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Characterization of neurosteroid effects on hyperpolarizing current at $\alpha 4\beta 2\delta$ GABA_A receptors

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Abstract

Rationale—The neurosteroid 3 α ,5 β -THP (3 α -OH-5 β -pregnan-20-one, pregnanolone) is a modulator of the GABA_A receptor (GABAR), with $\alpha 4\beta 2\delta$ GABARs the most sensitive. However, the effects of 3 α ,5 β -THP at $\alpha 4\beta 2\delta$ are polarity-dependent: 3 α ,5 β -THP potentiates depolarizing current, as has been widely reported, but decreases hyperpolarizing current by accelerating desensitization.

Objectives—The present study further characterized 3 α ,5 β -THP inhibition of hyperpolarizing current at this receptor and compared effects of other related steroids at $\alpha 4\beta 2\delta$ GABARs.

Methods— $\alpha 4\beta 2\delta$ GABARs were expressed in HEK-293 cells, and agonist-gated current recorded with whole cell voltage-clamp techniques using a theta tube to rapidly apply agonist before and after application of neurosteroids.

Results—The GABA-modulatory steroids (30 nM) 3 α ,5 α -THP (3 α -OH-5 α -pregnan-20-one, allopregnanolone) and THDOC (3 α ,21-dihydroxy-5 α -pregnan-20-one) inhibited hyperpolarizing GABA(10 μ M)-gated current at $\alpha 4\beta 2\delta$ GABARs similar to 3 α ,5 β -THP, while the inactive 3 β ,5 β -THP isomer had no effect. Greater inhibition was seen for current gated by the high efficacy agonist gaboxadol (THIP, 100 μ M) than for GABA (0.1 – 1000 μ M), consistent with an effect of 3 α ,5 β -THP on desensitization. Inhibitory effects of the steroid were not seen under low [Cl[−]] conditions or in the presence of calphostin C (500 nM), an inhibitor of protein kinase C. Chimeras swapping the IL (intracellular loop) of $\alpha 4$ with $\alpha 1$, when expressed with $\beta 2$ and δ , produced receptors (α [414] $\beta 2\delta$) which were not inhibited by 3 α ,5 β -THP when GABA-gated current was hyperpolarizing, while α [141] $\beta 2\delta$ exhibited steroid-induced polarity-dependent modulation.

Conclusions—These findings suggest that numerous neurosteroids exhibit polarity-dependent effects at $\alpha 4\beta 2\delta$ GABARs which are dependent upon protein kinase C and the IL of $\alpha 4$.

Keywords

pregnanolone; allopregnanolone; THDOC; $\alpha 4$; δ ; GABA_A receptor; intracellular loop; gaboxadol; protein kinase C

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Introduction

The steroid 3 α -OH-5 α pregnan-20-one (3 α ,5 α -THP or allopregnanolone) is a metabolite of the ovarian steroid progesterone (P) (Compagnone and Mellon 2000) synthesized through enzymatic conversions by 5 α -reductase and 3 α -hydroxysteroid oxidoreductase. Circulating levels of this steroid parallel those of P, increasing during the luteal phase of the menstrual cycle (Havlikova et al. 2006), on the afternoon of proestrus and diestrus1 of the estrous cycle (Corpechot et al. 1997; Palumbo et al. 1995) and during pregnancy (Concas et al. 1998; Luisi et al. 2000) as well as before puberty (Shen et al., 2007). However, 3 α ,5 α -THP is also released by certain types of sustained stress (Barbaccia et al. 1996; Girdler et al. 2001; Mukai et al. 2008; Purdy et al. 1991), when it can also be formed in the brain and is thus considered a neurosteroid (Purdy et al. 1991). The enzymatic requirements to synthesize this steroid from cholesterol are in place in many principal neurons in a variety of CNS sites, including the pyramidal cells of the CA1 hippocampus (Agis-Balboa et al. 2006).

Unlike most steroids, 3 α ,5 α -THP does not act via nuclear receptors to produce genomic effects. Instead, this steroid is best known as a positive modulator of the GABA_A receptor (GABAR) (Majewska et al. 1986). This receptor mediates most inhibition in the brain and is a pentameric membrane protein typically comprised of 2 α , 2 β and 1 γ subunit (Chang et al. 1990) which generates a chloride conductance. However, many subunit combinations exist from a pool of 6 α , 3 β , 3 γ as well as δ , ϵ , ρ and subunits (Olsen and Sieghart 2009), yielding receptors with distinct biophysical and pharmacological properties. 3 α ,5 α -THP acts at a transmembrane binding site on α 1 β 2 γ 2L distinct from other GABA modulators (Hosie et al. 2006) which can be independent of subunit (Bracamontes et al. 2012). The steroid increases the mean open time of the receptor while decreasing the mean closed time duration (Akk et al. 2010).

The α 4 β δ GABAR is considered the most sensitive target for neurosteroids such as 3 α ,5 α -THP, its active isomer 3 α ,5 β -THP (3 α -OH-5 β -pregnan-20-one or pregnanolone) and THDOC ((3 α ,5 β)-3,21-dihydroxypregnan-20-one) (Belelli et al. 2002; Bianchi and Macdonald 2003; Brown et al. 2002; Wohlfarth et al. 2002) as is the α 1 β 2 δ GABAR (Zheleznova et al. 2008). Unlike most GABARs, δ -GABARs are extrasynaptic (Wei et al. 2003), localized to both the soma and dendrites of neurons (Shen et al. 2010; Wei et al. 2003), and generate a tonic, inhibitory current (Stell and Mody 2002) because they have a high sensitivity (EC₅₀ ~ 0.5 μ M GABA) to low concentrations of ambient GABA (Brown et al. 2002) found far from the GABA synapse (~1 μ M) (Song et al. 2011). GABA is a partial agonist at δ -GABARs (Bianchi and Macdonald 2003), where single channel studies have identified two open states of the channel (Wohlfarth et al. 2002). Addition of THDOC increases receptor efficacy (Zheleznova et al. 2008), increasing the maximal GABA-gated current and adding an additional, longer open state of the channel (Wohlfarth et al. 2002). A number of full agonists of δ -GABARs have been identified, including gaboxadol (THIP) (Brown et al. 2002; Meera et al. 2011), a synthetic GABA agonist, as well as endogenous agonists β -alanine (Bianchi and Macdonald 2003) and taurine (Jia et al. 2008). THDOC modulation of current gated by maximal concentrations of β -alanine are reduced compared to its modulation of GABA-gated current, while desensitization is increased, suggesting that

the efficacy state of the receptor plays a role in neurosteroid modulation of δ -GABARs (Bianchi and Macdonald 2003).

Recent studies also suggest that the effect of $3\alpha,5\alpha$ -THP in modulating current gated by $\alpha4\beta2\delta$ GABARs is also dependent upon the polarity of the current. Many studies have shown that neurosteroids such as the THP isomers and THDOC increase inward current (outward Cl^- -flux or depolarizing current) in recombinant receptors (Belelli et al. 2002; Brown et al. 2002; Wohlfarth et al. 2002). In dentate gyrus granule cells, $\alpha4\beta2\delta$ GABARs generate a shunting inhibition which underlies a tonic current shown to be increased by THDOC (Stell et al. 2003). Neurosteroid-induced increases in this tonic inhibition reduces neuronal excitability in response to electrical stimulation (Stell et al. 2003).

However, $3\alpha,5\alpha$ -THP was recently shown to reduce outward (hyperpolarizing) current gated by $\alpha4\beta2\delta$ GABARs by accelerating receptor desensitization (Shen et al. 2007). Consistent with this finding, earlier studies reported that desensitization of δ -containing GABARs is polarity-dependent, with faster and a greater degree of desensitization observed for hyperpolarizing current versus depolarizing current (Bianchi et al. 2002), while steroids are reported to increase desensitization of recombinant δ -containing GABARs (Bianchi et al. 2002) and native GABARs in regions with high δ expression (Zhu and Vicini 1997). The polarity-dependent effect of $3\alpha,5\beta$ -THP requires a positively charged residue, arginine 353, in the transmembrane3 (TM3)-TM4 intracellular loop of the receptor (Shen et al. 2007), suggesting that it may serve as a putative Cl^- modulatory site (Shen et al. 2007). Although mutation of this site to a neutral glutamine prevents the inhibitory effect of $3\alpha,5\beta$ -THP on current gated by $\alpha4\beta2\delta$ GABARs, it does not prevent potentiation of inward currents, suggesting that other residues may be involved (Shen et al. 2007).

This study addresses some of the issues which still remain, first by examining the role of the TM3-TM4 intracellular loop in producing the inhibitory effect of $3\alpha,5\alpha$ -THP on current gated by $\alpha4\beta2\delta$ GABARs transiently expressed in HEK-293 cells. Because temperature has been shown to influence the rate and degree of receptor desensitization (Bright et al. 2011), we also compare the temperature dependence of changes in steroid-induced current. In addition, receptor efficacy is also considered by examining effects of $3\alpha,5\beta$ -THP on current gated by gaboxadol (THIP, 4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol), a high efficacy agonist at $\alpha4\beta2\delta$ GABARs (Brown et al. 2002; Meera et al. 2011). Finally, the effect of similar steroids were examined to determine the structural specificity of polarity-dependent effects of steroids at $\alpha4\beta2\delta$ GABARs.

Materials and Methods

HEK-293 Cells

Human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA) were grown in Dulbecco's Modified Eagle's Medium (DMEM/F-12, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) in 35 mm cell culture dishes at 37°C in a humidified incubation chamber (5% CO_2 , 95% O_2).

cDNA

cDNA for GABAR subunits mouse $\alpha 4$ (N.L. Harrison, Columbia U., New York), rat $\beta 2$ (J. Bracamontes, Washington U, St. Louis) and human $\alpha 4$ and δ (P. Whiting, Merck, Sharp and Dohme, UK) were used for all studies. (Mouse, rat and human cDNA sequences for $\beta 2$ are nearly identical.) Results using mouse and human $\alpha 4$ were identical and have been pooled. The expression vector was pcDNA3.1.

Chimeric receptors

For the generation of $\alpha[141]$ and $\alpha[414]$ chimeric subunits (Fig. 1), chimeric $\alpha 1\alpha 4$ and $\alpha 4\alpha 1$ plasmids were first constructed in pcDNA3.1 vectors using polymerase chain reaction (PCR) technology (Genewiz, South Plainsfield, NJ). This generated two chimeras beginning at the start of the TM3-TM4 IL for each of the respective subunits. Then site directed mutagenesis (QuikChange II Site-Directed Mutagenesis kit, Agilent Technologies, Santa Clara, CA) (Shen et al. 2007) was used to introduce a unique ClaI restriction site at the 5' terminus of TM4. For $\alpha 1$, this involved two series of mutations using primers (forward, F; reverse, R): (1) F, 5' - GGA TCT GGC ACA AGT AAA ATC GAC AAA TAT GCC CGT ATT CTC - 3'; R, 5' - GAG AAT ACG GGC ATA TTT GTC GAT TTT ACT TGT GCC AGA TCC - 3'; (2) F, 5' - GGA TCT GGC ACA AGT AAA ATC GAT AAA TAT GCC CGT ATT CTC - 3'; R, 5' - GAG AAT ACG GGC ATA TTT ATC GAT TTT ACT TGT GCC AGA TCC - 3'. The primers for $\alpha 4$ were: F, 5' - GCG TCA GCA AAA TCG ATC GAC TGT CAA GAA TAG C - 3'; R, 5' - GCT ATT CTT GAC AGT CGA TCG ATT TTG CTG ACG C - 3'. Primers were synthesized by Integrated DNA Technologies (San Diego, CA). Chimeras were generated by cutting out the fragment spanning the region from TM4 to the C terminus with restriction enzymes ClaI and SmaI (NEB, Ipswich, MA) followed by gel electrophoresis. DNA fragments were isolated from the gel using a Qiagen purification kit (Qiagen, Valencia, CA), and the [TM4-C terminus] fragments from $\alpha 1$ and $\alpha 4$ ligated to the $\alpha 1\alpha 4$ and $\alpha 4\alpha 1$ restriction digests, respectively (Quick Ligation Kit, NEB, Ipswich, MA) to produce the final chimera products $\alpha[141]$ and $\alpha[414]$ (Fig. 1). Chimera sequencing and all mutations were confirmed by double-stranded sequencing (Genewiz, South Plainsfield, NJ).

Transfection

Cells were transfected with $\alpha 4$, $\alpha[414]$ or $\alpha[141]$ with $\beta 2$ and δ cDNA (1:1:1; $\alpha 4$: $\beta 2$: δ) using the Nucleofector method (Amaxa/Lonza, Walkersville, MD) with reagents and protocols optimal for HEK-293 cells (Kuver et al. 2012). (Transfection of 1:1:0.1 yielded no GABA-gated current when recorded with whole cell patch clamp procedures; therefore, this transfection ratio was not used.) A total of 5 μ g of cDNA was used per 100 μ l of Nucleofector transfection reagent. Cells were additionally co-transfected with 2 μ g eGFP cDNA (Amaxa/Lonza) for detection of transfected cells under fluorescence microscopy. The transfection efficiency was 70–80% and did not vary across treatments.

Electrophysiology

Pharmacological tests—Currents were recorded in response to GABA or gaboxadol at room temperature (21–22°C) or near physiological temperature (30°C) at a holding potential

of -30 mV using whole cell voltage clamp techniques on a Nikon Diaphot inverted microscope (Shen et al. 2007). The bath solution contained (in mM): NaCl 120, CsCl 5, CaCl₂ 2, MgCl₂ 1, HEPES 10 and glucose 25, pH 7.4, 320 mOsmol. Patch pipets (filament-capillary tubes, Sutter Instruments, Novato, CA) were fabricated from borosilicate glass using a Flaming-Brown puller to yield open tip resistances of 3 – 5 M Ω . The pipet solution contained (in mM): N-methyl-D-glucamine chloride (N MG-Cl) 120, Cs₄, BAPTA 5, Mg-ATP 5, and an ATP regeneration system (20 mM Tris phosphocreatine and creatine kinase). In some cases, internal [Cl⁻] was varied, using gluconate as the anion (N MK-Cl 13, K-gluconate 107), to alter the direction of Cl⁻ flow, corrected for the junction potential. Currents were recorded using an Axopatch 1D amplifier (Axon Instruments, Union city, CA) filtered at 2 kHz (four-pole Bessel filter) and detected at 10 kHz (pClamp 8.2). Agonist-gated currents were recorded before and after application of the indicated steroids.

Some cells were recorded in the presence of 1 μ M ZnCl₂ to block current from binary receptors which may have formed (Meera et al. 2011); because current responses were similar for all cells, results were pooled. For the temperature-dependence experiment, agonist delivery was accomplished with a solenoid-controlled micropipette array 50 μ m from the cell to deliver agonist for approximately 400–500 ms exposure times with 200–250 ms onset of application (Smith et al. 1998). For all other experiments, a piezoelectric-controlled (Burleigh Instr., LSS-3100) double-barreled theta tube (Sutter Instruments, 80–100 μ m dia. tip) containing GABA or gaboxadol and bath solution was used to rapidly apply agonist continuously for 5 s to transfected HEK-293 cells to allow for rapid onset and offset of agonist (Smith and Gong 2005). In these cases, steroids were both pre-applied in the bath solution for 30 s and co-applied in the theta tube in order to determine modulatory effects. Solutions were exchanged in the theta tube using a pinch valve system. Analysis of peak current was accomplished with pClamp 10.1 (Axon Instruments, Union City, CA) and Origin (Microcal, Piscataway, NJ) software packages. In all cases, 2–3 current traces were averaged for each agonist or agonist + steroid group.

In experiments to test the effects of low internal [Cl⁻], the intracellular solution contained (in mM), cesium-methanesulfonate (Cs-MeSO₃) 115 and N MG-Cl 5 instead of N MG-Cl 120. The extracellular solution contained Na-methanesulfonate 80 and NaCl 40 instead of NaCl 120.

Drugs

Except where indicated, drugs were obtained from Sigma Chemical Co. (St. Louis, MO). Cs₄-BAPTA was from Calbiochem (San Diego, CA) and the steroids 3 α ,5 α -THP, 3 α ,5 β -THP, 3 β ,5 β -THP and THDOC were from Steraloids, Inc. (Newport, RI).

Statistical analysis

Differences between groups were assessed using the Student's t test or ANOVA followed by the Tukey's post-hoc analysis, for two or multiple groups, respectively. Effects of a steroid on the same cell was tested using the paired t-test. Differences were judged to be significant when $p < 0.05$.

Results

Neurosteroid inhibition of hyperpolarizing current at $\alpha 4\beta 2\delta$ GABARs: Structural specificity

Because previous results suggested that $3\alpha,5\beta$ -THP exhibits polarity-dependent modulatory effects at $\alpha 4\beta 2\delta$ (Shen et al. 2007), other neurosteroids were tested for their effects on hyperpolarizing current at these receptors transiently expressed in HEK-293 cells. The active isomer of $3\alpha,5\beta$ -THP, $3\alpha,5\alpha$ -THP (30 nM), produced similar inhibitory effects, reducing hyperpolarizing GABA(10 μ M)-gated current by a mean 44% (versus 54%, $3\alpha,5\beta$ -THP, Fig 2). In addition, the related neurosteroid THDOC produced nearly identical effects on the hyperpolarizing current gated by $\alpha 4\beta 2\delta$ GABARs, reducing it by a mean 52%. In contrast, the inactive isomer of $3\alpha,5\beta$ -THP, $3\beta,5\beta$ -THP (30–300 nM), did not produce any significant effect on this current.

Agonist and concentration-dependent effects of $3\alpha,5\beta$ -THP at $\alpha 4\beta 2\delta$ GABARs

$3\alpha,5\beta$ -THP-induced modulation of current at $\alpha 4\beta 2\delta$ GABARs was compared across a range of concentrations of GABA (0.1 – 1000 μ M). Greater modulation was seen at higher concentrations of GABA, where the steroid potentiated depolarizing current, with a peak 120% increase at 100–1000 μ M GABA, consistent with its effect to increase receptor efficacy (Bianchi and Macdonald 2003, Fig 3). Similarly, the inhibitory effects of the steroid on hyperpolarizing current at this receptor were greater with higher concentrations of GABA. Peak effects of 57% inhibition were observed at 100–1000 μ M GABA.

Because GABA is a partial agonist at $\alpha 4\beta 2\delta$ GABARs (Brown et al. 2002), we also tested the effects of $3\alpha,5\beta$ -THP on hyperpolarizing current gated by a saturating concentration of the full GABA agonist gaboxadol (Fig 3). $3\alpha,5\beta$ -THP reduced hyperpolarizing gaboxadol-gated current by a mean 93%, a significantly ($P<0.001$) greater effect than its maximal reduction of GABA-gated current (60%).

Time-course and temperature dependent effects of $3\alpha,5\beta$ -THP at $\alpha 4\beta 2\delta$ GABARs

In order to further characterize the polarity dependent effects of $3\alpha,5\beta$ -THP at $\alpha 4\beta 2\delta$ GABARs, we tested the time-course of inhibitory effects on hyperpolarizing current gated by 10 μ M GABA. Significant inhibition (~25%) of current was observed by 30s after exposure to $3\alpha,5\beta$ -THP with peak inhibition (60%) observed after 5–10 min (Fig 3). Full recovery of current amplitude was observed after wash-out of the steroid. The time-course and amplitude of the effects of the steroid were not altered by temperature, with similar findings at 20° and 30°C (Fig 4).

Effects of low $[Cl^-]$ on THP-induced inhibition of $\alpha 4\beta 2\delta$ GABARs

In order to test the effect of Cl^- ion on the observed THP inhibition of hyperpolarizing current gated by $\alpha 4\beta 2\delta$ GABARs, recordings were performed using low $[Cl^-]$ conditions both intra and extracellularly by partially substituting $MeSO_3$ for Cl^- for both intra- and extracellular solutions, without changing the ratio of $[Cl^-]_o/[Cl^-]_{in}$. Under these conditions, THP did not significantly alter GABA-gated current (Fig. 5), suggesting that its polarity-dependent effects require sufficient Cl^- .

The intracellular TM3-TM4 loop of $\alpha 4$ mediates the polarity-dependent inhibition of $3\alpha,5\beta$ -THP at $\alpha 4\beta 2\delta$ GABARs

Our previous findings suggest that polarity-dependent effects of $3\alpha,5\beta$ -THP at $\alpha 4\beta 2\delta$ GABARs are dependent upon $\alpha 4$ because they are not observed at $\alpha 1\beta 2\delta$ GABARs (Shen et al. 2007). The intracellular TM3-TM4 loop (IL) of $\alpha 4$ has a very low homology with $\alpha 1$ (<10%), and is more than two-fold longer than the IL of $\alpha 1$, containing 16 basic charges (R and K) which are unique to the IL of $\alpha 4$ (Fig. 1). Therefore, we tested the role of this IL in mediating the polarity dependent effects of $3\alpha,5\beta$ -THP. To this end, we constructed chimeras which swapped the IL of $\alpha 1$ and $\alpha 4$ producing $\alpha[141]$ (IL of $\alpha 4$) and $\alpha[414]$ (IL of $\alpha 1$). When co-expressed with $\beta 2$ and δ , these constructs yielded functional receptors, which were differentially modulated by $3\alpha,5\beta$ -THP (Fig 6): This steroid reduced hyperpolarizing current at $\alpha[141]\beta 2\delta$ by a mean 52% and potentiated depolarizing current by a mean 80%. In contrast, the steroid potentiated both hyperpolarizing and depolarizing current at $\alpha[414]\beta 2\delta$ by a mean 78% and 83%, respectively. Thus, these findings suggest that the IL of $\alpha 4$ mediates the polarity-dependent effects of $3\alpha,5\beta$ -THP at $\alpha 4\beta 2\delta$ GABARs.

Protein kinase C is necessary for the polarity-dependent inhibition of $3\alpha,5\beta$ -THP at $\alpha 4\beta 2\delta$ GABARs

Recent studies have demonstrated a role for protein kinase C in some of the actions of neurosteroids (Abramian et al. 2010). For this reason, we tested whether it plays a role in neurosteroid inhibition of hyperpolarizing current at $\alpha 4\beta 2\delta$ GABARs. To this end, effects of $3\alpha,5\beta$ -THP were tested on GABA-gated current recorded from $\alpha 4\beta 2\delta$ GABARs before and after bath application of 500 nM calphostin C, which, at this concentration, is a selective inhibitor of protein kinase C. Calphostin C prevented the inhibition of hyperpolarizing GABA-gated current by $3\alpha,5\beta$ -THP typically observed at $\alpha 4\beta 2\delta$ GABARs (Fig 7), producing instead a 20% potentiation. In contrast, it did not alter the 25–30% potentiation of depolarizing GABA-gated current by $3\alpha,5\beta$ -THP at these receptors. These findings suggest that protein kinase C is required for the polarity-dependent inhibition of $\alpha 4\beta 2\delta$ GABARs by $3\alpha,5\beta$ -THP.

Discussion

The results from this study show that polarity-dependent effects of neuroactive steroids at $\alpha 4\beta 2\delta$ GABARs are not limited to $3\alpha,5\beta$ -THP but are also evidenced with its active isomer $3\alpha,5\alpha$ -THP, and the related neurosteroid THDOC. This suggests that neurosteroid-induced reduction in inhibition via $\alpha 4\beta 2\delta$ GABARs may be a widespread phenomenon produced by a host of neurosteroids released by stress. In addition, the present findings also suggest that this effect is not likely to be due to receptor internalization because of the lack of temperature dependence. The mechanism for neurosteroid reduction of hyperpolarizing current at $\alpha 4\beta 2\delta$ resides at the intracellular TM3-TM4 loop of $\alpha 4$ because chimeras produced using the loop of $\alpha 1$ did not show polarity-dependent effects of $3\alpha,5\beta$ -THP at $\alpha 4\beta 2\delta$.

The results from the present study have relevance for states when expression of $\alpha 4\beta 2\delta$ GABARs is increased, such as puberty (Shen et al. 2007; Shen et al. 2010) and following

exposure to the stimulant drug methamphetamine (Shen et al. 2013) when $\alpha 4\beta\delta$ GABARs increase in CA1 hippocampus. In both cases, $3\alpha,5\beta$ -THP reduces the tonic, inhibitory current produced by these receptors, which is hyperpolarizing at puberty (Shen et al. 2007) and after withdrawal from methamphetamine (Shen et al. 2013). This reduction of inhibition increases neuronal excitability, as evidenced in the hippocampal slice preparation, and at puberty facilitates activation of NMDA receptors (Shen et al., 2010). The results from the present study suggest that both $3\alpha,5\alpha$ -THP and THDOC would be expected to have the same effect under these conditions because they are also able to reduce hyperpolarizing current gated by $\alpha 4\beta\delta$ GABARs.

$3\alpha,5\beta$ -THP's inhibition of hyperpolarizing current at $\alpha 4\beta\delta$ GABARs is in contrast to the usual effect of this class of steroids to increase inhibition. In both pre-pubertal and adult rodents, the dentate gyrus granule cell has high levels of $\alpha 4\beta\delta$ GABARs (Wei et al. 2003; Wisden et al. 1992), which express extrasynaptically and generate a tonic inhibition (Stell and Mody 2002). This tonic GABAergic current is a shunting inhibition despite the fact that the current is depolarizing (Staley and Mody 1992) with sufficient conductance (Song et al. 2011). This is because the reversal potential for chloride is below the threshold for generating an action potential. Thus, neurosteroids potentiate this shunting inhibition via the $\alpha 4\beta\delta$ GABARs expressed extrasynaptically here and reduce neuronal excitability, as has been reported (Stell et al. 2003). During stress, however, hypothalamic CRH-containing neurons generate a depolarizing current mediated by $\alpha 4\beta\delta$ GABARs which is excitatory because the chloride reversal potential is above the threshold for generating an action potential (Sarkar et al., 2001). In this case, THDOC potentiates the depolarizing current, producing an excitatory effect on neuronal activity. This suggests that the net effect of steroids on current generated by $\alpha 4\beta\delta$ GABARs is dependent not only on whether the current is hyperpolarizing or depolarizing, but also on the relationship between the chloride reversal potential and the threshold for action potential generation (Smith, 2013). In contrast to their complex actions at $\alpha 4\beta\delta$ GABARs, neurosteroids exclusively potentiate current at $\alpha(1-5)\beta 2\gamma 2$ GABARs (Belelli et al. 2002; Shen et al. 2007).

In addition, $3\alpha,5\beta$ -THP exclusively potentiates current generated by $\alpha 1\beta 2\delta$ GABARs (Shen et al. 2007; Zheleznova et al. 2008), which express on the interneurons in the dentate gyrus (Glykys et al. 2007); thus neurosteroids could also potentially increase neuronal excitability in the dentate gyrus via disinhibition.

Both $3\alpha,5\alpha$ -THP and THDOC are considered stress hormones; They are released in humans (Droogleever Fortuyn et al. 2004; Girdler et al. 2001) by social and performance stress, and in rodents by restraint and CO_2 inhalation stress (Mukai et al. 2008; Purdy et al. 1991). Although the adrenal gland may also be involved, these steroids can be synthesized directly in the brain, where the enzymatic machinery is localized in many principal cells, including the pyramidal cells of CA1 hippocampus (Agis-Balboa et al. 2006). Stress-induced release of these steroids is observed in adrenalectomized animals, consistent with a CNS site of synthesis (Purdy et al. 1991). Although only the $3\alpha,5\alpha$ isomer of THP is formed in mice (Porcu 2010), circulating levels of $3\alpha,5\alpha$ -THP and $3\alpha,5\beta$ -THP are similar in men (Porcu et al., 2010) and women (Havlikova et al. 2006; Porcu et al., 2009), and they are also altered in

parallel in response to anti-depressant treatment (Girdler et al. 2012) suggesting that both isomers may play a role in the stress response in humans.

Previous findings suggested that the mechanism for the $3\alpha,5\beta$ -THP-induced inhibition of $\alpha 4\beta 2\delta$ was to accelerate receptor desensitization (Shen et al. 2007), an effect which is known to be affected by receptor efficacy. The results from the present study suggest steroid-induced increases in receptor efficacy are not part of this mechanism because $3\alpha,5\beta$ -THP successfully reduced current at $\alpha 4\beta 2\delta$ GABARs gated by the high efficacy agonist gaboxadol. In addition, this effect was not dependent upon temperature, suggesting that it does not involve receptor internalization. Importantly, the effect of the steroid to reduce hyperpolarizing current at $\alpha 4\beta \delta$ GABARs is as robust at physiological temperature as it is at room temperature. The peak effect is time-dependent, however, consistent with the present findings showing that protein kinase C is a necessary factor because it was blocked by calphostin-C which is a selective inhibitor of protein kinase C at the concentration used (Savage et al. 1995). Recent studies have shown a role for protein kinase C in the function of $\alpha 4\beta \delta$ GABARs: PKC- γ co-immunoprecipitates with $\alpha 4$ (Kumar et al. 2002), while PKC- δ is necessary for surface trafficking of the receptor following exposure to neurosteroid and GABA (Kuver et al. 2012). Both may be important for effects of ethanol on the receptor (Choi et al. 2008; Kumar et al. 2002). Phosphorylation of serine(443) on the $\alpha 4$ subunit stabilizes the receptor in the endoplasmic reticulum which facilitates trafficking to the surface (Abramian et al. 2010). In addition, phosphorylation of this residue prevents run-down of current at $\alpha 4\beta 2\delta$ GABARs (Abramian et al. 2010) suggesting that phosphorylation of these receptors can control multiple functions.

Previous findings demonstrated that a positively charged residue in the TM3-TM4 IL, arginine 353, is necessary for the inhibitory effect of $3\alpha,5\beta$ -THP at $\alpha 4\beta 2\delta$ (Shen et al. 2007). Mutation of this residue to a neutral glutamine prevented the inhibitory effect of the steroid but did not permit the typical potentiating effect of the steroid at this receptor. This IL is the least homologous portion of the $\alpha 4$ subunit, when compared with $\alpha 1$, which is not inhibited by $3\alpha,5\beta$ -THP when expressed as $\alpha 1\beta 2\delta$. The present findings suggest that swapping the loop of $\alpha 1$ alone completely restores the response of the receptor to the steroid, where potentiating effects, and not inhibition, are observed for hyperpolarizing current.

The mechanism for the polarity-dependent effect of neurosteroids at $\alpha 4\beta 2\delta$ GABARs is not known but may involve changes in membrane potential produced by the Cl^- flux. Polarity-dependent effects have been shown for receptor desensitization at δ -containing GABARs previously which are consistent with more rapid desensitization for hyperpolarizing current (Bianchi and Macdonald 2003), in contrast to $\alpha 5\beta 3\gamma 2$ GABARs which desensitize more quickly when the current is depolarizing (Burgard et al. 1996). Studies of other systems also suggest that ions can serve as a sensor or trigger; Cl^- triggers a cation conductance in hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Chen et al. 2000) and cations trigger phosphorylation in a novel membrane protein lacking an ion pore (Ramsey et al. 2006). Additional studies have shown that phosphorylation of the intracellular loop is involved in desensitization of serotonin receptors (Turner and Raymond 2005). Taken together, these findings suggest that ions regulate receptor function in many ways beyond ion conductance, which may include regulation of desensitization via an effect on

phosphorylation of the intracellular loop. This possibility is the most likely as a potential mechanism for the observed inhibitory effect of neurosteroids on $\alpha 4\beta 2\delta$ GABA_ARs. Less likely is the possibility that Cl⁻ may act in a modulatory capacity, as shown in in vitro studies (Olsen and Snowman 1982; Trifiletti et al. 1984; Houston et al. 2009).

In conclusion, these findings suggest that neurosteroids exert polarity-dependent effects on current gated by $\alpha 4\beta 2\delta$ which are dependent upon the TM3-TM4 IL of $\alpha 4$ and phosphorylation by protein kinase C.

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a

YFTKRGYAW $\alpha 1$
YFTNIQMOK $\alpha 4$
 ***:

DGKSVVPEKPKKVKDPLIKKNNTYAPTATSYTPNLARGDPGLATIAKSAT $\alpha 1$
AKKKISKPPPEVPAAPVLKEKHTETSLQNTNANLNMKRKTNALVHSESDV $\alpha 4$
 :. * : * * : * : . : : * . . : * .

IEPKVKPETK----- $\alpha 1$
KSRTEVGNHSSKTSAVQESSEATPKAHLASSPNPFSRANAAETMSAAAR $\alpha 4$
 : . ** . : .

----- $\alpha 1$
GLSSAASPSPHGTLRPASLGSASTRPAFGSRLGRIKTTVNTTGAAGNVS $\alpha 4$

-----PPEPKKTFNSVSKIDR $\alpha 1$
ATPPPPAPPPSGSGTSKIDK $\alpha 4$
 ** * : . . . *****:

Fig. 1. Chimera construction

a, Sequence alignment, TM3-TM4 intracellular loop (IL) of $\alpha 1$ (top, red) and $\alpha 4$ (bottom, blue). Homologies are indicated by “*”; conserved substitutions (similar amino acid groups) by “:” and semi-conserved substitutions (similar shapes) by “.”. The TM3-TM4 ILs of $\alpha 1$ and $\alpha 4$ are less than 10% homologous, and $\alpha 4$ has 16 unique basic residues (R and K) that $\alpha 1$ does not. **b**, Top, Schematic diagrams of $\alpha 4$ and $\alpha 1$ with their respective TM3-TM4 ILs indicated by cross-hatching. Bottom, Chimeras were constructed substituting the TM3-TM4

IL of $\alpha 1$ into $\alpha 4$ (upper diagram) to produce $\alpha[414]$ and substituting the TM3-TM4 IL of $\alpha 4$ into $\alpha 1$ (lower diagram) to produce $\alpha[141]$.

Fig. 2. Structural specificity of steroid inhibition of hyperpolarizing current at $\alpha 4\beta 2\delta$ GABARs
a, Representative currents and **b**, averaged data depict effects of 30 nM concentrations of indicated neurosteroids on GABA(10 μ M)-gated current recorded from recombinant $\alpha 4\beta 2\delta$ GABARs transiently expressed in HEK-293 cells. The 3 α OH-THP isomers 3 α ,5 α -THP and 3 α ,5 β -THP produced similar effects to reduce hyperpolarizing GABA-gated current as did the related neurosteroid THDOC. However, the inactive 3 β OH, 5 β -THP isomer had no effect on GABA-gated current at either 30 or 100 nM. * $P < 0.05$ vs. pre-steroid GABA-gated current, $n = 8-10$ cells/group.

Fig. 3. Agonist-specific and concentration-dependent modulatory effects of 3 α ,5 β -THP on current gated by α 4 β 28 GABARs

THP, 3 α ,5 β -THP. **a**, Concentration-response relationship for GABA-gated current showing greater effects of THP at higher concentrations of GABA, both for depolarizing current and hyperpolarizing current, where the steroid exerted potentiating or inhibitory effects, respectively, on α 4 β 28 GABARs. $n=5-6$ cells/group. **b**, Representative currents and **c**, averaged data showing inhibitory effects of THP in modulating hyperpolarizing current gated by the high efficacy agonist gaboxadol (GBX) at α 4 β 28 GABARs. **d**, Averaged data comparing THP effects on current gated by either GABA or gaboxadol at α 4 β 28 GABARs. These data show that THP inhibition of hyperpolarizing current is greater when gaboxadol is the agonist. * $P<0.05$ vs. GABA-gated current. $n=7-8$ cells/group.

Fig. 4. Time-course and temperature dependence of 3 α ,5 β -THP inhibition of hyperpolarizing current at α 4 β 2 δ GABARs

THP, 3 α ,5 β -THP. **a**, Representative current (30°C) and **b**, averaged data (20° and 30°C) showing the time-course of effects of THP on GABA(10 μ M)-gated current. Significant effects of THP are observed after 30 s with peak effects at 5 min after exposure of HEK-293 cells to the steroid. A similar time-course was observed at both bath temperatures. Complete recovery to pre-steroid amplitudes was observed in all cases. n=5–6 cells/group.

Fig. 5. Effect of low $[Cl^-]$ conditions on $3\alpha,5\beta$ -THP inhibition of hyperpolarizing current at $\alpha 4\beta 2\delta$ GABARs

THP, $3\alpha,5\beta$ -THP. MeSO₃ was partially substituted for Cl^- in both intracellular and extracellular solutions to achieve low $[Cl^-]$ both intra- and extracellularly. Under these conditions, THP had no significant effect on GABA(10 μ M)-gated current. **a**, Representative currents, **b**, averaged data. n=12 cells.

Fig. 6. The TM3-TM4 intracellular loop of $\alpha 4$ mediates the polarity-dependent inhibition by 3α , 5β -THP at $\alpha 4\beta 2\delta$ GABARs

THP, 3α , 5β -THP. **a**, Representative currents and **b**, averaged data reveal that substitution of the TM3-TM4 intracellular loop (IL) of $\alpha 4$ with IL of $\alpha 1$ ($\alpha[414]$) prevents THP inhibition of hyperpolarizing current at $\alpha[414]\beta 2\delta$ GABARs resulting instead in potentiation. In contrast, substitution of the TM3-TM4 IL of $\alpha 1$ with the IL of $\alpha 4$ ($\alpha[141]$) yields a $\alpha[141]\beta 2\delta$ GABAR which is inhibited by THP when GABA-gated current is hyperpolarizing, but potentiates depolarizing current, similar to $\alpha 4\beta 2\delta$ GABARs. Hyperpol I_{GABA} , hyperpolarizing GABA-gated current; Depol I_{GABA} , depolarizing GABA-gated current. * $P < 0.05$ vs. pre-steroid GABA-gated current; ** $P < 0.05$ vs. other groups; $n = 5-6$ cells/group.

Fig. 7. Protein kinase C is necessary for 3 α ,5 β -THP inhibition of hyperpolarizing current at α 4 β 2 δ GABARs

THP, 3 α ,5 β -THP. In order to test the role of protein kinase C, the selective blocker calphostin C (Cal C, 500 nM) was applied after 30 nM THP, and effects of THP on GABA(10 μ M)-gated current were determined using both hyperpolarizing (hyperpol I_{GABA}) and depolarizing (depol I_{GABA}) current. **a**, Representative currents and **b**, averaged data reveal that Cal C prevented THP inhibition of hyperpolarizing current, but not its potentiation of depolarizing current. * $P < 0.05$ vs. other groups; $n = 5-6$ cells/group.