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Adolescent female rats exhibiting activity-based anorexia express elevated levels of $GABA_A$ receptor α4 and δ subunits at the plasma membrane of hippocampal CA1 spines

Chiye Aoki[1,*], **Nicole Sabaliauskas**[1], **Tara Chowdhury**[1], **Jung-yun Min**[1], **Anna Rita Colacino**[2], **Kevin Laurino**[2], and **Nicole C. Barbarich-Marsteller**[2,3]

[1]Center for Neural Science, New York University, 4 Washington Place, New York, NY 10003

[2]Department of Psychiatry, College of Physicians and Surgeons of Columbia University, 1051 Riverside Drive, Unit 98, New York, NY 10032

[3]New York State Psychiatric Institute, 1051 Riverside Drive, Unit 98, New York, NY 10032

Abstract

Activity-based anorexia (ABA) is an animal model for anorexia nervosa that has revealed genetic links to anxiety traits and neurochemical characteristics within the hypothalamus. However, few studies have used this animal model to investigate the biological basis for vulnerability of pubertal and adolescent females to ABA, even though the great majority of the anorexia nervosa cases are females exhibiting the first symptoms during puberty. GABAergic inhibition of the hippocampus strongly regulates anxiety as well as plasticity throughout life. We recently showed that the hippocampal CA1 of female mice undergo a dramatic change at puberty onset – from expressing virtually none of the non-synaptic α4βδ $GABA_A$ receptors (GABARs) pre-pubertally to expressing these GABARs at approximately 7% of the CA1 dendritic spine membranes at puberty onset. Furthermore, we showed that this change underlies the enhanced modulation of anxiety, neuronal excitability and NMDA receptor-dependent synaptic plasticity in the hippocampus by the stress neurosteroid, THP (3a-OH-5α[β]-pregnan-20-one or [allo]pregnanolone). Here, we used quantitative electron microscopy to determine whether ABA induction in female rats during adolescence also elevates the expression of α4 and δ subunits of α4βδ GABARs, as was observed at puberty onset for mice. Our analysis revealed that rats also exhibit a rise of α4 and δ subunits of α4βδ GABARs at puberty onset, in that these subunits are detectable at approximately 6% of the dendritic spine membranes of CA1 pyramidal cells at puberty onset (postnatal day 32–36; P32–36) but this drops to about 2% by P40 – P44. The levels of α4 and δ subunits at the CA1 spines remained low following exposure of P40 females to either of the two environmental factors needed to generate ABA - food restriction and access to a running wheel for four days (P40 to P44). This pattern contrasted greatly from those of ABA animals, for which the two environmental factors were combined. Within the hippocampus of ABA animals, 12% of the spine profiles were labeled for α4, reflecting a six-fold increase, relative to hippocampi of age-matched (P44) control females [$p<0.005$]. Concurrently, 7% of the spine profiles were labeled for δ, reflecting a 130% increase from the control values of 3% ($p=0.01$). No measurable change was detected for spine size. The observed magnitude of increase in the α4 and δ subunits at spines is sufficient to increase both tonic inhibition of hippocampus and anxiety during stress, thereby likely to exacerbate hyperactivity and weight loss.

[*]**Corresponding author**: ca3@nyu.edu; FAX: 212-995-4011; tel (212) 998-3929.

Keywords

anorexia nervosa; receptor trafficking; anxiety; mood; stress; food restriction; hippocampus; hyperactivity; exercise; tonic inhibition; puberty; adolescence; eating disorder; allopregnanolone; pregnanolone; progesterone; THP; hippocampus; electron microscopic immunocytochemistry

INTRODUCTION

Anorexia nervosa is a psychiatric illness with no accepted pharmacological treatment (Powers and Bruty, 2009) and with one of the highest mortality rates among mental illnesses (10–20%) (Birmingham et al., 2005; Bulik et al., 2007; Sullivan, 1995). Even when not fatal, anorexia nervosa can cause life-long damage to multiple organ systems, creating an increased burden on the health care system. The onset of anorexia nervosa is most common during puberty and adolescence, with 90–95% of the cases occurring among females (DSM-IV-R) (APA, 2000), and particularly among the population that exhibits anxiety disorders starting in childhood (Kaye et al., 2004). This developmental pattern suggests that hormonal changes associated with puberty may trigger developmental changes in brain connections that increase an individual's vulnerability to stress, anxiety, and anorexia nervosa.

The hippocampus is a key brain structure regulating anxiety. Anxiety is regulated, in part, by GABAergic inhibition of the limbic system, and especially of the hippocampus. This idea is based on the observation that local application of either benzodiazepines (BZD) or the β-carboline inverse agonists into the dorsal hippocampus of rodents leads to anxiolytic and anxiogenic effects, respectively (Huttunen and Myers, 1986; Kataoka et al., 1991; Talaenko, 1993).

Of the $GABA_A$ receptors (GABARs) in the hippocampal CA1, the most prevalent are those containing α1 and γ2 subunits (Farrant and Nusser, 2005; Wisden et al., 1991). Although much less abundant, the GABARs containing the α4 and δ subunits play a unique and important role in CA1 excitability, due to their unusual pharmacological profile and subcellular distribution (Shen et al., 2007). The α4- and δ-containing GABARs are unique in being insensitive to BZD (Smith et al., 2007). The α4βδ GABARs localize to sites removed from GABAergic axon terminals (Mangan et al., 2005; Nusser et al., 1998; Shen et al., 2010; Wei et al., 2004; Wei et al., 2003), yet are highly sensitive to GABA (Brown et al., 2002) and resist desensitization, thereby making them well-suited to mediate tonic inhibition via ambient GABA (estimated to be about 1 μM) that bathes the neuropil. By their localization to dendritic spine membranes in the CA1 (Shen et al., 2007), these receptors can also mediate shunting inhibition during activation of glutamate receptors, and thus potently modulate NMDA receptor-dependent synaptic plasticity and hippocampus-dependent spatial memory tasks (Shen et al., 2010). During adulthood and pre-pubertally, the α4βδ GABARs occur in low abundance in the hippocampal CA1 of female mice, being detected in no more than 3% of dendritic spines (Shen et al., 2007, 2010). However, at puberty onset, the expression of α4βδ GABARs in the CA1 increases transiently in response to the rapid rise, followed by the decline in the levels of THP (3α-hydroxy-5α[[β]-pregnan-20-one or [allo]pregnanolone), a stress steroid and a metabolite of progesterone (Shen et al., 2007). Although tonic inhibition is increased at puberty onset due to the elevation of α4βδ GABARs, the presence of the δ subunit also confers strong modulation by THP. In the CA1, THP inhibits α4βδ GABARs, thereby enhancing excitability of CA1 pyramidal cells whenever THP level is elevated (Shen et al., 2007). This acute, inhibitory effect of THP upon the α4βδ GABARs in the CA1 is strikingly different from its acute effect upon the α4βδ GABARs in the dentate gyrus. In the dentate gyrus, these GABARs are expressed constitutively and are enhanced by THP (Stell et al., 2003). These contrasting effects of THP

upon α4βδ GABARs across the two regions is due to differences in the direction of Cl^- flux across the two regions: the Cl^- flux is inward in the CA1 and outward in the dentate gyrus (Shen et al., 2007). The acute effect of THP upon the α4βδ GABARs in the CA1 is also different from its effect upon the more commonly occurring α1βγ2 GABARs (Smith et al., 1987; Twyman and Macdonald, 1992), the latter of which is to also enhance the Cl^- flux, thereby reducing excitability of CA1 pyramidal neurons. In short, the α4βδ GABARs are highly distinctive, in that these can enhance or dampen excitability of CA1 pyramidal cells, depending on the circulating levels of THP and the direction of Cl^- current.

From a previous study, we learned that the anatomically detectable change in the levels of α4 and δ subunits was modest from prepuberty to puberty, showing a rise from 2.3% to 7% in the proportion of spines immunolabeled for the α4 subunits and from 0.6% to 2.5% in the proportion of spines immunolabeled for the δ subunits (Shen et al, 2010 and unpublished observations). Still, the behavioral manifestation was profound, causing a reversal in the physiological effect of THP, from being anxiolytic pre-pubertally to anxiogenic at puberty onset (Shen et al., 2007; Smith et al., 2009). Moreover, this apparently small increase of α4 and δ subunits at onset of puberty impairs spatial memory (Shen et al., 2010), exacerbates stress-induced anxiety (Shen et al., 2007) and strongly affects excitability of CA1 pyramidal neurons (Shen et al., 2007). One key finding supporting these ideas is that genetic deletion of δ-subunits restores the pubertal decline in spatial memory to pre-pubertal levels and prevents the pubertal increase of stress-related anxiety (Shen et al., 2007).

The stereotypical age of onset of anorexia nervosa (puberty and adolescence) among females suggests that age and sex are critical factors in the vulnerability to the disorder. Based on the knowledge gained regarding the GABAR subunit switch at puberty onset and of its link to THP and stress-induced anxiety, we sought to determine whether the neurobiological basis of pubertal females' vulnerability to anorexia nervosa may be revealed by examining the state of GABAergic inhibition in the hippocampus.

ABA - activity based anorexia - is an animal model of anorexia nervosa that yields robust anorexic phenotypes, such as hyperactivity (running wheel activity, even during the limited period of food access) (Davis et al., 1999), reduction of food anticipatory activity and food intake (Gelegen et al., 2008), link to anxiety disorders (Gelegen et al., 2007; Gelegen et al., 2008), hypothermia (Gutierrez et al., 2006; Gutierrez et al., 2009), hypoleptinemia (Verhagen et al., 2009), severe body weight loss, and ultimately, death, unless rescued from the environmental factors (running wheel and restricted food access) (Casper et al., 2008). This animal model has also revealed increased expression of dopamine D2 receptors and brain-derived neurotrophic factor (BDNF) (Gelegen et al., 2008), as well as the reduction of hippocampal cell proliferation (Barbarich et al., submitted). The programmed food restriction in ABA promotes hyperactivity that results in dramatic weight loss and increasingly greater levels of hyperactivity, thereby resembling a maladaptive behavioral pattern of starvation-induced hyperactivity seen in some individuals with anorexia nervosa. Moreover, ABA animals have the opportunity to consume more food during the period of food access and to minimize activity levels, but instead continue to run without an increase in energy consumption, providing a model of self-imposed starvation. The goal of this study was to determine whether ABA induction is accompanied by elevated expression of the α4 and δ subunits at non-synaptic sites within dendritic spines of the hippocampal CA1, from which we would be able to infer the level of expression of the α4 and δ-containing extrasynaptic GABARs. Our quantitative electron microscopic data reveal that, indeed, food restriction and wheel access have a measurable impact upon both the α4 and δ subunit expression at the spinous plasma membrane of hippocampal CA1.

METHODS

Primary antibody information

The rabbit antibody used to detect the δ subunit of GABARs was a generous gift from the laboratory of W. Sieghart (Medical University Vienna). The immunogen used to produce this antibody corresponds to the extracellular N-terminus amino acids 1–44 of the rat origin peptide sequence (MDVLGWLLLP LLLLCTQPHH GARAMNDIGD YVGSNLEISW LPNL). This antibody was produced in rabbit, affinity purified and characterized for its specificity by Western blot analysis, in which it recognized a single band (Fritschy and Mohler, 1995; Jechlinger et al., 1998). Application of this antibody to brain tissue from δ KO mice generated no staining, indicating that the antibody is specific (Peng et al., 2002). The particular lot of antibody that we used was exactly the same lot used to characterize the specificity of this antibody in the previously published works.

The goat antibody used to detect the α4 subunit of GABARs was produced by using an immunogen corresponding to amino acids 32 through 50 from the N-terminus of human α4 subunit of GABARs (ESPGQNSKD EKLCPENFTR). This antibody was purchased from Santa Cruz (catalog # SC-7355), together with the immunogen, (#SC-7335p), shown by Rockefeller University's Proteomics Resource Center to consist of amino acids 32–50 from the N-terminus, with amino acid #50 being the residue that immediately precedes the first transmembrane domain. This antibody has been shown to recognize a single band at 67 kD by Western blotting (Griffiths and Lovick, 2005; Sanna et al., 2003) and to also disappear, following preadsorption of the antibody with a peptide corresponding to the target sequence (Sanna et al., 2003). In the current study, further tests of this antibody's specificity were conducted through preadsorption control and application onto brain tissue from α4-knockout (KO) mice. Details of these procedures are described below.

Immunocytochemical controls were conducted, using hippocampus of female mice that were at the transition from pre-puberty to puberty onset, which is when α4 and δ subunits' immunoreactivity emerge at the spine plasma membrane of hippocampus (Shen et al., 2007; Shen et al., 2010). For both α4 and δ subunit antibodies, a no-primary antibody control was conducted. This procedure was to incubate hippocampal sections of mouse hippocampi in a standard buffer (PBS-BSA-Azide, consisting of 0.01 M phosphate buffer, pH 7.6, containing 1% bovine serum albumin and 0.05% sodium azide) lacking the primary antibody, but otherwise maintaining all other aspects of the immunocytochemical procedure identical to the condition detailed below under the header, "Immunocytochemistry." This control yielded no immunolabeling, as assessed by electron microscopy (not shown). No further controls were conducted for the δ-subunit antibody, since previous reports had demonstrated specificity using brain tissue deficient in the δ subunit (Peng et al., 2002), thereby demonstrating that this antibody is highly specific.

For the α4-subunit immunocytochemistry, two additional controls were run, also using the hippocampus of female mice that were at the transition from pre-puberty to puberty onset. One was the preadsorption control, in which brain sections encompassing the CA1 field of pubertal female mice were incubated in PBS-BSA-Azide buffer containing the preadsorbed primary antibody. For this control, the primary antibody was a at a dilution of 1:100, but was preadsorbed for 1 hour at 30°C, then for an additional 24 hours at room temperature, using a control peptide (SC 3775p, synthetic peptide with identical amino acid sequence as the immunogen) at a concentration of 106 ng/ml. Two hundred spinous profiles, occurring within stratum radiatum of the CA1 field of one pubertal female mouse were analyzed, strictly in the order of encounter along the vibratome surface, so as to ensure random sampling within a zone where exposure to the immunocytochemical reagents would have been maximal. This analysis showed that 0% of the spines exhibited immunolabeling by

silver-intensified immunogold (SIG) on the plasma membrane of spinous profiles and only twice in the cytoplasm of the spinous profiles. In the tissue from another pubertal female mouse's CA1, again, 0% of the 150 randomly encountered spines exhibited SIG on the plasma membrane or in the intracellular portions of spinous profiles. This frequency was significantly different from that observed for semi-adjacent sections treated with the primary antibody without preadsorption. Without preadsorption, 5 out of the 100 randomly encountered spinous profiles exhibited SIG particles, of which 3 occurred along the plasma membrane. The frequency of encounter of SIG particles of the other pubertal female CA1 was even higher (12% ± 2% at the plasma membrane; 21% intracellularly within spines). The Kolmogorov-Smirnov two-sample test and the Mann-Whitney U tests indicated a significance of the difference ($p<0.005$ and $p=0.003$, respectively) for the spine membrane labeling as well as for the spine cytoplasmic labeling ($p<0.001$ by the Kolmogorov-Smirnov two-sample test and $p=0.0002$ by the Mann Whitney U test).

The third control for α4 subunit-immunolabeling was to demonstrate that the α4-antibody staining within brains of α4 subunit knockout (KO) mice is significantly diminished. For this test, brain sections obtained from the CA1 field of the hippocampus of wildtype (WT) female mice at puberty onset, as described above, were compared against corresponding portions of the hippocampus of female mice at puberty onset but of the α4-KO genotype, generated by deletion of exon 3 of the α4 gene of $GABA_A$ receptor, as was described previously (Chandra et al., 2006). In brief, heterozygous α4 KO mice on a C57BL/6J × Strain 129X1/S1 genetic background of the F6 generation were imported from the laboratory of Dr. Gregg Homanics at University of Pittsburgh to the laboratory of Dr. Sheryl Smith at SUNY Downstate. Heterozygous mice were interbred to generate homozygous offspring, confirmed to be so by Southern blot (Chandra et al., 2006). For this study, the homozygous KO offsprings were subsequently bred at SUNY Downstate with each other to produce the α4 KO female mice at puberty onset. Immunocytochemistry using the WT and KO brain tissue at puberty onsetconfirmed substantial reduction of labeling of α4-KO tissue (0.0%, 0.5% and 1.4% of spines labeled at the membrane), compared to values obtained from age-matched WT tissue (3% to 12%). These differences were significant, as were indicated by the Kolmogorov-Smirnov two-sample test ($p<0.025$) and the Mann-Whitney U test ($p=0.005$, $z=2.81202$).

Other chemicals

The secondary antibodies were donkey anti-goat IgG, conjugated to 0.8 nm colloidal gold (catalog #25220, from Electron Microscopic Sciences) and goat anti-rabbit IgG, also conjugated to 0.8 nm colloidal gold (catalog #25101, Electron Microscopic Sciences). The silver-intensification kit used to enhance 0.8 nm colloidal gold particles were purchased from KPL (Kirkegaard & Perry Laboratories, Inc.).

Epoxy resin, grids, fixatives and most other electron microscopic supplies were purchased from Electron Microscopic Sciences, while chemicals, such as bovine serum albumin, buffers and salts were purchased from Sigma Chem.

Animals used and their general housing conditions

Female rats were purchased as a group of 10 and 12 from Taconic and delivered to the New York State Psychiatric Institute's animal facility on postnatal day 21 (P21). Upon arrival, animals were individually housed in a reverse dark:light cycle in the absence of males. All procedures described here and below for ABA induction and behavioral controls were in accordance with the Institutional Animal Care and Use Committees of the New York State Psychiatric Institute, Columbia University (Animal Welfare Assurance No. A3007-01) and New York University (Animal Welfare Assurance number A3317-01).

ABA induction and behavioral controls

Two groups of animals, one consisting of 10 females and another consisting of 12 females, were allocated for ABA induction or its behavior control. Body weight, food intake, and wheel running activity (where applicable) were measured daily within 20 minutes of the start of the dark cycle starting on P36 and through P43 or 44, the day that they were euthanized. On P37, each of the group of 10 or 12 rats that were delivered together were divided into 4 treatment groups: 1) Control (CON): 24 hour per day food access with no wheel access; 2) Exercise (EX): 24 hour per day food and wheel access; 3) Food-restricted (FR): one hour per day food access with no wheel access; 4) Activity-based anorexia (ABA): one hour per day food access and 24 hour per day wheel access.

Animals with access to a running wheel were housed in a standard home cage with an activity wheel attached (Med Associates, Inc., St. Albans, VT). Baseline wheel running activity with 24 hour per day access to food was recorded for the EX and ABA groups from P37 to P39; running was quantified based on the number of wheel rotations per day, which was converted to km of running per day. On P40, restricted food access began for FR and ABA groups, with animals receiving unlimited access to food for one hour per day at the onset of the dark cycle.

In addition to the behavioral control groups described above, we also collected brains from 12 animals that were never co-housed with ABA animals. These CON animals ranged in age from P32–36 (N=6), corresponding to female rats' puberty onset, and P40–43 (N=6), which are still pubertal, non-estrous cycling, and passed the pubertal onset.

Preparation of brain tissue for immunocytochemistry

All animals from a single delivery group were euthanized on the same day by transcardial perfusion with fixatives within a period spanning from 4 hours to 30 min before the time that the room light was scheduled to be turned off for the light-dark cycle, on P44. One animal's brain was found with its cerebellum severely atrophied. Therefore, this animal's brain tissue was excluded from further processing and the animal's behavioral data were also excluded from data analyses.

In order to assess the effect of pubertal age, alone, upon α4 immunoreactivity, twelve additional animals ranging in age from P32 to P43 and receiving the CON treatment only, were euthanized by transcardial perfusion with fixatives during the hours ranging from one to three hours before the time that the room light was scheduled to be turned off for the light-dark cycle. Animals from this third CON group were not housed in the same room as the ABA, FR or EX groups. Perfusions of animals from this third group spanned across four days, with perfusions being conducted for three to four animals per day.

Prior to perfusions, all animals were deeply anesthetized using urethane (34%; 0.65 – 0.85 cc/185 g body weight, i.p.). Animals were transcardially perfused with 50 ml of saline containing heparin (4000 U/liter) at a flow rate of 50 ml/min. This was followed, without interruption, by perfusion with 500 ml of 4% paraformaldehyde, buffered with 0.1M phosphate buffer (pH 7.4), over an 11 min period and at a flow rate of 50 ml/min. A subset of animals was perfused over the same 11 min period with the same fixative, but with at a flow rate reduced from 50 ml/min to approximately 10 ml/min after the 5th minute. Preliminary studies indicated that both perfusion conditions yield good ultrastructural preservation and retention of antigenicity, even without the use of glutaraldehyde. For a few animals, the perfusion with heparin-saline was prolonged, thereby causing a delay in the delivery of the fixative by 4 to 5 min. This had no apparent effect upon immunoreactivity. These perfusion procedures were in accordance with Institutional Animal Care and Use

Committees of the New York State Psychiatric Institute, Columbia University and New York University.

Within 30 min following transcardial perfusion, the entire brain was extracted from the skull and post-fixed for up to 9 hours in the same fixative solution used for perfusion. Coronal brain sections containing the hippocampal formation were prepared using a vibratome, set to a thickness of 40 to 60 μm. These sections were stored in new plastic multiwell plates (Corning, Sterile, Model 3338) at 4°C, suspended in a buffer consisting of saline (0.9% NaCl), buffered with 0.01M phosphate buffer (pH 7.4) and containing 0.05% (w:v) of sodium azide (PBS-azide), shown to prevent bacterial growth and allow excellent morphological preservation for months to years (Dumitriu et al, 2011).

Immunocytochemistry

Vibratome sectioning—Immunocytochemistry was performed upon vibratome sections to detect α4 subunits of GABARs within the hippocampus of the rats. Another set of tissue underwent immunocytochemical detection of δ subunits. Quantitative electron microscopic comparisons were conducted only upon vibratome sections that were processed strictly in parallel, so as to ensure that the immunocytochemical labeling conditions were equalized with respect to post-fixation of the brain, the number of days that the tissue were stored in the coldroom (different by no more than 8 days), exposure time to immunoreagents, ambient temperature, concentrations and lots of the immunoreagents, and chemical quality. The entire immunocytochemical procedure was repeated to verify that the immunolabeling pattern remains consistent.

Primary antibody dilutions—The anti-α4 subunit antibody was diluted to 2 ng/ml (1:100), using PBS-BSA-Azide (described under the section ‘Primary Antibody Information’, above) as the diluent buffer. The anti-δ subunit antibody was diluted to 1:400 (0.5 ng/ml), also using PBS/BSA/Azide as the diluent.

Tissue permeabilization—Detergents are not compatible with ultrastructural preservation. Therefore, in order to enhance access of immunoreagents to the antigenic sites, vibratome sections from each of the perfused animals were freeze-thawed eight times in PBS containing 20% DMSO, then rinsed in fresh PBS containing 0.05% sodium azide. Tissue from all animals planned for quantitative electron microscopic comparisons were freeze-thawed in parallel, so as to ensure that this tissue permeabilization procedure was equalized across the treatment groups.

Tissue incubation in immunoreagents—These sections were then incubated, in parallel with sections of other brains, with either of the two primary antibodies for three days at room temperature, under constant agitation. On the third day, the unbound primary antibodies were removed through multiple rinses in PBS. Sections were then incubated overnight in PBS-BSA-Azide containing the secondary antibody. The secondary antibody that followed incubation by the α4-subunit antibody was donkey anti-goat IgG, conjugated to 0.8 nm colloidal gold. The secondary antibody applied following incubation in the δ-subunit antibody was goat anti-rabbit IgG, also conjugated to 0.8 nm colloidal gold. The dilution for both secondary antibodies was 1:100.

Immunolabel development—Following an overnight incubation at room temperature and constant agitation, tissue was rinsed in PBS, then post-fixed for 10 min at room temperature, using 2% glutaraldehyde as the fixative and buffered with PBS (pH 7.4). Fixation was terminated by rinsing sections multiple times in PBS. In order to prepare these sections for silver-intensification, which enlarges the 0.8 nm colloidal gold particles to sizes

large enough to be detectable under the electron microscope, sections were immersed for one min in citrate buffer (pH 6.0, 0.1 M), then immersed in the silver-intensification solution for 12 min at room temperature. The silver-intensification step was terminated by rinsing for one min in the citrate buffer, then in PBS.

Preparing tissue for electron microscopy—These sections were processed through the osmium-free steps of fixation, so as to avoid loss of silver-intensified gold particles (SIGs) by oxidation. The osmium-free steps involved exposure to tannic acid, iridium tetrabromide, and uranyl acetate, as was described previously (Shen et al., 2010). Ultrathin sections were prepared from vibratome section surfaces, oriented tangentially to the vibratome section surface, so as to maximize capture of surface-most portions of vibratome sections, where exposure to the immunoreagents would be maximal.

Electron microscopic quantification

All parts of the quantitative analysis, including image acquisition, were conducted with the experimenters blind to the animals' environmental treatments or age. Additional personnel, unaware of the working hypothesis, were also recruited for image acquisition and quantification steps. Dendritic spine profiles within ultrathin sections spanning stratum radiatum of the CA1 field of the dorsal hippocampus were identified as oval profiles, approximately 0.2 – 0.5 μm in diameter, free of microtubules, vesicles or mitochondria, with a characteristic narrowing of the profile (the spine neck), and with a thick postsynaptic density (PSD) along the portion of the plasma membrane that is in apposition to an axon terminal containing numerous vesicles. These spinous profiles were subjected to quantitative electron microscopic analyses.

Care was taken to sample portions of the vibratome section that were within one μm of the surface, where exposure to the immunoreagents would be maximal. Vibratome section surface, as opposed to cracks in tissue created during the steps subsequent to incubation with antibodies, was identified by the characteristic serrated edges formed by the vibratome blade during brain tissue sectioning. We systematized the random sampling strategy by analyzing all spines, strictly in the order that they were encountered, along the serrated edges of these vibratome section surfaces. In this way, the serrated edges were like the grids that are randomly laid over the sample to assign points for sampling. Usually, only one ultrathin section was analyzed from any one vibratome section, so as to ensure that any one spine did not get sampled more than once. For those occasions where more than one ultrathin section needed to be sampled from a single vibratome section, care was made to sample ultrathin sections that were more than 3 μm apart in the z-axis, so as to avoid sampling any spine more than once. Analysis was terminated at the point of encountering the 210^{th} spine profile, so as to equalize the sampling across the animals.

Receptor subunits occurring at the plasma membrane may or may not reflect the presence of functional GABARs, since receptor function is dictated not only by the location of its subunits but also by the formation of subunit complexes into functional pentamers (two α subunits, two β subunits, and either a δ or γ subunit) (Barnard et al., 1998; Farrant and Nusser, 2005). Conversely, receptor subunits occurring at sites removed from the plasma membrane are clearly not functional. Therefore, immunolabeled spine profiles were subdivided into those with or without plasmalemmal expression of α4 or δ subunits (Figs. 1 & 5). Since both the α4 and δ subunit antibodies were generated by using peptide antigens corresponding to the extracellular, N-terminus of the subunit, SIG particles reflecting antibodies bound to receptor subunits with functional membrane topography would be expected to localize along the extracellular surface of the plasma membrane. Therefore, those SIG particles associated with the extracellular surface of the plasma membrane were

counted and compared to the levels of SIG occurring at intracellular, non-plasmalemmal sites. SIG particles were categorized to be non-membranous and intracellular, when they were displaced from the plasma membrane by greater than 20 nm (equal to the thickness of a unit membrane) at a direct magnification of 40,000×. Examples of SIG particles categorized to be occurring near but not at the plasma membrane are highlighted with white arrows in Figs. 1 and 5. Occasionally, an SIG particle occurred over the plasma membrane of both the dendritic spine and the axon terminal or within the synaptic cleft (see Fig. 1 for examples). In these cases, the SIG particle was included in the tally of axon terminal labeling as well as the dendritic spine labeling.

Two counting procedures were performed to assess immunolabeling at the spine plasma membrane. One was to measure the proportion of dendritic spine profiles immunolabeled at the plasma membrane for the α4 or δ subunit. To this end, for every group of 10 spines that were randomly encountered, the number of spine profiles immunolabeled at the plasma membrane was assessed. This assessment of the proportion of spine profiles labeled was repeated 21 times for a single source of tissue, to obtain a mean value of 21 assessments, representing the analysis of 210 spine profiles. Any single spine profile was categorized as labeled, so long as it contained one or more SIG particles at the plasma membrane. Occasionally, a single spine profile from the ABA tissue contained more than one SIG particle and as many as three. Therefore, a second type of counting was performed, so to assess the level of immunoreactivity. For this assessment, the number of SIG particles at the plasma membrane per group of 10 spine profiles was determined. This procedure was also repeated 21 times, to obtain a mean value representing the analysis of 210 spine profiles per animal.

Statistical analyses

Our design consisted of four treatment groups – CON, FR, EX and ABA. For the analysis of α4-subunit immunolabeling, four mean values, reflecting the proportion and SIG counts of membranous and intracellular immunolabeling in spinous profiles were obtained for each animal, and these mean values were compared across the four treatment groups, with the N-value reflecting the number of animals included in the statistical analyses. For the analysis of δ-subunit immunolabeling, statistical tests were conducted only for the ABA and CON groups.

Normality of distribution of the mean values of the spine proportion and SIG counts were evaluated by the Kolmogorov-Smirnov Normality test, as well as the Lilliefors and Shapiro-Wilk's W tests, using N=number of animals from which brain sections were collected. The normality tests indicated that both the proportion of spines labeled and the number of SIG particles occurring along the plasma membrane of spine profiles were normally distributed for the tissue immunolabeled for the α4 subunit but not those immunolabeled for the δ subunits. Therefore, the statistical analyses of tissue immunolabeled for both the α4 and the δ subunits used nonparametric tests. These were the Kolmogorov-Smirnov two-sample test and Mann-Whitney's U test for comparisons between two groups, and the Kruskal-Wallis ANOVA by ranks and median test, as well as the multiple comparison of z- and p-values. One-way ANOVA and Tukey's LSD *post hoc* comparisons were also performed for the tissue immunolabeled for the α4 subunit (since these values were normally distributed), to determine whether the treatment had an effect on the proportion or levels of spinous labeling.

Brains were also collected from a third group of animals, all CON, and never housed in the same room with ABA, FR, or EX animals. These were grouped into two, based on their age, so as to assess whether α4 immunoreactivity differed for the puberty onset group (P32–36) relative to the older pubertal group (P40–43). The values obtained from these tissue were

normally distributed. Thus, both the Student's unpaired t-test as well as the Mann-Whitney U test were conducted to assess statistical significance.

All data from the electron microscopic immunocytochemical quantification were analyzed using the software Statistica (version 10.0, released from StatSoft), while experimenters were kept blind to the treatment condition of each animal. Differences were accepted as significant for p-values < 0.05.

RESULTS

Behavior of the ABA rats

Induction of ABA was assessed using two parameters: activity on the running wheel and body weight loss. Nine ABA animals were exposed to the combination of food restriction and access to the running wheel. The ABA animals were compared against 4 CON animals that had neither the access to the running wheel nor food restriction, 3 EX animals that had access to the running wheel but without food restriction and 5 FR animals that were food-restricted but without access to the running wheel. Data from one FR animal was excluded, because its brain was found to be missing the cerebellum during the post mortem tissue processing. Compared to the 3 EX animals, the ABA animals began exhibiting slight hyperactivity within the first day of food restriction (P41, 5^{th} day of the study). The activity increased five-fold by the 6^{th} day of the study (P42) ($p<0.05$ Student's unpaired t-test, t-value = 2.308) (Fig. 2A). This change in behavior by the ABA group was accompanied by a 9% loss of body weight within the first day following food restriction (5^{th} day of study, P41) and led to a 16% weight loss by the 4^{th} day in the ABA-inducing environment (P44). Since the CON animals continued to gain weight from P41 to P44, the difference of the mean body weights of the ABA group and CON group was even greater, resulting in a 24% difference between the two groups by P44. This difference was confirmed to be statistically significant [$F(3,17)=18.927$, $p<0.0001$ by one-way ANOVA; $p<0.0005$ by Tukey's *post hoc* analysis, comparing the values of ABA to CON animals].

The FR animals also lost 10% of their body weight within the first day of food restriction and this progressed to a 15% weight loss by the 4^{th} day of food restriction (P44), resulting in a statistically significant difference in mean body weight of the FR group, relative to the CON group ($p<0.005$ by Tukey's *post hoc* analysis). The weight loss by the ABA group exceeded that of the FR group, although the difference between the ABA and FR groups was not statistically significant ($p=0.7$ by Tukey's *post hoc* analysis, comparing of weights on the 7^{th} day of study when animals were P43 of age; $p=0.6$ on the 8^{th} day of the study, when animals were P44 of age) (Fig. 2B). Given these similarities in weight loss between the FR and ABA groups, the FR group provides an appropriate control for teasing apart the independent effects of weight loss (which occurs to a similar extent in both groups) and the combined effects of weight loss and hyperactivity (which only occurs in the ABA group).

α4 immunoreactivity within stratum radiatum of control pubertal female animals

It was previously shown that pre-pubertal female mice exhibit low levels of α4 immunoreactivity in the hippocampus but that this immunoreactivity rises at the onset of puberty (Shen et al., 2007; Shen et al., 2010) and is triggered by rapidly fluctuating levels of THP (Smith et al, 2007). Furthermore, this change was detected specifically at the plasma membrane of dendritic spine profiles (Shen et al., 2010). It has been shown that in both humans and rodents, a rapid change (rise, then the fall) in the circulating levels of progesterone is paralleled by changes in its metabolite, THP (Fadalti et al., 1999; Mannan and O'Shaughnessy, 1988; Palumbo et al., 1995; Smith et al., 2007) We suspected that the rise in the level of α4 immunoreactivity would to be transient, based on another earlier

finding indicating that α4βδ GABAR expression rises, then returns to low, baseline levels within 3 to 4 days after the pharmacological treatment that simulates THP withdrawal at puberty onset (Smith et al., 2007). We tested this idea by examining α4 immunoreactivity in the CA1 pyramidal cells of female rats starting from puberty onset and for several days subsequently. For rats, puberty onset can be assessed by vaginal opening, and typically occurs around P32–35 (Frisch et al., 1975; Frisch et al., 1977). We analyzed CA1 pyramidal cells of female rats that had never been housed in the same room with ABA, FR or EX females or with males. At P40–43 (N=6), the frequency of encounter with dendritic spine profiles immunolabeled at the plasma membrane for the α4 subunit was low (0.18 ± 0.03 per 10 spines, Fig. 3A), which corresponds to 1.8% of all (approximately 1200) spines that were randomly encountered and analyzed. The number of SIG particles residing at the plasma membrane of spine profiles was also low (0.2 ± 0.05 SIG particles per 10 spines, Fig. 3B), or 2 SIG particles for every 100 spines. In contrast, the frequency of spine profiles labeled at the plasma membrane was higher for the puberty-onset group (P32–36, N=6 animals; 0.64 ± 0.05 per 10 spines) (Fig. 3A), corresponding to 6.4% of all (approximately 1200) spines randomly encountered and analyzed. This difference represented a 250% difference, relative to the P40–43 group. Unpaired t-test indicated that this difference in the proportion of spines labeled was statistically significant ($p<0.00005$ by Student's t-test, t-value = 7.339046; $p=0.005$ by the Mann-Whitney's U test, z-value=2.812092).

In contrast to the small but highly significant presence of immunoreactivity at the plasma membrane at puberty onset, those SIG particles residing intracellularly did not exhibit a developmentally regulated change in levels (Fig. 3A and 3B, right).

α4 immunoreactivity in stratum radiatum of animals exposed to food restriction and wheel access for four days

Immunoreactivity for the α4 subunit was assessed for the CA1 pyramidal cells of the same 21 animals whose activities and body weights are shown in Fig. 2. The CON animals of this set were distinct from the CON animals used to characterize α4 immunoreactivity across the pubertal ages (Fig. 4), in that these were housed in the same room as the ABA, FR and EX animals. All animals were euthanized at P44.

As expected, electron microscopy revealed that the pyramidal cells of CON animals exhibit low levels of α4 immunoreactivity at the plasma membrane of spine profiles (0.23 profiles immunolabeled per 10 spines encountered, Fig. 4, equal to 2.3% ± 1.1% of spine profiles). In contrast, the proportion of spine profiles labeled specifically at the plasma membrane was 12.3 % ± 1.8% for the pyramidal cells of ABA animals (1.23 spines per 10, Fig. 4A), reflecting the presence of 14 ± 2 SIG particles per 100 spine profiles (i.e., 1.4 SIG particle per 10 spines, Fig. 4B). This difference was significant (Kruskal-Wallis ANOVA by ranks, $H(3,N=21)=13.17119$, $p=0.0043$; $p<0.00001$ and z-value > 4.84 comparing values of ABA animals to those of CON, FR or EX animals). α4 immunoreactivity of FR and EX spine profiles was also elevated at the plasma membrane of spine profiles, relative to the values of CON animals, but these changes did not reach statistical significance (Fig. 4). The proportion of spines labeled intracellularly did not exhibit any effect of the treatments that was significant (Fig. 4, right) - not even of the ABA animals.

δ subunit immunoreactivity within spines of animals exposed to food restriction and wheel access for four days

The localization of α4 subunits to non-synaptic plasmalemmal sites (with respect to GABAergic afferents) suggests that these could reflect GABARs containing δ subunits. However, the association of α4 subunits with γ2 subunits at peri- and non-synaptic sites is also possible, (Zhang et al., 2007). Importantly, GABARs composed of α4 and γ2 subunits

would be expected to exhibit much less sensitivity to both GABA and the neurosteroid, THP, than would the GABARs containing δ with the α4 subunits (Shen et al., 2007).. Therefore, we probed the hippocampi from the brains of the same ABA animals for the presence of δ subunits at non-synaptic plasma membrane sites on spines. These were then compared against the tissue from same CON animals that exhibited basal levels of α4 subunits. Tissue from all four CON animals that underwent α4 analysis were analyzed for δ subunit immunoreactivity and these were compared against tissue from from all nine ABA animals.

Electron microscopy confirmed that δ subunits also occur non-synaptically along the plasma membrane of dendritic shafts and spines as well as intracellularly (Fig. 5). Among the spines encountered among the 4 sources of tissue of CON animals, the mean frequency of labeling at the plasma membrane was 2.8 ± 0.6% (i.e., 0.28 ± 0.6 spines labeled per group of 10 spines, Fig. 6A). In contrast, the proportion of spines labeled at the plasma membrane from the tissue of 9 ABA animals was 6.5 ± 0.1% (i.e., 0.65 ± 0.1 spines labeled per group of 10 spines, Fig. 6A). Using animal number as the N-values, the Kolmogorov-Smirnov two-sample test and the Mann-Whitney U tests revealed statistical significance in the proportion of spines labeled at the plasma membrane ($p<0.05$; $z=2.546005$) and the number of SIG particles occurring at the plasma membrane ($p<0.01$; $z=2.623157$) (Fig. 6B). In contrast, the intracellular labeling of spines for δ subunits did not reveal an ABA effect.

Spine profile sizes within stratum radiatum of control and ABA animals

Since our sampling procedure was restricted to 2 dimensions, the probability of encounter with α4 and δ-immunoreactive spine profiles would be dependent on the size of spine profiles as well as the number of spine profiles. In order to eliminate the dependence on the number of spine profiles analyzed, the number of spines sampled was kept constant across the animals. In order to identify the potential contribution of spine size upon the rise in the encounter with α4- and δ-immunoreactive spine profiles, the plasma membrane lengths and areas of spine profiles were measured. This analysis was conducted for the same population of spine profiles for which δ-immunoreactivity was analyzed. The experimenters conducting these measurements were blind to the treatment conditions. Analysis of 800 spine profiles, derived from 4 ABA animals compared against the 800 spine profiles derived from 4 CON animals revealed no difference in the areas ($p=0.231963$, Fig. 7B) or spine plasma membrane lengths ($p=0.8154$, Fig. 7A) between the two populations of spines. Analysis only of the δ-immunoreactive spine profiles also revealed no difference between those found in brains of CON versus ABA animals (Fig. 7C and 7D). This result indicated that the greater encounter with immunoreactive spine profiles within brains of ABA animals was also not due to increases in the sizes of labeled spines within brains of ABA animals. These outcomes indicate that the increase in encounter with α4 and δ-immunoreactive spine profiles within brains of ABA animals was due entirely to an increase in the proportion of immunoreactive profiles. Finally, the mean size of all spine profiles (Fig. 7A and 7B), approximately 90% of which were unlabeled, was less than the mean size of labeled profiles (Fig. 7C and 7D). This observation indicates that we were able to identify spine profiles adequately, even in the absence of labeling.

DISCUSSION

Electron microscopic immunocytochemistry demonstrates that exposure of pubertal female rats to the ABA-inducing environment for four days evokes increased immunoreactivity of hippocampal CA1 pyramidal cells to the α4 and δ subunits of GABARs at non-synaptic plasma membranes of spines. Analysis of the two environmental manipulations used to induce anorexic behavior indicates that neither food restriction nor wheel access, alone, increases α4-immunoreactivity of spines sufficiently to reach statistical significance, but that

the combination of the two environmental factors yields robust increases of both α4 and δ immunoreactivities, even beyond that observed at puberty onset.

Although the preferred partner for α4 subunits are the δ subunits (Sur et al., 1999), α4 can also form pentamers with γ2 subunits, yielding α4βγ2 GABARs (Hsu et al., 2003; Wafford et al., 1996; Zhang et al., 2007). The presence of γ2 subunits within the GABAR pentamer confers, although does not 'guarantee', synaptic localization through its interaction with gephyrin (Essrich et al., 1998). Conversely, the presence of δ subunits confers non-synaptic localization, due to the lack of interaction of the δ subunits with gephyrin. By using electron microscopy to focus our analysis upon the non-synaptic, yet plasmalemmal location of α4 and δ-subunit immunoreactivity within spines, we were able to follow the rise of receptors that most likely subserve the physiologically functional population of non-synaptic α4βδ GABARs following ABA induction. We discuss below the putative mechanisms through which α4 and δ subunits of α4βδ GABARs may have risen and the physiological significance of this rise.

The use of EM to identify the non-synaptic plasmalemmal location of α4 and δ-subunits

A large portion of the SIG particles were associated with the extracellular portion of the plasma membrane. This is as expected, since the immunogens used to produce the antibody corresponded to the portion of the α4 and δ subunits spanning the N-terminal extracellular domain. Besides these SIG particles along the plasma membrane, an additional population of SIG particles was located in the cytoplasm of dendritic shafts and spines, either contacting the intracellular surface of the plasma membrane or removed altogether from the plasma membrane. The number of SIG particles residing in the cytoplasm (i.e., removed from the plasma membrane), was reduced by preadsorption and by deletion of exon 3 of the α4 subunit, indicating specificity of immunolabeling. Based on the proximity of these SIG particles to the cytoplasmic smooth membranous vesicles (e.g., Fig. 6B), which most likely are segments of the smooth endoplasmic reticulum, these SIG particles are likely to reflect antibodies bound to α4- or δ-containing GABA receptors or even of the subunits yet to be formed into pentamers, residing as reserve pools of GABARs. Alternatively, these may reflect the degradative pools of GABARs.

Putative mechanism through which α4 and δ subunits may have risen in CA1 pyramidal cells of ABA animals

The mechanism underlying the rise of α4 and δ- in the hippocampus remains to be explored, but one possibility is through the rise in the circulating levels of a neurosteroid, THP. In both males and females, THP rises in response to stress (Purdy et al., 1991) and THP levels are elevated among adolescent individuals with anorexia nervosa (Golubchik et al., 2007). Therefore, the large increase of α4 immunoreactivity that we observed among the ABA animals may have been in response to the 4-days of food restriction, which caused stress and with it, the rise of THP. The possibility that food restriction is linked to stress and the THP rise is indicated by the observation that spinous profiles of the CA1 pyramidal cells of the FR animals also exhibited a rise of α4 subunits, although not to the degree seen within brains of ABA animals and not statistically significant. The slightly greater level of α4 in ABA brains, relative to those of FR animals, albeit not statistically significant, may be due to the stronger stress effect of food restriction, in face of the much greater energy demand caused by hyperactivity. Exercise is well-known to induce the release of BDNF (brain-derived neurotropic factor), (although this effect may depend on the developmental stage of the animal (Hopkins et al., 2011)) and others have, in turn, reported that BDNF release stimulates the expression of α4 subunits in the dentate gyrus (Roberts et al., 2006). Thus, it is also possible that the rise of α4 subunits is greater in the hippocampal CA1 of ABA

animals, relative to that of the FR animals, due to the synergistic effect of stress and voluntary exercise.

The reason we analyzed the hippocampus of pubertal females is because anorexia nervosa arises most frequently among females entering puberty. Why is this so? Adolescence and puberty, in particular, are marked by increased emotional lability and this has been proposed to be due to hormonal surges. One possible link between the hormonal surges at puberty onset and emotional lability may be fluctuations of THP levels, which regulate the expression and activity of α4βδ GABAR subunits in the hippocampus (Shen et al., 2005; Smith et al., 1998; Smith et al., 2007; Shen et al., 2007). THP levels naturally fluctuate at the onset of puberty both in humans (Fadalti et al., 1999) and in rodents (Palumbo et al., 1995; Shen et al., 2007). Accordingly, our most recent results (Shen et al., 2010) indicate that the plasmalemmal expression of α4 and δ subunits, measured by electron microscopic immunocytochemistry, and with it, the electrophysiologically detectable level of α4βδ GABAR within spines of female mouse hippocampi, increases at puberty onset. This observation was confirmed in the present study of the female rat hippocampi at puberty onset (P32–36). We therefore hypothesized that the combined environmental factors of physical activity with food restriction may interact synergistically with pubertal changes in circulating THP, resulting in brain circuitry changes, such as the increased localization of α4βδ GABAR that lead to ABA vulnerability.

The rat brains that were used for this study were P43 to P44, approximately 10 days past entry into puberty.Our results indicate that, by the age of P43 to P44, the α4 subunits at the hippocampal plasma membrane of CON and EX animals are at levels that are lower than at puberty onset (P32–36). Based on these observations, we are able to conclude that factors other than puberty onset stimulated the localization of the α4 subunits to the plasma membrane of CA1 pyramidal cell spines of the ABA animals at ages P44. We did notice high levels of intracellular α4 immunoreactivity in the spines of P43 CON animals (cf Fig. 3). These may serve as the reserve pool that supports trafficking of α4βδ GABARs to the plasma membrane under conditions favoring their plasmalemmal localization.

The proportion of spines labeled intracellularly did not exhibit any significant effect of the treatments - not even in the ABA animals. The contrasting outcome of plasma membrane versus intracellular immunolabeling suggest that the combined ABA treatment of food restriction and running wheel access may be affecting the trafficking of the cytosolic pool of α4-containing GABARs to the plasma membrane, with the net gain in the insertion of these receptors into the plasma membrane, while the cytosolic pool is being replenished by trafficking from the parent dendrite.

The functional significance of the rise of α4 and δ subunits in the CA1 at puberty onset

As a first step towards identifying changes that may be evoked by the ABA-inducing environment during puberty and adolescence, we began analyzing the occurrence of α4 and δ subunits in the CA1 pyramidal cells of the hippocampus. The pyramidal neurons in the CA1 field were chosen for the study, because it is one of the few brain regions that have undergone physiological characterization of α4βδ GABAR function, with the other brain regions being the thalamus and dentate gyrus (Chandra et al., 2006). As for the characterization of these receptors' physiological impact specifically within the female brain during puberty and adolescence, the hippocampal CA1 is the only region so far analyzed by any group. Pre-pubertally and in adulthood, acute (up to a few hours) rise of THP compensates for stress by augmenting α1βγ2 GABAR activity in the CA1 and the α4βδ GABAR activity in the dentate gyrus, both of which contribute towards the anxiolytic effect of THP (Shen et al., 2007). In contrast, at puberty onset, the CA1 expresses elevated levels of α4βδ GABARs and as a result, the THP modulation of α4βδ GABARs dominates over its

effect upon the α1βγ2 GABARs. Importantly, the effect of THP upon the α4βδ GABARs expressed by CA1 pyramidal cells has an opposite effect – namely, to reduce Cl^- fluxes gated by α4βδ GABARs, possibly through desensitization of the GABARs, thereby increasing excitability of the pyramidal cells and increasing stress steroid-induced anxiety (Shen et al., 2007).

Putative Biophysical correlates of α4βδ GABAR elevation in CA1 of ABA animals

Although our study demonstrates that ABA induces elevated expression of both the α4 and δ subunits, this does not prove that the two combine to form α4βδ GABARs. However, even though α4 subunits can potentially form complexes with either the δ or γ2, the two compete for partnership with α4, and of the two, δ may be the preferred partner (Sur et al., 1999). This, together with the concomitant rise of both the α4 and δ expression and specifically at non-synaptic portions of the plasma membrane, suggests that this neurochemical change reflects the rise of functional α4βδ GABARs at the plasma membrane.

The rise of α4 and δ subunit immunoreactivities within ABA hippocampi was highly significant, statistically, but was the increase of sufficient magnitude to underlie a physiological change? Based on results of earlier studies that correlated changes in plasmalemmal immunoreactivity of α4 and δ-subunits to excitability of hippocampal pyramidal neurons, it is likely to be physiologically significant. Specifically, we previously observed that an increase in immunoreactivity of the α4 subunits at spine membranes from pre-puberty to puberty (from 2.3% of spines being labeled, to 7%) was accompanied by a dramatically reduced excitability of the CA1 pyramidal cells in the absence of THP but also an increased excitability of pyramidal neurons in the presence of THP (Shen et al 2007, 2010). What we observed in the current study was a four-fold increase in the immunoreactivity of α4-subunits, from approximately 3% of spines being labeled in controls, to 12%. This change is comparable to that seen from pre-puberty to puberty, if not more. The increase of δ-subunits was smaller – 138% increase for both the proportion of spines immunolabeled and the number of SIG particles at the plasma membrane (from 2.8% of the spines being labeled in controls, up to 6.5%) – but this may be sufficient to accompany the greater increase of α4 subunits, since the stoichiometry of GABARs is two α subunits for every one δ subunit (Barnard et al., 1998; Farrant and Nusser, 2005). On the other hand, it is possible that some of the non-synaptic α4 subunits at the plasma membrane within the hippocampus of ABA animals reflect pentamers formed with the γ2 subunits, contributing towards reduced excitability of the CA1 pyramidal neurons.

The encounter rate of immunolabeled spines may seem low, but this is likely to reflect, at least in part, the procedure we used to measure immunoreactivity: our procedure was to measure encounter rates within single, non-overlapping ultrathin sections, rather than serially collected and stacked images rendered into 3-D reconstructions. Since the mean of spine head profiles had diameter of 300–350 nm (based on the mean area and perimeter measurements, Fig 7) with postsynaptic densities that were at least 1/2 that diameter (150–175 nm), most ultrathin sections of thickness 70 nm captured no more than one-half of the volume of average-sized spine heads and much less than one-half of the volume of larger-sized spine heads. Thus, assuming that each spine contains only one immunoreactive site, and keeping in mind that the SIG immunolabel is not diffusible (unlike HRP-DAB reaction products, which are diffusible), the rate of encounter of 12% with immunolabeled spine profiles is likely to reflect an underestimation of the actual prevalence of the α4- containing GABARs. Therefore, it is likely that the increased expression of α4 and δ subunits following ABA induction has physiological consequences similar to those shown at puberty onset, namely reduced excitability of the CA1 pyramidal cells at rest and heightened level of steroid stress hormone-induced anxiety.

α4βδ GABARs at spine heads

As was described earlier, the δ subunit of α4βδ GABARs confers non-synaptic localization, which leads to its diffuse location, including spine heads and other sites removed from GABAergic axon terminals. Spines are better known as targets of excitatory inputs. In cortex, the spines known to receive direct (i.e., synaptic) inhibitory input are a specialized subset that also receive direct excitatory input from the thalamus (Kubota et al., 2007). In the hippocampus, thalamic afferents are mostly in the stratum lacunosum moleculare (Herkenham, 1978), a region that was excluded from our analysis. Due to its non-synaptic location (with respect to GABAergic axon terminals) within layers removed from stratum lacunosum moleculare, α4βδ GABARs occurring on spines of CA1 stratum radiatum are more likely to be activated by the ambient, lower (micromolar) levels of GABA that bathe the synaptic neuropil and exert tonic (chronic) inhibition. At times when THP is not rising, these spinous α4βδ GABARs may interfere with excitatory input through shunting inhibition, thereby impairing plasticity at excitatory synapses and hippocampus-dependent learning. This idea was put forth by us earlier for female mice at puberty onset (Shen et al., 2010) and may apply to ABA animals as well. We observed that some of the SIG particles associated with spine heads were also overlying the plasma membrane of presynaptic axon terminals. We interpreted these SIG particles to be reflective of spine labeling, because we are not aware of any study demonstrating the effect of activity of GABARs containing α4 and δ subunits upon glutamate release. On the other hand, we cannot exclude the possibility that some of these SIG particles reflect immune-labeling of glutamatergic axon terminals and that neurotransmitter release from glutamatergic axon terminals is also modulated by ambient GABA. This is an idea that can be tested in future biophysical studies.

Future directions

Regular exercise is known to be anxiolytic (Cakir et al., 2010; Greenwood and Fleshner, 2008; Greenwood et al., 2003; Sasse et al., 2008). The voluntary hyperactivity exhibited by ABA animals may be a manifestation of the animals' method to 'cope' with heightened anxiety when faced with food restriction, even though it has a maladaptive effect of exacerbating the negative energy condition caused by food restriction. Since α4- and δ-containing GABARs are also BDZ-insensitive (Smith et al., 2007; Wafford et al., 1996; Wisden et al., 1991), the anxiolytic response of BDZ would also be expected to be blunted at puberty onset and among ABA animals. If individuals with anorexia nervosa also exhibit increased expression of α4-containing GABARs in the hippocampus, then BDZs might not be effective in the treatment of anorexia nervosa. To date, these compounds have not been systematically tested in the clinical population. In support of this idea, adult female rats that have been induced to express elevated levels of α4 subunits by the administration of progesterone or THP over a course of 48–72 hr exhibit blunted anxiolytic response to BDZ (Gulinello et al., 2001; Gulinello and Smith, 2003). We intend to explore the behavioral effects of drugs other than BDZ that compete with THP to modulate α4βδ GABARs, in hopes that these would reduce anxiety associated with stress and with it, the maladaptive hyperactivity.

Estrogen is another hormone linked to puberty and an obvious candidate involved in anorexic behavior (Eastwood et al., 2002; Gao et al., 2007; Geary et al., 2001; Rosenkranz et al., 1998; Young, 1991) as well as the enhanced excitability of the hippocampus (Smith and Woolley, 2004). The interplay of estrogen and progesterone/THP, via the excitatory and inhibitory neurotransmitter receptors at the dendritic spines of the hippocampus is of great interest to us and would be another subject to study in the future.

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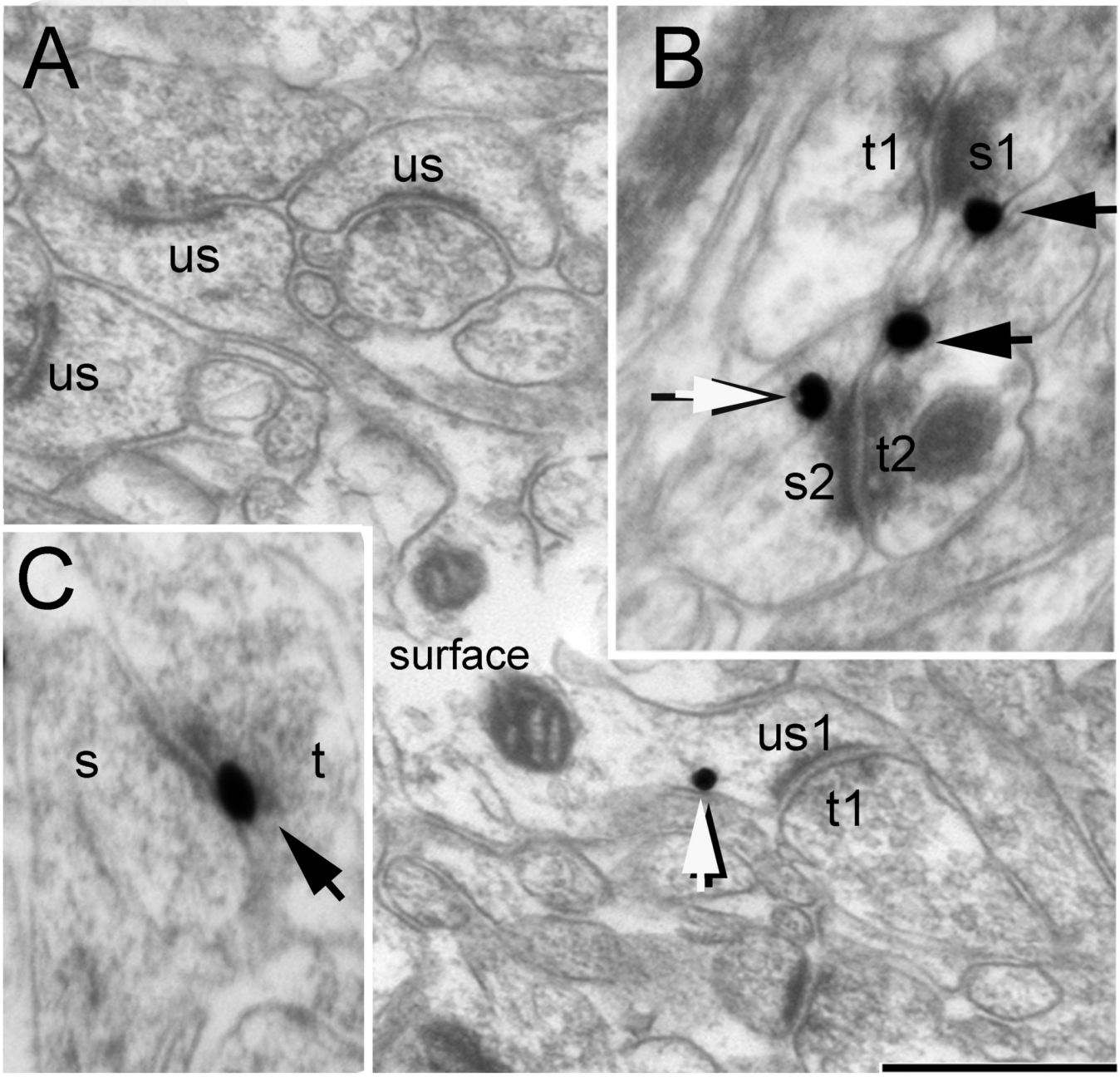

Fig. 1. Categorization of the ultrastructural location of α4 immunoreactivity, as revealed by electron microscopic immunocytochemistry

Electron microscopic immunocytochemistry, using silver-intensified gold (SIG) as immunolabels, revealed a variety of subcellular locations for the α4 subunit of GABAR. The subcellular location of SIG was categorized as plasmalemmal versus intracellular, and as occurring within spine versus shaft domains of dendrites. **Panels A through C** show examples of α4 immunoreactivity occurring at the plasma membrane of dendritic spine profiles (black arrows). Here and in other panels, 'us' indicates an unlabeled spine profile. In Panel A, the SIG particle associated with the synapse is localized along the intracellular surface of the plasma membrane (white arrow). Such localizations were excluded from the

counts of plasmalemmal labeling and were counted as intracellular. **Panel B** shows two SIG particles spanning the plasma membrane of dendritic spines s1 and s2 (black arrows). Both of these SIG particles are categorized as occurring along the extracellular surface of the plasma membrane and would be included in the plasmalemmal labeling counts. In contrast, the white arrow in Panel B points to an SIG particle occurring near the postsynaptic density of s2 but is not in contact with the plasma membrane. This SIG particle is categorized as intracellular and excluded from counts of plasmalemmal labeling. **Panel C** shows another SIG particle located along the extracellular surface of the plasma membrane of a dendritic spine (black arrow). Panels A and C were taken from brains of ABA animals, while panel B was taken from a FR animal. Calibration bar = 500 nm.

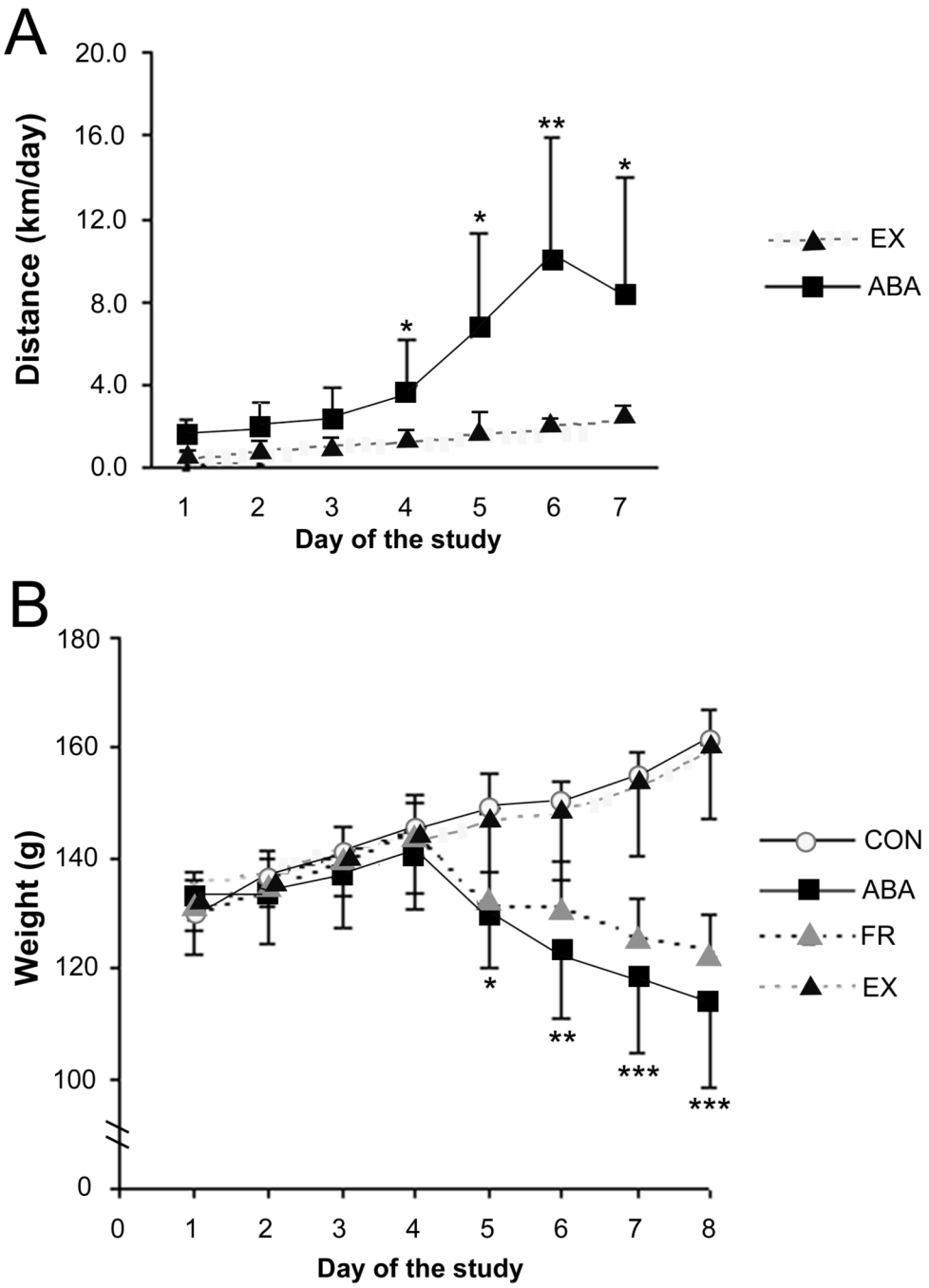

Fig. 2. Wheel activity and body weight of animals across the days of the study

Nine animals designated for ABA environment were monitored for their baseline wheel activity (**Panel A**) and body weight (**Panel B**) for 3 days, starting from postnatal day 37 (P37, indicated as 1st Day of the Study in the graphs) through P39 (3rd Day of the Study), then were shifted into the ABA environment on the 4th day of study, when they reached P40, and kept in this environment for 4 days (up to the 8th Day of the Study). Body weight of 4 control animals (CON), 5 food restricted animals (FR) and 3 exercise animals (EX) were also measured once per day within 15 min of the time that the 9 ABA animals were weighed. The EX and ABA animals were also monitored for their wheel activity per day, except the day on which they were perfused. In the Distance graph of Panel A, * indicates

significance of the difference in the distance ran by the ABA group, relative to the EX group with $p<0.05$ by the Student's t-test, while ** reflects a significance level $p<0.005$. The asterisks in the Weight graph of Panel B indicate significance of difference of the ABA group when compared to the CON group, with * indicating $p<0.01$; ** indicating $p<0.005$; and *** indicating $p<0.0005$. Values are reported as mean ± SD.

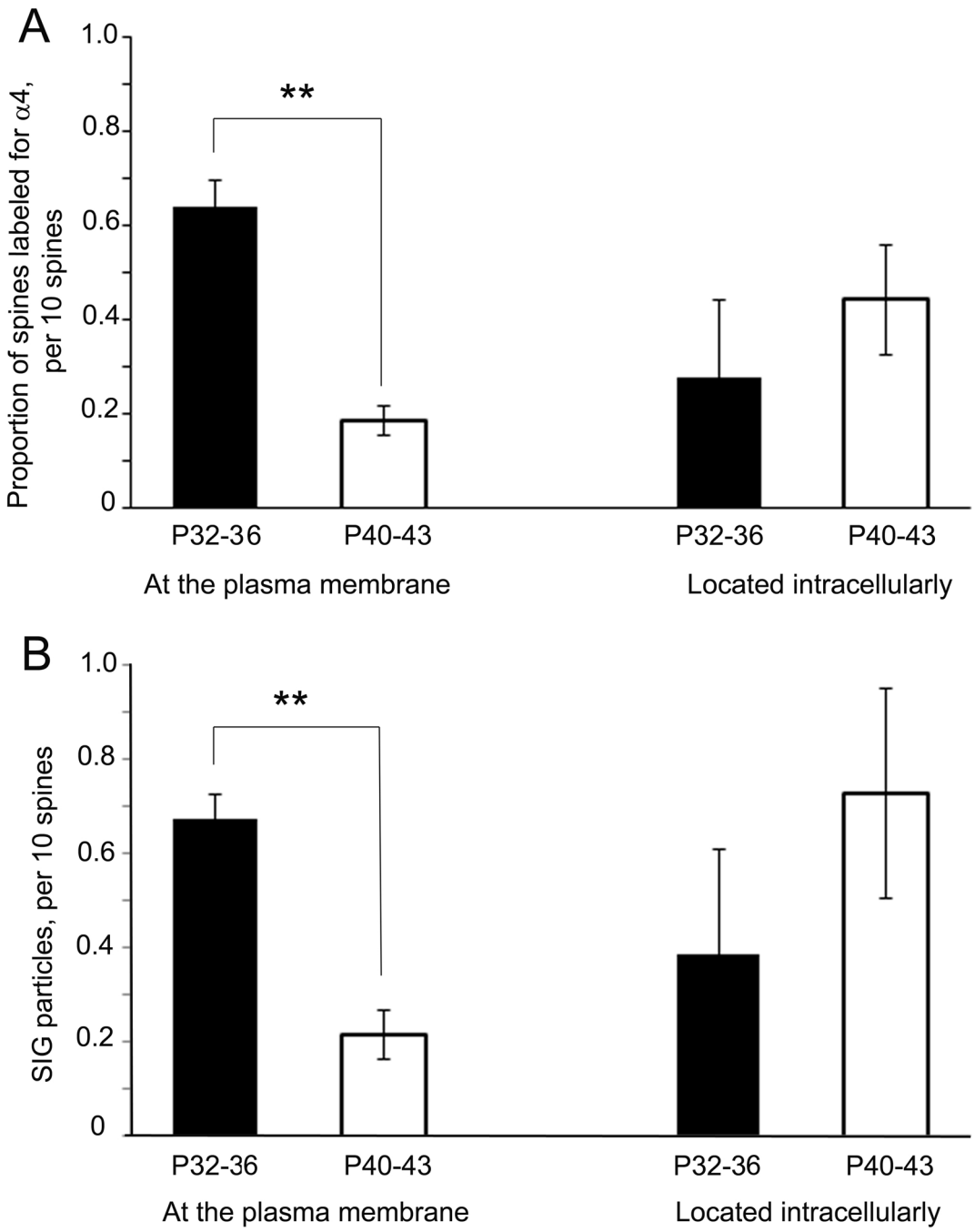

Fig. 3. Comparisons of immunoreactivity for the α4 subunit within the CA1 pyramidal cells of females at puberty-onset and older

Dendritic spine profiles from the CA1 pyramidal neurons of six animals spanning the ages of puberty onset (P32–36) and six more of slightly older age (P40–43) were compared for their immunoreactivity to the α4 subunit of GABARs. Two counting procedures were conducted: measurement of the proportion of all encountered spine profiles immunoreactive for the α4 subunit (**Panel A**) and the number of SIG particles occurring in spine profiles, which reflected levels of α4 immunoreactivity (**Panel B**). Only those SIG particles located at the extracellular surface were included in the plasmalemmal counts. From each animal, 210 spine profiles were analyzed. The two groups were compared, using the Kolmogorov-

Smirnov two-sample test and the Mann-Whitney U test. ** indicates p-value ≤0.005, by both tests. Values are reported as mean ± SEM.

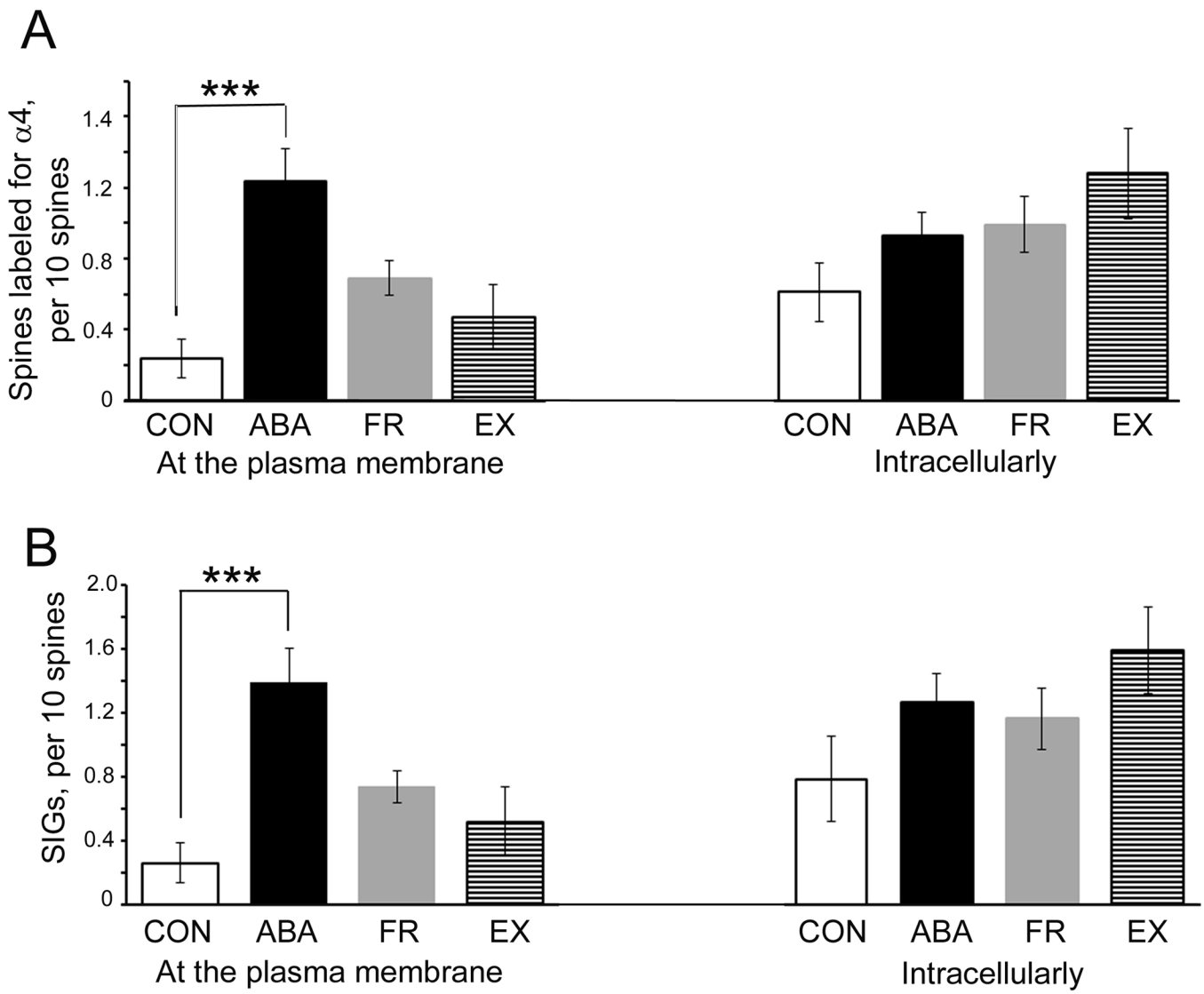

Fig. 4. Comparisons of immunoreactivity for the α4 subunit within the CA1 pyramidal cells following 4 days of treatment

The CA1 field from brains of 4 control animals (CON), 9 activity-based anorexia animals (ABA), 5 food restricted animals (FR) and 3 exercise animals (EX) were compared for α4 immunoreactivity. These tissues were obtained from the animals whose ante mortem data are shown in Fig. 2. The Kruskal-Wallis ANOVA by ranks and the Multiple comparison z- and p-values indicated a significant effect of ABA treatment upon α4 immunoreactivity ($p<0.005$), whether measuring the proportion of spine profiles labeled at the plasma membrane (**panel A**) or counting the SIG particles at the plasma membrane of spine profiles (**panel B**). *** indicates p-value of < 0.005. Values are reported as mean ± SEM.

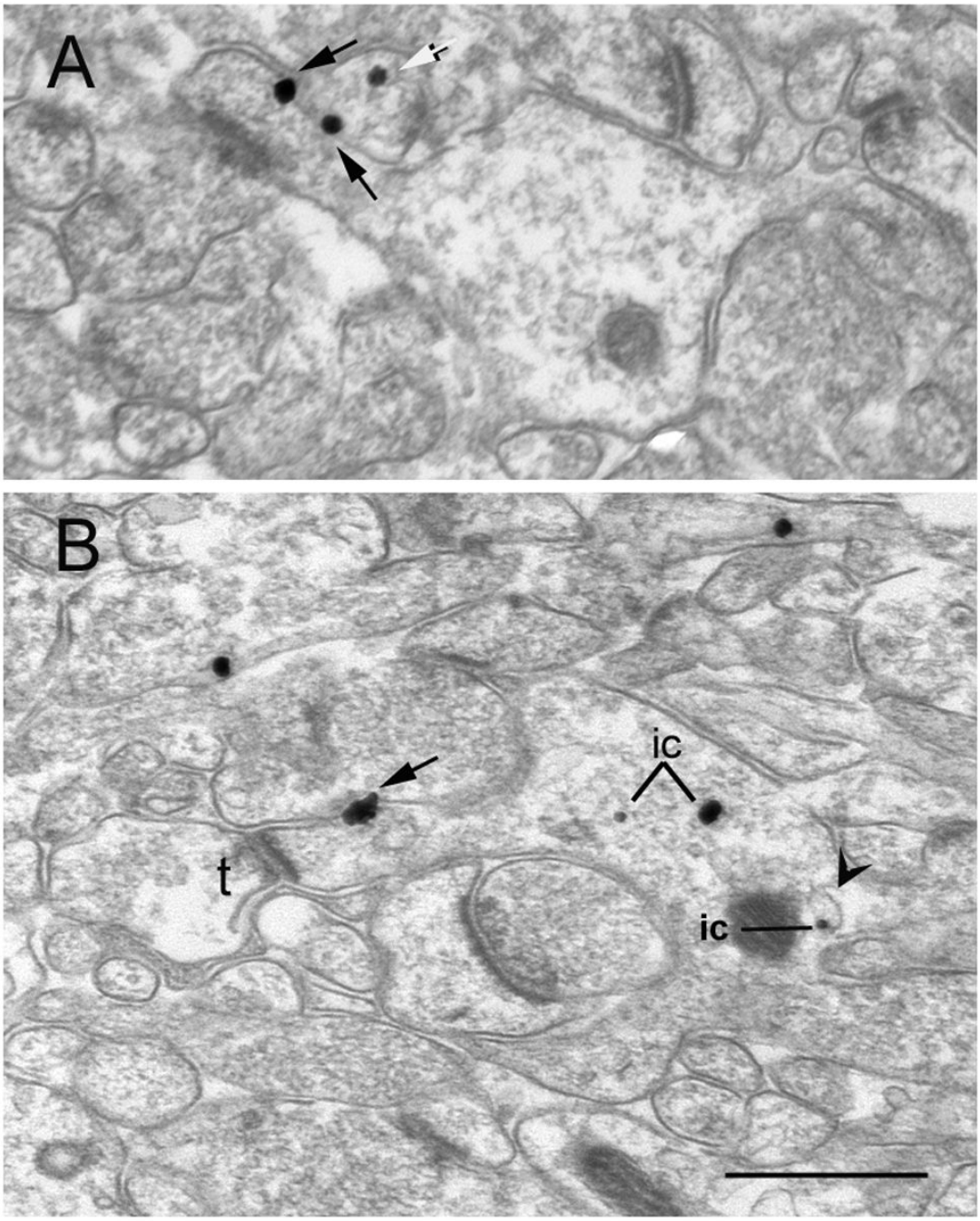

Fig. 5. Subcellular locations of δ subunits in the CA1 pyramidal cells following 4 days of ABA treatment

Panels A and B show examples of the subcellular distribution of δ subunits in relation to dendritic spine profiles of CA1 pyramidal neurons. The micrographs were taken from two different ABA animals. Both spinous profiles were sampled very near the surface of the vibratome section. The black arrow associated with synapses in panels A and B point to SIG clusters occurring along the plasma membrane and extending to the extracellular surface, while the white arrow in panel A points to an SIG particle that was counted as intracellular. 'ic' in Panel B points to SIG particles occurring intracellularly within the dendritic shaft and

associated with smooth endoplasmic reticulum, one of which is highlighted with an arrowhead. The calibration bar = 500 nm.

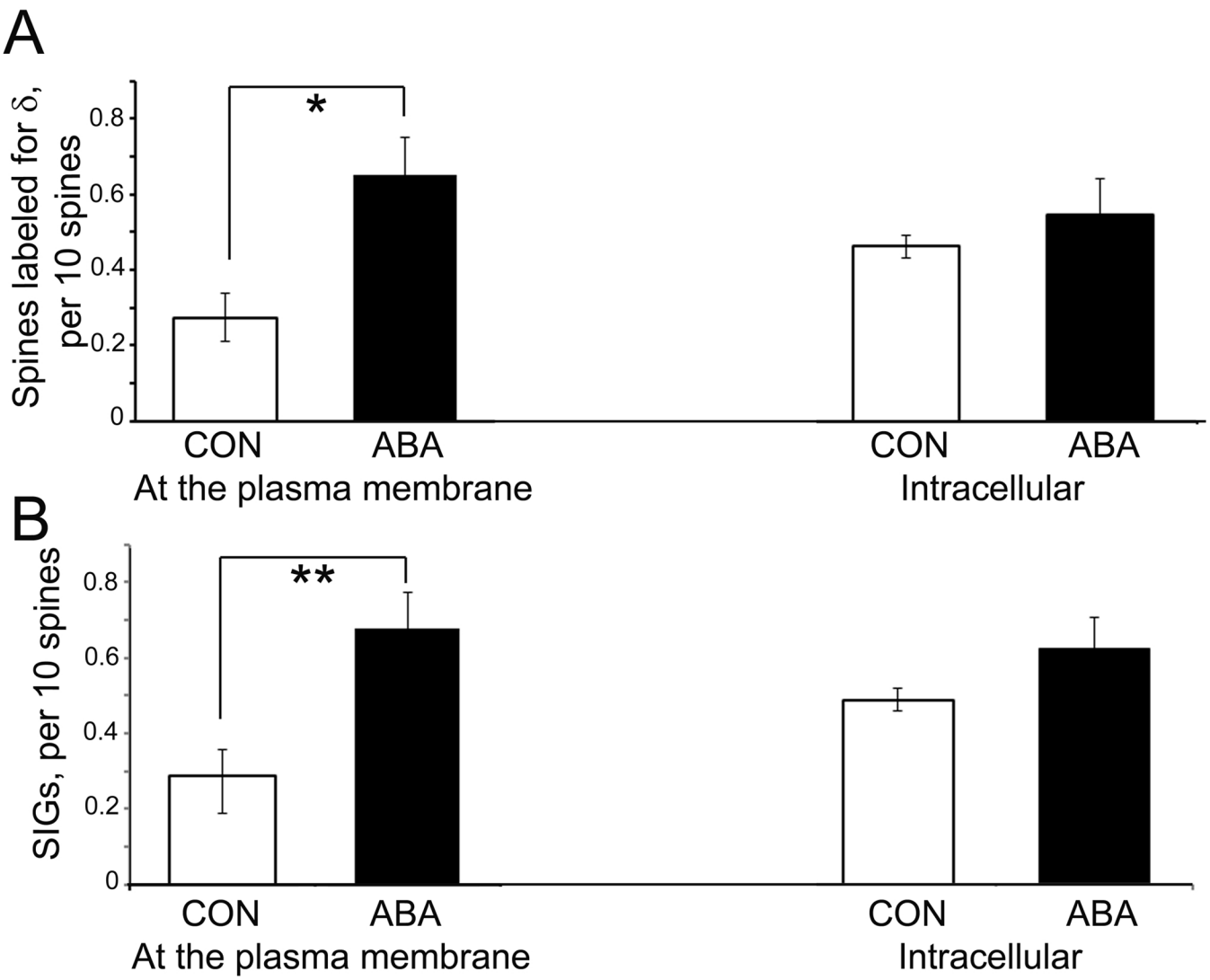

Fig. 6. Comparisons of immunoreactivity for the δ subunit within the CA1 pyramidal cells following 4 days of ABA treatment versus no treatment

Immunoreactivity to the δ subunit of GABARs in dendritic spine profiles of CA1 pyramidal neurons was compared between 9 ABA animals and 4 CON animals. Two counting procedures were conducted: measurement of the proportion of encountered spine profiles immunoreactive for the δ subunit (**Panel A**) and the number of SIG particles occurring in spine profiles, which reflected levels of δ immunoreactivity (**Panel B**). The quantification procedure was identical to that described for the α4 subunit, shown in Fig. 4. * indicates p-value of < 0.05; ** depicts p-value of <0.01. Values are reported as mean ± SEM.

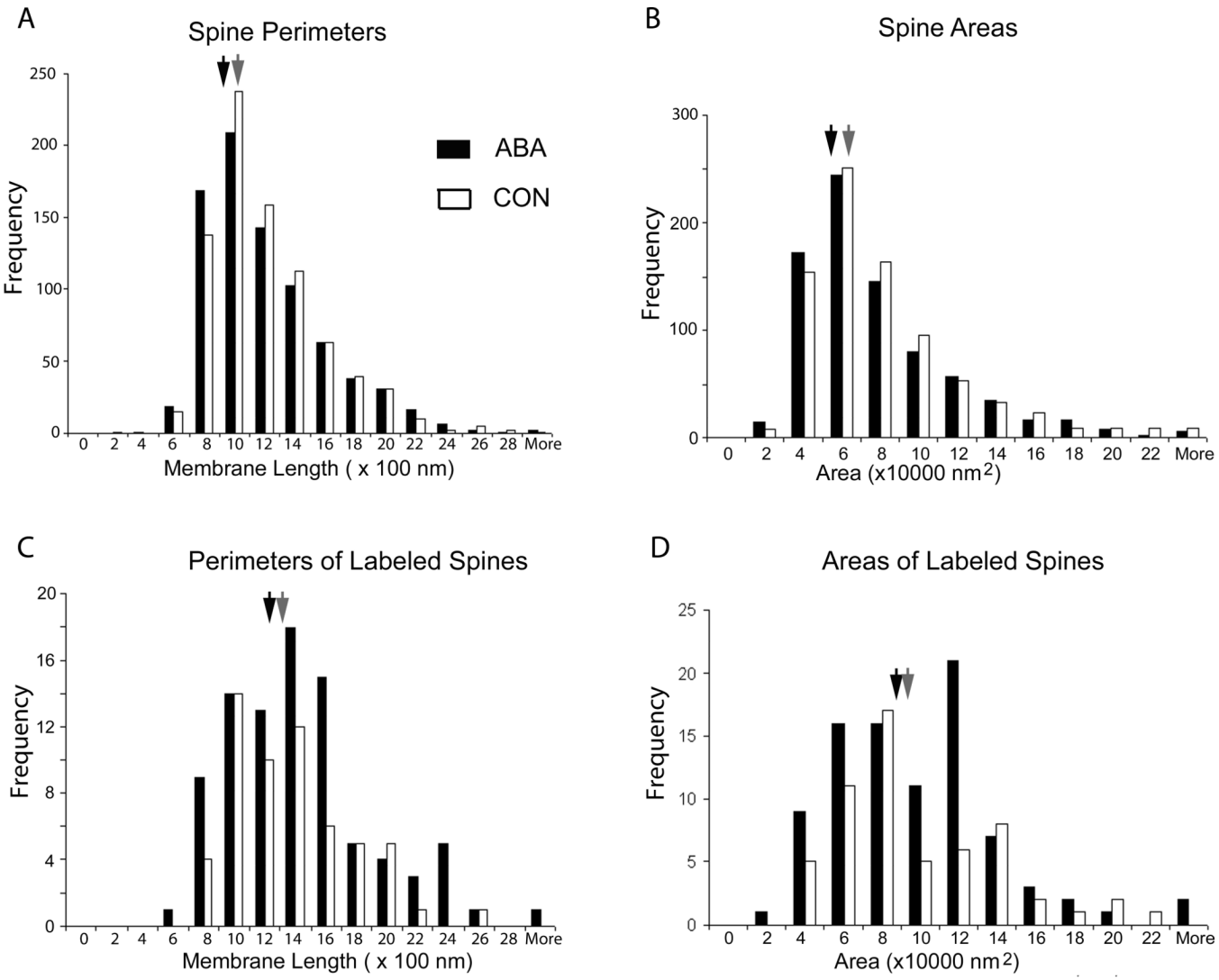

Fig. 7. Comparisons of the perimeters and areas of spine profiles obtained from electron microscopic images of CON and ABA animals

Sizes of spine profiles were compared between CON and ABA groups of animals, using the software, Ima g e J (NIH version 6). The spine profiles that underwent size measurements were the same ones analyzed for δ subunit immunoreactivity, consisting of 200 from each of 4 ABA and 4 CON animals, i.e., 800 spines from the CON and another 800 spines from the ABA tissue. The two populations of spine profiles were not significantly different from one another, using perimeter (**Panel A**, p=0.8, Unpaired t-test) or area (**Panel B**, p=0.2) to compare their sizes. Black arrows point to the mean size of the spine profiles from the ABA brains, while the grey arrows point to the mean size of spine profiles from the CON brains. Comparisons of spine sizes were repeated for the δ-immunoreactive subpopulation. Perimeters (**Panel C**) and areas (**Panel D**) of labeled spine profiles also showed no difference between the CON and ABA groups (p=0.33 for perimeters; p=0.92 for areas). In these panels, arrows also point to the mean values of the ABA (black) and CON (grey) labeled spine profiles.