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α4βδ-GABA$_A$ receptors in dorsal hippocampal CA1 of adolescent female rats traffic to the plasma membrane of dendritic spines following voluntary exercise and contribute to protection of animals from activity-based anorexia through its localization at excitatory synapses

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Abstract

In hippocampal CA1 of adolescent female rodents, α4βδ-GABA$_A$ receptors (α4βδ-GABA$_A$Rs) suppress excitability of pyramidal neurons through shunting inhibition at excitatory synapses. This contributes to anxiolysis of stressed animals. Socially isolated adolescent female rats with 8 days of wheel access, the last 4 days of which are restricted of food access, have been shown to exhibit excessive exercise, choosing to run instead of eat (activity-based anorexia, ABA). Up-regulation of α4βδ-GABA$_A$Rs in the dorsal hippocampal CA1 (DH), seen among some ABA animals, correlates with suppression of excessive exercise. We used electron microscopic immunocytochemistry to show that exercise alone (EX), but not food-restriction alone (FR), also augments α4βδ-GABA$_A$R expression at axo-spinous excitatory synapses of the DH (67%, $P=0.027$), relative to socially isolated controls without exercise or food-restriction (CON). Relative to CON, ABA animals' synaptic α4βδ-GABA$_A$R elevation was modestly elevated (37%), but, this level correlated strongly and negatively with individual differences in ABA vulnerability – i.e., food-restriction-evoked hyperactivity (Pearson's $R=-0.902$, $P=0.002$) and weight changes ($R=0.822$, $P=0.012$). These correlations were absent from FR and EX brains or ventral hippocampus of ABA brains. Comparison to CON of α4βδ-GABA$_A$R location in the DH indicated that ABA induces trafficking of α4βδ-GABA$_A$R from reserve pools in spine cytoplasm to excitatory synapses. Pair-housing control animals reduced cytoplasmic α4βδ-GABA$_A$R without reducing synaptic α4βδ-GABA$_A$R. Thus, exercise induces trafficking of α4βδ-GABA$_A$Rs to excitatory synapses, while individual differences in ABA vulnerability are linked most strongly to trafficking of α4βδ-GABA$_A$Rs in the reverse direction - from excitatory synapses to the reserve pool during co-occurring food-restriction.

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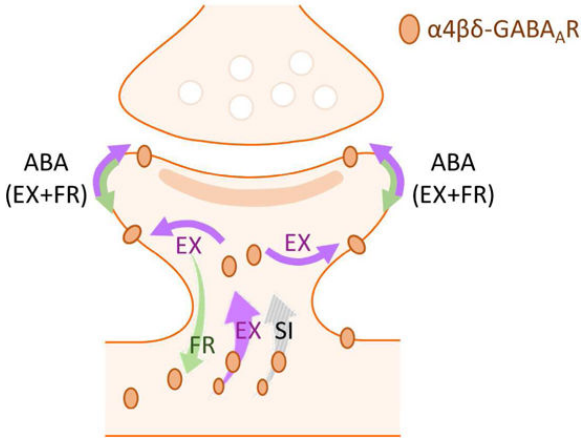
C. Aoki designed all parts of the experiment, participated in collection of behavioral data, analyzed all of the digitized images, participated in statistical analysis and figure preparation, and wrote all parts of the manuscript. Yi-Wen Chen designed and conducted the statistical analyses, prepared figures and participated in writing all parts of the manuscript. Tara Chowdhury participated in the design and collection of behavioral data and tissue processing. Walter Piper participated in acquisition of behavioral data, tissue processing, image acquisition, analysis of digitized images and participated in writing the manuscript.

CONFLICT OF INTEREST We have no conflict of interest to declare.

Graphical abstract



EM-immunocytochemnistry reveals that trafficking of α4βδ−GABA$_A$ receptors to synaptic clefts of excitatory synapses in the hippocampus is influenced by food restriction (FR), voluntary exercise (EX), and social isolation (SI). These α4βδ−GABA$_A$ receptors contribute towards adolescent female rats' resilience to activity-based anorexia (ABA), an excessive EX behavior induced by FR.

Keywords

Anorexia nervosa; Electron microscopic immunocytochemistry; GABA(A) receptor subunit delta; Wheel running; Exercise; Plasticity; Social isolation; Ventral hippocampus; Dorsal hippocampus; Anxiolysis; Receptor trafficking; GABA(A) receptor subunit delta; Tonic inhibition; Nonsynaptic; Neuromodulation

INTRODUCTION

GABA$_A$ receptors (GABA$_A$R) containing α4 and δ subunits (α4βδ-GABA$_A$Rs) exhibit properties that are unique among multiple GABA$_A$Rs that exist in the CNS (reviewed by (Smith et al. 2009; Smith and Woolley 2004)). Although both α4-containing nonsynaptic GABA$_A$Rs and α1-containing synaptic GABA$_A$Rs are pharmacologically blocked by bicuculline, bicuculline blockade leads to up-regulation of the α1-containing GABA$_A$Rs but not of the α4-containing GABA$_A$Rs. Additionally, α4βδ-GABA$_A$Rs are unresponsive to benzodiazepines, while the α1-containing synaptic GABA$_A$Rs are. Instead, α4βδ-GABA$_A$R expression is altered by changes in generalized network activity, such as to blockade of all action potentials by tetrodotoxin (TTX) (Joshi and Kapur 2009). This property fits well with its localization to excitatory synapses in the CA1, which is extrasynaptic with respect to GABAergic axon terminals (Shen et al. 2007; Shen et al. 2010). The extrasynaptic location of α4βδ-GABA$_A$R is due, in part, to the absence of the obligatory γ subunit required for clustering at GABAergic synapses (Crestani et al. 1999). At these extrasynaptic, excitatory synaptic sites, α4βδ-GABA$_A$Rs mediate shunting inhibition, which can reduce LTP (long-term potentiation) of excitatory synapses in the hippocampal CA1 and contribute towards spatial memory impairment (Shen et al. 2010).

α4βδ-$GABA_A$Rs in the hippocampus can also contribute to regulation of anxiety - both anxiolysis and anxiogenesis (Shen et al. 2007), due to the dual modulatory action of allopregnanolone (3alphaOH-5[alpha]beta-OH-pregnan-20-one). Allopregnanolone is a neuroactive steroid that rises in levels during stress (Purdy et al. 1991). In general, allopregnanolone is anxiolytic, anticonvulsant and sedative-hypnotic, targeting α4βδ-$GABA_A$Rs across multiple brain regions, including α4βδ-$GABA_A$Rs expressed by granule cells of the dentate gyrus (Frye et al. 2011; Majewska 1992; Olsen and Sapp 1995; Paul and Purdy 1992; Stell et al. 2003). Allopregnanolone's anxiolytic action can also be longer-lasting: α4βδ-$GABA_A$R expression in the hippocampal CA1 increases in response to fluctuating levels of allopregnanolone, whether experimentally induced or naturally, as during puberty onset (Gulinello et al. 2003; Shen et al. 2007; Shen et al. 2005; Smith et al. 1998; Smith et al. 2006; Sundstrom-Poromaa et al. 2002). In addition, allopregnanolone can be anxiogenic, due to its acute desensitizing action upon α4βδ-$GABA_A$Rs expressed by neurons where the Cl^- flux is inward (current is outward) such as the CA1 pyramidal neurons (Shen et al. 2007; Smith et al. 2009).

Because allopregnanolone is a metabolite of progesterone, the latter of which is secreted from the adrenal gland and ovaries (Fajer et al. 1971), allopregnanolone level in the hippocampus is closely linked to the circulating level of progesterone (Frye et al. 2000; Palumbo et al. 1995). For females, puberty is marked by fluctuating levels (i.e., both the rise and decline) of adrenal (progesterone) and gonadal (estrogen and progesterone) hormones. As a consequence, α4βδ-$GABA_A$Rs expression in the hippocampus changes from being barely detectable pre-pubertally to increasing transiently at puberty (Aoki et al. 2012; Shen et al. 2007; Shen et al. 2010). Animal models of post partum depression (Smith et al. 1998), premenstrual dysphoric disorder (Maguire et al. 2005; Smith et al. 2006; Stell et al. 2003; Sundstrom Poromaa et al. 2003) and stress-induced anxiety at puberty (Smith et al. 2007) support the idea that these affective symptoms are linked to altered expression or desensitization of α4βδ-$GABA_A$Rs in the hippocampus.

Previous work from this lab showed that an animal model of anorexia nervosa, called activity-based anorexia (ABA), also evokes increased expression of α4βδ-$GABA_A$Rs at excitatory synapses of stratum radiatum of the dorsal hippocampus of adolescent female rats (Aoki et al. 2012) and mice (Wable et al. 2015). ABA is an animal model that captures three hallmarks of anorexia nervosa: elevated anxiety (Wable et al 2015), excessive exercise, and voluntary food restriction (rev in (Aoki et al. 2016)). ABA is induced in animals by combining restricted food access with free access to a running wheel. Within 1 day of imposing restricted food access, laboratory rats that have previously acclimated to wheel running dramatically increase their voluntary wheel running. The running becomes excessive, since animals choose to run even during the limited periods of food access. This voluntary food restriction plus excessive wheel running leads to exaggerated weight loss which, to some, become life-threatening. As such, the food restriction-induced hyperactivity – ABA - captures the paradoxical voluntary food restriction and excessive exercise that is observed among 1% of the female adolescents of the human population that are diagnosed with the condition of anorexia nervosa (rev in (Aoki et al. 2016; Gutierrez 2013).

Since food restriction is stressful, food restriction, even without exercise, could evoke the release of neuroactive steroids that then modulate α4βδ-$GABA_A$R activity and expression. However, our previous examination of a small population of food restricted animals (N=5) (Aoki et al. 2012) did not reveal a significant change in α4βδ-$GABA_A$Rs specifically at the excitatory synapses of the dorsal CA1. Exercise, alone, evokes synthesis and secretion of brain-derived neurotrophic factor (BDNF) within the hippocampus (Neeper et al. 1996). Since BDNF increases trafficking of intracellular α4βδ-$GABA_A$R to the plasma membrane (Joshi and Kapur 2009), exercise, alone, may increase the level of α4βδ-$GABA_A$Rs at excitatory synapses of the CA1. A previous study, however, also failed to detect a change resulting from exercise alone, possibly due to the small sample size (N= 3) (Aoki et al. 2012). This study strived to re-examine the effect of food restriction, alone, and exercise, alone, and compare to the effect of ABA-induction upon α4βδ-$GABA_A$R expression within dendritic spines of CA1 pyramidal neurons.

Monitoring of individual animal's wheel activity required single housing. This led to another question that remained unanswered - whether the stress associated with single housing might increase the expression of α4βδ-$GABA_A$Rs at excitatory synapses. Previous studies have shown that social isolation during adolescence does not influence corticosterone levels (Lopez and Laber 2015) but alters neurotrophin levels in the hippocampus (Zhu et al. 2006), increases depression-like behavior (Leussis and Andersen 2008; Leussis et al. 2008), increases locomotion (Hall et al. 1998), and increases voluntary ethanol intake in adulthood (Lopez and Laber 2015). In this study, the putative influence of single housing, separate from the influence of wheel running or food restriction, was addressed by analyzing the hippocampus of animals that were pair-housed versus singly housed during adolescence but otherwise given food *ad libitum* and without wheel access.

Finally, previous studies examined the dorsal hippocampus, only, even though a number of studies have indicated that the ventral hippocampus may be involved more than the dorsal hippocampus in anxiety regulation (Bannerman et al. 2004; Fanselow and Dong 2010; McHugh et al. 2004) (however, see Bitran et al., 1999). Thus, one other goal of the current study was to re-assess the effect of ABA induction upon the expression of α4βδ-$GABA_A$R at excitatory synapses of the ventral hippocampus. Our findings indicate that 8 days of voluntary exercise, alone, during adolescence induces increased synaptic localization of α4βδ-$GABA_A$R in the dorsal but not ventral CA1 and that levels of synaptic α4βδ-$GABA_A$R in the dorsal hippocampus are tightly correlated with resilience of animals to the ABA-inducing environment.

MATERIALS AND METHODS

Animals

The brains analyzed for this study is the same set of brains used to analyze NMDA receptor expression in the hippocampus (Chen et al., 2016) and noradrenergic axons in the cerebellum (Nedelescu et al. 2016). Therefore, data on wheel activity and body weight can be found in these previous publications. We refer to those data in the Results section but restrict our reporting to new analyses on body weight and wheel running that relate to α4βδ-$GABA_A$Rs. All procedures involving the use of animals were in accordance with the NIH

Guide for the Care and Use of Laboratory Animals and also approved by the Institutional Animal Care and Use Committee of New York University (Animal Welfare Assurance No. A3317-01). All animals were female Sprague-Dawley rats, purchased from Taconic Farms and shipped to New York University at the age of P28. All animals were housed in a room with 12:12 light-dark cycle.

Rearing conditions

The rearing conditions for the ABA (Activity-Based Anorexia; i.e. combined food-restriction and exercise), the FR (Food-Restriction only), the EX (Exercise only) and the CON (Control; i.e. neither food-restriction nor exercise) animals were described in a previous publication (Nedelescu et al. 2016, Chen et al., 2016) and are re-described here briefly (Fig. 1 A). Thirty-one female rats of ages P28 were singly housed from P28, corresponding to the age at the time of arrival from Taconic Farms. On P35 or P36 (experimental day (ED) 1, and through the age of euthanasia on P43 or P44 (ED 8), animals designated for the ABA (n=8) and EX (n=8) groups were placed in a new shoe-box style cage with free access to a home cage running wheel of inner diameter 35 cm and running circumference per revolution of 110 cm (Med Associates, ENV-044). Wheel activity was measured continuously with 1 min temporal resolution. FR (n=8) and CON (n=7) animals were reared in the absence of a running wheel.

Animals designated for the ABA and FR groups were restricted of food access to the first hour of each ED, starting on ED 5, corresponding to the ages P39 or P40 and for four consecutive days, until ED 8. The amount of food available to ABA and FR animals during the first hour of each ED was unlimited. Thus, ABA animals were food-restricted for the last 4 out of the 8 days of wheel access. EX and CON animals were reared with *ad libitum* amount of food for all hours of the day.

All animals were weighed daily, just before the beginning of the dark phase, which corresponded to the end of each ED. The amount of food that they consumed was also measured daily. Wheel activity was also recorded manually, at the time of body weight measurement. All animals were euthanized at the end of ED 8, to collect their brains.

Singly versus pair-housed animals

Sixteen adolescent female Sprague-Dawley rats were shipped from Taconic Farms at the age of P28 and pair-housed within NYU's animal facility upon arrival. Eight of them began to be singly housed starting P36. Care was taken to allocate animals across the singly versus pair-housed groups, so that the mean body weights for the two groups were equalized and that the body weights of the co-housed animals were also minimally different. All animals were euthanized at the end of ED 8 to harvest their brains.

Electron microscopic immunocytochemical (EM-ICC) detection of α4 subunits of $GABA_AR$

Preparation of vibratome sections—All animals were euthanized by first anesthetizing them deeply with urethane (34%, 0.65 ml/185 g body weight, i.p.), then transcardially perfusing them with a solution consisting first of phosphate-buffered saline (PBS, pH 7.4) containing heparin (10,000 U per 500 ml), then of 4% paraformaldehyde in

0.1M phosphate buffer (PB) (pH 7.4) at a flow-rate of 50 ml/min over a 10-min period. After removing the brain from the skull, the brain was post-fixed for 24-30 hours in the perfusion fixative, then sectioned in the coronal plane using a vibratome at a thickness set at 50 μm. These vibratome sections were stored freely floating in PBS containing 0.05% sodium azide (PBS-azide) at 4-6 ° C until the day of the immunocytochemical procedure.

The immunolabeling procedure—The immunocytochemical procedure for detecting α4 subunits of $GABA_A$Rs was as described previously (Aoki et al. 2012; Aoki et al. 2014; Wable et al. 2014). In brief, vibratome sections were incubated in PBS-azide (pH 7.6) containing goat anti-α4 $GABA_A$R subunit antibody (Santa Cruz Biotechnology #SC7355, lot J1912, *RRID:AB_640770*). This antibody recognizes a single band at 67 kDa by Western blotting (Griffiths and Lovick 2005; Sanna et al. 2003) and has been shown to yield specific labeling for EM-ICC, based on reduction of immunoreactivity after preabsorption with the antigen corresponding to amino acids 32-50 from the N-terminus of human α4 subunit of GABARs (ESPGQNSKD EKLCPENFTR) and after application of the antibody to the hippocampal CA1 field of α4-knockout brains, instead of wildtype brains (Sabaliauskas et al. 2012). Vibratome sections were freeze-thawed 8 times to increase antibody penetration (Wouterlood and Jorritsma-Byham 1993), treated for 30 min with 1% hydrogen peroxide in PBS to reduce background staining, then incubated with the α4-subunit antibody at a dilution of 1:100, using PBS-azide containing 1% bovine serum albumin (PBS-BSA-azide) for 3 days at room temperature, under constant agitation. Immunoreactivity was detected by the pre-embed silver-intensified immuno-gold (SIG) procedure (Aoki et al. 2000), whereby the tissue was subsequently immunolabeled with a secondary antibody consisting of donkey anti-goat IgG, conjugated to 0.8 nm colloidal gold particles (EMSciences, Cat # 25801, temporary RRID:AB_2631210). To achieve this, sections were incubated in PBS-BSA-azide buffer containing the secondary antibody at a dilution of 1:100, overnight. These sections were post-fixed by immersing sections in PBS containing 2% glutaraldehyde (EMSciences, EM grade), then silver-intensified to enlarge the 0.8 nm colloidal gold particles for visualization under the electron microscope, using the Silver Enhancer Kit for Microscopy (#50-22-01, KPL, Inc, Maryland). Subsequently, these sections were processed osmium-free (Phend et al. 1995), so as to avoid loss of SIG particles. Sections underwent tissue processing for infiltration with plastic (EMBED-812, EM Sciences), which included a step of incubating in uranyl acetate (1% in 70% ethanol) to aid in ultrastructural preservation (Lozsa 1974; Terzakis 1968).

All 32 tissue, spanning the dorsal and ventral hippocampus from ABA, FR, EX and CON groups were processed strictly in parallel, so as to minimize artifacts originating from differences in immunoreagents or reaction times or room temperature. All 16 tissue from pair-housed and singly housed CON groups were also processed strictly in parallel but separately from the set of 32 tissue consisting of ABA, FR, EX and CON groups.

Electron microscopic imaging—Ultrathin-sections spanning the dorsal or ventral hippocampus were counterstained with uranyl acetate before viewing under the electron microscope (JEOL1200XL). Digitized images were captured using the AMT camera system, consisting of a 1.2 megapixel Hamamatsu CCD camera (Boston, MA) attached to

JEOLXL1200 electron microscope, at magnifications ranging from 25,000x to 60,000x. Alternatively, images were digitally captured, using a Phillips CM12 electron microscope with Gatan 4 megapixel digital camera at a magnification of 25,000x.

Ultrastructural analysis—Ultrastructural analysis began by first blinding the electron microscopist of the identity of animals from which the tissue was collected. Digital images of 200 dendritic spines spanning stratum radiatum of CA1 and forming asymmetric synapses were acquired, strictly in the order of encounter along tissue-plastic interface, representing the surface-most portions of vibratome sections, where SIG immunolabeling would be expected to be the greatest. Because imaging in each CA1 was completed after a constant number of dendritic spines had been encountered, the total area analyzed was variable, ranging from 250 μm^2 to 500 μm^2. The position of SIG particles was categorized to be of one of the following, each mutually exclusive of one another: in the cytoplasm of a dendritic spine, on the plasma membrane of the spine but removed from the synaptic cleft, or at the plasma membrane facing the synaptic cleft (Fig. 2). Three values, representing the frequency of SIG particles at each of the three potential sites, per 200 dendritic spines was determined for each animal. No attempt was made to quantify the level of immunoreactivity over other cellular elements of the grey matter, such as axons, dendritic shafts, neuronal cell bodies or glia.

Statistics

One-way ANOVA test was used to compare the level of immunoreactivity at the synaptic cleft, on the plasma membrane or in the cytoplasm, across the four experimentally reared groups, consisting of ABA, FR, EX or CON, followed by Fisher's Least Square Difference (LSD) *post hoc* analysis. Two-way ANOVA test was used to determine whether a main effect of exercise or of food restriction or of their interaction upon a4-immunoreactivity were significant. Two-way ANOVA test was used to determine whether the main effect of the brain region (dorsal vs ventral hippocampus) or of the treatment group (CON versus ABA) upon α4-immunoreactivity were significant or interacted. This was followed by Fisher's LSD *post hoc* analysis (which does not correct for the number of groups). Unpaired t-test was used to compare the level of immunoreactivity in the dorsal hippocampus of CON animals that were pair-housed versus singly housed. Pearson's correlation analysis was run to assess the significance of correlations between the SIG labeling frequencies, wheel running and body weight changes of each animal, across the eight EDs. All of these tests were preceded by tests for normality, using the Kolmogorov-Smirnov normality, the D'Agostino & Pearson normality, and the D'Agostino & Pearson normality tests. All variables were found to be normally distributed. The software used for statistical analyses and graph plotting was Prism (version 6.0 or 7.01, San Diego, Calif.).

RESULTS

Group differences in weight change and wheel running

ABA and EX groups of animals had free access to the running wheel and food for the first four experimental days (EDs), after which time, the ABA animals were restricted of food access to 1 hour per day while continuing to receive free access to the wheel. EX animals

remained in an environment with free access to both the wheel and food for the remaining four EDs. The FR animals never had access to the wheel and their access to food became limited to 1 hour, starting on the same day as that of the ABA animals.

During the first four EDs, i.e., preceding restricted food access, all four groups of rats exhibited steady daily increases in body weight, as reported in previous publications regarding this cohort of rats (Nedelescu et al. 2016; Chen et al., 2016). Based on a comparison of the animals with and without the wheel during the initial four 24-hr periods of acclimation (ED 1-ED 4), the to-be-food restricted-ABA and EX groups of animals gained weight daily in ways indistinguishable from those without wheel access, i.e., the CON and FR-to-be (P = 0.1, 0.2 and 0.2 for the first three 24 hr-periods spanning ED 1 through ED 4, Fig. 1 B). This was expected, since these animals are still growing and at the developmental stage of early to mid-adolescence (Spear 2000). As a consequence of food restriction beginning on ED 5, the ABA and FR groups of rats lost body weight, and this loss was the greatest for the first day of food restriction (FR1, from the end of ED 5 to ED 6, Fig. 1 B). Body weight loss continued for seven out of the 8 ABA rats, until the day of euthanasia, i.e., the fourth day of food restriction (FR4, from the end of ED 7 to ED 8). In contrast, the daily body weight change among the FR animals reversed by FR 4, meaning that all eight FR animals resumed daily weight gain. Two-way ANOVA revealed significant experimental day × group interactions for the daily body weight change ($F(9,81) = 11.69$, $p < .0001$). Two-way ANOVA revealed significant main effects of both experimental day ($F(3,81) = 42.73$, $p < .0001$) and group ($F(3,27) = 195.5$, $p < .0001$) for the daily body weight change. Post hoc analysis revealed that although the mean value of daily weight change did not differ during the first three food-restricted days for the FR versus ABA groups (Fig. 1 C, Fig. 1 D and Fig. 1 E), the two groups diverged during the last day of food restriction (Fig. 1F, −2.9 ± 1.13 g for ABA, 1.72 ± 0.37 g for FR, $t_{14} = -3.87$, $P = 0.005$).

ABA and EX group of rats increased their daily wheel running steadily throughout the EDs (Fig. 3). The ABA group of rats increased their daily wheel running more, relative to those of the EX group within 24 hours of food restriction, corresponding to ED5. Repeated measures two-way ANOVA, testing for the main effects of the experimental day and group (ABA versus EX) revealed significant experimental day × group interactions for the daily wheel running from day 3 to day 8 ($F_{5,70} = 6.152$, $P < .0001$). *Post hoc* analysis revealed that the group difference between ABA and EX groups in the daily running approached significance by ED6 (5.82 ± 1.05 km for ABA, 3.15 ± 0.71 km for EX, $t_{14} = 2.1105$, $P = 0.05$) and reached significance by ED7 (8.46 ± 1.75 km for ABA, 3.87 ± 1.09 km for EX, $t_{14} = 2.22853$, $P = 0.0427$). This increase contributed towards a significant difference in the ABA animals' running during the four food-restricted days ("ED 5 + 6 + 7 + 8" in Fig. 3) (27.18 ± 5.09 km for ABA, 13.97 ± 2.93 km for EX, $t_{14} = 2.24883$, P=0.0411). The extent of running by ABA animals that increased due to food restriction can be measured by calculating the difference in the extent of running during the food-restricted period (ED 5 through ED8) versus the period preceding food restriction and comparing to the corresponding values for the EX animals (i.e., ED 3 and 4). Repeated measures two-way ANOVA, testing for the main effect of experimental day and group (ABA versus EX) revealed significant interactions ($F_{1,14} = 5.417$, $P = .0354$). *Post hoc* analysis revealed that this difference the group difference was nearly significant during the first two days of food

restriction ("(ED 5 + 6) – (ED 3 + 4)" in Fig. 3, 6.16 ± 1.11 km for ABA, 2.54 ± 0.69 km for EX, t_{14} = 1.95898, P = 0.07,)) and reached statistical significance for the last two days of food restriction ("(ED 7 + 8) - (ED 3 + 4)" in Fig. 3, 14.94 ± 3.25 km for ABA, 5.24 ± 1.54 km for EX, t_{14} = 2.24883, P = 0.0411)).

Individual differences in food restriction-evoked wheel running and weight changes among ABA animals

As a cohort, all ABA animals increased wheel running (Fig. 4 A) and lost weight (Fig. 4B) during the four days of food restriction. The extent to which the animals lost body weight was highly correlated with their wheel running during the 24 hours before and after body weights were measured. This correlation was the strongest, when comparing the extent to which the eight animals lost body weight during the second day of food restriction (weight change from ED 5 to ED 6, Fig. 4 C), compared to their running during the 24 hours before (ED 5; Pearson's R = − 0.69, P = 0.0102) and to their running during the 24 hours after being weighed on ED 6 (R = −0.89, P = 0.003) (Fig. 4 C). Strong correlation between wheel running and weight loss was also evident for the third (R = − 0.69, P = 0.06) and fourth (i.e. last, R = −0.75, P = 0.03) days of food restriction. While this relationship held up across the animals, the extent to which each animal increased wheel running in response to food restriction varied greatly, especially during the last two days of food restriction (Fig. 4 A).

General description of α4 subunit immunoreactivity

The antibody used to immunolabel the α4 subunit of $GABA_A$Rs recognizes an extracellular N-terminus epitope and was established previously to be reflective of the location of α4βδ-$GABA_A$Rs (Aoki et al. 2012; Sabaliauskas et al. 2012; Shen et al. 2007). Accordingly and as was observed previously (Wable et al. 2015), immunoreactivity to the α4 subunit of $GABA_A$Rs occurred at the plasma membrane of dendritic spines in stratum radiatum of hippocampus (Fig. 2). α4 subunit immunoreactivity was also observed intracellularly, reflecting reserve and/or degradative pools. A subset of the plasmalemmal labeling occurred overlying the portion of the spine plasma membrane facing the synaptic cleft and in association with thick postsynaptic densities (PSDs). Thick PSDs and synaptic junctions formed on dendritic spines are hallmarks of glutamatergic excitatory synapses: they are where glutamatergic receptors anchor to the synaptic junction via PSD-95 and other PDZ-domain-containing anchoring proteins (Racz and Weinberg 2013). Thus, with respect to GABAergic synapses, these α4 subunits of $GABA_A$Rs were located extrasynaptically.

Group differences in the α4 subunit immunoreactivity at excitatory synapses of stratum radiatum of the dorsal hippocampus

While animals of all four groups exhibited α4 subunit immunoreactivity at the plasma membrane (Fig. 5 B) and synaptic cleft (Fig. 5 A), two-way ANOVA revealed a significant main effect of exercise upon synaptic cleft labeling ($F_{1,26}$ = 5.863, P = 0.0227), no main effect of food restriction and no interaction between exercise and food restriction ($F_{1,26}$=0.7836, P = 0.3841). Fisher's LSD *post hoc* analysis revealed that the EX group of animals exhibited significantly greater occurrence of α4 subunit immunoreactivity, compared to the CON (P = 0.027) and FR (P = 0.0141), specifically at synaptic clefts (compare Figs. 5A versus Fig. 5 B and 5 C) (2.1 ± 0.5 % for EX, 0.9 ± 0.2 % for CON; 0.8

± 0.2 % for FR at the synaptic cleft). Although ABA tissue also exhibited greater occurrence of α4 subunit immunoreactivity at synaptic clefts, relative to CON, this difference did not reach statistical significance, due to the large variance (Fig. 5 A). This observation led us to wonder whether the variance, especially for the ABA group, could be related to individual differences in reactivity to environmental factors. In order to test this idea, we determined Pearson's correlation between α4 subunit immunoreactivity and each animal's response to the two environmental factors that we imposed – food restriction and wheel access.

Correlation between weight loss, wheel running and α4 subunit immunoreactivity at excitatory synapses

For the tissue obtained from ABA animals, Pearson's correlation analysis revealed a strong, positive relationship between the frequency of α4 subunit immunoreactivity on the spinous, synaptic cleft and the extent to which each animal's body weight changed during food restriction, relative to its body weight just before food restriction began (body weight measurement on ED 4), calculated as the body weight change between ED4 and ED8 (Fig. 6 A, Pearson's $R = 0.78$, $P = 0.023$). This positive correlation indicates that the greater the animals expressed α4 subunit immunoreactivity at synaptic clefts, the more body weight they retained during the food-restricted period. Although the extent to which FR animals lost weight overlapped with the values seen for the ABA animals, α4 subunit immunoreactivity at synaptic clefts of FR animals did not correlate to their change in body weight (Fig. 6 B, $R = -0.14$, $P = 0.77$).

Pearson's correlation between the frequency of synaptic cleft immunoreactivity and food restriction-evoked wheel running, assessed by measuring the animals' wheel running during the four food-restricted days (ED 5 to ED 8) (Fig. 6 C, $R = -0.90$, $P = 0.002$) was even stronger than the correlation observed for weight change ($R^2 = 0.81$ for wheel running; $R^2 = 0.61$ for weight change). The negative correlation between wheel running and α4 immunoreactivity indicates that α4 immunoreactivity was greater among animals that became the least hyperactive following food restriction. This means that increased α4 immunoreactivity correlated with both of our measures of resilience to ABA – minimal weight loss and minimal hyperactivity.

The age-matched EX animals also exhibited daily increases in wheel running during the 8 days of wheel access. However, the extent to which they ran on the wheel during the last 4 days, analogous to the food restriction period for the ABA animals, did not correlate with the frequency of synaptic clefts showing α4 subunit immunoreactivity (Fig. 6 D, $R = -0.32$, $P = 0.44$).

Wheel running during the food-restricted period also correlated strongly with the frequency of labeling at nonsynaptic portions of spine plasma membrane of ABA animals (Fig. 7 C, $R = -0.73$, $P = 0.04$) but not for the EX animals (Fig. 7 D, $R = -0.39$, $P = 0.34$). Unlike the strong correlation observed between weight loss and synaptic cleft labeling of ABA animals, the extent of weight loss during the corresponding food-restricted period was not strongly correlated with the frequency of α4 subunit immunoreactivity at the plasma membrane for ABA animals (Fig. 7 A, $R = 0.45$, $P = 0.26$). FR animals also did not exhibit a strong correlation between weight loss during the food restricted period and plasmalemmal α4

immunoreactivity (Fig. 7 B, $R = 0.04$, $P = 0.68$). Although the extent of weight loss during the entire food-restricted period did not correlate strongly with nonsynaptic plasmalemmal α4 immunoreactivity for the FR or the ABA groups, the extent of weight loss on the second day of food restriction (weight change on FR 2, from ED 5 to ED 6) was correlated moderately with the frequency of plasmalemmal labeling at spines of ABA animals ($R = 0.69$, $P = 0.06$) and more strongly for the FR animals ($R = 0.80$, $P = 0.03$). The departure from this correlation after the second food-restricted day for nonsynaptic plasmalemmal labeling but not for the entire food-restricted period suggests a perturbation in the relationship between weight loss and α4 immunoreactivity that emerged over the last two days of food restriction. Accordingly, an increase in individual differences was especially evident for the ABA animals by examining the weight changes of individual animals during ED 7 and ED 8 (Fig. 4 B) and also by the drop in correlation of weight change to α4 subunit immunoreactivity at the plasma membrane for the FR group ($R = -0.07$, $P = 0.89$, for weight change on FR 3, from ED 6 to ED 7) and the ABA group ($R = 0.19$, $P = 0.65$).

Correlation of ABA and CON's α4 subunit immunoreactivity with body weight changes during the last experimental day

As was noted above, the frequency of α4 subunit immunoreactivity at the synaptic cleft correlated very strongly with ABA animals' degree of wheel running during the food-restricted period (Fig. 6 C) and this, in turn, correlated strongly with the extent to which animals lost body weight during the food-restricted days (Fig. 4 C). Accordingly, synaptic cleft labeling also correlated strongly with the degree to which animals lost body weight on the last day of food restriction, just prior to euthanasia (weight change on FR 4, from ED 7 to ED 8, Fig. 8 B, $R = 0.82$, $P = 0.012$). In comparison, the correlation between ABA animals' weight loss on the last day and the frequency of labeling at nonsynaptic portions of the plasma membrane of spines was weaker (Fig. 8 C, $R = 0.54$, $P = 0.17$), and completely absent for intracellular labeling (Fig. 8 D, $R = 0.09$, $P = 0.83$). When immunolabeling at all sites on dendritic spines were combined (at cleft, at nonsynaptic portions of the plasma membrane and intracellularly=total spine labeling), correlation was present and positive (Fig.8 A, $R = 0.71$, $P = 0.05$), due correlation with the synaptic cleft labeling.

Although total spine labeling for the CON tissue was also correlated strongly with weight changes during the last day, this correlation was negative (Fig. 8 E, $R = -0.86$, $P = .01$): the animals with the highest expression of α4 immunoreactivity were the ones that gained the least body weight. This strong correlation is an indication that some factor regulating body weight changes during adolescence may be regulating α4 subunit expression in the hippocampus as well. The negative correlation for the CON tissue may reflect an increase of total α4 in spines that is associated with maturation, as slight differences in maturity may be expected, even though all CON animals were P44 on the day of euthanasia.

Of the three categories comprising the total spine labeling, CON animals' weight change on the last day correlated the most with intracellular (Fig. 8 H, $R = -0.72$, $P = 0.07$) and plasmalemmal labeling (Fig. 8 G, $R = -0.86$, $P = .08$) and the least with synaptic cleft labeling (Fig. 8 F, $R = -0.30$, $P = .51$). This ABA-versus-CON difference in the subcellular location of α4 subunits with correlation to body weight changes suggests that the subcellular

location of α4βδ-$GABA_A$Rs can be maintained as the cytoplasmic reserve or nonsynaptic modulators under a sedentary state (i.e., CON state), while environmental factors associated with food restriction-evoked excessive exercise (i.e., ABA) prompt the trafficking of these receptors to and/or away from the synaptic cleft portion of the plasma membrane (Fig. 12). Apparently, the environmental factors associated with food restriction-evoked hyperactivity over-ride the factors linking body weight change and α4 subunit expression in spines that exists during a sedentary state.

α4 subunit immunoreactivity in the ventral hippocampus of ABA animals

Having observed that the dorsal hippocampus of the ABA animals exhibits increases in α4 immunoreactivity that correlates strongly with food restriction-evoked hyperactivity and weight loss, we next examined whether similar changes might be observed for the ventral hippocampus, previously reported to be more strongly linked to anxiety-like behaviors (Bannerman et al. 2004; Fanselow and Dong 2010). Contrary to expectation, the only correlation of statistical significance was between the food restriction-evoked hyperactivity during the first two days of food restriction and intracellular labeling at dendritic spines (R = 0.72, P = 0.0463, Fig. 9 B). Conversely, the dorsal hippocampus showed no correlation between these two measurements (R = 0.21, P = 0.6, Fig. 9 A). For the dorsal hippocampus, it was plasmalemmal labeling that correlated with the food restriction-evoked increase in activity during the first two days of food restriction (R = − 0.77, P = 0.03, Fig. 9C). Such correlation was absent at the synaptic cleft or nonsynaptic portions of spine plasma membranes in the ventral hippocampus (Fig 9 D). The most remarkable contrast between the dorsal versus ventral hippocampus was the diverging directions of the correlation between behavior and α4 subunit immunoreactivity: for the ventral hippocampus, the relationship between hyperactivity and α4 subunit immunoreactivity was positive, while for the dorsal hippocampus, the correlation was strongly negative. Similarly, synaptic cleft labeling in the ventral hippocampus exhibited only weak correlation with wheel running during the food restricted days (wheel running from ED 5 to ED 8) (R= 0.49, P = 0.2) and this weak correlation was in the opposite direction from that observed for the dorsal hippocampus (R=-0.90, P=0.002, Fig. 6 C). Correlation between nonsynaptic plasmalemmal labeling in the ventral hippocampus and wheel running during the four days of food restriction (ED 5 to ED 8) was absent altogether (R = 0.3, P = 0.5), even though this correlation was strong in the dorsal hippocampus (Fig. 7 C). In the ventral hippocampus, there was no correlation between weight loss during the four days of food restriction and synaptic cleft α4 subunit immunoreactivity (R = −0.3, P = 0.5). This, too, is in sharp contrast to the effect seen in the dorsal hippocampus (R = 0.78, P = 0.023, Fig. 6 A).

In the ventral hippocampus, CON tissue exhibited correlation between α4 subunit immunoreactivity with the weight increase during the last ED (Fig. 10 E-H). This correlation was the strongest for total spinous immunolabeling (Fig. 10 E, R = −0.86, P = 0.013), as was observed for the dorsal hippocampus of CON tissue (Fig. 8 E-H).

Two-way ANOVA revealed significant main effects of the environment (ABA versus CON) ($F_{1,26}$ = 4.505, P = .0435) and of brain region (dorsal versus ventral hippocampus) ($F_{1,26}$ = 8.534, P = .0071) upon the plasma membrane labeling of α4 subunits. Moreover, within the

ventral hippocampus, two-way ANOVA revealed significant main effects of both environment (ABA versus CON) ($F_{2,39}$ = 6.133, P = .018) and different portions of spine ($F_{2,39}$ = 8.904, P = .0007) for the α4 subunit expression. *Post hoc* analysis revealed that in the ventral hippocampus, ABA induction increased α4 subunit expression at nonsynaptic portions of spine plasma membranes (P = 0.0153, t_{39}=2.536, Fig. 10-I) but the pattern of perturbation of this expression was not in any way correlated with the animals' weight changes during the last ED (Fig. 10 A-D). In this way, the ventral hippocampus differs from the dorsal hippocampus, with only the dorsal hippocampus exhibiting a strong correlation between weight change and synaptic cleft labeling (Fig 8 B).

α4 immunoreactivity in the dorsal hippocampus of socially isolated versus pair-housed adolescent rats

All of the animals described above were reared from early to mid-adolescence under the condition of social isolation due to single housing, so as to be able to monitor wheel running activity accurately among the EX and the ABA groups. We examined the potential effect of social isolation due to single housing, alone, upon expression of α4βδ-$GABA_A$Rs in the dorsal hippocampus. Comparisons were made between the dorsal hippocampus of a group of eight adolescent female rats that were socially isolated from P36 to P44 (SI) versus the hippocampus of another group of eight age-matched pair-housed (PH) female rats. Both groups were reared without food restriction and without access to a running wheel. Two-way ANOVA to test for the main effect of housing condition and spine positions for α4 subunit expression (at cleft, at membrane or intracellular) revealed significant interaction between these two factors for the proportion of immunolabeling in the dorsal hippocampus ($F_{2,42}$ = 6.563, P = .0033). Two-way ANOVA also revealed a significant main effect of spine positions for the α4 subunit expression in the dorsal hippocampus ($F_{2,42}$ = 34.22, P < .0001). *Post hoc* analysis revealed that the frequency of axo-spinous excitatory synapses immunolabeled for α4 subunit at the synaptic cleft was similarly low for both groups (t_{14} = 0.16, P = 0.8, 0.7 ± 0.3 % for PH; 0.6 ± 0.2 % for SI) but the group difference approached significance for plasmalemmal labeling (t_{14} = 1.67258, P = 0.11, 1.6 ± 0.5 % for PH; 0.6 ± 0.2 % for SI) (Fig. 11). Immunolabeling was most abundant in the cytoplasm and was significantly greater for the SI group (t_{14} = 2.43341, P = 0.03, 3.9 ± 0.9 % for PH; 7.6 ± 1.2 % for SI) (Fig. 11).

DISCUSSION

Analysis of this cohort of ABA animals supports previous interpretation (Aoki et al., 2014), namely that increased expression of α4βδ-$GABA_A$Rs in spines contributes towards suppression of food restriction-evoked hyperactivity, thereby protecting animals from excessive weight loss. Dendritic spines are sites for excitatory synaptic input onto pyramidal neurons of the CA1 hippocampal pyramidal cells (Racz and Weinberg 2013). Since anxiety is positively correlated with wheel running (Wable et al., 2015), suppression of wheel running reflects anxiolysis, a behavioral response associated with α4βδ-$GABA_A$Rs that mediate shunting inhibition of excitatory inputs to CA1 pyramidal neurons (Shen et al., 2007). However, as noted under Introduction, α4βδ-$GABA_A$Rs can also mediate anxiogenesis, through desensitization by allopregnanolone, a neurosteroid that becomes

elevated during stress, if these receptors are expressed on plasma membranes with inward Cl^- flux (Smith et al., 2007, 2009). Apparently, α4βδ-$GABA_A$Rs within spines of ABA rats are not desensitized by allopregnanolone, either because allopregnanolone level is low due to food restriction, and/or the Cl^- flux across ABA rats' dendritic spine plasma membranes is outward. The direction of Cl^- flux is dictated by the expression of the neuronal K-Cl co-transporter, KCC2, relative to the NKCC1, since KCC2s pump Cl^- out, generating hyperpolarizing E_{GABA} and setting up the flux of Cl^- to be inward, while the NKCC1s do the opposite (Chamma et al., 2012). KCC2 has been shown to be highly localized at spines, regulate spine morphogenesis, maintain glutamatergic synapses and mediate cross-talk between excitatory and inhibitory transmission (Chamma et al., 2012). KCC2's additional role within dendritic spines may be to bi-directionally modulate α4βδ-$GABA_A$Rs-mediated shunting inhibition at axo-spinous junctions under conditions of stress, by changing the direction of Cl^- flux locally.

α4βδ-$GABA_A$R located at the synaptic cleft of excitatory synapses contributes most strongly to ABA resilience

Closer examination of α4βδ-$GABA_A$R distribution pattern within dendritic spines revealed that, albeit of low frequency, its accumulation at the synaptic cleft of excitatory synapses, more than at nonsynaptic portions of the plasma membrane, contributes strongly to the suppression of the food restriction-evoked wheel running and reduction of the weight loss by the ABA animals (compare outcomes from the correlation analyses in Figs 6 and 7). Both the suppression of hyperactivity on the running wheel and reduction of weight loss are important contributing factors to ABA resilience, since both reductions extend survival under the condition of ABA induction. Conversely, both excessive exercise and excessive weight loss are symptoms associated with anorexia nervosa (Aoki et al., 2016). There is at present no accepted pharmacological treatment for anorexia nervosa (Aoki et al., 2016). The new findings of this study suggest that treatments targeting activation of α4βδ-$GABA_A$Rs may be efficacious, especially if there is a way to target the drug to pyramidal neurons of the hippocampus.

Exercise alone increases the localization of α4βδ-$GABA_A$Rs to spine synapses

To our knowledge, this is the first study to examine the impact of exercise alone on α4βδ-$GABA_A$R expression in the hippocampus. The present study revealed that exercise alone for just 8 days during adolescence greatly increases α4βδ-$GABA_A$Rexpression in dorsal hippocampal spines. Such a change would be expected to reduce excitability of CA1 pyramidal neurons, which could serve to protect CA1 pyramidal neurons from excitotoxicity, particularly since this level of exercise also increases the expression of NR2A-NMDARs at spines (Chen et al., 2016).

Increased expression of α4βδ-$GABA_A$R could also decrease dorsal hippocampus-dependent behavior, such as spatial cognition, so long as this behavioral test is administered free of stress-induced anxiety (Shen et al., 2010). However, we have observed no detectable deficit or improvement in spatial memory (active place-avoidance) 3-4 days or 9-10 days following this regimen of exercise, relative to the level observed among age-matched CONs (Aoki et al. 2016; Chowdhury et al. 2015). Another previous study from this lab that used the same

exercise regimen revealed decreased dendritic branching of pyramidal neurons in the dorsal hippocampus of adolescent female rats, relative to CONs' (Chowdhury et al. 2014). Since decreased dendritic branching could increase input resistance of the neurons, so long as the expression of membrane channels and dendritic branch diameters remain unchanged, the morphological changes evoked by exercise may cancel the increased α4βδ-$GABA_AR$ expression evoked by exercise. Four weeks of exercise during adolescence is reported to improve male rats' ability to discriminate novel from familiar objects but only after a delay of two weeks (Hopkins et al. 2011). It is possible that the effect of exercise during adolescence upon memory will become detectable for female rats as well but only after such a delay or a longer regimen of exercise.

The localization of α4βδ-$GABA_AR$ to the synaptic cleft of excitatory synapses is modulated jointly by exercise and food restriction

Although exercise alone, but not ABA, evokes significantly greater expression of α4βδ-$GABA_AR$ at the synaptic cleft, compared to CONs (Fig. 5A), the *level* of expression of this receptor is not correlated with wheel running activity of the EX group (Fig. 6D). This indicates that trafficking of α4βδ-$GABA_AR$ to excitatory synapses is not simply a consequence of wheel running but is likely to be modulated by the stressful environmental context associated with food restriction. Since the synaptic cleft labeling is diminished by some of the ABA animals, relative to those that experienced exercise alone (i.e., the EX group), the weight loss and/or stress associated with food restriction within the ABA paradigm is likely to be an environmental factor contributing to individual differences in the curtailment of α4βδ-$GABA_AR$ there (Fig. 12).

α4βδ-$GABA_ARs$ trafficking to the synaptic cleft is likely to be a three-step process, involving trafficking of receptors into cytosol of spine heads, then from the cytosol to the plasma membrane, and finally from the plasma membrane to or away from the synaptic cleft (Fig. 12) This idea is supported by the tight correlation between two measurements within ABA tissue – α4βδ-$GABA_AR$ at the synaptic cleft and at nonsynaptic portions of the plasma membrane ($R = 0.81$, $P = 0.01$). This correlation between the two subcellular domains does not exist for the CON or the FR tissue and is weaker for the EX tissue ($R = 0.56$, $P = 0.15$), indicating that the final step of trafficking from nonsynaptic portions of the plasma membrane to the synaptic cleft is modulated by the combined effect of exercise and food restriction (Fig. 12).

Correlation between intracellular and nonsynaptic plasmalemmal levels of labeling is very strong for the EX and FR tissue ($R = 0.84$, $P = 0.01$ for EX; $R = 0.91$, $P = 0.002$ for FR), while this correlation is absent for the CON tissue ($R = 0.162$, $P = .701$). This indicates that trafficking of α4βδ-$GABA_AR$ between the cytoplasm and the plasma membrane is not a passive step but, instead, stimulated by environmental factors, such as food restriction and exercise. The plasmalemmal (Fig. 5 B) and cytoplasmic (Fig. 5C) levels of α4βδ-$GABA_AR$ were somewhat lower in the FR tissue than in the CON. Although this group difference did not reach statistical significance, the slight decline by the FR group contributed towards a significant difference, relative to the EX group's values at both subcellular sites (Fig. 5 B and Fig. 5 C). This suggests that food restriction alone may reduce the influx of α4βδ-

GABA$_A$R into the spine cytoplasm or of their *de novo* synthesis. The correlation between intracellular and nonsynaptic plasmalemmal levels of labeling is not strong for ABA tissue ($R = -0.502$, $P = 0.205$), probably because the plasmalemmal α4βδ-GABA$_A$R are mobilized from nonsynaptic to synaptic cleft sites, rather than dwelling at the nonsynaptic portion of the plasma membrane.

As for the cytoplasmic labeling, socially isolated CON brains exhibited greater levels than of the pair-housed group, suggesting that the stress associated with social isolation may have enhanced trafficking of receptors into spine heads and/or *de novo* synthesis of α4βδ-GABA$_A$R subunits. Together, these observations suggest that exercise and social isolation evoke net gain of α4βδ-GABA$_A$Rs into the dendritic spine cytoplasm, while exercise evokes additional trafficking of α4βδ-GABA$_A$Rs from the cytoplasm to the nonsynaptic portions of the plasma membrane, and that the combination of the exercise-evoked trafficking of α4βδ-GABA$_A$Rs from the cytoplasm to the synaptic cleft, ultimately yield the most effective shunting inhibition of excitatory inputs from CA3 pyramidal cells to CA1 pyramidal cells in stratum radiatum, so as to generate the behavior of suppressed wheel running and reduced weight loss (Fig. 12).

The present findings are consistent with the notion that individual differences in reactivity to ABA, measured based on the extent of excessive exercise and/or body weight loss, could arise from individual differences in reactivity to social isolation, food restriction, exercise or of their combination and that each of these environmental treatments, alone and in combination, influence α4βδ-GABA$_A$R expression and subcellular trafficking within spines. Among these factors, the effect of food restriction may be in the opposite direction (reducing synaptic α4βδ-GABA$_A$Rs), suggested by the trend towards dampened level of synaptic (Fig. 5 A), nonsynaptic plasmalemmal (Fig. 5B) and cytoplasmic (Fig. 5 C) levels within FR tissue relative to CON's and of the reduced levels at these sites within ABA tissue, when compared to the EX group's levels (Fig. 5).

The physiological impact of α4βδ-GABA$_A$R trafficking within spine heads

The biophysical impact of α4βδ-GABA$_A$Rs at the synaptic cleft, rather than at nonsynaptic portions of spine plasma membrane, is unknown. For glutamatergic and GABAergic receptors, those occurring at the synaptic cleft are recognized to sense phasically released neurotransmitters, while those that are removed from the synaptic cleft are proposed to sense ambient levels of the neurotransmitter that escape uptake by plasma membrane transporters. The localization of GABA and glutamate receptors at the synaptic cleft is ensured through an elaborate array of anchoring proteins and receptor subunit composition (Racz and Weinberg 2013; Crestani et al. 1999). However, α4βδ-GABA$_A$Rs are nonsynaptic with respect to GABAergic axon terminals, whether occurring at spines' asymmetric synaptic cleft or not. The additional "advantage" of α4βδ-GABA$_A$Rs being located at the synaptic cleft of glutamatergic synapses is a question that has not been posed before. Might the synaptic cleft population of α4βδ-GABA$_A$Rs improve the temporal resolution or amplitude of shunting inhibition? α4βδ-GABA$_A$Rs do not desensitize readily or to as great an extent as other GABA$_A$ receptors (Smith et al., 2009), but might the synaptic cleft population of α4βδ-GABA$_A$Rs be even better at escaping desensitization by ambient GABA? What

anchoring protein, if any, occurs at excitatory synapses for α4βδ-$GABA_A$Rs is also unknown. However, it would seem that some kind of an anchoring protein does exist, because the strong correlation of their levels specifically at synaptic clefts of spines to the animal's behavior is unlikely to be generated without a molecular mechanism that links animals' behavior to receptor trafficking.

One potential molecule linking these environmental factors to α4βδ-$GABA_A$Rs expression is BDNF, known to up-regulate in response to food restriction alone (Stranahan et al. 2009), exercise alone (Neeper et al. 1996), and by the combination of food restriction plus exercise (Gelegen et al. 2008; Stranahan et al. 2009). BDNF has also been shown to increase trafficking of α4βδ-$GABA_A$Rs from the cytosol to the plasma membrane of cultured hippocampal neurons (Joshi and Kapur 2009). Studies are under way to determine whether alterations in the bioavailability of BDNF affect exercise, food restriction and ABA-induced alterations in α4βδ-$GABA_A$R accumulation at excitatory synapses of the CA1 pyramidal neurons.

The weak negative correlation that we observed for the nonsynaptic plasmalemmal and cytoplasmic α4 subunit immunoreactivity, relative to body weight changes during the last day of CON tissue (Fig 8 for the dorsal hippocampus and Fig 10 for the ventral hippocampus), indicates that factors regulating body weight changes contribute towards the expression level of α4βδ-$GABA_A$Rs in the CA1 dendritic spines, even among sedentary animals fed *ad libitum*. For sedentary animals, those factors could be growth hormone, progesterone, and/or estrogen, the latter two of which have been shown to undergo age-dependent changes during adolescence (Vetter-O'Hagen and Spear 2012). Daily weight change at P44 is greater than in adulthood but less than that at P36 (Vetter-O'Hagen and Spear 2012). The slight differences in the body weight changes during the last ED (corresponding to P44) among our cohort of 8 CON animals may reflect slight differences in maturity among the CON animals, with the ones exhibiting less body weight changes being the relatively more mature and, based on our data, relatively more enriched of α4βδ-$GABA_A$Rs at cytoplasmic reserve pools and nonsynaptic portions of the plasma membrane of spines.

Lack of an ABA effect in the ventral hippocampus

The apparent lack of effect of ABA upon synaptic cleft localization of α4βδ-$GABA_A$Rs in the ventral hippocampus was contrary to expectation, as the ventral hippocampus is recognized to play a stronger role than the dorsal hippocampus in behavior associated with stress-induced anxiety (Bannerman et al. 2004; Fanselow and Dong 2010; McHugh et al. 2004). Based on the correlation that was observed between plasmalemmal labeling and food restriction-evoked increase in wheel running during the early phase of food restriction, plus the trend towards an increase in plasmalemmal labeling, relative to the CON spines of the ventral hippocampus, it is possible that the ventral hippocampus responds to environmental stressors but with greater delay than in the dorsal hippocampus. If so, a correlation between wheel running and synaptic as well as plasmalemmal labeling may emerge with a food restriction schedule that is longer than 4 days. The ventral hippocampus is recognized to regulate individuals' stress reactivity through inhibition of the HPA axis (rev in (Jankord and

Herman 2008)). Thus, even though the level of synaptic α4βδ-$GABA_A$Rs did not correlate with weight loss or wheel activity, other measurements of stress reactivity which we did not measure, such as the corticosterone level, may be correlated. Diet can influence BDNF (brain-derived neurotrophic factor) levels in the prefrontal cortex and ventral hippocampus concurrently and alter performance of behavior requiring the joint activation of the two brain regions, such as non-spatial discrimination reversal learning (Kanoski et al. 2007). It remains to be tested whether ABA animals' non-spatial learning remain intact, due to the lack of up-regulation of α4βδ-$GABA_A$Rs at synapses of the ventral hippocampus. On the other hand, animals that are better able to suppress food restriction-evoked hyperactivity through up-regulation of α4βδ-$GABA_A$Rs in the dorsal hippocampus may be 'paying the cost' of impaired spatial cognition, due to the reduction of synaptic plasticity in the dorsal hippocampus.

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ABBREVIATIONS

ED	Experimental Day
ABA	Activity-Based Anorexia
FR	Food-Restriction
EX	Exercise
CON	Control animals for ABA experiment
SI	Socially Isolated
PH	Pair-Housed

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SIGNIFICANCE

Anorexia nervosa (AN) has the highest mortality rate of any mental illness, surpassing depression, and is co-morbid with anxiety. There is currently no accepted pharmacological treatment to treat AN or to prevent its relapse. Using activity-based anorexia, an animal model of AN, we provide evidence that a risk factor to AN may be the failure to up-regulate α4βδ-$GABA_A$Rs at excitatory synapses of hippocampal CA1 pyramidal cells following stress. This may be due to stunted *de novo* synthesis and/or trafficking of α4βδ-$GABA_A$Rs from intracellular reserve pools to excitatory synapses. We propose the usage of α4βδ-$GABA_A$R agonists to treat AN.

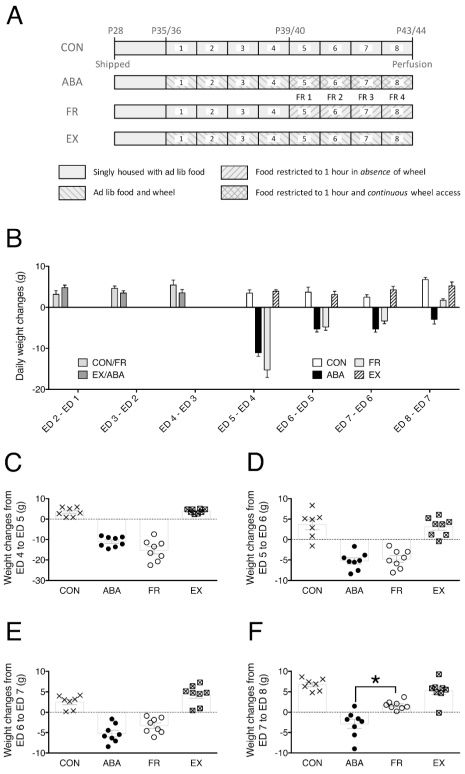

Figure 1. Daily weight changes of CON, EX, FR and ABA animals

Panel A shows the schedule of activity-based anorexia (ABA) and experimental controls**.** **Panel B** shows the group mean average of daily weight changes from ED 1 through ED 8. For the days preceding food restriction, the daily weight changes for the CON and FR groups were combined, while the daily weight changes for the EX and ABA groups were also combined, for assessing the potential effect of voluntary wheel running. This comparison revealed no difference. Upon food restriction, which began on ED4, the FR and ABA animals lost the greatest amount of weight during the first food restricted day (FR1, ED 5). ABA animals continued to lose weight up to ED8, while the FR animals no longer lost weight during the last food restricted day, FR4, from the end of ED7 to the end of ED8. Bars represent mean ± SEM. **Panels C through F** show single daily values of weight change for each animal of each group. The group difference between FR and ABA was significantly different for the last day of food restriction (the end of ED 7 to the end of ED 8, FR4). * denote $P<0.05$.

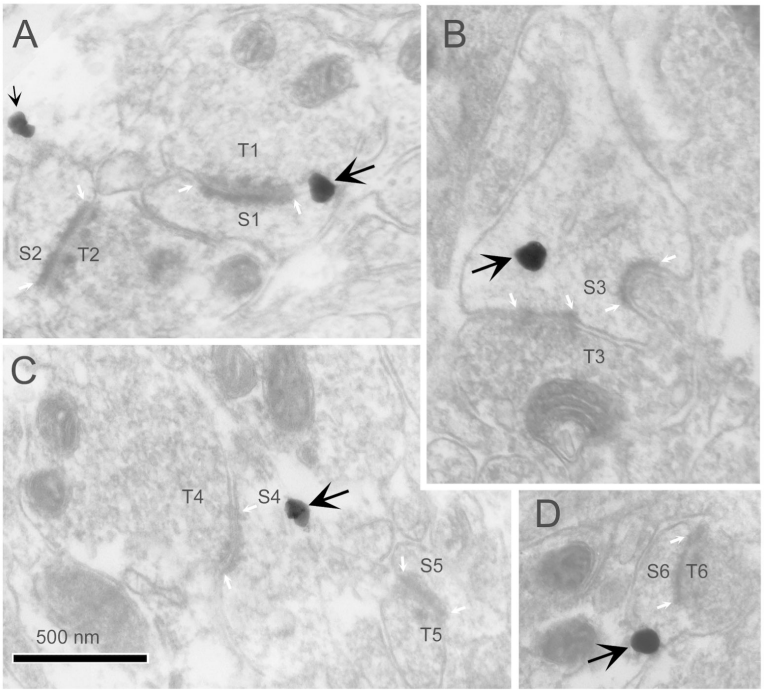

Figure 2. Electron micrographs showing examples of α4 subunit immunoreactivity within dendritic spines

All four micrographs were taken from the ventral hippocampus of the CON groups. Panel A shows the localization of silver-intensified gold (SIG) particle on the synaptic portion of the plasma membrane facing the synaptic cleft formed by axon terminal (T), T1 and dendritic spine (S), S1. The large black arrow points to the SIG particle, reflecting α4 subunit immunoreactivity, while the small white arrows point to the lateral extents of the thick postsynaptic density (PSD) demarcating the active zone of this excitatory axo-spinous synapse. The small black arrow to the left points to an example of SIG particles that were not identifiable, due to paucity of ultrastructural details surrounding them. The other excitatory synapses T2-S2 in panel A and T5-S5 in panel C are not immunolabeled for the α4 subunit. Panel B shows an example of an axo-spinous synapse T3-S3 exhibiting cytoplasmic SIG. Panels C and D show excitatory synapses with SIG that are associated with the plasma membrane of dendritic spines (S4 and S6) at portions that are not synaptic. Calibration bar equals 500 nm and applies to all four panels. These micrographs were captured at a magnification of 60,000x.

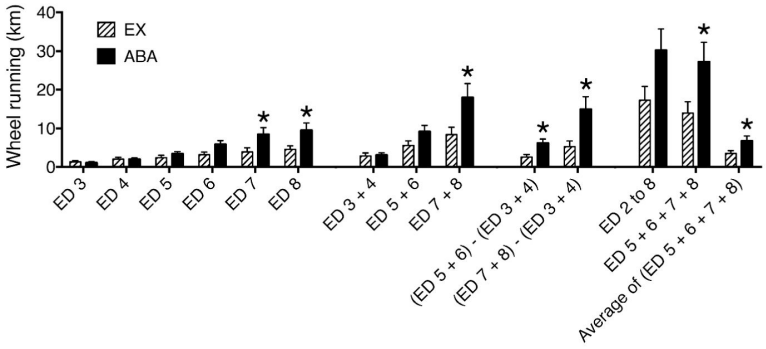

Figure 3. Daily wheel running activity of ABA and EX animals

Food restriction began on ED 5. Increases in wheel running evoked by food restriction were assessed by comparing ABA group's wheel running activity under food restriction to EX group's wheel running without food restriction in a variety of ways. ED 5 + 6, ED 7 + 8 and ED 5 + 6 + 7 + 8 depict wheel running during the food-restricted days. The difference of those running values to the running prior to food restriction (ED 3 + 4) reflect increases evoked by food restriction. Bars represent mean ± SEM. * denote $P<0.05$.

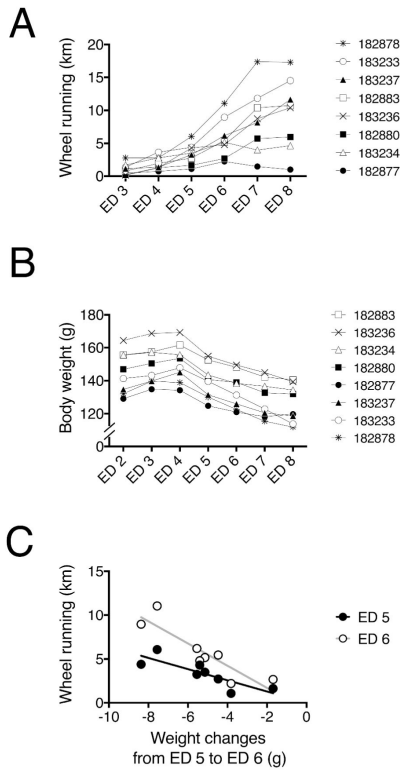

Figure 4. Individuality of food restriction-evoked hyperactivity and weight loss among the ABA animals

Panel A shows the daily wheel running activity of each ABA animal. While all animals increased daily wheel running activity, two animals decreased their daily running (animal 182877 and 183234) while others became much more hyperactive than others. For example, compare animal 182878 that ran 17-times more than animal 182877 during the last day of food restriction. **Panel B** shows the daily body weight of each ABA animal. **Panel C** shows the extent to which ABA animals became hyperactive due to food restriction varied greatly but was predictable, based on the extent to which the animal lost weight during the preceding day (FR1, corresponding to the end of ED4 to the end of ED 5), due to food restriction (filled data points). The extent that the animal lost body weight between the end of ED 5 and the end of ED 6 was also predictable, based on the extent that the animal ran during the second food restriction day, FR2, corresponding to the end of ED 5 and the end of ED 6 (unfilled data points).

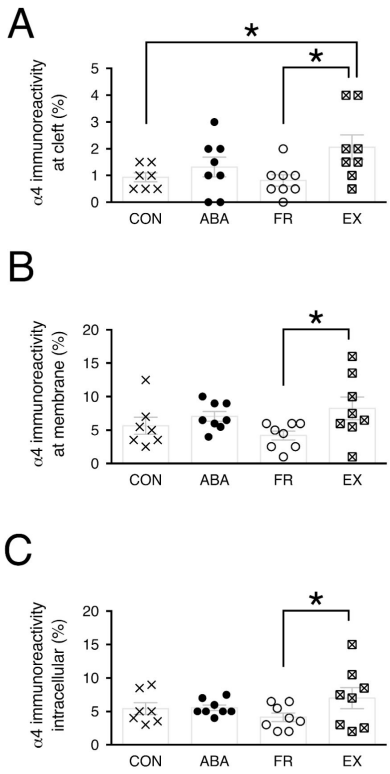

Figure 5. Group comparison of α4 subunit immunoreactivity at the synaptic cleft, at nonsynaptic portions of the plasma membrane and at intracellular sites of dendritic spines

Panel A: α4 subunit immunoreactivity was significantly increased at the synaptic membrane by exercise, relative to the CON and the FR tissue but not when exercise was combined with food restriction for the ABA group. ABA did not evoke a statistically significant difference from the CON in the membranous (**Panel B**) or intracellular (**Panel C**) labeling either, but exercise did.

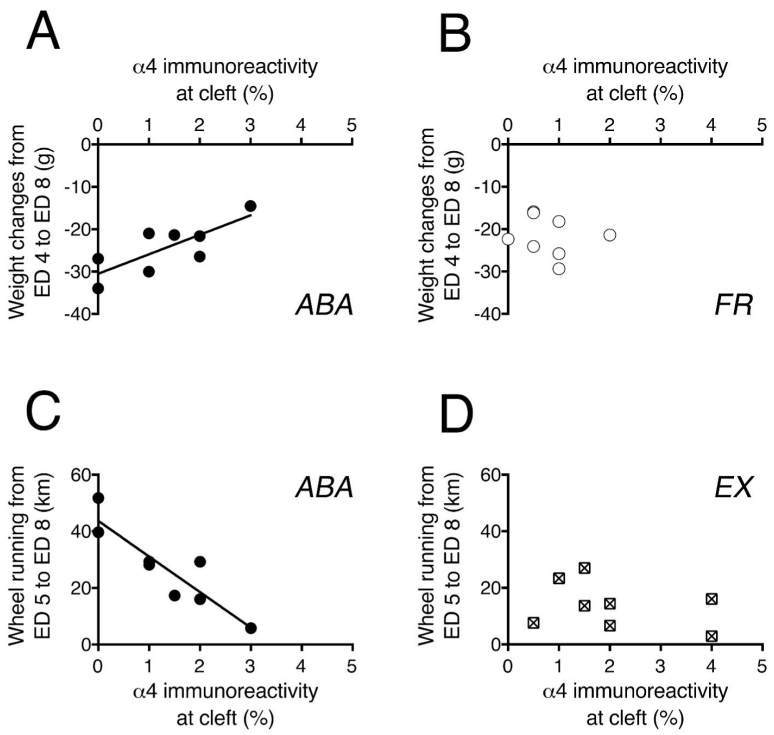

Figure 6. Correlation between α4 immunoreactivity at the synaptic cleft and weight loss or wheel running

Panel A: α4 subunit immunoreactivity at the synaptic cleft correlated strongly with the extent to which ABA animals lost body weight during the food restricted period, from the end of ED 4 to ED 8. The solid line in this and in panel C depict the best fit linear regression ($P = 0.023$, Pearson's $R = 0.78$ for panel A). Animals with highest levels of α4 subunit immunoreactivity lost the least amount of body weight. **Panel B:** α4 subunit immunoreactivity at the synaptic cleft does not correlate with the extent to FR animals lost body weight during the same food restricted period, from the end of ED 4 to ED 8, even though the extent to which they lost body weight is comparable. **Panel C**: α4 subunit immunoreactivity at the synaptic cleft correlated strongly with the extent to which ABA animals ran during the food-restricted period ($P = 0.002$, $R = -0.90$): the more that the animal expressed α4 immunoreactivity at the synaptic cleft, the less that they ran during the food-restricted days. **Panel D:** Although synaptic cleft labeling occurred at a higher frequency for the EX group, the extent to which this was augmented did not relate to the extent to which these animals ran during the EDs corresponding to ABA group's food restricted period.

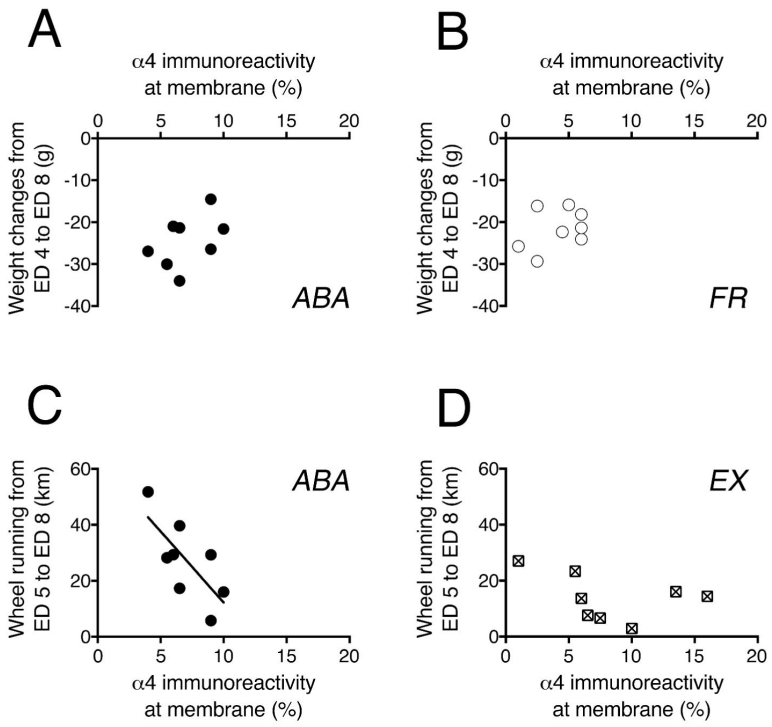

Figure 7. Correlation between α4 subunit immunoreactivity at the dendritic spine plasma membrane and weight loss or wheel running

α4 subunit immunoreactivity at the plasma membrane of dendritic spines did not correlate with the extent to which ABA animals or FR animals lost body weight during the food restricted period, relative to the beginning of the food restricted period (end of ED 4 to the end of ED 8) (**Panels A and B**). However, plasmalemmal α4 subunit immunoreactivity of ABA animals did correlate strongly with the extent to which the animals ran on the wheel during the food-restricted period (ED 5 to ED8) (**Panel C,** $P = 0.04$, $R = -0.73$). Plasmalemmal labeling of EX animals did not correlate with their wheel running during ED 5 to ED 8 (**Panel D**).

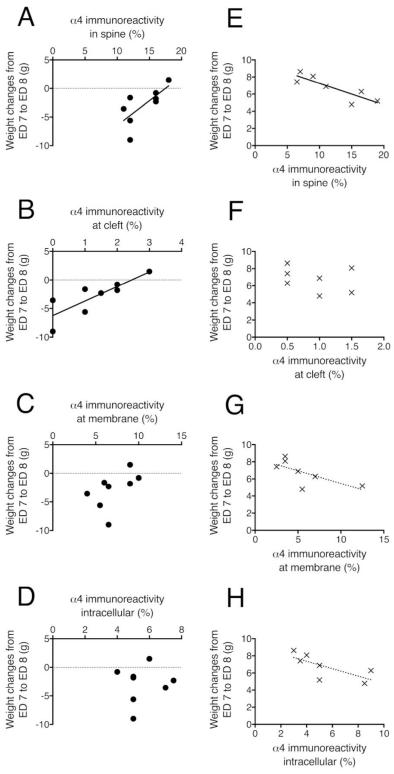

Figure 8. ABA and CON tissue differ in the subcellular compartment exhibiting correlation between α4 subunit immunoreactivity and body weight changes

Panels A through D show the results of correlation analysis between ABA animals' weight change during the day immediately preceding euthanasia (from the end of ED 7 to ED 8) and α4 subunit immunoreactivity across three distinct subcellular compartments – synaptic cleft (**Panel B**), at the plasma membrane (**Panel C**) and intracellularly (**Panel D**). **Panel A** shows the correlation between weight change and the sum of immunolabeling across the three compartments. **Panels E through H** show the results of correlation analyses for the same pairs of measurements but of the CON animals. Both CON and ABA tissue exhibit correlation between total α4 subunit immunoreactivity and weight change during the last day (**panels A and E**). However, the correlations are of opposite signs ($P = 0.05$, $R = 0.71$ for ABA; $P = 0.01$, $R = -0.86$ for CON). Moreover, the strong correlation is due to immunolabeling at the synaptic cleft for the ABA tissue (**panel B**, P=0.012, R=0.82) but is derived from intracellular ($P = 0.07$, $R = -0.72$) and nonsynaptic plasmalemmal sites (P=0.08, $R = -0.86$) for the CON tissue (**panels G and H**). This difference in the subcellular sites correlating with body weight changes suggests that ABA induction evokes redistribution of α4 subunit immunoreactivity from the cytosol and plasma membrane to the synaptic cleft.

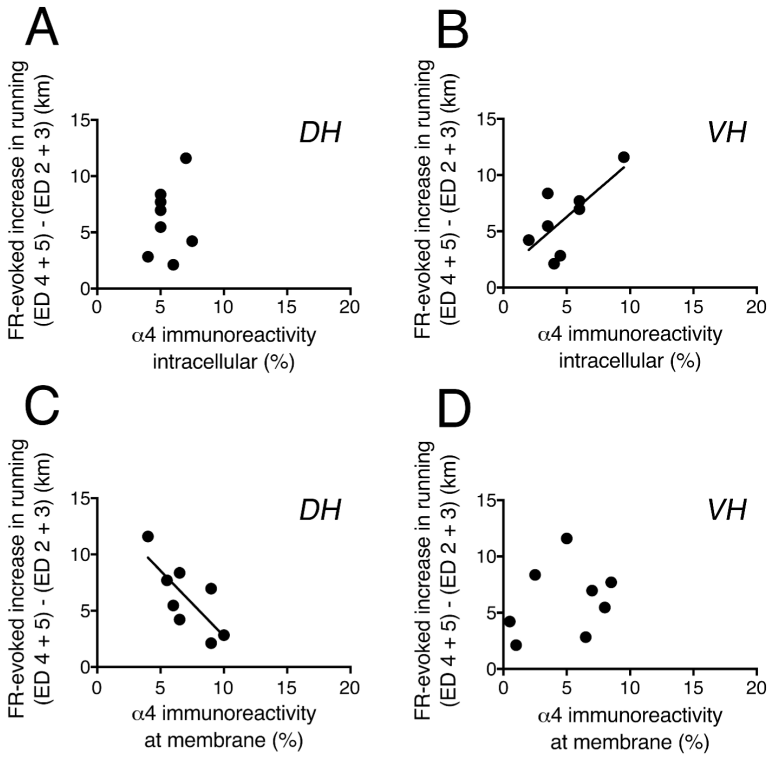

Figure 9. Comparison of α4 immunoreactivity in the dorsal versus the ventral hippocampus

Panels A and B compare correlations between intracellular α4 immunoreactivity and food restriction-evoked increase in wheel activity during the first two days of food restriction. The dorsal hippocampus (DH) exhibits no correlation (**panel A**), while the ventral hippocampus (VH) exhibits strong correlation (**panel B**, $P < 0.05$, $R = 0.72$, line depicts best fit for linear regression). The subcellular compartment of the dorsal hippocampus exhibiting correlation to food restriction-evoked increase in wheel activity is the plasma membrane (**panel C**, $P = 0.03$, $T = -0.77$). In contrast, the ventral hippocampus does not exhibit correlation between these two parameters (**panel D**).

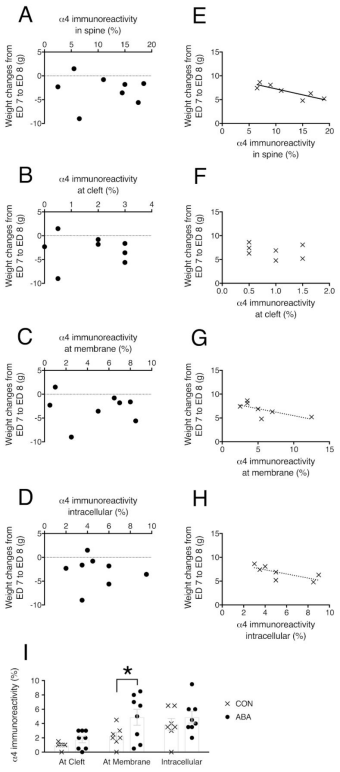

Figure 10. ABA disturbs the correlation between α4 immunoreactivity in the ventral hippocampus and body weight change

Panels E through H show correlations between weight changes during the last day before euthanasia (from the end of ED 7 to ED 8) and α4 subunit immunoreactivity in subcellular compartments of dendritic spines encountered within the ventral hippocampus of CON animals (cross symbols). Both the intracellular (**panel H**) and membranous (**panel G**) compartments exhibit weak correlation between α4 subunit immunoreactivity and weight changes during the last day before euthanasia, and the combined immunoreactivity is strongly correlated to the weight changes (**panel E**, P=0.013, R=-0.86). This pattern resembles that of the dorsal hippocampus of CON animals. **Panels A through D** show absence of correlation across the subcellular compartments of spines of ABA animals' ventral hippocampus (filled circles). **Panel I** shows comparisons of α4 subunit immunoreactivity in the ventral hippocampus of CON versus ABA tissue. The ABA rearing conditions evoked a change at the plasma membrane compartment that was significant. * indicates significant difference between group means ($P < 0.05$).

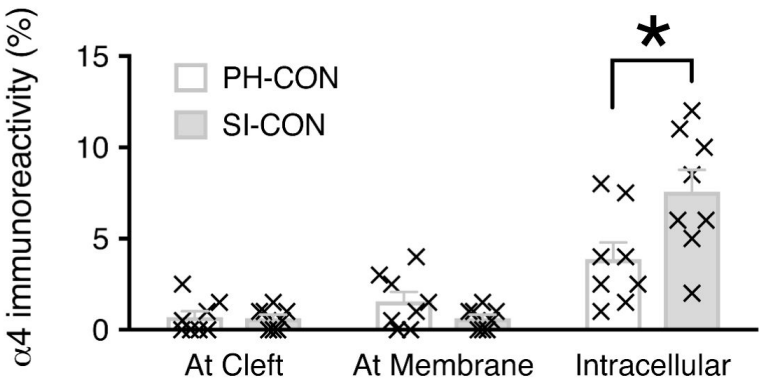

Figure 11. Comparison of α4 immunoreactivity in spines of animals socially isolated by single-housing versus pair-housed animals

α4 subunit immunoreactivity across the two rearing conditions were compared for the three subcellular compartments of spines: At the synaptic cleft, at nonsynaptic portions of the plasma membrane and at intracellular sites. The difference reached significance only for the intracellular domain (P=0.03, t_{14}=2.43341, 0.039 ± 0.009 % for PH; 0.076 ± 0.012 % for SI).

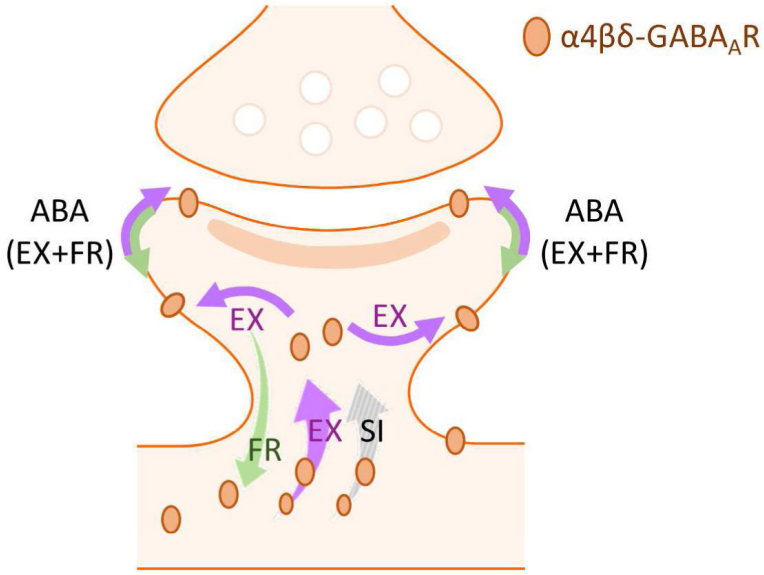

Figure 12. Hypothesized sequence of events leading to the up-regulation of α4βδ-GABA$_A$Rs at synaptic clefts of ABA tissue

This figure depicts a close-up of an axo-spinous excitatory synapse with a prominent postsynaptic density (PSD) and abutting presynaptic axon terminal, together defining the synaptic cleft portion of the spine's plasma membrane. Trafficking of α4βδ−GABA$_A$Rs (brown circles) to the synaptic cleft is hypothesized to be a three-step process: 1) from the dendritic shaft into the cytoplasmic reserve pool in the spine head; 2) from the cytoplasm to nonsynaptic portions of the spine's plasma membrane; then 3) towards the synaptic cleft portion of the spine plasma membrane. The arrows depict the environmental factors speculated to contribute towards trafficking of α4βδ−GABA$_A$Rs within spine heads, based on quantitative analyses of α4 subunit immunoreactivity within spines of the dorsal hippocampal CA1 of adolescent female rats. These animals were food restricted for four days (FR), housed with a running wheel to allow for exercise for 8 days (EX) or housed with a running wheel but also food restricted to induce ABA (activity-based anorexia). All animals were socially isolated (SI). In another cohort of adolescent female rats, the effect of social isolation was investigated by comparing α4βδ-GABA$_A$Rs within spines of socially isolated or pair-housed animals but otherwise given food *ad libitum* and no wheel access. Green arrows depict the hypothesized influence of food restriction, observed in the FR and ABA groups. Purple arrows depict the hypothesized influence of exercise, observed in the EX and ABA groups. Grey arrow depicts the influence of social isolation, observed in the socially isolated CON, when compared to pair-housed CON.