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 andro-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Jorge-Rivera, Juan Carlos, Kerry L. McIntyre, and Leslie P. Henderson. Anabolic steroids induce region- and subunit-specific rapid modulation of GABA<sub>A</sub> receptor-mediated currents in the rat forebrain. J Neurophysiol 83: 3299–3309, 2000. Anabolic-androgenic steroids (AAS) have become significant drugs of abuse in recent years with the highest increase reported in adolescent girls. In spite of the increased use of AAS, the CNS effects of these steroids are poorly understood. We report that in prepubertal female rats, three commonly abused AAS, 17α-methyltestosterone, stanozolol, and nandrolone, induced rapid and reversible modulation of GABAergic currents in neurons of two brain regions known to be critical for the expression of reproductive behaviors: 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 of 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-pregn-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>–2</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>3</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>3</sub>γ<sub>2</sub> recombinant receptors, while negative modulation was induced at α<sub>2</sub>β<sub>1</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.
METH O DS

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 transfected using Lipofectamine (Gibco) with plasmids expressing cDNAs encoding the α2, β3, and γ2 subunits or with ones expressing cDNAs encoding α2, β3, 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 post synaptic 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 the TCA were acquired. 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 MiniAnalysis Program (Jaejin Software; Lomia, 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 τ_1 time to peak versus the value of τ_2. The percentage of the peak current attributed to τ_1 versus the value of τ_1 to peak, or the value of τ_1 versus the percentage of the peak current attributed to τ_1 (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 (Burleigh 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 τ_1.
ANABOLIC STEROIDS AND GABA<sub>A</sub> RECEPTORS

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

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<th>n</th>
<th>τ&lt;sub&gt;1&lt;/sub&gt;, ms</th>
<th>Percent τ&lt;sub&gt;1&lt;/sub&gt;</th>
<th>τ&lt;sub&gt;2&lt;/sub&gt;, ms</th>
<th>Percent τ&lt;sub&gt;2&lt;/sub&gt;</th>
<th>τ&lt;sub&gt;3&lt;/sub&gt;, ms</th>
<th>Percent τ&lt;sub&gt;3&lt;/sub&gt;</th>
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<tr>
<td>VMN</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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<td></td>
<td></td>
<td>+ AAS 11.24 ± 0.38**</td>
<td>33.0 ± 2.0</td>
<td>43.04 ± 2.73**</td>
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<tr>
<td>mPOA</td>
<td>11</td>
<td>8.37 ± 0.96</td>
<td>17.7 ± 2.7</td>
<td>40.81 ± 3.63</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>+ AAS 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 current decays (sIPSCs) are given by τ<sub>1</sub> and τ<sub>2</sub>. Percent τ<sub>1</sub> indicates the percentage of the peak current amplitude attributed to the faster (τ<sub>1</sub>) 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.

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<sub>max</sub>/[1 + 10<sup>-LogEC50(Log[17α-meT])</sup>]) where I<sub>max</sub> 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.

RESULTS

GABAergic spontaneous inhibitory synaptic currents in the VMN and the mPOA

Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of glutamate 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, τ<sub>1</sub> and τ<sub>2</sub> (Table 1). A third component of current decay (τ<sub>3</sub> > 100 ms) was rarely observed and not included in the data analysis. In addition, the kinetics of current decay for sIPSCs were not significantly different from those estimated for miniature IPSCs (mIPSCs; data not shown). All responses were included in the analysis of 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.

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- or 5β-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 androstanole 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 decay of sIPSCs recorded from neurons of the VMN and significantly decreased the peak amplitude of sIPSCs recorded from neurons of the mPOA (Figs. 2 and 6). The fractions of responsive cells in the VMN were 14/14 (17α-meT), 12/15 (stanozolol), and 12/14 (nandrolone) and in the mPOA were 14/14 (17α-meT), 12/15 (stanozolol), and 12/14 (nandrolone) and...
AAS modulation of GABA_\text{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 17\alpha-meT using ultrafast perfusion techniques. GABA_\text{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_{\text{avg}} = 5.1 \pm 0.2 \, \text{pF} \text{ for VMN neurons; } n = 164) and 3.8 \pm 0.5 \, \text{pF} \text{ for mPOA neurons; } n = 152) with estimated diameters between 5 and 10 \, \mu \text{m}, 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_{\text{peak}}) elicited by 1 mM GABA from VMN neurons were comparable (648.31 \pm 71.66 \, \text{pA/pF}) with those elicited from mPOA neurons (635.76 \pm 68.64 \, \text{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, 17\alpha-meT, modulated GABA_\text{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 \mu M 17\alpha-meT with 1 mM GABA significantly increased I_{\text{peak}} (Figs. 3A and 6) and the value of τ_1 (Table 1) for neurons of the VMN, but decreased I_{\text{peak}} (Figs. 3A and 6) with no change in τ_1 or τ_2 (Table 1) for neurons of the mPOA. Modulation by 17\alpha-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 \mu M 17\alpha-meT also significantly increased the value of τ_3 (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_\text{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_\text{A} receptors (Brickley et al. 1996) may occur at significantly lower concentrations. Therefore the effects of 1 \mu M 17\alpha-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 \mu M, consistent with previous reports for \alpha_2-containing receptors (Lavoie and Twyman 1996).
and maximal currents were elicited by 10 mM GABA in both regions. EC$_{50}$ values for GABA were 32 
$nM$ for the VMN and 46 $nM$ for the mPOA, and the Hill coefficient was 1 for neurons from both regions.

Coapplication of 1 $mM$ 17$\alpha$-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 $\geq 10$ $mM$ (Fig. 4A). Similar concentration-response relationships on the VMN were obtained for the prolongation of current decay kinetics by 17$\alpha$-meT in the VMN (data not shown). The EC$_{50}$ values for GABA were shifted in response to coapplication of 1 $mM$ 17$\alpha$-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$_{A}$ 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 17$\alpha$-meT (Fig. 4B). For VMN neurons, significant potentiation of $I_{\text{peak}}$ was observed with $10^{-6}$ and $10^{-5}$ M 17$\alpha$-meT. Potentiation was not observed for responses from VMN neurons with $10^{-4}$ M 17$\alpha$-meT. For mPOA neurons, $I_{\text{peak}}$ was significantly decreased by 10$^{-6}$ to $10^{-4}$ M 17$\alpha$-meT (Fig. 4B). Assessment of concentration response relationships for neurons from both regions indicated that half-maximal effects on $I_{\text{peak}}$ (Fig. 4B) and decay kinetics (data not shown) were achieved by concentrations of 17$\alpha$-meT in the submicromolar range: EC$_{50}$ = 238 nM for the VMN and IC$_{50}$ = 857 nM for the mPOA. Application of 17$\alpha$-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$_{A}$ receptors expressed in the VMN is $\alpha_{2}\beta_{3}\gamma_{2}$ (Wisden et al. 1992) while the receptor type that predominates in the mPOA is $\alpha_{2}\beta_{3}\gamma_{1}$ (Herbison and Fénelon 1995; Wisden et al. 1992). Inclusion of a $\gamma_{1}$ subunit has been shown to confer unusual pharmacology for both native (Bormann and Ketten-
mann 1988; Nett et al. 1999) and recombinant GABA\(_A\) 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 \(\gamma_2\) versus \(\gamma_1\)-containing receptors in the VMN and the mPOA, respectively, we assessed modulation by 17\(\alpha\)-methyl-T of GABAergic currents elicited from HEK293 cells transiently transfected with cDNAs encoding the green fluorescent protein. As with native neurons, current decays of GABA \(A\) receptor-elicited currents in the VMN and the mPOA were best fitted by three exponential components (left) and co-application of 17\(\alpha\)-methyl-T reversibly enhanced currents elicited from cells transfected with \(\alpha_3\), \(\beta_3\), and \(\gamma_1\) cDNAs (middle) while reversibly diminishing currents from cells transfected with \(\alpha_2\), \(\beta_2\), and \(\gamma_1\) cDNAs (right). Scale bars: 500 pA; 100 ms.

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

While allosteric modulation of GABA\(_A\) receptors by endogenous neuroactive steroids is well-characterized (for review,
observed in the VMN with 50m similar dose-dependent (data not shown); the enhancement of current decay by 17a10, and 50m derivative, 3a of two endogenous neuroactive steroids, the progesterone derivative, 3a-androstane-3,17b-diol (3a-DIOL), to modulate sIPSCs in neurons in the VMN and the mPOA. Both 3a,5a-THP and 3a-DIOL potentiated sIPSCs in the VMN (3a,5a-THP: 5/5 cells; 3a-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 (3a,5a-THP: 6/10 cells; 3a-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 \( \Gamma_{A} \) receptor-mediated currents

Taken together, our results indicate that all three AAS induced rapid modulation of \( \Gamma_{A} \) 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 \( \Gamma_{A} \) receptors predominating in neurons of the mPOA. However, differences in the percent of potentiation between VMN neurons and HEK cells expressing \( \Gamma_{A} \)-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 \( \Gamma_{A} \) 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 \( \Gamma_{A} \) 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 \( \Gamma_{A} \) receptor function than do the endogenous neuroactive steroids.

DISCUSSION

Here we show for the first time that three commonly abused AAS, 17a-met, stanozolol, and nandrolone, can induce rapid and reversible modulation of \( \Gamma_{A} \) receptor-mediated synapses.
endogenous androgen metabolite, 3α-meT modulate GABA responses of isolated neurons. While the frequency of sIPSCs on application of AAS, suggesting the cellular mechanism by which these compounds may alter CNS synaptic 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<sub>A</sub> 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 τ<sub>1</sub> induced by nandrolone), in all responsive neurons from the mPOA. No significant differences were induced in the decay. The observed enhancement of sIPSC amplitude, when taken in conjunction with data from fast perfusion experiments demonstrating that concentrations as high as 10 mM GABA may be necessary to influence not only discrete GABAergic IPSCs but also the tonic actions of GABA that have been shown influence electrical excitability (Brickley et al. 1996).

In the VMN, our data indicate that both AAS and endogenous neurosteroids potentiated sIPSC amplitudes as well as prolonged the slower time constant (τ<sub>2</sub>) of synaptic current decay. The observed enhancement of sIPSC amplitude, when taken in conjunction with data from fast perfusion experiments indicating that modulation by AAS will occur in situ for both saturating (Jones and Westbrook 1995) and subsaturating (Frerking et al. 1995; Hill et al. 1998; Nusser et al. 1997) conditions at neuronal GABAA receptor-mediated responses in intact slices. Specifically, 17α-meT significantly enhanced peak current amplitudes and the value of τ<sub>1</sub> for neurons from the VMN but diminished peak current amplitudes with no change in τ<sub>1</sub> or τ<sub>2</sub> for neurons from the mPOA. For isolated neurons from the mPOA, 17α-meT also induce a significant prolongation of τ<sub>2</sub> 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 (Chery and DeKoninck 1999; Rossi and Hamann 1998). Concentration response relationships indicated that 1 μM 17α-meT 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 saturating (Jones and Westbrook 1995) and subsaturating (Frerking et al. 1995; Hill et al. 1998; Nusser et al. 1997) conditions at neuronal GABAA receptors. AAS may therefore act to influence not only discrete GABAergic IPSCs but also the tonic actions of GABA that have been shown influence electrical excitability (Brickley et al. 1996).

In the VMN, our data indicate that both AAS and endogenous neurosteroids potentiated sIPSC amplitudes as well as prolonged the slower time constant (τ<sub>2</sub>) of synaptic current decay. The observed enhancement of sIPSC amplitude, when taken in conjunction with data from fast perfusion experiments demonstrating that concentrations as high as 10 mM GABA were required to elicit maximal responses from these neurons, suggest GABA concentrations at synapses in the VMN may be subsaturating as has been reported for other brain regions (Frerking et al. 1995; Hill et al. 1998; Nusser et al. 1997). Conversely, although we did not include responses that were notably asynchronous and multiquantal in our analysis, small inflections in the rising phase may have not been detected and thus some degree of potentiation of sIPSCs by steroids may also reflect the integration of multiple mIPSCs whose durations are prolonged by AAS (Mody et al. 1994).

With respect to delineating the concentrations of AAS that induce significant modulation of GABAergic responses, we found that half-maximal effects of 17α-meT were in the hun-
dried 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<sub>A</sub> 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<sub>A</sub> 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<sub>1</sub> subunits underlies the negative modulation of currents in neurons of this region. The expression of γ<sub>1</sub>-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<sub>2</sub>-containing receptors underlying the positive modulation by AAS of currents in the VMN, 17α-mE induced significantly greater potentiation of responses from HEK cells transfected with α<sub>2</sub>, β<sub>1</sub>, and γ<sub>2</sub> subunit cDNAs than from VMN neurons. These data suggest that receptors other than α<sub>2</sub>β<sub>1</sub>γ<sub>2</sub> combination may contribute to AAS sensitivity. In particular, the role of α<sub>5</sub> (Fritschy and Mohler 1995; Wisden et al. 1992) and δ 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<sub>A</sub> 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<sub>A</sub> receptors (for review, Lambert et al. 1995) and that these compounds have significant effects on neuroendocrine behaviors (for review, Majewskas 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<sub>A</sub> receptors directly (for review, Lambert et al. 1995). In fact, the characteristics of AAS modulation of GABA<sub>A</sub> 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 (Rotwein 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<sub>A</sub> 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<sub>A</sub> 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 δ, and α<sub>2</sub> subunits (Davis et al., 2000) as well as the 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 γ 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 epoch. 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<sub>A</sub> 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 GABAA 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 endogenous neuroactive steroids, via actions at the GABAA receptor. While extrapolation from receptivity in rats to sexual behaviors in human abusers must be made with caution, cellular actions of AAS at the GABAA 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 cyclicity 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 benzo diazepine binding to GABAA receptors are not equivalent in female and male rats (Masonis and McCarthy 1995), suggesting that actions of these steroids at the GABAA receptor are also sexually dimorphic. Given that the most dramatic increases in AAS abuse are among women and especially young girls (Bahkre 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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REFERENCES


ANABOLIC STEROIDS AND GABA_4 RECEPTORS


