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Puberty, steroids and $GABA_A$ receptor plasticity

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Summary $GABA_A$ receptors (GABAR) mediate most inhibition in the CNS and are also a target for neuroactive steroids such as 3α,5[α]β-THP (3αOH-5[α]β-OH-pregnan-20-one or [allo]pregnanolone). Although these steroids robustly enhance current gated by α1β2δ GABAR, we have shown that 3α,5[α]β-THP effects at recombinant α4β2δ GABAR depend on the direction of Cl^- flux, where the steroid increases outward flux, but decreases inward flux through the receptor. This polarity-dependent inhibition of α4β2δ GABAR resulted from an increase in the rate and extent of rapid desensitization of the receptor, recorded from recombinant receptors expressed in HEK-293 cells with whole cell voltage clamp techniques. This inhibitory effect of 3α,5[α]β-THP was not observed at other receptor subtypes, suggesting it was selective for α4β2δ GABAR. Furthermore, it was prevented by a selective mutation of basic residue arginine 353 in the intracellular loop of the receptor, suggesting that this might be a putative chloride modulatory site. Expression of α4βδ GABAR increases markedly at extrasynaptic sites at the onset of puberty in female mice. At this time, 3α,5[α]β-THP decreased the inhibitory tonic current, recorded with perforated patch techniques to maintain the physiological Cl^- gradient. By decreasing this shunting inhibition, 3α,5[α]β-THP increased the excitability of CA1 hippocampal pyramidal cells at puberty. These effects of the steroid were opposite to those observed before puberty when 3α,5[α]β-THP reduced neuronal excitability as a pre-synaptic effect. Behaviorally, the excitatory effect of 3α,5[α]β-THP was reflected as an increase in anxiety at the onset of puberty in female mice. Taken together, these findings suggest that the emergence of α4β2δ GABAR at the onset of puberty reverses the effect of a stress steroid. These findings may be relevant for the mood swings and increased response to stressful events reported in adolescence.
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1. Introduction

It is well-known that the onset of puberty can be associated with mood swings (Buchanan et al., 1992; Huerta and Brizuela-Gamino, 2002) and irritability (Hayward and Sanborn, 2002), in contrast to pre-pubertal behavior. There is an increased response to stress (Modesti et al., 2006) at this time, and anxiety disorders, including panic disorder, typically first present at pubertal ages (Hayward and Sanborn, 2002), more likely to occur in girls than boys. There are also reports of increases in risk-taking behavior (Costello et al., 2007), including an increased probability of consumption of addictive substances. Although mood changes associated with premenstrual syndrome (Halbreich et al., 2007), postpartum depression and post-menopausal dysphoria (Mukai

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et al., 2008) have been investigated in clinical and preclinical studies, relatively little is known about the underlying CNS mechanisms which precipitate mood changes at puberty.

In females, the onset of reproductive puberty (gonadarche, menarche) is preceded by maturation of the adrenal gland (adrenarche) (Apter and Hermanson, 2002). Therefore, fluctuations in both adrenal (progesterone, androgens) and gonadal hormones (estradiol) are associated with female pubertal maturation in humans and rodents. Specifically, circulating levels of the steroid hormones progesterone and estradiol rise from very low levels earlier in development to peak immediately preceding the onset of puberty (Apter and Hermanson, 2002; Kahn et al., 2008; Safranski et al., 1993), defined in humans as the onset of menstruation and in female rodents as the time of vaginal opening. Steroid levels decline thereafter (Kahn et al., 2008; Fadalti et al., 1999; Shen et al., 2007) until ovarian cyclicity begins days later. It is this decline in steroid hormones which resembles the hormonal events associated with other hormonally-associated mood syndromes, such as premenstrual syndrome (premenstrual dysphoric disorder or PMDD) and post-menopausal dysphoria (Halbreich et al., 2007). Although ovarian hormones have been reported to exert an array of effects on mood and behavior, effects of neuroactive steroids on the $GABA_A$ receptor (GABAR) may play a role in pubertal anxiety disorders.

2. The $GABA_A$ receptor

The GABAR is a pentameric membrane protein that mediates most inhibition in the brain (Hevers and Luddens, 1998; Olsen and Sieghart, 2008), and is the target of most anxiety-reducing, sedative drugs, including benzodiazepines, barbiturates, anesthetics and, in some cases, alcohol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004; Glykys et al., 2007). The GABAR plays a pivotal role in regulating anxiety, as demonstrated by studies using transgenic mouse models (Rudolph et al., 1999). This receptor is typically composed of 2α, 2β and a γ subunit (Chang et al., 1990), but other subunits exist, including δ, ε, π, ρ and θ. Many subunit subtypes also exist (6α, 3β, 3γ) yielding a diverse array of possible GABAR isoforms (Hevers and Luddens, 1998; Olsen and Sieghart, 2008), with different biophysical and pharmacological properties that can differentially alter circuit properties in limbic structures and ultimately have an impact on behavior. Each subunit, in turn, is composed of 4 membrane-spanning α-helices, where TM2 surrounds a central Cl^- channel and the TM3–TM4 intracellular loop is a site for phosphorylation. GABA agonists bind at two specific sites between α and β to gate a Cl^- current, which can be modulated by a variety of agents, including steroids (Majewska et al., 1986), at distinct sites separate from GABA (Hosie et al., 2006). The most commonly expressed GABAR is $\alpha1\beta2\gamma2$. In contrast, GABAR of the form $\alpha4\beta\delta$ have relatively low expression in the brain (Wisden et al., 1992), but instead are remarkably plastic, increasing in response to hormonal fluctuations, either endogenously occurring across the ovarian cycle (Lovick et al., 2005; Maguire et al., 2005) or pregnancy (Maguire and Mody, 2009; Sanna et al., 2009) or exogenously administered (Griffiths and Lovick, 2005; Maguire and Mody, 2007; Shen et al., 2005; Smith et al., 1998a; Sundstrom-Poromaa et al., 2002). In particular, withdrawal from chronic administration of progesterone increases expression of $\alpha4\beta\delta$ in CA1 hippocampus (Smith et al., 2006; Sundstrom-Poromaa et al., 2002), an area which normally has very low expression of this receptor.

3. $\alpha4\beta\delta$ GABAR

The $\alpha4\beta3\delta$ GABAR is composed of 2α, 2β and 1δ subunit (Barrera et al., 2008), arranged as $\alpha\beta\alpha\delta\beta$, counter-clockwise when viewed from the extracellular space. δ-Containing GABAR are relatively unique in that they are not localized to post-synaptic sites, but rather are extrasynaptic or perisynaptic (Wei et al., 2003) where they generate a tonic current (Stell and Mody, 2002) in response to ambient concentrations of GABA regulated by GABA transporters (Wu et al., 2001) and spillover from synaptic release (Glykys and Mody, 2007). Recombinant δ-containing GABAR have a high sensitivity to low concentrations of GABA (Brown et al., 2002; Sundstrom-Poromaa et al., 2002) and little desensitization (Bianchi et al., 2002; Brown et al., 2002; Feng et al., 2006; Haas and Macdonald, 1999), making them ideally suited as an extrasynaptic receptor. They also possess a unique pharmacological profile, in that they are insensitive to benzodiazepine modulation (Brown et al., 2002), respond with high sensitivity to low concentrations of the GABA agonist gaboxadol (THIP) (Brown et al., 2002) and appear to be the most sensitive targets of steroid modulation (Belelli et al., 2002; Bianchi and Macdonald, 2003; Brown et al., 2002; Wohlfarth et al., 2002; Zheleznova et al., 2008). Specifically, steroids such as 3α,5[α]β-THP (3α-OH-5[α]β-pregnan-20-one or [allo]pregnanolone) and THDOC are potent positive modulators of recombinant δ-containing GABAR at physiological concentrations (10–30 nM), where they increase receptor efficacy (Bianchi and Macdonald, 2003; Zheleznova et al., 2008).

Although $\alpha4\beta\delta$ and the homologous $\alpha6\beta\delta$ GABAR have relatively low expression in most areas of the brain (Wisden et al., 1992), high levels of expression in cerebellar granule cells, dentate gyrus granule cells and thalamic relay nuclei generate a tonic inhibitory current (Belelli et al., 2005; Brickley et al., 2001; Herd et al., 2008; Jia et al., 2005; Mtchedlishvili and Kapur, 2006; Stell and Mody, 2002), while the tonic inhibitory current in CA1 hippocampus (Bai et al., 2000) is primarily mediated by $\alpha5$-containing GABAR (Caraiscos et al., 2004). Tonic current mediated by native δ-containing GABAR is highly responsive to low concentrations of gaboxadol, similar to recombinant δ-containing GABAR. In addition, a number of reports have also shown significant modulation of the tonic current by 10 nM concentrations of neurosteroids such as THP and THDOC in dentate gyrus granule cells (Stell et al., 2003; Maguire et al., 2005; Scimemi et al., 2006; Liang et al., 2008; Sanna et al., 2009; Spigelman et al., 2003). In addition, δ knock-out mice have reduced sensitivity to neurosteroids (Mihalek et al., 1999). However, other studies describe no or only modest effects of steroids on native δ-containing GABAR (Belelli and Herd, 2003; Hamann et al., 2002; Mtchedlishvili and Kapur, 2006) even at higher 250 nM concentrations (Porcello et al., 2003). These conflicting findings may be due to age, species or gender differences or may be the result of other factors such as phosphorylation state (Fancsik et al., 2000; Harney et al.,

2003), local neurosteroid metabolism (Belelli and Herd, 2003) or the ambient GABA level which could contribute to the ultimate effect of steroids on tonic current mediated by δ-containing GABAR.

4. The neurosteroid 3α,5[α]β-THP

3α,5[α]β-THP is formed via two enzymatic conversions from the ovarian/adrenal steroid progesterone (Mellon and Vaudry, 2001), and its levels fluctuate across the ovarian cycle and pregnancy. This steroid can also be formed directly from cholesterol in certain neurons, such as the CA1 hippocampal pyramidal cell (Agis-Balboa et al., 2006), via side chain cleavage enzyme. Thus, it is classified both as a neurosteroid (formed in the brain) as well as a neuroactive steroid (alters brain activity). Neurosteroid synthesis has been shown to be dramatically increased by stress (Purdy et al., 1991; Barbaccia et al., 1996). In particular, 20—45 min of restraint stress significantly increases CNS levels of 3α,5[α]β-THP 20-fold in rodents (Higashi et al., 2005; Mukai et al., 2008), while human studies have indicated that performance stress increases circulating levels of 3α,5[α]β-THP (Girdler et al., 2006; Drooglever Fortuyn et al., 2004).

Behaviorally, 3α,5[α]β-THP has been shown to reduce anxiety (Bitran et al., 1999) and possess anti-convulsant effects (Frye, 1995) in rodents and, at high doses, has sedative effects in both rodents (Lancel et al., 1997) and humans (van Broekhoven et al., 2006). In particular, the anxiety-reducing effects of this steroid have been demonstrated via systemic injection or direct infusion into the dorsal hippocampus (Bitran et al., 1999) in female rats. Thus, stress-induced increases in the steroid may serve as a compensatory response, because the resultant increase in GABAergic inhibition by the steroid would permit an adaptive decrease in the stress-induced anxiety response.

5. Puberty and α4βδ GABAR

Because responses to stress are increased at the onset of puberty (Modesti et al., 2006), we examined whether steroid/GABAR interactions were altered during this time in female mice as a result of changes in expression of their most sensitive target, α4βδ GABAR. To this end, we initially examined the expression of α4 and δ GABAR subunits in CA1 hippocampus, comparing results from female C57BL6 mice before puberty onset with those shortly after vaginal opening, the onset of puberty. In fact, hippocampal expression of both α4 and δ subunits was increased by 2—3-fold at the onset of puberty, assessed using Western blot techniques, compared to levels before puberty (Shen et al., 2007). In addition, immunocytochemical procedures revealed a striking increase in expression of both subunits along the apical dendrites of CA1 pyramidal cells compared to almost undetectable levels prior to puberty. Because δ-containing GABAR are highly sensitive to the GABA agonist gaboxadol (THIP) (Brown et al., 2002), we examined responses of CA1 hippocampal pyramidal cells to this drug using whole cell patch clamp recording in the hippocampal slice. In fact, gaboxadol application produced a significantly greater shift in the holding potential in pubertal slices compared to its effect before puberty (Shen et al., 2007). These results suggest that there is an increase in functional α4βδ GABAR at puberty.

Consistent with an increase in inhibitory tone at puberty, spontaneous spiking of CA1 pyramidal cells was decreased at this time (Shen et al., 2007) compared to pre-pubertal neuronal activity levels, assessed using cell-attached recording techniques. Taken together, these results suggest that puberty onset is a time of decreased levels of activity in limbic structures, which does not explain the increase in anxiety reported at this time (Buchanan et al., 1992; Hayward and Sanborn, 2002; Modesti et al., 2006).

6. 3α,5[α]β-THP effects at puberty

In order to investigate possible reasons for increased anxiety at puberty, we tested the hypothesis that the change in GABAR expression would alter responses to the stress steroid 3α,5[α]β-THP. This hypothesis was justified, in part, by clinical reports suggesting that mood changes in women with premenstrual dysphoric disorder were due to an abnormal response to progesterone (Schmidt et al., 1998), the precursor of 3α,5[α]β-THP. In addition several reports have indicated that in women with PMDD (Freeman et al., 2002) and post-menopausal dysphoria (Andreen et al., 2004), worsened mood was paradoxically associated with higher circulating levels of 3α,5[α]β-THP. These reports suggest the possibility that hormonally induced changes in mood might be the result of a negative response to 3α,5[α]β-THP.

When tested using the cell-attached recording paradigm, 3α,5[α]β-THP indeed produced the opposite effects at puberty (Shen et al., 2007), increasing spontaneous spiking of CA1 pyramidal cells, in contrast to its inhibitory effect before puberty. This excitatory effect of the GABA-modulatory steroid was not seen in pyramidal cells from δ knock-out mice, suggesting that effects at the α4βδ GABAR might be a likely target for this effect.

7. 3α,5[α]β-THP effects at α4β2δ GABAR

Numerous studies using recombinant δ-containing GABAR have demonstrated that this receptor is a highly sensitive target for steroids such as 3α,5[α]β-THP and THDOC (Belelli et al., 2002; Brown et al., 2002; Wohlfarth et al., 2002; Zheleznova et al., 2008), which normally increases GABA-gated current. However, most conventional recording is generally performed by assessing the outward flux of Cl^- ions, while in many areas of the mature CNS, Cl^- flux is inward (outward current) (Rivera et al., 1999). Thus, we tested the possibility that varying the direction of Cl^- flux might have an impact on the effect of 3α,5[α]β-THP at recombinant α4β2δ GABAR, expressed in HEK-293 cells. As expected, 30 nM 3α,5[α]β-THP produced robust increases in current gated by either α1β2δ or α4β2δ when Cl^- flux was outward (Fig. 1). However, in a separate set of cells, 3α,5[α]β-THP decreased GABA-gated current under conditions of inward Cl^- flux at α4β2δ GABAR (Shen et al., 2007), but not at α1β2δ receptors (Fig. 1). Substituting β3 for β2 yielded a receptor that was not inhibited by 3α,5[α]β-THP, nor was the steroid effective at inhibiting α4β2γ2, α1β2γ2 or α5β3γ2, suggesting that α4β2δ was uniquely sensitive to 3α,5[α]β-THP inhibition when Cl^- flux was inward. This is the likely receptor combination expressed endogenously, as suggested by studies conducted using the GABAR β2 knock-out mouse (Belelli et al., 2005; Herd et al., 2008), which reduces the tonic

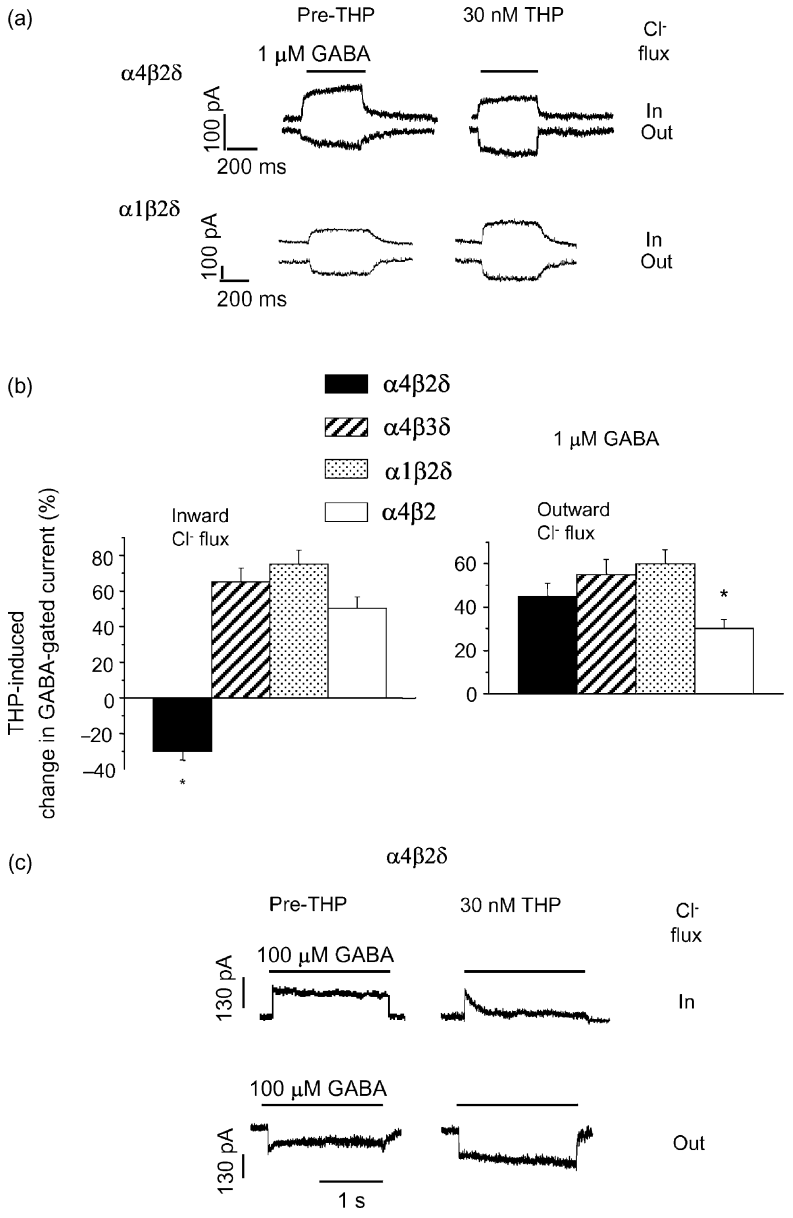

Figure 1 The neurosteroid 3α,5[α]β-THP decreases inward Cl^- flux at α4β2δ $GABA_A$ receptors. (a) Representative traces showing the effects of 30 nM 3α,5[α]β-THP (right) on current gated by 1 μM GABA (EC_{75}), under conditions of inward Cl^- flux (upper trace) and outward Cl^- flux (lower trace) for two δ-containing recombinant $GABA_A$ receptor subtypes. The direction of Cl^- flux was reversed by varying internal Cl^- (upper trace, ECl = −70 mV; lower trace, ECl = −30 mV), but using a constant holding potential of −50 mV. (b) Mean effects of 3α,5[α]β-THP on outward and inward Cl^- flux in response to 1 μM GABA (from 6 to 7 cells for each group; $^*P < 0.05$ vs. the other receptor subtypes). (c) 3α,5[α]β-THP effects on desensitization of inward (upper trace) and outward (lower trace) Cl^- flux through α4β2δ receptors. This effect is representative of 6 cells for each group. Reprinted from Fig. 1, Nat. Neurosci. 10, 469–477 (2007).

current in both dentate gyrus and thalamus, two areas with high levels of α4 and δ subunit expression (Wisden et al., 1992).

8. 3α,5[α]β-THP effects on desensitization of α4β2δ receptors

3α,5[α]β-THP did not change the reversal potential at α4β2δ receptors, suggesting that it did not alter conductances other than those generated by GABA, and its inhibitory effect increased with increasing concentrations of GABA (Shen et al., 2007), suggesting that it might attenuate current by increasing receptor desensitization. In fact, when tested directly using rapid agonist application, 3α,5[α]β-THP produced a significantly faster rate of desensitization and increased the extent of desensitization from 8 to 87%, when Cl^- flux was inward (Fig. 1; Shen et al., 2007). In contrast, 3α,5[α]β-THP did not increase desensitization with outward Cl^- flux, but, in fact, increased the peak current, suggesting that it increased receptor efficacy, as has been reported previously (Bianchi and Macdonald, 2003). Previous studies have suggested that steroids such as THDOC increase desen-

sitization of homologous α6β2δ GABAR (Bianchi et al., 2002; Haas and Macdonald, 1999), which exhibits a faster desensitization when Cl^- flux is inward (Bianchi et al., 2002). In contrast, α5-containing GABAR, which typically constitute the majority of extrasynaptic GABAR in CA1 hippocampus, desensitize more quickly with outward Cl^- flux (Burgard et al., 1996).

9. Mutagenesis studies

In order to determine a potential molecular mechanism for this polarity-dependent inhibition of α4β2δ GABAR by 3α,5[α]β-THP, it was first necessary to compare sequence homologies of α1 and α4, as α1β2δ GABARs are not sensitive to 3α,5[α]β-THP inhibition as are α4β2δ GABARs. The least homologous region of these subunits is the intracellular TM3—TM4 loop (Fig. 2), which is twice as long in α4, and possesses only a 10% sequence homology with the intracellular TM3—TM4 loop in α1 (Shen et al., 2007). In addition there are 16 unique positively charged residues in the α4 loop which are not present in α1, the most likely target for an effect dependent upon the negatively charged Cl^- ion. Site-directed mutagenesis of these individual residues revealed that arginine 353 in the loop, when mutated to a neutral glutamine, prevented the 3α,5[α]β-THP inhibition of α4[R353Q]β2δ GABAR (Fig. 2). This mutation did not change

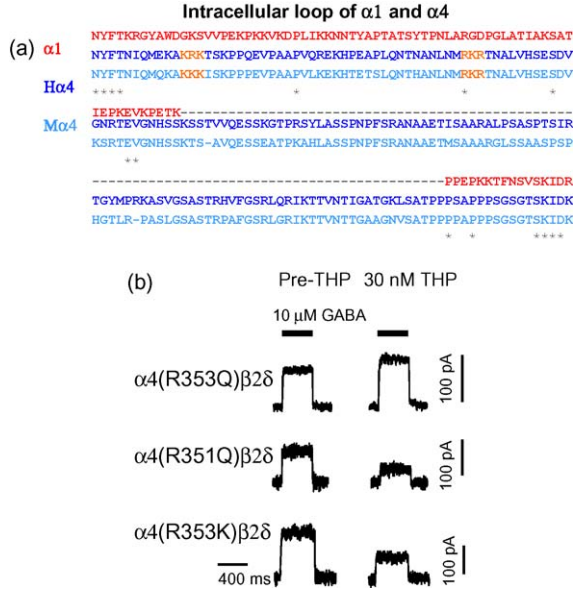

Figure 2 Arginine 353 in the α4 subunit is necessary for the direction-sensitive inhibition of α4β2δ $GABA_A$ receptors by 3α,5[α]β-THP. (a) Alignment of the intracellular loop of α1 and α4 (H, human; M, mouse) subunits reveals limited identity (<10%). *, identical residues for all three. (The sequences for human and mouse α1 are identical.) Orange, residues to be mutated. (b) Representative traces showing the effect of 30 nM 3α,5[α]β-THP on GABA (10 μM)-gated current at the indicated mutated α4β2δ $GABA_A$ receptors. Basic arginine (R351 or R353) residues in the α4 subunit were mutated to a neutral glutamine (Q) and/or a basic lysine (K). Reprinted from Fig. 2, Nat. Neurosci. 10, 469—477 (2007). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the concentration—response curve or current—voltage relationship, suggesting that it did not alter other conductances. In contrast, mutation of a nearby arginine (351) in α4 did not prevent 3α,5[α]β-THP inhibition of α4[R351Q]β2δ, suggesting that arginine 353 alone may be a putative Cl^- modulatory site.

10. Modulatory effects of ions

Modulatory effects of Cl^- have been noted before (Lo and Snyder, 1983; Olsen and Snowman, 1982), which are necessary for barbiturate and benzodiazepine enhancement of GABA binding. Recent studies suggest that ion sensor sites can regulate other events such as Cl^- activation of HCN subunits which mediate I_h (Chen et al., 2000). In addition, the recent discovery (Ramsey et al., 2006) of a cation-triggered phosphorylation event in a novel membrane protein lacking an ion pore suggests that ion sensor sites regulate neuronal function beyond ion conductance. In addition, the intracellular loop of the Cys-loop family of receptors is ion accessible (Kelley et al., 2003; Miyazawa et al., 2003), while for other membrane receptors this loop functions not only as a permeation pathway, but also as a site necessary for rapid desensitization (Turner and Raymond, 2005).

11. Tonic current

Because α4β2δ GABAR are increased at the onset of puberty, 3α,5[α]β-THP effects were tested on the tonic GABAergic current at this time. Initially, the polarity of the GABAergic current was verified using tight seal cell-attached recording (Perkins, 2006), which reflected an outward current (inward Cl^- flux) (Shen et al., 2007). Then, 3α,5[α]β-THP effects on the tonic current were tested using gramicidin perforated patch recording techniques to maintain the internal Cl^- milieu. Recordings were carried out in the presence of TTX, kynurenic acid and 1 μM GABA to isolate the post-synaptic GABAergic response. Under these conditions, application of 30 nM 3α,5[α]β-THP reduced the GABAergic current in slices from pubertal mice (Shen et al., 2007), but was without effect in slices from pre-pubertal animals. This effect of the steroid reversed when recordings were carried out in whole cell mode under conditions of outward Cl^- flux, suggesting a polarity-dependent effect. In contrast, 3α,5[α]β-THP had no effect on the tonic current recorded from δ knock-out mice recorded at puberty, consistent with an effect at α4β2δ GABAR. In addition, 3α,5[α]β-THP did not alter the reversal potential, suggesting that it was targeting GABA-gated current alone. In contrast to its effect on the tonic current, 3α,5[α]β-THP had no effect on synaptic current recorded at the onset of puberty, where sIPSC amplitude, frequency and τ were unchanged by steroid treatment.

12. 3α,5[α]β-THP effects on neuronal excitability

The functional impact of increased extrasynaptic GABARs along the dendrites at puberty was to reduce the input resistance of the neuron, assessed as the current response to a 10 mV step (Shen et al., 2007). By decreasing this tonic current, 3α,5[α]β-THP increased the input resistance of the neurons. Thus, 3α,5[α]β-THP would make the neuron more

responsive to depolarizing current at puberty. This was tested directly by assessing the threshold for evoking an action potential in response to depolarizing current, recorded with whole cell current clamp techniques from CA1 hippocampal pyramidal cells in the slice (Shen et al., 2007). In fact, 3α,5[α]β-THP reduced the threshold for triggering spiking and increased the number of spikes, consistent with its effect to reduce tonic inhibition at puberty (Fig. 3). This is in contrast to its effect before puberty, when it reduced spiking, and in the δ knock-out mouse where it had no effect, suggesting that the emergence of α4β2δ GABAR at puberty reverses the effect of this steroid to an excitatory role.

13. 3α,5[α]β-THP effects on anxiety

The reversal in 3α,5[α]β-THP effects at puberty was also reflected behaviorally, where 3α,5[α]β-THP increased anxiety in pubertal female mice (Shen et al., 2007), in contrast to its typical anxiety-reducing effect. Anxiety was tested using the elevated plus maze, a device elevated 3 ft above the floor, which assesses the preference of a rodent for the open versus the closed arms of the maze. A decrease in time spent in the open arm of the maze reflects an increase in anxiety (Pellow et al., 1995). In fact, administration of 10 mg/kg 3α,5[α]β-THP, i.p., significantly reduced the open arm time at puberty, reflecting an increase in anxiety, in contrast to its

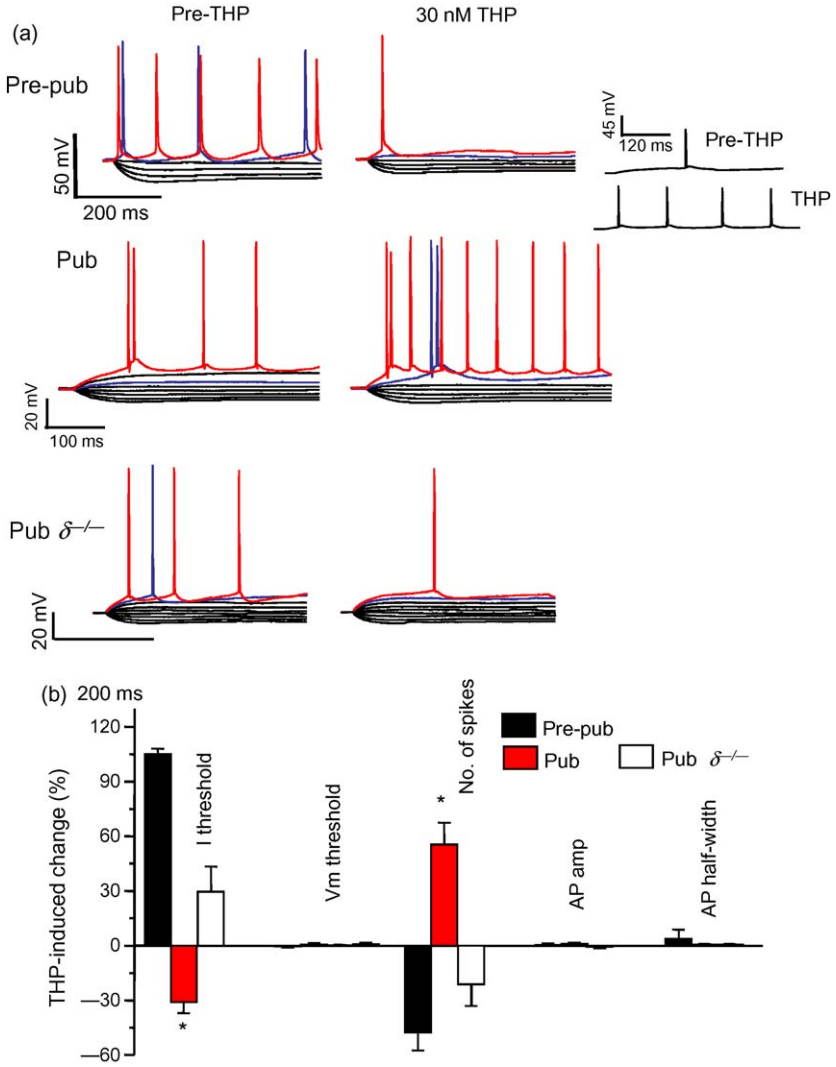

Figure 3 3α,5[α]β-THP lowers the current threshold for spiking of pyramidal cells at the onset of puberty. (a) Whole cell current clamp recordings conducted from CA1 hippocampal pyramidal cells. Voltage responses recorded in response to increasing 0.3 nA current injection (−1 nA, initial current) for slices recorded before puberty (Pre-pub), or at puberty in wild-type (Pub) or $\delta^{-/-}$ (Pub. $\delta^{-/-}$) mice. (The 3α,5[α]β-THP trace lacks the 800 pA current trace for ease of comparison.) Inset, spiking at threshold, 800 pA, pre-3α,5[α]β-THP; 500 nA, 3α,5[α]β-THP in a non-spiking pubertal cell. Red trace, equivalent current injection, threshold for the less excitable state. Blue trace, equivalent current injection, threshold for the more excitable state. (b) Mean ± SEM averaged from 7 to 8 cells for each group. Current threshold to spiking, *I threshold*; voltage threshold to spiking, *Vm threshold*; spike frequency, *No. of spikes*; action potential amplitude, *AP amp*; action potential half-width, *AP half-width*. Spike frequency was assessed at the minimum current required to produce spiking in both pre- and post-3α,5[α]β-THP traces. *$P < 0.05$ versus Pre-pub. Reprinted from Fig. 6, Nat. Neurosci. 10, 469–477 (2007). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

effect in pre-pubertal mice, where it significantly increased open arm time, reflecting a decrease in anxiety, as has been shown previously in adult rodents (Akwa et al., 1999; Bitran et al., 1993, 1999; Frye et al., 2009).

14. Stress effects at puberty

3α,5[α]β-THP is not only a metabolite of the ovarian/adrenal hormone progesterone, but is also released in response to stress (Purdy et al., 1991; Barbaccia et al., 1996; Girdler et al., 2006; Drooglever Fortuyn et al., 2004). 45 min of restraint stress produce maximal CNS levels of this steroid in rodents (Higashi et al., 2005; Mukai et al., 2008). When tested 20 min after this stress paradigm, pubertal mice decreased their time in the open arm of the maze (Shen et al., 2007), reflecting an increase in anxiety, again, in contrast to the pre-pubertal, as well as adult, female mice. Stress is a complex behavioral state, with multiple sequelae, including rapid increases in sympathetic tone (Goldstein and Kopin, 2008), with ensuing increases in cardiovascular and pulmonary function. Corticosterone is also released by stress and has multiple effects (Goldstein and Kopin, 2008), which include long-term changes in hippocampal neuronal function (McEwen, 2005). Therefore, in order to assess the role of 3α,5[α]β-THP in producing anxiety in pubertal mice, we pre-administered finasteride, a 5α-reductase inhibitor, to reduce stress-induced increases in 3α,5[α]β-THP levels. This enzyme inhibitor has been shown to effectively reduce 3α,5[α]β-THP levels produced by stress to almost undetectable (Mukai et al., 2008), as a dose-dependent effect. In fact, pre-administration of finasteride prevented the effect of stress on this behavioral outcome, in both pre- and pubertal mice (Shen et al., 2007). In addition, pre-administration of the inactive 3β-OH-THP isomer (Lundgren et al., 2003) prevented the anxiety-producing effect of 3α,5[α]β-THP, suggesting that alterations in anxiety level produced by sustained exposure to a behavioral stressor are mediated by 3α,5α-THP release. These results suggest that increases in 3α,5α-THP levels produced by stress increase anxiety at puberty, thus amplifying the stress response. In contrast, this steroid reduces anxiety in adults, consistent with results demonstrating 3α,5[α]β-THP-induced reductions in corticosterone levels in adult rodents (Guo et al., 1995).

Because the inhibitory effect of 3α,5[α]β-THP was selective for α4β2δ GABAR, we examined effects of restraint stress in mice lacking expression of the δ GABAR subunit. In fact, restraint stress did not alter anxiety level in δ knock-out mice at puberty (Shen et al., 2007), implicating δ-containing GABAR as the target for this paradoxical effect of the steroid. Taken together, these results suggest that the increase in expression of α4βδ GABAR at puberty reverses the effect of a stress steroid such that it reduces inhibition, thereby increasing anxiety. These results may be important for the increase in anxiety and mood swings reported at puberty.

15. Steroid withdrawal and δ-containing GABAR

In addition to puberty, hormonal fluctuations, either endogenous or exogenously administered, have also been shown to increase expression of the δ subunit in CNS areas (Lovick et al., 2005; Maguire and Mody, 2007; Sundstrom-Poromaa et al., 2002) such as CA1 hippocampus, dentate gyrus and the periaqueductal gray, although the effective timecourse varies in a regional and species-dependent manner. Short-term exposure to neurosteroids (Maguire and Mody, 2007; Shen et al., 2005), such as 3α,5[α]β-THP or THDOC, from 30 min to 48 h, increases expression of the δ subunit, in some cases in association with increased expression of the α4 subunit. These increases in δ expression were also associated with an increased responsiveness of the tonic inhibitory current to low concentrations of the GABA agonist gaboxadol, pharmacologically consistent with increased expression of functional δ-containing GABAR (Brown et al., 2002). In contrast, withdrawal from elevated circulating levels of 3α,5[α]β-THP also increase expression of δ subunit, in some cases, along with increased expression of the α4 subunit, in CA1 hippocampus (Shen et al., 2007), the periaqueductal gray (Griffiths and Lovick, 2005) and the dentate gyrus following pregnancy-induced elevations in steroids such as 3α,5[α]β-THP (Maguire and Mody, 2009).

16. Steroid withdrawal

Steroid withdrawal in rats is produced by cessation of chronic exogenous treatment (Smith et al., 1998a). However, steroid withdrawal in mice is accomplished using a 5α-reductase blocker (Smith et al., 2006) to block the normally high levels of this steroid. Mice exhibit circadian fluctuations in circulating levels of 3α,5α-THP (Corpechot et al., 1997), as recently also described in humans at puberty (McCartney et al., 2007), with levels which peak at high 30 nM concentrations in the early hours of the active phase (dark for rodents, light for humans). (Unlike humans, mice only produce 3α,5α-THP but not 3α,5β-THP.) Therefore, a withdrawal state can easily be induced in mice using a 5α-reductase blocker such as finasteride to prevent formation of 3α,5α-THP during its nocturnal surge (Smith et al., 2006).

17. 3α,5α-THP withdrawal

In fact, 3α,5α-THP withdrawal undertaken in pre-pubertal female mice produced increases in sensitivity of the tonic inhibitory current to application of gaboxadol (Shen et al., 2007) and inhibition by the trivalent cation lanthanum, results pharmacologically consistent with increased expression of α4βδ GABAR (Brown et al., 2002; Saxena et al., 1997), similar to findings at puberty (Shen et al., 2007). 3α,5[α]β-THP administration also reduced the tonic current, and increased spiking assessed in cell-attached mode following 3α,5α-THP withdrawal, in a manner similar to its effect at puberty (Shen et al., 2007). In addition, 3α,5α-THP withdrawal resulted in a state where acute in vivo administration of 3α,5[α]β-THP triggered anxiety, also similar to its effect at puberty (Shen et al., 2007).

18. Replacement 3α,5[α]β-THP and puberty

Interestingly, puberty was also associated with a decline ("withdrawal") in 3α,5α-THP (Shen et al., 2007) of similar magnitude to that seen with finasteride injection (Palumbo et al., 1995; Smith et al., 2006), well-correlated with the decline in progesterone which has been reported (Kahn et al., 2008). Thus, the increase in α4βδ GABAR expression

may represent a compensatory CNS mechanism to normalize the level inhibition in response to this decline in a GABA-enhancing steroid.

In order to test whether the decline in 3α,5α-THP at the onset of puberty triggered the increase in α4 and δ expression, we administered replacement 3α,5[α]β-THP to pubertal mice across a 48 h period. This treatment normalized α4 and δ expression to control levels and reduced the response of the tonic current to gaboxadol (Shen et al., 2007), suggesting that expression of functional α4βδ GABAR was reduced to control levels. By normalizing the GABAR population, replacement 3α,5[α]β-THP restored the inhibitory effect of the steroid, which reduced spontaneous spiking of CA1 pyramidal cells. This normalizing effect of replacement 3α,5[α]β-THP was also evidenced behaviorally (Shen et al., 2007), where restraint stress resulted in decreased anxiety, similar to its effect in control pre-pubertal female mice. Thus, these findings suggest that the increase in α4βδ GABAR at puberty is a direct result of the declining levels of 3α,5α-THP seen at this time.

19. Premenstrual dysphoric disorder

The 3α,5α-THP withdrawal state in mice may also be relevant as a rodent model for premenstrual dysphoric disorder (PMDD), another hormonal transition state associated with mood disturbances, especially irritability, anxiety and depression (Endicott et al., 1999; Halbreich et al., 2007; Yonkers, 1997). In women with PMDD, dysphoric mood follows a period of declining levels of progesterone and 3α,5[α]β-THP (the follicular phase), but can frequently begin sometime after the onset of the luteal phase, when progesterone and 3α,5[α]β-THP levels are increasing. Recent studies (Freeman et al., 2002; Schmidt et al., 1998) have suggested that women with PMDD may have an atypical response to progestins, such as 3α,5[α]β-THP. Similarly, female mice undergoing 3α,5[α]β-THP withdrawal respond in an atypical manner to acute application of 3α,5[α]β-THP (Smith et al., 2006), where it increases anxiety in the elevated plus maze, when preceded by a brief electrical shock. In contrast, 3α,5[α]β-THP decreases anxiety in female mice not undergoing steroid withdrawal (Akwa et al., 1999; Bitran et al., 1993, 1999; Frye et al., 2009; Smith et al., 2006). However, a recent study (Kask et al., 2009) has suggested that 3α,5α-THP does not produce different effects in women with PMDD, suggesting that other factors play a role in this disorder. Alternatively, the effect of 3α,5α-THP may vary across the luteal phase when progestin levels are fluctuating, or as suggested in the rodent model (Smith et al., 2006), may only be seen when adverse mood is triggered by an external event.

20. Behavioral pharmacology and α4βδ GABAR

Because the increased expression of α4βδ GABAR following 3α,5α-THP withdrawal would alter GABAR pharmacology (Brown et al., 2002), the behavioral effects of a variety of drugs which are active at GABAR were tested for their effect in altering behavior on the plus maze. α4βδ GABAR are insensitive to modulation by conventional benzodiazepine agonists (such as lorazepam) (Brown et al., 2002), but have greater sensitivity to the GABA agonist gaboxadol (THIP) (Brown et al., 2002) and are inhibited by low concentrations of the benzodiazepine antagonist flumazenil (Dunn et al., 2003). In fact, these drugs differentially altered open arm time in controls and 3α,5α-THP withdraw mice in a manner consistent with upregulation of α4βδ GABAR after steroid withdrawal; that is, following 3α,5α-THP withdrawal, lorazepam was ineffective in increasing open arm time (Smith et al., 2006), which is its typical effect in control animals. In addition, gaboxadol produced a greater increase in open arm time after 3α,5α-THP withdrawal than in controls, while a low dose of flumazenil (2 mg/kg) decreased open arm time, suggesting that it increased anxiety after 3α,5α-THP withdrawal, but was ineffective in vehicle-injected controls. These pharmacological findings are in fact also consistent with clinical reports of women with PMDD, who exhibit a relative benzodiazepine insensitivity (Sundstrom et al., 1997), assessed by mood ratings as well as by eye saccade velocity, an objective measure of GABAergic tone. In addition, flumazenil precipitates panic attacks in women with PMDD (Le Melledo et al., 2000), but not in normal control subjects. Taken together, these findings suggest that 3α,5α-THP withdrawal in the female mouse may represent a useful model of PMDD.

21. Progesterone withdrawal and the periaqueductal gray

Recent studies in the rat have demonstrated that withdrawal from progesterone, as well as naturally occurring hormonal fluctuations across the ovarian cycle increase α4 and δ subunit expression in the periaqueductal gray, an area with a direct role in mediating the panic response (Lovick, 2000). The activity of the output neurons in this area is under the control of GABAergic inhibition. Recent studies (Lovick et al., 2005) have demonstrated that fluctuations in endogenous steroids across the estrous cycle alter the expression of GABAR subunit combinations in this region: α4, β1 and δ subunit expression was increased on PAG interneurons on the day of late diestrus. This stage of the cycle follows the secondary peak in progesterone and 3α,5α-THP which occurs on early diestrus, and could thus be considered a time of 3α,5α-THP ''withdrawal''. In fact, the authors have a second study demonstrating similar increases in α4, β1 and δ GABAR subunit expression following withdrawal from administered progesterone (Griffiths and Lovick, 2005). Because this increase in GABAR expression is on the inhibitory interneurons, it would effectively ''disinhibit'' the output neurons, as has recently been shown. In this case, both application of bicuculline as well as the panicogenic cholecystokinin ligand pentagastrin increased PAG neuronal activity to a greater extent on estrus and late diestrus (Brack and Lovick, 2006), suggesting a reduced GABAergic tone on the output neurons on these 2 days of 3α,5α-THP ''withdrawal'' across the estrous cycle. Activation of these PAG output neurons has been shown to elicit a panic reaction in both humans and rodents (Lovick, 2000), because it is part of an afferent pathway to brainstem respiratory neurons that control the rate and amplitude of respiratory signals (Voituron et al., 2005). These findings have important clinical significance as well

because many women with panic disorder exhibit premenstrual exacerbation during the decline in progesterone at the end of the luteal phase (Yonkers, 1997) (i.e., a 3α,5[α]β-THP "withdrawal" state).

22. Steroid withdrawal and α4βγ2 GABAR

In addition to increasing α4βδ GABAR expression, in some cases steroid withdrawal can also increase expression of α4βγ2 GABAR subtypes (Follesa et al., 2001; Smith et al., 1998a), including pregnancy and pseudopregnancy in rats (Sanna et al., 2009; Smith et al., 1998b). GABARs containing γ2 instead of δ exhibit a unique pharmacological profile (Wafford et al., 1996). Although both α4βδ and α4βγ2 GABAR are insensitive to modulation by benzodiazepine agonists, current gated by α4βγ2 GABAR is increased by the benzodiazepine antagonist flumazenil and the partial inverse agonist RO15-4513 (Wafford et al., 1996). In contrast, current gated by α4βδ GABAR is decreased by flumazenil (Dunn et al., 2003). Withdrawal from 3α,5α-THP following chronic administration of exogenous progesterone or 3α,5[α]β-THP to female rats increases α4βγ2 GABAR, as evidenced by these characteristic changes in GABAR pharmacology, recorded from CA1 hippocampal pyramidal cells (Smith et al., 1998a). Withdrawal from 3α,5α-THP could be produced by selective administration of a 5α-reductase blocker in the presence of high levels of progesterone (Smith et al., 1998b), confirming that 3α,5α-THP was the active component, even under conditions of high ovarian production in a pseudopregnancy model. Administration of other GABA-modulatory drugs, such as benzodiazepines and ethanol, increase expression of α4βγ2 GABAR, either after chronic administration or following withdrawal from the drug (Devaud et al., 1997; Follesa et al., 2002; Holt et al., 1996, 1997; Liang et al., 2004; Mahmoudi et al., 1997; Sanna et al., 2003), suggesting it may be an outcome of prolonged exposure to positive GABA-modulators, in general. It is not clear, however, why steroid withdrawal may lead to either increased expression of α4βδ or α4βγ2, although the background steroid level may play a role.

23. Receptor kinetics

The functional consequence of increased expression of α4βγ2 GABAR would be to result in synaptic receptors with a faster deactivation time constant (Smith and Gong, 2005), yielding less inhibitory current. In fact, recombinant α4β2γ2 GABAR deactivate more quickly than either α1β2γ2, α3β2γ2 or α5β2γ2 (Gingrich et al., 1995; Lavole et al., 1997; Smith and Gong, 2005), as do synaptic currents reflecting α4βγ2 GABAR (Hsu et al., 2003; Cagetti et al., 2003; Chandra et al., 2006). Unlike tonic current, faster synaptic current would decrease feedback inhibition (Hsu and Smith, 2003), resulting in hyperexcitability and increasing pharmacologically induced seizure susceptibility (Smith et al., 1998a). This decrease in feedback inhibition and increased seizure susceptibility was indeed observed after 3α,5α-THP withdrawal in female rats, an outcome reversed by suppression of α4 expression using intraventricular administration of antisense oligonucleotides (Hsu and Smith, 2003; Smith et al., 1998a).

24. Corticosterone, stress and puberty

In addition to 3α,5[α]β-THP, stress increases release of corticosteroids (Goldstein and Kopin, 2008), which constitute an important component of the stress reaction. Initially, activation of the HPA (hypothalamo-pituitary-adrenal) axis increases CNS release of CRH (corticotrophin releasing hormone), which has been shown to increase anxiety (Holsboer and Ising, 2008; Sahuque et al., 2006), although some reports suggest that this effect can be mediated by effects via the GABAergic system (Waselus et al., 2005). In addition, CRH acts to increase corticosterone release. Globally, corticosterone acts to increase circulating levels of glucose as fuel for the stress reaction (Goldstein and Kopin, 2008). However, corticosterone also affects hippocampal excitability, in both a concentration- and time-dependent manner, where fast effects are excitatory and longer-term effects are inhibitory (Birnsteil et al., 1995; Olijslagers et al., 2008; Rey et al., 1987). Effects of corticosterone depend on the area of hippocampus as well as the receptor subtype which is activated: in ventral hippocampus, activation of mineralocorticoid receptors decreases the frequency of sIPSCs, while activation of glucocorticoid receptors increases the amplitude of sIPSCs (Maggio and Segal, 2009). In dorsal hippocampus, however, corticosteroid acts only via the glucocorticoid receptors to increase sIPSC amplitude (Maggio and Segal, 2009). Corticosterone also affects both the amplitude and expression of L-type calcium channels in hippocampus (Chameau et al., 2007). In addition, long-term stress effects mediated via corticosterone produce genomic changes, such as alterations in kainate receptor mRNA in hippocampus (Hunter et al., 2009).

In addition to these complex effects of corticosterone mediated via selective receptor sub-types, corticosterone is also converted to THDOC, another positive modulator of the GABAR, which has been shown to be increased by stress (Barbaccia et al., 1996). Thus, the effects of this steroid are complex and involve both excitatory and inhibitory levels of control. Recent studies have demonstrated a greater corticosterone response to stress in pre-pubertal rats of both sexes than adults (Romeo et al., 2006, 2004), suggesting that this hormonal response also changes at puberty.

25. Conclusions

In conclusion, the onset of puberty is associated with a striking upregulation of α4βδ GABARs along the apical dendrites of CA1 hippocampal pyramidal cells from almost undetectable levels before puberty. In areas with normally high expression of these receptors, dentate gyrus and cortical pyramidal cells, Cl^- flux is outward, where 3α,5[α]β-THP would increase inhibition. However, α4β2δ GABAR are inhibited by 3α,5[α]β-THP under conditions of inward Cl^- flux, as would occur in mature hippocampus. Thus, this steroid decreases tonic inhibitory current at puberty, leading to increased neuronal excitability at the circuit level and increasing anxiety at the behavioral level. Because circulating levels of 3α,5[α]β-THP are increased by stress, these findings may be relevant for mood swings and the increased response to stress reported at the onset of puberty. Anxiety-producing effects of 3α,5[α]β-THP mediated by α4βδ GABAR may also be relevant for other

hormonally-related changes in mood, such as PMDD, because they are triggered by fluctuating levels of endogenous neuroactive steroids.

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Conflict of interest statement

None declared.

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