αOH-5αβ-OH-pregnan-20-one), a stress steroid and a metabolite of progesterone. Presumably, this opposing effect was less dominant for ABA females, due to food restriction that reduced circulating levels of gonadal hormones (Martin et al., 2008).

α4-KO females showed more profound responses to food restriction during the first day of food restriction, and in contrast, no genotype difference was found among males. These data further support the idea that the anxiolytic role of α4βδ-GABA$_A$Rs is sex-specific. As for the transience of the KO effect, we hypothesize that food restriction-evoked hyperactivity is related to two factors. Initially, it is the increased anxiety, due to the absence of α4βδ-GABA$_A$Rs, that contribute to heightened anxiety and hyperactivity. On subsequent days (FR2 and FR3), the superior cognitive ability of α4-KO mice, as reported in other studies (Moore et al., 2010; Shen et al., 2010), may have helped these animals to better suppress hyperactivity, thereby counteracting the heightened anxiety due to the absence of α4βδ-GABA$_A$Rs. An alternative view is that the absence of the genotype effect beyond the first day of food restriction is due to the additive effects of compensatory expression of other GABA$_A$R subunits associated with phasic inhibition, which may be more robust for α4-KO mice. It has been shown that while δ-subunits are substantially decreased in α4-KO mice, other subunits increase, including α1 and γ2 subunits that occur at GABAergic synapses and mediate phasic inhibition (Peng et al., 2014). The precise role of GABA$_A$R containing α1 and γ2 subunits in the dorsal hippocampus and in other brain regions better known for their role in controlling food intake control, such as the parabrachial nucleus (Wu et al., 2009) should be investigated in future studies of ABA.

The changes that we've detected in the hippocampus following ABA and not by exercise alone is likely to be part of a global brain response to starvation. On the other hand, strong correlation of α4-subunit expression and wheel running suggests that the hippocampus may be one important node for linking the experience of hunger to an innate foraging-like behavior as well as the subsequent suppression of this foraging-like behavior via α4-GABAR activity, once an animal learns that wheel running is maladaptive.

α4-subunit expression at spine plasma membranes contributed to suppression of food restriction-evoked hyperactivity during FR1 but not on subsequent days of food restriction. Since spines are sites specialized

for excitatory synaptic inputs onto pyramidal neurons, the transience (FR1-only) of the contribution by spinous α4βδ-$GABA_A$Rs upon suppression of hyperactivity may be due to the influence by glutamatergic receptors that take over on subsequent days (FR2 and FR3). In support of this idea, NMDA receptor subunits in the hippocampal CA1 of adolescent female ABA rats become upregulated within dendritic spines of pyramidal neurons by the 4th food-restriction day, especially for those individuals that lose the most amount of body weight (Chen et al., 2017b).

Unlike α4βδ-$GABA_A$Rs at spines, α4βδ-$GABA_A$Rs at the dendritic shaft plasma membranes correlated with suppression of food restriction-evoked hyperactivity during all days of food restriction and more so for the latter days of food restriction. This temporal difference in α4βδ-$GABA_A$Rs expression across different subcellular structures (spine versus dendritic shaft) suggests that α4βδ-$GABA_A$Rs at dendritic shafts contribute towards anxiety suppression through tonic suppression of hippocampal excitability in ways that are relatively more independent of episodic, event-related excitatory afferent inputs impinging upon dendritic spines. Returning to the idea about the participation of α4βδ-$GABA_A$Rs in two factors affecting wheel behavior – anxiety and cognition, anxiety regulation may be mediated by α4βδ-$GABA_A$Rs at dendritic shafts, while cognitive control over maladaptive behavior, such as suppression of hyperactivity during starvation, may be mediated by α4βδ-$GABA_A$Rs expressed adjacent to excitatory synapses on dendritic spines.

4.4. Conclusion

Our analysis of adolescent mice undergoing ABA revealed that males and females exhibit vulnerability nearly equally, but with different origins (Fig. 9). Food restriction-evoked hyperactivity, which exacerbates weight loss of food-restricted animals, is more prolonged for females, but females exhibit more adaptive feeding behavior under restricted food-access schedule. Anorexia nervosa is a mental illness with the highest mortality rate, yet with no accepted pharmacological treatments, due to paucity of knowledge about its etiology. Our data support the idea that α4βδ-$GABA_A$Rs expressed by pyramidal cells of dorsal hippocampus may confer resilience against ABA for females but not for males. These sex-related differences reveal that designs of pharmacological treatments for anorexia nervosa may need to be tailored to individual differences in vulnerability and sex.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcn.2018.04.008.

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Conflict of interest

We declare no competing financial interests in relation with the work described.

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