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 anabolic potency with negligible androgenic effects (for review, Kochakian 1993), have become significant drugs of abuse in recent years, especially among elite athletes, and among a growing number of adolescents (Johnson 1990; Yesalis et al. 1997) and girls (Bahkre et al. 1998). It has been noted that long-term effects of AAS abuse are greater in women than in men (Franke and Berendonk 1997; Hickson and Kuowski 1986; Honor 1997; Strauss 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. 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-butylibicyclophosphorothionate (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), 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 in the VMN. 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>−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 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>1</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 responses was elicited for α<sub>2</sub>β<sub>1</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.
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 cyanoacrylic 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, rat 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 plasmid, 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%O2–5%CO2-saturated artificial CSF (ACSF) containing 125 mM NaCl, 4 mM KCl, 26 mM NaHCO3, 2 mM CaCl2, 1 mM MgCl2, 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 MgCl2, 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 (VH) 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; Instrotech; 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 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 (VH = −80 mV) using a LSS-3100 high-speed positioning system (Burrleigh 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.
ANABOLIC STEROIDS AND GABA\textsubscript{A} RECEPTORS

TABLE 1. Effects of 17α-meT on the kinetics of GABA\textsubscript{A} receptor-mediated currents

<table>
<thead>
<tr>
<th></th>
<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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<tbody>
<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>
<td></td>
<td></td>
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</tr>
<tr>
<td>+ AAS</td>
<td>11</td>
<td>11.24 ± 0.38**</td>
<td>33.0 ± 2.0</td>
<td>43.04 ± 2.73**</td>
<td></td>
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<tr>
<td>nPOA</td>
<td>11</td>
<td>8.37 ± 0.96</td>
<td>17.7 ± 2.7</td>
<td>40.81 ± 3.63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ AAS</td>
<td>11</td>
<td>9.32 ± 1.05</td>
<td>17.0 ± 2.0</td>
<td>46.52 ± 8.05</td>
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Ultrafast perfusion

| VMN | 54  | 8.23 ± 0.91  | 18.9 ± 2.9       | 62.28 ± 3.92 | 49.9 ± 3.9     | 356.05 ± 63.01 | 31.2 ± 4.6      |
| + AAS | 47  | 12.49 ± 1.89* | 11.3 ± 3.1       | 67.54 ± 5.20 | 50.8 ± 4.2     | 392.82 ± 29.34 | 37.9 ± 4.5      |
| nPOA | 47  | 10.97 ± 0.76  | 27.9 ± 4.0       | 51.33 ± 2.79 | 50.3 ± 3.6     | 369.93 ± 31.98 | 19.4 ± 1.9      |
| + AAS | 47  | 9.09 ± 1.23  | 28.5 ± 5.2       | 57.17 ± 3.70 | 53.6 ± 4.5     | 517.50 ± 44.36** | 18.4 ± 2.4      |

Values given represent means ± SE for GABA\textsubscript{A} receptor-mediated responses in neurons of the VMN or the nPOA in the absence (control) or presence (+AAS) of 1 μM 17α-meT. Time constants for spontaneous postsynaptic current decays (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 ** $P < 0.01$.

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 $I = I_{\text{max}}l + 10^{\text{LogEC50+Log}17\alpha\text{-meT}}$ 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.

RESULTS

GABA\textsubscript{ergic} spontaneous inhibitory synaptic currents in the VMN and the nPOA

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 amplitude 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 nPOA decayed with biexponential kinetics described by two time constants, $\tau_1$ and $\tau_2$ (Table 1). A third component of current decay ($\tau_3 > 100$ 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 infections 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 nPOA

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-hydroxyl at C3 and a keto group at either C20 or C17 in the androstane 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\textsubscript{A} receptor-mediated synaptic transmission, neurons in the nPOA 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 of sIPSCs recorded from neurons of the VMN and significantly decreased the peak amplitude of sIPSCs recorded from neurons of the nPOA (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 nPOA were 11/14 (17α-meT), 13/16 (stanozolol), and

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results produced by these two 17α-methyltestosterone (17α-met) (data not shown). In agreement with previous reports (Brussaard et al. 1996), 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-metT, 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-metT 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-metT 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-metT 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_{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-metT were also assessed for responses elicited by brief (3 ms) pulses of 10^{-4} to 10^{-2} M GABA (Fig. 4A). Measurable responses were not evident with concentrations of GABA ≤5 μM, consistent with previous reports for α_{2}-containing receptors (Lavoie and Twyman 1995).
and maximal currents were elicited by 10 mM GABA in both regions. EC_{50} values for GABA were 32 μM for the VMN and 46 μM 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_{peak} in VMN neurons and diminishing I_{peak} in mPOA neurons for concentrations of GABA $\leq$ 10 μM (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 EC_{50} values for GABA were shifted in response to coapplication of 1 mM 17α-meT to 11 μM in the VMN and 19 μM 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 17α-meT (Fig. 4B). For VMN neurons, significant potentiation of I_{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_{peak} was significantly decreased by 10 μM 17α-meT (Fig. 4B). Assessment of concentration response relationships for neurons from both regions indicated that half-maximal effects on I_{peak} (Fig. 4B) and decay kinetics (data not shown) were achieved by concentrations of 17α-meT in the submicromolar range: EC_{50} = 238 nM for the VMN and IC_{50} = 857 nM for the mPOA. Application of 17α-meT alone at concentrations between 1–50 μM 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-
Currents elicited by a 3-ms pulse of 1 mM GABA elicited reversibly diminishing currents from cells transfected with the \( \alpha \) subunit. As with native neurons, current decays of GABA \( \alpha \) receptor-elicited currents in the VMN and the mPOA were best fitted by 3 exponential components (left). Co-application of \( \alpha \) and the green fluorescent protein with inclusion of \( \beta \) and \( \gamma \) subunits in recombinant GABA \( \alpha \) receptors (for discussion, Sieghart 1995). Consistent with inclusion of \( \gamma \), as well as \( \alpha \), \( \beta \) subunits in recombinant receptors, the benzodiazepine, diazepam, was found to reversibly potentiate the peak current attributed to each kinetic component. Representative responses illustrating that co-application of the AAS, \( \alpha \)-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). Numbers in parentheses indicate the percentage of the total peak current attributed to each kinetic component. Representative responses illustrating that co-application of the AAS, \( \alpha \)-meT reversibly enhanced currents elicited by 1 mM GABA from human embryonic kidney (HEK) 293 cells transiently transfected with cDNAs encoding the \( \alpha_1 \), \( \beta_2 \), and \( \gamma_2 \) subunits and the green fluorescent protein. As with native neurons, current decays were best fitted by 3 exponential components (left) and co-application of \( \alpha \)-meT reversibly enhanced currents elicited from cells transfected with \( \alpha_2 \), \( \beta_3 \), and \( \gamma_2 \) cDNAs (middle) while reversibly diminishing currents from cells transfected with \( \alpha_3 \), \( \beta_3 \), and \( \gamma_1 \) cDNAs (left). Scale bars: 500 pA; 100 ms.

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

While allosteric modulation of GABA \( \alpha \) receptors by endogenous neuroactive steroids is well-characterized (for review,
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<sub>A</sub> 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 from 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<sub>A</sub> receptor-mediated currents

Taken together, our results indicate that all three AAS induced rapid modulation of GABA<sub>A</sub> 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 α<sub>3</sub>β<sub>3</sub>γ<sub>2</sub> receptors predominating in neurons of the mPOA. However, differences in the percent of potentiation between VMN neurons and HEK cells expressing γ<sub>2</sub>-containing receptors (Fig. 6), as well as previous assessments of subunit mRNAs (Wisden et al. 1992) and protein levels (Fritschy and Mohler 1995), suggest that receptor subtypes other than α<sub>3</sub>β<sub>3</sub>γ<sub>2</sub> 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<sub>A</sub> 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<sub>A</sub> 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<sub>A</sub> receptor-mediated synapses in native forebrain neurons of the VMN and the mPOA. Both 3α,5α-THP and 3α-DIOL, to modulate sIPSCs in neurons in the VMN and the mPOA. The enhancement was reversible (not shown). The enhancement then diminution of peak current amplitude (right). Scale bars: 20 pA; 20 ms.

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-androstanediol (3α-DIOL) and 3α,5α-DHT, cDNAs (A) and the mPOA and HEK293 cells transfected with α3β2γ2 cDNAs (B), indicating the relative enhancement or diminution of peak current amplitudes induced by 1 μM concentrations of both the endogenous neuroactive steroids, 3α-DIOL and 3α,5α-DHT, and the AAS, 17α-methyltestosterone (17α-mT), stanozolol, and 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-mT on 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 in mean AAS values with control (*P < 0.05; **P < 0.01; ***P < 0.001).

FIG. 6. Comparison of steroid modulation of peak currents. Cumulative histograms for data acquired from the VMN and HEK293 cells transfected with α3β2γ2 cDNAs (A) and the mPOA and HEK293 cells transfected with α3β2γ2 cDNAs (B), indicating the relative enhancement or diminution of peak current amplitudes induced by 1 μM concentrations of both the endogenous neuroactive steroids, 3α-DIOL and 3α,5α-DHT, and the AAS, 17α-mT, stanozolol, and 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-mT on 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 in mean AAS values with control (*P < 0.05; **P < 0.01; ***P < 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_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 % γ1 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-mT to modulate GABA responses of isolated neurons. While the endogenous androgen metabolite, 3α-DIOL, also induced reversible modulation of GABA_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-mT 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-mT on responses elicited by brief (3 ms) pulses of 1 mM GABA indicated that this AAS modulated GABA_A receptor-mediated responses from isolated cells in a manner comparable with that observed for synaptic responses in intact slices. Specifically, 17α-mT significantly enhanced peak current amplitudes and the value of τ_e for neurons from the VMN but diminished peak current amplitudes with no change in τ_e or τ_2 for neurons of the mPOA. For isolated neurons from the mPOA, 17α-mT also induce a significant prolongation of τ_e 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_A 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-mT 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 conditions at neuronal GABA_A receptor-mediated responses from isolated cells in a manner comparable with that observed for synaptic responses in intact slices.
potentiation of responses from HEK cells transfected with a sion of modulation of currents in neurons of this region. The expres-
ing receptors underlying the positive modulation by AAS of a data suggest that receptors other than a receptors directly (for review, Lambert et al. 1995). In fact, the characteristics of AAS modulation of GABA_A 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 benzo-
diazepine 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_A 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_A receptor subunit composition in the hippocampus have been shown to result in significant decreases in the sensitivity of granule cells to the endogenous neurosteroid, 3a, 21 dihydroxy-5a-pregn-20-one (THDOC), between PN10-PN20; a change that may arise from the late development-
mental expression of the ß subunit (Cooper et al. 1999; Zhu et al. 1996). Previous studies have demonstrated that expression of mRNA encoding the ß and ß subunits (Davis et al., 2000) as well as the ß subunits (Zhang et al. 1991) undergo develop-
tmental 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 ß subunit in the mPOA during the first two postnatal weeks. Both ß and ß subunits have been shown to influence the sensitivity of recombinant receptors to 3a, 5a-THP (Lambert et al. 1999; Maitra and Reynolds 1998; Puia et al. 1993), and ß subunit expression, as we have shown here, has significant effects on AAS modulation. Assessment of the percent mod-
ulation 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 neuro-
steroids may change over this epoch. However, a conclusive timeline for developmental changes in the sensitivity of neu-ons in these regions to steroid modulation will require a more comprehensive study. In particular, it will be of interest to determine if there are subsequent changes concomitant with the onset of puberty.

The ubiquitous expression of GABA_A receptors throughout the brain suggests that AAS will have widespread effects on a broad range of CNS functions; however, the data presented
endogenous neuroactive steroids, via actions at the 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 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 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 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 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

Given that the most dramatic increases in AAS abuse are among women and especially young girls (Bahrke et al. 1998), we thank Dr. Stefano Vicini for providing us with GABA \( \alpha \) receptor subunit cDNAs and Drs. Ann Clark and Anita Prasad for a critical review of the manuscript. This research was supported by the National Institute of Neurological Disorders and Stroke (NS-28668 to L. P. Henderson) and the National Science Foundation (DBI-9707826 to J. C. Jorge-Rivera). Address for reprint requests: L. P. Henderson, Dept. of Physiology, Dartmouth Medical School, Hanover, NH 03755. We thank Dr. Stefano Vicini for providing us with GABA \( \alpha \) receptor subunit cDNAs and Drs. Ann Clark and Anita Prasad for a critical review of the manuscript. This research was supported by the National Institute of Neurological Disorders and Stroke (NS-28668 to L. P. Henderson) and the National Science Foundation (DBI-9707826 to J. C. Jorge-Rivera). Address for reprint requests: L. P. Henderson, Dept. of Physiology, Dartmouth Medical School, Hanover, NH 03755. We thank Dr. Stefano Vicini for providing us with GABA \( \alpha \) receptor subunit cDNAs and Drs. Ann Clark and Anita Prasad for a critical review of the manuscript. This research was supported by the National Institute of Neurological Disorders and Stroke (NS-28668 to L. P. Henderson) and the National Science Foundation (DBI-9707826 to J. C. Jorge-Rivera). Address for reprint requests: L. P. Henderson, Dept. of Physiology, Dartmouth Medical School, Hanover, NH 03755. We thank Dr. Stefano Vicini for providing us with GABA \( \alpha \) receptor subunit cDNAs and Drs. Ann Clark and Anita Prasad for a critical review of the manuscript. This research was supported by the National Institute of Neurological Disorders and Stroke (NS-28668 to L. P. Henderson) and the National Science Foundation (DBI-9707826 to J. C. Jorge-Rivera). Address for reprint requests: L. P. Henderson, Dept. of Physiology, Dartmouth Medical School, Hanover, NH 03755. We thank Dr. Stefano Vicini for providing us with GABA \( \alpha \) receptor subunit cDNAs and Drs. Ann Clark and Anita Prasad for a critical review of the manuscript. This research was supported by the National Institute of Neurological Disorders and Stroke (NS-28668 to L. P. Henderson) and the National Science Foundation (DBI-9707826 to J. C. Jorge-Rivera). Address for reprint requests: L. P. Henderson, Dept. of Physiology, Dartmouth Medical School, Hanover, NH 03755. We thank Dr. Stefano Vicini for providing us with GABA \( \alpha \) receptor subunit cDNAs and Drs. Ann Clark and Anita Prasad for a critical review of the manuscript. This research was supported by the National Institute of Neurological Disorders and Stroke (NS-28668 to L. P. Henderson) and the National Science Foundation (DBI-9707826 to J. C. Jorge-Rivera). Address for reprint requests: L. P. Henderson, Dept. of Physiology, Dartmouth Medical School, Hanover, NH 03755. We thank Dr. Stefano Vicini for providing us with GABA \( \alpha \) receptor subunit cDNAs and Drs. Ann Clark and Anita Prasad for a critical review of the manuscript. This research was supported by the National Institute of Neurological Disorders and Stroke (NS-28668 to L. P. Henderson) and the National Science Foundation (DBI-9707826 to J. C. Jorge-Rivera). Address for reprint requests: L. P. Henderson, Dept. of Physiology, Dartmouth Medical School, Hanover, NH 03755.
ANABOLIC STEROIDS AND GABA<sub>A</sub> RECEPTORS


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