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α4βδ $GABA_A$ receptors trigger synaptic pruning and reduce dendritic length of female mouse CA3 hippocampal pyramidal cells at puberty

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Abstract

Synaptic pruning during adolescence is critical for optimal cognition. The CA3 hippocampus contains unique spine types and plays a pivotal role in pattern separation and seizure generation, where sex differences exist, but adolescent pruning has only been studied in the male. Thus, for the present study we assessed pruning of specific spine types in the CA3 hippocampus during adolescence and investigated a possible mechanism in the female mouse. To this end, we used Golgi-impregnated brains from pubertal (~PND 35, assessed by vaginal opening) and post-pubertal (PND 56) mice. Spine density was assessed from z-stack (0.1μm steps) images taken using a Nikon DS-U3 camera through a Nikon Eclipse Ci-L microscope and analyzed with NIS Elements. Spine density decreased significantly ($P<0.05$) during adolescence, with 50–60% decreases in mushroom and stubby spine-types ($P<0.05$, ~PND35 vs. PND56) in non-proestrous mice. This was associated with decreases in Kalirin-7, a spine protein which stabilizes the cytoskeleton and is required for spine maintenance. Because our previous findings suggest that pubertal increases in α4βδ GABAA receptors (GABARs) trigger pruning in CA1, we investigated their role in CA3. α4 expression in CA3 hippocampus increased 4-fold at puberty ($P<0.05$), assessed by immunostaining and verified electrophysiologically by an increased response to gaboxadol (100nM), which is selective for α4βδ. Knock-out of α4 prevented the pubertal decrease in Kalirin-7 and synaptic pruning and also increased the dendritic length, demonstrating a

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functional link. These data suggest that pubertal α4βδ GABARs alter dendritic morphology and trigger pruning in female CA3 hippocampus.

Keywords

CA3 hippocampus; α4βδ $GABA_A$ receptors; adolescence; synaptic pruning; dendrite morphology; mushroom spines

Introduction

The loss of dendritic spines in adolescence ("synaptic pruning") is widespread in the CNS (Huttenlocher, 1979, Zehr et al., 2006). Adolescent pruning is thought to play a critical role in developing optimal levels of cognition in adulthood because it is disrupted in disorders such as autism (Hutsler and Zhang, 2010) and schizophrenia (van Spronsen and Hoogenraad, 2010), where cognition is impaired (D'Cruz et al., 2013). Many studies have documented this process in areas such as the CA1 hippocampus (Meyer et al., 1978, Yildirim et al., 2008, Afroz et al., 2016) and prefrontal cortex (Huttenlocher, 1979, Petanjek et al., 2011) where spine density decreases by half in adolescence. Studies have also documented that decreases in spines (Meyer and Ferres-Torres, 1978) and synapses (Shi et al., 2015) of CA3 hippocampal pyramidal cells occur in adolescence but these are limited to the male rodent where neither the spine types involved nor the underlying mechanism are known. Sex differences have been reported in the regulation of spine density in the CA3 hippocampus (Mendell et al., 2017), thus suggesting that synaptic pruning in the female may not parallel that of the male. Our previous study (Afroz et al., 2016) showed that the emergence of α4βδ GABAA receptors (GABARs) in CA1 hippocampus triggers synaptic pruning at puberty via regulation of the spine protein kalirin-7 (Kal-7), a Rho guanine nucleotide exchange factor (GEF) required for spine maintenance (Ma et al., 2003). Therefore, we examined pruning and tested the role of these receptors in triggering pruning in female CA3 hippocampus in the present study.

The CA3 hippocampus plays a pivotal role in the generation of seizure activity, documented both in vitro and in vivo (Cherubini and Miles, 2015), where sex differences have been reported (Christensen et al., 2005). This region is an auto-associative network where recurrent excitatory collaterals produce bursts and high frequency oscillations which result in hyperexcitability (Le Duigou et al., 2014), and under pathological conditions, seizure activity. One recent study (Yu et al., 2016) has confirmed that these excitatory connections are necessary for epileptiform activity and behavioral seizures. Thus, spine density of CA3 pyramidal cells may be tightly correlated with the convulsant potential of this region. Furthermore, adolescent spine pruning of this region may also be a possible mechanism for the high remission rate reported for childhood epilepsy associated with the temporal lobe (Callenbach et al., 2010).

Unlike the CA1 hippocampus, the CA3 hippocampus, along with the dentate gyrus, also plays a unique role in pattern separation (Leutgeb et al., 2007, Bakker et al., 2008), where non-overlapping sets of encoding ensembles can separate similar patterns of input into distinct output ensembles. This can be evidenced as behavioral or spatial pattern separation

as has been reported in both rodent and human studies (Leutgeb et al., 2007, Bakker et al., 2008) and is dependent upon selective hippocampal responses (Jung and McNaughton, 1993, Suthana et al., 2015). The selectivity of response may be significantly impacted by spine density, reflecting the abundance of inputs, as well as by dendrite length. Recent studies (Runyan and Sur, 2013) suggest that a shorter dendrite length is associated with more selective responses of cortical interneuron populations. In the present study, we also tested the role of α4βδ GABARs in regulating dendrite length at puberty.

It is not known whether α4βδ GABARs increase expression at puberty onset in the CA3 hippocampus and what role, if any, these receptors play in adolescent synaptic pruning or regulation of dendritic length in this region. This study addressed these issues by first examining α4 expression in CA3 hippocampus before and at puberty onset. α4 expression has a high degree of plasticity (Smith, 2013), but is not increased at puberty in some brain regions (Piekarski et al., 2017), suggesting that the changes in its expression during are selective during adolescence. Dendritic spine density and spine types were then characterized across adolescence in wild-type and α4−/− female mouse CA3 hippocampus. We tested the hypothesis that spine pruning in CA3 hippocampus would be observed for the stubby and mushroom spine-types, which utilize NMDA receptor-dependent Ca++ influx (Mainen et al., 1999), but not for the thorny spines, unique to this region (Gaiarsa et al., 1992, Zhao et al., 2012), which do not (Reid et al., 2001), because α4βδ GABARs impair NMDA receptor activation (Afroz et al., 2016) as a mechanism to trigger synaptic pruning. Finally, dendritic length and branching were compared post-pubertally in wild-type mice and after α4 knock-out to test the role of α4βδ GABARs in regulating dendritic morphology.

We present data demonstrating that α4βδ expression increases at puberty in CA3 hippocampus, and that the inhibition generated by these receptors triggers synaptic pruning during adolescence. Kal-7 levels also declined at puberty in this region, an effect not seen after α4 knock-out. In addition, dendrite length was increased after α4 knock-out, suggesting that α4βδ GABARs trigger synaptic pruning and limit dendritic length in CA3 hippocampus at puberty.

Experimental procedures

Animals:

Female C57BL6 mice were used: both wild-type (WT, a total of 41) and (α4−/− (a total of 15). Animals were kept under reverse light:dark conditions (12h:12h). All testing was performed in the dark. α4−/− mice were bred from α4+/− mice (G. Homanics, (Univ. of Pittsburgh). The data from +/+ and WT C57BL6 mice were pooled because they were statistically similar. α4−/− mice were used rather than δ−/− mice to avoid changes to the interneuron population which express α1βδ GABARs (Ferando and Mody, 2013). Animals were tested for spine density changes at the onset of puberty (~PND 35, assessed by vaginal opening) and post-pubertally (PND 56). Animals were tested for α4 and kalirin-7 (Kal-7) immunoreactivity pre-pubertally (~PND 28–32) and at the onset of puberty. Estrous cycle stage was assessed by vaginal lavage in PND 56 mice. The non-proestrous group included estrous, metestrus and diestrous (1 and 2) mice. We used ovarian hormone administration to

induce proestrus (17β-estradiol [E_2] 8 μg/kg, i.p. for 3 d, followed by progesterone [P], 25 mg/kg, i.p., 2 h after the final E_2 injection) in order to ensure consistency in hormonal levels across mice. These proestrous mice (euthanized 5 h after P) were assessed separately from other stages in order to test separately any potential effect of circulating levels of 17β-estradiol (E_2) and progesterone (P) on spine density (Woolley and McEwen, 1994, Mendell et al., 2017). However, α4 expression is not altered by the estrous stage during the pubertal period (PND 35–44) (Shen et al., 2010), when α4βδ expression levels are elevated compared to other ages. In addition, α4−/− mice cycle normally (Sabaliauskas et al., 2015), and the age of puberty onset (i.e., vaginal opening) is similar to wild-type mice (~PND 35) (Shen et al., 2010). All experiments were conducted with the personnel blinded to the mouse groups. The authors certify that the studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 1996 and the SUNY Downstate Institutional Animal Care and Use Committee.

Golgi stain:

Whole brains were prepared using the Golgi stain (FD Rapid Golgi Stain kit, FD Neurotechnologies, Columbia, MD). Following staining, brains were sectioned at 200 μm using a vibratome (Leica VT 100M). Images of CA3 pyramidal cells were taken with a Nikon DS-U3 camera mounted on a Nikon Eclipse Ci-L microscope at 100X oil. Z-stack projection photomicrographs (0.1 μm steps) were captured with NIS-Elements AR 4.40.00 software for spine count analysis.

Spine density measurement:

Spine density on the basal dendrites was assessed with Neurolucida 2017.02.1. Dendritic crossings were assessed with the built-in Sholl analysis every 10 μm from the soma. The dendrites were analyzed proximally (up to 1/3 of the distance from the soma) or distally (up to 1/3 of the distance from the dendrite termination). Spine types included: stubby (< 1.1 length:width ratio), mushroom (≥ .35 μm head width ratio, >2 head width:neck length ratio), thin (<1.2 head width: neck length ratio, >3 length:width ratioi) (Arellano et al., 2007) or thorny (>1 head) (Arellano et al., 2007),.

Immunohistochemistry:

Following anesthesia with urethane (0.1 ml 40%), mice were perfused with saline (12–15 mls/min) and then with 4% paraformaldehyde followed by post-fixation of brain in 4% PFA (48 h, 4oC). The hippocampus was sectioned at 40 μm ((Leica VT 100M vibratome), and sections washed in PBS for (10 min, (3x) followed by blocking for 2 h in 10% donkey serum (0.4% Triton, .05% sodium azide in phosphate buffered saline). For immunohistochemistry, either anti-Kal-7 (ab52012, Abcam, 1:200) or anti-α4 (sc7523, Santa Cruz, 1:20) and, in some cases, anti-MAP2 (microtubule-associated protein, ab5392, Abcam, 1:1000) diluted in the blocking solution overnight at 4°C were used. The fluorescent secondary antibody, either (Alexa fluor 488 or 594 (1:500) was applied for 2 h, and washed in PBS for 10 min (3x). Images were analyzed using FIJI (Image J) software after acquisition with Olympus FluoView TM FV1000 confocal inverted microscope Olympus, Tokyo, Japan.

Slice preparation:

Brains were initially incubated in (in mM): NaCl 124, KCl 2.5, $CaCl_2$ 2, $NaH2PO_4$ 1.25, $MgSO_4$ 2, $NaHCO_3$ 26, and glucose 10, (95% O_2, 5% CO_2, 7.4 pH, 4o C, aCSF) and sectioned at 400 μm (Leica VT1000S vibratome).

Electrophysiology:

Following a 1 h incubation in oxygenated aCSF, CA3 hippocampal pyramidal cells were recorded using whole cell patch voltage clamp techniques (Shen et al., 2007) in voltage clamp mode. The slice was mounted on the stage of a Leica differential interference contrast (DIC)-infrared upright microscope. Recordings were carried out at 26–30o C at a −60 mV holding potential (Axopatch 200B amplifier, pClamp 9.2) using pipets (2–4 MΩ using a Flaming-Brown puller) containing (in mM): CsCl 140, HEPES 5, EGTA 5, CaCl2-H2O 0.5, QX-314 5, Mg-ATP 2, Li-GTP 0.5 , 5 mM QX-314 (pH 7.2, 290 mOsm). 50 μM kynurenic acid and 0.5 μM TTX were added to the bath to block excitatory current and to block the pre-synaptic component, respectively. 100 nM gaboxadol (GBX, THIP) was used to assess the tonic current (change in holding current). This concentration of GBX is selective for δ-containing GABAR (Brown et al., 2002, Meera et al., 2011).

A 10 kHz sampling frequency was used (2 kHz 4-pole Bessel filter) and access resistance monitored so that an increase > 10% resulted in rejection of the recording.

Drugs:

QX-314 was from Calbiochem (Billerica, MA), and all other drugs were from Sigma Chemical Co. (St. Louis, MO).

Statistics:

All data are presented as the mean ± the standard error of the mean (S.E.M.). For the electrophysiology experiments, each mouse yielded one recording. Electrophysiology, immunohistochemistry, dendritic branching, dendrite length: Comparisons between groups were analyzed with a Student’s t-test (2 groups) or and analysis of variance (ANOVA) followed by a post-hoc Fisher’s test (>2 groups). Spine density, spine-types (2 observations per animal): A generalized mixed linear model was constructed, with dependent variable spine count modeled as Poisson-distributed; a logarithmic link function was specified, and log (dendrite length) used as an offset variable. Fixed factors were age, genotype and their interaction. Mouse ID was introduced as a random effect. Kenward-Roger adjustments to standard errors and denominator degrees of freedom were applied. Heteroskedasticity was noted, so a separate dispersion-correction parameter was applied to each age-genotype combination. Ratio of the model’s generalized chi-square statistic to denominator degrees of freedom was used as an indicator of model fit. Model residuals were inspected for outliers. All interaction effects and additional details are presented in Appendix A. A $P < 0.05$ was considered significant.

Results

α4 GABAR immunostaining increases at puberty onset on CA3 hippocampal pyramidal cells

We have previously reported significant increases in α4 GABAR expression in CA1 hippocampus at the onset of puberty in female mice (Afroz et al., 2016). In the present study, we assessed whether similar increases in α4 GABAR expression occurs at the onset of puberty on CA3 hippocampal pyramidal cells. To this end, we assessed α4 immunostaining in CA3 hippocampal slices from pre-pubertal and pubertal female mice. There was a ~4-fold increase ($t(8)=7.02$, $P \leq 1 \times 10^{-4}$) in α4 immunostaining on CA3 pyramidal cells at puberty compared to pre-pubertal levels, which co-localized with immunostaining for MAP2 (microtubule-associated protein 2), a marker for the dendritic cytoskeleton (Fig. 1A, B).

CA3 pyramidal cells have increased responses to the GABA agonist gaboxadol at puberty, suggesting increased functional expression of α4βδ GABARs

The GABA agonist gaboxadol (i.e., THIP) is selective for α4βδ GABARs at low concentrations (100 nM) (Brown et al., 2002, Jia et al., 2005, Meera et al., 2011). Therefore, we assessed whether the increases in α4 immunoreactivity in CA3 hippocampus at puberty represented increased expression of functional α4βδ GABARs. To this end, we recorded the current responses of CA3 pyramidal cells to 100 nM gaboxadol using whole cell voltage clamp techniques in hippocampal slices from pre-pubertal and pubertal mice. Gaboxadol responses were more than 3-fold greater at puberty (*$t(9)=3.9$, $p \leq 0.004$; Fig. 1C,D) than before puberty, suggesting increased expression of α4βδ GABARs at this time.

Synaptic pruning in CA3 hippocampus during adolescence

In order to assess whether synaptic pruning occurs in female CA3, we assessed spine density in CA3 hippocampal pyramidal cells at puberty onset (~PND 35, vaginal opening) and post-pubertally (PND 56) in non-proestrous mice using Golgi-stained hippocampal slices. Significant decreases in spine density (~30–40%) were observed post-pubertally compared to puberty (Fig 2A,B,C) in both the proximal (Type III ANOVA, $F(1,23)=5.22$, $P \leq 0.05$) and distal (Type III ANOVA, $F(1,23)=5.22$, $P \leq 0.0005$) sections of the basal dendrites (Appendix A.1, statistics). In contrast to the pruning observed in non-proestrous mice across adolescence, post-pubertal proestrous mice did not exhibit a lower spine density than pubertal proestrous mice (Fig. 3; Appendix A.2). In fact, spine density in the proximal dendrite was significantly greater in post-pubertal proestrous mice compared to proestrous pubertal mice (Fig. 3 A-C; Type III ANOVA, $F(1, 7.77)=8.01$, $P \leq 0.05$), with no significant differences in spine density of the distal dendrites across groups.

Knock-out of α4βδ GABARs prevents synaptic pruning of CA3 pyramidal cells at puberty

Our former studies suggest that adolescent pruning of CA1 neurons is dependent upon α4βδ GABARs (Afroz et al., 2016). Therefore, we tested the hypothesis that these inhibitory receptors also play a role in adolescent synaptic pruning of CA3 pyramidal cells. To this end, spine density was quantified at the onset of puberty and post-pubertally in Golgi-stained

hippocampal sections of α4 −/− mice. Our results show that there is no significant change in spine density of either the proximal or distal dendrites of α4 −/− CA3 hippocampal pyramidal cells post-pubertally compared to puberty (Fig. 2A, B, C). However, the interaction effect (age by genotype) was significant only for the distal dendrites (Type III ANOVA, $F(1,16.92)=5.28$, $P\leq0.05$) but not for the proximal dendrites (Type III ANOVA, $F(1,16.92)=4.11$, $P\geq0.05$, Appendix A.1), although this reflected a trend. Furthermore, CA3 spine density of pubertal and post-pubertal α4 −/− mice was not significantly different than CA3 spine density of pubertal WT mice.

Synaptic pruning of CA3 hippocampal pyramidal cells is restricted to selective spine-types

Because individual spine types reflect selective input and functional properties, we identified and quantified spine types as either mushroom, stubby, thin or thorny in CA3 hippocampal sections from pubertal and post-pubertal female mice to test whether pruning was also selective for spine type in CA3 hippocampus. Indeed, significant ~50–60% decreases in spine density for only mushroom (Type III ANOVA, $F(1, 17.47)=11.10$, $P\leq 0.01$) and stubby spine (Type III ANOVA, $F(1, 15.89)=10.72$, $P\leq 0.005$) types were observed post-pubertally compared to puberty (Fig. 4A, B). In contrast, no significant changes were seen across pubertal state for either thin or thorny spines (Fig. 4C, D) or for any spine-types in CA3 hippocampus of α4 −/− mice (Fig. 4A-D; Appendix A.3, statistics). In contrast to these findings in non-proestrous mice, the density of selective spine types on CA3 pyramidal cells of proestrous mice did not change from puberty to post-puberty (Figure 3D, Appendix A.4).

Dendritic length and dendritic crossings increase after α4 knock-out in post-pubertal mice

In order to quantify dendritic branching, Sholl analysis was used to assess the number of dendritic crossings as a function of distance from the soma for post-pubertal CA3 pyramidal cells, comparing WT with α4 −/− mice. There was a significantly greater number of basal dendritic crossings 40 μm ($t(16)=2.34$, $*p\leq0.05$ vs. WT) and 50 μm ($t(16)=2.13$, $*p\leq0.05$ vs. WT) from the soma for post-pubertal α4 −/− mice compared to post-pubertal WT (Fig. 5A,B). Although there were no significant differences in the number of dendritic crossings for the apical dendrites across the length of the dendrite, there was a bimodal distribution of peak crossings for post-pubertal α4 −/− pyramidal cells, at 100 μm and at 200–250 μm from the soma in contrast to the single peak observed for the post-pubertal WT (Fig. 5C).

Dendrite length was also significantly greater for apical dendrites of the post-pubertal α4 −/− compared to the post-pubertal WT (210 ± 13 μm, α4 −/− versus 167 ± 7.4 μm, WT, $t(15)=2.96$, $p\leq0.01$) but not for the basal dendrites (116.7 ± 6.5 μm, α4 −/− versus 125.6 ± 13.4 μm, WT, $t(15)=0.60$, $p\geq1.0$). Because the α4 −/− apical dendrites were longer than WT, we also assessed total dendritic crossings which were increased by ~50% for the post-pubertal α4−/− ($t(14)=2.2$, $*p\leq0.05$ vs. WT) compared to the post-pubertal WT CA3 hippocampus (Fig. 5A, D).

The spine protein Kal-7 decreases at puberty, an effect prevented by α4 knock-out

Kal-7 is a spine protein necessary for spine maintenance (Ma et al., 2003). Therefore, we tested whether Kal-7 expression was altered by puberty onset in CA3 hippocampus of female mice. To this end, we assessed Kal-7 immunostaining in CA3 hippocampal sections

of WT and α4 −/− mice. Kal-7 immunoreactivity was significantly decreased (ANOVA, $F_{(2,12)}=9.51$, $P \leq 0.005$, Fisher's test $P \leq 0.05$; Fig. 6A, B) at puberty on CA3 hippocampal pyramidal cells of WT mice compared to pre-puberty. However, Kal-7 expression in pubertal α4−/− CA3 hippocampus was significantly greater than for pubertal WT (ANOVA, $F_{(2,12)}=9.51$, $P \leq 0.005$, Fisher's test $P \leq 0.001$), and not significantly different than pre-pubertal WT levels. These results suggest that reductions in a spine protein necessary for spine maintenance, Kal-7, occur at puberty, and that this decrease is dependent upon increased expression of α4-GABARs (Appendix A.5, statistics).

Discussion

Our findings suggest that adolescent pruning occurs in the CA3 hippocampus of the female mouse. Although spine pruning occurs across widespread areas of the CNS, previous studies have only shown this in male CA3 hippocampus (Meyer and Ferres-Torres, 1978, Shi et al., 2015). Our study also shows that this pruning is triggered by extrasynaptic α4βδ GABARs, which also reduce dendritic length at puberty.

The present results demonstrate that α4βδ GABAR expression increases on CA3 pyramidal cells of female mice at the onset of puberty, from relatively low levels of expression before puberty. This was demonstrated both by visualization and quantification of increases in α4 immunoreactivity in the CA3 region, as well as by electrophysiological verification of functional expression of α4βδ GABARs evidenced by increased responses of CA3 pyramidal cells to the GABA agonist gaboxadol at a concentration selective for α4βδ, showing increases in tonic inhibition. 100 nM gaboxadol exhibits a selective effect at α4βδ with little or no response from recombinant α1β2δ, α4β2γ2 or α1β2γ2 GABARs (Brown et al., 2002, Jia et al., 2005, Meera et al., 2011). Other studies have confirmed that expression of α4 is low in CA3 hippocampus of non-pubertal animals (Sperk et al., 1997), although δ is abundantly expression on parvalbumin-containing interneurons of this region (Sperk et al., 1997, Ferando and Mody, 2013, 2015). However, it is well established that α4βδ GABARs have a high degree of plasticity and can alter expression after alterations in ovarian hormones (Griffiths and Lovick, 2005, Maguire et al., 2005, Shen et al., 2007). In contrast to findings in hippocampus (Shen et al., 2007, Shen et al., 2010), gaboxadol-generated tonic inhibition is reduced in the anterior cingulate cortex after puberty (Piekarski et al., 2017) suggesting that increases in pubertal α4βδ expression is selective for certain brain regions.

α4βδ receptors are extrasynaptic (Wei et al., 2003) and generate a tonic inhibition (Stell and Mody, 2002) because they are activated by the ambient concentration of GABA (<1 μM) (Brown et al., 2002), which is maintained by GABA transporters (Wu et al., 2003), and desensitize little under steady-state conditions (Brown et al., 2002). The tonic inhibition generated by α4βδ receptors (Shen et al., 2010) impairs NMDA receptor activation in CA1 hippocampus at puberty, which in turn decreases expression of the spine-associated protein Kal-7 (Afroz et al., 2016). Kal-7 activates the small GTPase Rac1 which, via P21-activated kinases, controls the actin cytoskeleton to maintain spine rigidity (Penzes and Cahill, 2012, Ma et al., 2014, Afroz et al., 2016). This process is the likely trigger for spine pruning because the decrease in Kal7 does not take place after knock-out of α4 (Afroz et al., 2016).

In addition, knock-out of Kal-7 prevents synaptic pruning in the CA1 hippocampus (Afroz et al., 2016), confirming its role in pruning. In the present study, Kal-7 expression was also reduced at puberty in WT but not α4−/− mice, suggesting that a similar mechanism underlies synaptic pruning in the CA3 hippocampus triggered by α4βδ-generated inhibition of NMDA receptor currents. However, because of the diversity of spine proteins which subserve spine dynamics (Carlisle and Kennedy, 2005), we cannot rule out the possibility that other spine proteins play a role in spine pruning in this region.

Although this study focused on the female, our preliminary findings suggest that synaptic pruning of CA3 pyramidal cells in the male is also prevented by knock-out of the α4 subunit, implicating α4βδ GABARs. Greater regional differences were seen in the male where only the distal dendrites displayed significant (~30%, $F(2,8)=29.23$, $P<0.0005$) decreases in spine density at puberty (spine density, 12.9 ± 0.84, puberty versus 8.79 ± 9.74, post-puberty, $P<0.05$, Fisher's test), similar to the female, which were prevented by α4 knock-out (spine density, 16.0 ± 0.53, post-puberty α4−/−, $P<0.05$ vs. other groups, Fisher's test). The proximal dendrites did not display significant decreases in spine density across adolescence. Thus, although this process is observed in both sexes, it is more extensive in the female.

In contrast to what we have reported in CA1 hippocampus (Afroz et al., 2016), knock-out of α4βδ GABARs also increased dendritic branching and dendritic length for the basal and apical dendrites of CA3 pyramidal cells, respectively. Although a length-dependent increase in apical branching was not observed, the total number of dendritic crossings was markedly greater for the post-pubertal α4 −/− compared to the post-pubertal WT because the apical dendrites of α4 −/− mice were longer than WT values and thus branching extended further from the soma. α4βδ GABARs express both on the dendritic shaft and spine (Shen et al., 2010) where they would be expected to shunt excitatory current and mitigate the faciliatory effect of excitatory input mediated by NMDA receptors which have been shown to increase dendritic branching (Sepulveda et al., 2010).

The decreases in dendrite length and total spine density which occur in adolescence in the CA3 hippocampus may have repercussions for the pattern separation and pattern completion functions of this region. Studies conducted in rodents, monkeys, and, most recently, humans (Leutgeb et al., 2004, Leutgeb et al., 2007, Bakker et al., 2008, Sakon et al., 2014, Kyle et al., 2015, Knierim and Neunuebel, 2016) have indicated that the dentate gyrus (DG) and CA3 hippocampus play a pivotal role in pattern separation, a process whereby the neuronal output pattern minimizes overlap between similar input patterns (Rolls, 2013). Although the DG receives abundant input from the entorhinal cortex, it sends relatively few mossy fiber projections to the relatively sparse CA3 pyramidal cell population (Treves and Rolls, 1994). These orthogonalized representations allow for distinct responses of the DG/CA3 to similar inputs and permit the individual to discriminate between similar, but not identical stimuli (Treves and Rolls, 1994). In contrast, pattern completion, which may involve multiple areas, including CA3, CA1 and entorhinal cortex (Kesner et al., 2004, Bakker et al., 2008), performs the opposite function, by increasing the overlap between similar inputs to re-establish full memories from partial or distorted cues. Theoretical models have suggested that sparse inputs, as reflected by the total spine count, bias the DG/CA3 circuit to a pattern

separation function (Treves and Rolls, 1994). Indeed, a recent study has shown that dendrite length, and thus total spine number, can impact the selectivity of neuronal response (Runyan and Sur, 2013) which underlies pattern separation (Santoro, 2013). In highly tuned parvalbumin+ interneurons of the visual cortex, dendrite length is shorter than for broadly tuned neurons (Runyan and Sur, 2013). Thus, pubertal α4βδ GABARs in the CA3 hippocampus may play a role in regulating the selectivity of response to input by limiting the length of the dendrite and thereby enhancing pattern separation. However, a shorter dendrite with reduced total spine number would reduce the inputs required for maximizing overlap of representations, and thus hinder pattern completion.

The CA3 hippocampus functions in spatial memory in some cases in conjunction with the CA1 hippocampus, to which it projects via the Schaffer collaterals (Amaral and Witter, 1989). The CA3 pyramidal cells also project out of the hippocampus directly via the fimbria, fibers which innervate the diagonal band of Broca and both medial and lateral septum (Gaykema et al., 1991). Recent studies have determined a selective role of the CA3 in rapid processing of novel associations between places and objects (Lee and Kesner, 2002, 2003) and sequential processing of information important for spatiotemporal coding, shown also in humans (Deuker et al., 2014). Thus, these functions are both distinct, but in some cases complementary, with the functions of the other regions of the CA1 hippocampus. The CA3 hippocampus is a site with high seizure susceptibility likely enhanced by the recurrent axon collaterals here (Cherubini and Miles, 2015, Musto et al., 2015) which underlie an auto-associative network function of the region (Kesner, 2007). Abnormal pruning resulting in high spine density could increase seizure susceptibility and in fact a number of studies have linked seizure states with abnormal spine formation (Musto et al., 2015). Conversely, normal pruning of CA3 hippocampal pyramidal cells may be relevant for the 50% remission rate (Berg et al., 2014) observed for childhood epilepsy which abates in adolescence.

In the present study, decreases in spine density were selective for two classes of spines: mushroom and stubby, as we have shown for the CA1 hippocampus (Afroz et al., 2016). In contrast, no decreases in thin or thorny spines were observed. The mushroom spines are typically known as “memory” spines, and the thin spines as “learning” spines based on evidence from two-photon studies showing that thin spines become mushroom spines after LTP induction (Kopec et al., 2006, Harvey and Svoboda, 2007, Hill and Zito, 2013). Increased mushroom and reduced thin spine density is also seen after spatial learning in both CA1 (Beltran-Campos et al., 2011, Afroz et al., 2016) and CA3 hippocampus (Mahmmoud et al., 2015). These larger mushroom spines have an enlarged spine head with abundant AMPA receptors (Matsubara et al., 1996, Dumitriu et al., 2010). Mushroom and stubby spines also have high expression of NMDARs and NMDAR-mediated Ca++ influx (Yuste et al., 1999), which is impaired by the shunting inhibition of α4βδ GABARs (Shen et al., 2010), shown to trigger pruning in CA1 pyramidal cells (Afroz et al., 2016). In contrast, thorny excrescences, the target of mossy fiber inputs, have low expression of NMDARs (Reid et al., 2001). In these complex spines, Ca++ influx is mediated by voltage-activated Ca++ channels triggered by AMPA receptor-induced depolarizations (Reid et al., 2001). Thus, it is not surprising that α4βδ-generated shunting inhibition at puberty does not trigger pruning of these spines on CA3 pyramidal cells in the present study, and that knock-out of α4 does not significantly alter thorny spine density. However, because dendrite length was

longer in the α4−/−, the total number of thorny spines in post-pubertal CA3 hippocampus would be higher after α4 knock-out compared to wild-type. Our findings relate spine density changes not only to the adolescent period, but specifically to the onset of puberty when declining levels of the neurosteroid THP (3α-OH, 5α-pregnan-20-one) increase expression of α4βδ GABARs (Shen et al., 2007, Shen et al., 2010). A number of recent studies have also pinpointed puberty onset as the trigger for synapse loss in medial amygdala (Zancan et al., 2018), medial prefrontal cortex (Drzewiecki et al., 2016) and dorsomedial frontal cortex (Boivin et al., 2018). Interestingly, puberty onset is associated with an increase in spine density of gonadotropic hormone-containing neurons in the hypothalamus (Li et al., 2016).

The decrease in spine density observed in non-proestrous mice during adolescence was not observed on late proestrus. In fact, CA3 hippocampal pyramidal cells of proestrous post-pubertal mice had a higher spine density in the proximal region of the dendrite than their pubertal counterparts. This likely reflects the effect of ovarian hormones on spine density, and suggests that once pruning is complete by PND 56, estrous cyclicity produces a higher spine density than observed at puberty. Previous studies have shown that CA1 hippocampal pyramidal cells have significantly higher spine density on the late afternoon of proestrous (Woolley and McEwen, 1994), an effect due to the actions of 17β-estradiol (E_2). Similar effects of ovarian hormones may account for these effects in the CA3 hippocampus. Although some studies have not seen significant effects of the estrous cycle or ovarian hormones on spine density in CA3 hippocampus (Gould et al., 1990, Mendell et al., 2017), this discrepancy may be due to a species difference or the circadian cycle. The impact of this higher spine density of proestrous mice may have implications for seizures because E_2 has been shown to exacerbate seizure activity (Nicoletti et al., 1985).

Circulating levels of E_2 increase by 2-fold approximately 5 d before the onset of puberty (Ahima et al., 1997). These elevated levels of serum E_2 may also have an impact on spine density prior to puberty onset. A recent study (Tsurugizawa et al., 2005) reports that E_2 rapidly and selectively decreases the abundance of thorny spines suggesting the possibility that the thorny spines may be pruned at an earlier time-point than the mushroom and stubby spines.

Although less studied than other hippocampal sub-regions, the CA3 hippocampus has been the focus of several recent studies showing abnormalities in a variety of neurocognitive disorders, including autism, schizophrenia and Alzheimer's disease. In both autism and schizophrenia, reduced neuronal size in CA3 is reported (Zaidel et al., 1997, Saitoh et al., 2001, Kolomeets et al., 2007, Lawrence et al., 2010), in association with reduced volume (Aylward et al., 1999), unaccompanied by alterations in the CA1 region (Saitoh et al., 2001). A reduction in spines/spine proteins in CA3 hippocampus has also been reported (Law et al., 2004, Kolomeets et al., 2007, Tsamis et al., 2010), suggesting that this area may underlie, at least in part, some of the cognitive impairments in these disorders.

Pruning of dendritic spines is one pivotal event occurring during the adolescent period (Huttenlocher, 1979, Zehr et al., 2006, Petanjek et al., 2011, Koss et al., 2014) which may serve to "reset" learning potential (Chechik et al., 1998) at this major change in the life cycle. Our previous findings established that increases in inhibition generated by α4βδ

GABARs (Shen et al., 2007) reduce neuronal excitability, increase the threshold for triggering an action potential and, due to their localization to dendritic spines, also impair learning (Shen et al., 2010), in both in vitro models, such as long-term potentiation, as well as in spatial learning paradigms, such as the active place avoidance task. The adolescent period is also known as a developmental stage particularly vulnerable to stress (Modesti et al., 1994, Romeo and McEwen, 2006), when the stress/neurosteroid THP can trigger anxiety (Shen et al., 2007). Stress during adolescence can have far-reaching implications during adulthood (Dahl, 2004, RD et al., 2006, Gomes and Grace, 2017). Adolescence is also a period when a number of neuropsychiatric disorders first emerge, including anxiety (Hayward and Sanborn, 2002, Kessler et al., 2005), depression (Wang et al., 2016) and schizophrenia (Hennig et al., 2017, Owens et al., 2017)

Adolescent pruning is thought to remove unnecessary synapses to make room for new learning in adulthood. An optimal dendritic spine density thus allows optimal learning as suggested by theoretical analysis (Chechik et al., 1999), as well as by recent behavioral studies (Afroz et al., 2016), while dendrite length may be relevant for response selectivity (Runyan and Sur, 2013). The present findings reveal a GABAR-associated mechanism to regulate spine density as well as to limit dendritic length. Both end-points would be expected to enhance selectivity of CA3 responses and optimize certain functions such as pattern separation.

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Appendix A

Table A.1

Proximal spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	23.21	5.22	0.032 *
Genotype	1	23.21	2.43	0.132
Age * Genotype	1	23.21	4.11	0.054
Distal spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	16.92	10.67	0.005 *
Genotype	1	16.92	6.86	0.018 *
Age * Genotype	1	16.92	5.28	0.035 *

Fixed effects and adjusted P values for individual comparisons of spine density at puberty (~PND 35, assessed by vaginal opening) and post-pubertally (PND 56) in non-proestrous wild-type and α4 −/− mice. Statistics were performed using a multi-level model.

*Statistically significant.

Table A.2

Proximal spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	7.77	8.01	0.023*
Distal spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	8.72	0.02	0.904

Fixed effects and adjusted P values for individual comparisons of spine density at puberty and post-pubertally in proestrous wild-type mice. . Statistics were performed using a multi-level model.

*Statistically significant.

Table A.3

Mushroom spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	17.47	11.10	0.004*
Genotype	1	17.47	7.68	0.013*
Age*Genotype	1	17.47	12.10	0.003*
Stubby spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	15.89	4.05	0.062
Genotype	1	15.89	1.87	0.191
Age*Genotype	1	15.89	10.72	0.005*
Thin spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	14.68	0.93	0.352
Genotype	1	14.68	0.06	0.804
Age*Genotype	1	14.68	0.10	0.762
Thorny spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	70.42	0.02	0.893
Genotype	1	70.42	3.79	0.056
Age*Genotype	1	70.42	0.02	0.893

Fixed effects and adjusted P values for individual comparisons of spine types at and post-pubertally in non-proestrous wild-type and α4 −/− mice. Statistics were performed using a multi-level model.

*Statistically significant.

Table A.4

Mushroom spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	27.96	0.68	0.416
Stubby spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	8.07	4.85	0.059
Thin spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	7.97	1.87	0.209
Thorny spine density				
Effect	**Num *df***	**Den *df***	***F*-value**	***p*-value**
Age	1	7.92	0.04	0.839

Fixed effects and adjusted P values for individual comparisons of spine types at and post-pubertally in proestrous wild-type mice. Statistics were performed using a multi-level model.

Table A.5

Analysis variable: Kalirin-7 pixel intensity (averaged over 2 observations/animal)					
N Obs	**Minimum**	**Lower Quartile**	**Median**	**Upper Quartile**	**Maximum**
5	662	676	952	987	1048
5	547	573	598	639	763
5	758	1049	1092	1141	1262
		P values for Individual comparisons			
		Pre-pub WT v pub WT		0.032 *	
		Pre-pub WT v pub α4 −/−		0.056	
		pub WT v pub α4 −/−		0.016 *	

Upper table, Minimum, median, upper and lower quartile values for comparisons of kalirin-7 pixel intensity for pre-pubertal (pre-pub) and pubertal (pub) wild-type (WT) and pubertal (pub) α4 −/− female mice using an exact 2-sided Kruskal-Wallis test, p=0.003. N Obs, number of observations. Lower table, P values for individual comparison obtained using the post-hoc pairwise 2-tailed exact Wilcoxon rank-sum test comparisons.

*Statistically significant.

Abbreviations:

DG	dentate gyrus
E_2	17β-estradiol
GABAR	$GABA_A$ receptor
GBX	gaboxadol (THIP)

Kal-7	kalirin-7
PND	post-natal day
Rho Gef	Rho guanine nucleotide exchange factor (GEF)
WT	wild-type
THP	allopregnanolone (3α-OH, 5α-pregnan-20-one)
TTX	tetrodotoxin

Glossary

Gaboxadol (THIP)	A GABA agonist selective for α4βδ $GABA_A$ receptors at low concentrations (100 nM)
Kalirin-7	A Rho guanine nucleotide exchange factor (GEF) required for spine maintenance
Pattern separation	The process of making similar patterns of neural activity more distinct; behaviorally, differentiating between 2 similar environments/stimuli
Synaptic pruning	A decrease in dendritic spine density which generally occurs during adolescence
Tonic inhibition	A constant level of inhibition provided by extrasynaptic GABAA receptors, evidenced by a deflection in the holding current electrophysiologically (and distinct from phasic inhibition provided by synaptic $GABA_A$ receptors)

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Highlights

- α4βδ GABAA receptors increase at puberty on CA3 hippocampal pyramidal cells.
- Density of stubby and mushroom spines, but not thorny spines, decreases after puberty in CA3 hippocampus.
- Pruning was associated with decreases in expression of the spine protein kalirin-7.
- Knock-out of α4 prevented pruning and the decrease in kalirin-7 suggesting a link.
- Knock-out of α4 increased dendrite length and branching which may impact response selectivity.

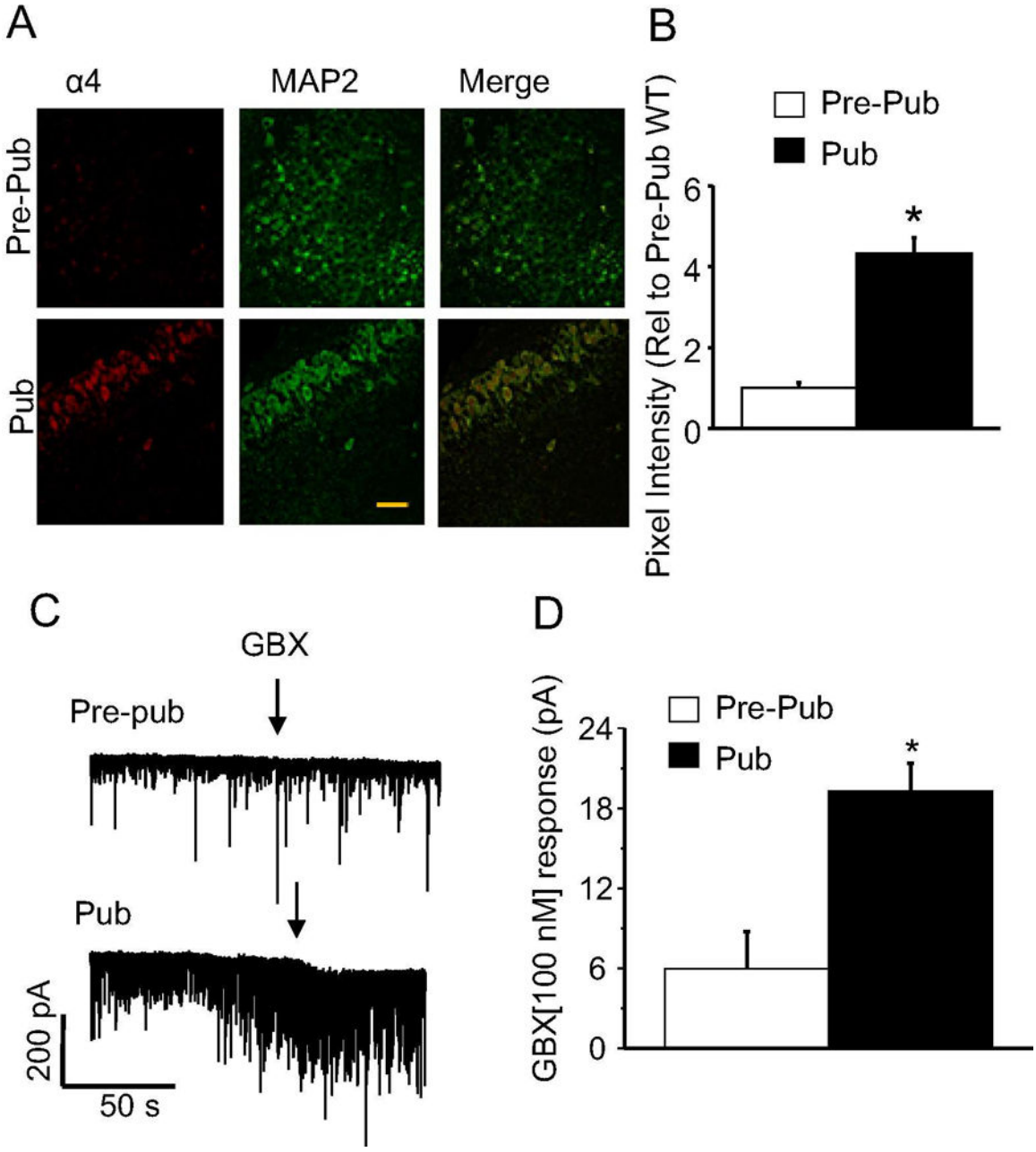

Fig. 1. α4 expression is increased on pyramidal cells of CA3 hippocampus at puberty.

Immunohistochemistry: A, Representative images from pre-pubertal (pre-pub, upper panel) and pubertal (pub, lower panel) female CA3 hippocampus showing α4 (red, left), MAP-2 (green, middle) and merged (yellow, right). Scale, 50 μm. B, Averaged data, *P=0.008, Exact 2-sided Wilcoxon rank-sum test (n=10, 5 mice/group). Pharmacological verification of α4βδ GABAR expression: C, Representative whole cell voltage clamp recordings of CA3 pyramidal responses to 100 nM gaboxadol (THIP) in slices from pre-pubertal and pubertal female mice. This concentration of gaboxadol is selective for α4βδ GABARs. D, Averaged data, *t (9)=3.9, p≤0.004. (n=5–6 cells/group).

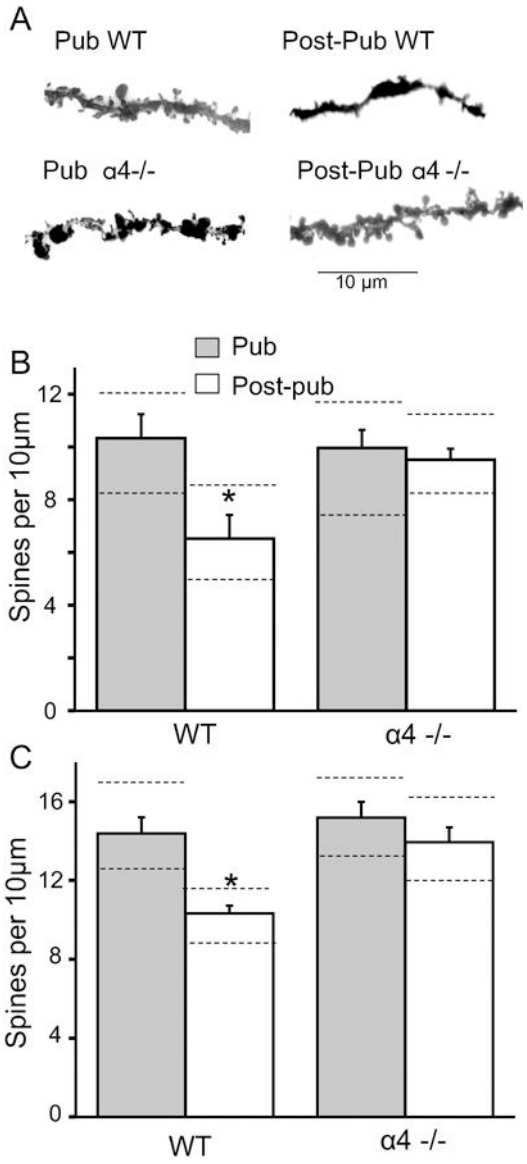

Fig. 2. Dendritic spines on CA3 pyramidal cells decrease from puberty to post-puberty in wild-type but not α4 −/− mice.

CA3 neurons were visualized by Golgi stain and using Nikon Elements and Neurolucida. A, Representative high contrast Z-stack images; scale 10um. B, C, Averaged data for spine quantification (mean ± S.E.M.). Synaptic pruning was seen both proximally (B) and distally (C) in WT post-pubertal mice. Knock-out of the GABAR α4 subunit prevented the post-pubertal decrease in spine density observed in wild-type. *p<0.05 vs. other pubertal/genotype groups. Proximal, WT, pub vs. post-pub, *P=0.014; α4−/−, pub vs. post-pub, P=0.825; post-pub, WT vs. α4−/−, *P=0.031. Distal, WT pub vs. post-pub, *P=0.001, α4−/−, pub vs. post-pub, P=0.509; post-pub, WT vs. α4−/−, *P=0.003. Dashed lines, upper and lower 95% confidence interval. Interaction effects, Appendix A.1. (n=21 dendrites, 5 mice/group).

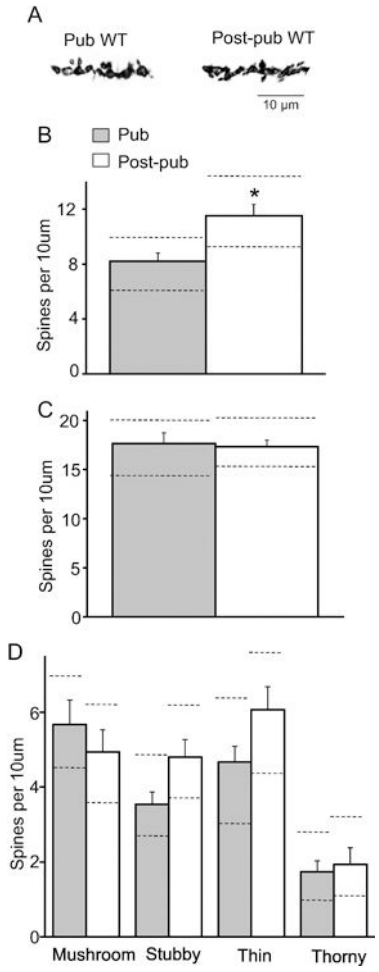

Fig. 3. Dendritic spines on CA3 pyramidal cells do not decrease from puberty to post-puberty in wild-type, proestrous mice.

A. Representative, high contrast Z-stack images; scale 10um. B, C. Decreases in spine density were not seen either proximally (B) or distally (C) in WT post-pubertal proestrous mice. Proximal, *P=0.023, reflecting an increase in spines post-pubertally. Distal, P=0.904 (Appendix A.2, n=10 mice, 5 mice/group). D. Changes in spine type from puberty to post-puberty were not statistically significant during proestrus. (Appendix A.4, n=15 dendrites, 5 mice/group). Dashed lines, upper and lower 95% confidence interval.

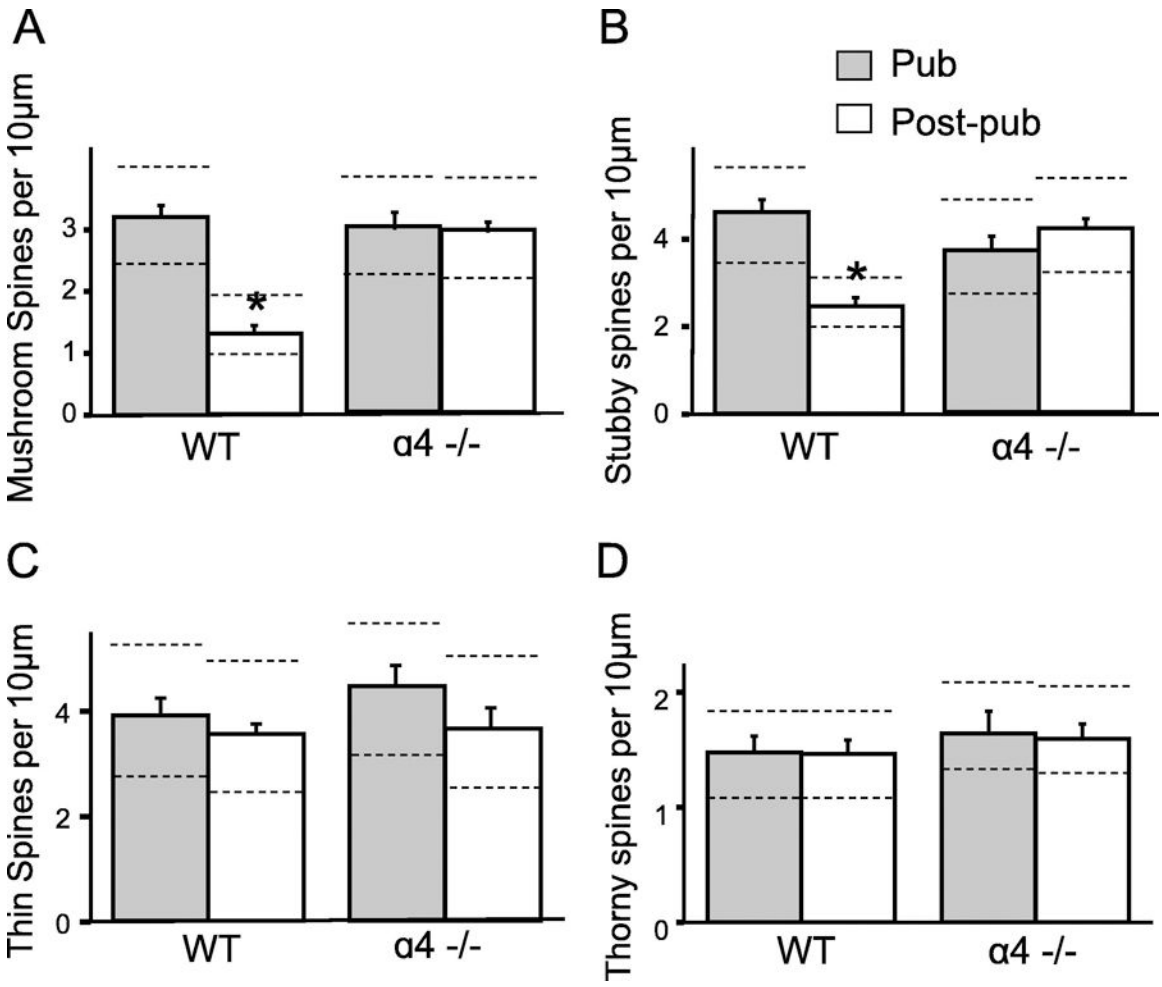

Fig. 4. Changes in CA3 spine types during adolescence are prevented by α4 knock-out

A-D, Averaged data, Quantification of spine types from Golgi-stained CA3 slices. Decreases in mushroom spines (A) and stubby spines (B) were observed post-pubertally, an effect prevented by α4 knock-out. *p<0.05 vs. other pubertal/genotype groups. A, Mushroom, WT, pub vs. post-pub, *P<0.001; α4−/−, pub vs. post-pub, P=0.913; post-pub, WT vs. α4−/−, *P<0.001. B, Stubby, WT, pub vs. post-pub, *P<0.002; α4−/−, pub vs. post-pub, P=0.382, post-pub, WT vs. α4−/−, *P=0.005. (n=20 dendrites, 5 mice/group). Dashed lines, upper and lower 95% confidence interval. Interaction effects, Appendix, A.3.

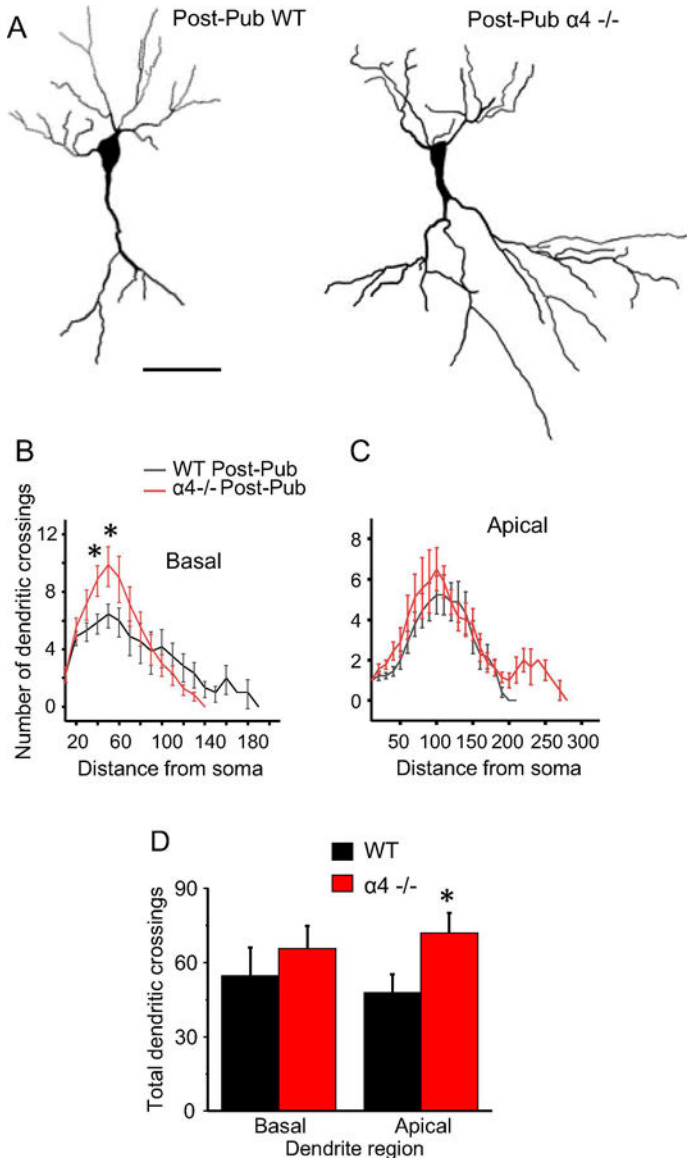

Fig. 5. Dendritic branching of CA3 pyramidal cells is increased post-pubertally in the α4 −/− mouse

A, Representative Neurolucida images for post-pubertal (Post-Pub) WT, left, and Post-Pub α4 −/−, right, CA3 pyramidal cells. Scale, 50 μm. B-D, Averaged data, Post-Pub WT versus Post-Pub α4 −/− mice: # of dendritic crossings versus distance from the soma for basal (B) and apical (C) dendrites, assessed with Scholl Analysis. D, Averaged data for the total number of dendritic crossings. B, 40 μm, t (16)=2.34, *p≤0.05 vs. WT; 50 μm, t (16)=2.13, *p≤0.05 vs. WT. D, apical, t (14)=2.2, *p≤0.05 vs. WT. n=8–9/group.

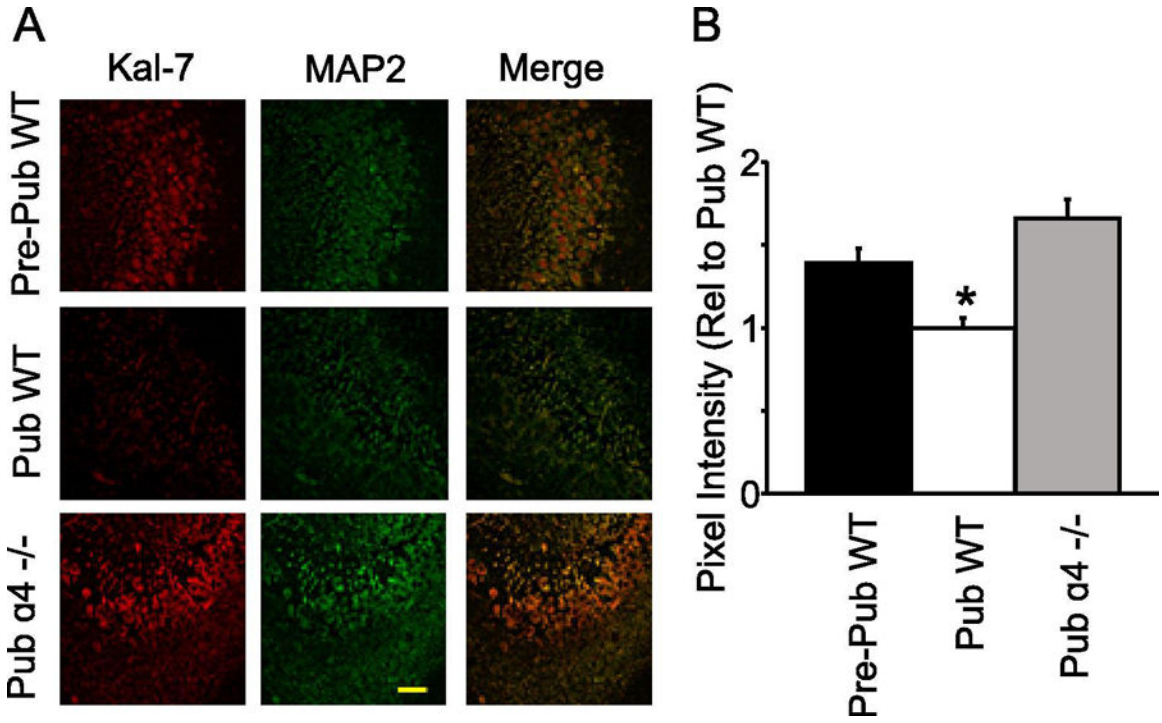

Fig. 6. The spine stabilizing protein, Kalirin-7, decreases at puberty in wild-type but not α4 −/− CA3 hippocampus

Kalirin-7 (Kal-7) is a Rho guanine nucleotide exchange factor necessary for spine maintenance. A, Representative Kal-7 images from pre-pubertal wild-type (pre-pub WT, upper panel), pubertal WT (pub WT, middle panel) and pub α4 −/− (lower panel) female CA3 hippocampus showing Kal-7 (red, left), MAP-2 (green, middle) and merged (yellow, right) immunostaining. Scale, 50 μm. B, Averaged data. Kal-7 expression showed a decrease at puberty in WT CA3 hippocampus but not in α4−/−. Mean pixel intensity, WT, pre-pub vs. pub, *P=0.032; WT pre-pub vs. α4−/− pub, P=0.056; WT pub vs. α4−/− pub, *P=0.016. (n=10, 5 mice). Additional statistics, Appendix A.5.