Anabolic Steroids Induce Region- and Subunit-Specific Rapid Modulation of GABA<sub>A</sub> Receptor-Mediated Currents in the Rat Forebrain

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INTRODUCTION

Anabolic-androgenic steroids (AAS), synthetic derivatives of testosterone originally designed to provide enhanced androgenic potency with negligible androgenic effects (for review, Kochakian 1993), have become significant drugs of abuse not only among elite athletes, but among a growing number of adolescents (Johnson 1990; Yesalis et al. 1997) especially young girls (Bahkre et al. 1998). It has been noted that long-term risks from AAS abuse are greater in women than in men (Franke and Berendonk 1997; Hickson and Kuowski 1986; Honor 1997; Stratus and Yesalis 1993) and that AAS use in both women and female rodents is associated with irregular cyclicity (Blasberg et al. 1997; Bronson 1996; Bronson et al. 1996; Clark et al. 1998a; Franke and Berendonk 1997), accelerated reproductive senescence (Bronson 1996), and changes in both aggressive and sexual behaviors (Bronson 1996; Bronson et al. 1996). While adverse effects of long-term AAS treatment on estrous cyclicity can be attributed to signaling through androgen receptors (Blasberg et al. 1998), recent studies demonstrating that AAS alter Cl<sup>-</sup> flux in synaptosomes, as well as binding of t-butylcyclcoporphorothionate (TBPS) and benzodiazepines to the γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor (Masonis and McCarthy 1995; 1996), suggest that these compounds may have acute effects in the CNS that are mediated by nongenomic actions at the GABA<sub>A</sub> receptor.

Here we show for the first time that three commonly abused AAS, 17α-methyltestosterone (17α-met), stanozolol, and nandrolone, induced rapid modulation of GABA<sub>A</sub> receptor-mediated synaptic currents in the ventromedial nucleus of the hypothalamus (VMN) and the medial preoptic area (mPOA). All three AAS significantly enhanced peak synaptic current amplitudes and prolonged synaptic current decays in neurons from the VMN. Conversely all three AAS significantly diminished peak current amplitudes of synaptic currents from neurons of the mPOA. The endogenous neuroactive steroids, 3α-hydroxy-5α-pregnan-20-one and 5α-androstane-3α,17β-diol, potentiated currents in the VMN as did the AAS. In contrast to the negative modulation induced by AAS in the mPOA, the endogenous steroids potentiated responses in this region. To determine the concentration response relationships, modulation by the AAS, 17α-methyltestosterone (17α-met), was assessed for currents evoked by ultrafast perfusion of brief pulses of GABA to acutely isolated neurons. Half-maximal effects on currents elicited by 1 mM GABA were elicited by submicromolar concentrations of AAS for neurons from both brain regions. In addition, the efficacy of 10<sup>−5</sup> to 10<sup>−7</sup> M GABA was significantly increased by 1 μM 17α-met. Previous studies have demonstrated a striking dichotomy in receptor composition between the VMN and the mPOA with regard to γ<sub>2</sub> subunit expression. To determine if the preferential expression of γ<sub>2</sub> subunit-containing receptors in the VMN and of γ<sub>1</sub> subunit-containing receptors in the mPOA could account for the region-specific effects of AAS in the two regions, responses elicited by ultrafast perfusion of GABA to human embryonic kidney 293 cells transfected with α<sub>2</sub>, β<sub>2</sub>, and γ<sub>2</sub> or α<sub>2</sub>, β<sub>1</sub>, and γ<sub>1</sub> subunit cDNAs were analyzed. As with native VMN neurons, positive modulation of GABA<sub>A</sub> responses was elicited for α<sub>2</sub>β<sub>2</sub>γ<sub>2</sub> recombinant receptors, while negative modulation was induced at α<sub>2</sub>β<sub>2</sub>γ<sub>1</sub> receptors as in the mPOA. Our data demonstrate that AAS in doses believed to occur in steroid abusers can induce significant modulation of GABAergic transmission in brain regions essential for neuroendocrine function. In addition, the effects of these steroids can vary significantly between brain regions in a manner that appears to depend on the subunit composition of GABA<sub>A</sub> receptors expressed.

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METHODS

Animal care and preparation of neuronal tissue

Prepubertal Sprague Dawley female rats [postnatal day PN (3–14)] were employed in this study (n = 245). Animal care procedures were approved by the Institutional Animal Care and Use Committee at Dartmouth and adhere to both the National Institutes of Health and the American Veterinary Medical Association guidelines. For slice recordings, animals were rapidly decapitated, the brains were quickly dissected and placed in ice-cold saline. A thick coronal section was mounted with cyanoacrylate ester (Krazy glue) on the chuck of a Campden Vibroslice microtome (Stoelting, Wood Dale, IL), and 300-µM slices at the level of the VMN or the mPOA were prepared. For isolation of acutely dissociated neurons, the VMN or the mPOA was quickly microdissected from animals of comparable ages. Tissue was minced into small pieces and transferred through several washes and incubated in 0.25% trypsin (Worthington Biochemical, Freehold, NJ) in Opti-MEMI (Gibco Laboratories; Grand Island, NY) at 37°C for 10 min followed by 8-min incubation in 0.2 mg/ml DNase (Sigma Chemical, St. Louis, MO) in the same trypsin-containing medium. Enzyme activity was inhibited by incubation at room temperature in Opti-MEMI containing 5% charcoal-stripped fetal bovine serum (FBS; Gibco) for 8 min. Medium was removed, the tissue triturated, and the cells were plated onto 35-mm tissue-culture dishes coated with Cell-tak (Collaborative Biomedical Products; Bedford, MA). Recordings were made 30–60 min after plating.

Transfection of HEK293 cells

Human α2, rat β3, γ2, and human γ1 GABA A receptor subunit cDNAs individually subcloned into the pcDM8 or M13 expression vectors were provided by Dr. Stefano Vicini (Georgetown University Medical Center, Washington, DC). Human embryonic kidney (HEK) 293 cells, provided by Dr. Lee Witters (Dartmouth Medical School, Hanover, NH), were transfectected using Lipofectamine (Gibco) with plasmids expressing cDNAs encoding the α2, β3, and γ2 subunits or with ones expressing cDNAs encoding α2, β1, and γ1 subunits (1 µg of each construct). Cotransfection of the plasmids pGreenLantern (Gibco), allowed for selection of transfected cells expressing the green fluorescent protein (GFP) under fluorescent optics.

Acquisition and analysis of spontaneous inhibitory postsynaptic currents (sIPSCs)

Recordings were made as described previously (Nett et al. 1999) using an Olympus BX50 microscope equipped with a Dage VE1000 CCD camera system (Optical Analysis, Nashua, NH) from slices superfused with 95%O 2-5%CO 2-saturated artificial CSF (ACSF) containing 125 mM NaCl, 4 mM KCl, 26 mM NaHCO 3, 2 mM CaCl 2, 1 mM MgCl 2, and 10 mM glucose supplemented with 10 µM CNQX and 20 µM CPP to block glutamatergic transmission (Smith et al. 1996). Pipette saline consisted of 153 mM CsCl, 1 mM MgCl 2, 5 mM EGTA, and 10 mM HEPES to which 2 mM MgATP was added each day. All chemicals were purchased from Sigma with the exception of diazepam (RBI; Natick, MA) and nandrolone (Steraloids; Wilton, NH). Recordings were made at 20–22°C, at a holding potential (V H) of ~80 mV. Modulators were dissolved in DMSO (0.01% final concentration) and applied to the bath via gravity flow. Three to 5 min of predrug data were acquired, the bath was then changed to steroid-containing ACSF (1 µM steroid), and 3–5 min later data were again acquired for 3–5 min. The bath was then switched back to ACSF alone, and 3–5 min were allowed to pass before postdrug data were collected. Recordings were made using series resistance compensation of 50–75%. Data were acquired with a List EPC-7 amplifier (ALA Scientific Instruments; Westbury, NY) and a PowerMac 8600 and analyzed using HEKA software (PulseFit; Instrutech; Great Neck, NY) and the Mini Analysis Program (Jaejin Software; Leonia, NJ). More than 50 sIPSCs with times to peak <2 ms were acquired, averaged, and analyzed for each drug condition for each neuron, and current averages were fitted under conditions in which the number of kinetic components, their magnitudes, and absolute values were not restrained, and the fits were optimized to give root mean square < 5%. No significant correlations were found between time to peak versus the value of τ1, time to peak versus the value of τ2, the percentage of the peak current attributed to τ1 versus the percentage of the peak current attributed to τ1 versus the time to peak, or the value of τ1 versus the percentage of the peak current attributed to τ1 (linear regression analysis gave multiple R values and slopes close to 0 for all comparisons) for sIPSCs recorded from neurons of these two regions.

Recording and analysis of responses from acutely isolated neurons

Responses were elicited by ultrafast perfusion of GABA or GABA plus steroids for both acutely isolated forebrain neurons and HEK293 cells in the whole cell configuration (V H = −80 mV) using a LSS-3100 high-speed positioning system (Burling Instruments, Fishers, NY) and solutions described in the preceding text. Assessment of open tip currents (Lester and Jahr 1992) indicated that 10–90% of the peak on and off responses that reflect time of solution exchange were achieved in <500 ms with this system and that applications were stable with repetitive exposures. Since steroids are known to be difficult to wash both from cell membranes and from tissue culture plastic, data were first acquired from separate populations of cells exposed either to 1 mM GABA alone or exposed simultaneously to 1 mM GABA plus 1 µM 17α-met. Subsequent experiments in which the same, individual cells were first exposed to GABA, then to GABA plus 17α-met, and finally to GABA alone again (wash) using solution switching techniques (Zhu and Vicini 1997) gave comparable results to those obtained from population studies, and the data have been pooled. No differences were noted between neurons exposed to modulator prior to GABA (preequilibrated) and those exposed to GABA and modulator simultaneously. For the experiments presented here, neurons were not preequilibrated with AAS.

Data acquisition and analysis of responses elicited by ultrafast perfusion to either isolated neurons or to HEK293 cells was made as described in the preceding text for synaptic currents with the following modifications. As previously described (Smith et al. 1996), initial assessment of current fits by eye indicated that the majority of responses were not well fitted by two exponential components with the largest error noted in the fastest component of current decay as described by τ1.
Therefore a least-squares fit using the simplex algorithm was generated for three exponential components of current decay with no restraints imposed on any individual component for each elicited response. Plots of all time constants indicated the presence of three distributions of time constants and the ranges of values for each distribution. Individual responses that, when initially fitted with three components, had more than one time constant that fell within a single distribution were subsequently refitted with two components. For each cell, time constants for individual responses were averaged, and these means then used to determine mean time constants for the populations of cells. Concentration-response curves were fitted using Prism software (Graphpad Software, San Diego, CA) using the equation 

\[
I = I_{\text{max}}/1 + 10^{(\text{LogEC}_{50} - \text{Log}[\text{drug}])}
\]

where \(I_{\text{max}}\) equals the current observed with 1 mM GABA alone. Data from both acutely isolated cells and slice recordings were digitized at 23.6 kHz and filtered at 4 kHz for analysis.

**Statistical analysis**

Values given are means ± SE. Statistical significance was determined using Kolmogorov-Smirnov test for cumulative distributions, paired and unpaired two-tailed Student’s t-tests for assessment of means, and by two-way ANOVA for analysis of concentration response curve fits. All statistical analyses were performed on non-transformed data.

**R E S U L T S**

**GABAergic spontaneous inhibitory synaptic currents in the VMN and the mPOA**

Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of glutamatergic antagonists (see METHODS). Responses were reversibly blocked by 10 μM bicuculline (data not shown), and >50 sIPSCs were averaged per each cell and each experimental condition. Peak current amplitudes of sIPSCs from VMN neurons (109.7 ± 6.7 pA) were comparable with those elicited from mPOA neurons (119.8 ± 10.1 pA). Data collected in the present study were consistent with previous reports (Nett et al. 1999; Smith et al. 1996), indicating that >90% of individual sIPSCs from the VMN and the mPOA decayed with biexponential kinetics described by two time constants, \(\tau_1\) and \(\tau_2\). Percent \(\tau_1\) indicates the percentage of the peak current amplitude attributed to the faster \((\tau_1)\) component of current decay. Responses from acutely isolated neurons (ultrafast perfusion) were elicited by brief pulses (3 ms) of 1 mM GABA in the absence (control) or presence (+AAS) of 1 μM 17α-meT. *n* = number of cells. Values during anabolic-androgenic steroid (AAS) exposure that were significantly different from control are indicated by *P < 0.05 and **P < 0.01.

**AAS effects on sIPSCs in the VMN and the mPOA**

Greater than 60 AAS have been reported to be available on the United States market (including both the generic and the black markets) (Kammerer 1993). While 17α-meT, stanozolol, and nandrolone represent three of the most commonly abused AAS (Kammerer 1993), the chemical structures of these compounds represent two structurally distinct groups, the 17α-alkylated derivatives (17α-meT and stanozolol) and the 19-nortestosterone derivatives (nandrolone). In addition to being chemically distinct from one another, all three AAS have important structural differences with the neurosteroids. In particular, active neurosteroids have been reported to possess a 5α-reduced steroid ring skeleton with an α-hydroxyl at C3 and a keto group at either C20 in the pregnane ring or C17 in the androstanate ring (Lambert et al. 1995), structural features that are not found in any of the three AAS examined here (Fig. 1).

To determine if AAS could modulate GABA<sub>A</sub> receptor-mediated synaptic transmission, neurons in the mPOA and the VMN 1 μM 17α-meT, stanozolol, or nandrolone, a concentration chosen to approximate that achieved in athletes abusing AAS (Wu 1997), significantly enhanced the average peak current amplitude for sIPSCs with the exception of those with inflections on the rising phase, indicative of asynchronous multiple events, and those events with rise times >2 ms, which may have been distorted due to cable filtering. Such excluded events accounted for >10% of those acquired.

**TABLE 1. Effects of 17α-meT on the kinetics of GABA<sub>A</sub> receptor-mediated currents**

<table>
<thead>
<tr>
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<th>n</th>
<th>(\tau_1), ms</th>
<th>Percent (\tau_1)</th>
<th>(\tau_2), ms</th>
<th>Percent (\tau_2)</th>
<th>(\tau_3), ms</th>
<th>Percent (\tau_3)</th>
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<td>VMN</td>
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<tr>
<td>Control</td>
<td>14</td>
<td>9.05 ± 0.46</td>
<td>31.7 ± 3.4</td>
<td>33.30 ± 1.32</td>
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<tr>
<td>+AAS</td>
<td></td>
<td>11.24 ± 0.38**</td>
<td>33.0 ± 2.0</td>
<td>43.04 ± 2.73**</td>
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<tr>
<td>Control</td>
<td>11</td>
<td>8.37 ± 0.96</td>
<td>17.7 ± 2.7</td>
<td>40.81 ± 3.63</td>
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<tr>
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<td>9.32 ± 1.05</td>
<td>17.0 ± 2.0</td>
<td>46.52 ± 8.05</td>
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Values given represent means and SE for GABA<sub>A</sub> receptor-mediated responses in neurons of the VMN or the mPOA in the absence (control) or presence (+AAS) of 1 μM 17α-meT. Time constants for spontaneous polysynaptic decay currents (sIPSCs) are given by \(\tau_1\) and \(\tau_2\). Percent \(\tau_1\) indicates the percentage of the peak current amplitude attributed to the faster \((\tau_1)\) component of current decay. Responses from acutely isolated neurons (ultrafast perfusion) were elicited by brief pulses (3 ms) of 1 mM GABA in the absence (control) or presence (+AAS) of 1 μM 17α-meT. *n* = number of cells. Values during anabolic-androgenic steroid (AAS) exposure that were significantly different from control are indicated by *P < 0.05 and **P < 0.01.
AAS modulation of GABA_A receptor-mediated currents, experiments were also performed on neurons acutely isolated from the VMN or the mPOA and exposed to GABA in the absence or presence of the 17α-meT using ultrafast perfusion techniques. GABA_A receptor-mediated responses (Fig. 3A) were elicited by brief pulses (3 ms) of 1 mM GABA, application parameters believed to approximate those in the synaptic cleft (Jones and Westbrook 1995). Acutely isolated neurons from these postnatal animals did not adhere tightly to tissue culture substrates, precluding formation of outside-out patches from these cells with an acceptable success rate. However, the neurons used for these studies were small (C_{avg} = 5.1 ± 0.2 pF for VMN neurons; n = 164) and 3.8 ± 0.5 pF for mPOA neurons; n = 152) with estimated diameters between 5 and 10 μm, values comparable with those reported for nucleated outside-out patches from embryonic mouse forebrain neurons (Sather et al. 1992). Responses elicited by brief pulses of 1 mM GABA were evident in nearly all cells (>90%) and rose rapidly (10–90% rise times <2 ms), consistent with previous reports (Nett et al. 1999; Smith et al. 1996). Peak current densities (I_{peak}) elicited by 1 mM GABA from VMN neurons were comparable (648.31 ± 71.66 pA/pF) with those elicited from mPOA neurons (635.76 ± 68.64 pA/pF), and as previously shown (Nett et al. 1999; Smith et al. 1996), the responses in neurons from both regions were in most cases best fitted by three kinetic components (Fig. 3A; Table 1). Specifically, for neurons from the VMN (n = 129), current decays for 50% of the responses were best fitted by three components. For neurons from the mPOA (n = 112), current decays for 71% of the responses were best fitted by three components. For both regions, in those neurons where current decays were best fitted by two components, it was the fastest component (τ_1) that was not observed.

AAS modulation of responses elicited by ultrafast perfusion of GABA to isolated neurons of the VMN and the mPOA

The AAS, 17α-meT, modulated GABA_A receptor-mediated currents from isolated VMN and mPOA neurons in a manner consistent with its effects on sIPSCs in the two brain regions. As with sIPSCs, coapplication of 1 μM 17α-meT with 1 mM GABA significantly increased I_{peak} (Figs. 3A and 6) and the value of τ_1 (Table 1) for neurons of the VMN, but decreased I_{peak} (Figs. 3A and 6) with no change in τ_1 or τ_2 (Table 1) for neurons of the mPOA. Modulation by 17α-meT was elicited in the absence of preequilibration with the steroid, supporting the assertion that the AAS act at an extracellular allosteric site on the receptor. For currents elicited by ultrafast perfusion of GABA to isolated mPOA neurons, 1 μM 17α-meT also significantly increased the value of τ_1 (Table 1), a kinetic component of current decay rarely observed in synaptic responses. Application of 0.01% DMSO alone, the carrier for AAS, did not elicit responses or modulate GABA_A receptor-mediated currents (data not shown, n = 5).

While millimolar concentrations of GABA are believed to reflect those in the synaptic cleft, tonic activation of both synaptic and extrasynaptic GABA_A receptors (Brickley et al. 1996) may occur at significantly lower concentrations. Therefore the effects of 1 μM 17α-meT were also assessed for responses elicited by brief (3 ms) pulses of 10^{-6} to 10^{-2} M GABA (Fig. 4A). Measurable responses were not evident with concentrations of GABA ≤5 μM, consistent with previous reports for α containing receptors (Lavoie and Twyman

Responses elicited by ultrafast perfusion of GABA to isolated neurons of the VMN and the mPOA

While analysis of synaptic responses in acutely isolated slices provides the most physiologically relevant system to assess how AAS may alter synaptic transmission in the brain, concentrations of modulators that reach synapses within the slice may be appreciably less than those in the external solution (Brussaard et al. 1997). To determine the concentration-response relationships for
1996), and maximal currents were elicited by 10 mM GABA in both regions. EC50 values for GABA were 32 mM for the VMN and 46 mM for the mPOA, and the Hill coefficient was 1 for neurons from both regions. Coapplication of 1 mM 17α-methyltestosterone (17α-meT) altered the efficacy of GABA, augmenting $I_{\text{peak}}$ in VMN neurons and diminishing $I_{\text{peak}}$ in mPOA neurons for concentrations of GABA $\leq 10$ mM (Fig. 4A). Similar concentration-response relationships were obtained for the prolongation of current decay kinetics by 17α-meT in the VMN (data not shown). The EC50 values for GABA were shifted in response to coapplication of 1 mM 17α-meT to 11 mM in the VMN and 19 mM in the mPOA; however, these shifts were not significant.

To establish the concentration range of AAS required to induce significant changes of GABA$\lambda$ receptor-mediated responses under conditions that mimic synaptic transmission, concentration response relationships were also determined for responses elicited by brief (3 ms) applications of 1 mM GABA and $10^{-8}$ to $10^{-4}$ M 17α-meT (Fig. 4B). For VMN neurons, significant potentiation of $I_{\text{peak}}$ was observed with $10^{-6}$ and $10^{-5}$ M 17α-meT. Potentiation was not observed for responses from VMN neurons with $10^{-4}$ M 17α-meT. For mPOA neurons, $I_{\text{peak}}$ was significantly decreased by $10^{-6}$ to $10^{-4}$ M 17α-meT (Fig. 4B). Assessment of concentration response relationships for neurons from both regions indicated that half-maximal effects on $I_{\text{peak}}$ and decay kinetics (data not shown) were achieved by concentrations of 17α-meT in the submicromolar range: EC50 $= 238$ nM for the VMN and IC50 $= 857$ nM for the mPOA. Application of 17α-meT alone at concentrations between 1–50 mM did not elicit responses in the absence of GABA ($n = 10$; data not shown).

AAS modulation of recombinant receptors expressed in HEK293 cells

Previous studies have indicated that the predominant isoform of GABA$\lambda$ receptors expressed in the VMN is α2β3γ2 (Wisden et al. 1992) while the receptor type that predominates in the mPOA is α2β3γ1 (Herbison and Fénélon 1995; Wisden et al. 1992). Inclusion of a γ1 subunit has been shown to confer unusual pharmacology for both native (Bormann and Ketten-
FIG. 3. Characteristics of responses elicited by ultrafast perfusion of 17α-meT. A: responses elicited from acutely isolated forebrain neurons by 3-ms pulses of 1 mM GABA. Representative response illustrating that current decays were best fitted by 3 exponential components indicated by the solid lines and described by the time constants $\tau_1$, $\tau_2$, and $\tau_3$ (left). Numbers in parentheses indicate the percentage of the total peak current attributed to each kinetic component. Representative responses illustrating that coapplication of the AAS, 17α-meT reversibly enhanced currents elicited by 1 mM GABA from neurons isolated from the VMN (middle) but reversibly diminished responses from neurons of the mPOA (right). Scale bars: 200 pA; 100 ms. B: responses elicited by 3-ms pulses of 1 mM GABA from human embryonic kidney (HEK) 293 cells transiently transfected with cDNAs encoding the α2, β3, and γ2 subunits and the green fluorescent protein. As with native neurons, current decays were best fitted by 3 exponential components (left) and co-application of 17α-meT reversibly enhanced currents elicited from cells transfected with α3, β2, and γ2 receptor cDNAs (middle). While reversibly diminishing currents from cells transfected with α5, β3, and γ1 cDNAs (left). Scale bars: 500 pA; 100 ms.

While allosteric modulation of GABAA receptors by endogenous neuroactive steroids is well-characterized (for review, J. C. Jorge-Rivera, K. L. McIntyre, and L. P. Henderson 1998; Nett et al. 1999) and recombinant GABAA receptors (Puia et al. 1991; Wafford et al. 1993). To test if the opposing modulation elicited by AASs in neurons of the VMN versus the mPOA arises from region-specific expression of γ2 versus γ1-containing receptors in the VMN, and the mPOA, respectively, we assessed modulation by 17α-meT of GABAergic currents elicited from HEK293 cells transiently transfected with cDNAs encoding the green fluorescent protein (GFP) and either cDNAs encoding the α2, β3, and γ2 subunits or the α3, β3, and γ1 receptor subunits. Previous studies have shown that >90% of cells expressing GFP also expressed GABAA receptors (Zhu et al. 1996), a result confirmed in the present study. Recordings were made from transfected HEK293 cells that were small in size (<5 pF) and not coupled to other cells. Currents elicited by a 3-ms pulse of 1 mM GABA elicited currents with $I_{\text{peak}} = 159 \pm 47.4$ pA/pF for cells transfected with the α2, β3, and γ2 cDNAs ($n = 5$) and 323.6 pA ± 51.5 pA/pF for cells transfected with the α2, β3, and γ1 cDNAs ($n = 7$). As with native neurons, current decays of GABAA receptor-mediated responses from HEK293 cells were described by three time constants as were currents from native receptors. Decay time constants were $\tau_1 = 3.80 \pm 0.39$ ms, $\tau_2 = 67.58 \pm 12.54$ ms, and $\tau_3 = 731.8 \pm 211.7$ ms for cells transfected with the α2, β3, and γ2 receptor subunit cDNAs. Time constants for cells transfected with the α2, β3, and γ1 receptor subunit cDNAs were $7.37 \pm 2.07$, $38.15 \pm 10.25$, and $228 \pm 20.6$ ms, respectively, for $\tau_1$, $\tau_2$, and $\tau_3$. Coassembly of a γ subunit is known to be required for benzodiazepine sensitivity of GABAA receptors (for discussion, Sieghart 1995). Consistent with inclusion of γ, as well as α, and β subunits in recombinant receptors, the benzodiazepine, diazepam, was found to reversibly potentiate $I_{\text{peak}}$ from both sets of transfected cells ($n = 6$; data not shown). Most important, as with currents elicited from neurons of the VMN, 17α-meT significantly ($P < 0.03$; $n = 6$) and reversibly enhanced currents from cells transfected with the α2, β3, and γ2 receptor cDNAs (Figs. 3B and 6). Conversely, 17α-meT significantly ($P < 0.03$; $n = 5$) and reversibly decreased currents from cells transfected with the α3, β3, and γ1 receptor cDNAs (Figs. 3B and 6), an effect that mirrors the modulation by this AAS for neurons from the mPOA. The magnitude of negative modulation of $I_{\text{peak}}$ induced in HEK cells transfected with cDNAs encoding the α3, β3, and γ1 receptor subunits was not significantly different from that observed for currents elicited by ultrafast perfusion of mPOA neurons (Fig. 6). In contrast, while 17α-meT enhanced current densities for both VMN neurons and HEK cells transfected with cDNAs encoding the α2, β3, and γ2 receptor cDNAs (Figs. 3B and 6), an effect that mirrors the modulation by this AAS for neurons from the mPOA. The magnitude of negative modulation of $I_{\text{peak}}$ induced in HEK cells transfected with cDNAs encoding the α2, β3, and γ1 receptor subunits was not significantly different from that observed for currents elicited by ultrafast perfusion of mPOA neurons (Fig. 6). Taken together with data from VMN and mPOA neurons, the data from analysis of responses elicited by ultrafast perfusion of GABA to transiently transfected HEK293 cells indicate that the opposing pattern of modulation by AAS can be attributed, at least in part, to the preferential expression of γ-containing neurons in the mPOA and of γ2-containing neurons in the VMN.

Neurosteroid effects on spontaneous inhibitory synaptic currents in the VMN and the mPOA

While allosteric modulation of GABAA receptors by endogenous neuroactive steroids is well-characterized (for review,
observed in the VMN with 50 mM point. In the mPOA (right n a the fit. The enhancement of current decay by 17 10, and 50 rivative, 3 of two endogenous neuroactive steroids, the progesterone de-

To determine if this was the case, we also assessed the ability between the AAS and the endogenous neuroactive steroids (Lambert et al. 1995), critical differences in chemical structure raised the possibility that these steroid compounds may have between VMN neurons and HEK cells expressing γ2-containing receptors (Fig. 6), as well as previous assessments of subunit mRNA (Wisden et al. 1992) and protein levels, suggest that receptor subtypes other than α3β2γ1 are also expressed at appreciable levels in this nucleus and may play a role in determining the sensitivity of VMN neurons to AAS modulation. Finally, our results indicate that endogenous neuroactive steroids also modulate GABA_A receptor-mediated currents in neurons from the VMN and the mPOA but enhance rather than diminish responses in the mPOA, which is in contrast to AAS in this region (Fig. 6). These data suggest that the AAS may have different mechanisms for altering GABA_A receptor function than do the endogenous neuroactive steroids.

**DISCUSSION**

Here we show for the first time that three commonly abused AAS, 17α-met, stanozolol, and nandrolone, can induce rapid and reversible modulation of GABA_A receptor-mediated syn-

FIG. 4. Dose-dependent effects of AAS on brief pulses of GABA. A: concentration-response relationships of average I_peak constructed from responses of neurons first exposed to concentrations of GABA from 1 μM to 10 mM (●) and subsequently to GABA plus 1 μM 17α-met (●) for the VMN (left: n = 5–9 cells for each data point) and the mPOA (right: n = 4–8 cells for each data point). Error bars indicate SE; * significant in comparison of mean AAS values with control (***0.01 < P < 0.05; **0.01 > P > 0.001; ****P < 0.001). B: concentration-response relationships were from neurons exposed to 1 mM GABA plus 10 mM to 50 μM 17α-met. In the VMN (left), 17α-met significantly enhanced I_peak at 1 μM and 10 μM. No potentiation was observed in the VMN with 50 μM 17α-met, and this point was omitted from the fit. The enhancement of current decay by 17α-met in the VMN was similarly dose-dependent (data not shown); n = 17–22 cells for each data point. In the mPOA (right), I_peak was decreased significantly by 17α-met at 1, 10, and 50 μM; n = 15–25 cells for each data point.

Lambert et al. 1995), critical differences in chemical structure between the AAS and the endogenous neuroactive steroids raised the possibility that these steroid compounds may have different mechanisms of interacting with the GABA_A receptor. To determine if this was the case, we also assessed the ability of two endogenous neuroactive steroids, the progesterone derivative, 3α,5α-THP, and the testosterone derivative, 3α-DIOL, to modulate sIPSCs in neurons in the VMN and the mPOA. Both 3α,5α-THP and 3α-DIOL potentiated sIPSCs in the VMN (3α,5α-THP: 5/5 cells; 3α-DIOL: 7/10 cells; Figs. 5 and 6). However, in contrast to the diminution produced by AAS in the mPOA, both of these neuroactive steroids significantly enhanced sIPSCs in the mPOA (3α,5α-THP: 6/10 cells; 3α-DIOL: 7/11 cells; Fig. 5, A and B, and 6). Testosterone itself, was without effect (Fig. 5B; n = 5). Enhancement of current amplitudes by endogenous neuroactive steroids and diminution by AAS was observed in the same neuron in the mPOA (Fig. 5B), indicating that the differences in responses to AAS and neurosteroids were not attributable to selective subpopulations of neurons within this region. These data show that synaptic transmission in forebrain neurons of the VMN and the mPOA can be modulated by both endogenous neuroactive steroids and AAS but that the modulation induced by the two classes of steroids for neurons of the mPOA is not equivalent.

Comparison of steroid effects on GABA_A receptor-mediated currents

Taken together, our results indicate that all three AAS induced rapid modulation of GABA_A receptor-mediated currents but that the pattern of AAS modulation differs for neurons of the VMN versus the mPOA (Fig. 6). Both the pattern and the extent of modulation in native neurons and HEK cells is consistent with α3β2γ1 receptors predominating in neurons of the mPOA. However, differences in the percent of potentiation between VMN neurons and HEK cells expressing γ2-containing receptors (Fig. 6), as well as previous assessments of subunit mRNA (Wisden et al. 1992) and protein levels, (Fritschy and Mohler 1995), suggest that receptor subtypes other than α3β2γ1 are also expressed at appreciable levels in this nucleus and may play a role in determining the sensitivity of VMN neurons to AAS modulation. Finally, our results indicate that endogenous neuroactive steroids also modulate GABA_A receptor-mediated currents in neurons from the VMN and the mPOA but enhance rather than diminish responses in the mPOA, which is in contrast to AAS in this region (Fig. 6). These data suggest that the AAS may have different mechanisms for altering GABA_A receptor function than do the endogenous neuroactive steroids.

**DISCUSSION**

Here we show for the first time that three commonly abused AAS, 17α-met, stanozolol, and nandrolone, can induce rapid and reversible modulation of GABA_A receptor-mediated syn-

FIG. 5. Modulation of sIPSCs by endogenous neuroactive steroids. A: representative sIPSCs recorded from neurons of the VMN or the mPOA during perfusion with ACSF and with ACSF containing 1 μM of the testosterone derivative, 5α-androstane-3α,17β-diol (3α-DIOL), or the progesterone derivative, 3α-hydroxy-5α-pregn-20-one (3α,5α-THP), indicating that both neuroactive steroids increased sIPSC amplitudes in neurons from both regions. The enhancement was reversible (not shown). B: representative sIPSCs recorded from an mPOA neuron in ACSF and in ACSF plus 1 μM testosterone showing no effect of the parent steroid (left). Representative sIPSCs illustrating that sequential application of 1 μM 3α-DIOL, followed by return to control (not shown) and then 1 μM 17α-met in the same mPOA neuron induced first enhancement then diminution of peak current amplitude (right). Scale bars: 20 pA; 20 ms.
endogenous androgen metabolite, 3α-DIOL and 3α,5α-THP, and the AAS, 17α-methyltestosterone, nandrolone. Modulation is illustrated for synaptic currents from neurons in brain slices (sIPSCs) and for currents elicited by ultrafast perfusion of 1 mM GABA (3 ms) and 1 μM 17α-methyltestosterone to acutely isolated cells (perfusion), either native neurons of the VMN or the mPOA or transiently transfected HEK293 cells (HEK) expressing recombinant receptors. Control values are indicated by 100%. Error bars indicate SE; *, significance in comparison of mean AAS values with control (*0.01 < \( P \leq 0.05 \); **0.01 < \( P \leq 0.001 \); ***\( P \leq 0.001\)).

aptic transmission in neurons from the mammalian brain. These data provide direct evidence that the AAS join a long list of allosteric modulators of the GABA\(_{\text{A}}\) receptor and suggest a cellular mechanism by which these compounds may alter CNS function. We found that peak current amplitudes and decay kinetics of sIPSCs were enhanced by these three AAS in all responsive neurons of the VMN. In contrast, peak current amplitudes of sIPSCs were decreased, without concomitant changes in decay kinetics (with the exception of a decrease in tau\(_1\) induced by nandrolone), in all responsive neurons from the mPOA. No significant differences were induced in the frequency of sIPSCs on application of AAS, suggesting the primary site of action of these compounds is postsynaptic, a conclusion further supported by the ability of 17α-methyltestosterone to modulate GABA responses of isolated neurons. While the endogenous androgen metabolite, 3α-DIOL, also induced reversible modulation of GABA\(_{\text{A}}\) receptor-mediated sIPSCs, application of testosterone did not, indicating that the effects of these androgens were not due to nonspecific membrane effects of steroids. Results indicating that ultrafast perfusion of 1–50 μM 17α-methyltestosterone in the absence of GABA to dissociated cells had no effect similarly argue against nonspecific effects of these steroids.

To obtain concentration response data for AAS under conditions where both the concentration of GABA and the concentration of the AAS could be accurately controlled, currents were also elicited from acutely isolated neurons of the VMN and the mPOA by ultrafast perfusion of GABA in the absence or presence of AAS. Initial assessment of a single concentration (1 μM) of 17α-methyltestosterone on responses elicited by brief (3 ms) pulses of 1 mM GABA indicated that this AAS modulated GABA\(_{\text{A}}\) receptor-mediated responses from isolated cells in a manner comparable with that observed for synaptic responses in intact slices. Specifically, 17α-methyltestosterone significantly enhanced peak current amplitudes and the value of tau\(_1\) for neurons from the VMN but diminished peak current amplitudes with no change in tau\(_1\) or tau\(_2\) for neurons of the mPOA. For isolated neurons from the mPOA, 17α-methyltestosterone also induce a significant prolongation of tau\(_1\) of responses elicited by ultrafast perfusion of GABA. The low frequency of occurrence of this component in synaptic responses precluded a comparison of AAS effects on this parameter between the two experimental paradigms, and the relevance of this component in currents elicited by ultrafast perfusion remains unclear. It is possible that there are limitations in solution exchange time with direct perfusion techniques that arise from the use of the whole cell configuration (Jonas 1995), even with the small neurons used in this study, and although this component was consistently observed, it may not reflect a physiological process that is relevant to synaptic transmission in the brain. Conversely, this slow component of decay may also arise from extrasynaptic receptors that would not be activated under conditions of low, spontaneous synaptic activity but may contribute to physiologically relevant GABA receptor-mediated responses in intact slices under conditions of high-frequency release and spillover to extrasynaptic receptors (Cherry and DeKoninck 1999; Rossi and Hamann 1998). Concentration response relationships indicated that 1 μM 17α-methyltestosterone significantly altered the efficacy of a broad range of GABA concentrations (10 μM to 10 mM), suggesting that modulation by AAS will occur in situ for both synaptic transmission in the brain. 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dred nanomolar range in both the VMN and mPOA. While the physiological range of circulating endogenous androgens in women has been determined to be appreciably below this level (2 nM) (Wu 1997), plasma concentrations of AAS in steroid abusers have been estimated to be 100- to 1,000-fold higher than those of these endogenous androgens (Wu 1997). Therefore concentrations of AAS that produced significant modulation of GABAergic currents are likely to be reached in individuals who abuse these drugs.

GABA_α receptors are expressed throughout the mammalian brain; however, there is a broad range of structural heterogeneity that in turn gives rise to a plethora of region-specific differences in GABA_α receptor function (for review, Sieghart 1995). Our results demonstrate that although the AAS produce significant modulation of GABAergic currents in both the VMN and the mPOA, these steroids induce an opposing pattern of modulation in these two brain regions. In conjunction with previous studies indicating that the VMN and the mPOA differ dramatically in the expression of γ subunit isoforms (Clark et al. 1998b; Herbison and Fénelon 1995; Wisden et al. 1992; Ymer et al. 1990), the data presented here from analysis of recombinant receptors expressed in heterologous cells strongly suggest that the preferential expression in the mPOA of receptors containing γ_1 subunits underlies the negative modulation of currents in neurons of this region. The expression of γ_1-containing receptors is restricted to a handful of brain regions involved with the production and affective components of reproductive behaviors (for discussion, see Nett et al. 1999), suggesting that the negative allosteric modulation induced by AAS may have unique actions on neuroendocrine functions. While the data from all experiments performed here are consistent with the predominant expression of γ_2-containing receptors underlying the positive modulation by AAS of neurons in the VMN. 17α-methylinduced significantly greater potency of responses from HEK cells transfected with α_2β_3 and γ_2 subunit cDNAs than from VMN neurons. These data suggest that receptors other than α_2β_3γ_2 combination may contribute to AAS sensitivity. In particular, the role of α_3 (Fritschy and Mohler 1995; Wisden et al. 1992) and β subunits (Davies et al. 1997; Whiting et al. 1997) should be explored since these receptor subunits are expressed at high levels in the VMN and have been implicated in determining the actions of other classes of allosteric modulators (Davies et al. 1997; Whiting et al. 1997; for review, Sieghart 1995). In addition, our data suggest that receptor heterogeneity will need to be considered as an important determinant of how AAS alter GABA_α receptor function in brain regions that play a significant role in mediating aggression and anxiety, behaviors affected by AAS in both rodents (Bitran et al. 1993; Bronson 1996; Bronson et al. 1996) and in human abusers (Moss et al. 1992; Pope and Katz 1988).

There is a wealth of data demonstrating that endogenous neuroactive steroids act as allosteric modulators of GABA_α receptors (for review, Lambert et al. 1995) and that these compounds have significant effects on neuroendocrine behaviors (for review, Majewska 1987). The results presented here, however, do not support the assertion that the AAS act equivalently to the endogenous neuroactive steroids in the CNS. Specifically, the pattern of modulation induced by AAS in the mPOA was significantly different from that observed with exposure to the endogenous neuroactive steroids, 3α,5α-THP and 3α-DIOL. All three AAS induced negative modulation of mean current amplitude in the mPOA, while 3α,5α-THP and 3α-DIOL enhanced the average peak current in this region. Moreover, no responses were elicited by concentrations of AAS as high as 50 μM in the absence of GABA, whereas high concentrations of neurosteroids are known to activate GABA_α receptors directly (for review, Lambert et al. 1995). In fact, the characteristics of AAS modulation of GABA_α receptors in mPOA neurons are more reminiscent of those reported for the benzodiazepine site modulator, zolpidem, (Nett et al. 1999) than to those elicited by these endogenous steroids. For example, not only do AAS induce negative modulation in mPOA neurons as does zolpidem, but the dose-response curve for VMN neurons is bell-shaped as has been reported previously for both positive and negative modulators acting at the benzodiazepine site (Rotyra and Ben-Ari 1993; Sigel et al. 1990; Stevenson et al. 1995). It is noteworthy that key structural elements shown to be common to all active neurosteroids (Fig. 1) (for review, Lambert et al. 1995) are absent from the three AAS tested here. While the precise mechanisms by which AAS alter GABA_α receptor function remain to be determined, our results indicate that AAS may mirror the actions of other modulators, such as the benzodiazepines, more closely than those produced by the endogenous neuroactive steroids.

Analysis of AAS and neurosteroid effects on sIPSCs in both the VMN and the mPOA were performed on animals that were an average age of PN13 (range PN10-PN17). Developmental changes in GABA_α receptor subunit composition in the hippocampus have been shown to result in significant decreases in the sensitivity of granule cells to the endogenous neurosteroid, 3α, 21-dihydroxy-5α-pregn-20-one (THDOC), between PN10-PN20; a change that may arise from the late developmental expression of the β subunit (Cooper et al. 1999; Zhu et al. 1996). Previous studies have demonstrated that expression of mRNAs encoding the α_1 and α_2 subunits (Davis et al., 2000) as well as β subunits (Zhang et al. 1991) undergo developmental changes in expression in the VMN and the mPOA during this postnatal period. Moreover, unpublished data from our laboratory (A. S. Clark, S. Robinson, and L. P. Henderson) indicate that there are significant changes in expression of the γ subunit in the mPOA during the first two postnatal weeks. Both α and β subunits have been shown to influence the sensitivity of recombinant receptors to 3α,5α-THP (Lambert et al. 1999; Maitra and Reynolds 1998; Puia et al. 1993), and γ subunit expression, as we have shown here, has significant effects on AAS modulation. Assessment of the percent modulation of sIPSCs as a function of development for the data in this study suggests that the magnitude of modulation induced in the VMN and the mPOA by both the AAS and the neurosteroids may change over this period. However, a conclusive timeline for developmental changes in the sensitivity of neurons in these regions to steroid modulation will require a more comprehensive study. In particular, it will be of interest to determine if the sensitivity to either class of these steroid modulators changes not only during the period of active synaptogenesis within the first few weeks of development, but also if there are subsequent changes concomitant with the onset of puberty.

The ubiquitous expression of GABA_α receptors throughout the brain suggests that AAS will have widespread effects on a broad range of CNS functions; however, the data presented
here have particular relevance with regard to delineating mechanisms by which AAS may alter reproductive behaviors. For example, GABAergic transmission in both the mPOA and the VMN regulates the expression of sexual receptivity in female rodents (for review, Pfaff et al. 1994), and neurosteroid modulators of GABA\(_\alpha\) receptors (for review, McCarthy 1995), including the endogenous androgen, 3α-Diol (Frye et al. 1996) facilitate female sexual behaviors when acutely infused into the VMN and its efferent targets. Although chronic AAS exposure may modify sexual behaviors via a number of signaling pathways, including actions mediated by nuclear androgen receptors (Blasberg et al. 1998), our data suggest that the AAS may also have acute effects on sexual behaviors, as do the exogenous neuroactive steroids, via actions at the GABA\(_\alpha\) receptor. While extrapolation from receptivity in rats to sexual behaviors in human abusers must be made with caution, cellular actions of AAS at the GABA\(_\alpha\) receptor may underlie some of the reported changes in sexual performance and libido in steroid abusers (Franke and Berendonk 1997). In addition, GABAergic control of gonadotropin-releasing hormone (GnRH) pulsatility by neurons of the mPOA is essential both for the onset of puberty (for review, Ojeda and Urbanski 1994) and for establishing estrous cycle in adult females (for review, Freeman 1994). AAS interactions with GABAergic control neurons of the mPOA may contribute to the changes in gonadotropin secretion (Bronson et al. 1996), irregular cyclicity (Blasberg et al. 1997; Bronson 1996; Bronson et al. 1996; Clark et al. 1998a), and accelerated reproductive senescence (Bronson 1996) reported with AAS use.

Although few studies have been carried out on either women or female rodents, the available data suggest that females are more sensitive to the actions of AAS than males (Bronson 1996; Hickson and Kurowski 1986). Previous studies have demonstrated that AAS effects on benzodiazepine binding to GABA\(_\alpha\) receptors are not equivalent in female and male rats (Masonis and McCarthy 1995), suggesting that actions of these steroids at the GABA\(_\alpha\) receptor are also sexually dimorphic. Given that the most dramatic increases in AAS abuse are among women and especially young girls (Bahrke et al. 1998), defining how these steroids act in both sexes, as well as determining if they have significantly different actions in prepubertal versus adult subjects, will be essential for understanding the adverse effects of these steroids in the CNS and the implications their use has for reproductive health.

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