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ESTROUS CYCLE VARIATIONS IN $GABA_A$ RECEPTOR PHOSPHORYLATION ENABLE RAPID MODULATION BY ANABOLIC ANDROGENIC STEROIDS IN THE MEDIAL PREOPTIC AREA

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Abstract—Anabolic androgenic steroids (AAS), synthetic testosterone derivatives that are used for ergogenic purposes, alter neurotransmission and behaviors mediated by $GABA_A$ receptors. Some of these effects may reflect direct and rapid action of these synthetic steroids at the receptor. The ability of other natural allosteric steroid modulators to alter $GABA_A$ receptor-mediated currents is dependent upon the phosphorylation state of the receptor complex. Here we show that phosphorylation of the $GABA_A$ receptor complex immunoprecipitated by β_2/β_3 subunit-specific antibodies from the medial preoptic area (mPOA) of the mouse varies across the estrous cycle; with levels being significantly lower in estrus. Acute exposure to the AAS, 17α-methyltestosterone (17α-MeT), had no effect on the amplitude or kinetics of inhibitory postsynaptic currents in the mPOA of estrous mice when phosphorylation was low, but increased the amplitude of these currents from mice in diestrus, when it was high. Inclusion of the protein kinase C (PKC) inhibitor, calphostin, in the recording pipette eliminated the ability of 17α-MeT to enhance currents from diestrous animals, suggesting that PKC-receptor phosphorylation is critical for the allosteric modulation elicited by AAS during this phase. In addition, a single injection of 17α-MeT was found to impair an mPOA-mediated behavior (nest building) in diestrus, but not in estrus. PKC is known to target specific serine residues in the β_3 subunit of the $GABA_A$ receptor. Although phosphorylation of these β_3 serine residues showed a similar profile across the cycle, as did phosphoserine in mPOA lysates immunoprecipitated with β2/β3 antibody (lower in estrus than in diestrus or proestrus), the differences were not significant. These data suggest that the phosphorylation state of the receptor complex regulates both the ability of AAS to modulate receptor function in the mPOA and the expression of a simple mPOA-dependent behavior through a PKC-dependent mechanism that involves the β_3 subunit and other sites within the $GABA_A$ receptor complex.

Key words: anabolic steroid, $GABA_A$ receptor, phosphorylation, PKC, medial preoptic area, inhibitory postsynaptic current.

Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone initially developed for the treatment of chronic wasting diseases but whose use is now predominantly illicit and for ergogenic purposes (for review, Basaria, 2010; Kanayama et al., 2010; Wood and Stanton, 2012). Secondary to their potential to build muscle mass and enhance athletic performance, AAS are known to impose significant changes on neural function and on behavior, and such changes are recapitulated in animal models (for review, Clark and Henderson, 2003; Wood, 2008; Oberlander et al., 2012a,b). Many of the behavioral actions of AAS arise with long-term exposure and involve signaling mediated by classical nuclear hormone receptors. However, the AAS can also elicit rapid changes in neural function through direct allosteric modulation of ion channels, in particular modulation of the $GABA_A$ receptor, raising the possibility that even with prolonged exposure, rapid and non-genomic actions may underlie some of the behavioral actions of these synthetic steroids (for review, Henderson and Jorge, 2004; Henderson, 2007; Oberlander et al., 2012b).

Allosteric modulation of $GABA_A$ receptors by neurosteroids, naturally occurring derivatives of progesterone, testosterone and corticosterone, is an extensively studied phenomenon (for review, Vicini, 2004; Mitchell et al., 2008). The $GABA_A$ receptor is a target for a wide range of kinases (for review, Tasker, 2000, 2004; Brandon et al., 2002; Song and Messing, 2005), and the phosphorylation state of the receptor (and/or the receptor and its associated proteins) has complex, but significant, effects on the ability of the neurosteroids to modulate the receptor. For example, phosphorylation mediated by protein kinase C (PKC) diminishes the modulation of the $GABA_A$ receptor by

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Abbreviations: 17α-MeT, 17α-methyltestosterone; 3α-diol, 5α-androstane-3α, 17β-diol; AAS, anabolic androgenic steroids; BSA, bovine serum albumin; E_2, 17β-estradiol; mIPSC, miniature inhibitory postsynaptic current; mPOA, medial preoptic area; PKC, protein kinase C; PN, postnatal; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sIPSC, spontaneous inhibitory postsynaptic current; TBST, Tris-buffered saline with Tween-20.

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neurosteroids that enhance currents (positive neurosteroids) in spinal cord neurons of young rats of undetermined sex (Vergnano et al., 2007), in pyramidal neurons of the piriform cortex of male rats (Kia et al., 2011), and in supraoptic nucleus neurons of pregnant female rats (Brussaard et al., 1997; Koksma et al., 2003). In contrast, PKC-dependent phosphorylation augments modulation by positive neurosteroids in supraoptic neurons of young, male rats (Fáncsik et al., 2000) and in dentate granule cells of young rats (of either sex) (Harney et al., 2003). Moreover, inhibition of PKC has been shown to antagonize the ability of positive neurosteroids in the ventral tegmental area to enhance lordosis; an effect likely reflecting a change in the modulation of $GABA_A$ receptors in that area (Frye and Walf, 2008). PKC binds directly to the intracellular domains of the β subunits and phosphorylates specific serine residues in these regions (serine 409 in β_1, serine 410 in β_2 and serines 408/409 in β_3) (Brandon et al., 1999, for review, Brandon et al., 2002), suggesting that these may be key sites for PKC-dependent regulation of neurosteroid modulation of the $GABA_A$ receptor.

In regions of the forebrain and hypothalamus, the sensitivity of the $GABA_A$ receptor to neurosteroid modulation has also been shown to vary significantly with hormonal state (for review, Brussaard and Herbison, 2000; Maguire and Mody, 2009; Smith et al., 2009). Such differences in neurosteroid modulation have most often been associated with underlying changes in the expression of specific $GABA_A$ receptor subunits. For example, the insensitivity of supraoptic neurons in rats at the time of parturition to modulation by allopregnanolone was initially attributed to a switch in the relative expression of α_2 to α_1 subunits (Brussaard et al., 1997), although this finding was later recanted (Koksma et al., 2005). Differences in the sensitivity across the estrous cycle of mPOA neurons to the positive neurosteroids allopregnanolone and 3α-diol were suggested to correlate with the expression of the ε subunit in this region (Jorge et al., 2002). The best characterized relationship between subunit expression and neurosteroid sensitivity has been with regard to the δ subunit. In the hippocampus and other forebrain regions, it has been proposed that as neurosteroid levels rise over the estrous cycle, they upregulate the expression of δ subunits (Shen et al., 2005; Maguire and Mody, 2007), thus creating a homeostatic mechanism (Maguire and Mody, 2009) whereby these neurosteroids regulate their own impact by altering the expression of the subunit that most strongly determines the sensitivity of the $GABA_A$ receptor to their action (for review, Henderson, 2007; Mitchell et al., 2008). Such neurosteroid-induced changes in δ subunit expression have also been proposed to reciprocally regulate the sensitivity of forebrain regions to allosteric modulation by these neurosteroids with other changes in hormonal state, such as puberty (Shen et al., 2007, 2010) and pregnancy/parturition (Maguire et al., 2009). While the preponderance of studies implicate changes in subunit composition as a major mechanism underlying steroid-dependent changes in sensitivity to allosteric modulators, Koksma et al. (2003) provide evidence that steroid-dependent changes in sensitivity of $GABA_A$ receptors to neurosteroid modulation can be elicited in juvenile males or postpartum females (cycling females were not examined) by manipulating the activity of PKC, or serine/threonine phosphatases, without changes in subunit composition. Taken together with studies demonstrating the importance of phosphorylation in neurosteroid modulation (for discussion, Tasker, 2000, 2004), these data support the assertion that steroid-dependent changes in posttranslational modification of the $GABA_A$ receptor may also be an important mechanism by which the sensitivity of the receptor to allosteric modulation by neuroactive steroids varies with hormonal state.

Although both neurosteroids and the AAS share a common core ring structure, there are marked structural and functional differences between these two classes of steroid molecules. In brief, the AAS lack key residues that are present in the positive neurosteroids that are required for modulatory capacity of the neurosteroids; the AAS do not directly gate the receptor (as do the neurosteroids); the two classes of steroids exhibit different mechanisms of receptor gating; and they do not show parallel dependence on subunit composition (Henderson, 2007; Oberlander et al., 2012a,b). Thus, one cannot extrapolate from studies of the neurosteroids to know if phosphorylation of the receptor will have an impact on AAS modulation, the structural basis for any effects of that phosphorylation on AAS modulation, or its functional repercussions. Moreover, no studies have shown that the phosphorylation state of the receptor varies with the natural changes in hormonal state, such as those that occur during adolescence or in cycling females. Thus, the goals of the current study were twofold. First, to determine if there are significant differences in the extent of phosphorylation of the $GABA_A$ receptor complex between female and male mice prior to the onset of puberty and in adult female mice over the course of the estrous cycle, and second to determine if such differences alter the functional output of an identified population of neurons in response to acute exposure to the AAS, 17α-MeT.

EXPERIMENTAL PROCEDURES

Animals

C57Bl/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) or were obtained from an in-house strain on a C57Bl/6J background (Oberlander et al., 2012b). Animals were group-housed (4/cage) with food and water *ad libitum* in a temperature-controlled and 12-h light cycle facility with lights on starting at 0700 h. Care was taken to minimize the discomfort and the number of animals used, and all procedures were approved by the Dartmouth College Institutional Animal Care and Use Committee and conducted in accordance with guidelines from the National Institutes of Health. Estrous cycle stages in adult female mice were determined by daily vaginal lavage (Cooper et al., 1993; Penatti et al., 2011). Experiments were performed on adolescent male and female mice from postnatal day (PN) 38–42 and on adult females (> PN55).