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Research article

Pubertal hormones increase hippocampal expression of α4βδ $GABA_A$ receptors

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ABSTRACT

CA1 hippocampal expression of α4βδ $GABA_A$ receptors (GABARs) increases at the onset of puberty in female mice, an effect dependent upon the decline in hippocampal levels of the neurosteroid THP (3α-OH-5α-pregnan-20-one) which occurs at this time. The present study further characterized the mechanisms underlying α4βδ expression, assessed in vivo. Blockade of pubertal levels of 17β-estradiol (E_2) (formestane, 0.5 mg/kg, i.p. 3 d) reduced α4 and δ expression by 75–80% ($P < 0.05$) in CA1 hippocampus of female mice, assessed using Western blot techniques. Conversely, E_2 administration increased α4 and δ expression by 50–100% in adults, an effect enhanced by more than 2-fold by concomitant administration of the 5α-reductase blocker finasteride (50 mg/kg, i.p., 3d, $P < 0.05$), suggesting that both declining THP levels and increasing E_2 levels before puberty trigger α4βδ expression. This effect was blocked by ICI 182,780 (20 mg/kg, s.c., 3 d), a selective blocker of E_2 receptor-α (ER-α). These results suggest that both the rise in circulating levels of E_2 and the decline in hippocampal THP levels at the onset of puberty trigger maximal levels of α4βδ expression in the CA1 hippocampus.

1. Introduction

The α4βδ $GABA_A$ receptor (GABAR) mediates a tonic inhibition in hippocampus which is sensitive to modulation by neurosteroids [65]. Regulation of the expression of this receptor is sensitive to ovarian hormone fluctuations [31,34,37,57]. Previous findings from this laboratory have shown that the onset of puberty in female mice results in increased expression of α4βδ GABARs on the dendrites and dendritic spines of pyramidal cells in CA1 hippocampus [57,60] for a period of ˜10 d [6]. This increase in receptor expression was mimicked by administration of a 5α-reductase blocker to reduce levels of the neurosteroid THP (3α-OH-5α-pregnan-20-one or allopregnanolone) and create a "THP withdrawal state" in pre-pubertal female mice [57]. This suggests that the decline in hippocampal levels of THP at puberty serves to trigger α4βδ GABAR expression.

Expression levels of α4 and δ are also increased on CA1 hippocampal pyramidal cells on the proestrous stage of the estrous cycle when circulating levels of E_2 peak [51]. Circulating levels of E_2 rise ˜5 d before the onset of puberty in female mice [4]. Thus, this peri-pubertal E_2 rise is a potential mediator of the pubertal increase in α4βδ GABARs, where it could act independently or synergistically with THP withdrawal. E_2 can act via two different estrogen receptors (ERs), ERα and ERβ [30,40]. Both of these receptor subtypes are localized to hippocampal neurons [41] where they mediate genomic actions for a variety of functions. In particular, ERα triggers activation of brain derived neurotrophic factor (BDNF), which has been shown to increase expression of the GABAR α4 subunit [49]. Thus, we also examined the role of ERα in mediating α4βδ GABAR expression. In addition, we tested whether mimicking the pubertal hormonal milieu could increase α4 and δ expression at times other than puberty, during pre-pubertal and adult life stages.

2. Materials and methods

2.1. Animals

Female C57/BL6 mice (Jackson Labs) were housed in a reverse light:dark cycle (12 :12, lights off at 1100 hs) and euthanized or injected during the light phase of the cycle (2 h before dark, 0900 hs). Mice were evaluated as to pubertal stage by vaginal opening during the

Abbreviations: 3α-HSD, 3α-hydroxysteroid oxidoreductase; BDNF, brain-derived neurotrophic factor; E_2, 17β-estradiol; ER-α, E_2 receptor-α; Fin, finasteride; Form, formestane; GABAR, $GABA_A$ receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICI, ICI 182,780 (i.e., fulvestrant); PKC, protein kinase C; PND, post-natal day; THP, 3α-OH-5α-pregnan-20-one (allopregnanolone); TrkB, tropomyosin receptor kinase B; VGCC, voltage-gated calcium channel

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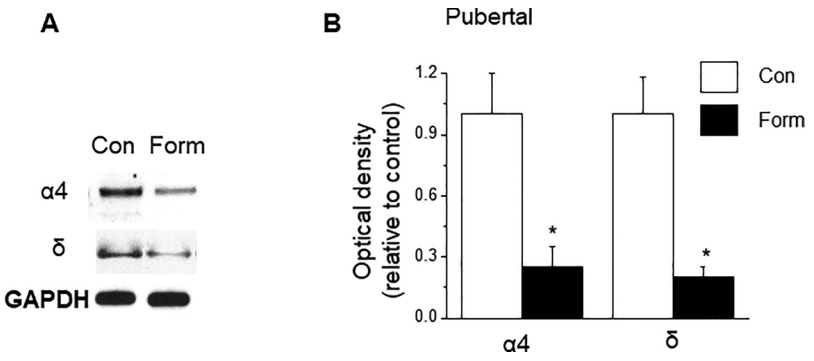

Fig. 1. Increases in pubertal expression of $GABA_A$ receptor α4 and δ subunits in hippocampus is reduced by blockade of estrogen synthesis.
In order to determine if elevated levels of the ovarian steroid 17β-estradiol (E_2) during the pubertal period contribute to the increase in hippocampal α4 and δ expression, the aromatase blocker formestane (Form) was administered in vivo (0.5 mg/kg, i.p., 3d). A, Representative Western blot. B, Summary figure: Form decreased hippocampal expression of α4 and δ subunits at puberty in female mice. α4, $t(10) = 3.35$, $^*P = 0.007$; δ, $t(10) = 4.28$, $^*P = 0.0016$. N = 6, performed in triplicate.

light phase. For the following experiments, pre-pubertal (PND 27–32) and pubertal (PND 40–44) mice were used as well as adult female mice (3 months of age) in the estrous stage of the estrous cycle. Estrous cycle stage was determined by the vaginal cytology in adult animals with established regular cycles [51]. All procedures were in accordance with the SUNY Downstate Institutional Animal Care and Use Committee.

2.2. Western blot [57]

Crude membranes from hippocampus were prepared as follows: After removal and hemi-section of the brain, the hippocampus was dissected from each hemisphere on ice. The cortex was peeled back laterally and the underlying hippocampus rolled out with the micro-spatula. Samples were homogenized using a polytron on ice in 0.0625 M Tris, pH 6.8, 2% SDS, 10% glycerol, 5% dithiothreital (DTT), 1 mM EDTA and a cocktail of protease inhibitors (GE healthcare). Crude membranes were prepared after centrifugation at 1000xg for 15 min to remove cellular debris and then collecting the pellet (P2) after centrifugation of the supernatant (S1) at 200,000xg for 30 min.

Samples were first normalized according to protein content using standard techniques and protein concentrations used were used in the linear range (4–10 mg); then, proteins were separated using SDS gel electrophoresis, transferred to nitrocellulose membranes and probed with selective antibodies for the rat α4 (67 kDa, SC-7355, Santa Cruz Biotechnology, 1:20 or 1:100) or δ (54 kDa, Novus Biologicals, 1:1000). Bands were detected with a highly sensitive chemiluminescence substrate (Pierce Supersignal West Femto substrate) for visualization and quantified using Adobe Photoshop. The results were standardized to a Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 36 kDa) housekeeping protein.

2.3. Drugs

The ovarian hormone 17β-estradiol (E_2) was injected at a dose of 8 μg/kg, i.p., to adult female mice for 3 d beginning on the day of estrus. In some cases the 5α-reductase inhibitor finasteride (Fin, 50 mg/kg, i.p.) was injected for 3 d alone or concomitant with E_2 to pre-pubertal or adult mice to create a THP withdrawal state [63]. In other cases, the aromatase blocker formestane (Form) was administered for 3 d (0.5 mg/kg, i.p., 3d) in order to prevent formation of endogenous E_2. In the final experiments, adult mice were treated for 3 d with a blocker of estrogen receptor-α (ERα) ICI 182,780 (ICI, 20 mg/kg, s.c.) concomitant with E_2 plus finasteride. In all cases the vehicle was oil, and vehicle-injected mice served as controls. All steroids and steroid blockers were obtained from Steraloids (Newport, RI).

2.4. Statistics

Comparisons of the degree of change across groups for all experimental procedures were analyzed with a student's unpaired *t*-test (2 groups) or one-way analysis of variance (ANOVA, 3+ groups). Post-hoc comparisons for the ANOVA were made with a Tukey's test. For all tests, the level of significance was $P < 0.05$. Complete statistics for all experiments are included in the figure legends.

3. Results

3.1. Peri-pubertal increases in circulating E2 levels contribute to the pubertal increase in hippocampal α4 and δ GABAR subunit expression

Because previous reports suggest that E_2 may increase α4 and δ expression depending on the existing hormonal milieu [70], we tested whether the pubertal rise in circulating E_2 [4] played a role in the increase in α4βδ expression at this time. To this end, we used the aromatase blocker formestane (0.5 mg/kg, i.p.) which has been shown to reduce E_2 formation and mimic the ovariectomized state [17]. Formestane was administered across a 48 h period (3 injections) because we have previously shown that this time-course is maximal for α4βδ GABAR expression [31]. Formestane treatment of pubertal mice reduced α4 and δ expression by 75–80% ($P < 0.05$, Fig. 1) suggesting that peri-pubertal increases in E_2 are necessary for maximal expression of α4 and δ subunits in CA1 hippocampus at puberty.

3.2. THP withdrawal increases α4 and δ GABAR subunit expression in the pre-pubertal hippocampus: an E2-dependent effect

Circulating E_2 levels rise 5 d before the onset of puberty [4] when circulating and hippocampal levels of THP are also elevated [57]. We used finasteride to reduce THP levels to simulate the pubertal THP withdrawal state in order to test whether THP withdrawal in pre-pubertal mice would increase α4 and δ expression and, further, whether pre-pubertal E_2 could play a role in their expression. Finasteride (50 mg/kg, i.p., 3d) was injected using a dosing regimen shown to reduce hippocampal THP levels by ~50% [63]. In some cases, late pre-pubertal mice (~5 d before vaginal opening) were also injected with the aromatase inhibitor formestane concomitant with finasteride to test the effect of reducing E_2 levels on α4 and δ expression. Finasteride treatment increased α4 and δ expression by ~200%, an effect prevented by formestane (Fig. 2) suggesting that both E_2 and THP withdrawal are necessary for maximal increases in α4βδ expression in pre-pubertal hippocampus.

3.3. E_2 administration increases α4 and δ GABAR subunit expression in adult hippocampus

In order to further investigate the effects of pubertal hormones, we tested whether E_2 could increase α4 and δ expression in adult hippocampus. To this end, we administered E_2 (8 υg/kg, i.p., 3d) to adult, female mice in the stage of estrus. This treatment increased δ and α4 expression by ~50 and 100% compared to vehicle-treated values ($P < 0.05$, Fig. 3), suggesting that E_2 alone can alter expression of α4βδ GABARs.

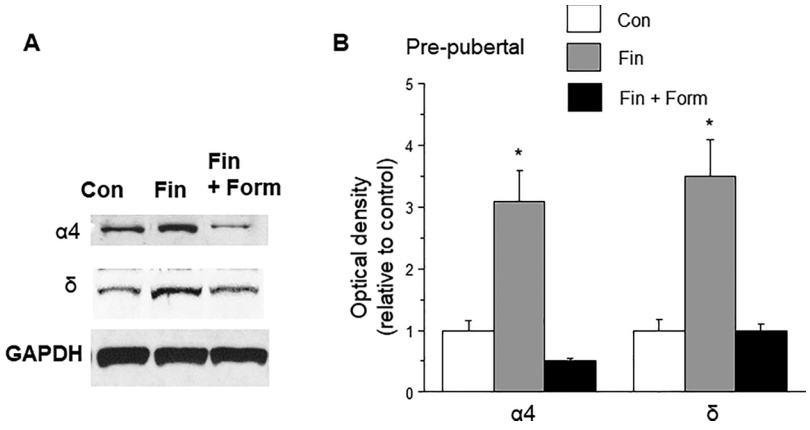

Fig. 2. Inducing "THP withdrawal" in pre-pubertal mice increases expression of hippocampal α4 and δ subunits: An effect prevented by blockade of estrogen synthesis.
"THP withdrawal" was induced in pre-pubertal mice by blocking THP formation with finasteride (Fin) administered in some cases with formestane (Form) to block E_2 synthesis. A, Representative Western blot. B, Summary figure. α4, $F(2,15) = 20.5$,*$P < 0.0001$; δ, $F(2,15) = 15.7$, $P = 0.0002$.*$P < 0.05$ vs. vehicle-treated control. N = 6, performed in triplicate.

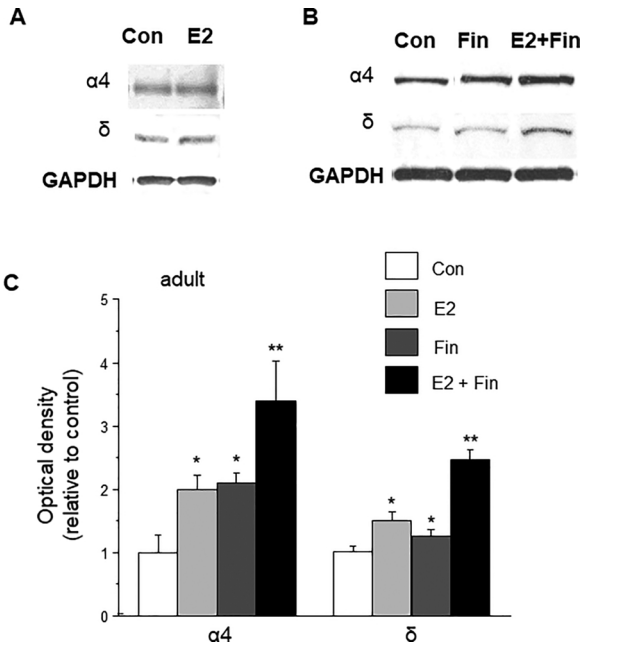

Fig. 3. Mimicking the pubertal hormonal milieu produces maximal expression of α4 and δ in the adult, female mouse.
E_2 (8 μg/kg, i.p.) and/or finasteride (Fin) were administered to adult mice for 3 d beginning on estrus. A, Representative Western blots show that both drugs significantly increased α4 and δ expression, but together produced optimal expression of these subunits. B, Summary figure. α4, $F(3,20) = 4.62$,*$P = 0.013$; δ, $F(3,20) = 3.23$, $P = 0.0442$.*$P < 0.05$ vs. vehicle-treated control, **$P < 0.01$ vs. other groups. N = 8 mice/group, performed in triplicate.

3.4. E_2 plus THP withdrawal produce peak levels of α4 and δ GABAR subunit expression in adult hippocampus

In order to determine the effects of THP withdrawal on α4βδ GABAR expression in the adult, we administered finasteride (50 mg/kg, i.p., 3d) to adult female mice in estrus. THP withdrawal produced by finasteride increased α4 and δ expression by 100% and 30%, respectively ($P < 0.05$, Fig. 3). However, administration of E_2 concomitant with finasteride increased α4 and δ expression significantly more, by 250% and 125% ($P < 0.05$, Fig. 3), suggesting that maximal levels of α4 and δ expression require both E_2 and THP withdrawal.

3.5. E_2 effects on α4 and δ GABAR subunit expression are mediated by estrogen receptor-α

E_2 can act through two different nuclear receptors (ERα and ERβ) [30,40] which have genomic effects, as well as via membrane effects. In order to determine whether E_2 effects on α4βδ GABAR expression were due to actions mediated by ERα, which mediates most effects of the hormone, we administered the selective ERα blocker [32] ICI182,780 (ICI) concomitant with treatment with E_2 and finasteride. ICI was injected using a dosing regimen (20 mg/kg, s.c., 3 d) that has been shown to block effects of E_2 on uterine weight in a manner similar to ERα knock-out [22]. ICI treatment prevented the increase in α4 and δ expression produced by E_2 plus finasteride (Fig. 4), suggesting that E_2 acts through ERα to increase α4 and δ GABAR subunit expression.

4. Discussion

4.1. Summary

Our findings suggest that the pubertal increase in α4βδ GABARs is

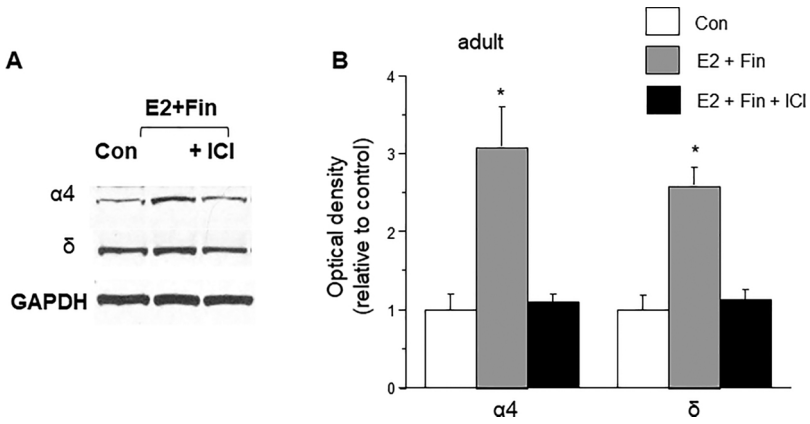

Fig. 4. E_2 increases expression of GABAR α4 and δ subunits via ER-α.
E_2 (8 μg/kg, i.p.) and finasteride (Fin) or vehicle (Con) were administered to adult mice for 3 d in some cases along with a blocker of estrogen receptor-α (ERα) ICI 182,780 (ICI, 20 mg/kg, s.c.). A, Representative Western blots show that blockade of ERα prevented the E_2 + Fin-induced increases in α4 and δ expression. B, Summary figure. α4, $F(2,21) = 14.0$,*$P = 0.0001$; δ, $F(2,21) = 23.2$, $P < 0.0001$.*$P < 0.05$ vs. vehicle-treated control, **$P < 0.01$ vs. other groups. N = 8 mice/group, performed in triplicate.

due to both declining levels of THP as well as peri-pubertal elevations in circulating E_2. In addition, we show that use of these ovarian hormones to mimic the changes which occur at puberty can also increase α4 and δ expression to maximal levels at times other than puberty. The effect of E_2 was mediated by ERα and potentiated by the effects of THP withdrawal.

4.2. Pubertal hormones

Our results suggest that optimal levels of α4 expression are achieved with sustained exposure to E_2 in combination with THP withdrawal, mimicking the ovarian milieu of the peri-pubertal period. The onset of puberty in the female mouse is preceded by increases in circulating E_2, with two-fold increases observed 5 d before vaginal opening which persist across the beginning of the pubertal period [4].

In addition, both brain and circulating levels of THP are increased during the late pre-pubertal period and decrease by ~70% at the onset of puberty in the female mouse [57]. Early studies established that this change in steroid levels is due to altered enzyme activity in the ovary [16,38]. THP is formed from P via two enzymatic conversions mediated by 5α-reductase and 3α-hydroxysteroid oxidoreductase (3α-HSD) [13,28,43]. In addition to an ovarian site of production, this steroid can be produced in the adrenal gland, with release into the general circulation before puberty onset [11]. Recent studies also suggest that THP can be produced de novo directly in the brain from cholesterol via side chain cleavage enzyme; thus, it is also classified as a neurosteroid [13]. This can occur in a variety of CNS sites, in both glia and neurons, including the CA1 hippocampal pyramidal cell [3]. In the mouse, THP levels peak during the dark phase of the circadian cycle [14]. This peri-pubertal pattern of ovarian hormonal fluctuations is also observed in the human: E_2 levels rise prior to puberty onset and circulating levels of THP fall at puberty onset, when they fluctuate in a circadian manner [7,25,39].

4.3. E_2 and α4βδ GABAR expression

The present results suggest that E_2 alone increases α4 and δ expression in addition to enhancing the effect of THP withdrawal. In our previous studies, increased α4βδ GABAR expression initiated at puberty was maintained for 10 d before undergoing cyclic expression patterns in female CA1 hippocampus [6,57]. This prolonged pubertal period has been noted before, where high circulating E_2 levels are maintained for many days after puberty onset despite estrous cycle variations in the vaginal cytology [23]. Thus, this pubertal plateau in E_2 levels may underlie the prolonged period of α4βδ expression at puberty.

Consistent with the present results, a number of studies have confirmed the role of E_2 in increasing expression of α4 and/or δ expression, which has been noted in cell lines [47,70] as well as in the hippocampus, where both ERα and β are localized [32], after increases in exogenous or endogenous E_2 levels [40]. The role of E_2 in increasing α4βδ GABAR expression at puberty and in the in vitro model is likely via its increase in brain-derived neurotrophic factor (BDNF), which activates the α4 promoter via expression of early growth response factor-3 (EGR-3) [50]. BDNF also increases membrane trafficking of δ-GABARs via TrkB receptors (tropomyosin receptor kinase B) [27].

E_2 increases both BDNF protein and mRNA in CA1 and CA3 regions of the hippocampus in ovariectomized rats [55,69]. BDNF immunoreactivity is also increased on the proestrous stage of the estrous cycle, during the peak in circulating levels of E_2 in the dendrites of CA1 and CA3 pyramidal cells [56]. More recent studies have established that E_2 increases phosphorylated TrkB receptors, via ERα, as well as ERβ, in hippocampal neurons [64]. ERα generates genomic events via classic nuclear steroid receptors [45], but can also mediate membrane-initiated signaling to activate kinase cascades in a more rapid time-course [5]. These genomic effects of E_2 likely underlie the E_2 (ERα)-mediated increases in α4 expression observed in the present study.

4.4. THP withdrawal and α4βδ GABAR expression

THP withdrawal was shown in the present study to result in peak levels of α4 and δ expression in the presence of high circulating levels of E_2. Our previous study showed that at puberty onset, the declining levels of THP trigger expression of α4βδ GABARs in CA1 hippocampus [57]. Administration of replacement THP prevents this increase in receptor expression, confirming the pivotal role of THP withdrawal in regulating α4βδ GABAR expression. Other studies have reported that declining levels of THP or its parent compound progesterone, either administered exogenously or as a result of the ovarian cycle and pregnancy, also increase expression of α4 or α4βδ GABARs in a variety of CNS sites [20,34,36,62].

In the present study, α4 and δ expression were assessed in the whole hippocampus which would include both the CA3 hippocampus and the dentate gyrus in addition to the CA1 hippocampus. Unlike the CA1 and CA3 hippocampus, the dentate gyrus expresses high levels of α4βδ GABARs at times other than puberty [8,9]. However, α4βδ expression is increased at puberty in the CA3 [46] and, preliminary data suggest, also in the dentate gyrus. Therefore, the pubertal hormone conditions used in the present study to increase α4 and δ expression may be relevant for all sub-regions of the hippocampus.

4.5. α4βδ GABAR expression as a compensatory response

The underlying mechanism for increases in α4βδ GABARs after THP withdrawal may be a result of a compensatory change in inhibitory tone as a result of the loss of this GABA modulatory neurosteroid. α4βδ GABARs generate a tonic inhibition due to their extrasynaptic location [66], high sensitivity to GABA [11,42] and relative lack of desensitization under steady state conditions [11]. Their relative plasticity suggests that their expression may serve a compensatory role; their expression is increased in certain seizure states [19], during chronic administration of the stimulant methamphetamine [59], in response to traumatic brain injury [44] and excitotoxic challenge [54]. Their increase after THP withdrawal, as demonstrated here, may also represent a compensatory increase in response to declining levels of this positive GABAR modulator which would decrease inhibition. One possibility is that removal of THP, which inhibits L-type voltage-gated calcium channels (VGCCs) [15], then increases $Ca++$ influx, which can increase expression and trafficking of α4βδ GABARs [52,53]. Conversely, α4βδ expression is decreased during pregnancy when circulating levels of THP are elevated [36]. This increase in α4βδ expression across hormonal transitions (i.e., the estrous cycle, pregnancy and puberty onset) reduces seizure susceptibility [35,67].

4.6. THP administration and α4βδ GABAR expression

In contrast to THP withdrawal, 48 h administration of THP also increases expression of α4βδ GABARs [1,31,58] by increasing surface expression of the receptor, with no effect on internalization [31]. This effect was mediated by PKC, including PKC-δ, targeting serine 443 in α4 [1,31]. The reason that both sustained exposure to and withdrawal from THP increase α4βδ expression is not clear, but may be related to the diverse effects of the steroid on triggering phosphorylation [1] and inhibiting VGCCs [15] which would increase receptor expression after administration or withdrawal, respectively.

4.7. α4βδ GABAR expression and puberty: effects on anxiety and panic response

Anxiety disorders, which are more prevalent in females, are most likely to emerge during or after puberty [21]. Our previous findings suggest that α4βδ GABAR expression at puberty onset are associated with alterations in mood. At the onset of puberty, when α4βδ GABARs are increased, the stress steroid THP [48] produces anxiety [57], due to

a paradoxical decrease in α4βδ-generated current, in contrast to its typical anti-anxiety effect [10]. This effect is dependent upon the polarity of the GABA-generated Cl- current [18,57] and is not observed in α4 knock-out mice. In addition, estrous cycle-dependent changes in α4βδ expression on midbrain interneurons have been shown to generate panic responses, suggesting a role for this receptor in panic disorder [33] as well as stress-induced anxiety.

4.8. α4βδ GABAR expression and puberty: effects on cognition and synaptic pruning

More recent studies from this laboratory [60,61] have shown that the expression of α4βδ on the dendritic shaft and spines of CA1 pyramidal cells at puberty impairs cognition acutely and prevents induction of long-term potentiation, an in vitro model of learning. This outcome may define the end of an optimal period for learning at the onset of puberty which has been described in the human for certain tasks such as learning a second language [26]. The impact of puberty onset on learning is complex, however, as it also is a time when stress can enhance cognition [23], which may be related to corticosterone effects or to the effects of THP [60], which is also released after stress [48].

One of the long-term consequences of α4βδ expression on the CA1 spines during adolescence is to trigger synaptic pruning [2], a phenomenon where dendritic spines are removed from many areas of the CNS [24,29,68], and is thought to be essential for optimal cognition in adulthood [12]. In the absence of synaptic pruning, as observed in the α4-/- mouse, spine density is increased and cognitive flexibility is impaired [2].

4.9. Conclusions

The results from this study demonstrate that pubertal increases in expression of α4βδ GABARs are due to both peri-pubertal increases in circulating levels of E_2 and declining levels of THP ("THP withdrawal"). These effects were mediated by ERα. Because α4βδ GABARs are associated with changes in anxiety and synaptic function, the results from the present study may be relevant for understanding the processes which contribute to neuropathologies associated with mood and cognition.

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